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Journal of Pharmaceutical and Biomedical Analysis 31 (2003) 647–654

JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS

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A semi-automated 96-well protein precipitation method for the determination of montelukast in human plasma using high performance liquid chromatography/fluorescence detection

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Received 9 June 2002; received in revised form 20 September 2002; accepted 15 October 2002

Abstract

A simple, semi-automated, protein precipitation assay for the determination of montelukast (SINGULAIR™, MK-0476) in human plasma has been developed. Montelukast is a potent and selective antagonist of the cysteinyl leukotriene receptor used for the treatment of asthma. A Packard MultiPROBE®II EX is used to transfer 300 µl of plasma from sample, standard, and QC sample tubes to a microtiter plate (96-well). After addition of the internal standard by a repeating pipettor, a Tomtec QUADRA 96® adds 400 µl of acetonitrile to all plasma sample wells, simultaneously, in the microtiter plate. The Tomtec is also used to transfer the acetonitrile supernatant from the plasma protein precipitation step, batchwise, to another microtiter plate for analysis by HPLC with fluorescence detection. This assay has been validated and implemented for a clinical study of over 1300 plasma samples and is comparable to manual assays in the LLOQ (lower limit of quantitation, 3 ng/ml) and in stability. This is the first semi-automated protein precipitation assay published for the analysis of montelukast in human plasma and it results in significant time savings over the manual methods, both in sample preparation and in HPLC run time.

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Keywords: Automated protein precipitation; Montelukast

1. Introduction

Montelukast is a potent and selective antagonist of the cysteinyl leukotriene receptor used for the treatment of asthma (SINGULAIR™, Merck & Co., Inc.). Leukotriene inhibitors (anti-LTs) are a new pharmacologic class of compounds for asthma management. Their discovery has had a significant impact on treatment strategies for the management of asthma [1,2]. Montelukast is the first LT inhibitor approved by the FDA for use by children [3].

Historically, antiasthma agents have been corticosteroids, beta (2)-agonists, and methylxanthines. Although these agents are safe and well tolerated

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doi:10.1016/S0731-7085(02)00723-9

when used properly, adverse effects may occur with use at higher dosages [1]. Montelukast and other anti-LTs have been administered concomitantly with inhaled corticosteroids and demonstrate complementary effects [4–6]. Recent clinical trial results suggest there may also be a role for anti-LTs as first-line therapy in children with mild asthma [5]. A 4-mg chewable tablet, administered once daily, was determined to be safe and effective in asthmatic children 2–5 years of age [7].

Here we report the first semi-automated high-throughput method, based upon a previously published manual method [8], for the determination of montelukast in plasma. A generic method for automated protein precipitation has been previously published [9]. We report a method that combines the advantages of both methods [8,9], has advantages over previously reported methods [3], and is applicable to large clinical studies involving montelukast, such as bioequivalence studies involving combination therapies.

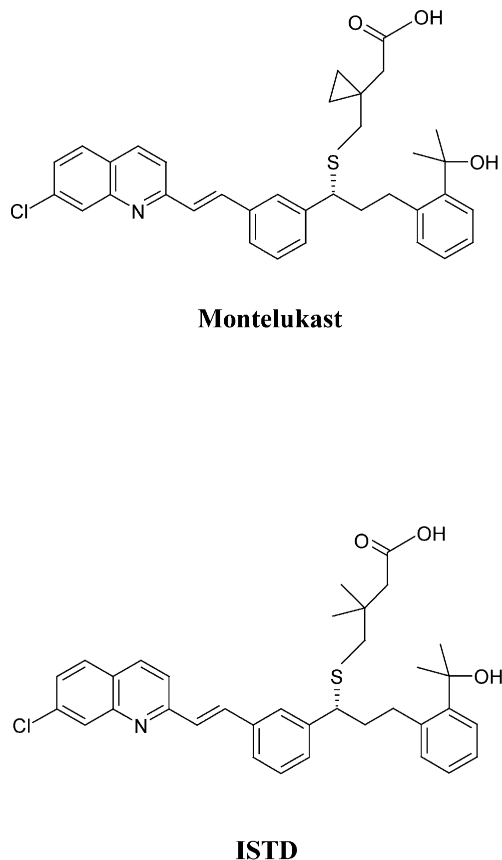

Fig. 1. Structures of Montelukast and ISTD.

2. Experimental

2.1. Chemicals

Montelukast and the internal standard (ISTD) were synthesized at Merck Research Laboratories (Rahway, NJ, USA) and can be seen in Fig. 1. Optima grade acetonitrile, ACS grade ammonium phosphate, HPLC grade *o*-phosphoric acid, and optima grade methanol were purchased from Fisher Scientific (Pittsburgh, PA, USA). Purified, filtered water was obtained from a Milli-Q system (Millipore, Milford, MA, USA). Human control plasma was purchased from Sera-Tec Biologicals (North Brunswick, NJ, USA).

2.2. Instrumentation and chromatographic conditions

A Hewlett-Packard Series 1100 Liquid Chromatograph with fluorescence detection (Agilent, Wilmington, DE, USA) was used with a Varian Pro Star 430 autosampler (Varian, Woburn, MA, USA). Chromatography was performed at 40 °C using an Apex C18 (4.6×50 mm, 3 μm) HPLC column (Jones Chromatography, Lakewood, CO, USA) with a 1.5 ml/min flow rate. The fluorescence detector was set to excitation and emission wavelengths of 350 and 400 nm, respectively. Data were collected, stored, and analyzed by a Turbochrom Navigator Client/Server version 6.1 (Perkin–Elmer, Cupertino, CA, USA). An Eppendorf EDOS 5222 repeating pipettor was used with a 1.25 ml Combitip (Brinkmann Instruments, Westbury, NY, USA) for adding the internal standard. The 96-well plates were vortexed using a TITERMix 100 (Brinkmann Instruments, Westbury, NY, USA). The 96-well plate centrifugation system was a Sigma 4K15C, from Qiagen, (Valencia, CA, USA) was used to separate the denatured plasma proteins from the acetonitrile supernatants. The 96-well plates (1- and 2-ml/well) were obtained from Matrix Technologies (Lowell,

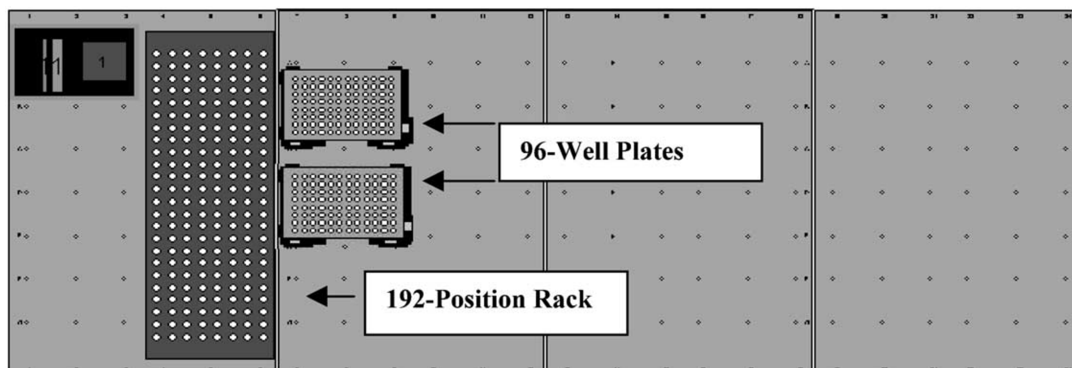

Fig. 2. Deck layout of the Packard MultiPROBE®II EX.

MA, USA) and the mats were purchased from Orochem (Westmont, IL, USA).

The mobile phase was composed of acetonitrile–ammonium phosphate (pH 3.5; 0.02 M) (65:35, v/v) and was filtered and degassed through a 0.22 μm Magna-R nylon filter (Whatman International, Maidstone, England). The ammonium phosphate buffer (pH 3.5; 0.02 M) was prepared by initially dissolving 66.03 g ammonium phosphate into 1.0 l Milli-Q H_2O and adjusting the pH to 3.5 with *o*-phosphoric acid resulting in a 0.5 M buffer stock. A volume of 40 ml of this 0.5 M stock was then diluted with 960 ml Milli-Q water (v/v) to achieve a final concentration of 0.02 M ammonium phosphate pH 3.5.

A Packard MultiPROBE®II EX Robotic Liquid Handling System (Packard Instruments, Downers Grove, IL, USA) was used for automated pipetting during sample transfer. The deck layout for this assay from the WinPREP software can be seen in Fig. 2. The MultiPROBE® was fitted with four liquid level sensing fixed tips, 1-ml syringes, and a tip wash solution made up of a 20% methanol solution.

A Tomtec QUADRA 96®, Model 320 (Gaithersburg, MD, USA) was used for adding the acetonitrile, aspirating the supernatant, and transferring the supernatant to a new 96-well plate for the automated protein precipitation. The QUADRA 96® Model 320 is a 96-well high throughput pipetter equipped with 96 disposable tips (1–450 μl volume range) and a six station automated shuttle that is able to transfer solvents to or from all 96 wells simultaneously. Fig. 3 shows a front view of the QUADRA 96® with the appropriate shuttle positions labeled on the deck.

1. Tip Reservoir
2. Acetonitrile Reservoir
3. Supernatant Destination Plate (1-mL/well)
4. Original Sample Plate (2-mL/well)

Fig. 3. Front view of the Tomtec QUADRA 96® model 320 with the utilized shuttle positions labeled.

Verification of accurate and precise volume transfers by the MultiPROBE® involved weighing replicate ($n = 5$) aliquots of Milli-Q water (Bedford, MA, USA) at each volume used in sample transfer with each Packard syringe ($n = 4$ syringes).

2.3. Preparation of standards and quality control (QC) samples

Stock standard solutions of montelukast were prepared under yellow light at a concentration of 1.0 mg/ml free acid in MeOH–H_2O (7:3, v/v). The stock standard of montelukast was diluted again with MeOH–H_2O (7:3, v/v) to yield a series of working standards. Plasma calibration standards were prepared by diluting the working standards with control human plasma to obtain concentrations of 3, 5, 10, 20, 50, 100, 200, 500, and 1000 ng/ml. The internal standard was prepared to a concentration of 200 μg/ml in MeOH–H_2O (7:3, v/v) and later diluted to 5 μg/ml for the working internal standard. The internal standard was stored in 4.5 ml polypropylene conical tubes (Sarstedt, Newton, NC, USA) at −70 °C until use.

QC samples were prepared by using a separate weighing of montelukast to yield a QC stock solution concentration of 1.0 mg/ml free acid in MeOH–H_2O (7:3, v/v). Working standards at 0.75, 30, and 75 μg/ml were prepared by further diluting the stock solution. The final QC concentrations were attained by adding 300 μl of respective working standard to 29.7 ml control human plasma to yield concentrations of 7.5, 300, and 750 ng/ml. Sample aliquots of 400 μl were transferred to 4.5 ml polypropylene conical tubes (Sarstedt, Newton, NC, USA) at −70 °C until use.

2.4. Sample preparation

Due to the light sensitivity of montelukast, all work on standard solutions and clinical samples was performed under yellow light. All plasma samples (including plasma standards and QC samples) were vortexed and centrifuged at room temperature at $2000 \times g$ for 10 min to remove fibrin. Plasma samples, standards, and QCs were all stored in 4.5 ml tubes (12×75 mm) that were decapped and placed into a 192-position rack on the deck of the Packard MultiPROBE®II EX (see Fig. 2). Deep well microtiter plate(s) (96-well, 2-ml/well) were also placed on the deck of the Packard. The Packard was programmed to transfer 300 μl of the standards, QCs, and samples from the 192-position rack to the 2-ml microtiter plates. The plates were then removed from the Packard and 25 μl of ISTD was added to each well using an Eppendorf EDOS 5222 repeating pipetter with a 1.25-ml Combitip. The microtiter plate containing the plasma and ISTD was then placed onto the deck of a Tomtec Quadra 96® (see Fig. 3 for locations of tips, acetonitrile reservoir, and the sample microtiter plates). After all 96 pipette tips were picked up from the tip reservoir, 400 μl of acetonitrile was aspirated from the acetonitrile reservoir and dispensed to each well of the microtiter plate for plasma protein precipitation. The plate was then removed from the deck of the Tomtec, covered with a mat, vortexed for approximately 30 s to mix acetonitrile with plasma, and centrifuged at $4000 \times g$ for 20 min at 0 °C to remove fibrin. The mat was then carefully removed from the plate and the plate was returned to the deck of the Tomtec. On the Tomtec, 350 μl of the acetonitrile supernatant was aspirated from all 96 wells from the original sample plate simultaneously and transferred to a separate microtiter plate (96-well, 1-ml/well). This plate was removed from the Tomtec, covered with a mat, and placed in a 5 °C autosampler. A volume of 50 μl of sample was injected onto the HPLC.

In a typical daily run, duplicate QC samples at each concentration were analyzed immediately before and after the clinical samples. If more than two 96-well plates were required for a daily run, a set of QC samples at each concentration were inserted into the extra 96-well plate to ensure that each plate contained a set of QC samples. Acceptance of the daily run was contingent upon the mean calculated QC concentrations falling within 20% of the nominal concentration for each of the three concentrations.

2.5. Method validation (precision, accuracy, specificity, stability, and recovery)

Intraday precision and accuracy were determined by analyzing replicate calibration curves ($n = 5$) in five different lots of human plasma. The peak heights generated by Turbochrom for montelukast and the ISTD were used to generate peak height ratios at each standard concentration of the

Table 1
Representative intraday precision and accuracy data for the determination of montelukast in five different lots of human plasma

Nominal concentration (ng/ml)	Mean[a] concentration (ng/ml)	Precision[b] CV (%)	Accuracy[c] (%)
3	2.95	11.36	98.33
5	5.00	9.85	100.08
10	10.42	5.76	104.20
20	20.98	1.71	104.92
50	47.84	1.05	95.67
100	105.22	2.57	105.22
200	183.99	2.14	92.00
500	482.23	1.82	96.45
1000	1029.36	0.59	102.94

[a] Mean concentrations calculated from the weighted ($1/x$) linear least-squares regression curve using all five replicates at each concentration.
[b] Percent coefficient of variation (CV) of peak height ratios ($n = 5$).
[c] Expressed as [(mean observed concentration/nominal concentration) $\times$ 100] ($n = 5$).

calibration curve. The calibration curves were constructed by weighted ($1/x$) linear least-squares regression: peak height ratios versus nominal concentrations.

QC samples were used to determine intraday assay variability and accuracy and interday variability. A set of QC samples at low, medium, and high concentrations were analyzed in triplicate with the daily standard curve and clinical samples. QCs were placed immediately before the first clinical sample, in the middle of the batch, and after the last clinical sample. If the set was large enough to require an additional plate, the middle QCs were placed within the middle plate to ensure that every plate has a set of QCs. Acceptance of the daily analytical run was based upon acceptable QC results for that run.

The stability of montelukast in human plasma at room temperature and 5 °C was assessed at three concentrations (4.8, 48.2, and 482 ng/ml) over 3 and 24 h, respectively. Plasma QC samples ($n = 5$ at each concentration) were processed and analyzed after sitting at room temperature at 0 and 3 h. The mean concentration ratios at each concentration were assessed to determine the stability. A similar stability study was performed at 5 °C (autosampler storage temperature) at 0 and 24 h.

The mean recoveries of montelukast (5 and 500 ng/ml, $n = 5$) and the ISTD (417 ng/ml, $n = 9$) were determined by comparing the peak height ratios of extracted standards versus neat standards. The extracted and neat standards were both prepared and processed as described above using control human plasma, except in the neat standards where proportionate amounts of the montelukast and ISTD standards were not added until after the supernatant was transferred to the 1-ml 96-well plate.

3. Results and discussion

The use of automation combined with microtiter format eliminates the need for labor-intensive manual pipetting steps and the labeling of tubes and vials by allowing samples to be processed in a batchwise fashion rather than individually. The Packard MultiPROBE®II EX is able to transfer four samples simultaneously (eight samples with the new MultiPROBE®II HT EX) and allows the analyst time to perform other duties (i.e. decapping sample tubes, preparing the Tomtec, etc.). The Eppendorf EDOS 5222 is a versatile pipettor that can accommodate Combitips, an 8-channel attachment, and the common single pipette tip attachments. The Tomtec QUADRA 96® Model 320 is able to transfer solvents to and from all 96 wells at the same time. The software allows a pause to be placed into the program to allow the analyst time to vortex and centrifuge the samples prior to transferring the supernatant to a new microtiter plate.

Table 2
Initial intraday analysis of QC samples ($n = 5$) for the determination of montelukast in human plasma

Nominal concentration (ng/ml)	Assayed concentration[a] (ng/ml)	Precision[b] CV (%)
7.5	6.51 ± 0.44	6.04
300	310.75 ± 0.89	0.28
750	759.57 ± 5.92	0.78

[a] Mean concentrations calculated from the weighted ($1/x$) linear least-squares regression curve using all five replicates at each concentration.
[b] Percent coefficient of variation (CV) of peak height ratios ($n = 5$).

By using two separate automated systems, the analyst is able to transfer additional samples using the MultiPROBE® while performing the protein precipitation batchwise on the Tomtec. This can result in higher throughput when compared to other automated sample preparation methods where the acetonitrile and supernatant transfer are not performed 96 at a time [9]. Steps such as vortexing and centrifuging, when using the 96-well plates, are not easily automated, but the throughput is much higher by using this format.

Other advantages of this method over previously published methods are, in general, reduced run time and throughput. This method has a run time of 4.5 min as opposed to 6–10 min run times for other methods [3,8]. This results in a 25–55% increase in sample throughput. The total time involved for sample preparation is comparable to the manual method (approximately 3.5 h), but many more samples are able to be processed within that period of time (> 240 samples versus approximately 100 manually). By performing manual sample preparation, the number of samples able to be processed in 1 day is often limited by the capacity of the autosampler. Most autosamplers used with manual methods are limited to around 100 injections, whereas the autosamplers for microtiter plates can typically hold 3–6 microtiter plates (288–576 injections). This feature alone can increase throughput by over 500%.

Intraday accuracy and precision for montelukast were 92–105% of nominal and 0.59–11.36% (% CV), respectively, from $n = 5$ standard replicates at each of nine different concentrations ranging from 3 to 1000 ng/ml (Table 1). The mean r^2 for the five standard curves was 0.998. The calculated concentrations were determined from linear regression using $1/x$ weighting and the equation $y = mx + b$ where y is the concentration of montelukast; m, the slope; x, peak height ratio; and b, the intercept.

Table 3
Interday precision and accuracy of plasma montelukast QCs spanning a 30-day period

Nominal	QC Concentrations (ng/ml)		
	7.5	300	750
Mean[a]	6.65	310.7	769.0
SD	0.67	6.23	14.59
% CV[b]	10.14	2.01	1.90
% Accuracy	88.7	103.55	102.53

[a] Concentrations calculated from the weighted ($1/x$) linear least-squares regression curve. Mean of concentrations from eight separate analyses occurring on eight separate days ($n = 3$).
[b] Percent coefficient of variation (CV) of peak height ratios ($n = 8$ separate analyses).

Intraday precision for the QCs ranged from 0.28 to 6.04 % CV (Table 2). Interday accuracy and precision for QCs ranged from 89 to 104% of nominal and 1.9 to 10.1% (% CV), respectively, for eight different sample analyses spanning 30 days (Table 3). These accuracy and precision data are comparable to those given for the manual method [8]. The absolute recoveries for 5 and 500 ng/ml montelukast in plasma were 102 and 91% with 9.1

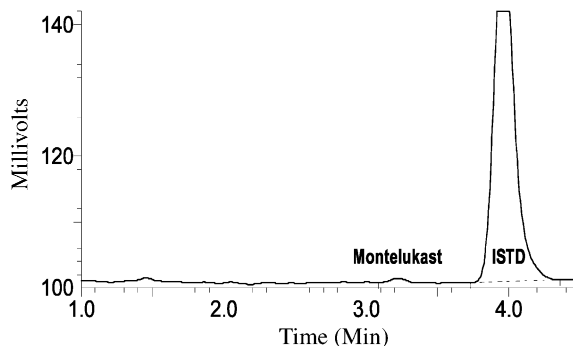

Fig. 4. Representative chromatogram of a 3 ng/ml plasma Montelukast standard.

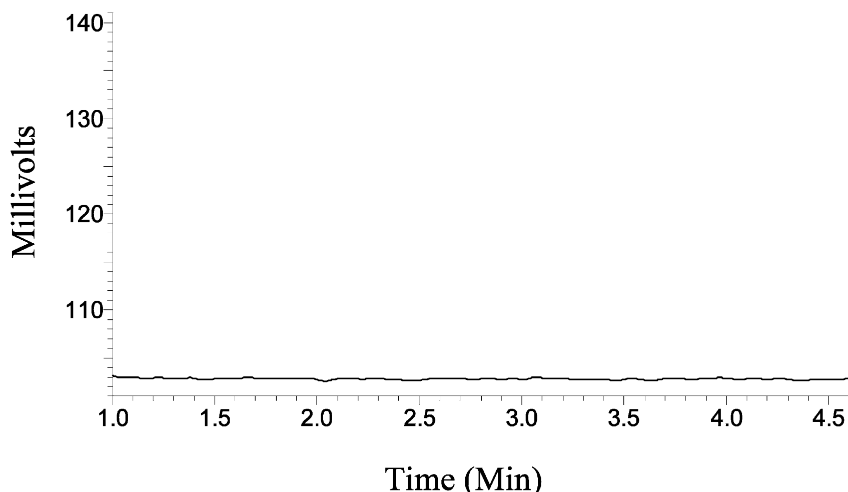

Fig. 5. Representative chromatogram of a clinical plasma sample predose.

and 2.5% CV, respectively. The absolute recovery of the ISTD at 417 ng/ml was 92% with 7.7% CV.

As previously reported [8], montelukast was found to be stable for more than 19 months in human plasma stored at −70 °C, and three cycles of freezing and thawing showed no apparent degradation of the compound.

Montelukast was found to be stable (≥96% remaining) after 3 h at room temperature in plasma at 4.8, 48.2, and 482 ng/ml. The mean percent of montelukast remaining after 3 h ranged from 96 to 101%. The % CV at the 0 and 3 h time points at each concentration ranged between 1.4 and 8.0%. On the autosampler at 5 °C, montelukast was found to be stable (≥95% remaining) after 24 h. The mean percent remaining after 24 h ranged from 95 to 101%. The % CV at the 0 and 24 h time points at each concentration ranged from 1.3 to 3.7%.

Representative chromatograms of a known standard, predose sample, and a post-dose sample are shown in Figs. 4–6. The retention times for montelukast and the ISTD are approximately 3.2 and 3.9 min, respectively. No interfering peaks

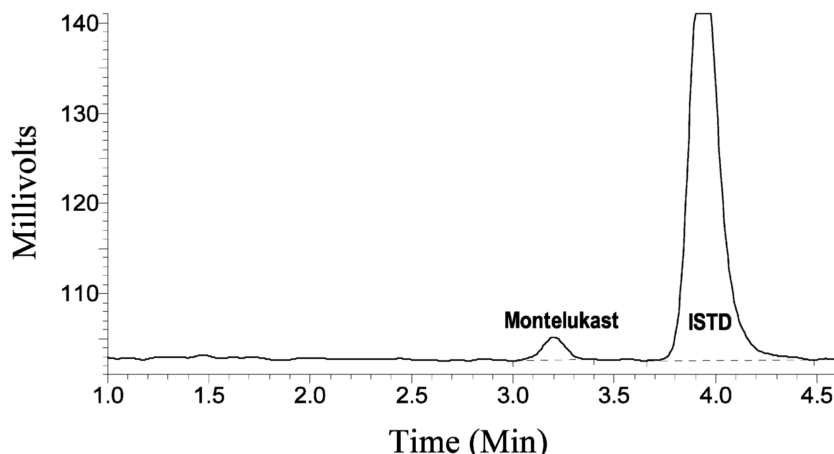

Fig. 6. Representative chromatogram of a clinical plasma sample at 16 h following a single oral dose of 10-mg montelukast coadministered with 10-mg loratadine (16.7 ng/ml montelukast).

were present during a study involving 36 patients (1300 samples). Assay specificity for montelukast in the presence of loratadine (and its metabolite descarboethoxyloratadine), chemical entities that may be administered concurrently with montelukast, was also demonstrated (Fig. 6).

4. Conclusions

Automation of the protein precipitation HPLC method for montelukast increased sample throughput approximately three times over manual protein precipitation methods [3,8]. In this assay, a Packard MultiPROBE® was used for pipetting during sample transfer and a Tomtec Quadra 96® and 96-well plate centrifuge were used to batch the protein precipitation. This assay uses two separate automated systems, thereby allowing the analyst to perform two automated steps at the same time (i.e. transfer additional samples on the MultiPROBE® while performing the protein precipitation batchwise on the Tomtec). Using this method, batch sample preparation of over 240 samples was easily accomplished in approximately 3.5 h compared to approximately 6–8 h with the manual method, provided that the autosampler for the manual method is able to hold 240 vials. This method also reduced the HPLC run time compared to other published methods (4.5 versus 6 min or more [3,8]) which also contributed to a higher sample throughput. Additionally, this semi-automated method can easily be applied to other manual protein precipitation methods using fairly standard automation equipment.

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