An Automated Method for the Determination of Montelukast in Human Plasma Using Dual-Column HPLC Analysis and Peak Height Summation of the Parent Compound and Its Photodegradation Product

Glenn A. Smith,[1] Cynthia M. Rawls,[1] and Robert L. Kunka[2,3]

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Purpose. To develop an assay to evaluate the bioequivalence of overcoated and marketed montelukast formulations, the former to be used for future blinded clinical studies.

Methods. The method used automated 96-well sample preparation and dual-column HPLC analysis for increased throughput. Regression analysis was performed using the total peak height of montelukast and its photodegradent, a *cis*-ethenyl geometric isomer. This approach successfully compensated for montelukast's light sensitivity, allowing both clinical specimen handling and bioanalytical laboratory analysis to be conducted without extensive precautions being taken to protect samples from UV light. To ensure a molar equivalent fluorescence response between the *cis* (Z) and *trans* (E) isomers, the emission wavelength and detector attenuation were both increased just prior to the elution of the montelukast peak (i.e., the *trans* isomer), effectively dampening the response of the stronger fluorophore. Plasma proteins were precipitated using acetonitrile, and 50 μl of supernatant was injected onto an HPLC system consisting of two C18 analytical columns connected to a 10-port switching valve. Injections were overlapped on alternating columns allowing twice as many samples to be processed during each analytical run.

Results. The calibration curve was linear from 5 to 2000 ng ml^{-1}. The inter-day and intra-day precision expressed as coefficient of variation (%CV), were 1.1–6.1% and 3.1–6.7%, respectively. The accuracy, reported as percentage bias, was less than or equal to ±9.1%. The absolute recovery was determined to be 94.3% and 98.1% at 15 and 1500 ng ml^{-1}, respectively.

Conclusions. This assay represents a rapid, accurate, and sensitive method for the determination of montelukast in human plasma. The method has been successfully used to demonstrate the bioequivalence of the overcoated montelukast formulations to their equivalent marketed tablets.

KEY WORDS: asthma; bioequivalence; fluorescence; HPLC; montelukast.

INTRODUCTION

Montelukast sodium (Singulair®), also known as MK-0476 [sodium 1-(((1-(3-(2-(7-chloro-2-quinolinyl)-(E)-ethenyl)-phenyl)(3-(2-(1-hydroxy-1-methylethyl)phenyl)propyl)thio)-methyl)-cyclo-propane) acetate] is a potent and selective leukotriene D_4 receptor antagonist used in the prophylaxis and treatment of chronic asthma (1,2). In solution, the compound is photosensitive which traditionally necessitates special handling precautions to protect specimens from light. Upon exposure to even very low levels of UV radiation, the (E)-ethenyl moiety of montelukast (**1**; Montelukast; Fig. 1) readily rotates to the (Z) geometric configuration (**2**; *cis* isomer; Fig. 1).

A method for the determination of montelukast levels in human plasma, using acetonitrile protein precipitation and HPLC with fluorescence detection was previously reported (3). Although the analytical technique used was simple and had a quick 5 min cycle time, the lower limit of quantitation (LLOQ) of 30 ng ml^{-1} was not adequate to support current clinical studies involving lower doses of 5 and 10 mg tablets. A subsequent method used direct plasma injection and column switching to address the problems of assay sensitivity and potential photo-degradation of sample extracts (4). Despite achieving the goal of lowering the LLOQ to 1 ng ml^{-1} and further reducing the likelihood of photo-degradation during analysis, the longer cycle time of 30 min would be quite rate limiting when supporting large pharmacokinetic studies. The increased risk of system carryover and the reduced robustness commonly reported when using on-line extraction techniques were also seen as drawbacks.

This report illustrates a simple and novel approach for analyzing an unstable compound. The methodology described allows for the very accurate determination of montelukast concentrations in plasma without the inconvenience and uncertainty involved when attempting to completely protect specimens from light. Also, improved accuracy of reported data can be expected by monitoring and correcting for any photo-degradation of the clinical blood and plasma samples, analytical reference standards, stock solutions, calibration standards, quality control samples, and final sample extracts. The paper details the laboratory automation equipment used to expedite the extraction of plasma samples and a simple switching valve technique used to cut chromatographic analysis times in half.

MATERIALS AND METHODS

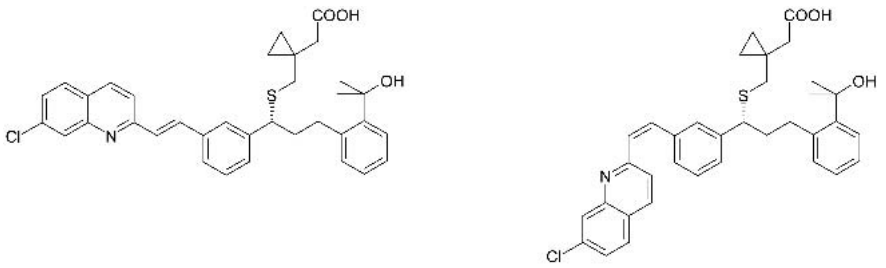
Materials

The analytical montelukast reference material, Calcium 1-(((1-(3-(2-(7-chloro-2-quinolinyl)-(E)-ethenyl)-phenyl)(3-(2-(1-hydroxy-1-methylethyl) phenyl)propyl)thio)-methyl)-cyclopropane) acetate was obtained from Custom Synthesis Services (Madison, WI). Acetonitrile and methanol (HPLC grade) were purchased from EM Science (Gibbstown, NJ). Acetic acid (glacial) was purchased from Sigma (St. Louis, MO). Human plasma (sodium heparin) was purchased from BioReclamation Inc. (Hicksville, NY). Milli-Q de-ionized water was used throughout. Deepwell plates (polypropylene, 1ml) were purchased from Porvair Sciences Ltd. (Shepperton, UK).

Instrumentation

The robotic sample preparation system consisted of a Zymate XP robotic arm and gripper hands, a ZP710 centrifuge, and a RapidPlate 96-channel disposable tip pipetting

[1] Department of Drug Metabolism and Pharmacokinetics, GlaxoSmithKline, Research Triangle Park, North Carolina 27709, USA.
[2] Department of Clinical Pharmacology, GlaxoSmithKline, Research Triangle Park, North Carolina 27709, USA.
[3] To whom correspondence should be addressed. (e-mail: Glenn.A.Smith@gsk.com)



1 **2**

Fig. 1. Chemical structures of isomers **1** and **2**.

station, which were all purchased from Zymark Corp. (Hopkinton, MA). Other integrated modules included a Bellco Glass Co. mini-orbital shaker (Vineland, NJ) and a Mettler PM-4000 top loading analytical balance (Hightstown, NJ) that were modified to work with deepwell plates.

The analytical system was comprised of an SCL-10Avp controller, an SIL-10ADvp auto-injector, two LC-10ADvp liquid chromatographs, a DGU-14A solvent degasser (Shimadzu Scientific Instruments Inc., Columbia, MD), a Lab-PRO 2-position/10-port switching valve (Rheodyne, Rohnert Park, CA), a column heater (Sys-Tec Corp.), and a FP-920 fluorescence detector (Jasco Corp., Tokyo, Japan).

Chromatography

Reverse phase chromatography was carried out at 25 ± 1°C under isocratic reverse phase conditions on two 10 × 0.3 cm, 3 μm, Genesis Lightning C18 columns (Jones Chromatography Inc., Lakewood, CO). The mobile phase consisting of acetonitrile-water-glacial acetic acid (75:25:0.25, v/v/v) was delivered to each of the analytical columns at a flow rate of 0.5 ml min^{-1}. The columns were connected to a 2-position, 10-port switching valve that allowed for 'stacking' of injections. Although the empirical chromatographic time was 10 min, injections were made every 5 min on alternating columns. The first 5 min of eluant from each injection was diverted to waste while the last 5 min of eluant - from the other column - was being monitored by the fluorescence detector. A schematic of the switching valve is shown (Fig. 2). Since the first half of each chromatograph is diverted to waste and not observed, the absolute retention times of the *cis* and *trans* isomers, 6.1 and 8.1 min, respectively, elute at the relative times of 1.1 and 3.1 min respectively. The cis-ethenyl isomer is monitored at λ_{EX} = 350 nm, λ_{EM} = 400 nm, gain = 1000, and an attenuation of 128. At 2.15 min the parameters are switched to λ_{EX} = 350 nm, λ_{EM} = 440 nm, gain = 1000, and an attenuation of 256 for the detection of the montelukast peak. Representative chromatograms are shown in Figs. 3 and 4.

Preparation of Calibration Standards

Duplicate parent stock solutions were prepared by dissolving montelukast calcium in methanol to yield concentrations of 200 μg ml^{-1} (free acid). Each of these primary stocks was further diluted to concentrations of 20 and 2 μg ml^{-1} in acetonitrile-water (75:25, v/v). The 2 μg ml^{-1} solutions were each injected into the HPLC system, in replicates of six, to demonstrate equivalency. All solutions were prepared in amber glassware, divided into several aliquots, and stored in amber tubes at –70 ± 5°C. Calibration standards (STDs) were prepared in control human plasma (sodium heparin) at concentrations of 5, 10, 50, 100, 500, 1000, and 2000 ng ml^{-1} (free acid) using the first set of spiking solutions. Likewise, quality control samples (QCs) were prepared in plasma at concentrations of 5, 15, 150, 1500, and 2000 ng ml^{-1} using the second set of spiking solutions. The 5 and 2000 ng ml^{-1} QCs were used only during method validation runs for the purpose of determining precision and accuracy at the lower and upper limits of quantitation. All STDs and QCs were transferred in 0.25 ml aliquots to 2 ml amber polypropylene microcentrifuge tubes and stored at –70 ± 5°C. Fresh aliquots were removed from the freezer and used with each new analytical batch and discarded after each run.

Automated Sample Processing

Plasma samples were extracted on a customized Zymark robotics system. Each station of the automated platform is configured to use microplate format labware (i.e. 96-well

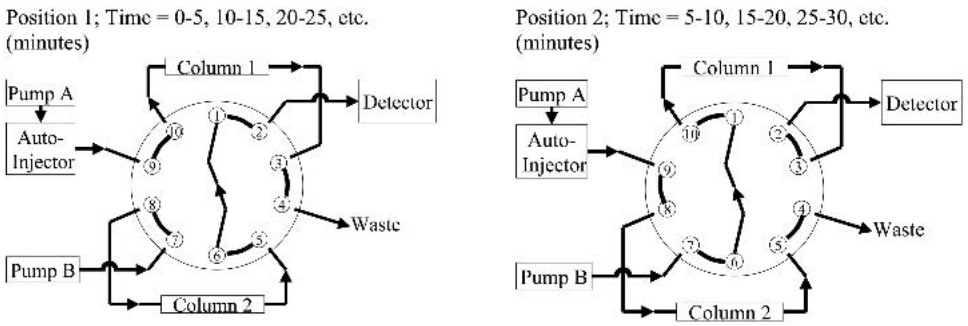


Fig. 2. Diagram of switching valve assembly.

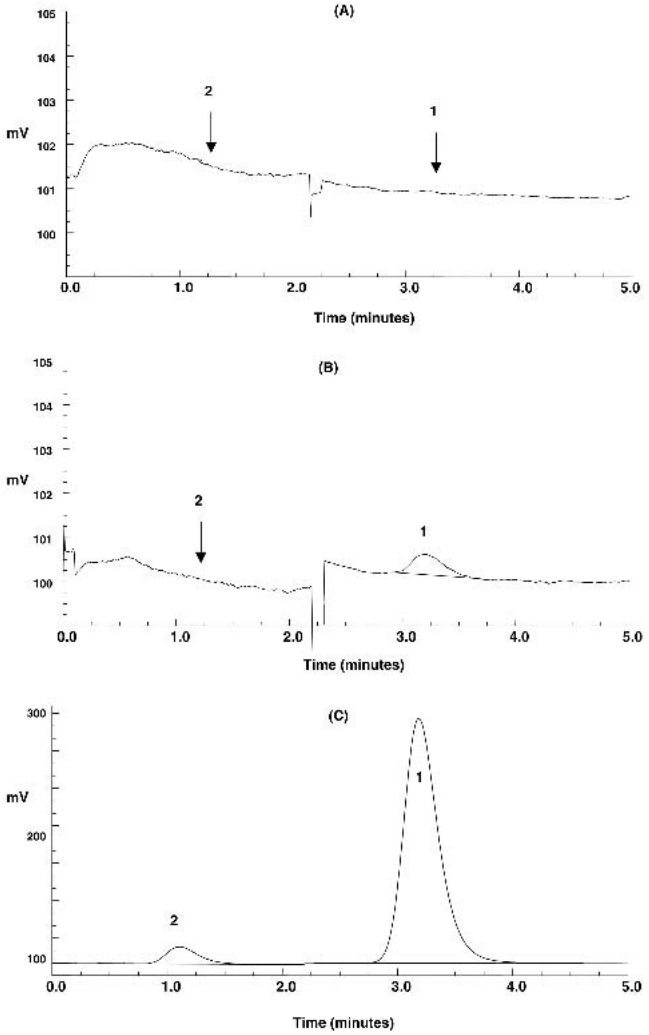


Fig. 3. Typical chromatograms from (A) drug-free plasma; (B) a 5 ng ml^{-1} standard; (C) a 2000 ng ml^{-1} standard.

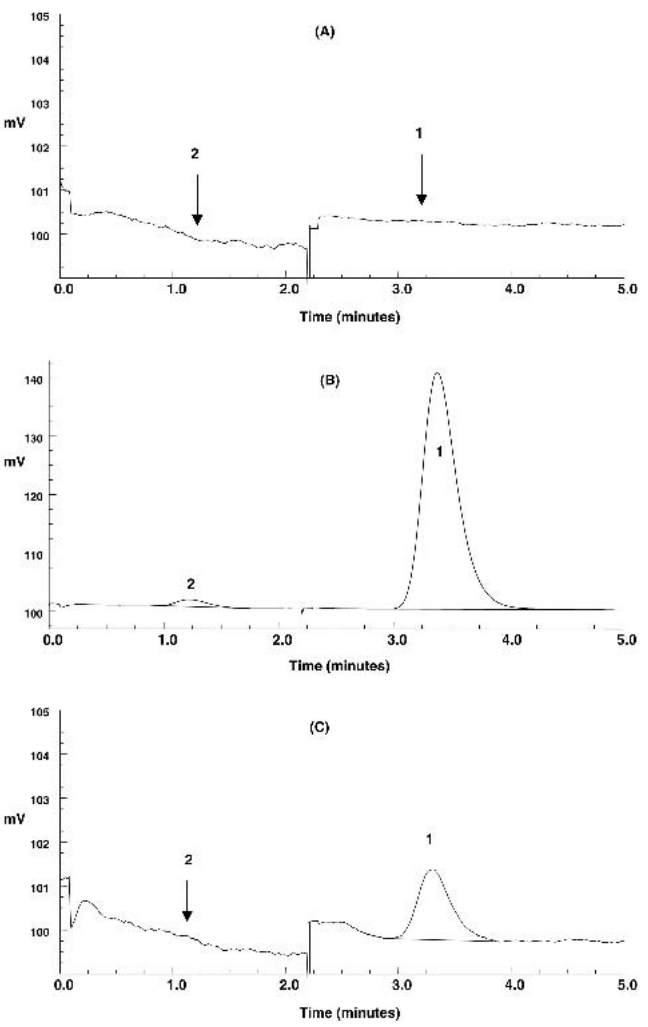


Fig. 4. Representative chromatograms from (A) a pre-dose clinical sample; (B) a 2-h post-dose clinical sample following a 10 mg dose; (C) a 24-h post-dose clinical sample following a 10 mg dose.

plates). A schematic of the individual modules and overall system layout is shown in Fig. 5. Using the 96-channel pipetting station, 300 μl of acetonitrile was added to all 96 wells of a 1 ml round bottom plate. At the same station, 100 μl of plasma standards, quality controls, and clinical samples were then immediately transferred from their individual microcentrifuge tubes to the deepwell plate, using fresh disposable tips. The plate is then moved to the vortexing station where samples are mixed at 1000 rpm for 1 min to ensure complete denaturing of the plasma proteins. The plate is centrifuged for 5 min at 2500 rpm (approximately 1600 rcf) then returned to the pipetting station where new tips are used to transfer 250 μl of supernatant to a clean deepwell plate. The plate is sealed with an adhesive free mat and placed into the auto-injector. The procedure and specific operation times are itemized in Table I.

Assay Validation

On four consecutive validation days, duplicate standards and quality control samples in replicates of six were processed at each concentration level. Calibration curves were derived using a least square linear regression with weighting of $1/x^2$. Calculations were based on the sum of the combined peak heights of both the *cis* and *trans* isomers. Additional samples were included in the various runs for the determination of assay specificity, extraction recovery, freeze/thaw stability, ambient stability in plasma, stability of extracted samples, and light sensitivity.

Assay specificity was determined by screening human plasma for chromatographic interferences from six individual donors.

Extraction recovery was determined at 15 and 1500 ng ml^{-1} using 6 replicates of both reference and test samples at both concentration levels. Reference samples were prepared by spiking montelukast into supernatant from extracted drug-free plasma at concentrations of 3.75 and 375 ng ml^{-1} which account for the 4-fold dilution factor associated with sample extraction.

Both the inter-day and intra-day precision and accuracy were determined at all quality control levels, including the low and high concentration limits of the calibration range, using a 1-way ANOVA analysis on log transformed data. The observed mean, standard deviation, % bias, and % coefficient of variation (%CV) were calculated.

All stability experiments were conducted by comparing

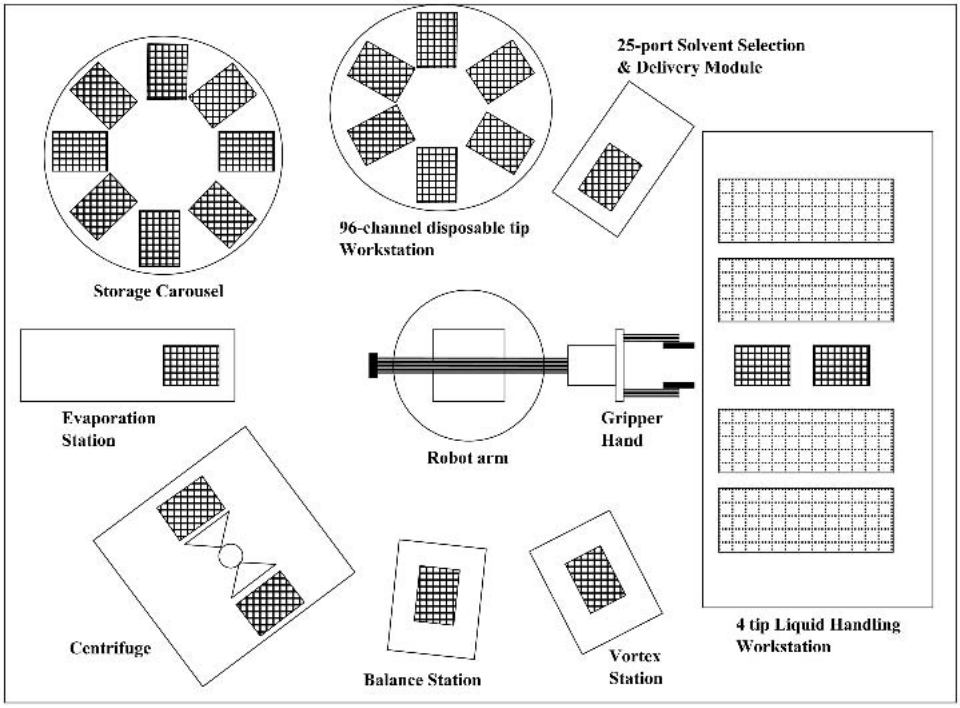


Fig. 5. Automated Sample Preparation System (top view schematic). Not all modules were used for this assay.

either the back-calculated concentrations or peak height summations of stability test samples to those of the control group samples. Both the stability test samples and the reference control samples were analyzed in replicates of 6. Stability was assessed using a one-sided *t*-test with 15% limits to determine if equivalency was met with at least 95% confidence.

Montelukast's light sensitivity was evaluated by exposing a stock solution to ambient laboratory light (primarily fluorescent) over a 6-h period. After timed sampling and HPLC analysis was performed, both the individual peak heights and their combined heights were plotted against exposure time.

Clinical Study

The assay was developed to support a clinical study examining the comparative bioavailability of overcoated 5 mg chewable and 10 mg swallow tablets to the marketed products. Twenty-three healthy male and female subjects (21–46 years old) were enrolled in a single dose, randomized, open-label, four-way crossover study where subjects received a 10 mg dose of each of the treatments following an overnight fast with 240 mL water that was used to rinse the mouth before being swallowed. Subjects remained fasted for 4 hours after dosing. Treatments were separated by at least 5 days. Five milliliter blood samples were obtained at the following times to determine plasma montelukast levels: pre-dose, and 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12, 16, 20, and 24 h. Peak concentrations (C_{max}), time of peak (t_{max}), terminal elimination rate constant, elimination half-life, area under the plasma curve to the last measurable concentration (AUC_{last}) and area to infinity (AUC_{∞}) were estimated for each subject using standard noncomparmental methods (5).

The research followed the tenets of the Declaration of Helsinki, was approved by the institutional review board, and informed consent was obtained from each patient or parent.

RESULTS AND DISCUSSION

Photodegradation

Previous papers describing methods for the determination of montelukast concentrations in human plasma have

Table I. Automated Procedure and Time Table

Automated step	Station used	Time required (min)
Add 300 mcl acetonitrile to deepwell plate	96-channel pipetting station	2
Transfer 100 mcl of plasma samples from cryotubes to the deepwell plate	96-channel pipetting station	2
Vortex plate at 1000 rpm for 1 min	Vortex Station	1
Prepare 2nd plate if processing 97–192 samples	96-channel pipetting station	4
OR		
Prepare counter-balance plate for centrifuge if processing 96 or fewer samples	Balance and solvent delivery module	4
Centrifuge at 2500 rpm for 5 min	Centrifuge Station	6
Transfer 250 mcl of supernatant to clean deepwell plate(s)	96-channel pipetting station	2 min if single plate 4 min if two plates

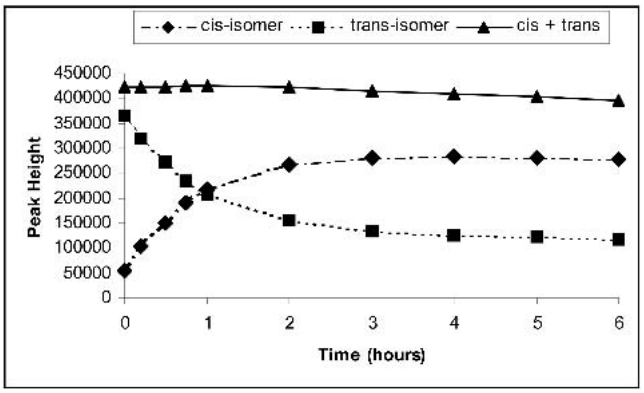


Fig. 6. Photodegradation plot.

drawn special attention to the compound's photoisomerization and have detailed specific lighting restrictions and sample handling requirements. Montelukast's *ex vivo* half-life in human plasma, under normal conditions, has been reported to be between 3 and 8 h, depending mostly on the ambient light conditions and sample containers. More importantly, the compound was noticed to be even less stable when in solution, such as stock solutions and plasma sample extracts.

Early in the development of the HPLC assay, it was observed that the original montelukast *trans* isomer had a stronger fluorescent response than its light degradation product, the *cis* isomer. To address this, a stock solution was split in two and one of the fractions was exposed to light until about half of the parent analyte had converted to the cis configuration. Repeated injections of each of the two fractions were made while making stepwise adjustments (increases) in the emission wavelength and the attenuation setting, during the elution of the *trans* isomer. The final detector settings for each of the isomers were chosen once the total peak height of both isomers was the same in both fractions.

A plot showing the photo degradation of a 2000 ng ml^{-1} stock solution prepared in acetonitrile-water (75:25, v/v), upon exposure to ambient laboratory light, is shown in Fig. 6.

The graph illustrates how the sum of the peak heights remains constant as montelukast undergoes light degradation and the *cis*/*trans* ratio increases. Upon prolonged exposure to UV light (i.e., greater than 1 h under our test conditions), a very slight drop in total peak height over time is observed which indicates that secondary degradation begins to take place. This observation allows the photo-isomerization limit of the assay to be set at a conservative cis/trans ratio of ≤ 1. Although the methodology would correct for up to 50% montelukast photo degradation, all STDs, QCs and unknown clinical samples, analyzed during validation and clinical study analysis (over 2000 samples), were observed to be well below this threshold level of *cis*-ethenyl isomer.

Automated Sample Processing and High-Throughput Analysis

The use of parallel processing on a fully automated sample preparation robotics system allowed for up to 192 samples (two 96-well plates) to be extracted simultaneously in less than 30 min. Once extracted, the deepwell plates were sealed to prevent sample evaporation and placed inside the enclosed autosampler protected from light. The use of a 10 port switching valve with dual columns and stacked injections allows the LC cycle time to be reduced from 10 min to 5 min. The LC analysis time required to inject 288 samples (3 plates) was only 24 h, which allowed for large batches to be run on a daily basis during analysis of the study samples.

Validation Results

The recovery of montelukast was determined to be 94.3% and 98.1% at 15 and 1500 ng ml^{-1}, respectively. The assay was determined to be linear, reproducible and accurate throughout the validation range of 5–2000 ng ml^{-1}. The summary statistics of QC precision and accuracy from the 4 day validation exercise are presented in Table II.

Montelukast extracts were shown to be stable in the autosampler for at least 24 h. Plasma samples were also determined to be stable through 3 freeze/thaw cycles, during processing, and for 4 h on the lab bench under ambient conditions. However, plasma samples failed our 24-h bench stability test, as did extracted samples stored for 48 h in the autosampler due to degradation other than photo-isomerization.

Clinical Results

Plasma montelukast concentration-time curves following administration of the two chewable treatments were similar in shape to each other and plasma concentration-time curves from both swallow treatments were also similar in shape to each other. However, absorption following swallow treatments was later than chewable treatments. Plasma montelukast concentrations following marketed and blinded swallow tablet administrations were appreciably lower during the first few hours compared to chewable tablet administration. However, montelukast concentrations after 6 h were similar. The median plasma montelukast concentrations following each treatment are presented in Fig. 7.

Geometric mean ratios (blinded/marketed) and 90% confidence intervals for AUC_{∞} and C_{max} were all well within the acceptance range (0.80–1.25) for chewable and swallow tablet comparisons. Chewable tablet AUC_{∞} and C_{max} com-

Table II. Validation Results for QC Samples

	QC 5	QC 15	QC 150	QC 1500	QC 2000
Target concentration (ng ml^{-1})	5.00	15.0	150	1500	2000
N	24	24	24	24	24
Mean	5.455	14.55	146.0	1375	2015
SD	0.4746	0.857	9.76	69.4	78.7
Accuracy (% bias)	9.1%	–3.0%	–2.7%	–8.3%	0.7%
Intra-assay precision (%CV)	6.7%	5.5%	4.7%	4.2%	3.1%
Inter-assay precision (%CV)	6.1%	2.1%	6.0%	1.1%	3.0%

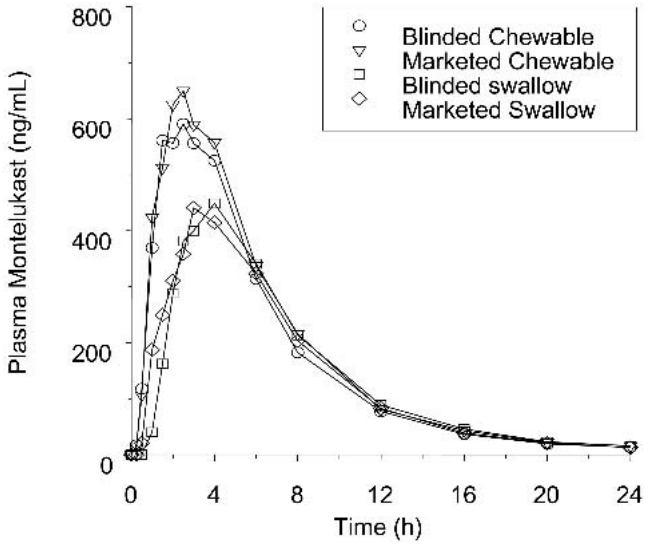


Fig. 7. Median plasma montelukast concentrations.

parisons were 0.94 (0.87, 1.02) and 0.96 (0.85, 1.08), respectively. Swallow tablet AUC_∞ and C_{max} comparisons were 1.02 (0.95, 1.10) and 1.06 (0.94, 1.19), respectively. No significant differences were found between t_{max} from blinded and marketed products. t_{max} averaged 2.7 h after blinded and marketed chewable tablet administration, 3.5 h after blinded swallow tablet administration, and 3.9 h after marketed swallow tablet administration.

CONCLUSIONS

A practical and high-throughput method for the determination of montelukast in human plasma has been developed. The approach used for the analysis of this photosensitive compound simplifies specimen handling and corrects for the greatest source of analytical error—photoisomerization. Simultaneous monitoring of the drug and its primary light degradation product, under separate fluorescence conditions that ensure a molar equivalent detector response, allows original plasma montelukast concentrations to be accurately calculated even though samples were exposed to moderate amounts of ambient light. Automated sample extraction and dual column HPLC analysis allows for large batches of up to 288 samples per day to be easily processed within the stability timeframe restrictions of the compound. Using only 100 μl of sample, the assay had adequate sensitivity as well as good precision and accuracy of less than 10% throughout the 5–2000 ng ml^{-1} linear range. The assay was used to demonstrate the bioequivalence of blinded montelukast formulations used for clinical studies.

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