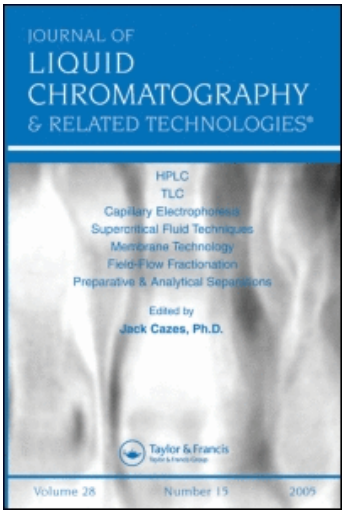

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An Improved HPLC Procedure for the Quantitation of Diclofenac in Plasma

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AN IMPROVED HPLC PROCEDURE FOR THE QUANTITATION OF DICLOFENAC IN PLASMA

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ABSTRACT

A rapid, simple and sensitive high performance liquid chromatographic (HPLC) assay for the quantitation of diclofenac (DF) in dog plasma has been developed. Mefenamic acid (MA) was used as the internal standard. After acidification, DF and MA were extracted from plasma into chloroform. Separation was achieved using a C18 reversed phase column. The retention times of DF and MA were 3.8 and 6.3 min., respectively at the flow rate of 1.5 ml/min. The DF interday standard plots (n=4) were highly linear (r>0.99) over the concentration range of 0.01 to 10 μg/ml. DF mean recovery was 98% ± 5.5, and the % CV of intra- and inter-day sample analyses ranged from 2.6 to 10.8% for the entire calibration range. The limit of quantification of DF in plasma was 0.01 μg/ml with the CV of 9.4%. The method was applied for the determination of the pharmacokinetic parameters of DF given by oral and iv bolus administration to dogs.

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INTRODUCTION

Diclofenac sodium (voltaren, [0-(2,6-dichloroanilino)-phenyl]acetate) is a nonsteroidal anti-inflammatory drug (NSAID), available in many countries since 1974. It is currently marketed in the USA for treatment of various inflammatory conditions such as rheumatoid arthritis, osteoarthritis, ankylosing spondylitis and other related symptoms (1-2). The drug has also analgesic and antipyretic activities, and is used for the relief of dental or minor surgical pain and headache. The anti-inflammatory activity of the drug and its other pharmacological effects are generally thought to be related to its inhibition of prostaglandin synthesis (3). Diclofenac competes with arachidonic acid for binding to cyclo-oxygenase, thereby decreasing the synthesis of prostaglandin (3,4).

Diclofenac is rapidly and efficiently absorbed after oral administration when given as a solution or as an enteric coated tablet (5,6). The drug undergoes a significant first-pass metabolism and only about 60% of the drug reaches the systemic circulation unchanged (5). The peak plasma concentrations of the drug usually occur 1.5 to 2.5 hrs after oral ingestion in fasting subjects, while peak times vary widely (2.5-12 hrs) under fed conditions (7). Diclofenac is eliminated principally by hepatic metabolism and subsequent urinary and biliary

excretion (8). In healthy volunteers, the mean plasma clearance of diclofenac is 16 L/hr and the elimination half-life is approximately 1.5 hr (5,6).

In view of its wide clinical use, different analytical methods have been developed for the quantitation of diclofenac in pharmaceutical preparations, blood, plasma, urine and other biological matrices. These methods include spectrophotometry (9,10), high performance liquid chromatography (HPLC) (11-21), gas-liquid chromatography with electron-capture detector (GC) (22-28), thin layer chromatography (TLC) (29), radioactive isotope (30) and GC/mass spectrophotometry (GC/MS) (31,32). The HPLC methods reported in the literature (11-21) were often developed with a simplified sample preparation steps and direct UV detection without derivatization. Some of these methods employ a single extraction step (11-15,18), multiple extraction (16,20) or an automated robotic extraction (19,21). The HPLC methods that depend on either repeated extraction or reextraction, although yielding higher sensitivity, are generally more complex, time consuming and involve tedious extraction steps (16,20). The GC with electron-capture detection methods are based on the formation of an indolone of a methyl or ethyl ester derivative (22-28). The reported TLC method (29), beside being less accurate and time-consuming, lacks the

sensitivity required for pharmacokinetic and bioavailability studies. The use of radioactive isotopes (30) is limited because of the potential hazards of radioactivity in man and lack of specificity for the intact compound.

The aim of this work was to develop an improved assay of unchanged diclofenac in plasma using HPLC. The new method is simple and highly sensitive with a limit of quantitation of 10 ng/ml of DF in plasma, rapidity with the assay time of approximately 2 hrs for 10 replicate samples and essentially complete recoveries (>98%) for the entire calibration range of 10 ng to 10 μg of DF in ml of plasma. The method also offers specificity without any interfering peaks near the drug or the internal standard from common anti-inflammatory drugs or endogenous plasma components. The inter- and intra-day reproducibilities as shown by % CV were excellent ranging from 2.6 to 10.8 for the entire calibration range. The method was successfully applied for the determination of the pharmacokinetic parameters of DF in dogs for up to 24 hrs after receiving a single dose of the drug (5 mg/kg) given by oral and i.v. bolus doses.

EXPERIMENTAL

Chemicals and Reagents

Diclofenac sodium, mefenamic acid, ketoprofen, ibuprofen, naproxen sodium and indomethacin were

purchased from Sigma Chemical, St. Louis, MO. Aspirin was obtained from Amend Drugs and Chemical, Irvington, NJ. Acetonitrile, methanol, chloroform and phosphoric acid were purchased from J.T. Baker Chemical, Inc., Phillipsburg, NJ. The solvents were all of HPLC grade. Water was deionized and filtered.

Chromatographic Conditions and Equipment

The chromatographic system used consists of a Beckman Pump (Model 110A), operated at a flow-rate of 1.5 ml/min, a variable-wavelength detector (SpectroMonitor III, Model 1204 A) set at 278 nm, and a fixed volume (50 μl) injector (Rheodyne, Model 7125) and the separation was achieved on a stainless steel reversed phase Novapak C_{18} (150 x 3.9 mm) column (Phenomenex, Model PP/9400 A) with a C_{18} pre-column (30-40 μm pellicular packing). A mobile phase consisting of acetonitrile and water (50:50% V/V) adjusted to pH 3.5 with glacial acetic acid was degassed using an ultrasonicator (Fisher Scientific, Model 14). The samples were centrifuged using IEC-Centrifuge (Damon ISC, Model CU-5000). Vortexing was achieved by Fisher Mini-Shaker (Fisher Scientific, Model 58). Chromatograms were recorded on a strip chart recorder (Esterline Anug, Rainin Instrument Model) at a speed of 2.5 mm/min.

Standard Calibration Curves

In screw-capped centrifuge tubes, 0.5 ml of dog plasma was mixed with 10 μl of the DF reference solutions

to produce concentrations of 10 ng to 10 μg of DF in ml of plasma. The tubes were vortexed for 10 sec. After adding 10 μl of the MA stock solution to each tube to yield the final MA concentration of 1 μg/ml, acidification was achieved by adding 50 μl of phosphoric acid (85.2%) to each tube, then the tubes were vortexed for 10 sec. The extraction of DF and MA was carried out by the addition of 3 ml of chloroform to each tube followed by vortexing for 1 min, then the tubes were centrifuged for 5 min at 6000 rpm. The organic layer was transferred into a 4 ml glass tube and evaporated to dryness under a stream of nitrogen at 45°C. The residue was reconstituted in 200 μl of the mobile phase and vortexed for 10 sec. A 50 μl of this solution was loaded into the HPLC sample loop. The calibration curves were obtained by plotting the peak height ratios of DF/MA versus their respective concentrations of DF.

Assay of Dosed Samples

The same extraction and chromatographic procedures described for the preparation of the calibration plots were used for the quantitation of DF in the dosed dog plasma samples excluding the addition of diclofenac sodium.

Animal Studies

A single dose of 5 mg/kg of diclofenac sodium was administered to two beagle dogs via bolus intravenous

injection after dissolving in 3 ml of normal saline, and orally in a gelatin capsule to two other beagle dogs. After two weeks of washout period, the animals were crossed-over to receive the other formulation. The dogs were fasted overnight with water freely available prior to dose in the early morning and continued fasting for 4 hrs after drug administration. The jugular vein was used to collect the blood samples (4-5 ml) into commercial blood collecting tubes containing a standard amount of anticoagulant (sodium heparin). The blood samples were obtained at the intervals of 0, 0.33, 0.67, 1, 2, 3, 5, 7, 9, 12 and 24 hrs post dose. Plasma was separated by centrifugation for 10 min at 2000 rpm and stored in refrigerator at -20°C until analysis which was usually done within one week.

RESULTS AND DISCUSSION

Chromatographic Specificity and Sensitivity

Typical chromatograms of (a) blank dog plasma spiked with mefenamic acid (1 μg/ml) and plasma spiked with the internal standard and diclofenac sodium (b) 5 ng/ml and (c) 10 ng/ml are shown in Figure 1. The chromatograms of plasma sample taken (a) before administration of DF and plasma taken (b) 12 hrs and (c) 24 hrs after oral administration of DF powder (5 mg/kg) to a beagle dog are shown in Figure 2. The specificity of the method was

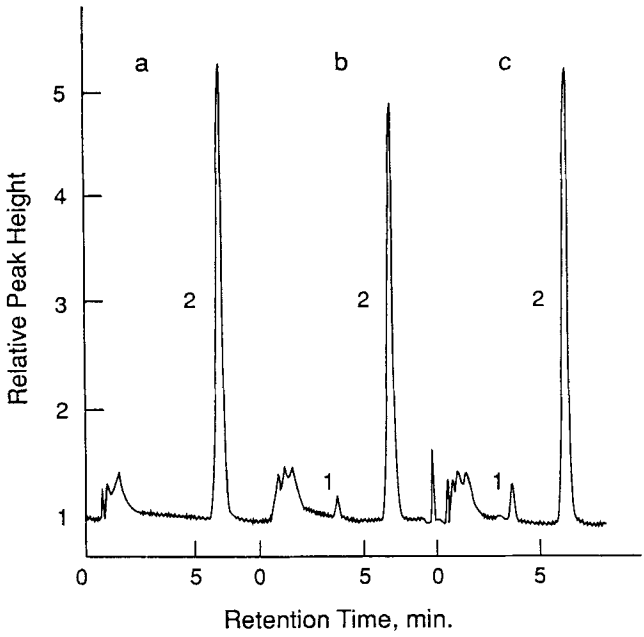

Figure 1. Typical chromatogram of blank dog plasma spiked with (a-2) mefenamic acid (1 μg/ml) and plasma spiked with the internal standard and diclofenac sodium (b-1) 5 ng/ml and (c-1) 10 ng/ml.

demonstrated by the lack of interferences at the retention times of DF (3.8 min) and MA (6.3 min). Both peaks were sharp and symmetrical with good baseline resolution, thus facilitating accurate measurement of the peak height ratio. The sensitivity of assay defined as the minimum concentration that can be quantitated with a statistically acceptable coefficient of variation (10%) in the peak height ratio was 10 ng/ml with the CV equal to 9.4% (see Table 1-A). The minimum detectable amount

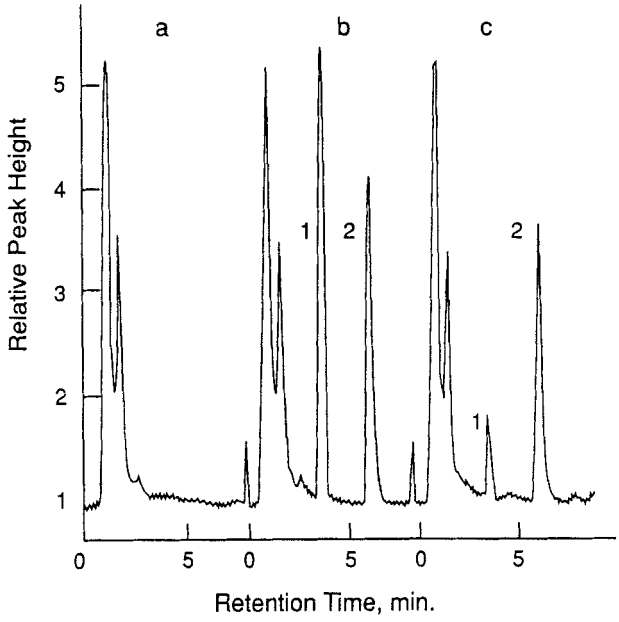

Figure 2. Chromatogram of plasma sample taken at time zero (a) and plasma taken (b-1) 12 hrs and (c-1) 24 hrs after oral dose of DF powder (5 mg/kg) to a beagle dog.

defined as the amount in nanograms that gives peak height of DF equal to twice the background noise at the most sensitive instrument setting used in the study was 5 ng of DF.

Selection of the Mobile Phase

A mobile phase consisting of acetonitrile-water (50:50% V/V) adjusted to pH 3.5 with glacial acetic acid gave the optimum resolution of DF and MA with a flow rate of 1.5 ml/min. According to Sayed et al. (18), the composition and pH of the mobile phase drastically

affected the retention times of the compounds. In this study, similar results were observed. Increasing the percentage of acetonitrile decreased the retention times of the analytes and visa versa. However, an opposite effect was observed with the pH change, where the retention times of DF and MA were increased with increasing the pH.

Selection of the Extraction Solvent

Several organic solvents were initially tested for the extraction of DF and MA from plasma samples. The solvents tested were: benzene, chlorobenzene, ethyl ether, petroleum ether, chloroform, and n-hexane. Of these solvents, chloroform showed the highest recovery without interfering peaks at the retention times of both DF and MA. When plasma samples (1 μg/ml) of both DF and MA were extracted using the above solvents, chloroform consistently gave the highest peaks for DF and MA at given concentrations.

Retention Times and Selectivity of the Assay

By using the assay procedure, the retention times of DF and MA were 3.8 and 6.3 min, respectively. As shown in Figure 1, there were no interferences due to the endogeneous plasma components at the retention times of the drug and the internal standard. The intercepts of the calibration plots showed no significant deviation

from zero, indicating that blank plasma has negligible interference for the analyte. Potential interferences to the peaks were also evaluated by injecting some common anti-inflammatory drugs to the HPLC system. Solutions of the following drugs (aspirin, ketoprofen, ibuprofen, naproxen, diclofenac, mefenamic acid and indomethacin were separately prepared in the mobile phase and directly injected onto the HPLC column, and their respective retention times were 1, 2, 4.3, 2, 3.8, 6.3, and 3.8 min. Except indomethacin, no compounds tested exhibited the same retention times of DF and MA.

Linearity of the Calibration Plots

Least-squares analyses of the inter-day calibration curves gave excellent linear responses to the tested concentration range (10 ng to 10 μg/ml) of DF in plasma (Table 1). A typical standard plot of DF in plasma can be described by the equation: $y = 1.87x + 0.049$ with a correlation coefficient (r) of greater than 0.99, indicating a good fit to the least-squares linear regression model.

Reproducibility of the Assay

The inter-day reproducibility of the assay was evaluated by comparing the linear regression analyses of the four standard plots obtained from spiked dog plasma samples at four different days over a period of two weeks (Table 2). Least-squares regression analyses of the

Table 1-A: Inter-day Reproducibility[1] of Calibration Curves of Diclofenac in Dog Plasma.

Plasma Conc (μg/ml)	Mean Peak Height Ratio (SD)[2]	% Coefficient of Variation, n=4
0.01	0.064 (0.006)	9.37
0.025	0.105 (0.011)	10.47
0.05	0.150 (0.008)	6.00
0.1	0.245 (0.019)	7.75
0.2	0.388 (0.002)	5.67
0.5	0.983 (0.077)	7.83
1	1.921 (0.063)	3.27
2	3.800 (0.198)	5.21
5	9.433 (0.491)	3.94
10	18.756 (0.670)	3.57

[1] Determined from four sets of standard curves on four different days over a period of two weeks.
[2] Standard deviation

Table 1-B: Inter-day Calibration Data.

Calibration Curve[1]	Slope[2]	Intercept	Correlation Coefficient, r
1	1.856	0.027	>0.99
2	1.914	0.086	>0.99
3	1.914	0.008	>0.99
4	1.778	0.096	>0.99

[1] Prepared over a period of two weeks.
[2] Mean slope (% CV) = 1.872 ml/μg (3.85)

calibration curves gave linear responses over the tested concentration range of DF (10 ng to 10 μg/ml). The average slope of the four standard plots was 1.87 ml/μg with a standard deviation (SD) equal to 0.064, and a day-to-day coefficient of variation (CV%) of 3.43, indicating excellent inter-day reproducibility. The correlation coefficient (r) was typically higher than 0.99.

Table 2-A: Intra-Day Reproducibility[1] of Calibration Curves of Diclofenac in Dog Plasma.

Plasma Conc (μg/ml)	Mean Peak Height Ratio (SD)[2]	% Coefficient of Variation, n=3
0.01	0.063 (0.005)	7.46
0.025	0.101 (0.010)	10.89
0.050	0.154 (0.012)	7.79
0.1	0.243 (0.013)	3.26
0.2	0.398 (0.022)	5.52
0.5	1.014 (0.061)	6.01
1	1.924 (0.050)	2.59
2	3.766 (0.098)	2.60
5	9.258 (0.561)	6.05
10	18.238 (0.770)	4.22

[1] Determined from three sets of standard curves on the same day.
[2] Standard deviation.

Table 2-B. Intra-Day Calibration Data.

Calibration Curve[1]	Slope[2]	Intercept	Correlation Coefficient, r
1	1.778	0.092	>0.99
2	1.746	0.073	>0.99
3	1.821	0.077	>0.99

[1] Calibration curves prepared on the same day.
[2] Mean slope (% CV) = 1.782 ml/μg (2.11)

The intra-day reproducibility was determined by comparing the linear regression analyses of three standard plots obtained from spiked dog plasma samples in the same day (Table 2). Least-squares regression analyses of the three calibration curves gave linear responses in the tested concentration range of DF (10 ng to 10 μg/ml). The average slope of the three plots were

1.78 ml/μg with a standard deviation (SD) of 0.038 and a coefficient of variation of 2.12, indicating good within-day reproducibility. The correlation coefficient (r) was also higher than 0.99 for each standard plot.

Recovery of Diclofenac from Plasma

Absolute recoveries of DF and MA from plasma were determined by comparing peak height ratios of DF/MA from the extracted plasma samples containing DF (0.01 to 10 μg/ml) to those ratios (DF/MA) obtained from direct injection of standard solutions prepared in the mobile phase at equivalent concentrations of DF (0.01 to 10 μg/ml) and the internal standard (1 μg/ml). In Table 3, the overall mean recovery of the assay was shown to be 98% with a standard deviation of 5.47 and overall coefficient of variation (CV%) of 5.58, indicating excellent extraction efficiency and reproducibility.

Accuracy and Precision of the Assay

Relative recovery data (Table 4) were used to assess the overall accuracy of the assay. Relative recovery of DF and MA was calculated by comparing peak height ratios of DF/MA from the spiked plasma samples containing DF (0.01 to 10 μg/ml) to those (DF/MA) obtained from the spiked water at the equivalent concentrations of DF and MA which were treated with the same procedure. Inter-day reproducibility of the recovery data were used to determine the precision of the method. The overall mean

Table 3: Recoveries of Diclofenac in Spiked Dog Plasma.

Plasma Conc (μg/ml)	% Recovery[1,2]	Coefficient of Variation, %
0.01	98.2 (9.89)	10.07
0.025	102.8 (7.09)	6.90
0.05	104.1 (3.65)	3.51
0.1	95.3 (7.67)	8.04
0.2	95.4 (5.37)	5.63
0.5	92.7 (7.30)	3.39
1	108.9 (3.69)	7.87
2	92.2 (4.94)	5.36
5	95.1 (5.65)	5.94
10	95.4 (3.87)	4.06

[1] Overall mean recovery (% CV) = 98.0% (5.58)
[2] Each value represents the mean % recovery (± SD) of 4-6 samples

Table 4: Inter-day Accuracy and Precision Data for Diclofenac Assay in Dog Plasma.

Plasma Conc. (μg/ml)	% Recovery[1,2]	Coefficient of Variation, %
0.01	95.81 (4.73)	4.94
0.025	102.93 (6.01)	5.84
0.05	101.87 (3.40)	3.33
0.1	94.42 (7.06)	7.48
0.2	98.45 (5.68)	5.77
0.5	102.93 (5.21)	5.06
1	98.92 (4.18)	4.23
2	97.36 (5.05)	5.18
5	93.41 (6.82)	7.30
10	96.18 (4.99)	5.19

[1] Overall mean relative recovery (% CV) = 98.2% (5.43)
[2] Each value represents the mean % recovery (± SD) of 3 samples

recovery for diclofenac for the calibration samples was 98.2% ± 5.31 (SD). In Table 4, the day-to-day coefficients of variation were 5.41% at the lower limit of quantitation (10 ng/ml) and 9.4-3.3% over the entire concentration range (10 ng-10 μg/ml).

The validation of the method was evaluated by analyzing DF in spiked plasma samples over the entire calibration concentration range (10 ng to 10 μg/ml) in a blind fashion where the analyst did not know the concentrations of the sample (Table 5). The peak height ratios of DF/MA were determined and the corresponding concentrations were calculated using the standard plot of DF in plasma (Table 1-A). In the blind study, accuracy and precision were remarkably good as measured by the overall mean recovery of 99.9% with a mean coefficient of variation (CV%) of 6.61% in the concentration range of 10 ng-10 μg/ml in plasma as shown in Table 5.

Table 6 summarizes the sensitivity, retention times, type of extraction solvents, recoveries and the coefficients of variation (CV%) of selected recent publications describing HPLC analysis of diclofenac in biological matrices. The proposed method clearly offers advantages over the existing HPLC procedures with respect to the extraction times, overall recoveries and inter- and intra-day variations. For example, Chan et al. (13) described a HPLC method which was claimed to be superior

Table 5: Validation of Assay for Diclofenac in Spiked Dog Plasma.

Actual Conc (μg/ml)	Measured Conc (μg/ml)	% Recovery	% Error[1]	% CV
0.01	0.009	90.0	10	9.35
0.025	0.028	112.0	12	4.12
0.05	0.053	106.0	6	4.12
0.1	0.103	103.0	3	5.80
0.2	0.180	90.0	10	1.75
0.5	0.493	98.0	1.5	6.40
1	0.999	99.9	0.1	3.36
2	1.999	99.9	0.05	1.82
5	5.014	100.3	0.28	6.10
10	9.995	99.9	0.05	4.20

[1] % Error = (Actual Conc - Measured Conc)/Actual Conc x 100
[2] % CV = Standard Deviation/Mean x 100

Table 6: Comparison of the Proposed Method with Published HPLC Methods of Analysis of Diclofenac in Biological Matrices.

Method	Sensitivity (ng/ml)	Retention Time (min)	Extraction Time (min)	Extraction Solvent	Mean Recovery (%)	CV (%)
Proposed Assay	10	3.8	6 (1[a]+5[b])	Chloroform	98	5.5
Nielsen-Kudsk (1980)	-	10.1	5	ppt[c] with acetonitrile	-	-
Yaginuma (1981)	-	5.8	12 (10[a]+2[b])	Benzene	-	-
Chan (1982)	5	3.9	25 (15[a]+10[b])	Hexane-IPA[d]	95	8.2
Godbillon (1985)	10	5.8	20 (10[a]+10[b])	Hexane-IPA[d]	98	3.1
Battista (1985)	-	~28	repeated	Diethyl ether	-	-
El-sayed (1988)	25	6.5	11 (1[a]+10[b])	ppt[c] with acetonitrile	90-98	2.5-4.6
Mascher (1989)	10	~1.5	reextraction	Heptane IPA[d]	78	2-12
Grandjean (1989)	20	2	8 (3[a]+5[b])	Hexane-IPA[d]	-	-
Brunner (1991)	5	2	30 (15[a]+15[b])	Hexane-IPA[d]	99.8	0.5-11.1

[a] Shaking Time
[b] Centrifugation Time
[c] Precipitation
[d] Isopropyl Alcohol

to other methods for analysis of DF in plasma. However, the reproducibilities as indicated by the coefficients of variation which were 20% at 5 ng/ml (three replicate samples analyzed) and 22% at 10 ng/ml (four replicates) were relatively low. The recoveries reported for the calibration range varied widely between 67% and 144%. In addition, the analytical procedure was relatively tedious involving 15-min shaking for extraction prior to freezing the aqueous layer by dipping the tubes into a dry ice bath. The method described by Godbillon et al. (15), Battista et al. (16), Sayed et al. (18) and Mascher (20), despite their high sensitivity, are rather complex and time consuming as compared to the proposed assay (Table 6). While having very high sensitivity (20 ng/ml and 5 ng/ml, respectively), the automated methods described by Grandjean et al. (19) and Brunner et al. (21) required longer extraction times than the current method.

In Vivo Application of the Assay

Figure 3 shows the individual plasma concentration-time profile of a single dose (5 mg/kg) of DF given to four adult beagle dogs via oral route. The drug was rapidly absorbed after oral administration as indicated by the short peak time (~20 min). After the peak concentration, the plasma levels of DF exponentially declined following both iv bolus and oral administration. The pharmacokinetic parameters of DF in the dogs after iv

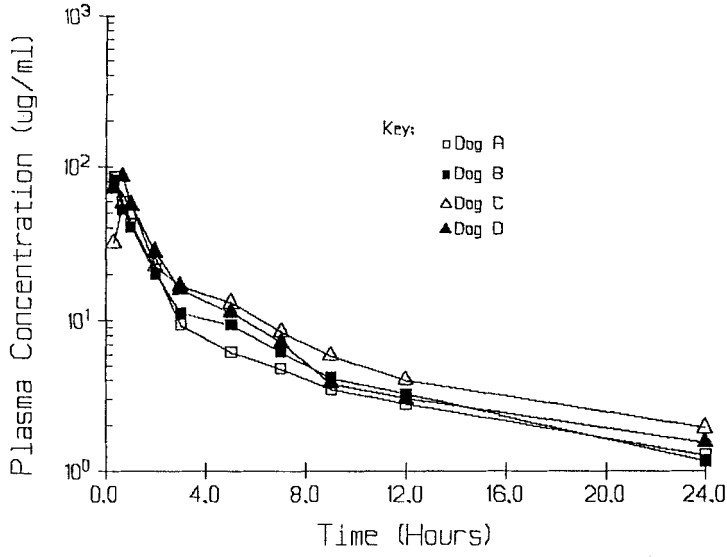

Figure 3. Plasma concentration versus time profile of diclofenac after a single oral dose (5 mg/kg) to four dogs.

bolus administration were calculated using the computer program (R-Strip) for the equation:

$$C = A_1e^{-\alpha t} + A_2e^{-\beta t}$$

The two half lives of the biexponential equation were 0.54 and 6.98 hr for α and β phases, respectively.

The pharmacokinetic parameters after the oral administration of DF to four dogs were calculated after fitting to the two-compartment model using the same computer program. These data are shown in Table 7. The mean peak plasma concentration (C_{max}) was found to be 78.2 μg/ml (± 12.5 SD). The time required to reach this

Table 7: Pharmacokinetic Parameters of Diclofenac after Oral Dose (5 mg/kg) to Dogs Based on the Two-Compartment Model.

Parameter	Dog A	Dog B	Dog C	Dog D	Mean	SD[1]
AUC[2] (μg.hr/ml)	183.84	199.79	223.69	199.48	199.48	17.41
MRT (hr)	4.35	4.65	5.89	4.43	4.83	0.71
CL_t (L/hr/kg)	0.14	0.15	0.16	0.16	0.15	0.01
Vd_{ss} (L/kg)	1.12	1.03	1.36	0.94	1.11	0.18
F[3] (%)	57.38	71.29	69.64	68.42	66.68	6.31
C_{max} (μg/ml)	86.17	82.41	58.10	86.13	78.21	12.52
t_{max} (hr)	0.33	0.33	0.66	0.01	0.44	0.19

[1] Standard deviation
[2] AUC over 0-24 hr
[3] $F = AUC_{oral}/AUC_{iv} \times 100$

concentration (t_{max}) was 0.4 hr (± 0.19 SD). The absolute bioavailability (%F) was calculated from the equation:

$$F = AUC_{oral}/AUC_{iv} \times Dose_{iv}/Dose_{oral} \times 100$$

The mean % F was 66.7 (± 6.31 SD). The relatively low value of F is attributed to a significant first pass metabolism of the drug (2,3).

CONCLUSIONS

The HPLC method described herein has sufficient sensitivity to determine the pharmacokinetic parameters of diclofenac in plasma following a single oral or iv bolus dose in a usual therapeutic range. The method is simple, rapid, accurate, and reproducible, representing a significant improvement over many of the recently published HPLC methods for the quantitation of this drug

in plasma. The pharmacokinetic parameters of diclofenac after a single oral or iv bolus administration (5 mg/kg) in dogs were determined.

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