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Automated column-switching high-performance liquid chromatographic determination of flumequine and oxolinic acid in extracts from fish[a]

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ABSTRACT

Two methods for automated analysis of extracts from edible muscle tissue of Atlantic salmon are described. Oxolinic acid and flumequine are extracted with phosphate buffer pH 9, and the extracts are analysed by high-performance liquid chromatography using a column-switching system One method applies on-line concentration and clean-up of the extracts on a precolumn packed with polystyrene-divinylbenzene This method was useful for the analysis of oxolinic acid and flumequine in the μg/g range The other method was based on on-line dialysis and concentration of the dialysate on the polymeric precolumn This method was shown to be a reliable method for residue analysis, and the limit of detection was 2 ng/g for oxolinic acid and 3 ng/g for flumequine with fluorescence detection

INTRODUCTION

Infectious diseases have become a serious problem to the fish farming industry and the use of chemotherapeutics, such as oxolinic acid and flumequine, for the treatment of infections in fish is growing. There is a demand for automated methods for carrying out pharmacokinetic studies and for residue control of these drugs in Atlantic salmon.

Several methods based on high-performance liquid chromatography (HPLC) have been published for the determination of oxolinic acid and flumequine in fish tissue [1–7]. All methods apply extraction of the compounds with organic solvents, and the manual work-up procedures include treatments such as liquid–liquid extraction [1–6], solid-phase extraction [5–7] and evaporation of solvent [1–6]. These methods are too lengthy for routine analysis of large series of samples. However, by employing aqueous extraction of the drugs, sample prepara-

[a] Dedicated to Prof. Dr A. Baerheim Svendsen, Division of Pharmacognosy, Leiden University, Leiden, The Netherlands, on the occasion of his 70th birthday

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tion can easily be automated by using HPLC in combination with a column-switching system. An automated HPLC procedure for the determination of flumequine and oxolinic acid in plasma has recently been described [8]. In this procedure plasma samples were cleaned on a polymeric precolumn. After column-switching the analytes were separated on an analytical column and detected with a fluorescence detector. On-line dialysis combined with trace enrichment of the dialysate on a precolumn is another possibility for automated sample preparation [9]. Dialysis is a technique able to separate macromolecular compounds from small molecules, and the technique has been used successfully for automated clean-up of a variety of samples including residues in food [10,11].

This paper describes two automated HPLC methods for clean-up and concentration of aqueous muscle extracts. In one method on-line sample concentration and clean-up was achieved on a polystyrene-divinylbenzene precolumn. The other method applies on-line dialysis and trace enrichment of the dialysate on the polymeric precolumn. After column-switching the analytes were separated on an analytical column and detected with UV detection or fluorimetric detection. Both methods are easily carried out, and the performance of the methods is discussed.

EXPERIMENTAL

Chemicals

Oxolinic acid was obtained from Sigma (St. Louis, MO, U.S.A.) and flumequine was purchased from Solchem Italiana (Mulazzano, Italy). Sodium dihydrogenphosphate, sodium hydroxide and orthophosphoric acid were supplied by E. Merck (Darmstadt, F.R.G.). HPLC-grade acetonitrile and tetrahydrofuran were from Rathburn (Walkerburn, U.K.). HPLC-grade water was obtained from a Milli-Q (Millipore, Bedford, MA, U.S.A.) water purification system.

Preparation of standards

Standard solutions of flumequine and oxolinic acid (1 mg/ml) were prepared in 0.01 *M* sodium hydroxide. Working standards (50 μg/ml) were prepared by dilution in 0.05 *M* phosphate buffer and in 0.02 *M* orthophosphoric acid (0.001–5 μg/ml). Spiked muscle standards were prepared from the working standards. Muscles from Atlantic salmon were kept at −20°C until analysis.

High-performance liquid chromatography

The liquid chromatograph was an LC 6A (Shimadzu, Kyoto, Japan) equipped with a Model SPD-6A (Shimadzu) UV detector with a 8-μl flow cell operated at 260 nm or a Model RF 535 (Shimadzu) fluorescence detector with a 12-μl flow cell operated at an excitation wavelength of 325 nm and an emission wavelength of 365 nm. Peak heights were recorded on a Chromatopac C-R3A integrator (Shimadzu). The analytical column (150 mm × 4.6 mm I.D.) was packed with 5 μm particle diameter polystyrene-divinylbenzene PLRP-S (Polymer Labs., Church Stretton, U.K.).

Trace enrichment and clean-up on the precolumn

Samples were injected with a Model 231 auto-sampling injector (Gilson Medical Electronics, Villiers-le-Bel, France) equipped with a 401 dilutor with a 1-ml syringe and a Model 7010 automatic six-port valve (Rheodyne, Berkeley, CA, U.S.A.). The precolumn connected to the six-port valve was a Chrompack (Middelburg, The Netherlands) cartridge with a cartridge precolumn (10 mm × 2 mm I.D.) packed with 36 μm particle size polystyrene-divinylbenzene particles (Dynospheres, Dyno Particles, Lillestrøm, Norway).

Trace enrichment and clean-up procedure

Extract (200 μl) was injected into the precolumn. Sample concentration and clean-up were achieved with a total of 4 ml 0.02 *M* phosphate buffer pH 5 delivered at a flow-rate of 1.5 ml/min from the dilutor. The samples were back-flushed on to the PLRP-S analytical column with a mobile phase consisting of acetonitrile–tetrahydrofuran–0.02 *M* orthophosphoric acid (20:15:65, v/v) at a flow-rate of 0.7 ml/min and detected with UV detection. After 3 min the solvent stream through the precolumn was switched back to the inject position. The precolumn was regenerated with 5 ml of phosphate buffer at a flow-rate of 1.5 ml/min, and a new sample was injected. After preconcentration and clean-up the switching of the analytes into the analytical column was performed every 13 min.

On-line dialysis and concentration of the dialysate on the precolumn

The sample preparation system was a Gilson ASTED (Automated Sequential Trace Enrichment of Dialysates) unit consisting of a Model 231 auto-sampling injector, two 401 dilutors equipped with 1-ml syringes and one flat-bed dialyser with a donor channel volume of 370 μl and a recipient channel volume of 650 μl, fitted with a cuprophan membrane, 15 000 molecular weight cut-off. An automated Model 7010 six-port valve (Rheodyne) connected the precolumn either with the recipient channel of the dialyser or with the analytical column of the HPLC system. The trace enrichment column (10 mm × 2 mm I.D.) from Chrompack was packed with 36 μm particle size polystyrene-divinylbenzene (Dyno Particles).

ASTED procedure

The ASTED was operated in the concurrent mode with injections every 14 min. One dilutor was used to inject 370 μl of extract into the donor channel of the dialyser. The sample was held static in the donor channel while the other dilutor transported 4 ml of 0.02 *M* phosphate buffer pH 5 in the pulsed mode through the recipient channel of the dialyser into the precolumn. The volume of recipient solution transported through the dialyser was divided in 6.15 pulses of 650 μl. After each pulse the recipient solution was held static in the recipient channel for 33 s. The ASTED used 22 s for the delivery of each pulse making a total time of 6 min and 24 s for the dialysis of each sample. Upon switching of the six-port valve the precolumn was eluted for 2 min with the mobile phase to bring the analytes

into the analytical column. The mobile phase was acetonitrile–tetrahydrofuran–0.02 *M* orthophosphoric acid (20:14:75, v/v) delivered at a flow-rate of 0 7 ml/min. The compounds were detected with fluorescence detection. The six-port valve was then switched to bring the precolumn back to the recipient channel of the dialyser. The recipient side of the dialyser and the precolumn was then washed with 2 ml of 0.02 *M* phosphate buffer. The donor side of the dialyser was simultaneously washed with 2 ml of 0.02 *M* phosphate buffer pH 5 containing 100 mg/l Triton X-100.

Extraction of muscle

Phosphate buffer (0.05 *M*, pH 9) was used for extraction of the drugs from the edible muscle tissue of Atlantic salmon. Flumequine was used as internal standard for the determination of oxolinic acid and oxolinic acid as internal standard for flumequine. Internal standard (50 μg/ml in buffer) was used for determinations in the concentration range 0.5–5 μg/g while 5 μg/ml internal standard in buffer was used for determinations in the concentration range 0.05–0.5 μg/g. To 5 g of muscle were added 200 μl of internal standard solution, 50 ml of buffer and 10 ml of hexane. The mixture was homogenized for 2 min on an Ultra-Turrax T 25 homogenizer (Ika Werk, Staufen, F.R.G.) and thereafter sonicated for 3 min. The mixture was then centrifuged for 5 min (1920 *g*) The upper hexane phase, which also contained the tissue material, was discarded, and 1.5 ml of the aqueous extract were again centrifuged for 1 min (8800 *g*). The extract was then filled into autosampler vials and automatically injected.

Evaluation of extraction efficiency

During method development the recovery and reproducibility of the extraction procedure were investigated using buffers of pH 7–10. Muscle (5 g) spiked with flumequine and oxolinic acid (2 μg/g) were extracted. A 200-μl extract was directly injected into the trace enrichment column. After column-switching and separation of the analytes on the analytical column the peak heights were recorded and the recovery and the reproducibility were calculated. The standard curve for measuring the recovery was based on peak-height measurements of external standards of flumequine and oxolinic acid prepared in 0.02 *M* orthophosphoric acid. These external standard solutions were injected directly into the analytical column.

Validation of the procedures

The accuracy and precision of the methods were determined by analysis of spiked muscle samples. The calibration curves were based on measuring the peak-height ratios relative to the internal standard.

RESULTS AND DISCUSSION

Precolumn and HPLC system

Trace enrichment and clean-up of the analytes on the precolumn is affected by the polarity of the drugs, the eluent composition and the packing material. The polymeric packing material used in this investigation retained the acidic analytes strongly when acidic aqueous eluents of pH 2–5 were used for enrichment and clean-up. No breakthrough of the analytes was observed when 30 ml of 0.02 *M* phosphate buffer pH 5 were pumped through the precolumn after injection of a 500-μl sample solution pH 9. Injection of larger volumes of the alkaline sample solution results in breakthrough of the analytes. No matrix effects on the breakthrough was observed. The same peak height were obtained after injection of the analytes in buffer as in drug-free muscle extract spiked with the analytes. Consequently, the trace enrichment and clean-up procedure using phosphate buffer pH 5 as eluent for enrichment and clean-up of 200-μl muscle extract gave complete retention of the analytes on the precolumn. Direct injection of extracts into the precolumn could therefore be used for determination of the recovery and reproducibility of the extraction procedure.

The analytical HPLC system previously described for on-line preconcentration and clean-up of plasma samples [8,12] also proved to be reliable for the analysis of tissue extracts. The analytical column was remarkably stable and the same column has been used in our column-switching systems for more than two years without significant loss of efficiency.

Extraction procedure

Flumequine and oxolinic acid have low solubility in water but the solubility increases in alkaline solutions. During method development borate, EDTA and phosphate buffers were investigated. Hexane was added to extract lipids which could otherwise contaminate the precolumn. The recovery and reproducibility were measured after direct injection of extracts into the precolumn. It appeared

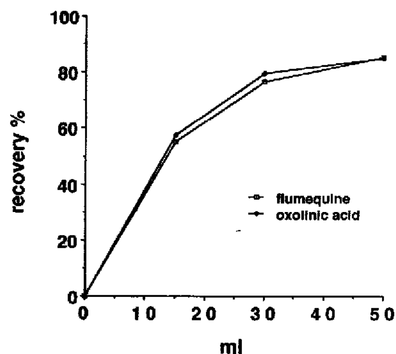

Fig. 1. Recovery of oxolinic acid and flumequine extracted from fish muscle with various volumes of 0.05 *M* phosphate buffer pH 9.

TABLE I

RECOVERY AND REPRODUCIBILITY AFTER EXTRACTION OF 5 g OF MUSCLE WITH 50 ml OF 0.05 *M* PHOSPHATE BUFFER

Compound	Recovery (mean ± C V., n = 6) (%)		
	pH 7	pH 8	pH 9
Flumequine	76.8 ± 2 8	83 8 ± 1.9	85 1 ± 0 9
Oxolinic acid	76 0 ± 3.7	84 4 ± 3.9	85.3 ± 1 2

that the pH was the most important parameter, and no significant difference in recovery was observed between the buffers. Phosphate buffer was selected for further studies, and 0.05 *M* buffers of pH 7–10 were investigated. To reduce handling time a single extraction was desirable. The recovery increased by increasing the pH of the buffer. However, by increasing the pH the amount of impurities extracted also increased. Impurities extracted at pH 10 interfered with the analysis.

Fig. 1 shows the recovery of the analytes extracted with various volumes of 0.05 *M* phosphate buffer pH 9, and Table I shows the recoveries and the reproducibilities obtained after one extraction with 50 ml of buffer pH 7–9. A 50-ml buffer pH 9 gave a recovery of the analytes of 85% with a coefficient of variation of 0.9% for oxolinic acid and 1.2% for flumequine. Due to the better recovery and reproducibility, 50 ml of 0.05 *M* phosphate buffer pH 9 was selected for extraction of 5 g of muscle. In extracts of spiked fresh and unfrozen muscle samples the same recoveries were found as in muscle samples which had been stored at −20°C for one year. Extraction of spiked muscle samples which were first stored for 24 h at room temperature and then at −20°C for one week before extraction also gave the same recoveries. By storing the extracts in the autosampler for 24 h no degradation of flumequine and oxolinic acid was observed.

On-line trace enrichment and clean-up of extracts on the precolumn

Injection of extracts directly into the precolumn was primarily used for the determination of the extraction efficiency. However, the procedure was also useful for the determination of oxolinic acid and flumequine in muscle in the μg/g range. Fig. 2 shows chromatograms of an extract of drug-free muscle and of an extract of muscle spiked with 1 μg/g oxolinic acid and flumequine. Results from validation of the procedure in the concentration range 0.5–4 μg/g are shown in Table II. A satisfactory coefficient of variation of 2.2–5.7% was obtained. The calibration graphs (peak-height ratio *versus* concentration in muscle) were linear in the concentration range 0.5–5 μg/g with r = 0.999. The precolumn was washed with 200 μl of water followed by 200 μl of acetonitrile after each tenth sample.

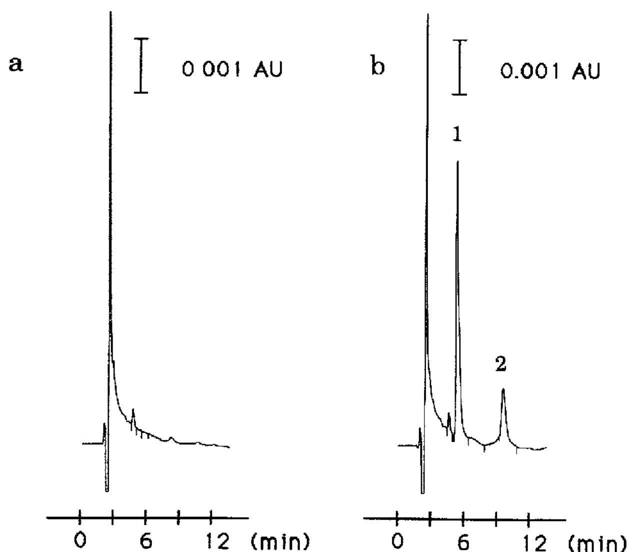

Fig. 2. Chromatograms of (a) an extract of drug-free muscle and (b) an extract of muscle spiked with 1 μg/g oxolinic acid and flumequine after clean-up on a precolumn packed with polystyrene-divinylbenzene Detection, UV at 260 nm. Peaks 1 = oxolinic acid; 2 = flumequine. For chromatographic conditions see text.

Following these conditions no increase in back-pressure was observed, and the precolumn was stable for fifty injections of 200 μl of extract before it was re-packed. For residue analysis, however, the precolumn gave insufficient clean-up of the extracts. Particularly impurities eluting in the front of the chromatogram gave high background signals which interfered with the analysis. By employing

TABLE II

ACCURACY AND PRECISION OF THE METHOD AFTER CONCENTRATION AND CLEAN-UP ON THE PRECOLUMN

Compound	Concentration added (μg/g of muscle)	Concentration found (mean ± C.V , n = 6) (μg/g of muscle)
Oxolinic acid	4.00	4.01 ± 2.9
	1 00	0.99 ± 4.2
	0.50	0.48 ± 2.2
Flumequine	4.00	4 02 ± 2.7
	1.00	1.00 ± 3.1
	0.50	0 51 ± 5 7

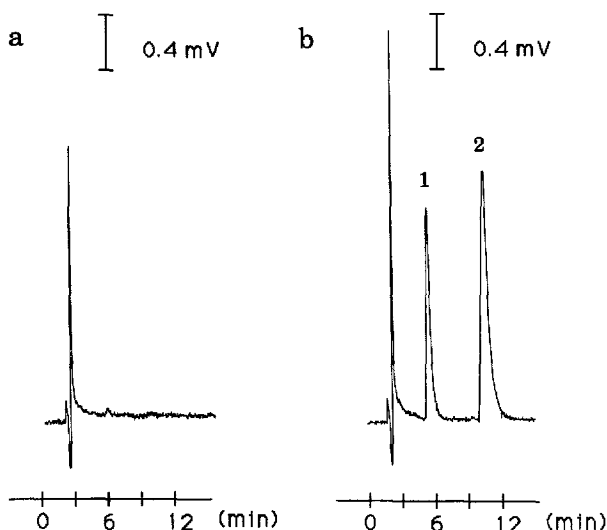

Fig. 3. Chromatograms of (a) an extract of drug-free muscle and (b) an extract of muscle spiked with 50 ng/g oxolinic acid and 100 ng/g flumequine after on-line dialysis and concentration of the dialysate on a precolumn packed with polystyrene-divinylbenzene Detection, fluorescence with excitation at 325 nm and emission at 365 nm Peaks· 1 = oxolinic acid; 2 = flumequine For chromatographic conditions see text

fluorescence detection these background signals increased strongly, and severe quenching of the fluorescence of oxolinic acid and flumequine was observed. Quenching has also been observed by others when analysing oxolinic acid and flumequine in salmon tissue [6].

On-line dialysis and trace enrichment of the dialysate on the precolumn

The problem encountered with impurities observed by direct injections of extracts into the precolumn was completely eliminated by on-line dialysis. It is therefore likely that these impurities were high-molecular-mass compounds which do not diffuse through the cuprophan membrane.

Chromatograms of an extract of a drug-free muscle and of an extract of muscle with 50 ng/g oxolinic acid and 100 ng/g flumequine are shown in Fig. 3. The front of the chromatogram is almost eliminated, and no impurities interfere with the analytes.

The dialysis process operates according to Fick's law which states that the number of molecules passing through the membrane per unit of time is related to the membrane surface area, the concentration gradient per unit of time and the diffusion coefficient. To increase the recovery a large area of membrane contact is essential. In this procedure the membrane surface area is given by the dialysis cell. By connecting two dialysers in series the sample recovery can be increased. A high gradient across the membrane is maintained by removing dialysate from the

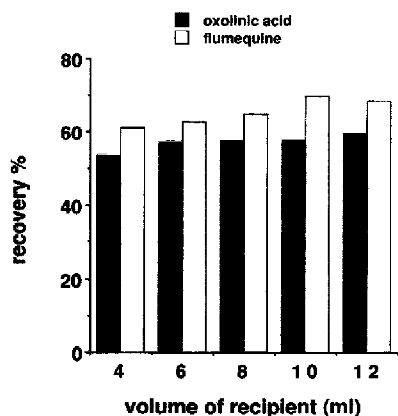

Fig. 4. Recovery of oxolinic acid and flumequine plotted against the volume of recipient solvent aspirated in the pulsed mode through the recipient channel of the dialyser in 6 min and 24 s

dialyser. In this procedure the sample was held static in the donor of the dialyser while the recipient solvent was delivered in a pulsed mode. The volume of recipient solvent aspirated at each pulse was equal to the volume of the dialyser recip-

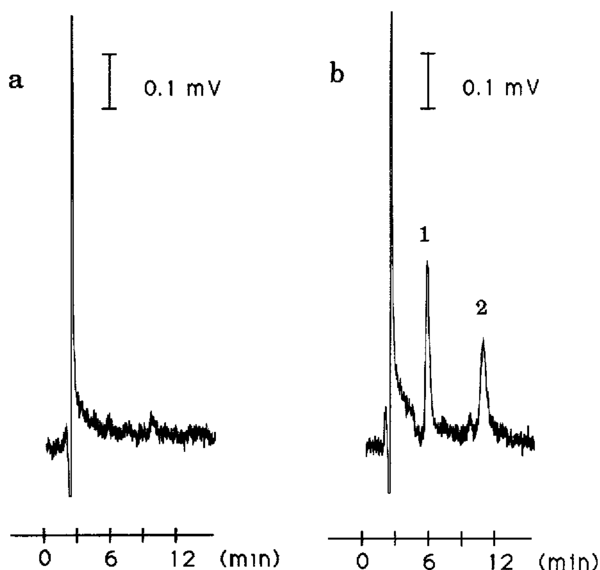

Fig. 5. Chromatograms of (a) an extract of drug-free muscle and (b) an extract of muscle spiked with 5 ng/g oxolinic acid and 10 ng/g flumequine after on-line dialysis and concentration of the dialysate on a polystyrene-divinylbenzene precolumn. Detection, fluorescence with excitation at 325 nm and emission at 365 nm Peaks: 1 = oxolinic acid; 2 = flumequine For chromatographic conditions see text.

ient channel. After each pulse, the recipient was held static in the dialyser channel. In the concurrent mode the fastest process will wait for the slowest process, the latter being the HPLC separation of the analytes in this procedure. The whole ASTED cycle was finished within each chromatogram with a dialysis time of 6 min and 24 s.

Fig. 4 shows the recovery obtained when 4–12 ml recipient solution were aspirated through the recipient channel in the pulsed mode for 6 min and 24 s. The recovery was calculated after injection of standards into the analytical column. The recovery increased slightly when the recipient volume was increased from 4 to 12 ml. In this procedure the use of a large volume of recipient was not recommended. Even traces of hydrophobic impurities in the recipient solution may accumulate on the precolumn, and particularly when the detector is operated in the highest sensitivity these impurities may interfere with the analysis. To avoid these problems 4 ml of recipient solution were selected and all solutions were prepared with pure water and with pure chemicals.

Fig. 5 shows chromatograms where the detector was operated at the highest sensitivity of an extract of drug-free muscle and of an extract of muscle spiked with 5 ng/g oxolinic acid and 5 ng/g flumequine. From this figure the limit of detection at a signal-to-noise ratio of 3 was estimated to be 2 ng/g for oxolinic acid and 3 ng/g for flumequine. Fluorescence detection with excitation at 325 nm was preferred because a better selectivity was obtained as compared to excitation at the absorption maximum of the compounds at 260 nm. Results from validation of the procedure are shown in Table III. The internal standard added to the sample before extraction with phosphate buffer corrects for loss of recovery after extraction and dialysis. The coefficient of variation was 1.9–4.3% for the determination of samples spiked with 50 and 100 ng/g oxolinic acid and flumequine, respectively. The calibration graphs obtained by plotting peak-height ratios *versus* concentration in muscle were linear in the concentration range 50–500 ng/g with $r = 0.999$.

TABLE III

ACCURACY AND PRECISION OF THE METHOD AFTER ON-LINE DIALYSIS AND CONCENTRATION OF THE DIALYSATE ON THE PRECOLUMN

Compound	Concentration added (ng/g)	Concentration found (mean ± C.V., n = 6) (ng/g)
Oxolinic acid	50.0	51.9 ± 3.5
	100.0	100.7 ± 4.3
Flumequine	50.0	50.3 ± 1.9
	100.0	101.7 ± 1.9

CONCLUSION

These results show that oxolinic acid and flumequine can be extracted from muscle of Atlantic salmon with phosphate buffer pH 9, and that sample preparation of the extract can be automated by HPLC with a column-switching system. On-line sample clean-up on a precolumn can be used for analysis in the $\mu g/g$ range. On-line dialysis combined with concentration of the dialysate on a precolumn was shown to be a highly reliable technique for residue analysis.

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