Extraction of Oxytocin and Vasopressin from Serum Using EVOLUTE® EXPRESS ABN Prior to LC-MS/MS Analysis

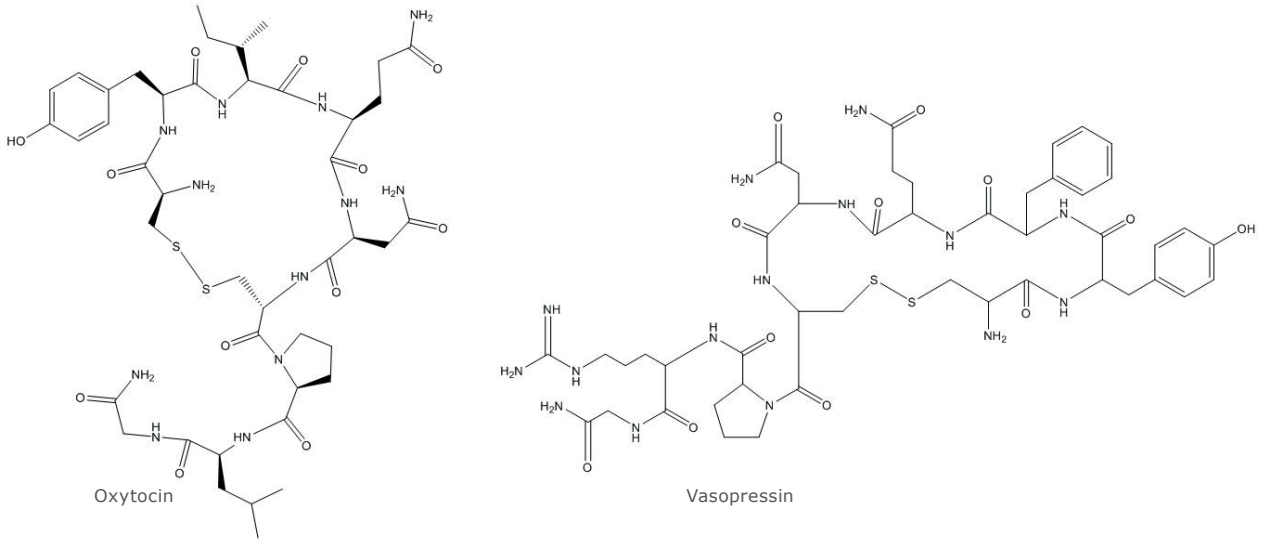

Figure 1. Structure of Oxytocin and Vasopressin.

Introduction

The method described in this application note achieves high reproducible extraction recoveries of the peptides oxytocin and vasopressin from serum using a fast, simple EVOLUTE® EXPRESS Load-Wash-Elute SPE procedure.

Extraction from serum was performed using EVOLUTE EXPRESS ABN 96-well plates, utilizing a highly aqueous elution solvent, and minimizing co-extractable material in the form of proteins, lipids and phospholipids.

Analytes

Oxytocin and vasopressin

Sample Preparation Procedure

Format

EVOLUTE EXPRESS ABN 30 mg plate, part number 600-0030-PX01.

Sample Pretreatment

Serum was spiked with with oxytocin and vasopressin at 0.2-500 ng/mL (200 µL) then diluted 1:1 (v/v) with 1% formic acid (aq).

Condition (Optional)

Condition each well with methanol (1 mL). This step is not required with the EVOLUTE EXPRESS Load-Wash-Elute procedure.

Equilibration (Optional)

Equilibrate each well with 0.1% formic acid (aq) (1 mL). This step is not required with the EVOLUTE EXPRESS Load-Wash-Elute procedure.

Sample Loading

Load 400 µL of pre-treated serum into each well

Wash 1

Elute interferences with 0.1% formic acid (aq) (1 mL).

Elution

Elute analytes with 5% formic acid in acetonitrile /H_2O (20/80, v/v, 200 µL).

This highly aqueous elution solvent delivers high recoveries of oxytocin and vasopressin, but minimises levels of co-extracted matrix components in the final extract. If desired, the extract can be injected directly into the analytical system, without additional processing. However, to minimise ion suppression (see post column infusion experiment, page 4), the following steps were performed prior to analysis:

Post Elution

Evaporate to dryness at 40 °C in a stream of air or nitrogen using a Biotage® SPE Dry.

Reconstitution

Reconstitute the extract with 0.1% formic acid in acetonitrile/H_2O (10/90, v/v, 200 µL).

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UPLC Conditions

Instrument

Waters ACQUITY I-Class

Column

ACE 3 1.7 µ C18-300 column (50 x 2.1 mm id)

Mobile Phase

A: 0.1% formic acid (aq)

B: 0.1% formic acid in acetonitrile

Flow Rate

0.4 mL/min.

Table 1. Gradient Conditions.

Time	% A	% B	Curve
0	90	10	1
1.50	67	33	6
1.6	90	10	1

Curve 6: Lineat Gradient

Injection Volume

10 µL

Sample Temperature

20 °C

Column Temperature

40 °C

Mass Spectrometry Conditions

Instrument

Xevo TQ-S triple quadrupole mass spectrometer equipped with an electrospray interface for mass analysis.

Desolvation Temperature

500 °C

Ion Source Temperature

150 °C

Collision Cell Pressure Temperature

3.7 e^{-3} mbar

Positive (+ve) ions acquired in the multiple reaction monitoring (MRM) mode:

Table 2. MRM Conditions.

Compound	MRM Transition	Cone Voltage (V)	Collision Energy (eV)
Oxytocin (Qual)	1007.3 > 285.2	30	25
Oxytocin (Quant)	504.2 > 285.2	30	15
Vasopressin (Qual)	542.8 > 757.2	30	10
Vasopressin (Quant)	542.8 > 328.2	30	15

Results

Good retention and chromatographic peak shape was obtained using the C18–300 column. Figure 2 demonstrates signal intensity and peak shape attained from serum spiked at 5 ng/mL.

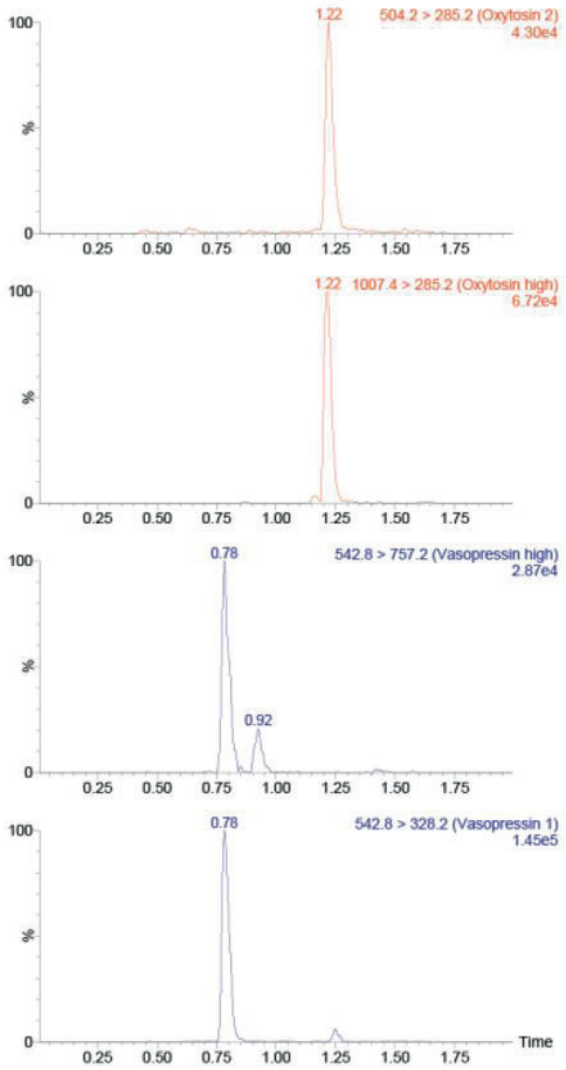

Figure 3. Chromatography obtained from serum spiked at 5 ng/mL.

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Recovery

Serum was spiked at various concentrations from 0.2–500 ng/mL for recovery determination. High reproducible recoveries > 70 % with corresponding RSDs < 10 % were demonstrated. Typical recovery data for both full SPE and Load-Wash-Elute SPE procedures from spiked serum at 2000 pg/mL is shown in Figure 3. Data generated using both direct injection and evaporation/reconstitution approaches is shown (see protocol on page 1 and notes for more information).

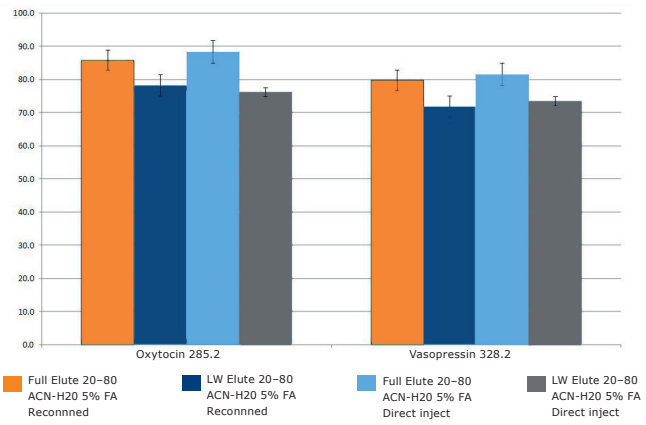

Figure 3. Spiked serum recovery profile for oxytocin and vasopressin extracted using full SPE and Load-Wash-Elute SPE protocols (evaporation/reconstitute or direct injection methods).

Extract Cleanliness

Phospholipid Removal

Phospholipids were investigated to provide an indication of extract cleanliness. The most abundant phospholipids (previously selected from full scan, SIR and precursor ion scanning experiments) using MRM transitions monitoring the common 184 product ion. Figure 5 demonstrates phospholipid content comparing 100 µL of protein precipitated serum with the final EVOLUTE® EXPRESS ABN extraction protocol using 200 µL of matrix.

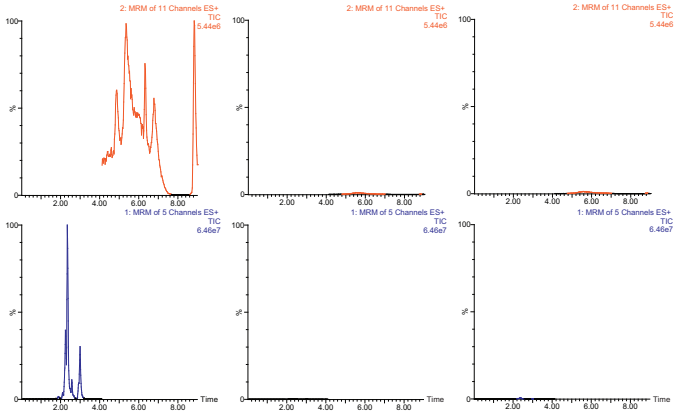

Figure 5. Phospholipid MRM TICs for final serum extraction protocol.

Calibration Curves

Calibration curves were generated using serum spiked at concentrations from 0.2-500 ng/mL. Good linearity, coefficients of determination (r^2 > 0.99) and sensitivity were obtained. Serum matrix demonstrated low endogenous levels of the analytes which contributed to a slight intercept on the calibration curves.

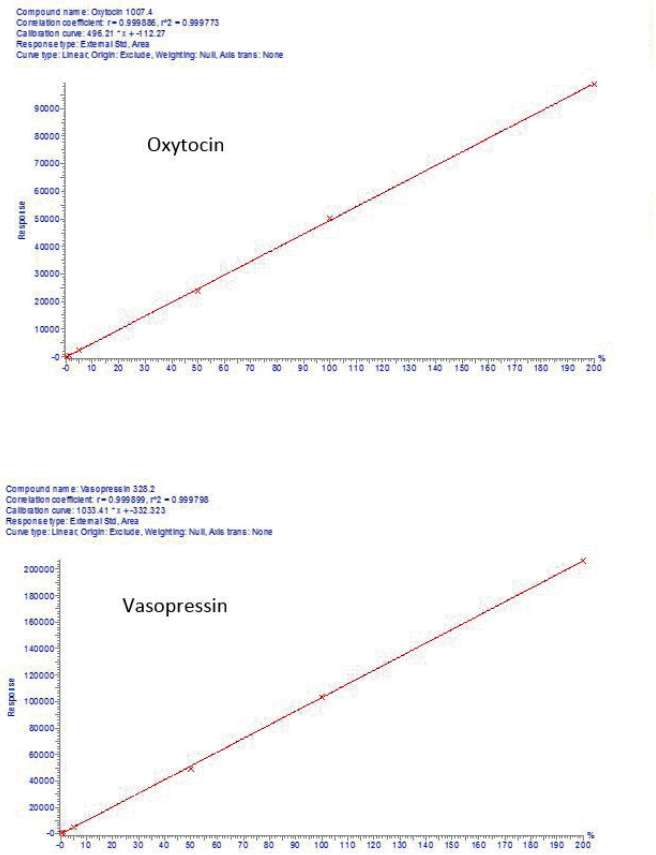

Figure 4. Serum quantifier ion calibration curves spiked from 0.2–500 ng/mL.

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Post Column Infusion

Extract cleanliness was also investigated using post-column infusion (PCI) experiments. Mobile phase and blank serum extracts were injected onto the LC-MS/MS setup whilst teeing in oxytocin and vasopressin. Infusion was used to determine regions of suppression for each technique, as demonstrated in Figure 8.

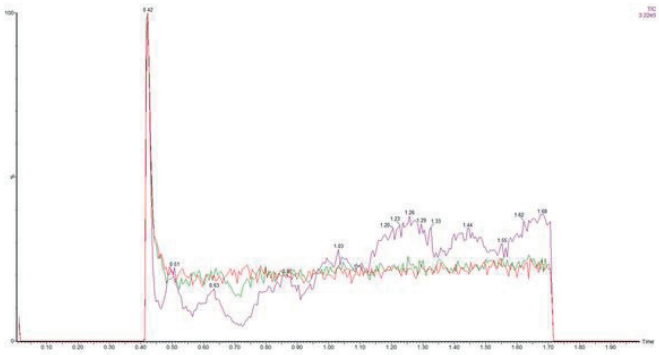

Figure 8. PCI baselines comparing blank solvent (red) to extracted blank serum using full SPE (green) or direct inject procedures (purple).

Additional Notes

Buffer Preparation

1. 0.1% formic acid aq: Measure 99.9 mL of H_2O and add 100 µL of formic acid (99.9% concentration).
2. 1% formic acid aq: Measure 99 mL of H_2O and add 1 mL of formic acid (99% concentration).
3. 5% formic acid in acetonitrile/H_2O (20/80, v/v). Measure 75 mL of H_2O; add 5 mL of formic acid followed by 20 mL of acetonitrile.
4. 0.1% formic acid in acetonitrile/H_2O (10/90, v/v). Measure 89.9 mL of H_2O; add 100 µL of formic acid followed by 10 mL of acetonitrile.

SPE considerations

1. During method development, EVOLUTE® EXPRESS ABN was compared to both strong and weak cation exchange sorbents. EVOLUTE EXPRESS WCX (weak cation exchange) also provided good analyte recoveries. However, better extract cleanliness was obtained using the optimized elution solvent in combination with EVOLUTE EXPRESS ABN.
2. Acidic pH conditions were used for sorbent conditioning (full SPE method) and sample pre-treatment in order to suppress ionization of the analyte, making it less polar, and improving initial analyte retention.
3. Due to high analyte polarity, interference elution steps were kept highly aqueous to prevent analyte losses.
4. Analyte elution conditions were optimized to use a highly aqueous elution solvent, which resulted in massively reduced phospholipid content in the extracts. Elution using 5% formic acid in H_2O/acetonitrile (20/80, v/v) demonstrates good removal of phospholipids and delivered high analyte recoveries.
5. Minimum elution volume was optimized at 200 µL using 5% formic acid in H_2O/acetonitrile (20/80, v/v). other elution solvents may require larger volumes.
6. It was possible to eliminate evaporation and move to direct inject using the selected chromatographic mobile phases, however, there was an increase in regions of suppression observed (see post column infusion experiments).

Mobile phase and ionization considerations

1. Ionization is performed using +ve ion mode for both oxytocin and vasopressin using precursor ions at m/z 504.2 and 542.8, respectively, corresponding to the $[M + 2H]^{2+}$ charge state.
2. Acidic mobile phase additives were used to facilitate protonation in positive ion mode.
3. Acetonitrile was selected for the organic eluent as a polar aprotic option for optimum ionization. Acetonitrile provided shaper peaks and as a result better signal to noise ratios compared to methanol.

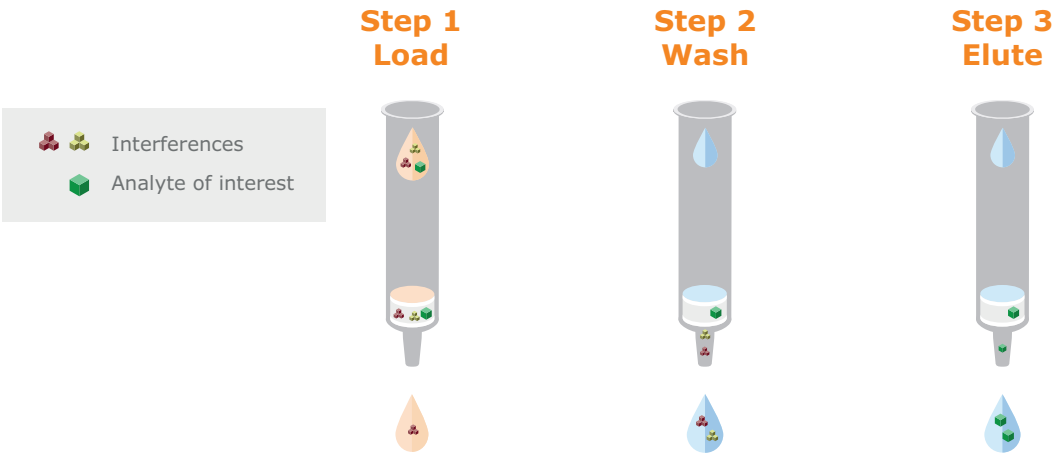

Figure 8. EVOLUTE® EXPRESS Load-wash-elute SPE.

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Ordering Information

Part Number	Description	Quantity
600-0030-PX01	EVOLUTE® EXPRESS ABN 30 mg Fixed Well Plate	1
PPM-96	Biotage® PRESSURE+ 96 Positive Pressure Manifold	1
121-5203	Collection plate, 2 mL, square	50
121-5204	Piercable sealing cap	50
SD-9600-DHS-EU	Biotage® SPE Dry 96 Sample Evaporator 220/240V	1
SD-9600-DHS-NA	Biotage® SPE Dry 96 Sample Evaporator 100/120V	1

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