Universidade de Aveiro
2021

MARIANA AMARAL FERNANDES

RELATÓRIO DE ESTÁGIO EM INVESTIGAÇÃO NA BLUEPHARMA INDÚSTRIA FARMACÊUTICA, S.A.

Universidade de Aveiro Departamento de Química
2021

MARIANA AMARAL FERNANDES

RESEARCH INTERNSHIP REPORT AT BLUEPHARMA INDÚSTRIA FARMACÊUTICA, S.A.
RELATÓRIO DE ESTÁGIO EM INVESTIGAÇÃO NA BLUEPHARMA INDÚSTRIA FARMACÊUTICA, S.A.

Relatório apresentado à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica Clínica, realizada sob a orientação científica da Professora Doutora Maria Teresa Herdeiro do Departamento de Ciências Médicas da Universidade de Aveiro e do Doutor António Lucas Nunes da Bluepharma Indústria Farmacêutica, S.A.

A ti, por ti e para ti, minha mãe.

o júri

presidente	Prof.ª Doutora Rita Maria Pinho Ferreira Professora auxiliar da Universidade de Aveiro
	Prof. Doutora António Lucas Nunes Head of Research and Technological Innovation, Bluepharma Indústria Farmacêutica S.A.
	Prof.ª Doutora Branca Margarida Almeida Silva Cientista Titular, Universal Farma S.L. – Insud Pharma Group

agradecimentos

Quero agradecer ao António por todo o apoio ao longo deste estágio. Porque nem só de ciência se trabalha, ajudaste-me a crescer enquanto pessoa.
Quero agradecer à professora Teresa, por me ter ajudado e apoiado para que tudo fosse possível.
Alain, por toda a ajuda, pela (imensa) paciência, pelo alento, pelo apoio, obrigada. A todos os outros colegas da Bluepharma, foram essenciais para me ajudar a crescer profissional e pessoalmente, muito obrigada.
Quero agradecer à minha mãe, pelo apoio incondicional, pelas sopas, pelos telefonemas e por acreditar em mim quando eu não fui capaz. Ao meu pai por todas as vezes que me mostrou orgulho se pode escrever de muitas formas e que desistir não é uma opção.
Obrigada à minha Diana, pela ajuda, pelas conversas, pelas gargalhadas, e por me ouvir de tal forma que parece que sei o que ando a fazer.
Obrigada ao Mano, à Tina, porque são sempre casa, porque não preciso de pedir ajuda para ela aparecer. Obrigada Matias, por todas as vezes que és o único a ouvir as explicações da Madrinha, por me fazeres sentir uma referência para ti.
Obrigada Sónia e Tiago, sempre me deram a mão, mesmo quando não sabiam porque precisavam de a segurar.
Madrinha, Tio Luís, se orgulho se expressasse em pessoas, seriam vocês. Por todas as marmitas, todas as ajudas, todos os "já falta pouco", obrigado não chega.
Obrigada Padrinho, Tia Lúcia, porque sei que estão sempre lá, para tudo.
Obrigada Felícia, Cátia, André e Sérgio, porque me mostraram que isto também é divertido.
Obrigada Tia Rosário, desde o dia 1, a professora que me encaminhou tantas vezes quantas eu precisei. Horacinho, por todas as vezes que nem precisei de pedir, porque já tinhas oferecido.
Obrigada minha Ariana, a eterna housemate, por seres sempre sinónimo de casa.

palavras-chave

Biorelevância, injetáveis de ação prolongada, libertação prolongada, técnicas de caracterização, libertação *in vitro*

resumo

Este relatório pretende descrever o trabalho desenvolvido durante o estágio na Bluepharma Indústria Farmacêutica, S.A., no setor de Investigação e Inovação. O primeiro objetivo deste estágio consistia em realizar uma revisão da literatura sobre os injetáveis de ação prolongada (LAI) e a sua caracterização, incluindo testes de liberação *in vitro*. Assim, é apresentada uma visão geral sobre os LAI, os seus métodos de caracterização, incluindo métodos de libertação *in vitro*, possíveis membranas ou barreiras usadas e características biorelevantes aplicáveis ao estudo. O segundo objetivo envolvia o apoio ao desenvolvimento de uma metodologia adequada para avaliar o perfil de libertação de uma tecnologia LAI com libertação prolongada de pelo menos 96 horas desenvolvida internamente. Assim, na segunda parte do relatório é apresentada uma descrição das atividades desenvolvidas, expondo o racional para a escolha do método, o desenvolvimento analítico do método HPLC-PDA para quantificação do princípio ativo (DS) e sua pré-validação de acordo com as diretrizes das autoridades. Embora o método analítico já tenha sido estudado, o desenvolvimento da formulação não tinha sido concluído até o término do estágio, pelo que o método analítico, bem como o método escolhido para determinação do perfil de libertação *in vitro* (incluindo *apparatus*, barreira, etc.) podem necessitar de otimização com o produto final.

keywords Biorelevance, Long-Acting Injectables (LAI), extended-release, characterization techniques, *in vitro* release

Abstract

This report intends to describe the work developed during the internship at Bluepharma Indústria Farmacêutica, S.A., in Research and Innovation sector. The first aim of this internship was to complete a review about long-acting injectables (LAI) and their characterization, as well as *in vitro* release. Thus, an overview of LAIs, their characterization methods, including *in vitro* release methods, possible membranes or barriers, and biorelevant characteristics is presented. The second aim was to support the development of the most appropriate methodology for assessing the release profile of LAI technology with an extended release of at least 96 hours develop internally. So, a description of developed activities is stated, presenting the rationale behind the choice of the method, the analytical development of the HPLC-PDA method to quantification of the drug substance (DS) and its pre-validation according to the authorities guidelines. Although the analytical method is already studied, the formulation development was not completed until the end of the internship, whereby the analytical method as well as the chosen method (including apparatus, barrier, ect.) to assess the *in vitro* release could need optimization with the final drug product.

Contents

Abbreviation index 9
Figures index 10
Tables index 12
1. Introduction 13
  1.1. About Bluepharma Indústria Farmacêutica, S.A. 13
2. State of the art 14
 2.1. Long-acting injectables 16
  2.2.1. Parenteral drug delivery systems 16
  2.2.2. Major routes of parenteral administration for long-acting injectables 16
  2.2.3. Long-acting injectables formulations 17
  2.2.4. Long-acting injectables drug release mechanisms 22
  2.2.5. Long-acting injectables advantages and disadvantages 23
  2.4.6. Therapeutic indications of long-acting injectables 24
 2.2. LAI characterization – quality assessment 26
  2.2.1. Universal tests 27
  2.2.2. Specific tests for parenteral formulations 31
 2.3. LAI characterization – development of microspheres and *in situ* formulations 35
  2.3.1. Tests proposed for microspheres 35
  2.3.2. Tests proposed for *in situ* gel formulations 37
 2.4. *In vitro* drug release for long-acting injectables formulations 38
  2.4.1. Authorities guidelines 38
  2.4.2. IVR methods 40
  2.4.3. IVR barriers 46
  2.4.4. *In vitro–in vivo* correlation 51
  2.4.5. Biorelevant conditions 52
  2.4.6. Accelerated *in vitro* release test 53
3. Internship: Laboratorial developed activities 57
 3.1. IVR Development 57
  3.1.1. PBS pH 7.4 stability 58
  3.1.2. Agarose gel as a barrier to IVR test 62
 3.2. Hight-Performance Liquid Chromatography-Photodiode Array (HPLC-PDA) 69
  3.2.1. Analytical method development 73
  3.2.2. Method pré-validation 75
  3.2.3. Stability 84
4. Conclusion 87
5. Bibliography 89

Abbreviation index

DS	Drug substance
EMA	European Medicines Agency
FDA	Food and Drug Administration
HPLC	High performance liquid chromatography
LAI	Long-acting injectable
LAR	Long-acting release
ICH	International Council for Harmonisation
IM	Intramuscular
IV	Intravenous
IVIVC	*In vitro–in vivo* correlation
IVR	*In vitro* release
MS	Mass spectrometry
PDA	Photodiode-Array Detection
Ph. Eur.	European Pharmacopoeia
RF	Response factor
RT	Retention time
SC	Subcutaneous
USP	United States Pharmacopeia

Figures index

Figure 1. Different types of classification of pharmaceutical dosage forms according to USP, 2020.......... 14
Figure 2. Parenteral drug delivery division. 16
Figure 3. Routes of parenteral administration of drugs. Legend: SC: subcutaneous; IV: intravenous; IM: intramuscular (Adapted from [15]) 17
Figure 4. Strategies to formulate a controlled release injectable product as LAI formulations. 18
Figure 5. Advantages and disadvantages of long-acting injectables. 24
Figure 6. Universal and specific tests for parenteral formulations according to ICH Topic Q6A – *Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Chemical Substances*. 27
Figure 7. Some tests proposed in literature during the development of microspheres and *in situ* formulations 35
Figure 8. Image of shaking flask method, using orbital agitator; this apparatus is then placed in an incubator with controlled temperature. 40
Figure 9. Representation of Franz diffusion cells system (from [134]). 42
Figure 10. Representation of USP Dissolution Apparatus II. 43
Figure 11. Dialysis methods. (a) dialysis sac method; (b) reverse dialysis sac method; and (c) glass basket dialysis method (from [140]). 48
Figure 12. Differences between the USP Apparatus I (baskets apparatus) (left) and the glass baskets (right) (from [147]). 49
Figure 13. Representation of set-up for IVR from microspheres using the Float-a-Lyzer. (from [142]) 49
Figure 14. IVR method using agarose gel in cuvettes a) Schematic of the layers with formulation and agarose gel followed by PBS within a cuvette. b) Actual cuvette with the agarose gels. (from [150]). 50
Figure 15. Schematic illustration of the agarose gel envelope (light grey), incorporating the dosage forms a) films, b) microspheres, c) Rod-shaped extrudates and d) implant (dark grey) (from [154]). 51
Figure 16. Graph of pH measurement as a function of time (days) for the three preparations of PBS pH 7.4, stored at Room temperature (RT). 60
Figure 17. Graph of pH measurement as a function of time (days) for the three preparations of PBS pH 7.4, stored at 37ºC. 60
Figure 18. Graph of osmolality measurement as a function of time (days) for the three preparations of PBS pH 7.4, stored at Room temperature (RT). 61
Figure 19. Graph of osmolality measurement as a function of time (days) for the three preparations of PBS pH 7.4, stored at 37ºC. 61
Figure 20. Apparatus used: well-plate with small holes in each well and the cover with the cylinders (syringe protection caps). 62
Figure 21. Schematic presentation of the method A1 for agarose gel shaping. 64
Figure 22. Schematic presentation of the method A2 for agarose gel shaping. 64
Figure 23. Application of method A2 for agarose gel shaping. 65
Figure 24. Schematic presentation of method B for agarose gel shaping. 66
Figure 25. Schematic presentation of method C for agarose gel shaping. 66
Figure 26. Apparatus used to asses agarose integrity: test tube rotator with 45 mL PBS pH 7.4 and agarose gel shape using method A2. 68
Figure 27. Schematic structure of the HPLC equipment, presenting the detector, the pump, the injector, the column, and the computer/software (from [165]). 70
Figure 28. Representation of the separation of two compounds, Green and Blue, between the mobile phase and stationary phase in the HPLC method, focusing in the column 71
Figure 29. Schematic diagram for a photodiode-array (PDA) UV detector (from [167]). 73

Figure 30. Graph of concentration of mobile phases A and B as a function of time (minutes) for the method of assay, with chromatogram at WVL-1 and WVL-2 for injection of DS A and DS B at 100%. 74
Figure 31. Graph of concentration of mobile phases A and B as function of time (minutes) for the method for IVR test, with chromatogram at WVL-1 and WVL-2 for injection of DS A and DS B at 100%. 75
Figure 32. Chromatogram at WVL-1 for injection of PBS pH=7.4. 76
Figure 33. Chromatogram at WVL-2 for injection of PBS pH=7.4. 77
Figure 34. Chromatogram at WVL-1 for injection of placebo. 77
Figure 35. Chromatogram at WVL-2 for injection of placebo. 77
Figure 36. Chromatogram at WVL-1 for injection of DS A and DS B at 5%. 78
Figure 37. Chromatogram at WVL-2 for injection of DS A and DS B at 5%. 78
Figure 38. Chromatogram at WVL-1 for injection of DS A and DS B at 100%. 78
Figure 39. Chromatogram at WVL-2 for injection of DS A and DS B at 100%. 79
Figure 40. Linear regression for DS A. 82
Figure 41. Linear regression for DS B. 83

Tables index

Table 1. Validation characteristics of each analytical procedures that are validated (from [87]). 31
Table 2. Summary of specific tests for parenteral formulations according to ICH Topic Q6A – *Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Chemical Substances* definition and method. 32
Table 3. Advantages and disadvantages of IVR methods. 45
Table 4. Advantages and disadvantages of the dialysis methods. 47
Table 5. Advantages and disadvantages of the reverse dialysis methods. 48
Table 6. Mechanisms and disadvantages of some parameters that can provide accelerated IVR test. 55
Table 7. Parameters and rationale of IVR test for BlueLAI. 58
Table 8. Results of pH measurement of PBS pH 7.4 (of the three prepared batches) for 4 days of study, stored at Room temperature (RT) and in the incubator at 37ºC. 59
Table 9. Results of osmolality measurement of PBS pH 7.4 (of the three prepared batches) for 4 days of study, stored at Room temperature (RT) and in the incubator at 37ºC. 61
Table 10. Measures of the well-plate and the cavity formed by the cylinder to put the LAI formulation. 63
Table 11. Advantages and disadvantages of the well-plate used to shape the agarose gel 67
Table 12. Types of chromatography, mobile and stationary phases characteristics. 69
Table 13. Normal phase and reverse phase chromatography characteristics. 71
Table 14. System precision results for DS A 100% standard solution. 80
Table 15. System precision results for DS B 100% standard solution. 80
Table 16. System precision results evaluation. 80
Table 17. Linearity results for DS A. 82
Table 18. Linearity results for DS B. 83
Table 19. Linearity results evaluation for DS A and DS B. 83
Table 20. Stability test results for DS A in standard solution with both DS at 100% in vials kept inside the autosampler at 15ºC. 84
Table 21. Stability test results for DS B in a standard solution with both DS at 100% in vials kept inside the autosampler at 15ºC. 85
Table 22. Stability test results for DS A in a standard solution with both DS at 100% at laboratory bench at room temperature. 85
Table 23. Stability test results for DS B in a standard solution with both DS at 100% at laboratory bench at room temperature. 85
Table 24. Results evaluation for DS A in a standard solution in vials kept inside the autosampler at 15ºC. 86
Table 25. Results evaluation for DS B in a standard solution in vials kept inside the autosampler at 15ºC. 86
Table 26. Results evaluation for DS A in a standard solution at the laboratory bench at room temperature. 86
Table 27. Results evaluation for DS B in a standard solution at the laboratory bench at room temperature. 86

1. Introduction

Within the scope of the master degree in Bioquímica Clínica, I attended an internship at Bluepharma Indústria Farmacêutica, S.A. from September 2020 to June 2021. This internship was in Research and Innovation department. There were two main objectives in this internship:

- Carry out systematic research work on the methodologies used to characterize long-acting injectables as well as evaluate the *in vitro* release profile of these formulations;
- Support the in-house development of the most appropriate methodology for assessing the prototype release profile of LAI technology developed internally (named throughout this report as BlueLAI technology).

This report intends to describe the work developed during the internship, both the review about LAI and their characterization and *in vitro* release of BlueLAI technology. First, an overview of long-acting injectables, their characterization methods, including *in vitro* release methods, membranes, and the use of biorelevant parameters is presented. Second, a description of developed activities is exposed, presenting the choice of the method, the development of the method for quantification of the drug substance (DS), and the optimization that may be required obtain better results.

1.1. About Bluepharma Indústria Farmacêutica, S.A.

This internship was accomplished at Bluepharma Indústria Farmacêutica, S.A., which is a pharmaceutical company founded in 2001, and based in São Martinho do Bispo, Coimbra.

The Bluepharma Group has 20 companies and employs more than 700 employees. It is present in 4 countries (Spain, Angola, Mozambique and USA) and exported, in 2019, 88% of its production, mainly generic pharmaceutical products, to more than 40 countries.

Bluepharma's activities are divided into 3 distinct areas: producing pharmaceutical drugs for Bluepharma and other companies; research, development, and registration of pharmaceutical drugs and marketing of generic pharmaceuticals.

Throughout its 20 years, Bluepharma achieved several certifications of quality, ISO Norms 9001, environmental, ISO 14001 and occupational health and safety, OHSAS 18001. It is also certified in Good Manufacturing Practices (GMP) by European Medicines Agency (EMA), Food and Drug Administration (FDA) and ANVISA Agência Nacional de Vigilância Sanitária (2016).

2. State of the art

To better understand what are long-acting injectables (LAI), a classification of LAI as pharmaceutical dosage forms according to route of administration and release system provided by regulatory authorities is presented.

Contextualization on regulatory authorities

Pharmaceutical dosage forms can be classified according to the route of administration and release system, as showed in **Figure 1**.

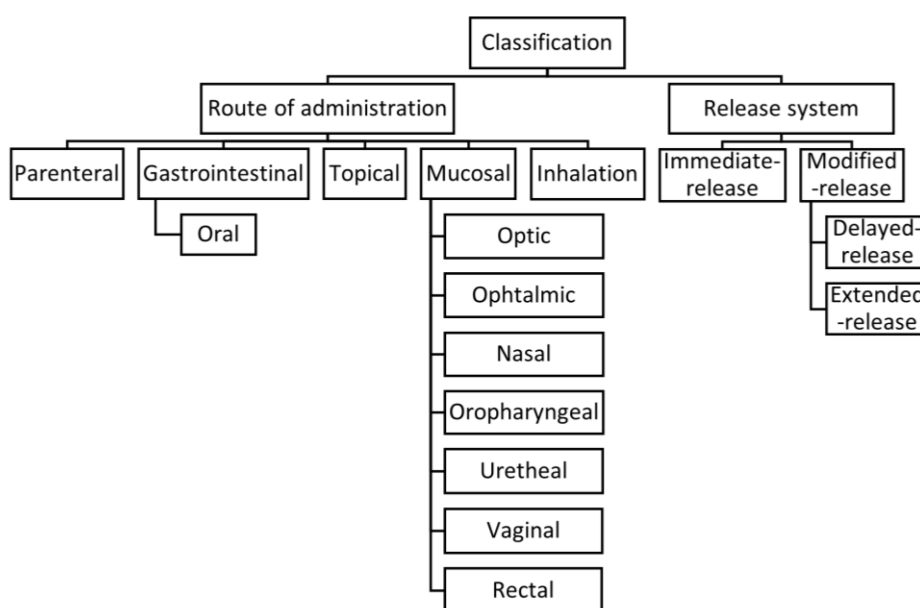

Figure 1. Different types of classification of pharmaceutical dosage forms according to USP, 2020.

According to the United States Pharmacopeia (USP), the major routes of drug administration are parenteral, gastrointestinal, topical, mucosal and inhalation [1].

- Parenteral route includes injections through the skin, or other external boundary tissue, or implanted drug products. These routes allow the direct administration of drug substance (DS) into blood vessels, organs, tissues, or lesions, ensuring complete bioavailability [2].
- The gastrointestinal route is orally administrated and lead to systemic and/or local effect in the oral cavity and/or gastrointestinal tract [3].
- The topical route can be applied to achieve systemic action when absorbed through the skin into the blood circulation, or local action, which can occur on the surface of the application site (e.g., stratum corneum), in the underlying tissues (e.g., epidermis and/or dermis) or subcutaneous tissues (e.g., muscle or joint) [4].

- The mucosal route of drug administration can be used to local action or systemic absorption and is subdivided into seven membrane surfaces: optic, ophthalmic, nasal, oropharyngeal, urethral, vaginal and rectal [5].
- In the inhalation route, the drug is delivered into the lungs by oral inhalation, using aerosols, powders, sprays, solutions, suspensions, or solutions [6].

Relatively to the release system, the United States Pharmacopeia (USP) and European Pharmacopoeia (Ph. Eur.) distinguish two types [1], [7]:

- Immediate-release when the drug substance release profile has not been modified;
- Modified-release when the onset, rate, and/or place of release of the active substance(s) are changed from the conventional drug product with the same route of administration. Within modified-releasing, there are two different types:
    - Delayed-release when DS releasing is delayed for some time after administration.
    - Extended-release when a slower release is achieved of the DS compared to a conventional release dosage form administered by the same route.

USP defines prolonged-release, repeat-action, controlled-release, long-acting and sustained-release as synonymous, but only extended-release should be used in official documents [1].

Historical contextualization of Long-Acting Injectables

Between 1950 and 1980 emerged the first generation of drug delivery systems, the basics of extended-release, which focus was developing oral and transdermal extended release systems and understanding drug release mechanisms. Later, in 1980-2010, the second generation appeared with “smart” delivery systems, including polymers and hydrogels environment-sensitive and self-regulated released, zero-order release, biodegradable depot, and the latter part, nanoparticles. Long-acting injectables are second-generation systems. Third-generation systems started in 2010 and onward, are characterized by modulated delivery systems [8].

Patient non-adherence with treatment and/or non-compliance with dosage regimen have a direct impact on the efficacy of therapies of several diseases. Some patients refuse or are unable to take their medicines, like mental patients. Antipsychotics emerged in the 1950s, but patients non-adherence and consequent release led to the development of LAI as antipsychotic by G. R. Daniels. In 1966, began LAI-fluphenazine enanthate and, 18 months later, LAI-fluphenazine decanoate, corresponding to first generation formulations due to its dissolution system [9]. The first proof of effectiveness of antipsychotics LAI just appears in 1971, by J. Denham and L.

Adamson [10]. In another medical field, the difficulty of remembering to take daily pills and the need for discrete methods, because of the stigma related to birth control, led to the start of LAI-contraception clinical studies in 1963 [11]. Along with these, other LAI appeared due to the necessity of long-term therapy in chronic diseases, like acromegaly.

2.1. Long-acting injectables

Overall, non-compliance with drugs can compromise successful treatment outcomes in several diseases, especially in chronic ones. Long-acting injectables (LAI) appeared under this context as extended-release formulations, whose route of administration is parenteral, usually intramuscular or subcutaneous. However, no clear definition for LAI is provided by the regulatory agencies European Medicines Agency (EMA) and Food and Drug Administration (FDA).

2.2.1. Parenteral drug delivery systems

Parenteral drug delivery is divided into injectables, implants, and infusion devices. Injections can be solutions, colloidal dispersions (liposomes, niosomes, polymeric micelles or nanoparticles), or microparticles (microspheres or microcapsules). On the other hand, implants are either solid or *in situ* depot forming [12]. **Figure 2** shows the parenteral drug delivery division.

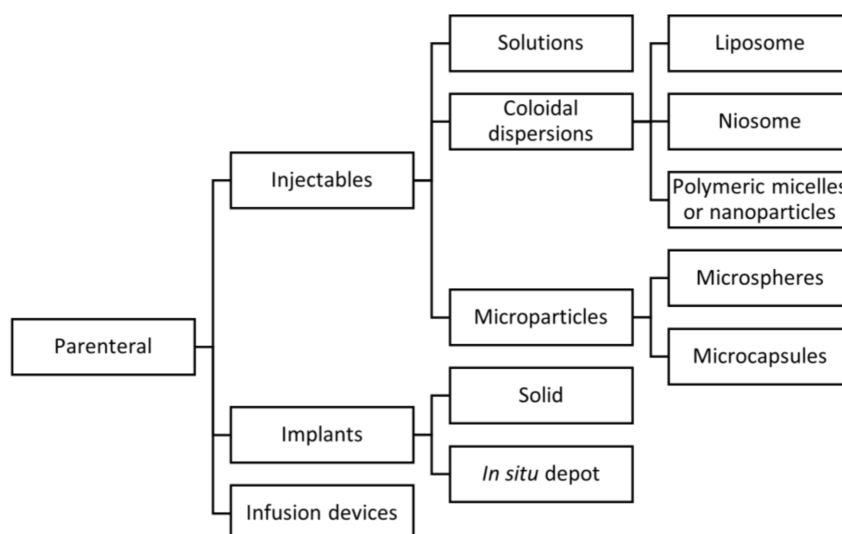

Figure 2. Parenteral drug delivery division.

2.2.2. Major routes of parenteral administration for long-acting injectables

As describe, LAI are parenterally administered. When systemic action is intended normally is used subcutaneous (SC) or intramuscular (IM) routes, but is also possible to use the intravenous route [13], [14]. These routes are represented in **Figure 3**.

- Intramuscular (IM): when the drug is injected into the muscle tissue, in the upper part of the arm, glutes or external thigh face [15]–[17]. These locations admit about 2ml, 7-8 ml and 5mL, respectively [15].
- Subcutaneous (SC): also called hypodermic administration, consists of injecting drugs under the skin into the adipose layer below the dermis. This route usually allows smaller injection volumes than IM route [15].
- Intravenous (IV): when a drug solution is introduced directly into a vein. This is the most rapid method of achieving therapeutic effect once the drug enters directly into systemic circulation without the delay related to absorption. IV injections supports volumes up to 10 ml [15].

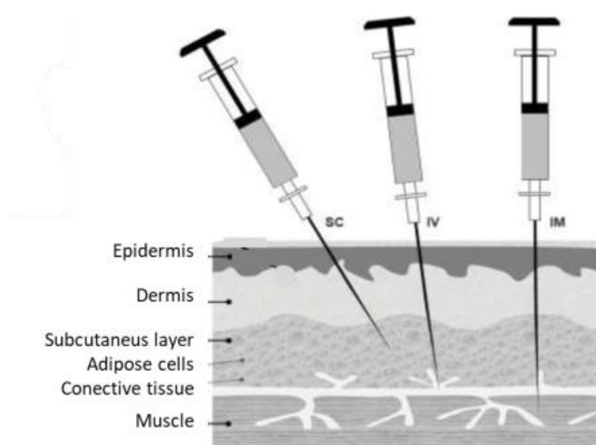

Figure 3. Routes of parenteral administration of drugs. Legend: SC: subcutaneous; IV: intravenous; IM: intramuscular (Adapted from [15])

When local treatment is wanted, intraarticular or intraocular injections can also be used. Intraarticular consists of direct administration of the drug on a joint. Intraocular injection inserts drug directly into the vitreous cavity, in vitreous humor gel [13], [17].

2.2.3. Long-acting injectables formulations

There are several strategies to formulate a controlled release injectable product, as LAI (Figure 4) that will be approached in this sub-chapter.

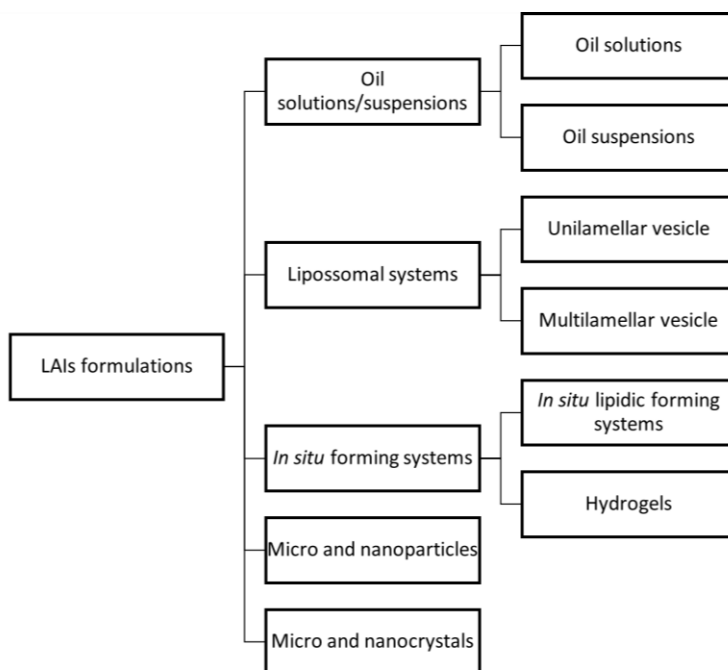

Figure 4. Strategies to formulate a controlled release injectable product as LAI formulations.

2.2.3.1. Oil solutions or oil suspensions

In oil-based LAIs the drug is dissolved in an oil carrier either as suspensions or as a solution but only lipophilic drugs (or hydrophilic drugs modified) can be used. There are some pharmaceutical oils available like castor, mineral, safflower, and soybean oil allowed by USP [18].

The rate-limiting step for drug absorption is drug-releasing in the oil solvent, whereby manipulating transport group for prodrug design and/or vehicle composition (like oil viscosity) could prolong the absorption process [19].

Fluphenazine decanoate is a LAI solution in sesame oil used in patients requiring prolonged parenteral neuroleptic therapy, such as schizophrenics [20]. It was approved in 1987 by the FDA. This LAI formulation presents a half-life of approximately 7–14 days and maximal plasma levels within 24 h of intramuscular injection [21].

Posimir® is a bupivacaine solution indicated in adults for administration into the subacromial space under direct arthroscopic visualization to produce post-surgical analgesia for up to 72 hours following arthroscopic subacromial decompression. It was approved in 1972 by the FDA. However, safety and effectiveness have not been established in other surgical procedures, including soft tissue surgical procedures, other orthopedic procedures, including for intra-articular administration, and bone procedures, or when used for neuraxial or peripheral nerve blockade [22].

2.2.3.2. Liposomal Systems

Liposomes are first described by hematologist Alec D Banghamin 60s [23]. They can have a single lipid bilayer, named as unilamellar vesicle, or an onion-like multilayered structure, designed as multilamellar vesicle (MLV). Unilamellar vesicles can also be classified into large unilamellar vesicles (LUV) and small unilamellar vesicles (SUV) [24]. This structure can entrap lipophilic drugs in the lipid membrane and hydrophilic agents in the aqueous core. Liposomes have low water solubility [2].

Some studies show that liposomal encapsulation of drugs leads to less toxicity. For example, doxorubicin used in metastatic breast cancer treatment presents cardiotoxicity. Liposomal doxorubicin HCl (commercial name Caelyx™ in Europe and Doxil® in USA) provides comparable efficacy to conventional doxorubicin, with significantly reduced cardiotoxicity, myelosuppression, vomiting, and alopecia, in a phase III trial [25]. Doxil® was approved in 1995 by FDA and Caelyx™ was then approved by EMA in 2005.

As another example, liposomal long-acting bupivacaine (Exparel®) is used for postsurgical local analgesia and as an interscalene brachial plexus nerve block to produce postsurgical regional analgesia, since its approval by the FDA in 2011 and by EMA in 2020. It is administered as a single dose that provides 96 hours effect after local infiltration or 120 hours after interscalene brachial plexus nerve block [26]. Hadzic *et al*. showed that Exparel reduces cumulative pain intensity scores and opioid consumption through 72 hours postsurgery compared with placebo, in patients undergoing a femoral nerve block [27].

2.2.3.3. *In situ* forming systems

The *in situ* forming systems are liquid formulations that generates solid or semisolid depot after administration into specific therapeutic targets [2].

- ***In situ* lipidic forming systems**

Vesicular phospholipid gels (VPGs) consist on highly concentrated liposomal dispersions where the liposomes construct a three-dimensional network. VPGs encapsulate macromolecular hydrophilic drugs, inside the vesicles or lipophilic or amphiphilic between the vesicles [28], [29]. Zhang *et al*. uses VPGs exenatide (an incretin mimetic for the treatment of type II diabetes) as a LAI and achieved extended release of exenatide for over 10 days and consequent hypoglycemic effect, without significant initial burst [29].

Another example is an injectable phospholipid-based phase transition gel (PPTG) for prolonging the release of ropivacaine for local anesthesia that was prepared by mixing phospholipids,

medium-chain triglyceride and ethanol. After subcutaneous injection, the liquid formulation rapidly forms an *in situ* gel. Li *et al*. showed that this depot may prolong analgesia, helping in the control of post-surgical pain, without causing systemic toxicity [30].

FluidCrystal is a technology that allows depot injection. This is an oily liquid solution in absence of water that, by absorbing interstitial aqueous body fluid, triggers gelification and allow a prolonged release of DS [31]. The use of FluidCrystal with some DS is being evaluated, such as Buprenorphine (for chronic pain) and octreotide (for acromegaly and neuroendocrine tumors) that are currently in Phase 3 studies [32]. FluidCrystal technology is also used in Buvidal products that are extended-release solutions for injection with buprenorphine, commonly used in the treatment of opioid dependence. These products were approved by EMA in 2018. Therapeutic levels are maintained over 1 week or 1 month after one subcutaneous injection [33].

- **Hydrogels/*In situ* polymer-based systems**

Hydrogels are a macromolecular three-dimensional network of polymers, which is hydrophilic and capable of absorbing a big quantity of water or biological fluids. There are two types of cross-linking on polymers: chemical, by covalent bonds or physical, by physical association between polymeric chains or nanoparticles. These two cross-linking types can co-exist in a single hydrogel [34].

When injected, the organic solvent disperses into tissue and, at the same time, water penetrates, inducing phase separation and precipitation of the polymer which creates a depot at the injection site. Aqueous body fluid penetrates the organic phase and slowly releases the drug entrapped in the gel. The drug release happens as polymers degradation occurs [35].

There are also some stimuli-sensitive hydrogels, in which solidification occurs because of an environmental change. For example, low pH or elevated temperature can be used to control the specific site where the drug is delivered. Hydrogels can be responsive to temperature, pH, electric signal, light, pressure, specific molecules, such as glucose or antigens, or ions, or thrombin-induced infection [36].
Antibody and antigen interactions are highly specific, whereby using this interaction could be advantageous in immunoassays and biosensor technology of hydrogels. Lu *et al*. created an antigen-responsive hydrogel containing antibody Fab’ fragments incorporated into the hydrogel matrix, which have efficient tumor targeting *in vivo*. Significant reversible volume changes occur when the hydrogel is exposed to antigens fluorescein (FL) and polyamidoamine dendrimer (PAMAM)-fluorescein (FD) [37].

2.2.3.4. Polymeric nanoparticles and microparticles

Nano/microparticles are composed of a matrix with proteins or synthetic, usually biodegradable, polymers in which DS is incorporated. These devices can encapsulate many types of drugs, including small molecules, proteins and nucleic acids. When the particles have a spherical form, they are designated as nano/microspheres, depending on the size in the nano/micro range [38], [39]. The most commonly polymers used in nano/microparticles include polyethylene glycol (PEG), poly(lactic acid) (PLA), poly(lactic-co-glycolic acid) (PLGA), poly(ε-caprolactone) (PCL), alginate, chitosan, and gelatin base [39].

For example, Risperdal® Consta® is an atypical antipsychotic agent indicated for the treatment of schizophrenia, approved by the FDA in 2003. This formulation consists of risperidone encapsulated on microspheres of polylactide-co-glycolide (PLG) that are suspended in a diluent before injection. It is administrated by deep IM deltoid or gluteal injection every 2 weeks [40].

The particles can be in solution or associated with an *in situ* forming system. Eligard® is a polymeric nanoparticle formulation of leuprolide acetate that is allied to Atrigel®, a polymeric biodegradable delivery system with PLGA for SC injection. This drug product, approved in 2002 by the FDA, is used in the palliative treatment of advanced prostate cancer [41].

In the FY2016 Regulatory Science Report about Long-Acting Injectable Formulations, FDA refers to LAIs as formulations that include biodegradable injectable microspheres and *in situ* geling implants [42].

2.2.3.5. Nanocrystals and microcrystals

Nano/microcrystals formulations are composed of mainly hydrophobic drugs with a small amount of excipient or surfactant. They are dispersed in a buffered aqueous vehicle and stabilizing excipients (to avoid particle agglomeration) and form nano/microsuspensions. In the suspension, the rate-limiting step for drug absorption is the speed for drug particle dissolution in the formulation or in body fluid, whereby factors like particle size and aggregation should be analyzed [39], [43].

Xu *et al*. are using acid-sensitive stearoxyl-ketal-dexamethasone microcrystals for long-acting intraarticular injection on chronic arthritis (inflammatory joints pH is acid), which prove to be effective in rats [44].

A microcrystals-based LAI formulation of iloperidone was patented in 2011 by Vanda Pharmaceuticals. Iloperidone is a second generation antipsychotic used for the treatment of

schizophrenia. This product is still in development and is currently ongoing a pharmacokinetic study in preparation for a Phase III program [45], [46].

2.2.4. Long-acting injectables drug release mechanisms

LAI can be classified into four categories based on the mechanism involved in the controlled release: dissolution controlled release, esterification, encapsulation, and adsorption. One or a combination of those mechanisms can be used to develop a LAI formulation [12], [47], [48].

2.2.4.1. Dissolution controlled release

In this method, drug absorption is controlled by the slow dissolution of drug particles within the formulation. The rate-limiting step in this mechanism is the dissolution of the drug particle [12], [47], [48]. Two approaches can be used. Salts with poor aqueous solubility or insoluble complexes will dissociate after injection and provide an extended-release profile. Suspension of macrocrystals can also be used to control drug dissolution, once macrocrystals dissolve very slowly compared with small crystals because the surface area of drug particles is directly proportional to dissolution [12], [47], [48].

2.2.4.2. Esterification

In this type of LAI, a drug is esterified to form a prodrug that will form a reservoir when injected. The number of hydrolytic enzyme (esterase) at the injection site can be used to slow release of the drug. The rate of drug absorption is controlled by the cross of drug esters from the reservoir to the tissue fluid and the rate of bioconversion of drug esters (prodrug) into active drug molecules, whereby rate-limiting step is the number of hydrolysing enzymes [12], [47], [48].

2.2.4.3. Encapsulation

In this method, mainly used for small drug molecules and proteins, a drug is encapsulated within a permeation barrier or disperses in a diffusion matrix. The release of the drug molecule is controlled by the rate of diffusion across the permeation barrier and the rate of biodegradation of the barrier macromolecules. Both permeation barrier and diffusion matrix are fabricated from biodegradable or bioabsorbable macromolecules, such as gelatin, dextran, polylacticacid, lactide-glycolide copolymers, phospholipids, and long-chain fatty acids and glycerides. The rate-limiting step in this mechanism is the permeability across permeation barrier [12], [47], [48].

2.2.4.4. Adsorption

In this mechanism, drug molecules are bonded to adsorbents. In this case only the unbound, free drug molecules can be absorbed. When unbound drug molecules are absorbed and a fraction of the bound drug molecules is released the equilibrium is established, which means

the rate-limiting step is the drug absorption rate. This depot preparation is used in vaccines in which the antigens are bound to highly dispersed aluminium hydroxide gel to sustain their release and hence prolong stimulation of antibody formation [12], [47], [48].

2.2.5. Long-acting injectables advantages and disadvantages

LAI formulations have been a growing presence in the pharmaceutical industry due to several advantages that make them so appealing (Figure 5). As presented below, LAIs can:

- Provide slow and continuous DS release, reducing drug dosing and, consequently, increasing patient compliance and adherence to treatment [49]–[51];
- Be useful for patients that are unable to adhere to treatment, such as those suffering from mental disorders [13], [51];
- Enhance the bioavailability, because limits the first-pass metabolism and increased compliance; this is specially advantageous in low bioavailability drugs [52], [53];
- Prevent accidental use of medicines due to the need for specialized professionals [12];
- Maintain steady plasma drug concentration for a longer period, with less and lower fluctuations, increasing safety margins for highly potent drugs with a narrow therapeutic index [12], [54], [55]. [39], [43].

C. Leucht *et al*. meta-analysis showed LAI antipsychotics significantly reduced relapse when compared with oral formulations [56]. However, this is not consensual. T. Kishimoto *et al* defend set of LAI antipsychotic did not differ from oral formulation, but LAI-fluphenazine isolated present superiority unlike LAI-olanzapine show inferiority [57]. In other study, H. G. Roozen suggests that enhanced compliance related to LAI-naltrexone compared with oral pill results in better treatment effectiveness [58]. In summary, LAI appear to obtain better outcomes but more studies are needed.

Moreover, Wei suggests that low tissue distribution and non-accumulation in main tissues in LAI-curcumin didecanoate prodrug could avoid organ toxicity [59].

LAI also have some disadvantages (**Figure 5**), that can lead doctors to be reluctant to indicate their use or patients not willing to accept. As presented below, LAIs:

- Can induce pain and erythema at the site of injection [12], [60];
- Require health care experts for administration [16];
- Have the possibility of subtherapeutic action owing to prolonged lower drug concentrations [12];

- Need a long time to achieve steady state levels, which can be problematic in acutely ill individuals [60];
- Can lead to dose dumping/burst release, that is, very high initial release of DS;
- Present a high cost due to the use of polymers, or other specific excipients, on manufacturing [12].

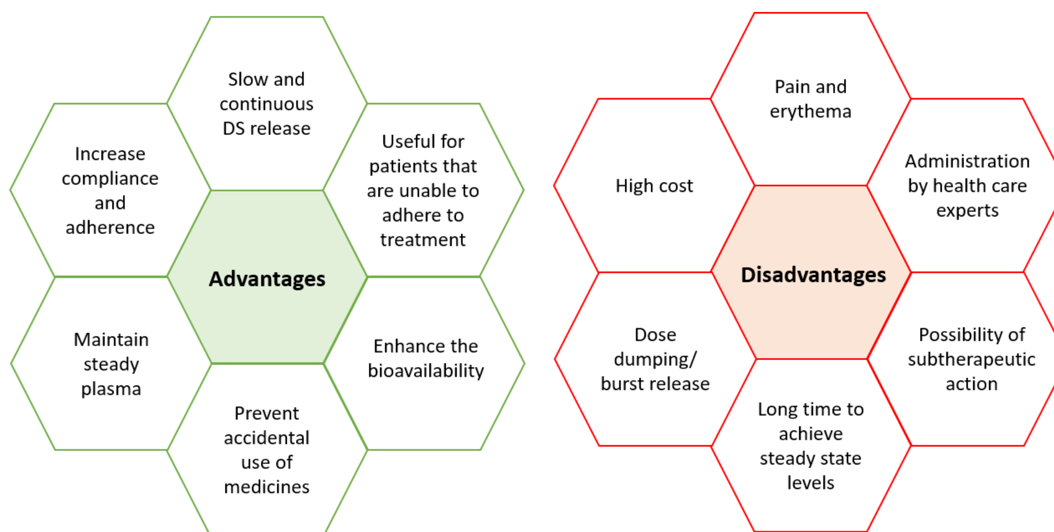

Figure 5. Advantages and disadvantages of long-acting injectables.

2.4.6. Therapeutic indications of long-acting injectables

Non-compliance with drugs can compromise successful treatment outcomes in several diseases. Thus, the promotion of patient adhesion and compliance can be quite useful in several diseases, like chronic diseases when patients need long-term treatment and non-cooperant patients. LAI can also decrease the occurrence of adverse events by controlling the drug plasma concentrations within the therapeutic range. These characteristics lead to the development of LAI formulations in several therapeutic indications as presented below.

2.4.6.1. Mental disorders

Bipolar Disorder (BD) is characterized by manic, hypomanic, or mixed episodes, and alternating episodes of depression. Schizoaffective disorder (SAD) consists of symptoms of schizophrenia and affective disorders (depression and/or mania) simultaneously. Nonadherence in BD and SAD patient's bipolar type is estimated between 10% and 60% even during euthymic periods which increases the risk of relapse and suicide as well as the risk of rehospitalization. LAI antipsychotics improve treatment adherence in patients with psychiatric illness requiring long-term treatment [61]. In schizophrenia, LAI can enhance adherence, improve treatment outcomes and quality of life, and help prevent a relapse [60].

2.4.6.2. Hormonal Therapy

2.4.6.2.1. Hormonal contraception

Long-acting hormonal contraception includes subdermal implants and intrauterine devices as well as long-acting reversible contraceptives (LARCs). LARCs currently on market can provide 1 to 3 months of contraception and are injected via IM or SC [62]. LARCs minimize problems with compliance and are very effective [50]. LARS formulations include microcrystal suspension, prodrug obtained via esterification, drug-loaded microsphere suspension, implants, and *in situ* forming depots [63]. Some LAI contraceptives that could provide contraception for 6 months are currently being developed [64].

2.4.6.2.2. Growth hormones

Growth hormone (GH) is used to treat GH deficiency (GHD), chronic kidney disease, Turner syndrome, AIDS wasting, Prader-Willi syndrome, small for gestational age, idiopathic short stature, small bowel syndrome, SHOX deletion, and Noonan syndrome [65]. Cutfield *et al*. showed that GHD patients are frequently non-compliance and obtain a significantly worst growth in this situation [66]. Several LAI-GH formulations are in clinical development, allowing better compliance and consequently better results. Y. Yang *et al*. meta-analysis showed LAI-GH significantly improved height velocity and IGF-1 serum values [65].

2.4.6.2.3. Acromegaly

Acromegaly is a rare condition that need long-term treatment to reduce the higher morbidity and mortality rates [67], [68]. Long-acting somatostatin analogue is used since the 1980s [67]. Due to diverse adverse effects and no effect on some patients, other drugs were developed, like LAI-pasireotide, a somatostatin multireceptor ligand that presents more biochemical disease control than octreotide LAR, a first-generation somatostatin receptor ligand [68].

2.4.6.2.4. Prostate cancer

Androgen deprivation therapy is used as an alternative to prostatectomy and consists of using LHRH (luteinizing hormone-releasing hormone) and GnRH (gonadotropin-releasing hormone) to block the release of natural LHRH, and consequently, avoiding testosterone releasing [69]. Long-acting GnRH agonists triptorelin and leuprolide are used since the 1990s and favorable tumor response (no progression) occurred in the majority of patients [70], [71]. Monthly LAI new formulations of leuprorelin (such as microspheres) continue to be used and studied [69].

2.4.6.3. Substance abuse

Substance abuse and dependence, especially on opioids and alcohol, are key health problems all around the world. Several pharmacological treatments can be used, but conventional agents have a relatively short duration of action and non-adherence to daily oral pharmacotherapy is frequent, which can lead to relapse [72], [73]. LAI-naltrexone, IM injection with 1 month release, showed reduction in heavy drinking in alcohol-dependent patients during 6 months of therapy [73]. Another study suggests that patients who were given naltrexone depot (1 month release) are more likely to abstain from using opioids and other drugs of abuse, with the possible exception of cocaine and cannabinoids [74]. Buprenorphine depot was also demonstrated to provide effective relief from opioid withdrawal [75].

2.4.6.4. Human Immunodeficiency Virus

Non-adherence or non-compliance with antiretrovirals complicates the treatment regimen and increases Human Immunodeficiency Virus (HIV)/Acquired Immunodeficiency Syndrome (AIDS) mortality. One recent trial showed the successful treatment of HIV-1 infection with monthly injections of LAI-cabotegravir and rilpivirine as an alternative to daily oral treatment. Participants who received the long-acting therapy reported greater satisfaction and preferred the regimen over previous oral therapy. Moreover, the frequency of serious adverse events was similar in the two treatment groups [76].

Pre-exposure prophyalxis is used for HIV prevention and is highly effective. LAI can provide 2 to 3 months dosing intervals, instead of daily oral medication currently available [14], [77]. Some clinical trials for LAI-rilpivirine (non-nucleoside reverse transcriptase inhibitor) are being developed to improve dosing frequency, lower injection volumes, and higher tissue distribution. J. Hilaire *et al.* develop nanoformulated rilpivirine prodrug that increases the half-life and improves tissue biodistribution [77]. Long-acting IM cabotegravir (HIV-1 integrase strand transfer inhibitor) obtained safety and pharmacokinetic positive results. Efficacy studies for HIV treatment and prevention are in progress [78].

In October 2020, EMA has recommended marketing authorizations for two LAI antiretroviral, Rekambys (rilpivirine) and Vocabria (cabotegravir), to be used together for the treatment of patients with HIV type 1 (HIV-1) infection [79].

2.2. Long-acting injectables characterization – quality assessment

The characterization techniques are used to determine the various physicochemical properties of a drug product and ensure consistency in formulation processing and performance after

commercialization. To control quality, safety and efficacy, International Council for Harmonisation (ICH) defines some guidelines that need to be fulfilled. According to ICH, specifications are a set of tests, references to analytical procedures, and acceptance criteria which can be numerical limits, ranges or other. These tests allow establishing standards to which a drug product is considered acceptable for its proposed use. Specifications are proposed and justified by the manufacturer and approved by regulatory authorities [80].

According to ICH Q6A – *Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Chemical Substances*, two types of tests can be considered (**Figure 6**) [80]:

- Universal test, when applies to all drug substances or drug products; e.g., appearance, identification, assay, and impurity tests;
- Specific test, when applies to particular drug substances or drug products depending on their specific properties and/or proposed use.

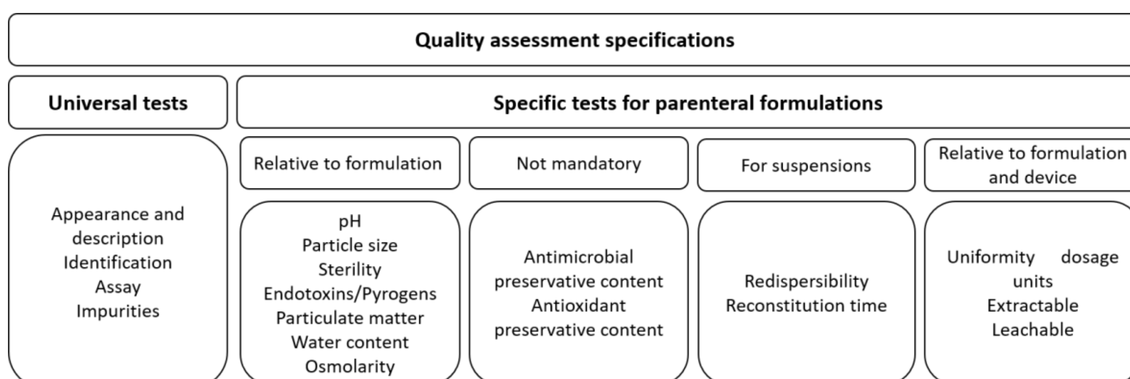

Figure 6. Universal and specific tests for parenteral formulations according to ICH Topic Q6A – *Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Chemical Substances*.

Universal and specific tests for parenteral formulations will be discussed. However, different LAIs formulations could need some tests not included, whereby in the next chapter microparticles and *in situ* formation characterization examples will be presented.

2.2.1. Universal tests

1) Appearance and description

Appearance is a qualitative test where is described the physical state (e.g., solid, liquid), color, size, and shape of a drug product, by visual inspection. The acceptance criteria should include the final standard appearance [80], [81].

UPS ⟨1790⟩ provides a visual inspection guide for injections, including container integrity defects such as cracks, misplaced stoppers, or incomplete seals [82]. Eur. Pharm. refers to containers for

parenteral formulations that should be made whenever possible from materials sufficiently transparent to permit the visual inspection of the contents [83]. The analysis of visible particulates in injections is discussed in USP ⟨790⟩ and Eur. Pharm. 2.9.20 [84], [85].

2) Identification

Identification test should be able to identify the DS(s) in the formulation, as well as discriminate between composites with a similar structure that could be present. This test should be specific for the DS [80].

According to ICH Q2 – Validation of Analytical Procedures: Text and Methodology, some tests need to be validated, including the identification [86]. The validation characteristics presented by ICH Q2 are accuracy, precision (repeatability and intermediate precision), specificity, detection limit, quantitation limit, linearity, and range. The main objective of validation is to demonstrate that the procedure is suitable for its intended purpose [86].

The validation of identification is done for its specificity. The specificity ensures that the presence of DS is explicitly assessed between other substances which may be present, including impurities, degradants, matrix, etc. When is not possible to validate that an analytical procedure is specific for a particular analyte, a combination of two or more analytical procedures should be performed to achieve complete discrimination [86]. Two chromatographic procedures with different separation principles or a combination of tests into a single procedure, such as HPLC/UV diode array, HPLC/MS, or GC/MS, are generally acceptable [80].

Spectroscopic tests, like infrared, near-infrared, and Raman spectroscopy, are presented in USP as identification tests currently performed [87].

3) Assay

Assay is a quantitative test that indicates the strength/content of the DS(s) in the drug product. Sometimes the same procedure can be used to assay and impurities test, for example, high-performance liquid chromatography (HPLC). Content uniformity test can also be done with assay if the method is adequate for both analyses [80].

The analytical procedure used in the assay needs to be validated according to ICH Q2 for accuracy, precision (both repeatability and intermediate precision), specificity, linearity, and range [86]. Each validation characteristic is explained below, as presented in ICH Q2 [86]:

- The accuracy, also called trueness, expresses the closeness of agreement between the conventional true value or an accepted reference value and the experimental value.

- The precision expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample. Precision can be evaluated in three points: repeatability, intermediate precision, and reproducibility. The precision can be expressed as the variance, standard deviation, or coefficient of variation of a succession of measurements.
    - Repeatability, also called intra-assay precision, expresses the precision under the same operating conditions over a short interval of time.
    - Intermediate precision expresses within-laboratories variations, like different days, different analysts, different equipment, etc.
- Specificity is the capacity to evaluate clearly the analyte regardless of the presence of other substances, such as impurities, degradants, matrix, etc. When associated with assay, specificity aims to provide an exact result that allows an accurate statement about the content or potency of the analyte in a sample. When a non-specific assay is used, other analytical procedures should be used to demonstrate specificity.
- The linearity is the capacity (within a given range) to achieve results directly proportional to the concentration of analyte in the sample. If there is a linear relationship, the results should be evaluated by appropriate statistical methods, and correlation coefficient, y-intercept, slope of the regression line, and residual sum of squares should be presented. To establish linearity, a minimum of 5 concentrations is recommended.
- The range is the interval between the upper and lower concentration (including concentrations themselves) of the analyte for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy, and linearity. The range is normally derived from linearity studies. For assay, the range is normally from 80 to 120% of the test concentration.

4) Impurities

According to ICH Q6A, impurity is any component of the drug product that is not the chemical entity defined as the DS or an excipient. Degradation products, both organic and inorganic, and residual solvents can be considered impurities. Manufacturing process derivatives impurities also need to be measured. Acceptance limits should be defined for each degradation product, including identified (that is, an impurity with a defined structural characterization) and unidentified (that is, an impurity defined by qualitative analytical properties only), and total degradation products [80].

According to ICH Q3B – Impurities in New Drug Products, the analytical procedures need to be validated and suitable for the detection and quantitation of impurities, using as guidance the ICH Q2 [88]. The impurities test includes both quantitative test and limits test and should accurately reflect the purity characteristics of the sample. Quantitative test and limit test have different validation characteristics. In a quantitative test accuracy, precision (both repeatability intermediate and precision), specificity, quantitation limit, linearity, and range are the evaluated characteristics. On the other hand, in a limit test specificity and detection limit are required as validation characteristics [86]. The validation characteristics not exposed in the assay are defined below, as presented in ICH Q2 [86]:

- When associated with impurities, specificity aims to guarantee that the analytical procedures performed to allow an accurate evaluation of the content of impurities of an analyte, i.e. related substances test, heavy metals, residual solvents content, etc.
- The quantitation limit is the lowest quantity of analyte in a sample which can be quantitatively determined with suitable precision and accuracy.
- The detection limit is the lowest quantity of analyte in a sample which can be detected but not necessarily quantitated as an exact value.
- In the impurities assessment, the range should be from the reporting level of impurity to 120% of the specification.

When performed in a microsphere formulation, is necessary to use an appropriate extraction method to ensure acceptable recovery of DS(s), impurities, and/or degradation products. When possible, HPLC is used to measure impurities and assay, at the same time [81].

When chromatographic methods are used, representative chromatograms should be presented to validate specificity and individual substances should be suitably labeled. Other separations techniques require similar considerations [86].

There are two approaches possible:

- Discrimination of analytes where impurities are available:
  - For the assay, it implies prove the discrimination of the analyte in the presence of impurities and/or excipients; in practice, samples of pure DS are compared with DS with impurities and/or excipients.
  - For the impurity test, the discrimination may be established by spiking active substance or product with appropriate levels of impurities and demonstrating the separation of these impurities individually and/or from other components in the sample matrix.

- Discrimination of the analyte where impurities are not available
  - The specificity may be demonstrated by comparing the test results of samples containing impurities or degradation products to a second well-characterized procedure. As appropriate, this should include samples stored under relevant stress conditions: light, heat, humidity, acid/base hydrolysis, and oxidation. For the assay, the two results should be compared. For the impurity tests, the impurity profiles should be compared. Peak purity tests may be valuable to prove that the analyte chromatographic peak is not attributable to different components.

Table 1. Validation characteristics of each analytical procedures that are validated (from [88]).

Type of analytical Procedure / Characteristics	Identification	Testing for Impurities		Assay (dissolution or content/potency)
		Quantitative	Limits	
Accuracy	○	●	●	●
Precision Repeatability Interm. Precision	 ○ ○	 ● ● (1)	 ● ●	 ● ● (1)
Specificity (2)	●	●	●	●
Detection Limit	○	○ (3)	●	○
Quantitation Limit	○	●	○	○
Linearity	○	●	○	●
Range	○	●	○	●

Legend: ○ signifies that this characteristic is not normally evaluated; ● signifies that this characteristic is normally evaluated; (1) in cases where reproducibility has been performed, intermediate precision is not needed; (2) lack of specificity of one analytical procedure could be compensated by other supporting analytical procedure(s); (3) may be needed in some cases

2.2.2. Specific tests for parenteral formulations

Some specific tests for parenteral formulations are proposed according to ICH QA6. A survey was carried out following this guideline, explaining tests and their methods using some American and European pharmacopeias chapters, which are summarized in the table below.

Table 2. Summary of specific tests for parenteral formulations according to ICH Topic Q6A – *Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Chemical Substances* definition and method.

Specific tests for parenteral according to ICH QA6	Definition	Method	Observations	Ph. Eur.	USP
pH	Measure the acidity or alkalinity of a solution.	Potentiometric sensor with a temperature probe	-	[89]	[90]
Particle size/particle size distribution	Indicates what sizes of particles are present in a relative amount	Laser light diffraction technique; Scanning electron microscopy (SEM); Dynamic light scattering (DLS); or Static light scattering	-	[91][70]	[92]–[94]
Sterility	Demonstrates the presence or absence of extraneous viable contaminating microorganisms	The test is performed using a medium that allows aerobes, anaerobes, and fungi growth during 14 days of incubation. Fluid Thioglycollate Medium for anaerobic and aerobic bacteria and Soybean–Casein Digest Medium for fungi and aerobic bacteria or equivalent commercial mediums can be used.	A good result only shows that no microorganism has been found in the examined sample. The sterility is guaranteed by sterilization process validation or by aseptic processing procedures.	[95]	[96]
Endotoxins/Pyrogens	Pyrogens are substances that can produce a fever, e.g. endotoxins. Endotoxin is a component of the exterior cell wall of Gram-negative bacteria.	Bacterial Endotoxins Test (BET): use amoebocyte lysate from the horseshoe crab	When justified, a pyrogenicity test may be proposed as an alternative.	[97]	[98]
Particulate matter	Consists of mobile undissolved particles, gas bubbles not included, unintentionally present in the solution	Light obscuration particle count	-	[85]	[99]

Osmolarity	Refers to the concentration of osmotically active particles in a solution	Calculated from the experimentally measured value of osmolality (using an osmometer).	Should be performed when a drug product presents tonicity	-	[100]
Water content	Assesses the quantity of water contained in a formulation	When the formulation only contains water: Method I (Titrimetric), Method II (Azeotropic), or Method III (Gravimetric)	It is common for non-aqueous parenterals and for parenteral products for reconstitution	-	[101]
Antimicrobial preservative content(1)	Assesses the quantity of antimicrobial in a formulation	Gas and liquid chromatographic methods or polarographic method	-	-	[102]
Antioxidant preservative content(1)	Assesses the quantity of antioxidant in a formulation	Not specified	-	-	-
Uniformity of dosage units	Dosage units are dosage forms containing a single dose or a part of a dose of drug substance in each unit	The units are individually analyzed using an appropriate analytical method and the accepted value is calculated as described in the pharmacopeias.	-	[103]	[104]
Extractable	Is an organic and inorganic constituents released from a pharmaceutical packaging/delivery system, packaging component, or packaging material of construction into an extraction solvent under laboratory conditions	For extraction maceration, reflux, soxhlet, sealed vessel, instrument-based solvent extraction, and sonication can be used. Extract characterization into divides four phases: scouting, discovery, identification, and quantification (using spectroscopy, wet chemical, gas, liquid or ion chromatography, spectrometry, or atomic spectroscopy).	-	(2)	[105]

Leachable	Is organic and inorganic compounds that leach into the drug product from the packaging/delivery system, packaging component, or packaging material of construction under normal conditions of storage and use or during accelerated drug product stability studies.	Analytical techniques are the same as those performed to extractables characterization	-	(2)	[106]
Functionality of delivery systems	Proves the functionality for parenteral formulations packaged in pre-filled syringes, auto-injector cartridges, or other similar devices	Syringeability, pressure, seal integrity (leakage), and/or parameters such as tip cap removal force, piston release force, piston travel force, and power injector function force	-	(3)	(3)
Redispersibility	Ability of a settled suspension to form a homogeneous mixture	Mechanical or manual procedure(4)	Applies to suspensions that sediment during storage(4)	-	-
Reconstitution time	Reconstitution is returning a powdered medicine (lyophilized) to its liquid form by adding an appropriate diluent	Not specified	-	-	-

(1) Just performed when formulation has antimicrobial or antioxidant preservative.
(2) There are some monographs regarding different materials that delimit their extractables acceptance criteria.
(3) There are some monographs regarding different components and materials.
(4) Information of ICH QA6.

2.3. Long-acting injectables characterization – development of microspheres and *in situ* formulations

In addition to the tests proposed by ICH, other tests can be carried out, both in the development phase and to assure product performance. In this chapter, it will be presented some tests proposed in the literature for the development of microspheres and *in situ* formulations (**Figure 7**), once FDA specifically includes these formulations as LAI.

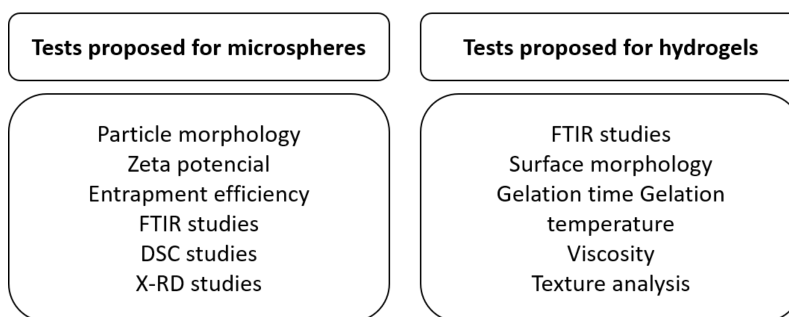

Figure 7. Some tests proposed in literature during the development of microspheres and *in situ* formulations

2.3.1. Tests proposed for microspheres

1) Particle morphology

The surface morphology evaluates shape, more specifically roundness, and the surface of microspheres, like smoothness. Particle morphology can be tested at the same time as particle size or individually, using for example SEM or light microscopy (LM) [107]–[112].

2) Surface charge/Zeta potential

Zeta potential is defined as the charge established at the interface between a solid surface and its liquid medium. It can be measured by Laser Doppler Electrophoretic Mobility [107].

3) Entrapment/encapsulation efficiency

When it refers to microspheres, the assay can also be mentioned as encapsulation or entrapment efficiency and is described as the extent of total drug in milligrams encapsulated per 100 mg of microspheres. Moreover, assay testing should be able of distinguishing encapsulated DS from un-encapsulated one [81].

The DS is extracted by dissolving a known quantity of microspheres in a suitable solvent and then is quantified using a suitable method that can be validated. The encapsulation efficiency is calculated as the quotient of the total amount added while preparation with the amount of drug present in the supernatant, as showed in the formula below [107], [108], [112]:

$$\%\, Drug\ Entrapment = \frac{Actual\ content}{Theorical\ content} \times 100 \qquad \text{(Equation 1)}$$

4) Bulk density

Bulk density is defined as the mass of particles divided by the total volume they occupy. It is evaluated by weighing a specific amount of microsphere and registering volume, which is the initial volume (V_0). Bulk density is calculated using the following formula [108], [110], [113]:

$$Bulk\ Density = \frac{Microspheres\ mass\ (W)}{Initial\ microspheres\ volume\ (V0)} \quad \text{(Equation 2)}$$

5) Tapped density

Tapped density is obtained by mechanically tapping a graduated measuring cylinder with a specific amount of sample previously weighed. The volume of tapped microspheres is registered and tapped density is calculated by using the formula presented below [108], [113].

$$Tapped\ density = \frac{Mass\ of\ the\ microspheres\ (W)}{Tapped\ volume\ of\ microspheres\ (Vf)} \quad \text{(Equation 3)}$$

6) Compressibility index

The compressibility index represents the changes that could occur in the packing arrangement of microspheres due to the tapping procedure. It is calculated using bulk and tapped densities, using the formula presented below [108], [113].

$$\%CI = \frac{Tapped\ density}{Bulk\ density - tapped\ density} \times 100 \quad \text{(Equation 4)}$$

7) Flow properties

To define the flow properties of microspheric formulation is used the angle of repose, with the fixed funnel method. The height and diameter of the heap were noted and the angle of repose can be calculated by using the formula below [108], [113].

$$Tan\ \emptyset = \frac{2\ height}{Diameter\ of\ hep} \quad \text{(Equation 5)}$$

8) Degree of swelling

The degree of swelling or swelling index (SI) is evaluated by measuring the extent of swelling of microspheres in a suitable solvent. Initially, dried microspheres are weighted and are then suspended in solvent for a certain time. The excess surface adhered liquid drops were removed by blotting and swollen microspheres are weighed. The degree of swelling is calculated by the following formula [109], [110].

$$SI = \frac{Swollen\ microspheres\ weight - Dried\ microspheres\ weight}{Dried\ weight\ microspheres} \times 100 \quad \text{(Equation 6)}$$

9) Fourier transforms infrared studies

The Fourier Transforms Infrared (FTIR) study allows confirming the cross-linking of polymers and the absence of chemical interaction between drug and polymers [109], [112], [114].

10) Differential scanning calorimetric (DSC) studies

DSC is used to ensure molecularly dispersion of DS in the polymer matrix and evaluate thermal properties and the physical state of DS within microsphere formulation [109], [114].

11) X-ray diffraction studies

X-ray diffraction (X-RD) analysis is performed to analyze the crystallinity of the DS in cross-linked microspheres. DS can be dispersed in the amorphous or crystalline state [109], [114].

2.3.2. Tests proposed for *in situ* gel formulations

1) Fourier transforms infrared studies

As for microspheres, FTIR is performed to study the compatibility between DS and excipients, as well as the interaction between DS and polymers used in gel [115]–[117].

2) Surface morphology

The surface characteristics of the gel formulation with and without DS can be analyzed using SEM [117].

3) Gelation temperature

Gelation temperature study is performed by placing a tube with the formulation in a thermostatically regulated water bath and increasing the temperature at a consistent rate, with soft shaking. The temperature at which it was distinguished from liquid formulation to solid gel upon the inversion of the tube is considered the gelation temperature [115], [116].

4) Gelation time

Gelation time is defined as the time necessary to liquid formulation become solid gel when placed at gelation temperature. This test is performed by placing a tube with the formulation in a thermostatically controlled water bath at the previously determined gelation temperature with soft shaking. The time at which it was distinguished from liquid formulation to solid gel, using flow or no-flow criterion with the test tube inverted, is considered the gelation time [115], [116].

5) Syringeability test

Syringeability test can be performed using vertical support for a filled syringe held by vertical support with a pan placed on the piston of the syringe. The time (seconds) at which the gel is completely removed from the syringe is considered as syringeability time [115]. Syringeability test can also be an evaluation of the ease with which the formulation passes through the syringe [118]. Another possible approach is to consider syringeability as the force required to push the formulation through the syringe needle. For this test, an universal syringe rig can be used [117].

6) Rheological studies

The most common rheological study is viscosity which is measured using a viscometer, at a specific temperature. This study can be performed for final formulations and structured gel [115], [118].

7) Texture analysis

The texture analysis includes firmness, consistency, and cohesiveness of formulation and is performed using a texture analyzer. Higher values of adhesiveness allow good contact with surface like tissues, which is desirable [118].

2.4. *In vitro* drug release for long-acting injectables formulations

In vitro release (IVR) test is a key test for LAI as it investigates the release profile of this type of formulation. IVR is often performed both during pre-clinical development and after approval as a control quality test. IVR test can be performed to evaluate methods of manufacture, to identify critical factors that may affect bioavailability, for batch quality control, to monitor and predict the effect of formulation changes, and principally to predict the expected bioavailability characteristics [119], [120].

2.4.1. Authorities guidelines

Despite the widespread use of LAI, no specific guidance has been presented for their dissolution/*in vitro* release test in USP or Eur. Ph.

The USP chapter ⟨711⟩ Dissolution presents the dissolution requirements for dosage forms administered orally. Four apparatus are available: Apparatus 1 (Basket Apparatus), Apparatus 2 (Paddle Apparatus), Apparatus 3 (Reciprocating Cylinder), and Apparatus 4 (Flow-Through Cell). The procedure and the possible interpretation for immediate, delayed, and prolonged-release dosage forms, separately, are presented [121].

The USP chapter ⟨724⟩ shows the dissolution requirements for transdermal systems (TDS) and other dosage forms. Three apparatus options are offered: Apparatus 5 (Paddle over Disk), Apparatus 6 (Cylinder), and Apparatus 7 (Reciprocating Holder). This chapter shows the procedure of sample preparation for osmotic pump tablets, for TDS, and other dosage forms. It also presents the media, procedure, time, and interpretation guidelines [122]. However, these techniques were designed for TDS, whereby do not offer particular advantages for IVR of injectables, like LAI.

Chapter 2.9.3. "Dissolution test for solid dosage forms" of Eur. Ph. provides a guideline for the dissolution of solid dosage forms (for example, tablets, capsules, and suppositories), presenting the apparatus available: Basket Apparatus, Paddle Apparatus, and Flow-Through Cell Apparatus. There is an explanation for each apparatus method, some considerations for dissolutions medium, and some aspects concerning the sampling and evaluation. It also refers to what should be mentioned for the dissolution test [123]:

- The apparatus used;
- The composition, volume, and temperature of the dissolution medium;
- The rotation speed or the flow rate of the dissolution medium;
- The timing, the method, and the amount of sampling;
- The analytical method of analysis;
- The quantity or quantities of DSs required to dissolve within a prescribed time.

The USP chapter ⟨1092⟩ presents guidelines for the development and validation of dissolution procedures, focusing on solid oral dosage forms. The USP chapter ⟨1088⟩ approaches several explanations and guidelines for *in vitro* and *in vivo* evaluation of dosage forms. According to these chapters, three-time points are needed for pharmacopeic purposes. The first time point, usually 1–2 h, proves the absence of burst release. The intermediate time point defines the *in vitro* release profile. The last time show essentially complete release of the drug (>80%). However, additional sampling times may be required for drug approval [124], [125].

Although there are not guidelines in USP or Ph. Eur, some entities participate, in February 2003, in a workshop with the aim of "Assuring Quality and Performance of Sustained and Controlled Release Parenterals" sponsored by the European Federation of Pharmaceutical Scientists (EUFEPS), in association with the American Association of Pharmaceutical Scientists (AAPS), the EMA, Ph. Eur., the FDA and the USP. A report was written with some conclusions. For example, it is shown that the method of IVR test must show batch consistency/variability, be able to monitor product stability with time, evaluate the effect of process changes, and demonstrate acceptability for use. USP apparatus 1 and 2 were designed for immediate- and modified-release oral formulations, however, when used to LAI formulations, problems with sample containment, microsphere aggregation, violation of sink conditions may occur [126]. According to the Ph. Eur., sink conditions occur in a volume of dissolution medium that is at least 3-10 times the saturation volume [127]. That is, if the maximum concentration of the DS in the dissolution medium is less than 3 times the saturation solubility, the sink conditions are assured. Moreover, the need for large volumes could not be pertinent for small volumes of IM or SC injections. USP apparatus 5,

6, and 7 do not offer any advantages for LAIs due to their design for TDS. The authorities in this workshop recognize USP apparatus 4 as the most suitable of the currently available USP apparatus for parenteral controlled and sustained release. They also talk about alternative apparatus, such as small sample vials and vessels, with and without agitation used to these formulations [126].

Although the previously referred USP chapter ⟨1092⟩ affirms that rotating bottles or dialysis tubes may be useful for microspheres and implants [124]. Although some reports try to harmonize the procedures, such as the presented one, the authorities do not present compendial solutions for the realization of this test, whereby non- or modified-compendial methods are used to evaluate *in vitro* release for LAI formulations.

2.4.2. *In vitro* release methods

As explained before, there are no compendial methods for IVR test for LAIs. However, some techniques are commonly used in literature and on already approved drug products, such as shaking flask, sample and separate, Franz cell, paddle apparatus, and flow-through methods. These techniques and their advantages and disadvantages will be present below.

2.4.2.1. Shaking flask method

The shaking flask method consists of containers with a release medium where the formulation is placed with constant agitation, as shown in **Figure 8**. The formulation is suspended in a suitable release medium at a controlled temperature, with constant agitation and the samples are collected at certain time points. An equal amount of fresh medium is added to the release medium container to maintain the total volume and sink conditions. Then, samples are quantified by a suitable and validated method [128].

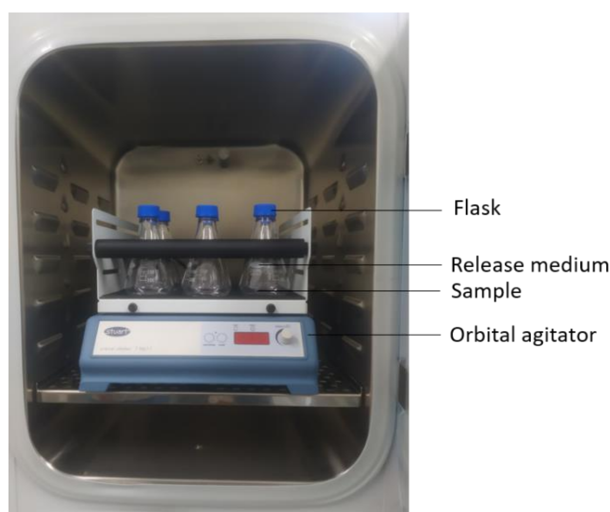

Figure 8. Image of shaking flask method, using orbital agitator; this apparatus is then placed in an incubator with controlled temperature.

This method allows some modifications, such as the size of the container and the rotation speed. The size of the container depends upon the amount of media required for dissolution, to maintain sink conditions. So, microfuge tubes, test tubes, or even large flasks/bottles can be used. The medium agitation is obtained by an orbital agitator or a rotary shaker. The temperature can be controlled using a water bath or an incubator [128].

This method is applied to several studies, such as the presented next. H. Kamali *et al*. evaluated the *in vitro* release behavior of an *in situ* forming gel with levothyroxine using PLGA-PEG-PLGA (triblock) polymer. The IVR test is performed injecting the formulation directly into each vial containing dissolution media (phosphate buffer pH=7.4). The vials were kept in a reciprocal shaking water bath. This method allows discriminating the differences of using different concentrations of triblock, including burst release assessment [129].

On the other hand, E. Khodaverdi *et al*. *in situ* forming gel chitosan/glycerol-phosphate (chitosan/Gp) solution loaded with insulin. Unlike the example above, the gel was incubated after solidification. The IVR test allows concluding that the release of insulin from chitosan/ Gp thermosensitive gel decreases by increasing Gp salt and initial insulin concentration [130].

2.4.2.2. Samples and separated method

The sample and separate technique is similar to the shaking flask method, although it implies the separation of the DS from the carriers using ultracentrifugation or filtration before drug quantification. So, in this method, the formulation is suspended in a suitable release medium at a specific temperature, with constant agitation and the samples are collected and centrifuged or filtrated to separate the medium from the settled microspheres (if any). An equal amount of fresh medium is added to the release medium container to maintain the total volume and sink conditions. Then, the samples are quantified by a suitable and validated method.

Modifications of this method include the size of the container, use of agitation, and sampling methods [129], [131]. These tests can be performed in tubes or vials when small volumes (<10 mL) or in bottles or Erlenmeyer flasks when larger volumes (100-400 mL) of medium are required. Agitation can be achieved using a magnetic stirrer at a fixed speed, a wrist shaker rotating at 360°, an incubator shaker, a shaking water bath, a tumbling end-over-end, a high-speed stirring/revolution of bottles, or even USP 2 paddle apparatus [129].

In the separation of the drug from the carriers using filtration, the medium contents are passed through a filter with an appropriate pore size to ensure complete separation of the supernatant from particulates or polymer fragments, followed by an analysis of either the supernatant or the

particulates. When centrifugation or ultracentrifugation is used, the supernatant is analyzed or the remaining drug due to instability in the release media [132].

2.4.2.3. Franz cells techniques

The Franz diffusion cells system consists of a Franz cell, also called vertical diffusion cells (VDC), with a suitable membrane that separates the donor compartment from the acceptor compartment, as presented in **Figure 9**. In this method, a suitable membrane previously chosen is placed in the upper donor chamber and then formulation is placed on the membrane. The acceptor compartment is filled with the release medium and the system is closed to prevent medium evaporation. The whole diffusion cell is submerged in a thermostatic bath, allowing a controlled temperature, as well as constant agitation in the acceptor compartment. The samples are withdrawn at predefined times and the same volume of fresh medium is replaced. The samples are quantified using a suitable and validated method, like HPLC-PDA [133].

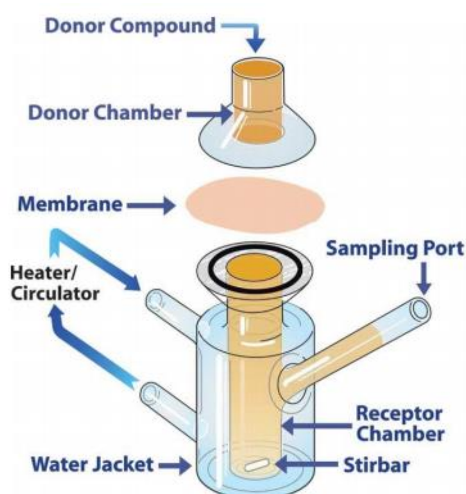

Figure 9. Representation of Franz diffusion cells system (from [134]).

As an example of the application of the method, Herrera *et al*. consider Franz cell system a good IVR method for microspheres once the formulation is in contact with the membrane and does not move, instead of in constant agitation, like dialysis methods. Thus, *in vitro* behavior is more similar to *in vivo* behavior, as microspheres are confined to an area but completely submerged in the release medium [133].

2.4.2.4. Paddle Apparatus

Paddle Apparatus, also known USP Dissolution Apparatus II, designed for dissolution tests in tablets, can also be used in IVR tests to some LAI formulations. **Figure 10** represents the USP apparatus II. The paddle apparatus consists of a metallic or suitably inert, rigid blade and shaft. In the method applicable to this apparatus, the formulation is dissolved into a suitable medium

in the vessel at controlled temperature and a constant rotation speed. The samples are withdrawn at predefined times and the same volume of fresh medium is replaced. The samples are quantified using a suitable and validated method, like HPLC-PDA [117].

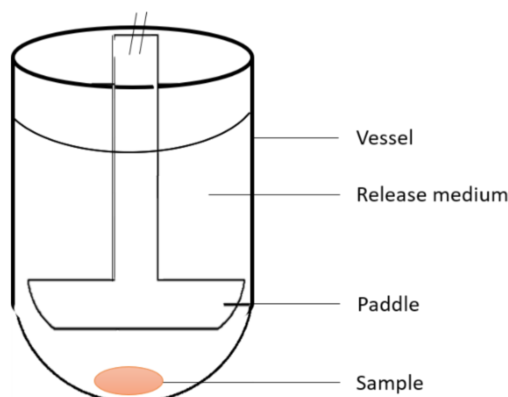

Figure 10. Representation of USP Dissolution Apparatus II.

This apparatus can also be used in combination with the dialysis bags, both in normal or reverse dialysis. The differences will be explained in chapter 2.4.3.1.

2.4.2.4. Flow-through method

The flow-through concept is presented in the USP and Ph. Eur. as the flow-through cell (USP Apparatus 4). In this technique, the medium is continuously circulated through a column containing the formulation followed by the analysis of the eluent. In the flow-through method, glass beads are placed into a suitable cell and the formulation is added on top of the beads or if specified on a wire carrier. The filter head is assembled, and the parts are fixed together using a suitable clamping device. The cell is immersed in a water bath with controlled temperature and constant flow. Collect the eluate by fractions at each of the times stated. The samples are quantified using a suitable and validated method, like HPLC-PDA [121], [131].

According to USP and Ph. Eur., the apparatus consists of [121], [123]:

- A flow-through cell with a conical lower and a cylindrical upper part is perfused by dissolution medium. USP and Ph. Eur. describe different types of cells (with 12.0 and 22.6 mm diameter and a lipophilic solid dosage forms specific one), but other non-compendial cells can be used;
- A reservoir for the dissolution medium;
- A pump that drives the dissolution medium from the reservoir to the cell;
- A bath and a heat exchanger coil.

The most important factor in the release of the drug from microparticulate delivery systems is hydration of the polymer matrix, which leads to drug release by diffusion and erosion. So, in

polymeric formulations, the low flow rate leads to incomplete release, while higher flow rates present cumulative release greater than 85%, whereby flow rate should be weighted when the flow-through method is used. The volume of the buffer also should be considered, which depends on drug solubility and assay sensitivity [129].

The top of the cell usually has a filter that prevents the exit of undissolved material. Thus, when formulations contain very small particles the filter resistance can increase due to little size reduction, which may lead to back-pressure inside the cells [129].

Morihara *et al*. showed different results on dissolution tests according to tablet position: on a holder, on top of a layer of glass beads, or within the glass bead layer [135]. Shiko *et al*. also showed the orientation and position of the tablet in the flow cell modify the releasing, due to the effect of table orientation on the dissolution profiles [136]. Thus, the LAI formulation position may also be important when USP apparatus 4 is used.

A FDA letter talks about an IVIVC established for risperidone microspheres and naltrexone microspheres with a rabbit model. An IVR test using USP apparatus 4 showed exceptional discriminatory ability and potential to predict the *in vivo* performance of these formulations [42].

Bhardwaj and Burgess developed a dialysis adapter for USP apparatus 4 for IVR test of dispersed system dosage forms. The dialysis adapter consists of a hollow cylinder and the base and top of the cylinder are made of circular Teflon with groves for O-rings seals. The USP apparatus 4 method with the adapter can discriminate between solution, suspension, and liposome formulations of dexamethasone, unlike the dialysis method [137].

Table 3 compares the advantages and disadvantages of the IVR methods presented before.

Table 3. Advantages and disadvantages of IVR methods.

IVR method	Advantages	Disadvantages	References
Shaking flask	Simple and easy to set up Adjustable parameters such as sample vial/vessel size and agitation speed Medium replacement assures sink conditions	Aggregation of the particulate dosage Variability in the shape and size of flask/ container may lead to inter as well as intra laboratory variations in drug release study	[128], [130]
Sample and separated	Simple set up Adjustable parameters such as sample vial/vessel size, agitation speed, and sampling methods	Product aggregation, mainly with microspheres Loss of sample during the separation Disruption of the formulation due to centrifugal force Redispersion of the degraded particles after centrifugation is difficult	[129], [131], [138]
Franz cells method	Reproducible due to complete control over the area of the diffusion membrane, given by the diameter of the cell	Possibility of particles agglomeration in the donor compartment Difficult sample collection	[133]
Paddle apparatus method	Prevented aggregation	Large medium volumes Absence of sample containment/membrane	[129], [131]
Flow-through method	Simulate the *in vivo* environment by constantly unidirectional circulating Long sampling time Automated process: samples are continuously sampled and analyzed along with buffer replacement	High volume of release medium Variation in the flow rate due to clogging of the filter (because of polymer degradation) leading to a high-pressure buildup in the system	[129], [139]

2.4.3. *In vitro* release barriers

Some barriers can be used to contain the formulations in each technique presented, such as dialysis membranes (such as dialysis bag, float-a-lyzer), and agarose gel. These barriers and their advantages and disadvantages will be present below.

2.4.3.1. Dialysis barriers

There are several dialysis barriers such as **dialysis membranes**, **dialysis bag**, and **float-a-lyzer** that can be applied in some techniques presented above. These techniques can use two compartments separated by a dialysis membrane, as a dialysis bag or a float-a-lyzer with appropriate molecular weight cut-off which retains the formulation allowing the transfer of the released drug into a receiver compartment with the release medium.

The dialysis membrane method, also called side-by-side dialysis method, consists of a small donor compartment (5-8 mL) separated from a large acceptor compartment (1000 mL) by a dialysis membrane, which creates a driving force for drug transport to the acceptor compartment [129]. In this method, the formulation is placed in a donor container separated from the receptor container by a dialysis membrane. Both containers have a suitable medium, at controlled temperature and continuously shaking, using for example the Franz Cell apparatus. The dialysis membrane traps the particles and allows the drug to be transferred to a recipient compartment. The samples are collected from the receptor container at predefined time intervals and replaced by a fresh release medium and then are quantified using a suitable method, such as HPLC [140].

On the other side, the formulation can be placed in a dialysis bag, also called dialysis tubing or dialysis sac, and it is sealed and is put in a receptor container with a suitable release medium. The whole set-up was put in a shaker at a controlled temperature and continuously shaking. The USP apparatus II, USP apparatus IV, or shaking flask apparatus can be used. The dialysis bag traps the particles and allows the DS to be transferred to the release medium in the container. A sample is collected from the container at predefined time intervals and replaced by fresh release medium and is then quantified using a suitable and validated method, such as HPLC [114], [141].

The medium agitation can minimize unstirred water layer effects, preventing the accumulation of polymer degradation products or DS. Agitation can be provided using a horizontal shaker or the USP paddle apparatus. The medium is selected according to drug solubility and stability during the release test [129], [141].

These barriers have points in common: the appropriate membrane molecular weight cutoffs (MWCO) should be chosen according to the studied DS. The membrane MWCO needs to be sufficiently high for not to be a limiting factor for drug diffusion [129], [131]. Moreover, the membrane should present a low affinity for the DS so that it is not binding or adsorbs to the membrane [142].

In addition to the MWCO, also the membrane surface area is a key parameter when characterizing the IVR profile of the DS. The dialysis membrane presents a small membrane surface area available for drug passage, which leads to a long time to achieve equilibrium with the receptor medium. This could limit the analysis of initial drug levels in formulations with burst release. So, using dialysis bags or a float-a-lyzer, with more surface area, it is possible to obtain more realistic results [129], [131].

The techniques using dialysis present some advantages and disadvantages, as presented in **Table 4**.

Table 4. Advantages and disadvantages of the dialysis methods.

Advantages	**Disadvantages**
Ease sampling and medium replacement [129], [138] Simple and economic [139]	Cannot be used with drugs that interact with the dialysis membrane [143] When the rate of drug release from the carriers is faster than the rate of diffusion out of the dialysis membrane, the data do not completely reflect the *in vivo* behavior Violation of sink conditions within the dialysis bag [129], [138], [139] Major errors may be introduced by the membrane [144]

Moreno-Bautista and Tam demonstrated that using the dialysis technique as IVR test for colloidal particles does not provide an exact release profile from drug carriers, principally when the release rate is very fast. Although this method can be used to provide an overall indication of the release rate, the obtained profile just should be used if it has been mathematically proven that the drug release is sufficiently slow for its measurement not to be impacted by the dialysis membrane [144]. However, Kostanski and DeLuca showed a good correlation between *in vitro* and *in vivo* data using the dialysis bag method with a peptide-loaded biodegradable microsphere

system [145]. That is, this method may be feasible for some formulations but it does not present viable results for others.

According to USP, sink conditions allows a more predictable dissolution results of the dosage form [124]. Thus, to overcome the disadvantage related to violation of sink conditions, Chidambaram *et al*. presented the **reverse dialysis** method in which the drug is placed directly in the larger container with medium and the samples are withdrawn from dialysis bags submerged into the container [146]. **Figure 11** represents the different dialysis methods.

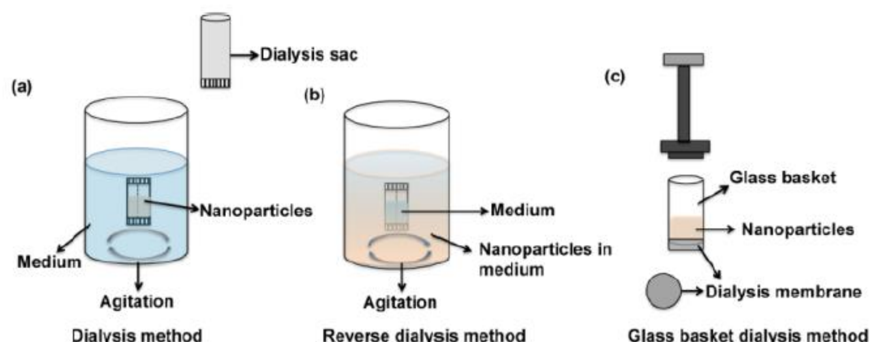

Figure 11. Dialysis methods. (a) dialysis sac/bag method, the formulation (represented by nanoparticulate systems) are placed inside the dialysis bags; (b) reverse dialysis bag method, the formulation is added to the medium outside the dialysis bags and the bag medium is analyzed; and (c) glass basket dialysis method, a glass cylinder with its bottom sealed by a dialysis membrane is used (from [140]).

Reverse dialysis techniques present some advantages and disadvantages, as presented in **Table 5**.

Table 5. Advantages and disadvantages of the reverse dialysis methods.

Advantages	Disadvantages
No violation of sink conditions [146] Long sampling time [139] Simple and economic [139]	High dilution of formulations in the medium may result in the loss of its discriminatory ability [137] Rate-limiting membrane [139]

M.M.A. Abdel-Mottaleb *et al*. uses a glass basket dialysis method to evaluate lipid nanocapsules systems and colloidal drug carriers (liposomes, polymeric, and lipid nanoparticles). This method consists of a modification of the USP Apparatus I, where the basket is replaced by a glass cylinder closed at the lower end by a dialysis membrane [147], [148]. According to the authors, glass basket dialysis, shown in **Figure 12**, is simple, cheap, easy, and reproducible. It also proved to be

suitable for the comparison purposes between different formulations [147]. However, J. Shen and D. Burgess affirm that violation of sink conditions may occur with this method [140].

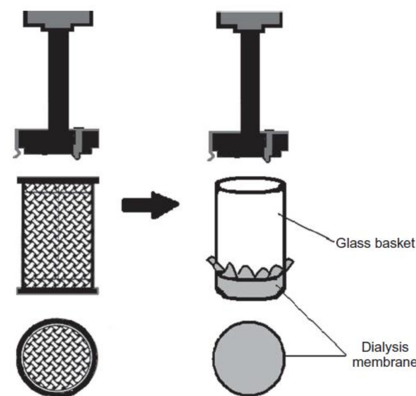

Figure 12. Differences between the USP Apparatus I (baskets apparatus) (left) and the glass baskets (right) (from [147]).

Prabhu *et al*. studied *in vitro* release of PLGA microspheres with calcitonin using two different techniques: separate method with agitation and dialysis bag. Although both methods showed complete release, the dialysis technique proved to be more reproducible but slower [149].

S. Souza and P. DeLuca used a regenerated cellulose membrane dialysis apparatus, a Float-a-Lyzer, presented in **Figure 13**, which has a larger membrane surface area, for IVR test for 1-month parenteral leuprolide PLGA microspheres. Float-a-Lyzer was capable of accurately assessing a low initial burst release. It was also stable to elevated temperatures, whereby it could be used for a short-term release study using high temperatures. According to the authors, this apparatus mimics the *in vivo* condition after SC or IM administration, where the microspheres are immobilized in the tissue [142].

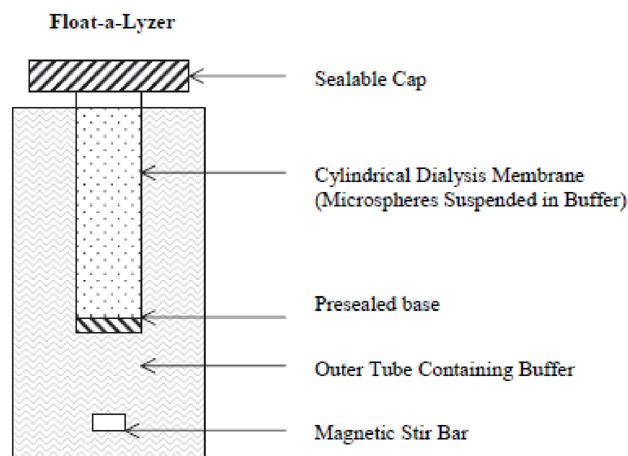

Figure 13. Representation of set-up for IVR from microspheres using the Float-a-Lyzer. (from [142])

2.4.3.2. Agarose gel

When IV administration is used, the drug is immediately in contact with bulk fluid in the systemic circulation. However, in SC and IM administrations, after injection, the formulation is in contact with an extracellular matrix consisting of a fibrous polymer framework of collagen and hyaluronic acid components surrounding physiological tissue. So, using bulk fluid in contrast with the use of agarose gel can lead to differences in mass transport and diffusion of the drug, affecting the IVR profile [150], [151]. In fact, these tissues have very slowly moving extracellular fluids [152].

Agarose gel mimics subcutaneous tissues due to its rheologic nature (as viscosity) and water content, allowing a close simulation of *in vivo* drug release of formulations administrated subcutaneously [152], [153].

T.H. Thi *et al*. compared two IVR methods: a sample and separate method using bulk fluid and into agarose gel. They categorized the agarose gel method as more biorelevant and discriminatory for drug release measurements from bone implants, despite its greater complexity [152].

D. H. Leung *et al*. developed an IVR method using agarose gel in cuvettes, PBS pH=7,4 as release medium upon the gel (as shown in **Figure 14**) and a continuous real-time data collection using mass spectrometric. They conclude that this method can be used for parenteral formulation optimization of various formulations with large and small molecules. Moreover, it is capable of predicting the *in vivo* behavior, when compared with the same dose concentration after being administered SC in rats [150].

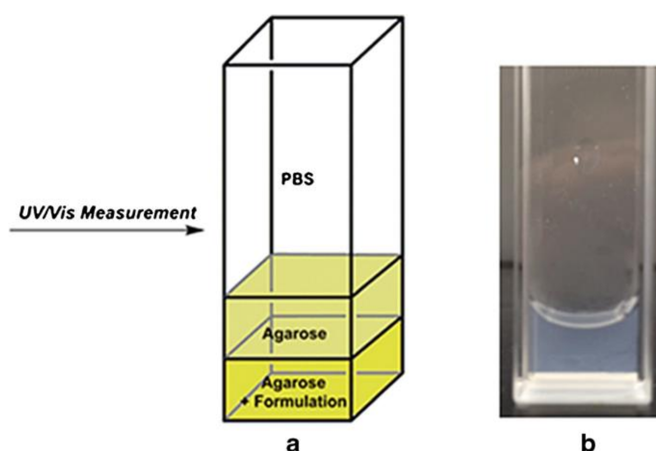

Figure 14. IVR method using agarose gel in cuvettes a) Schematic of the layers with formulation and agarose gel followed by PBS within a cuvette. b) Actual cuvette with the agarose gels. (from [150]).

J. Kozak *et al*. developed an IVR method to parenteral formulations using an agarose gel envelope, as showed in **Figure 15**, in other to mimic tissue firmness. They used different formulations (films, microspheres, and two different cylindrical implants); prepared from various polymers and loaded with different drugs (flurbiprofen, lidocaine, or risperidone). They determined that the release profile differs in most formulations in the use of agarose or release medium. Since this gel mimics physical conditions better, this method can better mimic *in vivo* results, but studies awaiting confirmation by *in vivo* behavior [154].

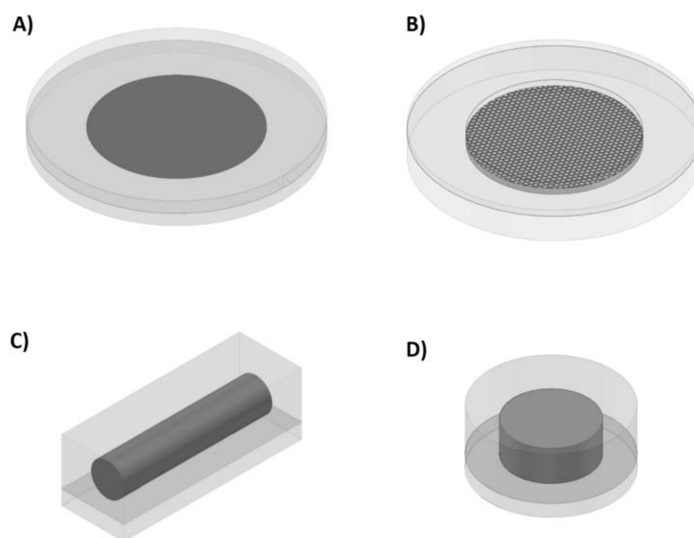

Figure 15. Schematic illustration of the agarose gel envelope (light grey), incorporating the dosage forms a) films, b) microspheres, c) Rod-shaped extrudates and d) implant (dark grey) (from [154]).

Semmling B. *et al* done a comparison between alginate, agar, agarose, polyacrylamide and poly(vinyl alcohol) gels for biorelevant dissolution test of drug-eluting stents. They consider agarose gels the most suitable because they are easy of handling, have a negligible small degree of swelling, maintain their cylindrical shape for periods of at least 28 days, are sufficiently transparent to allow monitoring of formulation placement in the gel lumen, are diffusible and elastic, maintain their mechanical stability under long-term test conditions for 28 days [155].

2.4.4. *In vitro–in vivo* correlation

As explained in USP chapter ⟨1088⟩, all the characterization studies aim to discover the relationship between the physicochemical and pharmacological properties of DS to the pharmacokinetic properties and *in vitro* performance of the final drug product [125]. For that, after IVR evaluation, a suitable *in vitro–in vivo* correlation (IVIVC) should be proposed. According to FDA, IVIVC can be defined as “a predictive mathematical model describing the relationship between the in-vitro property of a dosage form and the anticipated *in vivo* response” [156].

In vitro release methods are well established for oral dosage forms, as well as FDA Guidance about IVIVC, including its relevance, approaches, and classifications [156]. IVIVC is a good tool when the rate-limiting step in the absorption of the drug *in vivo* is its dissolution. However, *in vivo* factors, such as fluid volume, viscosity, tissue barriers, phagocytosis, inflammation, etc. can also affect the *in vivo* release and absorption of these formulations [157]. For this reason, the lack of standard approaches for IVIVC specified for dosage forms such as injectables depot proposed by EMA or FDA is a problem.

2.4.5. Biorelevant conditions

When used to predict the *in vivo* behavior, the IVR test should be biorelevant, which means that it should be performed in conditions that simulate specific characteristics of the physiological environment where the drug will be released, such as osmolarity, pH, or buffer capacity [139], [158]. This ensures that the release test data is clinically meaningful and predictive. The biorelevant *in vivo* parameters that usually should be considered are temperature, blood flow rates, tissue barriers, osmolarity, and pH [120].

The IM or SC administrations present tissue barriers *in vivo* that are not present in IV administration, in which the drug is directly in the systemic circulation. To mimic those conditions *in vitro* some artificial barriers can be used, like agarose gel presented in the chapter "IVR membranes".

The chosen apparatus should also mimic the blood flow around the tissue *in vivo* where the drug is injected. Some changes can be done to the standardized apparatus to reach a more biorelevant and predictive model for LAIs, as presented in the chapter "IVR methods".

As told above, LAI are parentally administrated whereby the dissolution medium should have an ion concentration, osmolarity, and pH similar to the human plasma. Marques *et al*. review the suitable medium to each route of administration and present a simulated body fluid and human blood plasma that can be used in IVR tests to parental formulations [159]. However, the dissolution medium will also depend on drug solubility. For example, Simon *et al*. developed a discriminative dissolution test for betamethasone sodium phosphate (BSP) and betamethasone dipropionate (BD) for intramuscular or intra-articular injectable suspension. As BP is poorly water-soluble, four biorelevant medium were evaluated to assess in which BP present better solubility: sodium phosphate buffer, simulated body fluid, simulated muscular fluid, simulated synovial fluid. The *in vitro* dissolution method using sodium phosphate buffer with a stirring speed of 50 rpm can discriminates between different BD power solutions [160].

Phosphate-buffered saline solutions (PBS) PBS pH 7.4 is widely used in IVR assays [109], [118], [149], [161]. Sindhu *et al*. used phosphate buffer pH 6.8 in IVR studies for injectable in situ gel matrix with thermosensitive polymer Pluronic F 127, carbopol 934P, hydroxyl propyl methylcellulose (HPMC), and sodium CMC, containing metoprolol succinate [116].

A biorelevant IVR method allows obtaining an *in vitro* release profile with a good discriminatory ability and similar to the *in vivo* release profile, using the IVIVC. Thus, using the IVR release profile, it is possible to assess what is the drug concentration in function of the time. It allows enhancing the formulation to avoid burst release, adjust the release profile to the wanted one (increase or decrease the number of days that DS is available on the body, for example), and avoid releases outside the therapeutic zone. For example, S. K Sindhu *et al*. observed that the concentration of polymers (Pluronic F 127 (20%) together with carbopol 934P, HPMC, and SCMC) affected the drug release from the in-situ geling matrix system using IVR studies [116]. C. Koulouktsi *et al*. saw the same phenomena in microspheres, where increasing of polycaprolactone content led to decreasing DS's release rates [114]. F. Damiri *et al*. identified several factors that influence the drug release from the polymer matrix, like degree of deacetylation (DDA), molecular weight, drug's charge [161].

2.4.6. Accelerated *in vitro* release test

Real-time IVR test is essential in formulation development and understanding and helps to reduce the regulatory burden of bioequivalence testing. However, accelerated IVR tests can be important in quality control once real-time release tests of LAI can last weeks to months, which would extend the time to batch release and hence reduce the effective shelf life [129], [162]. Moreover, a long IVR test needs the addition of preservatives and can lead to some problems such as stability and compatibility of the components of the release device, like tubings and membranes [129]. To develop accelerated IVR several parameters could be changed to induce the accelerated release such as temperature, pH, surfactants, solvent, ionic strength, enzymes, and agitation rate [129], [162]. Nevertheless, these conditions could accelerate the rate of drug release but also change the mechanism of drug release whereby the drug release mechanism and how accelerated parameters may affect it should be studied [162].

Elevated temperature can be used for accelerated IVR test since high temperature can increase molecular mobility. At temperatures above the polymer glass transition temperature, the increased polymer mobility results in the substantial rushing of DS release via diffusion. Furthermore, high temperature can enhance hydration and degradation of polymers, leading to erosion and faster drug release [162].

Accelerated IVR test using high temperatures may not correctly predict the “real-time” burst release due to a combination of two competing factors. Elevated temperature can increase polymer mobility, thus resulting in increased drug release via diffusion. However, the increased polymer mobility can cause microsphere surface morphology changes (e.g. pore closure), which in turn may decrease drug release. Consequently, an initial “real-time” IVR test may be needed. Also, high temperature may result in accelerated degradation of media components and the DS [162].

The pH can affect the hydrolysis kinetics of biodegradable polyesters, resulting in accelerated drug release from systems like polymeric microparticles. However, the acceleration of drug release is not as great as that achieved at high temperatures. Moreover, some DSs are sensitive to extreme pH conditions[162].

The addition of surfactants or organic solvents into the release medium can also accelerate drug release. In the case of lipid implants, the presence of surfactants (e.g. Tween 20) can facilitate wetting and buffer penetration, and/or increase drug solubility in the media (via micelle solubilization), resulting in faster drug release. Moreover, some surfactants (e.g. 0.1% Tween 81) may interact with the lipid matrix and induce the formation of cracks in the lipid matrix, thus accelerating drug release. Organic solvents (e.g. ethanol, acetonitrile) can also achieve accelerated drug release. Acetonitrile can increase the porosity of PLGA-based stent matrices and therefore result in accelerated drug release [162].

Parameters like agitation conditions and interfacial can also accelerate drug release. For example, the drug oil-water distribution coefficient is a key parameter influencing drug release from oil depot formulations [162].

Table 6 resumes the most common parameter used to provide accelerated IVR test, their mechanism and disadvantages.

Table 6. Mechanisms and disadvantages of some parameters that can provide accelerated IVR test.

Parameter	Mechanisms	Disadvantages
Temperature	Increase molecular mobility Enhance hydration and degradation of polymers	High temperatures may result in accelerated degradation of media components and the drug
pH	Affect the hydrolysis kinetics of biodegradable polyesters	Acceleration of drug release is not as significant as that achieved at high temperatures Not suitable for drugs sensitives to extreme pH conditions
Surfactants	Facilitate wetting and buffer penetration Increase drug solubility in the media (via micelle solubilization) Induce the formation of cracks in the lipid matrix	Only suitable for some formulations

In the previously referred workshop, in 2004, with the aim of "Assuring Quality and Performance of Sustained and Controlled Release Parenterals" the accelerated IVR was approached. It was suggested to use the time to plateau of release at approximately 100% to determine if a relationship can be established for products with different real time release rates, for predict an association between accelerated and real time IVR. It was also suggested to investigate if a relationship exists between *in vitro* accelerated release and *in vivo* release [126]. Moreover, accelerated release testing should be capable to serve as a discriminatory tool in quality control assessment [162].

J. V. Andhariya *et al*. developed an accelerated in vitro release testing method for naltrexone-loaded PLGA microspheres. First, a real-time IVR test was developed. They tested two approaches: sample-and-separate and USP apparatus 4. USP apparatus 4 method appeared to be more suitable and very reproducible. Since naltrexone is not stable under extreme pH conditions, an elevated temperature accelerated in IVR method at 45ºC was developed. However, high temperatures increase naltrexone degradation whereby medium was frequently replaced and 0.0625% (w/v) sodium ascorbate was added to the release medium successfully prevented naltrexone from degradation. A linear correlation between real-time and accelerated conditions was observed for the USP apparatus 4 method. According to the authors, this

suggests that the drug release mechanisms (the combination of polymer erosion and drug diffusion) at both temperatures may be similar. The accelerated USP apparatus 4 method is both discriminatory and reproducible. Accordingly, it can be used as a suitable fast quality control tool for naltrexone microspheres [163].

3. Internship: Laboratorial developed activities

The second aim of this internship was to develop an appropriate methodology for assessing the *in vitro* release profile of a LAI technology that allows a prolonged release of two DSs during at least 96 hours. This LAI is intended for local infiltration in a surgical incision, for post-surgical pain relief. In this report, the LAI technology will be called BlueLAI and the active pharmaceutical ingredients will be called DS A and DS B.

3.1. IVR Development

To assess what is the best method for IVR test of BlueLAI technology the advantages and disadvantages of each method were considered, including reproducibility, cost, and ease of sampling. The IVR test of some similar products already approved were compared too. Although the flow-through method appears to be a first choice to simulate the *in vivo* environment by constantly unidirectional circulation, the use of this apparatus was discarded due to limited access during my internship. Alternatively, the shaking flask method was the method chosen as, as presented above, it´s simple and easy to use and allows the adjustment of several parameters such as sample vial/vessel size and agitation speed in order to meet our requirements. Moreover, it is a method commonly used in LAI formulations, including some already approved drug products, such as Zynrelef™.

The parameters of the preliminary IVR test (based on chapter 2.9.3. of Ph. Eur.) are described in **Table 7**, as well as the rationale behind the choice.

Table 7. Parameters and rationale of IVR test for BlueLAI.

Parameter	Choice and rationale
Method:	**Shaking flask** – It is simple and easy to use. Moreover, the medium replacement can be tuned to assures sink conditions.
Apparatus:	**Orbital agitator** – Allows the use of various container sizes and agitation speeds. **Incubator** – allows setting the temperature to 37 ± 0.5 ºC, the average temperature of the body.
Barrier:	**Agarose gel** – mimic subcutaneous tissues once this gel has a similar rheologic nature (as viscosity) and water content to the *in vivo* conditions.
Release medium:	**PBS pH 7.4** – Phosphate Buffered Saline at pH 7.4 mimics the pH and the osmotic pressure of blood. It is widely used in the literature, as well as in drug products already approved.
Sampling technique:	**Sampling with replacement –** Release medium is collected for analysis and replaced with a fresh medium. Medium replacement can be tuned to assures sink conditions. In the beginning, samples are collected with closer time points to detect possible burst release.
Analytical method:	**HPLC-PDA –** High-performance liquid chromatography coupled with a photodiode array allows to separate and analyze each DS in a complex matrix. The method should be validated for Specificity, System Precision, Linearity and Accuracy.
Quantity of BlueLAI:	**200 µL** – A small volume allows a lower cost. Moreover, similar approved drug products use the same volume.

3.1.1. PBS pH 7.4 stability

As explained before, an IVR method for the BlueLAI with a prolonged release during at least 96 hours has been developed. It is important to assure that the conditions that could change the release profile or compromise the biorelevance of the test are maintained, such as temperature, agitation rate, and the release medium. Thus, the PBS pH 7.4 should present a shelf life of at least 96 hours.

At Bluepharma, solutions prepared with 100% of ultrapure water, as is the case with PBS pH 7.4, have a shelf life of 3 days. The stability of PBS pH 7.4 was evaluated in two conditions: room

temperature (about 18 to 25ºC) and the temperature at which the IVR test is performed (37±0.5ºC). Three parameters were used: pH, osmolality, and appearance. This release medium is chosen because it confers biorelevance to the method, once the pH and osmolality values are similar to those of the human body. So, it is important to assure that the pH and osmolality are between the acceptance range and the biorelevant conditions are guaranteed during all the IVR test. Moreover, the appearance analysis allows detecting some precipitate or color changes that could indicate contamination in the solution.

The PBS pH 7.4 solutions were prepared according to the following procedure: 8.0 g of sodium chloride (NaCl), 0,20 g of potassium chloride (KCl), 1.44f of disodium hydrogen phosphate (Na_2HPO_4) and 0.24g of potassium dihydrogen phosphate (KH_2PO_4) were weighted into a 1000mL clean and dry volumetric flask and dissolved with 900 mL of ultrapure water. The pH was adjusted to 7.4 with hydrochloric acid (HCl) 37% and then the volume was makeup with ultrapure water. Three different solutions of PBS pH 7.4 were prepared to carry out this study. About 90 mL of each prepared PBS pH 7.4 solution is placed in the benchtop in the laboratory, at room temperature and the other 90 mL is placed in the incubator at 37±0.5ºC. The appearance, pH, and osmolality tests were determined after preparation (t0) and daily during 96 hours (4 days), the time of the IVR test.

For pH measurement, about 3mL of each solution was placed into a falcon tube. The solutions stored in the incubator need to be at room temperature for the pH measurement (pH measurement should be between 20 and 25 ºC, according to Ph. Eur). The acceptance limit of pH is 7.4±0.05 pH units. The analysis is made concerning the target pH value of 7.4. The results of pH measurements are exposed in **Table 8** and schematized in a graph in **Figures 16** and **17** for room temperature and 37ºC, respectively.

Table 8. Results of pH measurement of PBS pH 7.4 (of the three prepared batches) for 4 days of study, stored at Room temperature (RT) and in the incubator at 37ºC.

	pH					
	#1		**#2**		**#3**	
Time (days)	RT	37ºC	RT	37ºC	RT	37ºC
0	7.401		7.393		7.396	
1	7.397	7.395	7.391	7.397	7.395	7.398
2	7.404	7.409	7.399	7.401	7.402	7.407
3	7.391	7.397	7.398	7.393	7.399	7.398
4	7.399	7.405	7.400	7.402	7.399	7.402

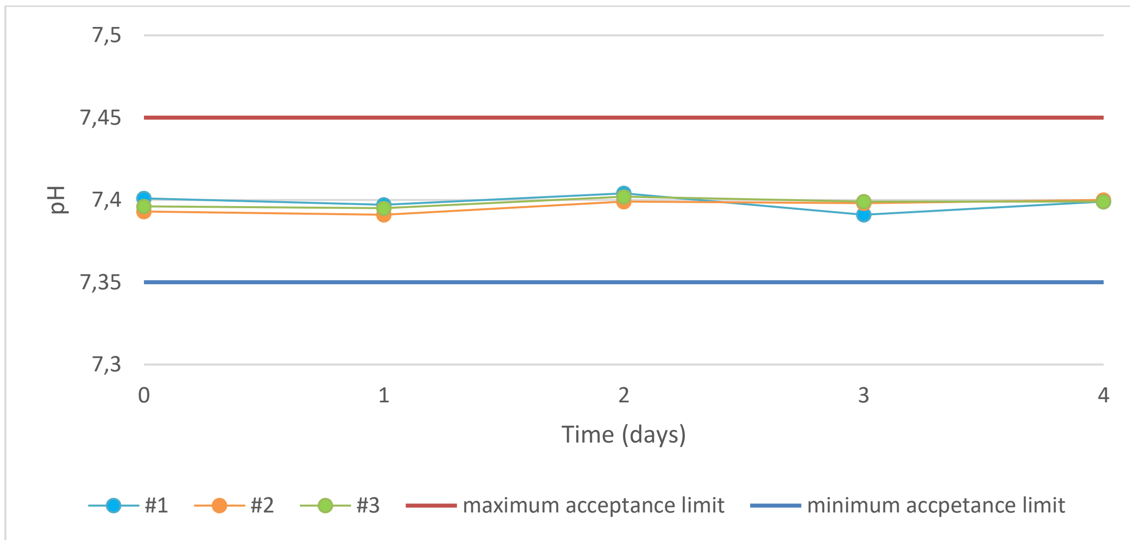

Figure 16. Graph of pH measurement as a function of time (days) for the three preparations of PBS pH 7.4, stored at Room temperature (RT).

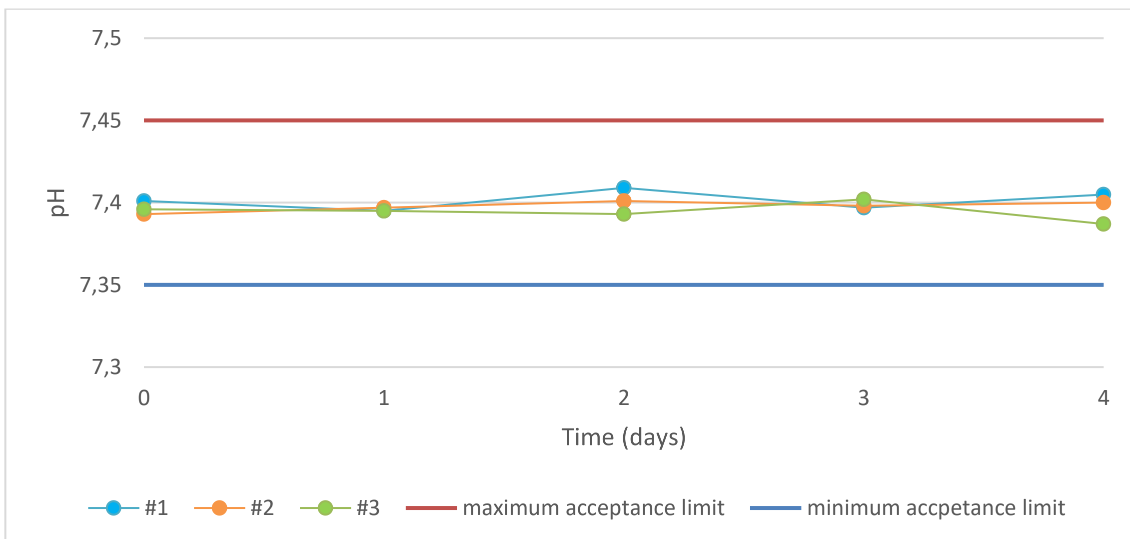

Figure 17. Graph of pH measurement as a function of time (days) for the three preparations of PBS pH 7.4, stored at 37ºC.

Assuming that the acceptance range for pH measurements is 7.4± 0.05 pH units, it is observed that the pH values measured on the different days of analysis comply with the limit of acceptance for the target pH value. It should be noted that the temperature of the solutions varied in general between 20.6 and 21.0ºC, with no major changes being observed.

For osmolality assessment, 100 µL of each solution were placed into the tube and the osmolality was read in an osmometer. The measurement is repeated 4 times for each solution. The solution stored in the incubator should also be at room temperature for the osmolality measurement. The acceptance limit of osmolality is between 275 and 300 mOsm/kg, the same osmolality of the human plasma. The analysis of osmolality is made concerning the target osmolality value (275-300 mOsm/kg). The results are exposed in **Table 9** and schematized in **Figures 18** and **19**.

Table 9. Results of osmolality measurement of PBS pH 7.4 (of the three prepared batches) for 4 days of study, stored at Room temperature (RT) and in the incubator at 37ºC.

	Osmolality (mOsm/kg)					
	#1		**#2**		**#3**	
Time (days)	RT	37ºC	RT	37ºC	RT	37ºC
0	286		285		285	
1	286	286	285	282	286	284
2	285	287	285	286	284	284
3	285	286	284	284	283	284
4	285	283	286	284	284	284

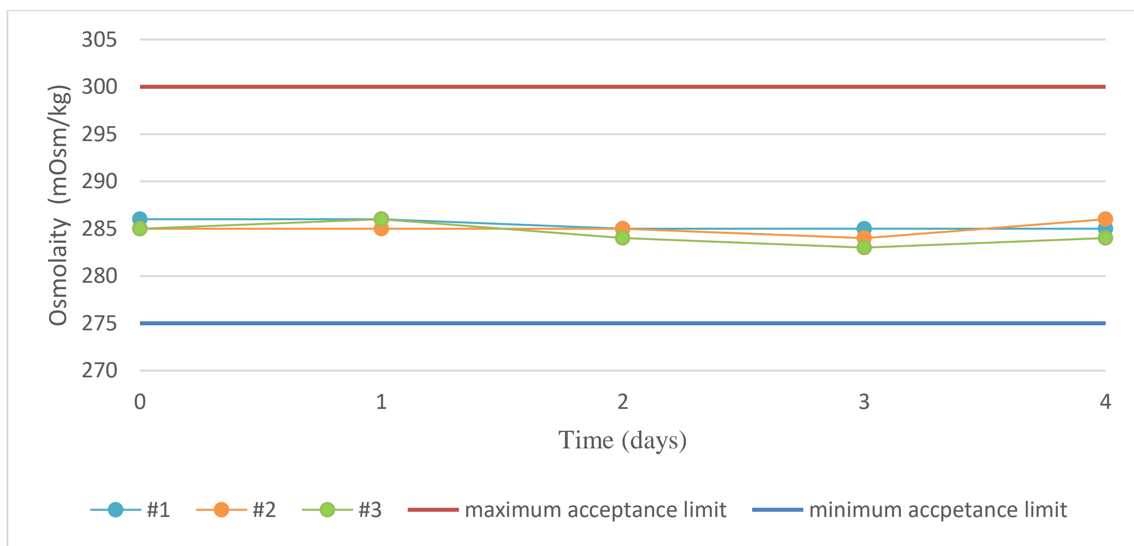

Figure 18. Graph of osmolality measurement as a function of time (days) for the three preparations of PBS pH 7.4, stored at Room temperature (RT).

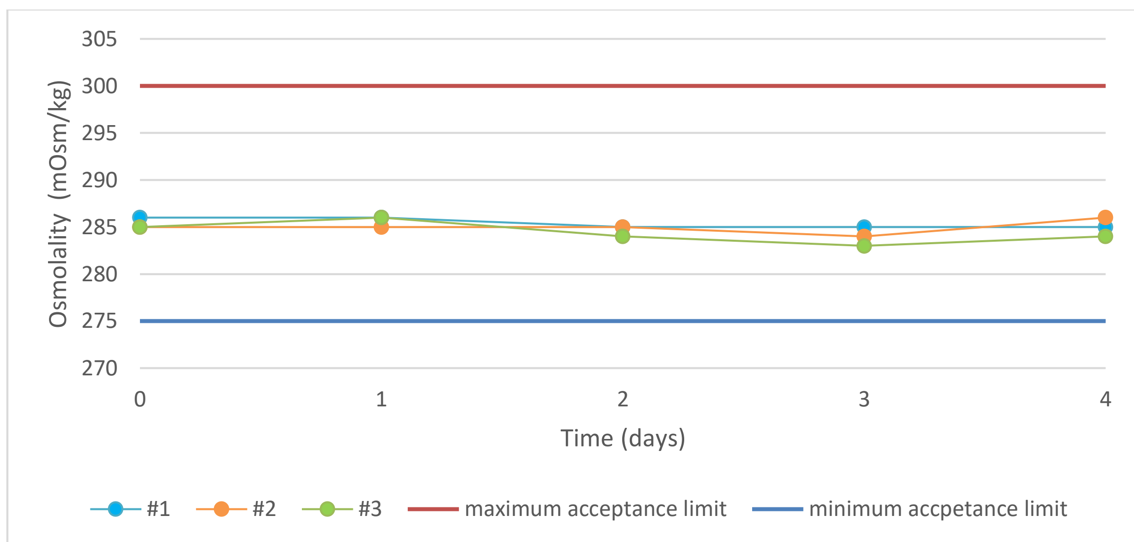

Figure 19. Graph of osmolality measurement as a function of time (days) for the three preparations of PBS pH 7.4, stored at 37ºC.

Assuming that the acceptance range for osmolality measurements is 275-300 mOsm/kg, it is observed that the osmolality values measured on the different days of analysis comply with the limit of acceptance for the target osmolality value.

The appearance consists of a visual inspection of the solution that focuses on the clarity of the formulation, the color, the presence of particles of foreign matter, the precipitation occurrence, phase separation, and deposition. During the 4 days, the three preparations of PBS pH 7.4 under study did not show the evolution of the color and clarity of the solution. There was no deposition or phase separation.

Through this study and evaluating the three parameters under study (appearance, pH and osmolarity) of three PBS pH 7.4 solutions it is possible to extend the shelf life of the PBS pH 7.4 to 4 days to use in the IVR test without impact in the study, both in room temperature and 37ºC.

3.1.2. Agarose gel as a barrier to IVR test

The rationale for the use of agarose gel as a barrier in this IVR method is explained in chapter 2.4.3.2. This current chapter will be discussing the process used to create agarose gel with a cavity with an expected volume of 200 µl to put BlueLAI formulation.

The agarose solution was prepared in two concentrations: 1% and 2%. The agarose power is added to PBS pH 7.4 at 85ºC, and dissolved with constant agitation (about 15 minutes) to avoid gelification.

Two methods were tested for agarose gel shaping using well-plates, both to maintain the formulation evenly separated from all ends of the gel. The apparatus was the same for both methods and consists of well-plate cell culture with small holes in each well used to inject air and remove the gel after gelification. As proof-of-concept syringe protection caps were used to make the cylindrical cavity. They were attached to the cover of the well-plate cell culture in other to ensure their immobilization, as showed in **Figure 20**.

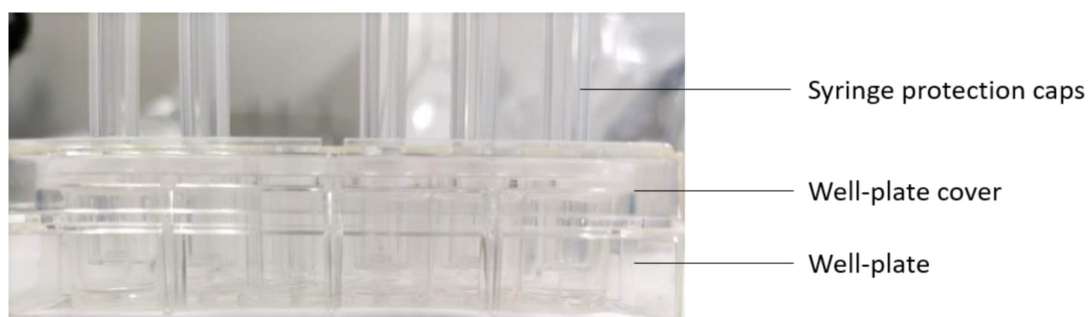

Figure 20. Apparatus used: well-plate with small holes in each well and the cover with the cylinders (syringe protection caps).

As presented above, 200 µl of BlueLAI will be used in each test. For that is necessary a volume of 200 µl in the cavity. The ideal measures of the cylindrical object used to create the cavity were calculated, following the rationale showed in **Table 10**.

Table 10. Measures of the well-plate and the cavity formed by the cylinder to put the LAI formulation.

	Well measures	**Cavity measures**
Radius (mm)	5.5	2.52
Height (mm)	17	10
Volume (µL)	1615.6	199.5

The cavity must be evenly separated from each end of the agarose gel. Knowing the height occupied by the formulation and the total height, the height at which the cavity is at the top and bottom was calculated, using the following formula:

$$Vd = \frac{Height_{well} - Height_{cavity}}{2} = \frac{17 - 10}{2} = 3.5\ mm$$

$$Hd = \frac{Diameter_{weel} - Diameter_{cavity}}{2} = \frac{11 - 5}{2} = 3\ mm$$

Where:

- Vd - Vertical distance between cavity and gel;
- Hd - Horizontal distance between cavity and gel.

The cavity needs to be 10mm (height of the cavity) plus 3.5mm (the top distance of the cavity to the top point), which is 13.5 mm. So, the syringe protection caps need to be immobilized 13.5 mm from the top and 3mm from the edges.

3.1.1.1. Method A for agarose gel shaping

This method is divided into A1 and A2, represented in **Figures 21** and **22**, respectively. The difference between the two methods is the volume of agarose solution used in the first layer, and consequently, the second volume. The final volume in each case is equal. In method A1, the cavity has only the BlueLAI volume, unlike the A2, in which a little space remains in the cavity after BlueLAI addiction. Thereby, in method A2, is formed a kind of stopper (with an indent).

The holes were covered with self-adhesive tape and agarose solution is added into the well (about 1000µL in method A1 and 1200µL in method A2). The cover with the syringe protection caps attached with 13.5 mm of depth and 2.5 mm from the edges is added. After gellification (about 20 minutes at room temperature) the syringe protection cap is removed. BlueLAI (200

µL) is placed in the cavity and the remaining agarose solution is added into the well (about 400µL in method A1 and 200µL in method A2). After remove the self-adhesive tape, using a syringe air is pushed through the hole and the agarose gel is removed. The cylinder of agarose gel with the BlueLAI inside is then placed in a container with 100-300 mL of PBS pH 7.4 at 37ºC, starting the IVR test. At each collection time point, release medium is collected for analysis and replaced with a fresh medium.

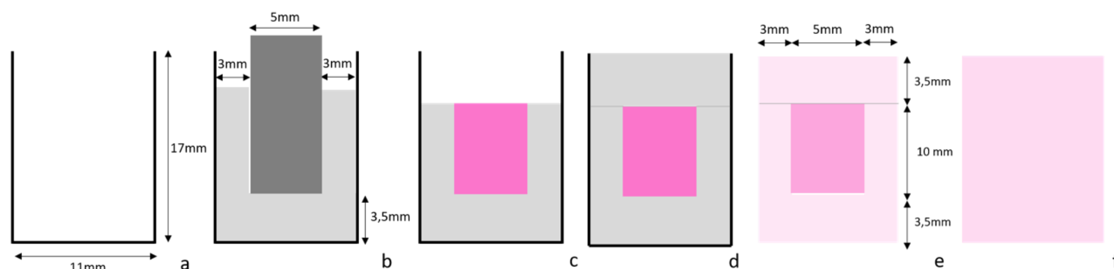

Figure 21. Schematic presentation of the method A1 for agarose gel shaping. a) Well dimensions b) Addition of first layer of agarose gel (light grey) with syringe protection caps (dark grey) to do the cavity for the formulation c) Addition of BlueLAI formulation (pink) d) Addition of the second layer of agarose gel (light grey) e) Final agarose gel with the agarose on light grey and formulation in pink f) Agarose gel after diffusion.

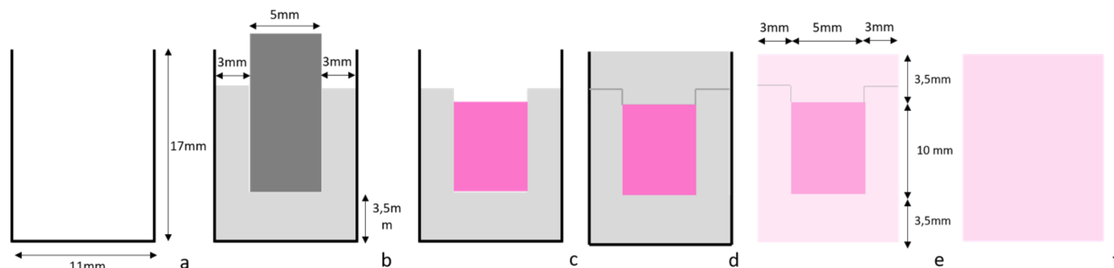

Figure 22. Schematic presentation of the method A2 for agarose gel shaping. a) Well dimensions b) Addition of first layer of agarose gel (light grey) with syringe protection caps (dark grey) to do the cavity for the formulation c) Addition of BlueLAI formulation (pink) d) Addition of the second layer of agarose gel (light grey) e) Final agarose gel with the agarose on light grey and formulation in pink f) Agarose gel after diffusion.

Figure 23 shows the application of the method A2 for agarose gel shaping. A pink dye was used in a simulated formulation to visually represent the diffusion of the DS in the gel over time.

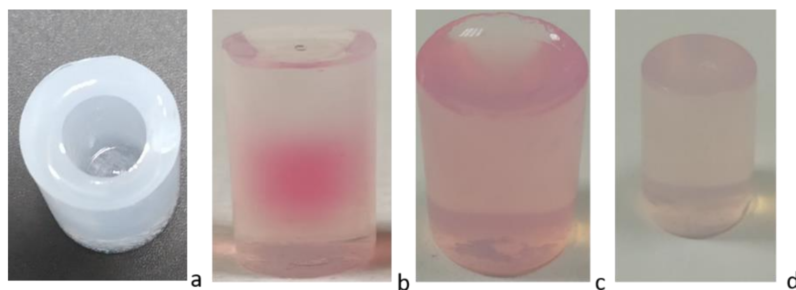

Figure 23. Application of method A2 for agarose gel shaping. a) First layer of agarose gel b) Addition of formulation (pink) and the second layer of agarose gel c) Agarose gel after 24 hours d) Agarose gel after 7 days in PBS pH 7.4.

The major disadvantage of this method is that if the BlueLAI does not gelificate instantaneously or in a short time (as desired), the DS diffusion in the first layer of gel starts before the agarose gel is in the release medium, whereby the IVR testing will not start in timepoint 0.

3.1.1.2. Method B for agarose gel shaping

This method intends to delete the major disadvantage of method A, allowing the injection of BlueLAI in an already done cylinder of agarose gel with a cavity.

The holes were covered with self-adhesive tape and about 1200 µL of agarose solution is added into the well. The cover with the syringe protection caps attached with 13.5 mm of depth and 2.5 mm from the edges is added. After gelification (about 20 minutes at room temperature) the syringe protection cap is removed. After remove the self-adhesive tape, using a syringe air is pushed through the hole and the agarose gel is removed. The holes are re-covered with self-adhesive tape and about 200 µL of agarose solution was added to the well. Immediately next, the agarose gel is turned with the cavity face down carefully to prevent agarose solution from entering the cavity. A small hole in the gel was made to remove the air that remains in the well that prevents the first layer from descending against the second. After gelification, BlueLAI is injected into the cavity. After remove the self-adhesive tape, using a syringe air is pushed through the hole and the agarose gel is removed. The procedure is schematized in **Figure 24**. The cylinder of agarose gel with the BlueLAI inside is then placed in a container with 100-300 mL of PBS pH 7.4 at 37ºC, starting the IVR test. At each collection time point, release medium is collected for analysis and replaced with a fresh medium.

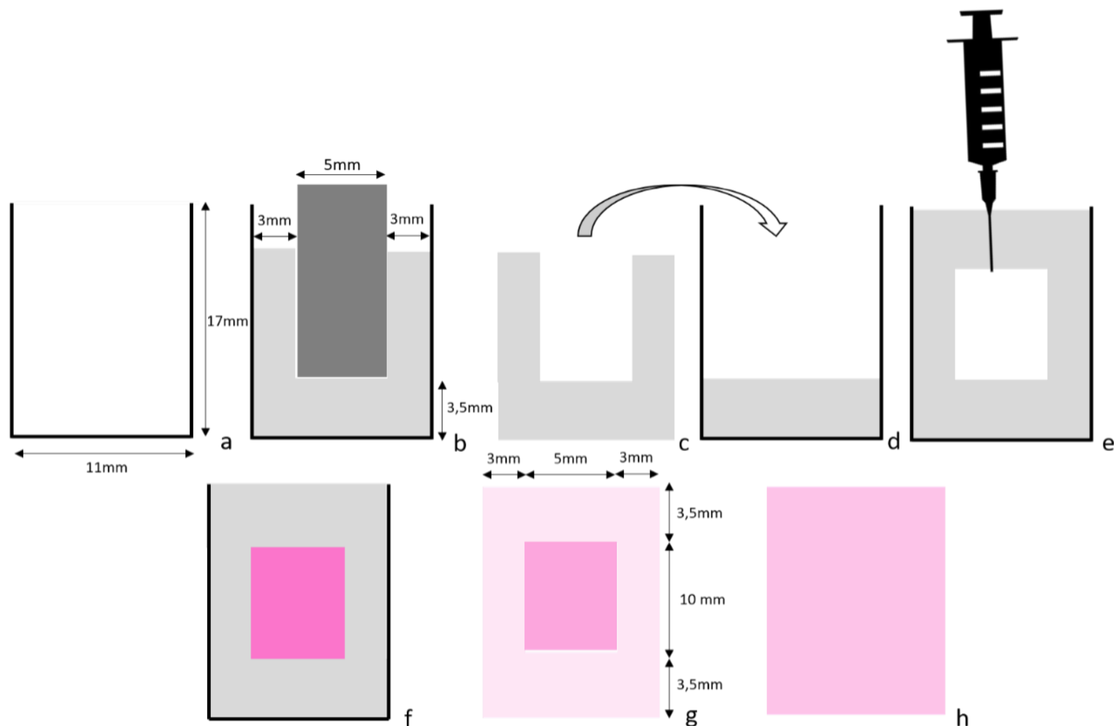

Figure 24. Schematic presentation of method B for agarose gel shaping. a) Well dimensions b) Addition of first layer of agarose gel (light grey) with syringe protection caps (dark grey) c) Removing of the first layer from the well d) Addition of second layer of agarose gel (light grey) e) Junction of the first inverted layer to form a cavity in the gel f) Addition of BlueLAI formulation (pink) g) Final agarose gel with the agarose on light grey and formulation in pink h) Agarose gel after dye diffusion**.**

3.1.1.3. Method C for agarose gel shaping

Another possible approach is to put the 200µL of BlueLAI formulation in the bottom of the container (approx. 300ml), wait until gelification, and then put a certain volume of agarose gel. After agarose gelification, the release medium is added, as illustrated in **Figure 25**.

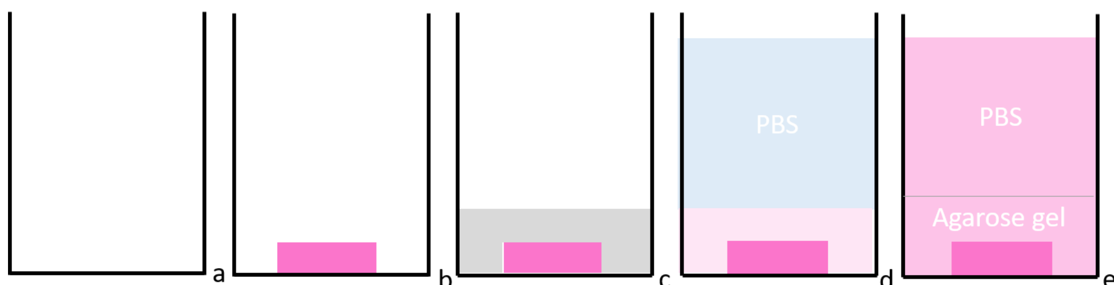

Figure 25. Schematic presentation of method C for agarose gel shaping. a) Container b) Addition of BlueLAI formulation (pink) c) Addition of agarose gel (light grey) d) Addiction of PBS pH=7.4 (blue) e) After diffusion.

This method does not allow the presence of release medium in all surfaces of agarose gel. On other hand, it is very reproducible and has slight human manipulation. Moreover, it allows the

BlueLAI gelification before the addition of agarose gel, allowing to start the IVR at the beginning of diffusion.

The advantages and disadvantages of using the well-plate to shape the agarose gel, both with method A and method B, and method C are presented in **Table 11**.

Table 11. Advantages and disadvantages of the well-plate used to shape the agarose gel**.**

	Advantages	**Disadvantages**
Method A1	Several volumes available Well-plate is reusable Homogeneous cylinders	If the BlueLAI does not gel instantaneously, the DS diffusion starts before the agarose gel is in the release medium
Method A2	Several volumes available Well-plate is reusable Homogeneous cylinders Separation of the two layers is more difficult	If the BlueLAI does not gel instantaneously, the DS diffusion starts before the agarose gel is in the release medium
Method B	Several volumes available Well-plate is reusable When the formulation is injected is immediately put in the release medium	Not homogeneous cylinders Formation of air bubbles between the two layers Hole made by the syringe to inject the formulation
Method C	Reproducible Easy	There is no medium in all surfaces Possibility of detaching from the base of the bottle (evaluated when the apparatus is in-house)

The method can only be chosen and validated when the final BlueLAI formulation is ready, approaching the advantages and disadvantages of each method.

3.1.2.1. Evaluation of agarose gel integrity in conditions used to IVR test

This study aims to evaluate the integrity of the agarose gel when exposed to IVR conditions. As explained before, the agarose gel, along with the BlueLAI is intended to stay in PBS pH 7.4 at 37ºC under constant agitation for at least 4 days during IVR test. It is important to assure that the BlueLAI formulation stays within the agarose gel for at least this period.

For this evaluation, method A2 was used to shape the agarose gel once this was the most promising method until the date of the test. However, when the apparatus (containers and orbital agitator) is available in-house, this study will be repeated with method C.

After modelling, each agarose gel cylinder was placed in a 50 mL falcon tube with 45 mL of pre-heated PBS pH 7.4, with 6 different samples. The tubes were then placed on a test tube rotator, as showed in **Figure 26**, under agitation. The rotator went to the incubator at 37ºC for 8 days. The tubes were daily observed and photographed.

Figure 26. Apparatus used to asses agarose integrity: test tube rotator with 45 mL PBS pH 7.4 and agarose gel shape using method A2.

Only the first falcon tube presented a thin gel layer released from the rest. However, there was a problem when this gel was done. When placing the second layer in the first well, a part was solidified at the tip, so more agarose solution had to be added. Due to the closeness between temperature of the agarose in the bath (45ºC) and the temperature of gelification (35-39ºC), it quickly gelified and the rest of the second layer was applied with the initial part already solidified, creating a third layer. This third layer went off the agarose gel cylinder. This reinforces the idea that using the method A2, in which a stopper is formed, the second layer is more attached to the first layer due to more contact area. The other tubes showed no problems of detachment or damaged edges. After 8 days, the PBS pH 7.4 of each tube was pink, as well as the agarose gel.

In the IVR test, at each collection time point, release medium is collected for analysis and replaced with a fresh medium. Then the DS is quantified by a suitable and validated method. The next chapter presents the method used.

3.2. Hight-Performance Liquid Chromatography-Photodiode Array (HPLC-PDA)

To establish the profile of *in vitro* release of the developed formulation, it is necessary to quantify the DSs during the release, in this case, DSs will be quantified using High-Performance Liquid Chromatography coupled with a Photodiode Array Detector (HPLC-PDA).

Chromatography is a process that separates chemical species which the fundamental driving force is the chemical equilibrium that results when a species distributes between two phases. The physical state of the phases variates according to the type of chromatography, as presented in **Table 12**. The HPLC technique is based on the sharing of the analyte between a liquid mobile phase and a solid stationary phase, fixed in a column [164].

Table 12. Types of chromatography, mobile and stationary phases characteristics.

Types of chromatography	Mobile phase	Stationary phase
Gas chromatography	Gas	Solid/Liquid
Liquid chromatography	Liquid	Solid/Liquid
Supercritical fluid chromatography	Supercritical fluid	Solid/Liquid

As illustrated in **Figure 27**, the HPLC consists of:

- A set of reservoirs containing the mobile(s) phase(s);
- A pump that forces the mobile phase to pass through the high-pressure system;
- An injector that introduces the sample into the mobile phase from an automatic sample collection device (autosampler);
- A chromatographic column suitable for analysis;
- An oven, which has the objective of adjusting the analysis temperature of the column;
- A detector that, when in contact with the analytes present in the eluent, emits electrical signals that are recorded in the form of peaks (there is detection by UV, fluorescence, mass spectrophotometry, etc.)
- A computer with data acquisition and processing software.

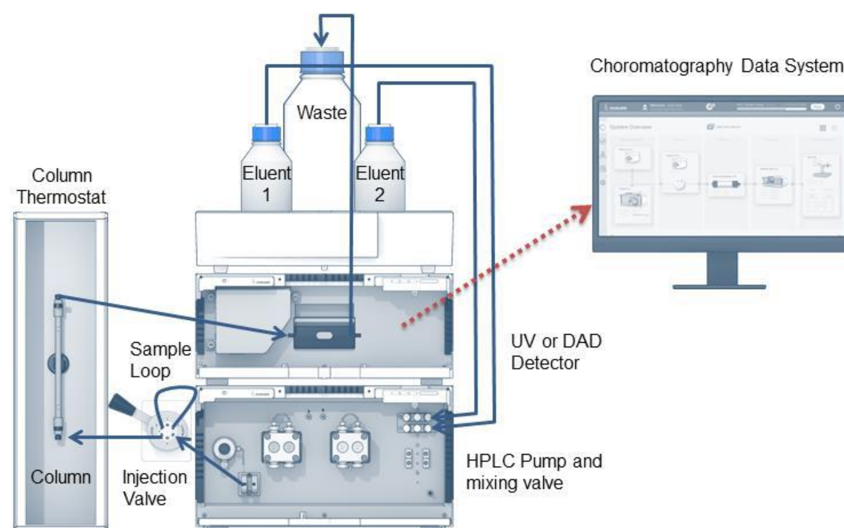

Figure 27. Schematic structure of the HPLC equipment, presenting the detector, the pump, the injector, the column, and the computer/software (from [165]).

The separation of the analytes in the HPLC method is illustrated in **Figure 28**. Basically, the rationale for separation is as follows:

1. The injector introduces the sample with two compounds (here represented as **Green** and **Blue**) into the mobile phase from the autosampler and the pump forces the sample with the mobile phase to pass through the high-pressure system.
2. The compounds are traveling at the same rate as the rate of flow on the mobile phase (t1 of **Figure 28**) until they reach the stationary phase. Then, **Green** and **Blue** are attracted with different forces to the stationary phase, which slows down the rate of travel.
3. The time used for a substance to travel in the stationary phase until it reaches the detector is the retention time (RT). The greater the affinity for the stationary phase, the longer the retention time.
4. If **Green** has a greater affinity to the stationary phase than **Blue**, **Green** will be more time in the stationary phase and travel more slowly (t2 of **Figure 28**). This results in a separation of **Green** and **Blue** (t3 of **Figure 28**).

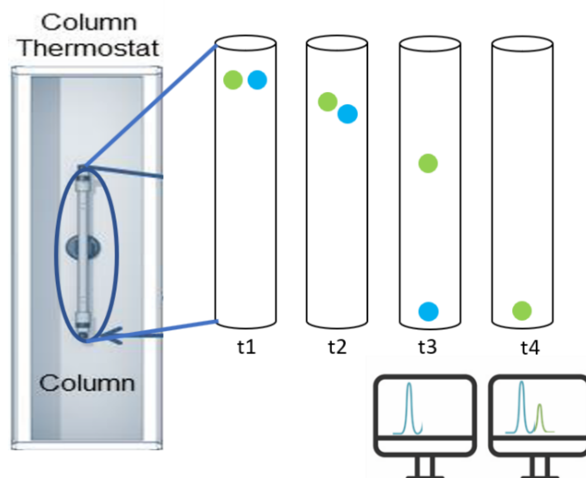

Figure 28. Representation of the separation of two compounds, Green and Blue, between the mobile phase and stationary phase in the HPLC method, focusing in the column

The HPLC types can be separated into normal phase HPLC and reverse-phase HPLC due to the polarity of the stationary phase and mobile phase, according to **Table 13**.

Table 13. Normal phase and reverse phase chromatography characteristics.

Types of chromatography	Mobile phase	Stationary phase
Normal phase	Polar	Non-polar
Reverse phase	Non-polar	Polar

In the normal phase HPLC, the stationary phase is polar and the mobile phase is apolar. The more polar analytes will have more affinity to the stationary phase than the less polar ones. The least polar analytes will eluate first and have a smaller retention time. In this case, the attractive forces that link the analyte to the stationary phase are dipole-dipole and hydrogen bonder (polar) interactions.

In the reverse phase HPLC, the most common one, the stationary phase is apolar and the mobile phase is polar. So, fewer polar analytes will be retained in the stationary phase, and the polar ones will eluate first. The attractive forces present in these interactions are non-specific hydrophobic interactions.

According to the concentration of the mobile phase that is pumped, HPLC analysis can operate in two modes: isocratic or gradient. In isocratic methods, the mobile phase has a constant concentration, while in gradient elution the mobile phase has a varying concentration. The mobile phase has two components: a weak solvent and a strong solvent. Weak solvent allows

the solute to elute slowly while strong solvent causes the rapid elution of the solute. In reverse phase chromatography, an aqueous solution is used as the weak solvent and an organic solution as the strong solvent [166].

Isocratic method disadvantages are a limited number of peaks that can be accommodated in the chromatogram, problems with samples containing analytes of varied polarities. Also, late eluters (such as dimers) are particularly difficult to quantitate in the isocratic analysis due to excessive band broadening with long retention times [164].

Gradient methods are suitable for complex samples and analytes of wide polarities. The gradient analysis yields better separation for early peaks and sharper peaks for late eluters. The disadvantages of gradient analysis are the requirements for greater skills in method development, longer run times, and difficulties in method transfer [164].

Finally, the analyte achieves the detector. The most common detectors are absorbance detectors such as UV/Vis or photodiode array detectors (PDA). However, other detectors can be used such as fluorescence, refractive index, evaporative light-scattering, electrochemical, conductivity, radioactivity, and mass spectrometry [164]. Unlike the UV detector, the Photodiode-Array Detection (PDA) or Diode-Array Detection (DAD) detects an entire spectrum simultaneously. So, UV and VIS detectors visualize the results in two dimensions (light intensity and time), while PDA has three dimensions (adding wavelength). This allows the analyst to determine which is the most suitable wavelength without repeating analyses [164].

Functionally, in a PDA detector, the light from the deuterium or tungsten lamp shine onto the flow cell and reaches the diffraction grating. In the diffraction grating, the light is directed onto a linear array of photodiodes and the diode array measures the intensity of light at each wavelength. The representation of a PDA detector is presented in **Figure 29**. Typically, 512 or 1024 diodes are used, allowing a spectral resolution of about 1nm and covering wavelengths from 190 nm up to 800 nm [164], [167].

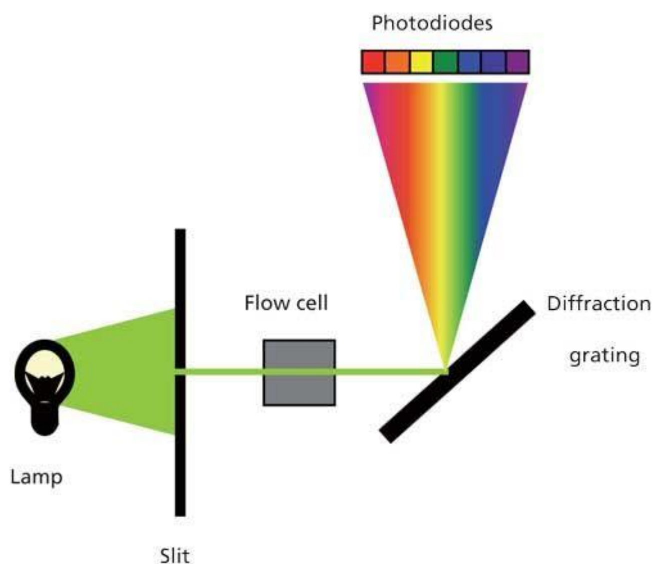

Figure 29. Schematic diagram for a photodiode-array (PDA) UV detector (from [167]).

The IVR method was developed in HPLC with reverse phase separation, running a gradient method and copled with a Phosphodiode Array Detector (PDA). Analytical development of this method will be presented in the next chapter.

3.2.1. Analytical method development

The HPLC-PDA method used to quantify the DSs in IVR test was based on a pre-existent method of Assay for the same project, already pre-validated to system precision, linearity, and selectivity (including degradation products). So, using this method some improvements were done.

The presented method presents a running time of 15 minutes, a suitable running time for the assay method for two DSs, considering the use of the gradient method. **Figures 30** shows the graph of concentration of mobile phase A and B (%) as a function of time (minutes), with chromatogram at wavelength-1 (WVL-1) and wavelength-2 (WVL-2) for detection of DS A and DS B at 100%, for the method of assay.

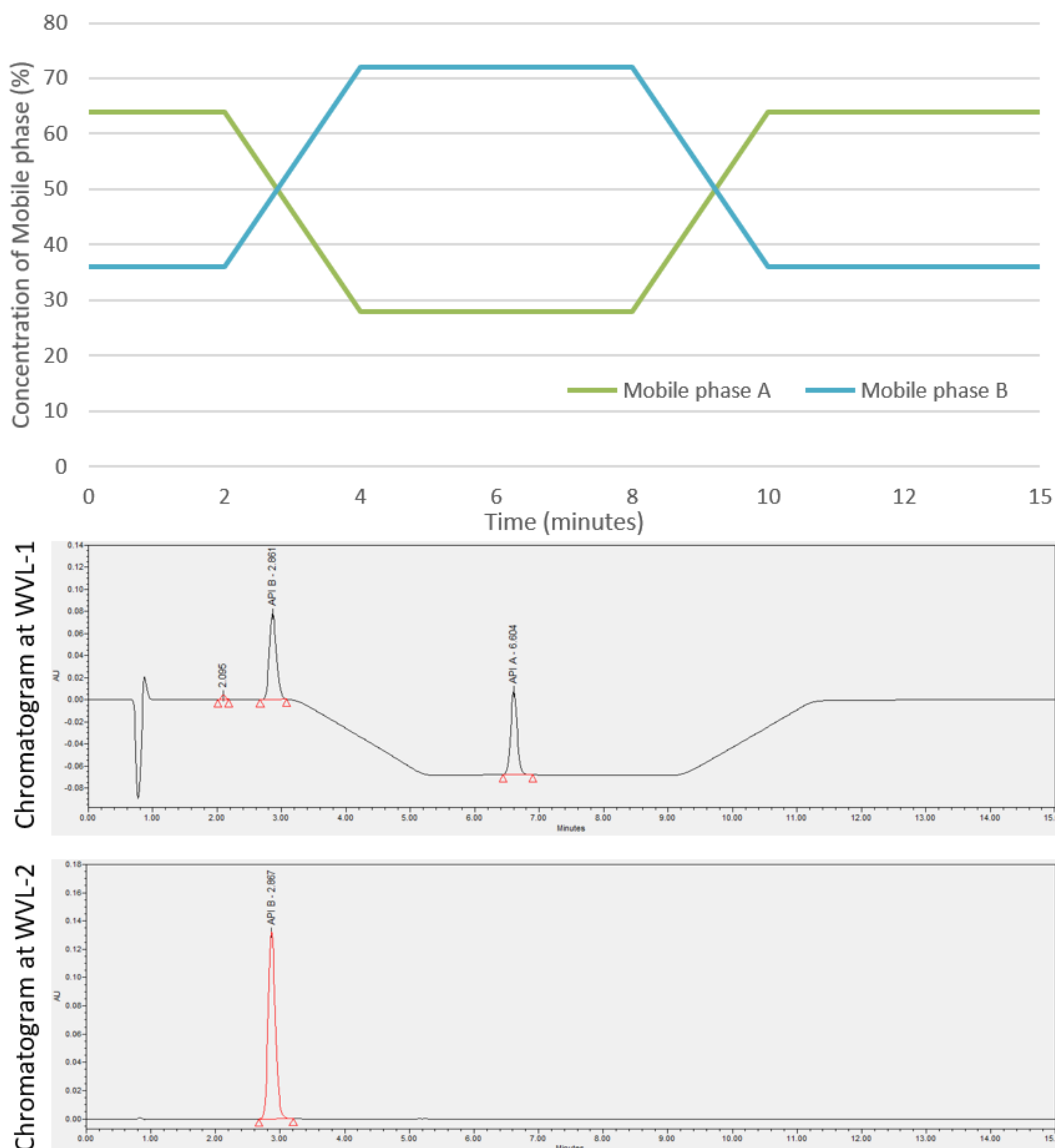

Figure 30. Graph of concentration of mobile phases A and B as a function of time (minutes) for the method of assay, with chromatogram at WVL-1 and WVL-2 for injection of DS A and DS B at 100%.

Although this method is suitable for the assay, the running time can be a problem for the IVR method. The IVR test is performed with 6-12 samples per time point, with an estimated collection of 15-20 time points per study. This represents a very high number of samples per study. Thereby, the reduction of the running time is a positive upgrade to the HPLC-PDA method development. The time was then reduced to 8 minutes but it was found that it was not enough to stabilize the pressure at the end of the chromatogram, before starting the new reading. Thus, it was increased to 10 minutes to achieve complete stabilization. So far, the 10 minutes seems to be a suitable running time and a good reduction from the initial 15 minutes. **Figures 31** shows

the graph of concentration of mobile phase A and B (%) as a function of time (minutes), with chromatogram at WVL-1 and WVL-2 for injection of DS A and DS B at 100%, for the IVR method.

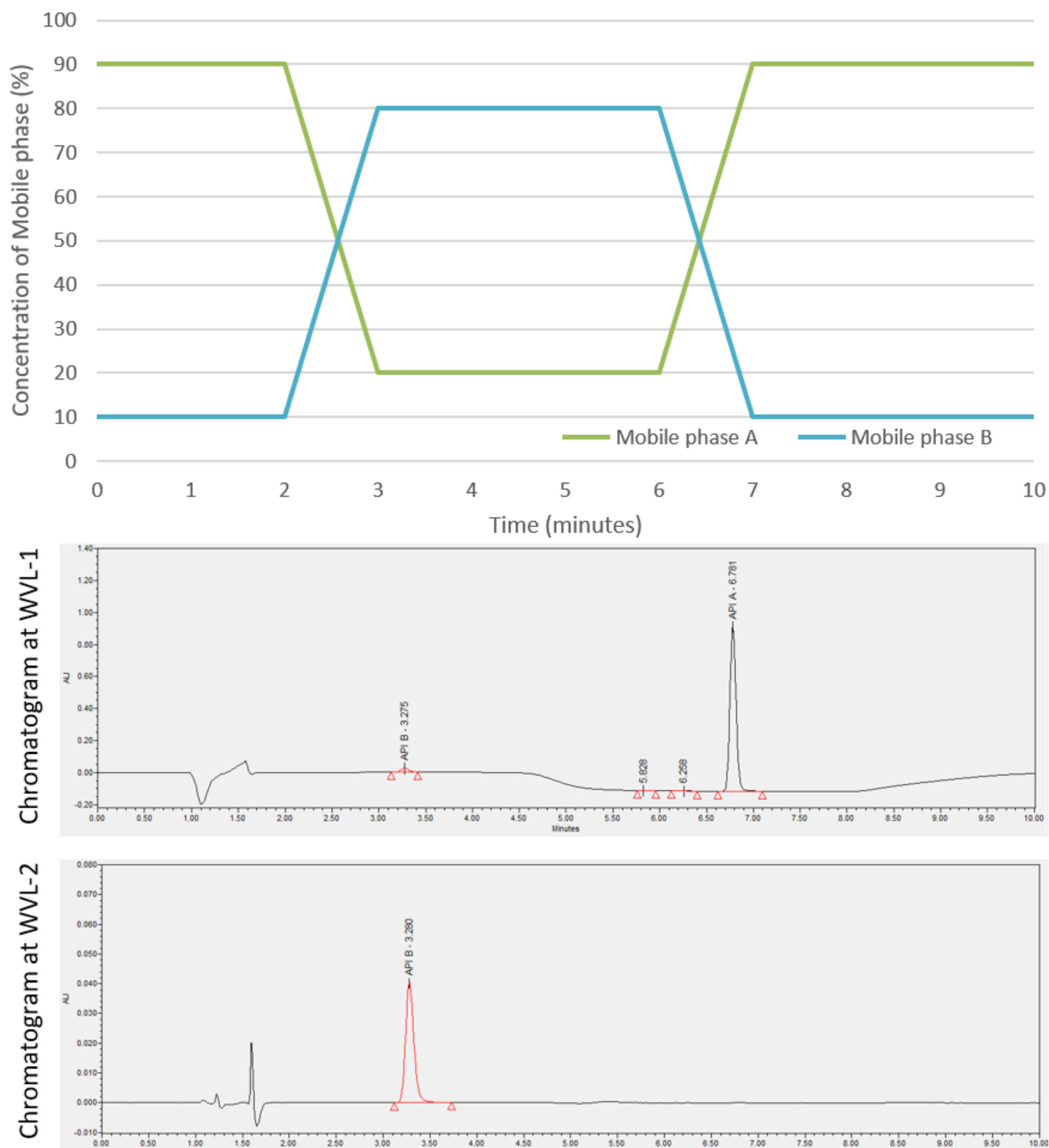

Figure 31. Graph of concentration of mobile phases A and B as function of time (minutes) for the method for IVR test, with chromatogram at WVL-1 and WVL-2 for injection of DS A and DS B at 100%.

3.2.2. Method pré-validation

As approached above, the HPLC method should be validated according to ICH Q2. The analytical procedure used in the IVR needs to be validated for specificity, precision, linearity, accuracy and stability.

3.2.2.1. Specificity

The specificity is the ability to assess unequivocally the active ingredient in the presence of components expected to be present in the sample matrix, including inactive ingredients, impurities, degradants, and matrix [86]. The IVR method does not need to be a stability indicator like the assay method that should detect the presence of impurities and/or degradation products in the formulation.

For chromatographic procedures, like HPLC-PDA, representative chromatograms must be presented to demonstrate specificity and individual components should be appropriately labelled [86]. For structural reasons, DS A and DS B are analyzed in channels with different wavelengths, here represented as wavelength-1 (WVL-1) and wavelength-2 (WVL-2), respectively.

The following solutions were injected and analyzed according to the analytical method:

- PBS pH=7.4;
- Preliminary placebo composition;
- Standard Solution at 5% for each DS (Quantification Limit – lowest concentration of a substance that is possible to be quantified);
- Standard Solution at 100% for each DS (maximum theoretical concentration present in the release medium in the IVR test).

The DS should have no interference from the excipients, presenting a peak completely separated from each other and any unknown peaks due to the excipients or solvent.

The chromatograms presented in **Figures 32** and **33** are obtained by the analysis of PBS pH=7.4, the release medium, which is used as blank in this analysis, extracted in both WVL-1 and WVL-2, respectively.

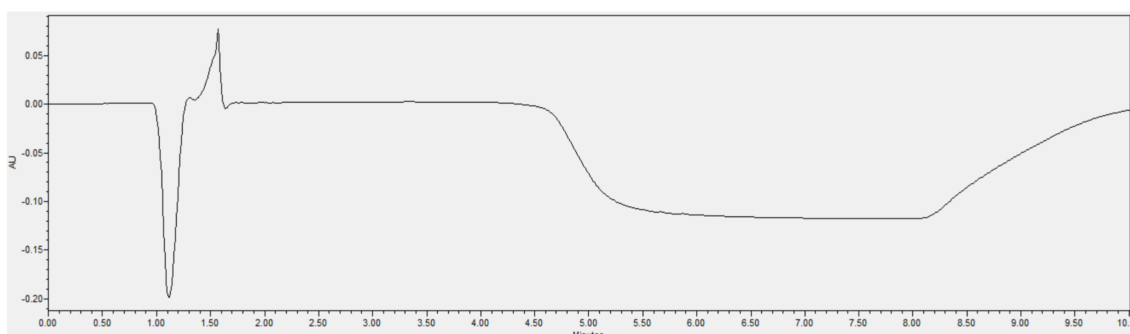

Figure 32. Chromatogram at WVL-1 for injection of PBS pH=7.4.

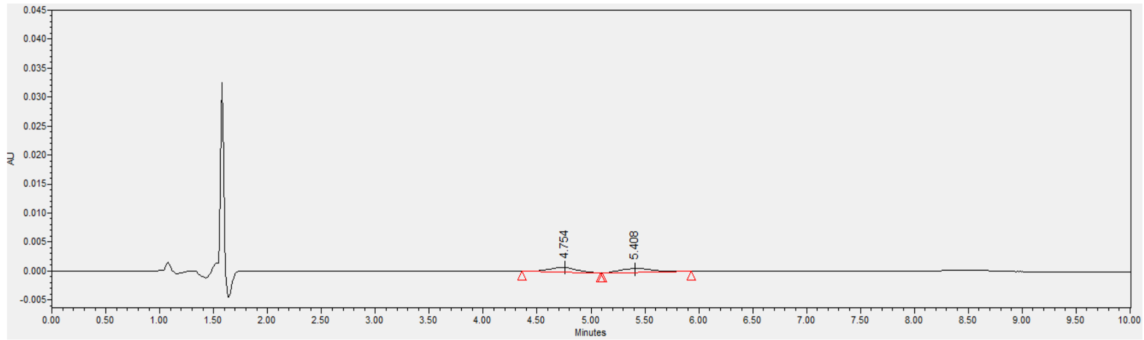

Figure 33. Chromatogram at WVL-2 for injection of PBS pH=7.4.

The chromatograms presented in **Figures 34** and **35** are obtained by the analysis of preliminary placebo composition, extracted in both WVL-1 and WVL-2, respectively.

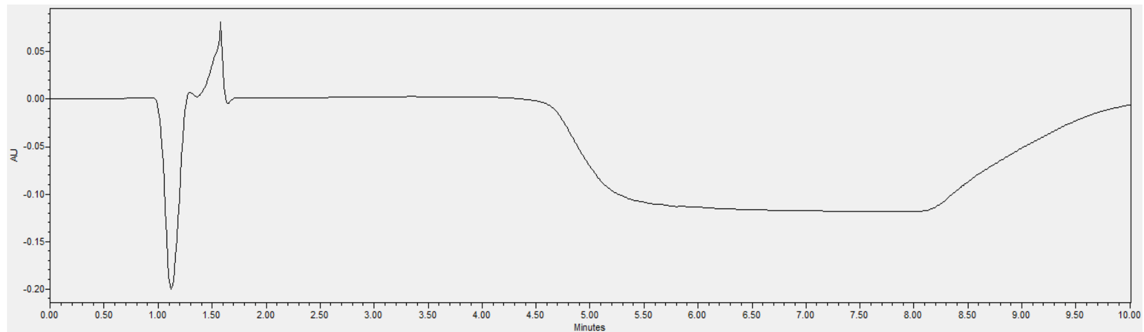

Figure 34. Chromatogram at WVL-1 for injection of placebo.

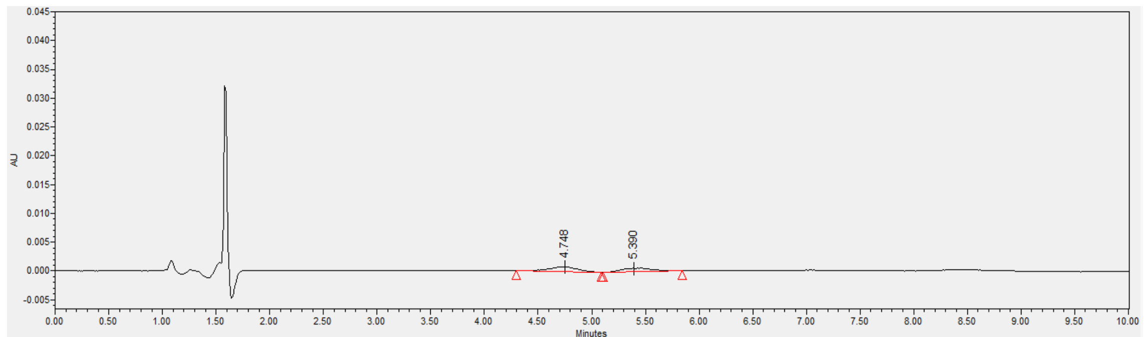

Figure 35. Chromatogram at WVL-2 for injection of placebo.

The chromatograms presented in **Figures 36** and **37** are obtained by analysis of stock solution with 5% of the concentration of DS A and DS B, extracted in both WVL-1 and WVL-2, respectively.

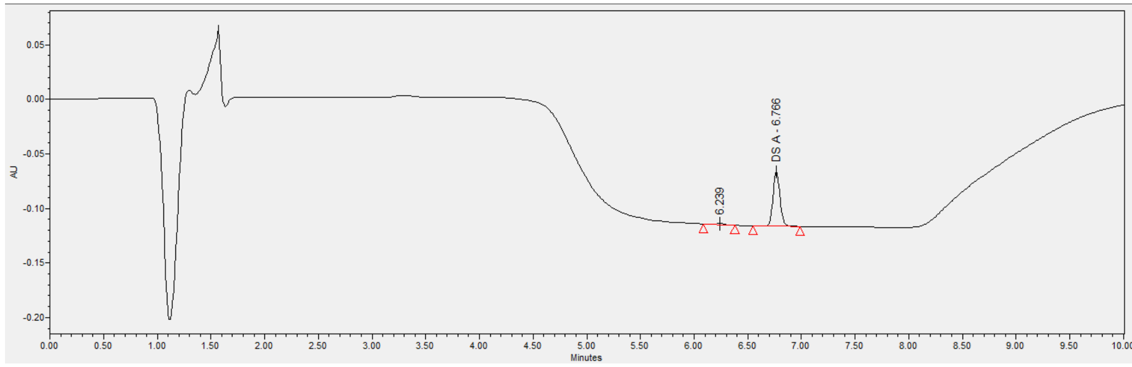

Figure 36. Chromatogram at WVL-1 for injection of DS A and DS B at 5%.

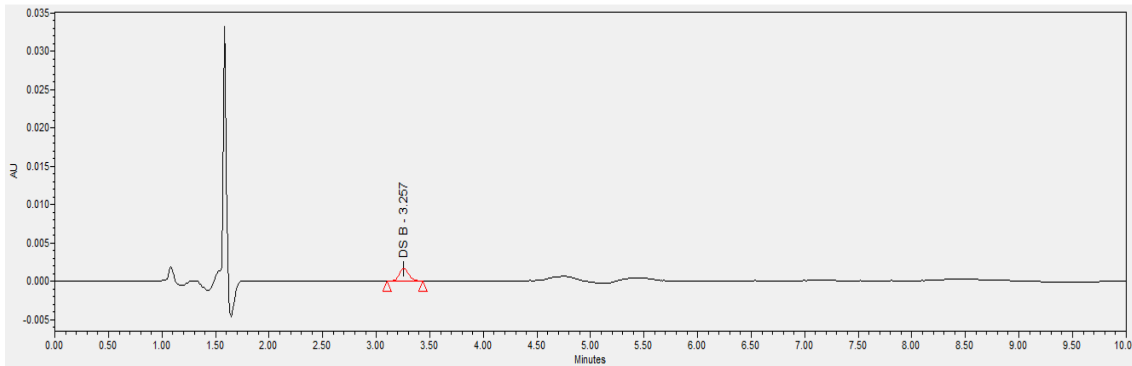

Figure 37. Chromatogram at WVL-2 for injection of DS A and DS B at 5%.

The chromatograms presented in **Figures 38** and **39** are obtained by analysis of stock solution with 100% of the concentration of DS A and DS B, extracted in both WVL-1 and WVL-2, respectively.

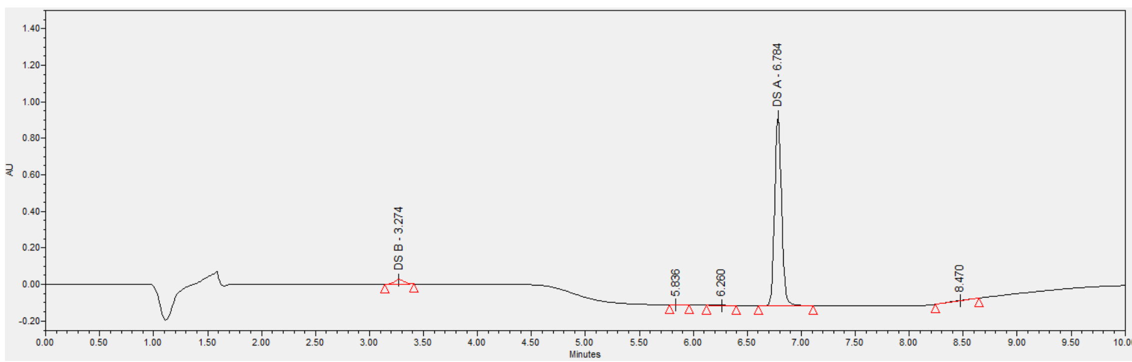

Figure 38. Chromatogram at WVL-1 for injection of DS A and DS B at 100%.

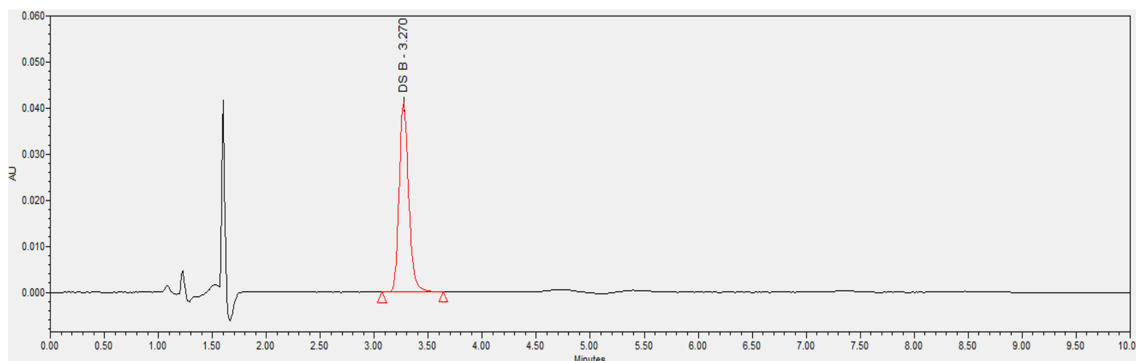

Figure 39. Chromatogram at WVL-2 for injection of DS A and DS B at 100%.

3.3.2.2. System Precision

The system precision expresses the closeness of the results obtained for a standard/sample using the same analytical method.

The precision of the HPLC system was demonstrated by performing 6 consecutive measurements in one day of one standard solution at 100% of the theoretical concentration of both DS.

The Response Factor (RF) is defined as the ratio between the concentration of the analyte and the response of the detector to it, in this case in the form of a peak area. So, RF is calculated by the following formula [168]:

$$Response\ Factor\ (RF) = \frac{Peak\ Area}{Concentration\ of\ the\ analyte} \qquad \text{(Equation 7)}$$

The RSD intra-day was reported and evaluated. The system is considered precise if the RSD is less than 0.85%.

The concentration measured and results obtained for the system precision test are expressed in **Table 14** and **Table 15**, for DS A and DS B, respectively.

Table 14. System precision results for DS A 100% standard solution.

Injection nº.	Concentration (mg/ml)	Peak area	RF	RT
1	0.033	4 703 587	1.40E+08	6.771
2		4 702 886	1.40E+08	6.767
3		4 701 327	1.40E+08	6.769
4		4 705 067	1.40E+08	6.766
5		4 700 104	1.40E+08	6.770
6		4 701 726	1.40E+08	6.766
Average		-	1.40E+08	6.768
SD			5.26E+04	0.002
RSD (%)			0.04	0.03

Table 15. System precision results for DS B 100% standard solution.

Injection nº.	Concentration (mg/ml)	Peak area	RF	RT
1	0.001	250 061	2.45E+08	3.246
2		250 478	2.46E+08	3.247
3		250 019	2.45E+08	3.252
4		250 535	2.46E+08	3.252
5		250 312	2.45E+08	3.253
6		249 874	2.45E+08	3.252
Average		-	2.45E+08	3.250
SD			2.64E+05	0.003
RSD (%)			0.11	0.09

The evaluations of the method system precision are expressed in **Table 16**.

Table 16. System precision results evaluation.

	Concentration	RSD Results	Limits	Evaluation
DS A	100%	0.04%	≤ 0.85%	Complies
DS B	100%	0.11%	≤ 0.85%	Complies

From the results obtained, it can be concluded that the system is precise for multiple measurements in the same standard solution.

3.2.2.3. Linearity

The linearity evaluates the ability of the analytical method to generate results directly proportional to the concentration of DS within the range of the analytical procedure.

For the establishing of the linearity, a minimum of 5 concentrations is recommended, equally distributed within the range of the analytical procedure, using at least 2 stock solutions. In this case, a set of 9 concentrations at 5%, 10%, 20%, 40%, 60%, 80%, 100%, 120% and 140% of the theoretical concentration of both DSs were prepared and analysed. The set of solutions was prepared from 2 independent stock solutions of DS A and DS B. This test was performed for active ingredients alone. Linearity was evaluated in 1 day.

The final solutions obtained were analyzed (single injection). Linear regression was computed from the data (concentration versus area units). The regression line parameters presented include:

- Linear equation: y=mx+b (m=slope, b=y-intercept)
- Correlation coefficient (r), whose acceptance criteria is r≥0.997
- y-intercept in percentage (%), related to the peal area of the solutions at 100% concentration level, is calculated by the following formula:

$$y - intercept\ (\%) = \frac{b}{A} \times 100 \qquad \text{(Equation 8)}$$

  Where: b= y-intercept; A= Area at 100%

  And present acceptance criteria of: y-intercept ≤ ±3%

- Residual sum of squares

The real concentration for each level, the obtained area and the RF for DS A are presented in **Table 17**. The linear regression that relates the concentration of DS A with the area is presented in **Figure 40**, with linear equation and determination coefficient.

Table 17. Linearity results for DS A.

Conc. Level (%)	Real concentration (mg/ml)	Area	RF
5%	0.0017	244 778	1.46E+08
10%	0.0033	445 020	1.34E+08
20%	0.0067	933 340	1.39E+08
40%	0.0133	1 803 733	1.35E+08
60%	0.0202	2 835 639	1.41E+08
80%	0.0267	3 687 362	1.38E+08
100%	0.0336	4 614 361	1.37E+08
120%	0.0400	5 491 597	1.37E+08
140%	0.0470	6 443 111	1.37E+08

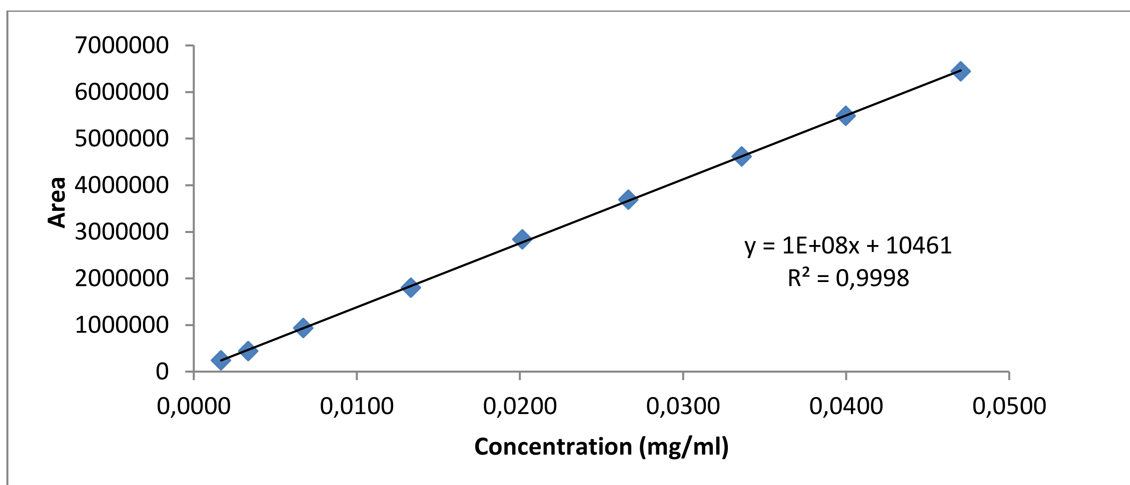

Figure 40. Linear regression for DS A.

The real concentration for each level, the obtained area and the RF for DS B are presented in **Table 18**. The linear regression that relates the concentration of DS B with the area is presented in **Figure 41**, with the linear equation and determination coefficient.

Table 18. Linearity results for DS B.

Conc. Level (%)	Real concentration (mg/ml)	Area	RF
5%	0.00005	10 129	1.99E+08
10%	0.00010	22 504	2.20E+08
20%	0.00020	44 152	2.16E+08
40%	0.00041	96 894	2.37E+08
60%	0.00061	138 939	2.27E+08
80%	0.00082	196 377	2.40E+08
100%	0.00102	239 935	2.35E+08
120%	0.00123	290 224	2.36E+08
140%	0.00143	342 897	2.40E+08

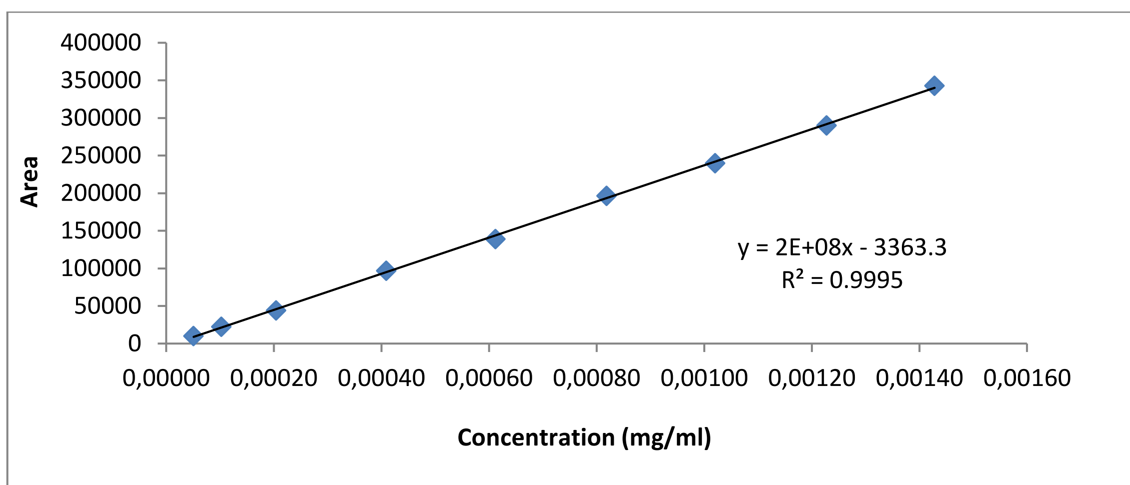

Figure 41. Linear regression for DS B.

The evaluations of the method linearity are expressed in **Table 19**.

Table 19. Linearity results evaluation for DS A and DS B.

	Limits	DS A	DS B	Evaluation
y-intercept (% rel. to the 100%):	≤ ± 3%	0.23	-1.40	Complies
Correlation coefficient	≥ 0.997	0.99992	0.99977	Complies
Residual Sum of Squares	–	6 171 895 272	56 022 847	–
Slope	–	137 194 657	240 449 704	–

From the results obtained, it can be concluded that the method can generate results directly proportional to the concentration of each DS within the range of the analytical procedure (5 to 140%).

3.2.3. Stability

The solution's stability was evaluated for a defined time in the concentration of 100%, using the developed method of HPLC-PDA. Standard solutions at 100% ($C_{DS\ A} \approx 0.033$ mg/ml and $C_{DS\ B} \approx 0.0010$ mg/ml) were prepared from two independent weighings. Standard solutions were analyzed (single injection) at t=0h. The standard solutions were kept:

- In vial inside the auto-sampler at 15ºC and re-injected at approximately t=7h, t=11h, t=24h, t=32h, and t=47h.
- At the bench laboratory at room temperature and injected at approximately t=7h, t=12h, t=24h, and t=47h.

Standard solution stability was determined in terms of the percentage of change in the average concentration of DS A and DS B in each time tested. The results were also compared with a *t-Student* test (paired two samples for mean), considering a confidence level of 95% (p-value>0.05).

The results obtained for standard solution stability in vials kept inside the autosampler at 15ºC are expressed in **Tables 20** and **21** for DS A and B, respectively.

Table 20. Stability test results for DS A in standard solution with both DS at 100% in vials kept inside the autosampler at 15ºC.

Sample no.	**DS A Standard Solution – Concentration (mg/mL)**					
	t = 0h	**t = 7h**	**t = 11h**	**t = 24h**	**t = 32h**	**t = 47h**
1	0.0338	0.0336	0.0325	0.0339	0.0339	0.0339
2	0.0335	0.0336	0.0341	0.0339	0.0338	0.0340
Average [ ]	0.0336	0.0336	0.0333	0.0339	0.0339	0.0339
t-Student test for average concentration (***p-value)***		0.7844	0.7776	0.2781	0.3125	0.3045
% Deviation (from t = 0h)		0.15	1.11	0.89	0.65	0.87

Table 21. Stability test results for DS B in a standard solution with both DS at 100% in vials kept inside the autosampler at 15ºC.

Sample no.	DS B Standard Solution – Concentration (mg/mL)					
	t = 0h	**t = 7h**	**t = 11h**	**t = 24h**	**t = 32h**	**t = 47h**
1	0.0010	0.0010	0.0010	0.0010	0.0010	0.0010
2	0.0010	0.0010	0.0010	0.0010	0.0010	0.0010
Average [ ]	0.0010	0.0010	0.0010	0.0010	0.0010	0.0010
t-Student test for average concentration (***p-value)***		0.3677	0.8614	0.8881	0.3875	0.4212
% Deviation (from t = 0h)		0.37	0.02	0.08	0.14	0.35

The results obtained for standard solution stability at the laboratory bench at room temperature are expressed in **Tables 22** and **23** for DS A and B, respectively.

Table 22. Stability test results for DS A in a standard solution with both DS at 100% at laboratory bench at room temperature.

Sample no.	DS A Standard Solution – Concentration (mg/mL)				
	t = 0h	**t = 7h**	**t = 12h**	**t = 24h**	**t = 47h**
1	0.0338	0.0346	0.0336	0.0339	0.0340
2	0.0335	0.0346	0.0335	0.0339	0.0340
Average [ ]	0.0336	0.0346	0.0336	0.0339	0.0340
t-Student test for average concentration (***p-value)***		0.5440	0.0989	0.537	0.1647
% Deviation (%) (from t = 0h)		0.24	0.22	0.74	1.13

Table 23. Stability test results for DS B in a standard solution with both DS at 100% at laboratory bench at room temperature.

Sample no.	DS B Standard Solution – Concentration (mg/mL)				
	t = 0h	**t = 7h**	**t = 12h**	**t = 24h**	**t = 47h**
1	0.0010	0.0010	0.0010	0.0010	0.0010
2	0.0010	0.0010	0.0010	0.0010	0.0010
Average [ ]	0.0010	0.0010	0.0010	0.0010	0.0010
t-Student test for average concentration (***p-value)***		0.7453	0.2903	0.5916	0.4397
% Deviation (%) (from t = 0h)		0.08	0.29	0.06	0.08

The evaluation of the stability of DS A and DS B in a standard solution in vials kept inside the autosampler at 15ºC is presented in **Tables 24** and **25**, respectively.

Table 24. Results evaluation for DS A in a standard solution in vials kept inside the autosampler at 15ºC.

	Limits	t = 7h	t = 11h	t = 24h	t = 32h	t = 47h	Evaluation
% Deviation t = 0h (%)	≤ 2%	0.15	1.11	0.89	0.65	0.87	Complies
t-Student test: p-value	> 0.05	0.78	0.78	0.28	0.31	0.30	Statistically similar

Table 25. Results evaluation for DS B in a standard solution in vials kept inside the autosampler at 15ºC.

	Limits	t = 7h	t = 11h	t = 24h	t = 32h	t = 47h	Evaluation
% Deviation t = 0h (%)	≤ 2%	0.37	0.02	0.08	0.14	0.35	Complies
t-Student test: p-value	> 0.05	0.37	0.86	0.89	0.39	0.42	Statistically similar

From the evaluation of results, it can be concluded that standard solutions are considered stable for, at least, 47 hours kept at 15ºC (inside the autosampler).

The evaluation of the stability of DS A and DS B in a standard solution at the laboratory bench at room temperature is presented in **Tables 26** and **27**, respectively.

Table 26. Results evaluation for DS A in a standard solution at the laboratory bench at room temperature.

	Limits	t = 7h	t = 12h	t = 24h	t = 47h	Evaluation
% Deviation t = 0h (%)	≤ 2%	0.24	0.22	0.74	1.13	Complies
t-Student test: p-value	> 0.05	0.65	0.79	0.35	0.23	Statistically similar

Table 27. Results evaluation for DS B in a standard solution at the laboratory bench at room temperature.

	Limits	t = 7h	t = 12h	t = 24h	t = 47h	Evaluation
% Deviation t = 0h (%)	≤ 2%	0.08	0.29	0.06	0.28	Complies
t-Student test: p-value	> 0.05	0.75	0.29	0.59	0.44	Statistically similar

From the evaluation of results, it can be concluded that standard solutions are considered stable for, at least, 47 hours kept at the laboratory bench at room temperature.

4. Conclusion

The pharmaceutical industry has shown an increasing focus on LAI technology as drug delivery system. The advantages of these formulations, particularly the decreasing of the frequency of administration, which promotes patient adhesion and compliance, and the control of the drug plasma concentrations within the therapeutic range, minimizing the occurrence of adverse events, make LAI an unique technology.

The increase in LAI use further reinforces the importance of the characterization techniques which should be used to determine the various physicochemical properties of the drug product and ensure consistency in formulation processing and performance. ICH specifications are essential to control quality, safety and product efficacy, but other tests, more specific for certain LAI formulations, can be required. Neither the FDA nor the EMA provide specific data regarding the procedure to be followed in characterizing most of these formulations.

As presented before, this internship had two main objectives, a systematic review on the methodologies used to characterize long-acting injectables as well as the evaluation of the *in vitro* release profile of these formulations and support for the in-house development of the most appropriate methodology for assessing the prototype release profile of BlueLAI technology.

Regarding the development of IVR method, the method, apparatus and parameters were chosen and the analytical method for IVR using HPLC-PDA were developed. Nevertheless, some parameters of the method can only be investigated using the final formulation, which was not possible to perform until the end of the internship. Thus, in the future, the *in vitro* release profile needs to be established and is expected that IVR analytical method may undergo fine-tuning adjustments, and thus few challenges may still arise. First, only with the final BlueLAI formulation is possible to guarantee that the HPLC-PDA method is suitable. For example, selectivity of BlueLAI formulation needs to be assessed and if it fails to accomplish the selectivity parameters, some alterations in the analytical method must be done. Second, the compatibility of agarose with the final formulation needs to be tested. The gelification time of the formulation can condition the use of the agarose gel as a barrier and the shaping method. Moreover, the quantity of agarose and the thickness of the layer that separates the BlueLAI formulation of the release medium need to be assessed. However, if the agarose gel is not possible, the use of dialysis bag or a float-a-lyzer can be tested as an alternative. Finally, the applicability of the method is tested with the final formulation, which could lead to some rearrangements, such as the sampling quantity and timing, the volume of sample used in the test, volume of release medium, and agarose gel dimensions.

The development of biorelevant IVR methods are essential to support the development of LAI formulations. Although the implementation of these methods presents some challenges, it will ensure knowledge of the *in vitro* release profile over time. This information will enable product developers to ensure the *in vivo* performance of the LAI, by establishing a good IVIVC, or even, if necessary, make small adjustments to improve its performance in the development phase.

5. Bibliography

[1] "〈1151〉 Pharmaceutical Dosage Forms," in *United States Pharmacopeia*, Rockville: MD, USA, 2020, p. 7929.

[2] "〈1〉 Injections And Implanted Drug Products (Parenteral)—Product Quality Tests," in *United States Pharmacopeia*, Rockville: MD, USA, 2019.

[3] "〈2〉 Oral Drug Products—Product Quality Tests," in *United States Pharmacopeia*, Rockville: MD, USA, 2020.

[4] "〈3〉 Topical And Transdermal Drug Products—Product Quality Tests," in *United States Pharmacopeia*, Rockville: MD, USA, 2020.

[5] "〈4〉 Mucosal Drug Products—Product Quality Tests," in *United States Pharmacopeia*, Rockville: MD, USA, 2018.

[6] "〈5〉 Inhalation And Nasal Drug Products—General Information And Product Quality Tests," in *United States Pharmacopeia*, Rockville: MD, USA, 2019.

[7] "Glossary," in *European Pharmacopeia*, 10.2., Strasbourg: Council of Europe, 2017.

[8] J.-A. L. Kelsey C. Martin Mhatre V. Ho, "The Controlled Drug Delivery Systems: Past Forward and Future Back," *J. Control Release*, vol. 190, pp. 3–8, 2014, doi: 10.1016/j.jconrel.2014.03.054.The.

[9] D. A. W. Johnson, "Historical perspective on antipsychotic long-acting injections," *Br. J. Psychiatry*, vol. 195, no. SUPPL. 52, pp. 7–12, 2009, doi: 10.1192/bjp.195.52.s7.

[10] L. Denham, John & Adamson, "The contribution of fluphenazine enanthate and decanoate in the prevention of readmission of schizophrenic patients," 1966.

[11] F. M. Benagiano, G. & Primiero, "Long acting contraceptives - Present status," *Drugs*, vol. 25, no. 6, pp. 570–609, 1983, doi: 10.1007/978-4-431-55462-2_6.

[12] K. Chaudhary, M. M. Patel, and P. J. Mehta, "Long-Acting Injectables : Current Perspectives and Future Promise," *Ther. Drug Carr. Syst.*, vol. 36, no. 2, pp. 137–181, 2019.

[13] L. Rahnfeld and P. Luciani, "Injectable lipid-based depot formulations: Where do we stand?," *Pharmaceutics*, vol. 12, no. 6, pp. 1–28, 2020, doi: 10.3390/pharmaceutics12060567.

[14] M. Landovitz, J. Raphael & Kofron, Ryan & McCauley, "The Promise and Pitfalls of Long Acting Injectable Agents for HIV Prevention," *Curr. Opin. HIV AIDS*, vol. 11, no. 1, pp. 122–128, 2016, doi: 10.1097/COH.0000000000000219.The.

[15] S. S. Ruiz, María Esperanza & Montoto, *Routes of Drug Administration: Dosage, Design, and Pharmacotherapy Success*, no. January. 2018.

[16] M. V. Seeman, "Drawbacks of Long-Acting Intramuscular Antipsychotic Injections," *J. Clin. Pract. Nurs.*, vol. 1, no. 1, pp. 12–22, 2018, [Online]. Available: www.openaccesspub.org.

[17] J. Huang, "Drug development & Delivery," *Rational Design & Development of Long-Acting Injectable Dosage Forms*, 2020. https://drug-dev.com/formulation-forum-rational-design-development-of-long-acting-injectable-dosage-forms/ (accessed Nov. 13, 2020).

[18] M. M. De Villiers, "Pharmaceutical Solvents and Solubilizing Agents," in *A Practical Guide to Contemporary Pharmacy Practice*, no. 15, 2009, pp. 190–202.

[19] S. Weng Larsen and C. Larsen, "Critical factors influencing the in vivo performance of long-acting lipophilic solutions-impact on in vitro release method design," *AAPS J.*, vol. 11, no. 4, pp. 762–770, 2009, doi: 10.1208/s12248-009-9153-9.

[20] APP Pharmaceuticals, "Fluphenazine decanoate Label," Schaumburg, 2010.

[21] T. R. E. Barnes and D. A. Cursori, "Long Term Depot Antipsychotics: A Risk-Benefit Assessment," *Drug Saf.*, vol. 10, no. 6, pp. 464–479, 1994, doi: 10.2165/00002018-199410060-00005.

[22] Food and Drug Administration, "Posimir - prescribing informations," 1972. [Online].

Available:
https://www.accessdata.fda.gov/drugsatfda_docs/label/2021/204803s000lbl.pdf.
[23] A. D. Bangham and R. W. Horne, “Negative staining of phospholipids and their structural modification by surface-active agents as observed in the electron microscope,” *J. Mol. Biol.*, vol. 8, no. 5, pp. 660–668, 1964, doi: 10.1016/S0022-2836(64)80115-7.
[24] A. Akbarzadeh *et al.*, “Liposome: Classification, preparation, and applications,” *Nanoscale Res. Lett.*, vol. 8, no. 1, p. 1, 2013, doi: 10.1186/1556-276X-8-102.
[25] M. E. R. O’Brien *et al.*, “Reduced cardiotoxicity and comparable efficacy in a phase III trial of pegylated liposomal doxorubicin HCl (CAELYX™/Doxil®) versus conventional doxorubicin for first-line treatment of metastatic breast cancer,” *Ann. Oncol.*, vol. 15, no. 3, pp. 440–449, 2004, doi: 10.1093/annonc/mdh097.
[26] Food and Drug Administration, “Exparel - prescribing information,” 2018. [Online]. Available:
https://www.accessdata.fda.gov/drugsatfda_docs/label/2018/022496s9lbl.pdf.
[27] A. Hadzic *et al.*, “Liposome bupivacaine femoral nerve block for postsurgical analgesia after total knee arthroplasty,” *Anesthesiology*, vol. 124, no. 6, pp. 1372–1383, 2016, doi: 10.1097/ALN.0000000000001117.
[28] M. Brandl, “Vesicular phospholipid gels: A technology platform,” *J. Liposome Res.*, vol. 17, no. 1, pp. 15–26, 2007, doi: 10.1080/08982100601186490.
[29] Y. Zhang *et al.*, “In vitro and in vivo sustained release of exenatide from vesicular phospholipid gels for type II diabetes,” *Drug Dev. Ind. Pharm.*, vol. 42, no. 7, pp. 1042–1049, 2015, doi: 10.3109/03639045.2015.1107090.
[30] H. Li *et al.*, “An in situ-forming phospholipid-based phase transition gel prolongs the duration of local anesthesia for ropivacaine with minimal toxicity,” *Acta Biomater.*, vol. 58, pp. 136–145, 2017, doi: 10.1016/j.actbio.2017.06.013.
[31] D. F. Joabsson, “FluidCrystal – a new lipid liquid crystal based LAI technology validated by recent product approvals.” https://kuleuvencongres.be/laiconference/documents/presentations/session-2-fluidcrystal-a-new-lipid-liquid-crystal.pdf (accessed Dec. 11, 2020).
[32] Camurus, “Development Pipeline,” 2021. https://www.camurus.com/pipeline/ (accessed Jun. 20, 2021).
[33] Committee for Medicinal Products for Human Use, “Buvidal Assessment report,” London, 2018.
[34] L. Yu and J. Ding, “Injectable hydrogels as unique biomedical materials,” *Chem. Soc. Rev.*, vol. 37, no. 8, pp. 1473–1481, 2008, doi: 10.1039/b713009k.
[35] N. Musmade, A. Jadhav, P. Moin, S. Patil, and A. Gupta, “An Overview of In Situ Gel Forming Implants: Current Approach Towards Alternative Drug Delivery System,” *J. Biol. Chem. Chronicles*, vol. 5, no. 1, pp. 14–21, 2019, doi: 10.33980/jbcc.2019.v05i01.003.
[36] A. K. Gupta, “Environmental Responsive Hydrogels: a Novel Approach in Drug Delivery System,” *J. Drug Deliv. Ther.*, vol. 2, no. 1, 2012, doi: 10.22270/jddt.v2i1.87.
[37] Z. R. Lu, P. Kopečkovci, and J. Kopeček, “Antigen responsive hydrogels based on polymerizable antibody Fab’ fragment,” *Macromol. Biosci.*, vol. 3, no. 6, pp. 296–300, 2003, doi: 10.1002/mabi.200390039.
[38] N. K. Varde and D. W. Pack, “Microspheres for controlled release drug delivery,” *Expert Opin. Biol. Ther.*, vol. 4, no. 1, pp. 35–51, 2004, doi: 10.1517/14712598.4.1.35.
[39] D. Guo, “New Developments in Long-Acting Injectable Nanoformulations,” *Glob. J. Pharm. Pharm. Sci.*, vol. 4, no. 2, 2017, doi: 10.19080/gjpps.2017.04.555633.
[40] Food and Drug Administration, “Risperdal Consta - prescribing information,” 2003.
[41] United States Pharmacopeia, “ELIGARD® - prescribing information,” 2002. [Online]. Available:
https://www.accessdata.fda.gov/drugsatfda_docs/label/2007/021731s005,021488s010

,021379s010,021343s015lbl.pdf.
[42] FDA, “FY2016 Regulatory Science Report: Long-Acting Injectable Formulations,” 2017. https://www.fda.gov/industry/generic-drug-user-fee-amendments/fy2016-regulatory-science-report-long-acting-injectable-formulations (accessed Nov. 09, 2020).
[43] N. Darville *et al.*, “Modeling the Time Course of the Tissue Responses to Intramuscular Long-acting Paliperidone Palmitate Nano-/Microcrystals and Polystyrene Microspheres in the Rat,” *Toxicol. Pathol.*, vol. 44, no. 2, pp. 189–210, 2016, doi: 10.1177/0192623315618291.
[44] Y. Xu *et al.*, “Intra-Articular Injection of Acid-Sensitive Stearoxyl-Ketal-Dexamethasone Microcrystals for Long-Acting Arthritis Therapy,” *Asian J. Pharm. Sci.*, pp. 0–25, 2020, doi: 10.1016/j.ajps.2020.07.002.
[45] D. Wieckhusen, A. Glausch, and M. Ahlheim, “Patent Application,” 2011/0212141 A1, 2011.
[46] “Vanda Pharmaceuticals: Corporate presentation 2019.” https://www.vandapharma.com/pdfs/vanda_corporate_presentation.pdf (accessed Dec. 16, 2020).
[47] P. D. Kilmer, “Controlled release injectable drug delivery: an overview,” *Asian J. Biomater. Res.*, vol. 11, no. 3, pp. 6–15, 2017, doi: 10.1177/1461444810365020.
[48] H. Bari, “A prolonged release Parenteral drug delivery system - An overview,” *Int. J. Pharm. Sci. Rev. Res.*, vol. 3, no. 1, pp. 1–11, 2010.
[49] S. Grover, S. Sahoo, S. BN, N. Malhotra, D. Dua, and A. Avasthi, “Attitude and perceptions of patients towards long acting depot injections (LAIs),” *Asian J. Psychiatr.*, vol. 44, pp. 200–208, 2019, doi: 10.1016/j.ajp.2019.07.052.
[50] B. Winner *et al.*, “Effectiveness of long-acting reversible contraception,” *N. Engl. J. Med.*, vol. 366, no. 21, pp. 1998–2007, 2012, doi: 10.1097/01.ogx.0000421455.21771.a1.
[51] E. N. Titus-Lay, E. D. Ansara, A. N. Isaacs, and C. A. Ott, “Evaluation of adherence and persistence with oral versus long-acting injectable antipsychotics in patients with early psychosis,” *Ment. Heal. Clin.*, vol. 8, no. 2, pp. 56–62, 2018, doi: 10.9740/mhc.2018.03.056.
[52] E. N. Nicole C. Ammerman, “Long-acting injectables for TB prevention in people living with HIV: prospects and challenges,” 2019. https://www.who.int/tb/areas-of-work/tb-hiv/croi-2019-ammerman_long-acting-injectables-for-tb-prevention.pdf (accessed Nov. 11, 2020).
[53] G. J. Remington and M. E. Adams, “Depot neuroleptic therapy: Clinical considerations,” *Can. J. Psychiatry*, vol. 40, no. 3 SUPPL. 1, 1995, doi: 10.1177/070674379504003s02.
[54] F. Approach, “Parenteral controlled drug delivery system,” *Int. J. Pharm. Nano Sci.*, vol. 2, no. 5, pp. 572–580, 2013.
[55] E. Mannaert, A. Vermeulen, B. Remmerie, P. Bouhours, and J. C. Levron, “Pharmacokinetic profile of long-acting injectable risperidone at steady-state: comparison with oral administration.,” *Encephale.*, vol. 31, no. 5 Pt 1, pp. 609–615, 2005, doi: 10.1016/S0013-7006(05)82420-0.
[56] C. Leucht, S. Heres, J. M. Kane, W. Kissling, J. M. Davis, and S. Leucht, “Oral versus depot antipsychotic drugs for schizophrenia-A critical systematic review and meta-analysis of randomised long-term trials,” *Schizophr. Res.*, vol. 127, no. 1–3, pp. 83–92, 2011, doi: 10.1016/j.schres.2010.11.020.
[57] T. Kishimoto *et al.*, “Long-acting injectable vs oral antipsychotics for relapse prevention in schizophrenia: A meta-analysis of randomized trials,” *Schizophr. Bull.*, vol. 40, no. 1, pp. 192–213, 2014, doi: 10.1093/schbul/sbs150.
[58] H. G. Roozen, R. De Waart, and W. Van Den Brink, “Efficacy and tolerability of naltrexone in the treatment of alcohol dependence: Oral versus injectable delivery,” *Eur. Addict. Res.*, vol. 13, no. 4, pp. 201–206, 2007, doi: 10.1159/000104882.

[59] X. L. Wei, Y. R. Han, L. H. Quan, C. Y. Liu, and Y. H. Liao, "Oily nanosuspension for long-acting intramuscular delivery of curcumin didecanoate prodrug: Preparation, characterization and in vivo evaluation," *Eur. J. Pharm. Sci.*, vol. 49, no. 2, pp. 286–293, 2013, doi: 10.1016/j.ejps.2013.03.010.
[60] O. Agid, G. Foussias, and G. Remington, "Long-acting injectable antipsychotics in the treatment of schizophrenia: Their role in relapse prevention," *Expert Opin. Pharmacother.*, vol. 11, no. 14, pp. 2301–2317, 2010, doi: 10.1517/14656566.2010.499125.
[61] I. Pacchiarotti *et al.*, "Long-acting injectable antipsychotics (LAIs) for maintenance treatment of bipolar and schizoaffective disorders: A systematic review," *Eur. Neuropsychopharmacol.*, vol. 29, no. 4, pp. 457–470, 2019, doi: 10.1016/j.euroneuro.2019.02.003.
[62] H. Gabelnick, "Long-acting hormonal contraception," *Women's Heal.*, 2015.
[63] L. Wu, D. R. Janagam, T. D. Mandrell, J. R. Johnson, and T. L. Lowe, "Long-acting injectable hormonal dosage forms for contraception," *Pharm. Res.*, vol. 32, no. 7, pp. 2180–2191, 2015, doi: 10.1007/s11095-015-1686-2.
[64] V. Halpern, R. M. Stalter, D. H. Owen, L. J. Dorflinger, A. Lendvay, and K. H. Rademacher, "Towards the development of a longer-acting injectable contraceptive: Past research and current trends," *Contraception*, vol. 92, no. 1, pp. 3–9, 2015, doi: 10.1016/j.contraception.2015.02.014.
[65] Y. Yang *et al.*, "Efficacy and safety of long-acting growth hormone in children with short stature: a systematic review and meta-analysis," *Endocrine*, vol. 65, no. 1, pp. 25–34, 2019, doi: 10.1007/s12020-019-01950-9.
[66] W. S. Cutfield *et al.*, "Non-compliance with growth hormone treatment in children is common and impairs linear growth," *PLoS One*, vol. 6, no. 1, pp. 5–7, 2011, doi: 10.1371/journal.pone.0016223.
[67] S. R. Sandler. L.M & Burrin, J.M. & Williams, G. & Joplin, G.F. & Carr, D.H & Bloom, "Effective long-term treatment of acromegaly with a long-acting somatostatin analogue," *Clin. Endocrinol. (Oxf).*, vol. 26, no. 1, pp. 85–95, 1987.
[68] D. Cuevas-Ramos and M. Fleseriu, "Pasireotide: A novel treatment for patients with acromegaly," *Drug Des. Devel. Ther.*, vol. 10, pp. 227–239, 2016, doi: 10.2147/DDDT.S77999.
[69] R. Sharifi and R. Browneller, "Serum testosterone suppression and potential for agonistic stimulation during chronic treatment with monthly and 3-month depot formulations of leuprolide acetate for advanced prostate cancer," *J. Clin. Oncol.*, vol. 13, no. 7, pp. 320–322, 2014, doi: 10.1016/S0022-5347(05)64560-0.
[70] J. M. Kuhn *et al.*, "A randomized comparison of the clinical and hormonal effects of two GnRH agonists in patients with prostate cancer," *Eur. Urol.*, vol. 32, no. 4, pp. 397–403, 1997, doi: 10.1159/000480796.
[71] M. C. Gittleman, S. D. Graham, P. B. Hudson, and B. Stein, "Leuprolide Acetate 22 . 5 mg 12-Week Depot Formulation in the Treatment of Patients with Advanced Prostate Cancer," *Clin. Ther.*, vol. 18, no. 4, pp. 647–657, 1996.
[72] A. Hegde, S. M. Singh, and S. Sarkar, "Long-acting preparations in substance abuse management: A review and update," *Indian J. Psychol. Med.*, vol. 35, no. 1, pp. 10–18, 2013, doi: 10.4103/0253-7176.112194.
[73] J. C. Garbutt *et al.*, "Efficacy and tolerability of long-acting injectable naltrexone for alcohol dependence," *J. Am. Med. Assoc.*, vol. 293, no. 13, pp. 1617–1625, 2005.
[74] S. D. Comer *et al.*, "Injectable, sustained-release naltrexone for the treatment of opioid dependence: A randomized, placebo-controlled trial," *Arch. Gen. Psychiatry*, vol. 63, no. 2, pp. 210–218, 2006, doi: 10.1001/archpsyc.63.2.210.
[75] S. C. Sigmon, D. E. Moody, E. S. Nuwayser, and G. E. Bigelow, "An injection depot formulation of buprenorphine : extended biodelivery and effects," *Addiction*, vol. 101,

no. 3, pp. 420–432, 2006, doi: 10.1111/j.1360-0443.2005.01348.x.
[76] S. Swindells *et al.*, "Long-Acting Cabotegravir and Rilpivirine for Maintenance of HIV-1 Suppression," *N. Engl. J. Med.*, vol. 382, no. 12, pp. 1112–1123, 2020, doi: 10.1056/nejmoa1904398.
[77] J. R. Hilaire *et al.*, "Creation of a long-acting rilpivirine prodrug nanoformulation," *J. Control. Release*, vol. 311–312, no. July, pp. 201–211, 2019, doi: 10.1016/j.jconrel.2019.09.001.
[78] R. J. Landovitz *et al.*, "Safety, tolerability, and pharmacokinetics of long-acting injectable cabotegravir in low-risk HIV-uninfected individuals: HPTN 077, a phase 2a randomized controlled trial," *PLoS Med.*, vol. 15, no. 11, pp. 1–22, 2018, doi: 10.1371/journal.pmed.1002690.
[79] EMA, "First long-acting injectable antiretroviral therapy for HIV recommended for approval," 2020. https://www.ema.europa.eu/en/news/first-long-acting-injectable-antiretroviral-therapy-hiv-recommended-approval (accessed Oct. 27, 2020).
[80] International conference on Harmonisation, "ICH Q6A: Specifications: Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Chemical Substances Step," 2000.
[81] R. Kumar and M. J. P. Jr, "Points to Consider when Establishing Drug Product Specifications for Parenteral Microspheres," *J. Am. Assoc. Pharm. Sci.*, vol. 12, no. 1, pp. 27–32, 2010, doi: 10.1208/s12248-009-9156-6.
[82] "⟨1790⟩ Visual Inspection of Injections," in *United States Pharmacopeia*, Rockville: MD, USA, 2017.
[83] "Parenteral Preparations," in *European Pharmacopeia*, 10.2., Strasbourg, 2015.
[84] "⟨790⟩ Visible Particulates in Injections," in *United States Pharmacopeia*, Rockville: MD, USA, 2016.
[85] "Particulate Contamination: Visible Particles," in *European Pharmacopeia*, 10.2., Strasbourg, 2020.
[86] International conference on Harmonisation, "ICH Q2: Validation of Analytical Procedures: Text and Methodology," 1995.
[87] "⟨197⟩ Spectroscopic Identification Tests," in *United States Pharmacopeia*, Rockville: MD, USA, 2020.
[88] International conference on Harmonisation, "ICH Q3B: Impurities in New Drug Products," 2006.
[89] "Potentiometric Determination of pH," in *European Pharmacopeia*, 10.2., Strasbourg, 2016.
[90] "⟨791⟩ pH," in *United States Pharmacopeia*, Rockville: MD, USA, 2020.
[91] "Particle Size Analysis by Laser Light Diffraction," in *European Pharmacopeia*, 10.2., Strasbourg, 2019.
[92] "⟨429⟩ Light Diffraction Measurement of Particle Size," in *United States Pharmacopeia*, Rockville: MD, USA, 2013.
[93] "⟨1430.3⟩ Analytical Methodologies Based On Scattering Phenomena—Dynamic Light Scattering," in *United States Pharmacopeia*, Rockville: MD, USA, 2020.
[94] "⟨1430.1⟩ Analytical Methodologies Based On Scattering Phenomena—Static Light Scattering," in *United States Pharmacopeia*, Rockville: MD, USA, 2019.
[95] "Sterility," in *European Pharmacopeia*, 10.2., Strasbourg, 2012.
[96] "⟨71⟩ Sterility Tests," in *United States Pharmacopeia*, Rockville: MD, USA, 2013.
[97] "Bacterial Endotoxins," in *European Pharmacopeia*, 10.2., Strasbourg, 2018.
[98] "⟨85⟩ Bacterial Endotoxins Test," in *United States Pharmacopeia*, Rockville: MD, USA, 2018.
[99] "⟨1788⟩ Methods for the Determination of Particulate Matter in Injections and Ophthalmic Solutions," in *United States Pharmacopeia*, Rockville: MD, USA, 2013.
[100] "⟨785⟩ Osmolality and Osmolarity," in *United States Pharmacopeia*, Rockville: MD, USA,

2020.
[101] “⟨921⟩ WATER DETERMINATION,” in *United States Pharmacopeia*, Rockville: MD, USA, 2013.
[102] “⟨341⟩ Antimicrobial agents—content,” in *United States Pharmacopeia*, Rockville: MD, USA, 2016.
[103] “Uniformity of Dosage Units,” in *European Pharmacopeia*, 10.2., Strasbourg, 2017.
[104] “⟨905⟩ Uniformity Of Dosage Units,” in *United States Pharmacopeia*, Rockville: MD, USA, 2013.
[105] “⟨1663⟩ Assessment of Extractables Associated with Pharmaceutical Packaging/Delivery Systems,” in *United States Pharmacopeia*, Rockville: MD, USA, 2020.
[106] “⟨1664⟩ Assessment of leachables Associated with Pharmaceutical Packaging/Delivery Systems,” in *United States Pharmacopeia*, Rockville: MD, USA, 2020.
[107] A. I. Journal and F. Paper, “Preparation, characterization and in vitro evaluation of rifampicin loaded biodegradable microsphere,” *ACAIJ*, vol. 13, no. 7, pp. 263–269, 2013.
[108] A. Verma and U. Pradesh, “Formulation and Characterization of Microspheres of Artemether,” *J. Pharm. Drug Deliv. Technol.*, vol. 1, no. 2, pp. 65–69, 2015, doi: 10.15272/ljpddt.v1i2.32.
[109] P. Sudhakar, S. N. Bhagyamma, S. Siraj, K. V Sekharnath, K. C. Rao, and M. C. S. Subha, “Preparation and characterization of microspheres for controlled release of anti HIV drug,” *J. Appl. Pharm. Sci.*, vol. 5, no. 02, pp. 51–57, 2015, doi: 10.7324/JAPS.2015.50208.
[110] S. J. R. I. Value, K. Hemlata, S. L. Harikumar, and K. Amanpreet, “Design and Characterization of sustained release Microspheres of Acarbose Full Length Research Manuscript Full Length Research Manuscript,” *Int. J. Drug Dev. Res.*, vol. 5, no. 1, pp. 70–82, 2013.
[111] C. Yang and J. Wang, “Preparation and Characterization of Collagen Microspheres for Sustained Release of Steroidal Saponins 2 . Material and Methods,” *J. Mater. Res.*, vol. 17, no. 6, pp. 1644–1650, 2014.
[112] M. Vidyavathi and S. Papishetty, “Design and evaluation of niacin microspheres with a biopolymer,” *Trends Biomater. Artif. Organs*, vol. 27, no. 2, pp. 81–87, 2013.
[113] M. Semalty and A. Semalty, “Development and evaluation of microcapsules of Verapamil hydrochloride,” *Indian Drugs*, vol. 48, no. 08, pp. 33–38, 2011.
[114] C. Koulouktsi, S. Nanaki, P. Barmpalexis, and M. Kostoglou, “Preparation and characterization of Alendronate depot microspheres based on novel poly (- ε - caprolactone)/ Vitamin E TPGS copolymers,” *Int. J. Pharm.*, vol. 1, no. 1, p. 100014, 2019, doi: 10.1016/j.ijpx.2019.100014.
[115] T. Das, V. M. Prakash, P. Kumar, and T. Math, “Injectable in situ gel of methotrexate for rheumatoid arthritis : Development , in vitro and in vivo evaluation,” *J. Appl. Pharm. Sci.*, vol. 9, no. 05, pp. 40–48, 2019, doi: 10.7324/JAPS.2019.90505.
[116] S. K. Sindhu, D. V Gowda, and V. Datta, “Formulation and Evaluation of Injectable In-Situ Gelling Matrix System for Controlled Drug Release,” vol. 2, pp. 89–92, 2014.
[117] Y. A. Alharby and K. M. El-say, “Depot injectable atorvastatin biodegradable in situ gel : development , optimization , in vitro , and in vivo evaluation,” *Drug Des. Devel. Ther.*, vol. 10, pp. 405–415, 2016.
[118] S. P. Nagam, G. Achanta, P. Chalimadugu, S. Lakshmi, J. Kala, and R. R. Nadendla, “Design and Development of a Weekly Shot of Injectable In-Situ Gel for an Antipsychotic Drug in the Treatment of Schizophrenia,” *Int. J. Res. Pharm. Chem.*, vol. 7, no. 3, pp. 221–231, 2017.
[119] D. Solomon, N. Gupta, N. S. Mulla, S. Shukla, Y. A. Guerrero, and V. Gupta, “Role of In Vitro Release Methods in Liposomal Formulation Development: Challenges and Regulatory Perspective,” *AAPS J.*, vol. 19, no. 6, pp. 1669–1681, 2017, doi: 10.1208/s12248-017-0142-0.

[120] D. Biswas and M. Halquist, "Using Biorelevant Testing to Characterize Release of Non Oral Dosage Forms as another Tool for Safety," *J. Pharmacovigil.*, vol. 04, no. 02, 2016, doi: 10.4172/2329-6887.1000e153.

[121] "<711> Dissolution," in *United States Pharmacopeia*, Rockville: MD, USA, 2016.

[122] "⟨724⟩ Drug release," in *United States Pharmacopeia*, Rockville, 2018.

[123] "Dissolution test for solid dosage forms," in *European Pharmacopeia*, 10.2., Rockville.

[124] "⟨1092⟩ The Dissolution Procedure: Development and Validation," in *United States Pharmacopeia*, Rockville: MD, USA, 2020.

[125] "⟨1088⟩ In vitro and in vivo evaluation of dosage forms," in *United States Pharmacopeia*, Rockville, 2013.

[126] D. J. Burgess, D. J. . C. A. S. Hussain, and M.-L. Chen, "Assuring quality and performance of sustained and controlled release parenterals: EUFEPS workshop report," *AAPS J.*, vol. 6, no. 1, 2004, doi: 10.1208/ps060111.

[127] "Recommendations on dissolution test," in *United States Pharmacopeia*, 10.2., Strasbourg, 2016.

[128] T. Dadhaniya, O. Sharma, M. Gohel, and P. Mehta, "Current Approaches for in vitro Drug Release Study of Long Acting Parenteral Formulations," *Curr. Drug Deliv.*, vol. 12, no. 3, pp. 256–270, 2015, doi: 10.2174/1567201812666150209143731.

[129] Z. Hanif Zadegan, "Optimization of in vitro test conditions for PLGA-based drug delivery systems," Freien Universität Berlin, 2017.

[130] E. Khodaverdi, M. Tafaghodi, F. Ganji, K. Abnoos, and H. Naghizadeh, "In vitro insulin release from thermosensitive chitosan hydrogel," *AAPS PharmSciTech*, vol. 13, no. 2, pp. 460–466, 2012, doi: 10.1208/s12249-012-9764-9.

[131] S. S. D'Souza and P. P. DeLuca, "Methods to assess in Vitro drug release from injectable polymeric particulate systems," *Pharm. Res.*, vol. 23, no. 3, pp. 460–474, 2006, doi: 10.1007/s11095-005-9397-8.

[132] S. D. Souza, "Injectables," in *In Vitro Drug Release Testing of Special Dosage Forms*, 1st ed., John Wiley & Sons Ltd, 2020, pp. 55–85.

[133] L. C. Herrera, M. V. D. Tesoriero, and L. G. Hermida, "In vitro release testing of PLGA microspheres with Franz diffusion cells," *Dissolution Technol.*, vol. 19, no. 2, pp. 6–11, 2012, doi: 10.14227/DT190212P6.

[134] FDA, "In Vitro Bioequivalence Data for a Topical Product: Bioequivalence Review Perspective," 2017. https://www.fda.gov/media/110389/download (accessed May 10, 2021).

[135] M. Morihara, N. Aoyagi, N. Kaniwa, N. Katori, and S. Kojim, "Hydrodynamic flows around tablets in different pharmacopeial dissolution tests," *Drug Dev. Ind. Pharm.*, vol. 28, no. 6, pp. 655–662, 2002, doi: 10.1081/DDC-120003856.

[136] A. M. Master, M. E. Rodriguez, M. E. Kenney, N. L. Oleinick, and A. Sen Gupta, "MRI Studies of the Hydrodynamics in a USP 4 Dissolution Testing Cell," *J. Pharm. Sci.*, vol. 100, no. 3, pp. 2386–2398, 2010, doi: 10.1002/jps.

[137] U. Bhardwaj and D. J. Burgess, "A novel USP apparatus 4 based release testing method for dispersed systems," *Int. J. Pharm.*, vol. 388, no. 1–2, pp. 287–294, 2010, doi: 10.1016/j.ijpharm.2010.01.009.

[138] J. V Andhariya and D. J. Burgess, "Recent advances in testing of microsphere drug delivery systems," *Expert Opin Drug Deliv*, vol. 13, no. 4, pp. 593–608, 2016, doi: 10.1517/17425247.2016.1134484.

[139] V. Gray *et al.*, "In vitro release test methods for drug formulations for parenteral applications," *Dissolution Technol.*, vol. 25, no. 4, pp. 8–13, 2018, doi: 10.14227/DT250418P8.

[140] J. Shen and D. J. Burgess, "In Vitro Dissolution Testing Strategies for Nanoparticulate Drug Delivery Systems: Recent Developments and Challenges," *Drug Deliv Transl Res*, vol. 3, no. 5, pp. 409–415, 2013, doi: 10.1007/s13346-013-0129-z.In.

[141] U. Bhardwaj and D. J. Burgess, “Physicochemical properties of extruded and non-extruded liposomes containing the hydrophobic drug dexamethasone,” *Int. J. Pharm.*, vol. 388, no. 1–2, pp. 181–189, 2010, doi: 10.1016/j.ijpharm.2010.01.003.
[142] S. S. D’Souza and P. P. DeLuca, “Development of a dialysis in vitro release method for biodegradable microspheres,” *AAPS PharmSciTech*, vol. 6, no. 2, 2005, doi: 10.1208/pt060242.
[143] V. G. Kadajji, G. V. Betageri, and N. Venkatesan, “Approaches for Dissolution Testing of Novel Drug Delivery Systems,” 2011. https://www.americanpharmaceuticalreview.com/Featured-Articles/36880-Approaches-for-Dissolution-Testing-of-Novel-Drug-Delivery-Systems/ (accessed Mar. 15, 2021).
[144] G. Moreno-bautista and K. C. Tam, “Evaluation of dialysis membrane process for quantifying the in vitro drug-release from colloidal drug carriers,” *Colloids Surfaces A Physicochem. Eng. Asp.*, vol. 389, no. 1–3, pp. 299–303, 2011, doi: 10.1016/j.colsurfa.2011.07.032.
[145] J. W. Kostanski and P. P. DeLuca, “A novel in vitro release technique for peptide-containing biodegradable microspheres,” *AAPS PharmSciTech*, vol. 1, no. 1, 2000, doi: 10.1208/pt010104.
[146] N. Chidambaram and D. J. Burgess, “A novel in vitro release method for submicron-sized dispersed systems,” *AAPS PharmSci*, vol. 1, no. 3, pp. 1–9, 1999, doi: 10.1208/ps010311.
[147] M. M. A. Abdel-mottaleb and A. Lamprecht, “Standardized in vitro drug release test for colloidal drug carriers using modified USP dissolution apparatus I,” vol. 37, no. 2, pp. 178–184, 2011, doi: 10.3109/03639045.2010.502534.
[148] M. M. A. Abdel-mottaleb, D. Neumann, and A. Lamprecht, “In vitro drug release mechanism from lipid nanocapsules (LNC),” *Int. J. Pharm.*, vol. 390, no. 2, pp. 208–213, 2010, doi: 10.1016/j.ijpharm.2010.02.001.
[149] S. Prabhu, J. L. Sullivan, and G. V. Betageri, “Comparative assessment of in vitro release kinetics of calcitonin polypeptide from biodegradable microspheres,” *Drug Deliv. J. Deliv. Target. Ther. Agents*, vol. 9, no. 3, pp. 195–198, 2002, doi: 10.1080/15227950290097633.
[150] D. H. Leung *et al.*, “Development of a Convenient In Vitro Gel Diffusion Model for Predicting the In Vivo Performance of Subcutaneous Parenteral Formulations of Large and Small Molecules,” *AAPS PharmSciTech*, vol. 18, no. 6, pp. 2203–2213, 2017, doi: 10.1208/s12249-016-0698-5.
[151] D. Klose, N. Azaroual, F. Siepmann, G. Vermeersch, and J. Siepmann, “Towards more realistic in vitro release measurement techniques for biodegradable microparticles,” *Pharm. Res.*, vol. 26, no. 3, pp. 691–699, 2009, doi: 10.1007/s11095-008-9747-4.
[152] T. H. Hoang Thi *et al.*, “Bone implants modified with cyclodextrin: Study of drug release in bulk fluid and into agarose gel,” *Int. J. Pharm.*, vol. 400, no. 1–2, pp. 74–85, 2010, doi: 10.1016/j.ijpharm.2010.08.035.
[153] S. Allababidi and J. C. Shah, “Kinetics and mechanism of release from glyceryl monostearate-based implants: Evaluation of release in a gel simulating in vivo implantation,” *J. Pharm. Sci.*, vol. 87, no. 6, pp. 738–744, 1998, doi: 10.1021/js9703986.
[154] J. Kožák, M. Rabišková, and A. Lamprecht, “In-vitro drug release testing of parenteral formulations via an agarose gel envelope to closer mimic tissue firmness,” *Int. J. Pharm.*, vol. 594, 2021, doi: 10.1016/j.ijpharm.2020.120142.
[155] B. Semmling, S. Nagel, K. Sternberg, W. Weitschies, and A. Seidlitz, “Long-term stable hydrogels for biorelevant dissolution testing of drug-eluting stents,” *J. Pharm. Technol. Drug Res.*, vol. 2, no. 1, p. 19, 2013, doi: 10.7243/2050-120x-2-19.
[156] Food and Drug Administration, “Guidance for Industry Extended Release Oral Dosage Forms: Development, Evaluation, and Application of In Vitro/In Vivo Correlations,”

Rockville, 1997.
[157] V. R. S. Uppoor, “Regulatory perspectives on in vitro (dissolution)/in vivo (bioavailability) correlations,” *J. Control. Release*, vol. 72, no. 1–3, pp. 127–132, 2001, doi: 10.1016/S0168-3659(01)00268-1.
[158] S. S. Iyer, W. H. Barr, M. E. Dance, P. R. Coleman, and H. T. Karnes, “A ‘biorelevant’ system to investigate in vitro drug released from a naltrexone implant,” *Int. J. Pharm.*, vol. 340, no. 1–2, pp. 104–118, 2007, doi: 10.1016/j.ijpharm.2007.03.032.
[159] M. R. C. Marques, R. Loebenberg, and M. Almukainzi, “Simulated biological fluids with possible application in dissolution testing,” *Dissolution Technol.*, vol. 18, no. 3, pp. 15–28, 2011, doi: 10.14227/DT180311P15.
[160] A. Simon, V. R. De Almeida Borges, L. M. Cabral, and V. P. De Sousa, “Development and validation of a discriminative dissolution test for betamethasone sodium phosphate and betamethasone dipropionate intramuscular injectable suspension,” *AAPS PharmSciTech*, vol. 14, no. 1, pp. 425–434, 2013, doi: 10.1208/s12249-012-9920-2.
[161] F. Damiri, Y. Bachra, C. Bounacir, A. Laaraibi, and M. Berrada, “Synthesis and Characterization of Lyophilized Chitosan-Based Hydrogels Cross-Linked with Benzaldehyde for Controlled Drug Release,” *J. Chem.*, vol. 2020, 2020, doi: 10.1155/2020/8747639.
[162] J. Shen and D. J. Burgess, “Accelerated in-vitro release testing methods for extended-release parenteral dosage forms,” *J. Pharm. Pharmacol.*, vol. 64, no. 7, pp. 986–996, 2012, doi: 10.1111/j.2042-7158.2012.01482.x.
[163] J. V. Andhariya, S. Choi, Y. Wang, Y. Zou, D. J. Burgess, and J. Shen, “Accelerated in vitro release testing method for naltrexone loaded PLGA microspheres,” *Int. J. Pharm.*, vol. 520, no. 1–2, pp. 79–85, 2017, doi: 10.1016/j.ijpharm.2017.01.050.
[164] S. Ahuja and M. W. Dong, *Handbook of pharmaceutical analysis by HPLC*, 1st ed. Elsevier Academic Press, 2005.
[165] “Problemas na bomba, no injetor ou no detector HPLC: como identifica-los e resolvê-los.” https://www.dctech.com.br/problemas-na-bomba-no-injetor-ou-no-detector-hplc-como-identifica-los-e-resolve-los/ (accessed May 15, 2021).
[166] Madhu, “Difference Between Isocratic and Gradient Elution,” 2020. https://www.differencebetween.com/difference-between-isocratic-and-gradient-elution/.
[167] J. W. Dolan, “How Does It Work? Part IV: Ultraviolet Detectors,” 2016. https://www.chromatographyonline.com/view/how-does-it-work-part-iv-ultraviolet-detectors (accessed May 15, 2021).
[168] “What is a Response Factor?,” *Chromatography today*, 2014. https://www.chromatographytoday.com/news/gc-mdgc/32/breaking-news/what-is-a-response-factor/31169 (accessed Apr. 10, 2021).