Quantitative analysis of erythromycin by reversed-phase liquid chromatography using column-switching

TH. CACHET,* K. DE TURCK, E. ROETS and J. HOOGMARTENS†

Katholieke Universiteit Leuven, Instituut voor Farmaceutische Wetenschappen, Laboratorium voor Farmaceutische Chemie, Van Evenstraat 4, B-3000 Leuven, Belgium

Abstract: A column-switching technique is described for LC of erythromycin. The method allows, in about 1 h, the separation of erythromycin A from all its known potential impurities, except erythromycin D, which is a minor impurity. The switching technique combines two columns (7.5 cm × 4.6 mm and 25.0 cm × 4.6 mm) both packed with RSil C 18 LL 10 μm. The mobile phase is acetonitrile–tetrabutylammonium sulphate (0.2 M, pH 6.0)–ammonium phosphate buffer (0.2 M, pH 6.0)–water (24:5:5:66, v/v/v/v). Temperature was 35°C, flow rate was 1.5 ml min^{-1}, detection was by UV at 210 nm. Results for a number of commercial samples of various origin are reported.

Keywords: *Reversed-phase liquid chromatography; column-switching; erythromycin.*

Introduction

The main component of the widely used macrolide antibiotic erythromycin is erythromycin A (EA). Originally, erythromycin B (EB) and C (EC) and the acid degradation products erythromycin A enol ether (EAEN) and anhydroerythromycin A (AEA) were considered as the main impurities of commercial erythromycin [1, 2]. In fact, commercial erythromycin can also contain a number of other components such as erythromycin D (ED) [3], *N*-demethyl-erythromycin A (dMeEA) [3], erythromycin F (EF) [4], erythromycin A *N*-oxide (EANO) [4] and erythromycin E (EE) [5].

Pseudoerythromycin A enol ether (psEAEN) and pseudoerythromycin A hemiketal (psEAHK), which are formed in neutral and alkaline solutions, may also be present [6]. The structures of the various components are shown in Fig. 1. Although several recent papers from other laboratories have been devoted to analysis of erythromycin by liquid chromatography (LC), no method separating all these components has been published [7–9]. Most of the recent papers refer to methods for the separation of erythromycin components in samples of biological origin [10–20]. These methods are not suitable for the assay or purity control of bulk erythromycin and preparations.

In this paper a LC method is described which has been derived from a previously published one [21] and which permits the separation and quantitation of most erythromycin components.

Experimental

Reference substances and samples

Part of the bulk samples were of known origin (Italy, France, PR China, Brazil or USA) while others, obtained through wholesalers, were of unknown origin. House standards of EA, EB, dMeEA, AEA and EAEN were available. EA house standard was obtained by crystallization of a commercial sample as described [22]. EB was obtained by preparative LC of mother-liquor concentrates from the industrial production of erythromycin [23]. AEA [24], EAEN [25] and dMeEA [26] were prepared from EA according to the described methods.

The base content of the standards was determined by non-aqueous titration with 0.1 N $HClO_4$ in acetic acid. Four independent series of titrations gave a mean of 95.25% m/m [total number of titrations $n = 27$; relative standard deviation (RSD) = 0.9%] for EA. The water content of the EA house standard was determined by Karl Fischer titration using a 10% m/v solution of imidazole in methanol as
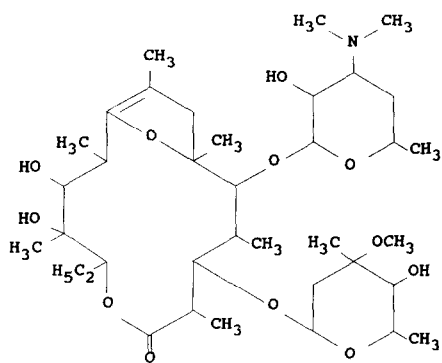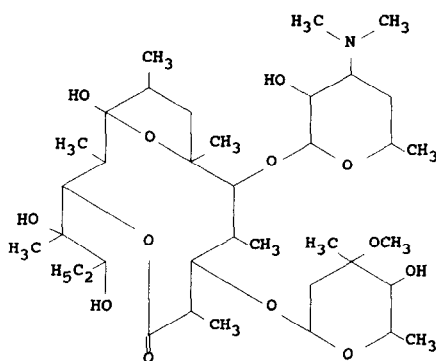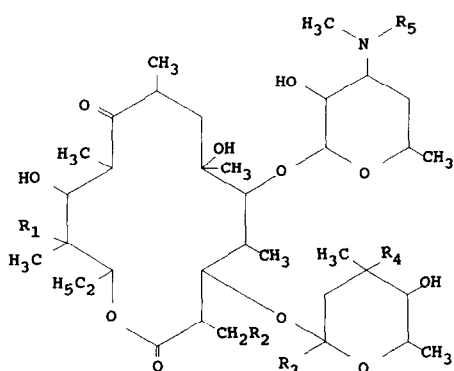
Erythromycin A enol ether (EAEN)
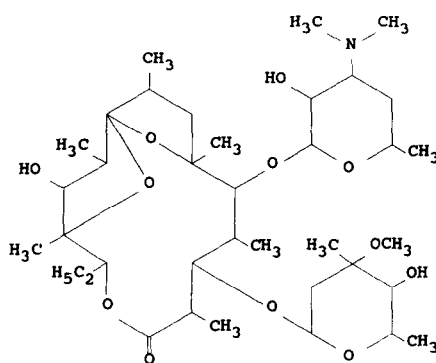
Anhydroerythromycin A (AEA)
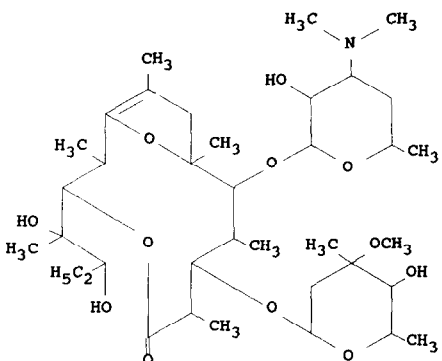
Pseudoerythromycin A enol ether (psEAEN)

Pseudoerythromycin A hemiketal (psEAHK)

	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 D (ED)	H	H	H	OH	CH_3
Erythromycin F (EF)	OH	OH	H	OCH_3	CH_3
Erythromycin E (EE)	OH	— O	—	OCH_3	CH_3
N-demethylery-thromycin A (dMeEA)	OH	H	H	OCH_3	H

Figure 1
Structures of erythromycin components.

the solvent [27]. The result was 4.75% m/m (n = 9, RSD = 2.7%). By chromatography the EA house standard was found to contain 0.5% of dMeEA and 0.05% of psEAEN. Therefore the house standard was accepted to contain 94.7% of EA [95.25% − 0.5% (dMeEA) − 0.05% (psEAEN)]. The other house standards were titrated at least three times resulting in a base content of 98.8% for

EB, 97.8% for dMeEA, 96.0% for AEA and 98.0% for EAEN. No correction was made for the small amounts of impurities found by LC. Small amounts of the other related substances EC, ED, EF, EE, EANO, psEAEN and psEAHK were also available. EC and ED were obtained by preparation LC of mother-liquor concentrates [23]. The isolation of EF and EE from commercial erythromycin has

been described [4, 5]. EANO [28], psEAEN and psEAHK [29] were prepared from EA as described.

LC apparatus

The LC equipment consisted of a Model 6200 pump (Merck–Hitachi, Darmstadt, Germany), a Marathon autosampler (Spark, Emmen, The Netherlands) fitted with a 50 μl loop, a Spectro Monitor 3100 UV detector (Milton Roy–LDC, Riviera Beach, FL, USA) set at 210 nm and a Model 3393 A integrator (Hewlett–Packard, Avondale, PA, USA). Water circulation was used to maintain the column temperature at 35°C. For column-switching an electrically actuated 10 port switching-valve (Valco, Houston, TX, USA) was used. Switching was programmed with the Marathon injector. Figure 2 shows the column assembly.

Columns of 25.0 cm × 4.6 mm i.d. were packed following a classical slurry packing procedure [30] with Spherisorb (Phase Separations, Queensferry, UK), Partisil (Whatman, Clifton, NJ, USA), Nucleosil (Macherey–Nägel, Düren, Germany), LiChrosorb (Merck), RoSil and RSil (RSL–Biorad, Eke, Belgium). For the quantitative analysis of erythromycin samples columns of 7.5 cm × 4.6 mm i.d. and 25.0 cm × 4.6 mm i.d. were packed with RSil C18 LL, 10 μm. These columns were conditioned prior to use for analysis of erythromycin. The columns were flushed with a mobile phase methanol–water–phosphoric acid 1 M (50:45:5, v/v/v), tightly closed and heated at 120°C for 1 h. A heating time of 1 h was sufficient to improve the chromatography without affecting the retention times of the separated components. More details on this conditioning procedure are described elsewhere [31, 32]. The short column was replaced after 150 analyses, the long column after 300 analyses.

Mobile phases

The mobile phase finally used for the analysis of erythromycin was acetonitrile–tetrabutylammonium sulphate (0.2 M, pH 6.0)–ammonium phosphate buffer (0.2 M, pH 6.0)–water (23:5:5:67, v/v/v/v). Acetonitrile (HPLC Grade S) was obtained from Rathburn (Walkerburn, UK). Ammonium dihydrogen phosphate and diammonium hydrogen phosphate were pro-analysi from Merck, 0.2 M solutions of these salts were mixed to prepare the buffer. Tetrabutylammonium hydrogen
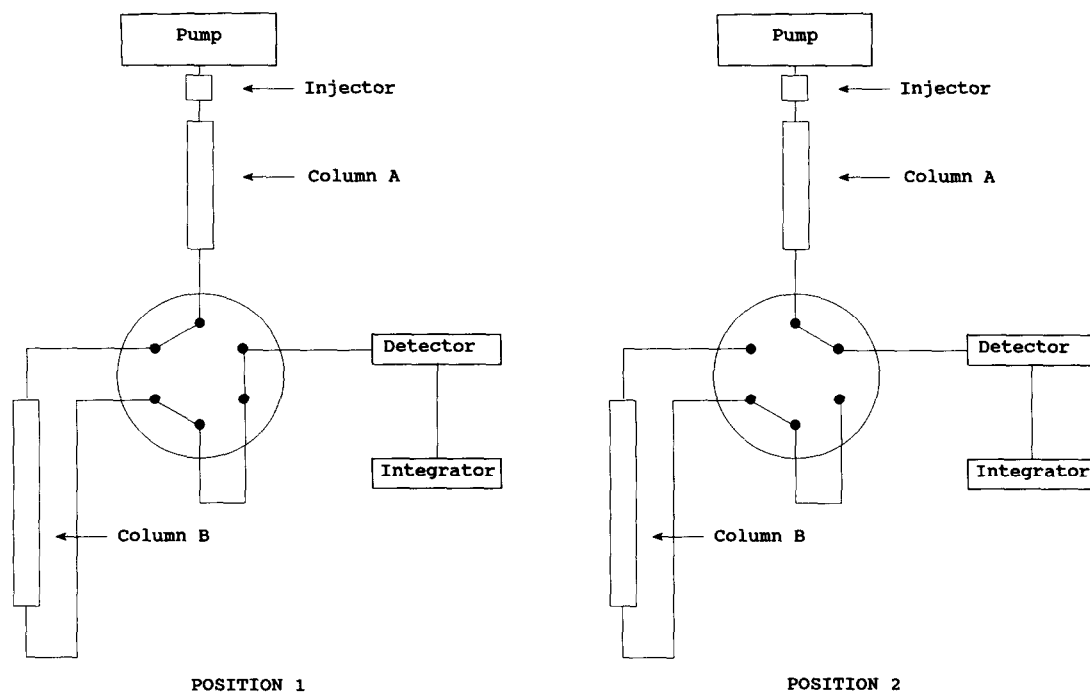
Figure 2
Column-switching assembly. Column A: 7.5 cm × 4.6 mm, column B: 25 cm × 4.6 mm. Both columns are packed with RSil C18 LL 10 μm. Position 1: chromatography through both columns. Position 2: chromatography through the short column only.

sulphate (TBA) (Janssen Chimica, Beerse, Belgium) was used to prepare 0.2 M TBA solutions. These solutions were adjusted to pH 6.0 with 40% m/v sodium hydroxide in water before the solutions were brought to the final volume. The mobile phase was degassed by ultrasonication. The flow rate was 1.5 ml min^{-1}.

Sample preparation and stability

Aliquots of commercial samples (100.0 mg) were dissolved in mobile phase and diluted to 25.0 ml with the same solvent. At room temperature the EA content in this solution was found to decrease at a rate of 0.02% h^{-1}.

Results and Discussion

Development of the HPLC method

As a starting point the LC method was used which was described for the separation of EC, dMeEA, EA, AEA, EB and EAEN [21]. This method used C8 or C18 reversed-phases and acetonitrile–ammonium phosphate buffer (0.2 M, pH 6.5)–tetramethylammonium phosphate (0.2 M, pH 6.5)–water (25–40:5:20:50: 50–35, v/v/v/v). Preliminary work demonstrated that the separation between EE and EA was most critical. EE, an inactive fermentation impurity, is present in a large number of commercial samples. By decreasing the acetonitrile (<25%) in the mobile phase and substituting tetramethylammonium phosphate by TBA it was possible to separate EE from EA on most C18 columns tested. Additional advantages were that less quaternary salt was required in the mobile phase and that the pH could be decreased to 6.0 without loss of

separation. The separation between EE and EA obtained on different C18 columns is shown in Table 1. Although EE was separated from EA on most columns, separation of EE resulted in a substantial increase of the retention time of stronger retained erythromycin components such as EB and EAEN. A typical chromatogram is shown in Fig. 3. Gradient elution, a normal approach for the improvement of the separation shown in Fig. 3 was not possible due to the low detection wavelength (<215 nm) and the presence of quaternary ammonium salts in the mobile phase.

Another option was the development of a method with a column-switching technique. This technique was also successfully applied in our laboratory for the analysis of a complex analgesic formulation [33].

Retention time increases with column length. For column length zero it can be assumed that the retention time will approach zero. An approximate relationship between retention time and column length can be obtained with only one experiment with a 25 cm column [33]. This is shown in Fig. 4 where retention times of the various erythromycin components were determined on a 25 cm column. From these data the approximate retention time of a particular component can be predicted for a given column length. The erythromycin derivatives can be divided into two groups based on elution speed. The faster eluted components are EF, EC, dMeEA, EE and EA. The second group contains the derivatives with a relatively high capacity factor, i.e. AEA, psEAEN, EB, EANO and EAEN. Separation of the latter group is not a major problem and does not

Table 1
Capacity factors of some erythromycin components on various stationary phases

	EF	EC	dMeEA	EE	EA	CH$_3$CN (x %)
LiChrosorb C18	1.9	5.0	10.3	10.5	12.2	19
Nucleosil C18	1.9	5.5	9.0	10.0	12.1	21
Partisil ODS 1	1.9	3.4	5.2	5.6	6.9	23
Partisil ODS 2	3.3	7.3	14.0	16.1	18.6	21
Partisil ODS 3	1.8	4.5	8.0	8.4	10.1	22
RosiL C18	1.3	3.2	5.7	6.2	7.9	24
RSiL C18 LL	1.8	4.2	6.9	7.3	8.8	23
RSiL C18 HL	1.5	4.3	6.9	7.5	9.3	25
Spherisorb ODS 1	2.2	5.7	10.3	11.0	13.0	20
Spherisorb ODS 2	3.1	9.0	16.5	17.5	21.3	17

Mobile phase: acetonitrile–TBA (0.2 M, pH 6.0)–ammonium phosphate buffer (0.2 M, pH 6.0)–water (x:5:5:90 − x, v/v/v/v). Columns (25.0 cm × 4.6 mm i.d.) were packed with 10 μm particles except for RoSil C18 (5 μm). Temperature: 35°C, flow rate: 1.5 ml min^{-1}, detection: UV at 210 nm.
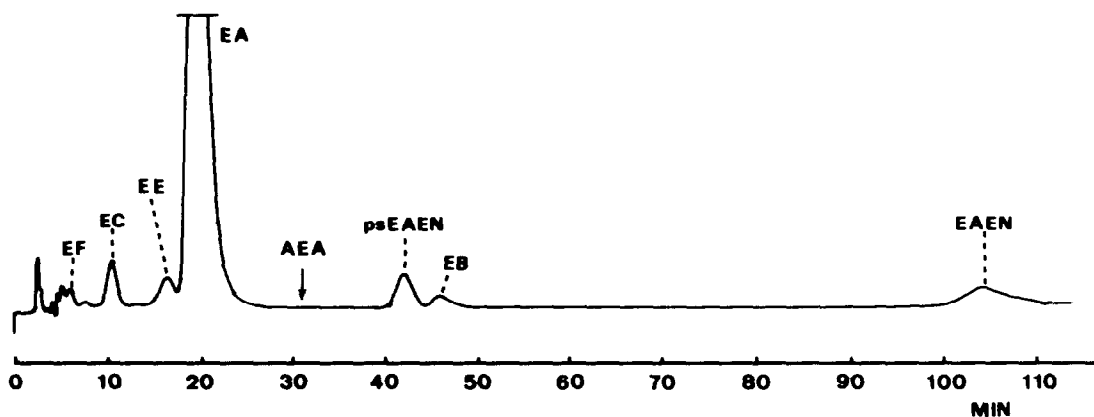
Figure 3

Typical chromatogram of a commercial erythromycin sample without column-switching. Column: RSil C18 LL, 10 μm, 25.0 cm × 4.6 mm i.d. Mobile phase: acetonitrile–TBA (0.2 M, pH 6.0)–ammonium phosphate buffer (0.2 M, pH 6.0)–water (23:5:5:67, v/v/v/v). Temperature: 35°C, flow rate: 1.5 ml min⁻¹, detection: UV at 210 nm. Sample injected: 200 μg. EF = erythromycin F, EC = erythromycin C, EE = erythromycin E, EA = erythromycin A, psEAEN = pseudo-erythromycin A enol ether, EB = erythromycin B and EAEN = erythromycin A enol ether.
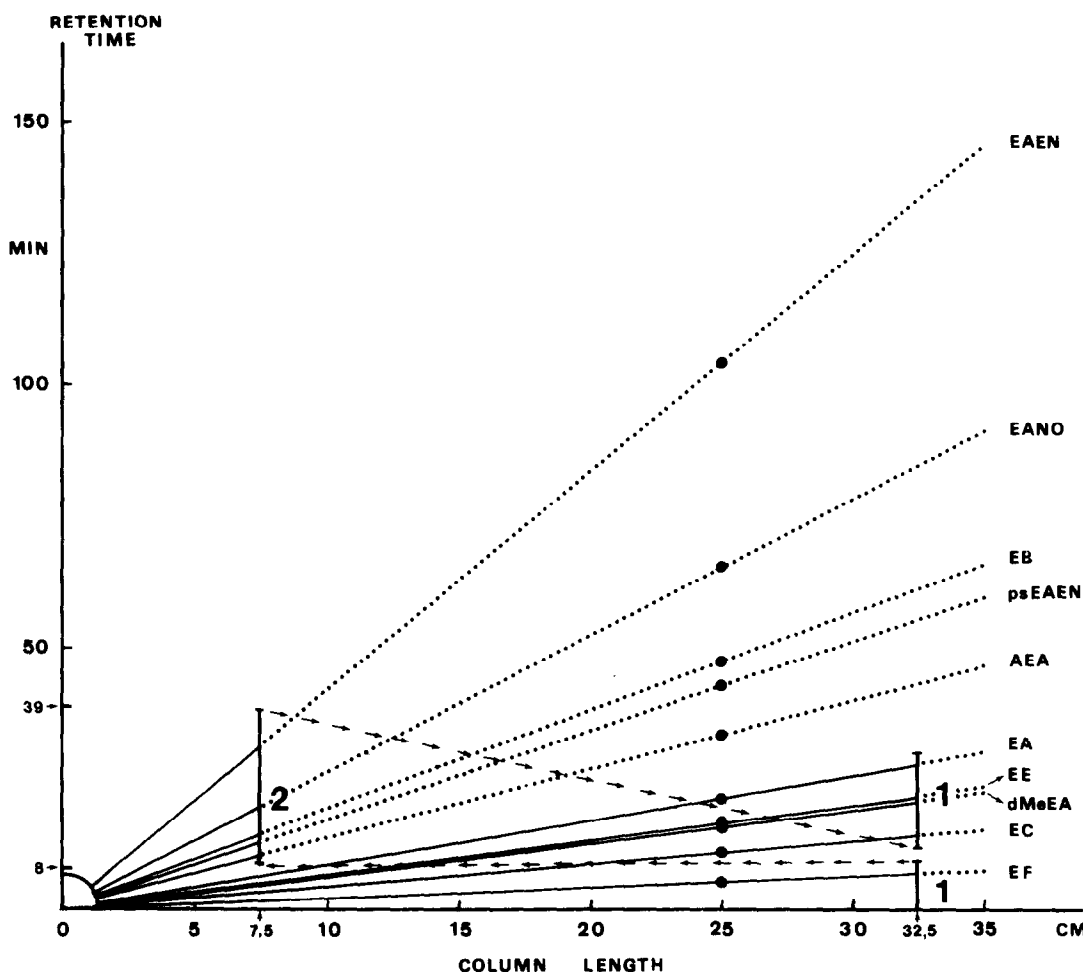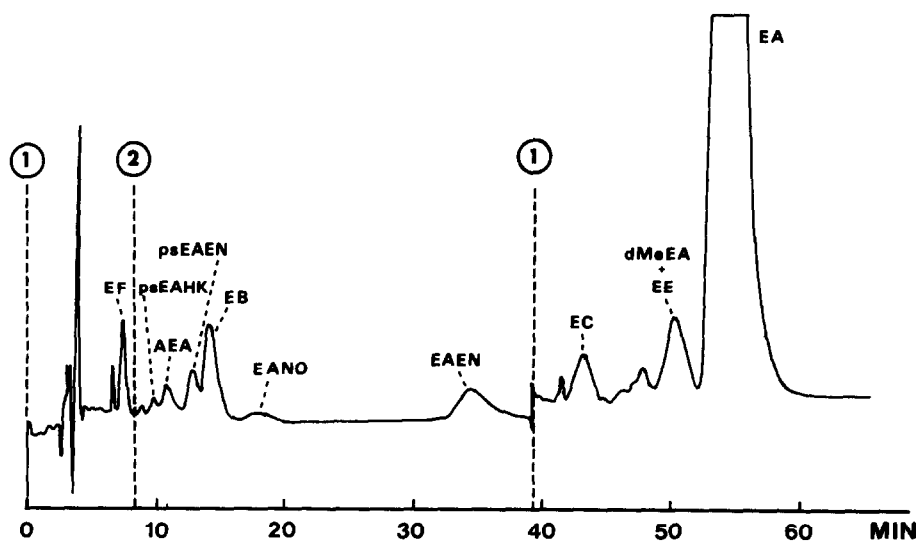

Figure 4

Retention times in relation with column length. ● = Experimental point. Column: RSil C18 LL, 25.0 cm × 4.6 mm i.d. Mobile phase: acetonitrile–TBA (0.2 M, pH 6.0)–ammonium phosphate buffer (0.2 M, pH 6.0)–water (23:5:5:67, v/v/v). For other conditions see Fig. 3. The retention times are extrapolated for zero column length. The arrows and the vertical bars indicate the order of elution, starting with EF and ending with EA, obtained by combining a 25 cm column and a 7.5 cm column (total length: 32.5 cm). The numbers 1 and 2 beside the vertical bars refer to the position of the switching valve. See Fig. 2.

require the full length of a 25 cm column as shown in Fig. 4. A column length of 7.5 cm seems to be sufficient. On the other hand, a 25 cm column is barely sufficient for the separation of the former group of components. By connecting a short column (7.5 cm) and a 25 cm column as shown in Fig. 2, a separation of the slower eluted group can be obtained on the short column while the separation of the faster eluted group is improved due to the increase of the total column length (32.5 cm). Ideally, with such a column assembly, the slower group is separated on the short column while the faster group is trapped on the second column. Hereafter the fast components are eluted from the long column. Unfortunately this sequence could not be realised since not all the fast eluted components could be trapped on the long column. Indeed, EF has already passed the whole system (32.5 cm) when AEA starts to elute (about 8 min) from the short column. Therefore, in the switching scheme applied, EF was allowed to move through the total column system (32.5 cm, position 1) while EC, dMeEA, EE and EA were collected on the 25 cm column. After 8 min the system was switched to position 2 (7.5 cm column) for elution of AEA, psEAEN, EB, EANO and EAEN. After complete elution of EAEN (39

min) the system was switched back to position 1 to elute the trapped substances EC, dMeEA, EE and EA from the 25 cm column. A typical chromatogram, obtained with the column-switching technique is shown in Fig. 5. The total analysis time is about 60 min compared to about 110 min for an ordinary technique without switching (see Fig. 3). The very small peak eluted right before AEA was identified as psEAHK. On this stationary phase dMeEA and EE were not separated. An additional advantage of the switching technique is that the limit of detection for the more strongly retained substances is lower.

Calibration curves and repeatability

Calibration curves were obtained with the house standard for EA, EB, dMeEA, AEA and EAEN. The following relationships were found, where y = peak area, x = amount of product injected (in μg), corrected for the purity of the standard, r = correlation coefficient, $S_{y,x}$ = standard error of estimate, R = range of injected mass examined. For EA: $y = 216036 + 185784\ x$, $r = 0.999$, $S_{.x} = 162327$, $R = 130–200\ \mu g$; EB: $y = 22658 + 135917\ x$, $r = 0.999$, $S_{y,x} = 26328$, $R = 4–30\ \mu g$; dMeEA: $y = 19817 + 181239\ x$, $r = 0.998$, $S_{y,x} = 101160$, $R = 2–35\ \mu g$; AEA: $y =$

Figure 5
Typical chromatogram of a commercial erythromycin obtained with the column-switching technique. Columns: 7.5 cm × 4.6 mm and 25.0 cm × 4.6 mm packed with 10 μm RSil C18 LL. Mobile phase: acetonitrile–TBA (0.2 M, pH 6.0)–ammonium phosphate buffer (0.2 M, pH 6.0)–water (23:5:5:67, v/v/v/v). Temperature: 35°C, flow rate: 1.5 ml min^{-1}, detection: UV at 210 nm. Sample injected: 200 μg. The numbers 1 and 2 indicate the position of the switching valve. See Fig. 2 for details. EF = erythromycin F, psEAHK = pseudo-erythromycin A hemiketal, AEA= anhydroerythromycin, psEAEN = pseudo-erythromycin A enol ether, EB = erythromycin B, EANO = erythromycin A N-oxide, EAEN = erythromycin A enol ether, EC = erythromycin C, dMeEA = demethylerythromycin A, EE = erythromycin E and EA = erythromycin A.

Table 2
Composition of bulk samples of erythromycin

Manufacturer	Sample No.	Age in months	EA	EB	EC	ED*	EE + dMeEA	EF	AEA*	psEAEN	EANO	EAEN	Sub-total	Water†	Total	Base‡
A	30	U	81.3 (0.7)	8.5	1.9	0.5	2.2	0.7	<0.5	<0.05	<0.5	0.05	95.2	4.8 (1.5)	100.0	94.6 (0.3)
	27	U	80.2 (0.1)	10.4	1.9	0.5	2.1	0.4	<0.5	<0.05	<0.5	0.05	95.5	4.6 (1.2)	100.2	94.9 (0.1)
B	1	U	86.4 (0.2)	1.2	3.6	<0.5	2.5	1.0	<0.5	0.08	0.5	0.08	95.4	2.6 (5.0)	98.0	95.3 (0.2)
C	57	U	88.6 (0.7)	1.5	2.2	<0.5	2.3	0.9	0.5	0.09	0.6	0.25	96.9	1.5 (7.0)	98.4	ND
D	55	U	89.5 (0.5)	1.2	0.9	<0.5	1.9	1.4	<0.5	0.07	<0.5	0.1	95.1	4.3 (2.2)	99.4	ND
	43	9	90.4 (0.3)	<0.5	3.2	<0.5	1.7	1.3	0.5	0.1	<0.5	0.05	97.3	1.5 (5.5)	98.8	97.5 (0.7)
E	23	107	83.4 (0.6)	7.1	3.0	<0.5	1.3	<0.4	0.5	0.05	<0.5	0.05	95.4	4.5 (1.8)	99.9	95.0 (0.3)
	60	U	88.7 (0.2)	0.9	3.9	<0.5	1.0	<0.4	0.5	0.05	<0.5	<0.05	95.0	4.8 (1.0)	99.8	ND
F	58	U	88.8 (0.4)	0.9	1.8	<0.5	1.2	1.1	0.5	0.06	<0.5	0.06	94.4	4.2 (2.0)	98.6	ND
G	15	46	82.4 (0.2)	3.3	1.4	<0.5	3.5	1.9	1.0	<0.05	<0.5	<0.05	93.5	4.4 (0.2)	97.9	94.2 (0.2)
	41	53	78.1 (0.1)	3.7	2.5	0.5	6.7	1.9	1.0	0.05	<0.5	<0.05	94.4	4.7 (3.0)	99.1	95.6 (0.2)
Wholesalers	3	U	84.7 (0.3)	<0.5	3.5	<0.5	4.3	1.5	<0.5	0.2	0.5	0.07	94.8	4.6 (3.3)	99.4	95.1 (0.3)
	7	105	91.8 (0.5)	<0.5	2.3	<0.5	1.7	<0.4	<0.5	<0.05	<0.5	0.07	95.8	3.5 (3.0)	99.3	95.2 (0.3)
	21	210	81.0 (0.6)	3.7	3.8	<0.5	6.0	1.0	<0.5	<0.05	<0.5	0.05	95.6	3.9 (2.0)	99.5	94.9 (0.3)
	18	19	81.1 (0.7)	2.7	2.0	0.5	4.0	1.9	1.0	0.5	<0.5	1.7	95.4	4.5 (1.1)	99.9	95.1 (0.5)
Secondary standard (n = 15)			81.8 (0.5)	3.3 (9.9)	2.2 (5.7)	<0.5	3.0 (12.4)	1.4 (8.0)	2.5 (15.5)	<0.05	0.5 (10.5)	0.02	94.7	4.4 (5.2)	99.1	94.5 (0.2)

Percentage values (m/m) are the mean of three analyses. RSD values are given in parentheses. U = unknown. ND = not determined due to limited amount of sample available.
* Evaluated by TLC.
† Evaluated by Karl Fischer titration, mean of at least three titrations.
‡ Determined by non-aqueous titration, mean of at least three determinations.

$99936 + 49744$ x, $r = 0.999$, $S_{y,x} = 103528$, $R = 3$–60 μg; EAEN: $y = 270031 + 2346189$ x, $R = 0.999$, $S_{y,x} = 399388$, $R = 0.3$–15 μg.

The calibration curve of EB was also used for the determination of EC and EF, the calibration curve of EAEN was also used for psEAEN and that for dMeEA was also used for EANO and for EE, from which dMeEA was not separated. The limit of quantitation, for an injected amount of 200 μg, was 0.4% for EF; 0.5% for EB, EC, dMeEA and EANO; 0.05% for EAEN and psEAEN and 1.0% for psEAHK and AEA. A secundary standard was analysed 20 times over a period of 5 days. The RSD for EA was 0.6%.

Analysis of commercial samples

More than 70 commercial bulk samples were analysed. Results of some samples are shown in Table 2. During these analyses a secundary standard was used, the composition of which is also given in Table 2. As it contained nearly all the impurities, it was at the same time useful for monitoring the quality of the LC separations. The EA content of the secundary standard was determined against EA house standard. The EA content of the bulk samples was calculated by comparison with chromatograms of the secundary standard, obtained before and after the chromatograms of the concerned sample. The content of impurities was calculated by means of the calibration curves. The results are a mean of at least three independent determinations. The RSD on the determination of EA was always lower than 1.0%. The RSD on the determination of impurities, only shown for the secundary standard, ranged from 5 to 25% depending on the concentration of the impurity. The EA content of the commercial samples ranged from 78.1 to 91.8%. Samples contained up to 10.4% EB, up to 6.7% dMeEA + EE and up to 3.9% EC. The most important impurities of erythromycin are thus EB, dMeEA + EE and EC, although some selected samples may contain as much as 2.3% of EF or 4.0% of EANO (samples not shown in table).

As ED was not separated from EA by LC and dMeEA was not separated from EE, the presence of ED and dMeEA was investigated using TLC on silica gel with as mobile phase ethyl acetate–methanol–25% ammonia (85: 10:5, v/v/v) [4, 5]. The detection limit was 0.5 μg or 0.5%. For ED this level was reached in only 10 samples indicating that ED indeed is

a minor component. dMeEA on the contrary was found in almost each sample at a level up to 2%, this means that the level of EE can reach up to 5–6%. But EE is not always present. TLC with a mobile phase dichloromethane–methanol–25% ammonia (90:9:1.5, v/v/v) was also used in order to achieve a slightly better detection limit for AEA and psEAHK (0.5% as compared with 1% for LC, for an injection of 200 μg). AEA was now detected in a number of samples, but not psEAHK. Owing to their stronger absorbance at 210 nm, EAEN and psEAEN were detected in a large number of the samples. The content of EAEN or psEAEN is usually lower than 0.1% although up to 1.8% of psEAEN (not shown in table) and up to 1.7% of EAEN were measured in some samples.

The sums of all detected erythromycin components and the water content of the samples, as measured by Karl Fischer titration, explain 98–100% of the total mass. The sums of detected components agree quite well with the results for the non-aqueous titration of the base content. This is an indication for the described LC method being appropriate for the analysis of erythromycin.

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