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ORIGINAL ARTICLE

Development and Validation of HPLC-UV Method for the Determination of Diclofenac in Human Plasma with Application to a Pharmacokinetic Study

Diklofenak'ın İnsan Plazmasından HPLC-UV ile Tayin Yöntemi Geliştirilmesi ve Validasyonu ile Farmakokinetik Çalışmaya Uygulanması

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ABSTRACT

A simple, rapid and reliable high performance liquid chromatography method (HPLC) with ultraviolet detection (UV) was developed and validated according to ICH guidelines, for quantitative analysis and therapeutic drug monitoring of diclofenac sodium (DS) in human plasma. Plasma samples (0.7 mL) were acid hydrolysis by 100 µL, 1 M hydrochloric acid. Analytes were concentrated from plasma by liquid–liquid extraction with 2 mL ethyl acetate by repeated twice, which allows to obtain good extraction yields (98.75%-99.32%). The separation was achieved by employing C18 analytical column (3.5 µm particle size, 150 mmx3.9 mm I.D.) under isocratic conditions using acetonitrile and NaH_2PO_4 mixture (42.5:57.5, v/v) as mobile phase (pH: 3.16) flow rate of 1.5 mL/min. Naproxen (3 µg/mL) was used as an internal standard (IS). The DS and IS were detected at 281 nm and eluted at 2.6 and 6.2 min, respectively. Total run time was 7 min. Method showed linearity with very good determination coefficients (r^2=0.999), over the concentration range of 50 - 1600 ng/mL. Limits of detection (LOD) and quantification (LOQ) were 8.95 ng/mL and 27.12 ng/mL, respectively. Intra-day precision and accuracy were between 0.93-5.27; 1.74-9.81, respectively. Inter-day precision and accuracy were between 2.71-6.64; 2.03-9.16, respectively. This method was successfully applied for determination of DS plasma concentrations during a pharmacokinetic study in healthy volunteers (n=12) after an oral administration of Voltaren® 75 mg/tablet and remarkable variations in DS levels were observed. In our study, on the contrary to equivalent doses of DS, the observed significant differences in plasma levels of DS, on 2nd, 4th and 6th hours, can be explained by pharmacokinetic differences, that arise from mainly polymorphisms of CYP2C9 and CYP3A4, which are major enzymes responsible for DS metabolism.

Key words: Diclofenac sodium, Validation, Plasma, High performance liquid chromatography, Ultraviolet detection

ÖZ

Diklofenak sodyumun (DS) insan plazmasında kantitatif analizleri ve terapötik ilaç izlemi için, basit, hızlı ve güvenilir bir ultraviyole dedektörlü yüksek performanslı sıvı kromatografisi (YPSK-UV) metodu geliştirildi ve ICH kurallarına göre valide edildi. Plazma örnekleri (0.7 mL) 100 µL, 1 M hidroklorik asitle hidroliz edildi. Analitler plazmadan iyi ekstraksiyon verimi (%98.75-%99.32) sağlayan sıvı-sıvı ekstraksiyonu metodu ile 2 mL etil asetatla iki tekrar ile elde edildi. Ayrım izokratik şartlar altında C18 analitik kolon (3.5 µm partikül büyüklüğü, 150 mmx3.9 mm I.D.), mobil faz asetonitril ve NaH2PO4 (42.5:57.5, v/v) karışımı (pH 3.16), akış hızı 1.5 mL/dk ile gerçekleştirildi. Naproksen (3 µg/mL) iç standart (İS) olarak kullanıldı. DS ve İS 281 nm'de ve sırasıyla 2.6 ve 6.2 dakikalarda tespit edildi. Toplam analiz süresi 7 dakika idi. Metot 50 - 1600 ng/mL konsantrasyon aralığında çok iyi bir belirlenme katsayısı ile (r^2=0.999) doğrusallık gösterdi. Gözlenebilme sınırı ve tayin sınırı sırasıyla 8.95 ng/mL ve 27.12 ng/mL idi. Gün içi kesinlik ve doğruluk sırasıyla 0.93-5.27; 1.74-9.81 aralığında idi. Günler arası kesinlik ve doğruluk ise sırasıyla 2.71-6.64; 2.03-9.16 aralığında idi. Bu metot sağlıklı gönüllülerin (n=12) tek doz oral Voltaren® 75 mg/tablet alımı ile farmakokinetik çalışma süresince DS plazma konsantrasyonlarının belirlenmesi için başarıyla uygulandı ve DS düzeylerinde dikkate değer varyasyonlar gözlendi. Çalışmamızda DS'nin eşit dozlarına karşın, 2., 4. ve 6. saatlerdeki plazma düzeylerinde gözlenen anlamlı farklılıklar, özellikle DS metabolizmasından sorumlu majör enzimler olan CYP2C9 ve CYP3A4 polimorfizmlerinden kaynaklanabilecek farmakokinetik farklılıklarla açıklanabilir.

Anahtar kelimeler: Diklofenak sodyum, Validasyon, Plazma, Yüksek performanslı sıvı kromatografisi, Ultraviyole deteksiyon

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INTRODUCTION

Diclofenac sodium (DS), 2-*[(2,6-dichlorophenyl)amino]phenyl-acetic acid,* (Figure 1a) is non-steroidal anti-inflammatory analgesic (NSAID) with potent cyclooxygenase (COX) inhibition activity (1,2). DS has a well documented safety profile, which is comparable to those of other NSAIDs. It inhibits prostaglandin synthesis by inhibition of enzymatic transformation of arachidonic acid into prostaglandins (3,4). This drug is widely used in clinical medicine for the pain control and treatment of rheumatoid arthritis, osteoarthritis, ankylosing spondylitis also acute injury (1). In addition to, it is used to treat chronic pain associated with cancer (2,5). DS has anti-uricosuric feature. The development of Alzheimer disease may be prevent if use of DS which low-dose in long-term (5).

DS is well-absorbed after oral administration with expensive hepatic metabolism. It is extensively (>99.7%) protein bound (albumin) in plasma and serum at therapeutic concentrations. Terminal half life is 1-2 hours. Food can cause a delay in the onset of absorption and a reduction in plasma levels of approximately 30%. After absorption, approximately half of the absorbed dose is metabolized immediately by the liver, due to first pass metabolism. 35% of absorbed DS enters enterohepatic circulation. The distribution volume (V_d) of DS is 1.4 L/Kg. C_{max} is reached at approximately 4 hours. T_{max} is approximately 0.5-1 h. Elimination is rapid with 90% of the drug clearance taking between 3 to 4 hours. The DS metabolism products, which are mainly 4'- hydroxy (OH) diclofenac and minor monohydroxy metabolites are 3'-OH diclofenac and 5'-OH diclofenac, are excreted by the urine (65%) and biliary (35%) (2,5-7).

The use of DS has been associated with occasional hepatic toxicity (8,9). Although the etiology of this toxicity is not known, clinical evidence suggests that it may be due to an immune (10,11) or a non-immune mechanism (9,12,13). In both cases, covalent modification of liver proteins may play an important role in the etiology of DS hepatotoxicity (14). The fact that, CYP2C9 was known to have a major role in the oxidative metabolism of DS (15,16). It seemed possible that CYP2C9 might metabolically activate DS into a reactive metabolite(s), which may have a role in DS hepatitis in humans. Naproxen which was used as an internal standart in our study, is a proprionic acid derivate related to the arylacetic acid group of nonsteroidal anti-inflammatory drugs. Naproxen is chemically, *(S)-6-methoxy-α-methyl-2-naphthaleneacetic acid* (Figure 1b) is commonly used for the reduction of moderate

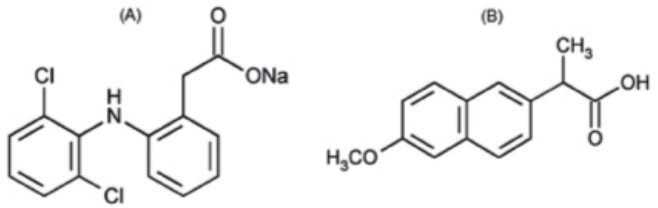

Figure 1. The chemical structure of diclofenac sodium (A) and naproxen (B)

to severe pain, fever, inflammation, and stiffness. It works by inhibiting both the COX-1 and COX-2 enzymes. It is antipyretic and analgesic effects were related to inhibition of both the COX-1 and COX-2 (17).

Bioavailability, bioequivalence and therapeutic drug monitoring studies have received major attention from the pharmaceutical industry, health authorities and clinic. These studies are performed to evaluate the safety and efficacy of a genetic structure. Which studies as well as drug product development studies require rapid, simple, sensitive and reliable bioanalytical methods to monitoring the target drug in human plasma sample. Also for clinical studies, it is essential to establish accurate, sensitive and selective analytical techniques that permit detection and quantitative measurement of drug entities in biological and pharmaceutical samples (1). Several methods have been reported for determination of DS including spectrophotometric (18,19), spectrofluorimetric (20,21), polarographic (22), conductometric (23), high-performance liquid chromatography (HPLC) (1,24-29), gas chromatography mass spectrometry (GC-MS) (30,31) and capillary electrophoresis (32) in human plasma and other biological fluids. Some of these methods are not suitable for routine analysis because they need sophisticated instruments, not yet available in many routine control laboratories. Since, HPLC-UV assays are reliable, inexpensive and widely utilized; it has appeared to fit best for performing simultaneous separation, quantification and clinical monitoring of DS as a primary concern of this paper.

Compared with other studies the advantages of present method are short run time (total run time 7 min) (26,28), using low biological sample volume (0.7 mL plasma) (26,27), high sensitivity (LOD 8,95 µg/mL) with small volume samples (1,2), include simple, efficient and inexpensive extraction procedure, high recovery value (98.75-99.32%) (26-28) and using inexpensive chemicals in analytical processes (27). Also this method is used to determine the DS in human plasma samples obtained from twelve healthy volunteers. The originality of this DS pharmacokinetic study is being the only research has ever been done with the contribution of the largest volunteers in Turkey. In addition to this, the method is efficient in analyzing large numbers of plasma obtained for pharmacokinetic study after therapeutic dose of diclophenac. In the present study, our objective is to develop and validate a reliable, simple, fast, and inexpensive HPLC method using UV detection for determination of DS in human plasma with the lower volume sample preparation according to ICH guidelines (33,34) and also to emphasize interpersonal differences in metabolization of DS for correct dosage of by simultaneously monitoring its levels in plasma. The developed method is validated by using linearity, precision, accuracy and sensitivity parameters according to ICH guidelines. For this purpose a group of volunteers, who are the employees of Gülhane Military Medical Academy, were contributed and DS levels in the plasma samples from volunteers were investigated.

EXPERIMENTAL

Chemicals and reagents

Ethyl acetate (EtOAc), acetonitrile (ACN), sodium dihydrogen phosphate dehydrate (NaH_2PO_4), hydrochloric acid (HCl), ortophosphoric acid (H_3PO_4) and methanol (MeOH) were purchased from Merck (Darmstadt, Germany). The standards of pharmaceuticals, diclofenac sodium (DS) and naproxen, which is internal standard (IS), were obtained from Sigma-Aldrich (St. Louis, MO, USA). Ultrapure water, which is prepared daily fresh, was used. Prescribed DS tablets, for volunteers, were obtained from pharmaceutical firm Novartis Medical Company (Istanbul, Turkey). All chemicals used in this study were of analytical-grade in the highest purity available, except MeOH and ACN that were HPLC grade.

Instrumentation and optimized chromatographic conditions

The separation and quantification were performed by HP Agilent 1100 (Santa Clara, CA, USA) high-performance liquid chromatography (HPLC) system, equipped with a UV detector.

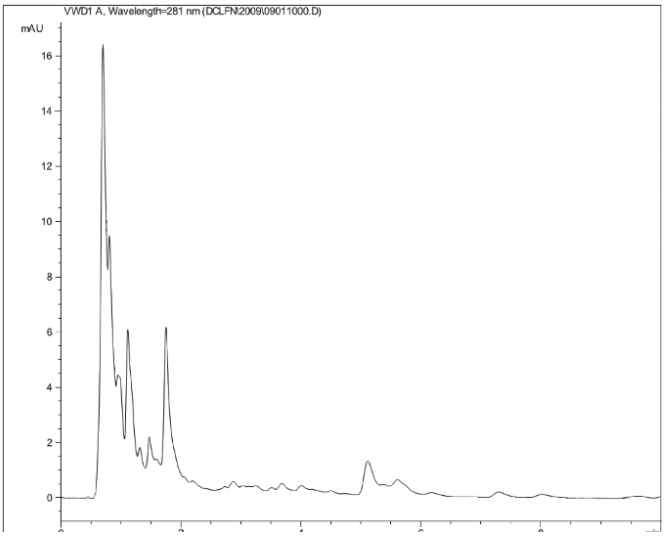

Figure 2. HPLC chromatogram of the blank human plasma

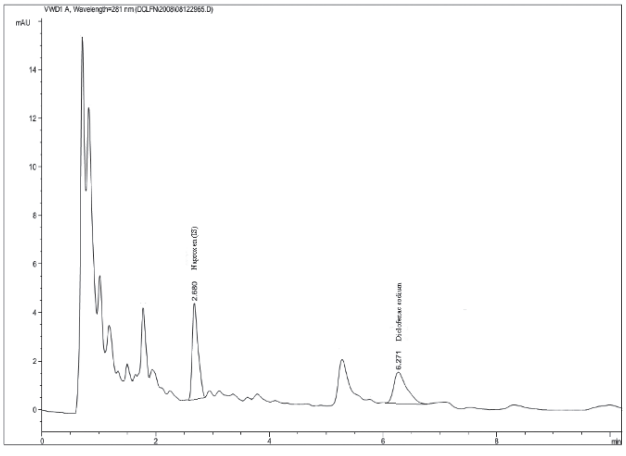

Figure 3. The HPLC chromatogram of DS (and internal standard) in human plasma spiked with 0.8 µg/mL DS (t_R=2.680) and 3 µg/mL naproxen (t_R=6.271) as an internal standard

Optimum analytic conditions were set after an optimization procedure was performed for column selection, content of mobile phase (MP) and wavelength. Prior to optimization, a standard assay for DS determination offered by the manufacturer was used and each parameter was adjusted while others were fixed.

The system consisted of isocratic pump, manual injector with a loop volume of 20 µL, and a Waters C18 column (3.5 µm, 150x4.6 mm I.D.) The wavelength at 281 nm was chosen for UV detection. The mobile phase, which consists of acetonitrile and Na_2HPO_4 buffer (42.5:57.5, v/v) adjusted to pH 3.16 by 0.1 M orthophosphoric acid. It was filtered through a 0.45 µm membrane (Alltech, IL, USA) and degassed in ultrasonic bath for 30 min. An isocratic solution was performed at a flow rate of 1.5 mL/min and at room temperature. Peak areas were measured and calculations were carried out considering the internal standard (IS) peak ratios.

Preparation of stock and working standard solutions

Stock solutions of DS and naproxen (IS) were prepared in methanol solution at the concentrations of 200 µg/mL and 30 µg/mL respectively. Working standards of DS, used for spiked plasma samples, were prepared weekly in the concentrations of 0.5, 1, 2, 4, 8 and 16 µg/mL and made by dilution of the stock solutions with methanol. Blank human blood samples, which were obtained from *Ankara University Serpil Akdağ Blood Center*, centrifuged at 3000 g for 5 min to separate the plasma. Human plasma samples and all working solutions were stored at -20°C until the analysis were carried out. These were checked chromatographically for purity before experiments and utilized (Figure 2) as quality control specimens for validation and optimization process. Their stability was checked before and after the injections of every sample set.

Preparation of sample

Blank plasma samples (0.7 mL) were placed into clean glass tubes which is containing 1 mL HCl (1 M). After that each of these tubes, 100 µL IS (30 µg/mL) and 100 µL of diclofenac sodium solutions, which were the concentrations of 0.5, 1, 2, 4, 8 and 16 µg/mL, were spiked. Reaching the total volume to 1.1 mL and achieving 10 times diluted mixtures; yielding analyte concentrations as calibration samples namely 50, 100, 200, 400, 800, 1600 ng/mL diclofenac sodium and 3000 ng/mL naproxen. Different from the calibration solutions, 100 µL MeOH was added instead of DS solution for the samples from patient's plasma.

Extraction procedure

Liquid-liquid extraction was applied for human plasma samples with acidic hydrolysis (35,36). A volume of 0.1 mL of 1 M hydrochloric acid was added to the samples and mixed at 900 g for 30 second. A volume of 2 mL of ethyl acetate was added to sample tubes and mixed at 900 g for 2 min and then centrifuged at 3000 g for 5 min. This extraction procedure

was repeated twice. Supernatant was collected to another sample tube and dried under nitrogen at 40 ˚C. Residue was dissolved in 50 µL volume of mobile phase, in order to be ready for injection, and then loaded into HPLC, by 20 µL which is loop volume.

Volunteers

Volunteers, who were consist of 12 healthy Gülhane Military Medical Academy employees, participated in this study. The average age of participants was 32±8.12. After ingestion of tablets, which including 75 mg DS, bloodletting was performed at 2nd, 4th and 6th hours. The plasma samples were collected severally and samples were stored at -20˚C. This study was approved by *Local Ethical Committee of Gülhane Military Medical Academy* in 12/11/2008 and decision number is 90.

RESULTS AND DISCUSSION

Selection of internal standard

Naproxen, which was developed in the 1970s, is a non-selective COX inhibitor is a nonsteroidal anti-inflammatory drug (NSAID) of the propionic acid class and is commonly used for relief of a wide variety of pain, fever, swelling and stiffness (17). Naproxen was chosen as internal standard due to its similar physico-chemical properties with diclofenac sodium (DS). On the other hand, the chromatographic pre-study have demonstrated that, naproxen might used as a reliable internal standard (Figure 1-3).

Validation of method

The analytical method was validated to demonstrate the selectivity, specificity, linearity, recovery, limit of

Table 1. Recovery of DS in human plasma (n=3)

Theoretical concentration ng/mL		Mean areas of diclofenac sodium (DS) and internal standard (IS)						
		First group extraction			Second group extraction			
		DS added before extraction	IS added before extraction	Areas (DS/IS)	DS added after extraction	IS added before extraction	Areas (DS/IS)	Mean Recovery (%)
200	1	4.59	33.78		4.87	33.34	0.146	
	2	4.64	30.40		4.99	33.35	0.150	
	3	4.83	31.92		4.72	32.29	0.146	
	Mean	4.69	32.03	0.146	4.86	32.99	0.147	99.32
400	1	10.3	31.7		10.99	33.33	0.330	
	2	10.02	33.47		10.01	32.35	0.309	
	3	10.72	33.25		10.65	33.65	0.317	
	Mean	10.35	32.81	0.315	10.55	33.11	0.319	98.75
800	1	21.12	31.92		20.4	32.28	0.632	
	2	20.11	31.53		21.78	32.23	0.676	
	3	20.58	32.36		20.62	32.11	0.642	
	Mean	20.60	31.94	0.645	20.93	32.21	0.650	99.23

DS: Diclofenac sodium, IS: Internal standard

Table 2. Precision and accuracy of the assay for DS (n=5)

Theoretical Conc. ng/mL	Intra-day				Inter-day			
	DS/IS				DS/IS			
	Estimated DS Conc.	SD	RSD%	RE%	Estimated Conc.	SD	RSD%	RE%
50	54.91	2.20	4.01	9.81	54.58	1.82	3.33	9.16
100	97.29	0.91	0.93	2.71	93.86	6.13	6.53	6.14
200	184.66	6.90	3.74	7.67	182.18	5.10	2.80	8.91
400	429.06	13.02	3.035	7.27	430.96	28.60	6.64	7.74
800	755.60	21.68	2.87	5.55	775.96	21.59	2.78	3.01
1600	1572.20	82.90	5.27	1.74	1567.60	42.44	2.71	2.03

DS: Diclofenac sodium, IS: Internal standard, SD: Standard deviation, RSD%: Percent relative standard deviation, RE%: Percent relative error

detection (LOD) and limit of quantification (LOQ). Intra- and inter-day validation protocol was applied considering reproducibility of method and instrument to obtain accurate and precise measurements in agreement with Conference on Harmonization Guidelines (33,34).

Recovery

The recovery of extraction procedures from human plasma is determined by comparing areas of DS and IS. DS and the IS first joined together in the plasma samples before extraction was performed three different extractions (200, 400, 800 ng/mL diclofenac sodium and 3000 ng/mL naproxen). Also DS and the IS joined together in the plasma samples after extraction in three different plasma samples. The areas of DS and IS are calculated and compared then % efficiency is obtained. Three individual replicates of spiked samples at mid-concentrations (200, 400, 800 ng/mL DS) were prepared with internal standard (n=3). The extraction procedure was conducted as described previously. Peak area ratios were compared and recoveries were calculated as 98.75%-99.32% for plasma (Table 1).

Precision

Precision, defined as relative standard deviation (RSD%), was determined by five individual replicates at six different concentrations (50, 100, 200, 400, 800 and 1600 ng/mL) (n=5). Interday and intraday precisions of method and the instrument were calculated. Results were showed that, RSD% were less than 6.7% both for intra- and interday precisions (Table 2). Analytical instrument precision was also displayed higher precisions namely less than 6.3% considering intra- and interday performances (Table 3).

Accuracy

Accuracy defines as the measure of how close the experimental value is to the true value. Accuracy is the degree of veracity while precision is the degree of reproducibility and accuracy expressed as the relative error (RE%) of the estimated concentrations. Table 2 shows RE% of estimated concentrations for intra- and interday accuracy of assays. The instrument was as accurate as assay which is displayed in Table 3. The accurate and precise measurements pointed at good reproducibility for the method and the instrument.

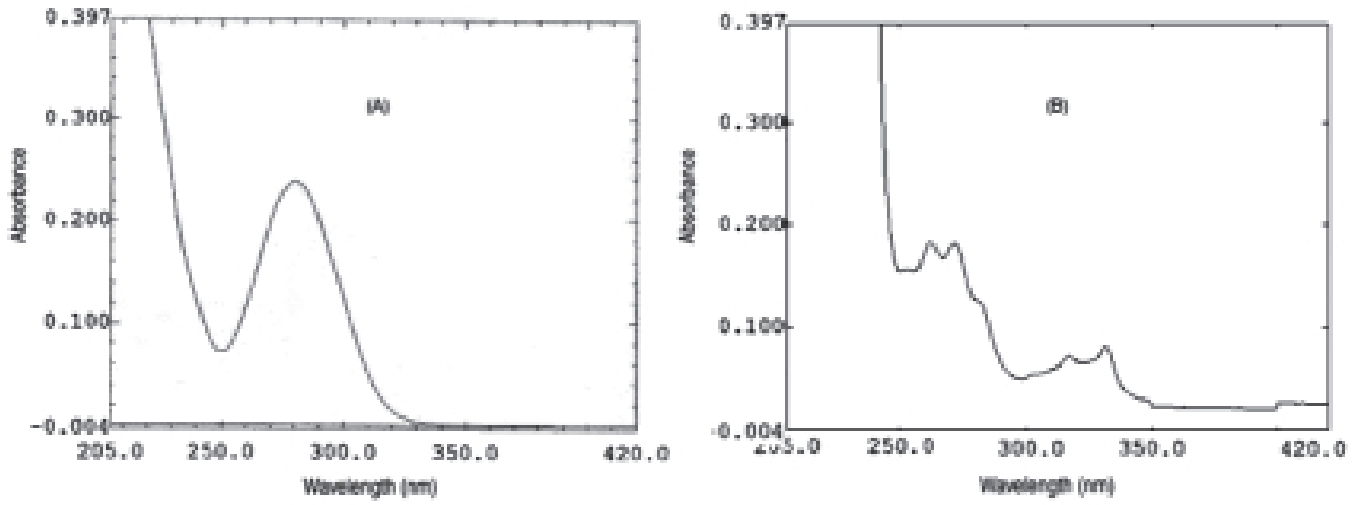

Figure 4. The obtained UV spectrums and maximum absorption wavelengths of DS (A) and Naproxen (B)

Table 3. Precision and accuracy of analytical instrument, HPLC – UV, for determination of DS (n=5)

Theoretical Conc. ng/mL	Intra-day DS/IS				Inter-day DS/IS			
	Estimated Conc.	SD	RSD%	RE%	Estimated Conc.	SD	RSD%	RE%
50	60.16	1.12	1.86	20.32	55.65	2.71	4.87	11.30
100	112.55	5.68	5.05	12.55	95.17	4.81	5.06	4.83
200	223.23	11.16	5.00	11.61	184.99	11.52	6.23	7.51
400	443.71	12.066	2.72	10.93	418.57	14.90	3.56	4.64
800	915.36	15.39	1.68	14.42	756.65	11.27	1.49	5.42
1600	1829.62	78.19	4.27	14.35	1577.49	40.15	2.55	1.41

DS: Diclofenac sodium, IS: Internal standard, SD: Standard deviation, RSD%: Percent relative standard deviation, RE%: Percent relative error

Specificity and selectivity

Method for plasma demonstrated excellent chromatographic specificity with no endogenous interference at the retention times of the IS and DS (2.6 and 6.2 min, respectively). Representative chromatograms for human plasma spiked with DS (800 ng/mL) and the IS (3000 ng/mL) are shown in Figure 2 and Figure 3. The most appropriate wavelength at 281 nm was chosen for UV detection of DS and IS (Figure 4).

Limit of detection and quantification

Limit of detection (LOD) and limit of quantification (LOQ) were determined based on the standard deviation of the response and the slope of the calibration curve, according to ICH guidelines (LOD= 3.3 σ/S, LOQ= 10 σ/S where σ is the standard deviation of the response and S is the slope of the calibration curve) (33,34). LOD and LOQ values were calculated from plasma samples and found as 8.95 ng/mL and 27.12 ng/mL respectively.

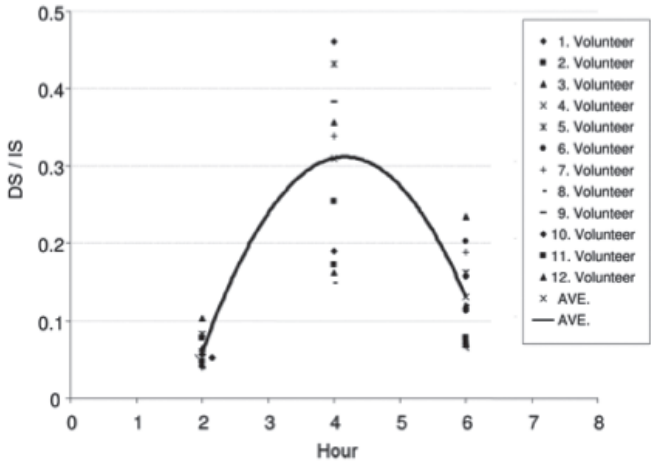

Figure 5. DS levels in plasma of volunteers after administration of Voltaren® SR 75 mg single oral dose

Table 4. Diclofenac sodium levels in 2nd, 4th and 6th times of volunteers plasma (ng/mL)

Number of volunteers	2nd hour	4th hour	6th hour
1	82.28	248.82	153.40
2	70.95	329.18	104.95
3	92.27	213.86	160.60
4	86.10	398.09	92.49
5	114.78	550.89	214.11
6	63.22	658.37	264.44
7	61.74	435.47	246.66
8	60.67	196.24	161.02
9	80.14	490.15	92.74
10	73.99	586.69	208.64
11	109.60	227.44	109.07
12	139.68	456.46	295.23
Mean	86.29	399.31	175.28

Linearity

After establishing the chromatographic conditions, separate calibration curves were prepared for plasma over a DS concentration range of 50-1600 ng/mL. For each concentration 5 individual replicates were injected and linearity was obtained for both methods with the determination coefficients (r^2) over 0.999.

CONCLUSIONS

In this study, simultaneous procedure of HPLC-UV method was proposed with simple extraction of sample yielding good recovery, selective chromatographic separation, and sensitive UV detection with enhanced sensitivity and accuracy of determination for analysis of DS. Therefore, to achieve quality separation of analyte in a reasonable analysis time, acceptable chromatographic factors were adjusted. The mobile phase composition and the pH were optimized. The mobile phase was a phosphate buffer adjusted to acidic pH and containing ACN as the organic modifier. Baseline separation of the analyte (and the IS) was achieved in less than 7 min. The method was validated in terms of linearity, accuracy, precision, reproducibility, quantification and detection limits in accordance with internationally accepted guidelines which are ICH. Analysis for all analytes demonstrates very precise and accurate results, even for inter-day assays which allow determining therapeutic and toxic concentration levels.

The study results showed that, established HPLC-UV method is applicable for the therapeutic drug monitoring bioavailability, bioequivalence study and applicable as a reference method in routine monitoring for toxicological and/or analytic purposes in research laboratories.

Diclofenac sodium levels of volunteers

Although considerable individual differences were observed, representative curves plotted by drug levels of each volunteer were similar. Variations in the DS levels detected in the volunteers were graphed and tabled (Table 4, Figure 5). After ingestion of DS, reaches the maximum level in plasma, at 4th hour. Even though, each volunteer was medicated with equivalent single oral dose, DS levels in plasma were quite dissimilar. Variations may arise from because of enzyme differences which are CYP2C9 and CYP3A4, mainly enzymes responsible for DS metabolism. In this respect, for an application of an effective dosage, the enzyme polymorphism should be taken under consideration in medical therapies.

According to monitored drug levels, differences in concentration rates of DS in human plasma can be expressed and the variation of plasma concentration can be based on hepatic enzyme polymorphism. DS is metabolized mainly by CYP2C9 and CYP3A4. The resulting data may also be considered as a pre-research for pharmacogenetic

polymorphismin this group of volunteers. The analytes were then quantified using HPLC technique, which provided good sensitivity and selectivity. The method has been shown to provide good reproducible recoveries and low limits of detection that allow the accurate quantification of the DS in plasma samples. As the DS level increases in biological material, the expected medical effects also increase. Since, the optimum drug use and maximum resultant effect is the main objective, this report offers clinical application of proposed method for monitorizations of DS in human plasma.

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