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## **Tavşan Plazmasında Naproksenin GC-MS Yöntemi ile Analizi**

Bilal YILMAZ

**ÖZET:** Naproksen günümüzde en çok kullanılan ilaçlardan biridir. Bu nedenle, naproksen tayini için yeni ve basit yöntemlerin geliştirilmesi önemlidir. Bu çalışmanın amacı tavşan plazmasında naproksenin analizi için bir gaz kromatografisi-kütle spektrometri yöntem geliştirmek ve farmakokinetik çalışmaya uygulamaktır. Tavşan kan örnekleri sıvı-sıvı ekstraksiyon yöntemi ile hazırlanmıştır. Naproksenin ayırımı HP-5 MS kolon ile yapılmıştır. Yöntemin kalibrasyon eğrisi 0.1 ve 5.0 µg mL<sup>-1</sup> arasında çizildi. Tavşan plazmasında naproksenin kesinlik sonuçları %4.17'den küçüktü ve doğruluk sonuçları %2.18'den daha iyiydi. Yöntemin tavşan plazmasındaki tüm örnekler için geri kazanım değerleri >%94.2'tür. Ayrıca, tavşanlarda naproksenin farmakokinetiğini incelemek için valide edilen yöntem uygulanmıştır. Maksimum naproksen plazma konsantrasyonu 42.1±4.243 µg mL<sup>-1</sup> idi. Naproksenin maksimum konsantrasyona ulaşma süresi ve eğrinin altındaki alan (AUC<sub>0-16 h</sub>) sırasıyla 1.50±0.196 h ve 566.3±41.72 µg mL<sup>-1</sup> idi.

**Anahtar Kelimeler:** GC-MS, ekstraksiyon, naproksen, farmakokinetik, tavşan

### **Analysis of Naproxen in Rabbit Plasma by GC-MS Method**

**ABSTRACT:** Naproxen is one of the most used drugs today. Therefore, it is important to develop new and simple methods for the determination of naproxen. The goal of this research is to develop a gas chromatography-mass spectrometry method for analyzing naproxen levels in rabbit plasma and apply this method to the pharmacokinetic study. The liquid-liquid extraction technique was used to prepare blood samples from rabbits. Separation of naproxen was achieved on an HP-5 MS column. The method's calibration curve was plotted between 0.1 and 5.0 µg mL<sup>-1</sup>. The accuracy results were better than 2.18% and the precision results were less than 4.17% in rabbit plasma for naproxen. The method had recovery values >94.2% for all samples in rabbit plasma. In addition, the validated method was used to study naproxen pharmacokinetics in rabbits. The maximum naproxen plasma concentration is 42.1±4.243 µg mL<sup>-1</sup>. The duration to attain the greatest naproxen concentration and the area under the curve from (AUC<sub>0-16 h</sub>) were 1.50±0.196 h and 566.3±41.72 µg mL<sup>-1</sup> h, respectively.

**Keywords:** GC-MS, extraction, naproxen, pharmacokinetic, rabbit

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## INTRODUCTION

The chemical formula structure of naproxen is 2-(6-methoxynaphthalen-2-yl) propanoic acid). It is a type of non-steroidal anti-inflammatory medication. Therefore, it has been widely used to manage chronic and acute pain, swelling, fever and inflammation. In addition, it is used to treat inflammatory rheumatic and other rheumatoid arthritis diseases (Camilo and Foley, 2021; Muneer et al., 2017; Sondnara et al., 2018). Because, naproxen is rapidly absorbed from the gastrointestinal tract (Yuan et al., 2018).

In literature research, high-performance liquid chromatography (HPLC) (Vittal et al., 2019; Hamid and Elsaman, 2017; Pushpa and Sailaja, 2020; Satterwhite and Boudinot, 1988; Upton et al.; 1980; Vree et al., 1992; Aresta et al., 2005; Hirai et al., 1997; Shimek et al., 1982; Navalon et al., 1999; Westerlund et al., 1979; Hsu et al., 2006; Mikami et al., 2000), liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Sultan et al., 2005; Pietruk et al., 2021) and gas chromatography-mass spectrometry (GC-MS) (Krokos et al., 2018; Goktas et al., 2020; Hlozek et al., 2014; Muthal and Snow, 2016) methods for determining naproxen in biological materials have been reached.

There are different chromatographic studies on the determination of naproxen in biological materials. Among these, the most used methods are usually HPLC (Vittal et al., 2019; Hamid and Elsaman, 2017; Pushpa and Sailaja, 2020; Satterwhite and Boudinot, 1988; Upton et al.; 1980; Vree et al., 1992; Aresta et al., 2005; Hirai et al., 1997; Shimek et al., 1982; Navalon et al., 1999; Westerlund et al., 1979; Hsu et al., 2006; Mikami et al., 2000), LC-MS/MS (Sultan et al., 2005; Pietruk et al., 2021) and GC-MS (Krokos et al., 2018; Goktas et al., 2020; Hlozek et al., 2014; Muthal and Snow, 2016).

In the previous research, two studies have been found that determine the naproxen level in human plasma by the LC-MS method (Sultan et al., 2005; Pietruk et al., 2021). The LOQ values of methods were  $2 \mu\text{g mL}^{-1}$  and  $20 \mu\text{g mL}^{-1}$ , respectively. Moreover, the analysis time of the methods is more than 15 minutes.

Studies on the determination of naproxen in biological materials by LC-MS have been reached. However, the LC-MS method is not always accessible because it is a very expensive method. Therefore, the GC-MS method developed in this study is cheaper than the LC-MS method.

In the literature, naproxen was extracted with a solid-phase extraction method from human plasma in previous studies (Aresta et al., 2005; Hirai et al., 1997; Mikami et al., 2000; Muthal and Snow, 2016). The solid-phase extraction technique is an expensive method. In our technique, the liquid-liquid method in plasma from rabbits is used as the extraction method. In addition, the extraction technique was very simple, cheap and could be done in one step.

In addition, it is seen that the GC-MS method developed and validated by us has a better mountain range in plasma than previous studies (Vittal et al., 2019; Hamid and Elsaman, 2017; Pushpa and Sailaja, 2020; Aresta et al., 2005; Shimek et al., 1982; Navalon et al., 1999; Westerlund et al., 1979; Hsu et al., 2006; Sultan et al., 2005; Goktas et al., 2020; Hlozek et al., 2014; Muthal and Snow, 2016).

However, the GC-MS method was not available for detecting naproxen in rabbit plasma. Therefore, a new and easy GC-MS approach for the analysis of naproxen in rabbit plasma was devised in this study. For this, a derivatizing agent *N-Methyl-N-(trimethylsilyl) trifluoroacetamide* compound is used to increase sensitivity. The developed method was then validated with respect to the Center for Drug Evaluation and Research guidelines (CDER, 2001).

The presented method is based on a simple and single extraction step in a short time using inexpensive chemicals. At the same time, the approach was also used to examine naproxen levels in rabbit plasma. For this, Alev tablet containing naproxen was given to six rabbits. Blood was drawn from

the rabbits at different times. The analysis was performed after extraction. From the results obtained, the blood pharmacokinetic parameters of naproxen were calculated.

## MATERIALS AND METHODS

### Chemicals and Reagents

The following ingredients were acquired from Sigma- Aldrich (St. Louis, MO, USA): Naproxen, ibuprofen, ethylacetate, *N-Methyl-N-(trimethylsilyl) trifluoroacetamide*, hexane and acetonitrile. From a pharmacy, Aleve tablet that included 220 mg naproxen was purchased.

### GC-MS System and Chromatographic Conditions

GC-MS analyses were performed with the GC-MS system. In this work, an HP-5 MS column (30 m × 0.25 mm, 0.25 μm) was used. Splitless injection mode was selected for the analysis. Helium was employed as the carrier gas, with a flow rate of 1 mL /min<sup>-1</sup>. For electron ionization, the MS detector was employed at 70 Ev. Fragment ions of naproxen and internal standard (IS) ibuprofen were selected as 185 and 73 (m/z), respectively.

### Preparation of Standard and Quality Control Samples

Acetonitrile was used to make a 1.0 mg mL<sup>-1</sup> naproxen solution. Naproxen standard solutions were diluted with acetonitrile. Standard calibration samples were prepared 0.1-5.0 μg mL<sup>-1</sup> (0.10, 0.25, 0.50, 1.0, 2.0, 3.0, 4.0 and 5.0 μg mL<sup>-1</sup>). The naproxen solutions were all kept at 4 °C. Naproxen quality control standard solutions were produced 0.3, 1.5 and 4.5 μg mL<sup>-1</sup> together with 1.0 μg mL<sup>-1</sup> IS.

### Extraction and Derivatization Procedure

The samples were extracted by solid phase extraction (SPE) cartridge (C<sub>18</sub>, 3 mL, 500 mg, Bond-elut, Agilent). The cartridge was conditioned with 3 mL methanol and 3 mL water. The frozen plasma samples (0.5 mL) were thawed at a temperature of 25 °C (room temperature) and 0.75 mL water and IS (0.1 mL at 1.0 μg mL<sup>-1</sup> concentration) were added to the plasma. The mixture was vortexed and transferred to SPE cartridge. Then, the cartridge was washed with a mixture of acetonitrile-water (2 mL, 15/85) and 3 mL hexane. Eluate was collected from with 3 mL acetonitrile. The eluate was evaporated at 50°C under nitrogen. The residue was dissolved in acetonitrile and *MSTFA* (50:50, v/v), and 1 μL volume was injected into GC-MS system. The extraction recoveries of naproxen from rabbit plasma were between 52.7 and 67.9%.

Therefore, the extraction procedure in rabbit plasma samples was carried out using the liquid-liquid extraction method. Butanol, acetonitrile, hexane, dichloromethane, chloroform and ethylacetate were tried to find the most suitable extraction solvent. In the end, hexane and ethylacetate mixture (3:2, v/v) was chosen.

0.5 ml rabbit plasma was used in the study. 0.1 mL naproxen standard solution (0.10, 0.25, 0.50, 1.0, 2.0, 3.0, 4.0 and 5.0 μg mL<sup>-1</sup>), 0.1 mL of IS solution (1.0 μg mL<sup>-1</sup>) and 0.5 mL H<sub>3</sub>PO<sub>4</sub> solution were added. Vortex procedure was performed for 5 seconds. 4 mL of hexane and ethylacetate (3:2, v/v) was added in rabbit plasma. The rabbit plasma sample was vortexed for 30 seconds, and the plasma sample was then centrifuged for 10 minutes at 4500 × g.

The organic phase was transferred into another plastic tube. Under nitrogen gas, the organic phase was vaporized. 100 μL *MSTFA* and acetonitrile mixture (50:50, v/v) were used to dissolve the dry residue sample. Then, GC-MS was used to examine a 1 μL plasma sample.

## Rabbits

Before starting the study, the ethics committee report was obtained from the Ethical Committee for Medical Experimental Research and Application Centre of Ataturk University (2009/122). Six rabbits were housed with free access to food and water, except for the final 2 h before experiment. After a single oral administration of 40 mg kg<sup>-1</sup> of naproxen, 2.0 mL of blood samples were collected from the marginal ear vein at 0, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0, 10, 12 and 16 h time-points into EDTA collection tubes. The blood samples were centrifuged at 4500 rpm for 10 min and the plasma was taken and stored at -20 °C until analysis. After that, they are analyzed by the GC-MS system.

## Statistical Analysis

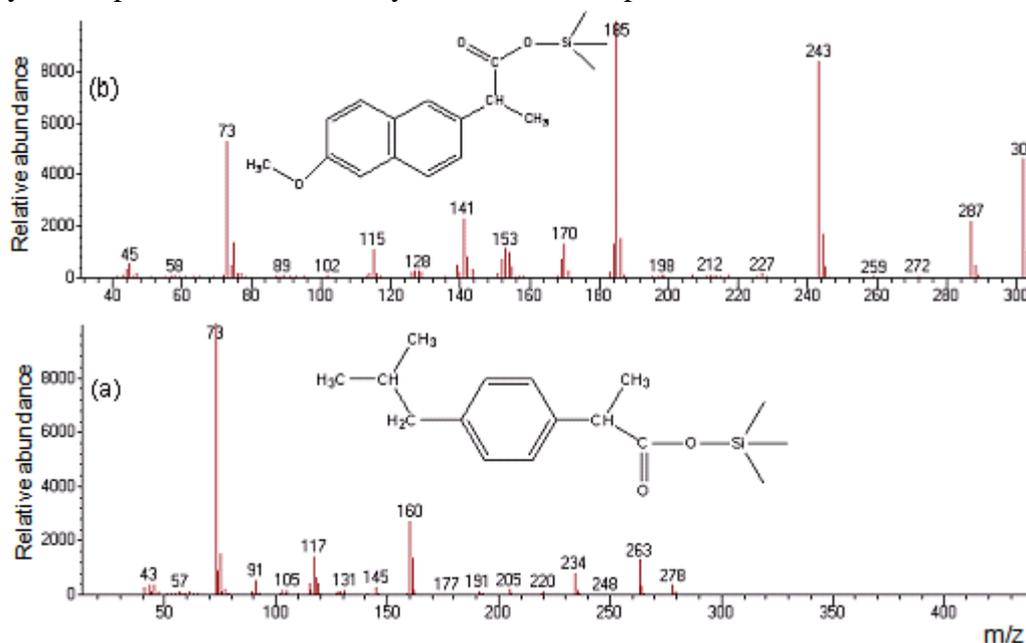
The statistical analyses were done with SPSS V.15.0 version at computer program. Regression analyses were used in the preparation of the naproxen standard line and calculations. For statistical significance, the results were given with the mean ± standard deviation.

## RESULTS AND DISCUSSION

### Method Development and Optimization

In this work, the HP-5 MS column and derivatization procedure were used because naproxen is a polar molecule. For the sensitivity of the GC-MS method, *MSTFA* compound was used to derivatize naproxen and IS (Figure 1). The hydroxy (-OH) groups of naproxen and IS were transformed to the corresponding silyl (-O-TMS) groups and then analyzed.

In addition, different column temperature programs were tested in this study. The optimum column temperature schedule is as follows. The starting temperature was 150 °C, left for 1 minute. It is brought to 220 °C by 20 °C per minute, and finally increases 30 °C per minute to 300 °C and holds 1 minute.



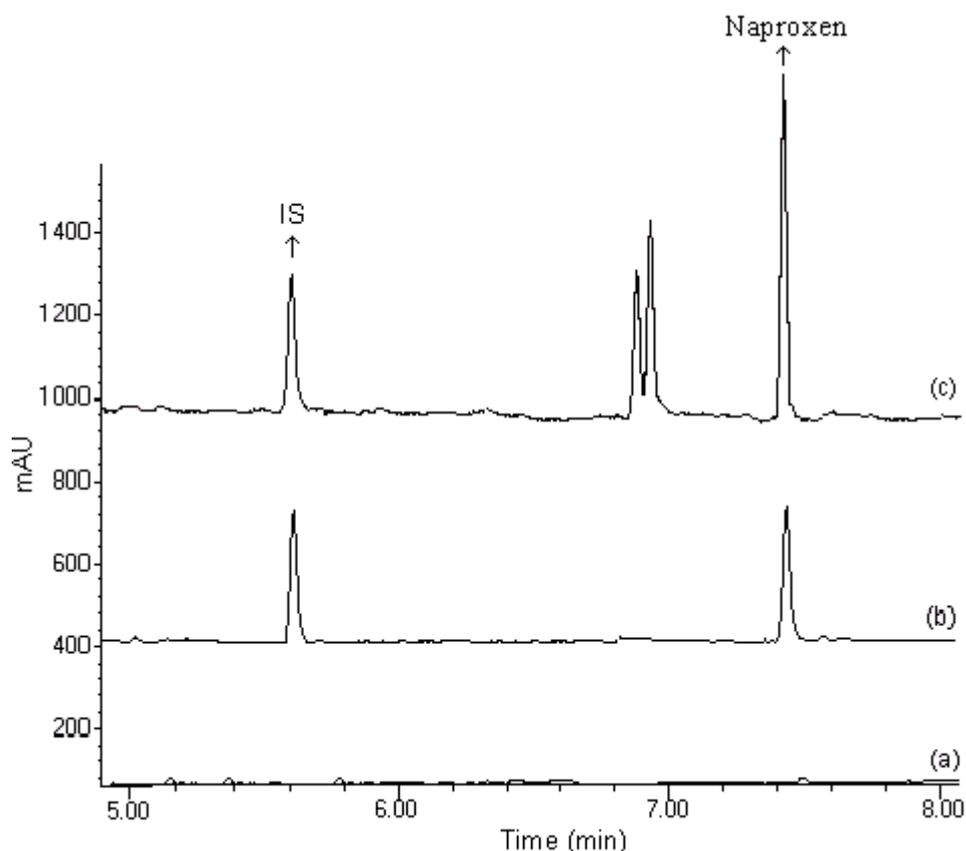
**Figure.1.** Mass spectra after naproxen (b) and ibuprofen (IS) derivatizations with MSTFA (a)

### Validation of the Method

GC-MS method was validated with validation parameters according to CDER. These parameters were selectivity, linearity, precision, accuracy, recovery, limits of detection (LOD) and quantification (LOQ), stability and dilution integrity.

### Selectivity

The selectivity of the developed technique was checked by comparing the chromatograms of spiked rabbit plasma with the blank rabbit plasma. Naproxen and IS retention times were approximately 7.4 and 5.6 min (Figure 2). The symmetry of the peaks and the short retention times are the advantages of the method. In addition, the blank plasma sample was analyzed. Endogenous interferences were not observed.



**Figure.2.** (a) Pre-drug rabbit plasma chromatogram, (b) rabbit plasma chromatogram spiked with  $5 \mu\text{g mL}^{-1}$  naproxen, (c) rabbit plasma chromatogram obtained 1 h after administration  $40 \text{ mg kg}^{-1}$  of naproxen

### Linearity

Standards calibration curves were drawn according to peak area ratio ( $y$ ) of naproxen and IS versus naproxen concentration. It was found to be linear over the  $0.1\text{-}5.0 \mu\text{g mL}^{-1}$  concentration range for naproxen (Figure 3). The mean calibration equation from three replicate experiments is  $y=1.9503x+0.0379$ . The correlation coefficient value was higher than 0.99 for the mean calibration curve.

### Precision and Accuracy

Intra-day and inter-day precision were used to assess the proposed method's precision. Six replicates for each of three different concentrations were analyzed to determine intra-day precision. The same samples of plasma were analyzed in three successive days to measure the intermediate precision. The percent relative standard deviation (RSD %) was used to assess precision.

In addition, the percentage relative error was used to assess the method's accuracy. The precision and accuracy for naproxen from plasma samples were gratifying. RSD is obtained as lower than 4.17%. In addition to this, accuracy is detected to be within  $\pm 2.18\%$  with relative error. From the results obtained, it is understood that both the precision and the accuracy of this method are good.

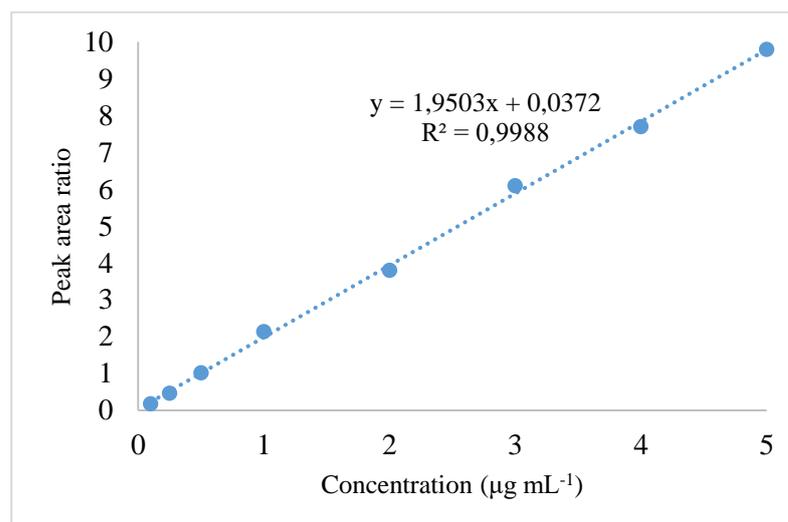


Figure.3. Standards calibration curve of naproxen in rabbit plasma

### Recovery

In order to prepare the samples at rabbit plasma, the liquid-liquid extraction technique was used for this work. Therefore, ethylacetate, hexane, acetonitrile, dichloromethane, butanol and chloroform were tried. In the end, hexane and ethylacetate mixture (3:2, v/v) was decided to be used as the most suitable solvent for extraction. Naproxen recovery values of rabbit plasma are between 94.2 and 99.3%.

In addition, in this investigation, the extraction recovery values of naproxen are great (Shimek et al., 1982; Navalon et al., 1999; Pietruk et al., 2021), processes of extraction are quick ((Aresta et al., 2005; Hirai et al., 1997; Mikami et al., 2000), additionally, the analysis time is short (Vittal et al., 2019; Upton et al., 1980; Shimek et al., 1982; Westerlund et al., 1979; Sultan et al., 2005; Pietruk et al., 2021; Goktas et al., 2020).

### Limits of Detection (LOD) and Quantification (LOQ)

The LOD value was evaluated as the minimum concentration in this work. The reason behind this is the fact that the signal-to-noise ratio of it is found to be 3 for naproxen at GC-MS chromatogram. In addition, the LOQ value was evaluated as the minimum concentration of the plasma spiked with naproxen. The LOD and LOQ values of the method were 0.03 and 0.10 µg mL<sup>-1</sup> in the work, respectively. However, if the LOQ value and retention time of the methods are compared with our study (Sultan et al., 2005; Pietruk et al., 2021). It is seen that the method we developed is more advantageous. Both precision and accuracy of the LOD and LOQ values were within the criteria (CDER, 2001).

### Stability

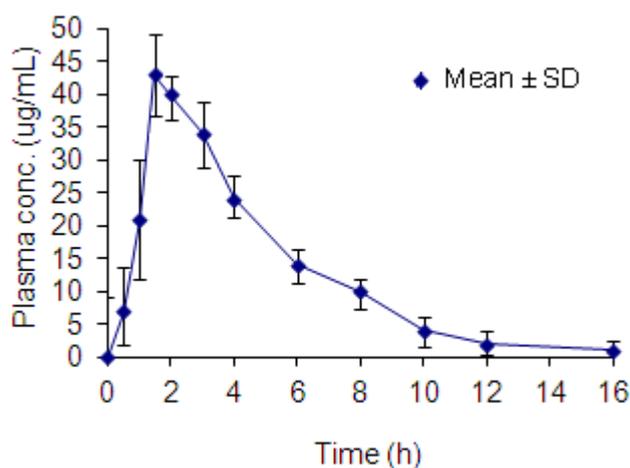
Naproxen stock solution stability was evaluated for at least 72 hours at room temperature. In addition, the stabilities of naproxen in rabbits were investigated under various storage conditions. By analyzing the low and high concentrated samples, the method's short-term stability was determined. Therefore, at room temperature, the samples were thawed. They were stored in room condition for 24 h. The low and high concentration samples were analyzed at -20 °C for three days to determine long-term stability. No significant degradation product of naproxen in these conditions.

### Dilution Integrity

The dilution integrity of the method was performed on higher naproxen concentrations above the upper LOQ. The accuracy and precision of naproxen were between 98.6 to 101.4 and 2.07 to 3.14 % for 1/5<sup>th</sup> and 1/10<sup>th</sup> dilution.

### Pharmacokinetic 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  $\ln(\text{plasma concentration})$  versus time curve. The elimination half-life ( $t_{1/2}$ ) was calculated using the pharmacokinetic relationship  $t_{1/2} = \ln(2)/k_{el}$ . Pharmacokinetic results of naproxen were calculated in rabbit plasma by the linear trapezoidal rule (Yilmaz et al., 2010). In addition, Figure 4 illustrates the average naproxen concentration-time curve for six rabbits.



**Figure.4.** Naproxen concentration-time profile in rabbit plasma (n=6).

The mean  $AUC_{0-16h}$ ,  $C_{max}$ ,  $T_{max}$  and  $t_{1/2}$  of naproxen obtained from six rabbits were  $566.3 \pm 41.72 \mu\text{g mL}^{-1} \text{ h}$ ,  $42.1 \pm 4.243 \mu\text{g mL}^{-1}$ ,  $1.50 \pm 0.196 \text{ h}$  and  $13.9 \pm 1.286 \text{ h}$ , respectively. Using this method, the obtained pharmacokinetics results are in agreement with the studies reported previously (Satterwhite and Boudinot, 1988).

### CONCLUSION

The GC-MS method is a widely used chromatographic method for the analysis of volatile or volatile substances. In this study, naproxen, a polar compound, was volatilized by derivatization. In this way, the sensitivity of the study was increased. With this, it was possible to determine the amount of naproxen in rabbit plasma even at low concentrations.

In addition to this, a new and fast GC-MS technique has been completely developed in order to analyze naproxen in rabbit plasma. Furthermore, validation parameters were used to validate the procedure. The advantages of the method are that 0.5 ml of plasma is sufficient for the application of the method and that it has an easy extraction method. In addition, the proposed method was applied to six rabbits. Therefore, the proposed method can be easily used in the plasma of people using naproxen in the clinic.

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