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HPLC Methods for the Determination of some Quinolones in Fish and Animal Tissues

Key words: Oxolinic acid, Ciprofloxacin, Enrofloxacin, Fish, Chicken, HPLC

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Introduction

Quinolones and fluoroquinolones are antibacterial agents with a broad spectrum against Gram-positive and Gram-negative bacteria. The most popular products are flumequine, oxolinic acid and enrofloxacin with its metabolite ciprofloxacin.

In Switzerland, the limit is 10 µg/kg for oxolinic acid (OA) and 30 µg/kg for enrofloxacin in milk, eggs and meat. Oxolinic acid is used in aquaculture since 1987 (1989, 12 600 kg in Norway (1)). Recently, a number of methods based on HPLC (2–3) or GC-MS (4) has been published. These methods require extensive cleanup. The aim of this work was to develop a simple and rapid method for the determination of oxolinic acid.

Material and methods

All solvents and chemicals were of analytical grade quality. Oxolinic acid (O–0877) and ciprofloxacin (C-4791) were purchased from Sigma and enrofloxacin was a gift from Bayer.

Extraction buffer solution

In a 1000 ml graduated cylinder, pour 6.0 g tris-(hydroxy-methyl)-aminomethane (Fluka 93350) and 200 ml distilled water. Adjust the pH to 8 with acetic acid (about 1.8 ml), complete to 250 ml with distilled water. Dilute to 1000 ml with methanol.

Analytical procedure for oxolinic acid (screening method)

Extraction

5 g of tissue (fish, chicken, veal, pork) were extracted with 10 ml of buffer, using an homogeniser (30 s at 15 000 rpm). After 30 min at 55 °C and 5 min in an ultrasonic bath, the mixture was centrifuged during 15 min at 4000 rpm. The clear solution was analysed by HPLC with fluorimetric detection.

Apparatus and conditions

Hewlett Packard 1090 M Liquid Chromatograph, Kontron SFM 25LC fluorescence detector, Nucleosil Column 100-5 C_{18} 5 µm 125 x 4 mm and Nucleosil Precolumn 100-5 C_{18} 7 µm 10 x 4 mm. Mobile phase: A ortho phosphoric acid 0.02 mol/l, B acetonitrile/tetrahydrofurane 1:1, a gradient was used at the end of the analysis to flush the column before the next sample:

Time	A	B	Flow rate
0.00	78%	22%	1.3 ml/min
3.50	78%	22%	1.3 ml/min
4.50	55%	45%	1.7 ml/min
6.00	55%	45%	1.7 ml/min
7.00	78%	22%	1.7 ml/min
8.00	78%	22%	1.3 ml/min

Injection volume was 100 µl and oven temperature 40 °C. Detection: excitation at 336 nm and emission at 375 nm. Retention time for oxolinic acid: 2.9 min.

Results

Typical chromatograms of control, spiked and positive samples are given in figures 1, 2 and 3. The mean recovery was 60% at spiking levels of 5, 10 and 30 µg/kg. 2 µg/kg were easily detected with this screening method and up to 48 samples can be analysed in one day.

Analytical procedure for oxolinic acid (clean up method)

Working with smoked salmon has led to interferences in the chromatograms, therefore a clean up has been developed as follows.

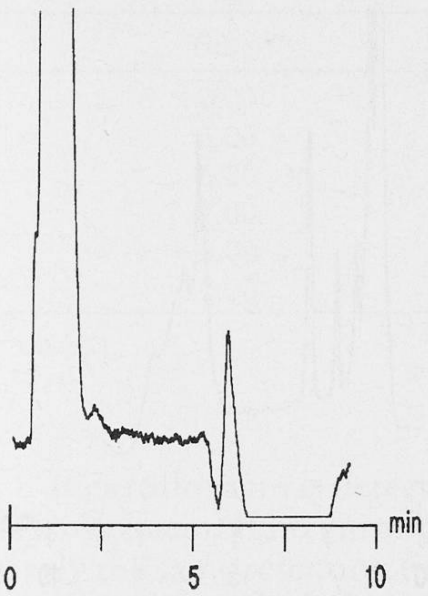

Fig. 1.
Blank salmon extract

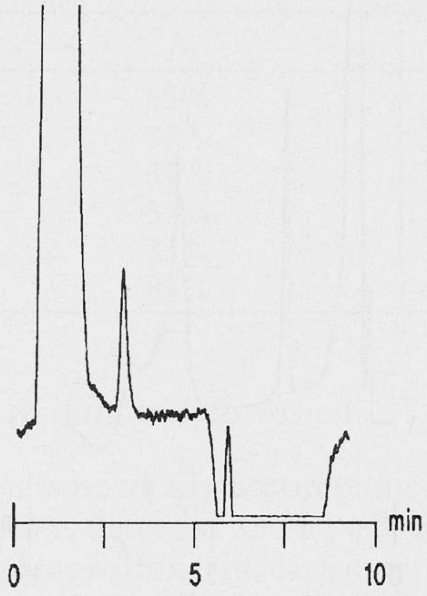

Fig. 2.
Salmon spiked at
10 µg OA/kg

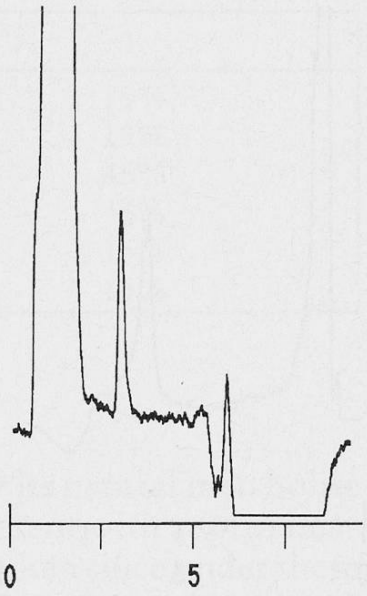

Fig. 3.
Positive salmon
sample with
12 µg OA/kg

Extraction

The extraction procedure was the same as in screening method, but with 20 ml of buffer. After the centrifugation, supernatant, to which 2–3 drops acetic acid were added, was partially evaporated (5–10 ml) and, after acidification with 20 ml of 0.5 mol/l phosphoric acid, extracted on a C_{18} cartridge (Chromabond 0.5 g, 3 ml, Macherey-Nagel). The eluate (2 x 2 ml of acetonitrile/triethylamin 9:1) was evaporated to dryness and the residue redissolved in 1 ml of mobile phase A.

Apparatus and conditions:

same as in screening method.

Results

The clean up procedure gives an excellent sensitivity and a good reproductibility. Typical recoveries were 58% (± 4%; n = 5), 59% (± 5%; n = 5) and 57% (± 5%; n = 5) at spiking levels of 5, 10 and 30 µg/kg. The detection limit is 1 µg/kg. Chromatograms obtained with this method are shown in figures 4, 5 and 6.

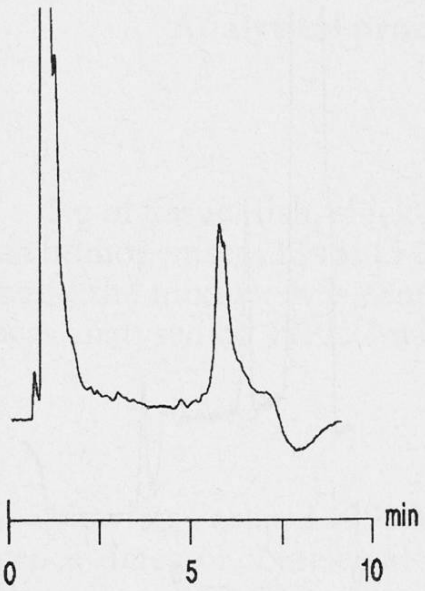

Fig. 4.
Blank salmon extract

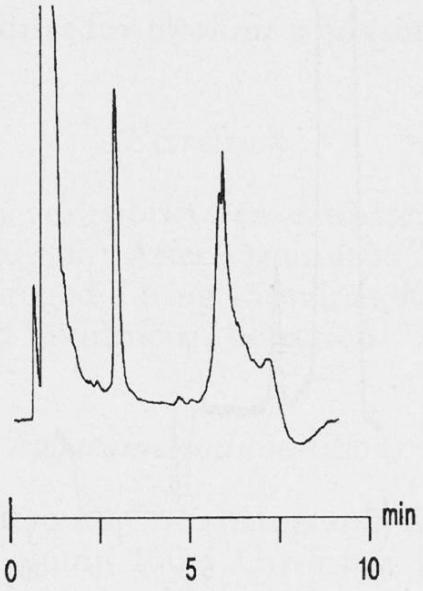

Fig. 5.
Salmon spiked at 10 µg/kg

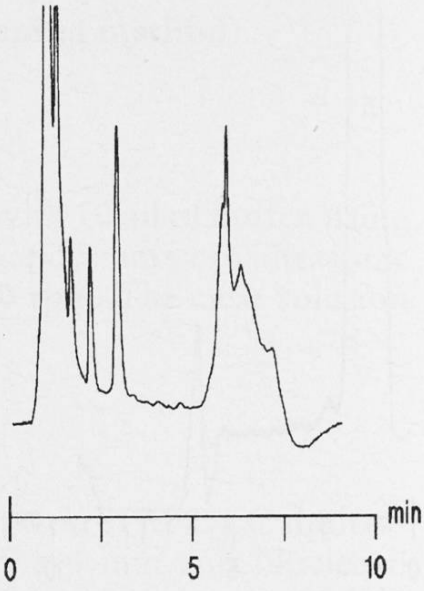

Fig. 6.
Positive salmon sample with 8 µg OA/kg

Analytical procedure for enrofloxacin and ciprofloxacin

Extraction

10 g of tissue (fish, chicken, veal, porc) were extracted twice with 40 ml of a mixture of ethanol/acetic acid 99:1 using an homogeniser (30 s at 15 000 rpm). After centrifugation (10 min at 4000 rpm), the combined solutions, to which 1 ml of triethylamine was added, were evaporated to dryness. After taking up with 2.5 ml of buffer solution diammonium-hydrogenphosphate pH 7.5, the solution was extracted three times with 2 ml of hexane and then transfered to a Chromabond C_{18} cartridge prewashed with 10 ml acetonitrile, 10 ml water and 10 ml buffer solution. The cartridge was washed with 2.5 ml of buffer solution and dried. The eluate (2 x 2 ml of acetonitrile/triethylamine 9:1) was evaporated to dryness and the residue redissolved in 1.0 ml of the mobile phase A.

Conditions

Mobile phase A: ortho phosphoric acid 0.05 mol/l to pH 3.0 with triethylamine, B: acetonitrile. Flow rate 1.5 ml/min). A gradient was used at the end of the analysis to rinse the column.

Injection volume 25 µl. Detection: excitation 278 nm, emission 443 nm. Retention times: ciprofloxacin 3.0 min and enrofloxacin 4.1 min.

Time	A	B
0.00	85%	15%
3.00	85%	15%
6.00	55%	45%
7.00	55%	45%
8.00	85%	15%
10.00	85%	15%

Results and discussion

If enrofloxacin is detected, it is normally accompanied by its natural metabolite ciprofloxacin. If no enrofloxacin is detected and a peak is present with approximatively the same retention time of ciprofloxacin, care must be taken since under these conditions, norfloxacin elutes at 2.7 min. Work on norfloxacin (a fluoroquinolone) determination with the present method is under way. The mean recoveries for enrofloxacin were 76.5% (± 4.3%; $n = 5$), 73.2% (± 3.3%; $n = 5$) and 75.6% (± 2.7%; $n = 5$) at spiking levels of respectively 5, 10 and 30 µg/kg. For ciprofloxacin, the recoveries were lower: 47.5% (± 5.0%; $n = 5$), 50.7 (± 4.2%; $n = 5$) and 53.2% (± 4.4%; $n = 5$) at the same spiking levels. The detection limits is 2 µg/kg for ciprofloxacine and 2 µg/kg for enrofloxacine.

This method was successfully applied in routine controls of one-day-old chicks. These controls are very useful to detect the eventual veterinary treatment of the young animals. The figures 7, 8 and 9 shows chromatograms of an untreated and a treated young animal.

Conclusion

The results show that the proposed method for OA is very suitable for screening purposes. The detection limit of 2 µg/kg is sufficient because the official tolerance is 10 µg/kg. With complex matrices such as smoked fish, we recommend to use the special clean up procedure. Our screenings of many different species of fishes with these methods show that positive samples for OA were mainly found in farmed trouts. A few samples of salmon were containing oxolinic acid or enrofloxacin with its metabolite, but the amounts detected were always lower than 10 µg/kg.

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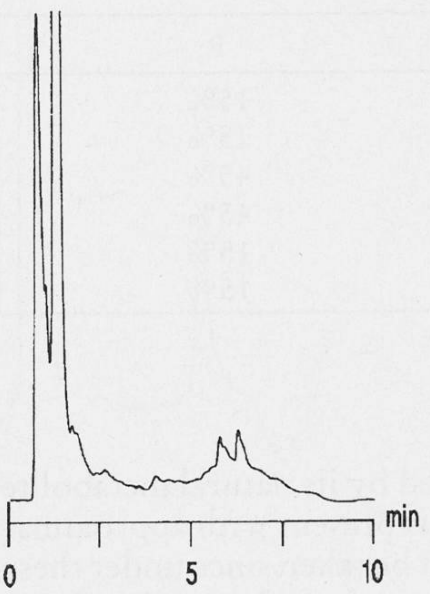

Fig. 7.
Untreated chick

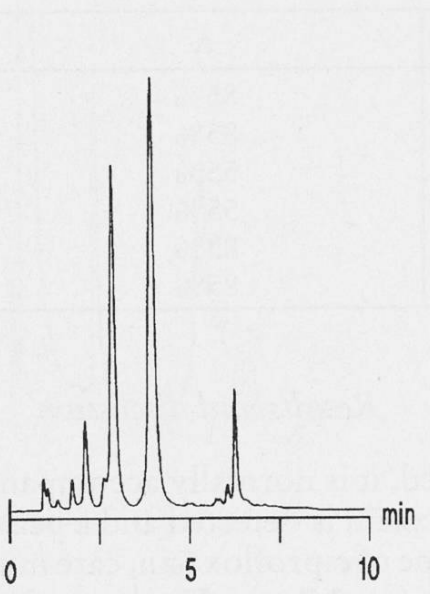

Fig. 8.
Treated chick contained 5 mg/kg Enroflox. and 7 mg/kg Ciproflox. (dilution 200 x)

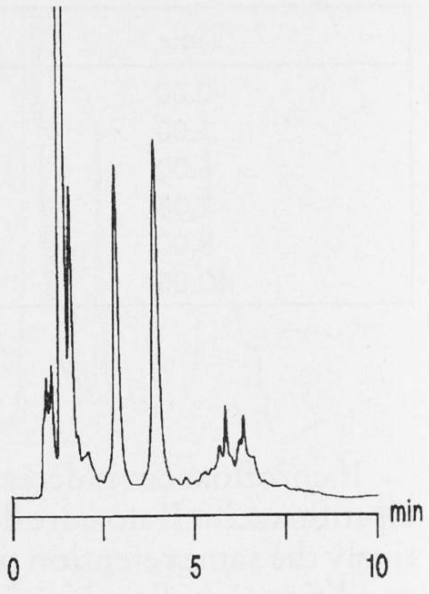

Fig. 9.
Chicken tissue spiked each with 10 µg/kg

Summary

A simple, fast screening HPLC method for the determination of oxolinic acid in animal tissues is presented. Up to 50 samples can be analysed in one day. A clean up procedure is also presented and the detection limit in tissues is 2 µg/kg.

Another method for the determination of ciprofloxacin and enrofloxacin has been developped and successfully applied in routine controls of fish, chickens and particularly of one-day-old chicks. The detection limit of this method is 2 µg/kg.

Zusammenfassung

Es werden zwei schnelle und einfache HPLC-Methoden zur Bestimmung von Quinolonen in Übersichtsuntersuchungen vorgestellt. Die erste dient zur Bestimmung von Oxolinsäure in tierischem Gewebe. Mit dieser Methode ist es möglich, bis zu 50 Proben pro Tag zu untersuchen. Ein Reinigungszwischenschritt kann eingefügt werden. Die Nachweisgrenze in Gewebe liegt bei 2 µg/kg.

Die zweite Methode für den Nachweis von Cipro- und Enrofloxacin wurde erfolgreich in der Routineanalytik von Fisch- und Pouletproben, speziell Eintagesküken, eingeführt. Die Nachweisgrenze dieser Methode liegt für beide Substanzen bei 2 µg/kg.

Résumé

Deux méthodes de détermination de quinolones sont présentées: La première est une procédure rapide de screening pour la détermination d'acide oxolinique dans les tissus

d'origine animale. Elle permet d'analyser 50 échantillons par jour. Dans cette méthode, une opération de clean up peut être incluse. La limite de détection se situe à 2 µg/kg.

Une deuxième méthode pour la détermination de ciprofloxacine et d'enrofloxacine a été développée et appliquée avec succès dans les contrôles de routine de poissons, poulets et particulièrement de poussins. La limite de détection se situe à 2 µg/kg pour les deux substances.

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