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Advances in the detection of growth hormone releasing hormone synthetic analogs

Siham Memdouh[1] | Ivana Gavrilović[2] | Kelsey Ng[1] | David Cowan[1] | Vincenzo Abbate[1]

[1]Department of Analytical, Environmental and Forensic Sciences, King's College London, London, UK

[2]Drug Control Centre, King's Forensics, Department of Analytical, Environmental and Forensic Sciences, King's College London, London, UK

Correspondence
Vincenzo Abbate and Ivana Gavrilović, Department of Analytical, Environmental and Forensic Sciences, King's College London, London SE1 9NH, UK.
Email: vincenzo.abbate@kcl.ac.uk; ivana.gavrilovic@kcl.ac.uk

Abstract

The administration of growth hormone releasing hormone (GHRH) and its synthetic analogs is prohibited by the World Anti-Doping Agency (WADA). Although there is evidence of their use, based on admissions and intelligence, they do not appear to have been found in anti-doping samples by WADA accredited laboratories. This might be due to their small concentration in urine and limited knowledge about their metabolism, especially for unapproved synthetic analogs. This study investigates the *in vitro* metabolism and detection of four of the larger GHRH synthetic analogs (sermorelin, tesamorelin, CJC-1295, and CJC-1295 with drug affinity complex) in fortified urine. Nineteen major *in vitro* metabolites were identified, selected for synthesis, purified, and characterized in house. These were used as reference materials to spike into urine together with commercially available parent peptides and a metabolite of sermorelin (sermorelin(3-29)-NH_2) to develop a sensitive liquid chromatography-tandem mass spectrometry method for their detection to help prove GHRH administration. Limits of detection of the target peptides were generally 1 ng/ml (WADA required performance limit) or less.

KEYWORDS
Anti-doping, GHRH synthetic analogs, *In vitro* metabolism, SPE, UHPLC-MS/MS

1 | INTRODUCTION

Human growth hormone releasing hormone (hGHRH), also known as somatocrinin, somatoliberin (human), somatorelin, or GRF(1–44) amidated, is a hypothalamic single strand peptide hormone consisting of 44 amino acids with a molar mass of 5,036.65 g/mol. It is released in a pulsatile manner from neurosecretory nerve terminals of the arcuate neurons of the hypothalamus and transported through the hypothalamic-hypophyseal system. GHRH reaches the anterior pituitary gland through a connecting stalk[1] where it leads to a pulsatile release of growth hormone (GH) from the pituitary gland that has a net anabolic effect on the body. The existence of GHRH had been suggested in the early 1960s by Reichlin, who deliberately caused lesions in the hypothalamus of rats to demonstrate a decline in GH secretion.[2]

GHRH synthetic analogs are prohibited substances under section S2 (peptide hormones, growth factors, and related substances) of the WADA Prohibited List.[3] Most of these analogs are non-approved drugs, which presents a great challenge for WADA accredited doping control laboratories due to the complexities associated with identifying relatively unknown and uncharacterized analytes. Moreover, clinical studies on non-approved peptides are strongly limited by ethical constraints. Therefore, there are limited data about their metabolism in humans. Hence, *in vitro* studies may be considered as a reasonable alternative to elucidate their metabolism.

The short half-life of hGHRH, largely due to proteolysis, presented a limitation for its use in therapy.[4] Several synthetic analogs of GHRH that have been designed to have increased resistance to proteolytic cleavage, and hence longer half-lives, have been studied.[5–7] For instance, tesamorelin, marketed as Egrifta®, is an FDA approved compound for the treatment of HIV-related lipodystrophy.[8] In 1997, sermorelin (Geref®) was approved for the treatment of growth retardation in children. However, in 2008, it was discontinued not due to safety or efficacy issues but rather to complications related to the manufacturing process of the peptide.[9] Other unapproved peptides such as CJC-1295 and CJC-1295 with DAC (drug affinity complex) were originally designed for therapeutic use but were never approved. However, illicitly manufactured CJC-1295 has been found in the black market.[10] Various preparations of these drugs are presumably produced illegally in clandestine laboratories. Hence, their pharmaceutical quality may not be reliable and fulfilling the standards of good manufacturing practice.[10] Since athletes are presumably using these peptides in an attempt to enhance their performance, their misuse may present an additional risk to their health.[11]

In 1986, Frohman *et al.*[4] conducted *in vitro* and *in vivo* metabolic studies using synthesized GHRH(1–44)-NH_2 and GHRH(1–40)-NH_2. These two peptide hormones are produced in the hypothalamus and by some tumors and stimulate the release of growth hormone.[4] *In vivo* studies suggested that the half-life of GHRH(1–44)-NH_2 was notably shorter than that of GHRH(1–40)-NH_2. Similarly, *in vitro* plasma incubation experiments showed similar results where GHRH(1–40)-NH_2 was demonstrated to be more resistant to plasma peptidase degradation than GHRH(1–44)-NH_2. The main enzymes involved were dipeptidyl peptidase IV (DPP IV) and trypsin-like enzyme (TL-1). DPP IV cleaves adjacent to the N-terminal peptide portion producing GHRH(3–44)-NH_2 and GHRH(3–40) by the loss of tyrosine and alanine (Figure 1). Trypsin-like degradation occurs in GHRH(1–44)-NH_2 between the arginine in position 11 and lysine in position 12. Paradoxically, formation of GHRH(3–44)-NH_2 occurs within 1 min after intravenous injection of GHRH(1–44)-NH_2, whereas GHRH(1–40) appears to be more stable.

Additionally, an *in vitro* plasma incubation of synthetic GHRH(1–44)-NH_2 and GHRH(1–40) as well as GHRH(1–32)-NH_2 and GHRH (1–29)-NH_2 was carried out.[12] All three synthetic analogs except GHRH(1–40) were rapidly cleaved between alanine in position 2 and aspartic acid in position 3 of the peptide sequence. In addition, this study demonstrated that C-terminal abbreviated fragments of GHRH of at least 29 residues were similarly degraded but with different rates over 60 min, depending on the composition of the sequence. D-amino acid substitution in position 2, [D-Ala*]-GHRH(1−29)-NH_2 inhibited the fast metabolism.

Detecting GHRH synthetic analogs in athletes' urines must meet a minimum required performance level (MRPL) criterion of 1 ng/ml established by WADA[13] according to identification criteria specified in WADA Technical Document TD2015IDCR.[14] A method to detect GHRH synthetic analogs has been published by Knoop *et al.*,[15] whereby serum detection and identification of four intact GHRH analogs (CJC-1293, CJC-1295, sermorelin and tesamorelin) was investigated, as well as two synthetic metabolites of sermorelin and CJC-1293. In order to develop a method for the simultaneous detection of intact peptides and their metabolites in serum, *in vivo* and *in vitro* studies were performed followed by immunoaffinity purification with a polyclonal GHRH antibody in concert with protein A/G monolithic MSIA™ D.A.R.T.'S™ (Disposable Automation Research Tips) and subsequent analysis by nano-ultrahigh performance liquid chromatography-high resolution mass spectrometry.[15] The amount of intact sermorelin showed a steady decrease over the duration of incubation (4 h) with a concomitant increase of GHRH(3–29)-NH_2. Conversely, over the same experimental time points, tesamorelin showed a considerable resistance to proteolytic cleavage in human plasma, and no relevant metabolite was identified. In addition, a single 0.5 mg dose of sermorelin was self-administered subcutaneously by a healthy male volunteer (59 years, 78 kg). Plasma samples were collected at 30, 90, and 270 min, intact sermorelin was not detected at any considered time point, and GHRH(3–29)-NH_2 metabolite was detected in plasma only at 30 min after administration.

Thomas *et al.*[16] published a simple sample preparation procedure for a large set of peptides with molecular weights >2 kDa by immunoaffinity purification with coated paramagnetic beads. Their study included the WADA prohibited peptides such as GHRH analogs (sermorelin, tesamorelin, CJC-1293, and CJC-1295), mechano-growth factors (human MGF, modified human MGF, and "full-length" MGF), synthetic IGF-I (long R3-IGF-I), synthetic/animal insulins (Lispro®, Aspart®, Glulisine®, Glargine®, Detemir®, Degludec®, and bovine and porcine insulin), and synthetic ACTH (Synacthen®). However, they were unable to distinguish CJC-1293 and sermorelin that had the same retention and elemental compositions differing only by the exchange of an L-alanine to a D-alanine at the N-terminus of the peptide. This study focused only on parent compounds of synthetic GHRH and not on their metabolites. Pont *et al.*[17] adapted and optimized the immunopurification with magnetic beads that had been developed by Thomas *et al.*[16] The optimization of sample preparation was carried out by using different solid-phase supports with different chemical affinities and binding capacities. The authors also considered the issue of the fast degradation of GHRH synthetic analogs, and therefore, the pH was adjusted, and a commercially available protease inhibitor was added to samples. However, this was applied only for spiked urine with the intact peptides (sermorelin, CJC-1295, and tesamorelin) and sermorelin(3–29)-NH_2 metabolite. According to Pont *et al.*,[17] the fast degradation of GHRH analogs may occur both in the body as well as in the preanalytical phase. Therefore, a thorough *in vitro* metabolism study is essential.

Even though immunopurification-based sample preparation combined with nano-spray MS seems to be a platform offering good sensitivity to detect large peptide administration, this platform requires skills and expertise in handling, which may not be available in every WADA accredited laboratory. Indeed, in 2019, only 5 of the 30 WADA accredited laboratories reported routine testing for GHRH analogs.[18] A simpler and more cost-effective detection

*The alanine in position 2 has a D configuration.

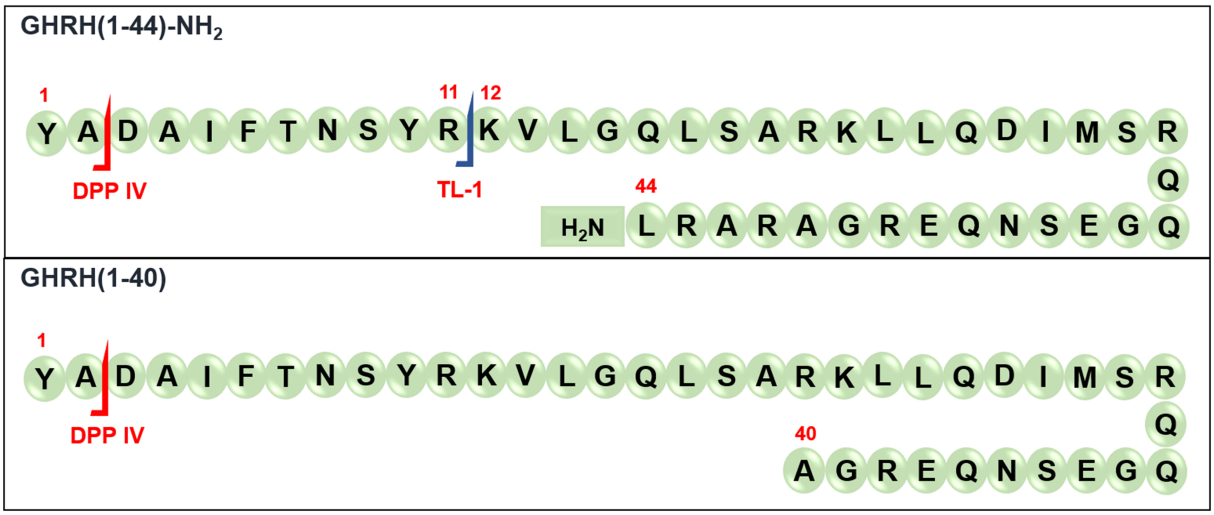

FIGURE 1 Sequence of GHRH (1–44)-NH_2 and GHRH(1–40) indicating potential sites of cleavage by plasma enzymes DPP IV and TL-1

technique might help more laboratories implement testing for these analytes. Currently, according to the last published "2019 Anti-Doping Testing Figures," no Adverse Analytical Finding (AAF) in blood or urine for a GHRH synthetic analog has been reported by any WADA accredited laboratory.[18] This might be due to their low concentration in urine. We questioned whether detection of the metabolites of GHRH synthetic analogs might be a more effective approach.

Here, we present the results of our investigation of the *in vitro* metabolism profile of the four GHRH synthetic analogs (tesamorelin, sermorelin, CJC-1295, and CJC1295 with DAC), the synthesis of a number of the identified metabolites, and their use in the development of an appropriate analytical method for their detection in urine samples.

2 | EXPERIMENTAL

2.1 | Chemicals and materials

Sermorelin, tesamorelin, CJC-1295, and CJC-1295 with DAC were purchased from UK-Peptides (Middlesbrough, UK). Sermorelin(3–29)-NH_2 was purchased from AusPep (Tullamarine, Australia). The two internal standards (IS) used in this study were d_3-GHRP-2 metabolite donated by the Institute of Biochemistry, German Sport University (Cologne, Germany) and acetyl-(Tyr^1, D-Arg^2)-GRF(1–29)-NH_2 purchased from Bachem (Bubendorf, Switzerland). Phosphate buffer (0.5 M, pH 7.4), pooled human liver microsomes (HLM), and S9 fraction were purchased from Corning (Woburn, MA, USA); pooled human kidney microsome (HKM) and S9 fraction were purchased from XenoTech (Kansas City, KS, USA). Four different resins purchased from Novabiochem (Darmstadt, Germany) were used for peptide synthesis: Fmoc-Rink Amide, Fmoc-Arg (PbF)-Novasyn R, Fmoc-Lys (Boc) Wang resin, and Nova PEG Rink Amide Resin. Fmoc-protected amino acids were purchased from Sigma Aldrich (Darmstadt, Germany). Coupling reagents used were ethyl (hydroxyimino) cyanoacetate (oxyma), N,N′-diisopropylcarbodiimide (DIC) and 1-[bis (dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate (HATU) were obtained from Alfa Aesar (Lancashire, United Kingdom). N,N-Diisopropylethylamine (DIPEA), piperidine, piperazine, picrylsulfonic acid, dimethylformamide (DMF), dichloromethane (DCM), methanol (MeOH), HPLC grade acetonitrile (ACN), trifluoroacetic acid (TFA), and formic acid (FA) were obtained from Fisher Scientific (Leicestershire, United Kingdom) and Sigma Aldrich (Darmstadt, Germany). Deionized water was collected at ~18 MΩ cm resistivity from an Elgastat Pure Lab Option system (High Wycombe, UK). Protein LoBind® tubes were obtained from Eppendorf (Stevenage, UK). For solid- phase extraction, Nexus WCX (60 mg, 3 ml) cartridges were obtained from Agilent Technologies (Santa Clara, United States) and Strata X-CW (30 mg, 3 ml) cartridges from Phenomenex (Macclesfield, Cheshire, UK).

Anonymized urine samples used for method development and validation were obtained from athletes who had provided their consent on the doping control form. Once these samples had been analyzed in the Drug Control Centre, King's College London (London, UK), they were stored at −20 °C for at least 3 months after negative analytical reports had been issued in accordance with WADA requirements.

2.2 | Instrumentation

2.2.1 | Peptide synthesis

An automated microwave peptide synthesizer Biotage® Initiator+ Alstra™ (Uppsala, Sweden) was used to synthesize the selected *in vitro* metabolites by an orthogonal strategy based on Fmoc solid- phase chemistry while the cleavage process was carried out manually.

2.2.2 | Peptide purification

Semi-preparative LC-DAD-MS was used to purify crude peptides with a Waters Auto purification System (Waters, Milford, MA, USA) consisting of a sample manager collection system (Waters 2767), binary gradient module (Waters 2545), system fluidics organizer (Waters), a HPLC Pump (Waters 515), and a dual photodiode array-mass spectrometry (DAD-MS) detector system. ESI--MS analysis was carried out on a Waters Micromass ZQ mass spectrometer operating in positive ionization mode from m/z 300 to m/z 1,500. The ESI conditions were capillary voltage 3.00 kV, cone voltage 20 V, source temperature 150 °C, desolvation temperature 250 °C, cone gas flow 50 L/h, and desolvation gas flow 500 L/h. The data acquisition and mass-triggered fraction collection were performed using MassLynx 4.1 software. The peak fractions of the peptides of interest were collected automatically by the sample manager collection system in polypropylene tubes according to the appropriate m/z values for the protonated molecular ions of each analyte. The column used for the chromatographic separation was a XTerraPrep C18 (Waters, Milford, MA, USA), particle size 5 μm, 125 Å pore size, 10 × 150 mm; the total run time was 53 min. The gradient started at 10 % B and was ramped linearly to 95 % B over 44 min at a flow rate of 10 ml/min. The injection volume ranged between 1 and 5 ml. The mobile phase consisted of A = 0.05 % TFA and 0.1 % FA in H_2O and B = 0.05 % TFA and 0.1 % FA in ACN.

Reversed-phase high-performance liquid chromatography (reversed-phase HPLC) was performed on a HP 1050 instrument (Agilent Technologies, Waldbronn, Germany) to assess the chromatographic purity of the synthesized peptides by analyzing the different fractions from the purification step (semi-preparative LC-DAD-MS). It was equipped with a quaternary pump, an autosampler, a DAD, and a Kontron DEG 104 degasser (Kontron, Tokyo, Japan). Agilent Chem Station software version A.10.02 was employed to interrogate the acquired data and to control the equipment. A C18 column, Zorbax, 80 Å, 3.5 μm, 2.1 × 100 mm was used with a total run time of 30 min. The gradient was 2 % B ramping to 90% B in 20 min at a flow rate of 0.3 ml/min. The injection volume was 10–30 μl. The detected UV wavelengths were 214, 220, 230, 254, and 281 nm. The HPLC mobile phases A and B were composed of 0.1 % TFA in H_2O and ACN, respectively.

2.2.3 | Peptide characterization

A Thermo Scientific Q-Exactive™ mass spectrometer (hybrid quadrupole orbitrap mass spectrometer) (Thermo Scientific, Bremen, Germany) coupled with a Thermo Scientific Dionex Ultimate™ 3000 UHPLC system was used to characterize the 19 synthesized peptides initially by direct infusion in positive ionization mode and then using the LC. The HESI-II source operating conditions using nitrogen were sheath gas flow rate 70; auxiliary gas flow rate 10; spray voltage 3.75 kV; capillary temperature 320 °C; S-lens RF level 55.0; and auxiliary gas temperature 350 °C. Full MS scan range was m/z 100–1,000 with a resolution of 70,000 FWHM and AGC target of 1×10^6. Xcalibur™ (version 4.1, Thermo Scientific) was used for data analysis. Collision induced fragmentation (CID) or all ion fragmentation (AIF) experiments with HRMS was performed at m/z 100–1,000 with a resolution of 35,000 FWHM and AGC target of 1×10^6. A 5 ppm extraction window was set for the extracted ion chromatograms of the protonated ($[M + nH]^{n+}$) precursor and product ions. The chromatographic separation was performed to analyze *in vitro* incubation samples by using an Acquity UPLC® BEH C18 reversed-phase column (130 Å, 1.7 μm 2.1 × 50 mm) (Waters, Elstree, United Kingdom), with a 10 μl injection volume. Mobile phase A was 0.3 % FA in H_2O, while mobile phase B was 0.3 % FA in ACN at a flow rate of 0.3 ml/min. The gradient started from 95 % A for 0.5 min, then to 80 % A over 3 min, 75 % A over 1.5 min and then 10 % A over 1 min, followed by a return to the initial condition in 0.1 min and equilibration over 1.9 min. The total run time was 10 min.

2.2.4 | Urine analysis for routine doping control

Waters Xevo TQ-S micro triple quadrupole mass spectrometer coupled with a Waters Acquity UPLC® Class system was used to develop the MRM transitions of 20 identified metabolites and four intact GHRH synthetic analogs by direct infusion of standard solutions (10 μg/ml) using a post-column infusion mode. Once the MRM transitions were optimized, neat standard solutions were injected, and the chromatographic separation was performed using a Waters Acquity UPLC® HSS T3 column (2.1 × 50 mm, 1.8 μm, 100 Å pore size) with a 10 μl injection volume and at a column temperature of 40 °C. Mobile phase A was 0.3 % FA in H_2O, while mobile phase B was 0.3 % FA in ACN at a flow rate of 0.4 ml/min. The gradient started from 95 % A and then 80 % A at 1 min, 70 % A at 4 min, 50 % A at 6 min, to 10 % A at 8 min. The total run time was 10.5 min. The MS conditions were positive electrospray ionization, desolvation gas (nitrogen) flow 1,000 L/h; desolvation temperature 500 °C; source temperature 150 °C capillary voltage 1 kV; the cone voltages; and collision energies were optimized for each MRM transition.

2.3 | *In vitro* metabolism studies

Metabolic *in vitro* studies were carried out, following a published procedure, with liver and kidney microsomes, S9 fraction and with human serum.[19] For the microsomal and S9 fraction incubation, each peptide stock solution (50 μl,100 μg/ml) was dried at 30 °C under a stream of nitrogen and reconstituted with phosphate buffer (100 μl, 0.5 M, pH 7.4) to obtain a peptide concentration of 50 μg/ml. The experiment was initiated by adding 2.6 μl of HLM, HKM, or S9 fraction (human liver and kidney) to obtain a total enzyme concentration of 0.5 mg/ml. The mixture was gently vortexed and incubated at 37 °C for 2, 4, and 24 h. To stop the incubation, ice-cold ACN was added (100 μl), and the tubes were transferred to an ice-bath for 15 min, and the sample was then centrifuged for 5 min at 12,000 *g*. The supernatant was then used for LC-HRMS analysis.

Peptide stock solutions (50 μl, 100 μg/ml) were dried at 30 °C under a stream of nitrogen and then resuspended in fresh serum (200 μl) and incubated at 37 °C with human serum (200 μl) for 2 h. Solid-phase extraction (SPE) with Nexus WCX cartridges (60 mg, 3 ml) was used for the purification and concentration of the resulting metabolites. Each cartridge was activated with MeOH (2 ml) and rinsed with H_2O (2 ml). Before loading, the sample was first diluted with H_2O (2 ml). After loading, the column was washed twice with H_2O (2 ml), and the analytes eluted with 5 % FA in MeOH (1 mL) into 15 ml plastic tubes. After evaporation under a stream of nitrogen at 65 ± 5 °C for 20 min, the residue was dissolved in 100 μl of H_2O: ACN:FA (95: 5: 0.3) for LC-HRMS analysis.

2.4 | BLAST analysis of peptide sequence alignment

The sequences of the 20 different selected metabolites as well as their parent peptides were subject to sequence alignment using BLAST analysis available at UniProt, ExPASy proteomics Website (https://www.uniprot.org/blast).

2.5 | Chemical synthesis of metabolites

All the selected metabolites underwent chemical synthesis producing a library of 20 metabolites (19 were synthesized in house, and 1 was purchased). Chemical synthesis was followed by their purification and characterization.

2.5.1 | General peptide synthesis

The chemical synthesis of peptides started from the C-terminal residue proceeding with the sidechain protected peptide sequence toward the N-terminal end. The general protocol for synthesizing peptides started by attaching the first Fmoc-protected amino acid on the most appropriate resin.[20] After the first amino acid was coupled, a site selective Fmoc deprotection was carried out. Then, the next amino acid with a previously activated carboxyl group was coupled to the alpha amino group of the amino acid attached to the solid support. Between each step, the resin was extensively washed with DCM and DMF to remove any excess of reagents or by-products. The cycle of coupling and deprotection was repeated until the desired peptide sequence was obtained. Afterward, the peptide was cleaved from the resin along with all sidechain protecting groups to afford the crude product.

2.5.2 | Automated microwave-assisted peptide synthesis

A total of 400 mg of the appropriate resin with a nominal loading between 0.18 and 0.31 mmol/g was loaded into a 10 ml fritted polypropylene syringe. The sequence to be synthesized was programmed by inserting the loading and the amount of the resin to be used. Four equivalents of the amino acid (0.5 M), DIC as activating agents and oxyma as a coupling additive were used. Each amino acid coupling was performed at 75 °C (50 W of microwave power) for 5 min. The Fmoc group removal was achieved by using 5 % piperazine in DMF. The deprotection reactions were performed on two cycles at room temperature for 13 min in total.

To synthesize the C-terminal amidated metabolites, Fmoc-Rink Amide or Nova PEG Rink Amide Resin was used to synthesize the following metabolites: sermorelin(12–29)-NH_2, sermorelin(21–29)-NH_2, sermorelin(22–29)-NH_2, sermorelin(23–29)-NH_2, sermorelin(8–29)-NH_2, sermorelin(16–29)-NH_2, tesamorelin(21–44)-NH_2, CJC-1295 (21–29)-NH_2, CJC-1295(23–29)-NH_2, CJC-1295(7–29)-NH_2, CJC-1295(8–29)-NH_2, CJC-1295(12–29)-NH_2, and CJC1295(16–29)-NH_2. On the other hand, carboxy-terminated peptides such as CJC-1295 (1–12), CJC-1295(1–21), tesamorelin(1–12), and tesamorelin(1–21) were synthesized by using Fmoc-Lys (Boc)Wang resins. Fmoc-Arg (PbF)-Novasyn was used to synthesize tesamorelin(1–20) and tesamorelin(1–11).

2.5.3 | Peptide cleavage procedure

After synthesis completion, peptides were all manually cleaved from the resin by using TFA under gentle agitation over a period of 2 h at room temperature. Standard cleavage solution used to cleave 10 mg of resin consisted of TFA (900 μl), tri-isopropylsilane (TIPS, 25 μl), ethane-1,2-dithiol (EDT, 25 μl), H_2O (50 μl) thioanisole (50 μl), and phenol (50 mg). Once the cleavage was completed, the crude material was filtered into a polypropylene centrifuge tube. Then, the crude material was concentrated by evaporating the solvent with a gentle stream of nitrogen at room temperature. Afterward, the crude peptide was precipitated by the addition of cold diethyl ether (4 ml), centrifuged, then washed three times with cold diethyl ether, dried under vacuum for 4 h, dissolved in ultrapure H_2O containing 0.1 % TFA then flash -frozen in liquid nitrogen, and lyophilized for 24–48 h.

2.6 | Metabolite purification

After the synthesis of all metabolites, each crude peptide was dissolved in ultrapure H_2O containing 10 % ACN and 0.1 % FA and subjected to a semi-preparative reversed-phase HPLC-MS. Afterward, the chromatographic purity of each fraction was examined using reversed-phase HPLC-DAD (HPLC data shown in Figure S2).

2.7 | Peptide identification and characterization

A solution (10 μg/ml) of each purified metabolite was infused into a Thermo Q -Exactive™ mass spectrometer to determine the accurate mass and full scan positive electrospray ionization spectrum. The

amino acid sequence was then confirmed using CID HRMS product ion spectra to reveal the b- and y-ion amino acid fragments. Various collision energies were used to enhance the fragmentation of the most abundant precursor ion of each peptide.

2.8 | General sample preparation

The extraction process followed the steps proposed by Thomas *et al*.[21] In brief, the weak cation exchange mixed mode Strata X-CW (30 mg, 3 ml) was used and activated with MeOH (1 ml), followed by H_2O (1 ml). Aliquots of urine (2 ml) were treated with phosphate buffer (0.1 M, pH 6.2, 2 ml) and loaded onto the cartridge after addition of deuterium labeled GHRP-2 metabolite (100 µl, 40 ng/ml) and acetyl-(Tyr^1, D-Arg^2)-GHRH(1–29)-NH_2 (20 µl, 200 ng/ml) as internal standards.[15] The column was washed with H_2O (1 ml), then with MeOH (1 ml). Then, vacuum was applied to dry the solid-phase bed. The analytes were eluted with 5 % FA in MeOH into clean polypropylene tubes and the solvent removed under a stream of nitrogen at 65 ± 5 °C for 20 min. The residue was then reconstituted in 100 µl 0.3 % FA in H_2O: ACN (95:5), vortexed, and centrifuged at 1,000 rpm for 5 min. The supernatant was transferred into an autosampler vial and injected (10 µl) into the LC-MS. Relative recoveries were assessed by repeating the whole procedure using Nexus WCX (60 mg, 3 ml).

2.9 | Sample preparation optimization

The methanol wash step was considered essential to remove interferences that might affect the detection of the targeted peptides. Therefore, a set of experiments was designed to optimize the MeOH washing step and to try and obtain better recovery and signal responses for all metabolites as well as for the intact parents. This optimization involved both cartridges.

The organic solvent strength of the washing step was carried out at different MeOH:H_2O ratios (40 % MeOH in H_2O, 60 %, 80 %,and 100 %) then elution with 5 % FA in MeOH for desorption of the analytes from the solid support.

2.10 | Method validation

The method validation was performed by using the described optimized SPE protocol and following the laboratory's protocol for the qualitative validation of WADA specified non-threshold compounds. As the name would suggest, non-threshold compounds do not have any maximum permissible level of the concentration, ratio, or a score in the sample. The developed method was validated by assessing carryover, selectivity, limit of detection (LOD), recovery, and matrix effect.

The carryover was checked by injecting two blank samples after analysis of spiked urine at 10 ng/ml (10 times the WADA MRPL).

The selectivity of the developed method was evaluated by extracting and analyzing 10 blank urine sample from different donors.

The limit of detection was experimentally determined by spiking blank urine samples with the four intact peptides and their respective 20 metabolites at three different approximate concentrations (nominally 0.1, 0.2, and 0.5 ng/ml since the synthesized peptide concentrations were estimated). The LOD was defined as the lowest concentration of each peptide detected with a signal-to-noise ratio (S/N) greater than 3.

Recoveries were evaluated by spiking three blank urine samples containing acetyl-(Tyr^1, D-Arg^2)-GHRH(1–29)-NH_2 (20 ng/ml, 2 ml of urine) as IS with all metabolites and their respective intact peptides at 10 ng/ml (n = 3). Other blank urine samples (n = 3) containing the same IS were extracted following the aforementioned procedure and then spiked with all the peptides before evaporation of the extracts to dryness and reconstitution in 100 µl of the mobile phase 0.3 % FA in H_2O: ACN (95:5). Data were reviewed using exported data from TargetLynx to Excel, and the recoveries were calculated based on the instrument response using the formula:

$$\%\text{Recovery} = \frac{\text{Peak Area of Pre} - \text{Extraction}}{\text{Average Peak Area of Post} - \text{Extraction}} \times 100,$$

where pre-extraction represents a known concentration of the analytes added into the blank urine before extraction and post-extraction is prepared by first extracting blank urine. After the elution step, the eluent is spiked with the analytes to give a known concentration.

Matrix effect (ion suppression) was evaluated by comparing the peak areas of three fortified blank urines to the fortified neat sample solution in H_2O:ACN:FA (95:5:0.3 %) at equivalent concentrations.

3 | RESULTS AND DISCUSSION

3.1 | Metabolite identification and confirmation of peptide sequence

The total ion chromatogram (TIC) obtained from the *in vitro* metabolism of each parent compound was compared with that from the blank sample (solution containing only enzymes). *In vitro* metabolites were initially predicted using ChemDraw software, considering peptide structure and hydrolysis of peptide-bonds as the main reaction of metabolite formation. The presence of each metabolite was established by assessing the full scan data and detecting the peak when extracting the respective exact protonated (or multiply charged) metabolite mass. Identified metabolites with larger relative abundance (peak counts greater than 10^5) were subject to targeted CID experiments using LC-MS/HRMS to confirm the proposed metabolites' amino acid sequences and their fragmentation patterns (within 5 ppm mass difference with respect to their exact masses). As a result, 19 major *in vitro* metabolites were identified and tentatively characterized (Figure 2). Results showed that the most common enzymatic

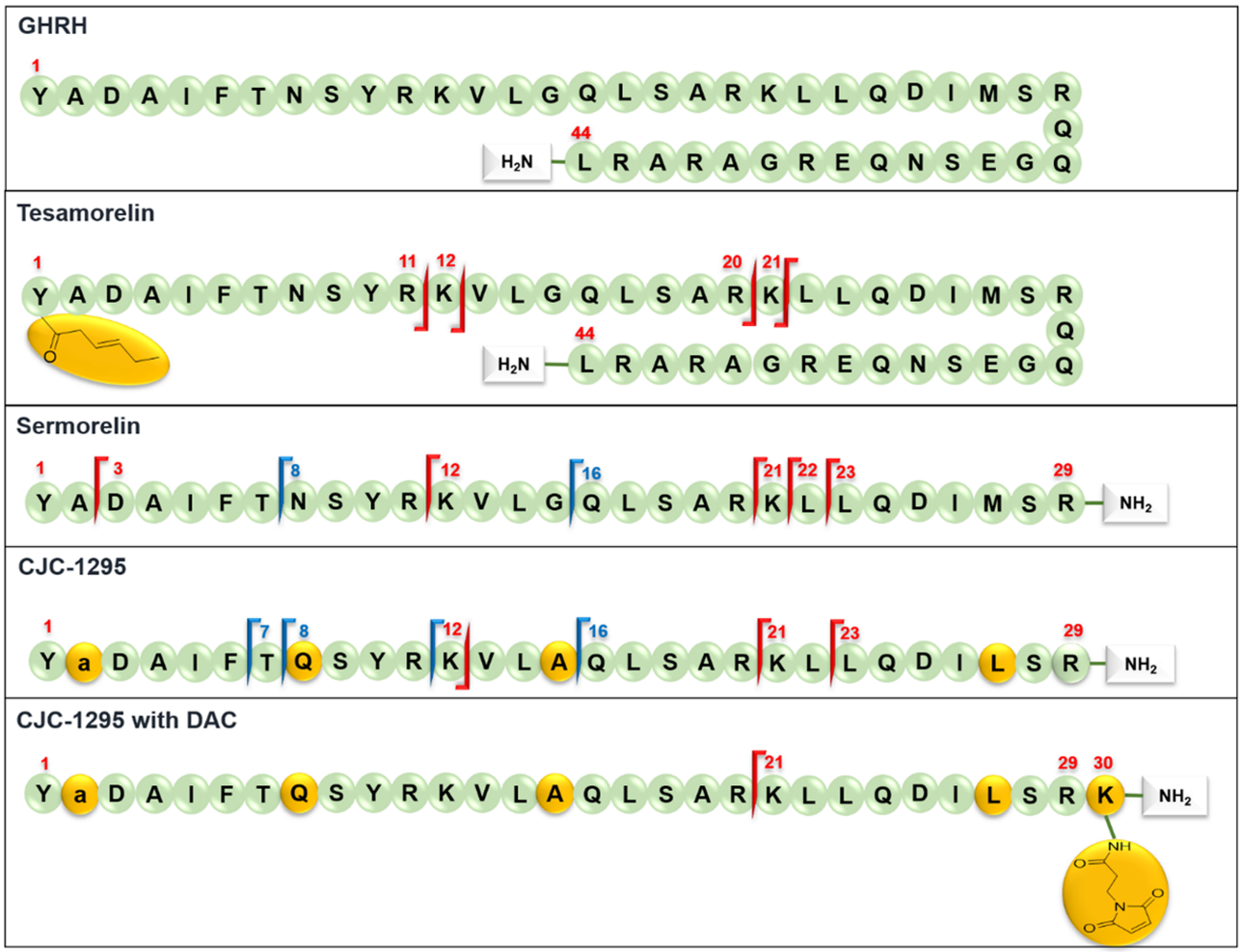

FIGURE 2 Indication of the major cleavage sites of the four GHRH synthetic analogs (the differences in chemical structure between hGHRH and the synthetic analogs are highlighted in yellow; colored markers are oriented toward the direction of the resulting metabolite; red markers represent those obtained using microsomes and S9 fraction and blue markers represent those obtained from serum, respectively)

degradation pathways for the four GHRH synthetic analogs involved cleavages at arginine, lysine, leucine, or valine residues. In addition, the incubation systems with HLM, HKM, S9 fraction and serum seemed to be optimal at 2 h incubation time in terms of metabolite abundance and variety (Table 1). The presence of the identified metabolites at 2 h was checked also at 4 and 24 h incubation times. The relative abundances of their respective peaks were found to be smaller compared with those obtained using a 2 h incubation time. The smaller abundances of the identified metabolites at 4 and 24 h suggests a further degradation of the same metabolites to even smaller fragments. Hepatic and renal fractions yielded similar metabolite profiles in terms of variety and abundance for tesamorelin, CJC-1295 and CJC-1295 with DAC. On the other hand, the liver S9 fraction gave greater metabolite abundance; this could be due to the larger peptidase content in the cytosolic enzymatic pool. As the *in vitro* studies generated several metabolites, only metabolites with large abundance in the mass spectrometry signal and acceptable stability over the considered incubation time were selected for full characterization and chemical synthesis.

For sermorelin incubation in HLM, HKM, and S9 fraction, seven metabolites were selected. CJC-1295 with DAC offered four major metabolites CJC-1295(1–21), CJC-1295(1–12), CJC-1295(21–29), and CJC1295(23–29) in HLM, HKM, and S9 models. CJC−1295(1–12) was also found as a metabolite of CJC-1295, while CJC-1295(21–29) and CJC-1295(23–29) are similar metabolites of CJC-1295 except for the amidated C-terminal. The free acid and amidated metabolite differ by 0.4920 Da. Thus, for convenience, the free acid metabolites were not selected as candidates among the list of metabolites to be synthesized as some degree of cross-talk in an MRM low-resolution method would be expected. In the serum incubate, these biotransformation products were not observed. This might be due to the presence of the DAC portion. According to Jetté *et al.*,[22] CJC−1295 with DAC has a prolonged half-life as it was detectable also after 72 h. In addition, in the same study, plasma samples were subjected to

TABLE 1 List of intact peptides and their metabolites identified with *in vitro* models at 2 h

	Peptide sequence	HLM	HKM	S9	Serum
Intact sermorelin	YADAIFTNSYRKVLGQLSARKLLQDIMSR-NH_2[a]	+[b]	+	+	+
Sermorelin metabolites	KVLGQLSARKLLQDIMSR-NH_2	-[c]	+	+	+
	KLLQDIMSR-NH_2	+	+	+	+
	LLQDIMSR-NH_2	+	+	+	+
	LQDIMSR-NH_2	+	+	+	+
	DAIFTNSYRKVLGQLSARKLLQDIMSR-NH_2	−	+	+	+
	NSYRKVLGQLSARKLLQDIMSR-NH_2	−	−	−	+
	QLSARKLLQDIMSR-NH_2	−	−	−	+
Intact tesamorelin	*Trans*-3-hexYADAIFTNSYRKVLG QLSARKLLQDIMSRQQGESNQERGARARL-NH_2	+	+	+	−
Tesamorelin metabolites	*Trans*-3-hex[d]-YADAIFTNSYRKVLGQLSARK	+	+	+	−
	Trans-3-hex-YADAIFTNSYRKVLGQLSAR	+	+	+	−
	Trans-3-hex-YADAIFTNSYR	+	+	+	−
	KLLQDIMSRQQGESNQERGARARL-NH_2	+	+	+	−
	Trans-3-hex- YADAIFTNSYRK	+	+	+	−
Intact CJC-1295	YaDAIFTQSYRKVLAQLSARKLLQDILSR-NH_2	+	+	+	+
CJC-1295 metabolites	Ya[e]DAIFTQSYRK	+	+	+	−
	KLLQDILSR-NH_2	+	+	+	+
	LQDILSR-NH_2	+	+	+	+
	TQSYRKVLAQLSARKLLQDILSR-NH_2	−	−	−	+
	QSYRKVLAQLSARKLLQDILSR-NH_2	−	−	−	+
	KVLAQLSARKLLQDILSR-NH_2	−	−	−	+
	QLSARKLLQDILSR-NH_2	−	−	−	+
Intact CJC-1295 with DAC	YaDAIFTQSYRKVLAQLSARKLLQDILSRK [N-(2-carboxyethyl maleimide)-NH_2]	+	+	+	+
CJC-1295 with DAC metabolites	YaDAIFTQSYRK	+	+	+	−
	KLLQDILSR	+	+	+	+
	LQDILSR	+	+	+	+
	YaDAIFTQSYRKVLAQLSARK	+	+	+	−

[a]Amidated C-terminal.
[b]Detected.
[c]Not detected.
[d]*Trans*-3-hexenoyl group.
[e]D-alanine.

Western blot analysis which showed that the CJC-1295 with DAC was covalently bound to albumin. In other published works, the maleimide group has been shown to react with the free thiol on albumin.[22] The prolonged plasma residence time, combined with the Western blot analysis, might explain why CJC-1295 with DAC did not generate many metabolites in serum.

3.2 | BLAST analysis of peptide sequence alignment

BLAST analysis and sequence comparison showed that 12 metabolites out of 20 were structurally different from possible fragments that could result from endogenous hGHRH, while three intact GHRH synthetic analogs out of the four studied had chemical modifications such as substitution, addition of amino acids and incorporation of chemical structures which confer different elemental compositions and hence isotopic mass from that of hGHRH. Tesamorelin has a similar backbone sequence compared with hGHRH, except for the hexanoyl moiety introduced on the N-terminus. The other synthetic analog, namely, CJC-1295 (composed of 29 amino acid residues), has a similar backbone sequence from positions 1 to 29 compared with hGHRH except for four amino acid substitutions in positions 2, 8, 15, and 27. CJC-1295 with DAC is similar to CJC-1295 with the addition of lysine and the DAC moiety in the C-terminal portion (Figure 2). The significant chemical substitutions and additions on tesamorelin, CJC-1295, and CJC-1295 with DAC might allow differentiation from possible fragments resulting from hGHRH.

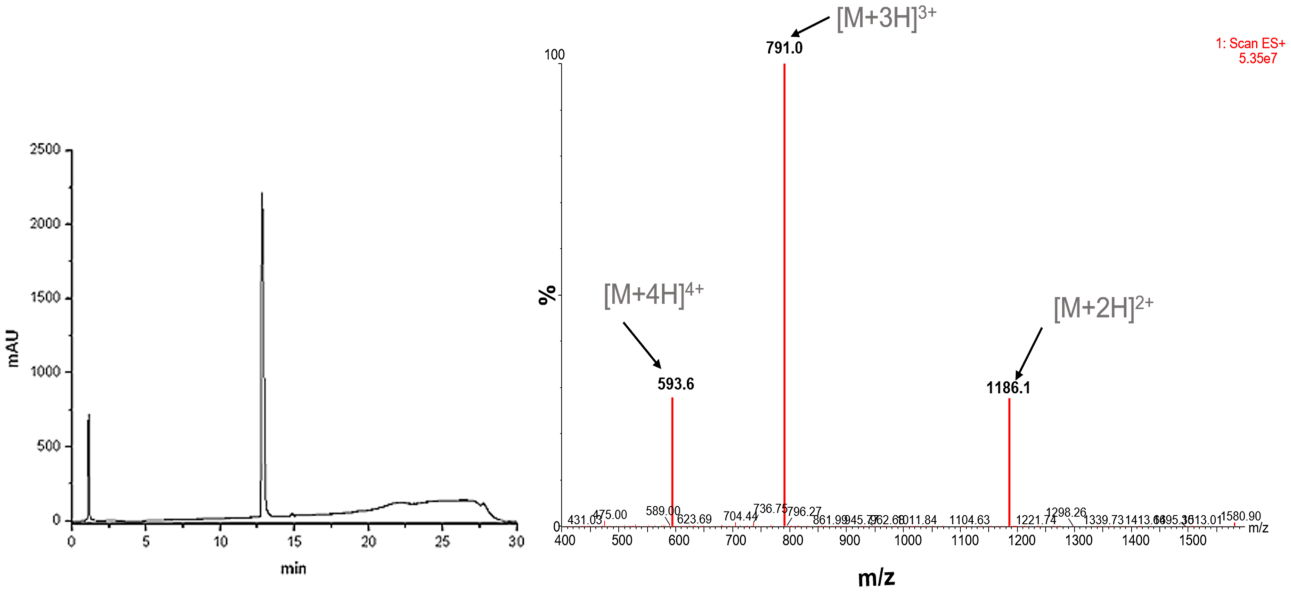

FIGURE 3 Chromatographic purity assessment of tesamorelin(1–20) using reversed-phase HPLC-DAD (214 nm detection wavelength) (left) and ESI$^+$ (single quadrupole) spectrum of tesamorelin(1–20) (right)

Conversely, sermorelin and its related metabolites suggested a large similarity with hGHRH. Therefore, sermorelin's metabolites are very likely to interfere with possible endogenously produced fragments from hGHRH. Sermorelin is a 29 amino acid fragment of hGHRH starting from the N-terminus but has a C-amidated terminal amino acid. This modification changes the exact mass for the intact compound as well as for some of the metabolites compared with those from hGHRH.

3.3 | Peptide synthesis, purification, and characterization

Based on the *in vitro* metabolism findings, a number of *in vitro* metabolites were identified via LC-HRMS as shown in Figure S1. The major ones based on mass spectrometric signal abundance were selected as candidates to be synthesized and purified in-house. The chromatographic purity of each crude synthetic material was examined using reversed-phase HPLC-DAD (data shown in Figure S2). The low-resolution ESI-MS spectra confirmed the presumptive identity of the purified peptides. As an example, the data obtained from tesamorelin(1–20) is presented in Figure 3, while that from all other metabolites are presented in the supporting material.

Accurate mass and full scan positive electrospray ionization spectra from the infusion of a solution of each purified metabolite into a Thermo Q-Exactive™ mass spectrometer was acquired; data from tesamorelin(1–20) are shown in Figure 4, while those from all other synthesized metabolites are shown in Figure S3. The amino acid sequence was then confirmed using all-ion fragmentation (AIF) in HRMS product ion spectra to reveal the b- and y-ion amino acid fragments (Figure 5). Different collision energies were used to enhance the fragmentation of the most abundant precursor ion of each peptide. The peptide sequences of the synthesized 19 metabolites were confirmed by evaluation of the spectra produced using a Thermo Scientific Q-Exactive™ mass spectrometer (Table 2).

The MRM, collision energy, and cone voltage of the Waters TQ-S Micro triple quadrupole were optimized for these metabolites, for a commercially available peptide sermorelin(3–29)—NH_2 and for the intact parent peptides (Table 3). A sensitive acquisition screening method for large GHRH synthetic analogs (>3 kDa) and their respective metabolites (0.8–3 kDa) was developed to detect the analytes with one MRM transition for each metabolite. In addition, confirmatory methods were developed to incorporate two to three transitions for each peptide. MS conditions including cone voltage (CV) and MRM transitions for acquisition and confirmatory methods are jointly presented in Table 3.

3.4 | Sample preparation optimization and recovery studies

The included metabolites are structurally diverse and consist of amino acids with hydrophobic and hydrophilic side chains as well as different numbers of readily ionizable amine groups. Hence a mixed mode weak cation exchange solid-phase extraction sorbent (Strata X-CW) was used. The sorbent's surface with carboxylic acid groups is activated at high pH, while it is expected to be neutral in acidic conditions. Therefore, at the pH of the loaded urine previously treated with phosphate buffer (pH 6.2), charged cationic peptides will be retained. On the other hand, peptides with hydrophobic side chains are expected to be retained by reversed-phase interactions.

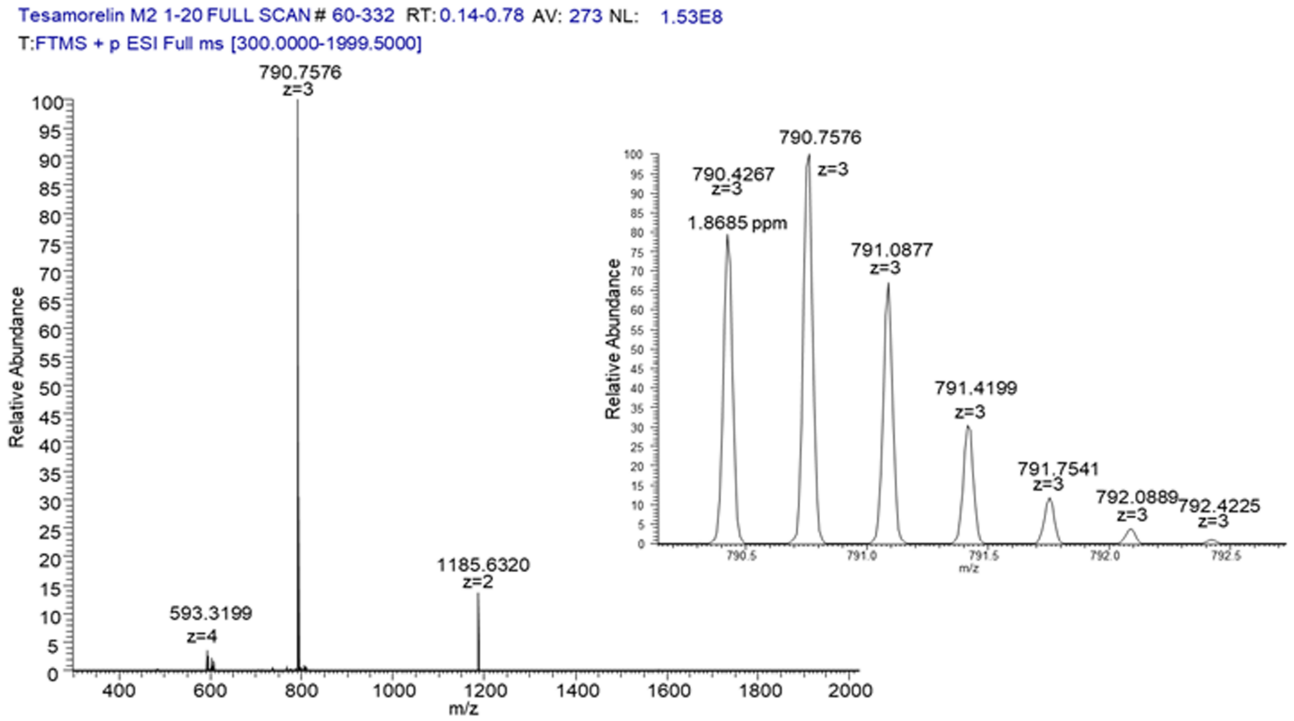

FIGURE 4 High-resolution full scan ESI^{+}-MS spectrum of tesamorelin(1–20) and a zoom view of the isotopic pattern of the $[M + 3H]^{3+}$ precursor

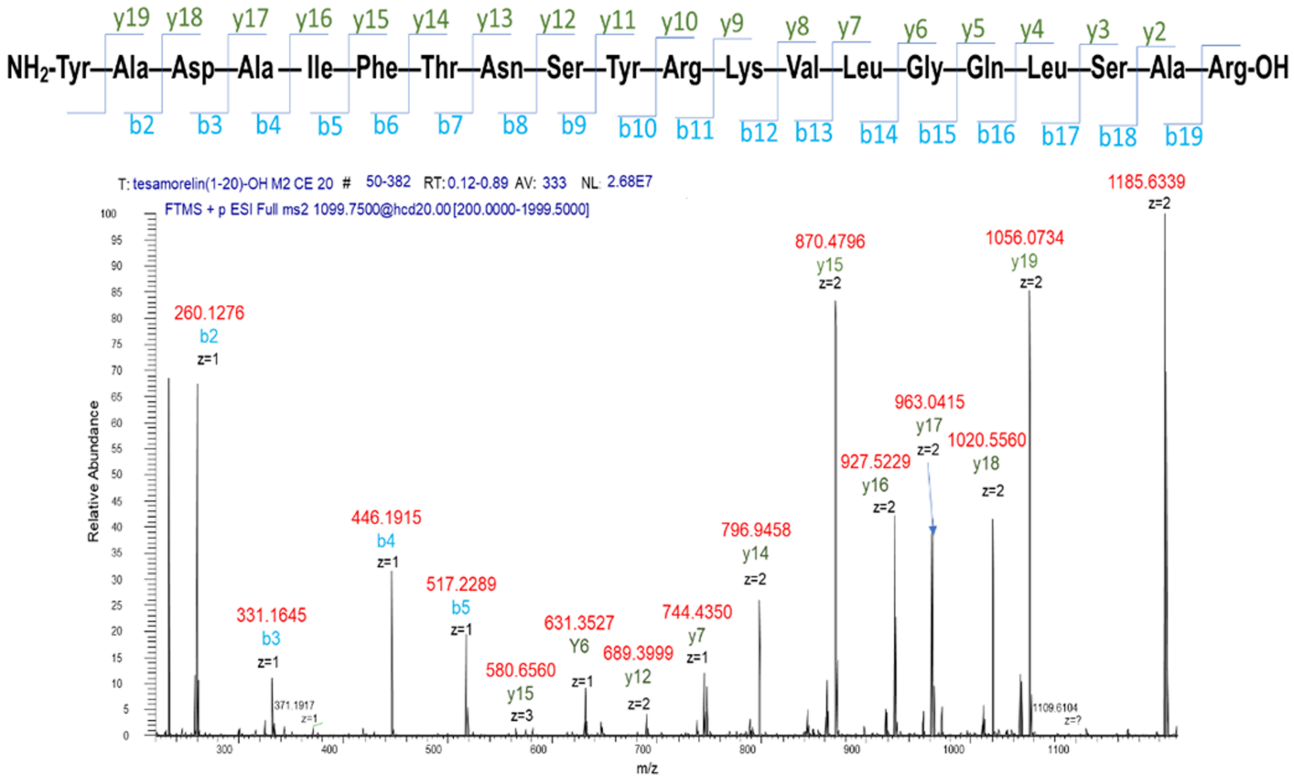

FIGURE 5 Product ion spectrum from the AIF of the tesamorelin(1–20) infusion in positive mode at a normalized collision energy of 20

TABLE 2 Measured and calculated accurate mass of the synthetic metabolites using HRMS

Identified metabolites	Elemental composition	Theoretical peptide content (%)[a]	Exact mass (m/z) $[M + nH]^{n+}$	Accurate mass (m/z) $[M + nH]^{n+}$	Charge state (n)[b]	Mass error (ppm)
Sermorelin(12–29)-NH_2	$C_{89}H_{163}N_{29}O_{24}S$	75	514.5609	514.5619	4	+1.8
Sermorelin(21–29)-NH_2	$C_{47}H_{87}N_{15}O_{13}S$	76	368.2182	368.2177	3	−1.3
Sermorelin(22–29)-NH_2	$C_{44}H_{75}N_{13}O_{12}S$	81	487.7762	487.7772	2	+2.0
Sermorelin(23–29)-NH_2	$C_{35}H_{64}N_{11}O_{12}S$	79	431.2342	431.2326	2	−3.7
Sermorelin(3–29)-NH_2	$C_{137}H_{232}N_{42}O_{39}S$	82	625.5516	625.5531	5	+2.4
Sermorelin(8–29)-NH_2	$C_{111}H_{195}N_{37}O_{31}S$	79	516.0988	516.0981	5	−1.4
Sermorelin(16–29)-NH_2	$C_{70}H_{128}N_{24}O_{20}S$	78	415.2437	415.2446	4	+2.2
Tesamorelin(1–21)	$C_{114}H_{181}N_{31}O_{32}$	85	625.0945	625.0934	4	−1.8
Tesamorelin(1–20)	$C_{108}H_{169}N_{29}O_{31}$	87	790.7597	790.7573	3	−3.0
Tesamorelin(1–11)	$C_{66}H_{93}N_{15}O_{20}$	93	708.8433	708.8437	2	+0.5
Tesamorelin(21–44)-NH_2	$C_{113}H_{199}N_{43}O_{37}S$	86	557.7026	557.7002	5	−4.3
Tesamorelin(1–12)	$C_{72}H_{105}N_{17}O_{21}$	87	772.8908	772.8896	2	−1.6
CJC-1295(1–21) with DAC	$C_{110}H_{177}N_{31}O_{31}$	81	486.8725	486.8724	5	−0.2
CJC-1295(1–12)	$C_{67}H_{99}N_{17}O_{20}$	87	488.2490	488.2498	3	+1.6
CJC-1295(21–29)-NH_2	$C_{48}H_{89}N_{15}O_{13}$	76	542.8455	542.8446	2	−1.7
CJC-1295(23–29)-NH_2	$C_{36}H_{66}N_{12}O_{11}$	79	422.2560	422.2562	2	+0.6
CJC-1295(7–29)-NH_2	$C_{118}H_{208}N_{38}O_{33}$	80	538.3233	538.3230	5	−0.6
CJC-1295(8–29)-NH_2	$C_{114}H_{201}N_{37}O_{31}$	79	517.9131	517.9145	5	+2.8
CJC-1295(12–29)-NH_2	$C_{91}H_{167}N_{29}O_{24}$	78	513.5757	513.5772	4	−2.9
CJC-1295(16–29)-NH_2	$C_{71}H_{130}N_{24}O_{20}$	78	410.7546	410.7542	4	−1.0

[a]Theoretical peptide content was calculated assuming that the counterions (TFA) are the only non-peptide components present. The percentages in the table were calculated using the formula below. The molecular weight of the peptide ($MW_{peptide}$) is divided by the sum of the number of TFA counterions (N_{TFA}) that are required to neutralize the peptide multiplied by the molecular weight of the TFA counterion (MW_{TFA}):
Teoretical peptide content $= \frac{MW_{peptide}}{MW_{peptide}+(N_{TFA}\times MW_{TFA})} \times 100$.
[b]Charge state based on the most abundant multiply charged ion.

The alternative cartridge to the Strata X-CW used was the Nexus WCX, which is also a mixed mode weak cation exchange sorbent that retains the analyte with the same mechanism but possesses a different bed mass, surface area, particle, and pore size. The sorbent of Nexus WCX has a lower surface-area and a lower resistance to flow due to the increased particle size. The samples were extracted similarly to the protocol presented in Section 2.8. However, after data analysis, it was found that peptides with a net charge of +1 or +2 were more susceptible to loss during the MeOH intermediate wash that might explain the observed poor recovery of these specific metabolites (data not shown). The best washing mixture was found to be MeOH:H_2O 80:20 for Nexus WCX, while for Strata X-CW, the recovery generally remained poor. Multiple reaction monitoring chromatograms of the four intact peptides and their metabolites spiked in urine at 1 ng/ml (the WADA MRPL), following the optimized solid-phase extraction protocol with Nexus WCX cartridges, are presented in Figure 6.

To carry out a verification of the recovery of the four intact GHRH synthetic analogs and the 20 metabolites, the performance of the Strata X-CW and the Nexus WCX cartridges were compared. Aliquots of three urine samples (with different pH and specific gravities) were spiked with all the peptides at 10 ng/ml prior to and after extraction (n = 3), and recoveries are presented in Figure 7. While Strata X-CW showed better recovery for only five metabolites, namely, sermorelin(22–29)-NH_2, sermorelin (23–29)-NH_2, tesamorelin(1–11), tesamorelin(1–12), and CJC-1295 (23–29)-NH_2, the Nexus WCX cartridge showed good recovery for all the included metabolites as well as for the intact parent peptides.

3.5 | Method validation

As the recovery was greater when using Nexus WCX cartridges, the method validation was carried out by using these cartridges with the optimized SPE protocol. The developed method was validated by assessing carryover, selectivity, limit of detection (LOD), recovery, and matrix effect.

Carryover was assessed by injecting two blank samples after spiking urine at 10 ng/ml (10 times the WADA MRPL). No interfering signal was detected in the blank samples.

TABLE 3 Mass spectrometry conditions on the Xevo TQ-S micro of the 20 identified metabolites and their respective intact synthetic analogs

Peptides	Precursor ion $[M + nH]^{n+}$ (m/z)	Charge state (n)	Product ion (m/z)	CV (V)	CE (eV)	Retention time (min)
Intact sermorelin	672.6	5	707.2	30	20	5.11
			787.9	30	18	
Sermorelin(12–29)-NH_2	515.1	4	510.3	25	17	3.91
			610.1	25	17	
			643.2	25	17	
Sermorelin(21–29)-NH_2	368.2	3	261.2	20	10	2.27
			392.1	20	10	
			598.4	20	10	
Sermorelin(22–29)-NH_2	487.8	2	199.1	20	16	2.36
			227.1	20	16	
			748.3	20	16	
Sermorelin(23–29)-NH_2	431.2	2	131.1	20	15	1.91
			242.3	20	15	
			620.4	20	15	
Sermorelin(3–29)-NH_2	625.8	5	670.4	40	18	5.01
			707.1	40	18	
			735.5	40	18	
Sermorelin(8–29)-NH_2	516.3	5	392.2	50	25	3.89
			594.7	50	15	
			616.3	50	15	
Sermorelin(16–29)-NH_2	415.7	4	392.2	20	13	4.31
			261.2	20	13	
Intact tesamorelin	734.8	7	751.9	30	20	5.23
			813.6	30	18	
Tesamorelin(1–21)	625.1	5	232.1	20	18	5.35
			260.3	20	18	
			746.7	20	18	
Tesamorelin(1–20)	790.7	3	260.3	20	30	5.60
			870.4	20	30	
			1055.7	20	30	
Tesamorelin(1–11)	709.3	2	630.2	15	20	5.34
			517.3	20	20	
			787.5	15	15	

TABLE 3 (Continued)

Peptides	Precursor ion $[M + nH]^{n+}$ (m/z)	Charge state (n)	Product ion (m/z)	CV (V)	CE (eV)	Retention time (min)
Tesamorelin(21–44)-NH_2	557.7	5	547.7	20	20	1.94
			767.9	20	20	
			810.7	20	20	
Tesamorelin(1–12)	773.3	2	260.1	70	25	4.50
			446.3	70	25	
			517.7	70	25	
Intact CJC-1295 with DAC	730.5	5	779.3	70	15	5.26
			854.2	70	15	
CJC-1295(1–21) with DAC	486.9	5	583.9	20	15	3.77
			632.8	20	13	
			670.5	20	15	
Intact CJC-1295	674.7		207.2	40	20	5.20
			709.6	40	18	
CJC-1295(1–12)	488.3	3	557.2	20	15	2.17
			615.0	20	15	
			650.4	20	15	
CJC-1295(21–29)-NH_2	362.6	3	261.3	20	10	2.54
			374.3	20	10	
			598.3	20	10	
CJC-1295(23–29)-NH_2	422.3	2	602.3	20	15	2.09
			242.2	20	15	
CJC-1295(7–29)-NH_2	538.6	5	261.3	30	19	4.28
			374.2	30	19	
			615.6	30	19	
CJC-1295(8–29)-NH_2	518.4	5	261.3	25	20	4.25
			374.3	25	20	
			571.2	40	18	
CJC-1295(12–29)-NH_2	514.0	4	374.2	30	20	4.33
			608.9	20	19	
			756.9	40	19	
CJC-1295(16–29)-NH_2	547.0	3	487.3	10	26	4.31
			602.7	10	26	
			700.2	10	26	

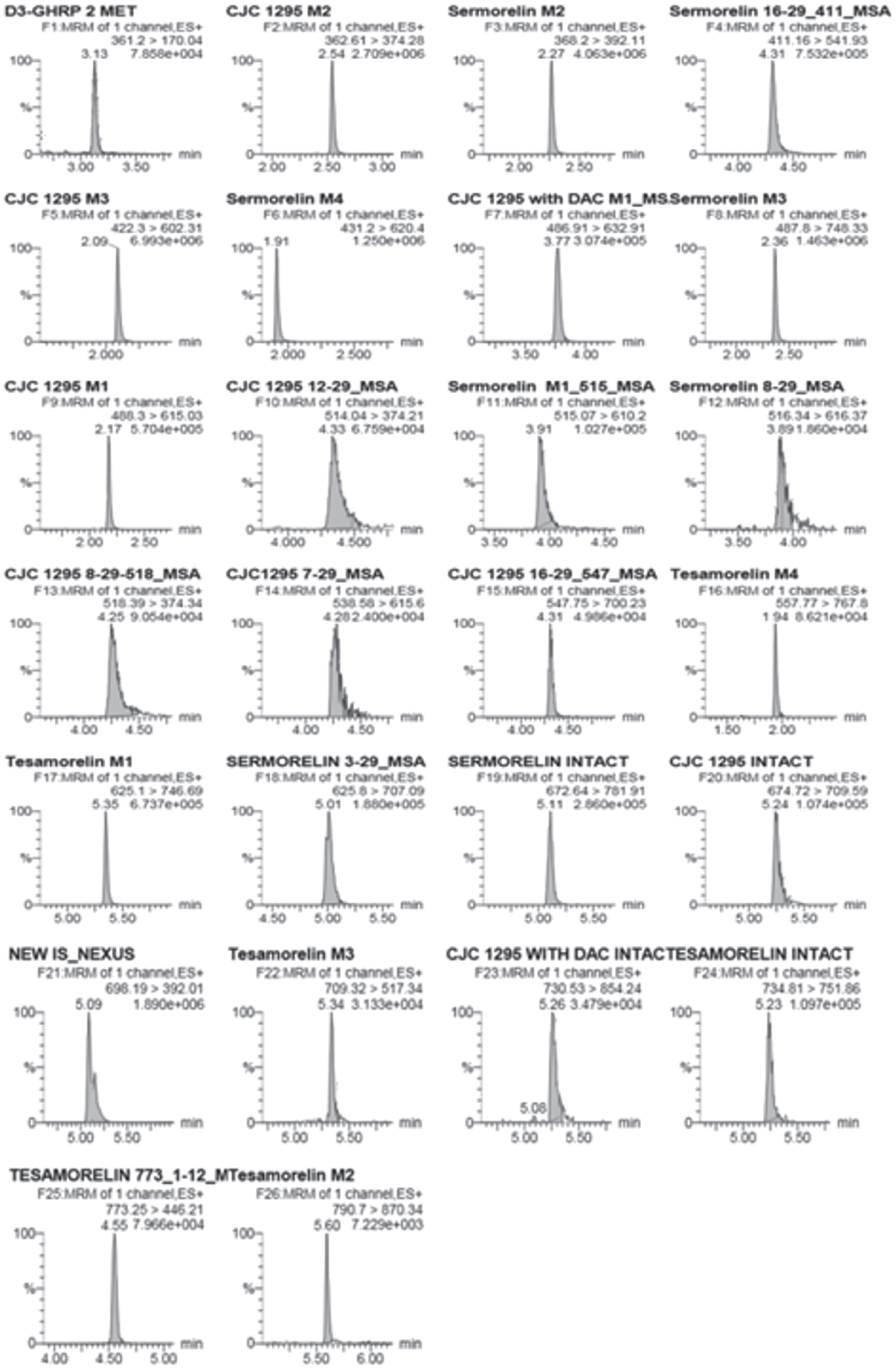

FIGURE 6 Multiple reaction monitoring chromatograms of the four intact peptides and their metabolites at 1 ng/ml (the WADA MRPL) in urine extracted using the Nexus WCX cartridge

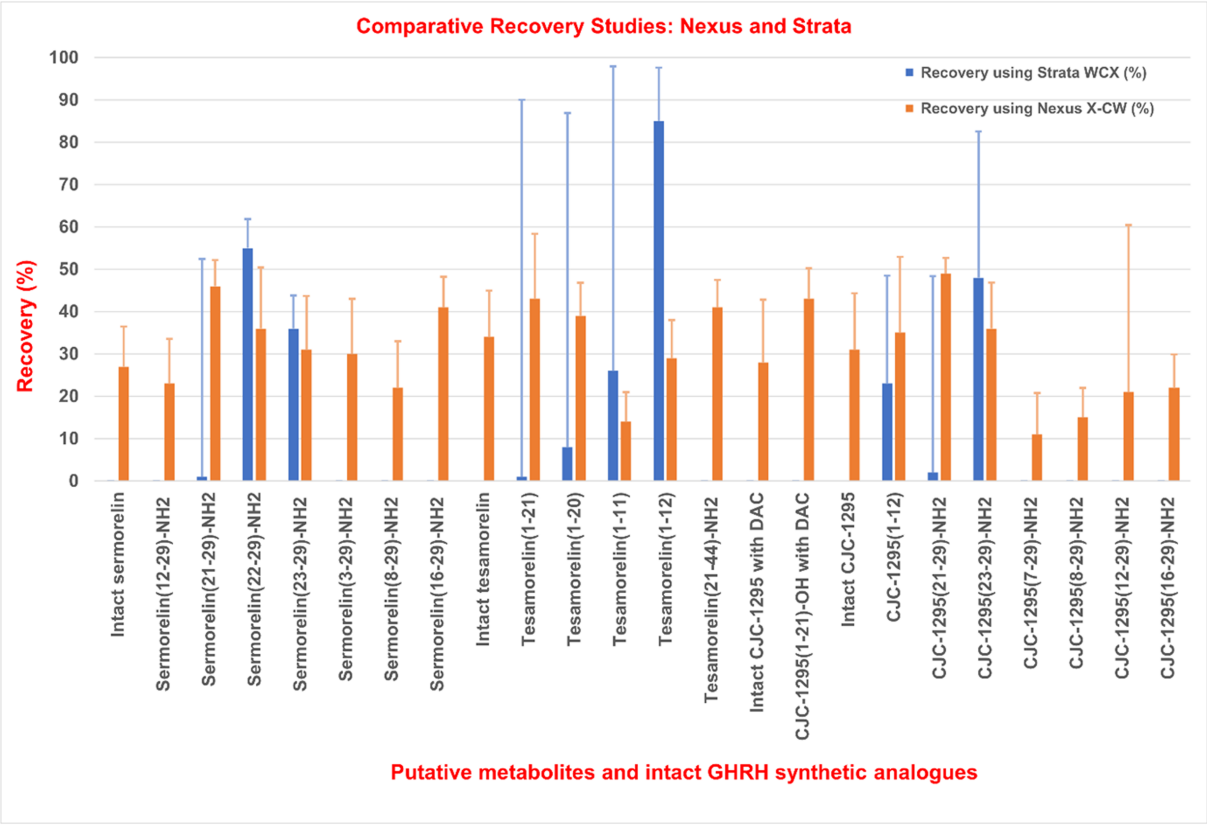

FIGURE 7 Comparative recovery studies between Nexus WCX and Strata X-CW cartridges. Recoveries estimated by comparing peak areas of the target compounds (three urines repeated in triplicate), spiked at 10 ng/ml, before and after extraction

TABLE 4 Recovery study and LOD results for the qualitative analysis of GHRH synthetic analogs in fortified urine samples using Nexus WCX SPE cartridges

Analytes	Recovery (%)	SD (%)	CV (%)	LOD, S/N > 3 (ng/ml)
Intact Sermorelin	27	0.03	9	0.2
Sermorelin(12–29)-NH_2	23	0.02	11	0.2
Sermorelin(21–29)-NH_2	46	0.03	6	0.2
Sermorelin(22–29)-NH_2	36	0.05	14	0.1
Sermorelin(23–29)-NH_2	31	0.04	13	0.2
Sermorelin(3–29)-NH_2	30	0.04	13	0.2
Sermorelin(8–29)-NH_2	22	0.02	11	0.5
Sermorelin(16–29)-NH_2	41	0.03	7	0.2
Intact Tesamorelin	34	0.04	11	0.2
Tesamorelin(1–21)	43	0.07	15	0.2
Tesamorelin(1–20)	39	0.03	8	0.2
Tesamorelin(1–11)	14	0.01	7	0.5
Tesamorelin(1–12)	29	0.03	9	0.5
Tesamorelin(21–44)-NH_2	41	0.03	6	0.2
Intact CJC-1295 with DAC	28	0.04	15	0.2
CJC-1295 with DAC(1–21)	43	0.03	7	0.1
Intact CJC-1295	31	0.04	13	0.2
CJC-1295(1–12)	35	0.06	18	0.1
CJC-1295(21–29)-NH_2	49	0.02	4	0.1
CJC-1295(23–29)-NH_2	36	0.04	11	0.1
CJC-1295(7–29)-NH_2	11	0.01	10	0.5
CJC-1295(8–29)-NH_2	15	0.01	7	0.5
CJC-1295(12–29)-NH_2	21	0.08	39	0.5
CJC-1295(16–29)-NH_2	22	0.02	8	0.2

Selectivity was tested by analyzing 10 different blank urine samples to check for interfering signals. The experiment confirmed the absence of interfering signals originating from blank urine.

LODs were estimated via signal to noise (S/N > 3) from spiked urine samples at concentrations of 0.1, 0.2, and 0.5 ng/ml and were considered to be the smallest concentration at which the metabolites and the intact parent peptides could be detected in six different urine samples (Table 4).

Matrix effect (ion suppression) was evaluated by comparing the peak areas of three fortified blank urines to the fortified neat sample solution in H_2O:ACN:FA (95:5:0.3 %) at equivalent concentrations. No ion suppression or enhancement was observed.

4 | CONCLUSIONS

The administration of GHRH synthetic analogs is prohibited in sport and their detection is required by WADA accredited laboratories at very low concentrations (≤1 ng/ml). We have investigated the *in vitro* metabolism of four GHRH synthetic analogs sermorelin, tesamorelin, CJC-1295, and CJC-1295 with DAC and suggested utilizing *in vitro* metabolites to facilitate the detection of GHRH synthetic analogs in urine. Sermorelin, CJC-1295, and CJC-1295 with DAC are unapproved drugs, and hence, their *in vivo* study is limited by ethical constraints. Nineteen metabolites were identified, synthesized in house and characterized. Urine was spiked with these metabolites as well as parent compounds and commercially available sermorelin (3–29)-NH_2. Solid-phase extraction with Nexus WCX cartridges was optimized and a selective and sensitive LC–MS analytical method developed and validated. Our compounds of interest were detected at or less than the WADA MRPL (1 ng/ml). Although our research did not include the analysis of real anti-doping samples where the misuse of GHRH synthetic analogs was suspected, we believe our method is simple, easily transferable to other WADA accredited laboratories, and has the potential to improve the detection of misuse of these compounds in sport.

ORCID

Ivana Gavrilović https://orcid.org/0000-0003-4942-6398
David Cowan https://orcid.org/0000-0001-6407-1113
Vincenzo Abbate https://orcid.org/0000-0002-3300-0520

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SUPPORTING INFORMATION

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