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On-line dialysis, liquid chromatography and post-column reaction detection of oxytetracycline in salmon muscle extracts

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Abstract: The development of a sensitive automated method for residue control of oxytetracycline (OTC) in salmon muscle is described. Tissue homogenate is dialysed and the dialysate enriched on a small on-line polystyrene column. OTC and the internal standard (tetracycline) are separated by HPLC on a polystyrene column using an ion-pair eluent system. The column effluent is mixed with sodium hydroxide and irradiated at 366 nm and the resulting derivatives monitored by means of a fluorescence detector (excitation: 358 nm, emission: 460 nm). By the method OTC is detected down to 5 ng g^{-1}. The standard curve was linear (r = 0.9999) over the range 50–1000 ng g^{-1}. Within-day and between-day relative standard deviations (n = 6) at 50 and 200 ng g^{-1} ranged from 1.0 to 1.7%.

Keywords: *Oxytetracycline; tissue extracts; on-line dialysis and HPLC; post-column derivatization; residue control; automated sample preparation.*

Introduction

Oxytetracycline (OTC) is widely used as an antibacterial in fish farming and as a consequence sensitive and specific methods for residue control of OTC in fish muscle are required. Many high-performance liquid chromatographic (HPLC) methods for the determination of OTC in muscle have been reported, which all include extensive manual sample work-up prior to HPLC with UV detection [1–12]. Residue control of antibiotics in fish is carried out in control laboratories as routine analysis, and, the number of samples being large, it is important to reduce sample handling to a minimum.

The ASTED™ system (automated sequential trace enrichment of dialysates) is able to process crude biological samples in line with HPLC analysis. Proteins and particles are removed by dialysis, in which a semipermeable membrane, having a molecular weight cut-off of 15 kD, separates small molecules (e.g. drug molecules) from a macromolecular matrix. The technique has been applied to plasma, serum, whole blood, skimmed milk, egg homogenate and tissue homogenate [13–19].

Fluorescence detection is more specific, and also in many cases more sensitive than UV detection. OTC shows little native fluorescence, but on pH adjustment and heating a more fluorescent product is formed. This has been applied in a spectrofluorometric assay for OTC in blood [20]. In the present HPLC method for OTC determination post-column derivatization and fluorescence detection is carried out by irradiating the base treated column effluent with a Beam Boost™ unit, giving a highly fluorescent product which is detected. The ASTED sample preparation system allows the direct injection of tissue homogenate.

Experimental

Chemicals and reagents

Oxytetracycline hydrochloride was obtained from Norsk Medisinaldepot (Oslo, Norway), tetracycline hydrochloride (TC) from Sigma (St Louis, MO, USA) and 1-heptanesulphonic acid (sodium salt, monohydrate, purum; 98%) from Fluka (Buchs, Switzerland). HPLC grade acetonitrile was used and other chemicals (orthophosphoric acid (85%), sodium dihydrogenphosphate, Triton X-100, *n*-hexane, sodium hydroxide and hydrochloric acid) were of analytical grade. Water was of Milli-Q grade.

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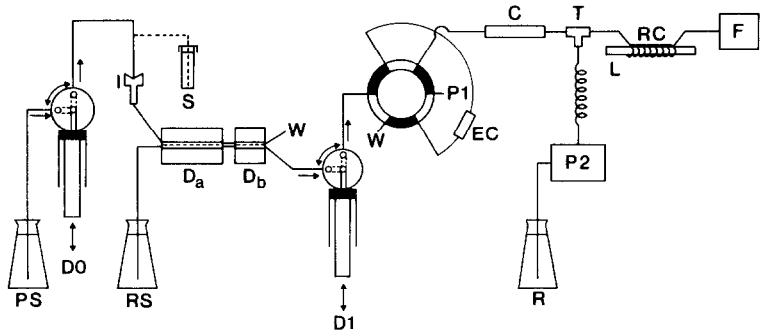

Figure 1
Schematic representation of the apparatus. PS = priming solution; D0 = dilutor 0; S = sample; I = injector; RS = recipient solution; D_a = dialyser, donor volume: 370 μl; D_b = dialyser, donor volume: 100 μl; W = waste, D1 = dilutor 1; EC = enrichment column; P1 = HPLC pump; C = column; R = reagent; P2 = reagent pump; T = mixing T-piece; L = UV lamp; RC = reaction coil; F = fluorescence detector.

Apparatus and conditions

A schematic representation of the apparatus is shown in Fig. 1. The ASTED system (Gilson, Villiers-le-Bel, France) comprised a sample injector (Model 231), two dilutors (Model 401) equipped with 1-ml syringes, plus two dialysis cells having donor volumes of 370 and 100 μl, respectively, connected in series. The dialysis cells were fitted with cellulose dialysis membranes (Cuprophan) with a molecular weight cut-off of 15 kD. A column (10 mm × 2.0 mm i.d.) (Chrompack, Middelburg, The Netherlands) packed with 36 μm polystyrene (Dynospheres, Dyno Particles, Lillestrøm, Norway) was mounted on a six-port valve (Rheodyne, Berkeley, CA, USA) Model 7010 that connected the enrichment column to the recipient channel of the dialysis cells or to the HPLC system when the valve was switched. The priming solution for the donor side of the system was 0.01% (w/v) Triton X-100 in water. The recipient solution was 0.02 M phosphate buffer pH 5, containing 0.005 M sodium heptanesulphonate. The chromatographic separation was carried out on a polystyrene analytical column (PLRP-S, particle size 5 μm, 150 mm × 4.6 mm i.d.) (Polymer Labs, Church Stretton, UK) using acetonitrile–aqueous 0.005 M sodium heptanesulphonate, 0.02 M orthophosphoric acid (23:77, v/v) as mobile phase, delivered at 0.7 ml min^{-1} by an LC-6A isocratic pump (Shimadzu, Kyoto, Japan).

Sodium hydroxide (2 M) was pumped at 0.15 ml min^{-1} using a Beckman Model 112 solvent delivery module (Berkeley, CA, USA). A 1000 μl stainless steel loop was placed between this HPLC pump and the mixing T-piece. Irradiation at 366 nm was carried out in a Beam Boost photochemical reaction unit (ict, Frankfurt, Germany). A knitted reaction coil of 10 m × 0.3 mm i.d. was used.

The RF-551 fluorescence detector (Shimadzu) was set at 358 nm excitation and 460 nm emission wavelength and the signals were recorded on a Chromatopac C-R3A integrator (Shimadzu).

Stock solutions, working standards and internal standard

Stock solutions of OTC and TC (1 mg ml^{-1}) were prepared in methanol–water (1:1, v/v). These solutions were stored at −20°C for up to 1 month. Working standards of OTC and TC internal standard solution were prepared in 0.01 M HCl and kept at 6°C for up to 1 week.

Samples

Drug-free muscle from Atlantic salmon was kept at −20°C until required for use. Spiking was carried out by adding the required amount of OTC in 0.1–0.5 ml of working standard to 5.0 g portions of thawed muscle that had been minced with a pair of scissors. The spiked muscle was allowed to stand for 1 h at ambient temperature before extraction.

Extraction procedure

The procedure was carried out at ambient temperature using solutions at ambient temperature. TC (125 μg ml^{-1}) was used as internal standard for the determination of OTC.

To 5.0 g of muscle was added 200 μl of internal standard solution, 50 ml of 0.05 M HCl and 10 ml of hexane. The mixture was

homogenized on an Ultra-Turrax T25 homogenizer (Ika Werk, Staufen, Germany) for 2 min and sonicated for 3 min. The mixture was then centrifugated for 5 min (1920*g*), and, using a pipette, ≈0.6 ml of aqueous extract was transferred to 0.8 ml polypropylene autosampler vials.

ASTED sample preparation procedure

Through dilutor D0 (Fig. 1) 530 µl of extract were aspirated and used to overfill the donor channels of the dialysis cells. There it was kept static for 7.4 min while 6 ml of recipient solution was pumped through the recipient channels in pulses by dilutor D1 (Fig. 1), which simultaneously pumps the dialysate into the enrichment column. The pulsation was accomplished by pumping seven portions of 875 µl (the volume of the recipient channels) into the dialysers, dilutor D1 being programmed to pump 1.7 ml min^{-1} and keeping them static for ≈36 s. Subsequently OTC and the internal standard were transferred to the analytical column by back-flushing with HPLC mobile phase for 2 min. At the same time, residual sample and dialysate were purged out of the dialyser by the two dilutors, using 2 ml of the respective solutions. Following elution the enrichment column was regenerated with 2 ml of recipient solution. In the concurrent operation of sample preparation and analysis, samples were prepared and the system purged and regenerated during the 13.5-min HPLC analysis of the previous sample.

Washing of the dialyser

The connecting tubing and the donor side of the dialysers were washed weekly with 1 M NaOH as recommended and described in the ASTED users guide.

Recording of excitation and emission spectra

Spectra were recorded after off-line irradiation of alkalized solutions of OTC and TC, respectively, in mobile phase. Quartz cuvettes (10 mm) containing the OTC or TC solution were irradiated for 1 min in a photo-irradiator consisting of a 900 W xenon arc lamp with an f3.4 monochromator (Applied Photophysics Ltd), operated at 360 nm with a bandwidth of 10 nm. Excitation and emission spectra of irradiated OTC and TC were recorded by means of a Perkin–Elmer LS 50 luminescence spectrometer.

Results and Discussion

Post-column derivatization

As the result of the irradiation of OTC in alkaline solution a highly fluorescent product is formed. Excitation and emission spectra of the product showed maxima at 358 and 460 nm for excitation and emission, respectively. The corresponding wavelengths for TC were 337 and 413 nm.

The irradiation time was optimized by varying the length of the reactor coil in the Beam Boost unit between 5 and 20 m. Maximum fluorescence was obtained for a 10 m reactor, corresponding to an irradiation time of 69 s. The fluorescence response also is dependent upon concentration and flow of sodium hydroxide. As shown in Fig. 2 the fluorescence response increased with the concentration of sodium hydroxide. However, only a small increase in fluorescence response of OTC was observed for sodium hydroxide concentrations of above 2.0 M. The fluorescence response also is dependent upon the flow rate of sodium

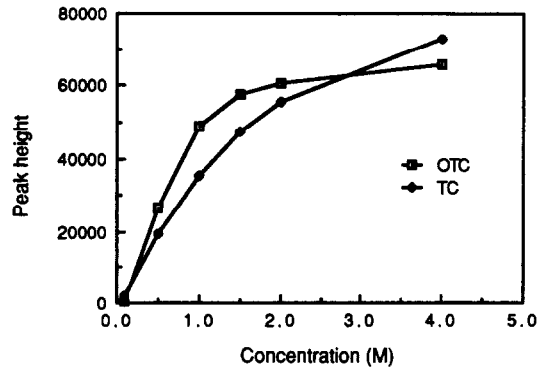

Figure 2
Peak heights for OTC and TC versus concentration of NaOH. NaOH flow rate: 0.15 ml min^{-1}.

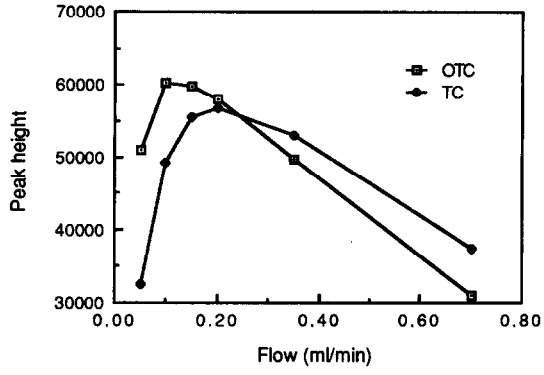

Figure 3
Peak heights for OTC and TC versus flow of 2 M NaOH.

hydroxide as shown in Fig. 3. The fluorescence response of OTC reached a maximum at 0.15 ml min^{-1} and then decreased due to dilution of the column effluent at higher reagent flow rates. From these results it was decided to use 2 M NaOH at 0.15 ml min^{-1}.

The difference in fluorescence with and without irradiation, respectively, is shown in Fig. 4. With irradiation a 10-fold increase in the fluorescence signal for OTC was achieved.

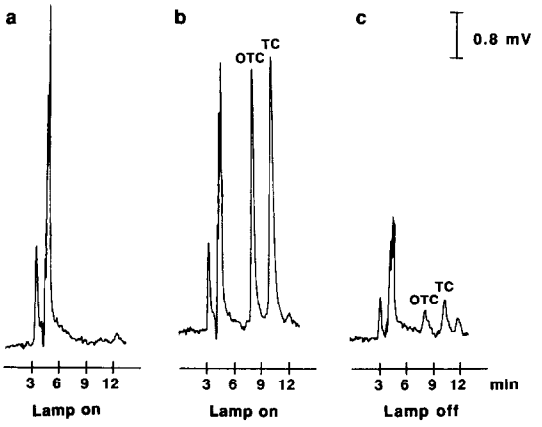

Figure 4
Chromatograms of extracts of (a) unspiked muscle, (b) muscle spiked with 50 ng g^{-1} of OTC and added 500 ng g^{-1} of TC and (c) same as (b) but without irradiation.

Extraction

Tetracyclines are highly soluble as their hydrochloride salts. This, along with the tissue bonding mechanisms discussed by Nelis and De Leenheer [21], makes hydrochloric acid a well-suited medium for extraction of tetracyclines from tissue. Several previous workers have used hydrochloric acid to extract tetracyclines from tissue, followed by further purification [1–4, 21–23]. In the present method only a simple extraction was found to be necessary. The recoveries of OTC and TC using phosphate buffer and hydrochloric acid, respectively, are compared in Table 1. The muscle was spiked with OTC (200 ng g^{-1}) and TC (2 μg g^{-1}). The best recoveries (100%) were obtained using 0.05 M HCl.

Dialysis and HPLC

In order to obtain high dialysis efficiencies in a short time, it is necessary to renew the recipient solution during the dialysis process. In the present method the sample is kept static for 7.4 min, whilst the recipient solution is pulsed through the dialyser. Keeping the dialysis time constant, the dialysis efficiency using 2, 4, 6 and 8 ml of recipient solution, respectively, were investigated. The efficiency increased with increasing volume, but from 6 to 8 ml there was no significant increase. Thus 6 ml appeared to be the optimal volume and was chosen for the method. Recoveries of 60.3% for OTC and 60.2% for TC were obtained using this volume. The recoveries were calculated by relating peak heights obtained after dialysis of spiked tissue extract to peak heights obtained after direct injection on the enrichment column of a solution of OTC and TC in 0.01 M HCl.

The conditions for enrichment and HPLC separation have been discussed elsewhere [13].

Chromatograms are shown in Fig. 4. The chromatogram of the blank extract contains no peaks that would interfere with OTC or TC detection. The chromatogram obtained after spiking of the muscle with 50 ng g^{-1} of OTC and addition of 500 ng g^{-1} of TC also is shown. At a signal-to-noise ratio of 3, OTC could be detected at 5 ng g^{-1} as shown in Fig. 5.

Stability of extract

OTC and TC are known to be slightly unstable in tissue extract at room temperature.

Table 1
Comparison of various extrants (n = 6)

	OTC		TC	
Extrant	Recovery (%)*	RSD (%)	Recovery (%)*	RSD (%)
HCl, 0.015 M†	90.1	1.3	89.0	1.7
HCl, 0.05 M‡	100.6	2.0	100.1	1.7
Phosphate buffer, pH 4, 0.4 M	84.6	1.8	80.4	1.4
Phosphate buffer, pH 2, 0.4 M	92.6	2.8	88.6	2.8

*Relative to a solution of OTC and TC in 0.01 M HCl.
†Giving pH 4 in extract.
‡Giving pH 2 in extract.

Table 2
Within-day and between-day accuracy and precision (n = 6)

Concentration added (ng g^{-1})	Concentration found (mean ±SD) (ng g^{-1})		RSD (%)	
	Within-day	Between-day	Within-day	Between-day
50	49.2 ± 0.5	49.4 ± 0.8	1.0	1.7
200	197.8 ± 2.2	199.0 ± 2.2	1.1	1.1

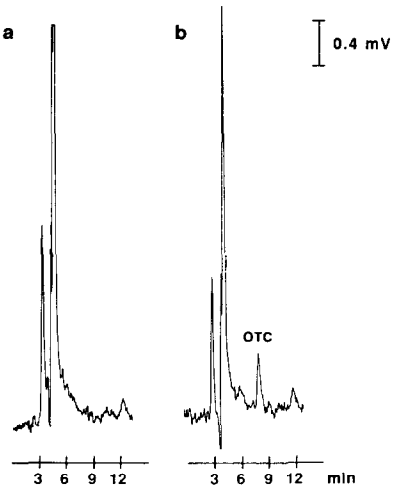

Figure 5
Limit of detection. Chromatograms of extracts of (a) unspiked muscle and (b) muscle spiked with 5 ng g^{-1} of OTC.

At 26°C ambient temperature the decrease in peak height after 6 h in the autosampler was 2.9% for OTC and 5.2% for TC. But when the extracts were kept at 6°C both OTC and TC showed no degradation after 48 h. It has been shown [24] that OTC in plasma samples that had been precipitated with trifluoracetic acid showed no degradation after 10 h at 15°C. In the present work samples were taken from the refrigerator and transferred to the autosampler every hour. For routine analysis it is recommended that a thermostatted sample rack should be used for cooling of the samples.

Validation

The standard curve was tested for linearity in the range 50–1000 ng g^{-1} by duplicate assays using standards of 50, 600 and 1000 ng g^{-1}, respectively. The correlation coefficient (r) was 0.9999 and the equation for the curve, given as mean ±SD, was $y = 0.00193\ (\pm 9.3 \times 10^{-6})x + 0.01014\ (\pm 0.00629)$, where y is peak height ratio and x is concentration. Within-day accuracy and precision were determined by analysing six extracts that had been spiked with 50 and 200 ng g^{-1}, respectively, whilst between-day accuracy and precision were determined by analysing one of the respective extracts on six successive days, keeping the sample vials at −20°C until analysis. The calibration curve that was prepared on the first day was used throughout the validation. Within-day and between-day accuracy and precision are shown in Table 2. Both accuracy and precision were found to be excellent.

Conclusions

A rapid and simple method for determination of OTC in muscle tissue has been developed. The sensitivity of the method is as good as that obtained with previously published methods using time-consuming manual work-up procedures. The present automated method is more convenient than such methods for the routine residue control of OTC in fish muscle. The system is capable of analysing 4.4 samples per hour and can be run without attention so that more than 100 samples can be analysed in 24 h.

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References

[1] J. Murray, A.S. McGill and R. Hardy, *Food Addit. Contam.* **5**, 77–83 (1987).
[2] I. Nordlander, H. Johnsson and B. Österdahl, *Food Addit. Contam.* **4**, 291–296 (1987).
[3] W.A. Moats, *J. Chromatogr.* **358**, 253–259 (1986).
[4] Y. Onji, M. Uno and K. Tanigawa, *J. Assoc. Off. Anal. Chem.* **67**, 1135–1137 (1984).
[5] G.J. Reimer and L.M. Young, *J. Assoc. Off. Anal. Chem.* **73**, 813–817 (1990).
[6] H. Oka, H. Matsumoto, K. Uno, K. Harada, S. Kadowaki and M. Suzuki, *J. Chromatogr.* **325**, 265–274 (1985).
[7] W.H.H. Farrington, J. Tarbin, J. Bygrave and G. Shearer, *Food Addit. Contam.* **8**, 55–64 (1991).

[8] A.R. Long, L.C. Hsieh, M.S. Malbrough, C.R. Short and S.A. Barker, *J. Assoc. Off. Anal. Chem.* **73**, 864–867 (1990).
[9] H. Björklund, *J. Chromatogr.* **432**, 381–387 (1988).
[10] A. Rogstad, V. Hormazabal and M. Yndestad, *J. Liq. Chromatogr.* **11**, 2337–2347 (1988).
[11] F. Kondo, S. Morikawa and S. Tateyama, *J. Food Prot.* **52**, 41–44 (1988).
[12] J.P. Sharma and R.F. Bevill, *J. Chromatogr.* **166**, 213–220 (1978).
[13] T. Agasøster and K.E. Rasmussen, *J. Chromatogr.* **570**, 99–107 (1991).
[14] T. Agasøster and K.E. Rasmussen, *J. Chromatogr.* **564**, 171–179 (1991).
[15] T.H. Hoang, A.T. Andresen, T. Agasøster and K.E. Rasmussen, *J. Chromatogr.* **532**, 363–373 (1990).
[16] J.D.H. Cooper, D.C. Turnell, B. Green and F. Verillon, *J. Chromatogr.* **456**, 53–69 (1988).
[17] L.G.M.Th. Tuinstra, P.G.M. Kienhuis, W.A. Traag, M.M.L. Aerts and W.M.J. Beek, *J. High Res. Chromatogr.* **12**, 709–713 (1989).
[18] M.M.L. Aerts, W.M.J. Beek and U.A.Th. Brinkman, *J. Chromatogr.* **500**, 453–468 (1990).
[19] D.C. Turnell and J.D.H. Cooper, *J. Chromatogr.* **395**, 613–621 (1987).
[20] B. Scales and D.A. Assinder, *J. Pharm. Sci.* **62**, 913–917 (1973).
[21] H.J.C.F. Nelis and A.P. De Leenheer, *Clin. Chim. Acta* **103**, 209–217 (1980).
[22] J.J. Ryan and J.A. Dupont, *J. Assoc. Off. Anal. Chem.* **57**, 828–831 (1974).
[23] K.W. Kohn, *Anal. Chem.* **33**, 862–866 (1961).
[24] B. Iversen, A. Aanesrud, A.K. Kolstad and K.E. Rasmussen, *J. Chromatogr.* **493**, 217–221 (1989).

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