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Short communication

Determination of flumequine and 7-hydroxyflumequine in plasma of sheep by high-performance liquid chromatography

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Abstract

A high-performance liquid chromatographic (HPLC) method for the simultaneous determination of flumequine and its metabolite 7-hydroxyflumequine in sheep plasma was described. The two compounds were extracted from 100 μl of plasma by liquid–liquid extraction. Aliquots (100 μl) were injected onto the HPLC system and separated on a LiChrospher Select B column with an isocratic system. The compounds were detected by fluorimetric detection for concentrations below 500 μg/l and by UV detection for the concentrations exceeding 500 μg/l. The range of the validated concentrations were 50 000 to 5 μg/l and 500 to 10 μg/l with mean recovery rates of 87±3% and 60±1% for flumequine and 7-hydroxyflumequine, respectively.

Keywords: Flumequine; 7-Hydroxyflumequine

1. Introduction

Flumequine (1H,5H-benzo[*ij*]quinolizine-2-carboxylic acid, 9-fluoro-6,7-dihydro-5-methyl-1-oxo), a second generation antibacterial quinolone, is used in veterinary medicine for treatment of animal diseases caused by a wide-range of Gram-negative bacteria (*Escherichia coli*, *Salmonella* and *Pasteurella*). Several methods to determine flumequine and its microbiologically active metabolite 7-hydroxyflumequine from humans and animals have been described [1–4]. Microbial and fluorometry methods were poor in selectivity and sensitivity. The high-performance liquid chromatography (HPLC) has been increasingly used to determine antibiotics in biological fluids. Harrison et al. [5] reported a HPLC method for the determination of flumequine and 7-hydroxyflumequine in human plasma and urine based on anion-exchange, using a mobile phase adjusted to pH 9.0, which was very critical for a silica chemically bonded phase. Furthermore, this method needed a relatively large plasma sample size (1 ml). Decolin et al. [6] described a HPLC method with UV–visible detection for the measurement of both flumequine and 7-hydroxyflumequine in human plasma and urine. This method was sensitive and selective, and the plasma sample size necessary for a measurement was only 200 μl.

In this paper, a method based on HPLC with UV–visible and fluorimetric detection for the separation and quantification of flumequine and its 7-hydroxy metabolite in sheep plasma was reported.

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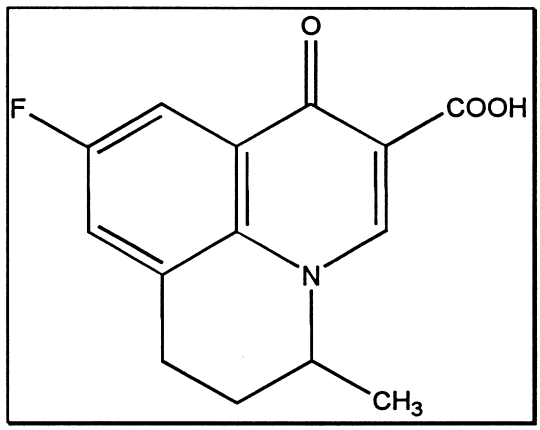
This method was used to determine the pharmacokinetic profiles of flumequine and its 7-hydroxy metabolite in sheep after intravenous and intramuscular administration of flumequine [7].

2. Experimental

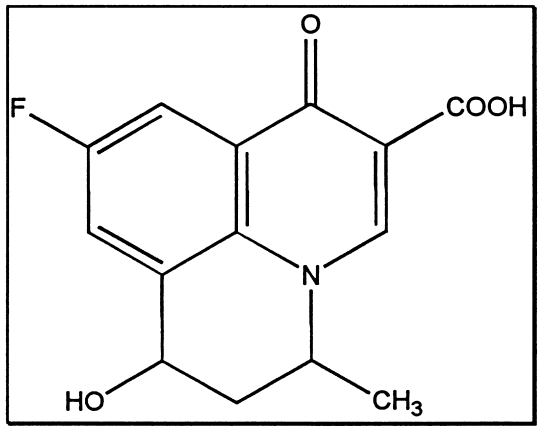
2.1. Reagents

Dimethylformamide, orthophosphoric acid, acetonitrile, chlorhydric acid, ethyl acetate, hexane, methanol (Merck, Nogent-sur-Marne, France) were all analytical-reagent grade.

Flumequine (batch 17248/B) and 7-hydroxyflumequine (batch EE 6964477780FR) were supplied by Sanofi Santé Nutrition Animale (Libourne, France). The structures of flumequine and 7-hydroxyflumequine are shown in Fig. 1.



Flumequine



7-hydroxyflumequine

Fig. 1. Structures of flumequine and 7-hydroxyflumequine.

2.2. Standard solutions

Stock solutions of flumequine at concentrations of 1 g/l and 0.2 g/l and 7-hydroxyflumequine at concentration of 0.1 g/l were prepared in methanol and stored at 4°C. Standard solutions of flumequine (0.005, 0.025, 0.05, 0.25, 0.5, 1, 5, 10, 50 mg/l) and of 7-hydroxyflumequine (0.01, 0.1, 0.5 mg/l) were prepared by dilution of stock solution in buffer phosphate, pH 7.8 and kept at 4°C. Solutions of flumequine (0.1, 0.5, 1, 5, 10, 20, 100 mg/l) and of 7-hydroxyflumequine (0.2, 2, 10 mg/l) were prepared by dilution of stock solutions in Ultrapure water and kept at 4°C until plasma spiking.

2.3. Spiked plasma

The blank plasma samples were obtained from different sheep in a slaughterhouse. The plasma samples were spiked with the flumequine–7-hydroxyflumequine solutions (95:5, v/v). The final concentrations in spiked samples were 0.005, 0.025, 0.05, 0.25, 0.5, 1, 5, 10 and 50 mg/l flumequine and 0.01, 0.1 and 0.5 mg/l 7-hydroxyflumequine. The spiked samples were stored at −20°C.

2.4. Chromatographic apparatus

The HPLC system consisted of the following components: a Varian Model 9010 solvent delivery system (Varian, Les Ullis, France), a Merck Model AS-2000A autosampler with a 100-μl injection loop (Merck), a fluorimetric detection system Jasco Model FP920 (Prolabo, Bordeaux, France) coupled to a UV detection system Varian Model 9050 (Varian) and a Varian Star computing program (Varian). The col-

umn used was a Lichrospher RP Select B (125×4 mm I.D.), packed with 5 μm Select B particle (Merck) and a C_{18} pre-column 4×4 mm (Merck) was used.

2.5. Extraction procedure

A 100-μl volume of spiked plasma sample was mixed with 100 μl of phosphate buffer, pH 6.0 and 1 ml of ethyl acetate in an Eppendorf tube. After shaking and centrifugation (20 000 *g*, 5 min), 800 μl of the organic phase was collected and dried under nitrogen at 45°C. A 300-μl volume of phosphate buffer, pH 7.8 and 300 μl of hexane was added to the residue. After shaking and centrifugation (20 000 *g*, 5 min), a 100-μl aliquot of the aqueous phase was injected into the HPLC system.

2.6. HPLC analysis

The UV detection system was set at 324 nm. Fluorimetric detection was obtained with excitation wavelength at 320 nm and emission wavelength at 365 nm. Quantification with UV detection was performed for the flumequine concentrations exceeding 500 μg/l in samples while fluorimetric detection was used for the flumequine and 7-hydroxyflumequine concentrations below this limit. Fluorimetric detection could not be used for concentrations greater than 1000 μg/l because the signal detector saturated. Furthermore, the limit of quantitation with the UV detector was 100 μg/l.

The mobile phase was a mixture of three solvents: orthophosphoric acid–water (25:75, v/v)–dimethylformamide–acetonitrile (54:28:18, v/v/v) at a flow-rate of 0.8 ml/min. The separation was performed at room temperature.

2.7. Recovery and precision studies

The procedure employed to validate the method follows the recommendations of Shah et al. [8]. At each concentration, two analyses (intra-day precision) were performed and repeated on five days (inter-day precision). An external standard was injected directly onto the analytical column. The observed plasma concentration of each analyte was calculated by comparison of the sample peak area with the external standard peak area. A linear regression equation was obtained by plotting the peak areas (corrected by the validated recovery) against concentrations.

3. Results and discussion

This paper describes a new HPLC method to quantify flumequine and its 7-hydroxyflumequine in sheep plasma. Contrary to the other methods previously published, the plasma sample volume required in our method is only 100 μl. Harrison et al. [5] used 1 ml and Decolin et al. [6] 200 μl of plasma. This advantage is useful for the pharmacokinetic studies where there are several small samples. The noise is also reduced. The limits of quantification of this method were 5 and 100 μg/l for flumequine and 7-hydroxyflumequine, respectively. Furthermore, the simple liquid–liquid extraction procedure is less expensive than an extraction with cartridges, and accordingly, the solvent volumes are below 1 ml. In addition, we used fluorimetric detection which improves the sensitivity of the analytical method to detect flumequine and its metabolite. The limits of detection achieved are a 20-fold decrease compared with limits reported by Mevius et al. [9]. Theses limits (2 and 3.32 μg/l) are smaller than the limits obtained by Harrison et al. [5] (0.5 and 0.1 mg/l) and by Decolin et al. [6] (2.5 and 0.5 mg/l) for flumequine and 7-hydroxyflumequine, respectively.

The mobile phase was optimized to separate flumequine and its hydroxylated metabolite on the same chromatogram within 10 min as shown in Fig. 2. The observed retention times were 3.7 and 6.4 min for 7-hydroxyflumequine and flumequine, respectively. We obtained the chromatographic separation of the 7-hydroxyflumequine diastereoisomers. There was no endogenous interference observed on the chromatograms in the blank plasma at the retention times of the respective analytes. The method used was selective for the flumequine compared to others quinolones (marbofloxacine, enrofloxacine, danofloxacine, ciprofloxacine, oxolinic acid and nalidixic

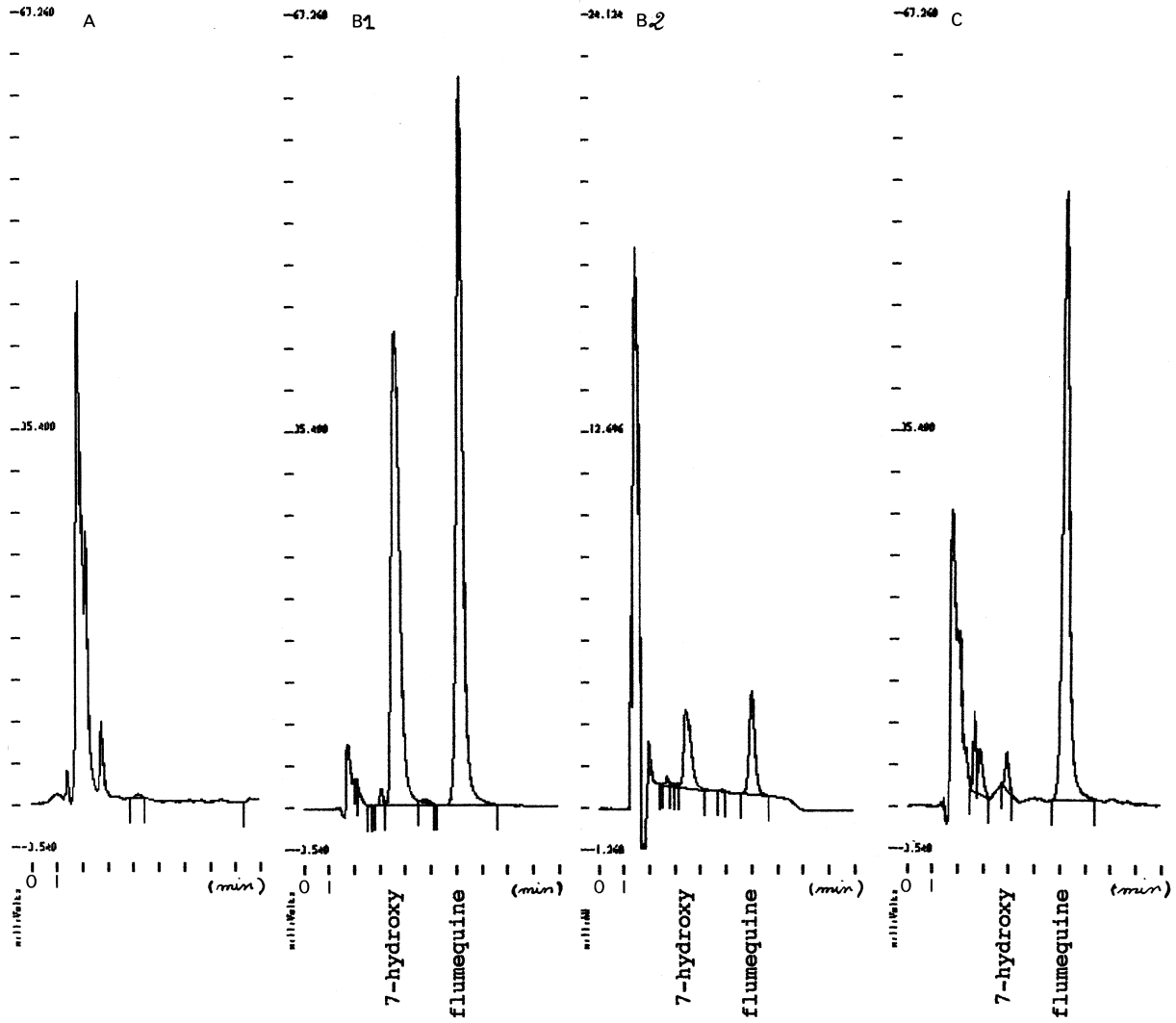


Fig. 2. Chromatograms of (A) blank plasma obtained by fluorimetric detection, of (B) plasma fortified with 1 mg/l of flumequine and of 7-hydroxyflumequine obtained (B1) by fluorimetric detection and (B2) by UV detection and of (C) plasma sample from a sheep treated with flumequine obtained by fluorimetric detection.

acid) but not for the 7-hydroxyflumequine which had a smaller retention time than flumequine. No precautions were required during the storage of flumequine and 7-hydroxyflumequine in plasma at room temperature during 4 h and at −20°C during six months according to our results of the stability study and in agreement with Guyonnet et al. [10].

The recoveries in plasma varied from 85 to 96% with UV detection and from 78 to 91% with fluorimetric detection for flumequine and from 58 to 79% for 7-hydroxyflumequine (Table 1). The mean recovery of flumequine was 87±3% and for 7-hydroxyflumequine was 60±1%. The mean, intra- and inter-day of reproducibilities of the procedure for both flumequine and 7-hydroxyflumequine are listed in Table 2.

In conclusion, this HPLC method described is selective and reproducible and therefore suitable for

Table 1
Recovery rates of flumequine and 7-hydroxyflumequine in spiked sheep plasma

	Flumequine conc. (μg/l)					7-Hydroxyflumequine conc. (μg/l)		
	500	250	50	25	5	500	100	10
Fluorimetric detection								
N	10	10	9	10	9	8	9	9
Recovery (%)	85	86	85	83	86	60	61	76
S.D.	2	1	3	2	3	1	1	3
	Flumequine conc (mg/l)							
	50	10	5	1	0.5			
UV detection								
N	10	10	10	10	10			
Recovery (%)	86	88	90	89	89			
S.D.	1	2	3	2	3			

N=Number of analyses.
S.D.=Standard deviation.

Table 2
Accuracy (mean concentration±S.D.), intra-day (CVr) and inter-day (CVR) precision of HPLC analyses

	Flumequine conc. (μg/l)					7-Hydroxyflumequine conc. (μg/l)		
	500	250	50	25	5	500	100	10
Fluorimetric detection								
N	10	8	8	10	8	8	8	8
Mean±S.D. (μg/l)	501.±9.6	252.2±3.9	49.9±1.5	24.3±0.5	5.1±0.2	495.6±9.8	101.2±2.5	12.6±0.5
CVr (%)	0.8	1.1	3.4	2.0	2.9	2.0	2.4	2.3
CVR (%)	2.0	1.1	3.4	2.0	4.5	2.0	2.4	4.2
	Flumequine conc. (mg/l)							
	50	10	5	1	0.5			
UV detection								
N	10	10	10	10	10			
Mean±S.D. (mg/l)	49.1±0.8	9.9±0.2	5.1±0.2	1.0±0.02	0.5±0.02			
CVr (%)	1.5	1.1	2.9	0.7	1.1			
CVR (%)	1.6	2.0	3.8	2.5	3.8			

N=Number of analyses during the study of the precision, CVr: the intra-day precision coefficient (%) and CVR: the inter-day precision coefficient (%).

the analyses of plasma sample during flumequine pharmacokinetic studies [7].

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