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New HPLC and UHPLC Assay Methods for Tetracycline Hydrochloride and 4-Epianhydrotetracycline Hydrochloride Impurity in Tetracycline Drug Products

INTRODUCTION

Tetracycline (TC) is a common antibiotic used to fight bacterial infections. It is used to treat urinary tract infections, acne, gonorrhea, and other conditions. Tetracycline yields a toxic degradation product, 4-epianhydrotetracycline (EATC). The United States Pharmacopeia and National Formulary (USP-NF) contains General Chapter 226 that prescribes an assay for EATC impurity in TC.¹ The monograph for epitetracycline and three other monographs for drug products containing tetracycline hydrochloride (TC-HCl) refer to General Chapter 226.²⁻⁵ This is an antiquated method—certainly a target for modernization—that uses a self-packed column eluted with chloroform. The EATC impurity elutes as a yellow band and is detected by its visible absorbance.

The TC-HCl drug substance monograph⁶ and some TC-HCl-containing drug products do not refer to General Chapter 226 to determine the amount of EATC but instead use a high-performance liquid chromatography (HPLC) method. This method, which is used both to determine the amount of EATC and to assay TC, uses a 4.6 × 250 mm, 5–10 μm L7 (C8) packing column, a mobile phase containing ammonium oxalate/dimethylformamide/dibasic ammonium phosphate pH 7.6–7.7, and a 280 nm detection wavelength.

This assay method is also a target for modernization because it uses the undesirable mobile phase component dimethylformamide, the dimethylformamide/ammonium oxalate/dibasic ammonium phosphate pH 7.6–7.7 mobile phase is tedious to prepare, and it uses a large resin bead size HPLC column.

This study reports HPLC and ultra HPLC (UHPLC) methods for assay of EATC in TC-containing drug products. These methods use Thermo Scientific Acclaim™ Polar Advantage II (PA2) columns. The HPLC method uses a 4.6 × 150 mm column packed with 3 μm resin and an acetonitrile/ammonium dihydrogen orthophosphate pH 2.2 mobile phase. The Acclaim PA2 column is made with high-purity spherical silica and—unlike a standard C8 column—can be used in a pH range of 1.5–10.

After developing the HPLC method, it was transferred to a UHPLC system using a 2.1 × 100 mm column packed with 2.2 μm packing material. This reduced the run time from 8 to 2 min. Both methods were evaluated using a TC-HCl drug product (TC-HCl capsules). The results from both methods exceed the specifications of the HPLC method in the USP TC-HCl monograph.

EQUIPMENT

Thermo Scientific Dionex UltiMate™ 3000 system including:
Integrated Vacuum Degasser SRD-3600 Solvent Rack
HPG-3400RS Pump
WPS-3000TRS Split-Loop Sampler
TCC-3000RS Column Compartment
DAD-3000RS Diode Array Detector
Sample Loop Size: 100 µL (conventional HPLC)
25 µL (UHPLC)
Mixer: 400 µL (conventional HPLC)
200 µL (UHPLC)
Flow Cell: 13 µL SST (conventional, HPLC)
2.5 µL SST (UHPLC)
Thermo Scientific Dionex Chromeleon™ Chromatography Data System (CDS) Software Version 6.80, SR10

CONDITIONS

Conventional HPLC

Column: Acclaim PA2, 3 µm, 4.6 × 150 mm
Mobile Phase: A: 20 mM NH₄H₂PO₄, pH 2.2
B: 50% CH₃CN in
20 mM NH₄H₂PO₄, pH 2.2
Gradient: 30% B from -5 to 0 min, ramp to
100% B in 6 min, hold 100% B for
1 min, and return to 30% B in 0.5 min
Flow Rate: 1.0 mL/min
Inj. Volume: 5 µL
Column Temp.: 40 °C
Sample Tray Temp.: 10 °C
Detection: UV 280 nm,
data collection rate 5 Hz,
response time 2.0 s

UHPLC

Column: Acclaim RSLC PA2, 2.2 µm,
2.1 × 100 mm
Mobile Phase: A: 20 mM NH₄H₂PO₄, pH 2.2
B: 50% CH₃CN in
20 mM NH₄H₂PO₄, pH 2.2
Gradient: 30% B from -1 to 0 min, ramp to
100% B in 1.4 min, hold 100% B
for 0.2 min, and return to 30% B
in 0.1 min
Flow Rate: 0.6 mL/min
Inj. Volume: 1 µL
Column Temp.: 40 °C
Sample Tray Temp.: 10 °C
Detection: UV 280 nm,
data collection rate 25 Hz,
response time 0.5 s

REAGENTS AND STANDARDS

Deionized water (DI), Type I reagent grade, 18 MΩ-cm resistivity or better
Acetonitrile (CH₃CN), HPLC grade (LAB-SCAN)
Ammonium dihydrogen orthophosphate (NH₄H₂PO₄), AR grade (Ajax)
Orthophosphoric acid, 85%, AR grade (ASP Finechem)
Tetracycline hydrochloride (TC-HCl), ≥ 95% (Sigma Aldrich)
4-Epianhydrotetracycline hydrochloride (EATC), 96.1% (Fluka)

PREPARATION OF SOLUTIONS AND REAGENTS

Mobile Phase A: 20 mM NH₄H₂PO₄, pH 2.2

Dissolve 2.3 g NH₄H₂PO₄ in 700 mL DI water. Transfer this solution to a 1 L volumetric flask and add DI water to bring to volume. Adjust to pH 2.2 with orthophosphoric acid (add ~3 mL of 85% orthophosphoric acid for 1 L preparation). Filter with a 0.2 µm filter (Whatman,® Cellulose Acetate, Cat. No. 7001 0004).

Mobile Phase B: 50% CH₃CN in 20 mM NH₄H₂PO₄

Mix 500 mL CH₃CN with 500 mL of 20 mM NH₄H₂PO₄, pH 2.2. Filter with a 0.2 µm filter.

TC-HCl Stock Standard Solution, 10,000 mg/L

Weigh 526 mg TC-HCl in a 50 mL volumetric flask. Add ~25 mL of mobile phase A and place in an ultrasonic bath until completely dissolved. Bring to volume with mobile phase A and mix well.

EATC Stock Standard Solution, 1000 mg/L

Weigh 10 mg EATC in a 10 mL volumetric flask. Add ~5 mL of mobile phase A. Place in an ultrasonic bath until completely dissolved. Bring to volume with mobile phase A and mix well.

Standard Solution for System Suitability Testing (Mixture of 100 mg/L TC and 25 mg/L EATC): Resolution Solution

Pipet 250 µL of TC-HCl stock standard solution and 625 µL of EATC stock standard solution into a 25 mL volumetric flask. Bring to volume with mobile phase A and mix well.

TC-HCl Working Standard Solutions

Transfer the appropriate volume of TC-HCl stock standard solution into a 10 mL volumetric flask and bring to volume with mobile phase A. A list of TC-HCl working standard solutions prepared in this study is presented in Table 1.

EATC Working Standard Solutions

Transfer the appropriate volume of EATC stock standard solution into a 25 mL volumetric flask and bring to volume with mobile phase A. The list of EATC working standard solutions prepared in this study is presented in Table 1.

Sample Preparation

Dissolve a capsule in 1 L of mobile phase A. Filter with a 0.2 µL syringe filter prior to injection.

Note: Prepare all standard and sample solutions just before analysis.

RESULTS AND DISCUSSION**Separation**

The Acclaim PA2 column contains a polar-embedded stationary phase that has similar selectivity to a standard C8 or C18 stationary phase but can be used under both lower and higher pH conditions as well as with highly aqueous conditions. Given these properties, the Acclaim PA2 column was chosen for developing a method for determining EATC in TC-HCl-containing drug products. Compared to the L7 column used in the TC-HCl monograph, a column smaller in terms of both length and resin bead size (3 µm, 4.6 × 150 mm) was chosen. With this column and a gradient separation using an ammonium dihydrogen phosphate pH 2.2/acetonitrile mobile phase, TC and EATC were well resolved (Figure 1). This separation exhibited a change in selectivity compared to the HPLC method in the TC-HCl monograph.

Table 1. Preparation of Working Standard Solutions

Compound	Stock Standard Solution Concentration (mg/L)	Stock Standard Solution Volume (µL)					Working Standard Solution Concentration (mg/L)				
		L1	L2	L3	L4	L5	L1	L2	L3	L4	L5
TC-HCl*	10,000	200	300	400	500	600	200	300	400	500	600
EATC**	1000	12.5	25	125	250	375	0.5	1	5	10	15

*Volume used to prepare 10 mL of working standard solution

**Volume used to prepare 25 mL of working standard solution

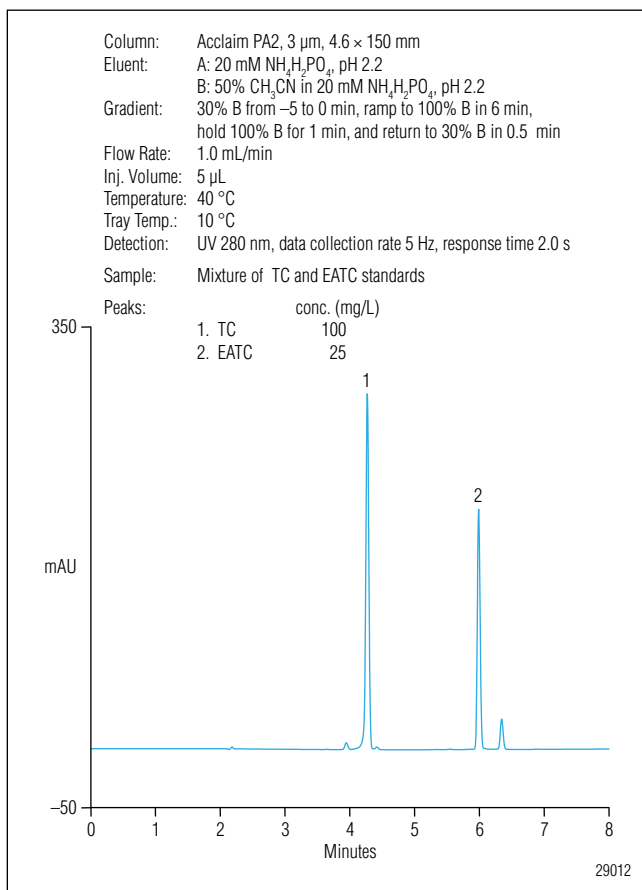


Figure 1. Chromatogram of the SST solution obtained using the HPLC method.

In the monograph, the relative retention times are 0.9 for EATC and 1 for TC. The separation in Figure 1 shows EATC eluting well after TC with a relative retention time of 1.4 min. The peak eluting before TC originates with the TC standard and the peak eluting after EATC originates with the EATC standard. The large retention time gap between TC and EATC makes the HPLC method ideal for transferring to a UHPLC system and accelerating it using a smaller Acclaim PA2 column format with smaller resin size packing material. The flow rate, column length, injection volume, and flow cell size are also reduced (Figure 2). With the UHPLC method, the run time is reduced from 8 to 2 min. The retention time gap and the higher pressure limits of the UHPLC system allow the flow rate to be reduced less than the proportional amount (i. e., a faster flow rate is possible). This results in a 75% reduction in run time while providing a 40% reduction in mobile phase consumption. The HPLC method uses significantly less mobile phase than the HPLC method in the TC-HCl monograph, which uses a 2 mL/min flow rate and a 25 cm column.

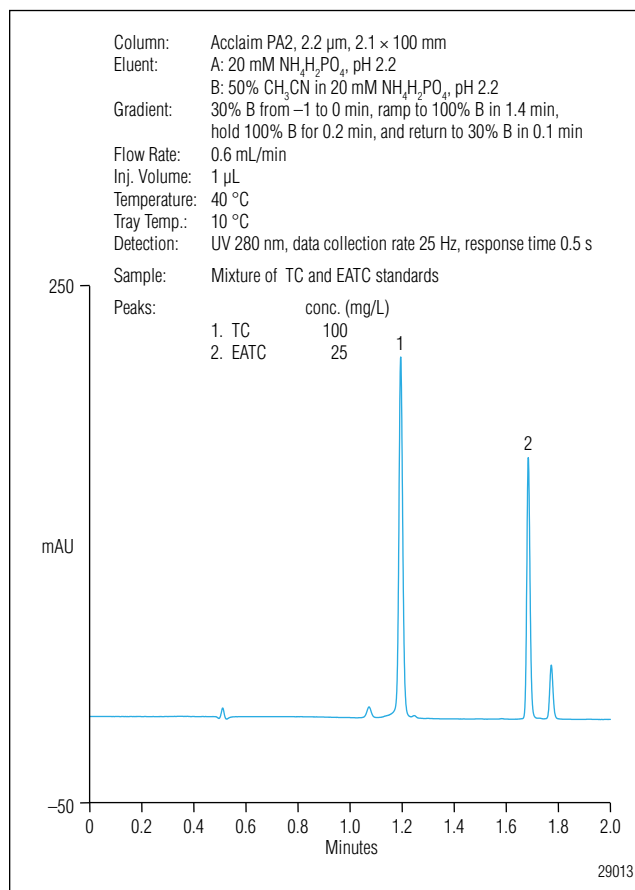


Figure 2. Chromatogram of the SST solution obtained using the UHPLC method.

System Suitability Testing

System suitability testing (SST) must be performed before analysis of a drug substance or drug product. Prepare a mixed standard of TC and EATC at concentrations specified in the USP TC-HCl monograph and inject five times. The monograph specifies that resolution between TC and EATC is not less than 1.2 in the resolution solution and the relative standard deviation (RSD) of peak response is not more than (NMT) 2 when analyzing a 0.5 mg/mL solution of TC-HCl. The resolutions of the HPLC and UHPLC methods are 20.8 and 19.9, respectively, exceeding the USP specification. The RSDs of TC peak area response in the resolution solution, where the concentration is only 0.1 mg/mL instead of the less challenging 0.5 mg/mL, are 0.60% and 0.45% from HPLC and UHPLC methods, respectively. These results also exceed the monograph specification.

Table 2. Reproducibility of Five SST Solution Injections Obtained from the HPLC and UHPLC Methods

Inj.No.	Area (mAU*min)			
	HPLC		UHPLC	
	TC	EATC	TC	EATC
1	16.05	10.03	3.644	2.185
2	16.08	10.03	3.645	2.177
3	16.07	10.03	3.656	2.194
4	16.18	10.01	3.616	2.171
5	16.28	10.04	3.656	2.187
Average	16.13	10.03	3.643	2.183
RSD	0.60	0.11	0.45	0.41
Resolution Between TC and EATC (USP)	20.8		19.9	
Relative Retention Time	1	1.4	1	1.4

Table 3. Calibration Concentrations and Calibration Results

Compound	Calibration Concentration (mg/L)					Calibration Results							
	L1	L2	L3	L4	L5	HPLC				UHPLC			
						Points	r ²	Offset	Slope	Points	r ²	Offset	Slope
TC	200	300	400	500	600	15	0.99968	3.4928	0.1505	15	0.99965	0.8230	0.0337
EATC	0.5	1	5	10	15	15	0.99996	0.0091	0.3908	15	0.99996	0.0005	0.0866

Additional results of the SST of the HPLC and UHPLC methods are shown in Table 2. The chromatography of the resolution solution by the HPLC and UHPLC methods is shown in Figures 1 and 2, respectively.

Method Detection Limits

To determine the sensitivity of both methods for determining EATC, the method detection limit (MDL) of EATC was studied. The calculated MDLs of the HPLC and UHPLC methods for EATC were 0.002 and 0.014 mg/L, respectively. These were calculated from seven injections of 0.01 and 0.05 mg/L standards for the HPLC and UHPLC methods, respectively, using the equation:

$$MDL = t(n-1, 0.99) (S)$$

where $t(n-1, 0.99)$ = the Student's t value appropriate for a 99% confidence level and a standard deviation estimate with $n-1$ degrees of freedom, S = standard deviation of the replicate analyses.

The following estimates of $3 \times$ signal-to-noise (S/N) were calculated from the concentration used for the MDL studies:

- For the HPLC method (0.01 mg/L), S/N was ~ 5 , MDL based on S/N was 0.006 mg/L
- For the UHPLC method (0.05 mg/L), S/N was ~ 10 , MDL based on S/N was 0.015 mg/L

Method Calibration

Both methods were calibrated before performing sample analysis. Five-point calibration curves were constructed for both TC and EATC separately, due to a small amount of EATC present in the TC-HCl standard. Standard concentrations and calibration results for both methods are shown in Table 3.

Table 4. Sample Results Obtained Using Each Method (Five Injections/Capsule)

Method	Capsule	TC			EATC		
		Average (mg/L)	RSD	% Assay (90–125%)	Average (mg/L)	RSD	% Concentration (NMT 3%)
HPLC	1	495	0.05	99.0	0.57	0.19	0.057
	2	480	0.04	96.0	0.71	0.20	0.071
	3	480	0.09	96.0	0.77	0.27	0.077
UHPLC	1	497	0.27	99.4	0.98	0.88	0.098
	2	510	0.59	102	0.82	0.81	0.082
	3	482	0.42	96.4	0.75	0.43	0.075

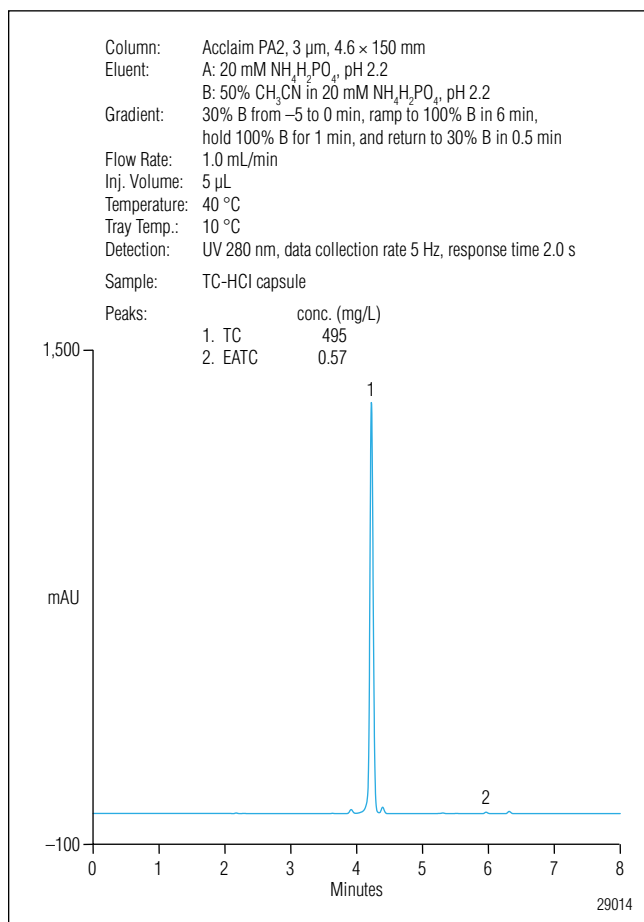


Figure 3. Chromatogram of the TC-HCl capsule sample obtained using the HPLC method.

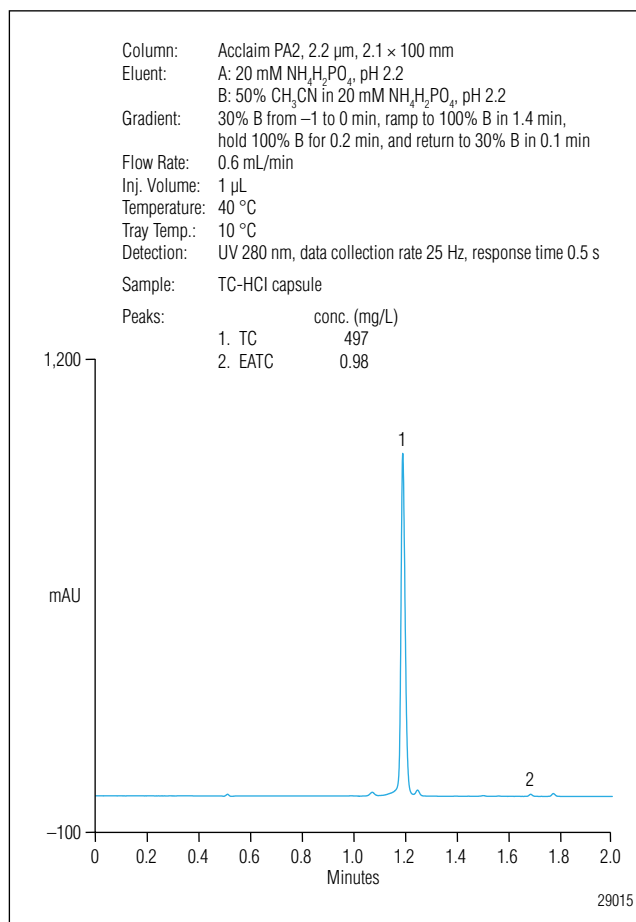


Figure 4. Chromatogram of the TC-HCl capsule sample obtained using the UHPLC method.

Sample Analysis

A TC drug product (TC-HCl capsules) was purchased from a pharmacy. The sample label indicated that a capsule contained 500 mg of TC. To assay this product, three sample preparations were performed and each sample preparation was injected five times.

The assay results (Table 4) obtained from both methods show that the capsules have the appropriate amount of TC to meet the USP specified range (90–125% of the labeled amount).⁷ The capsules also met the monograph requirement of NMT 3% EATC (Table 4). Figures 3 and 4 show chromatography of the sample obtained using the HPLC and UHPLC methods, respectively.

CONCLUSION

This study shows HPLC and UHPLC methods that can be used to assay TC and EATC in a TC-HCl capsule. The results from both methods meet or exceed the criteria in the appropriate USP monographs. Both methods are faster and show higher resolution than the current methods. They also generate less waste and use less hazardous organic solvents. These methods may be applicable to other TC-HCl-containing drug products.

REFERENCES

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5. United States Pharmacopeia and National Formulary USP 34 NF 29 (2011), Tetracycline Hydrochloride and Nystatin Capsules, p 4392.
6. United States Pharmacopeia and National Formulary USP 34 NF 29 (2011), Tetracycline Hydrochloride, p 4385.
7. United States Pharmacopeia and National Formulary USP 34 NF 29 (2011), Tetracycline Hydrochloride Capsules, p 4386.

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