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Determination of oxyntomodulin, an anorectic polypeptide, in rat plasma using 2D-LC–MS/MS coupled with ion pair chromatography

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ABSTRACT

Polypeptide therapeutics present a challenge for quantitative analysis when using immunoassays or recently, liquid chromatography–tandem mass spectrometry because of their structural similarities to endogenous proteins and peptides in plasma. In this assay, a Waters Oasis® mixed-mode anion exchange (MAX) microelution modified solid phase extraction (SPE) method coupled with two-dimensional reversed phase ion pair chromatography–tandem mass spectrometry was used for the validation and analysis of oxyntomodulin in rat plasma. Oxyntomodulin (OXM) and its isotope labeled internal standard were extracted from rat plasma and analyzed with a chromatographic run time of 8 min. Modified SPE, two-dimensional liquid chromatography coupled with 3-nitrobenzyl alcohol as a mobile phase additive, and monitoring of multiply charged SRM transitions (+7 charge state) of OXM were necessary to achieve a lower limit of quantification of 1 ng/mL. The method was validated with a linear range of 1–1000 ng/mL, with average R^2 of 0.992, and reversed calculated residuals between −8.6% and 6.0%. Precision and accuracy for inter- and intra-day were determined to be ±17%. Following a complete validation, the method was applied to show utility using rat plasma samples that were intravenously dosed with oxyntomodulin.

1. Introduction

While the prevalence of obesity is evident, as over one-third of adults in the United States are considered obese, at present, there are no effective pharmacotherapies to treat obesity or overweight and/or reduce its prevalence [1]. In this context, however, recent studies have advocated that manipulation of the gut–brain axis for appetite control can be physiologically natural as a means to reduce body weight, such that gut-secreting anorectic polypeptides produced post-prandially in response to meal intake have received a considerable attention as potential therapeutic drug entities in obesity [2]. Oxyntomodulin (OXM) is one of such anorectic polypeptides, composed of 37-amino acids with a molecular weight of 4449 Da [2,3]. In humans, its systemic blood levels under fasting condition are ∼50 pM (0.2 ng/mL), which is elevated to ∼150 pM (0.7 ng/mL) within 60 min, in response to meal intake [7,8]. Central and peripheral injections of OXM have demonstrated significant food intake suppression and reduced body weight in rodents and humans [2–6]. Nevertheless, OXM has also shown to be metabolized quite rapidly by dipeptidyl peptidase (DPP-IV) and neutral endopeptidase (NEP), apparently suffering from a short half-life in the systemic circulation and thus, pharmacological effects [7].

When OXM is to be developed as a therapeutic drug entity in obesity, one of the critical hurdles would be determination of OXM concentrations in biological matrices e.g., blood, to assess its pharmacokinetics following administration. Conventionally, for most polypeptide drugs like OXM, highly sensitive immunoassays such as radioimmunoassay and enzyme-linked immunosorbent assay (ELISA) have been used for this purpose, even though their cross-reactivities to related molecules and time-consuming procedures are disadvantageous [8]. This is primarily because liquid chromatography–tandem mass spectrometry (LC–MS/MS) commonly used for small molecule drugs has not been considered suitable for quantification of large molecule drugs such as proteins and peptides until recently [9–13].

LC–MS/MS quantitative analysis of peptides involves four processes, which include: (1) sample pre-treatment, (2) chromatography, (3) ionization, and (4) detection for quantification [14]. Sample pre-treatment from plasma has included protein precipitation (PPE), liquid–liquid extraction (LLE), solid phase extraction (one and two-dimensional SPE), immunoaffinity purification (IAP), and online extraction using two-dimensional high-performance liquid chromatography (2D-LC) [9,10,12,14–19]. Each sample preparation has its advantages and disadvantages, with difficulty, method development time, and overall costs considered in method development as illustrated in Fig. 1. Protein precipitation has been employed for peptide analysis previously; however, losses of target peptide in precipitate and/or insufficient

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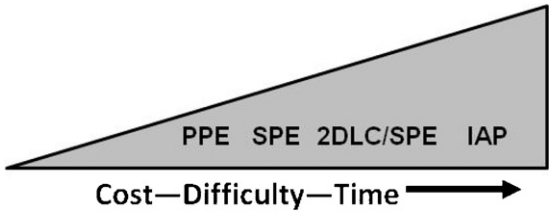

Fig. 1. Considerations for choosing peptide sample preparation.

reduction in matrix effects are disadvantages. Liquid–liquid extraction has limitations for sample pretreatment since it is more suitable for nonpolar compounds which are unlike peptides that have ionic activity [14]. Solid phase extraction provides sufficient reduction in matrix effects and improved recovery of PPE; however, multiple steps can require lengthy optimization. Immunoaffinity purification (IAP) requires antibodies that can be expensive, require significant optimization, and may not be necessary to use for smaller polypeptides.

In this sense, two-dimensional liquid chromatography (2D-LC) has recently emerged by virtue of its ability to provide separation of protein or peptide mixtures. Two-dimensional liquid chromatography (2D-LC) can provide separation of peptide mixtures and has emerged as the chromatographic method of choice [10,14,20]. One-dimensional reversed phase chromatography may be sufficient for chromatographic separation; however, 2D-LC offers the additional dimension that is used as an online sample purification to reduce matrix effects and improve sensitivity.

Ionization of peptides is accomplished using electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), atmospheric pressure photoionization (APPI), or matrix-assisted laser desorption ionization (MALDI). However, positive electrospray ionization (ESI) has been well established as the method of ionization for ionizing polar, large molecules such as peptides [21]. ESI enables multiply charged positive ions $[M+nH]^{n+}$ to form, which is suitable for the ionization of polypeptides. Formation of a multiple charge state ion will reduce the mass to charge ratio (m/z), which may allow for a polypeptide to be analyzed with a mass spectrometer with limited mass range.

Mass spectrometric detection of proteins and peptides has traditionally been done with ion trap, time-of-flight (TOF), or hybrid quadrupole time-of-flight (Q-TOF) mass analyzers. These mass analyzers offer accurate mass measurements for the identification and relative quantification of proteins and peptides [22]. However, these mass analyzers lack the sensitivity necessary for absolute quantification of peptides due to their significantly lower duty cycle compared to a tandem mass spectrometer operating in selection reaction monitoring mode [22]. The duty cycle difference of 100% active for tandem mass spectrometers versus 20% active for TOF type instruments can result in a 5–10-fold difference in sensitivity [22]. van den Broek et al. revealed in a recent review that larger peptides (>3200 Da) normally do not employ SRM (or multiple reaction monitoring: MRM) for quantification of peptides in biological fluids. It is understandable that the formation of MRM transitions for large peptides is challenging due to the potential to produce too many fragments from high collision energy, which yields lower sensitivity [23].

In this paper, as an alternative to immunoassays, OXM determination in rat plasma samples by LC–MS/MS method has been developed and validated using the FDA bioanalytical guidelines [24]. The method was unique in the use of an optimized microelution anion exchange SPE, two-dimensional LC–MS/MS, and reversed phase ion pair chromatography, while being sensitive and selective. The method requires 100 μL of rat plasma to obtain a lower limit of quantification of 1 ng/mL and a chromatographic run time of less than 10 min. A stable isotope-labeled internal standard was spiked into each sample. Following validation, the method was used for determination of the pharmacokinetic profile of OXM in rats following intravenous injection.

A) HSQGTFTSDYSKYLDSRRAQDFVQWLMNTKRNRNNIA
B) HSQGTFTSDYSKYLDS[Arg($^{13}C_6$; $^{15}N_4$)[Arg($^{13}C_6$; $^{15}N_4$)]AQDFVQWLMNTK[Arg($^{13}C_6$; $^{15}N_4$)]N[Arg($^{13}C_6$; $^{15}N_4$)]NNIA.

Fig. 2. Amino acid sequences in letter form for (A) native oxyntomodulin and its (B) stable isotope internal standard.

2. Experimental

2.1. Chemicals and reagent

Oxyntomodulin (OXM: molecular weight of 4449.9 g/mol) was purchased from Bachem Americas (Torrance, CA, USA). Isotope-labeled OXM (OXM_{IL}) was synthesized by Open Biosystems, Inc. (Huntsville, AL, USA), replacing all arginines (R) at 17th, 18th, 31st and 33rd positions with heavy arginine isotopes (>98% HPLC purity). The sequences for native OXM and its labeled internal standard are shown in Fig. 2. This labeling resulted in an average mass of 4491.0 g/mol, which was approximately a +40 Da shift from native OXM. DPP IV inhibitor was purchased from Millipore (St. Charles, MO, USA). Formic acid, trifluoroacetic acid (TFA), 3-nitrobenzyl alcohol (3-NBA), sodium hydroxide, hydrochloric acid, bovine serum albumin (BSA), and ammonium hydroxide were obtained from Sigma Aldrich (St. Louis, MO, USA). Deionized water was obtained in-house using a Nanopure Diamond water system from Barnstead International (Dubuque, IA, USA). Methanol, isopropanol, and acetonitrile were purchased from Burdick and Jackson (Muskegon, MI, USA). Sodium heparinized rat plasma was obtained from BioChemed Services (Winchester, VA, USA). All reagents were HPLC grade unless otherwise noted.

2.2. Materials and equipment

Heparinized rat plasma samples were aliquoted into 2 mL conical bottom 96-well plates (VWR International, Westchester, PA, USA). Oasis® brand mixed-mode anion exchange (MAX) microelution plates (30 μm, 2 mg/well; Waters Corporation, Milford, MA, USA) were used for solid phase extraction (SPE) of the samples using automated pipetting on a Quadra 96 Model 320 Tomtec (Hamden, CT, USA). Centrifugation and mixing, respectively, were carried out using an Eppendorf 5804R centrifuge (Hamburg, Germany) and a multi-tube vortex mixer (VWR International or a Talboys Advanced Model 1000MP Microplate Shaker (Troemner: Thorofare, NJ, USA). A syringe pump from Harvard Apparatus (Holliston, MA, USA) was used to infuse OXM solutions for tuning optimization and post-column infusion studies.

2.3. Chromatography and mass spectrometry equipment

The first dimension (loading step) of 2D-LC was performed on a Waters Acquity UPLC®. The second dimension (eluting pump) used high performance liquid chromatography (HPLC) with the following equipment: Shimadzu system controller SCL-10A VP, two LC-10AD VP pumps, solvent degasser DGU14A (Shimadzu, Kyoto, Japan). A 10-port Cheminert switching valve and a microelectric actuator obtained from Valco Instruments Co. Inc. (Houston, TX, USA) was used to perform on-line column trapping. The mass spectrometer was an API 4000Qtrap hybrid triple quadrupole/linear ion trap from AB Sciex (Foster City, CA, USA) with Analyst 1.5 data acquisition software. The data analysis was performed using Quantitation Wizard processing software that accompanies

Analyst. Bioanalyst software AB Sciex (Foster City, CA, USA) was used for peptide reconstruction to evaluate charge state distribution during tuning optimization. All nitrogen gas was generated from a Parker Hannifin (Haverhill, MA, USA) Tri-Gas Generator LC/MS 5000.

2.4. Charge state distribution and product ion formation study of oxyntomodulin

Electrospray ionization is a soft ionization technique which may form multiply charged analyte ions. The charged state distribution of an intact biomolecule will normally yield mass to charge ratios (m/z) between 500 and 3000 Da depending on the zero charge mass [25]. This offers an advantage to the analysis of large molecules using lower mass range mass analyzers (<3000 Da) such as a triple quadrupole mass spectrometer. Part of this investigation was to evaluate the factors that may affect charge state distribution and product ion formation of OXM. Given that the sequence of OXM contains several basic amino acid residues, positive ions tend to be localized on these residues and it will most likely ionize better with positive polarity [26]. It has been established that multiple factors can affect charge state distribution such as solvents, instrument parameters, primary structure and conformation of a protein [26]. Solvents such as glycerol and 3-nitrobenzyl alcohol have been shown to increase charge state and improve fragmentation [26,27]. Therefore, we investigated the charge state distribution of oxyntomodulin to determine the feasibility for quantification of the intact biomolecule using a triple quadrupole mass spectrometer. Our intention was to maximize the detectability of a particular charge state (m/z) and form product ions for selected reaction monitoring (SRM).

The first part of this study was to determine the charge state distribution of OXM in various solvent mixtures and optimize precursor ions. Different combinations of acetonitrile (ACN) or methanol (MeOH), water, 3-nitrobenzyl alcohol (3-NBA), glycerol, and formic acid (FA) were prepared. Each solvent combination was used as a diluent for OXM to prepare a final concentration of 5 μg/mL. The solvent (S1–S6) ratios consisted of: 25:75 (ACN:H_2O, S1), 25:74:1 (ACN:H_2O:3-NBA, S2), 25:74:1 (ACN:H_2O:glycerol, S3), 25:74:1 (ACN:H_2O:FA, S4), 25:73:1:1 (ACN:H_2O:3-NBA:FA, S5), 25:73:1:1 (ACN:H_2O:glycerol:FA, S6). Note that methanol was used to replace acetonitrile in each of the six solvent mixtures to make the total of twelve solvent mixtures. The solvent mixture was infused into the mass spectrometer at a flow rate of 10 μL/min. Mobile phase was also tee'd into the infusion experiment at a flow rate of 0.200 mL/min. with 80% acetonitrile and 20% 0.1% formic acid. Infusion experiments were performed with and without mobile phase flow by evaluation of Q1 (1st quadrupole) MS scans (i.e. full scan). Each mixture underwent optimization of each instrument parameter, which included: ramping declustering potential and entrance potential, optimization of gases, source temperature, and turbo ion voltage. Following the observation of maximum Q1 full scan intensity, a peptide reconstruction was performed using Bioanalyst to determine the charge state distribution of the mass spectrum obtained. The list of ions reconstructed from the mass spectrum was evaluated for the presence of OXM (MW = 4449 Da), and the charge state distribution was observed. Following optimization of precursor ions for each solvent, formation of product ions was determined for the minimum, maximum, and most abundant charge state of OXM. In product ion mode, instrument parameters were again ramped for optimal performance. Collision energy was ramped slowly from 0 to 150 due to the potential to generate multiple fragments with weak intensity that are from multiply charged biomolecules [28]. As a final tuning optimization, selected reaction monitoring (SRM) transitions were evaluated for maximum intensity with optimal parameters applied for each solvent and respective charge state that formed product ions.

2.5. Optimization of reversed phase ion pair chromatography

Reversed phase chromatography was investigated with and without the presence of an ion pairing reagent. It has been observed that incorporation of modifiers into reverse phase chromatography may improve sensitivity and resolution for intact proteins [29]. Traditionally, 3-NBA ($C_7H_7NO_3$), with a pK_a of 13.82, has been used as a matrix for fast atom bombardment and matrix assisted laser desorption ionization (MALDI) [30,31]. Following optimization of SRM transitions, it was observed that signal intensity improved more than an order of magnitude in the presence of 3-nitrobenzyl alcohol (3-NBA) in the solvent mixture. Therefore, 3-NBA was evaluated as a potential ion pairing agent to improve chromatography and enhance sensitivity. Initially, 3-NBA was added into the aqueous (mobile phase A) and organic mobile phase (mobile phase B) at seven molar concentrations (0.0, 1.3, 3.3, 6.5, 13.1, 32.7, 65.3 mM). A slow gradient (5% A over 1 min, 80% B from 1 to 5 min, hold for 1 min at 80% B, and equilibrate column for 2 min again at 5% A) onto the eluting column described in Section 2.6 was used to evaluate response and chromatographic performance. Following the evaluation of each molar concentration of 3-NBA, six pH adjustments (pH = 2.3, 4.7, 5.7, 6.4, 7, and 10) to the mobile phase were evaluated.

2.6. Gradient method for 2D-LC analysis of oxyntomodulin

Samples were injected (30 μL injection) with a Waters Acquity UPLC® integrated autosampler. In the first dimension of 2D-LC, the loading column was an ACE C8, 5 μm, 2.1 mm × 50 mm, 300 Å (MacMod, Chadds Ford, PA, USA). The second dimension of 2D-LC used a Waters XBridge BEH300 C18 Column, 5 μm, 2.1 mm × 100 mm, 300 Å as the analytical (eluting column). Mobile phase A consisted of 0.1% (6.5 mM) 3-NBA (pH 4.7) (v/v) and mobile phase B consisted of 0.1% (6.5 mM) 3-NBA in acetonitrile (v/v). The Acquity employed two washes: a weak wash consisted of 1:1 acetonitrile:water and the strong wash consisted of 80:20 acetonitrile:water with 0.1% 3-NBA and 0.1% formic acid. In the first dimension, the samples were loaded onto the ACE C8 column maintained at 50 °C using a flow of 0.250 mL/min. and mobile phase composition of 95% A for 1 min. After a 1 min loading time, the diverter valve switched to position B and the gradient initiated. Mobile phases A and B were coupled together with a mixer and the elution conditions started with 5% mobile phase B (0.1% 3-NBA in acetonitrile) and increased to 90% B over 3.5 min. Isocratic conditions were held for 2 min at 90% B, followed by a quick switch (5.6 min) back to the original conditions of 95% A. The columns were equilibrated for approximately 2.5 min for a total run time of 8 min. During the elution step while the diverter valve was in position B from 5.6 to 7 min, the loading column was washed at a mobile phase composition of 50% A at a flow of 0.4 mL/min. Continuous flow was also permitted into the mass spectrometer with the eluting pumps.

2.7. Mass spectrometry

The mass spectrometer was operated in the positive electrospray ionization (ESI) mode with selected reaction monitoring (SRM) of OXM and its isotope labeled internal standard (OXM_{IL}). Charge state distribution and evaluation of the most intense SRM transitions was finalized following the investigation discussed in Section 2.4. Tuning was performed on several potential SRM transitions and all parameters were re-evaluated such as gases, source temperature, declustering potential, collision energy, ion spray voltage, and collision exit potential. A 2 μg/mL solution of OXM or its labeled internal standard was prepared in solvent 2 (25:74:1

Table 1
Selected reaction monitoring (SRM) transitions and selected parameters.

Peptide	Q1	Q3	Q3 ion description	DP (v)	Collision energy (v)	Dwell time (ms)
OXM (+7)	636.9	666.9	SKYLDS-28	70	27	350
OXM_{IL}	642.3	676.6	SKYLDS-H_2O	76	29	200
OXM $(+8)^{m1}$	557.4	666.9	SKYLDS-28	70	27	200
OXM $(+8)^{m2}$	557.4	225.4	HS-b ion	45	54	200
OXM $(+7)^{m3}$	636.9	110.1	H-immonium ion	45	78	200

m1, m2, and m3 = SRM's used as monitoring transitions and not quantified. Q3 ion description reflects fragment sequence pattern.

ACN:H_2O:3-NBA) and was used as a tuning solution for direct infusion at 10 μL/min. Tee'd with mobile phase at a flow rate of 0.20 mL/min using a composition of 80% mobile phase B. The mass spectrometer parameters were tuned and optimized to achieve maximum sensitivity for each SRM transition which resulted in the following parameters: source temperature 325 °C, ion spray voltage 5500 V, gas 1 (GS1) 66, gas 2 (GS2) 30, collision activated dissociation (CAD) was set on high, entrance potential (EP) was set to 9 V, collision exit potential (CXP) 16 V, and channel electron multiplier (CEM) was set at 2500 V. Oxyntomodulin and OXM_{IL} eluted at approximately 3.95 and 3.91 min, respectively. SRM transitions, collision energies (CE), declustering potential (DP), Q3 fragment ion descriptions, and dwell times used during this study are shown in Table 1. Each peptide was given a name with its precursor ion charge state $(M + nH)^{n+}$ in parentheses.

2.8. Stock solution and working solution preparation

OXM was diluted with 1% BSA in deionized water to prepare a stock solution at 0.5 mg/mL. This stock solution was subsequently diluted with 1% BSA to prepare working solutions. All solutions were subaliquoted and stored at −70 °C. The working solutions were used to prepare fresh calibration standards and frozen quality controls. As OXM_{IL} was provided at 5 pmol/μL in 5% acetonitrile (22.45 μg/mL) by Open Biosystems, 10 aliquots of 0.2 mL were prepared and stored at −70 °C. OXM_{IL} spiking solution was prepared fresh daily at 2.25 μg/mL by diluting the 0.2 mL aliquots with 2 mL of 5% acetonitrile. All working solutions were prepared just before spiking into rat plasma.

2.9. Preparation of calibration standards and quality control samples in rat plasma

Pooled sodium heparin rat plasma from at least two donors was used to prepare the calibration standards and quality controls. A volume of 0.020 mL of each OXM intermediate solution (20, 40, 100, 200, 500, 1000, 2000, 5000, 10,000, and 20,000 ng/mL) was spiked into 0.4 mL of rat plasma for each calibration standard. A total of ten calibration standards were freshly prepared for each analytical run at concentrations of 1, 2, 5, 10, 25, 50, 100, 250, 500, and 1000 ng/mL. Quality controls were prepared in two different pools of rat plasma at the same concentrations. One pool was the same rat plasma used to prepare the calibration standards. The other plasma pool contained DPP-IV inhibitor at a concentration of 20 μL per milliliter of rat plasma. A volume of 0.4 mL of a 20,000 ng/mL OXM intermediate solution was spiked into 10 mL of rat plasma in a volumetric flask to obtain the highest quality control (HQC = 800 ng/mL). A 10,000 ng/mL intermediate solution was spiked (0.060 mL) into a 10 mL volumetric flask and diluted to 10 mL with rat plasma to make a medium QC (MQC) at 60.0 ng/mL. A 1000 ng/mL intermediate solution was used to prepare the lower limit of quantitation (LLOQ) QC and the low QC (LQC), by spiking 0.025 mL into a 10 mL volumetric flask for the LQC (2.5 ng/mL) and 0.010 mL into a 10 mL volumetric flask to prepare the LLOQ (1.0 ng/mL). A dilution control was prepared at 5 times the highest calibration standard (5000 ng/mL), to evaluate dilution of samples into the calibration range. The dilution control was prepared by performing a ten-fold dilution with blank rat plasma to obtain a concentration within the calibration range (50.0 ng/mL). Quality control samples were freshly prepared for validation to determine the day zero nominal value for storage stability studies. QC samples were sub-aliquoted for daily use (0.250 mL) into lobind polypropylene tubes (Eppendorf, Hauppauge, New York, USA) and stored at −70 °C until analysis.

2.10. Sample preparation

All samples were thawed in an ice water bath and vortex mixed. A 100 μL sample of rat plasma was aliquoted into a 2 mL conical bottom 96-well plate, along with 100 μL of 4% phosphoric acid, and 20 μL of internal standard spiking solution (2.25 μg/mL). While samples mixed for 10 min, the MAX microelution was conditioned with 200 μL of methanol followed by 200 μL of 4% phosphoric acid. Samples were centrifuged prior to loading, and then slowly loaded onto the SPE (2 × 110 μL) by increasing the vacuum pressure from 0 to 5 psi after each loading step. The SPE was then washed with 200 μL of 5% ammonium hydroxide and 200 μL 5% acetonitrile. The plate was blotted dry and samples were eluted with 5% TFA in 75:25 acetonitrile/water (v/v) (2 × 25 μL). Eluent was collected into a 1 mL 96-well plate (Waters Corp.). Finally, samples were diluted with 25 μL of 25:74:1 acetonitrile/water/3-NBA and mixed briefly. A 25 μL sample was injected onto the LC–MS/MS.

2.11. Validation

2.11.1. Matrix effects evaluations and recovery

Post-column infusion studies, post-extraction addition, and evaluation for phospholipids as potential matrix effects were evaluated. A post-column infusion study was performed by injecting an extracted blank rat plasma sample while infusing a 2 μg/mL oxyntomodulin solution at 10 μL/min. The resulting profile was evaluated for chromatographic co-elution with OXM and changes in the ESI response of OXM that may indicate suppression or enhancement. During the post-column infusion study, phospholipids were monitored using the in source-SRM transition 184 → 184 as suggested by Little et al. [32]. As suggested by Matuszewski et al., a more quantitative approach to assessing matrix effects was carried out using a post-extraction addition study, where a percent matrix effects (%ME) is determined [33]. Unextracted "clean" samples were prepared as final extracted concentrations (3.33, 80.0, and 1067 ng/mL) from the LQC (2.5 ng/mL), MQC (60 ng/mL), and HQC (800 ng/mL) in the final sample solvent (75% of: 5% TFA in 75:25 acetonitrile/water/25% of: 25:74:1 acetonitrile/water/3-NBA) and compared to post-spiked blank extracts with the same quality control concentrations. A percent matrix effect was determined using the following equation: %ME = ((peak area response of post-extracted sample/peak area response of clean sample) − 1) × 100.

A relative recovery experiment was also performed for OXM in rat plasma, by comparison of pre- and post-spiked extracted quality

control samples. QC's were extracted $n = 6$ and percent recovery was calculated.

2.11.2. Linearity

A total of ten calibration points were freshly prepared as described in Section 2.9 in sodium heparin treated rat plasma. Each analytical run employed duplicate analyses of calibrators. Peak area ratios of OXM:OXM_{IL} and each concentration level was used to construct the calibration curve. Calibration curves used linear regression with a $1/x^2$ weighing factor for the determination of OXM concentrations.

2.11.3. Precision and accuracy

Three quality controls were used to assess inter-day precision and accuracy, which were analyzed in at least three separate runs. QC concentrations were calculated from the calibration curves analyzed in the same run. Intra- and inter-assay precision and accuracy were determined by extracting LLOQ, low, medium, and high quality controls in six replicates for intra-assay performance. Furthermore, a dilution control (5000 ng/mL) was evaluated for intra-assay performance with six replicates. Quality controls were extracted in triplicate in five additional runs.

2.11.4. Selectivity

Six individual rat plasma samples were used to assess selectivity. Each rat subject was extracted in duplicate with and without the presence of internal standard as described in Section 2.10. Acceptable selectivity was established by the fact that chromatographic peaks that co-elute with OXM were less than 20% of the peak area of the average LOQ samples. No more than 5% contribution from OXM to its isotope labeled internal standard signal was acceptable. This experiment was performed by extracting a double blank with no internal standard added as well as a high standard (1000 ng/mL) without internal standard.

2.11.5. Stability evaluations and carryover

Stock solution stability was evaluated in a stress test. The stress test experiment was performed by comparison of a 0.5 mg/mL stock solution kept at −70 °C to an aliquot of the same stock solution that was left at room temperature for 4 h. Both aliquots of the stock solution were diluted in the final extraction solvents described in Section 2.10 and injected in triplicate. Average peak area responses of the diluted stock solutions were compared.

The first validation run was used to determine day 0 nominal concentrations of quality controls for long term storage stability. Each QC was stored at −70 °C in sub-aliquots for daily use. Storage stability was determined by using the day zero nominal values established for the low and high QC's. Following an appropriate storage time, low and high QC's were analyzed and concentrations were determined with freshly prepared calibrators. Post-preparative stability (PPS) was performed to determine an extended amount of time that extracted samples could be left in the refrigerated autosampler in the event that re-injection was necessary due to instrument malfunction. Carryover was assessed by injecting final extraction solvent samples immediately after the highest calibration standards (1000 ng/mL) in an analytical run.

2.11.6. Cross technology validation of OXM immunoassay

As part of this study, we also compared sample results using a peptide immunoassay versus our validated LC–MS/MS assay. A commercially available competitive immunoassay was used according to the manufacturer's instructions for rat serum and plasma (Bachem Americas; Torrance, CA, USA).

2.12. Analysis of dosed rat blood samples

This validated method was used to determine the plasma OXM concentration versus time profiles in 4 Sprague-Dawley rats following intravenous bolus injection at a dose of 0.5 mg/kg. Under pentobarbital anesthesia, 0.1 mL of OXM solution in saline was directly injected to the right jugular vein. Subsequently, blood samples (0.1 mL) were withdrawn from the left jugular vein at 2, 5, 10, 15, 20, 30, 45, 60, 90 and 120 min following injection. These samples were immediately centrifuged at 10,000 rpm for 2 min and stored at −70 °C prior to analysis.

3. Results and discussion

In this study, we employed a modified solid phase extraction coupled with 2D-LC/MS/MS to monitor a multiply charged peptide for quantitative analysis of OXM in rat plasma samples. The combination of these parameters allowed for a robust method to quantify OXM in rat plasma with a lower limit of quantification (LLOQ) at 1 ng/mL (Fig. 3(A)). Optimization of solid phase extraction, multiply charged SRM, and two-dimensional reversed phase ion pair chromatography was necessary to meet the demands of quantifying this polypeptide.

3.1. Mass spectrometry of oxyntomodulin

Charge state distribution and product ion formation were investigated using different solvents as described in Section 2.4. The objective was to achieve conditions that would reduce the m/z to achieve a more suitable mass range for the mass spectrometer and ultimately improve intensity for the formation of SRM's. Following the optimization of mass spectrometer parameters, solvent effects proved to play a role in charge state distribution and product ion formation. Lavarone et al. demonstrated this with cytochrome c using 3-NBA and glycerol to increase charge state and abundance [34]. This increase in charge states may be the result of using a low vapor pressure solvent, which may determine the electrospray droplet evaporation characteristics [35]. Solvent additives such as 3-NBA have a high surface tension, which may play a role in the increased charge states and abundances [26]. The solvent mixture (S2) which contained 25:74:1 (ACN:H_2O:3-NBA), improved intensity of the most abundant charge state by as much as one order of magnitude. The charge state distribution for this solvent mixture consisted of a lowest charge state equal to +4, a highest charge state equal to +10, the most abundant charge state was +8. All solvent mixtures resulted in the +6 or +8 charge state being the most abundant; however, the lowest and highest charge state varied between each solvent and the formation of reliable product ions. The charge state distributions of each solvent mixture are highlighted in Table 2. Each charge state was evaluated for the formation of product ions; however, the most abundant charge states yielded more product ions that ultimately lead to useful SRM transitions. Solvents 2 and 5 produced the most intense product

Table 2
Charge state distribution of oxyntomodulin in different solvent mixtures.

Solvent mixture (S1–S6)	Lowest and highest charge state	Most abundant charge state
25:75 ACN:H_2O	+4, +8	+6
25:74:1 ACN:H_2O:3-NBA	+4, +10	+8
25:74:1 ACN:H_2O:glycerol	+4, +8	+8
24:75:1 ACN:H_2O:formic acid	+5, +7	+6
24:74:1:1 ACN:H_2O:3-NBA:formic acid	+5, +11	+8
24:74:1:1 ACN:H_2O:glycerol:formic acid	+4, +8	8

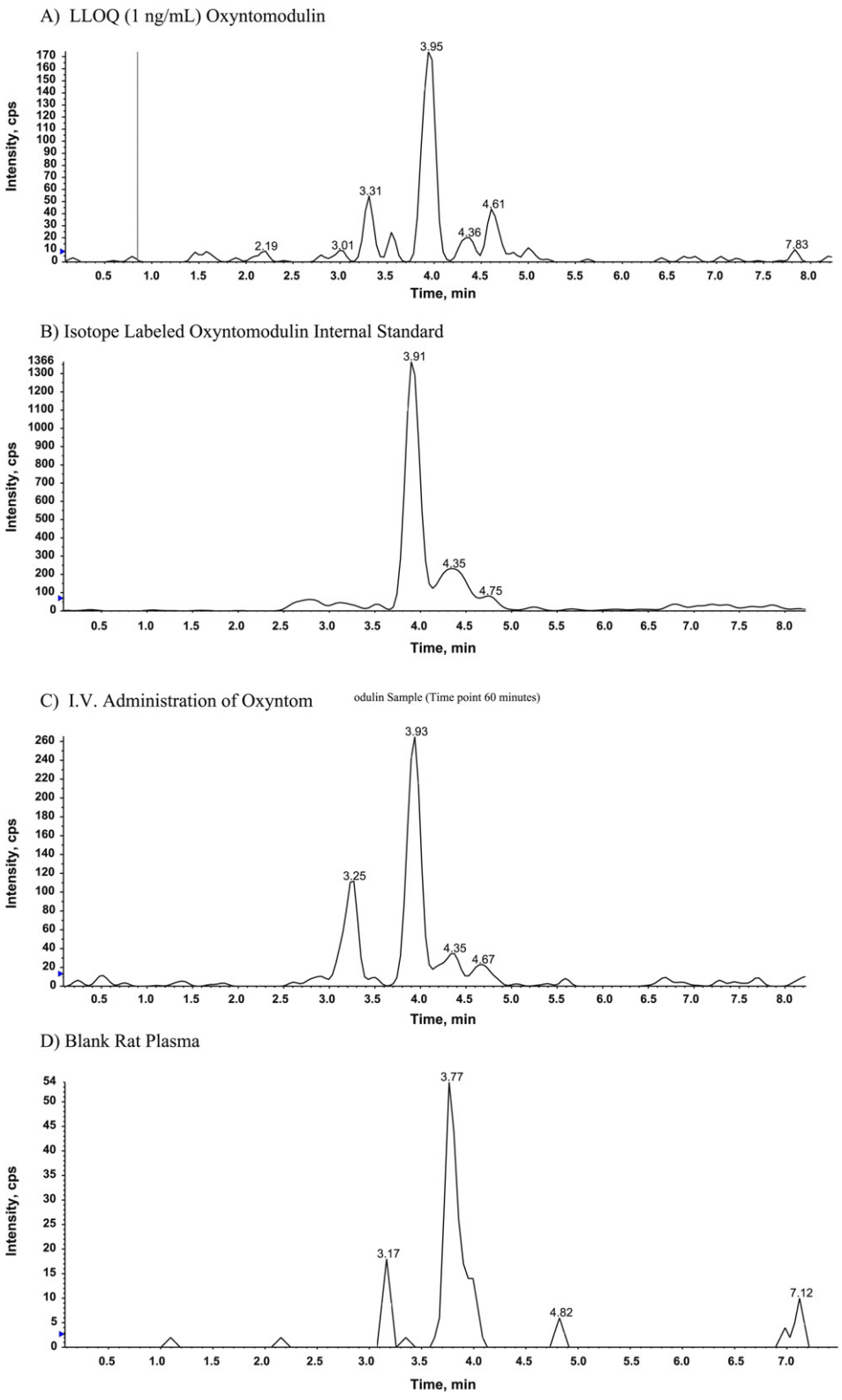

Fig. 3. Representative chromatograms of (A) LLOQ (1 ng/mL) oxyntomodulin, (B) isotope labeled oxyntomodulin internal standard, (C) rat sample following I.V. administration of oxyntomodulin (time point 60 min) and (D) blank rat plasma.

ions to form SRMs using the +7 (636 m/z) and +8 (557 m/z) charge states of oxyntomodulin. The immonium ion 110 m/z was the most intense product ion. However, it was not selective for quantitative analysis due to interferences present. Following evaluation of SRM transitions, each was monitored to determine the most sensitive and selective SRM. This investigation resulted in a transition of 636.9 → 666.9 m/z for quantification purposes. Other SRM's in Table 1 were monitored as qualification transitions.

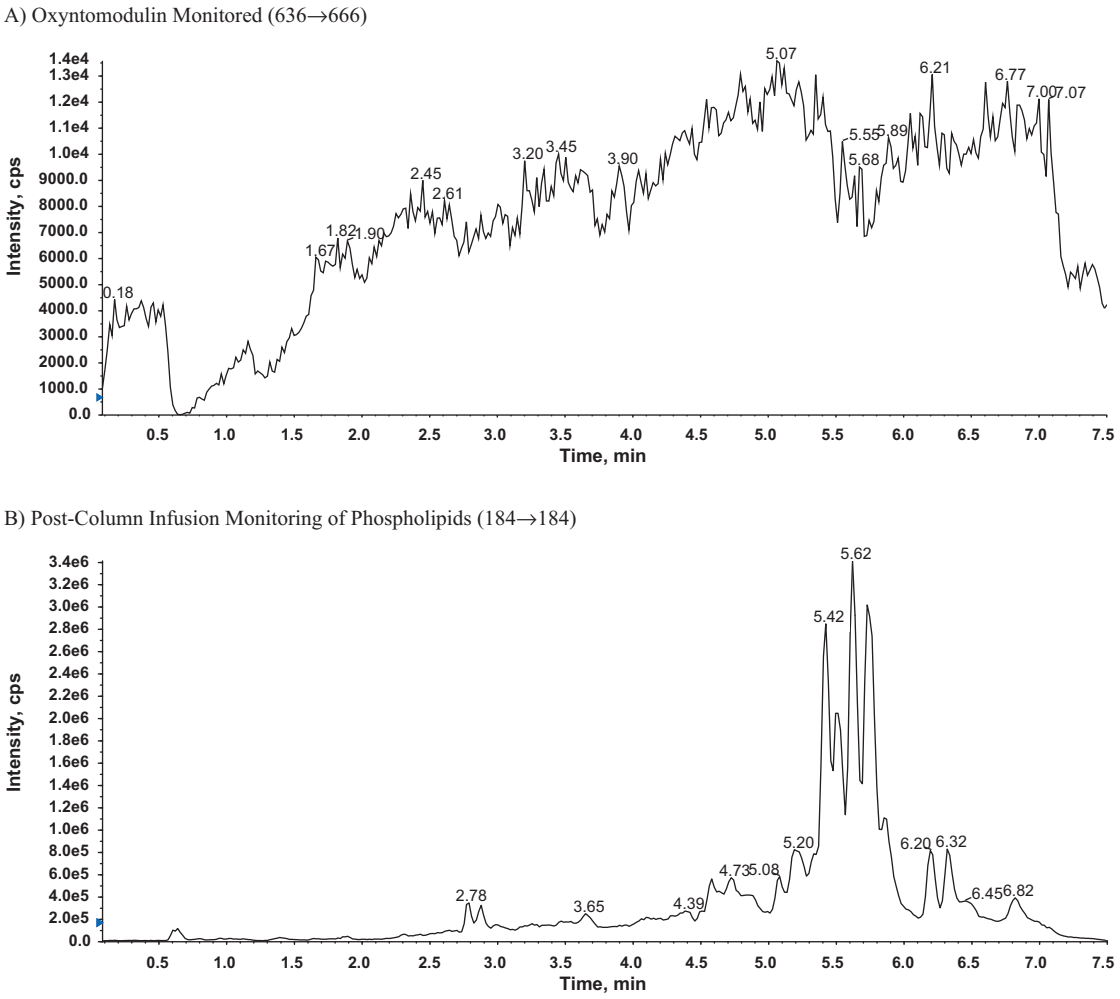

Fig. 4. Post-column infusion study of oxyntomodulin (A) oxyntomodulin post-column infusion profile; oxyntomodulin monitored (636 → 666) and (B) phospholipids post-column infusion profile; post-column infusion monitoring of phospholipids (184 → 184).

3.2. 2D-LC–MS/MS using reversed phase ion pair chromatography

Two-dimensional reversed phase chromatography is the standard of practice for quantification of peptides [10,14]. Use of column trapping allows for an additional sample purification online, which should reduce matrix effects and improve robustness. The results from the charge state distribution discussed in Section 3.1 lead us to use 3-NBA as a mobile phase additive [29]. The addition of 3-NBA allows provides for ion pairing with the zwitterionic peptide species in an equilibrium process. Peak shape, response, and reproducibility were investigated during the optimization of molar concentration of 3-NBA in the mobile phase. Consistent peak shape and the most intense response were produced with 0.1% (6.5 mM) 3-NBA in the mobile phase. The pH of the mobile phase can also play a role in ion pair chromatography; therefore, three molar concentrations (3.3, 6.5, and 65 mM) of 3-NBA were then evaluated for pH adjustment (pH 2.3, 4.7, 5.7, 6.4, 7, and 10) as shown in Fig. 5. The most intense and reproducible peak shape was generated using 6.5 mM 3-NBA with the pH adjusted to 4.7. During this study, various gradients were attempted with single and two-dimensional reverse phase chromatography. The increase to 90% organic (0.1% 3-NBA in acetonitrile) provided ideal retention (Tr = 3.93 min), good peak shape, and the highest peak area response. Part of this investigation also revealed that the presence of 3-NBA in the final sample extract must be optimized. Three different proportions of 3-NBA (0.05: 3.3 mM, 0.1: 6.5 mM, and 1.0%: 65 mM) were evaluated in the final sample diluent, which contained 25% acetonitrile. The thought was having more ion pair reagent (3-NBA) would help force the equilibrium to the ion pairing of the peptide and 3-NBA. The results in Fig. 6 show that the peak area response was more than 2.5-fold higher with more 3-NBA (1.0%: 65 mM) in the final sample extract.

During chromatographic investigations, a single reversed phase C18 UPLC® column was originally used for oxyntomodulin with a gradient similar to that described in Section 2.5. However, during sample extraction development, this column quickly (<200 injections) showed poor peak shape, high back pressure, and proved to be inefficient for quantification purposes. Our previous successes with column trapping lead us to investigate

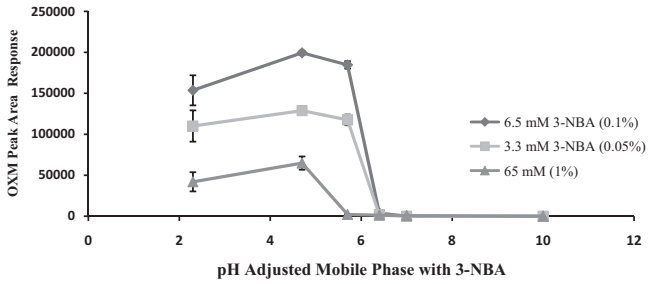

Fig. 5. Assessment of pH adjustment to mobile phase containing 3-NBA versus peak area response of oxyntomodulin.

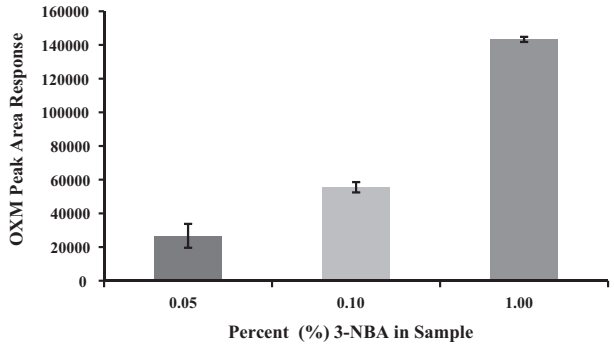

Fig. 6. Optimization of percent 3-NBA present in sample.

2D-LC for chromatographic separation of OXM. Initially, C18 guard columns were attempted as the loading column to provide a short loading time as demonstrated previously [36]. The mobile phase optimization study of 3-NBA described in Section 2.5 provided compelling evidence to continue use of the Waters XBridge BEH300 C18 for the analytical column. A Phenomenex Gemini C18 (4.0 mm × 2.0 mm) security guard, an Agilent ZORBAX 300SB-C3 Guard 5um, 2.1 mm × 12.5 mm, and a Waters Acquity HSS C18 VanGuard Pre-column, 1.8 μm, 2.1 mm × 5 mm were evaluated as the loading column. All loading columns showed minimal retention of oxyntomodulin, with the maximum retention time being 42 s. Therefore, it was decided to investigate a more traditional length (50 mm) column that may retain OXM long enough to remove unwanted matrix components prior to switching to the analytical column. A C8 and C18 column (both 2.1 mm × 50 mm, 5 μm, and 300 Å) were evaluated. It was concluded that the C8 provided better overall performance with consistent retention, and ruggedness after multiple injections. The combination of an ACE C8 column in the first dimension with a loading time of 1.5 min, followed by a switching valve to change the direction of the mobile phase and initiate the gradient onto the analytical column (Waters XBridge BEH300 C18) was sufficient for methods validation. The loading and analytical column washes post-elution increased the longevity of the column, which allowed for more than 900 injections. Chromatographic examples of the lower limit of quantification (A, 1 ng/mL), isotope labeled internal standard (B), rat sample (C), and blank rat plasma (D) are presented in Fig. 3.

3.3. Sample preparation

During this investigation protein precipitation (PPE) and solid phase extraction (SPE) were evaluated. Precipitating reagents acetonitrile, perchloric acid (7%) and methanol were tested as solvents for sample purification. Glucagon, the first 29 amino acids of OXM, has been quantified using a protein precipitation with acetonitrile in rat plasma [15]; however, the recovery in all precipitating reagents tested for OXM was low (<20%). Similar recovery results were observed when using reversed phase SPE and ion-exchange SPE. Mixed mode weak cation (WCX) and anion (MAX) microelution solid phase extractions were further optimized to improve recovery. Recovery using the manufacturer's protocol's for MAX and WCX was approximately 10–15%, respectively. An investigation was performed to determine where the loss was occurring. The first investigation included collection of all loading, washing, and elution solvents to evaluate the presence of OXM in any of these solvents. It was observed in the manufacturer's protocol for wash 2 (20% acetonitrile) that the presence of OXM was prevalent in the collected wash; therefore, wash 2 was reduced to 5% acetonitrile, which yielded negligible recovery of OXM during analysis. The loading application for each SPE was 4% phosphoric acid, which may not adequately disrupt OXM from other proteins. Consequently, nonspecific protein binding was investigated. It was first observed that recovery of OXM in water as a matrix was significantly lower (5% versus 15%) than rat plasma, thus nonspecific binding may be an issue. Chaotropes such as guanidine hydrochloride and UREA were added to the plasma to facilitate disruption of OXM binding to proteins; however, these reagents had negligible effects as compared to the 4% phosphoric acid that was currently being added to the plasma prior to loading onto the SPE. Lastly, a stronger elution solution was tried. Instead of 1% TFA in the eluent, the TFA percentage was increased to 5% which resulted in improved recovery.

3.4. Validation results

3.4.1. Linearity

Linearity was determined using the peak area ratio of OXM and its isotope labeled internal standard as a function of concentration over the range 1–1000 ng/mL. Calibration curves had acceptable reverse calculated residuals between −8.6% and 6.0%. Inter-run precision measured in terms of percent relative standard deviation ranged from 6.5% to 11.0% over six analytical runs.

3.4.2. Selectivity

Six different lots of rat plasma were evaluated for selectivity of the method. No significant (<10% of LLOQ) endogenous peaks at the retention time of OXM were observed for any of the lots. Fig. 3(D) shows a representative blank rat plasma chromatogram. Qualification SRM's were monitored throughout the entire study, which are listed in Table 1. The SRM transition for OXM 636.9 → 666.9 was chosen over the other monitored transitions due to superior selectivity and reproducibility. Transitions 557.4 → 225.4 and 636.9 → 110.1 had higher response but were not reliable for quantification due to imprecision most likely from matrix effects. A blank sample with internal standard and the upper calibration standard (1000 ng/mL) were analyzed for analyte interference and found to have only a negligible effect on OXM or internal standard response.

The post-column infusion study described in Section 2.11.1 resulted in no obvious suppression or enhancement at the retention time of OXM. Fig. 4(A) reveals the ion profile for OXM during the post-column infusion, which shows no change in response at the retention time of OXM (Tr = 3.93 min.) Fig. 4(B) represents the chromatographic profile of phospholipids monitored during the post-column infusion, which indicated their presence following extraction; however, all peaks were chromatographically resolved from OXM. The late eluting peaks present in Fig. 3(B) are well resolved from OXM and the run time was increased to 8 min to avoid late elution phospholipids on subsequent injections. Matrix effects were further evaluated with a post-extraction addition study performed as described in Section 2.11.1. The presence of matrix effects was −33.4% for LQC, −23.2% for MQC, and −40.2% for HQC.

3.4.3. Recovery and carryover

Recovery was performed following optimization of extraction and chromatographic procedures during the validation. Six extracted replicates of LQC, MQC, and HQC were compared to post-spiked matrix residue samples as described in Section 2.11.1. Calculations were obtained by dividing the matrix pre-spiked extracted samples by the post-spiked extracted samples and multiplying by 100. The results revealed 54.5%, 64.9%, 58.7% recovery for the LQC, MQC, and HQCs, respectively. No response in solvent blanks following the high calibration standard was observed at the retention time of OXM.

Table 3
Inter and intra-assay precision and accuracy.

QC (ng/mL)	Average measured concentration (ng/mL) ±SD	Inter-assay % bias[a]	Intra-assay % bias	Intra-assay precision (% RSD)	Inter-assay precision (% RSD)[a]
LLOQ (1.00)	0.88 ± 0.13	−11.7	−13.10	15.26	14.30
LQC (2.50)	2.81 ± 0.31	12.40	6.74	11.14	11.03
MQC (60.00)	66.7 ± 2.67	11.16	8.22	5.60	4.01
HQC (800.00)	817.23 ± 59.91	2.15	5.11	8.26	7.33
DQC (5000.00)	5102.31 ± 363.28	N/A	2.04	7.12	N/A

[a] QCs analyzed $n \geq 3$ in six separate analytical runs.

3.4.4. Precision and accuracy

Table 3 summarizes the inter- and intra-day precision and accuracy results. Inter-day results were determined based on six separate runs, whereas intra-day results were determined in one run with six replicates of the LLOQ QC, LQC, MQC and HQC samples. Accuracy (% bias) for inter-day was −11.7% to 12.4% for all quality controls including the LLOQ. Intra-day accuracy resulted in −13.1% to 8.2% bias. The relative standard deviation (precision) for inter-run results ranged from 4.0% to 14.3% and intra-day precision was 5.6–15.2% RSD. The LLOQ was within ±20% for precision and accuracy, and all other controls were within ±15% for inter and intra assay results.

3.4.5. Stability and incurred sample re-analysis (ISR)

Stability assessments included: post-preparative stability (PPS), storage stability, stock stability, and freeze–thaw stability. PPS was determined from quality controls stored in the autosampler for approximately 39 h at 5 °C. These stored QC's proved to be stable with accuracy results all within ±12.4%. Freshly prepared calibration standards were used to calculate the accuracy of stored QC's. Storage stability results ranged from −13.3% to −15.1% biases for LQC and HQC with and without DPP-IV inhibitor samples stored at −70 °C for 64 days. The stress test for stock solution held at room temperature compared to a fresh aliquot of the same stock showed acceptable peak area response differences (5.2% bias). Freeze–thaw stability was assessed for three cycles, with LQC and HQC resulting in −18.1% and −16.3% biases, respectively. One freeze–thaw cycle of the LQC and HQC's resulted in acceptable accuracy (±15%). Therefore, samples should be sub-aliquoted for daily use and be thawed in an ice water bath as a precaution.

Ten rat subject samples were analyzed a second time to evaluate incurred sample re-analysis. The latest European Medicines Agency bioanalytical guidance was used to determine the acceptability of ISR results [37]. This guidance indicates 67% percent of the ISR samples must be within 20% of the mean of the original and repeat sample. Results indicated that 7 out of 10 (70%) of the repeated rat samples met these criteria.

4. Method utility

The validated method provided a mean pharmacokinetic profile as shown in Fig. 7, which provides further evidence that this method is applicable to OXM analysis in rat plasma.

5. Cross technology immunoassay validation results

A peptide immunoassay kit was validated using reagents provided with the kit procedure. Calibration curves resulted in R^2 values of 0.988 and 0.991, respectively, with a linear range from 0.1 to 7 ng/mL. The curve fitting was a four parameter fit with $y = ((A - D)/(1 + (x/C)^B)) + D$. Precision (%CV) of these calibration curve replicate points were from 0.4% to 24.5%. The same rat samples that were used for the pharmacokinetic study described in Section 2.12 were analyzed with the LC–MS/MS method and

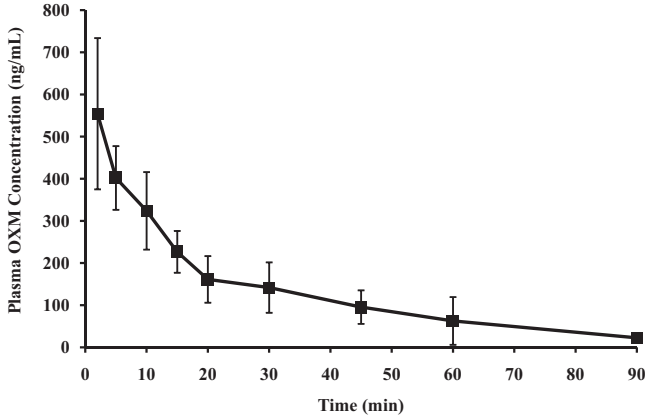

Fig. 7. Plasma oxyntomodulin (OXM) concentration versus time profile in rats following bolus injection at 0.05 mg/kg. Data represent mean ± standard deviation (SD) with $n = 4$.

this immunoassay. The results of the rat samples analyzed with the immunoassay were significantly different than the LC–MS/MS results and almost all time points yielded no detectable response for OXM. Furthermore only one rat had a similar pharmacokinetic profile. However, some of the individual time points did not match with this rat either (percent difference as much as 200% at the 5 min time point). An investigation followed where successive runs with calibration standards and quality controls were evaluated in two different matrices. It was suspected that the rat plasma from the rat species may contain a matrix effect that would hinder this assay. Thus, another investigation was performed using dilution to reduce the suspected matrix effect. The kit contains a peptide-free rat serum for preparation of calibration standards, quality controls, and samples. This rat serum was also used as a diluent to prepare quality control samples (low, 0.2 ng/mL and high, 6 ng/mL), which may dilute out the potential matrix effect from rat plasma obtained from the same rat species used in the pharmacokinetic study. Dilutions of 1, 10, and 100 of blank rat plasma with the kit matrix (peptide free rat serum) supplied with the kit were evaluated at the same concentrations. The results indicate there may be a matrix effect because no result was obtained for 1- and 10-fold dilutions. The 100-fold dilution was acceptable for the high QC (6 ng/mL) with a percent bias of 17.3%; however, no result was obtained for the low QC. Therefore, a significant dilution of this rat plasma may be necessary to achieve reliable results whereas too much dilution would result in poor sensitivity.

6. Conclusions

Polypeptides that are approaching protein sizes are challenging molecules to quantify in the presence of a complex biological matrix such as plasma. Matrix effects and non-specific binding proved to be a challenge during method development and validation. Characteristics of the peptide such as isoelectric point (p*I*), hydrophobicity,

and individual amino acid residues must be considered for separation, storage, and ionization. All of these characteristics affected the development and optimization for oxyntomodulin. The use of 3-NBA proved to be beneficial for ionization of the peptides and as a mobile phase additive for reversed phase ion pair chromatography. The use of 2D-LC was found to be necessary for peptide quantification due to its reliable removal of matrix effects and superior robustness. Stability experiments indicate that peptide stability can be a concern and storage precautions such as daily use aliquots for samples, quality controls, and stock solutions must be necessary. Another concern was the inability of the immunoassay to yield results that could be correlated with the LC–MS/MS analysis. These results could be an anomaly, but prove the value of using LC–MS/MS as an alternative to immunoassays.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2012.06.047.

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