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# TISSUE DISTRIBUTION AND RESIDUE DEPLETION OF FLUMEQUINE IN THE RABBIT

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**Abstract:** Flumequine is a fluoroquinolone derivative used in food-producing species to control systemic infections caused by susceptible microorganisms, in particular Gram negative species such as *Escherichia coli*, *Salmonella* spp. and *Pasteurella* spp. Our study was carried out in order to evaluate the distribution and residue depletion of flumequine in rabbits. Tissue distribution was defined administering a single oral dose of 15 mg of flumequine per kg body weight. Residue depletion was determined administering the drug *via* drinking water at the ranging dose of 15 mg per kg body weight for 5 days. The tissue concentrations were quantified using a HPLC method, with a quantification limit of 25 $\mu g.kg^{-1}$ for muscles, fat and lungs and of 50 $\mu g.kg^{-1}$ for livers and kidneys. The experimental results show that in rabbits flumequine reaches effective tissue concentrations rapidly after oral treatment. At the moment of sacrifice (withdrawal time 0 hours) the residue depletion study showed the highest concentrations in the kidney and the liver (2064 with SD 1571 and 388 with SD 25 $\mu g.kg^{-1}$, respectively), while in the other tissues analysed (muscles, fat and lungs) the residues were much lower (27 with SD 30, 38 with SD 12, 60 with SD 34 $\mu g.kg^{-1}$ in muscles, fat and lungs, respectively). The residue concentrations decrease quickly and fall below the maximum residual limits, as defined by the European Authorities (200, 250, 500 and 1000 $\mu g.kg^{-1}$ for muscles, fat, livers and kidneys, respectively), within 24 hours from the cessation of medication. Considering the tissue concentrations observed after the repeated administration it can be concluded that at the dose employed (15 $mg.kg^{-1}$) potentially effective drug concentrations are recorded only in the liver and the kidney.

**Key words**: flumequine, rabbit, residues, tissue distribution.

## INTRODUCTION

As reported in a previous paper (VILLA *et al.*, 2001), in all food-producing species, it is very important to guarantee the correct use of drugs in order to ensure the production of safe foodstuff for human consumption. A proper pharmacological

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therapy is based on various aspects; however, there are two main problems that must be considered. The definition of dosing regimens must be to achieve prefixed therapeutic objectives in the target species, while also protecting the environment and reducing the cost of therapy. Besides, the determination of the withdrawal times must be adequate in order to ensure the absence of tissue residues higher than the maximum residual limits (MRLs) in food intended for human consumption.

As stated in the Report by the Committee for Veterinary Medicinal Products (CVMP) (EMEA, 1997), these aspects are investigated principally in the so-called major species and scarcely in the minor animal species (equine, ovine-caprine, poultry [nonbroiler], rabbits, fishes [non-salmonidae]). In relation to the world-wide food-stuff production at world-wide, minor species, have a reduced importance; however, locally, in well-defined geographical areas, they are very important.

Few experimental studies on tissue distribution and residue depletion of the drugs used in this animal species have been undertaken and published. In this study the distributive and excretive behaviour of flumequine, a synthetic antibacterial drug that is increasingly being used on rabbits, was investigated. Structurally related to nalidixic acid (MEVIUS *et al.*, 1990a; REYNOLDS, 1996; DELMAS *et al.*, 1997), Flumequine (9-fluoro-6,7-dihydro-5-methyl-1-oxo-1H,5H-benzo[i,j]quinolizine-2-carboxylic acid) is a second generation quinolone developed for use in veterinary medicine. Flumequine (FLU) is never administered on humans because of the availability of more active compounds with broader antimicrobial spectrum and a better tissue distribution (LEMELAND *et al.*, 1981; CRUMPLIN, 1988). Conversely, in several animal species (ruminants, swine, fowl, fish) the drug is diffusely used to control systemic infections caused by Gram negative microorganisms (*e.g., Escherichia coli, Salmonella* spp. and *Pasteurella* spp.) (DORRESTEIN *et al.*, 1983; PIJPERS *et al.*, 1989; MEVIUS *et al.*, 1990b; WERKMAN, 1996). The minimal inhibitory concentrations, such as $MIC_{90}$ (the least amount of antibiotic that will inhibit the growth of 90% of the test organisms) for the above reported strains, ranges from about 0.1 to 0.8 $\mu g.mL^{-1}$, (CHEVALIER *et al.*, 1982; ZIV *et al.*, 1986; ATEV *et al.*, 1987; HANNAN *et al.*, 1989; PIJPERS *et al.*, 1989).

FLU is slightly metabolised by the liver, and its principal metabolite, identified in faeces and urine, is hydroxylated. The extent of this metabolism ranges from 10 to 20%. For this reason only flumequine as parent compound is considered to be the Marker Residue in the definition of the MRL.

Several studies are available on the kinetics of FLU in ruminants and fowl when administered at doses ranging from 5 to 15 $mg.kg^{-1}$ body weight (b.w.) (GOREN *et al.*, 1982; ZIV *et al.*, 1986; MEVIUS *et al.*, 1990a; DELMAS *et al.*, 1997). Conversely, like the information pertaining to most antimicrobials administered to rabbits, FLU information for this species is scarce. Consequently, the clinical protocols and the withdrawal times for FLU are often defined on the basis of experimental studies performed on other species.

The objective of this study is to define the tissue distribution and the residue depletion of FLU in rabbits orally treated. This was done in order to verify the usefulness of the drug in controlling systemic infections by susceptible pathogens and to determine its distributive ability in the most important tissues while calculating an appropriate withdrawal time considering the MRL values (200, 250, 500 and 1000 $\mu g.kg^{-1}$ for muscles, fat, livers and kidneys, respectively) as established by the European Authorities (EMEA, 2002).

## MATERIALS AND METHODS

The experiment was carried out on 52 "French crossbreed" food-producing rabbits from the Perego farm in Milano, Italy.

### Distribution study

*Animals*: 24 rabbits of both gender, with a mean weight of 2.7 kg and clinically healthy, were used. The animals were caged individually and submitted to a 12-h light/dark cycle in accordance with European requirements (EEC, 1986). They were housed for an acclimatisation period of seven days before the start of the experiment.

During periods of acclimatisation and experimentation, the animals were fed only commercial pellets with no active ingredient potentially interfering with the FLU titration. The pelletted feed was produced by Martini & Co. in Forlì, Italy. The animals had free access to food and water.

*Treatment*: Four animals out of 24 were not treated in order to obtain negative controls. The other rabbits were treated individually by gavage feeding at a dose of 15 $mg.kg^{-1}$ b.w., a commercial formulation (*Flumexil granulato idrosolubile 10%,* A.T.I., Italy) being administered. During the gavage feeding process, a mouth gag prepared from the plastic case of a 5 ml syringe and a urinary catheter (Rusch Nelaton 40 CH.14) were used.

*Sacrifice and Sample collection:* the 20 animals treated were allocated *ad random* to 5 groups (4 animals per group, two males and two females). The treated groups were sacrificed at 1, 3, 6, 9 and 12 hours post-treatment; the control animals were divided ad random into 2 groups of 2 subjects (1 male and 1 female) and sacrificed at the time-point of 3 and 6 hours post-treatment. All the animals were euthanised in a $CO_2$ chamber in accordance with the requirements set out in Recommendations for Euthanasia of Experimental Animals (Commission of the European Communities, 1993). From each animal, samples of muscles, livers, kidneys, fat and lungs were collected and stored at – 80°C pending assay.

**Residue depletion study**

*Animals*: 28 clinically healthy rabbits of both genders, with a mean weight of 2.5, were used. As reported in the distribution study, the animals were caged individually, acclimatised, maintained and fed. During the whole experimental period food and water consumption was registered daily.

*Treatment*: 4 animals were not treated in order to obtain negative controls. The other 24 animals were treated for 5 days with medicated drinking water (150 ppm of FLU) prepared daily using a commercial-type formula (*Flumexil granulato idrosolubile 10%,* A.T.I., Italy). In order to ensure a daily intake of about 15 mg of

FLU per kg b.w., the amount of the drug dissolved in drinking water was calculated in accordance with the recorded water consumption. Medicated water was administered to rabbits from individual bottles and the individual water consumption and the consequent drug assumption were recorded daily.

*Sacrifice and Sample collection*: after the administration, the animals were randomly allocated in 4 groups of 6 animals (3 males and 3 females) and sacrificed at 0, 24, 48 and 72 hours; the control animals were randomly divided in 2 groups of 2 animals (1 male and 1 female) and sacrificed at time-point 0 and 24 hours post-treatment. The animals were euthanised as reported in the distribution study. Furthermore, the samples collected and the storage conditions were the same as in the previous trial.

**Method of analysis**

HPLC was used for the analyses. Samples (5 g) were homogenised with 20 ml of extraction buffer (A: an aqueous solution of metaphosphoric acid 1%; B: methanol [A 60%: B 40%]). After sonication in a water-bath (30 min at 50°C) and centrifugation (10000 *g* per 10 min at 4°C), the liquid phase was cleaned up using a SPE column Sep-Pak VAC C18 (Waters, Millipore, Italy). Elution was carried out using 10 ml of methanol, which was dried under nitrogen flow and then added with 1 ml of ortophosphoric solution 0.02M. The eluates were assayed under the following chromatographic conditions: ortophosphoric acid 0.02M, acetonitrile and tetrahydrofuran (69:18:13) during the mobile phase; 1 $ml.min^{-1}$ flow rate; 325 nm excitation wavelength; 365 nm emission wavelength; chromatographic column: 5 ODS (3) Prodigy, 5 mm, 250 x 4.6 mm (Phenomenex, Torrance, USA). The limits of quantification (LOQ), which are the lowest concentrations at which the method of analysis is able to quantify the substance with sufficient linearity, accuracy and reproducibility, were 25 $\mu g.kg^{-1}$ for muscles, fat and lungs and 50 $\mu g.kg^{-1}$ for livers and kidneys. The limits of detection (LOD), which are the lowest concentrations at which the method is able to recognize, though without quantifying the substance, were 5.9, 5.9, 4.7, 4.5 and 4.1 $\mu g.kg^{-1}$ for muscles, livers, kidneys, fat and lungs, respectively. The method, validated intra-laboratory, was specific, linear, reproducible and accurate. Mean recoveries were 99.5, 94.6, 100.9, 92.7, 90.1 %

for muscles, livers, kidneys, fat and lungs, respectively.

**Statistical analysis**

The statistical analysis of the groups was undertaken using the Kruskal-Wallis test (non-parametric ANOVA) with Dunn's post test, performed with GraphPad InStat Version 3.00 for Windows 95/NT, GraphPad Software (San Diego, CA, USA).

## RESULTS

The LOQs were set taking into account the suggestions of the CVMP (EMEA, 1996) for a LOQ having at least half the value of the established MRL. Our method was also linear at concentrations far below half the MRL, and therefore we decided to set the LOQs at the lowest tissue concentrations where the validation criteria were satisfied (25 $\mu g.kg^{-1}$ for muscles, fat and lungs and 50 $\mu g.kg^{-1}$ for livers and kidneys). The LOD values were calculated as mean of the noise threshold of 20 chromatograms of blank samples plus 3 times the standard deviations. In Tables 1 and 2 the residue values, presented as mean ± standard deviation, are sometimes below the LOQ defined for that tissue. The reason for this is that in the calculation of the mean values the results below LOQ (<LOQ) were considered as the LOQ value and the ND (not detected) as 0.

The experimental data obtained following individual administration by gavage feeding is reported in Table 1. In all the tissues investigated, mean peak concentrations were achieved about 1 hour after treatment. Thereafter the kinetic profiles of liver and kidneys showed a constant decrease until the 12$^{th}$ hour post-treatment (200 ±8 $\mu g.kg^{-1}$ and 632±173 $\mu g.kg^{-1}$, respectively). For muscles, FLU was measured only until the 6$^{th}$ hour post-treatment (27±21 $\mu g.kg^{-1}$); then, in all the other samples FLU concentrations were not detectable or below LOQ. For lungs, after the peak levels recorded at the first collection time, the FLU concentrations resulted quite constant at the 3h and 6h time-points, and then decreased below the LOQ as mean value at the 9h collection time. Finally, for fat FLU concentrations also resulted low at the

**Table 1:** FLU concentrations ($\mu kg^{-1}$) after gavage administration at 15 $mg.kg^{-1}$.

| Sacrifice Time | Animal | Liver | Kidney | Muscles | Lungs | Fat |
|---|---|---|---|---|---|---|
| 1h | ♀ | 1382 | 4440 | 28 | 196 | 51 |
| | ♂ | 1677 | 1536 | 95 | 207 | 38 |
| | ♂ | 2758 | 6625 | 176 | 287 | <LOQ |
| | ♀ | 2446 | 6325 | 56 | 254 | 51 |
| Mean | | 2066(§) | 4732(§) | 89(#) | 236 | 41(§)(#) |
| SD | | 644 | 2340 | 64 | 42 | 12 |
| 3h | ♀ | 478 | 4473 | 49 | 55 | ND |
| | ♂ | 1716 | 4245 | 47 | 202 | <LOQ |
| | ♂ | 2065 | 5382 | ND* | 212 | 33 |
| | ♀ | 1187 | 3754 | 76 | 114 | 40 |
| Mean | | 1362 | 4464(§) | 43 | 146 | 25 |
| SD | | 691 | 682 | 32 | 75 | 17 |
| 6h | ♂ | 861 | 2887 | ND | 96 | <LOQ |
| | ♂ | 832 | 1291 | 49 | ND | <LOQ |
| | ♀ | 1988 | 1232 | <LOQ** | 353 | ND |
| | ♀ | 818 | 1970 | 34 | 107 | ND |
| Mean | | 1125 | 1845 | 27 | 139 | 13 |
| SD | | 576 | 771 | 21 | 150 | 14 |
| 9h | ♀ | 366 | 1850 | ND | <LOQ | ND |
| | ♂ | 410 | 1251 | ND | 31 | ND |
| | ♀ | 165 | 738 | ND | ND | ND |
| | ♂ | 397 | 897 | ND | <LOQ | ND |
| Mean | | 335 | 1184 | 0 | 20 | 0 |
| SD | | 115 | 493 | | 14 | |
| 12h | ♂ | 191 | 423 | <LOQ | <LOQ | ND |
| | ♂ | 210 | 842 | ND | <LOQ | ND |
| | ♀ | 198 | 664 | ND | <LOQ | ND |
| | ♀ | 201 | 598 | <LOQ | <LOQ | ND |
| Mean | | 200 | 632 | 13 | 25 | 0 |
| SD | | 8 | 173 | 14 | 0 | |

LOQ muscles, fat and lungs= 25 $\mu g.kg^{-1}$; LOQ liver and kidney = 50 $\mu g.kg^{-1}$; *ND = not detected, considered as 0 in the statistical analyses; ** <LOQ = residues below the LOQ were considered as LOQ in statistical analyses; (§)= significantly different from concentrations at 12h ($P<0.05$); (#)= significantly different from concentrations at 9h ($P<0.05$); SD = standard deviation.

first collection time (41±12 $\mu g.kg^{-1}$), decreasing rapidly until reaching values either below LOQ or undetectable between 3h and 6h time-points.

The relevant variations of the rabbits recorded in this trial could not be attributable to differences in the individual drug assumptions, which were homogeneous. However, the substantial individual variability could be attributed to physiological and metabolic differences among the animals and by the presence of different quantities of food in the digestive tract.

With regard to the 5-day administration of medicated water (residue study), the higher tissue concentrations are recorded at the suspension of treatment, as reported in Table 2. The decrease of FLU concentrations was very rapid, arriving at values lower than the LOQs already at 24 hours of withdrawal time in 5 out of 6 rabbits in livers and in 2 out of 6 rabbits in kidneys. FLU was not quantified in muscles, fat and lungs following the first collection time, with the exception of one lungs sample in one rabbit sacrificed at the 24h time-point (77 $\mu g.kg^{-1}$). FLU titrable concentrations were not observed in any of the tissues collected at the 48h and 72h time-points, with the exception of a sample of liver containing 139 $\mu g.kg^{-1}$ at the 48h time-point.

Many individual variations were also observed in this experimental phase, but, as mentioned above, these could not be attributed to the different individual drug assumptions that, as reported in Table 3, were quite homogeneous. Additionally, individual variability and digestive tract repletion differences in this trial are probably the main reasons for the substantial differences observed within the groups.

In the tissue distribution study, the livers, kidneys and fat showed significant differences in the concentrations assayed only between 1h and 12h ($P<0.05$), muscles and fat between 1h and 9h ($P<0.05$), and in the kidneys significant differences were also observed between 3h and 12h ($P<0.05$).

In the residue study, the statistical analysis of the livers and kidneys showed a significant difference between the concentration assayed at 0h and those assayed at

**Table 2:** FLU concentrations (µg.kg$^{-1}$) after repeated administration (5 days) of medicated water (150 ppm).

| Sacrifice Time | Animal | Liver | Kidney | Muscles | Lungs | Fat |
|---|---|---|---|---|---|---|
| 0h | ♂ | 636 | 3016 | ND* | 53 | 50 |
| | ♀ | 249 | 2606 | ND | <LOQ** | <LOQ |
| | ♀ | 716 | 4489 | ND | 31 | 41 |
| | ♂ | 272 | 1045 | 55 | 118 | 36 |
| | ♂ | 227 | 668 | 60 | 56 | 52 |
| | ♀ | 228 | 559 | 47 | 79 | <LOQ |
| Mean | | 388(§)(#) | 2064(§)(#) | 27 | 60(#)(£)(&) | 38(§) |
| SD | | 225 | 1571 | 30 | 34 | 12 |
| 24h | ♂ | <LOQ | 101 | ND | 77 | ND |
| | ♂ | <LOQ | <LOQ | ND | ND | ND |
| | ♂ | <LOQ | <LOQ | ND | ND | ND |
| | ♀ | 80 | 67 | ND | ND | ND |
| | ♀ | <LOQ | 51 | <LOQ | ND | ND |
| | ♀ | <LOQ | 55 | ND | ND | ND |
| Mean | | 55 | 54 | 4 | 13 | 0 |
| SD | | 12 | 29 | 10 | 31 | |
| 48h | ♂ | ND | <LOQ | ND | ND | ND |
| | ♂ | 139 | <LOQ | ND | ND | ND |
| | ♂ | <LOQ | <LOQ | ND | ND | ND |
| | ♀ | ND | <LOQ | ND | ND | ND |
| | ♀ | ND | <LOQ | ND | ND | ND |
| | ♀ | ND | <LOQ | ND | ND | ND |
| Mean | | 32 | 50 | 0 | 0 | 0 |
| SD | | 56 | 0 | | | |
| 72h | ♂ | ND | <LOQ | ND | ND | ND |
| | ♂ | ND | <LOQ | ND | ND | ND |
| | ♂ | ND | <LOQ | ND | ND | ND |
| | ♀ | ND | ND | ND | ND | ND |
| | ♀ | ND | <LOQ | ND | ND | ND |
| | ♀ | ND | <LOQ | ND | ND | ND |
| Mean | | 0 | 42 | 0 | 0 | 0 |
| SD | | | 20 | | | |

LOQ muscles, fat and lungs = 25 µg.kg$^{-1}$; LOQ liver and kidney = 50 µg.kg$^{-1}$; *ND = not detected, considered as 0 in the statistical analyses; ** <LOQ = residues below the LOQ were considered as LOQ in statistical analyses; (§) = significantly different from concentrations at 48h ($P$<0.01); (#) = significantly different from concentrations at 72h ($P$<0.001); (£) = significantly different from concentrations at 24h ($P$<0.001); (&) = significantly different from concentrations at 48h ($P$<0.001); ($) = different from concentrations at 72h ($P$<0.05); s.d. = standard deviation.

48h ($P<0.01$) and at 72h ($P<0.001$). With respect to the lungs, the concentrations at 0h were always significantly different from all the other time points ($P<0.001$). As for the fat, a significant difference in the residues was only observed between 0h and 72h ($P<0.05$), while in the muscles no statistical differences were observed.

## DISCUSSION

FLU concentrations recorded for kidneys and livers in the distribution study were found to be in accordance with our expectations. These concentrations were greater than the $MIC_{90}$ values available in the literature on the most susceptible microorganisms for the entire 12h experimental period in kidney samples and for 6 hours in liver samples (Table 4). The FLU levels recorded in the other tissues investigated was lower. In particular, for muscles, fat and lungs the drug concentrations were never higher than $MICs_{90}$. For tissue collected at the 6h time-point FLU was detectable at measured levels in two out of four muscles samples and in three out of four lungs samples, while the drug was not quantified in fat

**Table 3:** FLU assumption ($\mu g.kg^{-1}$) during the 5-days period of administration of medicated water (150 ppm ).

| Group | T0 | T24 | T48 | T72 |
|---|---|---|---|---|
| ♂ | 15.9 | ♂ 13.3 | ♂ 17.8 | ♂ 18.3 |
| ♀ | 18.8 | ♂ 17.4 | ♂ 18.1 | ♂ 16.1 |
| ♀ | 18.0 | ♂ 18.7 | ♂ 15.6 | ♂ 17.6 |
| ♂ | 16.6 | ♀ 18.4 | ♀ 13.0 | ♀ 17.2 |
| ♂ | 14.8 | ♀ 16.0 | ♀ 20.0 | ♀ 16.6 |
| ♀ | 16.1 | ♀ 17.4 | ♀ 17.6 | ♀ 15.8 |
| Mean | 16.7 | 16.9 | 17.0 | 16.9 |
| SD | 1.5 | 2.0 | 2.4 | 0.9 |

SD = standard deviation.

samples. These results reveal a very short duration of FLU efficacy in the control of systemic infections sustained by susceptible bacteria.

However, the experimental data recorded in the distribution study shows that the antibiotic diffuses rapidly (1 hour post-administration), although in different concentrations, in all the tissues studied.

The FLU levels observed at the first time of sacrifice (1 hour) following the gavage administration of the single dose of 15 $mg.kg^{-1}$ were higher than those recorded at the first time-point (0h) following treatment with medicated water. These results are probably attributable to the different types of treatment: administration by gavage in the distribution study and by continuous assumption (medicated water) during the 5-day period in the residue depletion study. In fact, in the first type of treatment the drug dose was administered as a *bolus* in a unique administration, whereas in the second type of treatment the same dose was assumed during a 24h period. The drug was, therefore, available for a longer period but at smaller concentrations.

**Table 4:** Minimal inhibitory concentrations $MIC_{90}$ ($\mu g.mL^{-1}$) of flumequine for bacterial strains isolated from several species (from CHEVALEIER *et al.*, 1982; ZIV *et al.*, 1986; ATEV *et al.*, 1987; HANNAN *et al.*, 1989; PIJPERS *et al.*, 1989).

| | $MIC_{90}$ |
|---|---|
| *Salmonella* spp. | 0.4 - 1 |
| *Escherichia coli* | 0.4 - 1 |
| *Pasteurella* spp. | 0.25 - 0.8 |
| *Staphylococcus aureus* | 64 |
| *Streptococcus* spp. | >64 |
| *Pseudomonas aeruginosa* | 32 |
| *Mycoplasma* spp. | 2.5 - 10 |

The analytical method used for the detection of FLU in the different matrixes was specific, linear, reproducible and accurate. The extraction procedure is easily applicable, but not very rapid as only a few samples can be processed in a day.

In conclusion, the results of the experiment enable us to suggest that in the rabbit an oral dose of 15 $mg.kg^{-1}$ administered as a *bolus* by gastric gavage is adequate to ensure effective concentrations for at least 6-8 hours only in livers and kidneys, whereas this dose seems to be insufficient to control respiratory, muscoloskeletal and adipose infections sustained by susceptible bacteria.

The same conclusions can also be drawn a considering the results of the residue study in which FLU was administered *via* drinking water. In fact, at the cessation of treatment (5 consecutive days), potentially effective drug concentrations were recorded only in the livers and kidneys.

Finally, the results deriving from the residue depletion study allow us to conclude that a 2-day withdrawal time for FLU preparations administered *via* medicated water for about 5 days at the dosing regimen, which is normally adopted in the commercial breeding sector, can be considered sufficient to guarantee the respect of established MRLs.

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