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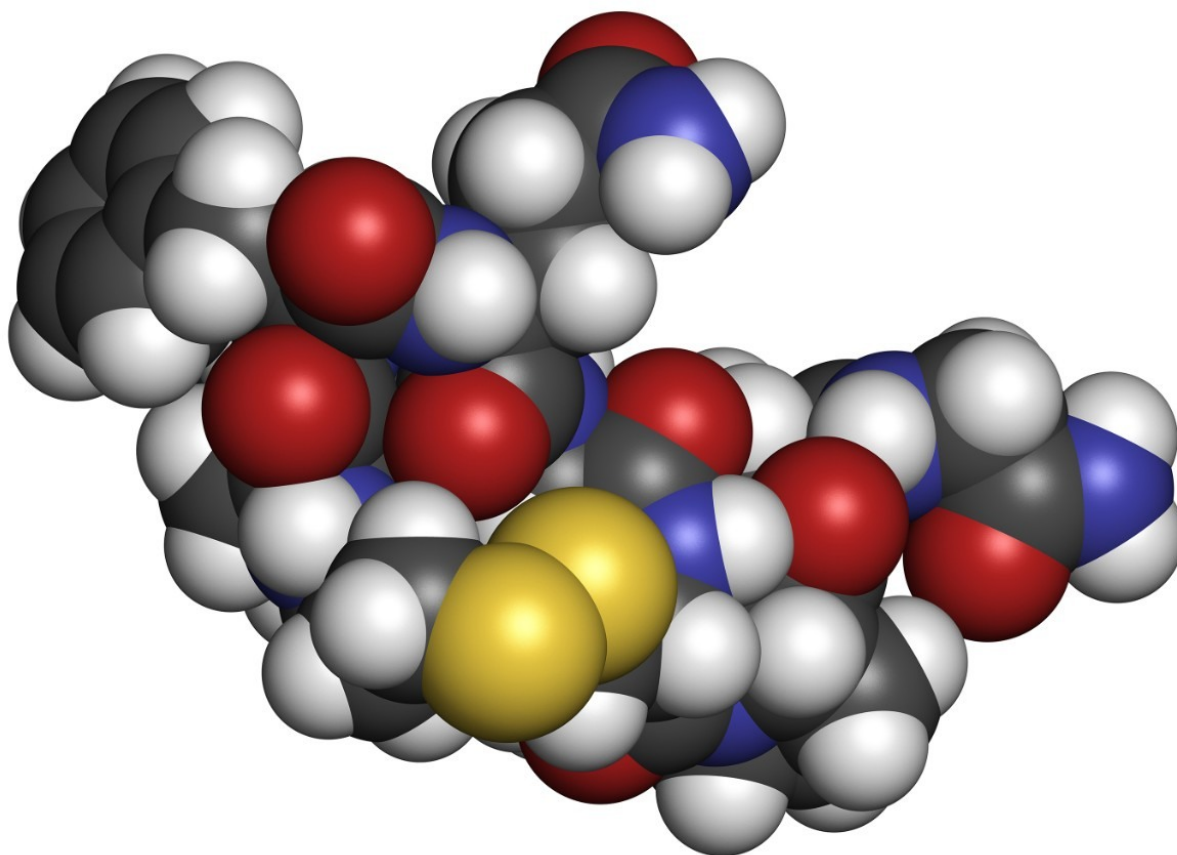
應用手冊

# 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  $\mu$ L of plasma
- Use of the ionKey/MS System facilitates detection limits of 2.5 pg/mL from only 25  $\mu$ L of plasma
- Use of mixed-mode solid-phase extraction (SPE) reduces matrix interferences and enhances selectivity of the extraction in plasma
- 96-well  $\mu$ Elution 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.<sup>1</sup> Therefore, to achieve low pg/mL quantification limits, large plasma sample volumes (0.2–1 mL) and sample injection volumes are often required.<sup>2–6</sup> 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.<sup>7-9</sup> The general properties of these peptides are shown in Table 1. Using a combination of selective  $\mu$ Elution 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  $\mu$ L.

## 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 $\mu$ m, 100 $\text{\AA}$ , 150 $\mu$ m x 100 mm iKey (p/n 186007261)
Trap column:	ACQUITY UPLC M-Class Symmetry C <sub>18</sub> , 5 $\mu$ m, 300 $\mu$ m 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 $\mu$ L/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)

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elute of trap onto the analytical column)

Analytical gradient:	See Table 2
Elution flow rate:	3.0 $\mu\text{L}/\text{min}$
iKey temp.:	75 $^{\circ}\text{C}$
Sample temp.:	15 $^{\circ}\text{C}$
Injection volume:	5 $\mu\text{L}$
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 $^{\circ}\text{C}$
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-NH <sub>2</sub> [Disulfide Bridge: 1-6]	1069	8.6	16.8
Vasopressin	Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH <sub>2</sub> [Disulfide Bridge: 1-6]	1084	9.1	7.6

Table 1. Peptide chemical properties.



Figure 1. ionKey Source.

Gradient:	Time (min)	Profile		Curve
		%A	%B	
	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  $\mu\text{L}$  of human plasma was diluted 1:1 with 4%  $\text{H}_3\text{PO}_4$  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  $\mu\text{Elution}$  plate that contained samples.

#### Oasis WCX $\mu\text{Elution}$ Protocol

Part number: <a href="#">186002499</a>
<b>Condition:</b> 200 $\mu\text{L}$ MeOH
<b>Equilibrate:</b> 200 $\mu\text{L}$ $\text{H}_2\text{O}$
<b>Load:</b> 200 $\mu\text{L}$ Diluted Plasma
<b>Wash 1:</b> 200 $\mu\text{L}$ 5% $\text{NH}_4\text{OH}$ in $\text{H}_2\text{O}$
<b>Wash 2:</b> 200 $\mu\text{L}$ 10% ACN in $\text{H}_2\text{O}$
<b>Elute:</b> 2 x 25 $\mu\text{L}$ of 2% FA in 50:50 ACN: $\text{H}_2\text{O}$
<b>Dilute:</b> 50 $\mu\text{L}$ $\text{H}_2\text{O}$

Figure 2. Oasis  $\mu\text{Elution}$  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  $y_3^{1+}$  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] <sup>2+</sup>	535.4>328.2	40	12	$y_3^{(1+)}$
Vasopressin	[M+2H] <sup>2+</sup>	542.7>328.2	40	14	$y_3^{(1+)}$
Octreotide	[M+2H] <sup>2+</sup>	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- $\mu\text{m}$  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  $\mu\text{m}$ , 100 $\text{\AA}$ , 150  $\mu\text{m}$  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  $\mu\text{L}$  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.



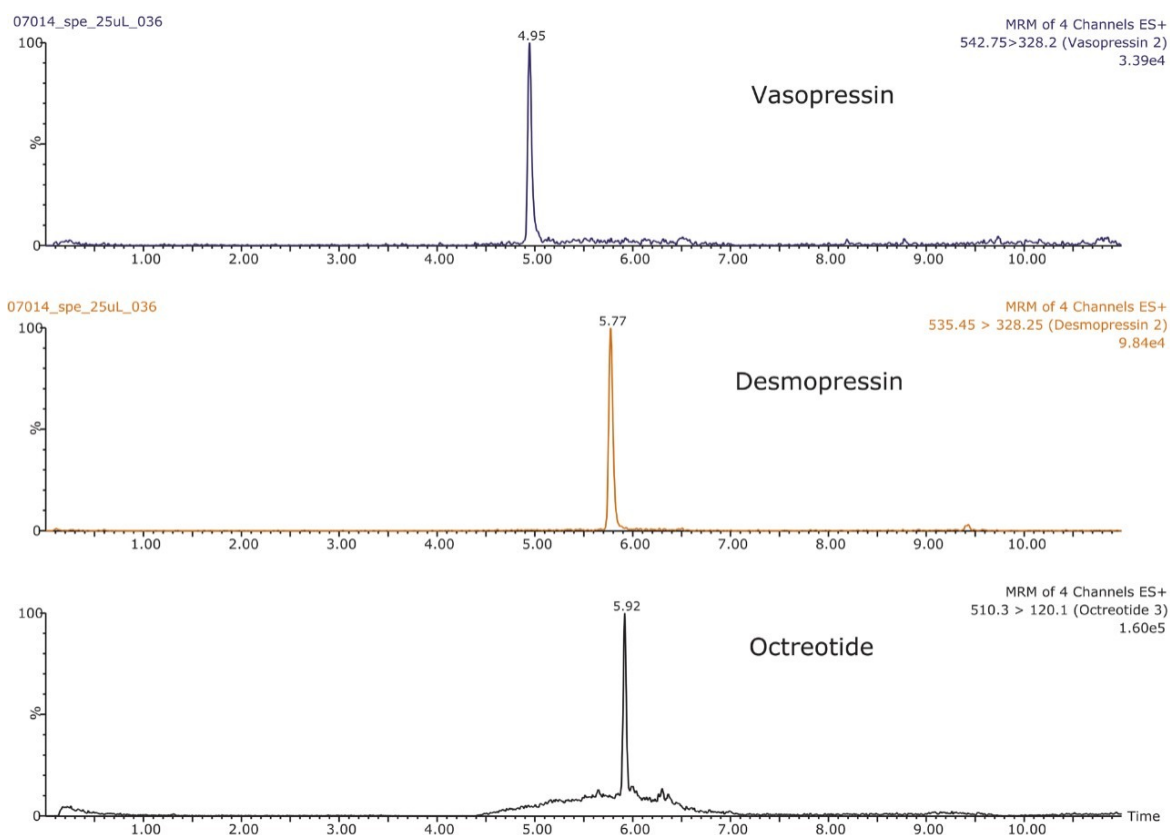


Figure 4. UPLC separation of desmopressin, vasopressin, and octreotide, using the iKey HSS T3, 1.8  $\mu\text{m}$ , 100 $\text{\AA}$ , 150  $\mu\text{m}$  x 100 mm (p/n 186007261).

## 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  $\mu\text{L}$  human plasma, using injection volumes  $\leq 10$   $\mu\text{L}$ .

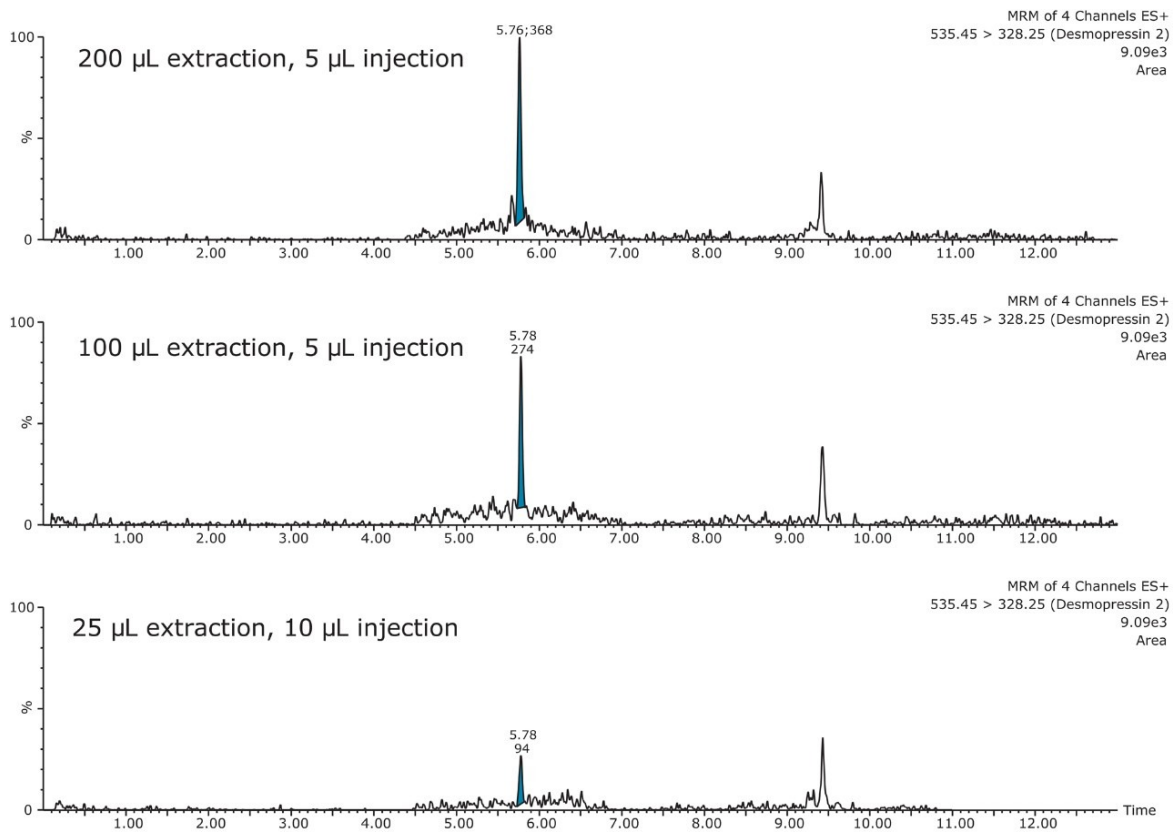


Figure 5. Enhanced sensitivity using the ionKey/MS System: Extraction volume comparison of desmopressin (2.5 pg/mL) from human plasma.

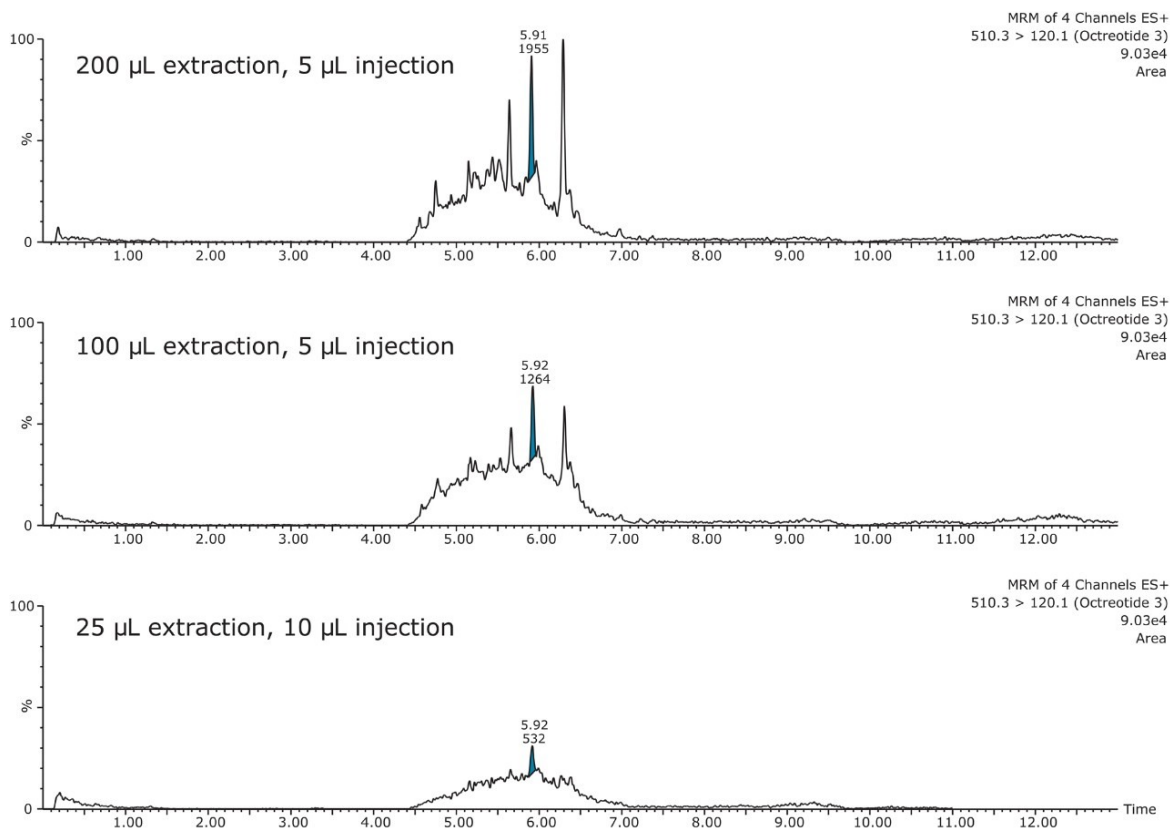


Figure 6. Enhanced sensitivity using the ionKey/MS System: Extraction volume comparison of octreotide (2.5 pg/mL) from human plasma.

## 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.

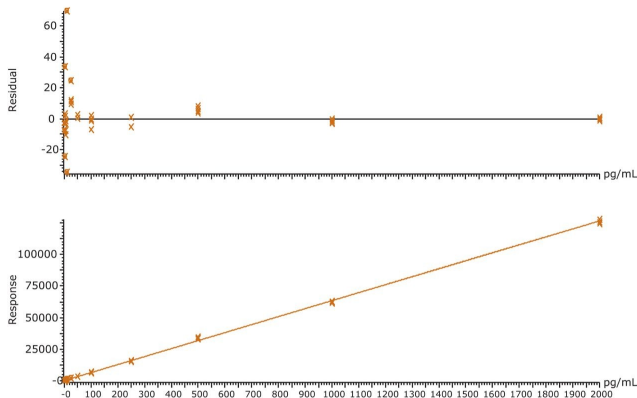
The 96-well Oasis  $\mu$ Elution Plate format facilitates fast sample processing (under 30 minutes), and is compatible with automation by most liquid-handling robotic systems, improving sample throughput. Additionally, this format also provides the ability to elute in very small sample volumes, minimizes 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  $\mu$ L of plasma, calibration lines were obtained for each peptide and are shown in Figure 7, panels A, B, C.

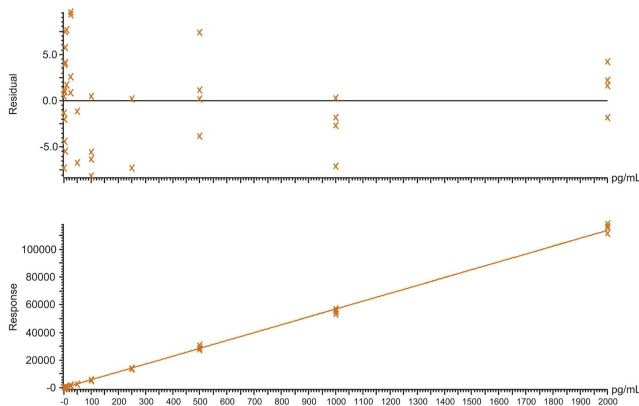
Compound name: Desmopressin  
 Correlation coefficient:  $r = 0.999596$ ,  $r^2 = 0.999192$   
 Calibration curve:  $63.2499 \cdot x + 154.958$   
 Response type: External Std. Area  
 Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None

A



Compound name: Vasopressin  
 Correlation coefficient:  $r = 0.999367$ ,  $r^2 = 0.998735$   
 Calibration curve:  $56.8185 \cdot x + 51.2769$   
 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:  $610.829 \cdot x + 19.8713$   
 Response type: External Std. Area  
 Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None

C

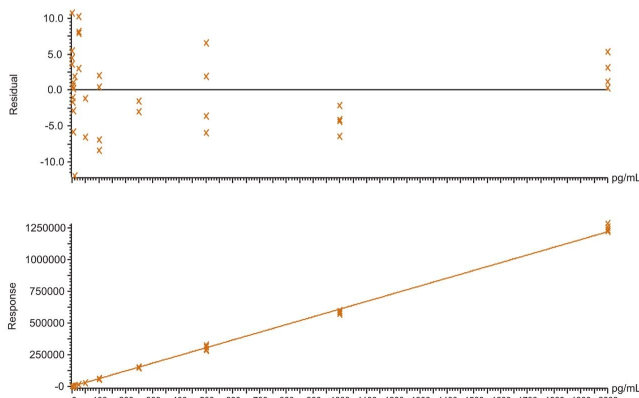


Figure 7. Calibration lines obtained for the

quantification of desmopressin (A), vasopressin (B), and octreotide (C) in extracted plasma.

A summary of standard curve performance is shown in is shown in Table 4. Using 1/X regressions, quantification was linear from 1–2,000 pg/mL with  $R^2$  values of  $>0.99$  for all 3 peptides monitored. Representative

~~chromatograms for extracted desmopressin, vasopressin, and octreotide plasma standard samples are shown in~~

Figures 8-10.

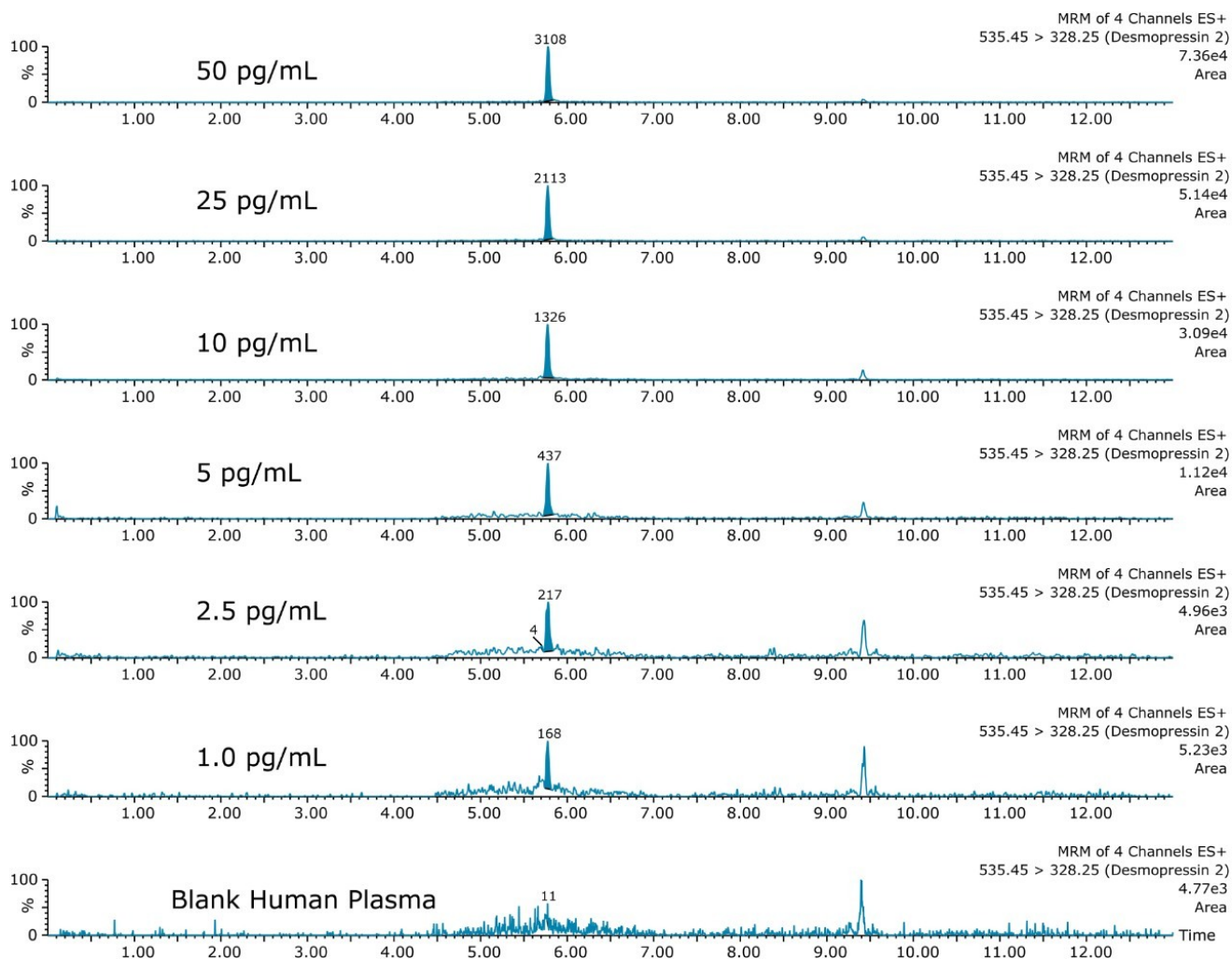


Figure 8. Representative chromatograms from desmopressin extracted from plasma at 1, 2.5, 5, 10, 25, and 50 pg/mL, compared to blank plasma.

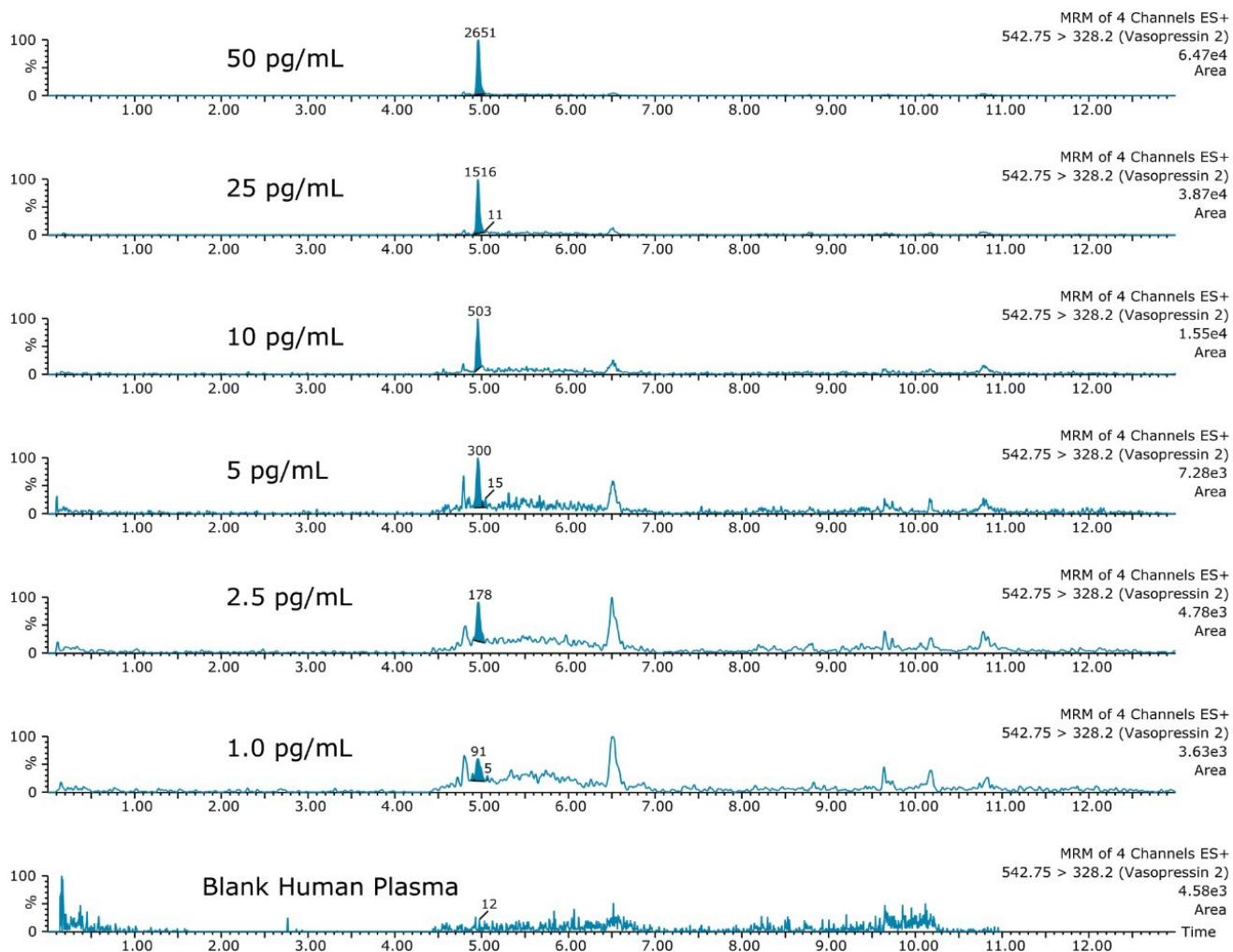


Figure 9. Representative chromatograms from vasopressin extracted from plasma at 1, 2.5, 5, 10, 25, and 50 pg/mL, compared to blank plasma.

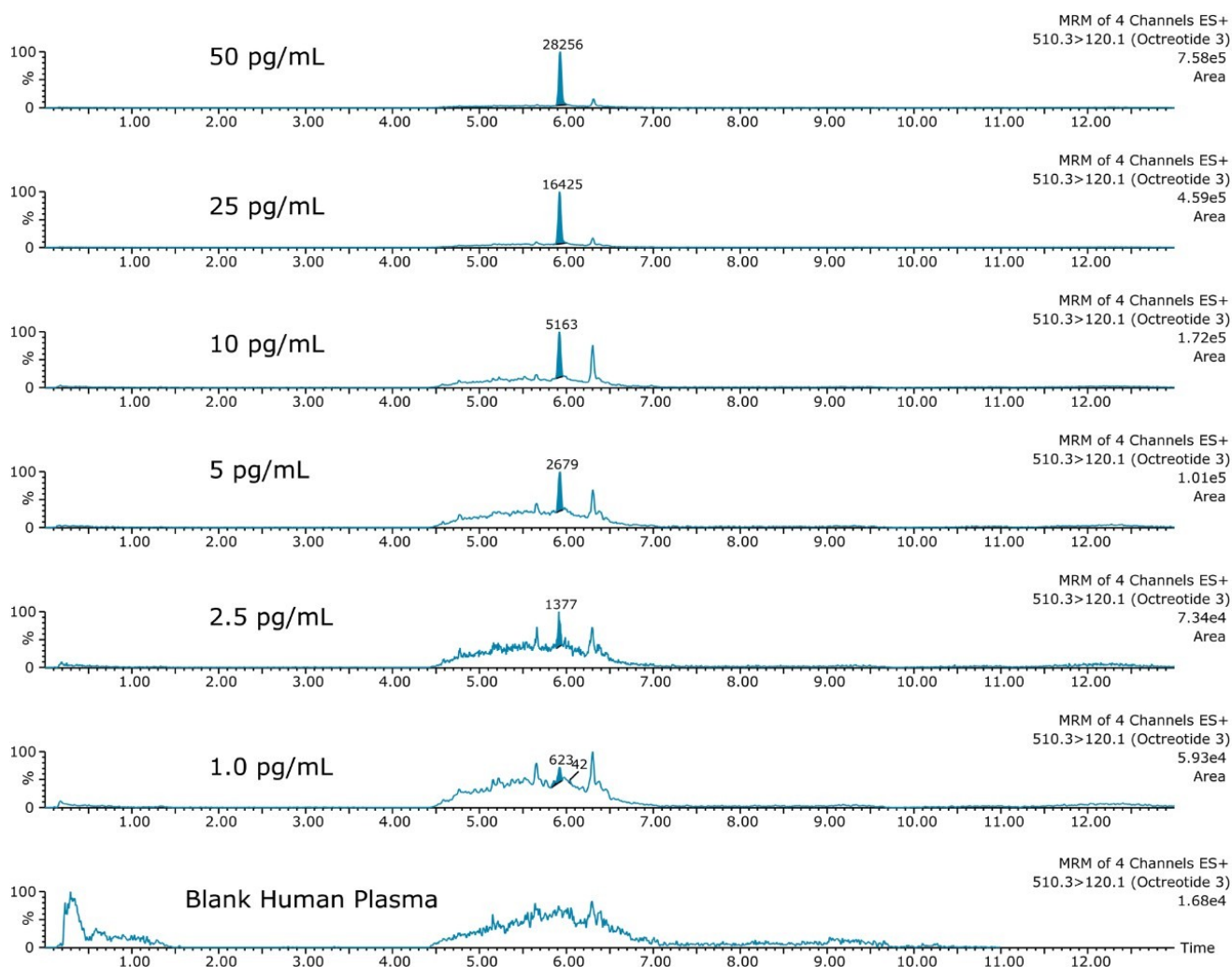


Figure 10. Representative chromatograms from octreotide extracted from plasma at 1, 2.5, 5, 10, 25, and 50 pg/mL, compared to blank plasma.

Desmopressin plasma concentration (pg/mL)	Area	Calculated desmopressin concentration (pg/mL)	Mean accuracy	Vasopressin plasma concentration (pg/mL)	Area	Calculated vasopressin concentration (pg/mL)	Mean accuracy	Octreotide plasma concentration (pg/mL)	Area	Calculated octreotide concentration (pg/mL)	Mean accuracy
1.0	215	0.95	94.8	1.0	107	0.98	98.9	1.0	668	1.06	106.0
2.5	319	2.58	103.5	2.5	193	2.50	99.9	2.5	1474	2.38	95.3
5.0	453	4.70	94.3	5.0	344	5.15	103.1	5.0	3017	4.91	98.1
10.0	898	11.75	117.5	10.0	646	10.50	104.8	10.0	5820	9.50	95.0
25.0	1965	28.63	114.5	25.0	1551	26.38	105.6	25.0	16404	26.82	107.3
50.0	3363	50.70	101.5	50.0	2781	48.05	96.1	50.0	29385	48.08	96.2
100.0	6376	98.35	98.4	100.0	5454	95.08	95.1	100.0	59131	96.77	96.8
250.0	15625	244.60	97.9	250.0	13751	241.10	96.5	250.0	149267	244.34	97.8
500.0	33594	528.68	105.7	500.0	28805	506.05	101.2	500.0	304504	498.48	99.7
1000.0	62242	981.60	98.2	1000.0	55252	971.53	97.2	1000.0	584899	957.52	95.8
2000.0	126145	1991.95	99.6	2000.0	115450	2030.98	101.5	2000.0	1251918	2049.51	99.7

Table 4. Summary of standard curve performance.



## Conclusion

The combination of the ionKey/MS System and mixed-mode  $\mu$ Elution SPE provided enhanced selectivity and increased sensitivity, whilst simultaneously significantly reducing sample volume requirements. Use of  $\mu$ Elution format SPE eliminated the need for evaporation, reducing peptide losses due to adsorption and non-specific binding. The 150  $\mu$ m iKey Separation Device enabled the development of a highly sensitive, low flow quantitative MRM method that simultaneously detects vasopressin, desmopressin, and octreotide with LOD <1pg/mL, and dynamic ranges from 1–2,000 pg/mL. The current analysis uses 100  $\mu$ L of plasma and provides a significant improvement in sensitivity and S:N over analytical scale (2.1 mm I.D.) analysis. Furthermore, detection limits of 2.5 pg/mL are achievable from only 25  $\mu$ L of plasma. In addition, the ionKey/MS System reduces solvent and sample consumption, thereby reducing cost and allowing for multiple injections of samples for improved accuracy or to meet the guidelines for incurred sample reanalysis (ISR).

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