A highly sensitive quantification method for trace level of oxytocin in urine using mixed-mode solid phase extraction coupled with LC-MS/MS

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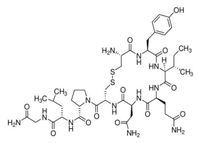
Introduction

Oxytocin (OT) is a 9-amino acid peptide hormone known to regulate social behaviors in many mammalian species by peripheral actions. OT is released into the blood stream from the posterior pituitary, then diffuses into the saliva and urine. To investigate its role in human and animal behavior, OT is often measured in blood, urine, and saliva. Compared to blood collection, urine collection is less invasive, and urine has been used to measure the effects of social interactions on peptide secretion. However, less than 1% of OT is cleared in urine. Background interference in urine specimens is also high.

Fig. 1. Structure of Oxytocin



Study Aim: *To develop a sensitive, selective, and high-throughput LC-MS/MS method to quantify trace level of OT in human urine.*

Experimental Conditions

Standard: OT was obtained from Sigma-Aldrich (St. Louis, MO, USA) for use as an analytical standard. The internal standard OT-d10 was synthesized by Cayman Chemical (Ann Arbor, MI, USA).

Sample Preparation: Phenomenex Strata-X-Drug-B, a mix-mode solid phase extraction (SPE) cartridge, was used for OT extraction after urine pH adjustment by using 0.2 M ammonium acetate (pH 3.0). After washing with ammonium acetate (pH 3.0), water, and acetonitrile to remove interferences, OT was eluted with 2X 1mL 0.2% ammonium hydroxide in methanol and concentrated under nitrogen gas flow.

Fig. 2. Experimental workflow: Solid phase extraction → LC-MS/MS

UPLC Conditions:

- **Instrument:** Shimadzu Nexera 40 Series
- **Column:** ACE Excel C18: 2.1 mm×100 mm, 1.7 µm
- **Mobile Phase**: A: water containing 0.1% (v/v) formic acid; B: acetonitrile containing 0.1% (v/v) formic acid
- **Flow rate**: 0.5 mL/min
- **Gradient**: 0.00–1.00 min (10–20% B), 1.00–2.00 min (20% B), 2.00–2.70 min (20–30% B), 2.70–3.60 min (30% B), 3.60–3.65 min (30–98% B), 3.65–5.00 min (98% B), 5.00–5.10 min (98–10% B) and a 1.5 min post-elution period for re-equilibration
- **Column Temperature**: 40 °C
- **Autosampler temperature**: 4 °C
- **Injection volume:** 15 µL

Mass Spectrometry:

Instrument: Shimadzu 8060NX triple quadrupole mass spectrometer equipped with an electrospray interface under multiple reaction monitoring (MRM) mode was used for the detection, with parameters shown in Tables 1 and 2.

Table 1. Ion source/Gas Parameters

Parameters	Value
Nebulizing Gas Flow	3 L/min
Heating Gas Flow	15 L/min
Interface Temperature	270 °C
DL Temperature	250 °C
Heat Block Temperature	170 °C
Drying gas flow	3 L/min

Table 2. Optimized MRM conditions for OT and D_{10}-OT

Compounds	MRM transitions (m/z)	Q1 Pre Bias (volts)	CE (volts)	Q3 Pre Bias (volts)
OT	1007.10→723.30	-48	-31	-26
D10-OT (IS)	1017.30→723.15	-50	-31	-26

Results

Column and Mobile Phase Optimization:

- OT sensitivity and resolution were compared among six reversed-phase columns and one HILIC HPLC column (Table 3).
- Mobile phase additives was optimized using ACE Excel C18 column (Table 4).
- The highest sensitivity and best resolution were achieved with ACE Excel C18 column (2.1mm×100 mm, 1.7 µm particle) and the mobile phase composed of water with 0.1% (v/v) formic acid (A) and acetonitrile with 0.1% (v/v) formic acid (B).

Table 3. LC columns optimization for 3 pg/mL OT

No.	Brand	Type	Dimensions	S/N	Peak area (counts)	Selected
1	ACE	Excel 2 C18-PFP	2.1×50mm	65.8	4.79e3	
2	Phenomenex	Kinetex 1.7 µm C18	2.1×100mm	9.75	3.42e3	
3	ACE	Excel 1.7 C18	2.1×100mm	87.5	6.91e3	√
4	Agilent	Zorbax XDB C18	4.6×50mm	35.7	6.09e3	
5	Shimadzu	Nexcol C18	2.1×50mm	45.5	4.01e3	
6	Waters	Atlantis HILIC Silica	2.1×50mm	NA	NA	
7	Phenomenex	Kinetex C8	2.1×50mm	37.2	5.76e3	

Table 4. Mobile phase additives optimization using ACE Excel C18 column

	Mobile phase A	Mobile phase B	S/N	Peak area (counts)	Selected
Combo 1	0.1% formic acid in Water	0.1% formic acid in Acetonitrile	87.5	6.91e3	√
Combo 2	10mM ammonium acetate in Water (pH 3.0)	10:90 (v:v) 10mM ammonium acetate in Water: Acetonitrile (pH 3.0)	35.8	4.03e3	

Extraction Optimization:

- Three SPE columns and corresponding protocols were compared for OT extraction (Table 5).
- Phenomenex Strata-X-Drug-B (reversed-phase and strong anion exchange mix-mode SPE) was most effective for both removing matrix impurities and preconcentrating OT for analysis.

Table 5. Comparison of three SPE columns for OT analysis in human urine

	Protocol 1	Protocol 2	Protocol 3
Cartridge	Phenomenex Strata X	Biotage Evolute Express ABN	EVOLUTE® EXPRESS WCX
Activation	2ml Methanol	2 ml Methanol	2 ml Methanol
Equilibration	2 ml Water, 2ml 0.2M Ammonium Acetate pH3	2 ml 0.1% Formic acid	2 ml Water
Sample loading	5.5 ml 0.2M Ammonium Acetate pH3	3 ml 1% Formic acid	3 ml 4% Phosphoric acid
Washes	2 ml 0.2M Ammonium Acetate pH3, 2ml water, 2ml ACN	2 ml 0.1%Formic acid	2 ml 5% (v/v) aqueous ammonium solution, 2ml acetonitrile-water (75:25, v/v)
Elution	2 ml 0.2% ammonium hydroxide in methanol	500 µL 20% Acetonitrile with 5% Formic acid	2 mL acetonitrile-water (75:25, v/v) solution containing 1% (v/v) formic acid
Recovery	79.6%	68.7%	2.7%
Matrix effect	107.2%	61.8%	3.3%

Surrogate Material Evaluation:

- Using surrogate matrices for standard curve preparation is one of the methods for quantitative analyses of endogenous compounds (e.g., OT).
- Four surrogate matrices were evaluated, including 1) Diluted urine by PBS (1:4, v:v); 2) Diluted urine by DI water (1:4, v:v); 3) Diluted urine by DI water (1:2, v:v); and 4) DI water.
- Calibration curve was prepared and tested using the four matrices. Two QC urine samples at low and high levels were tested and used for the evaluation.
- DI water met the accuracy criteria within ±15% and thus was selected as the surrogate material.

Results

Method Validation:

- The developed method was validated for calibration curve performance, accuracy, precision and stability referring to bioanalytical method validation guidelines of the US Food and Drug Administration.

LLOQ: The lower level of quantification (LLOQ) of OT in urine by this method was 3pg/ml (Figure 3).

Linearity: The assay was linear over the concentration ranges of OT from 3pg/ml to 1 ng/ml with coefficients of determination being 0.998 (r^2) (Figure 4).

Fig. 3. Chromatograms of OT at LLOQ (3pg/ml) and D_{10}-OT at 100 pg/ml in calibration sample

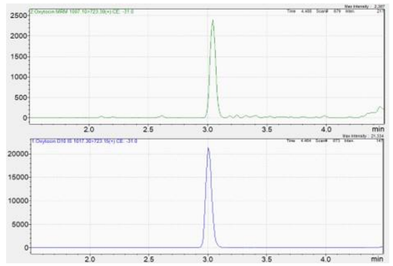
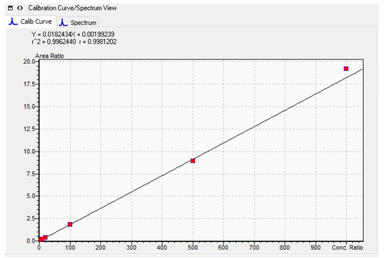


Fig. 4. Typical calibration of OT



Accuracy/Precision: The intra-day and inter-day precision for all quality control (QC) levels, including the LLOQ, ranged from 8.0% to 13.0% and 6.1% to 10.9%. The intra-day and inter-day accuracy biases, based on QC data, ranged from −2.7% to 9.4% and 1.1% to 5.3%.

Stability:

Processed sample stability: To estimate the stability of the processed samples for LC-MS/MS analysis, QC samples of low and high OT levels were extracted as described above. The accuracy (% nominal) at each level were within 15% (Table 6).

Autosampler stability: To evaluate autosampler stability, QC samples of low and high OT levels were analyzed after storage in autosampler for 24 hours. The accuracy (% nominal) at each level were within 15% (Table 6).

Table 6. Results for processed sample stability and autosampler stability of OT

	Accuracy	
	Low QC samples	High QC samples
OT processed sample stability	109.4%	97.3%
OT autosampler stability	104.7%	97.7%

Summary

- In this study, a specific UPLC-MS/MS method was developed and validated for the quantification of trace level of OT in human urine.
- The combination of an efficient mixed-mode solid phase sample extraction and a Shimadzu UPLC coupled to a Shimadzu 8060NX triple quadrupole mass spectrometer proved to be a sensitive method for the detection of OT at the endogenous levels in urinary samples derived from non-lactating and non-pregnant women.
- This developed method has been successfully applied to more than 1,000 of human urine samples.

Acknowledgement

- This study was supported by the NIH OD grant P51 011132 to the ENPRC.