Characterization and quantification of phosporothioated (PS) oligonucleotides in horse plasma for gene doping control

Using IDA and MRM^{HR} on the SCIEX TripleTOF 6600 system

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The recent advances in veterinary medical technologies have enabled the development of therapeutic oligonucleotides, such as oligomers or polymers of nucleic acids and nucleic acid analogues, to enhance athletic performance. Gene doping, the misuse of gene therapies for performance enhancement, is known to significantly increase body weight and muscle mass, resulting in a significant improvement in speed, muscle strength, endurance and overall physiological performance. The use of these gene delivery technologies has become a rising concern in sports, and more specifically in horse racing.[1,2] The administration of these gene therapies has been shown to significantly increase the racing performance of race horses.

Phosphorothioated oligonucleotides (PSOs) are a class of modified therapeutic oligonucleotides commonly used as gene doping agents. These modified oligonucleotides have proven to be effective in their ability to maintain their structural integrity in the bloodstream and as a result have been commonly used in horse racing doping. As these synthetic oligonucleotides are gaining popularity as doping agents, effective methods capable of detecting, monitoring and quantifying PSOs in a biological matrix are critically needed. Specific and sensitive detection methods for gene doping are urgently needed to maintain the integrity of horse racing and equestrian sports in general.

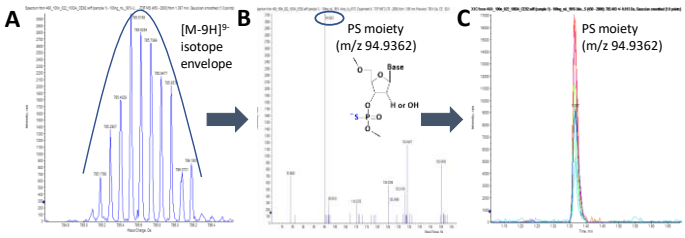

Figure 1. Non targeted IDA analysis of a PSO standard by monitoring the PS moiety for gene doping control. Full TOF MS mass spectrum of phosphorothioated oligonucleotide (PSO) showing the [M-9H]9- isotope envelope using precursor ion scan mode (A), TOF MS/MS spectrum of the PSO showing unique fragment ions including the most intense fragment ion at *m/z* 94.9362 (B) and a set of extracted ion chromatograms (XICs) of the *m/z* 94.9362 fragment ion derived from the PSO (C).

In this technical note, the use of high-resolution mass spectrometry (HRMS) for the detection of a marker of PSO use in horse plasma is demonstrated. The use of both the IDA and the MRM^{HR} workflow enabled the development of a specific assay and the comprehensive characterization and accurate quantification of a phosphorothioate (PS) moiety, a specific product ion of PSOs, as a marker for gene doping detection in horse racing.

Key advantages of LC-MS/MS method for monitoring PSOs in plasma

- A fast and simple sample preparation procedure enables deproteination of horse plasma samples
- A rapid, 4-minute LC run enables high-throughput separation of PSOs moiety from plasma matrix components
- Untargeted analysis using IDA enables characterization of the specific product ion of negatively charged PS as a suitable marker for the detection of PSOs
- Targeted analysis using MRM^{HR} provides accurate and sensitive quantification of PSOs by monitorin the PS moiety across a wide range of concentrations with a limit of detection (LOD) of 0.1 ng/mL
- The overall performance of the system resulted in excellent correlation (R^2>0.98) with inter-day accuracy (expressed as bias%) below 20%
- Presented workflow provides an easily implemented and accurate method for monitoring the marker of PSOs use for doping

Experimental details

Sample description: The phosphorothioated oligonucleotide of the antisense strand of exon 2 of the horse *myostatin* gene (MSTN), 5'-GAGATCGGATTCCAGTATACCA-3' (22-mer) was synthesized by GeneDesign, Inc (Osaka, Japan). All the nucleotides in the oligonucleotides were phosphorothioated (average mass: 7080.8, monoisotopic mass: 7075.692) then purified by high-performance liquid chromatography (HPLC). Oligo dT, 5'-TTTTTTTTTTTTTTT-3' (15-mer), which was not phosphorothioated was also synthetized by GeneDesign, Inc and used as an internal standard. The oligonucleotides used in this study were received lyophilised and reconstituted in 100 mmol/L triethylamine acetate (TEAA) buffer at a concentration of 20 µg/mL.

Plasma sample preparation: Horse plasma was used as the matrix in this study. Control plasma samples were prepared by collected blood in EDTA blood collection tubes. Plasma was quickly separated by centrifugation and transferred to a new tube using a pipette. Protein was removed from control horse plasma using the following protein precipitation procedure: 30 mL of LC-MS grade acetonitrile was added to 15 mL of control plasma in a 50 mL tube. The mixture was thoroughly mixed and stored at 4˚C for 15 minutes. The solution was then centrifuged at 3000 x g for 15 minutes. 40 µL of the supernantant was collected in a new tube and the solvent was evaporated using a gentle N_2 flow for 30 minutes. The final volume was adjusted to 10 mL using Milli-Q water. The deproteined horse plasma samples were stored at -20˚C until use.

Spiked plasma samples preparation: The control horse plasma samples were spiked with phosphorothioated oligonucleotide to create calibrator solutions ranging from 0.1 to 200 ng/mL. The 15-mer internal standard was added to each calibrator solution to a final concentration of 4.5 µg/mL. Figure 2 summarizes the sample preparation procedures used in this study.

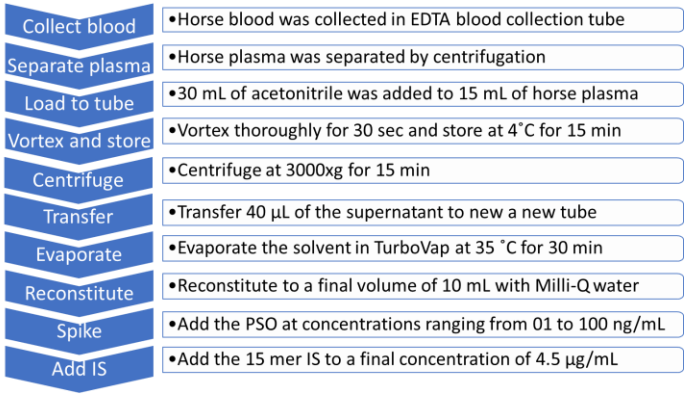

Figure 2. Proposed plasma deproteination and sample preparation procedures. An 10-step protein precipitation procedure was used to prepare the control horse plasma samples used in this study.

Chromatography: HPLC separation was achieved using an ACQUITY Oligo BEH C18 column (30 × 2.1 mm, 1.7 µm) held at 60ºC on a Prominence UFLC XR HPLC system. Mobile phase A consisted of 100 mM hexafluoro-2-propanol (HFIP) and 10 mM triethylamine (TEA) in water and mobile phase B consisted of methanol. The LC flow rate was 0.4 mL/min and the total run time was 4 min. The injection volume was 2 µL.

Mass spectrometry: MS and MS/MS data were acquired using negative electrospray ionization on the TripleTOF 6600 system equipped with a DuoSpray Turbo V ion source. Untargeted data acquisition was accomplished using information dependant acquisition (IDA) which consisted of a TOF MS scan to collect accurate mass precursor ions from 450 to 2800 Da, followed by a full scan TOF MS/MS on the selected charge states, with m/z range from 70 to 2800 Da to ensure all fragments were analyzed for identification. A maximum of 10 canditate ions per cycle were selected using the IDA criteria. Targeted data acquisition was accomplished using the MRM^{HR} algorithm using the precursor ion of *m/z* 785.18 corresponding to $[M-9H]^{9-}$ and the product ion of *m/z* 94.9362 derived from the PS moiety that was identified as a unique doping marker in the untargeted workflow. Data was acquired using Analyst software 1.7.1.

Data analysis: Data was processed in SCIEX OS software. Peak detection and integration were performed using the MQ4 algorithm. Mass spectra of precursor and product ion scans were viewed in the Explorer module of the software, then extracted ion chromatograms (XICs) could be directly. Quantitative analysis was performed in the Analytics module of the software where calibration curves, concentration calculations and assay accuracy statistics were automatically generated.

Characterization of PSOs and identification of phosphorothioate (PS) moiety using IDA

Developing and optimizing a robust method for the characterization of phosphorothioated oligonucleotides is key to identify markers of gene doping in biological matrix. The use of an untargeted data acquisition approach such as IDA is an appealing strategy for detection of oligonucleotides with no *a priori* sequence knowledge. Method optimization was performed by flow injection analysis (FIA) by exhaustively scanning the selected PSO in prepared horse plasma using the IDA criteria.

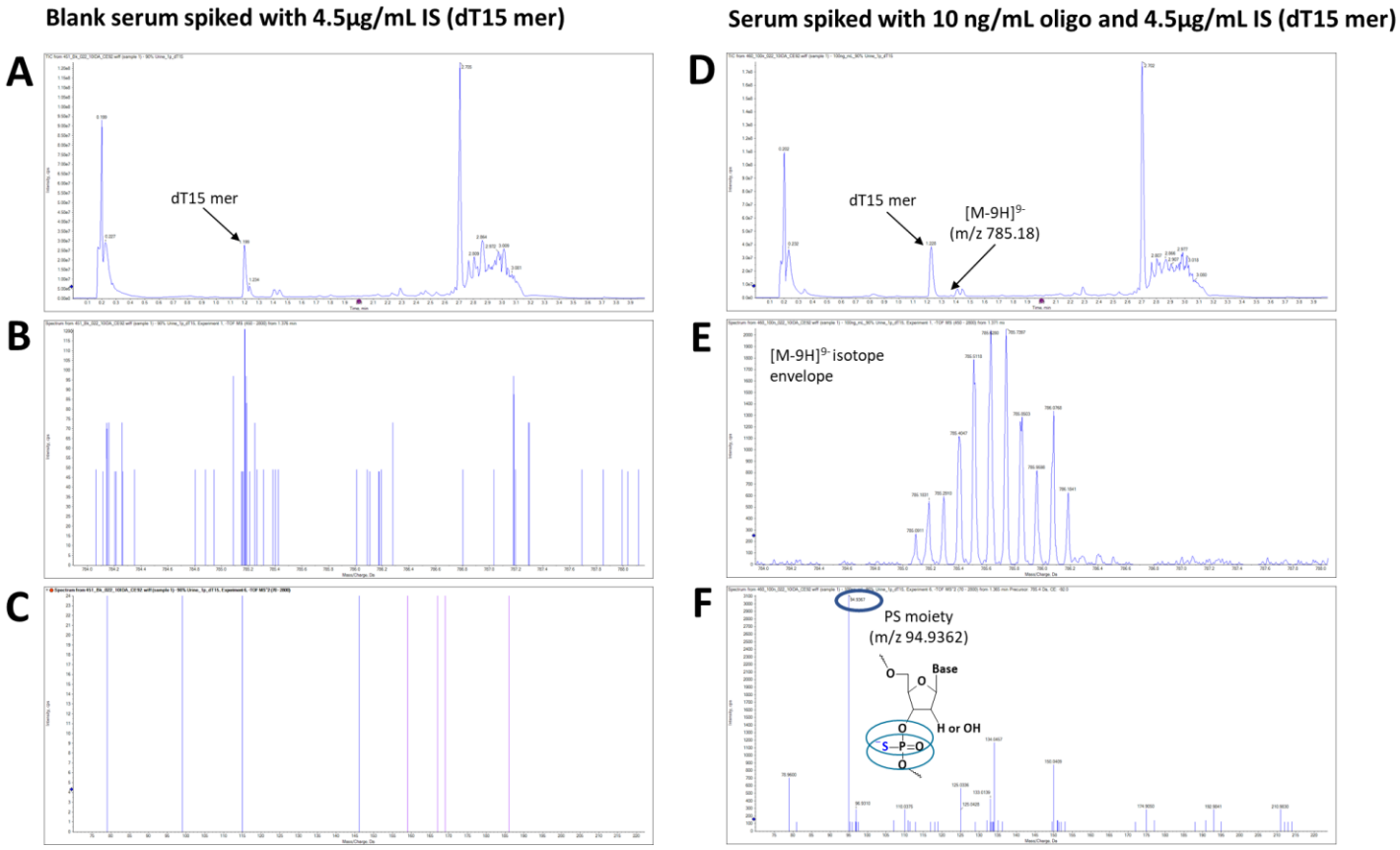

Figure 3. Untargeted analysis of phosphorothioated oligonucleotides (PSOs) in prepared horse plasma using IDA. Total ion chromatograms (TICs) of the blank matrix sample spiked with the PSO and the dT15-mer (D) and the blank matrix sample spiked the dT15-mer internal standard only (A) using precursor ion scan mode. TOF MS spectra centered at m/z 785.18 showing the charge state envelope [M-9H]9- of the sample containing the PSO (E) and the absence of peaks in the blank matrix sample containing the dT15-mer internal standard only. TOF MS/MS spectrum of the PSO sample showing unique fragment ions including the product ion at *m/z* 94.9362 derived from the PS moiety (F) and TOF MS/MS spectrum of the blank matrix sample containing the dT15-mer internal standard only showing no fragments (C).

Multiply charged ions with charge state ranging from 3 to 10 were detected (data not shown). The most intense charged ion detected at 1.37 min with charge state 9 isotope envelope ([M-9H]$^{9-}$, theoretical mass of *m/z* 785.09 was selected as a unique PSO precursor ion for the analysis. Figure 1 A shows the full scan TOF MS mass spectrum of a standard phosphorothioated oligonucleotide (PSO) showing the [M-9H]9- isotope envelope using the precursor ion scan mode. Figure 1B is the TOF MS/MS spectrum of the PSO showing the most intense fragment ion at m/z 94.9362 used as a diagnostic marker of PSO use. Figure 1C shows the extracted ion chromatograms (XICs) of m/z 94.9362, the most specific and abundant product ion derived from the PSO.

In order to ensure no endogenous components in the deproteinated horse plasma matrix contained any PSOs, two samples were prepared: a blank matrix sample containing the PSO and the dT15-mer and a blank matrix sample containing only the dT15-mer internal standard. Both samples were injected to compare the resulting spectra acquired with IDA.

Figure 3 shows the total ion chromatograms (TICs) of the blank matrix sample spiked with the PSO and the dT15-mer (D) and the blank matrix sample containing the dT15-mer internal standard only (A). Plotting the full TOF MS revealed the presence of the charge state 9 isotope envelope [M-9H]$^{9-}$ centered around the theoretical mass of *m/z* 785.18 in the sample containg the PSO (Figure 3 E). No peaks were detected in the TOF MS spectrum of the blank matrix sample containing the dT15-mer internal standard only (Figure 3 B). Figure 3 C and F show the TOF MS/MS spectra of the blank matrix sample containing the dT15-mer internal standard only and the blank matrix sample containing the PSO and the dT15-mer, respectively, extracted at 1.37 min. No fragments were observed in the TOF MS/MS product ion scan of the blank matrix sample containing the dT15-mer internal standard only (Figure 3 C). However, unique fragment ions at *m/z* 78.9600, 94.9367, 125.0336, 134.0457 and 150.0409 were observed in the TOF MS/MS product ion scan of the blank matrix sample containing the PSO and the dT15-mer (Figure 3 F). The most intense fragment observed at *m/z* 94.9367 derived from the PS moiety

was chosen as a diagnostic fragment ion to detect and monitor PSOs in horse blood for gene doping since they are not derived from endogenous substances in animal body.

Accurate and sensitive quantification of the PS moiety using the MRM^{HR} workflow

Based on the results of the untargeted analysis of spiked plasma, the specific and most abundant product ion of *m/z* 94.9362 derived from the PS moiety can be used as a diagnostic marker of PSO use in biological matrix. Therefore, targeted quantification can be performed through high-resolution extraction of the unique fragment ion derived from the PS moiety from MS/MS spectra. The transition of m/z 785.18→94.9362 was monitored with the MRM^{HR} workflow, then the diagnostic fragment was extracted post-acquisition using a 20 mDA window. Here, the series of 10 calibrator plasma solutions spiked with the PSO at concentrations ranging from 0.1 to 100 ng/mL were injected ten different days to assess the linearity and quantitative performance of the MRM^{HR} workflow for gene doping control.

Figure 4 shows the calibration curves plotted across the nine calibrator solutions ranging from 0.1 to 100 ng/mL for the two consecutive days. The quadratic calibration curves of PSO spiked in deproteinated horse plasma demonstrate excellent correlation and linearity with correlation coefficient (R^2 values) above 0.98 for both days.

The quantification performance of the MRMHR workflow was investigated by determining the lower limit of quantification (LLOQ) and the inter-day accuracy (expressed as bias%). The limit of detection (LOD) for the PSO spiked in deproteined horse plasma was estimated to be less than 0.1 ng/mL. In addition, the inter-day accuracy was calculated to be between 82 and 118% across the two consecutive days of analysis. Table 1 summarizes the accuracy of the assay across

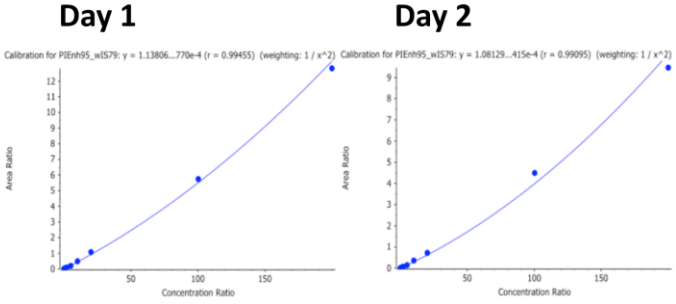

Figure 4. Excellent linearity for the detection of PSO spiked in deproteinated horse plasma. Regression curves resulting from the calibration series from 0.1 to 100 ng/mL for the *m/z* 785.18→94.9362 MRM transition. R^2 values greater than 0.98 were observed on both days.

Table 1. Assay accuracy. Statistical results showing the accuracy of measurements across two consecutive days for the 10 calibrator levels and 2 quality control samples.

Sample Type	Concentration (ng/mL)	Accuracy (%bias) Day 1	Day 2
Standards (STD)	*0.1*	*103.0*	*108.5*
	0.2	*103.5*	*93.4*
	0.5	*82.5*	*82.0*
	1	*89.8*	*86.6*
	2	*91.6*	*94.2*
	5	*98.7*	*90.9*
	10	*113.1*	*117.4*
	20	*114.7*	*117.2*
	100	*104.7*	*109.8*
	200	*97.4*	*95.4*
Quality controls (QC)	*20*	*111.5*	*119.3*
	200	*96.1*	*102.4*

the two consecutive days for the 10 levels of calibrator ranging from 0.1 to 100 ng/mL and the two quality control samples analyzed using the MRMHR workflow.

Conclusions

A comprehensive workflow for the detection of a marker of PSO use in horse plasma was successfully developed using the SCIEX TripleTOF 6600 system. Untargeted analysis using IDA enabled characterization of PSOs using the PS moiety as a diagnostic fragment ion in deproteinated horse plasma samples using precursor ion scan mode. Identification of a unique fragment ion at *m/z* 94.9362 derived from the PS moiety was used as a gene doping marker for the presence of PSOs in horse blood. The information extracted from the IDA workflow was used to develop an MRM^{HR} acquisition method which enabled sensitive and accurate quantification of the PSO spiked in deproteinated horse plasma at concentrations ranging from 0.1 to 100 ng/mL. The assay showed an LLOD of 0.1 ng/mL with excellent inter-day accuracy (below 20%), demonstrating that the product ion *m/z* 94.9362 resulting from the PS moiety is an effective marker that can be accurately quantified with high accuracy and used for the detection of non-approved PSOs used for doping. Overall, the SCIEX TripleTOF 6600 system was shown to be a flexible, specific and sensitive platform with multiple acquisition workflows that can be leveraged for the analysis of unknown PSOs used for gene doping.

References

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2. Tozaki, T. ; Hamilton, N. (2021) Control of gene doping in human and horse sports. *Gene Ther.* 2021.

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