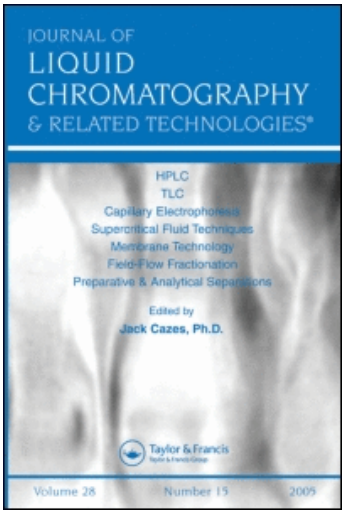

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Determination of Flunixin and Tiamulin Hydrogen Fumarate in Meat and Toltrazuril and the Metabolite Toltrazurilsulfon in Meat and Eggs Using LC/MS

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ABSTRACT

A liquid chromatographic-atmospheric pressure ionization ion spray method is described for the determination of flunixin (FLU), tiamulin hydrogen fumarate (TIA) in meat, and toltrazuril (TOL) and the metabolite toltrazurilsulfon (TOLS) (Ponazuril) in meat and egg. The method can also be used for the determination of flunixin in milk. Samples were extracted with acetone–tetrahydrofurane, after which the organic layer was separated from water with dichloromethane and evaporated to dryness. The dry residue was diluted in methanol-l-heptane sulfonic

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acid and fat was extracted with hexane, filtered, and injected into the LC–MS. The lower limit of quantification for flunixin and toltrazuril were 5 ng/g and for tiamulin and toltrazurilsulfon in meat 2 ng/g.

Key Words: Flunixin; Tiamulin; Toltrazuril; Food; LC/MS.

INTRODUCTION

Flunixin (FLU) is a non-steroidal anti-inflammatory and analgesic drug used in veterinary practice. Tiamulin hydrogen fumarate (TIA) is used for the treatment of swine dysentery and pneumonia. Toltrazuril (TOL) is used in the prophylaxis and treatment of coccidiosis in chicken and turkey, and neonatal pig. The drug, TIA, must not be used together with other polyether ionophores such as lasalocid, monensin, narasin, and salinomycin,[1] because of drug incompatibility.[2,3] When used in food-producing animals, residues of FLU, TIA, TOL, and toltrazurilsulfon (TOLS) represent a potential health risk to consumers.

Several methods have been published describing the determination of TIA in food and swine liver.[1,4,5] These methods are, however, time-consuming, have poor sensitivity, and require the use of large quantities of chemical reagents. No methods have been published for the determination of FLU, TOL, and TOLS. Simultaneous determination of two or more drugs is desirable in drug residue control programmes, because of the saving both in time and money.

The purpose of the present study, was to develop a time saving, cost-effective, and sensitive method for the determination of FLU, TIA in meat, and TOL and the metabolite TOLS in egg and meat. The sensitivity should at least meet the requirement of quantitative detection at the Maximun Residues Levels (MRL), as set by the EU Committee for Veterinary Medical Products. The MRLs for FLU in bovine and swine meat are 20 and 50 ng/g, respectively. In milk the MRL is 40 ng/mL. For TIA in meat the MRL is 100 ng/g. The MRL for the sum of TOL and TOLS in meat is 100 ng/g.

EXPERIMENTAL

Materials and Reagents

Samples of drug-free chicken and swine meat and egg were used in the spiking experiments.

All chemicals and solvents were of analytical or HPLC grade. Flunixin was donated by Norbrook Laboratories Limited (Northern Ireland), TIA was supplied by Sigma Co. (St. Louis, MO). Toltrazuril and TOLS were donated by Bayer (Norway). Stock solution (1 mg/mL) of FLU was prepared by dilution with water, and TIA by dilution with acetone–tetrahydrofurane (6 + 4) and further with water. Working solutions of mixed FLU and TIA (1.0 and 0.1 μg/mL) was prepared by diluting the stock solutions with water. Stock solutions (1 mg/mL) and working solutions (1.0 and 0.1 μg/mL) of a mixture of TOL and TOLS were prepared by dilution with methanol. Stock and working solutions (standards) were stored in a refrigerator (+4°C).

Spin-X centrifuge filter units (0.22 μm, nylon type) from Costar (Cambridge, MA) were also used.

Solution A was a mixture of 70% methanol and 30% 0.022 M l-heptane sulfonic acid sodium salt (Avocado Research Chemicals, UK) and 0.01 M di-sodium hydrogenphosphate (Ferax, Germany). The aqueous part of this mixture was prepared by dissolving 4.45 g/L heptane sulfonate and 1.8 g/L di-sodium hydrogenphosphate di-hydrate in c. 750 mL of water. The pH was then adjusted to c. 6.3 with 3 M H_3PO_4 and to 6.0 with 0.5 M H_3PO_4. The solution was then diluted with water to 1 L and, finally, the pH was adjusted to 6.0 with 0.5 M H_3PO_4.

Chromatographic Conditions

The analyses were performed on a Applied Biosystems LC–MS system, consisting of a Series 200 quaternary pump and a Series 200 autosampler (Perkin Elmer). The acquired data were entered into a Model 8500 Apple Power Macintosh, and processed with either Multiview 1.4 or MacQuan 1.6 software packages (Applied Biosystems), for spectral information and quantification data processing. An API 100 LC–MS system (Applied Biosystems) single quadrupole mass spectrometer with a Turbo-Ion Spray Inlet for the API LC–MS system was employed for this study. The turbo probe of the instrument was maintained at 150°C, the probe air flow-rate was 6 L/min. The LC–MS was set to collect single-ion data in negative ion mode for the ion at m/z 295.2, 424, and 456 for FLU, TOL, and TOLS and positive ion mode for the ion at m/z 494.3 for TIA. The entrance electrode voltages were adjusted to provide optimum intensity for the molecular ion.

The analytical column (Chromolith Performance RP-18e, 100 × 4.6 i.d. mm) and the guard column (5 × 4.6 mm i.d.) (Merck, Germany) were operated with a constant temperature of 26°C. The guard column was connected to an A-318 precolumn filter on line, with an A-102X frits (Upchurch Scientific, USA). The mobile phase for TIA was a mixture of methanol–0.1% acetic acid in water (55 + 45). The pump was operated

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isocratically at a flow rate of 1.2 mL/min. The mobile phase for FLU, TOL, and TOLS consisted of a mixture of acetonitrile–0.1% acetic acid in water (Table 1).

Beforehand, the LC eluent was split, post-column, ~1 : 20 with a flow rate of 1 mL/min with water–methanol (1 : 1), so that c. 50 μL flowed into the Ion-Spray ion source.

Sample Pretreatment

Volumes of 0.5 mL water and 0.5 mL methanol (1 : 1) or standard (the total volume should always be 1 mL), and 8 mL acetone-tetrahydrofuran (6 + 4) were added to 3 g/mL of sample. The mixture was homogenized for ~6 sec with an Ultra-Turrax TP 18/10 (Janke & Kunkel KG, Germany). For milk, the mixture was shaken vigorously for ~10 sec. After centrifugation for 5 min at 5000 rpm, 1.6 mL of supernatant (corresponding to 0.4 g/mL sample) was transferred to a glass-stoppered centrifuge tube, followed by 3 mL CH_2Cl_2. The mixture was shaken vigorously for ~10 sec, and after being centrifuged for 3 min at 3500 rpm. The organic layer (CH_2Cl_2–acetone) was transferred to another glass-stoppered tube with a Pasteur pipette to avoid water residues. The organic layer was evaporated to dryness under a stream of air, using a Reacti-Therm heating module at 45°C and a Reacti-Vap evaporating unit (Pierce, Rockford, IL). The dry residue was dissolved (vortex-mixed) in 0.6 mL solution A, and then 0.5 mL hexane was added. The mixture was shaken for 6 sec. After centrifugation for 3 min, the upper layer (hexane) was discarded, and the hexane washing was repeated. The methanol based phase was filtered through a Spin-X centrifuge filter. Two hundred microliter of the clear filtrate was diluted with 200 μL water and mixed. Aliquots of 20 μL were injected into the LC–MS at intervals of 7 min for the determination of

Table 1. Mobile phase operating conditions for FLU, TOL, and TOLS.

Step	Time (min)	Flow (μL/min)	Acetic acid (0.1%) in water (%)	Acetonitrile (%)
0	0.1	1,200	65	35
1	4.0	1,200	30	70
2	3.0	2,000	65	35
3	1.0	1,200	65	35

Note: Beforehand, the LC eluent was split post-column ~1 : 20 with a flow rate of 1 mL/min with water–methanol (1 : 1) so that c. 5O μL flowed into the Ion-Spray ion source.

TIA in swine meat. For the determination of FLU in swine meat and TOL/TOLS in chicken meat and egg, 10 μL were injected into the LC–MS at intervals of 8 min.

Calibration Curves and Recovery Studies

The precision, recovery, and linearity for FLU, TIA, TOL, and TOLS was determined from meat spiked with standard solutions to yield 0, 2, 5, 10, 20, 30, 50, 100, and 150 ng/g. Duplicate samples were used. The recovery rates were determined by comparing analysis of spiked meat with those of standard solutions. The linearity of the standard curves for FLU and TIA in meat, and for TOL and TOLS in meat and egg, was calculated using peak height measurements.

For the determination of recovery rates, the corresponding doses of standard solutions were diluted with solution A and water (1 + 1). For example, for a sample of 10 ng/g, 300 μL of a mixture of FLU/TIA and 300 μL of a mixture of TOL/TOLS standard solution of 0.1 μg/mL and 200 μL methanol and 200 μL water were added to 3 g of meat, as described for sample pretreatment. The equivalent standard for FLU/TIA and TOL/TOLS was calculated to be 40 μL standard solution of 0.1 μg/mL. From sample pretreatment, 1.6 mL supernatant corresponds to a 0.4 g sample; that is 7.5 part of the total volume of 12 mL. The corresponding ratio applied to the standard solution gives 300 μL standard: 7.5 = 40 μL standard, diluted with 560 μL with solution A and 560 μL water, the total volume is 1.2 mL.

RESULTS AND DISCUSSION

For FLU in meat and TOLS in egg and meat, the standard curves were linear in the investigated areas from 2 to 150 ng/g. For TIA in meat and TOL in meat and egg from 5 to 150 ng/g. The corresponding correlation coefficients for FLU, TIA in meat, and TOL and TOLS in meat and egg were 0.9996. The recovery and repeatabilities for FLU, TIA, TOL, and TOLS are shown in Table 2.

Chromatograms obtained from drug-free meat samples, and from the corresponding samples spiked with TIA, are shown in Fig. 1. Chromatograms of drug-free meat and the corresponding samples spiked with FLU, TOL, and TOLS are shown in Fig. 2. Chromatograms of drug-free egg, and a real egg sample from a poultry farm, 10 days after accidentally being contaminated with TOL are shown in Fig. 3.

Table 2. Recovery and repeatability for flunixin, tiamulin, toltrazuril and toltrazurilsulfon from spiked samples.

Sample	*n*	Amount of drug (ng/g)	FLU (%)		TIA (%)		TOL (%)		TOLS (%)	
			SD	RC	SD	RC	SD	RC	SD	RC
Meat	8	30	0.7	97	1.9	105	1.2	91	0.8	93
3 g	8	100	0.9	98	1.7	109	0.5	98	0.4	97
Egg	8						1.0	93	0.9	92
3 g	8						0.6	96	0.5	96

Note: SD, standard deviation; RC, recovery.

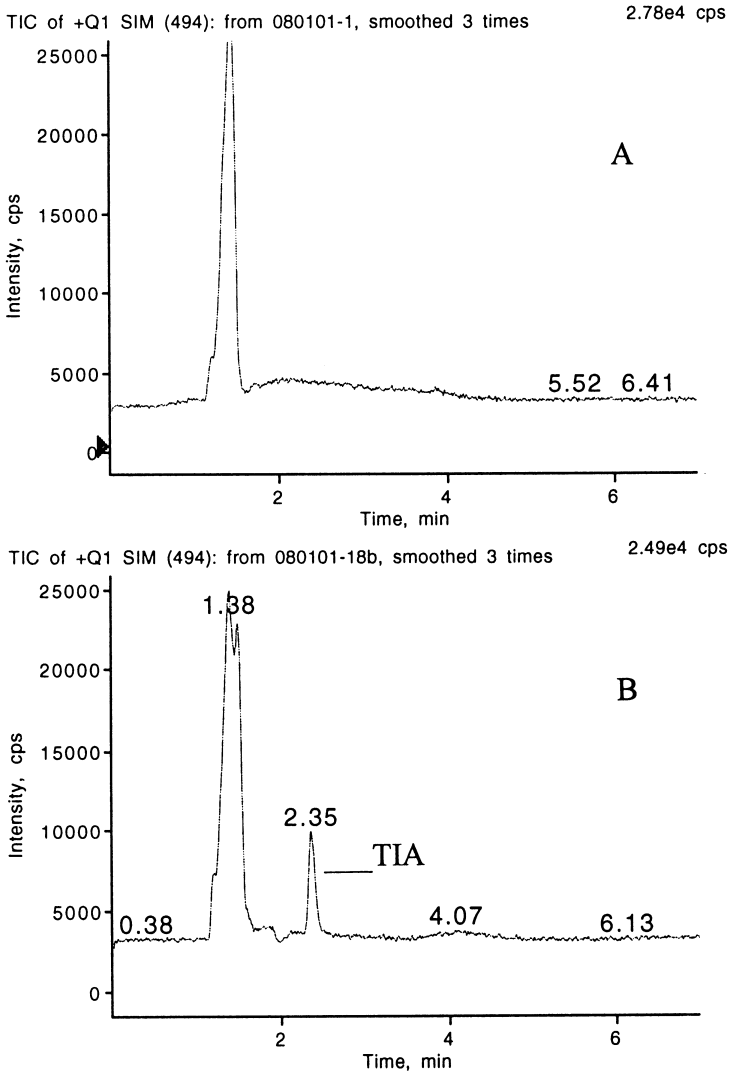

Figure 1. Chromatograms of extract from swine meat. (A) Drug-free meat, (B) meat spiked with TIA (10 ng/g).

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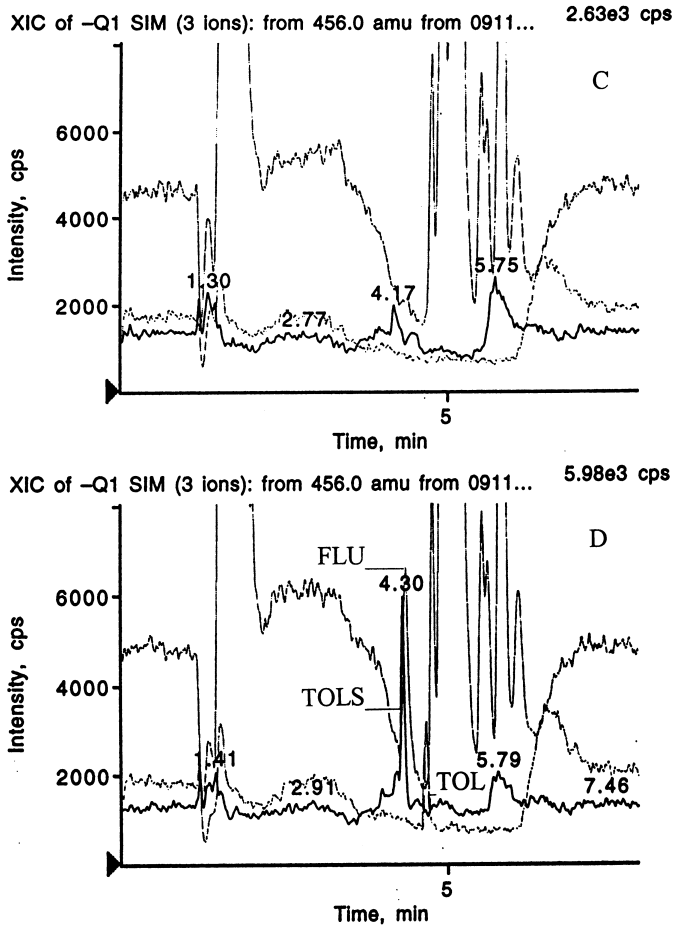

Figure 2. Chromatograms of extract from chicken meat. (C) Drug free-meat, (D) meat spiked with FLU, TOL, and TOLS (20 ng/g).

The Chromolith Merck analytical column is a new generation analytical column, developed on the basis of a new gel process for the preparation of monolithic porous silica rods using highly pure-metal free alkoxysilanes. The silica rod possesses a biporous structure, typically consisting of macropores and mesopores in the skeleton, providing a higher porosity compared with particulate columns.

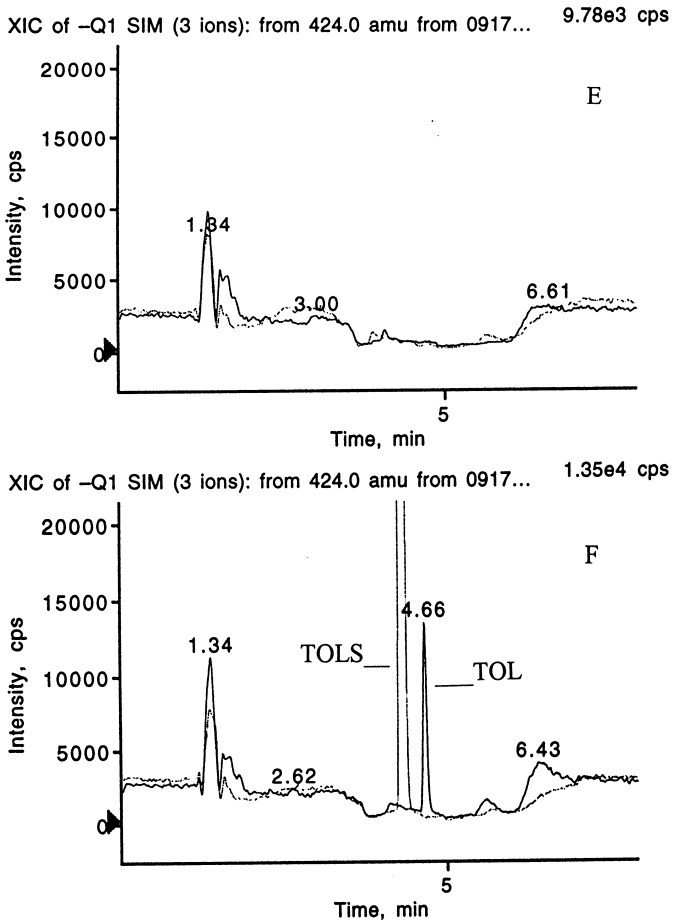

Figure 3. Chromatograms of extract from egg. (E) Drug-free egg, (F) real egg contaminated with TOL (quantified to 47 and 185 ng/g for TOL and TOLS, respectively).

Initially, the addition of acetone–tetrahydrofurane and dichloromethane in the sample pretreatment was performed using an automate-pipette with tips. However, this technique gave irregular volumes. This problem was avoided with a bottle top dispenser, which gave good results.

In many laboratories, a stream of nitrogen is used to evaporate drug samples to dryness. Nitrogen and air produced from a central air compressor

(generator) were compared for evaporating the samples of FLU, TIA, TOL, and TOLS from muscle and milk. No differences were found. The use of air is an economically favourable alternative compared with nitrogen.

The method presented in this paper is selective, robust, sensitive, and accurate. It can also be used for the determination of FLU in milk with a good baseline resolution, but the method is not validated.

The detection limit of the assay was calculated to be three times the baseline noise from drug-free tissue. No interference was seen during analysis, neither when calibrating the curves, nor when performing the recovery studies.

The limit of detection for TOLS and TIA in swine meat was close to 1 ng/g, while the limit of quantification was 2 ng/g, for FLU and TOL. The limit of detection in swine meat was close to 2.5 ng/g, while the limit of quantification was 5 ng/g. The limit of detection and quantification for TOL and TOLS in egg were similar to meat.

The detection limit of the assay depends mainly on the sensitivity of the LC/MS. This, in turn, could be influenced by such factors as the position of the ion spray inlet, the composition of the mobile phase, and the flow-rate of the mobile phase into the ion source.

The described assay offers a number of significant advantages compared to previously published methods for the detection and quantification of TIA. The detection limits are good. The extraction procedure is simple and no derivatization is required.

The advantage of the LC/MS technique lies in the combination of the separation capabilities of HPLC with the power of MS as an identification and confirmation method with high sensitivity, and quantitative capability. Quantification using selected ion monitoring has high selectivity, sensitivity, and broad dynamic range. Thus, LC/MS seems to provide a better alternative than GC or HPLC.

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