

High performance liquid chromatographic determination of diclofenac sodium in plasma of the rat

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Abstract

A rapid and sensitive high performance liquid chromatographic method has been developed for the determination of diclofenac sodium in rat plasma. The assay was performed after liquid-liquid extraction with 1M orthophosphoric acid and a mixture of hexane:isopropyl alcohol. Chromatographic separations were performed on C₁₈ stationary phase with a mobile phase composed of acetonitrile: deionised water: orthophosphoric acid (45:54.5:0.5,v/v) as mobile phase with final pH of (3.5). Analytes were detected at wave length of 276nm. This method was validated for specificity and linearity with a correlation coefficient, $r=0.99$.

Keywords: Liquid chromatographic, Diclofenac sodium, Rat.

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استشراب السائل عالي الاداء لقياس عقار الدايكلوفيناك صوديوم في مصل دم الجرذان

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الخلاصة

يوضح البحث سرعة و حساسية طريقة الاستشراب السائل عالي الاداء المنجزة لقياس تركيز الـ diclofenac sodium في مصل الدم للجرذان. التجربة أنجزت بعد عملية الاستخلاص بواسطة 1 مولر من محلول مادة أورثوفوسفورك أسيد ومحمول الخليط المتكون من مادتي الهكسان و أيزوبروباييل الكحول للدواء من مصل الدم. وقد تم الفصل بواسطة استخدام الطور الثابت من نوع C18 و الطور المتحرك المتكون من المواد التالية أسيتونايتريل:ماء لا أيوني:أورثو فوسفورك أسيد بنسبة 45:54.5:0.5% بمعيارية مقدارها 3.5، وقد قيست العينات على الطول الموجي 276 نانوميتر لتحديد تركيزها. هذه الطريقة أثبتت دقتها و استقامة قياساتها بمعامل ارتباط مقدارها 0.99. وصف في هذا البحث طريقة سريعة و حساسة وهي طريقة الاستشراب السائل عالي الاداء لقياس تركيز دواء الـ diclofenac sodium في مصل الدم للجرذان في المختبر.

Introduction

Diclofenac sodium is a member of the nonsteroidal anti-inflammatory drugs (NSAIDs). It has been shown, in pharmacologic studies, that it has anti-inflammatory, analgesic and antipyretic activities (1). The primary mechanism that is responsible for its action is related to inhibition of cyclooxygenase (COX) enzyme that results in the reduction of prostaglandin synthesis at the site of

inflammation (2). This drug is commonly used for reducing pain, fever and inflammation in humans and animals and it is usually used as an adjunct to antimicrobial therapy in veterinary practice (3,4). The simultaneous measurement of this drug in biological samples is required in therapeutic monitoring, pharmacokinetic and bioavailability studies. Multiple separation and analysis techniques have been developed for the simultaneous and single entity determination of this drug in biological fluids such as

spectrophotometric, fluorometric, potentiometric and thin layer chromatographic methods (5). In this study, the high performance liquid chromatographic method have employed at variable UV detector with no special assembly which proved to be convenient, inexpensive and suitable for routine work, and was successfully applied for the determination of the drug simultaneously in rat plasma samples.

Materials and methods

Chemicals and reagents

Purified free base of diclofenac sodium for research purposes was provided by NDI/Nenava Drug Industry/Iraq.

All solvents used were HPLC grade, and all chemicals were analytical grade: HPLC-grade acetonitrile Scharlau/Spain and deionised water NDI/Iraq.

Analytical grade orthophosphoric acid was from GCC company/UK, hexane was from THOMAS BAAER/UK and isopropyl alcohol was from EVANS/England.

Instrumentation

The analyses were carried out using a chromatographic system from Shimadzu Corporation (Japan) that is available in AL-Kindy state company. This instrument consisted of a pump, a UV-visible detector, a system controller, and a manual injector. Software was used to control the LC system and data acquisition.

Analysis of diclofenac sodium was performed at room temperature on a (GL Sciences Inc.) C₁₈ column (4.6mm × 250mm I.D., 5µm particle size) under isocratic conditions using acetonitrile: deionised water: orthophosphoric acid (45:54.5:0.5,v/v) as a mobile phase with final pH of (3.5) and flow rate of 1.2 mL/min. UV detector was operating at 276nm. The mobile phase was filtered through a millipore membrane filter (0.45µm) (Steril-R/USA) and degassed ultrasonically prior to use.

Preparation of stock solution and working standards

Stock solution of diclofenac sodium was freshly prepared in deionised water at the concentration of (10mg/10mL). Working standards of diclofenac sodium were freshly prepared in the concentrations of (0.05, 0.1, 0.2, 0.4 and 0.8µg/mL) and made by the dilution of the stock solution with mobile phase.

Animals and Sample preparation

Adult albino rats were used in this work that have been taken from animal house of the College of Veterinary Medicine, University of Mosul. This study was carried out on 4 animals (both sexes), their weights were between 250-350 g. The work was done at laboratory of the College of Veterinary Medicine, University of Mosul.

One ml of blood samples were collected from healthy adult rats, not taking any kind of drug, in heparinized glass tubes, then blood samples were collected from each animal after (15, 30, 60min) of administration of therapeutic dose of diclofenac sodium (2.5mg/kg) was given by i.m. route to each animal (6,7).

The blood samples were centrifuged at 3000rpm for 15min and the plasma was frozen and stored at -20°C, no longer than 72h.

Extraction of the samples (liquid-liquid extraction) LLE

To 0.5mL of plasma, was added 0.5mL of 1M orthophosphoric acid, and 2.5mL of a mixture of hexane: isopropyl alcohol (90:10), vortexed and centrifuged at 2000 rpm for 3min. the supernatant was separated and evaporated to dryness. The residue was reconstituted with 100µL of mobile phase and 20µL aliquot of the resulting solution was injected into HPLC (3).

Chromatographic conditions

Several chromatographic conditions, such as mobile phase, type of column and its length, mobile phase pH, flow rate, temperature and volume of injection were studied to obtain a satisfactory chromatographic separation (good resolution and efficacy) for the compound. In addition, the total time required for the analysis was also an important factor because the analysis could be unfeasible since interfering compounds could elute close to the diclofenac sodium, modifications were performed in order to reduce the analysis time.

Various solvents or mixture of solvents at different compositions were used to extract the diclofenac sodium from rat plasma using LLE.

To optimize the HPLC parameters, several mobile phase compositions were tried: Methanol (10), 10mM sodium dihydrogen phosphate buffer: acetonitrile (67:33) (8), and acetonitrile: deionised water: orthophosphoric acid (45:54.5:0.5) (3).

The final mobile phase was the preferred one of the above, its pH was adjusted finally to (3.5) by sodium hydroxide to give the best result of peak elution without any interference of other chemical and endogenous substances.

The wave length was adjusted also to increase and compare the highest sensitivity for eluting the typical peak of the drug, so the detector was first operated at 283nm then at 276nm which expressed the best results compared to the first one.

For extraction, we also tried several methods to choose the best one that expressed the optimal results (the peak of the drug) without interfering with any peak of the chemicals used for extraction and endogenous substances: single step LLE procedure using combination of acetonitrile and trichloroacetic acid TCA (50:50,v/v) as protein

precipitating agent added to the plasma, vortexed, centrifuged and the clear supernatant was separated and injected into HPLC column, and acetonitrile added to the plasma, vortexed, centrifuged and the clear supernatant was separated, mixed with HPLC grade water filtered and injected into HPLC column.

These two methods show high interference of both chemicals and endogenous materials of the plasma with the peak of the drug alone. But the methods used in this work show the best resolution with the best peak shape and low retention time.

Results

A satisfactory separation and good peak symmetry was found in a mixture of acetonitrile: deionised water: orthophosphoric acid in the ratio of 45:54.5:0.5%,v/v at a flow rate of 1.2mL/min. The optimum wavelength for detection was set at 276nm at which much better detector response for drug was obtained. The retention time was 5.6min for diclofenac sodium and no interferences were observed in formulation sample, also with a better reproducibility.

Quantification was achieved with UV detection at 276nm based on the peak area. Better resolution of the peaks with clear base line separation is found as shown in (Table 1) and (Figure 1).

Specificity

The specificity of method was performed by comparing the chromatograms of blank, standard and sample. It was found that there is no endogenous interference and also found good correlation between the retention times of standard and sample as shown in (Table 2), (Figure 2), (Figure 3) and (Figure 4).

Table 1: Optimized chromatographic conditions for estimation of Diclofenac sodium.

Mobile phase	acetonitrile: deionised water: orthophosphoric acid 45:54.5:0.5%,v/v
Pump mode	Isocratic
Diluent	Mobile phase
Column	C18 column (4.6 × 250mm, 5µm)
Column temp	Ambient
Wavelength	276nm
Injection volume	20 µL
Flow rate	1.2mL/min
Run time	10min
Retention time	5.6min

Table 2: Specificity study (retention time of blank, standard and sample).

Name of the solution	Retention time in Min (Rt)
Blank	No peak
standard	5.6
sample	5.6

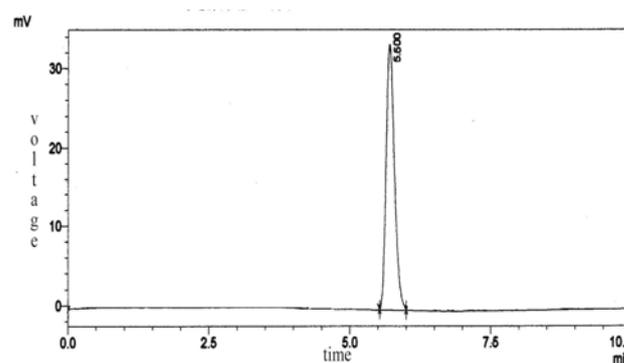


Figure 1: Chromatograms of validation of the method for Diclofenac sodium.

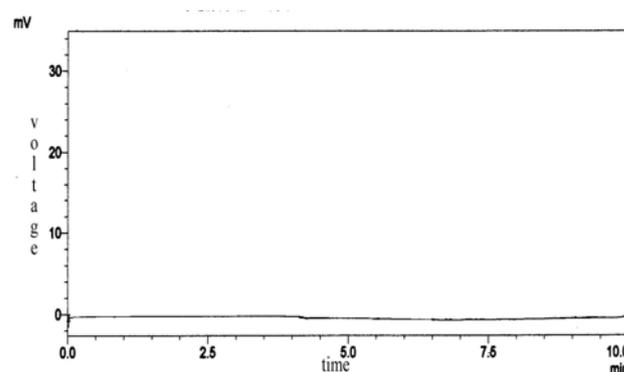


Figure 2: Chromatogram of blank.

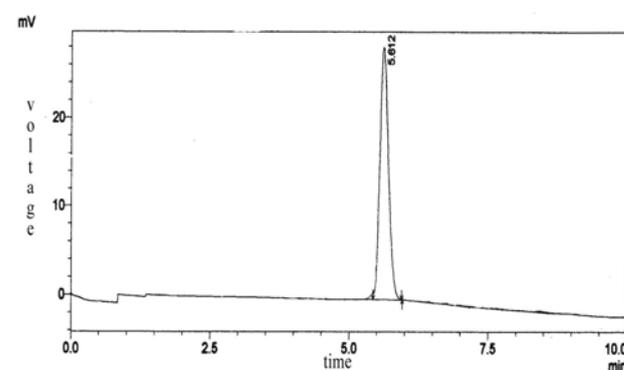


Figure 3: Chromatogram of standard Diclofenac sodium.

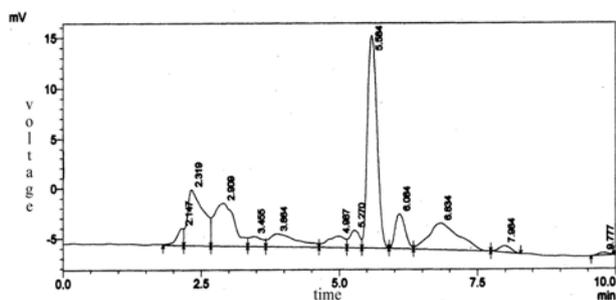


Figure 4: Chromatogram of plasma sample containing Diclofenac sodium.

Linearity

Linearity was performed by preparing standard solutions of Diclofenac sodium at different concentration levels including working concentration mentioned above. Twenty micro liters of each concentration was injected into the HPLC system. The response was read at 276nm and the corresponding chromatograms were recorded. From these chromatograms, the peak areas were calculated and linearity plot of concentrations over the peak areas were constructed. The regression of the plot was computed by least square regression method. Linearity results were presented in (Table 3), calibration plot was shown in (Figure 5) and calibration plots of the samples was shown in (Figure 6).

Table 3: Linearity Results.

Levels	Concentration of Diclofenac sodium in (µg/mL)	Peak area (mV)
1	0.05	28324
2	0.1	77358
3	0.2	143722
4	0.4	283518
5	0.8	512302
Range:0.05 to 0.8		Correlation coefficient 0.997

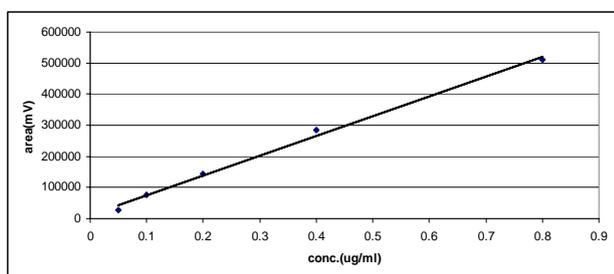
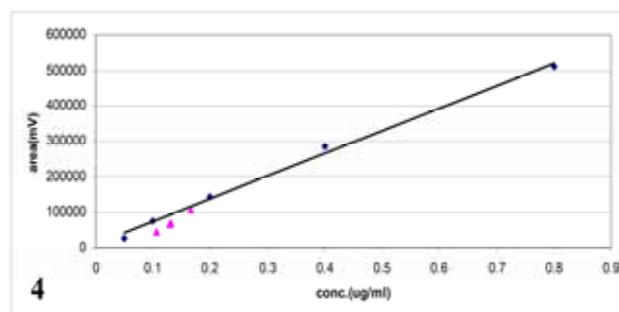
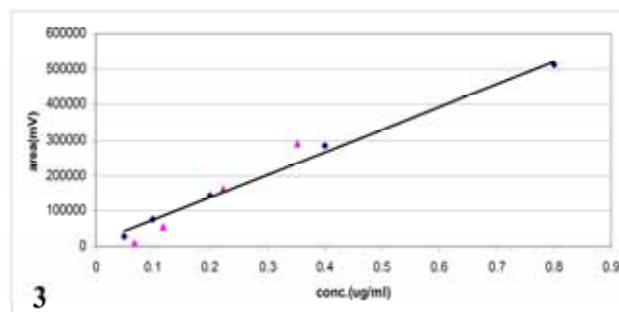
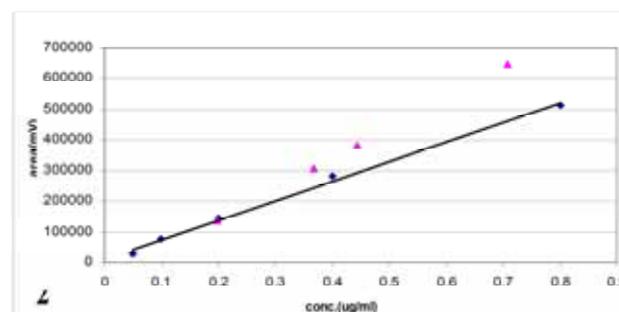
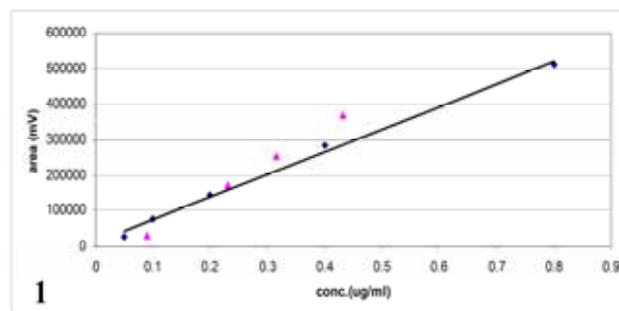


Figure 5: Calibration plot for Diclofenac sodium standards on X axis concentration (µg/mL) and on Y axis peak area (mV).



▲ concentration of samples
■ concentration of standards

(1,2,3,4) the number of animal used

Figure 6: Calibration curves were found to be linear with correlation coefficient (0.997), and Calibration plots for diclofenac sodium plasma concentration-area at (0,15,30,60min) after i.m. administration of the drug in 4 animals.

Discussion

Different techniques have been developed for the determination of diclofenac sodium such as spectrophotometric, fluorometric, potentiometric and Thin layer chromatography are available methods, but they have disadvantages that they are lacking of sensitivity and selectivity, slow, difficult in application and required different conditions for optimal use (8,9). While the HPLC method has been the technique of choice for the separation and determination of diclofenac sodium in the tablet and biological fluids (11,12). Although, several HPLC methods have been described to analyze diclofenac sodium in various human body fluids using different clean-up procedures including direct injection, protein precipitation and liquid-liquid extraction (LLE) with different UV detectors (3) but, some times in both protein precipitation and direct injection procedures, endogenous compounds overloaded the column and resulted in a very noisy baseline which may interfere with the peak of interest or appears as late eluting peaks which consequently lead to long run time.

In the present study, it was of interest to develop a reliable, simple and economical HPLC method using UV detection and LLE for determination of diclofenac sodium in rat plasma with improved sensitivity.

Other studies have been described in the literature for analysis of NSAIDs in human plasma by HPLC (8) who describes the disadvantage of LLE. In our study, increasing the sensitivity of the HPLC for diclofenac sodium assay has been controlled very well. Thus, this work expresses a specific and a precise HPLC method for the determination of diclofenac sodium in the plasma of the rat.

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