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Dedicated to Professor Florin Dan Irimie on the Occasion of His 65th Anniversary

VALIDATED LC-MS/MS METHOD FOR THE DETERMINATION OF THE NONSTEROIDAL ANTI-INFLAMMATORY DRUG (NSAID) DICLOFENAC FROM HUMAN PLASMA

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ABSTRACT. The purpose of this study was the development and validation of an LC-MS/MS method, for the determination of diclofenac from human plasma. The sample workup involved a simple protein precipitation procedure. A core/shell type analytical column (50×2,1 mm, 2.6 Å) was used with C18 stationary phase. The mobile phase consisting of 52.5% acetonitrile and 47.5% water provided good peak shape, accuracy and precision (stable ionization). The mass spectrometer was operated in negative electrospray ionization mode for analyte and internal standard. The following parameters were evaluated for validation purpose: Selectivity, sensitivity, matrix effect, anticoagulant effect, linearity, precision and accuracy, recovery, short and long term analyte/IS stability in solvent/matrix and carryover. The validated calibration range was 3.9-1194 ng/ml. The correlation coefficient R^2 was at least 0.999 in all validation batches. The validated method has been successfully used for the evaluation of bioequivalence of a generic diclofenac potassium formulation of 12.5 mg strength.

Keywords: *diclofenac, NSAID, method validation, bioequivalence trial, LC-MS/MS*

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INTRODUCTION

Diclofenac 2-[(2,6-dichlorophenyl)amino] benzeneacetic acid monopotassium salt with the empirical formula $C_{14}H_{10}Cl_2NKO_2$, is a nonsteroidal potent anti-inflammatory agent, an effective PGE (prostaglandin E) synthase inhibitor, platelet aggregation inhibitor, present analgesic and anti-pyretic effects. [1,7]. Inhibition of prostaglandin synthesis is considered fundamental to its mechanism of action, as prostaglandins play a major role of inflammation, pain and fever triggering. Diclofenac is generally used in form of sodium or potassium salt (Figure 1).

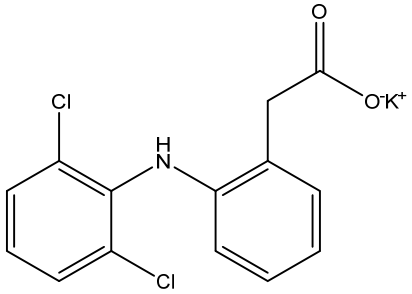

Figure 1. Structure of diclofenac potassium salt

RESULTS AND DISCUSSION

Determination of acquisition parameters

There are some methods known in the literature for the determination of diclofenac in human plasma or pharmaceutical formulations including topical products, using LC/UV [2,4,6] or LC-MS/MS methods [3,5,8].

The m/z transitions used for multiple reaction monitoring (MRM) were chosen based on the spectra from Figures 2 and 3. The monitored transitions should not interfere in their m/z value, specific for a given analyte. Their intensity should be convenient for the qualifiers, and the qualifier/quantifier ratio should remain stable over the time. Taking into account the considerations above the following transitions were chosen for the quantitative assay method:

Diclofenac: m/z 294.0→250.0, (296.1→252.0 qualifier ion) CE 5V,

Diclofenac acetophenyl ring-$^{13}C_6$ (IS): m/z 300.1→256.1

(302.1→258.1 qualifier ion) CE 5V. (CE – Collision Energy)

For analyte and IS (Internal Standard) the single charged molecular ions were used as precursors.

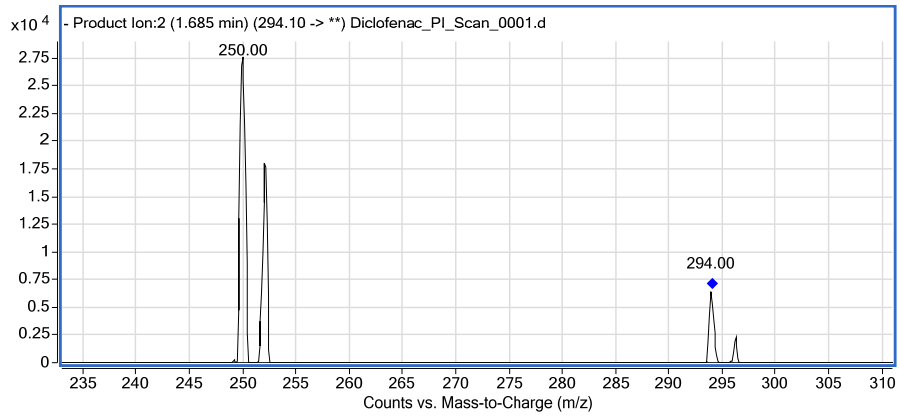

Figure 2. ESI(-) Spectrum of Diclofenac

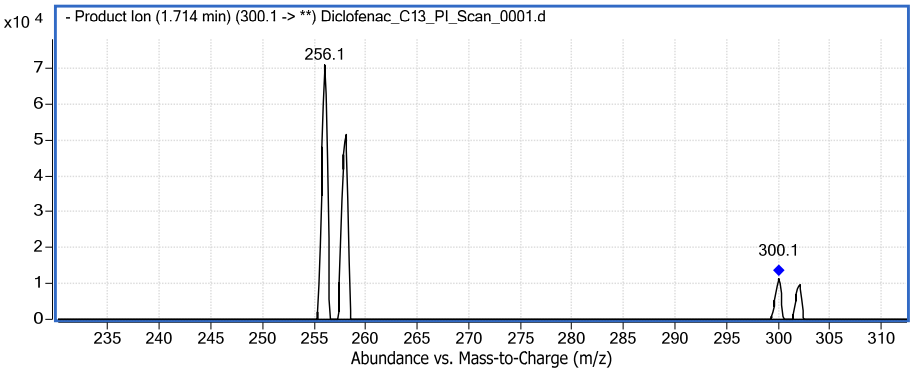

Figure 3. ESI(-) Spectrum of Diclofenac acetophenyl ring-$^{13}C_6$ (IS)

Figure 4 shows a typical MRM total ion chromatogram for an ULOQ (upper limit of calibration) sample. The analyte and IS are practically co-eluting at ca. 1.75 min. Values are back calculated concentrations for each analyte.

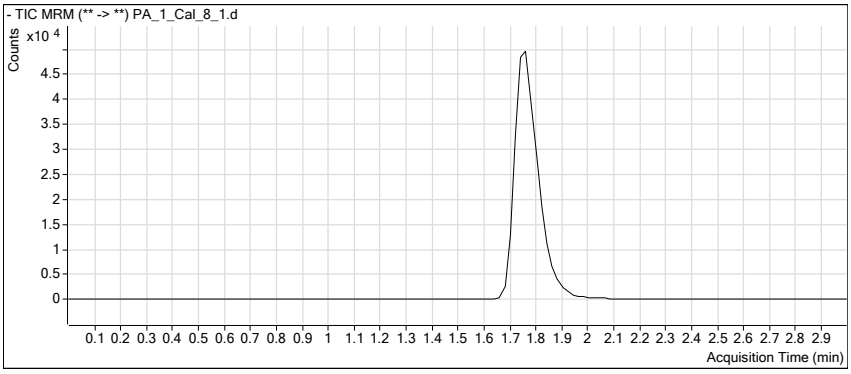

Figure 4. MRM chromatogram of Cal_8_1 (Diclofenac 1194 ng/ml, IS 58.9 ng/ml)

The use of the stable ^{13}C isotope labelled internal standard automatically leads to co-elution with the analyte. This is not an inconvenient in tandem mass spectrometry, also minimization of the matrix effect is achieved. It's noticeable, that no significant spectral response has been observed at the retention time of the analyte/IS in matrix blank samples (Figure 5.).

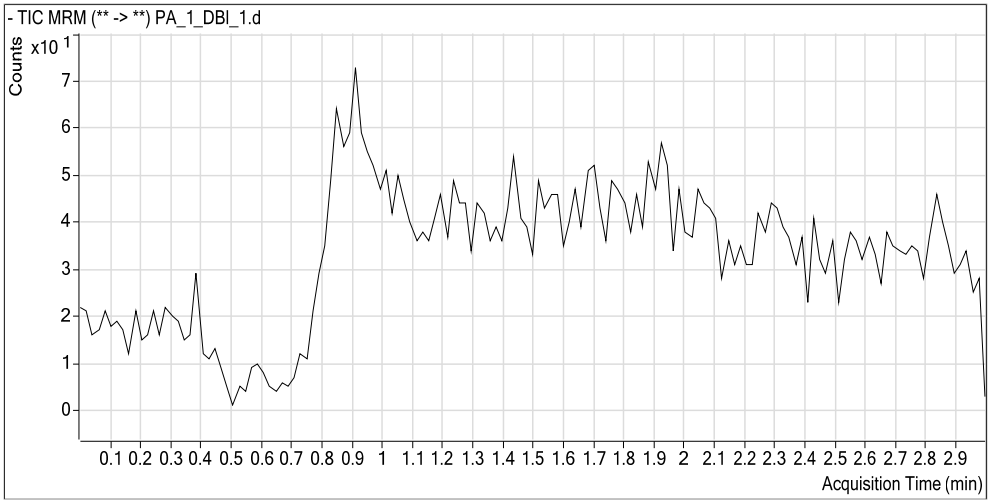

Figure 5. MRM chromatogram of DBI1 (matrix blank 0 ng/ml analyte/IS)

Bioanalytical method validation

The analytical method was validated according to the EMEA/CHMP/EWP/192217/2009 Guideline on validation of bioanalytical methods [11].

The tested parameters were: selectivity, sensitivity, matrix effect, intra/interbatch precision and accuracy, recovery, short/long term stability of stock solutions of analyte, short term stability of working solutions of analyte, bench top stability in biological matrix, freeze thaw stability in biological matrix, injector/autosampler stability of the processed samples, stability during delayed processing, dilution integrity, carryover. All tests were performed using 6 replicates at the mentioned QC (Quality Control) levels.

The calibration curve range is established according to literature data about plasma concentrations of the analyte. $C_{max\ average\ from\ literature}$ for diclofenac was found of ca. 450-600 ng/ml, after administration of a 25 mg dose. [2,9,10]

A summary of main results of validation batches is presented in Table 1.

The validated calibration range was 3.9-1194 ng/ml. The calibration curves were obtained using a linear weighted (1/x) regression analysis of the peak area ratio (analyte/internal standard) versus the nominal concentration of the calibration standards. The lower limit of quantitation was set smaller than 5% of expected average C_{max} values. A typical calibration curve is presented in Figure 6.

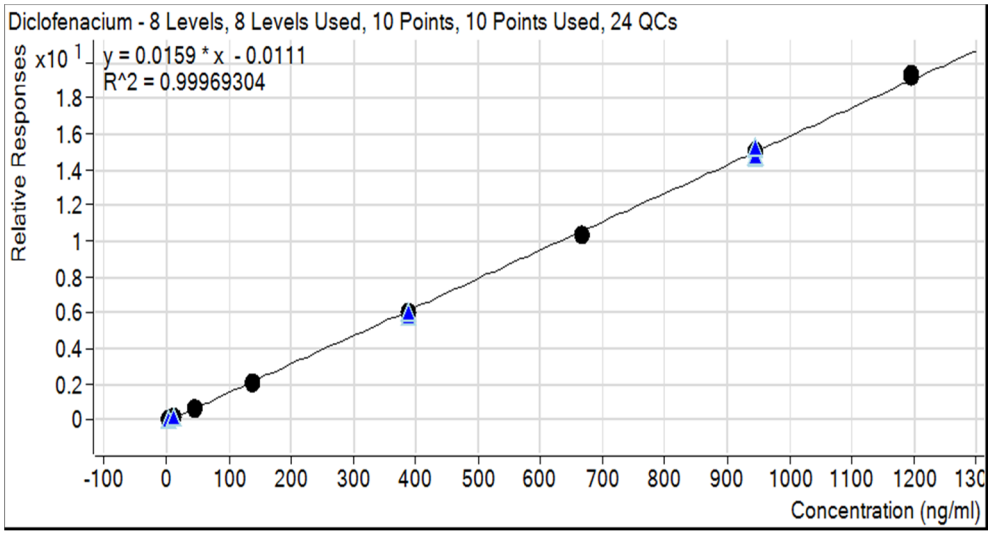

Figure 6. Calibration curve for diclofenac

Linearity summary results for diclofenac are presented in Table 2. The limit of quantitation was 3.9 ng/ml and the linear dynamic range of the curve was from 3.9-1194 ng/ml.

Summary of method validation

Table 1. Bioanalytical method validation summary for diclofenac

Calibration concentrations (ng/ml)	3.89, 11.11, 44.44, 138.88, 388.86, 666.62, 944.38, 1194.37
Lower limit of quantitation (ng/ml)	LLOQ, 3.89 Accuracy 103.28 %, RSD 4.52
QC Concentrations (ng/ml)	LLOQ-QC, LQC, MQC, HQC 3.89, 11.11, 388.86, 944.38
Between-run accuracy (%)	LLOQ-QC, LQC, MQC, HQC 104.67, 100.25, 101.47, 102.90
Between-run precision (RSD)	LLOQ-QC, LQC, MQC, HQC 1.49, 1.35, 2.28, 2.02
IS normalized Matrix factor (MF) RSD	LQC, HQC 1.06, 1.01 5.35, 0.95
Recovery (%)	LQC, MQC, HQC 94.04, 99.68, 98.60
Long term stability of stock solution and working solutions (Observed change %)	Confirmed up to 39 days at +4 °C LQC Stab. 98.99, change –1.01 % HQC Stab. 106.53, change +6.53 % IS Stab. 95.01, change – 4.99%
Short term stability in biological matrix at room temperature or at sample processing temperature. (Observed change %)	Confirmed up to 20.81(6) h LQC Stab. 104.91, change +4.91 % HQC Stab. 102.05, change +2.05 %
Long term stability in biological matrix (Observed change %)	Confirmed up to 125 days at –50 °C LQC Stab. 101.44, change +1.44 % HQC Stab. 96.21, change –3.79 %
Autosampler storage stability (Observed change %)	Confirmed up to 80.5(6) h LQC Stab. 104.87, change +4.87 % HQC Stab. 101.36, change +1.36 %
Freeze and thaw stability (Observed change %)	-50 °C, 3 cycles LQC Stab. 101.58, change +1.58 % HQC Stab. 101.14, change +1.14 %
Dilution integrity	Concentration diluted (2-fold) 101.26 %; RSD 1.04 % Concentration diluted (4-fold) 104.02 %; RSD 0.98 %

PA – Precision and Accuracy batch
LLOQ-QC/LQC/MQC/HQC – Lower Limit of Quantitation/Low/Medium/High Quality Control sample

Table 2. Linearity summary results for diclofenac

Calibration level	Nominal conc. (ng/ml)	Mean conc.±S.D. (ng/ml) n=3	RSD %	Accuracy %
Cal_1_1	3.89	4.08±0.20	4.97	104.96
Cal_1_2	3.89	4.23±0.32	7.60	108.74
Cal_2	11.11	10.45±0.32	3.03	94.02
Cal_3	44.44	41.93±1.83	4.37	94.35
Cal_4	138.88	136.85±3.12	2.28	98.54
Cal_5	388.86	386.87±3.48	0.90	99.49
Cal_6	666.62	655.00±11.88	1.81	98.26
Cal_7	944.38	949.32±8.34	0.88	100.52
Cal_8_1	1194.37	1198.59±21.73	1.81	100.35
Cal_8_2	1194.37	1203.49±23.86	1.98	100.76

CONCLUSIONS

A rapid, sensitive, and robust method has been developed and validated for the determination of diclofenac in human plasma. The quantitation was performed on an Agilent 1200 series HPLC system, coupled to an Agilent 6410 triple quadrupole mass spectrometer, using electrospray ionization technique. The components were detected in negative ionization mode. The method was successfully used for the evaluation of bioequivalence of a generic formulation of diclofenac potassium 12.5 mg film-coated tablets in human subjects. The administered dose was 25 mg (two tablets).

EXPERIMENTAL SECTION

Solvents and reference materials used

All used solvents are of HPLC grade. Acetonitrile was purchased from VWR, methanol from LGC Standards, formic acid and hydrochloric acid from Merck KGaA, ammonium acetate was purchased from Sigma-Aldrich, HPLC water was obtained using a Millipore Simplicity UV water purification system. Certified reference materials of Diclofenac sodium and Diclofenac acetophenyl ring-$^{13}C_6$ sodium salt heminonahydrate (internal standard-IS) were obtained from Sigma-Aldrich and are of analytical standard grade. Blank human plasma was obtained from Innovative Research/Dunn Labortechnik GmbH.

Instrumentation and working parameters

An Agilent 1200 series HPLC system with a Phenomenex Kynetex C18 column (50 × 2.10 mm) equipped with Phenomenex Security Guard (4 ×2.0 mm) was used for separation. The used mobile phase was an isocratic mixture of 52.5:47.5 acetonitrile:water (containing 1 ml 5% ammonium acetate and 1 ml formic acid per 1L of water). The used flow rate was 0.3 ml/min., the column temperature was set to 35 ºC. An Agilent 6410 triple Quadrupole Mass Spectrometer (Agilent Technologies, USA), equipped with electrospray ion source was used for the LC-MS/MS analyses. The runtime was 3 min/sample. The data acquisition and processing were carried out using MassHunter software. The whole system (software and hardware) was validated. The mass spectrometer was operated in negative ionization mode for analyte and IS. Nitrogen was used as nebulizing gas and collision cell gas. The temperature of the ESI source was set to 350 ºC, and the needle voltage to 4000V.

The quantitation was performed using MRM (multiple reaction monitoring) of the transitions: m/z 294.0→250.0, (296.1→252.0 qualifier ion) CE 5V, for diclofenac and 300.1→256.1 (302.1→258.1 qualifier ion) collision energy 5V for Diclofenac acetophenyl ring-$^{13}C_6$ (IS).

The mass spectrometer was operated at unit resolution with a dwell time of 300 ms per transition.

Stock and working solutions preparation

Stock solutions of diclofenac (1.0 mg/ml) were prepared in acetonitrile/water 50/50 (w/w) dissolving accurately weighed amounts of reference material. Stock solutions of IS (1.0 mg/ml) were prepared in acetonitrile/water 50/50 (w/w) dissolving accurately weighed amounts of diclofenac acetophenyl ring-$^{13}C_6$. They were stored between 2-8 °C. Correction factors were applied to the weighed amounts of reference materials to calculate the content of the pure substance (Table 3). Correction factors are derived from the purity and the chemical form (salt). Water content will be substracted from the purity.

Table 3. Correction factors for reference materials

Reference material	Diclofenac	Diclofenac $^{13}C_6$
Purity (%)	99.9	99.9
Water (%)	n/a	19.9
Chemical form correction factor	0.9309	0.9291
Correction factor	**0.9300**	**0.7432**

Working solutions of analyte and internal standard were prepared freshly before use by successive dilutions from stock solutions to appropriate levels, using acetonitrile/water 50/50 (w/w) as solvent. They were used for spiking human plasma used for calibrators and QC samples preparation.

Calibrators and QC samples preparation

To 400 µl of blank human plasma, 50 µl of spiking solution of analyte and 50 µl of spiking solution of internal standard were added in polypropylene tubes, to yield final concentrations of 3.89, 11.11, 44.44, 138.88, 388.86, 666.62, 944.38, 1194.37 ng/ml for diclofenac.

Sample preparation (workup)

To precipitate plasma proteins, 2 ml of methanol (containing 5 ml of 25% HCl/2.5 L) was added to the spiked samples, then vortexed for 10 minutes at 1500 rpm. Further the samples were centrifuged at 4 °C for 10 minutes at 4000 rpm. 500 µl of supernatant was transferred to HPLC autosampler vials diluted with 200 µl of water and injected into the analytical system (25 µl/sample).

Calibration curve parameters

The linearity of the method was evaluated using spiked plasma samples in the concentration range mentioned above using the method of least squares. Three linearity curves were analyzed.

Each calibration batch (curve) consisted of: blank samples in duplicate, zero samples (blank with IS) in duplicate and eight non-zero concentration levels, of which the lower and upper limit of quantitation samples were in duplicate. The calibration curves were obtained by using a linear weighted (1/x) regression analysis of the peak area ratio (analyte/internal standard) versus the nominal concentration of the calibration standards. Study samples concentrations were obtained by interpolation from the calibration curve.

The linearity results are summarized in Table 2 in the ‘Results and Discussion’ section.

ACKNOWLEDGMENTS

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