# Introduction

Tuberculosis is very common in India. It is still increasing in Indian population (Ramkrishnan and Chandrashekhar, 1999). Similarly its spreading rate is also alarming in U.S.A. and other European countries. It can be cured by some of the effective antibiotics. Rifampicin is one the drug of choice in the regimen of tuberculosis treatment. It has been noted that single drug treatment not only develops resistance against drug in bacteria but also associates with some side effects (The Pharmaceutical Codex, 1979; Martindale-The Extra Pharmacopoeia, 1989). Multiple drug therapy used in recent years is also not without the side effects (Gupta *et al,* 1983). In drug replacement therapy rifampicin is used often but it also shows toxic side effects (Its toxicity is known and reviewed in following paragraphs) as an individual drug or as a part of multiple drug system (Gupta *et al,* 1983). Being a potent drug; use of rifampicin against tuberculosis infection, if modulated by any other drug which will spare it from the toxic side effects and simultaneously allow it to retain its potency against M. tuberculi; will help to increase its use and will be a good weapon in the hands of physicians. Mandur bhasma one of the Ayurvedic drug which is proven hepatoprotective (Devarshi *et al,* 1986; Kanase *et al,* 1992,

1997). It is recommended against TB in Ayurvedic Texts (Mishra, 1984). Therefore it was selected for testing its potency against rifampicin toxicity. The details about the relevant information related to the work are considered in reviewed literature.

*Rifampicin:*

Rifamycins are group of closely related antibiotics produced by *Streptomyces mediterrani.* Sensi *et al* (1959) isolated the antibiotic and showed their preferential activity against prokaryotes. Rifamycins together with streptovaricins and polpomycin form a new unusual class of antibiotics. All of them are ANSA compounds containing an aromatic ring system, which is spanned by an aliphatic bridge. Some times they are called as anasamycins. Rifamycins can be described as derivatives of an α-aminonaphthohydroquinone. The aliphatic bridge from position 2 to 5 is composed of acetic acid and propionic acid residues. In position 4 Rrifampicin B contains a glycollic acid residue the hydrogen in position 3 can very easily substituted by other residues. Thus substituting this hydrogen derived a synthetic product by a 4-(methyl)-piperzinnyl-iminomethyl residue.

Extensive reviews on the chemical and biological properties of rifampicin are available (Frontali and Tecce, 1967a, b; Silvestri, 1970; Goldberg and Friedman, 1971; Binda *et al,* 1971 Kersten and

Kersten, 1974) and Riva and Silvestri (1972) have published pharmacological properties of rifamycins and reviewed chemical, biochemical and biological effects of rifampicin. The Pharmaceutical Codex (1979) and Martindale - The Extra Pharmacopoeia (1989) has also listed its therapeutic properties and adverse effects in human and on laboratory animals.

Rifampicin is the semi-synthetic; highly active derivative of rifamycin B. It preferentially inhibits the synthesis of cellular RNA in susceptible bacteria, DNA synthesis remaining unaffected (Goldberg and Friedman, 1971)

Rifampicin is odorless, brick, red, crystalline powder, slightly soluble in water, acetone, alcohol and ether and freely soluble in chloroform and methyl alcohol. It is stored in airtight conditions at low temperature.

Rifampicin is effective against *Mycobacterium tuberculosis* and may be active when resistance to other drug has developed. It is also active against large number of Gram positive and Gram negative organisms including Staphylococci and Bacterioids and is occasionally used to treat infections with these organisms. It is bactericidal, but should not be used alone in the treatment of tuberculosis, because relatively rapid induction of bacterial resistance may occur. It is usually administered in conjunction

with one or two other antitubercular drugs, such as isoniazid, streptomycin and ethambutol for the treatment of tuberculosis (primary and re-treatment also) and is also used for the treatment of leprosy in combination with dapsone and cloflazimine. It is administered orally approximately 10 mg/kg body wt. to 600 mg/kg body wt. Evaluation of rifampicin in clinical use especially in tuberculosis treatment is given in The Pharmaceutical Codex (1979) and Martindale - The Extra Pharmacopoeia (1989). The place of rifampicin in the treatments of patients who have had relapse during or after a course of treatment with other drugs is important and hence it is necessary to improve its status by decreasing its toxicity in addition to other approaches.

Rifampicin is readily absorbed from the gastrointestinal tract and peaks plasma concentrations of 7 to 9 μg /ml 2 to 4 hr after a dose of 600 mg, but there may be considerable individual variations; food may reduce or delay the absorption. Half life for rifampicin have been reported to range initially from 2 to 5 hr. The longest elimination time occurs after the largest doses. Rifampicin induces its own metabolism. Elimination time may decrease by 40 % during first two weeks thus showing half -lives of 2 to 5 hr. It may be highly bound to plasma proteins. Rifampicin, but not its metabolite under goes enterohepatic circulation. It is widely distributed in body tissues and body fluids (Sunahara and

Nakagwa, 1972; Winsel *et al*, 1976; Shaha *et al*, 1983). It is rapidly metabolized in the liver mainly to active desacetyl rifampicin and is excreted in the bile and about 60 % of a dose eventually appear in the faeces (Patel *et al*, 1981; Yan, 1982). The amount exceeds in the urine and increases with increasing doses and up to 30 % of 900 mg may be excreted in the urine, about half of it within 24 hr. The metabolite formyl rifampicin is also excreted in the urine. It crosses the placenta and is excreted in breast milk (Acocella, 1983; Kenny and Strates, 1981; Venkatraman *et al*, 1988). Numerous papers deal with the pharmokinetics of rifampicin administering the drug to the volunteers and check its half-life, its levels in plasma and other body fluids (Mahajan and Rohtagi, 1988). Avachat *et al* (1992) and Le Guellec *et al* (1997) studied stability of rifampincin. Bioavailability of rifampicin was also studied (Dahanukar, 1983; Elard and Fowrie, 1999).

*Adverse effects of refampicin*:

Adverse effects of rifampicin in-patients are reviewed in the pharmacentical codex (1979), Gupta *et al* (1983) and Martindale-The Extra Pharmacopoeia (1989). They include hepatotoxicity, renal toxicity, skin reactions, eosinophilia, transient leucopenia, thrombocytopenia, purpura, haemolysis and thrombophlebitis. Other side effects reported include confusion, drowsiness,

headache, muscular weakness, ataxia, dizziness, generalized numbness and visual disturbances.

*The metabolism of Rifampicin:*

The liver is a versatile organ which is responsible for metabolism of chemicals and maintenance the regulation of three major metabolisms and energy and hence internal chemical environment. Exogenous and endogenous nutrients and chemicals (xenobiotics) are absorbed, concentrated, modified and processed by the liver for clearance through kidney or gastrointestinal tract (Roullier, 1969; Gupta, 1983; Hinton and Grasso, 1995; Timbrell, 1995). After a xenobiotic has been absorbed into a biological system, it may undergo a biotransformation that leads to rapid excretion and therefore elimination of the compound from the animal. The highly lipophilic substances enter in the body by passive diffusion, are mostly poorly excreted, and hence remain in an animal body for many years. The products of the metabolism are usually more water-soluble than the original compound. In animals biotransformation seems to be directed at increasing water-solubility and hence excretion. The biotransformed compounds may cause the toxicity rather than the detoxification thus metabolic fate of the compound influences its toxic potential, disposition in the body and its excretion, leading various kinds of

lesions in liver (Timbrell, 1995) and to the excretory organs kidney (Lock, 1995).

Rifampicin being hydrophobic and tend to accumulate in the body parts. It is also biotransformed leading to the hepatotoxicity and renal toxicity. Hepatotoxicity of antituberculus regimens containing rifampicin has been reviewed by Nessi *et al* (1976) Girling (1978); Girling and Hitze (1979); Sallie *et al* (1991). Rifampicin induced renal failure that was reviewed by Martindale-The Extra Pharmacopoeia (1989). These show risk factor with interrupted therapy and need appropriate supportive care, but precaution never to re-administer the drug to the patients with renal failure or rifampicin should be replaced by other appropriate drug. The mechanism of renal failure is not revealed, but sometimes it is associated with shock and haemolysis. Renal biopsies in some patients showed tubular necrosis, tubulointerstitial lesions, cortical necrosis, mild tubular damage or glomerulonephritis (Katz and Lor, 1986). The gastrointestinal tract is also affected. There are reports of pseudomembranous colitis associated with use of rifampicin (Fekety *et al*, 1983).

*Effects of rifampicin studied in rats:*

Oral administration of 25 and 50 mg/kg doses of rifampicin to rats showed 60 to 85 % systemic absorption. The

tissue availability of the antibiotic was high in oral administration than intravenous administration [Firsov *et al* 1986]. High doses of Rifampicin [400 mg/kg for1, 2, 4, 6 and 8 days] resulted in fatty liver showing ultrastructural changes in endoplasmic reticulum, Golgi apparatus, and Mitochondria [Piriou *et al,* 1987]. In 12-day experimental study of rifampicin administration to male rats [Zitkova *et al,* 1982] showed many fold high concentration of rifampicin in liver and comparatively low concentration was noted in epididymal fat and blood. This was coupled with disorder of metabolic equilibrium, intake and resorbtion of nutrients [lipids and sugars]. Rifampicin administration showed 34 to 72 % of antibiotic in bound form in various organ homogenates and liver contents of rifampicin were very high [Iakovlev, 1978]. Rifampicin [60 mg/kg body weight/per week oral or intra-muscular] administration to rats for four weeks resulted in accumulation of the drug in various tissues [Bruzzese *et al,* 2000]. Rifampicin 10 mg per kg body weight given intra bronchially resulted in 10 to 12 times high levels of rifampicin in lung tissue after two hours. It was nearly 10 times higher as compared to enteral administration. But rifampicin levels were very low in liver in the same experimental condition [Shapovalov *et al,* 1982]. Rifampicin influences prenetal development of rodents [Tuchmann-Duplessis and Mercier-Parot, 1969]

The role of N-acetyleysteine (100 mg/kg per day for 3 weeks) a glutathione precursor was investigated (Attari *et al*, 2000) in protection against rifampicin + isoniazid (Both 50 mg/kg per day) induced oxidative hepatic injury in young Wistar rats. The oxidative stress was observed, with increased lipid peroxidation and histological lesions that ranged from interlobular inflammation to patchy necrosis. Status of liver protein and energy mal nutrition played important role in the pathogenesis of isoniazid + rifampicin (50 mg/kg of each intraperitonially) induced hepatic injury in weanling rats (Sodhi *et al* 1998), growing rats (Rana *et al*, 1996; Sodhi *et al*, 1997a,b) and young rats (Sodhi *et al*, 1996). Serum transaminases were increased coupled with hepatic necrosis while hepatic thiols blood glutathione were decreased; similarly anti-oxidative enzymes superoxide dismutase, catalase, glutathione peroxidase and glutathione-S- transferases were significantly declined while lipid peroxidation was elevated. Oxidative injury was closely associated with significant decline of glutathione and related thiols in hepatoprotection as antioxidative profile. Fatty liver induction was studied by high doses of rifampicin in rats [Piriou *et al* 1979]. It may be the consequences of inhibition of RNA polymerizes and blockage of very low-density lipoproteins' biosynthesis or secretion in eukaryotic cells. Liver steatosis was also induced by high doses of rifampicin in rats [Truhaut *et al*,

1978]. An anabolic steroid, 19-nortestosterone phenylpropionate did not show protective effects on the fatty liver induced by high doses of rifampicin in the male rat [Piriou *et al*, 1980].

*Why toxicity modulation studies:*

The reduction in the frequency in the side effects or the toxic effects can improve the place and potency of rifampicin. Therefore improvement in toxicity problems will give efficacy to rifamycin in its clinical use. This will also reduce the dose of rifamycin and cost of treatment, this requires numerous animal trials followed by clinical trials. Therefore toxicity studies are important in achieving this goal. Toxicity studies in humans are restricted to serum, urine studies. Toxicity studies in laboratory animals allow the extension of studies unto organ and cellular levels in *in vivo* conditions and provide the data more near to *in situ* conditions. Thus it is essential to study the toxicity of rifampicin in animal model/s. Different parameters studied provide the probable metabolisms in toxicity and will be helpful to the physicians in planing the treatment.

Animals including the albino rats are used to study the pharmacokinetic levels of parent compounds and its metabolites in plasma and other body fluids coupled with its accumulation in organs (Rodriguez *et al*, 1973; Filippos'iants *et al*, 1980; Zwolska-

Kwiek *et al*, 1980; Venturini 1983; Firsov *et al*, 1986 and Bruzzese *et al*, 2000). Akimoto *et al* (1970) studied the absorption, metabolism and excretion of rifampicin in rat. Zitkova et *al* (1982) showed manyfold-high concentration of rifampicin in liver than blood and other organs and it was harmful to the hepatoparenchymal cells. Even fat concentration was half as compared to the epididymal fat. In total it affects the nutrition.

The hepatotoxicity is studied *in vivo* and *in vitro* also and covers very broad range of studies and hence alterations in albino rat males are reviewed here. $LD_{50}$ for rifampicin in rat for oral route is 2650 mg/kg with confidence limits upto 3350 mg (Frontali and Tecce, 1967). Subacute and chronic toxicities were tested in rat males using 50 to 200 mg/kg doses (Maffii *et al*, 1961). High doses of rifampicin induced steatogenic liver Truhaut *et al* (1978). Piriou *et al* (1979) administered 200 mg and 400 mg/kg/24 hr doses were given to for 8 days. The treatment resulted into increase in hepatic triglycerides, phospholipids and cholesterol but decreased in blood. VLDL blockage may be the result of synthesis and secretion. Khedun *et al* (1992) studied the isolated liver perfusion model with supra therapeutic doses of rifampicin. The rate and the concentration of glucose decreased. The ratio of lactate : pyruvate was increased. The fatty changes were present in the liver. Strolin and Dostert (1994) reviewed the induction and autoinduction

properties of rifampicin in animals and human. The rat hepatic cytochrome P-450 system seems to be resistant to the action of rifamycin unless very high doses are used. Histological evidence of hepatic damage was noted in rats. Olatunde *et al* (1999) showed 8.6 mg/kg rifampicin administered intraperitoneally for 5 days increased serum triglycerol, aniline hydroxylase, aminopyrin- N-demethylase and ρ-nitroanisole –O- demethylase were elevated. Induction of these hepatic enzymes may lead to hypetriglyceredemea. Intrabronchial administration of rifampicin (10 mg/kg body wt) exhibited the highest rifampicin levels in lungs at all periods. At 2 hr after administration 10 to 12 times higher levels were noted in lung as compared to its enteral use. Thus as compared to enteral use intrabronchial administration achieved high levels of rifampicin within a short period, while the levels were low in liver. The levels of rifampicin in blood were not significantly affected by its route of administration (Shapovallov *et al,* 1982). Skakun and Shaman'ko (1985) showed synergistic effect of rifampicin on hepatotoxic effect of isoniazid as judged by high levels of transaminases, alkaline phosphatases, higher rate of inhibition of biliary secretion and synthesis and excretion of bile acids, bilirubin and cholesterol with bile. Lipid peroxidation was also enhanced. Skakun and Tabachuk (1992) showed inhibition of bile products and bioenergy, activation of AST and ALT, alkaline

phosphatase levels were elevated in serum. $Na^+$-$K^+$- ATPase, succinic dehydrogenase and cytochrome oxidase were reduced in liver. The rate of bile secretion, excretion of bile acids, bilirubin and cholesterol were lowered. Skakun and Slivaka (1992) showed cytolytic liver injury, hypertransferacemea initiation of lipid peroxidation, suppression of antioxidant system and lipid peroxidation. Similar results were obtained with rifampicin (50 and 250 mg/kg) given for 15 days in equimolecular doses with isoniazid (Slivaks, 1989). In growing rats rifampicin induced hepatic injury when given 50 mg/kg/day; Superoxide dismutase, catalase, glutathione peroxidase were decreased. Hepatic thiol bound to protein showed significant elevation. Non protein thiols remained unchanged. Glutathion-S-transferases exhibited significant elevation. Histopathological alterations showed increased fatty infiltration with protein malnutrition. The results indicated altered oxidative and antioxidative profile (Sodhi *et al*, 1997). The oxidative stress was increased in isoniazid- rifampicin (50 mg/kg body wt each) induced hepatic injury in young rats in two weeks. Liver showed microvescicular fat deposition with mind portal triaditis. Glutathione, superoxidative dismutase, glutathione peroxidase, catalase and glutathione-S-transferase were decreased Sodhi *et al* (1997). Protein and energy malnutrition enhanced the oxidative hepatic injury in young rats (Sodhi *et al*, 1998). Strolin and Dostert

(1994) reviewed the induction and autoinduction properties of rifampicin in animals and human. Rifampicin acts as non-competitive inhibitor of hepatic microsomal aryl hydrocarbon hydroxylase in *in vitro* at or below 0.10 mM concentration. It acts as non-competitive inhibitor of intestinal arylhydrocrbon hydroxylase at or below 0.075 mM. The results indicated rifampicin undergoes biotransformation (Wu and Stohs, 1983). Polasa and Krishnamurthy (1983) studied *in vitro* binding of proteins to rifampicin. Protein binding of drugs is clinically important because it is only the free drug that exerts the pharmacological action (Levy, 1980; Wise, 1983). *In vitro* binding to the proteins was influenced by albumin and in presence of other anti-tubercular drugs rifampicin binds the most to the proteins.

*Dose of rifampicin:*

Fifty-mg rifampicin/kg body wt/day for two weeks induces hepatotoxicity (Sodhi *et al*, 1997; 1998). When the above dose was given orally for 30 days resulted in histological necrosis in hepatocytes in centrolobular, periarterial and transitory regions. It had created tubular necrosis in kidney also. Thus 50-mg rifampicin/kg-body wt/day treatment was used to induce the toxicity in male albino rats and its modulation by mandur bhasma was studied with reference to liver and kidney in present work.

*Selection of Mandur Bhasma:*

Our body is composed of lipids, proteins, carbohydrates, nucleic acids, and some other components. Proteins form the major structure of the body with lipids and carbohydrates. The proteins are not only structural entities but functional also. They are acting as receptors, carriers, transporters, and secretions. Many of the proteins function as catalysts in the cellular reactions and are called as enzymes. Most of the metabolic reactions are carried out by catalytic molecules; enzymes. Most of them depend upon one or more non-proteins components called as cofactors. The cofactor may be a metal ion or an organic molecule called a coenzyme, while some enzymes require both. The catalytically acting enzyme complex is called as holoenzyme, and without cofactor as apoenzyme. There are numerous enzymes which contribute to the body metabolisms need one or the other cofactor or ion/s. There are non-enzyme proteins also that carry metallic ions for their reactions and are called as metalloproteins Iron containing enzymes and proteins and cofactors are very common in cell *viz.* hemoglobin, cytochromes, ferritins, hemocyanins, porphyrins etc. (Lehninger, 1984).

Mandur bhasma is derived from iron ore and thus contains iron in metallochelate forms. For present work it was prepared as

described in Material and Methods Chapter. The quality of mandur bhasma was tested according to method described in Ayurveda.

Since herbs are used in its preparation, a defined structure is not known, but the process of preparation may be increasing the therapeutic value and bioavailability of mandur (iron) and may be eliminating its toxicological effects. In narrating the properties of mandur bhasma in Ayurvedic text, it is described as a rasayan, which is capable of remodeling the body (Sharma, 1977). The modern researchers are trying to develop drugs containing these elements as their importance is realized. Many of the metal bound organo-complexes are flooded in the drug market. It is a continuous process as per marketing strategy, which is adapted by western companies. Mandur bhasma being indigenous drug its influences on rifampicin toxicity will be of help to those Physicians who believe in integrated medicine.

Mandur bhasma mediated hepatoprotective metabolisms have been studied against $CCl_4$ induced hepatitis (Devarshi *et al*, 1986; Kanase *et al*, 1992) and hepatocurative effects against $CCl_4$ induced hepatotoxicity (Kanase *et al*, 1997).

Through the same view and the reasons discussed above, present project is designed to test the claims of mandur bhasma against the rifampicin toxicity. To ensure the quality of

bhasma, it was prepared in laboratory (Sharma, 1977). In Ayurvedic text it is mentioned that, mandur bhasma is used against liver disease, heart diseases, skin diseases, kidney diseases, spleen diseases, respiratory diseases and for maintenance of overall health.

*Dose of Mandur Bhasma:*

The dose of Mandur bhasma was selected by primary studies on histology of liver. Ten mg mandur bhasma/kg body wt was already tested as hepatoprotective effective dose against $CCl_4$ induced hepatotoxicity (Devarshi *et al*, 1986; Kanase *et al*, 1992) and hepatocurative effects against $CCl_4$ induced hepatotoxicity (Kanase *et al*, 1997). When it was given orally simultaneously with rifampicin showed nearly normal histological architecture of centrolobular, periarterial and transitional regions of the liver after $30^{th}$ day of the treatment. Similarly renal protection was also observed at histological level by this dose. Therefore, 30 day duration was used for the treatment.

*Selection of Albino Rat as an Animal Model:*

Albino rats are breed and maintained through out the world to carry out the research in Biochemistry, Physiology, Cell and Molecular Biology, Immunology, Toxicology, Pharmacology *etc.* Breeding of albino rats and maintaining them in animal houses is

well established and reviewed (Farris and Griffith, 1971; Melby and Altman, 1977). Their nutritional demands, genetic strains and behavior are well studied and well documented. Rat feed is commercially available in the market. Its composition is also known. The defined composition of food required for nutritional research is also provided in the market. Well-documented literature is available on rat handling, maintenance, breeding and other necessary aspects. They are comparatively small in size, which can be easily handled, housed within comfortable space in aluminum or steel cages. They can be maintained in semi sterile conditions in animal house/s. Their breeding period is small and it is possible to arrange for the required number of animals of same age and weights. Thus among the rodents used as laboratory animals albino rat was selected. The rats are used in many laboratories for experimental work that involves behavioral, physiological, biochemical, toxicological and immunological studies. Numerous works will be sited in the following pages. They are also used to produce transgenic animals, mutant animals. Cell cultures from rat cells are developed for *in vitro* studies. The scientific magazines are filled with the advertisements that market the transgenic and mutant albino rats; different cell lines derived from the rats. All these are evidences of the popularity of rat as laboratory animal

and hence it was used in the present work. To avoid the hormonal variations male were selected for the studies.

*Selection of organs*

*LIVER*

The textbooks and reviews on liver are availble (Roullier, 1963; Elias and Sherrick, 1969; Talwar, 1980; Guyton, 1992).

For convenience in routine light microscopic observations in the sections liver is divided into small territorial units about 1 mm in diameter, which is centered around the smallest roots of the hepatic veins. These are called as the hepatic lobules. The lobular boundaries are usually imaginary considering the lobular areas of neighboring veins. The histological pictures shows three regions i) Centrolobular region. ii) periarterial region iii) interlobular region.

Most of the liver is formed of hexagonal cells called parenchymal/hepatic cells. Though the cells appear homogenous in appearance they exhibit functional heterogeneity depending on their location in the liver.

In liver the Kupffer cells and the sinusoidal cells can be identified by the methods described. Thus the normal and altered organization of the liver can be evaluated by cellular appearance of

hepatocytes, Kupffer cell distribution. It can be used to judge the physiological status of the liver.

*Functions of liver:*

On absorption from the intestinal tract, most of the nutrients pass directly to the liver - the major center for distribution of nutrients in mammals. As compared to any organs perhaps the liver shows the greatest metabolic flexibility since nutrients reach the liver first and must therefore adjust its metabolic mixture and to the discontinuous or intermittent nutrient intake. The regulations of balance between carbohydrates, lipid and protein metabolisms are maintained through food intake in liver.

Urea formation by the liver is well studied (Guyton, 1992). The ammonia formed during deamination is removed from the blood almost entirely by conversion into urea. Ammonia results in hepatic coma. In the other excretory functions of liver is the glyco- and tauro- conjugation of bile acids, cholic acid, chenodeoxycholic acid. The catabolic products of hemoglobin through macrophage phagocytotic degradation especially by liver Kupffer cells are excreted in bile as bile pigments (Guyton, 1992).

Other major role of hepatocytes is modification and excretion of the variety of hydrophobic compounds in body. These

reactions involve both endogenous body components/constituents e.g. haem or steroids, nutrients absorbed with the diet or produced by the body's own microflora. In general the physiological role of the reactions is to prepare the compounds for excretion. The enzymes involved are conventionally divided into two groups- I) Reactions involve chemical modification of the reactant normally by oxidation. ii) Reactions are biosynthetic, generally involving conjugations with a hydrophobic moiety (Conjugates such as glucuronic acid or glutathione). These conversions in normal conditions may be harmless or useful, but incase of foreign compounds and specified type of conversions the products may be harmful leading to pathological conditions, may be carcinogenic. Recent works in toxicological studies has been revealed that, many of the conversions are cytochrome P-450 and cytochrome $b_5$ system dependent. The new species of enzymes linked to cytochrome P-450 dependent may be induced depending upon the xenobiotics. The changes occurring may be hepatotoxic, organotoxic. Some activities of cytochrome P-450 expression are also sex dependent. Liver cytochrome P-450-$4A_2$ is regulated in a manner similar to (but not identical to) P-450-$3A_2$ a male specific testosterone-6-beta-hydroxylase. The male - specific expression of liver P-450-$4A_2$ is thus under the control of distinct pituitary dependent hormones acting in a tissue specific manner (Sundseth and Waxman, 1992).

Simultaneous to these results glutathione, glucuronic acid production may be increased or some times some new system may be evolved (Hinton and Grasso, 1995).

From this review on the liver it is obvious that, liver is involved in the numerous metabolisms including drug metabolism and is vital to the body. In such a dynamic organ the hepatotoxicity was induced by rifampicin and tried to study the influence of mandur bhasma as a protective agent.

*Reasons to select kidneys for studies in the present project:*

Liver produces the metabolic waste - urea, which is taken through the blood to the kidney for filtration. Similarly some of the products of drug metabolism also are carried through the blood for excretion. This may affect the kidney adversely. Kidney is a vital organ that removes the waste from the body and hence must be studied for the renal toxicity also.

To understand the toxicological changes one must study the normal structure and function of kidney.

*Kidney:*

Roullier and Muller (1969) provide a detailed histologial account. It also provides anatomical details of the kidney. Talwar (1980) and Guyton (1992) have also reviewed the structure and

function of kidney. In these two layers the nephrons are arranged and their parts can be identified under light microscope. Thus the mammalian kidney is an extremely complex organ, both anatomically and functionally and plays an important role in the control and the regulation of homeostasis. In this regard it has key role in the regulation of extracellular fluid volume and electrolyte composition their role in blood borne wastes and other hormonal influenced metabolisms is affected, when metabolites of some drug are excreted or the lipophilic drug enter in metabolic routines. Many functions of kidneys are reviewed by Maunsbach *et al*, 1980; Dworkin and Brenner, 1985.

*Selection of parameters:*

Following parameters were used to adjudge the effects of mandur bhasma against rifampicin induced toxicity in male albino rats:

1. Serological Tests.
    A. AST
    B. ALT
    C. Alkaline phosphatase
    D. Bilirubin
    E. Urea
    F. Creatinin

2. Histology.
3. Rifampicin content.
4. Other parameters.
    A. Tissue Weights
    B. Protein Contents.
    C. Lipid peroxidation
    D. Formaldehyde
    E. Glutathione
    F. Protein oxidation

*1. Serological/ Serum tests:*

Serological parameters especially liver function tests are included under this category. The tests include GOT/AST, GPT/ALT, alkaline phosphatase (ALP), bilirubin, Singh (1980), Guyton (1992), Stonard and Evans (1995) reviewed the details of these tests.

Usually physicians depend on these tests for the status of liver the tests are used as diagnostic tests for hepatitis. Thus tests were included in the studies of the project.

*Liver function tests: A, B, C, D .*

In patients limited availability exists in order to determine the presence and severity of liver disease. The tests can give

differential diagnosis of jaundice, judgement of prognosis of the patient and evaluation of the response to the treatment. Most of the tests evaluate the efficiency of hepatocytes.

The liver with a constant rate produces bile. Among the most important products of bile is bilirubin formed as a breakdown of hemoglobin. The parenchymal cells absorb it. Here it undergoes conjugation. Eighty per cent of the conjugates are glucuronides and perhaps 20 % are sulfates; and are water-soluble and excreted in biliary canaliculi, where they form a part of bile and are excreted in gastrointestinal tract through stool in processed form. Its secretion-absorption and reabsorption cycle also continues. With the failure of the parenchymal cell functions the enzyme activities in serum are increased and thus their serum levels can be used to deduce the well being of hepatocytes and liver. In rat especially they can be used to detect cholestasis. There are two types of bilirubin direct (unconjugated) and indirect (conjugated). Direct bilirubin is primarily increased, when it reaches certain levels, the protein bound prehepatic bilirubin is released in plasma. It is not filtered through kidney, as it is not water-soluble.

Thus serum enzymes GOT/AST, GPT/ALT, ALP and bilirubin (direct and indirect) levels are used for the preliminary hepatotoxicity determinations as well as hepatoprotection and cure.

*The kidney function tests: E, F.*

In the other serological tests are included urea and creatinin content the metabolic end products of proteins.

The damage to the kidneys may causes the interference in the clearance of urea and creatinin, which appear in the serum. Their levels are used to realize the disfunctioning of kidney.

*2. Histology*

The normal histology of liver and kidney is well studied. Light microscopic stucture and ultrastructures are well known (Roullier, 1963; Roullier and Muller, 1969). The function of any organ is reflected through its histological structure. Zoological, Physiological studies have revealed a close and strong relationship between structure and functions of organs. Even the alterations that occur during development are also observed in their histological pictures. This is also true in case of ultrastructure. The staining specificities are also altered which can be observed using various histochemical techniques. Physiological stress is also mirrored in the appearance of tissue and staining patterns in histochemical tests. Rifampicin induces fatty degeneration is known and it can be used to evaluate the status of hepatic protection.

Thus histological pictures of liver and kidneys are more direct and conclusive evidences of their functional status where they may be under stress, pathologically altered, protected or cured.

*Refampicin content :*

Detailed reviews of the clinical pharmacokinetics of rifampicin are cited in Pharmacopoeia (1979; 1989). Most of these works are either on patients (Krishna *et al*, 1984) or in volunteers (Avachat *et al*, 1992). They are mostly studied in plasma, urine, saliva and other body fluids (Sunahara and Nakagawa, 1972). The absorption of the drug in various tissues irrespective of the route of the entry is well known. The oral feeding and the absorption excretion cycles in gastrointestinal tract and its absorption and retention in organs on this path (Pharmacopoeia 1979; 1989 Iakovlev, 1978 Shapovalov *et al*, 1982); and its excretion through faecal excretion is important. The content of rifampicin has been studied to reveal the influence of mandur bhasma on these cycles.

*Other parameters.*

*G. Weights of tissue*

*H. Protein content*

*I. Lipid peroxidation*

*J. Formaldehyde*

*K. Glutathione*

*L. Protein oxidation*

During biotransformation most lipid soluble compounds are changed into polar products to facilitate their secretion into bile or hydrophilic compounds excretion through urine. These reactions control the biological activity of a foreign compound; its duration of action and accompanying toxic symptoms.

*Cellular Involvement in Biotransformation :*

Microsomes are studied *in vivo* and *in vitro* and are shown to involve in the transformation of xenobiotics. There are many of the compounds that are transformed from original compounds (Krishnamurthy , 1985).

The enzymes involved in this reactions are present in microsomes (Nebert and Negishi, 1982; Nebert *et al*, 1983). These enzymes are generally referred to as mixed function oxygeneses (MFO) and require a reducing agent (which is known to be NADPH), cytochrome P-450 and atmospheric oxygen. The reaction can be known as

$$RH + NADPH + H + O2 \text{ ----- } ROH + NADP + H2O.$$

This is well known microsomal electron transport system. The reviews indicate that mixed function oxygenases of microsomes

are present in vertebrate liver, Malpighian tubules and digestive tract of insects. Mixed function oxygenases activities are more in mammals as compared to birds and fishes. They have been studied in rat and are known to show high activities of enzymes in male than in female. The microsomal enzyme system exhibits poor specificity and requires only high lipophilicity in the molecule to be biotransformed. Cytochrome P-450 that is obligatory for the enzyme activities is inactivated by carbon monoxide and also inactivates the enzymes. Oxidised cytochrome P-450 is reduced by NADPH providing the reducing potential and is catalysed by the flavoproteins cytochrome-C-reductase. The cytochrome $b_5$ electron transport depending upon NADPH is somehow interconnected with P-450 pathway.

Although mixed function oxygenases are nonspecific to xenobiotics to be qualified properties including polarity, electron nature and stereo or spatial configuration. Substrates to compete for sites on mixed function oxygeneses (Krishnamurthy, 1985; Waxman and Azaroff, 1992). The multiplicity of enzymes is known which is evidenced by the distinction between cytochrome P-450 dependent oxygenation and flavoprotein dependent N-oxidation. Isolation of cytochrome P-450also decides the multiplicity. The electron transport is studied well; the components of which are

NADPH - Cytochrome - C - reductase, cytochrome P-450, a heat soluble factor and lipids.

The binding of xenobiotics to the microsomal membrane is a complex process involving both binding sites and catalytic sites on NADPH cytochrome-C-reductase. The rate limiting step in monoxygenation is the rate of reduction of oxidised cytochrome P-450 substrate complex . Two electron equivalents are required for the coupled reaction of oxygen and the oxidation of the substrates. Cytochrome P-450 is one-electron carriers. The flavoprotein NADPH-cytochrome P-450 reductase mediates this reduction. The synergistic effect of NADPH on NADPH catalysed xenobiotic oxidation and the alterations in the steady state level of cytochrome $b_5$ during NADPH initiated non-oxygenation have led to the assumption that reduced cytochrome $b_5$ interacts with oxygenated cytochrome $b_5$ interacts with oxygenated cytochrome P-450. Mixed function oxygenase activity is inducible. Many drugs, food additives and chlorinated hydrocarbons can induce the activity. Three commonly used inducers are 3-methylchlolanthrene, phenobarbital and DDT. The inductions are coupled with significant morphological changes in liver and the inductions are considered as the adaptive responses of mammals to deal with increasing entry of chemicals in body; but long-term effects are not studied.

*Extra hepatic monoxygenases*

The increasing awareness of exposure to xenobiotics and better understanding of biochemistry of other organs *viz.* Lungs, kidneys, placenta *etc.* have stimulated these studies in various organs. Kidney cortex microsomes catalyse hydroxylations, liver normally carries that out.

The integrity of microsomes is essential component in enzyme activities of mixed function oxygenases. Therefore, membranes of microsomes are essential entity. The drugs that lead the phospholipid disintegrity of microsomal membranes also show rapid turnover in lipids. In this condition the membranes are disintegrated by lipid peroxidation (LPO). The induction of LPO by the metabolic activities induced by drugs concurrently destructs the enzyme activities of microsomes. NADPH-dependent LPO destructs cytochrome P-450 activity also. This increase in LPO and decrease in MFO are strongly exhibiting the microsomal functioning and membrane integrity.

*Lipid peroxidation: LPO*

Lipid peroxidation is well studied in many hepatotoxic drugs (Recknagel and Glende, 1978; Mehendale, 1985). Lipid peroxidation occurs as follows. Rifampicin has shown to induce the

lipid peroxidation in rat and in present project it is used to adjudge the protection.

The concept of LPO damage of membranes was advanced by Slater (1978) as the principle mechanism in $CCl_4$ induced liver injury. It was further studied and accepted on the basis of ample amount of experimental evidence (Recknagel and Glende, 1978; Mehendale, 1979, 1985) which showed coupled distruction of cytochrome P-450 and glucose-6-phosphatase activity *in vivo* and *in vitro* preparation, representative microsomal monoxygenase activities, protein synthesis, formation and secretion of low density lipoproteins. This indicated the induced injury to endoplasmic reticulum (ER) altering its function.

In addition to above biotransformations, non-oxygenative oxidations in biotransformations are also studied. These studies also include the reductions, which are catalyzed by liver alcohol dehydrogenase and NADH/NADPH as electron donor. The hydrolytic activities are also studied. Mammalian esterases are widely distributed and exhibit divergent properties of clearing and are directly involved in hydrolytic reactions. Biotransformations may occur as combinations of xenobiotics with readily available endogenous substrates (*e.g.* glucuronic acid, sulfate, acetyl, methyl, glycine) to form conjugates; which can be eliminated through the kidney. The transfer of the endogenous substrates

mediate through coenzymes (UDP, CO-A, glutathione) to which they are bound by specific enzymes. There are some miscellaneous biotransformations, which include ring scission, cyclization, dehalogenation etc.

*Glutathione*

The above review indicates the biotransformations of xenobiotics. In recent years there are other alterations are also worked out which play an important role in the protection of cells (liver, kidney and other organs). Some of the substances present in tissue/cells play the role as endogenous protective ligands.

Among these important is glutathione reduced. Decreased levels of glutathione in case of irreversibly binding compounds is reported by many workers (Mehendale, 1985). In this review role of glutathione is well discussed.

Glutathione is known to undergo conjugation reactions with electrophilic reactants either via enzymatically catalysed reactions by a family of glutathione transferases or via non-enzymatic reactions. Increased toxicities associated with depleted glutathione levels was explained on the basis of diversion of biotransformation pathway via the increased accumulation of active intermediates which interacted with tissue macromolecules to cause toxicity.

In the condition of biotransformation of xenobiotics reduced glutathione levels may be increased by the administration of glutathione precursors such as cysteine or methionine to provide protection against bromobenzine and acetaminophen induced hepatic necrosis. Experimental manipulation of glutathione levels has become a standard experimental protocol in attempts to explain the toxicity of a verity of toxic chemicals.

It is important to realize that although depletion of protective agents such as glutathione can result in diversion of the biotransformation pathway to a more toxic route, quantitatively such alteration only becomes important when depletion is more severe and when it falls below a certain critical level.

Similarly it is possible that the availability of sulfate and glucuronic acid are important ligands in the detoxification of a verity of foreign chemicals and may be important in the expression of toxicity.

There are other types of ligands also viz. Vitamin E and ascorbic acid, which can protect against bioactivation mediated toxicities such as the free-radical mechanisms (Recknagel and Glende, 1978; Slater, 1978; Mehendale, 1985).

*Formaldehyde*:

A variety of NADPH and oxygen dependent reactions catalyzed by the microsomal fraction of many tissues has been recognized to yield formaldehyde as a product. The most common and frequently studied reaction resulting in the liberation of formaldehyde as a product. The most common and frequently studied reaction studied reaction resulting in the liberation of formaldehyde is the N-demethylation of secondary and tertiary amines. These reactions are mediated by cytochrome P-450 the terminal oxidase of the microsomal mixed function oxygenase system (Cooper *et al,* 1965). The reactions are catalyzed by cytochrome P-450, amino oxidase and catalase.

The reaction is initiated by an amine oxidase which catalyses N- oxidation of certain hydroxylamines. The highly unstable hydroxylamine oxides formed undergo rapid dehydration and the resulting nitrone intermediates liberate formaldehyde upon nonenzymic hydrolysis (Poulsen *et al,* 1974). A third reaction sequence resulting in the formation of formaldehyde has been demonstrated to be associated with method oxidation.

These reactions may involve the cytochrome P-450 containing electron transport system as a source of H-O (Hildebrandt *et al.*1975), which in the presence of methanol is

reduced peroxidatically by catalase (Oshino *et al.*1973) contaminating many microsomal preperations.

*Protein oxidation :*

Protein oxidation is defined as the covalent modification of a protein induced either directly by reactive oxygen species or indirectly by reaction with secondary by-products of oxidative stress. Oxidative modification of proteins can be induced experimentally by a wide array of prooxidant agents and occurs in vivo during aging and in certain disease conditions. Oxidative changes to proteins can lead to diverse functional consequences, such as inhibition of enzymatic and binding activities, increased susceptibility to aggregation and proteolysis, increased or decreased uptake by cells, and altered immunogenicity. There are numerous types of protein oxidative modification and these can be measured with a variety of methods. Protein oxidation serves as a useful marker for assessing oxidative stress *in vivo* (Shacter, 1999). Introduction of carbonyl group in amino acids of proteins is hallmark for oxidative modifications (Levin *et al*, 1990 For this reason along with the lipid peroxidation protein oxidation is also included in the studies.

Results obtained from the experimental schedules in present project are presented in this thesis consisting of following chapters.

I. *Introduction*

It includes reasons to take the problem, selection of animal, drug, parameters, organs and experimental approach. It also contains reviews on rifampicin toxicity on liver and kidney. The other relevant things are included to introduce the subject of study.

II. *Material and methods*

The chapter incorporates details about animals used, experimental protocol, methods to estimate different parameters *viz*, proteins, liver and kidney function tests, rifampicin, histology and other parameters *viz*. Formaldehyde, lipid peroxidation, protein oxidation and glutathione.

*III. Serological studies:*

Under this Chapter the serum parameters are studied. They include liver function tests and kidney function tests. The alterations are related to the toxicity status of liver and kidney.

IV. *Histology:*

The livers and kidneys of all the experimental animals were studied for their histological details and based upon it the rifampicin toxicity and mandur bhasma mediated modulation is evaluated.

V. *Rifampicin content:*

Rifampicin being hydrophobic there is tendency to accumulate in the soft parts and since it is fed the fecal excretion is also of importance. In this Chapter the rifampicin content is studied in gastroentestinal tract and associated glands and some important organs. The alterations are discussed as per the modulations.

*VI. Other parameters:*

Among other parameters some parameters related with the drug metabolisms. The parameter related to oxidation includes - Lipid peroxidation and glutothione are known to increase as a rifampicin metabolism. They are used to evaluate both toxicity and mandur bhasma mediated alterations. In addition formaldehyde formed and protein oxidation is also studied since proteins are also known to occur as a result of drug induced oxidations.

*VII General Discussion*

All the results are considered together and mode of action of the mandur bhasma can be evaluated. These results may give new modes and possibilities that can be exploited to develop other drugs. The possibility to use mandur bhasma as the integrated plan of treatment also exists.