A STUDY OF THE BEHAVIOR OF PROPIONYL ERYTHROMYCIN IN BLOOD BY A NEW CHROMATOGRAPHIC METHOD*

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By means of a two-step chromatographic technique propionyl erythromycin and erythromycin can be separated from the normal blood components and then from each other. The relative amounts of each form of the antibiotic can be determined on bioautograph plates by comparison of the zone sizes with those of a set of reference standards. Using this method, we have found that when propionyl erythromycin is added to blood there is an initial rapid hydrolysis to erythromycin. The hydrolytic rate decreases markedly with time, however, and overall is much slower than in buffer solution at the same pH. The rate of hydrolysis in blood differs with different animal species.

In an earlier paper[1] we reported that after the ingestion of erythromycin estolate** the antibiotic appears in the blood stream as a mixture of erythromycin and propionyl erythromycin.

The previous work involved the use of solvent extraction to remove the antibiotic from the blood and paper chromatography to separate and identify the components in the extracts. We have now developed a direct chromatographic method that is more reliable and convenient for determination of the amounts of both the ester and erythromycin in whole blood, serum, or any other body fluid. In this process, all of the antibiotic is measured and there is little if any ester hydrolysis during development. Since the standards used are subjected to the same development procedures as the test samples, any slight hydrolysis in the samples during development is compensated for by a similar change in the standards.

Materials and Methods

Subjects

Blood samples for laboratory experiments were obtained from laboratory personnel. Subjects were given five doses (250 mg at 6-hour intervals), from commercial lots of erythromycin estolate before the blood samples were drawn. The tests were run as soon as possible, and many times the chromatograms were in the first solvent system within

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** Erythromycin estolate is the generic name for the lauryl sulfate salt of the propionyl ester of erythromycin. The Lilly trademark for this compound is Ilosone®.

one-half hour after the blood was drawn. The samples were never kept at room temperature for more than a few minutes.

Preparation of Assay Samples

Whole blood, or the cellular fraction from heparinized blood, containing propionyl erythromycin and erythromycin was diluted with deionized water prior to spotting. Five to 25 μl samples (1~5 μl of undiluted blood) were spotted on Whatman No. 1 paper (19 × 46.5 cm). Serum or plasma was applied directly to the chromatograph without dilution. Urine was diluted with 24 volumes of water, and then 1~2 μl of this solution was used. Saliva, 10~20 μl, was applied directly to the paper.

Preparation of Standards

Reference standards were prepared by mixing 1 μg each of erythromycin base and propionyl erythromycin with 1 ml of normal human serum. A fresh standard solution was prepared each day. Reference standards were usually applied in a range of 0.5~5.0 μl, which was equivalent to 0.0005~0.005 μg of each component. In general, they were run and plated separately, with 10 lanes of standards on a single plate.

Paper Chromatography

Prepared samples of whole blood, serum, plasma, urine, and saliva were applied to Whatman No. 1 paper as noted above. After the samples had been air dried, the chromatogram was developed by descending chromatography in absolute methanol (solvent system No. 1). Additional methanol was placed at the bottom of the chromatography chamber in a beaker. After approximately one hour the chromatograms were removed and air dried. The upper portion of the chromatogram was cut off approximately 5 cm (2 inches) below the point of application to remove the residual blood. The lane numbers were transposed to a point 6.35 cm (2.5 inches) from the upper end of the cut chromatogram. Development of the chromatogram was continued by descending chromatography with solvent system No. 2 (dissolve 12.5 g of NH_4Cl and 35 g of NaCl in 100 ml of distilled H_2O; add 25 ml of dioxane and 12.5 ml methyl ethyl ketone; dilute to 1 liter with distilled H_2O and adjust to pH 5.7 with 1 N NH_4OH). The chromatogram was removed from the chamber when the solvent front neared the lower end of the tape. It was then air-dried and placed on nutrient agar of the following composition: Beef Extract (Difco), 1.5 g; Peptone (Difco), 6.0 g; Yeast Extract (Difco), 3.0 g; agar, 15 g; distilled water, 1 liter. Each bioautograph plate (25 × 45 cm) was poured with 250 ml of agar at pH 8.7~8.9, which was seeded with a 1 % suspension (55 % light transmission using Lumetron colorimeter model 402E, green filter, 530 mμ) of a *Sarcina lutea* culture (ATCC 9341) preserved in liquid nitrogen. By using only the central portion of the chromatogram, it was possible to put two tapes on one bioautograph plate. After 20 minutes, the chromatogram sheet was removed, and the plate was incubated at 37°C for 16 hours. The concentration in the test samples was determined by comparing the zones of inhibition of the test samples with those of the known standards.

Extraction Experiments

Heparinized whole blood, to which a mixture of erythromycin and propionyl erythromycin had been added, was combined with an equal volume of ether. After thorough mixing, the ether was separated by centrifugation and removed by means of a syringe or pipette. A second fraction of ether was added along with enough 1 N NaOH, to give a pH of 8.5~9.5. The blood was then extracted as before. A third extraction was performed without further pH adjustment. The combined extracts were dried over anhydrous sodium sulfate and transferred to a round-bottom flask, and the solvent was removed by the use of a rotary evaporator. The residue was dissolved in a volume of acetone equal to the original blood-sample volume, and this solution was used for chromatographic analysis. Samples of the original and extracted bloods were diluted with 4 volumes of water and were spotted on the same tape as the extracts.

A similar extraction was done in duplicate using serum. Again the pH was adjusted to 8.5~9.5 after the first extraction. Samples of the serum (2 μl), the extracts (2 μl) and the residual serums (20 μl) were then developed on the same chromatographic tape.

Results

When samples of diluted whole blood or cells, serum, or plasma containing erythromycin and propionyl erythromycin were spotted on chromatographic paper and developed in methanol, the antibiotic fraction was completely removed from the residual blood components and moved with the solvent front (Fig. 1). If an aqueous system such as solvent No. 2 was used directly, satisfactory separation was not achieved. That the extraction was complete with methanol but not with other common organic solvents is shown on Fig. 2. There was no separation of the antibiotic components during this process (left half of Fig. 3). Before transferring the partially developed chromatogram to the second system, the upper portion of the tape was cut off to remove the major blood constituents, and, since it no longer contained any active antibiotic fraction, it was discarded. In the second system the antibiotic components were separated (right half of Fig. 3). Their relative concentrations were then estimated from the zone sizes resulting from a *Sarcina lutea* bioautograph as compared with the zones produced by standard amounts of the components in serum developed by the same method.

Fig. 1. Schematic drawing of the paper chromatography procedure.

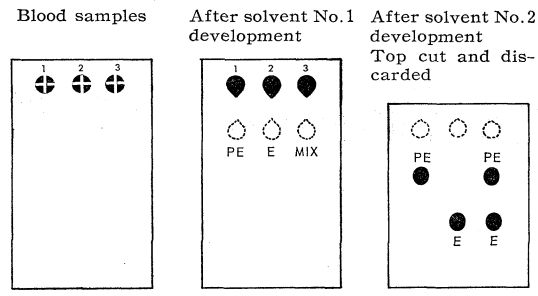
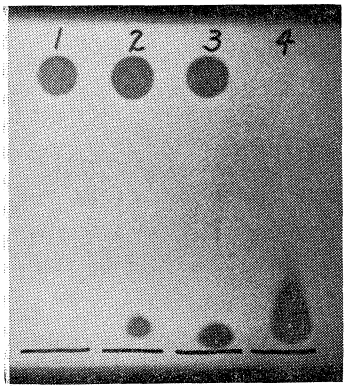
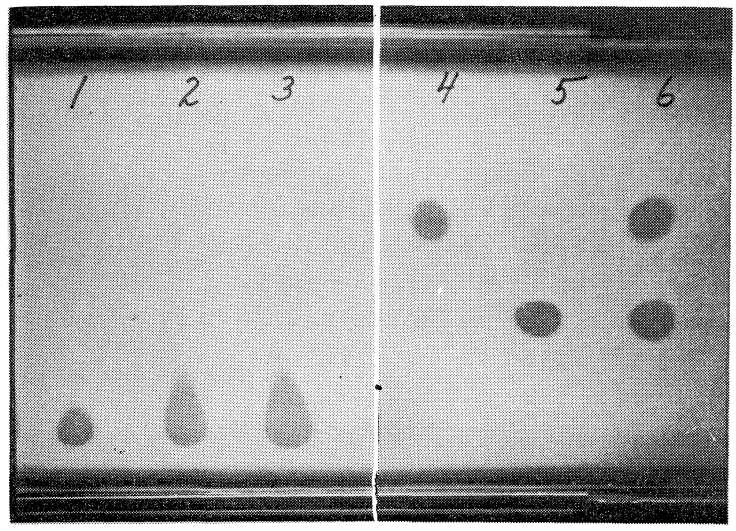


Fig. 2. Bioautograph of whole blood containing propionyl erythromycin and erythromycin developed on individual paper strips then plated together on a *Sarcina lutea* inoculated assay plate.



Lane 1 Developed with chloroform
2 Developed with ethyl acetate
3 Developed with acetone
4 Developed with methanol

Fig. 3. Antibiotic samples applied as acetone solutions.



Lanes 1 and 4, propionyl erythromycin
2 and 5, erythromycin
3 and 6, mixture

Fig. 4. Whole blood extraction

A mixture of 1/3 erythromycin and 2/3 propionyl erythromycin was added to fresh whole blood. Percent erythromycin found is indicated below.

Lane 1. Whole blood applied directly. 39 % (2 μl)
2. Ether extract at pH 8.5. 23 % (2 μl)
3. Residual blood (pH 8.5). 70 % (2 μl)
4. Ether extract (pH 9.5). 20 % (2 μl)
5. Blood after extraction (pH 9.5). 60 % (20 μl)

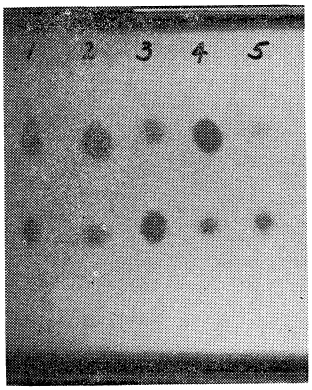


Fig. 5. One part erythromycin and two of propionyl erythromycin in serum. Percent erythromycin is indicated below.

Lane 1. Serum before extraction. 32 % (2 μl)
2. Ether extract (1). 29 % (2 μl) pH 8.5
3. Ether extract (2). 34 % (2 μl) pH 8.5
4. Residual serum (1). (20 μl)
5. Residual serum (2). (20 μl)

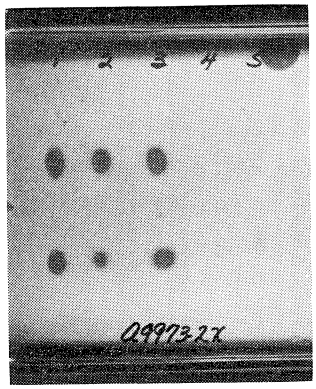
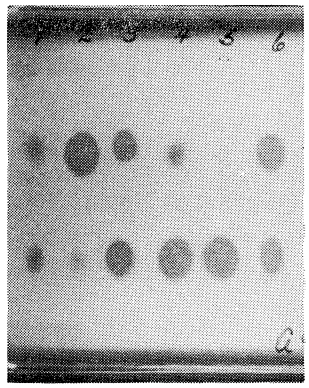


Fig. 6. Kinetic study in pH 7.4 phosphate buffer.

Propionyl erythromycin was added to pH 7.4 phosphate buffer and kept at 37°C for the time indicated then frozen quickly until completion of the experiment.

Lane 1. Standards 0.001 μg each
2. 0 hour. 5 % E
3. 1/2 hour. 60 % E
4. 1 hour
5. 2 hours. >95% E
6. Standards at 0.003 μg each



Good separation of the components in saliva occurred only if the methanol development was utilized. Urine samples may be run with the aqueous system alone, but in the experiment reported both systems were used because the urine was spotted on the same tape with the blood samples.

Extraction Studies

A triple ether extraction of whole blood to which a mixture of erythromycin and propionyl erythromycin had been added resulted in incomplete removal of the antibiotic from the blood sample (Fig. 4). The pH was raised to 8.5~9.5 between the first and second extractions. The residual activity was predominantly erythromycin while the extract contained a higher proportion of ester than the original blood. It appeared also that at the higher pH (9.5) some antibiotic activity was destroyed.

Fig. 7. Kinetic study in whole blood.

Propionyl erythromycin was added to whole fresh blood and held for the times indicated at 25 and 37°C. The room temperature samples were chromatographed immediately on paper strips, while the 37°C samples were frozen overnight and then chromatographed together.

25°C (chromatographed immediately)		37°C (frozen overnight)	
1. 0 minute	10 % E	6. 0 hour	10 % E
2. 15 minutes	10 % E	7. 1 hour	30 % E
3. 30 minutes	12 % E	8. 2 hours	40 % E
4. 60 minutes	15 % E	9. 4 hours	50 % E
5. 120 minutes	23 % E	10. 6 hours	55 % E

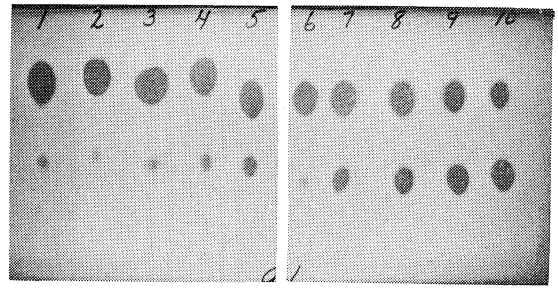


Table 1. Propionyl erythromycin in serum

	Propionyl erythromycin (%)			
Added	80	70	60	50
Found (Ave.)	74	67	59	49
Corrected	77.5	69	60	49

When serum was extracted (Fig. 5) by the same method essentially all of the activity was removed.

Kinetic Studies

When propionyl erythromycin was dissolved in pH 7.4 phosphate buffer, there was a rapid conversion to the unesterified antibiotic, and after two hours at 37°C the hydrolysis was essentially complete (Fig. 6).

When propionyl erythromycin was added directly to whole blood (Fig. 7) and chromatographed immediately, or frozen overnight and then chromatographed, about 10 % of the activity appeared as erythromycin. After two hours at 25°C about 25 % base was measured, while at 37°C about 40 % of the antibiotic had hydrolyzed to erythromycin. After six hours at 37°C the hydrolysis in whole blood appeared to be at about the same point as that in pH 7.4 buffer after one-half hour. Thus the overall hydrolysis rate in blood was much slower than that in pH 7.4 buffer. The rate of hydrolysis, which was at least 15 % per hour in the first 2 hours at 37°C, slowed to less than 3 % per hour in the 5th and 6th hours.

Fig. 8. Kinetic study in serum.

Serum drawn from a subject two hours after the fifth dose of erythromycin estolate and held for the times indicated.

23℃	37℃
1. 0 hour	6. 0 hour
2. 2 hours	7. 2 hours
3. 4 hours	8. 4 hours
4. 6 hours	9. 6 hours
5. 8 hours	10. 8 hours

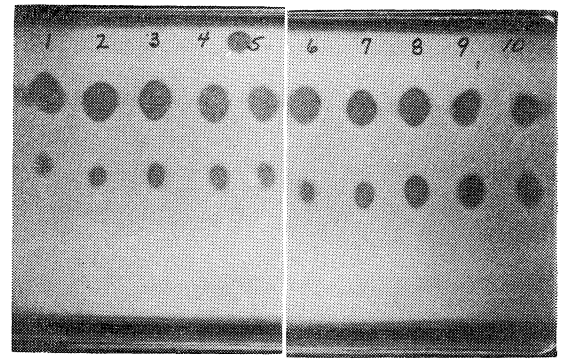
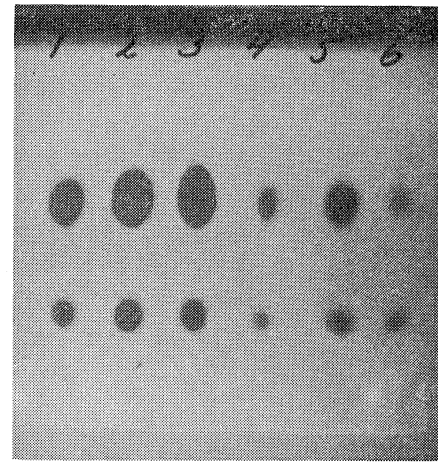


Fig. 9. Bioautograph from body fluids two hours after the fifth dose of erythromycin estolate. Whole blood (lane 1), serum (lane 2), plasma (lane 3) and urine (lane 5) all contained about 1/4 erythromycin and 3/4 ester. The cell fraction (lane 4) contained about 1/3 and the saliva about 1/2 erythromycin.

1. Whole blood 3 μl	4. Cell fraction 3 μl
2. Serum 3 μl	5. Urine 0.06 μl
3. Plasma 3 μl	6. Saliva 15 μl



The results of a similar study with serum from an individual who had taken erythromycin estolate two hours before are shown in Fig. 8. Hydrolysis occurred more rapidly at 37°C than at 23°C. Again the rate was much slower than in phosphate buffer. There was little change in the first two hours at either temperature and over the 8-hour period the rate of hydrolysis was about 1 % per hour at 23°C and 3 % per hour at 37°C. The rapid initial hydrolysis observed in the *in vitro* whole blood experiment (Fig. 7, lanes 1, 2, and 3) was not observed in this or other experiments in which the ester entered the blood *in vivo*. The hydrolytic rate was, however, similar to that in the *in vitro* studies two hours after the antibiotic was added to the blood.

There was no evidence of increased hydrolysis when the assay results from fresh

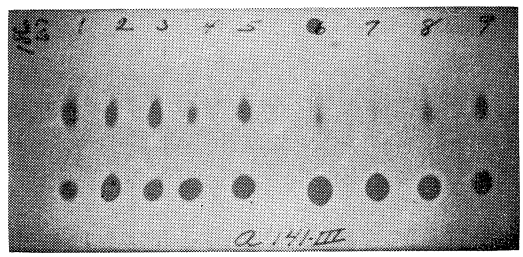
samples were compared with those from the same samples after overnight freezing or even after two months at −20°C. Hydrolysis was more rapid in the diluted blood samples; therefore, such samples were chromatographed as quickly as possible after dilution.

Whole blood, serum, plasma, cellular fraction, urine, and saliva from one subject were collected 2 hours after the fifth dose. Fig. 9 is a picture of the bioautograph of these samples. In this study the whole blood, serum, plasma, and urine contained about 20~25 % erythromycin and 75~80 % propionyl erythromycin. The cellular fraction contained about one-third erythromycin, suggesting that the antibiotic base was adsorbed preferentially by the cells or was formed on the cellular surface. While for this subject the urine ratio was similar to that of blood, we have found that this is not always true, due perhaps, to pH variations, *etc.* Saliva levels were low (usually about 10 % of the whole blood level) but were 50 % or more erythromycin. In some subjects only erythromycin appeared in the saliva and in general, the saliva contained a greater percentage of erythromycin than the blood.

Fig. 10. Bioautograph from whole bloods to which propionyl erythromycin was added. Samples were then held at 37°C for three hours. Lanes 1, 5 and 9 were from bloods of three different humans.

Hydrolysis of propionyl erythromycin in whole blood of 7 different species during 3 hours at 37°C.

Lane 1. Human
2. Dog
3. Mouse
4. Rat
5. Human
6. Horse
7. Rabbit
8. Monkey
9. Human



Propionyl erythromycin was added to whole blood from a number of animal species and from three human subjects. The samples were kept at 37°C for three hours, frozen quickly, and kept cold prior to spotting on the chromatographic tapes. The rate of hydrolysis varied markedly among species (Fig. 10). The most rapid hydrolysis appeared in rabbit blood. There was a less rapid rate in horse, monkey, rat, dog, mouse and human bloods.

There was some variation in the hydrolytic rate in the bloods from different individuals but not as much as between species. Variability in the ratio of erythromycin to propionyl erythromycin also was seen in the bloods from individuals who had taken erythromycin estolate. Bloods from several groups of individuals drawn at intervals of one-half to 14 hours after the last antibiotic was administered showed only a slight increase in the percentage of erythromycin in the later samples. Overall about 20~35 % of the total antibiotic present was erythromycin and 65~80 % was propionyl erythromycin.

To evaluate the accuracy of the method for determining relative amounts of erythromycin and propionyl erythromycin in serum, samples containing varying amounts of the two components were analyzed. The experiments were run on two successive days and the amounts were determined by two individuals. The results are summarized in Table 1. No significant day-to-day or analyst-to-analyst variation was observed.

The correction factor used was determined from the amount of hydrolysis (9 %) observed when propionyl erythromycin alone was added to serum. When a 1:1 ratio was added, the sample and standard were identical, and the correction at that point was then zero. As the percentage of ester increased, a proportionately greater correction factor would apply. Since this correction is to compensate for the rapid initial hydrolysis when propionyl erythromycin is added to blood *in vitro*, its use would not be warranted in assaying *in vivo* samples.

Discussion

The addition of propionyl erythromycin to whole blood results in a rapid, but limited, hydrolysis of the ester, followed by a significant slowing of the hydrolytic process. When the ester is introduced into the blood *in vivo*, this initial hydrolysis takes place in the body and such blood or serum shows only a very slow, steady increase in erythromycin content on standing at 25°C or below. This results in a relatively constant base/ester ratio throughout the time the antibiotic is in the blood. Since the ratio of erythromycin to propionyl erythromycin remains relatively constant, the rate of the continuing hydrolytic process must approximate that of metabolism and/or excretion of the base.

The blood is primarily a transport system. Antibiotic present in the blood in the form of ester still has ample opportunity to be hydrolyzed to the free base in the interstitial fluid. The presence of bacterial cells may also have a profound effect on the hydrolytic rate. Nowhere in the body, except in the gastro-intestinal tract, would one expect the percentage of base to be lower than in the blood itself. Since only about 4 % (for the estolate) of the total antibiotic is eliminated in the urine[1], this is not a major avenue for loss of the ester before it is hydrolyzed. The erythromycin proportion in the saliva is higher than in the blood and in some individuals appeared to be 100 %. The total antibiotic concentration in saliva was approximately 10 % of the level in blood.

Summary

1. Blood, or other body fluids, containing erythromycin or its esters can be examined chromatographically for its antibiotic components by the described procedure.

2. With a normal dosage regimen only very small samples are needed, since 1~5 μl portions usually contain sufficient activity for this test.

3. In blood, propionyl erythromycin hydrolyzed much slower than in buffer solution at the same pH. The rapid initial hydrolysis *in vitro* decreases significantly with time to about 3 % per hour in the 5 th and 6 th hours. A similar slow hydrolytic rate was observed in serum from subjects who had taken erythromycin estolate orally.

4. When blood samples, drawn from one-half to 14 hours after the fifth and last dose of erythromycin estolate, were examined about 20~35 % erythromycin and 65~80 % propionyl erythromycin were found.

Literature Cited

1) Lee, C. C.; R. C. Anderson & V. C. Stephens: Further pharmacological studies on propionyl erythromycin ester lauryl sulfate. Antibiot. & Chemoth. 11 : 110~117, 1961