Journal of Chromatography, 157 (1978) 185–196

CHROM. 10,977

ELEVATED COLUMN TEMPERATURE FOR THE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF ERYTHROMYCIN AND ERYTHROMYCIN ETHYLSUCCINATE

KIYOSHI TSUJI and JOHN F. GOETZ

Control Analytical Research and Development, The Upjohn Company, Kalamazoo, Mich. 49001 (U.S.A.)

(Received February 21st, 1978)

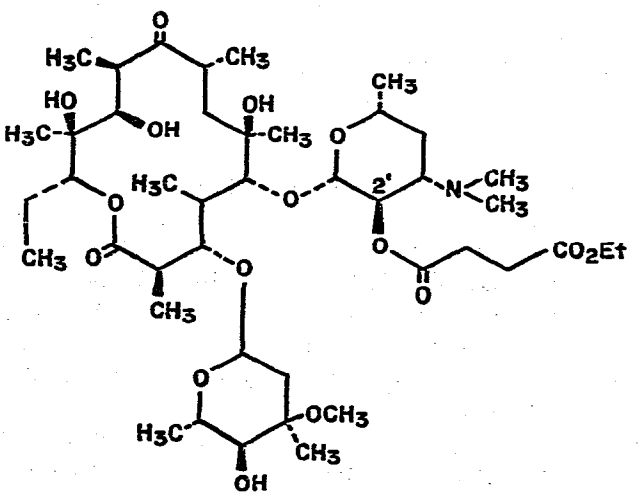
SUMMARY

An elevated column temperature has been found to be an ideal tool not only in shortening chromatographic analysis time, but to increase resolution of erythromycin ethylsuccinate peaks and drastically decrease the height equivalent for a theoretical plate (HETP). When the logarithms of HETP were plotted against the column temperature, a linear relationship was obtained with a correlation coefficient of 0.998.

The high-performance liquid chromatographic method thus developed can separate erythromycin, erythromycin ethylsuccinate and its degradation compounds with a relative standard deviation of 1.4% for the assay of erythromycin ethylsuccinate.

INTRODUCTION

Erythromycin ethylsuccinate is a pro-drug for pediatric use. The erythromycin ethylsuccinate (EES) is esterified at the 2′ position of the desosamine moiety of erythromycin (E) with ethylsuccinyl chloride to stabilize erythromycin in gastric fluid



Erythromycin Ethylsuccinate
Emp. Formula: $C_{43}H_{75}O_{16}N$
Formula Weight: 862.04
Monohydrate, M.W.: 880.01

and to improve taste for oral, pediatric use. The esters of erythromycin, *e.g.* EES, erythromycin propionate, etc., are microbiologically inactive and must be hydrolyzed *in vivo* or by an assay microorganism during incubation to exhibit biological activity[1]. In addition to erythromycin A ethylsuccinate, (EES-A), a commercial preparation of EES may contain erythromycin A (E-A), erythromycin B ethylsuccinate (EES-B), erythromycin C ethylsuccinate (EES-C), anhydroerythromycin ethylsuccinate, 8,9-anhydro-6,9-hemiketal erythromycin ethylsuccinate, etc. The microbiological assay method as accepted by FDA[2] is incapable of differentiating EES-A from E-A or any other impurities, thus, is not an ideal stability nor efficacy-indicating assay method. This paper reports the development of a high-performance liquid chromatographic (HPLC) method which is capable of making that distinction.

EXPERIMENTAL

Analytical instruments

A modular liquid chromatograph (Laboratory Data Control, Riviera Beach, Fla., U.S.A.) equipped with a high-pressure mini-pump and a variable-wavelength detector (Spectro Monitor I) at 215 nm was used. The attenuation of the detector was either 0.04 or 0.08 a.u.f.s. depending on sample concentration. A sample of EES was injected through a Rheodyne injector (Model 70-10, Berkeley, Calif., U.S.A.) with a 100-μl fixed loop. A reversed-phase column [either μBondapak C_{18} (Waters Assoc., Milford, Mass., U.S.A.) 300 × 3.0 mm I.D. or LiChrosorb RP-18 5 μm (Brownlee Lab., Santa Clara, Calif., U.S.A.) 250 × 4.6 mm I.D.] with a pre-column of 51 × 2.1 mm I.D. stainless steel, packed with μBondapak C_{18} was used.

The mobile phase was pumped at a flow-rate of 0.9 ml/min (1100 p.s.i.). The column temperature was maintained at 70° ± 0.1° by a rubber water jacket connected to a Lauda K-2/R controlled-temperature circulating water bath (Brinkmann, Lauda, G.F.R.). Both the pre-column and the analytical column were placed inside the water jacket. The columns were mounted vertically with the inlet side up to avoid possible channeling due to dissolution of silica gel under the chromatographic conditions used. Under continuous usage, a few millimeter void may be noted at the inlet side of the pre-column in two to three weeks. However, the analytical column will not be affected. The pre-column may need replacement when the total column pressure suddenly exceeds over 4000 p.s.i. The analytical column was stable over months of continuous use.

Mobile phase

The composition of the mobile phase used was as follows: acetonitrile–0.2 *M* ammonium acetate–water (60–57:10:40–33), pH 7.0, for μBondapak C_{18} and (65–60:10:25–30), pH 7.0, for LiChrosorb RP-18 packing. The acetonitrile used was UV grade, distilled-in-glass obtained from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.). The 0.2 *M* ammonium acetate was prepared by weighing 15.5 g of reagent grade ammonium acetate into a 1-l measuring cylinder and adding water to the volume. The amount of the solvent in the mobile phase may have to be modified to obtain the maximum performance of the column.

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. After the end of analysis, the column was throughly rinsed with methanol and stored in methanol until used.

EES reference standard solution

U.S.P. EES reference standard powder (5–6 mg) was accurately weighed into a 10-ml volumetric flask. Just prior to analysis, the mobile phase was added to volume and sonicated to facilitate dissolution. For routine analytical laboratory work, about 60 mg of EES should be weighed into a 100-ml volumetric flask for convenience.

For the analysis of EES in formulation, the 10 ml of the reference standard solution must be washed with 20 ml of heptane for 2 min prior to analysis. This simulated sample preparation step is needed since acetonitrile is slightly miscible in heptane thereby affecting quantification.

Erythromycin reference standard solution

Pharmacopoeia (U.S.P.) erythromycin reference standard (8–9 mg) 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.

For the analysis of the EES formulation, the 10 ml of the reference standard solution must be washed with 20 ml of heptane for 2 min prior to analysis. This simulated sample preparation step is needed since acetonitrile is slightly miscible in heptane thereby affecting quantification.

Sample preparation

EES bulk powder. The powder (5–6 mg) 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. For routine laboratory work, about 60 mg of EES powder should be weighed into a 100-ml volumetric flask for convenience. Just prior to the analysis, the mobile phase is added to volume and sonicated to facilitate dissolution.

EES formulation: 200 mg and 400 mg per 5 ml. Shake vigorously and take approximately a 3-ml sample using a disposable syringe or a pipette. Weigh the filled syringe before and after the sample has been transferred into a 250-ml volumetric flask to obtain the weight of sample by difference. When a pipette is used to quantitatively transfer the sample, thoroughly rinse the pipette with the mobile phase; no weighing or correction with the use of specific gravity of the sample is needed. Just prior to the analysis, the mobile phase is added to volume and sonicated to facilitate dissolution.

Pipette 10 ml of the diluted sample into a 10-dram vial and shake vigorously with 20 ml of heptane for 2 min to remove interfering compounds. Centrifuge the vial for 1 min at 1500 *g*. Remove the top heptane layer by aspiration and inject the bottom aqueous layer.

Calculation

The following equations were used to calculate the quantity of EES in powder:

$$\text{EES-A } (\mu\text{g/mg}) = \frac{A}{At} \times \frac{Wt_1}{W} \times F_1 \times 1000 \qquad (1)$$

$$\text{EES-B } (\mu g/mg) = \frac{B}{At} \times \frac{Wt_1}{W} \times F_1 \times 1000 \quad (2)$$

$$\text{EES-C } (\mu g/mg) = \frac{C}{At} \times \frac{Wt_1}{W} \times F_1 \times 1000 \quad (3)$$

$$\text{E-A } (\mu g/mg) = \frac{E}{Et} \times \frac{Wt_2}{W} \times F_2 \quad (4)$$

where A, B and C are the peak areas of EES-A, -B and -C, respectively, in a sample; E is the peak height of E-A in a sample; At the peak area of EES-A in the U.S.P. EES reference standard; Et the peak height of E-A in the U.S.P. E reference standard; W the weight of a sample; Wt_1 and Wt_2 the weights of the U.S.P. EES and U.S.P. E reference standards, respectively; F_1 the purity of the U.S.P. EES reference standard; and F_2 the assigned potency of the U.S.P. E reference standard (990 μg/mg for the Issue H). For the calculation of EES and E-A contents in the formula, multiply the eqns. 1–4 with a suitable dilution factor to obtain the quantity in a 5-ml dosage unit. When the E-Mycin Liquid® is weighed instead of measured by volume, the specific gravity of the E-Mycin Liquid must be a factor in eqns. 1–4 to correct for volume–weight difference.

RESULTS AND DISCUSSION

Establishment of chromatographic condition

Column temperature was found to be an ideal tool not only in shortening chromatographic analysis time but to increase resolution of peaks and to decrease drastically the height equivalent for a theoretical plate (HETP) of EES. The relationship between HETP and column temperature was linear when the logarithms of HETP were plotted against the column temperature (Fig. 1), $\ln y = -0.023\,x - 0.462$ with a correlation coefficient of 0.998. Fig. 2A and B dramatically illustrate the improvements of chromatography by the increase in column temperature from 23° to 70°.

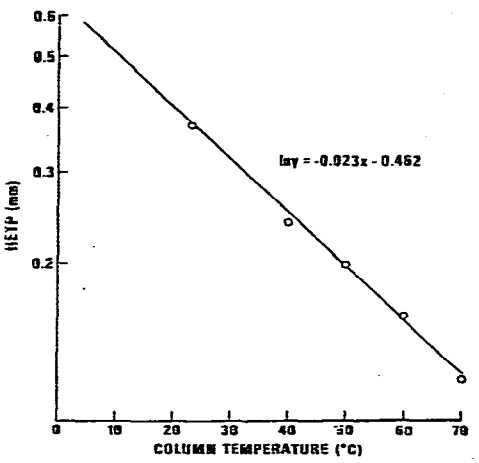


Fig. 1. Effect of column temperature on the HETP using μBondapak C_{18} for the analysis of EES

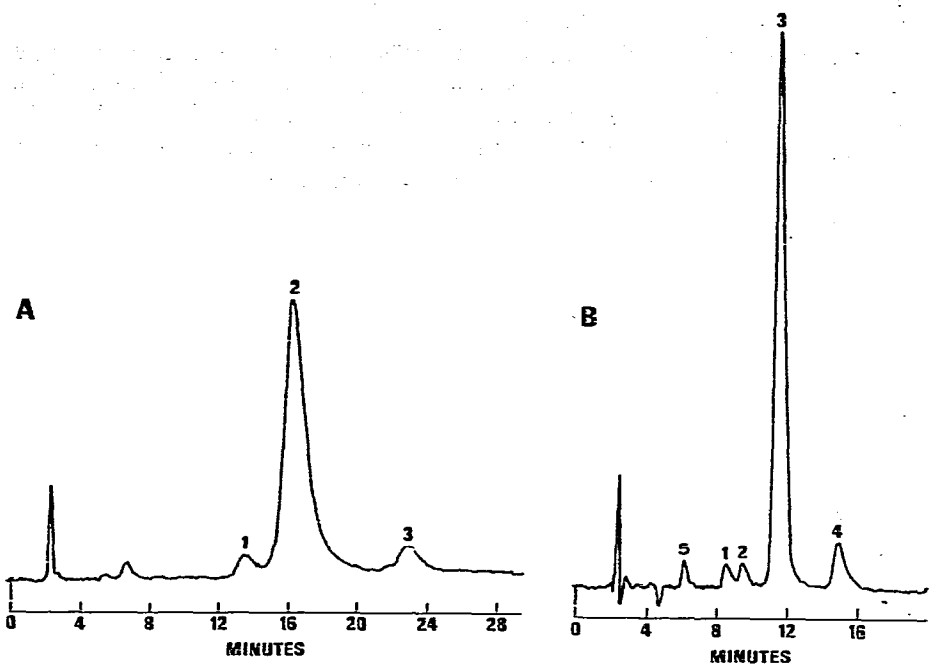


Fig. 2. (A) Effect of column temperature on the chromatographic performance at 25°. Peaks: 1 = E-A and EES-C; 2 = EES-A; 3 = EES-B. (B) Effect of column temperature on the chromatographic performance at 70°. Peaks: 1 = EES-C_1; 2 = EES-C_2; 3 = EES-A; 4 = EES-B, 5 = E-A.

Although Karger *et al.*[3] observed that increase in column temperature (from 25 to 60) caused a small decrease in the hydrophobic selectivity of alcohols on the reversed-phase column, many investigators[4–8] reported significant decrease in HETP by increase in column temperature.

Increase in temperature has been shown to reduce viscosity of a mobile phase, and increase in the mass transfer or diffusion of the solute to the stationary phase[8,9]. Giddings has shown the effects of viscosity of moving phase and diffusion coefficient on theoretical plate heights in the following equation[9]:

$$H = \frac{q\,R(1-R)\,d^2\,u}{D_s}$$

where H is the plate height; d the viscosity of the moving phase; D_s the diffusion coefficient of the sample to the stationary phase; q the constant dependent on the geometry of the stationary phase; R the fraction of the sample in the mobile phase; and u the average mobile phase velocity.

The column temperature of 70° was selected for the analysis of EES, since a temperature above 70° is too close to the boiling point of acetonitrile and any bubble which may form in the mobile phase would adversely affect the efficiency of the column and stability of a UV monitor.

Effects of pH on the elution volume of E-A and EES

The pH of the mobile phase was shown to affect greatly the elution volume and separation between erythromycins A, B, and C[10]. However, pH of the mobile phase has been shown to have little effect on the elution volume of EES (Fig. 3). These phenomena are partly attributable to the difference in the pK values of E-A and EES. Therefore, the pH of the mobile phase has been used as a factor to effect separation between E-A and EES-C peaks.

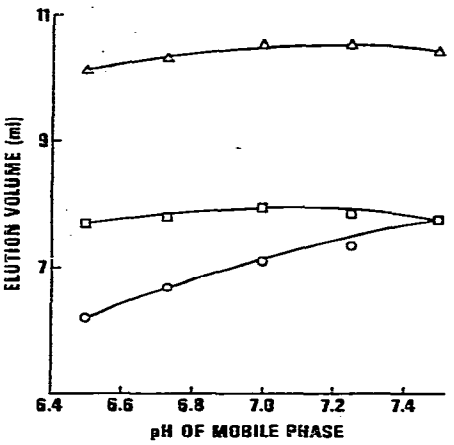


Fig. 3. The effect of pH of the mobile phase on the elution volumes of (△) EES-A, (□) EES-C, and (○) E-A using a μBondapak C_{18} column. Mobile phase, acetonitrile–0.2 M ammonium acetate–water (58:10:32).

Effects of particle size of the reversed-phase column support

A 5-μm silica gel column support (LiChrosorb RP-18) and a 10-μm silica gel support (μBondapak C_{18}) were used to examine the effects of particle size on chromatography of EES.

Use of smaller particle-size support contributed to about 50% increase in theoretical plates (from 11,000 per m for 10-μm particle size to 16,000 per m for 5-μm particle size). This increase in theoretical plates which resulted in increase in peak height obtained by use of the 5-μm particle-size support has been considered as an added advantage to detect and to quantify minor impurities and degradation compounds. The K' value also increased when 5-μm particle size was used. Therefore, increase in acetonitrile concentration in the mobile phase, from about 60 to 65%, is necessary to shorten the chromatographic time.

Since an elevated column temperature is used for the analysis, only a slight increase in column pressure has been experienced (600 and 1000 p.s.i. for 10-μm and 5-μm particle-size column, respectively).

Identification of impurities and degradation compounds

As may be seen in Fig. 2B, commercial preparations of EES contain various amounts of impurities and degradation compounds. In order to identify the major impurities and degradation compounds, a small-scale preparative LC instrument was

constructed using a 122 cm × 7 mm I.D. stainless-steel column packed with μBondapak C_{18}–Porasil B. The column was jacketed to maintain a temperature of 70°.

About 100 mg of EES bulk powder was dissolved in 1 ml of methanol and injected on to the pre-column via a 100-ml loop injector. The column effluents corresponding to those peaks of interest were collected into 50-ml round-bottom flasks immersed in a methanol-dry ice bath. The procedure was repeated for 10 times. The effluents thus collected were freeze-dryed overnight. Immediate freezing of column effluents was found to be essential to minimize degradation of the compounds collected.

A few milligram quantities of powder thus obtained were analyzed by mass spectrometry using the electron impact ionization mode. Identification of the peaks was also made by matching the elution volume (LC) and R_F values (thin-layer chromatography) of the peaks with authentic compounds synthesized by reacting erythromycin B, erythromycin C, and anhydroerythromycin A with ethylsuccinyl chloride. Anhydroerythromycin A ethylsuccinate and 8,9-anhydro 6,9-hemiketal erythromycin ethylsuccinate were also prepared using the methods as outlined by Stephens and Conine[11].

The mass spectra of the column effluents are shown in Fig. 4A–D. The examination of the mass spectrum (Fig. 4A) corresponding to the major peak, erythromycin A ethylsuccinate, indicated that the molecular ion observed at *m/e* 862 was weak, presumably due to low volatility and ready fragmentation of the glucosidic bonds of the sugars. Other weak ions were found at *m/e* 844 (M—H_2O),776$C_{43}H_{73}NO_{15}$ (M—$CH_3CH_2CHCCH_3OH$ of erythronolide), 716 $C_{37}H_{65}NO_{12}$ (M—ethylsuccinyl), 685 $C_{35}H_{59}NO_{12}$ (M—cladinose), 558 $C_{29}H_{50}O_{10}$ (M—desosamine), 383 $C_{21}H_{34}O_6$ (M—cladinose—desosamine). Much stronger peaks were observed at *m/e* 304 $C_{14}H_{25}NO_6$, and 286 $C_{14}H_{24}NO_5$ (desosaminyl ethylsuccinate) and 158 $C_8H_{16}NO_2$ (desosaminyl). The fragmentation pattern observed was similar to that of erythromycin reported by Rinehart *et al.*[12].

The mass spectra of column effluents corresponding to erythromycin C ethylsuccinate, anhydroerythromycin ethylsuccinate, 8,9-anhydro 6,9-hemiketal erythromycin ethylsuccinate peaks showed similar pattern of fragmentation (Fig. 4B–D). The mass spectrum of erythromycin C ethylsuccinate failed to yield the parent ion, but the *m/e* 829 (M—H_2O) peak was strong. Weak peaks were observed at *m/e* 685 $C_{35}H_{59}NO_{12}$ (M—cladinose), 382 $C_{21}M_{34}O_6$ (erythronolide), and 303 $C_{14}H_{25}NO_6$ (desosaminyl). Stronger peaks were observed at *m/e* 287 $C_{14}H_{25}NO_5$ (desosaminyl ethylsuccinate), 158 $C_8H_{16}NO_2$ (desosaminyl), and 146 $C_7H_{14}O_3$ (cladinose C).

The mass spectra of anhydroerthromycin ethylsuccinate yielded *m/e* of 844 (M), 685 $C_{35}H_{57}NO_{12}$ (M—cladinose), 365 $C_{21}H_{32}O_5$ (anhydroerythronolide), 303 $C_{14}H_{25}NO_6$ (desosaminyl ethylsuccinate), 287 $C_{14}H_{25}NO_5$ (desosaminyl ethylsuccinate). The mass spectra of 8,9-anhydro 6,9-hemiketal erythromycin ethylsuccinate was similar to that of anhydroerythromycin ethylsuccinate.

Stability of erythromycin ethylsuccinate at various pH values

In order to investigate the stability of EES in various pH solutions, EES was dissolved in 10% methanolic 0.02 *M* phosphate buffer solution and the pH was adjusted from 2.0 to 8.0. The solution thus prepared was placed in a 37° water bath and analyzed periodically by LC.

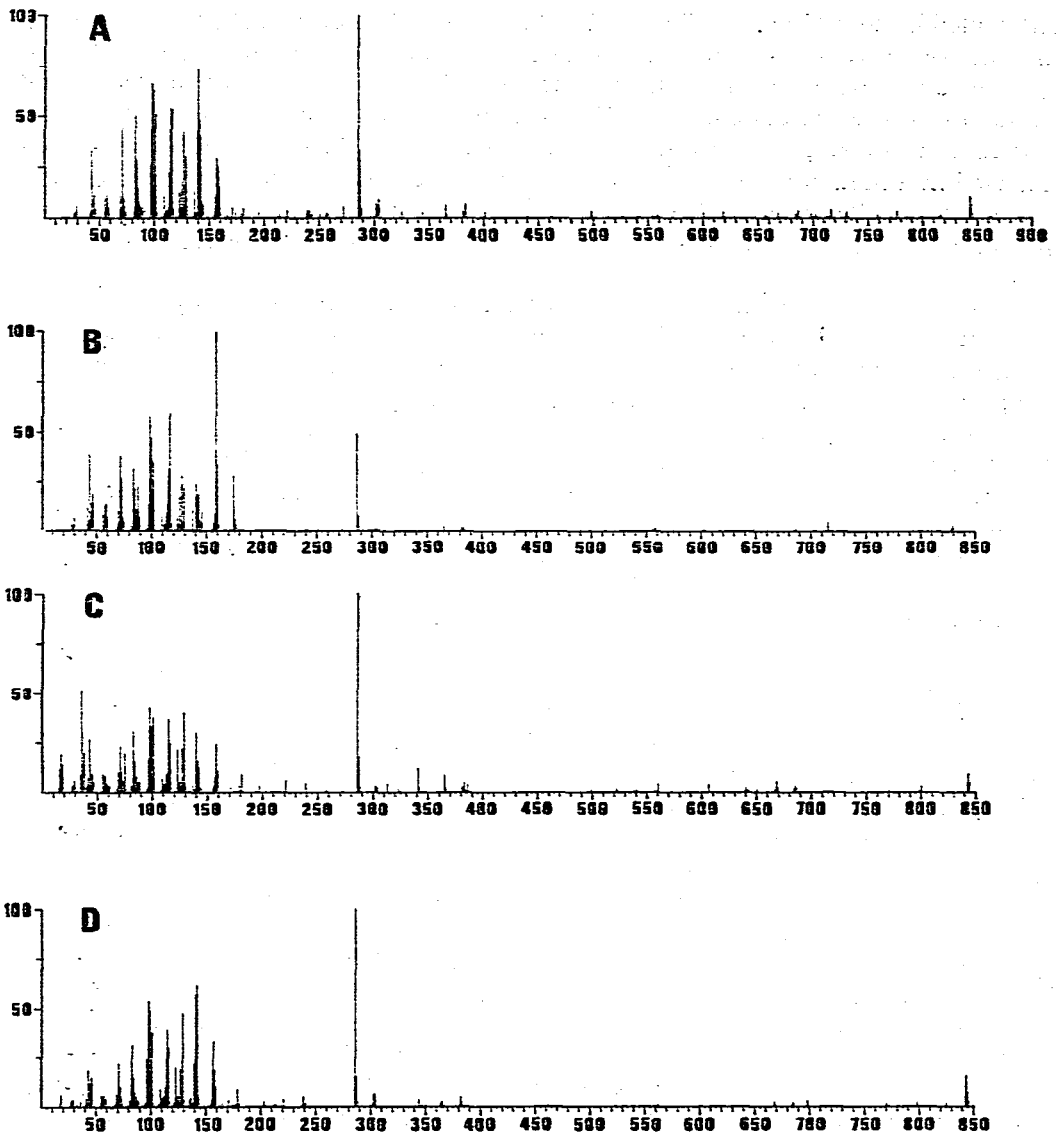


Fig. 4. Electron impact mass spectra. (A) EES-A, (B) EES-C, (C) anhydroerythromycin ethylsuccinate, (D) 8,9-anhydro 6,9-hemiketal erythromycin ethylsuccinate.

Contrary to popular belief, EES was most stable at pH 5.0 (Table I). At high pH the EES readily hydrolyzes to form E-A and at low pH the EES rapidly anhydrates to form anhydroerythromycin ethylsuccinate and 8,9-anhydro 6,9-hemiketal erythromycin ethylsuccinate. A typical chromatogram indicating separation of degradating compounds is shown in Fig. 5.

Precision of the HPLC method

Precision of the HPLC method for quantification of EES-A in EES bulk drug

TABLE I

STABILITY OF EES AT VARIOUS pHs

6.0 mg EES per 10 ml of 10% methanol in 0.02 *M* phosphate buffer at 37°.

pH	*Time*	*EES (%)*	*E (%)*	*Anhydro EES*	*EES hemiketal*
8.0	0	100	0	0	0
	17 min	52.8	24.2	0	0
	32 min	46.1	25.4	0	0
	46 min	43.0	34.0	0	0
	1 h 33 min	33.9	41.7	0	0
	2 h 31 min	19.5	60.6	0	0
7.0	5 min	86.3	13.7	0	0
	25 min	43.3	43.1	0	0
	38 min	27.9	51.8	0	0
	52 min	18.6	58.5	0	0
	1 h 05 min	13.5	60.6	0	0
	1 h 25 min	9.4	64.5	0	0
6.0	5 min	97.0	3.0	0	0
	20 min	88.9	7.7	0	0
	39 min	80.3	13.8	0	0
	1 h 22 min	62.2	25.1	0	0
	2 h 48 min	27.6	44.8	0	0
5.0	5 min	98.7	0	1.3	0
	21 min	97.2	0	2.6	0
	44 min	93.4	0	5.2	2.0
	2 h 41 min	74.7	0	16.7	9.5
	5 h 31 min	54.5	0	27.9	16.0
4.0	5 min	100	0	0	0
	22 min	73.3	0	15.5	7.0
	50 min	57.2	0	27.7	15.2
	1 h 15 min	50.5	0	32.1	17.1
	3 h 15 min	24.1	0	54.5	27.9
	6 h 45 min	10.1	0	68.5	23.0
3.0	5 min	44.5	0	40.0	16.8
	34 min	0	0	73.7	12.0
	1 h 03 min	0	0	76.8	8.3
	2 h 35 min	0	0	77.7	7.6
2.0	5 min	5.6	0	57.6	11.7
	1 h 29 min	0	0	62.8	7.0

was determined by analyzing 6 individually weighed and prepared EES powders. The relative standard deviation of the method is 1.4% (Table II).

Quantitative determination

Since molar absorptivity of E-A is approximately 2.4 times less than that of EES (Table III), the U.S.P. erythromycin reference standard must be assayed each time to precisely quantify E-A content in EES.

Analysis of bulk powder

Table IV lists the results of HPLC analysis comparing the quality of EES

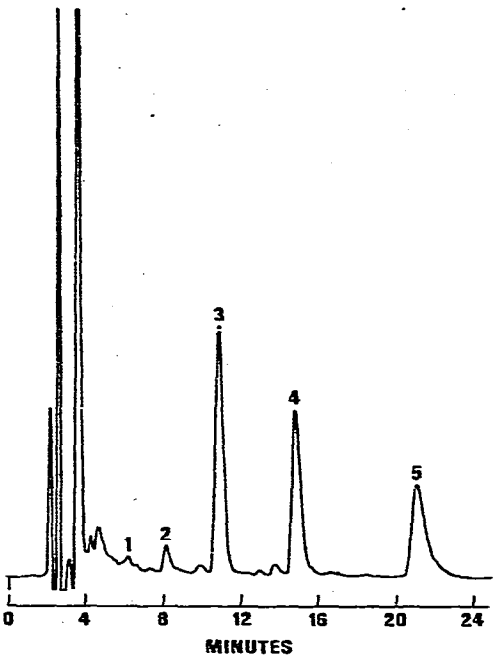


Fig. 5. Chromatogram showing separation of compounds of erythromycin ethylsuccinate degraded by exposure in simulated gastric fluid. Peaks: 1 = E-A; 2 = EES-C, 3 = EES-A, 4 = anhydro-erythromycin ethylsuccinate, 5 = 8,9-anhydro 6,9-hemiketal erythromycin ethylsuccinate.

TABLE II

PRECISION OF HPLC ASSAY FOR EES POWDER

Weight of EES (mg per 10 ml)	*Height of EES peak*	*Ratio of peak height–weight of sample*
15.075	48.40	3.21
16.085	51.85	3.22
16.050	52.50	3.27
15.430	50.45	3.27
14.596	47.85	3.28
15.920	53.25	3.34
	Relative standard deviation	1.43%

TABLE III

RELATIVE MOLAR ABSORPTIVITY OF E-A AND EES AT 215 nm

Assay day	*Weight of U.S.P. E-A corrected for purity*	*Area of erythromycin peak*	*Area–weight ratio*	*Weight of U.S.P. EES corrected for purity*	*Area of EES-A peak*	*Weight–area ratio*	*Relative molar absorptivity*
1	8.999	817898	90888	5.583	1146439	205345	2.26
2	9.227	806792	87438	5.775	1299246	224978	2.57
3	9.092	866058	95255	5.255	1232139	234469	2.46
						Average	2.43

TABLE IV

HPLC ANALYSIS OF EES BULK POWDER

Supplier	*Sample*	*E-A (μg per mg EES)*	*EES-A (μg per mg EES)*	*EES-B (μg per mg EES)*	*EES-C (μg per mg EES)*	*Anhydro EES-A (μg per mg EES)*	*Hemiketal (μg per mg EES)*
Company A	1	26	915	—	—	—	—
	2	36	910	—	—	—	—
Company B	1	73	769	74	43	—	—
	2	29	780	138	38	—	—
	3	26	779	134	26	—	—
	4	27	805	99	22	—	—
Company C	1	50	857	—	48	—	—
	2	55	817	—	58	—	—
	3	30	831	—	62	—	—
	4	34	844	—	115	6	9

bulk drugs purchased from various suppliers. All the EES powder analyzed contained a varying degree of E-A. In addition, EES powder from company B contained a substantial quantity of EES-B and EES-C and EES powder from company C contained EES-C but no detectable EES-B. One lot of EES from company C contained a trace quantity of anhydro EES and hemiketal EES. A typical chromatogram of EES from company B is shown in Fig. 6.

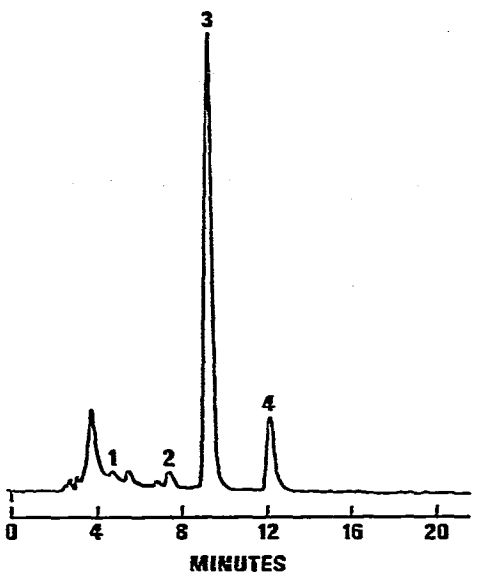


Fig. 6. A typical chromatogram of erythromycin ethylsuccinate manufactured by company B. Peaks: 1 = E-A; 2 = EES-C; 3 = EES-A; 4 = EES-B.

Analysis of EES formulation

When the placebo of the EES formulation was injected on the HPLC system after a simple dilution in a mobile phase, a small peak with the identical retention volume as EES-A was detected. However, the sample preparation procedure developed which include a 2-min heptane extraction effectively eliminated the interfering peak.

The precision of the HPLC method for quantification of EES-A in the formulation was determined by analyzing 6 individually weighed samples of both the 200 mg per 5 ml and 400 mg per 5 ml dosage forms. The relative standard deviations of the assay method are 1.32 and 0.67%, respectively.

Recovery of EES-A from E-Mycin Liquid was investigated by quantitatively adding EES into a placebo of both 200 mg per 5 ml and 400 mg per 5 ml dosage forms at 80, 100 and 120% of the production target. The average recovery for the 200 mg per 5 ml formulation was 99.3% and that for the 400 mg per 5 ml formulation was 98.9%, respectively.

Since the HPLC method is capable of separating and quantifying erythromycin, erythromycin ethylsuccinate and their degradation compounds, the method is of value as a stability indicating assay method.

ACKNOWLEDGEMENTS

The authors would like to acknowledge L. Baczynskyj for the mass spectrometric analysis, and E. J. Hessler for preparation of EES-A, EES-B, EES-C, and anhydroerythromycin ethylsuccinate.

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