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Probing for peptidic drugs (2–10 kDa) in doping control blood samples

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Abstract

Bioactive peptides with a molecular mass between 2 and 10 kDa represent an important class of substances banned in elite sports, which has been recognized with an increasing number and variety of substances by anti-doping organizations. Also, the annually renewed list of prohibited substances of the World Anti-Doping Agency (WADA) explicitly mentions more and more of these peptides, and efficient testing procedures are required. Even under simplified sample preparation conditions, liquid chromatography coupled to high-resolution mass spectrometry (with resolution properties > 100,000 full width at half maximum) offers suitable conditions for this task and can therefore be used as an initial testing procedure. In contrast to urine, blood analysis essentially relies on the detection of intact peptide hormones, and the expected concentrations are commonly higher in blood samples than in urine. This facilitates the analysis, and a generic sample preparation by means of mixed-mode solid-phase extraction could be realized in this study. Co-extraction and analysis of several different peptides such as insulins (human, lispro, aspart, glulisine, tresiba, detemir, glargine, bovine insulin and porcine insulin), growth hormone releasing hormones (sermorelin, CJC-1295 and tesamorelin), insulin-like growth factors (long-R_3-IGF-I, R_3-IGF-I and Des_{1-3}-IGF-I) and mechano growth factors (human MGF and MGF-Goldspink) with criteria that fulfil the requirements of the WADA documents (TD2022 MRPL) for doping controls. The proof of principle was shown by the analysis of post administration samples after treatment with synthetic insulin analogues.

KEYWORDS
high-resolution mass spectrometry, LC-MS/MS, sports drug testing

Abbreviations: FWHM, full width at half maximum; GH-RH, growth hormone-releasing hormone; IGF, insulin-like growth factor; ISTD, internal standard; LOD, limit of detection; MGF, mechano growth factor; MRPL, minimum required performance level; PS, particle size; SPE, solid phase extraction; TD, technical document; tSIM, targeted single ion monitoring; WADA, world anti-doping agency.

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1 | INTRODUCTION

The World Anti-Doping Agency (WADA) has acted on the increasingly important role of peptidic drugs by recognizing a considerable number of these in the annually updated prohibited list and, in accordance with criteria applicable to urine samples, has also introduced minimum required performance levels (MRPLs) for blood samples (serum or plasma). These MRPLs are set to sub ng/ml levels for synthetic insulins (0.3 ng/ml) and growth hormone releasing hormone analogues (0.3 ng/ml), while higher concentrations apply in the case of IGF-I analogues (2 ng/ml).[1,2] In general, the analysis of blood samples is favoured because for most (if not all) banned peptides the pharmacologically relevant concentrations in blood are established, whereas the renal clearance and urinary concentrations in many cases are largely unknown and sometimes unidentified degradation products (metabolites) warrant consideration. Additionally, the stability of most peptide hormones in blood samples (e.g. ethylenediaminetetraacetic acid [EDTA] plasma or serum) is presumably superior compared to urine samples, especially in situations when frozen conditions (e.g. sample transport) are not guaranteed. Extensive degradation of the prohibited peptides synachten (synthetic adrenocorticotropic hormone analogue) and long-R_3-IGF-I in urine was described previously when stored at room temperature or 4°C.[3–6]

Although the expected concentrations of the target peptides in blood samples are generally higher than in urine samples, the available volume compensates for this. With regular urine samples usually available in 100 ml containers, blood samples are commonly limited to less than 2 ml of serum or plasma. The volumes required for blood analytical assays usually range between 50 and 500 µl, while for urine most assays are designed to operate with 1 to 5 ml. Nevertheless, the analysis of doping control blood samples represents a promising approach, especially to uncover the misuse of prohibited peptide hormones (2–10 kDa) by means of liquid chromatography-mass spectrometry (LC-MS). Besides the low concentration of the target analytes, the coexisting and complex mixture of high-abundant proteins complicates a simple analysis with established standard protocols. Existing methods include time-consuming sample preparation steps such as immunoaffinity purification before LC-MS.[5,7–15] These methods are very specific and selective due to the complementary combination of immune extraction, liquid chromatographic separation and detection by (high-resolution/tandem) MS. But due to the laborious sample preparation steps, recently also more simplified approaches were developed, which also meet the criteria outlined in mandatory WADA documents.[16–19] Most of these assays focus on urine analysis or are limited to one class of peptides (e.g. insulins) only; conversely, in the present study the applicability of a mixed-mode anion-exchange solid-phase extraction (SPE) was shown to allow for an effective sample preparation adequate for subsequent LC-MS analysis of doping control blood samples for several different prohibited peptides. Noteworthy, not all target peptides were recovered satisfactorily with the chosen SPE-based strategy and, thus, for example synacthen was not considered in this study. On the other hand, the method is per definition not limited to the included target peptides and also other substances (e.g. lower molecular mass peptidic drugs) are co-extracted.

2 | METHODS

2.1 | Chemicals and reagents

Acetic acid (glacial), acetonitrile and methanol were obtained from Merck (Darmstadt, Germany). Ammonium hydroxide solution (25% in water) and formic acid were purchased from Sigma (Schnelldorf, Germany). The mixed-mode SPE cartridges (HR-XA, 3 ml, 60 mg) were from Macherey&Nagel (Düren, Germany). For all dilution steps and preparation of aqueous solutions, ultra-pure water of MilliQ-quality was used. Insulin analogues lispro (Humalog), aspart (Novolog), glulisine (Apidra), detemir (Levemir) and degludec (Tresiba) were supplied by Eli Lilly (Indianapolis, IN), Novo Nordisk (Princeton, NJ) and Aventis (Kansas City, MO). Long-R^3-IGF-I, porcine insulin and bovine insulin were from Sigma (Schnelldorf, Germany). GH-RH_{1-29} (Geref) was purchased from BMFZ (Düsseldorf, Germany). Tesamorelin, and Acetyl-(Tyr_1, D-Arg_2)-GRF_{1-29}(ISTD2) were from Bachem (Bubendorf, Switzerland). CJC-1295((D-Ala_2, Gln_8, Ala_{15}, Leu_{27})-GRF amide), CJC-1293((D-Ala_2)-GRF amide) and the metabolites of Geref (GRF_{3-29}) and CJC-1293((D-Ala_2)-GRF_{2-29} amide, purity 91%) were custom-synthesized by Centic Biotec (Jena, Germany). Des_{1-3} -IGF-I and R_3-IGF-I were obtained from IBT Biosystems (Reutlingen, Germany). The glargine metabolite ($DesB_{31-32}$ glargine) was obtained from IBA (Warsaw, Poland, purity > 90%) and the stable isotope-labelled insulin internal standard [[$^2H_{10}$]-Leu $^{B6, B11, B15, B17}$]-Insulin (human) (ISTD1) was purchased from PeptaNova (Sandhausen, Germany). MGF human and MGF "Goldspink" were obtained from Phoenix Pharmaceuticals, Inc (Karlsruhe, Germany), and ^{15}N-labelled IGF-I used as ISTD3 was purchased from Prospec (Santa Clara, CA). All reference standards own a purity > 95% unless otherwise stated.

2.2 | Sample preparation

All target peptides were purified from 200 µl of plasma or serum by means of mixed-mode anion-exchange SPE. Two hundred microliters of plasma (or serum) were fortified with 10 µl of ISTD solution (containing 0.5 ppm of ^{2}H-labelled human insulin (ISTD 1), ^{15}N-labelled IGF-I (ISTD 3) and acetyl-(Tyr_1, D-Arg_2)-GRF_{1-29}(ISTD 2) and 10 µl of ammonium hydroxide solution (5% in water). After vortex, the samples were precipitated with 550 µl of a mixture of ice-cold methanol/acetonitrile (50/50, v:v) and centrifuged at 17,000 g for 10 min. The supernatant was diluted with 1 ml of water in a new Eppendorf tube and transferred to the mixed-mode solid-phase cartridge (HR-XA), which was preconditioned with 1 ml of methanol and 1 ml of water. After sample loading, the cartridge was washed with 2 ml of water and 2 ml of methanol/water (50/50, v:v). Elution into an Eppendorf tube followed using 1.2 ml of methanol (acidified with 5% of formic acid). After

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evaporation in a vacuum centrifuge (approx. 60 min at 40°C), the samples were reconstituted in 70 µl of formic acid (3%) and injected with 20 µl into the LC-MS.

2.3 | Blood specimens and administration samples

The validation was carried out with EDTA plasma and serum samples from 10 healthy male and female volunteers without any medication within the last 24 h. Additionally, a commercially available plasma pool (Octaplas, Octapharma GmbH, Langenfeld, Germany) was used for selected validation parameters (e.g. recovery) due to the absence of endogenous insulin. Post-administration serum samples were obtained from insulin-dependent diabetics who regularly administer different synthetic insulin analogues (male volunteer, diabetes mellitus type I, subcutaneous injection via insulin-pen, insulin aspart: 8 IU, 2 h before sample collection, insulin detemir: 17 IU, 3 hours before sample collection). Written consent of the volunteers and approval by the local ethics committee (DSHS No.: 139/2021) was obtained for this study.

2.4 | Liquid chromatography

The chromatographic separation of the peptides was performed by means of high-performance LC using a Vanquish system (Thermo, Bremen, Germany). The system was equipped with a dual-pump set-up and initial trapping of the target analytes on an Accucore Phenyl/Hexyl, 3 × 10 mm, 2.7 µm PS (Thermo) trapping column using water with formic acid (0.1%, solvent A_1) and acetonitrile (with 0.1% formic acid, solvent B_1). Trapping was performed for 2 min at 99% of solvent A_1 before switching the flow to the analytical column (Poroshell C18 3 × 50 mm (Agilent, Karlsruhe, Germany)). As solvent buffers, A_2 and B_2 for the gradient, water with formic acid (0.1%) and dimethyl sulfoxide (DMSO, 1%) as solvent A_2 and acetonitrile with DMSO (1%) and formic acid (0.1%) (solvent B_2) were used. The flow was set to 400 µl/min and the gradient started at 95% of A_2 and decreased to 60% of A_2 within 8 min. Within the next 2 min, the gradient decreased to 20% of A_2 for cleaning the column. Finally, the system was re-equilibrated for 5 min at starting conditions. The resulting overall run time was 15 min, and the injection volume was 20 µl. The column compartment was set to 25°C and the autosampler cooled to 10°C.

2.5 | Mass spectrometry

High-resolution MS was performed using an Orbitrap Exploris 480 (Thermo) equipped with a heated electrospray ion source. The instrument operated in positive ionization mode acquiring data in full scan mode (mass-to-charge ratio (m/z) = 400–1700, resolution 60,000 full width at half maximum [FWHM]) and targeted single ion monitoring (tSIM) with data dependent MS^2 by means of an inclusion list. tSIM experiments were performed for the multiply protonated molecules of the target peptides (see Table 1) with a resolution at 120,000 FWHM and multiplexed 5 times with a quadrupole isolation window of 3 m/z units. The data-dependently triggered targeted MS^2 experiments were acquired with a resolution of 15,000 FWHM and a quadrupole isolation window of 2 Da. The instrument was calibrated according to the manufacturer's recommendations using a calibration mixture (consisting of caffeine, the tetrapeptide MRFA and Ultramark). The gas supply consisted of nitrogen (N_2-generator; CMC, Eschborn, Germany). Ionization in positive mode was accomplished at a voltage of 3 kV, and the temperature of the ion transfer tube was adjusted to 320°C. Xcalibur Software used was: Foundation 3.1 SP7 QF1 and Xcalibur 4.4 (Thermo).

2.6 | Validation

The method was validated according to the requirements of WADA, described in the international standards for laboratories and the technical document for the MRPL considering an initial testing procedure for non-threshold substances.[2,20] The parameters were specificity (10 blank plasma samples), reliability at the MRPL (10 different plasma samples fortified at the respective MRPL), the limit of detection (LOD, six different samples at MRPL 50%, 25% and 10%), carryover and stability in the autosampler (24 h). In addition to these recommended parameters, also the recovery was determined. Here, the loss of the respective peptides during the sample preparation is characterized by analysing six technical replicates fortified at the MRPL before the preparation in comparison to six replicates fortified after the sample preparation just before the injection. The carryover of a highly concentrated sample (4 × MRPL[20]) to a following blank sample was evaluated with three repetitions. The chromatograms of the blank sample after injection of the high concentrated sample (4 × MRPL) was evaluated for the presence of occurring peaks in the respective retention time window. For testing the stability after preparation, 10 samples at the MRPL were reinjected after 24 h storage in the autosampler. The robustness was tested for serum instead of plasma with six different serum samples analysed as blank and fortified at the respective MRPL.

3 | RESULTS AND DISCUSSION

3.1 | Validation

Due to the recently introduced MRPLs for several prohibited peptide hormones in blood samples, all validation parameters were performed in accordance with the WADA requirements fixed in the international standard for doping control laboratories.[20] The main results are summarized also in Table 2. Chromatograms were investigated at the respective retention times (window ~30 s) with a mass window of 10 ppm. The detection rate in all blank samples was 0/10 except for human insulin and IGF-I, which was present as endogenous hormones in all samples (10/10). The specificity was fulfilled accordingly. The same set of 10 blank samples was also fortified at the respective MRPLs of the different peptide classes (see Table 2) and here all peptides were detected with 100% detection rate (10/10 samples). Thus,

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TABLE 1 Amino acid sequences, masses, precursor ions and retention times of target peptides

Peptide	Amino acid sequence	Monoisotopic mass (Da)	Precursor tSIM (*m/z*)	Dominant charge state	~Ret. time (min)
Human insulin	GIVEQCCTSICSLYQLENYCN – FVNQHLCGSHLVEALYLVCGERGFFYTPKT	5803.6	1162/1452	4+/5+	6.9
Bovine insulin	GIVEQCCASVCSLYQLENYCN - FVNQHLCGSHLVEALYLVCGERGFFYTPKA	5729.6	1147	5+	6.8
Lispro	GIVEQCCTSICSLYQLENYCN – FVNQHLCGSHLVEALYLVCGERGFFYTKPT	5803.6	1162	5+	6.9
Aspart	GIVEQCCTSICSLYQLENYCN – FVNQHLCGSHLVEALYLVCGERGFFYTDKT	5821.6	1166	5+	6.9
Glulisine	GIVEQCCTSICSLYQLENYCN – FVKQHLCGSHLVEALYLVCGERGFFYTPET	5818.6	1166	5+	6.9
Glargine Met	GIVEQCCTSICSLYQLENYCG – FVNQHLCGSHLVEALYLVCGERGFFYTPKT	5746.6	1151	5+	6.9
Porcine insulin	GIVEQCCTSICSLYQLENYCN – FVNQHLCGSHLVEALYLVCGERGFFYTPKA	5773.6	1156	5+	6.9
Detemir	GIVEQCCTSICSLYQLENYCN- FVNQHLCGSHLVEALYLVCGERGFFYTPK-Myr	5912.8	1479	4+	8.9
Degludec	GIVEQCCTSICSLYQLENYCN – FVNQHLCGSHLVEALYLVCGERGFFYTPK-γ-L-Glu-Pal	6099.8	1527	4+	8.1
Geref	YADAIFTNSYRKVLGQLSARKLLQDIMSR-NH_2	3355.8	840	4+	7.2
CJC-1293	YdADAIFTNSYRKVLGQLSARKLLQDIMSR-NH_2	3355.8	840	4+	7.2
CJC-1295	YANAIFTQSYRKVLAQLSARKLLQDILSR-NH_2	3365.9	842	4+	7.3
Geref Met	AIFTNSYRKVLGQLSARKLLQDIMSR-NH_2	3121.7	781	4+	7.1
CJC-1293 Met	dADAIFTNSYRKVLGQLSARKLLQDIMSR-NH_2	3192.7	799	4+	7.1
Tesamorelin	Hex-YADAIFTNSYRKVLGQLSARKLLQDIMSRQ QGESNQERGARARL-NH_2	5133.7	734	7+	7.3
LongR_3-IGF-I	MFPAMPLSSLFVNGPRTLCGAELVDALQFVCGDRGFYFNK PTGYGSSSRRAPQTGIVDECCFRSCDLRRLEMYCAPLKPAKSA	9105.4	1307	7+	7.4
R_3-IGF-I	GPRTLCGAELVDALQFVCGDRGFYFNKPTGYGSS SRRAPQTGIVDECCFRSCDLRRLEMYCAPLKPAKSA	7670.6	1099	7+	6.4
IGF-I	GPETLCGAELVDALQFVCGDRGFYFNKPTGYGSS SRRAPQTGIVDECCFRSCDLRRLEMYCAPLKPAKSA	7643.6	1093	7+	6.6
Des1-3-IGF-I	TLCGAELVDALQFVCGDRGFYFNKPTGYGSSSRRAPQ TGIVDECCFRSCDLRRLEMYCAPLKPAKSA	7360.5	1053	7+	6.5
MGF human	YQPPSTNKNT KSQRRKGSTF EERK	2866.5	574	5+	3.2
MGF "Goldspink"	YQPPSTNKNT KSQRRKGSTF EEHK	2846.5	570	5+	3.2

the reliability at the MRPL is given. The recoveries are calculated in a range between <10% (e.g. hMGF) to >80% (e.g. insulins). The LOD for each target peptide is defined with a detection rate of 95%. It was found that for 12 out of the 18 target peptides the LOD range at 25% of the MRPL and for 16 out of the 18 target peptides at 50% of the respective MRPL. Noteworthy, hMGF and insulin detemir were reliably detectable at 100% MRPL only. At 10% of the MRPL all target peptides were detected only sporadically. Reinjection of the samples fortified at the MRPL after storage in the autosampler (set to 10°C) for 24 h showed good stability with a detection rate of 10/10. No carryover in the chromatographic system from a sample fortified at 4 × MRPL level to the next blank sample was observed for all target analytes. The serum samples tested for robustness showed no interfering signals in the blank samples (0/6) and 100% detection rate at the MRPL (6/6). Noteworthy, the here presented method is designed as initial testing procedure to enable an effective first analysis of doping control samples. The final identification of the prohibited peptides according to the technical document WADA TD_IDCR2021.[21]

3.2 | Liquid chromatography-MS

The obtained results indicate sufficient selectivity, specificity and sensitivity of the approach, which fulfils the criteria for state-of-the-art

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TABLE 2 Validation results

Peptide	Specificity	Recovery (%)	MRPL (ng/ml)	Detection rate at MRPL	at MRPL 50%	at MRPL 25%	at MRPL 10%	Carryover (%)	Stability detection rate after 24 h at MRPL
Human insulin	10/10	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Bovine	0/10	82	0.3	10/10	6/6	6/6	6/3	n.o.	10/10
Lispro	0/10	78	0.3	10/10	6/6	6/6	6/5	n.o.	10/10
Aspart	0/10	86	0.3	10/10	6/6	6/6	6/3	n.o.	10/10
Glulisine	0/10	86	0.3	10/10	6/6	6/6	6/3	n.o.	10/10
Glargine Met	0/10	88	0.3	10/10	6/6	6/6	6/2	n.o.	10/10
Porcine	0/10	84	0.3	10/10	6/6	6/6	6/1	n.o.	10/10
Detemir	0/10	79	0.3	10/10	6/4	6/1	6/0	n.o.	10/10
Degludec	0/10	96	0.3	10/10	6/6	6/6	6/2	n.o.	10/10
Geref/CJC-1293	0/10	35	0.3	10/10	6/6	6/4	6/0	n.o.	10/10
CJC-1295	0/10	56	0.3	10/10	6/6	6/6	6/2	n.o.	10/10
Geref Met	0/10	31	0.3	10/10	6/6	6/6	6/1	n.o.	10/10
CJC-1293 Met	0/10	35	0.3	10/10	6/6	6/6	6/1	n.o.	10/10
Tesamorelin	0/10	21	0.3	10/10	6/6	6/4	6/0	n.o.	10/10
LongR_3-IGF-I	0/10	43	2	10/10	6/6	6/5	6/1	n.o.	10/10
R_3-IGF-I	0/10	15	2	10/10	6/6	6/2	6/0	n.o.	10/10
IGF-I	10/10	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Des1-3-IGF-I	0/10	33	2	10/10	6/6	6/6	6/2	n.o.	10/10
MGF human	0/10	5	2*	10/10	6/4	6/1	6/0	n.o.	10/10
MGF "Goldspink"	0/10	25	2*	10/10	6/6	6/6	6/3	n.o.	10/10

n.d.: not determined; n.o.: not observed.
*MRPL not explicitly shown in TD2022 MRPL.

doping control analysis. Figure 1 shows the chromatograms of a blank sample fortified at the respective MRPLs. Additionally, in Figure 2 a blank sample is shown. Nevertheless, considering established hybrid assays combining immuno-extraction with subsequent LC-MS analysis, the herewith yielded extracts are much more complex with a lower degree of purification. This has a direct impact on the HRMS data with a high number of interfering (or at least visible) signals in the tSIM mass spectra at the respective retention time. This phenomenon was already described earlier for insulin-specific assays.[14] Especially in low concentrated samples (at MRPL or less), the evaluation of the mass spectra and the corresponding extracted ion chromatograms are less straight forward compared to the hybrid assay data reported previously.[11,15] In order to enable specific detection, the resolution of the orbitrap analyser was set to 120,000 FWHM in the SIM experiments. Thus, extraction of very narrow mass ranges (~2 ppm) were used for data evaluation, and employing the multiplex option for the SIM precursors allowed for the generation of a sufficient number of data points per peak (with a typical peak width of 10 s). In case of confirmatory analysis, extraction with specific antibodies is strongly recommended in order to avoid result misinterpretations.

The growth hormone releasing hormones Geref (Sermorelin) and CJC-1293 differ by the exchange of one D-amino acid at position 2 ($dAla_2$) only. With the present approach it is not possible to differentiate both peptides, due to identical mass, retention time, and product ion spectrum (see also Table 1).[22] This was shown already in earlier studies and the differentiation might be enabled by diagnostic metabolite pattern.[15,23,24] The metabolites of Geref and CJC-1293 were included in the validation accordingly. For the differentiation of human insulin and insulin lispro, the ddMS2 spectra enable the identification of the synthetic insulin analog with the diagnostic product ion at *m/z* 217(corr. to $(B)y_2$-ion, see Figure 1).[11,15,17] The proof-of-principle was shown with post administration samples obtained from a patient suffering from *diabetes mellitus* following the regular treatment with two synthetic insulin analogues. In Figure 3 the extracted ion chromatograms for all synthetic insulin analogues are shown with abundant signals for the short acting insulin aspart (at 6.9 min) and long acting insulin detemir (at 8.9 min). Additionally, the corresponding mass spectrum from the SIM experiment for insulin aspart (showing the 5-fold protonated precursor at *m/z* 1166) and the triggered ddMS2 spectrum for insulin detemir (derived from the 4-fold protonated precursor at

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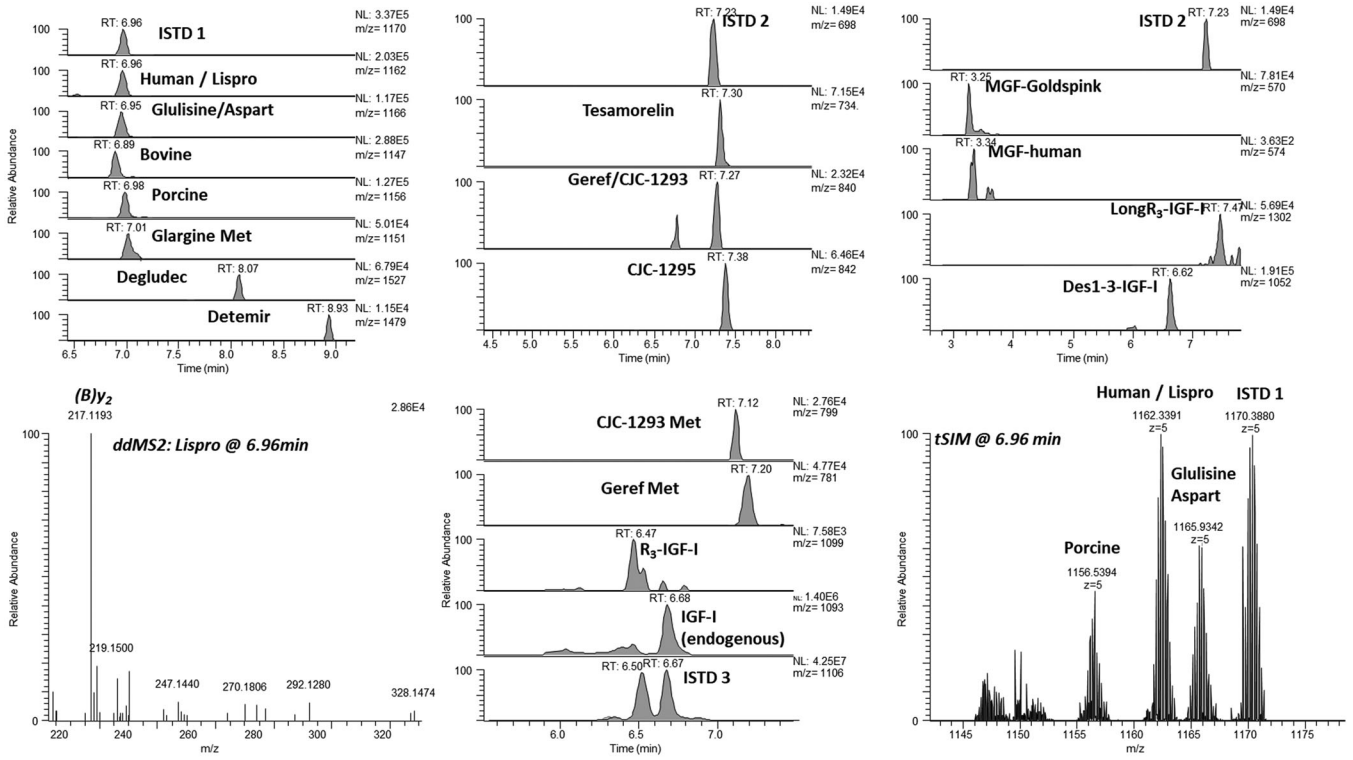

FIGURE 1 Extracted ion chromatograms (from targeted single ion monitoring [tSIM]) of a blank plasma sample fortified at the respective minimum required performance levels (MRPLs) of different insulins (0.3 ng/ml), GH-RH (0.3 ng/ml), MGF (2 ng/ml) and IGF-I analogues (2 ng/ml)

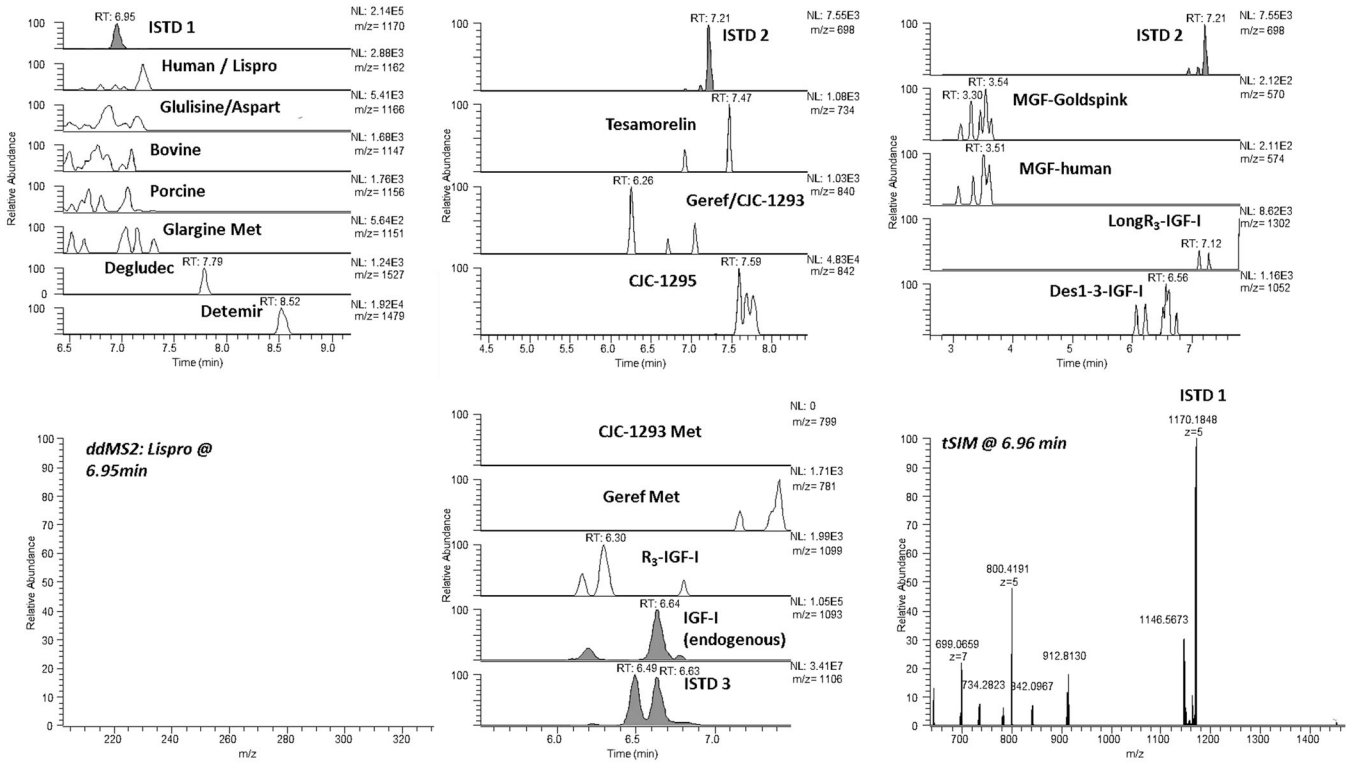

FIGURE 2 Extracted ion chromatograms (from targeted single ion monitoring [tSIM]) of a blank plasma (Octaplas) with the diagnostic ion traces of the different insulins, GH-RH, mechano growth factor (MGF) and insulin-like growth factor (IGF)-I analogues

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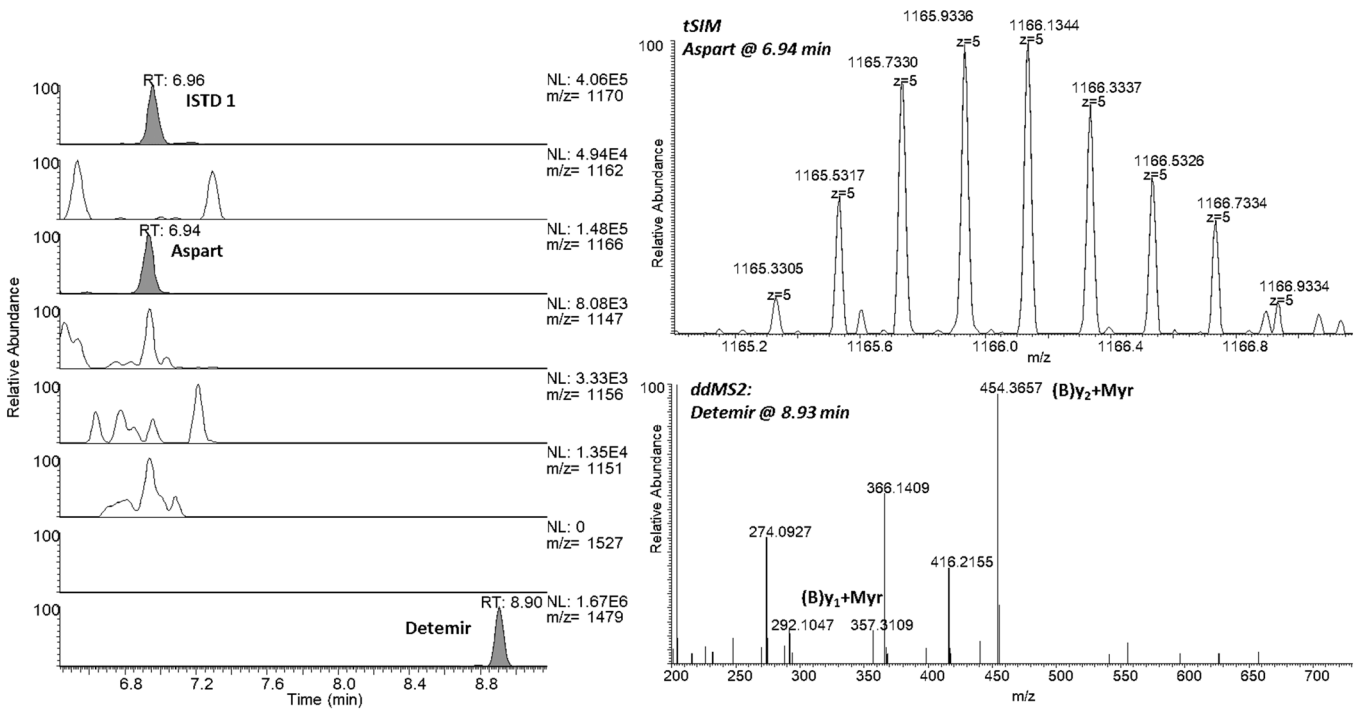

FIGURE 3 Extracted ion chromatograms (left, from targeted single ion monitoring [tSIM]) showing a sample from a diabetic patient after administration (regular treatment) of the fast-acting synthetic insulin aspart and the long-acting insulin detemir. Mass spectra (right) of insulin aspart (SIM, top), showing 5-fold protonated precursor ion and of insulin detemir (ddMS$_2$, bottom) with two diagnostic product ions including the attached myristic fatty acid

m/z 1489) are shown on the right. Under evaluation of the acquired data, the presence of these two synthetic insulins is clearly indicated.

3.3 | Doping control aspects

Especially in sports drug testing, the simplicity and the speed of the method represent a clear benefit when analysing a high number of samples in a short time. Due to the low number of adverse analytical findings in doping controls, confirmatory reanalysis of suspicious samples is rare and, thus, effective (fast and sensitive) initial testing methods are favoured. Here, the presented approach offers obvious progress to established assays. Even with respect to the low MRPL values, bioactive peptides are detectable after parental administration for several hours up to a few days only.[8,10–12,14,24] Thus, frequent out-of-competition sampling represents a more promising doping control approach, compared to classical in- (or after) competition testing. This is especially true with most of the prohibited peptides providing no direct benefit when used during the competition. Also, the expected dosages in cheating athletes and the respective plasma concentrations are very hard to foresee, because some of the target peptides lack medical approval yet (e.g. GHRH- or IGF-I analogues) or produce direct life-threatening effects in case of high dosages (e.g. insulins). Thus, as long as no additional information about the misused peptides is available, the recommended MRPLs were accepted for effective doping control analysis.

Not all prohibited peptides were purified with the same recovery and the method represents a compromise between comprehensiveness on the one hand and selectivity on the other hand. All insulins were well recovered, while the GH-RHs, IGFs and MGFs show significantly lower values for recovery. Nevertheless, all WADA requirements are met. In principle, the method is open also for further target peptides or metabolites, but, noteworthy, also some prohibited peptides (e.g. synacthen, isoelectric point [pI] = 11) yielded inadequate results and were not considered accordingly. The extraction quality is largely correlated to the number of acidic respectively basic amino acids in the peptide sequence and the resulting pI. On the other hand, also peptides <2 kDa, such as gonadoliberin and analogues are potentially co-extracted with the present approach. Data for gonadoliberin (LH-RH, MW: 1181 Da, not included in this communication) yielded recoveries at approx. 30% and an estimated LOD < 0.1 ng/ml. Hence, the method is potentially expandable to other prohibited peptides (of lower molecular mass) without changes in the sample preparation procedure.

4 | CONCLUSION

Along with the development of increasingly powerful mass spectrometers with very high resolution (> 100,000 FWHM), it is now possible to simplify sample preparation to the extent that less pure extracts can be analysed without jeopardizing the required specificity.[25] Samples prepared in this way are generally not suitable for analysis with nano-LC

systems, but modern normal-flow systems have demonstrated sufficient sensitivity and robustness to achieve the mandatory MRPLs in doping controls.[7,8,14,16,19] The present method is designed as an initial testing procedure, which ideally covers different (if not all) prohibited peptides in one analytical approach. Although this was not entirely achieved, the developed assay offers a clear improvement considering simplicity and speed without compromising the analytical sensitivity. This straightforward approach (without the need for handling antibodies) can be readily implemented and allows for meeting mandatory WADA requirements for initial testing procedures; however, for confirmatory analyses (which may result in formal adverse analytical findings), methods employing immunoaffinity purification prior to MS are still recommended.

AUTHOR CONTRIBUTIONS

Andreas Thomas was associated with method development and conceptualization, and wrote the original draft and supervision. **Sam Thilmany** and **Amelie Hofmann** were associated with methodology, formal analysis and investigations. **Mario Thevis** was associated with supervision and reviewed and edited the final manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The datasets generated and analysed during the current study are available from the corresponding authors on reasonable request.

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