

## RESEARCH ARTICLE

### Depletion of Amikacin Residues in Fresh, Boiled and Frozen Rabbit's Tissues Using High Performance Liquid Chromatography

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Article History: Received: 08/03/2022 Received in revised form: 02/04/2022 Accepted: 06/04/2022

#### Abstract

Amikacin is a semisynthetic bactericidal aminoglycoside derived from kanamycin A with a broad spectrum of activity. The aim of this work is to investigate the withdrawal of amikacin residues in the tissues of rabbits and the effect of boiling and freezing on amikacin residues using high performance liquid chromatography (HPLC). About 15 rabbits were injected with an intramuscular dose of amikacin (15 mg/kg BW) for 7 successive days. Samples were obtained from kidneys, liver and breast muscle on the 1st, 3rd, 5th, 7<sup>th</sup>, and 10th day after the last dose. Sample extraction was carried out using solid phase extraction and derivatization process. Reversed HPLC analysis included fluorescence detection at an excitation wave-length 360 nm and emission 435 nm and mobile phase mixture (69: 31 v/v) of methanol, water with a flow rate 1.5 min. Our results revealed that residues of amikacin in the fresh kidneys, liver and breast muscle were  $14.2 \pm 0.57 \mu\text{g/g}$ ,  $9.02 \pm 0.45 \mu\text{g/g}$ , and  $8.23 \pm 0.18 \mu\text{g/g}$ , respectively in the first day post-treatment. The residues' level was declined to  $0.19 \pm 0.02$  in kidney and not detected in liver and breast muscle on the 10<sup>th</sup> day post treatment. Residues level in rabbits' kidneys, liver and breast muscle was recorded as a reduction % of 26.3%, 32.35%, and 44.13% after boiling treatment and 11.74%, 20.18%, and 22.27% after freezing, respectively compared to the residues in fresh tissues in the first day post treatment. The obtained results concluded that the withdrawal time of amikacin residues from the tissues of rabbits is 10 days.

**Keywords:** Amikacin, residues, HPLC, Rabbits, boiling.

#### Introduction

Aminoglycosides are important antibacterial drugs used for the treatment of various infections in humans and are extensively used in different animal species, including both food-producing animals and companion animals [1].

Amikacin is a semisynthetic bactericidal aminoglycoside antimicrobial derived from kanamycin A with a broad spectrum of activity including gram-positive and gram-negative aerobic bacteria. It acts by impairing bacterial protein synthesis through binding to the 30S ribosomal subunit [2]. It is employed in the treatment of several bacterial infections such

as *Escherichia coli*, *Enterobacter*, *Klebsiella*, *Proteus mirabilis*, *Serratia*, *Mycobacterium*, *Staphylococcus aureus*, *Corynebacterium equi*, *Streptococcus zooepidermicus* and *Actinobacillus* in various animal species [3].

The main difference between amikacin and kanamycin A molecular structure is an extra 4-amino-2-hydroxybutyl side chain allowing amikacin to be less susceptible to be inactivated by plasmid-mediated enzymes secreted by aminoglycosides resistant bacteria. Subsequently, amikacin has a significant efficacy against many kanamycin, gentamicin and tobramycin-resistant gram-negative bacteria [4, 5].

The pharmacokinetics of amikacin was studied in different species of animals specially farm animals as calves [6], sheep [7], goats [8], camels [9] and chickens [10]. However, there is low information about amikacin residues and its withdrawal time in rabbits. This work aimed to investigate the withdrawal of amikacin residues following intramuscular administration in rabbits and the effect of boiling and freezing on amikacin residues using high performance liquid chromatography (HPLC).

## Materials and Methods

### Drugs and chemicals

Amikacin standard and Amikacin vial with a commercial name (Amikacin®) were provided from Amoun Company, Cairo, Egypt. Methanol and water were HPLC grade and obtained from Lab scan chemical industries, Poland. Methylene chloride, Trichloroacetic acid (TCA) and EDTA tripotassium salt were obtained from Sigma Aldrich, Egypt.

### Animals

Eighteen clinically healthy New Zealand rabbits with an average weight of 2 kg and 2 months age were obtained from experimental animals' research unit, Faculty of Veterinary Medicine, Zagazig University. The rabbits were housed at batteries at post graduate research laboratory at Faculty of Veterinary Medicine, Zagazig University and fed on balanced ration with free access to water for 3 weeks without any treatment before starting the experiment. The rabbits were divided into a control group (3 rabbits) used for the preparation of blank and spiked samples for method validation and an experimental group (15 rabbits) which were then injected with an intramuscular dose of amikacin (15 mg/kg BW) for 7 successive days.

### Sampling

Three rabbits were sacrificed on the 1st, 3rd, 5th, 7th and 10th day after the last intramuscular dose. Breast muscle, liver and kidney samples (about 15 gm of each tissue) were obtained from each rabbit and divided into 3 equal parts. Five gm from each of which were frozen at

-4°C until time of analysis, 5 gm were boiled for 15 minutes then frozen at -4°C until time of analysis and the last 5 gm were frozen at -20°C until time of analysis for determination of antibiotic residues.

### Analytical method

#### a. Sample extraction

Amikacin was extracted from tissue as described by Santos *et al.* [11] and Li *et al.* [12] using solid phase extraction and derivatization process. Briefly, one gram of tissue was put in Falcon 50 mL conical centrifuge tubes to which 5 mL of TCA was added, followed by shaking for 30 min and then centrifugation for 5 min at 3200 rpm. Supernatant was separated, and then 5 mL of 1 M NaOH, 10 mL 0.250 M phosphate buffer followed by 2 mL methylene chloride which were shaken for 30 min and then centrifuged at 3200 rpm / 5 min. After that, 5 mL of methanol and 5 mL of water were used for pre-conditioning of CBX cartridges. The sample was loaded for solid phase extraction and eluted with 5 mL mobile phase followed by evaporation using nitrogen evaporator till dryness. Then 0.1 mL of methanol was added, and the residue was stored in a dark place at 4°C until derivatization procedure.

Finally, 1 mL of derivatizing reagent, 500 mg anhydrous Na<sub>2</sub>CO<sub>3</sub> and 0.5 mL of 2-propanol were added to the sample, shaken, and centrifuged at 3200 rpm for 5 min. Then, 50 µL of 2-propanol extract was injected to HPLC.

#### b. Chromatographic conditions

Amikacin residues were analyzed using HPLC (1200 series, Agilent, Germany) with fluorescence detection at an excitation wavelength of 360 nm and emission of 435 nm. The mobile phase (69: 31 v/v) of methanol, water, and 2.2 g of EDTA tri potassium salt was filtered then degassed using ultrasound bath (3510 Branson). Separation was carried out using C18 column (10µm, 100x4.6 mm), Agilent, Germany with flow rate 1.5 min. Quantification of residues in samples was obtained and calculated from areas under curves extrapolated automatically by the software (Chemstation).

### c. Method validation

HPLC analytical method was validated according to the guidelines of the International Council for Harmonization (ICH) of technical requirements for pharmaceuticals for human use.

### d. Calibration curve

Calibration curve was prepared by using concentrations of 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, 10 and 20 µg/ml of amikacin in eluent and spiked tissues.

### Statistical analysis

Different variables were analyzed using Dunnett's test. All values were presented as means ( $\pm$ ) standard error (SE) [13].

## Results

Clinically, no adverse effects were observed after administration of amikacin intramuscularly (15 mg/kg BW for 7 consecutive days in rabbits).

### Method validation

The method for amikacin separation is precise as the Relative Standard Deviation (RSD) of amikacin test solution is 0.47. There is no interference between the amikacin in the samples and peaks of any impurities or extracted solvents. The retention time of amikacin is 7.84 minutes. The calibration curve of amikacin displayed a linear relationship over the range of 0.05–20 µg/mL with a correlation coefficient 0.999 (Figures 1, 2). The recovery of spiked tissue samples ranged from 80-104 %, the limit of detection (LOD) was 0.01 µg/mL and the limit of quantification (LOQ) was 0.05 µg/mL.

### Tissue residues

Following intramuscular administration of amikacin (15 mg/kg BW) for 7 consecutive days in rabbits, residues level of amikacin in the fresh kidneys was ( $14.2 \pm 0.57$  µg/g) on the 1<sup>st</sup> day then displayed a significant decrease on the 3<sup>rd</sup>, 5<sup>th</sup>, 7<sup>th</sup> day until reach  $0.19 \pm 0.02$  µg/g

on the 10<sup>th</sup> day post-treatment. Amikacin residues in rabbits' kidneys displayed a reduction percent of 26.3%, 41.54%, and 38.33% after boiling and displayed a reduction percent of 11.74%, 25.6%, and 44.28% after freezing compared to the residues in fresh kidneys on the 1<sup>st</sup>, 3<sup>rd</sup>, and 5<sup>th</sup> days post-treatment, respectively. Residues' level remained under the detectable level on 7<sup>th</sup> and 10<sup>th</sup> days for the boiled samples and under the detectable level on 10<sup>th</sup> day for frozen ones (Table 1) and Figures (3,4)

Amikacin residues level in rabbits' fresh liver declined significantly from ( $9.02 \pm 0.45$  µg/g) on the 1<sup>st</sup> day to ( $0.58 \pm 0.04$  µg/g) on the 7<sup>th</sup> day while amikacin residues were not detected on the 10<sup>th</sup> day post-treatment. Amikacin residues' level in rabbits' liver reduced by 32.35%, 65.33%, and 98.12 % after boiling and displayed a reduction percent of 20.15%, 31.27%, and 70% after freezing compared to residues' level in fresh liver on the 1<sup>st</sup>, 3<sup>rd</sup>, and 5<sup>th</sup> days post-treatment respectively, while residues were not detected on the 7<sup>th</sup> and 10<sup>th</sup> days post-treatment (Table 2).

Amikacin residues' level in rabbits' fresh muscle displayed ( $8.23 \pm 0.18$  µg/g) on the 1<sup>st</sup> day and remained within the detectable level until 7<sup>th</sup> day post-treatment. After boiling amikacin residues in rabbits' muscle decreased by a reduction percent of 44.13%, 72%, and 98.3% compared to residues in fresh muscle on the 1<sup>st</sup>, 3<sup>rd</sup>, and 5<sup>th</sup> days post-treatment respectively, while residues were not detected on the 7<sup>th</sup> and 10<sup>th</sup> day post treatment. Amikacin residues level in frozen rabbits' muscle reduced by 22.27%, 38%, and 73.43% compared to residues in fresh muscle on the 1<sup>st</sup>, 3<sup>rd</sup> and 5<sup>th</sup> days post-treatment respectively while residues were not detected in the 7<sup>th</sup> and 10<sup>th</sup> day post treatment (Table 3).

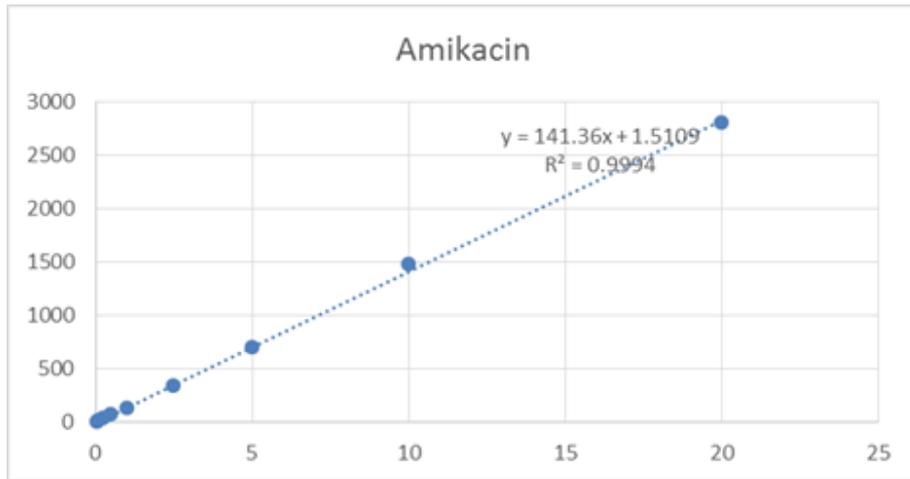


Figure 1: Calibration curve of amikacin

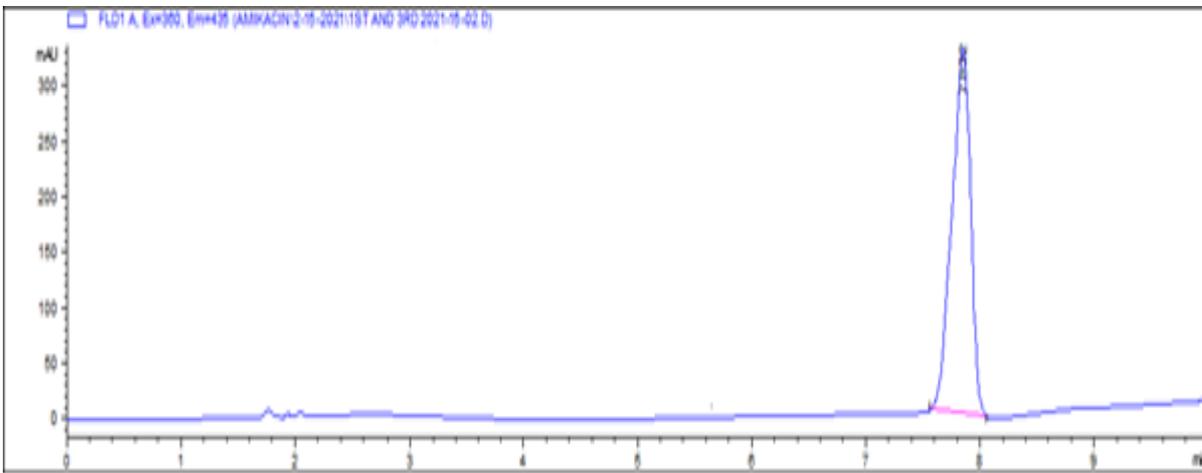


Figure 2: Liquid chromatogram of 1 µg/gm. injection of amikacin standard

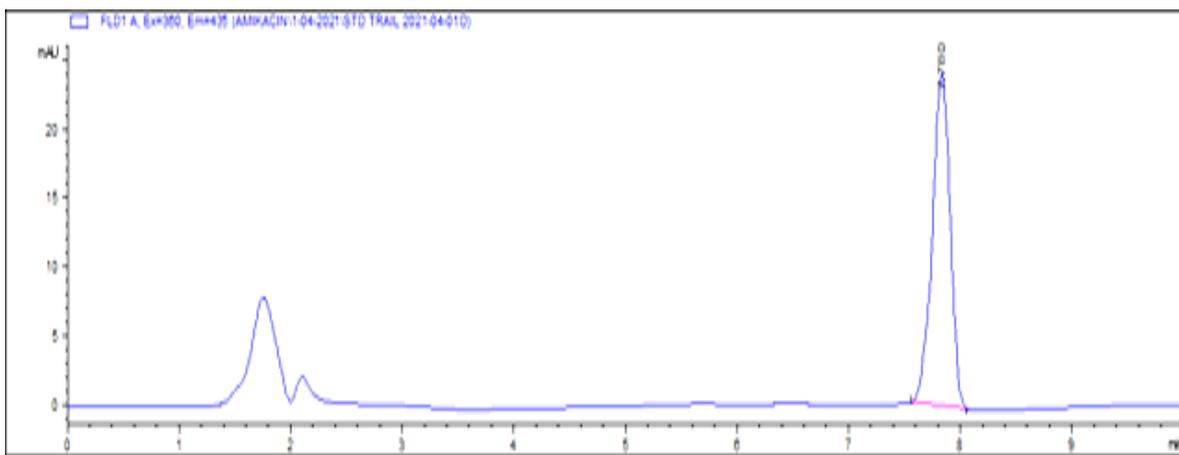
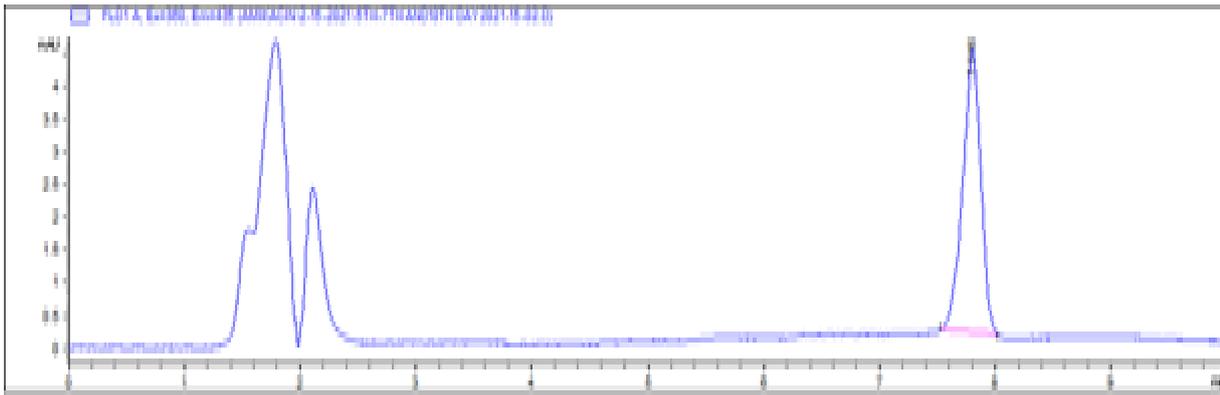


Figure 3: Liquid chromatogram of amikacin extracts in rabbit's kidney 1 day post administration of 15 mg/kg BW amikacin intramuscularly for 7 successive days.



**Figure 4: Liquid chromatograms of amikacin extract in rabbit's kidney 10 days post administration of 15 mg/kg BW amikacin intramuscularly for 7 successive days.**

**Table 1. Amikacin residues' level in fresh, boiled and frozen kidneys post administration of 15 mg/kg BW amikacin intramuscularly for 7 successive days in rabbits.**

Days Post administration	Amikacin residual level ( $\mu\text{g}/\text{gm}$ )		
	Fresh Kidney	Boiled Kidney	Frozen Kidney
1	14.2 $\pm$ 0.57	10.46 $\pm$ 0.87	12.53 $\pm$ 0.78
3	8.47 $\pm$ 0.87**A	4.95 $\pm$ 0.50**A	6.3 $\pm$ 0.82**A
5	6 $\pm$ 0.32***B	3.7 $\pm$ 0.25**B	3.34 $\pm$ 0.25***B
7	2.4 $\pm$ 0.15***C	Not detected	0.06 $\pm$ 0.01***C
10	0.19 $\pm$ 0.02***D	Not detected	Not detected

\* P<0.05      \*\* P< 0.01      \*\*\* P< 0.001      Means that carry A, B, C or D letter denoted significant difference compared with 1<sup>st</sup> day within the same column.

**Table2. Amikacin residues' level in fresh, boiled, and frozen livers post administration of 15 mg/kg BW amikacin intramuscularly for 7 successive days in rabbits.**

Days Post administration	Amikacin residual level ( $\mu\text{g}/\text{gm}$ )		
	Fresh liver	Boiled liver	Frozen liver
1	9.02 $\pm$ 0.45	6.1 $\pm$ 0.56	7.2 $\pm$ 0.40
3	5.5 $\pm$ 0.38**A	1.91 $\pm$ 0.27**A	3.78 $\pm$ 0.44**A
5	3.5 $\pm$ 0.33***B	0.06 $\pm$ 0.00**B	1.05 $\pm$ 0.03***B
7	0.58 $\pm$ 0.04***C	Not detected	Not detected
10	Not detected	Not detected	Not detected

\* P<0.05      \*\* P< 0.01      \*\*\* P< 0.001      Means that carry A, B or C letter denoted significant difference compared with 1<sup>st</sup> day within the same column.

**Table3. Amikacin residues' level in fresh, boiled and frozen muscles post administration of 15 mg/kg BW amikacin intramuscularly for 7 successive days in rabbits.**

Days Post administration	Amikacin residual level ( $\mu\text{g}/\text{gm}$ )		
	Fresh muscle	Boiled muscle	Frozen muscle
1	8.23 $\pm$ 0.18	4.6 $\pm$ 0.56	6.4 $\pm$ 0.31
3	5 $\pm$ 0.40**A	1.4 $\pm$ 0.21**A	3.1 $\pm$ 0.59**A
5	3.12 $\pm$ 0.17***B	0.053 $\pm$ 0.00**B	0.83 $\pm$ 0.03***B
7	0.43 $\pm$ 0.04***C	Not detected	Not detected
10	Not detected	Not detected	Not detected

\* P<0.05      \*\* P< 0.01      \*\*\* P< 0.001      Means that carry A, B or C letter denoted significant difference compared with 1<sup>st</sup> day within the same column.

## Discussion

Aminoglycosides antibiotics have a broad spectrum of therapeutic applications in both human and animals. The use of aminoglycosides is extended for many purposes in farm animals as growth promoters and for prevention of bacterial infections besides animals' treatment. The economic price of aminoglycosides will encourage the overuse of this category of drugs which already represent 3.5% of the overall antibiotics that are used in veterinary field. The uncontrolled use of aminoglycosides will lead to the increase of drug residues in farm animals and subsequently antibiotic resistance in some strains of bacteria especially enteric bacteria [1, 14].

The analysis of aminoglycosides is a complicated process because of their chemical properties such as high polarity, polyatomic nature and lack of chromophores. Liquid chromatography (LC) remains the gold standard in the separation of aminoglycosides prior to their determination. It is a well-established technique high resolution, selectivity, and sensitivity [14].

In the current study, the highest level of amikacin residues  $14.2 \pm 0.57 \mu\text{g/g}$  was recorded in fresh kidneys of rabbits on the first day post-treatment followed by a significant decrease on the following days until the 10<sup>th</sup> day post-treatment when it reached  $0.19 \pm 0.02 \mu\text{g/g}$ . In this respect, Kornguth and Kunin [15] injected rabbits intramuscularly with gentamicin and amikacin (15 mg / kg), and the antibiotic levels in tissues were determined 20 h after either a single or multiple injections. Their results revealed that the kidney is the major site of antibiotic deposition. Similar results were obtained for kanamycin residues in kidney of rabbits where the kanamycin residues' level reached 10160  $\mu\text{g/kg}$  and 210  $\mu\text{g/kg}$  at 10 and 50 days, respectively, following subcutaneous administration of 15 mg/kg kanamycin twice daily for 5 consecutive days. Also, the highest concentrations of kanamycin were detected in kidney of calves (16380  $\mu\text{g/kg}$ ) and piglets (12210  $\mu\text{g/kg}$ ) at 10 days following kanamycin intramuscular administration [16]. Moreover,

spectinomycin's residues displayed highest concentration in kidneys of cattle and pigs [17] and gentamicin's residues displayed highest concentration in kidneys of calves and piglets [18].

In the present experiment, the mean amikacin residues' level in rabbits' liver and muscle post treatment was  $9.02 \pm 0.45 \mu\text{g/g}$  and  $8.23 \pm 0.18 \mu\text{g/g}$  in the first day, respectively, declined on the third, fifth, and seventh days and was not detected on the 10<sup>th</sup> day post-treatment. Our results are consistent with that reported in calves treated with repeated intramuscular doses of 4 mg of gentamicin per day for 3 days. Significant amounts of residues were detected in liver at 7, 30, 60, 70, and 80 days after the last injection [18]. Similar results obtained for gentamicin in pigs treated with a dose of 4 mg/kg BW intramuscularly for 3 days where the gentamicin's residues were detected in liver at 10, 20, 30, 40, 50, 60, and 70 days after the last injection [18]. Also, our results are in agreement with that reported for kanamycin's residues in liver of rabbits 960  $\mu\text{g/kg}$ , calves 3810  $\mu\text{g/kg}$  and piglets 4190  $\mu\text{g/kg}$  at 10 days and declined to lower than 100  $\mu\text{g/kg}$  at 40 days following parenteral administration for 5 consecutive days. In muscle kanamycin residues were always below the limit of detection (100  $\mu\text{g/kg}$ ) in rabbits, close the limit of detection of the microbiological analytical method (100  $\mu\text{g/kg}$ ) 10 days after the last administration in calves and below the limit of detection at 20 days after the last administration in piglets [16].

Moreover, Ahmed and Jwher [19] declared that streptomycin residues were found in 54.5% of the examined slaughtered sheep and distributed as following; 22.72 % in liver, 36.36 % in Longissimus dorsi muscle, 45.45 % in diaphragm muscle and, 50 % in kidney. Also, El Tahir *et al.* [20] reported that tylosin and gentamicin residues were the highest concentration among 5 antibiotics residues in 100 chicken liver samples and were the lowest ones at the same time in 100 chicken breast samples collected from five poultry farms.

In this study, results revealed that mean amikacin residues' level in rabbits' kidneys,

liver and muscle after boiling were  $10.46 \pm 0.87 \mu\text{g/g}$ ,  $6.1 \pm 0.56 \mu\text{g/g}$ , and  $4.6 \pm 0.56 \mu\text{g/g}$ , respectively, recording a respective reduction by 26.3 %, 32.35%, and 44.13% compared to the residues in fresh tissues in the first day post treatment, and were not detected on the 7<sup>th</sup> day post treatment. Our findings are compatible with previously reported boiling effect on tetracycline drugs where the most unstable one was oxytetracycline [21] . Our observed results are conflicted with that reported by Shaltout *et al.* [22] who found that quinolones' residues in chickens are very stable during thermal procedures with ultra-high temperatures and had nearly no effects on degradation. Moreover, Lolo *et al.* [23] recorded that cooking procedures did not reduce enrofloxacin residues in chicken muscles; therefore, this residue retained its stability during heating

Furthermore, our investigations revealed that mean amikacin residues' level in rabbits' kidney, liver and muscle following freezing were  $12.53 \pm 0.78 \mu\text{g/g}$ ,  $7.2 \pm 0.40 \mu\text{g/g}$ , and  $6.4 \pm 0.31 \mu\text{g/g}$  recording a decreasing percentage of 11.74%, 20.18%, and 22.27% compared to the residues in fresh tissues in the first day post treatment, respectively but were not detected in the 7<sup>th</sup> day post treatment except in frozen kidney. Similar results obtained by Pavlov *et al.* [24] who carried out studies on the residues level of tobramycin in poultry tissues frozen at  $-18^{\circ}\text{C}$ . The residues of tobramycin were determined over a period of 60 days revealing a decreasing level of this drug during this period of storage. The highest residual levels were recorded in the liver, followed by breast and thigh muscles, residues level was not detected in the muscles on the 30<sup>th</sup> day. Our results agreed also with that reported by Shaltout *et al.* [22] who stated that freezing for 6 months causes a reduction of 62.62% for ciprofloxacin and 2.05% for oxytetracycline. After 12 months of freezing, Ciprofloxacin was completely disappeared while oxytetracycline was reduced by 32.38%. The maximum residual limits (MRL) of amikacin are not established until now. Taking into consideration MRL of kanamycin (2500  $\mu\text{g/kg}$  in kidney, 600  $\mu\text{g/kg}$  in liver and 100

$\mu\text{g/kg}$  in muscle) [16] . The withdrawal time for amikacin rabbits is 10 days.

### Conclusion

It is concluded that the withdrawal time for amikacin residues from tissues of rabbits is 10 days taking into consideration MRL of kanamycin. Cooking and freezing processes only reduced amikacin residues in rabbit kidneys, muscles and livers and did not ensure a complete break-down of amikacin residues in such tissues except in samples taken on the fifth day post-treatment for muscle and liver and on the seventh day for kidney.

### Conflict of interest

All the authors have no conflict of interest to declare.

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## الملخص العربي

### سحب بقايا أميكاسين من أنسجة الأرانب الطازجة والمغلية والمجمدة باستخدام جهاز الفصل الكروماتوجرافي السائل عالي الأداء

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1 - قسم الأدوية - كلية الطب البيطري - جامعة الزقازيق

2- قسم الأدوية - كلية الطب البيطري - جامعة المنصورة

3- قسم مراقبة الأغذية - كلية الطب البيطري - جامعة الزقازيق

أميكاسين هو مضاد للميكروبات من مجموعة الأمينوجليكوسيدات نصف مُصنَّع مشتق من كاناميسين أ ، ذو تأثير واسع المدى ضد الميكروبات . الهدف من هذا العمل هو دراسة سحب بقايا الأميكاسين في الأرانب وتأثير الغليان والتجميد على بقايا الأميكاسين باستخدام جهاز الفصل الكروماتوجرافي السائل عالي الأداء . تم حقن 15 أرنباً بجرعة عضلية من الأميكاسين (15 مجم / كجم وزن) لمدة 7 أيام متتالية. تم الحصول على عينات من الكلى والكبد والعضلات في اليوم الأول والثالث والخامس والسابع والعاشر بعد آخر جرعة. تم استخدام جهاز الفصل الكروماتوجرافي السائل عالي الأداء لتحديد تراكيز متبقيات الأميكاسين وذلك بعد إجراء اختبار صلاحية الطريقة واستخلاص العقار من الأنسجة. أظهرت النتائج أن تركيز بقايا الأميكاسين في أنسجة الكلى والكبد والعضلات الطازجة كان  $0.57 \pm 14.2$  ميكروغرام / غرام ،  $0.45 \pm 9.02$  ميكروغرام / غرام و  $0.18 \pm 8.23$  ميكروغرام / غرام في اليوم الأول بعد إعطاء الجرعة النهائية على التوالي. انخفض مستوى المتبقيات إلى  $0.02 \pm 0.19$  في الكلى ولم يتم تسجيل أي متبقيات في الكبد والعضلات في اليوم العاشر بعد إعطاء الجرعة النهائية. سجل مستوى متبقيات الأميكاسين في أنسجة الكلى والكبد والعضلات انخفاضاً بنسبة 26.3% و 32.35% و 44.13% بعد الغليان، وسجل انخفاضاً بنسبة 11.74% و 20.18% و 22.27% بعد التجميد مقارنة بالمتبقيات في الأنسجة الطازجة في اليوم الأول بعد العلاج على التوالي. وخلصت الدراسة إلى أن وقت سحب بقايا الأميكاسين في الأرانب هو 10 أيام.