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Routine determination of flumequine in kidney tissue of pig using automated liquid chromatography

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Abstract

A high-performance liquid chromatographic assay is described as a routine analytical method for the determination of flumequine (FLU) and its hydroxylated metabolite (OH-FLU) in pig kidney tissue. Kidney samples (2 g) containing FLU and OH-FLU were extracted by liquid–liquid extraction with ethyl acetate (10 ml). Analytical separations were performed by reversed-phase HPLC with fluorometric detection at 252 nm excitation and 356 nm emission under gradient conditions. The mobile phase was acetonitrile– $2.7 \cdot 10^{-3}$ M oxalic acid in water (pH 2.5). The assay is specific and reproducible within the flumequine range of 0.050–2.5 $\mu\text{g/g}$ and recovery at 0.050 $\mu\text{g/g}$ was 94.8%.

Keywords: Flumequine; Hydroxyflumequine

1. Introduction

Flumequine (formerly called 1*H*,5*H*-benzo[*ij*]-quinolizine-2-carboxylic acid, 9-fluoro-6,7-dihydro-5-methyl-1-oxo-) is an anti-infective, quinoline-derived agent which belongs to the quinolone family. Flumequine is used in mammals for treatment and prevention of *Escherichia coli*, *Salmonella spp* and *Pasteurella spp* infections [1–3]. In man [4], rat, dog [5] and pigs [6] flumequine is glucuronidated and to a lesser extent, hydroxylated to 7-hydroxyflumequine. To register a new animal drug or to support an actual market drug for use in food-producing pigs, the sponsor of the compound must demonstrate that drug-related residues in the edible tissues of treated animals constitute no potential hazard when consumed by humans. Thus, the deple-

tion of drug from the tissues must be assessed in order to determine how long it takes before the antibiotic has disappeared from tissue and when the treated animal can be safely consumed. To this end, the sponsor must develop information on the amount, persistence and chemical nature of the drug, and routine methods are especially required in the target tissue. A dossier for the determination of the Maximal Residue Limits (MRL) of flumequine has been submitted to the regulatory authorities in order to establish the tolerance level for this molecule in foodstuffs of animal origin. Findings had shown that flumequine levels in kidney were systematically higher than in other tissues. Thus, kidney was proposed as the target tissue for routine controls.

Flumequine has been assayed by microbial assay [7]. More recently a high-performance liquid chromatographic method has been devised, but the procedure describes the separation of flumequine

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without its metabolite and no appropriate internal standard [8]. None of these methods appear satisfactory for the determination of flumequine residues in pig tissues. This study describes a routine analytical procedure for detection and determination of flumequine (FLU) and 7-hydroxyflumequine (OH-FLU) in the target tissue of pig (i.e. kidney) using a high-performance liquid chromatographic (HPLC) assay method for flumequine and utilizes a reversed-phase C_{18} column with specific fluorometric detection and ibafloxacin as internal standard. Sample preparation involves the use of liquid-liquid extraction with an organic solvent.

2. Experimental

2.1. Chemicals and reagents

Flumequine (Fig. 1) was obtained from Sigma (Saint Quentin Fallavier, France), 7-hydroxyflumequine and ibafloxacin were obtained from 3M Riker Laboratory (Pithiviers, France). Acetonitrile was obtained from Distilab (Leuven, Belgium), ethyl acetate, hexane and oxalic acid dihydrate from Prolabo (Manchester, UK). Sodium sulfate anhydrous sulfate was obtained from Merck (Darmstadt, Germany). The water was purified using a Milli-Q system (Milford, MA, USA).

2.2. Drug standards

Stock solutions of flumequine, 7-hydroxyflumequine and ibafloxacin (1 mg/ml) were prepared in a $2 \cdot 10^{-2}$ M solution of sodium hydroxide and kept at 4°C. Intermediate and working solutions

were prepared immediately before use by diluting these stock solutions with distilled water.

2.3. Kidney working standards

Drug-free kidney tissue was prepared from freshly killed pig. Tissue was minced in small pieces and samples were stored at -20°C . A series of thawed drug-free kidney samples were fortified with flumequine and 7-hydroxyflumequine to give final concentrations of 0.05, 0.1, 0.25, 0.5, 1.0 and 2.5 $\mu\text{g/g}$. Internal standard was added in order to obtain a final concentration of 0.25 $\mu\text{g/g}$. The overall volume of drug solutions added to kidney working standard was 200 μl in total.

2.4. Chromatography

The chromatographic system consisted of a Varian 9010 gradient pump (Walnut Creek, CA, USA), a Shimadzu RF-551 fluorometric detector set at 252 nm excitation and 356 nm emission, an Ultrabase octadecyl analytical column (particle size 5 μm , 250 \times 4.6 mm I.D., Shandon, UK), a Varian 9090 automated injector and a Perkin-Elmer Nelson 1020 integrator (Norwalk, CT, USA) for recording of the peak areas. The mobile phase was a mixture of acetonitrile (eluent A) and 0.0027 M oxalic acid in water (pH 2.5) (eluent B). At $t=0$, the mixture consisted of 10% A and 90% B, which changed linearly in 20 min to 70% A and 30% B. Then, the system remained stable during 5 min and returned to its initial state in 5 min. The flow-rate was 0.8 ml/min, and drugs were detected by measuring the peak area.

2.5. Sample preparation procedure

Both whole kidneys are used as test sample, and 2-g quantities of pooled and minced kidney tissue are used as test portion (i.e. quantity of material drawn from the test sample and on which the test is carried out). This test portion (2 g) was introduced into a 30-ml disposable screw-cap polyethylene tube with 100 μl of internal standard and mixed until homogenization with 4 g of anhydrous sodium sulfate. Next, 10 ml of ethyl acetate were added and the mixture

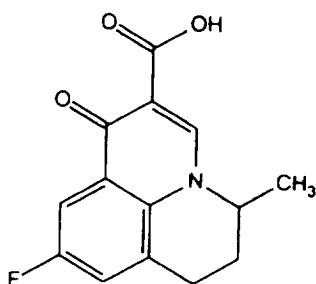


Fig. 1. Structure of flumequine.

was mixed for 10 min on a mechanical shaker and centrifuged at 1500 g for 10 min. The upper organic ethyl acetate layer was transferred into a 15-ml disposable tube. This liquid–liquid extraction was repeated a second time on the tissue pellet and all organic phases were pooled and evaporated under a stream of nitrogen at 50°C. The total residue was dissolved into 1 ml of acetonitrile–oxalic acid solution (50:50, v/v), the mixture was vortex-mixed and placed in an ultrasonic water bath for 5 min. Next, the residue was filtered through a 0.45- μm filter (GHP Acrodisc GF, Gelman Sciences, Ann Arbor, MI, USA) into an HPLC vial. Volumes of 100 μl were injected onto the HPLC system.

2.6. Calibration and calculation

Analyses of drug-free pig kidney tissue spiked with known amounts of flumequine and 7-hydroxy-flumequine were carried out applying the above-described procedure. Linearity was evaluated from six calibration graphs prepared and run on six different days within the flumequine range 0.050–2.5 $\mu\text{g/g}$. Precision was evaluated by repeated analyses of flumequine at three concentrations on the same day. The accuracy and reproducibility of the method were tested by repeatedly injecting a set of kidney working standards on six different days. To evaluate the extraction recovery, the calculated concentrations of flumequine and 7-hydroxyflumequine in kidney tissue were compared with those obtained with a drug working solution injected directly onto the chromatographic system.

All chromatograms obtained were evaluated by peak-area measurement. The concentrations of flumequine were calculated with the calibration curve generated on each day by unweighted linear regression of the peak-area ratio between drug on internal standard against the nominal concentrations.

2.7. Animal study

A study of the depletion of flumequine in pigs was carried out after administration of the product Flumiquil Poudre A 3% in therapeutic conditions of use: a loading dose of 15 mg of flumequine/kg of body weight for dose 1 followed by nine maintenance doses of 7.5 mg/kg given every 12 h for five

consecutive days to twenty-four healthy pigs which were divided into six groups of four animals each. This dosage corresponded to 0.50 g of product per kg of body weight for dose 1 and 0.25 g for doses 2–10 blended in feed. Four pigs were slaughtered at each fixed time starting from the last administration (i.e., 12, 24, 36, 48, 72 and 96 h), and the kidney of the pig was assayed using the present method.

3. Results and discussion

3.1. Sample preparation and HPLC separation

Flumequine is a small molecule and its structure is characterised by a pyridone ring associated with a carboxyl group. Flumequine is soluble in polar organic solvents: methanol, ethanol, ethyl acetate, diethyl ether, chloroform but not in hexane. Flumequine differs from the other quinolones by its hydrophobicity, as determined by its partition coefficient between *n*-octanol and 0.1 M phosphate buffer (pH 7.2) (i.e., partition coefficient of 13.0 for flumequine, 0.33 for ofloxacin and 0.01 for norfloxacin) [9]. Its partition coefficient indicates that this molecule can a priori be determined in a conventional chromatographic system. The molecule is thermostable in aqueous, acidic and basic media and was not degraded by light and by oxygen: no precaution is required during its storage (unpublished results). As a moderately hydrophobic compound, flumequine can usually be extracted with solid–liquid extraction. Solid-phase extraction columns have been extensively used for clean-up of flumequine from biological material such as fish tissues [10]. Non-polar sorbents such as C₁₈, C₈ and C₂ were assayed, but led to low recovery rates [10]. Due to these difficulties, a more traditional liquid–liquid extraction was used with an appropriate solvent and both solubility properties of flumequine and 7-hydroxylated metabolite were taken into account in the selection of ethyl acetate as the best organic solvent, giving a recovery rate above 90% [11]. Thus, a rapid liquid–liquid extraction was performed from a 2-g kidney sample. An appropriate system proved to be an Ultrabase C₁₈ column with acetonitrile–0.0027 M oxalic acid in water as mobile phase, using an elution gradient mode. An Ultrabase C₁₈ column allows work to be carried out at extreme

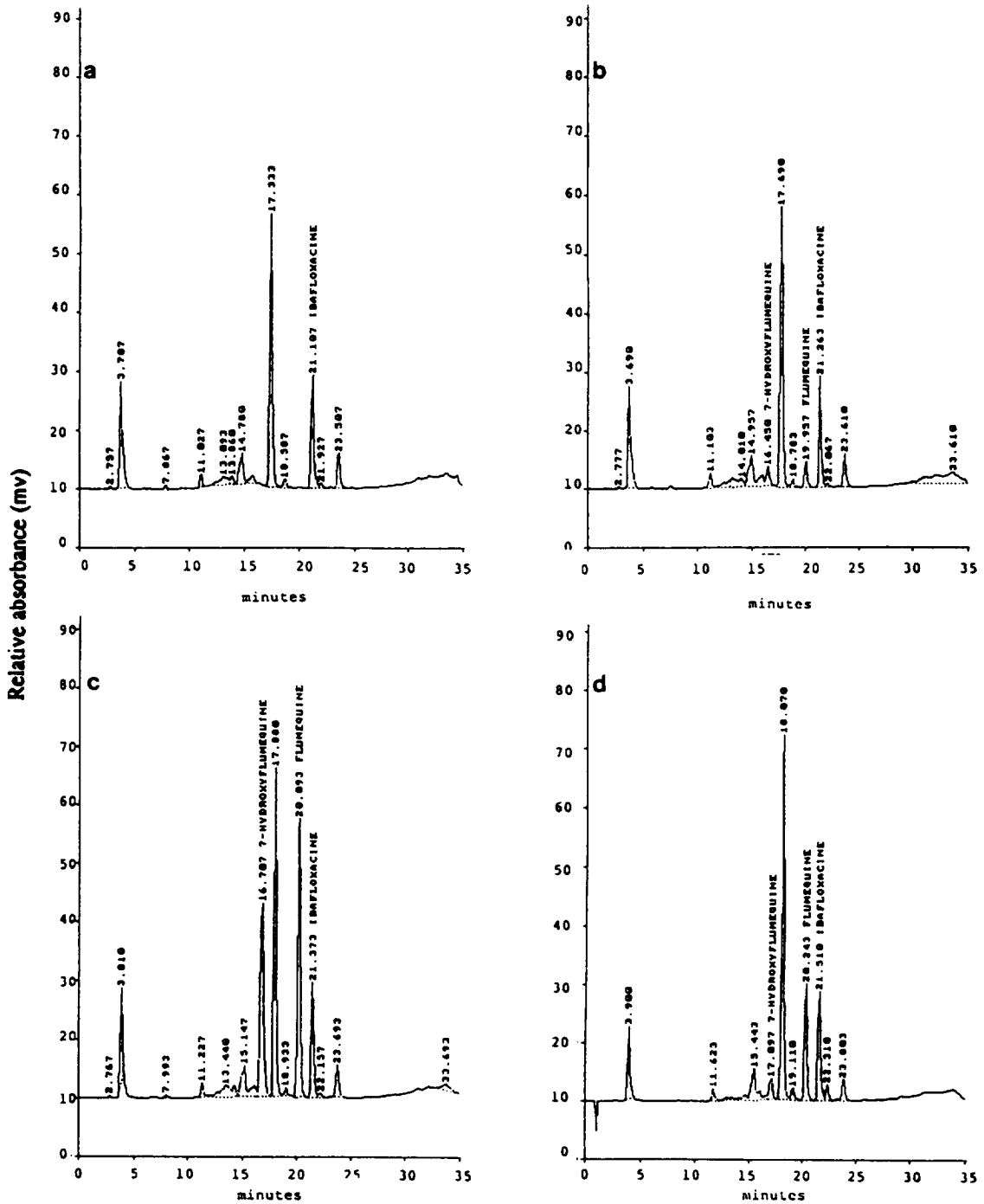


Fig. 2. Chromatograms after addition of (a) drug-free pig kidney tissue, (b) pig kidney tissue spiked with 0.1 µg/g flumequine, 7-hydroxyflumequine and internal standard, (c) pig kidney tissue spiked with 1.0 µg/g flumequine, 7-hydroxyflumequine and internal standard, (d) pig kidney tissue from one animal obtained after 5 days of treatment (7.5 mg/kg/12 h), 24 h after the last dose of flumequine.

Table 1
Within-day precision of flumequine and 7-hydroxyflumequine

Nominal concentration ($\mu\text{g/g}$)	Flumequine area ratio (mean \pm S.D., $n=12$)	C.V. (%)	7-Hydroxyflumequine area ratio (mean \pm S.D., $n=12$)	C.V. (%)
0.050	0.116 \pm 0.005	4.4	0.093 \pm 0.009	9.2
0.500	1.177 \pm 0.050	4.3	1.001 \pm 0.065	6.5
2.500	5.464 \pm 0.225	4.1	4.462 \pm 0.185	4.1

pH values, ranging from 2 to 8, and improves the separation of chromatographic peaks. Chromatograms obtained after extraction of kidney tissue of

pig spiked with 0.1 and 1.0 $\mu\text{g/g}$ of flumequine, 7-hydroxyflumequine and internal standard are shown in Fig. 2.

Table 2
Day-to-day precision and accuracy of flumequine

Nominal concentration ($\mu\text{g/g}$)	Concentration found (mean \pm S.D., $n=6$) ($\mu\text{g/g}$)	C.V. (%)	Accuracy (%)
0.050	0.051 \pm 0.003	6.0	1.5
0.100	0.102 \pm 0.006	5.7	1.9
0.250	0.246 \pm 0.004	1.5	-1.8
0.500	0.528 \pm 0.015	2.9	5.5
1.000	1.020 \pm 0.028	2.8	2.0
2.500	2.486 \pm 0.012	0.5	-0.6

Several excitation wavelengths have been tested for optimizing the response against the noise, and a 252-nm wavelength was selected as excitation with a 356-nm emission. The drug-free sample did not contain substances that would interfere with the detection of the drug at 252 nm excitation and 356 nm emission.

In addition, this method was selective between the following products (i.e. oxolinic acid and nalidixic acid), ciprofloxacin, enrofloxacin and norfloxacin were not detected under these conditions.

Table 3
Day-to-day precision and accuracy of 7-hydroxyflumequine

Nominal concentration ($\mu\text{g/g}$)	Concentration found (mean \pm S.D., $n=6$) ($\mu\text{g/g}$)	C.V. (%)	Accuracy (%)
0.050	0.054 \pm 0.002	4.6	7.5
0.100	0.104 \pm 0.005	4.5	3.5
0.250	0.251 \pm 0.002	0.9	0.4
0.500	0.528 \pm 0.013	2.5	5.6
1.000	1.028 \pm 0.033	3.2	2.8
2.500	2.482 \pm 0.012	0.5	-0.7

3.2. Limit of quantification

The limit of quantification in kidney tissue of pig is the lowest concentration which is measured within the limits of precision and accuracy set for the method (15% of precision, -30 to +10% of percentage difference). This limit was determined to be 0.050 $\mu\text{g/g}$ at 252 nm detection and 356 nm emission wavelengths. The overall intra-assay ($n=12$) and inter-assay ($n=6$) coefficients of variation (C.V.) at this concentration were 4.4% and 6.0% for flumequine and 9.2% and 4.6% for OH-FLU with deviations from the theoretical value of -1.5% and

Table 4
Recovery rate of flumequine and 7-hydroxyflumequine

Nominal concentration ($\mu\text{g/g}$)	Flumequine (mean \pm S.D., $n=6$) ($\mu\text{g/g}$)	Recovery (%)	7-hydroxyflumequine (mean \pm S.D., $n=6$) ($\mu\text{g/g}$)	Recovery (%)
0.050	0.047 \pm 0.001	94.8	0.048 \pm 0.003	95.0
0.500	0.446 \pm 0.016	89.1	0.465 \pm 0.018	93.0
2.500	2.145 \pm 0.038	85.8	2.111 \pm 0.035	84.4

7.5% for FLU and OH-FLU respectively. For the purpose of flumequine depletion in the kidney tissue of pig, a quantification limit of $0.050 \mu\text{g/g}$ is considered satisfactory.

3.3. Limit of detection

The limit of detection in kidney tissue is the smallest concentration from which it is possible to deduce the presence of the analyte with reasonable statistical certainty. The limit of detection is equal to the mean of the measured content of representative blank samples ($n=20$) plus three times the standard deviation of the mean [12]. This limit was determined to be $0.015 \mu\text{g/g}$ and $0.024 \mu\text{g/g}$ for FLU and OH-FLU, respectively.

3.4. Assay validation

Analyte concentrations were determined from their peak-area ratio, using calibration standards made from the same biological material as the samples. The calibration standards (0.050 to $2.5 \mu\text{g/g}$) gave correlation coefficients of 0.9954 and 0.9928 for both drugs. The results in Table 1 shows that FLU and OH-FLU are determined with good precision.

The day-to-day variation was determined by analysing one calibration curve for six days over a period of two weeks. The day-to-day precision was determined over the concentration range 0.050 – $2.5 \mu\text{g/g}$ in kidney tissue. The results (Table 2 and Table 3) show good precision.

Mean recovery of flumequine was calculated after a comparison of extracted sample with a working

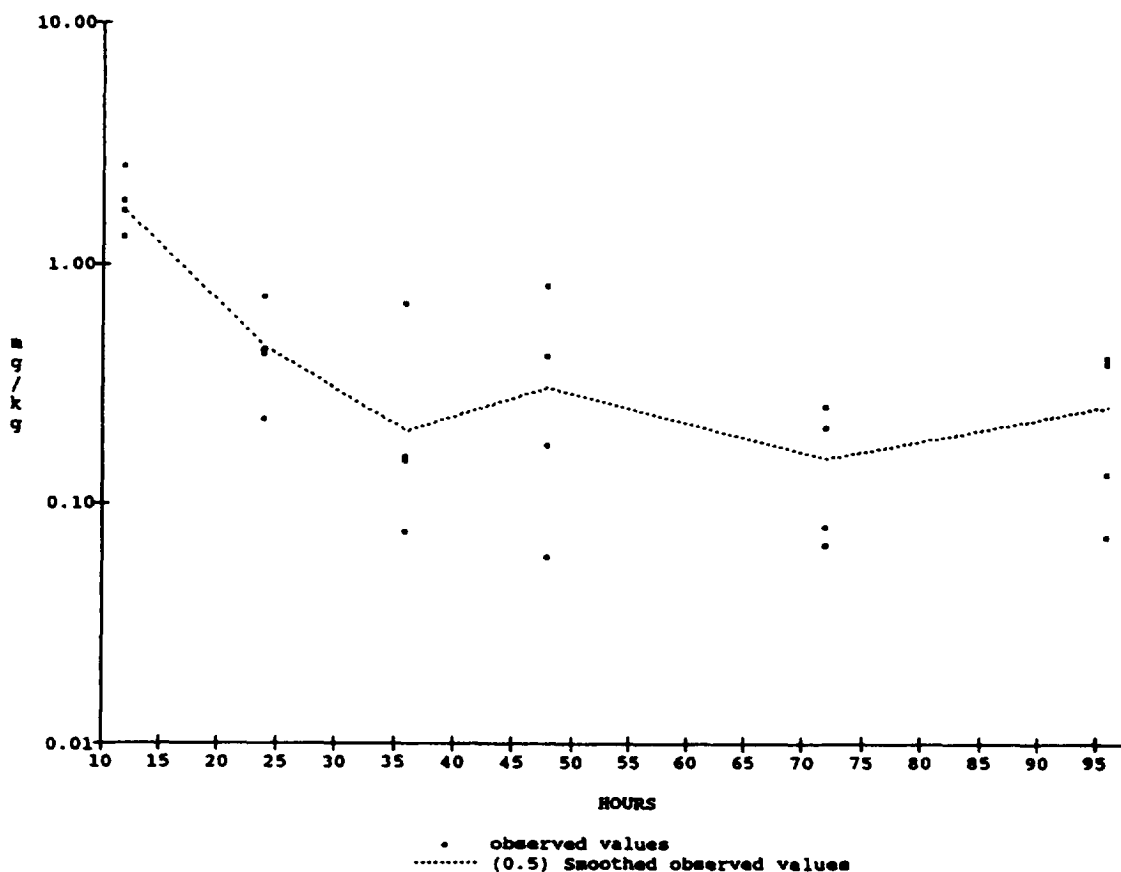


Fig. 3. Semilogarithmic plot of flumequine depletion against slaughter times after repeated twice daily oral administration of flumequine at $7.5 \text{ mg/kg}/12 \text{ h}$ over five consecutive days in twenty four pigs.

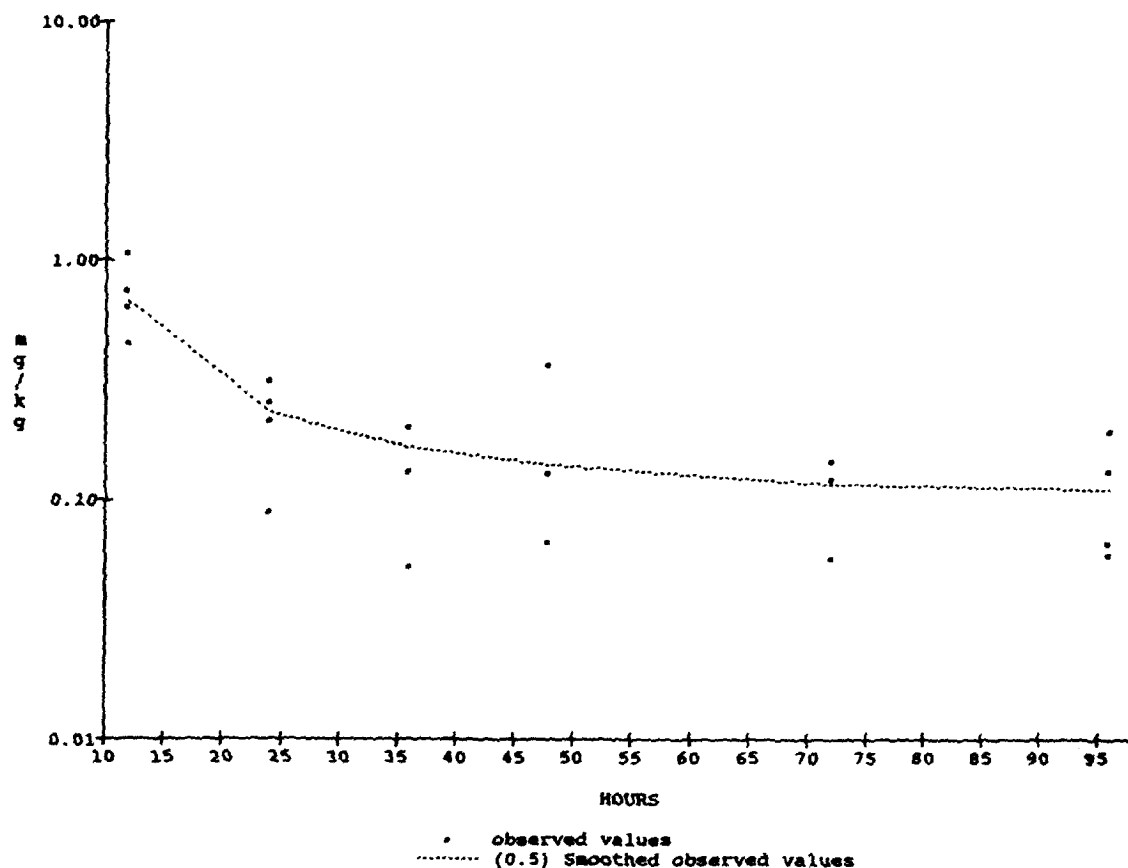


Fig. 4. Semilogarithmic plot of 7-hydroxyflumequine depletion against slaughter times after repeated twice daily oral administration of flumequine at 7.5 mg/kg/12 h over five consecutive days in twenty four pigs.

solution rather than a traditional spiked blank extract. As preliminary experiments gave similar results with both assays, it was concluded that co-extracted biological material does not have significant effects on the recovery. Overall recovery rates for FLU and OH-FLU were 89.9% and 90.8%, respectively (Table 4).

3.5. Animal study

Residue depletion studies show that flumequine is ingested in pigs. At 12 h after the last administration, the concentration of flumequine (Fig. 3) and its metabolite (Fig. 4) were $1.879 \pm 0.541 \mu\text{g/g}$ (FLU) and $0.737 \pm 0.263 \mu\text{g/g}$ (OH-FLU); 24 h after the last administration, findings were $0.460 \pm 0.209 \mu\text{g/kg}$ (FLU) and $0.221 \pm 0.096 \text{ mg/kg}$ (OH-FLU); 96 h

after the last administration, significantly lower concentrations were observed in kidney for flumequine and 7-hydroxyflumequine (i.e. $0.253 \pm 0.174 \mu\text{g/g}$ and $0.115 \pm 0.065 \mu\text{g/g}$).

4. Conclusion

Using the combination of an inexpensive liquid-liquid extraction with a simple HPLC system, flumequine can be determined with high precision and reproducible recovery. The simplicity of the method, the high sample throughput capability of at least 300 injections on each column prior to chromatographic deterioration, and the quantification limit of $0.050 \mu\text{g/g}$ enable this system to be used as a suitable analytical method for the routine determi-

nation of flumequine and its metabolite in the kidney tissue of pig. This routine method of analysis was also assayed from other edible tissues of pig with success.

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