Short review on determination of nusinersen for spinal muscular atrophy treatment

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Abstract

Nusinersen, an antisense oligonucleotide, is an active ingredient of the first drug approved by the Food and Drug Administration for the treatment of spinal muscular atrophy, a genetic condition characterized by progressive muscle weakness and atrophy. A deficiency in the survival motor neuron (SMN) protein causes the disease. SMN protein is required for the functioning of motor neurons and the transmission of nerve signals to muscles. Using nusinersen therapy determines the need for a pharmacokinetic and metabolic analysis of the drug. Consequently, this requires the use of various analytical techniques to study biological samples. Based on current scientific literature, encompassing publications from 2007 to 2024, the sample preparation methods for nusinersen involve liquid-liquid extraction, solid-phase extraction, dispersive solid-phase extraction, and microextraction by packed sorbent or hybridization, which facilitate the removal of interfering components and ensure the efficient recovery of the analyte. Liquid chromatography combined with mass spectrometry is currently the primary tool used for the determination of nusinersen and its metabolites. This review discusses the latest developments in analytical methods used in nusinersen research, presents techniques for the purification of biological samples, and compares their efficiency. The use of ultra-performance liquid chromatography coupled with mass spectrometry for the determination of oligonucleotides is also described. The results obtained using different chromatographic modes (reversed-phase high-performance liquid chromatography, ion-pair reversed-phase high-performance liquid chromatography, and hydrophilic interaction liquid chromatography) are then summarised and compared.

Keywords: biological samples; extraction; liquid chromatography; nusinersen; spinal muscular atrophy; therapeutic oligonucleotides

1. Introduction

Spinal muscular atrophy (SMA) is a genetic neuromuscular disease that results in the gradual deterioration of motor neurons in the spinal cord, leading to muscle weakness and atrophy [1]. The earliest known descriptions of this disease date back to the nineteenth century, when medical practitioners began to document cases of children exhibiting progressive muscle weakness, which resulted in difficulties with movement and even breathing. For decades, there was no effective treatment for SMA, which was associated with high mortality and a significant decrease in the quality of life of patients [2]. The advent of three novel therapeutic modalities in the 21st century—gene therapy, antisense therapy, and oral therapy—has led to a notable enhancement in the functional capacity of patients with SMA. As a result of these developments, it is now feasible not only to decelerate the advancement of the disease but also to partially reinstate impaired motor abilities [1]. Nevertheless, the factor in the efficacy of SMA treatment is accurate diagnosis and monitoring of therapy, which requires advancing these techniques for the purification of biological samples, such as serum or cerebrospinal fluid. The purification process enables the isolation of analytes, including active drug ingredients and their metabolites. These are then subjected to a comprehensive analysis [3]. Properly preparing biological samples, including extracting, purifying, and concentrating analytes, is paramount in ensuring the reliability and repeatability of chromatographic analysis [4]. The development of analytical methods, such as liquid chromatography, with the appropriate detection, allows for the precise separation and sensitive detection of chemical compounds in biological samples, is particularly important.[1]. Such analytical techniques play a pivotal role in the advancement of increasing methodologies for the assessment of pharmacokinetics and pharmacodynamics associated with therapeutic interventions [1]. The ultimate objective of these endeavors is to develop diagnostic instruments that will guarantee the efficacy and security of treatment and facilitate the advancement of SMA therapy [5].

This review addresses topics in SMA, including the historical outline of the disease, the nusinersen molecule, recent advances in analytical methods used in nusinersen research, methods for preparing biological samples containing nusinersen and its metabolites, and the use of different liquid chromatography-mass spectrometry modes in the determination of nusinersen. The article systematically organizes the latest literature (2007 – 2024) concerning extraction methods as well as separation and determination conditions for nusinersen. This review provides a well-structured summary of existing findings, aiming to cover these topics comprehensively for the first time. The widespread use of SMA therapies, ongoing studies, and

the need for continuous clinical monitoring leads to a better understanding of nusinersen and its metabolites, providing better patient treatment efficacy.

2. Spinal muscular atrophy (SMA)

SMA is a relatively uncommon genetic disorder. The estimated incidence is 1 to 2 cases per 100,000 people, with an estimated incidence of 1 in 11,000 births [1]. It is inherited as an autosomal recessive trait [1,6]. The mutation in question affects chromosome 5q13 and has a deleterious effect on the Survival of Motor Neuron 1 (SMN1) gene, resulting in a deficiency of survival motor neuron (SMN) protein. This, in turn, gives rise to motor neuron degeneration and a reduction in motor nerve signal transmission to proximal muscles [7,8]. Degeneration of motor neurons affects the processes of breathing, swallowing, and movement [1].

The SMN1 gene encodes the full-length SMN protein, which is critical for the proper functioning and viability of motor neurons. A complete absence of SMN protein is embryonically lethal, underscoring the protein's indispensable function [1]. Additionally, humans possess a second gene, designated SMN2, which is nearly identical to SMN1. However, a critical single-nucleotide variation in SMN2 results in truncated and unstable SMNΔ7 protein. In SMA, the loss of SMN1 results in deficient levels of full-length SMN protein to support motor neuron function. The number of SMN2 copies serves to partially compensate for this loss, as each additional copy increases the amount of functional SMN protein produced. A more significant number of SMN2 copies is typically associated with a milder SMA phenotype and a later onset of symptoms due to the increased production of functional SMN protein [1,7,9].

The historical classification of the disease has been based on the occurrence of symptoms and the degree of development of the child's motor functions, which have been identified as the primary determinants of the disease's progression [2,10]. This has led to the delineation of five stages (0-IV) in the disease's natural history (

89 **Table *1***).

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Table 1. Characteristics of SMA types (I-IV) [2,9]

Type 0	The most severe form of the disease; manifests itself immediately after birth and often results in death within the first few weeks of life.
Type I	This condition affects approximately 60% of births and is characterized by motor weakness that typically manifests within the first six months of life. Respiratory and nutritional support is usually required to ensure the infant's survival. Prior to the advent of effective treatment, the mortality rate among affected infants was high, with most dying before reaching two years of age.
Type II	The condition affects approximately 30% of births and is characterized by motor weakness that typically manifests within the first 18 months of life. Individuals tend to demonstrate superior motor development, reaching the sitting milestone at an earlier age. Respiratory, swallowing, and muscle contracture difficulties occur.
Type III	The condition affects approximately 10% of births and typically appears after 18 months. Most patients achieve all developmental milestones, although lower limb weakness is a common manifestation that can occur at different ages. Life expectancy is generally in line with the average of the general population.
Type IV	The rarest and mildest form of spinal muscular atrophy (SMA), it presents with symptoms in adulthood and is characterized by mild impairments in motor skills.

The classification above pertains to the typical progression of the disease. Following the introduction of a novel drug for SMA in 2016, the landscape shifted considerably. At present, the patient's classification is based on their age and functional status at the commencement of treatment or the number of copies of the SMN2 gene [1].

3. SMA drugs

Nowadays, three different SMA therapies are known to exist and are used for the treatment of patients. The drugs used in therapy are Spinraza, Zolgensma, and Evrysdi; their summary is presented in **Błąd! Nie można odnaleźć źródła odwołania.** Despite the differences between them, such as the active substance, method of administration, dosage, or mechanism

of action, the use of each of them aims to increase the production of functional SMN protein in the body [7,9].

Including their active substances, years of market introduction, administration methods, mechanisms of action, and recommended dosages. All three SMA drugs received regulatory approval. However, they are also the subject of ongoing clinical studies, which aim to refine their application, explore new patient populations, and improve understanding of their long-term outcomes.

4. Nusinersen - the first drug for SMA therapy

Oligonucleotides are short fragments of nucleic acids composed of several dozen of nucleotide monomers. They occur naturally in the cells of living organisms. The development of the synthesis of these compounds has facilitated their novel applications, including antisense therapy. It is based on introducing a complementary oligonucleotide, which results in its binding to specific fragments of mRNA and inhibition of translation. Nusinersen is an 18-mer modified antisense oligonucleotide with the following modifications: 1. replacing one of the non-bridging oxygen atoms in phosphate group with sulfur; 2. introduction of a methoxyethyl group in the 2' position of ribose; 3. methylation of cytosine and uracil [16–19]. For illustrative purposes, part of its structure is shown in **Błąd! Nie można odnaleźć źródła odwołania.**.

The chemical modifications introduced to the structure of nusinersen have been shown to affect the physicochemical properties of the molecule. All of them increase the stability of nusinersen, reducing its susceptibility to degradation by enzymes such as ribonuclease and prolonging its activity in cells. In addition, modifications enhance the specificity of action, resulting in more precise binding to target mRNA sequences. Consequently, the combination of these modifications contributes to the improved stability, efficacy, and specificity of nusinersen, thereupon enabling its long-term and effective treatment of SMA [16].

Nusinersen is an oligonucleotide with a molecular weight of 7126.23 Da that forms multiply charged ions (polyanions) [20]. It undergoes degradation under extreme pH conditions. In acidic conditions (pH 2), hydrolysis of phosphodiester bonds occurs, whereas in alkaline conditions, base-catalyzed decomposition occurs, leading to strand fragmentation [20]. Furthermore, stability tests have demonstrated that nusinersen is sensitive to factors such as oxidation, elevated temperatures, and exposure to light. Photostability studies of nusinersen, conducted in accordance with ICH Q1B guidelines, have shown that photodegradation occurs slowly, without significant formation of impurities under typical manufacturing and handling

conditions. No special light protection measures are needed during production, storage, or handling [20].

The ultraviolet absorption spectrum of nusinersen exhibits a maximum at approximately 260 nm, attributable to the presence of nitrogenous bases in its structure. The phosphate groups present in the structure of nusinersen have low pKa values, meaning that they are ionized at pH above 2 (including physiological conditions (pH~7.4)) [20]. The nitrogenous bases differ in their pKa values: adenine (pH~3.5) and cytosine (pH~4.2) are protonated in acidic media, while guanine is protonated at pH~9.2 [20].

It is characterized by good solubility in water and mixtures of organic solvents, such as acetonitrile (ACN) and buffers containing phosphoric acid or triethylamine (TEA) [21]. The research results have demonstrated that initiating treatment at an early stage of the disease causes a notable improvement in motor skills (e.g., sitting independently, reduced necessity for ventilatory assistance) and prolonging survival. Consequently, the drug's efficacy is contingent upon its administration's timing, and for more pronounced outcomes, treatment should start at a very early stage. Therefore, screening genetic tests are of great importance [22].

5. Nusinersen extraction from biological samples

A comprehensive understanding of the metabolic pathway of nusinersen, following administration, is primary for evaluating its efficacy and safety. An analysis of them requires their extraction from biological samples, such as blood or cerebrospinal fluid [23]. Biological matrices typically comprise organic and inorganic components (e.g. protein, lipids, carboxyhydrates, vitamins, salts, etc.), which can potentially interfere with analytes. Furthermore, the binding of antisense oligonucleotides to plasma proteins represents an additional challenge [24]. Therefore, the samples to be analyzed must be prepared appropriately before analysis.

5.1. Extraction of nusinersen metabolites from serum

Liquid-liquid extraction (LLE) is the most commonly applied for the extraction of antisense oligonucleotides, such as nusinersen [4]. Studzińska et al. [4] performed LLE of nusinersen metabolites from serum samples using a phenol/chloroform/isoamyl alcohol (25:24:1 v/v/v) mixture. Alkalized phenol leads to protein denaturation, chloroform facilitates the separation of the aqueous and organic phases, and isoamyl alcohol acts as an anti-foaming agent. As a result, two phases are obtained after extraction - an aqueous phase containing nucleic acids and an organic phase containing protein and lipid impurities. An additional LLE

extraction step was performed with chloroform (1:4 v/v) to remove residual phenol. An analogous method was used by Vosáhlová et al. [18]. In the initial phase of the study, the direct extraction of ON (oligonucleotide) standards from diluted human serum was examined. The highest recoveries, irrespective of ON modification, were obtained for LLE, although it did require additional purification [4,18]. Alternatively, the enzymatic degradation of proteins using proteinase K may be employed [4,18].

Solid phase extraction (SPE) is a commonly used technique for sample preparation in bioanalytical analysis. Sorbents used in SPE may show different properties and consequently various interaction mechanism between the analyte and the sorbent. This is one of parameters determining the success of the extraction. Oasis HLB and Clarity OTX® are usually applied in the case of antisense oligonucleotides [4]. Zhang et al. used hydrophilic-lipophilic Oasis HLB sorbent to achieve high-yield isolation of nusinersen from rabbit plasma. The procedure starts by mixing plasma with Clarity OTX buffer and 4% H_3PO_4, which allows efficient release of nusinersen from proteins. Next, SPE proceeded in ion-pair mode using ion-pair reagents, namely alkylamines, such as TEA, dimethylbutylamine (DMBA), and hexylamine (HA). The SPE cartridge was conditioned with a mixture of TEA and hexafluoroisopropanol (HFIP) to absorb TEA at the adsorbent surface. Next, the sample was mixed with the same reagents (to form ion pairs between nusinersen oligonucleotide and TEA) and loaded into Oasis HLB material. The SPE column was washed with a mixture of water, HFIP, and TEA, followed by nusinersen elution with an acetonitrile/TEA mixture. The resulting extracts were evaporated to dryness at 28°C and then dissolved in ACN/H_2O (1:1 v/v) to concentrate them. The recovery of nusinersen ranged from 66.7% to 76.8%, depending on the analyte concentration. Enhanced recoveries of nusinersen observed at elevated concentrations can be attributed to its adsorption within the SPE column and polypropylene tubing, which occurs at low sample concentrations. The authors claimed that the method effectively eliminated matrix interference (no significant ionization suppression or enhancement) [25].

SPE with Oasis HLB was also used to extract nusinersen metabolites from human serum samples. The method was an additional purification step for LLE extracts [21]. The analysis demonstrated that LLE was not sufficiently adequate for the purification of the serum sample (matrix effect observed), but it enabled effective protein removal. SPE was performed in ion pair mode, similarly as in the case of Zhang et al. [25] study. However, different ion-pair reagents were used during conditioning and sample load: DMBA and HFIP. Nusinersen metabolites were eluted from Oasis HLB adsorbent using methanol. Application of this

purification method yielded the highest metabolite recoveries (over 90%), yet the reproducibility was low [21]. Two other extraction methods were additionally used to purify LLE extracts after extraction of nusinersen metabolites. The first one was microextraction by packed sorbent (MEPS) with polystyrene-divinylbenzene (SDVB) and octadecyl (C18) adsorbents used in ion-pair mode with DMBA/HFIP. The procedure was similar to SPE, while the volume of sample and solvents was significantly lower, as usual in MEPS. Moreover, magnetic dispersive solid phase extraction with nanoparticles coated with ionic liquid co-polymers was also used for subsequent purification and concentration of the LLE extracts [21]. The surface of these adsorbents has been modified with imidazolium ionic liquid cations with functional groups that can attach a proton or become ionized depending on the pH of the solvent. This was used to conduct extraction in a weak ion exchange mode. Nusinersen metabolites were adsorbed in a low-pH solution and desorbed with a high-pH salt The described procedure does not apply any of toxic solvents (nor organic solvents or amines), moreover, the whole procedure is carried out in one eppendorf. For this reasons it may be classified as 'green' (environmentally friendly) methodMEPS employing an SDVB sorbent yielded lower recoveries (approximately 80%) yet gave high repeatability and purification efficiency. Conversely, utilizing MNPs yielded the lowest recoveries (approximately 65%) and the highest standard deviations [21]. Consequently, MEPS was the optimal method because it combines high repeatability with efficient sample purification, facilitating further precise determination of nusinersen metabolites [21].

Figure 1.

Studzińska et al. [4] used dispersive solid-phase extraction (dSPE) with novel AMINO/CARBOXYL adsorbent to isolate nusinersen metabolites from serum samples. The adsorbent surface was modified with aminopropyl groups and aspartic acid. Consequently, terminal amino and carboxyl groups were present. Utilization of this adsorbent allows adsorption and desorption to be controlled by changing the pH (low pH during sample load and basic pH for elution of nusinersen metabolites). For the extraction, the serum sample was first diluted with 10 mM ammonium acetate (pH 4.5) and loaded onto the material. Elution was performed by changing the pH of salt to 9.5. The dSPE method provided high efficiency for the isolation of nusinersen metabolites from both enriched serum extracts and direct extraction from the serum of patients. The technique was simple, fast, and environmentally friendly: without ion-pairing reagents (long conditioning) and organic solvents - this can be considered a green

chemistry technique [4]. However, it required the synthesis of new, specially designed materials.

Hybridization was another method used to extract nusinersen metabolites from serum [26]. It is based on using an adsorbent with an immobilized oligonucleotide of sequence complementary to the target one. Hybridization using modified silica with an immobilized complementary strand allowed selective adsorption of nusinersen analogs, while desorption was carried out in water heated to 80°C. The method may be considered environmentally friendly (green), since it uses only water and aqueous solutions of inorganic salts. However, this method had limitations. Although the method provided high selectivity, its effectiveness was limited in the case of short metabolites of nusinersen due to their poor ability to bind to the capture strand. The method is also expensive due to the need to prepare a sorbent containing strands complementary to the target ONs. In addition, oligonucleotide modifications, such as phosphorothioates or 2'-methoxyethyl groups, negatively affected the recovery efficiency, which ranged from 57% to 60%. Compared to other methods, such as dispersive solid phase extraction (dSPE), hybridization was less effective, especially for short metabolite fragments [4].

To sum up, the nusinersen extraction methods used for serum preparation LLE are highly efficient but require additional purification; hybridization is selective but less effective for short metabolites.dSPE allowed simple and efficient isolation, especially of short metabolites, with high reproducibility [4].

5.2. Extraction of nusinersen metabolites from cerebrospinal fluid

So far, only one paper has been published on the extraction of nusinersen and its metabolites from cerebrospinal fluid [27]. Three methods for preparing cerebrospinal fluid (CSF) samples were applied for nusinersen and its metabolites. The LLE, similarly to serum, was used to remove proteins. Unfortunately, only a few nusinersen metabolites were detected in the extracts after LLE due to their low concentration and the need to concentrate the sample. Therefore, SPE with an Oasis HLB cartridge was additionally used. CSF samples were mixed with a solution of DMBA/HFIP (5 mM/150 mM) and applied to the SPE column previously conditionedwith this solution . The elution was performed using 10% DMBA/HFIP solution and 90% methanol. Following this, the extract was evaporated to dryness, and the residue was dissolved in the DMBA/HFIP solution. This attempt allowed for 40-fold enrichment of the sample and, consequently, detection and identification of a greater number of metabolites compared to LLE [27].

Nusinersen and its metabolites were extracted from cerebrospinal fluid samples using the MEPS, employing SDVB sorbent [27]. The extraction procedure was the same as in the case of SPE, but the volumes of sample and solvents were lower. The samples were then evaporated to dryness, and the residue was dissolved in DMBA/HFIP solution. MEPS was an effective method for the purification of samples in the case of serum. However, determination of nusinersen metabolites in CSF involves larger sample volumes and the use of additional sorbent. Despite the attempt to enhance sample enrichment, nusinersen metabolites remained undetectable. The primary limitations of the method were identified as inadequate sample volume and constrained adsorbent capacity. MEPS was inadequate for metabolite analysis in CSF [27].

It was concluded that using a two-step extraction is unnecessary in the case of CSF. Nevertheless, given the scarcity of CSF metabolites, SPE must substantially enrich theanalyte . The authors emphasize that the developed procedure ensures effective recovery and purification of metabolites. However, further studies are required to enhance the selectivity and enrichment level of the samples to detect a more significant number of metabolites, given that 40-fold enrichment is regarded as inadequate [27].

A summary of the advantages and disadvantages of sample preparation and determination methods is presented in ***2***.

Table 2. Comparison of the advantages and disadvantages of sample preparation and determination methods

HILIC-MS/MS	- low resolution of metabolites; no ion-pair reagents needed; - stable and MS-compatible	- lower sensitivity compared to IP-RP; - adducts from alkali metal
RP-UHPLC-MS	- separation of metabolites lower than for IP-RP but higher compared to HILIC; - increased ionization efficiency; - improved metabolite detection	- reduced sensitivity compared to IP-RP - the presence of adducts at the mass spectra
IP-RP-UHPLC-MS	- the highest separation efficiency for the mixture of metabolites; - the highest sensitivity; - best for the determination of low-concentration of metabolites	- requires ion-pair reagents

6. Liquid chromatography coupled with mass spectrometry as an analytical method for the determination of nusinersen

Liquid chromatography, mainly ultra-high-performance liquid chromatography (UHPLC) coupled with mass spectrometry (MS), is a key technique for analyzing oligonucleotides, allowing their separation, identification, and quantification. The reversed-phase chromatography and ion exchange separate molecules by hydrophobicity or charge, particularly useful in gene therapy and diagnostic research [28]. Nusinersen and its metabolites extracted from biological samples have been analyzed using three UHPLC modes. These were reversed phase (RP), ion pair chromatography (IP-RP), and hydrophilic interaction liquid chromatography (HILIC). Zhang et al. [25] developed a HILIC hyphenated with tandem mass spectrometry (HILIC-MS/MS) method to analyze nusinersen. The XBridge™ Premier BEH Amide column (50 × 2.1 mm, 2.5 µm) was used. The mobile phase consisted of acetonitrile and aqueous ammonium acetate solution, and gradient elution was used. Ammonium acetate was selected because it gave a better peak shape and lower baseline noise compared to ammonium formate. The retention time of nusinersen increased with increasing salt concentration, but simultaneously, MS signal intensity decreased and the limit of quantification (LOQ). As a result, a 20 mM concentration was chosen. Additionally, the observed effect concerns the impact of the pH of salt on nusinersen retention and MS signal intensities. The ionization efficiency was the highest when the mobile phase pH was up to 10, contrary to decreased retention time. The authors suggested that this effect may be attributed to a more significant interaction between nusinersen and ammonium cations than with the amide stationary phase. Moreover, it was pointed out that when the concentration and pH of ammonium acetate are low, only a portion of the phosphorothioate groups in the nusinersen is deprotonated, resulting in a low mass spectrum response. The finally optimized chromatographic method achieved good linearity in the 30-10,000 ng/mL range. In comparison, the lower limit of quantitation (LLOQ) was 30 ng/mL, with intra- and inter-assay precision of 12.3% and 9.9%, respectively. The HILIC-MS/MS method proved reliable and was successfully applied to the pharmacokinetic determination of nusinersen in rabbit plasma [25] it may also be helpful to the study of nusinersen metabolites extracted from serum samples, as shown by Vošáhlová et al. [18]. The same stationary phase was used, namely amide (Acquity Premier BEH Amide, 1.7 µm, 2.1 mm × 50-150 mm). The mobile phase contained a mixture of acetonitrile and 10 mM ammonium acetate (pH 6.8) (gradient elution). These conditions were different compared to Zhang et al. [25] methodology, but some general remarks remain the same: greater sensitivity for ammonium acetate; low pH of mobile phase provides broad peaks and long retention;

separation improved for higher concentrations of buffer; the effect of temperature was negligible. Since the unknown metabolites of nusinersen were studied, a time-of-flight analyzer had to be selected [25]. The developed method allowed for identifying metabolites, which were very short, modified, and structurally similar fragments of nusinersen. For this reason, their separation by HILIC was challenging despite the application of UHPLC. Utilizing a 50 mm column provided the coelution of four metabolites in one peak and three in the other. Changing the column length to 150 mm led to obtaining a more extended analysis time (increased retention) and higher resolution of all 11 metabolites (2-4 mers) extracted from the serum of SMA patients (treated with Spinraza). HILIC proved to be an effective method for the separation and identification of nusinersen metabolites without the need for ion pair reagents application [18]. Nevertheless, HILIC has one significant disadvantage: alkali metals (sodium and potassium) adducts present at the mass spectra of nusinersen and its metabolites [18]. They contribute to the distribution of ion current to a greater number of signals, which decreases the signal intensity and sensitivity in MS detection [18,25]. Zhang et al. [25] pointed out that despite the higher sensitivity of the IP-RP-LC-MS/MS method compared to HILIC-MS/MS, its use is associated with significant problems. In contrast, HILIC-MS/MS has greater stability and compatibility with mass spectrometry, making it a more reliable choice for the analysis of nusinersen if the assay requirements are met [25].

These findings were confirmed in another study devoted to the comparison of three different chromatographic modes: reversed-phase, ion pair, and hydrophilic interaction for the determination of nusinersen metabolites in the serum extracts of patients treated with Spinraza. Octadecyl stationary phase was applied for RP-UHPLC and IP-RP-UHPLC, while silica gel was applied for HILIC. Ammonium formate was used as a mobile phase component in HILIC and RP-UHPLC, while DMBA and 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) were used in IP-RP-HPLC. In the case of RP-UHPLC, some general tips can be collected: the retention of nusinersen metabolites decreases with increased salt pH, but the number of detected metabolites increases (greater ionization efficiency). Surprisingly, similar tendencies were noted for HILIC, but only half of the metabolites were detected compared to RP-UHPLC. Despite eliminating ion pair reagents, this method detected the fewest metabolites, and broad, asymmetric peaks reduced its sensitivity [4]. The most excellent sensitivity was observed for IP-RP-UHPLC, as a result, the greatest number of metabolites were identified. This method allowed the detection of 15 metabolites and achieved MS signal intensities several times higher than the other techniques, making it the most sensitive and suitable for low metabolite concentrations,

355 although it requires the use of ion-pair reagents [4]. The authors suggested that mobile phases
356 buffered with acetic or formic acid salts have limited compatibility with MS, contrary to ion-
357 pair reagents buffered with HFIP [4].

358 A more complex chromatographic study on nusinersen metabolites by IP-RP-UHPLC
359 was presented in another study [21]. The impact of three different alkylamines (DMBA,
360 diisopropylamine - DIPEA, dipropylamine - DPA) and their mixtures with HFIP on the
361 separation of metabolites and their ionization process in MS was presented. The lowest
362 sensitivity was observed for DPA due to ionization suppression. Regarding the application of
363 two other amines, the authors concluded they might be used interchangeably since the
364 sensitivity for nusinersen metabolites is similar. These effects were assigned to the structure of
365 amines: DPA is a secondary amine, while DMBA and DIPEA are tertiary ones (higher MS
366 signals for nusinersen metabolites). Moreover, sensitivity also depends on Henry's constant law,
367 which has the lowest values for DMBA and DIPEA. To sum up, amine structure and Henry's
368 low constant are the two key parameters for consideration when mobile phase composition is
369 selected for IP-RP-UHPLC determination of nusinersen and its metabolites [21]. On the other
370 hand, the separation of metabolites is also important. Consequently, the proper selection of
371 chromatographic columns is crucial. Of the three columns tested, the highest resolution was
372 noticed for octadecyl with a phenyl group, while the lowest for octadecyl with incorporated
373 polar groups, probably due to hydrogen bonding between nusinersen metabolites and polar
374 group in the structure of the stationary phase. Authors suggested that additional aryl group
375 impacts the resolution because of π…π interactions responsible for increasing the retention of
376 nusinersen metabolites. The developed IP-RP-UHPLC Q-TOF-MS method was successfully
377 used for analysis of serum extracts from patients treated with Spinraza (nusinersen) [21].

378 HILIC-MS/MS methods were found to be particularly useful for the simplified
379 identification of impurities and metabolites without the use of ion-pair reagents, although they
380 require further optimization for the separation of short metabolites [18,25]. The highest
381 sensitivity was achieved with IP-RP-HPLC methods. IP-RP-UHPLC methods, on the other
382 hand, are characterized by high precision, and competitive costs, making them a promising tool
383 in pharmacokinetics [4,21]. Further work should focus on combining the advantages of existing
384 approaches - simplifying the analysis while maintaining high sensitivity and efficient separation
385 of metabolites.

7. Metabolites of nusinersen

Methods for the extraction, separation, and determination of nusinersen have been successfully developed and are being used to study its metabolism. These are critical analytical tools and an important research direction.

Fifteen different metabolites were identified in serum, mainly short nusinersen fragments (2-5-mers). These were formed by the activity of 3'-exo- and 5'-endonucleases, which successively shorten both the 3' and 5' ends of the nusinersen oligonucleotide. Results demonstrated a predominance of low-molecular-weight fragments in the serum samples. Longer nusinersen fragments (e.g., 17-, 16-, 15-mers) are likely ineffective in crossing the blood-brain barrier. The low penetration of large molecules and longer metabolites into the blood is due to their relatively high molecular weight and numerous negative charges [4]. Different metabolites were identified in the cerebrospinal fluid (CSF) of children with spinal muscular atrophy (SMA). Ten 3’ and 5’ short mers of nusinersen formed due to 3' and 5' endonuclease activity (gradual degradation of phosphodiester bonds between nucleotides). The detected compounds included fragments truncated from both the 3' end (e.g., 3'N-8) and 5' end (e.g., 5'N-10, 5'N-6), as well as metabolites truncated from both ends simultaneously (e.g., 5'N-5 + 3'N-1). Furthermore, depurination and depyrimidination products were detected, indicating alternative degradation pathways. The metabolism of nusinersen is probably more active in the bloodstream, while in the central nervous system, it may be slower or lead to the accumulation of the active form of the drug [27].

8. Conclusion and perspectives

The development and implementation of a new therapy for treating (SMA) represents progress in medicine, potentially increasing the quality of life for patients markedly. The treatment inhibits the disease's progression and simplifies the development of vital functions in patients, including movement, respiration, and swallowing. However, administering a drug to the patient's body is associated with its metabolism, making monitoring the body's metabolism necessary. The metabolic processes may vary depending on the individual predispositions of patients. Therefore, developing new methods for purifying biological samples and improving analytical techniques to facilitate accurate pharmacokinetics and pharmacodynamics assessment is challenging. LLE is the most commonly used technique, providing high recovery of oligonucleotides. However, it requires additional purification steps, and the matrix effect may affect its efficiency. SPE is a method that eliminates interferences from the sample matrix, thereby increasing the recovery efficiency. It should be noted that the efficiency of SPE

decreases at low analyte concentrations. MEPS is a more efficient method with high repeatability and recoveries. Dispersive dSPE utilizing novel adsorbents offers simplicity, ecological characteristics, and high efficiency, particularly in the case of short metabolites, though it necessitates the synthesis of specialized materials. Developing novel materials is an ongoing process, with continuous improvement necessary to recover the substance from the biological sample completely. Alternatively, the hybridization method, based on selective adsorption using complementary oligonucleotides, provides high selectivity. Still, its effectiveness is limited in the case of short or modified metabolites and is additionally associated with high costs. Studies have demonstrated that there is no requirement for a two-step extraction process in the case of cerebrospinal fluid, but significant enrichment of samples is crucial. The methods employed, including RP and IP-RP, HILIC, facilitate separation through diverse mechanisms. Albeit HILIC-MS/MS is less sensitive, it is effective in the simplified identification of metabolites and impurities. It is important to note that optimization is required for short metabolites. Although, IP-RP-UHPLC-MS attains the highest sensitivity and resolution, becoming the favoured method in pharmacokinetic and metabolomic analysis, despite the necessity for ion pair reagents. Studies indicate the key role of pH, mobile phase composition, and column parameters in method optimization, enabling better separation and detection of nusinersen metabolites. Any of these factors may serve as a foundation for further studies. Another development pathway is the synthesis of new materials that improve the selectivity of nusinersen adsorption. The growing interest in developing green extraction methods that reduce the use of harmful solvents highlights the need to establish new procedures. Although techniques such as IP-RP-UHPLC-MS are already quite effective, additional efforts should focus on increasing their sensitivity and resolution. Here, once again, the synthesis and application of new stationary phases is interesting and promising direction of further studies.

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Author Contributions

Conceptualization – S.S.; writing - original draft preparation, K.O., S.S.; writing - review and editing, K.O., N.B., S.S. All authors have read and agreed to the published version of the manuscript.

References

[1] J.W. Day, K. Howell, A. Place, K. Long, J. Rossello, N. Kertesz, G. Nomikos, Advances and limitations for the treatment of spinal muscular atrophy, BMC Pediatr 22 (2022). https://doi.org/10.1186/s12887-022-03671-x.

[2] L. Belter, J. Jarecki, S.P. Reyna, R. Cruz, C.C. Jones, M. Schroth, C.M.C. O'Toole, S. O'Brien, S.A. Hall, N.B. Johnson, A.D. Paradis, The Cure SMA Membership Surveys: Highlights of Key Demographic and Clinical Characteristics of Individuals with Spinal Muscular Atrophy, J Neuromuscul Dis 8 (2021) 109–123. https://doi.org/10.3233/JND-200563.

[3] D.C. Schorling, A. Pechmann, J. Kirschner, Advances in Treatment of Spinal Muscular Atrophy - New Phenotypes, New Challenges, New Implications for Care, J Neuromuscul Dis 7 (2020) 1–13. https://doi.org/10.3233/JND-190424.

[4] S. Studzińska, J. Szymarek, M. Mazurkiewicz-Bełdzińska, Improvement of serum sample preparation and chromatographic analysis of nusinersen used for the treatment of spinal muscular atrophy, Talanta 267 (2024). https://doi.org/10.1016/j.talanta.2023.125173.

[5] L. Unger, A. Ronco-Campaña, P. Kitchen, R.M. Bill, A.J. Rothnie, Biological insights from SMA-extracted proteins, Biochem Soc Trans 49 (2021) 1349–1359. https://doi.org/10.1042/BST20201067.

[6] I. Welian, K. Gendek, I. Jastrzębska, K. Maliszewska, M. Chrościńska-Krawczyk, SMA Therapy in Poland: New Hopes and Challenges, Journal of Education, Health and Sport 23 (2023) 28–34. https://doi.org/10.12775/jehs.2023.23.01.003.

[7] A. Roos, L.I. Schmitt, C. Hansmann, S. Hezel, S. Salmanian, A. Hentschel, N. Meyer, A. Della Marina, H. Kölbel, C. Kleinschnitz, U. Schara-Schmidt, M. Leo, T. Hagenacker, Alteration of LARGE1 abundance in patients and a mouse model of 5q-associated spinal muscular atrophy, Acta Neuropathol 147 (2024). https://doi.org/10.1007/s00401-024-02709-x.

[8] L.D. Brollier, M. Matuszczak, T. Marri, J.G. Carbajal, A.T. Moorman, E.M. Sorial, R. Jain, Anesthetic management of pediatric patients undergoing intrathecal nusinersen administration for treatment of spinal muscular atrophy: A single-center experience, Paediatr Anaesth 31 (2021) 160–166. https://doi.org/10.1111/pan.13964.

[9] I. Welian, K. Gendek, I. Jastrzębska, K. Maliszewska, M. Chrościńska-Krawczyk, SMA Therapy in Poland: New Hopes and Challenges, Journal of Education, Health and Sport 23 (2023) 28–34. https://doi.org/10.12775/jehs.2023.23.01.003.

[10] I.E.C. Verhaart, A. Robertson, I.J. Wilson, A. Aartsma-Rus, S. Cameron, C.C. Jones, S.F. Cook, H. Lochmüller, Prevalence, incidence and carrier frequency of 5q-linked spinal muscular atrophy - A literature review, Orphanet J Rare Dis 12 (2017). https://doi.org/10.1186/s13023-017-0671-8.

[11] R.N. Singh, N.N. Singh, Mechanism of splicing regulation of spinal muscular atrophy genes, in: Adv Neurobiol, Springer New York LLC, 2018: pp. 31–61. https://doi.org/10.1007/978-3-319-89689-2_2.

[12] U.S. Haque, T. Yokota, Recent Progress in Gene-Targeting Therapies for Spinal Muscular Atrophy: Promises and Challenges, Genes (Basel) 15 (2024). https://doi.org/10.3390/genes15080999.

[13] T.H. Chen, New and developing therapies in spinal muscular atrophy: From genotype to phenotype to treatment and where do we stand?, Int J Mol Sci 21 (2020). https://doi.org/10.3390/ijms21093297.

[14] J. Kakazu, N.L. Walker, K.C. Babin, K.A. Trettin, C. Lee, P.B. Sutker, A.M. Kaye, A.D. Kaye, Risdiplam for the Use of Spinal Muscular Atrophy, Orthop Rev (Pavia) 13 (2021). https://doi.org/10.52965/001C.25579.

[15] H. Kletzl, Y. Cleary, P. Grimsey, M. Gerber, R.S. Scalco, Risdiplam▼: pharmacokinetic, pharmacodynamic, safety and efficacy exposure response analyses , 2022. https://www.roche.com/media/releases/med-cor-2022-05-31.

[16] A. Kilanowska, S. Studzińska, In vivoandin vitrostudies of antisense oligonucleotides - a review, RSC Adv 10 (2020) 34501–34516. https://doi.org/10.1039/d0ra04978f.

[17] S. Studzińska, R. Rola, B. Buszewski, The impact of ion-pairing reagents on the selectivity and sensitivity in the analysis of modified oligonucleotides in serum samples by liquid chromatography coupled with tandem mass spectrometry, J Pharm Biomed Anal 138 (2017) 146–152. https://doi.org/10.1016/j.jpba.2017.02.014.

[18] Z. Vosáhlová, K. Kalíková, M. Gilar, J. Szymarek, M. Mazurkiewicz-Bełdzińska, S. Studzińska, Hydrophilic interaction liquid chromatography with mass spectrometry for the separation and identification of antisense oligonucleotides impurities and nusinersen metabolites, J Chromatogr A 1713 (2024). https://doi.org/10.1016/j.chroma.2023.464535.

[19] P.A. Lobue, M. Jora, B. Addepalli, P.A. Limbach, Oligonucleotide analysis by hydrophilic interaction liquid chromatography-mass spectrometry in the absence of ion-pair reagents, J Chromatogr A 1595 (2019) 39–48. https://doi.org/10.1016/j.chroma.2019.02.016.

[20] Committee for Medicinal Products for Human Use (CHMP) Assessment report: Spinraza (nusinersen). European Medicines Agency; 2021 April 17. Procedure No. EMA/289068/2017.

[21] S. Studzińska, M. Mazurkiewicz-Bełdzińska, B. Buszewski, Development of the Method for Nusinersen and Its Metabolites Identification in the Serum Samples of Children Treated with Spinraza for Spinal Muscular Atrophy, Int J Mol Sci 23 (2022). https://doi.org/10.3390/ijms231710166.

[22] B. Cavaloiu, I.E. Simina, C. Vilciu, I.A. Trăilă, M. Puiu, Nusinersen Improves Motor Function in Type 2 and 3 Spinal Muscular Atrophy Patients across Time, Biomedicines 12 (2024). https://doi.org/10.3390/biomedicines12081782.

[23] Z.J. Lin, W. Li, G. Dai, Application of LC-MS for quantitative analysis and metabolite identification of therapeutic oligonucleotides, J Pharm Biomed Anal 44 (2007) 330–341. https://doi.org/10.1016/j.jpba.2007.01.042.

[24] Ł. Nuckowski, A. Kaczmarkiewicz, S. Studzińska, Review on sample preparation methods for oligonucleotides analysis by liquid chromatography, J Chromatogr B Analyt Technol Biomed Life Sci 1090 (2018) 90–100. https://doi.org/10.1016/j.jchromb.2018.05.025.

[25] X. Zhang, C. Sha, W. Zhang, F. Zhao, M. Zhu, G. Leng, W. Liu, Development and validation of an HILIC/MS/MS method for determination of nusinersen in rabbit plasma, Heliyon 10 (2024). https://doi.org/10.1016/j.heliyon.2024.e31213.

[26] S. Studzińska, J. Szymarek, M. Mazurkiewicz-Bełdzińska, Improvement of serum sample preparation and chromatographic analysis of nusinersen used for the treatment of spinal muscular atrophy, Talanta 267 (2024). https://doi.org/10.1016/j.talanta.2023.125173.

[27] S. Studzińska, O. Błachowicz, S. Bocian, O. Kalisz, A. Jaworska, J. Szymarek, M. Mazurkiewicz-Bełdzińska, Study of nusinersen metabolites in the cerebrospinal fluid of children with spinal muscular atrophy using ultra-high-performance liquid chromatography coupled with quadrupole-time-of-flight mass spectrometry, Analyst 149 (2024) 3739–3746. https://doi.org/10.1039/d4an00436a.

[28] D.R. Scoles, E. V. Minikel, S.M. Pulst, Antisense oligonucleotides: A primer, Neurol Genet 5 (2019). https://doi.org/10.1212/NXG.0000000000000323.

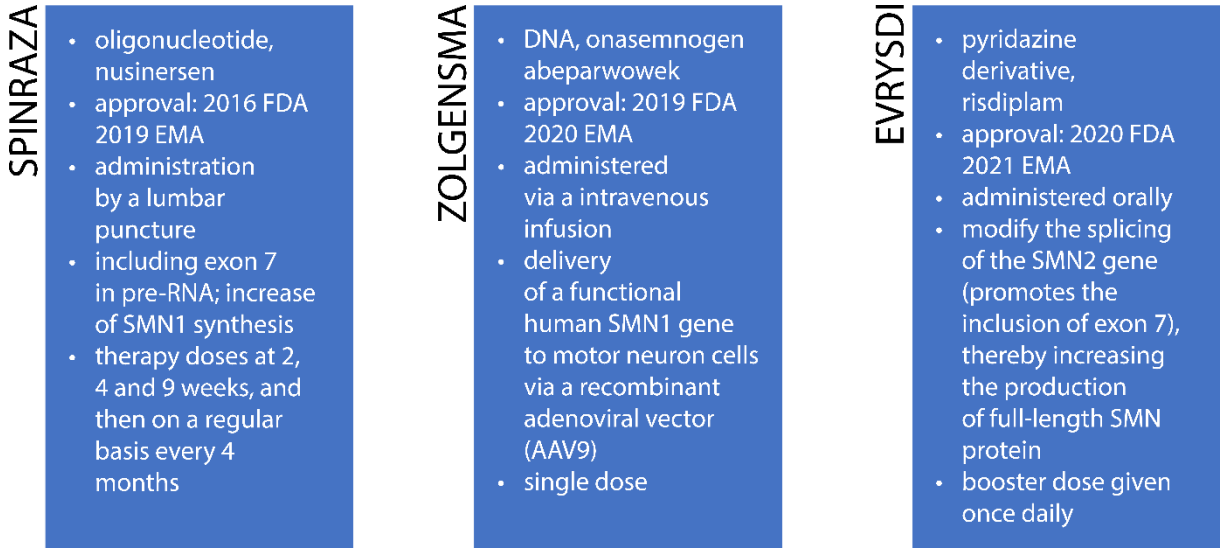


Figure 2. The concise overview of the drugs employed in SMA therapy (Spinraza [1,8,11], Zolgensma [1,12,13], Evrysdi [14,15]Including their active substances, years of market introduction, administration methods, mechanisms of action, and recommended dosages. All three SMA drugs received regulatory approval, but they are also the subject of ongoing clinical studies, which aim to refine their application, explore new patient populations, and improve understanding of their long-term outcomes.

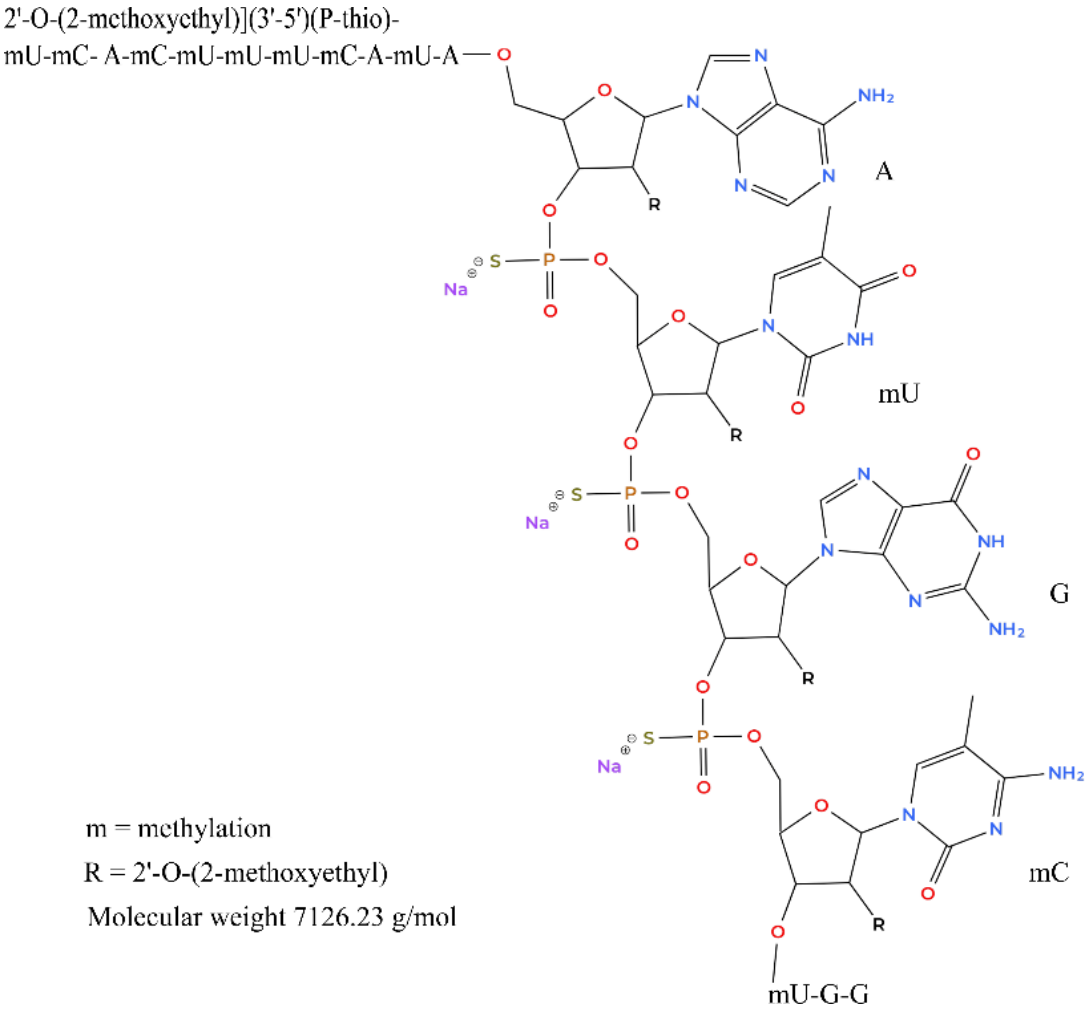
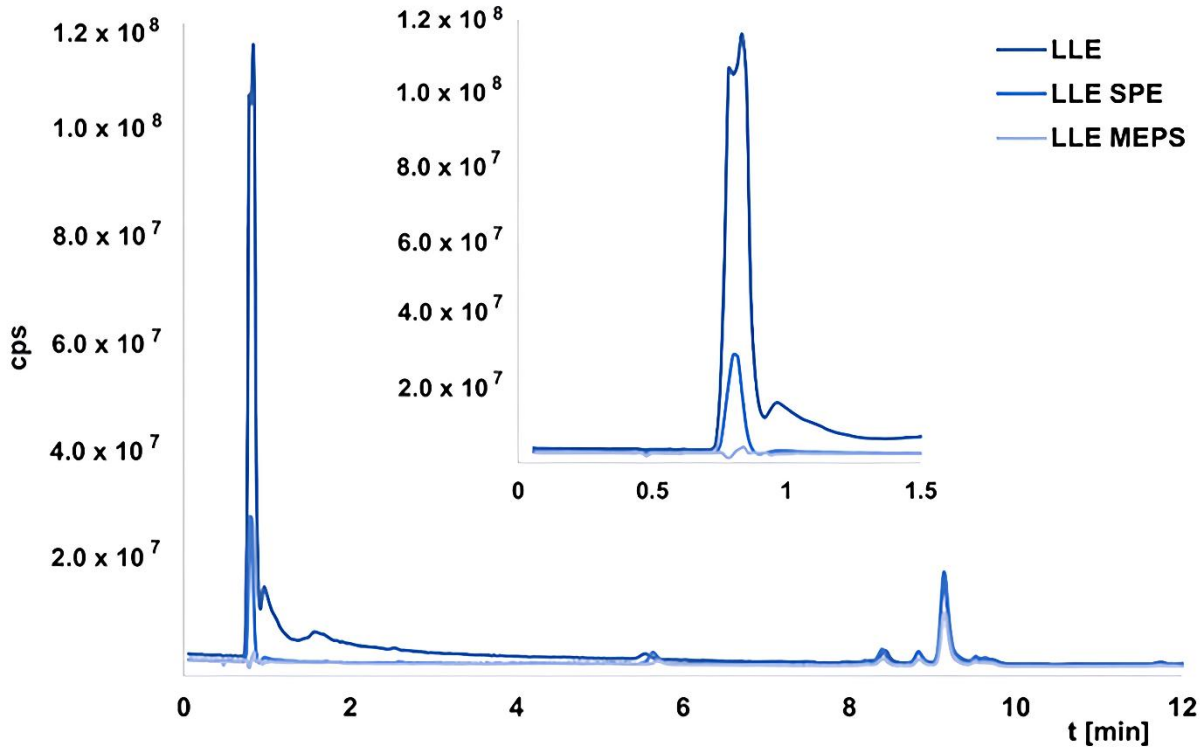


Figure 3. Fragment of the structure of nusinersen (Spinraza).



579

580 **Figure 4.** TIC chromatograms. They refer to serum extracts obtained using three extraction
581 methods: LLE, LLE followed by SPE, and LLE followed by MEPS (for SDVB). [Reprinted
582 from International Journal of Molecular Sciences, Vol 23(17):10166; S. Studzińska,
583 M. Mazurkiewicz-Bełdzińska, B. Buszewski, "Development of the Method for Nusinersen and
584 Its Metabolites Identification in the Serum Samples of Children Treated with Spinraza for
585 Spinal Muscular Atrophy," Copyright (2022), under Creative Commons Attribution:
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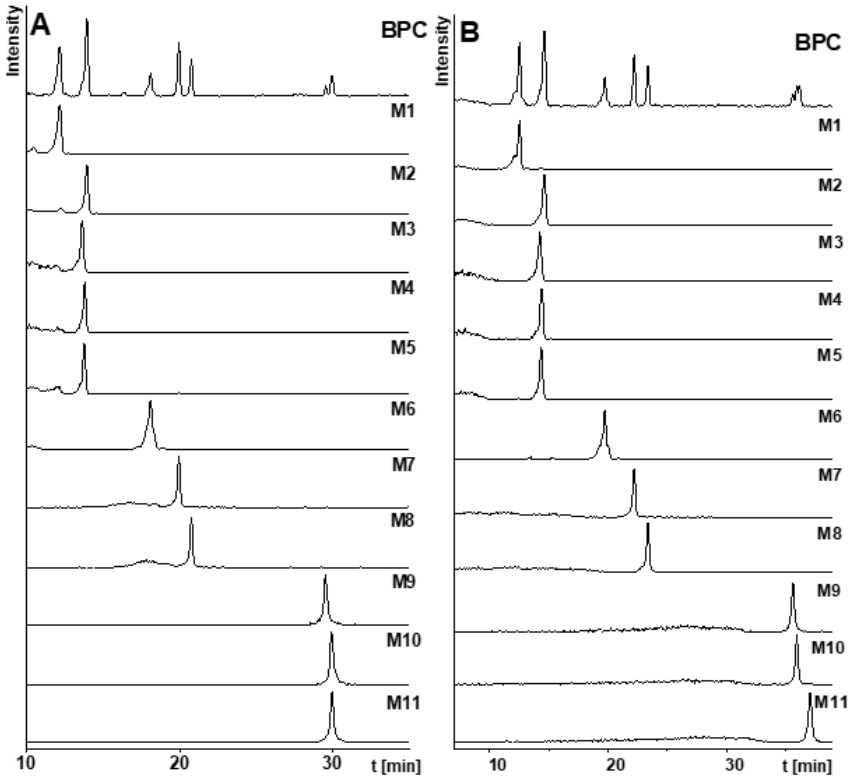

588

589 **Figure 5.** Base peak chromatograms and extracted ion chromatograms for metabolites extracted
590 from serum samples of an SMA patient treated with nusinersen. (A) gradient elution program
591 0–1 min 100% MP A, 1–30 min 100–85% MP A (20 min re-equilibration); (B) 0–1 min 100%
592 MP A, 1–60 min 100–80% MP A (20 min re-equilibration). [Reprinted from Journal of
593 Chromatography A, Vol 1713 / 464535; Z.Vosáhlová, K. Kalíková, M. Gilar, J.Szymarek,
594 M. Mazurkiewicz-Bełdzińska, S. Studzińska, "Hydrophilic interaction liquid chromatography
595 with mass spectrometry for the separation and identification of antisense oligonucleotides
596 impurities and nusinersen metabolites," Copyright (2024), with permission from Elsevier.
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