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# Development and validation of a sensitive method for tetracycline in gingival crevicular fluid by HPLC using fluorescence detection

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**Abstract**

Increased interest in the clinical use of antibiotics for periodontal therapy required the development of a sensitive assay for the quantitation of tetracycline in gingival crevicular fluid (GCF). An HPLC method was developed and validated for tetracycline which separates and identifies the degradation component epi-tetracycline. The HPLC assay employs a C18 reversed-phase Hypersil column with a mobile phase composed of methanol and sodium acetate buffer containing $CaCl_2$ and EDTA disodium salt. The chromatographic separation was monitored by a fluorescent detector with an excitation wavelength of 375 nm and an emission wavelength of 512 nm. Tetracycline was extracted from GCF collected on Periopapers by addition of a methanol solution containing the internal standard, doxycycline, and the mobile phase buffer (25:75, v/v). The mean percent recovery for the extraction method was 107.8% with all the %R.S.D. below 7.5%. The mean inter- and intra-batch accuracy was 104.1 and 105.3%, respectively with a coefficient of variation of less than 9.5%. The lower limit of detection was 2.5 ng on the Periopapers. The typical GCF volumes collected were 0.1–1 μl. The method was validated for the linear concentration range 2.5–1000 ng of tetracycline on the Periopaper. This assay for tetracycline was shown to be an accurate, precise and rugged method. 1997 Published by Elsevier Science B.V.

*Keywords:* Tetracycline; Gingival crevicular fluid; Fluorescence detection; HPLC

## 1. Introduction

Actisite® is a ethylene vinyl acetate fiber which contains approximately 13 mg of tetracycline (TC) per 23 cm of fiber [1]. Therapeutically, Actisite® is used to treat periodontal disease by placing the fiber in the periodontal pocket at the site of disease. It releases TC at a constant rate into the surrounding gingival crevicular fluid (GCF) in the periodontal pocket during a 10-day treatment period [1]. Samples of GCF are collected from the site using small filter-like papers called Periopapers (Fig. 1) [1–4]. GCF is expressed from the gingival pocket and was collected at the intersection of the tooth and the gingival tissue [5]. Due to the low flow rate of GCF, limited volumes are obtainable (0.1–1 μl)

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Fig. 1. Diagram of a Periopaper (actual size 1.4 cm × 0.2 cm). Dotted area = plastic handle held by tweezers; blank area below line = filter paper area used for GCF collection. Once GCF is collected the Periopaper is cut into a tube on or below the black line so that only the blank area is extracted.

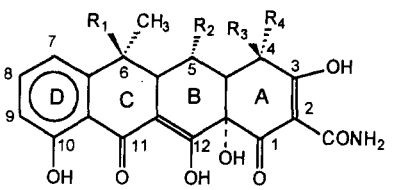


Fig. 2. Structure of TC, ETC and doxycycline. For TC, $R_1 = OH$, $R_2 = H$, $R_3 = H$, $R_4 = N(CH_3)_2$; for doxycycline, $R_1 = H$, $R_2 = OH$, $R_3 = H$, $R_4 = N(CH_3)_2$; for ETC, $R_1 = OH$, $R_2 = H$, $R_3 = N(CH_3)_2$, $R_4 = H$.

thus assays measuring levels of drugs in this fluid must have a greater sensitivity than assays measuring drugs in serum or plasma.

Typically, the amount of TC at the site in GCF is measured using a Bacillus microbiological method which is linear from 10–750 ng of TC on the Periopaper [1]. Microbiological methods lack sensitivity and require 37°C incubation [6–8] which promote degradation of TC to epi-tetracycline (ETC). There are numerous HPLC UV methods for TC in biological fluids [9–15] but most lack the sensitivity required for this application. The few that do have the sensitivity also have lengthy extraction procedures and do not separate ETC [16]. Fluorescence detection methods offer increased sensitivity and reduced interferences that the UV methods lack [6,17–19]. TC demonstrates very little native fluorescence, however, an increased response is achieved by the chelation of TC to divalent or trivalent cations [6].

An HPLC fluorescent method was developed and validated for the analysis of TC in GCF with a limit of quantitation (LOQ) of 2.5 ng of TC on the Periopaper (12.5 ng $ml^{-1}$ in extraction solvent) and excellent precision and accuracy. Separation and identification of ETC was an important part of the method since ETC exhibits less than 5% of the antimicrobial activity of the parent TC [20]. Fig. 2 shows the structure of TC, ETC and the internal standard doxycycline. Demonstration that $CaCl_2$ and EDTA disodium salt (EDTA) both enhance the fluorescence of TC and ETC is presented along with clinical data produced using this method.

## 2. Experimental

### 2.1. Materials and reagents

Methanol, sodium acetate trihydrate, EDTA disodium, $CaCl_2$ dihydrate and 50% (w/w) NaOH solution were supplied by J.T. Baker (HPLC or ACS grade) and used for the mobile phase preparation. Standard solutions were prepared using TC–HCl and doxycycline hyclate USP reference standards. Drug free human serum was purchased from Valley Biomedical (Winchester, VA). Additionally 4-epi-tetracycline, anhydrotetracycline and 4-epi-anhydrotetracycline were purchased from Janssen Chemical and used for peak identification. Periopapers and the Periotron used for quality control (QC) preparation, standard preparation and clinical sample collection were purchased from I.D.E. Interstate Dental Supply (Amityville, NY). Drummond Microcaps (1 μl) used to spike the standard and QC solutions were purchased from VWR Scientific.

### 2.2. Apparatus

The HPLC system was comprised of a Hewlett Packard HP1050 equipped with a Hitachi model L7480 fluorescence detector. The column was an Alltech Altima, C18, 250 mm × 4.6 mm, 5 μm column proceeded by a Brownlee New Guard, RP-18, 7 μm, 15 × 3.2 mm guard column. All biological sample preparations were done in a biosafety hood, Sterilchem GARD class II, type B2, The Baker Company.

### 2.3. Solution preparation

#### 2.3.1. Preparation of the internal standard solution

A 4.0 μg ml$^{-1}$ doxycycline hyclate USP reference standard solution was accurately prepared in HPLC grade methanol.

#### 2.3.2. Standard solution preparation

*2.3.2.1. TC–HCl stock solution (TC stock).* A 20 mg ml$^{-1}$ USP TC–HCl reference standard solution was accurately prepared in HPLC grade methanol.

*2.3.2.2. Working standard curve.* Working standard curve solutions at the following concentrations; 50, 100, 200, 500, 2000, 10 000 and 20 000 μg ml$^{-1}$ were prepared from the TC stock in methanol.

*2.3.2.3. Serum standard curve.* Each working standard solution was diluted by a factor of 20 with serum into a 1.5 ml microcentrifuge tube to obtain the following concentrations; 2.5, 5.0, 10, 25, 100, 500 and 1000 μg ml$^{-1}$. The serum standard solutions sat at room temperature for 3 h before preparation of the final standard curve on Periopapers. GCF was not used to prepare standards or QC samples because it was not commercially available nor easily collectible in large volumes.

*2.3.2.4. Preparation of the final standard curve on Periopapers.* The bottom tip of a Periopaper (Fig. 1) was placed into a 1.5 ml microcentrifuge tube and 1 μl of each serum standard was spiked on the Periopaper using a 1 μl Drummond Microcap. This spiking process was repeated in triplicate for each serum standard.

*2.3.2.5. Quality control (QC) samples.* QC samples were made as described for the serum standard curve solutions starting with a different TC stock of the same concentration. The following four concentrations of QC samples were prepared, 2.5 ng (QC1), 4.998 ng (QC2), 499.8 ng (QC3) and 749.6 ng (QC4). The QC spiked samples were prepared in advance in bulk for storage at −70°C for use in method validation and clinical sample analysis batches.

#### 2.3.3. Clinical sample collection

Samples were collected from patients with periodontal disease using Periopapers. Extreme care was taken to minimize GCF contamination with tooth surface debris such as supragingival plaque and saliva. To minimize this the tooth surface above the periodontal pocket collection site was wiped with a cotton swab to remove any debris. The Periopaper was held with tweezers at the intersection of the tooth and the gingival tissue at an angle that would minimize contact with the tooth surface and gingival mucosa. The GCF fluid flowed from the gingival pocket up the Periopaper by capillary action. The lower portion of the Periopaper was placed between the jaws of the Periotron and a score was obtained. Sample volumes are determined by interpolation from a volume versus Periotron score calibration curve. GCF volumes were used to report TC as concentration per unit volume. Typical volumes obtained were between 0.1–1.0 μl. The Periopapers were cut below the black line (Fig. 1) into microcentrifuge tubes, capped and stored at −20°C until shipment.

### 2.4. Extraction procedure

The spiked standard curve, QC and clinical samples were extracted as follows. Samples were examined to verify the Periopapers were at the bottom of the microcentrifuge tubes. Doxycycline internal standard solution (50 μl) and buffer A solution (150 μl) were added to each tube. Samples were vortexed and filtered through 0.45 μm millipore syringe filters into a 100 μl autosampler vials and injected into the HPLC.

### 2.5. HPLC conditions

The mobile phase consisted of methanol and a 0.1 M sodium acetate buffer containing 25 mM EDTA and 35 mM $CaCl_2$ dihydrate (buffer A, pH 6.5) (40:60, v/v) at a flow rate of 1.0 ml min$^{-1}$. The injection volume was 50 μl and the run time was 15 min. The fluorescence detector had an

Table 1
TC and ETC test solutions

| Compound | Concentration of sodium acetate (M) | Concentration of EDTA (mM) | Concentration of $CaCl_2$ (mM) |
|---|---|---|---|
| TC | 0.1 | — | — |
| TC | 0.1 | 25 | — |
| TC | 0.1 | — | 35 |
| TC | 0.1 | 25 | 35 |
| ETC | 0.1 | — | — |
| ETC | 0.1 | 25 | — |
| ETC | 0.1 | — | 35 |
| ETC | 0.1 | 25 | 35 |

All solutions were 40% methanol and 60% buffer mixture

excitation wavelength of 375 nm and a emission wavelength of 512 nm.

## 3. Results and discussion

Four quality control pools were prepared at LOQ (QC1), 2 xLOQ (QC2), 50% of upper limit of quantitation (ULOQ) (QC3) and 75% of ULOQ (QC4) in pre-screened human serum and 1 μl was spiked on Periopapers to validate this method for TC in GCF. All quality control samples and standard curve samples were prepared using human serum instead of GCF because GCF was not commercially available nor easily collectible.

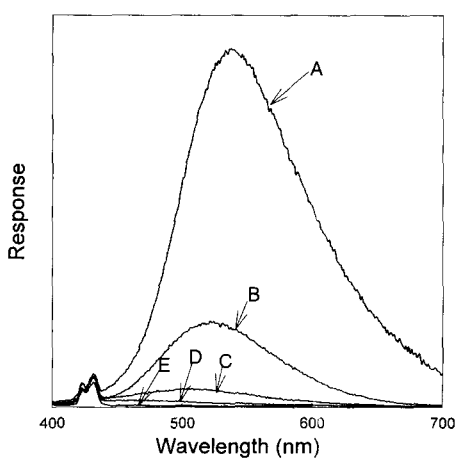


Fig. 3. Emission spectra of TC at 500 ng $ml^{-1}$ in various solutions. A: TC, 40% MeOH and 60% buffer with $CaCl_2$ and EDTA; B: TC, 40% MeOH and 60% buffer with $CaCl_2$; C: TC, 40% MeOH 60% buffer with no $CaCl_2$ and EDTA; D: 40% MeOH and 60% buffer with $CaCl_2$ and EDTA; E: TC, 40% MeOH and 60% buffer with EDTA.

Validation of the method involved 4 separate batches, 3 precision and accuracy batches (P&A) and 1 method evaluation batch. Two P&A batches consisted of a serum standard curve in triplicate and 6 replicates of QC1–QC4 and the third consisted of the same samples run on a column with a different lot number. The method evaluation batch consisted of 111 samples including a serum standard curve run in triplicate, a methanol standard curve run in triplicate to determine percent recovery, and triplicate samples of QC2–QC4 exposed to various conditions to investigate solution and freeze thaw stability.

### *3.1. Effect of $CaCl_2$ and EDTA on the fluorescence response of TC and ETC*

Eight solutions of TC and ETC were prepared at a concentration of 500 ng $ml^{-1}$ in a solution of 40% methanol and 60% buffer with varying amounts of $CaCl_2$ and EDTA (Table 1). Emission spectra of all solutions were performed on a SPEX Flourolog equipped with a SPEX 1680, 0.22 mm emission monochromator and a SPEX 1681, 0.22 mm excitation monochromator. TC alone without either $CaCl_2$ or EDTA gave a small peak for the emission spectra. Addition of $CaCl_2$ increased the intensity of the TC peak by approximately 5 times and addition of both $CaCl_2$ and EDTA increased the intensity of the TC peak by approximately 24 times. EDTA alone quenched

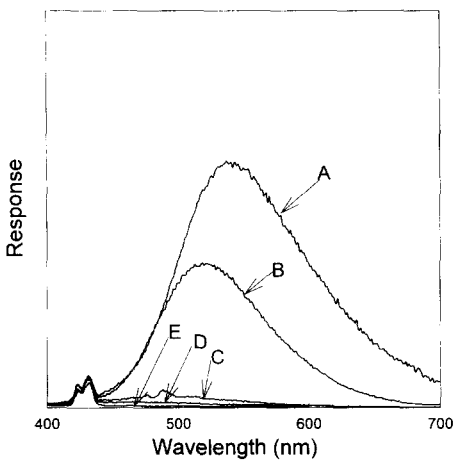


Fig. 4. Emission spectra of ETC at 500 ng $ml^{-1}$ in various solutions. A: ETC, 40% MeOH and 60% buffer with $CaCl_2$ and EDTA; B: ETC, 40% MeOH and 60% buffer with $CaCl_2$; C: ETC, 40% MeOH and 60% buffer with no $CaCl_2$ and EDTA; D: 40% MeOH and 60% buffer with $CaCl_2$ and EDTA; E: ETC, 40% MeOH and 60% buffer with EDTA.

the fluorescence producing a flat line. Identical trends were observed for both TC and ETC, however, the overall intensity of TC was approximately 1.5 times larger than ETC at the same concentration (Figs. 3 and 4). This suggested that calcium chelated to both TC and ETC forming rigid planar complexes thus increasing the fluorescence. The role of EDTA is also very important for fluorescence response and it is possible a trimer complex formed with TC, $Ca^{2+}$ and EDTA causing a further increase in response. EDTA also had an effect on the chromatography and without it the TC peak became very broad. Iwaki et al. attributed the chromatography problems to interactions between TC chelates and the column packing [14].

### 3.2. Linearity

The linear range for TC in human serum was validated using 7 standards covering the range 2.5–1000 ng of TC on the Periopaper. Standard curves were generated using a weighted $(1/x)$ linear least squares regression of the TC to internal standard (IS) peak area ratio versus concentration. Table 2 summarizes the correlation coefficients, slope, intercept and the pooled percent errors for all validation standard curves. The data demonstrates the linearity and the reproducibility of the standard curves for the developed method.

### 3.3. Precision and accuracy

Table 3 summarizes the precision and accuracy results for the method. The precision (%R.S.D., relative standard deviation: (S.D./mean) × 100) for all batches at all QC concentrations was below 9.5%. The accuracy (%REC, relative recoveries: (back calculated value/nominal value) × 100) for QC1 (the LOQ) was within ±20% and QC2, QC3 and QC4 were all within ±15%. The accuracy of the method varies from 95 to 116% within individual batches and from 97 to 111% among all batches.

### 3.4. Recovery

The recovery was measured over the linear range of 2.5–1000 ng of TC on the Periopaper by comparing a triplicate standard curve prepared in methanol to a triplicate standard curve prepared in serum spiked Periopapers. The results are shown in Table 4 and the percent recovery ranges from 101.5 to 123.3% with an average at 107.7%. The recovery determined from the slopes of the two standard curves is 104.4%.

### 3.5. Specificity

The specificity of the method was demonstrated by running chromatograms of human serum from 11 drug free patients. All chromatograms demonstrated no peaks which would interfere with the TC, ETC or internal standard peaks.

### 3.6. Ruggedness

Ruggedness of the assay was shown by running a P&A batch on two columns with different lot numbers. Both P&A batches 2 and 3 passed the specifications and the results are shown in Table 2.

Table 2
Summary of standard curve results

| | P&A batch 1 | P&A batch 2 | P&A batch 3 | ME batch | Acceptance criteria |
|---|---|---|---|---|---|
| $R^2$ | 0.99886 | 0.99878 | 0.99859 | 0.99624 | ≥0.990 |
| Slope | 0.00916 | 0.00917 | 0.00928 | 0.00962 | NA |
| *Y*-intercept | −0.00226 | −0.00297 | −0.00044 | −0.00809 | NA |
| %PRE | 4.5 | 3.5 | 4.6 | 8.8 | ≤10% |

%PRE, pooled percent relative error of all back calculated values from nominal concentrations ($[\Sigma(\%RE)^2/(n-1)]^{1/2}$)
%RE, the percent difference between the back calculated standard concentrations from the nominal concentrations (((calculated conc − nominal conc)/nominal conc) × 100)

### 3.7. Application of the method

This method was used to determine TC concentration in GCF samples from 2 human patients exhibiting periodontal disease participating in a clinical pharmacokinetic study. Both subjects were treated with Actisite® at five separate tooth sites. Each patient was sampled 9 times from the tooth sites for GCF over a 21 day period. See Fig. 5 for a typical chromatogram obtained from a patient. The pharmacokinetic profile shown in Fig. 6 demonstrates that a constant level of TC remained in the GCF fluid throughout a 10 day period and then immediately dropped below the LOQ once the Actisite® fiber was removed. The average TC concentration for subject 1 was 3203 ug $ml^{-1}$ and subject 2 was 2568 ug $ml^{-1}$. The clinical data results were in agreement with previously published data on ethylene vinyl acetate fiber treatments for periodontal disease patients [17].

Table 3
Precision and accuracy results for QC samples

| | QC1 | QC2 | QC3 | QC4 |
|---|---|---|---|---|
| Nominal concentration (ng) | 2.500 | 4.998 | 499.8 | 749.6 |
| Batch 1 | | | | |
| Intra-mean (ng) | 2.87 | 4.75 | 522 | 762.8 |
| S.D. | 0.27 | 0.15 | 18.8 | 60.8 |
| %R.S.D. | 9.5 | 3.1 | 3.6 | 8.0 |
| %REC | 114.8 | 95.1 | 104.4 | 101.7 |
| *n* | 6 | 6 | 6 | 6 |
| Batch 2 | | | | |
| Intra-mean (ng) | 2.56 | 4.74 | 508.6 | 788.2 |
| S.D. | 0.12 | 0.20 | 14.8 | 50.6 |
| %R.S.D. | 4.8 | 4.3 | 3.0 | 6.4 |
| %REC | 102.4 | 94.8 | 101.7 | 105.1 |
| *n* | 6 | 6 | 6 | 6 |
| Batch 3 | | | | |
| Intra-mean (ng) | 2.89 | 5.06 | 492.6 | 757.4 |
| S.D. | 0.13 | 0.25 | 17.0 | 15.8 |
| %R.S.D. | 4.6 | 5.0 | 3.5 | 2.1 |
| %REC | 115.7 | 101.2 | 98.6 | 101.0 |
| *n* | 5 | 6 | 6 | 6 |
| Pooled Batches | | | | |
| Inter-mean (ng) | 2.78 | 4.85 | 507.8 | 769.4 |
| S.D. | 0.24 | 0.24 | 20.0 | 45.8 |
| %R.S.D. | 8.6 | 5.0 | 4.0 | 6.0 |
| %REC | 111.2 | 97.0 | 101.6 | 102.6 |
| *n* | 17 | 18 | 18 | 18 |

Intra-mean, the mean within the batch.
Inter-mean, the mean among all the batches.

### 3.8. Conclusion

The validated method for TC in GCF described in this paper is precise, accurate and rugged and

Table 4
Recovery results

| Standard number | Mean percent recovery ($n = 3$) |
|---|---|
| STD1 | 123.3 |
| STD2 | 112.9 |
| STD3 | 101.5 |
| STD4 | 104.9 |
| STD5 | 101.8 |
| STD6 | 104.8 |
| STD7 | 104.8 |
| Average[a] | 107.7 |

[a] This average is calculated over the linear range 2.5–1000 ng of TC on the Periopaper.

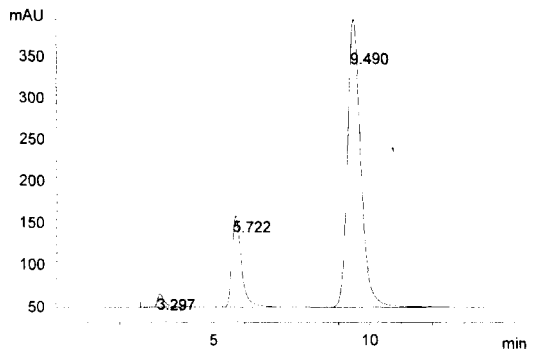


Fig. 5. Typical chromatogram of a clinical sample. Identification of peaks from left to right: ETC, TC, doxycycline. Concentration of TC 1000 ug $ml^{-1}$.

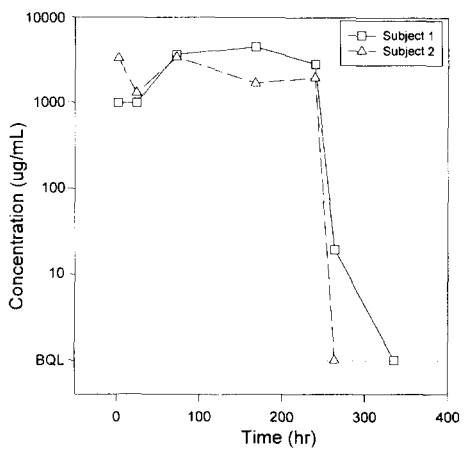


Fig. 6. Concentration of TC in GCF after Actisite® application to two subjects. The logarithm of tetracycline concentration is plotted as a function of time.

was shown to be more sensitive than the existing microbiological method used (LOQ of 2.5 ng compared to 10 ng of TC on the Periopaper) for previous clinical studies and produced equivalent results. This method is suitable for use as the standard method for analysis of TC in GCF for future products and product modifications for the treatment of periodontal disease.

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