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Antibacterial Activities of Erythromycins A, B, C, and D and Some of Their Derivatives

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The MICs of erythromycins A, B, C, and D and some of their derivatives were determined against 21 gram-positive and 15 gram-negative microorganisms. Antibacterial activity was confined to gram-positive and very few gram-negative bacteria. Erythromycin B was somewhat less active than erythromycin A, and erythromycin C and D showed about half that activity or even less. Most other derivatives had negligible activity. Determination of potency by diffusion and turbidimetric assays were in line with MICs. The examination of the results of these assays, however, revealed that there are differences between the data of different laboratories, depending on the microorganisms and conditions used.

During the determination of the structure of erythromycin A (14), it has been found that related products like erythromycin B (15) and erythromycin C (13) were also produced by *Streptomyces erythreus*. Recent analytical methods, like gas-liquid chromatography (11) and especially high-pressure liquid chromatography (10), have shown that these compounds were present in commercial samples of erythromycin. With this technique, it has also been found (10) that some degradation products which are formed in weakly acid medium, like erythromycin enolether (8) or anhydroerythromycin (3), may be present. During our thin-layer chromatographic studies (7, 12), we observed that besides these components, erythromycin D and des-*N*-methylerythromycin could be detected. These products either had been isolated (9) before (erythromycin D) or had been prepared (2) by chemical transformation (des-*N*-methylerythromycin).

In most textbooks (4, 5), erythromycin is considered as a single entity. In all pharmacopoeiae, except the latest edition of the European Pharmacopoeia (1), there is no control of the amounts of these related components. Very little is known about the antibacterial activity of these components. Some MICs against *Bacillus subtilis* were published (9). In the microbiological assays, the potencies of these products probably are different, depending on microorganism and technique used. The use of a single conversion factor from chemical assay to potency (10, 11) seems questionable. We have examined the antibacterial activities of these different substances against a series of microorganisms. The potency was determined in different laboratories under a variety of conditions.

MATERIALS AND METHODS

Samples. Erythromycin B, C, and D were obtained by preparative high-pressure liquid chromatography of mother liquor concentrates of an industrial production of erythromycin. Their identity was checked by high-pressure liquid chromatography (10) and thin-layer chromatographic comparison (7, 12), with reference samples provided by Abbott Laboratories, North Chicago, Ill. Erythromycin enolethers A and B (8), anhydroerythromycin A (14), and erythralosamine (3), and des-*N*-methylerythromycin A (2) were prepared by published procedures. The identity of all samples was confirmed by mass spectrometry.

Purity. By high-pressure liquid chromatography (10) and thin-layer chromatography (7, 12), it was shown that the products were free of any detectable contaminant (less than 1%). The purity of erythromycin A, B, C, and D was determined by titration in acetic acid with 0.025 N perchloric acid in acetic acid with quinaldine red (0.1% [wt/vol] in acetic acid) as indicator. The result was checked by a determination of water by Karl Fischer titration (1).

Susceptibility tests. Laboratory 3. The microorganisms used were American Type Culture Collection strains or clinical isolates. The MIC determination was done by the plate dilution method in a Mueller-Hinton medium containing 5% lysed horse blood (pH 7.4). All antibiotics were dissolved to a concentration of 1,280 μg/ml and thereafter diluted with water in twofold dilutions to 0.625 μg/ml. From these dilutions, 0.5 ml was added to 10 ml of molten medium in petri dishes, giving final concentrations in the range of 0.03 to 64 μg/ml.

The test organisms were grown in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) overnight and diluted in such a way that after inoculation with a multipoint inoculator (Dynatech Industries, Inc., McLean, Va.), 10^4 to 10^5 CFU were obtained. *Neisseria* spp., *Haemophilus* spp., and viridans group streptococci were grown on a solid medium and brought afterwards into a homogeneous suspension in the broth. The MICs were read as the lowest concentration without visible growth after an incubation period of 24 h at 36°C.

Potency determinations. Laboratory 1. (i) Diffusion method. An assay medium containing (in grams): peptone, 6.0; beef extract, 1.5; yeast extract, 3.0; glucose, 1.0; agar, 15 (Difco Laboratories, Detroit, Mich.); and water to produce 1,000 ml was used (medium A [1] without pancreatic digest of casein). The pH after sterilization was 8. The petri dishes, containing a 4-mm layer of the assay medium, were inoculated with *B.*

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TABLE 1. MICs of erythromycins and derivatives against gram-positive microorganisms

Organism	MIC (μg/ml) of:								
	Erythromycin A	Erythromycin B	Erythromycin C	Erythromycin D	des-*N*-methylerythromycin A	Erythromycin A enolether	Erythromycin B enolether	Anhydroerythromycin A	Erythralosamine
Streptococcus pneumoniae 1	0.03	0.03	0.03	0.03	0.12	0.25	0.5	2	>64
Streptococcus pneumoniae 2	0.03	0.03	0.25	0.03	0.12	0.25	0.25	2	>64
Streptococcus faecalis 1	>64	>64	>64	>64	>64	>64	>64	>64	>64
Streptococcus faecalis 2	32	>64	>64	>64	>64	>64	>64	>64	>64
Streptococcus faecalis ATCC 19433	0.5	0.12	0.5	0.5	4	4	8	>64	>64
Streptococcus agalactiae 1	0.03	0.03	0.03	0.03	0.5	1	1	8	>64
Streptococcus agalactiae 2	0.03	0.03	0.03	0.03	0.12	0.5	0.5	4	>64
Streptococcus pyogenes 1	0.03	0.03	0.03	0.03	0.25	0.5	0.5	4	>64
Streptococcus pyogenes 2	0.03	0.03	0.03	0.03	0.12	0.25	0.25	2	>64
Streptococcus pyogenes ATCC 19615	0.03	0.06	0.06	0.12	1	2	4	16	>64
Streptococci viridans (group 1)	0.03	0.03	0.03	0.03	0.03	0.12	0.12	1	>64
Streptococci viridans (group 2)	0 03	0.03	0.03	0.03	0.5	1	1	4	>64
Micrococcus luteus ATCC 9341	0.03	0.03	0.03	0.12	2	2	2	8	>64
Streptococcus epidermidis ATCC 12228	0.06	0.06	0.25	0.25	1	4	4	32	>64
Staphylococcus aureus ATCC 25923	0.06	0.06	0.25	0.25	4	4	4	32	>64
Staphylococcus aureus peni-S 80/9	0.06	0.12	0.25	0.25	2	4	4	32	>64
Staphylococcus aureus oxa-R 80/5	>64	>64	>64	>64	>64	>64	>64	>64	>64
Staphylococcus aureus peni-R 80/48	>64	>64	>64	>64	>64	>64	>64	>64	>64
Staphylococcus aureus peni-R 80/57	0.06	0.06	0.25	0.25	2	4	4	32	>64
Bacillus subtilis ATCC 6633	0.03	0.03	0.06	0.12	2	2	2	8	>64
Bacillus anthracis	0.5	0.25	2	1	8	8	8	64	>64

subtilis ATCC 6633 (2×10^5 spores per ml) or *Staphylococcus aureus* ATCC 6538P (5×10^5 cells per ml).

The erythromycins were dissolved in methanol in a concentration of 1 mg/ml and diluted with 0.2 M phosphate buffer (pH 8) to contain a concentration of 0.5 μg/ml for the assay with *B. subtilis* or 0.66 μg/ml for *S. aureus*. Two further dilutions were made in 1.5 logarithmic progression. The solutions were put in wells (8-mm diameter) cut in the

TABLE 2. MICs of erythromycins and derivatives against gram-negative microorganisms

Organism	MIC (μg/ml) of:								
	Erythromycin A	Erythromycin B	Erythromycin C	Erythromycin D	des-*N*-methylerythromycin A	Erythromycin A enolether	Erythromycin B enolether	Anhydroerythromycin A	Erythralosamine
Neisseria meningitidis 1	0.03	0.03	0.03	0.03	0.12	0.12	0.12	2	>64
Neisseria meningitidis 2	0.5	1	4	4	32	32	64	>64	>64
Haemophilus influenzae	1	2	4	4	64	64	>64	>64	>64
Pasteurella multocida	2	4	16	16	>64	>64	>64	>64	>64
Pasteurella ureae	0.12	0.25	1	2	4	8	8	64	>64
Serratia marcescens ATCC 8100	>64	64	>64	>64	>64	>64	>64	>64	>64
Salmonella typhimurium ATCC 14028	>64	>64	>64	>64	>64	>64	>64	>64	>64
Pseudomonas aeruginosa ATCC 27853	32	64	64	64	>64	>64	>64	>64	>64
Klebsiella pneumoniae ATCC 13883	16	32	64	64	>64	>64	>64	>64	>64
Escherichia coli ATCC 25922	32	>64	>64	>64	>64	>64	>64	>64	>64
Enterobacter cloacae ATCC 23355	64	>64	>64	>64	>64	>64	>64	>64	>64
Acinetobacter calcoaceticus ATCC 19606	32	32	>64	>64	>64	>64	>64	>64	>64
Citrobacter freundii ATCC 8090	>64	>64	>64	>64	>64	>64	>64	>64	>64
Enterobacter aerogenes ATCC 13048	>64	>64	>64	>64	>64	>64	>64	>64	>64
Shigella sonnei ATCC 25931	64	>64	>64	>64	>64	>64	>64	>64	>64

TABLE 3. Potencies of erythromycin A, B, C, and D expressed as percentage of erythromycin A potency

Drug	Drug potency for indicated laboratories and organisms by:										
	Diffusion						Turbidimetry				
	B. subtilis ATCC 6633				*S. aureus* ATCC 6538P		*K. pneumoniae* ATCC 10031	*K. pneumoniae* ATCC 9997		*S. aureus* ATCC 6538P	
	1	2a[a]	2b[a]	3	1	2a	1	2a	2b	1	2a
Erythromycin B	78.6 ± 3	73.2	77.1	83.9 ± 4.9	60.4 ± 9	73.1	83.4 ± 2	104.3	108.5	61.4 ± 4	71.0
Erythromycin C	31.3 ± 2	42.0	45.7	44.6 ± 4.3	51.4 ± 4	39.7	67.2 ± 2	56.0	63.7	55.3 ± 3	37.5
Erythromycin D	34.2 ± 5	39.7	41.7	47.1 ± 5.0	35.1 ± 2	37.0	42.7 ± 2	56.5	61.1	42.0 ± 6	36.0

[a] In laboratory 2, the tests were performed two times with an interval of about one year. The fiducial limits of error of laboratory 2 are ±5%.

agar. A 3 × 3 assay design with erythromycin A as reference substance was applied. For each assay, 10 dishes were used, and the incubation was at 30°C for 18 h.

(ii) Turbidimetric method. The medium contained (in grams): heart extract, 1.5; yeast extract, 1.5; peptone casein, 5.0; glucose, 1.0 g (Difco); sodium chloride, 3.5; dipotassium hydrogen phosphate, 3.68; potassium dihydrogen phosphate, 1.32; potassium nitrate, 2.0; and water to produce 1,000 ml (medium D [1]). This medium (150 ml) was inoculated with 1 ml of a suspension of *S. aureus* ATCC 6538P (4 × 10^4 cells per ml) or *Klebsiella pneumoniae* ATCC 10031 (3 × 10^{10} cells per ml) and incubated for 1 h 30 min at 37°C. For the assay, the medium was inoculated with the suspension (30:1,000).

The erythromycins were dissolved in methanol at a concentration of 1 mg/ml and diluted with 0.2% phosphate buffer (pH 8) to obtain a concentration of 10 μg/ml for the assay with *K. pneumoniae* or 1 μg/ml for that with *S. aureus*. Three further dilutions were made in 1.5 logarithmic progression.

For the assay, 0.3 ml of the solution of the antibiotic was added to 2.7 ml of the inoculated medium. A 4 × 4 assay design was applied with three replicates per assay. After incubation for 3 h 30 min at 37°C, the A_{630} was measured in each tube.

Laboratory 2. (i) Diffusion method. The assay medium was the same as that used in laboratory 1 but contained in addition 4.0 g of pancreatic digest of casein. The concentration range of erythromycin for *S. aureus* was 0.5 to 2 μg/ml, and for *B. subtilis* it was 0.1 to 0.4 μg/ml.

(ii) Turbidimetric method. Medium and bacteria were the same as those used in laboratory 1 except that *K. pneumoniae* ATCC 9997 was inoculated. The concentration range of erythromycin was 2.5 to 5.0 μg per tube for *K. pneumoniae* and 0.5 to 1.0 μg per tube for *S. aureus*.

Laboratory 3. Diffusion method. The assay medium (Oxoid antibiotic medium 1, medium A [1]) was brought to pH 7.8 with 1 N NaOH, inoculated with *B. subtilis* spore suspension (Difco 0453-36) at 1 ml/liter, and distributed in 18-ml amounts in 90-mm petri dishes. The erythromycins were dissolved in alcohol (2.56 mg/ml) and diluted with water to contain 8 μg/ml. Further dilutions were made up to 1 μg/ml. The solutions were put in 5-mm wells cut in the agar, and the plates were incubated for 18 h at 36°C.

RESULTS AND DISCUSSION

The MICS of erythromycin A and B against gram-positive bacteria were very similar (Table 1), in agreement with previously published data (6). The antibacterial activity of erythromycin C and D was lower for most microorganisms. A comparison of the activity against *B. subtilis* has been determined for the four related substances (9). Our results with this microorganism are similar to those published previously. The activity of erythromycin against gram-negative bacteria except *Neisseria* spp. is very low (Table 2). None of the related components had a higher activity than erythromycin A. The MICs of des-*N*-methylerythromycin and the acid degradation products, i.e., the enolether, the anhydro-derivative, and erythalosamine, are much higher and do not contribute to the antibacterial activity.

The potencies of erythromycins B, C, and D were determined with erythromycin A as a reference compound (Table 3). The assay of potency was performed with greater precision than the determination of MIC. The precision of the potency of an antibiotic was lower for substances which contain related compounds. When a substance B is assayed against reference substance A, variations occur which are due to the method used (diffusion or turbidimetry) and the microorganism and culture conditions which are applied. These differences are clearly in Table 3. Even within the same laboratory (laboratory 2), some differences occurred when assays were performed with an interval of 1 year. Random variations seem to be greater than when one substance is assayed against an identical reference compound.

The precision of the assay of erythromycin is influenced by the presence of related substances, which may be present in rather large amounts, e.g., up to 13% of erythromycin B (10). The precision of the assay can be improved by limiting the amount of three impurities. The European Pharmacopoeia (1) now has a limit of about 5% for these related substances.

Another conclusion of this study is that the use of a single factor for converting the amount of erythromycin B and C, which were determined by a physico-chemical method, and which was used in some publications (10, 11) is rather arbitrary. The factor depends on the microorganism and conditions of the bioassay.

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