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Note

Bioautography of erythromycin and its esters

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Erythromycin is poorly soluble in water, has a persistent bitter taste and is readily inactivated in strong acid media such as found in the stomach[1,2]. As such it is therefore unsuitable for oral administration. Enteric coated or buffered preparations of erythromycin gave inconsistent or unreliable blood levels[3]. To overcome this problem more acid-stable and more palatable chemical derivatives of erythromycin have been prepared. The most effective derivatives are the stearate salt of erythromycin and the sodium lauryl sulphate salt of its propionyl ester, more commonly called erythromycin estolate.

After oral administration the stearate dissociates and is absorbed, primarily from the duodenum, as the free erythromycin base. The estolate is absorbed as the propionate[4], which is known to be inactive[5], and must be hydrolysed *in vivo* to release the active base. There have been numerous reports in the literature of erythromycin estolate giving higher and more prolonged blood levels than an equivalent dose of the stearate[6,7] yet, therapeutically, there appears to be little significant difference between the two forms. Although, according to standard microbiological assay, the estolate appears to produce higher and more effective blood levels than the base or stearate, this cannot immediately be assumed for the following reasons:

(i) The estolate is adsorbed in the inactive propionyl ester form, which must be hydrolysed *in vivo* to release the base, but the rate of hydrolysis of the ester in man is not known[8].

(ii) After ingestion of the estolate the percentage of the active base and inactive ester forms cannot be determined by the current standard assay methods, since the ester is hydrolysed during the analytical procedure. Subsequently total erythromycin activity is measured, giving unrealistic, high readings for the microbiological activity of the test sample.

In order to overcome these problems, it is necessary that the assay method can quantitatively distinguish the base and the ester. Neither chemical nor spectrophotometric means can successfully be used to distinguish the two forms at low concentration. Thin-layer (TLC) and paper chromatography have been successfully used to separate erythromycin from its derivatives qualitatively[9,10,11].

In 1969 Stephens *et al.*[12] used a two-step paper chromatographic technique to separate propionyl erythromycin and erythromycin from each other in blood

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samples. They assayed the relative amounts of each form of the antibiotic from bioautographs by comparing zone sizes with those of a set of reference standards. Attempts to reproduce this work were unsuccessful as the chromatography paper caused the appearance of large blank areas in the bioautograph, thus occluding any zones of inhibition which might have been produced by the antibiotics. Preliminary washing of the paper with solvent or sodium thiosulphate failed to eradicate the problem. Other attempts to reproduce this work have similarly been unsuccessful[13].

Both Richard *et al.*[14] and Radecka *et al.*[15] have used TLC to separate, and densitometry to assay, the antibiotics.

In an attempt to increase the sensitivity of the assay to usual plasma concentrations of the antibiotic, the TLC separation technique, similar to the method described by Richard *et al.* and Radecka *et al.*, has been used in conjunction with bioautography.

EXPERIMENTAL

Preparation of plates

Precoated silica gel G plates (Merck; aluminium backed), 20 × 20 cm, 0.25 mm thick, were washed overnight in methanol–0.02 *N* aqueous sodium acetate solution (120:30). Further spraying of the plates with 0.02 *N* aqueous sodium acetate solution after washing considerably improved the separation of the two forms of erythromycin. The plates were then dried in a hot air oven at 60° for 20 min and allowed to cool before spotting with the test solution.

Chromatographic procedure

Using S.G.E. microsyringes the plates were streaked with 10-μl samples of the antibiotics in phosphate buffer (pH 7.4) (U.S.P.) across $\frac{1}{2}$-cm lines perpendicular to the direction of solvent flow. This improved the range of the assay over the normal spotting procedure as it allowed better diffusion of the antibiotics on the plate and created larger zones of inhibition on the subsequent bioautographs.

The plates were developed to a height of 16 cm in the solvent system, methanol–0.02 *N* aqueous sodium acetate (4:1), and were then removed from the tank and air dried.

Preparation of the test solutions, the spotting procedures and development of the plates were all carried out at 4° to avoid the rapid hydrolysis of the estolate which occurs in aqueous solution at room temperature. These precautions are not necessary for methanolic test solutions of the antibiotic.

Assay procedure

After development the plates were placed on a large microbiological assay plate. A 2.7-mm layer of molten B.B.L. base agar antibiotic medium 2 with added tryptone and glucose, which had been adjusted to pH 8.4 before sterilization and seeded with *Sarcina lutea* ATCC 9341[16], was then poured gently over the surface. After the gel had set the assay plates were incubated overnight at 37°. Under these conditions the microbiologically "inactive" estolate hydrolyses to the "active" base, and visible zones of inhibition develop in the seeded agar directly above the antibiotic spots on the chromatogram. The TLC plate and adjacent agar were then re-

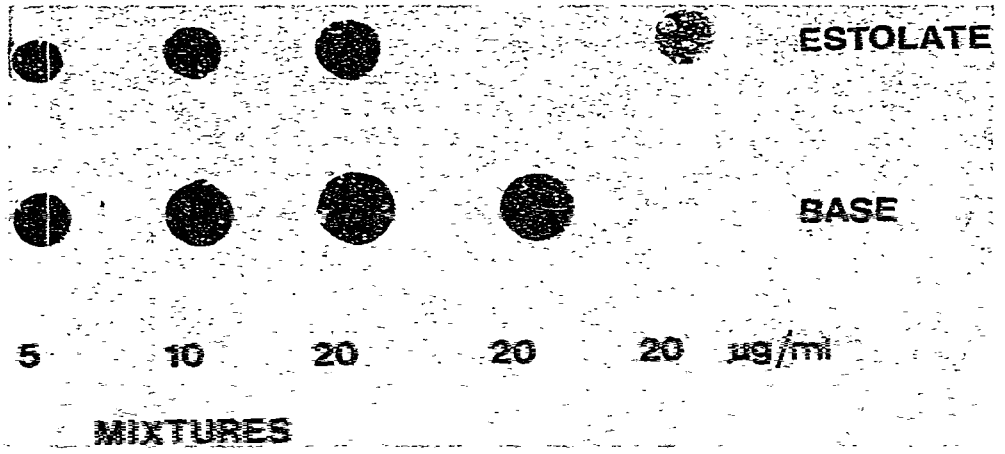

Fig. 1. Bioautograph of erythromycin base and erythromycin estolate, separately and in mixtures of various concentrations.

moved from the assay plate and placed face down on a sheet of glass. The plate was then peeled off and any remaining silica gel removed. Fig. 1 shows a direct contact print of the preparation at this stage with single zones of inhibition for base and estolate alone and two zones of inhibition, corresponding to the base and estolate components, for the various mixtures. The estolate does not hydrolyse during the chromatography procedure and, as can be seen, the two forms separate completely.

The areas of the zones of inhibition were measured viewing the glass plate through a projector (magnification 12) and tracing the images of the inhibited areas on the screen.

RESULTS AND DISCUSSION

There is a linear relationship between the logarithm of concentration and the square root of the area of the zone of inhibition in the range 3–20 µg/ml for the

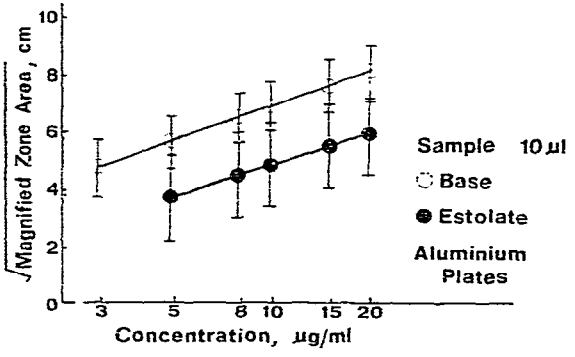

Fig. 2. Results of analytical procedure using aluminium-backed TLC plates. Sample size, 10 µl. ○, Base; ●, estolate.

TABLE I

A SUMMARY OF SOME OF THE DISCRIMINATORY ASSAY PROCEDURES FOR ERYTHROMYCIN AND ITS ESTERS

PC = paper chromatography; TLC = thin-layer chromatography; Al. = aluminium-backed plates; Pl. = plastic-backed plates.

Reference	*Chromatographic procedure*	*Detection method*	*Sensitivity (μg)*
12	PC	Bioautography	0.0005 (Base and ester)
14	TLC	Densitometry	20.0 (Base, stearate and esters)
15	TLC	Densitometry	5.0 (Base and estolate)
Current work	TLC (Al.)	Bioautography	0.03 (Base, stearate) 0.05 (Estolate)
	TLC (Pl.)	Bioautography	0.015 (Base, stearate) 0.025 (Estolate)

erythromycin base and 5–20 μg/ml for the estolate. The upper concentration limit is determined by the low solubility of the estolate in aqueous solutions.

Fig. 2 shows the pooled results (with 95% confidence limits) from a number of aluminium-backed TLC plates. The concentrations correspond to 0.03–0.2 μg and 0.05–0.2 μg of base and estolate, respectively, in the sample spots, which are within the range of normal plasma concentrations of these antibiotics. The R_F value of the base and stearate was 0.177 and that of the estolate 0.436.

The sensitivity of this assay can be greatly improved by using Eastman plastic-backed silica gel TLC plates following the same procedure. By using these plates 0.015 μg of the base and 0.025 μg of the estolate can be detected. Further work has been hindered by the presence of an antibacterial agent present in later batches of these plates which causes inhibition of growth of the *Sarcina lutea*. Attempts to remove or trace this substance have been unsuccessful and it has not been possible to use these plastic-backed plates for routine assay work.

Table I shows some of the results of other workers and compares the sensitivities obtained with that of the current method.

This method can be used to assess the percentage base (*i.e.*, true antibacterial activity) in plasma following administration of erythromycin estolate and to determine the rate of hydrolysis of the estolate *in vivo*. It may be used in conjunction with protein binding studies to determine the degree of plasma protein binding of the two forms. The assay appears to be useful for estimating various other erythrocymin esters such as the ethyl succinate or ethyl carbonate forms.

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