Short review on currently used sample preparation and determination methods of risdiplam

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Abstract

Risdiplam is a new therapeutic agent developed to treat spinal muscular atrophy (SMA), a genetic neurodegenerative disease caused by mutations in the SMN1 gene. Unlike previous invasive therapies, risdiplam offers the advantage of oral administration, significantly improving patient comfort and accessibility. The review provides information on an SMA historical overview, breakthrough therapies of the development, and design of the methods used to treat SMA. We then focus on its structure and physicochemical properties. The analysis of risdiplam concentrates on developing improved analytical methods for the precise quantification of risdiplam and its metabolites by high-performance liquid chromatography with mass spectrometry in biological samples using octadecyl stationary phases. For sample preparation, only the protein precipitation method was used. Challenges associated with the risdiplam analytics include developing a highly sensitive and selective method in biological matrices and dealing with potential interferences from the biological matrix. Future research should focus on improving analytical methods, investigating metabolite activity, and expanding our knowledge of its long-term effects.

Keywords: risdiplam; spinal muscular atrophy; liquid chromatography; mass spectrometry; protein precipitation

Introduction

Spinal muscular atrophy is a rare genetic disease characterized by the progressive loss of motor neurons in the spinal cord, leading to muscle weakness and atrophy(1). This autosomal recessive disease is caused by mutations in the SMN1 gene, which encodes a motor neuron survival protein (SMN) essential for the health and function of motor neurons. Although the second gene, SMN2, also produces the SMN protein, it is not fully functional. SMA affects approximately 1 in 10,000 live births and remains the leading cause of infant mortality in genetic diseases. Previously, treatment options were limited to symptomatic treatment aimed at prolonging life and alleviating complications (1,2). Over the past decade, there has been a breakthrough in disease-modifying therapies that target the genetic mechanisms underlying SMA. These currently include nusinersen (Spinraza) (3), onasemnogen abeparvovec (Zolgensma) (4), and the most recent drug risdiplam (Evrysdi) (Figure 1). Risdiplam represents a significant advance in treating SMA due to its oral formulation, which offers greater convenience and accessibility compared to invasive therapies. Approved by the European Medicines Agency (EMA) in 2020 (Figure 1), risdiplam is the first SMA drug to be administered at home (5,6)., Unlike other SMA therapies, risdiplam is mainly metabolized in the liver by cytochrome P450 enzymes and flavin-containing monooxygenases. These enzymes facilitate oxidative metabolism, producing excreted metabolites in the feces and urine (7–9). The bioavailability of the drug and its ability to cross the blood-brain barrier make it uniquely effective in combating the multifaceted symptoms of SMA (10). Determining the concentration of risdiplam in biological samples from patients is crucial for assessing the efficacy of therapy and adjusting dosage. For this purpose, liquid chromatography coupled to mass spectrometry (LC-MS) is used, due to its high sensitivity and selectivity for the determination of risidplam and its metabolites (9).

This review focuses on the risdiplam molecule, sample preparation methods used for risdiplam extraction, and application of liquid chromatography coupled with mass spectrometry to analyze risdiplam and its metabolites. To date, there has been no such literature review. This one is the first to provide the reader with a structured and condensed knowledge of the structure of risdiplam and its metabolites, the available method of extraction, and the conditions of analysis. Knowledge of risdiplam is fundamental given the ongoing research and clinical monitoring that will guarantee the safety and efficacy of the treatment of patients with spinal muscular atrophy.

Risdiplam as an active substance of Evrysdi drug

Risdiplam is the third drug approved in 2020 (Figure 1). It was developed for disease-modifying treatment and is available as an oral syrup once daily (6). Risdiplam is a pyridine derivative designed to modify the splicing of SMN2 pre-mRNA and increase the functional levels of SMN protein (5). Risdiplam significantly improved motor function across SMA type 1, 2, and 3 groups, with improvements observed even after 24 months of treatment. It demonstrated good tolerance among patients and a favourable benefit-risk profile. The oral administration method offers a convenient and functional treatment option (6). Therapy that can be successfully performed at home is less burdensome than therapies that require regular, invasive procedures performed by trained and specialized medical personnel in a hospital. Based on current knowledge and ongoing research, risdiplam is approved for patients over two months with SMA Types 1, 2, or 3 or presymptomatic patients with no more than four copies of the SMN2 gene. Risdiplam is an active ingredient of Evrysdi. Evrysdi is a product ready to be administered to the patient in the form of a powder for an oral solution containing 0.75 mg/ml of risdiplam as an active substance. The excipients in the syrup are: isomalt (E953), mannitol (E421), strawberry flavour, sodium benzoate (E211) as a preservative to prevent the growth and degradation of microorganisms, sucralose, ascorbic acid (E300) as an antioxidant, tartaric acid (E334), macrogol/polyethylene glycol 6000 and disodium edetate dihydrate as a stabilizer (9).

Risdiplam molecule

Risdiplam is a pyridazine derivative, a chemical compound containing an aromatic ring with two nitrogen atoms in positions 1 and 2. The full chemical name is 7-(4,7-diazaspiro[2,5]octan-7-yl)-2-(2,8-dimethylmidazo[1,2-b]pyridazine-6-yl)-4H-pyrido [1,2-a] pyrymidin-4-one) with a molecular formula of $C_{22}H_{23}N_7O$ and a molecular weight of 401.46 g/mol (9). Its structure is illustrated in Figure 2 (11). Risdiplam is a non-hygroscopic, yellow, crystalline powder (9). It is weak acid with pKa = 9.41 ± 0.20 (2). It exhibits pH-dependent solubility, dissolving at pH 1 and transforming into insoluble at neutral pH (Figure 2). Risdiplam in an acidic environment with pH<7 can potentially attach protons; protonation occurs mainly in functional groups having nitrogen atoms that can act as bases. Different protonation sites in risdiplam can be active depending on the pH of the environment, primarily nitrogen atmos (Figure 2). In a strongly acidic environment, several nitrogen atoms can undergo protonation simultaneously (2).

Risdiplam is photostable only in the solid (not in solution). Therefore, it is necessary to store it in amber polypropylene vials. The solubility of this compound in water depends on pH (dissolves at pH=1 and transforms into an insoluble form at neutral pH) (9). This compound dissolves well in organic solvents such as ethanol, methanol, DMSO, and water acidified with 0.1% formic acid, while it is not soluble in acetonitrile and non-polar solvents. It exhibits a shelf life of 24 months at 30 ºC and 75% humidity, and 72h at -20 ºC, 4 ºC and 25 ºC. Its UV absorption occurs between 240 and 400 nm with maximum absorbance at 290 nm (2).

Sample preparation methods used for risdiplam extraction

Therapeutic drug monitoring is a process of monitoring drug concentrations in blood and other body fluids to adjust the drug dose to optimize the patient's individual needs (2,8). Pharmacokinetic analysis is based on the determination of the concentration of the active substance of a drug in the biological matrix (blood, plasma, serum, urine, cerebrospinal fluid, saliva) (2,8,9). Risdiplam should be isolated from biological samples to obtain the correct pharmacokinetic model of the drug and to study its metabolites. It is necessary to isolate risdiplam from the substances that are part of the matrix and from other pharmaceutical preparations administered to patients, which could affect and interfere with the results of the tests. Currently, extraction of risdiplam is based mainly on the removal of proteins by precipitation using an organic solvent such as methanol, acetonitrile, or ethanol (2). The organic solvents displace the hydration water envelope surrounding a protein. Consequently, protein denaturation may be observed as a reduction of its solubility, and therefore, a precipitate is formed in the sample. Risdiplam is soluble in most organic solvents and invariably remains in the solution (12). Generally, protein precipitation is the only method used so far for the sample preparation of risdiplam.

Protein precipitation with ice-cold acetonitrile (1:1) was used during an in vitro risdiplam metabolism study after incubation with human liver microsomes (HLMs) suspension. Except for acetonitrile, ethanol/acetonitrile mixtures may also be successfully used to remove high molecular weight compounds from biological samples to analyze risdiplam (8,13). Protein precipitation may also be used in vivo studies to isolate risdiplam from human plasma samples. In a comprehensive study, the effects of both the type of organic solvent (methanol, acetonitrile), sample volume (400-1000µl), freezing time (0-15min), and ultrasound time (0-10min) on the extraction efficiency of risdiplam from plasma was tested. Extraction

efficiencies were compared by comparing risdiplam peak areas on chromatograms for the extracts. The results showed that the application of methanol offers greater efficiency compared to acetonitrile. On the other hand, freezing and precipitation time also affected risdiplam extraction efficiency. The highest recoveries were obtained for 1000 µL of methanol with an ultrasound time of 5 minutes without freezing. The sample preparation was simple, using readily available precipitants and a short processing time (2).

Application of liquid chromatography coupled with mass spectrometry to the analysis of risdiplam and its metabolites

Due to the fact that Evrysdi has been used for 4 years, the number of scientific publications presenting the results of chromatographic analysis of risdiplam in biological samples is still limited. To our knowledge, there are 3 papers (2,7,13). There is a limited amount of information on preparing risdiplam standard solution for chromatographic analysis. Based on its solubility, stock solutions may be prepared by dissolving the standard in using dimethyl sulfoxide. Next, the working solutions of risdiplam may be prepared by diluting stock solutions in methanol. On the other hand, risdiplam can be dissolved in water acidified with formic acid, and working solutions can be prepared by diluting the stock solution with water (2).

During the precipitation process, which is the initial preparation of the sample for analysis, risdiplam may be lost through adsorption onto the walls of the vial. To eliminate this problem, the sample is recommended to use LoBind polypropylene amber vials. To conduct a more accurate risdiplam analysis, it is recommended that column debris be removed with an optimized gradient, rinse time, and increased flow rate, as well as regular rinsing of the autosampler. To improve work with risdiplam samples, it is recommended to abandon the randomization of samples, use blanks after high concentrations, monitor the course of each sample, and repeat it (14).

Risidplam is typically analyzed using ultra high-performance liquid chromatography (UHPLC) in reversed-phase mode. To date, each research has used a non-polar octadecyl stationary phase with a particle size lower than 2 µm. As for detection, tandem mass spectrometry in positive ionization mode is used. The mobile phase consisted of a mixture of water and acetonitrile. Methanol was also tested, but it resulted in higher pressure in the system for the same flow rate. The chromatographic columns play a key role during chromatographic analysis therefore, a comprehensive comparative study on the analysis of risdiplam with three

different octadecyl (C18) columns (Kinetex XB C18; Agilent SB C18, Agilent Eclipse plus C18) provided interesting results in terms of peak shape. The greatest symmetry and the shortest retention time of risdiplam was observed for the Kinetex XB C18 column (50 × 3 mm, 2.6 μm). The method was easy to use, rapid, and effective in analyzing risdiplam concentrations in extracts from blood samples from SMA patients. The authors of the publication determined risdiplam concentrations in human blood serum extracted from six patients. The detected blood concentrations of risdiplam showed high variability despite administering identical doses. The analysis time was short, with a retention time for risdiplam of 2.02 min and the internal standard of 2.81 min. Furthermore, the method also showed satisfactory accuracy, precision, reproducibility, and high linearity in the range of 1.95-125.00 ng/ml (2).

The liquid chromatography-tandem mass spectrometry (LC-MS/MS) was also used for the determination of concentrations of risdiplam and its metabolites M1, M2, M5, M7, M9, M10, M18, and M26 (Figure 3) in plasma and tissue samples. Acceptable linearity, precision, accuracy, and specificity of risdiplam tested were observed in the range of 0.5 to 2500 ng/ml in plasma or 0.100 to 100 ng/ml in cerebrospinal fluid of monkeys (9).

LC-MS/MS was used also for the non-clinical absorption, distribution, metabolism, and excretion (ADME) profile for risdiplam. Through the application of this strategy, the clinical development of risdiplam was accelerated, and drug regulatory requirements were fulfilled. Once again, a C18 column (200 × 2 mm, 2.7 μm) was used at each study stage. However, the column was kept at a high temperature of 70 °C. The mobile phase consisted of water with the addition of 0.5% formic acid and methanol (gradient elution). It should be noted that using a long column ensured good resolution, which is particularly important in identifying unknown metabolites. On the other hand, however, the analysis times were long (risdiplam eluted at 18 minutes and some of the metabolites at 35 minutes) (7). This method was also applied for the quantitative determination of risdiplam during in vitro metabolism studies using human liver microsomes, hepatocytes from humans, rats, cynomolgus monkeys, mice, and rabbits and using recombinant human P450 enzymes. The chromatographic method with an Orbitrap detector was applied for an in vivo metabolism study in healthy male individuals following a single oral dose. Figure 3 presents the identified circulating bloodstream metabolites (7). All the results obtained during the research prove that the use of reversed-phase HPLC coupled with mass spectrometry is a handy tool for studying risdiplam and its metabolites in vitro and in vivo (7).

Another study provided very similar results during the in vivo study of risdiplam after administration of 0.6 mg to healthy volunteers. Plasma and urine samples were collected and

risdiplam was successfully quantified using UHPLC-MS/MS. The limit of quantification (LOQ) was equal to 0.250 ng/ml for the calibration range 0.250-250 ng/ml. The precision was good and lower than 6.6% for plasma and ≤ 6.5% for urine. Surprisingly, however, the authors do not provide information on the analysis conditions, such as the composition of the mobile phase or the type of stationary phase. Nonetheless, based on the results indicated in this study, it can be concluded that the application of LC-MS/MS in risdiplam analytics is wide (8).

RP HPLC with C18 column was also used during pharmacokinetic studies of risdiplam with adult subjects aged 18-70 years. Nevertheless, the mobile phase differed compared to other studies, since it was composed of aqueous ammonium carbonate, acetonitrile, 2-propanol and acetone. Still, it was compatible with MS/MS, and risdiplam was determined in positive ion multiple reaction monitoring mode. Both the precision and accuracy were at a good level (13).

Metabolites of risdiplam

Risdiplam is well absorbed from the gastrointestinal tract and widely distributed in the body, including the central nervous system, which is crucial for protecting motor neurons. Risdiplam is metabolized by human liver, kidney, and intestinal microsomes, but mainly in the liver by cytochrome P450 enzymes, particularly CYP3A4, and by the flavin-containing monooxygenases (FMOs) FMO1 and FMO3 (7). Unlike cytochrome P, FMOs do not readily react with other chemicals and produce reaction products that are readily excreted, thus less likely to cause potentially harmful side effects (8). The main metabolic pathway is oxidative metabolism at the cyclopropyl and piperazine moiety. The metabolite M1 is the only exception since hydroxylation at one of the nitrogen atoms occurs. Metabolic processes lead to the formation of metabolites that are subsequently excreted from the body. Risdiplam is excreted mainly in feces (approximately 53%) and, to a lesser extent, in urine. The most common primary metabolites of risdiplam are M1, M2, and M5 (Figure 3). The metabolite M1 (Figure 4)has been identified as the primary metabolite of risdiplam and can be generated by both FMO1 and FMO3, as well as by CYP. Participation of FMO enzymes contributed more (>60%) than by CYP enzymes (<10%). There are four low-concentration metabolites (M2, M7, M9 and M26) (Figure 3) formed by biotransformation of the piperazine group and the carboxylic acid metabolite M10 (Figure 3), as well as a number of very low-concentration metabolites (7–9).

Future perspectives and conclusions

Risdiplam has contributed to significant developments in the treatment of SMA and is a non-invasive and convenient therapy for the patient. Future studies should focus on finding analytical methods that will improve the accurate and reproducible determination of risdiplam in samples from SMA patients. Further interdisciplinary research on risdiplam will be crucial to understanding its full potential and addressing existing challenges in SMA therapy. Future research should focus on optimizing the methods used in high-performance liquid chromatography applied to the analysis of risdiplam. The focus should be on testing a more significant number of stationary phases, which will likely increase the resolution of mixtures of risdiplam and its metabolites (Figure 5). On the other hand, it is also essential to use. In addition, other sample preparation methods should be developed for the additional purification extracts obtained after protein purification. They are needed to efficiently isolate risdiplam and its metabolites while minimizing interference from the biological matrix.

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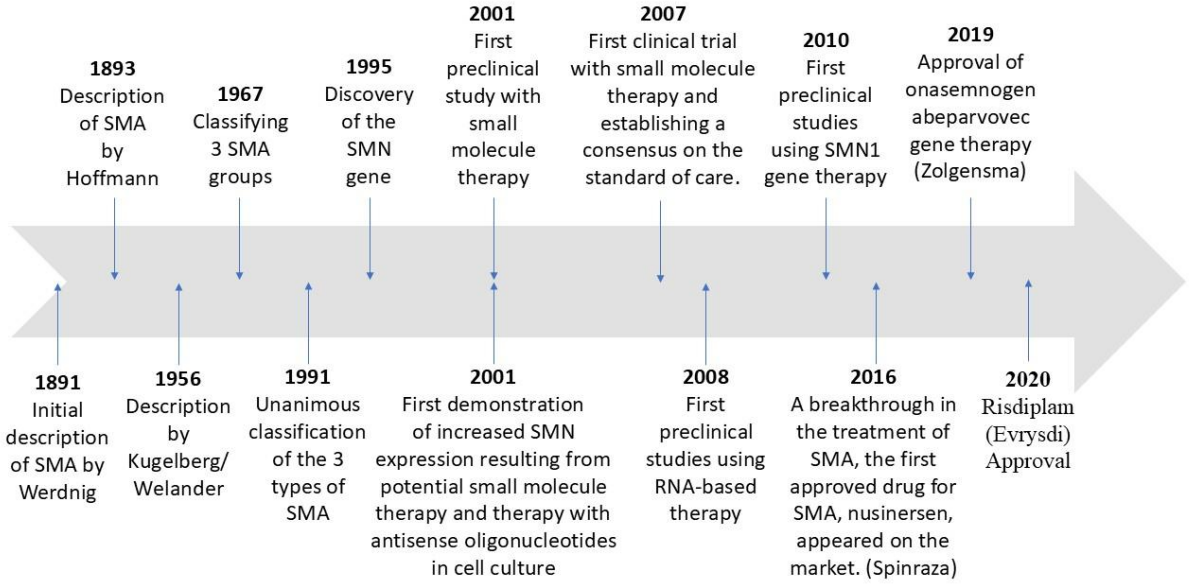

Figure 1. Historical overview of SMA (1–9,15–22).

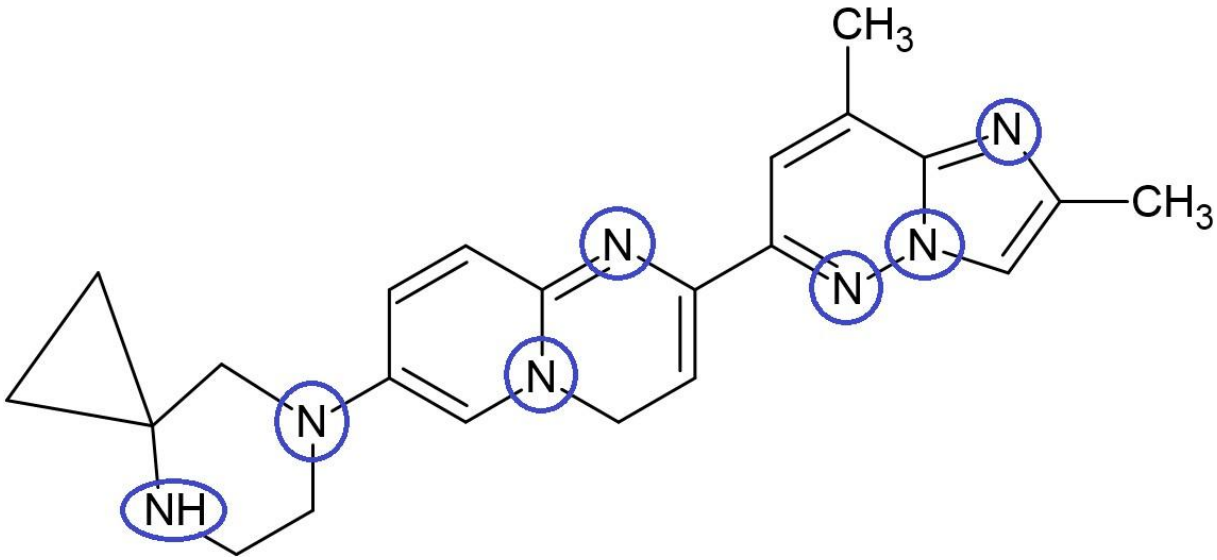

Figure 2. Risdiplam structure.

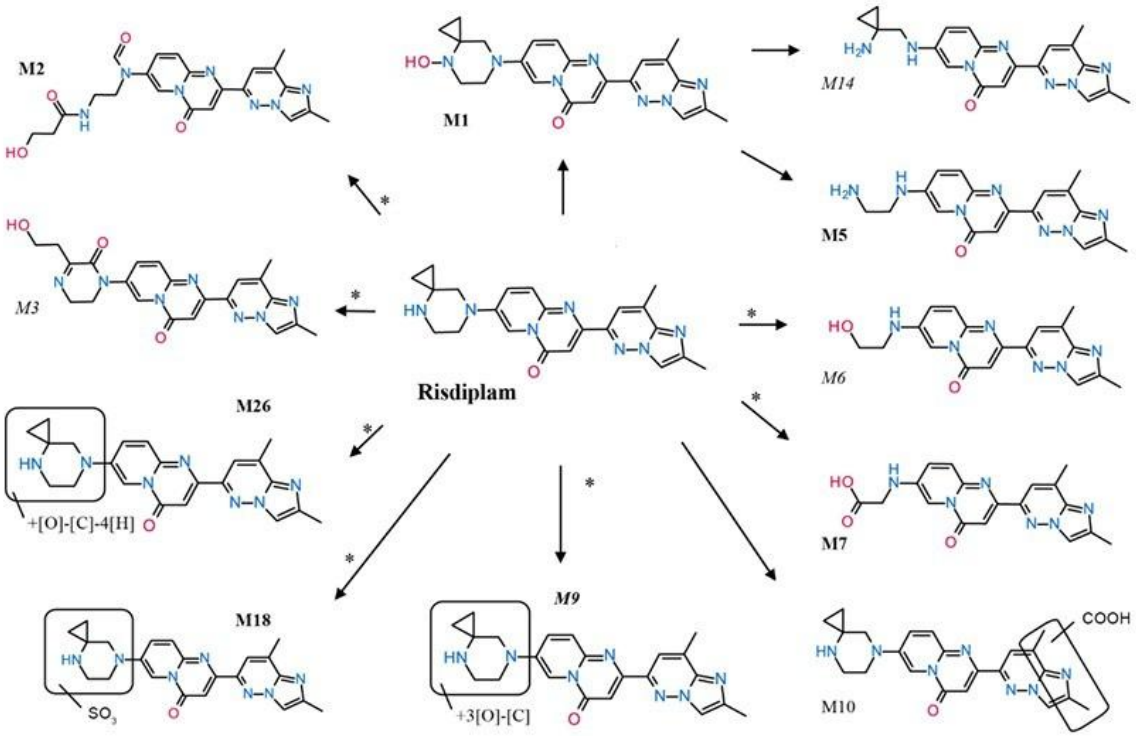

Figure 3. A review of the major metabolite structures of risdiplam identified in a healthy male and in vitro after a single oral dose of 18 mg. [Reprinted from Stephen Fowler, Andreas Brink, Yumi Cleary, Andreas Gunther, Katja Heinig, Christophe Husser, Heidemarie Kletzl, Nicole Kratochwil, Lutz Mueller, Mark Savage, Cordula Stillhart, Dietrich Tuerck, Mohammed Ullah, Kenichi Umehara, and Agnes Poirier, Addressing Today's Absorption, Distribution, Metabolism, and Excretion (ADME) Challenges in the Translation of In Vitro ADME Characteristics to Humans: A Case Study of the SMN2 mRNA Splicing Modifier Risdiplam, Drug Metabolism and Disposition, 2022, 50, 65-75, with permission from Elsevier, license number: 5958860233161]

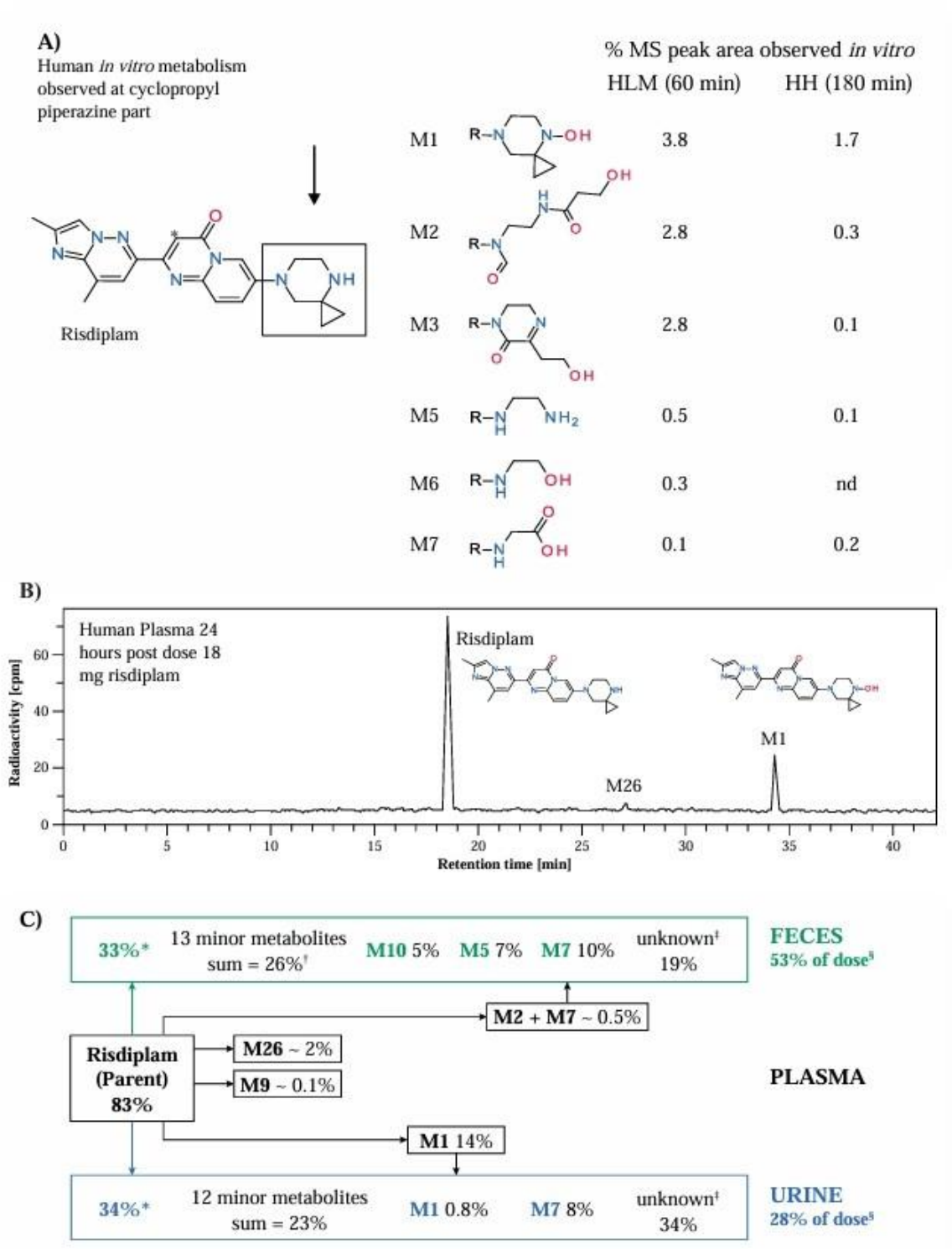

Figure 4. Overview of risdiplam disposition metabolism and excretion in man after a single oral dose of 18 mg. A) in vitro metabolites identified in incubations of HLM and HH after 60 and 180 minutes, respectively. B) high performance liquid chromatography and microbeta-scintillation count analysis of the metabolite profile of risdiplam in pooled human plasma of six healthy individuals at 24 h after oral administration (18 mg) of [14C]-risdiplam. C) Overview

of risdiplam disposition, metabolism and excretion after a single oral dose of 18 mg in six heathy volunteers [Reprinted from Stephen Fowler, Andreas Brink, Yumi Cleary, Andreas Gunther, Katja Heinig, Christophe Husser, Heidemarie Kletzl, Nicole Kratochwil, Lutz Mueller, Mark Savage, Cordula Stillhart, Dietrich Tuerck, Mohammed Ullah, Kenichi Umehara, and Agnes Poirier, Addressing Today's Absorption, Distribution, Metabolism, and Excretion (ADME) Challenges in the Translation of In Vitro ADME Characteristics to Humans: A Case Study of the SMN2 mRNA Splicing Modifier Risdiplam, Drug Metabolism and Disposition, 2022, 50, 65-75, with permission from Elsevier, license number: 5958860233161]

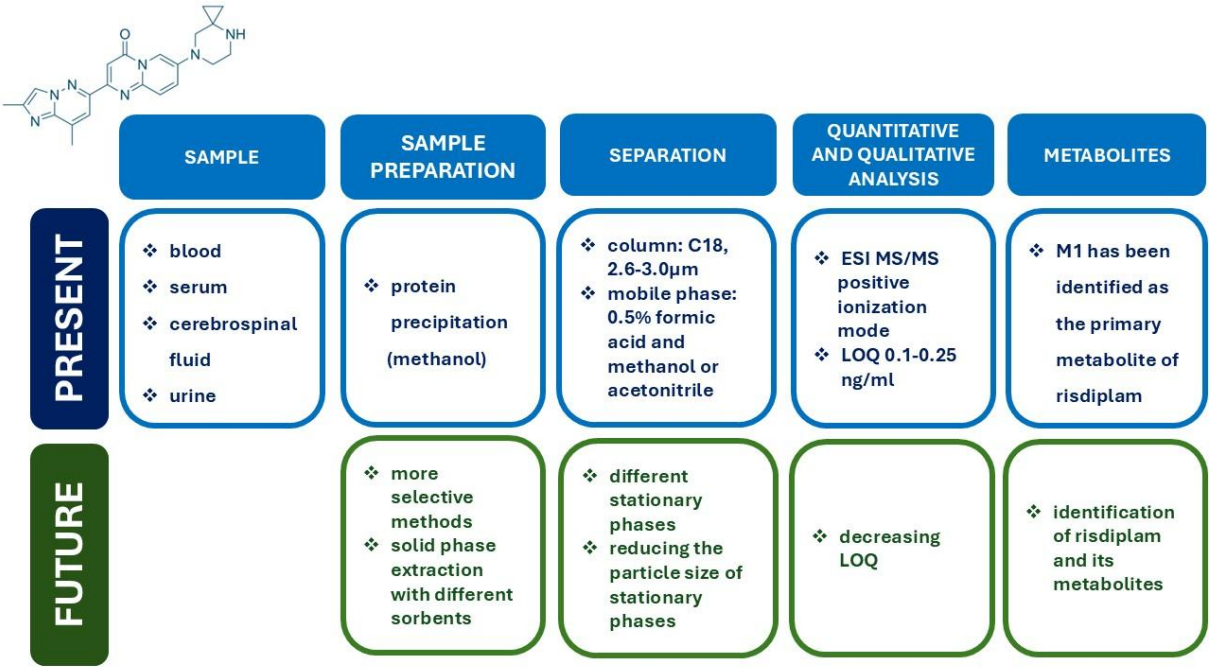

Figure 5. Future perspectives and conclusions of risdiplam.