Journal of Pharmaceutical & Biomedical Analysis
Vol. 11, No. 2, pp. 121–130, 1993
Printed in Great Britain

0731–7085/93 $6.00 + 0.00

Simple methods for the qualitative identification and quantitative determination of macrolide antibiotics

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Abstract: Pyrolysis–gas chromatography is shown to be a rapid straightforward method for the qualitative differentiation of the macrolide antibiotics erythromycin, oleandomycin, troleandomycin, spiramycin and tylosin. Organic salts do not interfere and identification of erythromycin and troleandomycin in commercial products is viable. Spectrophotometric quantitation of these same five antibiotics after reaction with concentrated sulphuric acid is studied at about 470 nm. Reaction conditions such as acid concentration, time and temperature are provided. The sugar moieties of the antibiotics are proposed as the reactive sites. Detection limits are about 0.2–1.0 ng ml^{-1} and analysis of pharmaceutical products should be possible.

Keywords: *Erythromycin; oleandomycin; troleandomycin; tylosin; spiramycin; pyrolysis–gas chromatography; spectrophotometry.*

Introduction

Macrolide antibiotics, all composed of a 14- or 16-membered oxygenated ring with several sugars attached, have shown to be effective antibacterial agents for many years. The structures of three 14-membered ring and two 16-membered ring macrolide antibiotics are shown in Fig. 1. Erythromycin is certainly the most well-known of the group however troleandomycin is also available as a pharmaceutical product. Tylosin is used in veterinary applications.

Qualitative methods for the differentiation of macrolide antibiotics are somewhat limited because these compounds have a similar aliphatic structure with few functional groups. UV spectroscopy is only possible at wavelengths less than 220 nm and IR spectroscopy can mainly provide identity of the ester group [1]. Conventional mass spectrometry is difficult due to the nonvolatility of these fairly high molecular weight organic compounds [2]. Mass spectrometry with a thermospray interface can give molecular weight information for macrolide antibiotics [3]. One relatively simple technique, pyrolysis–gas chromatography (PY–GC), has been utilized for the qualitative identification of a wide variety of nonvolatile high molecular weight organic compounds. Organic polymers, proteins, carbohydrates, microorganisms, and various geological samples have all been characterized by PY–GC [4–6]. Samples having smaller molecular weight biochemical compounds such as amino acids, nucleotides, and peptides have also been analysed by PY–GC [7]. Primarily, pharmaceuticals such as penicillins [8] and sulphonamides [9] have been examined by PY–GC. The antibiotic lasalocid will undergo reproducible pyrolysis in the GC injection port [10]. The macrolide antibiotics have not been previously characterized by this technique.

Easy quantitative methods such as molecular spectroscopy have mainly focused on the determination of erythromycin and oleandomycin. A UV method at 240 nm could determine these drugs after alkaline hydrolysis [11, 12]. Hydrolysis of erythromycin [13] or oleandomycin [14] in concentrated sulphuric acid produced a yellow product that could be measured spectrophotometrically. Tetrazolium blue can react with a reducing agent such as erythromycin to form a dark blue pigment [15]. Complex formation with bromcresol purple has been a successful approach for the colorimetric determination of erythromycin [16]. A variety of other dyes have also been used for the ion pair determination of erythromycin in chlorinated solvents [17]. Recently, erythromycin and tylosin have been determined at 842 nm using tetracyanoquinodimethane [18].

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A colorimetric method at 532 nm for oleandomycin using dizaotized sulphanilic acid has also been reported [19]. Fluorometric methods for erythromycin [20] and oleandomycin [21] have more recently been published. A chemiluminescence method for erythromycin is also an option [22]. Although some experimental conditions have been previously provided regarding the reaction of erythromycin with sulphuric acid, the other four macrolide antibiotics have not been analytically investigated with respect to this reaction.

We report here the possibility of using PY–GC and visible spectrophotometry for the characterization of the macrolide antibiotics shown in Fig. 1.

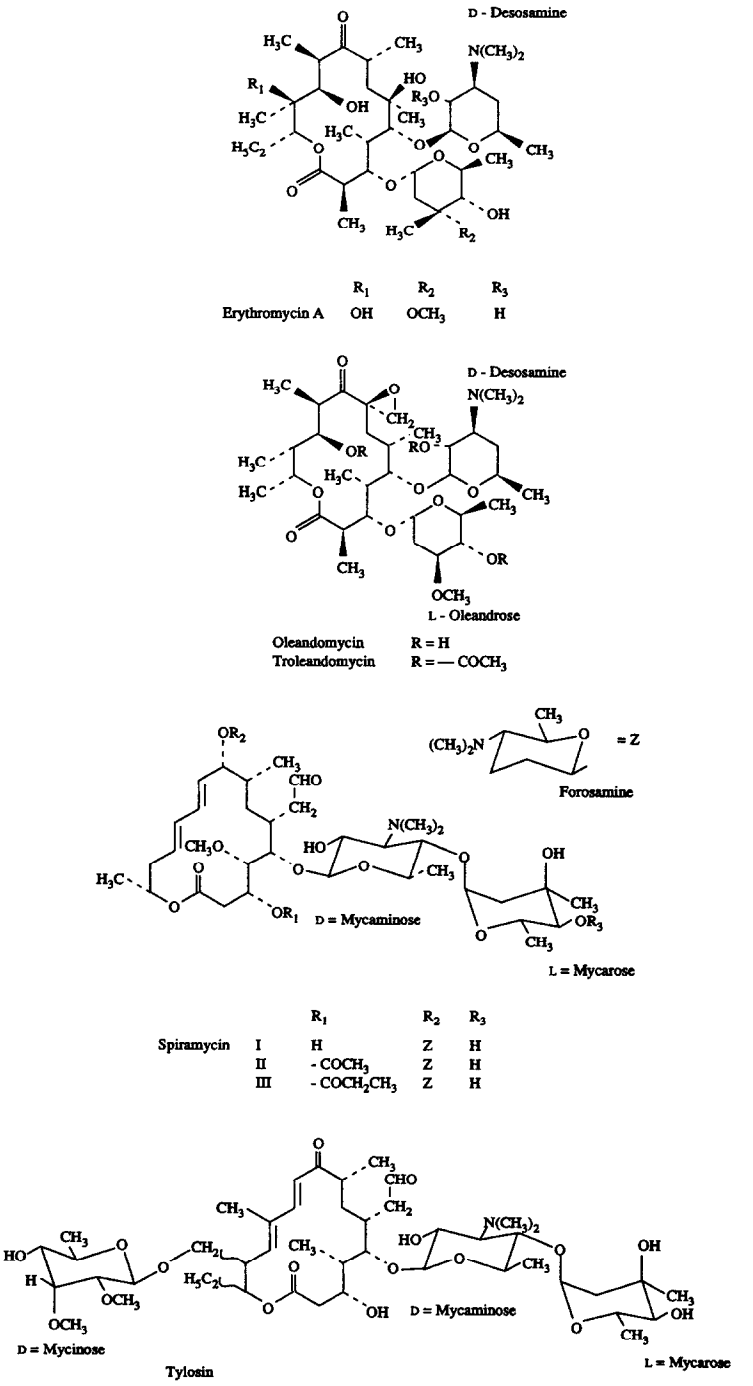

Figure 1
Structures of macrolide antibiotics.

Experimental

Chemicals

All chemicals used were reagent grade or better. The sulphuric acid was purchased from Fisher Scientific (Cincinnati, OH, USA) and the triply distilled water was obtained from a Barnstead Nanopure distillation unit (Sybron/Barnstead, Dubuque, IA, USA). The macrolide antibiotics erythromycin A, oleandomycin phosphate, spiramycin (a mixture of I, II, and III), troleandomycin, and tylosin tartrate were all purchased from the Sigma Chemical Co. (St. Louis, MO, USA). The erythromycin ethylsuccinate capsules (Parke–Davis, Morris Plains, NJ, USA) were formulated at 250 mg. The Tylan 50 solution (Eli Lilly, Indianapolis, IN, USA) contained 50 mg ml^{-1} of tylosin in a 50% propylene glycol–50% H_2O solvent. Both were purchased locally. The TAO (250 mg troleandomycin) tablets were a gift from Roerig–Pfizer (New York, NY, USA).

Equipment

A Perkin–Elmer (Stamford, CT, USA) Model 8500 gas chromatograph equipped with a flame ionization detector and the Omega software data station was used. A Supelco (State College, PA, USA) SPB-20 (1.0 μm thick phenysilicone stationary phase) glass capillary column (0.75 mm i.d. by 30 m) was installed in the GC oven. Pyrolysis of the solid samples was carried out using a Chemical Data Systems (Oxford, PA, USA) pyroprobe 100. A Varian (Humboldt, CA, USA) DMS 90 UV–vis spectrophotometer was employed for the determination of the sulphuric acid hydrolysed antibiotics.

Procedure

Generally a 1 mg or less sample of the antibiotic was weighed into a glass capillary (1 mm i.d. × 20 mm) closed on one end. The capillary was then placed into a platinum coil probe taking care that the Pt coil did not touch the probe itself. The probe was directly inserted into the GC injection port (250°C) and was fired at 500°C for 5 s using a 1 ms ramp. Higher pyrolysis temperatures of 650 or 850°C produced similar pyrograms however a lower temperature of 250°C generated far fewer peaks. The GC detector was heated to 300°C and the column flow rate was adjusted to 5 ml min^{-1}. The column temperature was initially set at 125°C for 1 min and then ramped at 15°C min^{-1} to a final temperature of 250°C where it was held for 12.6 min.

Appropriate dilutions of concentrated (36 N) sulphuric acid were made and these solutions were allowed to cool. An equivalent volume of a standard antibiotic solution was added to the acid and the mixture was allowed to react a certain time before the absorbance was recorded. Generation of the calibration data was carried out in the same manner using the optimum reaction time. A temperature profile as a function of reaction time for each antibiotic was also carried out.

Erythromycin was isolated from the capsule by sonication of the orange and white beads in about 75 ml of water for 3–4 h. An aliquot of the following solution was then taken for further dilution. The contents of the troleandomycin capsule were emptied into a 500 ml solution containing 250 ml of 1.3 M H_2SO_4 and 350 ml of 95% ethanol. After sonication, an appropriate aliquot was taken for further dilution. A 1 ml portion of the Tylan 50 solution was pipetted into a 500 ml flask and then diluted to the mark.

Results and Discussion

It was first established that the presence of an organic anion or dye with the macrolide antibiotic would not make qualitative identification of these antibacterials by PY–GC confusing. Tartrate salt mixed with NaCl at the same stoichiometric ratio as for tylosin tartrate did not pyrolyse at these conditions to give any significant retained peaks (Fig. 2). In addition, the dye tartrazine did not show any major peaks upon pyrolysis at 500°C. Pyrograms for the 16-membered macrolide antibiotics, tylosin and spiramycin, are shown in Figs 2 and 3, respectively. The main structural difference between these two compounds was the third substituted sugar moiety in tylosin. Although both pyrograms show strong peaks at about 6.5 and 7.5 min, an additional strong peak at 8.5 min was evident only in the tylosin figure. A distinctive broad peak at 13 min was also noted in the spiramycin pyrogram. Pyrograms for two very structurally similar macrolide antibiotics oleandomycin and troleandomycin, are shown in Figs 3 and 4(A). Although the main difference between the two is the presence of an ester group for troleandomycin instead of a hydroxy group for oleandomycin, the two pyrograms were very different. Only one

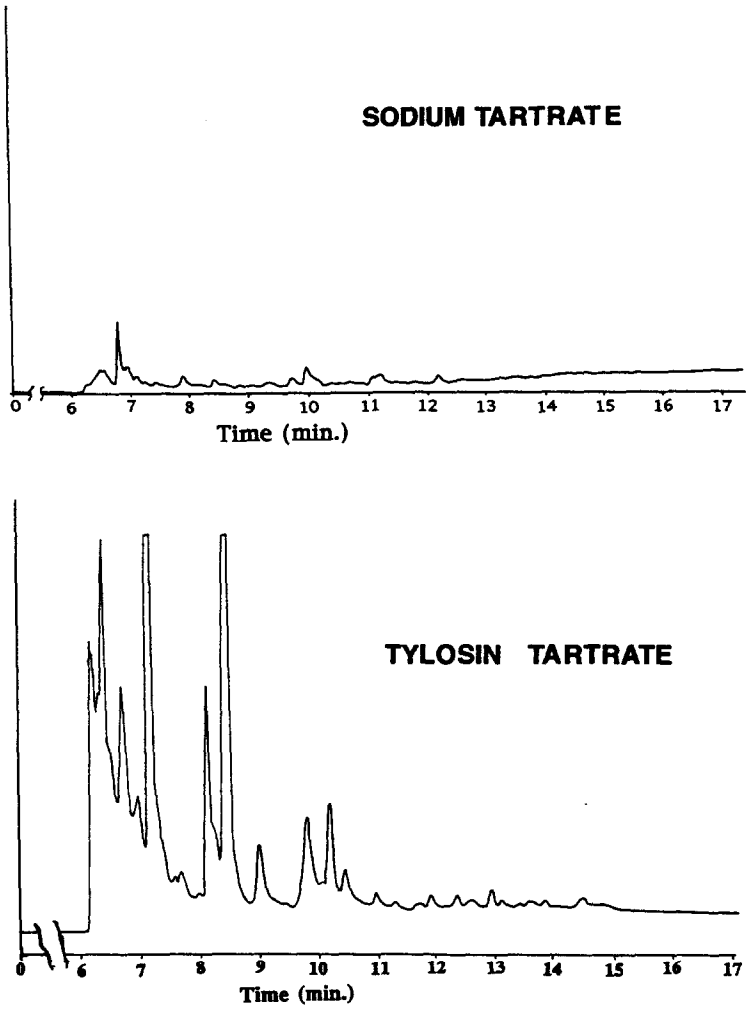

Figure 2
Pyrograms for sodium tartrate (2.5 mg) and tylosin tartrate (1.6 mg).

major retained component was seen for troleandomycin at about 10 min while several resolved peaks at 6.0, 6.7, 7.3, 8.0 and 9.2 min appeared in the oleandomycin pyrogram. These differences between oleandomycin and troleandomycin were not due to the fact that one is a phosphate salt and one is the free base. A sample of troleandomycin was mixed with a NaH_2PO_4 solution and the water was then allowed to evaporate at room temperature. The resultant salt showed the same pyrolysis pattern as that in Fig. 4. A pyrogram similar to the standard for the pharmaceutical product TAO was generated [Fig. 4(B)]. The pyrogram for erythromycin is shown in Fig. 5(A). Peaks at 7 min and a doublet at 8–9 min tended to dominate this pyrogram. The pyrogram of a pharmaceutical product, erythromycin ethylsuccinate, closely resembles that for the standard as seen in Fig. 5(B). An extra peak at 14.9 min and the absence of a peak at 11 min in the pharmaceutical product pyrogram are the main differences. A standard of erythromycin ethylsuccinate did not generate any significant different pyrolysis peaks from those seen for erythromycin.

Table 1 summarizes the primary features of the pyrograms for all five of these antibiotics. All of the antibiotics can be distinguished from each other with tylosin and erythromycin having the most similar features. Reproducibility of the major peaks from 6 to 17 min in the erythromycin pyrogram ($n = 3$) indicated RSD values of about 1–2%. Typical retention reproducibility from day to day was 2–4%. Although the pyrolysis temperatures for the troleandomycin standard and sample are different (Fig. 4), the primary retained peak

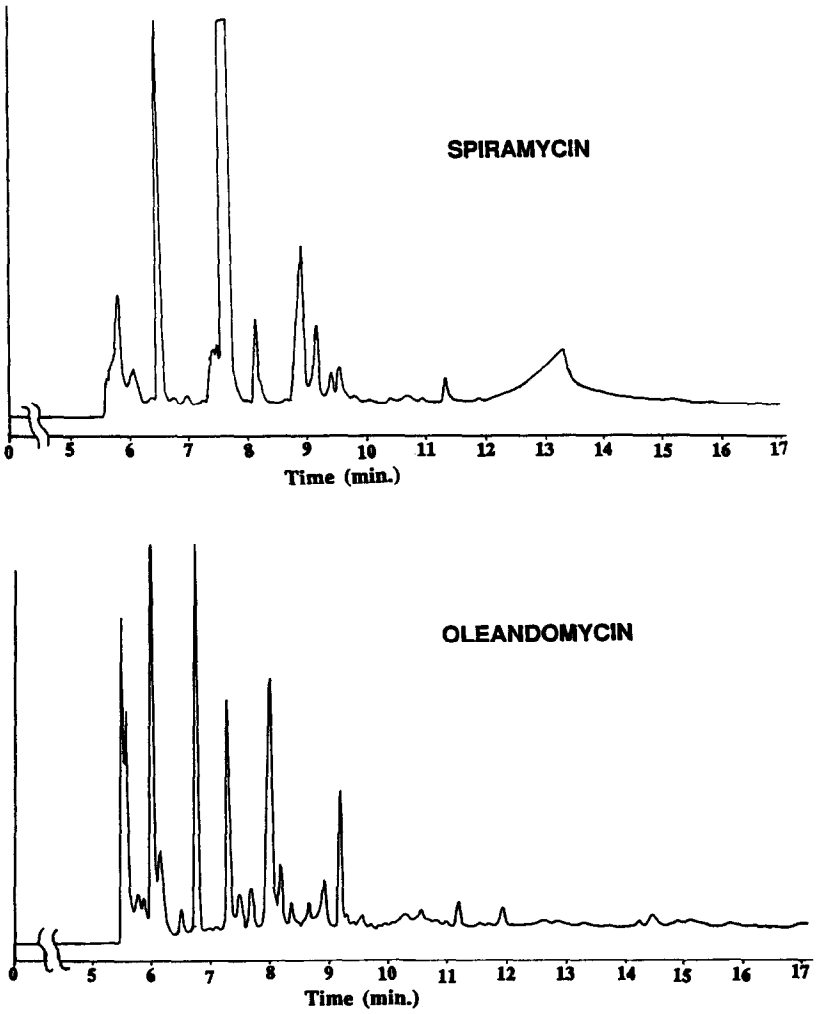

Figure 3
Pyrograms for spiramycin (0.7 mg) and oleandomycin (0.8 mg).

has the same retention time in both pyrograms. Only the first peak varied in retention time as expected, since smaller organic molecules would likely be formed at the higher pyrolysis temperature. Therefore, good reproducibility of the retained peaks in the pyrograms is likely even if the pyrolysis temperature may vary between 500 and 650°C. The pyrolysis of the sugars glucose and sucrose at the 2.5 mg level did produce two early eluting peaks but no significant retained peaks beyond 6 min. If these sugars were present in the pharmaceutical matrix, minimal interference would be expected upon pyrolysis. Clearly, PY–GC is a rapid, inexpensive method of identifying macrolide antibiotics as either the free base or a salt. Compounds close in structure or in a pharmaceutical matrix are also differentiated.

Initially, the reaction of a 10 ng ml^{-1} solution of each of the five antibiotics was carried out in 18 N H_2SO_4. A yellow colour was seen in all the solutions. The UV–vis spectra of the solutions were all very similar in shape to that previously found for erythromycin [13]. The wavelength maxima in the visible region for erythromycin, troleandomycin and oleandomycin are all about 470 nm. A strong absorbance peak in the moderate UV region is also evident. The corresponding wavelength maxima for the 16-membered macrolide antibiotics, tylosin and spiramycin, are slightly higher at 478 nm. To account for the similarity in reaction spectra, it is proposed that the sugar moieties react with H_2SO_4 to generate the yellow colour. The reaction of glucose with 18 N H_2SO_4 produced the same yellow colour as that for erythromycin with the acid. It is known that sulphonated naphthols will react

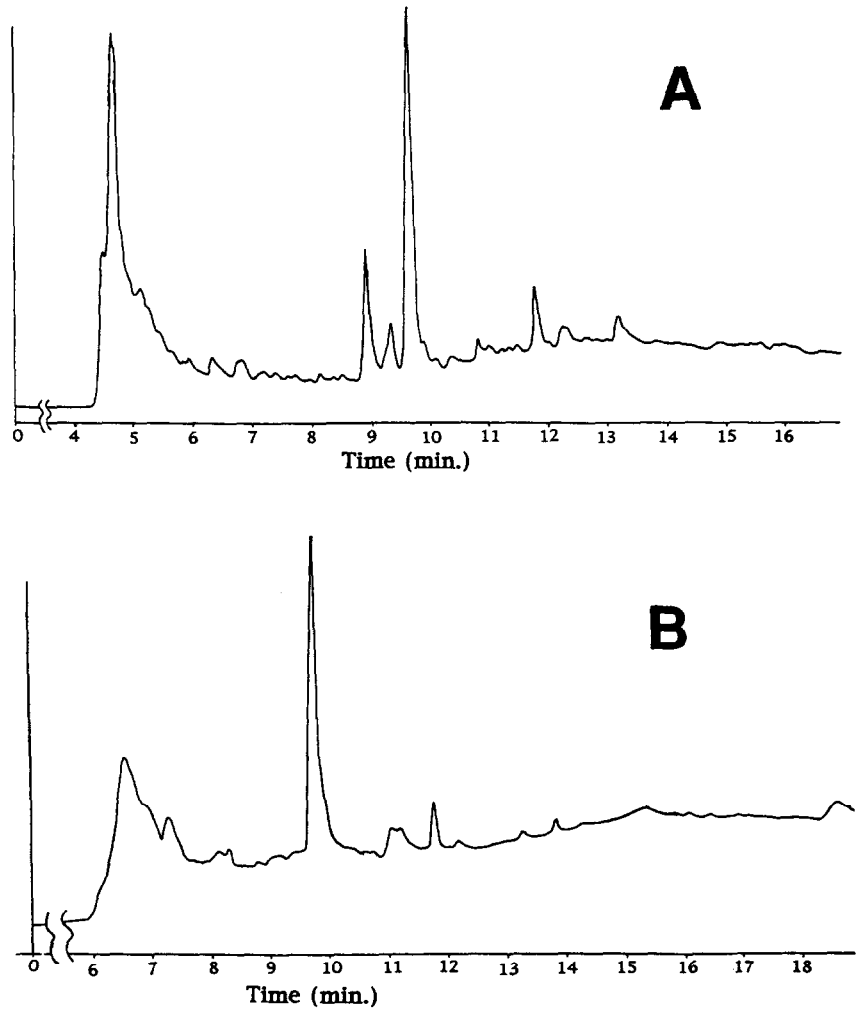

Figure 4
Pyrograms for (A) troleandomycin (0.6 mg) at 650°C and (B) crushed powder from a TAO tablet (2 mg) at 500°C.

with sugars in concentrated H_2SO_4 to form coloured products [23]. Both erythromycin and glucose solutions upon reaction of naphthol-disulphonate in H_2SO_4 formed identical yellow–brown colours. The possibility that the strong H_2SO_4 can convert the carbonyl group of the macrolide ring to a hydroxy and set up extended conjugation in the form of a trienylic cation [24] was also considered. A similar reaction using Fe^{3+} and concentrated acetic or sulphuric acid for cholesterol has been previously reported [25]. The reaction of erythromycin in a Fe^{3+}–acid solution did produce a greenish-blue colour. The visible spectrum taken against the blank does indicate prominent peaks at 480 and 590 nm. In concentrated acetic acid alone, no colour is seen when erythromycin is added. Therefore, ferric ion is important for this reaction involving the macrolide ring. Due to the similarity of the glucose and erythromycin reactions described above, the sugar groups are probably the primary reaction sites causing the colour involving concentrated H_2SO_4 as the reagent.

The optimum final concentration of the sulphuric acid was generally between 14 and 16 N for the antibiotics as shown with erythromycin and tylosin in Fig. 6. The acid profile for spiramycin plateaus between 14 and 18 N. The reaction with oleandomycin and troleandomycin is more favourable using concentrated H_2SO_4 as the reagent resulting in a final acid concentration of 18 N. Although the solutions were initially hot (about 65°C) after mixing, they all cooled in an exponential fashion to room temperature in about 30 min. However, the reaction time to generate the maximum absorbance not only is longer than expected

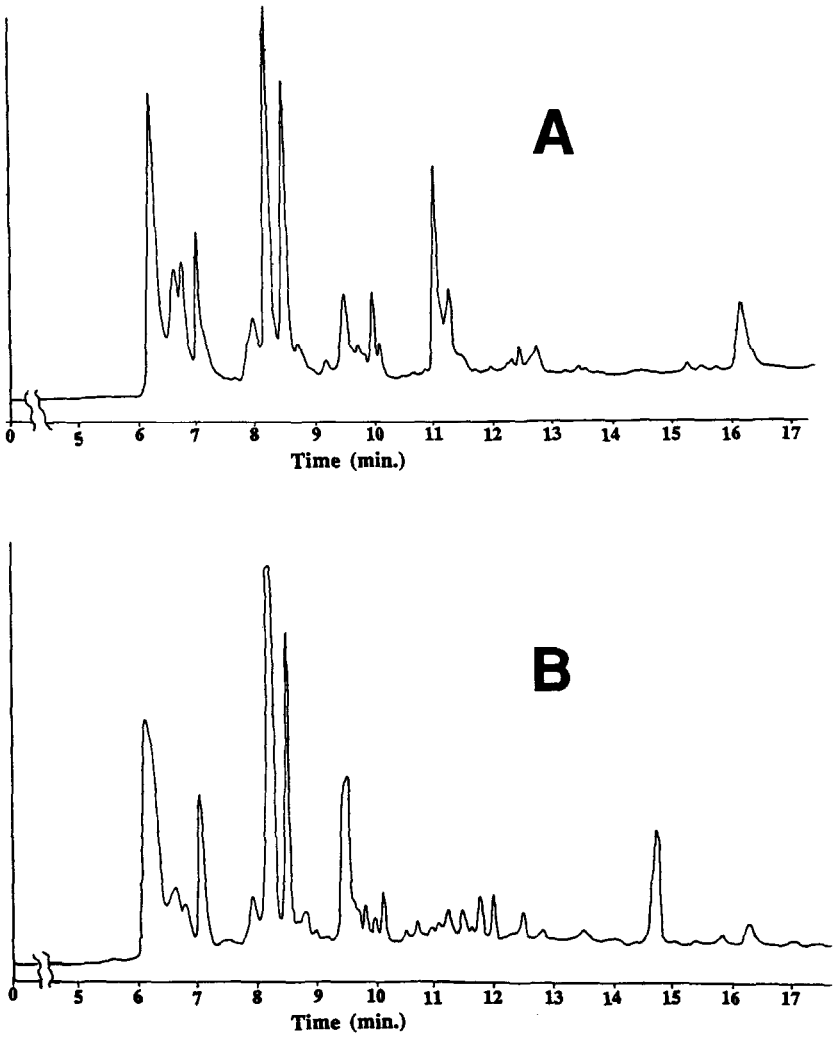

Figure 5
Pyrograms for (A) erythromycin (0.7 mg) and (B) crushed orange powder from erythromycin capsule beads (0.7 mg).

Table 1
Major PY–GC peaks of some macrolide compounds

Drugs	Major peaks (retention time range in min)							
	5–6	6–7	7–8	8–9	9–10	10–11	11–12	13–17
Erythromycin		6.4 S	7.1 M	8.3 S 8.6 S		10.0 W	11.1 S	16.1 BW
Oleandomycin	5.5 S	6.0 S 6.7 S	7.3 S	8.1 S	9.2 M			
Spiramycin		6.5 S	7.7 S	8.2 W 8.9 M	9.2 W			13.3 BW
Troleandomycin				9.0 M	9.8 S		11.8 W	
Tylosin		6.5 S 6.7 M	7.3 S	8.2 M 8.6 S	9.8 W	10.2 W		

S, strong; M, medium; W, weak; B, broad peak.

but varies quite a bit (Fig. 7). Spiramycin reacted in the shortest time of about 30 min with little change in absorbance while tylosin requires about 200 min to reach maximum absorbance. Troleandomycin also took about 200 min with the solution remaining fairly constant in absorbance, however, the reaction time for oleandomycin peaks at 150 min. The

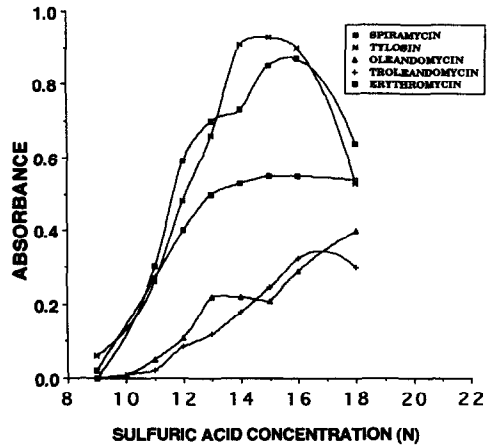

Figure 6
Absorbance of macrolide reaction products as a function of H_2SO_4 concentration.

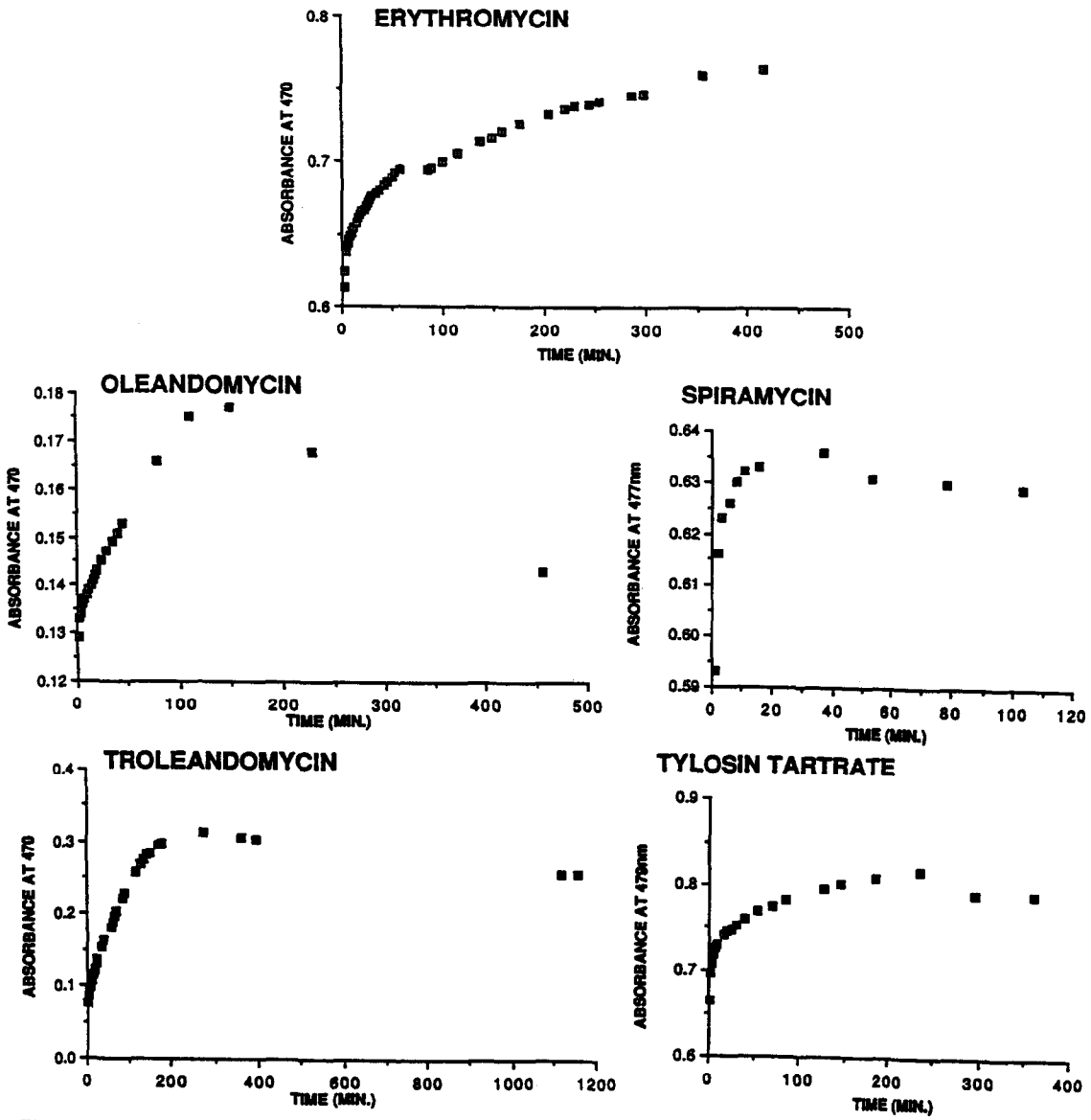

Figure 7
Absorbance of macrolide reaction products as a function of time.

Table 2
Spectrophotometric linearity of response for antibiotics after reaction with H_2SO_4

Compounds	Least squares (Equations $y = a + bx$)*	Corr. coeff.	Molar absorp.
Erythromycin	$a = 6.3 \times 10^{-3} \pm 1.4 \times 10^{-3}$ $b = 1.5 \times 10^{-2} \pm 1.2 \times 10^{-4}$	0.9998	11,200
Oleandomycin	$a = -4.3 \times 10^{-3} \pm 3.2 \times 10^{-5}$ $b = 8.3 \times 10^{-3} \pm 3.2 \times 10^{-5}$	0.9999	6,500
Troleandomycin	$a = -9.7 \times 10^{-3} \pm 2.4 \times 10^{-3}$ $b = 1.3 \times 10^{-2} \pm 1.1 \times 10^{-4}$	0.9993	10,400
Spiramycin	$a = -2.7 \times 10^{-3} \pm 2.7 \times 10^{-3}$ $b = 1.1 \times 10^{-2} \pm 1.3 \times 10^{-4}$	0.9986	10,100†
Tylosin	$a = -5.7 \times 10^{-3} \pm 1.5 \times 10^{-3}$ $b = 1.5 \times 10^{-2} \pm 7.3 \times 10^{-5}$	0.9997	13,600

*y = Absorbance; x = concentration (ppm).
†Calculated using the molecular weight of spiramycin II which is intermediate between spiramycin I and III.

absorbance for erythromycin increased steadily with time up to about 350 min. The rapid reaction time for spiramycin compared to the other macrolide antibiotics may be due to the presence of two amino sugars in spiramycin instead of one for the other compounds. These differences in reaction profiles of the five antibiotics could also provide information to assist in qualitative identification. In general, it is best to make the absorbance measurements of the samples and standards within a 15 min time frame. Alternatively, to shorten the reaction time, solutions of all five antibiotics were incubated at 65°C with sulphuric acid for 10, 20 and 30 min time periods. In general, all of the solutions reached their maximum absorbance in 10 min with no improvement at longer times. This procedure did reduce the reaction time by about a factor of 5 for oleandomycin, troleandomycin and tylosin. However, the heating step did not markedly help the erythromycin and spiramycin reactions.

Using the optimum acid, reaction time, and wavelength conditions, calibration curves are generated from the detection limit to 50 ng ml^{-1} for each antibiotic (Table 2). This linear range agreed with previous work [13] which showed some curvature from 50 to 200 ng ml^{-1}. The relative standard deviation (RSD) of the slopes range from 0.4 to 1.1% and very good correlation coefficients are found. The molar absorptivity values for four of the antibiotic reaction products are 10–13,000 while that for oleandomycin is about two-thirds in magnitude. The detection limit for erythromycin was about 0.2 ng ml^{-1}; no value for erythromycin had been previously cited [13]. Detection limits for spiramycin and tylosin are about 0.5 ng ml^{-1} while those for troleandomycin and oleandomycin are 1.0 ng ml^{-1}. Pharmaceutical capsules of erythromycin and troleandomycin can be assayed with a precision of 1–3% RSD ($n = 5$) with a 93–96% recovery based on the claimed amount. No colour was seen upon reaction of an acetaminophen tablet or capsule with concentrated H_2SO_4. Therefore, excipients other than sugars in such pharmaceutical products should not generally pose to be an interference. If a slight reaction did take place, dilution of the sample would likely prevent any problem. The veterinary tylosin solution is assayed with a precision of 2% RSD ($n = 5$) with a 110% recovery based on the labelled concentration. Attempts to automate this H_2SO_4 assay of macrolide antibiotics with flow injection analysis were unsuccessful due to the difficulties of pumping concentrated H_2SO_4 and of mixing an aqueous sample with the acid. However, this method could be adopted for confirmation of antibiotics fraction collected after preparative HPLC.

Acknowledgement — Summer support from Eli Lilly and Howard Hughes Medical Institute grants for the respective authors D.C.B. and D.H.K. is gratefully appreciated.

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[*Received for review 28 February 1992; revised manuscript received 12 August 1992*]