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Determination of Marbofloxacin residues in Rabbit Tissues by HPLC

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Abstract

The present study was designed to evaluate marbofloxacin residues in different rabbit tissues after multiple intramuscular administrations. For that purpose, rabbits were divided into two groups; the first group (n=21) administered 2mg/kg marbofloxacin for five successive days, while the second group (n=3) were untreated and served as controls. Tissues were collected at the 1st, 3rd, 5th, 7th, 9th, 15th and 21stday after the last dose post administration of the drug. Liver, kidneys, pectoral muscle and thigh muscles, spleen, heart, blood and lung from each rabbit were taken, extracted and marbofloxacin residues were analyzed using high-performance liquid chromatographic method with ultraviolet detection. Results indicated a widespread distribution of marbofloxacin in the most tested tissues. It remained within detectable level till the 5th day in liver and serum while it continues till the 7th in kidneys day following the last dose. Therefore, muscles of rabbits treated with marbofloxacin could be consumed safely following the 1st day post treatment, while liver and kidneys could be consumed safely in the 3rd day after treatment without any hazards on consumers as the residual level below the recommended MRL (150µg/kg).

Keywords: Marbofloxacin, Residues, Rabbits, HPLC

Introduction

The use of antibiotics that might result in residues in meat, eggs and milk should not be permitted in food intended for human consumption. If these antibiotics are necessary for treating or preventing animal diseases, a withholding period must be observed until these residues are negligible or no longer be detected. These residues may lead to direct toxicity to consumers or alteration of microflora causing diseases and possible development of resistant strains of microorganisms which cause failure of drug therapy in clinical situations [1].

Marbofloxacin is considered the third generation fluoroquinolone with broad spectrum activity against some gram-positive, gram-negative organisms and Mycoplasma [2-4]. It has bactericidal action by a concentration - dependent mechanism [5]. It inhibits bacterial DNA gyrase enzyme which is responsible for supercoiling of DNA within the cells [6]. In fact, marbofloxacin was shown to be the most effective drug against bacterial strains isolated from rabbits infected with upper respiratory tract diseases compared to doxycycline, enrofloxacin, danofloxacin and tetracycline [7]. Quinolone residues in food obtained from animal origin decreased its effectiveness in human treatment due to its emergence of drug-resistant bacteria [8]. Therefore, the analysis of the quinolone residues in products of animal origin is very important for human health because WHO and FAO established tolerances for drugs, pesticides and other chemicals in tissues of food producing animals. There are few experimental data for marbofloxacin residues in rabbit tissues so we compared our results with other food producing animals and birds.

Material and Methods

Animals

A total of twenty-four healthy male NewZealand rabbits ranging from 2-2.5 kg body weight were housed in batteries at postgraduate research laboratory, Faculty of Veterinary Medicine, Zagazig University. Rabbits were given ad libitum drug-free pelleted diet and water. No clinical abnormalities were observed on rabbits.

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Experimental design

Rabbits were divided into two groups. The first group (n=21) was weighed and intramuscularly administered marbofloxacin (Marbocyl 10 %®), Vetoquinol, France) at dose of 2 mg/kg BW for five successive days [9]. The second group (*n*=3) was kept as controls for preparation of blank and spiked samples. Three rabbits were slaughtered at the 1st, 3rd, 5th, 7th, 9th, 15th and 21stday after the last dose and samples from blood, heart, lung, liver, spleen, thigh muscle. Pectoral muscle and kidneys were collected for determining marbofloxacin residues.

Analytical procedures

High Performance Liquid chromatography (Surveyor, Thermo Scientific Company, USA) was used for the analysis, Central laboratory at faculty of Veterinary Medicine, Zagazig University. The collected blood samples were left to coagulate and centrifuged at 3000 rpm. for 15min. to obtain clear serum which was then transferred immediately to sterile tubes and stored at -20°C until analysis. Tissue samples were blended in a food processor for 20–30 seconds at high speed and were subjected to stirring for a uniform paste-like consistency then stored at -70°C until analysis.

Extraction was carried out according to Ding *et al.* [10]. Frozen samples were thawed at room temperature (23°C); 3gm of tissue were homogenized and 15 ml extraction solution (0.015 mol/L perchloric acid (SD fine-chem Limited, Mumbai, India) and 0.015mol/L phosphoric acid (Sigma Aldrich Co, Germany) in water-methanol (Fisher Scientific Co., Fairlawn, NJ, UK) (50:50 v/v)) was added (5 mL extraction solution was added for 1mL of serum) and centrifuged at 15000 rpm. for 8min. The samples were then hydrolyzed in 50°C water bath for 90min., cooled to room temperature and centrifuged at 5000 rpm. for 10 min. The supernatant solution (50 µL) was then added to auto-sampler vial for analysis.

Liquid chromatographic conditions

Injection volume: 50 µL, flow rate: 1mL/min., column temperature: 50°C, UV-detector: 295 nm and the mobile phase (12% acetonitrile (J.T. Baker Co, Deventer, Netherlands): 0.75% formic acid (Merk Co., Darmstadt, Germany): 0.4% triethylamine (Sigma Aldrich Co, Germany).

Quantification

Quantification of the residues was obtained and calculated from area under curves extrapolated automatically by the software.

Method validation

System Precision

It is conducted by using 5 replicates of the toluene standard solution. Acceptance criteria: Relative standard deviation (RSD) ≤1% according to the International conference on harmonization of technical requirements for registration of pharmaceuticals for human use [11].

Linearity and range

Linearity is performed by preparing at a minimum 6 different concentrations of drug standard. Linearity is defined by the squared correlation coefficient, which should be ≥ 0.99 (r^2) according to ICH, 2005.

Method Precision

It is conducted by using 5 replicates of marbofloxacin standard solutions. Acceptance criteria: RSD ≤ 1% according to ICH, 2005.

Selectivity and specificity

Verification of selectivity is conducted by evaluating the spiked standard response following extraction from different rabbit tissues. Acceptance criteria: there is no interference between the pure standard and peaks of any impurities or extracted solvents according to ICH, 2005.

Accuracy and recovery

The tissue samples of rabbits are spiked by adding known quantities of marbofloxacin. Those samples are analyzed against standard solutions of the same concentrations. The accuracy is then calculated from the test results as a percentage recovery [12].

Limit of detection (LOD)

It is the concentration which gives signal to noise ratio 3:1 according to (ICH, 2005).

Limit of quantification (LOQ)

It is the concentration which gives signal to noise ratio 10:1 according to (ICH. 2005).

Ruggedness

It is conducted by analysis of the same samples under different conditions such as different personnel and different times. Acceptance criteria: pooled RSD is not more than 6% in every change item.

Robustness

It is determined by observing how a method stands up to slight variations in normal operating conditions. Acceptance criteria: pooled RSD is not more than 6% in every change item.

Results

Method validation

The HPLC system was precise as the Relative Standard Deviation (RSD) of 5 replicates of toluene standard solution is 0.007%. High correlation coefficient was obtained that indicating linearity (r^2=0.99858).

The method for marbofloxacin separation is precise as the (RSD) of 6 replicates of marbofloxacin standard solution was 0.13%. There was no interference between the pure standard and peaks of any impurities or extracted solvents.The retention time (R.T.) of marbofloxacin was 14.274 min (Figure 1).

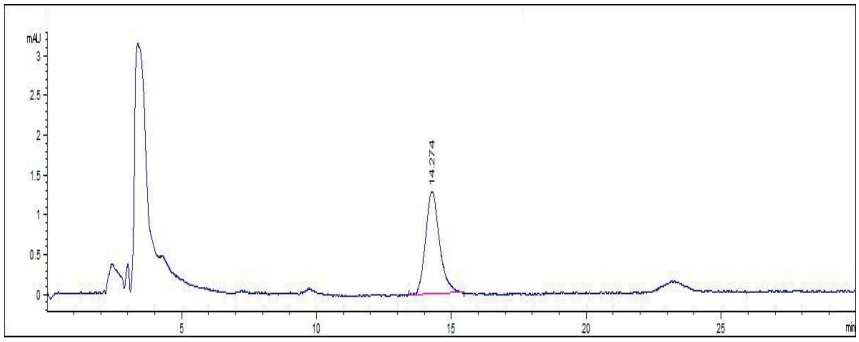

Figure 1: Chromatogram of Marbofloxacin fortified muscle at a concentration of 0.5 µg/gm.

The recovery percentage of marbofloxacin spiked samples ranged from 98-101%. The LOD for marbofloxacin was 0.003µg/mL, while, LOQ was 0.01µg/mL.The pooled RSD for marbofloxacin was 2.8% for Ruggedness and the Pooled RSD for Robustness was 2.1%.

Standard curve preparation

Marbofloxacin standard concentrations of 0.025, 0.050, 0.100, 0.200, 0.500 and 1 µg/gm were prepared in homogenized muscles of control rabbits (blank samples) then treated according to the extraction procedure and their corresponding peak responses are illustrated in Table (1) and Figure (2).

The calibration curve was calculated by linear regression equation method as y=87.1676184 X x-2.3664147 where y symbol refer to the area under peak and x symbol refer to the concentrations of marbofloxacin. Linearity existed within the range between 0.025-1 µg/gm with a correlation coefficient (r^2=0.99858).

Table 1: The concentrations of marbofloxacin spiked tissues (µg/gm) and their corresponding peak response automatically using HPLC.

RT*	Level	Amount (µg/gm)	Area
14.242	1	0.025	3.56
	2	0.050	6.346
	3	0.100	12.89
	4	0.200	22.432
	5	0.500	45.861
	6	1.000	88.915

*RT: Retention Time

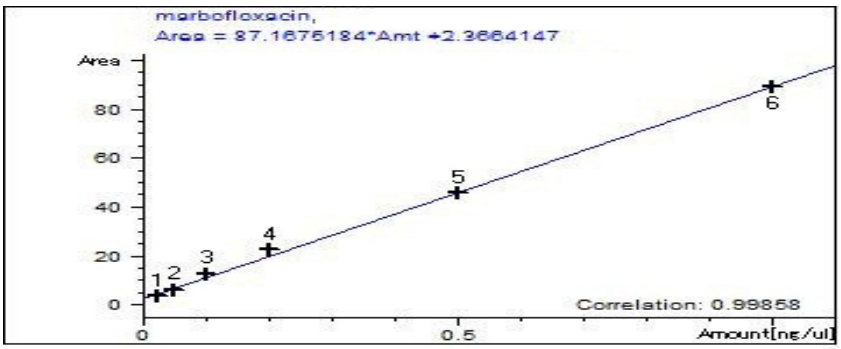

Figure 2: Standard curve of marbofloxacin using HPLC.

Tissue residues

Marbofloxacin distribution in serum and tissues was represented in Table (2). There was a widespread distribution of the drug in the tested serum and tissues. Marbofloxacin concentrations were 0.222±0.016, 0.306±0.007, 0.077±0.003, 0.0615±0.002, 0.296±0.01, 0.134±0.008, 0.048±0.002 µg/gm and 0.230±002 µg/mL. at the 1st day after the last dosage in liver, kidneys, breast muscles, thigh muscles, lung, spleen, heart and serum, respectively. Marbofloxacin remained within detectable limit till the 5th day in liver and serum while till the 7th in kidney post drug treatment (Table 2).

Discussion

Marbofloxacin is a third generation of fluoroquinolone with broad spectrum antimicrobial activity against some gram-positive, most gram-negative organisms and Mycoplasma [2-4] with a relatively low concentration [5,6]. It prevents the bacterial DNA replication and synthesis via inhibition of the A subunit of the bacterial DNA gyrase. Drug residues is defined as the very low amounts of drugs remained in animal products [13], that may lead to allergy, antibiotic resistance, disruption of intestinal flora and chronic toxic effects with prolonged administration [14]. Fluoroquinolones have health hazards as they cause gastro-intestinal toxicity, photo irritation and developmental alteration with joints inflammation [15].

Table 2: The concentrations of marbofloxacin in tissues of slaughtered rabbits at various intervals following treatment with 2 mg/kg BW once daily for 5 consecutive days (n=3) automatically using HPLC.

Tissue	concentration (µg/gm) after marbofloxacin administration Mean ± SE						
	1st	3rd	5th	7th	9th	15th	21th
Liver	0.222±0.016	0.062±0.003	0.024±0.006	ND	ND	ND	ND
Kidney	0.306±0.007	0.095±0.003	0.026±0.004	0.008±0.001	ND	ND	ND
Lung	0.296±0.01	0.084±0.009	ND	ND	ND	ND	ND
Spleen	0.134±0.008	0.077±0.005	ND	ND	ND	ND	ND
Pectoral muscles	0.077±0.003	0.019±0.004	ND	ND	ND	ND	ND
Thigh muscle	0.0615±0.002	ND	ND	ND	ND	ND	ND
Heart	0.048±0.002	ND	ND	ND	ND	ND	ND
Serum	0.230±0.002	0.072±0.002	0.012±0.001	ND	ND	ND	ND

ND: Not Detected.

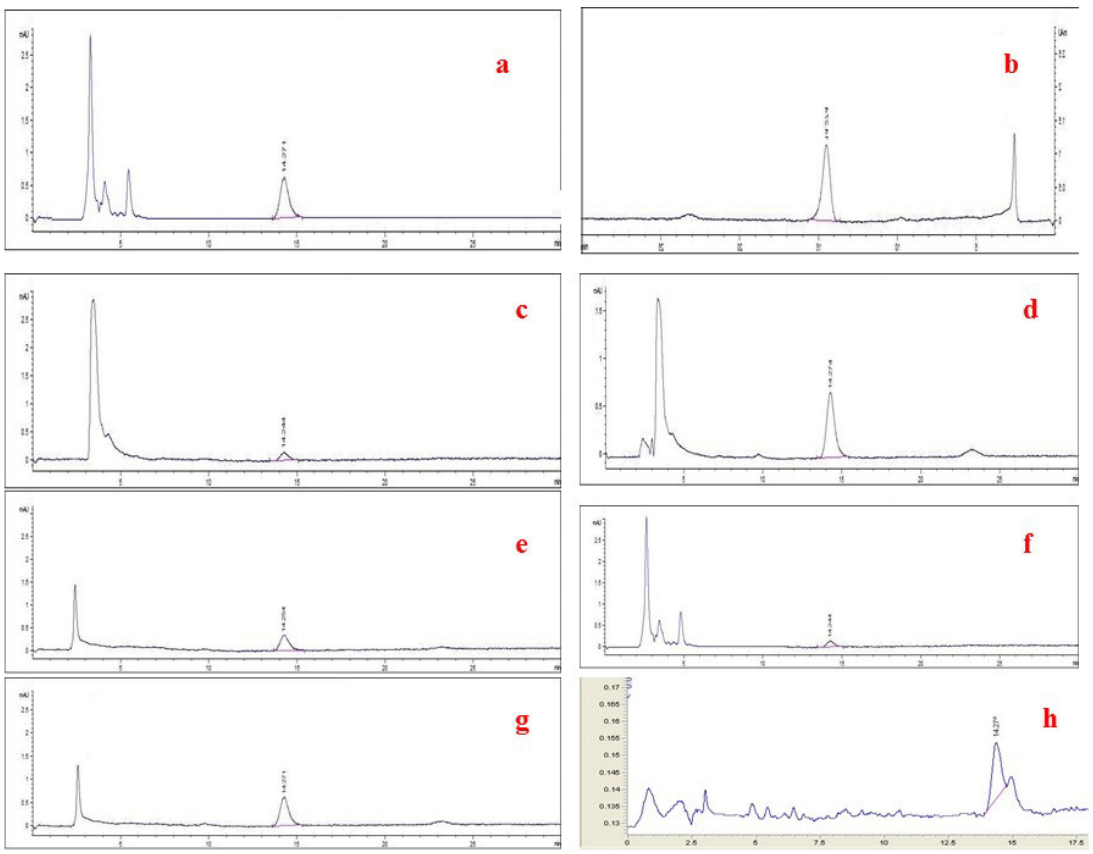

Figure 3: Chromatograms of marbofloxacin concentration in rabbit liver (a), kidney (b), pectoral muscle (c), lung (d), spleen (e), heart (f), serum (g) and thigh muscle and (h) at 1st day following last oral dose (2 mg/kg BW) using HPLC.

High concentrations of marbofloxacin were detected after 24 h from the last administered dose in both kidneys and liver which were much lower than those reported by Anadon *et al*. [16] who mentioned that marbofloxacin concentration in both kidneys and liver were 0.985 and 0.735 µg/gm, respectively in healthy Ross male broiler chickens received marbofloxacin at dose of 2mg/kg BW for 3 days. While our results were much higher than those reported by Ligabue *et al*. [17] who found that the concentration of marbofloxacin in both kidneys and liver were 0.016 ± 0.006 and 0.032±0.01 µg/gm, respectively in rabbits subcutaneously injected with marbofloxacin at a dose of 2mg/kg BW for 5 days. Also, our results were higher than those reported by Ding *et al*. [10] who mentioned that the marbofloxacin concentration in both kidneys and liver were 0.26 and 0.1 µg/gm, respectively in *Mycoplasma gllisepticum* infected chickens treated with marbofloxacin at dose of 5mg/kg BW for 3 days. The differences may be attributed to the different animal species, route of administration, dose, analytical techniques and pathological status [18].

The results obtained in the present study confirmed that the absorption and distribution of marbofloxacin administered to rabbits via intramuscular injection reach high levels in a short time in kidney, liver, lung and muscle which were in agreement with the previous reports [10,16,17,19]. Marbofloxacin remained within the detectable limit till the 5thday in most tested tissues, while in kidney it remained till the 7th day following last administration of the drug. A progressive order of marbofloxacin levels was detected in muscles, liver and kidneys, respectively. At all tested times post-treatment, marbofloxacin residues were probably higher in the kidneys than other examined tissues. This finding is in agreement with Ding *et al*. [10] and Anadon *et*

al. [16] who reported that marbofloxacin concentration in kidney was more than that in liver. But is contrast with Ligabue *et al*. [17] and Yang *et al*. [19] who reported that marbofloxacin concentration in liver was more than that in kidney.

Marbofloxacin concentration in muscles at the 1st day after injection was lower than that in liver and kidney. This finding is in agreement with Ligabue *et al*. [17] who reported that marbofloxacin in rabbits' muscle was lower than those measured in the liver and kidney after S.C injection at a dose of 2mg/kg BW/day for 5 days. We found that marbofloxacin residues concentrations in muscles at the 1st day and in kidneys and livers at the 3rd day were lower than the MRL (150 µg/kg) recommended by EMEA [20].

The withdrawal time for marbofloxacin orally administered in broiler chickens was ranged between 3 days at a dose of 2mg/kg BW for 3 days [15] and Yang *et al*. [21] reported 4 days at a dose of 5 mg/kg BW for 3 days that supported our results. The withdrawal period in rabbits was 2 days after S/C injection of marbofloxacin at a dose of 2mg/kg BW for 5 days as reported by Ligabue *et al*. [17]. The withdrawal period for chickens was 3 days after oral treatment of marbofloxacin at a dose of 2 mg/kg BW for 5 days [22]. According to EMEA [20], the microbiological acceptable daily intake (ADI) of 4.5µg of marbofloxacin/kg BW×60kg (standard body weight of human)=268 µg/person which can be ingested by human over a life time without appreciable risk.

Conclusion

Marbofloxacin residues were below the recommended MRL in pectoral and thigh muscle, spleen and heart of rabbits at the 1st day post treatment, while in liver, kidney and lung at the 3rdday post treatment. Therefore, muscles of rabbits could be eaten safely in the 1stday post treatment, while liver and kidneys of rabbits could be eaten safely in the 3rd day after treatment without any health hazard on consumers.

Conflict of interest

None of the authors have any conflict of interest to declare.

Acknowledgment

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الملخص العربي

تقدير بقايا الماربوفلوساسين في أنسجة الأرانب باستخدام جهاز الفصل الكروماتوجرافى السائل عالى الأداء

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استهدفت هذه الدراسة قياس بقايا الماربوفلوكساسين فى الأنسجة المختلفة للأرانب وذلك بعد الحقن العضلى المتعدد. ولهذا الغرض تم تقسيم الأرانب الي مجموعتين، الأولي بها ٢١ حيوان حيث تم الحقن العضلي للمجموعة الأولي بالماربوفلوكساسين بجرعة ٢ مجم/ كجم من وزن الأرانب لمدة ٥ أيام متتالية. أما المجموعة الثانيه قد تم استخدام عدد ٣ أرانب كمجموعة ضابطة. وقد تم تجميع الأنسجة من الحيوانات عند اليوم الأول، الثالث، الخامس، السابع، التاسع، الخامس عشر والحادى و العشرين بعد آخر جرعة للماربوفلوكساسين وأخذ الأنسجة المختلفة (الكبد، الكلى، العضلات، الطحال، الرئة، القلب والدم) ثم فحصها وقياس مستوى الدواء فيها بواسطة جهاز الفصل الكروماتوجرافى السائل العالى الأداءومن هذه النتائج نستنتج انتشار الدواء انتشارا واسعا فى أنسجة الأرانب المختلفة. قد تلاحظ وجود الماربوفلوكساسين فى الكبد والسيرم حتى اليوم الخامس من إعطاء الجرعة النهائية بينما في الكلي استمر وجود الدواء بها حتى اليوم السابع من إعطاء الجرعة النهائية. وعليه فإن عضلات الأرانب المعالجه بالماربوفلوكساسين يمكن أن تستخدم بأمان للإستهلاك الآدمي من اليوم الأول بعد العلاج بينما الكبد والكلي بعد اليوم الثالث حيث أنها أقل من المعدل المسموح به من MRL وهو ١٥٠ ميكروجرام/كجم.