Large Volume Injection with On-Column Enrichment and Gradient Focusing in HPLC and UPLC for Assay for Fluticasone Propionate in Fine Particle Dose Dissolution Studies

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Detection for assay of active ingredients from low dose pharmaceutical formulations is often challenging, particularly when large numbers of samples need to be analysed with fast turnaround as is the case for dissolution studies. In order to obtain the necessary speed short/narrow LC columns are usually employed and sample injection volumes are minimised in order to maintain the separation efficiency and peak shape on the small column format. However, typical dissolution media are usually aqueous in nature and therefore have weak elutropic strength. The use of predominantly aqueous diluents and gradient elution programs permits large injection volumes to be employed in UPLC using narrow diameter columns. Provided the column has good retention for the solute of interest it is possible to use extremely large injection volumes to increase the limit of quantification whilst maintaining good peak-shape, separation speed and efficiency. In this study the large volume injection / gradient focusing technique was applied to the measurement of fluticasone propionate to support dry powder inhaler fine particle dose dissolution studies. The absolute amount of compound being analysed is small and that the loading capacity of a column in these situations is more than large enough for the compound being analysed.

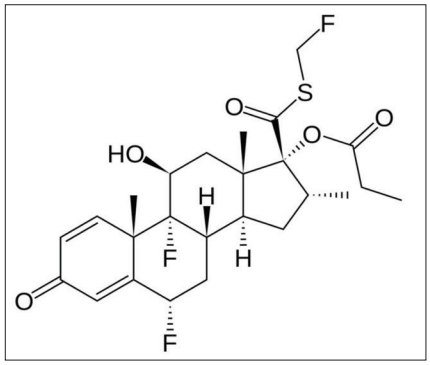

Figure 1: Structure of Fluticasone Propionate.

Introduction

Modern gradient liquid chromatography (LC) systems and column developments (including the use of porous monoliths, superficially porous phases and sub two micron porous particle packed columns) have enabled pharmaceutical labs to achieve unprecedented levels of throughput in recent years [1,2]. The use of high sample concentrations, small injection volumes and narrow columns is now commonplace in the industry. However, the assay of low-dose formulations can be a challenge with regard to achieving the necessary low limits of detection, particularly when test sample concentrations are low. In the development of dry powder and metered dose inhaled products the successful assay of aerosolised particle size and fine particle dose dissolution can be complicated by the large dilution factors used [3,4,5,6].

The predictive properties of low dose fine particle mass dissolution studies with regard to pharmacokinetic uptake of inhaled fluticasone propionate (FP) in the lung have been studied concluding that aerosolised fine particle doses from inhaled drug delivery devices are typically less than thirty micrograms [7]. Aerosolised particles are collected onto stainless steel mesh filters placed strategically into next generation impactors (NGI). After device actuation the filters are removed from the NGI apparatus and placed in traditional dissolution baths. In order to obtain sufficient sensitivity to discriminate between dissolution rates of different formulations the bath volume is large (400 mL) and the diluent is optimised such that dissolution occurs over a significant time (typically greater than 5 hours). Maximum concentrations are typically less than 50 ng / mL and early time-point samples will have API concentrations of 1 ng/mL or less. Such concentrations are too low to measure accurately using conventionally scaled LC-UV assay techniques where sample injection volumes, limited by the system injection configuration, typically ranging from 1 - 50 µL. With multiple device actuations and the need to perform large numbers of rapid assay measurements at different dissolution time-points it is necessary to operate the assay in high throughput mode in order to avoid sampling time delays which could bias dissolution results.

High sensitivity assay methods for FP have been published using tandem mass spectrometry utilising deuterated internal standards to overcome instrument variability [8,9,10]. Due to the lipophilicity (logP = 3.4) and lack of acid/base functionality of FP (Figure 1) the primary ionisation of the molecule at atmospheric pressure is challenging and only the most modern mass spectrometers are capable of achieving the necessary sensitivity to meet the requirements of the dissolution study without prior sample concentration or large volume injection. Experiments carried out in-house (data not shown) demonstrated that that the ionisation of FP was variable from day to day and a significant amount of tuning and optimisation was necessary in order to achieve the necessary limit of detections. It was concluded that LC-UV would be a more reliable and cost-effective approach to assay should a way to achieve the necessary limit of detection sensitivity be possible.

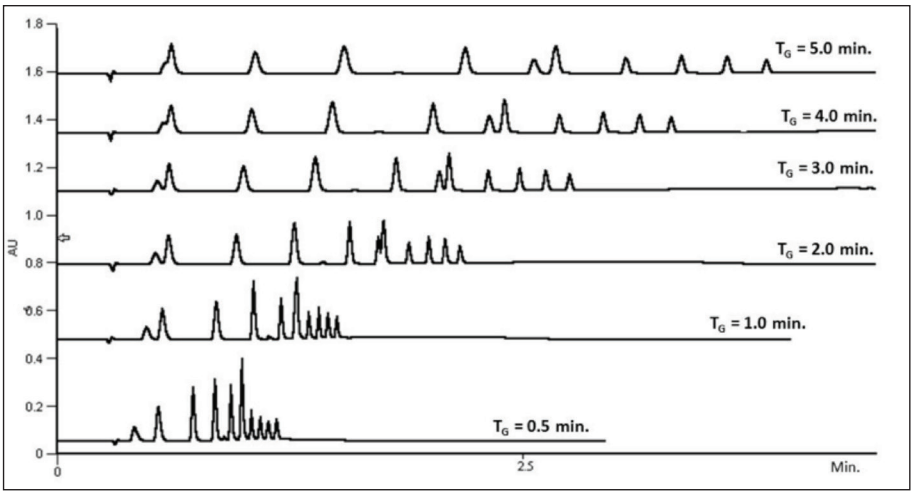

Figure 2: The influence of mobile-phase gradient ramp-rate on peak-shape and resolution of 12 solutes of widely differing polarity [16. Separations were performed on a 3.0 x 50 mm Luna C18 (1), 3.5 µm column with mobile phase flow-rate of 1 mL/min using the Agilent 1100 HPLC system (dwell volume 1.1 mL) and UV detection wavelength of 225 nm. The faster acetonitrile ramp-rates (5-95%) promote focusing and resolution of polar solutes at the expense of narrowing the elution window.

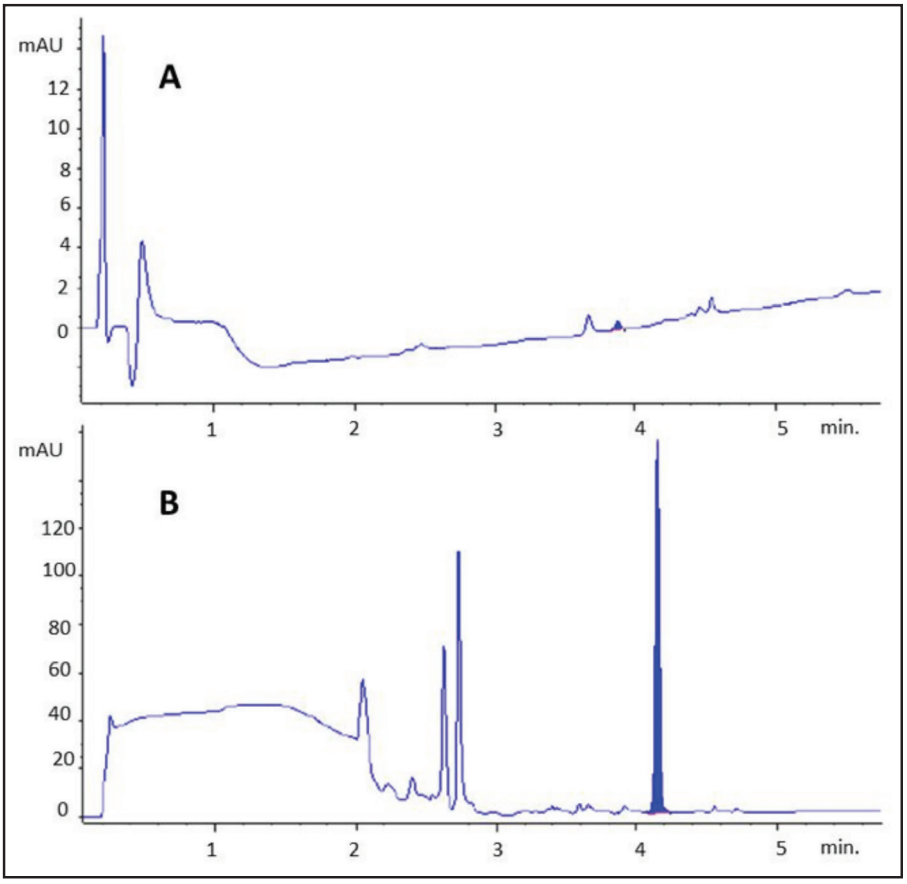

Figure 3: Overlaid chromatograms (242 nm) arising from HPLC analysis of a solution of FP (peak shaded, 100 ng/mL in dissolution media), showing a 400 fold enrichment of FP peak area as a result of increasing the injection volume. A = 5 µL injection volume, 500 pg injected through HPLC autosampler; B = 2 mL full-loop manual injection.

The hydrophobic nature of glucocorticoids, such as FP, facilitates their trace enrichment from aqueous solution using a reversed phase retention mechanism. This can be performed on-line to the chromatographic separation using automated solid phase extraction (SPE) [11], an on-line pre-concentration column [12] or directly onto the analytical column itself using large volume injection delivered via an oversized loop [13], injection pump [14] or by sampling from a flowing stream using electrokinetically driven cross-flow [15].

Provided the organic solvent content of the mobile phase remains below the concentration required to effect elution of solutes from the column then very large volumes can be injected. Interfering matrix components from the dissolution media (surfactants *etc*) can be separated without affecting the chromatographic performance of the column. In this approach the test solutes are enriched on the head of the column before a gradient elution program is invoked to elute and focus them before detection. The use of a rapid gradient programme is more effective at focusing polar solutes but longer gradient programs usually provide better overall resolution by widening the elution window as shown in Figure 2 where a published test mixture of neutral solutes [16] is separated using different gradient ramp times. In this study larger than usual injection volumes are employed in an attempt to reduce the limit of detection of for FP in dissolution media by a factor of 400 enabling direct, high-throughput analysis of low concentration samples from fine particle dose dissolution studies to be measured without sample pre-concentration.

Experimental

Reagents

Micronised FP was obtained from Pfizer (Sandwich, UK). Acetonitrile (HPLC gradient grade) was obtained from Sigma Aldrich (poole, UK) and water was obtained from a MilliQ A10 gradient system (Millipore, Billerica, USA). Dissolution media was prepared using sodium dodecyl sulphate (2 mM), PIPES (piperazine-N,N'-bis-2-ethanesulfonic acid (20 mM) and sodium chloride (134 mM). All dissolution media additives were obtained from Sigma Aldrich, Poole, UK).

Chromatography

Methods development using HPLC was performed using an Agilent 1100 system comprising a well-plate auto-sampler, quaternary pump, degasser and UV detector with standard flow-cell under Chemstation software control (Agilent, Waldbronn, Germany). HPLC separations were performed using Onyx monolithic C18 columns (50 x 2 mm) obtained from Phenomenex (Macclesfield, UK). Mobile phase was acetonitrile:water in gradient elution mode. The acetonitrile composition was maintained at 5% from 0 – 0.1 minutes after injection then ramped from 10 to 90% from 0.1 to 5 minutes, returning to 5% from 5 to 8 minutes. Larger volume HPLC injections were performed using a lab-Pro 10 port valve fitted with stainless steel loops of 250, 500 and 2000 µL volume. Gas-tight syringes (Hamilton, Reno, USA) of appropriate size

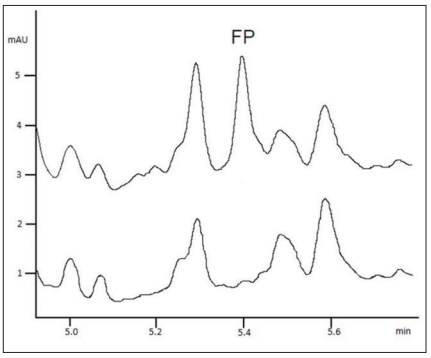

Figure 4: Low-level HPLC detection of FP in dissolution buffer showing baseline interference from trace contaminants in sample diluents arising from concentration of a 2 mL injection volume onto a 50 x 2 mm column. Blue chromatogram (242 nm) = 500 pg/mL spiked dissolution buffer and red chromatogram = blank dissolution media.

were used to manually load the loops with overfill factor of 1.5 times the loop volume.

The optimised UPLC assay for dissolution samples was performed using an Acquity system with variable wavelength detector fitted with high-sensitivity flow-cell under Empower software control (Waters, Milford, USA). Mobile phase was acetonitrile:water (containing 5 mM ammonium acetate (Sigma, Poole, UK)) in gradient elution mode. Separations were performed using a BEH C18 column (50 x 2.1 mm, 1.8 µm) also obtained from Waters, Milford, USA). The acetonitrile composition was maintained at 5% (v/v) from 0 – 0.5 minutes after injection then ramped from 5% to 55% at from 0.5 to 2.0 minutes, returning to 5% from 2.1 to 2.5 minutes. Further details are provided in legends to Figures.

Dissolution Testing

Fine particle dose dissolution samples were prepared by actuating proprietary development samples of an inhaled drug delivery device (Pfizer, Sandwich, UK) onto a modified next generation impactor (Copley Scientific AG, Therwil, Switzerland) collecting the FP fine particle dose onto a glass fibre filter disk. Following device actuation the filter disks were immersed into dissolution baths using a specially modified stainless steel holder and samples were taken periodically over a period of 15 hours for analysis by UPLC using a fraction collector. The VanKel dissolution apparatus and fraction collector were supplied by Varian, Palo Alto, USA.

Results and Discussion

Preliminary experimentation to assess the suitability of the large volume injection approach for assay of samples from fine particle dose dissolution studies were performed with a standard Agilent 1100 HPLC system. The auto-sampler was fitted with a 100 µL loop and syringe. Following optimisation of gradient elution profiles to ensure resolution of API from potentially interfering matrix components it was found that the injection volume of a 100 ng/mL solution of FP in dissolution buffer could be increased from 5 µL to 100 µL gaining a linear increase in peak response whilst maintaining good chromatographic efficiency for FP and matrix peaks. Further experiments were carried out using a lab-Pro 10 port valve as a manual injection system with loop volumes of 250, 500 and 2000 µL. Full loop injections were made and chromatographic performance was still very good with a linear increase in FP peak response using all four loops. Linearity of UV peak area response was plotted as a function of injection volume over the range of 5 to 2000 µL resulting from injection of a 100 ng/mL solution of FP in dissolution media achieved a linear regression result of $y = 0.1761x + 1.0252$ and $R^2 = 0.9999$. The positive intercept was biased by inclusion of the upper (2000 µL) calibration point which was 40 times the volume of the lowest injection volume used to construct the curve. Omitting this point and recalculating over the range of 5 to 1000 (µL) resulted in a linear regression result with reduced intercept bias of 0.1108X-0.0043 (R2-0.9995). Precision measurements (n = 5) were made using the 500 µL loop and injecting a 100 ng/mL FP solution in full-loop mode with 1.5 x overfill factor. The % RSD for the FP peak area was 0.78 and the % RSD for the FP peak apex retention time was 0.07.

The large loops were switched out of line after the sample was swept out to minimise gradient dwell volume effects. Shifts in retention time were observed corresponding to increased dwell volume from increased loop-size during the injection cycle. The quaternary HPLC system had a gradient dwell-time of 1.1 minutes so a one minute gradient delay-time was introduced to the HPLC method when the 2 mL loop was used to ensure that the gradient did not enter the loop before it was switched out of line. It is likely that even larger injections could be performed should the need arise and cycle-time and sample volume are not a limitation. Loops were flushed with 2 full loop volumes of acetonitrile between injections in order to reduce carry-over. A 400-fold increase in limit of detection, concordant with the increase in injection volume for FP was achieved using the 2 mL loop injection when compared to a 5 µL injection volume typically employed with assay measurements using a 50 x 2 mm column format. A shift in retention time was observed as a result of the increased system volume during loop emptying but peak shape and overall run time were unaffected (Figure 3).

With the largest (2 mL) loop the HPLC method solution concentrations could be measured with a limit of quantitation of 500 pg / mL. Baseline interference from enriched trace components in the sample diluents may become a limitation to even larger injection volumes (Figure 4). Following the initial method development using HPLC the approach was transferred to a Waters Acquity UPLC system fitted with a 250 µL syringe, 100 µL sample loop and high-

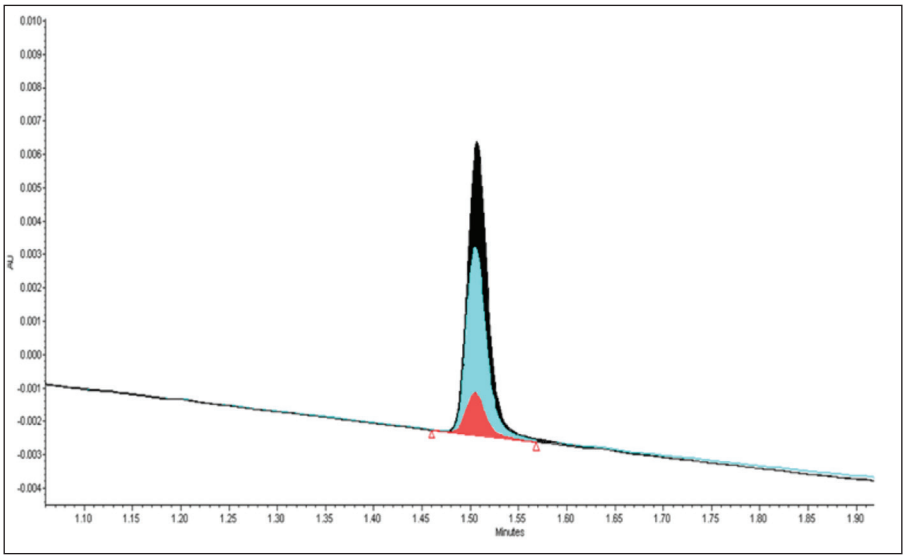

Figure 5: Example chromatograms (242 nm) from optimised high-speed UPLC method with high-sensitivity flow cell showing low-level detection of FP in dissolution samples using 100 µL injection with 2 mm I.D. column. Red chromatogram = time-point T1 (10 minutes, 6.1 ng/mL FP), cyan chromatogram = time-point T5 (90 minutes, 18.9 ng/mL FP) and black shaded chromatogram = time-point T10 (900 minutes, 29.1 ng/mL FP).

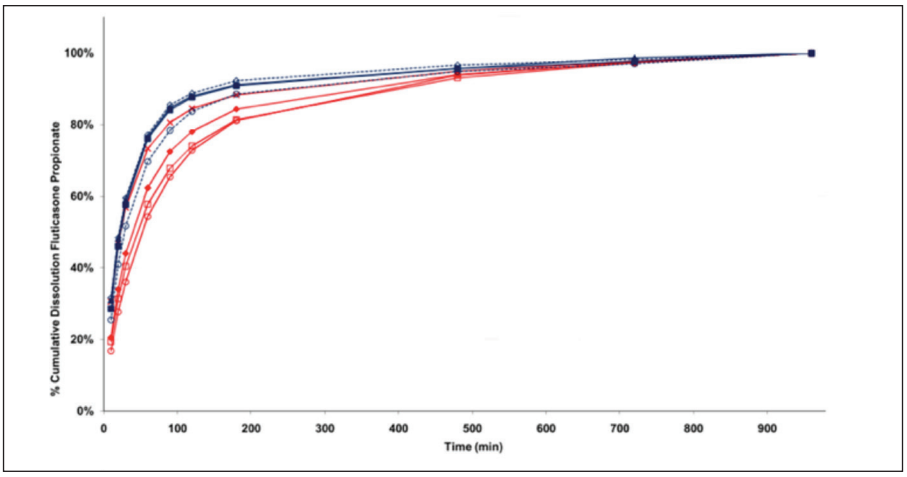

Figure 6: Example cumulative dissolution plots from single-shot FP fine particle doses (100 µg/dose) captured from actuations of eight different developmental dry powder inhaler devices onto filter-disks in NGI device, showing differences in dissolution rates at scales.

sensitivity flow-cell. With a full-loop (2.5 times overfill) injection mode the method was capable of detecting FP in dissolution samples down to a LOQ of 1 ng/mL (100 pg injected). Peak area % RSD (n = 6) with 25 ng/mL FP concentration in dissolution media was 0.34 and retention time % RSD was 0.29.

Low-level dissolution experiments were completed following capture of doses from a 100 µg dose of FP using different devices and formulations and discrimination between the dissolution-rate of batches was observed. The UPLC method with large (100 µL) injection volume had sufficient sensitivity to measure the low-concentration early time-point samples (Figure 5) and produce useful dissolution curves (Figure 6). It could be possible to increase the injection volume on the UPLC method (using a larger loop) to further reduce the FP LOQ to a projected 50 pg/mL. However, trace contamination of the dissolution medium would need to be carefully controlled to avoid baseline interference with detection of FP at such a low level.

Despite the presence of SDS in the dissolution media no problems with column-contamination or degradation of column performance were observed with over 1000 injections being completed on a single column. Despite the fact that the injection volumes were significantly greater than the calculated column volume ($V_m \approx 0.5Ld_c^2$) chromatographic performance was still very good due to strong retention of FP which was presumably concentrated as a tight plug at the head of the column and focused by the rapid gradient during elution. As the lipophilicity and retention of the solutes decreases then band-broadening due to a reduced gradient focusing effect increases. If the approach were to be used for less polar solutes then longer, more retentive columns incorporating phases with higher surface area may help to focus dispersed polar solutes prior to elution from the column. Furthermore, increasing the gradient ramp-rate can help to focus weakly retained and dispersed solute bands before they reach the detector (as in Figure 2). The high linear flow velocity of the mobile phase also facilitates the focusing effect by reducing dwell-time of the gradient and this could be increased further should the need arise though this will reduce UV response. Back-pressure with the monolithic HPLC column with a 1 mL/min flow-rate was 50 to 100 bar, depending on the mobile phase composition, so there is an opportunity to increase the mobile-phase velocity significantly to improve speed and efficiency of the focusing mechanism.

Conclusion

This work demonstrated that provided the sample diluent is of sufficiently low elutropic and the API is sufficiently well retained by the stationary phase it is possible to use injection volumes greatly in excess of the column volume to perform on-column sample enrichment. The on-column enrichment approach can result in sensitivity improvements of 400 fold or more when compared to standard injection volumes employed in LC but is limited to samples where sample matrix interferences are non-problematic. It greatly simplifies analysis as additional valves and enrichment columns are not required. In combination with more selective detection techniques (e.g. tandem mass spectrometry) this simple approach could be used to drive down detection limits when the need arises without resorting to more complicated and expensive alternative detectors or column-switching. It is particularly applicable to dissolution studies where sample volumes are rarely a limitation, test solute concentrations are often low and the dissolution medium is usually an aqueous buffer. The approach could also be applied with mixed-mode and ion exchange columns [17,18] to help retain and enrich more polar analytes. However, for routine operation modifications to auto-samplers would need to be made to enable larger than usual injections including the necessary flow diversion and wash functions.

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