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RESEARCH ARTICLE

Residues of Diclofenac Sodium in Rabbit Tissues

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Abstract

Diclofenac sodium is one of the non-steroidal anti-inflammatory drugs (NSAIDs), which is used for treatment of rheumatic diseases and pain relieve.The current study aimed to determine diclofenac sodium residues in different rabbit tissues (liver, muscles, kidneys, spleen, heart and lungs) following its intramuscular injection. Tissues were extracted and diclofenac residues were assayed using high performance liquid chromatography (HPLC). A total of twenty healthy male New Zealand white rabbits were divided into two groups; the first group (n=15) was intramuscularly injected with diclofenac sodium for four successive days at a dose of 1.5mg/kg body weight (BW) twice daily, while the second group (n=5) remained untreated (negative control). The samples were collected at the 1st day, 3rd day, 5thday and 7th day post injection**.** The results showed that diclofenac remained within the detectable limit till the 3rd day post injection in serum (0.0227±0.05 µg/mL) and rabbit issues as liver, heart, lung and spleen (0.043±0.078 µg/g, 0.146 ±0.064 µg/g, 0.043±0.012 µg/g and 0.075±0.035 µg/g, respectively) but it remained in muscles (0.034±0.0603 µg/g) and kidneys (0.0507±0.0146 µg/g) till the 5th day following the last dose of drug administration. In conclusion, rabbits treated with diclofenac must be slaughtered after the 5th day from last dose of repeated administration for complete withdrawal of diclofenac residues from all tissues of treated rabbits to be safe for human consumption.

Keywords: Residues, Diclofenac, Rabbits, HPLC.

Introduction

Rabbit's meat is very important as it is profitable for its dietary properties since it is lean, enriched with protein of high essential value and low in cholesterol content. So, rabbits have an economic importance as its meat is more advantageous than other meat and is used in people nutrition [1].

Non-steroidal anti-inflammatory drugs (NSAIDs) are utilized in food producing animals to relieve the pain either major or minor [2] and has antibacterial effect to improve some quality characteristics of meat [3]. To avoid residues in food producing animals, the member's states make surveillance programs of these residues in the edible parts to be safe for human consumers [4].

Diclofenac is one of the NSAIDs, which has been used in human pharmacy - therapy from a long period. 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 [5]. Diclofenac possesses structural characteristics of arylalkanoic acid agents and displays anti-inflammatory, analgesic and antipyretic properties [6].

Moreover, diclofenac sodium could be used for the treatment of rheumatic diseases, secondary and moderate pain, and used after surgery as analgesia in medicine [7].

The utilization of diclofenac sodium in veterinary medicine is nearly constricted, and

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there isn't much information about diclofenac pharmacokinetics in the different species of animals. Veterinarians used diclofenac in different inflammatory conditions, disorders as result of degenerative post – injury, as well as treatment of lameness in foals, cattle and swine [8, 9].

The misuse of anti-inflammatory drugs has adverse effects: (i) increase the body weight gain in female rabbits, (ii) increase the level of prolactin hormone, (iii) decrease the level of luteinizing hormone and (iv) increase blood sugar levels [10] .

The presence of drug residues in different tissues of treated animals may increase the risk of resistance to other drugs or adverse effects on people consuming meat and/or animal by-products [11]. World Health Organization (WHO) and Food Agriculture Organization (FAO) established maximal residual limits (MRLs) for residues of drugs, pesticides and other chemicals in the relevant tissues of food producing animals to protect and safeguard human health [12].

There is no experimental data available on diclofenac residues in different tissues of treated rabbits. Therefore, the aim of the present work was to assess residues of diclofenac in different tissues (heart, lung, kidney, spleen, muscles and liver) of rabbits injected with diclofenac. Moreover, estimation of the withdrawal time of the drug in rabbit tissues was carried out.

Materials and Methods

Drug and chemicals

Diclofenac sodium was obtained as a solution (2.5%) for injection from Veterinary Pharmaceutical Company, FATRO, Cairo, Egypt. Chemicals used for HPLC were analytical grade including HPLC-grade acetonitrile (Honeywell Co, Germany), deionized water (Millipore, Molsheim, France), analytical grade Ortho-phosphoric acid (GCC Company, UK), Hexane (THOMAS BAAER Company, UK) and isopropyl alcohol (EVANS Company, England).

Experimental design

A total of 20 healthy male New Zealand white rabbits (2-2.5 kg BW) were used in this study. The animals were housed in batteries and provided with a drug-free pelleted diet and ad libitum water for at least 15 days before the study. No clinical abnormalities were observed on rabbits during the experimental period. The rabbits were classified into 2 groups, the first group (n= 15) were injected with diclofenac sodium at a dose of 1.5 mg /kg BW twice per day for 4 consecutive days [13]. The second group (n=5) saved as negative control (they were utilized for making the blank and the spiked samples). The experimental study was approved by the Committee of Animal Welfare and Research Ethics, Faculty of Veterinary Medicine, Zagazig University, Egypt.

Tissue Samples

Three rabbits were slaughtered on the 1st, 3rd, 5th and 7th day after the last dose of drug administration. Samples were collected from blood, heart, lung, liver, muscles and kidneys for determining diclofenac residues.

Analysis of diclofenac residues

Instrumentation and chromatographic conditions

The high performance liquid chromatography device used was Agilent 1200 series (Agilent Technologies, USA) equipped with 4 mobile phase channels, automatic degasser, quaternary gradient pump, auto- sampler, C8 separation column with adjustable controlled heater and Ultra DAD detector.The aforementioned device is controlled through computerized chemo – station software. Liquid chromatography operating conditions included diclofenac injection (20µL), with a flow rate of 1.2µL/min and UV -VIS detector adjustmentat276 nm. The chromatographic separation was performed using a mobile phase of 45% acetonitrile, 54.5% deionized water and 0.5% Ortho -phosphoric acid (v/v), pH 7.0 ± 0.1, at room temperature according to Brunner and Luders [14]. Measurement of residues in the examined samples was performed and determined from area under the curve (AUC), and appeared automatically by the software

Preparation of blank, stock, and working solutions

Blank standard solution of diclofenac sodium (2.5%) was prepared. Individual stock

solution (1000 ppm) of diclofenac sodium was freshly prepared in deionized water at the concentration of 2.5 mg/10mL. Working standard solution, an intermediate solution containing 100 ppm, was prepared from the stock solution by diluting 10 mL stock solution of diclofenac to 10 mL deionized water. This solution was stored at calibrated refrigerator (4°C).

Standard Curve concentration

Diclofenac standard calibration curve was set up at concentrations of 0.125 µg/mL, 0.25 µg/mL, 0.5 µg/mL, 1 µg/mL 2.5 µg/mL, 10 µg/mL and 20 µg/mL.The calibration curve was determined by an equation representing linear- regression - method as y = 47.283x – 1.56 where Y symbol indicated the area under peak and X symbol indicated diclofenac concentration. Linearity existed ranged between 0.125 and 20 µg/mL with a correlation co- efficient r2 = 0.99999 (Table 1and Figure 1). The percentage recovery of diclofenac ranged from 95-97%, while the retention time of diclofenac was 15.76 min (Figure 2).

Table 1: Concentrations of diclofenac standard (µg/mL) and their corresponding peak response automatically using HPLC chromatogram system

Retention time (Minutes)	Number of standards	Area under peak	Concentration (µg/mL)
	1	6.553	0.125
	2	9.859	0.250
	3	18.798	0.500
15.76	4	35.030	1.000
	5	177.000	5.000
	6	350.300	10.000
	7	701.530	20.000

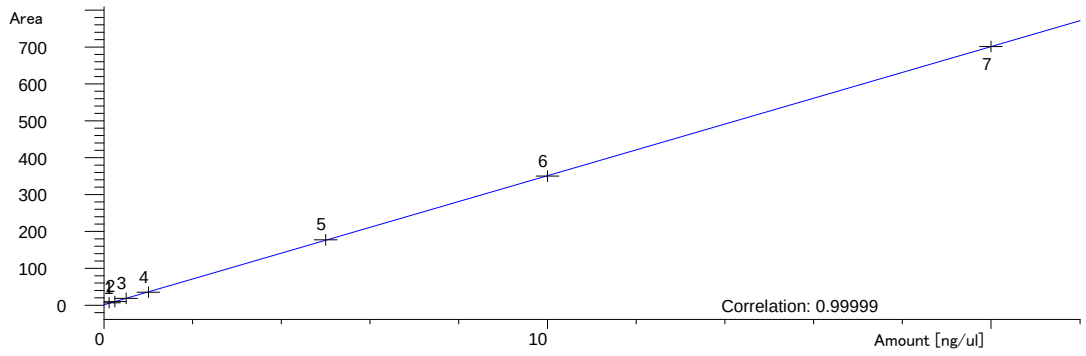

Figure 1: Standard curve of diclofenac automatically using HPLC chromatogram system.

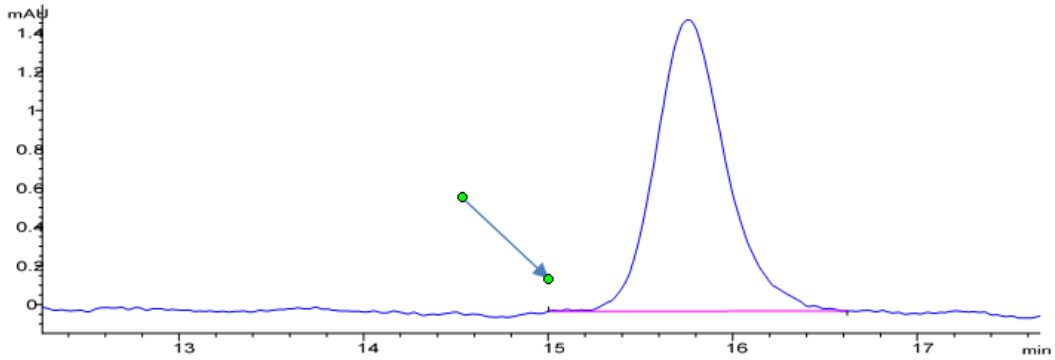

Figure 2: Chromatograms of diclofenac pure standard (2 µg/mL) determined automatically using HPLC chromatogram system with a retention time 15.76 minutes

Extraction and preparation of samples

Preparation of serum

In a sterile Wasserman tube, 3-5 mL of blood was collected from the ear vein without an anticoagulant. The sample was allowed to coagulate, and then the serum was separated by centrifugation at 3000 rpm for 10 minutes and stored at -20 °C in sterile Eppendorf tubes until used for estimation of diclofenac residues [15]

Assay of serum samples

Diclofenac residues were assayed in the serum by HPLC method as described previously [16]. Diclofenac was extracted from rabbit serum using liquid – liquid extraction method; 0.5mL of serum was added to 0.5 mL of 1 M Ortho-phosphoric acid and 2.5 mL of a mixture of Hexane to Isopropyl alcohol [90:10], vortexed then centrifuged at 2000 rpm for 3 min. The supernatant (upper layer) was separated and evaporated to dryness. Residue was reconstituted with 100 µL of mobile phase; then 20µL aliquot of the resulting solution was injected into HPLC.

Assay of tissue samples

The extraction of diclofenac from rabbit tissues was carried out as described previously [17]. The samples (2g) of well minced tissue were added to 4 mL of 2.5 M Ortho-phosphoric acid, then diclofenac was extracted three times by adding 5 mL Hexane to Isopropyl alcohol (90 : 10) . The sample was vortexed for fifteen min, then centrifuged for 15 min at 3.600 ×g. The collected organic phases were dissipated using liquid nitrogen at 45 ºC using 100 µL of mobile phase solution and the residue was reconstituted. The sample was vortexed for 1 min and infused into the chromatographic system (HPLC).

Validation method

The methods of analysis of diclofenac was tested for accuracy and sensitivity by determination of limit of detection (LOD) and limit of quantification LOQ as described previously [18]. The value of LOD in the analysis method of diclofenac concentration was 0.0034 µg/mL, while that of the LOQ was 0.0011µg/mL.

Results and Discussion

Diclofenac distribution in both serum and tissues of normal healthy rabbits after intramuscular administration (1.5 mg/kg BW/ 4 days) was examined and the obtained results are presented in Table 2**.** The data emphasized a widespread distribution of the drug in the examined samples (kidneys, spleen, liver, muscles, serum, heart and lungs). The diclofenac concentrations in tissue were 0.675 ± 0.084 µg/g, 0.513 ± 0.093 µg/g, 0.473 ± 0.038 µg/g, 0.250 ± 0.086 µg/g, 0.210 ± 0.057 µg/g, 0.403 ± 0.096 µg/g and 0.061 ± 0.028 µg/mL, on the 1st day post administration in liver, kidney, muscles, lung, spleen, heart and serum, respectively. Diclofenac remained detectable till the 3rd day in most examined tissues, except muscle (0.034± 0.0603 µg/g) and kidney (0.0507± 0.0146 µg/g), it persisted till the 5th day post treatment.

Table 2: Diclofenac concentrations in tissues of rabbits on various intervals post-treatment with 1.5 mg/kg BW twice daily for 4 consecutive days automatically using HPLC (Mean ± SE) (n=3)

Tissues (µg/g)	Time (day)			
	1st	3rd	5th	7th
Serum	0.061 ± 0.028	0.0227 ± 0.05	N.D	N.D
Muscle	0.473 ± 0.038	0.113 ± 0.086	0.034± 0.0603	N.D
Liver	0.675 ± 0.084	0.043 ± 0.078	N.D	N.D
Kidney	0.513 ± 0.093	0.160 ± 0.065	0.0507± 0.0146	N.D
Lung	0.25 ± 0.086	0.043 ± 0.012	N.D	N.D
Heart	0.403 ± 0.096	0.146 ± 0.064	N.D	N.D
Spleen	0.210 ± 0.057	0.075 ± 0.035	N.D	N.D

N.D non detectable

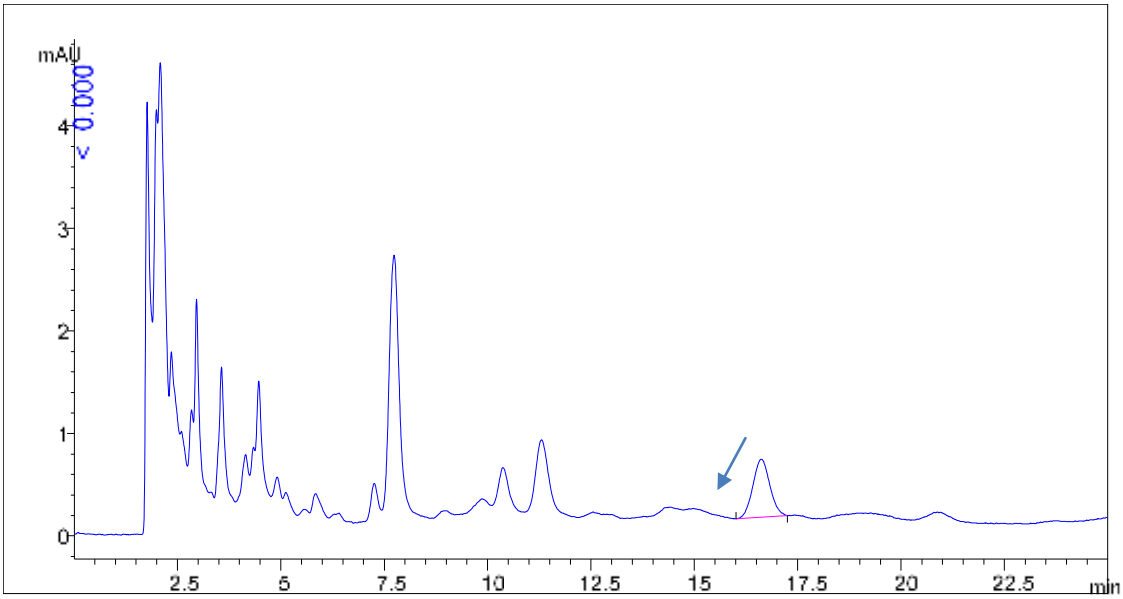

Figure 3: Chromatograms of diclofenac extract of rabbit muscle sample post the last dose (1.5 mg/kg BW twice daily) determined automatically using HPLC chromatogram system with a retention time 15.76 minutes .

High concentration of drug residues in edible animal tissues is a consequence of extra-label use of drugs or non-compliance withdrawal period [19]. In most cases, diclofenac residue cause renal failure, visceral gout and cytotoxicity by excessive metabolizing of drug [20].

The present investigation revealed that muscles, liver and kidneys contained the highest drug residue concentrations, while the lowest concentrations were found in spleen and heart on the 1st day after the last dose administration. Similarly in a previous study, the diclofenac residue was detected in pig tissues using HPLC at 15, 72, and 120 h after the administration of diclofenac sodium intramuscularly. At 15 h after administration, diclofenac was detected with high concentration in the kidney (0.614 mg / kg), while its concentration decreased in the liver (0.316 mg /kg). Diclofenac concentration at the injection site was 0.432 mg/kg [21].

Our result agreed with another study [22], in which the high concentrations of diclofenac were detected in liver followed by kidneys, while the low concentrations appeared in the lungs and heart. As this drug shows a high percentage of plasma protein binding, its elimination through the hepatic metabolism and urinary excretion are mainly dependent on the plasma concentration of the free drug.

The obtained result showed that residues in the liver remained detected till the 3rd day post treatment. This result similar to a previous study in cow [23] in which, diclofenac was identified in the liver as long as 71 h after medication (a single intramuscular dose of

diclofenac; 1000 µg/ Kg BW), while in plasma, the half-life was 12.2 h.

It could be assumed that all out diclofenac clearance get in rabbits is primarily through hepatic excretion as the highest concentration was in liver on 1st day post last dose of drug administration, while in humans, diclofenac was excreted mainly by hepatic metabolism [24-26]

Serum diclofenac concentration declined quickly in pigs following intramuscular administration of a single dose of diclofenac-sodium (2.5 mg/kg BW). Diclofenac serum concentrations were determined by HPLC. Maximum serum concentration (Cmax) of the drug in blood was less than 10% after 6-9 h, but still detectable after 24 h post dosing. The short elimination half-life (1.67 h) demonstrated fast clearance of diclofenac from the plasma because of diclofenac is characterized by quick distribution and excessive digestion in pig's body [27].

According to the European Medicines Agency (EME), the established MRL of diclofenac is 5µg/kg for muscle and liver and 10µg/kg for kidney. In the current study, it has been found that diclofenac residues in muscles and kidneys after the last injection were above the MRLs on the 5th day post injection. Also, diclofenac value in liver was above MRL on 3rd day post administration. The results showed that withdrawal time of diclofenac was five days after the last dose administration. The obtained results are supported by a previous study [28] in which the withdrawal time of diclofenac was 120 h in all tissues of pigs. Conversely, in another study, after diclofenac administration of single dose in pig (2.5 mg / kg per day intramuscularly for continuous 3 days), the withdrawal time in the tissues of liver, kidney, serum, muscles and injection site were 9.892, 5.116, 14.205, 5.444 and 8.818 days, respectively. According to the double-sided 95% confidence interval, diclofenac withdrawal period should be 15 days [29].

However, diclofenac Sodium was eliminated rapidly from sheep blood [30] with a terminal T1/ 2λ of 2: 3 h for two routes of administration intramuscularly and intravenously. Total diclofenac clearance after intravenous and intramuscular administration was 87.86 ± 24.10 and 85.69 ± 40.76 mL/ kg/h, respectively. The bioavailability of diclofenac absolutely appeared around 100%.

Conclusion

After intramuscular injection of diclofenac to rabbits at a dose of 1.5 mg / kg twice daily for 4 consecutive days, diclofenac remained within detectable limit in liver till the 3 $^{rd.}$ day and continued to 5th day in muscles and kidney. Rabbits treated with diclofenac must not be slaughtered before the 5th day to withdraw the drug residues from all tissues of treated rabbits to be safe for human consumption.

Conflict of interest

The authors have no conflict of interest to declare.

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الملخص العربي

بقايا الديكلوفيناك صوديوم فى أنسجة الارنب

جمال الدين امين شمس [١] ,نجاح السيد محمد عمر[١], الهام أحمد مبارز [٢] و سارة عبدالله عبدالوهاب *[٣]
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الديكلوفيناك واحد من مضادات الالتهاب التى تستخدم فى علاج الامراض الروماتيزميه وكذلك يستخدم كمسكن للالام. استهدفت هذه الدراسة قياس بقايا الديكلوفيناك فى الأنسجة المختلفة للأرنب (الكبد ,العضلات ,الكلي, الطحال, القلب والرئه) بعد الحقن العضلى للدواء تم استخلاص الانسجه وقياس بقايا الدواء عن طريق استخدام جهاز الفصل الكروماتوجرافى السائل العالى الاداء. تم استخدام عدد عشرون ذكر أرانب من النوع النيوزلاندى وتقسيمهم الى مجموعتين : المجموعة الاولى وتتكون من خمسه عشر أرنبا تم حقنهم بالديكلوفيناك صوديوم بجرعة ١.٥مجم/كجم من وزن الارنب مرتين يوميا لمدة أربعه أيام متتالية , المجموعة الثانية عبارة عن خمسة أرانب غير معالجين تم استخدامهم كمجموعة ضابطه . تم تجميع العينات عند اليوم الاول , الثالث ,الخامس و السابع بعد أخر جرعة دواء . اظهرت النتائج أن الديكلوفيناك ظل مستمرا حتى اليوم الثالث بعد اخر جرعة دواء فى المصل (٠.٠٢٢٧ ± ٠.٠٥ ميكروجرام/مللى) وكذلك فى الكبد ,القلب ,الرئة والطحال (٠.٠٤٣ ± ٠.٠٧٨, ٠.١٤٦ ± ٠.٠٦٤, ٠.٠٤٣ ±٠.٠١٢, ٠.٠٧٥ ± ٠.٠٣٥ ميكروجرام /جرام على التوالى) بينما ظل متواجدا فى العضلات والكلى حتى اليوم الخامس من اخر جرعة دواء (٠.٠٣٤ ±٠.٠٦٠٣ و ٠.٠٥٠٧ ± ٠.٠١٤٦ ميكروجرام /جرام على التوالى) . لذلك ننصح بذبح الارانب التى تم معالجتها بالديكلوفيناك بعد اليوم الخامس من اعطاء الجرعة النهائية للدواء حتى يتم سحب الدواء نهائيا من انسجه الارنب المعالجه لتصبح صالحة للاستهلاك الادمى .