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# A Rapid Method for the Ultra-Sensitive Quantification of Fluticasone Propionate and Salmeterol Xinafoate from Human Plasma

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## APPLICATION BENEFITS

- Ultra-sensitive quantification of fluticasone propionate and salmeterol xinafoate
- Simple, selective, and fast sample preparation using Oasis™ PRiME HLB SPE in a µElution™ Plate format
- ACQUITY UPLC I-Class PLUS System with FTN and ACQUITY™ UPLC™ BEH $C_{18}$ Columns for optimal chromatographic performance
- High sensitivity using Xevo™ TQ-XS Mass Spectrometer

## WATERS SOLUTIONS

ACQUITY UPLC I-Class PLUS System

Xevo TQ-XS Mass Spectrometer

ACQUITY UPLC BEH $C_{18}$ Column

Oasis PRiME HLB 96-well µElution Plate

96-well Sample Collection Plate

## KEYWORDS

Fluticasone propionate, salmeterol xinafoate, µElution SPE, Oasis PRiME HLB, Xevo TQ-XS, BEH $C_{18}$

## INTRODUCTION

Fluticasone propionate (Figure 1A)[1] is a synthetic trifluorinated glucocorticoid receptor agonist with antiallergic, anti-inflammatory and antipruritic effects.[2] It binds and activates glucocorticoid receptors, resulting in the activation of lipocortin. Lipocortin, in turn, inhibits cytosolic phospholipase A2, which triggers a cascade of reactions involved in the synthesis of inflammatory mediators, such as prostaglandins and leukotrienes. Salmeterol xinafoate (Figure 1B)[3] is a highly selective, long-acting beta-2 adrenergic agonist with bronchodilatory activity.[4] It is used in the maintenance and prevention of asthma symptoms and maintenance of chronic obstructive pulmonary disease (COPD) symptoms.

Both fluticasone propionate and salmeterol xinafoate are inhaled compounds that are often co-administered in the treatment of asthma and COPD. Both compounds were designed to act on the lungs and airways with limited to zero systemic exposure.[5] Most of the systemic exposure that occurs is due to the patient swallowing the dose and the medicine entering the bloodstream vial the portal vein and liver. The compound levels are extremely low with peak concentrations of sub pg/mL reported, and therefore require a very high sensitivity assay to detect them.

Due to the extremely low circulating levels of these drugs, there is always a need to develop high sensitivity assays to better understand trough concentrations. A previously published method for these molecules has achieved LLOQ's of 0.2 pg/mL and 0.1 pg/mL for fluticasone propionate and salmeterol xinafoate, respectively.[6] This application note describes a quick and simple optimized workflow that uses Oasis PRiME HLB µElution Plates, ACQUITY UPLC I-Class PLUS System, and Xevo TQ-XS Mass Spectrometer, and achieves LLOQ of 0.1 pg/mL and 0.05 pg/mL for fluticasone propionate and salmeterol xinafoate, respectively.

## EXPERIMENTAL

### Sample preparation

Four hundred microliters of samples were pre-treated with 400 µL of 40:60 (v/v) 0.1 M $ZnSO_4$ in water:10% ammonium hydroxide in water, and mixed. These pre-treated samples were then extracted using Oasis PRiME HLB 96-well µElution Plates employing the protocol below.

### SPE protocol

| | |
|---|---|
| Load sample: | Pre-treated sample was loaded onto the extraction plate in two steps of ~400 µL each |
| Wash: | 200 µL of 50% methanol in water |
| Elute: | 2 × 25 µL 10:90 isopropanol/methanol (v/v) |
| Dilute: | 50 µL water |

### LC-MS/MS conditions

### LC conditions

| | |
|---|---|
| System: | ACQUITY UPLC I-Class PLUS |
| Detection: | Xevo TQ-XS Mass Spectrometer, ESI+ |
| Column: | ACQUITY UPLC BEH $C_{18}$, 1.7 µm, 2.1 × 50 mm (p/n: 186003554) |
| Temp.: | 60 °C |
| Sample temp.: | 5 °C |
| Injection volume: | 10 µL |
| Mobile phase A: | .10% ammonium hydroxide in water |
| Mobile phase B: | 10:90 isopropanol:methanol (v/v) |

Gradient:

| Time (min) | Flow rate (mL/min) | %A | %B | Curve |
|---|---|---|---|---|
| 0.00 | 0.3 | 50 | 50 | 6 |
| 1.00 | 0.3 | 50 | 50 | 6 |
| 3.00 | 0.3 | 5 | 95 | 6 |
| 4.00 | 0.3 | 5 | 95 | 6 |
| 4.10 | 0.3 | 50 | 50 | 6 |
| 5.00 | 0.3 | 50 | 50 | 6 |

### Data management

| | |
|---|---|
| LC-MS software: | MassLynx™ v4.2 |
| Quantification software: | TargetLynx |

MS conditions

| | |
|---|---|
| Capillary: | 1 kV |
| Cone voltage: | 30 V |
| Desolvation temp.: | 500 °C |
| Cone gas flow: | 150 L/hr |
| Desolvation gas flow: | 1000 L/hr |
| Collision gas flow: | 0.15 mL/min |
| Nebulizer gas glow: | 7 Bar |

MRM transitions:

| Compound name | Precursor (*m/z*) | Product (*m/z*) | Collision energy (eV) | Cone voltage (V) |
|---|---|---|---|---|
| Fluticasone propionate | 501.3 | 293.3 | 15 | 30 |
| Salmeterol xinafoate | 416.4 | 232.2 | 20 | 30 |
| Fluticasone propionate-d3 | 504.3 | 293.2 | 15 | 30 |

## RESULTS AND DISCUSSION

All steps for the LC and MS sample preparations were optimized during method development to ensure that analytes of interest were adequately separated from other matrix components and that maximum sensitivity was achieved.

### SAMPLE PREPARATION

Fluticasone propionate is known to have extremely high protein binding (>99%).[7] Therefore, it is essential to dissociate the analyte from plasma proteins to ensure accurate quantification of circulating levels. This was achieved by diluting the plasma sample with a combination of ammonium hydroxide and zinc sulphate. An SPE protocol, previously developed by Mather et al.[6] was used as a starting point for the SPE method development. Various wash solvent compositions were evaluated from 10:90 up to 50:50 methanol:water (v/v). The use of 50:50 methanol:water as the wash solvent yielded the best area counts, and was employed for the final method (data not shown). Similarly, different elution solvents comprising of 10:90 to 30:70 isopropanol:methanol, 25:75 to 75:25 acetonitrile:methanol, 100% methanol, 100% acetonitrile, and 100% isopropanol were evaluated. Ten:ninety and 20:80 isopropanol:methanol gave the highest recovery and area counts. Ten:ninety IPA:methanol was used as the elution solution in the final method as it matched the LC gradient starting conditions (Figure 2). Oasis PRiME HLB SPE removes the need for conditioning and equilibration steps, making the sample preparation process simpler and quicker. For fluticasone propionate and salmeterol xinafoate, use of Oasis PRiME HLB SPE showed matrix effects <1% and recoveries of >90%, and was used in the final protocol.

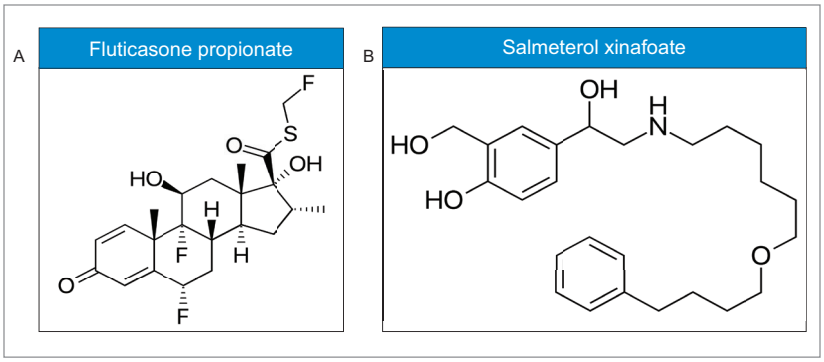


*Figure 1. (A) Structure of fluticasone free salt.[1] (B) Structure of salmeterol free salt.[3]*

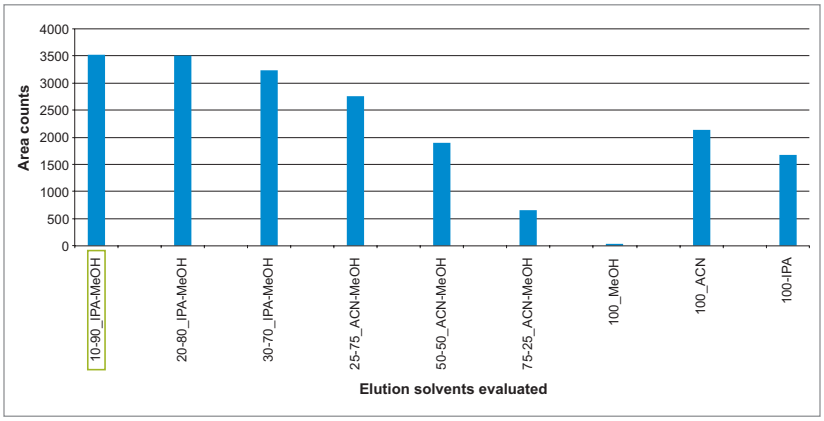


*Figure 2. Elution solvent evaluation: 10:90 IPA:methanol gives highest area counts for fluticasone propionate.*

### LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

The physico-chemical properties of fluticasone propionate and salmeterol xinafoate make them ideally suited for reversed-phase separations. Multiple reversed-phase columns, including ACQUITY UPLC BEH $C_{18}$, ACQUITY UPLC HSS T3, ACQUITY UPLC HSS $C_{18}$, and CORTECS™ $C_{18}$, were evaluated; and, the ACQUITY UPLC BEH $C_{18}$ Column gave the best chromatographic performance for both analytes. Additionally, flow rate and gradient conditions can have a significant impact on peak shapes and signal-to-noise ratios. After evaluating flow rates from 100–500 µL/min, and different gradient starting conditions, the flow rate of 300 µL/min and initial gradient condition of 50:50 mobile phase A:B were employed (Figure 3).

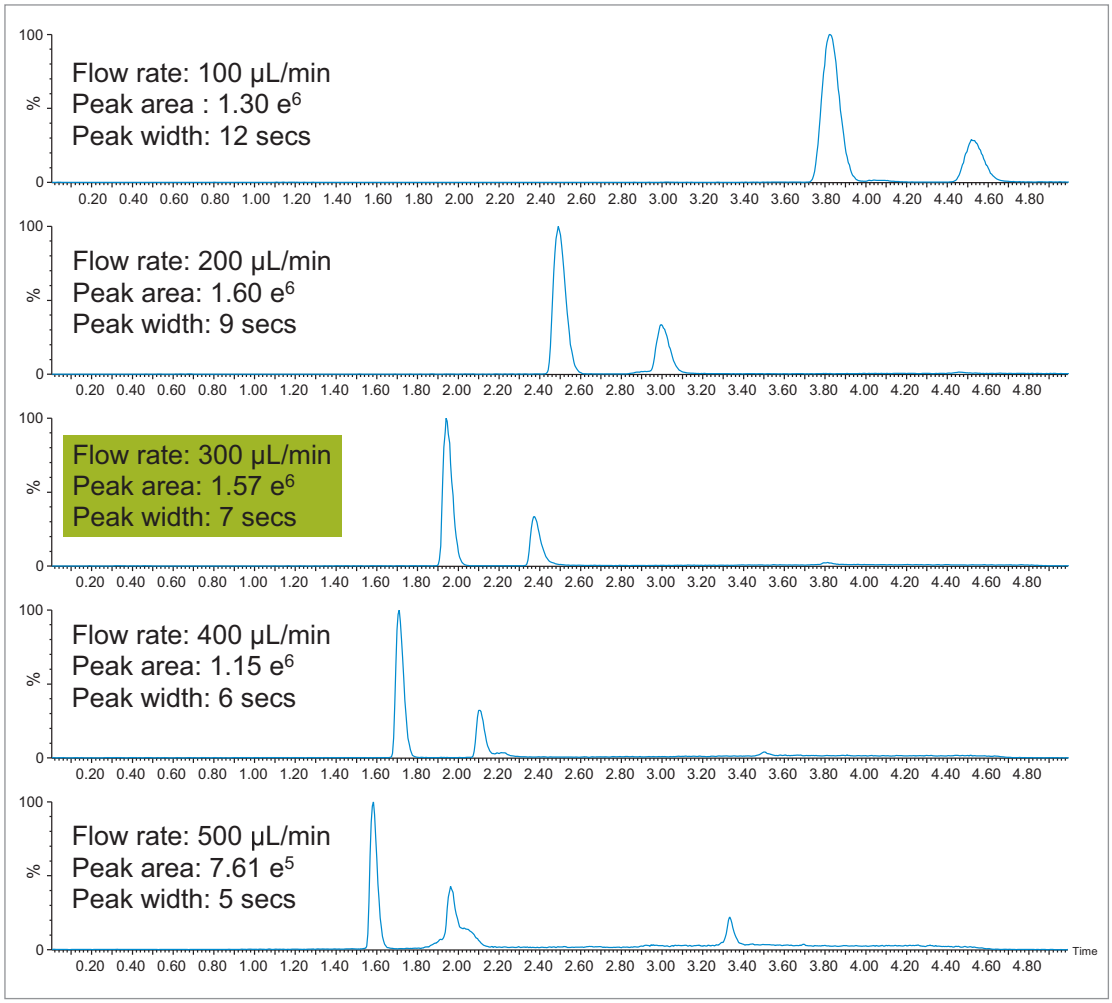


*Figure 3. Effect of flow rate on peak area and peak width for fluticasone propionate and salmeterol xinafoate.*

The Xevo TQ-XS Triple Quadrupole Mass Spectrometer, operating in a positive ion electrospray mode, was used to quantify fluticasone propionate and salmeterol xinafoate. Source conditions and tune page parameters were optimized, and the MRM transitions used are listed in the methods section.

## Linearity, precision, and accuracy

Using 400 µL of serum and aforementioned sample preparation, quantification limits of 0.1 pg/mL and 0.05 pg/mL (Figure 4) for Fluticasone propionate and Salmeterol xinafoate were achieved, respectively.

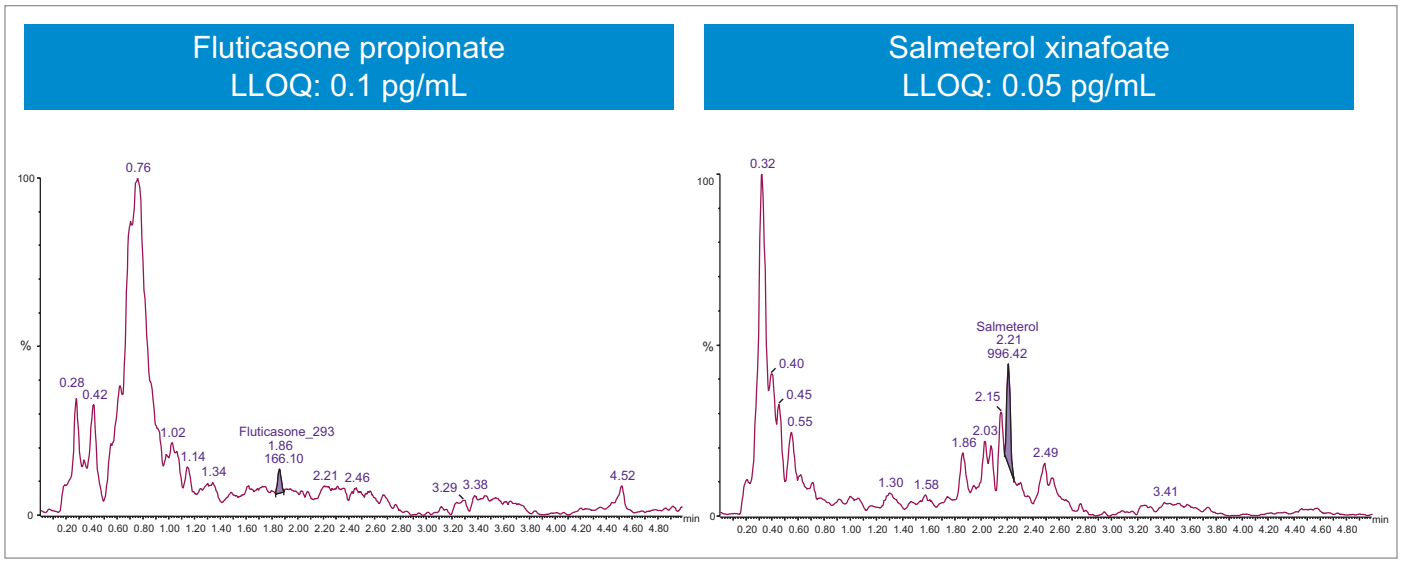


*Figure 4. LLOQ chromatograms for fluticasone propionate and salmeterol xinafoate.*

Calibration curves were linear with $R^2$ values >0.99 (1/x weighted regression) with inter-day mean accuracies of 100% and 99.32% for fluticasone propionate and Salmeterol xinafoate, respectively. A summary of standard curve performance is shown in Tables 1A and B. In addition, intra and inter-day precision and accuracy for both analytes was excellent with mean %RSDs all <10%. QC performance is highlighted in Tables 2A (fluticasone propionate) and 2B (salmeterol xinafoate), and a representative QC chromatogram for fluticasone propionate is shown in Figure 5.

*Table 1. Calibration curve performance for (A) fluticasone propionate and (B) salmeterol xinafoate.*

**A**

| Calibration curve | Day 1 | Day 2 | Day 3 | Inter-day |
|---|---|---|---|---|
| % Accuracy range | 86.9–112.7 | 81.8–114.3 | 83.8–115 | N/A |
| % Mean accuracy | 100 | 98.99 | 98.97 | 99.32 |

**B**

| Calibration curve | Day 1 | Day 2 | Day 3 | Inter-day |
|---|---|---|---|---|
| % Accuracy range | 82.3–114.5 | 85–113.4 | 85–110 | N/A |
| % Mean accuracy | 100 | 100 | 99.99 | 100.00 |

*Table 2. Intra and inter-day QC statistics for (A) fluticasone propionate and (B) salmeterol xinafoate.*

**A**

| | | Day 1 (N=3) | Day 2 (N=3) | Day 3 (N=3) | Inter-day |
|---|---|---|---|---|---|
| LQC (0.25 pg/mL) | % Accuracy | 90.03 | 98.37 | 112.79 | 100.40 |
| | % CV | 5.3 | 8.79 | 3.96 | 6.02 |
| MQC (1.0 pg/mL) | % Accuracy | 102.47 | 93.62 | 106.31 | 100.80 |
| | % CV | 1.384 | 3.87 | 5.82 | 3.69 |
| HQC (7.5 pg/mL) | % Accuracy | 89 | 114.45 | 91.32 | 98.26 |
| | % CV | 9.06 | 3.03 | 10.59 | 7.56 |

**B**

| | | Day 1 (N=3) | Day 2 (N=3) | Day 3 (N=3) | Inter-day |
|---|---|---|---|---|---|
| LQC (0.125 pg/mL) | % Accuracy | 86.19 | 89.87 | 103.47 | 93.18 |
| | % CV | 7.4 | 5.359 | 9.37 | 7.38 |
| MQC (0.5 pg/mL) | % Accuracy | 91.58 | 96.69 | 94.99 | 94.42 |
| | % CV | 4.63 | 0.372 | 8.83 | 4.61 |
| HQC (3.75 pg/mL) | % Accuracy | 98.6 | 98.63 | 95.31 | 97.51 |
| | % CV | 9.28 | 5.667 | 6.71 | 7.22 |

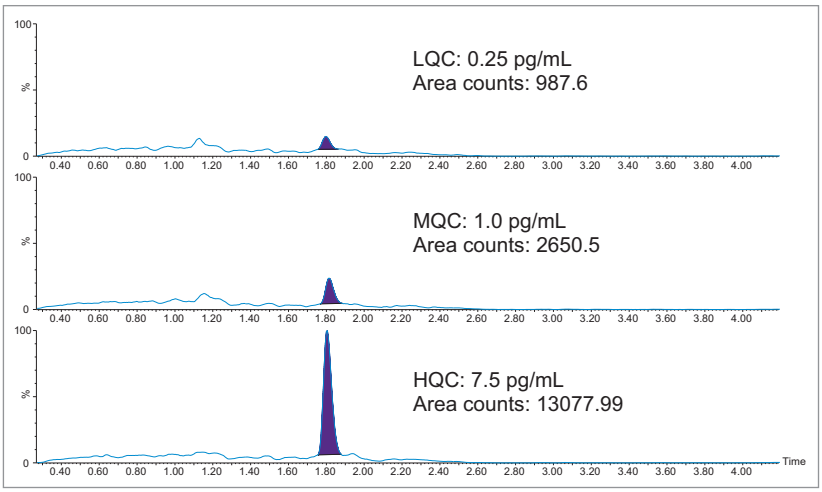


*Figure 5. Representative QC chromatograms for fluticasone propionate.*

## CONCLUSIONS

The method described employs a simple pretreatment and SPE strategy combined with analytical flow LC and tandem-quadrupole MS for the pg/mL level quantification of fluticasone propionate and salmeterol xinafoate from human plasma. The main features of the method include:

- Simple, fast, and inexpensive sample preparation with Oasis PRiME HLB SPE
- Use of a sub-2-µm BEH $C_{18}$ Column for excellent peak shapes and peak width

The analytical sensitivity (0.1 pg/mL and 0.05 pg/mL), linear dynamic range (0.1–10 and 0.05–5 pg/mL), and excellent reproducibility of the method described reliably measures low levels of fluticasone propionate and salmeterol xinafoate.

## References


1. Fvasconcellos. Wikipedia. [Online] September 2007. https://en.wikipedia.org/wiki/Fluticasone_propionate#/media/File:Fluticasone_propionate.svg.
2. Ji, A.J.; Zhou, D.; Zhaang, S.; Cawley, M.A.; Fang, X.; Wu, J. Ultrasensitive and Automated 1 pg/mL Fluticasone Propionate Assay in Human Plasma Using LC-MS/MS. *Bioanalysis*. **2013**, *5*(4), 423–435.
3. Ayacop. Wikipedia. PubChem. [Online] January 13, 2007. https://commons.wikimedia.org/wiki/File:Salmeterol.svg.
4. Samir, A.; Salem, H.; Abdelkawy, M. Simultaneous Determination of Salmeterol Xinafoate and Fluticasone Propionate in Bulk Powder and Seritide® Diskus using High Performance Liquid Chromatographic and Spectrophotometric Method. *Pharmaceutica Analytica Acta*. **2012**, *3*(8), http://dx.doi.org/10.4172/2153-2435.1000180 (accessed month date, year).
5. Sunwoo, J.; Rhee, S.; Lee, S.; Lee, S.W.; Jung, J.; Son, H.; Jang, I. Pharmacokinetic Characteristics of Fluticasone, Salmeterol and Tiotropium After Concurrent Inhalation. *Translational Clinical Pharmacology*. **2017**, *25*(2), 85–92.
6. Mather, J.; Graham, K.; Rodriguez Cabaleiro, D.; Chadwick, S.; Rainville, P.; Plumb, R.Quantification of Fluticasone Propionate and Salmeterol Xinafoate in Plasma at the Sub pg/mL Level using UPLC/MS/MS. Waters Application Note, 720004340EN (2012).
7. Tayab, Z.R.; Fardon, T.C.; Lee, D.K.C.; Haggart, K.; McFarlane, L.C.; Lipworth, B.J.; Hochhaus, G. Pharmacokinetic/Pharmacodynamic Evaluation of Urinary Cortisol Suppression After Inhalation of Fluticasone Propionate and Mometasone Furoate. *British Journal of Pharmacology*. **2007**, *64*(5), 698–705.


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