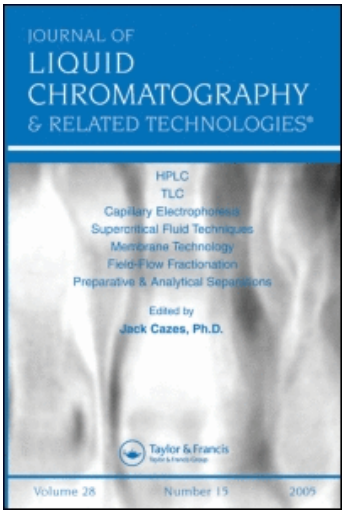

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HPLC Method for the Pharmacokinetics Study of Acemetacin in Human Plasma

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Abstract: A reproducible and specific high performance liquid chromatography (HPLC) method was developed to quantity plasma levels of acemetacin and its metabolite, indometacin in order to conduct a pharmacokinetics study. Plasma samples were prepared by liquid-liquid extraction using ethyl acetate. Extracted samples were analyzed on a reverse-phase C_{18} column with a mobile phase consisting of 20 mM phosphate buffer (pH 2.9) and acetonitrile (60:40, v/v) and UV detection at 254 nm. Using this method, the pharmacokinetics study was carried out on eight volunteers after a single oral administration.

Keywords: Acemetacin, Indometacin, High performance liquid chromatography (HPLC)

INTRODUCTION

Acemetacin (ACE), [1-(p-chlorobenzoyl)-5-methoxy-2-methyl-indole-3-acetoxy]acetic acid (Fig. 1) is a non-steroidal anti-inflammatory drug (NSAID), which is used for the treatment of arthritis and rheumatoid diseases.[1,2] Acemetacin is rapidly absorbed orally and hydrolysed by liver to produce indomethacin (IND) as its major metabolite. Another acemetacin metabolite is p-chlorobenzoic acid (PCBA), which is a minor metabolite.

HPLC and thin-layer chromatography (TLC) methods have been published for the determination of ACE in biological fluids in order to

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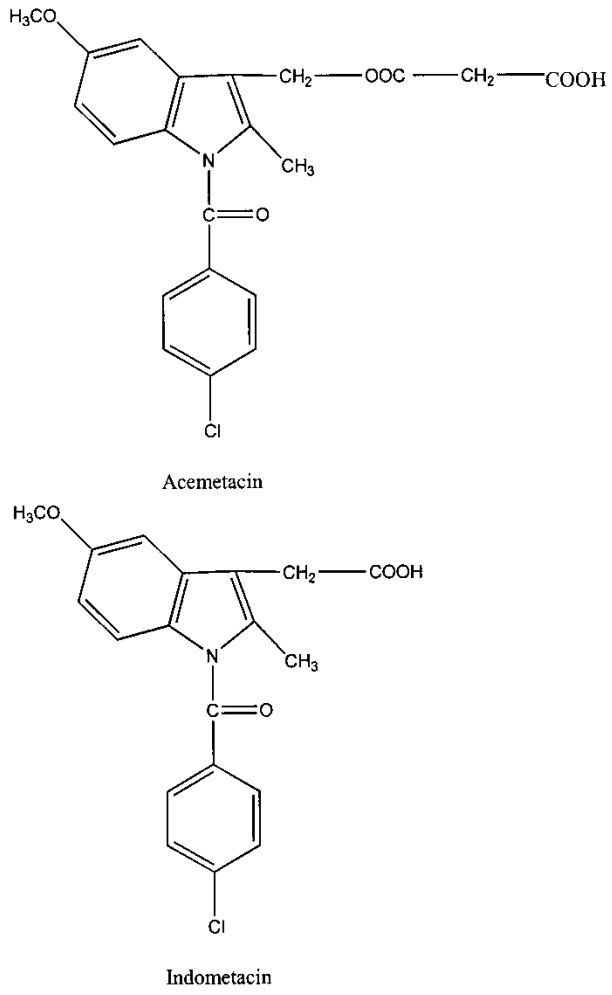

Figure 1. Chemical structures of acemetacin and indometacin.

study the pharmacokinetics of ACE.[3–5] A TLC method has the disadvantages of laborious and time-consuming steps, as well as high detection limits of 50 μg/mL.[3] HPLC methods have been reported in order to achieve higher sensitivity with less tedious sample handling, and showed sensitive and rapid analysis of ACE and its metabolites in plasma samples.[4,5] However, in our laboratory, these HPLC methods showed insufficient specificity and extraction to efficiently analyze ACE, IND, and internal standard (IS) simultaneously in plasma.

Therefore, there was a need to develop and validate a specific and reliable HPLC method for simultaneous determination of ACE and IND in human plasma for pharmacokinetic studies. We developed and validated a precise,

specific, and reproducible HPLC assay. Also, this method was used to apply the pharmacokinetic study of ACE and IND in human plasma after a single administration.

EXPERIMENTAL

Chemicals and Reagents

Standard acemetacin was obtained from Yu Han Pharmaceutical Co., Ltd. (Seoul, South Korea). Flurbiprofen, as internal standard, was provided by Sam IL Pharm. Co., Ltd. (Seoul, South Korea). Standard indometacin and potassium phosphate were purchased from Sigma Chem. Co. (St. Louis, MO, USA). Cetacin capsules (60 mg) were obtained from Hanyoung Pharm. Co. Ltd. (Seoul, South Korea). Acetonitrile and methanol were purchased from J.T. Baker (Phillipsburg, NJ, USA). All other reagents and solutions were either HPLC or analytical grade.

Standard Solutions Preparation

Stock solutions of 10 mg/mL acemetacin (ACE), indometacin (IND), and flurbiprofen as an IS, were prepared by dissolving in methanol. The analytical standards of ACE and IND were prepared by serially diluting the stock solution with mobile phase concentrations of 5, 10, 25, 50, and 400 μg/mL for ACE and IND. Also, the stock solution of IS was diluted with mobile phase to yield 25 μg/mL internal standard working solution. The stock solutions were stored at −20°C.

Plasma Samples Preparation

Heparinized blood samples were centrifuged at 2000 g for 10 min at 4°C. After centrifugation, plasma samples were separated and stored at −70°C prior to analysis. To 1 mL of plasma in a 15 mL test tube, 1 mL of 100 mM phosphate buffer (pH 2.0) and 20 μL of IS (25 μg/mL) were added and extracted with 9 mL of ethyl acetate, vortexed for 5 s, and centrifuged at 3000 g for 10 min. The supernatant was transferred to another clean glass tube and evaporated under a steam of nitrogen gas at 30°C. The residue was reconstituted by 125 μL of mobile solution, and 80 μL of the solution was injected into the HPLC system.

Chromatographic Conditions

ACE and IND were determined by HPLC with UV detection. The HPLC apparatus used was a Waters 2690 Separation Module (Alliance) system

with a Waters 2487 Dual λ Absorbance detector and Millenium[33] Chromatography Software (Waters, Milford, MA, USA). Chromatographic separation was performed using a Shiseido Capcell Pak C_{18} reversed phase column (4.6 × 150 mm, 5 μm, Shiseido, Tokyo, Japan). The plasma samples were separated by isocratic elution of the mobile phase consisting of 20 mM phosphate buffer (pH 2.9) and acetonitrile (60:40, v/v), at 1 mL/min, at room temperature. The eluents were monitored with a UV detector at 254 nm.

Validation of Assay Method

Specificity

The interference of endogenous compounds was assessed by analyzing standard ACE, IND, and IS drug-free plasma, plasma spiked with ACE, IND, and IS, and plasma obtained from subjects given ACE.

Sensitivity

The limit of quantification was defined as the low concentration yielding a precision less than 20% (relative standard deviation, RSD) and accuracy between 80 and 120% of the theoretical value.

Linearity

The linearities of calibration curves for ACE and IND were assessed in the range of 100–4000 ng/mL in plasma samples. Standard samples were prepared by adding ACE and IND to blank plasma at concentrations of 100, 200, 500, 1000, 2000, and 4000 ng/mL with 20 ng/mL IS, and these were extracted and analyzed as described above. Peak area ratios of each ACE and IND to IS were measured and the calibration curves were obtained from the least-squares linear regression (no weighing factor) presented with their correlation coefficients. The regression lines were used to calculate the respective concentrations of ACE and IND in the plasma samples from volunteers.

Precision and Accuracy

The inter-and intra-assay relative standard deviations and standard mean errors were used to validate the precision and accuracy of the assay by determining standard samples of ACE and IND in plasma. For inter-day validation, five sets of control samples at four different concentrations (100–4000 ng/mL) were evaluated on five different days. The ranges of relative standard deviation were reported. For intra-day validation, five sets of controls at four different drug concentrations were assayed with one standard curve on the same run. The range of the relative standard deviation was also

reported. Accuracy was determined by comparing the calculated concentration using calibration curves to known concentration.

Extraction Efficiency

Recoveries of ACE and IND from spiked samples were determined by comparing the peak areas obtained by extraction of freshly prepared plasma at low, medium, and high concentrations, in three replicates with those found by direct injection of aqueous standard solution at equivalent concentrations.

Stability

To test the short-and long-term stability of ACE and IND, three QC samples, containing low, medium, and high concentrations, were stored under different conditions; at 4°C, room temperature, and −20°C at 6, 12, and 24 h after preparation. Moreover, the stabilities of stock solutions were tested at room temperature for 6 h in the daylight. The compounds were considered stable if the variation of assay was less than 10% of initial time response.

Subjects

The protocol of the bioavailability study was approved by the Korean Food and Drug administration. A total of 8 healthy male subjects participated in this study after signing a consent form. The subjects had an age of 24 ± 1.3 year (22–26 years), body weight of 70 ± 8.6 kg (64–86 kg), and height of 174 ± 5.1 cm (170–182 cm). Subjects with a history of drug allergies or idiosyncrasies, renal or hepatic impairment, or drug or alcohol abuse were excluded. Subjects who used medications of any kind within 2 weeks of the start or during the study were also excluded.

Administration of ACE and Sample Collection

Subjects were advised not to take any medication for 2 weeks before the study, and were orally administered a single dose (180 mg) of three Cetacin® capsules. Fasting was maintained for 4 h after the drug administration, which was administered with 240 mL of water. Subjects were provided a standard meal at 4 h (lunch) and 8 h (supper) after during administration in each treatment. Heparinized blood samples (5–10 mL) were withdrawn from the forearm vein according to the time schedule, which included a blank before drug administration and then at 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 6, 8, and 12 hr. after administration of ACE. Blood samples were transferred to a Vacutainer tube (BD, NJ, USA) and immediately separated by

centrifugation at 2000–3000 g for 10 min. After centrifugation, plasma samples were separated and stored at −70°C prior to analysis.

Pharmacokinetic Analysis

Calculation of pharmacokinetic parameters was performed using the K-BA Test 2002 program. The following parameters were assessed for the period of 0–12 hr. The maximum ACE and IND concentration (C_{max}) and the corresponding peak time (T_{max}) were determined by the inspection of the individual drug plasma concentration-time profiles. The elimination rate constants (Kel) were obtained from the least square fitted terminal log-linear portion of the plasma concentration-time profile. The elimination half-life ($T_{1/2}$) was calculated as 0.693/Kel. The area under the curves to the last measurable concentration ($AUC_{0-12\,hr}$) were calculated by the linear trapezoidal rule.

RESULTS AND DISCUSSION

Chromatography

Reproducible chromatographic separation between ACE, IND, and IS were established at 20 mM phosphate buffer (pH 2.9)-acetonitrile, (60:40, v/v) after examining various chromatographic conditions by injecting the extracted plasma sample. Typical chromatograms of human blank plasma and plasma containing ACE, IND, and IS are shown in Fig. 2. In all the chromatograms, each compound showed symmetrical peak shape and good baseline resolution. Also, plasma matrix components did not interfere with the analysis. The retention time of IS, IND, and ACE were 21.0, 25.1, and 27.3 min, respectively. It can be seen, that this method enabled simultaneous determination for in vivo studies to evaluate ACE and IND pharmacokinetics, although the retention time was slower than those reported by previous studies.

Sensitivity

The limit of quantification was determined as a concentration of drug giving a signal-to-noise ratio greater than 10 with accuracy between 80 and 120% and with a precision RSD (%) less than 20%. The LOQ was estimated to be 100 ng/mL as shown in Table 1.

Linearity

The linearity of detector response was assessed for extracted plasma standards over the range of 100–400 ng/mL. The calibration curves for ACE and IND

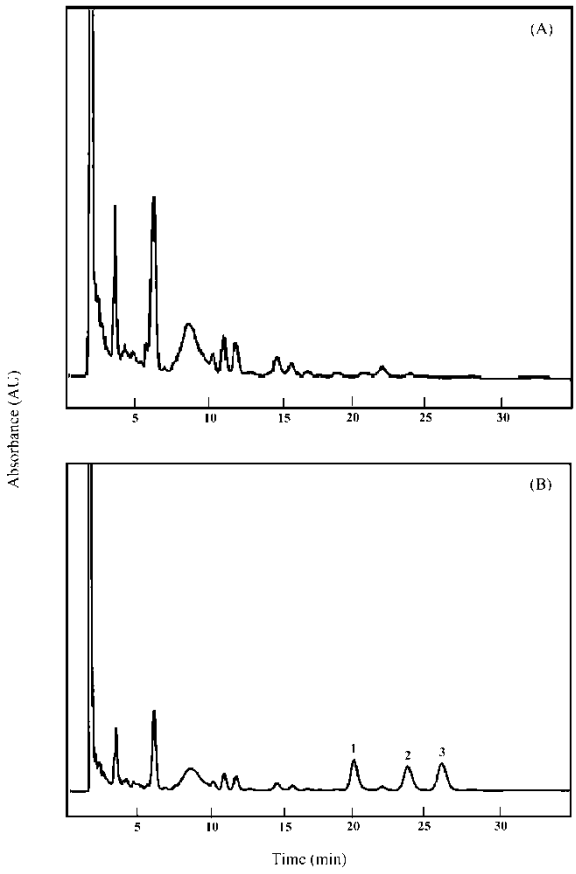

Figure 2. Typical chromatograms of (A) blank plasma and (B) blank plasma spiked with 5 μg/mL of flurbiprofen as internal standard, 1 μg/mL of acemetacin and 1 μg/mL of indometacin. Peaks: 1 = internal standard; 2 = acemetacin; 3 = indometacin.

were generated by linear regression of peak area ratios against their respective concentrations. The calibration curves showed good linearity between peak-area ratios against concentrations over the calibration ranges in plasma; $y = 0.00115\ \ x + 0.03571$, $r^2 = 0.9997$ for ACE, $y = 0.001194\ \ x + 0.034672$, $r^2 = 0.9998$ for IND.

Precision and Accuracy

The inter-day precision and accuracy for ACE and its metabolite IND were determined by analyzing plasma samples spiked at 100, 200, 1000, and 4000 ng/mL. Inter-day precision was determined by analyzing five calibration curves on five different days. The intra-day precision and accuracy was

Table 1. Assay specifications for the simultaneous determination of acemetacin and indometacin ($n = 5$)

Compound	Concentration (ng/mL)	Precision (%)		Accuracy (%)	
		Intra-day	Inter-day	Intra-day	Inter-day
Acemetacin	100	16.64	12.33	107.47	106.39
	200	3.98	6.80	98.09	107.07
	1000	10.72	7.35	100.02	97.38
	4000	14.40	13.41	100.12	100.14
Indometacin	100	15.82	19.51	104.93	106.35
	200	8.96	8.27	102.54	105.86
	1000	13.80	8.45	97.65	97.69
	4000	12.91	8.18	100.18	100.13

determined by analyzing plasma samples spiked at 100, 200, 1000, and 4000 ng/mL. The intra-day precision was determined by analyzing five replicates on the same day. The results of intra- and inter-day assay precision are expressed as relative standard deviations (RSD). The assay was reproducible with an average RSD of intra- and inter-day less than 10% except for the 100 ng/mL plasma sample. Those of 100 ng/mL were less than 20%. The accuracy of the method was higher than 90% for all the compounds at 200, 1000, and 4000 ng/mL. The results are presented in Table 1.

Recovery

Extraction of ACE and IND in plasma has been mainly reported by LLE using diethyl ether.[4–6] However, we couldn't obtain enough extraction efficiencies using ether. Therefore, we conducted several trials of extraction solvent in this study in order to obtain the optimal extraction condition. Efficient extraction of ACE and IND in plasma was achieved using ethyl acetate as extraction solvent.

Extraction efficiencies of ACE and IND were evaluated in blank plasma samples and mobile phase spiked with known amounts of the ACE, IND, and IS. Plasma samples were extracted as described above, and the recovery was calculated by comparing the peak area ratio of ACE and IND to internal standard obtained from the extracted working standard solutions in plasma and those resulting from the direct injection of the working standard solutions of ACE and IND, prepared in the mobile phase and having the same concentrations (100, 500, and 4000 ng/mL) of ACE, IND, and IS. Extraction efficiencies of ACE and IND measured in IS were higher than 90 and 87%, respectively. It is shown that this extraction method is suitable for the analysis of ACE and IND in plasma sample.

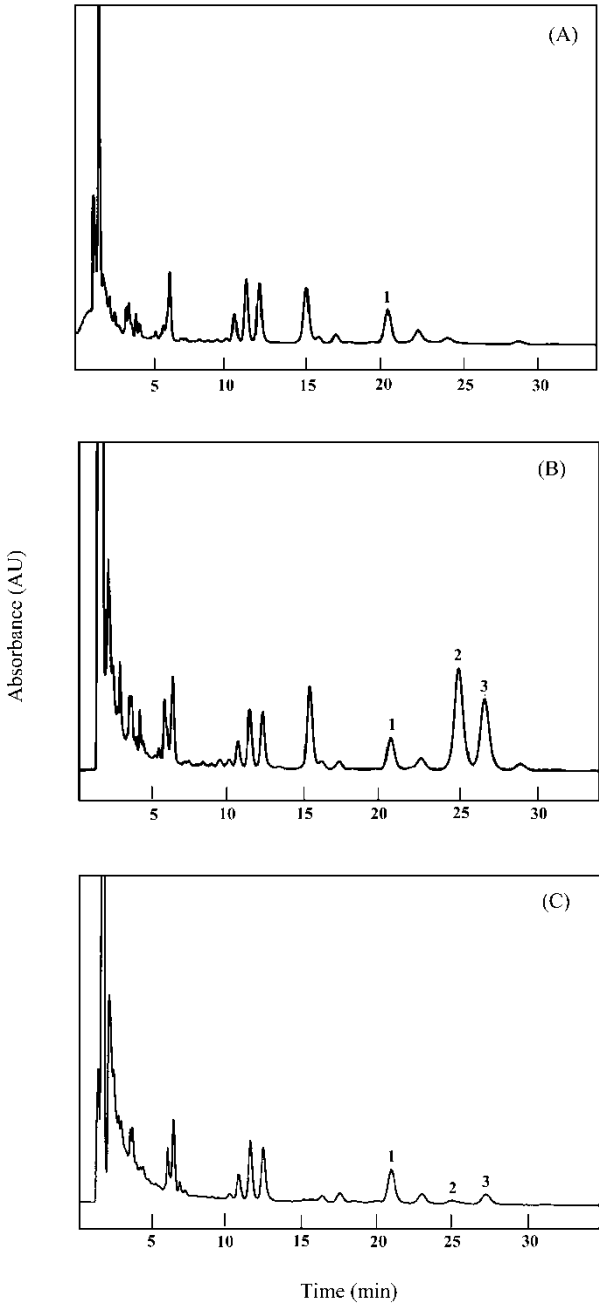

Figure 3. Chromatograms of a volunteers plasma at (A) 0, (B) 2.5, and (C) 8 hr after a single oral dose of 180 mg acemetacin. Peaks: 1 = internal standard; 2 = acemetacin; 3 = indometacin.

Stability

No degradation of ACE, IND, and IS at room temperature under daylight for 6 h was observed. Plasma samples spiked with ACE, IND, and IS showed no loss of analytes during 2 months at -70°C. In the short term stability study, they were also stable for 24 h at 4°C, room temperature and -20°C.

Pharmacokinetics of ACE and IND

This method was applied to a pharmacokinetics study of ACE capsule formulation. Eight healthy volunteers were administered a single oral dose of ACE capsules (60 mg, three capsules). Plasma samples were obtained during 12 hr after ACE administration. Figure 3 shows the change of the plasma concentration of ACE after oral administration. The human plasma concentration-time profiles of ACE and IND following oral administration of 180 mg ACE are shown in Fig. 4. ACE concentration, following oral administration of ACE tablets, was below the detection limit (100 ng/mL) in five subjects, among the eight subjects, at 6 hr, while their plasma IND level was considerably high. Concentrations of ACE were below the limit of quantitation (LOQ) at 8 hr after the administration. Therefore, ACE seems to be rapidly metabolized in the

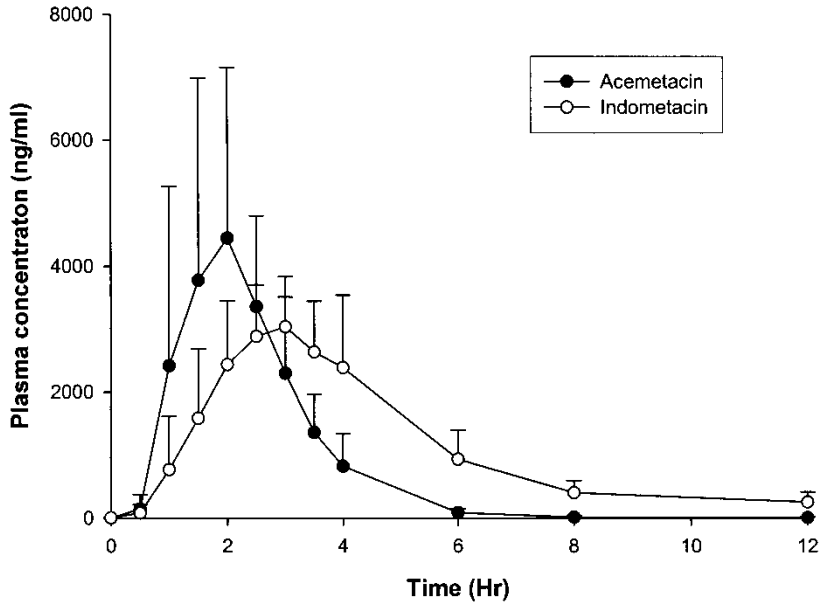

Figure 4. Plasma concentration-time profiles of indometacin and acemetacin following oral administration of acemetacin capsules (180 mg) to man. Each point represents the mean $\pm$ SD. Indometacin, n = 8; Acemetacin, $n = 8$.

Table 2. Mean pharmacokinetic parameters of acemetacin and indometacin after oral administration of acemetacin capsule (180 mg)

	Acemetacin ($n = 8$)	Indometacin ($n = 8$)
AUC ($ng \cdot min/mL$)	9192 ± 4192	12822 ± 2756
C_{max} (ng/mL)	6141 ± 1864	3578 ± 787
T_{max} (min)	108 ± 36	172 ± 48
$T_{1/2}$ (min)	59 ± 27	156 ± 48

body. Pharmacokinetic parameters are presented in Table 2. While $AUC_{0\text{-}12hr}$, C_{max}, T_{max} and $T_{1/2}$ of indometacin were $12822 \pm 2756\,ng \cdot min/mL$, $3578 \pm 787\,ng/mL$, 172 ± 48 min and 156 ± 48 min, those of ACE were $9192 \pm 4192\,ng \cdot min/mL$, $6141 \pm 1864\,ng/mL$, 108 ± 36 min and 59 ± 27 min, respectively. These values are comparable to the parameters reported by Seissiger et al.[7]

CONCLUSION

The proposed HPLC method has been shown to measure, precisely, the ACE and IND concentrations following single oral administration of ACE 180 mg capsules in human volunteers. The method has been found to be specific and reproducible. The pharmacokinetic parameters obtained using the present analytical method were found to be very much in agreement with the corresponding values cited in the literature. A simple, specific, and precise HPLC method has been developed, which is suitable for the simultaneous determination of ACE and its metabolite, IND, in plasma and it has potential for other clinical applications, including pharmacokinetic studies.

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