The silica-based sorbents for the mixed-mode extraction of risdiplam and its metabolite from serum samples

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Abstract

Analysis of risdiplam and its main metabolite is critical for pharmacokinetic studies and therapeutic drug monitoring in spinal muscular atrophy. However, current analytical methods face challenges due to low analyte stability, limited selectivity, and pronounced matrix effects, with only four studies published to date. In this work, a comprehensive analytical strategy was developed for the first time, combining optimized chromatographic separation with a novel sample preparation approach. The effects of methanol content, salt concentration, and pH on retention, peak symmetry, and resolution were systematically investigated. Chromatographic conditions were optimized using a central composite design, enabling rapid selection of mobile phase and achieving efficient separation of both compounds within 3.5 min. General trends in mobile phase composition were identified, indicating optimal performance at either low salt concentration and high pH or high salt concentration and low pH. For sample preparation, dispersive solid-phase extraction was introduced using newly synthesized hydrophobic and mixed-mode sorbents designed to provide hydrophobic, π–π, electrostatic, and hydrogen-bonding interactions. The extraction procedure was extensively optimized with respect to sorbent type and elution conditions (solvent composition and the addition of salt, formic acid, or ammonia). Compared with conventional non-polar sorbents, mixed-mode materials containing alkylamide, benzoic acid, cholesterol, and residual aminopropyl groups demonstrated superior recoveries and reproducibility. Finally, an alkylamide sorbent with acidified methanol-water elution was selected. The method enabled direct extraction from serum without protein precipitation, yielding recoveries of 86% and 53% for risdiplam and its metabolite, respectively. The proposed strategy provides a robust, selective, and efficient approach for risdiplam bioanalysis.

Keywords: risdiplam; metabolite; high performance liquid chromatography; dispersive solid phase extraction (dSPE); hydrophobic-hydrophilic sorbents; serum

1. Introduction

In recent years, numerous studies have been published on the analysis of compounds with unusual physicochemical properties in biological matrices. Recent studies indicate that conventional sample preparation methods are associated with certain limitations and the need to make trade-offs regarding reproducibility and recovery [1]. The literature emphasizes that the stability of many bioactive compounds in plasma is limited, which requires the use of specific stabilization strategies, as in the case of e.g. risdiplam and its metabolite [2]. Although classical SPE remains an effective and selective sample purification technique, dSPE is a good alternative due to lower solvent consumption, shorter preparation time, and higher process efficiency [3]. Recent literature reviews emphasize that the development of analytical methods for biopharmaceuticals requires not only the optimization of chromatographic conditions but also a systematic comparison of new sample preparation strategies with existing ones [4].

Risdiplam (RIS) is the active ingredient in Evrysdi, a drug used to treat spinal muscular atrophy (SMA). SMA is a neurodegenerative genetic disease that is inherited in an autosomal recessive manner [5,6]. Until 2016, it was a fatal disease; now we have three drugs for three different therapies, significantly changing the medical prognosis for patients [7–9]. Evrysdi is the only SMA drug that can be administered in a convenient syrup form, at home [10,11]. Unlike other SMA therapies, RIS is mainly metabolized in the liver by cytochrome P_{450} enzymes and flavin-containing monooxygenases. The main metabolic pathway is an oxidative metabolism at the cyclopropyl and piperazine moieties. The primary RIS metabolite, M1, is the only exception since hydroxylation at one of the nitrogen atoms occurs [10,12,13]. Clinical trial data have shown that M1 is the major and quantitatively dominant biotransformation product, and its exposure is relevant to the safety and efficacy of the therapy [14]. The remaining metabolites have been identified exclusively in in vitro models, such as hepatocytes and microsomal systems, and there is no evidence of their formation in the human body [11]. In accordance with the principles for evaluating metabolites relevant to humans, only metabolites confirmed in vivo and contributing significantly to exposure can be considered clinically relevant. At the same time, due to the numerous and unpredictable variables affecting in vivo metabolism, it is not possible to determine which metabolites will circulate in plasma and may reach or exceed the 10% threshold of total exposure to drug-related material based solely on in vitro data [11].

Consequently, M1 remains the only metabolite meeting the criteria for an analytically relevant metabolite, while metabolites detected only in vitro do not qualify for further toxicological evaluation and were therefore not included in our study[11].

Given that SMA therapy using RIS the methods that have been used worldwide for 3 years. There is limited information available on methods for determining SMA in biological samples. This issue is important because knowledge of RIS concentration and its metabolites is important for monitoring therapy and evaluating its effectiveness. To date, only four publications on RIS analytics have been released [10,11,14,15]. The research has focused primarily on developing fast, sensitive, and selective reversed-phase ultra-high performance liquid chromatographic methods hyphenated to mass spectrometry (RP UHPLC-MS) for the determination of RIS and its metabolites in biological matrices, using conventional octadecyl (C_{18}) columns under reversed-phase conditions [6,10,11,13,14]. Although RP UHPLC-MS provides high selectivity and low detection limits, there are no unambiguous recommendations for optimal chromatographic parameters, including mobile phase composition, gradient elution, column temperature, etc., especially in the context of metabolite analysis [6,10,11,14,15]. The predominant use of C18 columns limits the ability to separate structurally similar RIS metabolites, suggesting the need to test alternative columns. Moreover, the detected blood concentrations of RIS showed high variability despite administering identical doses, indicating the need for in-depth pharmacokinetic studies and identification of factors influencing the variability of bioavailability [15] The appropriate sample preparation method is crucial in this case; however, until now, the only method used in RIS and M1 extraction was protein precipitation (PP) using methanol, ethanol, and acetonitrile [11,14,15]. It allows isolation of both compounds from serum, but with some limitations, e.g., increased susceptibility to matrix effect. In addition, RIS tends to bind strongly to serum proteins, which may lead to analyte loss during sample preparation and may affect the accuracy of quantitative analyses [14]. The literature also indicates that the efficiency of RIS extraction is sensitive to extraction conditions, such as the type of solvent used, shaking time, or temperature, limiting repeatability and the possibility of interlaboratory validation [15]. In addition, it has been shown that the stability of RIS and M1 in biological matrices is limited, implying the need to standardize and improve extraction protocols [14]. A review of the scientific literature indicates the need to further improve RIS and M1 sample preparation processes. For this reason, an attempt was made to develop a dispersed solid-phase extraction (dSPE) method as a promising alternative for the isolation of RIS and M1 from serum samples.

The goal of the study was to optimize a chromatographic method for the analysis of RIS and M1 using a design of experiments (DOE) approach. Moreover, it was aimed to develop a dSPE method using newly synthesized adsorbents modified with various functional groups (aminopropyl, alkylamide, octadecyl, phenoxypropyl, benzoic acid, and cholesterol) for the first application to RIS and M1. In adsorbents with mixed hydrophilic and hydrophobic properties, the combination of different interaction mechanisms allows RIS and M1 to interact via different mechanisms (hydrophobic, π–π, hydrogen bonding, electrostatic interactions), resulting in greater selectivity and capacity than commercial non-polar or polar adsorbents. The potential of mixed-mode dSPE was studied for the first time for RIS and its metabolite. Such studies have not been conducted previously for either compound, either in solid-phase extraction or dispersive solid-phase extraction. The approach is novel and has proved to be useful for serum samples.

2. Materials and methods

2.1. Materials

RIS, with the full name 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, (purity≥ 99.51%) was purchased from Merck (Darmstadt, Germany) (Figure 1). The stock solution was prepared by dissolving 1 mg in 1 mL of 0.1% formic acid (FA). The solution was stored in a refrigerator at a nominal 4 °C. Subsequent dilutions were prepared by diluting the stock solution with deionized water (H_2O) or 10 mM ammonium acetate (AcAm) (pH 11). M1 with the full name 2-(2,8-Dimethylimidazo[1,2-b]pyridazine-6-yl)-7-(4-hydroxy-4,7-diazaspiro[2.5]oct-7-yl)-4H-pyrido[1,2-a]pyrimidin-4-one (purity≥ 95.2%) for HPLC was purchased from ALSA CHIM a Shimadzu Group Company (Illkirch, France) (Figure 1). The M1 stock solution was prepared by dissolving 1 mg in 1 mL of 0.1% FA. The working solutions were prepared by diluting the stock solution to 0.1 mg mL^{-1} with deionized H_2O. They were prepared freshly before use or stored at -20 °C (thawed at room temperature). As the analytes are light-sensitive, all preparations were performed in the dark, and solutions were stored in the dark. Standard solutions were stored in glass or polypropylene amber tubes. L-ascorbic acid (0.2 or 10 mM) was added to all RIS-M1 mixtures for stabilization. Their concentrations used during dSPE studies were equal 12 μg mL^{-1}. Dilutions of the stock solutions for the dSPE were carried out using either a conditioning solution (10 mM AcAm, pH 10) or an elution solution.

The following reagents were used for chemical modification of the silica support: (3-aminopropyl)trimethoxysilane, 3-phenoxypropyldimethylchlorosilane, benzoyl chloride (≥

99%), lauroyl chloride (≥ 99%), n-octadecyldimethylchlorosilane, triethylamine (≥ 99%), cholesteryl chloroformate (≥ 99%), and morpholine. They were purchased from Alfa Aesar (Haverhill, MA, USA). The organic solvents used during the synthesis, methanol, 2-propanol, and toluene, were purchased from J.T. Baker (Deventer, Netherlands).

The support for adsorbents synthesis was silica gel with an average pore diameter of 60 Å (52-73 Å), a pore volume of 0.7-0.85 cm^3/g, and a particle size range of 63-200 μm. It was purchased from Merck (Warsaw, Poland).

Mobile phases were prepared using gradient-grade methanol (MeOH), ammonium formate (AF) (purity ≥ 99.99%), FA (purity ≥ 99.99% for LC-MS) (Merck, Darmstadt, Germany), and a 25% solution of ammonia (Avantor, Gliwice, Poland). The washing solution for the autosampler was prepared using 2-propanol, acetonitrile, and HPLC-grade methanol (Sigma-Aldrich, Dorset, UK). Deionized H_2O was obtained from the Milli-Q H_2O purification system (Millipore El Passo, TX, USA).

The following solvents were used for sample preparation and dSPE extraction: AcAm (≥ 99%) (Merck, Warsaw, Poland), L-ascorbic acid (≥ 99%) (Avantor, Gliwice, Poland)25% solution of ammonia (Avantor, Gliwice, Poland), FA (purity ≥ 99.99%, for LC-MS) (Merck, Warsaw, Poland).

PP was done with the use of ethanol and acetonitrile (purity ≥ 99.99%) (Merck, Warsaw, Poland).

2.2. Apparatus and chromatographic conditions

The Thermo Vanquish Flex (Karlsruhe, Germany) chromatographic system equipped with a PDA (Photo-Diode Array) detector was used. Experimental data were collected using Thermo Scientific Chromeleon 7.0 software. UV detection was performed at the following wavelengths: 210, 240, 255, and 277 nm (the absorption maxima for RIS and M1). The 255 nm was selected for the quantitative analysis. Isocratic elution was used during the study with a mobile phase composed of 60/40 % v/v methanol (mobile phase B) and 60 mM $HCOONH_4$ (pH 4) (mobile phase A). The flow rate was 0.3 mL min^{-1}, and the injection volume equaled 2 μL. The column temperature was 30 °C, while the autosampler temperature was 10 °C. The study used a Discovery HS C_{18} column (3 μm, 2.1×50 mm) (Supelco, Darmstadt, Germany). The autosampler washing solution contained 2-propanol/H_2O/acetonitrile/methanol (25/25/25/25, v/v/v/v) with 0.1% ammonium hydroxide to reduce the autosampler carryover. Dead time was determined using the solvent peak. Retention factor (k) was calculated by using a standard equation (the difference between retention time and dead time divided by the dead

time). The peak asymmetry (f_{AS}) (at 5% peak height according to European Pharmacopeia definition) and resolution (according to European Pharmacopoeia and the United States Pharmacopoeia, measured at 50% of the peak height for the width of both peaks) were measured by Chromeleon 7.0 software.

The pH meter CP-505 Elmetron (Zabrze, Poland) was used to measure the pH of the mobile phases and the conditioning and absorption solutions. The Frontier Micro FC5515 centrifuge (OHAUS Europe GmbH, Nänikon, Switzerland) was used for centrifugation during dSPE.

Elementary analysis was carried out using a Vario MACRO CHN analyser from ELEMENTAR Analysensysteme GmbH (Langenselbold, Germany). Fourier-transform infrared (FTIR) spectra were recorded on an FT-IR Vertex 70V spectrometer equipped with a Hyperion 1000 microscope from Bruker (Leipzig, Germany) in the range of 4000-400 cm^{-1}. The nuclear magnetic resonance (NMR) spectra were obtained on a Bruker Avance III 700 MHz spectrometer (Bruker BioSpin, Billerica, MA, USA), equipped with a 3.2 MAS probe, and operating in a 13C resonance sequence at 176.10 MHz. Adsorbent samples were placed on a rotor of zirconium dioxide (ZrO2) with Kel-F caps, with a rotation frequency of 12 kHz. The spectra were obtained by collecting 4,096 datapoints, at an acquisition time of 27 ms and with a 4 s recycle delay. Spectral collection, elaboration, and integration of spectral regions for characteristic functional groups were performed using Bruker Topspin 3.2 Software.

2.3. Optimization of chromatographic separation using design of experiments

In our study, the chromatographic separation of RIS and M1 was optimized using a central composite design (CCD). The main objective was to investigate the effect of mobile phase composition, namely percentage part of methanol (30-70% v/v MeOH), salt concentration (10-50 mM $HCOONH_4$), and pH (4-7) on the resolution (R_s), f_{AS}, and k for both compounds. The CCD plan consisted of three parts: eight factorial points, six axial points, and one center point. All measurements were performed in duplicate. The data were processed and graphically presented using the Statistica package. The resulting models were presented as two-dimensional graphs (2D contour plots) to illustrate the influence of the variables on peak shape and resolution.

2.4. Synthesis and characterization of new adsorbents with various functional groups

We have synthesized and tested five silica-based sorbents modified with various functional groups, including alkylamide (AP), octadecyl (C18), phenoxypropyl (PhO), benzoic

205 acid (BA), and a cholesterol molecule (CHOL). All structures of synthesized materials are
206 shown in Figure 2.
207 Before chemical modification, 10 g of silica gel was dried at 180 °C under vacuum for
208 5 h to remove adsorbed H_2O. Next, the temperature was reduced to 70 °C, and (3-aminopropyl)
209 trimethoxysilane (8 mL) in toluene was added. The entire suspension was mixed for 16 h. The
210 synthesized aminopropyl material was washed with toluene, isopropanol, and methanol, and
211 dried 16 h at 80 °C in the oven. The sorbent was next used as a support for the synthesis of three
212 different sorbents (AP, BA, CHOL).
213 1.5 g of aminopropyl sorbent was mixed with 10 mL toluene, 1.2 mL of dodecanoyl
214 chloride, and 0.4 mL of triethylamine. The reaction was carried out at 70 °C for 16 h under
215 magnetic stirring. The reaction product was an AP adsorbent (Figure 2). It was washed using
216 toluene, 2-propanol, and methanol. The adsorbent with aromatic ring (BA) (Figure 2) was
217 prepared in a similar procedure, however, using different reagents: 0.9 mL of benzoyl chloride
218 in 10 mL of toluene and 0.4 mL of triethylamine. The CHOL adsorbent (Figure 2) was
219 synthesized using 1.5 g of aminopropyl material, 1.3 g of cholesteryl chloroformate in 10 mL
220 of toluene, and 0.4 mL of triethylamine. All the other conditions (temperature, time, drying, and
221 washing procedure) were similar to AP procedure.
222 The non-polar C_{18} adsorbent (Figure 2) was synthesized in a one-step procedure. The 5
223 g of dried silica gel was mixed with 10 mL of n-octadecyldimethylchlorosilane in 10 mL toluene
224 and 2 mL morpholine in 2 mL toluene. The synthesis was carried out at 70 °C for 16 h. The
225 adsorbent was washed with toluene, isopropanol, and methanol, and dried for 16 h at 80 °C.
226 The adsorbent with phenoxypropyl groups (PhO) (Figure 2) was synthesized in the
227 following way: 5 mL (3-phenoxypropyl) dimethylchlorosilane in 10 mL toluene and 1 mL
228 morpholine in 1 mL toluene were added to 5 g of dried silica gel. The reaction was carried out
229 at 70 °C for 16 h. The synthesized material was washed with toluene, isopropanol, and
230 methanol, and dried for 16 h at 80 °C.
231
232 *2.5. Development of dSPE procedure for the extraction of RIS and M1*
233 The dSPE procedure was carried out in 500 µL Eppendorf tubes using 2.0±0.1 mg of
234 adsorbents (each in two replicates).
235 The developed dSPE procedure included: 1. adsorbent conditioning, 2. sample load, 3.
236 washing, 4. elution. During adsorbent conditioning, the following solvents were applied: 200
237 µL of MeOH, 200 µL of purified H_2O, and 200 µL of the solution used during sample load. The
238 following solvents for adsorption were tested: H_2O, and 10 mM AcAm (pH 4 and 11). Sample

load was performed using 100 µL of 12 µg/mL solution of RIS and M1 with 0.2 mM L-ascorbic acid dissolved in the solvent for adsorption. The 100 µL of 1/9 (v/v) MeOH/10 mM AcAm (pH 4 or 11) was used for washing. The following parameters were tested during elution: the effect of the type of organic solvent (methanol, ethanol), the effect of the content of the organic solvent (1/1, 8/2 v/v), the addition of salt (10 mM, 50 mM AcAm), the addition of FA (0.1%, 1%) and ammonia (1%), on the recovery RIS and M1. Mixing using vortex and centrifugation for 15 min (14,000 rpm) was performed at each step of dSPE procedure. During the recovery determination, the peak area of the standard was compared with the eluate containing RIS and M1. For a proper assessment, the standard was dissolved in the same solution as the eluate.

Three approaches for extracting RIS and M1 were tested to verify their stability during extraction. The first involved adding ascorbic acid only to the elution solution, the second involved the addition of ascorbic acid during sample load, and the third involved spiking the sample with ascorbic acid during sample load and after elution (to eluate).

2.5.1. Optimum dSPE procedure

The developed dSPE procedure included AP adsorbent and: 1. conditioning using 200 µL MeOH, 200 µL H_2O and 200 µL of 10 mM AcAm (pH 10); 2. sample load using 100 µL of RIS and M1 (12 µg mL^{-1}) with 0.2 mM L-ascorbic acid in 10 mM AcAm (pH 10); 3. washing using 100 µL of 9/1 (v/v) 10 mM AcAm (pH 10)/MeOH; elution using 100 µL of 1/1 (v/v) MeOH/water with 1% FA and 0.2 mM L-ascorbic acid. The total sample preparation time is 90 minutes. It is possible to prepare 18 samples simultaneously.

2.6. Validation of chromatographic method

Linearity was determined by calculating the coefficient of determination (R^2) for the calibration curve in the range of 1-20 µg mL^{-1}. Validation of the chromatographic method was performed based on linearity, limit of detection (LOD), limit of quantification (LOQ), and intraday and interday precision. LOD and LOQ were calculated in the following way: LOD = 3(S/N) and LOQ = 3.3 LOD, where S is signal, while N - noise. The relative standard deviation (RSD) of the peak area for 10 injections on a single day at three concentrations (4, 10, and 16 µg mL^{-1}) was calculated to assess intraday accuracy. The relative standard deviation of the peak area for five injections at three concentrations (4, 10, and 16 µg mL^{-1}) on the first, third, and seventh days was calculated to assess repeatability.

2.7. Adsorption kinetics, desorption, and sorption capacity

These experiments were carried out for AP adsorbent. The experiment for the determination of time needed to reach adsorption equilibrium (adsorption kinetics) and desorption kinetics was carried out using 2 mg of AP and dSPE procedure described in section 2.5.1. For both attempts, the mixture of RIS and M1 (12 μg mL^{-1}) spiked with 0.2 mM L-ascorbic acid was used. Next, 20 μL of the solution above the adsorbent were taken at given time intervals (1, 2, 5, 10, and 20 min), centrifuged for 10 min at 14,000 rpm, and analyzed using a developed RP UHPLC method (determination of RIS and M1 adsorption). For desorption kinetics, the solution was collected at given time intervals (1, 2, 5, 10, and 20 min) and centrifuged for 10 min at 14,000 rpm.

Sorption capacity was determined using 1 mg of AP. The tests were performed using three different volumes (20, 50, and 100 μL) of 100 μg mL^{-1} solution of RIS. The same procedure was used for M1. The value of the adsorption capacity Q_e, [μg/mg] in equilibrium was calculated by the following equation:

$$Q_e = \frac{(C_0 - C_e) * V}{\mathrm{m}}$$

where C_0 is the initial concentration [μg mL^{-1}], C_e is the equilibrium concentration [μg mL^{-1}], V is the initial volume of sample solution [mL], and m is the mass of the adsorbent [mg].

2.8. Application of dSPE to the extraction of RIS and M1 from serum samples

Samples were taken in accordance with 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. KB/236/2025). Two different attempts were tested during the extraction of RIS and M1 from human serum samples: 1. PP, 2. dSPE.

PP was carried out by fortification of 100 μL of serum with RIS and M1 (to a concentration of 12 μg mL^{-1}) and L-ascorbic acid (to a final concentration of 10 mM). We observed that the biological matrix significantly accelerates the degradation of M1, which is susceptible to oxidation. Ensuring the stability of the RIS and M1 mixture required the use of a higher concentration of L-ascorbic acid than in the reference solutions. Next, 300 μL of an EtOH/ACN (1/4 v/v) with 10 mM L-ascorbic acid was added, mixed for 1 min, and centrifuged for 10 min at 14,000 rpm. The solution above the protein precipitate was analysed using the developed RP UHPLC method.

The dSPE procedure developed during the study was also used for RIS and M1 extraction from serum. First, 100 μL of the serum sample was spiked with RIS and M1 to a

concentration of 12 μg mL^{-1}, and L-ascorbic acid (to a final concentration of 10 mM). Next, the sample was diluted in a ratio of 1:3 (v/v) with the 10 mM AcAm (pH 10). The L-ascorbic acid was added to the samples (to the final concentration of 10 mM) to maintain M1 stability. Next, the entire dSPE procedure was carried out in accordance with the method described in section 2.5.1 (with the difference that the antioxidant concentration was 10 mM).

The matrix effect (ME) was determined using the post-extraction addition protocol presented in the literature data [9]. The blank serum was subjected to the entire dSPE procedure without standard fortification. Next, RIS and M1 were added to the obtained blank extract at the same concentrations as the standard. ME was calculated by comparing the peak areas of the standards with those of the blank extract fortified with standards. The following equation was used for this purpose:

$$ME = \frac{A1}{A2} * 100\%$$

where: ME-matrix effect; A1-peak area of RIS/M1 in post-extraction spiked matrix; A2-peak area of RIS/M1 standard.

3. Results and discussion

3.1 RIS and its M1 stability issues

The development of a method for the extraction and determination of RIS and its metabolite M1 may provide a useful tool for assessing pharmacokinetics and studying biotransformation pathways [6,10,14]. Currently, our knowledge of the analytics of RIS and M1 is very limited, and significant improvements are needed. For this reason, the first studies on optimizing the composition of the mobile phase for the separation and analysis of RIS and M1 mixtures were undertaken. These studies were conducted for the first time and are important because they expand our understanding of the rapid selection of optimal conditions for the analysis of both compounds. For the first time, an attempt was made to extract RIS and M1 from biological samples using dSPE, which should enable better purification compared to previously used PP.

The first obstacle in routine laboratory work with RIS and M1 was their low stability and susceptibility to degradation. M1 proved to be much more demanding in this respect. This was critical, especially during the development of dSPE procedure. Literature data confirm our observations and indicate that RIS stability is problematic, especially light sensitivity, carryover, and nonspecific binding [14]. The M1 is prone to oxidative degradation [11].

The solubility and stability of RIS and M1 were assessed in various solutions: H_2O, 0.1% FA, and 0.01% FA. Based on our laboratory practice and observations, we can conclude that both compounds are insoluble in water but soluble in acidified water. The stock solutions were prepared in 0.1% FA and the working solution was prepared by dilution of the stock with water. RIS remains stable for approximately four weeks when dissolved in 0.1% FA, stored at 4 °C, and protected from light. Regarding M1, its degradation in acidified H_2O was rapid. Based on previous research [14], L-ascorbic acid was added to the mixture to increase M1 stability. In samples prepared without L-ascorbic acid, the peak area of M1 decreased by approximately 43%, confirming its marked instability.

We conducted systematic experiments on the stability of the RIS and M1 mixture in the presence of 0.2 mM L-ascorbic acid. The samples were stored at room temperature and at 4 °C for 30 minutes and 1 hour, respectively. Chromatographic analyses of samples collected after these time periods revealed no degradation of RIS under any conditions. M1 degraded by 5% under 4 °C during 30 minutes. In contrast, significantly greater degradation of M1 was observed at room temperature: 15% after 30 minutes, and 30% after one hour. These results indicate that the RIS and M1 mixture in the presence of vitamin C exhibits limited stability at room temperature, whereas storage at 4°C significantly slows degradation.

Two concentrations, 0.2 mM and 5 mM, were tested. Results indicate that L-ascorbic acid effectively stabilized M1 at 0.2 mM, providing sufficient protection. However, 0.2 mM ascorbic acid was insufficient to maintain its stability when serum samples were used. Therefore, 10 mM was applied during the extraction of RIS and M1 from serum samples.

Finally, the stock solution of M1 (in 0.01% FA) was portioned into 150 µL and frozen (-20 °C). The portioned M1 was thawed on ice in a thermal box. The mixture of RIS and M1 with 0.2 mM ascorbic acid was prepared fresh, just before analysis or extraction.

3.2. Optimization of chromatographic separation RIS and M1 using a CCD

We used a design of experiments with CCD to optimize the chromatographic separation of RIS and M1. The CCD allows to expand beyond the initial experimental space, provides a wide range of information, and significantly improves method optimization. Moreover, accurate capture of nonlinear dependence between factors and responses is possible without testing each factor individually. CCD allows rapid (15 experiments in replicate) and effective selection of the mobile phase composition for the separation of RIS and M1, yielding symmetrical peaks in a short time. The main goal was to investigate the impact of MeOH content, salt concentration, and pH on R_s, k, and f_{AS}. These parameters were selected based on RIS and M1 properties,

primarily polarity, which increases upon protonation; consequently, to ensure proper peak symmetry and improve resolution, the changes included AF concentration and pH. The percentage of MeOH in the mobile phase allows optimization of the elution strength, enabling effective separation. The salt concentration minimizes analyte-stationary phase interactions, thereby affecting peak shape. Maintaining a controlled pH in the mobile phase results in reproducible retention times and better peak symmetry. These parameters were chosen to separate RIS and M1, which differ only by one additional hydroxyl (Figure S1). The tested parameters and values obtained in the experiments are summarized in Table S1. Based on these data, 2D contour plots were created (Figure 2 and S2). The graphs allowed determination of the range of values for the tested parameters, ensuring optimal separation, peak symmetry, and retention.

Considering peak asymmetry for RIS it will be high at high salt concentrations (50-60 mM), MeOH content (30-45%), and pH in the range of 6-8 (Figure 2A). Increasing the pH and salt concentration improves peak symmetry by reducing analyte interaction with residual silanol groups on the silica surface, a phenomenon typical of C_{18} columns under alkaline conditions [16]. Lowering the pH (below 3.5), AF concentration (below 30 mM), and percentage part of MeOH in the mobile phase reduces symmetry (f_{AS}>2). The effect may be related to the protonation of RIS and to a probable reduction in interactions with the non-polar C_{18} stationary phase. In the case of M1 (Figure 2B), symmetrical peaks can be obtained for the following parameter ranges: 45-55% (v/v) of MeOH, 30-45 mM AF, with pH in the range of 2-4. Each of these parameters affects the interaction of M1 with the surface of the stationary phase and, consequently, the peak symmetry and separation. Figure 3C shows the CCD results for the impact of mobile phase composition on R_s. The high R_s values (exceeding 35) for RIS and M1 will be achieved, but this leads to very long analysis times (over 100 min) (Figure 3C). For this reason, mobile phase conditions were selected to ensure R_s lower than 3. At high MeOH concentrations (≥70%), regardless of AF concentration or pH, RIS eluted close to the dead time. A decrease in R_s was observed with increasing pH and salt concentration. Obtaining R_s in the range of 1.5 to 4 is possible for AF concentrations from 20 mM to 60 mM with a simultaneous change in pH from 9 to 2. This means that the salt in the mobile phase should have either a low concentration and high pH or a high concentration and low pH. This effect is most likely due to the interaction of two mechanisms: (1) changes in the degree of protonation of the analytes, and (2) control of silanol surface activity through shielding. Residual silanol groups on the surface of the C18 stationary phase are a source of electrostatic and donor-acceptor interactions that significantly affect retention, peak symmetry, and resolution. Lowering the pH of the

mobile phase protonates silanol groups, thereby significantly limiting their ionization and reducing electrostatic interactions with analytes. Under the same conditions, RIS and M1 undergo protonation, which reduces their affinity for the C18 phase. An increase in the ionic strength of the mobile phase (i.e., higher salt concentration) reduces analyte-stationary phase interactions through strong screening of electrostatic interactions. Under basic conditions (pH≥7), the situation is reversed: silanols occur mainly in the ionized form, which increases their surface acidity and the potential for electrostatic interactions. At the same time, analytes undergo partial deprotonation, which increases their retention but also their susceptibility to interactions with ionized silanols. Under these conditions, even a low salt concentration can effectively shield the charge on residual silanols [17]. As a result, both low-salt concentration/high pH and high-salt concentration/low pH provide satisfactory selectivity and a resolution of 1.5-4.

As for the effect of MeOH, 45-60% (v/v) MeOH provides R_s below 4. These dependencies suggest that the separation of both compounds results from differences in their affinity to the stationary phase. A comparison of the graphs for f_{AS} and R_s shows that a mobile phase composition providing good peak symmetry may not allow for obtaining an adequate R_s (below 3). A compromise is needed in the mobile phase composition, allowing a reduction in fAS while ensuring an acceptable Rs within a short analysis time. Finally, CCD allowed us to select three different mobile phase compositions suitable for achieving separation and peak symmetry: 1. 40/60 (v/v) 60 mM AF (pH 4)/MeOH, 2. 40/60 (v/v) 60 mM AF (pH 7)/MeOH, 3. 40/60 (v/v) 80 mM AF (pH 8)/MeOH. The chromatograms shown in Figure 4 illustrate the effects of these three attempts. At 80 mM AF (pH 8), was observed very symmetrical peaks (f_{AS} for RIS 1.14, f_{AS} for M1 1.24), but R_s (1.7) is insufficient. By lowering the AF concentration to 60 mM and the pH to 7, an increase in R_s (12.6) and a decrease in peak symmetry (1.41 for R, and 1.25 for M1) were noticed (Figure 4). The highest R_s (13.1), with symmetrical peaks (1.37 for RIS and 1.20 for M1), and the shortest analysis time, was achieved using 60 mM AF (pH 4). The finally selected mobile phase composition was 40/60 (v/v) 60 mM AF (pH 4)/MeOH. These conditions provided the shortest analysis time (2.5 min) (Figure 4). The high salt concentration leads to symmetrical peaks and limited analyte interaction with the stationary phase, whereas pH 4 protonates RIS and M1, ensuring their chemical stability and short, reproducible retention times.

In our opinion, the greatest disadvantage of the selected method is the high salt concentration, which allows us to obtain symmetrical peaks, but cannot be used in mass spectrometry (MS) due to potential signal suppression and salt deposition in the ionization

source. Moreover, it requires the need to frequent rinsing of the instrument with H_2O. Due to these limitations, a new method was developed that enables the analysis of both compounds using gradient elution with a much lower salt concentration (10 mM AF, pH 4). A linear gradient of 40-90% (v/v) MeOH in 7 min was used (Figure S2). This method can be used for MS. However, this method is longer because re-equilibration after the gradient is necessary. For the development of the extraction method, the CCD-based method was used due to its short analysis time (3 min).

3.3. Sorbents

3.3.1. Justification for the selection of sorbents for the extraction of RIS and M1 using dSPE

In this study, dSPE was used for the first time to extract RIS and M1. Literature data on the application of PP showed some disadvantages (described in the Introduction), e.g. not complete purification (remaining potentially interfering compounds). Consequently, our goal was to develop a method to improve sample purification and selectivity, particularly with complex biological matrices. We assume that dSPE enables more effective purification of samples by selectively isolating RIS and M1. Instead of using commercially available adsorbents, new ones were synthesized due to their probable advantages for both analytes. However, we have synthesized one sorbent similar to commercially available ones, namely C18 (Figure 1, Figure 2), with bonded octadecyl, non-polar alkyl chains. It served as a reference material, against which was compared the results obtained for other sorbents with mixed, hydrophobic-hydrophilic properties. Synthesizing new sorbents enables us to tailor their properties for RIS and M1 adsorption and desorption, as our approach combines multiple interaction types, including hydrophobic, π-π, electrostatic, and hydrogen bonding. The rationale for this attempt is also based on the structures of RIS and M1 (Figure S1; Figure 1), which can interact with adsorbents or other molecules through various interactions. Synthesized sorbents were used for dSPE in mixed-mode, which has recently been gaining popularity [18]. It was expected that the reduced matrix effect and high, reproducible recoveries of RIS and M1, which are crucial for quantitative methods used in therapy monitoring.

The silica-based adsorbents were modified with the following functional groups: alkylamide (AP), phenoxypropyl (PhO), benzoic acid (BA), and cholesterol (CHOL). AP, CHOL, and BA contain residual amino groups and amide bonds that enable electrostatic interactions and hydrogen bonding. The use of different types of interactions in mixed-mode dSPE for RIS and M1 has not yet been reported in sample preparation for these compounds.

3.3.2. Instrumental characterization of adsorbents

All adsorbents were characterized using elemental analysis, FTIR and NMR. The first attempt allowed the determination of the percentage content of carbon, nitrogen, and hydrogen. The percentage content of carbon and hydrogen for AP, BA, and CHOL indicate that the ligands were bound to the surface of the support (aminopropyl silica-based material, abbreviated as NH_2). As expected, the nitrogen content remained at the same level as before modification, because in the second step of synthesis, the attached ligands did not contain nitrogen atoms (Table S2). Adsorbents differ significantly in their carbon and hydrogen content, which results from the types of attached functional groups. The highest carbon and hydrogen load was observed for CHOL and C18 adsorbents, which is consistent with the size of cholesterol molecule and the long aliphatic chain (C18). For the PhO adsorbent, the carbon and hydrogen content was lower than for CHOL and C18, while the lowest values were observed for AP and BA (Table S2). This is due to the presence of a shorter aliphatic chain and a relatively small ligand attached to the adsorbent surface.

The surface coverage density of the ligands was calculated using the Berendsen formula described by Bocian et al. [19] and included in the supplementary materials. Coverage density ranges from 1.50 to 3.08 μmol/m² (Table S2), indicating effective modification of the silica surface in all tested adsorbents. The highest coverage densities were observed for the PhO and C18 (3.08 and 2.28 μmol/m² respectively) (Table S2), suggesting effective attachment of ligands to the support surface. The high coverage density in this case may result from the small molecular size of the ligand (compared to e.g., CHOL) and its favorable spatial orientation, allowing for effective attachment to the silica surface. The lower coverage density was determined for BA, CHOL, AP (Table S2). In the case of materials synthesized using a two-step procedure, these values may be limited by steric interactions between neighbouring aminopropyl functional groups, which may reduce the availability of reactive sites. In summary, the coverage density values confirm the effective modification of the silica surface, with differences between sorbents mainly resulting from their molecular sizes and the availability of functional groups.

Regarding the FTIR characterization of adsorbents, the spectra are presented in Figure S3. The intense bands present in all spectra in the range of 1080-800 cm^{-1}, correspond to Si-O-Si vibrations (Figure S3). Additional signals for AP, BA, and CHOL appear in the range of 1550-1600 cm^{-1} and originate from the deformation vibrations of the -NH_2 group (Figure S2 A-C). There are bands in the range of 2920-2850 cm^{-1} corresponding to C-H vibrations of the aliphatic chain in the FTIR spectrum of the AP, CHOL, and C18 (Figure S1 A, C, D). The band

at 1650 cm^{-1} corresponds to C=O vibrations originating from the amide group in the case of AP, CHOL, and BA (Figure S3 A). Moreover, bands observed in the range of 1450-1600 cm^{-1} for BA and PhO correspond to aromatic ring vibrations (1600-1585 cm^{-1} C=C stretching vibrations in the aromatic ring;1470-1450 cm^{-1} C-H deformation vibrations in the aromatic ring) (Figure S3 B, E). The band in the 750-850 cm^{-1} range corresponds to out-of-plane deformational vibrations of the ring C-H bonds, which confirms the presence of an aromatic group. In the case of CHOL adsorbent, the FTIR spectrum shows bands in the range of 2800-3000 cm^{-1} (Figure S3 C). They correspond to the stretching vibrations of the C-H of the steroid and aliphatic groups. The bands at 1450 cm^{-1} and 1380 cm^{-1} correspond to the bending vibrations of CH_2 and CH_3 (Figure S3 C). Additionally, for CHOL, the C=C stretching vibration at around 1650 cm^{-1} confirms the presence of the cholesterol fragment.

The 13C NMR spectrum of AP (Figure S4 A) shows a signal corresponding to aliphatic chains (CH_2 groups) for chemical shifts of 29–32 ppm. Additional signals originating from the methylene group are observed at 33–35 ppm. The chemical shift of 45 ppm originates from CH_2–NH–C(O) group. The 13C NMR spectrum of BA shows four main signals, whose distribution is consistent with the expected structure (Figure S4 B). The chemical shifts between 170–175 ppm correspond to the carbonyl group in amide one (C=O). Moreover, signals originating from carbon atoms of the benzene ring (125–140 ppm), CH_2–OH group (55–65 ppm), CH_2 groups bonded with nitrogen (35–45 ppm) were recorded (Figure S4 B). The 13C NMR spectrum of CHOL (Figure S4 C) shows a series of signals confirming successful binding of a cholesterol molecule. The chemical shift of: 160 ppm corresponds to C=O; 120–140 ppm relates to C=C double bond (unsaturated centre in the steroid skeleton); 20–80 ppm corresponds to saturated carbons (Figure S4 C). The 13C NMR spectrum recorded for C18 shows a typical pattern for compounds containing a long, saturated alkyl chain (Figure S4 D): the terminal methyl group (CH_3) (14 ppm) and the –CH_2– groups (22–34 ppm). The 13C NMR spectrum for PhO also confirms successful bonding of this ligand to the silica surface, since chemical shifts characteristic for aromatic ring (160–165 ppm), carbon atoms of the aromatic ring (125–145 ppm), Ar–O–CH_2 group (70–80 ppm) were observed (Figure S4 E).

Both FTIR and 13C NMR confirmed the successful modification of the silica surface by the presence of bands characteristic of functional groups.

It is worth noting that the adsorbents used in this study were modified in our laboratory using silica supplied by the manufacturer. The nature of the introduced functional groups was confirmed using basic spectroscopic techniques (IR, NMR); however, these studies did not include a quantitative analysis of the ratio of these functional groups. Due to the need to work

with the exceptionally unstable M1 metabolite, the priority was to develop a method enabling stable, reproducible recovery of risdiplam and M1. In this context, the use of a sorbent with a confirmed structure, but without further analysis of the ratio of functional groups, was sufficient. At the same time, we would like to note that a systematic study of the influence of the proportions of individual functional groups on the sorption properties of the sorbent represents an interesting direction for future work.

3.3.3. Development of the dSPE procedure

The work aimed to develop an effective dSPE procedure for isolating RIS and M1 from biological samples. Was assumed that this approach could increase selectivity while simplifying the dSPE procedure (pH changes, reduction in the use of organic solvents, or the use of low-concentration salts) compared to previously used approaches.

3.3.3.1. Conditioning

The methanol was used as the first solvent, followed by water. This two-step conditioning was applied for C18, PhO, and CHOL. For BA and AP, a third conditioning step with 10 mM AcAm (pH 10) was required, as complete adsorption occurred at alkaline pH for both materials.

3.3.3.2. Sample load - adsorption step

Due to the ease of degradation of M1, was knew was might encounter problems during dSPE, which involves several steps. For this reason, the effect of adding L-ascorbic acid (0.2 mM) during adsorption and elution was evaluated. Adding an antioxidant only to the adsorption mixture did not provide complete stability of M1, as it undergoes oxidation during elution. On the other hand, adding L-ascorbic acid only to the elution solution did not protect the M1 at earlier stages, leading to its loss. The best results were obtained when L-ascorbic acid was added simultaneously to both the adsorption and elution solutions. This approach ensured the stability of the M1 throughout the procedure.

The effectiveness of RIS and M1 adsorption from water and aqueous solutions of AcAm (10 mM, pH 4, 9, 10, and 11) was evaluated for all sorbents. Solvents for adsorption were selected to test the impact of RIS and M1 protonation/deprotonation, as well as protonation of aminopropyl groups (CHOL, AP, BA). The complete adsorption of RIS and M1 was observed for C18, CHOL, and PhO under all tested conditions. The pH effect is negligible, similar to the effect of salt. This is an advantage of the synthesized materials, as they can be employed for

adsorption across a wide range of solvents, ensuring the complete adsorption of both RIS and its metabolite. For C18 and PhO at pH 4, only residual silanol groups can undergo protonation; at pH> 7, they are ionized. However, most were blocked during synthesis, so their impact is low. We assume that RIS and M1 adsorption is dominated by hydrophobic interactions for C18 and by π–π interactions for PhO.

For BA and AP adsorbents, complete adsorption was observed at 10 mM AcAm (pH 11). This is probably a consequence of electrostatic repulsion between RIS and M1, and between the residual aminopropyl groups, at pH below 9.5. Under these conditions, both analytes and amino groups are positively charged, leading to electrostatic repulsion and no adsorption. Above the pKa of the amino groups (upon deprotonation), RIS and M1 can probably adsorb through hydrogen bonding (AP and BA), hydrophobic (AP), or π–π interactions (BA). Aminopropyl groups are also present on the surface of CHOL, but in this case, adsorption of RIS and M1 occurred across the entire pH range tested. This is most likely due to the size of the cholesterol molecule and the shielding of the amino groups. The simultaneous participation of hydrogen bonds and hydrophobic interactions are responsible for RIS and M1 adsorption at the surface of this material.

Based on the obtained results, the following adsorption solvents were selected: water for PhO, CHOL, and C18; 10 mM AcAm (pH 10) for AP and BA. Ultimately, complete analyte adsorption was achieved with all five tested sorbents.

3.3.3.3. Washing step

The washing step involved the usage of 90/10 % v/v 10 mM AcAm (pH 11)/MeOH (AP, BA) or 90/10 % v/v water/MeOH (C18, PhO, CHOL). Under these conditions, no elution of RIS and M1 was observed.

3.3.3.4. Elution – desorption

The desorption of RIS and M1 from synthesized adsorbents was studied by modifying the composition of the eluent, including changing the type and proportion of organic solvent, salt concentration, and pH. MeOH and EtOH were selected based on RIS and M1 solubility (contrary to acetonitrile) and ability to weaken hydrophobic interactions. The acidification of the eluent leads to protonation of the amino groups in AP, CHOL, BA, and nitrogen in RIS and M1. Consequently, electrostatic repulsion occurs. Alkalization allowed us to study the impact of deprotonation. The application of salts aimed at modifying ionic strength and influencing the desorption of polar compounds from the surfaces of hydrophobic-hydrophilic sorbents. The

selection of these parameters enabled a comprehensive and controlled analysis of elution/desorption mechanisms of RIS and M1 from all tested sorbents and optimization of the process using sorbents for RP and mixed-mode dSPE. Figure 5 shows the effect of eluent composition on the recovery of RIS and M1.

In the first stage, the effect of acidification (using 0.1 and 1% FA) of the MeOH/H_2O mixture (1:1 v/v) was studied. A significant increase in RIS and M1 recovery (up to 40%) was observed after the addition of FA for all tested adsorbents (Figure 5). In the case of BA, AP, and CHOL, this effect results from the protonation of residual amine groups of the adsorbent and nitrogen atoms in the RIS and M1 structures. The electrostatic repulsion under these conditions promotes analyte desorption. Increased recovery for C18 and PhO can be attributed primarily to the protonation of nitrogen atoms in the structures of RIS and M1, which increases their polarity and weakens hydrophobic interactions in the case of C18 and π–π interactions between the analyte and PhO. Recoveries for both solvents (MeOH/H_2O, MeOH/H_2O with 1% FA) were repeatable, except for BA (Figure 5). In most cases, recoveries were greater than 67% for RIS and 55% for M1 when 1% FA was added to MeOH/H_2O (1:1 v/v) mixture, contrary to results for MeOH/H_2O (Figure 5).

A different effect was observed when 1% NH_4OH was added to the MeOH/H_2O (1:1 v/v) mixture. Decreases in recoveries for RIS and M1 were observed across all synthesized materials (Figure 4 and 5). These values were the lowest among all solvents tested during optimization of elution, since they were in the range of 12-69% for RIS and 12-42% for M1 (Figure 5). Under these experimental conditions (high pH of the eluent), neither the analytes nor the groups on the surface of the sorbents have a charge. As a result, electrostatic repulsion does not contribute to desorption, which may reduce recovery, as protonation provides more effective desorption of RIS and M1. This effect indicates that RIS and M1 are more strongly retained on adsorbent surfaces under alkaline conditions than under acidic conditions. It is probably related to other types of interactions, e.g., hydrogen bonds (AP, CHOL, BA), hydrophobic interactions (C18), and π–π interactions (PhO, BA), which participate in adsorption and reduce desorption. Reduction of recovery for an alkaline solution was the greatest for RIS and C18, and PhO (a decrease of 37-48%), as probably hydrophobic and π–π interactions are the most influential. Furthermore, the repeatability was satisfactory for RIS, but very low for M1, proving that 1:1 v/v MeOH/H_2O with 1% NH_4OH is not appropriate for elution (Figure 5).

Next, the impact of the MeOH percentage (50, 80% v/v) on RIS and M1 recovery was tested (Figure 4 and 5). The trends were similar for each adsorbent and analyte: recovery

increased with increasing MeOH content (Figure 4 and 5). Recoveries ranged between 18-78% for RIS and 29-60% for M1 when 1:1 v/v MeOH/H_2O was used for elution (Figure 5). Application of 8:2 v/v MeOH/H_2O provided 47-83% for RIS and 36-81% for M1. MeOH disrupts hydrophobic interactions and reduces hydrogen bonds; therefore, increased recovery at higher MeOH percentages suggests more effective disruption of both types of interactions during desorption/elution of RIS and M1. Although the general trend for each adsorbent remains the same, the recovery increases are different. For C18, and PhO, an increase by 30% was observed for RIS, suggesting the importance of hydrophobic and π–π interactions between these adsorbents and RIS and M1. In adsorbents with residual aminopropyl groups (AP, CHOL, BA), the increase in recovery is small (5-6%), as hydrophobic interactions are likely not the most influential. Repeatability of the results is high in most cases, but it remains low for M1 and AP, CHOL, and C18 (Figure 5).

We also tested the effect of another organic solvent on RIS and M1 recovery for an eluent consisting of 8:2 v/v EtOH/H_2O. For AP, CHOL, and C18, results were comparable for both organic solvents, while they increased for BA and PhO. Different results were observed for M1, where replacing MeOH with EtOH increased recovery (except for AP and BA) (Figure 4 and Figure 5). EtOH poses greater elution strength in reversed-phase extraction (C18 and PhO in our case); however, the effect may be similar for mixed-mode (AP, BA, CHOL), where desorption is based not only on the reduction of hydrophobic interactions, but also on others related to the polarity of analytes and sorbent surface. In the case of BA and PhO, the increase in RIS recovery for EtOH probably results from more effective weakening of the π–π interactions. Although π–π interactions are not directly broken by EtOH, they become less energetically stable because ethanol competes more effectively for space on the adsorbent, facilitating the desorption of RIS. The increase in M1 recovery for CHOL, PhO, and C18 is probably due to their higher polarity, leading to weaker hydrophobic interactions with the sorbent surface and greater elution strength of EtOH. Despite the high and reproducible recoveries obtained for RIS, the results for M1 were unsatisfactory in terms of repeatability (Figure 4 and 5). In the case of AP, where M1 desorption reproducibility was good, recovery was lower than with the other solvents. For this reason, this solvent was rejected as unsuitable for the extraction of both compounds. However, it should be emphasized that if only RIS needed to be extracted, EtOH could be used for both BA and AP.

The effect of salt and its concentration on the RIS and M1 recovery was also studied for solvent containing AcAm/MeOH with the addition of 1% FA (1:1 v/v) (Figure 5). Introducing the salt for the elution solution increased the ionic strength. However, the effect of concentration

is low (differences between recoveries for 10 and 50 mM were in the range of 1-7%) (Figure 5). These data show that there is no need to use high salt concentrations for elution, which is important for direct LC-MS analysis of extracts (decreased ionization efficiency at higher AcAm concentrations). The use of high salt concentrations would necessitate an additional desalting step. When comparing the effect of adding salt to the elution solution for R, it should be noted that for CHOL, BA, the results are comparable (or lower, but only by 2-5%), while for C18 and PhO, the recovery is lower, and for AP, it is higher. The decrease of recovery for hydrophobic, reversed-phase C18 and PhO sorbents may result from the salting-out effect, which increases the affinity of the analyte to hydrophobic ligands. For AP, the change in ionic strength likely weakens hydrogen bonds and electrostatic interactions, facilitating risdiplam desorption and increasing recovery. In the case of M1 decreased recovery was noticed for C18, PhO, CHOL, and AP (Figure 5). This effect may be due to the properties of M1 and possibly its lower solubility in AcAm solutions; however, we do not have data to confirm this conclusion. Although the repeatability of the extraction procedures was good in most cases (except BA and PhO) for both RIS and M1, the low recoveries for the metabolite led to the rejection of these solutions in the final dSPE procedure.

For all adsorbents, the recovery for M1 was lower than for RIS (Figures 4 and 5), which is probably due to its higher polarity caused by the presence of a hydroxyl group in the structure. Increased polarity promotes stronger interactions with the polar adsorbent surface, which may probably hinder its desorption.

To sum up, the lowest RIS and M1 recoveries were noticed for CHOL and PhO. Surprisingly, C18 also provided very low recoveries for M1 (below 40% in most cases) (Figure 5). This sorbent was chosen as a reference material, the most commonly used in extraction, and also the only type of stationary phase used to date for RIS and M1 analyses. However, our results indicate that C18 is not suitable for the extraction of both compounds, especially for the highly polar M1. C18 binds analytes by hydrophobic interactions, which are weak for polar RIS and M1, often resulting in low and unreliable recovery. Similar results were obtained for PhO. It can therefore be concluded that the use of dSPE in RP mode is not appropriate for RIS and M1. Higher recoveries of both compounds were obtained for sorbents used for mixed-mode dSPE (Figure 5). This may indicate that, in the case of RIS and M1 capable of various types of interactions, the use of sorbents with centers participating in equally diverse interactions is beneficial to dSPE. We are the first to present such research and conclusions for RIS and M1; no such research has ever been conducted. Adsorbents for mixed-mode dSPE utilize additional interactions, allowing RIS and M1 to be adsorbed and then desorbed more effectively by

altering the pH or composition of the eluent. As a result, for RIS and M1, mixed-mode dSPE provides higher recovery, better reproducibility, and greater control of the extraction process than the previously used C18. The use of adsorbents for mixed-mode dSPE is an effective solution for the simultaneous extraction of RIS and M1, which is important for their analysis in patient samples with SMA.

The highest recoveries for RIS were noticed for BA and AP, but repeatability is greater for AP. In the case of M1, the highest recovery was obtained with BA and 8:2 v/v MeOH/H2O, but this was accompanied by the lowest repeatability. The final conditions for dSPE of RIS and M1 were selected as a compromise between the recovery of both compounds and the reproducibility of the procedure, which is why AP and 1:1 v/v MeOH/H_2O acidified with 1% FA were chosen.

3.4. Sorption and desorption kinetics and sorption capacity

Sorption and desorption kinetics, as well as sorption capacity, were determined for the finally selected AP material. The adsorption study of RIS and M1 mixture showed that 100% adsorption occurs in 1 min. Rapid adsorption is particularly advantageous when working with M1, as it oxidizes quickly. Moreover, rapid adsorption reduces the overall procedure time. The desorption kinetics analysis indicated that the optimal elution time is 5 min. The sorption capacity equaled 8.9 μg mg^{-1} for RIS and 9.4 μg mg^{-1} for M1. This is an advantage because it allows for the adsorption of larger amounts of analyte without the risk of sorbent overload.

3.5. Validation of chromatographic method

The validation parameters of the developed chromatographic method were determined and collected in Table S3. The calibration curve showed very good linearity for both compounds (R^2 = 0.999 for RIS and R^2 = 0.997 for M1) over the concentration range used (1-20 μg mL^{-1}). The LOQ was 1 μg mL^{-1}, while the LOD was 0.33 μg mL^{-1}. The intraday precision values were below 4.42% for M1 and 0.74% for RIS, while the interday for M1 is 11.04% and 6.31% for RIS. To sum up, the chromatographic method had good linearity, precision, and repeatability.

3.6. Application of the dSPE method for RIS and M1 extraction from human serum samples

Two sample preparation methods were selected for the extraction of RIS and M1 from human serum, namely PP and our newly developed dSPE method. Until now, only PP has been used for isolating RIS from plasma, which is why this method was used as a comparative method [14,15]. At the same time, a new method developed by us was applied to assess its

suitability, sample purification efficiency, and analytical capabilities. Ascorbic acid was particularly important in the extraction of RIS and M1 from serum. Literature data show that RIS binds to proteins in blood, which may hinder extraction, reducing reproducibility and recovery [14]. Another challenge faced was the very low stability of M1 in serum and plasma samples, as demonstrated by previous studies and also observed by us during our experiment [14]. M1 in a human serum sample without the addition of L-ascorbic acid decomposed within an hour. The L-ascorbic acid inhibited the decomposition of M1 for 3 h. However, it was necessary to increase the concentration to 10 mM. This ensured that M1 would not degrade during the preparation of serum samples using both PP and mixed-mode dSPE. It should be noted that L-ascorbic acid was added directly to the serum samples before extraction. Moreover, the solvent used for PP also contained this antioxidant, as did the solutions used during mixed-mode dSPE. In each case, human serum was fortified with RIS and M1 to a concentration of 12 μg mL^{-1}. We would like to emphasize that, based on the experiences described by scientists from Roche [14], plasma that had been frozen only once (immediately after collection) was used. It has been shown that plasma freshness is particularly important for the stability (low oxidation) of M1 [14]. Similar effects were observed in our studies.

Regarding PP, the procedure described in a former study was applied [14]. The serum sample was first diluted with water in a 1:3 (v/v) ratio. Next, 1:4 (v/v) EtOH/ACN mixture containing 10 mM L-ascorbic acid was added, mixed, and centrifuged. The recoveries equalled 100±1% for RIS and 55±2% for M1. Figure 6 A presents a chromatogram of the PP extract The PP did not completely purify the sample (as shown in the chromatograms in Figure S5 for PP and dSPE extracts for a blank serum), leading to a possible reduction in the lifetime of the chromatographic columns. The results obtained indicate that the PP is effective and repeatable, but its efficiency depends on the structure of RIS and M1. M1 has one additional -OH group in its structure, and it may bind to proteins more strongly, potentially co-precipitating with them and leading to analyte loss, which could limit repeatability and recovery. This effect suggests the need for further optimization of the sample preparation conditions for M1 [20].

The main goal of the study was to show the applicability of the newly developed mixed-mode dSPE procedure for the extraction of RIS and M1 from serum. This attempt appeared to be successful for the serum sample (containing 10 mM ascorbic acid) fortified with RIS and M1 and diluted with 10 mM AcAm (pH 10) at 1:3 (v/v) ratio. The samples were directly loaded onto a conditioned AP adsorbent, and dSPE procedure described in section 2.5.1 was applied. The recoveries equalled 86±4% for RIS and 53±3% for M1, while the matrix effect was low (99%). The observed differences in recovery between RIS and M1 may result from differences

in their structures and polarities, as well as from M1 lower stability. The recovery of the M1 metabolite, at approximately 53%, is lower than expected. The most likely cause is the limited stability of the metabolite. For this reason, further optimization of the method is needed. Nevertheless, the method has good repeatability, enabling reproducible and reliable extraction of both compounds. Figure 6 B presents chromatograms obtained at various stages of dSPE procedure when the solution above the adsorbent (after sample load, washing and elution) was subjected to chromatographic analysis. Both RIS and M1 adsorbed onto the AP surface, as did the other compounds from the matrix. However, during the washing step, some of the compounds were washed off the surface of the material (Figure 6B, green line), unlike RIS and M1. This proves the selectivity of the selected adsorbent and mixed-mode dSPE for the tested compounds. The developed procedure is simple and selective since most compounds from serum adsorb at the AP surface (Figure 6B); however, only RIS and its metabolite are desorbed under selected experimental conditions. Moreover, dSPE more efficiently removes compounds originating from the matrix compared to PP, as presented in Figure S5. The chromatograms presented in this figure were recorded for extracts from blank serum, without RIS, M1 and antioxidant fortification. The application of ascorbic acid, which is eluted in the dead time, makes it impossible to determine the effectiveness of removing e.g., proteins from serum after PP or dSPE. For this reason, an extraction for blank serum was performed to highlight this effect (Figure S5). In our opinion, the most important advantage is direct application to human serum without the need for additional purification (e.g. protein removal), which significantly simplifies sample preparation.

3.7. Comparison of the newly developed method with previously published procedures.

A comparison of the sample preparation procedures presented in Table 1 reveals significant differences in terms of extraction method, applied solvents, sample volumes and matrix effect. Procedures 1 and 4 [11,14,15,21] allow for the extraction of RIS, whereas procedures 2, 3, and the method we developed enable simultaneous isolation of RIS and M1, a significant advantage in the context of pharmacokinetic studies. Our method is the first to use dSPE for the extraction of RIS and M1, thereby distinguishing it from all previously described procedures based exclusively on PP. Procedures 1 and 4 require large amounts of serum (180-200 µL), whereas procedures 3 and our method operate with much smaller volumes (10-100 µL), which is advantageous in medical bioanalytics (Table 1). The main disadvantage and limitation of our method is lower recovery compared to previously used PP methods (Table 1). However, dSPE exhibits a very low matrix effect (99%), comparable only to procedure 4 (Table

1), confirming its effectiveness in reducing matrix interference. The method we propose, despite lower recovery, ensures simultaneous extraction of RIS and M1, minimal matrix effect, and low demand for biological material, making it a competitive alternative to PP.

4. Conclusions

In this study, a chromatographic method based on CCD was developed for the separation of RIS and M1. Chromatographic studies showed that the salt in the mobile phase should have either a low concentration and a high pH or a high concentration and a low pH. Still, selecting the final conditions for a successful analysis of the RIS and M1 mixture must be a compromise, allowing a reduction in peak asymmetry while providing good separation in a short time (when isocratic elution is used). The results obtained provide a basis for further development of chromatographic methods used in SMA therapy monitoring.

The first application of hydrophobic (C18, PhO) and mixed hydrophobic-hydrophilic adsorbents (CHOL, AP, BA) for the dSPE extraction of both compounds yielded valuable results regarding interactions, recoveries, and applicability to serum samples. These adsorbents demonstrated the ability to interact with RIS and M1 via electrostatic, hydrophobic, π-π, and hydrogen-bonding mechanisms. In our opinion, it is an original approach that allows for a better match between sorption properties and the structure of analytes and provides a basis for further development in the design of functional sorption materials used for R. For the first time, it has been shown that adsorbents with mixed properties can effectively isolate RIS and its main metabolite. At the same time, it has been confirmed that their recovery depends mainly on the percentage of organic solvent and the pH of the eluent. Worth noting is that mixed-mode dSPE provided higher recovery, better reproducibility, and greater control of the extraction process than RP dSPE using conventional C18. This effect indicates that specific materials are required for isolating RIS and its metabolites. Application of AP material provided the highest recoveries for both compounds, together with good repeatability. Nevertheless, M1 recoveries are always lower compared to RIS, which we believe may be related to the greater polarity of M1 and its easier degradation through oxidation (by far the biggest challenge in its analytics).

The application of the developed mixed-mode dSPE procedure for the extraction of RIS and M1 from a human serum sample enabled direct extraction without the need for additional purification steps, such as PP using organic solvents. Direct loading of the sample onto the AP sorbent ensured complete adsorption of both analytes and their effective elution without matrix effects. This method is quick, easy to use, and reproducible. An additional advantage of dSPE is the ability to perform the entire process in a single centrifuge vial, which significantly

simplifies and speeds up sample preparation. In view of the growing need to improve sample preparation methods and search for new sorption materials, the presented studies respond to the current trends in the analytics of RIS and its metabolite, and may find potential application in the SMA therapy monitoring.

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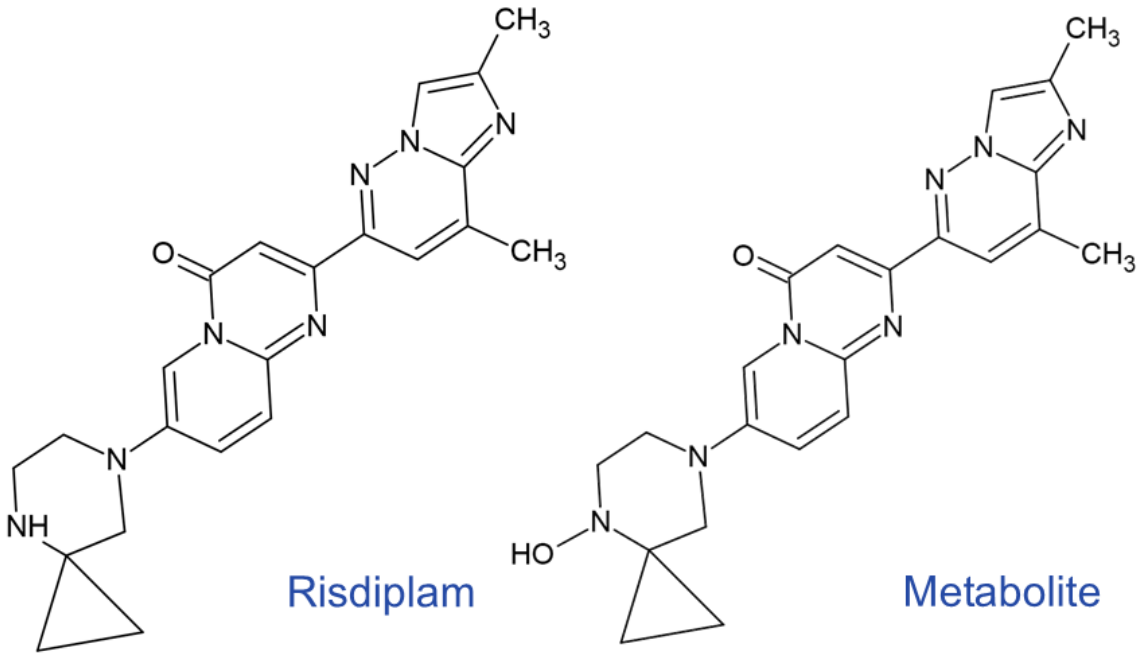

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964 **Figure 1.** Structure of risdiplam (RIS) and its metabolite (M1).

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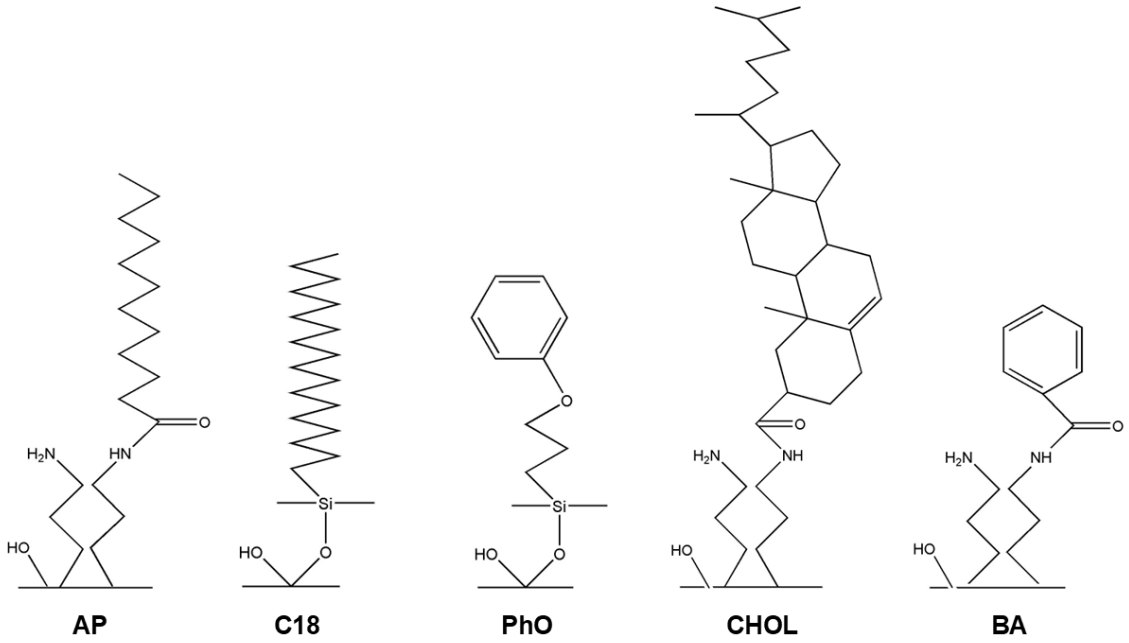

Figure 2. Chemical structures of new silica adsorbents used in the study for the dSPE extraction of RIS and M1.

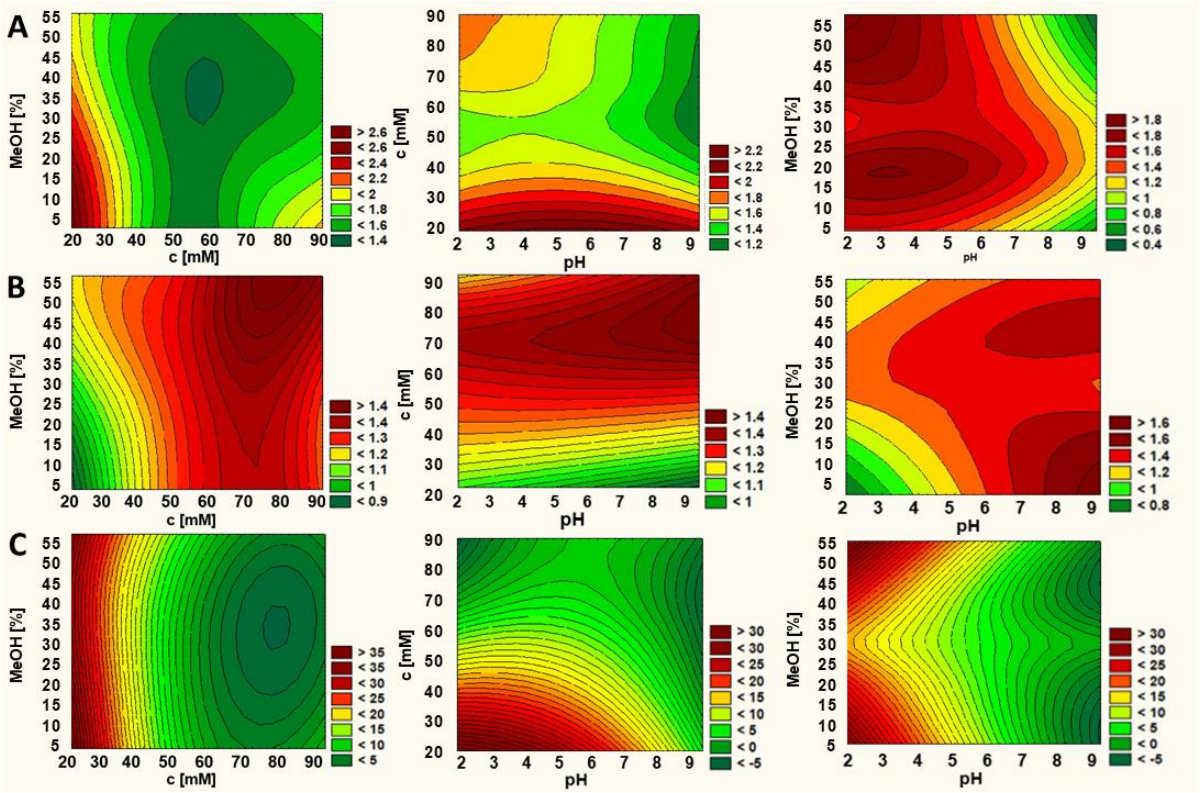

Figure 3. 2D contour plots showing CCD results for: peak asymmetry (f_{AS}) for RIS (A), peak asymmetry for M1 peaks (B), and resolution (R_s) (C).

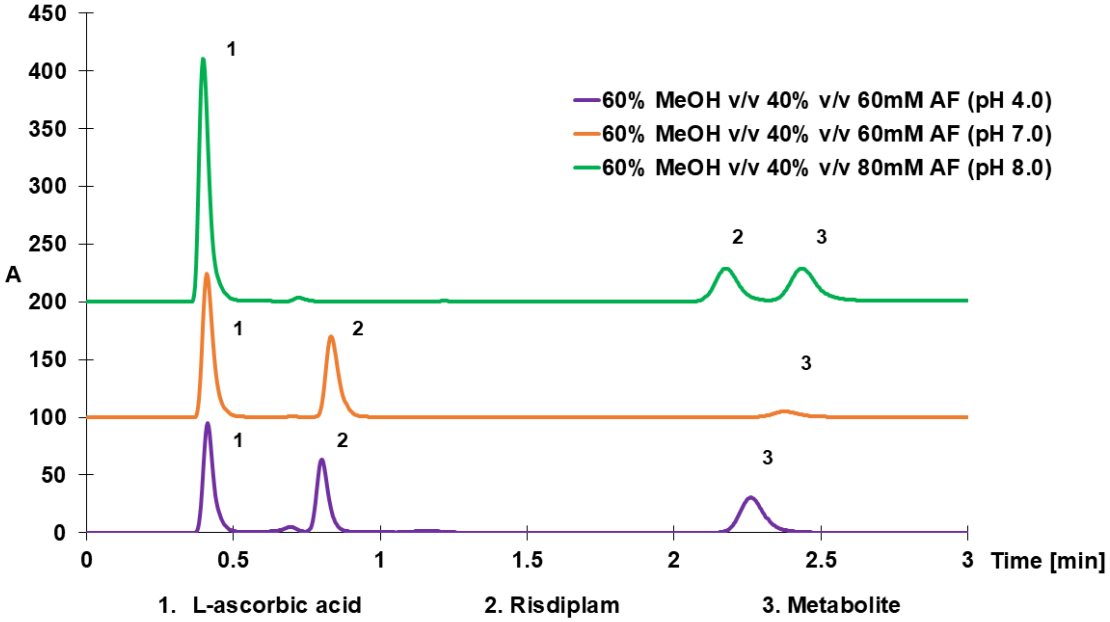

Figure 4. Chromatograms presenting RIS and M1 separation for a different composition of the mobile phase. Experimental conditions: mobile phase composition as indicated in the figure; UV detection at λ=255 nm; column temperature 30 °C; autosampler temperature 10 °C; flow rate 0.3 mL min $^{-1}$.

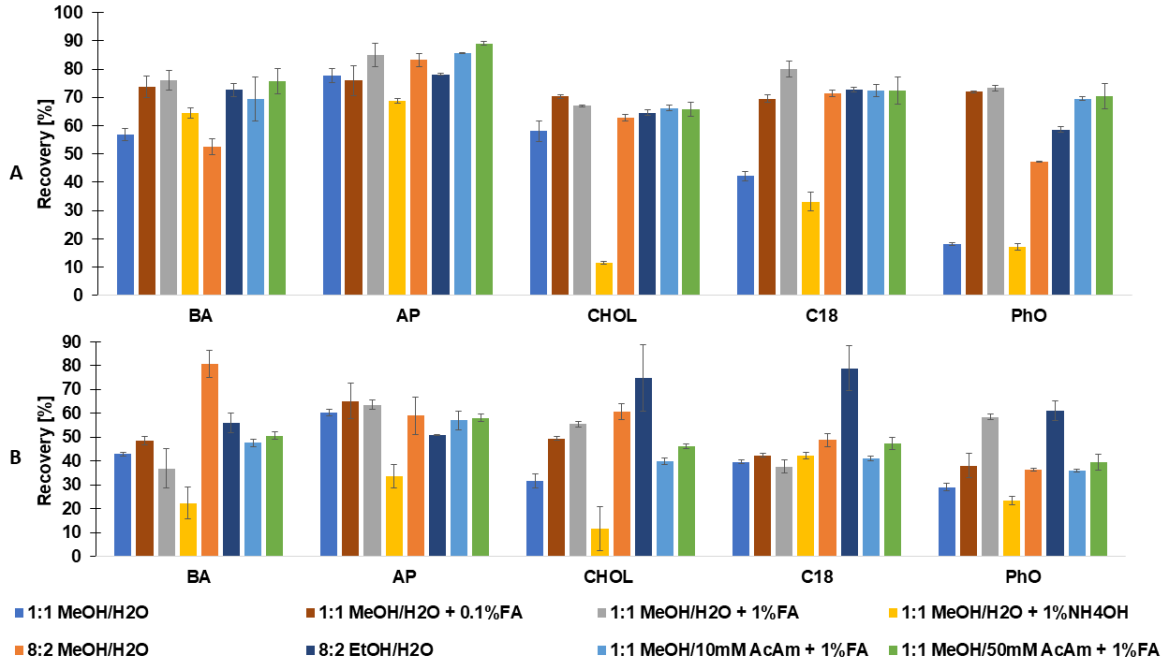

Figure 5. A comparison of the recoveries of RIS (A) and M1 (B) obtained using various eluents for the individual adsorbents used during the study.

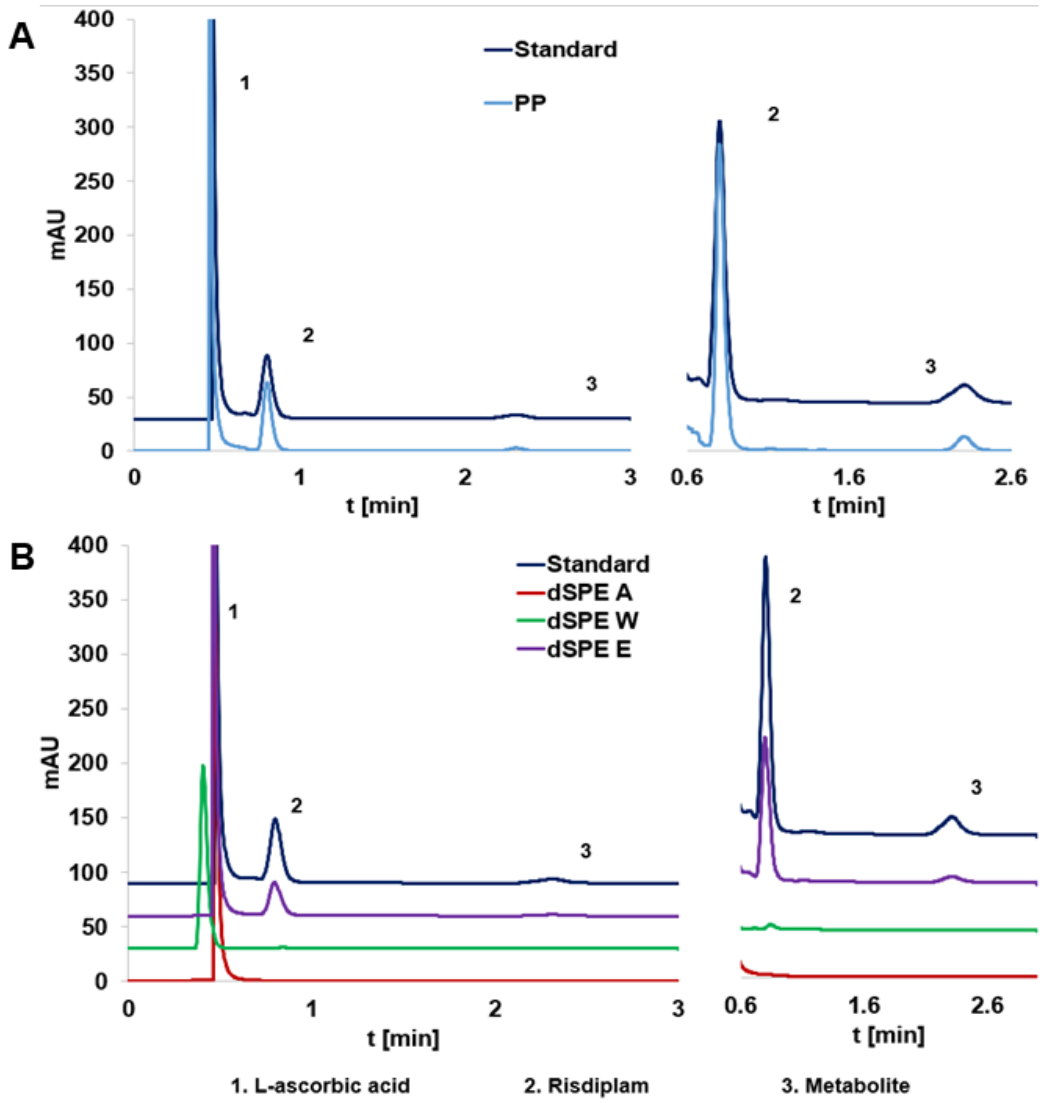

Figure 6. Chromatograms for serum extracts obtained during PP (A) and dSPE using AP adsorbent (B) at various stages (after sample load, washing, and elution). Experimental conditions: mobile phase composition 60/40% v/v of 60 mM AF (pH 4.0)/MeOH; UV detection at λ=255 nm; column temperature 30 °C; autosampler temperature 10 °C; flow rate 0.3 mL min^{-1}.

Table 1. Comparison of the newly developed method with previously published procedures.

Sample preparation					
Parameters	Procedure 1	Procedure 2	Procedure 3	Procedure 4	Our method
Analytes	RIS	RIS	RIS and M1	RIS	RIS and M1
Method	PP	PP	PP	PP	dSPE
Sample volume and solvents	180 µL of serum and 1 mL of MeOH	-	10 µL of serum and 30 µL of mixture EtOH:ACN (2:8 v/v)	200 µL of serum and 1.8 mL of 10% TFA	Adsorption: 74 µL 10mM AcAm pH 11 Desorption: 25 µL of serum; 75 µL of 60% MeOH 40% 60 mM AcAm pH 11
Recovery R (%)	90.89-101.44 %	92-99%	94.6-95.5% for RIS 92.4-95% for M1	90.79%	86% for RIS 53% for M1
Matrix effect	88.30-90.69%	-	85-95%	100%	99%
Advantages and disadvantages	- Large matrix effect, - Large sample volumes	- No description of the sample preparation method used	- Large matrix effect	- Large sample volumes	- Low recovery, - High salt concentration
	+ High recovery of RIS	+ High recovery of RIS	+ Small sample volumes	+ No matrix effect	+ Small sample volumes, + Low matrix effect
Reference	[15]	[11]	[14]	[21]	—