A RAPID METHOD FOR THE ULTRA-SENSITIVE QUANTIFICATION OF FLUTICASONE PROPIONATE AND SALMETEROL XINAFOATE FROM HUMAN PLASMA

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INTRODUCTION

Fluticasone propionate (Figure 1a) (1) is a synthetic trifluorinated glucocorticoid receptor agonist with antiallergic, anti-inflammatory and antipruritic effects (2). Fluticasone propionate binds and activates glucocorticoid receptor, 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.

These two inhaled compounds 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 is due to the patient swallowing the dose and the medicine entering the bloodstream via the portal vein and liver. There levels are extremely low with peak concentrations of sub pg/mL reported, thus requiring a very high sensitivity assay to detect these compounds.

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. Previously published method for these molecules have achieved LLOQ's of 0.2 pg/mL (6) and 0.1 pg/mL for Fluticasone propionate and Salmeterol xinafoate respectively. This poster describes an optimized, quick and simple workflow using Oasis HLB PRiME µElution, Acquity I-class plus and Xevo TQ-XS, achieving LLOQ of 0.1 pg/mL and 0.05 pg/mL for Fluticasone propionate and Salmeterol Xinafoate respectively.

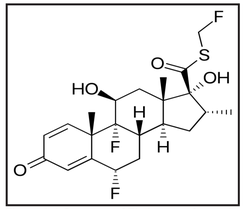

Figure 1a) Structure of Fluticasone free salt

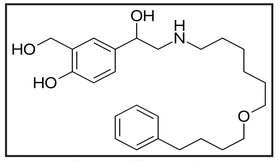

Figure 1b) Structure of Salmeterol free salt

METHODS

SAMPLE PREPARATION:

400 µL of samples were pre-treated with 400 µL of 40:60 (v/v) 0.1M $ZnSO_4$ in Water:10% Ammonium Hydroxide in Water and mixed. These pre-treated samples were then extracted using Oasis PRiME HLB 96 well µElution plates using 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 x 25 µL 10:90 Isopropanol/Methanol (v/v)
Dilute: 50 µL Water

LC CONDITIONS:

LC System:	ACQUITY UPLC® I-Class
Detection:	Waters Xevo TQ-XS Mass Spectrometer, ESI+
Column:	ACQUITY UPLC BEH C_{18}, 130Å, 1.7 µm, 2.1 mm x 50 mm
Temp:	60 °C
Sample Temp:	5 °C
Injection Volume:	50 µL
Mobile Phases:	A:10% Ammonium Hydroxide in Water 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

MS CONDITIONS:

Capillary (kV):	1
Cone Voltage (V)	30
Desolvation Temperature (°C):	500
Cone Gas Flow (L/Hr):	150
Desolvation Gas Flow (L/Hr):	1000
Collision Gas Flow (mL/min.):	0.15
Nebuliser Gas Flow (Bar):	7

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-d_3	504.3	293.2	15	30

RESULTS

Using 400 µL of plasma and the aforementioned sample preparation strategy, quantification limits of 0.1 pg/mL & 0.05 pg/mL (Figures 2a & 2b) for Fluticasone propionate and Salmeterol xinafoate respectively were achieved. 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 Table 1a & 1b. 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 3.

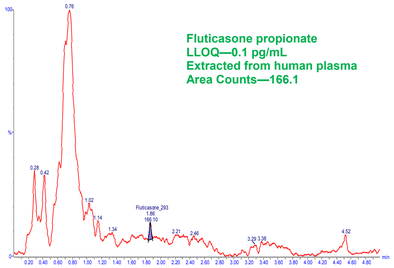

Figure 2a) Representative LLOQ chromatogram for Fluticasone propionate

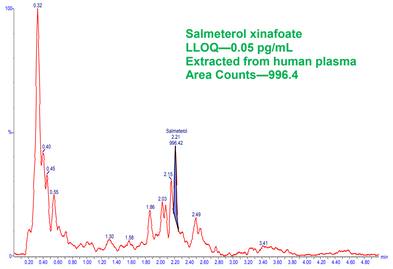

Figure 2b) Representative LLOQ chromatogram for Salmeterol xinafoate

DISCUSSION

Sample Preparation:

Fluticasone propionate is known to have extremely high protein binding (>99%) (7). Hence, 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. SPE protocol previously developed by Mather et al. (6) was used as the starting point for SPE method development. Various wash solvent compositions, starting from 10:90 up to 50:50 Methanol:Water (v/v) were evaluated. The use of 50:50 Methanol:Water as the wash solvent yielded the best area counts and was employed for the final method. 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. 10:90 and 20:80 Isopropanol:Methanol gave the highest recovery and area counts. 10:90 Isopropanol:Methanol was used was used as the elution solution in the final method as it matched the LC gradient starting conditions. Oasis PRiME HLB 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 showed matrix effects <1% and recoveries of >90% and was used in the final protocol.

Liquid Chromatography-Mass Spectrometry

The physico-chemical properties of Fluticasone propionate and Salmeterol xinafoate make them ideally suited for a reversed-phase chromatographic separation. Multiple reversed-phase columns, including BEH C_{18}, HSS T3, HSS C_{18} and CORTECS C_{18} were evaluated and BEH C_{18} gave the best chromatographic performance for both analytes. Additionally, flow rate and gradient conditions can also have a significant impact on peak shapes and signal to noise. After evaluating flow rates from 100 – 500 µL/min and different gradient starting conditions, flow rate of 300 µL/min and initial gradient conditions of 50:50 mobile phase A:B were employed (Figure 3). The Xevo TQ-XS tandem quadrupole mass spectrometer operating in positive ion electrospray mode was used to quantify Fluticasone propionate and Salmeterol xinafoate. Source conditions and tune page parameters were optimized and MRM transitions listed in the methods section were used.

Calibration Curve 0.1—10 pg/mL	Day 1	Day 2	Day 3	Inter-Day
% Accuracy range	82.3 - 114.5	85 - 113.4	85 - 110	NA
% Mean Accuracy	100	100	99.99	100.00

Table 1a) Calibration curve statistics for Fluticasone Propionate

Calibration Curve 0.05—5 pg/mL	Day 1	Day 2	Day 3	Inter-Day
% Accuracy range	86.9 - 112.7	81.8 - 114.3	83.8 - 115	NA
% Mean Accuracy	100	98.99	98.97	99.32

Table 1b) Calibration curve statistics for Salmeterol Xinafoate

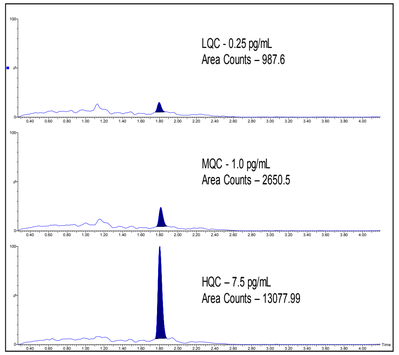

Figure 3) Representative QC chromatograms for Fluticasone Propionate

		Day 1	Day 2	Day 3	Inter-Day
LQC (0.250 pg/mL)	% Accuracy	86.19	89.87	103.47	93.18
	% CV	7.4	5.359	9.37	7.38
MQC (1.0 pg/mL)	% Accuracy	91.58	96.69	94.99	94.42
	% CV	4.63	0.372	8.83	4.61
HQC (7.5 pg/mL)	% Accuracy	98.6	98.63	95.31	97.51
	% CV	9.28	5.667	6.71	7.22

Table 2a) QC statistics for Fluticasone Propionate

		Day 1	Day 2	Day 3	Inter-Day
LQC (0.125 pg/mL)	% Accuracy	90.03	98.37	112.79	100.40
	% CV	5.3	8.79	3.96	6.02
MQC (0.5 pg/mL)	% Accuracy	102.47	93.62	106.31	100.80
	% CV	1.384	3.87	5.82	3.69
HQC (3.75 pg/mL)	% Accuracy	89	114.45	91.32	98.26
	% CV	9.06	3.03	10.59	7.56

Table 2b) QC statistics for Salmeterol Xinafoate

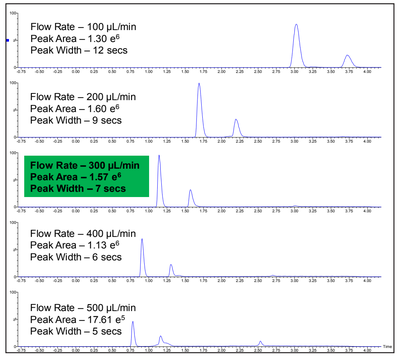

Figure 4) Representative chromatograms showing effect of flow rate for

CONCLUSION

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

- Simple, fast and cheap sample preparation with simple Oasis PRiME HLB
- Use of a sub 2µm BEH C_{18} column which provided excellent peak shapes and peak width

The analytical sensitivity (0.1 pg/mL & 0.05 pg/mL), linear dynamic range (0.1 – 10 & 0.05 - 5 pg/mL), and excellent reproducibility of the method described reliably measures low levels of Fluticasone propionate and Salmeterol Xinafoate making it suitable for bioanalysis.

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