Development of a chromatographic method for the analysis of risdiplam in serum extracts

Natalia Balińska[a], Anna Lemska[b], Maria Mazurkiewicz-Bełdzińska[b], Sylwia Studzińska [a, *]

[a]*Chair of Environmental Chemistry and Bioanalytics, Faculty of Chemistry, Nicolaus Copernicus University in Toruń, 7 Gagarin Str., PL-87-100 Toruń, Poland*
[b]*Department of Developmental Neurology, Medical University of Gdansk, 7 Dębinki Str., PL-80-952, Gdańsk, Poland*
*Corresponding author: kowalska@umk.pl

Ethical conduct of research statement

Samples were taken following the relevant guidelines of the Medical University of Gdańsk and approved by the Independent Bioethics Committee for Scientific Research at the Medical University of Gdańsk (permission no. NKBBN/778/2022).

Abstract

Background: Risdiplam has been used to treat spinal muscular atrophy for three years. There are limited number (4) of papers devoted to its analytics. Until now, risdiplam and its metabolites have only been analyzed using a C18 column, while the sample preparation method involved only protein precipitation.

Research design and methods: Risdiplam was analysed using reversed-phase UHPLC. The experiment was designed to compare the retention of risdiplam on five columns using various mobile phases. The protein precipitation was used as the sample preparation method.

Results: Risdiplam shows greater retention on phenyl columns, where π-π interactions take part in retention. The increasing of mobile phase pH caused increased risdiplam retention, while salt concentration had no significant effect. An octadecyl column with pentafluorophenyl groups was selected with a mobile phase containing 10 mM ammonium formate (pH 4) and acetonitrile. The method was characterized by good linearity, precision, repeatability, and short analysis time. It was applied to risdiplam analysis in serum samples after protein precipitation with different solvents. Finally, proteins were effectively precipitated using 10% TFA solution, providing 90% recovery.

Conclusions: The developed procedure of extraction and determination of risdiplam is simple, fast, accurate, and reliable. It may find application for routine monitoring of risdiplam or for quality control.

Keywords: risdiplam; liquid chromatography; stationary phases; retention of risdiplam; protein precipitation

1. Introduction

Spinal muscular atrophy (SMA) is a genetic disease inherited in an autosomal recessive manner [1–3]. Manifested by gradual muscle weakness and atrophy, patients gradually lose motor neurons in the spinal cord through a mutation in the SMN1 gene [2–5]. Since 2016, three therapies to treat SMA have been developed [6–8]. Determining the concentration of risdiplam in biological matrices is important for evaluating the efficacy of therapy, adjusting the dose that produces a therapeutic effect, and providing information on metabolism [2,5].

So far, only reversed-phase ultra high performance liquid chromatography (RP UHPLC) has been used for the determination of risdiplam in biological samples. Currently, there are only four papers devoted to risdiplam analysis using this technique [5–7,9]. All of them used a standard octadecyl (C18) stationary phase, however, the application of other UHPLC columns can provide greater selectivity [5,7]. Wu et al. [5] described the development of a method for the analysis of risdiplam concentrations in blood. Three different octadecyl columns (Agilent SB C18, Kinetex XB C18, Agilent Eclipse plus C18) were used with a mobile phase composed of water and acetonitrile. Kinetex XB C18 column provided the shortest retention time and greatest peak symmetry. The developed method was used to determine risdiplam in human blood serum extracted from SMA patients [5]. Fowler et al. [7] also used a C18 column, but the mobile phase consisted of 0.5 % solution of formic acid (FA) and methanol. It allowed for increased mass spectrometry sensitivity and improved the peak shape due to lowering the pH of the mobile phase. On the other hand, the analysis times were very long, risdiplam eluted at 18 minutes, and some of the metabolites as late as at 35 minutes. This chromatographic method was successfully used during in vivo study of risdiplam metabolism in healthy males after a single oral dose of the drug [7]. Sturm et al. [[6]] also conducted in vivo studies; however, no information on the experimental conditions was provided by the authors. Risdiplam was successfully quantified in urine and serum, with good precision, and the limit of quantification equalled 0.250 ng mL^{-1} [6]. Heining et al. [9] also used C18 column; however, the mobile phase composition was more complex compared to the previous studies (A: water/acetonitrile/1M ammonium hydrogen carbonate pH 9; B: water/acetonitrile/2-propanol/acetone) due to the asymmetric peak shapes observed for risdiplam and its main metabolite. This chromatographic method was successfully used to analyze both compounds in Animal K3-EDTA plasma, human K3-EDTA plasma, human urine, artificial CSF, cynomolgus monkey cerebrospinal fluid, and

brain tissue. The authors' challenges arose from the low stability and high polarity of risdiplam and its metabolite [9].

Analysis of risdiplam has to be preceded by a sample preparation step. So far, only protein precipitation has been used for the isolation of risdiplam from serum. To date, two papers describing risdiplam extraction have been published [5,9]. The optimization included the study of the impact of the organic solvent (methanol, acetonitrile), sample volume, freezing time, and ultrasound time on the extraction efficiency [5]. The results showed that application of methanol provides the greatest recoveries, but freezing and precipitation times were also important for the extraction of risdiplam [5]. Another study showed that when risdiplam and its main metabolite are to be extracted from plasma, a mixture of ethanol and acetonitrile (2:8 v/v) should be used [9].

The main goal was to develop a chromatographic proof-of-concept method rather than a full bioanalytical method. The present study focuses on evaluating the effects of different stationary phases on the retention of risdiplam. This is the first study of its kind for risdiplam; no similar studies have ever been conducted before [10,11]. The impact of both the stationary phase and the mobile phase composition on risdiplam retention was tested. Next, the most optimal chromatographic conditions were selected to develop a chromatographic method for the determination of risdiplam in an enriched human serum sample. Such extensive research has made it possible to expand the discussion on the retention mechanism of risdiplam. Moreover, the goal of the study was to test different solvents for protein precipitation during risdiplam extraction from serum samples. To the best of our knowledge, such comprehensive work describing risdiplam retention, analysis, and extraction has been presented for the first time.

2. Materials and methods

2.1. Materials

Risdiplam, with the full name of 2-(2,8 dimethylimidazo[1,2-b]pyridazine-6-yl)-7-(4,7-diazaspiro[2,5]acetate-7-yl)-4H-pyrido[1,2-a]pyrimidin-4-one, and a molecular weight of 401.46, was purchased from Merck (Darmstadt, Germany). The stock solution was prepared by dissolving 1 mg of R in 1 mL of 0,1% formic acid. Subsequent dilutions were prepared by diluting the stock solution with deionized water. The concentration of risdiplam used for the retention studies was 10 μg mL^{-1}. The solubility of risdiplam was assessed by visual inspection under laboratory conditions at room temperature, using the following solvents: water, ethanol,

methanol, acetonitrile, 0.1% formic acid, 0.1% ammonium hydroxide. The mobile phases were prepared using acetonitrile for HPLC (Sigma-Aldrich, Dorset, UK), ammonium formate (purity≥ 99.99%) and formic acid (purity ≥ 99.99%) (Merck, Darmstadt, Germany), 25 % solution of ammonia, hydrochloric acid (Chempur, Piekary Śląskie Poland). Deionized water was obtained from the Milli-Q water purification system (Millipore El Passo, TX, USA).

Protein precipitation was performed with the use of methanol, ethanol p. a. (Stanlab, Lublin, Poland), acetone (purity ≥ 99.99%) (Merck, Darmstadt, Germany), and 10% trifluoroacetic acid (Merck, Darmstadt, Germany).

2.2. Apparatus and chromatographic conditions

An UltiMate® 3000 Binary Rapid Separation LC (RSLC) chromatographic system equipped with a DAD-3000RS diode array detector (Dionex, Sunnyvale, CA, USA) was used. Experimental data were collected using Thermo Scientific Dionex Chromeleon 7.0 software.

The CP-505 Elmetron pH meter (Zabrze, Poland) was used to measure the pH of the mobile phases. Serum samples enriched with risdiplam were centrifuged using Frontier Micro FC5515 centrifuge (OHAUS Europe GmbH, Nänikon, Switzerland). The solvent after protein precipitation was evaporated to dryness using a CentriVap vacuum concentrator (Labconco, Kansas City, MO, USA).

Five different chromatographic columns were selected for the experimental work (Table 1). Risdiplam retention studies for each of the five columns were conducted in the isocratic elution mode. In this study, we used the isocratic elution mode as we wanted to minimize the stabilization time of the chromatographic system after the gradient. The gradient elution was not tested because the isocratic conditions allowed us to achieve adequate selectivity, symmetrical peaks, and a short time of analysis (4 min.). Acetonitrile was always used as mobile phase B, while mobile phase A was: water; 0.1% aqueous solution of formic acid (FA); ammonium formate ($HCOONH_4$) solution with different concentrations (10, 20, and 30 mM) and pH (3-8). The concentration of risdiplam for the retention studies was 10 µg mL^{-1}. The flow rate was 0.3 mL/min. The sample injection volume was 5 µL. The column temperature was 40 °C, while the autosampler temperature was 10 °C. UV detection was carried out at the following λ wavelengths: 210, 240, 260, and 270 nm (absorption maxima for risdiplam) (Figure 1). Dead time was determined using solvent peaks. The retention factor (k) was calculated by using a standard equation (the difference between the retention time and dead time divided by the dead

time). Peak asymmetry f_{AS} were measured by Thermo Scientific Dionex Chromeleon 7.0 software, at 5 % peak height.

135 **Table 1.** Characteristics of the stationary phases used in the study.

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Column	Abbreviation	Stationary phase	Silica particle size (µm)	Column size (mm)	Manufacturer	Pore size (Å)	Carbon load (%)
Waters ACQUITY Premier Oligonucleotide C18	C18	Octadecyl	1.7	2.1 × 100	Waters, Massachusetts, US	130	18
Avantor® ACE® Excel® C18-PFP, Analytical UHPLC	C18-PFP	Pentafluorophenyl	1.7	2.1 × 100	Avantor, Pennsylvania, USA	100	14.3
Evosphere C18/AR	C18/AR	Phenyl	1.7	2.1 × 100	Fortis Technologies, Warrington, UK	100	17
Evosphere Diphenyl	DiPh	Diphenyl	1.7	2.1 × 100	Fortis Technologies, Warrington, UK	100	15
Evosphere Phenyl-Hexyl	Ph	Phenyl	1.7	2.1 × 100	Fortis Technologies, Warrington, UK	100	15

2.3. Validation of chromatographic method

The calibration curve was plotted based on the results of peak areas measured for 10 different concentrations of risdiplam standard solutions (0.4, 0.5, 0.6, 0.8, 1, 2, 4, 6, 8 and 10 μg mL^{-1}). The determination coefficient (R^2) indicated whether the calibration curve showed linearity within this range. The relative standard deviation (RSD) of the peak area for ten injections on a single day, at three concentrations (0.8, 4, and 8 μg mL-1), was calculated to determine intraday accuracy. The relative standard deviation of the peak area for five injections at three different concentrations (0.8, 4, and 8 μg mL^{-1}) on the first, third, and fifth day was calculated to determine repeatability. The limits of detection (LOD) and quantification (LOQ) were determined by analyzing the peak-to-noise ratio on the chromatogram. LOD and LOQ were calculated based on dependents LOD = 3(S/N) and LOQ = 10(S/N), where S is signal, while N is noise.

2.4. Serum sample preparation and extraction of risdiplam

Human serum 180 μL was enriched with risdiplam standard solution to the final concentration of 2 μg mL^{-1} (final sample volume was 200 μL). Methanol, ethanol, acetone, and 10 % TFA were used sequentially to precipitate the proteins. The samples were mixed on a vortex for 2 minutes and placed in a centrifuge for 40 minutes at 14,000 rpm at room temperature (~22 °C). The supernatant was pipetted off and evaporated to dryness at 50 °C. The dry residue was dissolved in 100 μL 0.1% FA before analysis (Figure S1). Consequently, the sample was enriched two-fold. The samples were taken following the relevant guidelines of the Medical University of Gdańsk and approved by the Independent Bioethics Committee for Scientific Research at the Medical University of Gdańsk (permission no. NKBBN/778/2022). The matrix effect was determined based on the post-extraction addition protocol presented in the literature data [12]. First, the whole protein precipitation procedure was performed for the serum sample without its enrichment with risdiplam (blank extract). Next, the extract was enriched with risdiplam to the same concentration as in the case of the standard. Finally, both samples (the standard and enriched blank extract) were analysed using the developed chromatographic method. The peak areas of the standard and blank extract were compared and the matrix effect was calculated based on this ratio multiplied by 100%. The matrix effect was determined to assess the impact of plasma components on the analyte signal, which could lead to its amplification or reduction during analysis.

3. Results and discussion

Risdiplam is a pyridazine derivative [13]. Its structure and UV-Vis spectrum are presented in Figure 1.

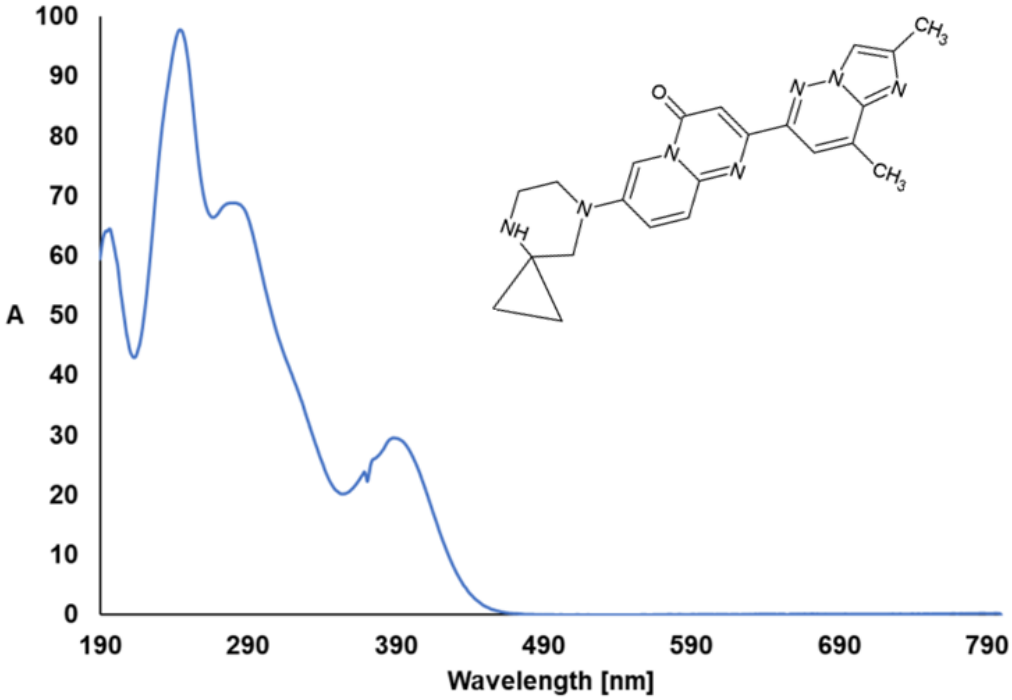


Fig. 1 Structure and UV-VIS spectrum of risdiplam. UV detection for λ from 190 to 800 nm.

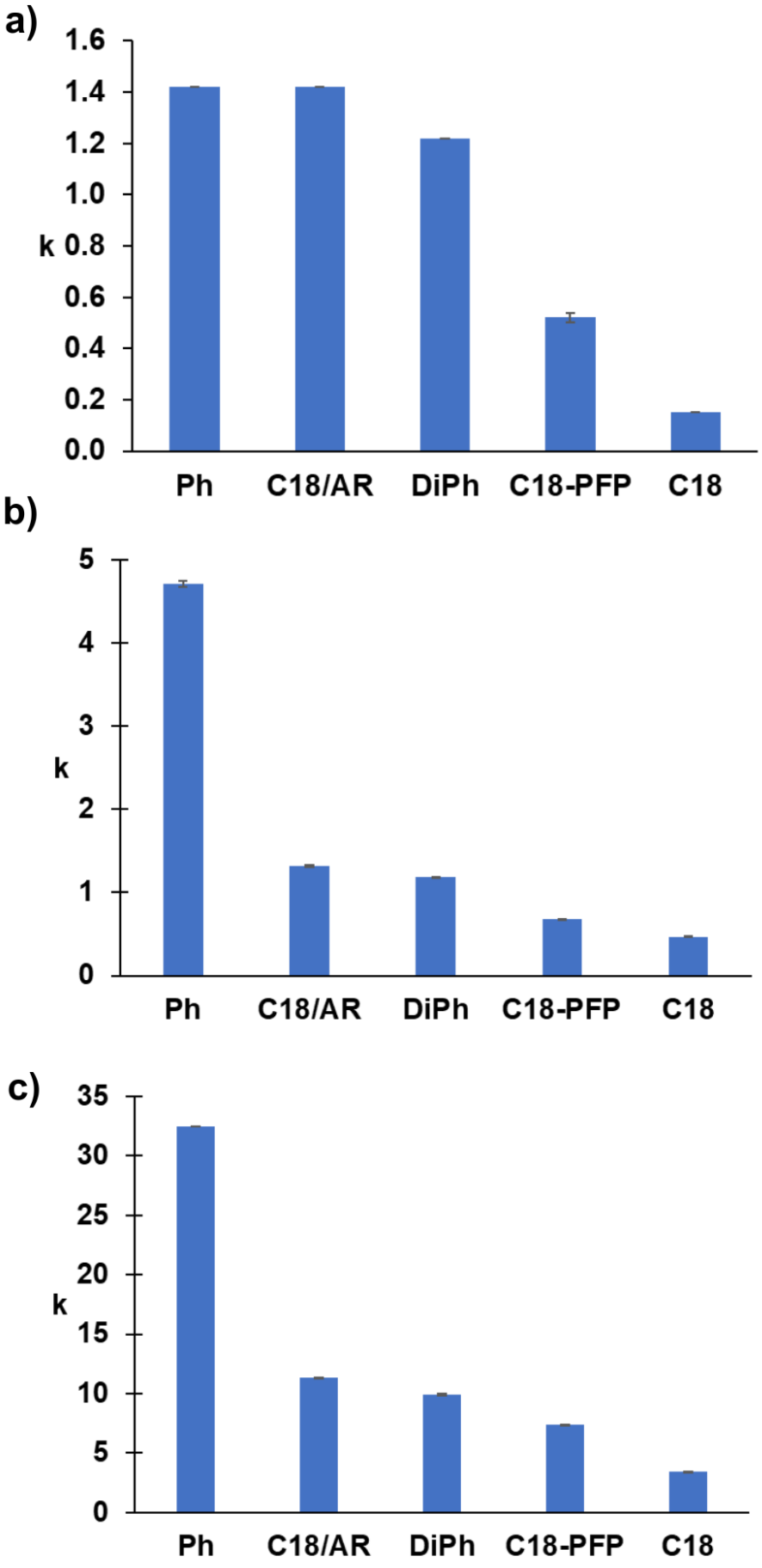
Risdiplam is insoluble in water, but it is soluble in acidified water. It attaches protons at low pH, mainly by nitrogen atoms. In a strongly acidic environment, more nitrogen atoms can undergo protonation. The risdiplam sample was prepared in different solvents and different volumes of these solvents. To the best of our knowledge, the solubility of risdiplam is problematic, but also critical during its analysis and sample preparation. We tested the solubility of risdiplam in acetonitrile, methanol, ethanol, water, acidified (0.1% formic acid), and alkalized water (0.1% ammonium hydroxide). The greatest solubility was obtained for 0.1% of formic acid, so it was chosen for the preparation of stock solutions, and then working solutions were prepared by dilution with water. Risdiplam was insoluble in acetonitrile.

The present research on risdiplam focuses on improving the previously known analytical methods for precise quantification of risdiplam in biological matrices RP UHPLC and selecting appropriate conditions of the protein precipitation method during the preparation of human serum. In the initial stage of the research, the most important task was to select a suitable solvent for both chromatographic testing and sample preparation. To this end, we tested the solubility of risdiplam under various conditions. It was assessed by visual inspection under laboratory conditions at room temperature. Initially, an attempt was made to dissolve 1 mg of the substance in 10 mL of water; the mixture was sonicated for 15 min, but the compound remained in suspension. Next, the risdiplam solubility was tested in organic solvents: ethanol, methanol, and acetonitrile. A clear solution was obtained for ethanol and methanol, contrary to acetonitrile. In the next step, 1 mL of 0.1% formic acid and 0.1% ammonium hydroxide was added to the 1 mg of risdiplam. The compound showed good solubility in the acidified solution, while it remained undissolved in ammonia. To sum up, risdiplam showed good (1mg mL^{-1}) solubility in 0.1% FA, ethanol, and methanol. Finally, we decided to prepare the stock solutions of risdiplam in 0.1% FA, while the working solutions were obtained by diluting the stock with water to the appropriate concentration.

3.1 Retention studies

3.1.1 Influence of the stationary phase type

Columns differing in stationary phases were selected to study the retention of risdiplam in RP UHPLC mode: octadecyl (C18); octadecyl with pentafluorophenyl ring (C18-PFP); octadecyl and phenyl group (C18/AR); phenylhexyl ligand (Ph) and diphenyl (DiPh) groups were selected (Table 1). They differ in structural properties and consequently can interact with the risdiplam differently. Figure 2 presents the effect of the type of stationary phases on the retention factor (k) of risdiplam for three different mobile phases.


a)
1.6
1.4
1.2
1.0
k 0.8
0.6
0.4
0.2
0.0
Ph
C18/AR
DiPh
C18-PFP
C18
b)
5
4
3
k
2
1
0
Ph
C18/AR
DiPh
C18-PFP
C18
c)
35
30
25
20
k
15
10
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0
Ph
C18/AR
DiPh
C18-PFP
C18

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Fig. 2 The *k* values for all of the stationary phases used in the study for the different mobile phase compositions. Experimental conditions: (a) 85 % v/v of 0.1% aqueous solution of FA, 15 % v/v of acetonitrile; (b) 75 % v/v of 20 mM $HCOONH_4$ pH=4 , 25 % v/v of acetonitrile; (c) 75 % v/v of 20 mM $HCOONH_4$ pH=8, 25 % v/v of acetonitrile; UV detection at λ = 260 nm; column temperature 40 °C; autosampler temperature 10 °C; flow rate 0.3 mL min^{-1}. Regardless of the mobile phase, the effect of the stationary phase on risdiplam retention is similar, meaning that interactions between risdiplam and the stationary phase are also similar. For each of the five stationary phases, it was observed that retention decreases in the mobile phase with acidic pH.

The data were compared for mobile phases containing 0.1% solution of FA or 20 mM solution of ammonium formate with pH 4 and pH 8. This experiment allows us to conclude that, regardless of the mobile phase used, the effect of the stationary phase on the retention of risdiplam is similar, which means that the interactions between risdiplam and the stationary phase are also similar. Generally, the highest *k* value was observed for Ph, while the lowest was for C18 and C18-PFP (Figure 2). For each of the five stationary phases, it was noted that retention for a mobile phase containing ammonium formate of pH 8 was higher than for pH 4. Moreover, in the case of all the columns, a significant decrease in *k* was observed when 0.1% solution of FA was applied (Figure 2).

Comparing risdiplam retention at several different stationary phases can provide information about the approximate interactions taking part between this compound and the surface of the stationary phase in RP UHPLC. Although it is just an estimation, without any additional experimental studies, we believe they are important. Therefore, we decided to present this discussion of approximate risdiplam interactions in the following paragraphs.

The greatest retention for Ph column may be related to the presence of an aromatic ring in its structure (Figure 2). Risdiplam also contains an aromatic ring (Figure 1), and consequently, π-π interactions may occur between this compound and stationary phase ligands. Surprisingly, the carbon load (values are taken from the manufacturer's specifications) for the Ph column is the lowest of the five columns used (14%) (Table 1), and still, the retention is the highest. This may confirm our assumption of the high impact of π-π interactions on risdiplam retention. Aryl groups are also present at the surface of DiPh, C18/Ar, and PFP, however *k* values are lower (Figure 2). DiPh has a relatively low carbon load (15%) (Table 1). We have assumed that π-π interactions should probably also be the dominant ones here. Nevertheless, *k* was lower than for Ph (Figure 2), probably due to a steric hindrance that may have occurred

between the phenyl ligands of the stationary phase. In π-electron systems, there are situations in which the electron distribution is limited by the spatial arrangement of the ligands, which affects the retention properties [14]. A different effect was noticed for C18/Ar with a higher carbon load (17%) and lower k compared to Ph (Table 1, Figure 2). Based on the structure of C18/Ar it may be concluded that both hydrophobic (alkyl chain) and π-π interactions (aryl group) can take part in the retention of risdiplam. Reduction of *k* values for C18/Ar compared to Ph may be related to the probable reduction of the number of aryl (phenyl) groups (π-π interactions centres) present in the structure of C18/Ar. We believe that this effect also indicates that π-π interactions have a greater impact on increasing risdiplam retention, and hydrophobic interactions will probably not be the dominant ones (Figure 2). Such an assumption is confirmed by the lowest *k* values determined for C18 column with the highest carbon load (18%) (Table 1, Figure 2). C18 has the highest hydrophobicity and interacts with analytes by hydrophobic interactions, and therefore reduction of *k* may suggest that neither hydrophobicity nor the alkyl chain is the key factor determining the strength of interactions of risdiplam with the stationary phase surface. What differentiates C18 and Ph is their structure. If the highest *k* was determined for the aryl group and the lowest for the alkyl chain, we can assume that π-π interactions will be the strongest and dominant for risdiplam, as opposed to hydrophobic interactions. Although there are no studies on risdiplam focusing on its interaction with stationary phases (our studies are the first of this kind), literature data is confirming that phenyl columns exhibit specific π-π interactions that can enhance the retention of aromatic compounds compared to alkyl stationary phases. Columns with a phenyl stationary phase exhibit increased retention of aromatic compounds due to an additional mechanism of π–π interactions between the aromatic rings of the analyte and the phenyl ligands of the stationary phase [15]. In particular, studies comparing C18 and Ph (or biphenyl) columns indicate that for compounds with complex π systems, the retention on Ph is significantly higher than on C18, despite lower carbon load [16] In the context of our results, risdiplam, as a compound with an aromatic structure, showed a significantly longer retention time for Ph compared to the C18 using the same mobile phase. This observation is consistent with the mechanism described in the literature [15,16]. Based on these data, we assume that the dominant interactions for risdiplam are π-π interactions.

The use of a C18-PFP with a pentafluorophenyl and octadecyl groups provides lower risdiplam retention compared to Ph, despite the presence of the aromatic ring in the structure (Figure 2). This effect may be due to the five highly electronegative fluorine atoms present in the aromatic ring acting as electron acceptors. These atoms weaken the electron density.

Moreover, risdiplam contains heterocyclic aromatic rings with nitrogen atoms; therefore, it can act as a proton acceptor, similarly to the pentafluorophenyl group.

The interaction of risdiplam with the stationary phase surface affects not only the retention but also the peak shape. To summarize, the greatest asymmetry factor (f_{AS}) was noticed for C18/Ar (Table S1). DiPh column showed a wide variation in peak asymmetry (f_{AS} =1.65-2.65) depending on mobile phase composition, but generally peaks were not symmetrical, which is similar to the case of Ph (long retention times reduced symmetry) (Table 1). The application of C18 with the lowest risdiplam retention provided suitable peak symmetry (Table S1).

Table S1. Peak asymmetry factor (f_{AS}) for all of stationary phases used in the study for the different mobile phase. Experimental conditions: 85% v/v of 0.1% aqueous solution of formic acid, 15% v/v of acetonitrile; 75% v/v of 20 mM $HCOONH_4$ pH 4 , 25% v/v of acetonitrile; (c) 75% v/v of 20 mM $HCOONH_4$ pH 8, 25% v/v of acetonitrile, UV detection at λ=260 nm; column temperature, 40°C; autosampler temperature, 10°C; flow rate, 0.3 mL min^{-1}; injection volume, 5 μL.

Application of C18/Ar and DiPh provided asymmetrical peaks depending on the composition of the mobile phase. The best symmetry was obtained for C18, while the peak shape for risdiplam for Ph and C18-PFP columns were acceptable.

Mobile phase composition	f_{AS}±SD for five different stationary phase				
	Ph	C18/Ar	DiPh	C18-PFP	C18
0.1% aqueous solution of formic acid	1.68±0.03	2.58±0.03	1.73±0.01	1.67±0.01	1.43±0.01
20 mM $HCOONH_4$ pH=4	1.69±0.01	1.77±0.04	1.65±0.02	1.96±0.04	1.59±0.03
20 mM $HCOONH_4$ pH=8	1.69±0.01	3.0±0.04	2.65±0.02	1.74±0.01	1.36±0.01

SD – standard deviation

The C18-PFP column was selected for further RP UHPLC analysis, since its properties proved to be an interesting direction for further research into the application of new stationary phases for risdiplam chromatographic analysis. This concerns both the short retention time, regardless of the mobile phase used, and the relatively symmetrical peaks (Table S1). Although the Ph column showed more symmetrical peaks under certain conditions (Table S1), its

retention times were up to three times longer than those of the C18-PFP column. Even though some f_{AS} values (Table S1) are less favourable than those of the C18, choosing C18-PFP represents a compromise between retention time and peak symmetry among all the columns tested. Moreover, C18-PFP is an interesting alternative to the conventionally used C18 column that offers different retention mechanisms. C18-PFP enables the combination of hydrophobic and π–π interactions. In the context of risdiplam's chemical structure, this variability in retention may improve selectivity compared to C18, which is particularly important when analyzing complex biological matrices or potential contaminants and degradation products. Due to the short analysis time, we minimize the consumption of the mobile phase. Additionally, short analyses enable the examination of multiple extracts in a single day, which is crucial in routine medical analyses.

3.1.2 The influence of the mobile phase composition

Next, the impact of different mobile phase compositions (mixtures of acetonitrile with 0.1% solution of FA, or $HCOONH_4$ at different concentrations and pH) on *k* and peak asymmetry for five different columns was studied.

When the mixture of water and acetonitrile (75/25% v/v) was used in the mobile phase, the peak of risdiplam was asymmetric, and the retention time was equal to 49 minutes. The addition of FA to the mobile phase caused a significant reduction in the retention time, up to 2 minutes (Figure 3), and the peak was symmetrical (f_{AS}=1.06).

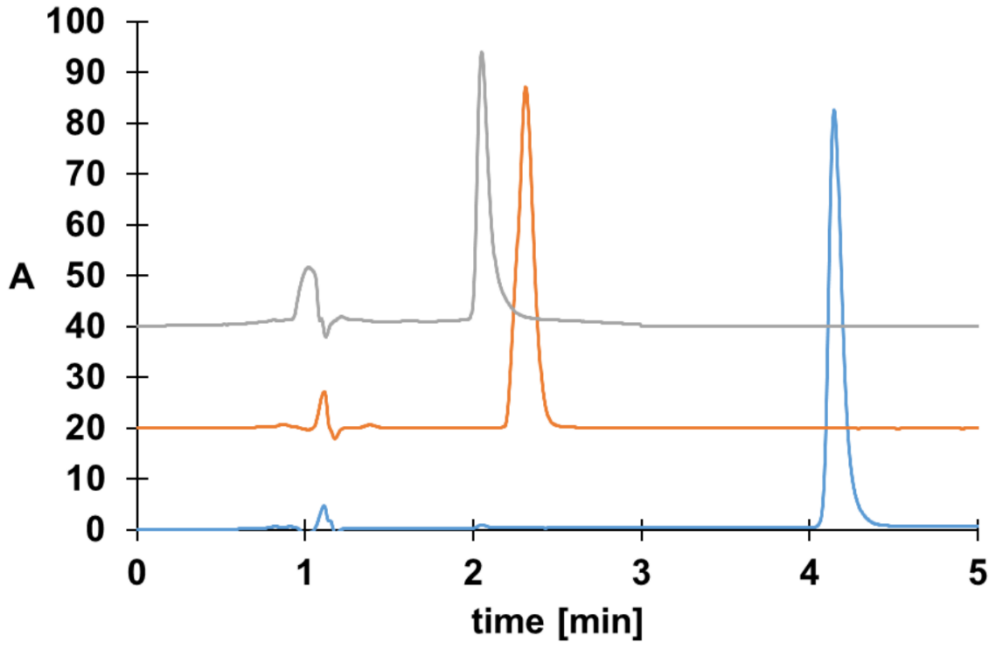


Fig. 3 Chromatograms of risdiplam for 0.1 % solution of formic acid, 20 mM $HCOONH_4$ of pH 3.0 and 10 mM $HCOONH_4$ of pH 4.0 on C18-PFP. Experimental conditions: orange chromatogram: 75 % v/v of 0.1% solution of formic acid, 25 % v/v of acetonitrile; blue chromatogram: 75 % v/v of 20 mM $HCOONH_4$ of pH 3.0, 25 % v/v of acetonitrile, grey chromatogram: 75 % v/v of 10 mM $HCOONH_4$ of pH 4.0, 25 % v/v of acetonitrile, UV detection at λ= 260 nm; column temperature, 40 °C; autosampler temperature, 10 °C; flow rate, 0.3 mL min^{-1}. The chromatogram of the standard risdiplam solution for the finally developed method is marked with a grey colour.

Nitrogen atoms in the structure of risdiplam in acidic pH can undergo protonation and consequently interact with the stationary phase to a lower extent. All the stationary phases used during the study contain residual silanol, which is likely to interact electrostatically with risdiplam at higher pH of the mobile phase. Silanols and risdiplam undergo protonation at acidic pH, causing electrostatic repulsion and leading to shorter retention times. Application of 20 mM $HCOONH_4$ (pH 3) in the mobile phase showed a significant deterioration in peak asymmetry, f_{AS} = 1.52, and an increase in retention time to 4 minutes. The addition of $HCOONH_4$ (pH 3) to the mobile phase resulted in greater interactions of risdiplam with the stationary phase than for acidified water, thus increasing the retention time. Prolonged elution is often the cause of deterioration in the peak symmetry.

Table 2 presents the impact of different salt concentrations on k values and f_{AS} of risdiplam for all the columns. In the concentration range used, there was no systematic change of k value with increasing concentration, indicating that this parameter does not significantly affect the interactions between risdiplam and the stationary phase. Moreover, salt concentration has little effect on peak asymmetry.

Table 2. Summary of the peak asymmetry (f_{AS}) values along with the retention factor (k) of risdiplam analyzed with five different stationary phases. Experimental conditions: 70% v/v $HCOONH_4$ 30% v/v of acetonitrile. No systematic changes in k values and fAS were observed with increasing concentrations, indicating that this parameter does not significantly affect the interaction between risdiplam and the steady state.

Column	10 mM		20 mM		30 mM	
	k	f_{AS}	k	f_{AS}	k	f_{AS}
C18	0.85±0.01	1.39±0.03	0.86±0.01	1.53±0.02	0.83±0.01	1.57±0.04
C18-PFP	1.33±0.01	1.48±0.01	1.45±0.01	1.37±0.03	1.76±0.01	1.45±0.04
C18/Ar	2.73±0.01	1.65±0.01	2.67±0.01	2.12±0.01	2.58±0.01	2.21±0.02
DiPh	3.05±0.01	2.66±0.01	2.73±0.01	2.28±0.01	2.59±0.03	2.28±0.03
Ph	10.90±0.02	1.48±0.03	9.80±0.01	1.72±0.02	9.10±0.02	1.64±0.01

Regardless of the column, the effect of changing the pH provided similar trends: risdiplam retention decreases as the pH decreases (Figure 4, Table S2).

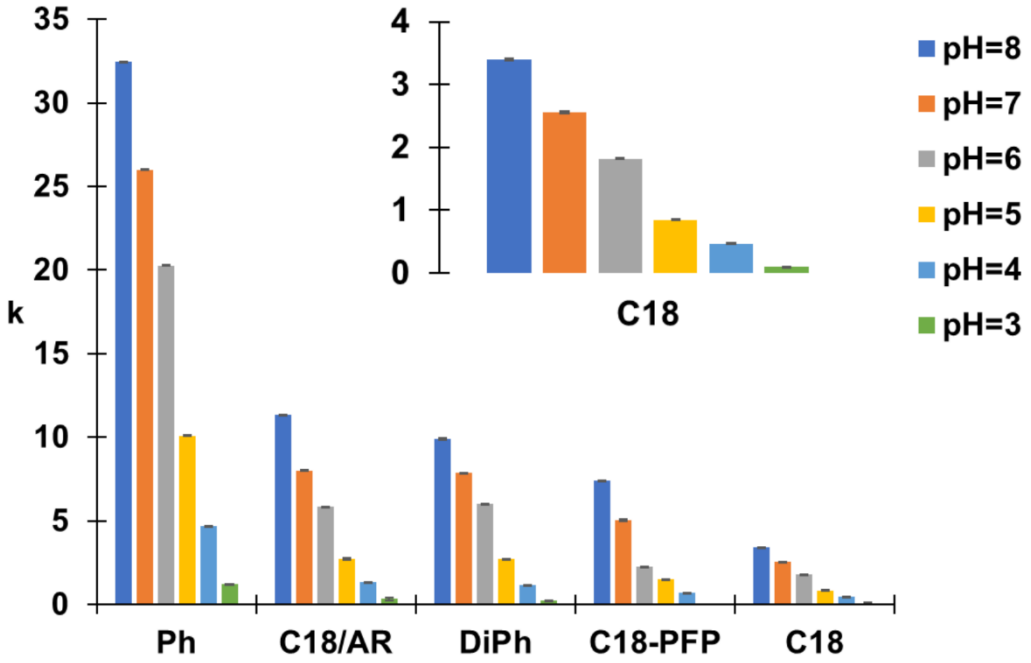


Fig. 4 The *k* values for all of the stationary phases used in the study for the different pH of mobile phase. Experimental conditions: 75 % v/v of 20 mM $HCOONH_4$ (pH range 3.0-8.0), 25 % v/v of acetonitrile, UV detection at λ= 260 nm; column temperature 40 °C; autosampler temperature 10 °C; flow rate 0.3 mL min^{-1}. Regardless of the column, the effect of changing the pH of the mobile phase on risdiplam retention was similar: it decreases as the pH decreases.

Table S2. Asymmetry factor (f_{AS}) for all of stationary phases used in the study for the different pH of mobile phase. Experimental conditions: 75% v/v of 20 mM $HCOONH_4$ (from pH 3 to 8), 5% v/v of acetonitrile, UV detection at λ=260 nm; column temperature, 40°C; autosampler temperature, 10°C; flow rate, 0.3 mL min^{-1}; injection volume, 5 µL.

Regardless of the column, the effect of changing the pH provided a similar tendency. Reducing the pH of the mobile phase causes greater peak symmetry.

Column	f_{AS}±SD across the pH range					
	3	4	5	6	7	8
Ph	1.48±0.03	1.69±0.01	2.28±0.01	1.95±0.04	1.91±0.01	1.69±0.01
C18/Ar	1.65±0.01	1.77±0.04	2.24±0.05	2.61±0.04	2.91±0.01	3.00±0.04
DiPh	1.52±0.01	1.65±0.02	2.24±0.04	2.67±0.01	2.77±0.01	2.65±0.02
C18-PFP	1.79±0.01	1.96±0.04	1.85±0.01	1.42±0.01	1.55±0.02	1.74±0.01
C18	1.58±0.09	1.59±0.03	1.58±0.04	1.33±0.01	1.29±0.03	1.36±0.01

SD – standard deviation

The *k* values are different for each column and pH, but the general tendency remains similar. In the structure of stationary phases, the only groups able to undergo protonation/ionization are residual silanols. At low pH, they become protonated, and at high pH, they can ionize. Risdiplam is a weak acid (pK_a = 9.41 ± 0.20), meaning that it can easily attach protons, while it will become the donor of one proton at pH greater than 9 (Figure 1). Residual silanols are most likely to be found in ionized form at pH>7, while at pH below 4, they can attach a proton. Since residual silanols are present in each of the stationary phases, the effect of their protonation will probably be similar for each of the stationary phases. On the other hand, each column differs markedly in silanol activity and ligand coverage density; therefore, various *k* values were determined for different stationary phases and similar pH values (Figure 4), e.g. for pH 7, *k* equaled ~26 for Ph and ~8 for C18Ar. On the other hand, when we compare the results noticed for individual columns, general trends are similar, i.e. increasing *k* with increasing pH. For these reasons, we assume that the impact of pH on risdiplam retention is connected with the changes in its structure (Figure 1). Reducing the pH of the mobile phase causes retention to decrease because risdiplam becomes a cation. The more positive charge it has, the more polar it becomes, and its interactions with non-polar stationary phases are lower compared to the mobile phase of high pH (Figure 4).

The 10 mM ammonium formate solution was chosen for the final method. The choice of pH 4 gave a shorter analysis time. Based on these data, 75 % v/v of 10 mM $HCOONH_4$, pH 4; 25 % v/v of acetonitrile was chosen as the mobile phase for a routine analysis. The chromatogram of the standard risdiplam solution for the finally developed method is presented in Figure 3 (grey chromatogram).

3.2 Validation of the chromatographic method

Validation of the chromatographic method was carried out based on linearity, LOD, LOQ, accuracy, and precision. Linearity was determined by calculating the correlation coefficient (R^2) for the calibration curve. The calibration curve showed very good linearity (R^2 = 0.999) over the concentration range used (0.4-10 μg mL^{-1}). The concentrations were selected to examine a wide range of values. LOD indicates the lowest concentration of risdiplam that can be detected, while LOQ indicates the lowest concentration at which risdiplam can be quantitatively analysed with acceptable precision and accuracy. The LOD was 0.3 μg mL^{-1}, while the LOQ was 0.4 μg mL^{-1}. As expected, the sensitivity of UV detection is lower compared to LC-MS/MS methods applied so far for risdiplam analysis (LOQ was 0.25 and 1.95 ng mL^{-1}) [5,9]. However, our priority was to develop a simple, inexpensive, and easily implementable approach to the routine analysis of risdiplam, where a UV detector may be sufficient despite its lower sensitivity. The developed method can be a simple alternative to LC-MS/MS when ultra-high sensitivity is not required. It may be used for qualitative and quantitative analysis in quality control laboratories, routine stability monitoring, and sample purity assessment. Furthermore, the presented method provides a promising basis for further development in the context of clinical monitoring (when coupled with MS).

The intraday and interday precision were determined and summarized in Table 3. The intraday precision values were less than 4.13%. Low RSD indicates the high precision of the method during the analysis. The repeatability was lower than 5%. The calculated RSD on different days allowed for assessing the stability of the method over time. Validation studies allow for determining the suitability of the developed method for determining risdiplam in biological samples

Table 3. The validation parameters for risdiplam analysis using developed chromatographic method.

Calibration curve					RSD [%]					
					Inter-day			Intra-day		
Concentration range [µg mL^{-1}]	Calibration curve equation	R^2	LOD [µg mL^{-1}]	LOQ [µg mL^{-1}]	0.8 [µg mL^{-1}]	4 [µg mL^{-1}]	8 [µg mL^{-1}]	0.8 [µg mL^{-1}]	4 [µg mL^{-1}]	8 [µg mL^{-1}]
0.3-10	y = 807.9x – 0.313	0.999	0.3	0.4	4.53	4.81	4.91	2.1	2.91	4.13

3.3. Extraction of risdiplam from serum samples

Protein precipitation was chosen for the extraction of risdiplam from human serum samples because it effectively removes proteins that significantly interfere with chromatographic analysis. Solvents for protein precipitation were selected based on the solubility of risdiplam. It has to be soluble in a selected solvent, contrary to proteins. The 10% aqueous solution of TFA was selected because it is well known that the solubility of risdiplam depends strongly on pH; moreover, this solvent leads to protein destabilisation and precipitation. From a safety perspective, TFA has high acid strength and, unlike stronger mineral acids, is safer for everyday use. Organic solvents, such as acetone, ethanol, and methanol, reduce the solubility of proteins in solution while risdiplam remains soluble. These four solvents were selected and used during the present study; two of them (TFA and acetone) were tested for the first time for the extraction of risdiplam.

Firstly, the enriched serum sample was diluted with each of the selected solvents at three different volume ratios: 1:3, 1:10, and 1:20. The total final volume of the samples (serum diluted with precipitation solvent) was 2 mL in each case. The final risdiplam concentration was the same for all the samples and was equal to 2 μg mL^{-1}. The blank samples were prepared similarly, however, without the addition of risdiplam. The samples were mixed using a vortex for 2 minutes and centrifuged for 40 minutes at 14,000 rpm. The supernatant was evaporated to dryness at 50 °C to remove solvents and concentrate the sample; this process plays a key role in obtaining accurate and reliable results. The dry residue was dissolved in 100 μL of 0.1% FA before analysis (Figure S1). Consequently, the sample was enriched twofold.

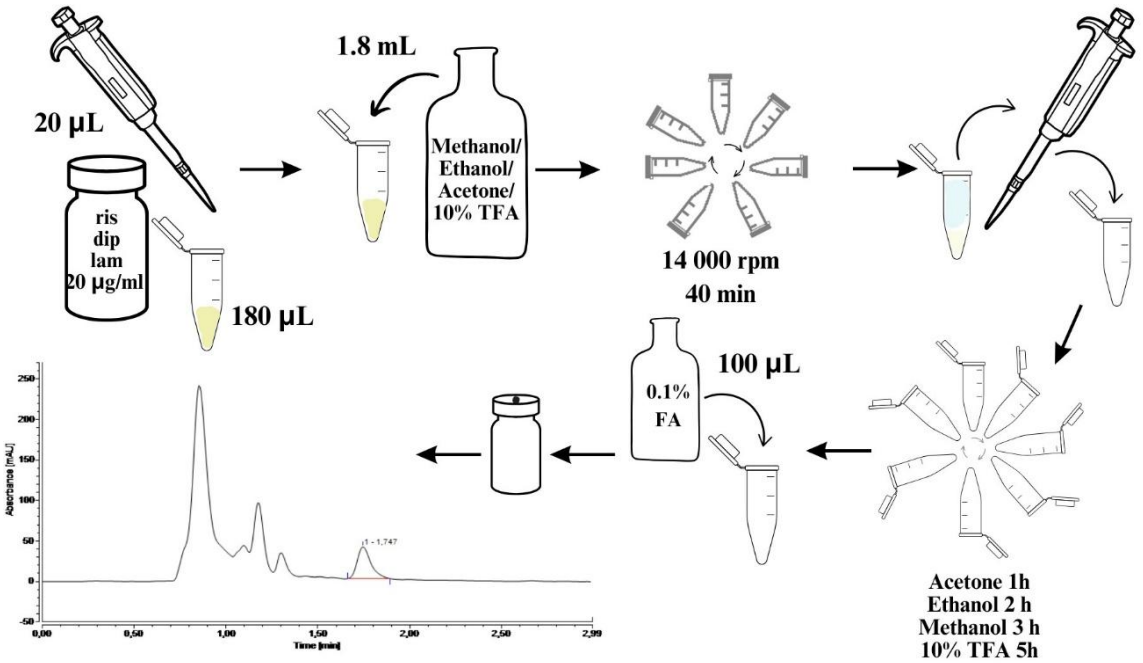
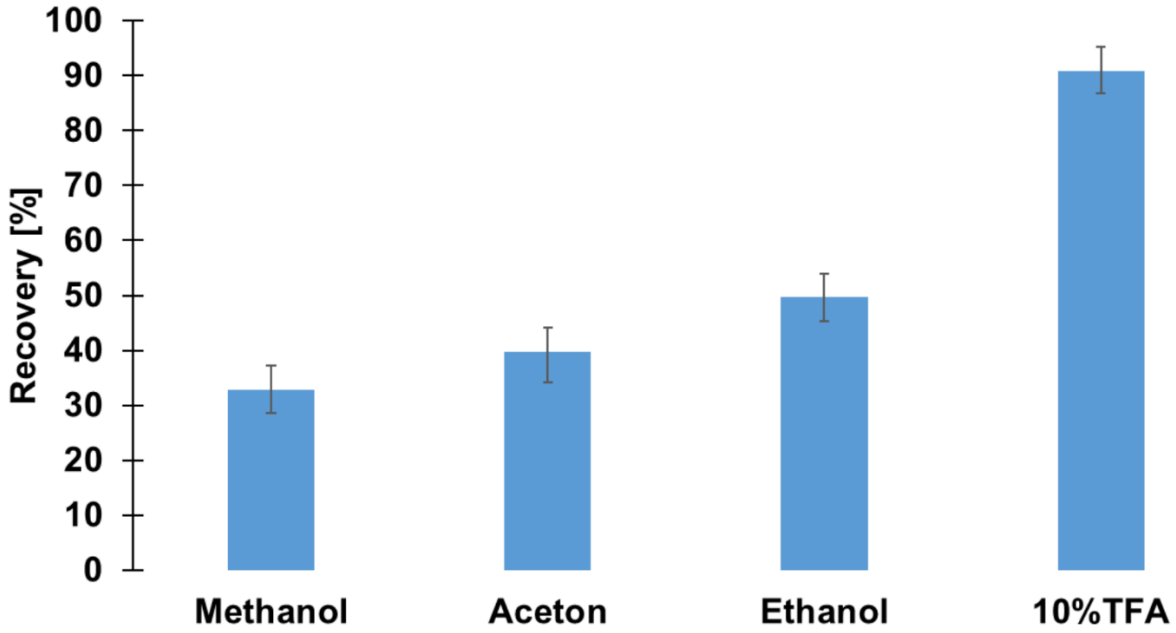


Fig. S1 General scheme for the extraction of risdiplam from an enriched human serum sample**.**

The first volume ratio used (1:3) did not provide effective protein precipitation for any of the solvents used during the study, because after evaporation to dryness, the large residue of protein deposit was visible in the Eppendorf. We have decided not to analyse these samples using the chromatographic method. Dilution of serum in the ratio of 1:10 and 1:20 allowed for more effective protein removal (no visible protein precipitate after solvent evaporation). Finally, for the extraction of risdiplam, we used 200 µL of serum and 1.8 mL of solvent for precipitation. No significant differences in the recovery between the samples for 1:10 and 1:20 serum: precipitation solvent ratios were observed. The recovery of risdiplam was determined using a developed chromatographic method. The results are collected in Figure 5.



462

463 **Fig. 5** Recovery of risdiplam from serum samples for different solvents used for protein
464 precipitation. The highest recovery was noticed for 10% TFA (~91), despite the high intensity
465 of the peaks originating from matrix. The recoveries differ (30-90%) depending on the
466 precipitation solvent.

467

468 The highest recovery, despite the high intensity of the peaks from proteins, was obtained
469 using a 10% of aqueous solution of TFA (90.79±4.04%). The recoveries were different (30-
470 90%) depending on the precipitation solvent, probably due to the affinity of risdiplam for the
471 proteins and the various solubilities of risdiplam in organic solvents. Based on our knowledge
472 of risdiplam solubility, we assume that the highest recovery was achieved in 10% TFA due to
473 its acidic nature and its preferential solubility in acidic solutions (Figure S2, Figure 5). The
474 matrix effect (Figure S3) was determined by comparing the peak areas of the standard solution
475 of risdiplam with the extract from a blank sample enriched with risdiplam to a similar
476 concentration and was 100%.

477

478

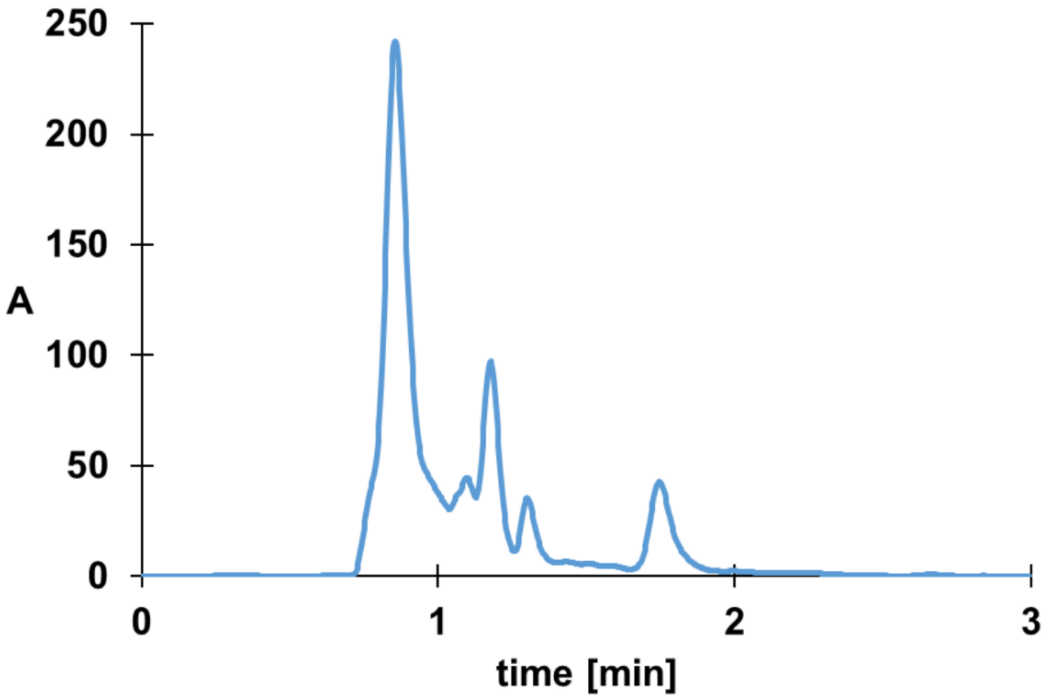


Fig. S2 Chromatogram of serum extracts obtained by precipitation of proteins with 10% TFA in 200 µL serum sample. Experimental conditions: 75% v/v of 10 mM $HCOONH_4$ pH 4, 25% v/v of acetonitrile, UV detection at λ=260 nm; column temperature, 40°C; autosampler temperature, 10°C; flow rate, 0.3 mL min^{-1}; injection volume, 5 µL.

The developed procedure is relatively simple and fast. It ensures effective removal of proteins, whose presence makes chromatographic analysis difficult and shortens column life. The solvents used are easily evaporated, which allows for the concentration of the sample. The disadvantage of the developed method is its time consumption and insufficient purification of the sample (remaining proteins), demonstrating the need for additional purification of the extracts. The developed chromatographic method can already be used in routine analyses of risdiplam impurities for quality control purposes. Although the current results relate to a preliminary assessment in the model matrix, the method could potentially be used for routine monitoring of risdiplam in patients when coupled with MS (increasing the sensitivity), enabling more accurate tracking of risdiplam concentrations in plasma, especially at low doses or in samples with limited volume. The chromatographic method we have developed under this study can be directly applied in LC-MS/MS, as the composition of the mobile phase is appropriate and the peak symmetry is satisfactory. Furthermore, the use of TFA (volatility) as a solvent for protein precipitation also makes the entire procedure MS-friendly. In summary, the advantage of the risdiplam extraction and determination procedure presented in the publication is the direct

applicability of the procedure to MS. Adapting the method to LC-MS/MS also offers the possibility of rapid analysis of multiple samples in laboratories, which could support therapeutic approaches and individualized dosage. Further research will aim to develop an effective sample preparation method that ensures the removal of all proteins and enables accurate risdiplam analysis in biological samples.

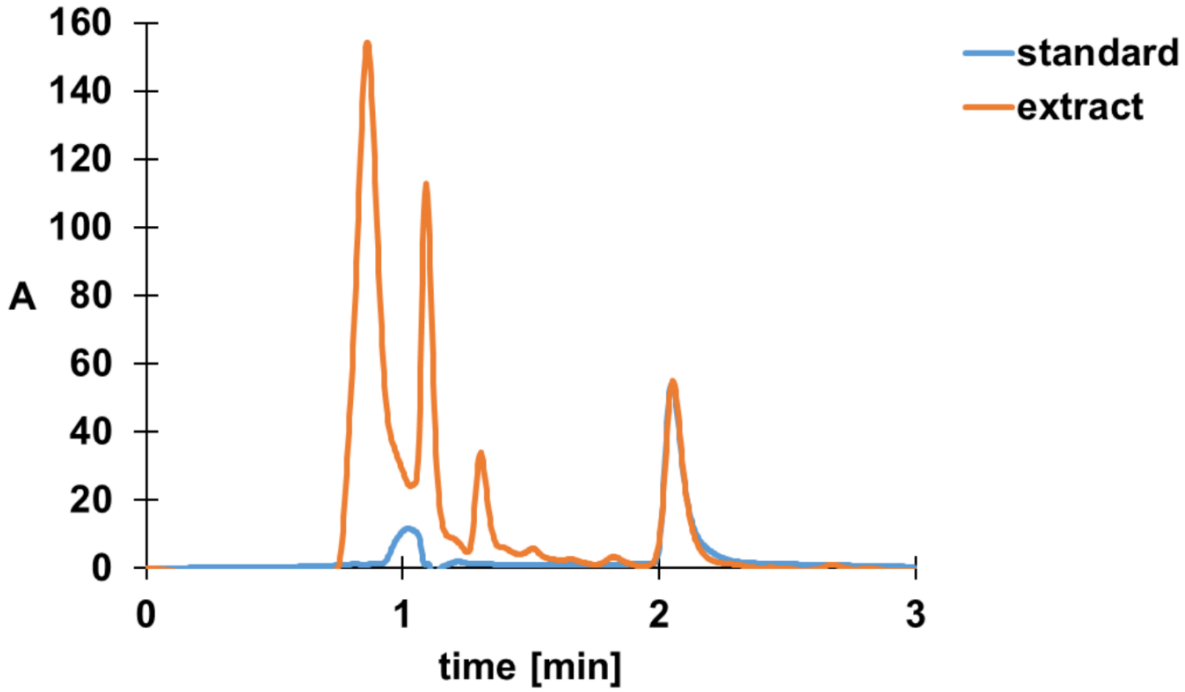


Fig. S3 Chromatograms of risdiplam standard solution (blue line) and extract from blank sample enriched with risdiplam standard the same concentration (orange line). Experimental conditions: 75% v/v of 10 mM $HCOONH_4$ pH 4, 25% v/v of acetonitrile, UV detection at λ=260 nm; column temperature, 40°C; autosampler temperature, 10°C; flow rate, 0.3 mL min^{-1}; injection volume, 5 µL.

3.4. Comparison of results with previously published procedures

To provide a better overview of the chromatographic and sample preparation methods, and to understand the main advantages of the presented methodology, Table 4, comparing the previously published studies involving analysis of risdiplam, is presented. This comparison includes three articles published to date. In our method, the C18-PFP column is used, because it provides shorter analysis time with high peak symmetry compared to C18. C18-PFP is an interesting alternative to the conventionally used C18 column that offers different retention

mechanisms. In contrast to all papers shown to date, we used an isocratic elution thus reducing the analysis time to 3 minutes, because no re-equilibration was needed. It can be concluded that our method provides comparable recovery to other methods, while being characterized by a low matrix effect. However, it should be noted that this is the only method that uses UV detection and, despite its lower sensitivity, it can be a simple alternative to LC-MS/MS, which can be used for qualitative and quantitative analysis in quality control laboratories, routine stability monitoring, and sample purity assessment.

Table 4. Comparison of our method with previously published procedures.

Chromatographic method				
Parameters	Procedure 1	Procedure 2	Procedure 4	Our method
Analytes	Risdiplam	Risdiplam and its metabolites	Risdiplam and its metabolites	Risdiplam
Column	Phenomenex Kinetex XB C18 (2.6 µm, 50 x 3 mm)	Supelco Ascentis Express C18 (2.7 µm, 200 x 2 mm)	YMC Triart C18 (3 µm, 50 × 2 mm)	Avantor® ACE® Excel® C18-PFP, Analytical UHPLC (1.7 µm, 100 × 2.1mm)
Mobile Phase	A: 0.7% formic acid B: acetonitrile	A: 947.5/47.5/5 v/v/v water/methanol/formic acid B: methanol	A: 935/50/15 v/v/v water/acetonitrile/1M ammonium hydrogen carbonate pH 9; B: 5/75/10/10 v/v/v/v water/acetonitrile/2-propanol/acetone	A: 10 mM ammonium formate pH 4; B: methanol
Elution mode	Gradient	Gradient	Gradient	Isocratic
Detection	MS	MS	MS	UV
LOQ	1.95 ng mL^{-1}	-	1.95 ng mL^{-1}	0.4 µg mL^{-1}
Analysis time	6.5 min	40 min	4 min	3 min
Method	PP	PP	PP	PP
Solvents	methanol	-	mixture of ethanol and acetonitrile (2:8 v/v)	10% solution TFA

Sample volume	180 µL of serum and 1 mL of solvent	-	10 µL of serum and 30 µL of solvent	200 µL of serum and 1.8 mL of solvent
Recovery R (%)	90.89-101.44 %	92-99%	93-95%	90.79%
Matrix effect	88.30-90.69%	92-99%	85-95%	100%
Advantages and disadvantages	- Long analysis time, - Matrix effect, - Large sample volumes	- Long analysis time, - Matrix effect,	- High salt concentration shortening the life of the column	- Low sensitivity - Large sample volumes
	+ High sensitivity	+ High sensitivity, + Analysis of risdiplam and metabolite	+ Short analysis time, + Small sample volumes, + High sensitivity, + Analysis of risdiplam and metabolite	+ Short analysis time + Isocratic mode does not require stabilization of the chromatographic system + Low salt concentration + No matrix effect + Can be a simple alternative to LC-MS/MS when ultra-high sensitivity is not required.
Reference	[5]	[7]	[9]	

528

4. Conclusions

In the present study, we conducted retention studies of risdiplam using five stationary phases selected based on structure, and several mobile phases differing in the presence of salt, its concentration, and pH. The results showed that the retention of risdiplam for phenyl columns was higher than that of C18, probably due to π-π interactions. Moreover, it was noted that the retention of risdiplam depends mainly on the pH of the mobile phase. This effect is probably related mainly to the changes in the structure of the studied compound. It was shown that salt concentration did not significantly affect risdiplam retention and peak shapes. The C18/PFP column was used in the finally developed chromatographic method together with a mobile phase composed of 10 mM $HCOONH_4$ (pH 4) and acetonitrile. The method was sensitive, with good linearity over the concentration range used (0.4-10 μg precision and repeatability). It was applied to the analysis of risdiplam in the extracts from enriched human serum samples. Protein precipitation was used as a method for efficient protein removal using 10% aqueous solution of TFA (recovery 90%). Lower recoveries were obtained for three other organic solvents, consequently, they were excluded from the study. In our opinion, methanol, ethanol, and acetone should not be selected for protein precipitation during the extraction of risdiplam from serum. It may be due to the limited solubility of risdiplam in organic solvents.

Such comprehensive retention studies have provided key information about risdiplam and found application in the determination of its content in a biological matrix, which provides an opportunity for the development of previously used chromatographic methods. The signals observed on the chromatograms corresponding to protein residues indicate that the current sample preparation procedure does not ensure complete removal of high-molecular-weight components. Residual proteins can negatively affect the lifetime of the chromatographic column and the selectivity of the separation. Therefore, it is necessary to develop a more effective sample purification step that will completely reduce the protein content and increase the stability of the method.

Article Highlights

1. First comprehensive chromatographic and sample preparation study of risdiplam.
2. Comprehensive study of risdiplam retention under varied conditions using RP-UHPLC.
3. π–π interactions are crucial in the retention mechanism for risdiplam.

4. Mobile phase pH significantly affects risdiplam retention.
5. Precipitation extraction 10% TFA allowed the recovery of 90% of risdiplam.
6. Developed RP-HPLC method enables sensitive and precise risdiplam analysis

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Declarations

The samples were taken following relevant guidelines of the Medical University of Gdańsk and approved by the Independent Bioethics Committee for Scientific Research at the Medical University of Gdańsk (permission no. NKBBN/778/2022). We obtained consent for participation from all the patients.

Disclosure Statement

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

Writing assistance disclosure

No writing assistance was utilized in the production of this manuscript.

Open repository

The data that support the findings of this study are openly available in the repository: 'Risdiplam retention studies and development of extraction from serum' at https://doi.org/10.18150/TKNNOP.

Author contribution statement:

Natalia Balińska: Methodology; Investigation; Data Curation; Writing - Original Draft; Visualization

Sylwia Studzińska: Conceptualization; Methodology; Resources; Data Curation; Writing - Original Draft; Project administration; Funding acquisition

Anna Lemska: Writing - Review & Editing

Maria Mazurkiewicz-Bełdzińska: Writing - Review & Editing

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'' – of interest, or '"**" – of considerable interest, with a brief sentence explaining why in each case.*