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High Performance Liquid Chromatographic Analysis of a New Proton Pump Inhibitor KR60436 and Its Active Metabolite *O*-Demethyl-KR60436 in Rat Plasma Samples Using Column-Switching

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A fully automated high performance liquid chromatography with column-switching was developed for the simultaneous determination of KR60436, a new reversible proton pump inhibitor, and its active metabolite *O*-demethyl-KR60436 from rat plasma samples. Plasma sample (50 μl) was directly introduced onto a Capcell Pak MF Ph-1 column (10 × 4 mm I.D.) where primary separation was occurred to remove proteins and concentrate target substances using acetonitrile-potassium phosphate (pH 7, 0.1 M) (2:8, v/v). The drug molecules eluted from MF Ph-1 column were focused in an intermediate column (10 × 2 mm I.D.) by the valve switching step. The substances enriched in intermediate column were eluted and separated on a Vydac 218MR53 column (250 × 3.2 mm I.D.) using acetonitrile-potassium phosphate (pH 7, 0.02 M) (47:53, v/v) at a flow rate of 0.5 ml/min when the valve status was switched back to A position. The method showed excellent sensitivity (detection limit of 2 ng/ml) with small volume of samples (50 μl), good precision and accuracy, and speed (total analysis time 24 min) without any loss in chromatographic efficiency. The response was linear ($r^2 \geq 0.999$) over the concentration range of 5-500 ng/ml.

Key words: KR60436, *O*-demethyl-KR60436, HPLC, Rat plasma, Column-switching

INTRODUCTION

KR-60436, 1-(2-methyl-4-methoxyphenyl)-4-[(2-hydroxyethyl)amino]-6-trifluoromethoxy-2,3-dihydropyrrolo[3,2-c] quinoline (Fig. 1a) was synthesized in Korea Research Institute of Chemical Technology (KRICT, Taejeon, Korea) in order to develop a new anti-ulcer drug. This acylquinoline derivative having a reversible H^+/K^+ ATPase inhibitory activity was found to be more effective against *in vivo* gastric acid secretion (ED_{50}=10 mg/kg) and ethanol-induced gastric lesion (ED_{50}=10 mg/kg) compared to SK&F 96067, the prototype of reversible proton pump inhibitor as well as other acylquinoline derivatives (Pope and Parsons, 1993).

In the *in vitro* studies with rat and human liver microsomes, KR60436 was extensively metabolized to produce at least seven metabolites (Lee *et al.*, 2000a). O-demethyl-KR60436,1-(2-methyl-4-hydroxyphenyl)-4-[(2-hydroxyethyl) amino]-6-trifluoromethoxy-2,3-dihydropyrrolo[3,2-c]quinoline (Fig. 1b), has the similar H^+/K^+ ATPase inhibitory activity to KR60436 (personal communication). The simultaneous determination of KR60436 and its active metabolite O-demethyl-KR60436 was necessary for the evaluation of the KR60436 pharmacokinetics.

To speed up process and reduce sample preparation efforts, the fast assays using column-switching HPLC have been published for the quantitation of drugs and meta-

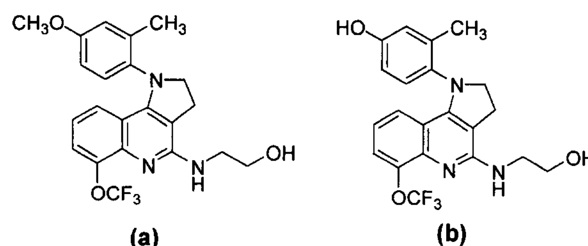

Fig. 1. Structures of KR60436 (a) and *O*-demethyl-KR60436 (b)

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bolites in rat or human plasma (Choi *et al.*, 1999; Jeong *et al.*, 2001; Lee *et al.*, 1989, 2000b). The present method describes a rapid, high-throughput, highly sensitive and a selective column-switching HPLC method for the simultaneous determination of KR60436 and O-demethyl-KR60436 in rat plasma samples. The applicability of the method was proved in the study of the pharmacokinetics of KR60436 and its active metabolite O-demethyl-KR60436 in rats after a single intravenous or oral administration of KR60436.

MATERIALS AND METHODS

KR60436 and O-demethyl-KR60436 were synthesized by KRICT. HPLC grade methanol and acetonitrile were purchased from Burdick & Jackson, Inc. (Muskegon, MI, USA). Stock solutions of KR60436 and O-demethyl-KR60436 were prepared by dissolving in acetonitrile (1 mg/ml) and aliquots were spiked to drug-free rat blank plasma to obtain the calibration plasma standards at six concentrations of 5, 10, 50, 100, 200, and 500 ng/ml. Plasma samples were filtered with low protein binding membrane syringe filter (0.22 μm, PVDF, Millipore, Bedford, MA, USA) before HPLC injection.

Column-switching system and HPLC conditions

The configuration of the column-switching system using triple columns was shown in Fig. 2 and consisted of the Nanospace SI-1 series (Shiseido, Tokyo, Japan), i.e., two 2001 pumps, a 2013 fluorescence detector, a 2003 autosampler, a 2004 column oven, a 2012 high pressure switching valve, and a 2009 degassing unit. The system was operated by Syscon (Shiseido) and the signals were processed by S-MicroChrom (Shiseido).

In order to remove proteins and concentrate KR60436 and O-demethyl-KR60436 in plasma samples, plasma was pre-separated on Capcell Pak MF Ph-1 cartridge (10 × 4 mm I.D., Shiseido) using 1.0 ml/min of acetonitrile-potassium phosphate (pH 7, 0.1 M) (2:8, v/v). The drug molecule fractions from primary separation were transferred to an intermediate column (Capcell Pak C_{18} UG 120, 10 × 2 mm I.D.) and the final separation was performed on Vydac 218MR53 column (5 μm, 250 × 3.2 mm I.D., Vydac, USA) using acetonitrile-potassium phosphate (pH 7, 0.02 M) (47:53, v/v) at a flow rate of 0.5 ml/min. The column temperature was 30°C and the effluent was monitored with fluorescence (excitation at 340 nm and emission at 485 nm).

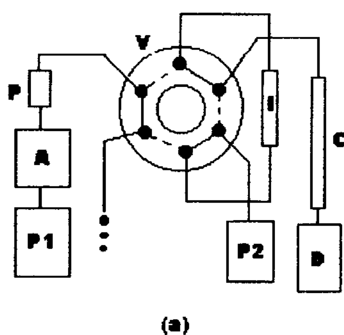

Fig. 2. The schematic diagram of triple column-switching system. A: autosampler; C; Vydac 218MR53 column; D: fluorescence detector; I: Capcell Pak C18 UG120 (10 × 2 mm I.D.); P1: pump 1; P2: pump 2; P: Capcell Pak MF Ph-1 (10 × 4 mm I.D.); V: switching valve. —: position A, ---: position B

Analytical procedure

Step 1 (0-2.6 min, valve position A): Plasma sample (50 μl) filtered with 0.22 μm membrane filter was introduced onto a Capcell Pak MF Ph-1 column where plasma proteins, KR60436 and O-demethyl-KR60436 were separated using acetonitrile-potassium phosphate (pH 7, 0.1 M) (2: 8, v/v) at a flow rate of 1.0 ml/min. The intermediate column and analytical column were equilibrated using the mobile phase.

Step 2 (2.6-6.4 min, valve position B): When the valve status was changed to B, target drug-containing zone separated in Capcell Pak MF Ph-1 precolumn was focused onto the top of an intermediate C_{18} column using acetonitrile-potassium phosphate (pH 7, 0.1 M) (2:8, v/v) at a flow rate of 0.5 ml/min. The analytical column was equilibrated using the mobile phase.

Step 3 (6.4-24 min, valve position A): The analytes trapped in the intermediate C_{18} column were transferred to a Vydac 218MR53 column and separated by using 0.5 ml/min of acetonitrile-potassium phosphate (pH 7, 0.02 M) (47:53, v/v) when the valve status was switched back to the A position. In the meanwhile, the MF Ph-1 column was equilibrated with a washing solvent.

Method validation

Limit of quantitation (LOQ) for KR60436 and O-demethyl-KR60436 was determined as the concentration of drug giving a signal to noise ratio greater than 5:1. Six KR60436 and O-demethyl-KR60436-spiked plasma standard samples over the concentration range of 5-500 ng/ml were quantified to evaluate the recovery, linearity, precision [the coefficient of variation (C.V.) of replicate analysis] and accuracy (the bias between theoretical and actual concentration).

Animal experiments

A 10 mg/kg oral dose of KR60436 dissolved in 50% transcutol was administered to male Sprague-Dawley rats (200-250 g). Following dose, blood samples (approximately 250 μl each) were withdrawn from the jugular vein at 0, 1, 3, 10, 15, 30 and 45 min and 1, 1.5, 2, 3,

4, 6, 8, 12 and 24 h. Plasma samples were then harvested by centrifugation (3000 rpm, 10 min, 4°C) and were stored at -20°C until drug analysis.

RESULTS AND DISCUSSION

The efficiency of different bonded stationary phase including a Capcellpak UG C_{18}, Capcellpak phenyl, and Vydac 218MR53 column in the simultaneous determination of KR60436 and *O*-demethyl-KR60436 was evaluated. In octadecyl and phenyl column, the retention of KR60436 increased severely but the retention of *O*-demethyl-KR60436 was not affected compared to Vydac 218MR53 column. The Vydac 218MR53 column was chosen for the simultaneous determination of KR60436 and O-demethyl-KR60436 in plasma because of excellent resolution, short analysis time, selectivity and good sensitivity (Fig. 3). The increase in pH of the mobile phase from 2.0 to 7.0 increased the retention times of KR60436 and *O*-demethyl-KR60436, and therefore, the mixture of acetonitrilepotassium phosphate (pH 7.0, 0.02 M) (47:53, v/v) was used as the mobile phase. The use of fluorescence detection resulted in many advantages such as increased sensitivity (100 pg) and specificity over the UV detection.

Column-switching technique is on-line trace enrichment system that can directly analyze biological samples in the hundreds of microliters without any pre-purification steps, loss in the sensitivity increase and chromatographic efficiency (Choi *et al.*, 1999; Jeong *et al.*, 2001; Lee *et al.*, 1989, 2000b). To establish the column-switching system for the simultaneous determination of KR60436 and O-demethyl-KR60436 from plasma, the choice of precolumn, washing solvent and valve-switching time must be considered.

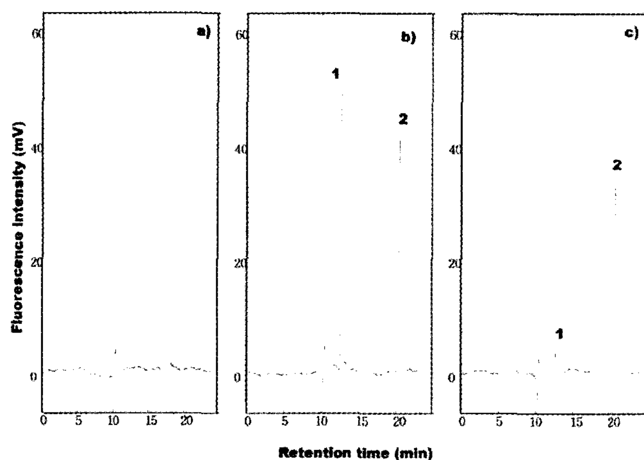

Fig. 3. Chromatograms of (a) blank plasma, (b) blank plasma containing 50 ng/ml of KR60436 and O-demethyl-KR60436, and (c) plasma sample obtained after a 10 mg/kg KR60436 oral dose. Peaks: 1=O-demethyl-KR60436 (t_R= 12.6 min), 2=KR60436 (t_R=20.4 min).

Capcell Pak MF Ph-1 precolumn was appropriate to remove proteins and concentrate KR60436 and *O*-demethyl-KR60436 from plasma because the MF Ph-1 phase consists of hydrophilic polyoxyethylene groups and hydrophobic phenyl groups bonded on the surface of 80A silica. KR60436 and *O*-demethyl-KR60436 are basic drugs, and therefore, the retention of them on MF Ph-1 column increased at higher pH. The mixture of acetonitrile and potassium phosphate (pH 7, 0.1 M) was appropriate for the deproteinization and analyte fractionation to obtain good recovery and clean chromatogram within relatively short time. To determine the appropriate time for column-switching, the separation profile of KR60436 and *O*-demethyl-KR60436 in plasma on MF Ph-1 column (10 × 4 mm I.D.) was evaluated using acetonitrile-potassium phosphate (pH 7, 0.1 M) (2:8, v/v).

The analytes (equivalent to 1.9 ml volume) isolated from MF Ph-1 by the valve switching step were focused in the top of intermediate C_{18} column (10 ×2 mm I.D.) to obtain sharp peaks in the final separation and protect MF Ph-1 column from high pressure as well as the blockage due to high concentration of acetonitrile.

Typical chromatograms of blank plasma, plasma spiked with KR60436 and *O*-demethyl-KR60436, and plasma sample obtained after an oral adminsitration of 10 mg/kg KR60436 are shown in Fig. 3. The retention times of O-demethyl-KR60436 and KR60436 were 12.6 and 20.4 min, respectively. Chromatographic separation was excellent, with no interfering peaks from endogenous plasma constituents.

MF Ph-1 precolumn was exchanged after injection of 60 plasma samples (equivalent to 3.0 ml plasma). The intermediate and main columns showed no decrease in efficiency after more than 500 injections of plasma samples.

Mean recoveries of KR60436 and O-demethyl-KR60436 from plasma samples were 96.5 ± 2.4 % and 95.2 ± 2.1 %, respectively. The calibration curves of peak areas versus the concentrations of KR60436 and O-demethyl-KR60436 in plasma were linear giving a correlation coefficient of 0.999 in the range of 5-500 ng/ml. LOQ of KR60436 and O-demethyl-KR60436 was 2 ng/ml using 50 μl plasma. The intra- and inter-day precision and accuracy of the assay were shown in Table I. Actual concentrations were deviated from 2.0 % to 2.0 % of the theoretical concentrations in the spiked plasma samples and the assay was precise because C.V. was less than 6.2 %.

The suitability of this method was demonstrated in the study of the pharmacokinetic disposition of KR60436 in male rats. Fig. 4 shows mean plasma concentration-time profiles of KR60436 and its active metabolite O-demethyl-KR60436 in rats after a single oral administration of KR60436 to rats. The mean elimination half-life ($t_{1/2}$), maximum plasma concentration (C_{max}) and time to maximum plasma concentration (t_{max}) of KR60436 were 7.4 h, 108 ng/ml and 0.5 h, respectively. The mean t_{max} and

Table I. Reproducibility of KR60436 and O-demethyl-KR60436 in rat plasma samples (n=6)

Theoretical concentration (ng/ml)	concentration found (ng/ml)		C.V. (%)	
	KR60436	O-demethyl-KR60436	KR60436	O-demethyl-KR60436
Intra-day				
5	5.0	5.0	3.6	4.7
10	10.2	10.1	1.2	3.2
50	50.4	50.1	1.5	2.4
100	100.8	100.3	1.2	1.9
200	200.7	200.3	0.6	1.2
500	502.7	500.1	1.1	0.9
Inter-day				
5	4.9	5.0	3.4	4.5
10	9.9	10.1	4.0	6.2
50	49.8	49.5	2.3	3.9
100	101.3	100.4	4.1	4.9
200	200.9	199.7	2.3	2.4
500	501.4	503.2	1.9	1.5

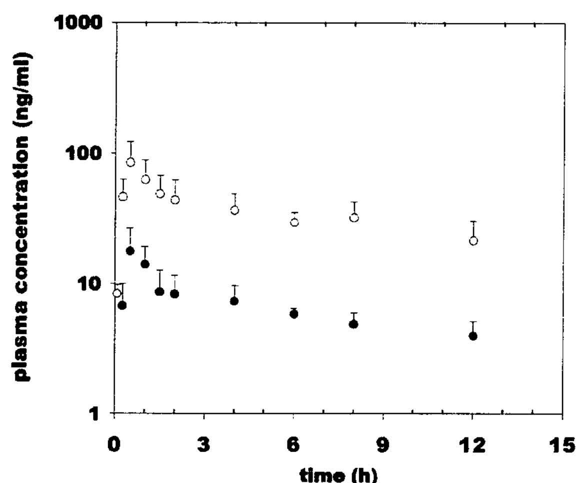

Fig. 4. Plasma concentration-time profiles of *O*-demethyl-KR60436 (●) and KR60436 (○) following a single oral dose of KR60436 (10 mg/kg) to male rats. Each point with vertical bar represents the mean and standard deviation of five rats.

C_{max} of O-demethyl-KR60436 were 0.7 h and 24 ng/ml, respectively, and the ratio of area under the plasma concentration time curve (AUC) of *O*-demethyl-KR60436 to AUC of KR60436 was 0.20, indicating that KR60436 was rapidly and extensively metabolized to the active metabolite, *O*-demethyl-KR60436.

In summary, an automated column-switching HPLC method with fluorescence detection has been developed for the simultaneous determination of KR60436 and O-demethyl-KR60436 from rat plasma samples. This assay method showed the excellent sensitivity (2 ng/ml), reproducibility, specificity, and speed (total analysis time 24 min) using small sample volume (50 µl).

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