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Effects of the matrix and sample preparation on the determination of fluoroquinolone residues in animal tissues

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Abstract

The effects of aqueous–organic solvent extraction and enzymic digestion sonication procedure on the isolation of spiked fluoroquinolones from poultry tissue have been studied. The highest recovery from spiked tissue was obtained using a mixture of trichloroacetic acid–acetonitrile (8:2) as extractant and an SDB1 cartridge for clean-up purposes. Validation data are presented for enrofloxacin, ciprofloxacin, sarafloxacin and difloxacin. The spiking procedure (spike contact time, spike solvent and matrix) had a small influence on the recovery of fluroquinolones from poultry muscle or liver. The effects of a different extraction on the determination of incurred enrofloxacin and its metabolite, ciprofloxacin, residues in poultry tissues have been investigated. The extraction procedures investigated – aqueous–organic solvent extraction, enzymic digestion or sonication – all gave similar results for incurred fluoroquinolone concentration in poultry muscle after correlation for spike recovery. The highest results were obtained in poultry liver when enzymic digestion has been used.

Keywords: Solid-phase extraction; Food analysis; Fluoroquinolones

1. Introduction

Fluroquinolones (FQs) are very effective chemotherapeutic agents in a wide range of infections and diseases. Experimental data indicate that distribution of FQs in animal tissues is rapid, and concentrations reached are higher than the corresponding plasma levels [1]. These characteristics make these drugs suitable for the treatment of livestock, especially poultry.

Animal treatment with FQs represents a potential hazard to consumers due to the persistence of residues in tissues. European Union (EU) countries established a maximum residue level (MRL) of 30 μg/kg of muscle, liver or kidney (sum of enrofloxacin and ciprofloxacin). The recommendations also include 10–100 μg sarafloxacin/kg, and 1900 μg difloxacin/kg, depending on the poultry tissue [2].

Many methods have been published for the determination of FQs in biological samples [3–6]. These methods usually utilize blending in organic or aqueous–organic extractans that followed solid-phase extraction (SPE) as a purification technique. However, clean-up involving ion-exchange, adsorption or reversed-phases (C_8 or C_{18}) can be reproduced with varying degrees of success that are strongly influenced by the manufacturer/batch of SPE column used.

Methodology for FQs with co-polymeric styrene–divinylbenzene support for clean-up has already been described by this laboratory [7]. The aim of this investigation was to expand this methodology to the

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determination of FQs in poultry tissues and a comparison of the efficiency of different extraction procedures on the assay of incurred enrofloxacin and its metabolite, ciprofoloxacin, in poultry muscle and liver. Additionally, the effect of the spiking procedure, and the optimal conditions for SPE have been studied. The work reported here has been carried out as a part of a project investigating the influence of a sample preparation on analytical determination of veterinary drug residues in animal tissues.

2. Experimental

2.1. Materials

Acetonitrile (ACN), trichloroacetic acid (TCA) and orthophosphoric acid were of analytical grade. 1-Heptanesulfonic acid was obtained from Sigma (Poole, UK). Sarafloxacin (SRFX), difloxacin (DIFX) were from Abbott Labs. (North Chicago, IL, USA). Enrofloxacin (ERFX) was from Union Quimico Farmaceutica (Barcelona, Spain) and ciprofloxacin (CRFX) was from Flavine (Madrid, Spain).

2.2. Stock solution

Individual stock standard solutions (1 mg/ml) of each standard were prepared in acetonitrile. The working solutions were mixtures prepared by the dilution of 1 ml of each stock solution to serial 10-fold dilutions in mobile phase of 100, 10, and 1 μg/ml. All solutions were stored in the dark at −4°C.

2.3. Samples

2.3.1. Blank tissue

Muscle and liver samples were obtained from poultry that had not been exposed to any FQs within the previous 4 weeks. Tissue samples were stored at −20°C until the time of the in vitro study. A sample of liver and muscle (5 g wet mass) was accurately weighed.

2.3.2. Incurred samples

Samples of liver and muscle were taken from broiler chicken dosed with enrofloxacin at a level of 10 mg/kg body mass, by intramuscular injection. The chicken was slaughtered 24 h after dosing. Samples were stored at −20°C before analysis.

2.4. Spiking procedures

In order to investigate the effect of spiking procedure on extraction efficiency, the fluoroquinolone standard solutions were prepared in water or in acetonitrile. Spiked samples were prepared at 0.50 μg/g by the addition of 0.1 ml of solution containing 10 μg of each fluoroquinolone/ml, to 5 g blank tissue.

2.5. Sample preparation procedures

2.5.1. Procedure I: standard procedure

The sample preparation was based on the method described in a previous paper [7]. To 5 g of ground muscle or liver was added a mixture containing 10% TCA–acetonitrile (8:2) and then the sample was homogenized. After homogenization the tube content was centrifuged at 3500 *g* for 10 min (4°C). The supernatant was filtered and diluted with 70 ml of water.

2.5.2. Procedure II: enzymic digestion

Poultry tissue (5 g) was homogenized with 8 ml of a suspension containing 0.8 mg/ml collagenase and 2 mg/ml protease in water. To the homogenate 2 ml of 0.1 *M* sodium acetate buffer, pH 5 was added and the mixture was incubated at 37°C for 24 h. Sodium acetate buffer, pH 5 was then added (8 ml) and the tube content was carefully mixed. The suspension was then centrifuged at 3500 *g* for 10 min at room temperature.

2.5.3. Procedure III: sonication

Poultry tissue (5 g) was sonicated with 20 ml 0.05 *M* sodium acetate buffer, pH 4 for 2 min. The ultrasonic probe used was a Vibra cell (Sonic & Materials, Danbury, CT, USA), fitted with a standard probe. The suspension was centrifuged at 10 000 *g* for 10 min at room temperature.

2.6. Solid-phase extraction

The cartridges Bakerbond octadecyl (C_{18}) 500 mg (catalogue No. 7020-03), Bakerbond octyl (C_8) 500 mg (7087-03), Bakerbond styrene–divinylbenzene (SDB1) 200 mg (7519-02), Bakerbond amine (NH_2) 500 mg (7088-03) and Bakerbond benzenesulfonic acid ($C_6H_5SO_3H$) 500 mg (7090-03) used in this study, were purchased from J.T. Baker (Deventer, The Netherlands). The cartridges were used as supplied.

At first, the extraction of blank liver tissue was performed according to the procedures described above. The filtered supernatants (5 ml) were used for the preparation of solutions containing 50 ng of each drug/ml. These solutions were processed with a vacuum manifold system through the cartridges, which previously have been conditioned with 3 ml of methanol followed by 3 ml of water. The cartridges were washed with 3 ml of water, and elution was accomplished using 2 ml of HPLC mobile phase.

2.7. Chromatography system

A Hewlett-Packard 1050 liquid chromatograph equipped with a fluorescence detection (FLD) system with excitation wavelength=278 mm and emission wavelength=440 mm was used to analyze the tested solutions. LC control, data acquisition and peak integration was performed by Hewlett–Packard HPLC ChemStation Software. The chromatographic analyses were performed on a LiChrospher 100 RP-8 (250×4 mm) column with mobile phase 0.025 m*M* orthophosphoric acid–acetonitrile (70:30) containing 2.5 m*M* 1-heptanesulfonic acid. Isocratic mode at a flow-rate of 1.0 ml/min was used for the separation of analytes. Aliquots of 20 μl were injected into the column.

3. Results and discussion

3.1. Optimization of TCA–acetonitrile extraction

In the previous paper [7], we reported a simultaneous determination of FQs antibacterials in bovine and porcine tissues where samples were extracted with TCA–acetonitrile, and the clean-up procedure used an SDB1 cartridge. There, we expended the use of such a method for the determination of FQs in poultry tissue. Table 1 shows the results of the recovery experiment on chicken muscle and liver tissue spiked with 0.25 μg/g of each drug, respectively.

Table 1
The effect of the organic solvent content in the extracting solvent on the recovery of enrofloxacin, ciprofloxacin, difloxacin and sarafloxacin from chicken liver[a]

Extracting solvent	Recovery (%)			
	ERFX	CRFX	DIFX	SRFX
5% TCA	39	44	45	40
5% TCA–acetonitrile (9:1)	69	75	72	70
5% TCA–acetonitrile (8:2)	85	87	83	80
5% TCA–acetonitrile (7:3)	84	82	80	82
5% TCA–acetonitrile (6:4)	81	81	82	80
5% TCA–acetonitrile (5:5)	80	81	82	82

[a] Samples were spiked with 0.25 μg/g of each drug. Mean results of six replicates.
TCA, Trichloroacetic acid.

The acetonitrile content at 30%, as was used previously, provided good recoveries, but many interfering compounds were isolated as well. Therefore, we used acetonitrile at 20% for poultry sample extraction. A decrease in acetonitrile content did not adversely influence recoveries, while minimizing the isolation of co-extractive components from matrix.

Table 2 shows the influence of the TCA concentration on the recoveries of FQs from biological samples. Isolation with 5% TCA–acetonitrile (8:2, v/v) was not suitable enough with regard to deproteinization. An increase in TCA content to 10% resulted in good deproteinization of poultry samples,

Table 2
The effect of the concentration of trichloroacetic acid in extracting solvent on the recovery of enrofloxacin, ciprofloxacin, difloxacin and sarafloxacin from chicken liver[a]

Extracting solvent	Recovery (%)			
	ERFX	CRFX	DIFX	SRFX
5% TCA–acetonitrile (8:2)	85	87	83	80
7.5% TCA–acetonitrile (8:2)	84	86	81	81
10.0% TCA–acetonitrile (8:2)	86	85	87	82
12.5% TCA–acetonitrile (8:2)	84	82	82	83

[a] Samples were spiked with 0.25 μg/g of each drug. Mean results of six replicates.

Table 3
The effect of the spiking procedure on the recovery of enrofloxacin, ciprofloxacin, difloxacin and sarafloxacin from chicken liver[a]

Tissue	Spike contact time (h)	Spike solvent	Recovery (%)			
			ERFX	CRFX	DIFX	SRFX
Muscle	0	Water	85	86	86	87
	0	Acetonitrile	84	84	83	84
	1	Water	83	85	85	88
	1	Acetonitrile	82	83	81	83
	24	Water	80	82	82	83
	24	Acetonitrile	82	83	83	81
Liver	0	Water	83	86	84	83
	0	Acetonitrile	85	82	86	84
	1	Water	80	84	84	82
	1	Acetonitrile	83	87	84	81
	24	Water	80	81	81	83
	24	Acetonitrile	81	82	83	88

[a] Samples were spiked with 0.50 μg/g of each drug. Mean results of six replicates.

and 10% TCA–acetonitrile (8:2, v/v) was used as the extractant in subsequent experiments.

3.2. Effects of the spiking procedure

Three elements of the spiking procedure – the spiking solvent, the contact time between spiking and extraction, and the tissue matrix – were investigated. The standard extraction procedure was used in these experiments. The results obtained are shown in Table 3.

The differences in recovery between the various procedures were all less than 10%. Analysis of variance (ANOVA) evaluation of the data suggested that there was no significant effect of spike solvent on recovery. The contact time caused a small but statistically not significant reduction in recovery. There was no significant difference between the recoveries from kidney and muscle tissue at zero contact time.

3.3. Validation

The recoveries of sarafloxacin, difloxacin, enrofloxacin and ciprofloxacin extracted from the spiked muscle and liver were calculated by comparison with a solution of suitable analytes. All FQ compounds were extracted with high efficiency (>80%) from biological matrices. As it was found in a previous paper [7], the highest recoveries were obtained from muscle. Table 4 shows the results of intra-assay (precision), and inter-assay (day-to-day variation) of the method. Average variabilities for all types of assays were calculated at less than 10%.

Table 4
The reproducibility of the HPLC assay fluoroquinolones in muscle tissue

Fluoroquinolone	Precision (RSD, %)[a]			
	Intra-day		Inter-day	
	0.25 μg/g	0.5 μg/g	0.25 μg/g	0.5 μg/g
ERFX	9	9	9	9
CRFX	9	9	10	9
DIFX	9	8	8	8
SRFX	10	9	10	9

[a] Samples were spiked with 0.25 μg/g of each drug.

3.4. Evaluation of SPE clean-up

In this study extracts of liver tissue samples were obtained using the methodologies supplied for isolation of veterinary drugs from samples of animal origin, and the SPE cartridges included for clean-up have been demonstrated to provide high recoveries [3,4,8,9]. In order to select a suitable cartridge, commercially available (C_8, C_{18}, SDB1, NH_2, and $C_6H_5SO_3H$) cartridges were compared for their ability to retain FQs.

To eliminate the isolation of fluoroquinolone from the matrix as a variable, the extracts were spiked with the drugs before loading to SPE cartridges, and the retention capacity for each drug on the different support was compared. The results are shown in Table 5.

Generally, the drugs were strongly adsorbed on (C_8, C_{18} and SDB1) cartridges after application in solutions prepared with sonication or enzymic digestion method. While C_8 or C_{18} cartridges were coupled with TCA–acetonitrile procedure, the recoveries were strongly lower. This is caused by the presence of acetonitrile in extraction solution. It was found that the organic solution did not have such an important effect when a styrene–divinylbenzene had been used as cartridge support.

The extracts obtained from sonication or enzymic digestion methods were pigmented and contained large amounts of co-extracted compounds from the matrix and generally were more ''dirty'' than from the standard procedure. In this study, a styrene–divinylbenzene SPE clean-up of the extract was the most effective in removing the majority of the co-extracted compound in the extract. The use of this support has been found suitable both for the sonication or enzymic digestion procedure.

3.5. Extraction from incurred tissue samples

Two extraction procedures involving protease digestion or sonication were compared with aqueous–organic extraction used routinely in this laboratory. These investigations were carried out using muscle and liver, which are the target poultry tissues for detection of fluoroquinolones.

Tables 6 and 7 show that the ultrasonic probe treatment of tissue homogenate had a minimal effect on the extraction of incurred enrofloxacin or ciprofloxacin from liver or muscle. The recoveries from spiked tissues compared with the standard extraction were slightly less.

The digestion with β-glucuronidase and protease was found to increase the amount of incurred enrofloxacin and its metabolite in liver compared to the standard procedure. The influence of enzymic

Table 5
The influence of extraction cartridges on the recovery of fluoroquinolone drugs from chicken muscle extracted with different methods[a]

Fluoroquinolone	Method	Recovery (%)				
		C_{18}	C_8	SDB1	NH_2	BSA
ERFX	TCA–acetonitrile	34	43	84	63	80
	Sonic	80	77	85	76	83
	Enzymic	76	77	81	73	80
CRFX	TCA–acetonitrile	41	41	86	70	72
	Sonic	76	79	84	69	77
	Enzymic	73	73	87	73	74
SRFX	TCA–acetonitrile	30	36	87	76	78
	Sonic	81	76	85	63	70
	Enzymic	76	73	83	57	71
DIFX	TCA–acetonitrile	46	48	82	70	74
	Sonic	81	82	81	79	73
	Enzymic	82	78	83	73	70

[a] Samples were spiked with 0.50 μg/g of each drug. Mean results of four replicates.

Table 6
The effect of the probe treatment on the determination of incurred enrofloxacin and ciprofloxacin in chicken liver

	Enrofloxacin			Ciprofloxacin		
	TCA–ACN	Enzymic	Sonic	TCA–ACN	Enzymic	Sonic
Spike recovery (%)	79.2	72.6	70.4	78.4	71.7	69.3
Amount (μg/g) of incurred extracted						
Uncorrected for recovery	0.46	0.49	0.32	0.05	0.05	0.03
Corrected for spike recovery	0.58	0.68	0.45	0.06	0.06	0.04
n	5	5	5	5	5	5

ACN, Acetonitrile.

Table 7
The effect of the probe treatment on the determination of incurred enrofloxacin and ciprofloxacin in chicken muscle

	Enrofloxacin			Ciprofloxacin		
	TCA–ACN	Enzymic	Sonic	TCA–ACN	Enzymic	Sonic
Spike recovery (%)	77.9	72.3	71.6	76.7	72.3	70.1
Amount (μg/g) of incurred extracted						
Uncorrected for recovery	0.18	0.16	0.10	0.02	0.02	0.01
Corrected for spike recovery	0.23	0.22	0.14	0.03	0.03	0.02
n	5	5	5	5	5	5

digestion on improved levels of both fluoroquinolones in muscle levels was not so clear.

4. Conclusions

The change of TCA concentration and the proportion between TCA and acetonitrile in the extraction procedure were effective in the deproteinization of poultry tissue samples and isolation of analytes from spiked samples. These studies have shown that the choice of spiking procedures has no influence on the determination of fluoroquinolones in animal tissues. It was found that a styrene–divinylbenzene SPE clean-up allows the removal of most co-extractive compounds that are isolated with the different extraction procedures.

The protease digestion or sonic probe treatments did not increase the amounts of enrofloxacin or ciprofloxacin from incurred residues in poultry tissues. The most likely explanation for the similarity in the results obtained using different extraction methods is that enrofloxacin and ciprofloxacin are readily extractable, in the solvent systems tested, from spiked or incurred tissues.

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