HPLC Method for Determination of Diclofenac in Human Plasma and Its Application to a Pharmacokinetic Study in Turkey

Bilal Yilmaz[1,*], **Ali Asci**[2], and **Saziye Sezin Palabiyik**[2]

[1]Department of Analytical Chemistry, Faculty of Pharmacy, Ataturk University, 25240, Erzurum, Turkey and [2]Department of Toxicology, Faculty of Pharmacy, Ataturk University, 25240, Erzurum, Turkey

Abstract

A simple high-performance liquid chromatography (HPLC) method has been developed for determination of diclofenac in human plasma. The method was validated on Ace C_{18} column using UV detection. The mobile phase consisted of 20 mM phosphate buffer (pH 7) containing 0.1% trifluoroacetic acid–acetonitrile (65:35, v/v). Calibration curve was linear between the concentration range of 75–4000 ng/mL. Intra- and inter-day precision values for diclofenac in plasma were less than 3.6, and accuracy (relative error) was better than 5.3%. The limits of detection and quantification of diclofenac were 25 and 75 ng/mL, respectively. Also, this assay was applied to determine the pharmacokinetic parameters of diclofenac in healthy Turkish volunteers who had been given 50 mg diclofenac.

Introduction

Diclofenac (Figure 1) is a nonsteroidal anti-inflammatory drug (NSAID) that is widely prescribed for the treatment of rheumatoid arthritis, osteoarthritis, musculoskeletal injuries, and post surgery analgesia in human and veterinary medicine. Patients are frequently given special formulations of diclofenac or a co-treatment agent as a therapeutic strategy to attenuate the gastrointestinal tract complications that limit the use of diclofenac and other NSAIDs (1–3). Many patients prescribed diclofenac for arthritis also take additional drugs for other chronic health problems such as hypertension (4,5).

Several methods have been reported for determination of diclofenac including gas chromatography-mass spectrometry (GC–MS) (6–9) high-performance liquid chromatography (HPLC) (10–25) and LC–MS–MS (26) in human plasma and other biological fluids.

In addition, no method is reported to date for determination of diclofenac by HPLC in humans in Turkey. In this paper a HPLC with UV method for determination of diclofenac in healthy Turkish volunteers using internal standard methodology is reported. The developed method was validated by using linearity, stability, precision, accuracy, and sensitivity parameters according to International Conference on Harmonization (ICH) guidelines (27).

The advantages of present method include simple and single step extraction procedure using inexpensive chemicals and short run time. Also, this method was used to assay the diclofenac in human plasma samples obtained from six healthy male volunteers. At the same time, the method was efficient in analyzing large numbers of plasma obtained for pharmacokinetic study after therapeutic doses of diclofenac.

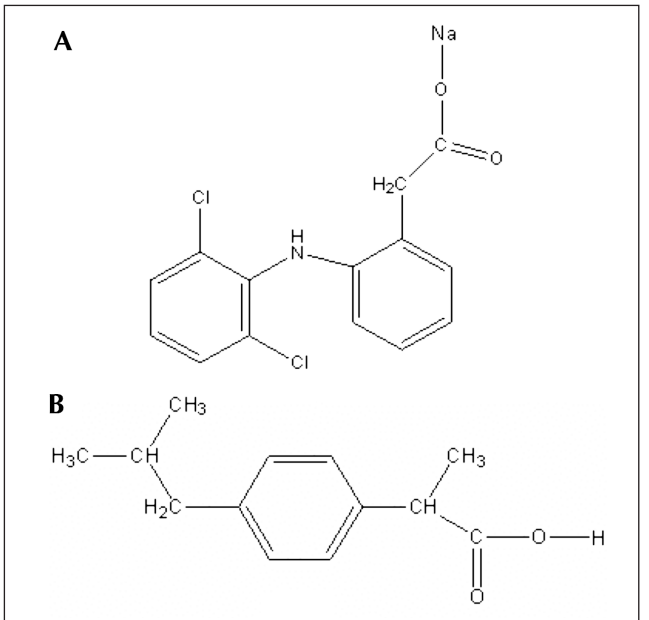

Figure 1. Chemical structures of diclofenac (A) and ibuprofen, IS (B).

*Author to whom correspondence should be addressed: email bilalylmaz@yahoo.com.

Experimental

Materials and reagents

Diclofenac and ibuprofen (internal standard, IS) were obtained from Sigma (St. Louis, MO). Ethylacetate, hexane and methanol were purchased from Sigma-Aldrich (St. Louis, MO). Dolorex dragee (50 mg diclofenac) was obtained by pharmacy (Erzurum, Turkey). HPLC-grade organic solvents were purchased from Merck (Darmstadt, Germany). All chemicals were of analytical grade. Distilled water was prepared as required by using aquaMAX ultra, Young instrument (Korea) ultrawater purification system. Human plasma was obtained from Yakutiye blood bank, Erzurum, Turkey.

Instrumentation

A Perkin-Elmer series 200 HPLC system equipped with programmable UV–vis detector and Total Chrom Chromatography Data System software was used (Perkin-Elmer Life and Science, Shelton, CT). The HPLC mobile phase was composed of 20 mM phosphate buffer (pH 7) containing 0.1% TFA-acetonitrile (65:35, v/v). Separation was achieved using an Ace C_{18} column (5 µm, 4.6 × 250 mm i.d.) with a guard column (4 mm × 3 mm i.d., Phenomenex) packed with the same material at a flow rate of 1.0 mL/min. The eluent was monitored by UV detection at 225 nm.

Preparation of stock and standard solutions

The stock solution of diclofenac (1.0 mg/mL) was prepared and diluted with methanol to give standard solutions of 75–4000 ng/mL. Standard calibration samples were prepared daily by spiking 1.0 mL of drug-free human plasma with 1.0 mL of appropriate diclofenac standard solutions to achieve final concentrations of 75–4000 ng/mL for plasma. The working solution of IS was prepared by dissolving in methanol to obtain a concentration of 20 µg/mL.

Preparation of quality control samples

The concentrations of diclofenac were 150, 750, and 3500 ng/mL in human plasma to represent low, middle, and high quality controls, respectively. Appropriate volumes from stock solution of diclofenac were added to normal human plasma to get low, middle and high quality control samples and stored at –20°C. The quality control samples were taken out from storage for analysis to determine intra- and inter-day precision and accuracy.

Extraction procedure

Blood samples were collected into the tubes containing disodium EDTA and centrifuged at 4500 × *g* for 10 min. A 1.0 mL of the resultant plasma sample was spiked with 1.0 mL of diclofenac, 0.1 mL of internal standard, and 0.5 mL H_3PO_4 solu-

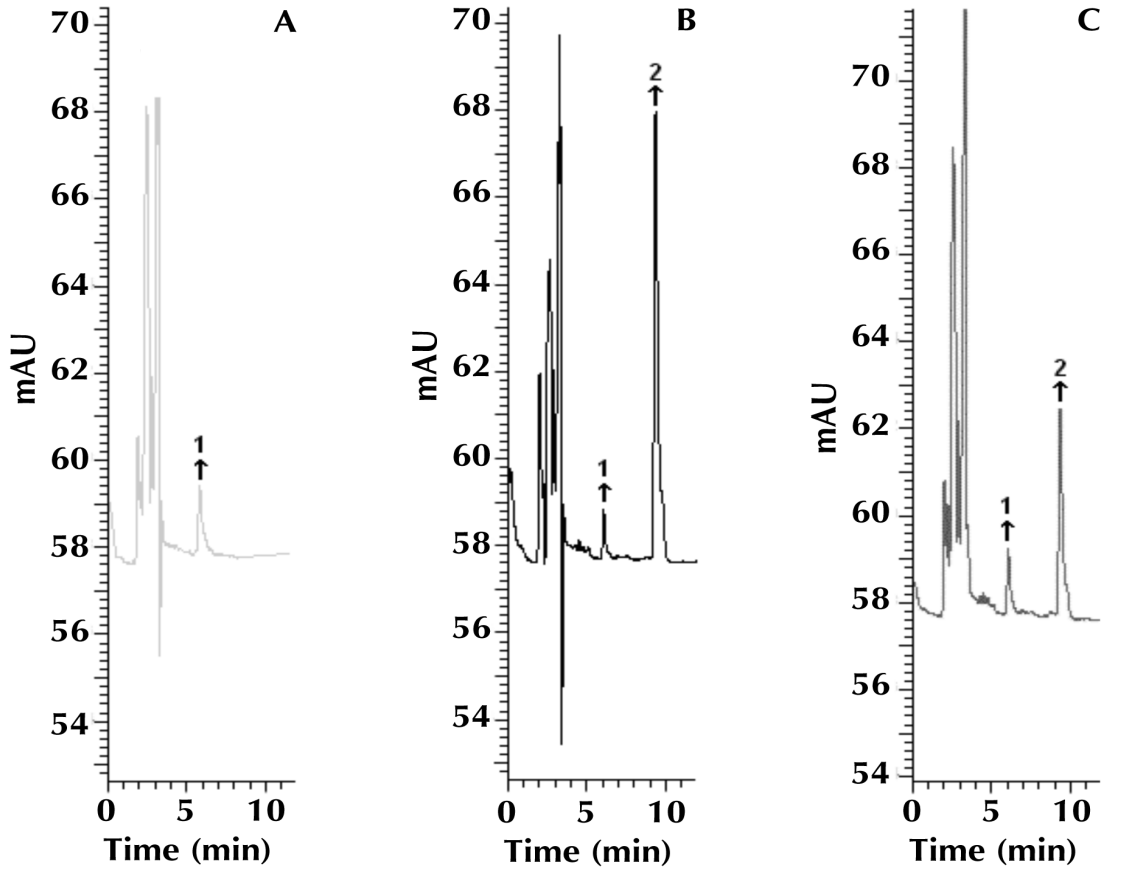

Figure 2. Representative chromatograms of (A) drug-free plasma, (B) the plasma spiked with diclofenac (2000 ng/mL) and IS (2000 ng/mL), (C) the plasma obtained at 45 min after a single oral dose of 50 mg diclofenac, (1) IS, (2) diclofenac.

tions were added. After vortex mixing for 5 s, 3 mL of ethylacetate and hexane (2:3, v/v) was added. The mixture was vortexed for 2 min and then centrifuged at 3000 × *g* for 3 min. The organic layer was transferred into another 5 mL tube and evaporated to dryness under stream of nitrogen gas at 40°C. The residue was reconstituted in 1.0 mL methanol, and a 10 µL aliquot was injected into the HPLC system.

Collection of samples

This study was approved by the Ethics Committee of Faculty of Medicine, Ataturk University, after having received written consents from all the volunteers. The validated method was applied to pharmacokinetic study of diclofenac in six healthy Turkish volunteers [aged between 29 and 38 years (34.8 ± 3.1), weighing between 62 and 85 kg (78.6 ± 8.2)] after an oral administration of 50 mg diclofenac (Dolorex dragee). The volunteers were fasted overnight before the study and for 4 h after the dosing. Venous blood samples (5 mL) were collected at 0, 15, 30, 45, 60, 75, 90, 120, 180, 240, and 300 min following administration. The blood samples were centrifuged at 4000 × *g* for 10 min and the plasma was taken and stored at –20°C until analysis.

Data analysis

The maximum plasma concentration (C_{max}) and the time to reach maximum concentration (T_{max}) were directly determined from the plasma concentration versus time curves. The area under the curve from 0 to t (AUC_{0-t}) was calculated by the linear trapezoidal rule. The area under the curve from 0 h to infinity ($AUC_{0-\infty}$) was estimated by summing the area from 0 to t (AUC_{0-t}) and t to infinity ($AUC_{t-\infty}$), where $AUC_{t-\infty} = C_t/K_{el}$, with C_t defined as the last measured plasma concentration at time t, and k_{el} the slope of the terminal portion of the ln(plasma concentration) versus time curve (28). The elimination half-life ($t_{1/2}$) was calculated using the pharmacokinetic relationship $t_{1/2} = \ln(2)/k_{el}$.

Results

Method development and optimization

Method development was focused on the optimization of column detection, sample preparation and chromatographic separation. Reversed-phase column (C_{18}) can be used for the separation of non-ionic as well as ion forming non-polar to medium polar substances while normal phase chromatography can be used for the separation of non-ionic and/or non-polar substances. Majority of the ionizable pharmaceutical compounds can be very well separated on C_{18} column (29). Thus, diclofenac can be satisfactorily separated by reversed phase chromatography.

Several tests were performed for optimizing the components of mobile phase in order to achieve good chromatographic peak shape and resolution. The test results showed that the solvent system of acetonitrile could improve the peak shapes of diclofenac. Good separation of target compounds and short run time were obtained using a mobile phase system of 20 mM phosphate buffer (pH 7) containing 0.1% TFA–acetonitrile (65:35, v/v). The retention time of diclofenac (9.3 min) was quite short than that studied in other papers (6–8).

Representative chromatograms of (A) drug-free plasma, (B) the plasma spiked with diclofenac (2000 ng/mL) and IS (2000 ng/mL) and (C) the plasma obtained at 45 min after a single dose of 50 mg diclofenac were given in Figure 2. There is no interference in the chromatogram of drug-free plasma.

Validation of the method

The validation of the method was carried out by establishing specifity, linearity, intra- and inter-day precision, accuracy, recovery, limit of detection (LOD), and limit of quantitation (LOQ) according to ICH guidance (27).

Specificity

Preparation of plasma samples was processed by liquid–liquid extraction procedure. The samples were chromatographed to determine to which extent endogenous plasma components may contribute to the peak interference at retention time of diclofenac and IS. Commonly prescribed drugs were analysed for possible interference. The retention times for these drugs under the chromatographic conditions for the diclofenac assay were determined.

As mentioned earlier, under the described analysis procedure, the peaks of diclofenac and IS were well resolved with good symmetry and desirable retention time from endogenous compounds in the blank human plasma. Representative chromatograms of human blank plasma and plasma samples spiked

Table I. Precision and Accuracy of Diclofenac in Human Plasma

	Intra-day			Inter-day		
Added	Found ± SD*	Precision % RSD†	Accuracy‡	Found ± SD*	Precision % RSD†	Accuracy‡
Plasma§						
150	142 ± 4.5	3.2	–5.3	143 ± 3.8	2.7	–4.7
750	739 ± 21.2	2.9	–1.5	762 ± 27.6	3.6	1.6
3500	3452 ± 50.4	1.5	–1.4	3528 ± 58.4	1.7	0.8

* SD = Standard deviation of six replicate determinations.
† RSD = Relative standard deviation, average of six replicate determinations.
‡ Accuracy: (% relative error) (found – added)/added × 100.
§ Plasma volume (1.0 mL).

Table II. Recovery of Diclofenac in Human Plasma (n = 6)

	Concentration (ng/mL)			
Sample	Added	Found (Mean ± SD)	%Recovery	%RSD
Plasma	250	242 ± 7.7	96.8	3.2
Intra-day	1000	1012 ± 28.9	101.2	2.9
	4000	3753 ± 98.7	93.8	2.6
Plasma	250	237 ± 8.7	94.8	3.7
Inter-day	1000	976 ± 25.9	97.6	2.7
	4000	3852 ± 109.4	96.3	2.8

with diclofenac and IS were shown in Figure 2. There were no interference peaks near the retention times of diclofenac and IS.

Linearity

Calibration curves were prepared by adding known amount of diclofenac (75, 250, 500, 1000, 2000, 3000, and 4000 ng/mL) to 1.0 mL of blank plasma. An aliquot of 0.1 mL of the IS solution (2000 ng/mL) was added to each sample. The samples were extracted as described above. The standard curves were constructed by plotting the peak area ratio of diclofenac and IS on *Y*-axis and concentration of diclofenac on *X*-axis. Linearity was assessed by a weighted (1/*C*) least square regression analysis. The calibration equation from three replicate experiments, $y = 0.035x + 0.016$ ($r = 0.999$), demonstrated the linearity of the method.

Precision and accuracy

Intra-day and inter-day precision and accuracy were determined by replicate analysis of six sets of samples spiked with three different concentrations of diclofenac (150, 750, and 3500 ng/mL) within a day or during three consecutive days. The precision was calculated from the ratio of the standard deviation to the mean (relative standard deviation, RSD). The accuracy of the method was examined by comparing the concentrations of spiked samples to the theoretical concentrations. Both values were expressed as percentage. The results of precision and accuracy were presented in Table I.

The intra-day precision and accuracy were varied between 1.5% and 3.2%, and 94.7% and 98.6%, respectively. The inter-day precision and accuracy ranged from 1.7% to 3.6% and 95.3% to 101.6%, respectively.

Sensitivity

The sensitivity was evaluated by the limit of quantitation (LOQ), the lowest concentration of the plasma spiked with diclofenac in the calibration curve. The LOQ was defined as the concentration producing a precision less than 20% and accuracy between 80% and 120% of the theoretical concentrations. The LOQ was determined to be 75 ng/mL. The intra-day precision and accuracy were 5.8% and 94.9%, respectively. The inter-day precision and accuracy were 9.2% and 102.4%, respectively.

Recovery

The recovery was determined by comparing peak area of diclofenac after extraction to that before extraction at concentrations of 250, 1000, and 4000 ng/mL. The mean extraction recovery of diclofenac from human plasma was 96.8%. The mean relative recovery for IS at 2000 ng/mL was 96.8 ($n = 6$). Recovery values are shown in Table II.

Stability

In bench top stability, three replicates of low and high controls of diclofenac (250 and 3000 ng/mL) were analyzed at 0 and 6 h at room temperature and the deviation was calculated. In autosampler stability, three replicates of low and high quality control samples were analyzed at 0, 12, and 24 h by keeping in autosampler at 10°C and the deviation was calculated.

In freeze-thaw stability, three replicates of low and high quality control samples of diclofenac were prepared, frozen at –20°C and analyzed after 1, 2, and 3 freeze-thaw cycles. In dry extract stability, three replicates of high and low quality control samples were prepared. After evaporating the organic phase the tubes were stored at –20°C and analyzed after 24 h by reconstituting with 1.0 mL of methanol and injected 10 µL in the HPLC system. The mean concentration of 24 h samples was compared with that of sample analyzed at 0 h long-term stability was done for 14 days by taking three replicates of high and low quality control samples. The mean concentration was taken into consideration which was compared with zero day sample concentration. The percentage variation observed in bench top stability, autosampler stability, three freeze-thaw cycles and dry extract stability were within the limit of 15% (Table III).

Discussion

Today, HPLC is a powerful technique for highly specific and quantitative measurements of low levels of analytes in biological samples.

The specificity of the method was verified by investigating the peak interference from the endogenous plasma substances. Representative chromatograms of blank plasma and plasma samples spiked with diclofenac and IS were shown in Figure 2.

Table III. Stability of Diclofenac in Human Plasma ($n = 3$)

	Recovery (Mean ± SD) Plasma conc. (ng/mL)	
Treatment	250	3000
Bench top stability for 6 h	93 ± 5.2	101 ± 3.8
Autosampler stability for 24 h	98 ± 5.4	90 ± 3.9
Three freeze-thaw cycles	91 ± 6.4	94 ± 4.2
Dry extract stability for 24 h	101 ± 5.2	96 ± 7.6
Stored at RT for 24 h*	93 ± 4.4	91 ± 6.1
Stored at –20°C for 24 h	90. ± 3.5	94 ± 4.5
Stored at –20°C for 2 weeks	87 ± 6.5	89 ± 5.6

* RT = room temperature.

Table IV. Mean Pharmacokinetic Parameters of Diclofenac for Six Volunteers after Oral Administration of Dolorex Dragee (50 mg)

Parameter	(Mean ± SD)	%RSD
Maximum plasma conc. C_{max} (ng/mL)	1136 ± 128	11.3
Time required for maximum plasma conc. (T_{max}) (min)	45 ± 12.3	27.3
Area under curve $AUC_{(0-300\ min)}$ (min ng/mL)	9164 ± 1963	21.4
Area under curve at infinite time $AUC_{(0-\infty)}$ (min ng/mL)	11626 ± 1539	13.2
Elimination rate constant (K_{el}) (min^{-1})	0.009 ± 0.001	11.1
Plasma half life ($T_{1/2}$) (min)	74 ± 20	27.0

When this method is applied to plasma samples, its sensitivity was found to be adequate for pharmacokinetic studies. The present method has the following advantages over the reported method.

Calibration curve of diclofenac was linear over the concentration range of 75–4000 ng/mL for plasma which is as good as or superior to that reported in other papers (12,14,18,22, 22,24,26). Diclofenac was extracted from plasma with a solid phase extraction procedure by Hirai et al. (14) and Arcelloni et al. (23). These methods are also the most comprehensive method which can extract diclofenac in a single extraction procedure. The mean recovery is better for plasma than those of the studies reported by Borenstein et al. (6), Kadowaki et al. (7), Roskar et al. (21), and Kaphalia et al. (22).

Sparidans et al. (26) have reported an LC method with tandem mass detection for the analysis of diclofenac in mause plasma. The calibration curve of LC–MS–MS method was linear for diclofenac in the range 20–10000 ng/mL. Intra- and inter-day precision values were lower than 13%. The maximum recovery of diclofenac was between 90 and 108%. Detection using LC–MS–MS would be a more sensitive approach but it is costly and not yet available for every laboratory.

In statistical comparison ($P > 0.05$) with other methods in the literature (11,17,18,20,21,24,26), the proposed method has indicated high accuracy and precision.

The sensitivity was evaluated by the limit of quantification (LOQ). The LOQ was determined to be 75 ng/mL. This method is as good or superior to that reported in the other papers (7,14,21,22,24).

Additionally, this method was applied to six Turkish volunteers who had been given an oral dragee of 50 mg diclofenac. The amount of diclofenac was determined between 0 and 6 hours in human plasma. The mean plasma concentration-time curve was shown in Figure 3. The mean values of pharmacokinetic parameters estimated by the computer program WinNonlin with noncompartmental method were shown in Table IV. The maximum plasma concentration of diclofenac in Turkish volunteers was in good agreement with the values reported by other groups (8,24). Arcelloni (23) previously reported that after an oral administration of 100 mg diclofenac slow-release tablet to healthy volunteers, AUC, C_{max} and T_{max} were 5340 ± 2840 h ng/mL, 630 ± 390 ng/mL and 6.0 ± 2.0 h, respectively. The mean AUC, C_{max}, and T_{max} of diclofenac obtained from healthy Turkish subjects (9164 ± 1963 min ng/mL, 1136 ± 128 ng/mL, and 45 ± 12.3 min, respectively) were higher than the reported values by Arcelloni under the comparable study design (23), at least in part, due to differences in ethnic groups, pharmaceutical forms and analytical method (HPLC).

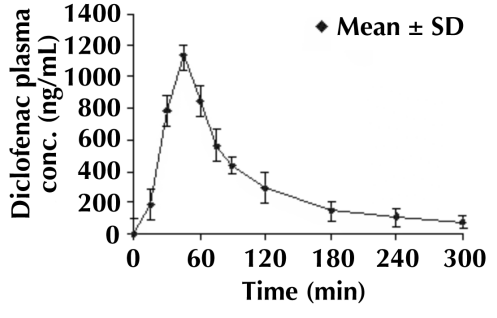

Figure 3. Mean plasma diclofenac concentration-time profile for six volunteers after a single oral dose of diclofenac, 50 mg.

Conclusion

The HPLC method was validated for simple, rapid, sensitive, and accurate determination of diclofenac in human plasma as well as for satisfying the bioanalytical method validation of ICH. Also, by applying this method to analyze the plasma samples of healthy Turkish subjects, we determined the pharmacokinetic parameters of diclofenac in Turkey, demonstrating the adequacy of this assay for clinical studies. Therefore, the method has potential applicability in pharmacokinetic and bioequivalence studies of diclofenac.

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