Detection of Basic Doping Agents in Equine Urine using Liquid Chromatography – Mass Spectrometry

By

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Abstract

The potential abuse of small molecule drugs and peptides in horseracing is an area of great concern due to their high potential to act as doping agents. These compounds include classes of agents as growth hormone-releasing peptides (GHRPs), beta-2-agonists and quaternary ammonium drugs that are a challenge to detect and regulate due to their chemical properties and potential rapid elimination following administration. The use of highly sensitive and selective analytical techniques such as liquid chromatography – mass spectrometry (LC-MS) is necessary to provide coverage of these substances and their potential metabolites. This study describes development and validation of methodology capable of the detection of over fifty different peptide-based doping agents, related secretagogues, quaternary ammonium drugs, and other challenging small molecules in equine urine following solid phase extraction using a weak cation exchange sorbent. Following sample extraction the compounds were detected using LC-MS with chromatographic separation via a reverse phase gradient at 0.4 ml/min and selective reaction monitoring based detection following introduction to a triple-stage quadrupole mass spectrometer using positive mode electrospray ionization. Validation parameters including limits of detection and quantitation, accuracy, precision, linear range, recovery, stability, and matrix effects were determined. Briefly, the limits of detection for most compounds were in the 0.05 – 1.0 ng/mL range with adequate precision and accuracy sufficient for an initial testing method. Stability studies indicated that most compounds were sufficiently stable to allow for effective screening using conditions commonly utilized in drug testing laboratories.

1.0 Introduction

Effective detection of potential doping agents in equine sport requires efficient initial screening procedures to process and review hundreds of compounds across a wide variety of chemical classes in a timely manner. There are several classes of molecules including peptide based molecules and quaternary ammonium drugs that may not be effectively screened using some routine approaches creating challenges for both laboratories and regulatory authorities [1]. The misuse of bioactive peptides has become an increasing area of concern due to their pharmacological effects on the cardiovascular, nervous, immune, and endocrine systems with over 80 peptide drugs approved for use in the United States. There are numerous websites that sell some of these agents or similar compounds without requiring a prescription [2, 3]. Likewise there are other small molecule compounds such as quaternary ammonium drugs that can impact similar physiological systems in the horse and require coverage to regulate potential abuse or use within established detection times for therapeutic agents such as glycopyrrolate or ipratropium[4, 5]. The Association of Racing Commissioners International and International Federation of Horse Racing Authorities have classified many of these agents as prohibited substances and monitoring for their use in equine sport is important to maintaining the safety and integrity of the sport.[6, 7]

The abuse of peptide-based drugs such as growth hormone releasing peptides (GHRPs), peptide analgesics (e.g., dermorphin), and other similar agents is documented in human and animal sport [1, 8-10]. For many of these naturally produced peptides, the half-life in the systemic circulation is very short (10 - 30 minutes) which makes use of these compounds as therapeutic agents challenging [11]. Accordingly, modifications of the chemical structures have been done to improve pharmacokinetics and pharmacodynamics with alterations of the N- or C-

terminus, use of d-enantiomers of amino acids, or use of substitutions such as the addition of pyrogluatmic acid, aminosiobutyric acid, methlytryptophan functional groups to reduce degradation [12, 13]. Further complicating the analysis of peptides that may be used as doping agents is the potentially complex metabolic pathways that they may undergo following *in vivo* administration necessitating the need for metabolite monitoring by laboratories [8, 12, 14-16]. While use of doping peptides is a more recently emerging threat to equine safety/welfare and racing integrity, there are other classes of agents such as quaternary ammonium drugs (QADs) and beta-2-agonists (β_2-agonists) that share some of the physiochemical properties as many of the peptides of concern that warrant inclusion with a laboratory's initial testing procedures. Quaternary ammonium drugs are permanently positively charged compounds that have been developed predominately as either short or long acting anticholinergic agents commonly used for the treatment of respiratory conditions such as chronic obstructive pulmonary disorder [4, 5]. β_2-agonists are another class of compounds that have clinical use in the treatment of respiratory conditions improving bronchodilation and at higher doses may be used as repartitioning agents [17]. These compounds represent a diverse array of chemical structures and pharmacological effects that require highly sensitive and selective analytical procedures to combat their potential abuse in horseracing.

There are a number of analytical approaches for the detection of peptides, quaternary ammonium drugs and β_2-agonists with liquid chromatography – mass spectrometry (LC-MS) using electrospray ionization being the preferred technique due to its sensitivity, speed and selectivity [8, 12, 14-16, 18-23]. While the use of highly sensitive and selective LC-MS based approaches has increased the ability to detect low levels of these agents in biological samples there are many considerations such as non-specific losses due to binding to glassware, enzymatic

degradation, chemical properties of the agents (polarity and zwitterion), and matrix effects that may impact the overall ability to detect a compound in a biological sample [12, 20, 24, 25]. The development of highly sensitive mass spectrometry based initial testing procedures is critical to addressing the potential abuse of compounds in horseracing. This thesis describes the development and validation of an analytical method focused on detection of doping peptides, related secretagogues, β_2-agonists, and quaternary ammonium drugs in equine urine following solid phase extraction (SPE) and detection using LC-MS on a triple stage quadrupole mass spectrometer. The following parameters were determined during method validation: limits of quantitation and detection, accuracy, precision, linear range, recovery, compound stability, and matrix effects.

2.0 Background

2.1 Peptides in Equine Doping

Peptide based drugs are comprised of covalently linked amino acids with minimal secondary or tertiary structure with less than 50 amino acids [26]. The chemical structure of these peptide doping agents can make their analysis challenging. In previous work by Kwok *et al*, zwitterionic nature of peptides has revealed difficulties with poor extraction during SPE [20]. For example, GHRP-2, is a multiply charged species at neutral pH. Lysine's sidechain has a pKa of 10.54 and an NH_2 pKa of 9.06, so at physiological pH it is polar (Figure 1). Newer SPE cartridges with anion and cation exchange columns to retain acidic or basic compounds have been developed. There are still issues with peptides that are slightly acidic or neutral when using the mentioned columns. For example, Deltorphin II has glutamate and no basic residues as shown in Figure 2. Therefore, it is not retained on the SPE columns designed for basic compounds although it can still reach low levels of detection but with poor recovery [18]. In

addition to the challenges associated with the peptides being zwitterions, peptide drugs have been shown to have instability issues and losses associated with non-specific binding [26, 27].

In recent years, there has been a large growth in the development of synthetic or semi-synthetic peptides that have the potential to impact performance [26]. For example, the development of GHRP peptides (GHRP 1 to GHRP 6) was originally intended to enhance the growth hormone axis by increasing GHRH release, amplifying GHRH signaling, reducing somatostatin release, and antagonizing somatostatin receptor signaling [28, 29]. The instability of peptide drugs was a concern due to their short systemic circulation and enzymatic degradation [26, 27]. Synthetic modifications such as amidation of the C-terminus, D-amino acid substitution, N-terminal acetylation, and cyclization enhance the peptide stability from enzymatic degradation [12, 13, 29, 30]. These chemical modifications increase their marketability as a performance-enhancing agent and they have since been used to enhance performance in human sports [9, 10].

The development of an LC-MS based screening method focused on GHRP's and their metabolites in urine relevant to anti-doping was first reported in 2012 [15]. The method was used to test samples from humans with no prior use of GHRP-2 and from a subject orally administered 10 mg of GHRP-2 [15]. The procedure involved pH treatment of urine samples to be 7±1 followed by an extraction with a mixed-mode solid-phase extraction cartridge and centrifugation before transferring the supernatant into an auto sampler vial for LC – MS [15]. In 2015, Cox and colleagues reported an LC-MS based method for the detection of GHRP-2, GHRP-6, and their metabolites in human urine and demonstrated the applicability in *in-vivo* samples [19].

These early reports encouraged the development of new screening methods for peptide-based drugs, but it also illustrated the challenges associated with developing a multi-analyte

screening methods focused on these peptide drugs used as doping agents. In the reports by Cox *et al*, urine collected following administration of peptides to human subjects did not have detectable presence of the unchanged peptides while their metabolites were detectable [19]. This conflicts with a previous study focused only on analysis of a single peptide where both GHRP-2 and its metabolite were detectable in urine [31]. Thomas *et al.* developed a screening method that expanded the scope of analysis to a larger group of compounds but in the process the limits of detection were higher for some molecules [32]. Currently, most detection methods still try to detect the parent compound due the unknown metabolism of peptides *in vivo,* which makes this difficult for drug detection [8, 14, 26]. *In vitro* studies have been performed and detected trace amounts of the intact molecule and metabolites [8, 14]. Other studies examined homogenized liver and kidney samples to identify metabolites formed by exopeptidase, a proteolytic enzyme occurring in the liver or kidneys [14, 33]. However, both approaches have their limitations but have allowed us to better understand peptide metabolites, degradation, and limitations on drug detection. Recently, a 40 bioactive peptide drug screen in equine urine was developed and validated on an ultra-high-performance liquid chromatography – high-resolution mass spectrometry (UHPLC – HRMS) [18]. The research group was able to obtain detection levels as low as 50 pg/mL for several compounds. They experienced poor recoveries with compounds that were determined to be slightly acidic or neutral [18]. Recent reviews have suggested that use of mass spectrometry based detection methods utilizing HRMS systems provides the best analytical methodology due to their high specificity and good sensitivity [26]. While HRMS provides high quality data, the high cost of instrumentation, slower scan speed, and the large data files make use of triple quadrupole based mass spectrometers an appealing alternative technology.

2.2 QADs in Equine Doping

Quaternary ammonium cations are permanently positively charged species independent of their environment with four carbons attached to a nitrogen Figure 3. They are synthesized by reacting quaternary ammonium salts with alkyl halides. Therefore many of these species consist of bromide or chloride salts. QADs have been predominately developed as either short or long acting as anticholinergic agents commonly used for the treatment of respiratory conditions such as chronic obstructive pulmonary disorder.

QADs compounds are polar at physiological pH due to their permanent positive charge and require specific methods of extraction from their matrices to obtain low detection limits. In past studies, liquid-liquid and SPE extraction have been utilized [21]. However, weak cation exchange simplifies extraction and clean-up of these polar compounds [21]. In 2011, Ho *et al* reported a new LC-MS based screening method targeting 38 QADs in equine urine using a triple-quadrupole mass spectrometer with acquisition using SRM based acquisition [21]. The LODs for most compounds were between 0.05 – 1.0 ng/ mL. In one study, ipratropium was clearly detected post administration using this approach [34].

2.3 Advancing Research

The use of doping agents including peptide based molecules, QADs, and other similar agents of interest to individuals trying to gain a competitive advantage and represent threats to the safety and integrity of horseracing. To detect the incidence of their use, a comprehensive drug screen is a strong approach for identifying their abuse. The aim of this study was to develop and optimize a method for detection of small peptides, QADs and other similar agents (Tables 1 and 2) in a single run for high throughput analysis suitable for routine use in an anti-doping laboratory.

3.0 Material and Methods

3.1.1 Reference Standards

Alarelin, AOD-9604, ARA-290, GHRP-4, GHRP-5, GHRP-6, GHRP-6 (2-5) free acid, GHRP-6 free acid, hexarelin, hexarelin (1-3) free acid, hexarelin free acid, ipamorelin, ipamorelin (1-4) free acid, ipamorelin free acid, lecirelin, LHRH (1-3) free acid, mechano-growth factor (MGF), and nafarelin (5-10) MET were purchased from Auspep (Victoria, Australia). Buserelin, deslorelin, leuprorelin, triptorelin, triptorelin free acid, and goserelin were obtained from Bachem (Torrance, CA, USA). Ibutamoren was purchased from Cayman (Ann Arbor, MI, USA). Deltorphin, deltorphin I (D-Ala2), deltorphin (D-Ala2) II, dermorphin (1-4) (D-Arg2, Sar4), dermorphin (1-4) amide (Arg2, Lys4), and dermorphin (1-4) amide (D-Arg2) were purchased from CPC Scientific (Sunnyvale, CA, USA). GHRP-1 and GHRP-2 were purchased from ProSpec (East Brunswick, NJ, USA). Capromorelin, dermorphin, histrelin, clidinium bromide, ipratropium bromide, mepenzolate bromide, N-butylscopolamine, neostigmine, oxyphenonium bromide, pancuronium bromide, pipenzolate bromide, pirbuterol, propantheline bromide, and tiotropium was purchased from Sigma-Aldrich (St. Louis, MO, USA). Bretylium bromide, edrophonium bromide, glycopyrrolate, and isopropamide iodide were purchased from USP (Lauderdale, Florida, USA). Methotrexate was purchased from Toronto Research Chemicals Inc. (Toronto, ON, CA). The internal standards benzyldimethylphenylammonium chloride was purchased from Acros (Pittsburgh, PA, USA), [^{13}C,^{15}N]-GHRP-2 (1-3) was purchased from Auspep (Victoria, Australia), and Glycopyrrolate-*d* was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA).

3.1.2 Reagents

Methanol optima grade, 2N sodium hydroxide solution, 2N hydrochloric acid solution, and ammonium formate certified grade, were purchased from Fischer Scientific (Pittsburgh, PA,

USA). Deionized nanopure water was obtained from a Nanopure system (Thermo Scientific, Waltham, MA, USA). Formic acid, acetonitrile and HPLC grade water were purchased from Honeywell - Burdick & Jackson (Muskegon, MI, USA).

3.2.1 Preparation of Standard Solutions

Most reference standards were prepared at 1 mg/mL in various concentrations of acetonitrile and water depending on their solubility. Deltorphin, deltorphin I, deltorphin II, LHRH 1-3 (free acid), MGF, and methotrexate were prepared at 2 mg/mL in various concentrations of acetonitrile and water depending on their solubility. The targeted compounds were mixed together into a working solution of 50/50 methanol/water containing most of the compounds at 1 ng/µL except for the reference standards that were prepared at 2 mg/mL where the working solution concentration was 2 ng/µL to allow for the preparation of a sample with different concentrations of compounds being present in it. The internal standards for the QADs, glycopyrrolate-*d3* and benzyldimethylphenylammonium, were diluted to 4 µg/mL and 50 µg/mL. The internal standard for the peptides, [^{13}C,^{15}N]-GHRP-2 (1-3), was diluted to 50 µg/mL. Glycopyrrolate-*d3*, Benzyldimethylphenylammonium, and [^{13}C,^{15}N]-GHRP-2 (1-3) were then added to 200 mL of 0.3M ammonium formate buffer (pH 7) to a final concentration of 0.050, 1.25, and 2.5 ng/mL respectively.

3.2.2 Stock Solutions for Sample preparation

A concentration of 0.3 M ammonium formate buffer, pH 7.0 was prepared by adding 19g of ammonium formate to 800 mL of DI water in a 1 L mixing cylinder and then bringing to volume after mixing thoroughly. To prepare 0.1 M ammonium formate buffer, pH 7.0, 6.3 g of ammonium formate were added to 800 mL of DI water in a 1 L mixing cylinder and then bringing to volume after mixing thoroughly. The pH of both solutions was adjusted with

ammonium hydroxide or hydrochloric acid as needed. A 5% formic acid solution was prepared daily by adding 95:5 methanol with formic acid.

3.3 Calibrator and Quality Control Preparation

Negative control urine was prepared from cleared post-race samples (>5 horses) that was pooled. After pooling the urine was stored frozen at -20°C until thawed in a refrigerator (2-8°C) overnight. A calibration curve was constructed from a urine sample (2.25 mL) that was spiked (0.25 mL) with the mixed working solution containing targeted compounds at either 1 or 2 ng/µL which was diluted in urine to achieve final concentrations of 0.005, 0.01, 0.05, 0.1, 0.5, 1, 2, 5, 10 ng/mL except for deltorphin, deltorphin I, deltorphin II, LHRH 1-3 (free acid), MGF, and methotrexate which were spiked at twice the concentration for each level. Quality control samples were prepared at low, mid, and high levels of 0.5, 2.5, and 7.5 ng/mL, respectively. For the compounds spiked at double the concentration, the quality control concentrations were 1.0, 5.0, and 15.0 ng/mL. For matrix effect experiments the neat standards were prepared by adding 7.5 µL of 10/20 ng/µL working solution into 92.5 µL of water with 0.2% formic acid. Then were diluted 1:10 in water with 0.2% formic acid to a final concentration of 7.5 ng/mL.

3.4 Sample Preparation

A 1 mL of urine was transferred into a 12 x 75 mm glass tube and 2 mL of 0.3 M ammonium formate, pH 7.0 containing the internal standards was added and pH verified to be 7.0 ± 1.0 using pH paper. The pH was adjusted with 2 N sodium hydroxide or 2 N hydrochloric acid (if needed) and the sample was vortexed, 30 seconds (Fisher Scientific, Pittsburgh, PA, USA). The urine samples were then placed in a water bath and sonicated (Yamato Scientific America Inc., Santa Clara, CA, USA) at 60º C for one hour. After incubation the samples were

centrifuged (Thermo Scientific, Waltham, MA, USA) at 4,000 revolutions per min at 4 °C for 5 minutes.

3.5 Solid Phase Extraction

Evolute® Express weak cation exchange solid-phase extraction (SPE) cartridges (100 mg, 3 mL, Biotage Inc., Uppsala, Sweden) were placed in a 48 well positive pressure manifold (Cera Inc., Baldwin Park, CA, USA). The SPE cartridges were conditioned with 3 mL of methanol at a flow rate 1mL/min. Then the pH of the cartridges was adjusted with 3 mL of 0.1 ammonium formate buffer at pH 7.0. The samples were then introduced into the preconditioned columns and passed through slowly to allow the deprotonated carboxylic acid to interact with the positively charged analytes of interest. The column was then rinsed with 3 mL of deionized water. Lastly, the columns were dried down with 1.5 mL of methanol and dried with high pressure for one minute. A prepared auto sampler rack was placed into the manifold and 1.8 mL of 95:5 methanol: formic acid was added. The extract was collected via gravity or low pressure if needed into auto sampler vials. The vials were then placed into a TurboVap LV Evaporator (Zymark Corporation, Hopkinton, MA, USA) and evaporated for approximately 14 minutes. The vials were then reconstituted with 100 µL of 5% acetonitrile in water both with 0.2% formic acid. Next, the vials were vortexed and ready for the UHPLC analysis.

3.6 LC-MS Analysis

LC-MS analysis used a Vanquish™ Duo HPLC system coupled to an Altis™ triple stage quadrupole mass spectrometer (Thermo Scientific, Waltham, Massachusetts, USA). The sample extract was introduced to the HPLC system with a 20 µL injection volume using positive mode electrospray ionization and compounds separated over 15 minute analytical run using a Accucore™ Vanquish™ C18 (1.5 µm, 2.1 mm x 50 mm) column with guard pre-column

installed (Thermo Scientific, Waltham, Massachusetts, USA) held at 20 °C. Mobile phase A is acetonitrile with 0.2% formic acid, and mobile phase B is water with 0.2% formic acid. The mobile phase composition was modified using a reverse phase gradient with initial conditions 0.00 min, 2% A; 1.00 min, 2% A; 4.50 min, 30% A; 8.50 min, 90% A; 9.50 min, 90% A; 15 min, 2 % A. The flow rate was 400 µL/min with a total run time of 15 minutes. The divert valve was on until 0.75 min and the mass spectrometer acquisition time began. Argon was used as the collision gas and it was set to 1.5 mTorr in Q2. The mass spectrometer was operated in selected ion reaction monitoring (SRM) mode with the precursor and product ions along with their corresponding collision energies for the monitored compounds shown in Table 3. SRM transitions and conditions were optimized using Xcalibur Tune software by infusion of neat standards (10 ng/µL) with 50/50 mobile phase A and B at 0.4 ml/min using a T set up. The source parameters were as follows: spray voltage 4,500V, sheath gas 55 arbitrary units, auxiliary gas 7 arbitrary units, sweep gas 2 arbitrary units, ion transfer tube 350 ºC, and vaporizer temperature 250 °C. The use of a formic acid modifier and a Thermo Accucore C18 column with a <2 µm particle size allowed for good peak shape and chromatographic separation for most compounds (Figure 4). The Q1 resolution was set at 0.7 and Q3 resolution was set at 1.2 full width at half-maximum height. The mass spectrometer was calibrated using Pierce™ extended mass range calibration solution (Thermo, Waltham, Massachusetts, USA) consisting of a mixture of imidazole, trimethylamine, trifluoroacetic acid, tetrmethylpiperidine, 1,8-bis(dimethylamino) naphthalene, 2,4,6-tri(trifluoromethyl)-1,3,5-triazine, acetic acid, dexakis(2,2,-difluroethoxy)phosphazne, hexakis(2,2,3,3-tetrafluroopropoxy)phosphazene, dexakis(1h,1h5h-octafluropropoxy)phosphazene, hexakis(1h,1h,5h-octafluoropentoxy)phosphazene, hexakis(1h,1h,7h-perfluroheptoxy)phosphazene, and hexakis(1h,1h9h-

perfluorononyloxy)phosphazene in water, acetonitrile and isopropanol (4/92/4 v/v). The LC-MS system was controlled using the Xcalibur (version 4.3) and Aria (version 2.6.13) software from Thermo (Waltham, Massachusetts, USA). Data review was accomplished using Quanbrowser software (version 4.3) from Thermo (Waltham, Massachusetts, USA).

3.6 Validation

The method was validated as a fit-for-purpose method based in part on ISO/IEC 17025 requirements to be used as an initial testing procedure in equine urine. As part of the validation, the following parameters were monitored: limits of detection, limits of quantitation, accuracy, precision, recovery, matrix effects, specificity, carryover, stability, linear range, and qualitative identification per Association of Official Racing Chemists criteria was conducted [35]. Accuracy (% accuracy) and precision (relative standard deviation) were assessed at each QC level (n=6/level) over 3 days. Matrix effects and recovery were assessed by comparing instrument response for the targeted compounds in extracted QC samples, extracted negative control samples spiked with the compounds after extraction, or neat standards diluted to equivalent concentrations as the QC levels. Specificity was assessed by monitoring 6 individual lots of negative control urine. Stability samples were prepared at the high quality control level and stability was assessed at various time points at room temperature, 2-8 ºC, -20 ºC, and -20 ºC with one freeze/thaw cycle. Assessment of linear range was determined from negative control urine samples containing the targeted analytes by linear regression analysis using the peak area ratio of the targeted compound and its corresponding internal standard. The limits of detection and quantitation were determined using a signal to noise ratio of more than 3:1 and 6:1 from baseline to the peak height, respectively. Additionally the limit of detection had the requirement that the

compound met AORC criteria for identification. Carryover was assessed by monitoring a solvent blank directing following the highest calibration sample.

4.0 Results

The method was validated per in-house criteria based in part on ISO/IEC 17025 requirements with recovery, matrix effects, carryover, limits of detection and quantitation were determined for each compound (Table 4). The linear range for each compound was evaluated with regression correlation coefficients, R^2, greater than 0.99 for most compounds (Table 4). Carryover was assessed by monitoring a solvent blank directly following the highest calibration sample with no detectable carry over observed at the concentration ranges monitored. Analyte recovery was determined by dividing the average peak area of QC (n=6) at 7.5 ng/mL with the peak area of a post-extraction spiked samples (n=6) at the same concentration as shown in Table 4. Matrix effects were determined by dividing the peak areas of post-extraction spiked samples with those of neat standards at the equivalent concentration with most compounds having minimal ion enhancement or suppression (Table 4). The limits of quantitation were defined as the lowest calibration standard used in the calibration curve that had a S/N >6 and qualitative ion ratios (figure 5), with most compounds ~2-5x greater than their corresponding limits of detection (table 4).

The inter- and intra-day accuracy (% accuracy) and precision (%CV) of the method was determined by analyzing quality control samples spiked at low, medium, and high (n=6/level) as shown in Table 5. The accuracy was calculated from the mean of each concentration level and the expected concentration. Overall, accuracies ranged from 61 to 109%, 82 to 125% and 75 to 127% for the low, mid, and high QC levels, respectively. The intra-day accuracies were comparable. Precision was determined by calculating the coefficient of variation for each

concentration level. Overall, the inter-day precision ranged from 2 to 45%, 1 to 26%, and 2 to 30% for the low, mid, and high QC levels, respectively. The intra-day precision across all 3 days was comparable.

The stability of each compound was assessed by spiking negative control urine at mid quality control concentrations followed by storage at room temperature (24 hr.), 2-8°C (24 hr.), -20°C (7 and 14 days), or -20°C (7 and 14 days) with one freeze-thaw cycle to mimic sample handling conditions commonly employed by anti-doping laboratories as shown in Table 6.

5. Discussion

Detection of prohibited substances such as doping related peptides and quaternary ammonium drugs requires sensitive and selective analytical methods that can be replicated for routine use within the laboratory. Previously the analysis of bioactive peptides and QADs was accomplished in urine or serum/plasma using various extraction approaches including liquid-liquid extraction and solid phase extraction using different sorbents [14, 15, 18-21, 31, 36]. QADs and bioactive peptide drug classes have been evaluated individually, but a single drug screening method would be useful in regulating the use of prohibited substances. The instrumentation has also varied from LC-MS analysis using a triple quadrupole or HRMS using either Time of Flight or Orbitrap mass spectrometers [12, 15, 18, 37]. Overall, these methods did not achieve sufficient sensitivities or utilized equipment that is too costly for routine screening. Analysis of bioactive peptides, QADs and related compounds using UHPLC-TSQ-MS/MS offers high sensitivity, selectivity, and high throughput. The methodology was developed as a qualitative screening tool for suspect positive samples. While the method was developed as a quantitative method, its ultimate use will be for initial qualitative screening to trigger further confirmatory analysis.

During method development, we initially used a Thermo LTQ linear ion trap mass spectrometer (Thermo Scientific, Waltham, MA, USA) in positive electrospray ionization mode (ESI). This instrument utilizes full scan ms^2 capabilities of the selected precursor ion's fragment via CID. The masses of the parent ions were determined by direct infusion of neat standards at 10ng/mL at a flow of 5 µL/min. A collision-induced dissociation (CID) energy ramp was applied during the infusion to find optimized CID energies for product ions. The peptide compounds co-eluted using the initial chromatography conditions; therefore, we applied several different mobile phase gradient compositions. In spite of the varied gradients, the compounds continued to co-elute which made analysis challenging given the limitations on scan speed of the instrument.

After varying the gradient, a Phenomenex Kinetex™ EVO C18 column for polar compounds was used in an attempt to increase the separation of the compounds. While the column chemistry proved to be better for some compounds, the majority of the peptides continued to co-elute. As the Kinetex column has a working pH range of 1-12, we attempted to utilize basic mobile phase conditions to achieve a better resolution between the compounds. At this pH range, we used negative electrospray ionization and found the corresponding precursor ion and fragments for each compound. Ammonium hydroxide was used with acetonitrile and water at 2mM concentration. The results showed clear changes in chromatographic performance with many of the highly basic polar compounds eluting at much later retention times although it did not significantly improve the chromatographic separation of most of the peptides.

After these initial attempts on the LTQ instrument, the method was transitioned to the Altis triple quadrupole mass spectrometer due to its rapid scan speed and high sensitivity. The use of a triple quadrupole makes co-elution of compound less impactful due to its ability to scan between compounds with a dwell time in the ~10 millisecond timeframe. The use of SRM

allowed for targeted analysis of multiple product ions from a single precursor ion each with its own optimized collision energy. Direct infusion in positive electrospray ionization was used to find the precursor ion, one to five product ions, collision energy, and RF voltage for each compound. After each direct infusion, neat standards were run to determine their retention times and ionization efficiencies for each transition. As the system was able to handle higher backpressures, we used an Accucore™ Vanquish™ C18 UHPLC column with a particle diameter of 1.5 µm compared to the 5 µm of the Kinetex™ EVO C18. The smaller particle size decreased the eddy diffusion inside the column, which allowed for a more symmetrical elution and greater separation between analytes.

Following optimization of the mass spectrometer and chromatographic systems, the targeted compounds were then spiked into urine from 0.05 ng/mL to 10 ng/mL and extracted using the WCX cartridge to examine how they behaved in actual matrix samples that were extracted. We compared the peak area and response for each compound's SRM scan from spiked urine, neat standards and blank matrix to determine the most selective ions to further refine the list of monitored SRM transitions. The earliest eluting compounds were highly polar compounds edrophonium and pirbuterol which had wider peak shapes as compared to the majority of the other compounds. Interestingly, the peak shape for dermorphin had a wider base as compared to other similar compounds and was investigated using different preparations of the reference material obtaining similar results (data not shown) with the dermorphin metabolites not having this effect. The majority of compounds eluted between 2 and 5 minutes. Several compounds (AOD-9604, Lecirelin, Leuprorelin – 605 *m/z*; GHRP1, Deltorphin – 478 *m/z*, GHRP6, GHRP6 free acid – 437 *m/z*) had similar precursor ion masses but were able to be qualitatively identified using a combination of retention time and/or the monitored product ions. For example, lecirelin

and leuprorelin were only separated by ~0.1 min and shared several of the same product ions but they could be differentiated by retention time as shown in Figure 6 & 7. While both compounds were able to be chromatographically separated they still eluted very close to each other and one should use caution when attempting to identify either compound in tested samples and ensure the methodology used can sufficiently separate the compounds.

As shown in Table 3, the limits of detection were below 0.1 ng/mL for all non-peptide compounds except methotrexate and bretylium which was not surprising given the fixed positive charge for most of the compounds allowing for good sensitivity via positive mode electrospray ionization. The limits of detection for the peptides were more variable although most were below 0.5 ng/mL and some such as alarelin were at 50 pg/mL (Table 3). The detection limits for deltorphin I, deltorphin II, and LHRH (1-3) free acid were above 0.5 ng/mL likely due to poor recovery (<10%) as a result of the neutral or acidic nature of the compounds at physiological pH which replicates similar results observed by Kwok et al [18]. The following compounds exceeded 20% CV for their lowest QC concentration AOD-9604, Deltorphin, Deltorphin I, Deltorphin II, GHRP-6 free acid, Hexarelin, Hexarelin free acid, Histrelin, Ipamorelin, Ipamorelin (1-4) free acid, Lecirelin, Leuprorelin, LHRH (1-3) free acid, MGF, and Methotrexate. This is likely due to their detection limits being at the low quality control level.

While some limits of detection and quantitation were at 5x different concentrations this may be a function of the concentration range utilized to asses LODs rather than the methodology and further refinement using additional concentrations between 0.01 and 0.5 ng/mL may be helpful in future studies. The recoveries for QADs ranged from 67-81%, and 2-77% for the other monitored non-peptide drugs with methotrexate having the worst recovery. Recovery for the peptides was good with most compounds having between 40-80% recoveries although

several compounds such as deltorphin and LHRH (1-3) that were not as basic had lower recoveries similar to results observed by others [18]. Ion suppression most impacted GHRP1 (~25%) while ion enhancement was most observed for ipramorelin (1-4) free acid at 165%.

The accuracy and precision for some peptides was larger than others likely due to their limits of detection or quantitation being at or near the lowest quality control level with AOD-9604, deltorphin, deltorphin I, deltorphin II, GHRP-6 free acid, hexarelin, hexarelin free acid, histrelin, ipamorelin, ipamorelin (1-4) free acid, lecirelin, leuprorelin, LHRH (1-3) free acid, MGF, and methotrexate being the most impacted. Unsurprisingly, the data for the non-peptide drugs except methotrexate was much tighter than the most of the peptides likely due to the higher signal observed on the LC-MS system. While the results shown in this report show quantitative results, none of the monitored compounds require quantitative analysis in urine per current ARCI or IFHA regulations and thus would be reported as qualitative detections (if applicable) [6, 7]. Ipratropium and N-butylscopolammonium are currently controlled by many regulatory authorities with international screening limits of 0.25 and 25 ng/mL, respectively. Thus our initial testing procedure would allow for these compounds to be easily monitored.

The recoveries of some compounds were greatly affected by the evaporation process in the absence of the matrix constituents found in an extracted urine sample. As mentioned previously, some peptides have shown non-specific adsorptive binding to glassware leading to losses during sample handling, evaporation steps and storage in autosampler vials [20, 22, 27]. Unfortunately, the non-specific binding of some peptides leads to losses which initially skewed the recovery and matrix effect experiments. The observed peak areas for some peptides, such as GHRP-1, were spiked in urine and extracted were 2 to 3 times higher than neat standards (Figure 8). However, neat standards should be significantly higher due to not going through the

extraction process. This was most pronounced for the larger molecular weight compounds with MGF hardly being observable following evaporation in the absence of matrix. Conversely the QADs, which are not affected by non-specific binding for recovery and matrix effect experiments agreed with our initial hypothesis. After observing this, the neat standards used for the recovery and matrix effects experiments were diluted without any evaporation steps prior to instrumental analysis (figure 9). Others have observed similar effects and steps should be taken to avoid these issues such as use of a carrier protein or molecule, coating of glassware prior to introduction of solvents containing the peptide drugs, low bind plastic ware, removal of evaporation steps or removal of solid phase extraction prior to instrumental analysis [27]. The compounds with low recoveries can be a concern. The compounds with low recoveries should be moved to an acidic extraction method. The compounds viable for this method can be seen in table 7.

Sample pre-treatment prior to extraction can impact recoveries of compounds following extraction and thus we utilized ammonium formate to adjust the urinary pH to be above the pKa of the carboxylic acid functional group of the sorbent found on the WCX cartridge. The wash steps are equally important when it comes to ion suppression due to removing unwanted urea, salts, organic, and inorganic compounds. The concentrations of analytes were slightly lower when using 3 mL of methanol due to being washed away. Using too little methanol would not wash the sample matrix enough, resulting in the baseline noise being significantly higher. Therefore, 1.5 mL of methanol was used to wash the samples which created a good balance of sample cleaning to response. After the validation was completed, a large batch of post-race urine samples was extracted using the WCX cartridge and methodology described in Section 3.4 with low IS response observed in ~5% of urine samples which caused some concern. This was

investigated and the data suggested either large ion suppression or poor recovery of compounds. After an investigation, we determined that the use of 3 mL of 0.1 M ammonium formate (pH 7) rather than 3 mL of water in the wash step following sample addition resulted in an increase of the internal standard response (data not shown) similar to that of the pooled negative control urine suggesting that the use of water did not fully control the pH of the sorbent leading to partial elution during the wash steps. This was further investigated by spiking one of the impacted samples at our QC high concentration and extracting using our procedures with either water or ammonium formate as the first wash solution followed by LC-MS/MS analysis which showed comparable responses for most compounds as compared to our pooled negative control urine used for the validation as observed in Figure 10.

The physiochemical properties, acid-base interactions, molecular size, solubility, non-specific losses due to adsorption to glassware, and potential low levels following administration make detection of the targeted classes of molecules in a single method challenging. The QADs, β_2-agonists, and most of the monitored peptides are positively charged at physiological pH suggesting that use of a cation exchange sorbent may be useful in obtaining a clean extract with good recoveries of these compounds. Complicating the analysis is that most of the peptides are zwitterions. Indeed, most recent studies utilizing solid-phase extraction have found success with weak cation exchange or mixed mode cation exchange sorbents [16, 20, 21, 23]. During our initial method development, we compared the Evolute Express WCX cartridge to the Isolute CBA cartridge (Biotage) to determine which cartridge would provide optimal results. The results, from our preliminary method development data suggested that the CBA cartridge provided comparable results to the WCX for the QADs but the WCX was superior for the peptides.

Urine is the preferred matrix for the analysis of most of the compounds monitored on our method due the larger sample volumes and generally higher concentrations of compounds found in urine. However, the properties of urine such as specific gravity, pH and concentration of proteins, especially after intense exercise can vary significantly which can make analysis challenging [24, 38, 39]. Sample pre-treatment prior to extraction can impact recoveries of compounds following extraction and thus we utilized an ammonium formate buffer to adjust the urinary pH to be above the pKa of the carboxylic acid functional group of the sorbent found on the WCX cartridge. The presence of high amounts of protein in equine urine following intensive exercise is a common occurrence in an equine anti-doping laboratory and can lead to challenges with sample extraction, accordingly we utilized a sonication step to help reduce viscosity of the sample prior to loading the cartridge while others have found success with protein precipitation with either an organic solvent or strong acid [40, 41].

Selection of an appropriate internal standard can greatly improve methodological performance with stable isotope labelled internal standards being preferable when using mass spectrometry. Stable isotopically labeled [^{13}C,^{15}N]-GHRP-2 (1-3) was used as an internal standard for the peptide-based drugs. Benzyldimethylphenylammonium chloride and glycopyrrolate-*d3* were used as the internal standard for the QADs. Both internal standards mimicked the chemical properties of their respective compounds. Testing of the internal standards was conducted through standard addition. Since the internal standards are not identical to compounds on the drug screen, we evaluated how they behaved in matrix by spiking at three concentrations within the linear range. The internal standards were paired with compounds of similar intensities and chemistries.

The compound's stability is an important factor when developing an analytical approach. The half-lives as previously mentioned are a few minutes to a couple hours at most [12, 13, 42]. This is further complicated with storage which can degrade the peptides significantly. We found the best storage for these compounds is -20 °C. A general decrease in analyte response can be seen due to compound degradation, but most compounds are relatively stable. The stability samples underwent the same extraction and preparation procedures as described above and were run in parallel with a calibration curve. At room temperature the majority of compounds were within ~25% of the expected concentration though AOD-9604, Deltorphin, GHRP-1, MGF, Tiotropium, and Triptorelin free acid were more impacted. Storage with refrigeration (2-8°C) showed improved stability with only MGF, AOD-9604, and Triptorelin free acid with >25% deviation from expected concentration. Storage at -20°C for 7 and 14 days along with a freeze/thaw cycle for each of those time points showed minimal degradation suggesting that dramatic losses in urinary concentrations following storage at conditions commonly employed by testing laboratories is not of concern.

6. Conclusion

A drug screening method to detect and quantitate 51 basic drugs was developed and validated using ultra high performance liquid chromatography – triple stage quadrupole mass spectrometry (UHPLC-TSQ MS/MS). Solid phase extraction using the Evolute WCX cartridge allowed for selective sample clean-up and pre-treatment of strongly basic substances and peptides in equine urine with a combination of weak cation exchange and reverse phase interactions. The method was able to achieve low limits of detection and quantitation suitable for routine analysis. Compound stability was also assessed with results indicating that storage of

collected urine at sub-ambient conditions will avoid large losses in analyte concentrations. The method has the potential to expand the coverage to additional compounds.

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9. Figure Legend

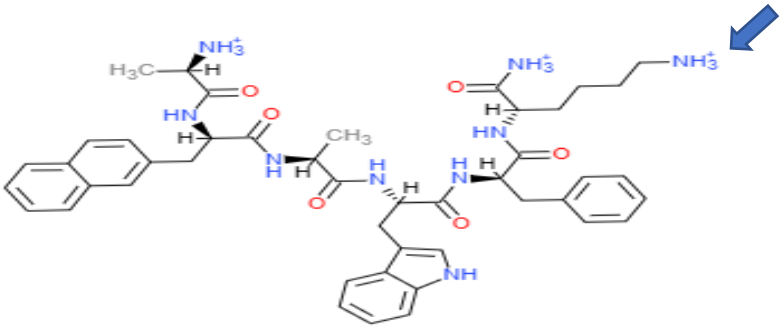


Figure 1. The chemical structure of GHRP-2 is shown. The primary amine present on lysine's side chain is positively charged at physiological pH.

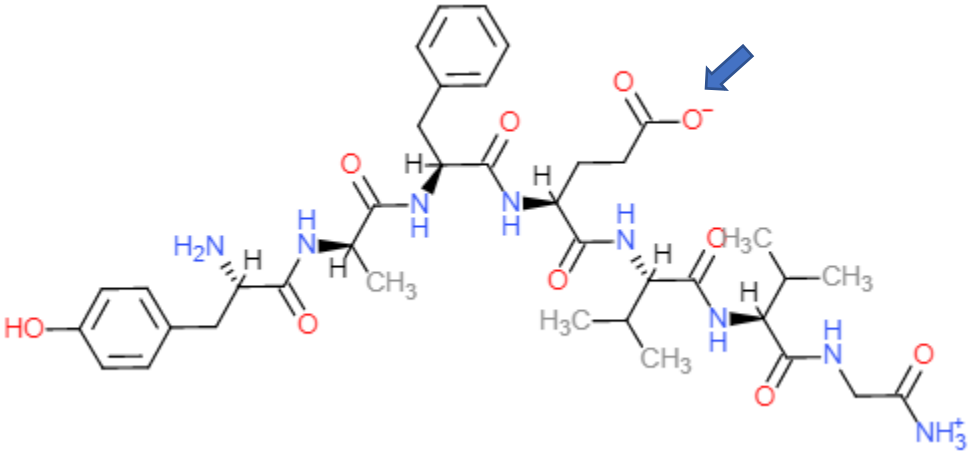


Figure 2. The chemical structure of deltorphin II is shown. At physiological pH, glutamate has a pKa side chain of 4.25 and is negatively charged while glycine's n-terminal end has a pKa of 9.60 and is positively charged.

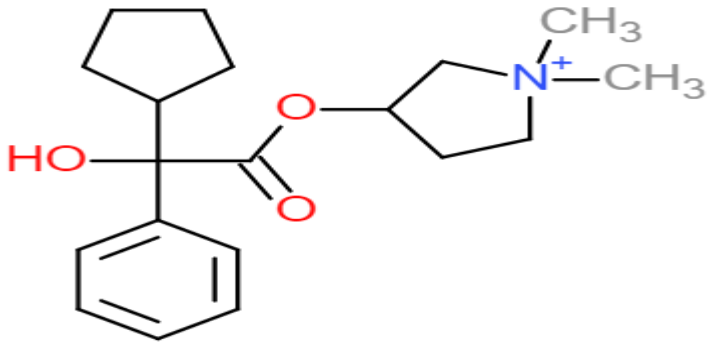
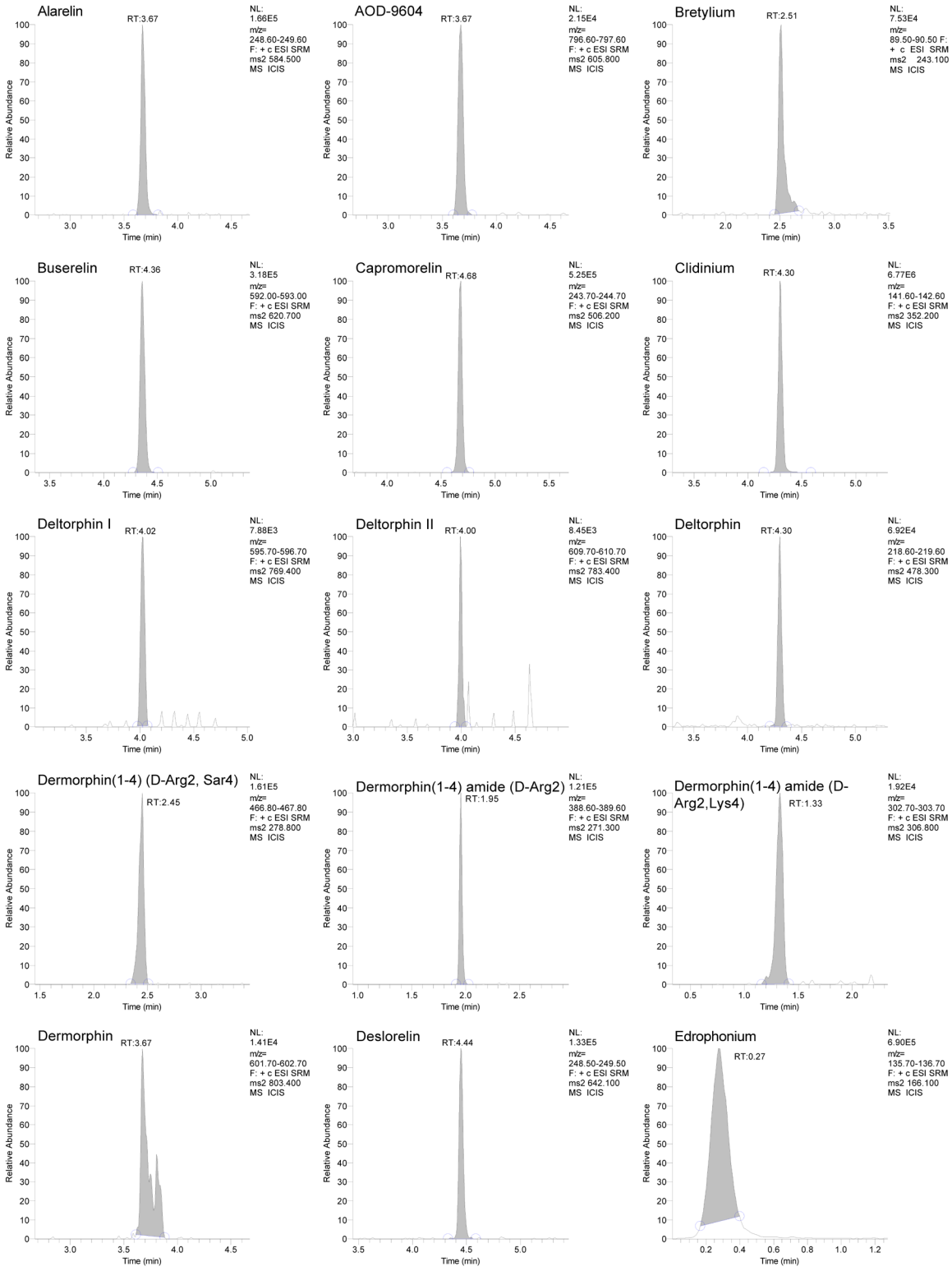


Figure 3. Glycopyrrolate structure is shown with a permanent positive charge.

29


Alarelin
RT:3.67
NL: 1.66E5 m/z= 248.60-249.60 F: + c ESI SRM ms2 584.500 MS ICIS
AOD-9604
RT:3.67
NL: 2.15E4 m/z= 796.60-797.60 F: + c ESI SRM ms2 605.800 MS ICIS
Bretylium
RT:2.51
NL: 7.53E4 m/z= 89.50-90.50 F: + c ESI SRM ms2 243.100 MS ICIS
Buserelin
RT:4.36
NL: 3.18E5 m/z= 592.00-593.00 F: + c ESI SRM ms2 620.700 MS ICIS
Capromorelin
RT:4.68
NL: 5.25E5 m/z= 243.70-244.70 F: + c ESI SRM ms2 506.200 MS ICIS
Clidinium
RT:4.30
NL: 6.77E6 m/z= 141.60-142.60 F: + c ESI SRM ms2 352.200 MS ICIS
Deltorphin I
RT:4.02
NL: 7.88E3 m/z= 595.70-596.70 F: + c ESI SRM ms2 769.400 MS ICIS
Deltorphin II
RT:4.00
NL: 8.45E3 m/z= 609.70-610.70 F: + c ESI SRM ms2 783.400 MS ICIS
Deltorphin
RT:4.30
NL: 6.92E4 m/z= 218.60-219.60 F: + c ESI SRM ms2 478.300 MS ICIS
Dermorphin(1-4) (D-Arg2, Sar4)
RT:2.45
NL: 1.61E5 m/z= 466.80-467.80 F: + c ESI SRM ms2 278.800 MS ICIS
Dermorphin(1-4) amide (D-Arg2)
RT:1.95
NL: 1.21E5 m/z= 388.60-389.60 F: + c ESI SRM ms2 271.300 MS ICIS
Dermorphin(1-4) amide (D-Arg2,Lys4)
RT:1.33
NL: 1.92E4 m/z= 302.70-303.70 F: + c ESI SRM ms2 306.800 MS ICIS
Dermorphin
RT:3.67
NL: 1.41E4 m/z= 601.70-602.70 F: + c ESI SRM ms2 803.400 MS ICIS
Deslorelin
RT:4.44
NL: 1.33E5 m/z= 248.50-249.50 F: + c ESI SRM ms2 642.100 MS ICIS
Edrophonium
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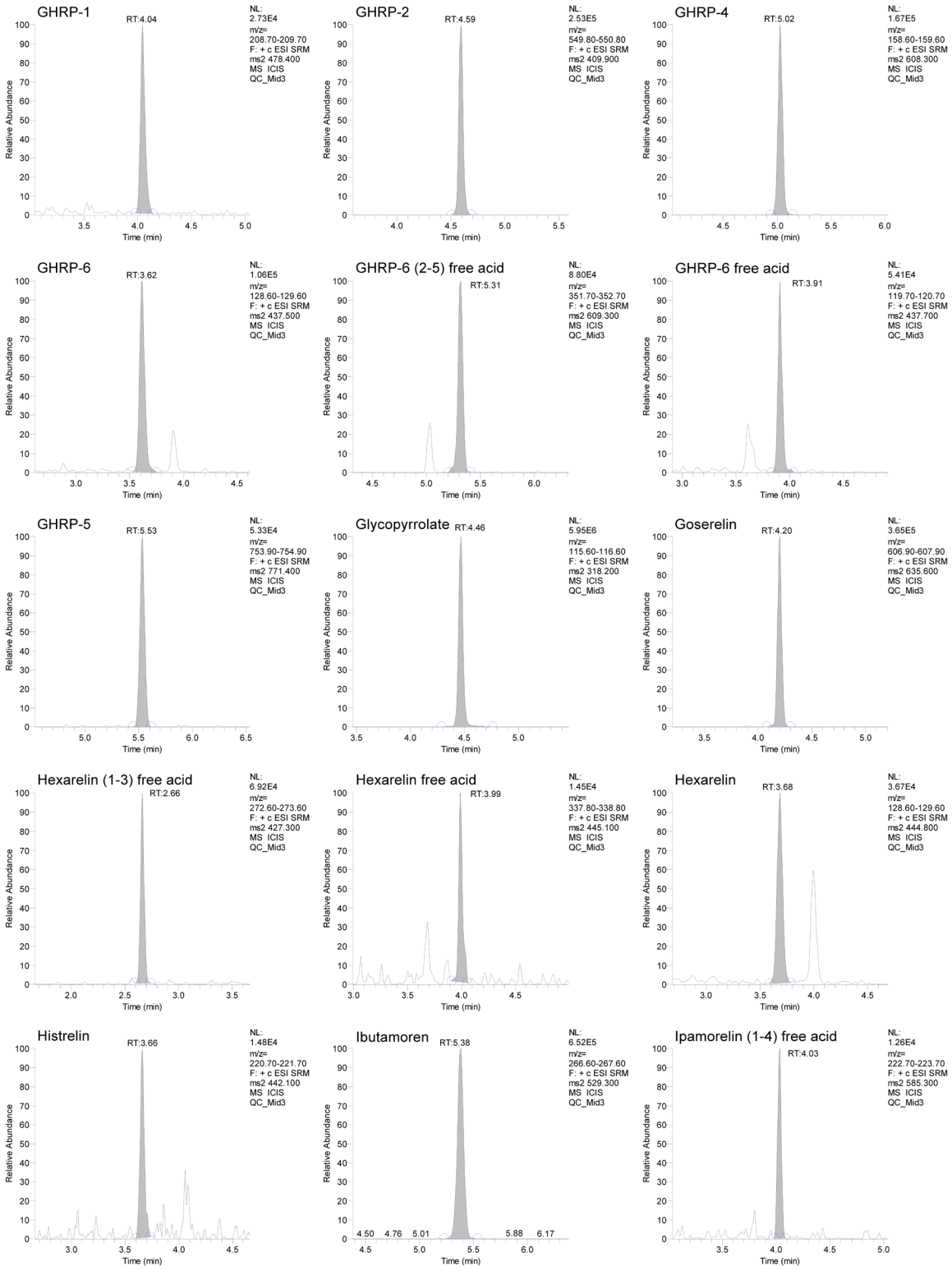
Relative Abundance
Time (min)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GHRP-2
RT:4.59
NL: 2.53E5 m/z= 549.80-550.80 F: + c ESI SRM ms2 409.900 MS ICIS QC_Mid3
GHRP-4
RT:5.02
NL: 1.67E5 m/z= 158.60-159.60 F: + c ESI SRM ms2 608.300 MS ICIS QC_Mid3
GHRP-6
RT:3.62
NL: 1.06E5 m/z= 128.60-129.60 F: + c ESI SRM ms2 437.500 MS ICIS QC_Mid3
GHRP-6 (2-5) free acid
RT:5.31
NL: 8.80E4 m/z= 351.70-352.70 F: + c ESI SRM ms2 609.300 MS ICIS QC_Mid3
GHRP-6 free acid
RT:3.91
NL: 5.41E4 m/z= 119.70-120.70 F: + c ESI SRM ms2 437.700 MS ICIS QC_Mid3
GHRP-5
RT:5.53
NL: 5.33E4 m/z= 753.90-754.90 F: + c ESI SRM ms2 771.400 MS ICIS QC_Mid3
Glycopyrrolate
RT:4.46
NL: 5.95E6 m/z= 115.60-116.60 F: + c ESI SRM ms2 318.200 MS ICIS QC_Mid3
Goserelin
RT:4.20
NL: 3.65E5 m/z= 606.90-607.90 F: + c ESI SRM ms2 635.600 MS ICIS QC_Mid3
Hexarelin (1-3) free acid
RT:2.66
NL: 6.92E4 m/z= 272.60-273.60 F: + c ESI SRM ms2 427.300 MS ICIS QC_Mid3
Hexarelin free acid
RT:3.99
NL: 1.45E4 m/z= 337.80-338.80 F: + c ESI SRM ms2 445.100 MS ICIS QC_Mid3
Hexarelin
RT:3.68
NL: 3.67E4 m/z= 128.60-129.60 F: + c ESI SRM ms2 444.800 MS ICIS QC_Mid3
Histrelin
RT:3.66
NL: 1.48E4 m/z= 220.70-221.70 F: + c ESI SRM ms2 442.100 MS ICIS QC_Mid3
Ibutamoren
RT:5.38
4.50 4.76 5.01 5.88 6.17
NL: 6.52E5 m/z= 266.60-267.60 F: + c ESI SRM ms2 529.300 MS ICIS QC_Mid3
Ipamorelin (1-4) free acid
RT:4.03
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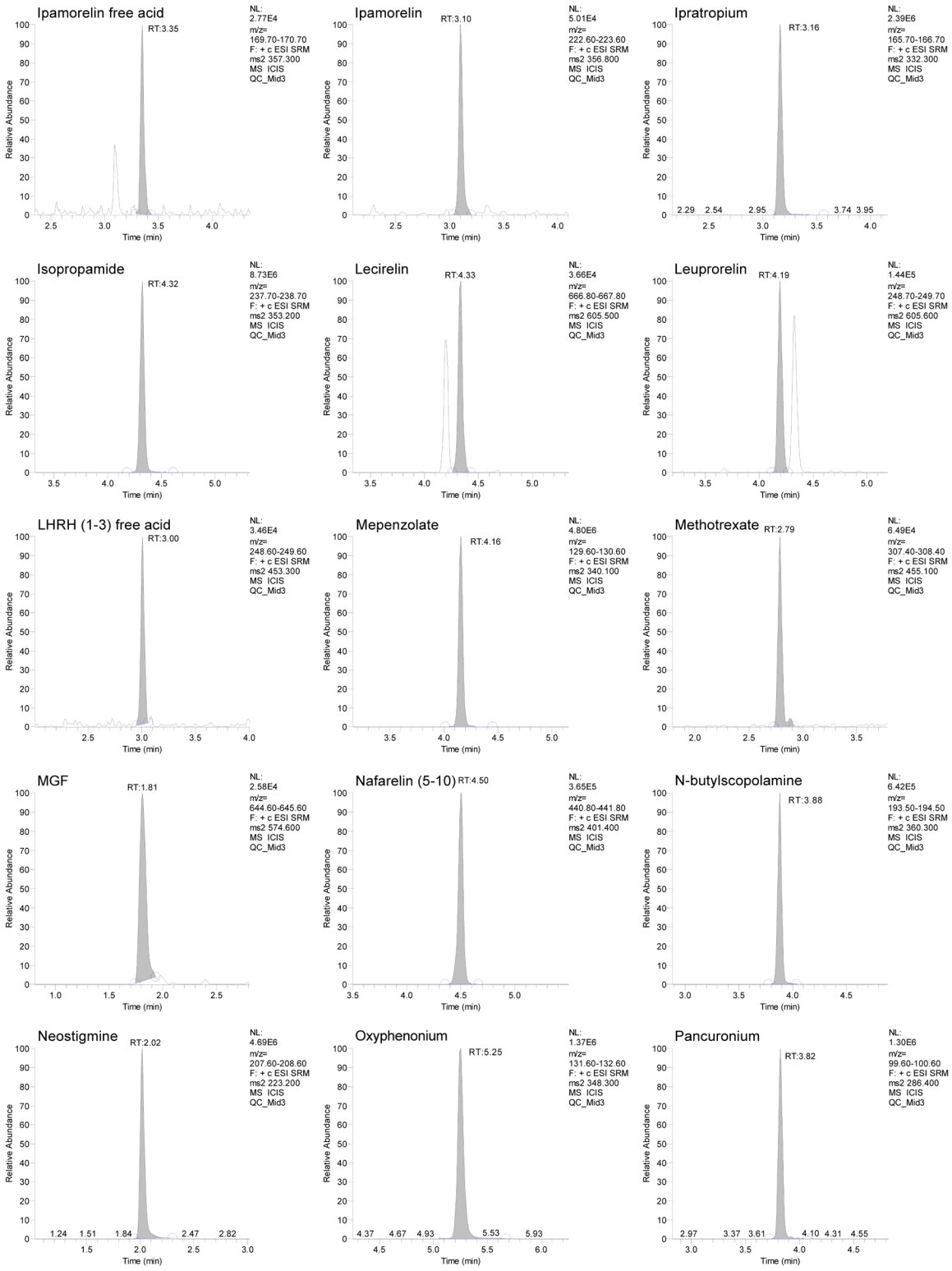
Relative Abundance
Time (min)


Ipamorelin free acid
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RT:3.35
Ipamorelin
NL: 5.01E4 m/z= 222.60-223.60 F: + c ESI SRM ms2 356.800 MS ICIS QC_Mid3
RT:3.10
Ipratropium
NL: 2.39E6 m/z= 165.70-166.70 F: + c ESI SRM ms2 332.300 MS ICIS QC_Mid3
RT:3.16
Isopropamide
NL: 8.73E6 m/z= 237.70-238.70 F: + c ESI SRM ms2 353.200 MS ICIS QC_Mid3
RT:4.32
Lecirelin
NL: 3.66E4 m/z= 666.80-667.80 F: + c ESI SRM ms2 605.500 MS ICIS QC_Mid3
RT:4.33
Leuprorelin
NL: 1.44E5 m/z= 248.70-249.70 F: + c ESI SRM ms2 605.600 MS ICIS QC_Mid3
RT:4.19
LHRH (1-3) free acid
NL: 3.46E4 m/z= 248.60-249.60 F: + c ESI SRM ms2 453.300 MS ICIS QC_Mid3
RT:3.00
Mepenzolate
NL: 4.80E6 m/z= 129.60-130.60 F: + c ESI SRM ms2 340.100 MS ICIS QC_Mid3
RT:4.16
Methotrexate
NL: 6.49E4 m/z= 307.40-308.40 F: + c ESI SRM ms2 455.100 MS ICIS QC_Mid3
RT:2.79
MGF
NL: 2.58E4 m/z= 644.60-645.60 F: + c ESI SRM ms2 574.600 MS ICIS QC_Mid3
RT:1.81
Nafarelin (5-10)
NL: 3.65E5 m/z= 440.80-441.80 F: + c ESI SRM ms2 401.400 MS ICIS QC_Mid3
RT:4.50
N-butylscopolamine
NL: 6.42E5 m/z= 193.50-194.50 F: + c ESI SRM ms2 360.300 MS ICIS QC_Mid3
RT:3.88
Neostigmine
NL: 4.69E6 m/z= 207.60-208.60 F: + c ESI SRM ms2 223.200 MS ICIS QC_Mid3
RT:2.02
1.24 1.51 1.84 2.47 2.82
Oxyphenonium
NL: 1.37E6 m/z= 131.60-132.60 F: + c ESI SRM ms2 348.300 MS ICIS QC_Mid3
RT:5.25
4.37 4.67 4.93 5.53 5.93
Pancuronium
NL: 1.30E6 m/z= 99.60-100.60 F: + c ESI SRM ms2 286.400 MS ICIS QC_Mid3
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2.97 3.37 3.61 4.10 4.31 4.55
Relative Abundance
Time (min)

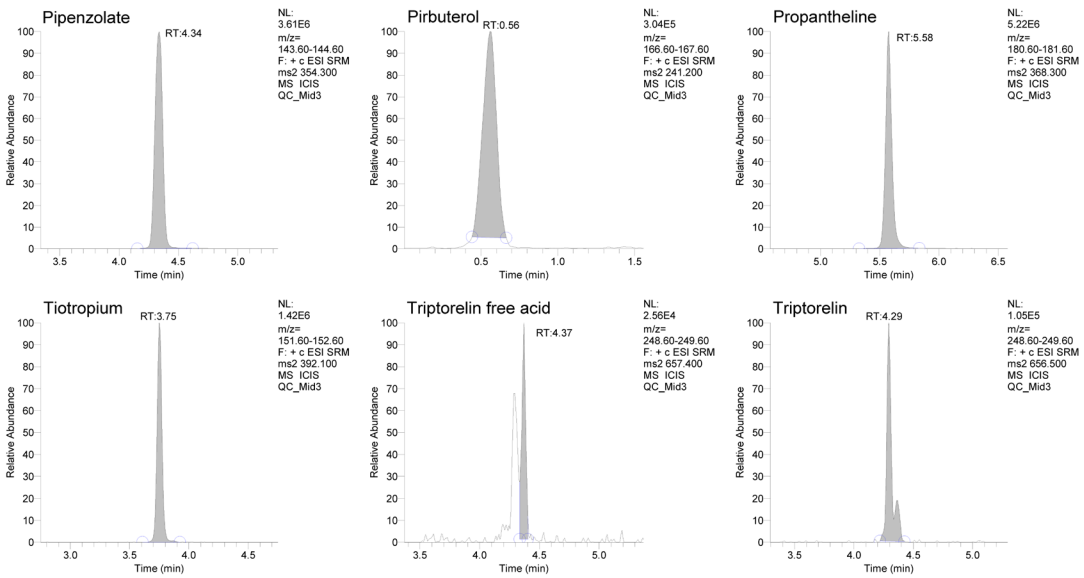

Figure 4. Extracted ion chromatogram of targeted compounds at mid quality control level. The free acid structure for GHRP-6, Hexarelin, Ipamorelin, and Triptorelin are detected in both forms.

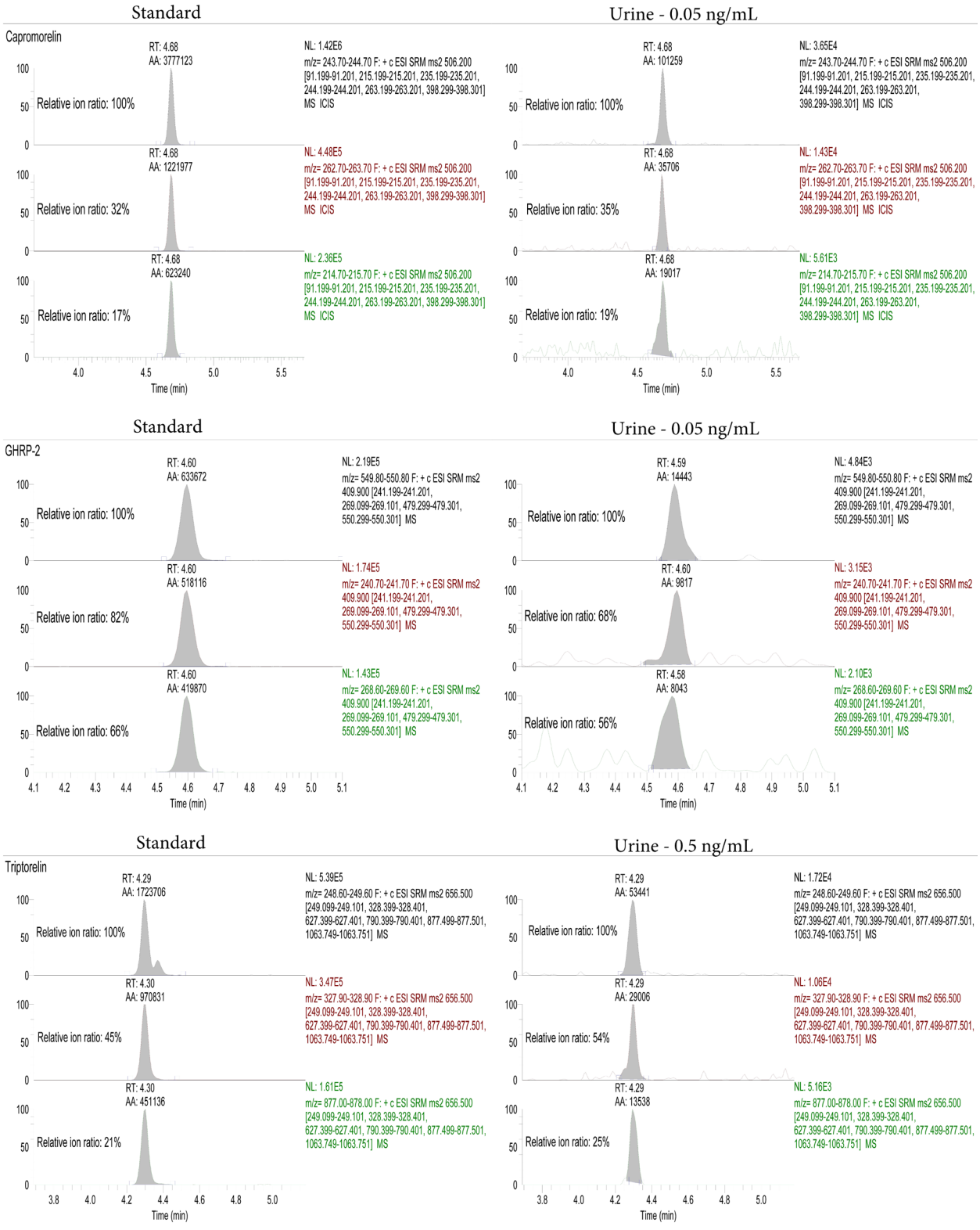

Figure 5. Extracted ion chromatogram shows the qualitative ion ratios at limit of detection for Capromorelin, GHRP-2, and Triptorelin.

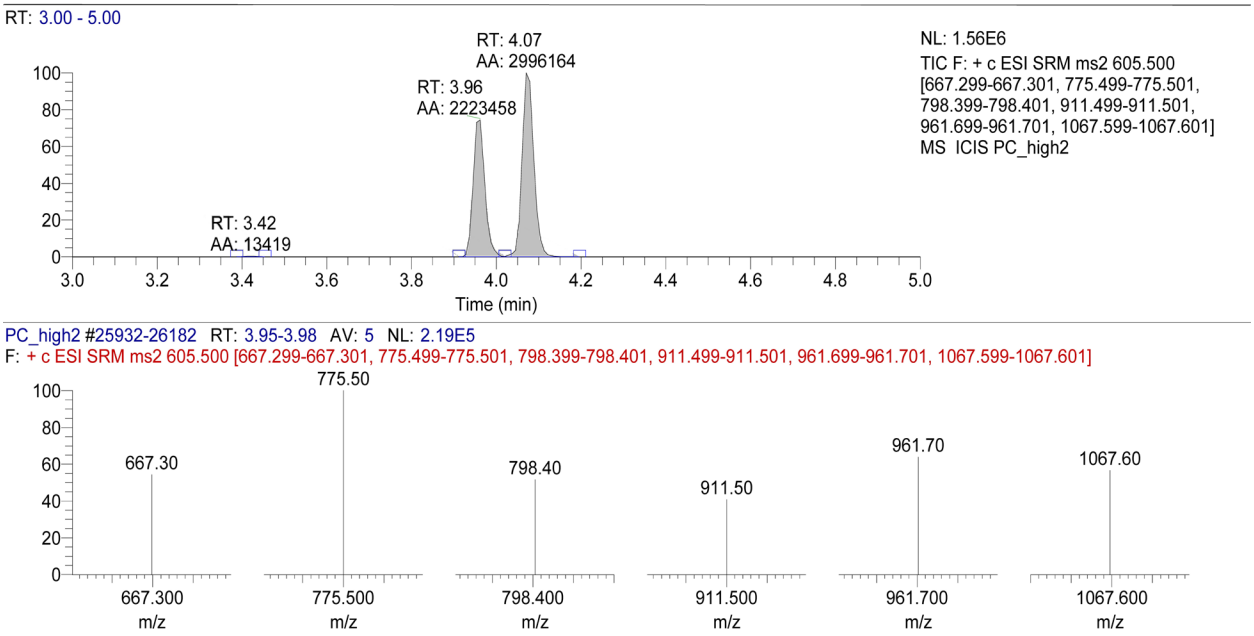

Figure 6. Leuprorelin extraction ion chromatogram retention time 3.96.

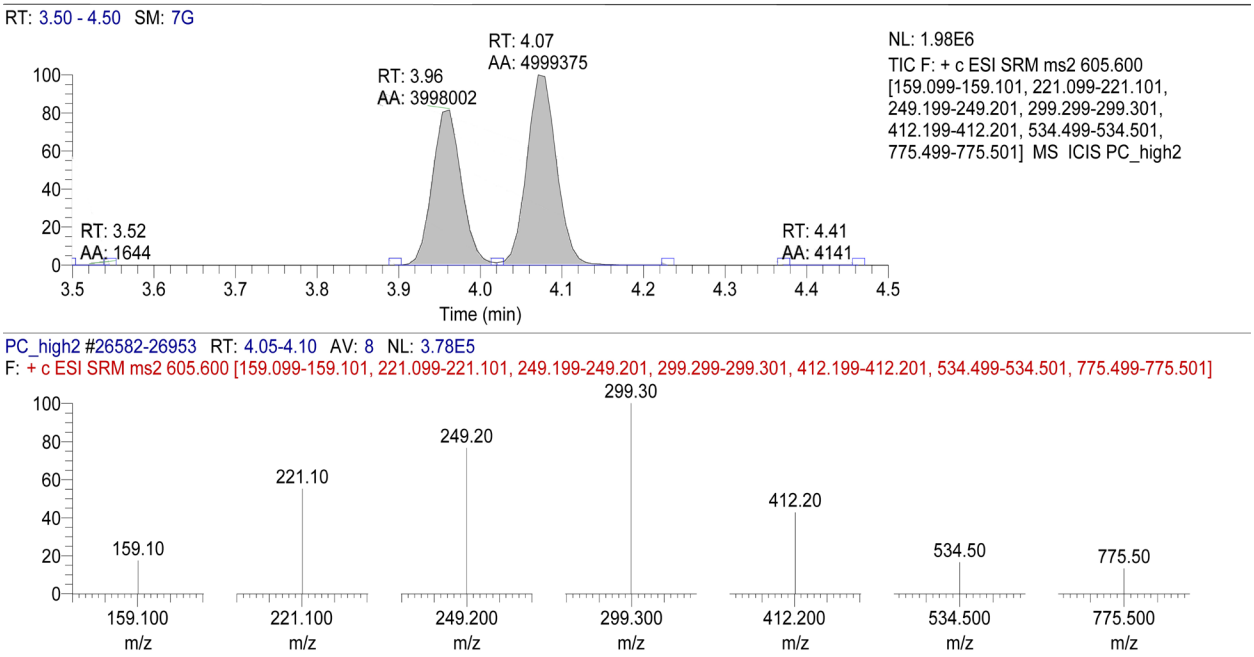

Figure 7. Lecirelin extracted ion chromatogram retention time 4.07.

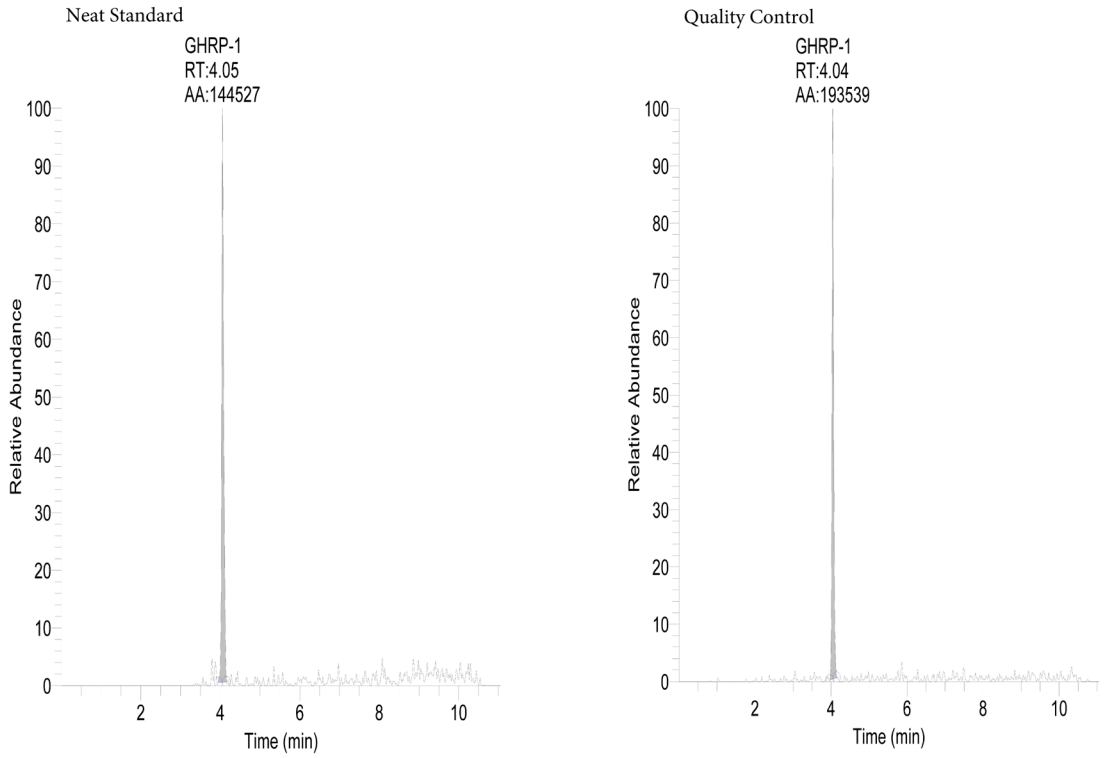

Figure 8. GHRP-1 total ion chromatogram neat standard at 7.5 ng/mL compared to QC at 7.5 ng/mL. The neat standard should have a higher peak area due to not going through the extraction procedure. The losses are a result of non-specific losses.

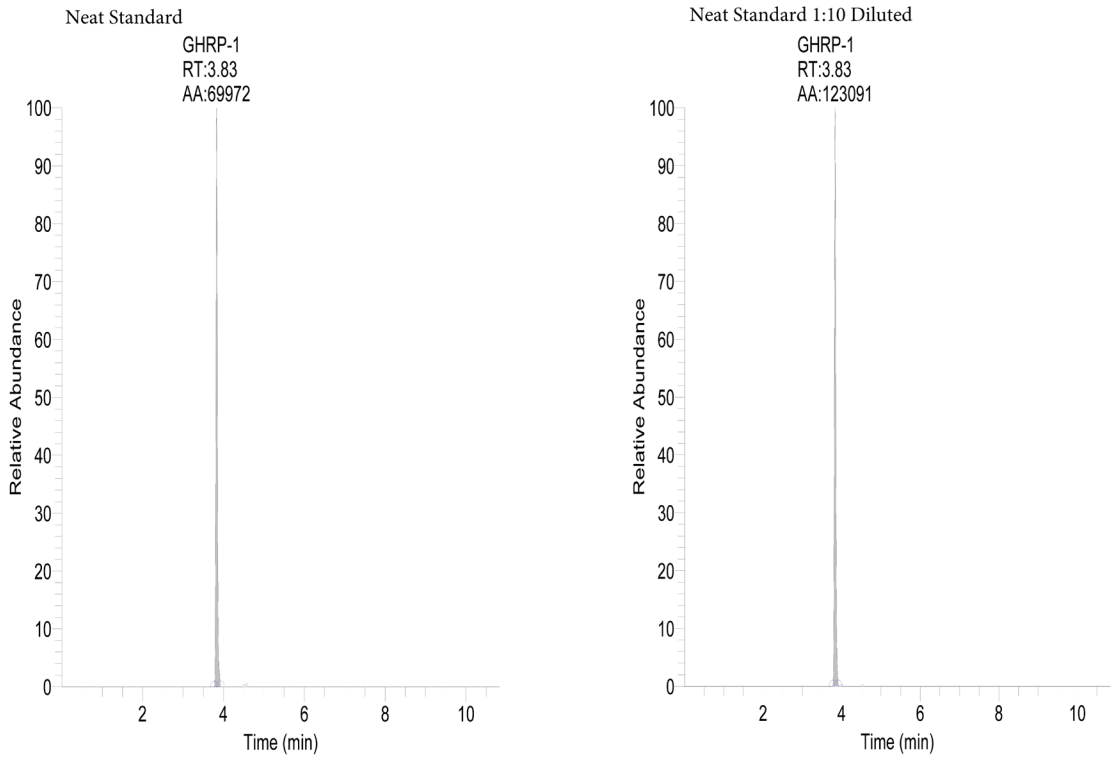

Figure 9. GHRP-1 neat standard at 7.5 ng/mL compared to the neat standard that was spiked into matrix also at 7.5 ng/mL. We see double the concentration when spiking into matrix rather than spiking into an autosampler and drying down.

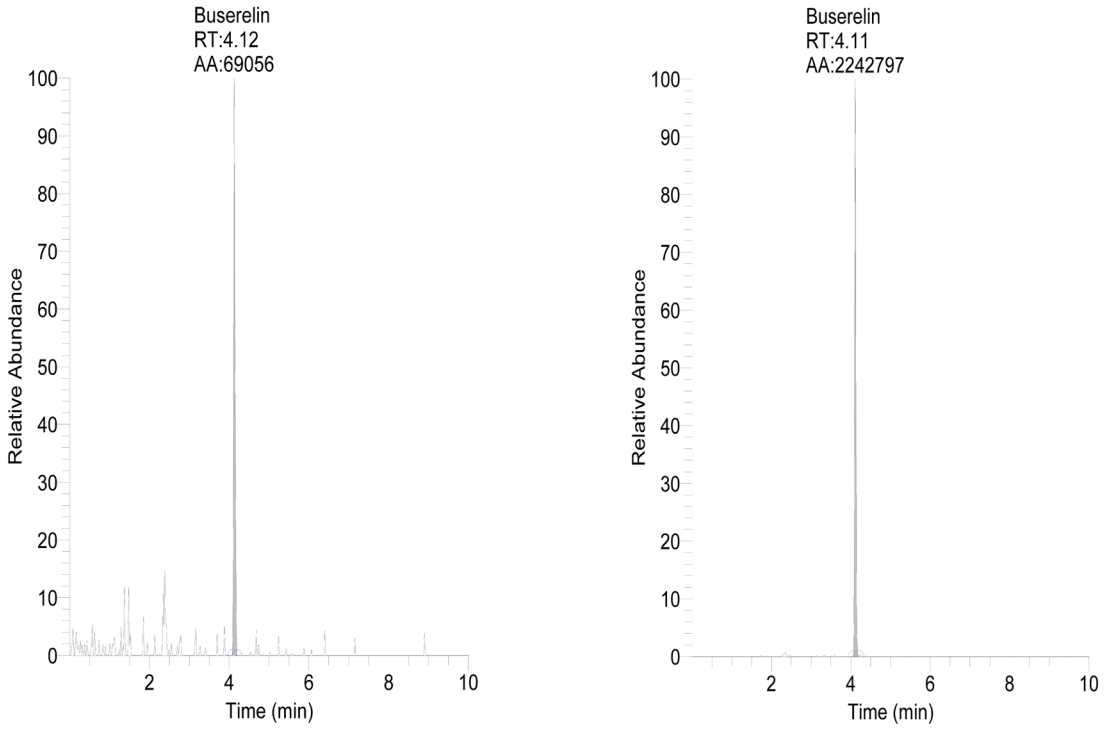

Figure 10. Shows the total ion chromatogram of buserelin in the impacted samples with ion suppression washed with water (left) versus when washed with 0.1 ammonium formate pH 7.0 (right).

Peptide	Amino Acid Sequence	Molecular Weight (g/moL)	Precursor ion charge state (*z*)
Alarelin	H-Pyr-His-Trp-Ser-Tyr-D-Ala-Leu-Arg-Pro-NHEt	1167.3	2
AOD-9604	H-Tyr-Leu-Arg-Ile-Val-Gln-Cys (1)-Arg-Ser-Val-Glu-Gly-Ser-Cys (1)-Gly-Phe-OH	1815.1	3
Buserelin	H-Pyr-His-Trp-Ser-Tyr-D-Ser(tBu)-Leu-Arg-Pro-NHEt	1239.4	2
Deltorphin	H-Tyr-D-Met-Phe-His-Leu-Met-Asp-NH2	955.17	2
Deltorphin I	H-Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH2	768.8	1
Deltorphin II	H-Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH2	782.9	1
Dermorphin	H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH2	802.9	1
Dermorphin (1-4) (D-Arg2, Sar4)	H-Tyr-D-Arg-Phe-Sar-OH	555.6	2
Dermorphin (1-4) amide (D-Arg2, Lys4)	H-Tyr-D-Arg-Phe-Lys-NH2	611.7	2
Dermorphin (1-4) amide (D-Arg2)	H-Tyr-D-Arg-Phe-Gly-NH2	540.6	1
Deslorelin	H-Pyr-His-Trp-Ser-Tyr-D-Trp-Leu-Arg-Pro-NHEt	1282.4	2
GHRP-1	H-Ala-His-D-2-Nal-Ala-Trp-D-Phe-Lys-NH2	955.1	2
GHRP-2	H-D-Ala-D-2-Nal-Ala-Trp-D-Phe-Lys-NH_2	818	2
[$^{13}C^{15}N$]-GHRP-2 (1-3)	H-D-Ala-D-2-Nal-Ala-OH	361	1
GHRP-4	H-D-Trp-Ala-Trp-D-Phe-NH2	607.7	1
GHRP-5	H-Tyr-D-Trp-Ala-Trp-D-Phe-NH2	770.9	1
GHRP-6	H-His-D-Trp-Ala-Trp-D-Phe-Lys-NH2	873.0	2
GHRP-6 (2-5) free acid	H-D-Trp-Ala-Trp-D-Phe-OH	608.7	1
GHRP-6 free acid	H-His-D-Trp-Ala-Trp-D-Phe-Lys-OH	874.0	2
Goserelin	H-Pyr-His-Trp-Ser-Tyr-D-Ser(tBu)-Leu-Arg-Pro-NHNHCONH2	1269.4	2
Hexarelin	H-His-D-Trp(2-Me)-Ala-Trp-D-Phe-Lys-NH2	887.0	2
Hexarelin (1-3) free acid	H-His-D-Mrp-Ala-OH	426.0	1
Hexarelin free acid	H-His-D-Mrp-Ala-Trp-D-Phe-Lys-OH	888.0	2
Histrelin	H-Pyr-His-Trp-Ser-Tyr-D-His(1-Bn)-Leu-Arg-Pro-NHEt	1323.5	3
Ipamorelin	H-Aib-His-D-2-Nal-D-Phe-Lys-NH2	711.9	2
Ipamorelin (1-4) free acid	H-Aib-His-D-2-Nal-D-Phe-OH	585.0	1
Ipamorelin free acid	H-Aib-His-D-2-Nal-D-Phe-Lys-OH	712.8	2
Lecirelin	H-Pyr-His-Trp-Ser-Tyr-D-Tle-Leu-Arg-Pro-NHEt	1209.4	2
Leuprorelin	H-Pyr-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-NHEt	1209.4	2
LHRH (1-3) free acid	H-Pyr-His-Trp-OH	452.5	1
MGF	H-Tyr-Gln-Pro-Pro-Ser-Thr-Asn-Lys-Asn-Thr-Lys-Ser-Gln-Arg-Arg-Lys-Gly-Ser-Thr-Phe-Glu-Glu-Arg-Lys-OH	2868.1	5
Nafarelin (5-10) MET	H-Tyr-D-2-Nal-Leu-Arg-Pro-Gly-NH2	801.0	2
Triptorelin	H-Pyr-His-Trp-Ser-Tyr-D-Trp-Leu-Arg-Pro-Gly-NH_2	1311.4	2
Triptorelin free acid	H-Pyr-His-Trp-Ser-Tyr-D-Trp-Leu-Arg-Pro-Gly-OH	1312.4	2

Table 1. Amino acid sequence, molecular weight (g/mol), and precursor ion charge state (*z*) of targeted peptides.

Compound	Chemical formula	Molecular Weight (g/mol)	Precursor ion charge state (*z*)
Benzyldimethylphenylammonium	$C_{15}H_{18}N$	212.3	1
Bretylium Bromide	$C_{11}H_{17}Br_2N$	323.1	1
Clidinium Bromide	$C_{22}H_{26}BrNO_3$	432.3	1
Edrophonium Bromide	$C_{10}H_{16}BrNO$	246.1	1
Glycopyrrolate	$C_{19}H_{28}BrNO_3$	398.3	1
Glycopyrrolate-*d*	$C_{19}H_{28}BrNO_3$	401.3	1
Ipratropium Bromide	$C_{20}H_{30}BrNO_3$	412.4	1
Isopropamide Iodide	$C_{22}H_{33}IN_2O$	480.4	1
Mepenzolate Bromide	$C_{21}H_{26}BrNO_3$	420.3	1
N-butylscopolamine	$C_{21}H_{30}NO_4$	440.4	1
Neostigmine	$C_{12}H_{19}N_2O_2$	223.2	1
Oxyphenonium Bromide	$C_{21}H_{34}BrNO_3$	428.4	1
Pancuronium Bromide	$C_{35}H_{60}Br_2N_2O_4$	732.7	2
Pipenzolate	$C_{22}H_{28}BrNO_3$	434.4	1
Propantheline bromide	$C_{23}H_{30}NO_3Br$	448.4	1
Tiotropium	$C_{19}H_{22}NO_4S_2$	392.5	1
Capromorelin	$C_{28}H_{35}N_5O_4$	655.7	1
Ibutamoren	$C_{27}H_{36}N_4O_5S$	528.6	1
Methotrexate	$C_{20}H_{22}N_8O_5$	454.4	1
Pirbuterol	$C_{12}H_{20}N_2O_3$	240.3	1

Table 2. Chemical formula, molecular weight (g/mol), and precursor ion charge state (*z*) of non-peptide compounds of interest.

Peptide	Precursor ion (m/z)	Product ion (m/z) with Collision Energy (EV)	T_R (min)
Alarelin	584.5	176.1 (45), 221.1 (35), **249.1 (26)**	3.67
AOD-9604	605.8	770.1 (18), **797.1 (18),** 825.6 (18)	3.69
Buserelin	620.7	249.1 (30), **592.5 (16)**, 749.5 (25)	4.36
Deltorphin	478.3	**219.1 (21)**, 285.2 (25), 398.3 (21)	4.32
Deltorphin I	769.4	497.2 (31), 596.2 (28), **695.3 (28)**	4.03
Deltorphin II	783.4	**610.2 (26),** 709.3 (26)	4.01
Dermorphin	803.4	455.3 (35), 574.3 (28), **602.2 (24)**	3.69
Dermorphin (1-4) (D-Arg2, Sar4)	278.8	303.2 (16), 320.2 (13), **467.3 (10)**	2.41
Dermorphin (1-4) amide (D-Arg2, Lys4)	306.8	275.8 (14), **303.2 (18),** 320.2 (16)	1.31
Dermorphin (1-4) amide (D-Arg2)	271.3	248.9 (11), 303.3 (18), **389.1 (13)**	1.94
Deslorelin	642.1	**249.0 (30),** 299.3 (22), 598.5 (23)	4.48
GHRP-1	478.4	**209.2 (25)**, 810.8 (17), 406.3 (24)	4.07
GHRP-2	409.9	241.2 (16), 269.1 (13), **550.3 (13)**	4.59
[$^{13}C^{15}N$]-GHRP-2 (1-3)	362.2	**170.1 (26)**, 241.2 (16), 269.1 (11)	3.90
GHRP-4	608.3	**159.1 (38)**, 591.2 (16)	5.04
GHRP-5	771.4	**754.4 (22)**, 421.2 (27)	5.56
GHRP-6	437.5	**129.1 (20)**, 159.2 (34), 728.5 (15)	3.63
GHRP-6 (2-5) free acid	609.3	**352.1 (18)**, 335.1 (25), 159.2 (37)	5.37
GHRP-6 free acid	437.7	110.2 (26), 120.2 (31), **248.1 (20)**	3.93
Goserelin	635.6	**607.4 (17)**, 249.1 (31), 221.1 (38)	4.20
Hexarelin	444.8	**129.1 (20)**, 144.1 (55), 248.3 (30)	3.73
Hexarelin (1-3) free acid	427.3	**273.1 (31)**, 310.2 (25), 338.3 (17)	2.68
Hexarelin free acid	445.1	**338.3 (22)**, 752.1 (15), 595.3 (17)	4.01
Histrelin	442.1	**249.2 (19)**, 221.2 (21), 538.6 (18)	3.68
Ipamorelin	356.8	110.1 (33), **223.1 (17)**, 420.2 (17)	3.14
Ipamorelin (1-4) free acid	585.3	**223.2 (25)**, 420.3 (20), 166.1 (31)	4.05
Ipamorelin free acid	357.3	170.2 (19), 129.1 (29), **335.1 (27)**	3.38
Lecirelin	605.5	911.5 (15), **667.3 (23)**, 775.5 (25)	4.33
Leuprorelin	605.6	**249.2 (30)**, 775.5 (25), 534.5 (19)	4.20
LHRH (1-3) free acid	453.3	**249.1 (20)**, 221.1 (27), 110.1 (42)	3.04
MGF	574.6	**645.1 (17)**, 574.5 (10)	1.85
Nafarelin (5-10) MET	401.4	333.2 (14), **441.3 (14)**	4.50
Triptorelin	656.5	**249.1 (29)**, 328.4 (21), 877.5 (26)	4.31
Triptorelin free acid	657.4	**249.1 (31)**, 329.8 (22), 628.8 (24)	4.38
Benzyldimethylphenylammonium	212.2	91.1 (22), **120.1 (31)**, 134.1 (15)	3.07
Bretylium Bromide	243.1	72.1 (19), **90.1 (43)**, 169.5 (24)	2.53
Clidinium Bromide	352.2	124.1 (26), **142.1 (31)**	4.30
Edrophonium Bromide	166.1	**136.2 (20),** 138.3 (16)	0.27
Glycopyrrolate	318.2	98.2 (25), **116.1 (27)**	4.47
Glycopyrrolate-*d*	321.2	101.2 (25), **119.1 (27)**	4.46
Ipratropium Bromide	332.3	124.1 (31), **166.2 (26**), 290.2 (26)	3.16
Isopropamide Iodide	353.2	116.2 (14), 142.2 (32), **238.2 (18)**	4.33
Mepenzolate Bromide	340.1	**130.1 (28)**	4.17
N-butylscopolamine	360.3	155.9 (29), **194.0 (30)**	3.89
Neostigmine	223.2	**208.1 (20)**	2.01
Oxyphenonium Bromide	348.3	**132.1 (29)**, 149.1 (24), 199.2 (24)	5.26
Pancuronium Bromide	286.4	**100.1 (26)**, 206.7 (17), 236.8 (13)	3.82
Pipenzolate	354.3	**144.1 (28)**, 239.1 (18)	4.35
Propantheline bromide	368.3	**181.1 (33),** 253.2 (17), 326.2 (18)	5.58
Tiotropium	392.1	**152.1 (28),** 170.1 (32)	3.77

Peptide	Precursor ion (m/z)	Product ion (m/z) with Collision Energy (EV)	T_R (min)
Capromorelin	506.2	215.2 (39), **244.2 (23)**, 263.2 (15)	4.68
Ibutamoren	529.3	**267.1 (20)**, 263.2 (17), 235.2 (22)	5.39
Methotrexate	455.1	175.1 (40), **307.9 (24)**	2.81
Pirbuterol	241.2	**167.1 (17)**, 185.2 (15)	0.55

Table 3. LC-MS settings for screening method. Precursor ion (*m/z*), product ion (*m/z*), confirmatory ion (*m/z*) bolded, collision energy (EV), and retention time (min) of targeted compounds with a 0.75 min divert valve.

Compound	Linearity			Recovery (%)	Matrix Effect (%)	LOD (ng/mL)	LOQ (ng/mL)
	Intercept	Slope	R^2				
Alarelin	-0.0006104	0.047961	0.999	72	94	0.05	0.10
AOD-9604	-0.0018621	0.030630	0.989	34	102	0.50	1.0
Buserelin	-0.0026306	0.056713	0.989	71	106	0.10	0.50
Deltorphin	-0.0083285	0.031038	0.999	17	89	0.50	1.0
Deltorphin I	-0.0001194	0.004940	0.994	6	98	1.0	2.0
Deltorphin II	-0.0002047	0.005753	0.994	5	99	1.0	2.0
Dermorphin	-0.0005654	0.029558	0.996	24	100	0.50	1.0
Dermorphin (1-4) (D-Arg2, Sar4)	0.0093924	0.265867	0.997	66	83	0.05	0.10
Dermorphin (1-4) amide (D-Arg2, Lys4)	-0.0022068	0.041656	0.999	61	97	0.10	0.50
Dermorphin (1-4) amide (D-Arg2)	0.0001944	0.019715	1.000	68	91	0.10	0.50
Deslorelin	-0.0026306	0.05671	0.989	72	69	0.50	1.0
GHRP-1	0.0036682	0.015096	0.991	44	25	0.10	0.50
GHRP-2	0.0015337	0.320210	0.987	62	83	0.05	0.10
GHRP-4	-0.0007559	0.20935	0.994	49	95	0.10	0.50
GHRP-5	0.0001918	0.074570	0.998	46	94	0.50	1.0
GHRP-6	0.0100297	0.164150	0.999	37	97	0.10	0.50
GHRP-6 (2-5) free acid	-0.0015498	0.126180	0.999	10	95	0.50	1.0
GHRP-6 free acid	0.0009814	0.014899	0.988	49	97	0.50	1.0
Goserelin	0.0001138	0.103559	0.998	73	99	0.10	0.50
Hexarelin	-0.0024775	0.018200	0.998	47	97	0.50	1.0
Hexarelin (1-3) free acid	0.0008203	0.077680	0.990	13	105	0.50	1.0
Hexarelin free acid	-0.0060030	0.019245	0.992	48	102	0.50	1.0
Histrelin	0.0008612	0.010260	0.983	68	96	0.50	1.0
Ipamorelin	-0.0005867	0.013803	0.999	65	104	0.10	0.50
Ipamorelin (1-4) free acid	-0.0000591	0.019036	0.997	5	163	0.50	1.0
Ipamorelin free acid	0.00420358	0.037686	0.996	69	101	0.50	1.0
Lecirelin	-0.0001056	0.04395	0.994	69	106	0.10	0.50
Leuprorelin	-0.0008015	0.062304	0.993	70	99	0.10	0.50
LHRH (1-3) free acid	0.0026919	0.022001	0.999	1	91	1.0	2.0

Compound	Linearity			Recovery (%)	Matrix Effect (%)	LOD (ng/mL)	LOQ (ng/mL)
	Intercept	Slope	R^2				
MGF	-0.0016027	0.022889	0.989	43	N/A	1.0	2.0
Nafarelin (5-10)	0.0013217	0.538370	0.991	72	63	0.05	0.10
Triptorelin	-0.0006044	0.014385	0.999	73	93	0.10	0.50
Triptorelin free acid	0.0025071	0.011104	0.933	47	109	0.50	1.0
Bretylium Bromide	-0.0014330	0.179460	0.991	78	72	0.50	1.0
Clidinium Bromide	-0.0195294	11.14940	0.999	77	80	0.005	0.01
Edrophonium Bromide	-0.0071873	2.57360	0.998	75	60	0.05	0.10
Glycopyrrolate	0.0145843	10.47180	0.998	81	69	0.005	0.01
Ipratropium Bromide	-0.0046490	3.961420	0.999	81	91	0.05	0.10
Isopropamide Iodide	0.0008360	14.99560	0.999	79	83	0.005	0.01
Mepenzolate Bromide	0.0113210	7.943180	0.999	79	72	0.01	0.05
N-butylscopolamine	-0.0044082	1.064410	0.998	81	76	0.10	0.50
Neostigmine	-0.0095701	8.175880	0.998	80	57	0.01	0.05
Oxyphenonium Bromide	-0.0047275	3.444770	0.999	80	85	0.10	0.50
Pancuronium Bromide	-0.0128133	1.677050	0.996	77	70	0.05	0.10
Pipenzolate	-0.0127810	10.40640	0.999	80	77	0.05	0.10
Propantheline bromide	0.0434520	11.22940	0.999	73	84	0.05	0.10
Tiotropium	-0.0071887	2.268197	0.996	67	67	0.01	0.05
Capromorelin	0.0011973	0.589339	0.993	18	98	0.05	0.10
Ibutamoren	0.0001943	1.233510	0.997	32	108	0.05	0.10
Methotrexate (non QAD)	0.0251424	0.056004	0.995	2	131	1.0	2.0
Pirbuterol	-0.0073100	1.179360	0.995	77	71	0.10	0.50

Table 4. Validation results including linearity (intercept, slope and regression coefficient), extraction recovery (%) and matrix effects (%) are shown. Limit of detection and quantitation were determined by the signal to noise ratio from the baseline noise to the peak height.

Compound	Day 1 (% CV)			Day 1 Accuracy (%)			Day 2 (% CV)			Day 2 Accuracy (%)			Day 3 (% CV)			Day 3 Accuracy (%)			Intra-day CV (%)			Intra-day Accuracy (%)		
	0.5 ng/mL	2.5 ng/mL	7.5 ng/mL	0.5 ng/mL	2.5 ng/mL	7.5 ng/mL	0.5 ng/mL	2.5 ng/mL	7.5 ng/mL	0.5 ng/mL	2.5 ng/mL	7.5 ng/mL	0.5 ng/mL	2.5 ng/mL	7.5 ng/mL	0.5 ng/mL	2.5 ng/mL	7.5 ng/mL	0.5 ng/mL	2.5 ng/mL	7.5 ng/mL	0.5 ng/mL	2.5 ng/mL	7.5 ng/mL
Alarelin	10.6	6.3	5.3	96.2	112	114	15.3	6.0	5.3	69.0	105	106	13.2	7.92	8.63	115	108	123	23.7	7.00	8.80	93.3	108	**114**
AOD-9604	43.5	5.8	5.6	121	129	130	28.3	10.2	6.7	78.0	126	129	47.3	9.30	8.88	58.6	111	109	51.2	10.4	10.5	85.8	122	**123**
Buserelin	12.2	8.1	8.3	128	146	146	8.7	8..1	5.4	92.7	129	132	9.9	10.7	16.0	94.0	96.4	104	19.8	19.0	16.8	105	124	**127**
Deltorphin*	17.6	8.3	4.5	94.6	116	115	23.3	8.1	9.2	95.1	110	106	20.9	13.3	6.8	79.3	109	119	21.5	9.8	8.1	89.7	112	**113**
Deltorphin I*	19.7	19.7	8.9	80.0	75.7	73.8	42.2	22.8	12.1	66.3	92.3	110	28.5	18.5	7.1	135	111	110	43.6	25.0	20.3	93.7	92.9	**98.1**
Deltorphin II*	65.0	26.5	10.9	68.0	90.9	77.0	89.3	21.1	15.4	96.9	80.1	99.9	65.0	26.5	10.9	76.1	106	125	66.6	22.0	22.5	80.3	92.3	**101**
Dermorphin	44.9	26.4	13.0	62.3	99.1	107	18.2	21.9	19.8	59.4	125	130	24.0	14.7	20.2	64.1	110	103	30.5	22.3	20.2	61.9	111	**113**
Dermorphin (1-4) (D-Arg2, Sar4)	4.5	5.9	4.4	104	124	121	9.8	7.7	7.2	94.5	112	120	8.1	7.9	14.1	98.8	96.2	101	8.1	12.6	11.7	99.1	111	**114**
Dermorphin (1-4) amide (D-Arg2, Lys4)	17.2	5.9	5.8	99.4	124	110	10.7	10.7	8.1	100	106	110	22.9	13.3	21.0	84.7	99.2	98.3	18.1	13.5	13.1	94.8	110	**106**
Dermorphin (1-4) amide (D-Arg2)	9.3	7.1	6.0	117	121	117	20.8	10.4	5.5	86.7	107	101	14.7	10.0	8.7	110	100	104	18.3	11.7	9.1	105	110	**107**
Deslorelin	21.1	9.4	5.1	101	128	131	9.8	10.3	11.1	93.4	135	130	14.6	9.9	12.2	104	102	99.7	16.5	15.1	15.4	99.4	122	**120**
GHRP-1	30.1	17.9	2.7	92.1	119	125	45.0	14.2	14.6	67.1	107	135	15.1	18.8	15.2	108	103	99.8	43.7	17.3	17.0	89.0	110	**112**
GHRP-2	10.4	9.9	5.3	117	140	139	13.3	5.9	3.6	96.5	128	136	4.9	10.2	14.7	92.4	97.0	98.1	15.1	17.4	17.1	102	122	**124**
GHRP-4	9.9	4.8	5.2	108	124	130	7.8	6.4	4.0	79.7	114	117	11.4	9.3	11.8	89.9	101	99.1	16.1	10.8	13.2	92.5	113	**115**
GHRP-5	20.9	9.8	9.8	112	127	131	12.4	6.4	13.3	88.5	127	124	11.3	11.3	10.6	92.6	101	108	18.4	13.9	11.3	97.6	118	**121**
GHRP-6	5.3	6.0	8.0	108	128	127	9.5	6.7	5.0	90.0	123	121	13.0	11.7	12.6	107	94.4	93.0	12.2	15.2	15.7	101	115	**113**
GHRP-6 (2-5) free acid	10.3	3.4	6.2	88.9	97.5	95.9	21.2	8.4	5.0	90.1	108	110	13.9	8.6	10.1	90.2	104	104	14.8	8.2	9.0	89.7	103	**103**
GHRP-6 free acid	43.9	20.1	8.9	62.6	124	120	33.5	6.2	9.6	44.3	113	116	46.8	18.7	8.2	98.9	111	90.2	57.9	16.3	15.1	68.3	116	**109**
Goserelin	11.5	6.2	4.9	115	136	128	9.4	10.9	7.9	96.4	109	105	13.5	11.3	7.8	102	98.7	105	13.5	16.7	11.7	104	115	**113**
Hexarelin	81.6	15.2	8.8	57.0	131	113	33.2	12.3	12.2	90.1	111	106	31.6	12.5	15.4	105	101	96.1	45.1	17.2	13.3	84.1	114	**105**
Hexarelin (1-3) free acid	13.7	12.2	10.5	90.0	97.5	93.5	18.8	11.5	7.8	76.2	180	105	18.8	10.1	6.2	82.8	95.2	95.8	17.4	34.5	9.3	83.0	124	**98.1**
Hexarelin free acid	46.3	22.9	4.5	98.5	109	125	19.0	17.7	7.8	72.1	127	115	40.2	11.4	13.3	58.1	82.6	90.9	57.4	25.2	15.5	76.2	107	**110**
Histrelin	19.6	13.5	23.0	178	124	118	40.1	25.5	12.7	158	126	130	49.9	21.9	21.0	47.9	114	119	55.8	20.1	18.4	128	121	**122**
Ipamorelin	27.1	11.9	9.4	86.2	132	126	28.8	15.5	6.4	86.1	105	97.8	15.6	9.8	6.3	109	87.5	98.3	24.8	18.2	14.8	93.7	111	**107**

Compound	Day 1 (% CV)			Day 1 Accuracy (%)			Day 2 (% CV)			Day 2 Accuracy (%)			Day 3 (% CV)			Day 3 Accuracy (%)			Intra-day CV (%)			Intra-day Accuracy (%)		
	0.5 ng/mL	2.5 ng/mL	7.5 ng/mL	0.5 ng/mL	2.5 ng/mL	7.5 ng/mL	0.5 ng/mL	2.5 ng/mL	7.5 ng/mL	0.5 ng/mL	2.5 ng/mL	7.5 ng/mL	0.5 ng/mL	2.5 ng/mL	7.5 ng/mL	0.5 ng/mL	2.5 ng/mL	7.5 ng/mL	0.5 ng/mL	2.5 ng/mL	7.5 ng/mL	0.5 ng/mL	2.5 ng/mL	7.5 ng/mL
Ipamorelin (1-4) free acid	36.2	18.8	12.2	115	112	106	56.6	15.8	7.1	85.9	106	107	20.2	12.0	9.4	126	92.6	90.2	37.8	17.2	12.2	109	103	**101**
Ipamorelin free acid	19.0	5.9	11.0	101	114	118	26.1	17.6	9.0	90.1	109	101	9.03	11.1	11.0	109	100	79.7	18.4	12.9	18.9	100	108	**99.3**
Lecirelin	20.9	12.1	6.1	108	135	126	20.3	9.9	8.7	81.5	135	125	22.6	13.2	14.7	105	120	119	24.5	12.3	22.6	98.3	130	**123**
Leuprorelin	14.6	8.7	6.4	115	127	130	23.6	8.3	6.1	77.9	109	116	11.4	5.8	15.8	86.8	101	99.0	22.6	12.4	14.4	93.4	112	**115**
LHRH (1-3) free acid*	24.7	11.8	13.9	71.6	92.3	89.1	45.0	5.8	10.3	57.7	95.0	92.0	16.0	12.3	8.60	107	81.7	74.0	38.5	11.6	14.4	78.9	89.7	**85.0**
MGF*	31.4	9.8	14.6	108	123	126	43.3	19.5	18.4	56.0	114	148	34.0	22.7	20.7	89.2	111	94.8	42.2	17.3	24.9	84.5	116	**122**
Nafarelin (5-10)	7.5	6.4	3.8	101	130	128	13.0	5.9	6.3	93.8	134	135	9.5	9.6	14.2	93.0	101	103	10.4	14.1	13.8	96.0	122	**122**
Triptorelin	11.7	5.2	7.1	93.6	122	112	15.2	14.8	11.8	96.7	113	101	24.7	14.7	5.7	101	120	119	17.8	12.0	10.5	97.2	118	**111**
Triptorelin fa	N/A	17.2	15.8	N/A	124	134	N/A	19.0	19.7	N/A	115	125	N/A	8.7	30.1	N/A	63.2	74.1	N/A	31.6	31.2	N/A	101	**111**
Bretylium Bromide	13.1	9.7	6.2	92.3	87.9	91.4	6.2	7.2	3.7	83.7	94.7	98.0	8.6	12.4	9.9	97.7	95.6	98.2	11.4	10.2	7.5	91.2	92.7	**95.9**
Clidinium Bromide	7.5	7.1	2.3	110	109	100	6.3	8.2	8.6	95.1	106	96.8	5.9	8.2	4.5	104	104	90.7	8.6	7.6	6.9	103	106	**96.0**
Edrophonium Bromide	11.6	5.9	3.2	97.9	104	97.3	8.4	5.8	4.0	84.4	103	91.2	6.0	8.4	8.4	96.0	99.4	94.8	10.7	6.6	6.0	92.8	102	**94.4**
Glycopyrrolate	5.7	6.6	4.9	101	104	105	7.4	5.9	3.4	97.3	113	105	8.0	7.7	6.6	99.1	99.3	97.4	6.7	8.3	6.0	99.0	106	**103**
Ipratropium Bromide	5.9	4.4	4.8	96.9	105	100	13.2	5.5	7.7	75.1	106	101	8.1	8.7	2.6	93.8	101	100	8.8	6.4	5.2	88.6	103	**100**
Isopropamide Iodide	4.5	1.5	4.9	111	125	116	4.2	6.7	9.0	80.2	95.6	91.7	10.0	8.1	4.9	96.1	102	100	14.6	13.4	11.6	95.6	107	**102**
Mepenzolate Bromide	5.0	7.9	5.6	103	101	102	5.3	6.4	5.0	85.9	97.7	91.7	6.7	8.0	5.6	102	104	96.2	9.8	7.5	7.0	97.1	101	**96.7**
N-butylscopolamine	6.3	6.6	2.5	94.8	104	105	6.1	6.0	7.8	69.8	100	101	8.4	11.0	7.0	93.8	96.6	96.5	9.0	8.2	6.7	86.1	100	**101**
Neostigmine	5.3	4.5	3.7	98.6	101	98.6	10.6	6.8	8.1	85.8	96.8	90.1	8.5	9.8	6.7	99.8	104	96.7	10.1	8.5	7.1	94.7	101	**95.1**
Oxyphenonium Bromide	4.2	7.7	5.0	91.0	97.9	99.5	7.3	7.2	6.5	78.6	95.0	95.6	7.4	9.2	4.9	93.7	95.6	92.9	9.6	7.7	6.0	87.7	96.2	**96.0**
Pancuronium Bromide	3.0	107	7.4	94.7	92.9	95.4	17.3	11.7	6.1	82.2	93.8	107	27.6	17.9	16.4	81.2	116	108	19.0	17.4	11.9	86.2	101	**103**
Pipenzolate	7.4	5.8	3.4	103	106	105	4.5	4.4	8.5	87.7	100	93.7	7.6	8.4	5.9	99.8	99.2	90.1	9.3	6.7	8.9	96.7	101	**96.3**
Propantheline	7.4	4.2	4.0	117	123	122	6.4	6.2	6.9	89.0	105	99.9	5.7	9.4	6.8	98.6	102	96.2	13.5	10.6	12.3	102	110	**106**
Tiotropium	2.1	8.9	7.4	92.9	99.8	96.3	15.5	4.2	5.4	70.2	101	103	8.2	8.5	8.2	92.6	95.3	94.5	9.4	7.5	7.6	85.2	98.7	**98.0**

Compound	Day 1 (% CV)			Day 1 Accuracy (%)			Day 2 (% CV)			Day 2 Accuracy (%)			Day 3 (% CV)			Day 3 Accuracy (%)			Intra-day CV (%)			Intra-day Accuracy (%)		
	0.5 ng/mL	2.5 ng/mL	7.5 ng/mL	0.5 ng/mL	2.5 ng/mL	7.5 ng/mL	0.5 ng/mL	2.5 ng/mL	7.5 ng/mL	0.5 ng/mL	2.5 ng/mL	7.5 ng/mL	0.5 ng/mL	2.5 ng/mL	7.5 ng/mL	0.5 ng/mL	2.5 ng/mL	7.5 ng/mL	0.5 ng/mL	2.5 ng/mL	7.5 ng/mL	0.5 ng/mL	2.5 ng/mL	7.5 ng/mL
Capromorelin	8.7	5.3	3.9	118	144	138	6.1	7.1	7.1	101	124	128	10.2	8.7	13.4	93.5	101	103	13.6	15.9	14.5	104	123	**123**
Ibutamoren	12.3	5.7	4.3	121	148	144	6.9	6.8	6.8	91.8	127	122	8.8	7.8	14.1	93.2	99.9	98.1	17.2	17.3	17.8	102	125	**121**
Methotrexate	49.3	13.2	9.9	43.6	94.5	97.8	160	16.1	19.0	16.9	75.6	68.8	13.3	9.04	20.4	134	76.5	59.1	85.5	16.5	26.8	64.8	82.2	**75.2**
Pirbuterol	8.7	5.8	4.4	84.8	97.3	102.9	11.2	6.0	5.6	75.7	97.3	95.7	7.3	9.2	4.8	91.5	99.5	98.6	11.4	6.8	5.5	84.0	98.1	**99.1**

Table 5. Mean, accuracy, and precision for low, mid, and high quality control concentrations. *These compounds Low, Mid, High concentrations were 1.0ng/mL, 5.0ng/mL, and 15ng/mL respectively.

Compound name	Stability RT – 7.5 ng/mL (% Accuracy)		Stability 2-8°C – 7.5 ng/mL (% Accuracy)	Stability -20°C – 7.5 ng/mL (% Accuracy)			
	0.0 hr.	24 hr.	24 hr.	7 days	FT 7 days	14 days	FT 14 days
Alarelin	114	99	103	110	109	108	107
AOD-9604	103	2	37	97	96	105	95
Buserelin	82	81	91	94	90	86	89
Deltorphin	96	40	84	91	100	104	106
Deltorphin I	92	99	105	78	88	94	90
Deltorphin II	86	111	107	106	116	100	99
Dermorphin	95	78	95	99	104	101	82
Dermorphin (1-4) (D-Arg2, Sar4)	77	84	91	98	89	87	88
Dermorphin (1-4) amide (D-Arg2, Lys4)	81	78	92	163	89	86	88
Dermorphin (1-4) amide (D-Arg2)	111	101	109	113	103	102	100
Deslorelin	79	90	91	104	89	97	94
GHRP-1	90	39	91	83	79	122	112
GHRP-2	78	84	88	91	87	107	95
GHRP-4	80	81	93	84	87	100	94
GHRP-5	74	85	86	89	100	100	87
GHRP-6	66	78	89	87	87	92	85
GHRP-6 (2-5) free acid	91	89	96	89	104	95	88
GHRP-6 free acid	70	69	75	99	99	79	71
Goserelin	114	105	109	104	109	98	92
Hexarelin	70	80	85	90	86	94	91
Hexarelin (1-3) free acid	111	101	104	96	105	107	105
Hexarelin free acid	105	99	98	89	84	99	86
Histrelin	78	79	85	97	95	88	79
Ipamorelin	113	106	105	100	116	109	110
Ipamorelin (1-4) free acid	80	83	105	69	99	102	104
Ipamorelin free acid	71	78	90	91	95	90	88

Compound name	Stability RT – 7.5 ng/mL (% Accuracy)		Stability 2-8°C – 7.5 ng/mL (% Accuracy)	Stability -20°C – 7.5 ng/mL (% Accuracy)			
	0.0 hr.	24 hr.	24 hr.	7 days	FT 7 days	14 days	FT 14 days
Lecirelin	82	89	95	93	94	107	115
Leuprorelin	83	85	97	95	87	95	96
LHRH (1-3) free acid	93	124	141	79	91	90	95
MGF	73	51	86	135	92	93	87
Nafarelin (5-10) MET	81	88	92	107	86	107	95
Triptorelin	107	95	93	103	110	107	111
Triptorelin free acid	73	30	69	106	123	102	91
Bretylium Bromide	98	100	102	94	103	92	82
Clidinium Bromide	99	88	89	91	94	79	84
Edrophonium Bromide	99	99	107	96	98	84	96
Glycopyrrolate	93	100	97	99	101	87	88
Ipratropium Bromide	96	100	101	98	103	94	95
Isopropamide Iodide	103	100	99	101	106	86	84
Mepenzolate Bromide	96	90	92	97	98	85	85
N-butylscopolamine	107	92	92	96	96	89	90
Neostigmine	94	99	96	98	103	92	92
Oxyphenonium Bromide	96	98	93	97	104	88	88
Pancuronium Bromide	97	77	84	93	98	97	90
Pipenzolate	97	94	91	95	100	87	86
Propantheline	97	78	85	93	98	87	90
Tiotropium	89	68	80	95	96	88	88
Capromorelin	92	84	83	80	82	87	86
Ibutamoren	89	81	85	81	89	98	91
Methotrexate	154	103	125	89	118	111	106
Pirbuterol	98	99	103	119	101	90	92

Table 6. Stability tests of mid quality control concentration, 7.5ng/mL, at RT, 4°C, -20°C and one freeze/thaw cycle at 7 and 14 days.

Compound	Recovery (%)	Matrix Effect (%)	LOD (ng/mL)	LOQ (ng/mL)
Alarelin	72	94	0.05	0.10
AOD-9604	34	102	0.50	1.0
Buserelin	71	106	0.10	0.50
Deltorphin	17	89	0.50	1.0
Dermorphin	24	100	0.50	1.0
Dermorphin (1-4) (D-Arg2, Sar4)	66	83	0.05	0.10
Dermorphin (1-4) amide (D-Arg2, Lys4)	61	97	0.10	0.50
Dermorphin (1-4) amide (D-Arg2)	68	91	0.10	0.50
Deslorelin	72	69	0.50	1.0
GHRP-1	44	25	0.10	0.50
GHRP-2	62	83	0.05	0.10
GHRP-4	49	95	0.10	0.50
GHRP-5	46	94	0.50	1.0
GHRP-6	37	97	0.10	0.50
GHRP-6 (2-5) free acid	10	95	0.50	1.0
GHRP-6 free acid	49	97	0.50	1.0
Goserelin	73	99	0.10	0.50
Hexarelin	47	97	0.50	1.0
Hexarelin (1-3) free acid	13	105	0.50	1.0
Hexarelin free acid	48	102	0.50	1.0
Histrelin	68	96	0.50	1.0
Ipamorelin	65	104	0.10	0.50
Ipamorelin free acid	69	101	0.50	1.0
Lecirelin	69	106	0.10	0.50
Leuprorelin	70	99	0.10	0.50
MGF	43	N/A	1.0	2.0
Nafarelin (5-10)	72	63	0.05	0.10
Triptorelin	73	93	0.10	0.50
Triptorelin free acid	47	109	0.50	1.0

Compound	Recovery (%)	Matrix Effect (%)	LOD (ng/mL)	LOQ (ng/mL)
Bretylium Bromide	78	72	0.50	1.0
Clidinium Bromide	77	80	0.005	0.01
Edrophonium Bromide	75	60	0.05	0.10
Glycopyrrolate	81	69	0.005	0.01
Ipratropium Bromide	81	91	0.05	0.10
Isopropamide Iodide	79	83	0.005	0.01
Mepenzolate Bromide	79	72	0.01	0.05
N-butylscopolamine	81	76	0.10	0.5
Neostigmine	80	57	0.01	0.05
Oxyphenonium Bromide	80	85	0.10	0.50
Pancuronium Bromide	77	70	0.05	0.10
Pipenzolate	80	77	0.05	0.10
Propantheline bromide	73	84	0.05	0.10
Tiotropium	67	67	0.01	0.05
Capromorelin	18	98	0.05	0.10
Ibutamoren	32	108	0.05	0.10
Pirbuterol	77	71	0.10	0.50

Table 7. The compounds this method is most viable for are shown.