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Short communication

Simultaneous determination of aceclofenac and its three metabolites in plasma using liquid chromatography–tandem mass spectrometry

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Abstract

A new LC/MS/MS-based method allows simultaneous determination of aceclofenac and its three metabolites (4′-OH-aceclofenac, diclofenac, and 4′-OH-diclofenac) in plasma. After acetonitrile-induced precipitation of proteins from the plasma samples, aceclofenac, 4′-OH-aceclofenac, diclofenac, 4′-OH-diclofenac, and flufenamic acid (an internal standard) were chromatographed on a reverse-phase C_{18} analytical column. The isocratic mobile phase of acetonitrile/0.1% formic $acid_{(aq)}$ [80:20 (v/v)] was eluted at 0.2 mL/min. Quantification was performed on a triple–quadrupole mass spectrometer employing electrospray ionization, and the ion transitions were monitored in multiple reaction-monitoring mode. The monitored transitions for aceclofenac, diclofenac, 4′-OH-diclofenac, 4′-OH-aceclofenac and flufenamic acid were *m*/*z* 352.9 → 74.9, 296.1 → 251.7, 311.8 → 267.7, 368.9 → 74.9, and 279.9 → 235.9, respectively. The coefficient of variation of the assay precision was less than 6.5%, and the accuracy ranged from 93% to 103%. The limits of detection were 2 ng/mL for aceclofenac and 0.2 ng/mL for both diclofenac and 4′-OH-diclofenac. This method was used successfully to measure the concentrations of aceclofenac and its three metabolites in plasma from healthy subjects after administration of a single 100-mg oral dose of aceclofenac. This analytic method is a very simple, sensitive, and accurate way to determine the pharmacokinetics of aceclofenac and its metabolites.

Keywords: Aceclofenac; 4′-Hydroxy-aceclofenac; Diclofenac; 4′-Hydroxy-diclofenac; Pharmacokinetics; LC/MS/MS

1. Introduction

Aceclofenac, 2-[2-[2-[(2,6-dichlorophenyl)amino]phenyl]-acetyl]oxyacetic acid is used as a potent analgesic and anti-inflammatory drug [1,2]. Although its mode of action is not well understood, it is known to suppress *cyclo*-oxygenase 2-dependent prostaglandin synthesis following long-term treatment. Aceclofenac is biotransformed to 4′-hydroxy-aceclofenac [4′-(OH)-aceclofenac] and diclofenac via cytochrome P-450 2C9 (CYP2C9)-mediated hydroxylation and hydrolysis, respectively. CYP2CP also mediates the hydroxylation of diclofenac to yield 4′-hydroxy-diclofenac [4′-(OH)-diclofenac] and the hydrolysis of 4′-OH-aceclofenac to 4′-OH-diclofenac [3].

A few HPLC methods have been used for quantitation of aceclofenac and/or diclofenac [4–7]. In addition, Hinz et al. published a method for the simultaneous determination of aceclofenac and its three metabolites in human plasma using HPLC with ultraviolet detection [8]. They used a liquid–liquid extraction procedure following acidification of the plasma sample to purify the analytes.

Here we present a simple and sensitive method for simultaneous determination of aceclofenac and its three metabolites in human plasma. This method uses liquid chromatography (LC) combined with tandem mass spectrometry (MS). Samples are prepared using a simple protein precipitation. This method is useful for characterizing the pharmacokinetics of aceclofenac and its metabolites in humans.

2. Experimental

2.1. Reagents and materials

Aceclofenac, diclofenac, and 4′-OH-diclofenac were kindly donated by Novartis Pharma AG (Basel, Switzerland). Flufenamic acid, which was used as an internal standard, and

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HPLC-grade acetonitrile were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Merck Co. (Darmstadt, Germany), respectively. All other chemicals and solvents were of the highest analytical grade available.

2.2. Preparation of standards and quality controls

Aceclofenac, diclofenac, 4′-OH-diclofenac, and flufenamic acid were dissolved in acetonitrile at 1 mg/mL. These standard solutions were serially diluted with acetonitrile and added to drug-free plasma to achieve final concentrations of 0.1, 0.2, 0.5, 1, 2, 5, or 10 μg/mL for aceclofenac and 0.5, 2, 5, 10, 50, or 200 ng/mL for diclofenac and 4′-OH-diclofenac. Calibration graphs in plasma were derived from a linear regression of the ratios of aceclofenac, diclofenac, and 4′-OH-diclofenac peak areas to that of flufenamic acid. The absolute concentration of 4′-OH-aceclofenac was approximated using the aceclofenac calibration curve, because 4′-OH-aceclofenac was not available from commercial or other sources.

Quality-control samples were prepared daily by adding 10 μL of standard solution to 90-μL aliquots of blank human plasma. To evaluate the inter- and intra-day precision and accuracy of this assay method, aceclofenac was prepared at 0.1, 0.5, 2, and 10 μg/mL, and diclofenac and 4′-OH-diclofenac were prepared at 0.5, 5, 10, and 100 ng/mL.

2.3. Characterization of the product ions using tandem mass spectrometry

To characterize the product ions of aceclofenac, diclofenac, 4′-OH-diclofenac, and flufenamic acid, 10 ng/mL solutions of each were separately infused into the mass spectrometer at a flow rate of 10 μL/min. The precursor ions ($[M–H]^-$) and the pattern of fragmentation were monitored using negative ion mode. The major peaks observed in the MS/MS scan were used to quantify aceclofenac, its metabolites diclofenac, 4′-OH-diclofenac, and flufenamic acid. The precursor ion and the fragmentation of 4′-OH-aceclofenac were inferred from those of aceclofenac, and the metabolite was detected using the same mass spectrometric conditions.

2.4. Analytical system

The concentrations of aceclofenac and its three metabolites in human plasma were quantified using LC–MS with a PE SCIEX API 4000 LC/MS/MS System (Applied Biosystems, Foster City, CA, USA) equipped with an electrospray ionization interface used to generate negative ions ($[M–H]^-$). The compounds were separated on a Zorbax C_{18} reverse-phase column (100 mm × 2.1 mm i.d., 3 μm particle size; Agilent, Wilmington, DE, USA) with an isocratic mobile phase consisting of acetonitrile/0.1% formic $acid_{(aq)}$ [80:20 (v/v)]. The mobile phase was eluted using an HP 1100 Series pump (Agilent) at 0.2 mL/min.

The turbo ion spray interface was operated in negative ion mode at 5500 V and 400 °C. The operating conditions were optimized by flow injection of a mixture of all analytes and consisted of: nebulizing gas flow, 1.04 L/min; auxiliary gas flow, 4.0 L/min; curtain gas flow, 1.44 L/min; orifice voltage, 80 V; ring voltage, 400 V; collision gas (nitrogen) pressure, 3.5×10^{-5} Torr. Quantitation was performed by multiple-reaction monitoring (MRM) of the deprotonated precursor ion and the related product ion for aceclofenac and its metabolites using the internal standard method with peak-area ratios. The mass transitions used for aceclofenac, 4′-OH-aceclofenac, diclofenac, 4′-OH-diclofenac, and flufenamic acid were *m/z* 352.9 → 74.9, 368.9 → 74.9, 296.1 → 251.7, 311.8 → 267.7, and 279.9 → 235.9, respectively. Quadrupoles Q1 and Q3 were set at unit resolution. The analytical data were processed using Analyst software (version 1.4.1; Applied Biosystems).

2.5. Sample preparation

Five hundred microliters of internal standard (10 ng/mL) were added to 0.1 mL of plasma, and the mixture was vortexed for 10 s. The mixture was then centrifuged at 13,200 rpm for 10 min, and 5 μL of the resulting supernatant were injected onto the column [9].

2.6. Validation procedure

The validation parameters were selectivity, precision, and accuracy. Five batches of blank, heparinized human plasma were screened to determine the specificity. The precision and accuracy of the intra- and inter-day assay validations were estimated using inverse prediction of the concentration of the quality controls from the calibration curves. To study the matrix effect, blank plasma samples were processed and spiked later to obtain the concentrations at 0.1 and 2 μg/mL. The response (area) was compared with the standard solutions directly injected at the same levels.

2.7. Clinical application

Twenty healthy subjects who gave written informed consent took part in this study. Candidates with health problems, drug or alcohol abuse, or abnormalities in laboratory screening values were excluded. After an overnight fast, all subjects received a single 100-mg oral dose of aceclofenac. Blood samples (5 mL) were drawn at various times (up to 24 h) after drug administration and stored at −80 °C until analysis.

3. Results and discussion

3.1. Mass spectra

Precursor and corresponding product ions for aceclofenac, diclofenac, 4′-OH-diclofenac, and the internal standard flufenamic acid were determined from spectra obtained during the infusion of standard solutions into a mass spectrometer using an electrospray ionization source. The instrument was operated in negative ionization mode with N_2 collision gas in Q2 of the MS/MS system. Aceclofenac, diclofenac, 4′-OH-diclofenac, and flufenamic acid predominantly produced

deprotonated molecules at *m/z* 352.9, 296.1, 311.8, and 279.9, respectively. After collision with N_2 in Q2, the corresponding product ions were scanned at *m/z* 74.9, 251.7, 267.7, and 235.9, respectively, in Q3. These are the most sensitive product ions for quantification (Fig. 1). The optimal collision energy to produce the *m/z* 74.9 fragment ion from aceclofenac was 20 eV, whereas 16 eV of collision energy was needed to detect the daughter ions of diclofenac, 4′-OH-diclofenac, and flufenamic acid in MRM mode.

3.2. Determination of aceclofenac and its three metabolites

The elution times for aceclofenac, 4′-OH-aceclofenac, diclofenac, 4′-OH-diclofenac, and flufenamic acid were 1.8, 1.3, 1.9, 1.3, and 2.1 min, respectively, with no apparent interfering peaks. Typical chromatograms for the various plasma samples are shown in Fig. 2; blank plasma is shown in the left column, plasma spiked with 0.1 μg/mL aceclofenac or 10 ng/mL diclofenac, 4′-OH-diclofenac, or flufenamic acid is shown in the middle column, and a plasma sample from one of the human subjects is shown in the right column.

Because we were unable to obtain 4′-OH-aceclofenac from commercial sources or from other researchers, its concentration was approximated from the calibration curve of aceclofenac instead. Nevertheless, the approximation seems reasonable because (1) the metabolite and parent compound were eluted under the same chromatographic conditions; (2) they were fragmented at the same collision energy (20 eV), and the same transition to *m/z* 74.9 was used for quantification; and (3) the introduction of oxygen at the 4′-position might have only minimal influence on ionization efficiency due to its distance from the cleavage site [10] (Fig. 1).

In a previous study, aceclofenac and its metabolites were purified from plasma samples by extraction with an organic mixture of *n*-hexane and diethyl ether under acidic conditions [8]. After evaporation of the organic layer, the residue was analyzed by HPLC with ultraviolet detection. The chromatographic analysis for one sample took longer than 80 min, and distinguishing diclofenac and 4′-OH-diclofenac from the baseline noise was difficult due to limited detection sensitivity, especially at low concentrations. On the other hand, the present method uses a simple protein precipitation for sample preparation, and analysis of the plasma sample is rapid, i.e., the total elution time is only 2.5 min. Furthermore, the limit of detection is markedly improved.

3.3. Linearity and detection limit

The plasma calibration curves provided a reliable response from 0.1 to 20 μg/mL for aceclofenac and from 0.5 to 200 ng/mL for diclofenac and 4′-OH-diclofenac. The mean equations of the regression lines in plasma were $y = (0.369 \pm 0.015)x + (0.052 \pm 0.008)$ for aceclofenac ($r^2 > 0.998$), $y = (4.122 \pm 0.011)x + (0.022 \pm 0.005)$ for diclofenac ($r^2 > 0.999$), and $y = (6.122 \pm 0.035)x + (0.030 \pm 0.011)$ for 4′-OH-diclofenac ($r^2 > 0.999$). The limits of detection were 2 ng/mL for aceclofenac and 0.2 ng/mL for diclofenac and 4′-OH-diclofenac at a signal-to-noise (S/N) ratio of 5.

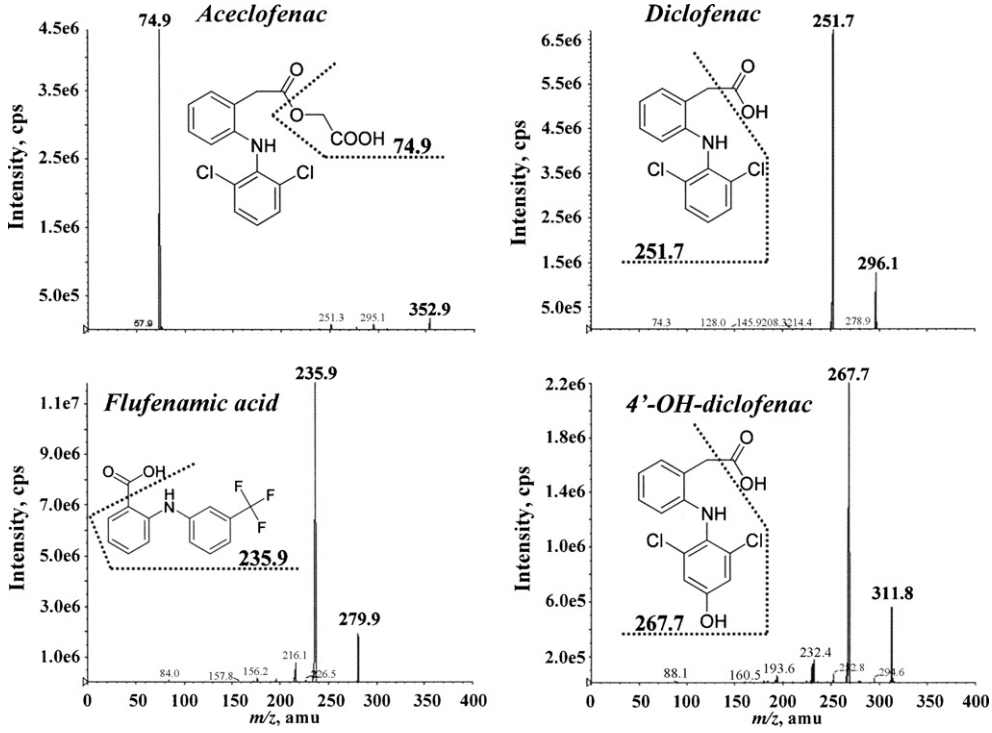

Fig. 1. Tandem mass spectra of aceclofenac, diclofenac, 4′-OH-diclofenac, and flufenamic acid obtained using electrospray ionization mode.

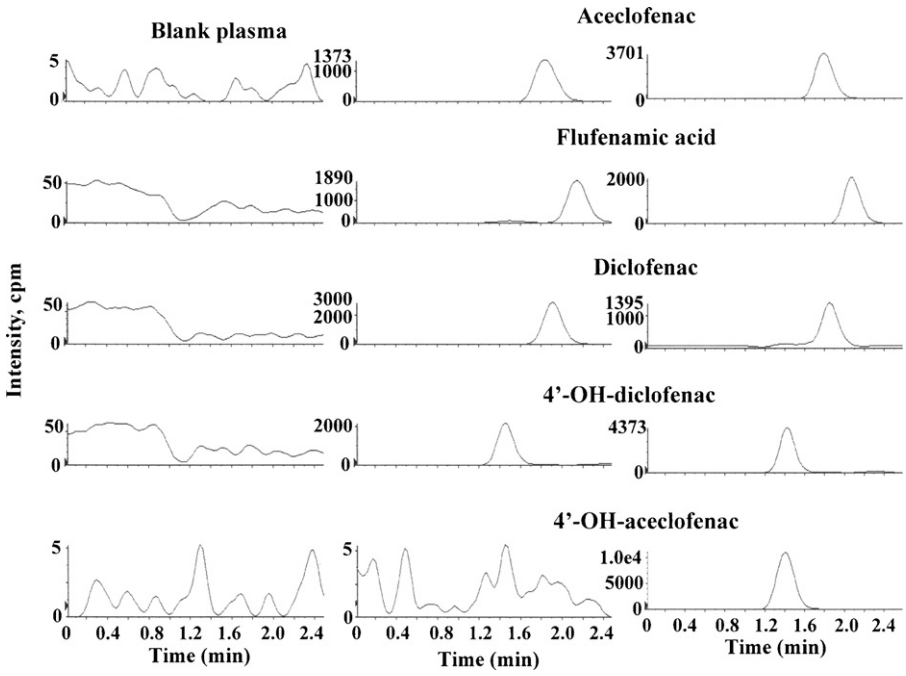

Fig. 2. Chromatograms of aceclofenac, its three metabolites, and flufenamic acid. Left: blank plasma; middle: plasma spiked with aceclofenac (100 ng/mL), diclofenac (10 ng/mL), or 4′-OH-diclofenac (10 ng/mL); right: plasma sample drawn from a subject 2 h after dosing with 100 mg aceclofenac.

3.4. Precision and accuracy

The intra- and inter-day precision and accuracy of our method are shown in Tables 1 and 2, respectively. The coefficients of variation of the precision of the intra- and inter-day validation were less than 5.3% and 6.5%, respectively. The accuracy of the method ranged from 93% to 110%. The ion suppressions due to the matrix components were about 15%, 18%, and 48% for aceclofenac, diclofenac, and 4′-OH-diclofenac, respectively.

3.5. Clinical application

Plasma concentration–time curves of aceclofenac, 4′-OH-aceclofenac, diclofenac, and 4′-OH-diclofenac obtained after oral administration of 100 mg aceclofenac are illustrated in Fig. 3. The pharmacokinetic parameters of aceclofenac and its metabolites are given in Table 3. The present results are comparable to the previous data provided by Bort et al. [3] and Hinz et al. [8], even though they studied in a small group (3–4 volunteers) and the sensitivity did not seem to be quite enough to

Table 1
Precision and accuracy of the intra-day assay ($n = 5$)

Concentration (μg/mL)	Aceclofenac (%)	Concentration (ng/mL)	Diclofenac (%)	4′-OH-diclofenac (%)
0.1	93.0 ± 4.9[a] (5.3)[b]	0.5	95.1 ± 4.2 (4.4)	95.8 ± 5.0 (5.2)
0.5	107.0 ± 2.3 (2.1)	5	99.3 ± 3.8 (3.8)	103.5 ± 4.6 (4.4)
2	100.5 ± 3.5 (3.5)	10	97.4 ± 4.3 (4.4)	96.5 ± 4.3 (4.5)
10	99.8 ± 1.6 (1.6)	100	95.1 ± 2.4 (2.5)	98.4 ± 2.4 (2.4)

[a] Accuracy (mean% ± S.D.).
[b] CV, coefficient of variance (%).

Table 2
Precision and accuracy of the inter-day assay ($n = 5$)

Concentration (μg/mL)	Aceclofenac (%)	Concentration (ng/mL)	Diclofenac (%)	4′-OH-diclofenac (%)
0.1	95.3 ± 4.9[a] (5.1)[b]	0.5	94.5 ± 5.6 (5.9)	92.5 ± 5.1 (5.5)
0.5	110.4 ± 7.2 (6.5)	5	103.2 ± 6.4 (6.2)	98.4 ± 4.8 (4.9)
2	103.5 ± 4.8 (4.6)	10	99.7 ± 5.1 (5.1)	92.9 ± 5.9 (6.4)
10	96.9 ± 3.5 (3.6)	100	98.5 ± 3.7 (3.8)	97.5 ± 4.2 (4.3)

[a] Accuracy (mean% ± S.D.).
[b] CV, coefficient of variance (%).

Table 3
Pharmacokinetic parameters of aceclofenac and its three metabolites in healthy subjects after single-dose oral administration of 100 mg aceclofenac

Parameter	Aceclofenac	4′-OH-aceclofenac	Diclofenac	4′-OH-diclofenac
T_{max} (h)	1.5 ± 0.6	2.6 ± 0.8	1.6 ± 0.7	2.4 ± 0.9
C_{max} (μg/mL)	7.1 ± 1.6	5.0 ± 1.1	0.09 ± 0.02	0.04 ± 0.01
AUC_t (μg h/mL)	21.0 ± 3.9	26.8 ± 8.1	0.26 ± 0.07	0.37 ± 0.13
$t_{1/2}$ (h)	1.4 ± 0.3	3.3 ± 0.7	1.0 ± 0.5	7.6 ± 4.7

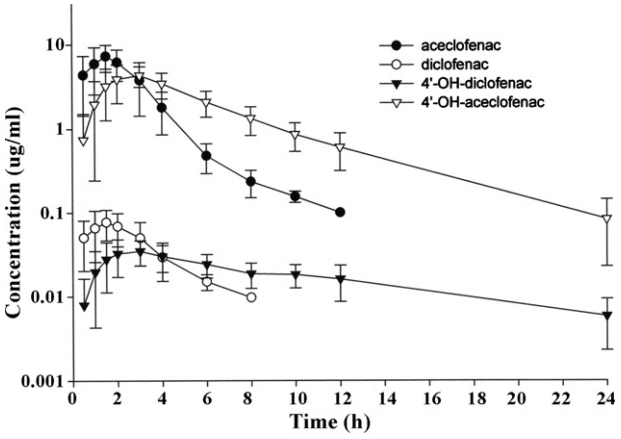

Fig. 3. Plasma concentration–time curves of aceclofenac and its three metabolites (diclofenac, 4′-OH-diclofenac, and 4′-OH-aceclofenac) in healthy subjects after a single 100-mg oral dose of aceclofenac. Each point represents the mean ± S.D. ($n = 20$).

determine the terminal phases of the plasma concentration–time curves of metabolites.

In conclusion, the LC/MS/MS method described here is a very simple, sensitive, and accurate procedure for simultaneous determination of aceclofenac and its three metabolites (diclofenac, 4′-OH-aceclofenac, and 4′-OH-diclofenac) in plasma. It is suitable for in vitro and in vivo pharmacokinetic studies of aceclofenac.

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