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Determination of erythromycin ethylsuccinate by liquid chromatography

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ABSTRACT

A method is described for the determination of erythromycin ethylsuccinate by liquid chromatography. A C_{18} reversed-phase column (25 cm × 4.6 mm I.D.) was used with acetonitrile–0.2 *M* tetrabutylammonium sulphate (pH 6.5)–0.2 *M* phosphate buffer (pH 6.5)–water [*x*:5:5:(90 − *x*)] as mobile phase. The proportion of acetonitrile (*x*) has to be adapted to the type of stationary phase used. For RSil C_{18} LL, 42.5% was used. The column was heated at 35°C, the flow-rate was 1.5 ml/min and UV detection was performed at 215 nm. The main component, erythromycin A ethylsuccinate, was separated from all other components which were present in commercial samples. The main impurities were erythromycin A and the ethylsuccinate esters of erythromycin B and C. The amide N-ethylsuccinyl-N-demethylerythromycin A was shown to be present in all the samples examined. The method was successfully applied to the analysis of specialties.

INTRODUCTION

Soon after the introduction of the antibiotic erythromycin in 1952, a number of esters were prepared and marketed [1]. Esters of erythromycin are biologically inactive prodrugs and have to be hydrolysed to produce antimicrobial activity. Especially the ethylsuccinate ester of erythromycin (EES) is nowadays used in various pharmaceutical forms.

At present, pharmacopoeias prescribe a microbiological method for the assay of erythromycin ethylsuccinate [2,3]. After hydrolysis of the ester, the total activity is determined against an erythromycin standard. Liquid chromatography (LC) allows the separation of the ester from erythromycin and other related substances and therefore allows the specific and accurate determination of the ester. An LC method for EES has already been described by Tsuji and co-workers [4,5]. The same method was applied to the fluorimetric determination of EES in serum [6]. A method using electrochemical detection to measure EES and erythromycin in plasma, urine and saliva has been reported [7]. Methods for the separation of the ester from biological fluids are less suitable for quality control as the main concern is the separation of the ester from the biological background. The method reported by Tsuji and Kane [5] for the analysis of bulk EES suffers from the use of an elevated column temperature (70°C) and a relatively high pH of the mobile phase (pH 7.4), needed to reduce peak tailing and analysis time. Such conditions severely affect the lifetime of the silica-based reversed-phase stationary phase.

More recently, an LC method for the determination of erythromycin was described in which moderate conditions of temperature (35°C) and pH (6.5) could be used after addition of quaternary ammonium ions to the mobile phase [8]. In this paper, a modification of this LC method is described for the determination of EES in bulk or in pharmaceutical forms.

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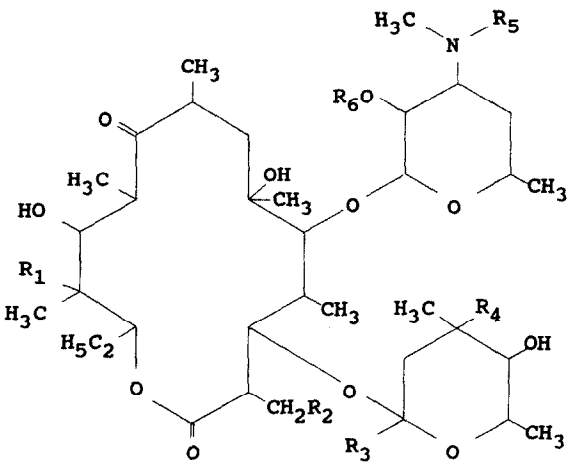
EXPERIMENTAL

Samples and reference substances

Bulk samples of EES were kindly provided by Proter (Milan, Italy) and Upjohn (Kalamazoo, MI, USA) or were a gift from Professor H. Vanderhaeghe (Rega Institute, Katholieke Universiteit Leuven, Belgium). Specialties containing EES were obtained from the Belgian market. Pure erythromycin A (EA) was obtained by crystallization of a commercial sample of erythromycin as described [9]. Anhydroerythromycin A (AEA) [10] and erythromycin A enol ethr (EAEN) [11] were prepared from EA according to previously described methods. Analogous methods were used to prepare anhydroerythromycin A ethylsuccinate (AEAES) and erythromycin A enol ether ethylsuccinate (EAENES) from EAES.

Details of the preparation of EAENES and AEAES and analytical data will be reported elsewhere. Ethylsuccinate esters of erythromycin B (EBES), erythromycin C (ECES), erythromycin E

(a)



	R1	R2	R3	R4	R5	R6
Erythromycin A (EA)	OH	H	H	OCH_3	CH_3	H
Erythromycin B (EB)	H	H	H	OCH_3	CH_3	H
Erythromycin C (EC)	OH	H	H	OH	CH_3	H
Erythromycin F (EF)	OH	OH	H	OCH_3	CH_3	H
Erythromycin E (EE)	OH	- O -		OCH_3	CH_3	H
N-desmethylerythromycin A (dMeEA)	OH	H	H	OCH_3	H	H

The corresponding ethylsuccinate esters (..ES) have R6 = $CO-CH_2-CH_2-COOC_2H_5$. N-ethylsuccinyl, N-demethylerythromycin A (ESdMeEA) corresponds to dMeEA with R5 = $CO-CH_2-CH_2-COOC_2H_5$.

Fig. 1.

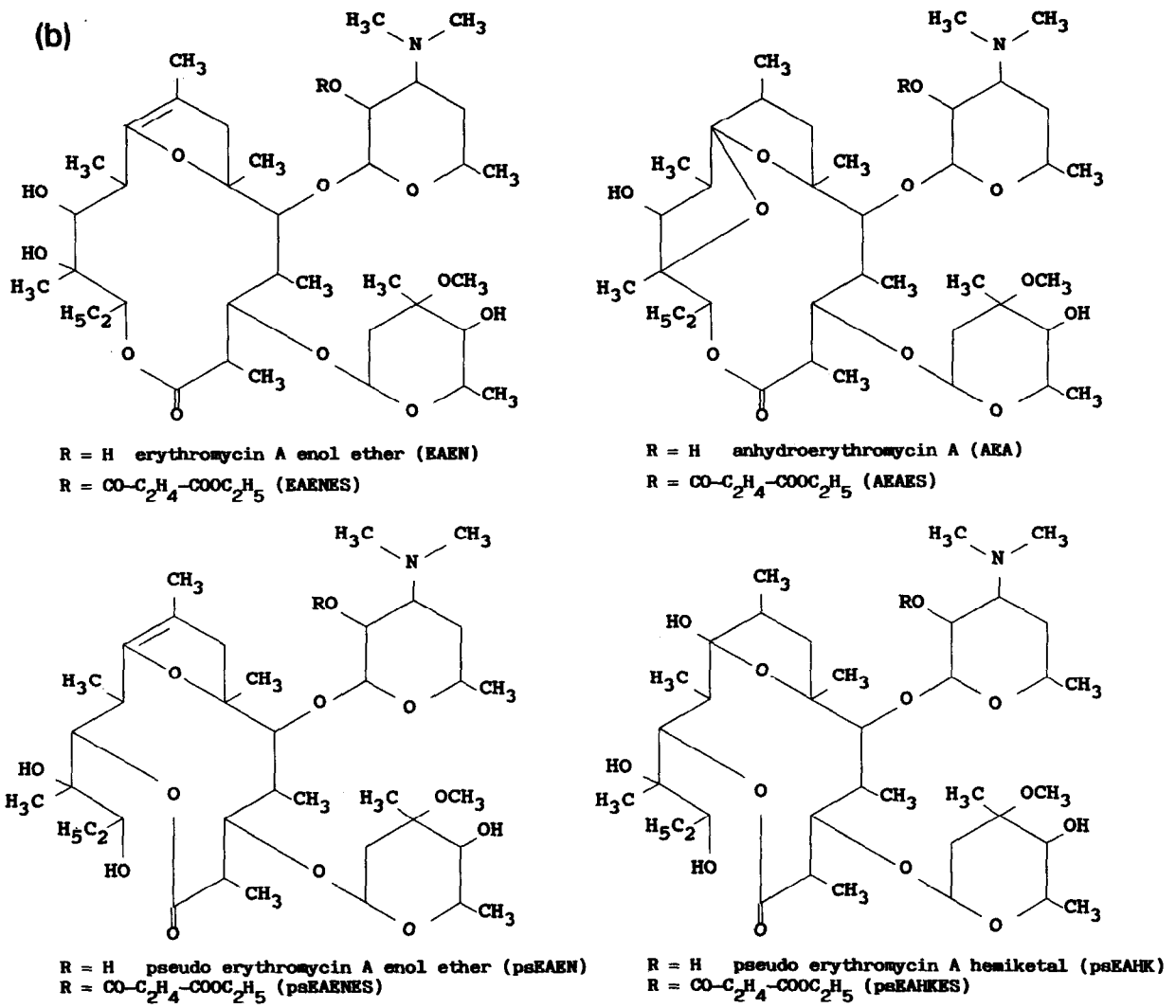


Fig. 1. Structures of the erythromycins examined.

(EEES), erythromycin F (EFES), N-demethylerythromycin A (dMeEAES), pseudo-erythromycin A hemiketal (psEAHKES) and pseudo-erythromycin A enol ether (psEAENES) were prepared by reaction with ethylsuccinyl chloride. The following typical procedure was used: to a mixture of 100 mg of starting material (*ca.* 0.15 mmol) dissolved in 10 ml of dry acetone and 500 mg of dry sodium carbonate, an equivalent amount of ethylsuccinyl chloride (Janssen Chimica, Beerse, Belgium) was added. The mixture was stirred for 2 h, filtered and evaporated under reduced pressure. Smaller amounts of starting material (up to 10 mg) were used to prepare the derivatives of EB, EC, EE and EF. The starting materials EB and EC were obtained by preparative LC of mother liquor residues from erythromycin production [12]. EE [13] and EF [14] were isolated from commercial erythromycin; dMeEA [15], psEAEN and psEAHK [16] were prepared according to described procedures. After analysis, the derivative obtained from dMeEA turned out to be an amide (N-ethylsuccinyl-N-demethylerythromycin A = ESdMeEA) and not a 2'-O-ester (dMeEAES). The determination of the structure is discussed below. Structures are shown in Fig. 1.

Instrumentation

The chromatographic system was composed of a Waters (Milford, MA, USA) M45 pump, a Valco (Houston, TX, USA) Model CV-6-UHPa N60 injection valve equipped with a 20-μl loop, a Merck–Hitachi Model 4200 variable-wavelength detector set at 215 nm (Merck, Darmstadt, Germany) and a Hewlett-Packard (Avondale, PA, USA) Model 3390 A integrator.

Mass spectra were recorded on a Kratos (Ramsey, NJ, USA) Concept 1H mass spectrometer at 8 kV accelerating voltage, 70 eV ionization energy and 150°C ion-source temperature. The sample was introduced by the direct insertion probe. ^{13}C NMR spectra were measured on an FX90Q 90-MHz Fourier transform instrument (Jeol, Tokyo, Japan). The sample was dissolved in C^2HCl_3.

Stationary phases

The stationary phases used are listed in Table I and were laboratory-packed in columns of 25 cm × 4.6 mm I.D. following a classical slurry packing procedure [17]. Spherisorb materials were obtained from Phase Separations (Queensferry, UK), Partisil materials from Whatman (Clifton, NJ, USA), μ-Bondapak from Waters and RSil from RSL–Bio-Rad (Eke, Belgium).

Solvents and mobile phases

Phosphate buffers (0.2 *M*) were prepared by mixing suitable amounts of 0.2 *M* solutions of ammonium dihydrogenphosphate and diammonium hydrogenphosphate (analytical-reagent grade, E. Merck). Tetrabutylammonium hydrogensulphate (TBA) (Janssen Chimica) was used to prepare 0.2 *M* TBA solutions. These solutions were adjusted to the required pH with 40% (w/v) sodium hydroxide solution before the solutions were brought to the final volume. LC-grade acetonitrile was obtained from Rathburn Chemicals (Walkerburn, UK). Water was distilled twice from glass. Mobile phases were degassed by sonication. Acetone was obtained from Janssen Chimica and was purified by distillation after refluxing in the presence of potassium permanganate.

Sample preparation and stability of the solutions

About 150 mg of EES bulk sample were dissolved in 6 ml of acetone and diluted to 10.0 ml with a mixture containing 12.5% (v/v) 0.2 *M* TBA (pH 6.5), 7.5% (v/v) 0.2 *M* phosphate buffer (pH 6.5) and water up to 100% (v/v).

TABLE I

INVESTIGATED STATIONARY PHASES

Stationary phase	Column No.	d_p (μm)	Particle shape[a]	Carbon content (%)
Spherisorb C_8	I	5	S	6
Spherisorb ODS 1	II	10	S	7
Spherisorb ODS 2	III	10	S	12
Partisil CCS/C_8	IV	10	I	9
Partisil ODS	V	10	I	5
Partisil ODS 2	VI	10	I	15
Partisil ODS 3	VII	10	I	10
μBondapak C_{18}	VIII	10	I	9
RSil LL C_{18}	IX	10	I	12

[a] S = spherical; I = irregular.

For dispersible powders and tablets, 10 ml of acetone were added to an amount corresponding to about 250 mg of EES. The suspension was sonicated for 5 min in a glass-stoppered test-tube and then centrifuged at 2500 *g* for 5 min. An aliquot of 6.0 ml of supernatant was diluted to 10.0 ml with the same aqueous mixture as described for bulk EES.

The stability of EAES in this solution was examined at room temperature and at 6°C using the described LC method. After 1 h the concentration of EAES was 96.2% of the initial concentration at room temperature and 98.7% at 6°C, owing to hydrolysis of EAES and formation of EA. Samples for analysis were therefore prepared immediately before use. Rapid hydrolysis of EAES to EA in neutral aqueous solution has been reported previously [18].

Quantitative analysis

Analysis of the samples was finally performed on an RSil LL C_{18} (10 μm) column (25 cm × 4.6 mm I.D.). For the determination of the main component a house standard of EAES was used. The standard was purified by four consecutive crystallizations from acetone–acetonitrile (1:1). By LC this standard was found to contain 2.1% EA, 0.2% ESdMeEA and 0.6% ECES. Titrations with perchloric acid in non-aqueous medium gave a mean value of 97.9% calculated as EAES with a relative standard devia-

tion (R.S.D.) of 0.2% for $n = 11$ determinations. When the titration result was corrected for the amount of EA, which is also titrated and which has a lower molecular mass than EAES, a total base content of 97.5% was obtained. The water content was determined by means of a Karl Fischer titration using a 10% (w/v) solution of imidazole in methanol as the solvent [19]. The result was 2.6% (R.S.D. = 1.5%, $n = 9$). The presence in the house standard of residual organic solvents was examined by gas chromatography under conditions where acetonitrile could be detected at the 0.05% (w/v) level and acetone at the 0.1% (w/v) level. No solvents were detected. The total mass (100%) was thus sufficiently explained by the base titration result (97.5%) and the water content (2.6%). Therefore, the standard was accepted to contain 100% − 2.9% (total impurities by LC) − 2.6% water = 94.5% EAES.

Regression lines for EAES, EA, ESdMeEA and EAENES are given in Table II. The regression line obtained with small amounts of EAES was used for the quantification of EBES or ECES. Regression lines obtained with EAES were corrected for a purity of 94.5%. Other curves were not corrected. Limits of quantification for an injected amount of 300 μg were 1 μg (0.3%) for EA, 1.5 μg (0.5%) for EB, 2 μg (0.6%) for AEA, EAES and AEAES, 0.15 μg (0.05%) for ESdMeEA and 0.5 μg (0.15%) for EAENES.

RESULTS AND DISCUSSION

Structure of N-ethylsuccinyl-N-demethylerythromycin A (ESdMeEA)

The amide structure of ESdMeEA was determined by mass and NMR spectrometry. The mass spectrum exhibited a weak molecular ion at *m/z* 847 (0.1%) and other weak ions at *m/z* 829 ($M-H_2O$, 0.2%), 802 ($M-C_2H_5O$, 0.1%) and 784 (829 − C_2H_5O, 0.5%). Prominent ions occurred at *m/z* 272 (desosamine, 100%), 113 (94%) and 101 (91%). The ^{13}C NMR spectrum in C^2HCl_3 clearly showed the 21 aglycone resonances, the 8 cladinose and the 6 ethylsuccinyl peaks at their expected positions. The remaining 7 desosamine signals (103.9, 71.5, 68.3, 54.1, 35.5, 28.6 and 20.7 ppm) showed a pattern indicative of an N-ethylsuccinyl moiety. Indeed, esterification of the 2′-OH function normally results in a characteristic 2–2.5 ppm upfield shift of C-1′ and C-3′ and a *ca.* 1 ppm downfield shift of C-2′. In this case, however, when compared with the spectrum of dMeEA [20], a 1 ppm downfield shift of C-1′ was observed together with a substantial upfield shift for C-3′ (−5.7 ppm), the N-CH_3 group (−3.4 ppm) and C-4′ (−1.2 ppm). Another strong argument in favour of the 3′-N-amide structure is the appearance of supplementary resonances at a 30% intensity level for almost all desosamine signals. These are probably due to the presence of different rotamers in solution as a result of restricted rotation of the amide bond.

Development of the mobile phase

As erythromycin ethylsuccinate (EES) is prepared by reaction of erythromycin with ethylsuccinyl chloride, the chromatographic conditions were examined in relation to the separation of EA and its decomposition products AEA and EAEN, which are formed in acid, and of the corresponding esters EAES, AEAES and EAENES. First, acetonitrile–

TABLE II

REGRESSION LINES

y = Peak area; x = mass injected (μg); $S_{y,x}$ = standard error of estimate; R = range of injected mass (μg) examined.

Compound	Slope	Intercept	Correlation coefficient	$S_{y,x}$	R
EAES	253 394	−412 491	0.999	635 562	230 –360
	255 940	−41 860	0.998	48 278	5 –60
EA	196 001	44 537	0.999	27 924	2 –16
ESdMeEA	1 242 087	102 394	0.998	99 786	0.4–3.5
EAENES	2 069 873	129 400	0.999	52 827	0.5–3.0

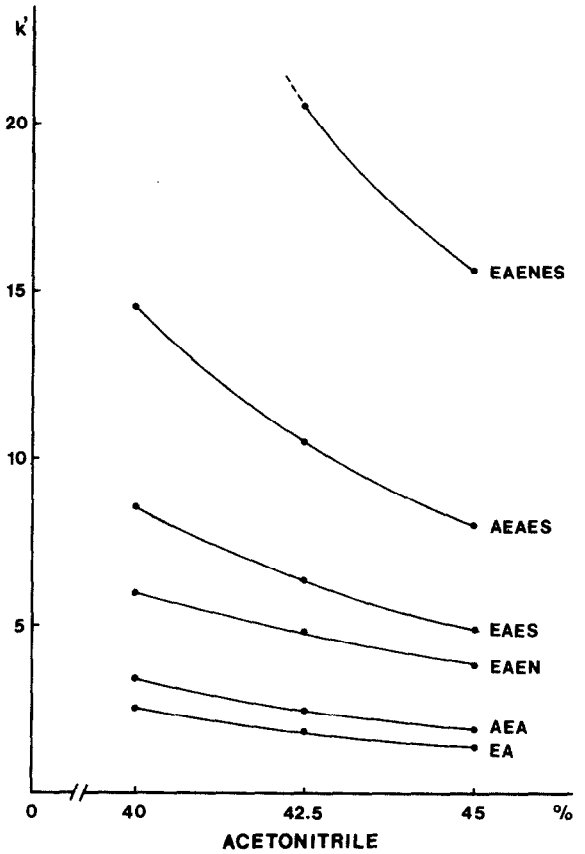


Fig. 2. Influence of the acetonitrile content on the separation. Mobile phase, acetonitrile–0.2 *M* TBA (pH 6.5)–0.2 *M* phosphate buffer (pH 6.5)–water (x:5:5:90 − x); column, Partisil ODS 3, 10 μm (250 mm × 4.6 mm I.D.); flow-rate, 1.5 ml/min; temperature, 35°C; detection, UV at 215 nm.

0.2 *M* TBA (pH 6.5)–0.2 *M* phosphate buffer (pH 6.5)–water (x:5:5:90 − x) was chosen as the mobile phase. This was derived from that previously used for erythromycin [8,21].

Owing to the stronger retention of the esters as compared with erythromycin, it was necessary to use mobile phases with a higher acetonitrile content.

In Fig. 2 it is demonstrated that on a Partisil ODS 3 column the proportion of acetonitrile should be at least 42.5% for EAENES to be eluted within a reasonable period of time. However, these increased levels of acetonitrile negatively affect the separation of the other components. Variation of the pH of the mobile phase between 6 and 7 did not have much effect on the separation. At pH 7 EAEN was eluted closer to EAES than at pH 6. A pH higher than 7 was not considered because it lowers the stability of the stationary phases. Below pH 6 the peak symmetry of the esters was severely affected and on most of the higher loaded stationary phases fronting was observed. Therefore, pH 6.5 was adopted in subsequent chromatography.

The influence of the TBA content of the mobile phase is shown in Fig. 3. The use of TBA influenced the order of elution and reduced the total analysis time. The effect of TBA on the retention times was less pronounced for the esters. This can be explained by their pK_b values. Esters (pK_b 6.3) are considerably less basic than erythromycin (pK_b 4.5) [18]. Beyond 5%, a further increase in TBA content did not substantially alter the separation. Therefore, this concentration was adopted. The influence of the concentration of the buffer in the mobile phase on

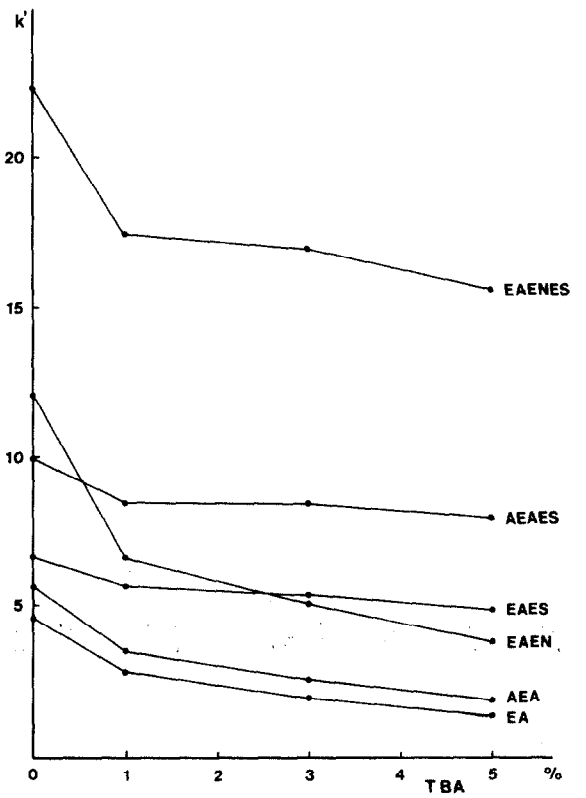


Fig. 3. Influence of the concentration of tetrabutylammonium ion (TBA) on the separation. Mobile phase, acetonitrile–0.2 *M* TBA (pH 6.5)–0.2 *M* phosphate buffer (pH 6.5)–water (45:x:5:50 − x); other conditions as in Fig. 2.

the chromatography was small. Only the separation between EAEN and EAES was improved slightly on increasing the buffer concentration from 1% to 10%. An intermediate value of 5% was adopted.

With the mobile phase acetonitrile–0.2 *M* phosphate buffer (pH 6.5)–0.2 *M* TBA (pH 6.5)–water (45:5:5:45), the capacity factors of EA, AEA, EAEN, EAES, AEAES and EAENES were determined on the stationary phases mentioned in Table I. The results are shown in Fig. 4. The same elution order was observed on all the columns. For this reason, the position of the substances is indicated by their elution number. The elution pattern is dependent of the stationary phase. The higher loaded phases show the strongest retention, but the carbon content alone does not explain the total analysis time, as is shown, for example, by columns V and VIII, nor does a higher carbon content guarantee better selectivity towards all the compounds examined. The total analysis time can be adapted by adjusting the acetonitrile content of the mobile phase. The chromatography of these erythromycin esters was not influenced by the age of the column, as was mentioned previously for erythromycins [8,22,23].

Quantitative analysis of bulk erythromycin ethylsuccinate

With the mobile phase acetonitrile–0.2 *M* TBA (pH 6.5)–0.2 *M* phisphate buffer (pH 6.5)–water (42.5:5:5:47.5) and an RSil C_{18} LL column, a number of commercial EES samples of various origin were analysed. Small amounts of EA were found in most samples whereas AEA, EAEN and AEAES were not present. EAENES was present in very small amounts. The chromatograms of most samples showed the presence of other impurities.

As EB and EC may be present in the erythromycin used for the preparation of the ester, the corresponding esters EBES and ECES can be present as impurities. The presence of EBES and ECES in commercial samples has already been mentioned by Tsuji and Goetz [4]. Meanwhile, we demonstrated that, in addition to EB and EC, commercial-grade erythromycin may contain appreciable amounts of EE, EF and dMeEA (up to 5%) and small amounts of the decomposition products EAEN and psEAEN (up to 1%). AEA and psEAHK were found in negligible amounts [21]. Therefore, the separation of the esters EBES, ECES, EFES, EEES, psEAENES and psEAHKES and of the amide ESdMeEA was also examined. The retention times of the various components relative to that for EAES are given in Table III. There was no separation between EEES and EAES or between EFES and ESdMeEA. Except for ESdMeEA, the order of elution of the various components was consistent with the order of elution of the parent components [22]. Based on these

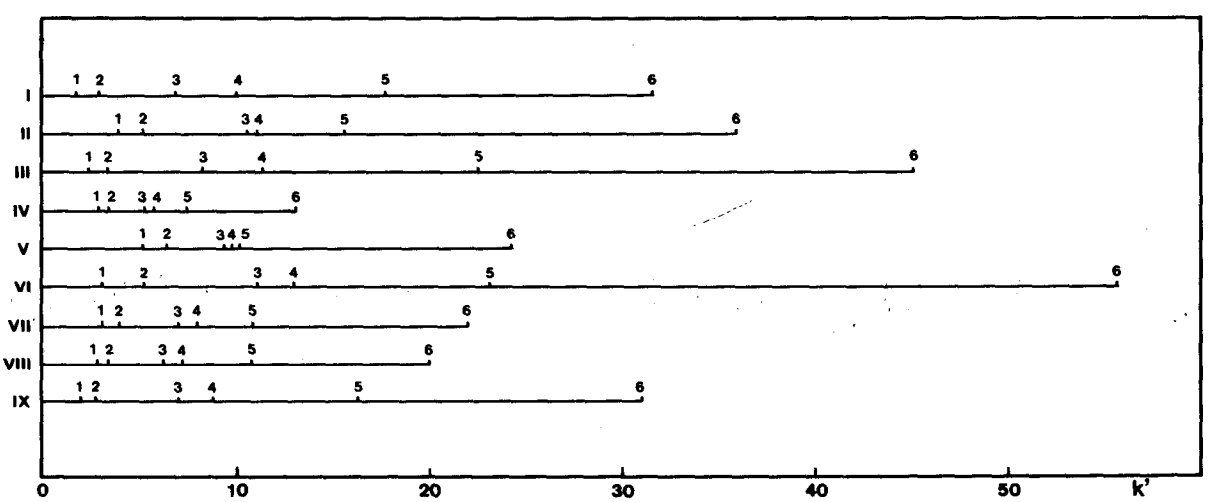


Fig. 4. Influence of the stationary phase on the separation. See Table I for column identification and characteristics. Mobile phase, acetonitrile–0.2 *M* TBA (pH 6.5)–0.2 *M* ammonium phosphate buffer (pH 6.5)–water (45:5:5:45), other conditions as in Fig. 2. 1 = EA; 2 = AEA; 3 = EAEN; 4 = EAES; 5 = AEAES; 6 = EAENES.

TABLE III

RELATIVE RETENTION TIMES OF THE VARIOUS ETHYLSUCCINATE DERIVATIVES

Column, RSil C_{18} LL, 10 μm (250 mm × 4.6 mm I.D.); mobile phase, acetonitrile–0.2 *M* TBA (pH 6.5)–0.2 *M* phosphate buffer (pH 6.5)–water (42.5:5:5:47.5); temperature, 35°C; flow-rate, 1.5 ml/min; detection, UV at 215 nm.

Compound	Relative retention time
Erythromycin F ethylsuccinate (EFES)	0.41
N-Ethylsuccinyl-N-demethylerythromycin A (ESdMeEA)	0.43
Erythromycin C ethylsuccinate (ECES)	0.65
Erythromycin E ethylsuccinate (EEES)	0.97
Erythromycin A ethylsuccinate (EAES)	1.00
Pseudo-erythromycin A hemiketal ethylsuccinate (psEAHKES)	1.51
Erythromycin B ethylsuccinate (EBES)	1.68
Anhydroerythromycin A ethylsuccinate (AEAES)	1.90
Pseudo-erythromycin A enol ether ethylsuccinate (psEAENES)	2.08
Erythromycin A enol ether ethylsuccinate (EAENES)	3.83

relative retention times we were able to identify EBES, ECES and ESdMeEA in a number of EES samples. Fig. 5 shows a typical chromatogram of a commercial EES sample. As there was no separation between EEES and EAES or between EFES and ESdMeEA, the samples were methanolysed and it was possible to show, by thin-layer chromatography (TLC), the presence of EF, but not of EE. This infers the presence of EFES in commercial EES samples. TLC of erythromycin has been extensively discussed elsewhere [14].

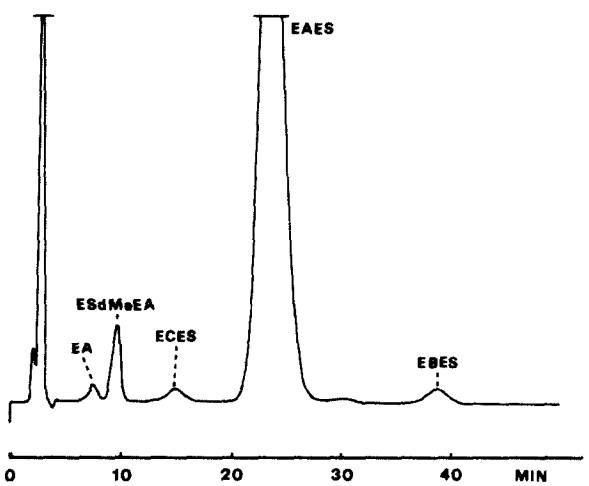


Fig. 5. Typical chromatogram of a bulk sample of erythromycin ethylsuccinate. Mobile phase, acetonitrile–0.2 *M* TBA (pH 6.5)–0.2 *M* phosphate buffer (pH 6.5)–water (42.5:5:5:47.5); column, RSil C_{18} LL, 10 μm (250 mm × 4.6 mm I.D.); other conditions as in Fig. 2. EA = erythromycin A; ESdMeEA = N-ethylsuccinyl-N-demethylerythromycin A; ECES = erythromycin C ethylsuccinate; EAES = erythromycin A ethylsuccinate; EBES = erythromycin B ethylsuccinate.

Determination of erythromycin ethylsuccinate

Table IV gives the results obtained in the analysis of commercial samples of bulk erythromycin ethylsuccinate. Some samples from manufacturer B contained large amounts of EBES (up to 16.4%). However, recent batches from the same manufacturer showed the highest content of EAES (94.9%). The content of ESdMeEA never exceeded 1.1%. Owing to the five times higher specific absorbance of ESdMeEA at 215 nm compared with EA, ESdMeEA gave relatively high peaks. In faster eluting systems ESdMeEA may be eluted together with EA, leading to overestimation of the EA content. EFES, which was eluted together with ESdMeEA, was determined by means of TLC. Only one sample contained a detectable amount of EAENES. By addition of the different components, figures close to 100% were obtained. The R.S.D. for EAES was never higher than 1.0%. Sample No. 1 was used as a secondary standard. It was analysed 47 times over a period of 20 days with an R.S.D. of 0.9% on the peak area corresponding to EAES.

TABLE IV

COMPOSITION (%, w/w) OF BULK SAMPLES OF ERYTHROMYCIN ETHYLSUCCINATE

Manufacturer	Sample No.	EA	ESdMeEA[a]	EFES[b]	ECES	EB	EAES (*n*, R.S.D., %)	EBES	EAENES	H_2O[c] (*n*, R.S.D., %)	Total
A	2	1.0	1.0	0.5	0.8	<0.5	92.8 (3, 0.1)	0.7	<0.15	1.7 (5, 2.2)	98.5
B	6	0.6	0.5	0.5	0.5	3.0	75.0 (3, 0.7)	16.4	<0.15	1.6 (4, 1.7)	98.1
	13	1.0	0.5	<0.5	0.5	<0.5	94.9 (3, 0.7)	2.3	<0.15	1.5 (3, 3.0)	100.7
C	9	0.7	0.4	<0.5	1.2	<0.5	94.6 (3, 1.0)	0.6	<0.15	1.8 (3, 1.5)	98.7
D	14	1.1	1.1	<0.5	1.7	<0.5	91.8 (3, 1.0)	2.8	0.4	1.4 (3, 4.6)	100.3
E	1	1.0	0.8	0.5	1.2	<0.5	93.1 (3, 0.9)	2.3	<0.15	1.4 (7, 5.3)	100.3

[a] No correction was applied for EFES, eluted together with ESdMeEA. Under the conditions used, the specific absorbance of ESdMeEA is about five times larger than that of EFES.
[b] Evaluated by TLC.
[c] KF titration.

Results obtained by analysis of specialties are reported in Table V. The content of EAES ranged from 90.4 to 112.9% of the label claim, with EA being the most important impurity (up to 6.3%). The higher content of EA in specialties compared with that in bulk samples suggests that some EA is formed during processing. This is consistent with the lability of the ester towards hydrolysis.

The results show that LC is very suitable for the determination of erythromycin ethylsuccinate in bulk samples and in preparations.

TABLE V

COMPOSITION OF SPECIALTIES AS A PERCENTAGE (w/w) OF LABEL CLAIM

Manufacturer	Sample No.	Pharmaceutical form[a]	EA	ESdMeEA	ECES	EAES (*n*, R.S.D.)	EBES	Total
A	19	G	4.0	0.4	0.5	91.6 (3, 0.4)	1.0	97.5
	21	T	1.0	0.8	1.5	95.3 (3, 0.4)	0.6	99.2
E	22	DP	0.4	0.9	1.7	96.6 (3, 0.8)	1.9	101.5
F	15	DP	6.3	0.4	0.5	112.9 (3, 0.6)	0.8	120.9
G	18	DP	3.1	0.3	0.7	90.4 (3, 1.0)	0.6	94.5

[a] DP = Dispersible powder, G = granules, T = tablets.

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