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Article

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

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A simple high-performance liquid chromatography method has been developed for the determination of naproxen in human plasma. The method was validated on an Ace C18 column using ultraviolet detection. The mobile phase consisted of 20 mM phosphate buffer (pH 7) containing 0.1% trifluoroacetic acid–acetonitrile (65:35, v/v). The calibration curve was linear between the concentration ranges of 0.10 and 5.0 μg/mL. Intra-day and inter-day precision values for naproxen in plasma were less than 4.84, and accuracy (relative error) was better than 3.67%. The extraction recovery values of naproxen from human plasma were between 91.0 and 98.9%. The limits of detection and quantification of naproxen were 0.03 and 0.10 μg/mL, respectively. Also, this assay was applied to determine the pharmacokinetic parameters of naproxen in six healthy Turkish volunteers who had been given 220 mg of naproxen.

Introduction

Naproxen (Figure 1), a non-steroidal anti-inflammatory drug (NSAID) derived from propionic acid, is widely used to moderate pain relief in the treatment of many diseases (1). NSAIDs, including naproxen, are commonly employed to reduce ongoing inflammation, pain and fever, because they are able to block (2) the cyclooxygenase (Cox) enzymes (Cox-1 and Cox-2) that both produce prostaglandins; these classes of compounds have several important functions, such as the promotion of inflammation, pain and fever (3). However, prostaglandins produced by the Cox-1 enzyme are also able to protect the stomach and to support platelets and blood clotting. Thus, NSAIDs can cause ulcers in the stomach and promote bleeding after an injury or surgery. Moreover, they are associated with other serious side effects, i.e., kidney failure, and with many minor side effects, such as nausea vomiting, diarrhea, constipation, decreased appetite, rashes, dizziness, headaches and drowsiness. When anti-inflammatory treatments become chronic, as in the case for rheumatoid arthritis, the patients are exposed to the drugs for prolonged time periods. The potential misuse and involuntary intake of naproxen as residues in food can pose a health risk in people; for example, causing allergies, severe gastrointestinal lesions, changes in renal function and nephrotoxicity (4).

Several methods have been reported for the determination of naproxen in plasma and other biological fluids, including high-performance liquid chromatography (HPLC) using ultraviolet (UV) (5–12) or fluorescence detection (13–17), liquid chromatography–tandem mass spectrometry (LC–MS-MS) (18, 19), gas chromatography (GC)–MS (20, 21) and GC–flame ionization detection (FID) (22, 23).

Various HPLC methods have been reported to determine naproxen by using different mobile phases and wavelengths (5–17). In addition, there several available methods to simultaneously separate NSAIDs by HPLC, but some lack suitable sensitivity.

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

When this method was applied to plasma samples, its sensitivity was found to be adequate for pharmacokinetic studies. The present method has the following advantages over the reported methods: the calibration curve of naproxen was linear over the concentration range of 0.10–5.0 μg/mL for plasma, which is as good as or superior to that reported in other HPLC methods (5–8, 11, 13–16).

Although many of these methods have been successfully applied, their use for the analysis of high sample numbers remains limited by several drawbacks. Both GC–MS and GC–FID require a suitable derivatization step due to the polar nature of naproxen, e.g., by use of N,O-bis(trimethylsilyl)acetamide (20), tetrabutylammonium hydroxide (21) or diazomethane (22, 23), incurring additional sample preparation time and costs. However, GC methods have not been used to quantitate naproxen in clinical samples since 1981.

To date, no method has been reported for the determination of naproxen by HPLC in humans in Turkey. Therefore, this paper reports an HPLC method with UV detection for the determination of naproxen in healthy Turkish volunteers by using internal standard methodology. The developed method was validated by using linearity, stability, precision, accuracy and sensitivity parameters according to the International Conference on Harmonization (ICH) guidelines (24).

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

Experimental

Materials and reagents

Naproxen sodium and the internal standard (IS), ibuprofen, were obtained from Sigma (St. Louis, MO). Ethyl acetate, hexane, dichloromethane, acetonitrile, butanol and chloroform were purchased from Sigma-Aldrich. Aleve tablets (220 mg of naproxen sodium) were obtained from a pharmacy (Erzurum,

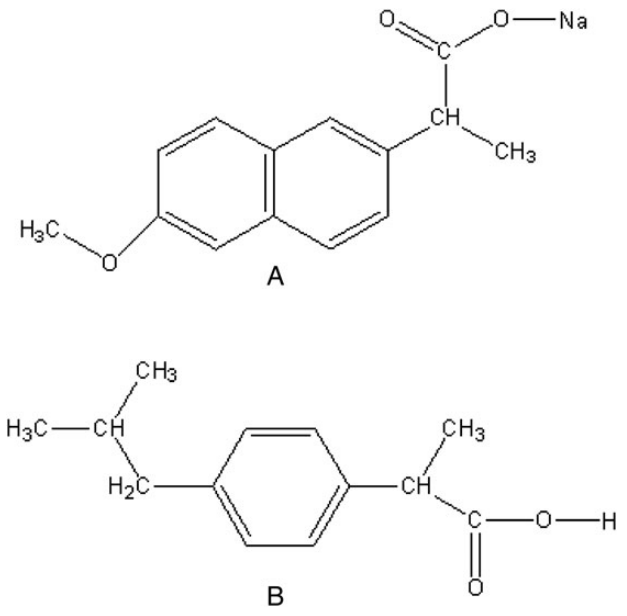

Figure 1. Chemical structures of atenolol (a) and metoprolol, IS (b).

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 an aquaMAX Ultra water purification system from Young Instruments (Korea). Human plasma was obtained from Yakutiye Blood Bank (Erzurum, Turkey).

Instrumentation

A Perkin Elmer series 200 HPLC system equipped with a programmable UV/Vis detector and Total Chrom Chromatography Data System software were used (Perkin Elmer Life Science, Shelton, CT). The HPLC mobile phase was composed of 20 mM phosphate buffer (pH 7) containing 0.1% trifluoroacetic acid (TFA)–acetonitrile (65:35, v/v). Separation was achieved by using an Ace C18 column (5 μm, 4.6 × 250 mm i.d.) with a guard column (4 × 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 naproxen (1.0 mg/mL) was prepared and diluted with acetonitrile to produce standard solutions of 0.10–5.0 μg/mL. Standard calibration samples were prepared daily by spiking 0.5 mL of drug-free human plasma with 10 μL of appropriate naproxen standard solutions to achieve final concentrations of 0.10–5.0 μg/mL for plasma. The working solution of the IS was prepared by dissolving the IS in acetonitrile to obtain a concentration of 100 μg/mL.

Preparation of quality control samples

The concentrations of naproxen were 0.3, 1.5 and 4.5 μg/mL in human plasma to represent low, middle and high quality controls, respectively. Appropriate volumes from the stock solution of naproxen were added to normal human plasma to produce low, middle and high quality control samples and stored at −20°C. The quality control samples were removed from storage for analysis to determine intra-day and inter-day precision and accuracy.

Extraction procedure

Naproxen is an acidic drug. It is bound in blood to a high degree (99%) to plasma protein (25). Therefore, phosphoric acid (H_3PO_4) was used for the release of naproxen from plasma proteins.

Blood samples were collected into the tubes containing disodium ethylenediaminetetraacetic acid (EDTA) and centrifuged at 4,500 × g for 10 min. A 0.5 mL quantity of the resultant plasma sample was spiked with 10 μL of naproxen, 10 μL of IS and 0.5 mL of H_3PO_4 solutions were added. After vortex mixing for 5 s, 3 mL of ethyl acetate and hexane (2:3, v/v) were added. The mixture was vortexed for 2 min and centrifuged at 3,000 × g for 3 min. The organic layer was transferred into another 5 mL tube and evaporated to dryness under a stream of nitrogen gas at 40°C. The residue was reconstituted in 1.0 mL of acetonitrile, and a 10 μL aliquot was injected into the HPLC system.

Collection of samples

Before the study, the clinical protocol was approved by the Ethics Committee of Faculty of Medicine, Ataturk University. All volunteers gave written informed consent to participate in the study according to the principles of the Declaration of Helsinki. The volunteers who agreed to attend this project were medically examined and three volunteers were selected (39.2 ± 2.65 years; 74.2 ± 1.87 kg; 172 ± 5.67 cm) for the pharmacokinetics study for naproxen. The subjects were required to abstain from taking any other drug for three days before the start of test. They were also forbidden to smoke or drink alcohol or xanthine containing beverages for 24 h from before the beginning of the study until its end. Six volunteers received an oral tablet (Aleve) containing 220 mg of naproxen. After this, they were allowed to drink water. The total amount of water drunk during the day was 1,500 mL. The volunteers sat during lunch and had normal activity (standing or sitting) during the study, but were never in a supine position during the 16 h after administration. Blood samples were placed into EDTA tubes at 0, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0, 10, 12 and 16 h after oral administration. Blood samples were centrifuged at 4,500 × g for 10 min and the plasma was separated and kept frozen at −20°C until analysis. Real samples were diluted, extracted and analyzed. The results were multiplied by the dilution factor.

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 time 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 areas 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 (26). The elimination half-life ($t_{1/2}$) was calculated by using the pharmacokinetic relationship $t_{1/2} = \ln(2)/k_{el}$.

Assay validation

Specificity

The plasma samples were prepared by a liquid–liquid extraction procedure. The samples were chromatographed to determine to which extent endogenous plasma components contributed to the peak interference at the retention times of naproxen and IS. Commonly prescribed drugs were analyzed for possible interference. The retention times were determined for these drugs under the chromatographic conditions for the naproxen assay.

Linearity

Calibration curves were prepared by adding known amounts of naproxen (0.10, 0.25, 0.50, 1.0, 2.0, 3.0, 4.0 and 5.0 μg/mL) to 0.5 mL of blank plasma. An aliquot of 10 μL of IS solution was added to each sample. The samples were extracted as described previously. The standard curves were constructed by plotting the peak area ratio of naproxen and IS on the *y*-axis and the concentration of naproxen on the *x*-axis. Linearity was assessed by a weighted ($1/C$) least-squares regression analysis.

Precision and accuracy

For the calculation of the intra-day precision and accuracy, six replicates of quality control samples (0.3, 1.5 and 4.5 μg/mL) were extracted as described previously and the concentrations were calculated from the standard curve. For the calculations of inter-day precision and accuracy, six replicates of quality control samples were analyzed on three consecutive days along with the standard calibration curve.

Limits of detection and quantification

The limit of detection (LOD) is the lowest amount of naproxen in a sample that can be detected, but not necessarily quantitated, as an exact value. The limit of quantification (LOQ) is the lowest amount of naproxen that can be quantitatively determined with suitable precision. The LOQ was defined as the concentration producing a precision less than 20% and accuracy between 80 and 120% of the theoretical concentrations.

Recovery

The recovery of naproxen was determined by comparing the peak areas measured after the analysis of spiked plasma samples with those found after the direct injection of standard solutions at the same concentration levels. The average value of area was taken into consideration to calculate the recovery. The recovery of the IS was also calculated in a similar way.

Stability

In bench top stability, three replicates of low and high controls of naproxen (0.5 and 3.0 μg/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 them in autosampler at 10°C and the deviation was calculated.

The quality control samples were frozen in the freezer at −20°C and thereafter thawed at room temperature. After complete thawing (approximately 8 h), samples were refrozen again applying the same conditions. At each cycle, samples were frozen for at least 24 h before they are thawed. The samples were analyzed after three freeze–thaw cycles. The observed concentrations were compared with their nominal values.

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 acetonitrile and injected 10 μL in the HPLC system. The mean concentration of 24 h samples was compared with that of samples analyzed at 0 h. Long-term stability was studied for 14 days with three replicates of high and low quality control samples. The mean concentration was taken into consideration, which was compared with the zero day sample concentration.

Matrix effect

The matrix effect was defined as the direct or indirect alteration or interference in response as a result of the presence of unintended analytes or other interfering substances in the sample (26). The matrix effect of naproxen was investigated by comparing the amount of naproxen solutions with the processed blank samples reconstituted with naproxen solutions. The blank plasmas used in this study were from six different batches of human blank plasma. If the ratios were <85 or >115%, a matrix effect was implied.

Results

Method development and optimization

Development of the method was focused on the optimization of column detection, sample preparation and chromatographic separation. The majority of the ionizable pharmaceutical compounds can be very well separated on a C18 column (27, 28). A reversed-phase column (C18) can be used for the determination of naproxen. Thus, naproxen can be satisfactorily separated by reversed-phase chromatography.

Several tests were performed for optimizing the components of mobile phase to achieve good chromatographic peak shape and resolution. The test results showed that the solvent system of acetonitrile could improve the peak shapes of naproxen. Good separation of the target compounds and short run time were obtained by using a mobile phase system of 20 mM phosphate buffer (pH 7) containing 0.1% TFA–acetonitrile (65:35, v/v). The retention times and run times of naproxen and the IS (5.2 and 7.1 min) were much shorter than those found in other papers (5, 9, 13, 15, 18, 19, 21).

Figure 2 shows representative chromatograms of drug-free plasma (Figure 2A), plasma spiked with naproxen (1.0 μg/mL) and the IS (1.0 μg/mL) (Figure 2B) and plasma obtained at 1.0 h after a single dose of 220 mg of naproxen (Figure 2C). No interferences were observed in the chromatogram of drug-free plasma.

Validation of the method

The validation of the method was conducted by establishing specifity, linearity, intra-day and inter-day precision, accuracy, recovery, LOD and LOQ according to ICH guidelines (24).

Specificity

Plasma samples obtained from volunteers were assessed by the previously described procedure and compared with respective plasma samples to evaluate the specificity of the method. Commonly prescribed drugs were analyzed for possible interference. The retention times for these drugs were determined under the chromatographic conditions for the naproxen assay. As mentioned previously, under the described analysis procedure, the peaks of naproxen and the IS were well resolved with good symmetry and desirable retention times from endogenous compounds in the blank human plasma. Representative chromatograms of human blank plasma and plasma samples spiked with naproxen and IS are shown in Figure 2. No interfering peaks were observed near the retention times of naproxen and the IS.

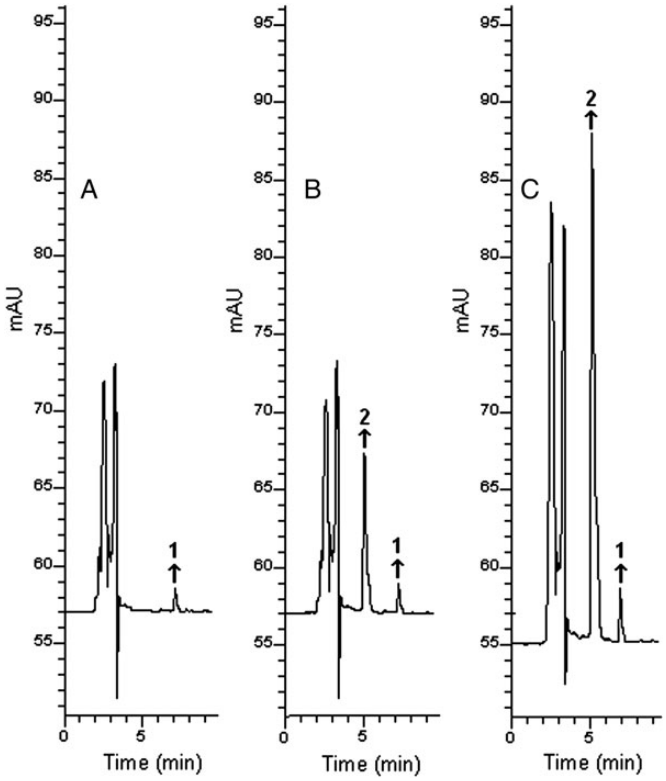

Figure 2. Representative chromatograms of (a) drug-free plasma, (b) the plasma spiked with atenolol (100 ng/mL) and IS (200 ng/mL), (c) the plasma obtained at 6 h after a single oral dose of 50 mg atenolol.

Linearity

The calibration equation from three replicate experiments, $y = 0.028x + 0.035$ ($r = 0.999$), demonstrated the linearity of the method. The linear regression equation was calculated by using the least squares method with Microsoft Excel. Standard deviations of the slope, intercept and correlation coefficient for the calibration curves were 0.014, 0.376 and 2.15×10^{-3}, respectively.

Precision and accuracy

The method indicated very good precision and accuracy. Data for the intra-day and inter-day precision and accuracy for naproxen from plasma samples are shown in Table I. The intra-day precision and accuracy varied between 2.89 and 4.47%, and 96.7 and 98.0%, respectively. The inter-day precision and accuracy ranged from 3.08 to 4.84% and 96.3 to 99.1%, respectively.

LOD and LOQ

The LOD and LOQ values of the HPLC–UV method were determined to be 0.03 and 0.10 μg/mL, respectively. For the LOQ, the intra-day precision was 4.39% relative standard deviation (RSD) and accuracy was 91.0%. The inter-day precision was 9.71% RSD and accuracy was 104.3%.

Recovery

The extraction recovery values of naproxen from human plasma were between 91.0 and 98.9%, as shown in Table II. Also, the extraction recovery of the IS from human plasma was 95.5%.

Stability

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).

Matrix effect

The relative matrix effect of naproxen at three different concentrations (0.5, 2.0 and 4.0 μg/mL) was less than ± 2.70% (Table IV). The results showed that no matrix effect of the analytes was observed from the matrix of plasma in this study.

Table I
Precision and Accuracy of Naproxen in Human Plasma

Added (μg/mL)	Intra-day Found ± SD*	Precision RSD† (%)	Accuracy‡	Inter-day Found ± SD	Precision RSD (%)	Accuracy
Plasma (0.5 mL)						
0.3	0.291 ± 0.013	4.47	−3.00	0.289 ± 0.014	4.84	−3.67
1.5	1.45 ± 0.042	2.89	−3.33	1.46 ± 0.045	3.08	−2.67
4.5	4.41 ± 0.178	4.04	−2.00	4.59 ± 0.195	4.25	0.89

*SD: standard deviation of six replicate determinations.
†Average of six replicate determinations.
‡Accuracy: (percentage of relative error) (found − added)/added × 100.

Table II
Recovery of Naproxen in Human Plasma

Added (μg/mL)	Found (mean ± SD*)	Recovery (%)	RSD† (%)
0.10	0.091 ± 0.004	91.0	4.39
0.25	0.232 ± 0.012	92.8	5.17
0.5	0.481 ± 0.032	96.2	6.65
1	0.964 ± 0.059	96.4	6.12
2	1.978 ± 0.136	98.9	6.87
3	2.896 ± 0.141	96.5	4.87
4	3.923 ± 0.263	98.1	6.70
5	4.782 ± 0.362	95.6	7.57

*SD: standard deviation of three replicate determinations.
†Average of three replicate determinations.

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

Treatment	Recovery (mean ± SD)	
	Plasma concentration (μg/mL)	
	0.5	3.0
Bench top stability for 6 h	94.6 ± 5.23	101.3 ± 3.84
Autosampler stability for 24 h	97.4 ± 5.42	90.4 ± 3.93
Three freeze–thaw cycles	96.9 ± 7.66	94.2 ± 4.25
Dry extract stability for 24 h	91.8 ± 6.11	91.6 ± 6.46
Stored at RT* for 24 h	93.2 ± 4.44	101.2 ± 5.27
Stored at −20°C for 24 h	90.5 ± 3.58	94.4 ± 4.52
Stored at −20°C for 2 weeks	92.1 ± 6.52	91.9 ± 5.68

*Room temperature.

Table IV
Evaluation of Matrix Effects of Naproxen and IS in Human Plasma ($n = 3$)

Samples	Concentration level (μg/mL)	Mean ± SD*	Mean ± SD†	Matrix effect (%)
Naproxen	0.5	0.478 ± 0.061	0.481 ± 0.058	99.4
	2.0	1.983 ± 0.194	2.023 ± 0.024	98.0
	4.0	3.918 ± 0.047	4.025 ± 0.042	97.3
IS	1.0	0.986 ± 0.088	1.032 ± 0.109	95.5

*The amount of naproxen and IS derivatized in reconstituted solution of a blank plasma sample (the final solution of blank plasma after extraction and reconstitution).
†The amount of naproxen and IS.

Dilution integrity

The dilution integrity experiment was performed to validate the dilution test to be conducted on higher analyte concentration above the upper limit of quantification, which may be encountered during real subject sample analysis. The precision and accuracy values for 1/5th and 1/10th dilution ranged from 3.47 to 7.28% and 96.2 to 105.7 for naproxen.

Discussion

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 naproxen and the IS are shown in Figure 2.

Naproxen was extracted from human plasma with a solid-phase extraction procedure in other papers (11, 12, 17). This method is also the most comprehensive method to extract naproxen in a single extraction procedure. The mean recovery is better for plasma than in other papers (13, 14, 19).

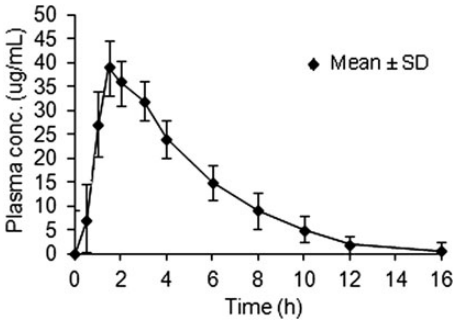

Figure 3. Mean plasma atenolol concentration-time profile for six patients after a single oral dose of atenolol, 50 mg.

Table V
Mean Pharmacokinetic Parameters of Naproxen for Six Volunteers after Oral Administration of Aleve Tablet (220 mg)

Parameter	Mean ± SD	RSD (%)
C_{max} (μg/mL)	39.1 ± 4.124	10.54
T_{max}	1.50 ± 0.176	11.73
AUC	513.4 ± 42.34	8.25
$AUC_{(0-16h)}$ (μg/mL/h)		
$AUC_{(0-\infty)}$ (μg/mL/h)	664.2 ± 51.14	7.69
$t_{1/2}$ (h)	13.8 ± 1.246	9.03

Sultan *et al.* (18) and Elsinghorst *et al.* (19) described two methods to simultaneously quantify and identify naproxen and other NSAIDs in human plasma, employing LC–MS in full-scan MS mode. Both methods were robust and reliable; however, their run times were approximately 15 min with rather high LOQ values of 2 and 20 μg/mL, respectively.

The sensitivity was evaluated by the LOQ, which was determined to be 0.10 μg/mL. This method is as good as or superior to that reported in the other papers (18, 19).

Additionally, this method was applied to six Turkish volunteers who had been given an oral tablet of 220 mg of naproxen. The amount of naproxen in human plasma was determined between 0 and 16 h. The mean plasma concentration–time curve is shown in Figure 3.

The mean values of pharmacokinetic parameters, estimated by the computer program WinNonlin with a noncompartmental method, are shown in Table V. Pharmacokinetic parameters reported that after an oral administration of a 220 mg naproxen tablet to healthy volunteers, AUC, C_{max} and T_{max} were 513.4 ± 42.34 μg/mL/h, 39.1 ± 4.124 μg/mL and 1.50 ± 0.176 h, respectively. The mean half-life was 13.8 h. The maximum plasma concentration of naproxen in Turkish volunteers agreed with the values reported by other groups (19, 29).

Conclusion

In the present work, a simple and sensitive HPLC method was developed for the determination of naproxen in human plasma. The method was completely validated by using sensitivity, stability, specificity, linearity, accuracy and precision parameters for the determination of naproxen in human plasma. Additional advantages of this method include a small sample volume (0.5 mL), good extraction recovery from plasma and a readily

available IS. Also, by applying this method to analyze the plasma samples of healthy Turkish subjects, the pharmacokinetic parameters of naproxen in Turkey were determined, demonstrating the adequacy of this assay for clinical studies. Therefore, the method has potential applicability in pharmacokinetic and bioequivalence studies of naproxen.

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