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THIN-LAYER CHROMATOGRAPHY OF MACROLIDE ANTIBIOTICS

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SUMMARY

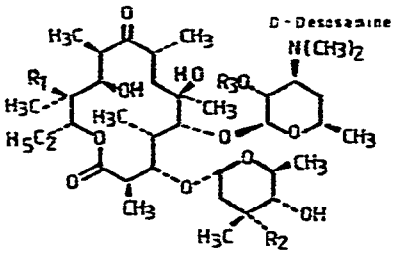
Various macrolides and macrolide esters, which are commercially available, were examined in different chromatographic systems. The most useful system for the identification of macrolides was ethyl acetate–ethanol (or isopropanol)–15% ammonium acetate adjusted to pH 9,6 (9:4:8) on silica gel plates. For the separation of erythromycin esters, chloroform–ethanol–15% ammonium acetate pH 7.0 (or 3.5% ammonia) (85:15:1) gave the best results. The different components of erythromycin were separated on silanised silica gel plates with methanol–water–ammonium acetate pH 7.0 (50:20:10). These systems also can be used for the separation and identification of various macrolides.

INTRODUCTION

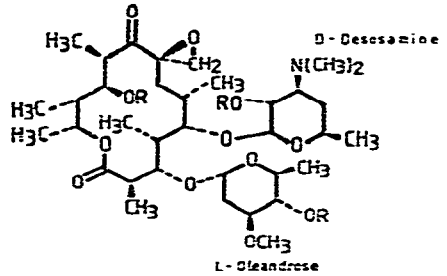
The recent introduction of some new macrolides has raised the problem of their identification. No systematic study seems to have been published.

We have not examined paper chromatography, because this method is tedious. Information about the use of this method for some macrolides can be found in recent reviews[1,2]. High-performance liquid chromatography (HPLC) can be used for the identification of drugs but this rather elaborate method will not be a first choice in a pharmacopoeial compendium. The assay of different components and impurities in erythromycin and its esters by HPLC has been published[3–5]. HPLC of other macrolides like leucomycin, tylosin and spiramycin also has been described[6–9]. Thin-layer chromatography (TLC) has been employed for some macrolides using the solvent systems: chloroform–methanol (1:1 and 95:5)[10]; *n*-butanol–acetic acid–water (3:1:1)[11,12] and chloroform–methanol–17% ammonia (2:1:1)[12]. Results with some of these chromatographic systems will be examined in this publication. TLC of erythromycin esters has also been reported and will be discussed later.

The stereochemical formulae of the macrolides have been presented in different ways. We have used the presentation of Masamune *et al.*[13]. The structures of erythromycin A[14], B[15], C[16] and D[17] have been determined by chemical methods. Configuration and conformation has been studied by different physico-chemical techniques[18–20]. The structure of oleandomycin was elucidated by classical methods[21,22] and the absolute configuration was determined by Celmer[23]. Both have been confirmed

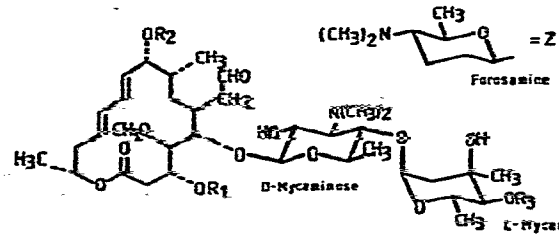


		R_1	R_2	R_3
Erythromycin	A	OH	OCH_3	H
	B	H	OCH_3	H
	C	OH	OH	H
	D	H	OH	H
Erythromycin A				
ethylcarbonate		OH	OCH_3	$-COOC_2H_5$
propionate		OH	OCH_3	$-COCH_2CH_3$
ethylsuccinate		OH	OCH_3	$-COCH_2CH_2COOC_2H_5$

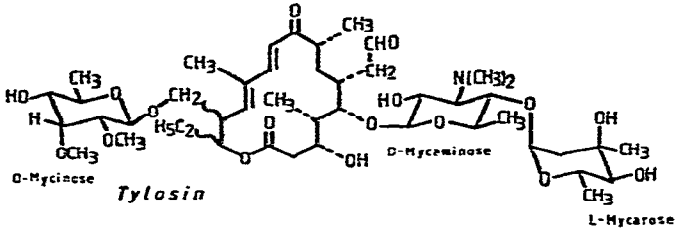


Oleandomycin	R = H
Troleandomycin	$R = -COCH_3$

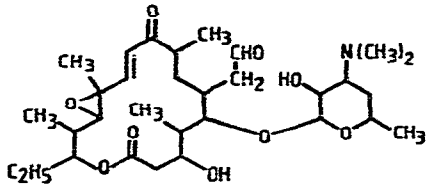
recently by X-ray diffraction[24]. Leucomycin (kitasamycin) is a mixture of a great number of components[6,25]. The structure of leucomycin A_1 was determined by Ōmura *et al.*[26]. Josamycin is identical with leucomycin A_3[27]. Spiramycin is a mixture of three related substances with spiramycin I as the main component[28]. Its structure has been studied in different laboratories and its relation with leucomycin has been examined[29]. The configurational assignment at C-9 of the leucomycins and spiramycins has been revised recently[30]. The conformation of leucomycin and spiramycin was studied by NMR[31,32]. Midecamycin (antibiotic SF-837) is related to leucomycin[33]. Tylosin is another 16-membered macrolide, whose structure and configuration was determined by various techniques[32,34]. Megalomicin and rosamicin are macrolides produced by Micromonospora[35,36]. The structure of megalomicin A[37] was revised recently[38]. The structure of rosamicin (at first named rosaramicin) was elucidated by Reimann and Jaret[39].



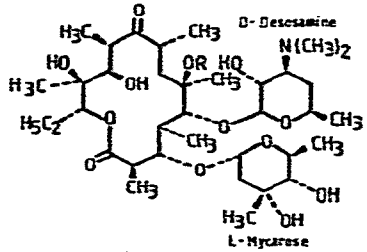
		R1	R2	R3
Leucomycin	A1	H	H	- $COCH_2CH(CH_3)_2$
	A_3 (= Josamycin)	- $COCH_3$	H	- $COCH_2CH(CH_3)_2$
Spiramycin	I	H	Z	H
	II	- $COCH_3$	Z	H
	III	- $COCH_2CH_3$	Z	H
Midecamycin, SF-837	A1	- $COCH_2CH_3$	H	- $CO\ CH_2CH_3$
Midecamycin ethylcarbonate		- $COCH_2CH_3$	- $COOC_2H_5$	- $CO\ CH_2CH_3$



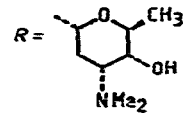
Tylosin



Rosamicin



Megalomicin A



L-Megosamine

EXPERIMENTAL

Products

Erythromycin samples of the firms Abbott, Lilly, Polfa, Proter, Roussel and Upjohn were examined. Samples of erythromycin B and C were donated by Abbott Labs. (Chicago, Ill., U.S.A.) and erythromycin D by Dr. J. Majer, Department of Biochemistry, Northwestern University Medical and Dental Schools, Chicago, Ill., U.S.A. Erythromycin esters used were: erythromycin ethylsuccinate (Abbott), erythromycin ethylcarbonate (Lederle, Pearl River, N.Y., U.S.A.) and erythromycin estolate (propionylerythromycin laurylsulphate) (Lilly, Indianapolis, Ind., U.S.A.). Josamycin was furnished by UCB (Brussels, Belgium), kitasamycin by Mr. E. Denis, Institut d'Hygiène et d'Epidémiologie, Brussels. Midecamycin was obtained from Clin-Midy (Paris, France), megalomicin and rosamicin from Schering (Bloomfield, N.J., U.S.A.), oleandomycin and troleandomycin from Pfizer (Brussels, Belgium). Spiramycin was extracted from Rovamycin^{R} tablets. Samples of spiramycin I, II and III, obtained from Specia (Paris, France), were given by Mr. G. A. Bens, University of Ghent, Ghent, Belgium. Tylosin was given by Lilly Labs.

Erythromycin spiroketal[40] and erythromycin 8,9-anhydro-6,9-hemiketal[41] were prepared using the described procedures.

Chromatographic procedure

Solutions (5 μl) containing 20 mg of the drug in 10 ml of methanol were applied to the plates by means of micropipettes and the plates were placed in a filter-paper-lined chromatographic chamber which had been saturated overnight.

Plates

Plates (0.25 mm thickness) were prepared with Kieselgel G (Type 60; Merck, Darmstadt, G.F.R.) and Kieselgel 60 HF 254 silanisiert (Merck). Pre-coated plates used were: Fertigplatten Kieselgel 60 F 254 (Merck), Stratocrom SIF 254 (Carlo Erba, Milan, Italy), Sil G-25 (Macherey, Nagel & Co., Düren, G.F.R.), Fertigplatten SIF (Riedel-De Haën, Hannover, G.F.R.), Fertigplatten Kieselgel 60 F 254 silanisiert (Merck) and Whatman RP KC18.

Spray reagents

(A) 50% Sulphuric acid. Heating at 130° for 10 min. All macrolides gave dark brown spots. This reagent can be used for the semi-quantitative estimation of impurities.

(B) A solution of glucose (2 g) in a mixture of 85% phosphoric acid (10 ml), water (40 ml), ethanol (30 ml) and *n*-butanol (30 ml). Heating at 150° for 5 min[42,43]. This reagent is more complex than 50% sulphuric acid and presents no advantages because all macrolides give similar colours.

(C) A 10% solution of phosphomolybdic acid in ethanol. Heating at 150° for 2 min[44]. All macrolides give green-blue spots on a yellow background. This reagent can have some use as a general detection reagent. It is much less sensitive than reagent D, which has the additional advantage of providing a variety of colours for different macrolides.

(D) Anisaldehyde–conc. sulphuric acid–ethanol (1:1:9). Heating at 110° for 2 min[17]. This spray gives a great variety of colours for different macrolides and it is the most suitable reagent for identification.

(E) Sulphuric acid–methanol (1:1) applied to plates which had been heated at 100° for 10 min[12]. This reagent yields different colours for various macrolides but the variety of colours is less marked than with reagent D.

(F) A 0.05% solution of xanthydrol in 30% sulphuric acid. Heating at 100° for 3 min. This reagent also gives some difference in colour between some macrolides but it is inferior to the reagent D.

(G) Dragendorff reagent[45] gave weak spots with all macrolides (20 μg).

(H) A solution of 20 mg 2,7-dichlorofluoresceine and 10 mg rhodamine B in 100 ml of ethanol. Examination under UV light of 365 nm or 254 nm. This reagent is used for the detection of lipids.

RESULTS AND DISCUSSION

One of the most useful chromatographic systems (Table I) was that described by Majer *et al.*[17]. The original mobile phase (I) was modified in that the ratios of

TABLE I

R_F VALUES IN THE MAJER TYPE MOBILE PHASES

R_F values obtained with Fertigplatten Merck silica gel 60 F254. Amount: 10 μg, except (a) where 5 μg and (b) where 15 μg were applied. Mobile phases: I = ethyl acetate–isopropanol–15% ammonium acetate (9:7:8); II = ethyl acetate–ethanol–15% ammonium acetate (9:7:8); III = ethyl acetate–isopropanol–15% ammonium acetate (9:4:8); IV = ethyl acetate–ethanol–15% ammonium acetate (9:4:8). In all systems, the 15% ammonium acetate was adjusted to pH 9.6 with conc. ammonia, and the upper phase was used after careful separation of the phases. Detection: spray D, except for (b) where spray G was used. —, Not examined.

	I	*II*	*III*	*IV*
Midecamycin ethylcarbonate	0.84 *	0.89 *	0.78 **	0.83 **
Midecamycin	0.83 *	0.88 *	0.76 *	0.80 *
Josamycin	0.83 *	0.88 *	0.76 *	0.80 *
Troleandomycin	0.73 **	0.78 **	0.61 **	0.63 **
Erythromycin ethylcarbonate	0.75 *	0.80 *	0.62 **	0.64 **
Erythromycin ethylsuccinate	0.74 *	0.79 *	0.58 *	0.62 *
Erythromycin propionate	0.74 *	0.79 *	0.58 *	0.62 * (b)
laurylsulphate	—	—	—	0.30 (b)
Tylosin	0.72	0.77	0.55	0.55
Spiramycin III	0.74	0.79	0.58	0.61
II	0.72	0.77	0.55	0.58
I	0.68	0.73	0.48	0.52
Erythromycin A	0.53	0.58	0.30	0.36
Rosamicin	0.50	0.55	0.27	0.32
Oleandomycin	0.50	0.55	0.24	0.29
Megalomicin	0.40	0.44	0.15	0.20
Erythromycin	—	—	0.30 (b)	0.36 (b)
stearate	—	—	0.20 (b)	0.15 (b)
Erythromycin A	—	0.58 * (a)	—	0.36 ** (a)
B	—	0.59 *** (a)	—	0.37 *** (a)
C	—	0.53 *** (a)	—	0.32 *** (a)
D	—	0.55 ** (a)	—	0.33 ** (a)

* No separation.

** Partial separation, sometimes visible because of the difference in colour with spray D.

*** Complete separation.

solvents were changed from 9:7:8 to 9:4:8 and that isopropanol was replaced by ethanol. The change of alcohols had little influence, but in some critical mixtures the separation was somewhat better. The lower amount of alcohol improved the separation on laboratory made plates and it gave excellent results with ready-made plates. Most chromatograms were run on Merck plates, but very similar R_F values were obtained with ready-made silica gel plates of Carlo Erba, Macherey, Nagel & Co. and Riedel-De Haën. Identification was aided by the difference in colour observed by detection with spray D (anisaldehyde–sulphuric acid): gray-green to brown for erythromycin and its esters; different shades of blue-violet to violet-gray for josamycin, megalomicin, midecamycin, spiramycin and tylosin; pale brown for rosamicin; red for oleandomycin and troleandomycin.

Although this chromatographic system (especially mobile phase IV) gave excellent results, it should be noted that josamycin and midecamycin remain together and that some erythromycin esters are not separated. A separation or partial separation of erythromycins A, B, C and D was observed when small amounts were applied. Their detection was aided by the difference in colour obtained with spray D: erythromycin A and C; gray-green; erythromycin B and D, violet-brown. The mobile phases I–IV were not suitable for the estimation of the components B, C and D in commercial erythromycin, where they are present in concentrations from 1% to more than 10%. The presence of erythromycin C could easily be detected, but erythromycin B was located in the upper part of the main spot (erythromycin A), where its presence was adumbrated by a difference in colour.

The separation of the erythromycin components by HPLC on reversed-phase columns[3] indicated that their detection should also be possible on reversed-phase plates. Separation of erythromycins A, B and C was obtained on Whatman KC18 and on silanised silica gel plates. Various combinations of solvents were examined but mobile phases V and VI gave the best results (Table II). Because of the lower price, silanised silica gel seems preferable at the moment. The colours obtained with spray D (anisaldehyde–sulphuric acid) were somewhat different from those observed on silica gel; most significant is the difference in colour for erythromycin A (and esters) and C (gray-brown) and erythromycin B and D (blue-green). The R_F value of erythromycin spiroketal on silanised plates with mobile phase VI is 0.35. The anhydrohemiketal has the same R_F as erythromycin B. Reversed-phase plates were the most appropriate ones for separation of the components of leucomycin. The separation of josamycin and midecamycin is a useful application of these plates. The various erythromycin esters and different macrolides, which were could be identified with the Majer type solvents, were not separated.

The separation of different erythromycin esters, which could not be obtained by previous mobile phases, was observed with a 80:15:1 mixture of chloroform–ethanol–ammonia or ammonium acetate (Table III). Similar results were obtained with different brands of ready-made silica gel plates and also with plates prepared in the laboratory, although the R_F values are higher in the last case. Mobile phase VII is somewhat better than VIII, because erythromycin ethylcarbonate and ethyl succinate can be separated. It is less suitable for the macrolides with lower R_F values, because some tailing occurs. Mobile phase VIII is useful to confirm the identification deduced from chromatography with one of the systems of Table I. It should be noted that solvents VI and VII are not suitable for the separation of the three components

TABLE II

R_F VALUES ON REVERSED-PHASE PLATES

W. RP = Whatman RP KC18; M. Sil. = Fertigplatten Kieselgel 60 F254 silanisiert (Merck). Mobile phases: V = methanol–acetonitrile–water–15% ammonium acetate pH 7.0 (30:40:10:20); VI = methanol–water–15% ammonium acetate pH 7.0 (50:20:10). Amount: 10 μg. Detection: spray D. For other symbols see Table I.

	W. RP, V	*M. Sil., V*	*M. Sil., VI*
Megalomicin	0.55 **	0.60 **	0.50 *
Oleandomycin	0.52 **	0.63 **	0.50 *
Rosamicin	0.49 **	0.60 **	0.50 *
Tylosin	0.48 *	0.60 *	0.50 *
Spiramycin I	0.50	0.62	0.54
II	0.45		
III	0.40	0.58	0.48
Erythromycin C	0.56	0.63	0.52
A	0.48	0.58	0.49
B	0.40	0.53	0.35
D	—	—	0.49
Midecamycin	0.29	0.57	0.38
Josamycin	0.21	0.54	0.31
Leucomycin	7 spots	5 spots	7 spots
Erythromycin ethylcarbonate	0.15 *	0.50 *	0.22 *
ethylsuccinate	0.15 *	0.50 *	0.22 *
propionate	0.15 *	0.50 *	0.22 *
Troleandomycin	0.12	0.40	0.20
Midecamycin ethylcarbonate	0.07	0.43	0.16

TABLE III

R_F VALUES IN VARIOUS MOBILE PHASES

M = Merck Fertigplatten silica gel 60 F254; L = laboratory made plates using silica gel G 60 (Merck). Mobile phases: VII = chloroform–ethanol–15% ammonium acetate pH 7.0 (85:19:1); VIII = chloroform–ethanol–3.5% ammonia (85:15:1); IX = chloroform–methanol–toluene (80:17:23); C = chloroform–methanol (75:25); XI = *n*-butanol–acetic acid–water (3:1:1). Amount: 10 μg, except (b) where 15 μg were applied. Detection: spray D, except (b) where spray G was used. (c) Elongated spots and double front in the lower part of the plate; (d) elongated spots. For other symbols, see Table I.

	M., VII	*L., VII*	*M., VIII*	*M., IX*	*M., X*	*M., XI*
Midecamycin ethylcarbonate	0.67	—	0.78	0.60	0.72	0.59 **
Midecamycin	0.63 *	—	0.72 *	0.52 ***	0.67 *	0.56 **
Josamycin	0.62 *	0.72	0.72 *	0.56 ***	0.67 *	0.59 **
Tylosin	0.50	0.55	0.60	0.35	0.52	0.51
Troleandomycin	0.53	0.65	0.67	0.53	0.70	0.40
Erythromycin ethylcarbonate	0.40 ***	0.55 *	0.58 *	0.43 *	0.58 *	0.47 *
ethylsuccinate	0.37 ***	0.55 ***	0.58 *** (b)	0.40 ***	0.53 ***	0.47 *
propionate	0.32 ***	0.50 ***	0.54 (b)	—	—	—
laurylsulphate	—	—	0.07 (b)	—	—	—
Spiramycin II + III	0.40 (c)	0.48	0.56	0.19 (d)	0.33 (d)	0.20
I	0.32 (c)	0.42	0.51	0.13 (d)	0.25 (d)	0.20
Rosamicin	0.27	—	0.37	0.15	0.24 (d)	0.39
Erythromycin	0.16 (c)	0.17	0.24 (b)	0.09 (d)	0.17 (d)	0.46
stearate	—	—	0.10 (b)	—	—	—
Oleandomycin	0.05	0.12	0.22	0.09 (d)	0.17 (d)	0.37
Megalomicin	0.05	—	0.10	0.03	0.05 (d)	0.26

of spiramycin. Replacement of ethanol in VI and VII by methanol or isopropanol gave mobile phases with lower separating power.

The separation of erythromycin esters from erythromycin has been effected on sodium acetate buffered silica gel plates with methanol–0.02 *N* sodium acetate (120:30)[42,43] and with chloroform–methanol–hexane (100:40:10) and methanol–water (50:50)[46]. Separation was also possible with an acid mobile phase, chloroform–methanol–acetic acid (90:10:1 and 90:5:5)[47,48]. It should be noted that the different erythromycin esters could not be separated in these solvent systems.

We examined *n*-butanol–acetic acid–water (3:1:1) as acid mobile phase (XI, Table III) because different macrolides had already been studied in this system[11,12]. As neutral solvents, we used chloroform–methanol (75:25) (X, Table III), similar to the mixtures used by Bickel *et al.*[10], and chloroform–methanol–toluene (80:17:23) (IX, Table III), selected by Gantes *et al.*[49] for the chromatography of oleandomycin esters. On these neutral mobile phases, several macrolides show tailing. The addition of ammonia is necessary for obtaining round spots.

The separation of erythromycins A, B and C has been described[50] on silica gel–Kieselguhr (1:1) plates impregnated with 15% formamide in acetone, and run in dichloromethane–*n*-hexane–ethanol (60:35:5)[50]. We always obtained bad tailing with this method. This was confirmed in other laboratories[51,52], although Dr. Calam has obtained satisfactory results[53].

Some erythromycin salts are also used in medicine, *i.e.*, erythromycin stearate and the laurylsulphate of propionylerythromycin (erythromycin estolate). For the detection of the fatty acid part we used rhodamine B. We found that a mixture of rhodamine B and dichlorofluoresceine (spray G) and examination under UV light gave much better results than rhodamine B–sodium hydroxide. With this spray, the erythromycin part was also visible on the plate. This detection was only examined with solvents IV and VIII. On silanised silica gel, erythromycin and stearic acid are not separated and give only one spot. This detection is more convenient than with dichromate–sulphuric acid[48].

In conclusion, the presently available macrolides and their esters can be identified by chromatography on silica gel in one of the Majer type solvents (I–IV), with solvents VII or VIII and on reversed-phase plate with solvents V or VI. Other solvent mixtures (Table III) may confirm the identification, but are not essential.

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