N-[2-naphthyl] glycine hydrazide—A potent inhibitor of *Mycobacterium tuberculosis* $H_{37}R_v$

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Abstract

N-[2-naphthyl] glycine hydrazide (compound MC 1415) was found to completely inhibit the growth of *Mycobacterium tuberculosis* $H_{37}R_v$ at 1 μg/ml when tested in Youman's medium. The dihydrochloride salt of the compound was prepared and found to have the same level of biological activity as the parent compound in equimolar amount. In the presence of human serum in the medium the activity of both against *M. tuberculosis* is reduced ten-fold (there is partial inhibition of growth of the organism at 10 μg/ml). The LD_{50} of the dihydrochloride in mice by the intraperitoneal and oral routes was found to be 246·6 mg/kg and 593 mg/kg respectively.

Key words : N-[2-naphthyl] glycine hydrazide dihydrochloride, *Mycobacterium tuberculosis*, anti-tubercular activity, acute toxicity.

1. Introduction

Substitutions in the *para* position of primary arylamines have yielded compounds with notable inhibitory activity against tubercle bacillii in *in vitro* tests. However, their high toxicity has limited their use in *in vivo* studies and therapy[1–2].

A large number of new N-arylglycines especially those bearing an alkyl, alkyloxy or halogen substituent in the *para* position and their corresponding hydrazides have been synthesized by Buu-Hoi *et al.*[3]. Several N-arylglycines bearing a bulky *para* substituent (especially a higher alkyloxy group) were found to exhibit an *in vitro* tuberculostatic activity against *Mycobacterium tuberculosis* $H_{37}R_r$ at a concentration of 10 μg/ml when tested in Dubos culture medium. But their corresponding hydrazides were found to be inactive[3].

In the present report the preparation of the dihydrochloride of N-[2-naphthyl] glycine hydrazide (compound MC 1415) and the activity of both the parent compound and its salt against *M. tuberculosis* $H_{37}R_v$ *in vitro* and *in vivo* toxicity are described.*

2. Experimental

Melting points are uncorrected. The microanalytical values for carbon, hydrogen and nitrogen were within ± 0·4% of the theoretical value. UV spectrum was obtained in double distilled water on a 'Unicam' Sp 700 A spectrophotometer. The IR spectra were recorded on a CARL ZEISS Jena Model UR 10 spectrophotometer.

The NMR spectrum was recorded on a BRUCKER WH–270 spectrometer using TMS as internal standard.

2.1. *Preparation of N-[2-naphthyl] glycine hydrazide*

N–[2-naphthyl] glycine hydrazide was prepared by the procedure of Tien, Buu-Hoi and Yuong[3]. The product was recrystallised twice from ethanol.

m.p. 151–52° (reported m.p. 152°)[3]

UV : λ_{max}^{water} 242 nm (52,500)

IR : ν_{max}^{KBr} pellet 1654 cm^{-1} (C=O stretching)
1600 cm^{-1} (–N–H– bending of hydrazide)
3360 cm^{-1} (–N–H– aromatic stretching).

NMR (270 MHz) (DMSO–d_6):

9·185 δ (1H, s, –CONH–, disappears on D_2O exchange), 6·6–7·67 δ (7H, m, aromatic), 6·2 δ [1H, t (J = 6Hz), aromatic NH, disappears on D_2O exchange], 4·278 δ (2H, s, $-NH_2$, disappears on D_2O exchange) and 3·725 δ [2H, d (J = 6Hz), $-CH_2-$, appears as a singlet on D_2O exchange].

2.2. *Preparation of the dihydrochloride*

To an ice-cold suspension of the parent compound (6 g) in dry isopropanol (240 ml), dry hydrochloric acid gas was passed (~ 20 g) with occasional stirring and the mixture cooled at 4° C (20 h). At the end of the period, volatile materials were removed by flash evaporation. The residue was dissolved in about 275 ml of dry methanol. Ether

* Preliminary studies on this compound were carried out by Jogibukhtha, M., Iyer, B. H., Lalithakumari, H. L. K., Ramananda Rao, G. and Sirsi, M. (Jogibukhtha, M., *Ph.D. Thesis*, I.I.Sc., 1970, p. 99).

was added to precipitate the hydrochloride salt. The crystalline material thus obtained was filtered and washed with ether. The yield of the material was 7·9 g.

The nature of the hydrochloride salt was found to be dihydrochloride by titration. m.p. 203–204° (with decomposition)

IR : ν_{max}^{KBr} pellet 1740 cm^{-1} (strong C=O stretching)
1654 cm^{-1} (medium –N–H– berding of hydrazide).
Broad absorption between 3000 cm^{-1} and 2700 cm^{-1} (due to salts of primary amine).
1620 cm^{-1} (secondary amine salts, N–H– bending)
Increase in absorption frequencies of C=O and –N–H groups are due to protonation of $-NH_2$ group of hydrazide.

3. Materials and methods

3.1. *Preparation of solutions of test compounds*

(i) Compound 1415 (20 mg) was dissolved in 2·0 ml of ethylene glycol and sterilized by passing through Millipore filter (0·45 μ). 0·5 ml of this was serially diluted (10 fold) in Youman's medium[4] to give concentrations ranging from 1000 to 0·1 μg/ml.

(ii) Compound 1415.2 HCl (26·6 mg) was dissolved in 2·0 ml of double distilled water and sterilized by passing through Millipore filter (0·45 μ). 0·5 ml of this was serially diluted (10 fold) in Youman's medium[4] to give concentrations of the base, ranging from 1000 to 0·1 μg (base)/ml.

3.2. *Microorganism*

M. tuberculosis $H_{37}R_r$ culture was obtained from NCTC (England) and maintained by regular subculture on Petrik's solid medium[5], was used to study *in vitro* inhibitory activity of compounds 1415 and its dihydrochloride.

3.3. *Susceptibility testing method*

The tubes containing varying concentration of test compounds in Youman's media were inoculated with 14-days old culture of *M. tuberculosis* $H_{37}R_r$ grown on the same medium. Approximately equal inocula were floated on the surface of media containing test compounds. Controls containing only the Youman's medium and solvent (ethylene glycol) were also inoculated in the same way. All the tubes were then sealed with paraffin and incubated at 37° for three weeks.

The inhibitory action of the compound was assessed by comparing the growth of the bacillii in the experimental tubes with that in the controls. Readings were taken at the end of each week up to three weeks. The results of experiments carried out in triplicate agreed and are presented in Table I.

Table I

The *in vitro* antitubercular activities of compound 1415 on *Mycobacterium tuberculosis* $H_{37}R_v$

Concentration of compound 1415 (μg/ml)	Growth at the end of								
	7 days			14 days			21 days		
	A	B	C	A	B	C	A	B	C
1000	−	−	−	−	−	−	−	−	−
100	−	−	−	−	−	−	−	−	−
10	−	±	−	−	+	±	−	++	±
1	−	±	±	−	++	±	−	+++	±
0·1	±	+	+	++	+++	++	++	++++	++
Control	+	++	++	++	+++	+++	++++	++++	++++

A: Youman's medium.

B: Youman's medium with 8% human serum.

C: Youman's medium with 8% bovine serum.

− = No growth; ± = Slight growth.

+ to ++++ varying grades of growth.

3.4. *Effect of serum on* 1415

The effect of bovine and human sera (freshly prepared) on the activity of compound 1415 was studied at a level of 8% (v/v). The data on the antitubercular activity of the compounds in the presence of sera are presented in Table I.

3.5. *Toxicity of compound* 1415.2 *HCl*

(i) *Intraperitoneal route*:

The required concentrations of the dose levels tested, as given in Table II for the compound 1415.2 HCl, were made in saline and then millipore (0·45 μ) filtered. This sterile solution was administered intraperitoneally to male (Swiss inbred) mice weighing between 18 and 22 g. The animals in groups of 8 were treated with the compound at five different dose levels (K = 5) only once and then kept under observation for a period of two weeks. Saline was administered for the control group. The mortality rate is taken into consideration in assessing the acute toxic level (Table II).

(ii) *Oral route*:

The required concentration of the dose level tested as given in Table II for the compound 1415 . 2 HCl was made in distilled water. This solution was administered orally to male mice (starved overnight) weighing between 18 and 22 g. The animals in groups of 10 were treated with the compound only once and then kept under observation for a period of two weeks. Distilled water was administered to the control group. Mortality rate was taken into consideration in assessing the acute toxic level (Table II).

Table II

Acute toxicity of compound 1415 . 2 HCl in male mice

Dose (mg/kg)	Mice killed		% expected from graph (E)		O–E	Contribution to $(Chi)^2$
	No.	% (O)				
Route: Intraperitoneal						
200	0/8	0·7*	2		1·3	0·009
225	2/8	25	18·8		6·2	0·025
250	4/8	50	55·0	*N'*	5·0	0·010
275	7/8	87	84·0		3·0	0·007
300	8/8	98·85*	96·5		2·35	0·016
					Total	0·067
Route: Oral						
500	0/10	3·6*	11·3		7·7	0·059
525	2/10	20	20·0		0	0
550	4/10	40	30·0	*N'*	10·0	0·048
600	5/10	50	54·0		4·0	0·006
650	7/10	70	73·8		3·8	0·007
700	9/10	90	90·0		0	0
725	10/10	97·4*	92·0		5·4	0·040
					Total	0·160

* Reference (6).

3.6. *Statistical analysis*

Percentage mortality was plotted against dose on graph paper arranged logarithmically along the x-axis and probability units along the y-axis. Figs. 1 and 2 represent graphs for intraperitoneal and oral routes respectively. LD_{50}* was computed from the graph. It is necessary to see how the results differ from the expected result, E, obtained from the graph in order to calculate the fiducial limits of LD_{50} by the method of Litchfield and Wilcoxon[6]. A correction was made for results 0 and 100 per cent. The values marked with an asterisk obtained from a table relating the value, E, expected from the graph with the value likely to be obtained when the value is 0 or 100 per cent as done by Litchfield *et al.*[6]. $(Chi)^2$ for a single item was calculated by the method of Litchfield *et al.*[6].

4. Results

4.1. *Antitubercular properties of compound* 1415

Compound 1415 was found to be a potent inhibitor of *M. tuberculosis* $H_{37}R_v$ in Youman's medium (Table I). It showed complete inhibition at 1 μg/ml and partial inhibition at 0·1 μg/ml at the end of 21 days.

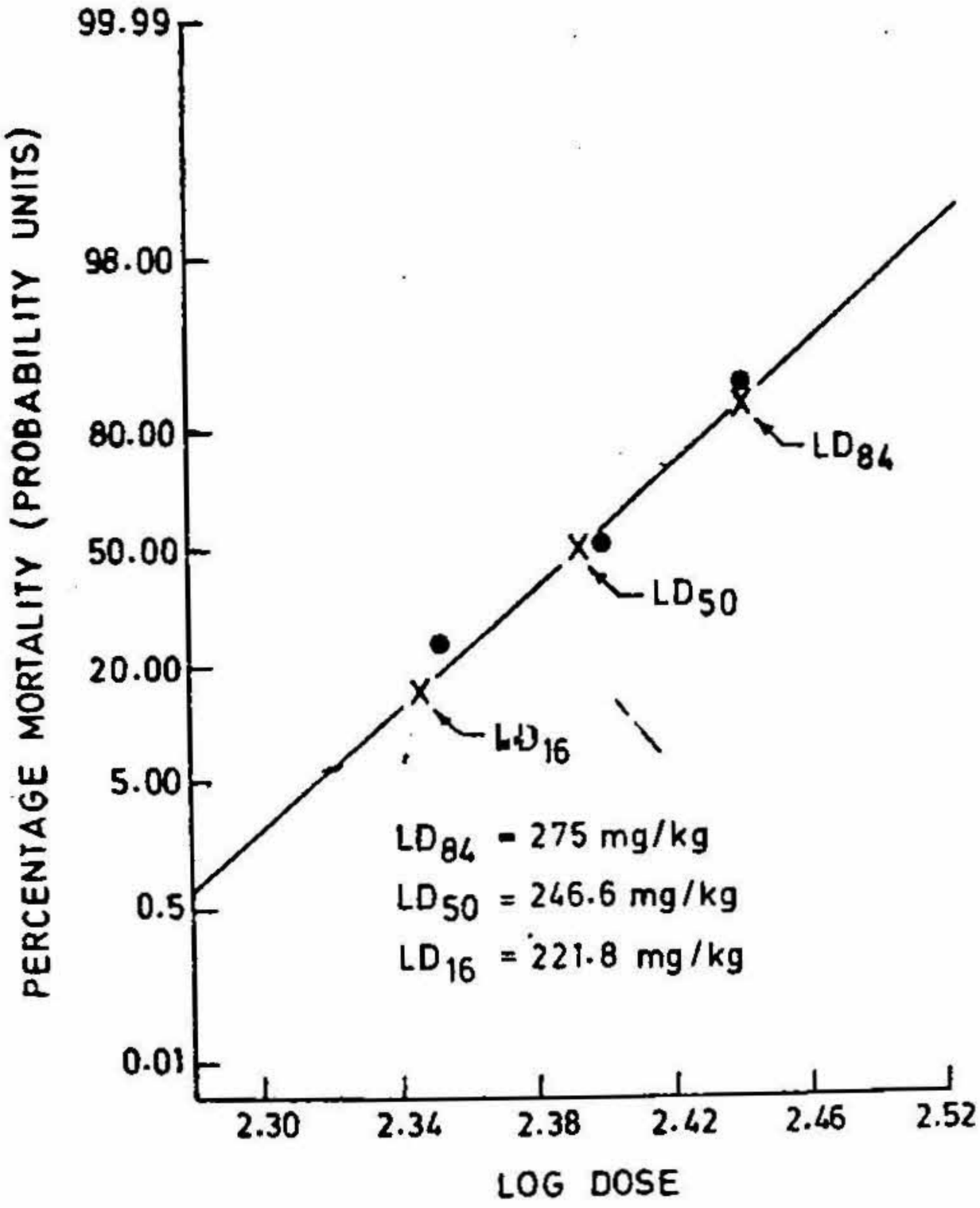

FIG. 1. Intraperitoneal toxicity; ● Percentage mortality; × Values derived from the graph.

*LD_{50}: Lethal Dose 50.

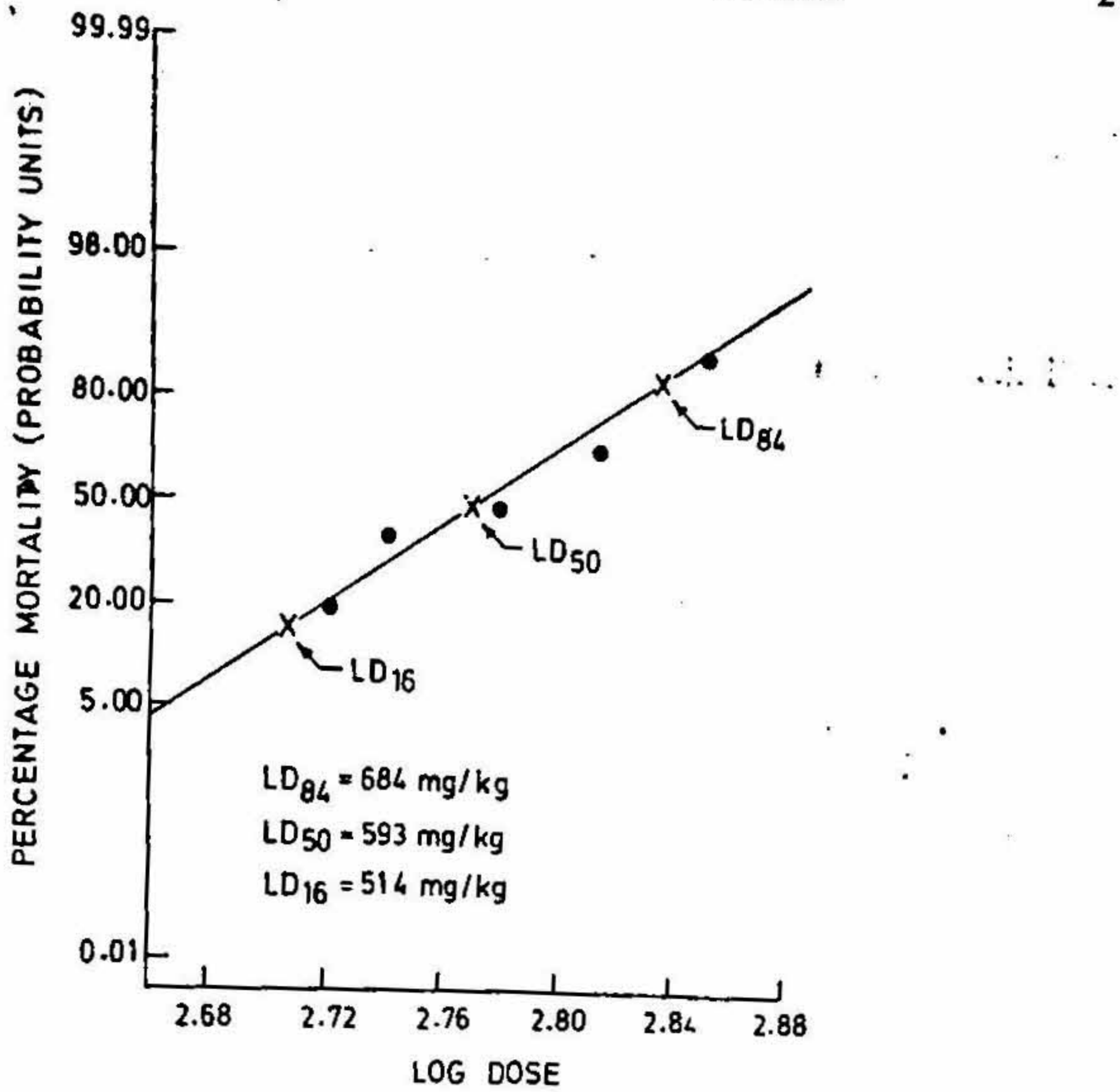

FIG. 2. Oral toxicity; ● Percentage mortality; × Values derived from the graph.

In the presence of human serum the results at the end of 21 days indicated that the compound 1415 is only partially active at 10 μg/ml. Readings at the end of 7 days indicated it to be a good inhibitor at 10 μg/ml and 1 μg/ml (Table I), but the effects lessen in subsequent weeks.

Compound 1415 shows more pronounced inhibitory action in the presence of bovine serum compared to human serum (Table I).

The results obtained for compound 1415.2HCl were identical with those obtained for compound 1415.

4.2. *Acute toxicity (LD_{50}) and statistical analysis*

(*i*) *Intraperitoneal route :*

From Fig. 1, we get LD_{50} as 246·6 mg/kg, LD_{16} as 221·8 mg/kg and LD_{84} as 275 mg/kg.

Slope function, S, is calculated.

$$S = \frac{LD_{84}/LD_{50} + LD_{50}/LD_{16}}{2} = 1{\cdot}11.$$

The number of animals tested for which the expected effect lies between 16 and 84, N', was next calculated (Table II) and $N' = 24$. The confidence limits of LD_{50} ($p = 0{\cdot}05$) are given by:

$$fLD_{50} = S^{\left(\frac{2{\cdot}77}{\sqrt{N'}}\right)} = 1{\cdot}11^{\left(\frac{2{\cdot}77}{\sqrt{24}}\right)} = 1{\cdot}11^{0{\cdot}57} = 1{\cdot}06.$$

The limits are therefore

$$246{\cdot}6 \times 1{\cdot}06 = 261{\cdot}4 \text{ mg/kg} \quad \text{and} \quad 246{\cdot}6 \div 1{\cdot}06 = 232{\cdot}3 \text{ mg/kg}.$$

The number of dose level tested, K, is equal to 5 and the degree of freedom, n, is equal to 3 ($n = K - 2$). Animals taken per dose is 8. Total $(Chi)^2$ is therefore $8 \times 0{\cdot}067 = 0{\cdot}536$ and $(Chi)^2$ from tables of Litchfield[6] for $n = 3$ is 7·82. At a level of probability $p = 0{\cdot}05$, for $n = 3$ gives a value of $(Chi)^2$ of 7·82, which is greater than the observed value, 0·536, so that results are not significantly heterogeneous and the line is a good fit.

(*ii*) *Oral route*

From Fig. 2, we get LD_{50} as 593 mg/kg, LD_{16} as 514 mg/kg and LD_{84} as 684 mg/kg. The confidence limits of LD_{50} ($p = 0{\cdot}05$) are 631 mg/kg and 557 mg/kg (confidence limits are calculated as above).

The number of dose levels tested, K, is equal to 7 and the degree of freedom, n, is equal to 5 ($n = K - 2$). Animals taken per dose is 10. Total $(Chi)^2$ is therefore $10 \times 0{\cdot}160 = 1{\cdot}6$ and $(Chi)^2$ from tables of Litchfield[6] for $n = 5$, is 11·1. At a level of probability $p = 0{\cdot}05$, for $n=5$ gives a value of $(Chi)^2$ of 11·1, which is greater than the observed value, 1·6, so the results are not significantly heterogeneous and the line is a good fit.

4.3. *Toxicity of isoniazid*

We have tested the toxicity of isoniazid in our laboratory mice (Swiss inbred). 50% mortality was observed at 150 mg/kg and 100% mortality at 200 mg/kg upon intraperitoneal administration of isoniazid. We have observed 63% mortality at 250 mg/kg and 100% mortality at 300 mg/kg upon oral treatment with isoniazid.

4.4. *Toxic signs observed*

During the study of acute toxicity of compound 1415.2 HCl other toxic signs were noted. Animals exhibited anorexia and malaise. They lost weight in the first three to four days after administration of the compound. All the above symptoms were more pronounced as the dosage of compound is increased. The surviving animals became completely normal four days after the administration of the compound. There were no epileptic convulsions observed unlike in the case of isoniazid administered animals.

5. Discussion

N-[2-naphthyl] glycine hydrazide was found to be a potent inhibitor of *M. tuberculosis* $H_{33}R_v$ in Youman's media. It is as good an inhibitor as isoniazid which was also screened for comparative studies. The compound shows good inhibitory activity in the presence of human serum at 1 μg/ml and 10 μg/ml levels at the end of the first week whereas at the end of the third week, the compound is totally inactive at the concentration of 1 μg/ml and partially active at 10 μg/ml. However, it is completely inhibitory at 100 μg/ml in the presence of human serum even at the end of the third week. The compound may be losing its inhibitory activity at lower concentrations probably due to binding to serum proteins. Identical results were obtained with N-[2-naphthyl] glycine hydrazide dihydrochloride.

LD_{50} of N-[2-naphthyl] glycine hydrazide dihydrochloride by intraperitoneal and oral routes is higher than that of isoniazid in mice. The acute toxicity of the compound is less than that of isoniazide. Further work on *in vivo* studies is in progress.

6. Acknowledgements

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