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Development and Validation of Bioanalytical Method for Determination of Flurbiprofen from Human Plasma by Liquid Chromatography

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1. Introduction

Flurbiprofen, is a chiral arylpropionic acid which is non steroidal anti inflammatory drug used in treatment of pain and inflammation. Flurbiprofen (FLU) is well absorbed orally, the peak concentration occur within 1-2 hour and conjugated in the liver [1]. It's half life in the plasma is about 6 hours. The major metabolic process is hydroxylation to form 4'-hydroxyflurbiprofen. FLU can be used as an in vivo probe for CYP2C9 activity [2].

Several HPLC-UV assays have been reported over the last 30 years for the determination of FLU from biological material [2-6]. FLU has been determined in human urine by using GC-MS [7]. FLU and its primary metabolites 4'-hydroxyflurbiprofen were determined by using HPLC-fluorescence system from serum [8]. Stereo specific analysis of FLU from plasma was developed by HPLC-UV system with chiral column [9].

LC/MS and also fluorescence method [10] and UVand MS detection[11] techniques have higher detection sensitivity. HPLC is a very common instrument for analytical laboratory and readily available and more economic instrument for measuring drug concentration in biological samples.

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Hutzler et. al. [2] and Giagoudakis et.al. [3] developed HPLC-UV method for the determination of FLU from biological samples, LOQ of their methods were 250 ng/ml and 100 ng/ml respectively.

In present study, a original validated HPLC method with UV detection for the determination of FLU from minimum amount of biological sample (200 µl plasma) with simple liquid-liquid extraction is presented.

The method developed for the determination of FLU from plasma which is reliable and robust. The validation results were included specificity, accuracy, extraction recovery, linearity and range. The assay can be applied successfully to the pharmacokinetic and bioequivalence studies.

2. Experimental

2.1. Chemicals and reagents

FLU and losartan (IS) were obtained from Aesica Pharmaceuticals Lmt. England and Chemo SA, Switzerland respectively. Methanol and isopropanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile was obtained from Lab-Scan (Gliwice, Poland). Orthophosphoric acid and sodium acetate were purchased from Merck (Darmstadt, Germany) and JT Baker (Deventer, Netherlands) respectively. All other chemicals were analytical grades. HPLC grade solvents were used. Drug free plasma sample was obtained from the Turkish Blood Centre. HPLC grade water was used throughtout the study (Millipore, Bedford, USA).

2.2 . Instrumentation

The HPLC system of Agilent 1100 series with UV detector were used for analyse Flurbiprofen. The HPLC system consisted of Agilent 1100 series G1379A pump, G1367A Auto sampler and Agilent 1100-G1315 detector. Chemstation ver:10.02 was used for data acquisition.

2.3. Chromatographic conditions

Chromatographic separations were carried out at room temperature using a reversed phase Nucleosil C18 (150 x 4.6 mm, 5 µm) column. The mobile phase consist of a mixture of 0.1 M sodium acetate and acetoni-

trile (65:35; v/v) and the pH of the mobile phase was adjusted to 6.30 by 85 % orthophosphoric acid. Flow rate of mobile phase was 1 mL/min. The detection wavelength, 248 nm was determined by scanning the maximum absorbance wavelength of FLU and losartan in the mobile phase. The injection volume was 25 µl.

2.4. Preparation of standard solutions and quality control samples

A stock solution of FLU (1mg/mL) and losartan (1mg/mL) was prepared using MeOH:dH_2O (1:1;v/v). Secondary standard solution of FLU and IS were prepared by diluting stock solution with MeOH:dH_2O (1:1;v/v). The seven calibration standard were prepared with spiked plasma (100, 500, 2500, 5000, 10000, 20000, 40000 ng/mL) independently. All solutions were stored at 4 °C during the study. The quality control (QC) samples at a concentration 100, 300, 2000, 30000, 40000 ng/mL were made by diluting the secondary standard solution with human blank plasma.

2.5. Plasma sample processing

FLU was extracted from plasma samples by using liquid-liquid extraction. 200 µL plasma samples in polypropylene tubes were spiked with 100 µL Internal standart (IS) solution (50 µg/mL) and vortexed for 15 sec.. 100 µL MeOH:dH_2O (1:1;v/v) were added to each tube and then added 500 µL 2.0 M orthophosphoric acid to precipitate the proteins. 1.5 mL extraction solvent (diethylether : dichloromethan: isopropanol; 3:1.5:0.5; v/v/v) was added and vortexed 1 min. to extract FLU. The tubes were centrifuged at 5000 rpm, 20 C for 10 min. The supernatants were transferred into clean tubes and evaporated to dryness under nitrogen atmosphere. After evaporation samples were reconstituted with 100µL mobile phase and injected to HPLC system.

2.6. Bioanalytical method validation

The method was validated according to FDA guidelines for validation of bioanalytical methods [12-15]. In order to show the acceptable nature of the analytical method, the following protocol was implemented during the method evaluation.

2.6.1. Selectivity and sensitivity

The selectivity of method was assessed by analyzing six different drug free human control plasma. Chromatograms were compared for any interference from the matrix or any of the assay reagents. The lowest standard 100 ng/mL on the calibration curve was identified as the lower limit of quantification (LOQ) with a precision of less than or equal to 20 %.

2.6.3. Linearity

The calibration curve was prepared from seven calibration spiked plasma samples within the range of 100-40 000 ng/mL, including LOQ. The acceptance criteria of back calculated standard concentration was 15 % deviation from nominal value except the LOQ (for LOQ less then or equal to 20 % deviation was applied). The calibration curve was obtained by plotting the area ratios of FLU and IS as a function of the FLU concentration using least squares linear regression analysis. The LOQ was defined as a reproducible lowest concentration with signal to noise ratio greater than 10.

2.6.4. Recovery

Recovery of the method was performed comparing the three quality control (QC) samples at low, medium and high concentrations (200, 4000, 80000 ng/mL). The recoveries of FLU and IS were determined by comparing peak area obtained for QC samples that were subjected to the extraction procedure with those obtained from blank plasma extracts that were spiked post extraction to the same nominal concentrations.

2.6.5. Accuracy and precision

Intra-day accuracy and precision were determined by analysis of six replicates of 5 concentrations including low, medium and high concentration QC samples. Inter-day accuracy and precision were determined by the analysis of these QC samples on three separate states. The overall precision of the method was expressed as percentage of coefficient of variation and the accuracy of the method was expressed in terms of relative errors.

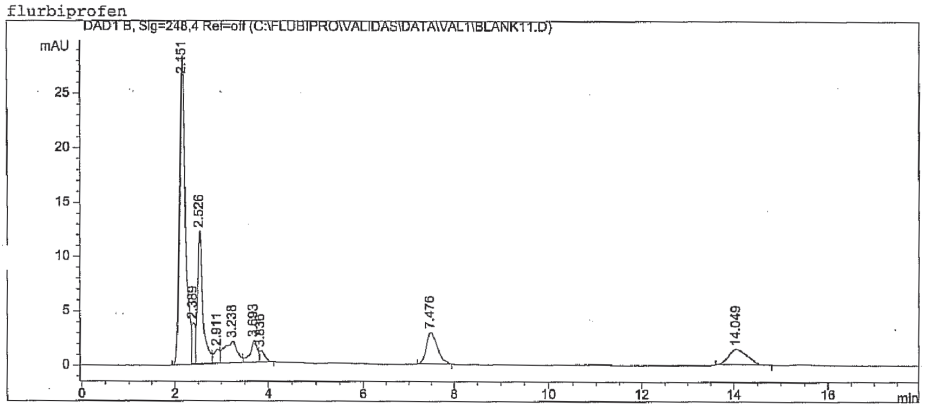

Figure 1a
Blank plasma chromatogram

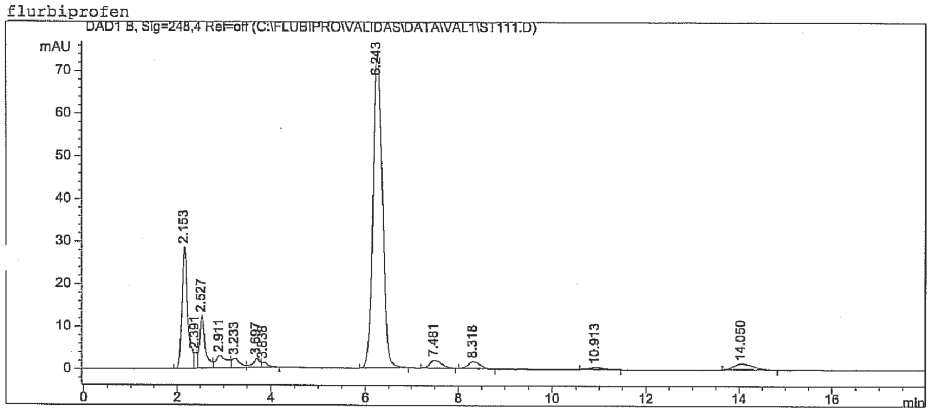

Figure 1b
Limit of quantitation of flurbiprofen from spiked plasma

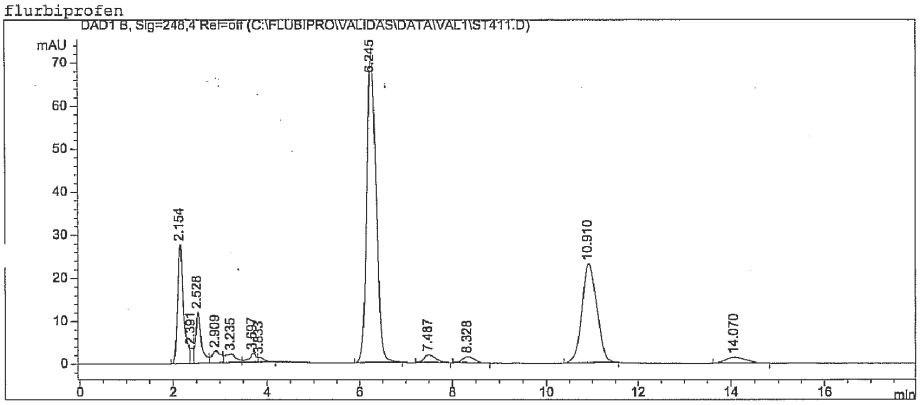

Figure 1c
Middle concentration of flurbiprofen from spiked plasma

2.6.6. Stability

The stability of the processed low, medium and high concentrations of QC samples were tested in autosampler, freeze-thaw, short-term, long term and stock solution stability. Auto-sampler stability was tested by analysis after storage in the auto-sampler for 48h at room temperature. Freeze-thaw stability of samples was obtained over four freeze-thaw cycles at room temperature. Short-term stability was evaluated by keeping QC samples in the laboratory banch at least analysis time, 6h and then reanalysing. Long term stability was tested by using QC samples stored at -80 °C for 66 days and then reanalysed every month to show the storage stability. Stock solution stability was determined immediately after preparation of stock solution of FLU and IS and at 12, 24 and 72 at room temperature and +4 °C.

3. Results and Discussion

3.1. Sensitivity and selectivity

Chromatographic separation of analyte and internal standard was optimized to provide acceptable resolution, good peak shape and intensity of the response. Mobile phase, 0.1 M sodium acetate and acetonitrile (65:35; v/v) with the pH:6.30 provided good resolution for FLU (t_R: 10.9 min) and IS (t_R: 6.2). No endogenous interference and matrix effect were found at the retention times of FLU and IS. Figure I shows representative for blank plasma and standards. The LOQ was defined as a reproducible lowest possible concentration linear with the calibration curve. The LOQ was found to be 100 ng/ml. The intra and inter day CV were found to be 5.3 and 12.0 respectively (Table I). The LOQ value of our analytical method was the same as that reported by Giagoudakis et.al.[3] using HPLC-UV detector and 2.5 times lower then that reported by Hutzler et.al. [2]. Their LOQ values were lower then proposed method. This method showed sufficient selectivity and sensitivity for analyzing FLU from plasma for pharmacokinetic and bioequivalence studies.

3.2. Linearity

The standart calibration curves was linear over the concentration range from 100-40 000 ng/mL with mean r^2=0.99934, n=6. The LOQ was 100 ng/mL. The calibration curve had a regression equation of y=0.00011x + 0.00164, where y is the peak area ratio of FLU to IS and x is the plasma concentration of FLU.

3.3. Accuracy and precision

The precision was expressed as the percentage of coefficient of variation. Intra and interday precision was less then 7.3 and 12.0 with the 96.2 and 100.8 CV % respectively. Intra and inter day accuracy (% relative errors) were less then 110.5 and 100.8 respectively. Table 1 gives a summary of the accuracy and precision at FLU concentrations 10-40 000 ng/ml. This suggests that the method developed was acceptable for bioequivalence and pharmacokinetic studies.

TABLE I

Precision and Accuracy for the determination of Flurbiprofen from plasma

Concentration added (ng/mL)	Precision (CV %)	Accuracy (error %)
Intra-day (n=6)		
100	5.3	110.5
300	2.5	103.8
2000	3.1	101.2
30 000	3.9	104.5
40 000	7.3	102.2
Inter-day (n=6)		
100	12.0	96.2
300	7.3	96.7
2000	3.1	100.3
30 000	4.6	100.3
40 000	6.2	100.8

3.4. Recovery

Diethylether:dichloromethane:isopropanol mixtures and diethylether: isopropanol mixtures were tested as a solvent for extracting flurbiprofen and IS from plasma. Diethylether:dichloromethan:isopropanol (3:1.5:0.5; v/v/v) mixture showed higher yield and clear baseline. Thus, the mixture was selected as a solvent for extraction.

The recovery of FLU in the Liquid-liquid extraction procedure from 200 µl of plasma was measured at low, medium and high concentrations. The mean relative recoveries of FLU from plasma ranged from 68.1 to 72.2 % with a CV between 2.0 and 7.3 %. These results indicate good recovery for FLU and allowed us to conclude that our method is able to quantify FLU in human plasma samples (Table II).

TABLE II

Recovery results of FLU from plasma

Concentrations(ng/mL)	Mean relative recovery (%) (n=6)
100	68.1
2000	68.4
40000	72.2

3.5. Stability

The stability was assessed under a variety of conditions and the data is shown in Table III. All samples stored at -80 °C. Short-term stability of FLU at room temperature for 6h had no effect on quantification. Auto sampler stability and stock solution stability were tested and found to be 44h and 72h respectively. Four freeze-thaw cycles of the quality control samples were appropriate for the quantification. Long-term stability of samples was found 66 days at –80 °C.

Pharmacokinetic application

The method was used to examine the pharmacokinetics of flurbiprofen. Maximum plasma concentration (C_{max}) of FLU 19 164.22 ng/mL were found. Areas under the plasma concentration time curve $AUC_{0-\infty}$ of

111 339.8 ng.h/mL were calculated. Figure 2 shows the representative plasma concentration- time profile of 12 healthy volunteers after administred a single oral dose of 100 mg FLU.

TABLE III

Stability data of flurbiprofen (n=12)

Stability	100 ng/mL (error %)	2 000 ng/mL (error %)	40 000 ng/mL (error %)
Autosampler	83.6	95.6	97.9
Freeze-thaw	84.5	93.3	94.9
Short term	118.5	109.6	103.8
Long term	93.9	97.6	103.4

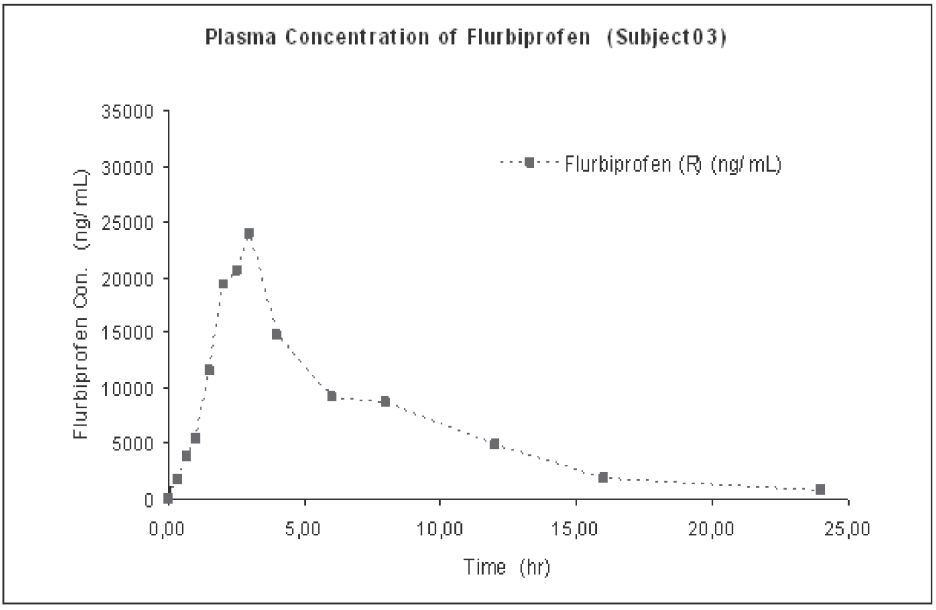

Figure 2
A representative Plasma concentration time profile of FLU

This study has approval of Yeditepe University Local Ethic Committee and Central Ethic Committee of Turkey. Clinical study of Flurbiprofen meets all requirements specified in the Decleration of Helsinki (2004), this decleration signed by all clinical investigators.

4. Conclusion

The developed method for the determination of flurbiprofen from biological material has been found accurate, precise, selective, and suitable for the bioequivalence and pharmacokinetic studies.

Özet

Flubiprofenin İnsan Plasmasından Sıvı Kromatografisi Yöntemi İle Analizinin Geliştirilmesi ve Validasyonu

Flurbiprofenin plasmadan analizi için Yüksek Basınçlı Sıvı Kromatografisi yöntemi ile UV dedektörü kullanılarak hassas bir yöntem geliştirilip valide edilmiştir. Flurbiprofenin plazmadan tüketilmesi için sıvı-sıvı tüketme yönteminde dietileter:diklorometan:izopropanol, 3:1.5:0.5, (v/v/v) karışımı tüketme çözücüsü olarak kullanılmıştır. Flurbiprofenin kromatografik analizinde losartan (IS) iç standart olarak kullanılmıştır. Zıt faz kromatografisinde Nucleosil C18 kolon (150x4.6 mm, 5mm) ile mobil faz olarak 0.1 M sodium asetat:asetonitril (65:35, v/v), pH:6.30 kullanılmış ve 248 nm de absorbansları ölçülmüştür. Mobil faz akış hızı 1 ml/dak ve enjeksiyon hacmi 25 ml dir. Kalibrasyon eğrisi 100- 40 000 ng/mL derişim aralığında doğrusal bulunmuştur. En düşük tayin limiti 100 ng/mL olarak tespit edilmiştir. Güniçi ve günlerarası hassasiyet sırasıyla 7.3 ve 12.0 dan az olarak hesaplanmıştır. Doğruluk ise % 2.5-7.3 aralığındadır. Flurbiprofen ve iç standartın geri kazanımı 200, 4000 ve 80000 ng/mL derişimlerinde % 68.1 ile % 72.0 aralığında bulunmuştur. Stabilite çalışmaları FDA'nın "Bioanalitik Yöntem Validasyonu" rehberinde belirtildiği koşullarda yapılmıştır. Geliştirilen bu yöntem Farmakokinetik çalışmalara başarı ile uygulanmıştır.

Summary

A sensitive high performance liquid chromatographic method with UV detection (HPLC-UV) for the determination of Flurbiprofen from plasma was developed and validated. Liquid-liquid extraction was used for extracting Flurbiprofen from plasma. Diethylether:dichloromethan:isopropanol, 3:1.5:0.5, (v/v/v) mixture was used as extraction solvent. The

chromatographic separation of Flurbiprofen and losartan (IS) was carried out using reverse phase Nucleosil C18 column (150x4.6 mm, 5mm) with mobile phase of 0.1 M sodium acetate:acetonitrile (65:35, v/v), pH:6.30 and UV detection at a wavelength 248 nm. The flow rate of mobile phase was 1 mL/min, injection volume was 25 ml. The calibration curve was linear within the concentration range 100- 40 000 ng/mL. The limit of quantification was 100 ng/mL with good accuracy and precision. Intra-day and interday precision were less than 7.3 and 12.0 respectively and the range of accuracy was 2.5-7.3 % . Recoveries of Flurbiprofen and IS from plasma ranged from 68.1 to 72.0% for 200, 4000 and 80000 ng/mL concentrations. The stability was assessed under a variety of conditions (FDA, Bioanalytical Method Validation Guidline) and found that appropriate for the quantification. The method developed has been applied successfully to pharmacokinetic study.

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