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RESEARCH ARTICLE

Determination of Ibuprofen in Rabbit Plasma by High-Performance Liquid Chromatography

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Summary

The aim of the present study was to develop and validate a procedure based on high-performance liquid chromatography (HPLC) for determination of ibuprofen in rabbit plasma. Separation of ibuprofen and naproxen (internal standard, IS) was achieved on an Ace C18 column (5 µm, 250x4.6 mm i.d.) using UV detection with λ=225 nm. The mobile phase consisted of 20 mM phosphate buffer (pH 7) containing 0.1% trifluoroacetic acid (TFA)-acetonitrile (65:35, v/v). The analysis was performed in less than 10 min with a flow rate of 1 mL/min. Excellent linearity was found between 0.5 and 40 µg/mL. Intra- and inter-day precision values for ibuprofen in plasma were less than 4.97, and accuracy (relative error) was better than 7.20%. The recoveries for all samples were >92.8%. The limits of detection (LOD) and quantification (LOQ) of ibuprofen were 0.10 and 0.25 µg/mL, respectively. The described HPLC method has adequate sensitivity and specificity to study pharmacokinetics of ibuprofen in rabbits, and could be adapted also to clinical pharmacokinetic study.

Keywords: *Ibuprofen, HPLC, Liquid-liquid extraction, Rabbit, Pharmacokinetic study*

Yüksek Performanslı Sıvı Kromatografisi Kullanarak Tavşan Plazmasında İbuprofenin Tayini

Özet

Bu çalışmanın amacı tavşan plazmasında ibuprofenin tespit edilmesi için yüksek basınçlı sıvı kromatografisine (YBSK) dayalı bir yöntem geliştirmek ve valide etmektir. İbuprofen ve naproksenin (internal standart, IS) ayırımı dalga boyu λ=225 nm olan UV dedektör kullanılarak C18 kolon (5 µm, 250x4.6 mm i.d.) ile yapılmıştır. Mobil faz %0.1 trifluoroasetik asit içeren 20 mM fosfat tamponu (pH 7)-asetonitril (65:35, h/h) den oluşmuştur. Analiz 1 mL/dk akış hızı ile 10 dakikadan daha az sürede yapılmıştır. 0.5 ve 40 µg/mL arasında çok iyi linearite bulunmuştur. Plazmada ibuprofen için gün içi ve günler arası kesinlik değerleri %4.97'den küçüktü, ve doğruluk (bağıl hata) %7.20'den daha iyiydi. Bütün örnekler için geri kazanım >%92.8 idi. İbuprofenin saptanabilen (LOD) ve ölçülebilen (LOQ) en düşük değeri sırasıyla 0.10 and 0.25 µg/mL'dir. Tanımlanan YBSK metodu tavşanlarda ibuprofenin farmakokinetiğini incelemek için yeterli duyarlılığa ve özgüllüğe sahiptir ve klinik farmakokinetik çalışmalara da uyarlanabilir.

Anahtar sözcükler: *İbuprofen, YBSK, Sıvı-sıvı ekstraksiyon, Tavşan, Farmakokinetik çalışma*

INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most commonly prescribed agents world-wide to treat a variety of pain-related conditions including arthritis and other rheumatic diseases. In addition, epidemiological studies have shown that long-term use of NSAIDs reduces the risk of developing Alzheimer's disease and delays its onset [1-3].

Ibuprofen (*Fig. 1*), 2-(4-isobutylphenyl)propionic acid, is a non-steroidal anti-inflammatory, analgesic and antipyretic drug. It is extensively used in the treatment of acute and chronic pain and many rheumatic and musculoskeletar disorders [4].

Several methods have been reported for determination

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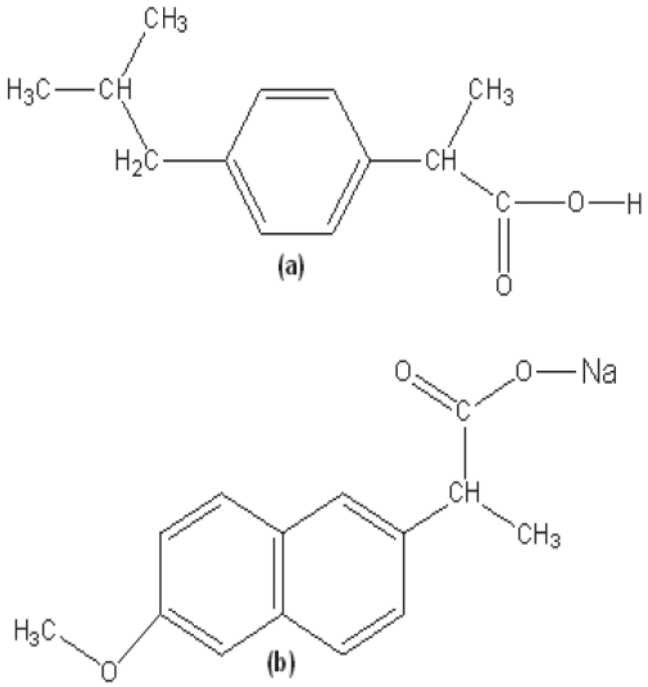

Fig 1. Chemical structures of ibuprofen (a) and naproxen, IS (b)
Şekil 1. İbuprofen (a) ve naproksenin (IS) (b) kimyasal yapıları

of ibuprofen including high-performance liquid chromatography (HPLC) [5-13], LC-MS-MS [14] and capillary electrophoresis (CE) [15-17] in biological samples.

In addition, no method is reported till date for determination of ibuprofen by HPLC in rabbits which had been given ibuprofen. Therefore, this report describes a simple and specific HPLC procedure with UV detection for determining ibuprofen in rabbit plasma. The developed method was validated by using linearity, stability, precision, accuracy and sensitivity parameters according to literature [18].

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 ibuprofen in plasma samples obtained from three rabbits which had been given an oral tablet of Artril tablet (600 mg ibuprofen).

MATERIAL and METHODS

Chemicals and Reagents

Ibuprofen and naproxen were obtained from Sigma (St. Louis, MO, USA). Ethylacetate, hexane and methanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Artril tablet (600 mg ibuprofen) was obtained Eczacıbaşı Pharmaceutical Industry (Istanbul, 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.

HPLC System

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, USA). 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 mL/min. The eluent was monitored by UV detection at 225 nm.

Preparation of Stock and Standard Solutions

The stock solution of ibuprofen (1 mg/mL) was prepared and diluted with methanol to give standard solutions of 0.5-40 µg/mL. Standard calibration samples were prepared daily by spiking 0.5 mL of drug-free plasma with 1.0 mL of appropriate ibuprofen standard solutions to achieve final concentrations of 0.5-40 µg/mL for plasma. Standard solutions were stored at +4°C. IS stock solution was made at an initial concentration of 1 mg/mL. The IS working solution (50 µg/mL) was prepared from the stock solution using methanol.

Extraction Procedure

Blood samples were collected into the tubes containing disodium EDTA and centrifuged at 4500 × *g* for 10 min. A 0.5 mL of the resultant plasma sample was spiked with 1.0 mL of ibuprofen, 0.1 mL of IS and 0.5 mL H_3PO_4 solution were added. After vortex mixing for 5 s, 3 mL of ethylacetate and hexane was added (2:3, *v/v*), 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 mL methanol, and a 10 µL aliquot was injected into the HPLC system.

Rabbits

The study was conducted in accordance with the Animal Ethical Guidelines for Investigations in Laboratory Animals and was approved by the Ethical Committee for Medical Experimental Research and Application Centre of Ataturk University (2009/122). The rabbits are male which is 4.8-5.2 kg weight. The rabbits were housed with free access to food and water, except for the final

2 h before experiment. After a single oral administration of 600 mg of ibuprofen (Artril tablet), 1.5 mL of blood samples were collected from the marginal ear vein at 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 10 and 12 h time-points into EDTA collection tubes. The blood samples were centrifuged at 4000 rpm for 10 min and the plasma was taken and stored at -20°C until analysis.

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(plasma concentration) versus time curve. The elimination half-life ($t_{1/2}$) was calculated using the pharmacokinetic relationship $t_{1/2} = \ln(2)/k_{el}$ [19].

RESULTS

The specificity of the method was verified by investigating the peak interference from the endogenous plasma substances. The chromatogram of the plasma spiked with ibuprofen and IS was compared to that of the blank plasma sample.

Representative chromatograms of (a) drug-free plasma, (b) the plasma spiked with ibuprofen (10 µg/mL) and IS (5.0 µg/mL) and (c) the plasma obtained at 4 h after a single dose of 600 mg ibuprofen was given in *Fig. 2*. There was no interference peak near the retention times of ibuprofen and IS.

The linearity of the method was evaluated by a calibration curve in the range of 0.5-40 µg/mL of the drug (n = 3). Drug-free plasma was spiked with ibuprofen standard solutions to achieve final concentrations of 0.5, 2.5, 5.0, 10, 20, 30 and 40 µg/mL. Calibration curve was obtained by plotting peak area ratios of ibuprofen to IS versus the ibuprofen concentrations with least-squares linear regression analysis. The calibration equation from three replicate experiments, $y = 0.03246x + 0.0335$

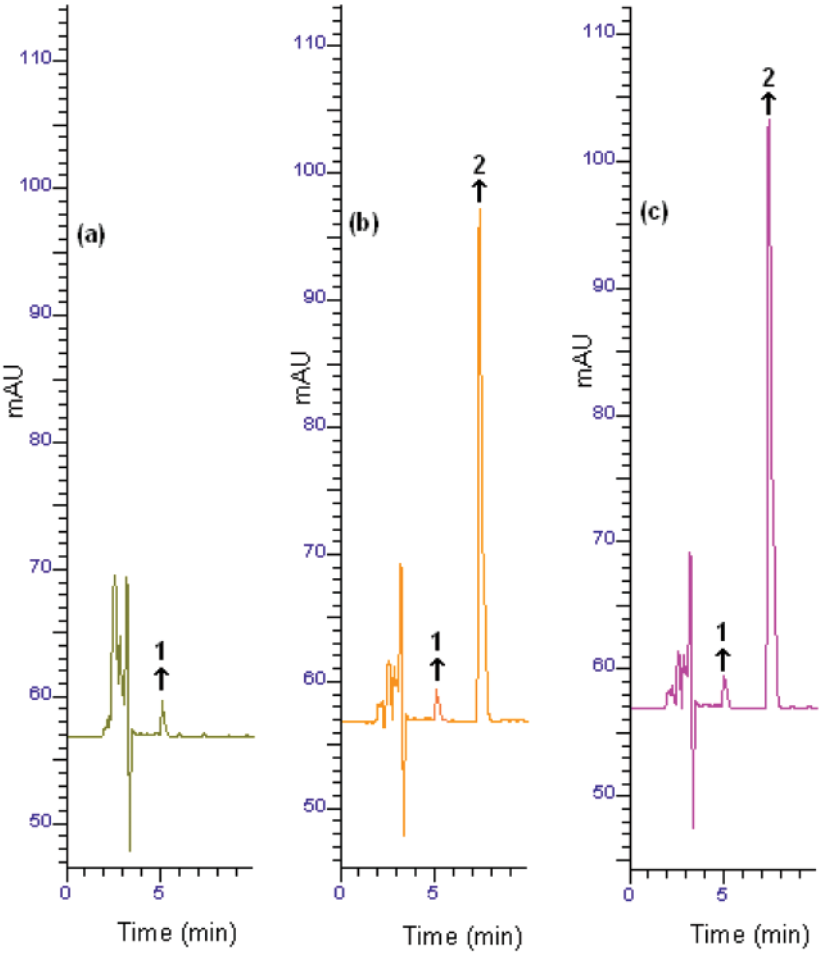

Fig 2. Representative chromatograms of (a) drug-free plasma, (b) the plasma spiked with ibuprofen (10 µg/mL) and IS (5.0 µg/mL), (c) the plasma obtained at 4 h after a single oral dose of 600 mg ibuprofen, (1) IS, (2) Ibuprofen

Şekil 2. Boş plazma (a), ibuprofen (10 µg/mL) ve IS (5.0 µg/mL) eklenen plazma (b), 600 mg ibuprofenin tek oral dozundan sonraki 4. saatte elde edilen plazmanın (c) örnek kromatogramları (1) IS, (2) İbuprofen

(r = 0.9998), demonstrated the linearity of the method.

Intra-day and inter-day precision and accuracy were determined by replicate analysis of six sets of samples spiked with three different concentrations of ibuprofen (1.5, 15 and 35 µg/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 1*. The intra- and inter-day precisions were measured to be within 1.74 and 4.97% for plasma.

Table 1. *Intra-day and inter-day precision and accuracy of ibuprofen in plasma (n=6)*

Tablo 1. *Plazmada ibuprofenin gün-içi ve günler-arası kesinlik ve doğruluğu (n=6)*

Sample	Concentration (µg/mL)		%RSD	%RE
	Added	Found (Mean±SD)		
Plasma	1.5	1.44±0.025	1.74	- 4.00
Intra-day	15	13.92±0.179	1.29	- 7.20
	35	32.83±0.322	0.98	- 6.20
Inter-day	1.5	1.47±0.073	4.97	- 2.00
	15	14.12±0.449	3.18	- 5.87
	35	33.81±0.371	1.09	- 3.40

Table 2. *Recovery of ibuprofen in plasma (n=6)*

Tablo 2. *Plazmada ibuprofenin geri kazanımı (n=6)*

Sample	Concentration (µg/mL)		%Recovery	%RSD
	Added	Found (Mean±SD)		
Plasma	1.5	1.44±0.025	96.0	1.74
Intra-day	15	13.92±0.179	92.8	1.29
	35	32.83±0.322	93.8	0.98
Inter-day	1.5	1.52±0.037	101.3	2.43
	15	14.29±0.195	95.3	1.36
	35	33.24±0.386	94.9	1.16

The sensitivity was evaluated by the limit of quantification (LOQ), the lowest concentration of the plasma spiked with ibuprofen 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 0.25 µg/mL.

The recovery was determined by comparing peak area of ibuprofen after extraction to that before extraction at concentrations of 1.5, 15 and 35 µg/mL. The mean extraction recovery of ibuprofen from plasma was 95.7%. The mean relative recovery for IS at 5.0 µg/mL was 93.4 (n = 6). Recovery data are shown in *Table 2*.

Ibuprofen and IS solutions were stable for 24 h at room temperature and 7 days at 4°C. No significant change in ibuprofen and base concentrations was found in plasma samples stored at -20°C after three freeze-thaw cycles or at room temperature for 24 h. The short-term stability of ibuprofen in plasma was stable for at least 1 week. In the long-term stability study, the plasma samples spiked with ibuprofen were stored for 2 weeks.

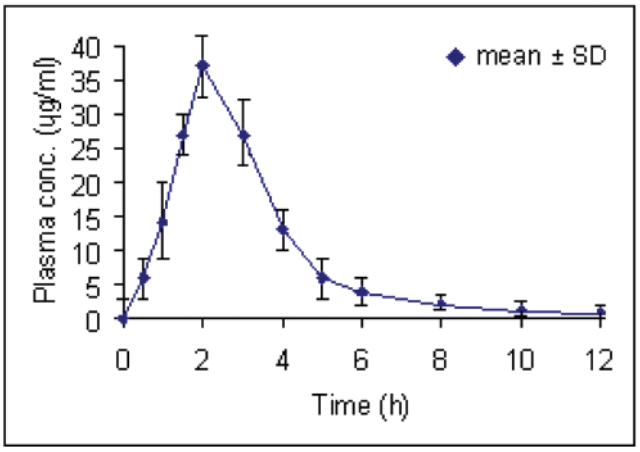

Fig 3. Mean plasma ibuprofen concentration-time profile for three rabbits after a single oral dose of ibuprofen, 600 mg

Şekil 3. 600 mg ibuprofenin tek oral dozundan sonra üç tavşan için ortalama plazma ibuprofen derişim-zaman profili

Table 3. *Mean pharmacokinetic parameters of ibuprofen for three rabbits after oral administration of Artril tablet (600 mg)*

Tablo 3. *Artril tabletin (600 mg) oral veriminden sonra üç tavşan için ibuprofenin ortalama farmakokinetik parametreleri*

Parameters	(Mean±SD)	%RSD
Maximum plasma concentration Cmax (µg/mL)	37.1±3.761	10.14
Time required for maximum plasma concentration (Tmax)	2.0±0.268	13.40
Area under curve $AUC_{(0\text{-}12\ h)}$ (µg/mL h)	118.4±22.43	18.94
Area under curve at infinite time $AUC_{(0\text{-}\infty)}$ (µg/mL h)	143.1±29.48	20.61
Plasma half life ($T_{1/2}$) (h)	2.59±0.648	25.02

The plasma samples obtained three rabbits were assayed with the validated method described above. The peaks of ibuprofen and IS were completely separated from endogenous peaks with similar retention times to those of the samples used for the validation studies *(Fig. 2).* The mean plasma concentration-time curve was shown in *Fig. 3.* The mean values of pharmacokinetic parameters estimated by the computer program WinNonlin with non-compartmental method were shown in *Table 3*.

DISCUSSION

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

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 [20]. Thus, ibuprofen 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 ibuprofen. 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 ibuprofen (7.3 min) was quite short than that studied in other papers [6-8].

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 [6,7,11]. CE methods are a little simpler and faster with respect to the deproteinization step and analysis time, but the reported detection limits of 1 µg/mL [16] and 8 µg/mL [17] are not sensitive enough for the pharmacokinetics studies of the drug in vivo. Calibration curve of ibuprofen was linear over the concentration range of 0.5-40 µg/mL for plasma which is as good as or superior to that reported in other papers [5,7,9,12,14,15,17].

Ibuprofen was extracted from plasma with a solid phase extraction procedure by Farrar et al.[12]. This method is also the most comprehensive method which can extract ibuprofen in a single extraction procedure. In this study, the recovery percentage of ibuprofen is high [12,14,17], extraction processes do not take much time [6-8]; additionally, the retention time is short which is an advantage [5,13-15].

Bonato et al.[14] have reported LC method with tandem mass detection for the analysis of ibuprofen in plasma. The calibration curve of LC-MS-MS method was linear for ibuprofen in the range 0.12-190 µg/mL. Intra- and inter-day precision values were lower than 15%. The maximum recovery of ibuprofen was 73.9%. The LOQ of method was found 0.12 µg/mL. 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 [5,6,12,13,15] the proposed method has indicated high accuracy and recovery.

In the proposed work, a simple and sensitive HPLC method has been developed for the determination of ibuprofen in rabbit plasma. Also, the method was completely validated by using sensitivity, stability, specificity, linearity, accuracy and precision parameters for determination of ibuprofen in plasma. Additional advantages of this method include small sample volume (0.5 mL), good extraction recovery from plasma and a readily available internal standard. To our knowledge, this is the first description of ibuprofen pharmacokinetics in rabbit plasma by HPLC method in the literature. It can be very useful and an alternate to performing pharmacokinetic studies in determination of ibuprofen for clinical use.

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