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Review Article

A Review of Analytical Methods for Adeno-Associated Virus Vectors' Biodistribution and Shedding Studies

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Abstract

Adeno-associated virus (AAV)-based gene therapy (GT) products can effectively deliver transgenes to replace lost or defective genes, providing hope for patients. However, there is limited research on analytical methods for pre-clinical and clinical studies which could impede their development success. During the development of AAV-based GT products, it is crucial to define the biodistribution and shedding profiles following treatment. In this review, we summarize PK studies for the AAV-based GT products approved so far by U.S. Food and Drug Administration and European Medicines Agency. We then comparatively review the analytical methods employed to evaluate AAV vectors' biodistribution and shedding of approved GT products. We further make recommendations on choice of methodologies for future GT product development based on the study data and the currently available regulatory guidance.

Key words: AAV; gene therapy; biodistribution; shedding; analytical methods

Abbreviation:

AAV: adeno-associated virus;

GT: gene therapy;

SMA: spinal muscular atrophy;

Adv: adenovirus;

CNS: central nervous system

PK: pharmacokinetic;

EMA: European Medicines Agency;

FDA: U.S. Food and Drug Administration;

NHPs: non-human primates;

SMN: survival motor neuron;

qPCR: quantitative PCR;

dPCR: digital PCR;

ISH: in situ hybridization;

ELISA: enzyme-linked immunosorbent assay;

IHC: immunohistochemistry;

IF: immunofluorescence.

Introduction

There are millions of patients around the world who suffer from inherited diseases, and gene therapy mediated by AAV offers a promising treatment option for certain patients with such conditions [1]. Various AAV-based GT products are currently available on the market, including Luxturna, which treats retinal diseases, and Zolgensma, a treatment for SMA [2]. In clinical applications, AAV vectors present multiple benefits such as wide tropism, low immunogenicity, ease of production, non-pathogenic characteristics, rare integration into the host chromosome and persistent transgene expression [3].

AAV belongs to the Parvoviridae family. It was first discovered in 1965 as a contaminant of AdV preparations, and its replication relies on a helper virus like AdV. Wild-type AAV is a non-enveloped, single-stranded DNA virus with either a sense or anti-sense strand genome [4,5]. Although not associated with any diseases, its ability to infect various types of cells is noteworthy, and different serotypes of AAV exhibit varying tissue tropisms. When utilized as a GT product, it is possible to optimize the capsid to enhance its tropism towards a target tissue [6].

AAV-based GT products can deliver DNA sequences of interest (e.g., gene replacement) to target cells in order to treat inherited diseases caused by monogenic deficiencies or mutations. AAV vectors have the same capsid structure as wild-type AAVs, but their viral sequences are replaced by a transgene except for the inverted terminal repeats at the ends of the viral genomes [5].

Systemic delivery is a common route to administrate AAV vectors. AAV transduction can be greatly impacted by systemic clearance and its interaction with various proteins in circulation, such as pre-existing

neutralizing antibodies [7]. In addition to systemic delivery, AAV vectors can also be administrated directly to target tissues using methods such as intramuscular, CNS delivery, or subretinal delivery [6]. In the course of AAV spreading and transduction (illustrated in Figure1), after vascular escape, AAV vectors first bind with cell surface receptors. The interaction between viral capsid proteins and cellular receptors triggers the internalization/entry of AAV vectors. Following viral intracellular trafficking, entry into nucleus and virion uncoating, the viral genome is released into the nucleus, where the single-stranded AAV genome is converted into double-stranded DNA (self-complementary AAV vectors do not undergo this process). Subsequently, the harbored transgene begins to transcribe into mRNA, which is further translated into a therapeutic protein. AAV genomes can be concatenated and circularized as episomes, persisting in transduced cells without integrating into host genomes [3]. However, the intracellular genomes and transgene expression of AAV may be diluted and even lost due to division and elimination of transduced cells [6, 8]. Meanwhile, AAV vectors can be shed from patients' bodies in different ways. For instance, AAV2 can be excreted through various excreta and secreta, such as urine, feces, saliva, nasal and oral secretions, semen, and tears, depending on the route of administration [9]. Overall picture of AAV GT products' spread within and out of patients' bodies upon administration is shown in Figure 1.

1. AAV GT products can be administrated to patients through systemic delivery (e.g., intravenous injection) or local delivery (e.g., intramuscular injection);

2. In blood stream, AAV particles probably interact with various proteins in circulation (e.g., neutralizing antibodies);

3. AAV particles spread to various tissues (e.g., liver and muscle) and biological fluids (e.g., cerebral spinal fluid) after vascular escape;

4. AAV particles may or may not disseminate to blood stream from the target tissues upon local delivery;

5. Within tissues, AAV particles bind to specific cellular receptors on cell surface and enter into cells;

6. AAV particles are transported within cell and imported into nucleus;

7. After uncoating, AAV genomes are exposed;

8. AAV single-stranded genomes are converted into double-stranded genomes (except scAAVs);

9. Transgene mRNAs are transcribed from AAV genomes;

10. Transgene mRNAs are transported to cytoplasm;

11. Therapeutic proteins are translated from transgene mRNAs;

12. AAV particles can be shed from various excreta (e.g., feces) and secreta (e.g., saliva, urine and tear) depending on administration route.
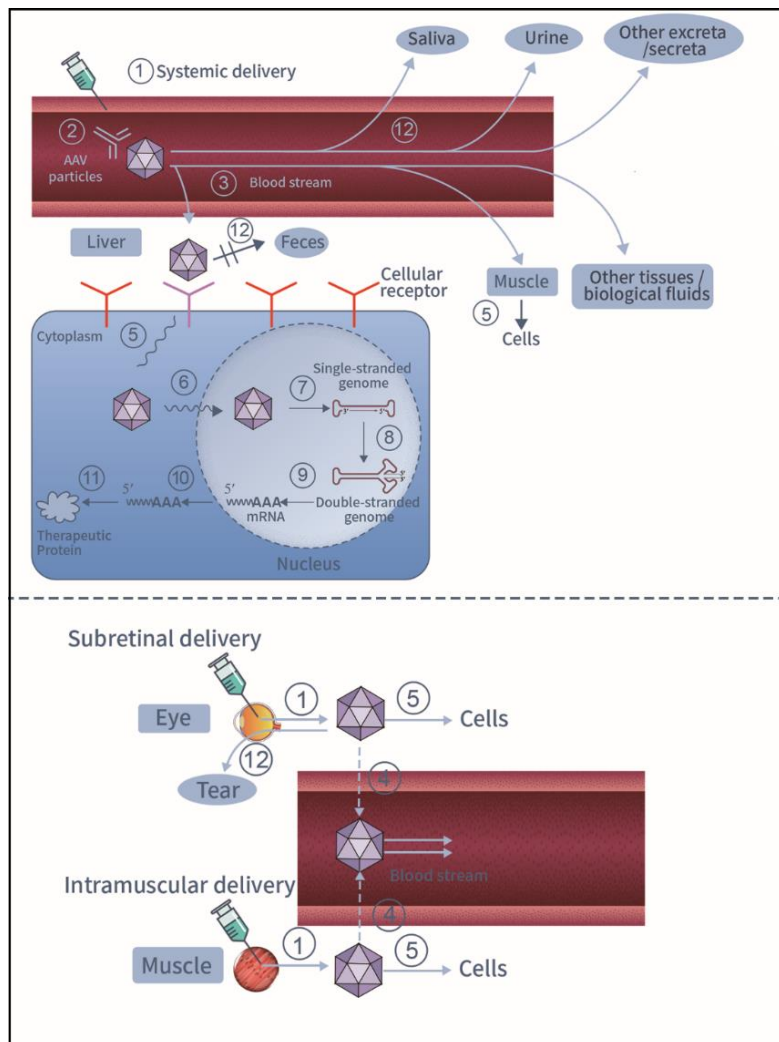

Figure 1: Overall picture of AAV GT products' spreading and transduction upon systemic (top) or local (bottom) administration

When conducting PK studies of a GT product, general scientific principles may apply. However, certain terms used in small molecular drug studies, such as absorption, distribution, metabolism, and excretion are not directly applicable [10]. The determination of the PK profile of a GT product is achieved through analyzing its biodistribution, which refers to how the product is distributed, how long it stays and how it is cleared from target and non-target tissues, as well as biofluids, following its administration. It is imperative to understand the biodistribution pattern of an AAV vector before any clinical trial is initiated [11, 12]. Biodistribution profiles from a preclinical study and/or an initial human trial help to interpret results of proof-of-concept studies, enhance safety assessments for GT products, and provide the required information for further designing other human trials (e.g., determining dose levels, dosing schedules and monitoring plans).

On the other hand, shedding refers to the release of a GT product from the dosed patient via one or all of the routes: excreta, secreta or skin (e.g., such as pustules, sores, or wounds). As all AAV-based GT products are derived from infectious viruses, shedding studies are necessary to evaluate the possibility of AAV transmission from treated to untreated individuals [13].

In this review, we present a detailed introduction of the analytical methods used in biodistribution and shedding studies of AAV-based GT products. In conjunction with pertinent regulatory guidance, we furnish comprehensive details regarding the applicability of each analytical method, experimental design considerations, challenges, and potential solutions for delineating the components of biodistribution investigations and shedding assays.

Overview of PK Studies for Approved AAV-based GT Products

Seven approved products are listed in Table 1. Their PK studies are briefly discussed in this section.

Product name	Serotype	Disease	Agency	Year of approval	Reference
Glybera	AAV1	lipoprotein lipase deficiency	EMA	2012	14
Luxturna	AAV2	inherited retinal dystrophy	EMA (and FDA)	2018	15
Upstaza	AAV2	aromatic L-amino acid decarboxylase deficiency	EMA	2022	16
Zolgensma	AAV9	spinal muscular atrophy	FDA (and EMA)	2019	17
Elevidys	AAVrh74	Duchenne muscular dystrophy	FDA	2023	18
Hemgenix	AAV5	hemophilia B	FDA (and EMA)	2022	19
Roctavian	AAV5	hemophilia A	EMA (and FDA)	2022	20

Table 1: List of AAV-based GT products approved by FDA or EMA

Glybera: Following an intramuscular administration of Glybera in mice, AAV vectors were transiently detected in circulation. The viral genome was detected eight days after administration at higher levels in injected muscle and draining lymph nodes, but at much lower levels in non-target tissues, such as liver, lung and brain. After this time point, the measured residual viral genome levels remained high in target tissues, while they were progressively declining in non-target tissues. Comparable distribution patterns of AAV vectors were observed upon administration in cats and rabbits. In clinical trials, elevated levels of viral genome were observed to persist for up to 12 months upon administering Glybera in injected leg muscle, but viral genome was not found in non-injected muscle. The viral genomes were found at the highest level in the serum, while the levels decreased by one to two logs per week. Although initially detected, AAV vectors became undetectable in the saliva, urine and semen of patients after 12, 10 and 26 weeks, respectively [14].

Luxturna: Following a subretinal administration to NHPs' eyes, AAV vectors were detected in intraocular fluids of injected eyes at the highest level, and in the optic nerve of the injected eye, optic chiasm, spleen, liver, stomach, and lymph nodes at lower levels for up to three months. In clinical studies, a low level of viral genome could be transiently detected in tear and serum samples from a portion of subjects after bilateral administration [15].

Upstaza: Similar to Glybera and Luxturna, Upstaza is also administered locally through intraputaminal administration. However, the AAV vector has not been demonstrated to distribute outside the CNS (target tissue) or shed in blood and urine after the CNS administration [16].

Zolgensma: It is a gene therapy prescribed for the treatment of SMA in children under the age of 2. The biodistribution of the AAV vector used in Zolgensma was evaluated in two patients who died 5.7 months and 1.7 months, respectively, following intravenous infusion. The viral genome was detected at the highest level in the liver, and also found in other organs such as the spleen, heart, pancreas, inguinal lymph node, skeletal muscles, peripheral nerves, kidney, lung, intestines, gonads, spinal cord, brain, and thymus. An immunostaining confirmed expression of the generalized SMN protein in spinal motor neurons, as well as in neuronal and glial cells of brain, heart, liver, skeletal muscles, and other tissues of the patients. Vector shedding was investigated over the course of 18 months. Like Glybera and Luxturna, the viral genome was found in saliva, urine, and feces, while the detected viral genome copies were decreased to undetectable levels in all the samples at different time points [17].

Other GT products: Elevidys, Hemgenix, and Roctavian are all administered via intravenous infusion. Like Zolgensma, AAV vectors were detected in samples such as serum/plasma, urine and saliva [18-20]. In Roctavian's PK studies, patients' plasma and semen samples were evaluated using an immunoprecipitation qPCR assay to detect capsid-wrapped viral genome, which could potentially be infectious, following conventional qPCR assay [20]. Encapsidated viral genome was detectable in plasma up to 10 weeks after administration, while its maximum time to clearance in semen was 12 weeks.

Analytical methods for PK studies of AAV-based GT products

Assessment of vector biodistribution

Samples collected in AAV vector biodistribution studies typically include tissues at the injection site (e.g., retina), as well as liver and gonad tissues, and biofluids such as blood and cerebrospinal fluid. The sampling time-points should be designed to cover the in vivo kinetics of AAV vectors, with additional time-points included to thoroughly capture the duration of the steady-state period or to estimate persistence. Vector biodistribution assessment typically involves detecting viral genome and transgene products [21]. In clinical trials, invasive techniques like biopsies and tissue collection may not always be practical or ethical to perform, resulting in limited number of clinical biodistribution studies [22]. Thus, biodistribution data about AAV

vectors are primarily obtained from preclinical studies performed in animals [23].

Test systems of preclinical biodistribution studies

Several animal species are susceptible to AAV infection and have been employed in preclinical studies of AAV vectors, such as rats, mice, NHPs, dogs, cats, and pigs [24]. Rodents are widely used due to their ease of genetic manipulation, fast reproduction and lower cost. However, due to apparent physiological and anatomical differences among species, it is not always possible to extrapolate findings from rodent models to larger animals, humans, or even other rodent species. For example, AAV PHP.B vector demonstrates superior brain transduction efficiency in C57BL mice compared to AAV9, whereas this is not confirmed in other animal species [3]. Humanized mouse and xenograft models were employed to overcome interspecies discrepancies, but it is yet to be determined if the experimental results obtained from these models can be translated into human clinical trials [25].

Compared to rodents, large animal models often exhibit greater similarities to humans and can therefore lead to more accurate predictions of AAV vector biodistribution profile in humans. For instance, in a study of AAV-based GT product for osteoarthritis, researchers utilized an equine model to investigate PK profile of the AAV vector after intra-articular administration. The choice of the equine model was due to the similarity in size and function of equine forelimb joints to human knees, which are also prone to osteoarthritis due to their weight-bearing nature. The data obtained from the equine model regarding vector biodistribution, transgene (IL-1Ra) expression, and dose-effect were proved highly translatable for clinical trials [26]. However, tissue collection at multiple time-points may not always be feasible for a large animal species due to the limited number of animals used in a study. The restricted availability of large animals can be somewhat alleviated by collecting biofluid samples at multiple time points over an extended duration, or by conducting experiments that incorporate both large and small animals [11, 22].

In addition to animal models, organoids can also be used for biodistribution studies involving AAV vectors. Organoids are derived from stem cells and can self-organize into 3D cultures that resemble organs in terms of their physiology, cell composition and functionality to a certain extent. A significant advantage of using organoids derived from humans is that there are no inter-species differences [27]. However, host immune responses, that likely affect AAV vector's transduction and biodistribution, cannot be accurately represented with organoids [22, 25].

Technical platforms for viral genome analysis

It is the genome of an AAV vector, but not its capsid, that carries the therapeutic transgene and can persist in transduced cells over an extended period. In addition, there is no reliable method available for measuring viral capsid levels in tissue samples. Thus, the viral genome is selected as a quantifiable surrogate for the whole vector in biodistribution studies [28]. qPCR is often utilized to detect the viral genome. For example, qPCR was used to analyze the biodistribution of an optimized scAAV9-SMN1 vector after intra-cerebroventricular administration in a SMA mouse model [29]. Similarly, qPCR was used in another study to investigate PK profile of a dual AAV vector for Usher syndrome type 1B in the retina of primates after subretinal administration [30]. Fluorescent dyes or probes can be incorporated into PCR reactions to monitor PCR process by tracing fluorescent signals after each cycle. By comparing the results with a standard curve, the copy number of viral genomes in a test sample can be determined. Linearized plasmids containing transgene or vector DNA are commonly used to prepare standard curves in AAV vector's biodistribution studies [31].

PCR specificity and sensitivity heavily rely on the design of the primers, and multiple software applications can aid in primer design [32]. In addition to the typical considerations of amplicon size and GC content, it is crucial that qPCR amplicons are specifically tailored to AAV vectors. To ensure specificity, it is often recommended to design the sequence of an amplicon at the junction of the open reading frame of the transgene and its 3' or 5' untranslated region on the vector. This strategy prevents an overestimation of viral load caused by co-presence of empty capsids. To further improve assay specificity, the primer sets should be submitted to vigorous Primer-BlAST test against the host genomic DNAs, to eliminate any non-specific cross-reactions [29, 33].

As qPCR is a highly sensitive assay, the procedure should be performed in a sterile environment for ensuring its specificity [31]. Moreover, disposable or sterile DNA-free sets of reagents and containers should be used during sampling to minimize contamination risks. It is a good practice for sampling to first collect the organs that are less likely to contain viral vectors, such as the gonads, prior to those organs, such as the liver, that are more likely to contain a large quantity of viral vectors. Injection sites should be harvested at the very end as they contain the highest levels of the viral vectors. Furthermore, it is important to apply perfusion or rinse the organ samples thoroughly in phosphate buffered saline solution to decrease contamination from blood [34]. DNA extraction and purification methods should be optimized to reduce contamination as necessary. To monitor any unintended amplification, it is recommended to include a negative control (i.e., no-template controls, NTCs) in the assay [35].

Per FDA recommendations, a qPCR assay performed for a tissue sample must demonstrate a limit of quantitation of <50 copies/µg genomic DNA. Each assay run should have a spiked control sample, in addition to those samples measuring AAV vectors' copy number, containing a known copy number of vector sequences to monitor assay sensitivity [31, 36]. Meanwhile, further experiments should be performed to assess potential PCR inhibition by measuring the copy number of an endogenous reference gene in tissue samples, or by adding a known copy number of the reference gene in plasma samples prior to qPCR reactions [30, 37].

dPCR is a relatively new techniques, which is also used to quantify AAV vector's copy number. For example, dPCR was used in a study to precisely quantify the viral load per cell in macaques' CNS tissue samples following intraparenchymal injection or cerebrospinal fluid-mediated administration [38].

In a dPCR assay, a reaction mixture (sample) is partitioned into numerous individual compartments. The number of compartments with a positive versus negative reaction is tallied and a Poisson distribution model is used to calculate the copy number of the target sequence in the original sample [39]. This method offers an advantage over qPCR in that it does not require the use of a standard curve, which is sometimes hard to construct due to lack of well-characterized reference materials. In addition, a dPCR-based method displays superior sensitivity, repeatability, accuracy, and greater tolerance to PCR inhibitors [40]. However, compared to qPCR platform, dPCR requires more time for sample analysis and method development. Furthermore, it is more expensive to perform a dPCR versus a qPCR, and dPCR has a narrower dynamic range [28, 33].

While qPCR and dPCR methods have been widely used to study PK profiles of AAV-based GT products, there remains a lack of regulatory guidance on the validation and implementation of such methods for GT products. The Global CRO Council in Bioanalysis has made recommendations regarding the acceptance criteria for a qPCR or dPCR method validation and sample analysis [41].

What qPCR and dPCR assays do not show is the cellular or spatial context necessary for a thorough analysis of AAV vector's biodistribution profile, such as the rates and types of infected cells, which can be supplemented by ISH [28, 42]. For example, a recent study introduced a new technique known as signal amplification by exchange reaction fluorescence in situ hybridization. ISH employs specific probes to localize and visualize target sequences in situ while preserving cell integrity. Together with image capture and analysis, this approach facilitated the visualization and measurement of individual AAV genomes in retinal cells following AAV subretinal delivery in mice [43]. In another case, upon intravenous bolus injection of AAV to Wistar Han rats and cynomolgus monkeys, the presence of viral genomes and transgene (SMN1) mRNA expression was confirmed in tissue samples via ISH to evaluate their relationships with toxicity findings

[44]. Proper controls measures are critical in the development of ISH methods. Certain molecules commonly expressed by most mammalian cells, for example actin, can serve as positive controls to check for nucleic acid preservation. Furthermore, a negative control can be made by excluding the specific probe in the ISH experiment. In addition, the elimination of an ISH signal should occur after pretreating the tissues with an appropriate nuclease, which can serve as another control for specificity [42].

Southern blotting can also be used to detect AAV vectors, though its use is limited in AAV vectors' biodistribution studies partially due to time-consuming and problematic procedures required to prevent contamination [23, 45]. Table 2 summarizes the advantages and challenges of all these viral genome detection techniques.

Techniques	Applications	Advantages	Challenges	References
qPCR	Quantification of viral genome in tissues or biological fluids	1.Ease and low cost to perform 2.Higher dynamic range	1.Primer design 2.Identification of control and assessment of potential contamination and inhibition in PCR reactions	29-37
dPCR		1.No standard curve required 2.Increased sensitivity, accuracy and tolerance to PCR inhibitors		38-41
ISH	Localization and visualization of viral genome in tissues	Spatial and cellular context crucial for biodistribution assessment	1.Probe design 2.Identification of appropriate controls	42, 43, 44

Table 2: Technical platforms for viral genome analysis

Technical platforms for transgene product detection

Upon AAV vector's entry into cells, a transgene must be transcribed into mRNA, and then translated into protein to exert its therapeutic function. The temporal and spatial pattern of transgene expression should, therefore, be investigated in biodistribution studies of an AAV vector [22]. Both qPCR and dPCR are commonly used for transgene mRNA detection, which require mRNAs to be reversely transcribed into cDNAs prior to PCR reactions [46, 47]. Compared to DNA samples, RNA samples are more prone to degradation, and RNase contamination poses a significant risk to RNA integrity. Thus, it is important to prevent RNase contamination from all sources, including reagents used in sampling, RNA extraction procedure, and other experimental procedures. Additionally, specific measures must be taken to maintain RNA integrity. For example, tissue samples were submerged entirely in RNA later reagent in sterile RNase-free tubes in a reported study [37]. In some cases, it is necessary for the primer set of a PCR assay to differentiate between transgene mRNA and its endogenous counterpart. In a study using a SMA mouse model, the primer set was specifically designed to detect SMN expression from the chicken-β-actin promoter of the AAV9 vector, and it did not amplify SMN originating from the SMN2 gene [48]. To ensure this required specificity, the primers must be submitted to a Primer-BLAST query against the host RNA database prior to use [31].

The detection of a transgene protein from complex mixtures of proteins extracted from tissues via Western blot is a widespread practice, where the target protein is separated from non-target ones via gel electrophoresis and probed by its specific antibody [45]. For example, SMN expression was analyzed via Western blot in the brain, spinal cord, and gastrocnemius muscle samples from wild-type, untreated SMA, and SMA mice treated with 2 types of AAV vectors. The results showed that SMN levels in the gastrocnemius muscles of both treated groups did not recover to wild-type levels, while the levels in the brain and spinal cord samples surpassed those of wild-type counterparts [47]. In another study, GAA expression in the liver

was demonstrated using Western blot in NHPs after hepatic gene transfer with AAV vector, and GAA uptake by various tissues was also shown [49]. Efficient protein extraction from tissue samples is critical for accurate Western blot analyses. The BCA Protein Assay Kit is commonly used for determining protein concentration after extraction. It is also crucial to include an endogenous reference protein such as tubulin and GAPDH to aid in the assessment of Western blot results and the quantification of target protein.

ELISA is commonly used for quantifying a specific protein in liquid samples based on the reaction between the target protein and its primary antibody [50]. ELISA has been employed to determine the concentrations of circulating hFVIII or hFIX protein in the plasma of treated animals after intravenous infusion of AAV vector harboring hFVIII or hFIX gene [46, 51]. In another study, human p75ECD levels in the brain and serum samples of Alzheimer's disease mice were analyzed with ELISA after AAV intramuscular administration [52].

To localize and visualize transgene expression in a tissue sample, one may utilize immunohistochemistry (IHC) or immunofluorescence (IF). Enzyme-linked antibody catalysis of the added substrate (in the case of IHC) or the use of fluorescence probe-conjugated antibody (in the case of IF) are commonly used in these techniques [45]. For example, assessed by IHC analysis of SMN expression in mice's lumbar spinal cords, SMN expression in α-motor neurons of SMA mice was shown to recover after AAV intracerebroventricular administration [29]. Similarly, using IF analysis, another study confirmed the recovery of SMN expression in motor neurons of SMA mice after AAV prenatal administration [47]. However, if the transgene protein is secreted or transported axonally, IHC or IF may not be able to provide an accurate depiction of the cellular transduction pattern or the origin of the transgene protein after AAV delivery. It is preferable to detect transgene mRNA via ISH in this case [53].

When selecting primary antibodies for transgene proteins used in these techniques, it is necessary to verify and prevent any potential cross-reactivity with endogenous host proteins [46, 52]. Alternatively, a tag can be encoded

in the transgene to differentiate between the transgene-derived protein and its endogenous counterpart [52, 54]. Table 3 summarizes the applications

and challenges of all these techniques available for detecting transgene products.

Techniques	Applications	Challenges	References
qPCR/dPCR	To quantify transgene mRNA in tissues	Difficulty in protection of RNA integrity	37, 46-48
ISH	To localize and visualize transgene mRNA in tissues		53
Western blot	To identify and quantify transgene protein	1.Protein extraction efficiency 2.Use of proper endogenous reference protein 3.Primary antibodies' specificity	47, 49
ELISA	To determine transgene protein concentration	Primary antibodies' specificity	46, 50, 51, 52
IHC/IF	To localize and visualize transgene protein in tissues	Primary antibodies' specificity	29, 47

Table 3: Technical platforms used for detection of transgene product

Assessment of vector shedding

Shedding refers to the spread of the viral vector through patients' secreta and excreta. Samples including urine, feces, and saliva are commonly collected for this assessment. While the spread of the viral vector in blood is necessary for its shedding, blood specimens are not typically collected for shedding assessment. The selection of sample type also depends on the route of AAV administration [13]. For example, if a viral vector is administrated via subretinal injection, it is recommended to collect tear samples from patients in addition to the commonly collected samples [55].

Regarding the sampling schedule, sampling should be more frequent in the first days after administration to examine a transient shedding profile. To ensure the reliability of the assessment, sampling should be performed until several consecutive samples are negative [56].

As in biodistribution studies, a qPCR technique is used to detect viral genome for assessing AAV vector shedding. Due to the replication deficiency of the AAV vector and its low potential to cause interpersonal transmission, qPCR is adequate as a primary assay to assess AAV vector shedding, especially when the viral genome copy number in samples decreases over time [13, 55]. For example, after intravenous injection of AAVLK03.hOTC for severe ornithine transcarbamylase deficiency, saliva, urine, and feces samples were collected from treated cynomolgus macaques and assessed for AAV shedding by qPCR. Viral genome was transiently detectable in all of those samples [57]. In a phase I/II study, patients received intra-articular injection of an AAV vector for inflammatory hand arthritis, and qPCR was used to detect the presence of viral genome in their saliva, urine, and feces samples [58].

It should be noted that the shedding assessment samples, such as feces, saliva, and nasal swabs, usually contain natural body flora, nucleases, and salts, which not only increase the risk of contamination but also inhibit PCR reactions. Thus, evaluation and assurance the specificity and sensitivity of the qPCR assay should be performed as done for biodistribution studies. If an interference is observed, sample dilution should be explored as the first solution to mitigate interference [13, 56].

As traditional qPCR cannot directly determine whether viral genomes in patient samples are infectious or not, the researchers have developed a novel immunoprecipitation qPCR assay as a solution. Using a highly specific monoclonal antibody against the AAV5 capsid, encapsidated viral genomes were captured, and then further purified from non-encapsidated ones by adding the nuclease, Benzonase. Post treatment, if viral genomes were still amplifiable in the subsequent qPCR, they should be wrapped by intact capsids and thus potentially infectious [20, 59]. In another study, researchers performed an infectious replication assay on PCR-positive samples to

determine if infectious particles were present [60], which was complicated and probably unnecessary given a simpler method is available.

Discussion

Biodistribution and shedding studies of an AAV-based GT product offer necessary data to support development and approval. Biodistribution results can establish if the viral vector successfully reaches targeted tissues and expresses the anticipated transgene protein. Furthermore, they aid in evaluating the potential connection between an unwanted effect and presence of the viral vector or transgene expression in specific cells or tissues. Additionally, studies on biodistribution offer valuable information for determining the first-in-human dosage [21], while viral shedding studies help researchers to address the safety concerns regarding the transmission potential of the vector from treated to untreated individuals [22]. To conduct meaningful biodistribution and shedding studies, analytical methods for these studies must be rationally designed and developed appropriately.

The techniques covered in this review typically involve sacrifice of animals and collection of tissue samples in order to assess the biodistribution profiles of AAV-based GT products. This can be problematic when animal availability is restricted, particularly in studies involving large animals. As a potential resolution, non-invasive in vivo imaging systems can be utilized to track AAV biodistribution patterns in live animals [45]. For example, researchers employed iodine-124 (I-124) radiolabeling of AAV capsid combined with positron emission tomography to analyze AAV biodistribution in organs of living NHPs following intravenous or intracisternal administration of the AAV vector for 1 to 72 hours [61]. In another study, a fluorescently biarsenical dye was used to label the core CCPGCC motif of tetracysteine sequence within AAV9 capsid, without compromising viral infectivity. The labelled AAV9 vector could be observed in anesthetized mice using intravital imaging after intravenous administration of the labeled virus [62]. To confirm successful infection and transduction in living animals' organs, fluorescent reporter or firefly luciferase imaging can be used to provide visual confirmation [47, 63]. As it is challenging to extrapolate biodistribution data from animal models to humans, coupled with difficulty in collecting biopsies or fluid samples in certain cases, in vivo imaging system offers a viable solution to obtaining valuable clinical data on AAV biodistribution in the future [22]. Radiolabeling and fluorescent dye tags on AAV particles, when combined with imaging techniques, may also provide a more convenient approach for detecting viruses in shedding studies.

Current biodistribution studies primarily focus on the transport of AAV vectors between tissues, while the "intracellular pharmacokinetics", that depicts how AAV particles are absorbed, deposited, and metabolized within cells warrants an increased attention in the future. Further understanding of the underlying mechanisms of AAV-mediated cell transduction and gene

expression requires an improved application of molecular and cellular biological techniques [25, 64]. In addition to gene replacement, strategies like gene silencing are being developed for AAV-based GT products [5, 65]. The guidance for their pharmacological studies needs to be updated since they differ from the current approved products which are all based on gene replacement.

To better interpret and apply PK data of AAV vectors in clinical studies, several other factors must be examined beyond a rational use of analytical methods. For example, discrepancies among the results of biodistribution and shedding studies could be attributed to variations in the titration and characterization of the AAV vector, such as empty-to-full capsid ratio. Therefore, minimizing these variations is crucial [25, 66]. Host immunity to AAV can affect the biodistribution of AAV vectors, and should be evaluated in a biodistribution study [21]. Other factors, such as galectin 3 binding protein, C-reactive protein and a serotype's blood clearance rate, can also influence vector biodistribution and should be taken into consideration during experimental design [7, 67]. Researchers are attempting to integrate all the PK pertinent factors into a novel physiologically based pharmacokinetic model, in order to address the interspecies discrepancies experienced while extrapolating AAV vectors' preclinical PK data to clinical use [64].

Conclusions

In brief, this review outlines the major analytical methods used to support the study of biodistribution and shedding of AAV-based GT products. The attainment of reliable data relies not only on the careful consideration of the appropriate application of these analytical methods and potential caveats, but also on strict adherence to the guidance and requirements set forth by regulatory agencies, including recommended sampling procedures and data acceptance criteria. This review offers comprehensive details about two aspects that can assist researchers in designing and/or selecting methods for future studies. To facilitate the development and approval of AAV-based GT products, particularly the newly emerging ones, an improved understanding of biodistribution and shedding must be obtained through adequate use of the analytical methods discussed in this review.

References

1. Mendell JR, Al-Zaidy SA, Rodino-Klapac LR, et al. (2021). Current Clinical Applications of In Vivo Gene Therapy with AAVs. Mol Ther. 29(2):464-488.
2. Li X, Wei X, Lin J, Ou L. (2022). A versatile toolkit for overcoming AAV immunity. *Front Immunol.* 13:991832.
3. Li C, Samulski RJ. (2020). Engineering adeno-associated virus vectors for gene therapy. Nat Rev Genet. 21(4):255-272.
4. Balakrishnan B, Jayandharan GR. (2014). Basic biology of adeno-associated virus (AAV) vectors used in gene therapy. *Curr Gene Ther.* 14(2):86-100.
5. Wang D, Tai PWL, Gao G. (2019). Adeno-associated virus vector as a platform for gene therapy delivery. Nat Rev Drug Discov. 18(5):358-378.
6. Naso MF, Tomkowicz B, Perry WL 3rd, Strohl WR. (2017). Adeno-Associated Virus (AAV) as a Vector for Gene Therapy. *BioDrugs.* 31(4):317-334.
7. Duan D. (2016). Systemic delivery of adeno-associated viral vectors. Curr *Opin Virol.* 21:16-25.
8. Colella P, Ronzitti G, Mingozzi F. (2017). Emerging Issues in AAV-Mediated In Vivo Gene Therapy. Mol Ther Methods Clin Dev. 8:87-104.
9. Brandon EF, Hermsen HP, van Eijkeren JC, Tiesjema B. (2010). Effect of administration route on the biodistribution and shedding of replication-deficient AAV2: a qualitative modelling approach. Curr Gene Ther. 10(2):91-106.
10. (2013). U.S. Food and Drug Administration. Preclinical Assessment of Investigational Cellular and Gene Therapy Products.
11. (2018). International Pharmaceutical Regulators Programme. Expectations for Biodistribution (BD) Assessments for Gene Therapy (GT) Products.
12. Gonin P, Gaillard C. (2004). Gene transfer vector biodistribution: pivotal safety studies in clinical gene therapy development. Gene Ther. 1: S98-S108.
13. (2015). U.S. Food and Drug Administration. Design and Analysis of Shedding Studies for Virus or Bacteria-Based Gene Therapy and Oncolytic Products.
14. (2012). European Medicines Agency. Glybera product information. 29.
15. (2019). European Medicines Agency. Luxturna product information.
16. (2022). European Medicines Agency. Upstaza product information.
17. (2023). U.S. Food and Drug Administration. Zolgensma package insert.
18. (2023). U.S. Food and Drug Administration. Elevidys package insert. Approved
19. (2022). U.S. Food and Drug Administration. Hemgenix package insert.
20. (2022). European Medicines Agency. Roctavian product information.
21. (2023). Center for Drug Evaluation. Nonclinical Biodistribution Consideration for Gene Therapy Products.
22. (2018). European Medicines Agency. Guideline on the quality, non-clinical and clinical aspects of gene therapy medicinal products.
23. Sun K, Liao MZ. (2022). Clinical Pharmacology Considerations on Recombinant Adeno-Associated Virus-Based Gene Therapy. *J Clin Pharmacol.* 62 S79-S94.
24. (2010). European Medicines Agency. Reflection paper on quality, non-clinical and clinical issues related to the development of recombinant adeno-associated viral vectors. 24.
25. Chowdhury EA, Meno-Tetang G, Chang HY, et al. (2021). Current progress and limitations of AAV mediated delivery of protein therapeutic genes and the importance of developing quantitative pharmacokinetic/pharmacodynamic (PK/PD) models. Adv Drug Deliv Rev. 170:214-237.
26. Watson Levings RS, Broome TA, Smith AD, et al. (2018). Gene Therapy for Osteoarthritis: Pharmacokinetics of Intra-Articular Self-Complementary Adeno-Associated Virus Interleukin-1 Receptor Antagonist Delivery in an Equine Model. Hum Gene Ther Clin Dev. 29(2):90-100.
27. Artegiani B, Clevers H. (2018). Use and application of 3D-organoid technology. *Hum Mol Genet.* 27(R2): R99-R107.
28. Chen N, Sun K, Chemuturi NV, Cho H, Xia CQ. (2022). The Perspective of DMPK on Recombinant Adeno-Associated Virus-Based Gene Therapy: Past Learning, Current Support, and Future Contribution. AAPS J. 24(1):31.
29. Armbruster N, Lattanzi A, Jeavons M, et al. (2016). Efficacy and biodistribution analysis of intracerebroventricular administration of an optimized scAAV9-SMN1 vector in a mouse model of spinal muscular atrophy. Mol Ther Methods Clin Dev. 3:16060
30. Ferla R, Dell'Aquila F, Doria M, et al. (2023). Efficacy, pharmacokinetics, and safety in the mouse and primate retina of dual AAV vectors for Usher syndrome type 1B. Mol Ther Methods Clin Dev. 28:396-411.
31. Ma H, Bell KN, Loker RN. (2020). qPCR and qRT-PCR analysis: Regulatory points to consider when conducting biodistribution and vector shedding studies. Mol Ther Methods Clin Dev. 20:152-168.
32. Thornton B, Basu C. (2015). Rapid and simple method of qPCR primer design. Methods Mol Biol. 1275:173-179.
33. Gorovits B, Marshall JC, Smith J, Whiteley LO, Neubert H. (2019). Bioanalysis of adeno-associated virus gene therapy therapeutics: regulatory expectations. Bioanalysis. 11(21):2011-2024.
34. Le Guiner C, Moullier P, Arruda VR. (2011). Biodistribution and

shedding of AAV vectors. Methods Mol Biol. 807:339-359.

35. Bustin SA, Benes V, Garson JA, et al. (2009). The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem.* 55(4):611-622.

36. (2020). U.S. Food and Drug Administration. Long Term Follow-Up After Administration of Human Gene Therapy Products.

37. Ferla R, Alliegro M, Marteau JB, et al. (2017). Non-clinical Safety and Efficacy of an AAV2/8 Vector Administered Intravenously for Treatment of Mucopolysaccharidosis Type VI. Mol Ther Methods Clin Dev. 6:143-158.

38. Kondratov O, Kondratova L, Mandel RJ, et al. (2021). A comprehensive study of a 29-capsid AAV library in a non-human primate central nervous system. Mol Ther. 29(9):2806-2820.

39. Long S, Berkemeier B. (2022). Ultrasensitive detection and quantification of viral nucleic acids with Raindance droplet digital PCR (ddPCR). Methods. 201:49-64.

40. Kojabad AA, Farzanehpour M, Galeh HEG, et al. (2021). Droplet digital PCR of viral DNA/RNA, current progress, challenges, and future perspectives. *J Med Virol.* 93(7):4182-4197.

41. Wissel M, Poirier M, Satterwhite C, et al. (2022). Recommendations on qPCR/ddPCR assay validation by GCC. Bioanalysis. 14(12):853-863.

42. Chu YH, Hardin H, Zhang R, Guo Z, Lloyd RV. (2019). In situ hybridization: Introduction to techniques, applications and pitfalls in the performance and interpretation of assays In situ hybridization: Introduction to techniques, applications and pitfalls in the performance and interpretation of assays. Semin Diagn Pathol. 36(5):336-341.

43. Wang SK, Lapan SW, Hong CM, Krause TB, Cepko CL. (2020). In Situ Detection of Adeno-associated Viral Vector Genomes with SABER-FISH. Mol Ther Methods Clin Dev. 19:376-386.

44. Palazzi X, Pardo ID, Sirivelu MP, et al. (2022). Biodistribution and Tolerability of AAV-PHP.B-CBh-SMN1 in Wistar Han Rats and Cynomolgus Macaques Reveal Different Toxicologic Profiles. Hum Gene Ther. 33(3-4):175-187.

45. Xu X, Jingwen Y, Yuanguo C. (2011). Pharmacokinetic Study of Viral Vectors for Gene Therapy: Progress and Challenges. InTech.

46. Elkouby L, Armour SM, Toso R, et al. (2021). Preclinical assessment of an optimized AAV-FVIII vector in mice and non-human primates for the treatment of hemophilia A. Mol Ther Methods Clin Dev. 24:20-29.

47. Rashnonejad A, Amini Chermahini G, Gündüz C, et al. (2019). Fetal Gene Therapy Using a Single Injection of Recombinant AAV9 Rescued SMA Phenotype in Mice. Mol Ther. 27(12):2123-2133.

48. Meyer K, Ferraiuolo L, Schmelzer L, et al. (2015). Improving single injection CSF delivery of AAV9-mediated gene therapy for SMA: a dose-response study in mice and nonhuman primates. Mol Ther. 23(3):477-487

49. Costa-Verdera H, Collaud F, Riling CR, et al. (2021). Hepatic expression of GAA results in enhanced enzyme bioavailability in mice and non-human primates. Nat Commun. 12(1):6393.

50. Hayrapetyan H, Tran T, Tellez-Corrales E, Madiraju C. (2023). Enzyme-Linked Immunosorbent Assay: Types and Applications. Methods Mol Biol. 2612:1-17.

51. Nair N, De Wolf D, Nguyen PA, et al. (2021). Gene therapy for hemophilia B using CB 2679d-GT: a novel factor IX variant with higher potency than factor IX Padua. Blood. 137(21):2902-2906.

52. Wang QH, Wang YR, Zhang T, et al. (2016). Intramuscular delivery

of p75NTR ectodomain by an AAV vector attenuates cognitive deficits and Alzheimer's disease-like pathologies in APP/PS1 transgenic mice. *J Neurochem.* 138(1):163-173.

53. Hunter JE, Gurda BL, Yoon SY, Castle MJ, Wolfe JH. (2019). In Situ Hybridization for Detection of AAV-Mediated Gene Expression. Methods Mol Biol. 1950:107-122.

54. Ye GJ, Budzynski E, Sonnentag P, et al. (2015). Safety and Biodistribution Evaluation in Cynomolgus Macaques of rAAV2tYF-CB-hRS1, a Recombinant Adeno-Associated Virus Vector Expressing Retinoschisin [published correction appears in Hum Gene Ther Clin Dev. 26(4):243. Neuringer, Martha [added]; McGill, Trevor [added]; Stoddard, Jonathan [added]]. Hum Gene Ther Clin Dev. 26(3):165-176.

55. Barnard AR, Rudenko AN, MacLaren RE. (2018). Vector Shedding and Immunogenicity Sampling for Retinal Gene Therapy. Methods Mol Biol. 1715:359-371.

56. (2009). European Medicines Agency. General Principles to Address Virus and Vector Shedding.

57. Baruteau J, Cunningham SC, Yilmaz BS, et al. (2021). Safety and efficacy of an engineered hepatotropic AAV gene therapy for ornithine transcarbamylase deficiency in cynomolgus monkeys. Mol Ther Methods Clin Dev. 23:135-146.

58. Vrouwe JPM, Meulenberg JJM, Klarenbeek NB, et al. (2022). Administration of an adeno-associated viral vector expressing interferon-β in patients with inflammatory hand arthritis, results of a phase I/II study. Osteoarthritis Cartilage. 30(1):52-60.

59. Sandza K, Clark A, Koziol E, et al. (2022).Ultra-sensitive AAV capsid detection by immunocapture-based qPCR following factor VIII gene transfer. Gene Ther. 29(1-2):94-105.

60. Farraha M, Kizana E. (2022). Assessing Recombinant AAV Shedding After Cardiac Gene Therapy. Methods Mol Biol. 2573:333-344

61. Ballon DJ, Rosenberg JB, Fung EK, et al. (2020). Quantitative Whole-Body Imaging of I-124-Labeled Adeno-Associated Viral Vector Biodistribution in Nonhuman Primates. Hum Gene Ther. 31(23-24):1237-1259.

62. Chandran JS, Sharp PS, Karyka E, et al. (2017). Site Specific Modification of Adeno-Associated Virus Enables Both Fluorescent Imaging of Viral Particles and Characterization of the Capsid Interactome. Sci Rep. 7(1): 14766.

63. Ezra-Elia R, Obolensky A, Ejzenberg A, et al. (2018). Can an in vivo imaging system be used to determine localization and biodistribution of AAV5-mediated gene expression following subretinal and intravitreal delivery in mice?. Exp Eye Res. 176:227-234.

64. Liu S, Chowdhury EA, Xu V, et al. (2023). Whole-Body Disposition and Physiologically Based Pharmacokinetic Modeling of Adeno-Associated Viruses and the Transgene Product *J Pharm Sci.* S0022-3549(23)00414-8.

65. Clarner P, Lau SK, Chowdhury T, et al. (2021). Development of a one-step RT-ddPCR method to determine the expression and potency of AAV vectors. Mol Ther Methods Clin Dev. 23:68-77.

66. Chen X, Lim DA, Lawlor MW, et al. (2023). Biodistribution of Adeno-Associated Virus Gene Therapy Following Cerebrospinal Fluid-Directed Administration. Hum Gene Ther. 34(3-4):94-111.

67. Kotchey NM, Adachi K, Zahid M, et al. (2011). A potential role of distinctively delayed blood clearance of recombinant adeno-associated virus serotype 9 in *robust cardiac transduction*. Mol Ther. 19(6):1079-1089.

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