Application of reversed-phase liquid chromatography and prepacked C$_{18}$ cartridges for the analysis of oxytetracycline and related compounds

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Abstract

The reversed-phase (RP) chromatographic separation of oxytetracycline (OTC) 4-epioxytetracycline (4-epiOTC), α-apooxytetracycline (α-apoOTC), and β-apooxytetracycline (β-apoOTC) has been accomplished on an Inertsil C$_8$ column at ambient temperature. Using the simplex method of solvent optimization, a 0.1 M ammonium acetate buffer (pH 3.0)–acetonitrile–tetrahydrofuran (72.5:12.5:15, v/v/v) mobile phase was found to give excellent separation of the compounds. OTC, 4-epiOTC, α-apoOTC and β-apoOTC were resolved in 35 min with calculated detection limits of 40, 20, 50 and 140 ng/ml, respectively. Solid-phase extraction (using RP C$_{18}$ cartridges) of OTC and OTC degradation compounds from distilled water and porcine muscle was tested at four concentration levels ranging from 200 to 2000 ng/ml (g); overall mean recovery of OTC from distilled water and porcine tissue was greater than 90% and 70%, respectively.

Keywords: Oxytetracycline; 4-Epioxytetracycline; $\alpha(\beta)$-Apooxytetracycline

1. Introduction

Oxytetracycline (OTC) is a broad-spectrum antibiotic that is commonly used in veterinary medicine as a growth promoter and for the treatment and prevention of bacterial infections in livestock. Four major dosage forms of OTC are available for animal health uses in Canada: (1) feed premixes; (2) injectables; (3) soluble powders; (4) tablets. When used at recommended dosage levels, the oral forms of OTC are regarded as having minimal potential for tissue residues [1]; however, studies have been published on the presence of OTC residues at injection sites after intramuscular injections of OTC formulations [2,3]. Regulatory testing in Canada has indicated that OTC is the second most commonly detected antibiotic residue in pork carcasses [4].

The formation of OTC degradation products (Fig. 1) in pharmaceutical preparations is believed to proceed by way of a dehydration reaction at the C6–OH group forming anhydrooxytetracycline. This compound is unstable in aqueous solution and breaks down to form α- and β-apooxytetracycline (α-apoOTC and β-apoOTC) [5]. Oxytetracycline also undergoes epimerization of the C4 dimethylamino group to form 4-epioxytetracycline (4-epiOTC). Oxytetracycline and 4-epiOTC are identical except for the configuration of the C4 dimethylamino group;
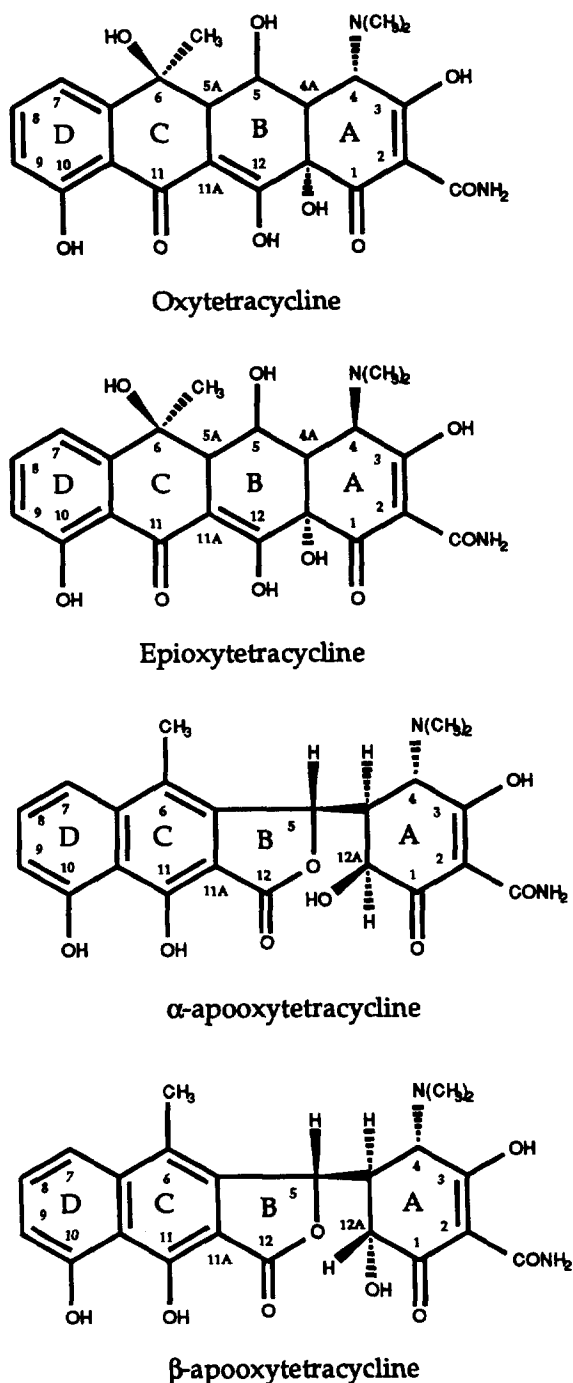
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Oxytetracycline

Epioxytetracycline

α-apooxytetracycline

β-apooxytetracycline

Fig. 1. Structures of oxytetracycline and its degradation compounds.

α- and β-apoOTC are identical except for the configuration of the C12a hydroxyl group.

The method currently used at the Health of Animals Laboratory (Saskatoon, Sask., Canada) for quantification of tetracycline, oxytetracycline, and chlortetracycline in animal tissue and whole egg (Method TTC-SP05 [6]) is based on that of Oka et al. [7], utilizing reversed-phase (RP) liquid chromatography and solid-phase extraction. This method, however, has not been applied to the extraction and chromatographic separation of the OTC degradation products, 4-epiOTC, α-apoOTC and β-apoOTC, which can be found in aged pharmaceutical preparations [5]. To the best of this author's knowledge, solid-phase extraction of OTC degradation products from different matrices has not been reported, and successful chromatographic separation of these compounds has only been accomplished on poly(styrene-divinylbenzene) columns using harsh conditions [8]. This report describes the optimization of a mobile phase for the RP liquid chromatographic separation of OTC and its degradation products by means of a statistical simplex design. The efficacy of Method TTC-SP05 [6] for the analysis of OTC degradation products in tissue has also been tested.

2. Experimental

2.1. Apparatus

The liquid chromatograph consisted of a Beckman Model 342 HPLC system (Beckman Instruments, Berkeley, CA, USA) containing a 20 μl injection loop, a Valco 2 μm precolumn screen filter (Supelco Canada, Mississauga, Canada), a C_8 Inertsil (50 × 4.6 mm I.D.) guard-column and C_8 Inertsil (250 × 4.6 mm I.D.) analytical column (Lab Link, Rockford, IL, USA) and a Waters 484 variable-wavelength detector (Millipore, Milford, MA, USA). A Hewlett Packard 3390A integrator (Hewlett Packard, Avondale, PA, USA) set at an attenuation of 2 mV was used for peak-area quantification.

2.2. Chemicals and materials

All organic solvents were of Omnisolv grade (VWR-Canlab, Edmonton, Canada); water was

purified by means of a Milli-Q purification system (Millipore, Mississauga, Canada). The aqueous buffer was prepared from 0.1 M ammonium acetate and adjusted to pH 3.0 using concentrated trifluoroacetic acid. Mobile phases were prepared by vacuum filtration of various portions (%, v/v) of the organic solvents and aqueous buffers through a 0.45 μm nylon filter. Separations were performed at ambient temperature (21 ± 2°C). Oxytetracycline was obtained from Sigma (St. Louis, MO, USA); 4-epiOTC, α-apoOTC and β-apoOTC were obtained from Spectrum Chemical (Gardenia, CA, USA). All other reagents were of analytical reagent grade. Bond Elut C_{18} (500 mg) cartridges were purchased from Varian Canada (Mississauga, Canada). Porcine semi-tendinosus muscle was obtained from a local supplier. Pretreatment samples of the tissue were used to initially verify the absence of OTC and degradation products.

2.3. Chromatography

A statistical simplex design [9] was used to optimize mobile phase selectivity for the compounds and the design is as shown in Table 1. The solvents selected for the study were methanol (MeOH) (x1), tetrahydrofuran (THF) (x2) and acetonitrile (ACN) (x3). The selectivity of these solvents is due to their relative differences in solvent polarity, proton acceptor contribution, proton donor contribution and dipole contribution [10] (Table 2). A solvent strength equivalent to 75:25 (v/v) buffer–acetonitrile was

Table 1
Ten-run simplex design for a triphasic organic solvent mixture

Run	Coded values for solvents			COF[a]
	Methanol	Tetrahydrofuran	Acetonitrile	
1	1.00	0.00	0.00	y1
2	0.00	1.00	0.00	y2
3	0.00	0.00	1.00	y3
4	0.50	0.50	0.00	y12
5	0.50	0.00	0.50	y13
6	0.00	0.50	0.50	y23
7	0.33	0.33	0.33	y123
8	0.66	0.17	0.17	y1123
9	0.17	0.66	0.17	y1223
10	0.17	0.17	0.66	y1233

Adapted from Snee [9].
[a]COF, chromatographic optimization function.

Table 2
Selectivity parameter values of organic solvents

Solvent	P'[a]	Xe[b]	Xd[c]	Xn[d]
Methanol	5.1	0.48	0.22	0.31
Tetrahydrofuran	4.0	0.38	0.20	0.42
Acetonitrile	5.8	0.31	0.27	0.42

Adapted from Snyder [10].
[a]P', solvent polarity.
[b]Xe, proton acceptor contribution.
[c]Xd, proton donor contribution.
[d]Xn, dipole contribution.

used as the starting point, as this is the recommended mobile phase in Method TTC-SP05 [6]. The chromatographic optimization function (COF) was calculated according to the formula of Glajch et al. [11]:

$$COF = \sum_{i=1}^{k} \ln(R_i / R_d) \qquad (1)$$

where R_i is the resolution of the ith peak pair in the chromatogram, R_d is the desired peak-pair resolution in the chromatogram, and k is the number of peak pairs. R_d was set to a value of 1.5; peak pair(s) with a resolution (R_i) greater than or equal to 1.5 were given equal value. All possible peak-pair combinations were taken into account during the calculation of the COF values. Data were fitted to quadratic and special cubic models and analysed using the Proc Reg procedure of the Statistical Analysis Systems (SAS) Institute Inc. [12] to determine the optimal mobile phase composition. Standards containing 1 μg/ml of the compounds were utilized for analysis.

2.4. Calibration curves and reproducibility

The calibration curves for OTC (calculated on its free base) and 4-epiOTC were obtained at 350 nm. Calibration curves for α- and β-apoOTC were obtained at 250 nm. Purity determination of the 4-epiOTC, α- and β-apoOTC preparations were not carried out; however, Khan et al. [13] reported the respective purities of preparations obtained from the same source to be 84.5%, 94.3% and 95.2%. These purities were not taken into account when determining the regression equations and were assumed to be 100% pure for calculation purposes.

2.5. Sample preparation

Sample and standards preparation are given in detail in Method TTC-SP05 of the Agriculture and Agri-Food Canada's Methods Manual [6]. Efficacy of the procedure outlined in Method TTC-SP05 for the extraction of OTC and degradation products from fortified distilled water and pork muscle was investigated at concentration levels of 2000, 1000, 200, and 100 ng/ml (g). Distilled water (5 ml) or homogenized meat samples (5 g) were fortified with appropriate amounts of 10 or 1 μg/ml methanol stock solutions of the compounds to give the final desired concentrations. The samples were blended with 20, 20 and 10 ml of 0.1 M Na_2EDTA McIlvaine buffer (pH 4.0), and centrifuged at 1000 × g for 10 min. The filtrate was applied to a 500 mg Bond-Elut C_{18} cartridge previously activated with methanol and water, and then washed with 20 ml of distilled water. The compounds were eluted with 7 ml of 0.01 M methanolic oxalic acid, and diluted to 10 ml with distilled water. Triplicate analyses for each concentration were obtained; the average of each analysis was determined from duplicate injections. Peak area quantification was used to determine the recovery (%) from the two matrices.

3. Results and discussion

3.1. Mobile phase optimization

According to the solvent classification scheme of Snyder [10] as cited by Glajch et al. [11], the calculated polarity strength of a 75:25 (v/v) buffer–acetonitrile mobile phase (assuming the aqueous buffer has the same eluting strength as water) would be 9.2. At a flow-rate of 0.6 ml/min, elution times for OTC and degradation products were: OTC, 10.5 min; 4-epiOTC, 11.2 min; α-apoOTC, 10.43 min; β-apoOTC, 31.2 min. Calculated compositions for equal polarity (equal to 75:25, v/v buffer–ACN) buffer–methanol and buffer–tetrahydrofuran mobile phases were calculated to be 78:22 (v/v) and 82:18 (v/v), respectively. With the calculated buffers as the mobile phase, elution of the primary compound (OTC) occurred at 40 min with buffer–methanol and 23 min with buffer–tetrahydrofuran, respectively.

Trial and error formulation of the buffer phases resulted in final binary-phase compositions of buffer–methanol 55:45 (v/v) (OTC, 10.8 min) and buffer–tetrahydrofuran 70:30 (v/v) (OTC, 12.0 min), corresponding to calculated polarity strengths of 7.9 and 8.3, respectively, lower than was calculated. This apparent discrepancy was reproducible, and may be attributed to the alteration of the normal bulk properties of the base solvent [11]. Differences in conformation have been shown to have dramatic effects on polarity of the tetracyclines, and are a function of their environment [14]. Casey and Yasin [15] as cited by Khan et al. [13] reported that in deuterated DMSO, the enone ring of the β-isomer assumes a half-chair conformation with the 4S, 4aS, and 12aR substituents in equatorial positions, whereas the α-isomer occurs as a mixture of conformers with at least one of the 4S, 4aS, or 12aS substituents in the axial position. This may result in different intramolecular interactions between proximal functional groups.

The actual mobile phase compositions tested according to the ten-run simplex design were as indicated in Table 3.

The COF values and adjusted retention times (unretained solvent time 3.9 min) for the four components were obtained (Table 4). Relative standard deviations for the adjusted retention times and COF values were less than 5% and 10% of the means, respectively. In general, it was observed that mobile phases incorporating tetrahydrofuran (strong dipole interaction, weak proton donor) effected a better peak separation than those without tetrahydrofuran.

Table 3
Mixture formulation for optimization experiment

Run	MeOH	THF	ACN	Buffer
1	45.00	0.00	0.00	55.00
2	0.00	30.00	0.00	70.00
3	0.00	0.00	25.00	75.00
4	22.50	15.00	0.00	65.00
5	22.50	0.00	15.00	62.50
6	0.00	15.00	12.50	72.50
7	15.00	8.33	10.00	66.77
8	30.00	4.17	5.00	60.83
9	7.50	16.70	5.00	70.80
10	7.50	4.17	20.00	68.33

Values in % (v/v).

Table 4
Adjusted retention times and chromatographic optimization function values for oxytetracycline and degradation compounds, and subsequent liquid chromatograph operating pressure ($n = 3$)

Run	Adjusted retention time (min)				COF	Pressure (kp.s.i.)
	OTC	4-Epi-OTC	α-ApoOTC	β-ApoOTC		
1	6.9	6.7	6.7	49.6	−9.35	2.36
2	8.1	9.2	4.9	35.1	−0.09	2.47
3	6.6	7.3	6.6	27.4	−3.30	1.49
4	8.6	9.3	5.2	38.8	−0.79	1.89
5	9.3	9.6	9.6	60.2	−7.11	2.50
6	7.2	8.1	4.8	31.0	−0.32	1.92
7	8.3	8.5	6.2	39.0	−0.51	2.09
8	8.3	8.5	6.2	45.4	−1.43	2.20
9	7.9	8.7	4.8	31.6	−0.38	1.75
10	7.9	8.6	6.2	35.8	−0.70	2.30

The COF values obtained from the experiments (runs 1–10) were fit to both quadratic and cubic equations. The response y as described by the quadratic model is:

$$E(y) = -8.86(x1) - 0.78(x2) - 3.21(x3)$$
$$+ 19.72(x1 \cdot x2) + 2.43(x1 \cdot x3)$$
$$+ 8.72(x2 \cdot x3) \qquad (2)$$

where $x1$, $x2$ and $x3$ are the coded values of methanol, tetrahydrofuran, and acetonitrile in the simplex design used for optimization. The response y as described by the cubic equation is:

$$E(y) = -8.74(x1) - 0.65(x2) - 3.08(x3)$$
$$+ 15.80(x1 \cdot x2) - 1.47(x1 \cdot x3)$$
$$+ 4.82(x2 \cdot x3) + 63.41(x1 \cdot x2 \cdot x3) \qquad (3)$$

Tabulated F-ratios ($\alpha = 0.05$) for the quadratic and cubic models were 2.49 and 2.40, respectively. Calculated F-ratios for lack of fit were 85 and 81, respectively, indicating that a significant lack of fit is present for both proposed models and that the models do not adequately describe the COF response as a function of the volumes of the three organic solvents. Quenouille [16] as cited by Snee [9] had stated that polynomial models do not account for components in the mobile phase that are inert or have additive effects and that a more complicated model would be needed to fit the data. Snee [9] had proposed several models that can overcome these

effects; these alternative models were not investigated as some of the tested mobile phases resulted in good chromatographic separation of OTC from the degradation compounds. The mobile phase with the lowest COF value (i.e. greatest resolution) was run 2 (Buffer–THF, 70:30). However, the operating pressure was high (2.47 kp.s.i.). Run 6 had the second lowest COF value (−0.32) and also had a reasonably low operating back pressure (1.92 kp.s.i.) (Fig. 2). Naidong et al. [8] achieved a good separation of the four compounds using a poly(styrene-divinylbenzene) column (250 × 4.6 mm I.D.) maintained at 60°C using a one-step gradient elution with a five-component pH 7.5 mobile phase. The mobile phase used in the present method offers the advantage of being simpler (triphasic mixture), and isocratic elution at ambient temperature is utilized. This mobile phase was subsequently utilized for further experiments.
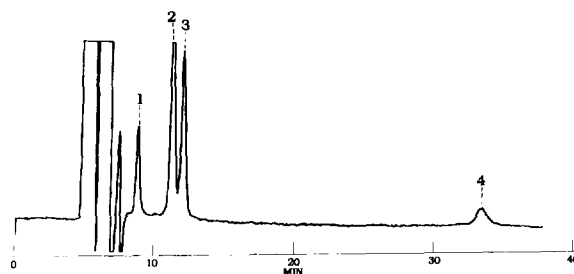

Fig. 2. Typical chromatogram for run 6. Peaks: 1, α-apoxytetracycline; 2, oxytetracycline; 3, 4-epioxytetracycline; 4, β-apooxytetracycline.

Table 5
Regression statistics for oxytetracycline, 4-epioxytetracycline, α- and β-apooxytetracycline for regression curves of peak-area counts as a function of concentration (ng/ml) ($n = 12$)

Regression statistics	OTC	4-EpiOTC	α-ApoOTC	β-ApoOTC
Intercept	3173.7	1951.2	4917.6	−20189.7
S.E.[a]	1523.0	698.6	5325.8	13482.6
x-variable	255.1	247.0	764.2	658.0
S.E.	2.7	1.2	9.5	24.0
R^2	0.999	0.999	0.998	0.987

[a]S.E., standard error.

3.2. Calibration curves

Regression statistics for the relationship between compound concentration and peak-area counts are shown in Table 5. The correlation coefficient exceeded 0.98 for all regression equations. The limits of detection for OTC, 4-epiOTC, α-apoOTC and β-apoOTC were 40, 20, 50, and 140 ng/ml, respectively.

3.3. Recovery experiments

Recovery data of OTC and degradation compounds from distilled water and pork tissue are reported in Table 6 and Table 7. Recovery of OTC was greater than 90% from distilled water for all concentrations investigated. Recovery of 4-epiOTC, α- and β-apoOTC were lower than OTC, and the percentage recovery appeared to be dependent upon the initial concentration. Recovery of OTC from tissue was lower than from distilled water. Recovery of 4-epiOTC was slightly reduced, whereas recoveries of α- and β-apoOTC were sharply decreased, with β-apoOTC recovery being reduced to less than 10% at concentrations of 2000 and 1000 ng/ml, and no detectable levels being found at concentrations of 200 and 100 ng/ml. Elution using acetonitrile, tetrahydrofuran and ethanol containing 0.01 M oxalic acid did not improve recoveries. The present findings agree with Law et al. [17] who had

Table 6
Recovery of oxytetracycline and degradation products from 5 ml of distilled water fortified at four different levels ($n = 3$)

Spike level (ng/ml)	Recovery (%) (C.V., %)			
	OTC	4-EpiOTC	α-ApoOTC	β-ApoOTC
2000	99.0 (0.7)	83.4 (1.3)	88.9 (5.6)	84.3 (16.9)
1000	90.5 (4.7)	79.4 (2.1)	52.6 (9.3)	58.1 (12.7)
200	93.4 (6.0)	83.9 (6.7)	17.7 (33.0)	62.9 (25.0)
100	90.1 (16.7)	66.1 (12.3)	ND[a]	ND

[a]ND, not detected.

Table 7
Recovery of oxytetracycline and degradation products from homogenized porcine tissue (5 g) fortified at four different levels ($n = 3$)

Spike level (ng/g)	Recovery (%) (C.V., %)			
	OTC	4-EpiOTC	α-ApoOTC	β-ApoOTC
2000	83.7 (2.4)	57.5 (4.6)	40.0 (3.7)	8.9 (11.4)
1000	69.9 (3.2)	52.9 (4.8)	29.8 (6.3)	6.5 (6.2)
200	74.1 (5.3)	61.8 (10.5)	26.0 (11.4)	ND[a]
100	74.6 (12.5)	49.3 (3.8)	29.9 (10.0)	ND

[a]ND, not detected.

reported that elution of some hydrophobic materials from RP C_{18} cartridges was more facile with methanol than acetonitrile. Hydrophobic interaction is therefore not the only retention mechanism operating in this recovery system and other secondary interactions are also present. Mulders and Van de Lagemaat [18] reported that the presence of residual silanols on C_{18} cartridges can lead to significant absorption of tetracycline drugs onto the cartridges, resulting in reduced recoveries. The mechanism of interaction can be ionic [17] or hydrogen bonding [19]. Drug recovery may also be reduced due to specific drug–matrix or cartridge–matrix interactions [20] whereby materials present in the matrix may interfere with the hydrophobic interaction between the compound and the C_{18} packing. Solid-phase extraction of OTC degradation products, particularly α- and β-apoOTC, does not appear to be feasible with the present method.

4. Conclusions

Oxytetracycline and three of its known degradation compounds have been chromatographically separated using an Inertsil C_8 column at room temperature, offering considerable advantage over previously published methods. Solid phase extraction of α- and β-apoOTC from distilled water and porcine tissue resulted in low, irreproducible recoveries, indicating that other interactions in addition to hydrophobic phenomena occur using this mode of extraction. Further work would be required to optimize Method TTC-SP05 before its use for analysis of OTC degradation compounds in different matrices.

Acknowledgments

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