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# THIN-LAYER CHROMATOGRAPHIC STUDY OF THE METABOLITES OF ERYTHROMYCINS IN THE WISTAR RAT

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## SUMMARY

The metabolites of erythromycin A, anhydroerythromycin A, N-demethylerythromycin A and erythromycin B in the Wistar rat were studied by thin-layer chromatography. In some experiments germ-free rats, rats with a cannulated bile duct and a gastrectomized rat were used. The erythromycins examined were shown to undergo two principal changes, N-demethylation and acid-catalysed degradation. It was demonstrated that the stomach and the liver are not the sole sites of acid degradation and demethylation of erythromycins, respectively. Erythromycin A gives three principal metabolites, anhydroerythromycin A, anhydro-N-demethylerythromycin A and N-demethylerythromycin A, and erythromycin A enol ether and N-demethylerythromycin A enol ether are present to a minor extent. 5-O-Desosaminylerythronolide A was also identified, suggesting the presence of an erythromycin glycosidase.

## INTRODUCTION

Erythromycin has proved to be a safe and effective antibiotic for the treatment of a number of infections and specific indications for its use are still increasing [1]. Several metabolites of erythromycin A (EA) have been described. N-Demethylerythromycin A (dMeEA) was reported first [2,3]. More recently, anhydroerythromycin A (AEA) has been identified in plasma and urine of dog [4] and in human serum [5]. Still other erythromycin metabolites have been found following the administration of erythromycin ethyl succinate to man: N-didemethyl-N-propionylanhydroerythromycin A [6] and erythromycin A enol ether (EAEN) [7] in urine and anhydroerythromycin A ethyl succinate, erythromycin A ethyl succinate enol ether and erythralosamine in serum [8].

During the early periods of investigation, one of the problems in the identification of metabolites of erythromycin was the lack of good separation methods.

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Following the development of an improved thin-layer chromatographic (TLC) method [9], we decided to investigate the metabolites formed after the administration of erythromycins A or B, AEA or dMeEA to the Wistar rat.

## EXPERIMENTAL

### *Erythromycins and derivatives*

EA was obtained by purification of a commercial sample as described previously [10]. Erythromycin B (EB) was obtained from mother liquor concentrates of the industrial production of erythromycin [10]. AEA or erythromycin A 6,9; 9,12-spiroketal [11] and dMeEA [12] were prepared according to described procedures. For administration to rats, the erythromycin bases were transformed into a water-soluble lactobionate salt. In a typical preparation, 4.5 g of EA was mixed with 2.0 g of lactobionic acid and 50 ml of water were added. The suspension was stirred magnetically for 1 h and then filtered through paper. The filtrate was freeze-dried to obtain 5.3 g of erythromycin A lactobionate (EA-LB). TLC showed the absence of any other erythromycin derivative.

EAEN or 8,9-anhydroerythromycin A 6,9-hemiketal [13], erythromycin B enol ether (EBEN) or 8,9-anhydroerythromycin B 6,9-hemiketal [13], anhydro-N-demethylerythromycin A (AdMeEA) or N-demethylerythromycin A 6,9;9,12-spiroketal [10], N-demethylerythromycin A enol ether (dMeEAEN) or 8,9-anhydro-N-demethylerythromycin A 6,9-hemiketal [14] and 5-O-desosaminylerythronolide A [15] were all prepared according to described procedures. Figs. 1–3 show the structures of the compounds. 5-O-Desosaminylerythronolide A, which is not represented, corresponds to EA lacking the neutral sugar (cladinose).

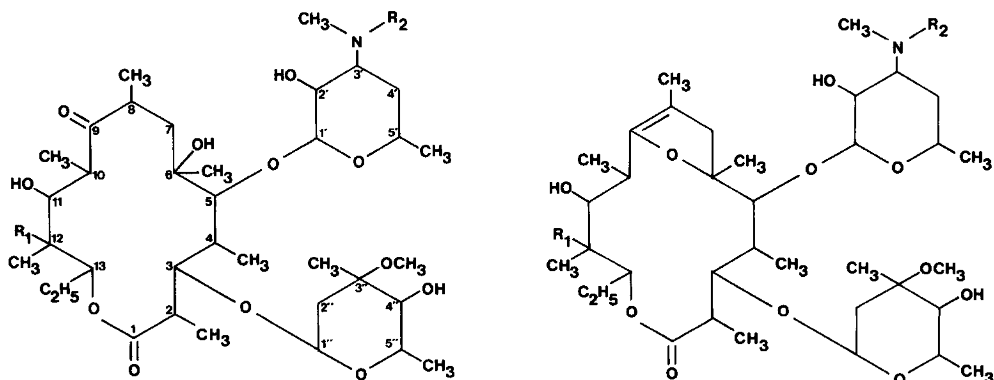

Fig. 1. Structure of erythromycins. Erythromycin A (EA): $R_1=OH$; $R_2=CH_3$. Erythromycin B (EB): $R_1=H$; $R_2=CH_3$. N-Demethylerythromycin A (dMeEA): $R_1=OH$; $R_2=H$. N-Demethylerythromycin B (dMeEB): $R_1=H$; $R_2=H$.

Fig. 2. Structure of enol ethers. Erythromycin A enol ether (EAEN): $R_1=OH$; $R_2=CH_3$. Erythromycin B enol ether (EBEN): $R_1=H$; $R_2=CH_3$. N-Demethylerythromycin A enol ether (dMeEAEN): $R_1=OH$; $R_2=H$. N-Demethylerythromycin B enol ether (dMeEBEN): $R_1=H$; $R_2=H$.

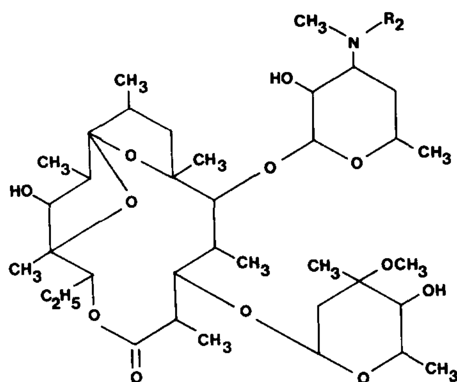


Fig. 3. Structure of spiroketals. Anhydroerythromycin A (AEA): $R_2=CH_3$. Anhydro-N-demethylerythromycin A (AdMeEA): $R_2=H$.

*Animals and dosing*

Male and female Wistar rats weighing 145–250 g were used. The animals had free access to a standard laboratory diet and tap water. Two germ-free rats (145 and 150 g body mass) were also used. Dosing solutions of EA-LB, EB-LB, AEA-LB and dMeEA-LB were prepared at concentrations equivalent to 75 mg of base per ml of water. Animals were dosed intravenously through a tail vein or intramuscularly at multisites on the hind limbs with 0.2 ml of a freshly prepared solution irrespective of body mass. For oral administration, 0.4 ml of a 1:2 diluted solution was used. In all experiments each animal received only one dose. When the same animal was used in several experiments, a seven-day wash-out period was allowed between successive experiments.

*Sample collection and extraction of metabolites*

*Urine and faeces.* Animals were housed in plastic metabolic cages designed for the separate collection of urine and faeces. Samples were collected at room temperature without addition of preservatives and extracted immediately.

Urine samples were diluted with twice their volume of acetonitrile and shaken vigorously. The precipitate was filtered off and the filtrate was diluted with an equal volume of water. The pH was adjusted to about 10 by adding saturated sodium carbonate solution and the mixture was extracted with an equal volume of ethyl acetate. The organic layer was dried over anhydrous sodium sulphate and evaporated to dryness under reduced pressure.

Faeces samples were homogenized mechanically in 20 ml of water and sonicated for 5 min. The homogenized mixture was shaken with 40 ml of acetonitrile for about 5 min and filtered. The filtrate was treated as described for urine samples.

For extraction, ethyl acetate could be replaced with dichloromethane or diethyl ether with similar results.

*Tissues from gastrectomized rat.* A male rat (250 g) was anaesthetized with sodium pentobarbital and its abdomen was opened. The oesophagus and duodenum were ligatured close to the stomach. After ligation of the main gastric arteries and veins, the stomach was excized and the abdominal opening was closed

with metal clips. While the animal was still under sedation, the appropriate dose of EA-LB was administered intravenously and 2 h later the animal was killed by cardiac puncture. Liver, kidney and bladder with contents were sampled. Organs were extracted immediately after separation or were stored at −15°C and extracted within 1 h. Each organ was finely powdered in liquid nitrogen using a mortar and pestle. Acetonitrile (20 ml) was added during the thawing period and the tissue was triturated for 5–10 min at room temperature to improve extraction. The mixture was filtered and the filtrate diluted with 10 ml of water. Saturated sodium carbonate solution (5 ml) was added and the mixture was extracted with 50 ml of ethyl acetate. The organic layer was dried over anhydrous sodium sulphate and evaporated to dryness under reduced pressure.

*Bile.* Bile from two male rats (about 250 g body mass each) was separately collected from polyethylene cannulae inserted through median abdominal incisions into the common bile ducts. The rats were dosed intravenously via a cannulated jugular vein at the moment of recovery from anaesthesia. Each rat was placed in a Bollman restricting cage, which in turn was placed in a larger cage maintained at 30°C. The animals were maintained for 24 h on a glucose infusion through the cannulated jugular vein. Bile was collected on ice first from 30 min before administration to 5 min after, and then over 30-min intervals up to 2 h after administration. Additional samples were obtained from 2 to 8 h and from 8 to 24 h. Samples up to 2 h were extracted at the end of the 2-h period and other samples at the end of the 24-h period. Each sample was diluted with an equal volume of saturated sodium carbonate solution and extracted with twice its volume of ethyl acetate by vortex-mixing for about 5 min. The organic layer was filtered through anhydrous sodium sulphate and evaporated to dryness under reduced pressure.,

*Control for the formation of metabolites in samples and for the efficiency of extraction.* As a control for the formation of metabolites during storage of samples, EA-LB was incubated for 24 h at room temperature with urine or faeces homogenates from rat at a final concentration corresponding to 1 mg/ml EA base. In a similar way EA-LB was incubated with homogenates of lung, muscle and kidney in distilled water at 37°C as a control for local formation of metabolites. All controls contained only negligible amounts of AEA.

The extraction efficiency was determined by TLC of 24-h urine and faeces samples before and after extraction. The erythromycin derivatives were shown to be extracted completely by the described procedures.

*Thin-layer chromatographic separation of the metabolites*

Extracts from samples were examined by TLC on pre-coated silica gel plates: Stratochrom SI $F_{254}$ (Carlo Erba, Milan, Italy) or silica gel 60 $F_{254}$ (E. Merck, Darmstadt, F.R.G.). Three mobile phases were used: (A) dichloromethane–methanol–25% ammonia (90:9:1.5, v/v); (B) ethyl acetate–methanol–25% ammonia (85:10:5, v/v); (C) diethyl ether–methanol–25% ammonia (90:9:2, v/v). Sample extracts were dissolved in 0.25 ml of dichloromethane and 10-$\mu$l volumes were spotted. After development over 15 cm in paper-lined chromatographic tanks, the plates were dried at 110°C for 5 min. Compounds were revealed by spraying

with 4-methoxybenzaldehyde–sulphuric acid–ethanol (1:1:9, v/v) and heating at 110°C for 1 min. Details of the chromatographic method have been published elsewhere [9].

TLC was used to identify and purify the metabolites and to determine their relative concentrations. To compare the relative amounts of erythromycins and metabolites, chromatograms were prepared with appropriate dilutions to obtain better spot sizes for visual assessment of spot intensity. All semi-quantitative determinations were performed on chromatograms obtained with TLC system A. Isolation of metabolites was performed mainly with TLC system A. When necessary, TLC systems B and C were used for further purification. After chromatography the band of interest was scraped off and the product was extracted with 10 ml of acetone or dichloromethane–methanol (1:1).

*Mass spectrometry*

Mass spectrometry (MS) was used to confirm the identity of the isolated metabolites. Mass spectra were recorded on an AEI MS-12 mass spectrometer (Kratos, Manchester, U.K.) operated at accelerating voltage 8 kV, trap current 100 μA, ionization energy 70 eV and ion-source temperature 150–170°C. Samples were introduced with the direct insertion probe.

## RESULTS

The chromatographic characteristics of all the compounds isolated from urine and faeces of rats dosed with EA or EB are presented in Table I. Their identities were confirmed by co-chromatography with reference compounds in the different TLC systems and further by MS. As no reference compounds were available for the N-demethylated EB derivatives, the presence of N-demethylerythromycin B (dMeEB) and N-demethylerythromycin B enol ether (dMeEBEN) was first suggested by analogy of their TLC behaviour with those of corresponding derivatives of EA. After isolation the identity was confirmed by MS. Moreover, after methylation with formaldehyde and cyanoborohydride by a procedure described for the methylation of dMeEA [16], the resulting substances were identified by TLC and MS as EB and EBEN, respectively.

Following administration of EA [orally, intravenously (i.v.) and intramuscularly (i.m.)], EB (i.v.), EAE (orally and i.v.) and dMeEA (orally and i.v.), the metabolites in 24-h urine and faeces of rats were identified and the relative amounts within each experiment were determined. The results of these experiments are reported in Table II. The presence of 5-O-desosaminylerythronolide A, which was formed in only very small amounts, is not considered further.

In another series of experiments, EA was administered i.v. to a number of rats under special experimental conditions. Faeces, urine, bile and a number of organs were examined. The results are given in Table III. As enol ethers and 5-O-desosaminylerythronolide A were always present in small amounts, they were not considered when the relative amounts of the metabolites within each experiment were calculated. As the relative amounts within each experiment are reported, the results do not allow the calculation of the total amount of metabolites re-

TABLE I

THIN-LAYER CHROMATOGRAPHIC CHARACTERISTICS OF ERYTHROMYCINS A AND B AND OF THE METABOLITES ISOLATED FROM URINE AND FAECES OF THE WISTAR RAT

See text for chromatographic conditions.

| Compound | $R_F$ value | | | Colour of spot |
|---|---|---|---|---|
| | System A | System B | System C | |
| Erythromycin A enol ether (EAEN) | 0.48 | 0.53 | 0.54 | Greyish green |
| Erythromycin A (EA) | 0.41 | 0.46 | 0.39 | Greyish green |
| 5-O-Desosaminylerythronolide A | 0.35 | 0.44 | 0.37 | Greyish yellow |
| Anhydroerythromycin A (AEA) | 0.36 | 0.42 | 0.41 | Greyish green |
| N-Demethylerythromycin A enol ether (dMeEAEN) | 0.29 | 0.37 | 0.33 | Greyish green |
| Anhydro-N-demethylerythromycin A (AdMeEA) | 0.25 | 0.34 | 0.31 | Greyish green |
| N-Demethylerythromycin A (dMeEA) | 0.21 | 0.29 | 0.21 | Greyish green |
| Erythromycin B enol ether (EBEN) | 0.47 | 0.53 | 0.55 | Violet blue |
| Erythromycin B (EB) | 0.44 | 0.50 | 0.45 | Violet blue |
| N-Demethylerythromycin B enol ether (dMeEBEN) | 0.23 | 0.34 | 0.15 | Violet blue |
| N-Demethylerythromycin B (dMeEB) | 0.21 | 0.29 | 0.13 | Violet blue |

TABLE II

RELATIVE AMOUNTS OF SUBSTANCES RECOVERED IN 24-h FAECES AND URINE OF RATS DOSED WITH VARIOUS ERYTHROMYCINS AND DERIVATIVES

| Sample* and route of adminis-tration | Source | Substance recovered** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | EA | AEA | EAEN | dMeEA | AdMeEA | dMeEAEN | EB | EBEN | dMeEB | dMeEBEN |
| EA (i.v.) | Faeces | ++ | + | + | ++ | + | << | | | | |
| | Urine | ++ | + | < | << | << | << | | | | |
| EA (i.m.) | Faeces | ++ | + | + | ++ | + | < | | | | |
| | Urine | ++ | + | < | < | < | << | | | | |
| EA (oral) | Faeces | + | ++ | << | + | ++ | << | | | | |
| | Urine | + | ++ | | + | + | << | | | | |
| AEA (i.v.) | Faeces | | ++ | | | +++ | | | | | |
| | Urine | | +++ | | | + | | | | | |
| AEA (oral) | Faeces | | +++ | | | ++ | | | | | |
| | Urine | | ++ | | | + | | | | | |
| EB (i.v.) | Faeces | | | | | | | +++ | << | +++ | << |
| | Urine | | | | | | | +++ | < | + | << |
| dMeEA (i.v.) | Faeces | | | | + | << | << | | | | |
| | Urine | | | | ++++ | +++ | + | | | | |
| dMeEA (oral) | Faeces | | | | ++ | ++++ | < | | | | |
| | Urine | | | | + | << | << | | | | |

*See Table I for compound abbreviations.

**The number of crosses indicates the relative amount of substance present; < and << indicate small and very small amounts, respectively.

TABLE III

RELATIVE AMOUNTS OF SUBSTANCES RECOVERED FROM RAT BILE, FAECES, URINE AND SOME ORGANS AFTER INTRAVENOUS ADMINISTRATION OF ERYTHROMYCIN A

| Experi-ment No. | Rat* | Sample | Time range (h) | Substance recovered** | | | |
|---|---|---|---|---|---|---|---|
| | | | | EA | AEA | dMeEA | AdMeEA |
| 1 | F and M | Faeces | 0-24 | ++++ | +++ | ++++ | +++ |
| | | Urine | 0-24 | ++++ | +++ | < | < |
| 2 | F and F, germ free | Faeces | 0-24 | ++++ | ++ | ++++ | +++ |
| | | Urine | 0- 6 | ++++ | ++ | < | < |
| | | | 6-24 | ++++ | ++++ | + | + |
| 3 | M and M with cannulated bile duct | Bile | 0- 2 | ++++ | | ++++ | |
| | | | 2- 8 | ++++ | + | ++++ | ++ |
| | | | 8-24 | +++ | ++ | ++++ | ++++ |
| | | Faeces | 0-24 | ++++ | +++ | ++++ | +++ |
| | | Urine | 0-24 | ++++ | ++++ | + | + |
| 4 | F and M | Upper small intestine and contents | 0- 2 | + | ++++ | +++ | < |
| | | Stomach and contents | 0- 2 | + | ++++ | ++ | ++++ |
| | | Kidney | 0- 2 | ++++ | ++ | + | < |
| | | Liver | 0- 2 | ++++ | ++ | +++ | + |
| 5 | M, gastrectomized | Kidney | 0- 2 | ++++ | + | + | < |
| | | Liver | 0- 2 | ++++ | ++ | +++ | + |
| | | Bladder and contents | 0- 2 | ++++ | ++ | < | < |

*F = female; M = male.
**The number of crosses indicates the relative amount of substance present; < indicates small amounts.

covered. Tables II and III do not allow a comparison with respect to recovery yields. Experiment 1 served as a reference experiment. In experiment 2, germ-free rats were used to check for the role of intestinal flora. In experiment 3, rats with cannulated bile duct were used. In experiment 4, organs of normal rats were excized for the investigation of metabolites. This experiment also served as a reference for experiment 5, where organs from a gastrectomized rat were examined.

## DISCUSSION

The results in Table I indicate that EA and EB are metabolized in rat to a mixture of N-demethylated compounds and also compounds whose origin must be attributed to acid-catalysed decomposition. It is accepted that in acidic media the erythromycins described here first degrade by attack of the C-6 hydroxy on the C-9 ketone group followed by dehydration to form the corresponding enol ether. The erythromycins with a C-12 hydroxy group are transformed by an irreversible addition of C-12 hydroxy to the double bond at C-8–C-9 into a spiroketal, generally called anhydroerythromycin [13]. Thus, both EA and EB give

enol ethers but only EA can yield a spiroketal. Examination of the urine and faeces of rat after administration of EA revealed six compounds, of which the metabolites EAEN, dMeEAEN, AdMeEA and 5-O-desosaminylerythronolide A are demonstrated for the first time. Except for the last compound, the presence of these compounds was predictable from previous identification of several demethyl and anhydro compounds after administration of erythromycin or its esters to rat or man [2–8]. The fact that not all the compounds reported here have been identified previously is probably due to the limited quality of the chromatographic methods used.

The formation of 5-O-desosaminylerythronolide A as a metabolite suggests the presence of a glycosidase. As the amounts detected increase when the same rat is used several times, the existence of an inducible glycosidase is postulated. Although the enzyme has not been definitively identified as a mammalian or microbial enzyme, detection of 5-O-desosaminylerythronolide A in faeces and urine of germ-free rats after a single dose indicates it to be a mammalian glycosidase.

The results in Table II confirm that EA readily undergoes acid-catalysed intramolecular cyclization to AEA and to a minor extent to EAEN [14]. Less of the intact drug is recovered after oral administration of EA than after i.v. or i.m. administration. The latter two routes give almost identical results. Oral administration of EA or dMeEA affords more anhydro derivatives and almost no enol ether derivatives as compared with i.m. or i.v. administration. It is generally accepted that the acid-catalysed reactions are a direct consequence of the stomachal acidity. Enzymic and non-enzymic mild acid catalysis elsewhere in the body are other hypothetical sources of spiroketal formation. The presence of more enol ether after i.m. or i.v. administration is in favour of the latter mechanism, as enol ethers are preferentially formed under mild acidic conditions [13].

Administration (i.v.) of EB afforded more intact substance than administration of EA, probably because the formation of anhydroerythromycin B is chemically excluded. EB and AEA are N-demethylated in the same way as EA, which is consistent with the previously reported low substrate specificity of the N-demethylating enzyme [17].

AEA is readily absorbed from the gastrointestinal tract (GIT) as it is present in the urine after oral administration. When dMeEA is administered i.v., nearly all of the metabolites (much AdMeEA and little dMeEAEN) are found in the urine, whereas after oral administration the faeces are the main excretion route. This confirms observations first made by Lee and co-workers [2,18] and proves that dMeEA is poorly absorbed from the GIT and that it is not efficiently excreted by the liver. On the other hand, dMeEA formed in the liver by demethylation of EA is very easily excreted through the bile, as is shown below. As the absorption of N-demethylated derivatives from the GIT is poor, the large amount of AdMeEA recovered from urine after an i.v. dose of dMeEA can be assumed to originate at sites other than the stomach.

In order to obtain more information about other possible site(s) in the body that favour the formation of anhydro derivatives, the experiments reported in Table III were carried out. As the results obtained with germ-free rats (experiment 2) are comparable to those in the reference experiment 1, involvement of

the activity of intestinal flora can be excluded. In experiment 3 using rats with a cannulated bile duct, it was observed that dMeEA appeared in the bile within 5 min following i.v. administration. This emphasizes the previous observation that the N-demethylation process occurs rapidly [17,19]. The proportion of anhydro derivatives in the bile increases with time. It is striking that faeces and urine show similar relative concentrations of the metabolites, as observed in the former experiments. This indicates that N-demethylation occurs also at site(s) other than the liver. The adrenal gland has been mentioned as showing some demethylating activity [17].

The presence in experiment 4 of relatively important amounts of N-demethylated metabolites in the stomach and contents is possibly due to a back-flow of bile contents from the duodenum into the stomach. The relative amounts of metabolites found in the bladder and contents of gastrectomized rats (experiment 5) are comparable to those observed in the urine of normal rats. The relative amounts of the four substances in the kidneys and liver of gastrectomized and normal rat 2-h post dose are similar. This indicates that stomachal acid catalysis is not the sole site of formation of anhydro derivatives.

A number of metabolites of erythromycins have been identified for the first time. Evidence has been forwarded that the stomach is not the sole site of formation of anhydro derivatives, because after i.m. or i.v. administration of EA or dMeEA relatively more enol ethers are formed than after oral administration. Moreover, an i.v. dose of dMeEA gives large amounts of AdMeEA in the urine, whereas it is known that N-demethyl derivatives, when not produced in the liver, are poorly excreted by the bile and poorly absorbed from the GIT. It also became clear that the liver is not the sole site of formation of N-demethyl derivatives because after i.v. administration of EA to a rat with cannulated bile duct, the faeces and urine contain the same relative amounts of N-demethylated substances as observed in normal rats.

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