Evaluation of release specifications for critical raw materials, drug substance and drug product in the production process of recombinant adeno-associated virus vectors

Master´s thesis by

Ina Alice Merikivi-Dahlqvist

Pharmaceutical Sciences

Faculty of Science and Engineering

ÅAU Supervisor: Tapani Viitala

Biovian Oy Supervisor: Artur Padzik

Åbo Akademi University

Turku, Finland

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Abstract

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Author: Ina Alice Merikivi-Dahlqvist	
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Supervisor: Tapani Viitala (Åbo Akademi University)	Supervisor: Artur Padzik (Biovian Oy)
In gene therapy, adeno-associated virus (AAV) vectors have shown to be efficient vehicles for delivering the gene of interest to the target site. To date, there are three AAV-based biologicals approved by authorities; Glybera, Luxturna and Zolgensma. The manufacturing process of AAV vectors, a recombinant form of AAV (rAAV), considered in this thesis is based on HEK293 cells. These producer cells are transfected by three plasmids containing *rep*/*cap* genes, helper genes and a gene of interest, turning the HEK293 cells into AAV-producing factories. Plasmid delivery into the producer cells is called triple transfection or transient transfection and is achieved using a transfection reagent. The upstream process that covers the production in producer cells is followed by a downstream process with several purification steps resulting in a purified rAAV drug substance and, after formulation and filling, in an rAAV drug product. Two critical raw materials in this process are plasmids and transfection reagent. Before these components can be added to the process, they must be qualified to ensure quality, safety, and efficacy. The same considerations apply to the purified drug substance before it can be released for filling and before the drug product can be released for clinical trials or to the market. Medicinal products are supervised and regulated by authorities. This thesis has collected the regulatory requirements and specifications for plasmids, transfection reagents, and the rAAV vector drug substance and drug product. In addition, associated assays have been defined. The result of this thesis is a suggestion of tests based on regulatory requirements, the quality by design (QbD) approach, and the reviewed literature, to be included in the specification of plasmids, transfection reagent and the AAV drug substance and drug product.	
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List of Abbreviations

AAV	Adeno-associated virus
CQA	Critical quality attribute
ddPCR	Droplet digital polymerase chain reaction
DNA	Deoxyribonucleic acid
DP	Drug product
DS	Drug substance
EC	European Commission
EMA	European Medicines Agency
EU	European Union
FDA	The Food and Drug Administration
GOI	Gene of interest
HEK293	Human embryonic kidney cells 293
ICH	The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
ITR	Inverted terminal repeats
kb	Kilobase
nm	Nanometer
PCR	Polymerase chain reaction
Ph. Eur.	European Pharmacopoeia
QA	Quality Assurance
QbD	Quality by design
qPCR	Quantitative polymerase chain reaction
QTPP	Quality target product profile
rAAV	Recombinant adeno-associated virus
RNA	Ribonucleic acid
TT	Transient transfection

1 Introduction

As it has been possible to identify disease-causing genes, the development of gene therapies has become important and gained much attention. Gene therapy aims at treating or even eliminating uncurable diseases. This treatment is implemented via correction of the mutated gene or normalization of the expression of genes. The disease-causing gene can be replaced with a correct copy of the gene. It is also possible to inactivate a disease-causing gene. Gene delivery systems are required to allow the uptake of the gene. (Naso et al., 2017)

To date multiple gene therapy products have been developed that are based on (FDA, 2018):

- plasmid DNA
- viral vectors
- bacterial vectors
- human gene-editing technologies
- patient-derived cellular gene therapy products

Among these, viral vectors belong to one of the earliest and most effective approaches in gene therapy. The properties of native viruses make viral vectors attractive vehicles. Viruses are highly efficient at delivering nucleic acid (their DNA or RNA) to specific cell types and, at the same time, capable of avoiding immunosurveillance by the infected host. Viral vectors are utilizing the structure and genome delivering capacity of viruses but replacing the viral genes with the gene of interest. (Naso et al., 2017)

The major virus types adopted for gene therapy applications are (Finer and Glorioso, 2017):

- Retrovirus (e.g., Lentivirus)
- Herpes Simplex Virus (HSV)
- Adenovirus
- Adeno-associated virus (AAV)

These viral reagents have different characteristics, and the choice depends on the desired profile.

One of the most actively studied vectors for gene therapy applications from the above list is the adeno-associated virus (AAV). It belongs to the parvovirus family. The name refers to the circumstances when it was first isolated, i.e., a contaminant in adenovirus preparation. An AAV particle has a size of approximately 25 nm in diameter and consists of a small and single-stranded genome. AAV is non-enveloped and is protected by a protein shell composed of VP1, VP2, VP3 subunits. The size of the genome is approximately 4.7 kb, and it contains three genes, *rep* (replication), *cap* (capsid) and *aap* (assembly). Three independent promoters, alternative translation start sites, and differential splicing allows the making of at least nine gene products out of these three genes. The *aap* gene product has been shown to provide a scaffold for capsid assembly, but it is crucial only for AAV2 and non-essential for AAV4, AAV5 or AAV11 (Earley et al., 2017) In the natural AAV virus, these genes are located between two inverted terminal repeats, ITRs, which are needed for the replication and packaging of the genome (Naso et al., 2017). The replication of natural AAV is dependent on a co-infection and, hence, needs a helper virus, e.g., adenovirus or baculovirus, because they contribute with the required E1a and E1b genes. (Barnes et al., 2021). At least 12 natural serotypes of AAV have been identified and studied, and they show different binding selectivity of capsid proteins to specific cell surface receptors, which enables cell wall penetration. AAV2 was the first AAV serotype identified and used as a reference when identifying other serotypes. (Hammond et al., 2017)

AAV has many advantages as a viral vector, e.g.:

- lack of apparent human pathogenicity
- low immunogenicity
- high gene transfer efficiency
- long-term expression due to the formation of stable episomes (Tustian and Bak, 2021)
- non-mutagenic, i.e., not integrating into the host cell genome
- tropism to selective tissue (Kimura et al., 2018)
- transfecting both dividing and non-dividing cells (Tustian and Bak, 2021)

In gene therapy, the natural AAV is not used but engineered into a recombinant AAV (rAAV) (Kimura et al., 2018). rAAV lacks viral genes and can be described as a protein-based nanoparticle built to penetrate the cell membrane and deliver genetic material into the nucleus (Naso et al., 2017). As mentioned earlier, the size of the AAV genome is about 4.7 kb, which also dictates the packaging capacity (Davidsson et al., 2020). The basic design of the rAAV vector consists of the gene of interest located between two ITRs packaged into a proteinous capsid. The *rep* and *cap* genes are removed, which means that the components needed for replication, packaging and capsid assembly have to be added from outside during production.

The way these components are added categorizes the manufacturing process into five types (Tanaka et al., 2020):

- plasmid DNA transiently transfected into producer cells
- mammalian stable producer cell lines
- infection of mammalian producer cells with recombinant herpes simplex viruses
- infection of mammalian producer cells with recombinant adenoviruses
- infection of insect producer cells with recombinant baculoviruses

The traditional and most widely used production method is based on DNA plasmid transfection of HEK293 cells (human embryonic kidney cells) with two or three plasmids, also called transient transfection. A transient transfection including three plasmids is called a triple transfection. (Barnes et al., 2021) One plasmid contains the sequence coding for the gene of interest, the second plasmid contains the AAV *rep*/*cap* genes, and the third one contains helper genes derived from adeno or herpes viruses (Naso et al., 2017). Initially, the HEK293 cells are transfected with the plasmids using a transfection reagent, e.g., polyethylenimine (PEI). After transfection, the HEK293 cells are expanded and harvested using microfiltration or centrifugation. The assembled viral particles are found both inside of HEK293 cells and in the growth medium. For this reason, both the supernatant and the cellular fraction are recovered (Tanaka et al., 2020). Subsequently, they are harvested using chemical or mechanical lysis of producer cells followed by a nuclease treatment which

digests any free DNA. Finally, the cell lysate undergoes several downstream processes, which result in the purified rAAV product. (de Rooij et al., 2019)

To date, three AAV-based biologics have been authorized to use. EMA approved Glybera in 2012 with the indication of familial lipoprotein lipase deficiency (LPLD). FDA approved Luxturna for a rare form of blindness in 2017 and EMA in 2018, and Zolgensma for spinal muscular atrophy by FDA in 2019 and 2020 by EMA. (Wörner et al., 2021) In addition to already approved drugs, multiple gene therapies based on AAV are actively being developed. The clinical trials database (ClinicalTrials.Gov) lists 264 clinical trials at various stages. AAV-based medicinal products are being investigated for various diseases ranging from cancer to neurological diseases.

The aim of this thesis was to investigate the regulatory requirements for specifications related to the manufacturing of rAAV. The process has two critical raw materials, plasmids and the transfection reagent. It generates a purified rAAV drug substance (DS), which results in a rAAV vector drug product (DP) after final formulation and filling. The purpose was to clarify and outline the specifications for the two critical raw materials, the DS and the DP, and to define associated assays to analyze them.

This thesis project was a collaboration with Biovian Oy, Turku. The thesis is written from the European point of view unless otherwise stated.

1.1 Background

Authorities regulate and supervise the pharmaceutical industry to ensure drugs' safety, quality, and efficacy (Olson, 2014). Before a medicinal product can be brought on the market, it must have a marketing authorization approved by the regulatory authority. European Medicines Agency (EMA) authorizes new medicinal products within the European Union, and on the national level in Finland, the authority is the Finnish Medicines Agency (FIMEA). Other markets, e.g., the United States of America or Japan, have their own regulatory frameworks. Bringing an investigational medicinal product from the pre-clinical phase into clinical trials within the EU requires a clinical trial authorization (CTA). The CTA form is submitted to FIMEA and to a regional ethics committee in Finland. (Eupati webpage) The regulatory requirements defined by the authorities need to be fulfilled in the case of both drug product that enters clinical trials or the market.

The regulatory authorities publish the regulatory rules and agreements in guidelines. In addition, The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) also provides internationally harmonized guidelines (EMA, 2016). Specifically for the quality control of medicines, European Pharmacopoeia consists of official standards for medicinal products in monographs (European Directorate for the Quality of Medicines & HealthCare (EDQM)). The regulatory authorities supervise that the pharmaceutical companies follow the regulatory requirements described in these guidelines and documents since it ensures high-quality, effective, and safe medicines (EMA, 2016).

According to the ICH Q11 guideline, all manufacturing processes related to drug substances must have a control strategy, which is a planned set of controls. ICH guideline Q6B states that "Specifications are part of a total control strategy to ensure product quality and consistency." (ICH Q6B guideline, direct citation) The ICH guideline Q6B definition of specifications is: "A specification is defined as a list of tests, references to analytical procedures, and appropriate acceptance criteria which are numerical limits, ranges, or other criteria for the tests described. It establishes the criteria to which a drug substance, drug product or materials at other stages of its manufacture should be considered acceptable for its intended use. "Conformance to specification" means that the drug substance and drug product, when tested according to the listed analytical procedures, will meet the acceptance criteria. Specifications are critical quality standards that are proposed and justified by the manufacturer and approved by regulatory authorities as conditions of approval." (ICH Q6B guideline, direct citation)

Based on this, every material intended to be added to the manufacturing process of a drug product has to be analyzed and meet the pre-defined acceptance criteria, i.e., a specification for each material must exist. Only a material that conforms to its specifications can be added to the process. After the manufacturing process and before a drug product can be released for clinical trials or on the market, the final drug product must be tested to ensure its safety, quality, and efficacy. The tests required are included in the release specifications of the final drug product and are a mandatory part of the marketing authorization application. (ICH Q6B and Project A-Gene, 2021)

For a new drug product, the quality control and specification considerations are done during the entire development of the drug. Product type and materials used for

production specify which tests should be included in the specifications. In this thesis, the considerations about specifications for materials/products are related to the manufacturing process of rAAV vectors. From a regulatory perspective, AAV-based gene therapy medicinal products belong to Advanced Therapy Medicinal Products, ATMPs. The group ATMPs include products based on genes, tissues, or cells.

The ATMP products can be classified into three main types (EMA webpage: Advanced therapy medicinal products: Overview):

- gene therapy medicines
- somatic-cell therapy medicines
- tissue-engineered medicines

and one additional type:

- combined ATMPs, if an ATMP contains one or more medical devices

2 Literature review

The conducted literature review included searching for relevant guidelines, becoming familiar with the manufacturing process, and studying the concept of quality control. Since the authorities emphasize and encourage the drug manufacturers to follow the concept of quality by design (QbD), the literature review includes the introduction to this approach.

2.1 Guidelines

EMA has seven scientific committees whose responsibility is to evaluate marketing authorization applications, provide scientific advice to pharmaceutical companies, prepare scientific guidelines and contribute to harmonizing regulatory requirements. One of these committees is the Committee for Advanced Therapies, CAT, whose responsibility is to work for the quality, safety, and efficacy of ATMPs. Since the regulatory requirements for medicinal products are published in guidelines, the guidelines related to AAV vectors had to be defined. On their webpage, EMA has collected and published a list of guidelines

related to ATMPs, both concerning gene therapy medicinal products and products related to cell-therapy and tissue engineering. (EMA webpage: Guidelines relevant for advanced therapy medicinal products) The list is intended to help pharmaceutical companies and individuals in the application process for marketing authorization for human medicines.

Among the list of guidelines concerning gene therapy medicinal products, the following guidelines were identified as relevant for the aim of this thesis:

- Guidelines on Good Manufacturing Practice specific to Advanced Therapy Medicinal Products
- Guideline on the quality, non-clinical and clinical aspects of gene therapy medicinal products (EMA/CAT/80183/2014)
- Reflection paper on quality, non-clinical and clinical issues related to the development of recombinant adeno-associated viral vectors (CHMP/GTWP/587488/2007 Rev. 1)
- The European Pharmacopoeia: 5.2.12. Raw materials of biological origin for the production of cell-based and gene therapy medicinal products and 5.14. Gene transfer medicinal products for human use and gene therapy medicinal products
- ICH quality guidelines

2.1.1 Guidelines on Good Manufacturing Practice specific to Advanced Therapy Medicinal Products

In the European Union, EudraLex collects rules and regulations covering medicinal products. Volume 4 of EudraLex guides how to interpret the principles and guidelines of good manufacturing practices (GMP) which is required for all medicinal products with a marketing authorization. Since AAV-based medicinal products and other ATMPs have special features and differ from other medicinal products, a separate GMP guideline specific for ATMPs was prepared and published by EMA in 2017. The ATMP GMP guideline

was added as part 4 (GMP requirements for Advanced Therapy Medicinal Products) in EudraLex Volume 4. This guideline highlights that other GMP guidelines do not apply to ATMPs unless mentioned in the ATMP GMP guideline. (ATMP GMP guideline, 2017)

The ATMP GMP guideline is comprehensive. It applies to both ATMPs with marketing authorization (authorized ATMPs) and ATMPs in clinical trials (investigational ATMPs) and for the development phase of all ATMPs. ATMPs are a versatile class of often very complex products with different degrees of variability in the finished product due to biological materials. Due to that, a risk-based approach applies to ATMPs, enabling a certain degree of flexibility in the GMP requirements. The ATMP manufacturer is still responsible for addressing the potential product and manufacturing process risks and related mitigation actions. (ATMP GMP guideline, 2017)

Relevant to this thesis is the ATMP GMP guideline chapter "6.2 Specifications and Instructions" which guides the content of specifications, e.g., raw materials and the finished product.

2.1.2 Guideline on the quality, non-clinical and clinical aspects of gene therapy medicinal products (EMA/CAT/80183/2014)

The Committee for Advanced Therapies published the guideline in 2018 as a revision of a document published in 2001 (Note for Guidance on the Quality, Preclinical and Clinical aspects of gene transfer medicinal products (CPMP/BWP/3088/99)). The guideline consists of scientific principles and guidance for Gene Therapy Medicinal Product development and evaluation. The guideline is considered the overarching guideline for human gene therapy medicinal products. (EMA, 2018)

The document comprises quality considerations related to gene therapy medicinal products divided into sections including drug substance, drug product and process development, analytical methods, stability, and adventitious agents. In addition to quality aspects, the document contains both non-clinical and clinical development considerations. (EMA, 2018)

2.1.3 Guideline on quality, non-clinical and clinical requirements for investigational advanced therapy medicinal products in clinical trials (EMA/CAT/852602/2018) (draft)

An important guideline that is still under preparation is the “Guideline on quality, non-clinical and clinical requirements for investigational advanced therapy medicinal products in clinical trials” the purpose of which is to guide the manufacturers of ATMPs when planning to apply permission for performing clinical trials. This guideline is needed because it is acknowledged that bringing an advanced therapy investigational medicinal product, ATIMP, to the clinical trial phase differs from other product types. It contains guidance for the structure and information required for the clinical trial application, CTA, and was first published for consultation in 2019 by CAT. The document is now a draft until it is approved. (EMA, 2019)

2.1.4 Reflection paper on quality, non-clinical and clinical issues related to the development of recombinant adeno-associated viral vectors (CHMP/GTWP/587488/2007 Rev. 1)

The Committee for the Medicinal Products for Human Use (CHMP) has published a reflection paper intended to discuss quality, non-clinical and clinical issues related to the development of medicinal products based on rAAV vectors. The paper contains information about requirements essential for the marketing authorization application: manufacturing methods, quality aspects, non-clinical aspects, environmental risks, and clinical aspects. (EMA, 2010)

2.1.5 The European Pharmacopoeia: 5.2.12. Raw materials of biological origin for the production of cell-based and gene therapy medicinal products and 5.14. Gene transfer medicinal products for human use

The European Pharmacopoeia, which The European Directorate established for the Quality of Medicines and HealthCare (EDQM), contains official quality standards. These standards are recognized scientific benchmarks and are applied not only in Europe. The content of the Ph. Eur. describes how human and veterinary medicines should be tested and quality controlled during manufacturing. (EDQM fact sheet, 2020) The official standards of the Ph. Eur. form a scientific basis for the quality control of drugs and the legally binding standards.

According to the EDQM webpage, the 10th edition of the Ph. Eur. is legally binding in 39 European countries and applied worldwide in more than 120 countries. (EDQM webpage: European Pharmacopoeia (Ph. Eur.) 10th Edition)

Chapter 5.2.12 contains considerations related to raw materials of biological origin to produce cell-based and gene therapy medicinal products. Chapter 5.14 lists gene transfer medicinal products for human use and considerations related to the design, manufacturing and quality control. (Ph. Eur. chapters 5.2.12 and 5.14)

2.1.6 ICH quality guidelines

The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use, abbreviated ICH, was founded in 1990. The aim is to work for an international harmonization related to regulatory authorities and the pharmaceutical industry by providing technical guidelines. (ICH, 2021) The guidelines contain uniform standards for technical requirements for pharmaceuticals for human use and are implemented by regulatory authorities. (mastercontrol.com) The relationship between EMA and ICH is that ICH harmonizes the scientific guidelines by EMA. The harmonization is needed to ensure the quality, safety and efficacy of medicines developed and registered worldwide. The harmonization is achieved by a collaboration between regulatory authorities and the pharmaceutical industry. (Ainslie, 2018)

The guidelines can be divided into four groups: quality guidelines, safety guidelines, efficacy guidelines, and multidisciplinary guidelines. Since this thesis project considers the quality requirements of rAAV production, a review of the quality guidelines has been conducted. The quality guidelines consist of 14 main topics containing one or more documents (Figure 1). For the objectives of this thesis, the following ICH quality guidelines are specifically applicable: Q6A, Q6B, Q8, Q9, and Q11. (ICH: Quality guidelines)

The ICH guideline Q5A, “Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin”, published in 1999, is currently revised to reflect the biotechnology development that occurred since its publication. (ICH: Final Concept Paper, 2019)

Figure 1. ICH quality guidelines consist of 14 topics, of which the relevant guidelines for this thesis are highlighted with yellow color. (ICH: Quality guidelines)

2.2 Manufacturing process of rAAV

This thesis considers the manufacturing process of recombinant adeno-associated virus vectors consisting of an upstream process where the viral particles are produced using HEK293 producer cells (Figure 2). Then, the process continues into a downstream part where the viral particles are purified, i.e., resulting in the AAV drug substance. Finally, through formulation and vialing, the drug product is produced.

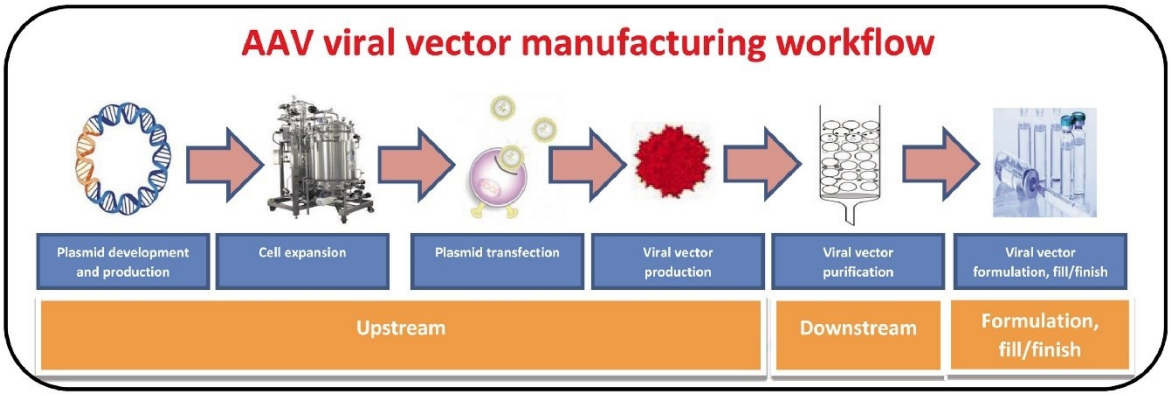

Figure 2. The workflow of the production process of rAAV. (Srivastava et al., 2021)

From a regulatory point of view, it is important to recognize that the process generates both a drug substance, DS, and a drug product, DP, since the information to be included in the marketing or clinical trial authorization applications usually requires considerations of these two parts separately. It is worth noticing that in EMA guidelines, the terms “drug substance” and “active substance” are used interchangeably, as is the case with the terms “drug product” and “finished product”. Further, it is highlighted that separating an ATMP into DS and DP is not always possible. “In those cases where the ATMPs production is a continuous process, it is not necessary to repeat the information that was already provided in the DS part, into the DP section”, as a direct citation from “Guideline on quality, non-clinical and clinical requirements for investigational advanced therapy medicinal products in clinical trials”. (EMA, 2019)

Regarding AAV-based products, the drug substance refers to the rAAV vector product after the purification steps before the final formulation. The DP process includes that part of manufacturing where the DS undergoes final formulation and filling into vials. Unlike EMA guidelines, which use the terms DS and DP, Ph. Eur. chapter 5.14 regarding AAV products uses the terms "purified harvest", "final bulk" and "final lot". The purified harvest refers to the DS, the final bulk describes the formulated AAV product, and the final lot refers to the formulated and aseptically vialed AAV drug product, which EMA guidelines calls the DP. (Ph. Eur. chapter 5.14)

When manufacturing AAV vectors of clinical grade, it is essential to know the complex methods of generation, purification, and characterization of AAV vectors. In

addition, the implementation of GMP and extensive product- and process-related controls are required. Further, considerations related to clinical grade manufacturing include the requirement of scalable methods, which should generate products of high safety, purity, potency, and stability to meet regulatory expectations. However, the stage of development and implementation of the manufacturing process should relate to the stage of clinical development. (Wright, 2008)

2.2.1 The upstream process

The upstream AAV manufacturing process consists of cell culture, transfection, and harvest. The initial step is the cell culture of producer HEK293 cells whose genome contains the E1a and E1b genes required for AAV replication. (Qu et al., 2015) The following components necessary to produce viable AAV particles are present on plasmids. The upstream process is based on the triple transfection of producer cells. Other production systems are also available and are based on the type of cell line used, the precursor materials required, and cell growth substrates.

The four main production systems used in the pharmaceutical industry are:

- triple transfection, also called transient transfection, TT
- baculovirus expression vectors, BEVS
- producer cell line systems, PCL
- HSV infection, HSVi

Triple/transient transfection remains the most used system and will be considered in this thesis project. (Dobrowsky et al., 2021) The triple transfection and the viral particle production is illustrated in Figure 3.

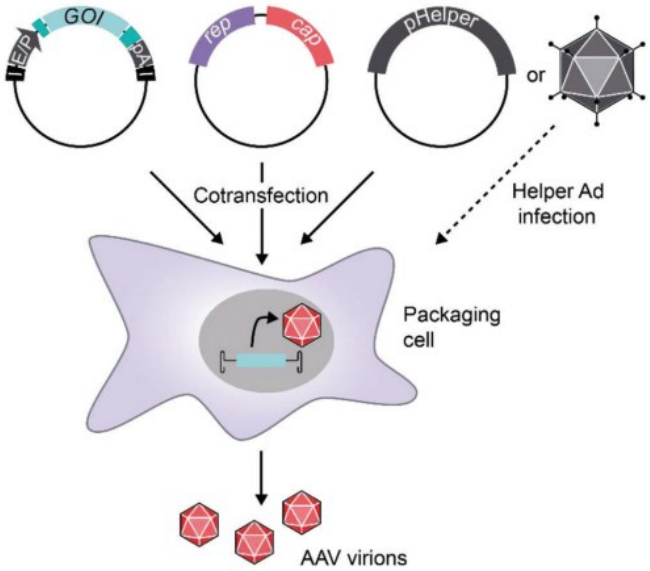

Figure 3. Illustration of the triple transfection and rAAV particle production. (Wagner et al., 2021)

After transfection, producer cells are allowed to produce viral particles for a certain amount of time (commonly 48-72 hours) and these are harvested using centrifugation or microfiltration. Both the cellular fraction and the supernatant are collected, since rAAV particles are present in both of them. The viral particles are harvested using mechanical or chemical cell lysis. (de Rooij, 2019) Polysorbate 20 can be used for lysing mammalian cells, but other alternatives also exist for chemical lysis of the producer cells; e.g., Triton X-100 has been used before its use was prohibited in EU in January 2021. (Florencio, G., 2015 and DiaSys International Newsletter) An endonuclease treatment with e.g., Benzonase is performed to remove any free DNA from the process. The remaining cell lysate will undergo viral purification in a downstream process. (de Rooij, 2019)

From a regulatory point of view, the upstream process contains two critical raw materials, plasmids, and transfection reagent which are described in detail below.

2.2.1.1 Plasmids

Since natural AAV cannot replicate on its own, a helper factor is required for replication. AAV can only release its genome inside host cells without this helper factor but can't replicate. Consequently, most gene copies will be cleared effectively, while only a small

amount will persist long-term. It is believed that AAV with long-term persistence occurs in a circular form, called episomal form. (Meier et al., 2020) Instead of using a helper virus, e.g., adenovirus, which will be considered a contaminant in the process, the helper gene (E2A, E4, and VA RNA), can be inserted into a plasmid. The second plasmid contains the sequence coding for the gene of interest flanked by ITR and is limited to the natural AAV genome size of around 5 kb. The third plasmid encodes the AAV *rep*/*cap* genes required for replication, packaging, and capsid assembly. (Naso et al., 2017) When all three plasmids are delivered into the same producer cell, they will produce rAAV vectors without the requirement of co-infection with wild type helper virus. (Smith, 2017)

In most cases, plasmids are circular, bacterial DNA molecules. Plasmids used to manufacture rAAV vectors are produced using E. Coli fermentation methods. (Ohlson, 2020) For economic large-scale plasmid production, plasmid copy number and biomass concentration have to be optimal. Factors affecting these parameters are related to the formula of cultivation media and cultivation methods.

The plasmid upstream production process is followed by a downstream process that covers their purification. It typically consists of the following steps: cell harvest, lysis, cell debris, affinity precipitation, adsorption, and buffer exchange. (Prather et al., 2003) For a successful transfection, the purity and quality of the plasmid DNA are critical. According to the responsible regulatory agencies, considering GMP production of rAAV vectors, plasmid DNA of high-quality grade is approved as a starting material. (Schmeer et al., 2017)

2.2.1.2 Transfection reagent

The transfection process of HEK293 producer cells requires a transfection reagent. The reason is that DNA plasmids, built of nucleic acids, have a negative charge and are repelled by the negatively charged HEK293 cell membrane. Polyethylenimine is a well-studied synthetic cationic polymer with a strong anion exchange capacity. (Virgen-Ortiz et al., 2017) The cationic PEI can condense DNA into positively charged particles, forming stable complexes called polyplexes, which will bind to negatively charged cell surfaces and will be efficiently internalized. (Longo et al., 2013) In addition, these complexes protect the nucleic acid from degradation by cellular nucleases. (Yuan and Li, 2017) Endocytosis and

endocytosis-like mechanisms are predominant mechanisms of polyplexes delivery. (Ita, 2020) Once endocytosed, PEI buffers the endosomal vesicle causing swelling and lysis. Consequently, DNA is released into the cytoplasm and translocated into the nucleus, where it is transcribed and translated into the protein product. (Zhang et al., 2011) Other methods also exist, such as calcium phosphate or liposomes, but the use of PEI results in high efficiency and reproducibility. (Crosson et al., 2018)

Polyethylenimine is available in two forms, linear and branched forms, based on the synthesized structure, and both are used in nucleic acid delivery. (Yuan and Li, 2017) The difference between these two PEI forms is related to amino groups; linear PEI contain primary and secondary amino groups while the branched form also contains tertiary amino groups. The size and shape of PEI affect its transfection efficiency and cytotoxicity. (Li and Ju, 2017) Studies have shown that the transfection efficiency of PEIs is related to their molecular weight, degree of branching, the charge ratios between PEI nitrogen and nucleic acid phosphate, and cell type. The safety considerations of PEIs are important for their clinical translation. Studies have shown that the positive charges of PEIs might lead to the induction of necrotic cell death or apoptosis, which causes toxicity. PEI can be chemically modified to reduce this toxicity, limiting its clinical use, and improving its transfection efficiency. (Yuan and Li, 2017) Among these chemical modifications, PEGylation is the most utilized. The method is based on the covalent coupling of the PEG (polyethylene glycol) molecule to PEI. (Grigoletto et al., 2017)

Several commercial PEI transfection reagents are available (e.g., from Polysciences, and Polyplus.) The products might differ in purity level or GMP/laboratory grade. (Zhang et al., 2004) In the manufacturing process of rAAV discussed in this thesis, the PEI transfection reagent is provided by Polyplus.

2.2.2 Downstream process

The main aim of the upstream process of rAAV production is high yield and purity, reproducibility of the process and economic sustainability. The vector productivity can be measured as vector genomes (v.g.) produced per cell. The amount has been shown to vary from $1x10^3$to $2x10^5$ v.g. per cell, while $1x10^5$ are considered a common yield. High

productivity is related to higher cost-effectiveness and efficient purification due to a higher concentration of vectors in the total biomass harvested. (Wright, 2008)

The process of packaging viral DNA is not error-free. (Wang et al., 2019) The downstream process aims to separate viral vector particles from different impurities originating from the process and the product, including e.g., empty or partial filled capsids, plasmid DNA and material from producer cells. (de Rooij, 2019) Due to their different physicochemical properties, multiple purification steps are required to achieve a pure rAAV product. (Dobrowsky et al., 2021)

In the downstream process, the first step is a clarification to remove solid and large size particles such as cell debris and cell fragments originating from the cell lysis. Methods for this purpose are centrifugation or filtration techniques, of which different filtration techniques are preferred for industrial-scale manufacturing. This step is followed by chromatographic techniques that wash away impurities while the AAV vectors bind to a matrix. (Hebben, 2018) For the removal of host cell proteins, affinity chromatography is a suitable method. Ion-exchange chromatography or caesium chloride gradient ultracentrifugation can separate full capsids from empty capsids. (Srivastava et al., 2021) Although it might be narrow, the difference in charges between full and empty capsids enables their separation, and ion-exchange chromatography seems to be the most promising alternative for full capsid enrichment. (Hebben, 2018)

The downstream purification process is essential for a successful rAAV clinical manufacturing since the goal is to produce a final clinical product with high purity, potency and titer. When considering the future marketing large-scale requirements, the selected process should ensure the highest recovery and consistency. (Clément and Grieger, 2016) For AAV-based investigational products in clinical development, the identification, characterization, and batch-to-batch control of impurities related to the process or products is a challenge, even for a highly purified product. (Wright, 2014)

When the impurities are removed, the product needs to obtain the correct vector concentration and the appropriate formulation. The buffer should support product stability and the administration to patients. Tangential flow filtration, TFF, is the standard

method for this manufacturing step before the product undergoes the final sterile filtration. (Hebben, 2018)

Impurities in the manufacturing of rAAV vectors are divided into two categories:

- Process-related impurities
- Product-related impurities

2.2.2.1 Process-related impurities

Impurities originating from the raw materials and components of the manufacturing process are considered process-related impurities and are presented and briefly explained below.

Residual plasmid DNA. To maximize the production of full capsids, an excess amount of plasmid DNA is required. Excess of free plasmid DNA in the product can be removed using, e.g., Benzonase, because plasmid DNA is sensitive to nucleases. (Wright, 2014)

Residual host cell DNA/RNA. During the production of rAAV particles, the cell lysis of the host cells to release the viral particles causes the appearance of host cell nucleic acid in the upstream product. As residual plasmid DNA, residual host cell DNA/RNA can be removed by the same nuclease treatment. (Wright, 2014)

Residual host cell protein. The cell lysis of producer cells to release AAV particles contributes to host cell material. Among cellular components such as lipids and polysaccharides, the most immunogenic are proteins. (Naso, 2017) In the drug product, host cell proteins, HCPs, might cause immunogenic reactions, inflammation, and even anaphylactic shock. Hence, the downstream process must ensure that residual host cell proteins are reduced to the minimum. (Hebben, 2018)

In addition to the impurities described above, process-related impurities include other substances such as residual helper components, residual cell culture components, residual nucleases, residual purification buffers, ligands, and growth media. (Wright, 2014)

2.2.2.2 Product-related impurities

Empty/partial capsids. The rAAV vector manufacturing process consists of many components, leading to a higher potential for generating product-related impurities. Not every producer cell will be transfected with all three plasmids during the transfection process. It is not unusual that a significant amount (50-90%) of the produced viral particles are empty. Subsequently, the empty capsids lack the required ITR-flanked transgenes. In addition to full and empty capsids, the manufacturing process can also produce partial capsids, which contain only fragments of the genome, see Figure 4. Naturally, only full capsids are desired since they contain the transgene. (Naso, 2017) The empty and partial capsids are referred to as product-related impurities. Since they closely appear like the desired full AAV capsid, they constitute a challenge in the purification process of rAAV. The upstream process must be optimized to reduce the creation of empty and partial capsids and the downstream process to recognize them to reduce or remove them. (Wright, 2014)

The presence of empty or partial capsids must be controlled due to their negative impact as an impurity on the rAAV product. Since empty/partial capsids appear like full capsids, they might compete with full capsids for receptor binding on target cells. This could lead to the need for an increased vector dose which is undesired, e.g., when administrating to smaller parts of the body such as the brain or the eyes. (White, 2021) The empty capsids also stimulate innate and adaptive immune responses, not adding anything to the therapeutic effect. (FDA, 2021) Many people have neutralizing antibodies against AAV, because they have been exposed to AAV earlier in life. Hence, the rAAV treatment can lead to a stronger adaptive immune response. (Ronzitti et al., 2020) Concerning the innate immune response, researchers debate whether the capsid protein itself is capable of activation and, hence, reduces the effectiveness of the treatment. (White, 2021)

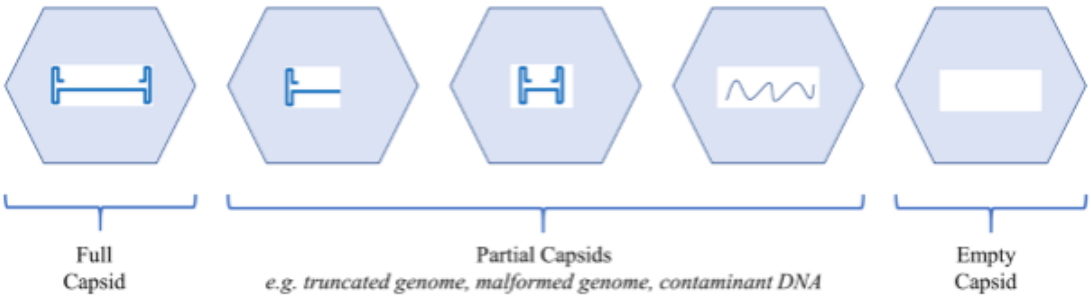

Figure 4. The produced viral capsids may be full or empty, or partial. (Tustian and Bak, 2021)

Encapsidated host cell DNA and helper component DNA. Fragments of both host cell DNA and helper component DNA (from plasmids or helper viruses) can be incorporated into AAV capsids during the production, forming viral particles with the same outer appearance as the desired AAV particles. Since host cell DNA or helper component DNA are internalized inside the protective capsid shell, they cannot be removed using nucleases due to their appearance similar to the desired vector product, the detection and removal of this class of product-related impurity is very difficult. Therefore, the focus should lie on the prevention of the production of these impurities. (Wright, 2014) Concerning the generation, it is believed that these DNA fragments contain sequences that unintentionally cause packaging into AAV. (Wright, 2008)

Replication-competent AAV. Another impurity is wild-type-like AAV which is commonly referred to as replication-competent AAV, rcAAV. Replication-competent AAV may appear during the production simultaneously with the desired rAAV vectors. The replication and packaging processes of viral DNA primarily take place in the nucleus of the host cells leading to the presence of a large number of AAV vector genomes. In combination with transcribed helper genes from the required helper plasmid, it is impossible to avoid the nonhomologous recombination between AAV *rep* and *cap* genes and ITRs. The described recombination results in the generation of rcAAV particles. The presence of these particles as an impurity in an AAV-based product may be harmful to the patient and may negatively affect the long-term transgene expression. (Dong et al., 2013)

Non-infectious AAV particles. Viral vectors must be able to infect the target cell to deliver the transgene and, hence, produce the gene product for the treatment of a disease. In this thesis, this class of impurity refers to rAAV capsids containing the intended transgene but lacking the capability of replicating in cell-based assays including helper sequences. However, it is very challenging to measure the infectivity of rAAV vector particles e.g., because the vector is designed not to replicate in transduced cells. The definition of non-infectious AAVs could also include many of the already mentioned product-related impurities, e.g., empty capsids. (Wright, 2014) As with empty or partially full capsids, the amount of non-infectious rAAV vector particles must be quantified, controlled, and reduced since they lead to unnecessary viral antigens. This directly, negatively affects the desired infectious particles' transduction and increases the risk for immunotoxicity. (Kuck et al., 2006) An rAAV vector-based product containing non-infectious viral particles lowers the infectivity and requires higher doses to achieve the desired efficacy. (Wright, 2014) Due to the disadvantages of non-infectious AAV vector particles, it is important to develop and improve the vector purification processes to remove this product-related impurity. (Kuck et al., 2006)

Aggregated AAV vectors. During different stages of manufacturing and purification and later formulation and storage, AAV vectors may form aggregates. Aggregated AAV vectors, often with a size over 100 nm, are classified as undesired product-related impurities. They lead to reduced yield during purification, reducing viral titer and, therefore, the efficacy of the product. (Rodrigues et al., 2019 and Technology Networks: Quantifying Adeno-Associated Virus Aggregation, 2019) The impact on vector transduction efficiency and biodistribution cause concerns as well. (Wright et al., 2005) In addition, aggregates of AAV vectors may contribute to increased immunogenicity following *in vivo* administration. (Wright, 2008) The formation of aggregates is possible to reduce by the use of excipients. Based on a study made by Wright et al. in 2004, the selection of excipients that maximize ionic strength is a promising approach for minimizing the generation of rAAV vector aggregates.

Degraded AAV vectors. The stability of AAV vector capsids is important in order to protect the encapsidated transgene until it reaches the target host cell. (Rayaprolu et al., 2013) It is a major challenge for AAV manufacturers to prepare stable viral vectors, prevent

their degradation during production and storage, and maintain long-term stability and efficacy. The degradation of capsid proteins can occur physically and chemically. It includes aggregation, proteolysis, oxidation, and deamidation. Actions during the downstream processes have to be taken to avoid those reactions, affecting the virus infection efficiency. (Srivastava et al., 2021)

2.2.3 Summary of the purification steps in the upstream and downstream processes

The production process of rAAV vectors can be summarized in the following steps:

- Harvest of producer cells
- Chemical or mechanical cell lysis
- Digestion of free DNA
- Particle separation
- Concentration, formulation, and sterile filtration

2.2.4 Fill and finish

The vector harvesting and purification results in the AAV vector drug substance, which undergoes final formulation. The last manufacturing step includes filling the vials and visual inspection, followed by labelling and packaging.

2.3 Principles of the Pharmaceutical quality system and quality control

Authorities are regulating the pharmaceutical industry. According to “EU Guidelines for Good Manufacturing Practice for Medicinal Products for Human and Veterinary Use” in EudraLex containing “The Rules Governing Medicinal Products in the European Union”, chapter 1 in part 1, states that the Manufacturing Authorization holder has to design and implement a Pharmaceutical Quality System including also Good Manufacturing Practice and Quality Risk Management. The manufacturer has to ensure that the manufactured medicinal products are appropriate for their intended use, that the marketing

authorization or clinical trial authorization requirements are complied with and that patients are not placed at risk due to lack of safety, quality, or efficacy. According to the guideline, the manufacturer has to establish and maintain a state of control among many aspects that need to be ensured through an appropriate pharmaceutical quality system. To achieve that, effective monitoring, and control systems for the performance of the process and the quality of the product, have to be developed and used. (Eudralex Volume 4, chapter 1, 2013)

One part of Good Manufacturing Practice is Quality Control which includes sampling, testing and specifications and procedures related to the organization, documentation, and release. These procedures ensure the conduction of the required tests and that only materials and products whose quality has been evaluated to meet the requirements, are released for use or for sale. (Eudralex Volume 4, chapter 1, 2013) These quality requirements are collected and constitute the specification of the material or product. The ATMP GMP guideline states that the pharmaceutical quality system should ensure that appropriate specifications for materials, bulk products, intermediates, and finished products are laid down. Specifications for these materials are intended to ensure compliance with the clinical trial or marketing authorization, product consistency and required quality level. (ATMP GMP guideline, 2017)

Regardless of whether applying for clinical trial authorization or marketing authorization, the applications require chemistry, manufacturing, and controls (CMC) information about the product through an electronic common technical document, eCTD, which is a standardized structure for the submissions. In the EU, the CTA submission requires a document called an Investigational medicinal product dossier, IMPD, which follows the eCTD structure. The eCTD structure includes modules with sections for the DS and the DP, and their specifications should be listed here. It is worth noting that as with biologics in general, it might be challenging to distinguish between DS and DP considerations due to an unclear delineation between the drug substance and drug product. (Project A-Gene, 2021)

More specifically, specifications consist of three key factors (Rathore, 2010):

- quality attributes
- references to associated analytical procedures to test these attributes
- qualitative or quantitative criteria for acceptance

The criteria can be of different types, e.g., numerical limits. The material should conform to these criteria to be accepted for its intended use. For example, only raw material batches that conform to its predefined specifications can be used, and only final product batches conforming to its release specifications can be released for use. (ICH Q6B guideline EMA, 1999)

Establishing relevant specifications requires extensive information about the material or product. For the drug product, the characterization should include determining physicochemical and immunochemical properties, biological activity, purity, and impurities. When considering the acceptance criteria for the specification, all data available should be utilized when establishing and justifying the criteria. When it is time to establish the specifications, data available is based on batches used in preclinical or clinical studies, batches from a demonstration of manufacturing consistency, stability studies, and development data. (ICH Q6B guideline EMA, 1999)

The test limits may vary for the drug product depending on whether the limits are applied to release testing (tighter limits) or shelf-life testing. The test limits at release ensure the desired quality at the end of shelf life, and if certain properties are affected by storage, this has to be considered when setting release limits. (EMA, 1992)

When considering raw materials and excipients, the quality should be appropriate for their intended use. Quality standards should be met to ensure a high-quality drug product. Hence, according to the ATMP GMP guideline, the quality of raw and starting materials is considered a key factor. Further, the importance of avoiding contamination and minimizing material variability, is highlighted. Chapter 5.2.12. in the European Pharmacopoeia named “general chapter on raw materials of biological origin for the production of cell-based and gene therapy medicinal products” have to be considered for

raw materials for ATMP manufacturing. (ATMP GMP guideline, 2017) The aim of this Ph. Eur. chapter was among others, harmonizing different practices and identifying the critical quality attributes of raw materials. (Pugieux-Amarantos, 2017)

2.3.1 Quality by design approach

The ICH guideline Q8(R2), considering the pharmaceutical development, was approved in 2009, and it highlights that the quality is not possible to test into products, i.e., increased testing will not improve the product quality. However, the required quality is achievable by successfully designing the product. This concept, called quality by design, QbD, is primarily based on the ICH guidelines Q8, Q9, Q10, and Q11. (ICH guideline Q8 R2, 2017) The quality by design for pharmaceuticals is a systematic approach to the development of high-quality medicinal products, by utilizing statistical, analytical, and risk-management methodology. (EMA webpage: Quality by Design) The process starts with predefined objectives and highlights the understanding and knowledge of the product and the process, and quality risk management. (Yu et al., 2014 and Darkunde, 2018)

The goals of the QbD approach are:

- To define relevant product quality specifications
- To minimize product variability and defects, and to improve process capability by improving the design, understanding, and control of the products and processes
- To increase the efficiencies of product development and production
- To improve the process of root cause investigation and “postapproval change management”

There are five main elements of QbD (Figure 5); a quality target product profile, QTPP, critical material attributes, CMAs, critical process parameters, CPPs, a control strategy and, the capability and continual improvement of the process. (Yu et al., 2014)

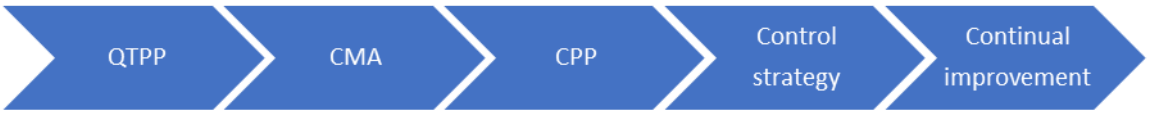

Figure 5. The five QbD elements.

The Quality Target Product Profile is used to identify the drug product's critical quality attributes, which is the basis for setting specifications. (Gentry, 2009)

QTPP considerations for drug products in general include (Yu et al., 2014):

- Route of administration
- Dosage form and strength
- Desired efficacy and safety
- Quality properties, e.g., purity and stability
- The pharmacological mechanism of action (MOA)

When the QTPP is formed, the next step is identifying the CQAs of the product. In product development, the QbD approach identifies quality attributes, QAs, that are critical to product quality from the patient's point of view. These properties also called potential critical quality attributes, pCQAs, are then evaluated using risk assessment and translated into critical quality attributes (CQAs) of the drug product. (Yu et al., 2014) ICH Q8 defines critical quality attributes as "a physical, chemical, biological, or microbiological property or characteristic that should be within an appropriate limit, range, or distribution to ensure the desired product quality." (ICH Q8 guideline, direct citation) The CQAs are properties that correspond to the product's identity, purity, potency, and safety. If they are outside their acceptance limits, they have a critical impact on the quality and efficacy of the product. (ICH Q8 guideline)

After identifying CQAs, the next step in the QbD approach is to define the functional relationship between CQAs and process parameters. It is critical to understand how changes in process parameters will affect the CQAs and, hence, affect the quality of the product. (Gimpel et al., 2021) The process parameters that will affect CQAs when changing are called critical process parameters, CPPs, and these should be controlled to ensure that the process results in products with desired quality. (ICH Q8 guideline) On the other hand, fast characterization of CQAs is beneficial when optimizing both upstream and downstream processes to achieve consistent drug products. (Gimpel et al., 2021)

All materials used in the manufacturing process impact the quality of the product and, hence, need to be controlled. These materials include the drug substance, excipients, and in-process materials, which all have their properties. Their critical properties that affect the quality are called Critical material attributes, CMAs. The term has been defined as “a physical, chemical, biological, or microbiological property or characteristic of an input material that should be within an appropriate limit, range, or distribution to ensure the desired quality of that drug substance, excipient, or in-process material.” (Yu et al., 2014)

The QbD approach, including considerations about material attributes and process parameters, is considered an enhanced quality by design approach from the ICH Q8 guideline point of view. It also defines the relationships between CMAs and CPPS to product CQAs. By using the enhanced QbD approach, the manufacturer can better understand the manufacturing process and the factors affecting it. (ICH Q8 guideline)

The QbD approach is used to gain important and required information enabling the development of high-quality drug products. (ICH Q8 guideline) One of the main goals of this approach is to define relevant product quality specifications. It is important to remember that specifications are chosen to confirm the quality of the drug substance and the drug product instead of acting as a complete characterization of them. Therefore, the specification should focus on testing those properties that through the QbD approach have been evaluated as essential and critical for the safety and efficacy of the critical raw materials and the particular drug product. For this purpose, CMAs and CQAs are in a key position. (ICH Q6B guideline)

When the first element of the specification, i.e., the quality attributes, are characterized, suitable and reliable analytical methods have to be selected and validated for testing these attributes. Many alternative analytical methods can be used to test the quality properties and generate different results. During the development of the drug, also the analytical procedures are evolving. When choosing the corresponding analytical procedure for a quality attribute, it is important that the data generated from different stages of the development, correlates with the analytical method chosen. In addition, to meet the requirements of authorities, the analytical methods have to be validated. (ICH Q6B guideline)

2.4 Project A-Gene document

Not a guideline, but significant support within gene therapy development is the Project A-Gene document. It is "a case study-based approach to integrating QbD principles in Gene Therapy CMC programs". Project A-Gene was released on June 24 in 2021 as a collaboration between Alliance for Regenerative Medicine, ARM, and The National Institute for Innovation in Manufacturing Biopharmaceuticals, NIIMBL, with over 50 industry experts from more than 20 leading drug developers. This four-year project aimed to support the implementation of the QbD approach to gene therapy manufacturing. The case study considers AAV vectors to be used in gene therapy. (The Alliance for Regenerative Medicine (ARM) press release, 2021)

The Project A-Gene contains the following chapters:

- Regulatory considerations
- Standards in Gene Therapy
- Generation of a Quality Target Product Profile (QTPP) for QbD AAV
- Process Development with QbD principles
- UPS and DPS process development
- Drug Product
- Process Control Strategy

- Comparability

The Project A-Gene document will be helpful since it covers considerations about the QbD approach, specifically concerning the development of AAV drug products. (Project A-Gene, 2021)

3 Aims

3.1 Goals

Before a raw material can be taken into use or a product can be released, the quality assurance, QA, unit must ensure that the material lot fulfils the requirements set in the specifications. Which are these requirements? This thesis investigates the regulatory requirements for setting specifications for the critical raw materials in rAAV production, i.e., plasmids and transfection reagents, and for the purified AAV-based product. With the definitions and conclusions made in this thesis, the goal is to create proposed specifications.

When the specification tests have been defined, each test needs a reference to an appropriate analytical method and an acceptance limit.

Further, specifications related to the drug substance and the drug product are a mandatory part of the CTD (Common Technical Document) required for clinical trial and marketing authorization applications. Therefore, they have to be defined when applying for permission for clinical trials and marketing authorization. Thus, the more comprehensive goal is to promote the future goals for rAAV projects at Biovian Oy.

3.2 Objectives

This thesis aims to build specifications for the materials by which they are possible to qualify, and which are required by the authorities. Since the ICH guideline Q8 emphasizes using the QbD approach, it will be applied for this thesis, i.e., for defining relevant quality specifications for the drug substance and the drug product.

The following objectives related to plasmids, transfection reagent, AAV drug substance and drug product are essential:

- Which are the regulatory requirements for the specifications?
- Which tests should be included in the specifications?
- By which analytical method should the tests be conducted?
- Which are the critical quality attributes for the AAV drug product?

4 Methodology

To investigate regulatory requirements for the above objectives, the primary goal has been to determine what regulatory documents exist and which of them are relevant for rAAV products. AAV-based gene therapy medicinal products belong to Advanced therapy medicinal products, ATMPs, and EC published a GMP guideline in 2017 specifically considering ATMPs. The European Pharmacopoeia contains chapter 5.14. named “Gene transfer medicinal products for human use” with a section dedicated only for adeno-associated virus vectors for human use. It consists of guidance covering the manufacturing steps and quality control of rAAV production.

In addition, ICH guidelines are comprehensive, considering the quality aspects, and they will be reviewed. EMA has also published a reflection paper considering quality issues related to recombinant adeno-associated virus vectors. EMA includes the Committee for Advanced Therapies (CAT), which is responsible for assessing the quality, safety, and efficacy of ATMPs; hence, CAT publications will also be reviewed.

To succeed in achieving the aims of this project, good knowledge and understanding of both the rAAV production and desired properties of the purified rAAV product are needed and these have been studied as well. ICH guideline Q8 contains an introduction and emphasizes the concept of QbD which provides a structured way to define the Critical Quality Attributes of a drug product. To build release specifications for the critical raw materials and the purified product, the regulatory requirements and the Critical Quality Attributes have to be defined. In addition, associated analytical assays have to be defined.

These methods aim to build specifications for plasmids, transfection reagents, the drug substance and the drug product. The specifications are crucial for ensuring the quality, safety, and efficacy of the finished rAAV product, possible to be used in clinical trials or released to the market.

The research for this thesis consisted of two parts of which the first one was searching and downloading the relevant guidelines, chapters, and articles. These documents were reviewed, and relevant data and knowledge were collected from them.

In addition, rAAV and its manufacturing process were studied. The second part was bringing the collected data together to evaluate the regulatory requirements and the critical quality attributes for both critical raw materials and the purified product. Based on these evaluations, suggested specifications were possible to create.

5 Results

The results of this thesis are proposed specifications built based on guidelines, the QbD approach, and available literature mentioned earlier in this document. Since specifications are product-specific, the results will be presented separately for the critical raw materials (plasmids and transfection reagent) and the rAAV drug substance and drug product. The result for each material starts with an overview of what is regulated or written in the guidelines published by regulatory authorities and in the reviewed literature.

Specifications: Quality attributes

Based on the ATMP GMP guideline, the European Pharmacopoeia, ICH guidelines and the reviewed literature, the results include the first part of a specification, i.e., quality attributes, to be tested for the release of the materials or products.

Specifications: Associated analytical methods

The second part of a specification is the associated analytical method by which the quality attribute should be tested. Since those quality attributes required in regulatory documents also contain accepted analytical methods, these are referred to as the method(s) of choice in the results. In addition, for quality attributes obtained from the reviewed literature, their associated analytical methods are suggested in the results section.

Considering the selection of analytical methods for release testing of ATMPs, the ATMP GMP guideline chapter 10.4. “Validation of test methods” highlights the importance of using validated test methods to ensure that the method is suitable for its purpose. A method is generally considered validated if described and carried out according to the European Pharmacopoeia, a member state pharmacopoeia or is part of a product-specific monograph. In addition, a verification of the suitability for the purpose should be performed on the selected method. It is worth noting that tests for investigational ATMPs have special features depending on the stage of clinical development. In the early stages

of clinical trials (First-in-man and exploratory), validation is required for sterility assays, microbial assays and assays, ensuring the safety of the patients. In pivotal clinical trials, validated methods are required if they are related to batch release and stability testing. Irrespective of the stage of clinical development, tests related to critical quality attributes need to be established but are not necessarily fully validated except for potency testing, which must be validated before pivotal clinical trials. (ATMP GMP guideline, 2017)

Specifications: Acceptance criteria

The guidelines and the European Pharmacopoeia do not present acceptance criteria for the quality attributes specific to AAV-based products. The manufacturer should determine the criteria based on the gained information about the properties of the product and the risks of the impurities in the product. (Hebben, 2018) Hence, the acceptance criteria for the specifications are beyond the scope of this thesis.

5.1 Specification considerations for plasmids

Whether the plasmids needed to produce AAV vectors are produced by the AAV vector manufacturer itself or by a contract manufacturer, the plasmids must be tested and qualified before they can be released for use. (ATMP GMP guideline, 2017) The following sections contain considerations about what should be included in the specification for plasmids.

5.1.1 Overview of the guidelines and the literature

Since plasmids are a raw material in the AAV vector production, the considerations about setting specifications are based on raw material regulation. In addition, from an AAV production point of view, plasmids are defined as potential critical raw material that involves certain additional considerations. The manufacturer assesses whether a raw material is considered critical or not, and the decision should be documented. (ATMP GMP guideline, 2017)

The ATMP GMP guideline states that the quality of raw materials is a key factor. Hence, the quality should be suitable for its intended use and lists information in the specification for raw materials. Since plasmids are a potential critical raw material, the specification should include quality requirements and acceptance criteria.

When an AAV vector product gains marketing authorization, these quality requirements should be agreed upon with the supplier. In the case of an investigational product, it should be done only if possible. These agreed quality requirements are called agreed specifications, and they should include considerations about the production, testing and control but also handling and distribution. By this agreed specification, the AAV vector manufacturer should ensure compliance with the material provided by the supplier but can also supervise and conduct further testing depending on the risks caused by the material. Only if the risks are fully understood and procedures exist to eliminate or mitigate the risks, it is acceptable that the manufacturer relies on the certificate of analysis provided by the supplier. This approach requires the qualification of the supplier. (ATMP GMP guideline, 2017)

In addition to the quality requirements, the specification should include the raw material description and information needed to avoid error risks such as internal codes. If the raw material has a biological origin, the specification should describe the identification and anatomical environment from which the material originates. As appropriate, sampling and testing should be instructed. The specification of raw materials should also contain information about the storage conditions and how long they can be stored, transport conditions, and precautions related to the raw material. (ATMP GMP guideline, 2017)

The ATMP GMP guideline highlights that the European Pharmacopoeia chapter 5.2.12 should be considered to the full extent for raw materials. The chapter named “Raw Materials of Biological Origin for the Production of Cell-Based and Gene Therapy Medicinal Products” contains considerations specifically for raw materials for the production of ATMPs. Is this chapter relevant for plasmids used in AAV production? The answer is no. This chapter does not cover plasmids as a raw material for rAAV vector production. (ATMP GMP guideline, 2017 and Ph. Eur. chapter 5.2.12 and 5.14)

However, the European Pharmacopoeia chapter 5.14 named “Gene transfer medicinal products for human use” regulates and guides the use of plasmids for rAAV vectors and is relevant for plasmid specification considerations. The section regarding AAV vectors for human use contains guidance on the production and the use of plasmids in rAAV vector production. The plasmids are produced using a bacterial cell-bank system. The master cell bank has to show compliance with requirements listed in the section Bacterial cells used to manufacture plasmid vectors for human use. Appropriate purification methods must be implemented for the plasmids. Chapter 5.14 regulates that only those plasmid batches complying with the requirements listed may be used to manufacture rAAV vectors. (Ph. Eur. chapter 5.14)

The Ph. Eur. chapter 5.14 requires testing of the following quality attributes (Ph. Eur. chapter 5.14):

- Identification

  The identification of plasmids is done by restriction enzyme analysis, sequencing, or nucleic acid amplification techniques, NAT (according to the Ph. Eur. chapter 2.6.21)

- Genomic integrity

  The verification of genomic integrity of the plasmid is done by methods suitable for this purpose, e.g., restriction enzyme analysis of the *rep*, *cap* and expression cassette regions

- Plasmid DNA

  The concentration of plasmid DNA must be determined. The method depends on the concentration. The proposed method for determining concentrations over 500 ng/mL may be using absorbance measurements at 260 nm. Based on the Ph. Eur., a double-stranded DNA solution of 50 microgram/mL has an absorbance value of 1 and a specific absorbance value of 200. If the concentration is less than 500 ng/mL, the determination is done after incubation with fluorescent dyes, which will specifically bind to double-

stranded DNA. The method requires a reference standard of DNA for creating a calibration curve. In addition to these two methods, using a reference standard could enable the use of liquid chromatography and in some situations, it may be acceptable to use capillary electrophoresis.

- Residual host cell DNA

  A suitable method for determining the amount of residual host cell DNA is required. Due to its specificity and sensitivity, quantitative polymerase chain reaction, qPCR, is recommended while other suitable methods may also be used. It is possible to avoid analyzing residual host cell DNA if the production process is validated to demonstrate desired clearance.
- Bacterial endotoxins

  Compliance to the test according to the Ph. Eur. chapter 2.6.14
- Sterility

  Compliance with sterility test according to the Ph. Eur. chapter 2.6.1

Regarding gene transfer medicinal products in general, the Ph. Eur. chapter 5.14. regulates that specifications should be established for substances used in the production to control their identity, potency if applicable, purity and safety related to microbiological quality and contamination of bacterial endotoxin. (Ph. Eur. chapter 5.14)

The ICH Guidelines Q6A and Q6B cover the aspects of specifications and the latter contains considerations related to biotechnological and biological products, making guideline Q6B relevant for rAAV vector products. The guideline does not define tests for raw materials but contains a chapter with considerations for setting specifications for raw materials and excipients. The quality should meet standards that are appropriate for the intended use. Further, it is highlighted that careful evaluation is required for biological raw materials and reagents when analyzing deleterious endogenous or adventitious agents. (ICH guidelines Q6A and Q6B)

When considering which tests to be included in the specification for plasmids, a review of relevant literature is helpful since the Ph. Eur. only contains the minimum

requirements. In addition, the quality of plasmids is also discussed in the A-Gene project, presenting a list of tests important for quality control.

In addition to the tests required by the Ph. Eur., the list contains the following:

- Appearance

  The appearance is tested by visual inspection
- pH and osmolality

  Pharmacopeial methods should be used
- Mycoplasma

  Testing for mycoplasma is done using qPCR methods
- Bioburden

  Bioburden is tested for total aerobes, anaerobes, spore-formers, and fungi
- Conductivity

  The conductivity is measured using a conductivity meter
- Residual Kanamycin

  The enzyme-linked immunosorbent assay, ELISA, is suggested to be used when determining residual amounts of Kanamycin
- Sterility validation

  The test is done according to pharmacopeial methods
- Host cell protein

  Micro bicinchoninic acid, Micro BCA, assay is suggested to be used when analyzing residual host protein
- Purity (Absorbance 260/280 ratio)

  The test is done using UV spectrophotometry
- Plasmid identity

  The suggested method is double-stranded primer walking sequencing when testing for plasmid identity

- Host cell RNA

  The suggested method is SYBR gold-stained agarose gel electrophoresis
- Restriction digest

  Ethidium bromide, EtBr-stained agarose gel electrophoresis is evaluated to be the method of choice

The analytical methods suggested for those tests listed in both Ph. Eur. and the Project A-Gene follow the Ph. Eur. requirements.

5.1.2 The proposed specification for plasmids

A proposed specification for plasmids was created after aggregating the data from the guidelines, see Table 1.

Table 1. The proposed specification for plasmids.

Source	**Attribute/Test**	**Associated analytical method**
Ph. Eur.	Identification	Restriction enzyme analysis, sequencing, or NAT (according to the Ph. Eur. chapter 2.6.21)
Ph. Eur.	Genomic integrity	Methods suitable for this purpose, e.g., restriction enzyme analysis
Ph. Eur.	Plasmid DNA concentration	UV Spectrophotometry
Ph. Eur.	Residual host cell DNA	qPCR
Ph. Eur.	Bacterial endotoxins	Ph. Eur. chapter 2.6.14

Ph. Eur.	Sterility	Ph. Eur. chapter 2.6.1
Project A-Gene	Appearance	Visual inspection
Project A-Gene	pH	Ph. Eur. chapter 2.2.3
Project A-Gene	Osmolality	Ph. Eur. chapter 2.2.35
Project A-Gene	Mycoplasma	qPCR
Project A-Gene	Bioburden	Testing aerobes, anaerobes, spore-formers and fungi
Project A-Gene	Conductivity	Conductivity meter
Project A-Gene	Residual Kanamycin	ELISA
Project A-Gene	Sterility validation	Pharmacopeial method
Project A-Gene	Host cell protein	Micro BCA
Project A-Gene	Purity (Absorbance 260/280 ratio)	UV Spectrophotometry
Project A-Gene	Plasmid identity	Double-stranded primer walking sequencing
Project A-Gene	Host cell RNA	SYBR gold-stained agarose gel electrophoresis
Project A-Gene	Restriction digest	EtBr-stained agarose gel electrophoresis

5.2 Specification considerations for transfection reagent

The transfection reagent considered in this thesis is polyethylenimine, PEI, a polymer product provided by a supplier. Before PEI can be released for use in the production of rAAV vectors, the raw material must be qualified to ensure its identity, quality, and safety.

5.2.1 Overview of the guidelines and the literature, and the proposed specification

PEI is a critical raw material in the AAV vector production and setting a specification for the material requires following the guidelines considering raw materials. From the standpoint of the ATMP GMP guideline, the considerations discussed for plasmids apply to the critical raw material transfection reagent as well, since the guideline does not discuss specific types of critical raw materials. The transfection reagent is a raw material and the same ATMP GMP guidelines as for DNA plasmid apply. (ATMP GMP guideline, 2017)

The relevance of the Ph. Eur. chapters for raw materials were discussed in the section regarding plasmids. Ph. Eur. chapter 5.14 does not include considerations related to transfection reagent. However, the chapter guides that all raw materials´ identity, potency, purity and safety should be controlled through specifications. (Ph. Eur. chapter 5.14)

Due to the lack of specificity regarding regulatory requirements for the transfection reagent, a review of the literature was relevant. Considerations related to the quality attributes for transfection reagent are included in project A-Gene. Identification of the transfection reagent consists of analyzing the polymer structure, molecular weight, polydispersity index, appearance, pH, and osmolality. The performance of the material measures the potency attribute. Testing for mycoplasma, endotoxins and sterility of the product contributes to describing the safety attribute and analyzing heavy metals contributes to the purity attribute. (Project A-Gene, 2021)

Even the relevance of ICH guidelines for setting specifications for raw materials was discussed earlier and it was concluded that guidelines regarding specifications (Q6A and Q6B) did not propose tests for raw materials. (ICH guideline Q6A and Q6B)

Since project A-Gene is the only found source considering specifications for the transfection reagent PEI, this information is used for the proposed specification, see Table 2.

Table 2. The proposed specification for the transfection reagent.

Attribute/Test	Associated analytical method
Polymer structure	Fourier-transform infrared spectroscopy or similar
Molecular weight	Size exclusion chromatography
Polydispersity index	Size exclusion chromatography
Appearance	Visual inspection
Performance	Suitable activity test
pH	Ph. Eur. chapter 2.2.3
Osmolality	Ph. Eur. chapter 2.2.35
Endotoxins	Ph. Eur. chapter 2.6.14
Mycoplasma	Ph. Eur. chapter 2.6.7
Sterility	Ph. Eur. chapter 2.6.1
Heavy metals	Ph. Eur. chapter 2.4.27

5.3 Specification considerations for the rAAV drug substance, DS

The purpose of the specifications is, as discussed earlier, to confirm the quality of the manufactured drug substance or the drug product. The drug substance of the rAAV vector manufacturing process is the purified rAAV vectors, in the Ph. Eur. called the purified harvest, generated through the upstream and downstream processes. The Ph. Eur. contains considerations and requirements for every rAAV vector harvest and the purified vector harvest. (Ph. Eur. chapter 5.14)

5.3.1 Overview of the guidelines and the literature

The ATMP GMP guideline states that specifications for bulk products should be in place where applicable. They should contain release criteria and the maximum time of storage accepted. (ATMP GMP guideline, 2017)

The Ph. Eur. chapter 5.14 provides more specific guidance containing requirements for each manufacturing step. The viral vector requirements are divided into each vector harvest, the purified vector harvest, and the final, formulated vector bulk, see Figure 6. Vector particles can be produced through several harvests, and chapter 5.14 describes the requirements for each harvest. Only harvests that comply with the requirements listed in the chapter are accepted for further processing. (Ph. Eur. chapter 5.14)

Figure 6. The levels of requirements for the AAV drug substance based on Ph. Eur.

The Ph. Eur. 5.14 requires testing of the following quality attributes of each harvest:

- Identification

  The identification of vectors is done using immunochemical methods according to the Ph. Eur. chapter 2.7.1, NAT according to chapter 2.6.21 or restriction enzyme analysis
- Vector concentration

  In every single harvest the titer of infectious vector and vector concentration is determined
- Extraneous agents

  According to the Ph. Eur. chapter 2.6.16

- Control cells

  Compliance of control cells to identification test in the Ph. Eur. chapter 5.2.3 and to test for extraneous agents in the Ph. Eur. chapter 2.6.16

The chapter further contains the section “purified harvest” with requirements for the AAV vector particles purified as part of the downstream process. Multiple single harvests can be combined before the purification process. (Ph. Eur. chapter 5.14)

The Ph. Eur. 5.14 requires testing of the following quality attributes of the purified harvest:

- Identification

  The identification of vectors is done using immunochemical methods according to the Ph. Eur. chapter 2.7.1, NAT according to the chapter 2.6.21 or restriction enzyme analysis.

- Genetic characterization

  Test 1: The whole vector genome is sequenced either on the purified harvest level or the final bulk level, followed by the comparison of the sequence to the theoretical sequence (based on databases and vector construction).

  Test 2: The genomic integrity of the vector DNA is analyzed by PCR.

- Vector concentration

  Both infectious vector titer and vector concentration is determined.

- Residual viruses used for production

  Plaque assays or tissue culture infective dose 50 (TCID50) are utilized. The manufacturing process considered in this thesis does not include the use of viruses in the production.

- Residual proteins (Host cell and/or viral proteins)

  A suitable immunochemical method, according to the Ph. Eur. chapter 2.7.1, unless a validated process demonstrates suitable clearance.

- Residual DNA (Producer-cell genome or plasmids)

  qPCR is recommended for the determination while other suitable methods may also be used. Testing is not needed if a validated process demonstrates suitable clearance.

- Residual reagents

  Tests for identifying residual reagents used during production, e.g., transfection reagent, are needed unless a validated process demonstrates suitable clearance. Project A-Gene suggests the use of ultra-high performance liquid chromatography with detection by charged aerosol detection, UHPLC-CAD.

- Residual antibiotics

  If the manufacturing contains antibiotics, the residual concentration is measured by a microbiological method adapted from the Ph. Eur. chapter 2.7.2 or e.g., liquid chromatography. Testing is not needed if a validated process demonstrates suitable clearance.

- Replication competent AAV (rcAAV)

  The Ph. Eur. defines that testing for rcAAV should be performed either on DS or DP level. Both Tustian and Bak, 2021 and Wright 2021 suggest that testing should be done on the DS. Based on the Ph. Eur., rcAAV is detected by a replication assay on a permissive cell line that has been infected with a helper virus. The replicative forms are analyzed by Southern blot on low-molecular-weight DNA, or the *rep* gene is detected by qPCR.

Under the heading “final bulk” it is described that a final AAV vector bulk batch can consist of several purified harvests. The final bulk batch must comply with the sterility testing to be accepted for use in the production of the final lot. (Ph. Eur. chapter 5.14)

- Sterility

  The final bulk before filling must comply with sterility testing described in the Ph. Eur. chapter 2.6.1

The ICH guideline Q6B lists specification tests valid for every drug substance and for rAAV vectors. The first test category is qualitative and called appearance and description which intends to describe the physical state and the colour of the drug substance. The identity test category is the next one, and according to the guideline, this test should be very specific for the drug substance in question. To identify the drug substance, several different tests may be required. (ICH guideline Q6B)

Testing the purity and testing for impurities can be challenging for ATMPs since the determination might be difficult, and the analyzing methods might affect the result. Several different methods can be used, and their goal is to separate the drug substance from impurities. The impurities are generally classified as process- or product related, as discussed earlier in this thesis. The potency of the drug substance should be tested through a relevant and validated potency assay. (ICH guideline Q6B)

The last test category for the drug substance mentioned in the ICH guideline is quantity. A suitable assay is chosen to determine the quantity of the drug product unless the product manufacture is based upon the potency and no further quantity testing is required. (ICH guideline Q6B)

In addition to the regulatory requirements, considerations for setting specifications for AAV vector DS are also included in the project A-Gene. It is worth noting that the project is conducted in the US and, hence, the requirements are mainly based on FDA and the US Pharmacopoeia. As the Ph. Eur. chapter 5.14, the considerations are divided into the harvest, the bulk drug substance, DS, and finally the drug product, DP. Chapter 6 of the project A-Gene document lists examples of release testing for a clinical AAV vector product. (Project A-Gene, 2021)

The harvest is suggested to be tested for adventitious viruses, mycoplasmas, and bioburden/sterility. This is in line with the Ph. Eur. requirements for every harvest, and testing is performed according to the Ph. Eur. chapter 2.6.16 (Extraneous agents) and chapter 2.6.1 (Sterility) (Project A-Gene, 2021 and Ph. Eur. chapter 5.14)

For DS, the tests suggested by Project A-Gene are (Project A-Gene, 2021 and Ph. Eur. chapter 5.14):

- Appearance

  The appearance of the drug substance is tested by visual inspection

- pH

  The pH of the drug substance is determined based on the Ph. Eur. chapter 2.2.3

- Osmolality

  The analytical method is according to the Ph. Eur. chapter 2.2.35

- Capsid confirmation and titer

  Project A-Gene suggests the use of ELISA, Western blot, but also the analysis of the ratio between empty and full capsids

- Payload sequencing

  Sequencing is carried out to confirm the identity of the payload. Project A-Gene suggests next-generation sequencing or Sanger sequencing

- Viral genome titer

  The concentration of the viral genome is determined. Tustian and Bak, 2021 suggest the use of droplet digital polymerase chain reaction, ddPCR, which is in line with another article published in 2022 (Escandell et al., 2022)

- Infectivity

  For the determination of the infectious titer, both Project A-Gene and Tustian and Bak, 2021 suggest TCID50 (Median Tissue Culture Infectious Dose) as the method of choice

- *In vitro* expression

  This is in line with the Ph. Eur. test “Expression of the genetic insert product” which is described for DP level testing. Cell cultures are inoculated with the product and the determination is done using biochemical assays, flow cytometry or suitable immunochemical method based on the Ph. Eur. chapter 2.7.1. (Ph. Eur. chapter 5.14).

- SDS-PAGE silver stain (Protein detection)

  The test consists of visualizing the capsid proteins VP1, VP2 and VP3 and ensuring the absence of other proteins

- Purity (Absorbance 260/280 ratio)

  The OD260/OD280 ratio describes the sample DNA purity and is done using UV spectrophotometry

- Residual host cell and plasmid DNA

  Residual DNA originating from the producer cells, or the plasmids are detected using qPCR or ddPCR

- Residual bovine serum albumin, BSA

  When bovine serum is used in the production process, the Ph. Eur. suggests testing for it on DP level by a suitable immunochemical method according to the Ph. Eur. chapter 2.7.1

- Residual HEK293

  Residues from producer cells may be analyzed with ELISA

- Residual benzonase

  The residues of nuclease used in the process, e.g., Benzonase, is analyzed using ELISA

- Residual caesium

  The residual substances from the purification process are analyzed using mass spectrometry (Wright, 2008)

- Aggregates

  Tustian and Bak, 2021 suggest that aggregates can be detected using dynamic light scattering, DLS, which provides the average particle size of a sample. This is in line with the Ph. Eur.

- Endotoxin

  Analyzing endotoxins is performed according to the Ph. Eur. chapter 2.6.14

- Sterility

  Sterility testing is performed according to the Ph. Eur. chapter 2.6.1

A review article from 2008 written by JF Wright, named “Manufacturing and characterizing AAV-based vectors for used in clinical studies”, contains the same classification as the project A-Gene. The tests for the harvest are suggested to be viral

contaminants and mycoplasma. The tests for the bulk drug substance are appearance, pH, osmolality, viral genome titer, infectivity, *in vitro* expression, proteins, OD260/OD280, residual host cell DNA, residual plasmid DNA, residual BSA, residual HEK293, residual Benzonase, residual caesium, endotoxin, sterility, and Wild-type AAV. (Wright, 2008)

Another review article named "Assessment of quality attributes for adeno-associated viral vectors" by Tustian and Bak from 2021 summarizes an example of release testing for AAV DS and DP. Tustian and Bak suggest that the DS release testing would include appearance and colour, pH, osmolality, bioburden, endotoxin, replication competent AAV, viral genome titer, infectious titer, capsid titer, genome identity, capsid identity, percentage of full capsids, aggregation, residual host cell protein, residual host cell DNA, residual plasmid DNA, residual endonuclease, residual affinity, residual transfection agent, trans-protein expression and functional activity. (Tustian and Bak, 2021)

5.3.2 The proposed specification for the rAAV drug substance

Based on the guidelines and literature, the following suggestion on the specification of the AAV DS was concluded, see Table 3.

Table 3. The proposed specification for the rAAV drug substance.

Source	**Attributes**
ICH	Appearance and description
ICH	Identity
ICH	Purity and impurities
ICH	Potency
ICH	Quantity

Source	Attribute/Tests	Associated analytical method
Ph. Eur.	Identification	Immunochemical methods according to the Ph. Eur. chapter 2.7.1, NAT according to chapter 2.6.21 or restriction enzyme analysis
Ph. Eur.	Genetic characterization	Genome sequencing method
Ph. Eur.	Vector concentration (incl. infectious vector titer and vector concentration)	Suitable method
Ph. Eur.	Residual proteins	Immunochemical method according to the Ph. Eur. chapter 2.7.1
Ph. Eur.	Residual DNA (Host cell and plasmid)	Primarily qPCR
Ph. Eur.	Residual reagent	Suitable method
Ph. Eur.	Residual antibiotics	Microbiological method either adapted from the Ph. Eur. chapter 2.7.2 or e.g., liquid chromatography
Ph. Eur.	Sterility	Ph. Eur. chapter 2.6.1
Ph. Eur.	Replication competent AAV (rcAAV) concentration	Replication assay followed by Southern blot or qPCR
Project A-Gene	pH	Ph. Eur. chapter 2.2.3
Project A-Gene	Osmolality	Ph. Eur. chapter 2.2.35
Project A-Gene	Appearance	Visual inspection

Project A-Gene	Capsid confirmation and titer	ELISA, Western blot
Project A-Gene	Infectivity	TCID50
Project A-Gene	*In vitro* expression	Suitable method
Project A-Gene	SDS-PAGE silver stain (Protein detection)	SDS-PAGE silver stain
Project A-Gene	Purity (Absorbance 260/280 ratio)	UV Spectrophotometry
Project A-Gene	Residual HEK293	ELISA
Project A-Gene	Residual benzonase	ELISA
Project A-Gene	Residual caesium	Mass spectrometry
Project A-Gene	Endotoxins	Ph. Eur. chapter 2.6.14
Project A-Gene	Aggregates	Light scattering techniques, e.g., DLS

5.4 Specification considerations for the rAAV drug product, DP

Releasing the finished AAV drug product for clinical trials or to the market is the final goal for the developer and the manufacturer. The release for use is based on fulfilling predetermined requirements, called specifications, which ensure the product's safety, identity, strength, purity, and quality. For drug products in clinical trials, the defined specification in the clinical trial application might be necessary to be modified as more information about the product is received during the clinical trials. (Tustian and Bak, 2021)

5.4.1 Overview of the guidelines and the literature

The ATMP GMP guideline lists information in the specification for finished products. However, the guideline highlights that for investigational ATMPs, the stage of development

affects the requirements for the specification. In addition, during clinical phases 1 and 1-2, the acceptance criteria are allowed to be wider as long as the risks are considered, and the clinical trial authority has given its approval. (ATMP GMP guideline, 2017)

Based on the ATMP GMP guideline, the specification should contain the name or identification of the product together with the description of the pharmaceutical form. Sampling and testing instructions should be included as well as both quantitative and qualitative requirements are within their acceptable limits. Further, the specification should include information related to how to transport and store the product and precautions. It is mentioned that the requirements at the cryopreservation stage should, when applicable, get special attention for ensuring product quality. Finally, the drug product shelf life should also be included in the specification. (ATMP GMP guideline, 2017)

As familiar at this point, the European Pharmacopoeia chapter 5.14 contains the requirements for AAV-based products. The formulated AAV product is called the final bulk in the Ph. Eur. and its requirement before being used to prepare the final lot is fulfilling the test "Sterility" based on the Ph. Eur. method 2.6.1. (Ph. Eur. chapter 5.14)

The requirements for the final lot, i.e., the AAV drug product, are in the Ph. Eur. divided into three categories which are "Identification", "Tests", and "Assay". The requirements are (Ph. Eur. chapter 5.14):

- Vector identification

  The identification of vectors can be based on immunochemical methods (2.7.1), NAT (2.6.21) or restriction enzyme analysis
- Osmolality

  The test is performed according to the Ph. Eur. chapter 2.2.35
- pH

  pH is determined based on the Ph. Eur. chapter 2.2.3
- Extractable volume

  Testing is based on the Ph. Eur. chapter 2.9.17
- Residual moisture

  Testing is based on the Ph. Eur. chapter 2.5.12
- Residual bovine serum albumin, BSA

If the production process contains the use of bovine serum, the amount must be tested by a suitable immunochemical method

- Replication-competent AAV

  The Ph. Eur. defines that testing for rcAAV should be performed either on DS or DP level. Both Tustian and Bak, 2021 and Wright 2021 suggest that testing should be done on the DS. Based on the Ph. Eur., rcAAV is detected by a replication assay on a permissive cell line that has been infected with a helper virus. The replicative forms are analyzed by Southern blot on low-molecular-weight DNA, or the *rep* gene is detected by qPCR. Since the literature suggest testing on the DS level, the conclusion in this thesis is that rcAAV testing is not included in the proposed DP specification

- Vector aggregates

  Tustian and Bak, 2021 suggest that aggregates can be detected using dynamic light scattering, DLS, which provides the average particle size of a sample. This is in line with the Ph. Eur.

- Sterility

  Sterility testing is done based on the Ph. Eur. chapter 2.6.1.

- Bacterial endotoxins

  Analyzing endotoxins is performed according to the Ph. Eur. chapter 2.6.14

- Vector concentration

  The concentration of vectors is determined by a suitable method e.g., qPCR and compared with a standard curve which is obtained using an AAV reference standard or recombinant AAV plasmid

- Infectious vector titer

  The determination is done through inoculation into cell cultures, and each assay is validated by titration by a suitable vector reference standard. The required result is not less than the concentration mentioned on the product label.

- The ratio of vector concentration to infectious vector titer

  The ratio is calculated based on the two above-described tests

- Expression of the genetic insert product

  The measurement is performed if possible. Appropriate immunochemical method (2.7.1) or flow cytometry method (2.7.24), or a biochemical method are possible alternatives. The expression is achieved by inoculating cell cultures with the product “at a predetermined multiplicity of infection”. (Ph. Eur. 5.14).

- Biological activity

  Biological activity is determined except in cases that were otherwise authorized or justified. A suitable *in vitro* or *in vivo* method is selected for the determination.

The ICH guideline Q6B lists tests to be included in the specification of all kinds of drug products. The test categories are appearance and description, identity, purity and impurities, potency, quantity, general tests, and additional testing for unique dosage forms. The appearance and description include qualitative tests for the colour, clarity, and physical state. The drug product properties must be considered when choosing identity tests since product-specific tests are required. When dealing with impurities, the guideline states that testing is considered based on the possible origin of impurities. A list of selected methods is built on the gained knowledge of the drug product, acceptance criteria and available analytical methods. The potency should be tested through a relevant and validated method and might for DP be replaced with an alternative method if potency testing is conducted for the DS. The quantity test includes measuring the amount of the DS in the DP, and a common method is based on protein content. General tests are relevant when evaluating the function of the drug product, including tests such as osmolarity and pH. The last aspect to consider for the specification of drug products is whether the dosage form is unique and, hence, requires other tests besides those already listed. (ICH guideline Q6B)

Similar to presenting an example of release testing for AAV harvest and DS, Project A-Gene lists corresponding tests for DP (Project A-Gene, 2021):

- Appearance

  The appearance of the drug product is tested by visual inspection

- pH

  The pH of the drug substance is determined based on the Ph. Eur. chapter 2.2.3

- Osmolality

  The analytical method is according to the Ph. Eur. chapter 2.2.35

- Viral genome identity

  The identification of the viral genome is included in the DP specification. The suggested method is PCR/restriction enzyme (RE) analysis (Project A-Gene, 2021 and Wright, 2021).

- Capsid confirmation and titer

  Project A-Gene suggests the use of ELISA, Western blot, but also the analysis of the ratio between empty and full capsids

- Payload sequencing

  Sequencing is carried out to confirm the identity of the payload. Project a-Gene suggests next-generation sequencing or Sanger sequencing

- Viral genome titer

  The concentration of the viral genome is determined. Tustian and Bak, 2021 suggest the use of ddPCR, which is in line with another article published in 2022 (Escandell et al., 2022)

- Infectivity

  For the determination of the infectious titer, both Project A-Gene and Tustian and Bak, 2021 suggest TCID50 (Median Tissue Culture Infectious Dose) as the method of choice

- *In vitro* expression

  This is in line with the Ph. Eur. test “Expression of the genetic insert product” which will be used in the specification. Cell cultures are inoculated with the product and the determination is done using biochemical assays, flow cytometry or suitable immunochemical method based on the Ph. Eur. chapter 2.7.1. (Ph. Eur. chapter 5.14)

- SDS-PAGE silver stain (Protein detection)

  The test consists of visualizing the capsid proteins VP1, VP2 and VP3 and ensuring the absence of other proteins

- Aggregates

  Tustian and Bak, 2021 suggests that aggregates can be detected using dynamic light scattering, DLS which provides the average particle size of a sample. This is in line with the Ph. Eur.

- Sterility

  Sterility testing is done based on the Ph. Eur. chapter 2.6.1.

- Endotoxin

  Analyzing endotoxins is performed according to the Ph. Eur. chapter 2.6.14

5.4.2 QbD approach

Considerations on the quality target product profile (QTPP) and therewith critical quality attributes (CQAs) based on the quality by design approach are useful when building specifications for the AAV vector product. Based on the number of on-going clinical trials, AAV-based vector products are widely studied, and the QbD approach has the potential to increase the quality of the products and process robustness. In addition, the QbD approach is emphasized by the regulatory authorities and at least FDA is requesting QbD considerations for new approvals.

5.4.2.1 QTPP

The first step in the QbD approach is defining the design criteria for the product, which is done by creating a quality target product profile, abbreviated QTPP. The aim with QTPP is to define which the desired properties of the drug product produced in the process are.

Based on the literature reviewed, QTPP considerations could include:

- Route of administration
- Dosage form and strength
- Desired efficacy and safety
- Quality properties, e.g., purity and stability
- Pharmacological mechanism of action (MOA)

- Container closure system

  (Yu et al., 2014)
- The vector and transgene information
- AAV concentration
- Dosage information
- Route of administration
- Container closure system

  (Tustian and Bak, 2021)
- Pharmacological mechanism of action (MOA)
- Expected efficacy and safety
- Route of administration
- Desired quality attributes
- Regulatory requirements

  (Tanaka et al., 2020)

- Suggestion for AAV (Tanaka et al., 2020):
  - Item: Mechanism of action. Target: Unique to the product
  - Item: Administration route. Target: Intravenous
  - Item: Dosage form. Target: Suspension for injection
  - Item: Storage condition. Target: Under -60
  - Item: Shelf-life. Target: 2 years.
  - Item: Empty capsid. Target: Less than 20%
  - Item: Replication-competent AAV. Target: Under detection limit

5.4.2.2 CQAs

Based on the QTPP, quality attributes for the AAV product are defined. Among them, the potential critical quality attributes, pCQA, are identified based on a selected risk assessment method. Based on the article “Optimization of the quality by design approach for gene therapy products: A case study for adeno-associated viral vectors” (Tanaka et al., 2020), the quality attributes, QAs, can be divided into target product QAs, product-

related impurities, process-related impurities, and adventitious agents. (The target product QAs are unique to the product and not part of the risk assessment) (Tanaka et al., 2020) The relationship between CQAs and the specifications are that product specifications are based on CQAs linked to the DS and DP. (Specification Design and Lifecycle Management Working Group, 2012)

As part of a case study for AAV products, Tanaka et al. list the following quality attributes (Tanaka et al., 2020):

- Target product QAs (e.g., viral genome titer)
- Product-related impurities
    - Empty capsids
    - rcAAV
    - Encapsidated plasmid DNA
    - Encapsidated host cell DNA
    - Aggregates
    - Glycosylated forms
    - Deamidated forms
- Process-related impurities
    - Host cell protein
    - Host cell DNA
    - Residual plasmid DNA
    - Residual raw materials
- Adventitious agents
    - Viruses
    - Bacteria
    - Mycoplasma
    - Bacterial endotoxins

A method to identify which of these quality attributes are critical for the product is required for defining the potential CQAs. ICH guideline Q9 presents several risk

management methods and among them, preliminary hazard analysis (PHA) is considered suitable for gene therapy products. The risk assessment using the PHA method is based on a scoring system in three steps: scores for severity, likelihood, and criticality.

Based on the scores, the following QAs are identified as potential CQAs (Tanaka et al., 2020):

- Encapsidated plasmid DNA
- Encapsidated hos cell DNA
- Empty capsids
- rcAAV
- Host cell DNA
- Aggregates
- Host cell proteins
- Deamidated forms

Adventitious agents are not included in the risk assessment since they are automatically considered CQAs (Tanaka et al., 2020 and ICH guideline Q9). Based on another article, potential CQAs for AAV products are virus titer, capsid content ratio, and aggregation (Gimpel et al., 2021).

As a summary, based on the literature reviewed, the following QAs are identified as CQAs for AAV products:

- Virus titer
- Encapsidated plasmid DNA
- Encapsidated host cell DNA
- Empty capsids
- rcAAV
- Host cell DNA

- Aggregates
- Host cell proteins
- Deamidated forms
- Viruses
- Bacteria
- Mycoplasma
- Bacterial endotoxins

5.4.3 The proposed specification for the rAAV drug product

When collecting requirements from guidelines and information from review articles, the tests to be included in the specifications for AAV vector DP are suggested below in Table 4.

Table 4. The proposed specification for the rAAV drug product.

Source	**Attributes**
ICH	Appearance and description
ICH	Identity
ICH	Purity and impurities
ICH	Potency
ICH	Quantity
ICH	General tests
ICH	Additional testing for unique dosage forms

Source	Attribute/Tests	Associated analytical method
Ph. Eur.	Identification	Immunochemical methods according to the Ph. Eur. chapter 2.7.1, NAT according to chapter 2.6.21 or restriction enzyme analysis
Ph. Eur.	Osmolality	Ph. Eur. chapter 2.2.35
Ph. Eur.	pH	Ph. Eur. chapter 2.2.3
Ph. Eur.	Extractable volume	Ph. Eur. chapter 2.9.17
Ph. Eur.	Residual moisture	Ph. Eur. chapter 2.5.12
Ph. Eur.	Bovine serum albumin	Immunochemical method
Ph. Eur.	Replication competent AAV (rcAAV) concentration	Replication assay followed by Southern blot or qPCR
Ph. Eur.	Vector aggregates	Light scattering techniques
Ph. Eur.	Sterility	Ph. Eur. chapter 2.6.1
Ph. Eur.	Bacterial endotoxins	Ph. Eur. chapter 2.6.14
Ph. Eur.	Infectious vector titer	Cell culture inoculation
Ph. Eur.	Ratio of vector concentration to infectious vector titer	Based on vector concentration and infectious vector titer
Ph. Eur.	Expression of the genetic insert product	Immunochemical method
Ph. Eur.	Biological activity	*In vitro* or *in vivo* method

Project A-Gene	Appearance	Visual inspection
Project A-Gene	Viral genome identity	PCR/RE analysis
Project A-Gene	Capsid confirmation and titer	ELISA, Western blot
Project A-Gene	Payload sequencing	Suitable sequencing method
Project A-Gene	Viral genome titer	ddPCR/qPCR
Project A-Gene	Infectivity	TCID50
Project A-Gene	SDS-PAGE silver stain (Protein detection)	SDS-PAGE silver stain

5.5 Summary of the results

Taken together, based on the requirements from various guidelines and information from review articles, the tests for specific attributes to be included in the specifications for plasmids, transfection reagent, DS and DP for rAAV vectors are integrated and collected in the tables below, see Tables 5-8.

Table 5. Plasmid specification.

Attribute/Test	**Associated analytical method**
Identification	Restriction enzyme analysis, sequencing, or NAT (according to the Ph. Eur. chapter 2.6.21)
Genomic integrity	Methods suitable for this purpose, e.g., restriction enzyme analysis
Plasmid DNA concentration	UV Spectrophotometry
Residual host cell DNA	qPCR
Bacterial endotoxins	Ph. Eur. chapter 2.6.14
Sterility	Ph. Eur. chapter 2.6.1
Appearance	Visual inspection
pH	Ph. Eur. chapter 2.2.3
Osmolality	Ph. Eur. chapter 2.2.35
Mycoplasma	qPCR
Bioburden	Testing aerobes, anaerobes, spore-formers and fungi
Conductivity	Conductivity meter

Residual Kanamycin	ELISA
Sterility validation	Pharmacopeial method
Host cell protein	Micro BCA
Purity (Absorbance 260/280 ratio)	UV Spectrophotometry
Plasmid identity	Double-stranded primer walking sequencing
Host cell RNA	SYBR gold-stained agarose gel electrophoresis
Restriction digest	EtBr-stained agarose gel electrophoresis

Table 6. Transfection reagent specification.

Attribute/Test	**Associated analytical method**
Polymer structure	Fourier-transform infrared spectroscopy or similar
Molecular weight	Size exclusion chromatography
Polydispersity index	Size exclusion chromatography
Appearance	Visual inspection
Performance	Suitable activity test
pH	Ph. Eur. chapter 2.2.3
Osmolality	Ph. Eur. chapter 2.2.35
Endotoxins	Ph. Eur. chapter 2.6.14
Mycoplasma	Ph. Eur. chapter 2.6.7
Sterility	Ph. Eur. chapter 2.6.1

Heavy metals	Ph. Eur. chapter 2.4.27

Table 7. Drug substance specification.

Attribute/Tests	**Associated analytical method**
Identification	Immunochemical methods according to the Ph. Eur. chapter 2.7.1, NAT according to chapter 2.6.21 or restriction enzyme analysis
Genetic characterization	Genome sequencing method
Vector concentration (incl. infectious vector titer and vector concentration)	Suitable method
Residual proteins	Immunochemical method according to the Ph. Eur. chapter 2.7.1
Residual DNA (Host cell and plasmid)	Primarily qPCR
Residual reagent	Suitable method
Residual antibiotics	Microbiological method either adapted from the Ph. Eur. chapter 2.7.2 or e.g., liquid chromatography
Sterility	Ph. Eur. chapter 2.6.1
Replication competent AAV (rcAAV) concentration	Replication assay followed by Southern blot or qPCR
pH	Ph. Eur. chapter 2.2.3
Osmolality	Ph. Eur. chapter 2.2.35
Appearance	Visual inspection
Capsid confirmation and titer	ELISA, Western blot

Infectivity	TCID50
***In vitro* expression**	Suitable method
SDS-PAGE silver stain (Protein detection)	SDS-PAGE silver stain
Purity (Absorbance 260/280 ratio)	UV Spectrophotometry
Residual HEK293	ELISA
Residual benzonase	ELISA
Residual caesium	Mass spectrometry
Endotoxins	Ph. Eur. chapter 2.6.14
Aggregates	Light scattering techniques, e.g., DLS

Table 8. Drug product specification.

Attribute/Tests	**Associated analytical method**
Identification	Immunochemical methods according to the Ph. Eur. chapter 2.7.1, NAT according to chapter 2.6.21 or restriction enzyme analysis
Osmolality	Ph. Eur. chapter 2.2.35
pH	Ph. Eur. chapter 2.2.3
Extractable volume	Ph. Eur. chapter 2.9.17
Residual moisture	Ph. Eur. chapter 2.5.12
Bovine serum albumin	Immunochemical method
Replication competent AAV (rcAAV) concentration	Replication assay followed by Southern blot or qPCR
Vector aggregates	Light scattering techniques

Sterility	Ph. Eur. chapter 2.6.1
Bacterial endotoxins	Ph. Eur. chapter 2.6.14
Infectious vector titer	Cell culture inoculation
Ratio of vector concentration to infectious vector titer	Based on vector concentration and infectious vector titer
Expression of the genetic insert product	Immunochemical method
Biological activity	*In vitro* or *in vivo* method
Appearance	Visual inspection
Viral genome identity	PCR/RE analysis
Capsid confirmation and titer	ELISA, Western blot
Payload sequencing	Suitable sequencing method
Viral genome titer	ddPCR/qPCR
Infectivity	TCID50
SDS-PAGE silver stain (Protein detection)	SDS-PAGE silver stain

6 Discussion and conclusions

Based on the number of ongoing clinical trials, there is a huge interest in, and high expectations on AAV-based drug products. Still, only three AAV-based products have been granted marketing authorization, which describes how challenging it is to get these complex products to the market. Viral vector products require a careful design, an optimal manufacturing process, an accurate quality control and a considered characterization. Specifications are essential for ensuring the quality, efficacy, and safety of the product.

The European Pharmacopoeia requirements for AAV-based products were considered minimum requirements which will probably not be enough for the definition of release specifications. When reviewing relevant literature regarding specifications, it was noticed that the proposed tests varied a lot depending on the source. Based on this, it can be assumed that the structure of the expected specifications is not clear. The specifications for the three approved AAV-based medicinal products were not accessed. It was even harder to find specifications for the raw materials, especially the transfection reagent, for which no specific requirements were mentioned in the European Pharmacopoeia.

There are several reflection papers and guidelines regarding AAV-based products, but for fast developing technologies such as viral vectors and gene therapy, they need to be updated on a regular basis. In fact, some guidelines are now being updated. Further, for the future of the whole field of ATMPs, the ATMP GMP guideline published in 2017, is serving as an important support for developing GMP level products. When searching for relevant reflection papers, guidelines etc., it was noticed that there would potentially be a need for more harmonization and collecting information from multiple sources to enable easier interpretation for developers and manufacturers, in order to get more AAV-based products to the market.

7 Summary in Swedish - Svensk sammanfattning

Bedömning av frisläppningsspecifikationer för kritiska råvaror, läkemedelssubstansen och slutprodukten i tillverkningsprocessen av rekombinant adenoassocierad virusvektor, rAAV

Avhandlingen behandlar rAAV-vektorers tillverkningsprocess, riktlinjer angående rAAV-vektorer publicerade av myndigheter samt relevant litteratur, vilka används som grund för definierandet av specifikationer för processens två kritiska råvaror (plasmider och transfektionsreagens) och för rAAV-läkemedelssubstansen och -slutprodukten.

1 Introduktion

I och med att det har blivit möjligt att identifiera gener som orsakar sjukdomar, har även olika former av genterapier utvecklats och fått mycket uppmärksamhet. Målsättningen med genterapi är att behandla eller bota sjukdomar som inte ännu har effektiv behandling. Med genbehandling kan man ersätta genen som orsakar sjukdomen med en normalt fungerande kopia av genen men det är även möjligt att inaktivera en gen som inte fungerar normalt. (Naso et al., 2017) Det finns olika klasser av produkter som används inom genterapin, t.ex. plasmid-DNA, virusvektorer och bakterievektorer. (FDA, 2018) Virusvektorer hör till de tidigaste och effektivaste och deras egenskaper gör dem attraktiva alternativ inom genterapi. (Naso et al., 2017) De huvudsakliga virustyperna för genterapi är retrovirus, herpes simplex-virus, adenovirus och adenoassocierat virus. (Finer och Glorioso, 2017)

Ett av de mest studerade virusen för användning som virusvektor inom genterapin är adenoassocierat virus, härefter förkortat AAV. Namnet kommer från de omständigheter där AAV först isolerades, nämligen som en förorening i preparat av adenovirus. AAV har en diameter på ca 25 nm och dess genetiska uppsättning är ca 4,7 kilobaspar som bildar tre gener som kallas *rep* (för replikering), *cap* (för bildande av viruskapsiden) och *aap* (för ihopsättning). Gensekvenserna finns mellan två områden som kallas *inverted terminal repeats*, förkortat ITR. (Naso et al., 2017) AAV är beroende av andra virus, t.ex. adenovirus för att replikera sig. Studier har visat att det finns olika

serotyper med olika selektivitet som kan utnyttjas inom genterapi. (Hammond et al., 2017)

Bland de många fördelar AAV har kan följande nämnas: orsakar inte sjukdom hos människan, har förmåga att effektivt överföra genetiskt material, orsakar endast låg immunrespons och har stabil och långverkande effekt pga. att det bildas stabila episomer. (Tustian och Bak, 2021)

Det naturligt förekommande AAV används inte som sådant utan modifieras till en rekombinant form, rAAV som saknar virusgenerna *rep* och *cap* mellan de två ITR-regionerna. (Kimura et al. 2018) Istället kan den önskade genen införas. rAAV vektorer kan tillverkas på olika sätt men i denna avhandling beaktas metoden där tre plasmider transfekterar HEK293-celler och börjar producera rAAV-vektorer innehållande den önskade genen. Plasmiderna innehåller a) gensekvensen som kodar för den önskade genen, b) AAV *cap* och *rep* generna och c) gener från ett sådant virus som krävs för replikering. (Naso et al. 2017) För transfektionen krävs en transfektionsreagens som klassas som en kritiskt råvara: i denna avhandling beaktas polyetylenimin, PEI. (Tanaka et al. 2020) Tillverkningsprocessen består av en process där vektorerna produceras i värdcellerna och en process där vektorerna renas från processrelaterade orenheter. (de Rooij et al, 2019)

Fram till idag har tre AAV-baserade läkemedel fått försäljningstillstånd, och forskning och utveckling av nya läkemedel sker kontinuerligt. (Wörner et al. 2021) Läkemedelsindustrin är strikt reglerad av myndigheter för att garantera säkra, kvalitativa och effektiva läkemedel. (Olson, 2014) Myndigheten som behandlar ansökningar för försäljningstillstånd och kliniska prövningar (*Clinical trial authorization,* CTA) i Europa är European Medicines Agency, EMA, och i Finland Säkerhets- och utvecklingscentret för läkemedelsområdet Fimea. Utöver dessa har t.ex. Japan och USA egna myndigheter. För att harmonisera olika områdens regelverk, har *The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use* (ICH) redan länge arbetat för internationella riktlinjer. (EMA, 2016) AAV-baserade läkemedel klassas av myndigheterna till *Advanced therapy medicinal products*, ATMP, och regler och instruktioner gällande dem och andra läkemedel publiceras i olika riktlinjer. (EMAs hemsida: Advanced therapy medicinal products: Overview)

För att få försäljningstillstånd måste myndighetskraven uppfyllas och därför är målet för denna avhandling att ta reda på vilka kraven på specifikationerna är för AAV-baserade produkter.

2 Litteraturöversikt

Avhandlingens litteraturöversikt inkluderade uppletande och genomgång av relevanta riktlinjer från myndigheterna samt studerande av tillverkningsprocessen, konceptet med kvalitetskontroll inom läkemedelstillverkning och *quality by design*-metoderna.

Riktlinjer

Riktlinjer relaterade till ATMP-produkter hanteras av en av de vetenskapliga kommitteerna i EMA, nämligen kommitteen för *Advanced therapies*, CAT. EMA har publicerat en lista med relevanta riktlinjer för ATMP-produkter på sin hemsida för att underlätta för läkemedelsföretagen. De riktlinjer som ansågs vara relevanta för AAV-baserade produkter är:

- ”Guidelines on Good Manufacturing Practice specific to Advanced Therapy Medicinal Products”
- ”Guideline on the quality, non-clinical and clinical aspects of gene therapy medicinal products” (EMA/CAT/80183/2014)
- ”Reflection paper on quality, non-clinical and clinical issues related to the development of recombinant adeno-associated viral vectors” (CHMP/GTWP/587488/2007 Rev. 1)
- Europeiska farmakopén: kapitel 5.2.12. ”Raw materials of biological origin for the production of cell-based and gene therapy medicinal products” och kapitel 5.14. ”Gene transfer medicinal products for human use and gene therapy medicinal products”
- ICH-riktlinjer

Tillverkningsprocessen

rAAV-tillverkningsprocessen består av en uppströmsprocess och en nedströmsprocess (från de engelska termerna *upstream* och *downstream*). Uppströmsprocessen som behandlas i detta projekt börjar med att odla HEK293-cellerna som för rAAV-produktionen bidrar med E1a- och E1b-generna som krävs för replikering. (Qu et al., 2015) HEK293-cellerna transfekteras sedan med en viss mängd av alla tre typers plasmider med hjälp av transfektionsreagensen PEI. (Dobrowsky et al., 2021) Eftersom plasmiderna innehåller DNA är de negativt laddade och kan inte interagera med de negativt laddade HEK293-cellerna. (Virgen-Ortiz et al., 2017) PEI är en syntetisk katjonisk polymer som har förmågan att kondensera DNA till positivt laddade partiklar som sedan kan interagera med den negativa laddningen på cellernas yta. (Longo et al., 2013) Ungefär efter 2-3 dygn från transfektionen samlar man cellerna med hjälp av centrifugering eller mikrofiltrering och för att sedan samla virusvektorerna, använder man mekanisk eller kemisk cellys. Slutligen används nukleaser, t.ex. Benzonase, för att avlägsna fritt DNA, innan produkten från uppströmsprocessen renas i nedströmsprocessen. (de Rooij, 2019)

I slutet av uppströmsprocessen är målet att ha så stor mängd virusvektorer som möjligt och enheten för dem är vektorgenom (v.g.). per HEK293-cell där 10^5 v.g. anses vara ett allmänt utbyte. (Wright, 2008) Uppströmsprocessen är inte felfri och målsättningen med nedströmsprocessen är att separera virusvektorerna från olika orenheter som härstammar från processen. De kan uppdelas i processrelaterade (t.ex. överbliven plasmid-DNA, proteiner och DNA/RNA från HEK293-cellerna) och produktrelaterade orenheter (t.ex. viruskapsider utan gen eller ofullständigt fyllda, viruskapsider fyllda med DNA från HEK293-cellerna eller AAV med förmågan att replikera.) (de Rooij, 2019) Det första steget i nedströmsprocessen är att avlägsna fasta partiklar som t.ex. cellfragment och detta kan göras via centrifugering eller filtreringsmetoder. Efter det används kromatografiska metoder vars funktion är att binda virusvektorerna och skölja bort orenheter. Jonbyteskromatografi kan användas till att skilja på tomma och fulla viruskapsider. Då orenheterna har avlägsnas behöver produkten uppnå rätt koncentration och formulering. (Hebben, 2018)

De renade virusvektorerna som uppkommer efter upp- och nedströmsprocessen utgör det verksamma ämnet eller läkemedelssubstansen som på engelska refereras till som

drug substance, DS. Den färdigt koncentrerade, formulerade och fyllda virusvektorprodukten utgör slutprodukten, på engelska *drug product*, DP. (Wright, 2008)

Kvalitetskontroll

Kraven på kvalitetskontroll kommer från de övervakande myndigheterna. I EU god tillverkningssed, GMP, står det att det ska finnas ett farmaceutiskt kvalitetssystem som innefattar GMP och ett system med hjälp av man hanterar kvalitetsrisker (*Quality risk management*). Tillverkaren ska försäkra sig om att produkterna är lämpliga för ändamålet, att kraven på produkten anmälda i försäljningstillståndet eller CTA följs och att produkten är trygg, effektiv och kvalitativ för patienten. (Eudralex vol. 4, kapitel 1)

Som en del av GMP, inkluderar kvalitetskontrollen (*Quality control*) provtagning, testningsförfarande, specifikationer och åtgärder relaterade till organisationen, dokumentation och frisläppning av produkterna. Kvalitetskontrollens uppgift är att se till att endast sådana produkter som uppfyller kraven används eller frisläpps på marknaden. (Eudralex vol. 4, kapitel 1) Dessa krav utgör materialets eller produktens specifikation. (ATMP GMP-guideline) Dessa specifikationer måste uppges då man ansöker om tillstånd för kliniska prövningar och försäljningstillstånd. (Project A-Gene, 2021) Specifikationerna består av tre delar, varav den första är den egenskap som undersöks, den andra är med vilken metod egenskapen testas och den tredje är godkändbara gränsvärden. (Rathore, 2010) Endast råvaror, läkemedelssubstanser och slutprodukter som uppfyller kraven i specifikationerna kan tas i bruk. (ICH Q6B, 1999)

Quality by design

Konceptet *Quality by design*, QbD, är baserat på ICH-riktlinjer och understryker att kvalitet inte går att testa in i produkten dvs. att intensifierad testning inte ökar kvaliteten utan produkten måste designas och utvecklas på ett sådant sätt att den önskade kvalitetsnivån nås. (ICH Q8, 2017) En av QbD:s målsättningar är att definiera relevanta specifikationer. Processen börjar med att göra upp en *Quality target product profile*, QTPP där produktens egenskaper listas. Med hjälp av denna kan man bedömma produktens kritiska kvalitativa

egenskaper som på engelska kallas *Critical quality attributes*, CQA och som från patientens perspektiv är kritiska för produktens kvalitet. (Yu et al., 2014) Användningen av QbD-konceptet rekommenderas i dagens läge av myndigheterna.

3 Målsättning

Projektets målsättning är att ta reda på vilka krav på specifikationer myndigheterna har, dvs. vilka tester som ska inkluderas i produktens specifikation. Detta gäller såväl de två kritiska råvarorna och läkemedelssubstansen och slutprodukten. Målet är att föreslå vilka test som ska ingå i respektive material eller produkt. Dessutom är målet att definiera med vilken testmetod de ska utföras. Med hjälp av litteraturen ska även CQA för slutprodukten definieras.

4 Metoder

För att lyckas med målsättningarna har det varit väsentligt att ta reda på vilka dokument och riktlinjer myndigheterna har publicerat. Metoderna består av att gå igenom dessa dokument för att använda relevant information ur dem. Relevanta artiklar och studier kommer också granskas. För att uppnå målet är det också väsentligt att bli bekant med tillverkningsprocessen och de önskade egenskaperna på produkten. Eftersom QbD-konseptet innefattar definierandet av CQA som i sin tur är viktig information då specifikationerna sätts, kommer även QbD-konceptet studeras.

Projektet bestod av två delar. Den första delen innefattade sökandet efter relevanta riktlinjer, kapitel och artiklar samt genomgång av dessa. AAV och dess tillverkningsprocess studerades. Den andra delen bestod av samlandet av all relevant information för att kunna föreslå specifikationer för råvarorna, läkemedelssubstansen och slutprodukten.

5 Resultat

Föreslagna specifikationer är avhandlingens resultat, som baserar sig på läkemedelsmyndigheternas riktlinjer, QbD-metoden och relevant litteratur, och de presenterades separat för plasmiderna, transfektionsreagensen, läkemedelssubstansen och slutprodukten eftersom specifikationerna är produktspecifika. Först beskrivs vad som står i läkemedelsmyndigheternas riktilinjer och i annan relevant litteratur, och för slutproduktens del har även QbD-metoden tagits i beaktande. Med hjälp av slutproduktens profil, QTPP, har produktens kritiska egenskaper, CQA, bedömts och dessa utgör grunden för skapandet av specifikationerna.

Specifikationernas första del är de egenskaper som ska undersökas. Den andra delen är med vilken analysmetod dessa egenskaper ska testas. ATMP GMP-riktlinjerna definierar att analysmetoderna ska vara validerade för att garantera lämplighet för sitt ändamål och att lämpligheten bör verifieras (ATMP GMP-guideline, 2017). Specifikationernas tredje och sista del är testernas gränsvärden, som lämnats utanför denna avhandling eftersom gränsvärdena speciellt för ATMP-produkter bör definieras av läkemedelsföretaget, baserat på den kunskap och information som erhållits under utvecklingsprocessen (Hebben, 2018.

5.1 Plasmider

Eftersom plasmiderna tillhör tillverkningsprocessens kritiska råvaror, följer kraven för plasmiderna de regler och riktlinjer som gäller råvaror. Europeiska farmakopéns kapitel 5.14 ”Gene transfer medicinal products for human use” innehåller kraven på plasmider som används i tillverkningen av rAAV-baserade läkemedel. Kraven är presenterade som de egenskaper som ska testas och tillhörande lämplig analysmetod. Förutom Europeiska farmakopén, har även ICH-riktlinjer, ATMP GMP-riktlinjerna och Project A-Gene-dokumentet granskats och slutresultatet är föreslagna specifikationer för plasmiderna, se Tabell 9.

Tabell 9. Föreslagen specifikation för plasmiderna.

Test	**Tillhörande analysmetod**
Identifiering	Analys med restriktionsenzym, sekvensering, eller nukleinsyratest (Nucleic acid amplification test, NAT) enligt Europeiska farmakopéns kapitel 2.6.21
Genetisk integritet	Lämplig metod för ändamålet, t.ex. analys med restriktionsenzym
Plasmid-DNA	UV-spektrofotometer
Resterande DNA från värdceller	Kvantitav polymeraskedjereaktion (Quantitave Polymerase Chain Reaction, qPCR)
Endotoxiner	Enligt Europeiska farmakopéns kapitel 2.6.14
Sterilitet	Enligt Europeiska farmakopéns kapitel 2.6.1
Utseende	Visuell granskning
pH	Enligt Europeiska farmakopéns kapitel 2.2.3
Osmolalitet	Enligt Europeiska farmakopéns kapitel 2.2.35
Mykoplasma	Kvantitav polymeraskedjereaktion (Quantitave Polymerase Chain Reaction, qPCR)
Biobördan	Test av aeroba och anaeroba organismer, sporbildare och svampar

Ledningsförmåga	Konduktivitetsmätare
Resterande Kanamycin	Enzymkopplad immunadsorberande analys (*Enzyme-linked immunosorbent assay*, ELISA)
Validering av sterilitet	Lämplig farmakopémetod
Proteiner från värdceller	Mikro bicinkoninsyrametod (Micro BCA)
Renhet (Absorbansförhållande 260/280)	UV-spektrofotometer
Plasmid identifiering	Dubbelsträngad ”primer walking” sekvensering
RNA från värdceller	SYBR-guldfärgad agaroselektrofores
Restriktionssmältning	Etidiumbromidfärgad agarosgelelektrofores

5.2 Transfektionsreagens

Även transfektionsreagensen tillhör kritiska råvaror i tillverkningsprocessen och därmed följer även de riktlinjerna för råvaror, vilka är inkluderade i t.ex. ICH-riktlinjerna och ATMP GMP-riktlinjerna. Europeiska farmakopén innehåller inte specifika krav på transfektionsreagensen men nämner att identifiering, potens om möjligt, renhet och säkerhet av alla råvaror bör kontrolleras. Detta kombinerat med granskandet av Project A-Gene-dokumentets information och föreslagna specifikationer för transfektionsreagensen (PEI) ledde till följande föreslagna specifikationer för transfektionsreagensen, se Tabell 10.

Tabell 10. Föreslagen specifikation för transfektionsreagens.

Test	Tillhörande analysmetod
Polymerstrukturen	Fourier transform infraröd spektroskopi eller motsvarande
Molekylvikten	Storleksuteslutningskromatografi
Polydispersitetsindex	Storleksuteslutningskromatografi
Prestanda/funktion	Lämpligt aktivitetstest
Utseende	Visuell granskning av klarhet och färg
pH	Enligt Europeiska farmakopéns kapitel 2.2.3
Osmolalitet	Enligt Europeiska farmakopéns kapitel 2.2.35
Endotoxiner	Enligt Europeiska farmakopéns kapitel 2.6.14
Mykoplasma	Enligt Europeiska farmakopéns kapitel 2.6.7
Sterilitet	Enligt Europeiska farmakopéns kapitel 2.6.1
Tungmetaller	Enligt Europeiska farmakopéns kapitel 2.4.27

5.3 Läkemedelssubstansen

Tillverkningsprocessens uppströms- och nedströmsprocesser genererar renade rAAV-vektorer som utgör produktens aktiva substans eller läkemedelssubstans. ICH Q6B-riktlinjerna innehåller allmänna krav som gäller alla läkemedelssubstanser medan Europeiska farmakopén innehåller specifika krav för rAAV-vektorer som

läkemedelssubstans. Dessa krav har jämförts med Project A-Gene-dokumentet och övriga relevanta artiklar gällande specifikationer för rAAV-baserade läkemedel för att skapa en föreslagen specifikation för rAAV-läkemedelssubstansen, se Tabell 11.

Tabell 11. Föreslagen specifikation för rAAV-läkemedelssubstansen.

Test	**Tillhörande analysmetod**
Vektoridentifiering	Immunokemisk metod enligt Europeiska farmakopéns kapitel 2.7.1, NAT 2.6.21 eller analys med restriktionsenzym
Genetisk karakterisering	Genomsekvenseringsmetod
Vektorkoncentration	Lämplig metod
Rester av proteiner	Immunokemisk metod enligt Europeiska farmakopéns kapitel 2.7.1
Resterande DNA (från värdceller och plasmider)	Kvantitav polymeraskedjereaktion (*Quantitave Polymerase Chain Reaction*, qPCR)
Resterande reagenser	Lämplig metod
Resterande antibiotika	Mikrobiologisk metod antingen från Europeiska farmakopéns kapitel 2.7.2 eller t.ex. vätskekromatografi
Sterilitet	Enligt Europeiska farmakopéns kapitel 2.6.1
Koncentrationen av replikeringskompetent AAV	Replikationsanalys följd av ”Southern blot”-metoden eller kvantitav polymeraskedjereaktion (*Quantitave Polymerase Chain Reaction*, qPCR)

pH	Enligt Europeiska farmakopéns kapitel 2.2.3
Osmolalitet	Enligt Europeiska farmakopéns kapitel 2.2.35
Utseende	Visuell granskning
Bekräftelse och mängd kapsider	Enzymkopplad immunadsorberande analys (*Enzyme-linked immunosorbent assay*, ELISA), ”Western blot”-metod
Infektivitet	TCID50-analys (*Median tissue culture infectious dose*, TCID50)
***In vitro*-uttryck**	Lämplig metod
SDS-PAGE silverfärgning **(Proteinbestämning)**	Silverfärgning efter natriumdodecylsulfate polyakrylamid gelelektrofores, SDS-PAGE
Renhet (Absorbansförhållande 260/280)	UV-Spektrofotometer
Resterande HEK293	Enzymkopplad immunadsorberande analys (*Enzyme-linked immunosorbent assay*, ELISA)
Resterande benzonase	Enzymkopplad immunadsorberande analys (*Enzyme-linked immunosorbent assay*, ELISA)
Resterande caesium	Masspektrometer
Endotoxiner	Enligt Europeiska farmakopéns kapitel 2.6.14
Aggregat	Ljusspridningsteknik t.ex. dynamisk ljusspridning (*Dynamic light scattering*, DLS)

5.4 Slutprodukten

Att kunna släppa ut den färdiga slutprodukten på marknaden eller för kliniska prövningar är den ultimata målsättningen för ett läkemedelsföretag. Denna frisläppning kan göras först då man försäkrat sig om att alla krav på produkten uppfylls. Precis som för den aktiva substansen, innehåller ICH Q6B-riktlinjerna allmänna krav som gäller alla typers slutprodukter medan Europeiska farmakopén innehåller krav specifika för rAAV-slutprodukter. Project A-Gene innehåller författarnas tolkning över vilka egenskaper som bör testas innan frisläppning och dessa har tagits i beaktande. För slutproduktens del har även *quality by design*-metoden beaktats och slutresultatet är föreslagna specifikationer för rAAV-slutprodukten, se Tabell 12.

Tabell 12. Föreslagen specifikation för rAAV-slutprodukten.

Test	**Tillhörande analysmetod**
Vektoridentifiering	Immunokemisk metod enligt Europeiska farmakopéns kapitel 2.7.1, NAT 2.6.21 eller analys med restriktionsenzym
Osmolalitet	Enligt Europeiska farmakopéns kapitel 2.2.35
pH	Enligt Europeiska farmakopéns kapitel 2.2.3
Extraherbar volym	Enligt Europeiska farmakopéns kapitel 2.9.17
Resterande fukt	Enligt Europeiska farmakopéns kapitel 2.5.12
Bovint serumalbumin	Immunokemisk metod enligt Europeiska farmakopéns kapitel 2.7.1

Koncentration av replikeringskompetent AAV	Replikeringsanalys följd av ”Southern blot”-metoden eller kvantitav polymeraskedjereaktion (*Quantitave Polymerase Chain Reaction*, qPCR)
Vektoraggregat	Ljusspridningsteknik t.ex. dynamisk ljusspridning (*Dynamic light scattering*, DLS)
Sterilitet	Enligt Europeiska farmakopéns kapitel 2.6.1
Endotoxiner	Enligt Europeiska farmakopéns kapitel 2.6.14
Koncentrationen av vektorpartiklar	Kvantitav polymeraskedjereaktion (*Quantitave Polymerase Chain Reaction*, qPCR)
Mängden infektiösa vektorer	Inokulation av cellkulturer
Förhållandet mellan koncentrationen av vektorpartiklarna och de infektiösa vektorerna	Utgående från de två ovannämda test
Uttryck av den insatta genprodukten	Immunokemisk metod enligt Europeiska farmakopéns kapitel 2.7.1, flödescytometri enligt Europeiska farmakopéns kapitel 2.7.24 eller en biokemisk metod
Biologisk aktivitet	Lämplig *in vitro*- eller *in vivo*-metod
Utseende	Visuell granskning
Identitet av viralt genom	Kvantitav polymeraskedjereaktion (*Quantitave Polymerase Chain Reaction*, qPCR) eller analys med restriktionsenzym

Bekräftelse och mängd kapsider	Enzymkopplad immunadsorberande analys (*Enzyme-linked immunosorbent assay*, ELISA), ”Western blot”-metod
Sekvensering av den insatta genen	Lämplig sekvenseringsmetod
Mängden viralt genom	Digital polymeraskedjereaktion (*Droplet digital polymerase chain reaction*, ddPCR)
Infektivitet	TCID50-analys (*Median tissue culture infectious dose*, TCID50)
SDS-PAGE silverfärgning (Proteinbestämning)	Silverfärgning efter natriumdodecylsulfat polyakrylamid gelelektrofores, SDS-PAGE

6 Slutsatser

Nya genterapier utvecklas i snabb takt men det faktum att hittills endast tre rAAV-baserade läkemedel i världen har blivit beviljade försäljningstillstånd innebär att det ännu saknas etablerad kunskap i hur läkemedelsföretagen ska få läkemedlet ut på marknaden. Teknologin utvecklas och kunskapen ökar, vilket även kräver att läkemedelsmyndigheter måste hålla sig uppdaterade. Som en del av denna uppdatering pågår omarbetning av flera av de i denna avhandling granskade riktlinjer.

För att definiera relevanta specifikationer för ett läkemedel krävs att företaget känner sin produkt väl. Detta betonas även av läkemedelsmyndigheter som rekommenderar användandet av QbD-metoden och speciellt för ATMP-produkter betonar vikten av riskbedömning p.g.a. att genterapiprodukternas mångfald och deras komplexa strukturer kräver en viss nivå av flexibilitet. Fullständig QbD-metod och riskbedömningar kan endast utföras av läkemedelsföretaget i fråga, eftersom det är där kunskapen om produkten finns.

Som grund för resultaten i denna avhandling låg dokument publicerade av läkemedelsmyndigheterna. Europiska farmakopéns krav är lagligt bindande för

medlemsländerna och dessa krav för rAAV-baserade produkter och dess råvaror inkluderades oförändrade i specifikationerna. Europeiska farmakopén innehåller inte krav på transfektionsreagens utan som grund för dess specifikation låg den vetenskapliga artikeln, eller snarare verket, ”Project A-Gene”. Under granskandet av artiklar som bedömt rAAV-baserade läkemedels specifikationer visade det sig att Europeiska farmakopén endast innehåller minimikraven d.v.s. att utöver dem kunde man motivera även andra testers inkluderande i specifikationerna. Det visade sig även att trots motsvarande tillverkningsprocess, fanns det varierande innehåll i de föreslagna specifikationerna. Detta utgjorde en utmaning för definierandet av resultaten även i denna avhandling men som samtidigt beskriver utmaningen som läkemedelsföretagen har med att tolka och uppfylla läkemedelsmyndigheternas krav.

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