ASSAY METHOD FOR BIOLOGICALLY ACTIVE ISONIAZID IN BODY FLUID BY AGAR DIFFUSION METHOD USING SAPROPHITIC MYCOBACTERIA AS TEST ORGANISM

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Biologically active isoniazid (INH) levels in plasma have been assayed by the tube dilution method[1] or by the vertical diffusion method[2,3] using human tubercle bacillus as the test organism. The cylinder plate method has been widely used as the standard procedure for the assay in the antibiotic field, because of its simplicity and accuracy. UMEZAWA *et al*[4] reported using this method with mycobacteria 607 as the test organism. This only assays INH in the ranges over 8 μg/ml and was thus useless for clinical purposes.

In the present paper, the authors intend to report their studies[6,7,8,9] of the assay method for biologically active INH in body fluids by the thin agar cylinder plate method[5] using rapidly growing, INH highly sensitive acid-fast bacterium as test organism.

Experimental Material :

Test organism : A saprophytic, non-chromogenic mycobacteria H-7 isolated from soil by the authors was used.

Culture media : KIRCHNER's agar medium containing 10% serum or albumin and 1.5% agar was used for the assay plate. The usual nutrient broth with 0.05% Tween 80 or DUBOS's oleic-acid albumin medium was used for the inoculum of the test organism.

Experimental Method .

Preparation of samples : Normal plasma containing INH in the following concentrations 8., 4., 2., 1., 0.5, 0.25, 0.125, 0.0625, 0 μg/ml were prepared as standards for testing of plasma taken after administration of INH.

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

Preparation of the plates : 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, the amount of the medium used is altered to provide the thin layer of agar.

Assy procedure : Cylinders were placed on the inoculated agar plates and filled with standard samples and test ones.

The plates were incubated at 37°C for approximately 48 hours.

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

Experimental Results :

The relation between the diameter of inhibition zone and the logarithmic concentration was linear in the range from 0.125 to 8.0 μg/ml, as shown in Table 1 and Fig. 1.

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

$$\text{Log}\,\theta = \frac{U-Sh}{Sh-SL}\,\text{Log}\,A$$

where θ was the ratio of high and test dose, A was the ratio of high and low dose and *Sh*, *Sl* and *U* were the diameters of standard high, standard low and test samples, respectively.

Table 1. Relation between concentration of INH and diameter of inhibition zones in plasma

Concentration of INH in plasma (μg/ml)	Diameter of inhibition zone (mm) Mean ± Fiducial limit
8	58.94±1.68
4	52.66±0.33
2	44.31±0.57
1	36.97±1.20
0.5	30.19±1.10
0.25	23.34±1.46
0.125	14.5±0.73
0.1	11.7
0.0625	—

$\alpha=0.01$, $n=8$

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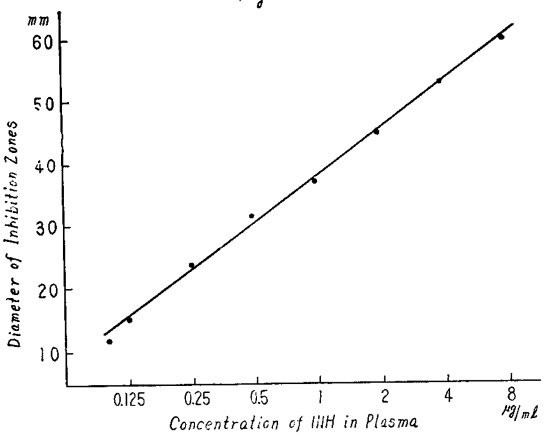
Fig 1



Experimental Error of the Assay Method :

From the results obtained by using 8 plates shown in Table 2, the couple of 4. and 1. μg/ml or the couple of 0.5 and 0.125 μg/ml were considered as standard and 2. or 0.25 μg/ml were calculated as test samples by means of the above mentioned formula, as shown in Table 3.

The mean (m) and reliable range were

$$M \pm u\sqrt{F\ {}^{N_1=1}_{N_2=7/8}(0.01)}$$

where, u was the sample standard deviation and n, the number of plates and F, the variance ratio.

And the probable maximum range (σ) of standard deviation was calculated as follows :

$$\frac{\sigma^2}{U^2} = F\ {}^{N_1=\infty}_{N_2=7}(0.01)$$

Then the maximum range (Mr) of the standard deviation of the mean obtained from the experiment

Table 2. Relation between concentration of INH in plasma and diameter of inhibition zone

Plate	Concentration of INH						
	8 μg/ml	4 μg/ml	2 μg/ml	1 μg/ml	0.5 μg/ml	0.25 μg/ml	0.125 μg/ml
1	60	52.75	45	37	31	24.5	14
2	59	52	44.25	38	31	21.5	14.25
3	61	52.75	43.75	34.75	28.5	25.5	15.5
4	60	52.75	44.5	36	29.5	22.75	15.25
5	55.5	52.5	44	37	30.5	25	14.75
6	58.5	53	44	38.75	31	23	14.75
7	58	53	43.75	37.25	31.25	22	13.5
8	59.5	52.5	45.25	37	28.75	22.5	14
Mean	58.94	52.66	44.31	36.97	30.18	23.34	14.5

Table 3. Concentration calculated from each plate

Plate	*μg/ml	**μg/ml
1	2.354	0.2527
2	1.822	0.2318
3	2.908	0.2499
4	2.070	0.2526
5	2.464	0.2338
6	2.017	0.2083
7	1.942	0.2216
8	2.183	0.2615
Mean	2.220	0.2390

*Concetration calculated from diameter, 2 μg/ml as the Test and 4, 1 μg/ml as the standard.

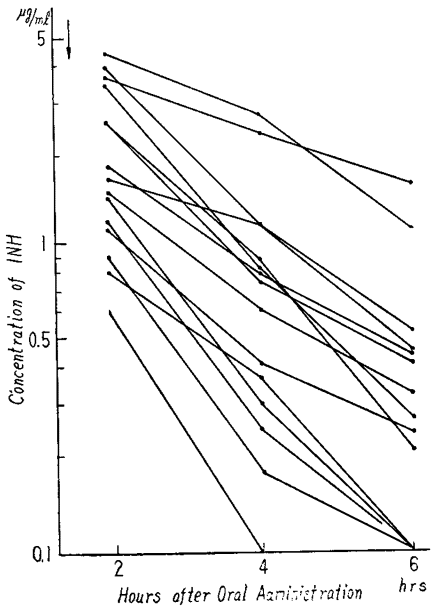
**Concentration calculated from diameter, 0.25 μg/ml as the Test and 0.5, 0.125 μg/ml as the standard.

Table 4. Maximum range of the standard deviation of the mean obtained from the experiments using 1~4 plates.

Number of plates used	* %	** %
1	120~83	146~69
2	114~88	133~78
3	112~90	127~82
4	110~92	123~84

* ** As described in Table 3

Fig. 2. Biologically active isoniazid level in serum after oral administration of Isoniazid 4 mg/kg in 15 adults



using n plates were calculated from the formula .

$$1 \pm \frac{\frac{\sigma^2}{\sqrt{N}}}{M \mp U\sqrt{F/N}} \gtrless Mr$$

as shown in Table 4.

Biologically active INH in plasma .

After oral administration of INH, biologically active INH levels in plasmas were determined in 15 adults. As shown in Fig. 2, metabolic patterns of active INH were ascertained to differ individually as reported previously by many workers.

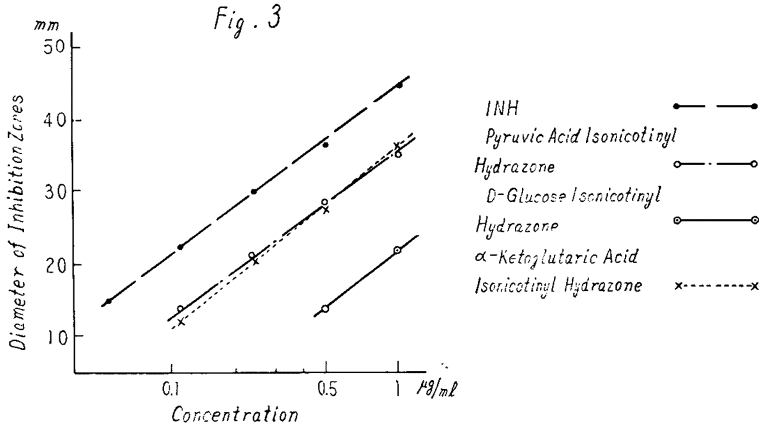
Antimicrobial Activity of Metabolic Substances of INH to the Test Organism :

Antimicrobial activity of metabolic substances of INH *e. g.* acetyl-INH, hydrazone types of INH and isonicotinic acid, to the M. H-7, were tested using the above mentioned agar diffusion method. As shown in Table 5 and Fig. 3, pyruvic acid isonicotiniyl hydrazone, α-ketoglutaric acid isonicotinyl hydrazone and D-glucose isonicotinyl hydrazone were approximately $\frac{1}{2}$, $\frac{1}{2}$ and 1/5 in antimicrobial activity in comparison with free-INH. Isonicotinyl acid and acetyl-INH, however, did not show any remarkable activity to M. H-7 strain.

Table 5. Relation between various kinds of metabolites of INH and the diameter of inhibition zone

Compounds	Concentration μg/ml in Aq					
	100	1	0.5	0.25	0.125	0.0625
		mm				
INH		42.5	36.25	30.0	22.25	14.5
Pyruvic Acid Isonicotinyl Hydrazone		35.25	28.25	21.0	14.0	0
D-Glucose Isonicotinyl Hydrazone		20.5	14.0	0	0	0
α-Ketoglutaric Acid Isonicotinyl Hydrazone		35.5	27.5	20.75	12.0	0
Isonicotinic Acid	0					
1-Isonicotinyl-2-Acetylhydrazine	0					

Fig. 3



Discussion

It seemed reasonable to complete the bioassay for INH as rapidly as possible, because the activity of free-INH might deteriorate to some extent in the medium with the lapse of time. The rapidly growing acid-fast bacillus M. H-7, highly sensitive to INH and having some properties in common with human tubercle bacillus appeared to be the most suitable test organism for assaying INH.

The cylinder-plate method is also applicable for the determination of the drug concentration in tissue or in body fluids *e. g.* urine or sputum, even though slightly contaminated.

The test organism M. H-7, being resistant to paraamino-salicylic acid, was eminently useful for the determination of active INH in the period of combined therapy with para-aminosalicylic acid. Moreover, a strain of streptomycin resistant M. H-7 obtained in the laboratory is available for the assay of active INH after streptomycin administration.

SUMMARY

(1) Biologically active INH was assayed to the lower limit of 0.125 μg/ml in plasma by the thin agar cylinder-plate method, in a two day period, using the saprophytic mycobacteria H-7 as the test organism.

(2) The test organism was more sensitive to free-INH than the metabolic hydrazone types of INH and resistant to acetyl-INH and isonicotinic acid.

(3) The experimental errors inherent in the method were also discussed.

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