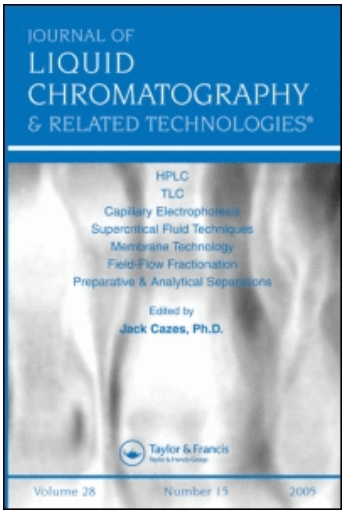

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High Performance Liquid Chromatographic Analysis of Erythromycin

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF ERYTHROMYCIN

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ABSTRACT

Liquid chromatographic analysis can provide a simpler and superior alternative to microbial assay of antibiotics. A number of liquid chromatographic methods for the analysis of erythromycin have been published in the past 17 years. However, many of these methods are complex and lack the selectivity needed for the assay of erythromycin in the presence of erythromycin derivatives and impurities. We examined several C_{18} based stationary phase columns and developed a significantly improved C_{18} liquid chromatographic method for the assay of erythromycin. In comparison to a recently published polymer (poly(styrene-divinylbenzene)) stationary phase LC method, our method is simpler, more rugged, faster, and more sensitive. The developed method has been successfully used for the analysis of erythromycin in commercial bulk samples. The chromatographic assay results correlate with microbiological assay.

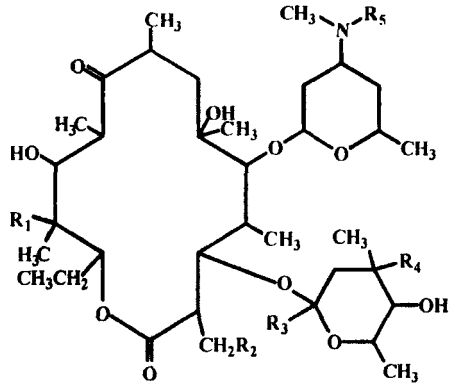
INTRODUCTION

Erythromycin is a widely used broad spectrum macrolide antibiotic. Erythromycin A (EA) is the main component of commercial bulk erythromycin. Erythromycin B (EB), erythromycin C (EC), erythromycin E (EE), N-demethyl erythromycin A (NDEA), erythromycin A enol ether (EEEA), anhydroerythromycin A (AE), and other erythromycin derivatives can be present as impurities. The antimicrobial activity of bulk erythromycin is due mainly to EA, EB & EC forms. Chemical structures of Erythromycin A and related substances are shown in Figure 1.

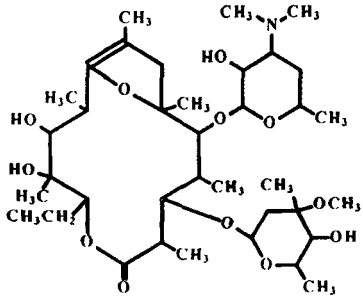
Several methods for the assay of Erythromycin A and related substances in biological samples have been published.[1-7] However, these methods are either not rugged or not suitable for the assay of erythromycin in both bulk and pharmaceutical dosage forms. In the last few years, several attempts were made to develop LC methods suitable for the assay of erythromycin in bulk and solid dosage forms.[8-12] In general, these methods lack the resolution needed for the separation of several related substances, such as EE, and common erythromycin impurities. Because of the presence of EE in commercial products and due to its low antimicrobial activity, a method capable of the separation of EE from other components is desired.[13] A column-switching technique was used by Cachet et al.[14] to separate EA from potential impurities. They succeeded in separating EE from EA, but NDEA was not separated from EE. Nilsson et al.[15] used a polymeric packing material and an alkaline mobile phase for the determination of erythromycin in plasma.

Recently,[16] a method utilizing poly (styrene-divinylbenzene) stationary phase, capable of the separation of EE and EA, was developed and adopted by the European Pharmacopoeia.[17] This European pharmacopoeial method is complex, delicate, and does not provide baseline separation of erythromycin isomers and related substances (such as EEEA), within a reasonable time. In short, the method succeeds in improving the selectivity at the expense of the simplicity and ruggedness needed for the routine assay of erythromycin.

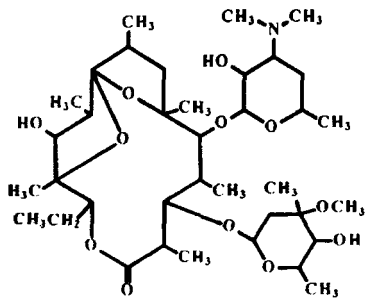
In this manuscript we are presenting a C_{18} based method that is simple, sensitive, rugged, and able to separate and assay erythromycin and most related substances commonly found in commercial samples. DryLab software[18] was used as a tool in method optimization. Computer chromatographic optimization software programs, such as DryLab, have been commercially available for a few years. In addition to speeding up chromatographic method development, these programs can be also utilized to assure the reliability, ruggedness and reproducibility of the separation.



	R_1	R_2	R_3	R_4	R_5
Erythromycin A (EA)	OH	H	H	OCH_3	CH_3
Erythromycin B (EB)	H	H	H	OCH_3	CH_3
Erythromycin C (EC)	OH	H	H	OH	CH_3
Erythromycin E (EE)	OH	—O—		OCH_3	CH_3
N-demethylerythromycin A (NDEA)	OH	H	H	OCH_3	H



Erythromycin A enol ether (EEEA)



Anhydroerythromycin A (AE)

Figure 1. Chemical Structures of Erythromycin A and related substances.

Table 1

Gradient System for the Erythromycin Assay

Time (min.)	%A[a]	% B[b]
0.0	78.0	22.0
39.0	26.0	74.0
47.0	26.0	74.0

[a] Mobile phase A (10% CH_3CN) was prepared by mixing 69 mL stock ammonium phosphate buffer (0.20 M, pH = 6.5), 60 mL stock tetrabutylammonium sulfate (0.20 M, pH = 6.5), and about 250 mL Milli-Q water. This was followed by the addition of 100 mL acetonitrile, diluting to 1 L with milli-Q water, mixing well and filtering through a 0.45 μm nylon membrane filter.

[b] Mobile phase B (50% CH_3CN) was prepared as in "A" with the only exception being the use of 500 mL acetonitrile in the mobile phase preparation.

EXPERIMENTAL

Chemicals and Reagents

USP erythromycin reference standard (RS) was used throughout the study. Other erythromycin standards (EB, EC, EE, NDEA, AE, and EEEA) were obtained from Abbott Laboratories, North Chicago, Illinois. In addition, Abbott Laboratories kindly provided samples of ten different batches of bulk erythromycin along with the relevant analytical data. Two bulk erythromycin samples were provided by the UPJOHN Company (Kalamazoo, Michigan). Several samples of bulk erythromycin were purchased from commercially available sources as identified in Table 4. All erythromycin samples were used without further treatment.

Ammonium hydrogen phosphate, ammonium hydroxide, and tetrabutylammonium hydrogen sulfate were purchased from different sources of the highest available purity and, were used without additional purification. Acetonitrile and methanol were HPLC grade and water was deionized and filtered through a Milli-Q™ water purification system (Millipore, New Bedford, MA).

Solutions

Stock 0.20 M ammonium phosphate buffer was made by dissolving the calculated amount of $(NH_4)H_2PO_4$ in Milli-Q water, the pH adjusted to 6.5 using ammonium hydroxide, and the solution filtered through a 0.45 μm nylon membrane filter. Stock 0.20 M tetrabutylammonium sulfate (mobile phase additive) was made by dissolving the calculated amount of $(C_4H_9)_4NHSO_4$ in Milli-Q water, the pH adjusted to 6.5 using ammonium hydroxide, and the solution filtered through a 0.45 μm nylon membrane filter.

Chromatographic Conditions

The HPLC system used in this investigation consisted of Spectra-Physics SP 8800 pump, Spectra-Physics SP 8880 autosampler, Spectra FOCUS Forward Optical Scanning detector set at 205 nm (unless specified otherwise), COMPAQ DESKPRO XL 5100 computer, and PC1000 System Software™ (Ver. 3.0). Two Prodigy 5 ODS-2 250 x 4.6 mm I.D. columns (Phenomenex, Torrance, CA) were used in the study. The gradient profile and mobile phase composition are described in Table 1. In all experiments, the concentrations of both the buffer and tetrabutylammonium sulfate were maintained constant in both mobile phases and the only difference between mobile phase A and B is the percentage of acetonitrile (10 vs 50, respectively). The gradient delay volume was determined to be 5.5 mL and no equilibration time was needed between injections. The mobile phase flow rate was set at 1.3 mL/min. The column temperature was controlled at 45°C with a block column heater (Jones Chromatography, Lakewood, CO) and the sample injection volume was 50 μL.

Samples were prepared by dissolving the weighed amount in a solvent made by mixing equal volumes of the mobile phases (50%A-50%B) to give a final concentration of 5-10 mg/mL. Samples were placed in an ultrasonic bath for about 5 minutes to enhance dissolution. In developmental experiments, different sets of chromatographic conditions were optimized, selected and employed (as in Figures 2 & 3).

RESULTS AND DISCUSSION

Developmental Experiments

The initial objective was to develop a simple and rugged HPLC method, preferably an isocratic reverse phase procedure, for the assay of erythromycin and most common isomers and impurities found in commercial bulk products.

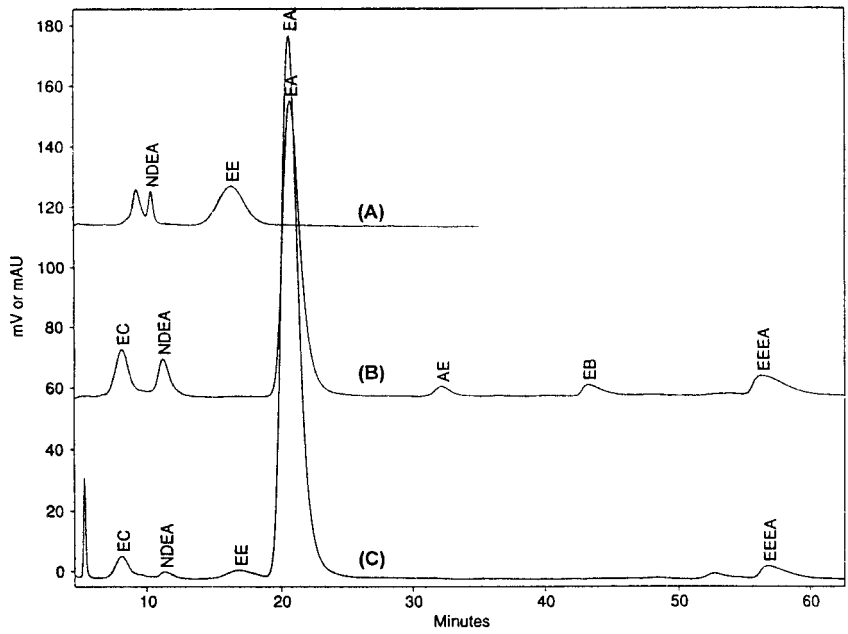


Figure 2. Typical chromatograms (monitored at 205 nm) obtained for (A) standard erythromycin E (EE), (B) a standard mixture of EC, NDEA, EA, AE, EB & EEEA, and (C) a commercial bulk erythromycin sample. Chromatographic conditions: mobile phase was made by mixing 255 mL CH_3CN, 60 mL of tetrabutylammonium sulfate (0.20 M, pH 6.5), 60 mL of ammonium phosphate buffer (0.20 M, pH 6.5) and diluting to 1.0 L with Milli-Q water; flow rate was set at 1.5 mL/min. and temperature was controlled at 45 °C.

After thorough review of the recent literature,[10-14] several commercially available C_{18} columns suitable for the assay of basic compounds, were examined.

Many of the tested C_{18} columns failed to provide baseline separation of NDEA, EE, and EA. Using a Zorbax RX-C_{18} (250 x 4.6 mm I.D.), we succeeded in developing a simple isocratic method capable of the separation of EA, EB, EC, EE, NDEA, AE, and EEEA. Figure 2 provides an illustration of the chromatographic separation achieved and a summary of chromatographic conditions. This method has distinct advantages over the recently adopted European Pharmacopoeial method.[17] The method is simpler and more sensitive.

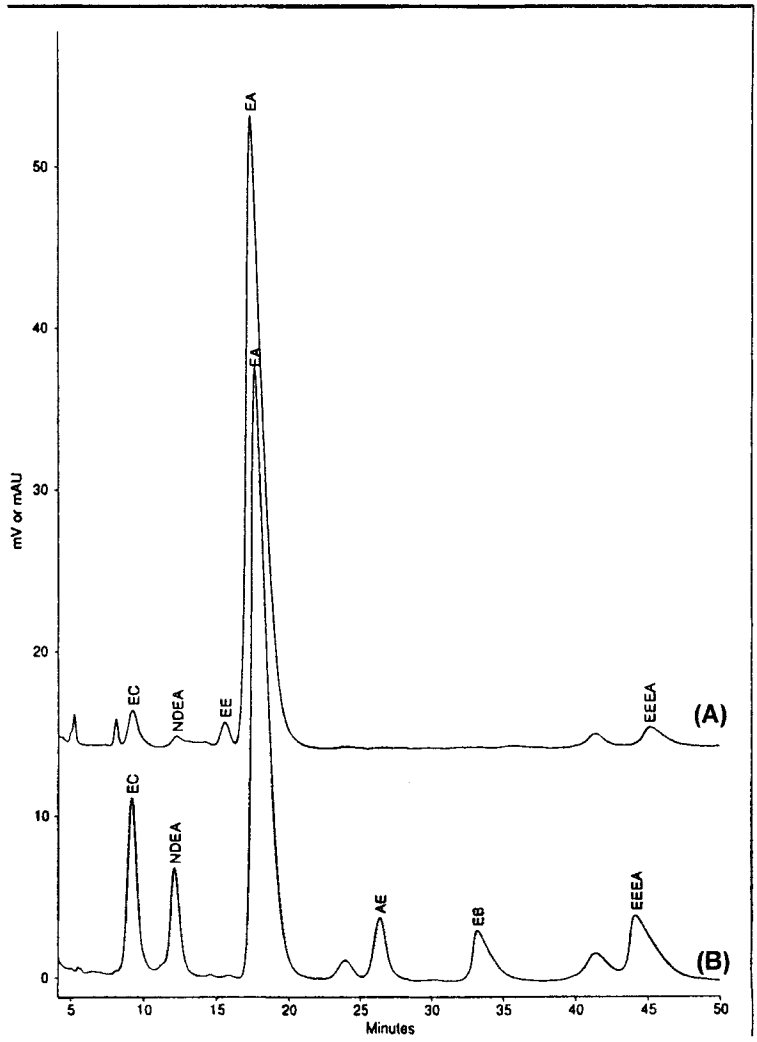


Figure 3. Typical chromatograms (monitored at 215 nm) obtained for (A) a commercial bulk erythromycin sample and (B) a standard mixture of EC, NDEA, EA, AE, EB & EEEA. Chromatographic conditions: mobile phase was made by mixing 500 mL CH_3OH, 100 mL of tetrabutylammonium sulfate (0.20 M, pH 6.5), 125 mL of ammonium phosphate buffer (0.20 M, pH 6.5) and diluting to 1.0 L with Milli-Q water; flow rate was set at 1.5 mL/min. and temperature was controlled at 45 °C.

Major components (including EEEA) are eluted in a reasonable time (less than 60 minutes) and peaks are more symmetrical. However, the EE peak and the late eluted peaks are broad, as expected, in a long isocratic run. In addition, the column life span under the assay conditions is short .

In order to improve method ruggedness and to overcome these disadvantages, another attempt was made utilizing the same Zorbax RX-C18 column. Methanol replaced acetonitrile as the organic modifier and both the buffer and tetrabutylammonium sulfate concentrations were adjusted for optimum separation. Typical chromatograms obtained are illustrated in Figure 3. Compared to acetonitrile, methanol has a similar effect on the system selectivity but the resolution between EE and EA is improved. Also, the use of methanol results in shortening the analysis time (50 minutes versus 60 minutes).

We had an initial success in applying this improved methanol method in the assay of erythromycin in bulk, as well as, finished solid dosage forms. However, the method shared most of the problems observed previously (acetonitrile method) including broad peaks (especially EEEA) and the column packing material instability under the employed chromatographic conditions. At this point, it was apparent that a different approach in C_{18} column selection was needed, aiming at enhancing the stability of the packing material without sacrificing the selectivity of the chromatographic system.

Gradient Method Development

Recently, several articles and application notes have been published, emphasizing the effect of high purity silica on the improvement of the resolution and peak shapes for basic compounds. New generation columns are made from high purity reagents and use improved bonding and packaging technologies . For example, Prodigy™ columns are made using high purity silica and manufacturer data strongly suggest greater stability (> 1000 hours) over a wide pH range (2.0 - 9.0).[19]

Kirkland et al.[20] have suggested recently that some silica-based C_{18} packings can be used for long periods at higher pH, without significant changes in packing material stability. A Prodigy™ column was selected for this investigation.

It has been shown, that the use of quaternary ammonium salts improve the quality of separation of basic compounds on C_{18} reverse phase chromatography, in addition to shortening analysis time possibly by preventing undesirable retardation effects commonly found in C_{18} columns.[10,12,21,22]

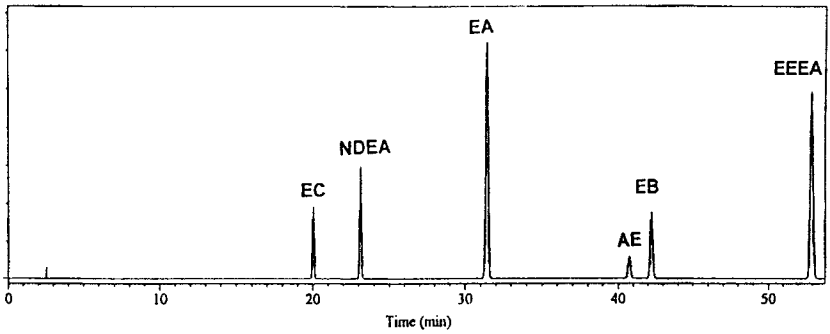


Figure 4. An optimized DryLab chromatogram of a standard erythromycin mixture(EA, EB, EC, NDEA, AE, and EEEA). Chromatographic conditions of preliminary gradient runs are described in Table II. t_0=2.56 min (estimated from preliminary runs), minimum resolution 4.89, and run time 54 min.

Table 2

Chromatographic Conditions used to Generate Data Needed for DryLab Method Optimization[a]

Time (min.)	%A[b]	% B[b]
0.0	80.0	20.0
t_G[c]	20.0	80.0

[a] HPLC system and Column were the same as described in the experimental section. The gradient delay volume was determined to be 5.5 mL, the temperature was controlled at 45 °C and flow rate was set at 1.0 mL/min.

[b] Mobile phases A and B are the same as described in Table 1.

[c] the gradient time (t_{G1}) for the first run was 50.0 minutes, whereas the gradient time for the second run (t_{G2}) was 100.0 minutes.

Tetrabutylammonium sulfate was the quaternary ammonium salt used in this study. Also, from the literature and our previous experience, it seemed that a phosphate buffer at pH 6.5 provides the optimum buffer for the separation without accelerating the degradation of erythromycin.

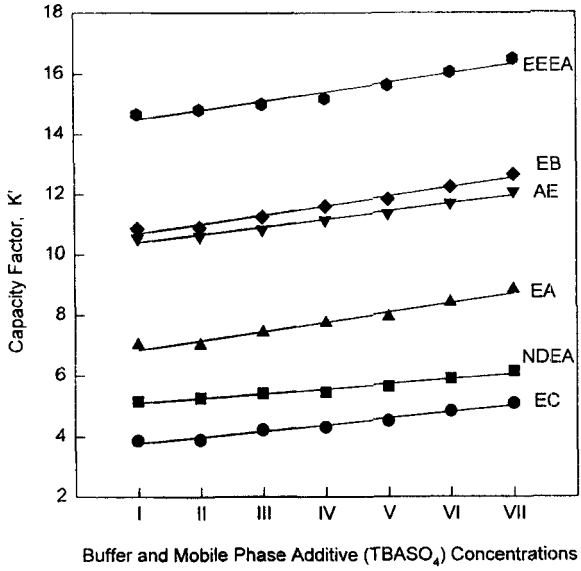


Figure 5. Effect of varying the concentrations of buffer (ammonium Phosphate, pH 6.5) and mobile phase additive (tetrabutylammonium sulfate, pH 6.5) on the separation of erythromycin isomers and related substances . The gradient profile (Table 1) was employed and the temperature was controlled at 45°C.

Mobile Phase	Buffer Conc., mM	Mobile Phase Additive Conc., mM
I	6.0	6.0
II	6.0	12.0
III	12.0	6.0
IV	12.0	12.0
V	12.0	24.0
VI	24.0	12.0
VII	24.0	24.0

DryLab software[18] was one of the tools used in method optimization. Two gradient runs of a standard mixture (made by mixing EA, EB, EC, AE, NDEA, and EEEA) were carried out under the chromatographic conditions described in Table 2 and, the data obtained was used by DryLab to generate a computer optimized separation (Figure 4). Chromatographic parameters generated by DryLab were then optimized further to produce the developed gradient assay

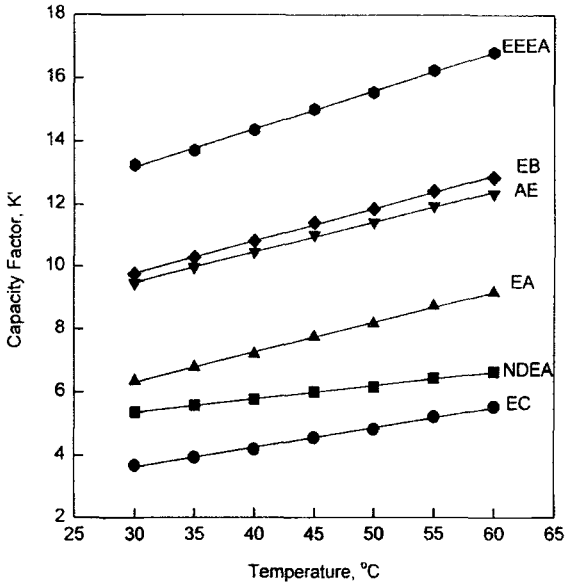


Figure 6. Effect of temperature on the separation of erythromycin isomers and related substances. Details of chromatographic conditions are described in the experimental section and Table 1. The concentrations of both the buffer and tetrabutylammonium sulfate were 12.0 mM.

method (as described in the experimental section and Table 1). Limited success was obtained in the conversion of the gradient method to an isocratic procedure. The isocratic procedure has some undesirable features such as: broad peaks, longer analysis time, and a noticeable decrease in sensitivity.

In order to confirm the ruggedness of the developed method, the effects of temperature, buffer concentration and the concentration of the mobile phase additive (tetrabutylammonium sulfate) on the developed gradient method (as described in the experimental section and Table 1) were investigated. The effect of varying the concentrations of both the buffer (ammonium phosphate, pH 6.5) and the mobile phase additive (tetrabutylammonium sulfate, pH 6.5) on the system selectivity was examined (chromatographic runs I-VII). Figure 5 summarizes the effect of varying the concentrations of the buffer and tetrabutylammonium sulfate on the separation of erythromycin and related substances. Erythromycin E (EE) was not used in the preparation of the

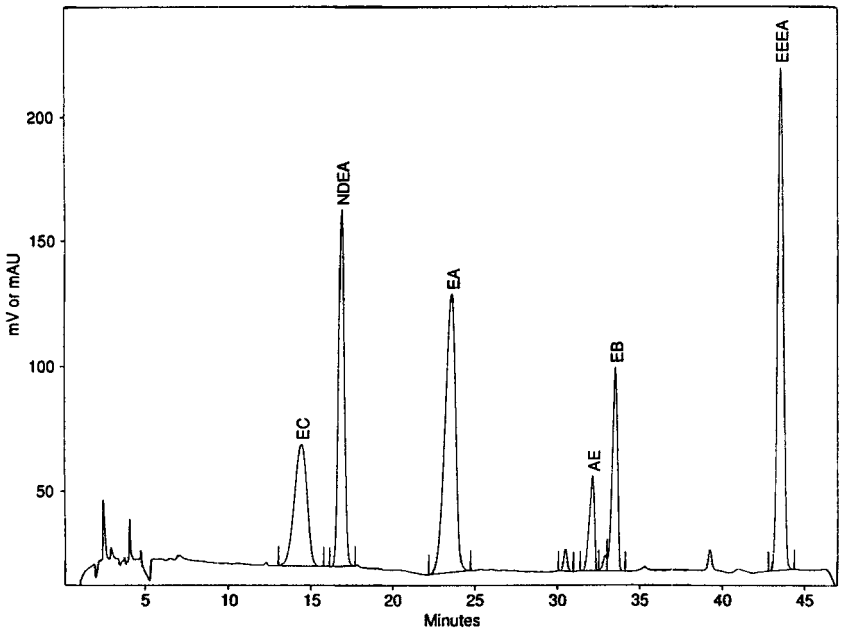


Figure 7. Typical chromatogram of a standard mixture containing EC, NDEA, EA, AE, EB, and EEEA. Details of chromatographic conditions are described in the experimental section and Table1.

standard mixture because only a very small amount (<1 mg) was available and it was used only as an HPLC marker (as in Figure 2). Mobile phase composition IV (12.0 mM buffer and 12.0 mM tetrabutylammonium sulfate) was selected. It provides optimum resolution and increasing the concentration of either component does not enhance the separation significantly.

From Figure 5, it is evident that a slight variation in the concentration of either the buffer or the additive (tetrabutylammonium sulfate) does not affect the selectivity. The effect of temperature on the separation of erythromycin isomers and related substances was also investigated (Figure 6). In order to optimize the separation of erythromycin and foster the stability of the column packing material, a problem encountered in our earlier experiments, the temperature was controlled at 45 °C.

Figure 7 provides an illustration of a typical chromatogram of a standard mixture analyzed, using the developed gradient method, as described in the experimental section and Table 1, whereas Figure 8 is a chromatogram of a

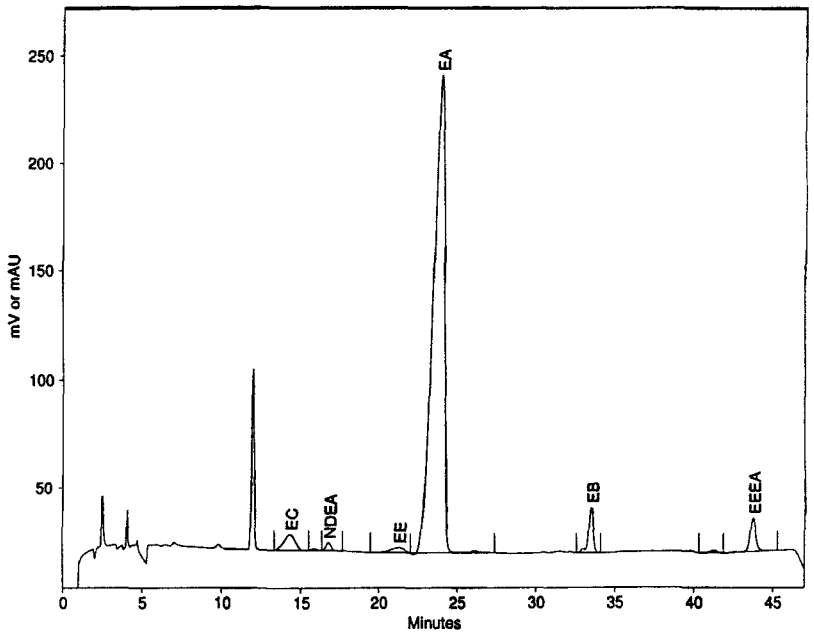


Figure 8. Typical chromatogram (monitored at 205 nm) of a bulk erythromycin sample (10.00 mg/mL) analyzed using the developed method. Details of chromatographic conditions are described in the experimental section and Table 1.

Table 3

HPLC Method Performance, Repeatability (n=5)

Components Found in a Bulk Erythromycin Sample	Retention Time, min. (% RSD)	Peak Area x 10^{-5} (% RSD)	Peak Ht x 10^{-3} (% RSD)
EC	14.4 (0.27)	3.50 (1.41)	7.24 (0.79)
NDEA	16.9 (0.18)	0.87 (2.15)	3.84 (1.45)
EE	21.4 (0.34)	1.68 (5.60)	2.54 (2.43)
EA	24.1 (0.25)	114.0 (0.37)	222.1 (0.28)
AE	32.1 (0.16)	0.30 (5.27)	2.01 (5.07)
EB	33.6 (0.17)	4.17 (0.48)	21.19 (0.43)
EEEA	43.8 (0.17)	3.53 (2.71)	14.79 (1.77)

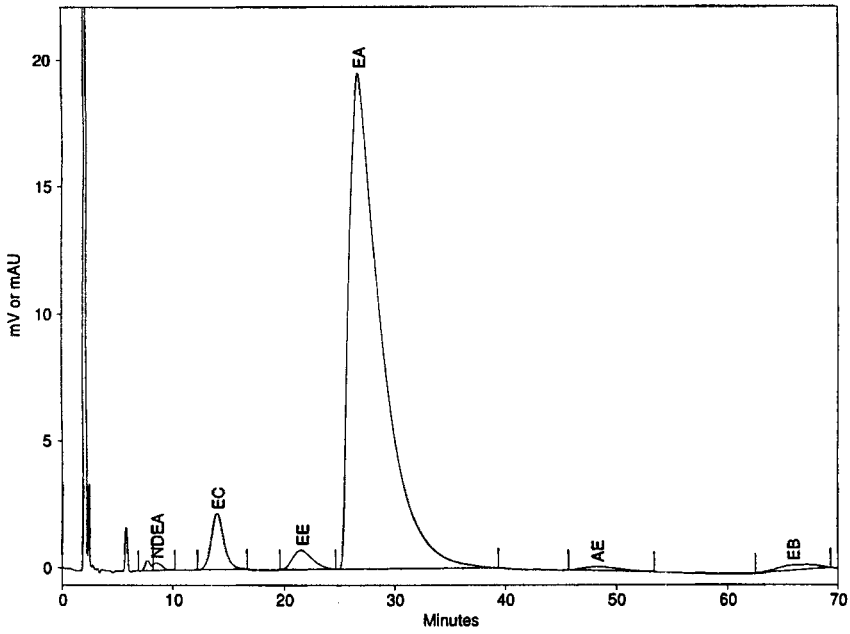


Figure 9. Typical chromatogram (monitored at 215 nm) of a bulk erythromycin sample (same sample as in Figure 8 at a concentration of 7.70 mg/mL) analyzed using the European Pharmacopoeial Method (17). EEEA was not observed due to its longer retention (greater than 80 minutes).

commercial bulk erythromycin sample. The result of method performance repeatability test (Table 3) illustrates the high precision obtained. In another test for system ruggedness, packing materia batch-to-batch evaluation was performed by using two Prodigy columns: column 1 (batch 17M2; serial number 103575) and column 2 (batch 19M; serial number 113448). The chromatography obtained in both cases was nearly identical and there was no apparent difference in system selectivity.

The same commercial sample was analyzed using the recently adopted European Pharmacopoeial method,[16,17] in which a Polymer Lab 250 x 4.6 mm I.D. PLRP-S column (1000 Å, 8 μ) was used at a flow rate of 1.0 mL/min (Figure 9). Due to high backup pressure and baseline noise, the flow rate was reduced from 2.0 mL/min as specified by the European Pharmacopoeia. Others involved in the validation of the European Pharmacopoeial method experienced

Table 4

HPLC Assay of Commercial Bulk Erythromycin Products

Commercial Bulk Products[a]	% Erythromycin[b,c] (% RSD)
Boehringer Manneheim, Lot No. 13742321-21	91.2 (0.34)
UPJOHN, Lot No. 306AA	96.7 (0.16)
UPJOHN (Micronized), Lot No. 159MH	96.4 (0.24)
Pharma-Tek ERYTHRO-R_x, Lot No. ZJ4E	94.1 (0.18)
ICN Biomedicals, Lot No. 50148	101 (0.19)
Fluka BiomChemika, Lot No. 333594/1	95.7 (0.12)
Spectrum, Lot No. KC122	96.9 (0.45)
SIGMA, Lot No. 31H0577	97.2 (0.22)
SIGMA, Lot No. 61H03496	99.0 (0.42)

[a] Samples were dissolved in a solvent made of 50%A and 50%B (as described in the experimental section) at concentrations of 5-10 mg/mL.
[b] Average of 3 runs.
[c] Calculated as the sum of erythromycins A, B, and C. Erythromycins B and C calculations were based on EA response factor.

the same difficulty.[23] Both the European Pharmacopoeial method and our developed method, succeed in the separation of erythromycin isomers and many of its impurities. Comparing Figures 8 and 9, the enhancement in sensitivity (ten fold), peak symmetry, selectivity, and resolution the developed gradient method provides should be noted. Also, the developed method is simpler and has a shorter anlaysis time.

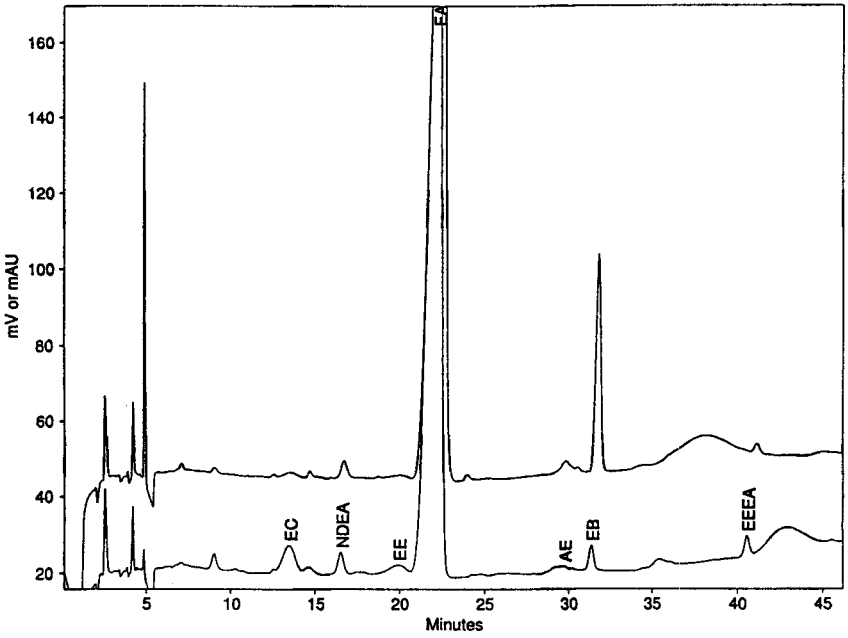


Figure 10. An illustration of detected differences in chromatographic patterns distinguishing two major United States bulk erythromycin manufacturers. Details of chromatographic conditions are described in the experimental section and Table 1.

Stability of Erythromycin in Sample Solutions

Recent literature addressed the stability of erythromycin solutions.[24,25] Erythromycin solutions were found to be relatively stable in neutral media (pH 6-8)[24] in both methanol and acetonitrile solutions.[25] However, acetonitrile solutions were more stable.[25] Since the solvents used in these studies[24,25] were different from the sample solutions (50%A - 50%B) used in this investigation, the stability of erythromycin in sample solutions was tested both at room temperature and 45.0 °C in order to accelerate any possible degradation. Erythromycin solutions were stable at room temperature for more than 48 hours and a degradation product with similar retention to EB was produced in measurable amounts after 5 days. At 45.0 °C, solutions were stable up to 4 hours, after which chromatographic interferences with EB was noticeable. In summary, erythromycin solutions are stable at room temperature many hours longer than needed under the described analysis conditions and the production of degradation product(s) should not affect the accuracy of the assay.

Table 5

HPLC Assay of Ten Different Lots of Abbott's Bulk Erythromycin

Lot Number	Pharm. Eur. (17)[a,b]	USP Bioassay[a]	HPLC Assay[b,c]
1	91.4%	90.6%	92.8%
2	93.4%	94.2%	90.2%
3	93.0%	96.1%	86.6%
4	91.5%	93.4%	88.1%
5	90.9%	91.8%	93.5%
6	89.8%	91.9%	90.8%
7	94.7%	93.2%	94.8%
8	90.9%	92.6%	93.2%
9	91.4%	93.6%	90.8%
10	89.2%	92.1%	89.3%

[a] Analytical data was provided by Abbott Laboratories.
[b] Calculated as the sum of erythromycins A, B, and C.
[c] Average of 3 runs.

Assay of Bulk Erythromycin Samples

Several commercial samples of bulk erythromycin were obtained and assayed using the developed method. The results of the assay are summarized in Table 4. The percentage of erythromycin in these samples was calculated as the sum of EA, EB, and EC. The absorptivities of erythromycin isomers and related substances in the sample solution (50%A - 50%B) at 205 nm were determined and, it was found that the relative absorptivity of EB/EA = 0.88 and EC/EA = 1.2. In spite of these differences, the concentrations of both EB and EC were calculated using the response factor of EA.

The ability of the developed method in the separation of erythromycin and its common impurities, coupled with the enhanced sensitivity especially for late eluted peaks (such as EB & EEEA), makes this method a useful technique in distinguishing different sources of bulk erythromycin.

Figure 10 provides an illustration of detected differences in chromatographic patterns of two major United States bulk erythromycin manufacturers. Chromatographic fingerprinting is becoming a useful tool in the identification of bulk pharmaceutical sources and in fraud investigations.[26]

Ten different samples, representing ten different batches, were obtained from Abbott Laboratories (North Chicago, Illinois) and analyzed using the developed gradient method. The results of their assay using this HPLC method, the European Pharmacopoeial method, and USP bioassay procedure are summarized in Table 5. The results in Table 5 illustrates the good correlation obtained for most batches between the developed method, with both the USP bioassay and the European Pharmacopoeial assay procedure.

ACKNOWLEDGMENT

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