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Application Note 234

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Simultaneous Determination of Pharmaceutical Peptides and Acetate by HPLC with UV Detection Using the Acclaim Mixed-Mode WAX-1 Column

INTRODUCTION

Peptides are active in a wide variety of physiological processes and are critical to regulatory and cell-signaling processes. Many peptides have been investigated as therapeutic pharmaceutical drugs and are active vasodilators, vasoconstrictors, hormones, and neuropeptides. Peptide analysis is necessary for any peptide to be considered as a drug product.

Oxytocin is a neuropeptide with therapeutic uses, including stimulating labor, control of post-partum hemorrhage, and inducing lactation.[1] It includes a disulfide bond between two cysteine residues forming a large-ringed structure (Figure 1). When present as a neutral compound, oxytocin is optimally stable between pH 3 and 5. In acidic solutions, the peptide hydrolyzes, and in neutral and basic conditions, it forms dimers and polymeric mixtures by the creation of intermolecular disulfide bridges that lead to peptide deactivation.[2]

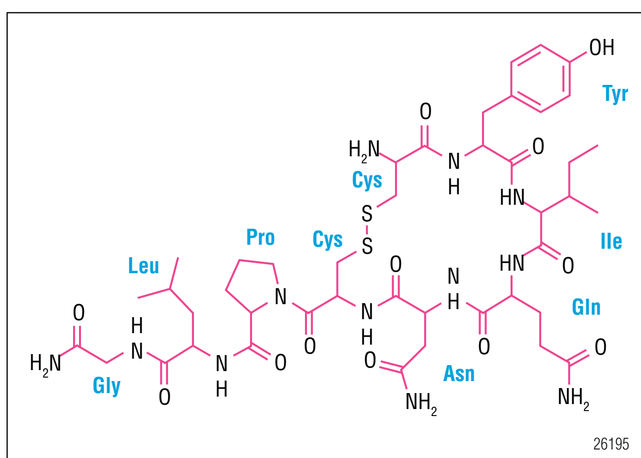

Figure 1. Structure of oxytocin.

Generally, oxytocin is available as an acetate salt. The United States Pharmacopeia (USP) and the European Pharmacopeia (EP) allow 6–10% by weight acetic acid in the solid peptide drug. The use of acetate as a counter-ion in peptide therapeutics is common and is found in a variety of small peptides. The current USP and EP monographs use two separate methods to assay oxytocin and the acetate counterion.[3-6] The USP monograph to assay oxytocin uses a gradient of sodium phosphate and acetonitrile on an L1 (C18) column with a 20 min run time and a flow rate of 1.5 mL/min. The EP oxytocin assay monograph is similar to the USP monograph, using a C18 column with a sodium phosphate/acetonitrile gradient. However, the EP method specifies a 1.0 mL/min flow rate and a run time of 30 min, with an additional 15 min of equilibration. The current USP and EP monographs for acetic acid in peptides are equivalent and are not specific to acetic acid in oxytocin. Similar to the oxytocin assay method, the acetic acid method uses an L1 column and a phosphate buffer/methanol gradient. This acetic acid method has a run time of 22 min at a flow rate of 1.2 mL/min, and uses a steep gradient to wash the peptide from the column after the acetate elutes. To fully analyze oxytocin acetate, it takes a minimum combined total of 42 min and two separate injections with different methods.

The Acclaim® Mixed Mode WAX-1 column is used, in this application note (AN), to separate oxytocin and acetate in a single chromatographic run. This column allows good retention of acetate and equivalent oxytocin elution times compared to a C18 column, leading to efficient determination of both acetate and oxytocin using a single injection. The broad applicability of this method to simultaneously determine acetate and peptides is demonstrated by the determination of acetate in four peptides: oxytocin, angiotensin I, bradykinin, and neurotensin. The Acclaim Mixed Mode column effectively separates the peptide of interest, acetate, and peptide impurities in less than 30 min. By using the 2.1 × 150 mm column format and simultaneously determining acetate and oxytocin, the proposed method provides faster total analysis time, uses only 13% of the solvent required for the USP methods, and generates less waste.

EQUIPMENT

Dionex UltiMate® 3000 Intelligent LC system consisting of:

- SRD-3200 Solvent Rack (Dionex P/N 5035.9250)
- HPG-3200M pump (Dionex P/N 5035.0018)
- WPS-3000TSL Micro autosampler (Dionex P/N 5822.0025)

Sample Loop, 25 µL (Dionex P/N 6820.2415)

TCC-3200 column compartment (Dionex P/N 5722.0025)

VWD-3400 detector (Dionex P/N 5074.0010)

- Semi-Micro Peek™ flow cell, 2.5 µL (Dionex P/N 6074.0300)

Chromeleon® 6.8 Chromatography Data System

Glass injection vials with caps and septa, 1.5 mL (Dionex P/N 055427) -or-

Polypropylene injection vials with caps and septa, 300 µL (Dionex P/N 055428)

Nalgene® Filter Unit, 0.2 µm nylon membrane, 1 L capacity (Nalgene P/N 164-0020)

REAGENTS AND STANDARDS

Deionized water, Type I reagent grade, 18 MΩ-cm resistivity or better

Methanol, HPLC grade or better (Honeywell P/N 230-4)

Potassium phosphate, monobasic, HPLC grade (Fisher Scientific P/N P286-1)

Tetrasodium pyrophosphate decahydrate (Fluka P/N 71515)

Phosphoric acid (EMD P/N PX0996-6)

pH buffers, 4.00 (VWR P/N 34170-127) and 7.00 (VWR P/N BDH5046-500mL)

Glacial acetic acid (USP P/N 1005706)

Chlorobutanol (VWR, Spectrum P/N CH1)

Samples

Angiotensin I (Sigma-Aldrich P/N A-9650)

Bradykinin (Sigma-Aldrich P/N B-3259)

Neurotensin (Sigma-Aldrich P/N N-6383)

Oxytocin (Sigma-Aldrich P/N O-6379)

CONDITIONS

Column: Acclaim Mixed-Mode WAX-1, 5 µm, 2.1 x 150 mm (Dionex P/N 067084)
Acclaim Mixed-Mode WAX-1, 5 µm, 2.1 x 10 mm guard column (Dionex P/N 069686) with Guard Holder (Dionex P/N 069580)

Mobile Phase: A: 50 mM potassium phosphate
B: methanol

Gradient: 98% A for 5 min, 2–50% B in 20 min,
50% B for 10 min, 5 min of equilibration prior to injection

Flow Rate: 0.21 mL/min

Temperature: 30 °C (column compartment)

Inj. Volume: 25 µL

Detection: Variable Wavelength UV-vis detector, 220 nm

Noise: ~0.06 mAU

System Backpressure: ~1400 psi

PREPARATION OF SOLUTIONS AND REAGENTS

Mobile Phase Solutions

Mobile Phase A

Dissolve 13.60 g of potassium phosphate, monobasic and 0.50 g of sodium pyrophosphate in 2000.0 g of deionized water. Add 200 µL of phosphoric acid to this solution and mix well. Filter the phosphate solution through a 0.2 µm nylon filter unit to remove insoluble particulates and sterilize the mobile phase. The pH of the mobile phase with this preparation should be 4.2.

Mobile Phase B

Transfer HPLC grade methanol into a 1 L media bottle and use without further treatment.

Autosampler Syringe Wash Solution

To prevent possible carryover from the autosampler, a wash solution of 50% methanol in DI water was used.

Standards and Sample Solutions

Acetic Acid Standards

Prepare a stock solution of acetic acid by transferring 1.87 g of glacial acetic acid to a 150 mL bottle and diluting to a total of 93.35 g with DI water, yielding a solution of 20.0 mg/mL of acetic acid. This can be done as follows: Preweigh a 125 mL capped bottle and record the weight. In a hood, transfer 1.5 mL of acetic acid to the bottle. Recap the bottle and record with weight of the bottle and acetic acid. Dilute the acid with an appropriate volume of water to prepare a solution containing 20.0 mg/mL of acetic acid. Standards should be prepared from the stock solution daily.

Chlorobutanol Resolution Stock

A stock solution of 0.5% chlorobutanol in 95/5 50 mM potassium phosphate/methanol was prepared as follows: a 50 mM potassium phosphate buffer was prepared by dissolving 1.70 g of potassium phosphate, monobasic and 0.07 g of sodium pyrophosphate in 250 g of deionized water. The solution was adjusted to pH 6.0 with sodium hydroxide and filtered through a 0.2 µm nylon filter unit.

A solution of 95/5 phosphate buffer/methanol was prepared by weighing 95.0 g of the 50 mM potassium phosphate buffer and adding 5 mL of methanol.

To prepare the 0.5% chlorobutanol solution, 0.52 g of chlorobutanol was placed in a 100 mL volumetric flask. The flask was filled to the mark with the 95/5 phosphate buffer/methanol solution. The chlorobutanol dissolved after four 10 min periods of sonication and flask inversion. This solution was used as a stock solution when preparing samples to confirm the USP resolution requirement between oxytocin and chlorobutanol.

Peptide Samples

Peptide stock solutions were prepared by dissolving 1.0 mg of peptide in 1.00 g of deionized water. These stocks were further diluted to 250 µg/mL with mobile phase A prior to analysis. Stocks and samples were stored at 4 °C.

Oxytocin with Chlorobutanol

A 250 µL volume of the stock solution of oxytocin was diluted in the chlorobutanol resolution stock to a total volume of 1 mL.

Precautions

The USP oxytocin reference standard should not be used for this method. The reference standard contains serum albumin which is not easily removed from the column under the method conditions. If USP oxytocin reference standard must be used, a column wash of 65% mobile phase B for a minimum of 15 min is recommended to remove the albumin from the column prior to the next injection.

Precautions must be taken to consistently prepare mobile phase A. Changes in either the ionic strength or pH of the mobile phase will shift analyte retention times. This is of particular importance as this phosphate solution is not at an optimal buffering pH. Acetate retention times were within ± 2%, using the mobile phase preparation described above.

To ensure mixing of samples, the tray shake option is highly recommended for samples that are stored for an extended time within the autosampler.

RESULTS AND DISCUSSION

Separation

Figure 2 shows the separation of acetate and oxytocin in a peptide sample on the Acclaim Mixed Mode WAX-1 column. The mobile phase pH was optimized at 4.2 after investigating a range of 3–6. A pH of 4.2 offers the best compromise between resolution, acetate retention time, and total run time. At lower pH values, acetate is weakly retained on the column, while at higher pH values (near 6) the resolution between oxytocin and peptide impurities is poor. At the optimized pH of 4.2, the acetate and oxytocin peaks are resolved from other impurities and easily quantified. The USP resolution between oxytocin and the next nearest peak is 2.60, which is greater than the USP monograph requirement of 1.5. An unknown impurity, Peak 3, is present in this peptide sample that elutes at 27 min. This impurity was not observed in injections of the USP reference material. If this impurity is absent, the run time of the method can be reduced to 20 min.

Chlorobutanol is a common preservative that is approved for use in injectable oxytocin solutions. To confirm that the presence of chlorobutanol does not interfere with oxytocin or acetate quantification, an oxytocin sample was prepared with chlorobutanol. As shown in Figure 3, there is significant resolution between oxytocin and chlorobutanol (USP R_s = 18.16). The USP resolution between oxytocin and the next nearest peak is equivalent to the resolution in the unpreserved sample. However, the high concentration of chlorobutanol coupled with the 25 µL injection volume, leads to excessive tailing of the chlorobutanol peak.

Quantification Assay, Linearity, Limits of Quantitation, and Limits of Detection

Determination of oxytocin by the EP method currently requires 250 µg/mL of oxytocin. The USP monograph assay specifies 10 USP Units/mL, which is equivalent to 25 µg/mL of oxytocin assuming an activity of not less than 400 Units/mg. The linear response of oxytocin determination by detection at 220 nm was investigated from 15 µg/mL to 250 µg/mL to cover the range of both monograph methods. The correlation coefficient across this range was determined to be 0.9997. The LOD and LOQ for oxytocin determination were also measured by injecting samples that produced a response 3 and 10 times the measured noise, respectively. These resulted in an LOD of 0.06 µg/mL and an LOQ of 0.2 µg/mL for oxytocin.

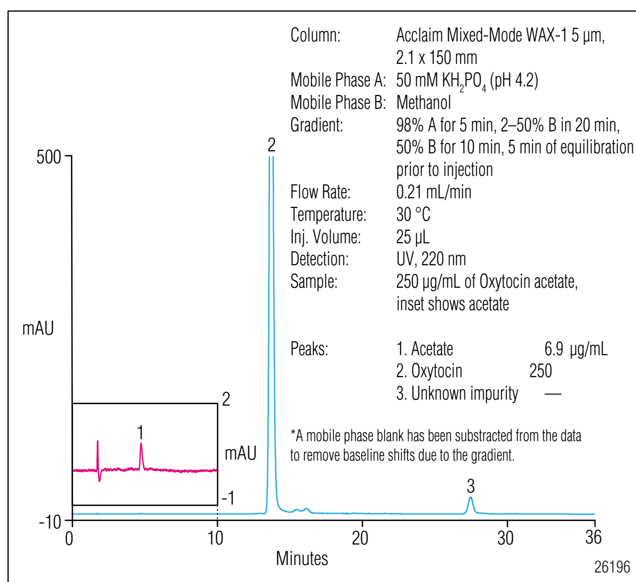

*Figure 2. Separation of acetate and oxytocin on the Acclaim Mixed-Mode WAX-1 column.**

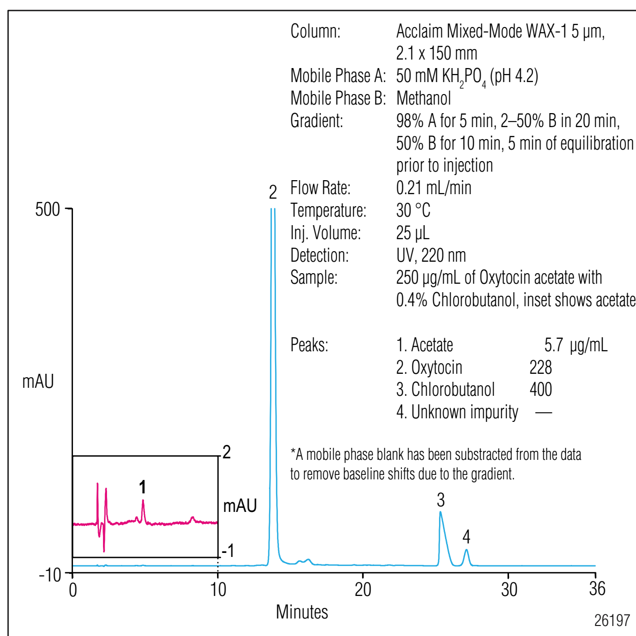

*Figure 3. Separation of acetate, oxytocin, and chlorobutanol on the Acclaim Mixed-Mode WAX-1 column.**

The concentration of acetate counterion in peptide preparations can vary greatly between peptides. The linearity of response of acetate was tested between 5.0 and 35 µg/mL to cover the expected range of acetate present in the peptides investigated. The correlation coefficient for acetate, the LOD, and the LOQ are presented in Table 1.

Table 1. LOD, LOQ, Linearity, and Precision for the Determination of Acetate and Oxytocin						
Analyte	**LOD (µg/mL)**	**LOQ (µg/mL)**	**Linear Range (µg/mL)**	**Correlation Coefficient r^2**	**Peak Area Precision[a] (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

[a] Precision was evaluated at 10 µg/mL (n=10) for acetic acid and 100 µg/mL (n=8) for oxytocin.

Table 2. Determination of Acetate in Four Different Peptides									
Peptide	**Est. Acetate Amount[b] (µg/mL)**	**Determined Acetate Amount (µg/mL)**	**Peptide RT (min)**	**Acetate RT (min)**	**Peptide Peak Area (mAU*min)**	**Acetate RT Precision (RSD)**	**Peptide RT Precision (RSD)**	**Acetate Peak Area Precision (RSD)**	**Peptide Peak Area Precision (RSD)**
Angiotensin I	12	13.0	17.80	4.74	252.4	0.16	0.17	1.94	0.11
Bradykinin	21	19.5	11.97	4.73	161.1	0.08	0.06	0.98	0.24
Neurotensin	20	24.0	18.27	4.72	357.5	0.08	<0.01	1.23	0.66
Oxytocin	6.2	6.7	13.83	4.74	292.6	0.08	<0.01	0.87	0.32

[b] The estimated acetate present in the peptide is based on the label estimates of 0.5 mol/mol acetate in oxytocin, 1.5 mol/mol acetate in angiotensin I, and 2 mol/mol acetate in bradykinin. Neurotensin samples contained 8% acetate, as detailed on the certificate of analysis for the neurotensin lot.

Precision and Accuracy of Acetate Analysis in Oxytocin and Other Peptides

The amount of acetate present in oxytocin as a counterion is quite small: ~0.5 moles of acetate per mole of oxytocin. However, the amount of acetate in other peptides can be as high as 2.5 mol/mol of peptide. To determine the concentration of acetate in the peptide solutions, all peptides were prepared at 250 µg/mL. Table 2 presents the acetate analysis of four peptides that incorporate acetate counterions. In all cases, the peptide peak area RSDs are < 2% and the method easily passes the USP acetate peak area RSD criteria of < 5%.

The determined acetate amounts in the peptide samples agree well with the labeled acetate-to-peptide mole ratio. The concentrations are within ± 10% of the estimated values, with the exception of neurotensin. The neurotensin sample contains 8% acetate based on the certificate of analysis for the lot, well over the 2.5 mole ratio of acetate to peptide that would predict 4% acetate based on the peptide purity and content. The determined amount of acetate in neurotensin was consistent with the value of 8% acetate, as listed in Table 2.

Figure 4 illustrates the separation of acetate from the peptides for oxytocin, bradykinin, angiotensin I, and neurotensin. In each case, both the acetate and the peptide are quantifiable using a single injection. Figure 5 shows an expanded view around the acetate peak. The acetate peak is easily integrated with no interferences from other components in the peptide samples.

Table 3. Recovery of Acetate in Peptide Preparations				
Peptide	**Amount of acetate determined in peptide (µg/mL)**	**Amount of acetate added (µg/mL)**	**Amount determined 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[c]	18.4	10.4	29.2	104
Oxytocin	6.7	11.2	18.4	105

[c] A different vial of neurotensin was used to prepare samples for acetate spiking than was used in Table 2. In addition, neurotensin was spiked with sodium acetate rather than acetic acid. Spiking with acetic acid led to unusually high recoveries (128-132%). Sodium acetate standards were tested and confirmed to have equivalent response compared to acetic acid standards.

Recovery of acetate in the four peptides was tested by spiking acetic acid into the peptide solutions, except neurotensin which was spiked with sodium acetate. The recoveries ranged from 98–105% (Table 3). Neurotensin was spiked with sodium acetate after spiking with acetic acid led to unusually high recoveries between 128–132%. It is suspected that the change in pH from spiking with acetic acid caused changes in the peptide, releasing additional bound acetate from the peptide. This effect was only observed in neurotensin samples.

Sample Stability

The stability of oxytocin in the diluent of mobile phase A was studied over five days at 4 °C, -20 °C, and ambient (~24 °C) temperature. Under each of these three conditions, no significant loss of oxytocin was observed. Peak area loss of oxytocin from the initial injections to the fifth day ranged from 2.8% for storage at -20 °C, 2.1% for storage at room temperature, and 1.3% for storage at 4 °C. The reduced peak area observed for storage at -20 °C is likely due to incomplete mixing during thawing of the sample in a 300 µL vial. It should be noted that upon storage there is potential for peptide adsorption to the vial walls, which may be partially responsible for the observed reduction in peak area.

CONCLUSION

The Acclaim Mixed-Mode WAX-1 column has been used to simultaneously determine peptides and the acetate counterion in a single injection. The method is fast, accurate, and has the sensitivity needed to determine the expected concentrations of acetate when using samples containing 250 µg/mL of peptide. Acetate has been successfully quantified in four different peptides, showing the flexibility of this method. The resolution and precision for replicate injections each exceeded the USP requirements for oxytocin and acetate. By using a 2.1 mm format column and combining the two methods into a single injection, this method saves time, reduces solvent use by a factor of five for the oxytocin assay alone, and minimizes waste.

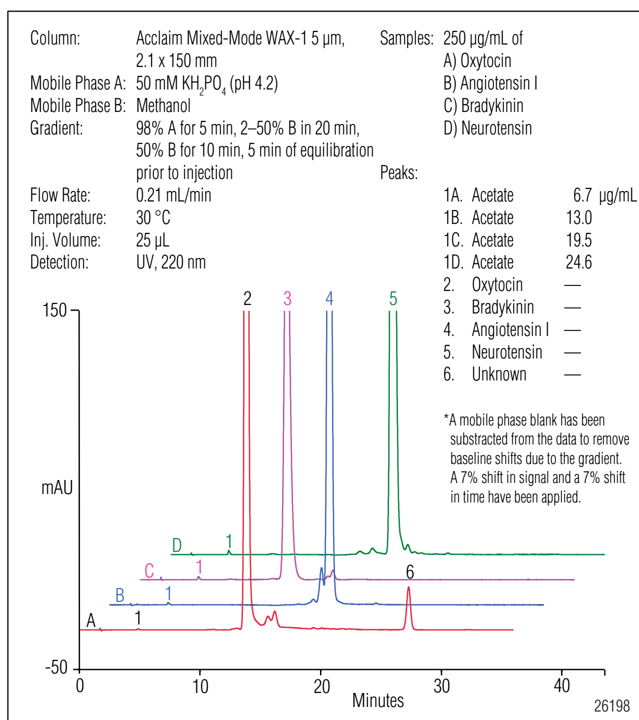

*Figure 4. Determination of acetate in oxytocin, angiotensin I, bradykinin, and neurotensin.**

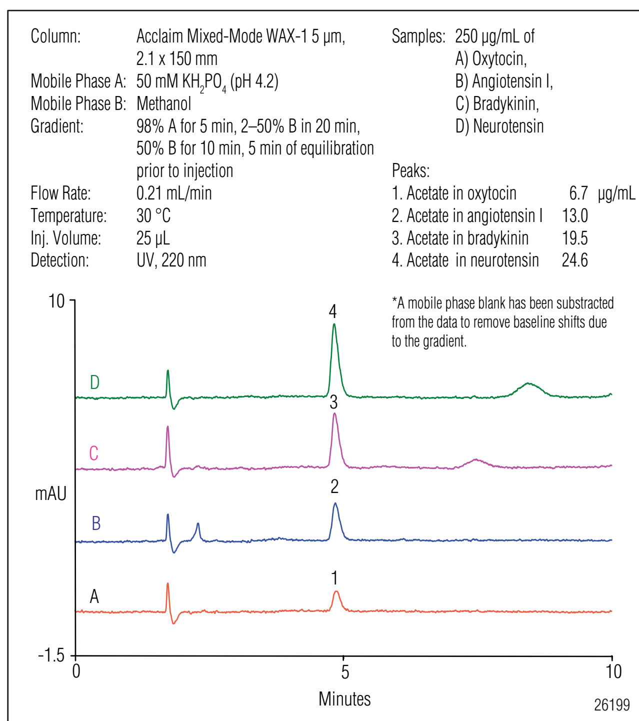

*Figure 5. Expanded view of acetate determined in oxytocin, angiotensin I, bradykinin, and neurotensin.**

LIST OF SUPPLIERS

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Sigma-Aldrich, P.O. Box 14508, St. Louis, MO 63178 USA. Tel: 800-325-3010. www.sigma-aldrich.com

U.S. Pharmacopeia, 12601 Twinbrook Parkway, Rockville, MD 20852, USA. Tel: 800-227-8772. www.usp.org

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LPN 2291 PDF 09/09

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