Simultaneous Determination of Pharmaceutical Peptides and Acetate Counterions by HPLC Using a Mixed-Mode Weak Anion-Exchange Column

Deanna C. Hurum, Brian M. De Borba, and Jeffrey S. Rohrer
Dionex Corporation, Sunnyvale, CA, USA

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INTRODUCTION

Peptides perform a wide variety of physiological functions; they are critical to regulatory and cell signaling pathways and have been used as pharmaceutical drugs. Oxytocin, a small peptide (Figure 1) has been used for many years to stimulate labor, control post-partum hemorrhage, and induce lactation. Synthesis of oxytocin incorporates an acetate counterion, and solutions of the drug often contain chlorobutanol as a preservative. Assay of both acetate and the active peptide is required before clinical use.

Current USP and EP methods for analysis of oxytocin and acetic acid use L1 (C18) columns with two independent gradient methods.[1-4] The first method quantifies the peptide, and the second determines the acetate counterion. The simultaneous determination of oxytocin and acetate in a single injection onto an L1 column can prove challenging because conditions that promote retention of acetate and resolution of peptide impurities lead to long retention times of the peptide, and therefore time-consuming analyses. Acetate is more strongly retained on a mixed-mode weak anion-exchange column, allowing better quantification. Using pH to control selectivity, a single method has been developed to simultaneously determine peptides and acetate counterions.[5] Table 1 summarizes the methods that are compared here.

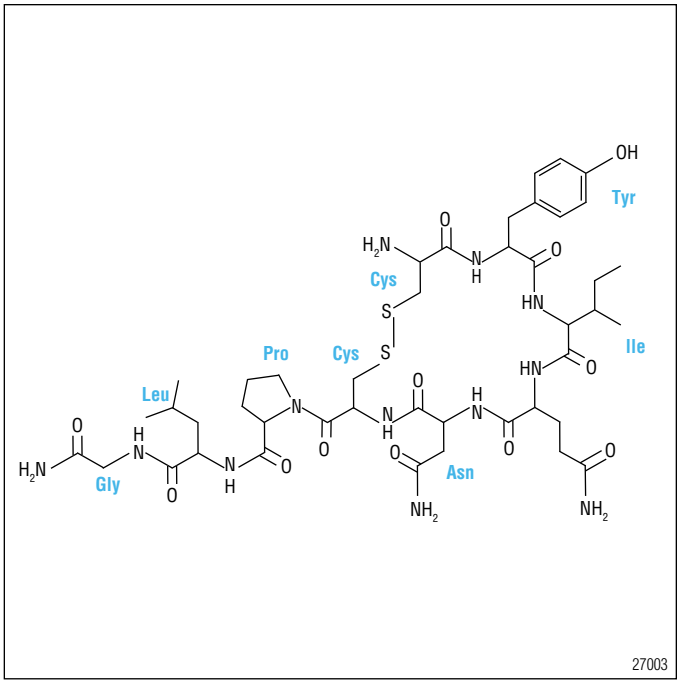


Figure 1. Structure of oxytocin.

Table 1. Summary of Compendial and Proposed Methods

Assay Details	USP and EP Acetic Acid in Peptides	USP Oxytocin	EP Oxytocin	Proposed Method
Column	C18 (4.6 × 250 mm)	C18 (4.6 × 120 mm)	C18 (4.6 × 125 mm)	Mixed-Mode WAX (2.1 × 150 mm)
Detection Wavelength (nm)	210	220	220	220 (210 is an option)
Mobile Phase A	0.7 mL/L Phosphoric Acid,(pH 3.0)	100 mM NaH_2PO_4	130 mM NaH_2PO_4	50 mM KH_2PO_4 (pH 4.2)
Mobile Phase B	Methanol	1:1 Acetonitrile/Water	1:1 Acetonitrile/Water	Methanol
Run Time with Equilibration (min)	22	35	45	40
Flow Rate (mL/min)	1.2	1.5	1.0	0.21
Mobile Phase (mL/Sample)	26.4	52.5	45	8.4

By following the proposed method, full characterization of the peptide product is faster, uses less mobile phase, and generates less waste than the traditional analysis.

EXPERIMENTAL

Dionex UltiMate® 3000 system comprising:

SRD-3200 Solvent Rack

HPG-3200M Pump

WPS-3000TSL Micro Autosampler

TCC-3200 Column Compartment

PDA-3000 Detector

Acclaim® Mixed-Mode WAX-1 column, 5 µm, 2.1 × 150 mm

Acclaim 120 C18, 5 µm, 2.1 × 150 mm column for USP methods

Chromeleon® Chromatography Data System was used for system control and data processing

RESULTS AND DISCUSSION

Existing Methods

Figures 2 and 3 show the results when using USP methods for determining acetic acid and oxytocin in an oxytocin sample. Using the oxytocin assay, acetic acid is poorly retained and difficult to determine (Figure 2). Using USP Method <503> acetic acid is better retained, but peptide impurities can interfere with quantification, as shown in the inset in Figure 3.

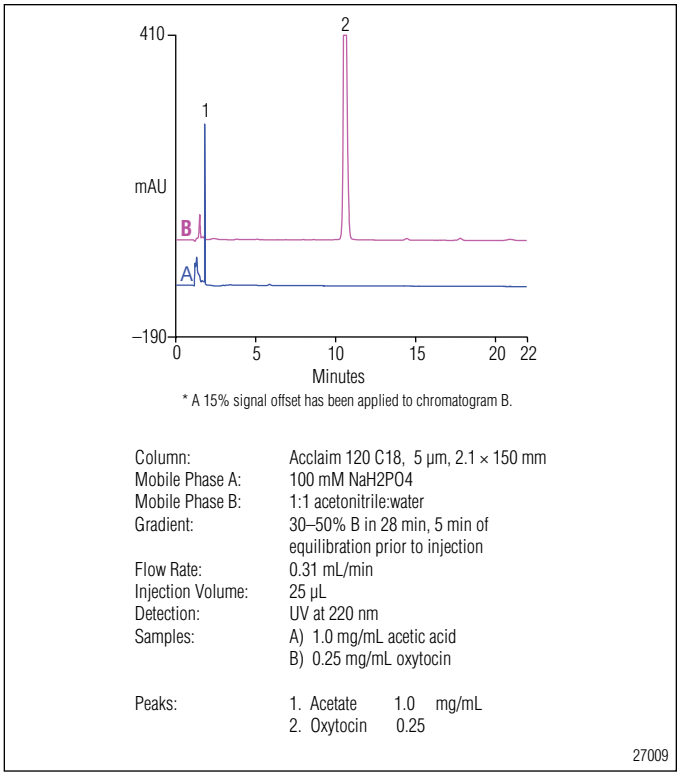


Figure 2. Results for determination of acetic acid and oxytoxin using the USP oxytocin monograph assay.

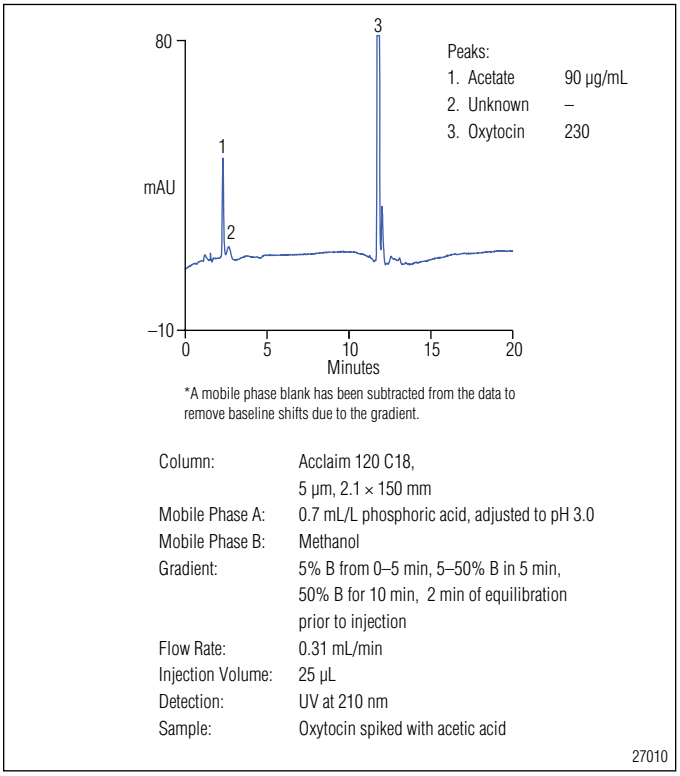


Figure 3. Results for determination of acetic acid and oxytocin using USP method <503>. Unknown peak 2 interferes with acetate quantification.

PROPOSED METHOD

Using the proposed method, acetic acid and oxytocin are easily determined, and oxytocin can be assayed in a single injection. Here, chlorobutanol was added to demonstrate that the preservative does not interfere with quantification. Acetic acid is well retained and well-resolved from other components.

The linearity of oxytocin was confirmed between the concentration specified in the EP and USP monographs. The linerity of acetic acid was confirmed in the range expected to be present in the peptides.

Acetic acid was determined in four representative peptides. For samples, acetic acid peak area precision, as RSD, was < 2. The USP/EP criteria is < 5. Retention time for acetic acid is very stable. Determined concentrations of acetate compare well to the estimated amounts calculated from those reported by the peptide manufacturer.

Recovery of acetate added to the samples confirms accuracy of the method. Recoveries range from 98–105% for the four representative peptides.

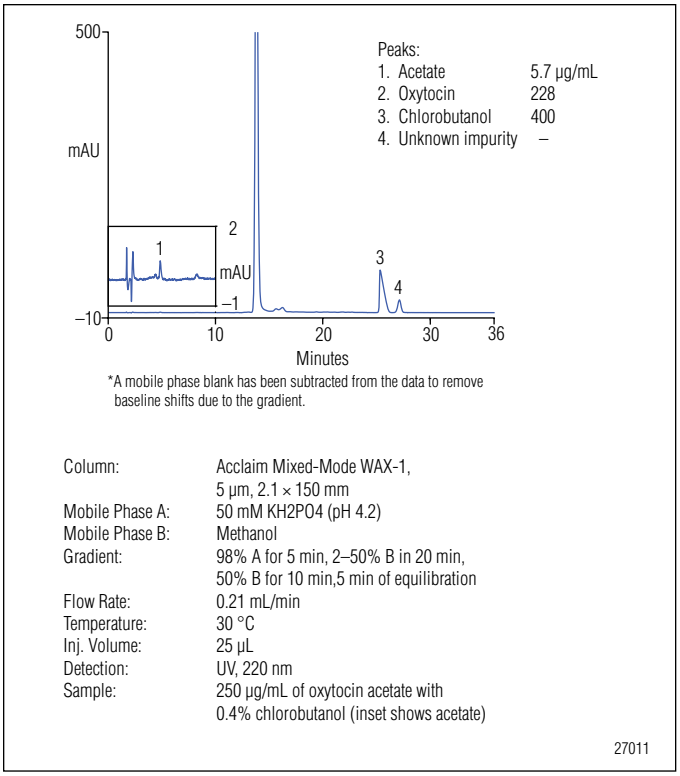


Figure 4. Separation of acetate, oxytocin, and chlorobutanol using the Acclaim Mixed-Mode WAX-1 column.

Table 2. Linearity, LOD, LOQ, and Precision of the Proposed Method

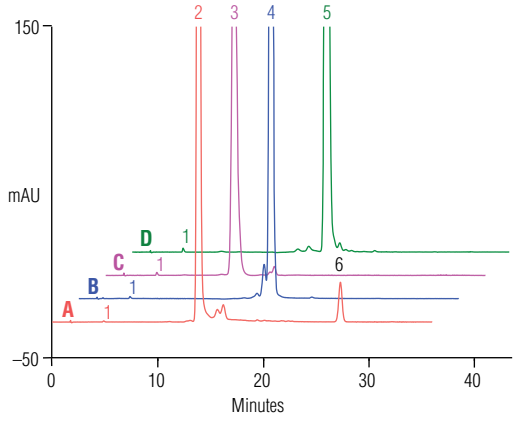
Analyte	LOD (µg/mL)	LOQ (µg/mL)	Linear Range (µg/mL)	Correlation Coefficient (r^2)	Peak Area Precision (RSD)	Retention Time (RT) Precision (RSD)
Acetic Acid	1.5	5	5.0 – 35	0.9994	2.40	0.08
Oxytocin	0.06	0.2	15 – 250	0.9997	1.13	0.11

Table 3. Determination of Acetate in Representative Peptides

Peptide	Est. Acetate Amount (µg/mL)	Determined Acetate Amount (µg/mL)	Acetate RT (min)	Acetate RT Precision (RSD)	Acetate Peak Area Precision (RSD)
Angiotensin I	12	13.0	4.74	0.16	1.94
Bradykinin	21	19.5	4.73	0.08	0.98
Neurotensin	20	24.0	4.72	0.08	1.23
Oxytocin	6.2	6.7	4.74	0.08	0.87

Table 4. Recovery of Acetic Acid in Peptide Samples				
Peptide	**Amount of Acetate Determined in Peptide (µg/mL)**	**Amount of Acetate Added (µg/mL)**	**Amount Determind in Spiked Sample (µg/mL)**	**% Recovery**
Angiotensin I	13.0	10.0	22.8	98.0
Bradykinin	19.5	15.0	35.2	104
Neurotensin	18.4	10.4	29.2	104
Oxytocin	6.7	11.2	18.4	105

The separation of peptides is shown in Figure 5.



Column:	Acclaim Mixed-Mode WAX-1	Peaks:	
Mobile Phase A:	50 mM KH_2PO_4 (pH 4.2)	1A. Acetate	6.7 µg/mL
Mobile Phase B:	Methanol	1B. Acetate	13.0
Gradient:	98% A for 5 min, 2–50% B in 20 min,	1C. Acetate	19.5
	50% B for 10 min, 5 min of equilibration	1D. Acetate	24.6
Flow Rate:	0.21 mL/min	2. Oxytocin	–
Temperature:	30 °C	3. Bradykinin	–
Inj. Volume:	25 µL	4. Angiotensin I	–
Detection:	UV, 220 nm	5. Neurotensin	–
Samples:	250 µg/mL of peptide	6. Unknown	–

* A mobile phase blank has been subtracted from the data to remove baseline shift due to the gradient. A 7% shift in signal and a 7% shift in time have been applied.

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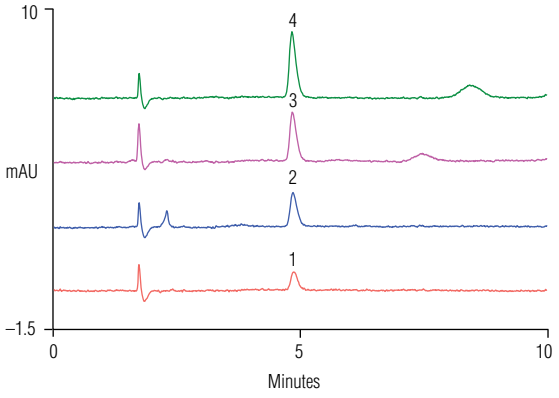
Figure 5. Separation of oxytocin, angiotensin I, bradykinin, and neurotensin acetates using the Acclaim Mixed-Mode WAX-1 column.

In each case, the peptides were determined with good retention time and peak area precision. Peptide peak area precisions (RSD) are < 1. Retention time precisions (RSD) were < 0.2. In each peptide, acetic acid is determined without interference from other components in the sample.

Table 5. Precision of Peptide Determination for Represenative Peptides			
Peptide	**Peptide RT (min)**	**Peptide RT Precision (RSD)**	**Peptie Peak Area Prescision (RSD)**
Angiotensin I	17.80	0.17	0.11
Bradykinin	11.97	0.06	0.24
Neurotensin	18.27	< 0.01	0.66
Oxytocin	13.83	< 0.01	0.32



* A mobile phase blank has been subtracted from the data to remove baseline shifts due to the gradient.

Column:	Acclaim Mixed-Mode WAX-1
Mobile Phase A:	50 mM KH_2PO_4 (pH 4.2)
Mobile Phase B:	Methanol
Gradient:	98% A for 5 min, 2–50% B in 20 min, 50% B for 10 min, 5 min of equilibration
Flow Rate:	0.21 mL/min
Temperature:	30 °C
Inj. Volume:	25 µL
Detection:	UV, 220 nm
Samples:	250 µg/mL of peptide

Peaks:		
	1. Acetate in oxytocin	6.7 µg/mL
	2. Acetate in angiotensin I	13.0
	3. Acetate in bradykinin	19.5
	4. Acetate in neurotensin	24.6

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Figure 6. Expanded view of acetate in oxytocin, angiotensin I, bradykinin, and neurotensin.

CONCLUSIONS

- The mixed-mode weak anion-exchange column was used to simultaneously determine peptides and the acetate counterion in a single injection, saving 17 min and 70.5 mL of mobile phase per sample.
- The proposed method is fast, accurate, and demonstrates sufficient sensitivity to determine acetate concentrations in 250 µg/mL peptide samples.
- The resolution and precision for replicate injections exceed the USP and EP requirements for both oxytocin and acetate.

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Dionex Corporation
1228 Titan Way
P.O. Box 3603
Sunnyvale, CA
94088-3603
(408) 737-0700

North America
U.S./Canada (847) 295-7500

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