

A rapid and sensitive modified HPLC method for determination of diclofenac in human plasma and its application in pharmacokinetic studies

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ABSTRACT

A sensitive, accurate and rapid reverse phase HPLC method was developed to quantitate plasma levels of diclofenac sodium in human plasma. The drug, internal standard (naproxene) and orthophosphoric acid 1 M, were added to plasma samples and vortexed for 20 sec. A mixture of hexane /isopropyl alcohol (90:10) was then added and vortexed for 2 min. Samples were centrifuged and the supernatant layer was separated, evaporated to dryness under nitrogen gas stream, reconstituted in mobile phase and an aliquot of 50 μ l was analyzed on a μ -bondapack C₁₈ (150 \times 4.6mm) column, with 45% acetonitrile in deionised water and 0.5% orthophosphoric acid, (pH = 3.5) at 276 nm. The standard curve covering 0.005 - 4 μ g/ml concentration range, was linear, relative errors were within 0.13 to 16 % and the CV % ranged from 1.24 to 8.75. The limits of quantitation and detection of the method were 0.005 μ g/mL and 0.002 μ g/mL, respectively. The method was suitable for bioavailability and pharmacokinetic studies of diclofenac in humans and applied in a randomized, two-way cross over bioequivalence study of two different diclofenac sodium preparations with twelve subjects and with a one-week washout period

Keywords: Chromatography, HPLC, Bioequivalence, Diclofenac

INTRODUCTION

Diclofenac sodium or Sodium [O-(2,6-dichlorophenyl)-amino-phenyl]acetate (Fig. 1) is a non-steroidal antiinflammatory analgesic with potent cyclooxygenase inhibition activity (1-4). This drug is commonly used for pain control and treatment of rheumatic diseases (5,4). Diclofenac is well absorbed after oral administration with extensive hepatic metabolism (6,7). This compound exhibits a terminal half life of 1-2 h, volume of distribution of 0.17 l/kg, 99% protein binding (8-10) and enters the synovial fluid (11). The drug is well absorbed orally and dissolves in the intestinal fluid (12). Food has no significant effect on the extent of diclofenac absorption but can cause a delay in the onset of absorption and a reduction in peak plasma levels of approximately 30%. (13). Bioavailability issues have been an increasing concern to drug regulatory authorities for assessment of safety and efficacy of synonym drug products (14). Local drug regulatory authorities have, therefore, issued guidelines to ensure adequate bioavailability studies in new drug applications for synonym drugs (15). In the light of these considerations, this study was conducted to compare the relative bioavailability of two brands of diclofenac sodium tablet preparations. A literature review revealed that the

HPLC method has been the technique of choice for separation and determination of diclofenac in biological fluids. Thus far, several HPLC methods have been described to analyze diclofenac sodium in various body fluids using different clean-up procedures including direct injection, protein precipitation (16-18), solid-phase extraction (SPE) (19-22), liquid-liquid extraction (LLE) (23-27), and column switching (28,29), either with UV (16-21, 28, 29), fluorimetric (18) or electrochemical detection (22, 23, 27).

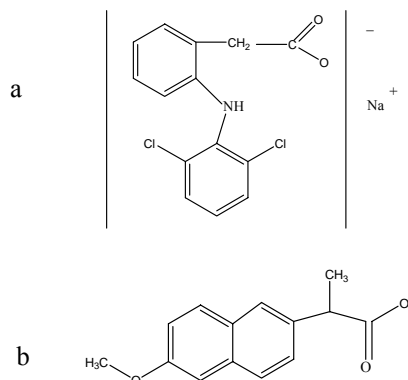


Figure 1. Chemical structure of diclofenac sodium (a) and internal standard, naproxen (b).

Each procedure has some advantages and some drawbacks. In the previous reported HPLC analyses of diclofenac, in both protein precipitation and SPE procedures, endogenous compounds overloaded the column and resulted in a very noisy baseline which interfered with the peak of interest (12) or appeared as late eluting peaks which consequently leads to long run time (20, 22). Different limits of quantification ranging from 0.005 to 1 µg/ml have been reported with SPE and UV detection using 1 ml of plasma (19,20), while, with SPE extraction and electrochemical detection, the limit of quantitation (LOQ) of 1 ng/ml has been reported (22). With LLE and electrochemical detection a LOQ of 10 ng/ml using 100 µl of biological matrices has also been reported (23, 27). Since SPE technique is an expensive procedure and electrochemical detector requires extremely pure and oxygen free mobile phase devoid of metal ions, these methods are especially well suited for very small volume of sample where a very sensitive assay is required and is not economical to use in routine pharmacokinetic studies. For LLE and UV detection a literature survey revealed (25) a HPLC method with LLE and UV detection at 278 nm reaching a sensitivity of 20 ng/ml by using 255 µl plasma samples. In a similar study, the LOQ of 45 ng/ml has been achieved using 1.2 ml of human synovial fluid (26). Although LLE may have some disadvantages, but has been the main procedure for isolation and determination of drug substances from biological matrices. In addition, increasing the sensitivity of the HPLC assays of diclofenac has also been the center of focus of these studies. In the present study, it was of interest to develop a reliable, simple and economical HPLC method using UV detection and LLE for determination of diclofenac in human plasma with improved sensitivity. The described method utilizes neither SPE nor electrochemical detector and yet sensitive, making the method rapid, simple and appropriate for pharmacokinetic and bioequivalency studies of this drug. The developed method was applied to a comparative bioavailability study.

MATERIALS AND METHODS

Reagent and chemicals

Sodium diclofenac and naproxen were from Sigma (Poole, UK), hydrochloric acid, tribasic sodium phosphate, monobasic sodium phosphate, methanol, orthophosphoric acid 85%, acetonitrile, hexane, isopropyl alcohol were from Merck (Germany). All reagents and solutions used were analytical grade except methanol and acetonitrile which were HPLC grades.

Study Products

Test: Sodium diclofenac 50-mg tablets (Sobhan Pharmaceutical Company, Iran)

Reference: Voltarol® 50-mg tablets

Apparatus and chromatographic conditions

The apparatus was a Waters HPLC system model 746 (USA), consisting of a model 515 intelligent solvent delivery pump, a 50 µl injection loop, a computerized system controller, and a Waters 487 UV detector. Chromatographic separation was performed using a µ-bondapack C18 (150 × 4.6mm) column. The mobile phase consisted of acetonitrile, deionized water, orthophosphoric acid (45:54.5:0.5) with final pH of 3.5. The aqueous phase was eluted at a flow rate of 1 ml/min and effluent was monitored at 276 nm. Quantitation was achieved by measurement of the peak area ratios of the drug to the internal standard.

Standard solutions of diclofenac and internal standard

A standard stock solution of sodium diclofenac (100 µg/ml) was prepared by dissolving 10.74 mg of drug (equal to 10 mg diclofenac base) in a 100-ml volumetric flask. A series of standard solutions at concentrations of 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 20, 30, and 40 µg/ml were prepared by further dilution of the standard solution in double-distilled water to obtain different working solutions. Stock of internal standard solution (100 µg/ml) of naproxen was prepared in methanol.

Calibration Procedure

To 1 ml of blank plasma, was added 100 µl of diclofenac standard solutions at concentrations of 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 20, 30, and 40 µg/ml and 100 µl of internal standard at fixed concentration of 100 µg/ml to obtain diclofenac standard concentrations ranging from 0.010 to 4 µg/ml. To the resulting solutions were added 1 ml of 1 M orthophosphoric acid, and 5 ml of a mixture of hexane isopropyl alcohol (90:10), vortexed and centrifuged at 2000 g for 3 min. The supernatant was separated and evaporated to dryness under nitrogen gas. The residue was reconstituted with 100 µl of mobile phase and 50 µl aliquot of the resulting solution was injected to HPLC. Two calibration curves (un-weighted regression line) were obtained by linear least-squares regression analysis by plotting peak area ratios (Diclofenac/I.S) versus two different ranges (low and high) of diclofenac plasma concentrations. Low and high diclofenac concentration ranges were 0.005-0.5 and 0.5-4 µg/ml, respectively.

Sample Preparation

To 1 ml of plasma of volunteers in a 10 ml test tube, were added 100 μ l of internal standard solution, 1 ml of 1M orthophosphoric acid, and 5 ml of a mixture of hexane isopropyl alcohol (90:10). All samples were taken through the extraction procedure. Final sample concentrations were calculated by determination of the peak area ratio of diclofenac related to internal standard and comparing the ratio with the standard curve, obtained after analysis of calibration samples. The presence of disturbing endogenous peaks was examined on twelve human plasma samples. These samples were pretreated according to the sample preparation procedure except for the addition of internal standard.

Recovery determination

The recoveries of diclofenac at the concentration range of calibration curve were evaluated by comparison of the peak areas obtained after extraction of known amount of diclofenac from plasma with those obtained from the same amounts of unextracted diclofenac in water.

Precision and accuracy

The intra- and inter-day variation of the assay were determined by replicate analysis ($n = 6$) of calibration samples of diclofenac at concentrations within the range of calibration curve (ranging from 0.005-0.5 and 0.5-4 μ g/ml) in a single analytical run on the same day and at seven different days, respectively, using the same stock solutions and plasma batches. The percent of relative standard deviations of results of the assay were determined.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The parameters LOD and LOQ were determined using the signal-to-noise ratio by comparing results of the test of samples with known concentrations of analyte to blank samples. The analyte concentration that produced a signal-to-noise ratio of 3:1 was accepted as the LOD. The LOQ was identified as the lowest plasma concentration of the standard curve that could be quantified with acceptable accuracy, precision and variability.

Application of the method

The present method was applied to a comparative bioavailability study. The ethics committee on human studies of the Isfahan University of Medical Sciences approved the study. Twelve healthy adult male volunteers aged between 21 to 30 years and weighing from 56 to 86 kg participated in the study. On the basis of medical history, clinical examinations and laboratory tests including hematology, blood biochemistry and urine analyses, no subject had a history or

evidence of hepatic, renal, gastro-intestinal or hematological deviations, or any acute or chronic disease or drug allergy. The subjects were instructed to abstain from taking any medication at least 2 weeks prior to and during the study period. Informed consent was obtained from the subjects after explaining the nature and purpose of the study. The protocol was the conventional, two-way, crossover study with twelve subjects and a one-week washout period. In the first trial period, after an overnight fasting, subjects were given a single oral dose of 50 mg diclofenac of either test or reference products in a randomized fashion with 200 ml of water. Approximately 2 ml of blood samples were drawn into heparinized tubes through an indwelling canola before (0 h) and at 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 6, 8, 10 h after dosing. The blood samples were centrifuged at 3500 rpm for 20 minutes; plasma was separated and kept frozen at -20°C in coded glass tubes.

Pharmacokinetic Analysis

The area under the curve to the last measurable concentration (AUC_{0-t}) was estimated by the linear trapezoidal rule and $\text{AUC}_{0-\infty}$ was calculated by equation $\text{AUC}_{0-t} + C_t / k_E$, where C_t is the last measurable concentration and k_E the elimination rate constant which was obtained from the least square fitted terminal log-linear portion of the plasma concentration-time profile. The peak plasma concentration (C_{max}) and corresponding time to peak (t_{max}) were determined by the inspection of the individual drug plasma concentration-time profiles.

Statistical Analysis

For the purpose of bioequivalence analysis, AUC_{0-t} , $\text{AUC}_{0-\infty}$, and C_{max} were considered as primary variables. Two-way ANOVA for crossover design was used to assess the effect of formulations, periods, sequences, and subjects on these parameters. A difference between two related parameters was considered statistically significant for a P-value equal to or less than 0.05. The 90% confidence intervals of the ratio of pharmacokinetic parameters of test to reference products as well as those of logarithmically transformed were also estimated (30). All statistical analyses were performed using SPSS 10.

RESULTS AND DISCUSSION

Chromatography

The use of LLE, as compared to SPE and direct protein precipitation, resulted in a considerable reduction in peaks corresponding to solvent front, early huge peaks, and interferences from normal plasma constituents. Fig 2 shows chromatograms of blank human plasma (A), blank plasma spiked

with naproxene as internal standard and drug at concentration of 4 $\mu\text{g/ml}$ (B), plasma sample obtained at 2.5 hr after a single oral dose of 50 mg diclofenac from a healthy volunteer containing 2.14 $\mu\text{g/ml}$ of diclofenac (C). The retention times for diclofenac and naproxen are 7.9 and 3.8 min, respectively. All samples were spiked with 100 μl of internal standard at a concentration of 100 $\mu\text{g/ml}$. All chromatograms were free from

interferences at the retention times of diclofenac or internal standard. Both compounds eluted as completely resolved peaks and no peak tailing was noticed enabling the use of either peak height or peak area in the calculation of standard curves. An optimum flow rate of 1 ml/min for the mobile phase resulted in the retention times of 7.9 min for diclofenac and 3.8 min for naproxen (internal standard).

Table 1. Within and between-day variability of the HPLC assay for determination of plasma diclofenac sodium concentrations.

C ($\mu\text{g/ml}$)	Within-day variability				Between-day variability			
	Mean	SD	Cv%	Error%	Mean	SD	Cv%	Error%
0.005	0.0058	0.00050	8.6	16.0	0.0056	0.00049	8.7	12.1
0.010	0.0104	0.00027	2.6	4.4	0.0107	0.000861	8.4	7.1
0.025	0.0267	0.00107	4.0	6.9	0.0264	0.00091	3.4	5.6
0.05	0.0478	0.00059	1.2	4.4	0.0444	0.00216	4.8	11.1
0.1	0.0961	0.00219	2.3	3.8	0.0962	0.00374	3.8	3.8
0.25	0.2541	0.00828	3.4	1.6	0.251	0.01254	4.9	0.7
0.5	0.4989	0.03139	6.3	0.2	0.492	0.03456	7.0	1.4
1	1.0173	0.04349	4.2	1.7	1.0568	0.04663	4.4	5.7
2	1.8597	0.06173	3.3	7.0	1.878	0.1371	7.3	6.1
3	2.9166	0.25126	8.6	2.7	2.911	0.1927	6.6	2.9
4	3.9947	0.2526	6.3	0.1	4.097	0.1911	4.6	2.4

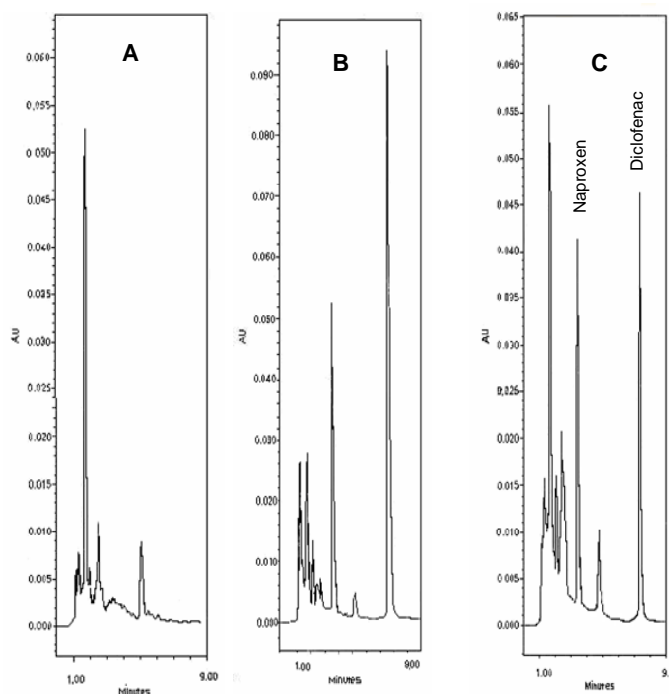


Figure 2. Chromatograms of blank human plasma (A), blank plasma spiked with internal standard, naproxen, and drug at concentration of 4 $\mu\text{g/ml}$ (B), plasma sample obtained at 2.5 hr after a single oral dose of 50 mg diclofenac from a healthy volunteer containing 2.14 $\mu\text{g/ml}$ of diclofenac (C). The retention times for diclofenac and naproxen are 7.9 and 3.8 min, respectively.

Table 2. Pharmacokinetic parameters of the two products of diclofenac (Generic and Brand tablets) administered orally to twelve healthy volunteers.

Parameters	Generic (mean ± SD)	Brand (mean ± SD)	Generic / Brand	CI _{90%} Generic / Brand	P-value Paired t-test
C _{max}	2.24 ± 0.29	2.42 ± 0.25	0.95 ± 0.14	0.978 - 1.061	(0.218)
Log C _{max}	0.35 ± 0.02	0.38 ± 0.01	0.91 ± 0.02	1.048 - 0.982	-
AUC ₀₋₁₀	3.47 ± 0.18	3.41 ± 0.11	1.01 ± 0.12	0.980 - 1.065	(0.918)
Log AUC ₀₋₁₀	0.54 ± 0.01	0.53 ± 0.01	1.01 ± 0.01	0.984 - 1.051	-
AUC _{0-∞}	3.51 ± 0.20	3.44 ± 0.11	1.01 ± 0.12	0.885 - 0.975	(0.881)
Log AUC _{0-∞}	0.54 ± 0.02	0.536 ± 0.01	1.016 ± 0.01	0.863 - 0.965	-
T _{max}	2.46 ± 0.56	2.31 ± 0.43	1.13 ± 0.55	0.964 - 1.202	(1.000)
T 1/2	1.85 ± 0.16	1.92 ± 0.15	1.015 ± 0.25	0.886 - 1.502	(0.934)

Linearity and reproducibility

Good linear relationships were found when the peak area ratios of diclofenac to the internal standard were plotted versus the diclofenac plasma concentration in both low (0.005-0.5 µg/ml) and high diclofenac concentrations (0.5-4 µg/ml). The linear regression equation for the low concentration range were $Y = 0.9268 X (\pm 0.0576) + 0.0135 (\pm 0.0022)$. Intra- and inter-day reproducibility for calibration curves were determined on the same day in replicate (n = 5) and on different days (n = 15) using same pooled plasma sample. The corresponding mean (\pm S.D.) coefficient of the linear regression analysis was 0.9997 ± 0.0075 . Results for calibration curves prepared at high diclofenac concentration range were as follows: $Y = 0.8861 X (\pm 0.0643) + 0.0206 (\pm 0.0028)$ and coefficient of the linear regression analysis of 0.9913 ± 0.0082 .

Limits of detection and quantification

The detection limit for diclofenac was approximately 2 ng/ml at a signal to noise ratio of 3:1 and the limit of quantitation corresponding with a coefficient of variation of less than 10% was 5 ng/ml using one ml of the plasma sample.

Precision, accuracy and recovery

The intra- and inter-day precision and accuracy of the assay were examined by analyzing replicate plasma samples spiked with different amounts of the drug within calibration curve range at the same day and 10 different days. The intra- and inter-days accuracy and precision values of the assay methods are presented in Table 1. Relative errors were within 0.13 to 16 % and the CV% ranged from 1.24 to 8.75. Coefficient of variation and percent of error indicate that the method is reproducible within and between days. The extraction efficiency was determined by comparison of the peak areas of the spiked plasma samples with those of un-extracted diclofenac solution in water. The mean recoveries were found to be 97.4% for diclofenac. The recoveries were consistent for the samples as demonstrated

by R.S.D. of less than 4.3%. Recoveries were not dependent on the concentration, and consequently resulted in good linearity of the calibration curve.

Assay application

Applicability of this method was confirmed by analyzing true samples obtained after administration of 50 mg of diclofenac sodium to healthy volunteers. This method was used for determination of plasma concentration of diclofenac in a randomized cross-over bioequivalence study following administration of 50 mg of the drug in 12 volunteers. Diclofenac was measurable at the first sampling time (0.25 h) in all volunteers. The concentration-time profile following oral administration of two different diclofenac sodium preparations are plotted in Fig. 3. The mean pharmacokinetic parameters for the brands of diclofenac sodium tablets are also summarized in Table 2. The AUC₀₋₁₀ and AUC_{0-∞} for the two products were not statistically different ($P > 0.05$), suggesting that the plasma profiles generated by test product were comparable to those produced by reference formulation. ANOVA for these parameters, after log-transformation of data, showed no statistically significant difference between the two formulations and did not reveal any considerable differences in periods, formulations, or sequences ($P > 0.05$). Ninety percent confidence intervals of the ratio of the AUC_{0-∞} of the two formulations (88.5 - 97.5 %) were found to be within the FDA acceptable range of 80 - 120% for evaluation of bioequivalency. The C_{max} values of two products were also analyzed with the ANOVA procedure, indicating no statistically difference between generic formulation and reference. Furthermore there was no significant difference with regards to periods and sequence ($P > 0.05$). The ninety percent confidence intervals of the ratio of C_{max} of two formulations were 97.8 - 106.1 %, which lies within the FDA acceptable range of 80 - 120 % (Table 2).

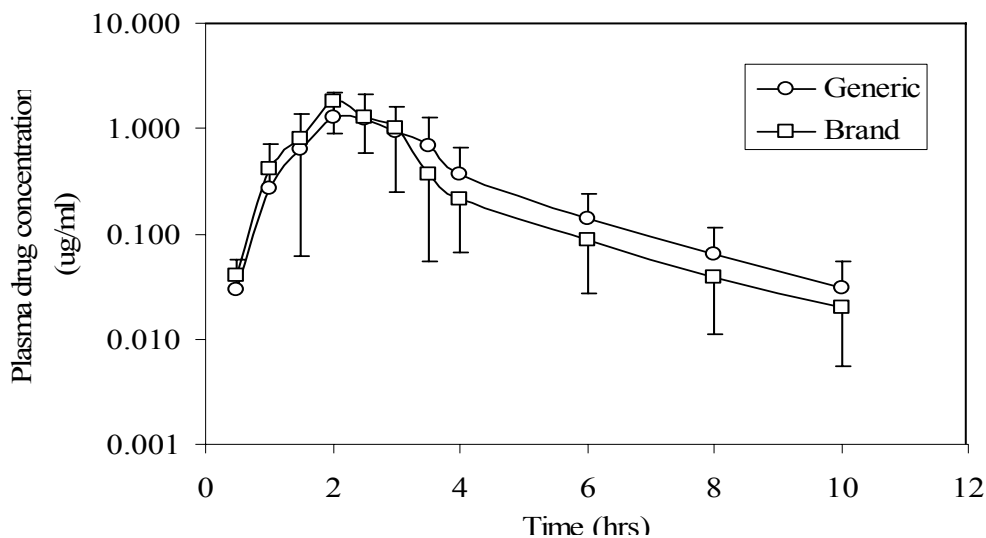


Figure 3. Plasma diclofenac levels vs time profiles following ingestion of a single dose of 50-mg tablet of the Generic and Brand to 12 healthy volunteers.

CONCLUSIONS

In summary, a rapid, practical and more sensitive HPLC method based on LLE with UV detection is described for determination of diclofenac in human plasma. The sensitivity of this method is lower than that reported for HPLC with electrochemical detection and SPE sample purification (22) where a highly sensitive method was required for analyzing diclofenac in cerebrospinal which is not economic to use in routine pharmacokinetic studies. In contrast, our HPLC method is more sensitive than other similar reported HPLC methods with LLE and UV detection (25, 26). A HPLC method with LLE and UV detection at 278 nm has been reported with a sensitivity of 20 ng/ml using 255 μ l plasma

samples (25) but the sensitivity of the assay could not be extrapolated to 1 ml of plasma. Moreover, the HPLC method presented here has the same sensitivity as HPLC methods with SPE and UV detection but by using 1 ml of plasma (12). The shorter time of analysis, simplicity, and sufficient sensitivity of the method make it particularly useful for the pharmacokinetic and bioequivalence studies of diclofenac sodium. Ultimately, the present method was applied in a bioequivalence study and it was found that the generic and brand dosage forms were bioequivalent.

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REFERENCES

1. Reynolds, JEF. Martindale, The Extra Pharmacopoeia, Vol. 33, pp 30-32, The Royal Pharmaceutical Society, Great Britain, London, 2002.
2. Furst DE, Munster T. Nonsteroidal anti-inflammatory drugs, disease modifying antirheumatic drugs, nonopioid analgesics and drugs used in gout. In: Katzung BG. Basic and clinical pharmacology, 8th ed. New York, Mc Graw – Hill, 2001, pp 598-99, 604.
3. Ku EC, Wasvary JM, Cash WD. Diclofenac sodium (GP 45840, Voltaren), a potent inhibitor of prostaglandin synthetase. *Biochem. Pharmacol.* 1985; 24: 641–643.
4. Menasse R, Hedwall PR, Kraetz J, Pericin C, Riesterer L, Sallmann A, Ziel R, Jaques R. Pharmacological properties of diclofenac sodium and its metabolites. *Scand. J. Rheumatol.* 1978; 22: 5–16.
5. Brogen RN, Heel RC, Pakes GE, Speight TM, Avery GS. Diclofenac sodium: a review of its pharmacological properties and therapeutic use in rheumatic diseases and pain of varying origin. 1980; *Drugs* 20: 24–48.
6. Hasan MM, Najib NM, Rawashdeh, NM, Sallam EN, Shubair MS, Alawneh Y. Comparative bioavailability of two tablet formulations of diclofenac sodium in normal subjects. *Int. J. Clin. Pharmacol. Ther. Toxicol.* 1991; 29: 178–183.
7. Fowler PD, Shadforth MF, Crook PR, John VA. Plasma and synovial fluid concentrations of diclofenac sodium and its major hydroxylated metabolites during long-term treatment of rheumatoid arthritis. *Eur. J. Clin. Pharmacol.* 1983; 25: 389–394.

8. Willis JV, Kendall MJ, Flinn RM, Thornik DP, Welling PG. The pharmacokinetics of diclofenac sodium following intravenous and oral administration. *Eur. J. Clin. Pharmacol.* 1979; 16: 405–410.
9. Benson MD, Aldo-Benson M, Brandt KD. Synovial fluid concentrations of diclofenac in patients with rheumatoid arthritis or osteoarthritis. *Semin. Arthritis Rheum.* 1985; 15: 65–67.
10. Chan KKH, Vyas KH, Brand KD. In vitro protein binding of diclofenac sodium in plasma and synovial fluid. *J. Pharm. Sci.* 1987; 76: 105–108.
11. Wallis WJ, Simkin PA. Antirheumatic drug concentration in human synovial fluid and synovial tissue: observation on extravascular pharmacokinetics. *Clin. Pharmacokinet.* 1983; 8: 496–522.
12. Arcelloni C, Lanzi R, Pedercini S, Molteni G, Fermo I, Pontiroli A, Paroni R. High performance liquid chromatographic determination of diclofenac in human plasma after solid-phase extraction. *J. Chromatogr. B* 2001; 763: 195-200.
13. Terhaag B, Gramatte T, Hrdlcka P, Richter K, Feller K. The influence of food on the absorption of diclofenac as a pure substance. *Int. J. Clin. Pharmacol. Ther. Toxicol.* 1991; 29: 418–421.
14. Eranko PO, Palva, ES, Konno K, Venho VMK. Regulatory control of synonym drug bioavailability: Bioinequivalency of Nifedipine preparations. *Pharmaceutic Med.* 1990; 4: 197-205.
15. Emami J, Varshosaz J, Flamarzian M, Tahvilian R. High performance liquid chromatographic determination, pharmacokinetic and comparative bioavailability studies of cisapride. *J. Pharm. Biomed. Anal.* 2003; 33: 513 – 520.
16. Avgerinos A, Karidas T, Malamataris S. Extractionless high-performance liquid chromatographic method for the determination of diclofenac in human plasma and urine. *J. Chromatogr.* 1993; 619(2): 324-329.
17. El-Sayed YM, Abdel-Hameed ME, Suleiman MS, Najib NM. A rapid and sensitive high-performance liquid chromatographic method for the determination of diclofenac sodium in serum and its use in pharmacokinetic studies. *J. Pharm. Pharmacol.* 1988; 40 (10): 727-729.
18. Moncrieff J. Extractionless determination of diclofenac sodium in serum using reversed-phase high-performance liquid chromatography with fluorimetric detection. *J. Chromatogr.* 1992; 577 (1): 185-189.
19. Hiari T, Matsumoto S, Kishi I. Simultaneous analysis of several non-steroidal anti-inflammatory drugs in human urine by high-performance liquid chromatography with normal solid-phase extraction. *J. Chromatogr. B Biomed. Sci. Appl.* 1997; 692: 375-88.
20. Millership JS, Hare LG, Farry m, Collier PS, McElnay JC, Shields MD, Carson DJ. The use of hydrophilic lipophilic balance copolymer SPE cartridges for the extraction of diclofenac from small volume pediatric plasma samples. *J. of Pharm. Biomed. Analysis.* 2001; 25: 871-879.
21. Mason JL, Hobbs GJ, A rapid high performance liquid chromatographic assay for the measurement of diclofenac in human plasma. *J. Liq. Chromatogr.* 1995; 18: 2045-2058.
22. Zecca L, Ferrario P, Costi P. Determination of diclofenac and its metabolites in plasma and cerebrospinal fluid by high-performance liquid chromatography with electrochemical detection. *J. Chromatogr.* 1991; 567(2): 425-432.
23. Torres-Lopez JE, Robles MB, Perez-Urizar J, Flores-Murrieta FJ, Granados-Soto V. Determination of diclofenac in micro-whole blood samples by high-performance liquid chromatography with electrochemical detection. Application in a pharmacokinetic study. *Arzneimittelforschung.* 1997; 47(9): 1040-1043.
24. Blaghrough IS, Daykin MM, Doherty M, Patrick M, Shaw PN. High performance liquid chromatographic determination of naproxen, Ibuprofen and diclofenac in plasma and synovial fluid in man. *J. Chromatogr.* 1992; 578: 251-257.
25. Giagoudakis G, Markantonis SL. An alternative high-performance liquid chromatographic method for the determination of diclofenac and flurbiprofen in plasma. *J. Pharm. Biomed. Anal.* 1998; 17(4-5): 897-901.
26. Roskar R, Kmetec V. Liquid chromatographic determination of diclofenac in human synovial fluid. *J. Chromatogr. B* 2003; 788: 57-64.
27. Plavsic F, Cullig j. Determination of serum diclofenac by high-performance liquid chromatography by electrochemical detection. *Hum. Toxicol.* 1985; 4(3): 317-322.
28. Lee HS, Kim EJ, Zee OP, Lee YJ. High performance liquid chromatographic determination of diclofenac sodium in plasma using column-switching technique for sample clean-up. *Arch Pharm.* 1989; 322: 801-806.
29. Lee HS, Jeong CK, Choi SJ, Kim BS, Lee MH, Ko GI, Sohn DH. Simultaneous determination of aceclofenac and diclofenac in human plasma by narrowbore HPLC using column switching. 2000; 23: 775-781.
30. Westlake WJ. Use of confidence intervals in analysis of comparative bioavailability trials. *J. Pharm. Sci.* 1972; 61(8): 1340-1341.