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Improved High-Pressure Liquid Chromatographic Method for the Analysis of Erythromycin in Solid Dosage Forms

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Abstract □ A stability-indicating high-pressure liquid chromatographic (HPLC) method for the assay of erythromycin in enteric film-coated tablets was developed. The method used a reversed-phase column at 70° with a mobile phase of acetonitrile–methanol–0.2 *M* ammonium acetate–water (45:10:10:35) at pH 7.0. The column effluent was monitored at 215 nm. Several reversed-phase columns were evaluated for the analysis of erythromycin. The HPLC method was also applicable for the analysis of salts and esters of erythromycin. The linearity and precision of the HPLC assay method for erythromycin in the solid dosage form were examined by spiking erythromycin into a tablet placebo at 60–120% of the label. The recovery of erythromycin was 99.9% with a relative standard deviation of <1%. The correction factors to express the results of HPLC in terms of antimicrobial bioequivalency against *Staphylococcus aureus* ATCC 6538P for erythromycins A, B, and C were determined to be 1.0, 0.92, and 0.48, respectively. Eight lots of tablets were assayed by the HPLC method, and the results, expressed in terms of erythromycin bioequivalency, showed no statistically significant difference from those of the microbiological assay method.

Keyphrases □ High-pressure liquid chromatography—improved method for the analysis of erythromycin in solid dosage forms □ Dosage forms—improved high-pressure liquid chromatographic method for the analysis of erythromycin □ Erythromycin—solid dosage forms, improved high-pressure liquid chromatographic method for analysis

Erythromycin, a macrolide antibiotic, is normally administered orally and is marketed in several forms. These include the free base, salts such as the stearate, and esters such as ethyl succinate and 2′-*O*-propionyl. Erythromycin free base is formulated as an enteric coated tablet to protect erythromycin from acid degradation and to allow absorption in the intestinal tract.

High-pressure liquid chromatographic (HPLC) methods for the determination of erythromycin free base and an ester of erythromycin, erythromycin ethyl succinate, have been reported (1–4). The HPLC assay method for erythromycin ethyl succinate uses an elevated column temperature at 70° to minimize peak tailing and to improve peak resolution (4). This HPLC method was adopted for monitoring the clinical blood level of erythromycin and erythromycin ethyl succinate by use of a postcolumn extraction and derivatization technique for fluorimetric detection (5).

Since the HPLC method reported for the assay of erythromycin uses ambient column temperature (1), considerable peak tailing and lack of peak resolution have been experienced. The method reported in this paper uses elevated column temperature for optimum peak resolution and minimum peak tailing for the assay of erythromycin in a solid dosage form.

EXPERIMENTAL

Apparatus—A modular liquid chromatograph equipped with a variable wavelength detector at 215 nm[1], a high-pressure pump[2], and a 100-μl fixed loop injector[3] were used. A reversed-phase HPLC column[4] was water jacketed and maintained at 70° with a circulating water bath[5]. The peak area was electronically determined by use of an electronic integrator[6].

Reagent—All the solvents used were distilled in glass and were UV grade[7]. Ammonium acetate used was analytical reagent grade. The mobile phase composed of acetonitrile–methanol–0.2 *M* ammonium acetate–water (45:10:10:35) was filtered through a membrane filter[8]. The flow rate of the mobile phase was ~1.0 ml/min.

The 0.2 *M* ammonium acetate was prepared by weighing 15.5 g of ammonium acetate[9] into a 1-liter graduated cylinder and adding water to volume.

[1] Model 1201 Spectromonitor I, Laboratory Data Control, Riviera Beach, Fla.

[2] Model 196-0066-02 High-Pressure Mini Pump, Laboratory Data Control.

[3] Model 70-10 Loop Injector, Rheodyne, Berkeley, Calif.

[4] 18-5A, LiChrosorb RP-18, Brownlee Labs, Santa Clara, Calif.

[5] Lauda K-2/R controlled-temperature circulating water bath, Brinkmann, Lauda, GFR West Germany.

[6] Chromatopac-E1A, Shimadzu Seisakusho, Ltd., Kyoto, Japan.

[7] Burdick and Jackson Labs, Muskegon, Mich.

[8] Catalog No. FHLP04700 Fluoropore Filter, Millipore Corp., Bedford, Mass.

[9] Mallinckrodt, Inc., Paris, Ky.

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Reference Standard Solution—USP erythromycin reference standard was dried at 60° for 3 hr under <5-mm Hg pressure. The dried bottle was capped and placed in a desiccator to cool. Approximately 10 mg of the reference standard was weighed accurately using an electronic balance[10] and placed in a 10-ml volumetric flask. The standard was dissolved and diluted to volume with the internal standard solution.

Internal Standard Solution—A sufficient quantity of megestrol acetate was dissolved in the mobile phase to give a final concentration of ~0.025 mg/ml.

Sample Preparation—Approximately 10 mg of erythromycin powder was accurately weighed into a 10-ml volumetric flask. The powder was dissolved and diluted to volume with the internal standard solution.

To assay solid dosage forms, 10 tablets were accurately weighed to compute an average tablet weight. Tablets were ground to a fine powder using a laboratory mill[11] with a 40-mesh screen. Approximately 18 mg of the ground tablet, or an equivalent quantity of the powder to contain ~10 mg of erythromycin, was accurately weighed using an electronic balance[10] into a 10-ml volumetric flask. The internal standard solution was added to the flask, sonicated for ~1 min, and shaken briefly to facilitate dissolution. The suspension was then centrifuged at 2000 rpm for ~2 min to remove tablet excipients.

Calculation—The erythromycin content in a tablet was calculated using:

$$\text{Erythromycin (mg/tablet)} = \frac{Pa + 0.9Pb + 0.5Pc}{Ta + 0.9Tb + 0.5Tc} \times \frac{W_t}{W_p} \times \frac{I_t}{I_p} \times F_1 \times \frac{F_2}{1000} \quad \text{(Eq. 1)}$$

where Pa is the peak area of erythromycin A of the sample; Pb is the peak area of erythromycin B of the sample; Pc is the peak area of erythromycin C of the sample; Ta is the peak area of erythromycin A of the reference standard; Tb is the peak area of erythromycin B of the reference standard; Tc is the peak area of erythromycin C of the reference standard; W_t is the weight of the reference standard in milligrams per milliliter; I_t is the peak area of the internal standard in the reference standard; I_p is the peak area of the internal standard of the sample; W_p is the weight of the sample in milligrams per milliliter; F_1 is an average tablet weight in milligrams per tablet; F_2 is the assigned potency of the reference standard in micrograms per milligram; and 1000 is a factor to convert the potency of the reference standard to a fraction.

Microbiological Assay Method—The turbidimetric assay method using *Staphylococcus aureus* ATCC 6538P as the test microorganism as described in the Code of Federal Regulations (6) was used to assay erythromycin.

RESULTS AND DISCUSSION

Chromatographic Conditions—The effect of mobile phase composition and pH on the chromatographic behavior of erythromycins A, B, and C have been reported (1). Increasing the concentration of acetonitrile or methanol or decreasing the pH of the mobile phase was found to reduce the retention times of erythromycins A, B, and C. The selection of pH for the mobile phase was determined by the type of sample under investigation, with pH 6.2 for the bulk drug and pH 7.8 for biological extracts.

Increase in the column temperature was found to significantly improve column performance for the assay of erythromycin. The effects of column temperature on the height equivalent to theoretical plate (HETP) for LiChrosorb RP-18[4] and μ-Bondapak C_{18}[12] columns are presented in Fig. 1. The HETP for the μ-Bondapak C_{18} column was significantly smaller than that of the LiChrosorb RP-18 at a column temperature <50°. However, lines of two-column performance intersected at ~70°. The HETP for the LiChrosorb RP-18 was slightly smaller than that of μ-Bondapak C_{18} at the column temperature of 80°. Although column performances increased up to 80°, 70° was selected for the assay of erythromycin. The 70° temperature represents a compromise between column performance and column stability. Most column manufacturers recommend that the column not exceed 70°. The HPLC column was stable for more than 1 month when operated at 70° if the mobile phase was saturated with silica.

Increase in temperature has been shown to increase the rate of mass transfer or diffusion of the solute to the stationary phase (7). This most likely has resulted in improved column efficiency.

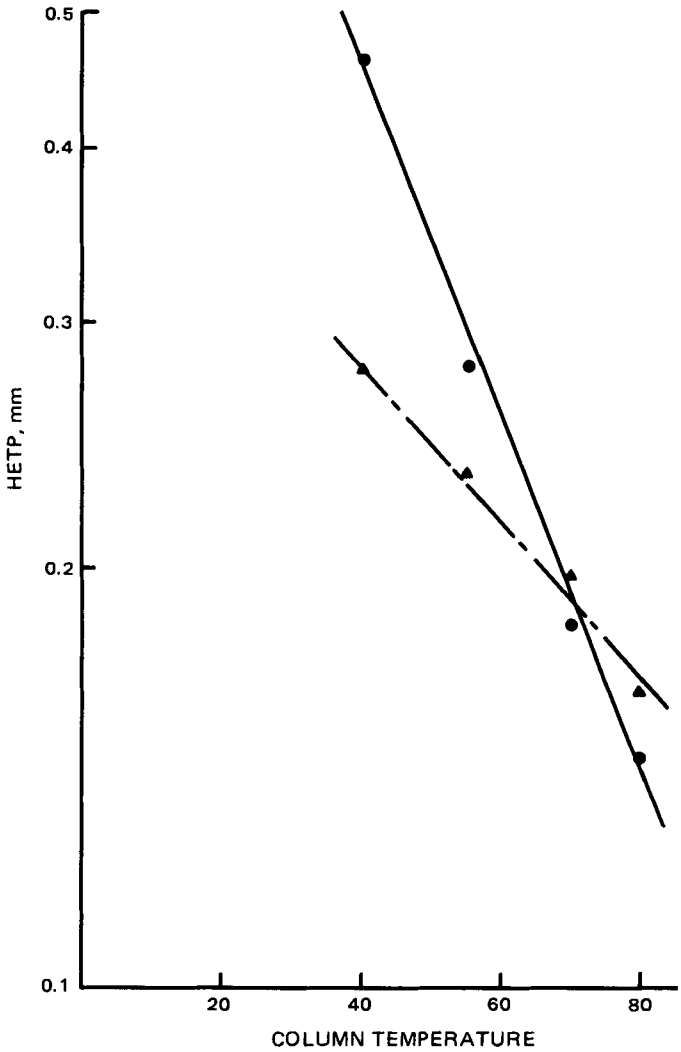

Figure 1—*Effect of column temperature on HETP. Key: (●) LiChrosorb RP-18; (▲) μ-Bondapak C_{18}.*

Selection of Column—Several HPLC columns were evaluated for analysis of erythromycin using the mobile phase composed of acetonitrile–methanol–0.2 *M* ammonium acetate–water (45:10:10:35) at pH 7.0. This pH was selected as a compromise between shorter retention times and better resolution. The following HPLC columns were evaluated: LiChrosorb RP-18[4], μ-Bondapak C_{18}[12], Spherisorb RP-18[13], Partisil ODS[14], Partisil ODS-3[14], and Zorbax ODS[15]. Erythromycin A eluted in 8–10 min from LiChrosorb RP-18, Partisil ODS, and Partisil ODS-3 columns. However, it was found necessary to increase the acetonitrile concentration to 60% to obtain comparative retention time for the erythromycin A peak from Spherisorb RP-18 and Zorbax ODS columns. These latter columns are packed with spherical particles.

The columns were evaluated on the basis of HETP, tailing factor for the erythromycin A peak, and the peak resolution between erythromycins A and C. The data are presented in Table I. The LiChrosorb RP-18 and μ-Bondapak C_{18} columns were judged to give good overall performance for the analysis of erythromycin. A typical chromatogram of erythromycin powder using the LiChrosorb RP-18 column is shown in Fig. 2.

The relative retentions of erythromycins A, B, and C, as well as various impurities and degradation products on the LiChrosorb RP-18 column, are listed in Table II. Although the relative retentions of these compounds were slightly different from those reported previously using a μ-Bondapak C_{18} column at room temperature (1), the order of elution was not affected.

A peak which frequently appears on GLC chromatograms using an

[10] Cahn 21 Automatic Electrobalance, Ventron Corp., Cerritos, Calif.
[11] Wiley Mill model 3383, A. H. Thomas, Philadelphia, Pa.
[12] Waters Associates, Milford, Mass.
[13] Brownlee Labs.
[14] Whatman Ltd., Clifton, N.J.
[15] DuPont Instrument Co., Wilmington, Del.

Table I—Evaluation of Various Reversed-Phase HPLC Columns for the Analysis of Erythromycin

Column at 70°	Column Parameters		
	HETP, mm	Tailing Factor[a]	Resolution[b]
LiChrosorb RP-18	0.182	2.0	2.5
μ-Bondapak C_{18}	0.198	2.0	2.5
Spherisorb RP-18	0.227	2.1	1.9
Partisil ODS	0.244	2.1	1.6
Partisil ODS-3	0.303	2.0	1.9
Zorbax ODS	0.530	2.8	1.7

[a] For erythromycin A peak. [b] Between peaks of erythromycins A and C.

Table II—Relative Retention of Various Erythromycins [a]

Compound	Relative Retention
Erythronolide B	0.73
Erythromycin C	0.78
Erythromycin A	1.00
8-Epi-10,11-anhydroerythromycin A	1.22
Erythralosamine	1.32
Anhydroerythromycin A	1.38
Erythromycin B	1.46
Dihydroerythromycin A	1.74
8,9-Anhydro-6,9-hemiketal erythromycin	2.20

[a] LiChrosorb RP-18 at 70°; mobile phase: acetonitrile–methanol–0.2 *M* ammonium acetate–water (45:10:10:35), pH 7.0.

OV-225 column and tentatively reported as an acid hydrolyzed erythromycin (8) was identified as 8,9-anhydro-6,9-hemiketal erythromycin (9). Although this compound is often inherently present in erythromycin powder, additional amounts can easily be formed from erythromycin under the silylation conditions employed for the GLC assay method. This was not a problem for this HPLC method.

Megestrol acetate, selected as the internal standard for the LiChrosorb RP-18 column, eluted between erythromycin B and 8,9-anhydro-6,9-hemiketal erythromycin peaks (Fig. 3). Calusterone, which elutes immediately after the erythromycin peak, would be suitable as an internal standard when a μ-Bondapak C_{18} column is used (Fig. 4).

Antimicrobial Activity—Since erythromycins A, B, and C differ in both peak response and antimicrobial activity, correction factors must be applied to each of these biologically active compounds so that the total antimicrobial potency of a sample as determined by HPLC correlates with that of the microbiological assay method. The correction factors for

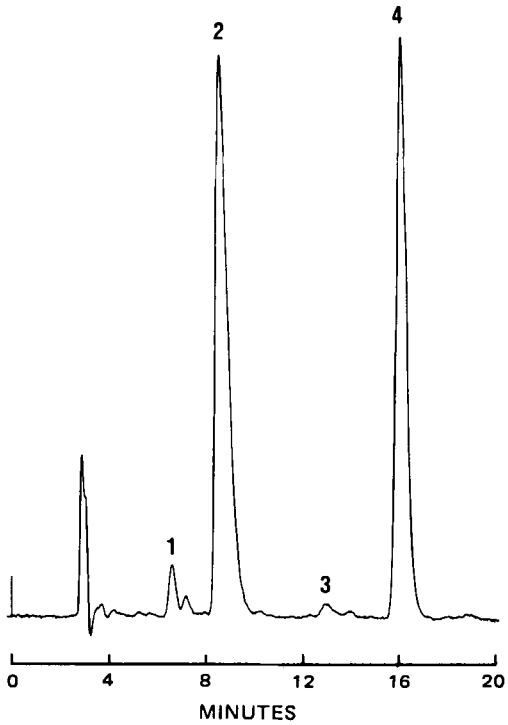

Figure 3—*Reversed-phase HPLC chromatogram of erythromycin USP with internal standard on LiChrosorb RP-18 at 70°. Mobile phase: acetonitrile–methanol–0.2 M ammonium acetate–water (45:10:10:35) at pH 7.0. Peak identification: 1, erythromycin C; 2, erythromycin A; 3, erythromycin B; 4, megestrol acetate.*

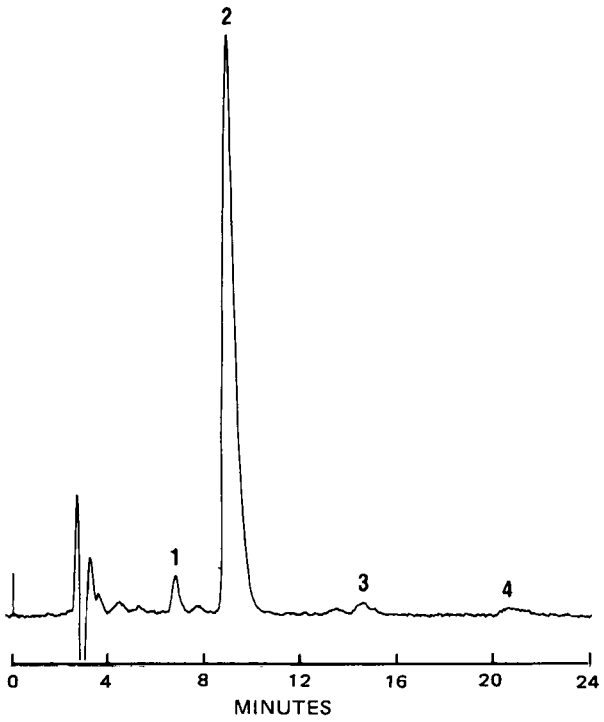

Figure 2—*Reversed-phase HPLC chromatogram of erythromycin USP on μ-Bondapak C_{18} at 70°. Mobile phase: acetonitrile–methanol–0.2 M ammonium acetate–water (45:10:10:35) pH 7.0. Peak identification: 1, erythromycin C; 2, erythromycin A; 3, erythromycin B; 4, 8,9-anhydro-6,9-hemiketal erythromycin.*

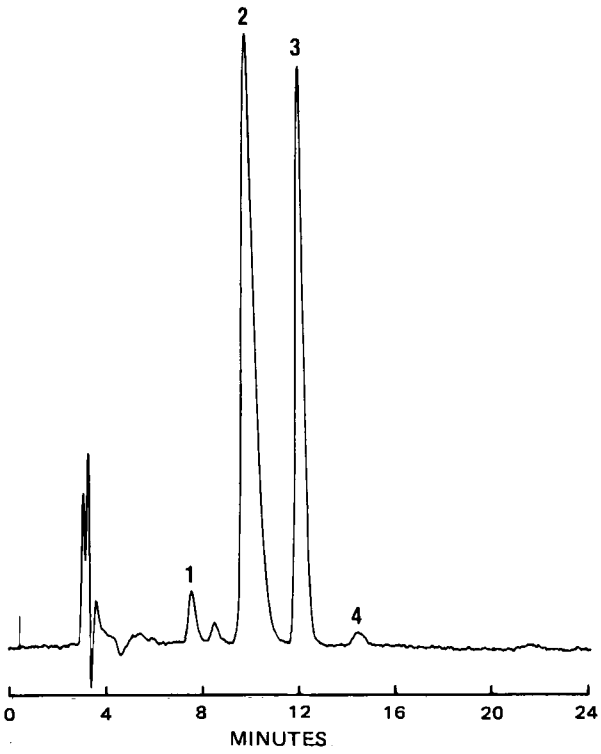

Figure 4—*Reversed-phase HPLC chromatogram of erythromycin USP with calusterone internal standard on μ-Bondapak C_{18} at 70°. Mobile phase: acetonitrile–methanol–0.2 M ammonium acetate–water (45:10:10:35) at pH 7.0. Peak identification: 1, erythromycin C; 2, erythromycin A; 3, calusterone; 4, erythromycin B.*

Table III—Correction Factors in the Determination of Erythromycin Bioequivalency

	Relative Response[a], area/weight	Potency[a], μg/mg	Correction Factor
Erythromycin A[b]	1.00	1000	1.00
Erythromycin B	0.86	787	0.92
Eryythromycin C	1.02	487	0.48

[a] Values obtained against *S. aureus* ATCC 6538P were corrected for purity determined by HPLC. Unidentified impurities were assumed to have an equal peak response. [b] USP Reference Standard Issue H-1.

Table IV—Relative Retention of Erythromycin Ethyl Succinate and Impurities[a]

Compound	Relative Retention
Erythromycin A	0.45
Anhydroerythromycin A	0.56
Erythromycin C ethyl succinate	0.69
8,9-Anhydro-6,9-hemiketal erythromycin A	0.78
Erythromycin A ethyl succinate	1.0
Erythromycin B ethyl succinate	1.4
Anhydroerythromycin ethyl succinate	1.7
8,9-Anhydro-6,9-hemiketal erythromycin ethyl succinate	2.2

[a] LiChrosorb RP-18 at 70°; mobile phase: acetonitrile–0.2 *M* ammonium acetate–water (60:10:30), pH 7.4.

erythromycins A, B, and C are 1.0, 0.92, and 0.48, respectively, when tested turbidimetrically using *S. aureus* ATCC 6538P (6) (Table III). These values were obtained by comparison of the relative chromatographic area responses and antimicrobial activities of erythromycins B and C with the erythromycin A reference standard. The relative response factors for erythromycins A, B, and C of 1.0, 0.5, and 0.4, respectively, reported previously were obtained by use of less pure samples of erythromycins B and C and a different test microorganism, *S. aureus* ATCC 9144 (8). Differences in the strain of *S. aureus* could account for the difference in the relative responses of erythromycin B and erythromycin A.

Assay of Erythromycins—Versatility of the HPLC method for the analysis of esters and salts of erythromycin has been demonstrated. HPLC chromatograms of erythromycin estolate, erythromycin ethyl-

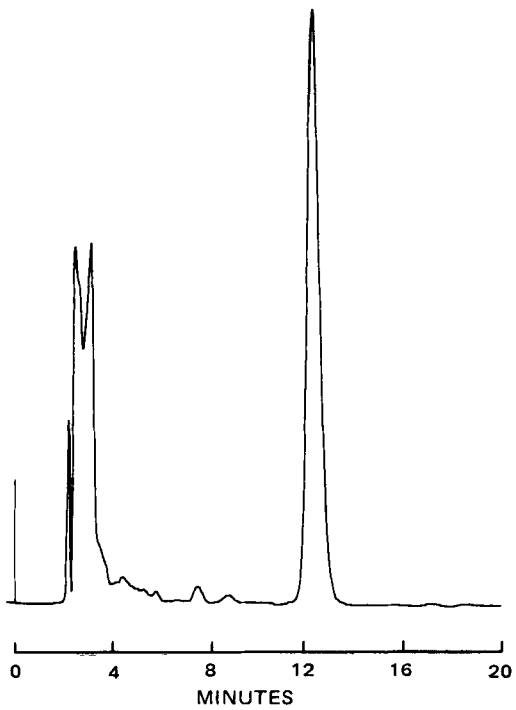

Figure 5—*HPLC chromatogram of erythromycin estolate on μ-Bondapak C_{18} at 70°. Mobile phase: acetonitrile–0.2* M *ammonium acetate–water (60:10:30) at pH 7.0.*

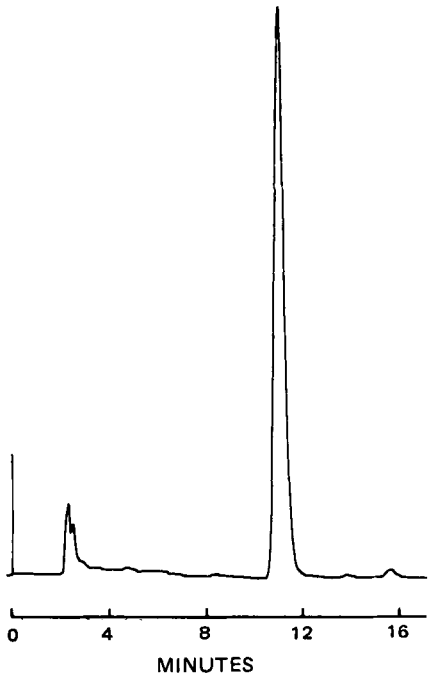

Figure 6—*HPLC chromatogram of erythromycin ethyl carbonate on μ-Bondapak C_{18} at 70°. Mobile phase: acetonitrile–0.2* M *ammonium acetate–water (60:10:30) at pH 7.0.*

carbonate, and erythromycin propionate are shown in Figs. 5–7. Chromatograms were obtained with a μ-Bondapak C_{18} column at 70° with mobile phase of acetonitrile–0.2 *M* ammonium acetate–water (60:10:30) at pH 7.0. The data shown in Table IV indicate the capability of HPLC

Table V—Precision of HPLC Assay for Erythromycin

Erythromycin, mg/ml	Peak Area Ratio, Erythromycin/Internal Standard	Peak Area Ratio/ Erythromycin Concentration
0.9256	1.039	1.123
0.8907	1.013	1.137
0.9126	1.030	1.129
0.8981	1.011	1.126
0.9042	1.015	1.123
0.9150	1.016	1.110
		RSD: 0.8%

Table VI—Linearity of Recovery of Erythromycin from Tablet Placebo

Erythromycin Added, mg	Erythromycin Recovered, mg	Recovery, %
6.242	6.273	100.5
7.928	7.841	98.9
9.969	10.069	101.0
11.949	11.830	99.0
13.993	14.035	100.3
		Mean: 99.9%
		RSD: 0.9%
		Correlation Coefficient: 0.9996
		$y = 0.9999x - 0.0058$

Table VII—Precision of Recovery of Erythromycin from Tablet Placebo

Erythromycin Added, mg	Erythromycin Recovered, mg	Recovered, %
9.969	10.069	101.0
9.974	9.884	99.1
10.123	9.981	98.6
10.359	10.266	99.1
9.984	10.014	100.3
10.276	10.297	100.2
		Mean: 99.7
		RSD: 0.9%

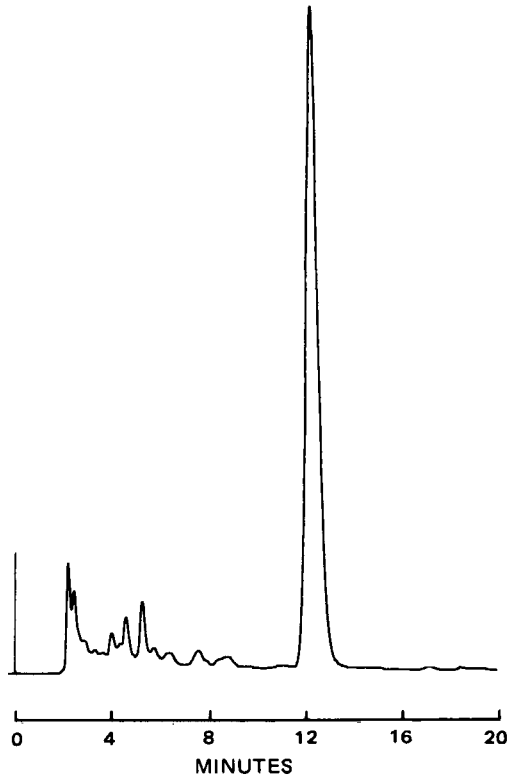

Figure 7—*HPLC chromatogram of erythromycin propionate on μ-Bondapak C_{18} at 70°. Mobile phase: acetonitrile–0.2 M ammonium acetate–water (60:10:30) at pH 7.0.*

to differentiate an ester of erythromycin, erythromycin ethyl succinate, from its impurities. The peaks, EES-C1 and EES-C2, reported previously (2) have been identified as erythromycin C ethyl succinate and 8,9-anhydro-6,9-hemiketal erythromycin A. For quantification of erythromycin, the HPLC response was linear over a concentration range of 0.5–1.3 mg of erythromycin/ml with a correlation coefficient of 0.9999. Six individually weighed and prepared samples were analyzed at an erythromycin concentration of 0.9 mg/ml. The relative standard deviation (*RSD*) of the assay was 0.8% (Table V).

Enteric film coated tablets were then examined for erythromycin content by HPLC. No interference from placebo was experienced. Linearity of recovery was studied by spiking erythromycin into placebo at ~60–120% of label. The average recovery was 99.9% (*RSD* 0.9%). The correlation coefficient for linearity was 0.9996 with the slope of 0.9999 (Table VI). The precision of the HPLC assay for tablets was determined by analyzing six replicate samples prepared at 100% of the label requirements. The average recovery was 99.7% (*RSD* 0.9%) (Table VII).

Table VIII—Comparison of HPLC and Microbiological Assay Methods for the Determination of Erythromycin in Enteric Film Coated Tablets

Lot Number	Erythromycin Content, mg/tablet	
	HPLC (bioequivalency)	Microbiology (potency)
A	263	257
B	261	250
C	267	250
D	262	260
E	251	251
F	263	246
G	335	347
H	328	347

Eight lots of tablets were assayed. The results of HPLC were expressed in terms of bioequivalency using the correction factors described earlier. The results of the HPLC and the microbiological assay methods are shown in Table VIII. No statistically significant difference was noted between the HPLC and the microbiological assay methods.

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