Tissue distribution of artemisinin in broiler chickens following single or multiple oral administration

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Abstract:

BACKGROUND: Artemisinin is commonly used for the treatment of malaria, but recently has been considered as a potential substance to control poultry coccidiosis. **OBJECTIVES:** The aim of the present study was to determine the tissue distribution of artemisinin following single or multiple oral administration of different doses in broiler chickens. **METHODS:** A total number of 390 one day old Ross broiler chicks were divided randomly into two main groups, in the first group 0, 1, 5, 25, 125, 250, 500 or 1000 mg/kg artemisinin as a single oral dose was administered on day 44, but the second group were treated with 0, 17, 34, 68 or 136 ppm artemisinin from day 8 to day 44. The HPLC system was used to determine the level of artemisinin in different tissue samples. Data were assessed using one way analysis of variance (ANOVA) followed by the Tukey's test ($p<0.05$). **RESULTS:** Maximum concentrations of artemisinin were found in the liver of chickens in both groups in a dose dependent manner. While, the minimum level was determined in the brain and the kidney of chickens received multiple artemisinin administration; in the spleen of those chickens a single oral dose was administered. The concentration of artemisinin in the brain reached a plateau at 68 ppm in multiple administration and 125mg/kg at single dose, no shift was found with dose increment. **CONCLUSIONS:** It can be concluded that tissue accumulation of artemisinin is time and dose dependent. Moreover, redistribution, saturation effect and tissue selectivity were also observed.

Introduction

Artemisinin, a natural product from *Artemisia annua*, has been used against malaria for decades in parts of the world where the disease is endemic. Artemisinin and its semisynthetic derivatives have also been shown to be anthelminthic, antidiarrheal, and antipyretic. Moreover, it is a cytotoxic agent against tumor cells (Moore et al; 1995; Efferth et al., 1996, 2001). Recently, in veterinary medicine, artemisinin, has been considered as a safe and effective agent to control poultry coccidiosis. Several studies have been carried out to explore anticoccidial effects of artemisinin in chickens (Allen

et al., 1997; Arab et al., 2006; Kaboutari et al., 2014). However, there are few studies to show the toxicity and pharmacokinetic features of artemisinin in different animals including birds. In an attempt to characterize the single dose toxicity of the drug following oral administration, it has been reported that artemisinin is a relatively safe drug that can be used for poultry coccidiosis. There was not any mortality at high doses, but some mild side effects were found in the kidney, the liver and particularly in the nervous system (Arab et al., 2009). Further study showed that multiple administration of high doses of artemisinin in broiler chickens was not effective on different organs including the heart, the lung, and the spleen; but it induced various changes in the liver, the kidney, and the brain. It was found that, while there was not any relationship between the severity of the liver alteration and the drug administration, the severity of lesions in the brain was dose dependent (Shahbazfar et al., 2011).

Toxicological studies conducted on human and a few animal species indicate that artemisinin has high margin of safety, and the pathological effects resulted from high doses of artemisinin in various tissues of different species are ambiguous. It seems that there is a saturation state in the artemisinin concentration in some tissues that cause resistance against toxic effects of artemisinin (Gordi and Lepist, 2004; Efferth and Kaina, 2010).

This study was conducted to determine the tissue distribution of artemisinin in broiler chickens following single or multiple oral administration of the drug in high doses. The study sought to examine the relationship between concentration of artemisinin and toxicological effects induced in different tissues of poultry.

Materials and Methods

Artemisinin administration: Artemisinin, as colorless crystals with the purity of 99%, was obtained from a commercial company in China (Sichuan Arts and Crafts Import and Export Corporation). The drug was dissolved in ethyl alcohol and then diluted by proper volume of water so that the maximum concentration of alcohol would not increase more than 10% of solution. The drug solution prepared for multiple administration was sprayed on feed with complete mixing (each time, 5 kg of feed for each group was prepared). The single doses of artemisinin were administrated to the chickens via gavage.

Animals and experimental groups: A total number of 390 one day old Ross broiler chicks were purchased from a local hatchery and housed in wire cages. The birds had free access to food and water. Light was on 24h a day throughout the study. Chickens were vaccinated against bronchitis, Newcastle disease, and bursal disease. The birds were divided randomly into two main groups on day 8. The first group was treated with a single oral administration of artemisinin. Chickens in the second group received the test compound for a period of 36 days. The birds in group one received a single dose of 0, 1, 5, 25, 125, 250, 500 or 1000 mg/kg artemisinin in 2 ml solution on day 44. The chicks in second group were continuously exposed to 0, 17, 34, 68, or 136 ppm of artemisinin from day 8 to day 44 (a period of 36 days). Each subgroup of doses consists of three replicates (n=10). All experiments were done in accordance with principles of the NIH Guidelines for Care and Use of

Laboratory Animals and approved by the Ethical Committee of the University.

Sample collection: To evaluate the distribution of artemisinin in different organs, animals were euthanized and tissue samples were collected from the liver, the kidney, the brain, the lung and the spleen of birds from both treated groups. Before euthanizing and tissue sampling, each animal was inspected for any gross changes. Animals in the first group were euthanized 1 hour after the administration of artemisinin on day 44, and animals in the second group were euthanized by the end of day 44. Tissue samples were wrapped in an aluminum paper and stored at -20˚C until analysis.

Sample preparation and analysis: The method used to extract artemisinin was based on the procedure described by Rawa-Adkonis et al., 2003 with some modification. One gram tissue was dissolved in 5ml of methanol/10%HCl (60/40, v/v), the solution was sonicated for 1.5 min in 3 cycles, and then centrifuged for 10 min. The supernatant was collected and dissolved again in 5 ml petroleum ether and the artemisinin was extracted and dried using a rotary evaporator at 40˚C. The residue was then dissolved in ethanol and hydrolyzed by NaOH as explained elsewhere (Liersch et al., 1986). The prepared samples were filtered and stored at -20˚C until analysis by HPLC system.

The HPLC analyzing procedure used to determine the amount of artemisinin in tissues was based on the isocratic analytical method described by Liersch et al., 1986 with some modifications. The system consisted of a Knauer 1001 HPLC pump, an h-2600 UV detector, a 10 cm × 4 mm Nucleosil C18 column, and an autosampler. The mobile phase was prepared from 20 mmol/l phosphate buffer (K2HPO4 + KH-2PO4) and methanol (60:40) adjusted at pH 7.9. The volume of each sample automatically injected into the loop of pump was 100 µl at flow rate of 1.5 ml/min.

Method validation of HPLC system: The method validation was conducted according to the guidelines on validation of analytical methods for selectivity, accuracy, precision, linearity, stability, limit of detection and extracted recovery (Shah et al., 1991). A series of standard solutions with concentrations ranging from 0.1 ng/ml to 500 ng/mL were prepared by dilution of artemisinin in methanol. Different blank and spiked blank samples were obtained from various tissues of six broiler chickens to test the selectivity of method. To ensure test accuracy and precision, the homogenate tissues were spiked with 1, 100 or 500 ng/ml artemisinin. The ratio between the results of the analysis of artemisinin in tissue samples and the standard samples dissolved in methanol were considered as acceptable range of accuracy (recycling percentage). To verify the accuracy of the method, each of the above mentioned concentrations was injected five times to the device. Precision of the method was determined by calculation of relative standard deviation (RDS). The RDS of less than 15% and the minimum detectable concentration of less than 20% were used as acceptable limits of accuracy.

The calibration curves were constructed using linear regression analysis based on the area under curve (AUC) of the peak and different known concentrations of the artemisinin. The curves were obtained from a blank sample and eight non - zero spiked samples covering the expected range including lower limit of quantification (LLOQ). The recovery extraction was determined as the

ratio of the peak area of the spiked samples to that of the reference samples prepared in methanol with the same concentrations. The homogenate tissues were spiked with artemisinin at concentrations of 1, 100 and 500 ng/ml, and then extracted according to the above-mentioned extraction procedures. The stability of the Artemisinin in biosamples was assessed at different storage conditions including long term stability, freeze and thaw stability and the room temperature stability. All the samples were analyzed and compared with freshly prepared samples.

Statistical analysis: The statistical analysis of data was performed using SPSS software (SPSS version 19.0 for Windows, Inc., Chicago, IL). Data obtained from analytical tests and in vivo experiments were expressed as Mean ± SEM from at least five (samples for each dose) experiments. The comparison of the mean values obtained from different experimental groups was performed by one way analysis of variance, and was followed by the Tukey's test ($p<0.05$).

Results

Method of analysis validation: Artemisinin was separated well with the retention time of approximately 4.6 min; no interferences were detected from endogenous substances. Figure 1 shows a representative chromatogram of standard artemisinin dissolved in methanol at a concentration of 100 ng/ml, liver tissue sample with no artemisinin (Blank) and liver tissue sample with 50 ng/ml of the artemisinin.

The range of relative recoveries obtained from different tissue samples was 76.4% to 103.98 %. The RDS of the intra-day precision and the inter-day precision were both less than 17.4%. The standard curves

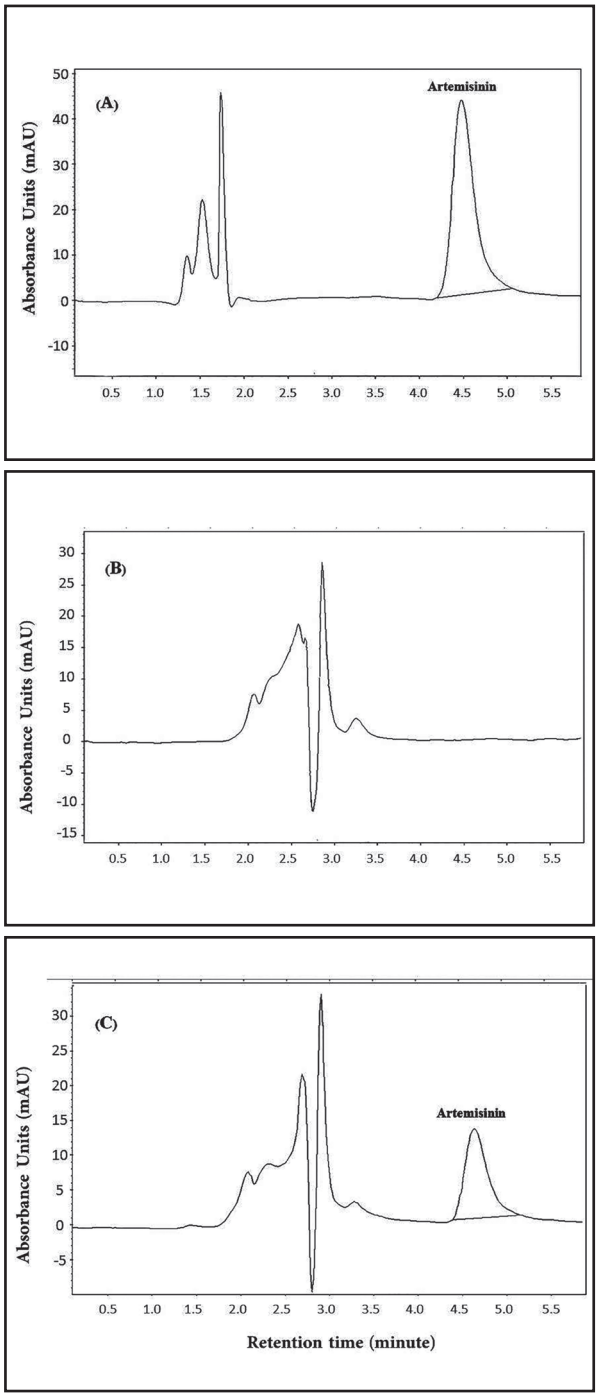

Figure 1. Representative chromatograms obtained from analysis of: A) Standard artemisinin dissolved in methanol at a concentration of 100 ng/mL, B) Liver tissue sample with no artemisinin (Blank), C) Liver tissue sample with 50 ng/mL of the artemisinin.

and correlation coefficients of the artemisinin in all tissue samples are listed in Table 1.The calibration plots of artemisinin were linear over the concentration range of 1- to 500 ng/ml. The values obtained in limits of

Table 1. Calibration curves for artemisinin in various tissues.

Biosamples	Liver	Kidney	Lung	Spleen	Brain
Calibration curves	y = 114.13x + 180.26	y = 96.347x + 486.9	y = 102.33x + 367.05	y = 87.583x + 820.02	y = 118.67x + 331.42
Calibration coefficients (R^2)	R^2 = 0.9976	R^2 = 0.9925	R^2 = 0.9946	R^2 = 0.9914	R^2 = 0.991

Table 2. Chickens Tissues concentration (ng/g tissue) of artemisinin following a single oral administration. The values presented are Mean ± SD ng/g dry tissue. In each row, different English characters show significant differences ($p<0.05$). In each column, different Greek characters show significant differences ($p<0.05$).

Dose / Tissue	1 mg/ kg	5 mg/ kg	25 mg/ kg	125mg/ kg	250 mg/ kg	500 mg/ kg	1000 mg/ kg
Liver	$2.85 \pm 0.96^{a\alpha}$	$3.70 \pm 1.34^{a\alpha\beta}$	$15.49 \pm 2.93^{a\alpha}$	$124.02 \pm 13.80^{b\alpha}$	$177.14 \pm 70.46^{b\alpha}$	$326.81 \pm 80.98^{c\alpha}$	$361.88 \pm 116.78^{c\alpha}$
Brain	$2.65 \pm 0.87^{a\alpha\beta}$	$3.28 \pm 1.01^{a\alpha\beta}$	$19.52 \pm 5.23^{a\alpha}$	$41.39 \pm 8.96^{b\beta}$	$47.34 \pm 12.67^{b\beta}$	$51.71 \pm 9.96^{b\beta\gamma}$	$48.77 \pm 13.26^{b\beta}$
Kidney	$3.13 \pm 1.22^{a\alpha}$	$5.168 \pm 1.86^{a\beta}$	$17.22 \pm 3.44^{ab\alpha}$	$46.81 \pm 8.30^{b\beta\gamma}$	$52.92 \pm 12.77^{b\beta}$	$119.38 \pm 22.17^{c\beta}$	$163.66 \pm 48.85^{d\gamma}$
Lung	$3.88 \pm 0.84^{a\alpha}$	$5.284 \pm 1.90^{a\beta}$	$32.50 \pm 6.49^{b\beta}$	$63.10 \pm 13.11^{c\gamma}$	$60.12 \pm 17.68^{c\beta}$	$52.18 \pm 9.69^{bc\beta\gamma}$	$64.83 \pm 19.35^{c\beta\gamma}$
Spleen	$1.13 \pm 0.25^{a\beta}$	$1.04 \pm 0.37^{a\alpha}$	$2.23 \pm 0.46^{a\gamma}$	$3.3 \pm 0.67^{a\gamma}$	$5.74 \pm 2.12^{a\beta}$	$21.65 \pm 7.14^{b\gamma}$	$23.46 \pm 5.60^{b\beta}$

Table 3. Chickens tissues distribution (ng /g tissue) of artemisinin following chronic oral administration. The values presented are Mean ± SD ng /g dry tissue. In each row, different English characters show significant differences ($p<0.05$). In each column, different Greek characters show significant differences ($p<0.05$).

Dose / Tissue	17 ppm	34 ppm	68 ppm	136 ppm
Liver	$21.24 \pm 4.14^{a\alpha}$	$77.86 \pm 14.54^{b\alpha}$	$71.416 \pm 13.01^{b\alpha}$	$112.23 \pm 23.61^{c\alpha}$
Brain	$12.65 \pm 2.46^{a\beta}$	$21.6 \pm 6.01^{a\beta}$	$44.236 \pm 10.42^{b\beta}$	$44.66 \pm 17.66^{b\beta\gamma}$
Kidney	$6.38 \pm 1.50^{a\beta}$	$34.356 \pm 12.72^{b\beta}$	$38.824 \pm 13.58^{b\beta}$	$29.67 \pm 8.04^{b\gamma}$
Lung	$7.29 \pm 3.19^{a\beta}$	$21.834 \pm 6.87^{a\beta}$	$37.378 \pm 12.28^{b\beta}$	$77.14 \pm 22.30^{c\beta}$
Spleen	$31.77 \pm 7.76^{a\gamma}$	$23.24 \pm 4.88^{a\beta}$	$56.55 \pm 19.31^{b\alpha\beta}$	$71.98 \pm 16.21^{b\beta}$

detection and limits of quantification tests were 0.2 ng/ml and 1 ng/ml, respectively. The level of artemisinin recoveries in liver tissues with three different concentrations (1, 100, 500 ng/ml) was 78.8%, 86.82% and 93.81%, respectively. The artemisinin content of the samples placed in different storage conditions and processed with various procedures was almost identical to those of freshly prepared samples (RSD<12.0%), indicating good stability of this method.

Tissue distribution of artemisinin: The levels of artemisinin determined in different tissue of broiler chickens following single and multiple oral administration are summarized in Table 2 and 3, respectively. The highest concentration of artemisinin following single oral administration was found in the liver. The amount of artemisinin measured in the liver increased significantly following dose increase (doses higher than 125 mg/kg) (Table 2).

The amount of artemisinin detected in the different tissues of chicks that received multiple administration of doses are shown in Table 3. As these show, the accumulation of artemisinin in the liver of chickens exposed to the multiple doses had an increasing pattern, and the groups fed 17 and 136 ppm had a significant difference with each

other ($p<0.05$) (Table 3).

Following single oral administration, distribution of artemisinin in brain increased following dose increment at doses lower than 125 mg/kg, however no shift could be found with dose increment at higher doses.

After multiple doses there was a significant difference between the groups fed 17 and 34 ppm with groups fed 68 and 136 ppm ($p<0.05$), and no additional increase was observed after increment of dosage from 68ppm to 136 ppm.

In the kidney following single administration, a significant accumulation was observed at doses higher than 250 mg/kg ($p<0.05$); it appears that at doses higher than 250 mg/kg the excess amount of drug was eliminated via renal routes. On the contrary, the concentration of the drug in kidney samples after multiple doses was even lower at the highest dose (136 ppm) than groups which were fed 34 and 68 ppm.

In the lung samples, following single oral administration, a significant increase was found following dose increment up to 125 mg/kg ($p<0.05$). No dose dependent increment was found at higher doses. However, a significant increase was observed following multiple dose study ($p<0.05$).

Very little artemisinin was found in the spleen samples following single oral administration at doses lower than 125mg/kg. However, at higher doses (500, 1000 mg/kg), concentration of artemisinin in spleen increases surprisingly. In contrast to single dose results, following multiple dose administration the concentration of the drug in the spleen increased significantly.

In general, single oral doses of artemisinin were seen to have a homogenous distribution between various tissues at doses lower than 25 mg/kg, with a significant decline towards the spleen. After single dose of 25 mg/kg, the tissue concentration was the highest in the lung. After that, the distant second was the brain which was closely followed by the liver, the kidney and the spleen. There was no significant difference between the liver, the brain and the kidney ($p>0.05$). At doses of 125 and 250 mg/kg the liver contained the highest dose followed by the lung, the kidney, the brain and the spleen. No significant differences were found between the kidney, the brain and the lung ($p>0.05$). At doses of 500 and 1000 mg/kg the tissue concentrations in various tissues from high to low were the liver, the kidney, the lung, the brain and the spleen. Significant differences were found between liver versus all other tissues and spleen versus all other tissues ($p<0.05$) (Table 2). After multiple administrations, in the group which was fed 17 ppm, the spleen was found to have the highest concentration. The highest level of artemisinin was found in the liver and followed by the spleen, the lung, the brain and the kidney in other groups (Table 3).

Discussion

The present study was conducted to test the relationship between artemisinin dose and tissue distribution following single or multiple administration of compound in broilers. The highest total amount of artemisinin, following multiple or single doses was found in the liver and the accumulation increased following dose increment. Orally administrated artemisinin is metabolized primarily in the liver over time (Ashton et al., 1998). Observations in the liver are considerably in agreement with different studies on tissue distribution of artemisinin in mice and rats (Genovese et al., 1999; Cheerama-

kara et al., 2008). Toxicopathologic study indicated that only the presence of drug was important in the frequency of lesions occurrences (Arab et al., 2009).

In the brain no increase was observed in the accumulation of artemisinin at high doses following single or multiple oral doses with dose increment. It suggests that binding sites in cerebral cortex and cerebral nuclei are being saturated, and that at high doses uptake into the brain tissues is limited by blood brain barrier. Neurotoxicity is considered as the main side effect of artemisinin and its derivatives, however, its incidence and severity is dependent on dose, period and route of administration (Fishwick et al., 1995),

Drug levels in the kidney were relatively low at doses lower than 250 mg/kg, but increased more at higher doses. Cheeramakara et al. 2008 reported that following intramuscular administration of arteether, an ethyl ether derivative of artemisinin, it was localized mainly in the kidney. The ratio of labeled arteether distribution in the blood: kidney: liver was 1: 5: 2. Higher kidney radioactivity was noted in the cytoplasmic cortex than the medulla (Cheeramakara et al. 2008).

In the present study, following multiple oral doses, a small amount of artemisinin distributed in the kidney. There is evidence that small amount of artemisinin is excreted through kidney without metabolism (Dien et al., 1997). It seems that artemisinin excreted via renal routes in broilers at high doses. This is in agreement with the previous study whichreported that in the microscopic observation the kidney tissues showed some pathologic lesions following single oral doses (Arab et al., 2009).

The distribution in the lung at doses lower than 250 mg/kg increased following an increase in the dose, however, no shift could be found with dose increment. Following single administration, at 25 mg/kg, the tissue concentration was significantly increased. The highest level was observed in the lung followed by the brain, the liver, the kidney, and the spleen. At doses of 125 and 250 mg/kg the liver contained the highest dose followed by the lung, the kidney, the brain and the spleen. No significant differences were found between the kidney, the brain and the lung. At doses of 500 and 1000 mg/kg the tissue concentrations in various tissues from high to low were the liver, the kidney, the lung, the brain and the spleen. Significant differences were found between the liver versus all other tissues and the spleen versus all other tissues.

The result of lung tissue samples from multiple dose study appeared to be inconsistent with the single dose administration. Results suggested that the lung deposition is time dependent and the long half-life of artemisinin in the lung tissue samples is one of the probable reasons.

In the spleen, the lowest level was found following single oral doses, in agreement with this, previous histopathologic studies showed no lesions in the microscopic sections of the spleen following administration of single or multiple doses of artemisinin (Arab et al., 2009; Shahbazfar et al., 2011). However, in the present study, considerable accumulation of artemisinin was observed following administration of multiple doses of artemisinin. It appeared that artemisinin redistributed into the spleen, as it cleared from other tissues, and distribution was time dependent. Results of tissue concentration in the spleen samples obtained from the chicks administrated multiple oral dos-

es are in agreement with the findings from other investigations in rats. Xie et al. 2009 reported that 1h after IV injection of labeled dihydroartemisinin in rats, drug distributed in tissues and after 24h, rapidly declined in all tissues with the exception of spleen (96h). It showed longest residence time in the spleen. This distribution pattern of artemisinin is understandable. The spleen, despite its small size is an important part of the reticuloendothelial system and receives a large percentage of the total cardiac output to filter aging erythrocytes (Smith et al., 1999), and besides, the affinity of artemisinin for heme group of hemoglobin is high (Woerdenbag et al., 1990). Thus the filtration of aging erythrocytes by the red pulp of spleen (Smith et al., 1999) can lead to the accumulation of artemisinin in the spleen following long exposure time.

In this study the tissue distribution of artemisinin in broilers following single or multiple oral administration at different doses was compared. Data obtained in this study allows a comprehensive tissue distribution of artemisinin in broiler chicken at different doses. In general, the results of this study demonstrated considerable complications in terms of artemisinin distribution in chicken tissues, so that tissue accumulation of artemisinin was time and dose dependent, and redistribution, saturation effect and tissue selectivity were observed. Mainly, the results showed highest accumulation in the liver, but the spleen levels were very low following single doses, in contrast, following multiple doses a considerable level of artemisinin was observed in the spleen. Moreover, data suggests that distribution of artemisinin in the brain is saturable, and the drug is eliminated by renal routs at higher doses.

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توزیع بافتی آرتمیزینین در طیور گوشتی پس از تجویز دارو به صورت تک دوز و دوزهای چندگانه

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چکیده

زمینه مطالعه: آرتمیزینین معمولاً برای درمان مالاریا اســتفاده می‌شــود ولی به تازگی به عنوان یک ماده بالقوه برای کنترل کوکسیدیوز طیور بکار می‌رود. **هدف:** هدف از مطالعه حاضر تعیین توزیع بافتی آرتمیزینین بدنبال مصرف خوراکی تک دوز یا چندگانه دوزهای بالای آن در جوجه های گوشــتی بود. **روش کار:** ۳۹۰ جوجه گوشــتی یک روز نژاد راس به دو گروه اصلی تقسیم شدند، در جوجه‌های گروه اول مقادیر mg ۰، ۱، ۵، ۲۵، ۱۲۵، ۲۵۰ ، ۵۰۰ و ۱۰۰۰ به ازای هر کیلوگرم وزن بدن آرتمیزینین به صورت تک دوز خوراکی در روز ۴۴ و در گروه دوم جوجه‌ها مقادیر ppm ۰ ، ۱۷، ۳۴، ۶۸ و یا ۱۳۶ آرتمیزینین از روز ۸ تا ۴۴ تجویز گردید. برای تعیین ســطح آرتمیزینین در نمونه‌های بافتی مختلف از سامانه HPLC و برای بررســی آماری داده‌ها از آزمون آنالیز واریانس یک طرفه (ANOVA) و آزمون تکمیلی، توکی (Tukey) استفاده گردید ($p<0/05$). **نتایج:** در هر دو گروه بیشترین غلظت آرتمیزینین در کبد جوجه‌های گوشتی دیده شد که به صورت وابسته به دوز بود. اما، کمترین سطح آرتمیزینین در گروه دریافت کننده دوزهای چندگانه دارو در در مغز وکبد و در گروه دریافت کننده تک دوز در طحال دیده شد. غلظت آرتمیزینین در مغز بدنبال تجویز چندگانه دوز ۶۸ppm و در تجویــز تــک دوز در دوز ۱۲۵ mg/kg به حالت ثابت رســیده بود و هیچ تغییری هم با افزایش دوز ایجاد نشــد. **نتیجه‌گیری‌نهایی:** می‌توان نتیجه گرفت که تجمع بافتی آرتمیزینین وابسته به زمان و دوز است. علاوه بر این، بازتوزیع، اثر اشباعی و انتخاب بافتی نیز دیده شد.

واژه های کلیدی: آرتیمزینین، وضعیت زیستی، جوجه گوشتی، دریافت چندگانه خوراکی، تک دوز

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