HPLC AS A RAPID MEANS OF MONITORING ERYTHROMYCIN AND TETRACYCLINE FERMENTATION PROCESSES

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(Received for publication January 9, 1978)

Reverse phase high performance liquid chromatography (HPLC) was used as a rapid means of monitoring the erythromycin and the tetracycline fermentation processes. The sample preparation process for tetracycline in the fermentation broth includes simple dilution and filtration through a Millipore filter prior to injection into the HPLC column. Fermentation broth samples showed no interference, and excellent separation for selective determination of tetracycline, 4-epitetracycline, anhydrotetracycline, chlortetracycline, and 4-epianhydrotetracycline was obtained. The relative standard deviation for the HPLC analysis for tetracycline is about one percent and the correlation coefficient between the HPLC and the spectrophotometric assay methods is better than 0.994.

The sample preparation procedure for erythromycin determination in fermentation broth requires solvent cleanup and extraction processes. The chromatographic analysis takes approximately 25 minutes, and the HPLC method is capable of separating and quantifying erythromycins A, B, C, and various epimers and degradation compounds. The correlation coefficient between the HPLC and the microbiological assay method is 0.970.

Just over seven years ago, HPLC began to be used in the pharmaceutical industry. With its ability to combine chromatographic separation at a rapid rate of analysis with selective determination of therapeutic agents in a complex biological matrix with minimal sample preparation, HPLC has become one of the most powerful tools for pharmaceutical analysis. Thus, several HPLC methods became available for the determination of complex, high molecular weight compounds, such as antibiotics[1~4].

At least three HPLC methods have been reported for the analysis of erythromycin[5~7]. With the exception of the latter, these reports failed to present data on quantitation and separation of numerous erythromycin epimers and degradation compounds.

For the tetracycline analysis, methods using the reverse phase[8~10] and ion-exchange modes[11~13] were reported. Advantages of the reverse phase mode over the ion-exchange have been amply demonstrated[9,10].

This paper demonstrates applicability of the HPLC methods[7,8] as a rapid means of monitoring the erythromycin and the tetracycline fermentation processes.

Experimental

Instruments

A Laboratory Data Control modular liquid chromatograph (LDC, Riviera Beach, Florida) equipped with a high pressure mini-pump was used. A reverse phase column (μBondapak C_{18}, Waters Assoc., Milford, Mass.) of 300 × 3.9 mm i.d. with a 50 × 2.1 mm i.d. stainless steel pre-column packed with a μBondapak C_{18} was used. A sample was injected through a Rheodyne injector (Model

7120, Berkeley, Calif.). The mobile phase was pumped at a flow rate of about 1.0 ml/min (1,700 psi) and the column was operated at room temperature.

For erythromycin determination, a variable wavelength detector (Spectro-Monitor I, LDC) at 215 nm was used at an electrometer setting of 0.04 absorbance units full scale (AUFS). A 280 nm fixed UV wavelength detector (LDC Model 1205) was used at 0.16 AUFS to monitor tetracycline.

Mobile Phases:

The solvents used were all UV grade, distilled in glass obtained from Burdick and Jackson Laboratories, Inc., Muskegon, Michigan. The amounts of the solvents and pH of the mobile phases may have to be slightly modified to obtain the maximum performance of the column. The mobile phase must be filtered through a Fluoropore filter (FHLP04700, Millipore Corp., Bedford, Mass.) and degassed under vacuum with sonication prior to use.

1. Erythromycin. A mixture of acetonitrile - methanol - 0.2 M ammonium acetate - water (45: 10: 10: 35) at pH 7.8 was used. As stated earlier[7], the pH of the mobile phase controls the elution volume of the erythromycin peak. The pH 7.8 was used to minimize interferences associated in the fermentation broth. The pH adjustment was made by the addition of either HCl, NH_4OH or NaOH. Due to its UV cutoff near 215 nm, acetic acid may not be used.

2. Tetracycline. A linear gradient elution, from the mobile phase A to B in 15 minutes, was used (LKB Ultragrade Gradient Mixer, Model 1130, LKB, Sweden). The composition of the mobile phases A and B were as follows:

A) Methanol - water - 0.2 M pH 2.5 phosphate buffer (30: 60: 10)
B) Methanol - acetonitrile - water - 0.2 M pH 2.5 phosphate buffer (50: 20: 20: 10)

The pH of the mobile phases A and B must be checked and adjusted to 2.5 with phosphoric acid to minimize the tailing of peaks. The 0.2 M pH 2.5 phosphate buffer was prepared by weighing 28 g of anhydrous, dibasic sodium phosphate into a one-liter measuring cylinder. Water was added to dissolve and the pH was adjusted to 2.5 with phosphoric acid, and finally water was added to volume.

The mobile phases described above are needed when the C_{18} (ODS) loading exceeds 10%; however, the mobile phases reported previously[8] are quite adequate when the C_{18} (ODS) loading is 5~7%.

Some reverse phase columns require pre-treatment with ethylenediamine tetraacetic acid (EDTA) to minimize the tailing of the tetracycline peak. This can be performed by pumping 0.1 M EDTA solution through a column for 2~3 hours. Once the column is properly conditioned, no further treatment is needed.

Routine Column Maintenance:

At the end of each working day, the column should be rinsed with deaerated water for about 30 minutes, followed by absolute methanol for about 30 minutes.

Sample Preparation:

1. Erythromycin.

Reference Standard Solution: Accurately weigh about 8~9 mg of the erythromycin reference standard, using an electro-balance, into a 10-ml volumetric flask. Just prior to the analysis, the mobile phase is added to volume and sonicated to facilitate dissolution. A 100-μl fixed loop has been used to inject both the standard and the sample solutions.

Fermentation Broth: Accurately weigh an appropriate quantity of the fermentation broth into a 100-ml beaker. Adjust pH to 8.9 with NaOH. The broth must be blended for uniform sampling if it has been frozen and thawed or kept in a refrigerator. Quantitatively transfer the content into a 250-ml volumetric flask and bring to volume with water. Shake the content vigorously to mix. Centrifuge the content at about 20,000 rpm and filter the supernatant through a 0.45 μm Millipore filter.

Pipet 25 ml of the solution into a 125-ml separatory funnel. Add 25 ml of heptane (UV grade) and shake for 5 minutes on a shaker. Collect the aqueous layer into a centrifuge tube. Wash the heptane layer twice with water and combine the washed solutions into a centrifuge tube. Add 10 ml of chloroform (UV grade) and shake vigorously for 5 minutes on a reciprocating shaker.

Centrifuge the tube for 5 minutes at 2,500 rpm. Depending upon the sample, an emulsion may form at the solvent interface. This interface must be gently dispersed with a glass rod and recentrifuged. Remove the aqueous layer with suction. Pipet a suitable quantity of the chloroform layer into a 10-dram vial and dry under a gentle stream of dry nitrogen. Just prior to the analysis add a suitable quantity of the mobile phase and dissolve the sample with sonication. A 100-μl fixed loop is used to inject the samples.

2. Tetracycline

Reference Standard Solution: Accurately weigh about 8 mg of the tetracycline hydrochloride reference standard into a 50-ml volumetric flask. Just prior to analysis, the standard is brought to volume with 0.01 M pH 4.5 phosphate buffer. Stability of tetracycline in pH 4.5 phosphate buffer has been described[14]. A 10-μl fixed loop is used to inject both the standard and the sample solutions.

Fermentation Broth: Accurately weigh an appropriate quantity of the fermentation broth into a 100-ml beaker. The broth must be blended for uniform sampling if it has been frozen and thawed or kept in refrigerator. Adjust pH to 1.7 with HCl or H_3PO_4. Quantitatively transfer the content into a suitable volumetric flask and bring up the volume with water. Centrifuge the contents for 5 minutes at about 3,000 rpm. Remove any oily layer with suction and filter the supernatant through a Millipore filter. A 10-μl fixed loop is used to inject the fermentation broth sample.

Calculation:

The formulae appearing in the references 7) and 8) were used to quantify the content of erythromycin and tetracycline, respectively.

Results and Discussion

Erythromycin

Although the gas-liquid chromatographic method is capable of selective determination of various erythromycin entities[15], the method requires at least 24 hours of derivatization and considerable

Fig. 1. A reverse phase chromatogram of erythromycin in the fermentation broth.
Mobile phase: acetonitrile - methanol - 0.2 M ammonium acetate - water (45: 10: 10: 35) at pH 7.8 as monitored at 215 nm.

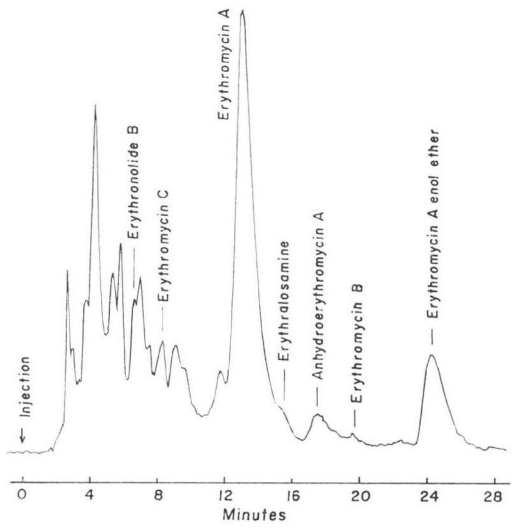


Fig. 2. The HPLC monitoring of erythromycin synthesis during the fermentation process.

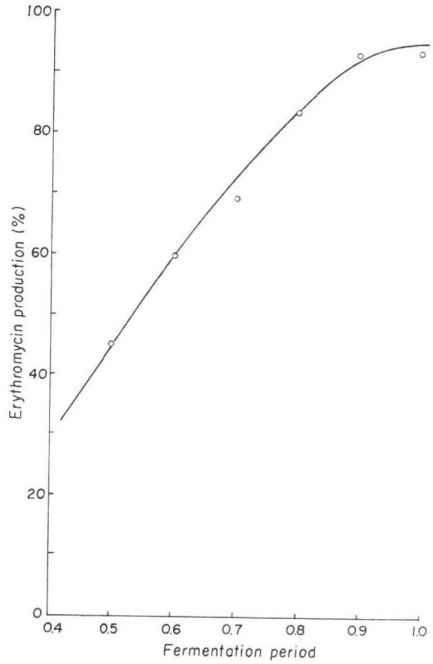


Table 1. Monitoring of erythromycin fermentation process

	Fermentation period	Erythromycin production (%)		Correlation coefficient
		HPLC	Microbioassay	
Run #1	0.5	48.4	—	0.989
	0.6	65.5	—	
	0.7	74.3	84.0	
	0.8	89.4	—	
	0.9	99.7	104.3	
	1.0	100.0	101.3	
Run #2	0.7	75.6	77.6	0.961
	0.8	84.0	—	
	0.9	96.7	91.9	
	1.0	100.0	101.3	
Run #3	0.5	47.4	—	0.961
	0.6	56.3	—	
	0.7	70.8	80.6	
	0.8	97.0	—	
	0.9	97.7	106.0	
	1.0	100.0	100.5	

sample cleanup procedures. The HPLC method can quantitate erythromycin in the fermentation broth in less than 30 minutes of chromatographic time with a minimum of sample preparation.

Efficiency of the fermentation broth cleanup procedure described in this paper for the recovery of erythromycin was examined. The recovery of erythromycin from the duplicate broth samples which were added to the known quantity of erythromycin were 103.3% and 99.0%, respectively.

Biosynthesis of erythromycin during the fermentation process was then monitored. The Fig. 1 is a typical chromatogram of erythromycin in the fermentation broth. Capability of the HPLC method to separate various entities of erythromycin has been demonstrated[7]. The fermentation process as monitored by the HPLC method is graphically illustrated in Fig. 2. HPLC data used to construct Fig. 2 were compared with data from the microbiological assay method[16]. As shown in Table 1, good correlation exists between the two methods (correlation coefficient of 0.970).

Tetracycline

In order to successfully use the Waters new end-fitting style μBondapak C_{18} column, packed with 10~18% C_{18} loading, it was found necessary to replace acetonitrile in the mobile phase A as reported in references[8,14] with methanol and to add methanol to the mobile phase B[8,14]. Both methanol and acetonitrile are required in the mobile phase B to elute anhydrotetracycline and 4-epianhydrotetracycline. The method reported[8,14] is still applicable to the reverse phase column packed with about 5~7% C_{18} loading.

As was shown previously[8], the pH of the mobile phase is a significant factor in controlling the tailing factor of the tetracycline peak. The tailing factor increases with an increase in the pH of the mobile phases A and B. Since the life of the reverse phase column can be significantly shortened when the pH of the mobile phase falls below 2.0, the pH of the mobile phase must be adjusted to 2.5.

Although the old end-fitting style Waters reverse phase column did not require an EDTA treatment[8], the new end-fitting style column must be treated with an EDTA solution to minimize the tailing of the tetracycline peak. Neither K′ nor α was affected by the treatment. Thus, far more metallic surface may be exposed in the new end-fitting style column than the old, resulting in a need for the EDTA treatment.

Fig. 3. A reverse phase chromatogram of tetracycline indicating separation of 4-epitetracycline (1), tetracycline (2), chlortetracycline (3), 4-epianhydrotetracycline (4), and anhydrotetracycline (5). Linear gradient from the mobile phase A to B in 15 minutes.
Mobile phase A: Methanol - water - 0.2 M pH 2.5 phosphate buffer (30: 60: 10),
mobile phase B: Methanol - acetonitrile - water - 0.2 M pH 2.5 phosphate buffer (50: 20: 20: 10).

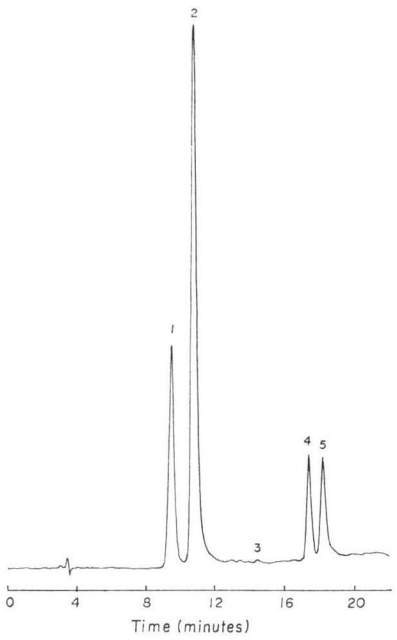


Fig. 4. Chromatogram of tetracycline in the fermentation broth.

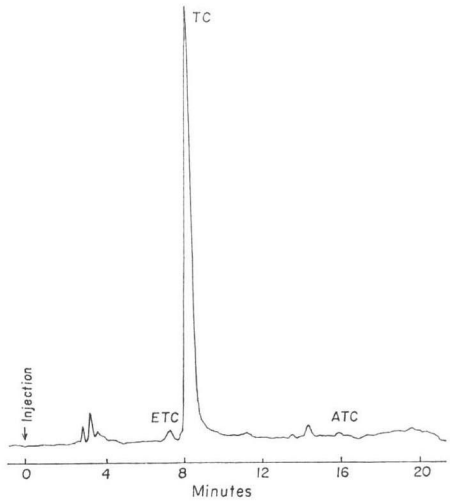
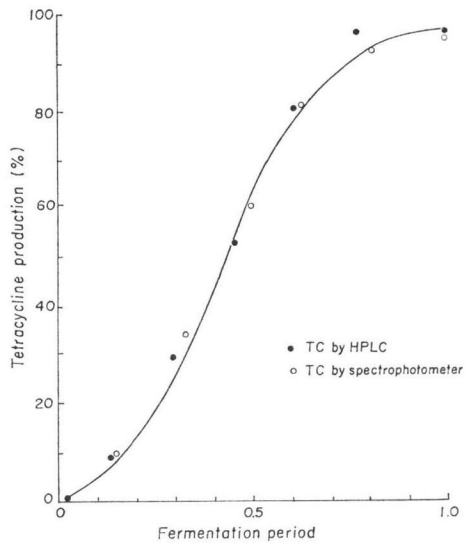


Fig. 5. Monitoring the tetracycline fermentation process by the HPLC and the spectrophotometric assay methods.



To our delightful surprise, a pre-column did not affect K′, α, Rs, nor the tailing factor. A typical chromatogram indicating separation of tetracyclines using the new mobile phase system is presented in Fig. 3.

Precision of the Modified HPLC system: Precision of the HPLC assay method with the modified mobile phase system was determined by analyzing 6 individually weighed tetracycline hydrochloride powders. The relative standard deviation of the method is about one percent (Table 2).

Monitoring of Fermentation Processes: Biosynthesis of tetracycline during the fermentation process was monitored by the HPLC method. The sample preparation involves only a simple dilution and filtration step. The chromatography takes about 20 minutes to complete. Fermentation broth showed no interference in the chromatographic analysis. A typical chromatogram of tetracycline in the fermentation broth is shown in Fig. 4.

Table 2. Precision of the HPLC assay method for tetracycline

Weight of tetracycline (mg)	Weight of tetracycline in solution (mg/ml)	Tetracycline peak height	Peak height/weight ratio
3.844	0.1538	42.65	277.3
3.798	0.1519	41.35	272.2
3.832	0.1533	41.75	272.3
3.782	0.1513	41.90	276.9
3.670	0.1468	41.10	280.0
3.755	0.1502	41.40	275.6

Relative standard deviation: 1.1%

Table 3. Monitoring of tetracycline fermentation process

	Fermentation period	Tetracycline production		Correlation coefficient
		HPLC	Spectrophotometric method	
Run #1	0.6	66.0	64.3	0.997
	0.7	89.2	85.3	
	0.85	100.2	98.5	
	1.0	100.	99.1	
Run #2	0.1	1.1	—	0.999
	0.3	17.0	16.5	
	0.4	30.8	32.3	
	0.6	53.3	53.2	
	0.7	76.9	76.3	
	0.85	95.1	94.2	
	1.0	100.	103.1	
Run #3	0.1	2.7	—	0.994
	0.3	18.1	17.6	
	0.4	36.3	37.3	
	0.6	51.5	60.2	
	0.7	91.6	90.6	
	0.85	95.4	97.5	
	1.0	100.	98.8	

The tetracycline contents in the broth during the fermentation processes were analyzed by the HPLC method and the values obtained were compared with those of the spectrophotometric assay method (Table 3). The spectrophotometric method converts tetracycline and 4-epitetracycline to their anhydro forms for measurement. As shown in Table 3, the values obtained by the HPLC method correlated extremely well with those of the spectrophotometric assay method (correlation coefficients are better than 0.994). Fig. 5 graphically illustrates the synthesis of tetracycline during the fermentation process. This figure includes the data obtained by the spectrophotometric method to show a good correlation with the HPLC method.

Thus, the HPLC methods have been proven as valuable tools to rapidly monitor fermentation processes.

Acknowledgements

J. W. EVANS, R. L. NIEWOONDER, C. Y. PEERY, J. G. SANTEK and J. VISSER are gratefully acknowledged for their assistance.

References

1) MECHLINSKI, W. & C. P. SCHAFFNER: Separation of polyene antifungal antibiotics by high-speed liquid chromatography. J. Chromatogr. 99: 619~633, 1974
2) TSUJI, K.: High-pressure liquid chromatography of antibiotics. *in* Methods Enzymol. (J. NASH, *ed.*) 43: 300~320, 1975
3) MAYS, D. L.; R. J. VAN APELDOORN & R. G. LAUBACK: High-performance liquid chromatographic determination of kanamycin. J. Chromatogr. 120: 93~102, 1976
4) MILLER, R. D. & N. NEUSS: High performance liquid chromatography of natural products. I. Separation of cephalosporin C derivatives and cephalosporin antibiotics: Isolation of cephalosporin C from fermentation broth. J. Antibiotics 29: 902~906, 1976
5) MONTOYA, E. F.: Assay of erythromycin antibiotic. Chromatography, Application, Spectra-Physics. Calif., 1975
6) ŌMURA, S.; Y. SUZUKI, A. NAKAGAWA & T. HATA: Fast liquid chromatography of macrolide antibiotics. J. Antibiotics 26: 794~796, 1973
7) TSUJI, K. & J. F. GOETZ: HPLC determination of erythromycin. J. Chromatogr. 147: 359~367, 1978
8) TSUJI, K. & J. H. ROBERTSON: Analysis of tetracycline in pharmaceutical preparations by improved high-performance liquid chromatographic method. J. Pharm. Sci. 65: 400~404, 1976
9) KNOX, J. H.; J. JURAND & A. PRYDE: Recent applications of high-performance liquid chromatography in pharmaceutical analysis. Proc. Analyt. Div. Chem. Soc. 13: 14~16, 1976
10) WHITE, E. R.; M. A. CARROLL, J. E. ZAREMBO & A. D. BENDER: Reverse phase high speed liquid chromatography of antibiotics. J. Antibiotics 28: 205~214, 1975
11) BUTTERFIELD, A. G.; D. W. HUGHES, W. L. WILSON & N. J. POUND: Simultaneous high-speed liquid chromatographic determination of tetracycline and rolitetracycline in rolitetracycline formulations. J. Pharm. Sci. 64: 316~320, 1975
12) FIKE, W. W. & N. W. BRAKE: Modified method for determination of tetracycline, 4-epitetracycline, and anhydrotetracycline in tetracycline base or hydrochloride. J. Pharm. Sci. 61: 615~617, 1972
13) LINDAUER, R. F.; D. M. COHEN & K. P. MUNNELLY: Determination of anhydrotetracycline in tetracycline by high-pressure liquid chromatography. Anal. Chem. 48: 1731~1734, 1976
14) TSUJI, K. & J. H. ROBERTSON: High pressure liquid chromatographic determination of tetracyclines. Anal. Chem. 46: 539~543, 1974
15) TSUJI, K. & J. H. ROBERTSON: Determination of erythromycin and its derivatives by gas-liquid chromatography. Anal. Chem. 43: 818~821, 1971
16) Code of Federal Regulations, Title 21. Parts 141 to 599, U.S. Government Printing Office, Washington, D.C., 1977