Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org
Original Research

# Pharmacokinetics and residues elimination of diclofenac sodium administration in pigs by a new HPLC/MS method

Yongjun Li[1], Haifeng Yang[1], Yaqin Wang[2], Shijin Bu[2,*]

[1] The School of Animal Pharmaceuticals, Jiangsu Agri-animal Husbandry Vocational College, Taizhou 225300, China
[2] Veterinary Medicine College / Jiangsu Co-innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonoses, Yangzhou Univercity, Yangzhou 225009, China

**Correspondence to:** bu_shijin810@yeah.net

**Received** February 8, 2018; **Accepted** January 7, 2019; **Published** January 31, 2019

**Doi:** http://dx.doi.org/10.14715/cmb/2018.65.1.7



**Abstract:** Diclofenac sodium (DS) was the third generation non-steroidal drugs with analgesic and antipyretic properties. Owing to taking action faster, long lasting potency, good efficacy and low side effects, DS was widely used in the pharmaceutical industry. To further ensure animal food safety and consumer health, the rational usages regulations of DS and DS withdrawal time should be provided. In the present study, a new high performance liquid chromatography tandem mass spectrometry (HPLC/MS) method was first established for extracting and validating diclofenac sodium levels in edible porcine tissues. Meanwhile, the pharmacokinetics characteristics and residue elimination of intramuscular DS administration in pigs were also studied. We found DS eliminated quickly and the distribution was poor *in vivo*. After a single dose of 2.5 mg/kg body weight per day for continuous 3 days, the withdrawal time in the tissues of liver, kidney, sebum, muscle and administration site were 9.892 days, 5.116 days, 14.205 days, 5.444 days and 8.818 days, respectively. According to the double-sided 95% confidence interval, DS withdrawal period should be 15 days. These experiment evidences lay a good foundation on the rational usages regulations of DS and DS withdrawal time, which will be helpful for the animal food safety and consumer health.

***Key words:*** Diclofenac sodium; Pharmacokinetics; Pig; Residue elimination, HPLC/MS.

## Introduction

Non-steroidal anti-inflammatory drugs were known as its antipyretic, analgesic, anti-inflammatory. Their chemical structures were similar with the chemical structure of adrenal cortex hormones without steroidal ring. They had nearly 100 years history since salicylic acid drugs used in clinical practice. With the rapid development of anti-inflammatory drugs, non-steroidal anti-inflammatory drugs have become the world's largest prescription drugs (1). Diclofenac (DF) was a classical non-steroidal, anti-inflammatory and an inhibitor of cyclooxygenase, which has been used in human pharmacotherapy for many years (2-4). Diclofenac inhibits prostaglandin biosynthesis and it could reduce leukotriene formation through promoting arachidonic acid binding triglyceride. These properties may contribute to its anti-inflammatory activity (5). Meanwhile, the diclofenac sodium (DS) could also prevent the process of arachidonic acid into prostaglandins by inhibiting cyclooxygenase activity. Furthermore, the drug has a short elimination half-life in most species, including humans; however, it accumulates at the site of inflammation, where its concentrations were higher than that in non-inflamed tissues, and similar to those achieved in plasma (2). The pharmacokinetics of DF has been well documented in humans and laboratory animals, but there is not much data in domestic pigs.

DF usage was limited in veterinary medicine, and there was a little data on the pharmacokinetics of DF in target animal species. In veterinary practice, DF was indicated and suitable for treatment of various inflammatory, degenerative post-trauma disorders and lameness in horses, cattle and pigs, as well as pre-operative treatment for cataract extraction (6, 7). Among them, the pharmacokinetics and metabolism have been studied through major ways of oral and intravenous administration. Meanwhile, the metabolism of diclofenac was also highly species dependent.

Though DS was convenient for clinical use, however, it was easy to cause veterinary drug residue without reasonable usage (8). In previous study (9, 10), the bad effects of DF in water on different organisms have been proved. For example, DF can change the fish's gills biological function (11) and can result in the death of zebra fish embryos (12). On the other hand, the determination methods of residual DF were chromatographic methods, and spectrophotometers and capillary electrophoresis. These methods were always expensive and low sensitivity, and the sample pretreatment was complex and time-consuming.

In order to provide the basis withdrawal period in clinical usage of pigs, the new DS from Yantai Green Leaf Animal Health Products Co., Ltd. was used for residue elimination experiments. In present study, the purpose was to estimate the pharmacokinetics and residual quantity of DS after a single therapeutic dose of intramuscular administration of 2.5 mg/kg body weight per day for continuous 3 days. These experiment evidences would lay a good foundation on the rational usages regulations

of DS and DS withdrawal time, which will be helpful for the animal food safety and consumer health.

## Materials and Methods

### Chemicals and DS administration in pigs

Diclofenac sodium administration (cat number was 121012) was provided by the Yantai Green Leaf Animal Health Products Co., Ltd. The control administration sample (cat number was 100334-200302) and naproxen (positive control, cat number was 100198-201205) were from China Pharmaceutical and Biological Products Institute.

27 healthy Yorkshire pigs (about 50 kg) were selected randomly and were used for experiments two weeks later after moving to new environment. The animals were kept on a farm, and the feed and water did not contain non-steroidal and anti-bacterial drug. Food was restricted 12 hours before and 4 hours after drug administration, and water 1 hours before and 2 hours after drug administration. With exception of these intervals, food and water were available at any time. Among 27 pigs, 25 were as experiment group, two pigs were used as control group. 25 health York white pigs with mean body weight of 50 kg received 3 consecutive intramuscular administration of 2.5 mg/kg body weight DS in the neck once a day.

### Sample collection and extraction

The pigs were sacrificed at 12 hours, 3 days, 5 days, 7 days and 12 days after intramuscular administration and related samples (muscle, sebum, liver, kidney and administration position) were collected at the same time as follows. After euthanatizing the pigs, muscle (from leg muscle, about 400 ~ 500 g), sebum (from skin fat, about 200 ~ 300 g), kidneys (from two longitudinal kidneys), liver (from the whole liver) and intramuscular administration samples (from the last administration of the administration point, a 10 × 10cm square and 6cm deep of the muscle) were collected from every pig.

For samples extraction, after being minced and homogenized, 1.0 g ± 0.1 g above various samples were first added into 10 μL internal standard solution (500 ng/mL) and 3 ml physiological saline, and been vortex for 10 seconds. Secondly, 5 mL ethyl acetate were added and been vortex for 3 minutes. Thirdly, the supernatant was transferred to another clean tube after centrifuged at 3500 rpm for 10 minutes, and then it was evaporated through dryness under a stream of nitrogen in a water bath at 50°C. Then, the residue sample was dissolved in 400 μl methanol for 1 min and centrifuged again at 13000 rpm for 10 minutes. In addition, the supernatant was dried at 40°C and the residue was rinsed with 200 μl mobile phase and centrifuged at 13,000 rpm for 10 min. Finally, the supernatant were transfer into the auto sample tube and was used as the mobile phase for high performance liquid chromatography (HPLC). Pharmacokinetic parameters were obtained by DAS2.1.1 pharmacokinetic analysis software.

### High performance liquid chromatography tandem mass spectrometry (HPLC/MS)

The chromatographic separation was performed using the Waters Xterra RP C18 column (150 x 2.1 mm, I.D. 3.5μm), and UV detector set at 226 nm. The mobile phase consisted of acetonitrile and 0.2 mM ammonium acetate solution (70: 30, volume / volume) and its rate was 0.2 mL/min.

The dilution solutions of serial standard working were added to the extraction of different blank tissues, and also been then dried. The last standard working fluid concentrations were reconstituted into 0.5, 1.0, 2.0, 5.0, 10, 20, 50 and 100 ng/ml. Meanwhile, naproxen (5 ng/ml) was used as the internal standard and analyzed by HPLC/MS. For the matrix matching standard curve, the ordinate was the ratio of characteristic ion mass chromatogram peak area and internal standard characteristic ion chromatographic peak area, and the abscissa was the corresponding matrix matching standard solution concentration. Each concentration was repeated four times. The standard curve regression equation and the correlation coefficient were also calculated and provided.

For the limits of detection (LOD) and limits of quantification (LOQ) experiments, a serial dilution standard working solution were added into the blank muscle, sebum, kidney and liver samples, and every group repeated 5 times in parallel. Meanwhile, we defined that the LOD is the concentration of detection limit when noise ratio (the signal / baseline noise value) was more than 3; The LOQ was the concentration when noise ratio (the signal / baseline noise value) was more than 10.

To study the accuracy and residua of DS, the concentration of 0.5, 5, 50 μg/kg DS were added into liver, kidney, muscle and sebum samples. Experiments were repeated three times in consecutive 3 days and every concentration group has 5 parallel samples. The batch and inter-batch precision were calculated and the recovery rate was calculated.

### The residues elimination of DS administration in pigs and statistics analysis

DF in edible tissues of pigs were extracted with ethyl acetate and determined by HPLC/MS method. The residues rate was equal to the detecting quantity (concentration of DS * volume of dissolving DS) / the DS quantity in different tissues. Among them, the concentration of DS could be calculated by the following formulate (Among them, A represented peak area of graph, C represented concentration, subscripts represented DS, subscript represented internal standard, x represented the residual quality of DS in samples, V represented the volume of DS when dissolving the samples, and m represented the quality of samples):

$$\frac{A_S}{A'_S} = a\frac{C_S}{C'_S} + b \text{ and } X = \frac{V}{m}$$

The data were analyzed with WT1.4 program. All data were presented as mean ± SD with a minimum of three independent experiments, and the differences between groups were evaluated using SPSS version 18.0 (USA). Significance was determined using a Student t test or one-way analysis of variance (Tukey test). Pearson correlation analysis and simple linear regression analysis was also performed in SPSS version 18.0 (SPSS, IL, USA) and Microsoft Office Excel 2007 (USA). Statistical significance was set at $P < 0.05$. Sta-

tistical extremely significance was set at $P < 0.01$.

## Results

### Standard curves by HPLC/MS

To extract and validate diclofenac sodium levels in edible tissues of pigs, a high performance liquid chromatography tandem mass spectrometry (HPLC/MS) method was first established. The peaks of DS and internal standard were narrow, sharp and symmetrical. All samples were separated and unique, which were consistent with the components of the biological material (Figure 1). The retention times for DF and the internal standard were about 3.2 and 2.3 minutes respectively, and total analysis time was about 4 minutes.

The concentration of diclofenac (0.5, 1.0, 2.0, 5.0, 10, 20, 50 and 100 ng/mL) showed a good liner relation with corresponding area in various tissues, which the linear correlation coefficients was more than 0.99 (Figure 2A, 2B, 2C and 2D). The limits of detection (LOD) and the limit quantification (LOQ) in edible tissues of pigs were 0.1μg/kg and 0.5μg/kg, respectively.

### Recovery experiments of DS

To measure the recovery rate, various concentrations of DS (0.5 μg/kg, 5 μg/kg and 50 μg/kg) were added in the muscle, sebum, liver and kidney tissues, respectively. After intramuscular administration, the drug was absorbed rapidly and efficiently. Mean recoveries of diclofenac in porcine tissues (muscle, liver, kidney and serum tissues) were from 70% to 120% at DS levels of 0.5, 5.50 μg/kg (Figure 3A), variation coefficients was less than 10% both in-day and between-day (Figure 3B).

### DS pharmacokinetic characteristics and residues elimination after intramuscular administration

After a single dose of DS intramuscular administration per day for 3 continuous days, the results indicated that DS was eliminated quickly. The results of residues elimination experiments showed that DS residues quantity in descending order was administration position, liver, sebum, muscle and kidney after 12 days since the last administration, and their residues quantity was 165.661 ± 21.509 μg/kg, 48.067 ± 14.117 μg/kg, 46.269 ± 23.135 μg/kg, 35.127 ± 14.720 μg/kg and 29.164 ± 7.064 μg/kg, respectively.

The above results showed that except for the administration site, the residual quantity of DS was highest in liver and lowest in kidney after 12 hours. After 5 days, the residual of DS in five porcine muscle tissues were all below the LOQ. The residues of DS in four porcine sebum tissues were all below the LOQ after 7 days, but it was still above the LOQ in both liver and kidney samples (Figure 4).

### Withdrawal period of DS in different tissues

In order to protect the consumer health, the European Union released the maximum residue limit (MRLs) standard of diclofenac in pig and cattle tissues in 2003 (13), and the MRLs in the pig's edible tissues were 5 μg/kg in the liver, 10 μg/kg in the kidney, 5 μg/kg in muscle, 1 μg/g in sebum. According to this standard, the experimental data of this experiment was fitted with WT1.4 calculation software. The results showed that

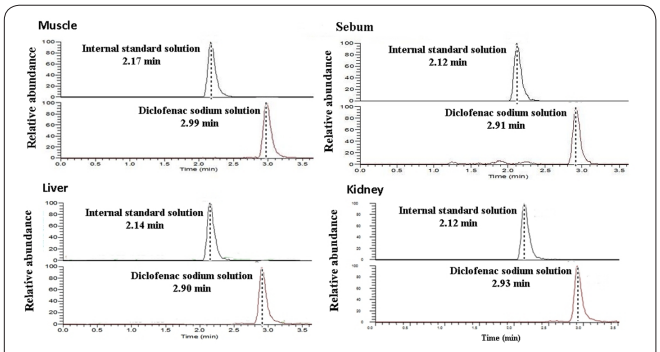


**Figure 1.** Total ion chromatogram of internal standard (upper) and diclofenac standard solution (down) in muscle, sebum, liver and kidney samples. X-axis represents time (minutes) and Y-axis represents relative abundance.

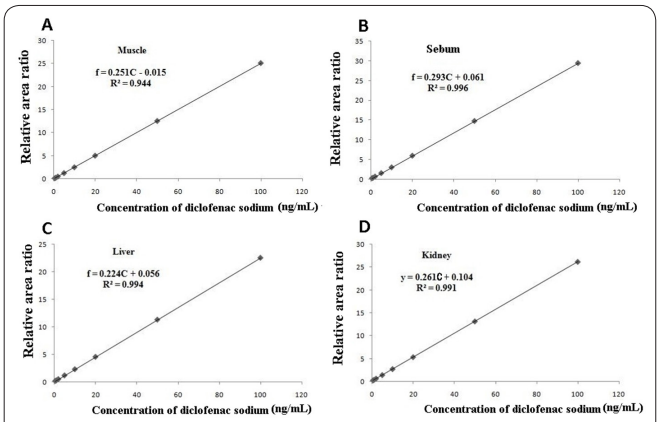


**Figure 2.** Standard curve regression equations in the four tissues; A: muscle tissue; B: sebum tissue; C: liver tissue; D: kidney tissue. X-axis represents the concentration of diclofenac sodium (0.5, 1.0, 2.0, 5.0, 10, 20, 50 and 100ng/ml) and Y-axis represents the corresponding area ratio of DS and internal standard solution in various tissues.

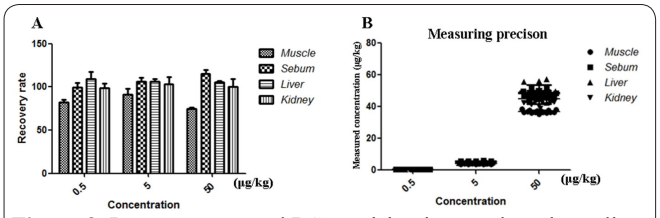


**Figure 3.** Recovery rate and DS precision in muscle, sebum, liver and kidney samples. (A) Recovery rate of DS in muscle, sebum, liver and kidney samples. (B) Measuring precision of DS in muscle, sebum, liver and kidney samples.

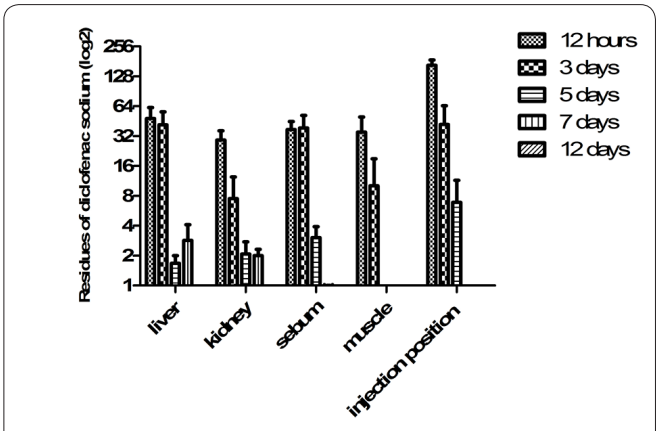


**Figure 4.** Residual quantities of DS in muscle, sebum, liver and kidney samples at 12 hours, 3 days, 5 days, 7 days and 12 days after DS intramuscular administration.

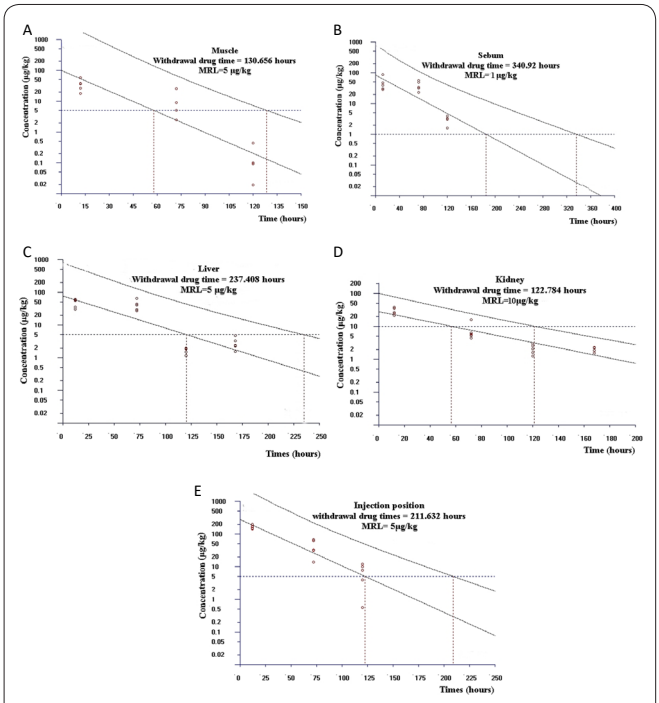


**Figure 5.** Withdrawal period fitting results of residual DS in muscle, sebum, liver, kidney and administration position.

withdrawal period of DS were 5.444 days in muscle, 14.205 days in sebum, 9.892 days in liver, 5.116 days in kidney and 8.818 days in administration position (Figure 5A, 5B, 5C, 5D, 5E ).

According to the double-sided 95% confidence interval calculation, when DS administration in pigs by 2.5 mg/kg body weight intramuscular administration, the proposed withdrawal period was 15 days.

## Discussion

DS was a potent non-steroidal and anti-inflammatory drug, which had antipyretic, analgesic and anti-inflammatory effects (14). It was suitable for porcine acute pain and fever symptoms which caused by operation and other factors. To extract and validate DS levels in edible tissues of pigs, a high performance liquid chromatography tandem mass spectrometry (HPLC/MS) method was first established. Our results showed that the LOD and LOQ in edible tissues of pigs were 0.1 μg/kg and 0.5 μg/kg, respectively (Figure 1 and Figure 2). The method complied with the requirements for diclofenac residue analysis.

On the one hand, some findings indicated that neck musculature administration may lead to greater variations in drugs' absorption, compared to the administration into the gluteus muscle (15). However, in present study, the neck musculature was chosen for drug administration, regardless of the fact that it might increase inter-individual variations in drug absorption, since this administration site was the most commonly used in practice when administering drugs to pigs. In addition, the pharmacokinetic of 5% DS administration were studied with cross-over experiment design. The pharmacokinetic parameters were obtained for each individual pig, and then combined to derive mean pharmacokinetic parameters. Owing to these protocols, the experiment data has no large deviation between the measured residual data of 5 pigs at the same time point of the same tissue samples, and there was no big variation in DS drugs' absorption among all samples (Figure 3B).

Residues DS decline rapidly in Figure 4, as well as visual inspection from the concentration times curves in Figure 5. These phenomena indicated a rapid distribution of DS were existed after intramuscular DS administration at the single dose of 2.5 mg/kg body weight per one times in consecutive 3 days. In administration positions, the DS residues quality at 3 day was absorbed quickly, and residues quality in 3 pigs (from total 5 pigs) levels were lower than the DS MRLs of European Union standard levels after 5 days. In 3 days after intramuscular administration, DS absorption rate in liver and sebum samples was much lower than that in other three samples. When at 7 days, except for sebum, DS residual quantity in all samples was lower than the MRLs of EU standard. In sebum, residual DS was still higher than the DS MRLs of EU standard levels after 5 days, and it was lower than the DS MRLs of EU standard levels until 12 days later after administration (Figure 4). These results showed that DS in muscle and sebum were decreased quickly and DS in liver and kidney were decrease slowly.

On the other hand, the European Union drug administration and European Medical Evaluation Agency reported that 2.5 mg/kg diclofenac was intramuscular administered to pigs for 3 days. Within 12 hours after the discontinuation of the drug, the concentration of diclofenac in the liver tissue of pigs was the highest, which consistent with the results of this study. However, 12 hours after drug withdrawal, the residues of diclofenac sodium in porcine edible tissues were not completely consistent with the results of this study. These differences may be due to the animal species difference, breeding environment, pharmaceutical materials, the administration interval time and other factors.

According to the results of DS pharmacokinetics and residues elimination in pigs, the withdrawal period should be more than 15 days when intramuscular DS administration at the dose of 2.5 mg/kg body weight per one times in consecutive 3 days.

### Ethics approval and consent to participate
The study was approved by the ethic committee. Informed consent was obtained.

### Consent for publication
Not applicable.

### Availability of data and material
Not applicable.

### Competing interests
The authors declare that they have no competing interests.

### Author Contributions
Shijin Bo was resposible for the concept and design, approval of article and manuscript review; Yongjun Li was resposible for the data analysis/interpretation, literature research, manuscript preparation, editing and review; Haifeng Yang and Yaqin Wang were resposible for clinical studies experimental studies and data collection. All authors were aggreed to review and approve

this manuscript.

**Acknowledgements**
This study was supported by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

## References

1. Da Costa BR, Reichenbach S, Keller N, Nartey L, Wandel S, Jüni P, et al. Effectiveness of non-steroidal anti-inflammatory drugs for the treatment of pain in knee and hip osteoarthritis: a network meta-analysis. The Lancet. 2017; 390:e21-e33.
2. Menasse R, Hedwall PR, Kraetz J, Pericin C, Riesterer L, Sallmann A, et al. Pharmacological properties of diclofenac sodium and its metabolites. Scandinavian journal of rheumatology. Supplement. 1978; 5-16.
3. Ku EC, Lee W, Kothari HV, Scholer DW. Effect of diclofenac sodium on the arachidonic acid cascade. The American journal of medicine. 1986; 80:18-23.
4. Riendeau D, Percival MD, Boyce S, Brideau C, Charleson S, Cromlish W, et al. Biochemical and pharmacological profile of a tetrasubstituted furanone as a highly selective COX-2 inhibitor. British journal of pharmacology. 1997; 121:105-117.
5. Kothari HV, Lee WH, Ku EC. An alternate mechanism for regulation of leukotriene production in leukocytes: studies with an anti-inflammatory drug, sodium diclofenac. Biochimica et biophysica acta. 1987; 921:502-511.
6. Altaher AY, Alkharfy KM, Al-Hadiya BM, Khan RM. Pharmacokinetics of diclofenac in sheep following intravenous and intramuscular administration. Veterinary anaesthesia and analgesia. 2006; 33:241-245.
7. Zorica P, Milena P, Milanka J. Pharmacokinetics of diclofenac in pigs after intramuscular administration of a single dose. Acta veterinaria. 2006; 56:323-331.
8. Taggart MA, Senacha KR, Green RE, Jhala YV, Raghavan B, Rahmani AR, et al. Diclofenac residues in carcasses of domestic ungulates available to vultures in India. Environment international. 2007; 33:759-765.
9. Sacher F, Lange FT, Brauch HJ, Blankenhorn I. Pharmaceuticals in groundwaters: analytical methods and results of a monitoring program in Baden-Württemberg, Germany. Journal of chromatography A. 2001; 938:199-210.
10. Kümmerer K. Pharmaceuticals in the environment: sources, fate, effects and risks. Springer Science & Business Media. 2008.
11. Triebskorn R, Casper H, Heyd A, Eikemper R, Köhler HR, Schwaiger J. Toxic effects of the non-steroidal anti-inflammatory drug diclofenac: Part II. Cytological effects in liver, kidney, gills and intestine of rainbow trout (Oncorhynchus mykiss). Aquatic toxicology. 2004; 68:151-166.
12. Dietrich D, Prietz A. Fish embryotoxicity and teratogenicity of pharmaceuticals, detergents and pesticides regularly detected in sewage treatment plant effluents and surface waters. Toxicologist. 1999; 48:151.
13. Wang Z, Li X, Su D, Li Y, Wu L, Wang Y, et al. Residue depletion of imidocarb in Swine tissue. Journal of agricultural and food chemistry. 2009; 57:2324-2328.
14. Munjal S, Gautam A, Okumu F, McDowell J, Allenby K. Safety and pharmacokinetics of single and multiple intravenous bolus doses of diclofenac sodium compared with oral diclofenac potassium 50 mg: A randomized, parallel-group, single-center study in healthy subjects. Journal of clinical pharmacology. 2016; 56:87-95.
Delmas JM, Chapel AM, Gaudin V, Sanders P. Pharmacokinetics of flumequine in sheep after intravenous and intramuscular administration: bioavailability and tissue residue studies. Journal of veterinary pharmacology and therapeutics. 1997; 20:249-257.