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Comparative *in vitro* and *in vivo* evaluations of oral sustained-release formulations of diclofenac sodium in beagle dogs

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Purpose: Two sustained release formulations (microspheres and Voltaren SR75®) were evaluated for their drug release characteristics in dissolution (*in vitro* study) and after oral administration to beagle dogs (*in vivo* study) by HPLC. *Methods:* The dissolution study was carried out according to the paddle method and the pharmacokinetic study was conducted using HPLC analysis in a crossover design in six female beagle dogs after oral administration of 75 mg diclofenac sodium (DFS). *Results:* The dissolution profiles showed 45% release for Voltaren SR75 and around 95% for the microspheres. Oral administration of DFS resulted in $AUC_{(0-24)}$ and C_{max} values of 20.4 μg.h/mL and 3.04 μg/mL for microspheres and 33.5 $\mu g \cdot h/mL$ and 5.59 μg/mL for Voltaren, respectively. The T_{max} was 3.0 h for both formulations. A significant difference in $AUC_{(0-24)}$ and C_{max} was observed for DFS absorption from microspheres and Voltaren. *Conclusions:* The results from the dissolution assay demonstrated the faster release of diclofenac sodium from microspheres. The bioavailability of DFS in microspheres was about 61% that of Voltaren, for the parameters AUC and C_{max}, and they are therefore not bioequivalent to Voltaren in relation to the extent of absorption. However, the rate of drug absorption (T_{max}) was similar for the two formulations.

1. Introduction

Diclofenac sodium is a non-steroidal anti-inflammatory drug (NSAID) commonly used for the long-term treatment of rheumatic disorders, such as osteoarthritis, rheumatoid arthritis and ankylosing spondylitis. Several unwanted adverse effects are generally associated with the long term oral administration of NSAIDs, including stomach ulcerations, abdominal burning, pain, cramping, nausea, gastritis, and even serious gastrointestinal bleeding and liver toxicity (Skoutakis et al. 1988).

This drug is rapidly and almost completely absorbed in the gastrointestinal tract but has a short biological half-life time ($t_{1/2} = 2$ h). Despite this short half-life, diclofenac also has a high percentage of protein binding and undergoes pre-systemic metabolism. Thus, diclofenac needs to be taken frequently to maintain its therapeutic activity (Tunçay et al. 2000a,b).

Due to its biopharmaceutical and pharmacological properties sustained release formulations of diclofenac are desirable. This approach should maximize the therapeutic benefits and reduce the unwanted side effects, since the frequency of administration is diminished, improving the therapeutic efficacy and patient compliance (Rattes and Oliveira 2007).

Recently, the association of polymers in matricial systems have been demonstrated to be a interesting strategy to modulate the drug release profile. The introduction of a second polymer in the matrix leads to changes in the permeability and degradation pattern of the system, providing a way to control the rate of the drug release. In this study cellulose acetate butyrate (CAB) was used in association with poloxamer Pluronic F68 for the preparation of the microspheres. This experimental controlled-released diclofenac formulation was compared to Voltaren SR75, chosen as the reference formulation.

A comparison between the *in vitro* release kinetics of different matrices and the *in vivo* performance is thus an important issue in relation to evaluating different formulations of diclofenac sodium (Sheng-Fang Su et al. 2003).

High-Performance Liquid Chromatography (HPLC) methods for measuring diclofenac sodium in plasma samples have been described by several authors (Santos et al. 1992; Hosny et al. 1996, 1997). So, an HPLC method with ultraviolet detection was chosen because it is sensitive, simple, rapid and economic.

2. Investigations and results

2.1. Dissolution study

In the *in vitro* study, no drug was released from the commercial enteric coated tablets (Voltaren SR75) during the first 2 h (Fig. 1). Conversely, the microspheres containing diclofenac sodium released approximately 15% in same

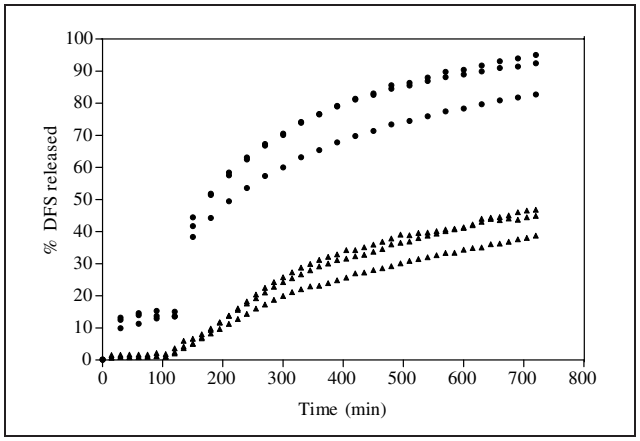

Fig. 1: Profiles of *in vitro* dissolution of diclofenac sodium from commercial enteric coated tablets (▲) and microspheres (●) in hydrochloric acid 0.1 M in first two hours and phosphate buffer pH 6.8 maintained at 37 °C at 100 rpm, according to paddle method (1)

time period. As the pH increased to 6.8, after 12 h of dissolution, approximately 95% of the DFS was released from the microspheres, whereas, around 45% was released from the reference formulation Voltaren SR75 in the same experimental time period.

Table 1: Precision (CV) and accuracy (analytical recovery) of HPLC analysis of DFS in dog plasma

DFS Concentration (μg/mL)	Accuracy (%) (Mean ± SD)	Within-day precision (%)
0.3	139.2 ± 16.8	12.0
3.0	93.5 ± 2.5	2.7
6.0	89.2 ± 5.5	6.1

2.2. Chromatographic validation

The calibration curve consisted of plasmatic concentrations of 0.1, 0.25, 0.5, 1.0, 2.0, 4.0, 8.0 μg/mL of DFS, and gave a mean correlation coefficient of 0.999 ($n = 9$). The CV values for these points were: 14.2, 15.3, 9.3, 6.2, 5.5, 3.2 and 1.0%, respectively (data not shown). The CV values obtained for within-day precision and accuracy are shown in Table 1. The LOQ was 0.1 μg/mL (CV $\leq$ 20%) and LOD was 0.05 μg/mL. The precision and accuracy data demonstrate that the method is acceptable.

The reverse-phase HPLC analysis with UV detection allowed the adequate separation of DFS and indomethacin (I.S.) in dog plasma samples. Figure 2 presents examples

Table 2: Pharmacokinetic parameters of microspheres and Voltaren SR75

Parameters	Microspheres	Voltaren SR75®
$AUC_{[0-24]}$ ($\mu g \cdot h \cdot mL^{-1}$)	20.4	33.5
Geom. mean	17.0–24.6	26.0–43.1
90% CI		
$AUC_{[0-\infty]}$ ($\mu g \cdot h \cdot mL^{-1}$)	22.3	35.5
Geom. mean	18.7–26.6	27.9–45.3
90% CI		
C_{max} (μg/mL)	3.04	5.59
Geom. mean	2.11–4.37	3.49–8.95
90% CI		
K_e (h^{-1})	0.177	0.187
Geom. mean	0.135–0.231	0.126–0.277
90% CI		
$t_{1/2}$ (h)	3.923	3.708
Geom. mean	2.997–5.134	2.501–5.498
90% CI		
T_{max} (h)	3.0	3.0
Median	0.5–6.0	3.0–6.0
Range		

Geom. mean – Geometric mean
CI – Confidence interval

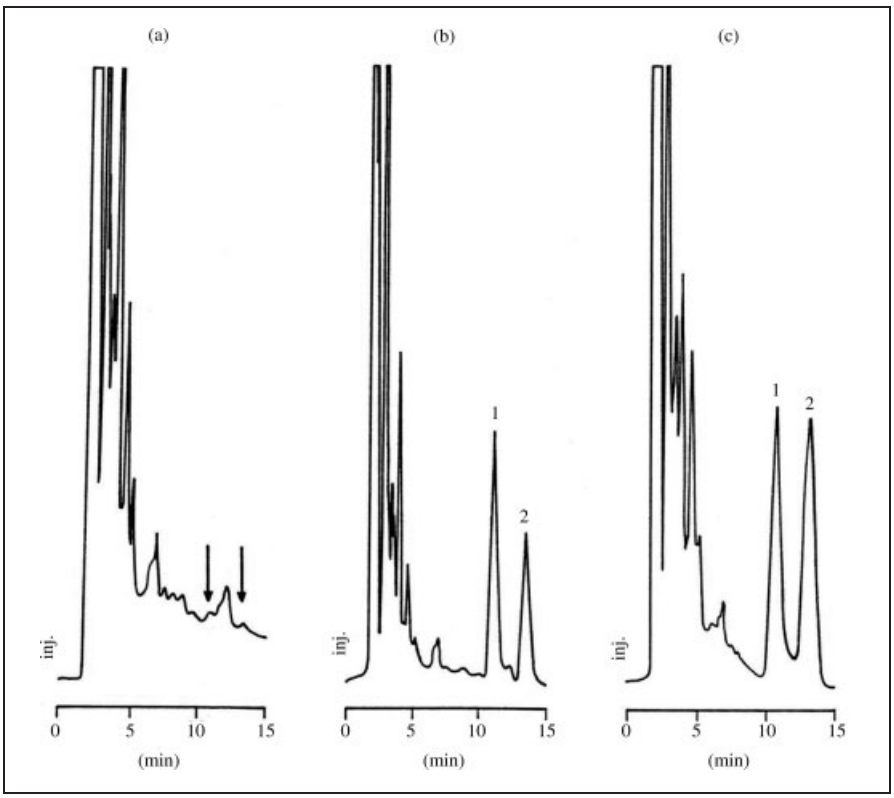

Fig. 2:
Examples of HPLC chromatograms obtained for. (a) blank plasma; (b) blank plasma spiked with DFS 2.0 μg/mL; and (c) plasma collected 30 min after oral administration of microspheres containing 75 mg DFS. 1 – DFS and 2 – Indomethacin, IS.

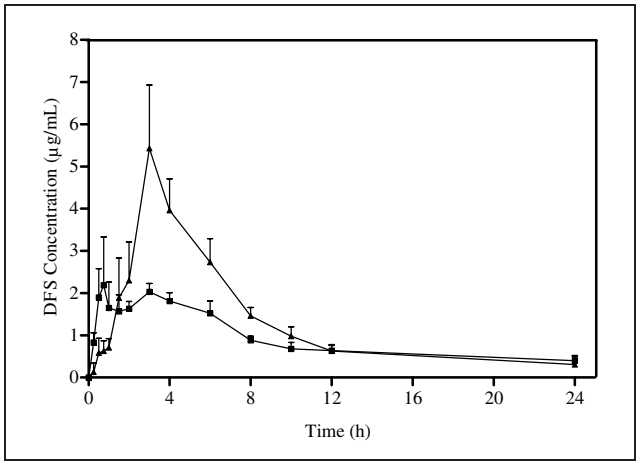

Fig. 3: Diclofenac plasma concentration *vs* time curves for six Beagle dogs orally administered with DFS-containing microspheres formulation (●) and Voltaren SR 75 (▲). Each point represents the mean ± S.E

of chromatograms under these conditions, for: (a) blank plasma; (b) blank plasma spiked with DFS 2.0 µg/mL; and (c) plasma collected 30 min after oral administration of microspheres containing 75 mg DFS. Under these conditions the retention times for diclofenac and indomethacin were 11.0 and 13.5 min, respectively, and a chromatographic run was completed in 15 min.

2.3. Pharmacokinetic study

Figure 3 shows the mean plasma diclofenac concentration vs. time curves for the Voltaren SR75 and microspheres. The major mean pharmacokinetic parameters derived from the plasma diclofenac concentration vs. time curves are given in Table 2.

Results for the parametric analysis of individual $AUC_{[0-24]}$, $AUC_{[0-\infty]}$, C_{max}, K_e and $t_{1/2}$ percent ratios and T_{max} differences between microspheres and Voltaren are shown in Table 3.

3. Discussion

The results from the *in vitro* dissolution tests indicate that the dissolution of diclofenac was pH dependent. At acidic pH, the DFS released from Voltaren SR75 tablets was insignificant, while from the microspheres the release was less than 15%. The *in vitro* DFS release improved with increasing pH for both formulations. The dissolution profiles show a release of 45% DFS from Voltaren SR75 and about 95% from the microspheres at pH 6.8. This result suggests that the low level of diclofenac release from both formulations at low pH was due to the insolubility of diclofenac in acid solution. The higher release of diclofenac observed for the microspheres at basic pH can be attributed to the formation of pores left by the elution of the Pluronic F68 during the dissolution assay, which can improve the drug diffusion. However, we cannot discard the possibility of an increase in the water content of the particles due to the greater hydrophilic propriety of Pluronic F68. Thus, both the increase in porosity and water content are considered to be responsible for the increase in the rate of drug released from aliphatic polyester matrices, when Pluronic F68 is added as a second polymer (Huatan et al. 1995; Yeh et al. 1996).

Table 3: Statistical analysis results for $AUC_{[0-24]}$, $AUC_{[0-\infty]}$, C_{max}, K_e and $t_{1/2}$ individual ratios and T_{max} individual differences for DFS

Microsphere/Voltaren	Parametric analysis	
	Geometric mean	90% CI
$AUC_{[0-24]}$ % Ratio	61.0	50.6–73.5
$AUC_{[0-\infty]}$ % Ratio	62.8	53.2–74.2
C_{max} % Ratio	54.4	35.8–82.4
K_e % Ratio	94.7	55.6–161.3
$t_{1/2}$ % Ratio	105.8	62.1–180.2
T_{max} Difference (h)	−0.75	−2.2–0.5

Microspheres = test formulation; Voltaren = tablets (reference formulation); CI = Confidence Interval; * = Arithmetic mean

With respect to the validation of the analytical procedures the calibration curves showed a satisfactory linearity, and the sensitivity was adequate for the intended objective, as shown by the LOD and LOQ values, it being possible to quantify concentrations higher than 0.1 µg/mL DFS. Values for precision and accuracy obtained through the CV for the between-day variation and the within-day variation assays were considered acceptable for the validation of this analytical method (as recommended in Shah et al. 1992).

The analytical recovery using acetonitrile for protein precipitation was higher than 50%. These values are within acceptable limits, especially in relation to the extraction of a drug from biological matrices (plasma). Although a higher recovery is desirable, *i.e.*, closer to 100%, such high values are not necessary to obtain good precision and accuracy if an adequate detention limit can be reached (Karnes et al. 1991).

Figure 3 shows the curves of the average plasma DFS concentrations *versus* time in Beagle dogs for microspheres and Voltaren formulations, under the described conditions. As can be noted, these curves have different profiles. The Voltaren curve profile is monophasic, while the microsphere curve has a two-phase (biphasic) profile. Regarding the first phase, the release might be attributed to DFS particles that are not entrapped but adsorbed on the surface of the microspheres, and the second phase may be due to the diffusion of DFS from the microspheres.

Parametric analysis of individual $AUC_{[0-24]}$ and $AUC_{[0-\infty]}$ percent ratios of DFS showed statistically significant differences between microspheres and Voltaren (Table 3), as shown by the non-inclusion of these ratios in the 80–125% interval (Hauschke et al. 1990). The microspheres had approximately a 61% bioavailability of DFS in comparison to Voltaren (reference formulation).

Hosny et al. (1997) using microspheres of sodium alginate and carboxymethyl-cellulose sodium containing 100 mg of DFS, administered to the same animal model, obtained $AUC_{[0-24]}$ values of 48.99 ± 11.07 µg/mL.h and 39.82 ± 26.61 µg/mL.h, respectively. The $AUC_{[0-24]}$ value for Voltaren Retard 100, used as a reference, was 83.02 ± 18.31 µg/mL.h. The results described by these authors indicate the same findings as the results obtained in this study, *i.e.*, the microsphere formulation has a reduced bioavailability in comparison to the tablet (reference formulation).

In this study, as was expected, the parameter C_{max} demonstrated a similar behavior to that of AUC. The bioequiva-

lence (relative bioavailability) was reduced for the microsphere formulation (test) in comparison to the Voltaren tablet (reference).

On the other hand, the T_{max} was 3.0 h for both formulations (Table 2). In this case, the confidence interval of the arithmetic mean of the individual differences of T_{max} (microspheres *vs.* Voltaren) includes the "zero" value (Table 3). However, we cannot draw conclusions regarding the bioequivalence of a pharmaceutical formulation based on the value of T_{max} alone. Since the value of this parameter did not differ between formulations it seems that the microsphere formulation has sustained release characteristics. In fact, formulations of immediate release have T_{max} values lower than 2 h.

In studies on bioavailability/bioequivalence, three parameters (AUC, C_{max} and T_{max}) are necessary in order to considered whether two formulations have a similar bioavailability, and to draw conclusions regarding the bioequivalence of pharmaceutical formulations. In this study, the AUC and C_{max} parameters, which evaluate the extension (intensity) of absorption, indicated a lower bioavailability of diclofenac sodium in microspheres than in Voltaren.

The parameters related to the elimination of the DFS in these assays (K_e and $t_{1/2}$) showed that the geometric mean ratios of the individual test/reference were within in the range of bioequivalence of 80–125% (Table 3). However, the 90% confidence intervals of this parameter exceed the lower and upper limits proposed in bioequivalence studies (Steinijans et al. 1991). Therefore, we can not draw conclusions regarding the bioequivalence of these pharmacokinetic parameters. It is possible that these discrepancies observed in our results would be reduced if the number of animals was increased to reduce the data variability.

4. Experimental

4.1. Materials

Diclofenac sodium (technical grade) and Voltaren SR75® (commercial product) were obtained from Novartis, Brazil. Cellulose acetate-butyrate, MW 70,000 and indomethacin were acquired from Sigma-Aldrich Chemical Co, USA. Pharmaceuticals Imosec® (loperamide hydrochloride, 2 mg tablets, Lot Number: 204646, expiry date: Nov 2005, from Janssen-Cilag Pharmaceutical Ltd, Brazil,) and Heparin® (Heparin sodium, 5,000 UI/mL, Lot Number: 02093506, expiry date: Sept 2004, from Cristália Pharmaceutical, Brazil) were bought in a drugstore. Pluronic F-68 (Basf Corporation, USA), Span 80 (Beraca Ind. Com. Ltd), mineral oil (Import. Química Delaware Ltd), n-hexane (Tedia, USA) were used in the experiments. Acetic acid, acetonitrile, hydrochloric acid, sodium hydroxide, sodium phosphate tribasic, and 85% orthophosphoric acid were purchased from Nuclear, Brazil. Acetone, methyl alcohol, dichloromethane, sodium chloride, sodium sulfate anhydrous were acquired from Vetec, Brazil. All products were of analytical grade or higher, except when stated otherwise.

4.2. Preparation of microspheres

The microspheres were prepared by the emulsification/evaporation method, using acetone and mineral oil as internal and external phases, respectively. For this, 100 mL of acetone solution of cellulose acetate butyrate MW 70000 and Pluronic F-68 (3 : 1 w/w), containing diclofenac sodium (1.25 g), were added to 100 mL of liquid vaseline containing Span 80 1% (w/v) as the stabilizer. The medium was stirred vigorously at 650 rpm for 24 h at room temperature to allow complete evaporation of acetone. The resulting microspheres were collected by filtration, washed with n-hexane, and dried in a stove under reduced pressure for 4 h.

4.3. Dissolution study

Both the microspheres and Voltaren were evaluated for their drug release characteristics. The studies were carried out using the dissolution test (Pharmatest model PTWS3, combined with a UV spectrophotometer HP 8452A) according to the paddle method (USP 23 1994) in 750 mL hydrochloric acid 0.1 M during the first 2 h. Beforehand, 250 mL of a phosphate buffer pH 6.8 solution maintained at 37 °C at 100 rpm were added. Samples of 3 mL each were collected with the assistance of a peristaltic pump at 0.0, 0.15, 0.30, 0.45, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0, 10.0 and 12.0 hours. To maintain the buffer level the volume removed was replaced after the sample was analyzed. The samples were detected at a wavelength of 278 nm.

4.4. Pharmacokinetic study

This study was performed in a two-way crossover design with a washout period of two weeks between two treatments. Six female beagle dogs, one year old and weighing 10–12 kg, were used in this study. In each experimental section the dogs received one formulation (crossover study) in a single oral dose equivalent to 75 mg diclofenac, and subsequently to 20 mL water. Four hours after administration of the formulations, the animals were allowed access to water and food *ad libitum*.

To make this experimental model suitable for pharmacokinetics studies on controlled-release formulations, animals received orally a 2 mg tablet of loperamide (Imosec®) 30 min before administration of the formulations (Yamada et al. 1995).

The study protocol was approved by the Ethics Commission for Use of Animals (CEUA) of the Federal University of Santa Catarina – UFSC (Certificate number 185).

Blood samples (2 mL) were collected using an intravenous catheter (Angiocath™ 20GA × 1.16 in., Becton Dickinson, Brazil) through the cephalic vein into 5 mL tubes containing 57 µl 15% K_3-EDTA solution, before the experiment and at 0.25, 0.50, 0.75, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0, 10.0, 12.0 and 24.0 h post DFS administration. The blood samples were immediately centrifuged at 4,000 rpm for 10 min and aliquots of plasma were stored at −20 °C for subsequent HPLC assay.

4.5. Chromatographic analysis

The plasma concentration of diclofenac was determined by reversed-phase high-performance liquid chromatography with UV detection. For sample preparation, 0.5 mL of plasma was transferred to a 15 mL centrifuge tube, adding 100 µl internal standard (10 µg/mL of indomethacin) and 200 µl orthophosphoric acid at 5% (v/v). After homogenizing the mixture, 4 mL of acetonitrile was added in the presence of Na_2SO_4 anhydrous (~1 g) and vortex mixing was carried out for 1 min. After centrifuging for 10 min, at 4,000 rpm, the supernatant was transferred to a 10 mL centrifuge tube and evaporated to dryness at 37 °C under a gentle current of N_2. The residue was reconstituted with 200 µl of acetonitrile. This was centrifuged for 10 min at 14,000 rpm and 80 µl was injected into the chromatograph. The concentrations of plasma diclofenac were interpolated from the calibration curve.

4.6. Instrumentation and chromatographic conditions

The HPLC system comprised a pump (model LC-10AS), a UV detector (model SPD-10A), and an integrator (model Chromatopac C-R6A), all manufactured by Shimadzu Co. Japan, and a manual injector (model 7125, Rheodyne, Cotati, USA), equipped with a 200 µL loop. Samples were run on an octadecylsilane (C18) reverse-phase analytical column (Supelcosil, 5 µm, 250 × 4.6 mm I.D.). A stainless steel guard-column (20 × 2 mm I.D.) was used to protect the analytical column which was packed with pellicular particles of octadecylsilane (Alltech Co., Milwaukee, USA).

The mobile phase was constituted of a 0.1 M acetic acid : acetonitrile mixture, 62 : 38 (v/v), adjusted to pH 5.6 with 5 M sodium hydroxide. After ultrasonic degasification and filtering under vacuum through a nylon membrane with 0.45 µm porosity, the mobile phase was isocratically pumped at a flow rate of 1.2 mL/min. at room temperature. The peaks were analyzed with detection at a wavelength of 280 nm with sensitivity adjusted to 0.005 AUFS.

4.7. Pharmacokinetic and statistical analysis

The individual plasma drug concentration-time curves were obtained for each animal, in each phase of the study. The maximum observed plasma concentration (C_{max}) and the time required to reach this maximum level (T_{max}) were obtained from these curves. The terminal elimination rate constant (K_e) was estimated by least squares regression of the points describing a terminal log-linear decay phase. The half-life ($t_{1/2}$) values were derived from K_e where $t_{1/2} = \mathrm{Ln}\ 2/K_e$. The areas under the diclofenac concentration vs. time curves from 0 to 24 h ($AUC_{[0-24]}$) were calculated using the trapezoidal method, and from 0 to infinity ($AUC_{[0-\infty]}$) were calculated using the trapezoidal method up to the time at which the plasma diclofenac concentration was above the limit of detection (0.05 µg/mL), with later addition of the C/K_e (C = last detectable diclofenac concentration) value.

The pharmacokinetic parameters obtained were expressed as geometric means with their respective 90% confidence intervals, except for T_{max} for which the arithmetic mean was determined. Individual ratios were statistically analyzed using the parametric method (one-way ANOVA for Ln-transformed data), with the exception of T_{max}, where individual non-transformed differences were analyzed.

The assessment of bioequivalence (relative bioavailability) as defined by Hauschke (1990) was also used to determine whether the microsphere formulation (test) was bioequivalent to Voltaren SR75 (reference). The bioequivalence range for the individual ratios of the Ln-transformed variables was defined as 0.8–1.25.

4.8. Validation of the chromatographic method

The HPLC method was validated before sample analysis. The calibration curves were prepared with seven points ranging from 0.10 and 8.0 μg/mL diclofenac adding to pooled blank plasma. Indomethacin was used as the internal standard. The calibration curve equations were estimated for the concentration range used by a linear least squares regression model, considering the ratio between the peak heights of the diclofenac and internal standard (Lau et al. 1987).

In order to assess between-day variation, the calibration curve was repeated every day for 9 days. The within-day variation was determined by assaying five plasma samples of low, middle and high concentration of DFS (0.3, 3.0 and 6.0 μg/mL, respectively) on the same day. The accuracy of the HPLC method, calculated for analytical recovery, was determined for comparison with standard solutions of DFS at equivalent concentration. The limits of detection (LOD) and quantification (LOQ) were considered as the lowest concentration measured in which the coefficient of variation (CV) was lower than 20% and 15%, respectively.

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