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Quantitative analysis of oxytetracycline and its impurities by LC-MS-MS

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Abstract

A liquid chromatographic-tandem mass spectrometric method using an Xterra MS C_{18} chromatographic column (100 mm × 2.1 mm i.d., 3.5 μm) that allows complete separation of oxytetracycline (OTC) and the impurities: 4-epi-oxytetracycline (EOTC), tetracycline (TC), 4-epi-tetracycline (ETC), 2-acetyl-2-decarboxamido-oxytetracycline (ADOTC), α-apo-oxytetracycline (α-AOTC) and β-apo-oxytetracycline (β-AOTC) was developed. Gradient elution was used and calibration curves were obtained using the scan mode selected reaction monitoring (SRM). Acceptable correlations were obtained for OTC, TC, EOTC and ADOTC whereas the correlations of α-AOTC and β-AOTC were less accurate resulting in higher limits of quantification (LOQ) and limits of detection (LOD) relative to the other compounds. The intraday and interday accuracy varied for all the compounds from 90 to 112% and the intraday and interday precision were lower than 7.1%. The method was applied for analysis of commercial available ointments containing OTC resulting in an acceptable quantification of OTC and the impurities in the drug preparations. The advantage of this method compared to the other separation methods is an empty separation window right after the large peak corresponding to OTC in the chromatogram, which facilitates an accurate determination of ADOTC and the other impurities.

Keywords: Oxytetracycline; Impurities; High performance liquid chromatography; Tandem mass spectrometry; Validation; Ointments

1. Introduction

Oxytetracycline (OTC) is a broad-spectrum antibiotic, which inhibits the protein synthesis in gram-positive and gram-negative bacteria. In Denmark, OTC is used for human treatment in combination with hydrocortisone and polymyxin B in preparations for use at the skin or in the eye. Recently, a preparation for oral use entered the Danish market. Similar preparations, but with different product names, are commercial available all over the world [1].

According to the European Pharmacopoeia (Ph. Eur.) oxytetracycline and oxytetracycline hydrochloride may contain the impurities 4-epi-oxytetracycline (EOTC), tetracycline (TC) and 2-acetyl-2-decarboxamido-oxytetracycline (ADOTC). Oxytetracycline

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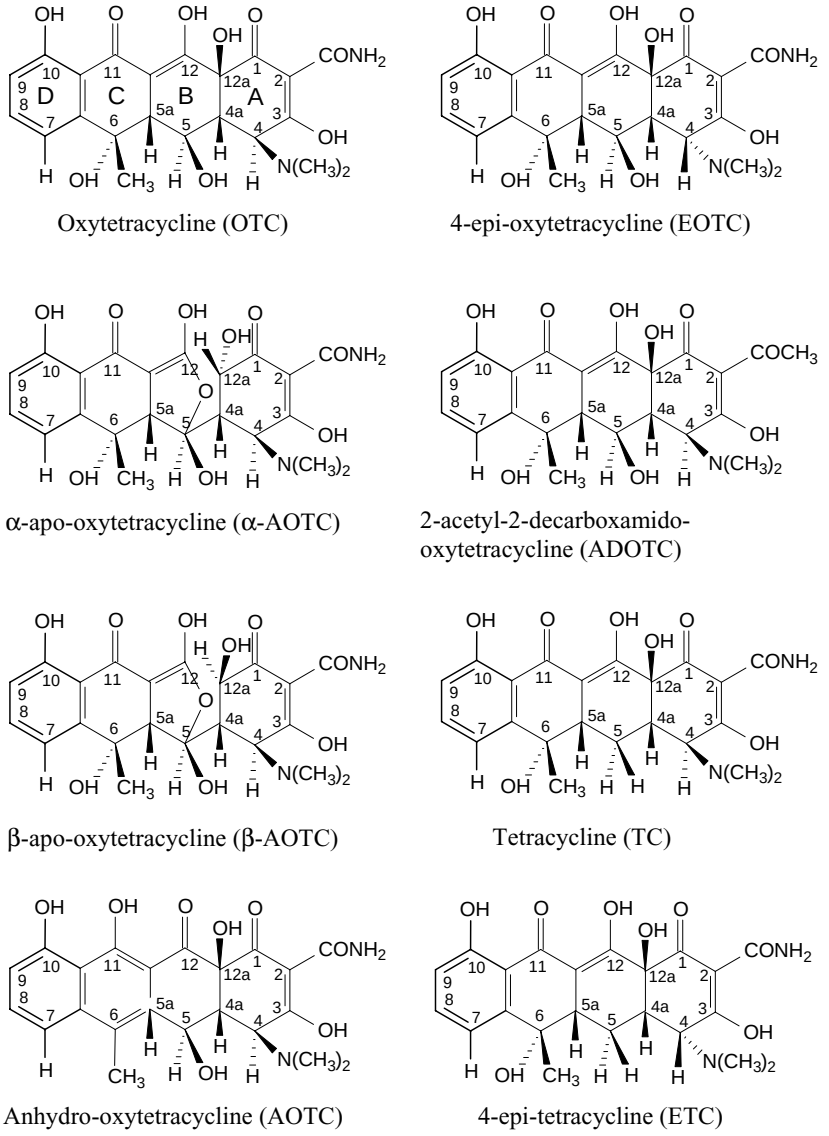

Fig. 1. Structures of oxytetracycline and its impurities.

hydrochloride is also allowed to contain anhydro-oxytetracycline (AOTC), α-apo-oxytetracycline (α-AOTC) and β-apo-oxytetracycline (β-AOTC). The structures are shown in Fig. 1. The claim for impurities in OTC is 2% of ADOTC, 2% of TC, 0.5% of EOTC and 2% in total of AOTC, α-AOTC and β-AOTC according to the Ph.Eur. [2].

OTC is produced by fermentation by certain strains of *Streptomyces rimosus*. TC and ADOTC are formed as by-products in the fermentation process of OTC and therefore a purification of OTC is necessary [3,4]. EOTC, AOTC, α-AOTC and β-AOTC are degradation products formed during the purification process of OTC. Under acidic condition AOTC is formed due to the elimination of water involving the hydrogen at position C-5a and the hydroxy group at position C-6. AOTC is very unstable in acidic solution due to the hydroxy group at C-5 resulting in scission of ring

B, to the more stable aromatic isomers α-AOTC and β-AOTC. In aqueous solutions OTC epimerises at position C-4, which results in the formation of EOTC [5]. OTC may also contain traces of 4-epi-tetracycline (ETC) as TC epimerises, even more willingly than OTC. ADOTC on the other hand does not epimerise [4].

Legislative demands (Ph.Eur.) limit the concentrations of the impurities to a low concentration level, as several of these are less potent than OTC. TC has similar potency as OTC, EOTC has a potency of 30% of that of OTC and α-AOTC, β-AOTC and ADOTC [4,6] has potencies of only 7–10% of that of OTC [5].

Contrary to most tetracycline impurities it is not possible to obtain ADOTC as a standard, and therefore the ADOTC used in the present investigation has been isolated in our laboratory facilities using preparative HPLC [7]. AOTC is too unstable to isolate, and thus it was not possible to obtain it as a standard.

TLC has formerly been used to analyse OTC and its impurities. The methods did generally provide a sufficient separation, but the method of Naidong et al. provided an inadequate separation of ADOTC and TC [8], and the method of Willekens did not include ADOTC and TC at all [9].

Khan et al. developed an HPLC method to quantify OTC and its impurities, but the method did not give a sufficient separation of ADOTC and OTC [3]. Nevertheless, this method is presently used in the monograph on OTC in Ph.Eur. [2].

Li et al. developed a capillary electrophoresis method that provided a sufficient separation of OTC and its impurities within an analysis time of only 15 min [10]. The capillary method developed by Tjørnelund and Hansen did not give a complete baseline separation of OTC and TC and the method did not include ADOTC and α-AOTC [11].

In this study, tandem mass spectrometry was used for quantification, because a very good selectivity was obtained using the scan mode selected reaction monitoring (SRM). This makes it possible to use a simpler sample preparation. CE has formerly given a very good separation of OTC and its impurities, but the electrophoresis buffer used in the method contains a detergent [10], which is not compatible with MS. The other CE-method contains high concentrations of magnesium [11], which should not be sprayed onto the MS-system. The TLC-methods [8,9] are difficult to couple with MS. Consequently HPLC is chosen, which is even easier to couple to MS than CE.

Previously, our laboratory developed an HPLC-MS-MS method able to follow the abiotic degradation of OTC in soil interstitial water [12]. The method provided an acceptable separation of several degradation products and impurities, but it was not developed to analyse OTC together with TC as required according to Ph.Eur. In this paper we therefore improved the separation of all major OTC impurities by modifying the temperature, the composition of the mobile phases and the gradient steps. The applicability of the developed method, as improved reference method for analysing OTC impurities in pharmaceutical formulations, was shown by analysing OTC and all major impurities in the two dermal OTC-containing ointments from the Danish market.

2. Materials and methods

2.1. Chemicals and reagents

The standards (purity in %) were purchased from the following companies: Oxytetracycline hydrochloride (95.7%) from Unikem (Copenhagen, Denmark), tetracycline (95%) from Sigma, 4-epi-oxytetracycline (97%), α-apo-oxytetracycline (97%) and β-apo-oxytetracycline (97%) from Acros Organics (Geel, Belgium). ADOTC was isolated at our own laboratory [7]. All other chemicals were of reagent grade or HPLC quality.

2.2. Analytical HPLC-system

An Agilent 1100 HPLC-system (Agilent Technologies, Palo Alto, CA, USA) controlled the gradient system. It was equipped with an auto sampler (operated at 4 °C), a pump and a thermostated column oven. The analytical column was an Xterra MS (Waters Corporation, Milford, MO, USA) C_{18} chromatographic column (100 mm × 2.1 mm i.d., 3.5 μm) operated at 35 °C.

The separation was performed using gradient elution. Mobile phase A: methanol–water (5:95, v/v) with formic acid (0.08 M) added. Mobile phase B: methanol–water (95:5, v/v) with formic acid (0.08 M) added.

The gradient was as follows: 0–6 min 89% A, 6–11 min a linear gradient to 50% A, 11–20 min 50% A, 20–25 min linear gradient to 89% A. This mixture was maintained until 30 min. The injection volume was 5.0 μl and the flow rate 0.25 ml/min.

2.3. MS-system

Mass spectrometric measurements were performed on a Sciex API 3000™ (Applied Biosystems, Foster City, CA, USA) equipped with an electrospray ionisation source. The instrument was operated in the positive mode and coupled to the outlet of the HPLC column via PEEK tubing. The temperature of the heated capillary was 500 °C, and the source voltage was set at 4.2 kV. Nebulizer gas, curtain gas, collision gas, declustering potential, focusing potential, entrance potential, collision energy and collision cell exit potential, were set at the following values: 8, 9, 4 psi, 41, 200, −10, 30 and 15 V, respectively.

SRM was used to quantify the compounds in the MS. To get a good sensitivity an abundant ion was selected from the product ion scans. Table 1 contains the most abundant product ions of OTC and its impurities. All compounds, except ADOTC, which contains a methyl–ketone group instead of the amide group at C-2 (see Fig. 1), lose NH_3 in the fragmentation. ADOTC, OTC, EOTC and TC eliminate water probably from the C-6 and C-5a position. The apo-compounds cannot eliminate water, as they are aromatic degradation products of OTC formed of anhydro-oxytetracycline as explained in the introduction. The product ions given in bold in Table 1 were used for quantification in SRM. However, mass 461/444 was used to quantify OTC, as it gave better calibration curves than mass 461/426 in the concentration range investigated.

Collection and treatment of data was done by use of Analyst™ software (Applied Biosystems, Foster City, CA, USA) in Windows NT® platform-based data processing.

2.4. Sample preparation

Stock solutions of OTC, EOTC, TC, ADOTC, α-AOTC and β-AOTC were prepared in methanol and kept at −18 °C for maximum 1 month. α-AOTC was dissolved completely by ultrasonication. A mixture of the stock solutions of the OTC impurities was further diluted in water giving a final methanol concentration of 10%. Calibration curves of all impurities as well as OTC were obtained at 5–6 concentration levels.

The following two OTC-containing dermal ointments at the Danish market produced by Pfizer (Ballerup, Denmark) were analysed: (A) hydrocortison med terramycin (30 mg OTC/g) and (B) terramycin–polymyxin B (30 mg OTC/g). The ointments were dissolved in hexane–0.08 M formic acid (1:1, v/v). The lipophilic excipients and the hydrocortisone were extracted to the hexane layer. The aqueous layer containing OTC was filtered through a filter (70 mm in diameter) from Merck Eurolab, and injected onto the HPLC.

3. Results and discussion

3.1. Separation of oxytetracycline and its impurities

The chromatographic method used in this work was a further development of an analytical HPLC-MS-MS method used to analyse the degradation of OTC in soil interstitial water [12]. The original method was

Table 1
Molecular related ions and the most abundant product ions of EOTC, OTC, ADOTC, TC, α-AOTC and β-AOTC produced using product ion scan

	Compound						Proposed structures of ions
	EOTC	OTC	ADOTC	TC	α-AOTC	β-AOTC	
Molecular ion (*m*/*z*)	461	461	460	445	443	443	$[M+H]^+$
Product ions (*m*/*z*)	444	444		428	**426**	**426**	$[M+H-NH_3]^+$
			442				$[M+H-H_2O]^+$
	426	**426**		**410**			$[M+H-NH_3-H_2O]^+$

The most abundant product ion of each compound are marked with numbers in bold.

not optimised for the separation of OTC and its impurities since β-AOTC was not eluted within 30 min and the method did not give a sufficient separation of TC, EOTC and OTC. The gradient was therefore further developed to improve the separation of these compounds.

The initial methanol concentration in the gradient was lowered to 15% to improve the separation of TC, EOTC and OTC and the final methanol concentration in the gradient was increased to 50% to elute β-AOTC within an analysis time of 30 min. These changes caused a poor resolution between ADOTC and α-AOTC. This resolution was improved by keeping the initial methanol concentration constant for 6 min and by raising the column temperature to 35 °C.

3.2. The HPLC-MS-MS method

The separation of an aqueous standard of OTC and its impurities is shown in Fig. 2. The method provides an improved separation with complete baseline separation of all the compounds and the selectivity in detection is further increased using SRM. The separation of OTC and its impurities in ointment A is shown in Fig. 3. A sufficient separation of the compounds is also obtained under these conditions; TC however, is on the front of the OTC-peak. This is of no particular importance for the quantification, because of the use of SRM, but it can involve some ion suppression. The ion suppression can have a minor influence on the determination of the concentration of TC. To avoid this source of error the standards of TC should be prepared and analysed in presence of OTC.

The samples contain a third compound with a molecular ion of *m*/*z* 443, which fragment to *m*/*z* 426 similar to the two peaks corresponding to α-AOTC and β-AOTC. The retention time of the unknown peak is 14.9 min, and it corresponds probably to AOTC, but it is not further investigated in this work.

The separation method developed provides a better separation of OTC and its major impurities than the HPLC method developed by Khan et al. intended for

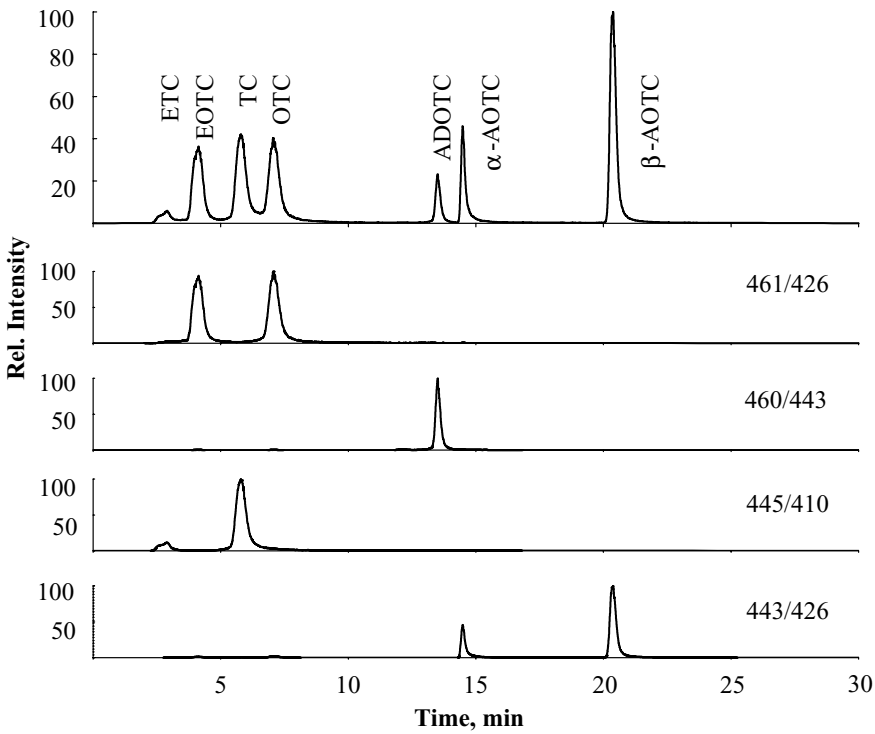

Fig. 2. LC-MS-MS chromatogram of standards of ETC, EOTC, TC, OTC, ADOTC, α-AOTC and β-AOTC using the scan mode selected reaction monitoring (SRM). The data are smoothed. The separation method is described under "Section 2".

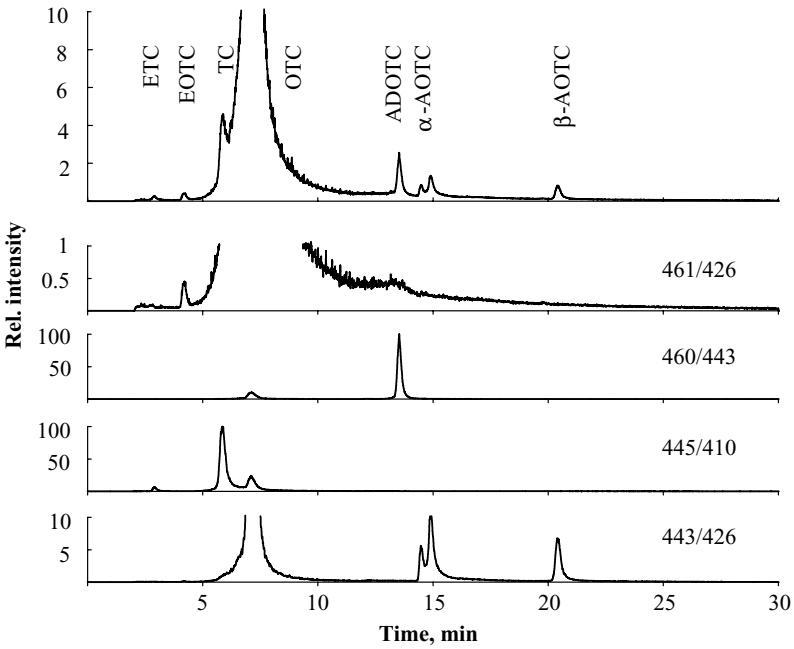

Fig. 3. LC-MS-MS chromatogram of an oxytetracycline-containing ointment using the scan mode SRM. The data are smoothed. The separation method is described under "Section 2".

the same propose [3]. This method did not separate ADOTC completely from OTC and the mobile phase contained a phosphate buffer, which is not compatible with MS. The advantage of the present method is the empty separation window right after the large peak corresponding to OTC in the chromatogram, which facilitates an accurate determination of ADOTC. The separation method is relatively fast with a total analysis time of 30 min and the selectivity is increased using MS-MS-detection, which reduces the need for laborious sample preparation. The combination of HPLC and MS-MS gives a reliable identification of the compounds compared to HPLC with only UV-detection.

3.3. Calibration curves and reproducibility

Calibration curves of OTC and its impurities were generated based on plotting peak-area versus the concentrations. Details of the calibration curves are listed in Table 2. All analytes show a linear response with acceptable correlation coefficients. Limits of quantification (LOQ) and limits of detection (LOD) are calculated from the standard deviations of the lowest standards.

For the quantification of OTC the product ion 461/444 was used, because the most abundant product ion 461/426 did not give a straight calibration curve

Table 2
Retention times and calibration curves of oxytetracycline and its impurities

	t_R	Slope ($\times 10^{-5}$)	R.S.D. (%)	Intercept ($\times 10^{-5}$)	R.S.D. (%)	r^2	Number of standards	Concentration range (mg/l)	LOQ	LOD (mg/l)
TC	4.0	15.196	0.57	−1.503	1.18	0.9993	24	0.095–9.5	0.07	0.02
EOTC	5.7	11.353	0.91	−0.250	8.45	0.9982	24	0.024–2.4	0.02	0.005
OTC	7.0	1.257	0.81	3.556	0.06	0.9986	24	4.8–480	2.60	0.78
ADOTC	13.4	4.013	0.34	2.838	0.99	0.9997	24	0.1–10	0.13	0.04
α-AOTC	14.4	1.917	1.80	0.244	3.10	0.9936	22	0.097–7.76	0.75	0.23
β-AOTC	20.4	4.132	2.10	−0.657	2.81	0.9913	22	0.097–7.76	0.47	0.14

Table 3
The intraday and interday precision and accuracy of the method for the determination of oxytetracycline and its impurities in the aqueous standards

Compound	Concentration (mg/l)	Mean found ± S.D. (mg/l)	Precision R.S.D. (%)	Accuracy (%)
Intraday, $n = 6$				
TC	0.95	0.90 ± 0.04	4.2	95
	7.60	7.63 ± 0.17	2.2	100
EOTC	0.24	0.24 ± 0.01	3.2	98
	1.94	1.91 ± 0.04	2.2	98
OTC	47.9	48.8 ± 0.3	0.7	102
	382.9	386.3 ± 7.9	2.1	101
ADOTC	1.00	1.06 ± 0.04	3.5	106
	8.00	8.02 ± 0.07	0.8	100
α-AOTC	0.97	1.04 ± 0.03	2.5	108
	7.76	7.78 ± 0.48	6.1	100
β-AOTC	0.97	0.89 ± 0.02	2.4	91
	7.76	7.86 ± 0.46	5.8	101
Interday, $n = 12$				
TC	0.95	0.92 ± 0.03	3.4	97
	7.60	7.60 ± 0.20	2.7	100
EOTC	0.24	0.24 ± 0.01	2.5	100
	1.94	1.92 ± 0.08	4.0	99
OTC	47.9	47.0 ± 2.0	4.2	98
	382.9	386.2 ± 5.6	1.4	101
ADOTC	1.00	1.03 ± 0.04	3.8	103
	8.00	8.05 ± 0.11	1.4	101
α-AOTC[a]	0.97	1.09 ± 0.06	5.4	112
	7.76	8.08 ± 0.57	7.1	104
β-AOTC	0.97	0.87 ± 0.03	3.2	90
	7.76	7.88 ± 0.38	4.8	102

[a] $n = 11$.

in the analysed concentration range. This resulted in very high LOQ and LOD of OTC compared to the other compounds. It was, however, necessary to analyse OTC in the concentration level used, to get sufficient high concentrations of the impurities.

The intraday and interday precision and accuracy of the aqueous standards are listed in Table 3. The intraday values were determined by measuring the compounds at two concentration levels, and the interday values were evaluated in the two concentration levels during 3 days. The values of the precision are in all cases lower than 7.1% and the intraday accuracy ranged from 91 to 108% and the interday accuracy varied from 90 to 112%. All the intraday and interday precision and accuracy values are considered acceptable.

3.4. Application of the developed method on commercial samples

The concentrations of OTC and its impurities in the two ointment samples and in the OTC standard are listed in Table 4. The concentrations of OTC in ointment A and B are 3.5 ± 0.7% and 4.6 ± 1.2% higher than declared, respectively. According to the Danish Standards of Drugs (DLS) the concentrations are only allowed to differ 5% from the declared concentration at the time of production. In the final product the lower

Table 4
Concentrations of OTC and its impurities in the ointments and in the OTC standard and the amount of the impurities expressed as percent of OTC

	Ointment A		Ointment B		OTC standard	
	Concentration ± S.D. (mg/g)	OTC (%)	Concentration ± S.D. (mg/g)	OTC (%)	Concentration ± S.D. (mg/l)	OTC (%)
OTC	31.1 ± 0.2	–	31.4 ± 0.3	–	386.3 ± 8.0	
TC	0.32 ± 0.008	1.00	0.34 ± 0.004	1.07	0.77 ± 0.04	1.99
EOTC	0.037 ± 0.005	0.12	0.038 ± 0.003	0.12	0.19 ± 0.002	0.48
ADOTC	0.50 ± 0.01	1.61	0.52 ± 0.006	1.65	5.15 ± 0.02	1.33
α-AOTC	1.02 ± 0.02	3.23	0.43 ± 0.10	1.35	<LOD	–
β-AOTC	0.26 ± 0.007	0.82	0.14 ± 0.05	0.45	2.17 ± 0.12	0.56

limit is extended to 10% of the declared concentration because of stability considerations [13]. The contents of the impurities TC, EOTC and ADOTC in both ointments fulfil the limitations stated in Ph.Eur. However, the content of α-AOTC and β-AOTC is too high in ointment A. The amounts of the impurities in the OTC standard analysed fulfil the limits of Ph.Eur., but the concentrations of TC and EOTC are very close to the upper limits. The contents of α-AOTC and β-AOTC are considerable lower in the OTC standard analysed than in the ointments.

4. Conclusion

The analysis method developed gives a robust separation of OTC and all of its impurities. It is the first method using MS-MS-detection to analyse OTC and its impurities, and the use of tandem mass spectrometry provides a very good selectivity. The analysis method gives a good quantification of the compounds, and it was shown that the method was applicable to ointments containing OTC.

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