[APPLICATION NOTE]

A HIGH SENSITIVITY UPLC/MS/MS METHOD FOR THE ANALYSIS OF FLUTICASONE PROPIONATE IN PLASMA

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INTRODUCTION

Fluticasone propionate (Figure 1) is a glucocorticoid indicated for the prophylactic treatment of asthma and is administered via inhalation from an aerosol-type device or power inhaler. The activity of fluticasone propionate when inhaled is due to the parent drug, with any metabolites formed being 2000 times less active.

Studies using oral dosing of labeled and unlabeled drug have demonstrated that the oral systemic bioavailability of fluticasone propionate is negligible (< 1%). This is primarily due to incomplete absorption and pre-systemic metabolism in the gut and liver, with the only reported circulating metabolite being the 17β-carboxylic acid derivative. Plasma fluticasone propionate concentrations, when delivered nasally, show a C_{max} average of 11.9 pg/mL and $AUC_{(0\text{-}t)}$ average of 8.43 pg hr/mL.

Due to the low circulatory levels of fluticasone propionate, it is necessary to have a high-sensitivity assay, in the < 10 pg/mL range, to correctly define the pharmacokinetics in plasma. Previous reports have demonstrated assays in the 10 to 500 pg/mL range for fluticasone propionate in plasma.[1,2]

The development of a high-sensitivity assay in biological fluid, such as plasma or serum, can be a time-consuming and complicated process. The plasma protein must be removed from the sample to prevent column fouling and improve assay ruggedness. This is normally achieved by protein precipitation or sample extraction, either liquid-liquid or solid-phase. Matrix components such as plasma lipids, amino acids, and other small molecular weight components can coelute with the analyte of interest and result in ion suppression or enhancement.

Of these components, phospholipids have been identified as being largely responsible for ion suppression in bioanalysis. Thus, in developing a bioanalytical assay there are three major challenges:

1. Determining a selective and specific MS/MS methodology
2. Developing a high-throughput LC process that does not compromise the specificity or sensitivity of the assay
3. Developing an extraction process that isolates and concentrates the drug from the plasma components

In this application note, we show a high-sensitivity method for the analysis of fluticasone propionate in rat plasma.

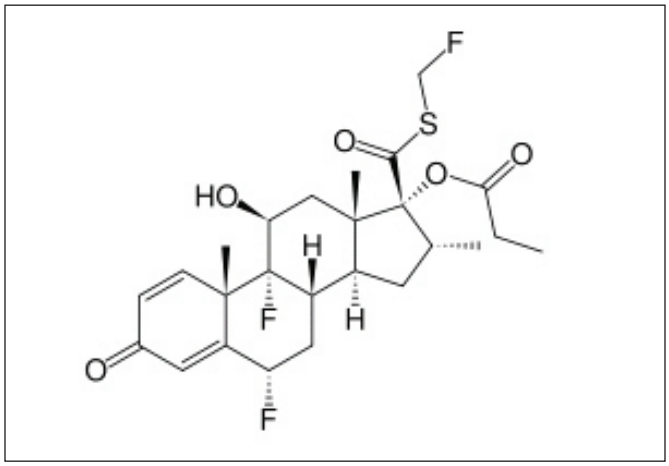

Figure 1. Structure of fluticasone propionate.

EXPERIMENTAL

LC /MS conditions

LC system:	Waters® ACQUITY UPLC® System
Column:	ACQUITY UPLC BEH C_{18} Column
	2.1 x 50 mm, 1.7 µm
Column temp.:	45 °C
Flow rate:	500 µL/min
Mobile phase A:	0.1% Ammonium Hydroxide
Mobile phase B:	Methanol
Gradient:	50 to 95% B/2 min
MS system:	Waters® Xevo™ TQ MS
MS/MS transitions:	Fluticasone 501.3 > 293.3
	Budesonide 431.3 > 323.2
Ionization:	Positive ion ESI
Capillary voltage:	1.5 KV
Collision energy:	18 eV
Cone voltage:	24 V

Solid-phase extraction

The plasma samples were extracted by diluting 375 µL of plasma with an equal volume of aqueous ammonium hydroxide (10%) and 40% 0.1 M zinc sulphate, which contained the internal standard budesonide at a concentration of 300 pg/mL. The samples were added to an Oasis® HLB µElution SPE 96-well plate which had been previously prepared by washing with methanol (200 µL) and water (200 µL). The sample was drawn through under vacuum then washed with 200 µL of 25% methanol/water solution. The sample was eluted with 2 x 25 µL of 50:50 acetonitrile/methanol, collected in a microtitre plate, and diluted with 50 µL of water in order to match initial gradient conditions.

UPLC/MS/MS method

The high efficiency of sub-2-µm particle LC, UPLC®, and the resulting improvements in analyte resolution, throughput, and sensitivity has made it the ideal partner for mass spectrometry. A systematic evaluation of column chemistries, mobile phase pH, and organic modifier showed that the use of an ACQUITY UPLC C_{18} BEH Column with a 50 to 95% methanol/ammonium hydroxide (0.1%) gradient over 2 minutes produced the best resolution and assay sensitivity.

RESULTS

The resulting chromatograms produced for the UPLC/MS/MS analysis of fluticasone propionate standard and the budesonide internal standard are shown in Figure 2. Here we can see that the fluticasone elutes with a retention time of 1.18 minutes and budesonide with a retention time of 1.09 minutes. The peak width at the base was in the range of 2 to 3 seconds. The analysis of these organo-aqueous standards of fluticasone propionate suggested that the required assay sensitivity was achievable.

The evaluation of a simple protein precipitation method revealed that it did not have sufficient sensitivity to address the assay needs nor did it remove the matrix effects. Previous reports have demonstrated

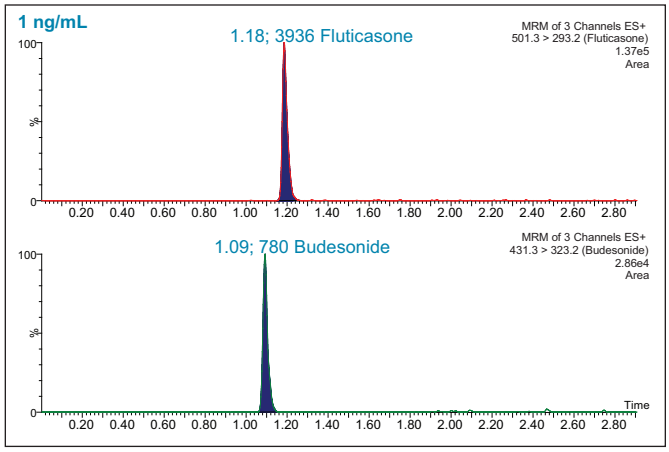

Figure 2. LC/MS/MS chromatogram of fluticasone propionate, top, and budesonide internal standard, bottom.

the efficiency of solid-phase extraction for the analysis of fluticasone propionate in plasma.[1] Consequently, an SPE methodology was developed for this assay. The use of a micro-elution plate approach allowed fluticasone to be extracted from the plasma sample and concentrated without the need for an evaporation step. This also eliminated the possibility of analyte loss, due to adherence to the surface of the collection vessel. The chromatogram displayed in Figure 3 illustrates the separation of the analytes from the choline-containing phospholipid matrix.

During the SPE method development process, the recovery of fluticasone propionate was determined to be > 95% when using aqueous standard solutions, however, with plasma samples the analyte recovery dropped to just 28%. This reduction in analyte recovery was attributed to plasma-protein binding that was not sufficiently disrupted by dilution with an acid or base buffer prior to SPE. The use of ammoniacal zinc sulphate 0.04 M was proven to be more than four times more effective than aqueous base alone at disrupting the protein binding.

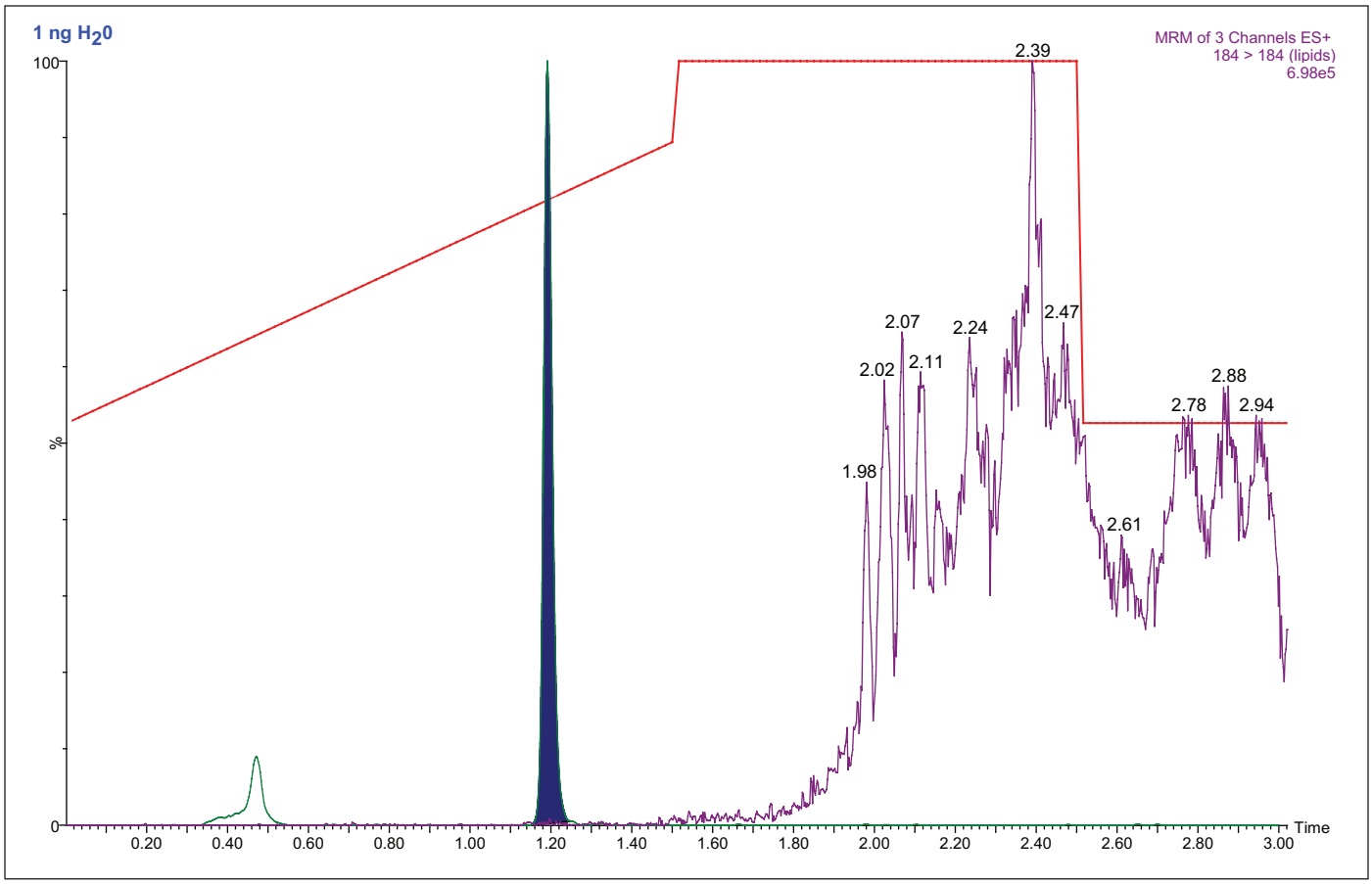

Figure 3. Separation of fluticasone, green trace, from endogenous choline-containing phospholipids, purple trace. The gradient profile utilized in the separation is shown, red trace.

By employing this step prior to SPE, the extraction efficiency was increased to > 95%, ± 2%, over four separate experiments. The overall assay sensitivity was determined to be 3 pg/mL for the limit of detection (LOD), and 5 pg/mL for the limit of quantification (LOQ), Figure 4. The overall assay was shown to be valid over the range of 5 to 1000 pg/mL, with an R^2 value of 0.994. Second-analyst, second-day value showed linearity over the same range with an R^2 value of 0.996.

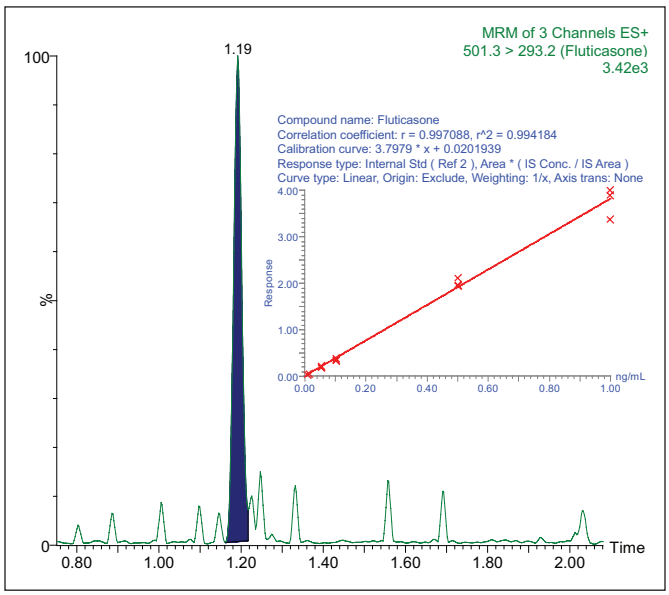

Figure 4. LC/MS/MS chromatogram of 5 pg/mL fluticasone propionate plasma standard and calibration line.

The overall usable sensitivity of the assay is dependent upon the carryover achieved by the LC system. The carryover was determined by the injection of an upper limit of quantification standard, followed by the injection of blank plasma. Any carryover observed must be less than 20% of the peak height of the lowest calibration standard. The data displayed in Figure 5 shows the resulting carryover observed; in this data the upper trace shows the LOQ standard at 5 pg/mL and the lower chromatogram shows a blank sample injection directly following the injection of a 1000 pg/mL standard. As we can see from this data, there was no carryover detected.

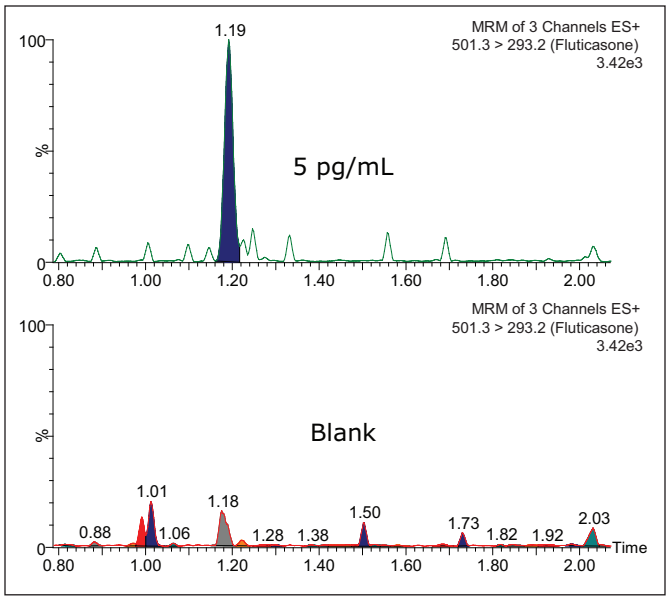

Figure 5. LC/MS/MS chromatogram of 5 pg/mL fluticasone propionate plasma standard and blank sample injection.

The very narrow peaks produced by ACQUITY UPLC and the sub-2-µm particles place a data collection constraint on the mass spectrometer. In order to accurately define the LC peaks, it is necessary to have 10 to 12 data points across the peak. For a peak that is 2 seconds wide, with the simultaneous collection of two MRM channels, a dwell time of 50 milliseconds is required, allowing for an inter-channel delay of 10 milliseconds. The Xevo TQ MS is equipped with a T-Wave™ collision cell that allows for rapid data collection, without loss in signal quality or data intensity. Using this collision cell, it is possible to have MS channel dwell time of just 5 milliseconds with an inter-channel delay as low as 3 milliseconds. This rapid data acquisition ensures that the LC/MS/MS peaks are correctly defined, that the apex of the peak is not missed, and that any peak shoulders are correctly defined.

CONCLUSION

- A high-sensitivity assay for fluticasone propionate in rat plasma was developed with an LOQ of 5 pg/mL.
- This sensitivity was achieved by using just 375 µL of plasma, which makes it suitable for low-sampling volume studies.
- The use of UPLC allowed for the development of a rapid-throughput assay while maintaining the resolution of the analyte peak from the endogenous matrix components.
- Oasis µElution plate technology was employed for the efficient isolation and concentration of the analyte from the plasma matrix.
- Carryover was determined to be well below the permitted limit.
- The rapid data capture capability of the Xevo TQ MS allowed for the narrow MRM peaks to be accurately defined and quantified.

References

1. Carter SJ, et al. J. Chrom. B. 2008; 876: 163-169.
2. Krishnaswami S, et al. J. Pharm. Biomed. Anal. 2000; 22: 123-129.

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