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Short communication

Determination of flumequine and oxolinic acid in fortified chicken tissue using on-line dialysis and high-performance liquid chromatography with fluorescence detection

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Abstract

Isolation of the fluoroquinolones, flumequine and oxolinic acid, from fortified chicken liver was achieved using liquid–liquid extraction, aqueous on-line dialysis and trace enrichment. Dialysis, trace enrichment and column switching were performed using the ASTED system. Separation of the isolated compounds in the tissue extracts was performed using reversed-phase HPLC and fluorescence detection. This procedure yielded excellent mean recoveries at the 50, 25, 10 and 5 ng/g spiking levels for flumequine (94–96%; 4–9% R.S.D.) and at the 25 and 5 ng/g spiking levels for oxolinic acid (98–99%; 4–6% R.S.D.). Clean chromatograms were obtained, allowing detection of 5 ng/g flumequine and 2.5 ng/g oxolinic acid to be easily made. Due to its lower organic solvent consumption, automation and on-line capabilities, this method may be a suitable replacement for conventional chemical extraction techniques for drug residues in animal tissues.

Keywords: Dialysis; Sample handling; Flumequine; Oxolinic acid; Antibiotics; Fluoroquinolones

1. Introduction

Compounds containing the 4-quinolone ring and 3-carboxylic acid group have been found to exhibit antimicrobial activity, especially against Gram-negative organisms. Several of these structurally-related nalidixic, piromidic and oxolinic acid (OXA) antimicrobial agents and a fluoro-containing derivative, flumequine (FMQ), have been extensively used to treat infections in fish raised by aquaculture [1]. The veterinary uses of these drugs have also been described [2]. More recently, the Food and Drug Administration approved the therapeutic use of sarafloxacin in poultry, making this fluoroquinolone (FQ) the first to be approved for use in food animals [3]. Other members of this class of drugs have been petitioned for similar use.

Common approaches for the extraction of FQs from blood and tissue matrices employ acid or base treatments followed by extraction into an organic solvent or aqueous buffer, respectively [4–6]. The vast majority of FQ liquid–liquid extraction and detection methods, however, pertain to fish tissues. An extraction method for FMQ in animal meat and kidney tissues yielded low recoveries and a relatively low detection limit [7]. Recently, Munns et al. [8] reported a modified version of an extraction method by Nose et al. [4] in which liquid–liquid extraction

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and HPLC–fluorescence detection was used to determine flumequine, oxolinic, nalidixic and piromidic acids in fortified and incurred fish muscle. At fortification levels of 5 and 10 ng/g, respectively, the overall recoveries of 83–94% were obtained. This is now an AOAC peer verified method [9].

Dialysis is an aqueous-based technique which separates macromolecules, such as proteins, from small molecules, such as drugs, as long as the two types of molecules are not bound or associated with each other. Because of the aqueous-based nature of dialysis, it is a potentially attractive alternative to conventional, solvent intensive, liquid–liquid extraction techniques. A commercial ASTED (automated sequential trace enrichment of dialysates) system is available that combines dialysis with trace enrichment and column-switching capabilities for HPLC separation and detection. The use of ASTED for preparing biological samples containing residual drug compounds have been thoroughly investigated and described earlier [10–12]. Quinolonecarboxylic acids, which contain both an acidic carboxylic acid and a basic nitrogen in a tertiary amine, lend themselves to dialysis because of their solubility in water. There have been reports [13–15] describing chemical extraction methods for FMQ and OXA in fish tissue coupled with automated cleanup of the residues using on-line dialysis. Our attempt to apply these extraction and dialysis methods for FMQ in chicken tissues resulted in poor recoveries.

In this paper, we describe a simplified version of a liquid–liquid extraction method adapted from Munns et al. [8] for the determination of FMQ and OXA residues in chicken liver employing aqueous on-line dialysis for sample cleanup and HPLC-fluorescence detection for separation and detection.

2. Methods

2.1. Chemicals and reagents

The antibiotics, FMQ and OXA, and the reagents sodium chloride and sodium phosphate (monobasic and dibasic) were purchased from Sigma Chemical Company (St. Louis, MO, USA). All solvents (acetonitrile, acetone, hexane and chloroform) were HPLC-grade and obtained from Burdick and Jackson (Muskegon, MI, USA). Tetrahydrofuran (HPLC-grade) was from Sigma–Aldrich (St. Louis, MO, USA; Milwaukee, WI, USA).

Stock solutions of FMQ and OXA (500 μg/ml) were prepared in acetonitrile and are stable for up to 1 year when stored at 4°C. Working solutions were prepared daily by diluting the stock solutions in 0.1 *M* sodium phosphate, pH 9.0, to final concentrations of 2.5, 0.5 and 0.25 μg/ml for FMQ and of 1.25, 0.25 and 0.125 μg/ml for OXA.

2.2. Tissue fortification and extraction

Minced liver from chickens fed a drug-free diet was homogenized using a Janke and Kunkel Ultra-Turrax T25 homogenizer (IKA Labortechnik, Staufen, Germany). One-gram portions of homogenized tissue were weighed into 50-ml plastic centrifuge tubes. A 20-μl aliquot each of the FMQ and OXA working solution was pipetted onto each tissue portion, giving a final concentration of 50, 10 or 5 ng/g FMQ or 25, 5 or 2.5 ng/g OXA, respectively. The tubes were shaken vigorously for 30 min at 25°C, after which they were allowed to sit overnight at 4°C to allow the drug to penetrate the homogenized tissue. Control tissue samples were treated the same way except 40 μl of 0.1 *M* sodium phosphate, pH 9.0, was added. The extraction method for fluoroquinolones from tissue was adapted from Munns et al. [8] and modified as shown in Table 1.

2.3. Dialysis

Dialysis was performed on the ASTED XL (Gilson, Villiers-le-Bel, France). The unit was comprised of the following components: autosampler; two 1-ml syringe pumps (401C); a 370-μl flat-bed dialysis block with a M_r 15 000 MWCO Cuprophan (cellulose acetate) membrane; trace enrichment cartridge (TEC) which was initially a PLRP-S (polystyrene–divinylbenzene) guard column, 5 mm×3 mm I.D. (Polymer Labs., Amherst, MA, USA) but was later replaced with a Hypersil ODS, 5.8 mm×4.6 mm I.D. (supplied with ASTED); two Rheodyne 6-port switching valves; and a controller keypad. Switching valve 1 is responsible for injection of the sample onto the dialyzer and then onto the TEC while valve

Table 1
A comparison of two methods for the recovery of flumequine and oxolinic acid from tissues

Peer verified method (Ref. [9])	On-line microdialysis method
1. Fortification: Add 50 μl drug solution to 1 kg tissue composite. Mix with paddle for 1–2 min. Repeat 2 more times with scraping in between mixing.	1. Fortification: Add 20 μl drug solution to 1 g tissue homogenate. Incubate with shaking for 30 min at 25°C Sit overnight at 4°C.
2. Extraction and cleanup: a. Deprotenize with 50 ml acetone and homogenize. b. Centrifuge; decant and filter supernatant. c. Repeat acetone extraction 2 more times. d. Add 15 ml *n*-propanol to combined extracts. e. Rotary evaporate to 15 ml. f. Defat with 20 ml acetone, 30 ml hexane; 60 ml 3% NaCl. g. Centrifuge; remove hexane layer. h. Repeat defatting process. i. Extract into 25 ml chloroform and separate phases, saving chloroform layer. j. Repeat chloroform step, combining all chloroform extracts. k. Add 25 ml 0.1 *M* NaOH and separate phases; discard chloroform. l. Wash aqueous layer with 25 ml chloroform and discard chloroform. m. Extract residue into 25 ml chloroform after acidifying with 25 ml 0.075 *M* H_3PO_4. n. Drain and filter chloroform layer. o. Repeat extraction with another 25 ml chloroform. p. Evaporate combined chloroform layers to dryness. q. Add 2 ml acetonitrile and evaporate to dryness again. r. Pipette 1.0 ml mobile phase and dissolve dried residue. s. Filter through 0.45 μm membrane if solution is cloudy. t. Inject onto HPLC.	2. Extraction and cleanup: a. Deprotenize with 5 ml acetone and homogenize. b. Centrifuge; decant and save supernatant. c. Repeat acetone extraction 1 more time. d. Defat with 2 ml acetone, 3 ml hexane; 6 ml 3% NaCl. e. Centrifuge; remove hexane layer. f. Extract into 25 ml chloroform and separate phases, saving chloroform layer. g. Add 2.5 ml 0.1 *M* sodium phosphate, pH 9.0+1 drop 1 *M* NAOH and separate phases; discard chloroform[a]. h. Wash aqueous layer with 2.5 ml chloroform and discard chloroform. i. Centrifuge aqueous layer to clarify solution. j. Inject onto ASTED/HPLC.

[a] NaOH was added to ensure complete conversion of the drug to the carboxylate ionized form (final pH>10).

2 controls the injection of TEC contents onto the HPLC column.

The system was manually primed by first rinsing the syringe pumps (PRIME) with the appropriate buffer, and then rinsing the dialysis membrane (INITIATE) with 0.1 *M* sodium phosphate, pH 9.0 (Buffer A) for the donor channel and 0.02 *M* sodium phosphate, pH 5.0 (Buffer B) for the recipient channel. The program used to execute the dialysis was as follows. (1) Wash TEC with 500 μl 50% acetonitrile. (2) Condition TEC with 500 μl Buffer B. (3) Dialyze in the pulsed donor/pulsed recipient mode. In this mode, one syringe pump delivered 370 μl of the sample onto the donor channel of the dialysis block at a rate of 0.3 ml/min and was then allowed to sit for 5 min. After the wait, 3×650 μl pulses of Buffer B were pumped through the recipient channel and deposited onto the TEC at a flow-rate of 0.6 ml/min. The whole donor/recipient process was repeated. Thus, a total of 740 μl of sample was dialyzed into a total of 3.9 ml of recipient volume which was then deposited onto the

TEC. (4) Wash the TEC with 500 μl Buffer B. (5) Inject contents of TEC onto the HPLC analytical column. (6) Wash the donor channel of the dialyzer with 2 ml 0.01% Triton X-100 in Buffer B and the recipient channel with 3 ml Buffer B. (7) Regenerate the dialysis membrane with 2 ml Buffer A and 3 ml Buffer B.

The time required for completion of dialysis of a 740-μl sample injection was 27.2 min. The wash step lasted 10.7 min. Therefore, sample injection into the ASTED proceeded every 37.9 min. The TEC breakthrough volumes for the FMQ and OXA were 8 ml for the PLRP-S guard cartridge and ~12 ml for the ODS cartridge. Tissue sample carryover on the dialysis membrane was minimal up to 20 injections following the indicated wash steps.

2.4. High-performance liquid chromatography

Reversed-phase chromatography was performed on the Rainin HPLC system using two HPXL pumps and computer-controlled by the Dynamax system software. A polymeric column (PLRP-S, 150×4.6 mm, Polymer Labs.) was used. The mobile phase was acetonitrile–tetrahydrofuran–20 m*M* sodium phosphate, pH 5.0 (20:15:65, v/v/v) pumped at a rate of 0.6 ml/min. Under these conditions, OXA had a retention time of ~7 min and FMQ a time of ~12 min.

Fluorescence detection (FL-750 HPLC-Plus spectrofluorimeter and 750-03 Universal lamp power supply, McPherson Instruments, Acton, MA, USA) was used to monitor the analyte. The excitation/emission wavelengths were 318/364 nm. Sensitivity was set at 0.003, the gain at 550 PMT/$h\nu$, and the lamp power at 7 amps. The minimal detectable level (MDL) was 2.5 ng for OXA and 5.0 ng for FMQ.

3. Results and discussion

We sought to simplify the multiresidue extraction method of Munns et al. [8,9] and to apply it to animal tissue so that it would be practical for use in a regulatory laboratory without sacrificing recovery or sensitivity. This goal was made possible by the automated on-line dialysis system. Chicken liver was the tissue of choice to test the dialysis method because of the recent FDA approval for the use of FQs in poultry and because of their high pharmokinetic distribution in the liver [15,16]. We also wanted to carry out the tissue fortification step in a manner that would best represent tissue incurred with the drug residue. Thus, we allowed for an extra lengthy drug–tissue homogenate incubation step of at least 16 h at 4°C.

A comparison between the method of Munns et al. [9] and the modified on-line dialysis version used in our laboratory is shown in Table 1. For a 1 kg tissue sample, Munns' method required 11 solvent extraction steps, 3 evaporation steps, and an overall solvent consumption of more than 370 ml. In order to reduce the need for large amounts of solvents, we used a smaller tissue sample. Our modified procedure then employed the same initial liquid–liquid extraction steps as was described previously [4,9]; i.e. deproteinizing with acetone, defatting with hexane, partitioning into chloroform, and base extraction into an aqueous buffer. However, from that point on (steps 2 m–s) we incorporated aqueous dialysis as a means to further cleanup the residues in the extract. Although the overall processing time was not shortened, a significant improvement was made with respect to the simplification of the liquid–liquid extraction procedure. Table 2 summarizes the recoveries (from 3 to 5 determinations) obtained after chemical extraction and dialysis of tissues fortified at 4 levels of FMQ and 3 levels of OXA. The overall

Table 2
Summary of recovery levels of flumequine and oxolinic acid from fortified chicken liver

Residue	Fortification level (ng/g)	Mean recovery (%)[a]	R.S.D. (%)
FMQ	50	94.0% (5)	9.2%
	25	96.4% (4)	4.4%
	10	95.5% (4)	3.6%
	5	95.3% (3)	7.1%
OXA	25	98.2% (4)	3.5%
	5	99.4% (5)	6.1%
	2.5	114.8% (3)	16.9%

[a] Recoveries were determined by comparing the extract peak areas relative to the peak areas obtained when a buffered solution of the drugs were carried through the same extraction, dialysis and chromatography procedures. Numbers in parentheses designate the number of determinations.

mean recoveries of the residues spiked from 5 to 50 ng/g were comparable to those reported by Munns et al. [8]. The R.S.D. values were also in good agreement with the values obtained by the original method. Linear calibration curves were obtained from overnight fortification of tissue homogenates with 0 to 80 ng/g FMQ ($y=3.400+6.685x$, $r^2=0.998$) and 0 to 40 ng/g OXA ($y=5.800+13.700x$, $r^2=0.998$). The HPLC–fluorescence detection profiles of fluoroquinolone-spiked chicken liver extracts using our extraction–dialysis method demonstrates the usefulness of this technique to resolve and identify these compounds at the 10 ng/g level — presented in Fig. 1.

Because FMQ and OXA molecules are highly bound to proteins, a deproteinization step prior to extraction is necessary. Nonorganic reagents, such as the protein precipitators/displacers, monochloroacetic acid (MCA) and trichloroacetic acid (TCA), are commonly used to disrupt drug–protein interactions in fluid matrices such as urine, whole blood or plasma [13,14]. Treatment of salmon whole blood and plasma spiked with flumequine, oxolinic acid and nalidixic acid with MCA or TCA has also been reported [17]. Animal tissue matrices, however, may pose a greater challenge for these aqueous reagents and it presently appears feasible to incorporate some rigorous organic solvent steps in an extraction method as a means of liberating protein-associated drug residues, thereby enhancing their chances of isolation from tissues.

A steep concentration gradient between the donor and recipient channels should be maintained to enhance mass transfer of solute across the membrane. In our dialysis method, we programmed the ASTED to dialyze samples in the pulsed donor/pulsed recipient mode. In this mode, the donor and recipient fluids are static and an equilibrium is set-up across the semipermeable membrane. Because constant removal of recipient buffer is required to enhance mass transfer, operating the dialyzer in either the static donor/continuous recipient or static donor/pulsed receipient modes would be more ideal and may result in improved dialysis efficiency and time.

Some early-eluting peaks are apparent in the chromatograms in Fig. 1; however, the two drugs are well removed from these potential interferences. Studies in our laboratory show that changing the pH of the mobile phase influences the retention time of

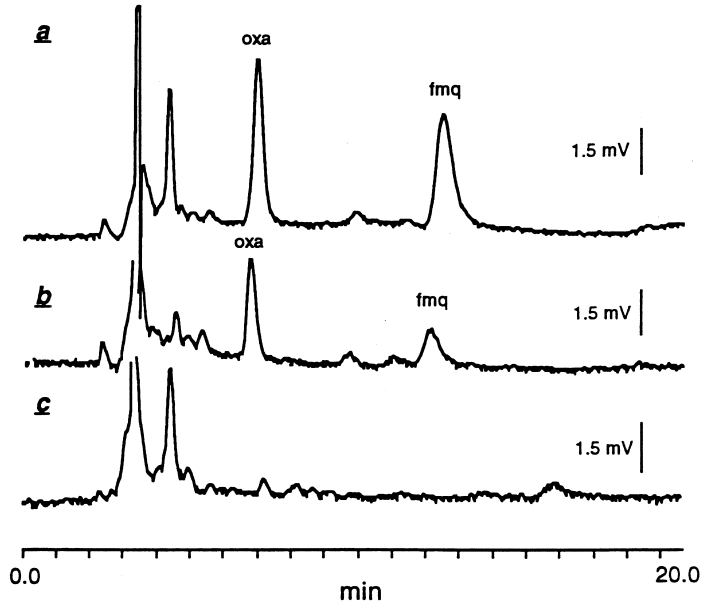

Fig. 1. Chromatograms of fluoroquinone-fortified and control chicken liver. (a) Chicken liver fortified with 50 ng/g flumequine and 25 ng/g oxolinic acid. (b) Chicken liver fortified with 10 ng/g flumequine and 5 ng/g oxolinic acid. (c) Control chicken liver.

FMQ on the polystyrene–divinylbenzene column. The retention time increased as the mobile phase pH decreased, presumably due to conversion of the molecule to its nonpolar unionized form. Oxolinic acid, on the other hand, was found to have a retention maximum close to its isoelectric point; thus, it possessed lower k' (capacity factor) values at pH values more acidic or alkaline than its isoelectric point [18]. These results suggest it may be possible to shift the drugs away from interfering extract components by merely adjusting the pH of the mobile phase.

4. Conclusion

We have successfully demonstrated that a published extraction method used for isolating FMQ and OXA from fish tissue can be modified for use in animal tissue employing significantly less organic solvent. We have also shown the efficacy and simplicity of aqueous dialysis and trace enrichment using the ASTED system for cleaning up animal tissue extracts containing residual levels of these drugs. Thus, ASTED, with its full automation and on-line capabilities was a valuable tool for replacing conventional chemical cleanup procedures without compromising analyte recovery and sensitivity.

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