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Evaluation of the stability of chlortetracycline in granular premixes by monitoring its conversion into degradation products

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Abstract

A methodology for the evaluation of the stability of chlortetracycline (CTC) in granular premixes is described. This methodology is based on the monitoring of the conversion of CTC into its degradation products by an improved gradient liquid chromatography (LC) method, based on one previously described by our laboratory. Sample preparation involves the extraction of CTC and its degradation products prior to LC analysis, using acidified methanol as extraction solvent. The gradient elution LC method proved to be very sensitive, especially towards the late eluting anhydro derivatives. The use of a Hypersil C8 BDS, 5 μm, 250 mm × 4.6 mm i.d. column is recommended since this column allowed a complete separation of the different impurities from each other and from the main component CTC. The applicability of this approach was demonstrated by the analysis of stability samples.
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Keywords: LC; Chlortetracycline; Premixes; Stability; Degradation products

1. Introduction

Tetracycline antibiotics such as chlortetracycline (CTC) are commonly veterinary therapeutic products to treat various infections. The CTC bulk sample used to prepare these products may contain various fermentation and degradation products. Moreover, during the manufacturing process and upon storage, degradation products can also be formed. The chemical structures of CTC and its related substances and degradation products are shown in Fig. 1. CTC, like other tetracyclines, undergoes epimerization at position C-4, forming 4-epichlortetracycline (ECTC) [1]. Due to the hydroxyl group at position C-6, CTC is liable to acid degradation forming anhydrochlortetracycline (ACTC) [2,3]. ACTC further epimerizes at position C-4 resulting in the formation of 4-epianhydrochlortetracycline (EACTC). CTC is not stable in alkaline medium [3]. Alkaline decomposition results in the formation of isochlortetracycline (IsoCTC), which further epimerizes to 4-epiisochlortetracycline (EIsoCTC) [1]. Some tetracycline (TC) and demethylchlortetracycline (DMCTC) may also be present in CTC samples [4]. Similar to CTC, TC undergoes epimerisation and acid degradation, with formation of 4-epitetracycline (ETC), anhydrotetracycline (ATC) and 4-epianhydrotetracycline (EATC). The degradation products show a different activity, toxicity and environmental profile compared to the parent compound [5]. EATC has for example been identified as an agent causing Fanconi-type syndrome [6]. To ensure the quality of veterinary medicated premixes containing CTC, a good analytical method is required. Previously, stability was often monitored by the microbiological potency or activity determinations, which are however not specific and generally overestimating the chemical stability [7].

Liquid chromatographic (LC) methods for the analysis of CTC have been described [8–12]. For most of these methods, the separation between IsoCTC, ECTC and CTC was not complete. The method described by Khan et al. [12] was the basis of the current LC method prescribed by the European Pharmacopoeia (Ph. Eur.) [13] for the analysis of CTC. This

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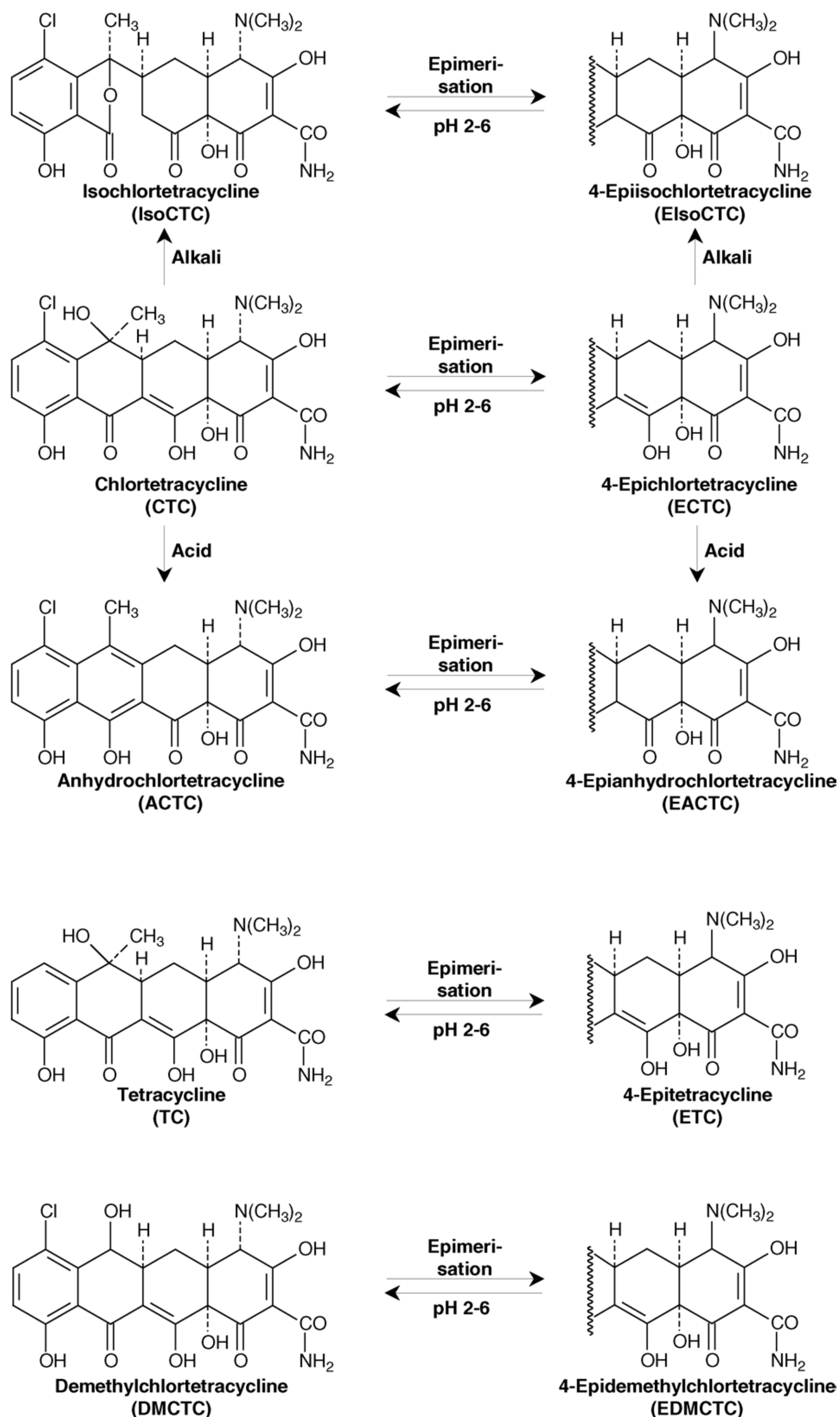

Fig. 1. Chemical structures of chlortetracycline and related substances.

method was also used as a basis for the method described in this paper. Thin layer chromatography (TLC) [14] and capillary electrophoresis (CE) [15,16] are alternative methods for the analysis of CTC.

To extract tetracyclines from animal feeds and premixes, an aqueous McBuffer is often used [10,17–21]. However, it has been reported that severe epimerization degradation of tetracyclines can occur in an aqueous McBuffer solution [21].

This work focuses on the evaluation of the stability of CTC in granular premixes. During pharmaceutical development, different formulations are put on stability in an early phase, while homogeneity and sampling procedures are not yet evaluated. Moreover, assay values are generally considered not sufficiently sensitive to detect degradation. Therefore, to obtain meaningful stability information, the methodology chosen is based on the monitoring of the conversion of CTC into its degradation products. Sample preparation involves the extraction of CTC and its related substances and degradation products, using acidified methanol as extraction solvent.

2. Experimental

2.1. Reagents

HPLC grade-acetonitrile and dimethyl sulfoxide (DMSO), analytical grade, were purchased from Acros Organics (Geel, Belgium). Perchloric acid, analytical grade, was from Ferak GMBH (Berlin, Germany). Methanol, HPLC grade, and concentrated hydrochloric acid, analytical grade, were purchased from Fisher Chemicals (Loughborough, Leics, UK). Water was purified in the laboratory by distillation of demineralised water.

2.2. Samples and reference substances

The samples analyzed are Aurofac® formulations, which are medicated granular premixes containing chlortetracycline hydrochloride (CTC·HCl) as active ingredient. These samples were made available by Alpharma (Antwerp, Belgium). General information about the Aurofac® formulations used in this study is given in Table 1. Reference substances of TC, ETC, ECTC, IsoCTC, ATC, EATC, ACTC and EACTC, were available from Acros Organics. DMCTC and 4-epidemethylchlortetracycline (EDMCTC) reference substances were available from the Ph. Eur. EIsoCTC was available in the laboratory. All these reference substances were hydrochloride salts. These reference substances were used for identification purpose, at a concentration of about 0.05 mg/ml. For quantitation purpose, CTC·HCl reference substance obtained from Alpharma was used. A 0.5 mg/ml solution of the CTC·HCl reference substance was prepared for the determination of the main compound CTC in Aurofac® samples. The samples to be analyzed were prepared at a final concentration of CTC·HCl corresponding to 0.5 mg/ml, calculated with regard to the label claimed content of CTC·HCl. In the final solutions to be analyzed, the solvent for the samples and the reference substances consisted of acidified methanol–water (1:1). The acidified methanol was prepared by mixing 99 volume-parts of methanol and 1 volume-part of 1 M HCl.

Table 1
Overview of the formulations used

Drug product name	Drug product formulation	
Aurofac® 10% CTC HCl	Chlortetracycline hydrochloride Inert carrier	10% qsp 100%
Aurofac® 25% CTC HCl	Chlortetracycline hydrochloride Inert carrier	25% qsp 100%
Aurofac® CTC 100[a]	Aurofac® 25% CTC HCl Vegetable diluent	43% qsp 100%

[a] Aurofac® CTC 100 is obtained by mixing 43% of Aurofac® 25% CTC HCl granule with an inert vegetable diluent, thus achieving a final concentration of 10% chlortetracycline.

2.3. Sample preparation

From the different laboratory samples, samples of 20 g were brought into an electrical grinder and mixed for 3 min in order to get homogeneous test samples. From these mixed test samples, a test portion of 1.000 g was accurately weighed into a volumetric flask and the extraction solvent (acidified methanol) was added up to volume. A 100 ml volumetric flask was used when the sample label claimed content of CTC·HCl was 10%, whereas a 250 ml volumetric flask was used when this content was 25%. This allowed to have, for all samples, a concentration of CTC·HCl corresponding to 1 mg/ml, calculated with regard to the label claimed content of CTC·HCl. The volumetric flask was wrapped with an aluminium foil. CTC was extracted by ultrasonication for 15 min in a ice cooled water bath. The extracts were filtered through 0.2 μm nylon filters (Alltech, Lokeren, Belgium). The filtrate was diluted with water (1:1) to obtain a concentration of CTC·HCl corresponding to 0.5 mg/ml. This dilution with water was necessary in order to achieve a good peak shape. The sample solutions were kept in a refrigerator until analysis. A refrigerated autosampler set at 4 °C was used to preserve the stability of these solutions during analysis.

2.4. Instrumentation and liquid chromatographic conditions

The LC apparatus consisted of an L-6200 Intelligent Pump (Merck Hitachi, Darmstadt, Germany), a TSP Spectra Series AS 100 autosampler (San Jose, CA, USA) equipped with a 20 μl loop, a variable wavelength Merck Hitachi L-4200 UV-VIS detector set at 280 nm and Chromeleon 6.50 software (Dionex Corporation, Sunnyvale, CA, USA) for data acquisition. For the experiments described in this paper, the columns (250 mm × 4.6 mm i.d., 5 μm) used were: Zorbax RX-C8 (Agilent Technologies, Waldbronn, Germany), XTerra RP18 (Waters, Milford, Massachusetts, USA), Hypersil C8 BDS (Thermo Electron Corporation, Runcorn, Cheshire, UK). The column temperature was maintained at 35 °C in a water bath heated by means of a Julabo EC thermostat (Julabo, Seelback, Germany).

In the final method, the mobile phases consisted of DMSO–1 M perchloric acid–water, (A) (450:50:500, v/v/v) and (B) (700:50:250, v/v/v). The mobile phases were

degassed by sparging helium. A gradient elution was performed at a flow rate of 1.0 ml/min. The gradient program was set as follows: 0–25 min, 0% of B (isocratic); 25–30 min, 0–40% of B (linear gradient); 30–43 min, 40% of B (isocratic); 43–45 min, 40–90% of B (linear gradient); 45–55 min, 90% of B (isocratic); 55–60 min, 90–0% of B (linear gradient); 60–75 min, 0% of B (isocratic).

An IKA electrical grinder from VWR (Leuven, Belgium) was used to pulverize the sample, prior to extraction. The mixer was cooled by passing a cooling liquid at 20 °C.

3. Results and discussion

3.1. Chromatography

Different LC methods were investigated for their suitability for the analysis of CTC and related substances. The LC method previously developed in our laboratory [12] and which was the basis for current method prescribed by the Ph. Eur. [11] for the analysis of CTC and related substances was selected as a starting point since it gave better selectivity for the compounds of interest. This method uses Zorbax octylsilyl silica gel (250 mm × 4.6 mm i.d.), 5 or 10 μm, as stationary phase, maintained at 35 °C. UV detection is performed at 280 nm. A mobile phase containing DMSO, perchloric acid and water is used at a flow rate of 1.0 ml/min. A typical isocratic chromatogram obtained with a commercial CTC bulk sample spiked with some related substances and the chromatographic conditions used are shown in Fig. 2. As can be seen, a baseline separation of the different compounds was obtained, except for the peak pair IsoCTC–ECTC and for this pair and the main peak CTC. The analysis takes more than 2 h. In order to speed up the elution of the strongly retained anhydro derivatives, the method was further combined with gradient elution, by increasing the amount of DMSO in the mobile phase. A typical chromatogram obtained by analyzing a commercial CTC sample spiked with some related substances and the gradient program applied are shown in Fig. 3. As can be seen, the analysis time was reduced and the sensitivity for the late eluting peaks was improved. Compared to the original method [12] where a different type of Zorbax material was used, an improved separation and a better peak shape were achieved with the method described here. However the separation of ECTC, IsoCTC and CTC was not yet optimum. In order to improve the separation, different C8 columns were investigated. XTerra RP-18 stationary phase was also investigated since this column was already used with success for the analysis of TC, oxytetracycline (OTC) and doxycycline (DOX) [22–24]. The use of this column also resulted in a good separation of CTC and its impurities; the separation is better than with the other columns investigated. A typical isocratic chromatogram is shown in Fig. 4. As can be seen, all the compounds of interest are well separated, including the critical separations IsoCTC–ECTC and the main peak. The peaks corresponding to EATC, ATC, EACTC and ACTC are not shown, as these compounds were not included in the sample analyzed. However, a gradual decrease of retention was observed in a very short period of use of the XTerra column, probably due to instability. This phenomenon was observed with several other columns as well.

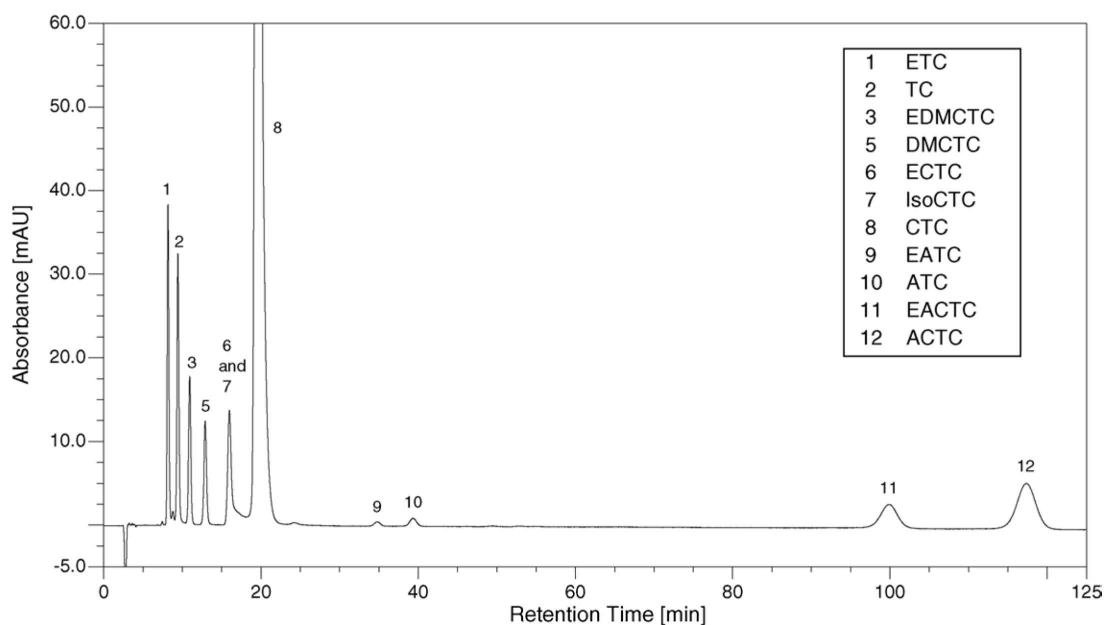

Fig. 2. Typical isocratic chromatogram of a 0.5 mg/ml commercial CTC bulk sample spiked with related substances, on a Zorbax RX-C8, 5 μm, (250 mm × 4.6 mm i.d.) column. Column temperature: 35 °C. Mobile phase: DMSO–1 M perchloric acid–water (500:50:450, v/v/v). UV detection: 280 nm. Flow rate: 1 ml/min. Injection volume: 20 μl.

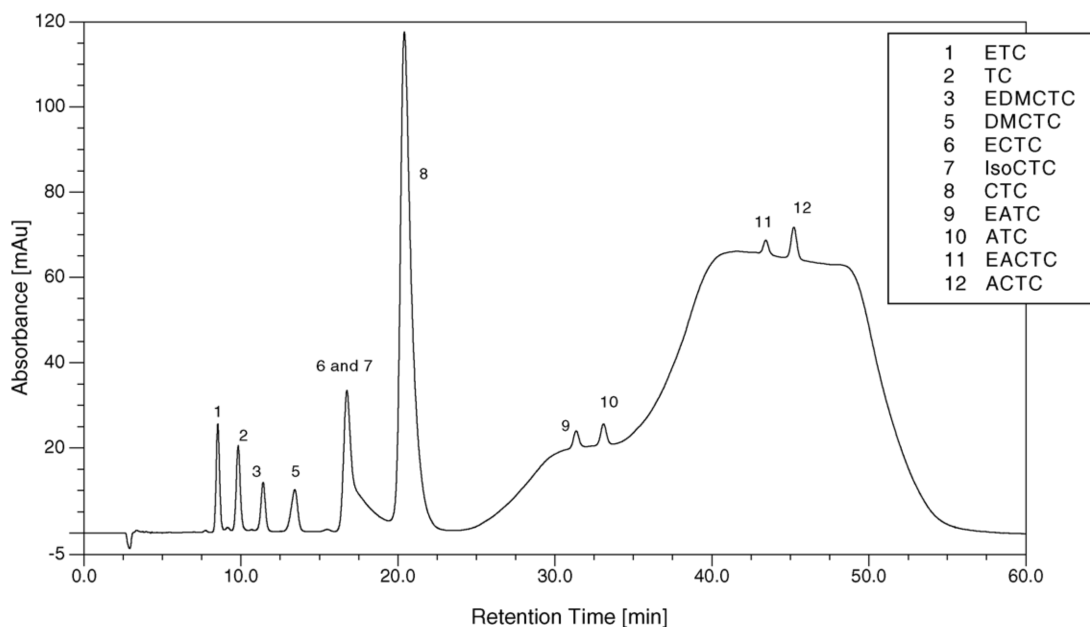

Fig. 3. Typical chromatogram of a 0.5 mg/ml commercial CTC bulk sample spiked with related substances on a Zorbax RX-C8 column, using gradient elution. The mobile phases consisted of DMSO–1 M perchloric acid–water (A) (450:50:500, v/v/v) and (B) (700:50:250, v/v/v). The gradient program was set as follows: 0–18 min, 0% of B (isocratic); 18–23 min, 0–40% of B (linear gradient); 23–28 min, 40% of B (isocratic); 28–33 min, 40–80% of B (linear gradient); 33–43 min, 80% of B (isocratic); 43–48 min, 80–0% of B (linear gradient); 48–60 min, 0% of B (isocratic). The other chromatographic conditions are as described in Fig. 2.

Further investigation of different columns led to an improved separation of ECTC, IsoCTC and CTC by using a Hypersil C8 BDS column. This stationary phase also showed good stability. Typical chromatograms obtained by analyzing a CTC bulk sample spiked with some of its related substances and an Aurofac® sample are shown in Fig. 5.

3.2. *Sensitivity limits for chlortetracycline and related substances*

The limits of quantitation (LOQ) for CTC and related substances were assessed by injecting solutions of the reference substances at various concentrations. Its determination was

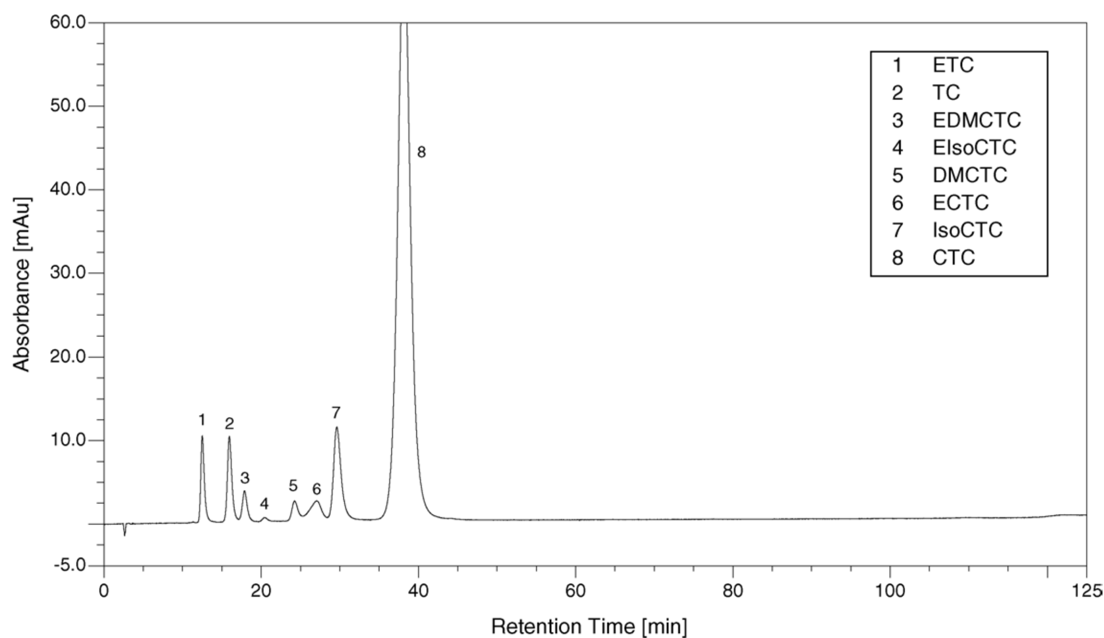

Fig. 4. Typical chromatogram of a 0.5 mg/ml commercial CTC bulk sample spiked with related substances. The chromatographic conditions are as described in Fig. 2 except that an XTerra RP18, 5 μm, (250 mm × 4.6 mm i.d.) column and a mobile phase consisting of DMSO–1 M perchloric acid–water (300:50:650, v/v/v) were used.

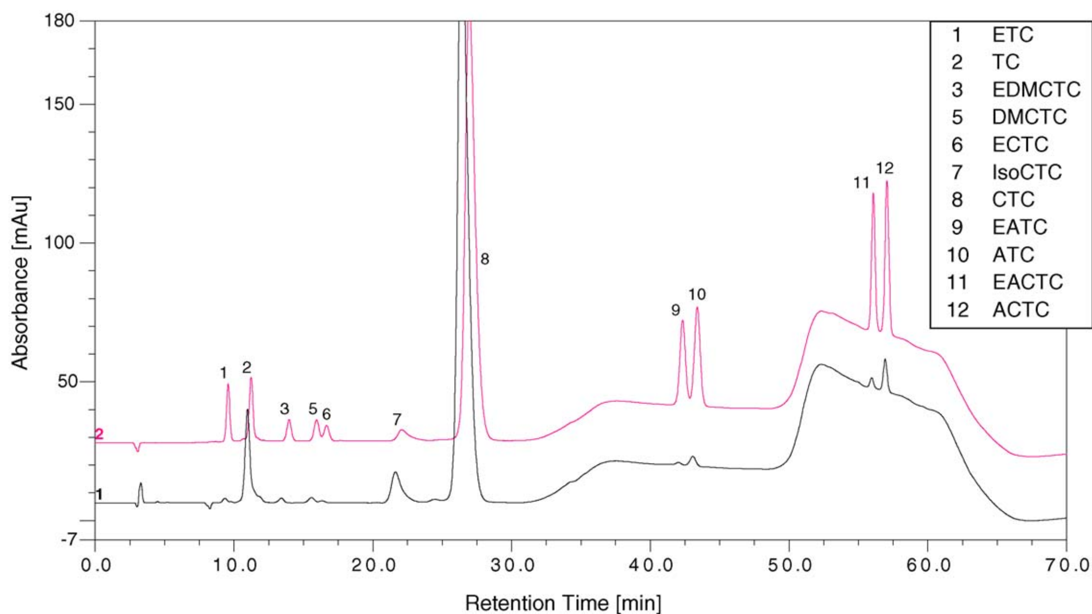

Fig. 5. Typical chromatograms obtained by analyzing (1) an Aurofac® CTC 100 T_6 (40 °C/75% RH) sample and (2) a 0.5 mg/ml commercial CTC bulk sample spiked with related substances. The Aurofac® sample was prepared as described in Section 2.3. Column: Hypersil C8 BDS, 5 μm, (250 mm × 4.6 mm i.d.). The chromatographic conditions were as described in Section 2.4.

based on a signal-to-noise ratio of 10. The results obtained, are summarized in Table 2. The percentages were calculated with respect to the CTC nominal value (0.5 mg/ml, corresponding to an amount of CTC on the column equal to 10 μg). As can be seen, the LOQ values for EATC, ATC, EACTC and ACTC are low, despite the late elution of these compounds. This is due to their higher relative response factor, as discussed below. In addition, the peaks were sharper and the sensitivity improved, as the amount of DMSO in the mobile phase increased with the gradient elution.

3.3. *Relative response factors for anhydrotetracycline and anhydrochlortetracycline*

It is often impractical or impossible to use reference standards to quantify each related substance in a sample. A common practice is to determine related substances relative to a diluted standard of the main component. Consideration of the response of the related substance relative to the main component is important when using this approach. A relative response factor, if significantly different from 1, can be applied to correct for differences in absorptivity at the detection wavelength in the mobile phase at elution. The anhydro derivatives of TC and CTC have higher UV absorption than the main component CTC under the applied experimental conditions. It was therefore decided to assess the relative response factors of these derivatives. This was done by analyzing a solution containing the same amounts of ATC, EATC, ACTC, EACTC and CTC. A response factor of 3 relative to CTC was obtained for all the anhydro derivatives. This relative response factor was therefore applied to correct for differences in absorptivity. No correction was required for the other derivatives for which a relative response factor of 1 was obtained.

3.4. *Evaluation of the precision and linearity of the method*

In order to evaluate the precision of the method, intra-day and inter-day assays were performed. From the Aurofac® CTC 100 stability sample, three test samples of 20 g were taken on 3 different days. Each test sample was mixed and

Table 2
Limits of quantitation for CTC and its related substances

	ETC	TC	EDMCTC	DMCTC	ECTC	IsoCTC	CTC	EATC	ATC	EACTC	ACTC
LOQ											
Percent (%)	0.029	0.031	0.032	0.042	0.064	0.052	0.054	0.023	0.023	0.024	0.024
Mass on column (ng)	2.9	3.1	3.2	4.2	6.4	5.2	5.4	2.3	2.3	2.4	2.4
R.S.D. (%) ($n = 6$)[a]	7.8	8.5	9.1	9.4	9.7	8.7	8.4	8.1	8.8	8.7	9.1

[a] For each compound, the R.S.D. values were calculated for six analyses of a solution corresponding to the LOQ.

Table 3
Results obtained analyzing Aurofac® CTC 100 (stability sample) sampled and mixed on 3 different days (samples I, II and III) using an electrical mixer

Sample	Day of analysis	Mean% recovery for 1 extraction[a] (R.S.D. for three analyses)			Mean% recovery for three extractions* (R.S.D. for nine analyses)	Inter-day R.S.D. (18 analyses)
Sample I	Day 1	137.8; (0.5)	136.4; (0.2)	135.4 (0.1)	136.5 (0.8)	1.5
	Day 2	140.5; (0.8)	136.6; (1.3)	134.7 (0.7)	137.3 (1.9)	
Sample II	Day 1	102.4; (0.3)	101.9; (0.2)	100.9 (0.3)	101.7 (0.7)	1.0
	Day 2	100.9; (0.1)	103.0; (0.3)	100.4 (0.2)	101.4 (1.2)	
Sample III	Day 1	117.2; (0.4)	115.6; (0.4)	115.4 (0.6)	116.1 (0.9)	0.8
	Day 2	117.6; (0.1)	116.1; (1.1)	117.2 (0.1)	116.9 (0.8)	

Each sample was analyzed on 2 different days.

[a] The different means were compared by analysis of variance (ANOVA). The results indicated a significant difference ($\alpha = 0.05$) between means from different samples, while no significant difference was observed between means from different extractions performed on the same sample.

regarded as a distinct sample from which CTC was extracted and analyzed on two different days. Each day, the extraction procedure was repeated three times for each sample and each extract was injected three times. The results obtained are summarized in Table 3. As can be seen, different recoveries of CTC were obtained for the different samples. However, for each sample, the variability of the results obtained for three extractions on the same day (nine analyses), is low. This demonstrates the good repeatability of the extraction procedure and the chromatographic procedure. The good precision of the method is also supported by the low variability observed for the inter-day precision. The high variability of the CTC assay results for the different samples is a typical example of sampling issue, where the conversion of the received laboratory sample to the test sample is not yet under control. From the test sample, 1 g test portions are weighed to obtain the solution for HPLC analysis, which is clearly under control. Although the Aurofac® sample was always shaken manually before sampling, good homogeneity of the different test samples was apparently not achieved. It was therefore decided to monitor the conversion of CTC into its degradation products using a normalisation procedure, awaiting further investigations on the sampling procedure.

The linearity of the chromatographic procedure was checked by analysing a CTC reference substance in the concentration range 0.054–125% (100% corresponds to 10 μg injected). Six different concentrations were prepared and each concentration was injected three times. The following results were found: $y = 1425x - 1441$; $r = 0.9996$ and $S_{y,x} = 2104$, where y = peak area; x = concentration in per cent; r = coefficient of correlation and $S_{y,x}$ = standard error of estimate. The results indicate that the method is linear.

Table 4
Summary of the normalized contents obtained for different batches of the Aurofac® samples analyzed

Set	Sample	Stress conditions	Normalized content	
			CTC[a]	Total impurities
Set I	Aurofac® CTC 100	No stress (T_0)	88.8 (0.6)	11.2
	Aurofac® CTC 100	6 months at 40 °C/75% RH (T_6)	87.5 (0.3)	12.5
Set II	Aurofac® 10% CTC HCl	No stress (T_0)	88.3 (1.1)	11.7
	Aurofac® 10% CTC HCl	12 months at 40 °C/75% RH (T_{12})	85.9 (0.3)	14.1
Set III	Aurofac® 10% CTC HCl	No stress (T_0)	88.5 (0.7)	11.5
	Aurofac® 10% CTC HCl	3 months at 25 °C/60% RH (T_3)	84.9 (0.4)	15.1
	Aurofac® 10% CTC HCl	6 months at 40 °C/75% RH (T_6)	85.5 (1.0)	14.5
	Aurofac® 10% CTC HCl	12 months at 40 °C/75% RH (T_{12})	84.8 (0.4)	15.2
Set IV	Aurofac® 10% CTC HCl	No stress (T_0)	88.9 (0.8)	11.1
	Aurofac® 10% CTC HCl	12 months at 40 °C/75% RH (T_{12})	85.1 (0.4)	14.9
Set V	Aurofac® 25% CTC HCl	No stress (T_0)	88.7 (0.7)	11.3
	Aurofac® 25% CTC HCl	1 month at 25 °C/60% RH (T_1)	88.2 (0.5)	11.8
	Aurofac® 25% CTC HCl	6 months at 40 °C/75% RH (T_6)	87.5 (0.7)	12.5

[a] The relative standard deviation (R.S.D.) values are given in parentheses. Number of analyses = 9.

3.5. Stability study of different Aurofac® samples

Using the conditions described above, the stability of a set of five Aurofac® samples was assessed by monitoring the conversion of CTC into its degradation products. For each sample, three different extractions were performed and each extract was analyzed three times. The results obtained were normalized. This was done by expressing the contents of individual compounds as a percentage of the overall content of CTC and its impurities. This normalization covering all possible related impurities allows to study the stability of CTC in different formulations without the need of having homogeneous analytical samples. The normalized contents obtained for the main compound CTC and for the total of related impurities are summarized in Table 4. Compared to the initial contents, the CTC content slightly decreases in function of time and stress, while the contents of almost all the individual degradants detected increased accordingly (data not shown). The major impurity TC also decreased with the stress conditions; it was converted into its degradation products, namely ETC, ATC and EATC. It is concluded that under the conditions examined including the accelerated VICH storage condition of 40 °C/75% RH for 12 months, the decrease of CTC content in the examined granular formulations remains below 5%, which is considered as a pharmaceutically not significant loss. Therefore, these formulations are considered as stable.

4. Conclusion

The stability of CTC in granular premixes was assessed by monitoring its conversion into degradation products, by gradient LC. A normalization procedure allows studying the stability without the need of having strictly homogeneous samples or of having a validated sampling procedure already in place.

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