THE THERAPEUTIC IMPORTANCE OF ISONIAZID METABOLISM IN ELDERLY PATIENTS.

A Thesis submitted for the degree of Doctor of Medicine (M.D.), in Clinical Pharmacology.

BY

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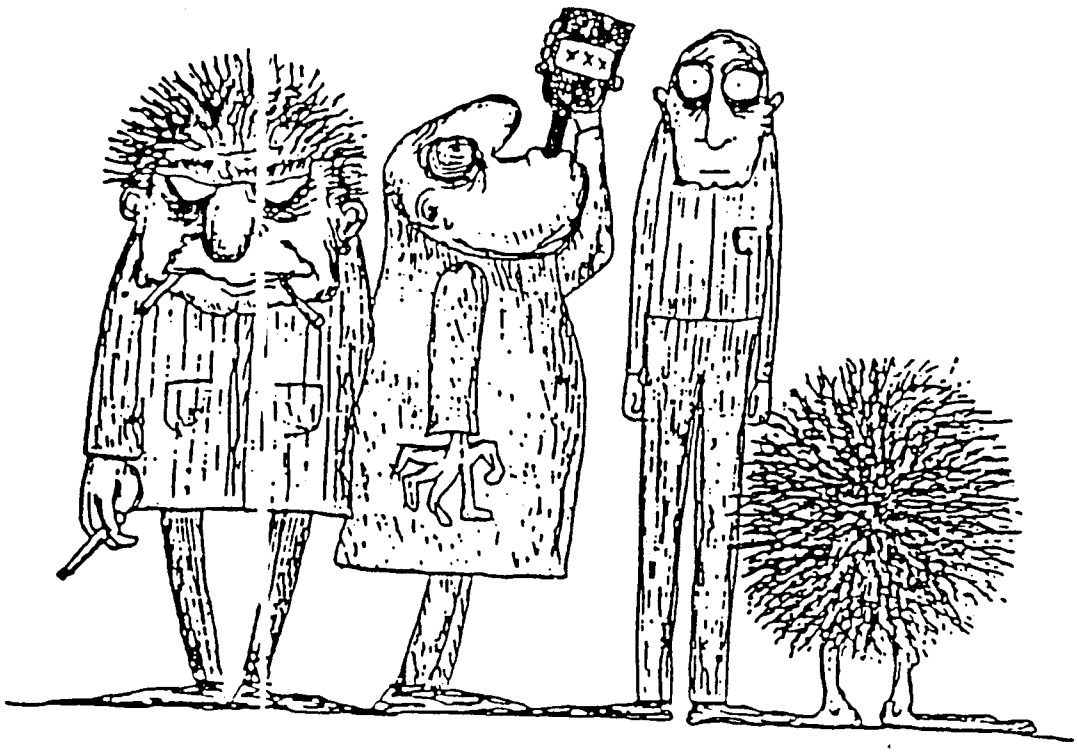
DECLARATION

I, Andrew WALUBO, declare that this thesis represents my own work and that it has not been submitted to this or any other institution in application for admission to a degree or any other qualification.

VARIABILITY IN DRUG RESPONSE

People vary not only in their appearance and philosophy but also in their physiology and biochemistry, and in their occupations and habits that may bear on the way in which they respond to drugs.



(Tucker, 1994)

ACKNOWLEDGMENT

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ABSTRACT

This is a study of the role of oxygen free radicals in isoniazid induced toxicity and its implications on the safety of isoniazid containing regimens in elderly patients. During isoniazid metabolism, toxic metabolites are produced which, together with isoniazid, may lead to toxicity by mechanism (s) yet unknown. Understanding the mechanism of toxicity is important for effective management and prevention of isoniazid induced toxicity. Elderly patients take priority for this consideration because isoniazid is more toxic in these people. Consequently, "the therapeutic importance of isoniazid metabolism in elderly patients" was found a suitable title for this work.

Isoniazid metabolites; acetylisoniazid, monoacetyl-hydrazine and diacetylhydrazine were synthesized and characterized by thin layer chromatography, high performance liquid chromatography (HPLC), ultra-violet absorption spectra and mass spectrometry. Thereafter, these compounds together with hydrazine were used in the subsequent experiments.

First, a comprehensive assay for determination of pyrazinamide, rifampicin, isoniazid and the four hydrazine metabolites is described. It involves organic solvent extraction of pyrazinamide and rifampicin, followed by derivatization of isoniazid, monoacetylhydrazine and hydrazine with salicylaldehyde and thereafter extracted into diethylether. Acetylisoniazid and diacetylhydrazine were hydrolyzed to isoniazid and monoacetylhydrazine, respectively, and processed as above. Using a gradient solvent programer, pyrazinamide and rifampicin were analyzed on a C_8 (5 um) column at 248 nm, while isoniazid and metabolites were analyzed on a C_{18} (5 um) ODS2 column at 280 nm.

In another experiment, the three drugs and four metabolites were screened *in vitro* for production of reactive oxygen species, in particular, superoxide anion and hydrogen peroxide radicals. The reaction mixture consisted of oxyhaemoglobin in K_2PO_4 buffer, at pH 9.2, 30°C with or without superoxide dismutase (SOD) or catalase or both. Isoniazid reaction was the most sensitive to inhibition by SOD while acetylisoniazid and monoacetylhydrazine reactions were sensitive to both SOD and catalase. Isoniazid, monoacetylhydrazine, acetylisoniazid and hydrazine activated

oxygen to reactive oxygen species while pyrazinamide, rifampicin and diacetylhydrazine did not. Therefore, only isoniazid, acetylisoniazid, monoacetylhydrazine and hydrazine were selected for further studies of reactive oxygen species.

Involvement of reactive oxygen species in isoniazid-induced hepatotoxicity was investigated by incubation of rat liver slices with isoniazid and each of the selected hydrazide metabolites (hydrazine, monoacetylhydrazine and acetylisoniazid), separately. Reactive oxygen species were detected by measuring lipid peroxides using the thiobarbituric acid-reacting-substances (TBARS) test while hepatotoxicity was assessed histologically. Hydrazine induced lipid peroxidation but isoniazid, acetylisoniazid and monoacetylhydrazine did not, most probably because the three compounds interfered with the TBARS test. Therefore, the TBARS test was found unsuitable for detecting lipid peroxidation induced by hydrazide compounds. Because hydrazine is a metabolite of isoniazid, it was concluded here that reactive oxygen species may contribute to isoniazid induced hepatotoxicity.

Since occurrence of toxicity depends on the amount of toxic agent in the body, disposition of the three drugs and four metabolites was

compared in 10 young (25 ± 1 years old) and 9 elderly (72 ± 3 years old) patients during treatment with pyrazinamide, rifampicin and isoniazid on days 1, 30, and 90. The pharmacokinetics of the three drugs and the four metabolites were similar on the three occasions in both young and elderly patients. For the metabolites, maximum concentration (Cmax), time to reach Cmax (Tmax) and area under curve (AUC) in both groups, were comparable. In general, during antituberculosis therapy with isoniazid, rifampicin and pyrazinamide, the metabolism of isoniazid to hydrazide metabolites is not related to age.

As plasma concentrations of the metabolites and drugs were lower than those used in the *in vitro* experiments, it was necessary show whether generation of reactive oxygen species *in vivo* occurs. Allantoin, a product of purine metabolism, was used to detect reactive oxygen species in patients. Oxidative stress during antituberculosis therapy was compared in 10 young and 9 elderly patients. Before treatment, allantoin plasma concentrations in young and elderly patients were similar. Administration of a combination of isoniazid, rifampicin and pyrazinamide increased the plasma concentration of allantoin in both groups of patients. Allantoin concentrations (mean ± s.e.) at six hours were higher (P

< 0.05) in elderly than in young patients on day one, (8.22 ± 1.50 vs 1.89 ± 0.98 ug/ml); day 30, (5.85 ± 0.82 vs 0.87 ± 0.57 ug/ml); and day 90, (4.84 ± 1.24 vs 0.52 ± 0.50 ug/ml). Although the total amount of allantoin excreted and the rate of allantoin formation at steady state were similar in both groups on the three occasions, allantoin clearance was nine times higher in young than in elderly patients. It was concluded that the high allantoin concentrations in the elderly was due to decreased renal excretion, while the increase in plasma allantoin concentration when the drugs were administered implies that these antituberculosis drugs induced oxidative stress in both the young and elderly patients.

In general, depending on the amount of hydrazine formed during isoniazid metabolism, reactive oxygen species may contribute to isoniazid induced toxicity.

PUBLICATIONS RELATED TO THIS PROJECT

1. **Comprehensive assay for pyrazinamide, rifampicin and isoniazid with its hydrazine metabolites in human plasma by column liquid chromatography.** J. Chromatogr. 1994; 658: 391-396.

2. **The generation of oxygen free radicals by antituberculosis drugs in vitro.** Proc. of the South African Pharmacology Society Congress, 22-24 Sept., 1994. Cape Town. Manuscript submitted to Biophysica et Biochemica.

3. **The disposition of isoniazid metabolites during anti-tuberculosis therapy.** Proc. of the 7th South East Asia Drug Metabolism Workshop, 6-9th Nov., 1994, Bangkok, Thailand. Manuscript submitted to The J. Clin. Pharmacol. Ther.

4. **Oxidative stress during antituberculosis therapy.** Proc. of the 12th International Congress of Pharmacology, 24-29th, July, 1994. Montreal, Canada. Can. J. Physio.& Pharmacol. vol.72, Suppl.1, pg 595, 1994. Also, published in J. Biomed. Envir. Sci., 1995; 8: 106 -113.

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CHAPTER ONE

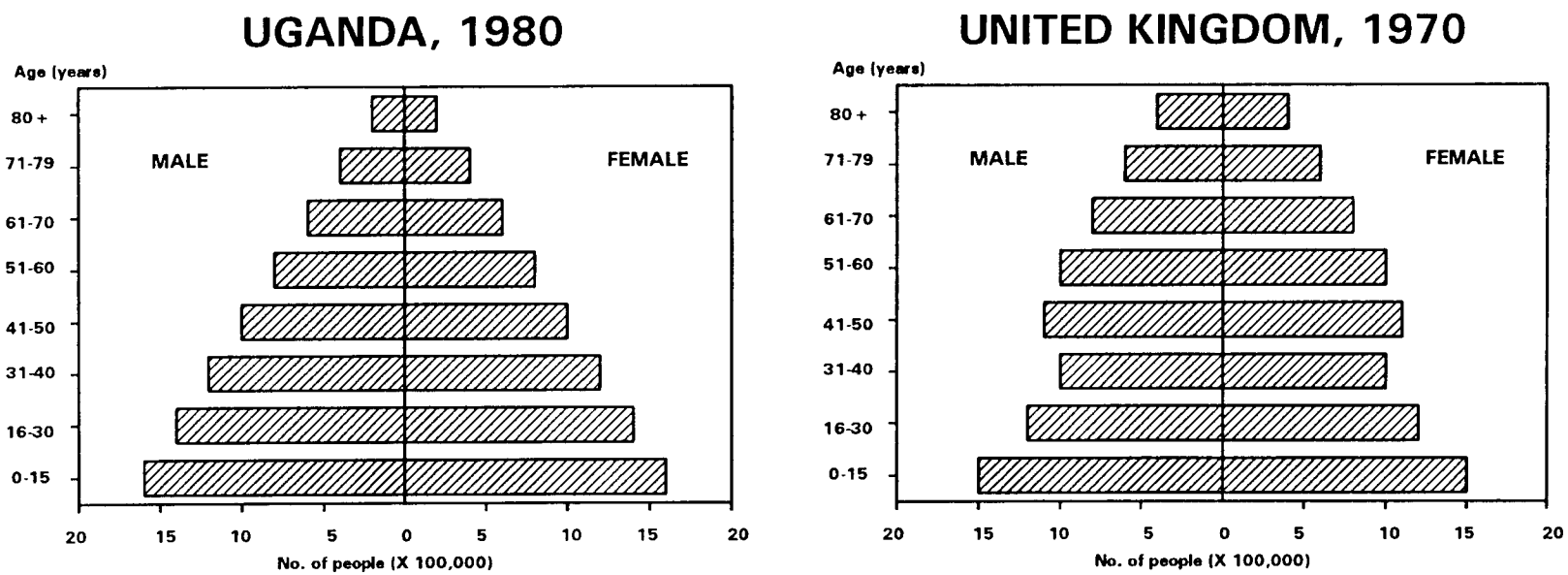
GENERAL INTRODUCTION

1.0 The elderly

"Elderly" refers to people aged 65 years and over. At a growth rate of 2.5 per cent per year, the world's elderly population is growing faster than the total population (Suzman, 1992). In thirty-two developed countries, it was estimated that 16 - 26% of the population will be elderly by the year 2020, versus 10 - 16% in 1990. For example, in the next forty years, twenty percent of the U.S. population will be elderly compared to 12.6% in 1990 (Suzman, 1992; Holden, 1990). The fastest growing segment of the elderly population are the "old old" (persons aged 80 years and over). The "old old" constitute 20% of the elderly population of the U.S.A. and this is expected to triple between 1980 and 2020 (U.S. Bureau of the Census, 1989; U.S. Senate Spec., 1986).

While developed countries are far ahead in the age demographic transition, developing countries have yet to show this trend. In developing countries, the number of elderly people is small compared to other age groups. This is depicted by the pyramidal shape of their population age structure (Fig. 1.1), a feature characteristic of communities with high infant mortality and fertility rates, and a low life expectancy (Macfadyen, 1992). However, due to effective World Health Organisation (WHO) programmes, some developing countries are beginning to experience a culture of demographic aging that has swept through the developing world.

Fig. 1.1 An illustration of a population demography for a developing country, Uganda, in 1980 (Baitera, 1980), and a developed country, The United Kingdom, in 1970 (Suzman, 1992).



Indeed, the fastest growing population of elderly people in the world is in developing countries. Of the 1.2 million annual world increase in people over 55 years, 80% occur in developing countries (Kane et al, 1990). For instance, in Indonesia, population change in people over 75 years of age from 1988 to 2005 (17 years) is estimated to be 12% while that of USA from 1990 to 2020 (30 years) shall rise by only 5.1% (Torrey et al, 1987; Kinsella, 1988; Brody, 1985; Suzman, 1992). Life expectancy over the same period (17 years) is to increase from 60.7 to 66.6 years for Asia and 52.6 to 59.8 years for Africa. In the U.S.A., life expectancy is to change in 10 years (1990 to 2000) from 72 to 73 years for males and 78 to 81 years for females (Natl. Res. Counc., 1987). Therefore, in developing countries the population of aged persons is growing fast and there will be a need in the coming years for all health personnel to pay attention to health issues associated with aging.

1.1 Tuberculosis

Tuberculosis is an infectious disease caused by *Mycobacteria* and is characterised by formation of tubercles and caseous necrosis in tissues (Friels, 1981). The disease has been in existence since the earliest times; a similar condition having been described by the ancient Chinese (Dubos, 1952), and was found in the Egyptian skeletons dating 2,500 B.C.(Morse, 1964). Nevertheless, it was only in 1882 that the causative organism was discovered by Dr. Robert Kochs as *Mycobacteria tuberculosis* (Kochs, 1882). Many other species have since been described but *Mycobacteria*

tuberculosis humanus remains the most common cause of tuberculosis in man. Infections due to *Mycobacteria bovis* and *avium* are rare. Occasionally *Mycobacteria kansasii* and other unclassified *Mycobacteria* are isolated in some atypical tuberculosis like infections.

1.2 Epidemiology of tuberculosis

Tuberculosis is a world wide problem. According to WHO, it was estimated that every year 30 million people are infected with tuberculosis, 10 million are newly infected and 3 million die from the disease (Bulla, 1981). Tuberculosis is more prevalent in developing countries where, even before the advent of human immunodeficiency virus (HIV), tuberculosis was one of the major causes of morbidity and mortality in young adults (Holdiness, 1985). Epidemiology of the disease has been well studied in developed countries. Tuberculosis was the leading cause of deaths among 38 communicable diseases reported in 1979 to the centres for Disease Control in the United States, and the number of deaths exceeded the combined total for the other 37 communicable diseases (Youmans, 1979). Due to good tuberculosis control programs in the U.S.A., in past 3 decades, the incidence of tuberculosis was falling at a case rate of 5 to 6% per year up to 1985 when the HIV epidemic struck. This fall was negatively correlated by an increase in tuberculosis cases in elderly people where between 1968 and 1982 the percentage of patients above 65 years with newly diagnosed tuberculosis increased from 18.9% to 29.1% (Stead et al, 1985). In the same period the percentage increase in the elderly

population was from 9.2% to 11.3%. In Arkansas state, tuberculosis case rate was 60 per 100,000 for the elderly versus 23 per 100,00 in those 40-60 years of age (Stead et al, 1985). Whether this was reactivated tuberculosis or nosocomial infection, it denotes a higher incidence of tuberculosis in elderly people of developed countries.

The implication here is that such catastrophes are higher in developing countries where the disease is highly prevalent and many cases and deaths are not reported or recorded. The prevalence of tuberculosis in elderly people of developing countries is not well documented. Nevertheless, there is a need to improve quality of life in elderly people during treatment for tuberculosis.

1.3 Tuberculosis in the elderly

Tuberculosis in the elderly is a syndrome whose features, except for age of the patient, are difficult to characterise. In spite of similar causative bacilli, the manifestations of tuberculosis in elderly patients are atypical (Alverez et al, 1987; Katz et al, 1987; Nagami and Yoshikawa, 1984; Rudd, 1985). A low incidence of fever, weight loss, night sweats, sputum production and heamoptysis was noted in the older group (n = 35; mean age 74.3 years) (Alvarez et al, 1987). In the same report, four elderly patients had no symptoms while all young patients (n = 29; mean age 45 years) were symptomatic. Tuberculosis related deaths occurred in 20% of the elderly (7 patients) versus 3% of the young (1 patient).

In another study, none of the elderly patients (n = 52; mean age 70 years) presented with heamoptysis (Katz et al, 1987). The prevalence of fever, anorexia, weight loss and cough in these elderly were similar to that in young patients (n = 17; mean age 51 years) patients. Radiographic findings also differed between young and elderly patients. Right lower lobe infiltrates were more prevalent in young patients (33%), than in elderly patients (9.6 %), (Khan et al, 1977; Hadlock et al, 1980; Miller and Macgregor, 1978).

Diagnosis of tuberculosis in old people is more often difficult because in the elderly, active pulmonary tuberculosis cannot be ruled out on the basis of absence of symptoms or a negative mantoux test. A high level of suspicion is necessary and a two-step (booster) mantoux test is recommended in anergenic patients or a therapeutic trial in difficult cases (Hadlock et al, 1980; Comstock, 1986).

Having made a diagnosis of tuberculosis in an elderly patient, administration of antituberculosis chemotherapy to this patient results in a pharmacological complex about which little is known. There is little information regarding a combination of tuberculosis, the elderly and antituberculosis drugs (Teale et al, 1993).

Treatment of tuberculosis in the elderly poses a challenge to tuberculosis therapeutic policy. As in the case of young patients, the standard antituberculosis regimen consists of isoniazid, pyrazinamide and rifampicin. This regimen is used despite reports of severe adverse reactions in elderly patients on this regimen (Woo

et al, 1987 and 1992; Teale et al, 1993). It is also known that the incidence of isoniazid induced hepatitis rises with age (i.e. rare under 20 years of age, up to 0.3% between 20 and 34 years, up tp 1.2% between 35 and 49 years, and up to 2.3% at 50 years and over) (Kopanoff et al, 1978; Riska et al, 1976), and that the risk of isoniazid toxicity is worsened by addition of pyrazinamide (8%; Hong Kong, 1976) and rifampicin (4%; Brouet, 1974). The regimen is still used and there is no hope for a substitute. There is a need to improve quality of life of elderly patients during treatment for tuberculosis.

In the present project, a mechanism of isoniazid induced toxicity is investigated. The pharmacokinetics of rifampicin, pyrazinamide, isoniazid and its hydrazide metabolites have been determined because these seven compounds co-exist during tuberculosis therapy, are potentially hepatotoxic (Girling, 1978), and have been associated with severe adverse effects in elderly patients (Woo et al, 1987 and 1992). Hydrazide metabolites of isoniazid were studied because they are more toxic than isoniazid, and were suggested to cause isoniazid related hepatotoxicity (Mitchel et, 1976; Sarma et al, 1986). Very little is known about the disposition of hydrazide metabolites in elderly patients during antituberculosis therapy and the mechanism of isoniazid induced toxicity is still unknown.

This project is based on the concept of understanding the mechanism of drug-induced toxicity as a prerequisite to formulating the most effective preventive measures against the drug's side effects.

CHAPTER TWO

ANTITUBERCULOSIS CHEMOTHERAPY.

PART 0

2.0 Introduction

Treatment of tuberculosis has improved during this century. The old fashioned course of observation, rest, sunshine and collapse therapy is no longer applied. The past 55 years have been marked by a series of inventions of new and modern antituberculosis chemotherapeutic agents with higher efficacy rates such that with the right treatment a cure rate of 100 per cent is predictable, in the shortest possible time of treatment and at the cost within reach of most countries.

In 1940 a sulphonamide promin (glucosulfone sodium), a derivative of dapsone, was demonstrated to have bacteriostatic effects on *Mycobacteria tuberculosis* (Youmans, 1979). Although this compound arrested progress of the disease in experimentally infected guinea pigs, it was abandoned because high concentrations were required for this action. Further investigations on antituberculosis activity of benzoic and salicylic acid related compounds lead to discovery of p-aminosalicylic acid (PAS) in 1946. Antituberculosis activity of streptomycin was demonstrated in animals in 1944 (Feldman, 1944), and has been used in man since 1947, the year when thiacetazone was introduced. Later in 1952, workers in three independent laboratories in America and Germany, reported the antituberculosis activity of isoniazid

(Bernstein, 1952; Fox, 1952; Offe, 1952). Isoniazid has proven a wonder drug in the treatment of tuberculosis; it is used for prophylaxis and is a keystone of the most effective antituberculosis regimens. Introduction of ethambutol in 1961, and rifampicin in 1968 (Crossland, 1980) has revolutionised antituberculosis chemotherapy not only by increasing efficacy but also by reducing the period of treatment and frequency of dosing.

Currently, there are over ten antituberculosis drugs on the market. They have been classified in different ways such as popular use, bactericidal or bacteriostatic, and naturally occurring or synthetic. Classification as first and second-line drugs is easier and is more frequently used. Isoniazid, rifampicin, pyrazinamide, streptomycin and ethambutol are considered first line drugs because of their higher potency, relatively low toxicity and ease of administration. P-aminosalicylic acid (PAS), thiacetazone, ethionamide, cycloserine, kanamycin, capreomycin, and viomycin are second line drugs because they are less potent and more toxic. They are used to supplement the actions of first line drugs and to guard against emergence of resistance.

Therefore, for treatment of tuberculosis, a combination of three to four drugs, preferably two or three first line drugs and one second line drug, are used for the first two or three months and there-after treatment is continued with two drugs for such a period depending on the form of tuberculosis and drugs being used. When isoniazid, streptomycin, and thiacetazone or PAS are used, treatment is continued for twelve to twenty four months, while, in case of

isoniazid, rifampicin, pyrazinamide, streptomycin and or ethambutol it is continued for six to twelve months. Tuberculosis of the central nervous system and milliary tuberculosis are treated for longer periods than other forms of tuberculosis. The long period of treatment for this disease is to avoid relapses due to the persistent semidormant bacilli which are killed slowly with time.

CHAPTER TWO; PART I

ISONIAZD AND HYDRAZIDE METABOLITES

2.1 ISONIAZID

2.1.1 Chemistry

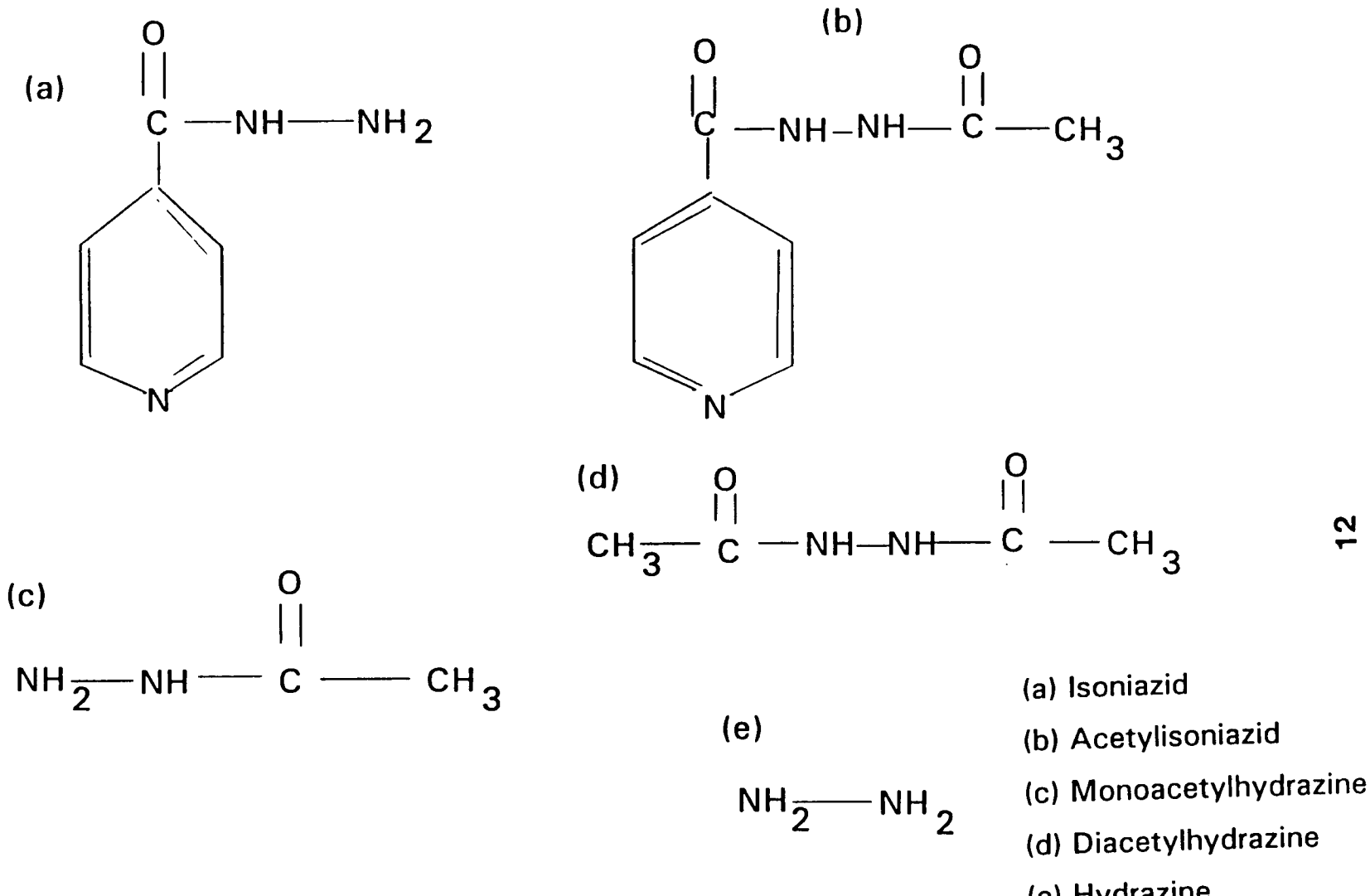
Isoniazid (isonicotyl hydrazine) is one of the most widely used antituberculosis drugs. It is used in the treatment of active disease as well as for prophylaxis in those at risk and below 35 years of age. The structure of isoniazid is as shown in Figure 2.1. It is a derivative of isonicotinic acid with a molecular weight of 137.2 and a melting point of 172°C. It is highly water soluble and has dissociation constants of 1.8, 3.5, and 10.8 at 20°C (Clarke's, 1986).

It is a white crystalline powder that is dispensed in form of tablets of 100 mg or 300 mg each, syrup of 10 mg/ml, and injection solution of 100 mg/ml. It is commonly given orally; 5-10 mg/kg/day in adults and 10-15 mg/kg/day in children less than 4 years. However, with the advent of short course and intermittent chemotherapy there is a wide variation in dosages today.

2.1.2 Antimycobacterial activity

Isoniazid is tuberculocidal against actively multiplying bacilli and tuberculostatic to slowly growing bacilli. *In vitro* minimum inhibitory concentration (MIC) for mycobacteria tuberculosis is of

Fig 2.1 THE STRUCTURES OF ISONIAZID AND ITS METABOLITES



(a) Isoniazid
(b) Acetylisoniazid
(c) Monoacetylhydrazine
(d) Diacetylhydrazine
(e) Hydrazine

order 0.025 to 0.05 μg/ml (Mandell, 1985). The drug is more effective against extracellular organisms at neutral pH. When isoniazid is used alone to treat tuberculosis, resistance develops within a few weeks. Fortunately, there is no cross resistance between isoniazid and other antituberculosis agents, making combined drug therapy essential to avoid emergence of resistant organisms.

The mechanism of action of isoniazid is not fully known. Takayama et al (1975) proposed that isoniazid inhibits synthesis of mycolic acid in Mycobacterial cell wall. This explains the highly selective action of isoniazid to mycobacteria. Later, Herman and Weber (1977) suggested that isoniazid also interferes with lipid and nucleic acid synthesis and the glycolytic processes of mycobacteria.

2.1.3 Absorption and distribution

Isoniazid is rapidly and completely absorbed in man after oral and parenteral administration (Barclay et al,1953; Des Prez and Boone, 1961; Elmendorf et al, 1952; Robson and Sulliva, 1963). It is absorbed mainly in the small intestines (Barley et al, 1972) and peak concentrations are achieved within 1 to 2 hours after ingestion (Robson and Sulliva, 1963). Absorption is reduced by presence of anti-acids like aluminium hydroxide gel (Hurtwitzand Schlozman, 1974), simultaneous intake of food (Melander et al, 1976) and some types of sugars (Rao et al, 1971). Plasma concentrations tend to be lower in fast acetylators than slow acetylators and it was proposed that there is appreciable first pass effect in rapid

acetylators because acetylator polymorphism is well expressed in the mucosal cell lining of small intestines (Hearse and Weber, 1973; Jenne, 1965).

Isoniazid is distributed in total body water with apparent volume of distribution of approximately 61 ± 11% of body weight (Jenne et al, 1961). Since there is no appreciable plasma protein binding (Boxenbaum et al, 1975) this indicates that the distribution is both extracellular and intracellular. Significant amounts of the drug were detected in cerebrospinal fluid, pleural effusion, saliva and faeces of healthy volunteers and patients with tuberculous meningitis (Barclay et al, 1953; Boxenbaum and Riegelman, 1973; Elmendorf et al, 1952). Nevertheless, data from these studies has been insufficient for proper use of isoniazid in treatment of tuberculous meningitis. Higher doses (> 10 mg/kg/day) are still used in the treatment of this form of tuberculosis (Lam et al, 1989). Since therapeutic effectiveness and toxicity of isoniazid depend on extent and intensity of exposure of the body to the drug, kinetic studies in this aspect are essential to understanding of how long and to what extent the drug stays in the central nervous system. This, in turn, leads to a rational approach on the use of isoniazid for treatment of tuberculosis of the central nervous system.

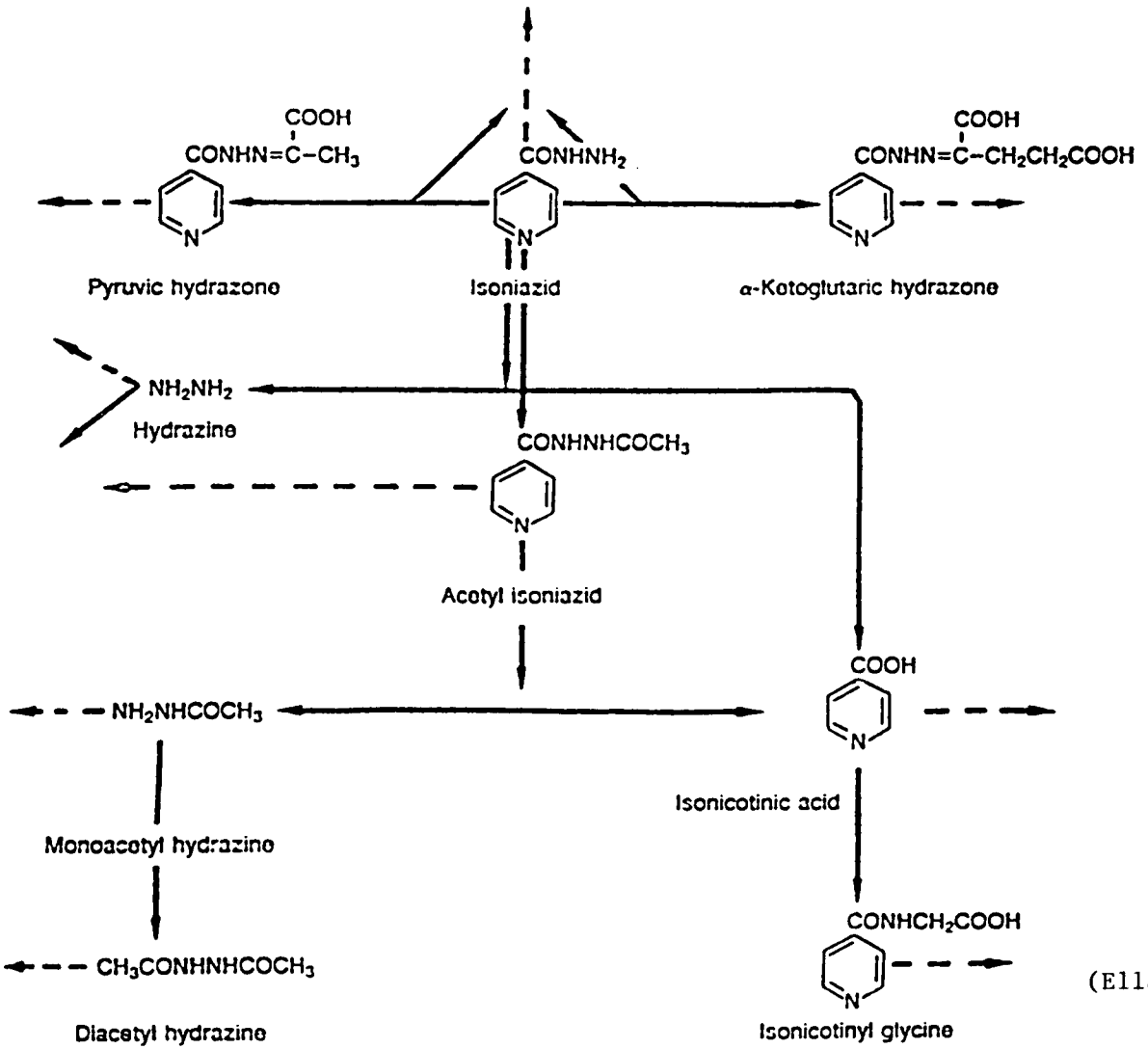
2.1.4 Metabolism and elimination

Isoniazid is cleared from plasma mainly by acetylation at a rate which is determined by type of the hereditarily polymorphic N-acetyltransferase enzyme an individual carries (Evan, 1965; Evan

and White, 1964; Glowinsk et al, 1978; Jenne, 1965). Individuals are therefore classified as either rapid or slow acetylators depending on the rate at which they eliminate isoniazid (Weber and Hein, 1979). Therefore, elimination half-life of isoniazid is determined by acetylator status. After an intravenous dose of isoniazid, rapid acetylators were considered as having isoniazid elimination half-life shorter, and slow acetylators longer than 110 minutes (Weber and Hein, 1979). However, different antimodes have been observed by other workers. Paulsen et al (1985) and Kergueris et al (1986) used antimode 120 minutes, most probably due to difficulties in classifying heterozygous (Rr) individuals when small populations are studied. Also, oral administration of drugs is associated with longer half-life values compared to values after intravenous administration (Chan and Wong, 1988; Walubo et al, 1991b). Man and rabbit species exhibit this acetylating capacity (Hearse and Weber, 1973) not only for isoniazid but other drugs as well, for example; hydrallazine, procainamide, and sulphonamides. The optimum pH for inactivation of isoniazid is 6.5 to 7.0. The enzyme is resistant to sulphhydryl inhibition and is insensitive to induction (Schulte et al, 1974). Furthermore, evidence from studies in rabbits suggests that rapid and slow N-acetyltransferase are structurally different enzymes (Weber et al, 1978).

Figure 2.2 shows important metabolic pathways for elimination of isoniazid (Boxenbaum and Reigelman, 1976; Ellard and Gammon, 1976). The major metabolic step is acetylation of isoniazid to acetylisoniazid (Mitchell et al, 1976). Acetylisoniazid is biotransformed to isonicotinic acid and monoacetylhydrazine (Ellard

Fig 2.2 Metabolic pathways for isoniazid.



(Ellard and Gammon, 1976)

et al,1972). Isonicotinic acid is then conjugated with glycine and excreted in urine. Most monoacetylhydrazine is acetylated to diacetylhydrazine which together with some free monoacetylhydrazine are excreted by the kidneys. Some isoniazid is conjugated to form pyruvic and ketoglutaric hydrazones, and a smaller proportion is hydrolysed to hydrazine and isonicotinic acid. Some hydrazine is lost via the renal route while the rest is presumably biotransformed by yet unexplained mechanism.

The metabolic fate of hydrazine and monoacetylhydrazine is still uncertain because of the low recovery of these compounds in urine and plasma (Weber and Hein, 1979). However, it has been shown that monoacetylhydrazine is the only metabolite which exhibits polymorphic metabolism in a manner similar to that of isoniazid (Ellard and Gammon, 1976). It was reported in the same communication, that there is little or no difference between plasma concentrations of monoacetylhydrazine in the two phenotypes. This contradicted earlier proposals that rapid acetylators produce more monoacetylhydrazine, and are therefore more prone to suffer from isoniazid induced hepatotoxicity (Black etal, 1975). It was demonstrated in rats that monoacetylhydrazine can be biotransformed by liver microsomal enzymes to a potent acylating agent that can cause hepatic necrosis, and thereby concluded, with clinical evidence (Mitchel et al, 1976), that it is the major toxic metabolite responsible for isoniazid induced liver toxicity. A lot of clinical evidence against this hypothesis has accumulated (Ellard et al, 1978; Girling, 1978; Ellard, 1984; Gurumurthy et al, 1984). It was suggested that, since a rapid acetylator also converts

monoacetylhydrazine more rapidly to diacetylhydrazine, the risk of liver toxicity should be the same in both phenotypes (Ellard and Gammon, 1976; Timbrell et al, 1977). Further studies on monoacetylhydrazine have shown that at concentrations of 1 μg/ml this metabolite can inhibit the antimycobacterial activity of isoniazid *in vitro* (Weber and Heine, 1979). The clinical significance of this is yet to be established.

Renal elimination of isoniazid is approximately 11 to 27 per cent of total body clearance for rapid and slow acetylators, respectively. This is considered unimportant compared to the metabolic pathway. About 75 to 95 per cent of a dose of isoniazid is excreted in urine within 24 hours as metabolites. N-acetylisoniazid and isonicotinic acid are the major urinary metabolites.

2.1.4.1 Hydrazine

Hydrazine is a metabolite of isoniazid that had been neglected on grounds that it is formed in negligible amounts. The structure of hydrazine is as shown in figure 2.1e.

Hydrazine is a potent toxin and carcinogen as classified by the American Conference of Government and Industrial Hygiene (Albert et al, 1977). Hydrazine was incriminated in causing lupus-like syndrome in man when hydrallazine and isoniazid were administered (Durant et al, 1980). In animal studies, hydrazine caused convulsions, hepatic toxicity, renal damage, blood injury and affected many metabolic processes (Back and Thomas,1970). It is associated with causation of choroidal carcinoma in man, and

questions arise as to whether it may contribute to hepatic angiosarcoma, which is a tumour associated with all hydrazide compounds (Daneshmend et al,1979; Toth, 1975 and 1976). Hydrazine is a potent inhibitor of many enzyme systems in the body and can cause hepatic necrosis. There is a variation in sensitivity to hydrazine toxicity in different species. Massive hepatic necrosis was observed in monkeys at doses that caused a less severe liver injury in rats (Back and Thomas, 1970). This suggests that humans may be more susceptible to hydrazine toxicity. As cited earlier, recovery of hydrazine metabolites in urine cannot account for the total amount presumably formed during isoniazid metabolism. Recently, it was reported that rifampicin increased hydrazine formation from isoniazid after two weeks of co-administration of the two drugs in healthy volunteers (Sarma et al, 1986). Therefore, in wake of the unproven role of monoacetylhydrazine in isoniazid related hepatotoxicity, hydrazine metabolite has been implicated as a possible culprit. Recent studies have shown that hydrazine is produced in significant quantities during treatment with isoniazid, rifampicin and pyazinamide (Gent et al, 1992; Walubo et al, 1991d). In one report, hydrazine was incriminated in the death of an elderly patient (Woo et al, 1992). Consequently, further studies on the kinetics of hydrazine as a metabolite of isoniazid are essential to explore and perhaps prevent its role in isoniazid related toxicity.

2.1.5 Side effects of isoniazid

The major side effect of this drug is liver toxicity which is clinically, biochemically, and histologically indistinguishable from viral

hepatitis. The risk of developing severe isoniazid induced hepatotoxity increases with age; it is rare in persons under 20 years of age, up to 0.3 percent in those 20 to 34 years of age, up to 1.2 per cent at 35 to 49 years, and up to 2.3 per cent at 50 years and over (Kopanoff et al, 1974). Stead et al (1981 and 1987) reported that the risk of developing isoniazid related hepatitis is about 5 per cent in persons above 65 years of age and over. The frequency of isoniazid induced hepatitis is not related to acetylator phenotype (Singapore Tuberculous Service, 1977). Therefore, it is recommended that base line liver function tests should be determined in patients on isoniazid containing regimens. Isoniazid can cause peripheral neuropathy by interfering with pyridoxine metabolism. This is common in patients with predisposing factors like chronic alcoholism, diabetes mellitus and malnutrition. Pyridoxine 10 mg/day has been prescribed as prevention therapy while 100 mg/day has been used to treat the established lesion. Other side effects include allergy, skin rashes, gastric upsets, febrile reactions and, rarely, red cell aplasia (Claiborne and Dutt,1985), psychosis, and depression.

2.1.6 Drug interactions

There is an increased risk of hepatotoxicity when isoniazid and rifampicin are used together (Bistritzer etal, 1980), and the induced toxic hepatitis is different from that by either drug (Gronhage-Riska et al, 1978). P-aminosalicylic acid led to elevated isoniazid plasma concentrations and prolonged the serum half-life of isoniazid (Schneck et al, 1979). Isoniazid inhibits phenytoin metabolism

predisposing some patients, especially slow acetylators, to phenytoin toxicity (Kutt et al, 1970). Many interactions with other drugs have been reported but their clinical significance is not yet known. This includes drugs like, warfarin, procainamide, carbamazepine, primidone, prednisolone, diazepam, 25-hydroxyvitamin D3, and enflurane to mention but a few.

2.1.7 Patho-physiological factors

Table 2.1 compares the pharmacokinetic parameters of isoniazid in relation to age and acetylator status (Advenier et al, 1980). For unexplained reasons the dose of isoniazid was higher in the elderly group than in the young group. There was no difference in the pharmacokinetics of isoniazid between elderly and young people (Advenier et al,1980; Paulsen and Nilsson, 1985; Kergueris et al, 1986). Although not proven by liver biopsy, it was concluded that age is an insignificant factor in the acetylator status of an individual (Evans et al, 1960; Advenier et al,1980). Nevertheless, as mentioned earlier, the risk of developing isoniazid induced hepatotoxicity increases with age. The reason for this phenomenon is not well explained. Liver disease in a patient is an indicator for reassessing and adjusting the dosage of isoniazid if deemed necessary. Renal disease has limited effect on isoniazid clearance such that dosage adjustment is not usually considered until creatinine clearance is less that 30 ml/min. However, some workers have demonstrated reduced acetylation of isoniazid in chronic renal failure (Kim et al, 1993). Chronic diseases such as diabetes mellitus (Mattila and Tiitinen, 1967), mongoloid subjects

Table 2.1 Pharmacokinetic parameters (mean ± s.d.) of isoniazid after intravenous administration in subjects under and over 60 years of age and in fast and slow acetylators (Advenier et al, 1980).

PARAMETER	SUBJECTS OVER 60 YEARS		SUBJECTS UNDER 60 YEARS	
Phenotype	Fast	Slow	Fast	Slow
Number (n)	5	6	3	7
Age (years)	89 ± 11	80 ± 10	39 ± 15	36 ± 13
Weight (kg)	54.2 ± 12	56.2 ± 11	61.4 ± 2	61.9 ± 10
Dose (mg/kg)	7.1 ± 0.5	6.7 ± 0.9	4.9 ± 0.2	5.0 ± 0.8
T1/2 (h)	1.4 ± 0.7*	3.1 ± 0.6	1.1 ± 0.1	3.1 ± 1.1
Ke (/h)	0.16 ± 0.1	0.14 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
Vz (L/kg)	0.15 ± 0.1	0.1 ± 0.03	0.2 ± 0.01	0.3 ± 0.03
AUC (μg/ml.h)	16.2 ± 6.6	38.3 ± 19*	12.1 ± 3.3	24.5 ± 9*
CLp (ml/min/kg)	8.4 ± 3.6	3.6 ± 1.8*	7.4 ± 2.0	3.7 ± 1.2*

T1/2 = Plasma elimination half life. Ke = Elimination constant rate. Vz = Apparent volume of central compartment. AUC = Area under the plasma concentration-timecurve. CLp = Plasma total clearance.

* = Statistical differerences between fast and slowacetylators; $p < 0.05$.

(Airaksinen et al, 1969), obesity (Reidenberg, 1973), tuberculosis (Tiitinen, 1969), and chronic alcoholism (Tiitinen, 1969) did not effect isoniazid metabolism. However insulin enhances the intestinal uptake of isoniazid (Daysz and Wisniewski,1970).

2.1.8 Acetylator phenotype

There are several procedures for determination of acetylator status of individuals (Weber et al, 1979). Two procedures have been used widely in the clinic because they require urine samples which is more convenient to the patient and clinician. One is by estimation of acetylisoniazid as a proportion of total hydrazides excreted in the urine 6 to 8 hours after oral administration of isoniazid (Eidus et al, 1973). The other classifies individuals according to proportion of total sulphadimidine excreted in acetylated form at 4 to 5 hours after oral ingestion of sulphadimidine (Evan, 1960; Weber and Brenner, 1974). In conditions where renal clearance is much reduced, determination of acetylator status is best done by estimating the metabolic clearance of a drug (Fine and Sumner, 1975). The racio-geographical distribution of acetylator phenotype shows a high prevalence of rapid acetylators in the far east populations; Chinese (85%), Japanese (90%), and Hong Kong Chinese (78%), and a low prevalence in the mid-eastern populations; East Africa (45%), and United States Caucasians (41%) (Weber and Heine, 1979).

2.1.9 Assay of isoniazid and metabolites

Further studies on isoniazid metabolism have been hampered by lack of a methodology for the assay of the drug and its metabolites in plasma for routine therapeutic monitoring. Secondly, the four hydrazine metabolites are not readily available on the market and their synthesis is difficult and costly. Isoniazid and its metabolites are highly ionic compounds which are difficult to extract from plasma and absorb UV-light poorly, necesstating derivatisation for optimal extraction and detection in plasma.

Several procedures for the assay of isoniazid and metabolites in biological fluids have been reported. Unfortunately, none of these procedures was favorable in our circumstances. The Gas chromatography mass spectrometer (GC-MS) procedure (Lauteburg et al, 1981) cannot be adopted in our laboratory, while the fluorometric assay by Ellard et al (1972) and liquid chromatography method by Von Sassen et al (1987) are complex and laborious. Generally, the three procedures are too expensive for routine practice.

The procedures described for assay of isoniazid with: acetylisoniazid (Holdiness, 1982;), acetylhydrazine (Jenner and Ellard, 1987) and hydrazine (Walubo et al, 1991a) cannot be used on a single plasma sample. Furthermore, the procedure by Holdiness (1982) required adverse chromatographic conditions (e.g. pH 2.5, flow rate 4.0 ml/min and injection volume 250 ul), while that by Jenner and Ellard

(1987) was an assay for monoacetylhydrazine in urine. The assay for isoniazid and hydrazine reported by Walubo et al (1991a) failed to show monoacetylhydrazine and suffered a poor limit of detection for isoniazid (0.2 ug/ml).

CHAPTER TWO; PART II

THE CHALLENGES OF TREATING TUBERCULOSIS IN THE ELDERLY.

2.2.1 Introduction

The incidence of tuberculosis and mortality from the disease increases with age , so that in some developing countries, it is the leading cause of death among the elderly (Chest Service, 1985; Mackay and Cole, 1984; Ramos, 1991). Although potentially serious adverse reactions are said to be uncommon with antituberculosis drugs (Girling, 1984), data on adverse reactions to various regimens had been gathered from ambulant middle-aged patients only (Hong Kong, 1976; Black et al, 1976). Thus it is unclear whether the same regimens apply to elderly patients and whether adverse effects are equally uncommon. It is known that adverse reactions to antituberculosis drugs are more common in the elderly, particularly isoniazid induced hepatitis (Mackay and Cole, 1984; Gulliford et al, 1986; Walubo et al, 1991c; Umeki, 1991; Riska, 1976; Woo et al, 1987 and 1992). Therefore it was found worth-while to review information available on variations in pharmacokinetics and pharmacodynamics of antituberculosis drugs with age, to emphasize the need for studying antituberculosis drug toxicity in elderly patients.

2.2.2 Pharmacokinetic changes with age

Of the commonly used antituberculosis drugs, isoniazid, rifampicin and pyrazinamide are predominantly metabolised by the liver (Holdness, 1984). Hepatic blood volume and blood flow decrease with age (Wyne et al, 1989), and the rate of hepatic metabolism may be reduced for certain drugs (Bach et al, 1981; Castleden et al, 1989). There are few studies on the effect of age on the pharmacokinetics of antituberculosis drugs, and most studies are of single drugs in isolation in healthy elderly individuals. For isoniazid, acetylator phenotype and half-life were not affected by age in one study (Weber and Heine, 1979), while in another study a positive correlation was found between age and plasma isoniazid concentrations when corrected for sex and weight (Iselius and Evans, 1983).

No age related changes in the pharmacokinetics of isoniazid, given alone, were observed in three studies (Advenier et al, 1980; Kergueris et al, 1986; Paulsen et al, 1985). One study of rifampicin given alone did not show any difference in pharmacokinetic parameters between six elderly individuals and five young subjects (Advenier et al, 1983). There are no reports on the influence of age on pharmacokinetic parameters for pyrazinamide given as a single drug in elderly individuals. Only one study examined the effect of age on the pharmacokinetic parameters of antituberculosis drugs given in combination in hospitalised patients with tuberculosis (Walubo et al, 1991c). No differences in parameters for isoniazid, rifampicin and pyrazinamide were observed

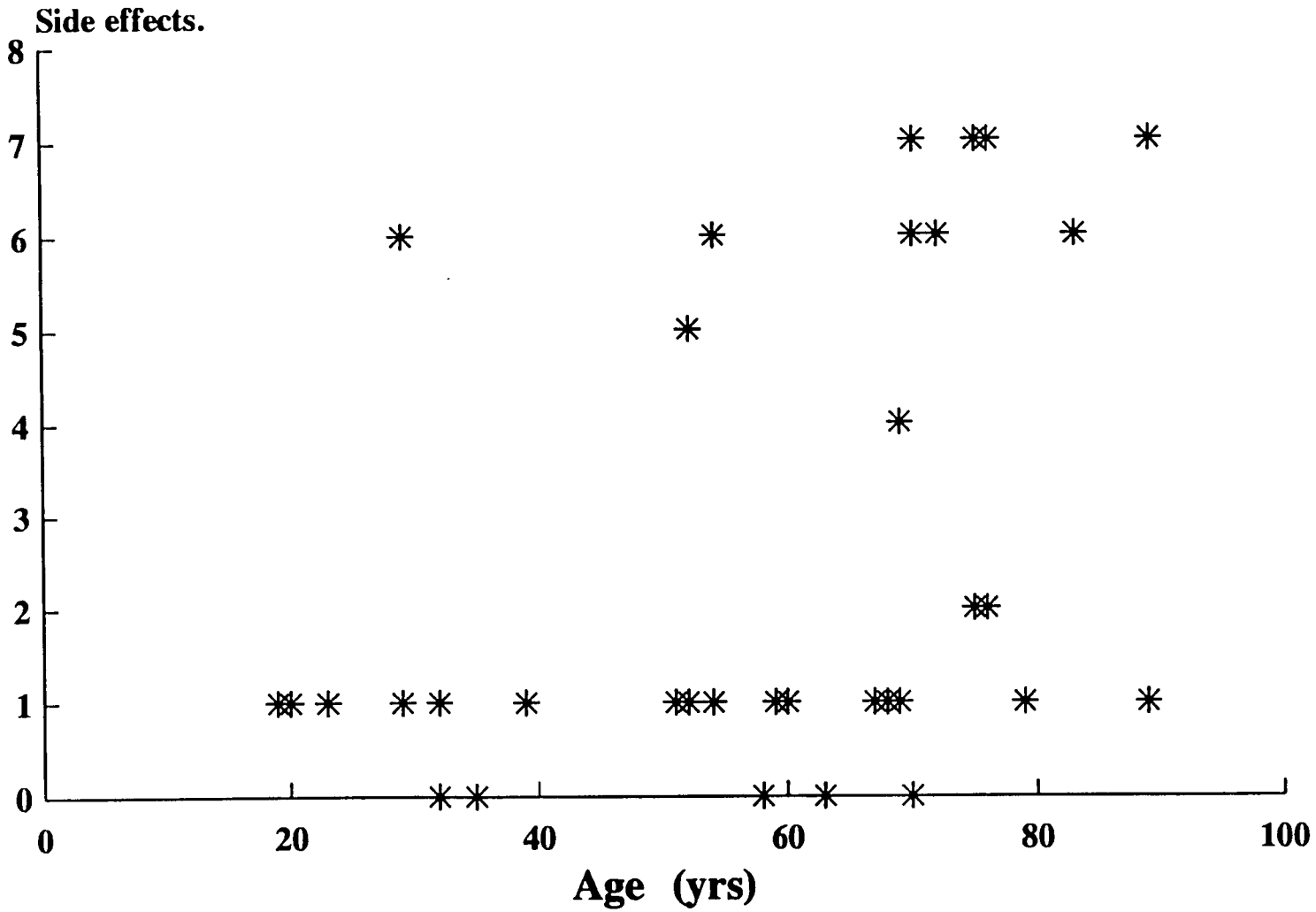
with initial dosing. However, one month after therapy, the clearances for isoniazid and rifampicin at steady state among subjects age 65 years and over were significantly lower compared to that at first dose, suggesting an interaction between the drugs. In that study, elevations in serum liver enzyme levels and other side effects were more common among elderly than younger patients (Fig 2.3).

2.2.3 Protein binding

Another factor which may affect the disposition of antituberculosis drugs in elderly patients is protein binding. In general, acidic drugs bind to albumin while basic drugs bind to alpha-1-acid glycoprotein, although there are exceptions (Kremer, 1988). In the elderly, chronic disease or malnutrition result in lower serum albumin concentrations, while alpha-1-acid glycoprotein increases with age (Woodford et al, 1964; Upton et al, 1984). Therefore, free drug concentrations of acidic drugs may rise, while that of basic drugs may fall with age. In healthy individuals, isoniazid is little bound to serum proteins, while the percentage bindings for rifampicin are quoted as 57 - 80% (Holdness, 1984). Protein binding data is not available for pyrazinamide.

The effect of age on protein binding of antituberculosis drugs has not been reported. Therefore, in spite of a similar pharmacokinetic profiles between young and elderly subjects described in the previous studies, there may still be a difference if free drug concentration were determined. Although the relationship between

Fig. 2.3 The distribution of side effects in 37 patients.



free drug concentration and toxicity or bactericidal effect has not been established for antituberculosis drugs, it is important to address this issue since dosage adjustment may be indicated with a view to minimising toxicity. For example in the study by Walubo et al (1991c), patients aged 65 years and over had lower serum albumin concentrations compared with those below 65 years of age, and although the drug plasma concentrations were the same in both groups, more side effects were noted in the elderly group (Fig. 2.3). Studies on the influence of age and disease on the percentage binding of antituberculosis drugs to individual serum proteins and the relationship of free drug concentrations to toxicity are needed to clarify these issues.

2.2.4 Toxic metabolites

The role of toxic metabolites in the age related increase in incidence of adverse effects of antituberculosis drugs is largely unexplored. Susceptibility to hepatotoxicity increases with age (Umeki, 1991; Riska, 1976), even in those with no previous liver disease. However it is unclear whether isoniazid, rifampicin or metabolites of these drugs are responsible. Isoniazid is metabolised to isonicotinic acid either by direct hydrolysis, or indirectly via acetylation to acetylisoniazid and then hydrolysis. In the direct pathway, a metabolite hydrazine is formed, while in the indirect pathway, monoacetylhydrazine is the product. Both metabolites are hepatotoxic. Plasma half-life of monoacetylhydrazine is five times longer than that of isoniazid which may result in greater accumulation following repeated doses (Gangadharam, 1986). Rapid acetylators may be expected to have a greater incidence of

hepatotoxicity as more monoacetylhydrazine will be formed; however, in these subjects, monoacetylhydrazine will also be more quickly acetylated to the less toxic metabolite diacetylhydrazine so that the risk of hepatic reactions during treatment with isoniazid is no greater in rapid than slow acetylators.

Hydrazine is a potent hepatotoxin and also affects many metabolic processes in the body (Back and Thomas, 1970). The age related reduction in acetylation rate may result in a greater proportion of isoniazid being metabolised to hydrazine, particularly in slow acetylator phenotypes. Hydrazine has been detected in plasma of healthy male volunteers taking isoniazid 300 mg daily for 2 weeks (Blair et al, 1985). In patients on antituberculosis therapy consisting of similar dosages of isoniazid, rifampicin and pyrazinamide per Kg body weight, the maximum concentration of hydrazine after the first dose was significantly higher in elderly than in young patients (Walubo et al, 1991d). Steady state hydrazine concentrations in one subject aged 72 years, who died of submassive liver necrosis 8 days after initiation of antituberculosis chemotherapy, exceeded twice the mean ± SD value for the group of elderly patients who did not develop hepatotoxicity (Woo et al, 1992).

2.2.5 Drug combination

The concomitant administration of several drugs may predispose to increased incidence of adverse side effects. It has been suggested that concomitant administration of isoniazid and rifampicin may

produce more hepatotoxicity than isoniazid alone (Lees et al, 1971; Centers for disease Control, 1980). Metabolic induction by rifampicin may result in increased production of hepatotoxic metabolites of isoniazid. However, pre-treatment with rifampicin did not modify the metabolism of acetylisoniazid (Jenner and Ellard, 1989). On the other hand, it has been suggested that concomitant administration of isoniazid and rifampicin may result in increased levels of hydrazine, particularly among slow acetylators (Sarma et al, 1986). It has been postulated that an age related difference in the hepatic microsomal drug detoxification system may account for the high incidence of isoniazid-rifampicin induced jaundice in children (Umeki, 1991). By analogy, a similar change could also occur in the elderly, so that concomitant administration of isoniazid and rifampicin may partly account for the increased occurrence of hepatotoxicity in the elderly.

2.2.6 Non-pharmacological factors

Non-pharmacological factors in the elderly may predispose to toxicity from antituberculosis drugs. Tuberculosis is commonly diagnosed in advanced stage in the elderly due to atypical presentation and difficulty in diagnosis (Umeki, 1991), so that miliary tuberculosis, for example, is more common among elderly people (Farer et al, 1979) Therefore, there may be a higher chance of liver involvement by *Mycobacteria*, predisposing to hepatotoxicity even in the absence of chronic liver disease or alcoholism. Poor nutritional status may be another predisposing factor, particularly in the absence of infection. Drug metabolism and toxicity is affected by dietary intake (Campbell and Hayes, 1974). Thus a higher

incidence of hepatotoxicity (3-22%) in patients in India compared to those in the USA (2-3%) may partly be explained by poor nutrition (Gangadharam, 1986).

2.2.7 Conclusion

In conclusion, during treatment of tuberculosis in elderly patients, the following factors must be considered in an effort to avoid toxicity: consideration of free drug concentrations, disposition of hepatotoxic metabolites, drug combinations, nutritional status, and hepatic involvement by the disease process. Available data on treatment regimens and adverse reactions may not apply to the sick elderly. Further studies on the above aspects are needed to determine whether the present combination therapy might be altered, with omission of one of the potentially hepatotoxic drugs without affecting bactericidal activity or recurrence rate. Clinicians should also be more aware of these serious adverse effects, monitor such patients closely, and provide nutritional support if necessary. The view that it is not necessary to monitor liver function tests as routine during antituberculosis therapy, unless the patient has liver disease, may not be appropriate in this group of patients. Moreover, cases of hepatotoxicity in the sick elderly were not mild transient biochemical abnormalities which permit continuation of drugs. As the absolute number of old people increases, together with the increasing incidence of tuberculosis in the elderly, such problems are likely to be encountered with increasing frequency. With further studies, more definite guidelines should be available on

the use of antituberculosis drugs in elderly patients with a view to reducing adverse effects, particularly hepatotoxicity.

CHAPTER TWO; PART III

FREE RADICALS, OXIDATIVE STRESS, AGING AND DISEASE

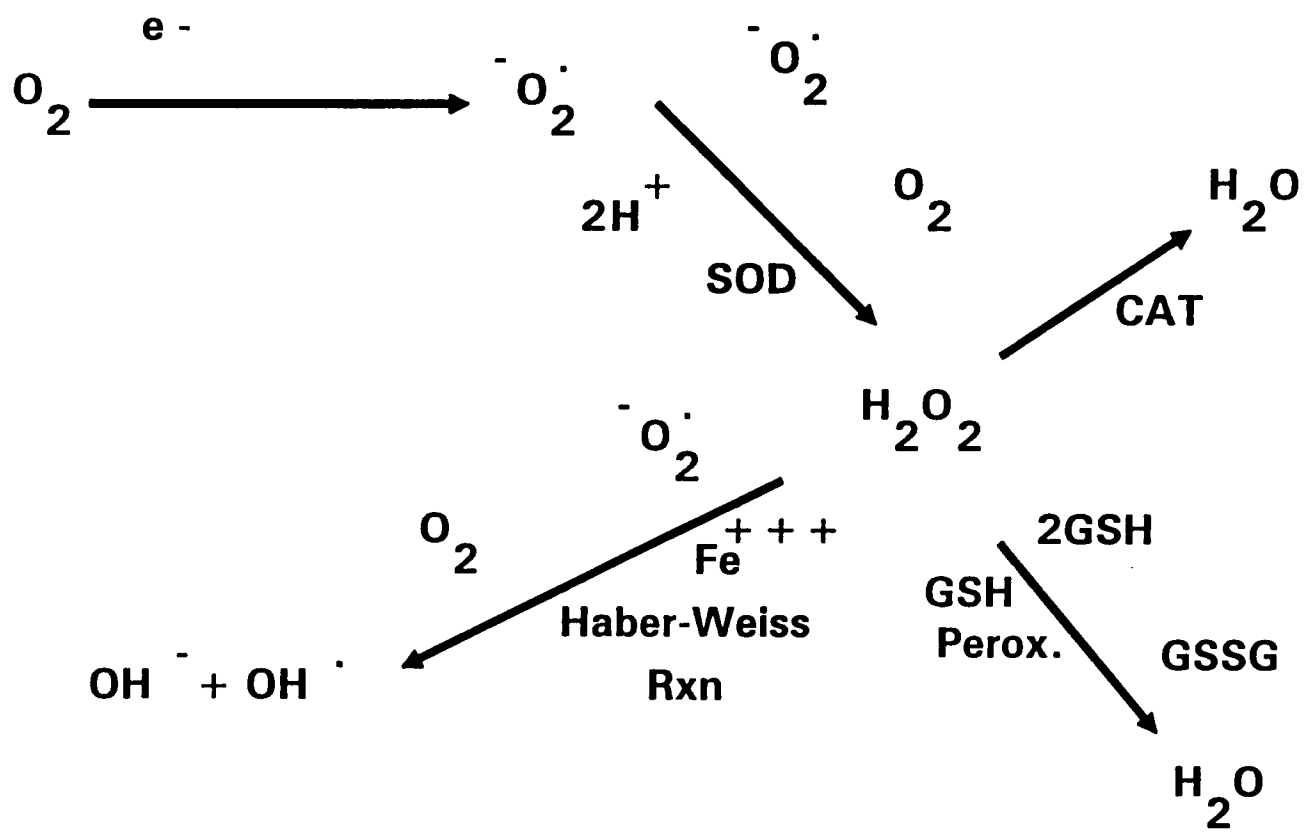
2.3.1 Introduction

A free radical is an atom or molecule with a free unpaired electron in its orbit. Free radicals are ubiquitous in the body and are continuously produced during normal metabolism and during metabolism of various chemicals and compounds including xenobiotics. Superoxide anion ($-O_2$), hydrogen peroxide (H_2O_2), hydroxyl ion (OH) and hypochlorous acid (HOCl) radicals are oxygen centered free radicals. Their generation requires molecular oxygen and an electron donor like NADPH of the respiratory chain system, hence they are called reactive oxygen species. Examples of non-oxygen centered radicals are; sulph-thyl (RS.; a sulphur centered radical), trichloromethyl(.CCL3; a carbon centered radical) and nitric oxide (NO; the free electron is situated between the two atoms).

Free radicals can react with other molecules in various ways, such as addition, subtraction and sharing of electrons, leading to a chain of reactions by generating other radicals that propagate the reaction (Leibovitz et al, 1980).

The initiating event in most biological free radical reactions is the production of a superoxide radical, which is formed from molecular oxygen by addition of a single electron (Fig. 2.4). Protonation of superoxide produces a hydroperoxy radical (HO_2) and addition of a

Fig. 2.4 The generation of superoxide anion and other oxygen radicals



second electron and protonation leads to production of hydrogenperoxide radical

On the other hand, addition of electrons to the oxygen molecule weakens the oxygen-oxygen bond which separates to form oxene (O) and oxide (O_2-). The oxide combines with two protons to produce water (H_2O) while oxene combines with a proton to form a hydroxyl radical. Under UV light hydrogen peroxide decomposes due to fission at the O-O bond to produce hydroxyl radicals;

$$H_2O_2 \text{ ----> } 2OH.$$

Formation of hydroxyl radicals from hydrogen peroxide may be catalysed by trace amounts of metal ions such as in the Fenton reaction;

$$Fe(II) + H_2O_2 \text{ --> } OH + OH = Fe(III)$$

or in the Haber-Weiss reaction;

$$O_2 + H_2O_2 = Fe(III) \text{ ---> } OH + OH + O_2 + Fe(II).$$

Other sources of oxygen centred radicals include hemolytic cleavage of water by ionising radiation. This produces a hydroxyl radical while the nitric oxide radical (NO) or the endothelium relaxing factor (EDRF) reacts with the superoxide radical to produce, after protonation, a hydroxyl radical and nitrogen dioxide;

$$O_2 + NO \text{ ---> } HOONO + H \text{ <---> } HOONO \text{ ---> } OH = NO_2).$$

Finally, hydrogen peroxide and hydroxyl radicals may react with halide compounds to produce halogen radicals. For example, during the respiratory burst in the neutrophil, H_2O_2 + Cl + neutrophil myeloperoxidase may result in the production of highly reactive

radicals such as hypochlorous acid (HOCl), hypochlorate and chlorine. These radicals can halogenate micro-organisms, tumour cells and normal tissue leading to tissue damage.

In terms of toxicity, as measured by activity as well as half-life, the hydroxyl radicals appear to exceed that of superoxide and hydrogen peroxide radicals. Other radicals that may be produced by the attack of superoxide or hydroxyl radical on biological molecules include, among others: lipid radicals, lipid peroxy radicals, pyrimidine radicals, and purine radicals. It is possible that many of the damaging effects attributed to the superoxide radical are a consequence of these secondary free radicals.

Superoxide is produced in many body reactions and processes during normal physiological functions in the body. Respiring cells are a major source of superoxide radicals. This led to a suggestion that the mitochondrion is the "molecular clock" of organisms whereby the maximal life span of species is related to the rate of oxygen consumption. In this regard, low oxygen concentrations have been shown to extend the life-span of cultured human diploid cells in vitro (Packer and Fuehr, 1977).

Also, the production of superoxide radicals has been associated with or required for the activity of a number of enzymes, including xanthine oxidase, aldehyde dehydrogenase, dihydrorotic acid dehydrogenase, indoleamine dioxygenase, 2-nitropropane dioxygenase, diamine oxidase, and ribulose-1,5-diphosphate

carboxylase (Bhargwat and Sane, 1978; Fridovich, 1978; Younes and Wester, 1978).

Superoxide radical production has also been shown during the autoxidation of a variety of molecules, including hydroquinones, flavins, catecholamines, thiols, tetrahydroptrines, ferrodoxins, dialuric acid and hemoglobin (Cohen and Heikkila, 1974; Fridovich, 1978; Heikkila and Cohen, 1973; Michelson, 1973; Misra and Fridovich, 1972). The autoxidation of various chemicals, including herbicides (paraquat), alkyl halides (carbon tetrachloride, bromotrichloromethane), and medicines (adriamycin, daunomycin) also produce superoxide radicals (DiLuzio. 1973; Henderson et al, 1978; Jose and Slater, 1972; Koster et al, 1978; Recknagel et al, 1977). Free radicals are also present in urban polluted air, in any smoke derived from the burning of organic matter (e.g. gasoline, paper, tobacco), and in ozone-contaminated air (Boenig, 1966). Superoxide radicals are also required for microbiocidal activity in neutrophils, monocytes and microphages and are produced in these cells during the respiratory burst which accompanies oxidative killing.

One consequence of free radicals is the production of lipid peroxides. Free radical attack on polyunsaturated fatty acids yields lipid radicals with allylic double bonds, which may add to molecular oxygen to produce lipid peroxy radicals which, by abstraction of a proton and electron, ultimately produce lipid peroxides. The significance of lipid peroxidation is that such lipid peroxides decompose to yield aldehydes which cross-link proteins, lipids and

nucleic acids. Other reactions include addition reactions, yielding covalent bonds as well as novel radicals. Scission reactions are important in regard to ionizing radiation, but also during chemical oxidations of adriamycin, daunorubicin and reducing agents. However, cross-linking reactions are of greater significance with regard to the aging process.

2.3.2 Aging and free radicals

The theory of aging is based on the action of free radical reactions which lead to cell membrane damage and cross-linking of biomolecules (Tappel, 1973). This results in decline in cellular integrity, primarily due to reduced enzyme activities, error-prone nucleic acid metabolism, damaged membrane function, and accumulation of aging pigments in lysosomes.

The cross-linking theory stems from the colloidal theory of aging originally proposed by Ruzika in 1924 (cf. Sinex, 1964). The basic concept is that unusual cross-links increase with age , and contribute to the loss of cellular integrity known to be associated with advancing age. Support for this concept was presented by Rickert and Forbes (1976), who found that collagen isolated from lungs of elderly persons had increased ratios of insoluble/acid soluble collagen compared with young persons. Cell membranes are also known to increase in rigidity with advancing age (Nagy, 1978), an effect possibly mediated by free radical-induced lipid peroxidation.

Cross-linking of biomolecules by aldehydes resulting from lipid peroxidation is well documented, and may be the most important mechanism in vivo. Lipid peroxides are known to decompose and yield a variety of products, including ethane, pentane and various aldehydes. The most important aldehyde produced is malondialdehyde (MDA; CHO-CH_2-CHO), which forms Schiff bases with amines of proteins, phospholipids and nucleic acids. The product of this reaction is a biomolecule many times the size of the original, which because of unusual bonds, is not digested in the lysosomes and accumulates there with age. These biopolymers are fluorescent with a characteristic excitation maxima at 360-380 nm, and an emmission maxima at 440-470 nm. Accumulation of these pigments, termed "aging pigments", lipofuscin or ceroid, is directly related to the age of the animal (Shimasaki et al, 1977); Tappel, 1973).

Therefore, the significance of lipid peroxidation and cross-linking in relation to aging is the reduction in cellular integrity. Lipid peroxides have been shown to inactivate certain enzymes such as isocitrate dehydrogenase (Green at al, 1971) which, when inactivated by linoleic acid hydroperoxide, yielded a decrease in sulfhydryl content of the enzyme. Variations in the ratio of sulfhydryl/disulfides may affect a wide variety of enzymes systems. For example, oxidised glutathione inhibits adenylcyclase activity of the rat brain as a result of increased disulfide levels in this enzyme. Lipid peroxidation also affects integrated membrane bound enzymes. For example the inhibition of oxidative phosphorylation of rat liver mitochondria by linoleate hydroperoxide (Naito et al,

1966). Unfortunately, there is no information on how lipid peroxidation affects drug metabolising enzymes.

2.3.3 Enzymatic defence systems.

Elaborate enzymatic systems have been evolved by anaerobic organisms to reduce the fluxes of superoxide and hydrogen peroxide radicals which, in turn, will reduce the production of hydroxyl radicals. Superoxide dismutase (SOD) is the major defense system which protects against elevations in levels of superoxide. SOD catalyses the dismutation of two molecules of superoxide radical to yield hydrogen peroxide and molecular oxygen. SOD is a cytoplasmic as well as mitochondrial enzyme and, in eukaryotes, contains either copper and zinc or manganese (Fridovich, 1978). Recently, extracellular SOD has been isolated and its role in antioxidant mechanism was considered insignificant (Jadot et al, 1995). The role of SOD in the aging process is not certain. A three fold decline with age in levels of SOD between young (6 months) and old (27 months) rats was reported (Reiss and Gershon, 1976).

Catalase and glutathione peroxidase protect against elevation in levels of hydrogen peroxide. Generally, catalase is of major importance in prokaryotes while glutathione peroxidase is of greater significance in eukaryotes. Glutathione peroxidase utilises the reducing power of glutathione (gamma-L-glutamyl-L-cysteinylglycine), and contains selenium as a coordinating mineral. Glutathione peroxidase also protects against the damaging effects

of lipid peroxidation, reducing lipid peroxides to fatty acid alcohols (Tappel, 1974). However, the activity of glutathione peroxidase is proportional to the concentration of selenium in the diet. Also, vitamin E appears to be required for activity of this enzyme (Chow, 1977; Fukuzawa and Tokomura, 1976; Scott et al, 1976). Furthermore, dietary ascorbic acid has also been shown to increase the levels of glutathione peroxidase (Combs and Pesti, 1976).

2.3.4 Non-enzymatic defense mechanisms

A variety of molecules have been shown to quench free radicals, reduce lipid peroxidation and detoxify hydrogen peroxide. These compounds include both dietary factors such as vitamin E, vitamin C, hydroquinonoes and sulfhydryl compounds as well as synthetic antioxidants including hydroxylated hydroxytoluene, butylated hydroxyanisole, sodium benzoate and ethanol.

Vitamin E is apparently the most important dietary free radical and lipid peroxide scavenger, due to its lipid solubility and occurrence in membranes. Vitamin E added to diet of animals results in a decrease in accumulation of aging pigments; conversely, vitamin E deficiency results in greater accumulation of these aging parameters (Csallany et al, 1977; Katz et al, 1978; Reddy et al, 1973; Sylven and Glavid, 1977; Tappel, 1972, Tappel et al, 1974). A novel estimation of aging is based on the exhalation of ethane and pentane, hydrocarbons derived from peroxidation of omega-3 and omega-6 polysaturated fatty acids, respectively. A combination of vitamin E and selenium deficiency in the rat increased ethane

production to 7.4 nmol/100 g body weight/24 hours; supplementation of the diet with vitamin E, selenium or both, reduced ethane evolution to 0.4, 3.1 and 0.2 nmol/100 g body weight/24 hours, respectively (Hafeman and Hoekstra, 1977). Lipid peroxidation *in vitro*, as measured by malondialdehyde production, is also related to vitamin E status. Therefore, the protective effect of vitamin E is due to its ability to quench free radicals such as the superoxide radical (Ozawa et al, 1978) and lipid peroxy radicals such as the methyl linoleate radical (Igarashi et al, 1976).

Ascorbic acid was shown to be oxidised by superoxide (Nishikimi, 1975) and hydroxyl radicals (Fessenden and Verma, 1978). Ascorbic acid may play a role as an extracellular defense against free radicals in such tissues as the lung, where approximately 59% of the ascorbic acid is found in the fluid lining of the respiratory epithelium in the rat (Willis and Kratzing, 1974). This is of considerable interest in that enzymatic defense mechanisms including SOD and glutathione peroxidase are found only intracellulary, and would not be available for reaction with extracellular free radicals or oxidants. Dietary ascorbic acid has been shown to reduce the extent of lipid peroxidation in both the mouse (Tappel et al, 1974) and the guinea pig (Chen and Chang, 1978). Although there are suggestions that low levels of ascorbic acid may induce lipid peroxidation in vitro, such phenomena has not been demonstrated in vivo. Therefore, ascorbic acid is a molecule which may quench free radicals without leading to further free radical reactions in vivo.

2.3.5 Levels of antioxidants as a function of age

A progressive decrease with advancing age for the following antioxidants has been observed; SOD (Reiss and Gershon, 1976), selenium (Thomson et al, 1977), mercaptan (Harman, 1960) and ascorbic acid (Attwood et al, 1978). Data on variation of vitamin E with age is not available.

The ability of antioxidants to quench free radical reactions should lead to enhanced longevity. Preliminary studies have reported that synthetic antioxidants extend the lifespans of AKR, C3H and LAF1 mice (Harman, 1957; Harman, 1961 and Harman, 1968).

2.3.6 Oxidative stress

Oxidative stress refers to a state where the pro-oxidant processes out-weigh the antioxidant mechanisms. This can occur when formation of reactive oxygen species is accelerated or levels of antioxidants are decreased or the protective mechanisms are impaired. However mild oxidative stress may render the cell resistant to further stress by increased synthesis of antioxidant systems such as SOD and heat shock proteins. It is the inhibition, deactivation and exhaustion of the antioxidant systems in acute oxidative stress which are of relevance in the events preceding a particular disease or in production of pathological symptoms of disease and drug toxicity.

2.3.7 Free radicals and disease

a) Atherosclerosis: This is a disease of the arteries. It is produced by local thickening of the intima and is thought to start with damage to vascular endothelium. One of the early processes in its pathogenesis is formation of phagocytic foam cells in fatty streaks. It has been shown that oxidised lipids, initiated by free radical reactions play a great role in this process. It is the damaging effects of reactive oxygen species combined with the cytotoxic effects of oxidised low density lipoproteins (LDL) that leads to endothelial damage and consequently the development of the atherosclerotic lesion (Streinbrecher et al, 1990;). Oxidation of LDL occurs only when endogenous antioxidants in the LDL particles are depleted (Jessup et al, 1990; Esterbaur et al, 1990). Clinical trials for antioxidant therapy for this disease have recently been suggested (Steinberg, 1992). Probucol, the hypocholesterolemic drug, has been show to have antioxidant activity.

b) Rheumatoid arthritis: This is a chronic inflammatory disease affecting mainly the joints. The destructive processes of rheumatoid arthritis have been related to increased production of reactive oxygen species. During an acute attack of rheumatoid arthritis, reactive oxygen species are produced in the inflammed joints by activated macrophages in the synovial membrane and by neutrophils in the synovial cavity. It is thought that reactive oxygen species then lead or propagate mechanisms that cause bone or cartilage damage (Unsworth et al, 1988). Lipid peroxidation is increased in synovial fluid of patients with rheumatoid arthritis and

the extent lipid peroxidation correlated with severity of the disease (Rowley et al, 1984). Also, the levels of ascorbate, a natural antioxidant, are below normal in synovial fluid and plasma of patients with rheumatoid arthritis (Lunec and Blade, 1985). Iron, in the form of ferritin and haemosederin is increased in the synovial membranes of these patients. Clinical trials with SOD and desferrioxamine have shown hope (Giordano, 1984).

c) **Cancer:** It has been shown that exposure to free radicals can lead to mutations, transformations, or activation of specific oncogenes (Weitzman and Stossel, 1981). Oxidative stress produces alterations in the c-abl gene methylation and also induction in the proto-oncogenes c-fos and c-myc in mouse epidermal cells (Weitzman et al, 1989; Crawford et al, 1988). In the same perspective, antioxidants have been found to inhibit neoplastic processes (Ames, 1983; Ames and Saul, 1987; Borek and Troll, 1983). SOD inhibits transformation induced by radiation and suppresses free radical induced tumor promotors, the phorbol esters (Borek and Troll, 1983). Similar protective roles have been shown for vitamins E and C, and selenium (Niki, 1987; Borek et al, 1986).

Many more disease processes, including aetiology and manifestations of diseases, where reactive oxygen species are involved, are being described. This is important because knowledge of the involvement of reactive oxygen species in disease or other toxic processes may help to provide information on factors that control these pro-oxidant systems with the aim of interfering

with a correct antioxidant system at the right time and at the right place to prevent and inhibit the disease or toxic process.

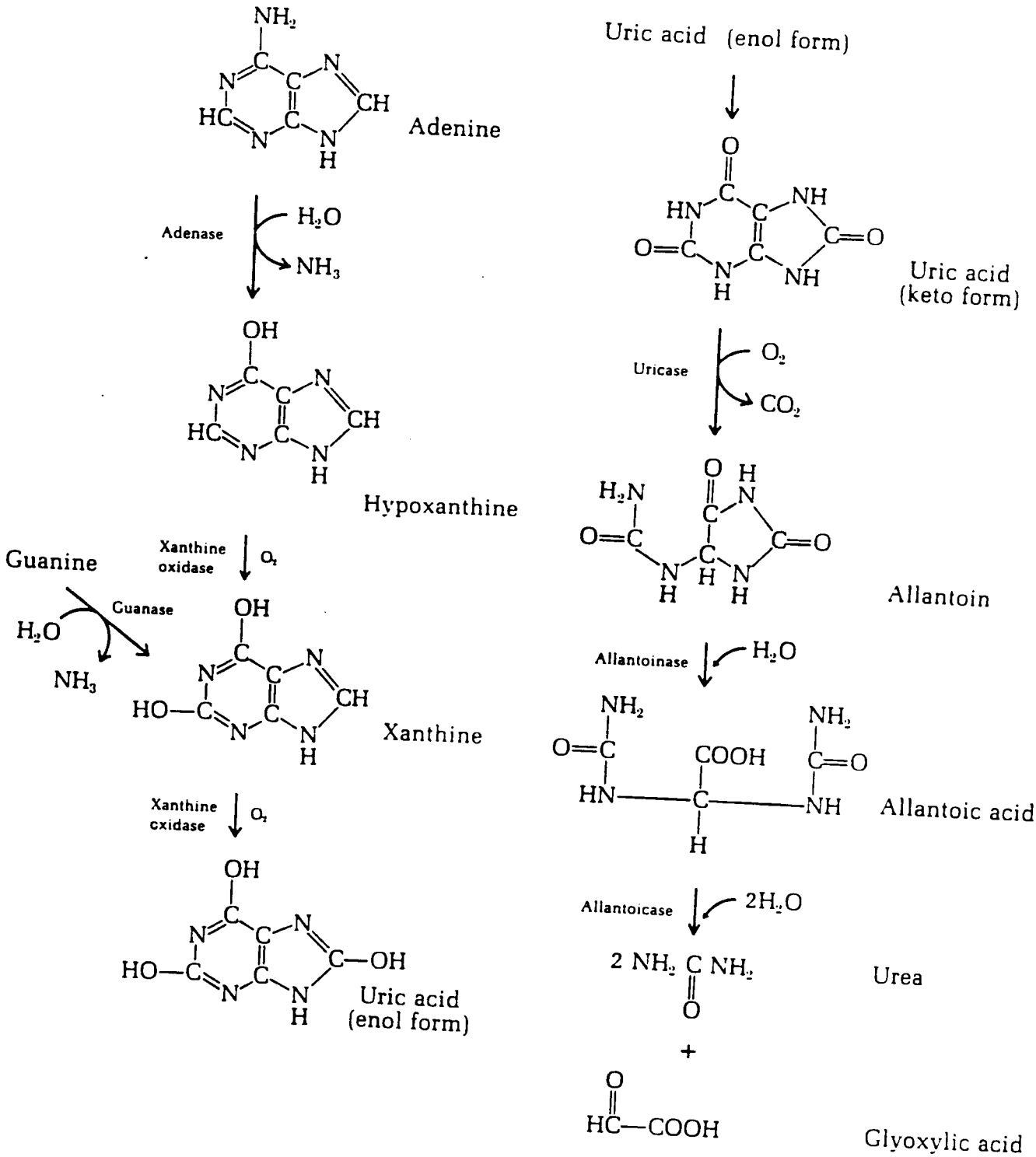
2.3.8 Assays for free radicals

a) Trapping: This is the identification of free radicals in biological systems by reacting free radicals with a trap molecule to give one or more stable products which are then measured. Reaction of free radicals with spin traps such as phenyl-tert-butyl nitrone, leads to formation of products that are detected by electron magnetic spin resonance (ESR). However, the currently available spin traps are not good detectors of reactive oxygen species in vivo, and they cannot be administered safely to man. Another trapping technique for free radicals is by measuring the products of aromatic hydroxylation of salicylates.

b) Uric acid oxidation: In primates, including man, uric acid is an end product of purine metabolism because urate oxidase enzyme (Uricase) is absent (Fig. 2.5). It has been suggested that uric acid acts as an antioxidant *in vivo* (Ames, 1981). Hence, measuring products of the interaction of reactive oxygen species with uric acid might be a potential marker of oxidative stress uniquely applicable to human beings. Uric acid is degraded on exposure to OH, HOCL and mixtures of haemoglobin (or myoglobin) with hydrogen peroxide but it is not affected by hydrogen peroxide alone, or by superoxide. The major product of uric acid oxidation by the first three systems is allantoin, but others include oxonic acid, oxaluric acid, cyanuric

FIG. 2.5

Degradation of purines to uric acid, allantoin, and urea.



acid and parabanic acid. Uric acid may be an important antioxidant defense against ozone in the upper respiratory tract (Hatch, 1991). Concentrations of uric acid oxidation products were increased in synovial fluid and serum of patients with rheumatoid arthritis (Grootveld and Halliwell, 1987). Such an observation is consistent with oxidative stress associated with this condition and illustrate an application of measuring uric acid oxidation products.

c) Fingerprint assays: These are methods where reactive oxygen species are detected by measuring products of their interaction with biological molecule e.g. DNA products (termed DNA-fingerprinting) and lipid peroxide assay. Measurement of end products of lipid peroxidation in human material is the assay most commonly used for detection of reactive oxygen species in human beings. However, there is no single fool-proof method of measuring lipid peroxidation. Each of the available methods measures only part of the oxidation process. Lipid hydroperoxides breakdown to other products and a huge number of different aldehydes. Also, other carbonyls and different compounds are produced by peroxide decomposition. To learn as much as possible about lipid peroxidation in human material, it is important to use techniques that give specific chemical information about what is present and to use more than one such technique. Some of the methods used to detect lipid peroxidation include (summary by Halliwell et al, 1992; Esterbauer et al, 1991; and Thomas et al, 1991):

1) Analysis of fatty acids by gas chromatography (GC) and high performance liquid chroatography (HPLC).

2) Measuring uptake of oxygen by carbon-centered radicals and during peroxide decomposition using the oxygen electrode.

3) Measuring lipid peroxides by iodine liberation, heme degradation of peroxides, cylooxygenase, fluoroscence and GC-mass spectroscopy (GC-MS).

4) Measuring excited carbonyl and singlet oxygen by light emission.

5) Measuring cytotoxic aldehydes by HPLC antibody techniques.

6) Measuring the diene-conjugated structures by diene conjugation.

7) Measuring thiobarbituric acid (TBA) reactive material by the TBA test.

Prostaglandin F_2 like compouds have been suggested as promising for detection of reactive oxygen species *in vivo* while measuring end products of oxidative damage to proteins is equally good.

CHAPTER TWO; PART IV

INTRODUCTION TO LIPOSOMES

Liposomes are stable microscopic lipid vesicles ranging in diameter from 0.1 to 20 μm. They are composed of one or more lipid membranes surrounding an internal aqueous compartment (Naessander et al, 1990; Ostro and Cullis, 1989). The lipid layers are similar in structure to those found in living cell membranes and it is for this reason that liposomes can be used to carry soluble substances across cell membranes. Also, liposomes can carry lipophilic substances within their bilipid layers just as sterols and hydrophobic proteins are intercalated into cell membranes.

Liposomes are classified according to their size viz:

1) The large multi-layered liposomes of variable size are termed multilamellar vesicles (MLV).

2) The large one-layered liposomes with homogenous particle size are called large unlamellar vesicles (LUV).

3) The small one-layered liposomes with particle size less than 1.5 μm are termed the small unilamellar vesicles (SUV).

After administration, the disposition of liposomes depends on, size, bilayer rigidity, temperature and charge. Temperature is important for stability of liposomes because at higher temperatures the phospholipids turn from a stable gel state to a leaky fluid-like crystalline state.

Cholesterol has been shown to improve the stability of liposomes, even in the fluid-like crystalline state (Scherphof et al, 1978). Positively charged and neutral liposomes circulate longer than those of similar size that are negatively charged.

The small liposomes (SUV) are taken up slowly by the mononuclear phagocyte system thus have a long circulation half-life (up to 24 hours). These SUV extravasate through fenestrations in the endothelium of liver sinusoids and interact with liver parenchymal cells. Therefore, their uptake by phagocytic cells is relatively low (Hwang et al, 1987; Senior, 1987). The larger liposomes (MLV and LUV) are rapidly and efficiently taken up by phagocytic cells of the mononuclear phagocyte system, mainly in the liver and spleen. These liposomes therefore, tend to have a shorter half-life in blood. Also, total lipid dose can influence half-life of liposomes because at higher lipid doses the mononuclear phagocyte system becomes saturated and the half-life of liposomes increases (Ostro and Cullis, 1989).

Uptake of liposomal associated drugs into the cell may occur through adsorption, followed by release of the drug into the extracellular fluid and diffusion of the drug into the cell, or liposomes may enter the cell intact by phagocytosis and then degraded by lysosomal enzymes to release the drug. Phagocytosis is the most important uptake mechanism for MLV and LUV. Also, liposomes may be taken up by pinocytosis.

Importance of liposomes

Liposomes have been used to transport therapeutic agents across cell membranes. With new technologies, modification of liposomal properties has led to use of liposomes as prolonged release drug reservoirs and for localisation of drugs within particular areas in the body to avoid or target specific tissue or subcellular sites. Amphotericin-B (Patterson and Andriole, 1990; Tollemar, 1990) and daunorubicin (Gabzon et al, 1989) are drugs where liposomal delivery is a success. Liposomal amphotericin-B is associated with a decreased risk of nephrotoxicity (Mufson et al, 1990; Wassan et al, 1990). The use of liposomal entrapped drug in the treatment of tuberculosis is still in experimental stage (Oronzco et al, 1990). Because SOD does not cross cell membranes, it is intended in this study, to use liposomal entrapped SOD to deliver this enzyme into the cells of rat liver slices.

CHARPTER THREE

OBSERVATIONS FROM THE REVIEW, HYPOTHESIS AND OBJECTIVES.

3.1 Hydrazine metabolites

Although isoniazid is considered a safe drug, the fatalities in elderly patients due to this widely used drug have warranted the need to re-assess its metabolism in this group of people. There is little information on the metabolism of isoniazid in patients over 65 years of age. Furthermore, the mechanism by which isoniazid induces toxicity remains unknown although its hydrazide metabolites have been implicated by several reports (Mitchell et al, 1976; Woo et al, 1992).

Isoniazid induced toxicity can be related to its hydrazide metabolites because:

1. Concentrations of isoniazid in plasma do not correlate with the occurrence of side effects (Bowersox et al, 1973).

2. There is no correlation of isoniazid toxicity to acetylator status (Weber and Heine, 1985)

3. Toxicological studies have shown that hydrazide metabolites of isoniazid are potentially more toxic than the parent drug (Mitchell et al, 1976; Back and Thomas, 1970).

4. Severe renal impairment can lead to significant elevation of isoniazid plasma concentration (Bowersox et al, 1973; Kim et al, 1993), yet isoniazid is only 11-20% renally excreted compared to metabolites which are >60% excreted by this route (Ellard and Gammon, 1976).

Metabolites of toxicological importance are; hydrazine, acetylisoniazid, monoacetylhydrazine, and diacetylhydrazine. Hydrazine is toxic to all tissues (Back and Thomas, 1970) while monoacetylhydrazine induces hepatotoxicity presumably by covalent binding to liver macromolecules and is thought to be the toxic intermediate for acetylisoniazid and diacetylhydrazine (Mitchell et al, 1976). Elimination of these metabolites is mainly by renal route with half-lives in young volunteers, after a single dose of isoniazid, of 4.9 and 8.0 times that of isoniazid, for monoacetylhydrazine and diacetylhydrazine, respectively (Ellard and Gammon, 1976; Lauterburg et al, 1985).

The long half-lives for these metabolites indicates their potential to accumulate during chronic dosing and especially in patients with altered metabolism or excretion of any of the metabolites. Since renal function falls with age (Schmucker, 1985), elderly people are more prone to suffer from the consequences of metabolite accumulation than young ones thereby obviating the need for more information on plasma profiles of isoniazid metabolites during antituberculous chemotherapy in elderly patients.

However, the incidence of isoniazid toxicity seem not to be related to renal function, implying that other factors have a major role in its

pathogenesis. Such factors include reduced ability to quench toxic products of drug metabolism (Leibovitz and Siegel, 1980) and poor cell regeneration mechanisms after injury in elderly people.

Therefore, during chronic treatment with isoniazid, the steady state concentration of hydrazide metabolites and isoniazid in elderly patients may be unsafe.

3.2 Mechanism of isoniazid induced hepatotoxicity.

The consequences of metabolite accumulation cannot be realized without adequate knowledge of the toxic potential of individual metabolites. Isoniazid and its hydrazide metabolites are potentially toxic and little is known about the mechanism of toxicity associated with these compounds. Mitchel et al (1976) explained that isoniazid induced hepatotoxicity is due to a metabolite, monoacetyhydrazine. This proposal has been widely criticized because it failed to explain the pattern of isoniazid related hepatitis in the population.

Isoniazid and the four metabolites (hydrazine, monoacetylhydrazine, diacetylhydrazine and acetylisoniazid) are hydrazides. They contain a hydrazine group, and this could explain many properties these compounds have in common, such as hydrophilia, basicity and toxicity (Martha, 1976). Hydrazine group has been implicated in lupus syndrome associated with isoniazid and hydrallazine (Durant and Harris, 1980), haemolysis by phenylhydrazine (Jain and Hochstein, 1979), P-450 inhibition by isoniazid and iproniazid (Muakkassah et al, 1981) and tumors induced by hydrazides in general (Toth, 1975). Unfortunately,

as for toxicity, the mechanism by which the above disorders occur remains unknown and understanding one mechanism may reveal a lot about the others.

It is possible that under certain conditions (pH, catalyst etc.), isoniazid and its hydrazine metabolites mentioned above can lead to generation of reactive oxygen species which may cause tissue injury. Therefore, when the defence system fails to quench free radicals, as in the case of excessive free radical generation due to xenobiotic metabolism or incompetent anti-oxidant defence mechanisms in the aged, the deleterious effects of free radicals begin to be observed. And, as for other injuries, the manifestations and severity of tissue injury (or oxidative stress) will depend on the amount of free radicals produced and tissues affected.

The possibility that isoniazid and its four metabolites can cause toxicity through oxygen reactive species has been entertained in view of the following observations:

1. Isoniazid and the four metabolites have a hydrazine group that can generate superoxide.

2. Phenylhydrazine, and acetylphenylhydrazine induce haemolysis by generation of superoxide via the hydrazine group (Jain and Hochstein, 1979).

3. The ability to generate superoxide by the hydrazine group is modified by molecular structures or other functional groups

attached to hydrazine group. For instance, haemolysis was greater with phenylhydrazine than acetylphenylhydrazine which in turn was greater than for hydrazine (Jain and Hochstein, 1979).

4. Mycobacterial action:

a) Action of isoniazid against *Mycobacteria* is reduced under hypoxic conditions (Youatt, 1958). Could this be due to insufficient oxygen and NADPH for free radical production?. The importance of oxygen in superoxide formation was shown by Packer and Fuehr (1977), who observed an extended life span of cultured human diploid cells in low oxygen concentrations *in vitro*. However, not all oxygen dependent reactions generate superoxide.

b) Action of isoniazid is enhanced in presence of trace amounts of copper (Maher et al, 1959). Copper is a powerful oxidant in metal catalyzed oxidation reactions leading to production of reactive oxygen species.

c) Mycobacterial sensitivity to isoniazid is associated with the presence of mycobacterial enzyme catalase and peroxidase. Although catalase is an anti-oxidant (destroys hydrogen peroxide), it can be a pre-oxidant through its heme nucleus, leading to enzymatic catalyzed oxidation reactions that generate radicals.

5. Inhibition of P-450 by isoniazid requires, and is enhanced in presence of, oxygen and NADPH, and is postulated to involve the hydrazine group (Muakkassah et al, 1981).

6. Enzyme inducers like phenobarbitone and 3-methylchlolanthrene increase production of superoxide by increasing activity of NADPH cytochrome-c reductase and other superoxide generating enzymes thereby out-witting the reducing potential of tissue anti-oxidants (Puntarulo and Cederbaum, 1992).

7. The fate of hydrazine group during isoniazid metabolism has not been adequately understood.

3.3 Oxidative stress and drug metabolism

Uric acid has been shown to be an antioxidant whereby, in the process, it is oxidized to allantoin (Ames et al, 1981). As humans do not have the enzyme uricase, that converts uric acid to allantoin, the amount of allantoin produced in the body may be an indicator of oxidative stress during antituberculosis chemotherapy. Uric acid products such as allantoin may be indicators of free radical reactions or oxidative stress during drug administration. The importance of uric acid in alleviating or predicting susceptibility to antituberculosis drug adverse reactions has not been evaluated.

3.4 Summary

In summary, It has been observed that;

1. A convenient and sensitive procedure for assay of isoniazid and metabolites is required for studying isoniazid metabolites in elderly patients and for therapeutic monitoring of patients in the clinic.

2. There is no information on the pharmacokinetics of hydrazide metabolites of isoniazid in elderly patients.

3. The role of oxygen radicals in isoniazid induced hepatotoxicity is not known.

4. The importance of uric acid metabolism in assessing oxidative stress during anti-tuberculous chemotherapy has not been explored.

5. The reason why isoniazid is more toxic in elderly patients is still not known.

3.5 Hypothesis:

`Isoniazid induces hepatotoxicity by generation of reactive oxygen species'.

By this hypothesis, it is intended here to show that oxygen reactive species are involved in isoniazid induced hepatotoxicity; that isoniazid metabolism leads to formation of metabolites which too can generate reactive oxygen species, and as a result, there is significant oxidative stress in elderly patients when on treatment with isoniazid containing regimen. This would indicate how isoniazid could pose a risk to elderly patients (i.e. the therapeutic importance of isoniazid metabolism in elderly patients).

This undertaking will involve a clinical study on the disposition of isoniazid and four of its hydrazide metabolites during antituberculsis treatment with isoniazid, rifampicin and pyrazinamide in young adults (20-30 years) and elderly (over 65 years) patients; and a study on the use of allantoin to estimate oxidative stress induced by the above antituberculosis drugs as well as its implications for antituberculosis chemotherapy in elderly patients. *In vitro* experiments will be done to pursue underlying mechanisms. It is only after establishing the toxic potential of isoniazid and its four metabolites, and the degree of oxidative stress they induce, in presence of other antituberculosis drugs, that it can be possible to determine how these compounds can be unsafe for elderly patients.

3.6 Objectives of the study.

Investigations will aim:

1. To develop a procedure for assay of isoniazid and metabolites, monoacetylhydrazine, acetylisoniazid, diacetylhydrazine and hydrazine in plasma by high performance liquid chromatography.

2. To compare the disposition of isoniazid metabolites in young and elderly patients during treatment with isoniazid, rifampicin and pyrazinamide after the first dose and at steady state.

3. To measure plasma concentration of the co-administered drugs; rifampicin and pyrazinamide (but not ethambutol) to elucidate their influence on isoniazid metabolism or vise versa.

4. To determine whether isoniazid and its four metabolites can generate oxygen reactive species.

5. To investigate, using rat liver slices, whether oxygen reactive species are involved in isoniazid and metabolites induced hepatotoxicity.

6. To compare oxidative stress in young and elderly patients during antituberculosis therapy, after the first dose and at steady state.

3.7 Expected results

The results of this study shall be:

1) A procedure for assay of isoniazid and hydrazide metabolites in plasma.

2) Understanding whether isoniazid and metabolites can generate oxygen free radicals.

3) Information on the pharmacokinetics of isoniazid metabolites in elderly patients.

4) Knowledge on the mechanism of isoniazid and metabolites induced hepatotoxicity. This is vital not only to understand risks associated with administration of isoniazid but is an aid to the prediction and therefore prevention of adverse effects by this drug. It is by understanding the mechanism of toxicity that accurate predictions on the toxicological consequeces of the observed concentrations of isoniazid and or metabolites can be made.

5) The use of allantoin to estimate oxidative stress due to anti-tuberculous drugs.

6) Understanding how isoniazid can be risk to the elderly person.

CHAPTER FOUR

A COMPREHENSIVE ASSAY FOR PYRAZINAMIDE, RIFAMPICIN, AND ISONIAZID WITH ITS HYDRAZINE METABOLITES IN HUMAN PLASMA BY COLUMN LIQUID CHROMATOGRAPHY.

4.0 SUMMARY

A comprehensive assay for determination of pyrazinamide, rifampicin, isoniazid and hydrazine metabolites is described. The assay involves organic solvent extraction of pyrazinamide and rifampicin, followed by derivatization of isoniazid, monoacetylhydrazine and hydrazine with salicyladehyde and thereafter extracted into diethylether. Acetylisoniazid and diacetylhydrazine were hydrolyzed to isoniazid and monoacetylhydrazine, respectively, and processed as above. Using a gradient solvent programer, pyrazinamide and rifampicin were analyzed on a RP C_8 column (Spherisorb, 250 mm x 4.6 mm I.D., 5 um) at 248 nm, while isoniazid and metabolites were analyzed on a RP C_{18} column (Spherisorb, 250 mm x 4.6 mm I.D., S5 ODS2) at 280 nm.

4.1 INTRODUCTION

Isoniazid, rifampicin and pyrazinamide form one of the most effective antituberculous regimens used in many countries, and as a result considerable effort has been spent on improving the efficacy of this regimen. Combined formulations like Rifater were introduced to improve acceptability and compliance, while intermittent short course therapy was to reduce adverse reactions and improve the quality of life. In spite of this, there is a continued threat of drug toxicity,

especially in the elderly and in patients with human immunodeficiency virus (HIV) (Barnes and Barrows, 1993), during treatment with these agents, and there has been a global increase in the prevalence of drug resistant tuberculosis (Iseman, 1993; Woo et al, 1992). Complicated tuberculosis in form of multidrug resistant tuberculosis or tuberculosis with HIV or tuberculosis in the elderly do not conform to the original tuberculosis syndrome for which the present regimens were prescribed. The effect of these complications on the disposition of anti-tuberculosis drugs is not yet clear and adjustments in treatment regimens during these complications is still empirical (Iseman, 1993). Knowledge of the drug plasma concentrations is important in case of drug resistance and toxicity, and concentrations of the toxic metabolites may be important in case of toxicity (Grosset, 1992). Therefore, drug monitoring during anti-tuberculosis therapy has become necessary in some patients and a methodology for use in therapeutic monitoring is needed.

Unfortunately, none of the existing procedures was suitable for this task because the assay conditions were favorable for only one (Jamaluddin et al, 1990; Swart and Papgis, 1992) or two drugs (Gaitonde, 1990) and no metabolites were measured. Furthermore, the use of separate procedures would require large sample volumes which could not be obtained at the time when there is more blood letting for other laboratory evaluations. Therefore, it was found necessary to develop a procedure that would be more helpful in the clinic and allow an optimum sample volume for processing to minimise interference with other investigations as well as inconvenience to the patient.

Using a modification of the previously reported procedures (Chan, 1988; Von Sassen et al, 1985; Jenner and Ellard, 1987; Walubo et al, 1991a) we describe a comprehensive methodology for determination of rifampicin, pyrazinamide and isoniazid with its hydrazine metabolites acetylisoniazid, hydrazine, monoacetylhydrazine and diacetylhydrazine in human plasma by high performance liquid chromatography (HPLC) for use in drug monitoring during treatment of complicated tuberculosis.

5.2 EXPERIMENTAL

5.2.1 Materials

Isoniazid, rifampicin, pyrazinamide, hydrazine hydrate, phenelzine, N-butarylaminophenol, heptane sulfonic acid, and acetic acid were obtained from Sigma Chemical Co.(U.S.A).

Acetylisoniazid, monoacetylhydrazine and diacetylhydrazine were synthesised in the laboratory according to the procedures described by Turner (1947). See below.

5.2.1.1. Synthesis of metabolites.

Monoacetylhydrazine: Hydrazine hydrate 25 ml (equivalent to 392 mmol of hydrazine) was reacted with 10 ml (98 mmol) of acetic anhydride. The mixture was stirred continuously for 12 hours at room temperature in a fume board. Excess hydrazine was removed by evaporation under vacuum and monoacetylhydrazine was recrystallised in a mixture of dichloromethane and n-hexane (2:1). As

monoacetylhydrazine is deliquescent, it was converted to a non-deliquescent hydrochloride form before use. 10 mg of dried monoacetylhydrazine was dissolved in 10 ml of a solution of hydrogen chloride and diethyl ether (1:1). The mixture was let stand for 1 hour and then filtered. The crystalline residue was dried for 10 hours at 80°C. Taking molecular weights of monoacetylhydrazine and monoacetylhydrazine-hydrogen chloride to be 74 and 109, respectively, 4.3 g (588 mmol) of monoacetylhydrazine were produced from which 3.2 g (29 mmol) of monoacetylhydrazine-hydrogen chloride was made.

Diacetylhydrazine: Hydrazine hydrate 25 ml (equivalent to 392 mmol of hydrazine) was reacted with 10 ml (98 mmol) of acetic anhydride. The mixture was stirred continuously for 12 hours at room temperature in a fume board. Excess hydrazine was removed by evaporation under vacuum and diacetylhydrazine was recrystallised in a mixture of dichloromethane and n-hexane (1:4). Approximately 1.2 g (10 mmol) of diacetylhydrazine was obtained (Mol. Wt. of diacetylhydrazine at 116).

Acetylisoniazid: 3.5 g (25 mmol) of isoniazid was reacted with 5 ml (196 mmol) of acetic anhydride at room temperature for 4 hours with continuous stirring. The product was recrystallised in diethyl ether. Taking the molecular weight of acetylisoniazid to be 179.6, approximately 1.5 g (8.3 mmol) of this metabolite was made.

5.2.1.2 Synthesis of salicylhydrazones.

The salicyl-hydrazones (HDZ) of isoniazid (isoniazid-hydrazone; INH-HDZ), phenelzine (phenelzine-hydrazone; PHEN-HDZ), monoacetylhydrazine (monoacetylhydrazine-hydrazone; mHYD-HDZ), and hydrazine (azine; Azine) were synthesised and identified as described earlier (Jenner and Ellard, 1987; Walubo et al, 1991a; Yale, 1953). Briefly, 7.3 mmol of each of the compounds (isoniazid 100 mg; phenelzine sulphate 100mg; hydrazine hydrate 35 mg; monoacetylhydrazine 54 mg; acetylisoniazid 130 mg; and diacetylhydrazine 85 mg) was shaken with 120 mg of the salicylaldehyde in 5 ml of ethanol in a tight screw-capped 10 ml centrifuge tube. The mixture was incubated in a water bath at 60°C for 30 minutes. The product were purified by crystallisation in ethylacetate .

Mass reaction ratio for derivatisation is 1:1 (Cram and Hammond, 1964) viz:

Hydrazide + Salicylaldehyde --- acid catalysis ---> Hydrazone + Water.

Molecular weights of the salicylhydrazones were assumed (not confirmed) to be; 241.4 for INH-HDZ, 240.4 for PHEN-HDZ, 240.5 for Azine and 178.4 for mHYD-HDZ.

All compounds and their salicylhydrazones were characterised by high-performance liquid chromatography, UV absorption, mass spectra, and thin-layer chromatography (TLC: system T1) (Curry and Clarke, 1975).

TLC procedure: The compounds, in methanolic solutions of 1 mg/ml, were spotted on a silica plate (coated with G 60, 0.2 mm thick; Sigma) 1.5 cm from the bottom line. The plate was placed in a tank containing ammonia-methanol (1.5:100) to a depth of 0.5-1 cm, and the tank was made air-tight with a lid. After 30 min the plate was dried in air and compounds were located by potassiumiodoplatinate (Sigma).

5.2.2. HPLC Apparatus

HPLC assays were done on SpectraPhysics (San Jose, CA, U.S.A) instruments which consisted of a pump model SP 8800, a degasser SCM 400, autosampler SP 8780 and integrator SP 4290 connected to a variable wavelength UV detector model 0200-4000, Linear Corpor. (Reno., Nevada, U.S.A.).

5.2.3. Mobile phase systems

System 1: 100 % acetonitrile in bottle A and 5 mM phosphate buffer at pH 3.5 in bottle B which were run at 2 ml/min on a gradient from 6% A to 90% A within 5 min and then continued at this concentration (90% A) for 12 min. A reversed-phase C_8 analytical column (Spherisorb, 250 mm x 4.6 mm I.D., 5 um; Phase Sep., U.S.A.) linked to a C_8 pre-column (30 um, 50 mm x 4.6 mm I.D.) was used. Maximum pressure was 124 Bars.

System 2: 100% acetonitrile in bottle A, 5 mM heptanesulphonic acid in acetonitrile-water-triethylamine (70:30:0.4 v/v) at pH 6 in

bottle B and 100% water in bottle C. These were run at 1 ml/min as: 75% B with 25% C for the first 5 min and then 85% B with 15% A for 12 min. A reversed-phase C_{18} analytical column (Spherisorb, 250 mm x 4.6 mm I.D., S5 ODS2; Phase Sep.) linked to a C_8 pre-column (30 um, 50 mm x 4.6 mm I.D.) was used. Maximum pressure was 103 Bars.

5.2.4. Sample preparation

a) Determination of rifampicin and pyrazinamide.

After addition of internal standards (50 ul of N-butarylaminophenol 50 ug/ml and 50 ul of phenelzine 50 ug/ml) to 2 ml of plasma in a 15 ml test-tube, 0.4 ml 10% (v/v) aqueous acetic acid was added to adjust pH to 4.2. Rifampicin and pyrazinamide were extracted by shaking with 7 ml of diethylether-dichloromethane (2:1) which, after centrifugation for 10 min at 2,059 g, was transferred to a tapered test-tube and evaporated under nitrogen (N_2) at 40 °C. The residue was dissolved in 200 ul of methanol of which 20 ul were injected into the HPLC and eluted with system 1. The eluate was detected at 248 nm.

b) Determination of isoniazid, monoacetylhydrazine and hydrazine

To the aqueous fraction, after extraction of rifampicin and pyrazinamide, further 10% aq. AA (0.6 ml) is added to lower the pH to 3.2. Ethanolic solution (0.3 ml) of 0.1% (v/v) salicylaldehyde was added and derivatisation was completed by incubation in a water bath at 60 °C for 30 min. The mixture was then cooled to room temperature (25 ± 1 °C), 1 ml of 1M K_2PO_4 added and extracted twice with 7 ml of diethylether which, after centrifugation for 10 min at 2,059 g, was transferred to a tapered test-tube and evaporated under nitrogen at 40 °C. The residue was dissolved in 200 ul of mobile phase B from system 2 of which 20 ul were injected into the HPLC and eluted with system 2. The eluate was detected at 280 nm.

c) Determination of acetylisoniazid and diacetylhydrazine

The aqueous fraction after organic extraction in (b) was transferred to a fresh 15 ml test-tube and 0.5 ml of 5 M HCL was added. Acetylisoniazid and diacetylhydrazine were hydrolysed to isoniazid and monoacetylhydrazine respectively by incubating in a water bath at 60 °C for 45 min. The mixture was then cooled to room temperature (25 °C) and 0.45 ml of 5 M NaOH was added to adjust pH to 2.2 followed by internal standard phenelzine of 50 ug/ml) and 0.3 ml of ethanolic solution of 0.1% (v/v) salicylaldehyde were added. Derivatisation was completed by incubation in a water bath at 60 °C for 30 min. The mixture was then cooled to room temperature (25 ± 1 °C), 1 ml of 1M K_2PO_4 added, extracted with 7 ml of diethylether which, after centrifugation for 10 min at 2059 g, was transferred to a tapered test-

tube and evaporated under nitrogen at 40°C . The residue was dissolved in 200 ul of mobile phase B of system 2 of which 20 ul were injected into the HPLC and eluted with system 2. The eluate was detected at 280 nm.

5.3 RESULTS

5.3.1. Characterisation of compounds

Figures 4.1 and 4.2, respectively, illustrate TLC R_F (retention factor) values and UV absorption spectra of the made compouds. The TLC R_F values are: isoniazid (INH) = 0.55, isoniazid-hydrazone (INH-HDZ) = 0.68, acetylisoniazid (acINH) = 0.55, monoacetylhydrazine (mHYD) = 0.65, monoacetylhydrazine- hydrazone (mHYD-HDZ) = 0.71, diacetylhydrazine (dHYD) = 0.45, hydrazine (HYD) = 0.09, Azine = 0.76, phenelzine (PHEN) = 0.60, phenelzine-hydrazone (PHEN-HDZ) = 0.78 and salicylaldehyde = 0.64.

The salicylhydrazone of each compound is less polar and has a longer TLC R_F. These hydrazones absorbed more UV light than their parent compounds. The mass-spectra for isoniazid and the three synthetic metabolites are shown in figure 4.3a-d. Acetylisoniazid has a peak at 179 (molecular weight is 179-180; Fig. 4.3b), monoacetyhydrazine at 69 and 70 (molecular weight is 76; Fig. 4.3c) and diacetylhydrazine at 116 (molecular weight is 116; Fig. 4.3d).

Fig 4.1

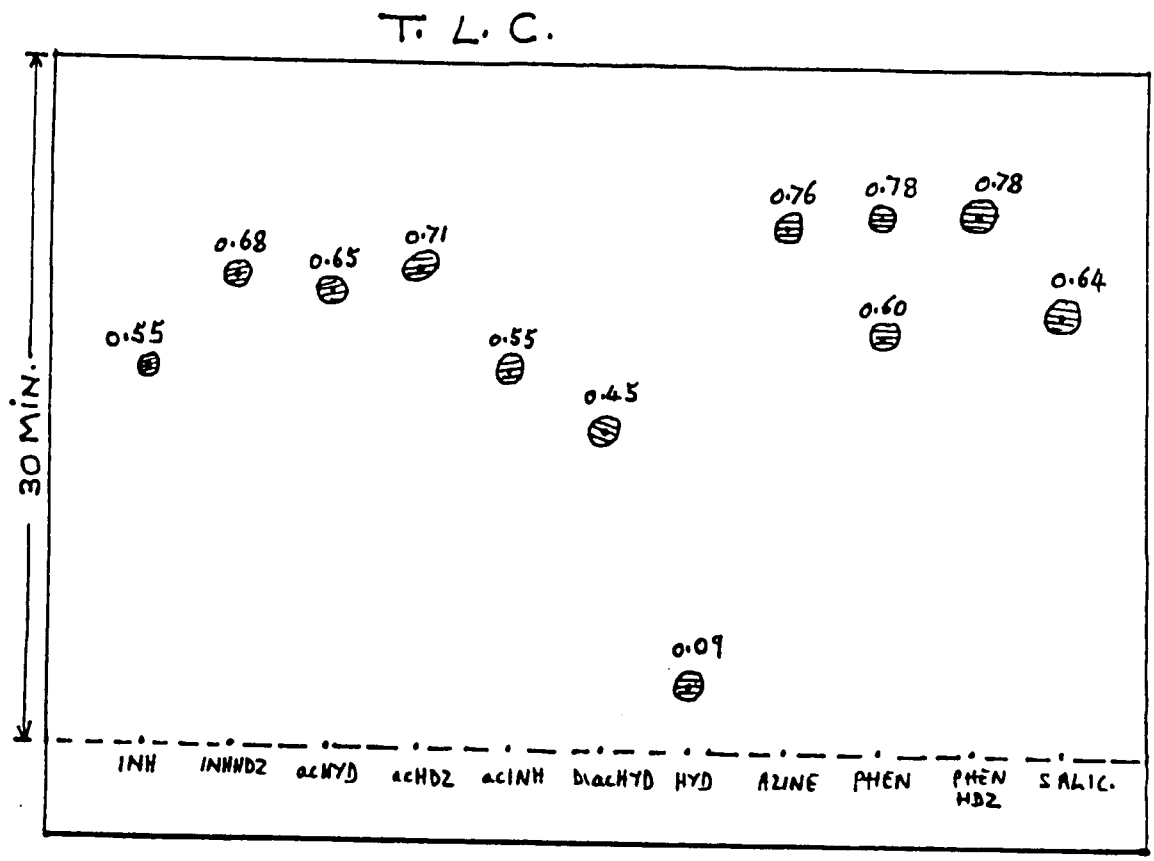


Figure 4.1 Thin Layer Chromatogram of the reference and parent compounds with retention factors in-printed.

Isoniazid (INH) and its hydrazone (INH-HDZ), monoacetylhydrazine (acHYD) and its hydrazone (acHDZ), diacetylhydrazine (diacHYD), hydrazine (HYD) and its hydrazone (Azine), phenelzine (PHEN) and its hydrazone (PHEN-HDZ) and salicylaldehyde (SALIC).

Figure 4.2 Ultra-Violet absorption spectra of the reference and parent compounds compared.

a) Isoniazid (INH) and its hydrazone (INH-HDZ),
b) Acetylisoniazid (acINH) and its hydrazone (INH-HDZ)
c) Monoacetylhydrazine (acHYD) and its hydrazone (acHDZ),
d) Hydrazine (HYD) and its hydrazone (Azine),
e) Phenelzine (PHEN) and its hydrazone (PHEN-HDZ),
f) Salicylaldehyde (SALIC) and
g) Acetic anhydride

Fig 4.2

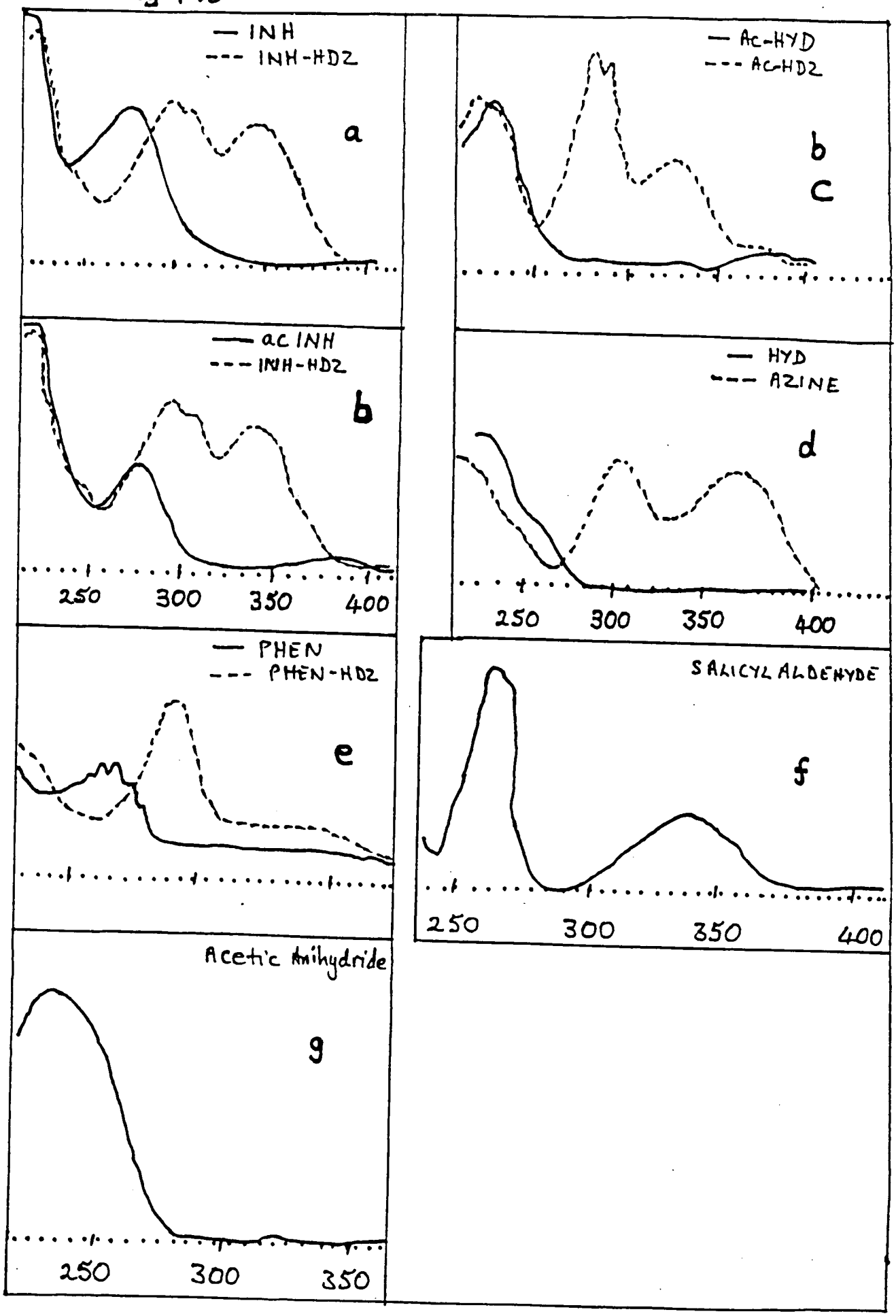

— INH
--- INH-HDZ
a
— Ac-HYD
--- Ac-HDZ
b
c
— ac INH
--- INH-HDZ
b
250
300
350
400
— HYD
--- AZINE
d
250
300
350
400
— PHEN
--- PHEN-HDZ
e
SALICYL ALDEHYDE
f
250
300
350
400
Acetic Anhydride
g
250
300
350

Figure 4.3 Mass spectrometry of

a) isoniazid (INH)

b) Acetylisoniazid (acINH)

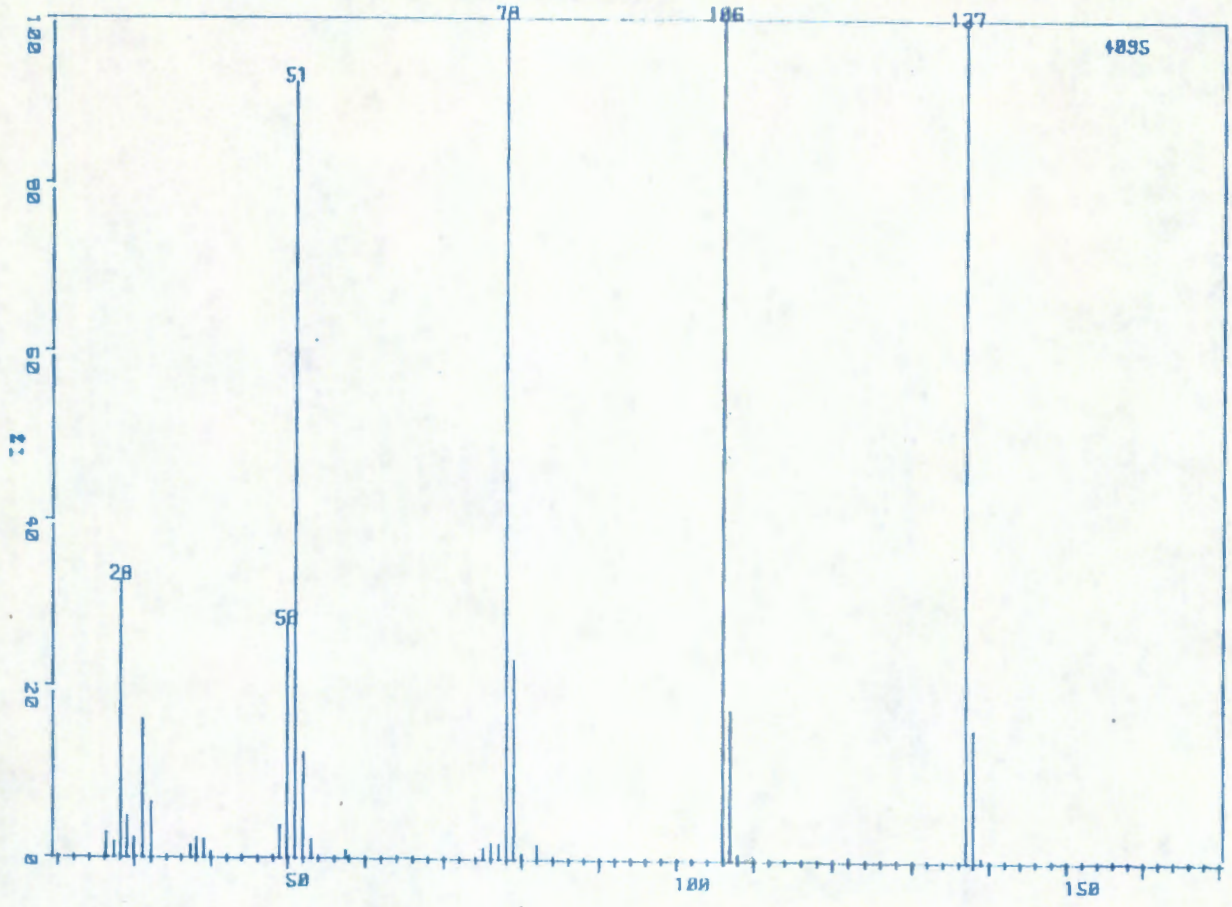
c) Monoacetylhydrazine (MonoacHYD) and

d) Diacetylhydrazine (DiacHYD)

CAL:CAL STA: INH

Fig 4.3a

21-JAN-1
0:48

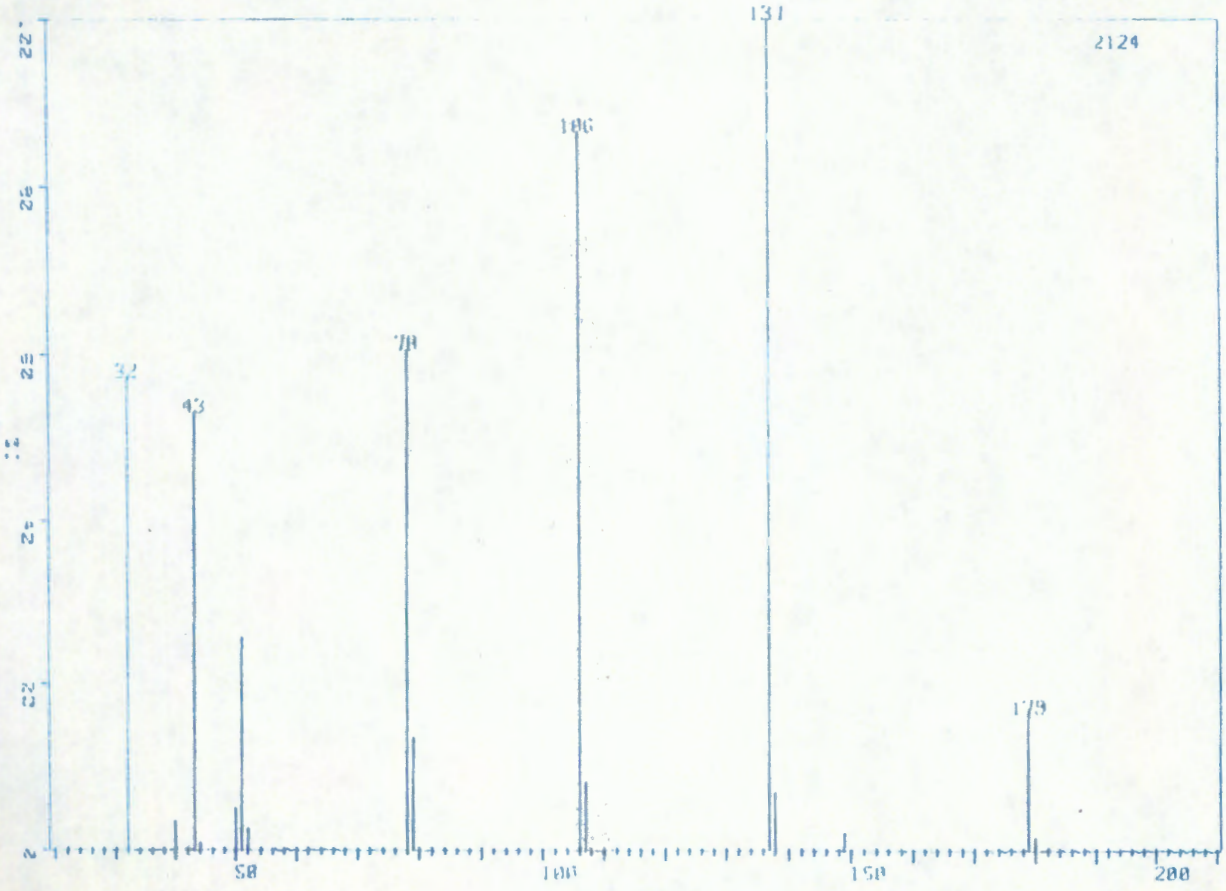


WAL1 14 C Ac INH

CAL:CAL STA: ~~Ac/NH~~

Fig 4.3b

25-FEB-1
0:53

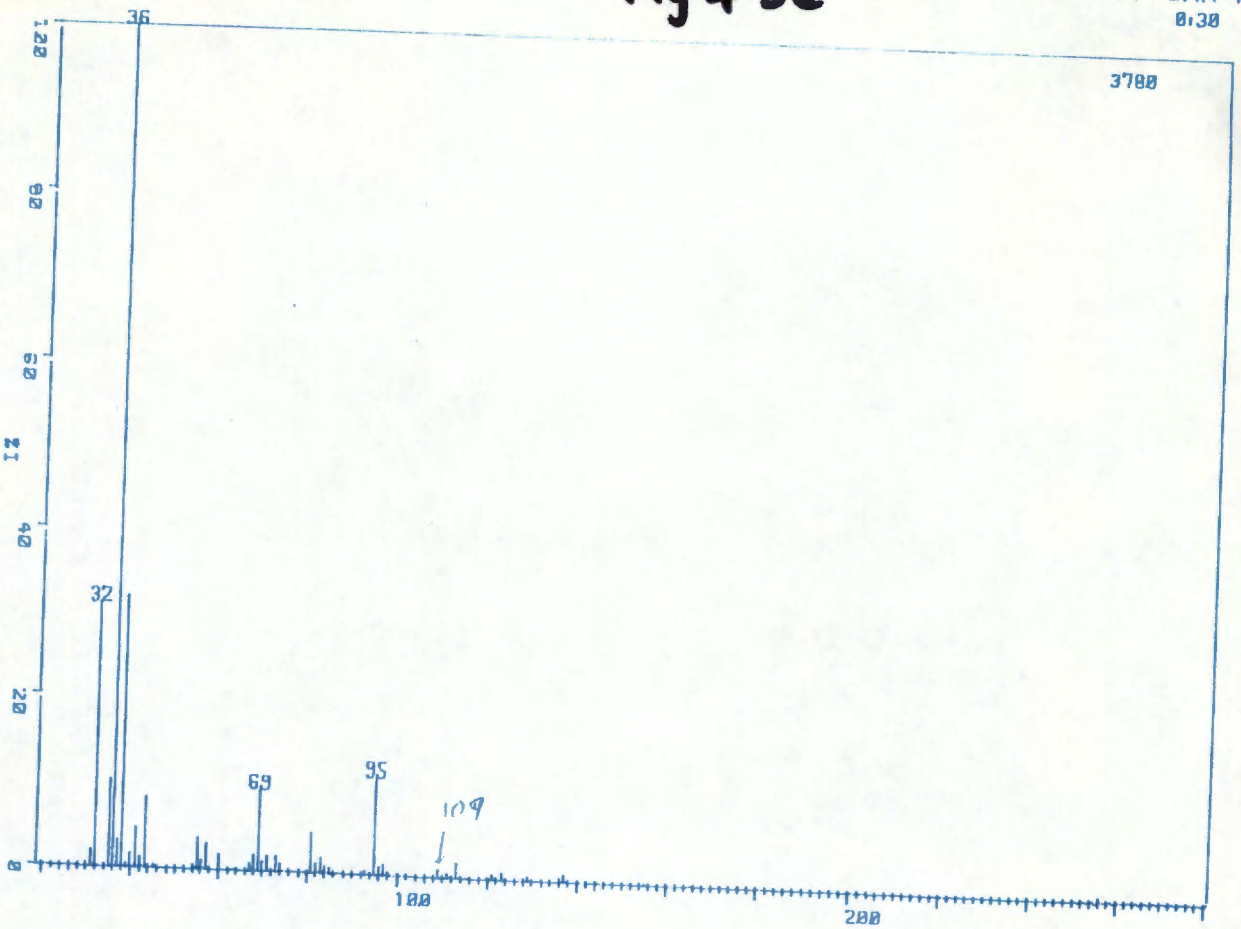


(Monoac HYD)

WAL2 7 A
CAL: CAL STA:

Fig 4·3C

21-JAN-11
0:30

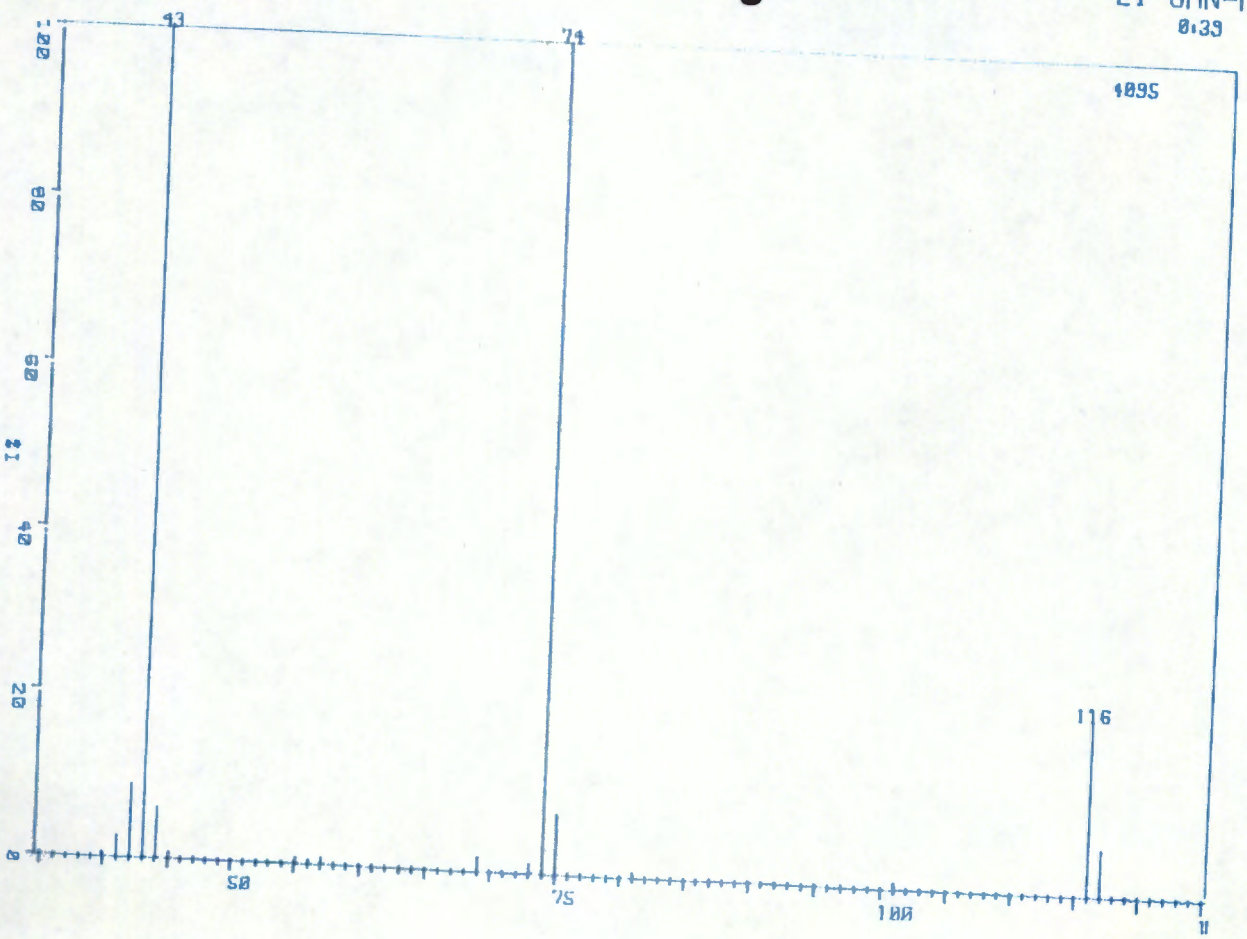


DiacHYD

WAL1 10 B
CAL: CAL STA:

Fig 4·3d

21-JAN-1
0:39



5.3.2. Performance of HPLC system

Figures 4.4 and 4.5 show the representative chromatograms of standard solutions, blank plasma and a plasma extract from a patient 6 hours after dosing, while table 4.1 illustrates the standardisation data of the assay. Retention times in min were: pyrazinamide = 2.91, rifampicin = 11.9 and N-butrylaminophenol = 4.22 (Fig. 4.4); monoacetylhydrazine = 3.75; isoniazid = 4.68; hydrazine = 13.77; phenelzine = 11.09, and excess salicylaldehyde = 5.78 (Fig. 4.5). The use of one internal standard did not affect linearity in the concentration ranges used. There was a great variation in the washing out of excess salicylaldehyde with 1M K_2PO_4 (Fig. 4.5B versus 4.5C and D). This was most probably due to difficulties in attaining the optimum conditions for this process (e.g pH). Nevertheless, this had no effect on the resolution or recovery of the relevant compounds.

The run time for the chromatograph of pyrazinamide and rifampicin was 15 min while that for isoniazid and metabolites was 17 min. The whole extraction and derivatisation procedure takes approximately 4 hours, a much shorter than if each drug were extracted and run separately using different procedures.

5.3.3. Recovery, precision, linearity and limit of detection

Recovery was assessed by comparing peak heights of the directly injected hydrazones (for isoniazid and metabolites) or drug (for pyrazinamide and rifampicin) with those obtained after derivatisation and extraction. Peak heights were evaluated automatically by the in

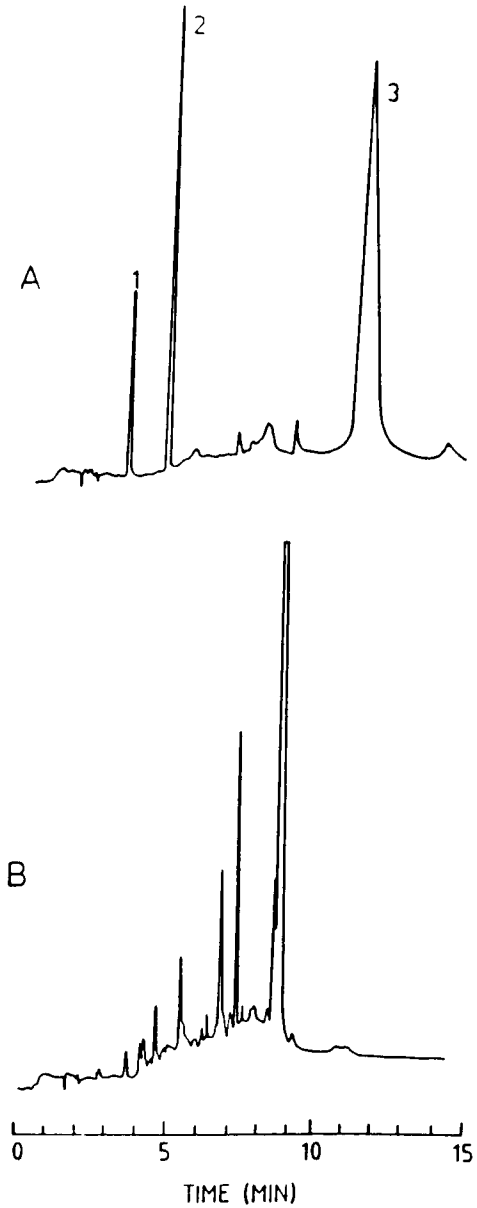
Figure 4.4. The representative chromatograms of standard solution (A), drug free plasma (B), and a plasma extract from a patient 6 hours after dosing (C).

1 = pyrazinamide (2.91 min); 2 = N-butarylaminophenol (internal standard, 4.22 min); 3 = Rifampicin (11.9 min)

Drug concentrations: A; pyrazinamide = 0.4 ug and rifampicin = 0.25 ug.

C; pyrazinamide = 24.3 ug/ml and rifampicin = 4.28 ug/ml.

Fig 4.4



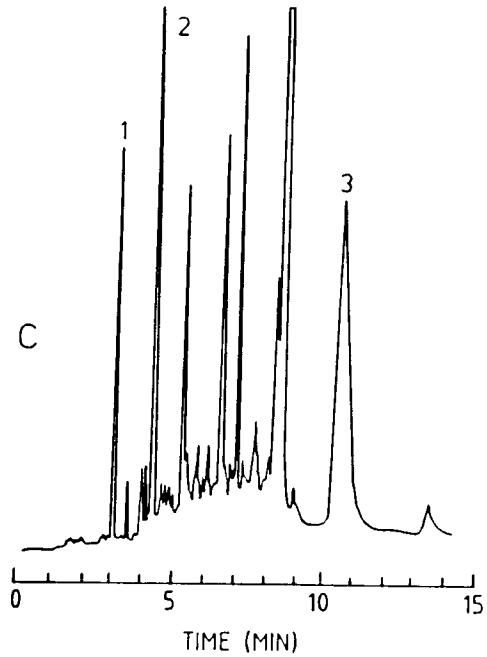


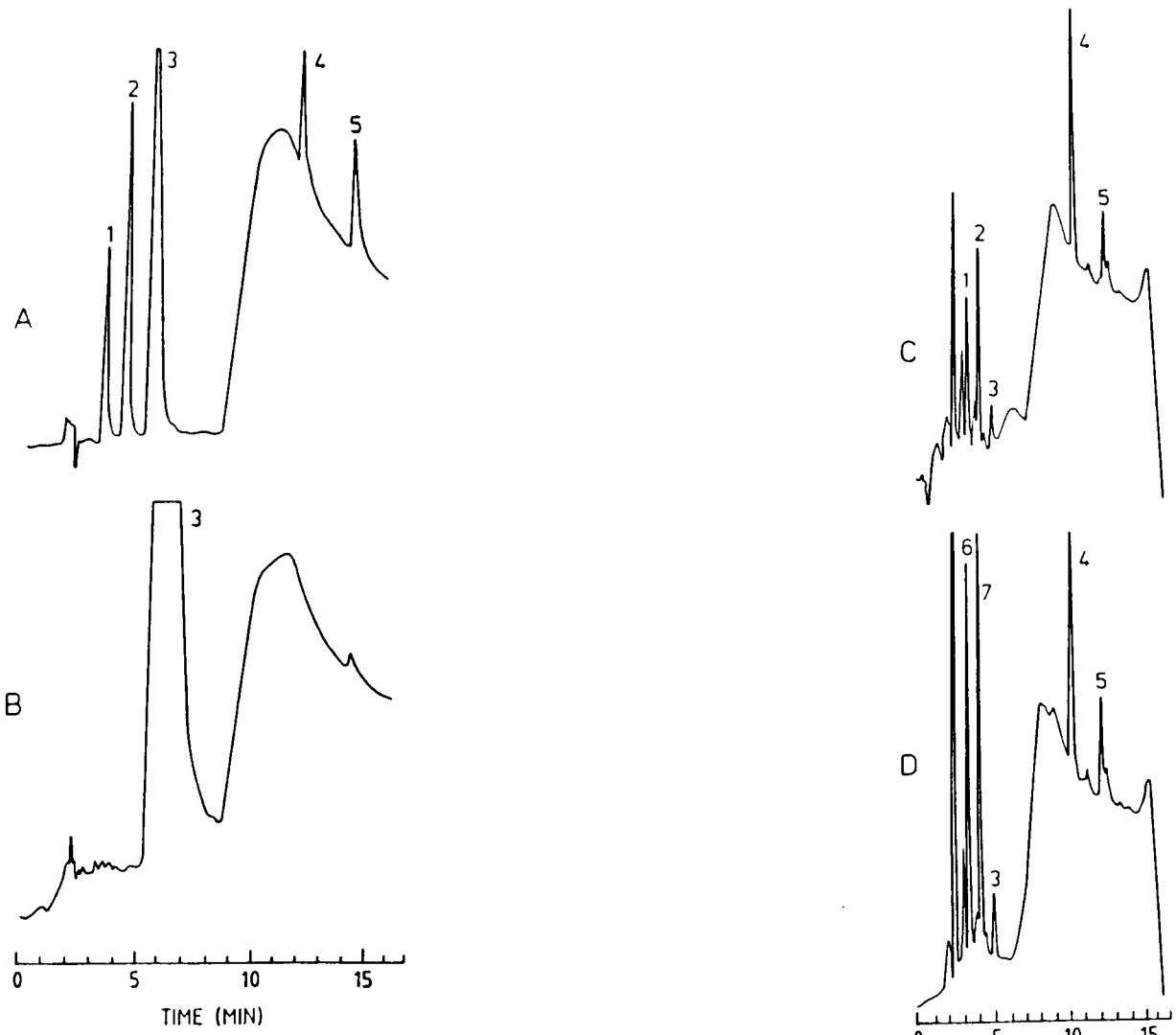
Figure 4.5. The representative chromatograms of standard solution (A), drug free plasma (B), and a plasma extract from a patient 6 hours after dosing, before (C) and after hydrolysis (D).

1 = mHYD (3.75 min); 2 = INH (4.68 min); 3 = salicylaldehyde (5.78 min); 4 = PHEN (internal standard, 11.09 min); 5 = HYD (13.77 min); 6 = dHYD (3.75 min); 7 = acINH (4.68 min).

Drug concentrations: A; INH-HDZ = 0.25 ug of INH, mHYD-HDZ = 0.1 ug of mHYD and Azine = 0.02 ug of HYD.

C and D; mHYD = 1.2 ug/ml, INH = 3.75 ug/ml, HYD = 0.28 ug/ml, dHYD = 0.41 ug/ml and acINH = 4.24 ug/ml.

Fig. 4.5



built intergrator and peak height ratios of the drugs to internal standard were used for deriving concentrations.

Concentration ranges of: 0.5, 1, 2, 5, and 8 ug/ml for rifampicin, isoniazid, acetylisoniazid, monoacetylhydrazine, and diacetylhydrazine; 0.1, 0.2, 0.5 and 0.8 ug/ml for hydrazine; and 5, 10, 15, 20, and 30 ug/ml for pyrazinamide were used for standardisation and regression analysis. Calibration equations of five standard curves were as follows; $y = 0.034x - 0.024$ for pyrazinamide; $y = 0.143x + 0.012$ for rifampicin; $y = 0.209x - 0.034$ for isoniazid; $y = 0.352x - 0.025$ for acetylisoniazid; $y = 0.463x + 0.009$ for monoacetylhydrazine; $y = 1.435x + 0.022$ for hydrazine and $y = 3.39x - 0.113$ for diacetylhydrazine. The mean regression coefficient (r) of the five standard curves and inter-assay coefficient of variation (CV%) at mid-point (5 ug/ml) of the curves are shown in table 1. For pyrazinamide and hydrazine the midpoint were 15 and 0.2 ug/ml, respectively.

Overall recovery at the mid-point of the standard curves is shown in Table 4.1. These recoveries, although lower, do not significantly differ from the original procedures. In the case of pyrazinamide and rifampicin, poor recovery could have occurred due to a single organic extraction step, failure to precipitate proteins before extraction and, for rifampicin, loss of drug due to autoxidation in the absence of vitamin C. Lower recovery for isoniazid and metabolites could have arisen from solubility limitations of the salicylhydrazones in the organic phase and adsorption to glass wares.

Table 4.1 A summary of standardisation data on recovery, linearity, and limit of detection.

Drug name	Lin. Regression (r) (n = 5)	Inter-assay C.V.% (at 5 ug/ml)	Recovery (%) (at 5ug/ml)	detection Lmt (ug/ml)
Pyrazinamide	0.997	2.3@	52.6 ± 3.2@	0.5
Rifampicin	0.996	9.8	54.4 ± 3.7	0.2
Isoniazid	0.998	9.8	46.3 ± 5.2	0.25
Acetylisoniazid	0.997	7.6	32.8 ± 3.5	0.25
Monoacetylhydrazine	0.999	6.2	49.2 ± 4.3	0.2
Diacetylhydrazine	0.999	12.5	34.6 ± 2.8	0.2
Hydrazine	0.999	6.2*	48.5 ± 3.5*	0.05

@ and * denote that evaluation for CV% and Recovery was done at 15 and 0.2 ug/ml, respectively.

Minimum reliable concentrations assayed are shown in Table 1. At concentrations lower than the limits stated, the coefficient variation is high such that the original procedures are preferred.

5.4. APPLICATION

The assay has been used to monitor patients with treatment problems one of whom is reported here. Briefly, she was a 54 year old patient who had been on antituberculosis therapy (rifampicin 8.8 mg/kg, isoniazid 8.8 mg/kg, ethambutol [not assayed] and streptomycin [for the first two months only]) for four months without improvement in spite of the organism sensitivity to the drugs. She was found to have moderate gastric outlet obstruction which was causing vomiting and it was not clear whether adequate plasma concentrations were being achieved. Five hours after dosing, plasma concentrations (ug/ml) were: rifampicin = 2.09, isoniazid = 4.77, acetylisoniazid = 6.89, monoacetylhydrazine = 1.8, diacetylhydrazine = 0.56 and hydrazine 0.35. Except for rifampicin, these levels were similar to those found in other patients in this setting where other assay procedures have been used, and they did not differ from the usual concentration ranges reported elsewhere (Iseman, 1993; Turner, 1947). The patient medications were adjusted to syrup preparations to improve absorption and compliance, and pyrazinamide was added to the regimen.

5.5. DISCUSSION

The assay described is beneficial in terms of sample utilisation and the limited amount of reagents used to analyse many compounds in a few

hours. It saves the burden of running different assays for each drug, and helps the clinician to evaluate all drugs and the relevant metabolites in time. The method is reproducible and can be adopted in any laboratory with a gradient programmer. For instance, System 2 is effectively run isocratically at 52.5% acetonitrile for the first five minutes, and then 74.5% acetonitrile for the next 12 minutes. It was also found that the column in system 2 can be used in system 1 without affecting the resolution of the compounds. The retention time for isoniazid hydrazone was pH sensitive whereby at a pH less than 4 it co-eluted with the salicylaldehyde. The low sensitivity with this procedure does not preclude its usefulness in the circumstances for which it has been developed. It is a useful clinical and research tool for quantitative assay of the compounds studied.

CHAPTER FIVE

THE GENERATION OF OXYGEN FREE RADICALS BY ANTITUBERCULOUS DRUGS AND HYDRAZIDE METABOLITES *IN VITRO*.

5.0 SUMMARY

Rifampicin, pyrazinamide and isoniazid with its hydrazide metabolites; monoacetylhydrazine, acetylisoniazid, hydrazine and diacetylhydrazine were screened for production of superoxide anion and hydrogen peroxide radicals. Isoniazid, monoacetylhydrazine, acetylisoniazid and hydrazine, activated oxygen to superoxide and hydrogen peroxide radicals while pyrazinamide, rifampicin and diacetylhydrazine did not Isoniazid reaction was most sensitive to superoxide dismutase (SOD) inhibition, while acetylisoniazid and monoacetylhydrazine reactions were sensitive to both SOD and catalase. In conclusion, isoniazid, monoacetylhydrazine, acetylisoniazid and hydrazine can activate oxygen to reactive oxygen species in presence of oxyhaemoglobin.

5.1 INTRODUCTION

Because of its importance in the treatment of tuberculosis, isoniazid is the most widely studied antituberculosis drug. Unfortunately, even after four decades in use, a lot remain unknown about its mechanism of action and toxicity. Understanding of the mechanism of toxicity is vital, not only for preventing occurrence of toxicity, but it may widen the use of isoniazid in patients where it is currently contraindicated.

Hydrazine metabolites have been implicated in isoniazid induced toxicity. Monoacetylhydrazine has been proposed as the most toxic metabolite (Mitchel et al, 1976). However, this theory emphasizes formation of organic radicals (acyl radical) and underscores the importance of other radicals (oxygen radicals) that are formed in the process.

Despite several reports showing that the action of isoniazid against mycobacteria is partly due to generation of free radicals (Shoeb et al, 1985a, 1985b and 1985c; Knox et al, 1956) and that hydrazine compounds can generate reactive oxygen species through the hydrazine group (Jain and Hochstein, 1979; Goldberg and Stern, 1974; Misra and Frodovich, 1976; Goldberg and Stern, 1976), there is no report relating isoniazid induced toxicity to this form of tissue injury. This is important because although isoniazid and the toxic metabolites are hydrazides, they are structurally different and thus bound to vary in reactivity and amount of each of the free radical species formed. Furthermore, as every free radical is potentially toxic, it is important to understand the extent of formation of acyl and oxygen free radicals formed by each of the metabolites and parent drug. For instance, it was shown that production of free radicals by isoniazid varied with reaction environment. At alkaline pH, horseradish peroxidase catalysed oxidation of isoniazid to superoxide radicals when oxygen was utilized, but organic radicals when hydrogen peroxide was consumed (Shoeb et al, 1985b). Therefore, during free radical reactions involving hydrazines, reaction environment may determine the dominant radical species produced.

I envisaged that during isoniazid metabolism, isoniazid and its metabolites (hydrazine, acetylhydrazine, acetylisoniazid and diacetylhydrazine) form reactive oxygen species which may lead to toxicity. However, because isoniazid is commonly prescribed together with pyrazinamide and rifampicin the two drugs were included in this evaluation. Using procedures described by Goldberg and Stern (1974), rifampicin, pyrazinamide, isoniazid and the four metabolites were screened for production of superoxide anion and hydrogen peroxide radicals by monitoring for oxidation of epinephrine in presence of oxyhaemoglobin (HbO) with or without superoxide dismutase (SOD) and catalase enzymes.

Haemoglobin (Hb), a haemo-protein like horse peroxidase and catalase, has been shown to oxidise hydrazine and form reactive oxygen species (Jain and Hochstein, 1979; Goldberg and Stern, 1974). It is ubiquitous in the body and is likely to come in contact with drugs and metabolites. Therefore, Hb was found most appropriate and toxicologically important to use in this investigation.

It is hoped that the result of this experiment will contribute to understanding the mechanism of isoniazid induced toxicity and allow the relocation of efforts to compounds that prove significantly positive by this mechanism.

5.2 METHODS

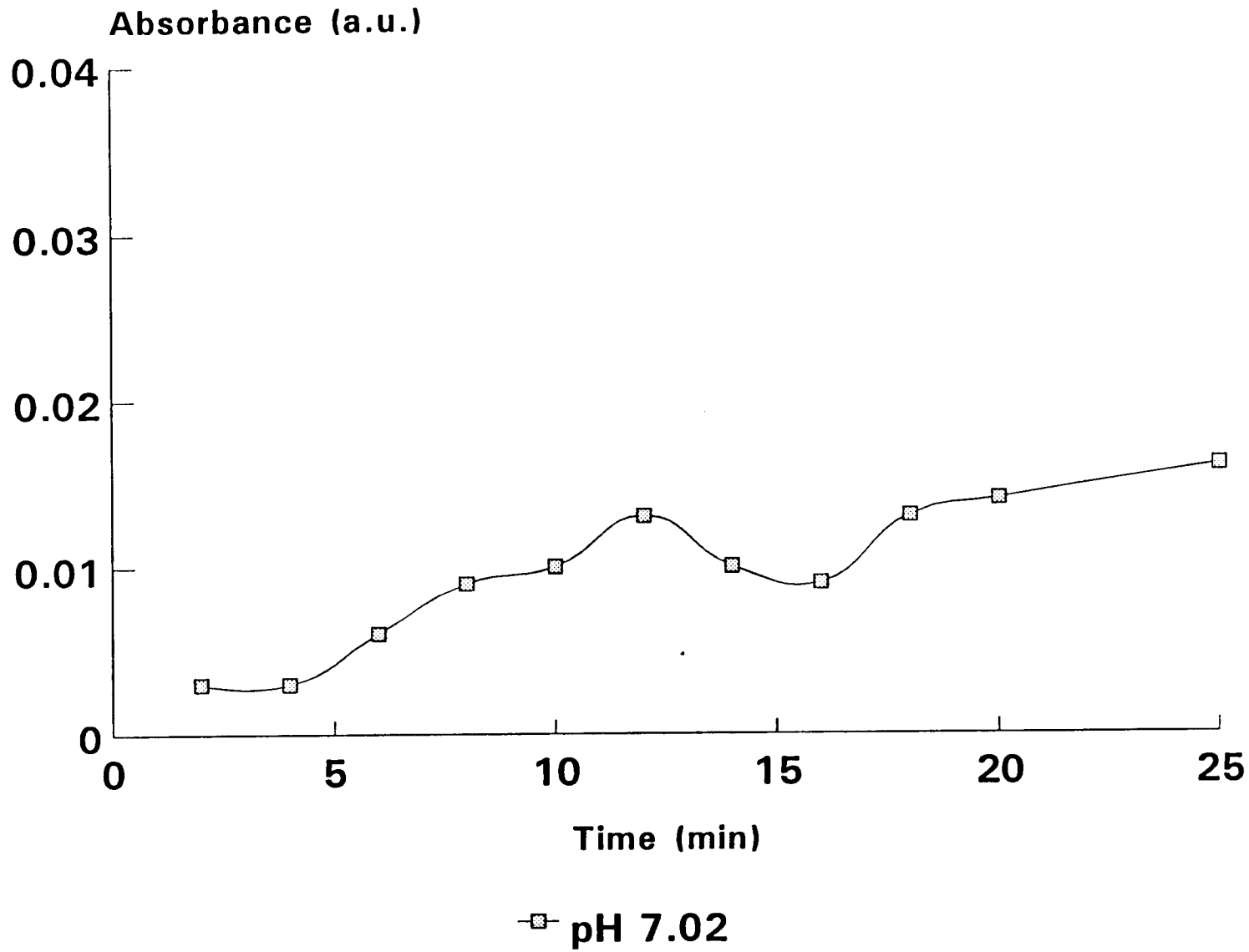
Isoniazid, rifampicin, pyrazinamide, SOD, catalase, Hb, epinephrine, and hydrazine hydrate were obtained from Sigma Chemical Co.(U.S.A, St. Louis), while acetylisoniazid, diacetylhydrazine and monoacetylhydrazine were synthesised in the laboratory as described earlier (chapter IV).

A stock solution of Hb (7.6×10^{-5} M) was shaken in O_2-CO_2 mixture (95%/5%) to yield HbO which was used in the experiment. The reaction volume was 4 ml of 100 mM EDTA in 50 mM sodium phosphate buffer containing HbO, 3.8×10^{-5} M; the test drug, 1.4×10^{-4} M; SOD, 7.7 μg/ml; Catalase, 3,500 i.u/ml; and epinephrine, 2.01×10^{-4} M.

When added to the reaction test tube alone, neither HbO nor the test drugs and metabolites could oxidise epinephrine. Oxidation of epinephrine occured only in presence of HbO with isoniazid or metabolites. At pH 7.02 the reaction between isoniazid and HbO was very slow (Fig. 5.1) while at pH 9.2 the reaction was faster and in most cases was complete by 14 minutes. Therefore pH 9.2 was used in further experiments.

Adrenochrome formation was monitored every 2 minutes by a spectrophotometer at 475 nm, 30°C and for 25 min. Timing was started when the test drug was added. Absorption was read against a control cuvette containing only epinephrine and HbO plus catalase and or SOD (i.e. without test drug). Three experiments were run for

Fig. 5.1 Adrenochrome formation by isoniazid



each compound and average net absorption was plotted on a graph versus time.

STATISTICAL ANALYSIS:

Parametric statistics was used. The student-Newman-Keuls Multiple test was used for testing level of significance at $P < 0.05$ and was analysed on the Instat computer package.

5.3 RESULTS

Figure 5.2 is a graphical illustration of adrenochrome formation during the reaction of HbO with isoniazid, acetylisoniazid, hydrazine and monoacetylhydrazine. In most reactions, adrenochrome formation was at maximum by 14 minutes.

Isoniazid (fig 5.2a): SOD inhibited adrenochrome formation by 74% while catalase had no effect. In presence of both enzymes the reaction was inhibited by 35% .

Acetylisoniazid (fig.5.2b): SOD inhibited adrenochrome formation by 92% while catalase increased the reaction by 143%. Both enzymes completely inhibited the reaction (100%).

Hydrazine (fig 5.2c): SOD inhibited the reaction by 87.8%, catalase by 53% and both enzymes 24.5%

Monoacetylhydrazine (fig 5.2d): There was minimal formation of reactive oxygen species. SOD inhibited the reaction by 62.5% and

Figure 5.2 Adrenochrome formation (absorption units; a.u.) under various conditions by;

5.2a. isoniazid

5.2b. Acetylisoniazid

5.2c. Hydrazine

5.2d. Monoacetylhydrazine.

KEY

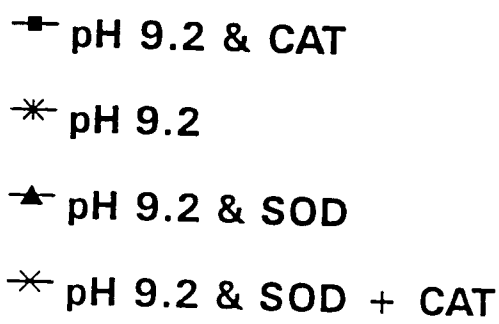


Fig. 5.2a Adrenochrome formation by isoniazid

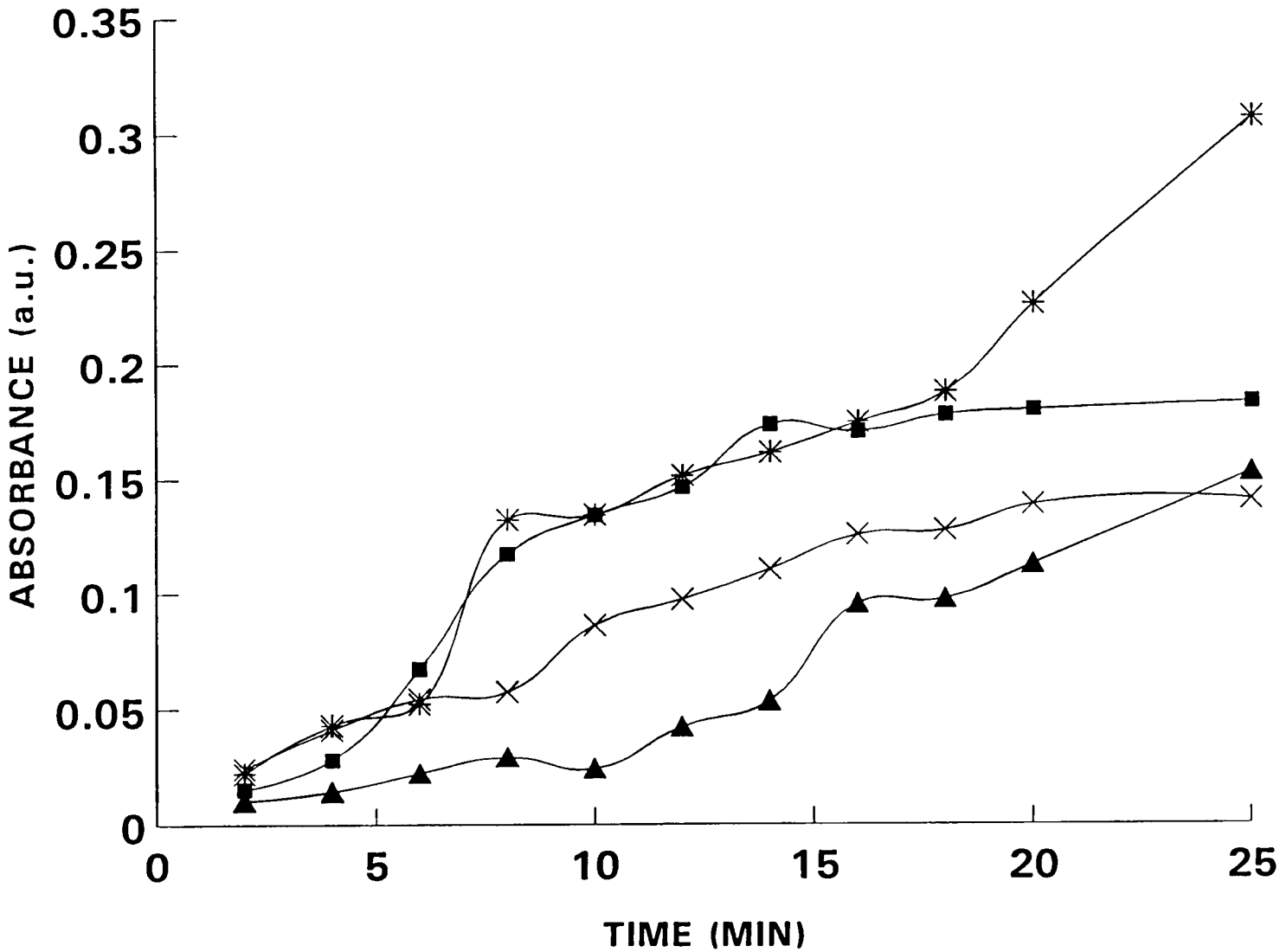


Fig. 5.2b Adrenochrome formation by acetylisoniazid

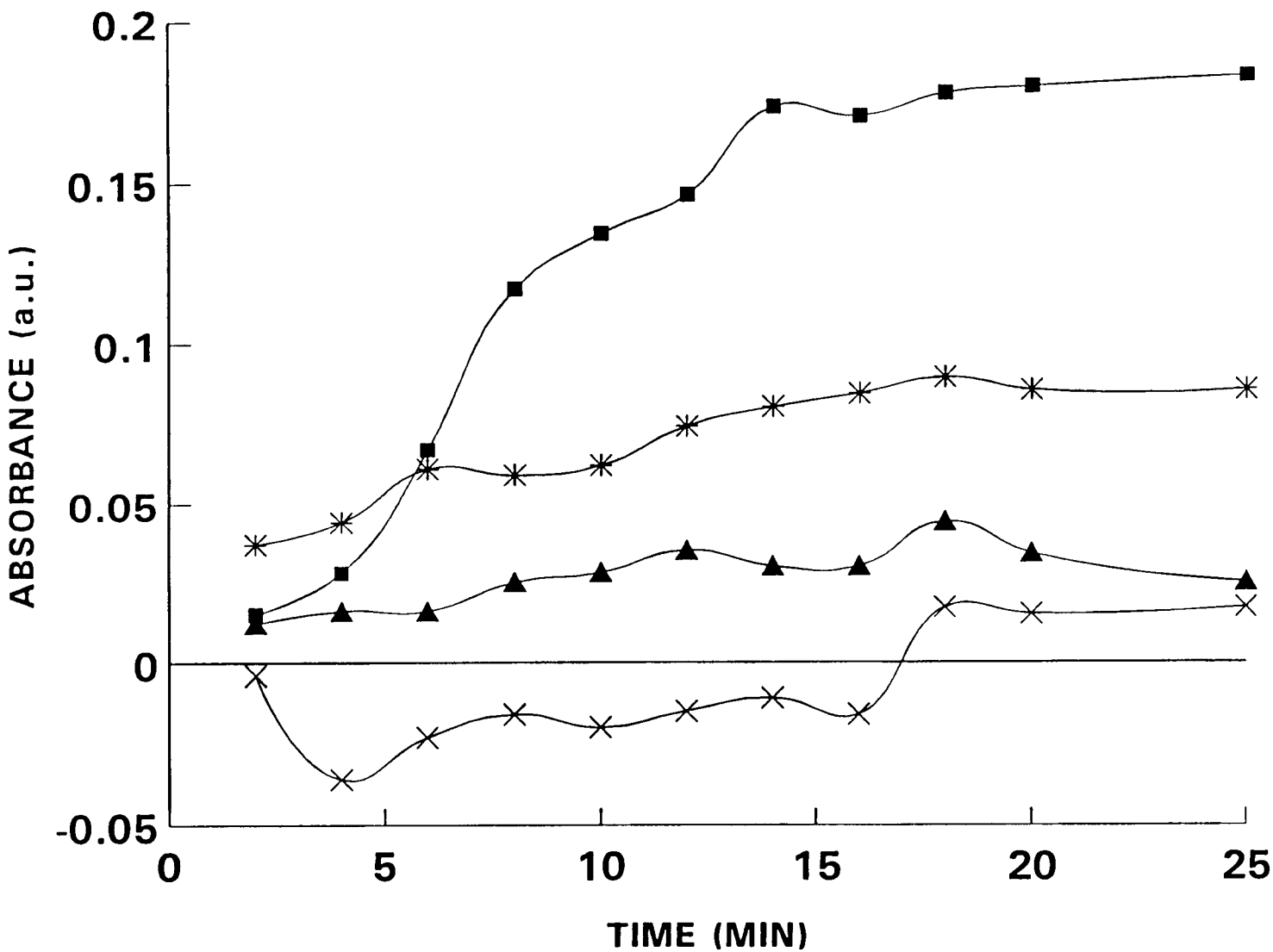


Fig 5.2c Adrenochrome formation by hydrazine

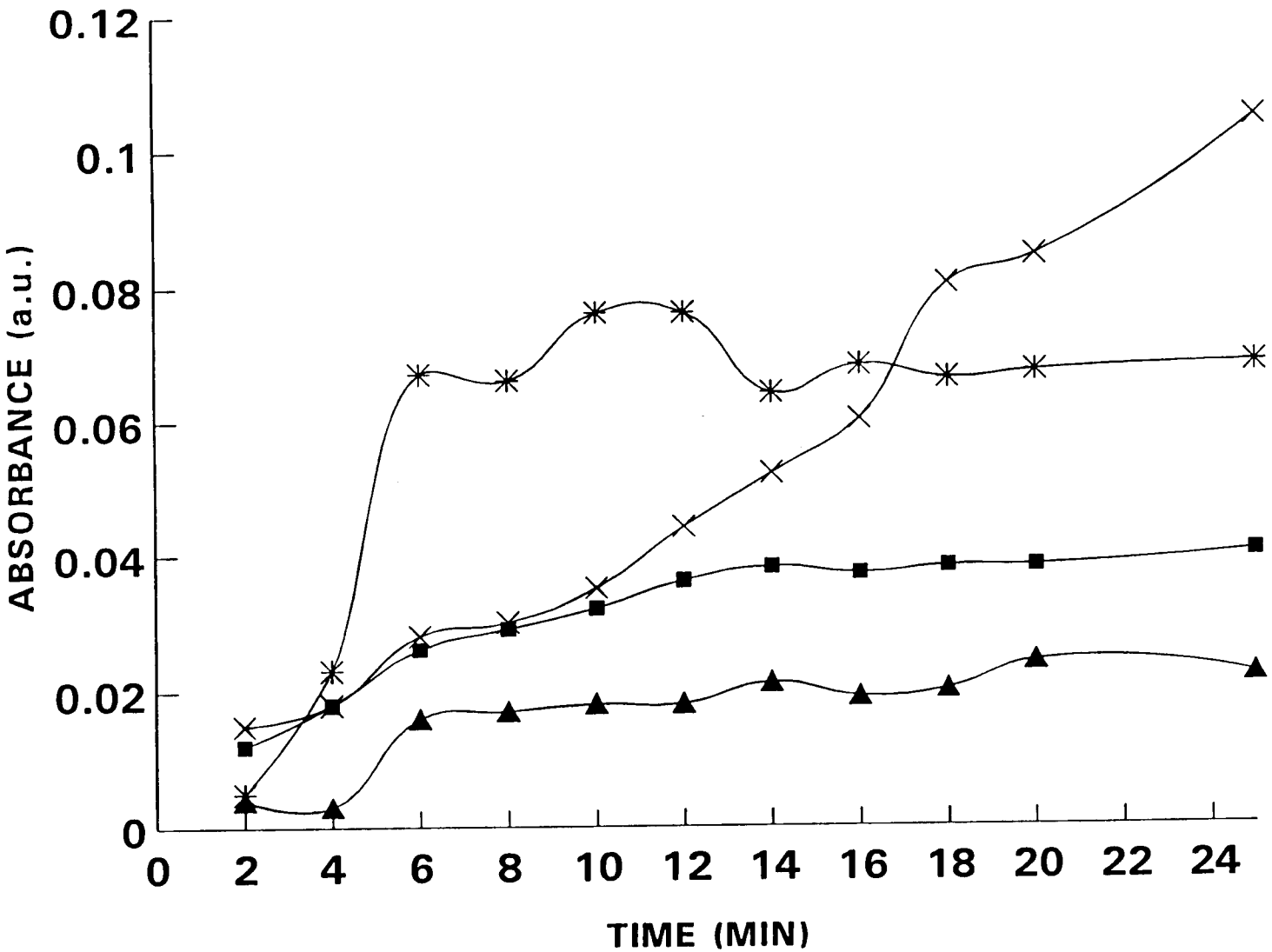
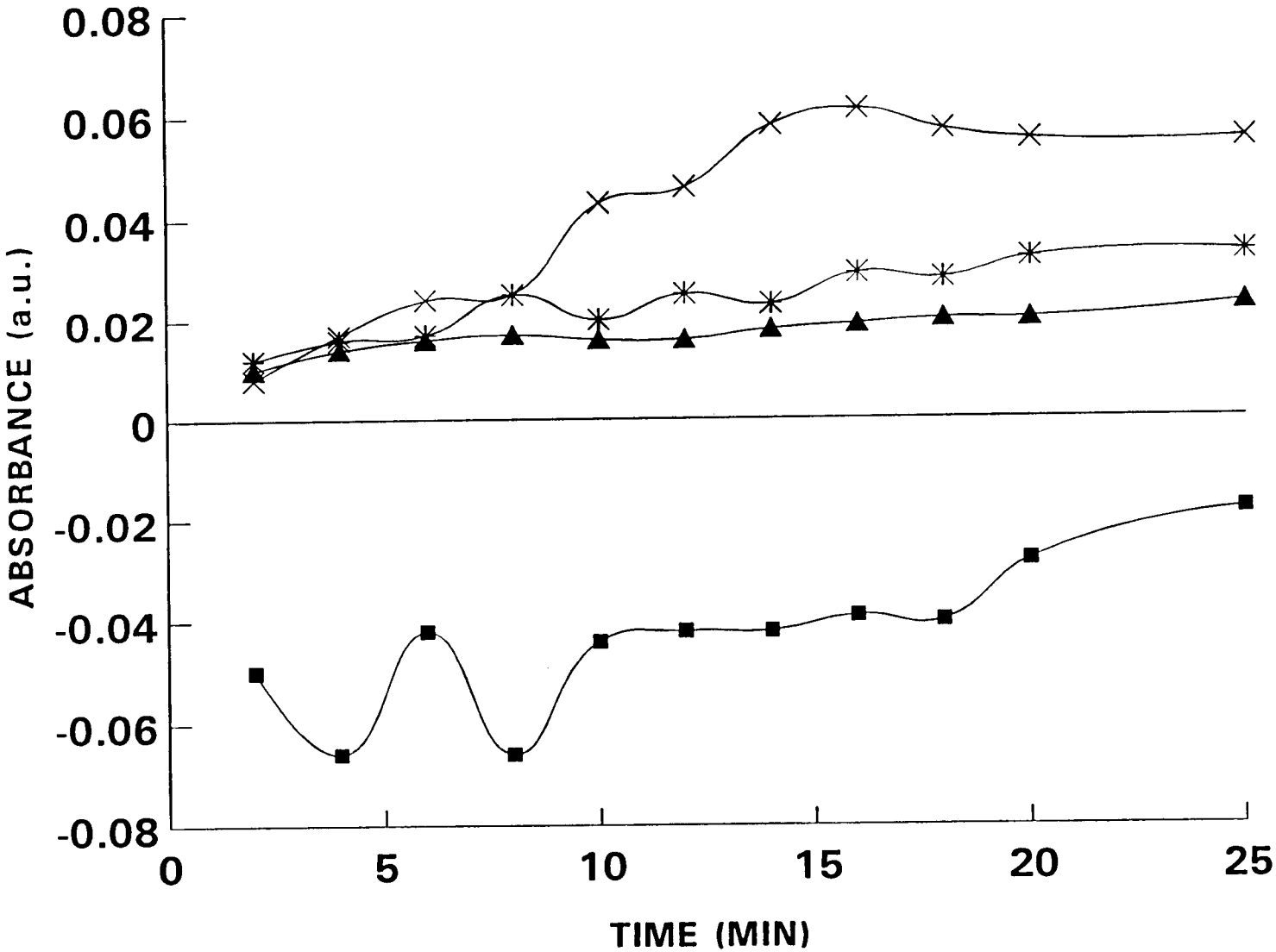


Fig. 5.2d Adrenochrome formation by monoacetylhydrazine



catalase caused almost total inhibition (100%), but both enzymes increased the reaction by 362.5%.

Diacetylhydrazine, pyrazinamide and rifampicin did not show adrenochrome production (fig 5.3a-c). At pH 9.2 and without enzymes, absorption reading at 14 minutes was -0.008 a.u. for diacetylhydrazine, 0.00 for pyrazinmide and -0.25 for rifampicin. This was not significant when compared to others viz: isoniazid, 0.146; acetylisoniazid, 0.065; hydrazine, 0.049 and monoacetylhydrazine, 0.008 (Table 5.1)

Figure 5.3 An illustration of lack of adrenochrome formation at pH 9.2 by;

5.3a. Diacetylhydrazine
5.3b. Pyrazinamide
5.3c. Rifampicin.

KEY

Fig. 5.3a Adrenochrome formation by diacetylhydrazine

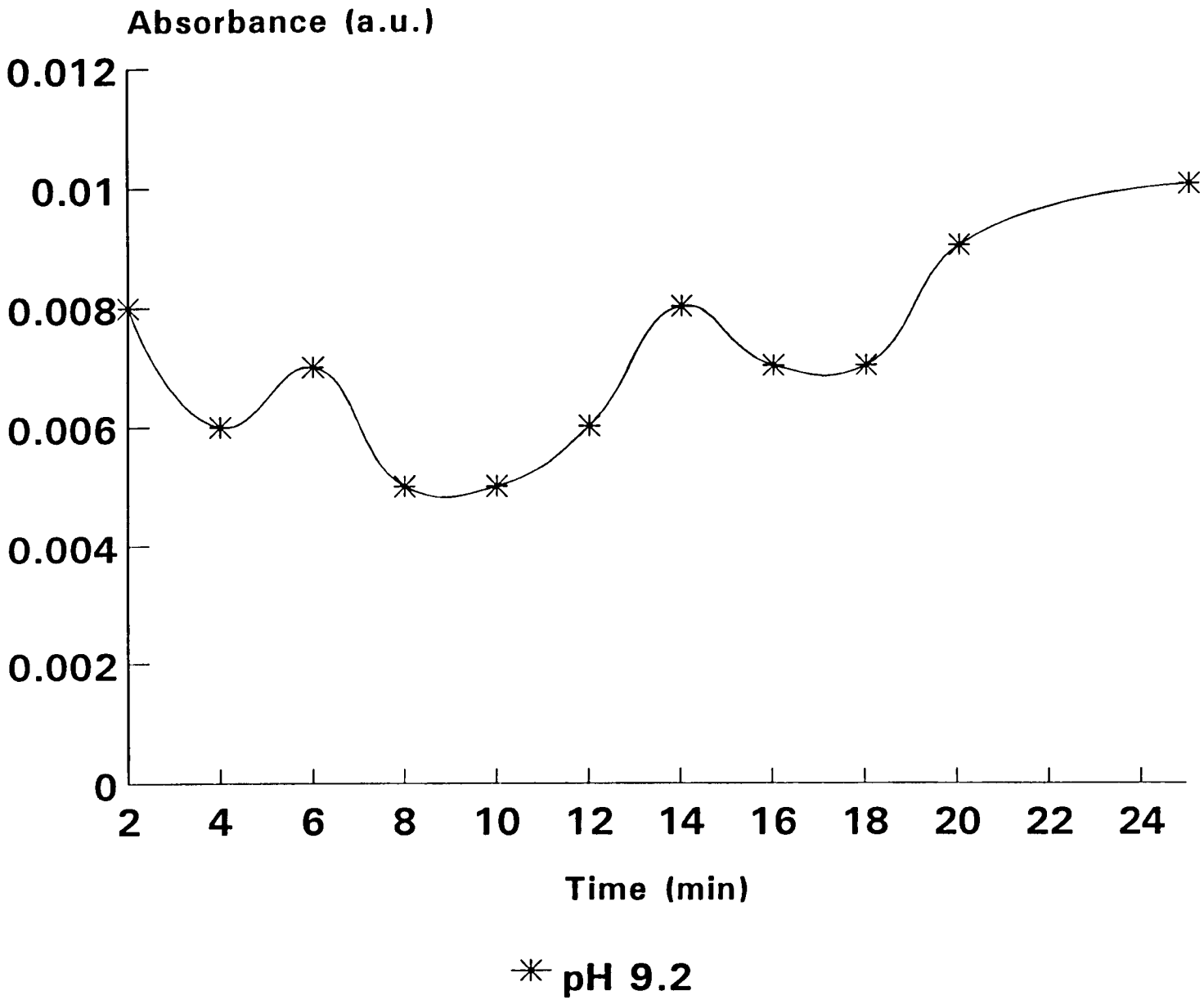


Fig. 5.3b Adrenochrome formation by pyrazinamide.

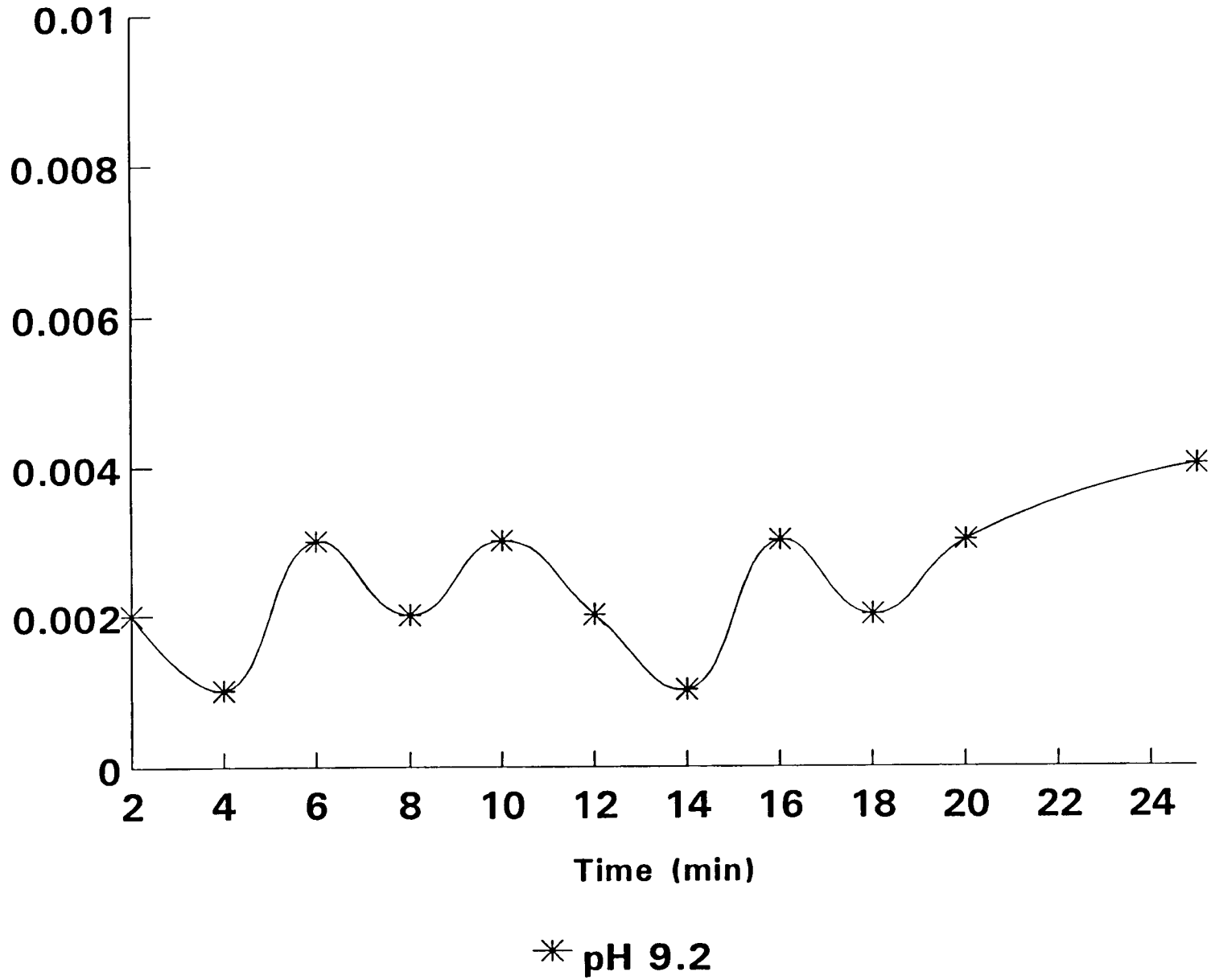


Fig. 5.3c Adrenochrome formation by rifampicin

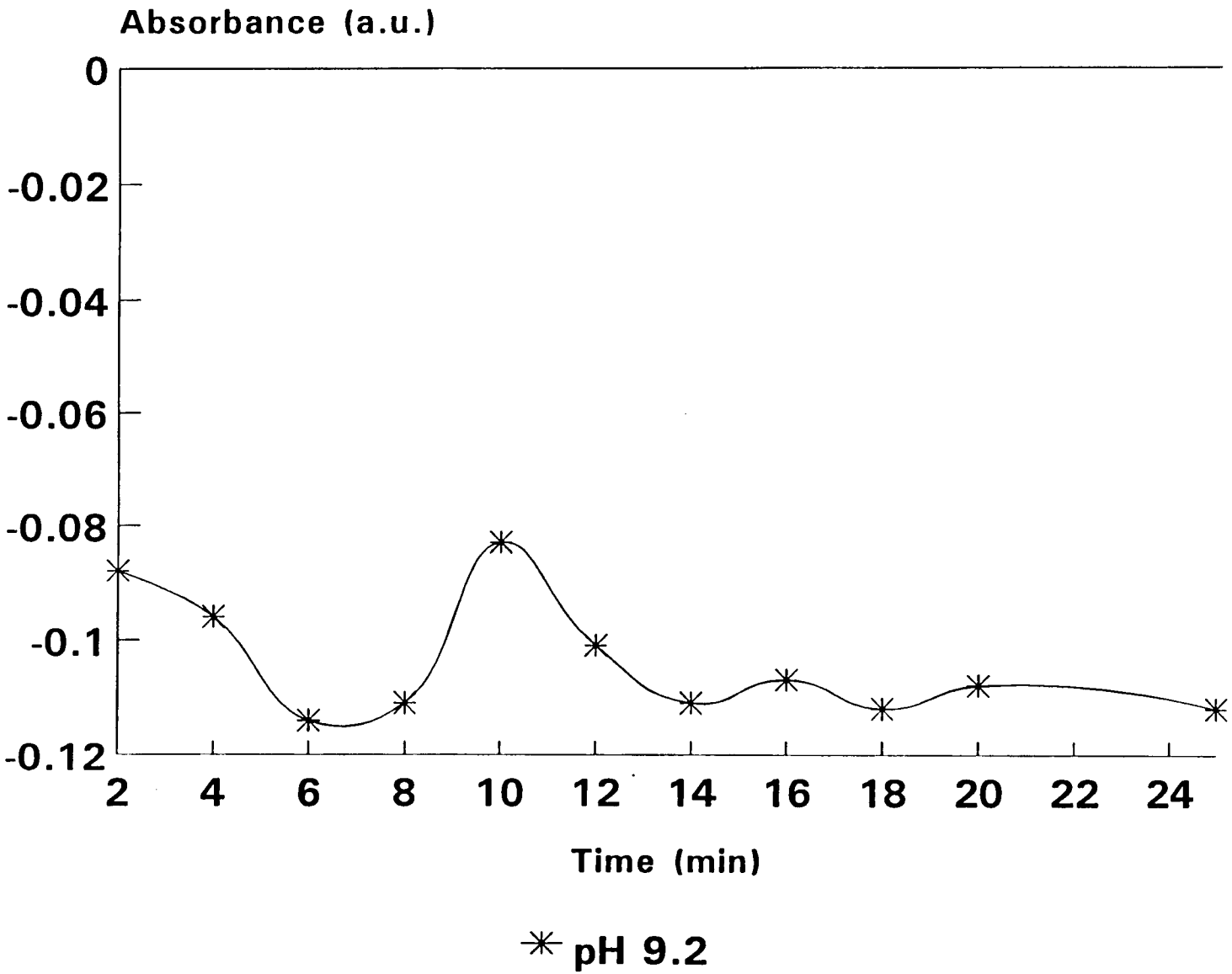


Table 5.1 adrenochrome formation (absorption units; mean ± SD) at 14 min. (pH 9.2) with and without SOD and CAT.

Added	Nil	SOD	CAT	CAT + SOD
Isoniazid	0.146 ±0.0015	0.038 ±0.002	0.158 ±0.003	0.095 ±0.004
Acetylisoniazid	0.065 ±0.003	0.005 ±0.003	0.158 ±0.005	-0.011 ±0.002
Hydrazine	0.049 ±0.003	0.006 ±0.001	0.023 ±0.002	0.037 ±0.003
Monoacetylhydrazine	0.008 ±0.001	0.003 ±0.001	0.002 ±0.001	0.037 ±0.002
Diacetylhydrazine	-0.008 0.002	—	—	—
Pyrazinamide	0.001 ±0.0002	—	—	—
Rifampicin	-0.001 ±0.003	—	—	—

SOD = Superoxide dismutase; CAT = Catalase

5.4 DISCUSSION

Inhibition of adrenochrome formation by SOD and catalase implies that superoxide anions and hydroperoxide radicals were produced. Isoniazid was the most reactive, then acetylisoniazid, hydrazine and monoacetylhydrazine, in that order. However, there was variation in the extent of free radicals produced as demonstrated by sensitivity of each reaction to either SOD or catalase. Isoniazid reaction involved superoxide anion while hydrazine, acetylisoniazid and monoacetylhydrazine reactions involved both superoxide anion and hydrogen peroxide. However, partial inhibition of adrenochrome formation by SOD and catalase meant that other free radicals were produced. Similary, the increase or persistance of the reaction when both enzymes were added to a reaction-mixer of isoniazid, monoacetylhydrazine and hydrazine denotes the free radical cascade proceeded in alternative pathways to generate radicals other than superoxide or hydrogen peroxide. These could be organic intermediates viz; akyldiimides, carbanion, acyl and peroxyl radicals or hydroxyl radicals (Rapport et al, 1972).

For acetylisoniazid, increase in the reaction with catalase suggests that either hydrogen peroxide reduces an intermediate or catalase interacts with an intermediate. Catalase had no effect on the isoniazid reaction. Most probably, hydrogen peroxide is not essential in this reaction.

The mechanism of oxidation of hydrazine in presence of HbO was described by Golberg and Stern (1976). It is either enzyme or base catalysed, and is a two step, one electron transfer from hydrazine (the

electron donor) to produce superoxide and hydrogen peroxide radicals, viz:

$NH_2\text{-}NH_2 + Hb_2 \text{-------} > Hb^+ + H^+ + H_2N\text{-}HN$

$NH_2\text{-}NH + O_2 \text{---} > {}^{\cdot}O_2 + H^+ + (NH{=}NH)$

${}^{\cdot}O_2 + H^+ + NH_2\text{-}NH_2 \text{---} > H_2O_2 + NH_2\text{-}NH$

$2O_2 + SOD + 2H^+ \text{---} > H_2O_2 + O_2$

The present experiment shows that at pH 9.2, isoniazid, acetylisoniazid, hydrazine and monoacetylhydrazine, in that order, reacted with HbO to produce reactive oxygen species. This reaction series relates to presence of a pyridine ring and keto groups. The electron rich pyridine ring of isoniazid meets the electron withdrawing effect of the keto group such that the nucleophilic properties of hydrazine group are unaffected. For acetylisoniazid, the electron withdrawing effect by a second keto group reduces the electron donating tendency of hydrazine. Hence, hydrazine was less reactive than isoniazid. This is more difficult for monoacetylhydrazine where the effect of one keto group is unopposed. Therefore for monoacetylhydrazine the hydrazine group cannot donate electrons easily and almost no reactive oxygen species were produced.

These results imply that isoniazid, acetylisoniazid, hydrazine and monoacetylhydrazine can interact with HbO to generate reactive oxygen species and other radicals. Whether this can occur *in vivo*, it is yet to be proven.

CHAPTER SIX

THE ROLE OF OXYGEN FREE RADICALS IN ISONIAZID INDUCED HEPATOTOXICITY

6.0 SUMMARY

Involvement of reactive oxygen species in isoniazid induced hepatotoxicity was investigated by incubating rat liver slices with isoniazid and each of its hydrazide metabolites; hydrazine, monoacetylhydrazine and acetylisoniazid, separately. Reactive oxygen species were detected by measuring lipid peroxides using the thiobarbituric acid-reacting-substances (TBARS) test while hepatotoxicity was assessed histologically. Hydrazine induced lipid peroxidation, but this was difficult to prove for isoniazid, acetylisoniazid and monoacetylhydrazine, because the three compounds interfered with the TBARS test. Hence, the TBARS test was foung unsuitable for detecting lipid peroxidation induced by hydrazide compounds. Because hydrazine is a metabolite of isoniazid, it was concluded that oxygen free radicals are involved in isoniazid induced hepatotoxicity.

6.1 INTRODUCTION

Hepatotoxicity is the most feared side effect of isoniazid, and is often used as parameter for estimating incidence and severity of isoniazid related toxicity. Isoniazid hepatitis occured in 16 to 20% of patients on isoniazid of whom 1-2% it was severe (Gangadharam, 1986; Girling, 1984). Isoniazid hepatitis is more frequent in elderly people, where fatal hepatic necrosis has been reported, and is rare below 20

years of age (Riska et al, 1976; Walubo et al, 1991c; Woo et al, 1987 and 1992).

Efforts to prevent isoniazid induced toxicity have been hampered by lack of knowledge of the mechanisms by which it occurs. There is no sine qua non symptom or sign of isoniazid toxicity; the biochemical, pathological and morphological features of isoniazid hepatitis are indistinguishable from other types of hepatitis such as viral and alcoholic hepatitis (Black et al, 1975; Mitchel et al, 1976).

Of the activated metabolites incriminated in isoniazid induced hepatic necrosis, monoacetylhydrazine has been considered the most toxic metabolite (Mitchel et al, 1976). It was explained that monoacetylhydrazine is metabolically activated to an acyl radical which binds to macromolecules and thus cause hepatic injury. In the same perspective, isoniazid and acetylisoniazid were thought to induce toxicity after hydrolysis to monoacetylhydrazine. It was thereby suggested that fast acetylators are more likely to suffer isoniazid hepatotoxicity than slow acetylators because they produce more acetylisoniazid and monoacetylhydrazine.

Unfortunately, the occurrence of isoniazid induced toxicity does not correlate with acetylator phenotype and the pharmacokinetics of isoniazid in elderly patients is similar to that in young patients. Secondly, it was shown that fast acetylators can acetylate monoacetylhydrazine in a similar fashion to isoniazid such that exposure to monoacetylhydrazine is not different from slow acetylators (Gangadharam, 1986).

It was envisioned here that the cause of isoniazid induced hepatotoxicity is multi-factorial whereby many mechanisms are involved, including host factors. Therefore, depending on the host factors, there should be variability in the propagation of one mechanism by different individuals leading to variations in occurrence and severity of isoniazid hepatitis in different patients. The theory of metabolic activation emphasized formation of organic radicals (acyl radical) and underscored the importance of other radicals (e.g. oxygen radicals) that could be formed in the process.

Despite several reports showing that isoniazid action against mycobacteria is partly due to generation of free radicals (Shoeb et al, 1985a, 1985b and 1985c; Knox et al, 1956), and that hydrazine compounds can generate reactive oxygen species through the hydrazine group (Jain and Hochstein, 1979; Goldberg and Stern, 1974; Misra and Frodovich, 1976; Goldberg and Stern, 1976), there is no report relating isoniazid induced toxicity to this form of tissue injury. I envisaged that during isoniazid metabolism, isoniazid and its metabolites (hydrazine, monoacetylhydrazine, acetylisoniazid and diacetylhydrazine) form reactive oxygen species which may lead to toxicity. Having demonstrated that isoniazid, acetylisoniazid, monoacetylhydrazine, and hydrazine, could activate oxygen in presence of oxyhemoglobin (see chapter five), we monitored formation of reactive oxygen species by each of these compouds in rat liver-slices.

It was hoped that the results of this study would clarify the role of reactive oxygen species in isoniazid related hepatotoxicity and help to combat isoniazid related toxicity because the only way of controlling

free radical reactions is by administering the right antioxidant at the right time and place.

6.2 METHODS

6.2.1 Materials

Sodium dodecylsulphate, Acetic acid, thiobarbituric acid, Butanol, Pyridine, TMP (Melanondaldehyde), L-dipalmitoyl-phosphodylcholine (DPPC;16:0), cholesterol, stearylamine, superoxide, SOD source 3000 i.u./mg protein, catalase

6.2.2. Procedures

The study was approved by the University Ethical and Animals Research Committee.

6.2.3. Preparation of liver slices

Sprauge Dawly male rats weighing 150 - 200 g were used. The animals were fasted overnight and, under ether anaesthesia, abdominal wall was opened and the liver was perfused in situ with cold Kreb's-Henseleit buffer (4°C, pH 7.4) to remove as much blood as possible. The liver was excised and cut into smaller blocks from which slices were prepared manually according to procedures described by Mcilwain, (1961). Briefly, a liver block was put on a filter paper on a cutting surface (top of a flat bottom bottle) and, using a microscope slide as the guide, a surgical skin graft blade was gently wedged into the liver block but against the glass slide to cut a thin slice.

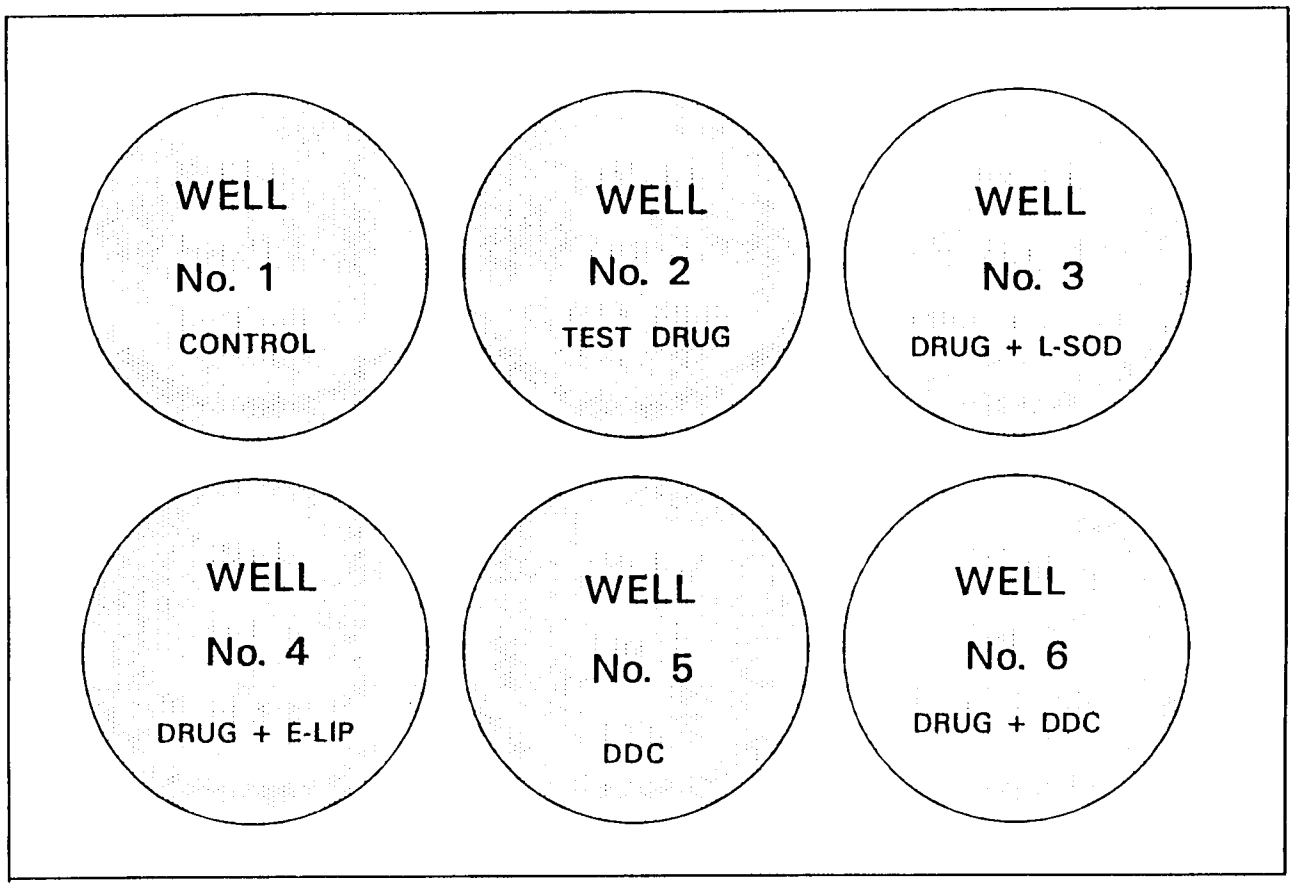
After cutting, the slice was immediately put in ice-cold Kreb's Henseleit buffer saturated with O_2-CO_2 mixture (95%/5%). As the slices were non-uniform, they were weighed and trimmed to 150 - 200 mg wet slice.

6.2.4. Culture of liver slices;

Five Six-well plates were used (Well size 3 cm diameter and 0.5 cm height) (Fig 6.1a and b). In each of the six wells (numbered 1 to 6), a liver slice was placed in 3 ml of ice-cold Eagle's MEM saturated with O_2-CO_2 mixture (95%/5%). Nothing was added to well no. 1 which was the control; well no. 2 contained the test compound; well no. 3 had liposomal SOD and test compound; no 4 had empty liposomes and test compound; no. 5, diethyldithiocarbamate (DDC) only; and no. 6, DDC with test drug. The plates were incubated in a water jacket incubator set at 37°C and gassed with O_2-CO_2 mixture (95%/5%). One plate was removed at intervals of 2, 4, 6, 8 and 10 hours of incubation for analysis of TBARS. Timing was started when the test compound was added.

The effect of test compound on formation of TBARS was monitored in well no. 1 while the role of SOD on production TBARS was monitored in wells 3 to 6. In wells 3 and 4, the effect of exogenous SOD on production of TBARS was assessed while in wells 5 and 6 the effect of inhibiting endogenous SOD with DDC was examined. Because SOD does not cross cell membranes, it was encapsulated into liposomes as described later. Hepatotoxicity was evaluated histologically.

Fig 6.1 An illustration of a six-well plate in which the liver slices were contained during incubation in 3ml of Eagle's MEM.



6.2.5. Histopathology preparation;

Slices were placed in 10% formalin and were processed and analysed blindly by an independent pathologist. Reported here are the ten hour incubated specimens lebelled A, B, C, D, E to F, and the non-incubated slice G. A was the Control. The others were incubated separately in 5 mmol/l of the respective compounds viz: A with isoniazid; C with acetylisoniazid; D monoacetylhydrazine and E with hydrazine. F was incubated with all four compounds at 2.5 mmol/l of each.

6.2.6. TBARS assay:

This was according to the method described by Ohkawa et al (1979). Briefly, a liver slice was washed three times in ice cold NaCl and there-after was homogenised with a Teflon coated homogeniser in 0.2 ml of 1.15% KCL buffer. A further 1.3 ml 1.15% KCL were added to make a 10% (w/v) tissue homogenate. To 0.2 ml of the 10% (w/v) homogenate, 0.2 ml 8.1% SDS, 1.5 ml 20% AA and 1.5 ml 0.8 thiobarbituric acids were added. Distilled water (0.6 ml) was added to make up 4 ml and this was heated in a water bath at 95°C for 60 min. The mixture was cooled with tap water. Distilled water (1 ml) and 5 ml of butanol:pyridine solution (BP;15:1 v/v) were added and the mixture shaken vigorously on a vortex mixture. It was then centrifuged at 400 g rpm for 10 min and absorbance of the organic layer was read at 540 nm immediately using BP as the blank.

TMP was used as external standard expressed as nmol of MDA. Calibration curve was made over a concentration range; 1.14, 5.63, 11.14, 22.7, 34.1, 45.4, and 68.1 nmol of MDA. The calibration

equation was y = 0.0102 + 0.0151x (regression coefficient r = 0.997). The total quantity of lipid peroxides in each liver slice was calculated and expressed as nmol/mg weight of the slice.

6.2.7. Characterisation of reference compounds

It was found necessary to clarify whether there was interaction between TMP and the test compounds, because such interaction may influence the results of small tissue samples used in this experiment. Liver slices have a limited reservoir of lipids such that encroachment on these lipids, of whatever magnitude, is likely to affect extent of lipid peroxidation leading to difficulties in interpretation of results.

An aqueous mixture of TMP 1.5 ml (8.87 mmol) and 2.22 mmol of each of the four compounds, separately (i.e. 300 mg of isoniazid, 110 mg hydrazine hydrate, 250 mg monoacetylhydrazine-hydrogen chloride and 400 mg of acetylisoniazid), was treated as described in the procedure except for SDS and trichloroacetic acid. The butanol extract was analysed by TLC, UV absorption and mass spectrum

6.2.8. Liposomal preparation:

Positive liposomes were prepared according to procedures described by Turrens et al (1982). A 15 ml mixture of L-dipalmitoyl-phosphotidylcholine (DPPC;16:0), cholesterol and stearylamine in chloroform in a molar ratio of 14:7:4 was made ((solutionX; this is equivalent to approximately 6.8 umol DPPC/ml or 5 mg DPPC/ml; i.e. 75 mg of DPPC, 19.74 mg of cholesterol and 7.8 mg of Stearylamine).

Solution X was mixed with 4.5 ml of SOD solution in 0.15 M NaCl, 10 mM KPO4 pH 7.4 (5 mg/ml; solution A) and was sonicated to form a homogenous emulsion. Chloroform was removed under vacuum by rotary evaporation at 46°C and residue was centrifuged at 105,000 g for 45 min. The supernatant (supernatant B) was used for validation of liposomal SOD encapsulation while the liposomes were resuspend in 2.5 ml of 0.15 M NaCl, 10 mM KPO4 pH 7.4 to yield a liposomal concentration of approximately 20 umol/ml. (liposomal concentrations are expressed per umol phospholipid (DPPC)).

Empty liposomes were prepared by reverse evaporation in presence 0.15 NaCl, 10 mM KPO4 pH 7.4. After centrifugation, the liposomes were resuspended in the same potassium buffer as for the enzyme containing liposomes.

Percentage of enzyme associated with liposomes was assessed by comparing the percentage inhibition of 6-OHDA oxidation by solution A with that of supertant B. Enzyme associated with liposomes includes both the encapsulated and the liposomal membrane bound SOD. Determination of encapsulated SOD was not done because it is more laborious and would exclude the SOD held within the liposomal membranes. Liposomes were prepared a day before the experiment and were kept at 4°C.

6.2.9. Estimating SOD associated with liposomes;

Appropriately diluted solution of SOD in buffer was used for calibration of SOD over concentration range; 10, 20, 40, 50, 80, and 100 ng/ml.

The samples were treated as described in the procedure below (Heikkila and Cabat, 1976). Percentage inhibition of 6-OHDA oxidation was plotted against SOD concentrations. Linear regression equation was y = 10.075 + 0.562x with a regression coefficient (*r*) of 0.993 (n = 4).

For estimating SOD associated with liposomes, a portion of the SOD solution A, 5 mg/ml was diluted to 50 ng/ml of SOD (linear range) and, together with supernatant B, were shaken for 5-15 min at 37°C to equilibrate with air before SOD assay.

SOD assay:

Using a solution of 10^{-2}M 6-hydroxdopamine (6-OHDA) in distilled water sparged with N_2, 50 ul of this solution were added to 450 μl of appropriately diluted tissue supernatant or enzyme solution to make a final concentration of 6-OHDA = 10^{-4}M. Absorption was read at 490nm at 20s and percentage inhibition of 6-OHDA oxidation by SOD was calculated using the buffer blank as the control (100%). The amount of SOD associated with liposomes was 16.8 ± 5.07% (n = 5)

6.2.10. Statistical analysis

Using the Instat statistical computer package, One Way Analysis of Variance (ANOVA) and the student-Newman-Keuls test were used to compare results ($P < 0.05$).

6.3. RESULTS

6.3.1. Characterisation of compounds

Figures 6.2 and 6.3, respectively, illustrate TLC R_F (retention factor) values and UV absorption spectra of the reference compouds. TLC R_F values are: isoniazid (INH) = 0.62; isoniazid + TMP (INH-TMP) = 0.81; acetylisoniazid (acINH) = 0.68; monoacetylhydrazine (mHYD) = 0.66; monoacetylhydrazine + TMP (mHYD-TMP) = 0.78; hydrazine (HYD) = 0.09; HYD-TMP = 0.76; TBA = 0.78; and TMP = 0.71.

Each of the four hydrazides reacted with TMP to produce derivatives that unlike the TBA derivative, did not absorb in 540 nm region (Fig. 6.3). The reaction was faster with the aromatic hydrazides of isoniazid and acetylisoniazid than with monoacetylhydrazine and hydrazine. When the three compounds were reacted with TMP in one test-tube, absorption at 540 nm was abolished or just diminished (not shown).

The mass-spectra for INH-TMP, acINH-TMP, mHYD-TMP and TBA-TMP are shown in figure 6.4a-d. As in the case of the TLC above, the final product when these compounds react with TMP is similar by weight.

6.3.2. Liver injury.

Table 6.1 shows lipid peroxidation in liver slices after incubation with varying concentrations of each compound. By 6 hours at 5.48 mmol/l of the drug, lipid peroxides were highest with isoniazid, then acetylisoniazid, monoacetyl-hydrazine and hydrazine in that order. Therefore, 5.48 mmol/l was used in subsequent experiments.

Figure 6.2 Thin Layer Chromatogram of the reference and parent compounds with retention factors in-printed.

Isoniazid (INH) and its derivative (INH-TMP), monoacetylhydrazine (mHYD) with its derivative (mHDZ-TMP), hydrazine (HYD) and derivative (HYD-TMP), Acetylisoniazid (acINH) and its derivative (acINH-TMP), 1,1,3,3-tetraethoxypropane (TMP), and thiobarbituric acid (TBA).

Fig 6.2

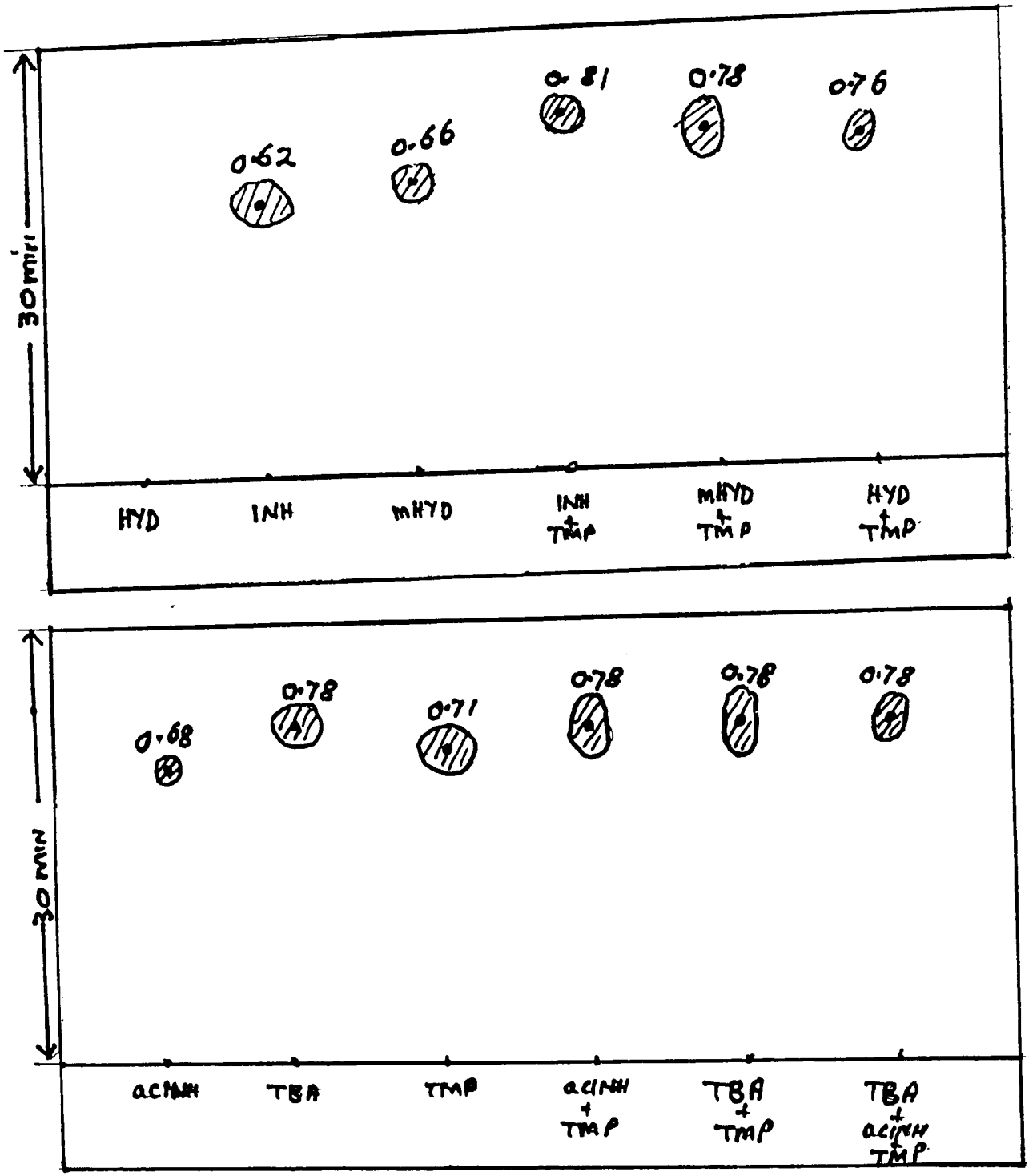

30 min
0.62
0.66
0.81
0.78
0.76
HYD
INH
mHYD
INH + TMP
mHYD + TMP
HYD + TMP
30 MIN
0.68
0.78
0.71
0.78
0.78
0.78
ACINH
TBA
TMP
ACINH + TMP
TBA + TMP
TBA + ACINH + TMP

Figure 6.3 Ultra-Violet absorption spectra of TMP derivatives with;

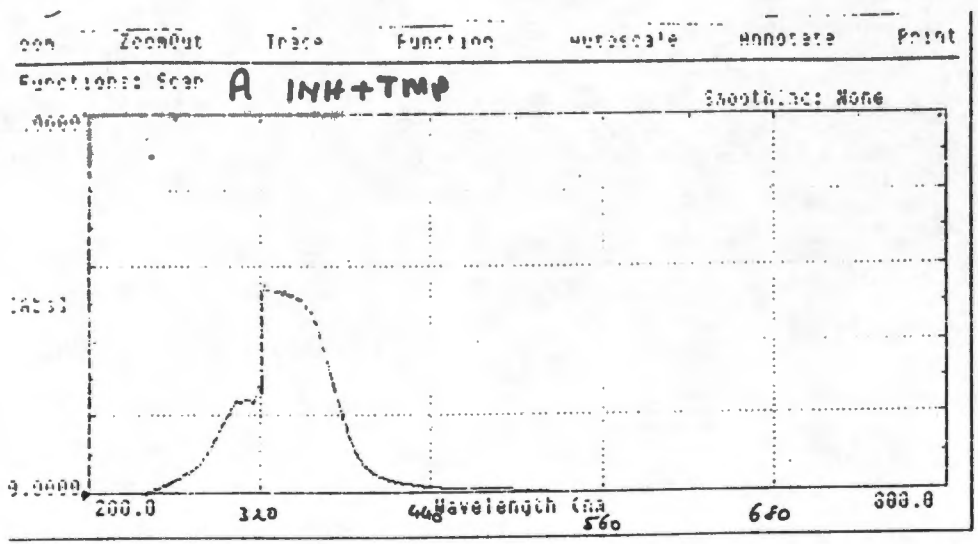
A) Isoniazid (INH-TMP).

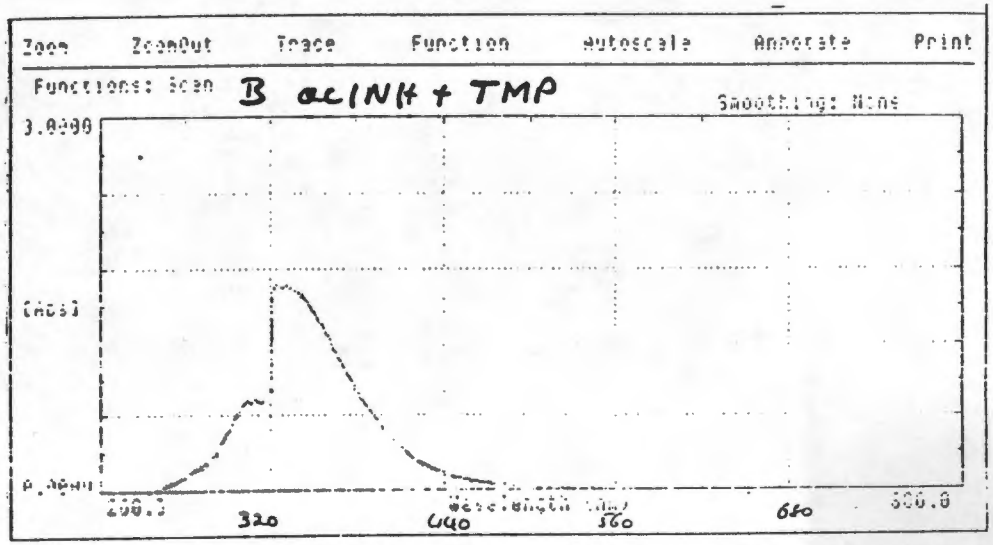
B) Acetylisoniazid (acINH-TMP).

C) Monoacetylhydrazine (acHYD-TMP).

D) thiobarbituric acid (TBA-TMP).

Fig 6.3


Zoom
ZoomOut
Trace
Function
Autoscale
Annotate
Print
Functions: Scan
A INH+TMP
Smoothing: None
[Abs]
200.0
320
440
560
680
800.0
Wavelength (nm)


Zoom
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Functions: Scan
B acINH + TMP
Smoothing: None
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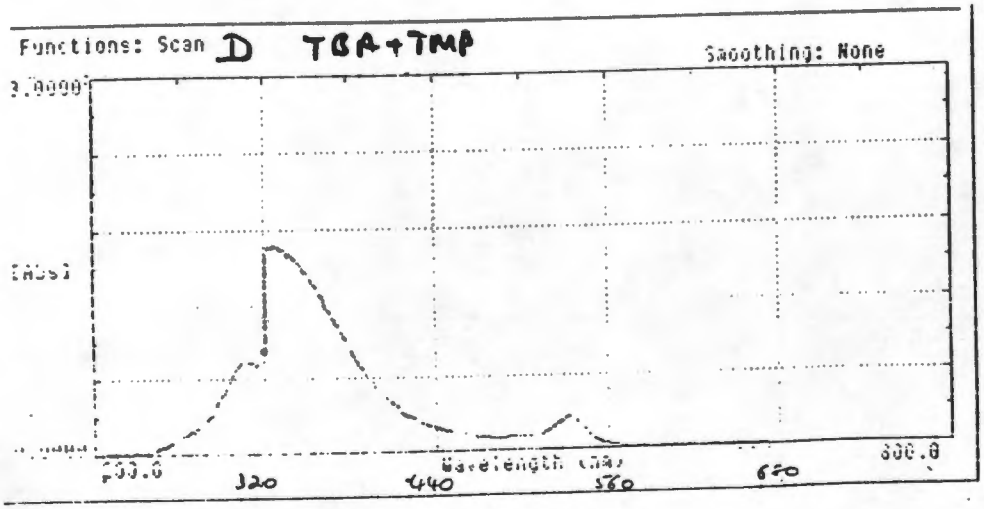

Functions: Scan
D TBA+TMP
Smoothing: None
[Abs]
320
440
560
680
800.0
Wavelength (nm)

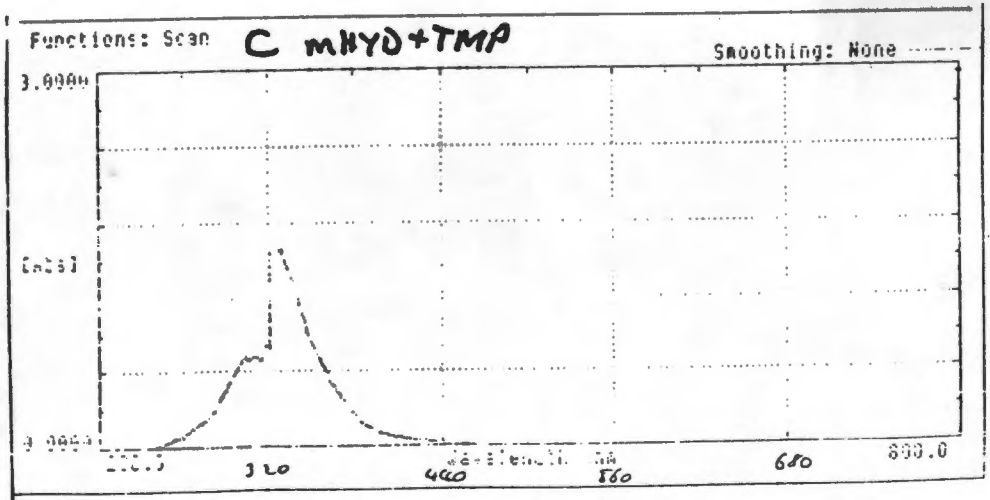

Functions: Scan
C mHYD+TMP
Smoothing: None
3.0000
[Abs]
320
440
560
680
800.0

Figure 6.4 Mass spectrometry of TMP derivatives with;

-Isoniazid -> INH-TMP (6.4a)

-Acetylisoniazid -> acINH-TMP (6.4b)

-Monoacetylhydrazine -> mHYD-TMP (6.4c)

-Thiobarbituric acid -> TBA-TMP (6.4d).

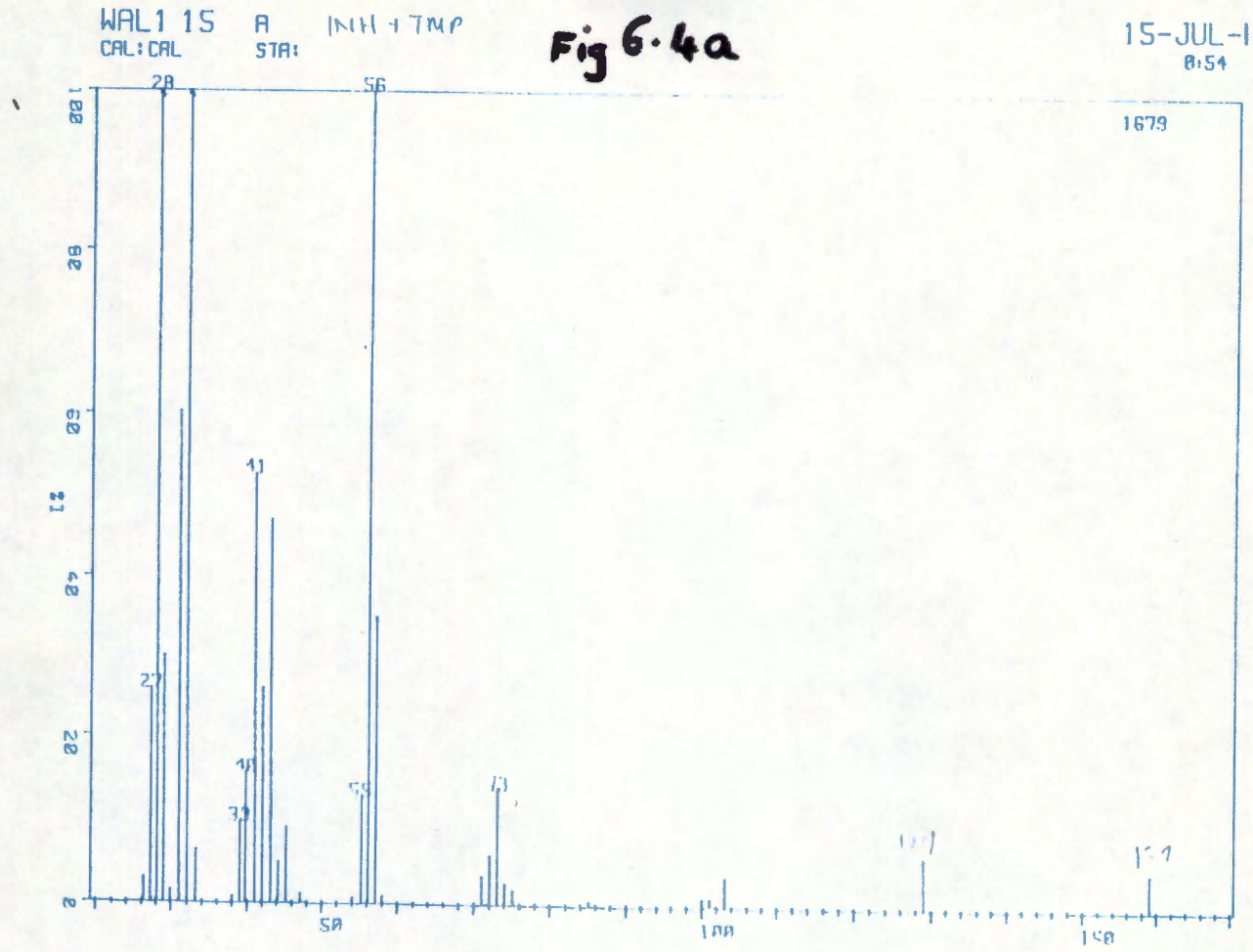


Fig 6.4a

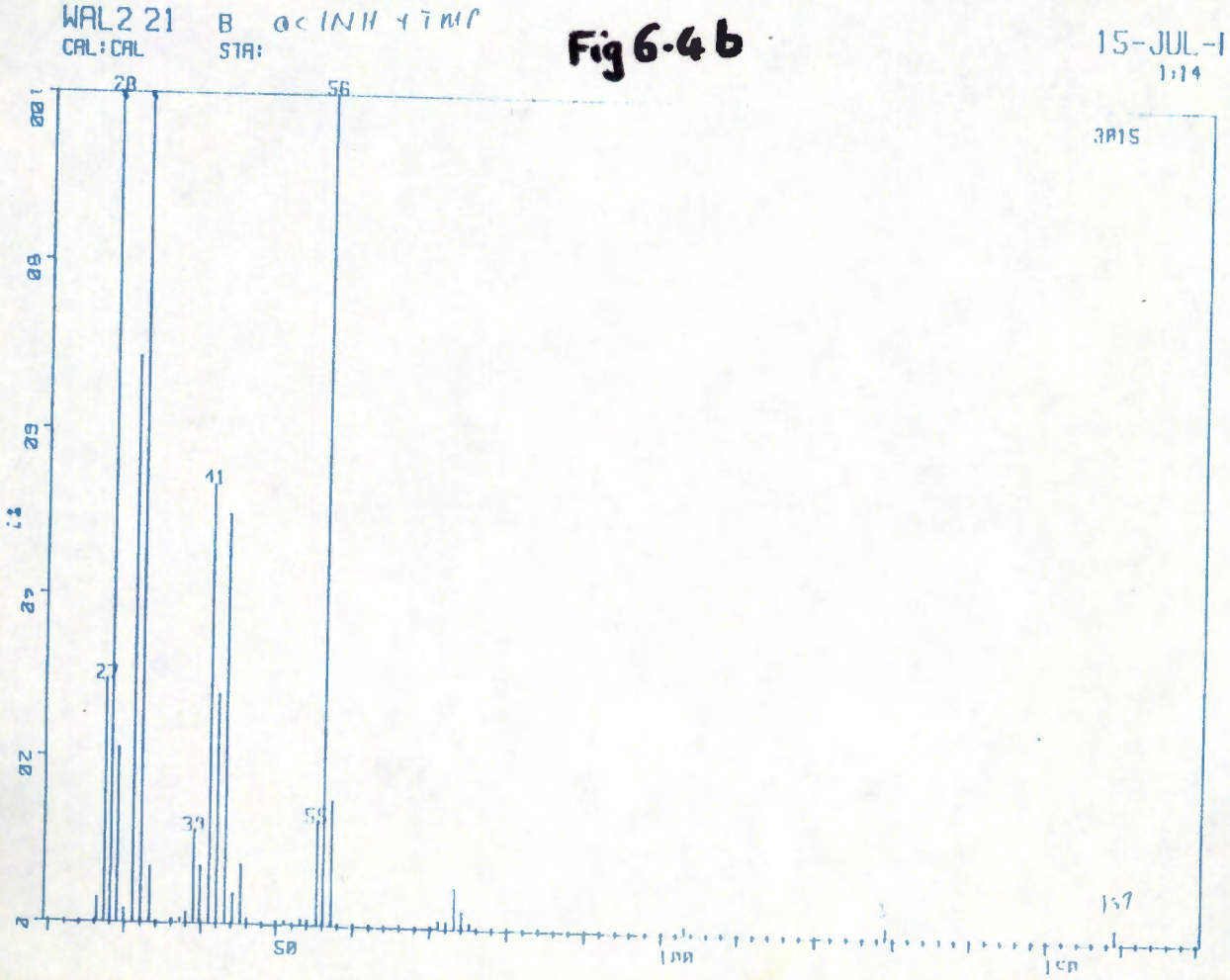


Fig 6.4b

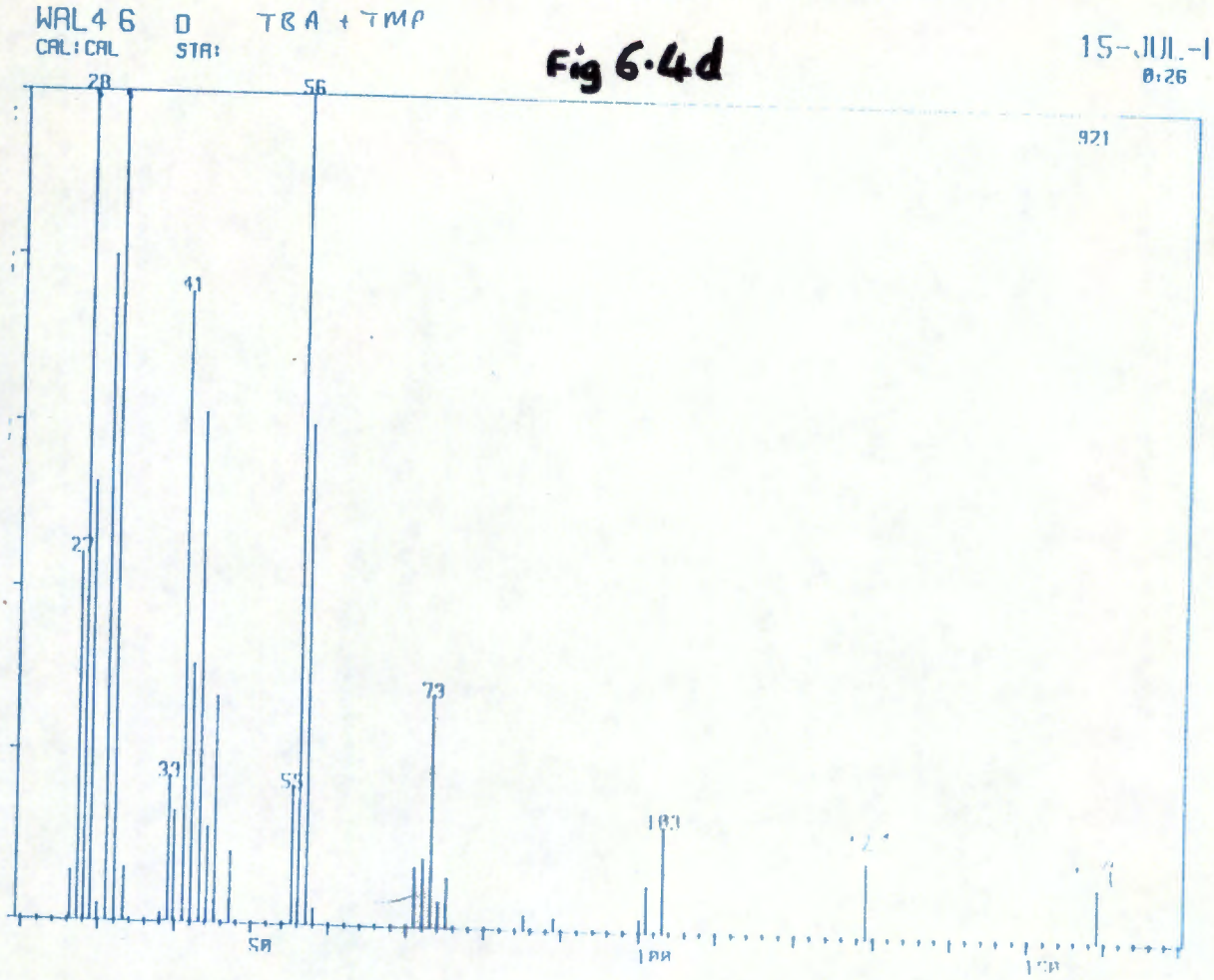


Fig 6.4d

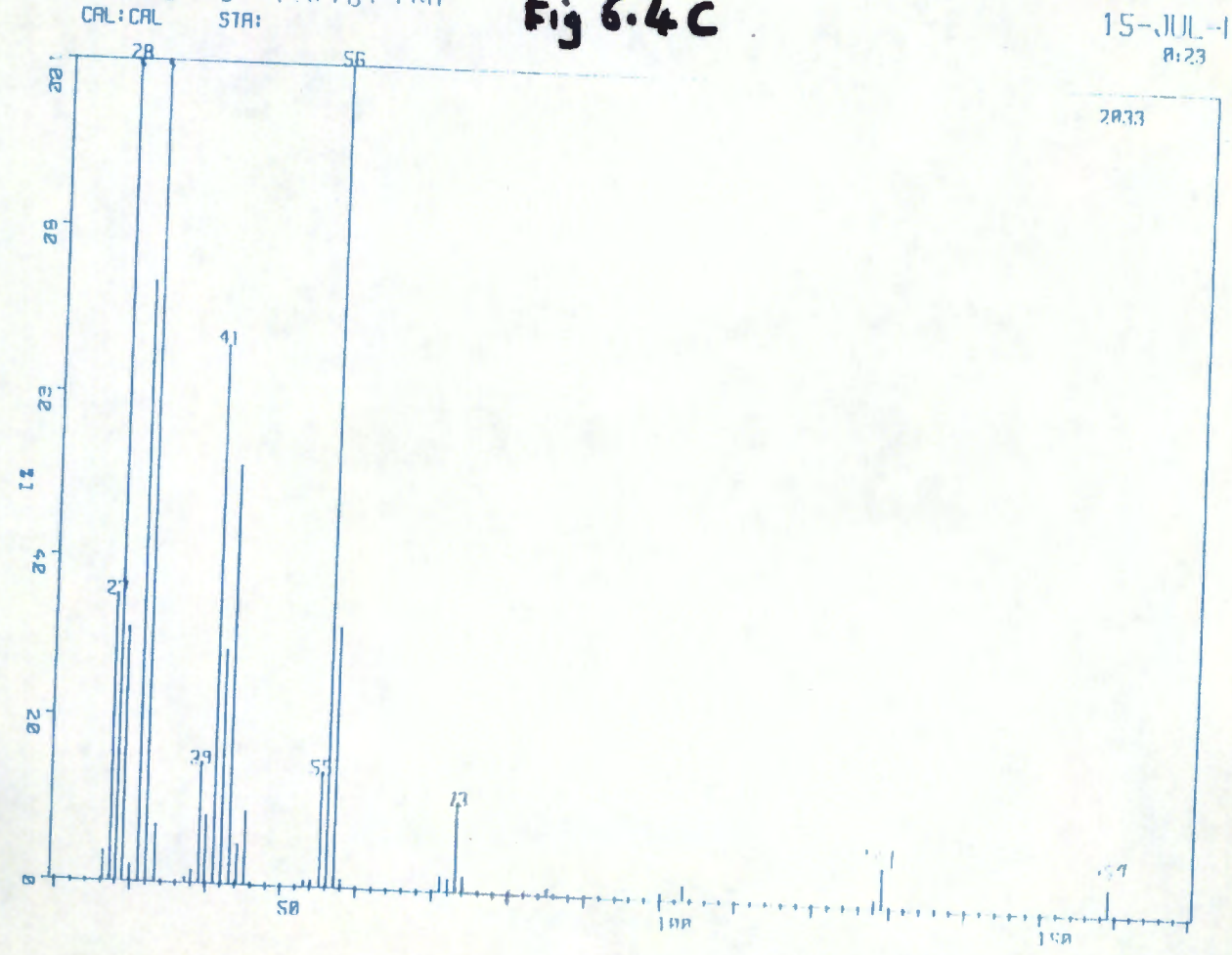


Fig 6.4C

Table 6.1 Lipid peroxides (mean ± s.d; nmol/mg) in liver slices after exposure to three different concentrations of each compoud.

Drug	INH		acINH		mHYD		HYD	
Time (hrs)	3	6	3	6	3	6	3	6
Drug Conc.								
0.37 mmol/l	0.27	0.30	0.11	0.24	0.23	0.35	0.47	0.72
1.1 mmol/l	0.27	0.95	0.19	1.35	0.25	0.67	0.84	1.07
5.48 mmol/l	0.27	1.72	0.22	1.43	0.42	1.38	0.86	1.86

INH = Isoniazid; acINH = Acetylisoniazid; mHYD = Monoacetylhydrazine; HYd = Hydrazine

Tables 6.2 to 6.5 (Appendices A1 to A4) illustrate hepatic injury measured by the amount of lipid peroxides during incubation of slices with isoniazid (Table 6.2); acetylisoniazid (table 6.3); monoacetylhydrazine (Table 4) and hydrazine (Table 6.5).

By the ANOVA, there was no difference between lipid peroxidation in the control wells and in those with isoniazid, acetylisoniazid and monoacetylhydrazine, with or without SOD, Liposomes and DDC. For hydrazine, there was significant variation in the means ($P = 0.0014$; see statistical calculations in appendix 2xi).

In general, there was a progressive increase in lipid peroxides with time of incubation in both the control and the test slices. Except for hydrazine, there was virtually no difference in the amount of lipid peroxides produced at 10 hours by the other componds compared to the control. At two hours, there was wide variation in the quantity of lipid peroxides formed by isoniazid compared to the control (Fig. 6.5a). Acetylisoniazid and monoacetylhydrazine did not vary from the control (Fig. 6.5b and c). There was significant lipid peroxidation by hydrazine at 10 hours ($P < 0.05$; Fig. 6.5d), but, as for the other three compouds, neither liposomal SOD nor DDC altered the formation of lipid peroxides.

Histopathology results: See the pathology report and the corresponding plates on page 128. The control slice exhibits a necrotic margin which not featured in the normal slice (plates G1, G3, A1 and A3). Isoniazid and acetylisoniazid exhibited a similar pattern of cell necrosis (plates B1 and C1). Monoacetylhydrazine caused extensive

vacuolation and disentegration of the nuclei, while, in the case of hydrazine, there was pyknosis of nuclei and loss of nuclei in necrotic cells (Plates D1, D3, E1 and E3). Vacuolation, pykinosis and massive cell necrosis were observed when the four compounds were combined at half strength (plates F1, F2 and F3).

Table 6.2 Lipid peroxides (mean ± s.d; nmol/mg) in liver slices after exposure to isoniazid.

Time (hours)	2	4	6	8	10
Control	0.92 ± 0.34	1.08 ± 0.26	1.07 ± 0.10	1.14 ± 0.12	1.12 ± 0.17
INH	0.60 ± 0.52	1.09 ± 0.17	1.12 ± 0.12	1.19 ± 0.27	1.28 ± 0.24
INH + SOD	1.08 ± 0.24	1.16 ± 0.14	1.13 ± 0.09	1.26 ± 0.23	1.26 ± 0.13
INH + E-LIP	0.98 ± 0.22	1.07 ± 0.15	1.04 ± 0.26	1.13 ± 0.02	1.15 0.07
DDC	0.96 ± 0.28	1.06 ± 0.15	1.02 ± 0.26	1.11 ± 0.02	1.21 ± 0.07
INH + DDC	1.03 0.07	1.04 0.25	1.28 0.48	1.12 0.03	1.18 0.06

Table 6.3. Lipid peroxides (mean ± s.d; nmol/mg) in liver slices after exposure to acetylisoniazid (acINH).

Time (hours)	2	4	6	8	10
Control	0.25 ± 0.47	0.24 ± 0.05	0.46 ± 0.26	0.55 ± 0.1	0.84 ± 0.42
acINH	0.21 ± 0.08	0.24 ± 0.08	0.60 ± 0.39	0.70 ± 0.25	0.88 ± 0.45
acINH + SOD	0.32 ± 0.12	0.23 ± 0.12	0.64 ± 0.52	0.47 ± 0.06	0.97 ± 0.62
acINH + E-LIP	0.29 ± 0.06	0.21 ± 0.06	0.71 ± 0.61	0.63 ± 0.19	1.02 ± 0.62
DDC	0.37 ± 0.06	0.36 ± 0.04	0.84 ± 0.51	0.88 ± 0.48	0.91 ± 0.42
acINH + DDC	0.31 ± 0.02	0.24 ± 0.11	0.71 ± 0.47	0.90 ± 0.53	0.94 ± 0.52

Table 6.4 Lipid peroxides (mean ± s.d; nmol/mg) in liver slices after exposure to monoacetylhydrazine (mHYD).

Time (hours)	2	4	6	8	10
Control	0.52 ± 0.23	1.00 ± 0.17	0.97 ± 0.13	1.14 ± 0.16	1.10 ± 0.12
Only mHYD	0.58 ± 0.42	1.17 ± 0.05	1.19 ± 0.22	1.09 ± 0.36	1. 43 ± 0.14
mHYD + SOD	1.05 ± 0.24	1.18 ± 0.18	1.16 ± 0.20	1.10 ± 0.30	1.25 ± 0.13
mHYD + E-LIP	0.89 ± 0.12	1.00 ± 0.07	1.19 ± 0.34	1.13 ± 0.22	1.39 0.16
DDC	1.10 ± 0.22	1.06 ± 0.07	1.16 ± 0.28	1.14 ± 0.27	1.35 0.19
mHYD + DDC	1.04 ± 0.08	1.12 ± 0.33	1.04 ± 0.28	1.18 ± 0.28	1.36 ± 0.25

Table 6.5 Lipid peroxides (mean ± s.d; nmol/mg) in liver slices after exposure to hydrazine (HYD).

Time (hours)	2	4	6	8	10
Control	0.86 ± 0.16	0.94 ± 0.12	0.97 ± 0.03	0.96 ± 0.12	1.13 ± 0.04
HYD	1.02 ± 0.01	0.97 ± 0.07	1.03 ± 0.15	1.15 ± 0.11	1.36 ± 0.02
HYD + SOD	0.96 ± 0.12	0.95 ± 0.08	0.98 ± 0.22	1.14 ± 0.05	1.30 ± 0.19
HYD + E-LIP	0.99 ± 0.09	0.88 ± 0.06	0.97 ± 0.25	0.89 ± 0.13	1.20 ± 0.14
DDC	0.87 ± 0.22	0.82 ± 0.21	1.05 ± 0.07	1.09 ± 0.13	1.39 ± 0.24
HYD + DDC	0.94 ± 0.24	0.93 ± 0.18	0.91 ± 0.12	0.96 ± 0.10	1.27 ± 0.15

Fig. 6.5a Lipid peroxidation by isoniazid.

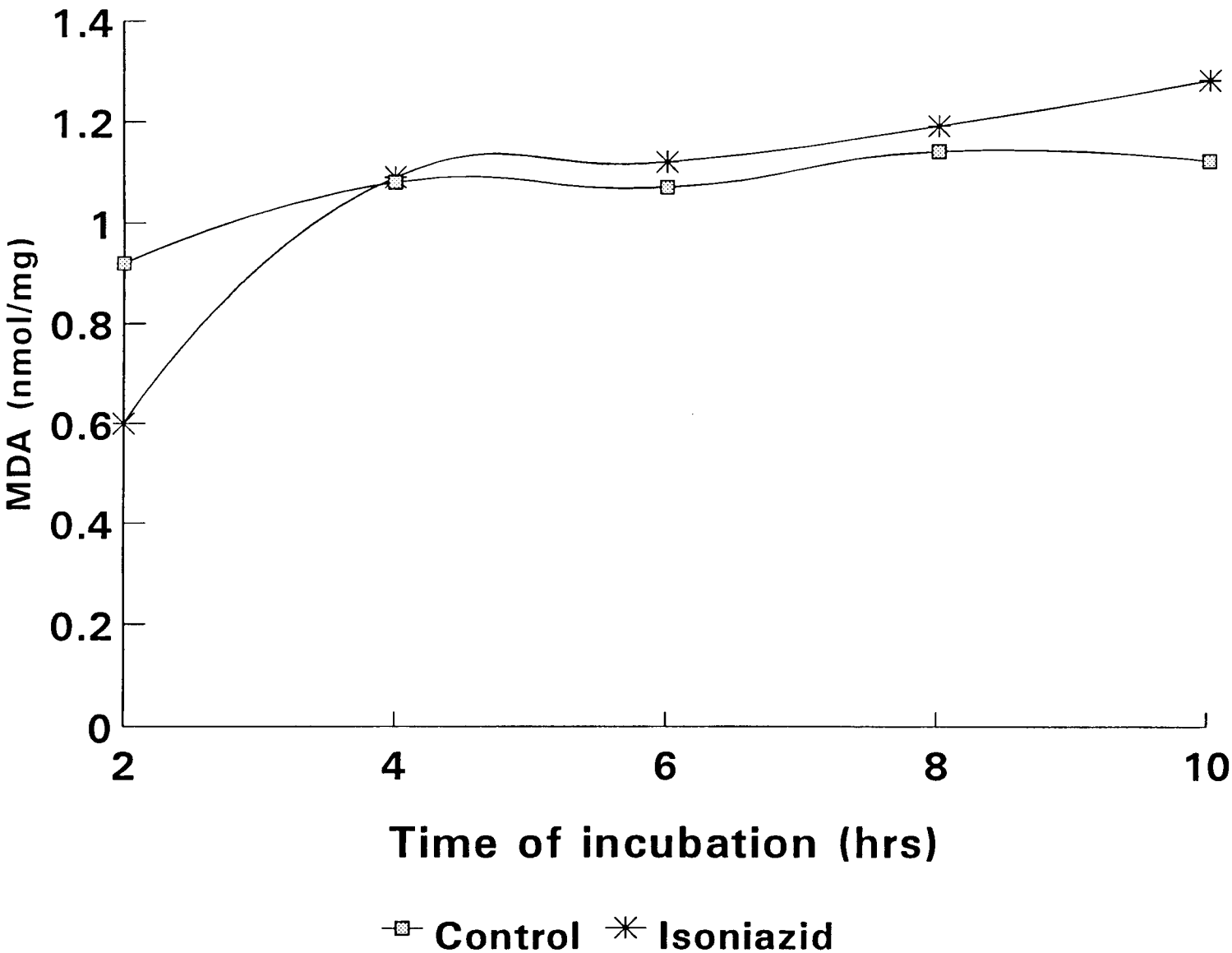


Fig. 6.5b Lipid peroxidation by acetylisoniazid

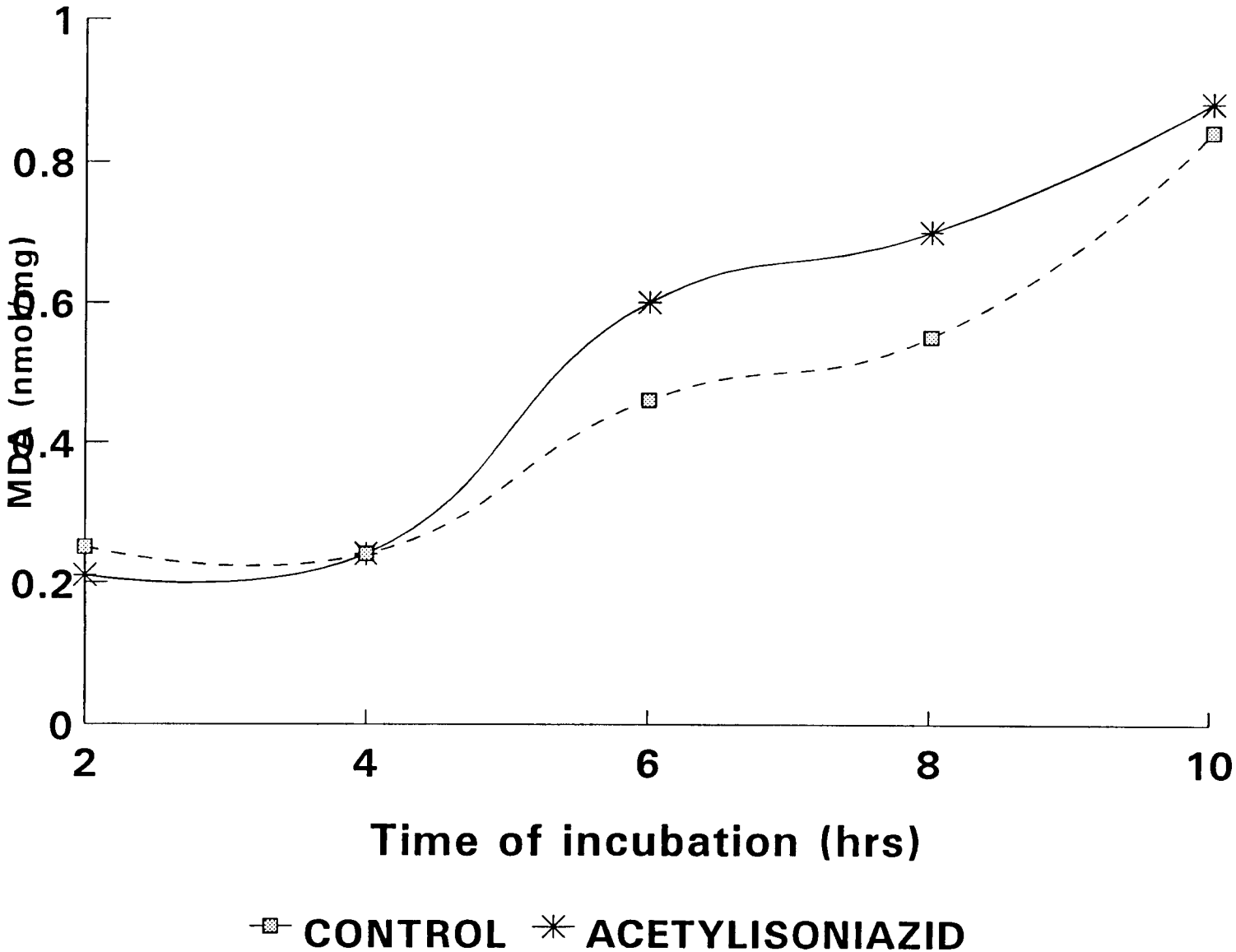


Fig. 6.5c Lipid peroxidation by monoacetylhydrazine

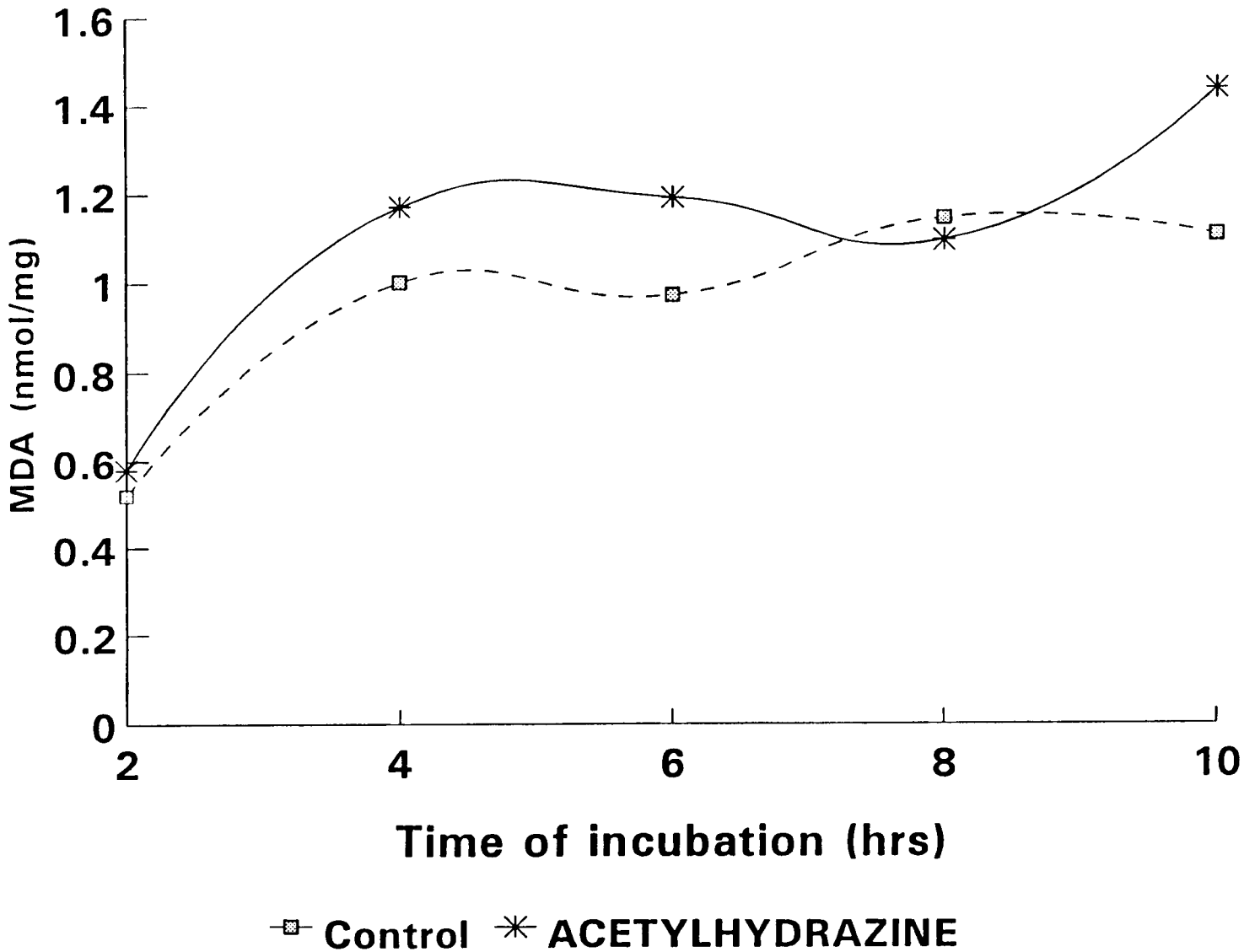
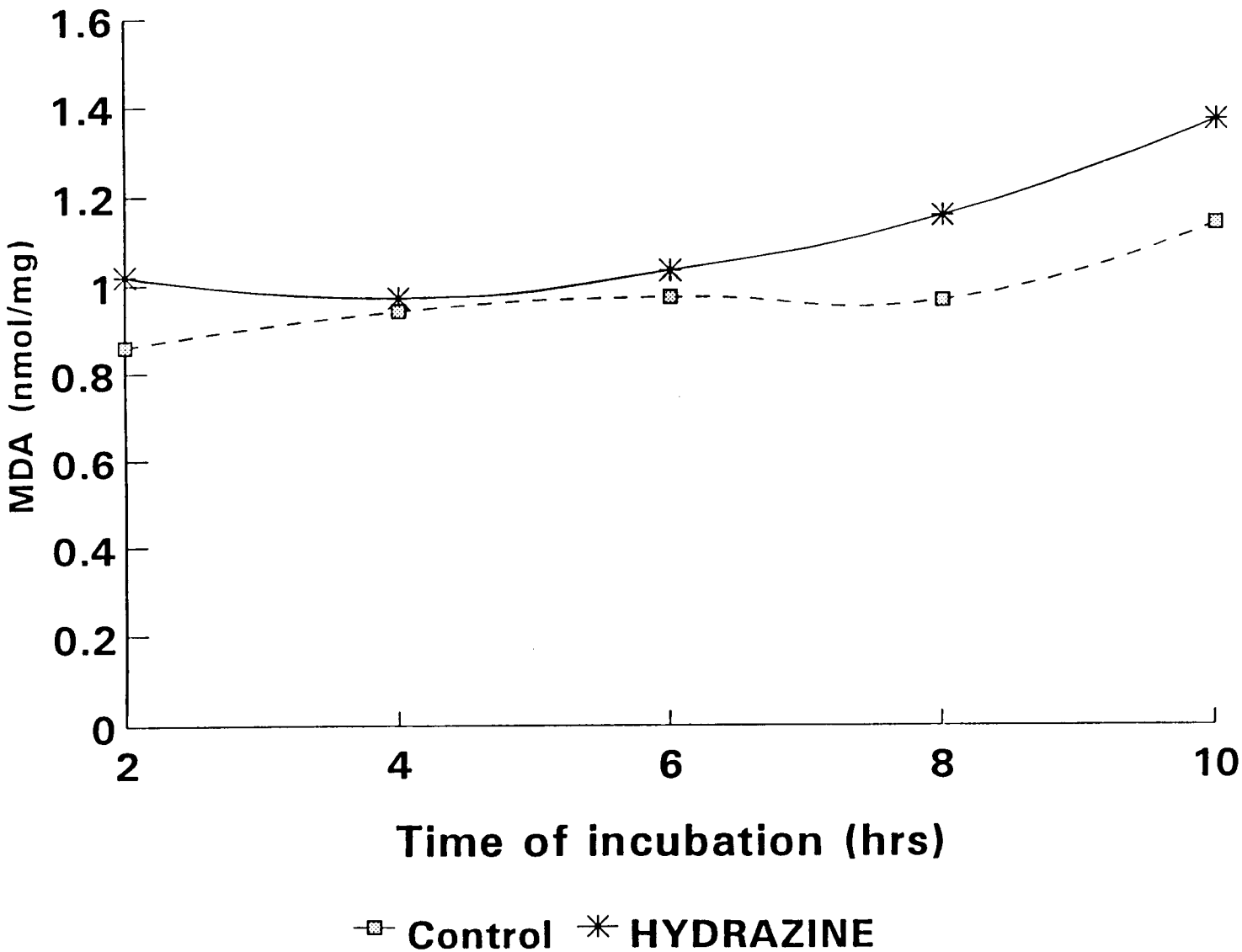


Fig. 6.5d Lipid peroxidation by hydrazine



PATHOLOGY REPORT ON THE LIVER SLICES

By: Dr. M. Arendse, Department of Anatomical Pathology, University of Cape Town.

Slide G:

There is no hepatic cell necrosis in this slide (plate G1, x100 and plate G3, x10). All hepatocytes and their nuclei are intact. Bile ducts, portal veins and the reticulin system (plate G2, x100) are normal. In general, all cells are normal.

Slide A:

There is a rim of hepatocellular necrosis on the outer perimeter of the sections (plate A1, x100 and plate A3, x10). Hepatocytes of the inner areas are normal. Bile ducts, portal veins and the reticulin system (plate A2, x100) are normal.

Slide B:

There is a rim of hepatocellular necrosis on the outer perimeter of the sections and patches of similar nature in the inner areas (plate B1, x100). Nuclei of some hepatocytes are pykinotic while in the necrotic areas the nuclei are distengrated and in a few cells vacuolated.
Bile ducts, portal veins and the reticulin system (plate B2, x100) are normal.

Slide C:

There is a rim of hepatocellolar necrosis on the outer perimeter of the sections and several patches of cell necrosis in the inner areas (plate

C1, x100). The nuclei of some hepatocytes are pykinotic while in the necrotic areas the nuclei are disentegrated. Necrosis in this section is slightly more than in B. Bile ducts, portal veins and the reticulin system (plate C2, x100) are normal

Slide D:

There is massive hepaticellular necrosis in the whole section with extensive nuclear vacuolation and distengration (plate D1, x100 and plate D3, x10). Cell necrosis in this slide is more than in A, B and C. Bile ducts, portal veins and the reticulin system (plate D2, x100) are normal

Slide E:

There is hepatocellular necrosis in most areas and a few patches of normal hepatocytes (plate E1, x100 and plate E3, x10). Unlike for slide D, most of the cell disentegration is in the cytoplasm. There is pyknosis of the nuclei and nuclear loss in some areas. Cell death here is less than for D, but more than for A, B. and C. Bile ducts, portal veins and the reticulin system (plate E2, x100) are normal

Slide F:

There massive hepatocellular necrosis with nuclear loss or distengration in all hepatocytes (plate F1, x100 and plates F2 and F3, x10). Virtually, no sign of a viable cell. Cell necrosis here is worse than in A to E. Bile ducts, portal veins and the reticulin system (plate F2, x100) are normal

PLATE A1

CONTROL (X100)

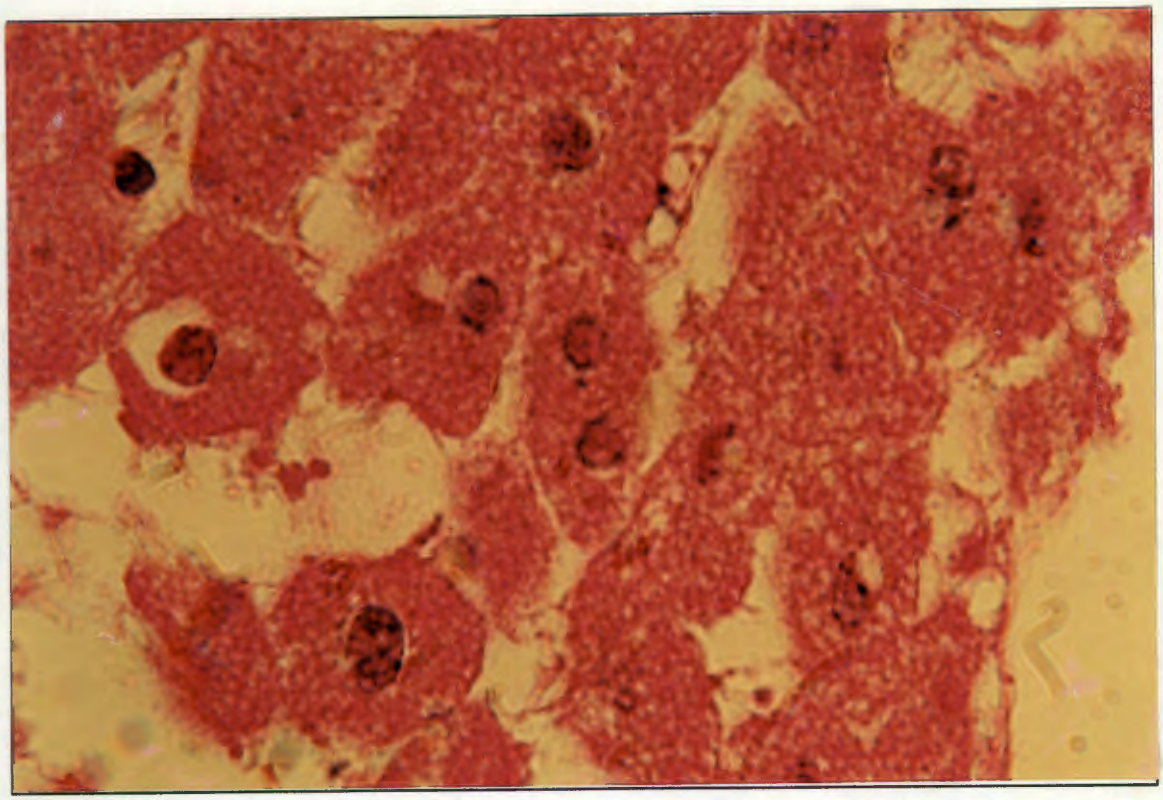


PLATE A2

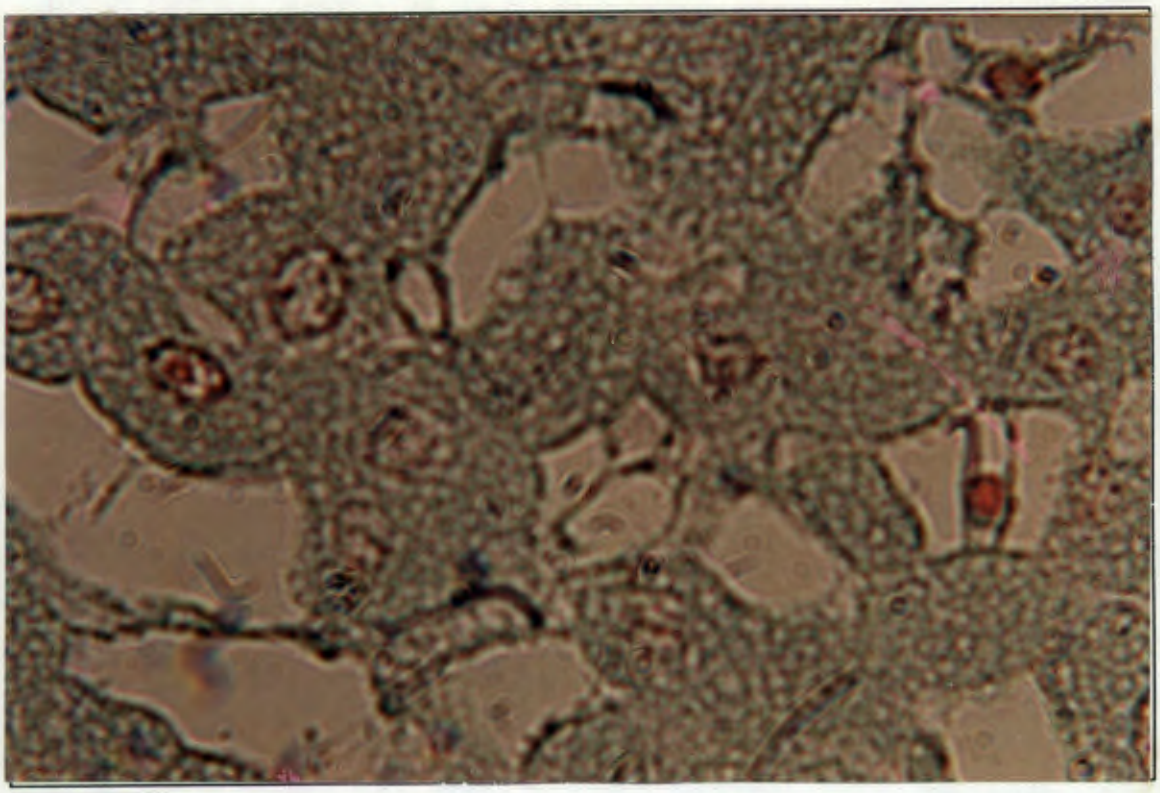


PLATE B1 ISONIAZID (X100)

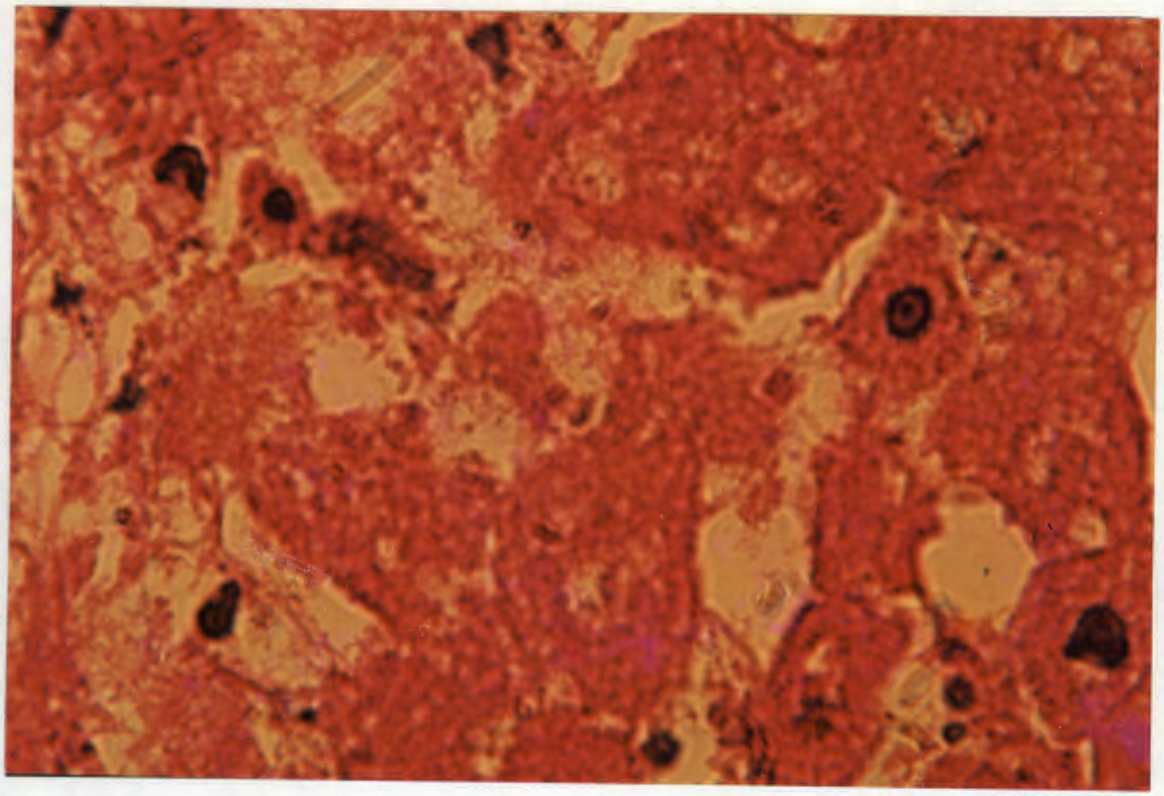


PLATE B2

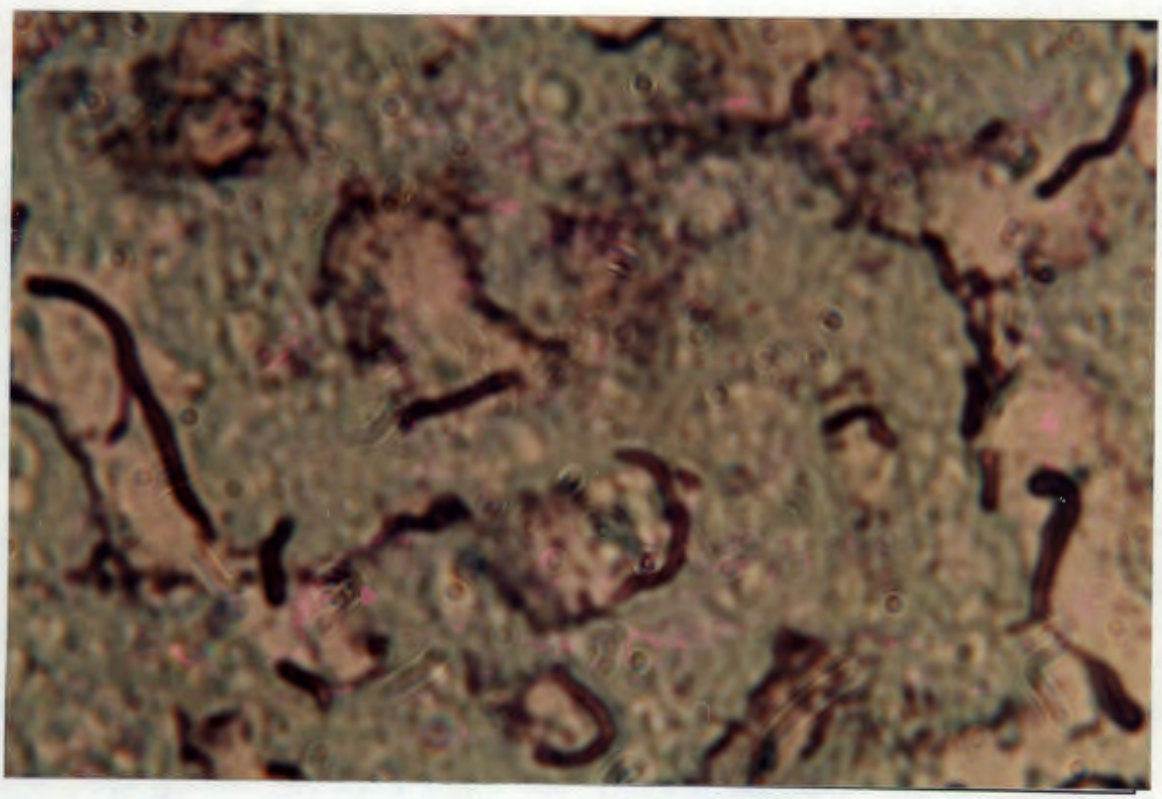


PLATE C1

ACETYLISONIAZID (X100)

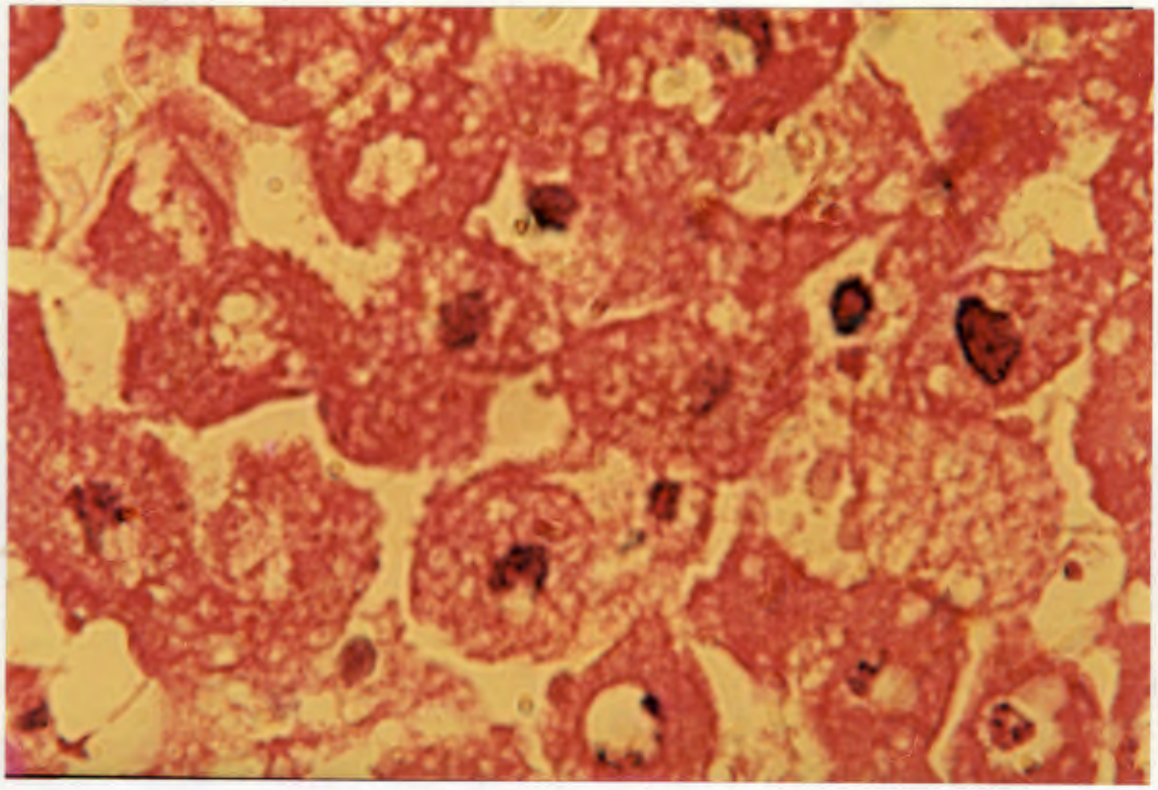


PLATE C2

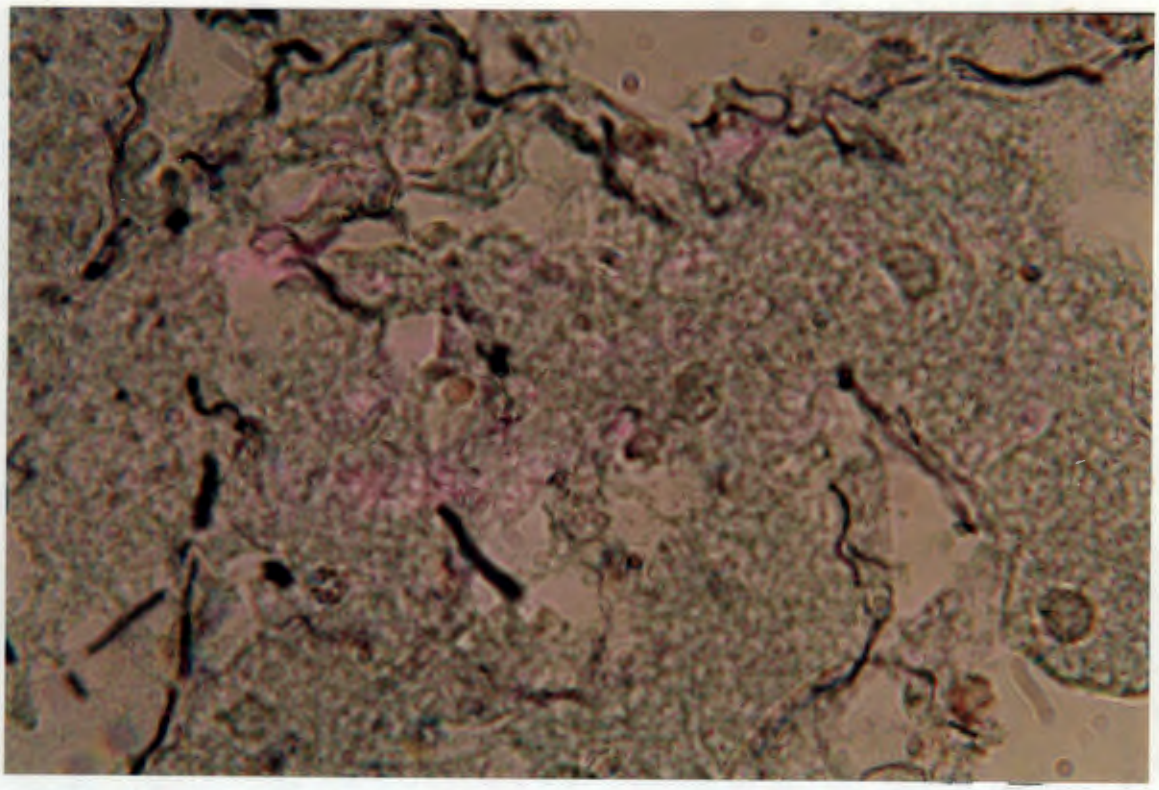


PLATE D1

MONOACETYLHYDRAZINE (X100)

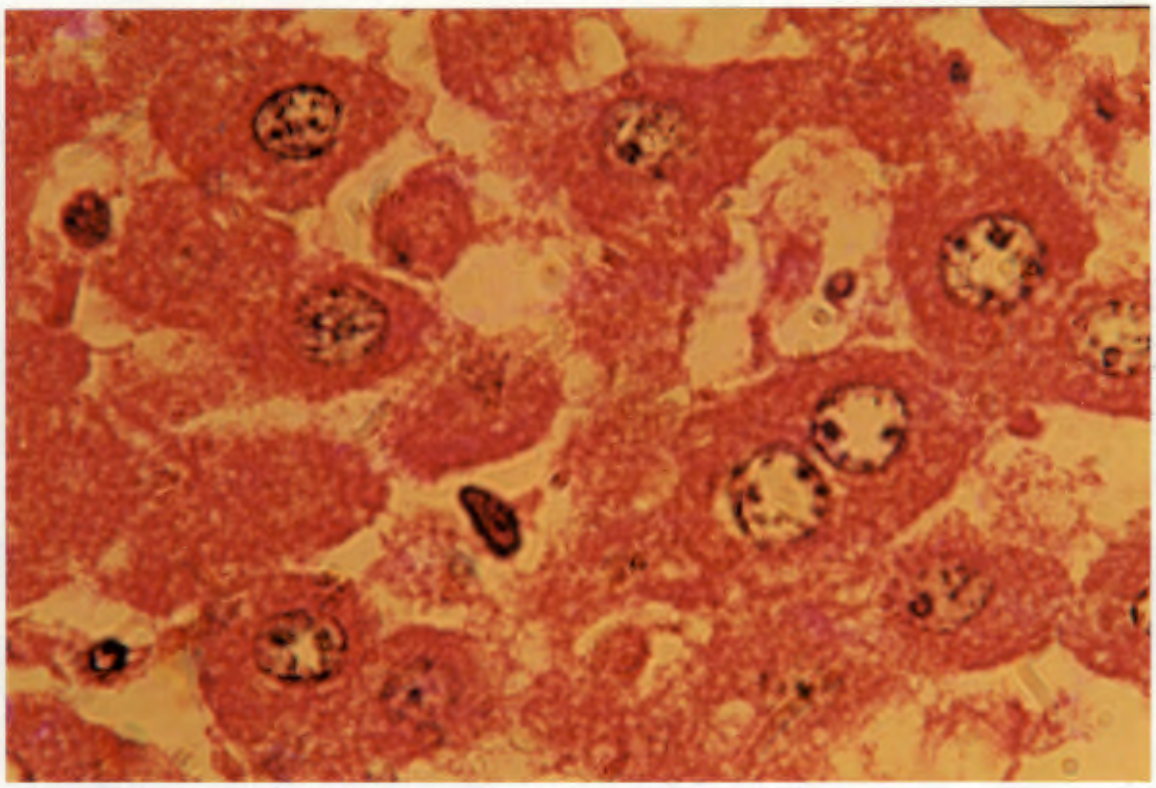


PLATE D2

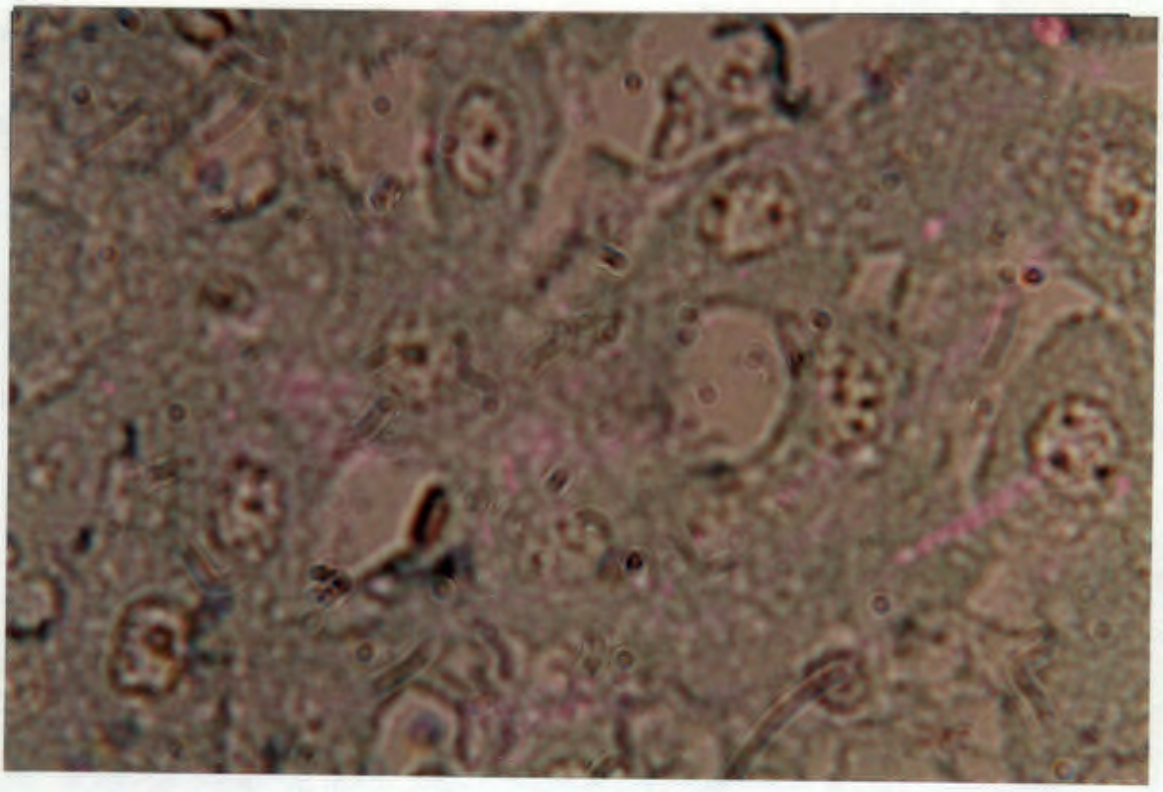


PLATE E1

HYDRAZINE (X100)

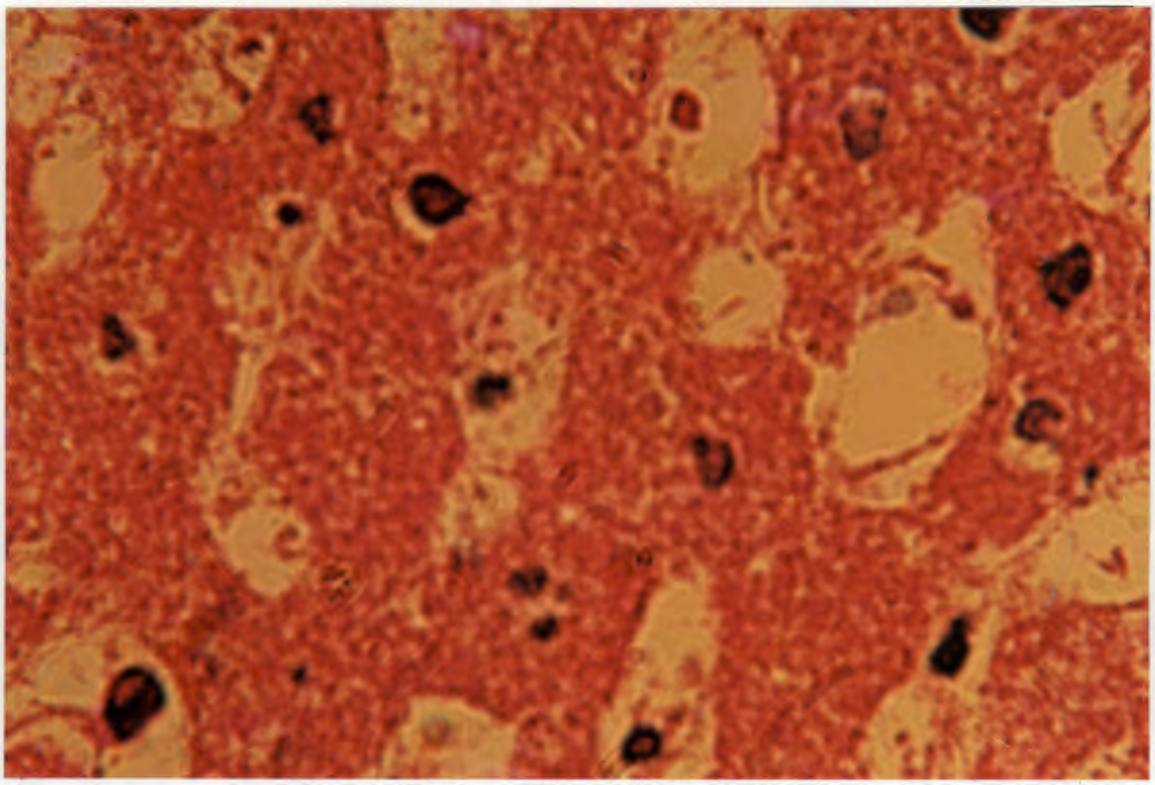
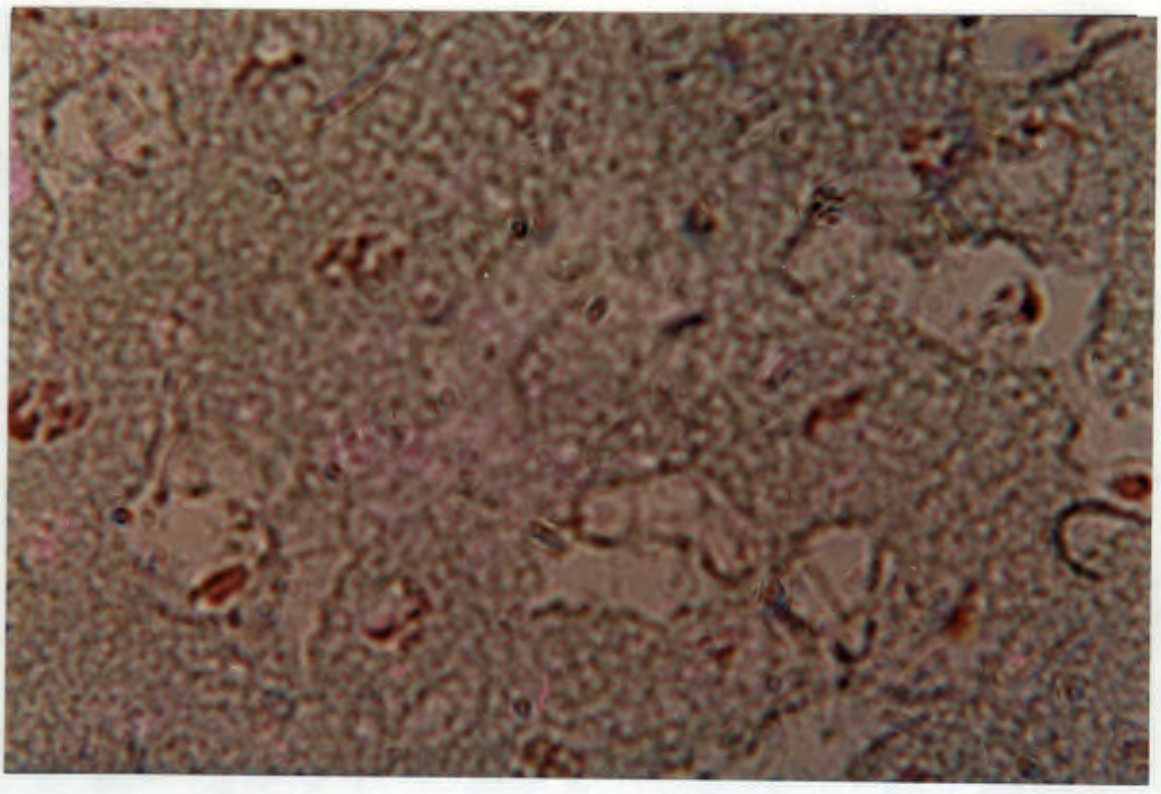


PLATE E2



ISONIAZID AND THREE COMPOUNDS (X100)

PLATE F1

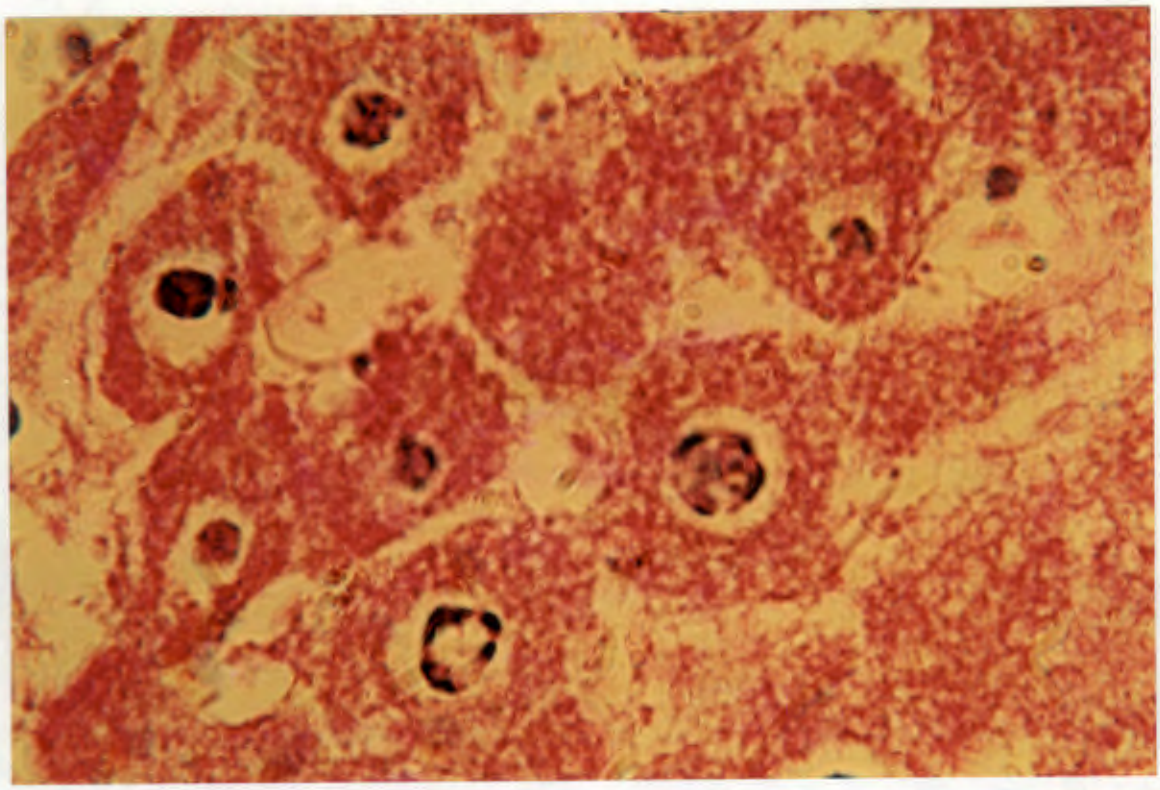


PLATE F2

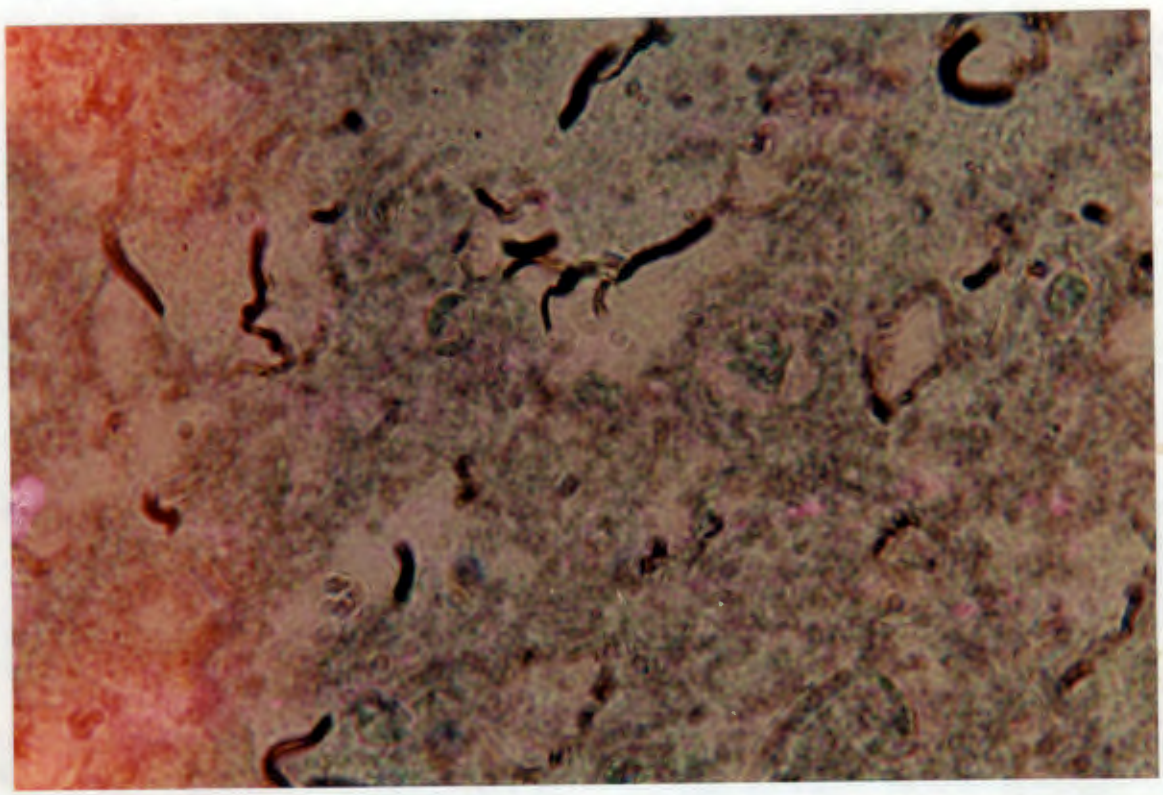


PLATE G1

FRESH LIVER.

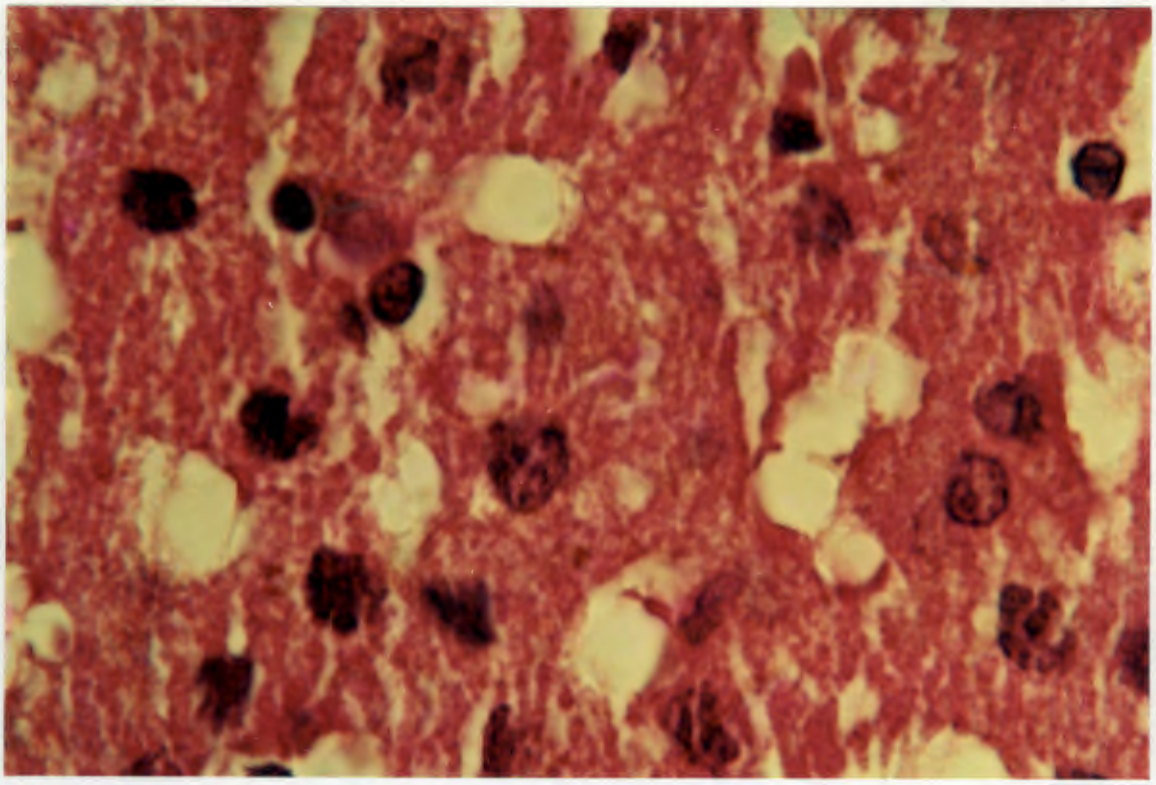


PLATE G2

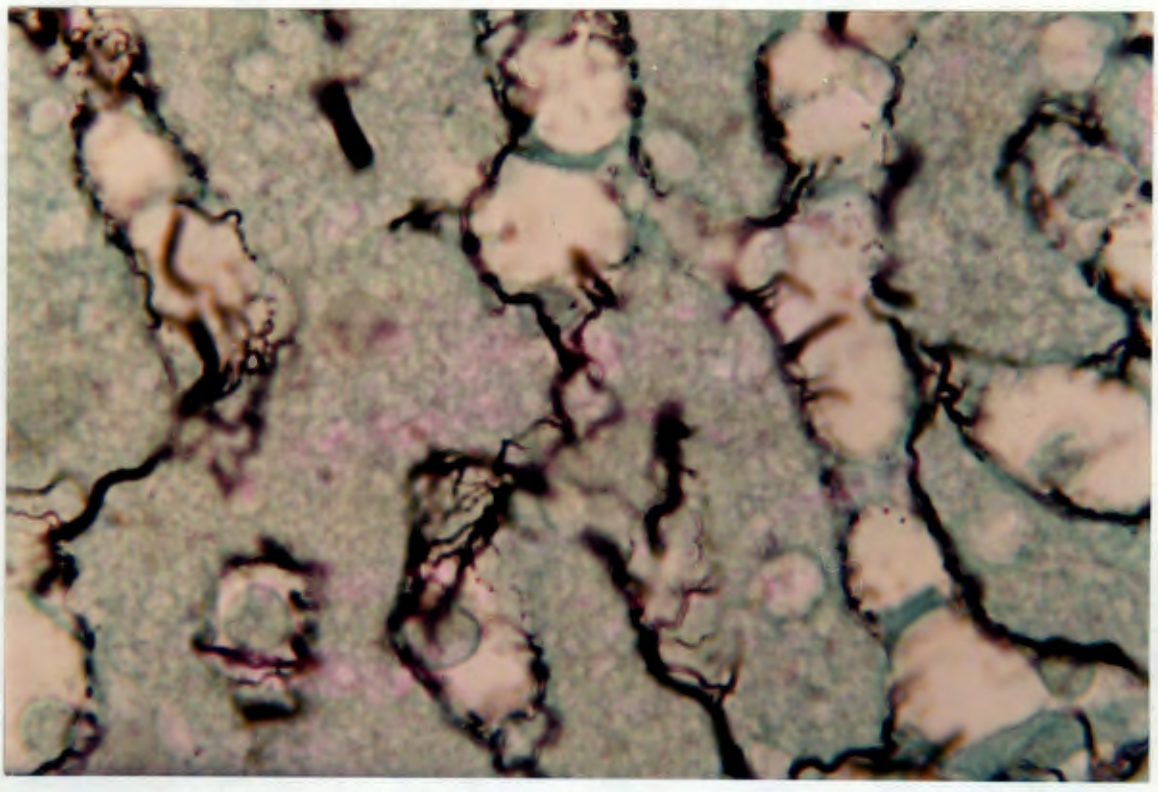


PLATE G3 FRESH LIVER (X10)

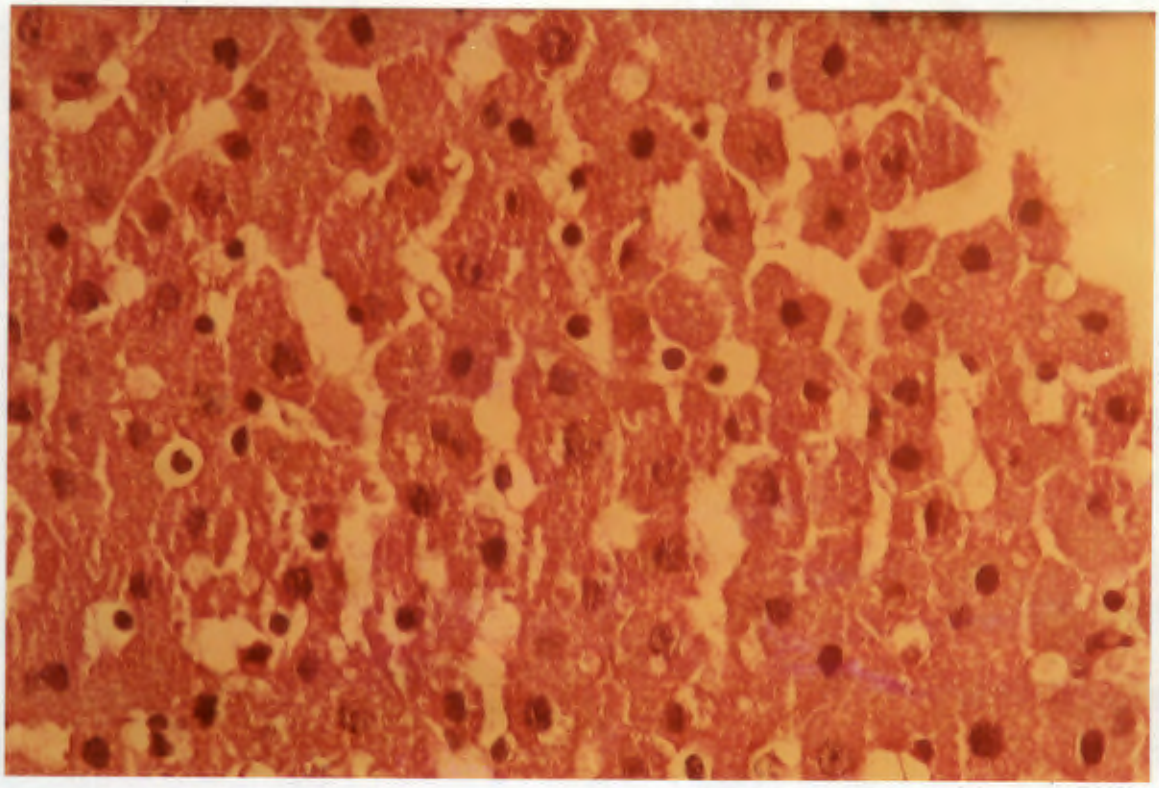


PLATE A3 CONTROL (X10)

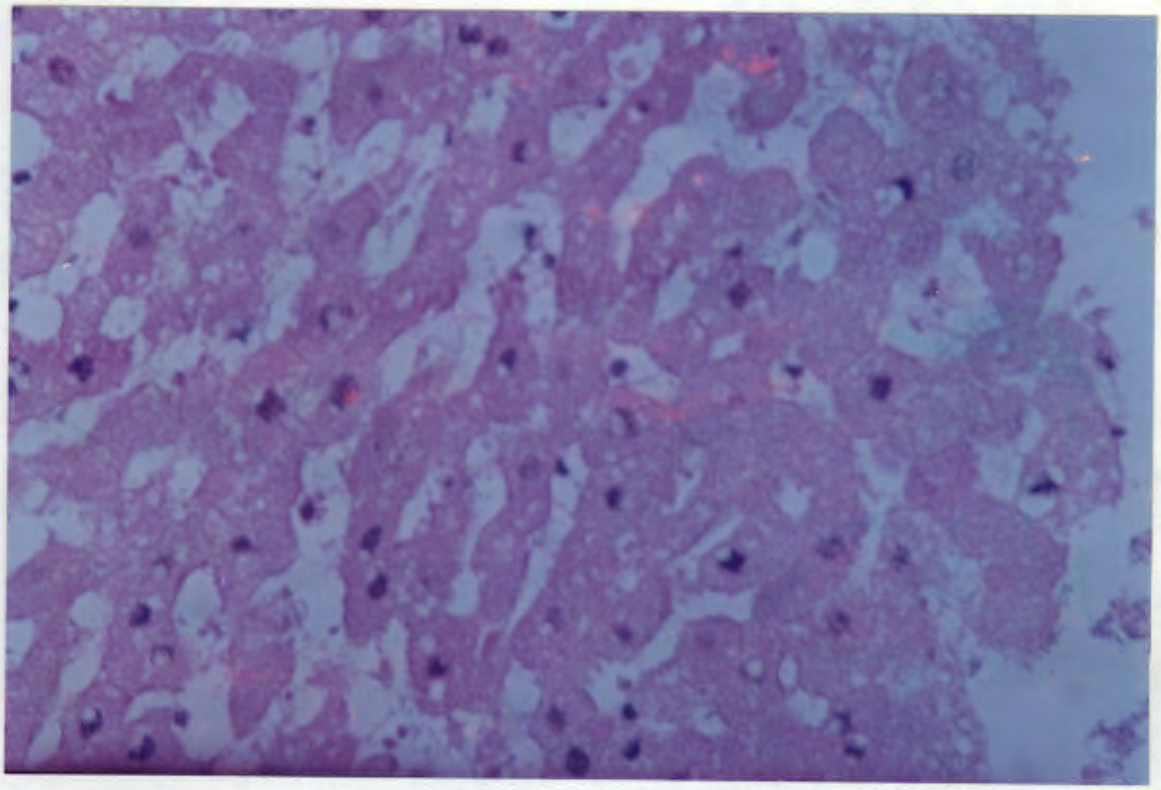


PLATE D3 MONOACETYLHYDRAZINE (X10)

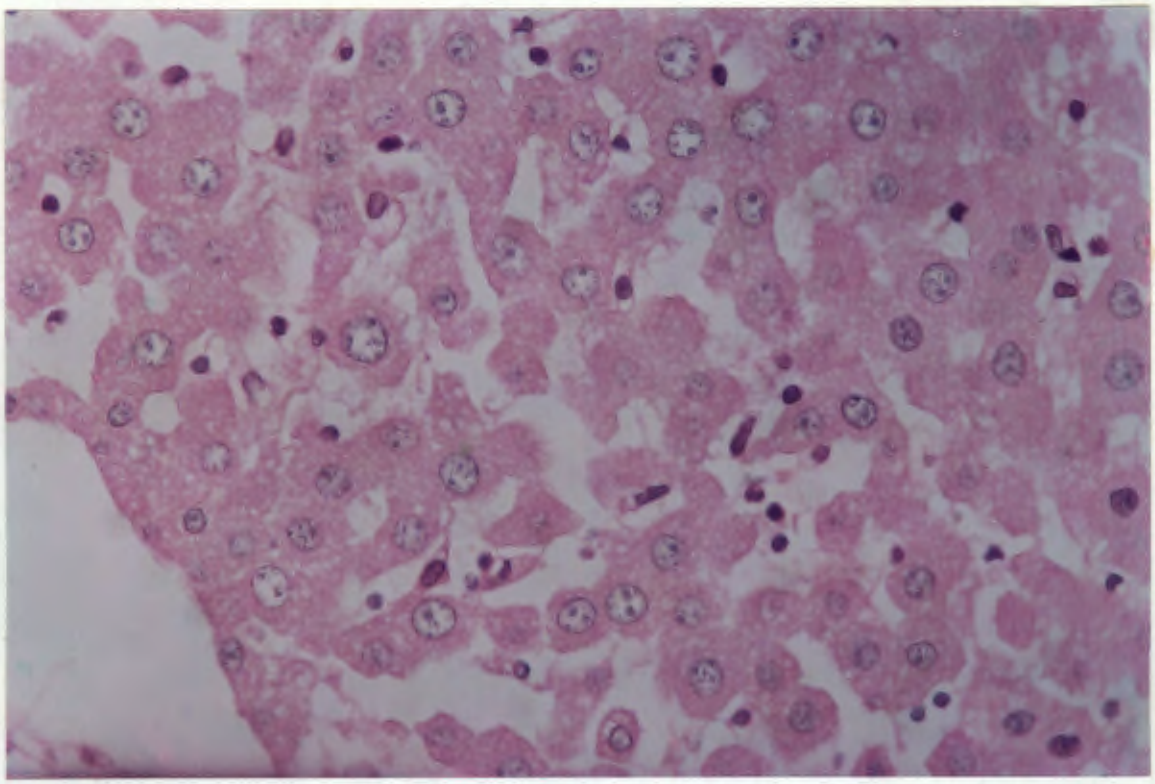
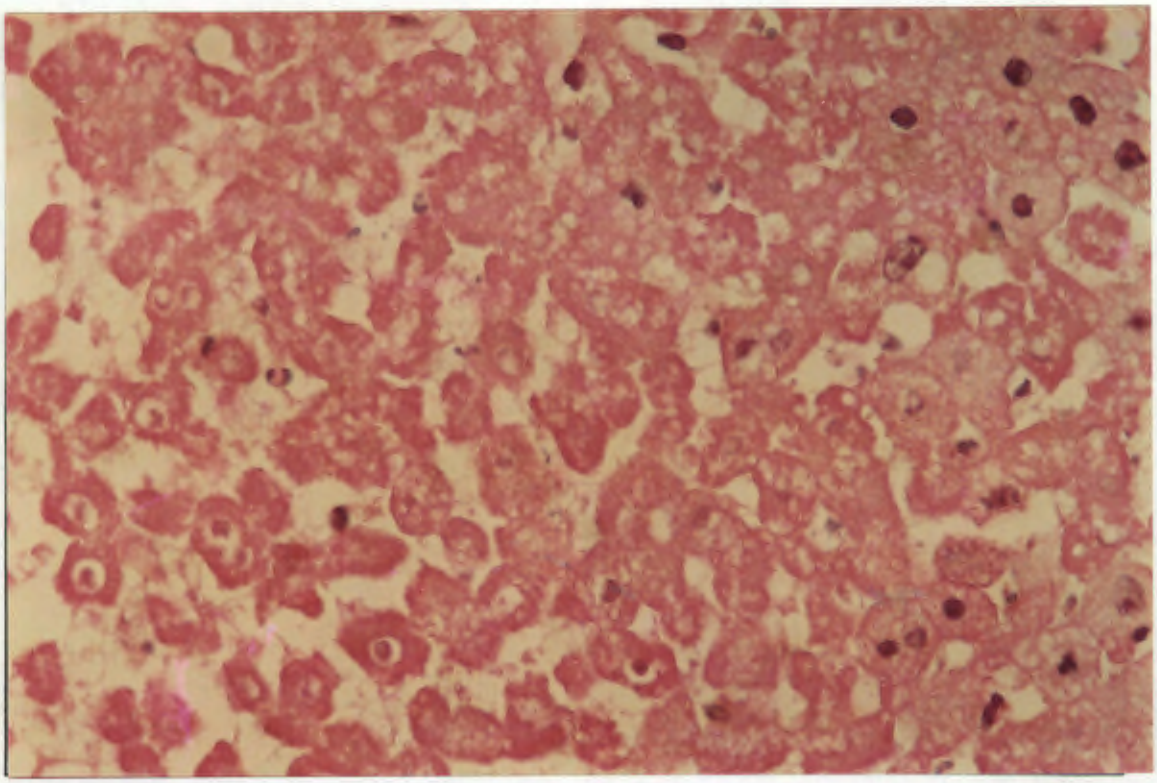


PLATE E3 HYDRAZINE (X10)



ISONIAZID AND THREE COMPOUNDS (X10)

PLATE F3

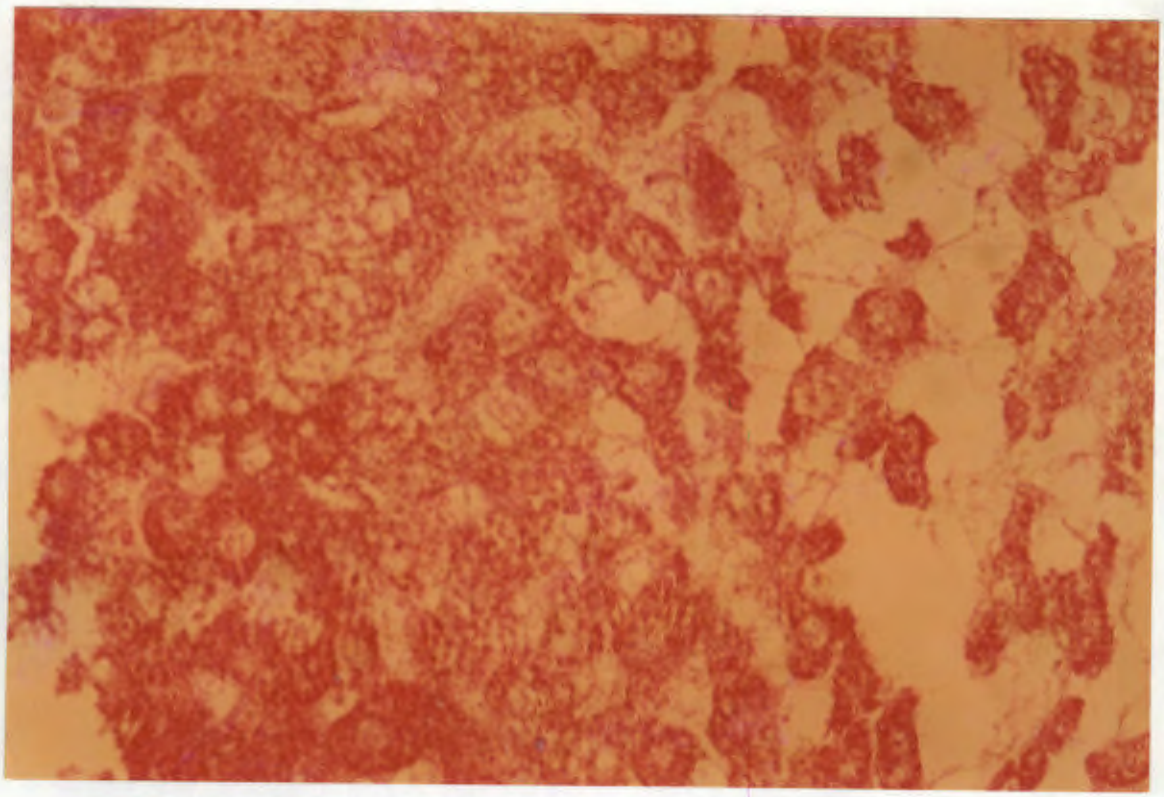
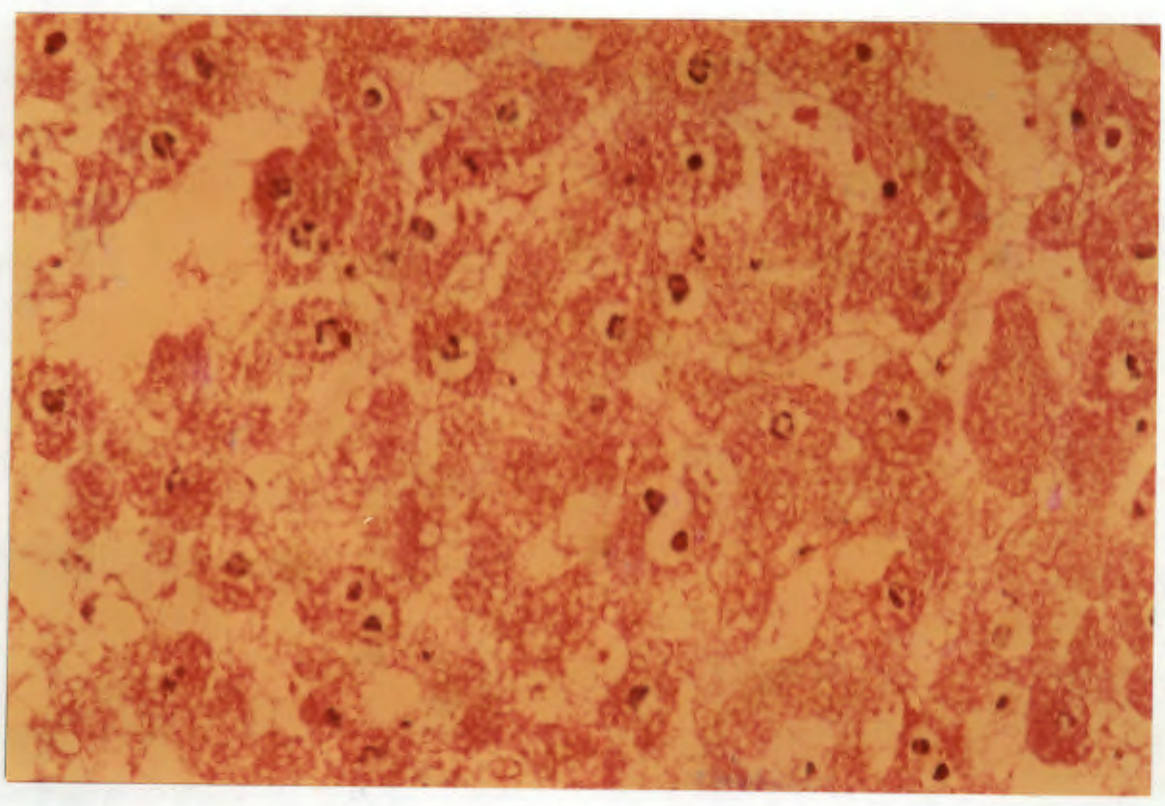


PLATE F4



6.4. DISCUSSION

Hydrazine led to increased lipid peroxidation while this was difficult to prove for isoniazid, acetylisoniazid and monoacetylhydrazine. As the hydrazine group is common to isoniazid, acetylisoniazid and monoacetylhydrazine, it was ironic that free hydrazine caused lipid peroxidation and the three compounds did not. Since the three latter compounds reacted with TMP to produce other products which, unlike the derivatives of TMP with TBA, do not absorb at 540 nm (fig 6.3), it is highly probable that the TBARS test was interfered with by this mechanism. The reaction of hydrazides compounds with TBARS is demonstrated by the use of 2-4-dinitrophenylhydrazine instead of thiobarbituric acid to detection lipid peroxides (Tomita et al, 1990). Despite the lack of signicance difference in the control and drug slices, it is possible that three compounds reacted with some products of lipid peroxidation thereby interfering with the TBARS test. On further incubation, the rise in lipid peroxidation, although not statistically significant, was probably due to increase in lipid peroxide species that could not react with isoniazid or acetylisoniazid. The smaller molecules of monoacetyl-hydrazine and hydrazine did react with TMP, but did not interfere with the TBARS test to the same extent as isoniazid and acetylisoniazid. This is expected because in the reaction with TMP, isoniazid and acetylisoniazid reactions were faster than those of monoacetylisoniazid and hydrazine.

The implications of statistical significance in this experiment need to be taken with reservations because extent of lipid peroxidation may be limited by;

i) the limited quantity of oxidisable (unsaturated) lipids in the small liver slices.

ii) the short a time of incubation for adequate induction of ROS cascade by hydrazine compounds.

iii) use of less sensitive assay to detect lipid peroxidation (TBARS).

The lack of effect by exogenous L-SOD and DDC on lipid peroxide formation implies that superoxide may not be involved in this reaction. However, other factors that could have led to this failure. The enzyme (L-SOD) may not have reached the intracellular space adequately due to difficulties in enzyme delivery across cell membranes viz; poor liposomal carrier efficiency (15%) including efficiency in release of their contents once inside the cell, and phagocytic activity of the host cells (Ostro MJ , 1987). After entry into the cell, the limiting factor is accessibilty of the enzyme to reaction sites. SOD is a large molecule which may exhibit limited access to reaction sites of the membrane bound oxidative enzymes (in case enzyme catalysed peroxidation) and other reactions (non-enzymatic) in similar locations.

Another limitation to the effect of SOD on lipid peroxidation is that SOD alone is an ineffective antioxidant in complex systems such as living tissues (Jadot et al, 1995). This is because SOD leads to production of hydrogen peroxide which can interact with transitional metals (Habers-Weiss reaction) or other groups to produce radicals (OH-) that lead to lipid peroxidation. Hence, a combination of SOD and catalase (or hydroxyperoxidase) was a more powerful antioxidant

than SOD or catalase alone. In this study, SOD and catalase enzyme combination was not used because of complexicity of the experiment, and it was uncertain whether the above observations would occur *in vitro* where there were no, or minimal, transitional metals or Hb.

The histopathology observations showed that although all the four compounds are toxic, the extent of cell injury differs for each compound. Monoacetylhydrazine lead to extensive vacuolation and disintegration of the nuclei, while hydrazine exhibited cytoplsamic toxicity and nuclei loss in the late stage. Isoniazid and acetylisoniazid exhibited both vacuolation and nuclear loss preceded by nuclear pyknosis. Cell necrosis by isoniazid and acetylisoniazid was less severe compared to monoacetylhydrazine and hydrazine. When the four compounds were combined, there was pykinosis, vacuolation, nuclear disintegration and massive necrosis of the cells, implying that the three compounds given together are more lethal than when given individually.

It appears that in this isolated system, the pathognomonic feature of monoacetylhydrazine hepatocellular injury is "vacuolation", while that of hydrazine is cytoplasmic disintegration (plate D3 and E3). This observation suggests that the two compounds may have different mechanisms of toxicity, and this is reaffirmed by observations that monoacetylhydrazine is less likely to induce oxygen free radical than hydrazine.

As only rat liver slices were used, the observations in this study may not apply to man. However, it can be predicted that the toxicological

profile may be worse in human tissue because rat hepatic tissue has a natural resistance to hydrazine induced toxicity (Back and Thomas, 1970). Most probably, this due to high levels of antioxidants such as SOD, reduced GSSG and CAT .

The liver slice model is ideal for studying mechanisms of toxicity, and, in light of these results, there is a need to run this experiment using human tissue. In South Africa, tissue slice techniques are still in their infancy, and human tissue slices are not available.

Whether liberation of reactive oxygen species by isoniazid and its metabolites occurs *in vivo*, is not yet known. To prove this, a method other than the TBARS test should be used for detection of reactive oxygen species.

In conclusion, hydrazine led to lipid peroxidation but this was difficult to prove in the case of isoniazid, acetylisoniazid and monoacetylhydrazine. Since hydrazine is a metabolite of isoniazid, it is hereby suggested that, depending on the amount of hydrazine produced during isoniazid metabolism, reactive oxygen species may be involved in isoniazid induced hepatotoxicity. The TBARS test is not suitable for assessing oxidative stress by hydrazide compounds because they may interfere with the test.

CHAPTER SEVEN

THE DISPOSITION OF ISONIAZID METABOLITES DURING ANTITUBERCULOSIS THERAPY.

SUMMARY

The disposition of pyrazinamide, rifampicin, and isoniazid with its hydrazide metabolites; acetylisoniazid, monoacetylhydrazine, diacetylhydrazine, and hydrazine, was compared in 10 young (25 ± 1 years old) and 9 elderly (72 ± 3 years old) patients during antituberculosis treatment with pyrazinamide, rifampicin and isoniazid on days 1, 30, and 90. Pharmacokinetics of the three drugs as well as the maximum concentration (Cmax), time to reach Cmax (Tmax) and area under curve (AUC) of the metabolites were similar in the two groups on the three occasions. In conclusion, during antituberculosis therapy with isoniazid, rifampicin and pyrazinamide, the metabolism of isoniazid to hydrazide metabolites is not related to age.

7.1 INTRODUCTION

Isoniazid is the most widely used drug for treatment and prophylaxis of tuberculosis. It forms the backbone of the most effective antituberculosis regimens such that no patient with tuberculosis ought to be denied the benefit of using this drug. Unfortunately, isoniazid has been associated with severe side effects which have limited its use in elderly patients (Black et al, 1975). The incidence of isoniazid

induced toxicity rises with age; it is highest in the elderly where some reactions have been fatal and tuberculosis prophylaxis with isoniazid is not recommended (Riska, 1976; Woo et al, 1987 and 1992). Of note however, the pharmacokinetics of isoniazid are similar in all age groups and the occurrence of adverse effects are not related to plasma concentrations of the drug (Advenier et al, 1980; Kergueris et al, 1986; Paulsen et al, 1985; Ellard, 1976 and 1984).

Despite the incrimination of hydrazine metabolites for isoniazid related toxicity, the mechanism of isoniazid induced toxicity is still unknown (Mitchel et al, 1976). The theory implicating hydrazine metabolites has not met wide acceptance because of the failure to explain clinical incidence and this is worsened by lack of information on these metabolites during tuberculosis treatment. Current knowledge on isoniazid metabolites is from studies in young healthy volunteers (Lauterburg et al, 1985; Timbrell et al, 1977; Blair et al, 1985), a few reports in children (Gent et al, 1992) and elderly patients (Walubo et al, 1991b). This is inadequate because either elderly people or some metabolites were excluded or antituberculosis agents commonly used with isoniazid were not given. As the number of elderly people with tuberculosis and cases of tuberculosis with HIV are on the increase, there is a growing need for information regarding plasma profiles of these metabolites during antituberculosis therapy in vulnerable difficult patients.

Described here, is the disposition of rifampicin, pyrazinamide and isoniazid with its metabolites acetylisoniazid, monoacetylhydrazine,

diacetylhydrazine and hydrazine in young and elderly tuberculosis patients on day one of treatment and at steady state.

7.2 METHODS

7.2.1. Materials.

The Rifater tablets containing (mg) isoniazid, 80; rifampicn, 120 and pyrazinamide 250 were from Mer-National (S. A.).

7.2.2. Clinical protocol

Nineteen patients (19) of the Xhosa tribe of South Africa participated in the study. They were newly diagnosed (sputum positive for acid fast bacilli) tuberculosis patients of whom 9 (5F & 4M) were elderly (72 ± 3 years of age; weight 46.7 ± 3.4 Kg) and 10 (4F & 6M) were young adults (25 ± 1 years of age; weight 56.1 ± 6.1 Kg). Antituberculosis drugs (Rifater tablets) were self-administered once daily and orally. Average daily doses in young and elderly patients were, respectively: 7.07 ± 0.72 and 7.07 ± 0.48 mg/kg for isoniazid; 10.61 ± 1.07 and 10.60 ± 0.72 mg/kg for rifampicin and 22.10 ± 2.23 and 22.08 ± 1.50 mg/kg for pyrazinamide.

Informed consent was obtained and the study was approved by the University Ethics Committee. All patients had normal liver, renal and haematological function tests before the study. Patients with history of treatment for tuberculosis and/or illnesses or disorders such as rheumatoid arthritis or diabetes mellitus were excluded.

After overnight fasting, an intravenous cannula connected to a heparin line (0.5 i.u./ml in normal saline) via a three-way tap was inserted into either arm. Blood samples (6 ml) were obtained before start of treatment and at 1, 2, 4, 6, 8, and 10 hours after ingestion of the drugs on treatment days one, thirty and ninety. Blood (5 ml) for haematological tests was drawn in a separate test tube.

On each occasion, patients emptied their bladder before starting the study and subsequent urine was collected in plastic containers for eight hours. No feeds were given for the first two hours and only fluids were allowed from 2 to 4 hours.

Blood samples were centrifuged immediately and plasma was stored at -80 °C until assay (within 10 weeks during which no significant loss of drugs and metabolites was observed) while urine was kept at 4 °C until end of collection (8 hours) when it was pooled, total volume determined, 5 ml stored as above and the rest was discarded. Urine was assayed for isoniazid and acetylisoniazid.

Compliance was achieved by home visiting and medicines were dispensed on a daily basis at tuberculosis clinics near home where patients swallowed the tablets under supervision of a tuberculosis nurse (this is standard practice in this region).

Analysis of compounds; A portion (2 ml of plasma) from each sample was analysed by HPLC for rifampicin, pyrazinamide, isoniazid and metabolites (acetylisoniazid, monoacetyl-hydrazine, diacetylhydrazine

and hydrazine) as described earlier (Chapter four; Walubo et al, 1994). From the remaining 1.5 ml, 0.5 ml was used for assay of allantoin (0.5 ml). The six hour sample was also analysed for TBARS (0.5 ml) and other laboratory parameters including uric acid (chapter 8).

7.2.3. Kinetic analysis

Pharmacokinetic parameters were obtained from plasma concentration-time points using standard formulae (Gibaldi 1984). The areas under plasma concentrations plotted against time (AUC) were calculated using the trapezoidal rule. Apparent first order rate constant for decline of plasma concentrations after administration (ß) was obtained by linear least square regression on log plasma concentration versus time. The apparent volume of distribution (V) was calculated according to equation:

$$V = \frac{DOSE}{ß * AUC}$$

Plasma clearance was derived from $DOSE/AUC_{(tot)}$.

7.2.4. Statistical analysis

The results were analysed by non-parametric methods using the StatGraphics computer package. The paired Mann-Whitney U test was used to test for level of significance at P value < 0.05.

7.3. RESULTS

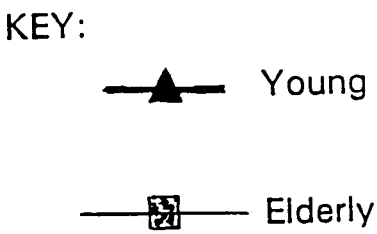
Figures 7.1a to 7.1c show the concentration time profiles of pyrazinamide, rifampicin and isoniazid on the three occasions while tables 7.1 and 7.2 illustrate the pharmacokinetic parameters of the three drugs on days 1 and 30 (Appendices B1 to B4). Concentrations on day 90 were unsuitable for regression analysis.

There was no difference in the pharmacokinetics of the three drugs in young and elderly patients. The median half-life for isoniazid on day 1 ranged from 2.29 to 5.57 hours in the young versus 2.68 to 4.98 in the elderly ($P = 0.216$). All patients were slow acetylators with half-life greater than 2 hours (Fig. 7.2; 10; Jackson et al, 1989) and acetyl-isoniazid to isoniazid ratio less than 1.14 (Appendix B5).
Rifampicin kinetics were similar in both age groups.

Whereas AUC for pyrazinamide on day 1 was similar ($P = 1.0$) in the two groups, AUC on day 30 was different ($P = 0.032$). AUC ranged from 422.82 to 876.87 mghr/L in the young versus 262.27 to 973.8 mghr/L in the elderly on day 1, and 354.37 to 938.37 mghr/L in the young versus 571.62 to 1003.0 mghr/L in the elderly on day 30.

Figures 7.3a to 7.3d show the plasma concentrations of metabolites on the three days, while tables 7.3 and 7.4 illustrate their AUC, maximum concentration, and time to maximum concentration (Appendices B6 to B8). The three kinetic parameters for each of the metabolites were similar in the two age groups. AUC for hydrazine on day 1 and diacetylhydrazine on day 30 were not different in the two groups. AUC for hydrazine on day 1 ranged from 2.24 to 20.2 mghr/L

Figure 7.1 Plasma concentrations of isoniazid (7.1a), rifampicin (7.1b) and pyrazinamide (7.1c) in young and elderly patients during TB treatment.



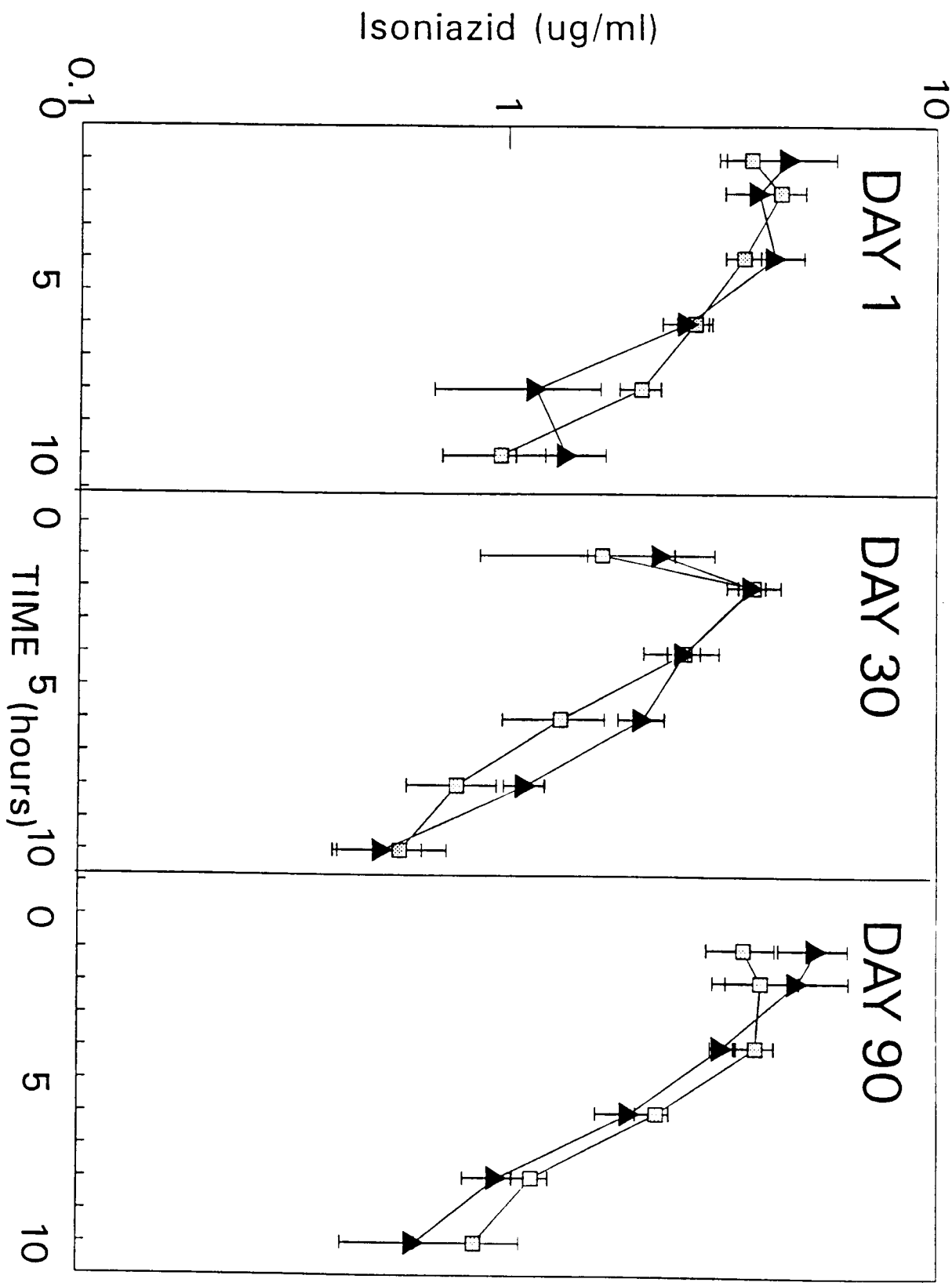


Fig. 7.1a

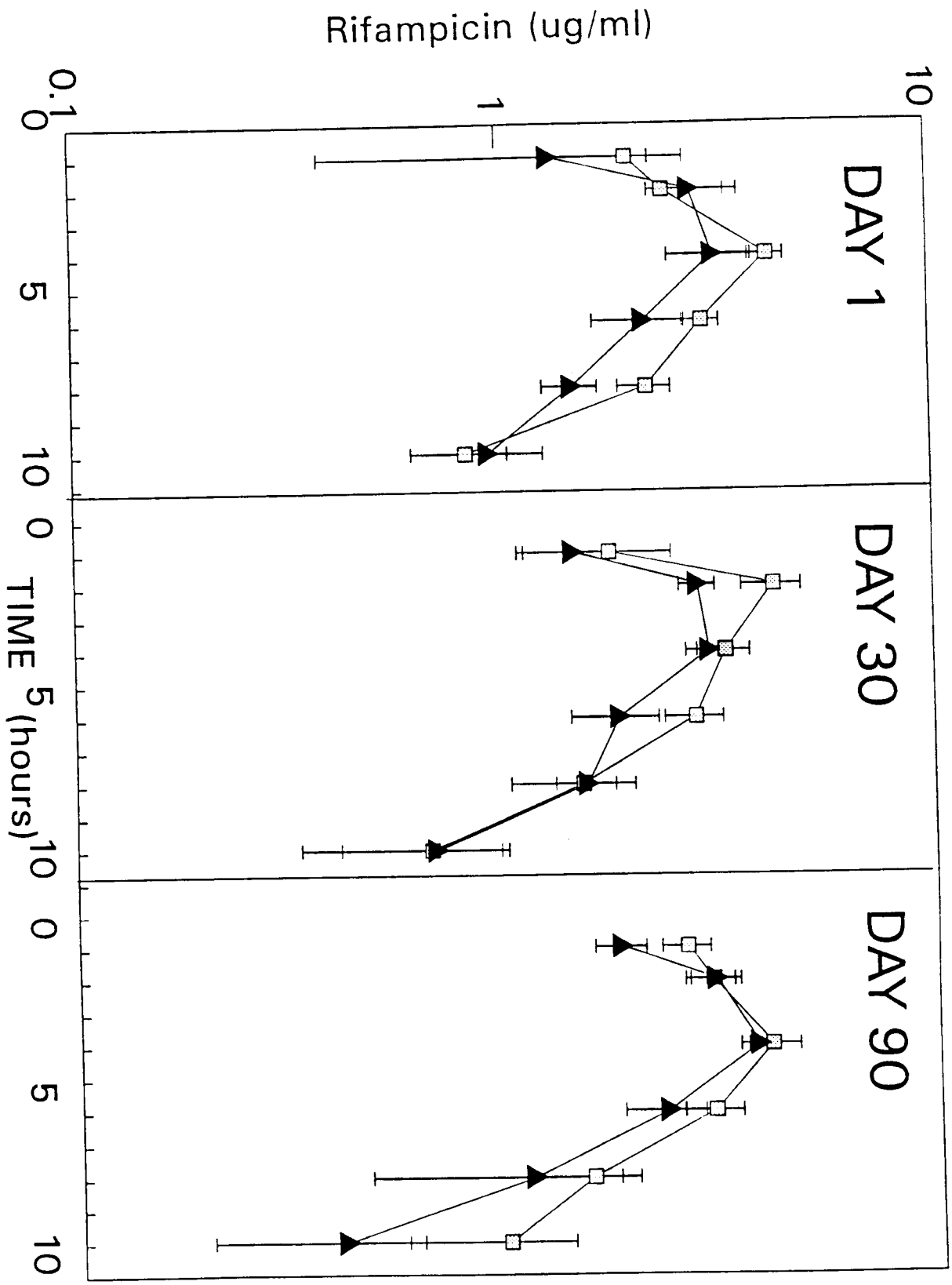


Fig. 7.1b

Fig. 7.1c

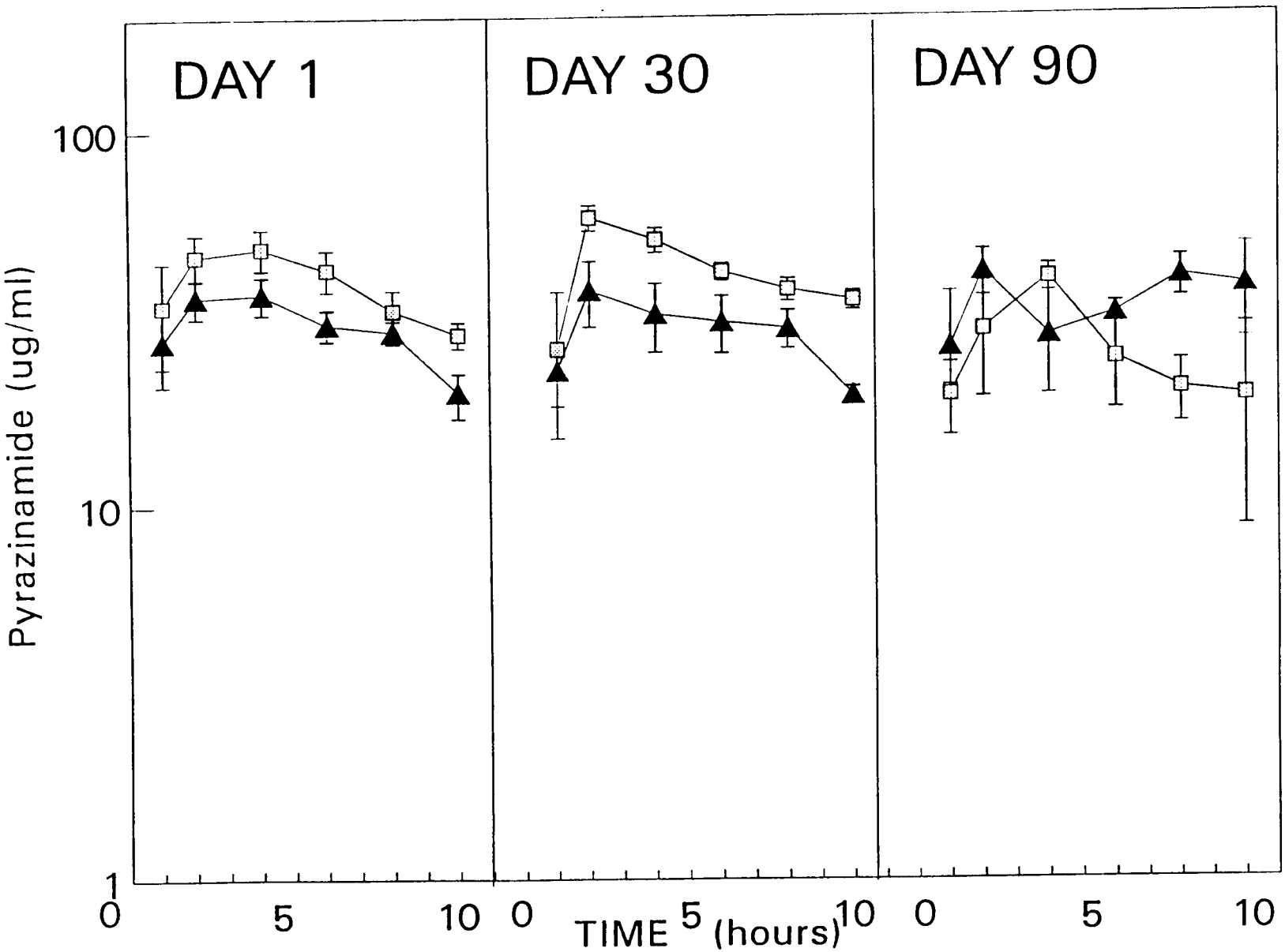

DAY 1
DAY 30
DAY 90
100
10
1
Pyrazinamide (ug/ml)
0
5
10
0
5
10
0
5
10
TIME (hours)

Table 7.1 Kinetic parameters (Mean ± s.d.) of INH, RIF, and PZA on day 1 of treatment.

	YOUNG						ELDERLY					
	AUC	$T_{1/2}$	CLp	Vd	C_{max}	T_{max}	AUC	$T_{1/2}$	CLp	Vd	C_{max}	T_{max}
INH	29.7	2.8	15.5	1.1	4.5	1.3	36.1	3.69	8.7	1.4	4.3	2.1
	±3.4	±0.3	±1.5	±0.1	±1.2	±0.8	±4.8	±0.4	±3.2	±0.1	±0.3	±0.6
RIF	31.7	3.23	19.0	2.29	3.4	3.6	32.3	4.14	14.9	1.97	4.3	3.4
	±4.9	±0.6	±3.0	±0.6	±0.7	±0.6	±5.3	±0.5	±2.8	±0.2	±0.6	1.2
PZA	511.9	9.2	2.45	0.5	36.8	2.1	694.5	6.9	1.8	0.38	48.9	2.3
	±65.7	±1.3	±0.2	±0.1	±4.3	±0.2	±96.1	±1.1	±0.4	±0.03	±6.1	±0.6

AUC = mghr/L; $T_{1/2}$ = hrs; CLp = L/hr; Vd = L/Kg; C_{max} = mg/L; T_{max} = hrs.

Table 7.2 Kinetic parameters (Mean ± s.d.) of INH, RIF, and PZA on day 30 of treatment.

	YOUNG						ELDERLY					
	AUC	$T_{1/2}$	CLp	Vd	C_{max}	T_{max}	AUC	$T_{1/2}$	CLp	Vd	C_{max}	T_{max}
INH	24.5 ±3.6	2.29 ±0.3	17.8 ±1.3	1.6 ±0.2	3.74 ±0.2	1.8 ±0.8	26.1 ±2.6	2.1 ±0.2	18.8 ±3.6	1.24 ±0.2	3.77 ±0.6	2.4 ±0.6
RIF	25.5 ±5.0	2.9 ±1.1	23.5 ±3.5	2.9 ±0.2	3.0 ±0.6	3.4 ±1.2	33.4 ±7.5	3.0 ±0.6	14.4 ±4.6	2.0 ±0.2	4.3 ±0.7	2.5 ±0.2
PZA	487.5 ±66.6	7.6 ±0.9	2.58 ±0.3	0.51 ±0.1	37.3 ±7.4	2.0 ±0.2	851.3 ±69.2	9.7 ±1.6	1.2 ±0.1	0.4 ±0.1	58.4 ±4.5	2.1 ±0.2

AUC = mghr/L; $T_{1/2}$ = hrs; CLp = L/hr; Vd = L/Kg; C_{max} = mg/L; T_{max} = hrs.

Figure 7.2.. The distribution of isoniazid plasma half-life in young and elderly patients during TB treatment on day 1

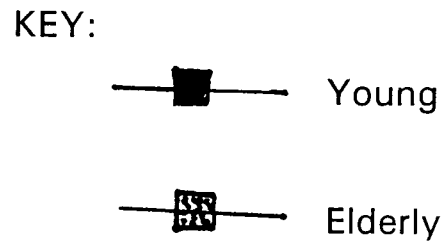


Fig. 7.2 Distribution of isoniazid half-life in young and elderly patients.

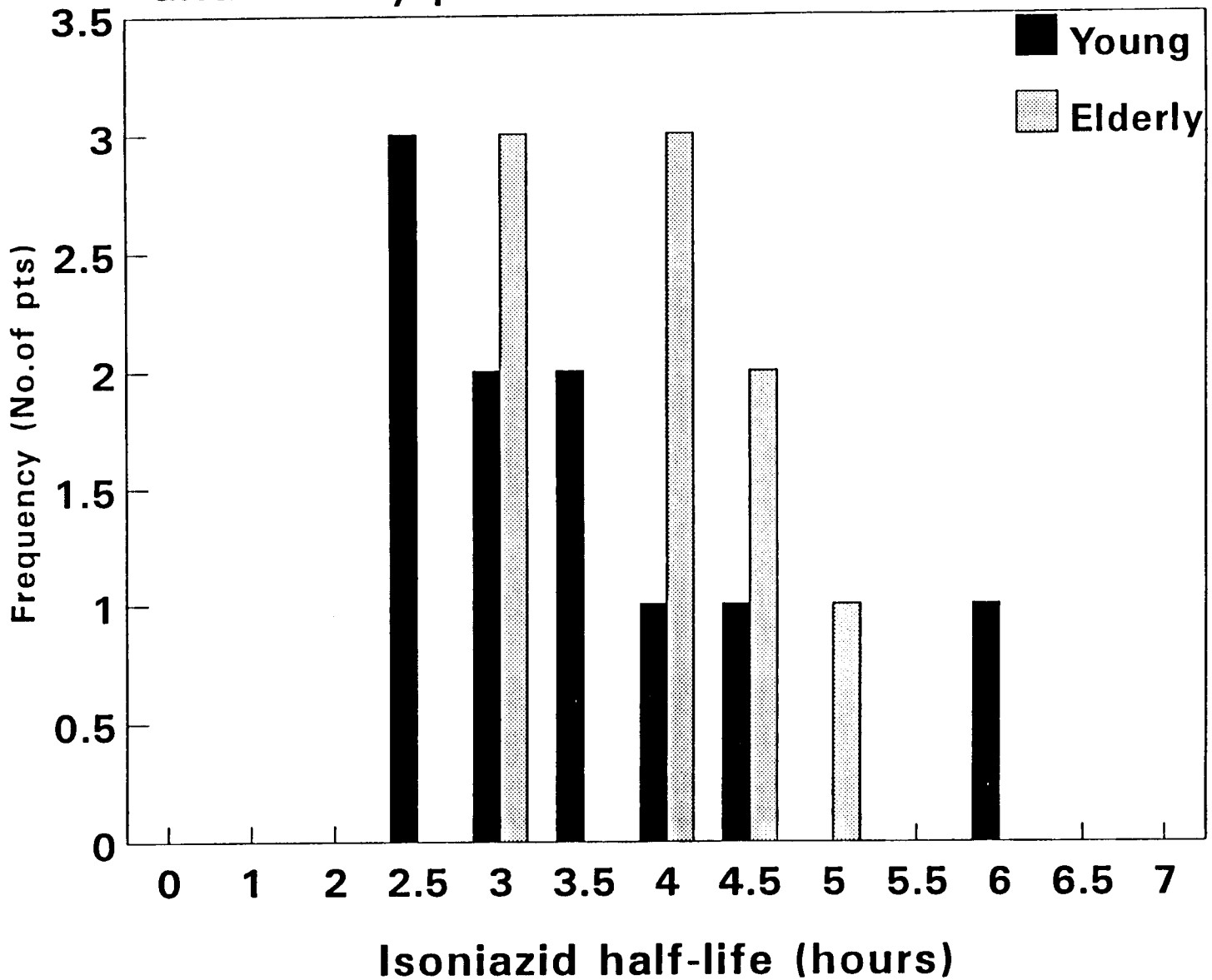


Figure 7.3 Plasma concentrations of acetylisoniazid (7.3a), mono-acetylhydrazine (7.3b), hydrazine (7.3c) and diacetylhydrazine (7.3d) in young and elderly patients during TB treatment.

KEY:

Young

Elderly

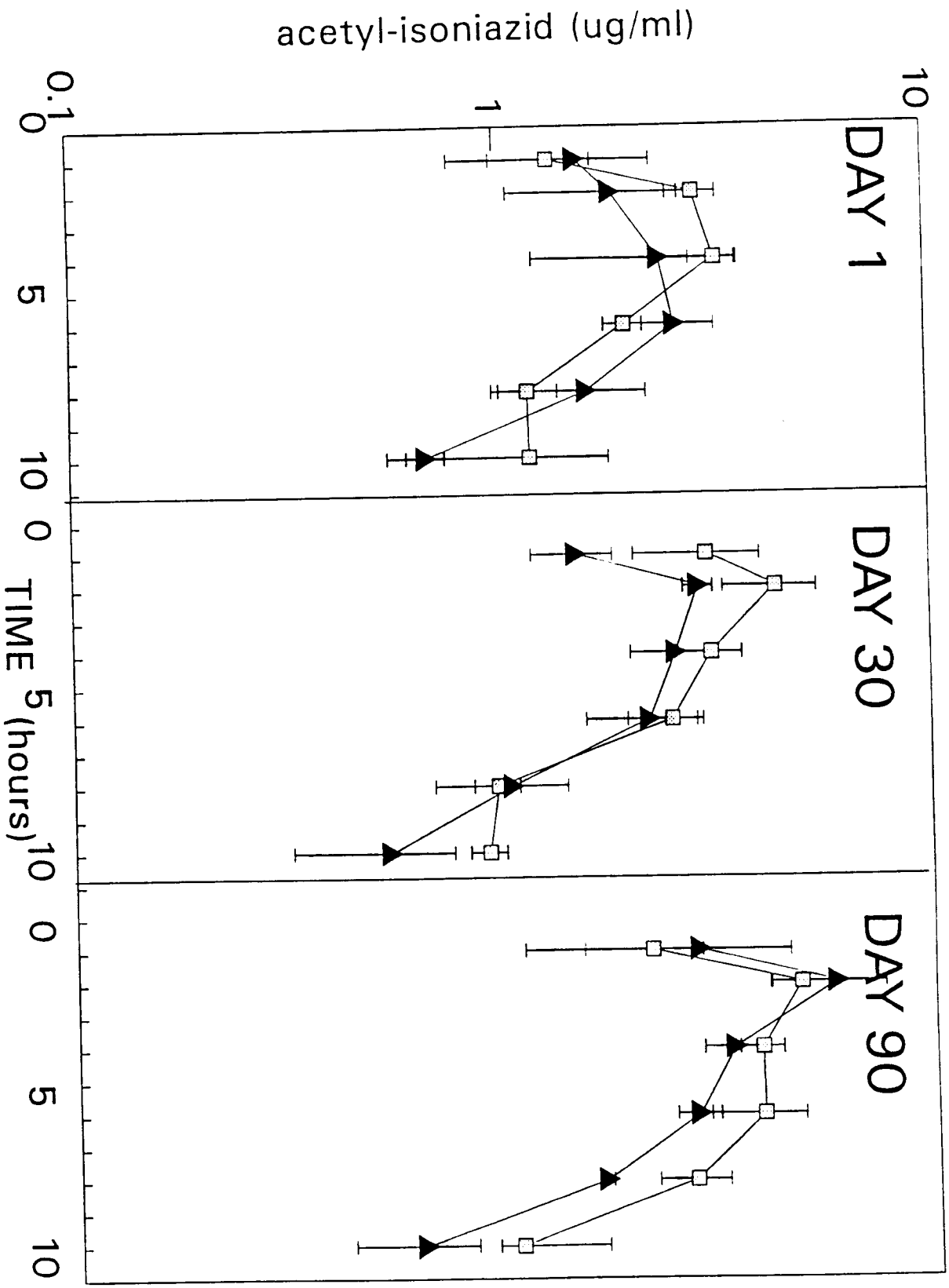

acetyl-isoniazid (ug/ml)
0.1
1
10
DAY 1
DAY 30
DAY 90
0
5
10
TIME (hours)

Fig. 7.3a

Fig. 7.3b

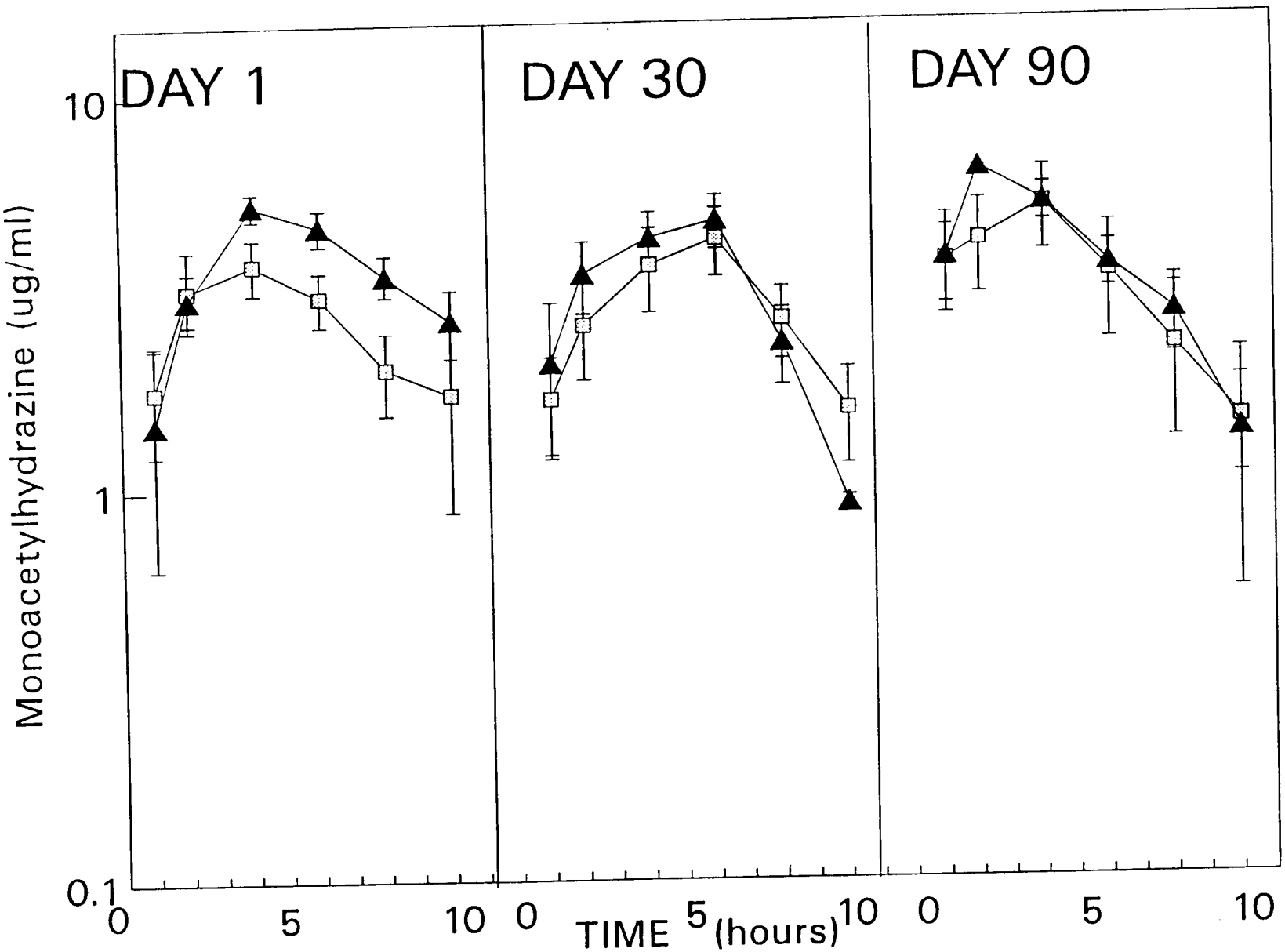

DAY 1
DAY 30
DAY 90
10
1
0.1
Monoacetylhydrazine (ug/ml)
0
5
10
TIME (hours)

Fig. 7.3c

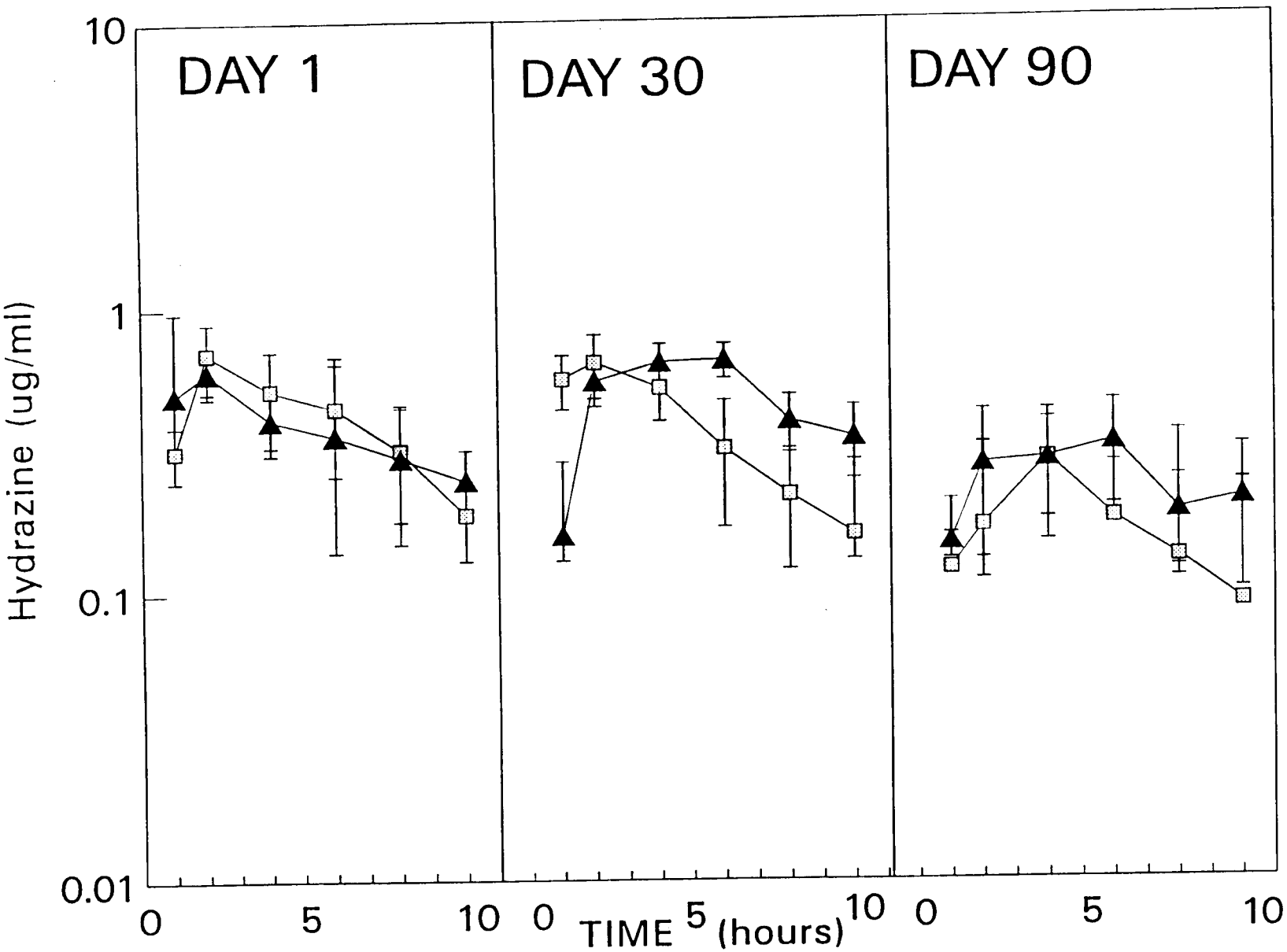


Fig 7·3d

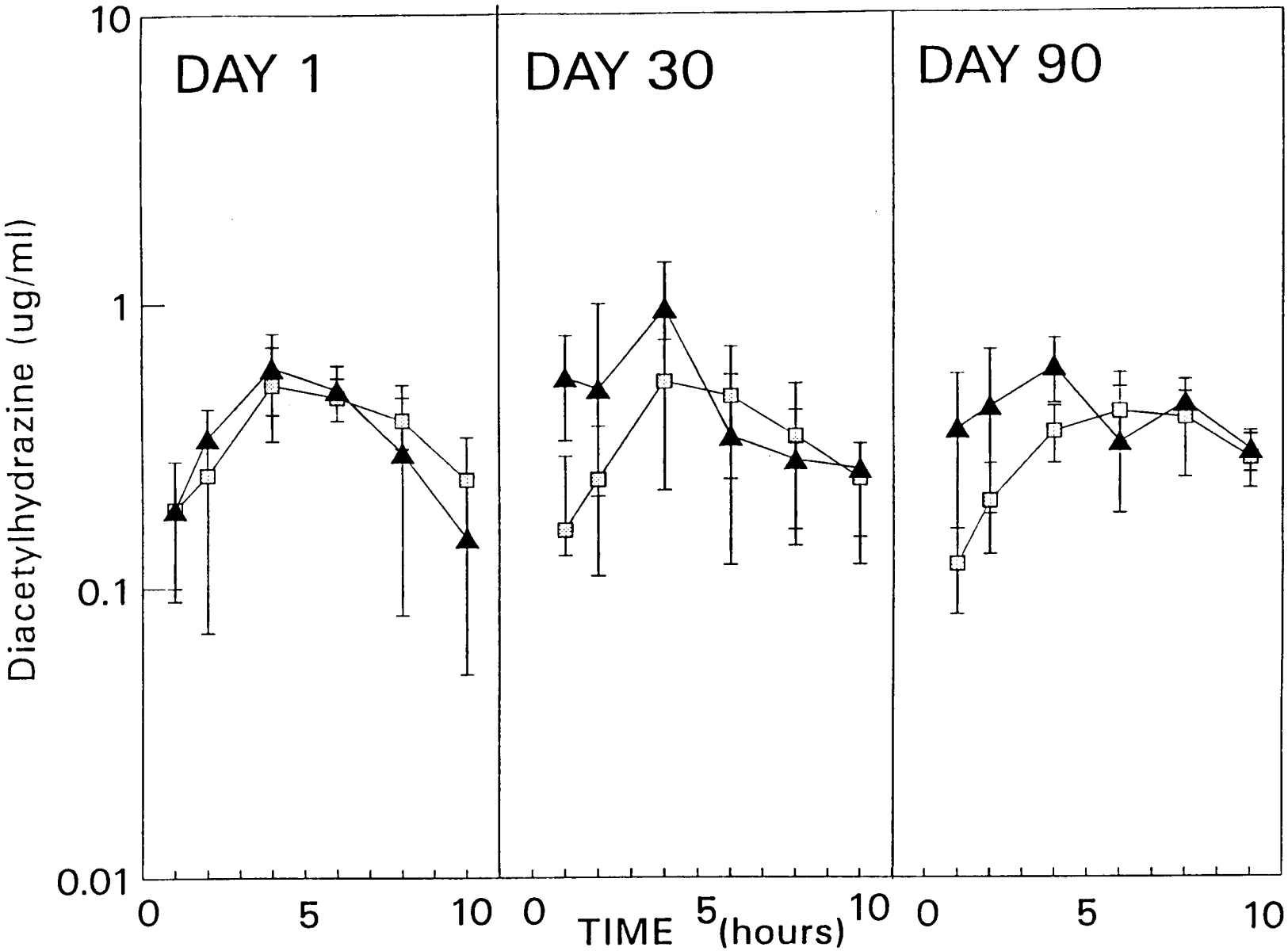

DAY 1
DAY 30
DAY 90
10
1
0.1
0.01
Diacetylhydrazine (ug/ml)
0
5
10
TIME (hours)

Table 7.3 Kninetic parameters (med ± s.d.) of metabolites on day 1 of treatment.

	YOUNG			ELDERLY		
	Cmax	tmax	AUC	Cmax	tmax	AUC
INH	4.54 ±0.3	1.3 ±0.8	29.7 ±4.8	4.34 ±0.6	2.1 ±0.6	36.0 ±3.4
acINH	2.64 ±0.6	5.6 ±1.3	20.6 ±6.1	3.28 ±0.4	5.1 ±1.2	19.9 ±1.8
mHYD	5.3 ±0.4	4.2 ±2.1	47.4 ±10.1	3.77 ±0.6	4.3 ±1.6	42.8 ±15.7
HYD	0.6 ±0.2	4.0 ±1.8	5.4 ±2.3	0.52 ±0.2	4.1 ±0.8	11.2 ±2.6
dHYD	0.6 ±0.2	5.1 ±1.2	6.3 ±1.5	0.52 ±0.2	4.8 ±0.6	8.5 ±2.0

Cmax = μg/ml; tmax = hours; AUC = μghr/ml.

Table 7.4 Kinetic parameters (mean ± s.d.) of metabolites on day 30 of treatment.

	YOUNG			ELDERLY		
	Cmax	tmax	AUC	Cmax	tmax	AUC
INH	3.74	1.22	4.5	3.77	2.23	4.4
	±1.2	±0.3	±3.6	±0.6	±0.2	±2.6
acINH	2.92	2.51	8.2	4.38	3.22	4.4
	±0.2	±0.8	±8.5	±1.1	±1.2	±5.4
mHYD	4.73	5.83	9.7	4.27	6.34	4.0
	±0.7	±2.1	±9.8	±0.8	±1.6	±15.8
HYD	0.7	2.8	10.7	0.6	2.61	2.8
	±0.1	±0.8	±4.9	±0.2	±0.8	±5.9
dHYD	0.95	4.5	6.8	10.53	4.81	3.6
	±0.4	±1.2	±1.9	±0.2	±0.7	±3.67

Cmax = mg/L; tmax = hours; AUC = mghr/L.

in the young versus 3.02 to 20.83 mghr/L in the elderly (P = 0.603), while AUC for diacetylhydrazine on day 30 ranged from 2.35 to 10.75 mghr/L versus 0.92 to 19.47 mghr/L in the elderly (P = 0.54).

Kinetic parameters such as half-life and clearance could not be determined from the concentration-time curves of metabolites because the profiles here define rate of formation and elimination.

Although the concentrations on day 90 appear to decline, they were at steady state such that C_{max} and AUC were not suitable for comparison with day 1 and 30.

The enzyme inducing effects of rifampicin were not significant at one month, and it is unclear whether this had a role in the increase in isoniazid clearance and AUC of hydrazine in young patients by day 30. Also, there was a slight drop, although not significant, in rifampicin clearance in both groups by day 30.

Overall, the concentration of metabolites ranged from 0.56 to 7.04 mg/L for acetylisoniazid, 0.45 to 6.8 mg/L for monoacetylhydrazine, 0.12 to 0.88 mg/L for hydrazine and 0.06 to 1.12 mg/L for diacetylhydrazine. Therefore, the most reliable concentrations for monitoring metabolites should be after the C_{max} (i.e. 6 hrs or later).

7.4 DISCUSSION

The disposition of isoniazid metabolites during antituberculosis therapy has been determined and concentration ranges obtained. This is important for assessing patients manifesting toxicity and drug resistance problems. Toxicity, because these metabolites are potentially toxic, and drug resistance, because at less than 1 μg/ml monoacetylhydrazine competitively inhibited isoniazid action against mycobacteria (Ellard and Gammon, 1976; Pope H, 1956).

One tribal group was used in the study to minimize genetic influence and the Xhosa tribe was chosen because they live within the vicinity of the university. Acetylator status for South African blacks was reported as 41 percent slow acetylators (Bach et al, 1976), however, the use of antimodes from other studies to classify acetylator status of these subjects may not be appropriate here. There are outstanding differences between this study and those from which these antimodes were determined, i.e. the formulation of isoniazid (Rifater tablets), co-administration with other drugs and disease (tuberculosis) might have modified and delayed drug absorption from the gut, leading to a 'slow release' kinetics. Characteristically a 'slow release' behavior results into a fictitiously prolonged half-life and reduced drug delivery such that urinary recovery of metabolites would be lower than expected e.g. acetylisoniazid. For the same reason patient 3E shows a discrepancy of a high metabolic ratio of 1.6 (Appendix B5) versus isoniazid half-life of 4 hours (Appendix B1) making it difficult to decide on the acetylator status. However, when the patients' own half-lives were used to phenotype, an antimode of 3.5 hours was obtained and the subjects

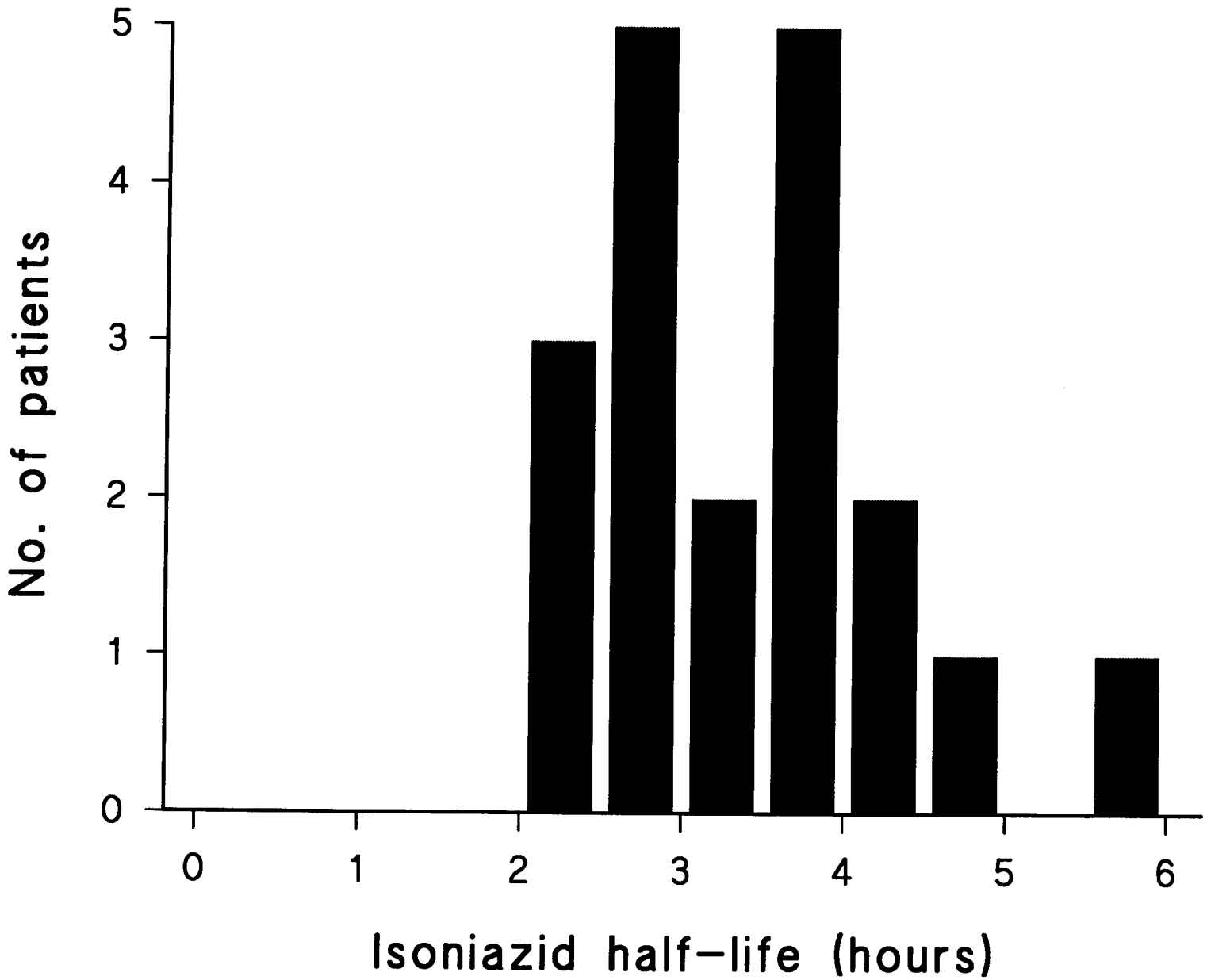
were reasonably divided into 10 fast acetylators (7Y and 3E) and 9 slow acetylators (3Y and 6E; Fig. 7.4).

In studies such as this one, acetylator status should be determined separately and prior to beginning of the study, but, owing to many blood letting in this prolonged study (over three months), and the standard practice of tuberculosis therapy where acetylator status is not routinely tested, preliminary tests for acetylator status were omitted to minimize inconvenience to the patients and avoid a high fallout. The study subjects did not receive any treatment different from other patients except hospitalization for blood sampling. Hence, this study depicts a clinical situation such that the difficulties in classifying acetylator status could be another reflection of the insignificant role of phenotypic status during antituberculosis therapy.

The four metabolites were produced in significant quantities. However, the maximum concentration (4.73 mg/L) and AUC (44.0 mghr/L) of monoacetylhydrazine are twice those reported by Lauterburg et al (1985) viz; 2.28 mg/L and 28.88 mghr/L, respectively. This could have been due to differences in assay procedures (HPLC vs GC-MS), dose of isoniazid (7.07 mg/Kg vs 300 mg oral bolus), presence of other drugs (pyrazinamide and rifampicin vs isoniazid), and disease (tuberculosis patients vs volunteers).

A similar controversy was observed with hydrazine concentration where, using the gas chromatography-mass spectrometry (GC-MS) methods, Beever et al (1981) and Blair et al (1985) reported C_{max} of hydrazine in healthy volunteers as 14.1 ng/ml and 9.9 $\pm$ 1.1 ng/ml,

Fig 7.4 The distribution of isoniazid half-life in 19 patients.



respectively. To the contrary, recent studies using HPLC assays have reported C_{max} of hydrazine as; (a) greater than 0.1 μg/ml in rabbits after 5 mg/kg of isoniazid; (b) 0.24 $\pm$ 0.08 μg/ml and 0.4 $\pm$ 0.2 μg/ml in young and elderly tuberculosis patients, respectively, during antituberculois therapy at 6.5 $\pm$ 0.77 mg/kg of isoniazid (Walubo et al, 1991c) and, (c) 0.1 μg/ml in one pediatric patient at 20 mg/kg of isoniazid (Gent et al, 1992).

It appears that other factors such as disease and the presence of other drugs may influence the extent of hydrazine formation whereby the enzyme inducing effects of rifampicin and a high catabolic state due to tuberculosis disease may increase hydrazine formation. Sarma et al (1984) reported a two-fold rise in the proportion of isoniazid metabolized to hydrazine (3% to 6%) when rifampicin was added to isoniazid in volunteers. Similarly in this study, the AUC of hydrazine in young patients on day 30 was higher than on day 1, most probably due to rifampicin induction of hydrolase enzymes. This phenomena was not exhibited by elderly patients where the AUC of hydrazine was similar on days 1 and 30. This is possibly due to chronic induction of enzymes over the years. Therefore, the AUC of hydrazine in elderly patients on day 1 was higher, although not statistically different, than in young patients. This was in agreement with previous observation of a higher C_{max} of hydrazine in elderly when compared to young Chinese patients (Walubo et al, 1991d). Neverthless, it is not known whether such observations make hydrazine metabolite the cause of a higher incidence of isoniazid induced toxicity in elderly than young patients.

By day 90, concentrations of hydrazine and diacetylhydrazine were at steady state, whereby elimination and production seem to be balanced. However, the concentrations remained high even after 5 hours, indicating accumulation of these metabolites with continued therapy. In the later months, pyrazinamide concentrations need to be checked because pyrazinamide concentrations increased with time causing high steady state levels in the elderly (figure 7.1).

Concentrations of the four metabolites varied over a wide range. As none of the study patients exhibited toxicity it would be logical to take these concentration ranges as the normal. However, it was difficult to predict whether concentrations observed in one patient would be safe in another. A study of these metabolites during isoniazid toxicity is needed to understand their role in this process.

In conclusion, the disposition of acetylisoniazid, monoacetylhydrazine, diacetylhydrazine and hydrazine during antituberculosis treatment are similar in young and elderly patients. The four isoniazid metabolites (acetylisoniazid, monoacetylhydrazine, diacetylhydrazine and hydrazine) were produced in significant quantities and tended to accumulate with continued treatment. Therefore, the occurrence of more isoniazid adverse reactions in the elderly when compared to young patients is due to factors other than disposition of isoniazid metabolites. It is hoped that the results of this study will aid the evaluation of difficult tuberculosis patients and improve understanding of the mechanism and management of isoniazid related toxicity.

CHAPTER EIGHT

OXIDATIVE STRESS DURING ANTITUBERCULOSIS THERAPY IN YOUNG AND ELDERLY PATIENTS.

8.0. SUMMARY

Using allantoin as a marker for reactive oxygen species, oxidative stress during antituberculosis therapy was compared in 10 young and 9 elderly patients. Before treatment, allantoin plasma concentrations in patients were similar to that of volunteers. Administration of a combination of isoniazid, rifampicin and pyrazinamide increased plasma allantoin in both groups of patients. Allantoin concentrations (mean ± s.e.) at six hours were higher ($P < 0.05$) in elderly than in young patients on day one, (8.22 ± 1.50 vs 1.89 ± 0.98 ug/ml); day 30, (5.85 ± 0.82 vs 0.87 ± 0.57 ug/ml); and day 90, (4.84 ± 1.24 vs 0.52 ± 0.50 ug/ml). Although the total amount of allantoin excreted and the rate of allantoin formation at steady state were similar in both groups on the three occasions, allantoin clearance was nine times higher in young than in elderly patients. It was concluded that the high concentrations of allantoin in the elderly was due to decreased renal excretion, while the increase in plasma allantoin concentration when the drugs were administered implies that these antituberculosis drugs induced oxidative stress in both the young and elderly patients.

8.1 INTRODUCTION

Oxidative stress refers to excessive generation of free radicals in the body. Oxygen free radicals or reactive oxygen species namely, H_2O_2, O_2 and -OH (ROS), have been incriminated in the pathophysiology of many biological reactions. When antioxidant defences are inadequate, ROS may lead to tissue damage. This is commonly due to disease but drug-derived radicals can also cause tissue damage (i.e toxicity).

The involvement of reactive oxygen species in aetiology and manifestation of disease and drug toxicity makes them worthwhile species to monitor. Such information may be helpful because administration of an appropriate antioxidant at the right time and in the right place is the best approach to controlling free radical activity. Unfortunately, there is no reliable test for timely detection of free radicals *in vivo*. Controversy reigns over the percentage molar ratio test (i.e. 9,11 linoleic:9.12-linoleic acid) because it may be influenced by disease (Jack et al, 1991) and diet (Britton et al, 1992). The desferrioxamine-chelatable iron test is unreliable because elements besides iron can propagate free radical activity. The thiobarbituric acid reacting substances (TBARS) test, a non-specific measure for lipid-peroxidation, is the most widely used indicator for free radical cascade. However, by measuring the end products of lipid peroxidation, detection of free radicals by this method is possible after considerable tissue damage has occurred. Secondly, we have observed in our laboratory that hydrazine compounds (isoniazid and metabolites in plasma) interfere with this test by reacting with some lipid peroxides. A test whereby free radicals can be detected before tissue damage occurs would be more valuable in the clinic.

Allantoin, a product of purine metabolism, was suggested to be a good marker for free radical activity. In humans, allantoin is formed by non-enzymatic oxidation of uric acid because there is no enzyme to metabolise uric acid. During this process, uric acid acts as an antioxidant that protects other molecules or tissues against attack by free radicals (Ames et al, 1981; Becker, 1993).

I envisaged that the amount of allantoin in the body is related to extent of free radical reaction with uric acid such that measurement of allantoin plasma profiles may help to predict the degree of oxidative stress during treatment with potentially toxic drugs. Such information may not only help to identify susceptible patients using the offending drug before organ damage occurs, but may be a clue to understanding the mechanism of toxicity.

The mechanism of isoniazid induced toxicity is still unclear but recent reports have shown that hydrazine compounds, including isoniazid, can generate free radicals in the presence of oxygen (Gayathri et al, 1975; Hussein et al, 1985; Sushil snd Hochstein, 1979). It was postulated here that during isoniazid metabolism, isoniazid and its metabolites (hydrazine, acetylhydrazine, acetylisoniazid and diacetylhydrazine) form free radicals which may lead to toxicity, and this could be demonstrated *in vivo* by variations in allantoin profiles in body fluids. As adverse reactions during treatment of tuberculosis with isoniazid containing regimens are more common in elderly patients, this study was undertaken to compare oxidative stress induced by a combination of isoniazid, rifampicin and pyrazinamide in young and elderly tuberculosis patients, with a hope that the result would shed light on

why elderly patients are more susceptible to antituberculosis drug toxicity.

8.2 METHODS

8.2.1. Materials.

The Rifater tablets containing (mg) isoniazid, 80; rifampicin, 120 and pyrazinamide 250 were used as in section 7.2.1. (Chapter seven).

8.2.2. Clinical protocol

As in section 7.2.2 (Chapter seven).

Allantoin was determined on all the samples, while only the pre-treatment and six hour samples were used for assay of uric acid, TBRAS, liver, renal and haematological function tests.

8.2.3. Analysis of samples

Allantoin plasma concentration was assayed by HPLC using a procedure described by Ora et al (1992), after a slight modification. Briefly, using acetic acid (5 ul of 10% aq. acetic acid) as internal standard, 20 ul of the ultrafiltrate from 0.5 ml of plasma or urine (0.1 ml urine diluted to 0.5 ml with water) was injected into the HPLC and eluted at 0.8 ml/min with 5 mM HSA in 5 mM phosphate buffer at pH 3.2. The eluate was detected by UV at 224 nm. Samples were run in batches of 10 and peak height ratios were used for deriving concentrations.

Plasma uric acid concentration, liver, renal and haematolcgy function tests were assayed in the hospital laboratory. Uric acid was not assayed by the HPLC method because the high uric acid concentrations needed dilutions to come into the linear calibration range which made allantoin detection difficult.

Antituberculosis drugs (isoniazid, pyrazinamide and rifampicin) and isoniazid metabolites (acetylisoniazid, monoacetylhydrazine, diacetylhydrazine and hydrazine) were tested and did not interfere with the assay (Walubo et al, 1994). Other metabolites such as pyrazinoic acid and desacetylrifampicin, although not tested would not appear in concentration similar to allantoin.
TBARS were assayed using a procedure described by Mitsuru et al, 1977.

8.2.4. Kinetic analysis

Renal clearances of allantoin, uric acid and creatinine were calculated using standard formula (Bennett, 1992):

$$\text{Clearance (ml/min)} = \frac{U_{fr}\ (\text{ml/min}) * U_c\ (\text{mg/dl})}{P_c\ (\text{mg/dl})}$$

Where U_{fr} is urine flow rate, U_c and P_c are urine and plasma concentrations of the reference compound, respectively.

To assess total allantoin formed, it was assumed that at steady: Amount formed = Clp_{ss} x C_{ss} = Amount excreted.

8.2.5. Statistical analysis

The results were analysed by non-parametric methods using the StatGraphics computer package. The unpaired Mann-Whitney U test was used to test for level of significance with a P value < 0.05.

8.3. RESULTS

Tables 8.1 and 8.2 show the average plasma concentrations of allantoin and uric acid as well as liver and renal function tests in control and tuberculosis patients before and during treatment, respectively. Before treatment the function tests in patients were within normal range for age and, together with allantoin and uric acid values, were comparable to those of control group (Table 8.1). Although alkaline phosphatase for elderly patients was in the upper range this did not reflect liver damage in the context of normal transferases. The liver enzymes and renal tests remained in the normal range over the study period and average weight gain by three months was 10.01 % for the young group and 6.08 % for the elderly (Table 8.2). The number of elderly patients on days 30 and 90 was less by two due to loss of the subjects by immigration back to their homelands (away from urban Cape Town) where they continued with their treatment. That this loss was not due to death was certified by their close relatives (children) or the ones that brought them in the city for medical care.

Table 8.1 Physiological parameters (mean ± s.d.) in control and tuberculosis patients before therapy.

PARAMETER	YOUNG		ELDERLY	
	CONTROL	TB PATIENTS	CONTROL	TB PATIENTS
No. & Sex	7 (4F & 3M)	10 (4F & 6M)	5 (2F & 3M)	9 (5F & 4M)
Age (years)*	24.1 ± 4.6	24.7 ± 2.9	78 ± 7.5	72.9 ± 8.3
Weight (Kg)	69.6 ± 5.8	56.1 ± 6.1	52.2 ± 5.5	46.7 ± 3.4
Allantoin (μg/ml)	2.46 ± 0.5	1.71 ± 1.5	3.51 ± 1.98	3.25 ± 2.3
Uric acid (mg/dl)	5.07 ± 0.92	6.49 ± 1.5	7.79 ± 1.89	9.27 ± 2.7
Urea (mmol/L)	4.07 ± 0.86	3.52 ± 0.92	5.96 ± 1.6	3.97 ± 1.8
CTN (mg/dl)	0.79 ± 0.13	0.68 ± 0.26	0.99 ± 0.36	0.86 ± 0.22
GGT.(mmol/L)	25.4 ± 11.5	39.8 ± 20.6	43.3 ± 23.3	41.3 ± 36.7
AST. (mmol/L)	24.3 ± 2.1	32.7 ± 9.5	29.2 ± 12.7	42.4 ± 27.5
ALT. (mmol/L)	9.4 ± 6.2	11 ± 5.8	11 ± 7.9	17.6 ± 10.4
ALKP (u./L)*	95.3 ± 24.5	33.6 ± 15.2	77.8 ± 24	115.9 ± 32.5
Total Bil (μmol/L)	12.4 ± 1.3	10.7 ± 3.5	13.6 ± 1.1	13.2 ± 6.3

Note: * significant difference between elderly and young patients' values ($P < 0.05$).

Table 8.2. Physiological parameters (median ± s.e.) of TB patients during treatment for tuberculosis.

PATIENT GROUP	10 YOUNG (25 ± 1 YRS) 4F & 6M			9 ELDERLY (72 ± 2 YRS) 5F & 4M		
TIME (DAYS)	1	30	90	1	30	90
Wt (Kg)	56 ± 2	60.6 ± 7	64.1 ± 8	46 ± 3.3	48.7 ± 8	51 ± 9
ATN (μg/ml)	1.9 ± 1	0.9 ± 0.6	0.5 ± 0.5	8.2 ± 1.5	5.9 ± 0.8	4.8 ±1.2
MDA (nmol/ml)	9.3 ± 1.4	9 ± 2	7.4 ± 2	10 ± 2	8.7 ± 0.5	8 ± 1
ESR (mm/h)	68 ± 25	47 ± 17	11 ± 3	88 ± 13	78 ± 21	50 ± 2
UA (mg/dl)	9.5 ± 2	8 ± 2	7.4 ± 2	10.4 ± 3	9 ± 2	9.3 ± 2
Hb (g/dl)	12 ± 1	13 ± 2	13 ± 2	12 ± 3	12 ± 2	11 ± 2
Urea (mmol/L)	3.4 ± 0.7	3.2 ± 0.1	3.4 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.7 ± 0.1
CTN. (mg/dl)	0.7 ± 0.3	0.6 ± 0.1	0.6 ± 0.3	0.9 ± 0.2	0.9 ± 0.2	0.9 ± 0.2
GGT. (mmol/L)	62 ± 20	29 ± 11	19 ± 10	29 ± 12	26 ± 12	37 ± 6
AST. (mmol/L)	23 ± 7	28 ± 13	22 ± 8	60 ± 24	40 ± 12	25 ± 9
ALT. (mmol/L)	15 ± 11	15 ± 10	15 ± 5	26 ± 17	22 ± 7	20 ± 6
ALKP (u./L)	55 ± 23	61 ± 23	71 ± 18	110 ± 39	109 ± 25	100 ± 10
TBil. (umol/L)	17 ± 4	13 ± 4	10 ± 4	17 ± 3	15 ± 2	16 ± 6

Normal ranges: Uric acid (UA) = 1.5 - 8 mg/dl; Urea = 3.6 - 7.1 mg.dl; Serum Creatinine (CTN) = < 1.5 mg/dl; Gamma Glutamyl transpeptidase (GGT) = 10 - 50 u./L; Aspartate transferase (AST) = 4 - 36 u./L; Alanine transefrase (ALT) = 0 - 40 u./L4; Alkaline phosphatase (ALKP) = 40 - 120 u./L; Bilirubin (Bil.) = 3.0 - 17 umol/L; Heamoglobin (Hb); 11.5- 16.5; melondaldehyde (MDA) ; Allantoin (ATN) Erythrocyte sedimentation rate (ESR) = 0 - 10.

Analysis of samples: Allantoin and acetic acid resolved at 3.91 and 6.08 minutes, respectively (Fig. 8.1). Regression coefficient (r) of the standard curves was 0.996 for plasma and 0.992 for urine while intrabatch coefficient of variation (CV) at 5 ug/ml (n = 8) was 9.3 ± 2.43% for plasma and 11.8 ± 2.2% for urine (n = 5).

Figures 8.2 and 8.3 illustrate, respectively, the plasma time profiles and age-distribution of allantoin over the three study days while table 8.3 shows the plasma concentrations of allantoin, uric acid, TBARS and creatinine at six hours after dosing (Appendices C, D and E).

In both groups, there was a rise in plasma allantoin after introduction of antituberculosis drugs and, the concentrations were higher in elderly than young patients on the three occasions. By day 90 allantoin concentrations were below the pre-treatment values for the young while that of elderly did not recover. Nevertheless, there was a general reduction in allantoin concentrations by day 90 in both groups.

Although uric acid is important for the formation of allantoin, variations in allantoin concentrations were not related to uric acid (Table 8.3). For instance, before treatment uric acid concentrations were different in the two age groups while that of allantoin was similar. Also, on the study days uric acid levels for the two groups were comparable while allantoin levels were different. Most probably, the concentrations of uric acid are above values that would influence allantoin levels.

In young patients, although uric acid concentration on day one was significantly higher than the pre-treatment value, subsequent

Figure 8.1 The representative chromatograms of standard solution (A), drug free plasma (B), and a plasma extract from patient (C).

1 = Allantoin (3.91 min.) 2 = Acetic acid (internal standard, 6.08 min.) X = plasma peak (5.39 min.)

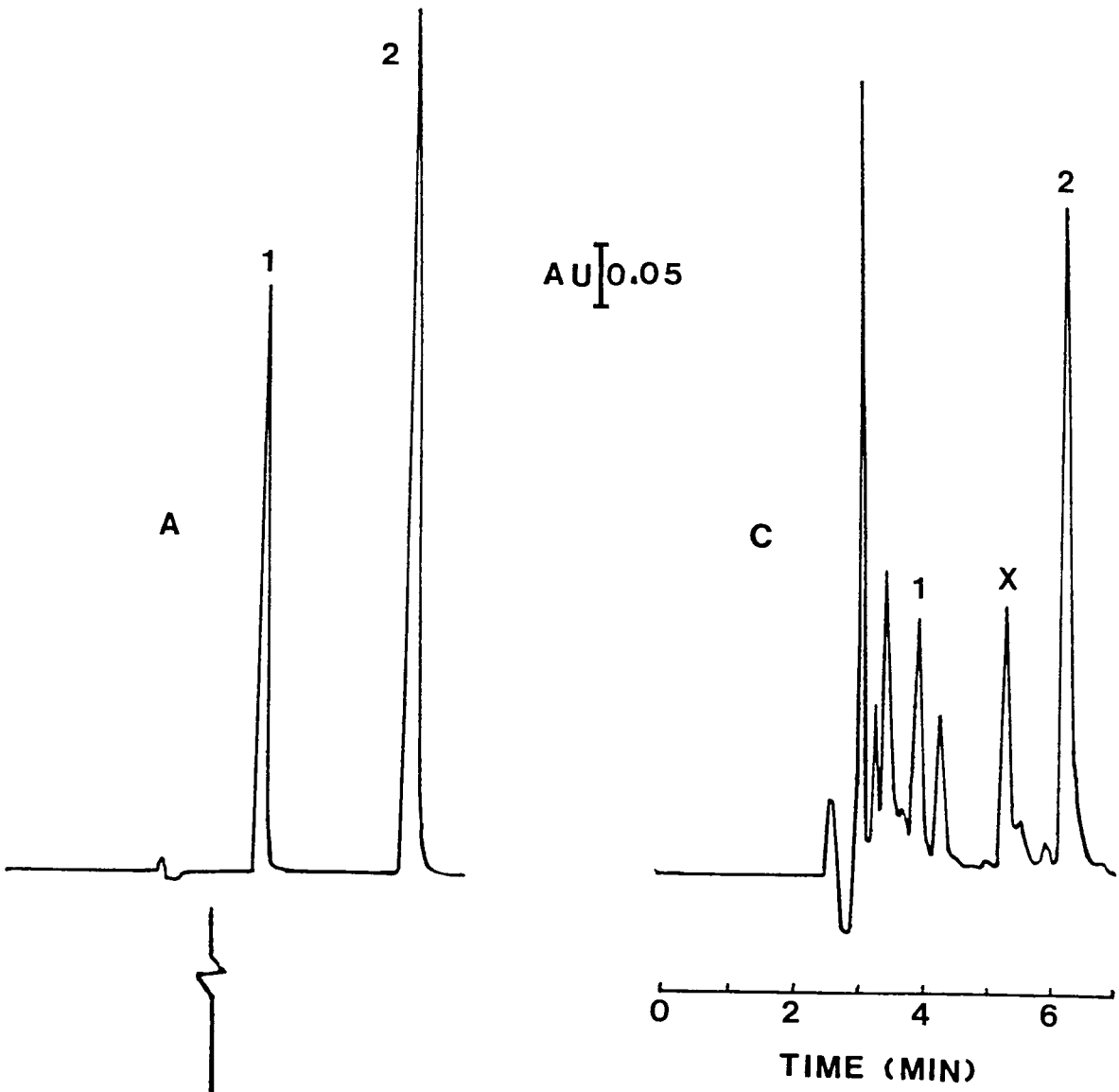


Fig 8.1

Figure 8.2. Plasma concentrations of allantoin in young and elderly TB patients during TB treatment on the three days.

KEY

Elderly patients.

Young patients

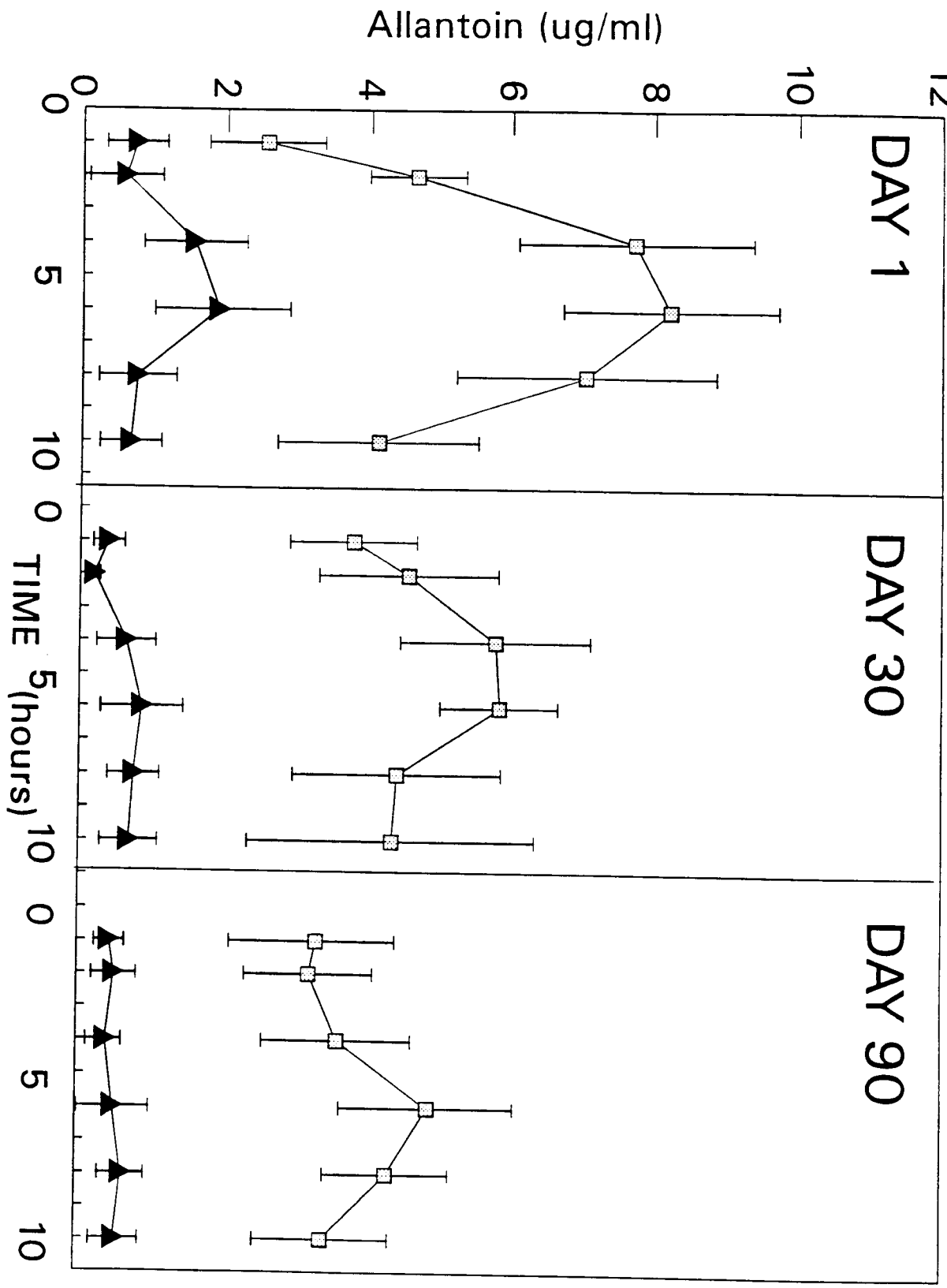
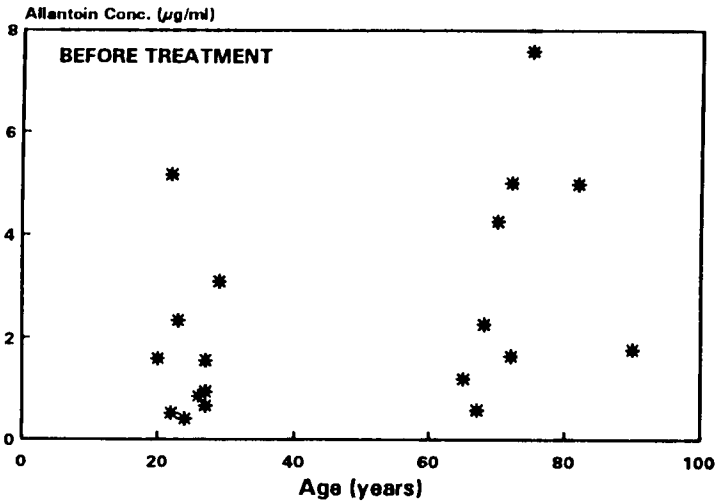
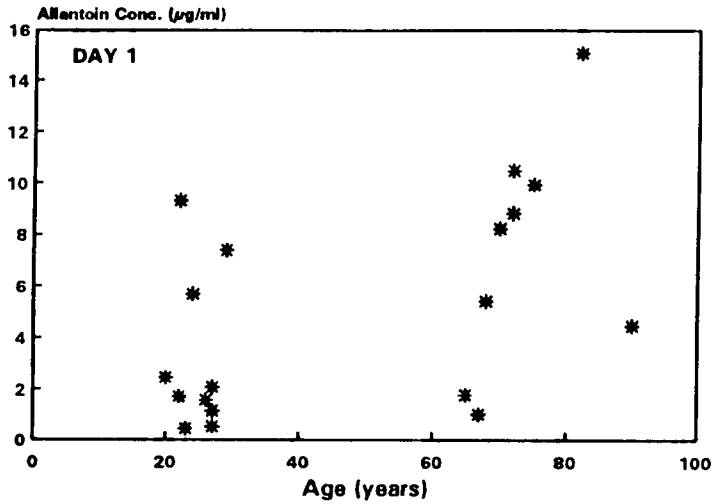
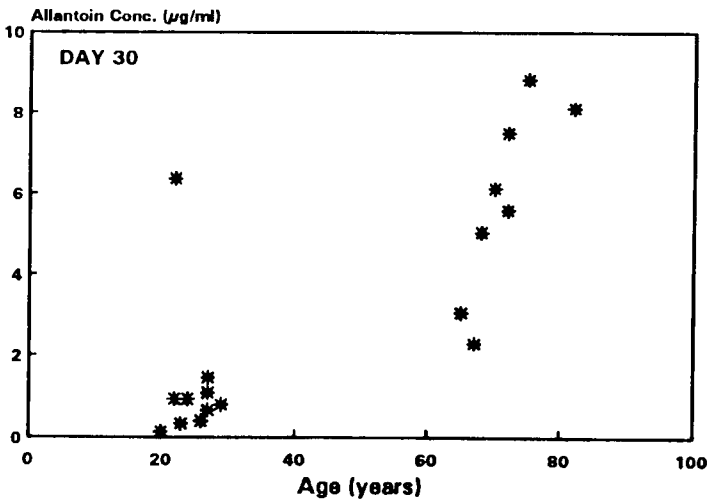


Fig. 8.2

Fig. 8.3 Plasma allantoin versus age before and during TB treatment.







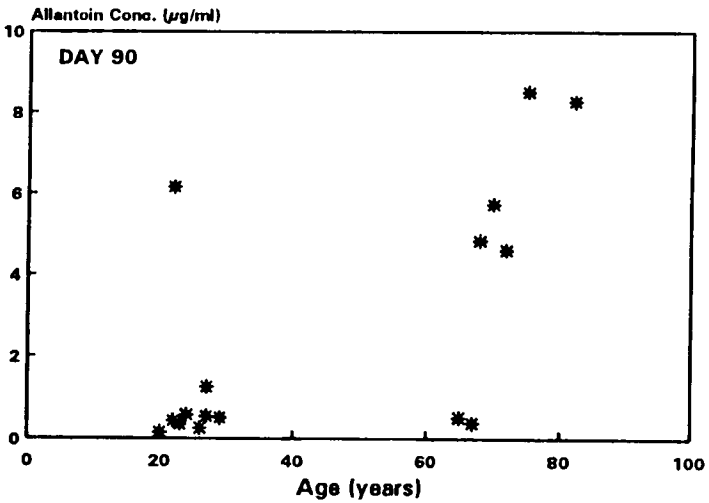


Table 8.3 Plasma concentrations (median ± s.e.) at 6 hrs for ATN, UA, TBARS and Cr during TB therapy.

PATIENT GROUP	YOUNG (20 -30 YEARS)			ELDERLY (OVER 64 YEARS		
TIME (DAYS)	1	30	90	1	30	90
Allantoin* (μg/ml)	1.89 ± 0.98	0.87 ± 0.57	0.52 ± 0.57	8.22 ± 1.5	5.85 ± 0.82	4.84 ± 1.24
Uric acid (mg/dl)	9.16 ± 0.52	7.59 ± 0.68	7.5 ± 0.6	10.78 ± 1.00	8.54 ± 0.75	9.45 ± 0.87
TBARS (nmol/ml)	9.32 1.43	8.9 1.73	7.38 1.87	9.88 1.98	8.65 0.54	8.12 1.13
Creatinine (mg/dl)	0.58 ± 0.06	0.59 ± 0.04	0.61 ± 0.03	0.80 ± 0.04	0.77 ± 0.03	0.76 ± 0.03

Note: * significant difference in the two groups ($p < 0.05$)

concentrations on days 30 and 90 were not. The elderly group did not exhibit this difference. Therefore, the rise in uric acid during antituberculosis therapy appears not to be significant.

The concentration of TBARS were similar in young and elderly patients on the three occasions and, as for other reports (Jack et al, 1994), there was no difference in the concentration of the three occasions. Creatinine concentrations remained in the normal range over the study period.

Table 8.4 illustrates the renal characteristics of allantoin, uric acid and creatinine in the two groups (Appendices F anf G). Renal function was better in the young than in elderly patients as indicated by higher urinary output, creatinine clearance and total creatinine excreted in the first 8 hours. Also, renal clearances for allantoin and uric acid were higher in young than elderly patients. Because of the high renal clearance of allantoin by young patients, total amount of allantoin excreted in the first 8 hours was similar to that excreted by the elderly.

Table 8.5 illustrates the relationship between allantoin concentrations and clearances of elderly and young patients while table 8.6 shows the formation amd excretion rates of allantoin on the three days. The clearance and concentration ratios were similar. The amount of allantoin formed in the two groups on each occasion was similar.

Table 8.4 Renal elimination of ATN, UA and creatinine (median ± s.e.) during TB therapy.

PATIENT GROUP	YOUNG (n = 10)			ELDERLY (n = 9)		
TIME (DAYS)	1	30	90	1	30	90
Total allantoin (μg/Kg) excreted in 8 hr	90.22 ± 12.25	83.22 ± 13.0	68.47 ± 13.34	44.7 ± 13	91.56 ± 9.87	82.12 ± 6.27
Allantoin renal* clearance (ml/min)	5.96 ± 1.7	11.8 ± 3.6	9.3 ± 5.7	1.1 ± 0.92	1.1 ± 0.35	2.4 ± 0.2
Total uric acid (mg) excreted in 8 hr	40.7 ± 28.2	58.0 ± 7.6	31.7 ± 7.2	21.9 ± 11.9	39.4 ± 7.9	33.9 ± 4.5
Uric acid renal* clearance (ml/min)	13.4 ± 3.4	8.6 ± 1.7	12.1 ± 1.5	0.68 ± 0.3	0.93 ± 0.5	0.57 ± 0.1
Total creatinine (mg)* excreted in 8 hr	455.2 ± 67.6	297.8 ± 35.6	218.3 ± 33.5	93.1 ± 12.5	144 ± 20.8	182.4 ± 28.9
Creatinine renal* clearance (ml/min)	147.8 ± 14.6	119.7 ± 10	138.4 ± 9.8	32.9 ± 12.8	43.4 ± 10.3	55.1 ± 15.6
Total urine output* in 8 hr (ml)	595.5 ± 93.5	610 ± 92.3	475 ± 49	150 ± 19.9	182.5 ± 30.4	290 ± 21.5

Note: * significant difference in the two groups ($p < 0.05$)

Table 8.5 Ratios of allantoin plasma concentration (Cp) and clearance (CL) in young (Y) and elderly (E) patients.

Day	1	30	90
Cp ratio (E/Y)	4.32 ± 3.7	6.72 ± 5.4	9.31 ± 4.8
CL ratio (Y/E)	5.42 ± 2.8	10.73 ± 3.6	3.88 ± 5.3

Table 8.6 A comparison of allantoin formation and excretion rates in young and elderly patients.

	YOUNG			ELDERLY		
Day	1	30	90	1	30	90
Excretion rate (μg/min)	7.7 ± 6.6	12.5 ± 9.9	7.4 ±4.0	6.9 ±4.8	9.3 ±6.0	8.6 ± 3.5
Formation rate (μg/min)	10.52 ± 6.4	10.51 ±7.6	9.14 6.8	4.28 ±5.8	9.29 ±6.5	8.73 ±5.8

8.3. DISCUSSION

It has been shown that a combination of isoniazid, rifampicin and pyrazinamide leads to increased production of allantoin in tuberculosis patients. As total allantoin formed in the first 8 hours was similar in both groups, the higher concentrations of allantoin in elderly patients are due to decreased clearance of allantoin in this group. This was paralleled by a low creatine clearance in the same people. However, according to current literature, the increase in allantoin concentration when antituberculosis drugs were administered in both young and elderly patients, is suggestive that ROS were produced. Hence, the subsequent fall in allantoin concentration during the treatment period would be due to improvement antioxidant defence mechanisms with clinical improvement.

Allantoin concentrations should be more indicative of the extent of free radical activity than the TBARS test because uric acid being a small and lipid soluble molecule has access to free radical production sites where it interacts directly with the free radicals. The TBARS test although an indicator of free radical activity is ineffective at estimating extent of free radical activity because it excludes many non-lipid structures such as proteins and uric acid that are oxidised by free radicals. Ascorbic acid depletion was not used for comparison because it is unreliable for detecting free radical activity. Ascorbic acid may exhibit anti- or pro- oxidant activity depending on the availabilty of transitional metal ions and in the aqueous media only (Halliwell and Gutteridge, 1992).

As antituberculosis therapy in these patients was uneventful and there is limited knowledge regarding the critical allantoin plasma concentrations which may have an effect, the results of this study indicate that; (i) oxidative stress occurs during antituberculosis chemotherapy with isoniazid, rifampicin and pyrazinamide regimen, and (ii) there degree of oxidative stress during antituberculosis treatment is similar in young and elderly patients. It suggests that antituberculosis drugs induce production of reactive oxygen species thereby underlining the importance of nutritive supplements like multivitamins used during antituberculosis therapy. Vitamins such as C, E and A are antioxidants which help the body to quench free radicals. Further studies are needed to address the implications of these observations, in particular, to examine the role of allantoin in predicting drug toxicity because changes in allantoin concentrations start at a time when liver and renal functions are relatively unchanged (i.e. before organ damage occurs).

8.3.1 Allantoin as a toxin (hypothesis)

The consequences of high plasma concentrations of allantoin cannot be realised at the moment, but recent reports on the use of allantoin in clinics have shown that allantoin has several effects on cell membranes. Allantoin is a component of cosmetics and several ointments used in treatment of many dermatological disorders including psoriasis (De Bumnan et al, 1987; Chadzynska, 1987). It is claimed to stimulate new skin growth and improve skin smoothness. This claim is supported by observations that when heparin preparations, with and without allantoin, were placed on the ear lobes of rabbits on opposite sides, those preparations containing allantoin showed a

several fold increase in thrombotic activity (Tauschel et al, 1984), indicating that allantoin improves permeability of cell membranes to drugs. In another study, movement of allantoin across cell membranes was associated with stimulation of Beta-receptors but not alpha-receptors (Sugino et al, 1984). In presence of calcium ions, allantoin is believed to induce Reye's syndrome (Marterns et al, 1986). Given these observations, it is possible that the high concentrations of allantoin in elderly patients may play a role in increasing susceptibility to drug toxicity by altering drug pharmacokinetics and or pharmacodynamics.

CHAPTER NINE

CONCLUSIONS

1. A procedure for assay of rifampicin, pyrazinamide, and isoniazid with four of its hydrazide metabolites (acetylisoniazid, monoacetylhydrazine, diacetylhydrazine and hydrazine) in human plasma was developed. By this method plasma profiles of isoniazid, the four metabolites and co-administered drugs (rifampicin and pyrazinamide) can be assessed from one sample. This approach makes pharmacological evaluations of patients easier.

2. Isoniazid and its metabolites; acetylisoniazid, monoacetylhydrazine and hydrazine can generate oxygen free radicals in the presence of oxyhaemoglobin. Although a pH of 9.2 does not occur in physiological situations, the physiological au milieu is a complex mixture of substances that can catalyse (enzymatically and non-enzymatically) reactive oxygen species formation at normal pH (7.2 - 7.4). Also, formation of other unidentified free radicals was noted.

3. Because hydrazine induced significant lipid peroxidation in rat liver slices, it can be concluded that, depending on the amount of hydrazine formed during isoniazid metabolism, reactive oxygen species may contribute to isoniazid induced hepatotoxicity. It is well established that hydazine is a metabolite of hydrazide compounds which includes isoniazid, acetylisoniazid,

monoacetylhydrazine and diacetylhydrazine. Hydrazine is a potent toxin with no safe levels in man (Back and Thomas, 1960) and was incriminated for the death of an elderly patient (Woo et al, 1992).

4. In this *in vitro* system, the pathognomonic feature of monoacetylhydrazine hepatotoxicity is vacuolation while that of hydrazine is cytoplasmic disintegration. This information is important not only to investigate toxicity but also during research for effective antidotes against either form of toxicity.

5. The pharmacokinetics of isoniazid metabolites; acetylisoniazid, monoacetylhydrazine, diacetylhydrazine and hydrazine are similar in young and elderly patients at first dose and steady state.

6. Administration of antituberculosis drugs increased plasma concentrations of allantoin in both young and eldelry patients. Therefore, antituberculosis drugs induced oxidative stress and this was of a similar degree in young and elderly patients.

7. The plasma concentrations of allantoin were higher in elderly than young patients due to decreased renal clearance of allantoin in the elderly group and a similar amount of allantoin was formed in the elderly as in the young patients.

8. The TBARS test, may not be a good marker for estimating oxidative stress during antituberculosis chemotherapy with isoniazid because the five hydrazide compounds may interfered with the test, making it less accurate.

9) During isoniazid metabolism, toxic metabolites (acetylisoniazid, monoacetylhydrazine, diacetylhydrazine and hydrazine) are produced. Of these, hydrazine metabolite induces formation of reactive oxygen species. This may be dangerous if anti-oxidant mechanisms of the body are compromised.

In general, depending on the amount of hydrazine formed during isoniazid metabolism, oxygen free radicals may contribute to isoniazid induced hepatotoxicity.

FUTURE STUDIES

1. This study opens a thought on the use of allantoin to predict isoniazid toxicity. Since oxidative stress precedes tissue damage, it would be plausible to monitor allantoin levels in tuberculosis patients and determine at what allantoin concentration (i.e. extent of oxidtative stress) does organ damage due to antituberculosis toxicity manifest. Then, it would be wise to take extra-precaution with antituberculosis chemotherapy in patients with high plasma level of allantoin.

2. There is a need to formulate appropriate remedial measures against pro-oxidant mechanisms during antituberculosis therapy to prevent toxicity and improve quality of life in elderly patients. For instance a search for effective antioxidants that can be administered together with antituberculosis drugs.

3. The use of tissue slices has not been well explored by South African researchers. This mode of experimentation can limit the use of animals, and may reduce extent of experimentation of new drugs in man. There is a need to open a tissue-slice bank in South African.

4. The effect of allantoin on the permiability of animal cells to antituberculosis drugs is not known. There will be a need to differentiate between the systemic effects of allantoin and drug side effects.

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APPENDICES

Appendix

File INHLIVER 2/ 4/93 Page 1

row	twocontrol	fourcontro	sixcontrol	eightcontr	tencontrol	twodrug	fourdrug
1	1.04	1.22	1.05	1.26	1.28	1.19	1.27
2	1.18	1.23	1.19	1.13	0.95	0.26	1.06
3	0.54	0.78	0.99	1.03	1.12	0.34	0.94

File INHLIVER 2/ 4/93 Page 1

row	sixdrug	eightdrug	tendrug	twosod	foursod	sixsod	eightsod	tensod	twolip
1	1.23	1.46	1.55	1.18	1.24	1.15	1.53	1.41	1.03
2	1.00	0.92	1.07	1.37	1.24	1.21	1.11	1.18	1.18
3	1.14	1.20	1.23	0.68	0.99	1.03	1.14	1.16	0.74

File INHLIVER 2/ 4/93 Page 1

row	fourlip	sixlip	eightlip	tenlip	twoddc	fourddc	sixddc	eightddc	tenddc
1	1.02	1.14	1.21	1.20	1.07	1.15	1.30	1.11	1.24
2	1.27	1.01	1.14	1.14	1.16	1.14	0.79	1.13	1.27
3	0.87	0.99	1.03	1.12	0.64	0.89	0.96	1.07	1.14

File INHLIVER 2/ 4/93 Page 1

row	twoddcdrug	fourddcdrug	sixddcdrug	eightddcdrg	tenddcdrug
1	1.24	1.07	1.83	1.10	1.24
2	1.18	1.27	1.02	1.16	1.13
3	0.66	0.78	0.99	1.11	1.16

File AINNALVER 2/ 4/93 Page 1

row	twocontrol	fourcontro	sixcontrol	eightcontr	tencontrol	twodrug	fourdrug
1	0.23	0.19	0.17	0.45	0.37	0.14	0.18
2	0.30	0.29	0.67	0.56	1.17	0.29	0.27
3	0.21	0.23	0.54	0.64	0.99	0.19	0.27

File AINNALVER 2/ 4/93 Page 1

row	sixdrug	eightdrug	tendrug	twosod	foursod	sixsod	eightsod	tensod	twolip
1	0.18	0.44	0.36	0.18	0.11	0.17	0.48	0.29	0.22
2	0.95	0.72	1.14	0.40	0.24	1.29	0.47	1.51	0.34
3	0.68	0.94	1.15	0.37	0.34	0.56	0.47	1.12	0.32

File AINNALVER 2/ 4/93 Page 1

row	fourlip	sixlip	eightlip	tenlip	twoddc	fourddc	sixddc	eightddc	tenddc
1	0.15	0.14	0.46	0.42	0.38	0.34	0.43	0.38	0.45
2	0.24	1.35	0.57	1.65	0.37	0.33	1.41	1.34	1.28
3	0.25	0.65	0.84	0.99	0.37	0.41	0.68	0.91	1.01

File AINNALVER 2/ 4/93 Page 1

row	twoddrug	fourddrug	sixddrug	eightddrg	tenddrug
1	0.33	0.11	0.20	0.36	0.39
2	0.27	0.30	1.13	1.41	1.42
3	0.31	0.30	0.81	0.92	1.02

File MHYDLVER 2/ 4/93 CONTROL = Well No 1

row	twocontrol	fourcontro	sixcontrol	eightcontr	tencontrol	[illegible]
1	0.78	0.82	0.88	0.96	1.01	[illegible]
2	0.40	1.16	0.92	1.29	1.15	[illegible]
3	0.37	1.02	1.12	1.33	1.04	[illegible]

File MHYDLVER 2/ 4/93 DRUG = well no 2 Page 1

row	tendrug	eightdrug	tendrug	twodrug	fourdrug	sixdrug	eightdrug	tendrug	twodrug
1	1.38	1.45	1.35	0.88	1.39	1.32	1.44	1.40	0.77
2	0.95	0.74	1.57	1.32	1.10	0.90	0.83	1.15	0.96
3	1.23	1.09	1.34	0.94	1.06	1.11	0.99	1.21	0.92

File MHYDLVER 2/ 4/93 Page 1

row	fourlip	sixlip	eightlip	tenlip	twoddc	fourddc	sixddc	eightddc	tenddc
1	0.94	1.59	1.38	1.43	1.34	1.12	1.49	1.44	1.57
2	1.07	0.99	0.97	1.53	1.03	1.06	1.00	0.91	1.28
3	0.99	1.00	1.06	1.21	0.92	0.99	1.00	1.06	1.21

File MHYDLVER 2/ 4/93 Page 1

row	twoddrug	fourddrug	sixddrug	eightddrg	tenddrug
1	1.13	1.49	1.33	1.49	1.62
2	1.01	0.87	0.78	0.94	1.34
3	0.99	0.99	1.01	1.12	1.12

File HYDLIVER 2/ 4/95 Page -

row	twocontrol	fourcontro	sixcontrol	eightcontr	tencontrol	twodrug	fourdrug
1	1.04	1.07	0.99	1.04	1.09	1.14	0.94
2	0.80	0.86	0.94	0.82	1.17	0.99	1.03
3	0.74	0.88	0.99	1.01	1.12	0.92	0.92

File HYDLIVER 2/ 4/95 Page -

row	sixdrug	eightdrug	tendrug	twosod	foursod	sixsod	eightsod	tensod	twolip
1	1.12	1.20	1.37	1.07	0.94	1.23	1.15	1.20	1.05
2	0.83	1.02	1.36	0.96	1.04	0.80	1.19	1.51	1.03
3	1.06	1.22	1.34	0.84	0.98	0.91	1.07	1.18	0.88

File HYDLIVER 2/ 4/95 Page 1

row	fourlip	sixlip	eightlip	tenlip	twoddc	fourddc	sixddc	eightddc	tenddc
1	0.95	1.23	0.94	1.09	1.05	1.04	1.05	1.24	1.19
2	0.84	0.74	0.73	1.36	0.95	0.62	1.12	1.01	1.65
3	0.84	0.94	0.99	1.14	0.62	0.79	0.99	1.02	1.31

File HYDLIVER 2/ 4/95 Page 1

row	twoddrug	fourddrug	sixddrug	eightddrg	tenddrug
1	0.99	1.01	0.96	1.02	1.23
2	1.08	0.93	0.77	0.84	1.44
3	0.74	0.84	0.99	1.01	1.14

Appendix A4

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ADRENOCHROME FORMATION (ABS.UNITS) AT pH 9.2

Column ID	A	B	C	D
Column Label	a1	b1	c1	d1
Mean	0.1462	0.0652	0.0486	0.0084
Sample Size	5	5	5	5
SD	0.001483	0.002775	0.002881	0.001140
SEM	0.0006633	0.001241	0.001288	0.0005099
Median	0.1460	0.06500	0.04800	0.008000
Lower 95% CI	0.1444	0.06176	0.04502	0.006985
Upper 95% CI	0.1480	0.06864	0.05218	0.009815
Minimum	0.1440	0.06200	0.04500	0.007000
Maximum	0.1480	0.06800	0.05200	0.01000

Column ID	E	F	G	H
Column Label	e1	f1	g1	
Mean	-0.0079	-0.00124	-0.11198	
Sample Size	5	5	5	0
SD	0.002012	0.0002074	0.001582	
SEM	0.0009000	9.274000E-05	0.0007074	
Median	-0.008000	-0.001200	-0.1119	
Lower 95% CI	-0.01040	-0.001497	-0.1139	
Upper 95% CI	-0.005402	-0.0009826	-0.1100	
Minimum	-0.01000	-0.001500	-0.1140	
Maximum	-0.005000	-0.001000	-0.1100	

Column ID	I	J	K	L
Column Label	a2	b2	c2	d2
Mean	0.0376	0.00492	0.0064	0.00268
Sample Size	5	5	5	5
SD	0.002074	0.003300	0.001319	0.001190
SEM	0.0009274	0.001476	0.0005899	0.0005324
Median	0.03800	0.005000	0.005800	0.002900
Lower 95% CI	0.03503	0.0008228	0.004762	0.001202
Upper 95% CI	0.04017	0.009017	0.008038	0.004158
Minimum	0.03500	0.001400	0.005000	0.001400
Maximum	0.04000	0.009200	0.008000	0.004000

Column ID	M	N	O	P
Column Label	a3	b3	c3	d3
Mean	0.158	0.1578	0.023	0.00224
Sample Size	5	5	5	5
SD	0.003162	0.004604	0.002121	0.0005727
SEM	0.001414	0.002059	0.0009487	0.0002561
Median	0.1580	0.1580	0.02300	0.002200
Lower 95% CI	0.1541	0.1521	0.02037	0.001529
Upper 95% CI	0.1619	0.1635	0.02563	0.002951
Minimum	0.1540	0.1520	0.02000	0.001600
Maximum	0.1620	0.1640	0.02500	0.003000

Column ID	Q	R	S
Column Label	a4	b4	c4
Mean	0.0954	-0.01092	0.0366
Sample Size	5	5	5
SD	0.003912	0.002448	0.003050
SEM	0.001749	0.001095	0.001364
Median	0.09500	-0.01000	0.03700
Lower 95% CI	0.09054	-0.01396	0.03281
Upper 95% CI	0.1003	-0.007881	0.04039
Minimum	0.09200	-0.01400	0.03300
Maximum	0.1020	-0.008400	0.04000

File INH1ELD 04/03/95

row	Hlife	Co	AUC	Clp	Vd	MRT	Ke
1	2.68	8.41	24.30	16.46	70.07	4.26	
2	3.69	8.20	36.01	8.89	46.15	5.20	
3	4.00	11.07	51.96	6.16	37.04	6.01	
4	3.84	9.14	40.30	7.94	45.90	5.78	
5	4.28	8.16	35.36	9.05	65.73	7.26	
6	4.98	5.72	36.66	8.73	61.69	7.07	
7	2.76	9.65	21.34	1.50	78.70	5.23	
8	3.67	8.89	39.10	8.18	45.81	5.60	
9	2.95	6.52	21.22	15.08	65.24	4.32	

File INH1YG 04/03/95

row	hlife	Co	AUC	Clp	Vd	MRT
1	2.35	9.98	25.83	15.48	53.00	3.42
2	4.08	8.08	40.25	9.94	58.44	5.88
3	3.28	8.22	29.74	13.45	66.43	4.94
4	2.29	23.07	40.53	28.06	62.11	4.86
5	2.54	18.50	39.15	10.22	45.90	4.49
6	3.71	17.81	58.67	6.82	49.11	7.20
7	5.57	1.60	11.04	36.23	79.94	8.14
8	2.48	8.29	22.38	17.88	65.93	3.69
9	2.80	6.86	19.72	20.28	90.05	4.64

File INH30ELD 04/03/95

row	Hlife	Co	AUC	CLp	Vd	MRT	Ke
1	1.87	7.22	12.35	32.38	96.32	2.97	
2	2.82	7.30	24.33	13.15	53.59	4.07	
3	2.06	11.15	16.45	19.45	75.22	3.87	
4	1.96	8.99	17.86	18.75	56.89	3.03	
5	2.55	10.26	26.23	12.20	50.18	4.11	

File INH30YG 04/03/95

row	Hlife	Co	AUC	Clp	Vd	MRT
1	3.24	6.90	24.52	16.13	81.20	4.98
2	1.73	36.44	33.67	11.88	45.21	3.81
3	2.61	8.35	22.08	18.12	74.43	4.11
4	3.73	4.56	19.76	20.25	110.15	5.44
5	5.07	5.02	31.12	12.85	94.50	7.35
6	3.35	5.27	21.69	18.44	89.47	4.85
7	4.59	4.64	24.70	16.19	114.80	7.09
8	2.67	7.68	22.42	17.84	75.03	4.20
9	3.92	3.45	53.91	24.98	140.66	5.63

File INH30ELD 04/03/95

row	Hlife	Co	AUC	CLp	Vd	MRT	Ke
1	1.87	7.22	12.35	32.38	96.32	2.97	
2	2.82	7.30	24.33	13.15	53.59	4.07	
3	2.06	11.15	16.45	19.45	75.22	3.87	
4	1.96	8.99	17.06	18.75	56.89	3.03	
5	2.55	10.26	26.23	12.20	50.18	4.11	

File RIF1YG 04/03/95

row	Hlife	Co	AUC	CLp	Vd	MRT	Ke
1	3.03	8.20	29.07	20.64	89.57	4.29	
2	1.93	35.46	34.76	17.26	81.02	3.69	
3	8.32	1.80	20.87	28.75	333.46	11.60	
4	2.80	11.31	66.82	35.67	265.66	7.45	
5	3.23	5.07	45.46	40.80	234.06	5.74	
6	3.01	9.40	25.09	23.91	128.36	5.37	
7	5.17	3.20	18.54	32.36	268.04	8.28	
8	4.69	6.94	38.77	15.48	112.69	7.28	
9	3.96	6.95	31.65	18.96	113.21	5.97	

File RIF1ELD 04/03/95

row	Hlife	Co	AUC	CLp	Vd	MRT	Ke
1	6.52	7.26	55.93	8.58	87.82	10.23	
2	4.58	11.46	61.96	7.75	53.22	6.87	
3	3.03	11.78	29.43	16.31	92.96	5.70	
4	4.18	9.83	41.12	11.70	84.35	7.21	
5	2.89	8.12	25.08	19.14	85.88	4.49	
6	5.19	4.84	31.54	15.22	116.50	7.65	
7	4.09	8.27	33.08	14.51	105.62	7.28	
8	1.70	21.45	18.51	25.92	102.83	3.97	

File RIF3OYG 04/03/95

row	Hlife	Co	AUC	CLp	Vd	MRT	Ke
1	4.03	8.67	34.72	17.28	119.84	6.93	
2	2.44	11.19	27.00	22.22	93.25	4.20	
3	5.63	3.66	25.48	23.54	197.57	8.39	
4	1.67	25.09	18.35	32.70	141.99	4.34	
5	12.02	3.95	64.12	9.36	163.55	17.48	
6	5.18	3.87	25.30	23.72	176.40	7.44	
7	2.45	5.81	14.47	41.45	170.24	4.11	
8	2.89	5.80	15.14	39.63	195.64	4.94	
9	2.69	13.22	29.37	20.43	99.77	4.88	

File RIF3OELD 04/03/95

row	Hlife	Co	AUC	CLp	Vd	MRT	Ke
1	3.52	7.26	55.93	10.73	109.77	10.23	
2	2.51	6.49	16.94	28.34	114.30	4.03	
3	2.98	10.20	33.42	14.36	63.63	4.43	
4	5.06	5.68	36.79	13.05	94.01	7.21	
5	1.48	11.29	14.33	33.49	74.11	2.21	

File PZA1YG 04/03/95

row	Hlife	Co	AUC	CLp	Vd	MRT	K
1	10.89	28.54	422.82	2.96	46.26	15.7	
2	12.70	45.31	783.51	1.60	29.29	18.4	
3	5.54	74.23	519.30	2.41	19.44	8.1	
4	7.47	49.70	481.40	2.60	28.35	10.9	
5	3.22	167.23	504.47	2.48	13.86	5.6	
6	13.10	51.43	847.42	1.47	30.09	20.4	
7	6.90	59.85	493.43	2.53	27.45	10.8	
8	11.94	55.56	876.87	1.43	25.23	17.7	

File PZA1ELD 04/03/95

row	Hlife	Co	AUC	CLp	Vd	MRT	Ke
1	8.52	68.07	757.19	1.65	20.68	12.53	
2	6.92	76.12	694.53	1.80	17.74	9.86	
3	10.18	97.74	931.82	1.07	15.73	14.66	
4	11.25	65.32	963.36	1.04	17.73	16.77	
5	4.05	120.43	486.70	2.05	14.32	6.97	
6	4.56	84.20	350.54	2.85	23.81	8.35	
7	11.68	107.13	973.60	0.58	9.78	16.76	
8	3.21	82.36	262.27	3.81	19.94	5.23	
9	5.61	44.72	329.03	3.04	24.15	7.95	

File PZA30YG 04/03/95

row	Hlife	Co	AUC	CLp	Vd	MRT	Ke
1	5.77	131.08	938.77	1.33	11.60	8.53	
2	6.91	96.32	800.18	1.56	16.79	10.75	
3	15.18	32.42	801.10	0.78	39.93	15.15	
4	8.25	47.99	516.99	2.42	29.17	12.07	
5	7.47	37.27	354.37	3.53	38.20	10.83	
6	8.86	41.12	455.72	2.74	37.20	13.56	
7	4.76	89.82	392.98	3.18	27.87	8.76	
8	7.94	40.61	415.63	3.01	35.29	11.73	
9	7.69	78.88	744.29	1.68	19.85	11.82	
10	5.90	70.25	457.96	2.73	17.00	9.88	

File PZA30ELD 04/03/95

row	Hlife	Co	AUC	CLp	Vd	MRT	Ke
1	9.70	65.43	836.86	1.49	21.27	14.24	
2	5.17	87.18	571.62	1.75	12.99	7.43	
3	8.45	77.86	853.38	1.17	14.66	12.51	
4	14.13	44.25	851.31	1.17	24.16	20.57	
5	12.24	61.17	1005.90	1.00	18.22	18.27	

Isoniazid and acetylisoniazid urinary excretion (mmol) and ratios.

PATIENT GROUP	10 YOUNG (25 ± 1 YRS) 4F & 6M			9 ELDERLY (72 ± 2 YRS) 5F & 4M		
NAME	INH	acINH	Ratio	INH	acINH	Ratio
1Y, E	0.855	0.179	0.21	0.433	0.191	0.44
2Y, E	0.601	0.372	0.62	0.691	0.126	0.18
3Y, E	0.976	0.55	0.65	0.197	0.315	1.6
4Y, E	0.791	0.217	0.27	0.847	0.44	0.52
5Y, E	0.99	0.408	0.41	0.834	0.393	0.47
6Y, E	1.18	0.163	0.14	0.903	0.424	0.47
7Y, E	0.796	0.146	0.18	0.702	0.259	0.37
8Y, E	1.08	0.311	0.29	0.684	0.347	0.51
9Y, E	0.415	0.286	0.69	0.643	0.335	0.52
10Y	0.573	0.29	0.51			

Key:

Y = Young, E = Elderly, INH = isoniazid, acINH = acetylisoniazid,

File AINH1YE 04/03/95 Page 1-

row	HlifeYG	CoYG	AUCyg	HlifeELD	CoELD	AUCeld
1	6.07	9.49	69.93	1.99	12.29	23.44
2	4.29	13.36	66.52	2.77	6.19	17.65
3	2.94	4.29	13.79	4.29	5.92	26.24
4	1.26	14.06	31.40	3.79	8.79	31.43
5	2.65	11.11	23.10	2.88	14.50	31.41
6	1.83	18.33	11.61	2.71	12.12	26.80
7	6.40	1.72	29.93	3.84	5.22	19.59
8	4.82	3.75	17.63	2.98	6.17	19.89
9	3.77	5.63	20.59	3.08	5.38	18.08
10	3.61	4.62	20.35			
11	1.67	39.16	18.89			

File AINH30YE 04/03/95 Page 1-

row	HlifeYG	CoYG	AUCyg	HlifeELD	CoELD	AUCeld
1	3.68	4.33	16.20	2.15	16.83	33.41
2	4.84	11.78	59.46	2.46	1.95	4.97
3	6.77	1.89	17.04	1.92	13.00	24.37
4	3.13	4.31	16.12	4.96	3.56	22.65
5	4.45	8.79	40.19	3.89	7.22	35.56
6	1.78	4.98	7.69			
7	8.07	3.43	85.82			
8	6.33	3.96	32.24			
9	2.32	7.90	17.16			

File MHYD1YE 04/03/95 Page 1-

row	HlifeYG	CoYG	AUCyg	HlifeELD	CoELD	AUCeld
1	5.95	8.52	35.19	3.30	5.90	21.27
2	5.90	9.52	34.52	3.97	9.21	42.82
3	17.84	5.58	136.18	13.85	6.49	121.31
4	2.97	26.93	48.96	3.05	16.49	40.64
5	2.38	26.25	45.90	3.12	10.64	27.85
6	4.25	15.05	55.14	20.45	4.16	117.85
7	4.38	11.25	50.18	10.11	4.67	62.79
8	3.86	5.51	21.49			
9	4.99	12.29	62.56			
10	3.55	8.53	32.71			

File MHYD30YE 04/03/95

row	HlifeYG	CoYG	AUCyg	HlifeELD	CoELD	AUCeld
1	7.69	12.70	118.14	1.70	3.76	3.71
2	3.56	15.82	52.40	2.93	17.14	44.00
3	4.60	11.66	64.09	3.70	17.33	45.99
4	6.82	7.92	69.09	6.12	5.48	98.34
5	5.33	3.52	23.74	5.52	3.37	23.01
6	3.50	11.63	42.09			
7	7.28	7.20	22.05			
8	4.09	3.87	16.52			
9	5.42	6.21	37.39			
10	3.78	5.40	20.21			

File HYD1YE 04/03/95

row	HlifeYG	CoYG	AUCyg	HlifeELD	CoELD	AUCeld
1	4.12	0.38	2.24	12.42	0.18	3.02
2	6.21	1.38	11.18	7.46	6.96	6.80
3	13.15	0.20	7.33	13.02	0.98	16.97
4	30.10	0.72	20.20	6.07	1.94	15.51
5	4.80	0.70	3.90	3.40	1.35	5.23
6	3.58	1.42	5.27	5.31	0.54	3.74
7	4.80	0.96	5.37	12.76	0.63	18.66
8				19.74	0.76	20.83

File HYD30YE 04/03/95

row	HlifeYG	CoYG	AUCyg	HlifeELD	Coeld	AUCeld
1	10.52	0.46	11.76	4.57	0.22	11.24
2	3.16	2.37	5.79	4.66	0.58	12.79
3	4.60	0.80	37.90	34.63	0.68	36.62
4	8.64	0.84	9.66	20.20	0.97	26.89
5	4.93	1.01	5.80	2.27	1.82	4.10
6	11.31	1.01	14.61			

File DHYD1YE 04/03/95

row	HlifeYG	CoyG	AUCyg	HlifeELD	CoELD	AUCeld
1	3.3	4.33	15.64	2.78	2.16	4.47
2	5.8	0.99	6.17	4.72	1.42	6.84
3	4.4	1.97	6.65	1.78	1.99	1.39
4	4.3	1.27	5.65	3.48	3.55	10.15
5	4.1	1.92	7.84	2.59	3.49	3.50
6	5.6	0.38	2.28	5.53	7.01	18.16
7	3.3	1.05	5.88	6.17	0.71	5.10
8	1.8	2.67	1.46	17.03	0.48	11.09
9				4.14	4.10	19.97
10				4.03	2.41	11.27

File DHYD30YE 04/03/95

row	HlifeYG	CoYG	AUCyg	HlifeELD	CoELD	AUCeld
1	1.69	5.95	8.75	2.78	2.16	4.47
2	2.90	5.23	10.75	27.71	0.48	17.84
3	3.89	1.04	4.87	5.91	0.12	0.92
4	2.99	0.89	2.35	37.40	1.14	59.47
5				5.33	2.57	13.57

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MEAN DATA FOR HYD CONTROL AND DRUG SLICES

Column ID	A	B	C	D
Column Label	2contrl	4contrl	6contrl	8contrl
Mean	0.86	0.93666667	0.97333333	0.95666667
Sample Size	3	3	3	3
SD	0.1587	0.1159	0.02887	0.1193
SEM	0.09165	0.06692	0.01667	0.06888
Median	0.8000	0.8800	0.9900	1.010
Lower 95% CI	0.4656	0.6487	0.9016	0.6603
Upper 95% CI	1.254	1.225	1.045	1.253
Minimum	0.7400	0.8600	0.9400	0.8200
Maximum	1.040	1.070	0.9900	1.040

Column ID	E	F	G	H
Column Label	10contrl	2drug	4drug	6drug
Mean	1.12666667	1.01666667	0.97	1.00333333
Sample Size	3	3	3	3
SD	0.04041	0.1124	0.07000	0.1531
SEM	0.02333	0.06489	0.04041	0.08838
Median	1.120	0.9900	0.9400	1.060
Lower 95% CI	1.026	0.7374	0.7961	0.6230
Upper 95% CI	1.227	1.296	1.144	1.384
Minimum	1.090	0.9200	0.9200	0.8300
Maximum	1.170	1.140	1.050	1.120

Column ID	I	J
Column Label	8drug	10drug
Mean	1.14666667	1.35666667
Sample Size	3	3
SD	0.1102	0.01528
SEM	0.06360	0.008819
Median	1.200	1.360
Lower 95% CI	0.8730	1.319
Upper 95% CI	1.420	1.395
Minimum	1.020	1.340
Maximum	1.220	1.370

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MEAN DATA FOR HYD CONTROL AND DRUG SLICES

One-way Analysis of Variance (ANOVA)

Source of variation	Degrees of freedom	Sum of squares	Mean square
Treatments (between columns)	9	0.5404	0.06005
Residuals (within columns)	20	0.2173	0.01087
Total	29	0.7577	

F = 5.526
The P value is 0.0007, considered extremely significant.
Variation among column means is significantly greater than expected by chance.

Bartlett's test for homogeneity of variances.

ANOVA assumes that all columns come from populations with equal SDs. The following calculations test that assumption.

Bartlett's test cannot be performed because a sample size is too small.

Student-Newman-Keuls Multiple Comparisons Test

Comparison	Mean Difference	q		P value
2contrl vs 4contrl	-0.07667	1.274	ns	P>0.05
2contrl vs 8contrl	-0.09667	1.606	ns	P>0.05
2contrl vs 4drug	-0.1100	1.828	ns	P>0.05
2contrl vs 6contrl	-0.1133	1.883	ns	P>0.05
2contrl vs 6drug	-0.1433	2.382	ns	P>0.05
2contrl vs 2drug	-0.1567	2.603	ns	P>0.05
2contrl vs 10contrl	-0.2667	4.431	ns	P>0.05
2contrl vs 8drug	-0.2867	4.763	ns	P>0.05
2contrl vs 10drug	-0.4967	8.252	***	P<0.001
4contrl vs 8contrl	-0.02000	0.3323	ns	P>0.05
4contrl vs 4drug	-0.03333	0.5538	ns	P>0.05
4contrl vs 6contrl	-0.03667	0.6092	ns	P>0.05
4contrl vs 6drug	-0.06667	1.108	ns	P>0.05
4contrl vs 2drug	-0.08000	1.329	ns	P>0.05
4contrl vs 10contrl	-0.1900	3.157	ns	P>0.05
4contrl vs 8drug	-0.2100	3.489	ns	P>0.05
4contrl vs 10drug	-0.4200	6.978	**	P<0.01
8contrl vs 4drug	-0.01333	0.2215	ns	P>0.05
8contrl vs 6contrl	-0.01667	0.2769	ns	P>0.05
8contrl vs 6drug	-0.04667	0.7754	ns	P>0.05

8contrl vs 2drug	-0.06000	0.9969	ns	P>0.05
8contrl vs 10contrl	-0.1700	2.825	ns	P>0.05
8contrl vs 8drug	-0.1900	3.157	ns	P>0.05
8contrl vs 10drug	-0.4000	6.646	**	P<0.01
4drug vs 6contrl	-0.003333	0.05538	ns	P>0.05
4drug vs 6drug	-0.03333	0.5538	ns	P>0.05
4drug vs 2drug	-0.04667	0.7754	ns	P>0.05
4drug vs 10contrl	-0.1567	2.603	ns	P>0.05
4drug vs 8drug	-0.1767	2.935	ns	P>0.05
4drug vs 10drug	-0.3867	6.425	**	P<0.01
6contrl vs 6drug	-0.03000	0.4985	ns	P>0.05
6contrl vs 2drug	-0.04333	0.7200	ns	P>0.05
6contrl vs 10contrl	-0.1533	2.548	ns	P>0.05
6contrl vs 8drug	-0.1733	2.880	ns	P>0.05
6contrl vs 10drug	-0.3833	6.369	**	P<0.01
6drug vs 2drug	-0.01333	0.2215	ns	P>0.05
6drug vs 10contrl	-0.1233	2.049	ns	P>0.05
6drug vs 8drug	-0.1433	2.382	ns	P>0.05
6drug vs 10drug	-0.3533	5.871	**	P<0.01
2drug vs 10contrl	-0.1100	1.828	ns	P>0.05
2drug vs 8drug	-0.1300	2.160	ns	P>0.05
2drug vs 10drug	-0.3400	5.649	**	P<0.01
10contrl vs 8drug	-0.02000	0.3323	ns	P>0.05
10contrl vs 10drug	-0.2300	3.822	*	P<0.05
8drug vs 10drug	-0.2100	3.489	*	P<0.05

Difference	Mean Difference	Lower 95% CI	Upper 95% CI
2contrl - 4contrl	-0.07667	-0.2542	0.1009
2contrl - 8contrl	-0.09667	-0.3120	0.1187
2contrl - 4drug	-0.1100	-0.3482	0.1282
2contrl - 6contrl	-0.1133	-0.3680	0.1414
2contrl - 6drug	-0.1433	-0.4109	0.1242
2contrl - 2drug	-0.1567	-0.4347	0.1214
2contrl - 10contrl	-0.2667	-0.5536	0.02029
2contrl - 8drug	-0.2867	-0.5813	0.007999
2contrl - 10drug	-0.4967	-0.7981	-0.1953
4contrl - 8contrl	-0.02000	-0.1975	0.1575
4contrl - 4drug	-0.03333	-0.2487	0.1820
4contrl - 6contrl	-0.03667	-0.2749	0.2015
4contrl - 6drug	-0.06667	-0.3214	0.1880
4contrl - 2drug	-0.08000	-0.3475	0.1875
4contrl - 10contrl	-0.1900	-0.4681	0.08805
4contrl - 8drug	-0.2100	-0.4970	0.07696
4contrl - 10drug	-0.4200	-0.7147	-0.1253
8contrl - 4drug	-0.01333	-0.1909	0.1642
8contrl - 6contrl	-0.01667	-0.2320	0.1987
8contrl - 6drug	-0.04667	-0.2849	0.1915
8contrl - 2drug	-0.06000	-0.3147	0.1947
8contrl - 10contrl	-0.1700	-0.4375	0.09752
8contrl - 8drug	-0.1900	-0.4681	0.08805
8contrl - 10drug	-0.4000	-0.6870	-0.1130
4drug - 6contrl	-0.003333	-0.1809	0.1742
4drug - 6drug	-0.03333	-0.2487	0.1820
4drug - 2drug	-0.04667	-0.2849	0.1915
4drug - 10contrl	-0.1567	-0.4114	0.09804
4drug - 8drug	-0.1767	-0.4442	0.09086
4drug - 10drug	-0.3867	-0.6647	-0.1086
6contrl - 6drug	-0.03000	-0.2075	0.1475

File ANDREW1 2/ 4/95 Plasma Allantoin

row	young	yage	ywt	yday0	yday1	yday30	yday90	elderly	eage	ewt	eday0	eday
1	1	27	53.	1.56	2.07	1.08	1.25	1	72	46.	5.00	6.80
2	2	26	70.	0.85	1.56	0.40	0.23	2	70	45.	-.25	8.22
3	3	24	54.	0.40	5.69	0.93	0.57	3	74	52.	7.57	9.92
4	4	27	55.	0.94	0.52	0.66	0.54	4	82	62.	4.97	13.07
5	5	22	50.	0.52	1.70	0.93	0.43	5	65	62.	1.19	1.74
6	6	27	60.	0.66	1.14	1.45	1.25	6	67	44.	0.88	1.60
7	7	20	61.	1.59	2.43	0.13	0.15	7	66	50.	1.25	3.41
8	8	23	50.	2.33	0.43	0.33	0.35	8	72	46.	1.64	10.48
9	9	27	58.	3.09	7.35	0.80	0.50	9	90	59.	1.77	4.48

File ANDREW1 2/ 4/95

row	eday30	eday90
1	5.58	4.61
2	6.12	5.74
3	8.82	8.51
4	8.10	8.27
5	3.07	0.51
6	2.30	0.38
7	5.03	4.84
8	7.48	
9		

File SERUM1 04/03/95 Page 1

row	seracry1	seracry30	seracry90	seracre1	seracre30	seracre90	serauay1
1	0.36	0.46	0.48	0.81			7.73
2	0.84	0.33	0.61		0.89	0.73	8.33
3	0.35	0.59	0.61	0.79	0.74		11.45
4	0.52	0.69	0.62	0.70	0.68	0.68	12.28
5	0.69	0.75	0.71	0.89	0.80	0.76	11.13
6	0.75	0.59	0.34	0.81	0.85	0.64	8.63
7	0.44	0.48	0.53	0.63			7.28
8	0.58	0.53	0.55		0.71	0.77	7.33
9	0.77	0.62	0.65				9.00
10	0.57	0.76	0.62				9.32

File SERUM1 04/03/95 Page 1

row	serauay30	serauay90	serauae1	serauae30	serauae90
1	6.67	8.27	11.02	9.60	7.60
2	6.92	9.38	11.80	12.47	9.43
3	4.80	4.67	7.98	6.38	5.62
4	8.43	7.50	11.68	7.20	12.62
5	12.57	7.85	6.92	6.91	10.38
6	9.30	7.00	10.78	10.83	9.67
7	6.52		16.80		
8	7.00	6.50	7.52	9.28	7.38
9	8.18	5.00	9.40	7.80	
10	9.65	10.07			

Appendix D

TBARS Check

File TBARS 04/03/93

row	YOUNG	YMDADAY1	YMDADAY30	YMDADAY90	ELDERLY	EMDADAY1	EMDADAY30	EMDADAY90
1	1	5.08	5.05	4.47	1	20.28	10.22	7.43
2	2	8.36	6.34	7.38	2	11.50	6.91	12.30
3	3	9.32	6.64	6.07	3	9.27	9.89	8.80
4	4	11.04	5.71	4.92	4	7.28	8.10	5.05
5	5	6.79	8.90	6.27	5	10.46	7.73	6.50
6	6	10.00	10.65	10.16	6	7.30	9.20	11.20
7	7	15.80	12.20	21.92				
8	8	18.74	15.30	14.22				
9	9	9.20	21.25	7.90				

Appendix E

File ANDREW2 2/ 4/95 Urine Allantoin

row	uatny	uatn1	uatny30	uatny90	uatne	uatne1	uatne30	uatne90	uatc	uatc
1	1	7982.4	1087.2	9120.0	1	2769.6	4602.3	4170.0	1	810
2	2	2644.0	4602.2	5558.4	2	3164.8	4771.0	4110.6	2	130
3	3	6312.0	4734.4	4699.0	3	6682.5	6066.0	5016.0	3	[illegible]
4	4	1992.0	2452.8	2776.0	4	4127.2	5207.3	3833.0	4	[illegible]
5	5	5169.6	6436.0	2060.0	5	1017.8	3428.4	2453.3	5	420
6	6	7968.0	8779.2	1363.2	6	2083.6	3750.0	3720.0	6	1045
7	7	2688.0	4094.4	4686.7	7	1990.0	2692.5	3406.5	7	[illegible]
8	8	4953.6	7512.0	1332.6	8	1243.9			8	[illegible]
9	9	4440.0	6412.6	4782.0	9	1760.3	3230.9		9	[illegible]
10	10	6542.4	6441.6	2995.2					10	870

File ANDREW2 2/ 4/95TTL Urine flow rate

Page -

row	ufrv30	uduty90	uduse	udute1	udute30	udute90	ucrt/	ucrty1	ucrty30	ucrt/90
1	140	300	1	280	225	300	1	415.36	243.74	265.50
2	675	610	2	200	350	300	2	701.19	293.96	340.31
3	600	400	3	140	125	200	3	146.57	131.31	207.66
4	640	450	4	220	270	250	4	111.03	368.15	218.25
5	400	420	5	140	140	290	5	409.27	160.40	130.32
6	1175	750	6	150	250	300	6	401.28	488.45	146.76
7	620	500	7	140	125	160	7	719.75	301.94	314.[illegible]
8	650	260	8	70			8	424.20	279.82	287.45
9	520	630	9	170	120		9	684.10	355.60	475.32
10	270	595					10	538.76	440.27	381.67

File ANDREW2 2/ 4/95 Urinary Creatinine

Page :

row	ucrte	ucrte1	ucrte30	ucrte90	uricy	uricy1	uricy30	uricy90	urice	urice1
1	1	189.42	227.96	294.9	1	35.36	51.52	33.99	1	51.3
2	2	87.70	131.74	104.4	2	93.34	76.48	84.91	2	20.4
3	3	67.14	52.18	69.6	3	64.52	92.00	58.68	3	112.7
4	4	140.49	188.73	222.5	4	10.55	55.49	22.40	4	18.2
5	5	86.39	77.21	169.1	5	23.23	43.00	23.00	5	16.8
6	6	128.96	162.75	227.5	6	22.64	70.50	15.00	6	20.0
7	7	87.88	160.14	182.4	7	46.02	37.94	31.65	7	23.3
8	8	93.10			8	97.00	111.18	31.51	8	63.0
9	9	117.96	128.63		9	24.48	39.88		9	
10					10	46.75	60.53	41.74		

File ANDREW2 2/ 4/95

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row	urice30	urice90

File CLEAREN 04/03/95 Allantoin, Creatinine & Uric acid clearances

row	acly	aclyl	acly30	acly90	acle	aclel	acle30	acle90	cclyl	crcly30	crcly90
1	1	9.80	25.05	57.97	1	1.78	2.32	2.83	240.65	113.54	147.5[illegible]
2	2	2.56	8.90	9.26	2	0.37	1.18	1.99	143.31	198.08	116.17
3	3	16.88	11.61	11.62	3	0.44	1.00		127.66	102.03	146.9[illegible]
4	4	7.95	7.72	6.22	4	1.53	1.41	0.98	101.62	110.67	111.8[illegible]
5	5	1.47	11.78	21.63	5	0.52	0.74	1.14	97.84	124.28	105.9[illegible]
6	6	2.91	19.68	8.17	6	7.86	1.14	21.15	111.10	172.62	146.8[illegible]
7	7	12.98	30.85	11.88	7	3.93	4.12	8.34	152.37	109.94	152.6[illegible]
8	8	1.11	2.46	1.31	8	0.60	1.12		173.80	176.54	314.1[illegible]
9	9	3.97			9		1.05		185.67	119.15	127.72
10	10	8.72	33.40	24.93					176.[illegible]	120.15	[illegible]8.28
11											

File CLEAREN 04/03/95

row	crclel	crcle30	crcle90	uaclyl	uacly30	uacly90	uaclel	uacle30	uacle90
1				19.62	5.40	6.41	0.782	1.060	
2		23.84	39.57	5.47	8.38	4.74	0.589	0.488	0.567
3	94.71	70.51		3.89	3.93	6.19	1.740	0.925	
4	41.97	33.25	70.68	8.63	8.82	6.83	0.328	2.433	0.787
5	23.57	19.99		15.51	6.83	13.08	0.383	0.719	
6	32.88	39.83		38.40	16.97	15.26	0.780	0.372	0.431
7	31.97			14.62	15.90	16.74	0.297		
8		46.91		12.19	18.92	12.54	2.932	3.588	
9				25.99	9.63	16.49			
10				7.11	4.07	11.49			
11									