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Simultaneous determination of aceclofenac and diclofenac in human plasma by narrowbore HPLC using column-switching

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Abstract

A fully automated narrowbore high performance liquid chromatography (HPLC) with column-switching was developed for the simultaneous determination of aceclofenac and diclofenac from human plasma samples. Plasma sample (100 μl) was directly introduced onto a Capcell Pak MF Ph-1 column (20 × 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) (14:86, v/v). The drug molecules eluted from MF Ph-1 column were focused in an intermediate column (35 × 2 mm I.D.) by the valve switching step. The substances enriched in intermediate column were eluted and separated on the narrowbore phenyl–hexyl column (100 × 2 mm I.D.) using acetonitrile–potassium phosphate (pH 7, 0.02M) (33:67, v/v) when the valve status was switched back to A position. The method showed excellent sensitivity (detection limit of 10 ng ml^{-1}) with small volume of samples (100 μl), good precision and accuracy, and speed (total analysis time 17 min) without any loss in chromatographic efficiency. The response was linear ($r^2 \geq 0.999$) over the concentration range of 50–10000 ng ml^{-1}.

Keywords: Aceclofenac; Diclofenac; Narrowbore HPLC; Human plasma; Column-switching

1. Introduction

Aceclofenac, 2-[(2,6-dichlorophenyl)amino]-phenylacetoxyacetic acid; (Scheme 1) is a new potent non-steroidal anti-inflammatory and analgesic drug of phenylacetic acid group used in the symptomatic treatment of pain and inflammatory or degenerative anthropathies with better gastric tolerance [1–4]. Aceclofenac is metabolized to 4′-hydroxyaceclofenac, diclofenac, and 4′-hydroxydiclofenac in rat, monkey and human [5–7]. Diclofenac, 2-[(2,6-dichlorophenyl)amino]phenylacetic acid, possesses similar anti-inflammatory and analgesic properties [1–3].

There was a high performance liquid chromatographic (HPLC) method for the simultaneous de-

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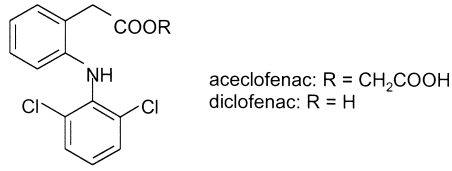
termination of aceclofenac, diclofenac and other metabolites in biological samples [5,6]. There are two techniques for the determination of aceclofenac in preparations: stripping voltammetry [8] and spectrometric and spectrofluorometric method [9]. Many analytical methods for the determination of diclofenac or together with its metabolites in biological fluids are based on gas chromatography (GC) [10,11], GC-mass spectrometry (GC-MS) [12–14], HPLC [15–22], and spectrometric methods [23]. Most of these methods involve laborious, time-consuming and tedious liquid–liquid extraction [5,6,10–13,15,18,20,21] or solid-phase extraction [14,17,19,22] as sample preparation procedures. In the previous paper [16], we described the column-switching HPLC method for the analysis of diclofenac in plasma.

For the simultaneous determination of aceclofenac and its active metabolite diclofenac from human plasma samples, the present method shows the advantages including increased concentration sensitivity, easier sample treatment and short total analysis time (17 min) by using semi-micro-columns, polymer-coated mixed function phase (MF Ph-1) and column-switching [24–29]. The applicability of the method was proved in the study of the pharmacokinetics of aceclofenac and its active metabolite diclofenac in human after a single oral administration of aceclofenac tablet.



Scheme 1. Aceclofenac, 2-[(2,6-dichlorophenyl)amino]phenylacetoxyacetic acid.

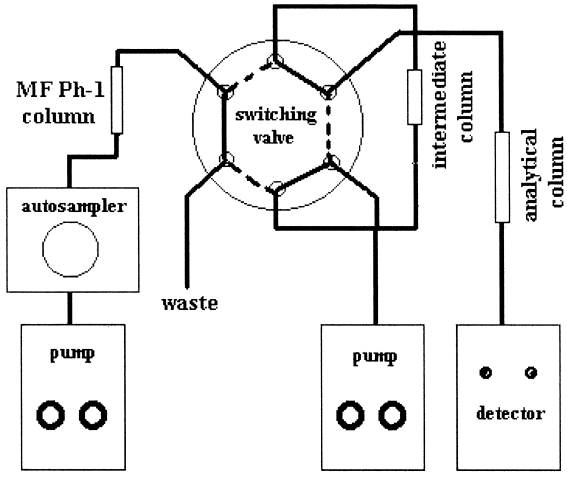


Fig. 1. Triple column system for the column-switching in semi-micro LC. Position A (– – –); Position B (-----).

2. Experimental

2.1. Materials and reagents

Diclofenac and aceclofenac were obtained from Young Poong Pharm. Co. (Incheon, Korea) and Daewoong Pharm. Co. (Seoul, Korea), respectively. HPLC grade methanol and acetonitrile were purchased from Burdick & Jackson, Inc. (Muskegon, MI, USA). Stock solutions of aceclofenac and diclofenac were prepared by dissolving in methanol (1 mg ml^{-1}) and aliquots were spiked to drug-free human blank plasma to obtain the calibration plasma standards at six concentrations of 50, 100, 500, 1000, and 5000, 10 000 ng ml^{-1}. Plasma samples were filtered with low protein binding membrane syringe filter (0.22 μm, PVDF, Millipore, Bedford, MA, USA) before HPLC injection.

2.2. Column-switching system and HPLC conditions

The configuration of the column-switching system using three columns was shown in Fig. 1 and consisted of the Nanospace SI-1 series (Shiseido, Tokyo, Japan), i.e. two 2001 pumps, a 2002 UV-VIS 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 aceclofenac and diclofenac from plasma samples, a large volume of plasma was pre-separated on Capcell Pak MF Ph-1 cartridge (20 × 4 mm I.D., Shiseido) using 0.5 ml min^{-1} of acetonitrile–

potassium phosphate (pH 7, 0.1 M) (14:86, v/v). The drug molecule fractions from primary separation were transferred to an intermediate column (Capcell Pak C_{18} UG 120, 35 × 2 mm I.D.) and the final separation was performed on Luna 2 Phenyl–hexyl column (3 μm, 100 × 2 mm I.D., Phenomenex, CA, USA) using acetonitrile–potassium phosphate (pH 7, 0.02 M) (33:67, v/v) at a flow rate of 0.2 ml min^{-1}. The column temperature was 30°C and the effluent was monitored at 278 nm.

2.3. Analytical procedure

Step 1 (0–6.0 min, valve position A): Plasma sample (100 μl) filtered with 0.2 μm membrane filter was introduced onto a Capcell Pak MF Ph-1 column where plasma proteins, aceclofenac and diclofenac were separated using acetonitrile–potassium phosphate (pH 7, 0.1 M) (14:86, v/v) at a flow rate of 0.5 ml min^{-1}. The intermediate column and analytical column were equilibrated using the mobile phase.

Step 2 (6.0–8.8 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) (14:86, v/v) at a flow rate of 0.5 ml min^{-1}. The analytical column was equilibrated using the mobile phase.

Step 3 (8.8–17 min, valve position A): The analytes trapped in the intermediate C_{18} column were transferred to a narrowbore phenyl–hexyl column and separated by using 0.2 ml min^{-1} of acetonitrile–potassium phosphate (pH 7, 0.02 M) (33:67, 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.

2.4. Method validation

Limit of detection (LOD) for aceclofenac and diclofenac was determined as the concentration of drug giving a signal to noise ratio greater than 3:1. Six aceclofenac and diclofenac-spiked plasma standard samples over the concentration range of 50–10 000 ng ml^{-1} were quantified to evaluate the recovery, linearity, precision [the coefficient of variation (CV) of replicate analysis] and accuracy (the bias between theoretical and actual concentration).

2.5. Application of the present method to human plasma samples

Two healthy male volunteers received a single oral dose of aceclofenac tablet (100 mg). Blood samples (2 ml) were withdrawn from the forearm vein at 0.5, 1.0, 1.5, 2, 3, 4, 6, 9 and 12 h post dosing, transferred to Vacutainer tubes and centrifuged. Following centrifugation (3000 rpm, 15 min, 4°C), plasma samples were transferred to eppendorf tubes and stored at −70°C prior to analysis. Drug concentrations were determined as the mean of duplicate samples. The peak concentration (C_{max}) and the time to peak concentration (T_{max}) of aceclofenac and its active metabolite, diclofenac were determined by visual inspection from each volunteer's plasma concentration-time plots for aceclofenac and diclofenac, respectively. Area under the plasma concentration-time curves (AUC) was calculated by the linear trapezoidal method from 0 to 12 h. Plasma elimination half-life ($t_{1/2}$) of aceclofenac and diclofenac was determined from the descending slope of the concentration-time profiles after logarithmic transformation of the concentration data.

3. Results and discussion

3.1. Chromatography and column-switching procedure

The efficiency of different bonded stationary phase including octadecyl, octyl, and phenyl–hexyl in the simultaneous determination of aceclofenac and diclofenac was evaluated. In octadecyl and octyl column, the retention of aceclofenac increased severely but the retention of diclofenac was not affected compared to phenyl–hexyl column. The phenyl–hexyl column was chosen for the simultaneous determination of aceclofenac and diclofenac in plasma because of

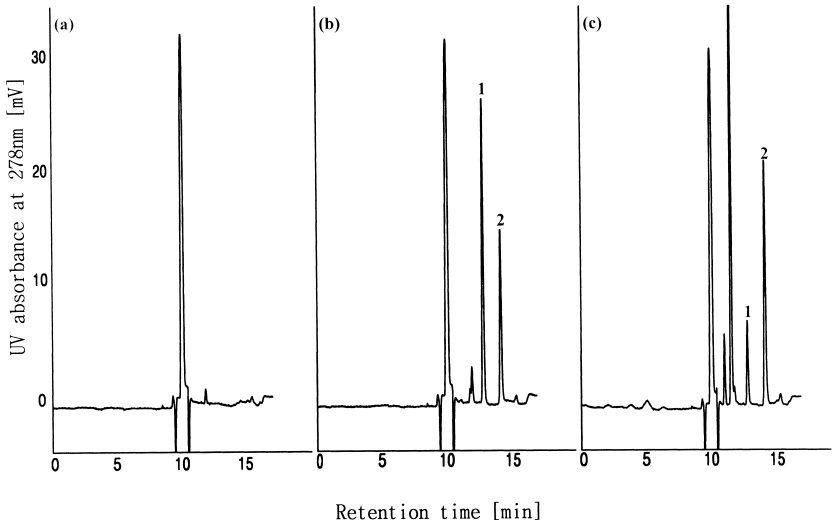


Fig. 2. Chromatograms of (a) blank plasma; (b) blank plasma spiked with aceclofenac and diclofenac (1 μg ml^{-1}), and (c) plasma sample at 3 h after an oral dosing of 100 mg aceclofenac to a volunteer. Peaks: 1, diclofenac; 2, aceclofenac.

excellent resolution, short analysis time, selectivity and good sensitivity (Fig. 2). Among other metabolites of aceclofenac, 4′-hydroxydiclofenac was not determined with the present method and 4′-hydroxyaceclofenac was suggested to be a 11.3 min peak in Fig. 2(c) but was not identified devoid of authentic standard. The use of narrow-bore column resulted in many advantages such as increased sensitivity (1 ng), high column efficiency, and lower solvent consumption over the conventional HPLC. The increase in pH of the mobile phase from 3.0 to 7.0 decreased the retention times of aceclofenac ($pKa = 4.7$) and diclofenac ($pKa = 4.0$), and therefore, the mixture of acetonitrile–potassium phosphate (pH 7.0, 0.02 M) (33:67, v/v) was used as the mobile phase.

Column-switching technique is a useful sample preparation system that can directly analyze biological samples in the hundreds of microliters without any loss in the sensitivity increase and chromatographic efficiency obtained by semi-microcolumns [24–29]. To establish the column-switching system for the simultaneous determination of aceclofenac and its active metabolite diclofenac from plasma, the precolumn packing material, washing solvent and valve-switching time must be chosen.

Capcell Pak MF Ph-1 precolumn was appropriate to remove proteins and concentrate aceclofenac and diclofenac from plasma because the MF Ph-1 phase consist of hydrophilic polyoxyethylene groups and hydrophobic phenyl groups bonded on the surface of 80A silica [24]. Aceclofenac and diclofenac are acidic drugs, and therefore, the retention of them on MF Ph-1 column increased at lower pH (Fig. 3). The mixture of acetonitrile and potassium phosphate (pH 7, 0.1 M) was

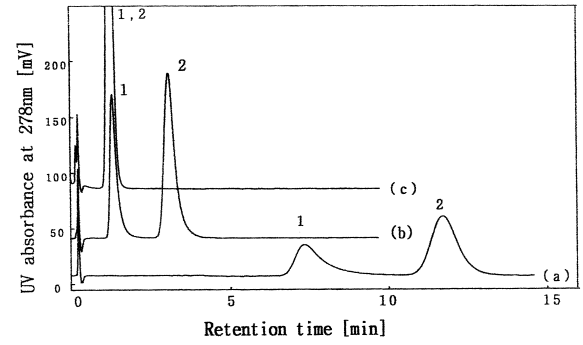


Fig. 3. Effect of pH of mobile phase on the retention behavior of aceclofenac and diclofenac on MF Ph-1 column (20 × 4 mm I.D.): (a) pH 3.0; (b) pH 4.5 and (c) pH 7.0. Peaks: 1, diclofenac; 2, aceclofenac. Conditions: mobile phase: acetonitrile-potassium phosphate (0.1 M) (2:8, v/v), flow-rate: 1.0 ml min^{-1}, injection volume: 100 μl.

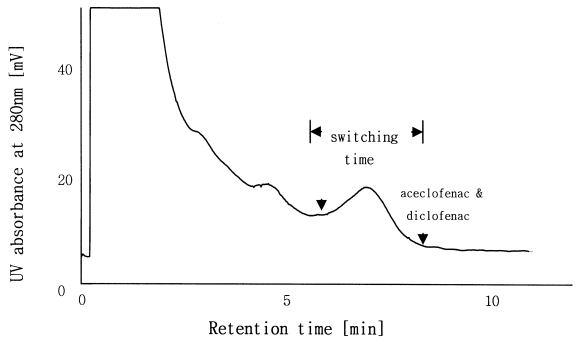


Fig. 4. Separation of aceclofenac and diclofenac-spiked plasma on MF Ph-1 column (20 × 4 mm I.D.). Conditions: mobile phase: acetonitrile-potassium phosphate (pH 7, 0.1 M) (14:86, v/v), flow-rate: 0.5 ml min^{-1}, injection volume: 100 μl

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 aceclofenac and diclofenac in plasma on MF Ph-1 column (20 × 4 mm I.D.) was evaluated using acetonitrile–potassium phosphate (pH 7, 0.1 M) (14:86, v/v) (Fig. 4).

The analytes (equivalent to 1.4 ml volume) isolated from MF Ph-1 by the valve switching step were focused in the top of intermediate C_{18} column (35 × 2 mm I.D.) to obtain sharp peaks in the final separation. The use of intermediate column resulted in the protection of MF Ph-1 column from high pressure as well as the saving of analysis time, that is, the focusing time was reduced from 7 min at the flow rate of 0.2 ml min^{-1} to 2.8 min at 0.5 ml min^{-1}.

MF Ph-1 precolumn (20 × 4 mm I.D.) was exchanged after injection of 40 plasma samples (equivalent to 4.0 ml plasma). The intermediate and main column showed no decrease in efficiency after more than 250 injections of plasma samples.

3.2. Method validation

Mean recovery of aceclofenac and diclofenac from plasma samples was 90.5 ± 2.5 and 90.2 ± 2.1%, respectively. The calibration curves of peak areas versus the concentrations of aceclofenac and diclofenac in plasma were linear giving a correlation coefficient of 0.999 in the range of 50–10 000 ng ml^{-1}. LOD of aceclofenac and diclofenac was 10 ng ml^{-1} using 100 μl plasma. The intra- and inter-day precision and accuracy of the assay were shown in Table 1. Actual concentrations were deviated from −1.0 to 2.0% of the theoretical concentrations in the spiked plasma samples and the assay was precise because CV was less than 3.0%.

Table 1
Reproducibility of aceclofenac and diclofenac in human plasma samples ($n = 6$)

Theoretical concentration (ng ml^{-1})	Concentration found (ng ml^{-1})		CV (%)	
	Aceclofenac	Diclofenac	Aceclofenac	Diclofenac
Intra-day				
50	50	51	2.8	2.9
100	99	102	2.5	2.8
500	498	497	2.1	2.4
1000	992	1020	2.2	1.9
5000	5018	4950	1.6	2.2
10 000	9980	10 080	2.1	2.3
Inter-day				
50	50	50	2.4	2.8
100	99	100	3.0	2.7
500	502	505	1.8	1.9
1000	1002	1002	1.9	2.1
5000	5007	4992	2.3	2.4
10 000	10 004	10 025	1.9	2.5

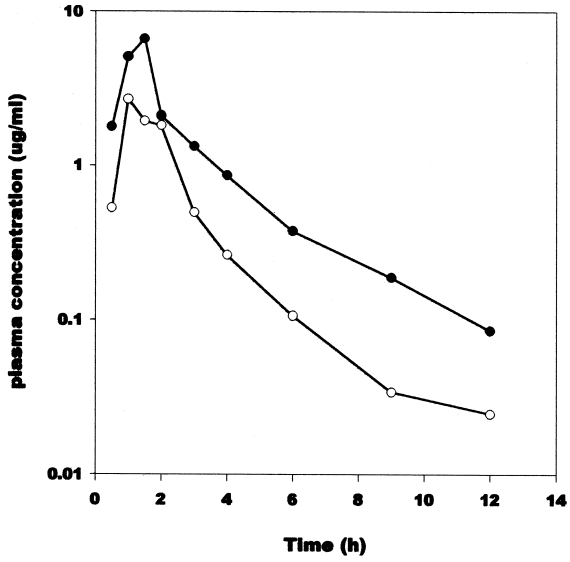


Fig. 5. Plasma concentration-time profiles of aceclofenac (●) and diclofenac (○) following a single oral administration of aceclofenac tablet (100 mg) in two male volunteers. The point represents the mean value.

3.3. Application of the present method to human plasma samples

The suitability of this method was proved in the pharmacokinetic study of aceclofenac after a single oral dosing of aceclofenac tablet (100 mg) to two healthy male volunteers. The plasma chromatogram of a human administered aceclofenac is shown in Fig. 2(c). Fig. 5 shows mean plasma concentration-time profiles of aceclofenac and its active metabolite diclofenac in two subjects. C_{max}, t_{max}, AUC and $t_{1/2}$ of aceclofenac were 6.6 ± 1.1 μg ml^{-1}, 1.5 h, 12.5 ± 1.9 μg·h ml^{-1}, and 2.3 ± 0.04 h, respectively. Aceclofenac was rapidly metabolized to the active metabolite, diclofenac (t_{max}: 1.3 ± 0.4 h) and C_{max}, AUC and $t_{1/2}$ of diclofenac were 3.0 ± 0.7 μg ml^{-1}, 5.1 ± 0.7 μg·h ml^{-1} and 2.1 ± 0.1 h, respectively.

4. Conclusion

An automated narrowbore HPLC method using column-switching has been developed for the simultaneous determination of aceclofenac and diclofenac from human plasma samples. The method shows excellent sensitivity (10 ng ml^{-1}), reproducibility, specificity, and speed (total analysis time 17 min). The suitability of the method was confirmed in the pharmacokinetic study of aceclofenac in man.

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