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Liquid chromatography–tandem mass spectrometric assay for diclofenac and three primary metabolites in mouse plasma

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ABSTRACT

The first liquid chromatography–tandem mass spectrometric assay for the simultaneous determination of diclofenac, 4′-hydroxy-diclofenac, 5-hydroxy-diclofenac and diclofenac-acyl-glucuronide in mouse plasma, using a simple sample pre-treatment procedure, was developed and validated. Analytes in plasma were stabilized using acetic acid and ascorbic acid. After addition of the internal standard D_4-diclofenac to a 10 μl sample volume and protein precipitation with acetonitrile, the supernatant was supplemented with an equal volume of water and injected into the chromatographic system. A polar embedded reversed-phase column with gradient elution using formic acid and ammonium acetate in water–methanol were used. The eluate was totally transfered into an electrospray interface with positive ionization and the analytes were quantified using triple quadrupole mass spectrometry. The assay was validated in the ranges 10–5000 ng/ml for 4′-hydroxy-diclofenac and 20–10,000 ng/ml for the other analytes, the lowest levels of these ranges (10 or 20 ng/ml) being the lower limits of quantification. Within day precisions were ≤10%, between day precisions ≤13% and accuracies were between 90 and 108%. The analytes were chemically stable under all relevant conditions. The assay was successfully applied in a pharmacokinetic study with diclofenac in mice.

1. Introduction

Diclofenac (DF, Fig. 1) is an important analgesic and anti-inflammatory drug, widely used in the treatment of post-operative pain, rheumatoid arthritis, and chronic pain associated with cancer. When given orally, absorption is rapid and complete in rat, dog, rhesus monkey, and man [1,2]. Extensive first pass metabolism (Fig. 1) combined with low enterohepatic circulation reduces oral bioavailability of DF in humans to 50–60% of the administered dose [3,4]. In rats and dogs, however, significant enterohepatic circulation due to biliary excretion of DF and its acyl-glucuronide (DF-G, Fig. 1) has been reported [1,5]. Biliary excretion of DF-G in rats is critically dependent on multidrug resistance protein (Mrp) 2, an efflux pump located at the canalicular membrane of hepatocytes [6]. To further evaluate the roles of Mrp2 and other ATP-binding cassette multidrug transporters, disposition of DF and its primary metabolites was studied at our laboratory in recently generated knockout mouse models [7,8]. To support this study, a sensitive bioanalytical assay for simultaneous quantification of DF and its three principal metabolites (Fig. 1) in small volumes of mouse plasma samples (ca. 25 μl) is required.

Chromatographic methods capable of quantifying diclofenac and one or more hydroxy-metabolites in human plasma [9–11] or urine [12–16] and in rat plasma [17] have been reported using LC-UV [9,14–17], GC-electron capture detection [10,12,13] and LC-electrochemical detection [11]. Lower limits of quantification (LLQs) in the range 5–25 ng/ml can be obtained using these techniques using 200–2000 μl of urine or plasma [9–11,13,14,17]. For sample pre-treatment liquid–liquid extraction [9,10,12–17], mostly using an ethereal solvent [9,10,12,14,15,17], is often used. Solid-phase extraction (SPE) was also reported as a suitable option [11]. No validated LC/MS(/MS) method has been published for diclofenac and these metabolites so far.

On the other hand, quantification of the acyl-glucuronide of diclofenac, the main metabolite of this drug, seems to be an almost unexplored field. Two concise method descriptions for the quantification of DF-G in microsomal incubation mixtures using LC/MS/MS [18,19] and one using LC-UV [6] could be found. The LC/MS/MS method of King et al. [19] only quantified DF-G (LLQ = 10 ng/ml)

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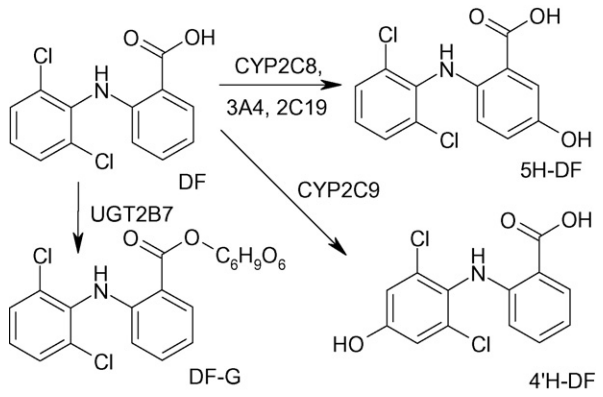

Fig. 1. Structures and biotransformation routes of diclofenac and its three most prominent primary metabolites with the responsible enzymes. CYP = cytochrome P450; UGT = uridine glucuronyl transferase.

after SPE while the method of Kumar et al. [18] was capable of quantifying DF, 4′-hydroxy-DF (4′-H-DF, Fig. 1) and 4′-H-DF-acyl-glucuronide at, depending on the analyte, 6 ng/ml or lower, using protein precipitation. Seitz et al. [6] did not report the sensitivity of their LC-UV assay for DF and DF-G using protein precipitation.

Therefore, a chromatographic assay for the simultaneous quantification of DF, 4′-H-DF, 5-hydroxy-diclofenac (5-H-DF, Fig. 1) and DF-G, with a simple pre-treatment procedure using electrospray-MS/MS as detection technique for 10 μl sample volumes of mouse plasma was developed and validated. The final aim was to support *in vivo* pharmacokinetic studies in mice, using small plasma samples obtained from the tail vein.

2. Experimental

2.1. Animals

Mice were housed and handled according to institutional guidelines complying with Dutch legislation. Animals used in this study were male wild-type mice of a FVB genetic background, between 9 and 15 weeks of age. Animals were kept in a temperature-controlled environment with a 12 h light/12 h dark cycle and received a standard diet (AM-II, Hope Farms, Woerden, The Netherlands) and acidified water (to pH 2.4–2.5 using hydrochloric acid to suppress bacterial growth) *ad libitum*.

2.2. Chemicals

DF was obtained from Sigma (Sigma–Aldrich Chemie, Steinheim, Germany), D_4-diclofenac (phenyl-d_4; 92 atm.% D) from CDN isotopes (Pointe-Claire, Quebec, Canada), DF-G from United States Biological (Swampscott, MA, USA) and 5-H-DF from Toronto Research Chemicals (North York, Canada); 4′-H-DF was a kind gift from Becton Dickinson Bioscience (Breda, The Netherlands). LC–MS grade water, methanol of HPLC quality and acetonitrile of HPLC-S gradient grade quality were obtained from Biosolve (Valkenswaard, The Netherlands). Water not used as eluent was home-purified by reversed osmosis on a multi-laboratory scale. Formic acid, L(+)-ascorbic acid and ammonium acetate were of analytical grade and originated from Merck (Darmstadt, Germany), acetic acid of analytical quality originated from Riedel-de Haën (Sigma Aldrich, Seelze, Germany). Pooled mouse heparin-sodium plasma was obtained from Innovative Research (Southfield, MI, USA) and plasma of individual mice was obtained from wild-type mice. Modified mouse plasma (MMP) was obtained by adding 4% (v/v) of 2 M acetic acid in water and 1% (v/v) of 0.5 M ascorbic acid in water to blank mouse plasma.

Table 1
MS/MS and LC parameters of individual compounds

Parameter	4′-H-DF	5-H-DF	DF-G	DF	(^{35}Cl-^{37}Cl-)D_4-DF
m/z precursor ion	312	312	296	296	302
m/z product ion	230	230	214	214	220
Tube lens offset (V)	83	83	74	74	83
Collision energy (V)	−35	−35	−35	−35	−35
Retention time (min)	3.5	3.9	4.5	5.2	5.2

2.3. Equipment

The LC/MS/MS equipment consisted of a DGU-14A degasser, a CTO-10Avp column oven, a Sil-HTc autosampler, two LC10-ADvp-μ pumps (all from Shimadzu, Kyoto, Japan) and a Finnigan TSQ Quantum Discovery Max triple quadrupole mass spectrometer with electrospray ionization (Thermo Electron, Waltham, MA, USA). For data recording and system controlling a Dell Optiplex GX270 personal computer, equipped with the Finnigan Xcalibur software (Version 1.4, Thermo Electron), was used.

2.4. LC/MS/MS conditions

Partial-loop injections (30 μl) were made on an Atlantis dC18 column (100 mm × 2.1 mm, d_p = 3 μm, average pore diameter = 10 nm, Waters, Milford, USA) with a Polaris 3 C18-A pre-column (10 mm × 2 mm, d_p = 3 μm, Varian Middelburg, The Netherlands). The column temperature was maintained at 50 °C and the autosampler was maintained at 4 °C. At 0.5 ml/min, the following gradient was used after 1 min of isocratic elution at 40% B, the percentage was raised linearly to 50% B during the next 2.5 min and raised further linearly to 75% during the following 1.5 min. After another isocratic minute at 75% B the column was flushed at the initial conditions for 2.5 min before starting the next injection, resulting in a total run time of 8.5 min. Solvent A contained 8.5 mM ammonium acetate and 0.0075% (v/v) formic acid in water and solvent B was methanol. The eluate was transfered into the electrospray probe, starting at 1.5 min after injection by switching the MS inlet valve. Tuning the ion spray at 50% solvent B, operated in the positive ionization mode, mixed with 5 μl/min of 4 μg/ml DF-G resulted in a 4700 V spray voltage and a 400 °C capillary temperature with the nitrogen sheath, ion sweep and auxiliary gasses set at 43, 2 and 0 arbitrary units, respectively; the up-front collision-induced dissociation was set off. The calibration of the quadrupoles was performed using phosphoric acid and three of its clusters (n = 6, 11 and 14) at m/z 98.98, 588.87, 1078.75 and 1372.68. The selected reaction monitoring (SRM) mode was used with argon as the collision gas at 1.5 mTorr. Compound dependent parameters are reported in Table 1. The mass resolutions were set at 0.7 full width at half height (unit resolution).

2.5. Sample pre-treatment

To a 10 μl plasma sample, pipetted into a 1.5 ml polypropylene tube standing on melting ice, 10 μl of the IS solution (250 ng/ml D_4-diclofenac in 50% (v/v) methanol in water) was added. The sample was shaken shortly on a vortex-mixer and 25 μl acetonitrile was added. The tube was closed and shaken vigorously for ca. 5 s using vortex-mixing. The supernatant (ca. 40 μl) was pipetted into a 250 μl glass insert placed in an autosampler vial after centrifugation of the sample at 10 × 10^3 *g* and 4 °C for 1 min. Forty microliters of water were added before closing the vial and 30 μl of the sample was injected onto the column.

2.6. Validation

A laboratory scheme based on international guidelines [20–22] was used for the validation procedure.

2.6.1. Calibration

Stock solutions of diclofenac sodium were prepared duplicately in 50% (v/v) methanol in water at exact concentrations of ca. 1.5 mg/ml. Stock solutions of the other analytes were prepared duplicately in methanol at exact concentrations in the 0.2–1 mg/ml range and one stock solution of the IS was prepared at 0.5 mg/ml in methanol. The stock solutions were stored at −80 °C (DF-G and 5-H-DF) or −30 °C (other three compounds). One set of stock solutions of the analytes was combined to obtain the highest calibration sample in MMP containing 5 μg/ml 4′-H-DF and 10 μg/ml of each other analyte. Calibration samples at lower levels were prepared daily at 5000, 2000, 1000, 200, 100, 40 and 20 ng/ml (half these levels for 4′-H-DF) in MMP. The 20, 40 and 10,000 ng/ml MMP calibration samples were processed in duplicate for each daily calibration, the levels in between only once. Least-squares double logarithmic regression was employed to define the calibration curves using the ratios of the peak area of the analytes and the IS.

2.6.2. Precision and accuracy

The second set of calibration samples was used to obtain validation (quality control (QC)) samples in MMP at 8000 (QC-high), 1000 (QC-med), 60 (QC-low) and 20 ng/ml (QC-LLQ) with half of these levels for 4′-H-DF. Precisions and accuracies were determined by sextuple analysis of each validation sample in three analytical runs on three separate days for all QCs (total: $n = 18$). Relative standard deviations were calculated for both, the within day precision (repeatability) and the between day precision (reproducibility).

2.6.3. Selectivity

Six individual MMP samples were processed to test the selectivity of the assays. These samples were processed with and without IS and with IS after spiking all analytes at the LLQ.

2.6.4. Recovery

The extraction recovery was determined in quadruplicate by comparing processed samples (QC-high, -med and -low) with blank MMP extracts spiked with the analytes at these levels. Ionization recovery (ion suppression) was assessed by comparing the spiked blank MMP extracts with reference material solutions in water–methanol–acetonitrile (11/1/4 (v/v/v)) at the three validation levels (QC-high, -med and -low). The extraction and ionization recoveries of the IS were assessed using identical procedures as for the analytes at the IS concentration used in the assay.

2.6.5. Stability

The stability of the analytes was investigated in QC-high and -low MMP samples. Quadruplicate analysis of these samples was performed after storage on ice (24 h at 0 °C), four additional freeze–thaw cycles (thawing on ice at 0 °C during ca. 1 h and freezing again at −80 °C for at least 1 day), and storage at −80 °C for 11 weeks, respectively. Furthermore, validation runs were re-injected after additional storage of the extracts at 4 °C.

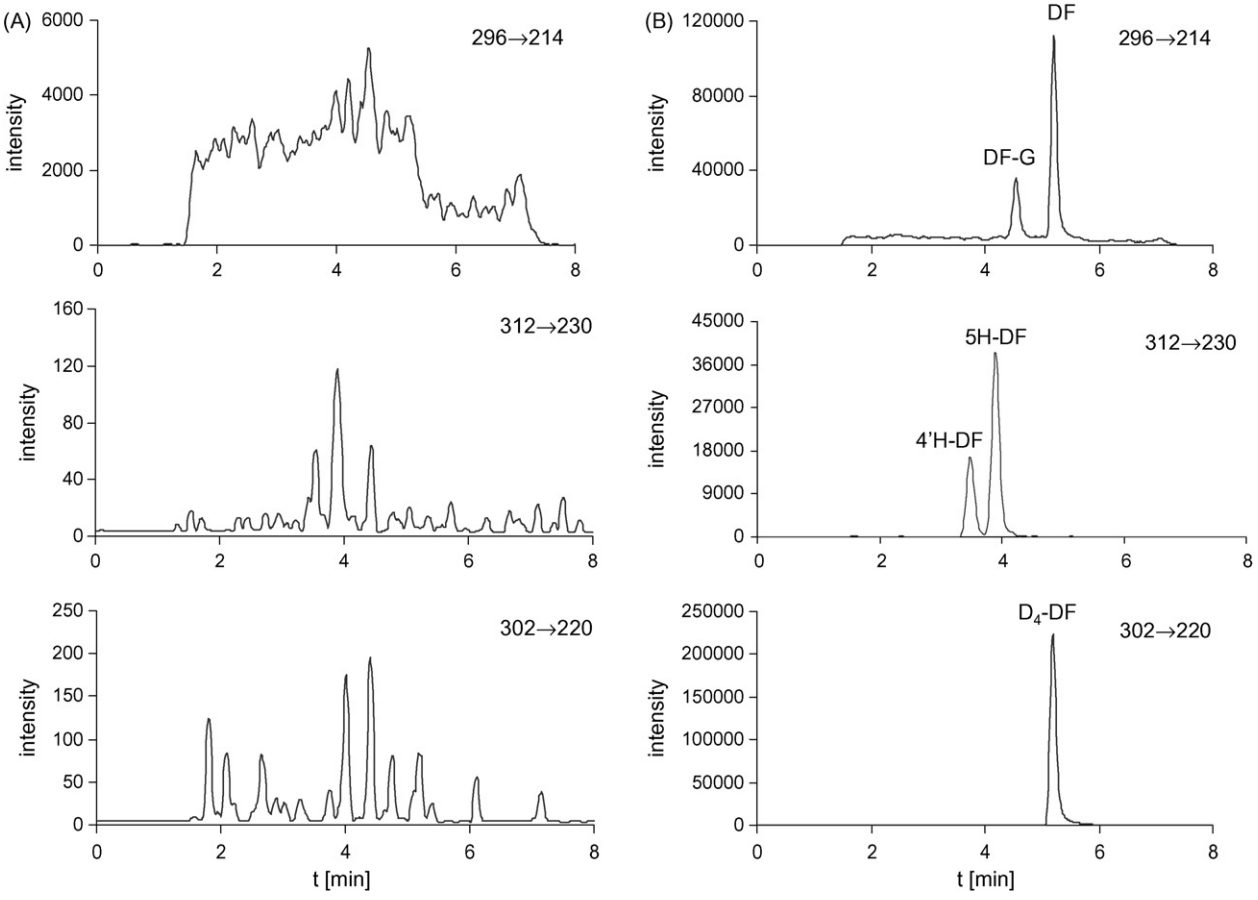

Fig. 2. SRM chromatograms of (A) blank MMP, (B) MMP spiked at the LLQ and (C) a MMP sample from a wild-type mouse 4 h after receiving 5 mg/kg diclofenac orally (data reported in Fig. 4). X and Y are probably hydroxy-glucuronide metabolites of diclofenac (data not shown).

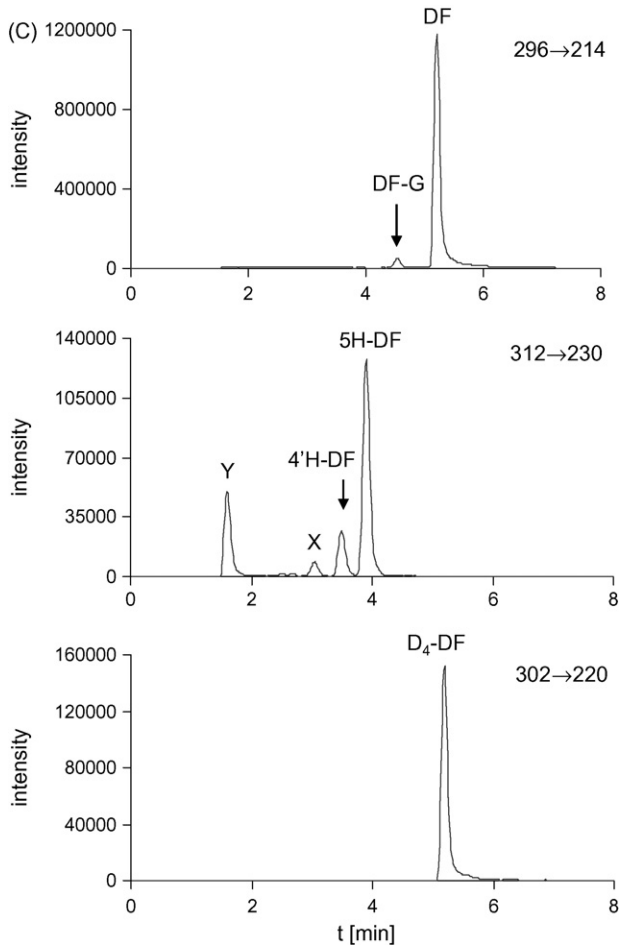

Fig. 2. (*Continued*).

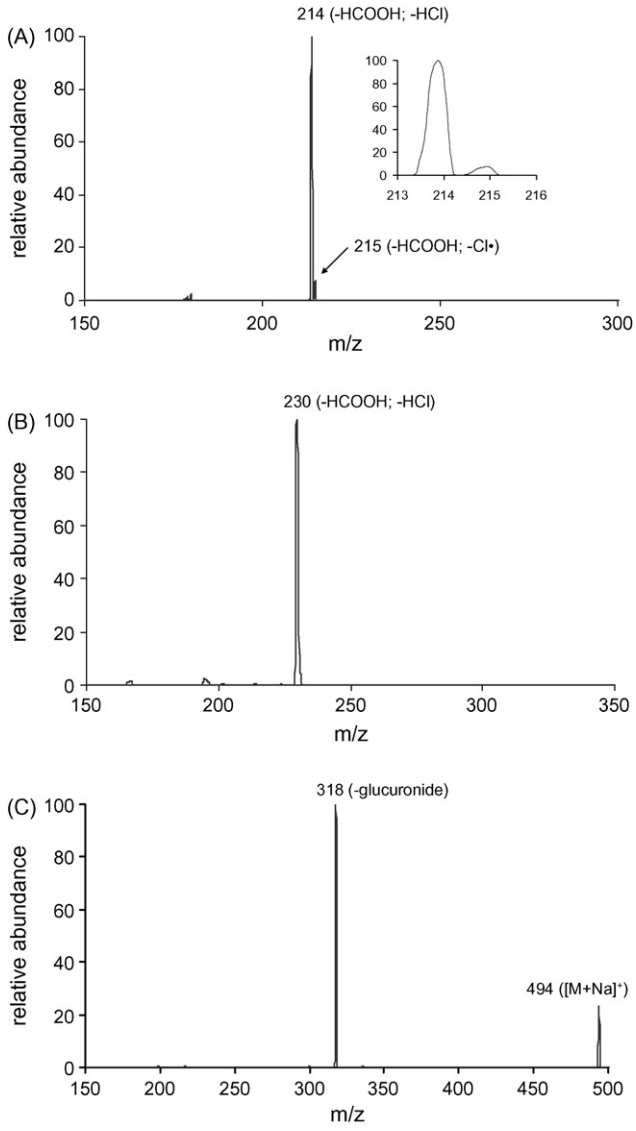

Fig. 3. Product spectra of (A) $[diclofenac+H]^+$ (296 at −35 V), including magnification of the main peaks, (B) $[4'\text{-H-DF+H}]^+$ (312 at −33 V) and (C) $[DF\text{-G+Na}]^+$ (494 at −20 V). Three standard solutions in 50% (v/v) methanol in water contained 7, 10 and 4 μg/ml of the analytes, respectively, and were infused at 5 μl/min and mixed with 0.5 ml/min of a mixture containing 50% (v/v) of 0.05% (v/v) formic acid and 50% (v/v) methanol before introduction into the electrospray source.

2.6.6. In vivo samples

A wild-type mouse was treated orally with 5 mg diclofenac/kg bodyweight. Diclofenac (Voltaren; 25 mg/ml) was 50-fold diluted with 5% glucose solution in water and a total volume of 10 ml/kg body weight was administered by gavage into the stomach using a blunt ended needle. Blood samples were collected in heparinized capillary tubes (Oxford Labware, St. Louis, USA) from the tail vein at 15 and 30 min and at 1, 2, 4 and 6 h after administration of the drug. Samples were kept on melting ice. After centrifugation at 2100 × *g* for 5 min at 4 °C, plasma was pipetted and supplemented with 4% (v/v) of 2 M acetic acid in water and 1% (v/v) of 0.5 M ascorbic acid in water and stored at −80 °C until analysis.

3. Results and discussion

3.1. Method development

Because of the high selectivity and sensitivity of the MS/MS detection, the simple pre-treatment procedure for a small sample volume could be used. Initially, an isocratic chromatographic system was developed using a mixture of 65% (v/v) methanol and 35% (v/v) of 0.05% (v/v) formic acid in water. The increased resolution of a 10 cm column was used to reduce the observed ion suppression by endogenous compounds using a 5 cm column and the 50 °C column temperature was used to reduce back pressure, thereby facilitating the 0.5 ml/min eluent flow on the LC-equipment. Unfortunately, this chromatographic system could not separate both hydroxy-metabolites of DF. Separation between 4′-H-DF and 5-H-DF was obtained using an initial eluent composition with a lower amount of organic modifier (40% methanol) and gradient elution. Further, 0.05% formic acid in the aqueous eluent A was partially replaced by 10 mM ammonium acetate. The amount of

Table 2
Regression parameters for the double logarithmic regression of diclofenac and its three metabolites for 6 calibrations (66 samples)

Method	Intercept	Slope	*P*-value[a]	Regression coefficient (r^2)	n[b]
DF	−2.06 ± 0.07	0.964 ± 0.021	0.07	0.9992 ± 0.0004	1
DF-G	−1.62 ± 0.06	0.974 ± 0.016	0.08	0.9994 ± 0.0003	1
4′-H-DF	−2.00 ± 0.04	0.958 ± 0.014	0.015	0.9991 ± 0.0006	2
5-H-DF	−1.99 ± 0.05	0.973 ± 0.014	0.06	0.9992 ± 0.0004	3

[a] Significance of non-linearity, slope smaller than 1 using a 1-tailed normal distribution.

[b] Number of samples excluded for the regression calculations because of exceeding the ±15% (±20% for the LLQ) level of their target values.

Table 3
Assay performance data of diclofenac and three metabolites resulting from 18 validation (QC) samples in 3 analytical runs

Nominal concentration (ng/ml)	Within day precision (%)	Between day precision (%)	Accuracy (%)
Diclofenac			
8000	3.9	5.3	98.6
1000	3.7	4.3	100.1
60	6.1	7.4	95.9
20	4.7	9.0	101.8
Diclofenac-glucuronide			
8000	7.8	8.1	106.2
1000	5.6	6.9	108.7
60	8.4	12.0	103.8
20	6.6	8.6	108.3
4′-Hydroxy-diclofenac			
4000	5.3	8.1	97.0
500	7.7	8.0	101.4
30	7.7	9.7	90.2
10	9.5	12.6	94.0
5-Hydroxy-diclofenac			
8000	5.0	7.1	90.8
1000	5.8	7.5	96.4
60	8.1	10.0	90.0
20	7.8	11.6	92.2

Table 4
Extraction recovery (%, ±S.D., $n = 4$) of the assay

Concentration (ng/ml)	4′-H-DF	Concentration (ng/ml)	5-H-DF	DF-G	DF
4000	92 ± 10	8000	97 ± 11	93 ± 10	103 ± 12
500	77 ± 9	1000	83 ± 9	82 ± 6	89 ± 8
30	88 ± 8	60	82 ± 10	82 ± 8	97 ± 13

formic acid solution replaced was optimised to obtain optimal resolution between 5-H-DF, 4′-H-DF and another metabolite of DF (Fig. 2C). The third known monohydroxy-metabolite of diclofenac, 3′-hydroxy-diclofenac, was not investigated because it was not commercially available.

3.2. Mass spectrometry

During electrospray ionization, diclofenac and the hydroxy-metabolites formed the corresponding protonated molecules. DF-G, however, formed protonated diclofenac most prominently and additionally it formed the DF-G-sodium adduct. The ratio of these ions ($[M+Na]^+/[M+H\text{-glucuronide}]^+$) increased when formic acid was partially replaced by ammonium acetate in the aqueous eluent A. In addition, this adjusted eluent reduced the response and increased the detector noise, both ca. a factor 2, for all analytes resulting in a sensitivity loss of ca. a factor 4. Product spectra of protonated DF and 4′-H-DF and of the DF-G-sodium adduct are shown in Fig. 3. The main fragments can be explained by loss of formic acid and hydrochloric acid (DF (Fig. 3A) and 4′-H-DF (Fig. 3B)) and by loss of glucuronide (DF-G (Fig. 3C)), respectively. Electrospray ionization-MS/MS properties of both investigated hydroxy-metabolites were almost identical, chromatographic separation was therefore required. Further, potential presence of 3′-hydroxy-diclofenac, the minor monohydroxy-metabolite of DF, in the mouse samples will probably not be noticed due to insufficient resolution. Liquid chromatographic separation between 3′-hydroxy-diclofenac and 4′-DF was not expected in our assay because it was shown to be very difficult to separate them resulting in only poor resolution using long columns and long run times [11,15,16,23] or in no separation at all [14].

Table 5
Ionization recovery (%, ±S.D., $n = 4$) of the assay

Concentration (ng/ml)	4′-H-DF	Concentration (ng/ml)	5-H-DF	DF-G	DF
4000	85 ± 6	8000	84 ± 6	88 ± 5	83 ± 6
500	104 ± 8	1000	104 ± 8	103 ± 6	106 ± 9
30	90 ± 7	60	95 ± 10	96 ± 10	94 ± 7

Table 6
Stability data (recovery (%); ±S.D.; $n = 4$) of diclofenac and three main primary metabolites under different storage conditions in MMP, reporting the percentage of the initial concentration (4000 and 30 ng/ml for 4′-H-DF; 8000 and 60 ng/ml for 5-H-DF, DF-G and DF)

Compound	Conditions	QC-high	QC-low
4′-H-DF	24 h on ice	91 ± 6	89 ± 4
	4 freeze–thaw cycles	116 ± 15	111 ± 9
	11 weeks at −80 °C	94 ± 6	95 ± 7
5-H-DF	24 h at on ice	91 ± 5	87 ± 4
	4 freeze–thaw cycles	107 ± 16	109 ± 6
	11 weeks at −80 °C	92 ± 4	98 ± 4
DF-G	24 h on ice	80 ± 3	84 ± 4
	4 freeze–thaw cycles	98 ± 12	106 ± 10
	11 weeks at −80 °C	93 ± 7	99 ± 5
DF	24 h on ice	91 ± 2	94 ± 1
	4 freeze–thaw cycles	98 ± 4	107 ± 8
	11 weeks at −80 °C	98 ± 3	97 ± 6

To eliminate isotopic interference of diclofenac with the IS-signal the mass transition of ^{35}Cl-^{37}Cl-D_4-diclofenac (Table 1) was monitored as IS-signal.

3.3. Stability

Half-lives of DF-G in buffer at pH 7.4 were reported to be ca. 0.5 h at 37 °C [24]. In mouse plasma, however, a half-live of 42 s at that temperature was observed during development of the present assay. DF-G was therefore protected from acyl-migration and hydrolysis by adding 4% (v/v) of 2 M acetic acid in water to mouse plasma, increasing the half-live of DF-G at 37 °C to 2–2.5 h and to ca. 25 h at ambient temperature. In addition, sample treatment was performed on melting ice to improve stability. This acidification of mouse plasma (to ca. pH 5.0) increased the concentration-dependent oxidative degradation rate of 5-H-DF (half-lives ca. 2 h at 0.4 μg/ml and 7 h at 10 μg/ml at 3 °C). The formation and degradation of the *p*-benzoquinone derivative [25] of 5-H-DF could both also be observed at retention time 3.1 min in transition 310 → 166 at −42 V, obtained from Chen et al. [25]. Therefore, 1% (v/v) of 0.5 M ascorbic acid in water was added to the samples and consequently, 5-H-DF degradation and its *p*-benzoquinone derivative formation was eliminated, the *p*-benzoquinone transition was monitored during the whole validation procedure without observing any peak.

3.4. Validation

SRM chromatograms of the analytes and the IS are depicted in Fig. 2, showing chromatograms of blank MMP, LLQ spiked MMP and MMP from a diclofenac-treated mouse, respectively.

3.4.1. Calibration

The response of all analytes deviated from a linear function with a minor significance (Table 2); therefore, a double logarithmic function was used for the assay calibration. For all target compounds in the calibration samples (66 samples in 6 calibration curves), the concentrations were back-calculated from the ratio of the peak area (of analyte and IS) using the calibration curves of the run is which

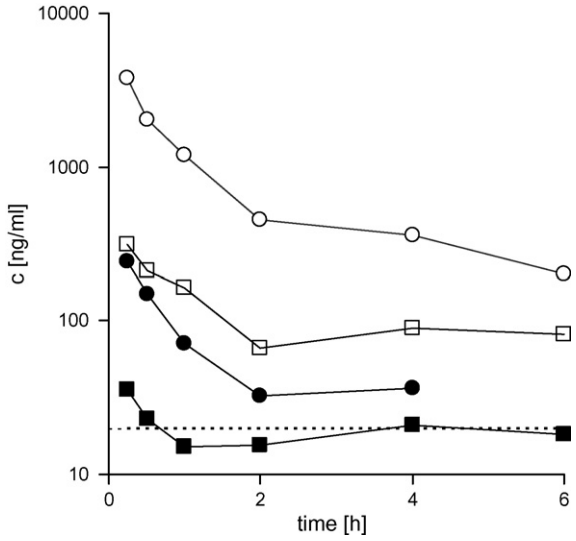

Fig. 4. Pharmacokinetic curves of diclofenac and three primary metabolites of a wild-type mouse after receiving 5 mg/kg diclofenac orally. - - - indicates LLQ level for all analytes but 4′-H-DF. (◯) DF; (□) 5-H-DF; (●) DF-G; (■) 4′-H-DF.

they were included and no deviations of the averages of each level higher than 7% were observed (data not shown), indicating the suitability of the regression model. The regression parameters of the double logarithmic regression functions are reported in Table 2. These data show reproducible calibration parameters.

3.4.2. Precision and accuracy

Assay performance data from the validation samples at 4 concentrations are reported in Table 3. No between day variations higher than 13% and no deviations of the accuracy higher than 10% were observed. Therefore, the upper limit of the calibration range can be assigned to the upper limit of quantification and precisions and deviations of the accuracy meet the required ±15% (±20% for the LLQ) [20–22] for all analytes.

3.4.3. Selectivity

The analysis of six batches of blank MMP showed no interfering endogenous peaks in all MS/MS traces, blank responses were therefore below 1%, 1%, 10% and 3% of the LLQ responses for 4′-H-DF, 5-H-DF, DF-G and DF, respectively. For the IS the blank response was below 0.05% of the regular signal. The average responses of the LLQ-spiked blank MMP samples ($n = 6$, ±S.D.) were 9.1 ± 0.7 ng/ml, 17.9 ± 0.7 ng/ml, 17.7 ± 0.7 ng/ml and 18.2 ± 1.0 ng/ml for 4′-H-DF, 5-H-DF, DF-G and DF, respectively. The LLQs are therefore 10 ng/ml for 4′-H-DF and 20 ng/ml for the other analytes [20–22].

3.4.4. Recovery

The recovery experiments showed some minor extraction losses (Table 4). Ion suppression (Table 5) could only be observed at the highest QC level for all analytes ($P < 0.01$, using a 1-tailed t-test). These data are very well suited for a chromatographic bioanalytical assay [20–22].

3.4.5. Stability

Recoveries of diclofenac and it metabolites in MMP after different storage procedures are shown in Table 6. DF-G in MMP seems to show some degradation during 24 h storage on ice, however, because sample treatment on ice does not take more than 2 h, these stabilities are all sufficient for standard use of the assay. Re-injection of calibration and validation samples after additional storage at 4 °C for 1–3 days resulted in successful performances, however with an increased number of QC samples exceeding the ±15% ranges of the accuracy for the three metabolites, additional storage at this temperature should therefore be limited.

3.4.6. In vivo samples

A concentration–time curve of diclofenac and its three most abundant primary metabolites in plasma in a wild-type mouse is shown in Fig. 4. Concentrations observed in plasma could all be assessed using the validated assay with only one exception (DF-G level at 6 h was <LLQ).

4. Conclusions

The first validated LC/MS/MS assay for the simultaneous quantitative analysis of diclofenac and three primary metabolites, DF-G, 4′-H-DF and 5-H-DF in plasma has been reported now. The assay uses a simple sample pre-treatment and meets common criteria for precision, accuracy and recovery. The sensitivity is in the same range as previous bioanalytical assays for only diclofenac and its hydroxy-metabolites but uses no more than 5% of the sample volume [9–11,13,14,17]. Sufficient stability of DF-G and 5-H-DF in mouse plasma, in addition to both other stable analytes, can be obtained by pH reduction and ascorbic acid addition, respectively. The assay will be a valuable tool in mouse pharmacokinetic studies, for studies in men partial re-validation will be required.

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