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Determination of montelukast sodium in human plasma by column-switching high-performance liquid chromatography with fluorescence detection

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Abstract

MK-0476 (montelukast sodium) is a potent and selective cysteinyl leukotriene receptor antagonist that is being investigated in the treatment of asthma. A simple and sensitive method for the determination of MK-0476 in human plasma was developed using column-switching high-performance liquid chromatography (HPLC) with fluorescence detection. A plasma sample was injected directly onto the HPLC system consisting of a pre-column (Capcell pak MF) and an analytical column (Capcell pak C18) which were connected with a six-port switching valve. The column eluate was monitored with a fluorescence detector (excitation at 350 nm; emission at 400 nm). The calibration curve was linear in a concentration range of 1–500 ng ml^{-1} for MK-0476 in human plasma. The intra-day coefficients of variation of all concentrations within the range was less than 9.2%, and the intra-day accuracy values were between 97.2 and 114.6%. This method was used to measure the plasma concentration of MK-0476 following oral administration of the drug in humans. © 1998 Elsevier Science B.V. All rights reserved.

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1. Introduction

MK-0476 (montelukast sodium) is a potent, orally active, specific cysteinyl leukotriene receptor antagonist that is being developed as a therapeutic agent for the treatment of bronchial asthma [1]. Recently, the pharmacological effects of MK-0476 have been evaluated in patients with chronic asthma. In a clinical study, MK-0476, compared with placebo, was reported to improve forced expiratory volume in one-second (FEV_1) and reduce symptom scores in asthma [2].

MK-0476 is a fluorescent compound, and a method was previously reported for the measurement of plasma levels of the drug using high-performance liquid chromatography (HPLC) with fluorescent detection [3]. This method requires the precipitation of plasma proteins by acetonitrile prior to HPLC analysis. The limit of quantitation (LOQ) was 30 ng ml^{-1}. A pharmacokinetic study after oral dosing of MK-0476 (50–400 mg man^{-1}) in healthy male Japanese volunteers was conducted using this method. In a therapeutic dose range-finding study, how-

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ever, doses lower than 50 mg were found to be adequately effective in the treatment of asthma. Therefore, a more sensitive method was required to determine MK-0476 at ng ml^{-1} levels for pharmacokinetic evaluations.

In the present study, we developed a method for the analysis of MK-0476 using column-switching HPLC. The column-switching technique has an advantage over other methods in sensitivity because biological fluid is injected directly into the HPLC column without any sample loss. In addition, this technique is especially useful for the sample clean-up of photo-sensitive compounds because on-line sample processing is done within an HPLC system under dark conditions [4–6]. Sample handling is minimal prior to analysis. MK-0476 is a photo-sensitive compound, and the *trans* form at the ethenyl moiety is converted to the *cis* form by UV irradiation. Therefore, it was thought that using column-switching HPLC in the determination of MK-0476 would effectively prevent this photo-isomerization.

We report the use of the column-switching method for plasma MK-0476 determination after oral administration of MK-0476 at doses of 2 and 10 mg in humans. Plasma samples were injected directly onto the pre-column, the commercially available Capcell pak MF column, whose solid phase consists of hydrophilic and hydrophobic functional groups covalently bound to silicone-coated silica beads [7,8]. This method was sensitive enough to quantitate 1 ng ml^{-1} without photo-isomerization.

2. Experimental

2.1. Chemicals

MK-0476, sodium 1-(((1(*R*)-(3-(2-(7-chloro-2-quinolinyl)-(*E*)-ethenyl)phenyl)(3-(2-(1-hydroxy-1-methylethyl)phenyl)propyl)thio-methyl-cyclopropane) acetate (Fig. 1) was supplied by Merck Frosst Canada Inc. (Montreal, Canada). Acetonitrile, methanol (both HPLC grade), tetrahydrofuran, and acetic acid (both reagent grade) were purchased from Junsei Chemical (Tokyo, Japan). Sodium acetate, ammonium acetate and sodium dodecyl sulfate (SDS) were of reagent grade from Wako Pure Chemical (Osaka, Japan). Water was purified using a Milli-Q system (Millipore-Japan, Tokyo, Japan).

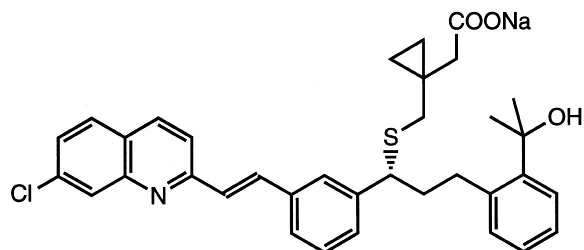

Fig. 1. Chemical structure of MK-0476.

2.2. Preparation of MK-0476 standard solution

A stock solution of 20 μg ml^{-1} of MK-0476 was prepared by dissolving the substance in methanol-water (7:3, v/v). This solution was stored below −20°C. MK-0476 standard solution was added to human control plasma to make standard samples for calibration curves. Plasma samples, containing 1, 2,10, 50, 100, and 500 ng ml^{-1} of MK-0476, were stored at −80°C until assay.

2.3. Sample preparation

The samples were prepared under a UV cut fluorescent lamp (FLR4OS AI/M National, Osaka, Japan) or a dark red lamp (Biolight, RH-525, National). Frozen samples were thawed in a water bath and were vortexed. Plasma (200 μl) was transferred into an Ultrafree filter unit (pore size 0.22 μm, UFC3OGVOO, Millipore-Japan) and was centrifuged at 4900 g at 5°C for 5 min. Aliquots of 100 μl of the filtered plasma were then injected onto an HPLC system.

2.4. Instrumentation

Fig. 2 shows a schematic diagram of the column-switching HPLC system. The system consisted of three pumps (Model 305, Gilson, Westwood, NJ, USA; 880-PU, Jasco, Tokyo, Japan), a manometric module (Model 805, Gilson), a column oven (860-CO, Jasco), an autosampler (Model 231, Gilson), a degasser (Model 563, M&S instruments, Tokyo, Japan), a six-port switching valve (Eicom, Kyoto,

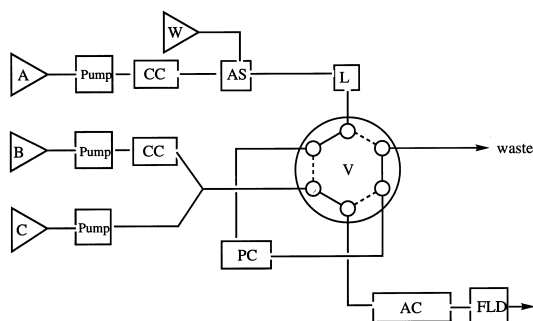

Fig. 2. Schematic diagram of the column-switching apparatus in the HPLC system.

Japan), a data processor (Quarter L pcx-320, Sony, Tokyo, Japan), a signal cleaner (SC77, SIC, Tokyo, Japan), a data module (Model 621, Gilson), and a fluorescence detector (RF-10A, Shimadzu, Kyoto, Japan). A Capcell pak MF Ph-1 column (PC; 50×4.6 mm I.D., Shiseido, Tokyo, Japan) was used for pre-separation and a Capcell pak MF Ph-1 column (GC;10×4.6 mm I.D., Shiseido) was used as its guard column (See Fig. 2). The analytical column was a Capcell pak C18 column (AC; 250×4.6 mm I.D., Shiseido). A Capcell pak C_{18} column (CC; 50×4.6 mm I.D., Shiseido) was used as a cleanup column to remove interferences from the aqueous mobile phase. An in-line filter (LRF-2, GL Sciences, Tokyo, Japan) was placed in front of the switching valve and the filter was changed daily.

The flow-rate was adjusted to 1.0 ml min^{-1} on each column. At the beginning of the analysis, the valve was placed in position 1 (Fig. 2), and a filtered plasma sample (100 μl) was directly analyzed by the HPLC system. The most polar components were removed by washing the pre-column with a mobile phase of acetonitrile–0.1 *M* ammonium acetate (10:90, v/v), while MK-0476 was retained on the column. At 7.5 min, the valve was switched to position 2, so the fraction containing MK-0476 was transferred from the pre-column to the analytical column with a mobile phase of acetonitrile–25 m*M* acetate buffer (pH 3.5) (45:55, v/v). At 15.5 min, the valve was switched back to position 1 and MK-0476 was eluted from the analytical column with a mobile phase of acetonitrile–25 m*M* acetate buffer (80:20, v/v). The column eluate was monitored with a fluorescence detector (excitation at 350 nm; emission at 400 nm). The pre-column and analytical column were equilibrated at the initial condition for more than 5 min before the next injection. The temperature of the analytical column was maintained at 40°C. The total run time per sample was 30 min. The temperature of the auto-sampler was set at 5°C, and tetrahydrofuran–0.1 *M* SDS (10:90, v/v) was used as the wash solution.

2.5. Assay validation

Calibration standards in human plasma were prepared by adding known amounts of MK-0476 to control plasma to produce standards with final concentrations of 1, 2,10, 50,100, and 500 ng ml^{-1}. The calibration curve for MK-0476 was generated by measuring peak height.

The intra-day coefficient of variation (C.V.) and accuracy of the quantitation were assessed for the assay by the analysis of five plasma samples spiked at the same concentrations as the calibration standards. The C.V. and accuracy for inter-day assay were evaluated at the same concentration, repeated for five different days. Recovery was determined by injecting an aqueous solution of MK-0476 (500, 50 and 5 ng ml^{-1}) directly onto the analytical column and by comparing its peak height with those produced by plasma samples.

3. Results and discussion

3.1. Photo-isomerization

MK-0476 is a photo-sensitive compound with a trans-ethenyl functional group. The *trans* form is converted to the *cis* form by UV light irradiation. Therefore, the stability of MK-0476 in human plasma was examined under two different light conditions. Under a UV cut lamp (185 lx), MK-0476 was stable at room temperature at least for 4 h at 100 ng ml^{-1} (99% remaining). Under a conventional fluorescent light (600 lx), the amount of MK-0476 remaining was 77% of the initial amount. These results indicate that protection from UV light is necessary during sample handling. However, sample handling under dark light in a dark room is laborious and is subject to mistakes. In this column-switching

method, the plasma was pretreated within the HPLC line. Accordingly, the sample handling time, i.e., irradiation time, was minimal. As a result, no peak caused by photo-isomerization was observed in the chromatogram of this method. Therefore, this method is suitable for quantitating photo-sensitive compounds like MK-0476 whose ethenyl group undergoes photo-isomerization. Sample handling consisted only of filtration under the UV cut light prior to injection onto the HPLC. Additionally, the time required for processing samples using this method was shortened compared with that using the previous method.

3.2. Chromatography

The Capcell pak MF column is one of the restricted access materials (RAM). It is not an internal-surface reversed-phase column, but a mixed-functional phase column. Both internal and external surfaces consist of a mixture of hydrophilic polyoxyethylene and hydrophobic phenyl phases coating on silicone polymer-coated silica beads. The column minimizes undesirable secondary interactions of plasma proteins with the silica surface and shows higher recoveries for injected proteins. No column deterioration was reported in serum sample injections of up to 10 ml [7]. Therefore, we used the Capcell pak MF Ph-1 column as the pre-column. The biological hydrophilic components such as proteins were eluted to waste, whereas small hydrophobic molecules such as MK-0476 were retained on the column and were subsequently eluted to the analytical column by increasing contents of acetonitrile in mobile phase.

A system carryover was observed when a blank plasma was analyzed after injection of a plasma sample containing 500 ng ml^{-1} of MK-0476; acetonitrile–water (10:90, v/v) was used as a wash solvent. This carryover was thought to be due to MK-0476 that was adsorbed to the HPLC line. Therefore, we tried to automatically wash the HPLC line between the injector and switching valve in every analysis. To effectively remove the system carryover, tetrahydrofuran–0.1 *M* SDS (10:90, v/v) was used as the wash solvent on the auto sampler. Tetrahydrofuran was more effective than methanol and acetonitrile. The SDS solution was used to resolve the precipitation attributed to plasma component denaturation on the line-filter [9]. The sample-loop volume of the injector was changed to 1 ml, and the autosampler was programmed to wash the line using the wash solvent (1 ml$\times$2) in position 2 (see Fig. 2) while transferring MK-0476 from the pre-column to the analytical column. After this improvement, no detectable carryover of MK-0476 was observed in the chromatogram of blank plasma. Based on the peak of limit of quantitation, it was determined that there was less than 0.2% carryover for MK-0476 between consecutive injections.

3.3. Calibration curve, coefficient of variation, and accuracy

The calibration curve was linear over the range of 1–500 ng ml^{-1} with correlation coefficients greater than 0.999. The typical calibration curve for MK-0476 was given by the equation, $y=153.8x+39.88$, where y indicates the peak height, and x represents the concentration of MK-0476 in ng ml^{-1}. Fig. 3 shows the chromatograms obtained from (a) 2-h post-dose plasma of a volunteer who received an oral dose of MK-0476 (10 mg), (b) plasma containing 1 ng ml^{-1} of MK-0476 and (c) drug-free plasma. No significant interference peaks such as endogenous compounds and metabolites were observed in the chromatograms. The concentration of MK-0476 in this post-dose plasma was determined to be 161 ng ml^{-1}. The results of intra-day and inter-day variation for analyses of MK-0476 are summarized in Tables 1 and 2, respectively. The intra-day coefficients of variation did not exceed 9.2%, and the intra-day accuracies were between 97.2% and 114.6% within the concentration range of the calibration curve. In the same way, the inter-day coefficients of variation did not exceed 14.1%, and its accuracies were between 97.2% and 106.6%. The limit of quantitation for MK-0476 was set to 1 ng ml^{-1}, which is the lowest concentration of the analyte that can be measured with a coefficient of variation and an accuracy less than 20%. The overall recoveries of MK-0476 at 500, 50 and 5 ng ml^{-1} were 91.5%,103.6% and 113.5%, respectively. MK-0476 in plasma samples was stable in the autosampler for at least 48 h at 5°C. Other stability data were reported previously; MK-0476 was stable for at

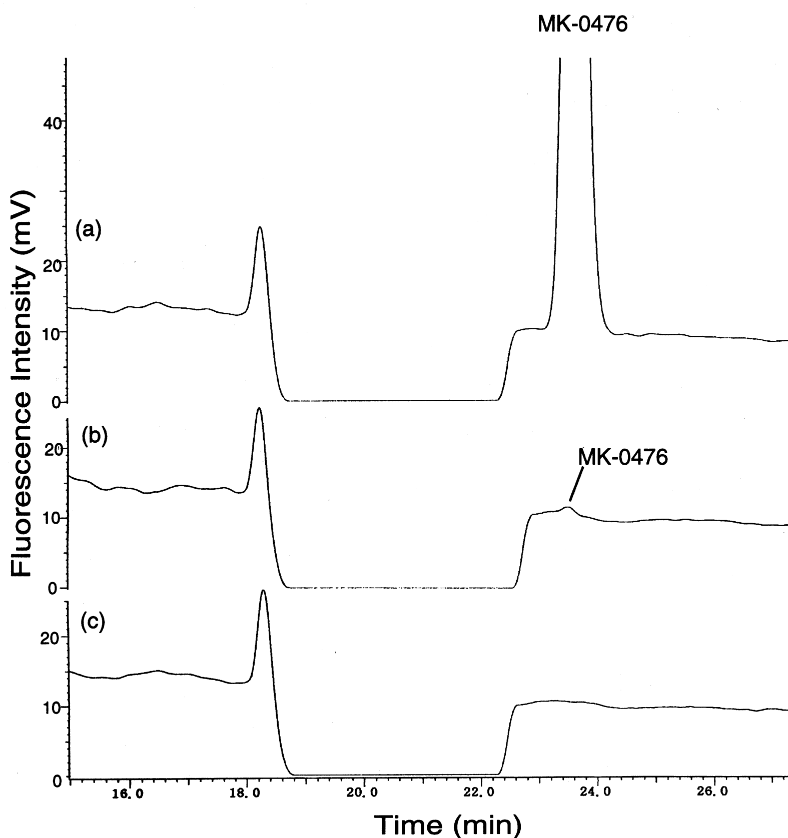

Fig. 3. Typical chromatograms obtained from (a) plasma collected at 2 h after oral administration of MK-0476 (10 mg, the estimated concentration was 161 ng ml^{-1}), (b) plasma containing MK-0476 (1 ng ml^{-1}), and (c) drug-free plasma.

least 19 months in human plasma at −70°C, and MK-0476 in plasma was stable after three repeated and freezing and thawing cycles [3].

Table 1
Intra-day coefficient of variation and accuracy for the determination of MK-0476 in human plasma (n=5)

Spiked concentration (ng ml^{-1})	Coefficient of variation (%)	Accuracy (%)
500.00	0.3	97.2
100.00	2.0	108.2
50.00	0.9	114.6
10.00	0.9	113.0
2.00	1.9	105.7
1.00	9.2	110.8

3.4. Application to clinical samples

The method described here was used to determine plasma levels of MK-0476 in humans who received

Table 2
Inter-day coefficient of variation and accuracy for the determination of MK-0476in human plasma (n=5)

Spiked concentration (ng ml^{-1})	Coefficient of variation (%)	Accuracy (%)
500.00	2.3	97.2
100.00	5.8	106.6
50.00	9.2	106.8
10.00	10.4	103.5
2.00	7.5	101.4
1.00	14.1	99.4

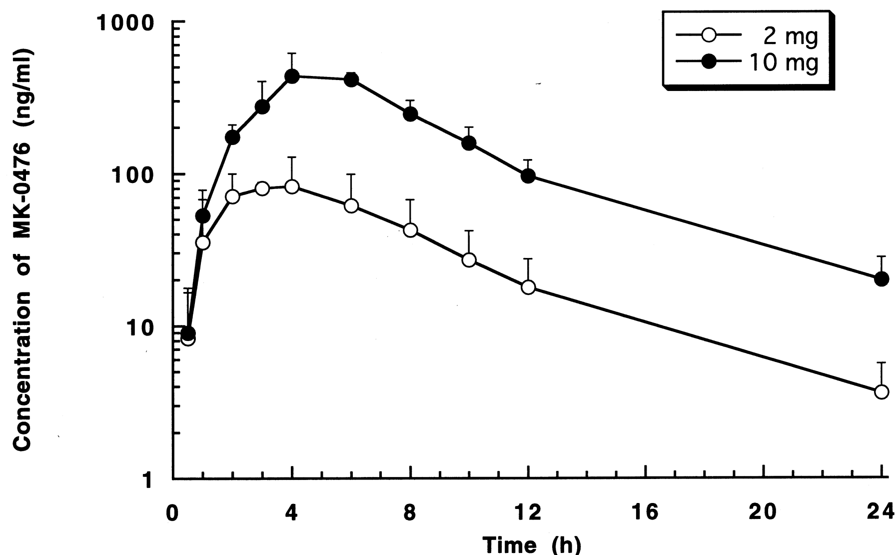

Fig. 4. Plasma concentration (mean+S.D.) of MK-0476 after single oral doses of MK-0476 (2 and 10 mg) in three healthy male volunteers.

an oral dose of MK-0476. Mean plasma concentration–time curves of MK-0476 after single oral doses of MK-0476 at dose levels of 2 and 10 mg in three volunteers are shown in Fig. 4. The mean C_{max} values for 2 and 10 mg of MK-0476 were 105 and 448 ng ml^{-1}, respectively. This method had adequate sensitivity and accuracy for the pharmacokinetic study of MK-0476 in plasma after oral administration in humans.

4. Conclusion

A selective and highly sensitive, direct injection column-switching HPLC method was developed for the quantitation of MK-0476 in human plasma. The method was simple, sensitive and suitable for the determination of photo-sensitive compounds. It was not necessary to use an internal standard because both accuracy and precision were adequate due to the simple sample handling process. This method did not cause any analyte loss during the determination, and only 100 μl of plasma was needed for the determination. The quantitation limit (1 ng ml^{-1}) was adequate for the determination of MK-0476 in human plasma after oral administration of MK-0476.

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