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Nota applicativa

Increasing Sensitivity and Minimizing Sample Volume for the Quantification of Therapeutic and Endogenous Cyclic Peptides in Plasma Using ionKey/MS

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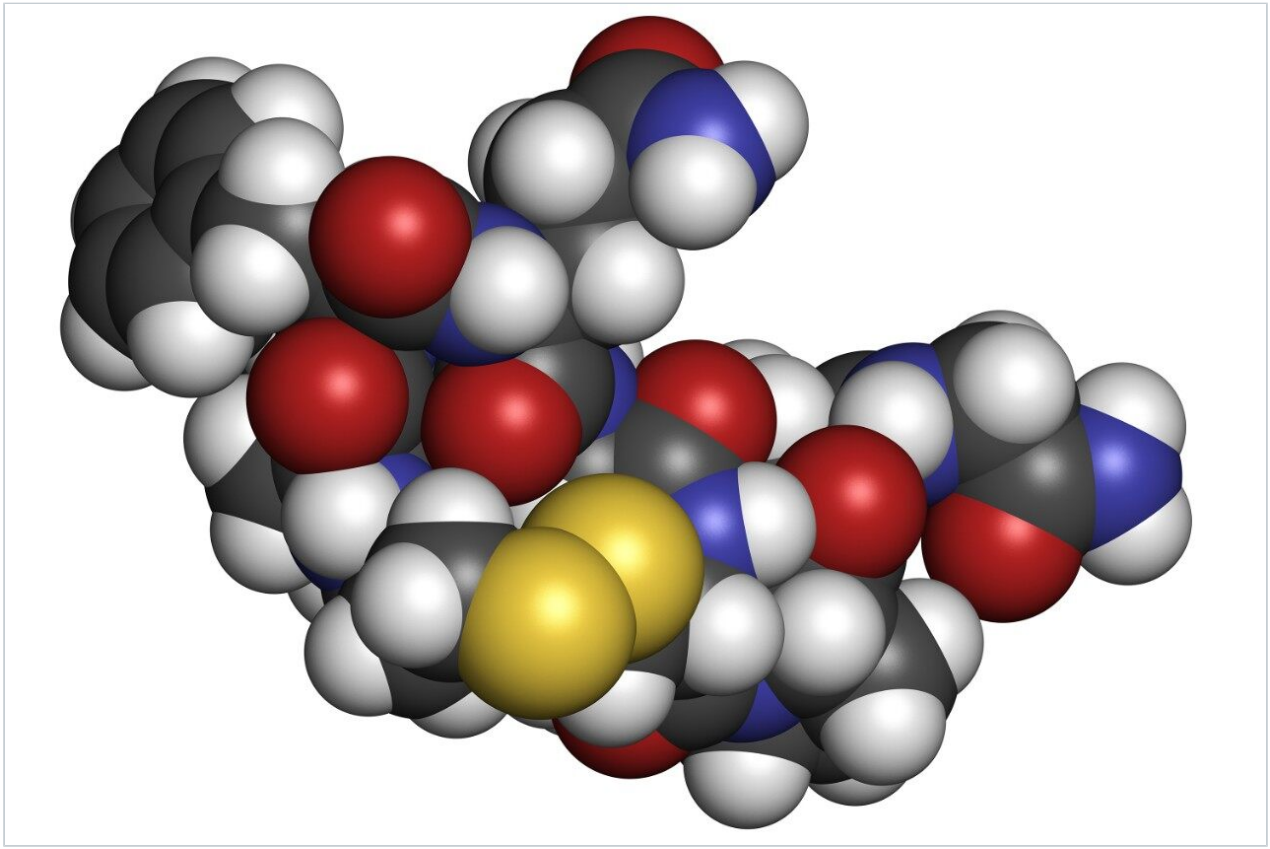


Abstract

This application note investigates the improved sensitivity and decreased sample volume requirements for the therapeutic and endogenous cyclic peptides: desmopressin, vasopressin and octreotide.

Benefits

- High sensitivity assay with LOQ of <1 pg/mL with only 100 µL of plasma
- Use of the ionKey/MS System facilitates detection limits of 2.5 pg/mL from only 25 µL of plasma
- Use of mixed-mode solid-phase extraction (SPE) reduces matrix interferences and enhances selectivity of the extraction in plasma
- 96-well µElution plate format enables concentration of the sample while maintaining solubility and minimizes peptide loss due to adsorption
- Selective, fast SPE extraction (<30 minutes) without time-consuming immuno-affinity purification
- Reduced solvent consumption (40X) compared to 2.1 mm scale means significant cost savings

Introduction

The use of peptides and proteins as therapeutic agents has increased significantly in recent years. Thus, the demand for their analysis for toxicokinetic and pharmacokinetic studies is increasing as well.

Historically, biologics have been quantified using ligand binding assays (LBAs). However, with recent advances in mass spectrometry (MS) and liquid chromatography (LC) technologies current approaches towards peptide quantification in biological fluids now include LC-MS/MS. This is in part driven by the fact that LBAs can suffer from significant cross-reactivity issues and lack of standardization. Additionally, LC-MS/MS also has the advantage of greater accuracy and precision, broader dynamic ranges, specificity, and speed of method development. However, accurate quantification of peptides by LC-MS/MS is often not without its own challenges. Peptides have diverse pharmacokinetic profiles, often low circulating plasma levels (pg/mL), generally low MS sensitivity, and require chromatographic resolution from endogenous isobaric matrix interferences.[1] Therefore, to achieve low pg/mL quantification limits, large plasma sample volumes (0.2–1 mL) and sample injection volumes are often required.[2-6] These volumes are often impractical

in discovery studies. Thus, the demand for quantitative bioanalytical assays that use decreased sample volumes, while maintaining or improving sensitivity are highly desired.

This application investigates the improved sensitivity and decreased sample volume requirements for the therapeutic and endogenous cyclic peptides: desmopressin, vasopressin and octreotide.[7-9] The general properties of these peptides are shown in Table 1. Using a combination of selective µElution mixed-mode SPE sample preparation, optimal MS precursor and fragment choice, and the ionKey/MS System (source shown in Figure 1), limits of quantification of 1 pg/mL in plasma were achieved. Capitalizing on the attributes of the ionKey/MS System facilitated reducing plasma sample required to 25–100 µL.

Experimental

Method conditions

UPLC conditions

LC system:	ACQUITY UPLC M-Class, configured for trap and back-flush elution
Separation device:	iKey HSS T3, 1.8 µm, 100Å, 150 µm x 100 mm iKey (p/n 186007261)
Trap column:	ACQUITY UPLC M-Class Symmetry C_{18}, 5 µm, 300 µm x 50 mm (p/n 186007498)
Mobile phase A:	0.1% formic acid in water
Mobile phase B:	0.1% formic acid in acetonitrile
Loading solvent:	98:2 mobile phase A:B, 25 µL/min for first two minutes, reverse valve
Valve position:	Initial position one (forward loading of trap), switch to position two at two minutes (back flush

	elute of trap onto the analytical column)
Analytical gradient:	See Table 2
Elution flow rate:	3.0 µL/min
iKey temp.:	75 °C
Sample temp.:	15 °C
Injection volume:	5 µL
Total run time:	12.0 min
Collection plates:	Waters 1 mL Collection Plates

MS conditions

MS system:	Xevo TQ-S
Ionization mode:	ESI positive
Capillary voltage:	3.8 kV
Source temp.:	120 °C
Cone gas flow:	100 L/hr
Collision cell pressure:	$5.5 \times 10^{(-3)}$ mbar
Collision energy:	Optimized by component, see Table 3
Cone voltage:	Optimized by component, see Table 3

Data management

Chromatography software:	MassLynx 4.1
Quantification software:	TargetLynx

Peptide	Amino acid sequence	MW	pI	HPLC index
Octreotide	8Phe-Cys-Phe-Trp-Lys-Thr-Cys-Thr-ol [Disulfide bridge: 2-7]	1019	9.3	40.8
Desmopressin	Mpa-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH2 [Disulfide Bridge: 1-6]	1069	8.6	16.8
Vasopressin	Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH2 [Disulfide Bridge: 1-6]	1084	9.1	7.6

Table 1. Peptide chemical properties.

Figure 1. ionKey Source.

Gradient:	Time (min)	Profile %A	%B	Curve
	0.0	98	2	6
	5.0	50	50	6
	5.5	50	50	6
	7.0	10	90	6
	8.0	10	90	6
	9.0	98	2	6

Table 2. LC gradient conditions.

Sample preparation

Sample pre-treatment

100 µL of human plasma was diluted 1:1 with 4% H_3PO_4 in water and mixed.

Sample extraction with Oasis WCX

Pre-treated plasma samples were extracted according to the protocol in Figure 2. All solutions are made up by volume. All extraction steps were applied to all wells of the µElution plate that contained samples.

Oasis WCX µElution Protocol
Part number: 186002499
Condition: 200 µL MeOH
Equilibrate: 200 µL H_2O
Load: 200 µL Diluted Plasma
Wash 1: 200 µL 5% NH_4OH in H_2O
Wash 2: 200 µL 10% ACN in H_2O
Elute: 2 x 25 µL of 2% FA in 50:50 ACN:H_2O
Dilute: 50 µL H_2O

Figure 2. Oasis µElution WCX extraction protocol.

Results and Discussion

Mass spectrometry

The 2+ precursors of desmopressin (*m/z* 535.45), vasopressin (*m/z* 542.75), and octreotide (*m/z* 510.30) were used for quantitation. Their corresponding fragments and optimal MS conditions are shown in Table 3. In this assay, the use of highly specific b/y ion specific fragments was more challenging due to the small size and cyclic nature of these peptides. The fragment at *m/z* 328.2, corresponding to a $y3^{1+}$ ion, was chosen for desmopressin and vasopressin. The fragment at *m/z* 120.1, corresponding to the phenylalanine immonium ion, was used for octreotide.

Peptide	Precursor	MRM Transition	Cone Voltage (V)	Collision Energy (eV)	Product Ion type
Desmopressin	$[M+2H]^{2+}$	535.4>328.2	40	12	$y3^{(1+)}$
Vasopressin	$[M+2H]^{2+}$	542.7>328.2	40	14	$y3^{(1+)}$
Octreotide	$[M+2H]^{2+}$	510.3>120.1	25	17	immonium ion (Phe)

Table 3. MS conditions for cyclic peptides.

Chromatographic separation

Chromatographic separation was achieved using the novel microfluidic chromatographic iKey Separation Device. The iKey Separation Device (Figure 3) is packed with UPLC-grade sub-2-µm particles that permit operation at high pressure and results in highly efficient LC separations. By integrating microscale LC components into a single platform design, problems associated with capillary connections, including manual variability, leaks, and excessive dead volume, are avoided. Use of the iKey HSS T3, 1.8 µm, 100Å, 150 µm x 100 mm (p/n 186007261) provided chromatographic retention, excellent peak shape, narrow peak widths (<4.5 seconds at base), and resolution from endogenous matrix interferences.

Figure 3. iKey Separations Device.

The peptides were eluted using a linear gradient from 2–50% B over 5 minutes, Table 2. Representative chromatograms are shown in Figure 4. The use of a trap and back-flush elution strategy, provided further sample cleanup and facilitated the loading of 5 µL of the high organic SPE eluate (required to maintain solubility of the peptides) without experiencing analyte breakthrough. Additionally, the ability to inject sample volumes typical for 2.1 mm analytical scale LC analysis on the iKey Separation Device can provide the substantial gains in sensitivity that are often required to accurately and reliably detect low pg/mL levels of peptides and proteins in complex matrices.

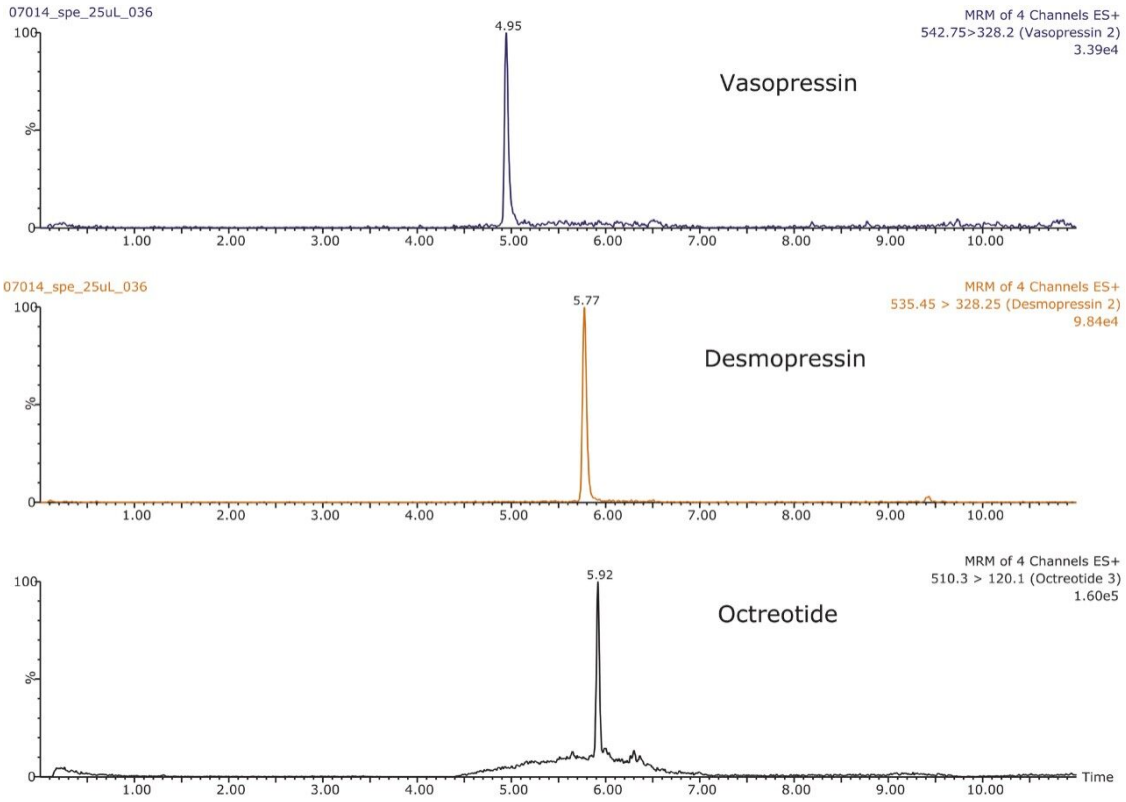


Figure 4. UPLC separation of desmopressin, vasopressin, and octreotide, using the iKey HSS T3, 1.8 µm, 100Å, 150 µm x 100 mm (p/n 186007261).

Enhanced sensitivity with the use of ionKey/MS

Versus analytical scale (2.1 mm I.D.), the ionKey/MS System generally offers increased sensitivity, making it ideal for high sensitivity peptide analysis. This also facilitates the use of smaller sample volumes whilst maintaining or improving sensitivity. In Figures 5 and 6, detection of 2.5 pg/mL of desmopressin and octreotide was easily obtained from extraction of 25, 100, or 200 µL human plasma, using injection volumes ≤10 µL.

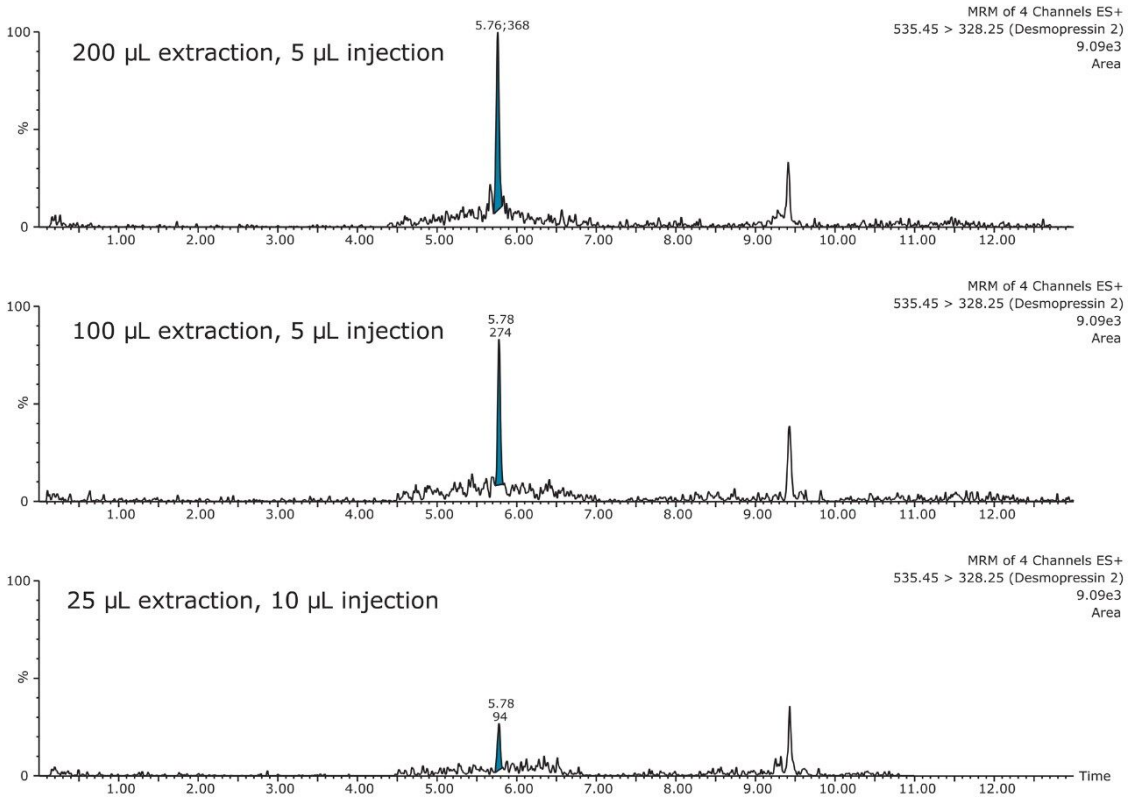


Figure 5. Enhanced sensitivity using the ionKey/MS System: Extraction volume comparison of desmopressin (2.5 pg/mL) from human plasma.

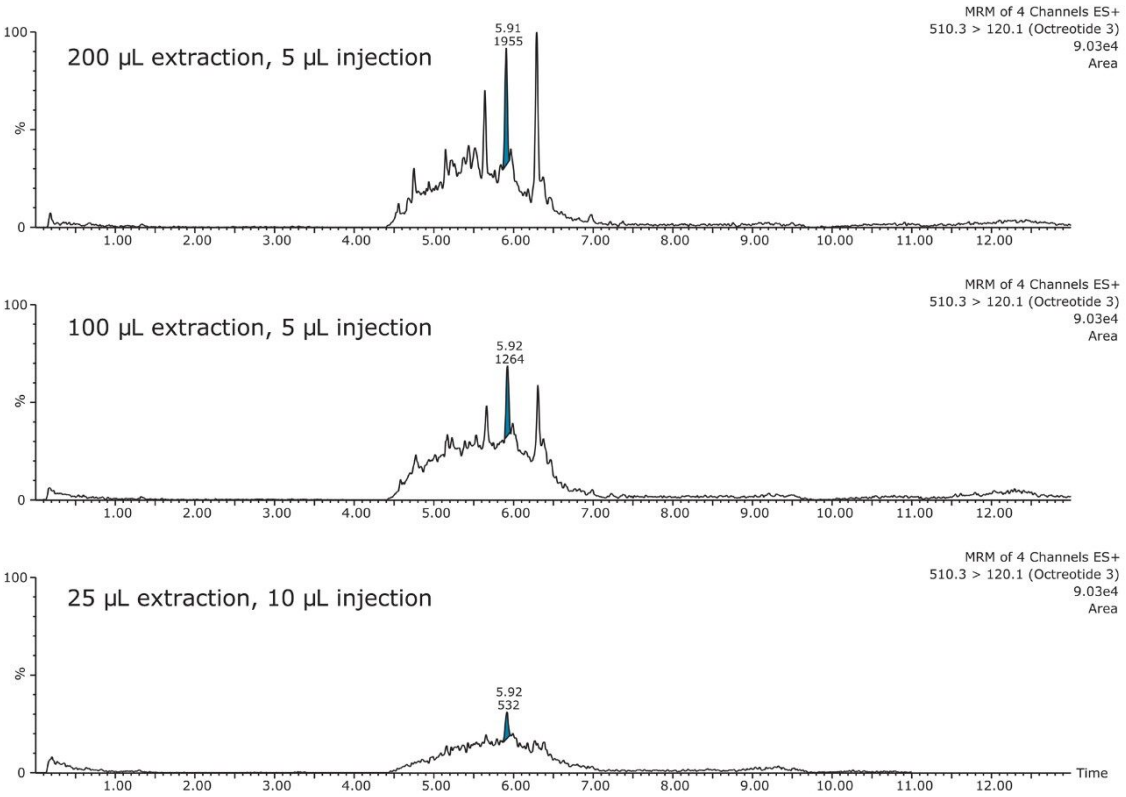


Figure 6. Enhanced sensitivity using the ionKey/MS System: Extraction volume comparison of octreotide (2.5 pg/mL) from human plasma.

Sample preparation

SPE was performed using Oasis WCX, which has both reversed-phase and ion-exchange modes of retention. The orthogonality introduced by the use of mixed-mode sorbents such as these enables greater sample cleanup, improved selectivity, and the sensitivity required for these peptides. Briefly, desmopressin, vasopressin, and octreotide were spiked at various concentrations into the plasma and mixed. These samples were then acidified with 4% H_3PO_4, which helped disrupt protein binding and reduce sample viscosity, improving contact time with the sorbent. Samples were loaded to the SPE device, and washed with 5% NH_4OH followed by 10% acetonitrile. The optimum elution solution was 50% organic, 25% water, with 2% formic acid.

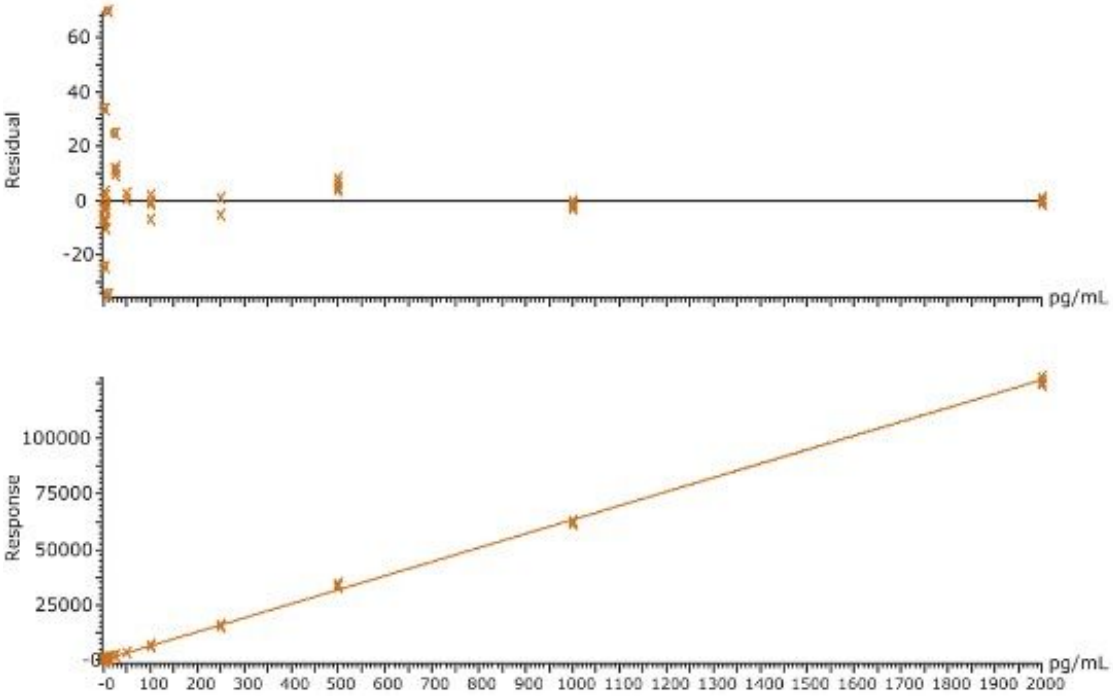
The 96-well Oasis µElution Plate format facilitates fast sample processing (under 30 minutes), and is compatible with automation by most liquid-handling robotic systems, improving sample throughput. Additionally, this format also provides the ability to elute in very small sample volumes, minimizes the potential for adsorptive peptide losses, as well as concentrates the sample for increased sensitivity.

Linearity, accuracy, and precision

To generate standard curves, human plasma was fortified with desmopressin, vasopressin and octreotide at the following final concentrations: 1, 2.5, 5, 10, 25, 50, 100, 250, 500, 1000, and 2000 pg/mL. SPE of the fortified plasma samples was performed as described above. The calibration curves were constructed using peak areas of the calibration samples by applying a one/concentration (1/x) weighted linear regression model. Using 100 µL of plasma, calibration lines were obtained for each peptide and are shown in Figure 7, panels A, B, C.

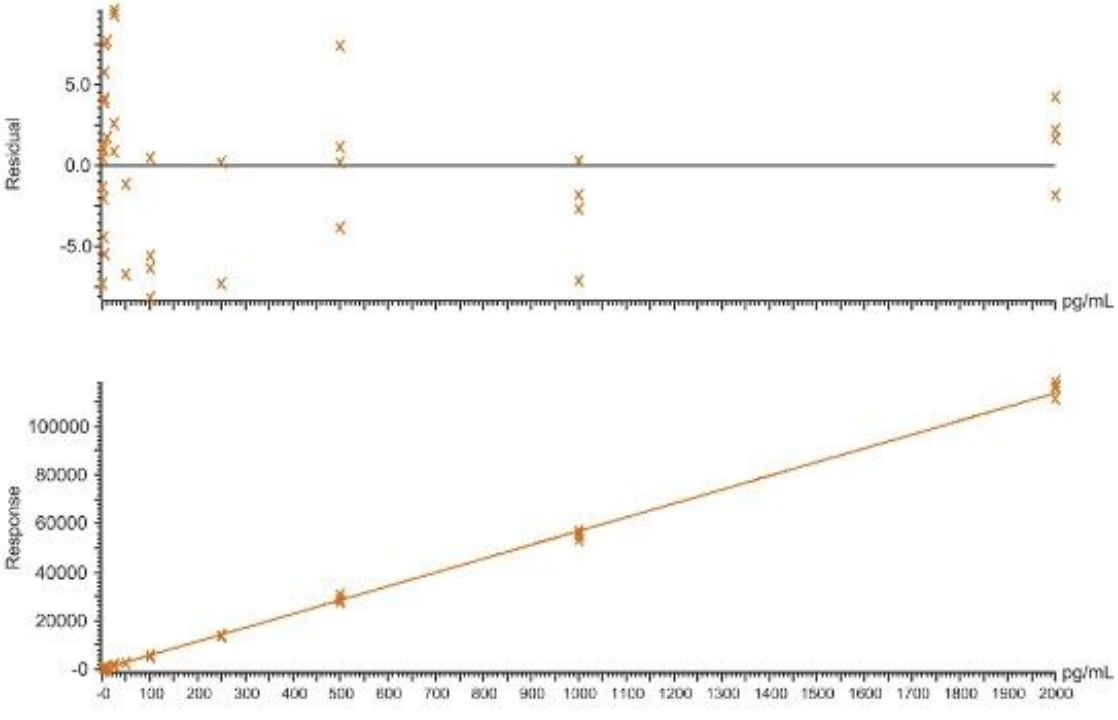
Compound name: Desmopressin
Correlation coefficient: r = 0.999596, r^2 = 0.999192
Calibration curve: 63.2499 * x + 154.958
Response type: External Std, Area
Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None

A



Compound name: Vasopressin
Correlation coefficient: r = 0.999367, r^2 = 0.998735
Calibration curve: 56.8185 * x + 51.2769
Response type: External Std, Area
Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None

B



Compound name: octreotide
Correlation coefficient: r = 0.999218, r^2 = 0.998437
Calibration curve: 610.829 * x + 19.8713
Response type: External Std, Area
Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None

C

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