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Short communication

A rapid and sensitive method for the quantitation of montelukast in sheep plasma using liquid chromatography/tandem mass spectrometry

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Abstract

A rapid LC–MS/MS method was developed and partially validated for the quantitation of montelukast in spiked sheep plasma. A total run time of 1.5 min was achieved using a short monolithic column and employing a rapid gradient. Sample preparation involved protein precipitation with twofold acetonitrile by volume during which a deuterated internal standard (montelukast D-6) was incorporated. The MRM transitions for montelukast and the deuterated internal standard were 586/422 and 592/427, respectively. A linear dynamic range of 0.25–500 ng/mL with a correlation coefficient of 0.9999 was achieved. Precision was below 5% at all levels except at the LOQ (0.36 ng/mL) which demonstrated an overall of R.S.D. of 8%. Post-column infusion experiments were performed with precipitated plasma matrix and showed minimal interference with the peaks of interest.

Keywords: Montelukast; Sheep; Plasma; Mass spectrometry

1. Introduction

Montelukast (Singulair^{TM}) is a potent leukotriene receptor antagonist approved for the treatment of asthma and seasonal allergic rhinitis [1,2]. Leukotrienes are produced and released from proinflammatory cells and are highly potent broncho-constrictors that lead to the development of asthma and associated symptoms [1]. Previously published bioanalytical methods for montelukast employed HPLC with fluorescence detection with total run-times ranging from 4.5 to 15 min [3–8]. The lowest reported LOQ's obtained using these approaches was 1 ng/mL in human plasma matrix [3]. Although the intrinsic fluorophore of montelukast can be exploited for highly sensitive methods, our efforts focused on developing a rapid LC–MS/MS method that would achieve a short analysis time of 1.5 min and provide further sensitivity improvements to support *in vivo* models. In order to achieve ultra-fast run times, the use of a 'ballistic' gradient was required in combination with high flow-rate and a short column. This type of approach has been reported by several authors [9,10] and was successfully applied herein.

While there are many benefits of ballistic gradients, the effect of fast chromatography can result in incomplete separation of matrix components from the analyte(s) of interest. Although the selectivity of MS/MS eliminates most potential interference issues, the plasma matrix can result in significant ion suppression/enhancement [11]. Accordingly, post-column infusion experiments were performed to investigate matrix effects for the LC–MS/MS analysis of montelukast in sheep plasma.

2. Experimental

2.1. Chemicals and reagents

HPLC-grade methanol and acetonitrile were obtained from EM Science (Darmstadt, Germany). Formic acid was purchased from Fluka (Buchs SG, Switzerland). Montelukast and montelukast-D-6 were obtained from the Merck Chemical Repository (Rahway, NJ, USA). Ammonium acetate was purchased from Sigma (Oakville, Canada). Deionized water was

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generated using a Millipore Milli-Q system (Bedford, MA, USA). Drug-free sheep plasma was obtained from a local farm.

2.2. Instrumentation

All analyses were performed using an HP1100 HPLC from Agilent (Palo Alto, CA, USA) coupled with a Leap Technologies HTS-PAL autosampler equipped with a valve self-wash system (Carrboro, NC, USA). A 20-μL loop was partially filled with 10 μL of sample at each injection using a 100 μL syringe. Chromatographic separations were carried out on a Merck KGaA Chromolith Flash RP18e, 4.6 mm × 25 mm monolithic column (Darmstadt, Germany). Mass spectrometric measurements were made using an Applied Biosystems API4000 (Foster City, CA, USA) mass spectrometer equipped with a Turbo-Ionspray source. Control of the LC-MS system as well as quantitation was accomplished using Analyst Version 1.4.1.

2.3. Standard and sample preparation

Standard solutions of montelukast and the D-6 internal standard (IS) were prepared in acetonitrile. All further dilutions of montelukast and the IS were made in acetonitrile. Working plasma standards were prepared by combining 50 μL blank sheep plasma, 50 μL working standard, 50 μL IS and vortexing for 10 min. The resultant mixture was centrifuged at 20,000 × *g* for 15 min and 100 μL of the supernatant was removed for injection. Quality control samples were prepared at four levels by spiking blank sheep plasma with stock solutions of montelukast in 50:50 methanol:water. Fifty micro-litres of the QC sample was combined with 50 μL acetonitrile, 50 μL IS and processed as above. The final QC concentrations were 0.36, 1.8, 45, 450 ng/mL.

2.4. HPLC conditions

Extracted plasma samples (10 μL) were injected on to the HPLC column, operated at a flow-rate of 2 mL/min. The column temperature was maintained at 45 °C. The mobile phase A and B consisted of 0.5% formic acid in water (v/v) and 0.5% formic acid in acetonitrile (v/v), respectively. The gradient program consisted of 30–95% B in 1 min followed by a plateau at 95% B for 0.2 min and re-equilibration to 30% B over 0.3 min. A divert valve program was used to divert the eluent at 0.9–1.4 min to the mass spectrometer. In order to minimize carryover, six needle washes were performed post-injection using 30% acetonitrile/30% methanol/40% DMSO (v/v) followed by 50% acetonitrile/50% water/0.1% formic acid (v/v). The valve assembly was cleaned with each of the preceding solvent mixtures for 20 s following each injection.

Post-column infusion experiments were performed by connecting a tee union after the column to allow a 15 μL/min syringe pump infusion of a 2.5 μg/mL montelukast standard into the mobile phase stream. Injections of either blank diluent (33% water/67%acetonitrile, v/v) or extracted blank sheep plasma were scheduled while monitoring montelukast by MS/MS.

2.5. Mass spectrometry conditions

The Turbo-Ionspray electrospray source was operated at 2500 V in positive ion mode. A zero-air generator was used for gas 1 and gas 2 at instrumental settings of 60 and 70, respectively. Multiple-reaction monitoring (MRM) mode was used to quantify montelukast (586/422) and the deuterated internal standard (592/427). The scans were performed using a dwell time of 40 ms and a pause time of 5 ms. The entire HPLC eluent flow was directed to the MS source without further splitting. The CAD, CUR, CE and TEM were maintained at 5, 25, 35 and 600 °C, respectively.

3. Results and discussion

3.1. Method development

Preliminary method development involved selection of the appropriate mass spectrometry conditions for detection of the analytes. Positive mode ionization was selected after evaluating both polarities from infusion of a montelukast standard in 50:50 10 mM ammonium acetate (pH 6.8):acetonitrile. Since montelukast contains a carboxylic acid functionality, negative mode was expected to provide equal or better response. In Q1 full-scan mode, both positive and negative showed similar response, however the negative mode product-ion was extensively fragmented and showed a greater than 10-fold reduction in intensity (data not shown). The transition 586/422 in positive mode was selected for this method. The selection of 0.5% formic acid (v/v) as the modifier in the mobile phase was then found to provide optimal sensitivity for positive-mode ionization of montelukast. Interestingly, the internal standard montelukast-D6 MRM transition was 592/427 and provided insight into the fragmentation mechanism. The product-ion scan of montelukast-D6 resulted in a 6 Da shift compared to montelukast for the fragment ions 574(568), 530(524), 462(456) and 446(440) but only 5 Da shift for 427(422) (Fig. 1A and B). The product-ion spectrum of 588 (^{37}Cl isotope of montelukast) was used to confirm the presence of chlorine in all fragment ions (Fig. 1C). A proposed mechanism for the formation of the major fragment ions of montelukast is shown in Fig. 2. By comparing product-ion spectra of montelukast and the deuterated internal standard, the mass shift indicates the isopropyl moiety is conserved in the fragment ions at 574(568), 530(524), 462(456) and 446(440). The 5 Da shift for 422(427) therefore stems from the dehydration of the isopropanol to propene, which conserves five of the original six deuterons for the internal standard. The proposed mechanism was also confirmed by obtaining MSMS spectra of 568(574) which resulted in product-ions 422(427) (data not shown).

Selection of the monolithic C18 column was completed after a quick evaluation of several manufacturers of short columns. In order to facilitate a rapid analysis time, all column lengths were restricted to ≤30 mm while using a steep gradient profile and short equilibration period. In general, the selectivity was essentially the same for all columns and each showed similar profiles in the post-column infusion experiments (data not shown). Therefore, the criteria used to select a column was

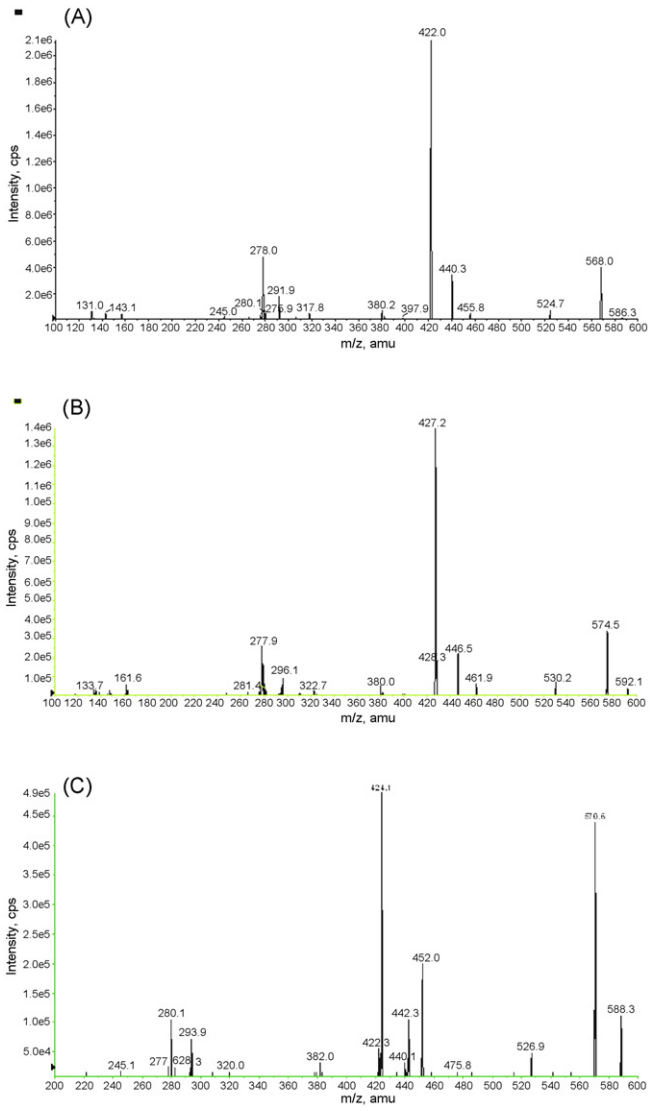

Fig. 1. Product-ion spectra of (A) montelukast $m/z = 586$, (B) montelukast-D-6 $m/z = 592$, (C) ^{37}Cl isotope of montelukast $m/z = 588$.

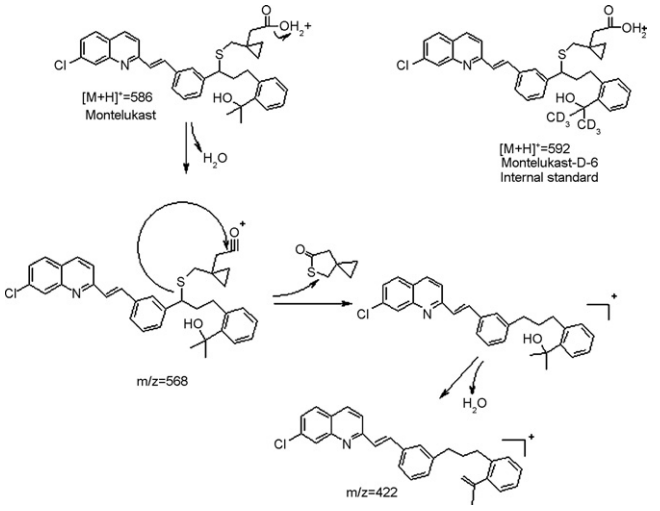

Fig. 2. Proposed mechanism for the CID fragmentation of montelukast to $m/z = 422$, and structure of deuterated internal standard.

Table 2
Representative precision and accuracy of montelukast standards in sheep plasma

	Montelukast standard curve						
Standard level (ng/mL)	0.2512	0.5024	1.0048	5.024	50.24	100.48	502.4
n	4	6	6	6	6	6	6
Accuracy[a] (%)	106.4	96.4	99.6	95.6	100.9	99.2	100.3
% R.S.D.	12.8	4.8	3.0	1.5	1.5	0.9	1.9

[a] Accuracy was back calculated from the calibration curve.

firstly, peak shape and secondly, column pressure which can be a concern when analyzing large amounts of plasma samples. Table 1 shows the performance results obtained from the different columns. The Magic Bullett™ column which uses a tapered diameter design (4 mm → 1 mm) was expected to produce a sharper peak shape since the linear velocity would increase (since $\mu \sim 1/\text{diameter}^2$) after separation at the front of the column, thereby reducing band broadening effects. However, the sharpest peak and lowest back-pressure was achieved using the monolithic column. The unique bimodal pore structure of monolithic columns enables high permeability and hence high mobile phase flow rates with low back-pressure while maintaining a large surface area for efficient separation capacity. The applicability of monolithic liquid chromatographic phases in the field of quantitative bioanalysis has been evaluated [12] and there are several recent examples of monolithic columns successfully used for bioanalysis [13–16]. These columns demonstrate less clogging in long-term usage for biological samples as compared to particulate columns [17].

3.2. Precision, accuracy, linearity and sample stability

Representative accuracy and precision of standards are shown in Table 2. All standards produced % R.S.D.s below 5% except

Table 1
Column performance from several different vendors

Column	Peak width at half height (min)	Peak width at baseline (min)	RT (min)	Pressure (bar)
Chromolith flash RP18e (4.6 mm × 25 mm) Merck KGaA	0.014	0.18	1.1	73
Magic C18 Bullett (4 → 1 mm × 25 mm) 3 μm Michrom Bioresources (Auburn, CA, USA)	0.015	0.20	0.9	258
Ace C18 (4.6 mm × 20 mm) 3 μm Canadian Life Sciences (Peterborough, CAN)	0.013	0.22	1.0	90
Prodigy Phenyl-3 (4.6 mm × 30 mm) 5 μm Phenomenex (Madrid, CA, USA)	0.022	0.28	1.1	84

Table 3
Control recovery of montelukast from spiked QC samples

	Montelukast QC recovery			
QC level (ng/mL)	0.36	1.80	45.00	450.0
Accuracy (%)	102.2	94.9	94.0	90.8
Intra-assay	8.0	4.4	3.0	3.1
% R.S.D.	(n = 12)	(n = 6)	(n = 9)	(n = 9)
Inter-assay[a]				
% R.S.D.	10.4	3.7	4.6	4.4

[a] Inter-assay precision was calculated using QC recovery from three separate calibration curves.

for the 0.25 ng/mL level which showed an overall % R.S.D. of 12.8. The signal-to-noise ratio was only 7 and therefore did not qualify as the LOQ. Linearity was demonstrated over the concentration range of 0.25–500 ng/mL with a slope of 0.0248x, and intercept of 0.000694 and a determination coefficient (r^2) of 0.9999. Recovery and precision of spiked quality control samples is shown in Table 3. Accuracy range from 91 to 102%, intra-assay precision was below 5% at all levels except for the LOQ (0.36 ng/mL) which showed an R.S.D. of 8% and signal-to-noise ratio of 10 (Fig. 3A). A short solution stability evaluation was performed. Montelukast extracts were stable in the cooled autosampler tray (8 °C) for 24 h. Low, medium and high level spiked QC samples were stable for at least one freeze–thaw cycle. Analytes were considered stable if the deviation in test results was within 15% of the freshly prepared sample result.

3.3. Matrix effects

Mass spectrometry detection is the technique of choice for bioanalysis due to high sensitivity and selectivity. However, matrix effects and ion-suppression are encountered when analyzing extracts of biological fluids [18,19]. In order to assess the extent of signal suppression/matrix effects with this method, post-column infusion experiments were performed. The montelukast signal from post-column infusion after injections of sheep plasma extract and blank water showed no significant suppression. In addition, extracts from six different blank plasma were injected with no observed interference at the expected retention time of the montelukast peak (Fig. 3B).

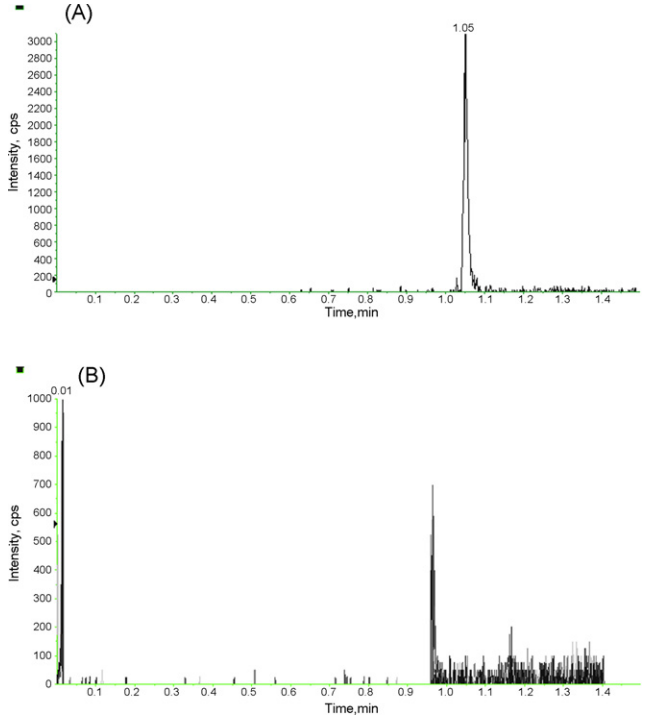

Fig. 3. Sample XIC (A) spiked LOQ control and (B) overlaid XIC of plasma blank extracts from six different sheep.

3.4. Carryover

In order to achieve an extended dynamic range, injections following ULOQ standard (500 ng/mL) initially showed unacceptable levels of carryover. The source of the carryover was isolated firstly by bypassing autosampler after completing the injection of the highest standard. A subsequent injection cycle without the autosampler confirmed that carryover did not come from the column, tubing or fittings. The procedure was repeated, whereby only the syringe was removed after completing the injection of the ULOQ standard. A subsequent injection cycle showed no peak, indicating injection valve, loop and autosampler fittings were not the source of carryover but rather the syringe itself. The needle wash solvent was also changed to a ternary mixture of DMSO:ACN:MeOH (4:3:3) followed by 50:50 ACN:water with 0.1% formic acid. Six wash cycles of each reduced carryover substantially. New generation autosamplers that use continuous flow-through design and inert materials should be employed to provide additional reduction in carryover.

4. Conclusions

A rapid, sensitive and robust LC/MS/MS assay was partially validated for the determination of montelukast in sheep plasma. It should be easily applied to plasma from other species with little or no modification. This tandem mass spectrometry method presents advantages over other published assays with respect to analysis time and detection limits. The method uses a simple one-step protein precipitation extraction and has a limit of quantitation of 0.36 ng/mL. The assay was linear over montelukast concentrations ranging from 0.25 to 500 ng/mL. Further improvements in sensitivity could be achieved by increasing the injection volume and employing lower-carryover autosamplers.

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