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ORIGINAL RESEARCH

Simultaneous determination of aceclofenac and three of its metabolites in human plasma by high-performance liquid chromatography

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ABSTRACT: Aceclofenac {[2-(2′,6′-dichlorophenyl)amino]phenylacetoxyacetic acid} is a phenylacetic acid derivative with potent analgesic and anti-inflammatory properties and an improved gastro-intestinal tolerance. In the present study, a liquid–liquid extraction-based reversed-phase HPLC method with UV detection was validated and applied for the analysis of aceclofenac and three of its metabolites (4′-hydroxy-aceclofenac, diclofenac, 4′-hydroxy-diclofenac) in human plasma. The analytes were separated using an acetonitrile–phosphate buffer gradient at a flow rate of 1 mL/min, and UV detection at 282 nm. The retention times for aceclofenac, diclofenac, 4′-hydroxy-aceclofenac, 4′-hydroxy-diclofenac and ketoprofen (internal standard) were 69.1, 60.9, 46.9, 28.4 and 21.2 min, respectively. The validated quantitation range of the method was 10–10,000 ng/mL for aceclofenac, 4′-hydroxy-aceclofenac and diclofenac, and 25–10,000 ng/mL for 4′-hydroxy-diclofenac. The developed procedure was applied to assess the pharmacokinetics of aceclofenac and its metabolites following administration of a single 100 mg oral dose of aceclofenac to three healthy male volunteers. Copyright © 2003 John Wiley & Sons, Ltd.

KEYWORDS: phenylacetic acid derivatives; diclofenac; 4′-hydroxyaceclofenac; 4′-hydroxydiclofenac; metabolic conversion; pharmacokinetics

INTRODUCTION

Over the past years, a number of new approaches have been pursued to reduce gastro-intestinal toxicity associated with long-term use of non-steroidal anti-inflammatory drugs (NSAIDs). Apart from the specific cyclooxygenase-2 (COX-2) inhibitors (Hinz and Brune, 2002; Werner *et al.*, 2002), the phenylacetic acid derivative aceclofenac, [2-(2′,6′-dichlorophenyl)amino]-phenylacetoxyacetic acid, has been launched as a potent anti-inflammatory and analgesic drug with similar efficacy and an improved gastro-intestinal tolerance compared with other NSAIDs (Pasero *et al.*, 1995; Ward *et al.*, 1995; Laudanno *et al.*, 2000; Dooley *et al.*, 2001). In contrast to the structurally similar diclofenac, aceclofenac bears an acetoacetoxy side chain.

Although aceclofenac has virtually no inhibitory activity against the enzymatic activity of the purified COX enzymes, long-term treatment of intact cells with aceclofenac has recently been shown to cause suppression of COX-2-dependent prostaglandin synthesis (Yamazaki *et al.*, 1997). However, the exact mode of action of aceclofenac in terms of inhibition of prostaglandin synthesis is far from understood, in particular with regard to the causal relation of biotransformation and pharmacodynamics (for review see Llenas, 1999). *In vivo*, aceclofenac is metabolized via cytochrome-P450-2C9-mediated hydroxylation at position 4′ or hydrolysis of the acetoacetoxy side chain to yield 4′-hydroxy-aceclofenac, diclofenac and 4′-hydroxy-diclofenac, respectively (Fig. 1; Bort *et al.*, 1996a,b). However, it is presently unknown whether the concentrations of the potent COX inhibitor diclofenac and the less active 4′-hydroxy-diclofenac, generated *in vivo* following oral administration of aceclofenac, are high enough to confer inhibition of COX-dependent prostanoid formation. To clarify this issue in pharmacokinetic studies, a sensitive and selective analytical method is needed for the simultaneous determination of aceclofenac and its metabolites 4′-hydroxy-aceclofenac, diclofenac and 4′-hydroxy-diclofenac.

Several HPLC methods have been published for the individual determination of aceclofenac (Zawilla *et al.*, 2002) and diclofenac (Hanses *et al.*, 1995) or for the simultaneous analysis of aceclofenac and diclofenac (Lee *et al.*, 2000) and diclofenac and its monohydroxylated metabolites (Landsdorp *et al.*, 1990), respectively. However, no validated analytical method has been published as yet that allows the simultaneous determination of aceclofenac and its metabolites 4′-hydroxy-

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Abbreviations used: COX, cyclooxygenase; HPLC, high-performance liquid chromatography; NSAIDs, non-steroidal anti-inflammatory drugs.

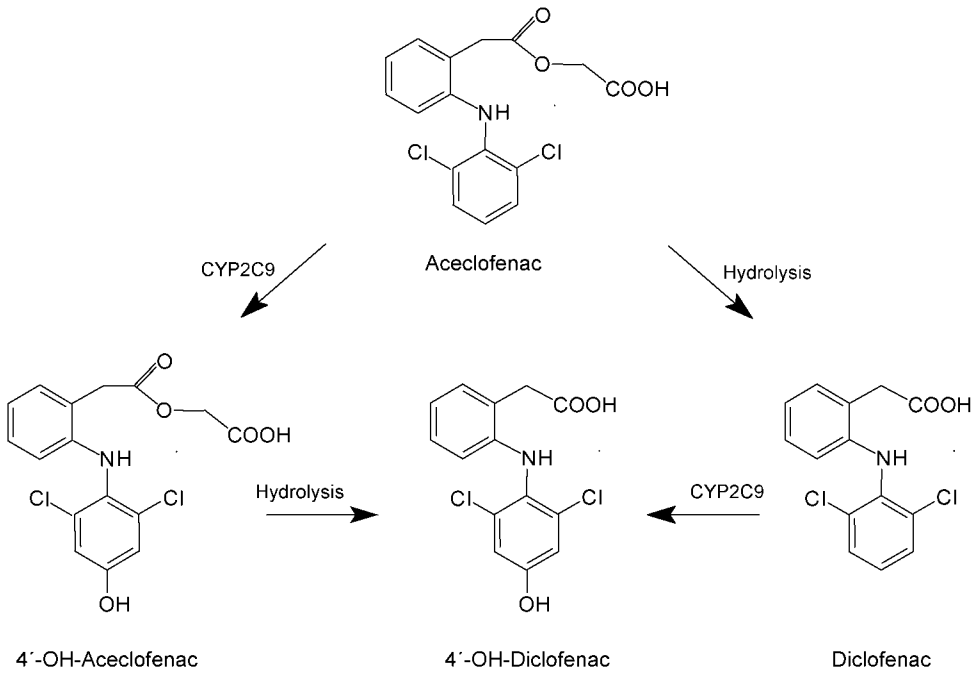

Figure 1. Metabolic pathways of aceclofenac in man (adapted from Bort *et al.*, 1996a,b).

aceclofenac, diclofenac and 4′-hydroxy-diclofenac. This paper describes a sensitive HPLC method with UV detection for the simultaneous analysis of the four phenylacetic acid derivatives in human plasma. Moreover, the developed and validated HPLC method was applied to assess the pharmacokinetics of aceclofenac and its metabolites, 4′-hydroxy-aceclofenac, diclofenac and 4′-hydroxy-diclofenac, following oral administration of a single 100 mg dose of aceclofenac to three healthy male volunteers.

MATERIALS AND METHODS

Chemicals and materials. Acetonitrile (gradient grade), disodium hydrogenphosphate dodecahydrat, *n*-hexane, methanol (gradient grade), phosphoric acid, potassium dihydrogenphosphate and tetrahydrofurane were purchased from Merck (Darmstadt, Germany). Diethylether (HPLC grade) was obtained from Fluka (Sigma-Aldrich, Taufkirchen, Germany). Aceclofenac was provided by Almirall Prodesfarma (Barcelona, Spain). Diclofenac, 4′-hydroxy-aceclofenac and 4′-hydroxy-diclofenac were provided by Novartis (Basel, Switzerland). Ketoprofen was obtained from Sigma (Deisenhofen, Germany).

Sample preparation. Samples were prepared by adding 0.5 mL sodium fluoride solution (40 mg/mL), 1 mL 1 mol/L phosphoric acid and 0.1 mL internal standard solution [0.05 mg ketoprofen–1 mL acetonitrile–0.01 mol/L phosphate buffer solution (pH 2.5; 95:5, v/v)] to 1 mL plasma followed by the addition of 5 mL *n*-hexane/diethylether (50:50, v/v). The tubes were capped, shaken for 30 min and then centrifuged at 4000 rpm for 10 min. The organic layer was transferred into a glass tube and evaporated to dryness under a nitrogen stream at room temperature. Prior to analysis, the residue was dissolved in 120 µL of a solution consisting of 72% 0.01 mol/L phosphate buffer, 15% acetonitrile, 10% methanol, 3% tetrahydrofurane (final pH 2.5). The injection volume of the extracted samples was 100 µL. Plasma calibration standards (10, 25, 50, 100, 250, 500, 750, 1000, 2500, 5000, 7500, 10,000 ng/mL) were prepared in the same manner. To achieve different final concentrations, plasma was spiked with increasing amounts of aceclofenac and metabolites. The stock solutions of all analytes were prepared with acetonitrile and stored at −80°C for a maximum of one month.

Chromatographic and detection conditions. For chromatography and detection of the analytes a gradient pump (Model PU 980; Jasco, Labor- und Datentechnik GmbH, Groß-Umstadt, Germany) and a detector (Model Spectra 100; Spectraphysics, San Jose, CA, USA) were used. The analytes were separated using a reversed-phase column (250/4 Nucleosil 120-5 C_{18}; Machery-Nagel, Düren, Germany) and a C_{18} precolumn insert. The column temperature was maintained at 30°C. Elution of aceclofenac and metabolites was achieved at a flow rate of 1 mL/min. A gradient separation was used with solvent A being 20% (v/v) 0.005 mol/L phosphate buffer, 80% (v/v) acetonitrile and solvent B being 88% (v/v) 0.01 mol/L phosphate buffer, 12% (v/v) acetonitrile. The 0.01 mol/L phosphate buffer was prepared by dissolving 7.1 g of disodium hydrogenphosphate dodecahydrat and 6.8 g potassium dihydrogenphosphate in 5 L distilled water. The gradient steps were as follows: 0–24 min, isocratic at 4% solvent A and 96% solvent B; 24–25 min linear gradient from 4 to 12% solvent A and from 96 to 88% solvent B; 26–40 min, isocratic at 12% solvent A and 88% solvent B; 40–41 min linear gradient from 12 to 15% solvent A and from 88 to 85% solvent B; 41–60 min, isocratic at

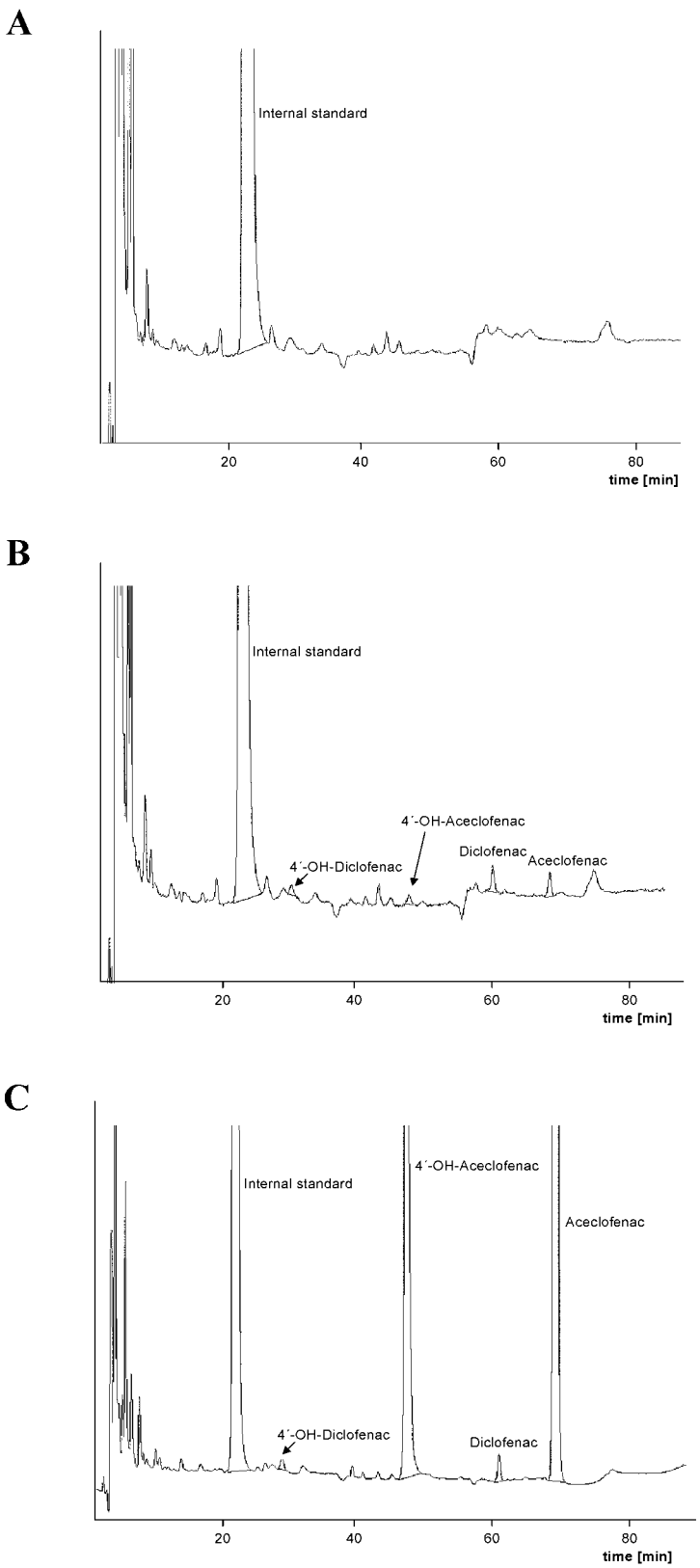

Figure 2. Chromatograms of (A) blank human plasma, (B) human plasma spiked with aceclofenac, 4′-hydroxy-aceclofenac, diclofenac and 4′-hydroxy-diclofenac at 25 ng/mL, and (C) plasma from an aceclofenac-treated human volunteer. Blood sampling (C) was performed 1.5 h after oral administration of 100 mg aceclofenac. Absorbance wavelength was 282 nm.

Table 1. Intra-day precision (coefficient of variations, CV) and accuracy for low, medium and high concentrations of aceclofenac and metabolites in human plasma

Added concentration (ng/mL)	Intra-day ($n = 5$ each) Measured concentration (mean ± SD, ng/mL)	CV (%)	Accuracy (%)	Intra-day ($n = 5$ each) Measured concentration (mean ± SD, ng/mL)	CV (%)	Accuracy (%)
	Acelofenac			*Diclofenac*		
10	10.8 ± 0.6	5.5	7.6	9.6 ± 0.4	4.4	−4.05
1000	1099 ± 28	2.5	9.9	938 ± 35	3.7	−6.3
10,000	10,717 ± 277	2.6	7.2	9429 ± 353	3.8	−5.7
	4′-Hydroxy-acelofenac			*4′-Hydroxy-diclofenac*		
10 (25)[a]	11.1 ± 0.5	4.7	10.9	26.6 ± 1.9	7.3	6.3
1000	949 ± 38	4.0	−5.1	974 ± 47	4.8	−2.5
10,000	10,185 ± 403	3.9	1.8	10,198 ± 301	2.9	2.0

[a] 4′-Hydroxy-aceclofenac, 10 ng/mL; 4′-Hydroxy-diclofenac, 25 ng/mL.

15% solvent A and 85% solvent B; 60–61 min, linear gradient from 15 to 4% solvent A and from 85 to 96% solvent B. The program ended with a 15 min re-equilibration at 4% solvent A and 96% solvent B. UV detection was set at 282 nm. Quantitation of peaks was achieved by the internal standard peak-area ratio method and linear regression.

Validation of the HPLC method. The validation of the method was performed according to Shah *et al.* (1991). The specificity of the method was examined by analyzing six human plasma samples from different volunteers who did not take any medication before blood sampling. Linearity of the standard curves was determined over 10–10,000 ng/mL (aceclofenac, 4′-hydroxy-aceclofenac, diclofenac) and 25–10,000 ng/mL (4′-hydroxy-diclofenac), respectively. The intra-day reproducibility of the method was tested by multiple analysis of human plasma samples spiked with different concentrations of the analytes on the same day. Inter-day reproducibility was assessed on three different days. Stability of analytes was evaluated at three concentrations (10, 1000, 10,000 ng/mL for aceclofenac, 4′-hydroxy-aceclofenac, diclofenac; 25, 1000, 10,000 ng/mL for 4′-hydroxy-diclofenac; $n = 5$ in each case) by injecting standards prepared from the same stock solutions stored at −80°C for one month.

Quantitation of the analytes. For quantitation of the samples a standard curve for aceclofenac and its metabolites over the validated range was generated for each analytical run. Furthermore, quality-control samples in duplicate in three different concentrations (10, 1000, 10,000 ng/mL for aceclofenac, 4′-hydroxy-aceclofenac, diclofenac; 25, 1000, 10,000 ng/mL for 4′-hydroxy-diclofenac) were incorporated into each run. The results of the quality-control samples provided the basis of accepting or rejecting the run.

Pharmacokinetic study. Three male volunteers (proband 1, 24 years, 73 kg; proband 2, 28 years, 79 kg; proband 3, 21 years, 77 kg) were included in the study. The study protocol was approved by the Ethics Commitee of the University of Erlangen-Nürnberg, and written informed consent was obtained from the volunteers. Volunteers were healthy based on medical history, physical examination, and routine laboratory screening. Probands were not permitted to take any other medication (including aspirin or other NSAIDs) for 2 weeks before and throughout the study. Volunteers with a history of coagulation disorders, a bleeding tendency, drug allergy or gastrointestinal disorders were excluded from participation in the study. The volunteers received 100 mg aceclofenac (Beofenac®, UCB Pharma, Kerpen, Germany) at a single oral dose between 08:00 and 09:00 after an overnight fast.

Table 2. Inter-day precision (coefficient of variations, CV) and accuracy for low, medium and high concentrations of aceclofenac and metabolites in human plasma

Added concentration (ng/mL)	Inter-day (three days, $n = 5$ each) Measured concentration (mean ± SD, ng/mL)	CV (%)	Accuracy (%)	Inter-day (three days, $n = 5$ each) Measured concentration (mean ± SD, ng/mL)	CV (%)	Accuracy (%)
	Acelofenac			*Diclofenac*		
10	10.2 ± 1.0	10.6	1.7	10.1 ± 1.1	10.5	0.7
1000	1010 ± 96	9.5	1.0	983 ± 105	10.7	−1.7
10,000	10,270 ± 1057	10.3	2.7	10,419 ± 1019	9.8	4.2
	4′-Hydroxy-acelofenac			*4′-Hydroxy-diclofenac*		
10 (25)[a]	10.9 ± 0.9	8.1	9.3	26.4 ± 2.4	8.9	5.6
1000	956 ± 58	6.0	−4.4	973 ± 50	5.1	−2.6
10,000	10,054 ± 322	3.2	0.5	10,053 ± 330	3.3	−0.5

[a] 4′-Hydroxy-aceclofenac, 10 ng/mL; 4′-Hydroxy-diclofenac, 25 ng/mL.

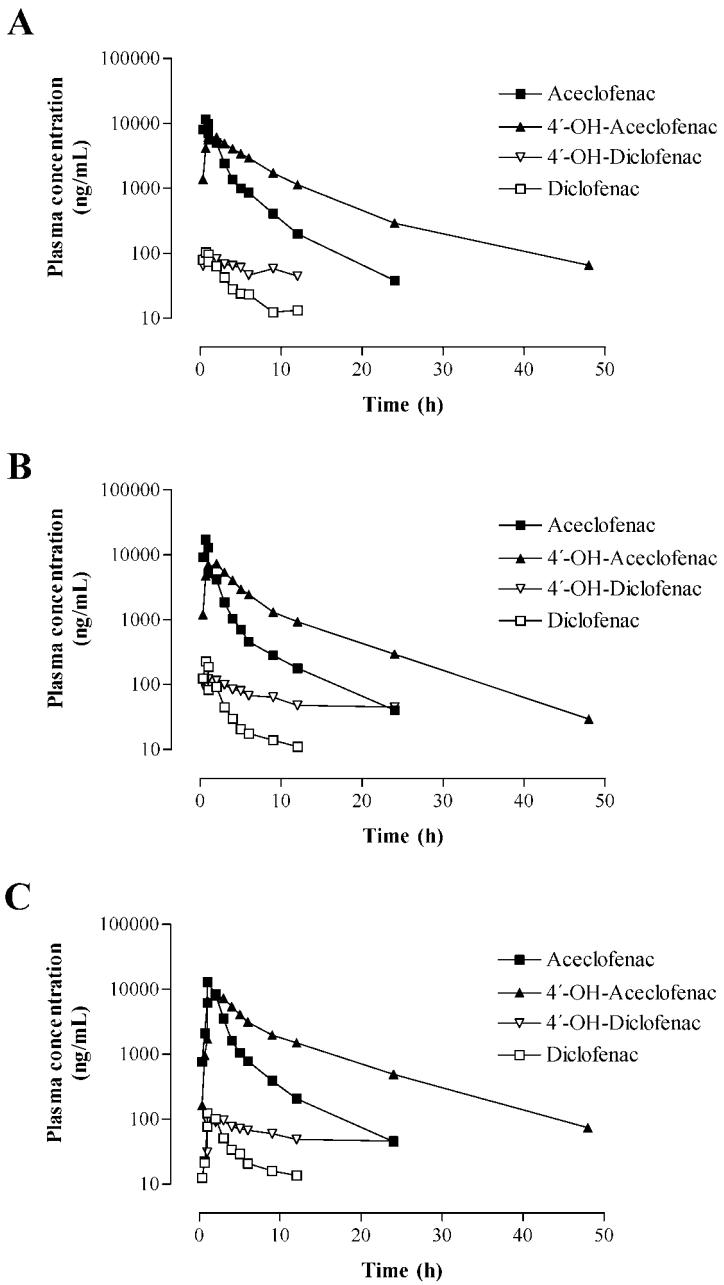

Figure 3. Individual plasma concentration–time curves (semilogarithmic plots) of aceclofenac, 4′-hydroxy-aceclofenac, diclofenac and 4′-hydroxy-diclofenac following a single oral administration of 100 mg aceclofenac to three healthy male volunteers (A, volunteer 1; B, volunteer 2; C, volunteer 3).

Intake of food was delayed for 2 h after medication. Peripheral venous blood samples were taken from each volunteer immediately before, at 20 and 40 min and at 1, 1.5, 2, 3, 4, 5, 6, 9, 12, 24 and 48 h after administration of aceclofenac. Heparinized blood samples were spun and, following addition of the esterase inhibitor sodium fluoride (Bort *et al.*, 1996a, 1996b), plasma aliquots were snap frozen. Plasma samples were stored at −80°C until analysis of aceclofenac and its metabolites for a maximum of one month.

Analysis of pharmacokinetic parameters. Plasma concentration-time curves of aceclofenac and its metabolites were evaluated by non-compartmental analysis using WinNonlin® Version 3.3 (Pharsight, Mountain View, CA, USA). Maximum plasma concentrations (C_{max}) and the time to C_{max} (t_{max}) were obtained directly from the individual plasma concentration vs time curves. The terminal half-life, $t_{1/2}$, was obtained from log–linear regression analysis of the plasma concentration-time curves in the terminal

Table 3. Individual pharmacokinetic data obtained from three healthy male volunteers following oral administration of 100 mg aceclofenac

	Volunteer	t_{max}, h	C_{max}, ng/mL (μmol/L)	AUC_t, ng h/mL (μmol h/L)	$t_{1/2}$, h
Aceclofenac	1	0.66	1624 (32.8)	27,430 (77.4)	4.6
	2	0.66	7234 (48.6)	26,993 (76.2)	5.4
	3	1.5	3056 (36.9)	27,324 (77.1)	5.0
4′-Hydroxy-aceclofenac	1	1.5	6166 (16.6)	49,893 (134.8)	8.4
	2	2	7301 (19.7)	47,644 (128.7)	7.2
	3	2	7944 (21.5)	60,349 (163.0)	8.2
Diclofenac	1	0.66	104 (0.35)	385 (1.3)	3.5
	2	0.66	230 (0.78)	497 (1.7)	8.8
	3	1.5	125 (0.42)	407 (1.4)	9.7
4′-Hydroxy-diclofenac	1	0.66	84 (0.27)	711 (2.3)	12.4
	2	1	121 (0.39)	1429 (4.6)	16.1
	3	3	96 (0.31)	1331 (4.3)	27.9

phase and calculated as $\ln 2/\lambda_z$, where λ_z denotes the time constant of the terminal slope. The area under the plasma concentration–time curve up to the last quantifiable plasma concentration (AUC_t) was determined according to the linear trapezoidal method.

RESULTS

Validation of the method

To get an optimal resolution of the analytes, a gradient system had to be employed. Figure 2 shows typical chromatograms for blank human plasma (drug-free), human plasma spiked with aceclofenac, 4′-hydroxy-aceclofenac, diclofenac and 4′-hydroxy-diclofenac (each at 25 ng/ml) and for plasma from a human volunteer obtained 1.5 h after administration of 100 mg aceclofenac. The chromatographic conditions described yielded retention times of 21.2 (internal standard ketoprofen), 28.4 (4′-hydroxy-diclofenac), 46.9 (4′-hydroxy-aceclofenac), 60.9 (diclofenac) and 69.1 min (aceclofenac), respectively [Fig. 2(B), (C)]. The peaks obtained were resolved with no interfering peaks.

Linearity of the calibration curves for the four compounds was achieved for concentrations between 10 and 10,000 ng/mL (aceclofenac, 4′-hydroxy-aceclofenac, diclofenac) and between 25 and 10,000 ng/mL (4′-hydroxy-diclofenac), respectively. All curves demonstrated a correlation coefficient of greater than 0.996. The intra- and inter-day precisions and accuracies are shown in Tables 1 and 2. All three control levels (high, medium and low) used for the validation were considered acceptable if the coefficient of variation (CV) and the error of accuracy were less than 15%, with an accepted tolerance of 20% at the lower limit of quantitation. The intra- and inter-day coefficients of variation as well as the errors of accuracy were within these ranges for all analytes at all three control levels. Limits of quantitation were determined to be 10 ng/mL for aceclofenac, 4′-hydroxy-aceclofenac, and diclofenac, and 25 ng/mL for 4′-hydroxy-diclofenac.

Average percentage recoveries were 44.4 ± 1.9, 63.5 ± 2.4, 29.0 ± 1.8 and 57.7 ± 2.1 for aceclofenac, 4′-hydroxy-aceclofenac, diclofenac and 4′-hydroxy-diclofenac, respectively (at 1000 ng/ml; mean $\pm$ SD, $n = 5$ in each case). Assay checks carried out over one month demonstrated that the stability of aceclofenac and metabolites in human plasma at three concentrations (10, 1000 and 10,000 ng/mL for aceclofenac, 4′-hydroxy-aceclofenac, diclofenac; 25, 1000 and 10,000 ng/mL for 4′-hydroxy-diclofenac; $n = 5$ in each case) and that the stability of the standard solutions of the analytes stored at −80°C was adequate (difference between measured value before and after freezing for one month <5% in each case).

Pharmacokinetics of aceclofenac and its metabolites in three healthy male volunteers following a single dose of 100 mg aceclofenac

To apply the developed and validated method, the pharmacokinetics of aceclofenac and its metabolites was assessed in the plasma of three male volunteers treated with 100 mg aceclofenac. Plots of aceclofenac, 4′-hydroxy-aceclofenac, diclofenac and 4′-hydroxy-diclofenac plasma concentrations as a function of time following oral dosing with aceclofenac are shown in Fig. 3.

The pharmacokinetic parameters of aceclofenac and its metabolites derived from non-compartmental analysis are summarized in Table 3.

DISCUSSION

In the present paper we have reported a sensitive analytical method to measure aceclofenac and three of

its metabolites simultaneously in human plasma. Among various isocratic and gradient mobile phases tested, the used acetonitrile–phosphate buffer gradient was found to be most effective to achieve separation of the peaks. Moreover, the usage of this gradient offered a time-saving procedure in terms of elution of all analytes. Specificity of the analytical method was indicated by the absence of interfering peaks as evaluated by chromatograms of blank human plasma and plasma spiked with aceclofenac, its metabolites and the internal standard ketoprofen. Linearity of standard calibration curves for aceclofenac and its metabolites was obtained over a range of 10–10,000 ng/mL (aceclofenac, 4′-hydroxy-aceclofenac, diclofenac) and 25–10,000 ng/mL (4′-hydroxy-diclofenac), respectively. An acceptable and reliable intra- and inter-day precision and accuracy of the method was demonstrated. For 4′-hydroxy-diclofenac, precision and accuracy data fulfilled the validation criteria as published by Shah *et al.* (1991) with a lower limit of quantitation of 25 ng/mL. However, for the purpose of our proband study, the validated concentration range was sufficient to assess the kinetics of this metabolite.

Determination of the stability of the analytes following a one-month-freezing period showed that all substances were fairly stable, and did not undergo significant degradation. To prevent esterase-mediated hydrolysis of the acetoxyacetic ester bonds of aceclofenac and 4′-hydroxy-aceclofenac, the esterase inhibitor sodium fluoride was added to plasma samples just before freezing (Bort *et al.*, 1996a,b). Low recovery for aceclofenac and diclofenac might have resulted from the use of a solvent system that was not quite suitable for extracting aceclofenac and diclofenac, and at the same time for extracting and analyzing the more hydrophilic 4′-hydroxy-aceclofenac and 4′-hydroxy-diclofenac. However, in view of the adequate precision and accuracy, the analytical method was found to be applicable for pharmacokinetic investigations.

In the past, several HPLC methods have been published for the individual determination of aceclofenac (Zawilla *et al.*, 2002) and diclofenac (Hanses *et al.*, 1995) or for the simultaneous assessment of aceclofenac and diclofenac (Lee *et al.*, 2000) and diclofenac and its monohydroxylated metabolites (Landsdorp *et al.*, 1990), respectively. In the present HPLC assay, however, four phenylacetic acid derivatives were determined simultaneously. This is a clear advantage compared to previously published methods. The procedure we supplied is relatively simple and gives a very low quantitation limit for aceclofenac and its metabolites. Among the methods for determination of aceclofenac and diclofenac, one paper has shown a better recovery and a shorter run time relative to our method (Lee *et al.*, 2000). However, in view of the necessary column-switching during HPLC operation in this study, our method offers a more accessible analytical procedure.

Evidence for method applicability was obtained by assessing the pharmacokinetics of aceclofenac and its metabolites following oral administration of 100 mg aceclofenac to three male volunteers. Analysis of volunteer plasma for aceclofenac and its metabolites yielded plasma concentrations within the range of the assay limits presented here. Pharmacokinetic data of aceclofenac were in agreement with previously published data (Creamer, 1992; Crema *et al.*, 1995). After oral dosing in man, the highest aceclofenac concentration was observed 0.66–1.5 h after administration. Aceclofenac yielded an AUC_t of 26,993–27,430 ng h/mL. Aceclofenac plasma levels declined with a terminal half-life of 4.6–5.4 h. According to our data aceclofenac is metabolized to at least three metabolites, namely 4′-hydroxy-aceclofenac, diclofenac and 4′-hydroxy-diclofenac. 4′-Hydroxy-aceclofenac was the major circulating metabolite, with an AUC value of 47,644–60,349 ng h/mL. However, of the identified metabolites, only diclofenac has been associated with significant inhibition of the activity of the COX enzymes (Yamazaki *et al.*, 1997). In our study analysis of plasma from volunteers treated with 100 mg aceclofenac yielded maximum diclofenac plasma concentrations of 103.5–230.2 ng/mL (ie 0.35–0.78 μmol/L). In this context it is interesting to note that diclofenac shows a high order of anti-COX-2 activity in human whole blood with an apparent IC_{50} of 0.024 μmol/L (Hinz *et al.*, in preparation) that is about one order of magnitude below the maximal diclofenac plasma concentrations determined in this study. In addition, diclofenac concentrations of 0.35–0.78 μmol/L are expected to cause only a partial suppression of platelet COX-1 activity given that diclofenac inhibits COX-1 with an apparent IC_{50} value of 0.43 μmol/L in the *in vitro* whole blood assay (Hinz *et al.*, in preparation). Thus, we strongly assume that diclofenac may contribute to the pharmacological action of aceclofenac by completely inhibiting COX-2-dependent prostaglandin formation while interfering with COX-1 activity to a minor extent. To address this issue and the question to what extent 4′-hydroxy-diclofenac contributes to the pharmacological activity of aceclofenac, the biological action of aceclofenac and its metabolites *in vivo* merits further investigation.

In summary, a reliable analytical method for the determination of aceclofenac and three of its metabolites has been developed and validated. Moreover, the assay presented has been found to be well suited for the determination of aceclofenac and metabolite concentrations in human plasma at a aceclofenac dose currently being employed in clinical use. The main advantage of the method is the ability to analyze the four compounds simultaneously under similar conditions, thereby saving time and expenses for sample preparation.

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