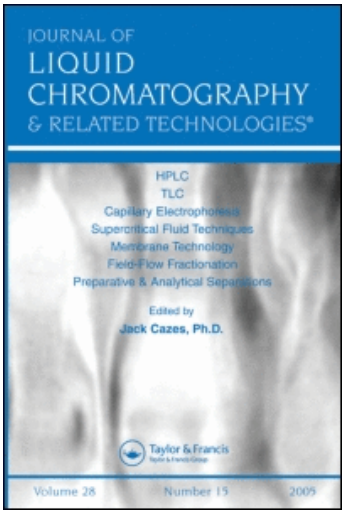

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OPTIMIZATION OF SOLID PHASE EXTRACTION OF OXYTETRACYCLINE FROM FISH TISSUE AND ITS DETERMINATION BY HPLC

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ABSTRACT

The extraction of oxytetracycline from fish tissues by use of solid phase extraction columns has been studied. The best recovery was obtained by using a C8 column and eluting with water/acetone (5% and 10% mixtures). The sensitivity of the method was 5 ng/g for muscle and 10 ng/g for liver. The repeatability of the assay on 10 samples at 1 µg/g and 0.1 µg/g was determined by the standard deviation equal to 4.2% and 6.7%, respectively. The recovery was 89.3% and 94.8%, respectively. The recovery of the internal standard (demeclocycline) was 82.8%.

INTRODUCTION

Oxytetracycline (OTC) is a widely used antibiotic in Norwegian fish farming. In 1986 about 85% of all antibiotics used for treatment of infections on cultured fish were OTC, and to ascertain safe withdrawal periods and monitoring residues in fish foods a sensitive assay for detecting OTC is needed.

Bioassays are most often used for determination of residual OTC in fish liver. However, their precision appears to be variable and the specificity is poor. When applying microbiological test systems there is always a possibility for non-specific inhibition zones.

Several chemical methods for analysing tetracyclines in biological tissue samples have been published (1-5). The clean-up procedures were time consuming and the chromatographic systems were insensitive. The use of solid phase extraction (SPE) columns in the clean-up of tissue samples for determination of drug residues has appeared to be efficient and time saving, and recently two methods using C18 SPE-columns for extraction of OTC from tissue were published (6,7). Oka et al. (6) published a method on animal liver and studied the difference between C18 cartridges from different suppliers and the effect of various deproteinizing agents and of tissue weight on the recovery of OTC. Norlander et al. (7) developed a method for fish tissues and obtained only 60-70% recovery of OTC. No internal standard was used in either of these methods. Moats (8) has in a recent study summerized problems on tetracycline analysis encountered by previous investigators and suggests a solid phase extraction and concentration on the analytical column.

In this study we have optimized the solid phase extraction of fish tissues for detection of OTC-residues by use of HPLC. The purpose was to develop a selective, sensitive, common and robust procedure applicable to the monitoring of residue levels. Also hide, slime and bile are important parts of the metabolic system of fish, and we have modified the clean-up method for assaying OTC-residue in those tissues to be able to perform a complete pharmacokinetic study (9).

EXPERIMENTAL

Chemicals and reagents

All chemicals were of analytical grade. Oxytetracycline hydrochloride (OTC) was supplied by Norsk Medisinaldepot (Oslo,

Norway), whereas demeclocycline hydrochloride (DMC) was purchased from Sigma Chemical Co (St. Louis, MO, USA).

Solvents were of analytical and HPLC grade. Stock solutions (1 mg/ml) of OTC and DMC were prepared in distilled water. The OTC-solution was stored in the freezer in dark stoppered flask, whereas the DMC-solution was freshly prepared every day. Extraction columns were packed in our laboratory. Sorbent material Bondesil™ type C18, C8 and C2, Bond Elut® empty columns (4 and 8 ml) and appropriate frits were supplied by Analytichem International (Harbor City, CA, USA). Bond Elut® adaptors and reservoirs of 125 ml (Analytichem) were connected to the columns when large volumes were applied. Bond Elut filter columns were also used in the method development.

Chromatographic conditions

The analyses were performed on a Perkin-Elmer HPLC system, consisting of a Series 400 solvent delivery system, an ISS 100 sampling system, an LC 85 UV detector and an LCI 100 laboratory computing integrator (Perkin Elmer, Norwalk, Conn., USA). The detector wavelength was set 357 nm. The analytical column (stainless steel, 15 cm x 4.6 mm I.D.) and guard column (stainless steel, 2 cm x 4.6 mm ID) were packed with 5 µm Supelcosil® LC-18DB (Supelco, Bellefonte, PA, USA). The mobile phase was 0.005M phosphate buffer (pH = 2) - acetonitrile-tetrahydrofuran (81:10:9) at a flow rate of 0.9 ml/min (10). Aliquots of 25 µl were injected onto the column.

Extraction and clean-up procedure (Fig. 1)

Liver tissue. To a sample of ground fish liver (5 g) was added internal standard (10µg), 1 g Na_2EDTA (ethylenediaminetetraacetic acid) and 5 ml of a hexane-dichloromethane mixture (1:3). The liver samples were blended three times in 20 ml of 0.1 M Na_2EDTA in 0.01M phosphate buffer (pH = 4.2) using a M.S.E. homogeniser (Measuring and Scientific Equipment, London, UK). The

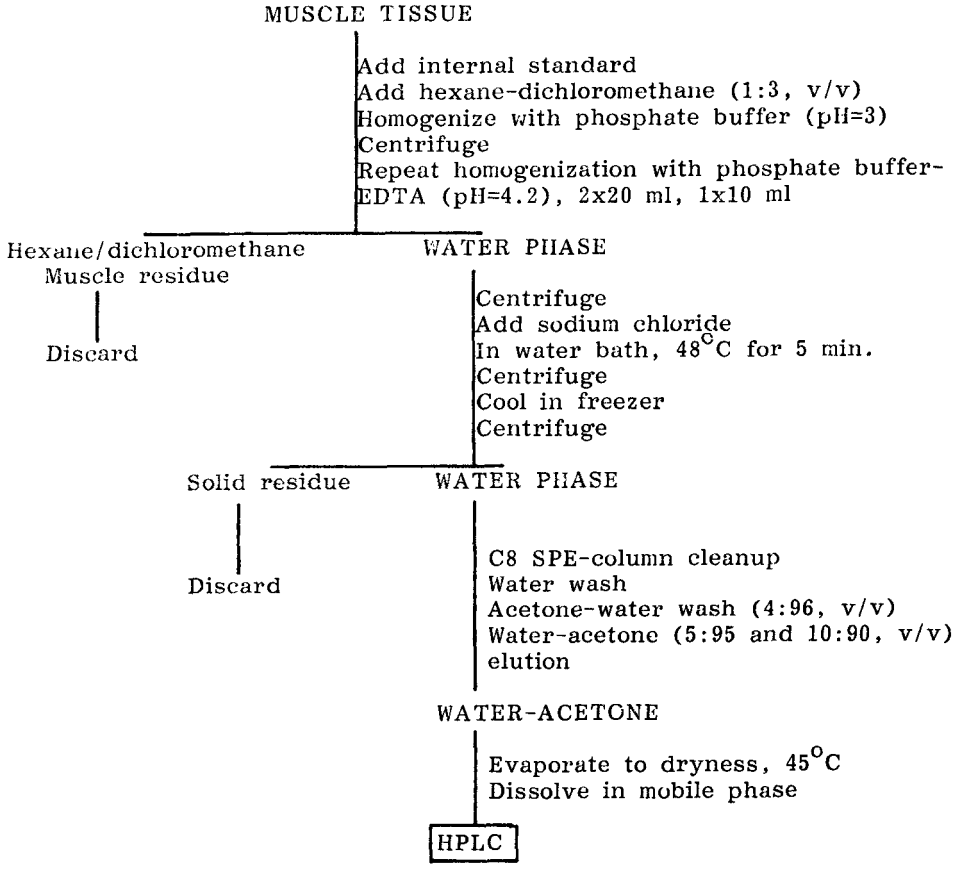

FIGURE 1

Extraction and Clean-up Procedure for Oxytetracycline (OTC) from Fish Muscle

homogenate was centrifuged at 3500 rpm for 5 min. Sodium chloride (5 g) was dissolved in the collected supernatants, which were warmed in a water bath at 48°C for 5 min. and then cooled rapidly in the freezer. After centrifugation the solution was loaded on a conditioned C8 column.

Muscle tissue. Sample of fish muscle (10 g) with added DMC (10 µg) and 5 ml hexane-dichloromethane (1:3) was homogenized once in 20 ml 0.01M phosphate buffer (pH = 3) and then three times in 20 ml 0.1 M Na_2EDTA in 0.01M phosphate buffer (pH = 4.2), 2x20 ml and 1x10 ml. After centrifugation sodium chloride was added to the collected supernatants which were warmed in a water bath (5 min, 48°C). The extract was cooled, centrifuged and loaded on a conditioned C8 column.

Sample of slime, hide and vertebra. Hide (2 g) and vertebra (2 g) samples were left overnight in 30 ml 0.1M Na_2EDTA in 0.01M phosphate buffer (pH = 4.2) and then homogenized after addition of DMC (10 µg) and 5 ml hexane-dichloromethane (1:3). The blending was repeated twice in 20 ml phosphate buffer-EDTA. Sodium chloride (5 g) was dissolved in the collected supernatants. The mixture was centrifuged and warmed up to 48° for 5 min and then cooled rapidly. After centrifugation the extract was loaded onto a conditioned C8 column.

After addition of DMC (10 µg) and 5 ml hexane-dichloromethane (1:3) slime (2 g) was homogenized three times in 20 ml phosphate buffer-EDTA. Further clean-up as mentioned above.

Clean-up on SPE-column. The column was activated with 4 ml methanol, 3x4 ml water and then loaded with 0.1 M Na_2EDTA in 0.01 M phosphate buffer prior to the extract of fish tissue. The column was washed with 1 ml 4% acetone in H_2O, except the liver extract, and then eluted with 1.5 ml 5% H_2O in acetone and 1.5 ml 10% H_2O in acetone. The collected eluates were evaporated to dryness under a stream of nitrogen (48°C) and dissolved in 150 µl of mobile phase. When large amount of OTC occurred in the tissue, the acetone was evaporated off and the residue (ca. 0.5 ml) was diluted with the mobile phase to appropriate volume.

Calibration curve and recovery studies

The calibration curve was made by spiking tissue (10 g muscle or 5 g liver) with standard solutions of OTC and DMC to yield 10, 5, 1, 0.5, 0.1 and 0.05 µg of OTC per sample and 10 µg of DMC. The samples were extracted using the above procedure. Each level was assayed in triplicate. The analyses of spiked tissues were compared with those of standard solutions to calculate recovery rates. Also the recovery of internal standard was examined.

RESULTS AND DISCUSSION

In previous papers (6,7) it is reported that the C18 solid phase extraction column is efficient for clean-up of fish tissue for chromatographic analysis of OTC. The recovery was, however, low and was dependent on the brand of column, the amount of tissue extracted and the deproteinizing agent. We found it necessary to optimize the clean-up procedure in order to develop a more robust procedure.

Application of SPE-cartridges to OTC standard solution

In our preliminary studies we compared C18, C8 and C2 SPE-columns from Analytichem. Standard solutions of OTC and DMC were tested. The columns were eluted with methanol. The effect of EDTA on the retention and elution properties of the columns was also tested. When OTC and DMC dissolved in water were loaded on a column which was preconditioned with methanol and water, only 10% of the compounds were eluted from the C8 and C18 columns, whereas nothing was eluted from the C2 column. About 10% of the compounds were unretained. Further experiments showed that addition of EDTA-solution and pH optimization of the solvent were essential for the retention and elution properties of OTC and DMC. EDTA dissolved in phosphate buffer was used either as solvent for OTC and DMC or as preconditioning agent to the SPE-columns instead of water. The pH was adjusted to 4.9 which is similar to that of a tissue extract. Both OTC and DMC were completely

retained on the three types of column material in the presence of EDTA (pH = 4.9). The elution efficiency of methanol appeared variable. From the C8 column 80.5 ± 2.5% OTC and 82.7 ± 5.4% DMC were eluted. From the C18 column 72.5 ± 6.8% OTC and 72.1 ± 9.1% DMC were eluted, whereas the values for the C2 column were 32.2 ± 2.8% and 50.7 ± 5.5% for OTC and DMC, respectively. According to Knox et al. (11) the doubly charged anionic form of EDTA predominates at pH-value above 3, whereas OTC exists predominantly in the zwitterion form. EDTA and the tetracyclines will form stable chelates whenever pH exceeds about 3. It must be the non-polar interactions that dominate when the chelate solution is applied on the C18, C8 and C2 columns, respectively. The non-polar interactions of these three types of sorbent material decrease in the order C18 > C8 > C2. The non-polar effect of C2 is weak and the polar effect is usually more pronounced. So far our studies showed that both C18 and C8 SPE-columns are applicable for cleaning and preconcentration of tetracycline extracts. Further evaluation of the two sorbent materials was performed with tissue extracts.

Sample treatment prior to column clean up

Tissue samples, spiked with OTC and DMC, were homogenized in a mild deproteinizing agent, due to the instability of DMC in acidic solution (pH<3). As tetracyclines are apt to bind to proteins it was very important to obtain complete precipitation. After extraction, solid sodium chloride was introduced as precipitating agent to the collected extracts which were warmed up to 48°C for 5 min and then cooled rapidly in the freezer. A considerable precipitation occurred, particularly in the muscle extracts. From muscle tissue the best recovery was obtained after homogenizing four times, whereas other types of tissue required only three times. A residue of approximately 6% OTC and 4.5% DMC was extracted in the fourth homogenization step from 10 g spiked muscle tissue. From 5 g spiked muscle tissue only 1.2% OTC and 2.5% DMC were

extracted in the fourth step. Our preliminary studies on the extraction of fish tissue showed that a high residual protein content in the extracts reduced the adsorption of OTC and DMC on the SPE-column. The recovery under such conditions was only 50-60%. This problem has been discussed by Oka and coworkers (6). After introduction of the additional precipitation steps such as sodium chloride addition and rapid cooling, the recovery appeared to be almost independent of tissue weight.

Column clean-up of tissue extracts

OTC and DMC in tissue extracts were completely retained on C8 and C18 extraction columns. The optimal quantity of C8 sorbent material in the column was studied and varied from 500-1000 mg. Tissue extracts containing a high quantity of residual proteins required 1.0 g sorbent material. The drugs were retained on 700 mg sorbent material when the protein precipitation was improved. When using methanol as eluting agent a coloured and impure eluate was obtained from the C8 column. The recovery was approximately 76%. Nordlander et al. (7) reported a recovery of only 66% OTC from muscle tissue when eluting the C18 column with methanol. To avoid a rapid contamination of the analytical column and obtain a better recovery, further experiments showed that acetone containing a small amount of water was a very efficient eluting agent. Both 5% and 10% mixtures of water in acetone were tested. A combination of the two solvent mixtures appeared to be the best, and the amount of elution solvent was reduced from 10 ml (6,7) to 3 ml. Figure 2 shows chromatograms of muscle extracts spiked with OTC. The figure clearly demonstrates better purification of the extract and enhanced recovery when using acetone/water as elution solvent instead of methanol/water.

Also a more efficient washing solution had to be assayed. When using 1-2 ml of 4% acetone in water we got rid of some more impurities, and the recovery of OTC and DMC in the eluate was enhanced. The small amount of acetone in the washing solution

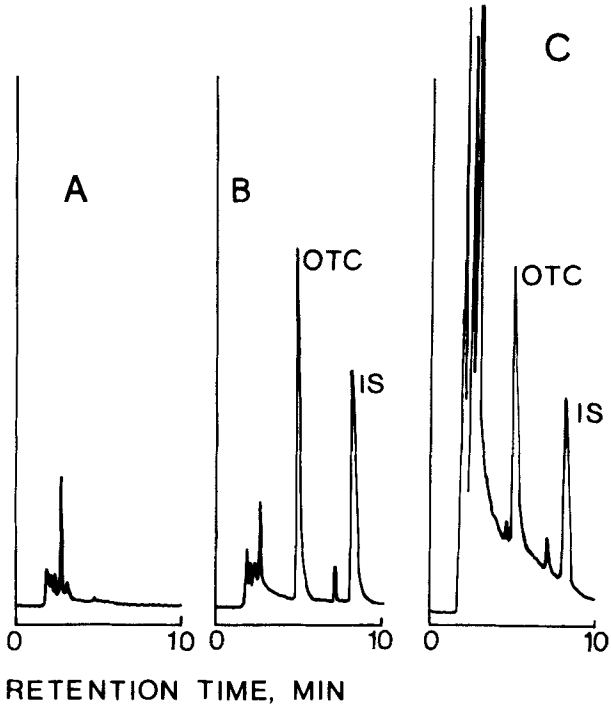

FIGURE 2

Chromatograms of extracts from 10 g fish muscle cleaned on C8 SPE-column.
A - acetone/water (90:10) eluate of unspiked muscle. B - acetone/water eluate of muscle spiked with 0.5 µg/g oxytetracycline (OTC) and 1 µg/g internal standard (IS). C - methanol/water (90:10) eluate of muscle spiked with OTC (0.5 µg/g) and IS (1 µg/g).

must have reduced the adsorption of OTC and DMC on the SPE-column, and the compounds were more easily eluted with a 5% water in acetone solution. The above washing solvent was not applicable to liver extracts as the elution of OTC and DMC from the C8 column started in the washing step. We assume that a higher content of residual proteins in the muscle extracts caused these

TABLE 1

Recovery of Oxytetracycline and Demeclocycline from Muscle and Liver of Farmed Fish

Tissue	No of samples	Amount (µg/g)	Recovery (%) OTC		DMC	
			Mean	SD*	Mean	SD
Muscle 10 g	10	1	89.3	4.2	82.8	4.4
	10	0.1	94.8	6.7	82.8	4.4
Muscle 5 g	6	1	95.2	4.2	88.7	4.5
Liver 5 g	6	1	100.2	3.6	93.2	4.4

*SD = Standard deviation

differences. The elution properties of the acetone-water mixtures were tested by stepwise elution with portions of one ml. In the fourth ml only 1% of the OTC and no DMC was eluted. When testing the C18 column an identical procedure was applied.

The results of the recovery study using C8 columns are listed in Table 1. The sensitivity of the method was found to be 5 ng OTC per gram muscle tissue and 10 ng per gram liver tissue. The calibration curve was linear over the range of 10 ng - 20 µg/g with correlation coefficient of 0.978 for muscle tissue. When using a C18 SPE-column to clean-up muscle (10 g) extracts the recovery was 69.9 $\pm$ 7.2% (n=8) and 73.4 $\pm$ 6.4% (n=8) for OTC and DMC, respectively.

The eluting solvent acetone-water was also tested on the C8 and C18 columns loaded with standard solutions of OTC and DMC. Only between 50 and 70% of the compounds were eluted. The retained amount of OTC and DMC was eluted with methanol and the total recovery was 100%.

CONCLUSIONS

Our results show that application of C8 SPE-column combined with acetone-water as elution solvent has improved the clean-up and

the recovery of oxytetracycline significantly (6,7). Internal standard has been introduced in the procedure to compensate for various analytical errors.

Further, our results demonstrate that interactions between tissue matrix (mainly proteins), tetracyclines, sorbent material and the washing and elution solvents are of great significance when evaluating the clean-up on solid phase extraction columns.

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