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Determination of montelukast (MK-0476) and its *S*-enantiomer in human plasma by stereoselective high-performance liquid chromatography with column-switching

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Abstract

A steoreoselective high-performance liquid chromatographic method was developed for the quantification of montelukast (free acid of Singulair™, or MK-0476), a potent and selective leukotriene D_4 ($cysLT_1$) recptor antagonist, and it *S*-enantiomer (L-768,232). The method involves protein precipitation and fluorescence detection. Chromatographic separation of the enantiomers from endogenous components in plasma and chiral resolution of the enantiomers are achieved by using column switching HPLC and an α-acid glycoprotein chiral column. The assay is linear in the range of 28.9–386 ng ml^{-1} of free acids of montelukast and L-768,232. The intraday precision (% relative standard deviation) values of this method were in the range of 2.5–9.1% for montelukast, and 2.4–6.8% for L-768,232, while the intraday accuracy values were in the range of 97–103% for montelukast and 96–104% for L-768,232. The interday precision values of this method at 48.2 and 193 ng ml^{-1} were 5.3 and 3.6%, respectively, for montelukast, and 4.2 and 3.7%, respectively, for L-768,232, while the interday accuracy values at these concentrations were 97 and 103%, respectively, for montelukast and 99 and 102%, respectively, for L-768,232. The utility of the methodology was demonstrated by analysis of plasma samples from a study in which healthy volunteers received 10 mg per day of montelukast orally for 7 days. Results of this study indicate that there is no apparent bioinversion of montelukast to its *S*-enantiomer in humans.

Keywords: Montelukast; Singular™; MK-0476; Chiral HPLC assay; Column-switching

1. Introduction

Montelukast (free acid of Singulair™), also known as MK-0476 [1-(((1(R)-(3-(2-(7-chloro-2-quinolinyl)-(E)-ethenyl)-phenyl) (3-(2-(1-hydroxy-1-methylethyl)phenyl)propyl)thio)-methyl)-cyclopropane) acetate], is a potent and selective leukotriene D_4 ($cysLT_1$) receptor antagonist [1,2]. This compound, which is currently under development for the treatment of chronic asthma, possesses a chiral center at the methine carbon of the thio side-chain and exists in *R*-enantiomeric form (**1**, Fig. 1). Studies have shown that many

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R- and *S*-enantiomers (e.g. *R*-ibuprofen [3] and other nonsteroid antiinflammatory drugs) undergo bioinversion in humans. To investigate the possibility of such a bioinversion for montelukast, a chiral assay method was needed for the quantitative determination of montelukast and its *S*-enantiomer (**2**, L-768,232, Fig. 1) in human plasma. This paper describes the development and validation of such a method and its application to the analysis of montelukast and L-768,232 in human plasma samples.

2. Experimental

2.1. Chemicals and reagents

Montelukast (dicyclohexylamine salt), L-768, 232 (sodium salt), and 1-(((1(R)-(3-(2-(7-chloro-2-quinolinyl)-(E)-ethenyl)-phenyl) (3-(2-(1-hydroxy-1-methylethyl)phenyl)propyl)thio)-methyl)-isopropane) acetate (**3**; L-705,254, sodium salt, internal standard; Fig. 1) were obtained from Merck Research Laboratories (Rahway, NJ, USA). Acetonitrile (optima grade), ammonium acetate (HPLC grade), and methanol (optima grade) were purchased from Fisher Scientific (Fairlawn, NJ, USA). Acetic acid (glacial, AR select grade) was purchased from Mallinckrodt (Paris, KY, USA). Human control plasma (heparinized) was purchased from Biological Specialty Corporation (Lansdale, PA, USA). Milli-Q de-ionized water was used throughout.

2.2. Apparatus

The chromatography system consisted of a pump (LC Pump 1) with an autosampler (Model 1050, Hewlett-Packard, Avondale, PA, USA), a Varian Model 9010 pump (LC Pump 2, Varian Analytical Instruments, Sunnyvale, CA, USA), and a six-port electrically actuated switching valve (Model EC6W, Valco Instruments, Houston, TX, USA). The extraction column (Column 1) was a Chromspher 5 Biomatrix extraction column (50 × 4.6 mm, 5 μm, Chrompack Inc., Raritan, NJ, USA) preceded by a Chromspher guard column (10 × 3 mm, 5 μm). The analytical column (Column 2) was a Chiral AGP column (100 × 4.0 mm, 5 μm, Regis Technologies, Morton Grove, IL, USA) preceded by a Regis Chiral AGP guard column (10 × 3 mm, 5 μm). The eluent was monitored with a variable wavelength fluorometric detector (Model RF-551, Shimadzu Scientific Instruments, Inc., Columbia, MD, USA). The fluorescence excitation and emission wavelengths were set at 350 and 400 nm, respectively. A Turbochrom II system (PE Nelson Systems, Cupertino, CA, USA) was used to perform data collection and switching valve control via a PE Nelson 960 analog-to-digital interface.

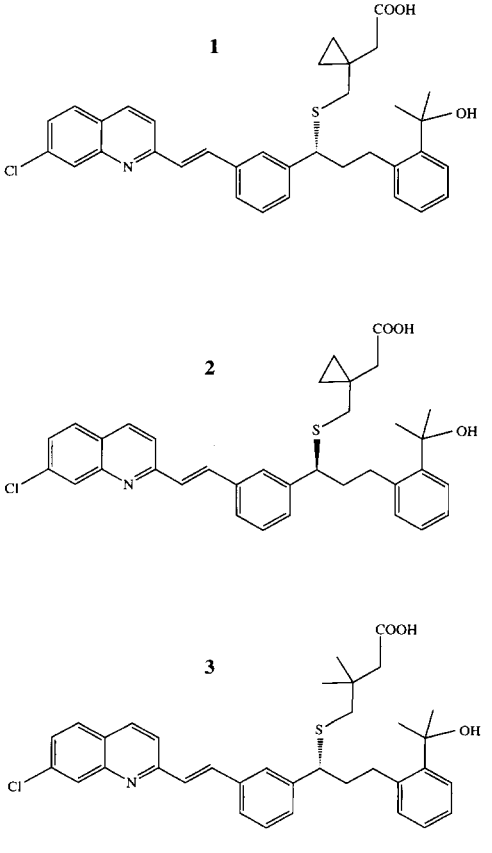

Fig. 1. Chemical structures of compounds **1**, **2** and **3**.

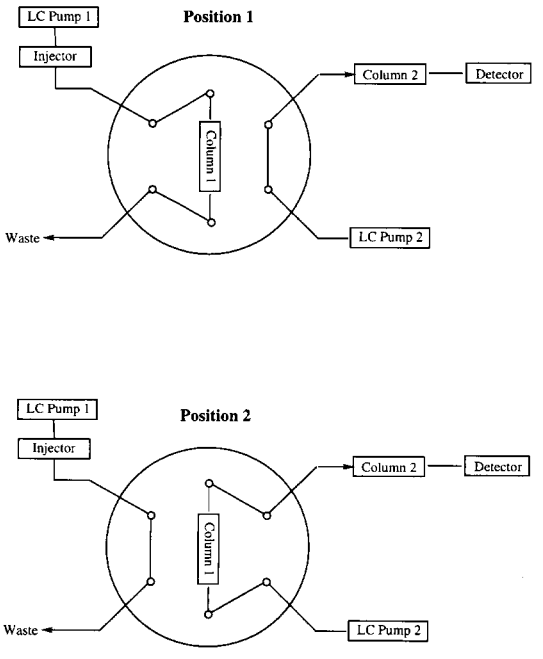

Fig. 2. Diagram of the column switching system.

2.3. Chromatographic conditions

The mobile phase (A) for LC Pump 1 consisted of acetonitrile and buffer solution 1 which was methanol-ammonium acetate (pH 3.6; 10 mM) (10:100, v/v, pre-mixed). Mobile phase (B) for LC Pump 2 consisted of buffer solution 2 which was acetonitrile-ammonium acetate (pH 5.8; 10 mM) (32.5:100, v/v, pre-mixed). The buffer solutions were prepared fresh daily. The LC flow rate was 1 ml min^{-1} for both LC pumps. Following the injection of plasma sample, the extraction column was washed with buffer solution 1 for 5 min to remove remaining plasma proteins and other endogenous components. Afterward another 5-min washing with acetonitrile-buffer solution 1 (12:88, v/v) was applied to provide additional washing, and more importantly, to bring the mobile phase condition closer to that on Column 2 to reduce the solvent front. During this period, the column switching valve was on Position 1 (Fig. 2). The valve was then switched to Position 2 and stayed for 3.5 min to backflush the analytes and internal standard from Column 1 into Column 2 with mobile phase (B). Afterwards, the switching valve was switched back to Position 1. Column 1 was then washed with acetonitrile-buffer solution 1 (80:20, v/v) to remove strongly retained compounds while Column 2 was continuously eluted with mobile phase (B). At 18.5 min of the run the mobile phases returned to their initial condition. The chromatography was performed at ambient temperature (~ 22°C). The LC run time for each injection was 35 min.

The following parameters were measured:

k': Capacity factor of an analyte: $k' = (t_R - t_0)/t_0$, where t_R is the retention time and t_0 is the mobile phase hold-up time.

R_s: Resolution factor between two analytes: $R_s(1,2) = 2(t_2 - t_1)/(w_1 + w_2)$, where w is the peak width at base.

α: Separation factor between two analytes: $\alpha = k'_2/k'_1$, $(k'_2 > k'_1)$

2.4. Preparation of standards

Stock solutions of montelukast, **2**, and **3** were prepared by dissolving the compounds separately in methanol-water (7:3, v/v) to yield a concentration of 0.964 mg (free acid) ml^{-1}. A diluted solution of montelukast and **2**, each at a concentration of 96.4 μg ml^{-1}, was prepared by mixing 200 μl of the stock solution of each analyte and diluting with 1.6 ml of methanol-water (7:3, v/v). Working standards containing 38.6, 19.3, 9.64, 4.82 and 2.89 μg ml^{-1} of each analyte were prepared by successive dilution of the 96.4 μg ml^{-1} solution with methanol-water (7:3, v/v). A 3 μg ml^{-1} internal standard working solution was prepared by diluting the internal standard stock solution with methanol-water (7:3, v/v). Plasma standards containing both analytes were prepared by adding 200 μl of each working standard to vials each containing 19.8 ml of human control plasma to give concentrations of 386, 193, 96.4, 48.2, and 28.9 ng ml^{-1}. Quality control samples were prepared by spiking the standard compounds (from separately prepared stock solutions) into human control plasma at concentrations of 193 and 48.2 ng ml^{-1} for each analyte.

2.5. Analytical and validation procedure

To each 1.5 ml polypropylene tube containing 300 μl of plasma standard, quality control or unknown plasma sample, 20 μl of internal standard working solution was added, followed by addition of 400 μl of acetonitrile. The contents were immediately vortexed and centrifuged at 10 000 rpm for 10 min. The supernatant from each tube was transferred to an amber autosampler vial, and 60 μl was injected onto the HPLC. Since montelukast, 2, and 3 are all light sensitive, protection of the plasma standards and study samples from exposure to light during sample preparation and injection is necessary. Precautions should also be taken during sample collection, storage and any other sample handling processes.

Calibration curves were constructed by using least-square linear regression analysis with a weighing factor of concentration^{-1}. Concentrations of analytes in each unknown sample were calculated by interpolation from the standard curves. All calculations were based on the peak height ratios of the analytes to internal standard. When a concentration of montelukast or **2** exceeded the upper limit of calibration range, the sample was diluted with blank control plasma and reassayed.

Individual plasma from 10 healthy subjects were analysed for possible chromatographic interference with montelukast, **2**, and **3**. Possible interference from endogenous substances in plasma was also evaluated by analysing predose samples.

The detection limit of the method for montelukast was determined as follows: 300 μl of human control plasma from each of the 10 healthy subjects was transferred into 10 separate culture tubes (blanks). To another 10 tubes, 300 μl of corresponding control plasma containing 9.6 ng ml^{-1} montelukast was added separately (spiked plasma). All of the samples were then analysed by the described assay method. The detector response at the retention time of montelukast from each sample was measured, and the difference in response from spiked vs. blank plasma pairs was evaluated with Student's t test for paired samples. The same procedure was used to determine the limit of detection for L-768,232.

The intraday precision and accuracy of the method were assessed by analysing five replicates of plasma standards with concentrations of 28.9, 48.2, 96.4, 193, and 386 ng ml^{-1} of montelukast and **2**. Interday precision and accuracy were determined by analysing five replicates of the quality control samples at concentrations of 48.2 and 193 ng ml^{-1} on three different days. The observed mean, percentage theoretical recovery and percentage relative standard deviation (%R.S.D.) were calculated.

3. Results and discussion

3.1. Method development

Previously a non-chiral HPLC assay involving protein precipitation and fluorescence detection was developed for the determination of montelukast in human plasma [4]. With this method, chromatography was performed on a C18 column (50 × 4.6 mm, 5 μm) with a mobile phase of acetonitrile-ammonium phosphate (pH 3.5; 50 mM) (62:38, v/v). The assay was linear in the range of 28.9–2890 ng ml^{-1} of montelukast. In developing an assay method for montelukast enantiomers, all the chromatographic conditions of the non-chiral method were re-evaluated.

A CHIRALCEL OD-R column (Chiral Technologies, Exton, PA, USA) and the Chiral AGP column were evaluated for the separation of montelukast enantiomers. With optimal mobile phase condition (acetonitrile-$NaClO_4$/$HClO_4$) suggested by the manufacturer for this type of compounds, no separation between montelukast enantiomers was achieved on the CHIRALCEL OD-R column. The Chiral AGP column, on the other hand, provided satisfactory separation between the enantiomers with an acetonitrile-ammonium acetate mobile phase, and therefore was chosen to be used in this method.

The chiral selectivity of the Chiral AGP column is based on the unique feature of the chiral stationary phase: the α_1-acid glycoprotein (α_1-AGP) that was immobilized on the supporting material. Proper selection of mobile phase, including the pH of buffer and content of organic modifier, can

Table 1
Effect of buffer pH on chiral separation of **1**, **2**, and **3**

pH	Retention time (min)			Peak width (min)			Capacity (k')			Resolution (R_s)			Separation factor (α)		
	1	2	3	1	2	3	1	2	3	1,2	1,3	2,3	1,2	1,3	2,3
3.6	10.6	7.71	15.30	2.60	2.00	3.40	4.89	3.28	7.50	1.26	1.57	2.81	1.49	1.53	2.28
4.0	9.57	7.25	13.70	2.00	1.80	2.80	4.32	3.03	6.61	1.22	1.72	2.80	1.43	1.53	2.18
5.0	8.22	6.16	13.17	1.80	1.40	2.80	3.57	2.42	6.32	1.29	2.15	3.34	1.47	1.77	2.61
5.5	5.78	4.20	8.94	1.40	1.40	2.00	2.21	1.33	3.97	1.13	1.86	2.79	1.66	1.79	2.98

enhance the enantio-resolving capability of this column. A recent study has shown that, using ammonium acetate and acetonitrile as mobile phase, the organic content in the range of 5–30% had little effect on the chiral resolution of montelukast enantiomers [5], and so did the acetate buffer concentration in the range of 5–30 mM. The buffer pH on the chiral column, however, had a large effect on the chiral separation of the enantiomers. This pH effect was therefore particularly evaluated. As shown in Table 1 and Fig. 3, using acetonitrile-ammonium acetate (10 mM) (28:72, v/v) as mobile phase, an increase in the buffer pH led to a decrease in retention time and an increase in separation factor for all three compounds. A maximum chiral resolution among **1**, **2** and **3** was achieved near pH = 5.0 (Fig. 4). The above study was performed on the chiral column alone with analytical solutions of the compounds. Later when coupled with column 1 using the column-switching technique, the buffer pH on the chiral column was found to be affected by the content transferred from column 1 and was no longer the same as the pH of buffer solution 2. Since the content transferred from column 1 was with low pH (buffer solution 1, pH 3.6), a pH higher than 5.0 for buffer solution 2 was needed to maintain the optimal pH on the chiral column. In addition, when applying the chromatographic condition with column-switching to plasma samples, adjustment of buffer solution 2 pH was also necessary to minimize matrix interference to one of the analytes (**2**). The optimal pH for buffer solution 2 was found, under such a condition, to be 5.8. The acetonitrile content was also adjusted, from 28 to 25%, for a better separation between **2** and the solvent front.

Further improvement to the chromatography and sensitivity of the method using liquid-liquid or solid phase extraction was found difficult due to the restriction from the ambient temperature stability of the compounds in aqueous solutions. The analytes were found degraded, probably by oxidation, after the concentrating steps in both extraction processes.

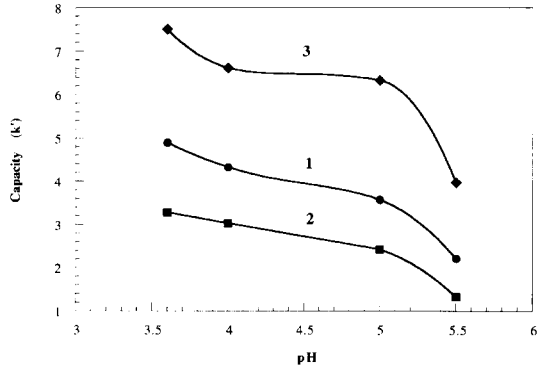

Fig. 3. Effect of buffer pH on the retentions of **1**, **2** and **3**.

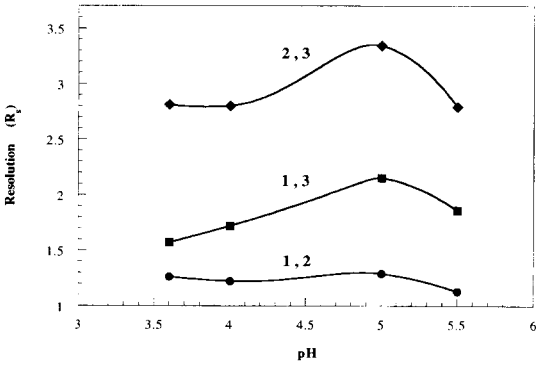

Fig. 4. Effect of buffer pH on resolution of **1**, **2** and **3**.

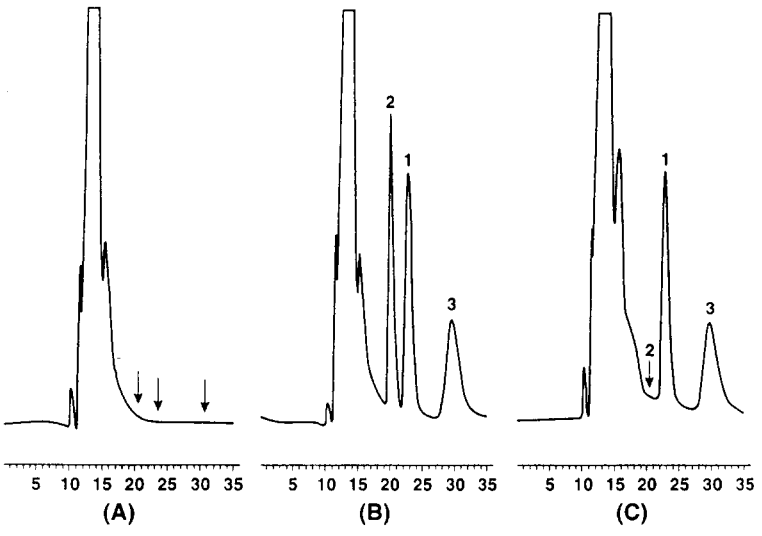

Fig. 5. Typical chromatograms of (A) a pre-dose plasma sample from a healthy volunteer; (B) a plasma standard spiked with 193 ng ml^{-1} of **1**, **2** and **3**; (C) a plasma sample from the same subject, collected 8 h after a 10-mg oral administration of montelukast. The arrows indicate peak positions for 1, 2 and 3.

Since the mobile phase used for Column 2 was low in organic content (25% acetonitrile), the supernatant (containing ~60% acetonitrile) from the protein precipitated sample could not be introduced onto this column directly. Furthermore, additional pre-injection sample treatments, such as extractions (solid-phase or liquid-liquid) and concentrating, were not desirable for the stability consideration as mentioned above. Under such a circumstance, the column-switching technique was found to be the choice to accomplish direct injection of samples after protein precipitation without producing disturbance to the chromatographic condition on the chiral column. In addition, the column-switching step also provides further sample clean-up. The 3.5 min backflushing window was determined by observing the highest sensitivity with minimum background and solvent front interference from the fluorescence detection.

Selection of the extraction column is also critical to the enantiomeric separation, as well as the sensitivity of the method. Compounds **1**, **2** and **3** should be fully retained on this column, and should be easily eluted upon elution with mobile phase (B) which was in low organic content. Since C18 column has been known to provide very strong retention of montelukast, requiring a mobile phase containing 62% acetonitrile to elute [4], several pre-columns or cartridges with C1, C2, C4, C8, and CN packings, and a Biomatrix extraction column were evaluated. Among these pre-columns or cartridges, the Biomatrix extraction (Chrompack Chromspher 5) column was found the easiest for the analytes to be eluted by mobile phase (B), while others required higher organic content. Furthermore, the Biomatrix column was designed for more efficient removal of plasma proteins and other endogenous components, providing a cleaner sample condition for the chiral column.

6-chloro montelukast (L-708,772) and other ten montelukast analogs were tested for the potential internal standard to be used in this method. Compound **3**, a dimethyl analog of montelukast, was selected in favor of its retention time. The retention times of other compounds were either too close to or too far from those of montelukast and **2**.

3.2. Method validation

Typical chromatograms obtained using this method are shown in Fig. 5. Satisfactory enantiomer separation was achieved with reasonable retention times. The retention times were about 20, 23, and 30 min for L-768,232, montelukast, and the internal standard, respectively. Interfering peaks at these retention times were not observed in the chromatograms of control plasma blanks and pre-dose plasma samples.

Good linearity was achieved with this method. The correlation coefficient was always greater than 0.99 for both analytes, with an average ($\pm$%R.S.D., $n = 6$) of 0.997 ± 0.13 for montelukast and 0.997 ± 0.23 for **2**.

The mean detector response for 9.6 ng of montelukast and **2** per millilitre of spiked plasma samples was significantly greater than that of the corresponding blank plasma samples ($p < 0.0001$). Thus 9.6 ng ml^{-1} was established as the detection limit of the method for both analytes (signal to noise ratio = 4:1).

The intraday and interday precision and accuracy data of this method are listed in Tables 2 and 3. The intraday precision values ranged from 2.5 to 9.1% for montelukast, and from 2.4 to 6.8% for **2**; while the intraday accuracy values were in the range of 97–103% for montelukast, and 96–104% for **2** (Table 2). The interday precision values at the concentrations of 48.2 and 193 ng ml^{-1} were 5.3 and 3.6%, respectively, for montelukast, and 4.2 and 3.7%, respectively, for **2**. The accuracy at these two concentrations was 97 and 103%, respectively, for montelukast, and 99 and 102%, respectively, for **2** (Table 3). Dilution of plasma samples with blank plasma was found to have no noticeable effect on the precision and accuracy of the method.

Table 2
Intraday precision and accuracy of the assay method ($n = 5$)

Nominal concentration (ng ml^{-1})	Mean concentration found (ng ml^{-1})	Mean recovery $\pm$ RSD (%)
Montelukast		
28.9	29.7	103 ± 9.1
48.2	47.4	98 ± 5.7
96.4	93.5	97 ± 2.5
193	198	102 ± 3.5
386	384	100 ± 5.2
L-768,232		
28.9	29.8	103 ± 6.8
48.2	46.2	96 ± 2.8
96.4	94.5	98 ± 2.4
193	201	104 ± 3.9
386	381	99 ± 4.5

Table 3
Interday precision and accuracy of the assay method ($n = 3$)

Nominal concentration (ng ml^{-1})	Mean concentration found (ng ml^{-1})	Mean recovery $\pm$ RSD (%)
Montelukast		
48.2	46.6	97 ± 5.3
193	199	103 ± 3.6
L-768,232		
48.2	47.8	99 ± 4.2
193	197	102 ± 3.7

Montelukast and **2** were both found to be stable in injection solution (plasma supernatant) for at least 24 h by reanalysing the same quality control samples at 24 h later. Previously [4], montelukast was found to be stable in human plasma for at least 19 months at -70°C, and after three freeze/thaw cycles. Also, the absolute recovery for montelukast after protein precipitation was found to be quantitative [4]. The same is expected to be true for **2**.

3.3. Application

The method has been used to evaluate the possibility of bioinversion of montelukast in humans. In a clinical study, plasma samples from six healthy volunteers receiving montelukast orally 10 mg per day for 7 days were collected on Day 7 and analysed. No detectable amount of L-768,232 was found in these samples, indicating that there is no apparent bioinversion of montelukast to its *S*-enantiomer in humans. Fig. 6 displays the mean plasma concentration-time profile of montelukast in healthy volunteers, showing that the peak

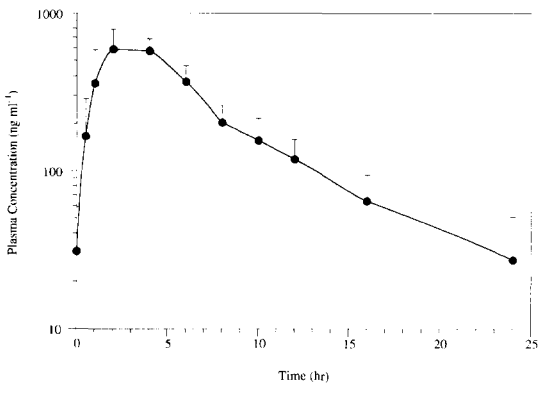

Fig. 6. Mean ($\pm$S.D., $n=6$) plasma concentration-time profile of montelukast on Day 7 in healthy volunteers receiving orally 10 mg day^{-1} montelukast for 7 days.

plasma concentrations averaged 0.67 ± 0.16 μg ml^{-1}, achieved at 3.0 ± 1.1 h post-dose on Day 7.

In conclusion, the combination of column-switching technique with stereoselective separation using a chiral column has proven to provide a simple and reliable assay method for the chiral analysis of montelukast and L-768,232 in human plasma, with a limit of quantitation similar to that of the non-chiral HPLC assay method [4].

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