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The Investigation of Bioequivalence of Some Enrofloxacin Formulations Following Parenteral Administration in Calves

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SUMMARY

In this study, the bioequivalence of two enrofloxacin preparations were investigated. Ten unmedicated female calves (Jersey strain, 46-50 day-old) were included. The calves were divided into two groups including five calves each. Reference drug and test drug were administered intramuscularly at a dose of 2.5 mg per kg to group 1 and group 2, respectively. Blood samples were taken before (0.0 min) and after the drug administration at 0.25, 0.5, 1, 2, 4, 8, 12, 18, 24 and 36th hours. Before the plasma drug concentration measurements, enrofloxacin study standards and the new standards that were obtained after adding initial standards to drug-free plasma were applied to high-performance fluid chromatography (HPFC). Standard curves were drawn, and the recovery value was calculated. The sensitivity of the extraction method was detected 0.05 µg/ml. The mean recovery value of extraction procedure was about 75-96 percent. The average retention time for enrofloxacin was obtained at 6.61-6.6th. Plasma enrofloxacin concentrations were measured by HPFC following acetonitrile extraction process. The plasma concentration-time curve for each animal showed that enrofloxacin followed the two-compartment open model. It was seen that the area under the curve and tmax value (µT/ µR=120) of the test drug were acceptable (80–125%). However, Cmax value was not in the acceptable range (0.80-1.25). Test Drug was determined to be used instead of the reference drug.

Key Words: *Bioequivalence, Calf, Enrofloxacin, Pharmacokinetic, HPFC*

ÖZET

Parenteral Kullanılan Bazı Enrofloksasin Müstahzarlarının Buzağılarda Biyoeşdeğerliğinin Araştırılması

Bu çalışmada, buzağılarda parenteral olarak kullanılan iki farklı enrofloksasin müstahzarının biyoeşdeğerliği incelendi. Çalışmada ilaç uygulanmamış, Jersey ırkı 46-50 günlük 10 dişi buzağı kullanıldı. Hayvanlar her birinde 5 hayvan bulunan iki gruba ayrıldı. Grup 1'e Referans İlaç, Test İlaç Grup 2'ye 2.5 mg/kg c.a dozunda, kas içi uygulandı. Hayvanlardan uygulama öncesi (0.0 dk), ilaç uygulamalarını takiben 0.25 saatten başlamak üzere 0.5., 1., 2., 4., 8., 12., 18., 24. ve 36. Saatlerde kan alındı. Plazma ilaç yoğunluğu ölçülmesinden önce; enrofloksasin çalışma standartları ve bu standartların ilaç içermeyen plazmalara katılarak elde edilen standartlar yüksek basınçlı sıvı kromatografisine (YBSK) uygulandı. Uygulamalar sonrası standart eğriler çizildi ve geriye kazanç hesaplandı. Yöntemin duyarlılığı 0.05 µg/ml olarak tespit edildi. Geriye kazanç %75-96 olarak bulundu. Enrofloksasinin 6.61-6.66 dakikalarda pik verdiği belirlendi. Plazmada enrofloksasin yoğunluğunun ölçülmesi için; plazmaların asetonitril ile özütleme işlemi yapıldı ve YBSK'ya uygulandı. Her hayvan için çizilen plazma yoğunluğu-zaman eğrisinden ilacın vücutta 2- bölmeli dışarı açık modele göre dağıldığı anlaşıldı. Test İlacın (plazma ilaç yoğunluk-zaman eğrisi altındaki kalan alan) EAA ve tdoruk (plazma ilaç yoğunluğunun doruk değere ulaşma süresi) değerinin (µT/ µR=120) kabul edilebilir sınır içerisinde olduğu (%80–125) ancak Test İlacın Ydoruk (plazma doruk ilaç yoğunluğu) değerinin kabul edilebilir sınırlara (0.80-1.25) uymadığı görüldü. Sonuç olarak Test İlacın Referans İlaç yerine kullanabileceği anlaşılmıştır.

Anahtar Kelimeler: *Biyoeşdeğerlik, Buzağı, Enrofloksasin, Farmakokinetik, YBSK*

INTRODUCTION

Bioequivalence is the property of two pharmaceutically equivalent products (test and reference) that possess similar bioavailability and produce the same effect at the site of physiological activity (Kaya 2000; Kayaalp 2008; Traş and Yazar 2002). For bioequivalence, the area under the curve (AUC) and maximum serum concentration (Cmax) data for the bioavailability of two drugs must be at the 90% confidence interval and between 80-125% (Hantash et al. 2008). Bioequivalence tests play a major role in the evaluation of drug formulations with similar plasma drug concentration-time curve and the same pharmacological and clinical

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effects (Toutain and Koritz 1997). It is a requirement that documents relating to bioavailability and pharmacodynamics studies should be included for the registration of drugs. Exploring the bioequivalence of the reference drug (drug produced by the pioneer company) and the generic drug (drug produced by any company after the reference drug was produced) is the most important factor in proving that the generic drug may be used instead of the reference drug as an equivalent drug (Öner 2003). The interchangeable usage, as well as the quality, efficacy, and safety of the drugs, are important in the licensing, in the freedom of the health professionals and also for the cost of the treatment.

Enrofloxacin is a bactericidal antimicrobial drug in the fluoroquinolone group, developed for use only in the veterinary field (Cabanes et al. 1992; Davidson et al. 1986; Kaya 2000). The spectrum of the effect of enrofloxacin is broad. It is effective against gram negative and Gram positive bacteria, Mycoplasma, Rickettsia, Ehrlichia and Chlamydia (Elmas et al. 2001; Elmas et al. 2000). Enrofloxacin is used in all pets, especially in the ruminant and poultry. In calves, enrofloxacin finds utilization in the respiratory system infections, septicemia caused by colibacillosis and in cases of intestinal inflammation by oral and parenteral ways (Bauditz 1987; Davidson et al. 1986; Kaartinen et al. 1997).

In this study, we aimed to investigate the bioequivalence of 2 formulations containing enrofloxacin in calves.

MATERIALS and METHODS

Chemicals, Drugs, and Solutions

The chemicals, drugs, and solutions that were used in the study are as follows

- Acetonitrile (ACN): Gradient Grade for Liquid Chromatograph, Merck 100030
- Triethylamine (C2H5)3N: Sigma T-0886
- Methanol: Gradient Grade for Liquid Chromatograph, Merck 100067
- Deionised water
- Enrofloxacin: effectiveness, 100.3%; production date: 01,01,2009, Expiry date: 01.01.2011; Serial Number: 176293; Lot 010001160374; Batch KP047PZ. Provided by Bayer Turk; Reference drug: 50 mg/ml injectable enrofloxacin, 20 ml per vial
- Test Drug: 100 mg/ml injectable enrofloxacin, 20 ml per vial

The reference and test drugs were provided directly by producing company. The production and expiry date of the drugs and serial numbers were noted. The amount of active ingredients of the drugs were established. These values were measured as 53.5 µg/ml for the reference drug and 95.6 µg/ml for the test drug. The amounts of active ingredient per volume were determined as normal.

Standards

Standard Solution: For enrofloxacin stock standard (1mg/ml) preparations, 10mg enrofloxacin was weighed and dissolved in 10ml methanol.

Enrofloxacin study standard 1 (0.1mg/ml): 100µl of 1mg/ml enrofloxacin stock standard was taken and prepared by filling to 1ml with methanol. 0.25 and 0.5µg/ml dilutions were prepared from this study standard solution.

Enrofloxacin study standard 2 (0.01mg/ml): 100µl of enrofloxacin study standard 1 was taken; filled to 1ml with methanol. 1, 2.5 and 5µg/ml dilutions were prepared from this study standard solution.

Trial Animals and Their Care-Feeding

The study was conducted with ten female calves of Jersey race and 46-50 days old (mean 40-45 kg) at Karaköy Agricultural Enterprise. The animals were taken to a separate environment 15 days in advance, and medication administration was restrained. Throughout the trial, the animals were fed with unmedicated feed. Calf growing feed, water, and hay were given freely as feed. They continued to be fed by 3 liters of milk twice a day. The study was reviewed by Ankara University Animal Trials Local Ethics Committee and approved with decision number 2007-7-17 and file number 2007-56.

Grouping of the Animals, Administrating the Drugs, Collecting Blood Samples and Drug Analysis in Plasma

The animals were weighed one day before the drug administration for accurate dosage purposes. The animals were divided into two groups (Group 1 and Group 2) each consisting of 5 animals. Group 1 was assigned to the reference drug group, and Group 2 was assigned to the test drug group. In both groups, the drugs were administered intramuscularly (m. semitendinosus, m. semimembranosus). The dose for all administrations was determined as 2.5mg/kg. 8-10 ml blood was collected from the animals through *V. jugularis* in vacuumed heparinized tubes before the administration (0.0 min.), and starting at 0.25th hour following the drug administration at 0.5th, 1st, 2nd, 4th, 8th, 12th, 18th, 24th and 36th hours. Plasmas were stored at -20°C until the drug analyses. The analyses were performed within two months. Sixty minutes after collecting the blood samples, the plasma was separated through centrifuge at 3000 rpm for 10 minutes. The extraction and sample measurement in enrofloxacin analyses in the plasma was conducted by Düzen Laboratories Group using HPFC. In the extraction and measurement of enrofloxacin from the plasma, the method used by Anadon et al. (1995) was taken as a basis. According to this, 200µL plasma was taken into Eppendorf tubes. 300µL acetonitrile was added. The tubes were capped and gently vortexed (2 min.). Then, they were centrifuged at 2500 rpm for 10 min. The acetonitrile part at the top was taken into vials with an insulin injector. 300µL deionized water was added and gently vortexed (0.5 min.). The vials were submitted to HPFC. The automated sampler was set to 280nm wavelength and to use 20µL from this extract. It was submitted to the device for 12 min. The flow rate of the mobile phase was set at 1ml/min and the column temperature as 30 ºC. Before the extraction, the retention time, recovery and sensitivity limit were calculated. The standard curves were drawn according to the results obtained. For these studies, enrofloxacin study standard solutions 1 (0.1mg/ml) and 2 (0.01mg/ml) were prepared with methanol. From these standard study solutions, 0.25, 0.5, 1, 2.5, 5 µg/ml dilutions of enrofloxacin were prepared with methanol and plasma.

HPFC parameters and instrument properties are as follows:

- High-Pressure Liquid Chromatograph (HPFC): Agilent Technologies, Model 1100 series, California) and attachments
- Detector: VWD, G1314A, Serial JP24020987
- Column heater: COLCOM, G1316A, Serial DE32133649
- Autosampler: ALS, G1329A, DE23910894
- Column: Nucleosil 100 - 5 C18 150, 4,6 µm Serial No: E 10021556, Macherey-Nagel

- Wavelength: 280 nm
- Mobile Phase Flow Rate: 1 ml/min
- Column Temperature: 30 °C
- Mobile Phase: 850ml deionized water + 150ml Acetonitrile + 5ml triethylamine (pH was set as 2.5 with orthophosphoric acid).

Pharmacokinetic Calculations and Statistical Calculations

The area under the plasma drug concentration-time curve (AUC), the time for plasma drug concentration to reach the peak value (Tmax) and plasma peak drug concentration (Cmax) were calculated. The other pharmacokinetic calculations using the Tmax and Cmax plasma drug concentration curve were made with Pharmacokinetic Calculation (PKCALC) program which is based on the equation reported by Shumaker (1986) and Wagner (1979). For the statistical calculations, the "SPSS 11.0 for Windows" statistics package program was used. Data were presented as an arithmetic mean±standard deviation or the pharmacokinetic data, One-Way Analysis of Variance (ANOVA) was used, and the differences between the groups were evaluated through Duncan test.

RESULTS

The standards prepared using the enrofloxacin active ingredient were injected to HPFC and the retention time was determined as 6.61-6.66 min. (Figure 1) The peak areas obtained were recorded. The extraction was performed by adding these standards to unmedicated plasma, and they were placed in the autosampler. The autosampler used 20µl of that solution. The standard curve was drawn using the peak areas obtained from the standards, and the equation of the resulting curve was calculated (Figure 2). By using these curves, the recovery value was found as 75-96%. The peak areas obtained from the plasmas were inserted into the equation, and the enrofloxacin in the plasmas was determined.

As a result of the trials conducted with enrofloxacin study standards, it was understood that 0.05µg/ml enrofloxacin might be measured with the method. The active ingredient amounts in the measurements for the control of Reference and Test Drugs used in this study were measured as 5.35 µg/ml for the Reference Drug and 9.56 µg/ml for the Test Drug.

The enrofloxacin concentrations measured in plasma samples following the administration of Reference and Test Drugs to the animals in Group 1 and Group 2 are given in Table 1, and the plasma drug concentration-time curve is presented in figure 3. In Table 1 and at 0.8-1.2nd hours following the administration of test Drugs, it is seen that the plasma enrofloxacin concentration reaches the peak value (1.116-1.664 µg/ml); the plasma drug concentration remains at ≥ 0.5 µg/ml for about 4 hours; and that there is 0.04 µg/ml plasma drug concentration at 36th hour.

The pharmacokinetic variances following the intramuscular administration of reference and test drugs to group 1 and group 2 are presented in Table 3.

Table 1. Plasma drug concentrations of enrofloxacin by time

Time (hour)	Enrofloxacin (µg/ml)	
	Reference Drug Group 1	Test Drug Group 2
0.25	0.80 ± 0.24	0.92 ± 0.28
0.5	1.01 ± 0.22	1.22 ± 0.37
1	1.10 ± 0.22	1.614 ± 0.43
2	0.80 ± 0.21	1.56 ± 0.28
4	0.58 ± 0.26	0.93 ± 0.42
8	0.16 ± 0.09	0.33 ± 0.11
12	0.08 ± 0.04	0.14 ± 0.04
18	0.05 ± 0.01	0.07 ± 0.02
24	0.04 ± 0.00	0.05 ± 0.01
36	0.04 ± 0.00	0.04 ± 0.00

Table 2. Some pharmacokinetic variables as a result of logarithmic transformation of reference and test Drugs

Variables	Reference Drug	Test Drug	µT/µR
AUC (µg. hour/L)	0.80 ± 0.45^{a} (0.69-0.94)	1.00± 0.40^{b} (0.86-1.11)	1.24
Cmax (µg/ml)	0.43 ± 0.03 (0.7-0.16)	0.21 ± 0.43 (0.08-0.29)	0.48
Acceptable Limit	0.80-1.25		

Table 3. Pharmacokinetic variances of reference and test drugs

Variables	Reference Drug	Test Drug
AUC (µg. hour/L)	6.54±0.69^{a} (4.87-8.63)	10.33 ± 0.89^{b} (7.23-12.7)
OKS (hour)	17.05±2.23 (9.05-21.45)	24.71±14.19 (9.91-81.47)
α (hour $^{-1}$)	0.35±0.05 (0.24-0.53)	0.32±0.04 (0.21-0.46)
β (hour $^{-1}$)	0.03±0.00 (0.02-0.07)	0.13±0.88 (0.04-0.49)
ka (hour $^{-1}$)	4.014±0.66^{a} (2.31- 5.38)	1.80±0.23^{b}(1.27-2.61)
t½α (hour^{-1})	2.09±0.27(1.30-2.88)	2.29±0.34 (1.48-3.23)
t½β (hour)	21.25±3.80 (8.91-31.48)	30.83±15^{b}.15 (14.0-91.4)
t½a (hour)	0.01±0.03^{a} (O.11-0 .29)	0 .408±0.04^{b} (0.26-0.54)
Tmax (hour)	1.00±0.27 (0.5-2)	1.20±0.20 (1.00-2.00)
Cmax (µg/ml)	1.12±0.10 (0.85-1.44)	1.66±0.154 (1.91-1.96)

AUC: Area under curve; a,b: The difference between the groups with different letters in the same row is significant (p<0.05); ca: In absorbed administration, the first degree absorption rate constant; a: Plasma drug concentration diffusion period rate constant; P: Plasma drug concentration elimination period rate constant; t½a : in case of oral administration, the half life of absorption from the digestive tract; t½α: Diffusion period half life

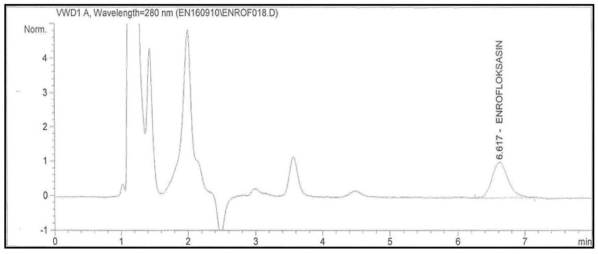

Figure 1. Chromatogram of enrofloxacin active ingredient (20µl from 1 µg/ml solution).

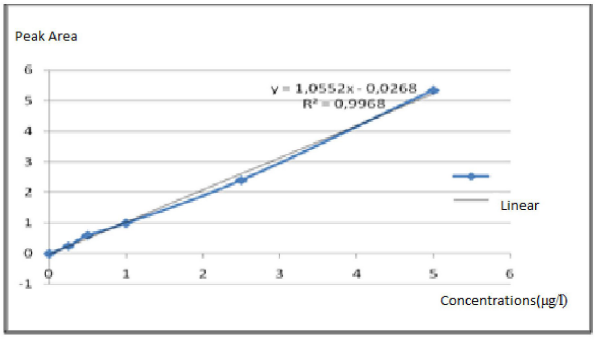

Figure 2. The standard curve prepared using the peak areas in high-pressure liqıid chromatography of enrofloxacin at a certain concentration.

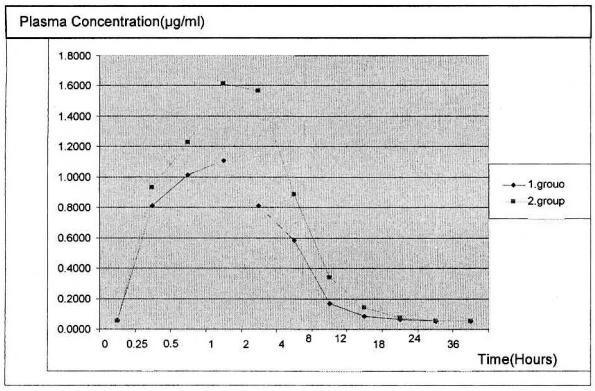

Figure 3. Plasma drug concentration-time curves of enrofloxacin drawn upon intramuscular administration of 2.5 mg/kg reference and test drugs.

DISCUSSION

Many drug preparations of enrofloxacin have a wide area of use in calves. This study regarding the bioequivalence of enrofloxacin has a specific quality as it contributes to bioequivalence research. The results to be obtained are intended to enlighten the research and development studies in this area. In Turkey, there are 382 enrofloxacin drug substances in various formulations which are suitable for parenteral administration in cattle and calves. (Anonymus, 2017) Despite that much preparations; there are no studies available regarding the bioequivalence of this drug in calves.

In the study, the sensitivity of the method was determined as 0.05 µg/ml for enrofloxacin. In this respect, this value is lower than Anadon et al.'s (Anadon et al. 1995) (0.003 µg/ml, HPFC); similar to Posyniak et al.'s (Posniyak et al. 2001) (0.02 µg/ml, HPFC); and higher that Şahan and Kaya's (Sahan and Kaya 2006) (0.1 µg/ml enrofloxacin; agar gel disk diffusion method) findings. The differences of the analysis method regarding sensitivity may be attributed to the analyses methods of the samples and the properties of HPFC instrument and the study conditions. In this study, the sensitivity limit of the method was found sufficient as it was possible to measure minimal values (50x) from the lowest effective concentration (<0.5 µg/ml) of enrofloxacin in the plasma.

The recovery ratio in the study was found as 80-96%. These values were obtained similar to the values determined by Posyniak et al. (2001) and Anadon et al. (1995) in plasma, and by Sumana et al. (2001) in serum (<90%, 87%, 92-97%, respectively). When compared with literature data, it was concluded that the recovery ratios found in the study were suitable enough to render the results reliable.

The area under the curve (AUC) was found 6.54±0.69 µg.hour/L for reference drug and 10.33±0.89 µg.hour/L for test drug. The AUC value of reference and test drugs administered intramuscularly was determined to be lower than the reference and test drug values found by Posyniak et al. (2001) by oral gastrointestinal administration (18.653±1.846 µg.hour/L, 17.934±1.636 µg.hour/L, HPFC, respectively), the value found by Elmas et al. (2001) (Group 2 intramuscular 19.07±02.43; HPFC), the value found by Kaya et al. (1996) (18.395±2.220 µg.hour/L for oral gastrointestinal administration for test drugs; 26.91±7.97 µg.hour/L, agar gel disk diffusion method); the value found by Parlar and Kaya (2005) (30.7±4.8 µg.hour/L for reference drug administered orally through drinking water, 41.3±3.4 µg.hour/L and 31.2±3.5µg.hour/L for test drugs; agar gel disk diffusion method).

Tmax of enrofloxacin in plasma was determined as 1.00±0.27 hours for the reference drug and 1.20±0.20 hours for the test drug. It was understood that it showed similarity with the value found by Elmas et al. (2001) (Group 2 intramuscular 1.09±0.28 hours, HPFC). The Cmax was found 1.12±0.10 µg/ml for reference drug and 1.66±0.154 µg/ml for test drug. In the study, Cmax value for reference and test drugs was determined to be similar to the reference and test drug values of Posyniak et al. (2001) (0.92±1.105 µg/ml; 0.98±0.099 µg/ml, respectively; gastrointestinal administration, HPFC) and of Anadon et al. (1995) (1.4 µg/ml; for enrofloxacin administered orally through drinking water; agar gel disk diffusion method); and it was determined to be lower than the value found by Elmas et al. (2000) (Group 2 intramuscular, 3.25±0.29 µg/ml, HPFC).

The drugs are accepted as bioequivalent in case the pharmacokinetic variables such as AUC, Cmax, and Tmax of the reference and test drugs are within the range of bioequivalence acceptance limits (0.80-1.25 or 80-125%). As Cmax value shows a wide variability depending on the sampling time, it may be accepted within 70-143% limits (Kayaalp 2008; Tras and Yazar 2002). When AUC and Cmax are evaluated by logarithmic transformation, the value obtained by dividing the AUC value of test drug (1.00±0.40 µg.hour/L) by reference drug value (0.80±0,45 µg.hour/L) is 1.24. The value obtained by dividing the Cmax value of the test drug (0.21±0.43 µg/ml) by the reference drug value (0.43±0.03 µg/ml) (µT/µR) is 0.48. The value obtained by dividing the test drug Tmax value, for which logarithmic transformation is not possible as it is a time-dependent parameter, (1.20±0.20 hours) by the reference drug value (1.00±0.27 hours) (µT/ µR) is 120. When the bioequivalence of the test drug is evaluated, it is seen that the Tmax (µT/µR=120) is within the acceptable limit (80–125%); and the AUC value of test drug when the logarithmic transformation is applied (µT/µR=1,24) is within the acceptable limits (0.80-1.25). However, it is

seen that the Tmax value of the test drug (μT/ μR=148 is μT/μR=0,48 after logarithmic transformation) is not within the acceptable limits.

As a result of evaluating the analyzed pharmacokinetic criteria (AUC, Cmax, Tmax) of reference and test drug products containing enrofloxacin, it was understood that the test drug was not bioequivalent in terms of Cmax, but it was bioequivalent in terms of AUC and Tmax, and that the test drug may be used as substitute for the reference drug. The higher Cmax value of the test drug compared to the reference drug indicates that it is better absorbed at the drug absorption point. This is considered to arise from the failure to adjust the exact dose, individual differences of the animals in the trial group, and from the analysis methods.

CONCLUSION

With this study, it is intended to promote the bioequivalence studies in veterinary drugs and to enlighten the research and development studies based on the results to be obtained. The limited number of the studies conducted in this area also restricts the path to follow, method and the interpretations of the results. Therefore, this study will constitute a resource for the bioequivalence studies in the veterinary field.

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