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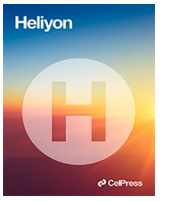

Research article

Development and validation of an HILIC/MS/MS method for determination of nusinersen in rabbit plasma

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ABSTRACT

A hydrophilic interaction liquid chromatography tandem mass spectrometry (HILIC/MS/MS) method was developed and validated for the quantitative analysis of the fully phosphorothioate modified oligonucleotide nusinersen. HILIC/MS/MS method is more robust and compatible with mass spectrometry than ion pair reversed-phase liquid chromatography-tandem mass spectrometry (IP-RP-LC/MS/MS). Various types and concentrations of additives and different pH of mobile phase affected the mass spectrometry response, chromatographic peak shape and retention of nusinersen. The optimized extraction method of nusinersen employs hydrophilic-lipophilic balance solid phase extraction, with a recovery of up to 80 %. Chromatographic quantification was performed using a gradient system on an amide column and the mobile phase consisted of ammonium acetate, acetonitrile and water in a certain proportion. The fully phosphorothioate modified nusinersen can obtain a high mass spectrometry response by providing greater peak symmetry and high ionization efficiency in a high-pH mobile phase. Moreover, the significant carry over interference was observed at the pH 6.3 of the mobile phase. Adjusting the pH value up to 10, and the carry over interference disappeared. The lower limit of quantitation of this developed HILIC/MS/MS assay was 30.0 ng/mL and the method was systematic methodology validated. This HILIC/MS/MS method provides an attractive and robust alternative for the quantitative analysis of nusinersen and was applied in the pharmacokinetic study of nusinersen in rabbits.

1. Introduction

Antisense oligonucleotides (ASOs) are therapeutic nucleic acids, generally formed from 15 to 20 nucleotide units [1]. Various chemical modifications have been designed to improve the stability and pharmacokinetic properties of ASOs. They are divided into three generations based on modification. Phosphorothioate backbone modifications are the major representatives of the first-generation ASOs and have sufficient resistance to nuclease degradation; the second generation mainly comprise 2′-O-methyl

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(2′-OMe) and 2′-O-methoxyethyl (2′-MOE) modifications, while the third-generation ASOs comprise modifications such as locked nucleic acids and phosphorodiamidate modified morpholino oligomers [2,3]. Nusinersen, an 18-mer ASO, belongs to the first and second generations with full phosphorothioate linkage modification and the 2′-MOE groups are substituted for 2′-hydroxy groups of all ribofuranosyl rings [4].

The quantitative analysis of ASOs is essential for drug development and therapeutic effect in clinic. An ion pair reversed-phase liquid chromatography tandem mass spectrometry (IP-RP-LC/MS/MS) method was developed earlier for the quantitative analysis of nusinersen [5]. The low ionization efficiency caused by the abundant phosphorothioate linkages of nusinersen was significantly improved, a high mass spectrometry response was obtained, and the lower limit of quantification was 3.0 ng/mL by adjusting the pH of the mobile phase to a high value of 9.6, resulting from the combination of 15 mM triethylamine (TEA) and lower concentrations of 1,1,1,3,3,3-hexafluoro-isopropanol (HFIP, 25 mM) in the mobile phase. However, the use of an ion-pairing mobile phase results in significant problems, including poor signal stability, gradually deteriorating sensitivity after extended use, and the ionization inhibition of samples in positive ion mode were observed, because TEA is strongly adsorbed onto the mass spectrometry, resulting in contamination of the mass spectrometry system [6–8,8]. Therefore, frequent instrument maintenance is required to remove ion-pairing residues.

Since Alpert [9] first proposed the hydrophilic interaction liquid chromatography (HILIC) mechanism in 1990, it has attracted widespread attention in the analysis of highly polar compounds. Compared with IP-RP-LC/MS/MS, "MS-friendly" mobile phase additives are used in HILIC, making it more compatible with mass spectrometry. HILIC-tandem mass spectrometry (HILIC/MS/MS) method is an alternative analytical tool for the analysis of oligonucleotides (OGNs) [10,11]. Sylwia Studzinska et al. [12] first attempted to separate and quantify phosphorothioate oligonucleotides in human serum using the HILIC/MS/MS method in 2017 and the lower limit of quantitation (LLOQ) was 142–165 ng/mL, which cannot fully characterize oligonucleotide elimination and pharmacokinetics [13–15,15]. Robert MacNeill et al. [4] developed an HILIC/MS/MS analytical method for determining an 18-mer unmodified oligonucleotide in human plasma, with an LLOQ of approximately 54 ng/mL. However, the concentration of drugs for real plasma samples in vivo had not been determined by this developed method. After that, owing to the limitations of sensitivity, there have been no relevant reports on the analysis of OGNs in biological matrices, especially for fully phosphorothioate modified OGNs with low ionization efficiency using the HILIC/MS/MS method. Inspired by the previous development of the IP-RP-LC/MS/MS method to separate and quantify nusinersen, we developed an HILIC/MS/MS method with high sensitivity to analyze fully phosphorothioate modified nusinersen by optimizing the pH of the mobile phase.

In this work, a relatively sensitive and "MS-friendly" HILIC/MS/MS method for the quantitative determination of full phosphorothioate modified ASO nusinersen was developed and systematically validated. The HILIC/MS/MS method was used to determine nusinersen levels in rabbit plasma samples.

2. Experimental methods

2.1. Materials

The phosphorothioate modified ASOs used in this study were synthesized from Genscript. The sequence of nusinersen is U*C*A*C*U*U*U*C*A*U*A*A*U*G*C*U*G*G. The sequence, base C and U modification, and phosphorothioate backbone modification of the internal standard (IS) are the same as nusinersen. The purity of both nusinersen and IS was 96.5 %. For mobile phase preparation, ammonium acetate, ammonium formate, formic acid, acetic acid, and ammonium hydroxide were purchased from Sigma-Aldrich (St. Louis, MO, USA), and H_3PO_4. LC–MS grade methanol and acetonitrile were obtained from Merck (Darmsadt, Germany). Deionized water was purified in-house using a Milli-Q system (Millipore, Bedford, MA, USA). Clarity OTX lysis buffer was obtained from Phenomenex Inc. (Torrance, CA, USA). New Zealand White rabbits were purchased from Qingdao Kangda Co. Ltd., in China. All procedures related to animal experiments in the study were approved by the Ethics Committee of Yantai University (IACUC No.20230507), and were implemented strictly in accordance with protocols of the relevant Institutional Animal Care and Use Committee (IACUC).

2.2. Instrument and LC–MS conditions

An API 5500 triple quadrupole mass spectrometer (Applied Biosystems Sciex, Ontario, Canada) was conducted to analyze analytes and IS in electrospray ionization of the negative ion mode and multiple reaction monitoring mode. The mass spectrum parameters were optimized as follows, the Declustering Potential was −50 to −80 V, the Collision Energy −150 V, the Entrance Potential −12 V, the Collision Gas 12 V, the Ion Spray Voltage −4500 V, and the drying gas temperature was set at 550 °C. Each compound carried multi-charged states, the charge ions with the most abundant and least matrix interference were selected to quantitative analysis. The multiple reaction monitoring for the nusinersen and IS were m/z 889.9 → 95.0 and 839.0 → 95.0, respectively.

An Agilent 1290 HPLC system and an XBridge™ Premier BEH amide column (50 mm × 2.1 mm, 2.5 μm) (Waters, Corp., Milford, MA, USA) was utilized to separate nusinersen in HILIC method. Mobile phase A consisted of H_2O-acetonitrile-ammonium acetate (20 mM) (70:20:10, v/v/v, pH 10) and mobile phase B consisted of acetonitrile-ammonium acetate (20 mM) (90:10, v/v, pH 10). A flow rate of 0.3 mL/min was used and the gradient elution was 0–0.5 min, 90 % B; 0.5–2.5 min, 90 % B–50 % B; 2.5–3.5 min, 50 % B; 3.6–5 min, 90 % B. The column temperature was set at 50 °C for the HILIC/MS/MS method.

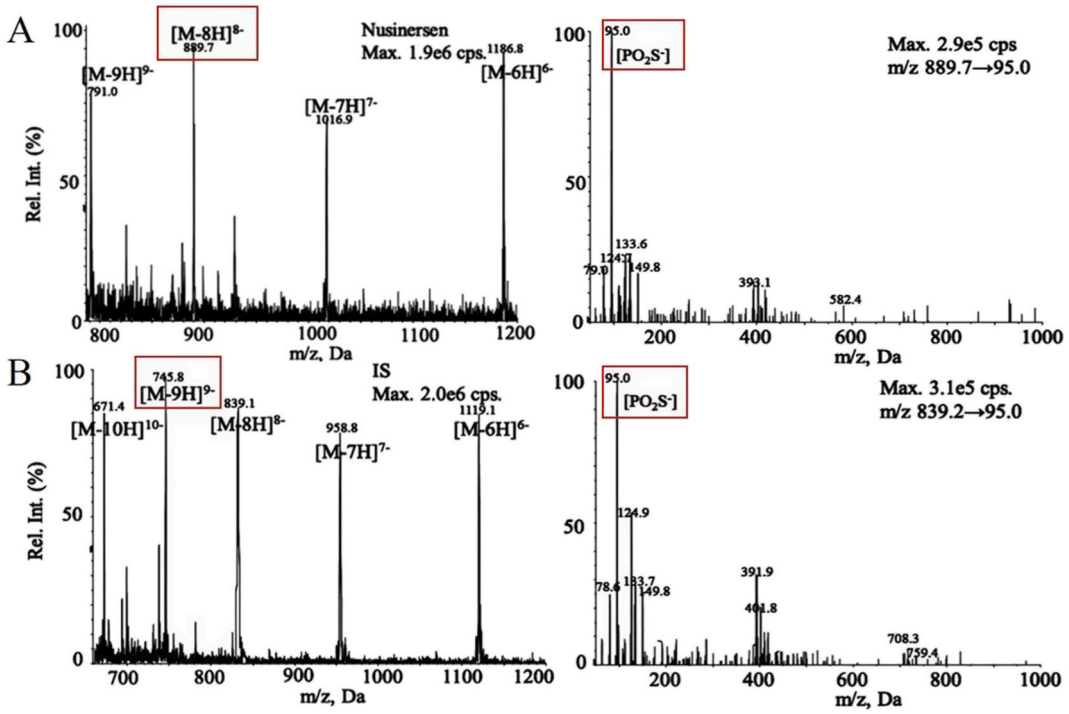

Fig. 1. Parent and product ion spectra for (A) nusinersen and (B) IS in HILIC/MS/MS method.

2.3. Preparation of standard and quality control (QC) samples

ASO stock solutions with concentration of 1 mg/mL were prepared by adding nusinersen and IS to appropriate amount of deionized water as working solutions. The working solutions were diluted with water in polypropylene tubes, the concentrations were 300, 500, 1000, 2000, 5000, 20000, 50000 and 100000 ng/mL for nusinersen, 500, 6000 and 80000 ng/mL for quality control (QC) solutions, and 20 μg/mL for the IS.

The analytical calibration samples were prepared at concentrations of 30, 50, 100, 200, 500, 2000, 5000 and 10000 ng/mL and at 50, 600 and 8000 ng/mL for QC samples by mixing 5 μL nusinersen and 5 μL IS working solution with 45 μL of blank rabbit plasma.

2.4. Sample preparation

Nusinersen extraction was performed using an Oasis® hydrophilic-lipophilic balanced (HLB) cartridge (Waters, MA, USA). Fifty μL plasma samples, 100 μL Clarity OTX Lysis Loading buffer, and 100 μL 4 % H_3PO_4 buffer were vortex mixed at least 3 min in 1.5 mL Eppendorf tubes before loading the sample. The HLB cartridge was equilibrated with 1 mL acetonitrile and 1 mL water-HFIP-TEA (100:2:0.2, v/v/v) solution. After the samples were injected into the extraction column, the wells were washed twice with 1 mL of water-HFIP-TEA (100:2:0.2, v/v/v). Immediately, the ASOs were eluted twice with 500 μL of a mixture of acetonitrile-water-TEA (60:40:1, v/v/v) and evaporated to dryness at 28 °C. The extract was reconstituted with 100 μL ACN-H_2O (1:1, v/v). Five microliters of the reconstituted solution was injected into the LC−MS/MS system.

2.5. Method validation

The developed HILIC/MS/MS method was validated according to published guidelines [16].

Selectivity was assessed by measuring nusinersen and IS concentrations in six different sources of rabbit plasma sources. Calibration curve and range was assessed by the concentration of the analyte to be easured as the horizontal axis, and the ratio of the peak area of the analyte to the area of the IS as the vertical axis, and calculated using weighted ($1/x^2$) least squares linear regression, of which accuracy was between 85.0 % and 115.0 % (80.0–120.0 % for LLOQ). Accuracy and precision were evaluated by preparing QC samples of low, medium and high concentrations (50, 600, and 8000 ng/mL), with at least 5 samples for each concentration, which were prepared continuously and measured for three days. Carry-over was evaluated by measuring the nusinersen peak area in a blank sample injected after the test sample to assess the upper limit of quantification (ULOQ). Extraction recovery of nusinersen from rabbit plasma samples was determined by comparing the peak areas of the analytes in rabbit plasma that was spiked with the analyte and processed, with the peak areas in a biological blank sample that is processed and then spiked with the analyte.

To evaluate the matrix effect, the corresponding peak areas in the extracted plasma (LQC and HQC) were compared with those of

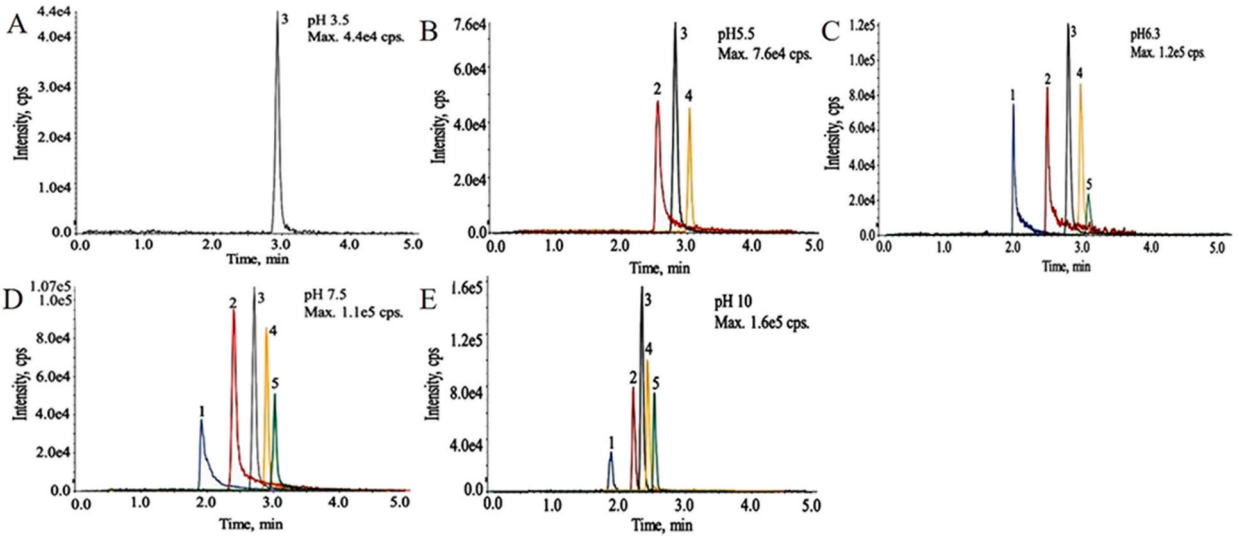

Fig. 2. Chromatogram of nusinersen at different concentrations of ammonium acetate (1) 5 mM ammonium acetate; (2) 10 mM ammonium acetate; (3) 20 mM ammonium acetate; (4) 30 mM ammonium acetate; (5) 50 mM ammonium acetate and different pH (A) pH3.5 (B) pH5.5 (C) pH6.3 (D) pH 7.5 (E) pH10 in the mobile phase.

the tested samples that were added to the pre-treated QC samples. Dilution integrity was evaluated by diluting high concentration (400 μg/mL) plasma samples prepared to HQC samples with blank rabbit plasma. To assess the stability of analytes in rabbit plasma, samples exposed to different temperatures and times including stability of the bench-top, in the HPLC auto sampler and three freeze/thaw cycles, as well as long-term (one month) were determined. The mean concentration at each tested QC level should be within ±15 % of the nominal concentration, and the precision of all tested QC concentrations should not be more than 15 %.

2.6. Application of the method

Six New Zealand white rabbits of similar body weight (2.5 kg) were selected to investigate the pharmacokinetics of nusinersen in vivo by the developed HILIC/MS/MS assay. Food and water were fed and a regular light/dark cycle to these rabbits normally during the experiment. The six rabbits were randomly divided into two groups, nusinersen was administered as an intrathecally (IT) bolus dose of 1.8 mg/kg to the first group, intravenously (IV) bolus dose of 1 mg/kg to the second group, respectively. Blood samples with K_2EDTA anticoagulation were withdrawn from the ear vein at 0 min (pre-dose), 5 min, 15 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h, and 12 h after IV and IT administration. The blood samples were centrifuged at 17500×*g* for 10 min and the supernatants of each were collected and kept frozen at −80 °C until analysis.

3. Results and discussion

3.1. Mass spectrometry

Nusinersen and the IS were expected to have multiple charges in API 5500 because of the large molecular weights of nusinersen (7126.23 Da) and the IS (6719.65 Da). The signal is distributed over several ions, which is the reason for the low sensitivity of OGNs [15,17]. Full and product ion scans of nusinersen and IS using chromatography coupled with mass spectrometry (API 5500) are shown in Fig. 1. The molecular ions $[M-6H]^{6-}$, $[M-7H]^{7-}$, $[M-8H]^{8-}$ and $[M-9H]^{9-}$ of nusinersen and $[M-6H]^{6-}$ to $[M-8H]^{8-}$ of IS were observed, nusinersen and IS exhibited the abundant ion signal when they had 8 charges, which were chosen to scan for product ions, resulting in generating major productions at *m/z* 95.0, with transitions of *m/z* 889.9 → 95.0 for nusinersen and *m/z* 839.0 → 95.0 for IS, respectively. The product ions 95.0 of nusinersen and the IS all corresponded to the phosphorothioate group, which was specific for the studied oligonucleotides.

3.2. Optimization of chromatographic experimental conditions

3.2.1. Chromatographic column selection

An XBridge™ BEH amide column was used for the HILIC/MS method, because the better separation and determination of phosphorothioate oligonucleotides was carried in the alkaline stationary phases.

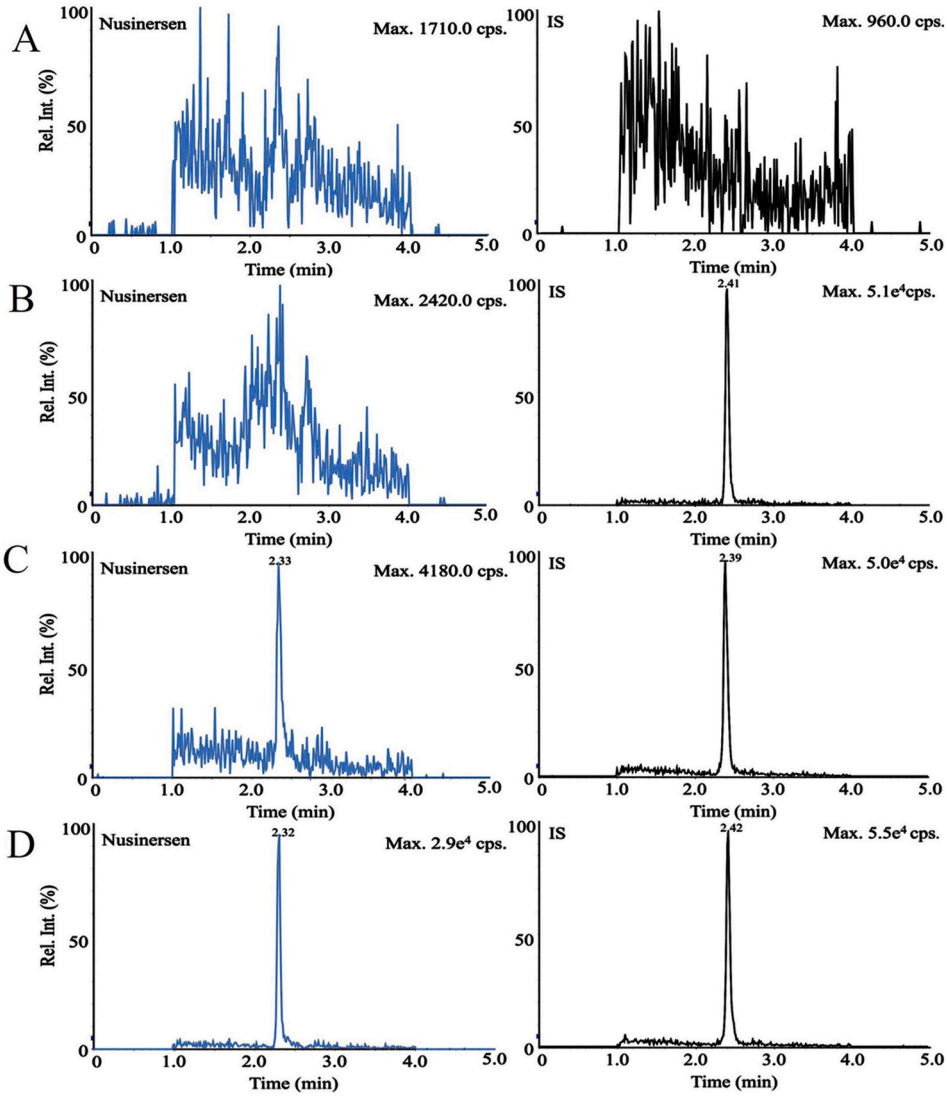

Fig. 3. Typical MRM chromatograms of specifity test for HILIC/MS/MS method.
(A) double blank rabbit plasma (drug and IS free), (B) blank rabbit plasma (only drug free), (C) LLOQ (30 ng/mL) of nusineren, (D) plasma samples obtained from a rabbit after injections of nusinersen (2 h).

3.2.2. Optimization of the mobile phase in hydrophilic interaction liquid chromatography

The HILIC/MS as an alternative method was developed, because mobile phase additives are more compatible with mass spectrometry. The impacts of different salt types, concentrations and pH values on the retention and response of nusinersen in the mobile phases were examined.

Ammonium formate and ammonium acetate were tested in the mobile phase. Similar mass spectrum responses from the two additives were observed, whereas ammonium acetate gave a better peak shape and lower baseline noise than ammonium formate, therefore, ammonium acetate was selected as an additive for subsequent investigations.

Fig. 2 shows the chromatographic spectra of nusinersen in different mobile phases. The retention time of nusinersen was extended with the increase of ammonium acetate concentration. However, the nusinersen signal first increased and then decreased with increasing ammonium acetate concentration. The maximum response was observed at 20 mM. For all concentrations of ammonium acetate, a minor decrease in the retention time and an increase in the mass spectrum response were observed with increasing mobile solution pH. Especially at pH 3.5 in the mobile phase containing a low concentration of ammonium acetate, the chromatographic peak was not observed. With increasing pH at the same concentration of ammonium acetate, nusinersen showed severe peak trailing and a low mass spectrum response.

The interaction between the phosphorothioate groups and the amide bond of the stationary phase was strong at low pH values and low concentrations of ammonium acetate, resulting in poor elution of nusinersen at pH 3.5 and a severe peak trailing at low ammonium acetate concentrations. The retention capacity enhanced of nusinersen in chromatographic column with increasing of ammonium

Table 1
Precision and accuracy for the determination of nusinersen in rabbit plasma of HILIC/MS/MS method.

Conc.level (ng/mL)	HILIC/MS/MS				
	Intra-day		Inter-day		Accuracy (%)
	Obsd conc. (ng/mL)	RSD (%)	Obsd conc. (ng/mL)	RSD (%)	
LLOQ	29.0 ± 3.1	10.6	29.7 ± 3.0	9.96	96.7
	28.5 ± 3.5	12.3			95.0
	31.7 ± 1.0	3.08			105.5
LOQ	51.2 ± 5.6	10.9	50.3 ± 4.1	8.19	102.4
	48.2 ± 1.8	3.68			96.4
	51.6 ± 3.8	7.42			103.1
	552.8 ± 12.2	2.21	565.9 ± 24.3	4.29	92.1
MOQ	571.5 ± 29.8	5.21			95.3
	573.3 ± 25.7	4.48			95.6
HOQ	8131.7 ± 328.8	4.04	8056.8 ± 426.7	5.30	101.6
	7988.7 ± 441.5	5.53			99.9
	8050.2 ± 550.9	6.84			106.3

acetate concentration, which was consistent with the findings of Studzińska S et al. [12]. The decrease in the retention time at high pH values can be attributed to the fact that there is a stronger interaction between nusinersen and NH_4^+ in the mobile phase than in the amide stationary phase due to more NH_4^+ at high pH values. At low ammonium acetate concentrations and low pH values, only a portion of the phosphorothioate groups in the nusinersen structure are deprotonated, resulting in low mass spectrum response of nusinersen [18]. With increasing ammonium acetate concentration and pH values, the negative phosphorothioate acid group in nusinersen was completely ionized, making the response higher, and improving the peak shape. However, at higher salt concentrations, there are a number of negative ions $HCOO^-$ and CH_3COO^-, and these negative ions compete with nusinersen for the surface position of droplets, resulting in ion inhibition [18]. Compounds are usually analyzed using the HILIC method under acidic or neutral conditions [19,19–21]. However, severe carryover interference with column blockage and increased column pressure were observed when the pH value of the mobile phase was at a lower level, especially at high concentrations of nusinersen. The reason probably was that sulfur atoms were less electronegative than oxygen atoms, and phosphorothioate precipitated easily at a low pH value (pH 6.3) and existed as a molecular state. The ionization efficiency of nusinersen is improved at high pH values, and carry over interference disappeared when the mobile phase pH was up to 10. Therefore, a mobile phase with a pH of 10 was chosen to analyze the fully phosphorothioate modified nusinersen.

3.3. Sample preparation

Based on previous investigations of liquid-liquid extraction and solid phase extraction (SPE) methods with weak anion exchange and hydrophilic lipophilic balance (HLB) mechanisms [5], nusinersen was extracted from plasma by HLB-SPE method with a relatively high recovery rate of 66.7%–76.8 %. In addition, Clarity OTX Lysis Loading buffer and 4 % H_3PO_4 were mixed with the plasma samples to sufficiently disrupt the binding of nusinersen to plasma proteins before the extraction of nusinersen by HLB-SPE method [22], due to the strong affinity of phosphorothioate OGNs with proteins.

3.4. Method validation

3.4.1. Selectivity and lower limit of quantification

The retention times of nusinersen and IS were 2.32 min and 2.40 min, respectively. In all analyzed rabbit plasma samples, typical chromatograms (shown in Fig. 3) showed no significant interference peaks at the retention times for nusinersen and the IS.

The LLOQ of the HILIC/MS/MS method was 30.0 ng/mL, with intraday and interday precision values (CV %) of 12.3 % and 9.9 %, respectively. The accuracy of LLOQ ranged from 86.3 to 115.0 % (n = 6). The LLOQ analyte responses are shown in Fig. 3.

3.4.2. Linearity of calibration curves

The quantitative concentration range of the developed standard curve was 30–10000 ng/mL based on 50 μL of plasma in the HILIC/MS/MS method. The typical linear regression calibration curve was y = 0.00282x - 0.0377 (r = 0.9964) for the HILIC/MS/MS method.

3.4.3. Carryover

The blank samples were prepared and measured continuously for three days after the standard curve ULOQ samples were injected into the system, and the response of the analytes in the blank sample were compared with the response of the LLOQ for the analytes. By adjusting the pH value of the mobile phase up to 10, the previously existing carryover problem was resolved.

3.4.4. Precision and accuracy

The results based on the calculated QC sample results are shown in Table 1. The intraday and interday precision was less than 12.3 % and 10.0 %, respectively. The accuracy ranged from −13.7 % to 15 % for all QC concentrations.

Table 2
Extraction recovery and matrix effects of nusinersen and IS at different concentration in rabbit plasma.

	Compound	Low	Medium	High
Recovery (%)	Nusinersen	66.7 ± 2.93	75.8 ± 8.33	76.8 ± 11.71
	IS		50.4 ± 8.38	
Matrix effect (%)	Nusinersen	99.8 ± 9.18	–	97.0 ± 9.34
	IS	100.4 ± 13.87	–	104.7 ± 8.37

Table 3
Stability of nusinersen in rabbit plasma stored under different conditions.

Conditions	Nusinersen					
	50 ng/mL			8000 ng/mL		
	Obsd conc. (ng/mL)	RSD (%)	Accuracy (%)	Obsd conc. (ng/mL)	RSD (%)	Accuracy (%)
Ambient, 25 °C, 4 h	49.6 ± 4.2	8.40	99.4	8170.3 ± 451.7	5.53	102.1
Autosampler, 8 °C, 24 h	49.3 ± 3.9	7.80	98.6	8272.2 ± 484.1	5.85	103.4
Freeze-thaw (three cycles)	50.9 ± 5.5	10.9	101.7	7792.0 ± 243.7	3.13	96.9
Long-term storage at −80 °C for 30 days	50.0 ± 5.2	10.3	100.1	8210.2 ± 508.6	6.19	102.6

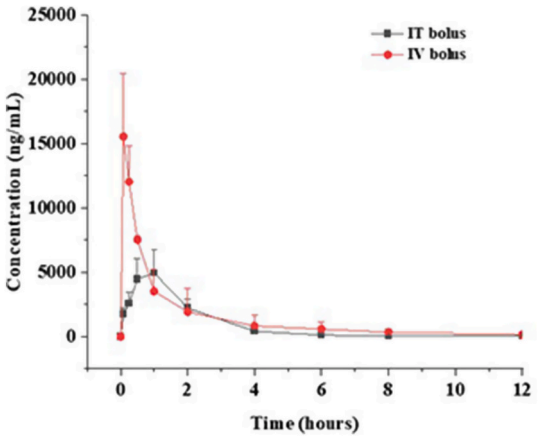

Fig. 4. Mean plasma concentration-time profiles of nusinersen in rabbits with IT and IV administration analysis by HILIC/MS/MS method.

3.4.5. Extraction recovery

The extraction recoveries of QC samples from rabbit plasma at 50, 600, 8000 concentration levels were 66.7 ± 2.93 %, 75.8 ± 8.33 % and 76.8 ± 11.71 %, respectively (nusinersen) and 50.4 ± 8.38 % (IS). The low recovery of nusinersen at lower concentrations may be attributed to the adsorption effect of SPE column and the polypropylene tube on compounds with low concentration was greater [18]. The recovery data are presented in Table 2.

3.4.6. Matrix effects

The calculated results about the ratio of the peak area for the analyte in the plasma samples to the corresponding peak area ratio for the pure solution at the same concentration are shown in Table 2 for nusinersen and the IS, respectively. The results showed no significant suppression or enhancement of ionization in the rabbit plasma matrix.

3.4.7. Stability

The stability of LQC and HQC concentration levels of samples were tested, the stability data was listed in Table 3. These results indicate that nusinersen is stable and meets the requirements for quantitative determination under all experimental conditions.

3.4.8. Dilution integrity

Plasma samples spiked with 400 μg/mL nusinersen were diluted 5-fold with blank rabbit plasma to HQC levels (80 μg/mL, n = 6), the precision was 5.2 %, and the accuracy was 97.1–111.3 %, indicating that the plasma samples with higher concentration than the ULOQ were diluted by adding blank plasma into the samples was accurate and reliable.

Table 4
Pharmacokinetic parameters of nusinersen in rabbits given as IV and IT injection obtained by HILIC/MS/MS method.

	HILIC/MS	
	IV(n = 3)	IT (n = 3)
$T_{1/2}$ (hour)	3.1 ± 0.60	2.4 ± 0.28
T_{max} (hour)	0.08	1
C_{max} (μg/mL)	15.6 ± 4.88	5.0 ± 1.88
C_0 (μg/mL)	17.6 ± 6.20	–
AUC_{last} (hour*μg/mL)	17.8 ± 4.10	10.6 ± 3.22
V_z (mL/kg)	280.9 ± 35.54	363.3 ± 70.92
Cl (mL/hour/kg)	61.2 ± 16.80	180.9 ± 63.17
MRT_{last} (hour)	1.9 ± 1.15	1.7 ± 0.17
V_{ss} (mL/kg)	138.5 ± 65.97	–

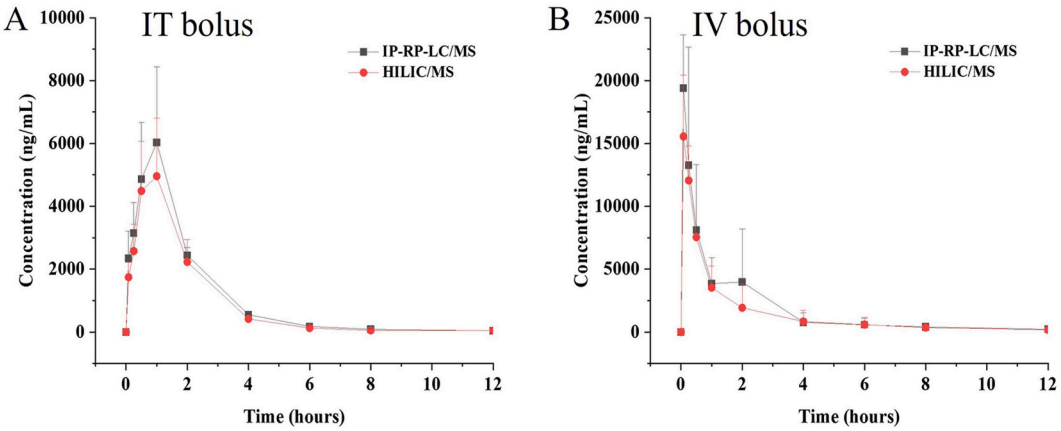

Fig. 5. Mean plasma concentration-time profiles of nusinersen in rabbits with (A) IT and (B) IV administration analysis by IP-RP-LC/MS/MS and HILIC/MS/MS methods.

3.5. Application of the methods to pharmacokinetic studies in rabbit

The developed HILIC/MS/MS method was used for the quantitative determination of nusinersen in rabbit plasma after a single IT (1.8 mg/kg) and single IV (1 mg/kg) injection. The data determined by HILIC/MS/MS method was plotted into the plasma drug concentration and time curve, as shown in Fig. 4. The pharmacokinetic parameters of nusinersen were calculated by WinNonlin 8.1 and summarized in Table 4. The C_{max}, AUC_{last} and C_0 of IV were 15.6 ± 4.88 μg/mL, 17.8 ± 4.10 h*μg/mL and 17.6 ± 6.20 μg/mL; the T_{max}, C_{max} and AUC_{last} of IT were 1 h, 6.0 ± 0.10 μg/mL and 12.4 ± 1.15 h*μg/mL, respectively. The calculated bioavailability of nusinersen was 33 %. The results obtained were compared with those determined using the IP-RP-LC/MS/MS method, as shown in Fig. 5. The plasma concentration-time profiles were anastomosed and the obtained pharmacokinetic results for nusinersen were similarly determined using the two developed methods. The sensitivity of the developed HILIC/MS/MS quantitative method was fulfilled to characterize the concentration changes of nusinersen in rabbits.

4. Conclusion

A more compatible with mass spectrometry HILIC/MS/MS method was developed for the analysis of the nusinersen concentration change in a biological matrix. The “MS-friendly” additive, 20 mM ammonium acetate, was included in the mobile phase, and the pH was adjusted to 10. High ionization efficiency and ideal peak shape were obtained at high pH, providing a strong mass spectrometry signal for nusinersen after detailed investigation of the optimal additive and pH value for the mobile phase. The significant carryover interference seen at lower pH disappeared at high pH in the HILIC/MS/MS method. Nusinersen was extracted from plasma at a recovery of between 67% and 77 % by employing an optimized Oasis HLB solid phase extraction method. The developed quantitative HILIC/MS/MS method was validated and the lower limit of quantification was 30.0 ng/mL and it was successfully applied to the pharmacokinetic study of nusinersen in rabbit plasma. Despite the fact that the developed IP-RP-LC/MS/MS method is more sensitive than the HILIC/MS/MS method, the contamination concerns of ion-pair reagents in mobile phase in the former, result in poor signal stability and frequent instrument maintenance. Conversely, the HILIC/MS/MS method is robust and more compatible with MS. In future research, if high sensitivity is needed, the IP-RP-LC/MS/MS method should be selected to analyze nusinersen; however, the HILIC/MS/MS method should be used if compatibility with mass spectrometry while meeting the determination requirements is a

concern.

Data availability statement

All data to support the conclusions have been either provided in article or are otherwise publicly available.

Ethics declarations

This study was reviewed and approved by the Ethics Committee of Yantai University, with the approval number: IACUC No.20230507.

CRediT authorship contribution statement

Xiao Zhang: Writing – original draft, Validation, Investigation, Data curation. **Chunjie Sha:** Writing – review & editing, Resources, Formal analysis. **Wei Zhang:** Validation, Resources. **Fengjuan Zhao:** Validation, Software. **Mingli Zhu:** Writing – original draft, Data curation. **Guangyi Leng:** Writing – original draft, Software, Resources. **Wanhui Liu:** Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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