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C_{18} columns for the simultaneous determination of oxytetracycline and its related substances by reversed-phase high performance liquid chromatography and UV detection

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Abstract

Simultaneous determination of oxytetracycline, 4-epioxytetracycline, α-apooxytetracycline, tetracycline and β-apooxytetracycline on C_{18} columns has been accomplished using a high performance liquid chromatographic method with UV detection. Separation was achieved on a Hypersil BDS RP-C_{18} column (250 mm × 4.6 mm) and on a Waters C_{18} Symmetry column (150 mm × 3.9 mm), 5 μm particle size each. These columns were equilibrated with mobile phases consisted of methanol–acetonitrile–0.1 M phosphate buffer pH 8.0 (12.5:12.5:75, v/v/v) and (15:15:70, v/v/v), respectively. The flow rate was 1.0 ml/min and the total elution time was 15 and 5 min, respectively. Both methods were applied to oxytetracycline raw material, human and veterinary formulations, where the excipients did not interfere. External standard calibration curves were linear for 4-epioxytetracycline, oxytetracycline, α-apooxytetracycline, tetracycline and β-apooxytetracycline in the concentration range of 0.27–200 μM, 0.05–200 μM, 0.03–200 μM, 0.35–200 μM and 0.20–200 μM on column A and 0.08–200 μM, 0.15–200 μM, 0.09–200 μM, 0.25–200 μM and 0.47–200 μM on column B, respectively. Day-to-day relative standard deviation of the determination for every component was less than 3%. Concerning the first column, limits of detection and quantification of the above compounds were in the concentration ranges of 10–106 nM and 30–352 nM, respectively, whereas on the second column these ranges became 27–144 nM and 81–475 nM, respectively. Recovery of the separated compounds was 95–105%.
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Keywords: Oxytetracycline; Related substances; C_{18} columns; Reversed-phase HPLC and UV detection; 4-Epioxytetracycline; Tetracycline; α-Apooxytetracycline and β-apooxytetracycline

1. Introduction

Oxytetracycline (OTC) is a broad-spectrum antibiotic that is commonly used in veterinary medicine as inhibits the protein synthesis in gram-positive and gram-negative bacteria. The European community has approved the use of OTC in a wide range of animal species like cattle, sheep, goats and pigs. The major dosage forms of OTC, which are available for animal health are feed premixes, injectables, sprays, soluble powders and tablets. On the other hand, OTC is used for human treatment alone or in combination with hydrocortisone and polymyxin B in preparations for oral use, use on the skin and in the eye. However, oxytetracycline (Fig. 1) and oxytetracycline hydrochloride may contain several impurities that should not exceed certain levels in the raw material according to Pharmacopoeia [1].

Although high-performance liquid chromatography (HPLC) on tetracyclines has been studied extensively, few articles have reported on the simultaneous separation of oxytetracycline, its degradation products and related substances, which may be formed during fermentation. The existing official method according to European and USP Pharmacopoeias [1,2] which is based on Khan's et al. work [3] includes an ion-pair RP-HPLC method at 60 °C, a complicated mobile phase, a copolymeric chromatographic column, which results in very wide peaks and a gradient elution with total elution time around 30 min. Moreover, Reenwijk et al. [4] have claimed that copolymeric columns are the only ones appropriate for such separations since silica-based materials present poor chromatographic efficiency and an apparently irreversible adsorption of tetracyclines.

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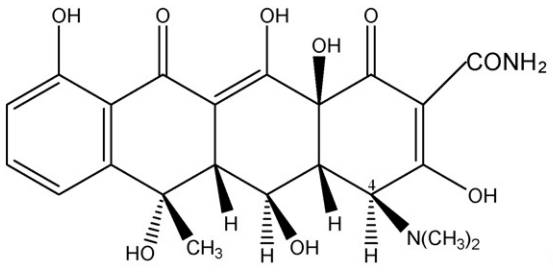
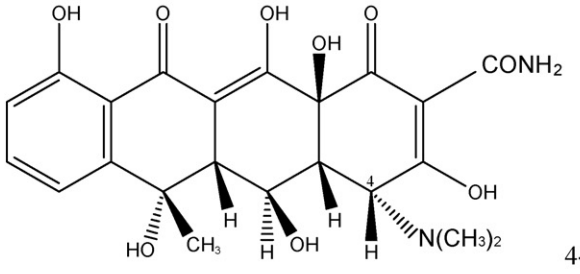


oxytetracycline



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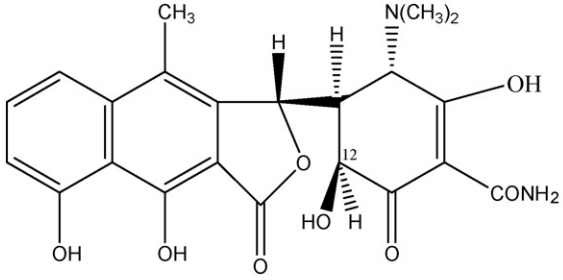
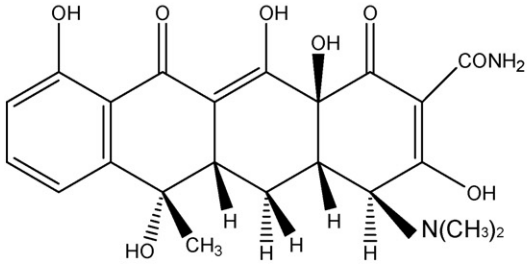
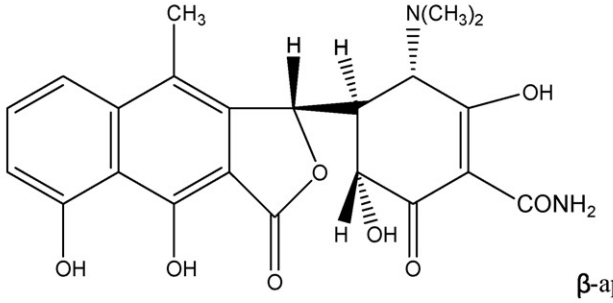


a-apooxytetracycline



tetracycline



β-apooxytetracycline

Fig. 1. Structures of oxytetracycline, 4-epioxytetracycline, α-apooxytetracycline, tetracycline and β-apooxytetracycline.

Very few workers have used C_8 columns with UV detection [5–8] but they have not separated all oxytetracycline related substances either simultaneously or adequately. Recently, some workers have proposed the XTerra RP-C_{18} column [9–12], a silica-based stationary phase with methyl end-capping, claimed to reduce silanol activity. However, even with that, simultaneous determination of all oxytetracycline related substances was not feasible [9] unless LC–MS–MS instrumentation [10–12] was used. As a consequence, no HPLC method has been reported in the literature using a C_{18} column with UV detection, for the successful simultaneous determination of oxytetracycline, 4-epioxytetracycline (EOTC), α-apooxytetracycline (α-AOTC), tetracycline (TC) and β-apooxytetracycline (β-AOTC).

The purpose of the present work was to develop a simple, fast, sensitive and reproducible isocratic RP-HPLC method with UV detection, performed on C_{18} columns, for simultaneous determination of OTC and its impurities in raw material, human and veterinary formulations.

The unique feature of the proposed method was its successful application on two kinds of C_{18} columns (Hypersil BDS and Waters Symmetry), which resulted in very short total elution time 15 and 5 min, respectively, time that was comparable or much less than that of the LC–MS–MS methods. At the same time, selectivity, precision and accuracy of the proposed method were very high, resulting in an accurate, precise and reliable method for determination of OTC and its related substances in any formulation.

2. Experimental

2.1. Instrumentation

The chromatographic system used, consisted of a Waters 600E multisolvent delivery system (a 600 controller, a pump and a U6K injector) and a Waters 486 tunable absorbance detector (Waters, Milford, MA, USA). The above system was controlled by the software package Millennium 2010.

2.2. Chemicals and reagents

All chemicals were of analytical purity grade. Highly purified water with a Milli-Q RG water purification system (Millipore Co., Bedford, MA, USA) was used in all procedures. Acetonitrile (ACN) and methanol (MeOH) of HPLC grade were purchased from Merck KGaA (Darmstadt, Germany). The standards (purity in %, w/w) of 4-epioxytetracycline (97%), α-apooxytetracycline (97%) and β-apooxytetracycline (97%) were purchased from Acros Organics (Geel, Belgium). Oxytetracycline hydrochloride (97%) and tetracycline hydrochloride (97%) were kindly donated by the pharmaceutical company Veterin Hellas (Athens, Greece). Human (powder and tablets) and veterinary (ointment, soluble powder and aerosol spray) formulations, analyzed in this study, were purchased from the pharmaceutical company Pfizer (Athens, Greece).

2.3. Chromatographic conditions

A Hypersil BDS RP-C_{18} column (250 mm × 4.6 mm), column A, and a Waters C_{18} Symmetry (150 mm × 3.9 mm), column B, 5 μm particle size both were used. Mobile phase, methanol–acetonitrile–0.1 M phosphate buffer pH 8.0 (12.5:12.5:75, v/v/v) and (15:15:70, v/v/v), respectively, was degassed with helium gas at a degassing rate of 30 ml/min. Flow rate of mobile phase was 1.0 ml/min. Injection volume was 20 μl. Experiments were performed at ambient temperature. Absorbance measurements were held at 253 nm. The total elution time was less than 15 and 5 min, respectively.

2.4. Solution preparation

Stock standard solutions 1.0 mM for OTC and 0.1 mM for EOTC, α-AOTC, TC and β-AOTC were prepared daily, dissolving 0.0020–0.0030 g in 50 ml of MeOH and shaken in an ultrasonic bath for 4 min each.

In order to construct the corresponding calibration curves and evaluate the precision of the proposed method, working standard solutions of EOTC (0.08–4.0 μM), OTC (20–140 μM), α-AOTC (0.03–0.5 μM), TC (0.25–6.0 μM) and β-AOTC (0.20–4.0 μM) were prepared. To establish the linearity range and calculate the limits of detection and quantification, working standard solutions of the above compounds were prepared in the concentration range of 0.27–200 μM for EOTC, 0.05–200 μM for OTC, 0.03–200 μM for α-AOTC, 0.35–200 μM for TC and 0.20–200 μM for β-AOTC on column A and 0.08–200 μM, 0.15–200 μM, 0.09–200 μM, 0.25–200 μM and 0.47–200 μM on column B, respectively.

Determination of OTC and its related substances in raw material, human (tablets and powder) and veterinary (powder, ointment and aerosol spray) formulations was performed five times using calibration curves. Solutions of these products were prepared following the same procedure as that for the preparation of stock standard solutions. A volume of 20–80 μl of them was diluted to 1 ml with mobile phase and filtered through 0.45 μm regenerated cellulose syringe filters. The final expected concentration of OTC in the injected solutions was around 100 μM.

Recovery studies of EOTC, OTC, α-AOTC, TC and β-AOTC were performed, both in standards and samples, using the standard addition method. A series of four solutions was prepared. The first solution was handled as described in the above paragraph, containing 100 μM oxytetracycline and its related substances more than 50 times lower than that. The other three solutions contained increasing amounts of OTC by 10 μM and of all related substances by 0.5 μM. The prepared solutions were then injected to the HPLC system.

2.5. Data analysis

Calibration curves of EOTC, OTC, α-AOTC, TC and β-AOTC were constructed. Regression equations were obtained through unweighed least squares linear regression analysis, using peak areas as a function of their concentration.

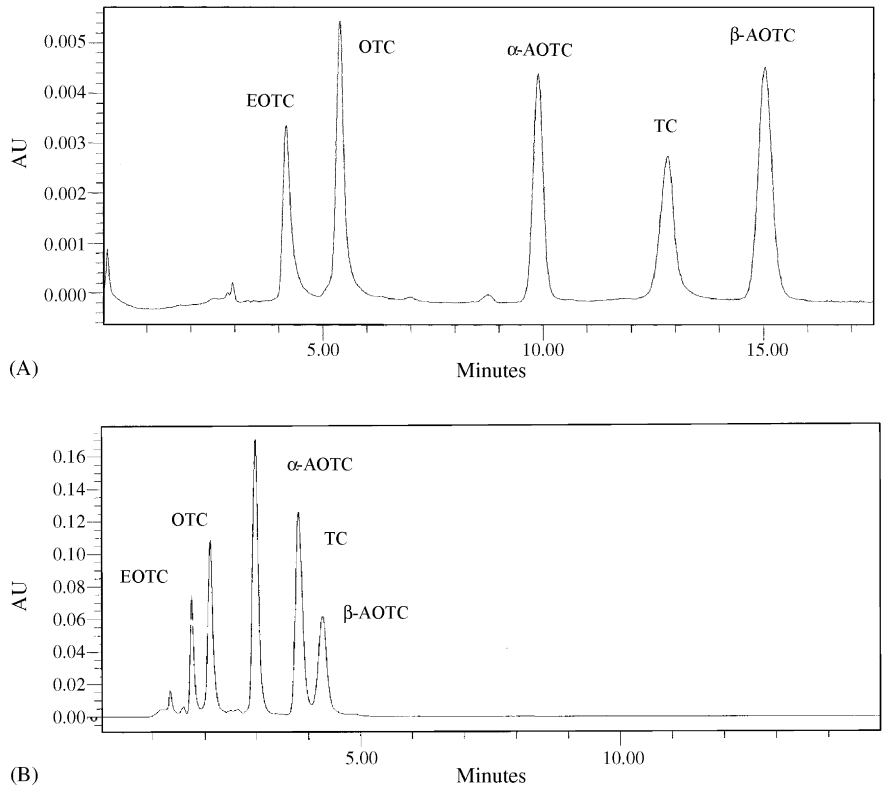


Fig. 2. Typical chromatograms of mixtures of standards on the Hypersil BDS RP-C_{18} column (250 mm × 4.6 mm) (A) and on the Waters C_{18} Symmetry column (150 mm × 3.9 mm) (B). Concentration of standards was in the range of 2–25 μM. The chromatographic conditions used were: mobile phase of methanol–acetonitrile–0.1 M phosphate buffer pH 8.0 (12.5:12.5:75, v/v/v) on column A and (15:15:70, v/v/v) on column B, flow rate 1.0 ml/min, detection wavelength 253 nm and room temperature.

3. Results and discussion

3.1. Mobile phase optimization

Several mixtures of solvents were tried on column A in order to achieve a sufficient separation of oxytetracycline and its related substances. The main difficulty of this study was the separation of OTC and EOTC as these compouds were identical in structure except from the configuration of C_4 on which dimethylamino group was located. A similar problem was faced with α-AOTC and β-AOTC as they were also identical except from the configuration of a hydroxyl group on C_{12}. On the

Table 1
Analytical parameters of working calibration curves of OTC hydrochloride and its impurities

Calibration method	Substance	Concentration range (μM)	Regression equation[a]		
			Intercept ($a \pm$ S.D.)[b] ($\times 10^{-1}$)	Slope ($b \pm$ S.D.) ($\times 10^{-10}$)[b]	$r(n)$[c]
External standards	Oxytetracycline	20–140	−7.8 ± 2.2	1.30 ± 0.04	0.9994(5)
	4-Epioxytetracycline	0.27–4.0	−42 ± 22	1.28 ± 0.14	0.9994(5)
	α-Apooxytetracycline	0.03–0.5	−0.5 ± 7.2	3.69 ± 0.23	0.996(5)
	Tetracycline	0.35–6.0	−11.3 ± 3.3	1.42 ± 0.04	0.9991(5)
	β-Apooxytetracycline	0.20–4.0	−23 ± 22	3.69 ± 0.30	0.9990(5)
Standard addition[d] method	Oxytetracycline	10–30	41.1 ± 1.6	1.30 ± 0.08	0.996(4)
	4-epioxytetracycline	0.5–2	41.1 ± 1.6	1.30 ± 0.08	0.996(4)
	α-apooxytetracycline	0.5–2	18.5 ± 4.0	3.63 ± 0.23	0.998(4)
	Tetracycline	0.5–2	41.6 ± 2.2	1.38 ± 0.11	0.993(4)
	β−apooxytetracycline	0.5–2	10.7 ± 2.0	3.98 ± 0.20	0.997(4)

The chromatographic conditions used were: Hypersil BDS-C_{18} column, mobile phase methanol–acetonitrile–0.1 M phosphate buffer pH 8.0 (12.5:12.5:75, v/v/v), flow rate 1.0 ml/min, detection wavelength 253 nm and room temperature.

[a] Linear unweighed regression analysis with a regression equation $y = a + bx$ where x is the concentration in M.

[b] S.D. is the standard deviation of intercept and slope.

[c] r is the correlation coefficient and n is the number of points in each calibration curve; each point is the mean of three experimental measurements.

[d] The first solution contained oxytetracycline in the concentration of 100 μM and the other three solutions contained increasing amounts of oxytetracycline by 10 μM and of all related substances by 0.5 μM.

Table 2
Analytical parameters of working calibration curves of OTC hydrochloride and its impurities

Calibration method	Substance	Concentration range (μM)	Regression equation[a]		
			Intercept ($a \pm$ S.D.)[b] ($\times 10^{-1}$)	Slope ($b \pm$ S.D.)[b] ($\times 10^{-10}$)	$r(n)$[c]
External Standards Calibration	Oxytetracycline	20–110	−8.9 ± 3.5	1.07 ± 0.12	0.998(5)
	4-Epioxytetracycline	0.08–4.0	−6.1 ± 3.0	0.70 ± 0.11	0.996(6)
	α-Apooxytetracycline	0.09–0.5	8.8 ± 9.6	3.88 ± 0.23	0.997(5)
	Tetracycline	0.25–6.0	−2.9 ± 4.7	1.07 ± 0.15	0.997(5)
	β-Apooxytetracycline	0.47–4.0	−49 ± 81	4.05 ± 0.23	0.996(5)
Standard addition[d] method	Oxytetracycline	10–30	44.8 ± 1.9	1.16 ± 0.10	0.993(4)
	4-epioxytetracycline	0.5–2	37.5 ± 1.1	0.72 ± 0.10	0.996(4)
	α-apooxytetracycline	0.5–2	143.2 ± 4.1	3.51 ± 0.23	0.996(4)
	Tetracycline	0.5–2	64.8 ± 1.1	1.23 ± 0.41	0.998(4)
	β-apooxytetracycline	0.5–2	165.3 ± 2.6	4.07 ± 0.22	0.998(4)

The chromatographic conditions used were: Waters C_{18} Symmetry column, mobile phase methanol–acetonitrile–0.1 M phosphate buffer pH 8.0 (15:15:70, v/v/v), flow rate 1.0 ml/min, detection wavelength 253 nm and room temperature.

[a] Linear unweighed regression analysis with a regression equation $y = a + bx$, where x is the concentration in M.

[b] S.D. is the standard deviation of intercept and slope.

[c] r is the correlation coefficient and n is the number of points in each calibration curve; each point is the mean of three experimental measurements.

[d] The first solution contained oxytetracycline in the concentration of 100 μM other three solutions contained increasing amounts of oxytetracycline by 10 μM and of all related substances by 0.5 μM.

course of optimization of HPLC experimental parameters, some compounds were not eluted and others showed poor resolution, inadequate selectivity and band broadening. It was a challenge to have a simple mobile phase composition for a fast and easy separation procedure. Thus, ion pairing reagents that have been used widely for similar determinations were avoided. Instead, a phosphate buffer of pH 8.0 in the eluent mixtures was utilized. Further optimization on mobile phase composition led to the final mixture of methanol–acetonitrile–0.1 M phosphate buffer pH 8.0 (12.5:12.5:75, v/v/v), mobile phase I, which resulted in a total elution time of about 15 minutes (Fig. 2A). The capacity factor k', the selectivity factor a and USP tailing were for EOTC 3.2, 1.6, 1.4, for OTC 4.4, 1.4, 1.2, for α-AOTC 8.9, 2.0, 1.1, for TC 12, 1.4, 1.0 and for β-AOTC 14, 1.2, 1.1, respectively.

This mobile phase was tried for the separation of the same substances on column B and it was observed that it needed minor optimization. In this column the most appropriate composition was methanol–acetonitrile–0.1 M phosphate buffer pH 8.0 (15:15:70, v/v/v), mobile phase II, resulted in a total elution time of less than 5 min (Fig. 2B). Under the optimized parameters on column B, values of the capacity factor k', the selectivity factor a and USP tailing of each chromatographic peak were for EOTC 0.8, 1.2, 1.1, for OTC 1.1, 1.5, 1.1, for α-AOTC 2.0, 1.3, 1.2, for TC 2.9, 1.4, 1.2 and for β-AOTC 3.3, 1.2, 1.1, respectively.

3.2. Quantification

Under the experimental conditions described in Sections 2.3 and 2.4 linear calibration curves were constructed in an every day basis for 4-epioxytetracycline, oxytetracycline, α-apooxytetracycline, tetracycline and β-apooxytetracycline in the concentration range of 0.27–4.0 μM, 20–140 μM, 0.03–0.5 μM, 0.35–6.0 μM and 0.20–4.0 μM on column A and 0.08–4.0 μM, 20–110 μM, 0.09–0.5 μM, 0.25–6.0 μM and 0.47–4.0 μM on column B, respectively. However, regression analysis revealed that calibration curves of EOTC, OTC, α-AOTC, TC and β-AOTC were linear in the concentration range of 0.27–200 μM, 0.05–200 μM, 0.03–200 μM, 0.35–200 μM and 0.20–200 μM on column A and 0.08–200 μM, 0.15–200 μM, 0.09–200 μM, 0.25–200 μM and 0.47–200 μM on column B, respectively. In the case of real samples, calibration curves were also constructed using the method of standard additions. Their slopes were statistically identical with those obtained by the external standards; this was an additional proof that there was no interference from the excipients in the human and veterinary formulations. The analytical parameters of representative calibration curves are summarized in Tables 1 and 2.

3.3. Validation

3.3.1. Selectivity

As shown in Fig. 2A and B retention times observed for EOTC, OTC, α-AOTC, TC and β-AOTC on column A were 4.1, 5.4, 9.8, 12.8 and 15.1 min, respectively, while those observed on column B were 1.8, 2.1, 2.9, 3.8 and 4.2 min, respectively. Good resolution between the examined chromatographic peaks was assured by the obtained values of R_S, with regard to the previous eluted peak, which were on column A for OTC, α-AOTC, TC and β-AOTC 3.7, 12.2, 5.9, 3.8, respectively, and on column B 1.6, 1.9, 3.6, 1.5, respectively.

3.3.2. Precision and accuracy

To verify the precision of the proposed HPLC method, within day and between days precision was obtained in measurements of OTC and its impurities in standard solutions as well as in human and veterinary formulations. Within day and between days relative standard deviation values (R.S.D.) were usually found less than 3%. Only close to limits of quantification, R.S.D. values were about 15%.

Table 3
Recovery studies for the simultaneous determination of OTC and its impurities in standard solutions, performed on a Hypersil BDS-C_{18} and on a Waters C_{18} Symmetry column

Substance	Spiked concentration μM[a], Hypersil BDS-C_{18}	Mean recovery ± S.D.[b], Hypersil BDS-C_{18}	Spiked concentration μM[a], Waters C_{18} Symmetry	Mean recovery ± S.D.[b], Waters C_{18} Symmetry
	10	97 ± 3	10	96 ± 4
OTC	20	101 ± 4	20	101 ± 2
	30	100 ± 2	30	97 ± 3
	0.5	102 ± 2	0.5	98 ± 3
EOTC	1	98 ± 4	1	98 ± 5
	1.5	99 ± 3	1.5	99 ± 4
	0.5	96 ± 2	0.5	99 ± 2
α-AOTC	1	99 ± 4	1	99 ± 4
	1.5	105 ± 5	1.5	104 ± 5
	0.5	100 ± 3	0.5	103 ± 4
TC	1	99 ± 4	1	96 ± 5
	1.5	100 ± 3	1.5	102 ± 4
	0.5	100 ± 3	0.5	101 ± 3
β-AOTC	1	101 ± 4	1	96 ± 4
	1.5	98 ± 5	1.5	95 ± 6

The chromatographic conditions used were: mobile phase of methanol–acetonitrile–0.1 M phosphate buffer pH 8.0 (12.5:12.5:75, v/v/v), flow rate 1.0 ml/min, detection wavelength 253 nm and room temperature.

[a] The first contained oxytetracycline in the concentration of 100 μM and the other substances more than 50 times lower than that. The other three solutions contained increasing amounts of oxytetracycline by 10 μM and of all related substances by 0.5 μM.

[b] S.D. is the standard deviation of the mean recovery.

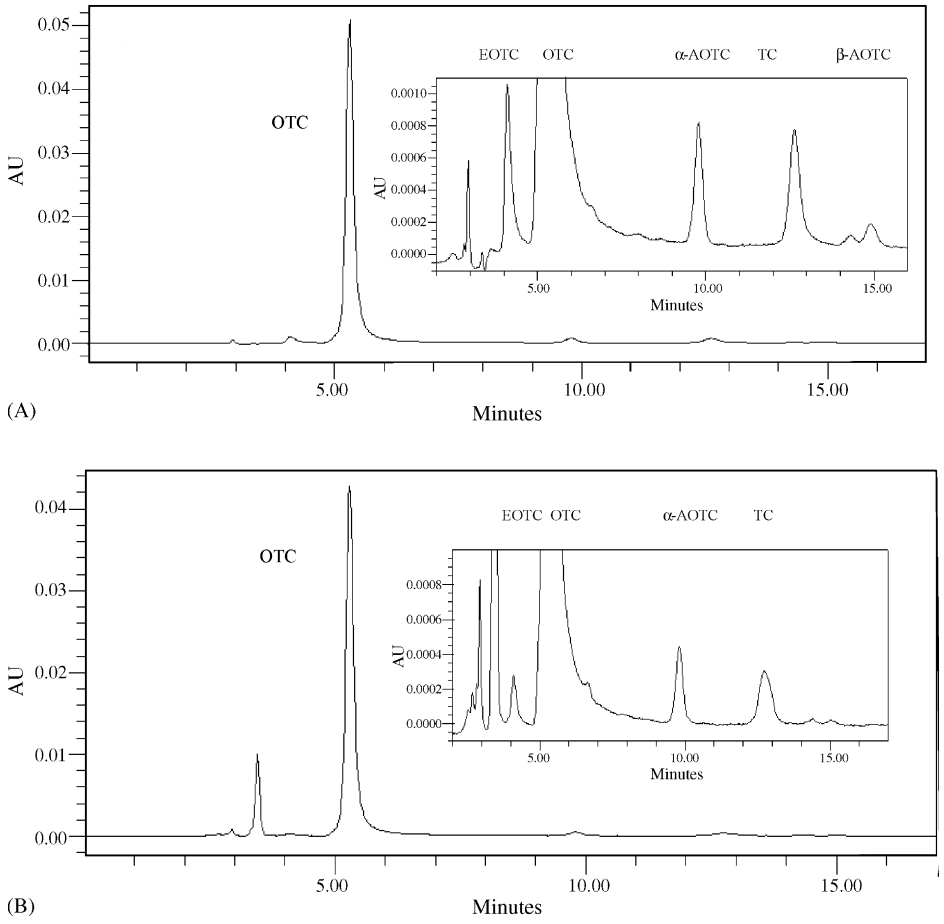


Fig. 3. Typical chromatograms of raw material (A) and a human tablet formulation (B) on the Hypersil BDS RP-C_{18} column (250 mm × 4.6 mm). Concentration of OTC was approximately 100 μM. The chromatographic conditions used were: mobile phase of methanol–acetonitrile–0.1 M phosphate buffer pH 8.0 (12.5:12.5:75, v/v/v), flow rate 1.0 ml/min, detection wavelength 253 nm and room temperature.

The accuracy of the developed method was examined by recovery studies conducted as described in Section 2.4. The mean recovery of the studied compounds was calculated and results are shown in Table 3.

3.3.3. Limits of detection (LOD) and quantification (LOQ)

The LOD and LOQ values were defined as follows [13]:

$$\text{LOD} = \frac{3.3 s_{y/x}}{b} \quad \text{and} \quad \text{LOQ} = \frac{10 s_{y/x}}{b}$$

where b is the slope and $s_{y/x}$ is the residual standard deviation of the regression line, calculated using working standards with concentrations close to LOD values.

Based on the above equations, the calculated LOD values for EOTC, OTC, α-AOTC, TC and β-AOTC were 83, 17, 10, 106 and 59 nM on the Hypersil BDS-C_{18} column and 27, 44, 30, 77 and 144 nM on the Symmetry C_{18} one.

Similarly, the LOQ values were found equal to 275, 55, 30, 352 and 195 nM on the first column and 81, 146, 90, 255 and 475 nM on the second one.

3.4. Application

Preparing the samples in the way that was described in Section 2.4, OTC was determined in raw material and in several human and veterinary formulations. Typical chromatograms of samples on BDS-C_{18} column are shown in Fig. 3A and B. The inserts present the relevant peaks of EOTC, OTC, α-AOTC, TC and β-AOTC magnified.

The method of standard additions was applied to all samples and the slopes of the obtained calibration curves were statistically identical to those obtained with the method of the external standard. This fact verified no interference from the drug excipients under the experimental conditions used. The resulting percentage of the determined compound OTC along with EOTC, α-AOTC, TC and β-AOTC detected are tabulated in Tables 4 and 5. Levels of related substances found were below the upper limits allowed by European Pharmacopoeia. The concentrations found within day and between days and the nominal concentrations are not significantly different according to the *t*-test at 0.05 level.

3.5. Robustness and ruggedness

Robustness of the proposed method was assessed with respect to small deliberate alterations in several experimental parameters. Determining OTC and its impurities in a spiked solution on the Hypersil BDS RP-C_{18} column, change of the content of the mobile phase from methanol–acetonitrile–0.1 M phosphate buffer pH 8.0 (12.5:12.5:75, v/v/v) to (13:13:74, v/v/v) and (12:12:76, v/v/v) did not change the results more than 2.2% for EOTC, 2.4% for OTC, 1.4% for α-AOTC, 2.5% for TC and 2.9%

Table 4
Concentration (% w/w) of OTC hydrochloride and its impurities in raw material, human and veterinary formulations

Sample[a]	Substance	Nominal concentration (%, w/w)	Concentration found ± S.D.[b]		
			External standard calibration		Method of standard addition
			Within day (5)	Between days (5)	Between days (5)
1	Oxytetracycline	97	98 ± 0.4	96 ± 0.9	97 ± 0.8
	4-Epioxytetracycline	–	0.3 ± 0.1	0.4 ± 0.1	0.3 ± 0.1
	Tetracycline	–	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
	α-Apooxytetracycline	–	0.6 ± 0.2	0.7 ± 0.2	0.7 ± 0.2
	β-Apooxytetracycline	–	0.12 ± 0.05	0.11 ± 0.05	0.10 ± 0.05
2	Oxytetracycline	65.4	66.8 ± 0.3	64.7 ± 0.4	65.7 ± 0.6
	4-Epioxytetracycline	–	0.51 ± 0.05	0.53 ± 0.06	0.4 ± 0.1
	Tetracycline	–	0.8 ± 0.1	0.7 ± 0.2	0.7 ± 0.2
	α-Apooxytetracycline	–	0.44 ± 0.05	0.41 ± 0.05	0.5 ± 0.1
3	Oxytetracycline	3.4	3.4 ± 0.1	3.4 ± 0.2	3.5 ± 0.5
	4-Epioxytetracycline	–	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
	Tetracycline	–	0.7 ± 0.1	0.5 ± 0.1	0.5 ± 0.2
4	Oxytetracycline	5.5	5.6 ± 0.1	5.6 ± 0.2	5.7 ± 0.5
	4-Epioxytetracycline	–	0.13 ± 0.05	0.1 ± 0.1	0.1 ± 0.1
	Tetracycline	–	0.4 ± 0.1	0.5 ± 0.1	0.4 ± 0.2
5	Oxytetracycline	3.4	3.3 ± 0.1	3.4 ± 0.1	3.2 ± 0.3
	4-Epioxytetracycline	–	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.1
	Tetracycline	–	0.8 ± 0.1	0.7 ± 0.2	0.7 ± 0.2
6	Oxytetracycline	4.0	4.1 ± 0.2	4.1 ± 0.2	3.9 ± 0.3
	4-Epioxytetracycline	–	0.24 ± 0.05	0.2 ± 0.2	0.2 ± 0.2
	Tetracycline	–	0.5 ± 0.1	0.4 ± 0.1	0.5 ± 0.1

The chromatographic conditions used were: Hypersil BDS-C_{18} column, mobile phase of methanol–acetonitrile–0.1 M phosphate buffer pH 8.0 (12.5:12.5:75, v/v/v), flow rate 1.0 ml/min, detection wavelength 253 nm and room temperature.

[a] (1) Oxytetracycline raw material; (2) human tablets; (3) human powder; (4) veterinary powder; (5) veterinary ointment; (6) veterinary aerosol spray.

[b] OTC percentage is referred to the sample, while related substances percentage is referred to OTC (%).

Table 5
Concentration (%, w/w) of OTC hydrochloride and its impurities in raw material, human and veterinary products

Sample[a]	Substance	Nominal concentration (%, w/w)	Concentration found ± S.D.[b]		
			External standard calibration		Method of standard addition
			Within day (5)	Between days (5)	Between days (5)
1	Oxytetracycline	97	97 ± 0.5	96 ± 0.6	96 ± 0.6
	4-epioxytetracycline	–	0.3 ± 0.1	0.4 ± 0.1	0.4 ± 0.2
	Tetracycline	–	0.5 ± 0.1	0.5 ± 0.1	0.6 ± 0.2
	α-apooxytetracycline	–	0.8 ± 0.2	0.7 ± 0.2	0.8 ± 0.2
	β-apooxytetracycline	–	0.11 ± 0.05	0.13 ± 0.05	0.11 ± 0.05
2	Oxytetracycline	65.4	66.2 ± 0.4	64.7 ± 0.5	65.2 ± 0.5
	4-epioxytetracycline	–	0.40 ± 0.05	0.4 ± 0.05	0.41 ± 0.05
	Tetracycline	–	0.6 ± 0.1	0.7 ± 0.1	0.7 ± 0.2
	α-apooxytetracycline	–	0.3 ± 0.05	0.3 ± 0.05	0.3 ± 0.1
3	Oxytetracycline	3.4	3.3 ± 0.1	3.4 ± 0.3	3.4 ± 0.4
	4-epioxytetracycline	–	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
	Tetracycline	–	0.5 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
4	Oxytetracycline	5.5	5.3 ± 0.1	5.6 ± 0.2	5.3 ± 0.5
	4-epioxytetracycline	–	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
	Tetracycline	–	0.5 ± 0.1	0.2 ± 0.1	0.4 ± 0.1
5	Oxytetracycline	3.4	3.5 ± 0.2	3.4 ± 0.3	3.3 ± 0.4
	4-epioxytetracycline	–	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
	Tetracycline	–	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
6	Oxytetracycline	4.0	4.1 ± 0.2	4.1 ± 0.3	4.3 ± 0.5
	4-epioxytetracycline	–	0.20 ± 0.05	0.22 ± 0.05	0.2 ± 0.1
	Tetracycline	–	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1

The chromatographic conditions used were: Waters C_{18} symmetry column, mobile phase of methanol–acetonitrile–0.1 M phosphate buffer pH 8.0 (12.5:12.5:75, v/v/v), flow rate 1.0 ml/min, detection wavelength 253 nm and room temperature.

[a] (1) Oxytetracycline raw material; (2) human tablets; (3) human powder; (4) veterinary powder; (5) veterinary ointment; (6) veterinary aerosol spray.

[b] OTC percentage is referred to the sample, while related substances percentage is referred to OTC (%).

for β-AOTC. A similar situation was observed changing the content of the mobile phase from methanol–acetonitrile–0.1 M phosphate buffer pH 8.0 (15:15:70, v/v/v) to (14.5:14.5:71, v/v/v) and (15.5:15.5:69, v/v/v) on the Symmetry C_{18} column and the results did not change more than 3%. These resulting changes were comparable to the R.S.D. of the method. During these changes the parameters t_R, a and R_S remained statistically the same. Similar observations were made, changing the flow rate from 1.0 to 1.1 ml/min and 0.9 ml/min and the pH from 8.0 to 7.8 and 8.1. pH did not exceed the value of 8.1 since the involved columns were silica-based. The results did not change more than the R.S.D. of the method and the parameters t_R, a and R_S of the chromatographic peaks remained statistically the same on both columns. It should be mentioned that in every change, new calibration curves were constructed under the new experimental conditions.

Ruggedness of the developed method was indicated by the between days precision because it included changes in reagents, chemicals and solvents.

4. Conclusions

Use of C_{18} columns was finally accomplished, for the simultaneous determination of OTC and its related substances EOTC, α-AOTC, TC and β-AOTC by HPLC with UV detection.

The proposed method includes a very short total elution time (down to 5 min), isocratic elution, simple mobile phase, short analysis time, sharp chromatographic peaks, peak resolution values in the range 1.5–12.2 and recovery values 95–105%.

In spite of what it has been written in the literature for unsuitability of silica-based material for such determination, presented results proved that this method, applied on certain C_{18} columns (probably with significantly improved silica material) is simple, fast, sensitive, reliable and robust. As a consequence, it can be useful for quality control of oxytetracycline in raw material and its formulations.

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