Determination of tetracycline and its major degradation products by chemiluminescence

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Abstract

A fast and simple flow injection chemiluminometric method for the determination of trace amounts of tetracycline (TC) and its major degradation products epitetracycline (ETC), epianhydrotetracycline (EATC) and anhydrotetracycline (ATC) based upon the action of potassium hexacyanoferrate(III) in alkaline solution has been developed. The analytes can be determined with 10σ limits of quantification of $0.5\,\mu g$ for ETC, $2.0\,\mu g$ for TC, $0.01\,\mu g$ for EATC and $0.04\,\mu g$ for ATC. The sensitivity for TC is greatly improved by acidic degradation prior to the chemiluminescence (CL) measurement. The method developed allows measurement within the ranges of $0.040–2.0\,\mu g$ TC and ATC, $0.50–5.0\,\mu g$ ETC and $0.01–2.0\,\mu g$ EATC. ©2000 Elsevier Science B.V. All rights reserved.

1. Introduction

Tetracycline (TC) (Fig. 1) is a broad-spectrum antibiotic used for medical purposes as well as in animal husbandry either as growth promoters in subtherapeutic doses, prophylactically for disease prevention or therapeutically for treatment of infections [1]. Epitetracycline (ETC), epianhydrotetracycline (EATC) and anhydrotetracycline (ATC) (Fig. 1) may be present in TC as impurities [2]. These compounds may form during storage under adverse conditions of temperature and humidity [3]. Anhydro derivatives may also be found in out-of-date samples of TC [4]. These com-

pounds are either inactive as antibiotics or toxic. The ingestion of degraded TC was reported to cause a reversible Fanconi-type syndrome [5]. Hence, the permitted concentrations of these impurities in pharmaceutical preparations fixed by the European Pharmacopoeia are 0.5% ETC and 0.05% EATC and ATC [6]. Therefore, it is very important to develop sensitive analytical methods for the measurement of degradation products in TC samples.

A number of spectrophotometric and fluorimetric methods have already been used successfully for the determination of TC and its major degradation products [7–9]. Several liquid chromatographic methods have been described [10–13] and UV detection is used by the European Pharmacopoeia [6].

The sensitivity of chemiluminescence (CL) has attracted attention for the development of analytical

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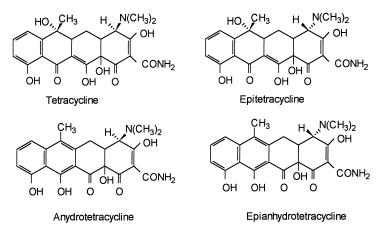*E-mail address:* calokerinos@chem.uoa.gr (A.C. Calokerinos).

Fig. 1. Structures of TC, ETC, ATC and EATC.

methods for TCs. Owa et al. have used peroxodisulphate for the oxidation of TC before mixing with 1,3-dibromo-5,5-dimethylhydantoin to initiate the CL reaction [14]. TC, oxytetracycline, doxycycline, chlortetracycline and demeclocycline have been determined in pharmaceutical preparations and honey samples by their chemiluminogenic reaction with N-bromosuccinimide [15]. TC, chlortetracycline and oxytetracycline have been determined in pharmaceutical preparations by the CL reaction with hydrogen peroxide catalysed by copper(II) ions [16]. The direct action of bromine [17] or potassium permanganate [18] on TC have also been reported to give CL. The action of lucigenin or hexacyanoferrate(III) after alkaline or acidic degradation of TCs, respectively, has also been found to exhibit CL [19]. Nevertheless, the CL behaviour of ETC, EATC and ATC, which are degradation products of TC, has never been reported.

In this paper, the CL behavior of TC and its major degradation products was studied in order to investigate whether the method can be used for screening the very low concentrations of degradation products allowed to be present in various samples.

2. Experimental

2.1. Reagents

All chemical reagents used were of analytical grade, were obtained from Merck (Darmstadt, Germany), unless otherwise stated and were diluted with distilled deionised water. Water was purified by distillation and passed through a Milli-Q System (Millipore, Bedford, MA).

A stock solution of 0.0500 M hexacyanoferrate(III) was prepared by dissolving 8.23 g of potassium hexacyanoferrate(III) in 0.5 M sodium hydroxide and diluting with the same alkaline solution to 500 ml.

All solvents used for liquid chromatography (LC) analysis were of LC grade and were purchased from Carlo Erba (Milan, Italy). The solvents and the water used were filtered through a 0.2 μm filter under vaccum and degassed by ultrasonication. The mobile phase consisted of 8.5% t-butanol, 10% (v/v) 0.20 M potassium hydrogenphosphate buffer, pH 9.0, 15% (v/v) of 0.020 M tetrabutylammonium hydrogensulphate and 10% (v/v) 0.010 M ethylenediaminetetraacetic acid sodium salt. The pH of the final solution was adjusted to 9.0 with sodium hydroxide solution.

TC was obtained from Sigma Chemical (Madrid, Spain). ETC, EATC and ATC were purchased from the European Pharmacopoeia (Strasbourg, France).

3. Preparation of standard solutions

Methanol was chosen as solvent for the TCs studied, due to its ability to dissolve the analytes, to mix with water and organic solvents and retard the degradation of TC [20]. Stock solutions of TC, ETC, EATC and ATC were prepared in methanol and were stored at −20°C in brown glass vials for a maximum period of

1 month. Working solutions were prepared daily by dilution of the stock solutions with methanol or water and kept in brown glass vials.

4. Acidic treatment of TC

100–1000 μg of TC was mixed with 1.0 ml of 1.0 M hydrochloric acid, diluted with water to ca. 20 ml and allowed to stand at 80°C for 30 min. The solution was cooled, transferred to a 100 ml volumetric flask and diluted with water.

5. Apparatus

5.1. Batch and flow injection chemiluminometer

The batch and flow injection (FI) manifolds were similar to those used previously [21,22]. The batch chemiluminometer consists of a 20 ml reaction cell in front of a photomultiplier tube (PMT) inside a light-tight housing. The operational voltage of the photocathode is −750 V. The solutions introduced into the cell are stirred continuously by means of a PTFE-coated magnetic bar. With the use of a shutter to shield the photocathode from ambient light, it is possible to wash the reaction cell while the PMT is still subjected to high voltage.

The FI analyser consists of a detector housing (THORN EMI S-MK2) and a flow-through system. The detector housing includes a coiled glass flow cell in front of the PMT (THORN EMI 9783R, S-5 response). The coil consists of 3.5 turns of glass tubing (2 mm i.d.) with total length and diameter 90 and 22 mm, respectively.

A peristaltic pump (Gilson Minipuls 3) pumps all necessary solutions at their optimum flow rates through PTFE flow tubes. The sample is injected into the carrier stream by a 500 μl loop of a low-pressure PTFE injection valve operated by a Rheodyne model 5701 pneumatic actuator controlled by two three-way solenoid air valves.

5.2. LC system

The LC system used was a Waters Associates liquid chromatograph 600 E with an auxiliary pump and solvent programmer connected to a Waters 991 photodiode array detector E Model 991 (Waters MA). The wavelengths employed were 360 nm for TC and ETC and 400 nm for EATC and ATC.

The column used was a PLRP-S column (250 mm × 4.6 mm i.d., 8 mm, 100 Å, Polymer Laboratories, Church Stretton, UK) and the results were acquired by a personal computer.

6. Results and discussion

6.1. Preliminary studies

The investigation of the chemiluminogenic behaviour of TC, ETC, EATC and ATC was carried out by using the batch chemiluminometer. No emission was recorded by mixing TC with an alkaline solution of hydrogen peroxide. When TC was mixed with potassium permanganate in polyphosphoric acid, a chemiluminescent glow was recorded. The intensity of this fast emission increased when the concentration of the oxidant was increased from 0.0010 to 0.10 M. Nevertheless, the blank emission due to the methanolic content of the solution was very high and the radiation could not be used analytically. The previously described reaction [19] of potassium hexacyanoferrate(III) with TCs in alkaline solution was found to be very sensitive without any effect of the presence of methanol. Hence, working solutions can be diluted either with methanol or with water without any effect on the emission intensity.

6.2. Optimisation of experimental parameters

The emission intensity depends on the concentration of potassium hexacyanoferrate(III) and sodium hydroxide. The effect of oxidant and alkali concentrations on the emission intensity from 0.200 and 0.400 μg of TC is shown in Figs. 2 and 3, respectively. Potassium hexacyanoferrate(III), 0.0500 M, prepared in 0.5 M NaOH was used for all further studies.

The optimised concentrations of oxidant and alkali were applied to the FI system which allows better repeatability and a shorter time of analysis. The flow rate of the alkaline oxidant was 2.0 ml min^{-1}.
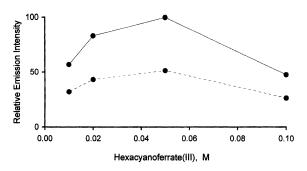
Fig. 2. Effect of concentration of hexacyanoferrate(III) on the relative emission intensity from 0.200 (dashed line) and 0.400 (solid line) μg of acid treated TC.
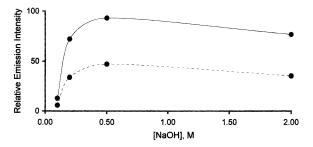

Fig. 3. Effect of concentration of sodium hydroxide on the relative emission intensity from 0.200 (dashed line) and 0.400 (solid line) μg of acid-treated TC.

6.3. Control of acidic treatment of TC

The emission intensity of TC was found to increase by about 100% when the compound was hydrolysed in acid prior to the CL measurement. TC epimerises at C4 to 4-ETC in weak acid conditions (pH 3–4.5). ATC may also be formed by thermal degradation of TC at pH <2. The formation of ATC and EATC by acid hydrolysis of TC at 80°C was confirmed by LC (Fig. 4).

6.4. Validation of the proposed method

The limits of quantification, linearity and precision of the method for the four compounds in study were evaluated. The analytical parameters for each TC ex-

amined by using batch and FIA CL are summarised in Tables 1 and 2 , respectively.

Within-day precision was determined by analysing five standard solutions at three levels (0.50, 1.00 and 2.00 μg injected) and was expressed as relative standard deviation (RSD). By using the batch chemiluminometer, the relative standard deviations for ETC, TC, EATC and ATC were 2.3, 4.1, 0.9 and 1.0% ($n = 5$), respectively. By using the FI chemiluminometer, the relative standard deviations were in the range 1.0–1.5 % ($n = 5$).

7. Proposed mechanism

Various mechanisms have been proposed for the chemiluminogenic reaction of TCs. Pre-oxidation of
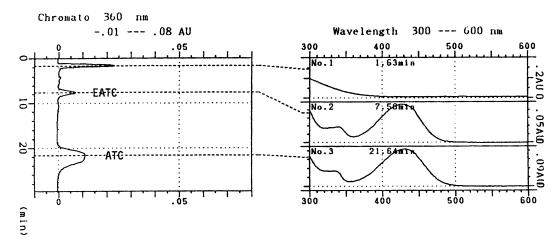

Fig. 4. Typical liquid chromatogram of TC after acid hydrolysis.

Table 1
Analytical characteristics for TC, ETC, EATC and ATC by using the batch chemiluminometer

Compound	LQ[a] (μg injected)	Linear range (μg injected)	SD of slope[c]	Regression equation[d]	r ($n = 5$)
ETC	0.50	0.50–5.00	0.11	$I = 2.14 + 21.8C$	0.9999
TC[b]	0.04	0.05–2.00	1.64	$I = 0.305 + 85C$	0.9992
EATC	0.01	0.01–2.00	6.35	$I = 3.23 + 238C$	0.9990
ATC	0.04	0.02–2.00	0.28	$I = 0.281 + 32.4C$	0.9998

[a] Limit of quantification (10σ).
[b] After acid hydrolysis.
[c] SD: standard deviation ($n = 5$).
[d] I: intensity, C: μg injected.

Table 2
Analytical characteristics for TC, ETC, EATC and ATC by using the flow injection chemiluminometer

Compound	LQ[a] (μg injected)	Linear range (μg injected)	SD of slope	Regression equation	r ($n = 5$)
ETC	0.500	0.500–5.00	0.11	$I = 0.258 + 7.3C$	0.9994
TC[b]	0.040	0.050–2.00	1.64	$I = 0.305 + 85C$	0.9993
EATC	0.010	0.010–2.00	2.94	$I = 0.177 + 110C$	0.9990
ATC	0.040	0.020–2.00	0.86	$I = 0.698 + 70C$	0.9997

[a] Limit of quantification (10σ).
[b] After acid hydrolysis.

TC with hydrogen peroxide and peroxodisulphate requires standing for 35 min before maximum emission in the presence of copper(II) as catalyst in an ammoniacal medium [16]. This was attributed to the conversion of TC to ATC which then chemiluminesces strongly due to breakage of the TC skeleton. Previous work [19] on the CL reaction of TCs with hexacyanoferrate(III) after acid hydrolysis has proved that a methyl group on C6 should be present to allow measurement of the corresponding compound and if a hydroxyl group is also present, then it should be removed prior to the chemiluminogenic reaction to allow maximum intensity to be generated. These results are further verified in this work. ATC and EATC react within seconds to generate strong CL by the action of hexacyanoferrate(III). Most probably, the reaction proceeds through a quinoidal intermediate which chemiluminesces before breaking down into various products.

The weak CL from TC is greatly increased by conversion to ATC. Dehydration of TC at C6 occurs in aqueous solutions at pH <2 [4] due to the presence of the hydroxyl group [23]. LC studies (Fig. 4) confirmed the formation of ATC from TC, but EATC is also present. This might be due to the presence of ETC in TC either as an impurity or due to epimerisation.

Nevertheless, ETC also degrades to EATC in acidic conditions [24].

8. Conclusions

The preliminary investigation of the CL determination of TC and its degradation products described in this work shows that the limits of quantification and sensitivity are within legal requirements and that the procedure should be further evaluated. The CL reaction seems suitable for measurement of TC and degradation products after liquid chromatographic separation since methanol, which is also used as elution solvent [25], does not interfere.

References

[1] W.H.H. Farrington, J. Tarbin, G. Shearer, Food Addit. Contam. 8 (1991) 55.
[2] J.R.D. McCormick, S.M. Fox, L.L. Smith, B.A. Bitler, J. Reichenthal, V.E. Origoni, W.H. Muller, R. Winterbottom, A.P. Doerschuk, JACS 79 (1957) 2849.
[3] R.F. Lindawer, D.M. Cohen, K.P. Munnely, Anal. Chem. 48 (1976) 731.

[4] A. Yasin, T.M. Jefferies, J. Pharm. Biomed. Anal. 6 (1988) 867.

[5] A. Azalos, Chromatographia 10 (1985) 313.

[6] Council of Europe, European Pharmacopoeia Secretariat, European Pharmacopoeia, 3rd Edition, 1997, Strasbourg, France.

[7] H. Oka, Y. Ikai, N. Kawamura, M. Yamada, K.I. Harada, S. Iko, M. Suzuki, J. Agric. Food Chem. 37 (1989) 226.

[8] P.P. Ascione, G.P. Chrekian, J. Pharm. Sci. 59 (1970) 1480.

[9] D. Hall, J. Pharm. Pharmacol. 28 (1976) 420.

[10] C. Hendrix, E. Roets, J. Crommen, J. De Beer, E. Porqueras, V.D. Bossche, J. Hoogmartens, J. Liq. Chromatogr. 16 (1993) 3321.

[11] P.D. Bryan, J.T. Stewart, J. Pharm. Biomed. Anal. 12 (1994) 675.

[12] H. Oka, J. Patterson, in: H. Oka, H. Nakazawa, K. Harada, J.D. MacNeil (Eds.), Chemical Analysis for Antibiotics Used in Agriculture, AOAC, Arlington, VA, 1995.

[13] N.H. Khan, P. Wera, E. Roets, J. Hoogmartens, J. Liq. Chromatogr. 13 (1990) 1351.

[14] T. Owa, T. Masujima, H. Yoshida, H. Imai, Bunseki Kagaku 33 (1984) 568.

[15] S.A.A. Halvatzis, M.M. Timotheou-Potamia, A.C. Calokerinos, Analyst 118 (1993) 633.

[16] X.R. Zhang, W.R.G. Baeyens, A. Van Den Borre, G. Van Der Weken, A.C. Calokerinos, S.G. Schulman, Analyst 120 (1995) 463.

[17] A.A. Al-Warthan, A. Townshend, Anal. Chim. Acta 205 (1988) 261.

[18] Z. Li, M. Feng, J. Lu, Z. Gong, H. Jiang, Anal. Lett. 30 (1997) 797.

[19] A.B. Syropoulos, A.C. Calokerinos, Anal. Chim. Acta 255 (1991) 403.

[20] G.D. Mack, R.B. Ashworth, J. Chromatogr. 16 (1978) 93.

[21] L.P. Palilis, A.C. Calokerinos, N. Grekas, Anal. Chim. Acta 333 (1996) 267.

[22] N.T. Deftereos, A.C. Calokerinos, C.E. Efstathiou, Analyst 118 (1993) 627.

[23] J.R.D. McCormick, E.R. Jensen, P.A. Miller, A.P. Doerrschuk, JACS 82 (1960) 3381.

[24] B.A. Hoener, T.D. Sokoloski, L.A. Mitscher, L. Malspeis, J. Pharm. Sci. 63 (1974) 1901.

[25] H. Oka, K. Uno, K. Harada, K. Yasada, M. Suzuki, J. Chromatogr. 298 (1984) 435.