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LC-MS/MS METHOD FOR THE QUANTIFICATION OF DICLOFENAC FROM HUMAN PLASMA AND ITS APPLICATION TO PHARMACOKINETIC STUDIES

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A simple, reversed-phase high performance liquid chromatography method with mass spectrometric detection (HPLC-MS/MS) was developed for determination of diclofenac in human plasma. The procedure involves a simple protein precipitation step by mixing 0.2 mL plasma with 0.6 mL methanol. After centrifugation, 5µl of the supernatant was injected onto a Gemini NX – C18 50×2.0 mm, 3µm column, and eluted with a mobile phase consisting in a mixture of water containing 0.2% acetic acid and acetonitrile 47:53 (v/v). Detection was in MRM mode, using an electrospray negative ionization. The ion transition monitored was: *m/z* (294+296) → *m/z* (250+252). The method was evaluated in terms of linearity, accuracy, precision, recovery, sensitivity. The LOQ was established at 24.2 ng/mL. The simple extraction procedure and short chromatographic runtime make the method suitable for therapeutic drug monitoring studies.

INTRODUCTION

Diclofenac, 2-(2',6'-dichloroanilino)-phenylacetic acid (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, combined with low enterohepatic circulation reduces oral bioavailability of diclofenac in humans to 50-60% of the administered dose.[3] Biliary excretion of diclofenac in rats is critically dependent on multidrug resistance protein (Mrp)2., an efflux pump located at the canalicular membrane of hepatocytes.[4]

Chromatographic methods capable to quantify diclofenac and one or more hydroxy- metabolites in human plasma[5] or urine[6,7] and in rat plasma[8] have been reported using LC-UV,[5-8] GC-electron capture detection[9] and LC-electrochemical detection.[10] Lower limits of quantification in the range 5-25 ng/mL can be obtained using these techniques, using 200-2000 µl urine or plasma. For sample pre-treatment liquid-liquid extraction,[5,8,9] mostly using an ethereal solvent, is often used. Solid phase extraction was also reported as a suitable option.[10] No validated LC/MS (/MS) method has been published for diclofenac determination from human plasma so far.

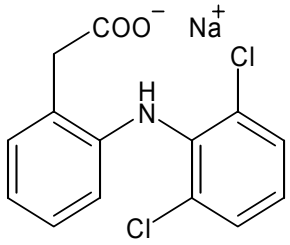

Fig. 1 – Molecular structure of diclofenac.

Therefore, the aim of the present study was to develop a fast HPLC-MS/MS method, able to

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quantify diclofenac in human plasma, by applying a simple protein precipitation extraction procedure. Finally, the developed and validated method was applied on a pharmacokinetic study on healthy volunteers.

RESULTS AND DISCUSSION

We propose a very simple and rapid pretreatment of plasma samples including only PP with methanol and direct injection into the chromatographic system from the supernatant obtained after centrifugation. In LC/MS assays, the sensitivity depends on the MS detection mode, but the method involved in sample preparation may influence the chromatographic background level and can generate a matrix suppression effect.

In order to avoid any interferences from other compounds (exogenous or endogenous) which may co-elute with the selected compound MS/MS detection was performed in MRM (multiple reaction monitoring) mode. Ionization of analyts was carried out using an ESI (electrospray ionization) ion source, operated in the negative ion mode. No interference at the retention time of diclofenac was observed.

In the case of diclofenac, the sum of ions from MS spectrum *m/z* (250, 252) was chosen to be quantified.

The applied calibration curve model $y = ax + b$ proved to be accurate over the concentration range 24.2 – 3100.8 ng/mL, with a correlation coefficient grater than 0.995.

The method had within- and between-run accuracy and precision (Tables 1 and 2) in agreement to international regulations regarding bioanalytical methods validation.[11-13]

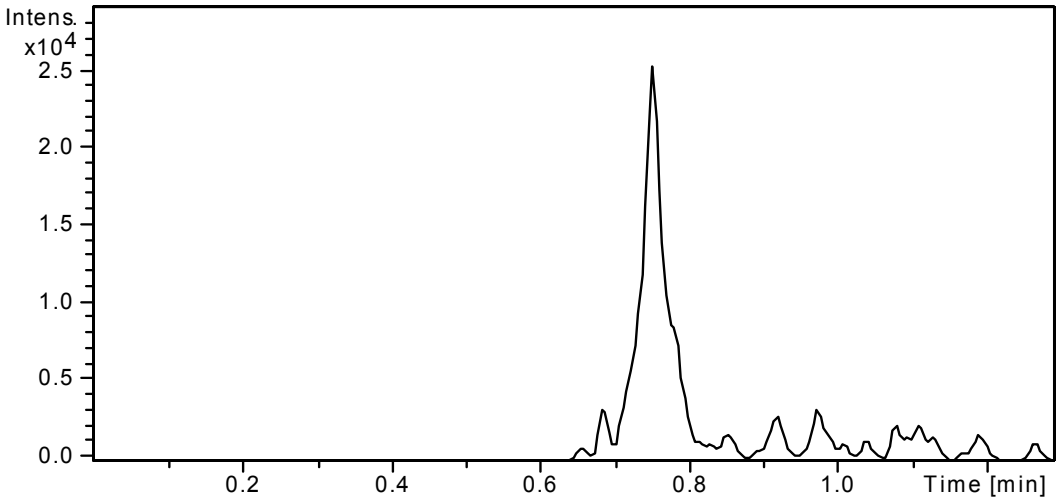

Fig. 2 – Chromatogram for LOQ plasma standard containing 24.2 ng/mL diclofenac.

Table 1

Within-run precision, accuracy and recovery for diclofenac (n = 5)

$c_{nominal}$ (ng/mL)	Mean c_{found} (ng/mL) (± S.D.)	C.V. %	Bias %	Recovery % (± S.D.)
24.22	26.94 (1.52)	5.7	11.2	90.1 (8.2)
146.25	154.67 (12.01)	7.8	5.8	106.0 (9.0)
292.50	288.26 (12.79)	4.4	-1.4	90.7 (4.1)
585.00	608.63 (29.71)	4.9	4.0	101.1 (5.0)

Table 2

Between-run precision, accuracy and recovery for diclofenac (n = 5)

$c_{nominal}$ (ng/mL)	Mean c_{found} (ng/mL) (± S.D.)	C.V. %	Bias %	Recovery % (± S.D.)
48.75	44.10 (6.44)	14.6	-9.5	83.4 (9.4)
146.25	155.79 (4.20)	2.7	6.5	103.1 (11.8)
292.50	302.03 (13.44)	4.4	3.3	99.9 (6.6)
585.00	621.46 (14.96)	2.4	6.2	100.9 (6.3)

The recovery was consistent and reproducible for the analyte (Table 1).

The validated method was verified during analysis of clinical samples. The method continued to perform in terms of accuracy, in each analytical run not more than two out of six QC samples being outside of ±15% nominal value, but not all two at the same concentration. The method was used to analyze all plasma samples from a pharmacokinetic interaction study between diclofenac and grapefruit juice on healthy volunteers. Figure 3 shows concentration profile for diclofenac administered alone and diclofenac administered with grapefruit juice respectively, for one volunteer.

In comparison with previously published HPLC methods (Table 3), the main advantage of the proposed method is the simple sample preparation by protein precipitation, without significant matrix effect. Besides its simplicity, the method is efficient for analysis of a large number of plasma samples (one run is completed in 1.3 min) and thus is more productive and cost effective.

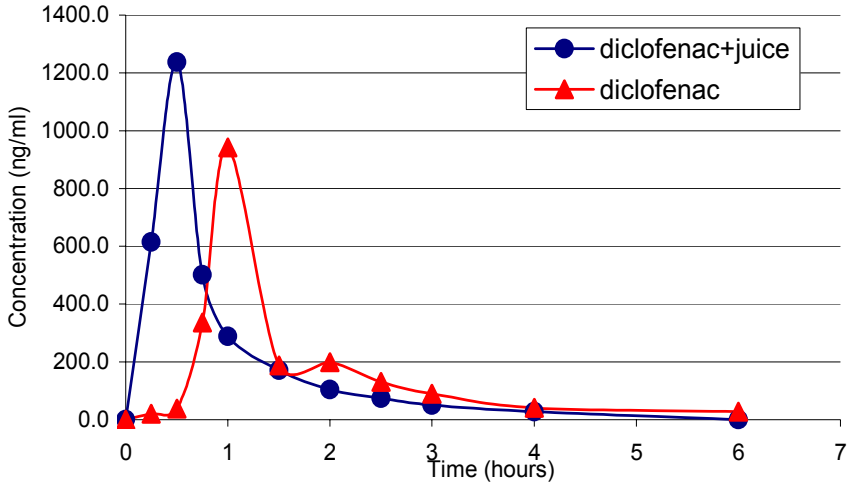

Fig. 3 – Concentration profile for one volunteer.

Table 3

Comparison between analytical characteristics for previously reported HPLC methods used for determination of diclofenac, with the proposed method

References	Detection	Species and matrix	Pretreatment/ extraction	LOQ (ng/mL)	Run Time (min)
H. S. Lee et al[14]	HPLC-column swiching	human plasma	PP	10	17
C. Arcelloni et al[15]	HPLC-UV	human plasma	SPE	5	7
R. B. Miller[16]	HPLC-UV	human plasma	SPE	2.5	6.4
A. Avgerions[17]	HPLC-UV	human plasma and urine	no extraction	200	2.8
D. Muntean et al	HPLC-MS/MS	human plasma	PP	24.2	1.3

SPE-solid phase extraction; PP-protein precipitation

EXPERIMENTAL

Reagents

Diclofenac, acetonitrile, methanol and acetic acid were purchased from Merck (Merck KgaA, Darmstadt, Germany). Distilled, deionised water was produced by a Direct Q-5 Millipore (Millipore SA, Molsheim, France) water system. The human blank plasma was supplied by the Local Bleeding Centre Cluj-Napoca, Roumania.

Standard solutions

A stock solution of diclofenac with concentration of 975 mg/mL was preparated by dissolving 10.3 mg diclofenac (as sodium salt) in 10 mL methanol-water 50/50 (v/v). A

working solution was prepared by diluting specific volume of stock solution with plasma. Than this was used to spike different volumes of plasma blank, providing finally eight plasma standards with the concentration ranged between 24.2 and 3100.8 ng/mL. Quality control samples (QC) were prepared in order to evaluate the precision and accuracy of the method during clinical samples analysis.

Chromatographic and mass spectrometry systems and conditions

The HPLC system was an 1100 series model (Agilent Technologies), consisted in a binary pump, an in-line degasser, an autosampler, a column thermostat and an ion trap VL mass spectrometer detector (Bruckner Daltonics GmbH, Germany). The ion transition monitored was: *m/z* (294+296) → *m/z* (250+252). Chromatographic separation was performed at 45ºC on a Gemini NX – C18 50x2.0 mm, 3µm column, protected by an in-line filter.

The HPLC system was coupled to an Agilent MSD VL Ion Trap detector (Bruker Daltonik, GmbH, Brehmen, Germany) equipped with an electrospray interface (ESI) operated in the negative ionization mode. Chromatographic and mass spectrometric data acquisition were performed using Chemstation software (Agilent Technologies, Palo Alto, CA, USA), version B.01.03 and LC/MSD Trap Control (Bruker Daltonik, GmbH, Brehmen, Germany), version 5.3, while data processing was performed using LC/MSD Quant Analysis (Bruker Daltonik, GmbH, Brehmen, Germany), version 1.7. Nitrogen was used as drying gas (12 L/min, 350 ◦C) and nebulizing gas (65 psi). The capillary voltage was set at 4000V, while the capillary exit potential was set at 100V. The trap drive was set at 52V.

Mobile phase

In order to find the suitable mobile phase, which provides a good ionization and a maximum sensibility of the analytical method, a solvent screening was achieved. In all cases the organic solvent used was acetonitrile, in proportion of 53% and to water phase were added salts or organic acids in order to modify the conductibility and the pH. It was compared the ratio signal/noise (S/N) for a standard solution containing diclofenac with concentration of 500 ng/mL, both in negative (MS^2) and positive (MS^3) ionization (Fig. 5).

The suitable mobile phase proved to be a mixture of water containing 0.2% acetic acid and acetonitrile 47:53 (v/v) using ESI negative ionization. The pump delivered it at 0.6 mL/min.

Sample preparation

Plasma samples were prepared as follows in order to be chromatographically analyzed: in an Eppendorf tube (max 1.5 mL), 0.2 mL plasma and 0.6 mL methanol were added. The tube was vortex-mixed for 10 s (Vortex Genie 2, Scientific Industries) and centrifuged for 6 min at 12000 rpm (2-16 Sartorius centrifuge, Osterode am Harz, Germany). The supernatant was transferred to an autosampler vial and 5µl were injected into the HPLC system.

Validation

As a first step of method validation,[11-13] specificity was verified using six different plasma blanks obtained from healthy volunteers who had not previously taken any medication.

The concentration of the analyte was determined automatically by the instrument data system. The calibration curve model was $y = ax + b$, weight 1/y linear response, where y-peak area and x-concentration. Distribution of the residuals (% difference of the back-calculated concentration from the nominal concentration) was investigated. The calibration model was accepted, if the residuals were within ±20% at the lower limit of quantification (LOQ) and within ±15% at all other calibration levels and at least 2/3 of the standards meet this criterion, including highest and lowest calibration levels.

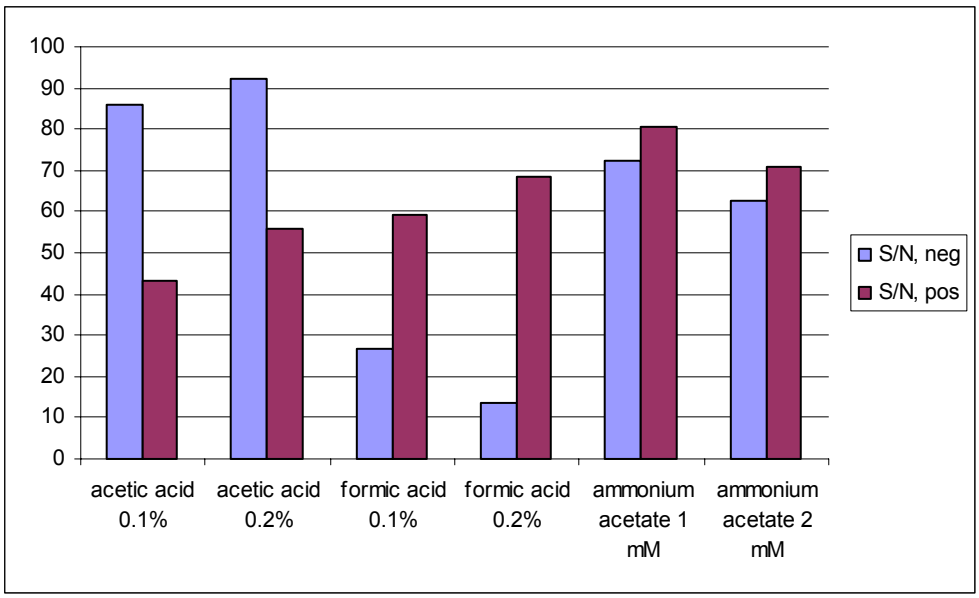

Fig. 4 – Solvent screening for a solution containing 500 ng/mL diclofenac.

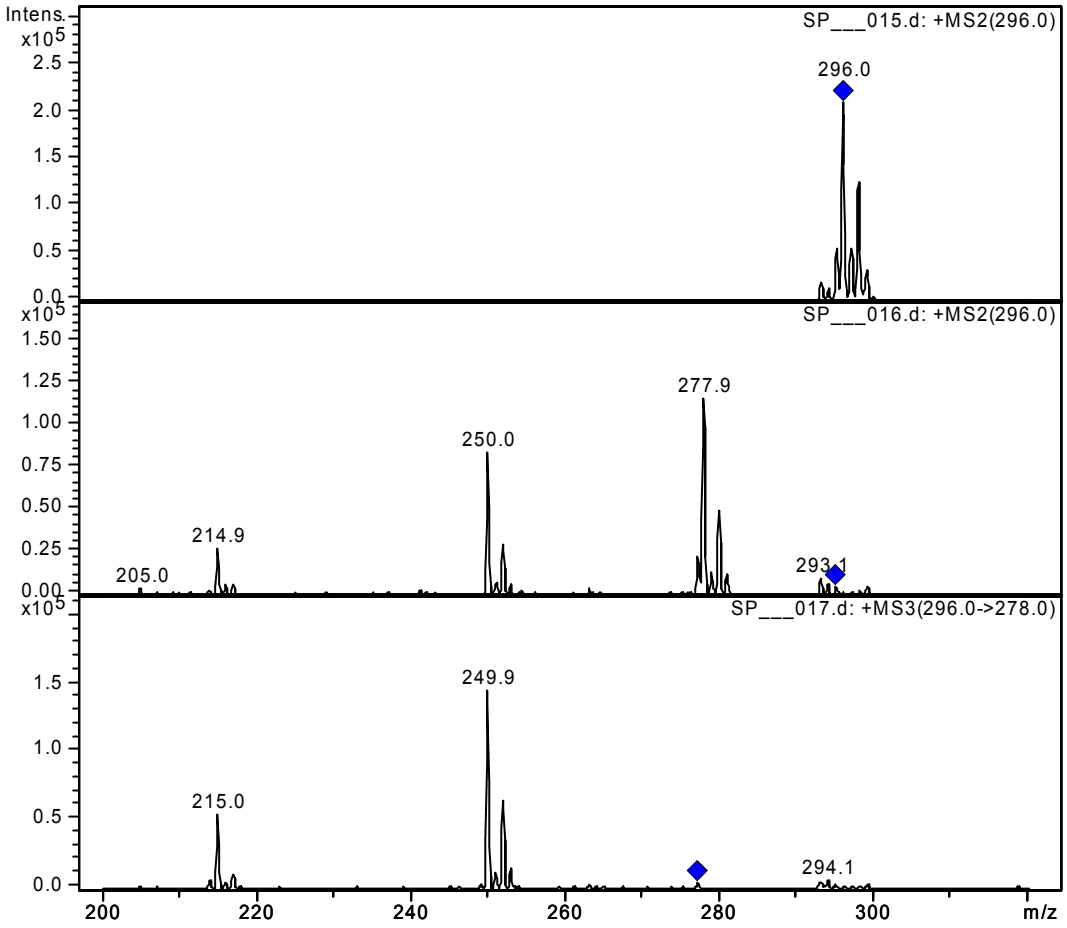

Fig. 5 – Mass spectra for diclofenac. ESI positive ionisation. Full scan spectrum (up), MS/MS spectrum (middle) and MS^3 (down).

The lower limit of quantification was established as the lowest calibration standard with an accuracy and precision less than 20%.

The within- and between-run precision (expressed as coefficient of variation, CV%) and accuracy (expressed as relative difference between obtained and theoretical concentration, bias%) of the assay procedure were determined by analysing on the same day five different samples at each of the lower, medium and higher levels of the considered concentration range and one different sample of each at five different occasions, respectively.

The stability study for diclofenac was not assessed, based on existing data in the literature.[18]

CONCLUSIONS

Our developed HPLC/MS/MS assay is simple, rapid, accurate, and inexpensive. In comparison with other published HPLC/UV assays for therapeutic level monitoring of diclofenac in human plasma, our method performed better in terms of speed (both sample preparation and chromatographic run time) and costs, which are essential attributes for methods used in routine analysis. The method was validated over the concentration range of 24.2 – 3100.8 ng/mL, which covers therapeutic plasma levels of diclofenac. The method is suitable for therapeutic drug monitoring studies and can also be used for pharmacokinetic studies conducted on healthy volunteers.

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