Supporting Information

First steps towards uncovering gene doping with CRISPR/Cas by identifying SpCas9 in plasma via HPLC-HRMS/MS

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Chemicals & Materials

Recombinant SpCas9 protein (TrueCut™ Cas9 Protein v2) used for method validation and Invitrogen TrueGuide™ sgRNA modified, targeting the myostatin (MSTN) gene in mice, as well as the magnetic beads (MagnaBind™ Goat Anti-Mouse IgG), Lipofectamine RNAiMAX transfection reagent and dimethyl sulfoxide (DMSO; organic eluent additive for HPLC-MS) were purchased from Thermo Fisher Scientific (Waltham, MA USA). Monoclonal anti-CRISPR-Cas9 antibodies [7A9-3A3] were obtained from Abcam (Cambridge, UK) and the customized peptide sequences of the diagnostic peptides T129 and T285[1] used for recovery determination, in addition to the peptide sequences used as internal standard ($ISTD_{T129}$ and $ISTD_{T285}$), were synthesized by GenScript (Piscataway, NJ USA). Sequencing grade modified trypsin was acquired from Promega (Madison, WI USA) and apheresis derived pooled human plasma was procured from Innovative Research (Novi, MI USA). Tris-(2-carboxyethyl)-phosphine hydrochloride (TCEP-HCl) was obtained from Carl Roth (Karlsruhe, Germany). Catalytically dead Cas9 (dCas9-3XFLAG™-Biotin protein), recombinant SpCas9 protein (CASPROT) utilized for the *in vivo* study, ammonium bicarbonate, iodoacetamide, glacial acetic acid and phosphate-buffered saline (PBS; 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4) as well as Amicon® Ultra-0.5 centrifugal filter units (100 K molecular weight cut off) and formic acid (eluent additive for HPLC-MS) were purchased from Merck (Darmstadt, Germany). LC grade acetonitrile was received from VWR®, part of Avantor (Radnor, PA USA) and ultrapure water for the preparation of all aqueous solutions was received from the Thermo Fisher Scientific Barnstead™ GenPure™ xCAD Plus water purification system.

Sample Preparation

Immunoaffinity Purification

The immunoaffinity purification procedure was adopted from Knoop A. *et al* [1] and adjusted for the current application. Thus, the magnetic beads, contained in a volume of 200 µL MagnaBind™ solution, were magnetically separated and the supernatant was discarded. Thereupon, the beads were vigorously washed with 200 µL of PBS and afterwards resuspended in 100 µL of PBS. Two µL of 1 mg/mL monoclonal anti-CRISPR/Cas9 antibodies were added, and the suspension was incubated for 30 min at RT with gentle vortexing every five min to prevent magnetic particles from settling and to promote attachment of the affinity ligands. Subsequently, the suspension was added to the previously prepared plasma sample and incubated for another 90 minutes at RT with slow end-over-end rotation. This was followed by threefold washing of the obtained bead-antibody-antigen complex solution with 300 µL of PBS. Elution and recovery of the analyte from the beads was achieved by adding 50 µL of 3% acetic acid and transferring the supernatant from the magnetically separated beads into a fresh LoBind tube. Finally, 7 µL of 100 ng/mL of $ISTD_{T129}$ and 5.5 µL of 100 ng/mL $ISTD_{T285}$ were added and the mixture was forwarded to enzymatic digestion.

Tryptic Digestion

To enhance the proteolytic digestion by making the cleavage sites more accessible for trypsin, reduction, neutralization and alkylation was performed as described elsewhere [2]. Reduction was achieved by adding 500 nmol of TCEP (5 µL of 100 mM solution) to the sample followed by incubation for 15 min at 60 °C and 900 rpm. Afterwards, the solution was neutralized with 15 µL of 2 M ammonium bicarbonate, and the cysteine residues were alkylated by adding 1625 nmol of iodoacetamide (6.5 µL of freshly prepared 250 mM solution) and incubation of the mixture for another 30 min at RT in the dark. Furthermore, 9 µL of acetonitrile were added to the mixture, as the addition of 10 – 20 % of organic solvent increases the protein's solubility and inhibits adsorption to the surface of the plastic tube, thus improving the enzymatic digestion[3]. Finally, 10 µL of 40 µg/mL trypsin solution were added and proteolysis was carried out over night at 37 °C and 500 rpm. The next day, tryptic digestion was terminated by the addition of 2 µL of glacial acetic acid and the prepared sample was transferred into a 0.3 mL plastic microvial, ready for HPLC-MS/MS analysis.

Qualitative Method Validation Parameters

The parameters for a comprehensive method characterization were determined as follows:

Selectivity: To furnish proof that the detection method is selective, ten plasma samples originating from ten different individual sources were analyzed as blanks (female n = 5; male n = 5) as well as zero samples (female n = 1; male n = 1) and checked for interfering signals.

Linearity: Evaluation of the linear relation between analyte concentration and measured response was conducted by fortifying six pooled plasma samples with 25, 50, 100, 250, 500, and 1000 ng/mL SpCas9 and generating a calibration curve with the ISTD-normalized peak areas. The obtained function was then assessed with simple linear regression analysis.

Precision: Precision was calculated as coefficient of variation of the ISTD-normalized peak areas of six replicates at the concentration levels of 100, 500, and 1000 ng/mL.

Limit of detection: The LOD was determined as the lowest concentration detectable with a signal-to-noise ratio (S/N) > 3 for the most intensive transition of each peptide. The evaluation was executed by processing six biological replicates prepared with the estimated concentration of 25 ng/mL.

Limit of identification: In this case, the LOI is defined as the lowest analyte content consistently identified in ten representative samples (identification rate = 100 %) meeting the criterion of the detection of two diagnostic precursor-product ion pairs per peptide to ensure unequivocal identification of the analyte. Therefore, ten different plasma specimens were fortified with the estimated concentration of 100 ng/mL SpCas9, processed and analyzed accordingly.

Recovery: Recovery was assessed in two different ways: as total recovery comprising the whole sample preparation and as partial recovery including only the enzymatic digestion. To evaluate both types of recoveries, ISTD-normalized peak areas of samples spiked with the analyte and processed as described before (totally extracted samples) as well as, ISTD-normalized peak areas of samples spiked with the analyte after the immunoaffinity purification (partially extracted samples), were compared to the ISTD-normalized peak areas of zero samples that were processed completely before adding the analyte (100 % samples). To obtain the totally extracted samples, plasma aliquots were directly fortified to a concentration of 1000 ng/mL SpCas9 (n = 6). Partially extracted plasma aliquots were spiked with the same amount of SpCas9 after sample purification with anti-CRISPR Cas9 antibodies (n = 6). The 100 % samples (n = 6) were prepared by dissolving the appropriate peptide contents in extracted zero plasma aliquots to correspond to the equivalent protein concentration of 1000 ng/mL.

Absolute matrix effects: To examine to which extent the matrix has an effect on the ionization of the analyte (ion suppression/ion enhancement) identical quantities of peptides, corresponding to a high protein concentration, were spiked into extracted zero plasma samples (n = 6) as well as in neat solution (99 µL 3 % acetic acid + 9 µL ACN, n = 6) and the resulting peak areas were compared.

Robustness: Robustness was assessed by testing whether the chromatographic separation can also be conducted on a C8 analytical column instead of a C18 column, as C8 columns are also frequently used

in doping control routine laboratories. Therefore, the six replicates used for the determination of the precision at the concentration level of 500 ng/mL were measured again with the application of a Poroshell 120 EC-C8 column analytical column (3.0 Int. ø x 50 mm, 2.7 µm particle size) from Agilent. Evaluation was achieved by calculating the bias of the ISTD normalized mean values of the peak areas between the samples separated on the C18 and C8 column.

Carryover: To ensure that the obtained results of the analytical method are not falsified due to carryover, three blank samples were analyzed directly after the highest concentrated sample containing 1000 ng/mL SpCas9 and checked for signals of the analyte and ISTD, respectively.

References

1. Knoop, A.; Thomas, A.; Bidlingmaier, M.; Delahaut, P.; Schaenzer, W.; Thevis, M., Probing for corticotropin-releasing hormone (CRH) in human blood for doping control purposes using immunoaffinity purification and LC-HRMS/MS. *Analytical Methods* **2017,** *9*, 4304-4310.
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3. Ewles, M.; Goodwin, L., Bioanalytical approaches to analyzing peptides and proteins by LC--MS/MS. *Bioanalysis* **2011,** *3*, 1379-97.