Assay method for biologically active ethambutol in body fluid by agar diffusion method using saprophytic mycobacterium as test organism

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Agar diffusion method *e.g.* cylinder-plate method has been widely used as the standard procedure for the assay in the antibiotic field, because of its simplicity and accuracy.

MIYAMURA *et al.*[1,2] reported the thin-agar cylinder-plate method as the assay procedure of antibiotics in body fluid, which was more sensitive than usual cylinder-plate method.

Authors[3~7] reported the thin-agar cylinder-plate techniques for the assay of biologically active levels of synthetic chemotherapeutics, *i.e.* sulfonamides, nitrofurans, isoniazid and ethionamide, in body fluids.

THOMAS *et al.*[8] reported an agar diffusion method for the assay of ethambutol* using *M. smegmatis* 607 as test organism, which was useful to determine the peak concentration in serum after drug administration.

In the present paper authors intend to describe the application of thin-agar cylinder-plate method to the assay of ethambutol in body fluids using ethambutol high sensitive mycobacterium.

EXPERIMENTAL MATERIALS AND METHODS

Test organism : A saprophytic, non-chromogenic mycobacterium H-7, which had been isolated from soil as the test organism for isoniazid.

Culture media : KIRCHNER's agar medium containing 10% serum or albumin and 1.5% agas was used for the assay plate. Usual nutrient agar medium with purified agar eliminated fatty acid was also useful.

Usual nutrient broth with 0.05% Tween 80 or DUBOS' albumin liquid medium was used for the inoculum of the test organism.

Preparation of samples : Normal plasma containing ethambutol** in the following concentration, 8, 4, 2, 1, 0.5, 0 μg/ml were prepared as standard for testing of plasma taken after drug administration.

Preparation of the inoculum of the test organism : Usual nutrient broth with Tween 80 or DUBOS' medium was inoculated with *M.* H-7 and incubated for approximately 24 hours at 37°C.

Preparation of the assay plate : KIRCHNER's agar medium melted and kept at 50°C was inoculated with 0.2% broth culture of *M.* H-7. Petri-dishes (inside diameter 90 mm) with plain bottoms were placed on a level surface and 5 ml of the inoculated medium was distributed evenly and allowed to harden. If dishes of other sizes were used, amount of medium in one plate was altered to provide thin layer of the inoculated agar medium.

Assay procedure : Cylinders were placed on the inoculated agar and filled with standard samples and test ones. The plates were incubated at 37°C for approximately 48 hours. Then diameter of each inhibition zone was measured and averaged.

Estimation of concentration : On semi-log paper, the diameter of the inhibition zone was plotted against the log of ethambutol concentration and the standard curve representing the relation between the drug concentration and the diameter of inhibition zone was drawn. Then the concentration of the test sample was estimated by comparison with standard curve.

EXPERIMENTAL RESULTS

Comparison between different kinds of mycobacteria as test organism : Effect of different kinds of mycobacteria *i. e. M.* 607, *M. grass*, [*M. phlei*, and

* Ethambutol is the generic name reserved for the dextrorotary form of 2, 2-(ethylendiimino)-di-1-butanol by Lederle Laboratories Division of American Cyanamid Company[8~10].

** Ethambutol was supplied by Kaken Chemical Company.

Fig. 1 Effect of different kinds of mycobacteria as test organisms on diameter of inhibition zone

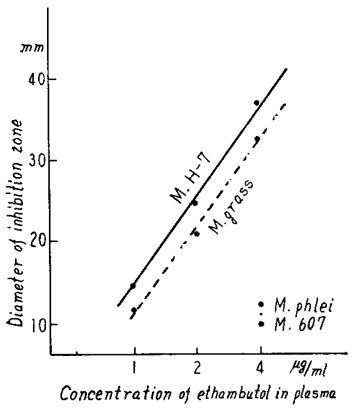
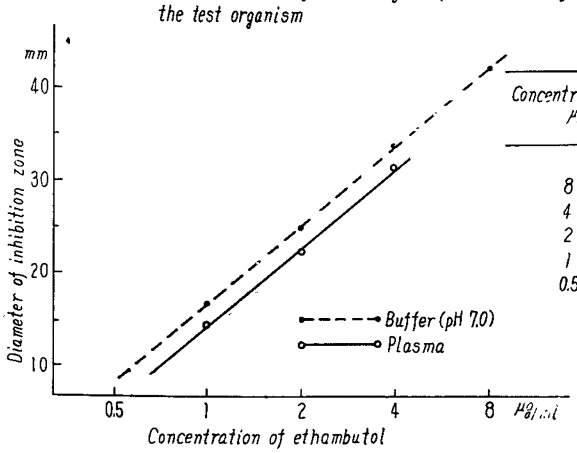


Fig. 2 Relation between the concentration of ethambutol and the inhibition zones by the thin agar cup method using M.H-7 as the test organism



Concentration μg/ml	Diameter of inhibition zone mm	
	in Plasma	in Buffer
8		41.2 ± 2.1
4	32.0 ± 1.8	33.1 ± 0.9
2	22.8 ± 0.9	24.4 ± 1.9
1	14.3 ± 2.4	16.3 ± 2.5
0.5	—	—

Average of 6 plates

M. H-7, on the diameter of inhibition zone were tested. *M.* H-7, growing clear zone, was most sensitive to the drug, *M.* 607, however, was less sensitive, as shown in Fig. 1.

Relation between the diameter of inhibition zone and the concentration of ethambutol : The relation between the diameter of inhibition zone and the logarithmic concentration was linear in the range from 1.0 to 8.0 μg/ml, as shown in Fig. 2.

Consequently, the concentration under test was calculated from the formula :

$$\log \theta = \frac{\sum U - \sum Sh}{\sum Sh - \sum Sl} \log A$$

where, θ was the ratio of highandand test dose, A was the ratio of high and low dose, Sh, Sl and U were the diameters of standard high, standard low and test samples, respectively. And the lower limit of the concentration measure was approximately 0.6 μg/ml.

Absorption and excretion of ethambutol . After single oral administration of 500 mg of ethambutol, plasma and urine levels were determined in 4 adults. As shown in Fig. 3 and Fig. 4 the drug showed active level in blood for approximately 8 hours period and was excreted in urine, in highly active level.

Influence of organ homogenates on the activity of ethambutol : Inactivation rate of the agent in organ homogenates *i.e.* lung, liver, and kidney of mouse were tested by using above mentioned assay method. No deterioration was observed in its activity in 2 hours at 37°C, as shown in Fig. 5.

Fig. 3 Blood level after oral administration of 500 mg ethambutol in 4 adults

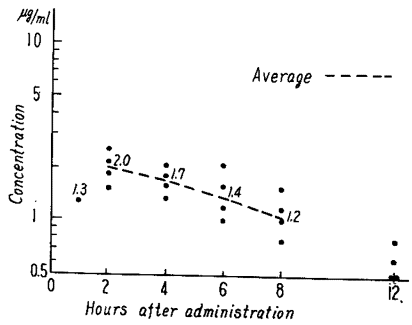
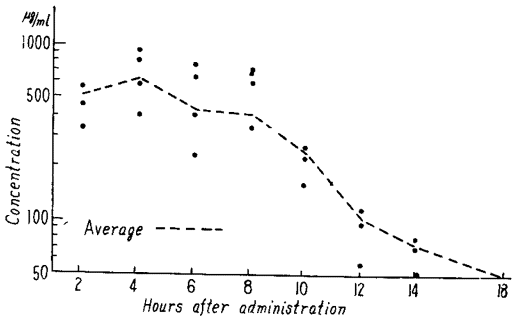


Fig. 4 Urinary level and urinary recovery after oral administration of 500 mg ethambutol in 4 adults



Urinary recovery in 24 hours

Case no.	mg	%	mean
1	360	72	
2	268	53	
3	299	60	65%
4	370	74	

Distribution of ethambutol in organs : Concentration of ethambutol in organs of mice following oral administration by intubation was determined. The data in Fig. 6 provided that the drug developed ac-

Fig. 5 Inactivation rate of ethambutol in organ homogenate of mouse

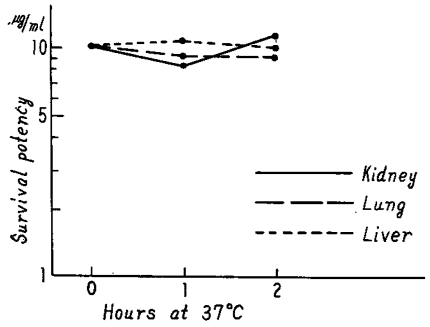
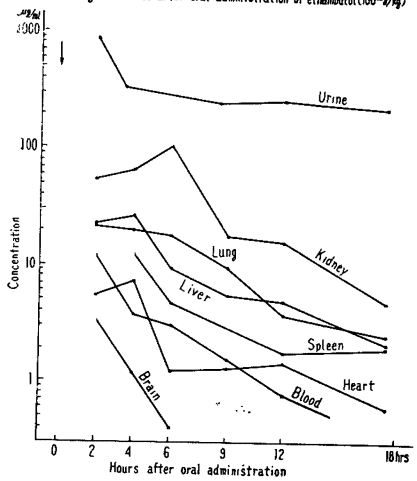


Fig 6 Biologically active concentration in body fluids and organs of mice after oral administration of ethambutol (100 mg/kg)



tive levels in kidney, liver and lung and then was excreted mainly in urine in active form.

Active level of ethambutol in resected lung tissue : Microbiologically active concentration in the lung tissues resected just after over 3 days' administration of ethambutol 1,000 mg per day eliminating other chemotherapy, was measured in 4 tuberdulous patients. Active levels were observed in the tissue of normal lung and cavity wall, but no trace in a caesous mass, as shown in Table 1.

DISCUSSION

The rapidly growing acid-fast bacillus *M.* H-7, highly sensitive to ethambutol and having some properties in common with human tubercle bacillus appeared to be the suitable test organism for assaying ethambutol, a new antituberculous chemotherapeutic.

From the results obtained by using the above mentioned assay method, it was indicated that

Table 1 Microbiologically active levels in terms of ethambutol concentration (μg/ml) in the resected lung tissues following over 3 days oral administration of ethambutol 1,000 mg per day in 4 adults.

Case No.	Cavity wall	Caseous mass	Normal lung
1	4.0, 2.4		5.2
2	1.1, 1.8	<0.6	1.2
3	2.0		2.5
4	3.3, 1.9		2.3

ethambutol was absorbed orally and distributed in organs and excreted in urine in active form. And the agent was assumed to be of effective in the treatment of lung, kidney and urinary tract tuberculosis, because it developed significant microbiologically active levels, especially in lung, kidney and urine.

SUMMARY

1) Microbiologically active ethambutol was assayed to the lower limit of 0.6 μg/ml in plasma using mycobacteria *M.* H-7 as the test organism.

2) After oral administration, ethambutol developed active blood level for several hours period and distributed in organ tissues including lung and kidney then excreted mainly in urine in biologically active form.

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