

CHAPTER-III

MATERIALS AND METHODS

## Outline of methods

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    - i) serum
    - ii) urine
  - b) tissues
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- C. Plasma protein binding study by Agarose-gel electrophoresis
- D. Preparation of microsomes with calcium
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### Details of methods

#### I. Biochemical methods

##### A. Estimation of rifampicin in -

- i) serum
- ii) biological fluids

##### a) Biochemical assay for serum rifampicin

#### Principle

The method of Shigeichi and Sunahara was followed. This method subserves extraction of rifampicin from the serum with isoamyl alcohol. The faint reddish-orange colour of the rifampicin-isoamyl alcoholic aliquot can be measured photometrically at wavelength 475 millimicrons.

#### Reagents

1. Isoamyl alcohol
2. Double distilled water
3. Phosphate buffer, M/15 (pH 7.0)

4. Stock standard solution of standard-reference rifampicin preparation: 100 mg of rifampicin per 50 ml of isoamyl alcohol (i.e. 0.5 ml/1000 ug).
5. Working standard solution of rifampicin.

Preparation of working reagents

- i) Phosphate buffer, M/15 (pH 7.)
  - a) Dissolve 9.073 gm of  $\text{KH}_2\text{PO}_4$  in, and make upto 1000 ml with distilled water,
  - b) Dissolve 11.866 gm of  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  and make upto 1000 ml with distilled water.

For use

Mix 38.9 ml of (a) and 61.1 ml of (b).

Adjust pH exactly to 7.0 (solution stable indefinitely on refrigeration).

- (ii) Working standard solution of rifampicin: To 0.5 ml of (4) i.e. stock standard solution, add 9.5 ml of (1), i.e. isoamyl alcohol.

Note

Total volume = 10 ml

10 ml = 1000 micrograms

Therefore, 1 ml = 100 micrograms

Stock and working standard solutions are unstable and fresh solutions were prepared every time. Results appeared to be satisfactory even when the solutions

were used 3 to 4 hours after preparation. This is due to the fact that rifampicin is auto-oxidisable in the presence of atmospheric oxygen. The stock of the working standard solutions was not stored in refrigerator for over a long period. The original colour was lost in due course of time.

#### Blood collection

Plain bulb (1.0 ml of serum is required).

#### Standard curve

It is to be plotted initially while working with fresh reagents and thereafter every 15 days.

TABLE-1

Reagents	Following volumes (in ml) were prepared into eleven test tubes labelled										
	0	1	2	3	4	5	6	7	8	9	10
Rifampicin concentration in micro-gram/ml	0	10	20	30	40	50	60	70	80	90	100
1. Isoamyl alcohol (ml) Reagent-1	3.0	2.9	2.8	2.7	2.6	2.5	2.4	2.3	2.2	2.1	2.0
2. Working standard solution of rifampicin (ml) Reagent-5	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
Total volume (ml)	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0

All the tightly capped tubes were mixed well on a vortex mixer/mechanical rotary mixer for one minute per tube, and allowed to stand at room temperature for five minutes. The absorbance (O.D.) of all the tubes (i.e. 1 to 10) was measured against tube labelled as "0" (zero) as blank, to set zero of the spectrophotometer at wavelength = 475 nm.

Procedure for serum rifampicin estimation

TABLE-2

Reagents	Pipetted into test tube as (B), (S) and (T)		
	Blank (ml)	Standard (ml)	Test (ml)
Distilled water	2.0	2.0	1.0
Phosphate buffer M/15 (pH 7.0)	1.0	1.0	1.0
Serum	-	-	1.0
Working standard solution (1 ml = 100 ug)	-	1.0	-
Isoamyl alcohol	3.0	2.0	3.0
Total volume (ml)	6.0	6.0	6.0

All the three tubes were centrifuged at 3000 rpm for 10 mins. The clear supernatant (viz. faint reddish-orange, rifampicin-isoamyl alcoholic aliquot) extract was removed.

The absorbance of the standard (S), and test (T) was read against blank (B) to set zero, at wavelength = 475 nm.

#### Calculation

$$\frac{\text{O.D. of the test}}{\text{O.D. of the standard}} \times 100$$

= concentration of rifampicin in ug/ml

#### Procedure for urine-rifampicin estimation:(I)(A) a-ii

1. For each pharmaceutical preparation of rifampicin, 24 hours accumulated urine sample was collected following oral administration of 450 mg of the antibiotic. (It should be noted that the urine samples of only those patients who were included under biochemical assay study, were collected).
2. The total 24 hours excreted urine volume was measured.
3. Rifampicin extraction

The procedure for rifampicin extraction from urine was similar to that for rifampicin extraction from serum, except that, 1 ml of diluted (1:5 dilution with distilled water) or undiluted urine was substituted for serum. Consequently, the estimated rifampicin concentration was expressed as mg/24 hours volume. Rifampicin in urine is almost completely extracted in the mixture solvent, consisting of an equal volume of benzene and hexane, while extraction of desacetyl



rifampicin in the solvent is nearly negligible. Concentration of desacetyl rifampicin was calculated as a difference in values between isoamyl alcohol extract (total) and benzene hexane extract (rifampicin).

4. Wavelength employed for urine-rifampicin estimation = 475 millimicrons.

Estimation of rifampicin in various tissues : (I)(A) b

Rats were stunned by giving a blow on the head and were sacrificed by decapitation. Various tissues (liver, lung, kidney, spleen, heart and skeletal muscle) were removed. Adhering muscle fibres and blood were cleaned. Tissues were blotted gently with filter paper after washing thoroughly in chilled 0.9% saline placed in a Petri-dish on ice. The weighed tissues were then cut finely into small pieces. Later, the tissues were individually homogenized in isoamyl alcohol using pestle and mortar and the filtrate from this was read at 475 nm against appropriate blanks on a Beckman UV speck spectrophotometer. Rifampicin concentrations in each tissue were expressed as ug/100 mg tissue.

Calculation

$$\frac{\text{O.D. of the test}}{\text{O.D. of the standard}} \times 100$$

= concentration of rifampicin in ug/ml

Tissue weight  $\longrightarrow$  0.5 gm was taken in 5 ml isoamyl alcohol = 100 mg/ml.

## B. Estimation of proteins

(a) Estimation of total serum proteins was done as per the routine procedure by the Biuret method (Varley, 1980).

### (b) Estimation of proteins in liver

I. Protein estimation: (Lowry et al., 1951)

Principle: Biuret reaction of protein with copper ions in alkaline conditions.

### Method

Aliquot of 0.05 ml of each of the diluted samples were taken for estimation. Exactly 3.0 ml of freshly prepared Lowry's reagent was added to all tubes. After 15 mins at room temperature, 0.5 ml of diluted Fohn's reagent (Lowry's E) was added. The contents of the tube were mixed well and the colour was allowed to develop in the next 30 mins. The blue colour obtained was read in spectrophotometer (Carl Zeiss, Jena) against a blank containing 0.05 ml 0.1 N NaOH, 3.0 ml Lowry's (and 0.05 ml Lowry's E reagent). Bovine serum albumin was used as a standard with different wavelength = 750 nm; range 10-100 p.

### Stability

The colour is stable for an hour after it develops. Different concentrations (20-120 mg) were used to prepare a standard graph.

C. Plasma protein-binding study by Agarose-gel electrophoresis: Determination of albumin and  $\alpha_2$  protein fraction concentration by Agarose-gel electrophoresis

Reagents

1. Barbitone-sodium barbitone buffer: pH 8.6, ionic strength 0.075

a) Barbitone : 2.76 gm

b) Sodium barbitone : 15.45 gm

Barbitone was dissolved in about 300 ml of boiling distilled water and sodium barbitone was dissolved in 400 ml of distilled water. Both were mixed and the final volume was made upto 1 litre. To this about 10 ml of isopropanol was added. The pH was adjusted to 8.6.

2. Agarose-gel, 1%

Agarose : 100 mg

Barbitone sodium- : 10 ml  
barbitone buffer

Agarose was dissolved by placing the mixture in boiling water bath.

3. Amido black-B stain for serum proteins: One percent solution in 10 ml of glacial acetic acid was made upto 100 ml with 90 percent methanol. This was filtered before use.
4. Fixing solution: 5% glacial acetic acid v/v.

5. Washing solution: 2% glacial acetic acid V/V.
6. Three glass slides or slabs of different sizes.
  - (a) 6 cms X 8 cms
  - (b) 6 cms X 15 cms
  - (c) 6 cms X 10 cms

### Procedure

#### I. Preparation of gel-slabs

- (a) Six ml of hot melted agarose-gel was poured on agarose gel on 6 cms X 8 cms glass slab and allowed to cool. The required number of such gel-slabs were prepared and used for electrophoresis of fasting serum sample.
- (b) Twelve ml of hot melted agarose solution was poured on 6 cms X 15 cms glass slab. This was allowed to solidify at room temperature. Four such gel-slabs were prepared and used for the in vivo electrophoresis experiment.
- (c) An even gel slurry was prepared by pouring 9 ml of hot melted agarose on 6 cms X 10 cms slab. Four slabs sufficed for each patient, for the in vitro experiment involving electrophoresis.

## II. Electrophoresis

### Case-1: (Fasting serum sample)

- (a) Concentration of serum charged - 20 micro-litres on 6 cm X 8 cm glass gel-slab
- (b) Time of run - 2 hours
- (c) Current - 25 mA

### Case-2: (4 and 24 hours serum samples for the in vivo experiment)

- (a) Concentration of serum charged - 0.1 ml on 6 cm X 15 cm gel-slab
- (b) Time of run - 8 hours (approximately)
- (c) Current - 25 mA

### Case-3: (4 and 24 hours serum samples for the in vitro experiment)

- (a) Concentration of serum charged - 20 micro-litres on 6 cm X 10 cm gel-slab
- (b) Time of run - 5-6 hours (approximately)
- (c) Current - 25 mA

## III. Fixation

Before staining all the gel-slabs with separated protein fractions, they were fixed in the fixing solution for 15 minutes.

#### IV. Staining

- (a) For case-1, the gel-slab was stained for 20 minutes in amido black-B.
- (b) For case-2, slab-1 of 4 hours and slab-1 of 24 hours were stained with the same serum protein stain for about  $\frac{1}{2}$ -1 hour.
- (c) For case-3, staining was carried out exactly in similar way as that described in case-1.

#### V. Washing

After staining, the slabs were first washed with plain water and then repeated washings were carried out with 2% acetic acid, till the background of the gel appeared to bear no tinge of stain. All the five stained serum protein fractions were rendered distinctly clear and ready for elution.

#### Method for elution of albumin and $\alpha_2$ protein fractions

- I. Elution was carried out soon after washing stage.
  - i) Albumin and  $\alpha_2$  bearing gel areas were dislocated and removed from those gel areas bearing  $\alpha_1$ , beta and gamma protein fractions with the help of gel-cutter.
  - ii) The "albumin-gel fraction" was placed into a suitable tube labelled (Alb).

The " $\alpha_2$ -gel fraction" was slid from the gel-slab into tube labelled ( $\alpha_2$ ).

For the blank, the "plain-gel" (which did not contain any protein or stain on its surface) was introduced into the tube labelled (B).

Further, treatment of the protein fractions with the chemicals was carried out as under -

TABLE-3

Reagents	Pipetted the following volumes (ml) into the three test tubes labelled -		
	B	T-Alb	T- $\alpha_2$
1. Protein fractions	Plain-gel	Albumin-gel	$\alpha_2$ -gel
2. N/100 NaOH	2.5	2.5	2.5
3. Glacial acetic acid	2.5	2.5	2.5

All the three tightly capped tubes were placed in the boiling water bath for merely 80 seconds. After cooling to room temperature, the absorbances of (T-Alb) and (T- $\alpha_2$ ) were used against blank (B) to set zero in the spectrophotometer at wavelength 630 millimicrons.

II. Similarly,  $\alpha_1$ , beta and gamma fractions were eluted and their respective absorbance was determined at 630 nm.

Calculation

- A. Optical density of total protein of the serum sample by the biuret method at 555 nm. gms/dl
- B. Therefore, total O.D. of all the five protein fractions by electrophoresis method of the same serum sample at 630 nm (i.e. O.D. of Alb + O.D. of  $\alpha_1$  + O.D. of  $\alpha_2$  + O.D. of beta + O.D. of gamma). gms/dl (same value of A)

Hence,

$$\begin{aligned}
 & \text{Concentration of albumin} \\
 & = \frac{(\text{O.D. of Alb}) \times (\text{gms/dl total protein})}{(\text{Total O.D. of all the five protein fractions})} \\
 & = \text{gms/dl Albumin}
 \end{aligned}$$

A similar calculation was likewise made for  $\alpha_2$  protein fraction.

Experimental technique for elucidation of relative-  
percentage binding of rifampicin to serum protein fractions

The experiment was carried out systematically as presented under the following steps -

Step-1

The fasting blood samples of the experimental subjects were withdrawn. They were administered 10 mg/kg rifampicin



orally. Blood samples were again withdrawn 4 hours and 24 hours following the oral administration.

#### Step-2

The serum total protein concentration of the fasting sample was determined by the biuret method.

#### Step-3

Twenty microlitres of serum of the fasting sample was subjected to electrophoresis and the concentrations of albumin and  $\alpha_2$  protein fractions were determined by gel elution method.

#### Step-4

The serum rifampicin concentration (g/ml) of the 4 and 24 hours serum samples respectively were determined by biochemical assay.

(Note: Steps-1a, 2, 3 and 4 are common for both, the in vivo and in vitro experiments)

#### Step-5

For the in vivo experiment

(a) Exactly 0.1 ml of serum on slab-1 and 0.1 ml of serum on slab-2, of the 4 hours blood samples were used for charging the electrophoresis. Electrophoresis was carried out for 8-10 hours approximately at the current strength of

order of 25 milliamperes for both the slabs. Slab-1 was stained with amido black-B.

(b) Both the slabs were aligned exactly in a line, such that, the charging point on slab-2 was in line with the charging point on slab-1. If this alignment parameter was imposed accurately, then consequently, the locations of the five stained protein fractions on slab-1 thus corresponded to the locations of the five unstained protein fractions on slab-2.

(c) After assuming this step with the help of gel cutter, the albumin and  $\alpha_2$  protein fractions from slab-2 were detached. The remainder portion of the gel was stained with amido black-B and after washing, it was confirmed whether only  $\alpha_1$ , beta and gamma fractions were retained on the gel or not.

(d) The concentrations of albumin and  $\alpha_2$  fractions were determined from the stained slab-1 by gel elution technique.

(N.B.: Concentrations of albumin and  $\alpha_2$  fractions determined in this step and in step-3 should be similar or identical).

(e) The detached albumin and  $\alpha_2$  protein fractions from slab-2 were then further treated as under -

TABLE-4

Reagents	Pipetted the following volumes (ml) into the four test tubes labelled -			
	Blank	Standard	Test (Alb)	Test (alpha <sub>2</sub> )
Distilled water	2.0	2.0	2.0	2.0
Phosphate buffer (pH 7, M/15)	2.0	2.0	2.0	2.0
Working standard rifampicin solution (1 ml 100 ug)	-	1.0	-	-
Protein fractions	-	-	Alb	Alpha <sub>2</sub>
Isoamyl alcohol	3.0	2.0	3.0	3.0

All the four tubes were covered with aluminium foil and test (Alb) and test (alpha<sub>2</sub>) tubes only, were placed in the boiling water bath for merely 80 seconds. Boiling dissolved agarose. Rifampicin, thus set free, was extracted by isoamyl alcohol. The isoamyl alcohol-rifampicin extracts of test (Alb) and test (alpha<sub>2</sub>) were then decanted into another corresponding test (Alb) and test (alpha<sub>2</sub>) labelled tubes. The aliquots were allowed to cool to room temperature.

Finally, the absorbances of standard, test (Alb) and test (alpha<sub>2</sub>) were read against blank to set zero at 475 nm.

Initially, concentrations were reported as -

ug of rifampicin per 0.1 ml of serum.

Final concentrations were reported as -

1. Micrograms of rifampicin bound to albumin fraction present in 100 ml of serum at 4 hours in vivo
2. Micrograms of rifampicin bound to  $\alpha_2$  protein fraction present in 100 ml of serum at 4 hours in vivo.

(f) For the blood samples collected at 24 hours, a similar procedure was followed for the in vivo determination of percentage binding of the antibiotic to albumin and  $\alpha_2$  protein fractions.

Final concentrations were reported -

1. Micrograms of rifampicin bound to albumin fraction present in 100 ml of serum at 24 hours in vivo.
2. Micrograms of rifampicin bound to  $\alpha$ -protein fraction present in 100 ml of serum at 24 hours in vivo.

#### Step-6

For the in vitro experiment

(a) To 2 ml of fasting serum sample in serological tube:  $V_1$ , 10 mg of rifampicin was added and mixed. This tube was placed in the incubator for 4 hours at  $37^\circ\text{C}$ .

(b) 10 mg of rifampicin was added to another 2 ml of fasting serum sample contained in serological tube:  $V_2$ . After mixing, incubation was done for 24 hours at  $37^{\circ}\text{C}$ .

(c) After 4 hours of incubation, tube  $V_1$  was removed from the incubator and this was centrifuged at 5000 rpm to obtain 4 hours serum-rifampicin incubated sample. The supernatant (serum-rifampicin aliquot) was decanted into another clean, dry serological tube labelled:  $t_1$ .

(d) Exactly 20 microlitres of the serum-rifampicin aliquot was charged in  $t_1$  on slab-1; and 20 microlitres of the same on slab-2. Electrophoresis was carried out for 5 hours approximately at 25 milliamperes, for both slabs.

At this stage, a faint yellowish-orange band was observed in the albumin region on both the slabs. Slab-1 was stained with amido black-B and further experiment was done according to the procedure given in step-5 (b,c,d,e).

Final concentrations were reported as -

1. Micrograms of rifampicin bound to albumin fraction present in 100 ml of serum at 4 hours in vitro.
2. No  $\alpha_2$  protein fraction is involved with the binding of rifampicin at 4 hours in vitro.

(e) After 24 hours of incubation, serological tube  $V_2$  was removed from the incubator, centrifuged at 5000 rpm and the supernatant was decanted into another serological tube labelled  $t_2$ .

(f) Step-6 (d) was followed. In this step, after electrophoresis, two faint yellowish-orange bands were observed - one in the albumin region and the other in the  $\alpha_2$  region.

(g) Step-5 was followed (b,c,d,e).

Final concentrations were reported as -

1. Micrograms of rifampicin bound to albumin fraction present in 100 ml of serum at 24 hours in vitro.
2. Micrograms of rifampicin bound to  $\alpha_2$  protein fraction present in 100 ml of serum at 24 hours in vitro.

#### Step-7

Percentage binding of rifampicin were calculated as follows -

$$\frac{\text{Micrograms of rifampicin bound to albumin/dl of serum}}{\text{+ Micrograms of rifampicin bound to } \alpha_2 \text{/dl of serum}} = 100\%$$

Therefore, micrograms of rifampicin bound to  
albumin/dl of serum = ? %.

= Micrograms of rifampicin bound to albumin/dl of  
serum X 100

-----  
Micrograms of rifampicin bound to albumin/dl of serum  
+ Micrograms of rifampicin bound to  $\alpha_2$ /dl serum  
= percentage rifampicin bound to albumin fraction.  
Similarly, the percentage value of rifampicin bound  
to  $\alpha_2$  fraction was calculated.

#### D. Preparation of microsomes with calcium

This method is better than the other conventional  
methods as can be seen below -

TABLE-5

Method	Disadvantage
1. Differential ultracentrifugation (Hogeboom et al., 1948; Schnerder et al., 1950).	Expensive and time consuming
2. Acid precipitation (Karker et al., 1968)	Inactivates a number of microsomal enzyme namely, cytochrome P-450, glucose-6-phosphatase, NADH cytochrome b <sub>5</sub> reductase, NADPH cytochrome <sup>5</sup> with reductase.
3. Gel filtration (Tangen et al., 1973)	Need for several columns, gel material, a fraction collector and time consuming

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In recent years, a method involving aggregation of  
microsomes with calcium, as one of the procedures for

microsomal isolation has been extensively studied (Kamath et al., 1971; Schenkman et al., 1972; Kamath et al., 1972; Unti et al., 1972; Kupfer et al., 1972).

Table-6 shows a list of enzymic activities and other microsomal membrane constituents that are unaffected by the  $\text{Ca}^{2+}$  aggregation method.

TABLE-6

Rat liver microsomal enzyme activities and microsomal membrane constituents shown to be unaffected by  $\text{Ca}^{2+}$  aggregation

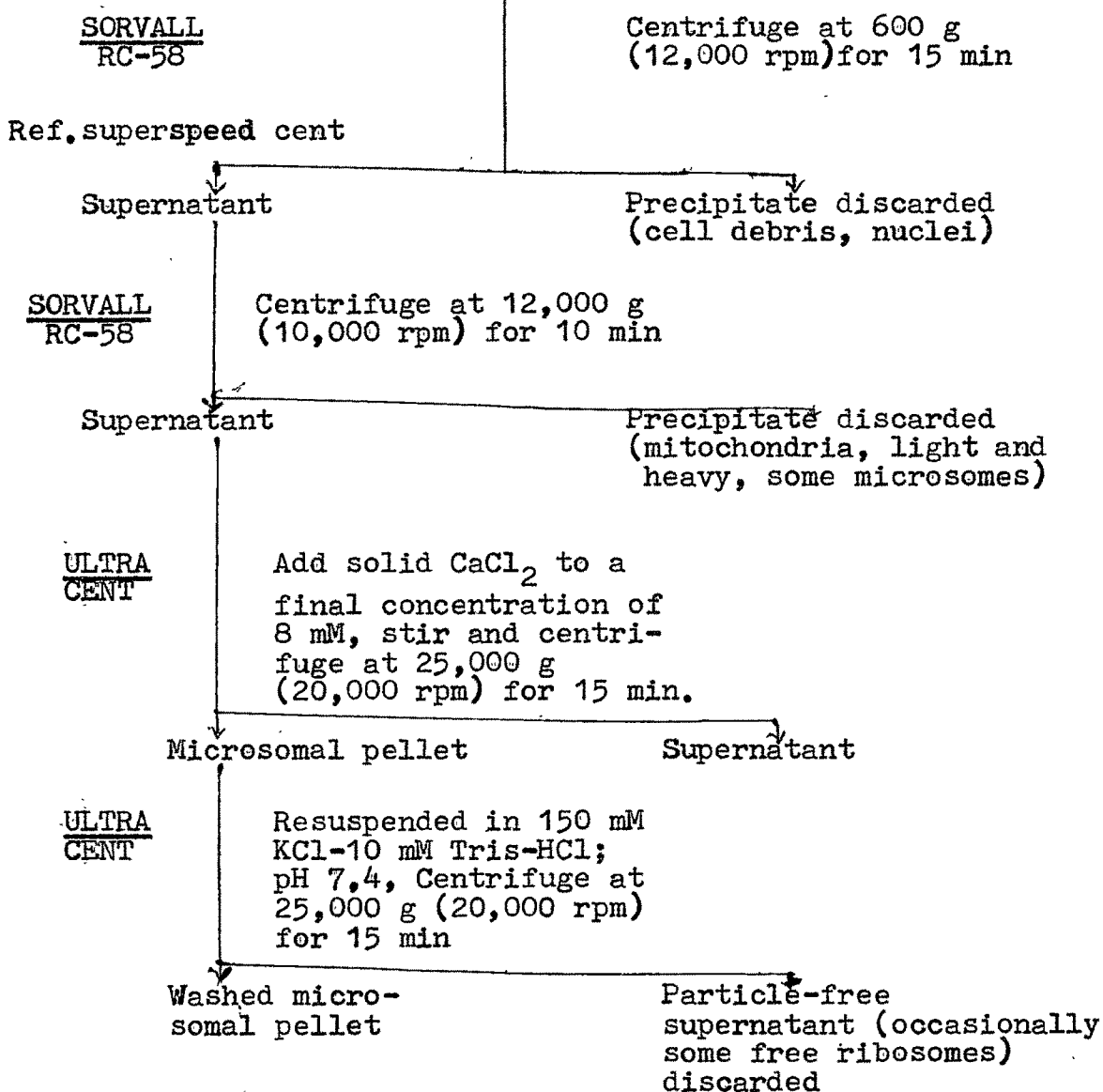
	Percent conventional activity	Reference No.
Glucose-6-phosphatase	97	85
Inosine diphosphatase	96	85
5'-nucleotidase	92	86
$\text{Mg}^{2+}$ - $\text{Na}^+$ $\text{K}^+$ -dependent ATPase	98	85
Cytochrome $\text{b}_5$	101	32
NADH-cytochrome $\text{b}_5$ reductase	100	32
NADH-cytochrome C reductase	102	32
Cytochrome P-450	107	32
NADPH cytochrome C reductase	100	32
NADPH cytochrome P-450 reductase	99	151
Aminopysine demethylase	96	85
Cholesterol	103	85
R N A	95	100
Tetrahydrocannabinol hydrolase	145	85
Total lipids	93	85
Phospholipids	101	85



Fig.1: Flow diagram of procedure for the calcium sedimentation of microsomes

(All steps are carried out A 0°-4°)

LIVER HOMOGENATE (with/without 0.5% Triton X-100)  
(10-20% in 250 mM sucrose - 10 mM Tris-HCl, pH 7.4)



### E. Estimation of cytochrome P-450

#### Principle:

Cytochrome P-450 is identified by the formation of a carbon monoxide (CO) complex of the reduced pigment with a maximum absorption peak at 450 mμ. The P-450 content in microsomes or purified preparation is assayed by the spectrophotometric measurement of the reduced CO complex (Omura et al., 1964; Vander Hoeve et al., 1974). The concentration of the cytochrome can be calculated from the CO difference spectrum of the dithionite reduced dilutions in 0.1 M potassium phosphate, pH 7.0 assuming an average extinction coefficient of ( $\text{mM}^{-1}: 450 \text{ m}\mu - 490 \text{ m}\mu$ ) =  $80 \text{ mM}^{-1} \text{ Cm}^{-1}$  for crude fractions and  $91 \text{ m}\mu^{-1} \text{ Cm}^{-1}$  for pure fractions, between 450 mμ and 490 mμ (Lu A.V.H. et al., 1976).

#### Method: Assay

Two ml of microsomal preparation containing 1 mg protein/ml, of 0.1 M phosphate buffer, pH 7.0 (1:100 dilution of original microsomal preparation) were placed in both the sample and reference tubes. 0.5 ml of 20% glycerol was added to both the tubes and CO was carefully bubbled through the solution placed in sample tube only for 1 minute (i.e. the reference tube was not exposed to CO) - this was sufficient to saturate the sample with the gas (Fig.2). Reduction of samples in both the tubes was effected with a few

mg of solid sodium dithionite ( $\text{Na}_2\text{S}_2\text{O}_4$ ). Cytochrome P-450 was assayed by the electrophotometric measurement of the reduced CO complex. The difference spectrum was recorded and the concentration of the cytochrome was calculated using an extinction coefficient of  $91 \text{ mM}^{-1} \text{ Cm}^{-1}$  for the absorbance at 450 nm minus the absorbance at 490 nm.

#### Assay system

TABLE-7

Reagent	Concen- tration	Concen- tration in assay system	Volume taken	
			Experi- mental	Control
1. 1:100 dilution of the sample with 0.1 M $\text{PO}_4$ buffer, pH 7.0	-	-	2.0 ml	2.0 ml
2. 20% glycerol	60%	20%	1.0 ml	1.0 ml
3. Sodium dithionite		1 mg (solid in both the tubes		
4. Carbon monoxide		Bubbled for 1 min.		
Total volume			3.0 ml	3.0 ml

Temperature: Room temperature ( $30^\circ\text{C}$ )

Wavelength : 450 nm and 490 nm

Stability : Stable on ice in the presence of glycerol but rapidly inactivated on standing otherwise.

The reaction was initiated by passing carbon monoxide through the sample and effecting its reduction by adding a few grains of sodium dithionite.

### Estimation of cytochrome $b_5$

The fresh microsomal preparation was diluted 1:100 in 0.1 M phosphate buffer, pH 7.0. Cytochrome  $b_5$  was determined from the difference spectrum between NADH reduced and air saturated microsomes since addition of an aliquot of an NADH solution to the contents of the sample tube results in the rapid reduction of cytochrome  $b_5$  characterized by an increase in absorbance at about 556 nm and 426 nm, and a loss of absorbance at about 409 nm. Therefore, by applying the millimolar difference extinction coefficient of 21 for the absorbance change at 556 minus 575 nm of 185 for the absorbance change at 426 minus 409 nm, the concentration of cytochrome  $b_5$  can be calculated.

### Assay system

TABLE-8

Reagent	Concen- tration	Concen- tration in assay system	Volume taken	
			Experi- mental	Control
1. Sample diluted 1:10 with 0.1 M phosphate buffer, pH 7.0	-	-	2.5 ml	2.5 ml
2. NADH	0.6 mM	0.1 mM	0.5 ml	-
3. GDW			-	0.5 ml
Total volume			3.0 ml	3.0 ml

Reaction is started by addition of 0.6 mM NADH to the experimental tube.

Temperature : Room temperature (30°C)

Wavelength : 426 nm and 409 nm

Stability : Cytochrome  $b_5$  is stable at room temperature for days and can be stored indefinitely at -20°C.

### Animal experiments

#### II. Experiments in rats

##### A. In vitro absorption study

Albino rats belonging to the Charles Foster strain bred and maintained in the departmental rat colony, were used for the investigation.

The pups were fed on stock diet (Table-9) for 21 days. The mothers were provided with food and water ad libitum. The pups had access to the maternal diet from about the 16th day after birth. After 21 days, only those rats, weighing more than 35 gm were chosen for the further procedure. The selected rats were divided into two groups. Post-weaning protein deficiency was induced in one group (test) by feeding them 7% casein diet while the other group (control) was fed a 22% casein diet. The rats were provided with the semi-purified diet (Table-10) and water ad libitum. The

weight gain of both the groups was noted at intervals of 7 days.

At 17 weeks, the two rat models were taken for the in vitro experiments. Rats were sacrificed by decapitation and part of small intestine 10 cm away from pyloric end of the stomach was taken. Adhering mesentery was removed and intestinal loops (3 cm long) were weighed.

Each loop was tied at one end. From the other end rifampicin solution (0.1 ml of 1 mg/ml solution) was filled inside the loop. The end of this loop was also closed. The prepared loops were placed in test tubes filled with Ringer solution (5 ml each) and placed in water bath, with temperature maintained at 37°C.

The test tubes were removed from the water bath at the following time points -

- 15 min
- 30 min
- 60 min
- 90 min
- 120 min
- 240 min

The collected Ringer solution (5 ml) was used to estimate rifampicin concentration to determine the rate of transport from intestine in undernourished and normal rats.

Preparation of standard solution

0.1 ml solution of rifampicin

+ 4.9 ml PSS

+ 3.0 ml amyl alcohol

---

8.0 ml mix  $\xrightarrow{2 \text{ min}}$  Centrifuge  $\xrightarrow{2 \text{ min}}$  supernatant \_\_\_\_\_  
 $\xrightarrow{475 \text{ nm}}$   $\xrightarrow{\text{read}}$  O.D.

Three ml isoamyl alcohol was added in each solution for rifampicin extraction. After centrifuging for 2 min, supernatant was taken and read at 475 nm on the spectrophotometer.

In each experiment, the standard reading was taken and calculation was done comparing it with the standard reading obtained on that day.

II.(B) Development of undernourished rat model

Animal models were developed according to the type of undernourished subjects which we had included in this study.

Undernourished models: Undernourished models were grouped according to the type and duration of drug treatment

- i) Rifampicin alone    - single dose
- multiple dose
- ii) Rifampicin + INH   - single dose
- multiple dose

Care was taken to ensure that all experiments conformed to the standards established in the "Guide for the Care and

Use of Laboratory Animals" (1978) (Institute of Laboratory Animal Researches, National Research Council).

Albino rats of either sex belonging to Charles Foster strain, bred and maintained in the rat colony (Department of Biochemistry, M.S. University of Baroda) were used. Newborn rats were reared by mothers fed on stock diet upto 21 days of age. The pups had access to the maternal diet from about the 16th day after birth (Table-9).

At this stage, rats were divided into two groups. Post-weaning protein deficiency was induced in the test group by feeding them a semi-purified diet containing 7% protein (low protein diet), while the control group was fed a semi-purified diet containing 22% protein (Table-10). Water was given ad libitum. At the end of 8th week of the dietary treatment, the drug was administered. A suspension of pure rifampicin powder (50 mg/kg) was fed to rats orally using rat-feeding cannula twice a week. INH (50 mg/kg) was simultaneously given orally once daily.

II.(D) Parameters studied were as follows -

1. Serum rifampicin concentration at 0,1,2,5,10 and 20 hours after drug administration.
2. Rifampicin concentration reached in tissues at the identical time points.



3. Liver cytochrome enzyme system
4. Urinary excretion of rifampicin and its major metabolite desacetyl rifampicin at 12,24,48 and 72 hours.

#### Metabolic studies

Seventeen week old rats from both the control and test groups were studied after a single dose of rifampicin alone or along with INH. Rats were placed in metabolic cages and the drug was administered.

Urine samples were collected at the end of 12, 24, 48 and 72 hours after the drug administration. Urinary excretion of rifampicin and desacetyl rifampicin were studied by the method of Nakagawa (1972). At the end of the treatment period, rats were sacrificed by decapitation and various tissues were removed and weighed. Tissue levels of rifampicin were determined by homogenizing the tissue in isoamyl alcohol using a pestle and mortar and the filtrate from this was read at 475 nm against appropriate blanks on a spectrophotometer.

Distribution of rifampicin in different tissues (liver, lung, kidney, heart, spleen, intestine, brain and skeletal muscle) of the rat was expressed as ug/100 mg tissue .

Liver protein was estimated by the method of Lowery et al. (1951). Microsomal fraction of hepatic tissue was prepared after perfusing the liver with saline and drying it on filter paper by the method of Cinti et al. (1972). Briefly, liver was minced in a Petri-dish on ice and 10% homogenate was prepared in 25 mM sucrose containing 10 mM Tris-HCl buffer (pH 7.4) using a Potter and Elvehjem homogenizer. The homogenate was centrifuged at 600 g for 5 minutes in a Sorvall refrigerated centrifuge. The supernatant was centrifuged at 12,000 g for 10 minutes, and to the resultant post-mitochondrial supernatant solid  $\text{CaCl}_2$  was added to a final concentration of 8 mM, before centrifuging at 25,000 g for 15 minutes on a Beckman ultracentrifuge. The resultant microsomal pellet was resuspended in 150 mM KCl 10 mM Tris-HCl buffer and centrifuged at 25,000 g for 15 minutes to get washed microsomal pellet. The latter was suspended in 0.1 M phosphate buffer (pH 7.0) to estimate microsomal cytochrome P-450 and protein.

Cytochrome P-450 and cytochrome  $b_5$  were assayed by the spectrophotometric measurement of the reduced carbon monoxide complex (Vader Hoever, 1974). The concentration of the cytochrome was calculated from the carbon monoxide difference spectrum of the dithionite reduced cytochrome-buffer, pH 7.0.

TABLE-9Composition of stock diet

Ingredients	Amount (g)
Wheat flour	350
Bajra flour	100
Bengal gram flour	110
Milk powder	210
Sprouted cowpeas	160
Groundnut oil	70
Fenugreek leaves*	70

\* Fenugreek leaves when in season, otherwise vitamin A acetate in oil (5000 IU/kg oil) was added.

These protein diets/concentrations were chosen since they have earlier yielded growth in rats, that were given 7% and 22% protein diets which are similar to the human low income group (LIG) and high income group (HIG) diets (Table-13) respectively. The rats were provided the semi-purified diet and water ad libitum.

TABLE-10Composition of semi-purified diet

Ingredients (g)	% dietary protein	
	7% protein diet	22% protein diet
Casein	7	22
Vitamin mixture*	2	2
Salt mixture**	4	4
Sago	80	65
Groundnut oil	7	7

\* Refer to Table-11

\*\* Refer to Table-12

Edible casein used in the diet was obtained from Amul Dairy, Anand.

Commercially available sago prepared tapioca flour (*Manihot utilissima*) was ground and used as starch source as it contains only 0.2% protein and no more than traces of vitamins and minerals. As tapioca flour is processed to some extent during the preparation of sago, the starch in the same is believed to be readily available (Booher et al., 1951).

TABLE-11Composition of vitamin mixture 1\*

Ingredients		Amount per kg mixture/diet
Thiamine hydrochloride		600 mg
Riboflavin		600 mg
Pyridoxine hydrochloride		700 mg
Nicotinic acid	2*	3 mg
D-calcium pantothenate		1600 mg
Folic acid		200 mg
D-biotin		20 mg
Cyanocobalamin (vitamin B <sub>12</sub> )		1 mg
Retinyl palmitate or acetate (vitamin A)	3*	
dl-Tocophenyl acetate (vitamin E)	4*	
Cholecalciferol (vitamin D <sub>3</sub> )	5*	2.5 mg
Menaquinone (vitamin K)	6*	5.0 mg
Sucrose, finely powdered		To make 1000 g

1\* Based on the NAS-NRC (1962) recommended levels for rats.

2\* Nicotinamide is equivalent

3\* As stabilized powder to provide 400,000 IU vitamin A activity or 120,000 retinal equivalents.  
(Added 5,000 IU vitamin A activity/kg oil)

4\* As stabilized powder to provide 5,000 IU vitamin E activity

5\* 100,000 IU may be in powder form

6\* Menadione

TABLE-12Composition of salt mixture\*

Ingredients	g/kg mixture
Calcium phosphate diabolic ( $\text{CaHPO}_4$ )	500
Sodium chloride ( $\text{NaCl}$ )	74
Potassium citrate monohydrate ( $\text{K}_3\text{C}_6\text{H}_5\text{O}_7 \cdot \text{H}_2\text{O}$ )	220
Potassium sulfate ( $\text{K}_2\text{SO}_4$ )	52
Magnesium oxide ( $\text{MgO}$ )	24
Manganous carbonate (43-48% Mn)	3.5
Ferric citrate (16-17% Fe)	6.0
Zinc carbonate (70% $\text{ZnO}$ )	1.6
Cupric carbonate (53-55% Cu)	0.3
Potassium iodate ( $\text{KIO}_3$ )	0.01
Sodium selenite ( $\text{Na}_2\text{SeO}_3 \cdot 5\text{H}_2\text{O}$ )	0.01
Chromium potassium sulfate ( $\text{Cr K}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ )	0.55
Corn starch - finely powdered	To make 1000 g

\* Based on NAS-NRC requirements for rats.

The diets were made/prepared once a week and vitamin mixture and groundnut oil were added at the time of feeding.

The weight gain of both the groups was noted at an interval of 7 days.

TABLE-13Food composition pattern in low and high income groups

Ingredients (gm)	Low income group	High income group	W.H.O.
Mild flour of wheat (Triticum sativum)	100	150	220
Rice (Oryza sativum)	50	100	-
Bajra (Pannicitum glaucum)	150	-	-
Kodri (Paspalum sorbiculum)	50	-	-
Moth bean (Phaseolus aconitifolius)	3	-	-
Bengal gram dal (Cicer anetenum)	12	10	-
Red gram dal (Cajunus cajan)	12	30	-
Green gram dal (Phaseolus mango)			
Milk powder (1)	12	65	65
Sugar	15	30	138
Jaggery (sugar)	10	15	-
Fat - groundnut oil	15	30	-
- hydrogenated oil (Dalda)	-	15	44
- butter (Amul)	-	-	25
Meat	10	(2)	250
Leafy vegetables/fruits and other vegetables	18 <sup>(3)</sup>	36	75
Common salt	10	10	10
Cheese (Cheddar)	-	-	12
Pea nuts	-	15	35

The growth rate of rats fed 6.8% of protein derived from casein was found to have similar growth rate to rats fed a low income group diet.

Report of Greaves and Hollingsworth for the British (1966)

1. Twelve gm with milk powder taken as 100 ml milk. Flour sweepings of milk powder were obtained from Amul Dairy, Anand.
2. Minced mutton was autoclaved and stored in polythene bags at 0°C. Since 80% of the families in the high income group are vegetarian, no meat was added to the diet of this group.
3. The monthly consumption pattern of vegetables and fruits was formulated on the basis of some surveys carried out in Baroda (Rajalakshmi, 1978). Vegetables and fruits were purchased in the proportion given in above Table-13. 100 gm of fresh vegetables gave on an average 15 gm of dry powder.



### III. Human experiments

This study was approved by Ethics Committee of the Institute.

#### A. Selection of subjects

For selection of human subjects, as far as possible, matched groups as to age, sex, weight, height, type of diet, dosage administered and drugs to be given were selected. In each group, the minimum number was six.

##### (a) Normal healthy volunteers

Normal healthy volunteers free from infections and diseases (age range 20 to 50 years and weight range 50 to 75 kg) were selected for this study, following a medical examination which included biochemical and haematological tests. The subjects were asked to abstain from the use of any drug or alcohol for atleast 3 weeks prior to the study and the drug was administered preprandially (minimum 12 hours fast). The healthy human volunteers were drawn from amongst doctors, medical students, staff members of S.S.G. Hospital and Medical College, Baroda.

##### (b) Patients

The patients proved cases of pulmonary tuberculosis as evidenced by X-ray examination and by sputum culture examination, who were receiving rifampicin for the first

time were picked up for this study from Smt. Padmavati Sanatorium and S.S.G. Hospital, Baroda.

Patients with certain abnormalities not specifically related to the disease state were excluded from this study. Rifampicin, a potent antituberculosis agent, is frequently combined with other antituberculous drugs or with drugs belonging to entirely different classes which may be required during a long period of antituberculous treatment and therefore, has a potential for drug interactions of practical clinical importance.

#### B. Plan of studies

Experiments were planned according to type of the treatment given -

- i) Rifampicin alone
- ii) Rifampicin + INH
- iii) Rifampicin + INH + streptomycin
- iv) Rifampicin + INH + ethambutol
- v) Rifampicin + INH + pyrazinamide

#### C. Parameters studied

Parameters studied were -

1. Plasma concentration curve after single oral dose of rifampicin.

2. Total plasma protein level at 0 hour blood collection samples and albumin/globulin ration.
3. Rifampicin binding to albumin/globulin
4. Detailed pharmacokinetic parameters -
  - Co - Concentration assumed to be at 0 hour
  - Cmax - Maximum concentration reached
  - Tmax - Time required to reach the maximum concentration
  - AVD - Apparent volume of distribution
  - El.rate const. - Elimination rate constant
  - AUC - Area under the curve
5. Urinary excretion of rifampicin/24 hours.

Selected patients of pulmonary tuberculosis were studied on the basis of body weight as an index of the nutritional status. Patients having (weight in kg/(height in cm)<sup>2</sup> X 100) ratio less than 0.18 were included in this study (Jayarao et al., 1972). The subjects were kept fasting for a period of two hours after the administration of the drug. A single dose of pure rifampicin powder (10 mg/kg) with 150 ml of drinking water was administered orally, to all the patients on empty stomach in the morning. Out of these, ten patients were also given rifampicin and INH (300 mg) orally and followed by rifampicin, INH and streptomycin (0.75 g im) or INH and pyrazinamide or INH and

ethambutol, with a wash out period of 4 days each time. Venous blood was collected at 0, 1/2, 1, 2, 4, 6, 8, 12 and 24 hours after the drug administration. Zero hour blood was collected to study total protein concentration by the Biuret method (Cornall et al., 1949). Serum albumin concentration was estimated by the method of Reinhold (1953). The difference between plasma and serum rifampicin concentration was taken as fibrinogen-bound rifampicin.

Serum half-life of rifampicin was derived from the slope of the curve, obtained by plotting serum concentration against time on a semi-logarithmic paper. The apparent volume of distribution (AVD) was determined by dividing the dose administered (mg/kg) by the expected concentration (ug) of the antibiotic per ml of plasma at zero hour. The latter was obtained by extra-polating the slow decline rate in plasma to zero hour (Kunin et al., 1959). The elimination rate was calculated from the standard formula  $k_r = 0.693/t_{1/2}$ . AUC was calculated by using the formula -

$$\begin{aligned} \text{AUC} &= \frac{1}{2} (C_1 + C_4) (t_2 - t_4) \\ &+ \frac{1}{2} (C_4 + C_6) (t_4 - t_6) \\ &+ \frac{1}{2} (C_6 + C_8) (t_6 - t_8) \end{aligned}$$

Urine samples were collected in sterilized bottles over a period of 24 hours. Estimation of rifampicin levels (ug/ml) in serum and urine was carried out by the method of Sunahara and Nakagawa (1972).

General plan of human experiments

Total No. 74

Healthy volunteers		Patients of pulmonary tuberculosis	
11		63	
Normally nourished	Poorly nourished	Comparatively better nourished	Poorly nourished
6	5	37	26
Nutritional score	0.180	Between 0.140 to 0.180	Below 0.140

Experiment No.							
1. Rifampicin alone	6	5	37	26			
2. Rifampicin + INH	6	5	10	15			
3. Rifampicin + INH + Streptomycin	-	-	5	5			
4. Rifampicin + INH + Ethambutol	6	5	6	2			
5. Rifampicin + INH + Pyrazinamide	-	-	6	2			

Plasma protein binding study in n = 15

a) in vivo n = 9

b) in vitro n = 6

P R O F O R M A

Name	Age	Sex
Regd.No.	Weight	
- Organ involved	(Extent of involvement) (to be graded)	
- Any other disease		
- Any other drug administered:	(if yes, the dose of drug and duration of therapy).	

## Nutritional status score

## Investigations

1. Plasma proteins, albumin/globulin ratio
2. Urine creatinine clearance

1. Date and time of rifampicin administration

Collection of sample

<u>S.No.</u>	<u>Time</u>	<u>Blood</u> Concentration of rifampicin	<u>S.No.</u>	<u>Time</u>	<u>Urine</u> volume	Concentration of rifampicin
1.	0 min		1.	0 hr		
2.	20 min		2.	2 hrs		
3.	40 min		3.	4 hrs		
4.	60 min		4.	6 hrs		
5.	90 min		5.	8 hrs		
6.	120 min		6.	12 hrs		
7.	240 min		7.	24 hrs		
8.	480 min		8.	48 hrs		
9.	600 min		9.	60 hrs		
10.	720 min		10.	72 hrs		
11.	960 min		11.	96 hrs		

$t_{1/2}$	Volume of distribution		Amount excreted
Co	Tmax	Cmax	Abs half time

2. Date and time of administration of rifampicin and INH

Collection of samples and table as above.

3. Date and time of administration of rifampicin and INH and alone of other antitubercular drugs.

Collection of samples and table as above.

Change of treatment will be started after the wash out period. The same patient will be studied again after recovery from undernutrition.

For this period, i.e. for 6 months the patient will be given supplementary protein rich diet.

<u>Treatment</u>	<u>Co</u>	<u>Cmax</u>	<u>Tmax</u>	<u>Abs <math>t_{1/2}</math></u>	<u>Eli. <math>t_{1/2}</math></u>	<u>AVD</u>	<u>AUC</u>
- Rif alone							
- Rifampicin and INH							
- Rifampicin with INH & streptomycin/ethambutol/pyrazinamide							
- Rifampicin with standard 3 drugs therapy after treating undernourishment							

6. List of important publications of the investigators in this field -

None.



#### V. Statistical analysis

Data were analysed using Students paired and unpaired 't' test. Values of 't' less than 0.05 were considered to be significant. The data <sup>were</sup> also subjected to regression analysis for finding out correlation.

#### VI. Drug and chemicals used

All the chemicals and reagents used were of research grade purity. Pure rifampicin powder was kindly supplied by M/s Ambalal Sarabhai Enterprises, Baroda, India.