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Determination of octreotide and assessment of matrix effects in human plasma using ultra high performance liquid chromatography–tandem mass spectrometry

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ABSTRACT

A selective UHPLC–MS/MS method for determination of the therapeutic peptide octreotide in human plasma was developed and validated. This assay used a UHPLC C_{18} column with 1.7 μm particle size for efficient separation and an ion-exchange SPE for selective extraction. Octreotide and its labeled internal standard, [$^{13}C_6Phe_3$] octreotide, were extracted from human plasma using a simple Oasis® WCX μElution SPE method and analyzed with a total chromatographic run time of 7.5 min. Matrix effects were studied during method development by direct monitoring of representative phospholipids. On-line removal of phospholipids using column switching and pre-column back-flushing was carried out to trap and remove any residual phospholipid matrix interferences. The UHPLC column provided baseline separation between the analyte and matrix peaks. The chromatographic conditions yielded optimal retention and excellent peak shape for both the analyte and internal standard. The assay was linear in the concentration range of 0.025–25.0 ng/ml, inter- and intra-assay precision and accuracy were within 6.1% and ±1.93%, respectively. Recovery was ~73%. Post-extraction addition experiments showed that matrix effects were less than 4%. This method for octreotide in human plasma has been validated and utilized to support of clinical pharmacokinetic studies.

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1. Introduction

Analysis of therapeutic peptides and proteins in bio-matrices is becoming very important in the pharmaceutical industry especially in the area of oncology therapy. Therapeutic peptides are generally either synthetic analogs of endogenous peptides, such as hormones or neurotransmitters, or are a derivative of the natural peptides [1]. Analysis of endogenous human serum peptides can be used for either diagnostic or treatment purposes [2]. Bioanalysis of smaller peptides is less complicated than analysis of large proteins, however, chromatographic separation and determination of peptides in digested protein samples can be a challenging procedure [3]. Immunoassay procedures have been used for the analysis of peptide therapeutics that can provide high sensitivity and rapid analysis; however, cross-reactivity and lengthy method development time have been considered main disadvantages of these methods. Liquid chromatography (LC) combined with ultraviolet (UV), fluorescence or electrochemical detection methods have been used for peptide analysis, although limited sensitivity and specificity, along with complicated sample pre-treatment steps, have been main drawbacks [1]. Two dimensional liquid chromatography coupled to tandem mass spectrometry may be the method of choice for peptide analysis due to high sensitivity, selectivity, reproducibility, rapid analysis, capability for the detection of co-eluting peptides, good chromatographic separation of complex peptide mixtures [1,4,5].

Octreotide [10-(4-aminobutyl)-19-(2-amino-3-phenylpropanoyl)-amino-16-benzyl-N-(1,3-dihydroxybutan-2-yl)-7-(1-hydroxyethyl)-13-(1H-indol-3-ylmethyl)-6,9,12,15,18-pentaoxo-1,2-dithia-5,8,11,14,17-pentazacycloicosane-4-carboxamide] (Fig. 1) is a synthetic long-acting cyclic octapeptide, which is a somatostatin analog that has a longer half life and more selectivity in inhibiting hormone secretion than somatostatin. It is used to control hormone hypersecretion in pituitary tumors, endocrine pancreatic tumors, and carcinoid tumors by inhibiting the release of growth hormone, insulin, glucagon, and other hormones [6,7]. Radio-immunoassay methods have been used for determination of octreotide levels in human blood, urine, and saliva [8] and in rabbit

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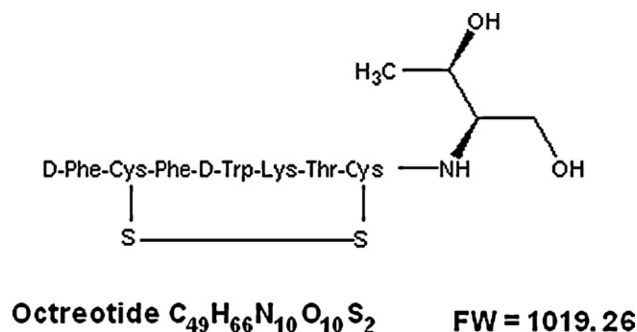

Fig. 1. Chemical structure, chemical formula and formula weight of octreotide.

plasma [9] after receiving of octreotide long acting formulation doses. A LC/MS method has been described for determination of octreotide in human plasma using selected ion monitoring (SIM) with a lower limit of quantitation (LLOQ) of 500 pg/ml [6]. A LC–MS/MS method has been reported for determination of octreotide in human plasma with a LLOQ of 50 pg/ml using triptorelin as an internal standard. The assay required a 400-μl plasma sample and used protein precipitation extraction followed by a dichloromethane wash [6]. Ultra high performance (or pressure) liquid chromatography (UHPLC) using small particle size stationary phases (less than 2 μm) provides many advantages including high separation efficiency, high sensitivity, and increased speed of analysis [10]. Assessment and elimination of matrix effects during LC–MS/MS method development has been shown to be essential for rugged and selective analysis [11–13]. Use of stable isotope-labeled internal standards [14], selection of the appropriate sample preparation technique and elimination of phospholipids have been found to be beneficial to ensure assay reliability. SPE methods have been found to provide clean extracts [15]. Two dimensional (2D) and multidimensional chromatographic techniques using switching valves have been used to improve peptide analysis [3,4,16]. In this study, a sensitive, selective and rapid method was developed and validated for determination of octreotide in human plasma using a simple μElution solid phase extraction and UHPLC–MS/MS. The method requires only 200 μL of human plasma and achieves a LLOQ of 25 pg/ml. A stable isotope-labeled octreotide internal standard was used to ensure optimum compensation for extraction and analysis variability. Direct MS monitoring phospholipids during method development and on-line removal of residual phospholipids in the final extracts was also employed to minimize matrix effects.

2. Experimental

2.1. Chemicals and reagents

Human plasma with tripotassium EDTA was purchased from Biological Specialty Corporation (Colmar, PA, USA), ammonium hydroxide (28–30%) and potassium hydroxide (45%) were from J.T. Baker (Philipsburg, NJ, USA). Formic acid (FA) and trifluoroacetic acid (TFA) were purchased from Sigma–Aldrich (ST. Louis, MO, USA). Acetonitrile (ACN), 0.1% formic acid in acetonitrile, methanol (MeOH), phosphoric acid (85%) and 0.1% formic acid in water, v/v, were all purchased from Burdicks & Jackson (B&J) (Muskegon, MI, USA). Oasis® WCX μElution 96-well plates, 30 μm (2 mg/well), were purchased from Waters Corp. (Milford, MA, USA). Isolute-96 100 mg C_{18} (EC) SPE plates were from Biotage (Charlotte, NC, USA). Octreotide acetate (M.W. 1019.26) and [$^{13}C_6Phe_3$] octreotide (cyclic) (M.W. 1025.17) were provided by PPD (Richmond, VA, USA).

2.2. Apparatus

The HPLC system consisted of an Agilent 1200 Series HPLC system (Agilent Technologies, Palo Alto, CA, USA) a Shimadzu, System Controller, SCL-10A Vp, Pump, LC 10AD Vp Solvent Degasser (DGU14A, Shimadzu, Columbia, MD, USA), and an CTC PAL autosampler (Zwingen, Switzerland). The Mass Spectrometer was an AB Sciex API 4000 system (Applied Biosystems Sciex, Ontario, Canada) using Analyst 1.4.2 software, which was operated in the electrospray ionization (ESI) positive multiple reaction monitoring (MRM) mode. The solid phase extraction was automated using a Tomtec Quadra 96 Model 320 liquid-handling system (Tomtec, Hamden, CT, USA).

2.3. Gradient method for the analysis of octreotide

The samples were analyzed using an ACQUITY UPLC® BEH C_{18}, 2.1 mm × 100 mm, 1.7 μm analytical column (Waters Corp, Milford, MA, USA), ACQUITY UPLC® BEH C_{18} VanGuard Pre-column, 2.1 mm × 5 mm, 1.7 μm (Waters Corp, Milford, MA, USA). Mobile phase A consisted of 0.1% formic acid, v/v, mobile phase B was 0.1% formic acid in acetonitrile, v/v, and mobile phase C (guard wash) was 10:45:45 water/acetonitrile/methanol, v/v/v.

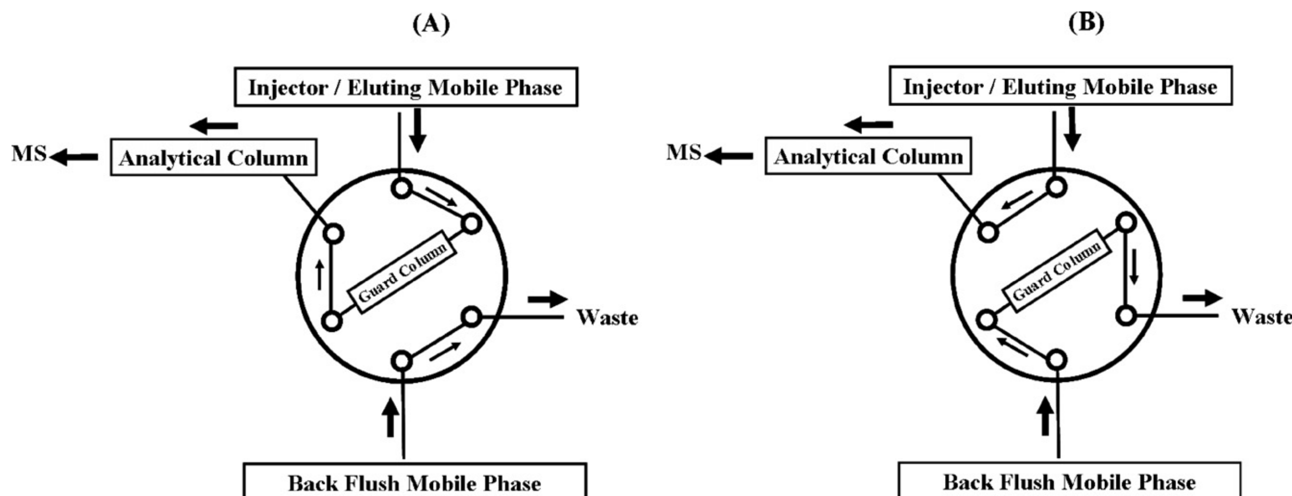

Fig. 2. Schematic diagram of the switching valve used to trap phospholipids on the guard column and transfer analytes onto analytical column (A), back flush the guard column into waste and elution of analytes from the analytical column onto the mass spectrometer (B).

Table 1
MRM parameters of octreotide and [$^{13}C_6Phe_3$] octreotide.

Analyte	t_R (min)	Dwell time (ms)	Q1 (m/z)	Q2 (m/z)	DP	CE	CXP	EP
Octreotide	4.15	200	510.5	120.1	60.0	48.0	6.0	9.0
Dummy transition[a]	N/A	20	600	400	60.0	48.0	6.0	9.0
[$^{13}C_6Phe_3$] octreotide	4.15	200	513.5	120.0	60.0	48.0	6.0	9.0
Dummy transition[a]	N/A	20	400	300	60.0	48.0	6.0	9.0

[a] Dummy transitions are used to prevent product ion crosstalk.

The autosampler utilized two rinse solutions, 0.1:10:90 formic acid/water/acetonitrile, v/v/v, and 20:80 acetonitrile/water, v/v, with a total run time of 7.5 min. The optimized chromatographic conditions were as described below:

The switching valve program consisted of 0–1.5 min at position A for loading sample and transferring analytes from the guard column into the analytical column, 1.5–4.5 min at position B for guard column back flush (at 1.5 ml/min) and 4.5–7.5 min position A for guard column and analytical column forward flush then re-equilibration (Fig. 2). The elution pumps ran a flow gradient as follows; 0–1.0 min 20% B at 0.25 ml/min flow rate, 1.0–4.7 min slow gradient from 20% to 40% B at 0.25 ml/min flow rate, 4.8–6.0 min 98% B at 0.3 ml/min flow rate and (6.1–7.5) min 20% B at 0.25 ml/min flow rate. The guard column back flush pump ran isocratic flow as follows; 0–1.5 min at 0.2 ml/min flow rate, 1.6–4.2 min at 1.5 ml/min flow rate and 4.3–7.5 min at 0.2 ml/min flow rate.

2.4. Mass spectrometry

The transitions (m/z 510.5 > 120.1) and (m/z 513.5 > 120.0) were used for octreotide and [$^{13}C_6Phe_3$] octreotide quantifications. The doubly charged ions of octreotide (m/z 510.5) and [$^{13}C_6Phe_3$] octreotide (m/z 513.5) were the main observed charged ions in the Q1 scan and were selected as the precursor ions for octreotide and its IS, respectively, and the fragment ions at m/z 120.0 were chosen for MS/MS acquisition. The MS/MS system parameters were: ion source temperature (500 °C), ion spray voltage (5000 V), electron multiplier CEM (2300 V) collision gas flow CAD (12), curtain gas flow CUR (35), nebulizer gas flow NEB/GS1 (50) turbo ion spray gas AUX/GS2 (30), deflector potential DF (−300), pause time (5 ms) mass transitions and MRM parameters as shown in Table 1.

2.5. Preparation of standards and quality control (QC) samples

Two separate 100 μg/ml stock solutions of octreotide were prepared in 0.1:20:80 formic acid/acetonitrile/water, v/v/v. The solutions were prepared by weighing approximately 1.0 mg of octreotide and quantitatively transferring it to an amber color glass bottle. The solution was diluted to approximately 10 ml using the appropriate solvent volume. After checking the octreotide responses from appropriate dilutions of these two solutions (the difference between the two stock solutions was less than 5%); one stock solution was used for preparation of calibration standards and the other was used for preparation of quality control (QC) samples. A 100 μg/ml stock solution of [$^{13}C_6Phe_3$] octreotide was prepared in 0.1:20:80 formic acid/acetonitrile/water, v/v/v, subsequent dilutions were made for preparation of the working internal standard (IS) solution at concentrations of 100.0 and 30.0 ng/ml in 0.1:20:80 formic acid/acetonitrile/water, v/v/v, for initial and modified methods, respectively. All solutions were stored at approximately 4 °C. The calibration standards were prepared by adding the appropriate amounts of the stock solution into pooled blank plasma with K_3EDTA. Nominal concentrations were 0.025, 0.05, 0.1, 0.4, 1.5, 6.0, 20.0, and 25.0 ng/ml of octreotide. QC samples (LLOQ, low, mid low, mid, mid high, high and a dilution QC) were prepared at concentrations of 0.025, 0.075, 0.2, 0.75, 3.0, 19.0, and 50.0 ng/ml of octreotide, respectively. Standards and controls were sub-aliquoted into polypropylene tubes and stored at approximately −20 °C. Another set of low and high QC samples were stored at approximately −70 °C to evaluate freeze/thaw and matrix stability at −70 °C.

2.6. Sample preparation method

A generic μElution extraction protocol suggested by the manufacturer for maximum peptide recovery has been applied. Human plasma samples were thawed at room temperature and vortex mixed. A 200-μl aliquot of each sample was transferred to a 96-position, 2.0-ml, square-well, conical-bottom, polypropylene plate. 200 μl of 4% phosphoric acid and 50 μl of 30.0 ng/ml internal standard working solution were added and vortex mixed briefly; samples were centrifuged for 5 min at 3000 rpm. The WCX μElution SPE plate was conditioned with 200 μl of methanol then equilibrated with 200 μl of water. Samples were loaded onto the SPE plate (2 × 225 μl). The sorbent was washed with 2 × 200 μl of 5% ammonium hydroxide, then with 2 × 200 μl 20% acetonitrile. Samples were eluted with 2 × 25 μl of 1% TFA in 75:25 acetonitrile/water, v/v, into a 96-position, 2.0-ml, square-well, conical-bottom, polypropylene plate. Samples were diluted with 150 μl of water and vortex mixed briefly. 50 μl of the resulting solution was injected onto the LC–MS/MS.

2.7. Monitoring phospholipids

Phospholipids were monitored using five specific phospholipid MRM transitions identified previously [15]. Parameters were also optimized for monitoring m/z 184 > 184 and m/z 104 > 104 as a common ion fragment for glycerophosphocholines and lysoglycerophosphocholines, respectively, without further fragmentation [17]. These ions were detected in plasma extracts during method development to monitor matrix effects using the same MS system parameters as mentioned above.

2.8. Validation

The resulting LC/MS/MS method for determination of octreotide in human plasma was validated according to the FDA guidelines [18] with a total run time of 7.5 min. The plasma calibration curve was constructed using eight calibration standards in duplicate over four validation runs. A linear, 1/concentration squared weighted least-squares regression algorithm was used to plot the peak area ratio of the analyte to its internal standard versus concentration.

Inter- and intra-assay precision and accuracy were calculated from quality control samples at five concentration levels (0.0250, 0.0750, 0.200, 0.750, 3.00, and 19.0 ng/ml) by analyzing each QC in duplicate in four validation runs. Intra-assay precision and accuracy were evaluated for each quality control pool, by multiple analyses (n = 6) of the pool in one validation run.

Extraction recovery of the analyte from human plasma was evaluated by comparing the analyte responses of pre-extraction spiked samples at the low, mid and high QC concentrations to those of post-extraction spiked samples representing 100% recovery.

Freeze/thaw stability was evaluated by analyzing two sets of low QC (0.075 ng/ml) and high QC (19.0 ng/ml) over five freeze/thaw cycles ($n = 6$). Samples were thawed at room temperature. One set was frozen at −20 °C and the other set was frozen at −70 °C for each freeze cycle. QC Samples for cycle one were frozen for at least 24 h then each consecutive cycle was frozen for at least 12 h before thawing at room temperature.

Analyte stability in thawed matrix was evaluated by removing a set of low QC (0.075 ng/ml) and high QC (19.0 ng/ml) samples from −20 °C storage, thawing and allowing to sit at room temperature for 24 h prior to extraction and analysis ($n = 6$).

Analyte stability in frozen matrix was evaluated by analyzing two sets of QC samples at low (0.075 ng/ml) and at high (19.0 ng/ml), QC samples. One set had been stored for 39 days at −20 °C and the other set had been stored for 39 days at −70 °C versus freshly prepared calibration standards.

Re-injection reproducibility was evaluated by analysis of calibration standards and quality control samples at five concentrations that were extracted and injected then stored for approximately 125 h at 2–8 °C prior to reanalysis.

Post-preparative extract stability was evaluated by re-injecting quality control extracts at low (0.075 ng/ml) and high (19.0 ng/ml) QC concentrations that were stored at 2–8 °C for approximately 125 h following their original extraction and analysis. The re-injected sample extracts were quantified versus freshly prepared calibrators.

Analyte and IS stability in solution under nominal storage and bench-top stress conditions were demonstrated. A whole blood stability evaluation was performed to assess the stability of octreotide at low QC and high QC concentrations in whole blood under the following sample collection and handling procedures: (1) time lapse between collection and storage (0–60 min), (2) use of an ice bath versus room temperature and (3) refrigerated versus non refrigerated centrifuge.

Human plasma samples, containing tripotassium EDTA, from six individuals were extracted and analyzed for interferences at the retention times of octreotide and its internal standard.

The ability to analyze samples with insufficient volume for a full aliquot was validated by analyzing six replicates of a 0.750 ng/ml QC sample as 4-fold dilutions with blank plasma. The ability to dilute samples originally above the upper limit of the calibration range was validated by analyzing six replicate of a 50.0 ng/ml QC sample as 10-fold dilutions with blank plasma.

Cross analyte interference was evaluated by analysis of 3 aliquots of blank human plasma or external solution that were fortified with the analyte only at the ULOQ or internal standard at the level of use.

The potential for carryover from a sample containing a high concentration of analyte to the following sample in an injection sequence was evaluated by injection of duplicate extracted matrix blanks immediately after the ULOQ calibration standards in each validation run.

The method has been used to determine octreotide in human plasma after a subcutaneous administration.

2.8.1. Post-extraction addition

Post-extraction addition experiments were conducted at low, mid and high QC concentrations. Post-spiked samples were prepared in which 200 μl of human blank plasma ($n = 9$) were aliquoted and extracted as described under Section 2.8 until the elution step. At the elution step, the designated low, mid and high wells ($n = 3$, each) were spiked with 50 μl of (0.3/30, 3.0/30 and 76/30 ng/ml) solutions of octreotide/[$^{13}C_6Phe_3$] octreotide, respectively, then diluted with 100 μl of water and vortex mixed briefly. 50 μl of the resulting solution was injected onto the LC–MS/MS. External samples were prepared in which 200 μl of water ($n = 9$) were aliquoted and extracted as described under Section 2.8 until the elution step. At the elution step the designated low, mid and high concentration wells ($n = 3$, each) were spiked with 50 μl of (0.3/30, 3.0/30 and 76/30 ng/ml) solutions of octreotide/[$^{13}C_6Phe_3$] octreotide, respectively, then diluted with 100 μl of water and vortex mixed briefly. 50 μl of the resulting solution was injected into LC–MS/MS.

2.8.2. Evaluation of matrix effects from multiple individuals (inter-subject variability)

Quality control samples ($n = 3$) at a concentration of 0.025 ng/ml of octreotide were spiked into 6 different human blank plasma samples from different donors, extracted and analyzed as described under Section 2.8. QC samples ($n = 3$) at a concentration of 0.025 ng/ml of octreotide were prepared in the matrix pool that was used to prepare the calibration standards and quality control samples, and analyzed for comparison.

3. Results and discussions

3.1. Method development for the analysis of octreotide

A Xterra MS C_{18} 2.1 × 50 mm, 5 μm analytical column (Waters Corp, Milford, MA, USA) and a Hypersil BDS C_{18} 2.1 × 20 mm, 5 μm guard column (Thermo Scientific, Waltham, MA, USA), using an ammonium hydroxide mobile phase were used initially to separate analyte and IS. Isolute-96 100 mg C_{18} (EC) SPE plate was initially used for sample extraction; poor sensitivity and broad peaks were observed at the LLOQ level, the analyte and IS peaks were eluted at 1.85 min with a 0.25 min peak width. The intra-assay precision ranged from 1.81 to 46.48% and the intra-assay accuracy ranged from −0.33 to 26.67%. This high variability could have been due to column instability at high pH or inconsistent analyte extraction recovery or matrix effects. It was found necessary to modify the original method to improve sensitivity, precision and accuracy; modifications included changing the sample preparation procedure, column and chromatographic conditions.

Mixed-mode cation exchange SPE has been found to provide cleaner extracts than polymeric reversed-phase and pure cation exchange SPE [15]. Oasis® WCX μElution SPE is recommended by the manufacturer for peptide analysis and is reported to provide high recovery with low residual matrix effects. Another advantage of μElution SPE is the small solvent volumes required for elution, which produces a high concentration extract, without the need for evaporation and reconstitution which can cause degradation or losses of peptide analytes. Oasis® WCX μElution SPE was used successfully for extraction of octreotide from human plasma with high recovery and minimum matrix effects.

UHPLC using a small particle size stationary phase (1.7 μm) provided advantages over conventional HPLC including high separation efficiency, higher sensitivity and rapid analysis [10] An ACQUITY UPLC® BEH C_{18}, 2.1 mm × 100 mm, 1.7 μm analytical column with an acidic mobile phase was used to separate octreotide from matrix components. Excellent peak shape (<6 s peak width), an approximate 4–5-fold increase in the peak height, and more than 2-fold increase in the peak area at the LLOQ concentration over the initial chromatographic conditions were obtained. The BEH column technology is reported to provide column stability through a wide pH range (1–12).

A shorter (5 cm) UHPLC C_{18} analytical column would be expected to provide lower backpressure and faster phospholipid removal at high flow rate as compared to a longer (10 cm) UHPLC C_{18} column. Although the ACQUITY UPLC® BEH C_{18}, 2.1 mm × 50 mm, 1.7 μm analytical column provided excellent peak shape, matrix interfering peaks were observed in the final Oasis® WCX μElution extracts which eluted very close to the tar-

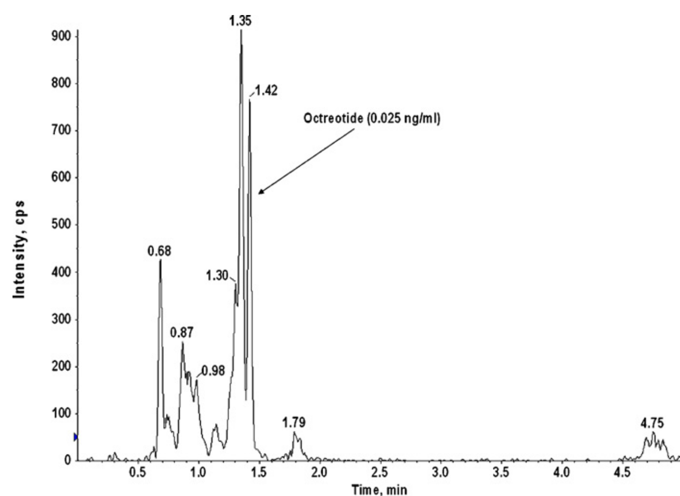

Fig. 3. MRM chromatogram of the LLOQ (0.025 ng/ml) of octreotide, *m/z* 510.5 > 120.1 using the ACQUITY UPLC BEH C_{18}, 2.1 mm × 50 mm, 1.7 μm analytical column and acidic mobile phase.

get analyte peak as shown in Fig. 3. Use of a longer analytical column (10 cm) and through optimization of the gradient conditions, the analyte and IS peaks were chromatographically resolved from interfering peaks (Fig. 4). No interfering peaks were observed from the extracts of the human plasma from six different individual sources.

3.2. Monitoring phospholipids

Monitoring phospholipids during method development has been found to be helpful to avoid matrix effects in LC/MS/MS bioanalysis [11,12]. Five characteristic mass transitions (*m/z* 496 > 184, 524 > 184, 704 > 184, 758 > 184, 786 > 184, and 806 > 184) have been identified for monitoring major plasma phospholipids [15]. Another approach for monitoring phospholipids is the in-source multiple reaction monitoring (IS-MRM) approach, using *m/z* 184 > 184 and *m/z* 104 > 104 to monitor all glycerophosphocholines (GPChos) and lysoglycerophosphocholines (Lyso-GPChos), respectively, using only one MRM channel for each group and without further fragmentation [17]. Both approaches were used to monitor phospholipids in the final extracts of the μElution WCX SPE carried out in this work.

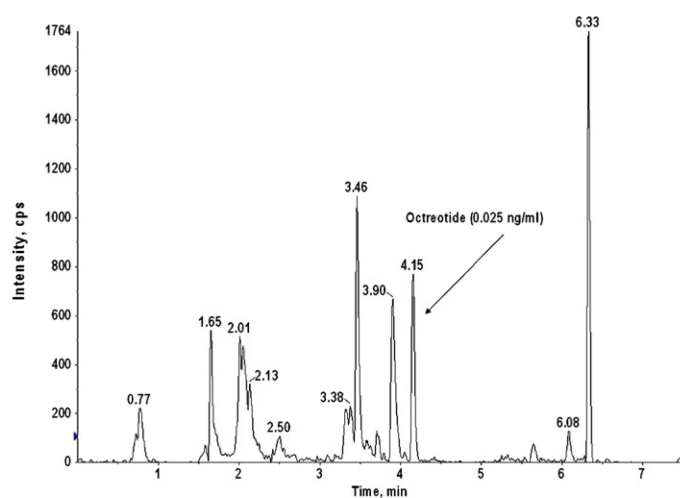

Fig. 4. MRM chromatogram of the LLOQ (0.025 ng/ml) of octreotide, *m/z* 510.5 > 120.1 using the ACQUITY UPLC BEH C_{18}, 2.1 mm × 100 mm, 1.7 μm analytical column and acidic mobile phase.

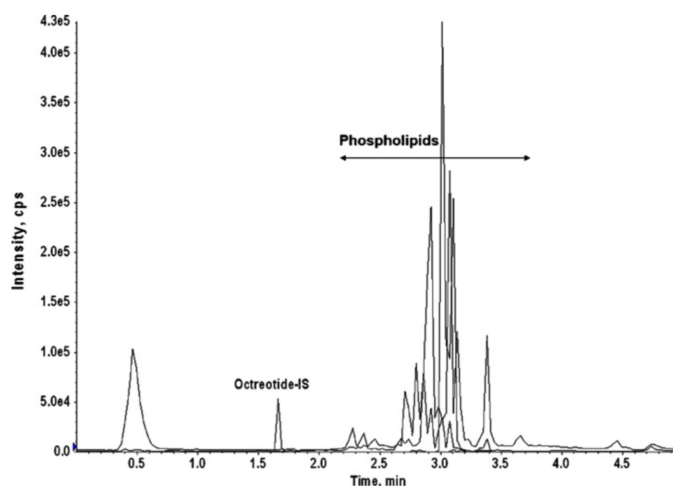

Fig. 5. XIC of +MRM (9 pairs) chromatogram of phospholipid elution profile and the octreotide internal standard peak (*m/z* 513.5 > 120.0) using the ACQUITY UPLC BEH C_{18}, 2.1 mm × 50 mm, 1.7 μm analytical column with an acidic mobile phase, flow rate 300–600 μl/min and valve switching at 3.2 min.

3.3. Online removal of residual phospholipids and optimization of chromatographic conditions

SPE methods are known to provide relatively clean extracts and a 100% methanol wash step in mixed-mode ion exchange SPE methods has been shown to remove endogenous matrix components and phospholipids [15]. However, the absence of this step in the manufacturer-suggested μElution SPE protocol for maximum general peptide recovery may be expected to lead to more phospholipids in the final extracts. Phospholipids are present at high concentrations in bio-matrices and residual phospholipids present in extracts following sample preparation may accumulate on analytical columns (especially on highly retentive reversed-phase columns). Complete elution of these phospholipids from analytical columns requires significant time with a high percentage of appropriate organic modifier in the mobile phase. If phospholipids are allowed to accumulate, they may slowly migrate and elute from the column in a subsequent sample injection, leading to variable matrix effects from one sample to another and imprecise and inaccurate results [11].

Fig. 5 shows phospholipid elution and the octreotide IS peak using the ACQUITY UPLC® BEH C_{18}, 2.1 mm × 50 mm, 1.7 μm analytical column. Phospholipids eluted from 2.2 to 3.5 min at a 400–600 μl/min flow rate and 98% of mobile phase B (0.1% formic acid in ACN). The phospholipids eluted were mainly Lyso-GPChos and most of the GPChos were still retained on the reversed phase column. These GPChos may elute in subsequent injections causing analyte response variability. Complete elution of phospholipids from the reversed phase analytical column may require long separation times, higher flow rates and/or different organic solvents (e.g. methanol or ACN/methanol mixture).

Online removal of phospholipids was found to be necessary in this study. This was accomplished by use of a guard-type precolumn column and a switching valve with a short transfer window to trap lipids on the guard column and then flush them into waste (Table 2). This was found to be a good approach to remove residual phospholipids and avoid accumulation of phospholipids on the reversed phase analytical column used in this study. Back flush of the guard column was carried out at high flow rate (1.5 ml/min) using a mobile phase consisting of 10:45:45 water/acetonitrile/methanol, v/v/v. No phospholipids peaks were observed after using the valve switching approach (Fig. 6). The early eluting peak at 0.5 min may be an endogenous interfering peak (e.g. un-retained inorganic salts). This peak appears only at the mass transition of *m/z* 104 > 104 and

Table 2
Valve switching program.

Time (min)	Position	Comments
Initial	A	• Load sample • Transfer analyte into analytical column
1.5	B	• Guard column back flush • Elute analyte from analytical column
4.5	A	• Forward flush of guard column and analytical column. • Re-equilibration

may be due to the low mass and inspecificity of the *m/z* 104 > 104 mass transition.

The optimized chromatographic conditions and valve switching using the ACQUITY UPLC® BEH C_{18}, 2.1 mm × 100 mm, 1.7 μm analytical column and ACQUITY UPLC® BEH C_{18}, 2.1 mm × 5.0 mm, 1.7 μm guard column to remove phospholipids and chromatographically resolve the analyte and IS peaks from interfering peaks were described under Section 2.4.

A short transfer window (1.5 min) was used to transfer analyte from the guard column to the analytical column at relatively low organic content (∼23% B) to minimize the transfer of phospholipids into the analytical column. In addition to the backward wash of, the guard column at high flow rate with high organic content, a forward washing step for the analytical column using 98% B was established after the analyte elution to ensure complete removal of any residual phospholipids that may have broken through the pre-column.

3.4. Validation

3.4.1. Linearity and calibration curve

The average correlation coefficient from four validation runs was >0.999. Precision of the standards (measured as the percent relative standard deviation %RSD) of back calculated standards was within 4.2%. Accuracy of the residuals (measured as the percent difference from the nominal concentration, %DFN) was within ±1.25%. The LLOQ was established as the lowest concentration on the calibration curve with accuracy and precision within ±20% (25.0 pg/ml) and linearity was obtained over the concentration range of (25.0 pg/ml to 25.0 ng/ml).

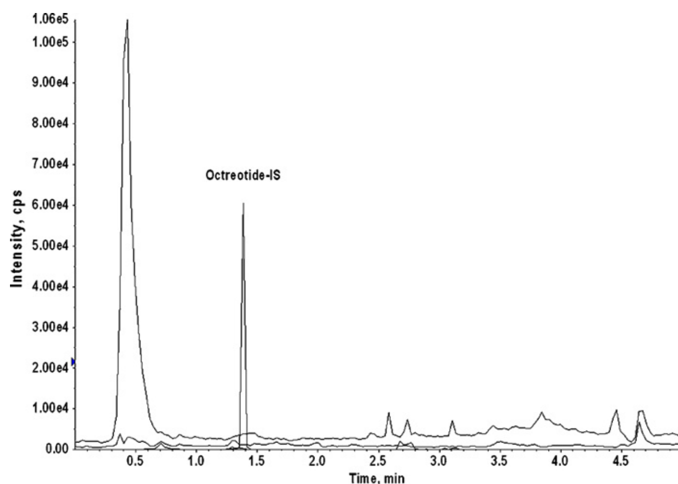

Fig. 6. XIC of +MRM (9 pairs) chromatogram of phospholipid elution profile and octreotide internal standard peak (*m/z* 513.5 > 120.0) using the ACQUITY UPLC BEH C_{18}, 2.1 mm × 50 mm, 1.7 μm analytical column with an acidic mobile phase, flow rate 400–600 μl/min and valve switching at 1.0 min to back flush phospholipids from the guard column to waste.

Table 4
Precision and accuracy calculated from stability experiments.

Experiment	%RSD	%DFN
Freeze/thaw stability (−20 °C, 5 cycles)	<2.10	<0.47
Freeze/thaw stability (−70 °C, 5 cycles)	<4.70	<1.50
Analyte stability in thawed matrix (24 h at RT)	<5.00	<−4.10
Analyte stability in frozen matrix (39 days at −20 °C)	<2.80	<3.10
Analyte stability in frozen matrix (39 days at −70 °C)	<1.90	<3.60
Analyte stability in whole blood	<2.70	<±2.00
Re-injection reproducibility	<3.00	<−1.20
Post preparative extract stability (125 h at 2–4 °C)	<2.90	<6.70

3.4.2. Precision and accuracy

Inter- and intra-assay precision was less than 3.2%, inter- and intra-assay accuracy was within ±1.93%. Inter- and intra-assay precision at the LLOQ was 6.06 and 4.42%, respectively (Table 3). Inter- and intra-assay accuracy at the LLOQ was −1.23 and 0.256%, respectively. The method showed accurate and precise results. The %RSD of the analyte absolute area responses at the LLOQ level ($n = 6$) was 5.2%, and the %RSD of the IS absolute area responses at the concentration used in the assay ($n = 36$) was 5.7%.

3.4.3. Recovery

Extraction recoveries of octreotide at 0.075, 0.75 and 19.0 ng/ml were 67.9%, 74.2% and 75.8%, respectively (the %RSD was 5.75%). The extraction recovery of the IS at the proposed concentration used in the assay ranged from 72.1% to 77.9% (the %RSD was 4.15%).

3.4.4. Stability experiments

All stability experiments showed precision within 5.0% and accuracy within ±6.7% (Table 4).

Octreotide acetate and [$^{13}C_6Phe_3$] octreotide (cyclic) stock solutions (100 μg/ml) were stable in 0.1:20:80 FA/ACN/water, v/v/v, solution at 2–8 °C for 33 and 430 days, respectively, and at room temperature for 6.0 h.

3.4.5. Specificity

There was no significant chromatographic peak (greater than 20% of the mean response at the lower limit of quantification) detected at the mass transitions and expected retention times of the analyte or its internal standard that would interfere with quantification.

3.4.6. Dilution integrity

The intra-assay precision and accuracy for the diluted QC pools was less than 3.3% and within ±2.8%, respectively.

Table 3
Intra- and inter-day precision and accuracy calculated from quality control (QC) samples.

QC conc. (ng/ml)	Intra-day ($n = 6$)				Inter-day ($n = 8$)			
	Mean (ng/ml)	SD	%RSD	%DFN	Mean (ng/ml)	SD	%RSD	%DFN
0.025	0.025	0.001	4.42	0.256	0.025	0.002	6.06	−1.23
0.075	0.076	0.002	3.15	0.607	0.076	0.002	3.04	0.949
0.200	0.198	0.002	1.09	−1.01	0.199	0.003	1.57	−0.696
0.750	0.740	0.015	1.98	−1.27	0.749	0.012	1.55	−0.076
3.00	2.94	0.037	1.26	−1.92	2.98	0.050	1.68	−0.524
19.00	18.80	0.218	1.16	−1.03	19.00	0.447	2.35	0.153

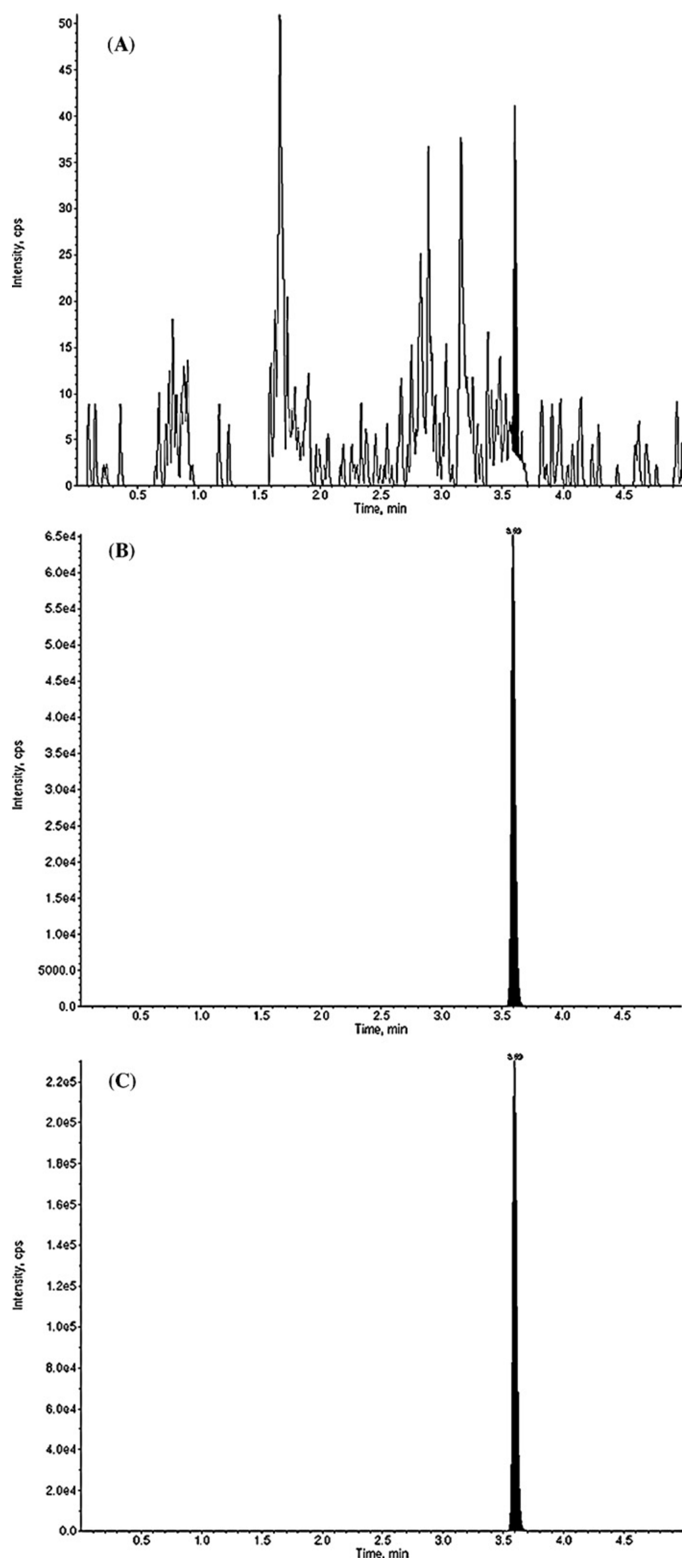

Fig. 7. MRM chromatograms of octreotide (*m/z* 510.5 > 120.1) in human plasma samples collected immediately before administration (A), after 30 min of subcutaneous injection (B) and after 24 h of subcutaneous injection (C).

3.4.7. Cross analyte interference and carry over

There were no significant chromatographic peaks detected at the mass transitions or expected retention times of the unfortified components. No significant cross analyte interference was observed from analyte to IS (less than 5% of the IS response at the level of use) or from IS to analyte (less than 20% of the analyte response at the LLOQ level).

No carry over was observed; there were no contributions from chromatographic peaks, at the expected retention time of the analyte in the blank samples (less than 20% of the analyte response at the LLOQ level).

3.4.8. Post-extraction addition experiments

Post-extraction addition experiments were conducted to quantitatively assess matrix-related ionization effects, by comparing analyte responses of the post-extraction spiked matrix samples to those of samples prepared at the same analyte concentrations (free from matrix components). At the low, mid and high QC concentrations, results showed that matrix effects were less than 4% for both the analyte and IS.

3.4.9. Evaluation of matrix effects from multiple individuals (inter-subject variability)

Variable matrix effects from different sources (i.e. donors) may seriously affect LC/MS/MS sample analysis, and acceptable precision and accuracy results in one plasma source does not ensure the validity of the method in multiple plasma sources [12]. The effects in different sources should be investigated during method development and validation. Inter-subject experiments showed that all subjects demonstrated acceptable accuracy and precision with precision less than 4.8% for all sources (3.08% for control) and inaccuracy less than 4.2% for 5 out of 6 sources (control, 2.0%) One source out of the six subjects demonstrated accuracy within 10.4%.

3.5. Pharmacokinetic study

This method has been utilized to support of clinical pharmacokinetic studies; 10 and 20 mg/ml octreotide were administrated subcutaneously, Fig. 7 shows the octreotide peaks in human plasma samples collected after 0, 0.5 and 24 h of administration.

4. Conclusion

A sensitive and selective UHPLC–MS/MS method for determination of octreotide in human plasma was developed and validated. The total run time was 7.5 min and the method provides a LLOQ of 25 pg/ml with 200 μl plasma. A simple μElution SPE method was used for sample preparation, and excellent peak shape was obtained with the optimized UHPLC conditions. It was found that monitoring phospholipids during method development was useful to assess and eliminate their matrix ionization effects, which were negligible with the final optimized method. Online phospholipid removal using a guard column and a switching valve were used successfully to avoid the accumulation of phospholipids on the reversed phase analytical column and ensure complete elution of the trapped phospholipids after each analysis.

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