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Note

Thin-layer chromatography of erythromycins and other macrolides

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The literature on the thin-layer chromatography (TLC) of macrolides has recently been reviewed[1-3]. An improved separation of the components of erythromycin has been reported by Vanderhaeghe and Kerremans[3]. The use of high-performance thin-layer chromatography in the analysis of some macrolides has been the subject of a recent publication[4].

The best TLC system known, described for the separation of erythromycins, uses plates coated with silanized silica gel and methanol–water–15% ammonium acetate buffer pH 7.0 (50:20:10) as the mobile phase[3]. This system (R-VI in Table I) allows very good separation of erythromycin A (EA), B (EB) and C (EC), but erythromycin D (ED) is not separated from EA. Small amounts of acid degradation products of erythromycin such as anhydroerythromycin A (AEA) and erythromycin A enol ether (EAEN) were found by high-performance liquid chromatography (HPLC) of commercial samples[5,6]. TLC with system R-VI did not separate AEA from EB. Preparative chromatography of the mother liquors of the erythromycin purification indicated the presence of des-N-methylerythromycin A (dMeEA). As this compound was not separated from erythromycin A with TLC system R-VI, other mobile phases were examined. In the present study a system using silica gel as the coating material and diisopropyl ether–methanol 25% ammonia (75:35:2) as the mobile phase was found to separate EA, EB, EC, ED, EAEN, AEA and dMeEA. The results obtained with several other mobile phases are also discussed, together with their application in the identification and purity control of other macrolides.

EXPERIMENTAL

Samples

Erythromycins A (EA), B (EB), C (EC) and D (ED) were obtained by preparative HPLC of a mother liquor concentrate using apparatus and packing material described previously[7]. Three mobile phases were successively used: ethyl acetate–methanol–25% ammonia (100:8:1), diethyl ether–methanol–25% ammonia (100:7:1) and dichloromethane–methanol–25% ammonia (100:5:0.5). After separation with the first mobile phase, the collected fractions were evaporated and purified further by chromatography with the second and then with the third mobile phase. The identity of the substances obtained by preparative HPLC was determined by comparison with reference samples in the different TLC systems mentioned below. Reference samples

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EA and EB were obtained by crystallization of commercial samples of erythromycin; EC and ED were gifts from Abbott laboratories (Abbott, North Chicago, IL, U.S.A.). More details on the preparative HPLC of erythromycins will be reported later. Erythromycin A and B enol ethers EAEN and EBEN[8], anhydroerythromycin A (AEA)[9], erythralosamine[10] and des-N-methylerythromycin A (dMeEA)[11] were prepared according to procedures mentioned in the literature. The identity of all the samples was confirmed by mass spectrometry.

Most erythromycin samples and all other macrolide samples were of the same origin as mentioned in a previous paper where the structures are also given[3]. Some other erythromycin samples of Asian, South American and European origin were obtained by courtesy of Gist-Brocades (Delft, The Netherlands).

Stationary phase

Laboratory-made plates (0.25 mm thick) were prepared with Kieselgel GF_{254} (Type 60) (E. Merck, Darmstadt, G.F.R.). After drying at room temperature, they were activated at 105°C for 1 h and stored without further precautions. Different brands of ready-made silica gel plates were used, *viz.*, Kieselgel Stratochrom SIF_{254} (Carlo Erba, Milan, Italy), Kieselgel F_{254} (Woelm, Eschwege, G.F.R.), Kieselgel 60 F_{254} (E. Merck) and Kieselgel SiF (Riedel-de Haën, Hannover, G.F.R.). Reversed-phase Fertigplatten Kieselgel 60 F_{254} silanisiert (E. Merck) were also used. All ready-made plates were used without prior activation.

Chromatographic procedure

Dichloromethane solutions (5 μl) containing 1 mg/ml of pure substance or 10 mg/ml of commercial sample were applied to the plates by means of a microsyringe. The plates were developed over a distance of 15 cm in filter-paper-lined chromatographic tanks which had been saturated for at least 2 h. The plates were then dried at 110°C for 5 min, cooled, sprayed with anisaldehyde–sulphuric acid–ethanol (1:1:9) and heated again for 1 min.

RESULTS AND DISCUSSION

Table I shows R_F values obtained for the erythromycin components and derivatives in different systems. Values for EBEN and erythralosamine are reported although they are not likely to be present in erythromycin samples. The results obtained with mobile phase R-VI correspond well with those published previously[3] although EB and EC migrate slightly further. EAEN is well separated from other components but AEA and EB are not separated. The component des-N-methylerythromycin A (dMeEA), which we found to be present in most commercial samples, is not well separated from EA.

Since we were also interested in the preparative HPLC of erythromycins, we were most attracted by straight-phase chromatography on silica gel. It is known that the capacity of silica gel is much higher than that of derivatized silica gel. Furthermore straight phase packing material is less expensive than reversed-phase material. The results obtained with the best straight-phase system yet published (system IV) are shown in Table I. Although the R_F values we obtained are slightly lower, the separation pattern of the erythromycins is the same as found previously[3]. This system is

TABLE I

R_F VALUES OF ERYTHROMYCINS AND DERIVATIVES

Stationary phases: Stratochrom SIF_{254} (Carlo Erba) with mobile phases ethyl acetate-methanol-25% ammonia (85:10:5) (I), diethylether-methanol 25% ammonia (90:9:2) (II), dichloromethane-methanol-25% ammonia (90:9:1.5) (III), ethyl acetate-ethanol-15% ammonium acetate buffer pH 9.6 (9:4:8), the ammonium acetate being adjusted to pH 9.6 with concentrated ammonia and the upper phase used (IV) and diisopropyl ether-methanol-25% ammonia (75:35:2) (V); Kieselgel $60F_{254}$ silanisiert (Merck) with mobile phase methanol-water-15% ammonium acetate pH 7.0 (50:20:10) (R-VI). Development at room temperature.

Compound	*Mobile phase*						*Colour of spot*
	I	*II*	*III*	*IV*	*V*	*R-VI*	
Erythromycin enol ether B (EBEN)	0.53	0.55	0.47	0.27	0.50	0.24	Violet-blue
Erythromycin enol ether A (EAEN)	0.53	0.54	0.48	0.27	0.50	0.32	Grey-green
Anydroerythromycin A (AEA)	0.42	0.40	0.36	0.28	0.47	0.43	Grey-green
Erythralosamine	0.43	0.42	0.35	0.25	0.43	0.50	Orange
Erythromycin B (EB)	0.48	0.43	0.43	0.23	0.43	0.42	Violet-blue
Erythromycin A (EA)	0.46	0.39	0.41	0.22	0.40	0.50	Grey-green
Erythromycin D (ED)	0.38	0.32	0.34	0.20	0.36	0.49	Violet-blue
Erythromycin C (EC)	0.35	0.28	0.31	0.18	0.33	0.56	Grey-green
Des-N-methylerythromycin A (dMeEA)	0.29	0.20	0.26	0.15	0.27	0.52	Grey-green

excellent for the chromatography of macrolides in general, but the erythromycins EA, EB, EC and ED are only partly separated. The mobile phase was then modified to make it a one-phase system. The composition of one-phase systems is independent of temperature effects and therefore the reproducibility of the results is better. Ethanol was replaced by methanol and the buffer was replaced by ammonia to give mobile phase I, ethyl acetate-methanol-25% ammonia (85:10:5). EA, EB, EC and ED were now better separated but AEA was not completely separated from EA. Replacing ethyl acetate by diethyl ether or dichloromethane to give mobile phase II, diethyl ether-methanol-25% ammonia (90:9:2), or III, dichloromethane-methanol-25% ammonia (90:9:1.5), did not sufficiently improve the separation. With mobile phase II, the separation of EA and EB was better but AEA was not separated from EA. With mobile phase III, AEA was separated from EA, but the separation of EA and EB was worse.

With the use of diisopropyl ether (mobile phase V in Table I) the separation of seven erythromycins and derivatives was finally achieved. With this system as with the other straight phase systems, EAEN and EBEN are not separated, but EBEN is not likely to be present in appreciable amounts since it is a derivative of a secondary component. Erythralosamine is not separated from EB but this impurity is not likely to be present either since it is only formed by more prolonged acid treatment of EA[10]; in mild acid conditions EAEN and AEA are formed in the first place[8]. It should be noted that, with mobile phase V, EA and EB are less separated than with mobile phase R-VI, but this drawback is compensated by a better resolution of the other impurities.

TABLE II

R_F VALUES OF ERYTHROMYCINS AND DERIVATIVES OBTAINED ON LABORATORY-MADE AND READY-MADE TLC PLATES FROM DIFFERENT MANUFACTURERS

Stationary phases: 1, Merck; 2, Carlo Erba; 3, Woelm; 4, Riedel-de Haën; 5, laboratory-made. Mobile phase V. Development at 7°C.

Compound	*Stationary phase*				
	1	*2*	*3*	*4*	*5*
Erythromycin enol ether B (EBEN)	0.56	0.49	0.50	0.51	0.62
Erythromycin enol ether A (EAEN)	0.55	0.48	0.49	0.50	0.62
Anydroerythromycin A (AEA)	0.48	0.44	0.45	0.45	0.57
Erythralosamine	0.45	0.40	0.41	0.41	0.54
Erythromycin B (EB)	0.45	0.40	0.41	0.42	0.54
Erythromycin A (EA)	0.41	0.36	0.38	0.38	0.51
Erythromycin D (ED)	0.35	0.30	0.32	0.32	0.46
Erythromycin C (EC)	0.31	0.27	0.28	0.28	0.43
Des-N-methylerythromycin A (dMeEA)	0.26	0.22	0.23	0.23	0.37

In order to improve the quality of the separations, the influence of temperature was examined and chromatograms were developed at different temperatures between room temperature and −18°C. At lower temperatures it was observed that the spots were more compact and the R_F values were slightly different, but no significant effect on the separation pattern was seen. A temperature of about 7°C was chosen as optimal since at lower temperatures the compactness of the spots did not improve further and because it is a common temperature in refrigerators. In Table II, results obtained at 7°C are shown. It is seen that the separation pattern is not affected by the brand of the plates used although some variations in R_F values are observed. Merck plates gave the most compact spots and therefore this brand was used for further experiments with commercial samples of erythromycin and of other macrolides.

Fig. 1 shows chromatograms of representative samples from different manufacturers and of some official standards, together with chromatograms of a mixture of pure reference substances and an erythromycin A sample purified by HPLC. Semi-quantitative interpretation of the chromatograms is possible after comparison with decreasing amounts of the reference substances. The spots shown in chromatogram R each correspond to 10% of the amount spotted in the other chromatograms. The detection limit for all substances is better than 0.5 μg or 1%. Quantitation by scanning at low wavelength, as described for other macrolides[4], is also possible. It is clear that the samples are of different composition. All contain some dMeEA (about 1%). This impurity was isolated by scraping off the corresponding band from a preparative TLC plate. By mass spectrometry, it was proved to be identical with dMeEA prepared from EA[11]. EC is also present in all the samples; sample K contains about 4%. EB is present in about half the samples and to about 6% in sample K. ED is less frequently present, 2–4% in sample K. Degradation products such as EAEN or AEA are rarely or not present in commercial samples, although sample K contains 2–4%.

TABLE III

R_F VALUES OF MACROLIDES IN VARIOUS MOBILE PHASES

Stationary phases: Kieselgel $60F_{254}$ silanisiert (Merck) with mobile phase R-VI; Kieselgel $60F_{254}$ (Merck) with mobile phases I–V (see Table I), chloroform–ethanol–15% ammonium acetate pH 7.0 (85:15:1) (VII) and chloroform–ethanol–3.5% ammonia (85:15:1) (VIII). Development at room temperature.

Compound	*Mobile phase*								*Colour of spot*
	I	*II*	*III*	*IV*	*V*	*R-VI*	*VII*	*VIII*	
1 Midecamycin ethyl-carbonate	0.74	0.76	0.76	0.80	0.79	0.19	0.69	0.71	Blue
2 Midecamycin	0.64	0.57	0.53	0.76	0.71	0.45	0.58	0.63	Blue
3 Josamycin	0.63	0.55	0.53	0.78	0.70	0.39	0.62	0.63	Blue
4 Tylosin	0.31	0.13	0.35	0.58	0.42	0.58	0.38	0.49	Violet-brown
5 Troleandomycin	0.65	0.53	0.67	0.60	0.63	0.25	0.57	0.60	Pink
6 Oleandomycin	0.36	0.21	0.32	0.24	0.36	0.57	0.08*	0.16	Pink
7 Leucomycin	7 spots	9 spots	6 spots	7 spots	7 spots	7 spots	7 spots	6 spots	Blue
8 Erythromycin ethyl-carbonate	0.58	0.45	0.48	0.60	0.63	0.28	0.44	0.52	Blue
9 Erythromycin estolate	0.56	0.45	0.47	0.56	0.63	0.27	0.38	0.47	Blue
10 Erythromycin ethyl-succinate	0.57	0.43	0.48	0.57	0.63	0.25	0.41	0.51	Violet-brown
11 Spiramycin I	0.38	0.21	0.37	0.47	0.45	0.57	0.18*	0.41	Violet-brown
11b Spiramycin II	0.54	0.39	0.45	0.54	0.57	0.55	0.24*	0.47	Violet-brown
11c Spiramycin III	0.58	0.46	0.47	0.57	0.61	0.53	0.30	0.47	Violet-brown
12 Rosamicin	0.32	0.13	0.47	0.29	0.31	0.57	0.23*	0.35	Grey-brown
13 Megalomycin	0.29	0.12	0.24	0.16	0.23	0.56	0.04*	0.12	Grey-brown

* Streaking spot.

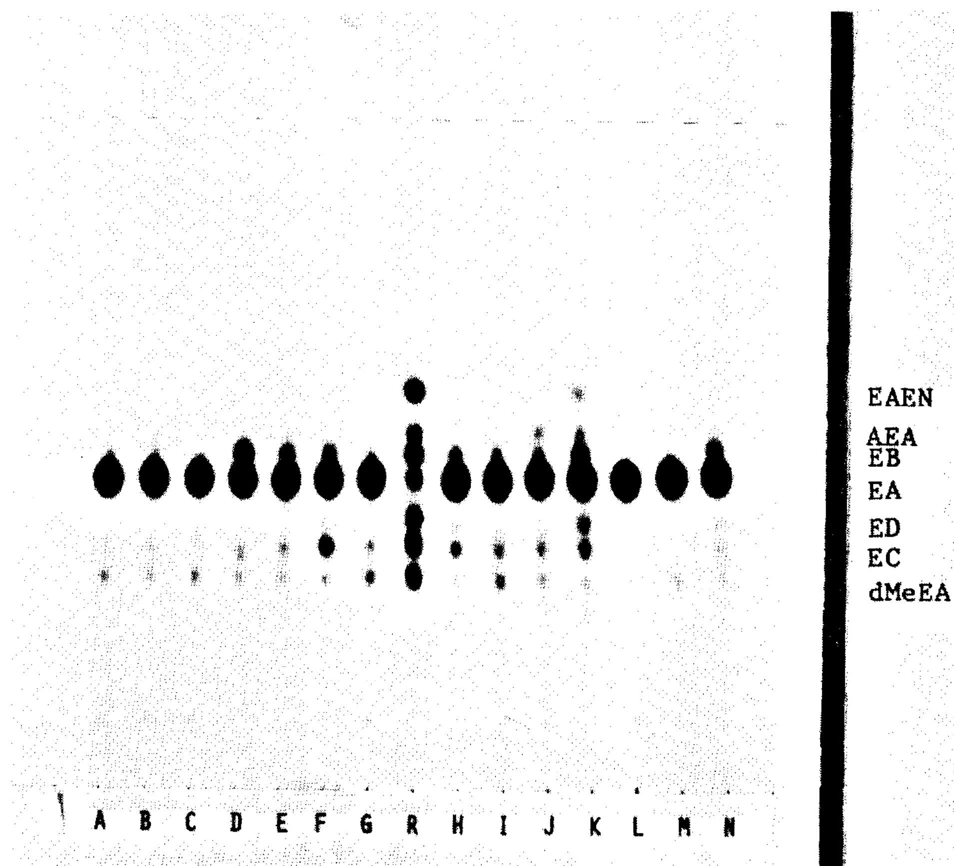

Fig. 1. Thin-layer chromatograms of commercial erythromycin samples and reference samples. Stationary phase: Kieselgel $60F_{254}$ (Merck). Mobile phase: diisopropyl ether–methanol–25% ammonia (75:35:2). Development at 7°C. Chromatograms: A–J = commercial samples; R = mixture of reference substances (from top to bottom) EAEN, AEA, EB, EA, ED, EC, dMeEA; K = old sample; L = EA purified by HPLC; M = USP erythromycin reference standard; N = European pharmacopoeia erythromycin reference substance. Amounts spotted: 50 μg except for R, 5 μg of each substance.

The United States Pharmacopoeia (USP) standard (M) consists of practically pure EA and the European standard (N) contains about 6% of EB. The EA sample obtained by preparative HPLC was observed to be of higher purity than the USP standard.

The new mobile phases I, II, III and V described above for erythromycins were also investigated for TLC of erythromycin esters and of other macrolides. Table III shows the R_F values obtained together with those obtained with mobile phases R-VI, IV, VII and VIII, described previously for TLC of erythromycin esters and other macrolides[3]. The combination of mobile phases IV and VII has previously been mentioned as very useful for identification of macrolides[3]. None of the newly developed systems was able to separate all erythromycin esters, which were best differentiated with mobile phase VII. The combination of phases II and VII is also

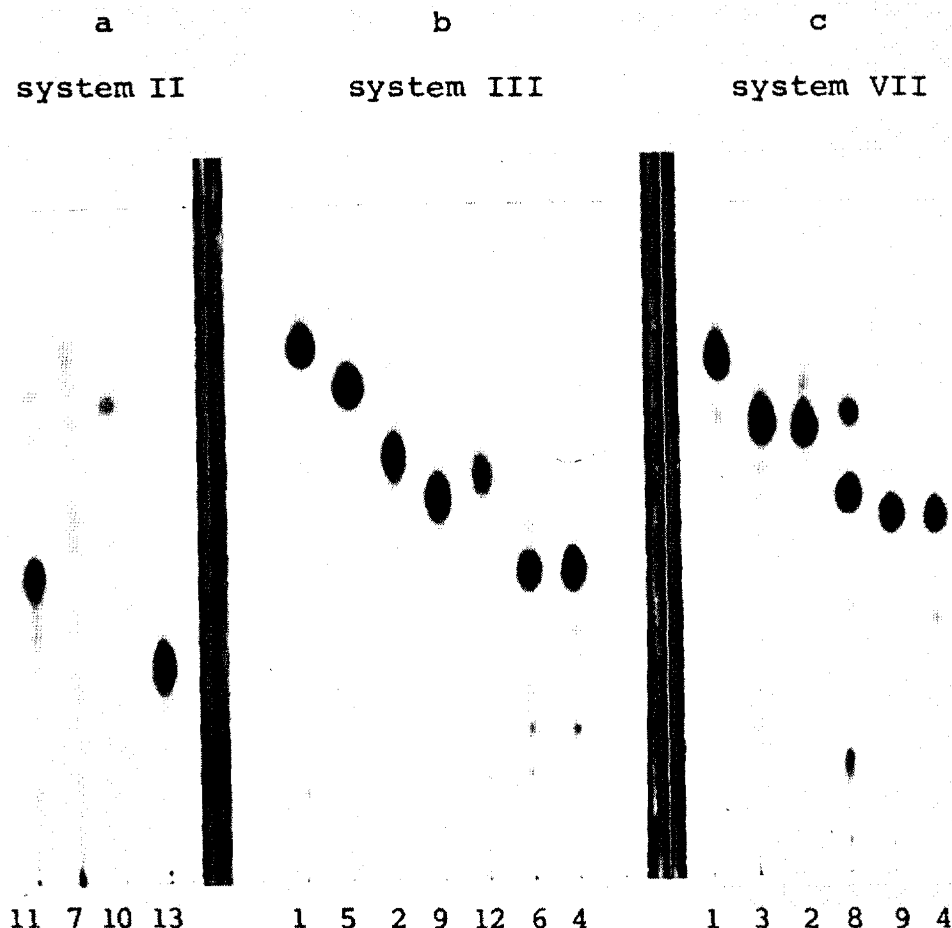

Fig. 2. Thin-layer chromatograms of macrolides. Stationary phase: Kieselgel $60F_{254}$ (Merck). Mobile phases: a, II, b, III; c, VII. Compounds: 1 = midecamycin ethylcarbonate; 2 = midecamycin, 3 = josamycin; 4 = tylosin; 5 = troleandomycin; 6 = oleandomycin; 7 = leucomycin; 8 = erythromycin ethylcarbonate; 9 = erythromycin estolate; 10 = erythromycin ethylsuccinate; 11 = spiramycin; 12 = rosamycin; 13 = megalomycin. Amount spotted: 50 μg.

considered as very useful for identification purposes, because macrolides which are not separated with II are easily differentiated with mobile phase VII.

The mobile phases in Table III were also investigated for testing the purity of the macrolides. That which separated the highest number of secondary spots from the main spot, and gave no streaking spots, was considered the best. Obviously the same mobile phase was not the best for all the macrolides. Mobile phases II, III and VII appeared to be most useful. Fig. 2 shows the chromatograms obtained with these mobile phases. Except for spiramycin (11) whose components are well separated, the identity of secondary spots was not investigated for lack of reference samples.

It can be concluded that mobile phase V is the best for testing the purity of

erythromycin. Mobile phases II and III, in combination with IV and VII, described previously, are useful for the identification or the purity control of erythromycin derivatives and other macrolides.

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