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**RESEARCH ARTICLE**

# The Bioequivalence Determination of Two Different Formulations of Enrofloxacin in Heifers Following Intramuscular Administration [1] [2]

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[1] *This work was summarised from same named PhD thesis*
[2] *This study was performed according to the ethic board approved by Faculty of Veterinary Medicine University of Selcuk, Konya, Turkey (Approve No: 2004/012)*

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## Summary

The aim of this study was to evaluate the bioequivalence (BE) of two medicinal products of enrofloxacin, which have been also marketed as 10% injectable solution in Turkey, after the intramuscular injection (IM) at a single dose of 2.5 mg/kg of BW in the heifers. The present study was performed on healthy 6 Swiss-Brown (12-18 months and 340-400 kg BW) heifers. This study was carried out on the based a single dose cross-over design. Blood samples were taken into sterilized tubes just before, and $10^{th}$, $20^{th}$, $30^{th}$, $45^{th}$, $60^{th}$ and $90^{th}$ min. and $2^{th}$, $3^{th}$, $4^{th}$, $6^{th}$, $8^{th}$, $12^{th}$ and $24^{th}$ h following injections. The plasma concentrations of enrofloxacin (ENR) were measured by high performance liquid chromatography (HPLC) following the extraction process. The plasma concentration-time curves for each animals showed that both products distributed according two-compartment open model. The basic pharmacokinetic parameters at this study were only the $AUC_{0-24}$ and $AUC_{total}$ were statistically significant ($P<0.05$) before logarithmic (log) transformation. Log transformed the $AUC_{0-24}$, $AUC_{total}$ and $C_{max}$ parameters and observed $t_{max}$ were used in BE evaluation. Minimum, maximum and mean $AUC_{0-24}$ $AUC_{total}$ and $C_{max}$ for A and B products were found in the acceptable ranges (70-143%). For the $t_{max}$ value log transformation has not been done and that were determined within the limits 80-125%. As a result; it is concluded that both products could be used instead of each other as an "inter-changeable drugs".

**Keywords:** *Bioequivalence, Enrofloxacin, Heifer*

# Enrofloksasin İçeren İki Müstahzarın Düvelerde Kas İçi Yolla Uygulama Sonrası Biyoeşdeğerliğinin Belirlenmesi

## Özet

Bu çalışmanın amacı, enrofloksasin içeren ve ülkemizde %10'luk enjektabl şeklinde satışa sunulan ürünlerden ikisinin sığırlarda tek doz (2.5 mg/kg.) olarak kas içi uygulama sonrası biyoeşdeğerliğini (BE) değerlendirmektir. Çalışma 6 adet sağlıklı (12-18 aylık 340-400 kg CA) İsviçre esmeri düvelerde tek doz çapraz dizayn esasına göre gerçekleştirildi. İlaç uygulaması öncesinde 0. dakika ve sonrasında 10, 20, 30, 45, 60 ve 90. dakikalar ile 2, 3, 4, 6, 8, 12 ve 24. saatlerde kan örnekleri toplandı. Plazma enrofloksasin düzeyleri yüksek performanslı likit kromatoğrafisinde (HPLC) ölçüldü. Her hayvan için ayrı ayrı çizilen konsantrasyon-zaman grafikleri iki kompartmanlı dışa açık modele uygunluk gösterdi ve tüm farmakokinetik parametreler bu esasa göre hesaplandı. BE'lik değerlendirmesinde temel alınacak parametrelerden sadece $EAA_{0-24}$ ve $EAA_{top}$ değerlerinin birbirlerinden istatistiksel olarak farklı ($P<0.05$) oldukları tespit edildi. Verilerden $t_{doruk}$ dışındakilerin hepsi log dönüşüm yapıldıktan sonra her iki ürünün değerleri birbirlerine oranlanarak $\mu_B/\mu_A$ %90 güvenle BE'lik için gerekli olan 0.7-1.43 aralığında olduğu görüldü. Log dönüşüm yapılmayan $t_{doruk}$ değerinin ise 0.8-1.25 sınırlar içinde olduğu tespit edildi. Netice olarak; bu çalışma sonuçlarına göre iki ürünün BE oldukları, endike oldukları alanlarda birbirlerinin yerine kullanılabilecekleri söylenebilir.

**Anahtar sözcükler:** *Biyoeşdeğerlik, Düve, Enrofloksasin*

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## INTRODUCTION

Bioequivalence (BE) techniques are scientific methods for the comparison of different veterinary medicinal products containing the same active substance, of different batches of the same veterinary medicinal products and, in broader sense, of different routes of administration [1-3]. The aim of BE testing is to demonstrate that plasma concentrations of two medicinal products are similar enough and thus to conclude that the systemic effects of the two products, in respect to efficacy (and possible safety), are the same. Consequently, BE is defined as "the absence of a significant difference in the rate and extent to which the active ingredient or active moiety in pharmaceutical equivalents or pharmaceutical alternatives becomes available at the site of drug action when administered at the same molar dose under similar conditions in an appropriately designed study" [2-5].

The determination of the BE of veterinary drug formulations has become an increasingly important subject in the European Community and other developed countries [1,2]. The European guideline states that, if BE exists between veterinary medical products, under identical and appropriate experimental conditions, the bioavilability (rates and extents of absorption) of the active ingredient differs within accepted limits [2,4]. In the veterinary medicine, multiple BE and/or pharmacokinetic studies of different therapeutics have been carried out in different animal species by some researchers. For example, in chicken [2,3], in cows [4], in cattle [6], in pigs [5,6], in Angora goats [7,8], in lactating cows [9], in horses [10], in newborn and one-week-old calves [11] and in calves [12].

ENR fluorinated quinolone carboxylic acid derivative, developed exclusively for veterinary use [3,7,8]. ENR is even effective on bacteries that have been resisting amino-glycosides, β-lactams, tetracyclines, folic acid antagonists and macrolids [13-17]. ENR is an ideal anti-bacterial agent that has long half-life (1-7 h), large distribution volume and can be penetrate all animal tissues [7,8]. In the veterinary practice it has been recommended that 2.5 or 5 mg BW/ per day oral or parenteral single administration is an appropriate dosage regimen [12,18,19]. After the SC or IM administration maximum serum concentration of ENR has been raising within 1 to 4 h [8,9,13,20]. In the systemic circulation its mean maximum concentration reaches 0.8-3 µg/ml, but this value varies with administration route, dosage regimen and animal species [7,8,12,20,21].

The objective of the present study is to determine plasma disposition kinetics and BE of two medicinal products containing ENR following intramuscular administration to heifers.

## MATERIAL and METHODS

### *Animals*

In this study six Swiss-Brown (12-18 months, 340-400 kg BW, Sultansuyu dairy cattle farm, Malatya) healthy heifers were used. All animals were clinically normal and did not receive any medication in the last two weeks before the commencement of the study. During the study, the animals offered high quality maize silage thrice daily and water was given *ad-libitum*. These conditions were kept on carefully till the end of study. In the present study the animal numbers, dose, administration route and study design were in accordance with the other studies [4,5,12]. All of the animals remained healty throughout the study and no adverse reactions were observed.

### *Experimental Design and Sample Collection*

The study was conducted at a single dose two period cross-over design. One day before injection of the products, the animals were individually weighed for dose adjustment and the test product (B®) was administered at a dosage of 2.5 mg/BW to the first three animals and the other product (A®) (reference) was administered to the second three animals at same dosage. After fifteen days of wash-out period, the study design was repeated. Intramuscular injections (app. 8.5-10 ml) were administered into a site on hind leg and this dose level was determined in accordance with body weight of the heifers. All injections were administered between 07.00 and 07.20 h. Blood samples (app. 10 ml) were collected into tubes with EDTA at $10^{th}$, $20^{th}$, $30^{th}$, $45^{th}$, $60^{th}$ and $90^{th}$ min and $2^{nd}$, $3^{rd}$, $4^{th}$, $6^{th}$, $8^{th}$, $12^{th}$, and $24^{th}$ h. The samples centrifuged at 2500 rpm for fifteen minutes within one h after collection and plasma was stored at -20°C until analyses.

### *Drug Assay*

Plasma concentrations of ENR were assayed by HPLC as described by Anadon et al. [17]. The ENR was extracted from the plasma with dichloromethane (Merck) and analysed by reverse-phase chromatography. Mobile phase: Acetonitrile (A): 0.025 M Orthophosphoric acid (B) (with triethylamine fixed to pH: 3); pumping programme. 20% A 80% B; Detector: Diode array detector (DAD); Column: C18 Thermo-Hypersil-Keystone 250x4.6 mm 5 µ Hypersil® BDS; wave length: 278 nm; flow rate: 1 ml/min. The plasma concentrations of ENR were measured in The Pendik Veterinary Control and Research Institute, İstanbul, Turkey.

### *Method Validation*

The method was validated in terms of linearity,

sensitivity, recovery, intra-day and inter-day precision. Drug-free plasma was used to prepare the calibration curves for ENR with eight concentrations of 0.010 to 10 µg/mL. The analysis of ENR in plasma exhibited excellent linearity through the coefficient of correlation $r^2$: 0.9984, for ENR. The percentage recovery of ENR was determined by comparing of the peak areas from spiked plasma samples with the areas resulting from direct injections of standards. The average recovery was 85% for ENR. The intra-day precision is referred to as the repeatability of the assay; the inter-day precision is referred to as the intermediate precision of the assay. The inter and intra-day precision in plasma was assessed by performing six replicated analyses of spiked plasma samples with ENR at three concentrations on three separate days. The method precision (relative standard deviation [RSD]) was assessed by expressing the standard deviation (SD) of repeated measurements as a percentage of the mean value. RSD for intra-day was 1.6 to 3.8% for ENR. RSD for inter-day was 2.8 to 5.1% for ENR. The limits of detection (LOD) was estimated on the basis of the concentration of the standard solution, which gives a signal of peak height three times the size of the background noise (S/N=3). The LOD was as 0.010 µg/mL and LOQ was 0.020 µg/mL for ENR.

### *Pharmacokinetic Analysis*

The plasma concentrations of ENR versus time curves for each animal were analysed with the PKCALC computer programme [22] by a least-squares regression analysis. For ENR, the appropriate pharmacokinetic model was determined by visually examining individual concentration-time curves and by Akaike Information Criterion (AIC) [23]. The pharmacokinetic characteristics were fitted to a two-compartment open model after the administration of ENR at recommended dose and administration route in same animals. Log trapezoid method were used for calculation of the $C_{max}$, $AUC_{0-24}$ and $AUC_{total}$. The $t_{max}$ was determined by direct investigation, $t_{1/2\beta}$ and the mean residence time (MRT) were calculated based on equation described by Wagner [24].

### *Statistical Analysis and Determination of the Bioequivalence*

All data were expressed as mean±SD. Differences at $P<0.05$ were considered significant. The means of $AUC_{0-24}$, $AUC_{total}$ and $C_{max}$ were calculated by using "two side t-test" (Minitab, Release 9.2, 1993). Before the BE decision, these parameters were compared the lower and upper limits of the confidence interval, the error variables of the intervals which had been detected within the ANOVA table previously. At the BE decision, according the AUC and $C_{max}$ parameters 90% confidence interval were within 0.7-1.43 limits after the log transformation [1,3,24]. Non-log transformed $t_{max}$ was considered as a second parameter and 90% confidence interval for this parameter was within 0.8-1.25 limits [1-3,24].

According to EMEA 2001 BE Guidelines, before performing the analysis of variance for AUC and $C_{max}$ parameters, log transformation of data is recommended, but for observed time dependent parameters (like $t_{max}$), this transformation is not applicable. The upper and lower limits of confidence interval must be within 0.8-1.25 or 0.7-1.43 for log transformed data and 0.8-1.2 or 0.7-1.3 for untransformed data [1].

## RESULTS

The pH values and amounts of active substance of products A and B were measured before study. The pHs were 10.910 and 11.145, the amounts of active substances were 95.7 mg/ml (A) and 106.0 mg/ml (B), respectively. After the pharmacokinetic calculations, the semilogarithmic plot of plasma concentration-time curves of two ENR preparations are shown in *(Fig 1)*. This graphic gave us an opportunity to directly observe the pharmacokinetic parameters.

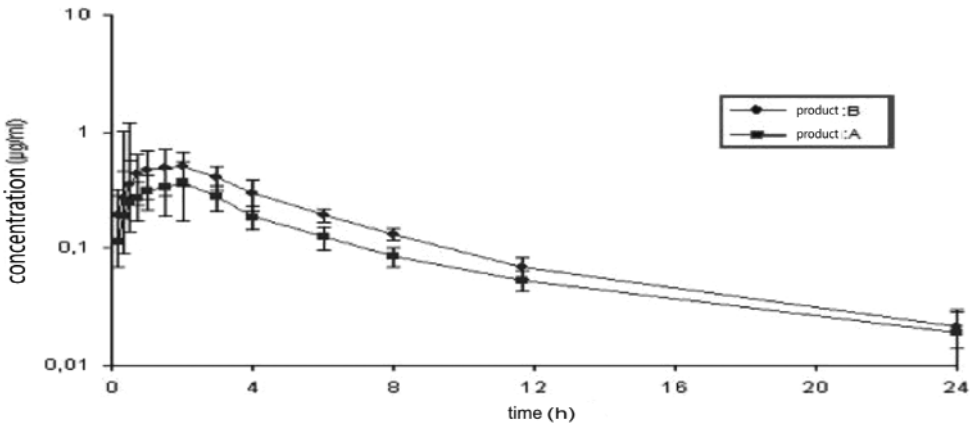


**Fig 1.** Semilogarithmic plot of plasma concentrations time curves of enrofloxacin formulations after single dose intramuscular (IM) administrations in heifers with (±SEM) (n=6)

**Şekil 1.** Enrofloksasin içeren ürünlerin düvelere kas içi yolla tek doz 2.5 mg/kg uygulanması sonrasında elde edilen yarı log ortalama (±SEM) konsantrasyon-zaman eğrileri (n=6)

In the present study, it was observed that the calculated parameters were eligible for direct observations of two compartment open pharmacokinetic model at the determined dose and administrative route.

Before log transformation, $AUC_{total}$ and $AUC_{0-24}$ were significantly different for $\mu_B$ and $\mu_A$ ($P<0.007$, $P<0.004$, respectively) *(Table 1)*. After log transformation this $\mu_B/\mu_A$ rates were found to be 1.34 and 1.29 respectively *(Table 2)*. The observed $t_{max}$ $\mu_B/\mu_A$ rate was (1.17) within the acceptable limits (0.8-1.25) *(Table 2)*. The MRT were found to be 6.35 h (B) and 7.66 h (A) *(Table 1)*. As defined above, all parameters were log transformed except $t_{max}$ and then, these parameters were proportioned ($\mu_B/\mu_A$) and discussed for the BE determination based on 0.7-1.43 interval *(Table 2)*.

slightly lower and this may be due to gender, age and breed. The $C_{max}$ has been determined and used as a second important parameter after the AUC in all BE trails [2-4,25-27]. For $C_{max}$ the ($\mu_B/\mu_A$) rates were found to be within 90% confidence interval at 0.7-1.43 limits and this was accepted to be satisfactory. $C_{max}$ has been determined and used as a second important parameter after the AUC in all BE trails [2-4,25-27]. For $C_{max}$ the ($\mu_B/\mu_A$) rates were found to be within 90% confidence interval at 0.7-1.43 limits and this was accepted to be satisfactory. For calculation of AUC and $C_{max}$ different methods have been used by certain authorities [2-6,25-28]. In similar trials, MRT [4,24], $t_{1/2\beta}$ [4] and $t>_{0.2}$ [2] parameters have been considered and used for BE determination in addition to $t_{max}$ [2,27]. In this study, the $AUC_{total}$, $AUC_{0-24}$, $C_{max}$ and $t_{max}$ were determined and used as principal evaluation criterias. After calculation, the data

***Table 1.*** *Before log transformed pharmacokinetic variables (mean±SD, minimum-maximum) obtained after single intramuscular (IM) administrations of enrofloxacin (2.5 mg/kg BW single dose) in six heifers (n=6)*

***Tablo 1.*** *Enrofloksasin içeren iki ürünün düvelere kas içi yolla tek doz 2.5 mg/kg uygulanması sonrasında log dönüşüm öncesi elde edilen ortalama (±SEM) farmakokinetik parametreleri (n=6)*

| Pharmacokinetic Parameters | Product (B) Mean±SEM (min-max) | Product (A) Mean±SEM (min-max) | P-Value |
|---|---|---|---|
| $C_{max}$ (ng/ml) | 548±74.0 (392-801) | 433±74.0 (211-728) | 0.326 |
| $t_{max}$ (h) | 1.75 | 1.50 | 1.000 |
| $AUC_{0-24}$ (µg.h/ml) | 3.379±0.232 (2.833-4.180) | 2.487±0.194 (2.097-3.035) | 0.004 |
| $AUC_{total}$ (µg.h/ml) | 3.469±0.224 (3.095-4.327) | 2.687±0.176 (2.196-3.141) | 0.007 |
| $t_{1/2\beta}$ (h) | 6.430±1.136(4.147-11.378) | 8.363±1.906(5.189-17.693) | 0.404 |
| MRT (h) | 6.349±0.616 (4.527-8.235) | 7.656±1.422(5.105-14.594) | 0.419 |

***$C_{max}$:*** *Maximum drug concentration,* ***$t_{max}$:*** *Time to reach $C_{max}$,* ***AUC:*** *Area under the concentration-time curve,* ***$t_{1/2\beta}$:*** *The half life of elimination,* ***MRT:*** *Mean residence time*

***Table 2.*** *After log transformed pharmacokinetic variables (mean±SD, minimum-maximum) obtained after single intramuscular (IM) administrations of enrofloxacin (2.5 mg/kg BW single dose) in six heifers (n=6)*

***Tablo 2.*** *Enrofloksasin içeren iki ürünün düvelere kas içi yolla tek doz 2.5 mg/kg uygulanması sonrasında log dönüşüm sonrası elde edilen ortalama (±SEM) farmakokinetik parametreleri (n=6)*

| Pharmacokinetic Parameters | Product (B) Mean±SEM (min-max) | Product (A) Mean±SEM (min-max) | $\mu_B/\mu_A$ |
|---|---|---|---|
| $C_{max}$ (ng/ml) | 2.739±0.056 (2.593-2.904) | 2.636±0.085 (2.324-2.862) | 1.04 |
| $t_{max}$ (h) | 1.75 | 1.50 | 1.17 |
| $AUC_{0-24}$ (µg.h/ml) | 0.525±0.029 (0.452-0.621) | 0.391±0.033 (0.322-0.482) | 1.34 |
| $AUC_{total}$ (µg.h/ml)) | 0.549±0.025 (0.491-0.636) | 0.425±0.029 (0.342-0.497) | 1.29 |
| $t_{1/2\beta}$ (h) | 0.779±0.069 (0.618-1.056) | 0.865±0.080 (0.715-1.248) | 0.90 |
| MRT (h) | 0.793±0.042 (0.656-0.916) | 0.798±0.022 (0.708-0.875) | 0.99 |

***$C_{max}$:*** *Maximum drug concentration,* ***$t_{max}$:*** *Time to reach $C_{max}$,* ***AUC:*** *Area under the concentration-time curve,* ***$t_{1/2\beta}$:*** *The half life of elimination,* ***MRT:*** *Mean residence time*

## DISCUSSION

Compared to the results of AUC on same dosage of ENR after IM administration in cows [9], the AUC's were

were verified *(Table 2)* and clinically relevant pharmacokinetic parameters as $t_{1/2\beta}$ and $V_d$ were similar to cows [4], and to other animal species [8,15,19,22]. The $C_{max}$ values product A and B were found to be similar with other studies [11,12] *(Table 1)*. After log transformation, differences

in $\mu_B/\mu_A$ rates (1.04) of $C_{max}$ values were found to be within acceptable limits and this further supports the BE decision in terms of the AUC. The aim of log transformation can be summarize like this; (a) to normalize the distribution of the parameter, (b) to ensure the additivity of the statistical model, (c) to stabilize its variance, and (d) to express the BE interval as a ratio (or percentage) [6,25].

The $\mu_B/\mu_A$ rate of observed $t_{max}$ was found within the acceptable limits (0.8-1.25) *(Table 2)* and this also supported the BE decision. However for this parameter, 0.7-1.43 limit can also be used, but in this study 0.8-1.25 limit was preferred. In studies carried out in different animal species, similar the results were obtained for $t_{max}$ values (for product A 1.5 h and B 1.75 h) [4,9,12]. In order to obtain the highest antibacterial efficiency and sustainibility, it is essential to reach the highest ENR concentration in general circulation. In this respect, it would be expected that $C_{max}$ and $t_{max}$ values of different preparations containing ENR to be similar to BE reference product and this is very important in terms of BE of these products [4].

The MRT played an important role for the determination and evaluation of possible differences on the absorption and elimination of any active substance(s) after administration. MRT is also important parameter for the comparison of different administration route and/or products [4-6,24]. In this study MRT obtained were found to be 6.35 h's (B) and 7.66 h's (A). For two products, the $t_{1/2\beta}$ values were calculated to be 6.43 h's (B) and 8.36 h's (A) and the $\mu_B/\mu_A$ ratio was 0.90 ($P>0.05$) *(Table 1)*. This was similar to results of the studies carried out in same animal species 5.68 h's [4] 5.9 h's [9]. The $t_{1/2\beta}$ was used as an additional parameter in some BE studies [4,26]. The ratio of $t_{1/2\beta}$ ($\mu_B/\mu_A$) was found to be 0.93 following log transformation *(Table 2)*, and using this parameters have supported the BE decision. However, the animal health is considered as a preference for the evaluation of the BE decision. Additionally, for the comparison of the products, AUC, $C_{max}$, $t_{max}$, $t_{1/2\beta}$, and minimal inhibitory concentrations (MIC) are generally used [4]. If $AUC_{total}$, $AUC_{0-24}$, $C_{max}$ and $t_{max}$ parameters are considered for BE decision, the ratio of $\mu_B/\mu_A$ of these pharmaceutical equivalent products should be within 0.7-1.43 limits at 90% confidence interval. The purpose of demonstrating the BE is to ensure similar clinical outcomes when changing formulations or using similar preprations or to prevent therapeutic gaps between formulations [25].

In conclusion, it is recommended that both products might be used as "inter-changeable drugs".

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