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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF ERYTHROMYCIN

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SUMMARY

An isocratic, reversed-phase high-performance liquid chromatographic (HPLC) method has been developed for the determination of erythromycin. The method uses a μBondapak C_{18} reversed-phase column with a mobile phase composed of acetonitrile–methanol–0.2 *M* ammonium acetate–water (45:10:10:35). The relative standard deviation of the method for quantification of erythromycin is about 0.6%, and the method is capable of selectively quantifying erythromycins A, B, C and at least nine other erythromycin epimers and degradation compounds in 15 min of chromatographic time. The bio-equivalent potencies of erythromycin powders calculated from the HPLC data agreed well with those of the microbiological assay method. The HPLC method developed is also applicable for the analysis of various derivatives of erythromycin.

INTRODUCTION

A commercial preparation of erythromycin usually contains erythromycins A, B, and C. Erythromycin A is the major component and is the most active antimicrobial agent[1]. Under acidic conditions, erythromycins degrade to anhydroerythromycin and 8,9-anhydroerythromycin 6,9-hemiketal[2]. Enzymes in microorganisms inactivate erythromycin and form erythralosamine, erythronolide, and other compounds[3].

Many oral-dosage forms of erythromycin are esterified at the 2′-position of the desosamine moiety to stabilize erythromycin in gastric fluid. These esters of erythromycin are inactive until hydrolyzed[4]; apparent activity of these esters is due to hydrolysis by a test microorganism during incubation period of microbioassay. Therefore, it is imperative to determine specifically erythromycin base in serum for *in vivo* rate of hydrolysis of a pro-drug for therapeutic efficacy study. A method is needed to separate and precisely quantitate erythromycin, its derivatives, and degradation compounds.

Several paper chromatographic[5,6] and thin-layer chromatographic[7,8] methods are available for separation of erythromycins; however, these methods are not suitable for precise quantification. A gas–liquid chromatographic (GLC) method reported by

Tsuji and Robertson[1,9] is capable of separating and quantifying erythromycins, their derivatives, and degradation compounds; however, the method requires at least 24 h derivatization and extensive sample clean-up processes to analyze erythromycin in a complex biological matrix.

At least two high-performance liquid chromatographic (HPLC) methods have been reported for the analysis of erythromycin[10,11]. The first method uses a Corasil II (silica gel) column with chloroform as mobile phase. Separation of anhydroerythromycin A from erythromycin A has been demonstrated. The second method uses a Jascopack SV-02-500 (reversed-phase) column with a mobile phase of methanol–1/15 *M* acetate buffer–acetonitrile (35:60:5) at pH 4.9. Separation of erythromycins A and B was reported. However, these reports failed to present quantification data nor demonstrate separation of numerous erythromycin epimers and their degradation compounds.

This paper reports the development of an HPLC method for separation and quantification of erythromycins, their epimers, and degradation compounds. It also demonstrates capability of the method to analyze erythromycin ester derivatives.

EXPERIMENTAL

Instrument

A Laboratory Data Control modular liquid chromatograph (LDC, Riviera Beach, Fla., U.S.A.) equipped with a high-pressure mini-pump and a variable-wavelength detector (SpectroMonitor I) at 215 nm was used. The attenuation of the detector used was either 0.08 or 0.04 a.u.f.s. A sample was injected through a Rheodyne injector (Model 71-20, Berkeley, Calif., U.S.A.) with a 100-μl fixed loop. A reversed-phase column (μBondapak C_{18}, Waters Assoc., Milford, Mass., U.S.A.), 300 × 3.9 mm I.D., with a 50 × 2.1 mm I.D. stainless-steel pre-column packed with μBondapak C_{18} were used. The mobile phase was pumped at a flow-rate of *ca.* 1 ml/min (1700 p.s.i.), and the column was operated at room temperature.

Mobile phase

The mobile phase used consisted of acetonitrile–methanol–0.2 *M* ammonium acetate–water (45:10:10:25). Depending upon the performance of a given column, methanol may be omitted without a significant loss of peak resolution. The solvents used are all UV grade, distilled-in-glass, obtained from Burdick and Jackson Labs. (Muskegon, Mich., U.S.A.). The amount of the solvent may have to be modified to obtain the maximum performance of the column.

The pH of the mobile phase may be adjusted to optimize the peak resolution and to control the elution volume, hence the speed of the assay. A pH of 6.0–6.5 has been used to analyze erythromycin base powders, erythromycin oxalate, and erythromycin ester derivatives where sample interference is minimum. In order to analyze samples of complex biological matrix, however, a mobile phase was adjusted to pH 7.0–7.8 to minimize interference. The pH adjustment was performed by the addition of either hydrochloric acid or sodium hydroxide. Due to its high UV cut-off, acetic acid may not be used. After wetting the filter with methanol, the mobile phase was filtered through a Fluoropore filter (FHLP04700, Millipore, Bedford, Mass., U.S.A.) and degassed under vacuum with sonication prior to use. The 0.2 *M* ammonium

acetate solution was prepared by weighing 15.5 g of reagent grade ammonium acetate into a one-liter measuring cylinder and adding water to the volume.

Reference standard solution

Ca. 8–9 mg of USP Erythromycin Reference Standard was accurately weighed into a 10-ml volumetric flask. Just prior to the analysis, the mobile phase was added to volume and sonicated to facilitate dissolution.

Sample preparation

Erythromycin base. Ca. 8–9 mg of the powder were accutarely weighed into a 10-ml volumetric flask. Just prior to the analysis, the mobile phase was added to volume and sonicated to facilitate dissolution.

Erythromycin oxalate. Ca. 30–32 mg of the powder were accurately weighed into a 25-ml volumetric flask. Just prior to the analysis the mobile phase was added to volume and sonicated to facilitate dissolution.

Calculation

The peak height was used to calculate both the bio-equivalent potency of the sample and the percent composition of the components.

$$\text{Potency } (\mu\text{g/mg}) = \frac{A + 0.5B + 0.4C}{W} \cdot \frac{Wt}{At + 0.5Bt + 0.4Ct} \cdot F_1 \cdot F_2$$

where A, B and C are peak heights of the erythromycins A, B and C, respectively, in a sample, W = weight of a sample, At, Bt and Ct are peak heights of the erythromycins A, B and C, respectively, in the reference standard, Wt = weight of the reference standard, F_1 = assigned potency of the reference standard (990 μg/mg for USP issue H), and F_2 = dilution factor.

$$\text{Percent composition} = \frac{P}{1000} \cdot \frac{I}{A + 0.5B + 0.4C}$$

where P = potency (μg/mg) of a sample as determined by the HPLC method, and I = peak height of the component of interest.

The peak height of anhydroerythromycin A, erythralosamine, erythromycin enol ether, and anhydroerythromycin B were divided by 10 prior to calcluating the percentage to compensate their molar absorptivity at 215 nm.

The factors 0.5 and 0.4 used for calculation of potency were reported in ref. 1. The relative molar absorptivities assigned to erythralosamine, anhydroerythromycin A, erythromycin A enol ether, etc., are tentative and may require further confirmatory study.

RESULTS AND DISCUSSION

Establishment of chromatographic condition

Concentration of both acetonitrile and methanol together with the pH of the mobile phase greatly affect the resolution and elution volume of various erythromycin

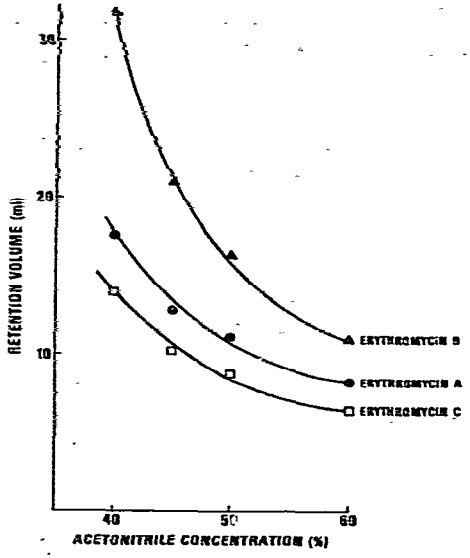


Fig. 1. Effect of acetonitrile concentration in the mobile phase (at pH 7.3) on the retention volume of various erythromycins.

peaks. As shown in Fig. 1, increase in acetonitrile concentration decreases the elution volume and resolution of the peaks. Addition of methanol into the mobile phase containing 45% acetonitrile again decreased peak elution volume (Fig. 2). The concentration of the acetonitrile and methanol at 45% and 10% was judged to give the optimum separation of the peaks with a reasonable chromatographic time.

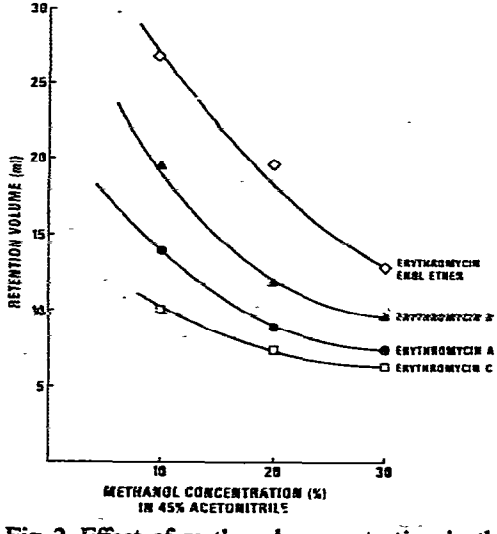
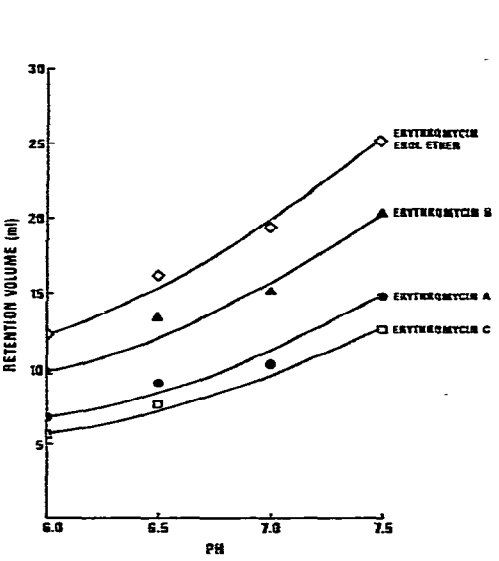


Fig. 2. Effect of methanol concentration in the mobile phase containing 45% acetonitrile (pH 7.8) on the retention volume of various erythromycins.

Changes in the pH of the mobile phase affect the retention volume of the erythromycin peaks (Fig. 3). However, the resolution of the peaks was only slightly affected. Therefore, the pH of the mobile phase was routinely adjusted to the type or the nature of the samples being analyzed. For example, pH 6.0–6.5 was used for the determination of relatively pure samples, *i.e.* erythromycin base powder, erythromycin oxalate powder, etc. On the other hand, pH 7.8 was used for the analysis of samples in complex biological matrix where removal of interference is essential for the chromatographic assay. A typical chromatogram of an erythromycin base powder of foreign origin indicating separation of various impurities is shown in Fig. 4.



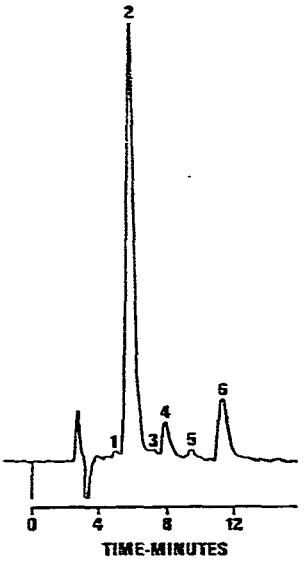


Fig. 3. Effect of pH of the mobile phase on the retention volume of various erythromycins.

Fig. 4. Reversed phase HPLC chromatogram of erythromycin base powder indicating separation of erythromycins A (2), B (4), C (1), anhydroerythromycin (3), erythromycin enol ether (6), and dihydroerythromycin A (5). Mobile phase: acetonitrile–methanol–0.2 *M* ammoniam acetate–water (45:10:10:35) at pH 6.2 as monitored at 215 nm.

The pH of the mobile phase affects stability of erythromycin (Table I). Erythromycin degrades mostly to enol ether at low pH (6.3) and to dihydroerythromycin at high pH. Since the HPLC chromatograph takes about 15 min to analyze one sample, effect of the pH of the mobile phase is minimum. However, where the maximum stability of the erythrocymin solution is required, *e.g.* in the automated HPLC sample injector system, the pH of the solution must be kept at or near 8.8.

Peak identification

Relative retention of various erythromycins, their epimers, and degradation compounds are shown in the order of elution in Table II. These compounds were received in relatively pure form from Abbott Labs.[12,13], Eli Lilly and Company, and W. J. Haak of The Upjohn Company. Table II has been used to identify compounds

TABLE I

STABILITY OF ERYTHROMYCIN AT VARIOUS pH VALUES

pH	*Period*	*Erythromycin (μg/mg)*			*Erythromycin A enol ether (%)*	*Anhydro-erythromycin A (%)*	*Dihydro-erythromycin A (%)*
		A	*B*	*C*			
6.3	0	93.5	4.2	1.3	0.07	0	0
	2 h	93.4	4.2	0.8	0.03	0	0
	6 h	94.2	4.6	1.1	0.35	0.05	0
	13 days	82.0	4.1	0.3	12.9	0.03	0
8.85	0	93.5	4.2	1.3	0.07	0	0
	5 h	94.9	4.3	1.0	0.05	0	0
	13 days	95.1	4.5	1.0	0.08	0.03	2.3
9.6	0	97.1	3.8	1.4	0.08	0.05	0
	5 h	98.4	4.7	1.4	0.06	0.06	0
	13 days	90.3	5.2	1.1	0.08	0.05	8.5
10.0	0	96.5	4.2	1.3	0.07	0	0
	2 h	96.8	4.8	0.5	0.05	0.05	0
	5 h	95.2	5.1	1.3	0.08	0.08	7.0
	13 days	63.4	4.5	1.3	0.43	0.08	19.3

TABLE II

RELATIVE RETENTION OF VARIOUS ERYTHROMYCINS

Mobile phase: acenitrile–methanol–0.2 *M* ammonium acetate–water (45:10:10:35) at pH 7.8.

Compounds	*Relative retention*
Erythronolide B	0.51
Erythromycin C	0.72
Erythromycin A	1
8-Epi-10,11-anhydroerythromycin A	1.11
Erythralosamine	1.16
Anhydroerythromycin A	1.22
4"-Acetylerythromycin A	1.34
Erythromycin B	1.40
Dihydroerythromycin A	1.53
8-Epi-11,12-epoxyerythromycin A	1.88
Erythromycin A enol ether	1.90
8-Epi-erythromycin B	2.28
2',4"-Diacetylerythromycin A	2.42
10,11-Anhydroerythromycin B	2.64

found in samples. In addition, column effluents corresponding to the peaks were collected and their identity was confirmed by field-desorption mass spectrometry (Fig. 5).

Quantitation of erythromycin

Precision of the HPLC method for quantitation of erythromycin A was determined by analyzing seven individually weighed and prepared erythromycin powders, USP Reference Standard, Issue H. The relative standard deviation of the assay method is 0.64% (Table III).

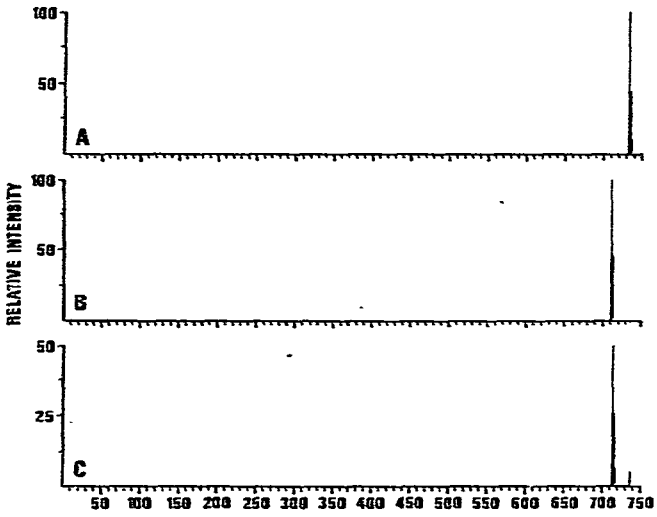


Fig. 5. Field desorption mass spectrograms of erythromycin A (A), erythromycin A enol ether (B) and dihydroerythromycin A (C).

TABLE III

PRECISION OF THE ERYTHROMYCIN HPLC ASSAY METHOD
R.S.D. = 0.64%.

Weight of erythromycin	*Peak height of erythromycin A*	*Peak height/wt. ratio*
0.9060	39.25	43.322
0.9990	43.10	43.143
0.9902	42.55	42.971
0.9864	42.30	42.883
0.9112	39.40	43.240
0.9592	41.35	43.109
0.9282	39.45	42.502

Various erythromycin base powders were then analyzed (Table IV). The bioequivalent potencies calculated from the HPLC data were compared with those of the GLC[1] and the turbidimetric microbiological assay methods[14]. The differences in the assay values were not statistically significant, and a significant correlation coefficient existed between the HPLC and the microbiological assay data.

The percentage of the erythromycin components as determined by the HPLC method gave reasonable correlation with that of the GLC method. It should be noted, however, that amounts of erythromycin B reported by the GLC method do include anhydroerythromycin A[1].

Erythromycin derivatives

The HPLC method for the analysis of erythromycin is also applicable for the determination of derivatives of erythromycin. The composition of the mobile phase may have to be modified depending upon the derivatives of interest. Fig. 6 represents

TABLE IV

ANALYSIS OF ERYTHROMYCIN

Erythromycin	*Potency (μg/mg)*			*Erythromycin (%)*			*Composition of erythromycins (%)*		
	Microbioassay	*HPLC*	*GLC*	*A*	*B*	*C*	*Erythromycin A enol ether*	*Anhydroerythromycin A*	*Dihydroerythromycin A*
USP issue G*	982	982	982	97.9	0	0.36	0.10	0.10	0
issue H*	990	990		99.0	0	0.1	0	0	0
Bulk Powder Lot 1	950	949		93.9	0	2.6	0.1	0.2	0.1
2	950	935	958	90.9	4.2	1.4	0.09	0.05	0
			(GLC	89.7)	(2.5)	(2.1)			
3	961	957	—	93.1	4.5	1.7	0.1	0.04	0
4	928	950	—	94.1	1.7	4.4	0.12	0.07	0
5	884	927	—	90.0	2.0	4.3	0.07	0.07	0.2
6	907	914	—	87.1	5.2	13.7	0.07	0.07	0
7	892	901	—	85.3	3.7	7.2	0.13	0.04	0
8	903	945	—	90.8	1.2	10.9	0.05	0.05	0
9	952	950	—	93.1	2.5	1.5	0.11	0	2.8
10	823	842	821	75.5	13.5	4.7	0.15	0.05	0.7
			(GLC	81.3)	(14.2)	(2.9)			
11	900	910	901	87.1	3.1	6.0	0.4	0	8.9
			(GLC	87.9)	(4.4)	(4.3)			
12	910	925	881	90.0	1.5	2.2	0.2	0.04	1.2
			(GLC	92.1)	(3.1)	(0.9)			
13	866	908	841	87.8	4.9	1.5	0.45	0.05	0.7
			(GLC	89.2)	(5.6)	(1.0)			
14	890	892	744	85.0	6.6	2.2	0.6	0	1.4
			(GLC	85.0)	(7.1)	(2.4)			
15	910	870	886	83.6	5.4	1.7	0.7	0.04	2.1
			(GLC	85.0)	(6.8)	(2.2)			
16	882	875	766	83.7	6.4	1.7	0.96	0.03	2.1
			(GLC	83.3)	(7.9)	(2.1)			
17	876	886	869	85.2	5.6	1.5	0.10	0.03	3.8
			(GLC	84.5)	(6.9)	(1.8)			

* Used as the standard for potency calculation.

a chromatogram of erythromycin ethyl carbonate. The HPLC method is capable of separating and analyzing erythromycin A base, the hydrolyzed compound, from the parent erythromycin ethyl carbonate.

Thus, the HPLC method is simple, fast, precise, and a good stability-indicating assay method. It would be of value in product formulation study, derivative synthesis, and clinical research.

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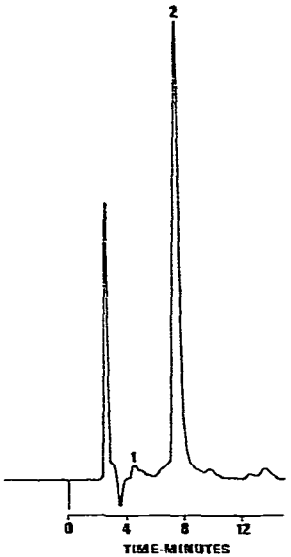

Fig. 6. Reversed-phase HPLC chromatogram of erythromycin ethyl carbonate (2) indicating separation of erythromycin A base (1). Mobile phase: acetonitrile–methanol–0.2 *M* ammonium acetate–water (45:20:10:25) at pH 6.0 as monitored at 215 mm.

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