ANALYTICAL AND METABOLIC STUDIES OF THE TRYPANOCIDAL PHENANTHRIDINES

A thesis submitted in accordance with the regulations governing the award of the Degree of Doctor of Philosophy in Pharmaceutical Sciences.

by

Justice Nii Addy Tettey B.Pharm (Hons), MSc.

Department of Pharmaceutical Sciences,
University of Strathclyde,
Royal College Building,
204 George Street,
Glasgow G1 1XW

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CONTENTS

ABSTRACT

A selective, reproducible and precise method for the determination of isometamidium chloride hydrochloride (ISM), in the presence of four process-related and degradation impurities, by RP-HPLC is described for the first time. An HPTLC system with UV densitometric evaluation, suitable for the separation and detection of these compounds is reported also. The Berthelot (Indophenol) reaction was modified for the quantitative determination of an inorganic impurity, ammonium chloride, in commercial samples of ISM (Samorin® and Veridium®). Application of the RP-HPLC method and the modified Berthelot reaction, for the determination of ISM and ammonium chloride respectively, to the analysis of preparations of ISM from different manufacturing sources demonstrated that there are marked qualitative and quantitative differences.

Confocal laser scanning microscopy (CLSM) showed that ethidium bromide (EBr) and ISM were taken up rapidly into the nucleoli and nuclear membranes of isolated rat hepatocytes. The use of HPLC and LC-MS established that EBr was biotransformed to at least six metabolites, by *N*-glucuronosylation (representing the major pathway), *N*-acetylation and also by *O*-glucuronosylation. In rats, the glucuronosylation pathways were differentially induced by phenobarbitone and 3-methylcholanthrene. Metabolites of ISM were not detected; however, its positional isomer (M&B4250, which possesses an unsubstituted 8-amino group; similar to that in EBr) was metabolised by *N*-glucuronosylation and *N*-acetylation.

Major interspecies variability was observed in the nature and the extent of the metabolism of EBr by rat, sheep and pig hepatocytes. *N*-glucuronosylation was the major pathway in all three species; with the *N*-acetylation pathway absent in sheep and pigs.

GLOSSARY

CLSM	Confocal laser scanning microscopy
COSY	NMR correlated spectroscopy
C8	Octylsilane
DDT	Dichlorodiphenyltrichloroethane
EBr	Ethidium bromide
ESI-MS	Electrospray ionisation mass spectrometry
FAB-MS	Fast atom bombardment mass spectrometry
h	Hour
HPLC	High performance liquid chromatography
HPTLC	High performance thin layer chromatography
ID	Column internal diameter
ISM	Isometamidium chloride hydrochloride
k	Solute capacity factor = $[(t_s - t_0) / t_0)]$ where t_s = retention time of solute and t_0 = retention time of unretained solvent
min	Minute(s)
m.p.	Melting point
3-MC	3-Methylcholanthrene
NMR	Nuclear magnetic resonance spectroscopy
NOESY	NMR nuclear Overhauser enhancement (NOE) spectroscopy
PEEK	Polyetheretherketone
R^2	Regression coefficient
R_F	Retardation factor = [distance travelled by solute from origin / distance travelled by solvent from origin]
RP-HPLC	Reversed phase high performance liquid chromatography

R_s	Resolution $= [2 (t_2 - t_1) / (W_2 + W1)]$ where t_1 and t_2 denote the retention times of compounds 1 and 2, and W_1 and W_2 denote the width at peak base of compounds 1 and 2 respectively)
SD	Standard deviation
SEM	Standard error of the mean
t_R	Retention time
UV	Ultraviolet light

The thing that hath been, it is that which shall be; and that which is done is that which shall be done: and there is no new thing under the sun.

Is there anything whereof it may be said, See, this is new? it hath been already of old time, which was before us.

Ecclesiastes 1: 9-10.

CHAPTER 1: INTRODUCTION

1. INTRODUCTION

1.1 Animal trypanosomiasis

The ravages of the tsetse fly on cattle and horses in Africa were brought to the attention of the scientific community in 1857 by the Scottish explorer, David Livingston. He believed that the fly injected a "lethal venom" into its prey leading to progressive emaciation, lethargy and eventually death. This medical condition, known as *Nangana* in Sub-Saharan Africa, is now termed African animal trypanosomiasis. The evidence for the involvement of a causative organism was first provided by Sir Robert Bruce (1895), who isolated the trypanosome in the blood of animals affected by the disease. He thus provided evidence that the trypanosome was in fact Livingston's "lethal venom" which was transmitted by the tsetse fly (*Glossina sp.*).

Trypanosomes are protozoan organisms of the Order Kinetoplastida, Family Trypanosomatidae and Genus *Trypanosoma.* The African animal trypanosomiases are a complex of related diseases. *Trypanosoma vivax*, *T. congolense* and *T. brucei brucei* affect cattle, sheep, goats and pigs; *T. simiae* affects pigs; *T. evansi* affects camels. In man, *T. brucei gambiense* produces mild initial symptoms followed by a chronic infection which may last for years, whilst *T. brucei rhodesiense* infections are generally acute and fatal within a few months. The species that cause human trypanosomiasis (sleeping sickness) also affect wild animals and can be transmitted from animals to humans (zoonotic infections). Wild hosts, mainly bovids, act as reservoirs of the trypanosomes, precluding their elimination by treating domestic hosts and man (Williams *et al.* 1993). Trypanosomes are then carried from one host to another by tsetse flies that feed on the blood of infected hosts.

The trypanosomes enter their mammalian host via a blood meal by the infected tsetse fly. They are transformed from the metacyclic forms in the salivary gland of the tsetse fly to

blood stream forms in the mammalian blood where they begin to multiply by asexual binary fission. The trypanosomes first infect the blood vessels and lymph glands, causing intermittent fever, a rash and swelling of the glands. Eventually the parasites invade the central nervous system. Inflammation of the outer membrane of the brain leads to lethargy, coma and death (Leach and Roberts 1981). Infected livestock develop fever, lose weight and become weak and unproductive; breeding animals may abort or become infertile (Jeffcoate and Holmes 1997). Many untreated animals die of anaemia, circulatory collapse or as a result of secondary bacterial infections (Williams *et al.* 1993).

Antigenic variation in trypanosomes is a highly sophisticated survival strategy involving switching between the transcription of one of an estimated thousand Variant Surface Glycoprotein (VSG) genes (Wang 1995). This antigenic variation results in irregular waves of rising and falling parasitaemia. The end result is a highly effective evasion of the immune responses of the host by the parasites. Thus the infected host can develop little protective immunity.

1.2 Epidemiology

Both human and animal trypanosomiases have an important impact on the development of rural areas, by reducing the labour force, livestock production and the availability of animal traction for farming. The socio-economic burden is important as both the human and the animal disease reduce family revenues and slow the economic growth of rural areas through the abandonment of fertile farming land. Accurate epidemiological data are not available on African animal trypanosomiasis. However, it is estimated that trypanosomiasis excludes cattle from seven million square kilometres of otherwise suitable range or farmland (Williams *et al.* 1993). Because the disease can be transmitted from

animal to man and vice-versa, epidemiological data in humans give a fair reflection of the scenario in animals.

Human African trypanosomiasis is rural and focal, with humans as the principal reservoir of infections of *T. brucei gambiense*, and domestic cattle and wild animals as important reservoirs of *T. brucei rhodesiense*. At the turn of the century, human trypanosomiasis accounted for over 200,000 deaths in Uganda. It was practically eliminated in the years 1960-1965 (Figure 1.1). The recrudescence of the disease occurred progressively from 1970 onwards in most of the old foci. The number of reported cases in 1995 parallels those in the 1940s and provides considerable cause for concern.

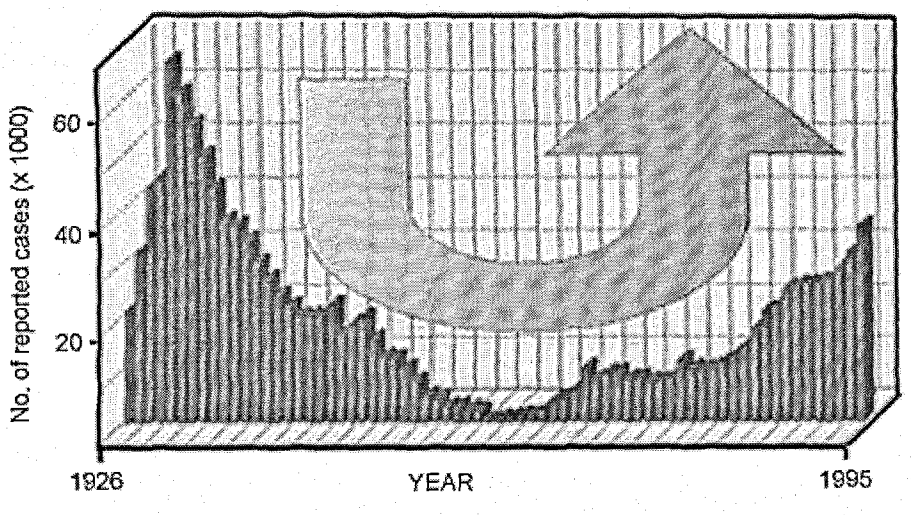


Figure 1.1 Number of reported cases of human trypanosomiasis in Sub-Saharan Africa from 1926 to 1995 (Source: World Health Organisation / Centre for Tropical Diseases 1998.)

Four main levels of endemicity have been identified:

- In Angola, the Democratic Republic of Congo, Sudan and Uganda the disease is considered epidemic due to a high prevalence and an important transmission level,

- Cameroon, the Central African Republic, Chad, Congo, Côte d'Ivoire, Guinea and Tanzania have been classified as countries of high endemicity where prevalence is increasing,
- Benin, Burkina-Faso, Equatorial Guinea, Gabon, Ghana, Guinea, Kenya, Mali, Mozambique, Nigeria, Togo and Zambia are considered of low endemicity although the epidemiological situation is poorly known in foci in several countries,
- Countries where the present epidemiological status is not well known, namely Burundi, Botswana, Ethiopia, Liberia, Namibia, Rwanda, Senegal and Sierra Leone.

It is estimated that 55-60 million people are exposed to the risk of becoming infected with trypanosomiasis, in 36 countries of Sub-Saharan Africa.

1.3 Control of animal trypanosomiases

The control of trypanosomiasis in both domestic animals and man has depended on the elimination of the tsetse fly, the breeding of trypano-tolerant species of domestic animals and, most importantly, on chemotherapy.

Elimination of the insect vector (Glossina sp.)

Earlier attempts to control the disease had the objective of eradicating the fly vector. In the first decades of this century, this was attempted by the clearance of vegetation to remove habitat essential to the tsetse fly. This method was abandoned when chemical control of the tsetse fly became possible due to the introduction of synthetic organochlorine insecticides such as dichlorodiphenyltrichloroethane (DDT) and dieldrin. Large-scale tsetse control operations were undertaken in the 1950s involving aerial and ground application of persistent deposits of organochlorine insecticides (Williams *et al.* 1993, Barrett 1997). Large-scale aerial spraying programmes of non-persistent insecticides, such as endosulphan,

have been conducted in Botswana, Somalia, Zambia and Zimbabwe in the last 15 years (Barrett 1997). Livestock can also be used as walking targets to reduce the fly population. Persistent insecticides, such as deltamethrin®, are applied directly to livestock as a dip-wash, spray or pour-on formulation (Bauer *et al.* 1995, Barrett 1997, Holmes 1997). Although the use of insecticides has successfully reduced the fly population in most affected areas, the technique has become widely unacceptable because of the adverse impact of such compounds (e.g. DDT) on the environment (Barrett 1997).

Insect traps and the release of sterile males, which are environmentally acceptable, have also been employed in affected areas to reduce the fly population. However, these methods (especially the sterile male release technique) are costly, rely on sophisticated technology and cannot be sustained for long periods of time (Williams *et al.* 1993).

Breeding of trypano-tolerant species

The key indicators of trypano-tolerance are the ability to control parasitaemia, to resist anaemia and to mount an effective immune response. Many wild hosts are innately resistant to the pathogenic effects of trypanosome infections (Leach and Roberts 1981). Breeds of cattle which are tolerant to trypanosomiasis, such as the N'dama, occur in some parts of Africa. However, the trypano-tolerant breeds are only a small proportion of the total cattle at risk of the disease and, under high tsetse fly challenge, even these can succumb to disease.

Chemotherapy of animal trypanosomiasis

The use of chemotherapeutic and chemoprophylactic agents remains the major method by which the disease is controlled in and outwith Africa (Wang 1995). Chemotherapy of African animal trypanosomiasis is dependent mainly upon the use of the aromatic diamidine, diminazene aceturate (Berenil®), and the phenanthridines, ethidium bromide (Novidium®,

homidium bromide) and isometamidium chloride hydrochloride (Samorin®/Trypamidium® and Veridium®) (Kinabo 1993). Isometamidium chloride hydrochloride remains the most important agent because of its chemoprophylactic properties (Kinabo and Bogan 1988a). These chemotherapeutic agents have various deficiencies ranging from poor oral absorption, acute toxicity, short duration of action and low efficacy (Hawking 1965). Strains of *trypanosomes* have emerged which have shown cross-resistance to ethidium, isometamidium and diminazene (Peregrine 1991, Codjia *et al.* 1993). In the face of emerging resistance to the pitiful handful of existing trypanocides, it should be appreciated that new drugs are unlikely to be developed for economic, rather than for scientific reasons. Therefore it is essential that the available drugs are used in ways that maximise their effectiveness.

1.4 Current status of trypanosomiasis research

Two major forces have driven research efforts in the control of trypanosomiasis. Primarily, the need to develop and achieve effective measures for control of the diseases in endemic areas. Secondly, the pursuit of an understanding of the unique biology of the trypanosomatids.

The main factor for the absence of new trypanocides has been the high cost of new drug development and the unlikely situation of research based organisations being able to recoup the substantial initial investment in a trypanocidal agent destined for the 'Third world'. Ironically, there is very little definitive information on the available trypanocides and their mechanisms of action (Wang 1995). The paucity of new drugs for either the human and animal diseases, combined with the increase in drug resistance, has led to a renewed interest in tsetse control (Kinabo 1993).

Measures to eliminate the tsetse fly seem to be the only avenue currently open to innovation. Recently the British-based Medical Emergency Relief, Merlin, announced the

introduction of a novel tsetse trap (Ball, 1998). The trap is made from a light fabric dyed a special iridescent blue colour. This shade of blue reflects light at a specific optical wavelength, which is irresistible to the tsetse fly and this device represents one of the many environmentally friendly measures designed to eliminate the fly.

In sharp contrast to the limited information on the mode of action and the metabolic fate of the few existing trypanocides, the African trypanosomes are among the most extensively studied protozoa to date. Many of their intriguing biological features have been documented and can be viewed as attractive targets for anti-trypanosomal chemotherapy. These features include trypanothione metabolism, polyamine metabolism, mechanisms for controlled protein degradation and the purine salvage enzymes (Wang 1995). The enormous potential of the trypanosome for antigenic variation has been the primary obstacle in the development of a trypanosomiasis vaccine.

It is obvious that, until a major breakthrough in anti-trypanosomal research produces a vaccine, the use of the few trypanocides (which are plagued by resistance) will have to be optimised. The mechanism of the development of resistance by the parasite is poorly understood, but it was proposed that the underlying mechanism of resistance to isometamidium was associated with reduced accumulation of the drug in trypanosomes (Sutherland and Holmes 1993).

1.5 Identification of possible causes for the development of parasitic resistance.

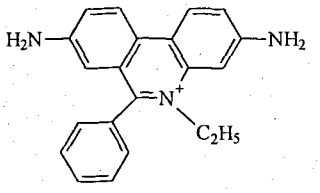
The resistance of organisms to otherwise therapeutic doses of chemical agents may be attributed to several factors, which include; an innate or natural resistance, development of resistance after exposure to the agent, and cross-resistance to structurally similar compounds.

Natural resistance of a strain or species to a drug is demonstrated by a variation in sensitivity to a drug which is not dependent on previous exposure to the drug concerned. As an illustration, *T. congolense* shows a higher rate of relapse infections than *T. vivax* after treatment with ethidium bromide (Leach and Roberts *1981*).

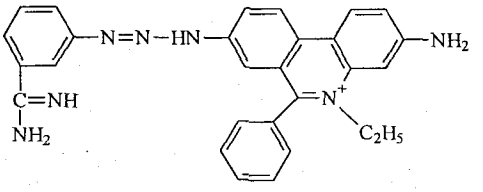
The conditions under which drug resistance develops in the field are all derived basically from administration of sub-therapeutic doses of the agents concerned. This may result from an incorrect estimation of body-weight or incorrect dosage when large numbers of animals are involved in mass treatment campaigns, or through the application of substandard formulations of therapeutic agents as a result of drug-counterfeiting or fraud in developing countries (Monteiro *et al.* 1998). Also, a high incidence of the disease renders normal therapeutic doses ineffective and irregular treatment with prophylactic drugs or the cessation of treatment while animals are still at risk can also contribute to the development of resistant strains.

Cross-resistance refers to the scenario when an organism which, by virtue of its innate or developed resistance to one compound, shows resistance to another compound to which it bears structural resemblance. A classical example is the widespread resistance shown by 'penicillinase-resistant' staphylococci to the series of structurally related penicillins (Mandell and Sande, 1990). Therefore, it is not surprising that cross-resistance in trypanosomes to

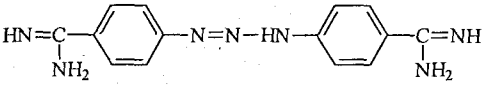
ethidium, isometamidium and diminazene has been reported (Codjia *et al.* 1993, Wang 1995), in view of their structural similarity (Figure 1.2).



Ethidium



Isometamidium



Diminazene

Figure 1.2 Chemical structures of the existing animal trypanocides, illustrating the structural similarities between ethidium and isometamidium, and between isometamidium and diminazene.

However, an assessment of the interactions that occur between trypanosomes, drugs and the host reveals a complexity of events which makes it difficult to offer a simple reason for the development of resistance. The trypanocidal action of the drug is countered by the ability of the organism to become resistant, which then requires another drug to produce an effective cure. The virulence of the trypanosome for the host provokes an immune response influenced by the breed of the host and the drug used for treatment. The immune response is countered in turn by antigenic variation and perhaps by antibody resistance in the trypanosomes. At the same time there are interactions between the drug and the host, because the drug is usually toxic to some extent and the host responds by storing, metabolising or excreting the drug. Biotransformation could lead to an increase or a decrease in therapeutic activity. Environmental factors, which affect the metabolic state of the host, can alter the biotransformation or excretion of the trypanocide. These factors include

exposure to heavy metals (e.g. lead, mercury, cadmium), industrial pollutants (e.g. polychlorinated biphenyls; solvents of the benzene and chlorinated hydrocarbon types, such as toluene, dichloromethane, chloroform) and insecticides and pesticides (e.g. DDT, dieldrin, parathion) (Gibson and Skett 1994). Meanwhile, the vector intrudes into the picture because it is responsible for natural incidence of trypanosomes; high incidence works against the drug by reducing the effective circulating concentration of trypanocides and against the host by swamping the immune response.

1.6 Chemoresistance – the unanswered questions

The foregoing exposé on the possible causes of resistance to trypanocidal chemotherapeutic agents has been based on the premise that the trypanocides circulating in international commerce are of acceptable quality and efficacy. The discussion has also been made without consideration of the metabolic fate of trypanocides in the host. Environmental effects, such as exposure to the pesticides concomitantly used to control the tsetse fly, on the metabolic state of the host have also not been evaluated. The mechanism of resistance of the parasite to trypanocides, which has been associated with reduced accumulation or diminished uptake of the drugs (Wang 1995), remains poorly understood. This leads to several unanswered questions:

1. Are the available trypanocides in international commerce suitable for their intended use in terms of the quality and efficacy of the product(s)?
2. Are these trypanocides extensively metabolised by the host?
3. How are the trypanocides taken up and metabolised by hepatic cells and trypanosomes?
4. Is the transport system for these drugs into trypanosomes altered by resistance?
5. Is the metabolism of trypanocides altered by environmental chemicals?

6. Are there species differences and/or similarities in the metabolism of trypanocides?
7. Does metabolism lead to activation or inactivation of the trypanocidal effects?

Veterinary drugs are subject to the requirements of quality, safety and efficacy, which are applied to human drugs. The lack (or the limitations) of resources available to most 'Third-world' Drug Regulatory Authorities has de-prioritised the control of veterinary pharmaceuticals. This situation has made markets in these parts of the world a haven for counterfeit/substandard drugs products. Monteiro *et al.* (1998) reported that commercial preparations of a veterinary anthelmintic, levimasole, on the Kenyan market contained varying amounts of the active ingredient (ranging from 0 to 118% of the label claim). The circulation of substandard products in a region, such as Kenya, where levimasole-resistant nematodes already exist may only serve to exacerbate chemoresistance.

Arguably isometamidium chloride hydrochloride (Figure 1.2) is the most important veterinary trypanocide available (Kinabo 1993). It is prepared by selectively coupling one of the two amino groups in ethidium chloride (Figure 1.2) with diazotized *m*-aminobenzamidine (Wragg *et al.* 1958). When two or more amino-groups are attached to different aromatic nuclei in the same molecule, as in ethidium, they can be considered for the purposes of diazotization as an assemblage of two or more different aromatic amines (Saunders 1949). This difference can be exploited, by controlling the reaction conditions, to enhance selective reaction of one of the amino groups. This has been the theoretical basis for the synthesis of isometamidium (see Section 2.1). However, the relatively unreactive amino group(s) can (and do) participate in the reaction to an appreciable extent, which depends on the conditions of reaction (Berg 1963a). This leads to the possibility of variation in the ratio, and also the nature, of diazoamino products of aromatic compounds with two or more amino groups (together with other by-products). The potential for the synthesis of isometamidium

to exhibit qualitative and quantitative differences in the composition of the final product is well documented (see Section 2.1). Therefore, it is surprising that, to date, the published literature and the official monographs in British and United States veterinary pharmacopoeia do not contain methods for the control of the quality of products containing isometamidium. For economic reasons, health authorities are increasingly supporting the use of generic substitution. This is laudable provided that chemical and therapeutic equivalence can be established between the innovator product and its generic substitute (i.e. that they are clinically interchangeable as a result of equivalent safety and efficacy (Meredith 1996). For a product like isometamidium, with a method of preparation which requires careful control (especially on an industrial scale) and the absence of methods to control the quality of the product, the following questions are fundamental;

1. How do we establish the quality (and hence the safety and efficacy) of these preparations of isometamidium which are in international commerce?
2. What is the basis for determining the chemical equivalence of a generic formulation to that of the innovator product?

The design and validation of analytical methods suitable for the control of the quality of available trypanocides would provide answers to these questions.

The biotransformation of xenobiotics in biological systems may have a significant effect on the observed therapeutic response. Biotransformation may result in an increase or decrease of the desired pharmacological action, detoxification or the generation of reactive or toxic species. Basic knowledge of comparative biotransformation in relation to drug elimination is of major importance for drug usage within the field of veterinary pharmacotherapy (van't Klooster 1992). The obvious reasons are the evaluation of safety and

efficacy of the therapeutic agent and the species-dependent estimation of the drug dosage regimen. Our knowledge of the metabolism of the phenanthridines is based on the conversion of ethidium in rats to acetylated derivatives (MacGregor and Clarkson 1971, Lecointe *et al.* 1981). However, there have been indications that the metabolism of the same agent may differ in other species (such as cattle and rabbits, in which acetylated derivatives have not been found to date) (Gilbert and Newton 1982, Murilla *et al.* 1996a).

Species differences in drug metabolism are found for both phase I and phase II metabolism and may be either quantitative or qualitative. As an illustration (Figure 1.3, adapted from Gibson and Skett 1994), the rat predominantly hydroxylates amphetamine, leading to conjugated products involving the phenol group; whereas the rabbit, guinea pig and man mainly deaminate amphetamine. The guinea-pig further oxidises the ketone to benzoic acid and excretes conjugates of benzoic acid. The rabbit reduces the ketone and subsequently excretes conjugates of the alcohol. Another example is found in the metabolism of sulfadimidine, where the hydroxylation pathway is important in goats, sheep and cattle; whereas in rats, acetylation is predominant (van't Klooster 1992). In addition to species differences, metabolism may also be affected by genetic/strain differences, age, sex, hormonal and disease states (van't Klooster 1992, Gibson and Skett 1994).

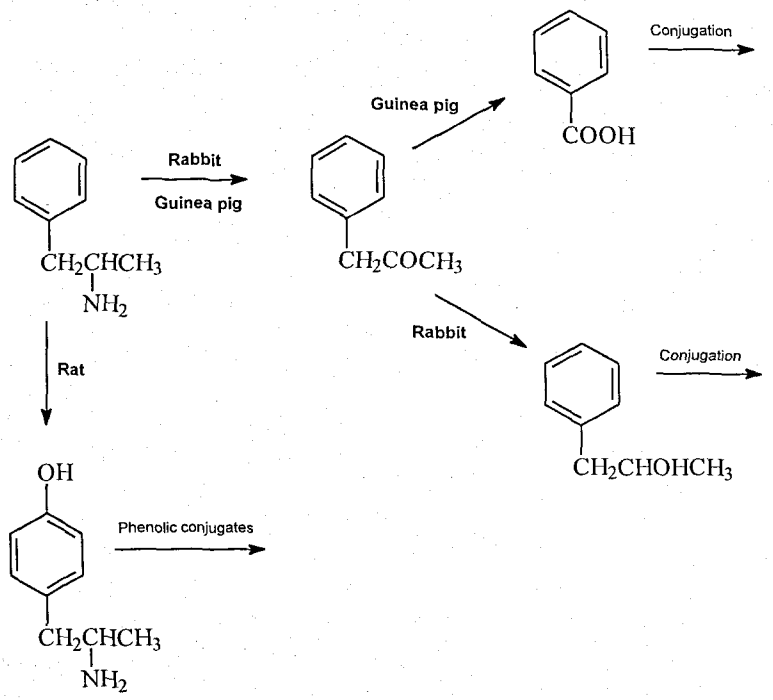


Figure 1.3 The metabolism of amphetamine in rabbit, guinea-pig and rat.

Similarities in the metabolism of compounds by different species are frequently encountered due to the presence of similar or identical enzyme systems and the primary sequence similarity for specific enzyme proteins. Examples of these enzyme proteins include the cytochrome P-450 and UDP-glucuronosyltransferase isoenzymes. In spite of the structural homology, these enzymes can show subtle differences in a small number of amino acid residues in different species. These small changes result in profound differences in substrate specificity and may form the basis for species and genetic differences in metabolism. The inability of the cat and the dog to form glucuronides and acetylated metabolites respectively of some compounds, the deficiency of glucuronosyltransferase activity in the Gunn rat and the presence of 'fast' and 'slow' acetylators are examples of species differences in biotransformation mediated by the presence of different isoenzymes (Weber *et al.* 1990, Timbrell 1991, Vatsis and Weber 1994, Lin 1995).

Considering the qualitative and quantitative differences which exist in the metabolism of many xenobiotics by different species, extrapolation of efficacy of a drug from one animal to the other is extremely complicated and difficult, if not impossible. Therefore, it is important that the metabolism of the phenanthridines is investigated in individual species to gain knowledge (which, hopefully, will be clinically significant as well as scientifically important) of differences and similarities in metabolism.

1.7 Aims and objectives of the thesis

1. This study aims to design and validate sensitive, precise and selective chromatographic methods for the determination of isometamidium in the presence of its possible synthetic impurities/related substances. Further, it aims to produce a protocol for the determination of chemical equivalence of those multi-source preparations which contain isometamidium. Variations in formulations from different sources may point to the possibility of compromised efficacy, which may contribute to the development of trypanosomal resistance.
2. Hepatocytes obtained from various species (rat, pig and sheep) will be used to define the metabolism of the prototype trypanocidal phenanthridine, ethidium bromide, with emphasis on species differences and similarities in metabolism. The effect of the classical metabolism-inducing agents (phenobarbitone and 3-methylcholanthrene) will be used to assess the possible effect of environmental chemicals, such as pesticides and polycyclic aromatic hydrocarbons, on the metabolism of ethidium bromide.
3. The metabolism of isometamidium chloride hydrochloride and a structurally related compound (M&B4250, Berg 1963b) will be examined using rat hepatocytes in order to investigate the structural requirements for the metabolism of the phenanthridines.

CHAPTER 2: DETERMINATION OF THE QUALITY OF PREPARATIONS OF ISOMETAMIDIUM CHLORIDE HYDROCHLORIDE

2. DETERMINATION OF THE QUALITY OF PREPARATIONS OF ISOMETAMIDIUM CHLORIDE HYDROCHLORIDE.

2.1 Introduction

In 1958, Wragg *et al.* described the chemical and biological properties of a new trypanocidal phenanthridine preparation, first designated M&B4404 and later assigned the name, metamidium chloride hydrochloride. Metamidium was prepared by the coupling of *m*-amidinobenzenediazonium chloride and 3,8-diamino-5-ethyl-6-phenylphenanthridinium chloride (ethidium chloride). The drug was the result of a long standing interest in the trypanocidal properties of the phenanthridinium salts and the aromatic diamidines in combining structural elements of the two classes (Browning *et al.* 1938, Watkins 1952, Watkins and Woolfe 1952, Fussgänger 1955). The introduction of a diazoamino bond into metamidium was intended to confer some of the structural features of another trypanocidal drug, diminazene aceturate (1,3-bis(4-amidinophenyl)triazene, Figure 1.2).

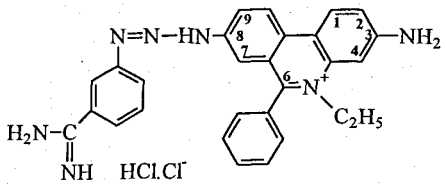
Metamidium was obtained as a mixture of red and purple isomers (45:55 w/w) to which the structures 3-(*m*-amidinophenyldiazoamino)-8-amino-5-ethyl-6-phenyl-phenanthridinium chloride and 8-(*m*-amidinophenyldiazoamino)-3-amino-5-ethyl-6-phenyl-phenanthridinium chloride respectively were assigned provisionally (Wragg *et al* 1958). The evolution of nitrogen which occurred when the red and purple isomers were treated with 3M sulphuric acid was given as evidence for the presence of a diazoamino group in both compounds.

Subsequent investigations (Berg 1960, 1963a and 1963b) showed that the red isomer, designated M&B4180A, was 8-(*m*-amidinophenyldiazoamino)-3-amino-5-ethyl-6-phenylphenanthridinium chloride and later the name isometamidium chloride hydrochloride

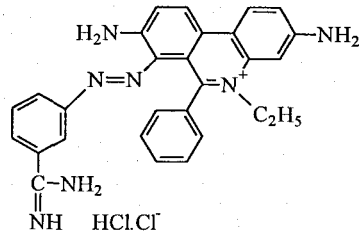
(ISM, Figure 2.1) was assigned to it. The evidence for the structure of ISM was obtained by comparing the physical properties and infrared spectrum of the desamino compound, produced by the deamination of ISM, with those of authentic 8-(*m*-amidinophenyldiazoamino)-5-ethyl-6-phenylphenanthridinium chloride (unambiguously synthesized by the coupling of diazotised 8-amino-5-ethyl-6-phenylphenanthridinium chloride with *m*-aminobenzamidine). In the purple isomer (designated M&B4250, Figure 2.1) the *m*-amidinophenylazo group is coupled directly to the phenanthridinium ring at one of the positions (*C*-7) *ortho*- to the *C*-8 amino group (Berg 1963b).

The relative amounts of ISM and the purple isomer produced were dependent on the temperature and the pH of the reaction medium. At an optimum pH of 1.8 to 2.2 and a temperature of 10-11°C, ISM was obtained in a fairly pure state in yields in excess of 70% (Berg 1963a, 1963b). It was established that the trypanocidal activity of metamidium was due primarily to ISM (Wragg *et al.* 1958, Brown *et al.* 1961).

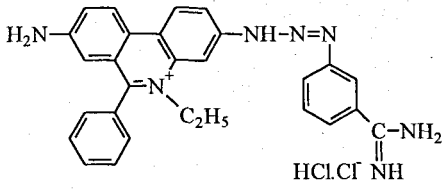
The product of this synthesis is marketed currently as Samorin® or Trypamidium® (Merial, Lyon, France), the originator product (*1960*), and latterly (*1996*) as Veridium® (Sanofi Animal Nutrition, Lyon, France). Clarke (1975) defines the innovator product, Samorin®, as a veterinary formulation containing ISM as the major component together with two impurities, namely 3,8-di(*m*-amidinophenyldiazoamino)-5-ethyl-6-phenylphenanthridinium chloride (the 3,8-disubstituted analogue of metamidium, designated M&B4596, IV, Figure 2.1) and 2-, 4-, 7- or 9-(*m*-amidinophenylazo)-3,8-diamino-5-ethyl-6-phenylphenanthridinium chloride (M&B4250, V). Subsequently, Kinabo and Bogan (1988a) reported that M&B4250 has the amidinophenylazo substituent at the *C*-7 position of the phenanthridine ring.



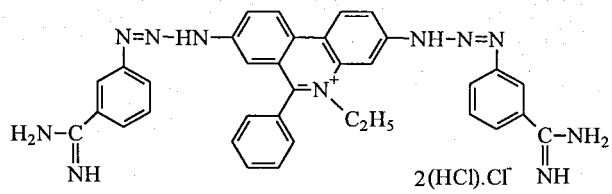
M&B 4180A (ISM, **II**)



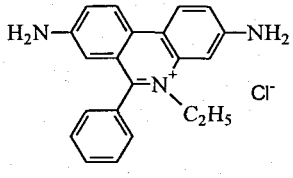
M&B 4250 (**V**)



M&B 38897 (**III**)



M&B 4596 (**IV**)



Ethidium Chloride (**I**)

Figure 2.1 Chemical structures of ISM (**II**) and its related substances in Samorin®

The presence of two other related substances, viz. 3-(*m*-amidinophenyldiazoamino)-8-amino-5-ethyl-6-phenylphenanthridinium chloride (pseudo-isometamidium, M&B38897, III, Figure 2.1) and ethidium chloride (I, Figure 2.1) in Samorin®, has been reported (Kinabo and Bogan, 1988a). The organic components of Samorin® namely, M&B4180A, M&B4250, M&B4596 and ethidium chloride (Figure 2.1) possess varying degrees of trypanocidal activity; with M&B4180A and M&B4596 possessing the highest curative and prophylactic activity respectively (Brown *et al.*, 1961). Ammonium chloride occurs as an inorganic impurity in Samorin® (Rhone Poulenc Rorer, Dagenham, *Personal*

Communication). This may originate as a source-impurity in *m*-amidinobenzenediazonium chloride (*ca.* 3%w/w) or as a process-impurity from its possible use as a 'salting-out' agent in the synthesis of ISM. The impurity profile of the innovator product, Samorin®, conforms to theoretical predictions made from the chemistry of the synthesis of ISM (Berg 1963a, 1963b, Berg *et al.*, 1963).

2.1.1 Synthesis of isometamidium: implications for the presence of related substances.

The coupling of a diazonium salt with an aromatic primary amine to give a diazoamino compound is an electrophilic substitution reaction involving the diazonium ion and the non-ionised amine. The particular synthesis of ISM results from the coupling of the diazonium salt, *m*-amidinobenzenediazonium chloride, to the 8-amino group of ethidium chloride. The presence of the amidino substituent enhances the reactivity of the parent benzenediazonium ion, leading to an increase in the range of the coupling reaction, with the resultant production of the other isomer (M&B38897) and the doubly coupled product (M&B4596). Also, the reactivity of the amidinobenzenediazonium ion results in the possibility of *C*-coupling at the 2-, 4-, 7- or 9-carbon atoms of the phenanthridinium ring to form purple phenyldiazo compounds (Berg *et al.* 1963). Berg (1963b) predicted that, because of the weakly basic nature of ethidium, *N*-coupling would be preferred in strongly acidic media; and it might be anticipated also that it would reduce the possibility of *C*-coupling. The validity of this prediction was demonstrated by a series of coupling reactions between *m*-amidinobenzenediazonium chloride and ethidium chloride at pH values from 1.5 to 6.0. *N*-Coupling at the 8-amino position was the most favoured reaction at low pH values, with *C*-coupling at the *C*-7 position being predominant at higher pH values. The relatively unreactive nature of the 3-amino group is probably due to resonance stabilisation of the

positive charge between the quaternary nitrogen and the 3-amino group (Figure 2.2), rendering the latter less susceptible to attack by electrophilic reagents (Berg, 1963b; Firth *et al.*, 1983).

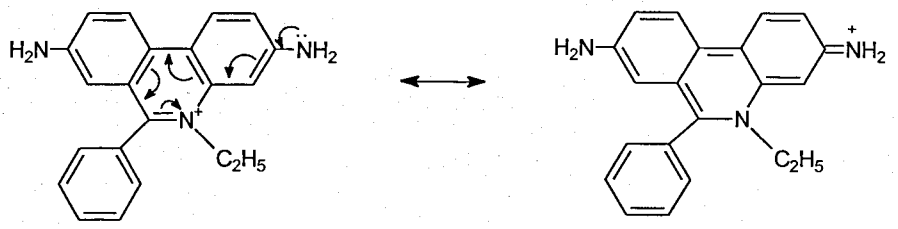


Figure 2.2 Participation of the electron pair of the 3-amino group in a resonance configuration with the quaternary nitrogen (Berg, 1963b; Firth *et al.*, 1983).

The presence of substantial quantities of ethidium chloride in the final product is an indication of an incomplete reaction sequence or the result of the cleavage of the acid-labile triazene bond in ISM (Philips *et al.* 1967, Kinabo *et al.* 1988a). It has been shown that the formation of 3,8-di(*m*-amidinophenyldiazoamino)-5-ethyl-6-phenylphenanthridinium chloride dihydrochloride (M&B4596), which possesses high prophylactic activity against *Trypanosoma congolense* (Brown *et al.* 1961), was least favoured by the reaction conditions (Berg *et al.* 1961, Berg *et al.* 1963) and thus it occurs as a minor impurity in the synthesis of ISM. However, as anticipated, a good yield of this bis-compound was obtained readily by the coupling of bis-diazotized ethidium chloride and *m*-aminobenzamidine monohydrochloride (Berg *et al.* 1961).

Maintenance of the nature and relative ratio of isomers/related substances in Samorin® (and consequently batch-to-batch therapeutic equivalence) is dependent on the optimisation, robustness and reproducibility of the synthetic process. The expiry of patent protection for Samorin® and the introduction of other formulations containing ISM (e.g.

Veridium®) necessitate the development of suitable methods for the complete characterisation of such products. Generic substitution of innovative products is being supported increasingly by health authorities worldwide for economic reasons (Meredith, 1996) and the feasibility of establishing the necessary bioequivalence between such products is well established in the case of generic substitution of single component products of defined chemistry (Nation *et al.* 1994, Marzo *et al.* 1995). However, in the case of multi-component pharmaceuticals (often resulting from complex synthetic chemistry) the maintenance of chemical equivalence may be a primary step in ensuring that there is essential similarity between products from different manufacturers. Chemical equivalence of preparations containing ISM is not only dependent on the quantity of the principal active component and the relative amounts of the related substances, but also on the nature and amount of inorganic impurities

The introduction of 'interchangeable multi-source formulations' containing ISM and increasing reports of the development of parasitic resistance to this agent (Codjia *et al.* 1993, Clausen *et al.* 1992, Peregine 1991) requires that different formulations be completely characterised to aid in product selection and maintenance of therapeutic activity. Characterisation of these products requires the development of accurate, sensitive, selective and robust methods for the determination of ISM in the presence of its related substances and other manufacturing impurities. Preferably, these methods would permit determination of the other components also.

2.1.2 Determination of isometamidium

Despite the extensive use of ISM in veterinary medicine over the past three decades (Kinabo and Bogan, 1988a), there is no published method available for the identification and quantitation of related substances and determination of the purity of ISM. As a result of

the difficulty in controlling the manufacturing process, accurate, specific and well characterised methods for the determination of ISM in the presence of its source, process and degradation impurities are necessary for development and quality control of the bulk manufacturing process and assessment of the stability of the bulk drug substance.

ISM possesses a quaternary nitrogen and a highly basic amidino moiety. The tenacity with which such basic compounds bind to residual silanols on silica-based chromatographic materials results in 'peak tailing' and poses a significant challenge in chromatographic analysis. Thus Clarke (1975), described a silica-based TLC system for ISM with a R_f of 0.05 and the occurrence of "a visible brown streak". Despite this detrimental effect due to interaction between the analyte and silanol (Nawrocki 1997), silica-based reversed-phase high-performance liquid chromatography (RP-HPLC) is still the most widely used method for the analysis of basic compounds because of the fast equilibration time, the selectivity characteristics and the reproducibility of retention (Vervoort *et al.* 1992). Over the years, numerous techniques have been developed to overcome analyte-silanol interactions in the analysis of basic compounds. These include; reduction of the number of free silanols (end-capping), suppression of ionisation of the silanol by use of mobile phases with low pH, reduction of the acidity of the silica backbone (base-deactivation) and elimination of the effects due to silanol by the addition of inorganic or organic salts (ion-pair or competing-ion chromatography).

In an attempt to overcome the chromatographic peak-tailing associated with the analysis of ISM, Kinabo *et al.* (1988b) developed a reversed-phase ion-pair chromatographic method using heptane-sulfonic acid and triethylamine as mobile-phase additives. Chromatographic resolution of a mixture of highly basic compounds would require the use of a high-efficiency column and this property is normally lost with ion-

pairing reagents because of the reduction in the apparent number of theoretical plates. As anticipated, the RP-HPLC method of Kinabo *et al.* (1988b) lacked selectivity for ISM in the presence of M&B38897 and both components of Samorin® eluted together as a single peak.

In recent years, several silica-based reversed-phase columns have been developed which are especially suitable for the chromatography of basic substances. It has been claimed that these 'silanol deactivated' materials have reduced silanol activity and produce better peak symmetry and efficiency. As a consequence, addition of mobile-phase additives (e.g. triethylamine) and use of ion-pairing reagents would become superfluous (Paesen *et al.* 1993, Nawrocki 1997). The correct choice of stationary phase, and the pH and composition of the mobile phase will aid in the development of a suitable chromatographic method for the determination of ISM in the presence of its related substances.

Very few reliable data on the pharmacokinetics of ISM have been available, owing to the lack of suitable analytical methods. Extensive binding of ISM to plasma and tissue macromolecules (Kinabo *et al.* 1987) results in low serum concentrations after administration of therapeutic doses of the product. This has led to the development of various bioanalytical methods designed to achieve reasonable sensitivity. These bioanalytical methods include ultraviolet spectroscopy (Philips *et al.* 1967, Braide *et al.* 1980, Ali *et al.* 1984), enzyme-linked immunosorbent assays (Whitelaw *et al.* 1991, Eisler *et al.* 1993, Eisler *et al.* 1996), measurement of total radioactivity (Mdachi *et al.* 1991, Sunderland *et al.* 1992, Murilla *et al.* 1996b), radioimmunoassay (Kinabo *et al.* 1988c) and HPLC (Perschke and Vollner 1985, Kinabo *et al.* 1988b). It has been shown that methods involving enzyme-linked immunosorbent assays are extremely sensitive (0.5 pg/ml, Whitelaw *et al.* 1991). However, although it has been reported that cross-reactivity between ISM and the structurally related trypanocides, EBr and diminazene, was not significant in

these enzyme-linked immunosorbent assays, the possible contribution of the other related substances (Figure 2.1) to the measurements has not been investigated. The HPLC method of Perschke and Vollner (1985), which involved the conversion of ISM to ethidium in acid media before analysis, makes it difficult to distinguish between the two compounds in admixture; a prime requirement for analytical selectivity.

Several authors have exploited the intrinsic fluorescence of ISM in bioanalytical (Kinabo *et al.* 1988b) and biological (Philips *et al.* 1967, Sutherland *et al.* 1991, Zilberstein *et al.* 1993, Wilkes *et al.* 1995) assays. In these assays it has been assumed that the fluorescence excitation and emission wavelengths of ISM (385nm and 590nm respectively, first reported by Philips *et al.* (1967)) are specific and selective for ISM. Whilst these authors used Samorin® in the assays, the contribution of the ISM-related substances to the observed fluorescence was not investigated. This raises concern about the validity of data presented on ISM which emanates from measurements of the total fluorescence of Samorin®. Such methods designed for ISM using Samorin® would require a performance characteristic of high selectivity in the presence of all possible interferents, including the known related substances.

2.1.3 Determination of ammonium chloride

The use of the Berthelot or indophenol reaction and modifications thereof in the determination of ammonium chloride in pharmaceuticals, agricultural, clinical and food samples has been reviewed extensively (Searle 1984). The Berthelot reaction refers to the reaction of ammonium ions and a phenol (lacking a substituent in the *para* position) which, under suitable oxidising conditions, results in the formation of an indophenol dye. Indophenol dyes are highly conjugated and absorb strongly light of wavelength between 630nm and 720nm.

Methods based on this reaction are sensitive and relatively specific for the ammonium ion. The British Pharmacopoeia (1993) describes a method based on the Berthelot reaction for the determination of ammonium chloride in Urea-Cream. Krom (1980) described the reaction sequence (Figure 2.3) involved in the formation of the coloured indophenol dye using sodium salicylate and sodium hypochlorite as the phenol source and oxidising agent respectively. Most methods based on the Berthelot reaction use nitroprusside as a catalyst although the exact mechanism is not completely understood (Searle 1984). Krom (1980) emphasised the complex inter-relationships existing between the reactants and reaction intermediates and a corollary is that the choice of optimum reagent concentrations and other reaction conditions is difficult. This is emphasised further by the large range of reagent conditions (e.g. pH, temperature and order of addition of reagents) used in the literature. It is evident that, for any one combination of reagents, there are probably several combinations of experimental conditions which give similar sensitivities and precision. The sensitivities of optimised analytical systems using different phenolic compounds have been reported as being essentially similar and a specific absorbance (A 1% 1cm) of the order of 1250 has been reported for sodium salicylate (Searle 1984). Therefore, the routine use of this procedure requires complete validation of the selected analytical parameters.

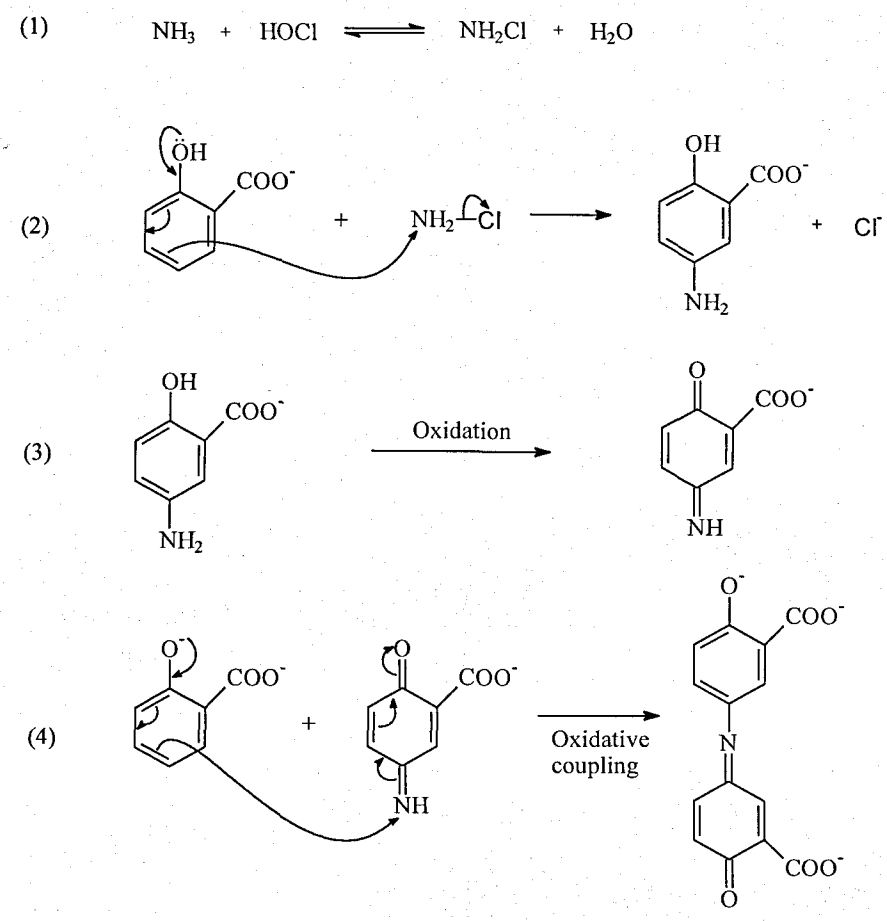


Figure 2.3 Formation of indophenol: reaction sequence according to Krom (1980)

2.1.4 Aims and Objectives

The previous sections in this chapter have highlighted the difficulties in the preparation of pure ISM (particularly on an industrial scale), the potential for a wide array of related substances to occur in commercial preparations and the lack of suitable methods for assuring the quality of the product. Therefore, the present study aims to;

1. Develop a sensitive reversed-phase HPLC method for the determination of ISM in the presence of its known process-related and degradation impurities. This will facilitate the determination of the absolute amounts of ISM in commercial preparations. In addition, the specificity of fluorescence detection in the analysis of ISM in Samorin® will be investigated.
2. Develop a planar chromatographic method, using HPTLC with UV densitometry, for the identification of ISM and its known related substances in commercial samples.
3. Modify and validate the British Pharmacopoeia (BP) 1993 method for the determination of ammonium chloride in urea, for use in determining the ammonium chloride content of commercial samples of ISM.
4. Design a protocol for establishing the chemical equivalence (or otherwise) of commercial preparations of ISM based on;

- determination of the absolute amount of the principal component (ISM)
- determination of the relative ratio of related substances
- content of the inorganic impurity (ammonium chloride).

2.2 Materials and Methods

2.2.1 Materials

Orthophosphoric acid (85% w/v), potassium dihydrogen orthophosphate, sodium thiosulphate (≥ 99.5% purity) and ammonium chloride (≥ 99.5% purity) were obtained from BDH Laboratory Supplies, Poole, UK. HPLC-grade methanol and acetonitrile were obtained from Merck Limited, Lutterworth, UK. Sodium nitroprusside (sodium nitroferricyanide (III) dihydrate, 99% A.C.S reagent) and sodium hypochlorite solution (10 - 15% available chlorine) were obtained from Sigma-Aldrich Co. Ltd., Dorset, UK. Sodium salicylate was obtained from Fisons Scientific Apparatus, Loughborough, UK. 3,8-Diamino-5-ethyl-6-phenylphenanthridinium bromide (ethidium, **I**) was a gift from Laprovet Ltd. (Tours Cedex, France). Samorin® Lot P517, 8-(*m*-amidinophenyldiazoamino)-3-amino-5-ethyl-6-phenylphenanthridinium chloride hydrochloride ((90%w/w), isometamidium, ISM, M&B4180A batch GHS331♣, **II**), 3-(*m*-amidinophenyldiazoamino)-8-amino-5-ethyl-6-phenylphenanthridinium chloride hydrochloride (M&B38897, **III**), 7-(*m*-amidino-phenyldiazo)-3,8-diamino-5-ethyl-6-phenylphenanthridinium chloride hydrochloride (M&B4250, **V**) and 3,8-di(3-*m*-amidinophenyltriazeno)-5-ethyl-6-phenylphenanthridinium chloride dihydrochloride (M&B4596, **IV**) were gifts from Rhône-Poulenc Rorer (Dagenham, UK). Samples (1g satchets) of Samorin® (Lots M157971, M158971, M159971 and M160971) and of Veridium® (Lots 1A2, 2A1, 3A1 and 4A1) were obtained from commercial sources.

♣ M&B4180 batch GHS331 (90%w/w ISM, < 0.1%w/w NH_4Cl) was used as the working standard of ISM.

Sodium thiosulphate solution (0.1M)

Sodium thiosulphate (12.41g) was placed in a 500ml volumetric flask and made up to volume with distilled water.

Standardisation of sodium thiosulphate (0.1M)

Sodium thiosulphate (25g) and sodium carbonate (0.2g) were placed in a 100ml volumetric flask and made up to volume with freshly boiled and cooled distilled water. Potassium bromate (0.2g) was placed in a 250ml volumetric flask and made up to volume with distilled water. Potassium iodide (2g) and 5ml of 2M HCl were added to an aliquot (50ml) of the potassium bromate solution. The resultant solution was titrated with sodium thiosulphate solution (0.1M) using starch mucilage, added towards the end of the titration, as indicator. Each ml of 0.1M sodium thiosulphate is equivalent to 0.002784g of potassium bromate.

Phosphate buffer (20mM, pH 3.0)

Phosphate buffer was prepared by accurately weighing KH_2PO_4 (1.36g) into a 500ml volumetric flask and making up to volume with water. The pH of the solution was adjusted to 3.0 with orthophosphoric acid.

Salicylate / Nitroprusside reagent:

Sodium salicylate (7.5g) and sodium nitroprusside (0.15g) were placed in a 100ml volumetric flask and made up to volume with water.

Sodium hypochlorite solution (containing 0.079%w/v free chlorine):

Potassium iodide (3g) was placed in a conical flask and 20ml of a 10%w/w solution of sulphuric acid and 20ml of water were added. Sodium hypochlorite solution (1ml, containing 10 -13% available chlorine) was added to the contents of the flask and the resultant solution titrated with sodium thiosulphate solution (0.1M) using starch mucilage, added towards the end of the titration, as indicator. The sodium hypochlorite solution was diluted to *V*ml with 0.2M sodium hydroxide to produce a solution containing 0.079%w/v free chlorine.

$$V = T \times F \times 4.49$$

Where T = volume (ml) of 0.1M sodium thiosulphate solution used in the titration and F = factor of sodium thiosulphate solution.

The factor of *4.49* was derived as follows;

1mole sodium thiosulphate ≡ 1mole chlorine

Moles of sodium thiosulphate used in titration = $F \times T \times 0.1 \times 10^{-3}$moles

$\Rightarrow$ 1ml of sodium hypochlorite solution ≡ $(F \times T \times 0.1 \times 10^{-3})$ moles of chlorine

≡ $(F \times T \times 0.1 \times 10^{-3} \times 35.5)$g of chlorine

$\Rightarrow$ concentration of available chlorine = $(T \times F \times 3.55 \times 10^{-1})$ % w/v

For *V* ml of solution containing 0.079% w/v of available chlorine, using 1ml of sodium hypochlorite solution;

$$V = ((T \times F \times 3.55 \times 10^{-1}) \times 1) / 0.079$$

$$V = T \times F \times 4.49$$

2.2.2 Sample preparation

Standard solutions of ISM:

For HPLC analyses, the standard sample of ISM (M&B4180A batch GHS331, 10 ± 1mg) was weighed accurately using a Mettler TA5 analytical balance (readability 0.01mg). The weighed sample was dissolved in, and made up to 100ml with a solution of 30%v/v acetonitrile in water to produce a 0.01%w/v stock solution. Portions of the stock solution were suitably diluted with 30%v/v acetonitrile in water to produce solutions containing 0.0001, 0.0002, 0.0003, 0.0004 and 0.0005%w/v ISM.

For HPTLC analysis, the standard sample of ISM (100mg) was weighed accurately into a 100ml volumetric flask and made up to volume with methanol to produce a 1mg/ml stock solution. Portions of the stock solution were suitably diluted with methanol to produce solutions containing 0.025, 0.050, 0.075 and 0.100mg/ml ISM.

Sample preparation for determination of ISM:

Samples (100mg) of Samorin® and Veridium® were weighed accurately, transferred to separate 100ml volumetric flasks and made up to volume with methanol. Aliquots (5ml) of these solutions were diluted to 100ml with 30% v/v acetonitrile in water. The resultant solutions were diluted ten-fold to yield solutions with a nominal concentration of 0.0005%w/v ISM. Solutions of the commercial samples of ISM were also prepared at a concentration of 0.002%w/v in 30% v/v acetonitrile in water for chromatographic profiling.

For qualitative HPTLC samples (100mg) of Samorin® and Veridium® were weighed accurately, transferred to 100ml volumetric flask and made up to volume with methanol to produce 1mg/ml solutions.

Standard solution of ammonium chloride:

Ammonium chloride (0.1g, W_{std}) was weighed accurately and placed in a 500ml volumetric flask; dissolved in and made up to volume with water. An aliquot (10ml) of the resultant solution was transferred to a 100ml volumetric flask and made up to volume with water.

Sample preparation for ammonium chloride determinations:

Commercial samples (0.05g, W_{sam}) containing ISM were weighed accurately into 250ml volumetric flasks, dissolved in and made up to volume with water.

2.2.3 Methods

2.2.3.1 NMR Spectroscopy

Proton NMR spectra were recorded on a Bruker AMX-400 FT-NMR spectrometer (Bruker Karlsruhe, Germany). Deuteriated dimethyl-sulphoxide (DMSO-d_6) was used as solvent and referenced at δ = 2.50ppm. Both one-dimensional and two-dimensional techniques were used in assigning signals due to ISM. Two-dimensional techniques used were; ^{1}H-^{1}H correlation spectroscopy (COSY) and ^{1}H-^{1}H nuclear Overhauser enhancement and exchange spectroscopy (NOESY).

2.2.3.2 HPTLC with UV densitometric detection

Solutions of samples were applied to Kieselgel 60 F_{254} plates (10 x 10cm, 0.25mm thickness, E. Merck, Darmstadt, Germany) using a Camag Nanomat III automatic spotter (Camag Ltd. Muttenz, Germany) equipped with 1µl capillary pipettes. Development of the plates was carried out in a Camag horizontal developing chamber (10 x 20cm; using 5min

for solvent saturation of the tank) at ambient temperature. A solvent system consisting of pyridine-acetonitrile-butanol-formic acid (6:6:4:1, v/v/v/v) was used.

A Shimadzu CS-9000 dual-wavelength flying spot scanner (Shimadzu Ltd., Kyoto, Japan) was used for densitometric evaluation of developed chromatograms with the following settings; photomode: absorbance/reflectance, λ: 320nm, Zero set mode: at start, beam size: 0.4 x 0.4mm, δy: 0.04mm, beam swing width: 4mm, scan-mode: zigzag, minimum spot-width detection: 0.1mm, minimum area: 0, smoothing: 7 points, output: area, b.c. accumulation: 8, linearizer: off.

2.2.3.3 HPLC

The chromatographic system consisted of a Spectra-Physics P100 isocratic pump equipped with a Spectra-Physics SP8450 UV-Vis detector (ThermoSeparations Inc., California, USA) set at 320nm. Fluorescence detection (λ_{exc}=385nm, λ_{em} = 590nm) was performed with a Model RF-530 fluorescence detector (Shimadzu Ltd, Kyoto, Japan). Chromatograms were acquired and analysed with a HP 3395 reporting integrator (Hewlett-Packard, Waldbronn, Germany) with the following settings; attenuation: 5, peak-width detection: 0.04, threshold: 4, chart speed: 0.3 cm/min. Chromatographic separations were performed at ambient temperature with a mobile phase flow-rate of 1ml/min. Samples were prepared in 30% v/v acetonitrile in water (see section 2.2.2) and injected through a fixed (20µl) PEEK or stainless steel loop (see section 2.3.3) onto a Lichrospher® 60 RP-select B column (Hewlett Packard, Waldbronn, Germany, 125 x 4mm i.d., 5µm particle size, 60Å) with a 20 x 2mm i.d. guard column packed with Lichrosorb C8, 5µm particle size (E. Merck, Darmstadt, Germany). The mobile phase was composed of acetonitrile- KH_2PO_4 (pH 3.0, 20mM)(22.5:77.5, v/v).

HPLC system suitability test

The suitability of the HPLC system was assessed using the following parameters;

1. Robustness of separation

The effect of small variations in the organic modifier content of the mobile phase (within a range of 20 to 30%v/v) on the degree of separation of the two positional isomers **II** and **III** was examined. That composition of the mobile phase which resulted in a R_S of ≥ 2.0 for the critical separation of **II** and **III** and a k_{max} of ≤ 15 was selected.

2. Linearity of detector response

This was determined by regression analysis of the relationship between the concentration of **II** and the detector response (peak area). The regression parameters used in confirming linearity were; low magnitudes of residuals, random distribution of data about the regression line and R^2 values of 0.99 or better.

3. Precision

This was determined as the relative standard deviation (RSD) of replicate injections ($n \geq 5$) of solutions of ISM (0.00025%w/v). A RSD of 2% or less was deemed suitable for quantitative analysis.

2.2.3.4 Colorimetric determination of ammonium chloride

Reference standard:

A volume (2ml) of a standard solution of ammonium chloride (Section 2.2.2) and 3ml of water were transferred to a 25ml volumetric flask. Salicylate/nitroprusside reagent (5ml) was added to the flask and the contents thoroughly mixed. An aliquot (5ml) of sodium

hypochlorite solution (containing 0.079%w/v free chlorine) was added and the resultant solution allowed to stand for 30min after mixing.

Reagent Blank:

The procedure for the reference standard was followed, replacing the standard solution of ammonium chloride with 2ml of water.

Sample:

An aliquot (1ml) of a sample solution (Section 2.2.2) and 4ml of water were transferred to a 25ml volumetric flask. Salicylate/nitroprusside reagent (5ml) was added to the flask and the contents thoroughly mixed. An aliquot (5ml) of sodium hypochlorite solution (containing 0.079%w/v free chlorine) was added. The resultant solution was shaken and allowed to stand for 30min.

Sample blank:

An aliquot (1ml) of sample solution and 9ml of water were transferred to a 25ml volumetric flask. An aliquot (5ml) of sodium hypochlorite solution (containing 0.079%w/v free chlorine) was added. The resultant solution was shaken and allowed to stand for 30min, after which the reaction mixtures were made up to volume with water and thoroughly mixed. The absorbances of the resultant solutions were measured against water at 665nm using 1cm glass cells. The content of ammonium chloride (% w/w) in the sample was calculated from the following equation;

$$\% \text{ Ammonium chloride} = [(A_{sam} - A_{sb} - A_{rb}) \times W_{std} \times 10] \div [(A_{std} - A_{rb}) \times W_{sam}]$$

Where

A_{sam} and A_{std} are the absorbances of sample and standard respectively

A_{sb} and A_{rb} are the absorbances of the sample blank and reagent blank respectively.

The assay for ammonium chloride was validated using the following criteria;

1. Establishment of a linear relationship between the amount of ammonium chloride present and the amount of indophenol (indicated by the absorbance of reaction mixture at 665nm) formed using regression analysis.
2. Interference from sample matrix was evaluated by measuring the absorbance at 665nm of sample solutions of Samorin® at the concentration used in the assay.
3. Efficiency of recovery of ammonium chloride from commercial ISM preparations spiked with different amounts of the analyte (1, 2, 3, 4, 5, 10 and 30%w/w).
4. Precision of replicate determinations (n = 5) of ammonium chloride in a commercial sample of ISM.

2.3 Results and Discussion

2.3.1 Confirmation of the structure of ISM.

The 400MHz ^{1}H-NMR spectra of ISM (batch GHS331) and that of the structurally related compound, EBr, are shown in Figures 2.4 and 2.5 respectively. All chemical shifts are reported in ppm with respect to the tetramethylsilane (TMS) signal used as an internal reference.

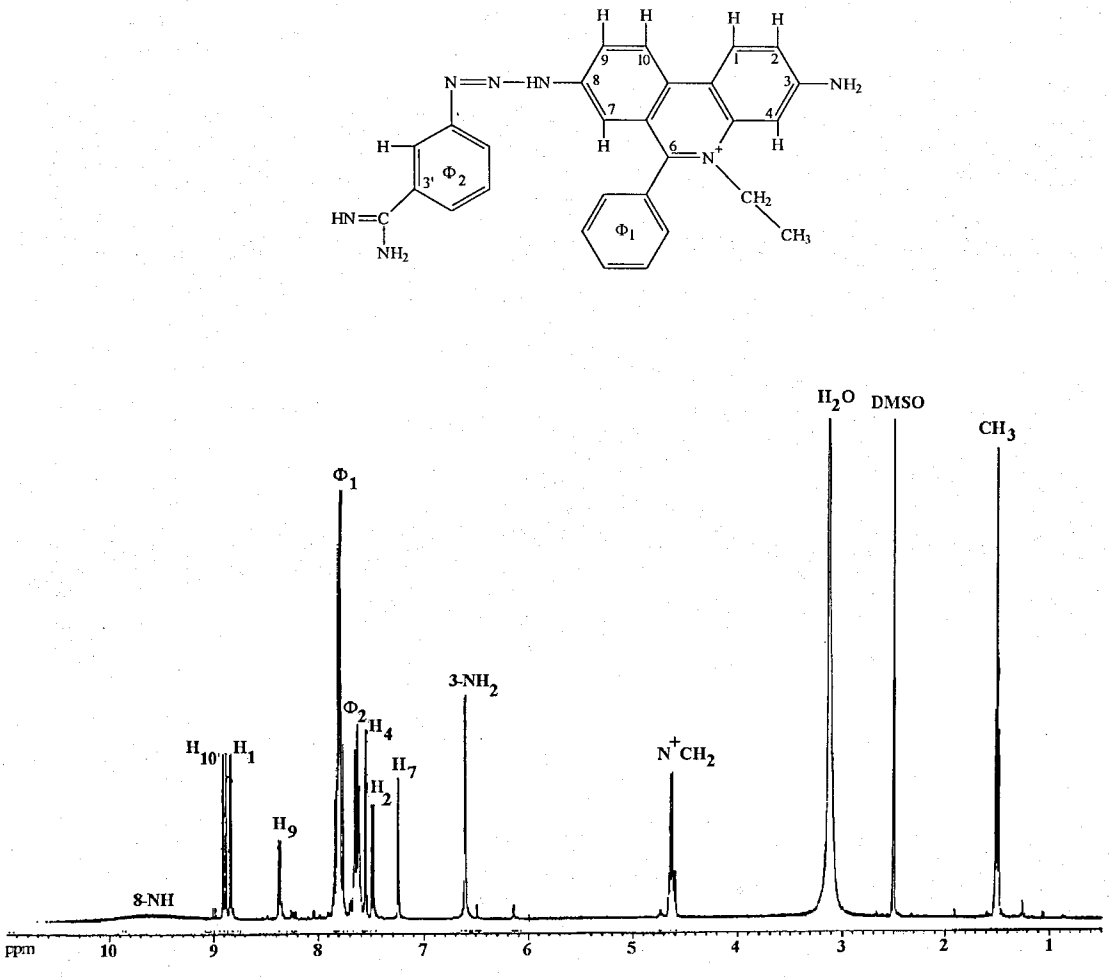


Figure 2.4 400MHz ^{1}H NMR spectra of ISM in DMSO-d_6; T = 330K

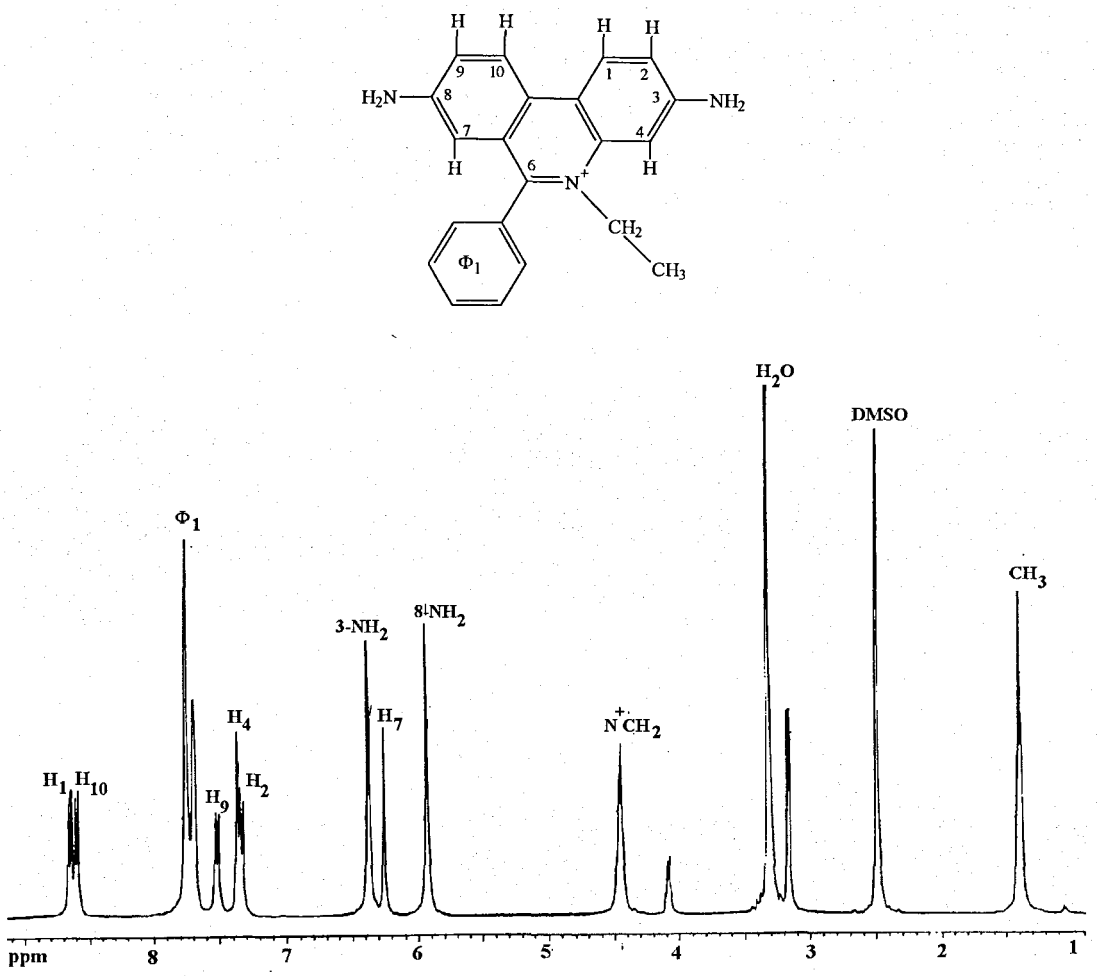


Figure 2.5 400MHz ^{1}H NMR spectra of EBr in DMSO-d_6; T = 330K

The ^{1}H NMR spectra of EBr and ISM were compared to facilitate assignment of the protons of the phenanthridine ring to the observed chemical shifts. The assignments for EBr (Figure 2.5) were consistent with those previously reported (Gaugain *et al.* 1981, Firth *et al.* 1983). Table 2.1 summarises the ^{1}H NMR chemical shifts for EBr and ISM. Substitution with the phenyldiazo moiety at the 8-amino group in ISM was reflected by the pronounced

deshielding effect on H_7 and H_9 and there is a similar effect on the proton(s) of the 8-amino group (*cf.* EBr, $\delta = 5.94$; ISM, $\delta = 9.60$, Figures 2.4 and 2.5). The proton at *C*-7 of EBr and ISM is shielded relative to that at *C*-9, which indicates (as anticipated) that Φ_1 is not co-planar with the phenanthridine ring. NOESY and COSY spectra of ISM (Appendix 1, Figures 2 and 3) confirmed the assignments for all the protons of the phenanthridine ring. The signal due to the protons of the phenyl group (Φ_2) of the *m*-amidinobenzenediazonium portion of the ISM molecule had an integral ratio of four, confirming di-substitution. The signal of these protons was manifest as two distinct resonances (Table 2.1). The more deshielded signal ($\delta = 7.76$) with a coupling constant of $J = 1.3$Hz provided evidence for *meta*-coupling in Φ_2. Because of the triazene bond, direct connections between the protons of the phenanthridine ring and those of Φ_2 could not be established. The resonances due to the amidino moiety on the Φ_2 ring were not manifest and would probably occur downfield of 10ppm (Figure 2.4).

Table 2.1 ^{1}H NMR chemical shifts (δ) for ISM and EBr in DMSO-d_6 solution

R	ISM	EBr
CH_3	1.50 (t)	1.41 (t)
CH_2	4.63 (q)	4.46 (q)
H_1	8.84 (d)	8.66 (d)
H_2	7.48 (dd)	7.34 (dd)
H_4	7.56 (d)	7.37 (d)
H_7	7.25 (d)	6.26 (d)
H_9	8.37 (dd)	7.52 (dd)
H_{10}	8.90 (d)	8.61 (d)
NH_2 (3)	6.61 (s)	6.38 (s)
NH_2 (8)	9.60 (broad)	5.94 (s)
Phenyl (Φ_1)	7.80 (m)	7.73 (m), 7.52 (m)
Phenyl (Φ_2)	7.77 (d, 1H), 7.76 (m, 3H)	

Where d = doublet, dd = doublet of doublet, t = triplet, s = singlet, q = quartet and m = multiplet.

2.3.2 HPTLC - UV Densitometry

Several solvent systems were evaluated in the separation of ISM and its related compounds on TLC plates coated with silica gel. The tenacity with which the phenanthridines bind to the silanol groups of silica was such that they could not be eluted with single solvents such as methanol. Successful planar chromatography required the presence of another quaternary ion in the mobile phase to compete with the phenanthridines for active silanol groups. However, compact spots were not obtained with solvent systems containing NH_4^+ because of the high water content. The pyridinium ion, which was generated *in situ* by the addition of formic acid and pyridine, proved to be a suitable ion to compete for silanol groups. The spots due to the analytes were compact, well separated and visible under normal light, since they were brightly coloured (Table 2.2). Analytes were identified by colour, direct comparison with authentic standards (**I**, **II**, **III**, **IV** and **V**, Figure 2.1) and reference to the published literature (Clarke, 1975, Kinabo *et al.* 1988a)

Table 2.2 HPTLC data of ISM (II) and related substances (I, III, IV and V)

Compound	Colour	R_f♣
I	Red	0.71 ± 0.02
II (ISM)	Orange	0.47 ± 0.02
III	Purple	0.35 ± 0.01
IV	Orange	0.53 ± 0.01
V	Yellow	0.22 ± 0.01

♣R_f values are mean ± SEM (n = 3)

The relationship between the amount of ISM applied to the plate (x μg), and the observed densitometer response (y) was established by non-linear regression analysis using a second-

order polynomial ($y = -ax^2 + bx + c$, where a and b are constants, Figure 2.6) with a mean correlation coefficient (R^2) of 0.999 (n = 4).

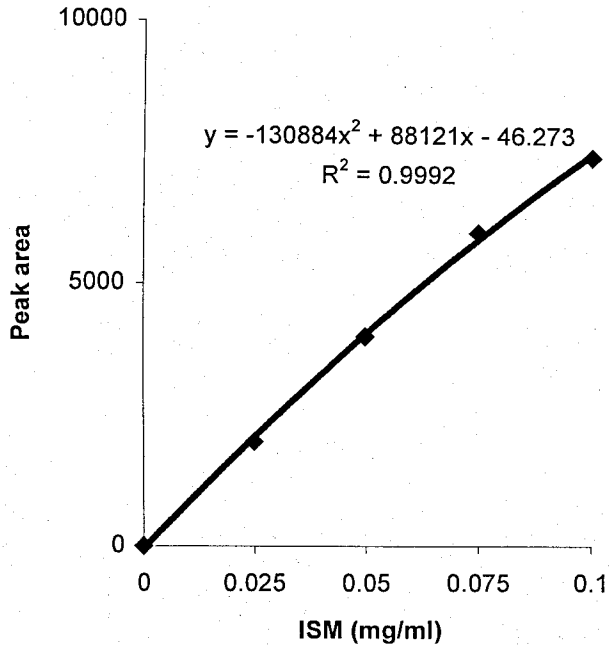


Figure 2.6 Second-order polynomial relationship between the concentration of ISM and the densitometric signal using the conditions described in Section 2.2.3.2.

The limit of detection of ISM using the parameters described in Section 2.2.3.2 was 20ng (S/N = 3). However, the non-linearity of calibration curves for **II** excluded the use of HPTLC in routine quantitative analysis. Calibration curves for *in situ* reflectance measurements are inherently non-linear (Poole *et al.* 1987). The linear dynamic range of the calibration curve is substance specific and, in favourable cases, may be adequate for quantitative analysis. Mathematical transformation of the data obtained with the densitometer using non-linear regression analysis based on second-order polynomials (as shown for ISM), or electronic transformations using the explicit hyperbolic solution of the

Kubelka-Munk model are required in the development of quantitative TLC methods, and these may introduce substantial error (Poole *et al.* 1987). However, the developed HPTLC method provides a rapid qualitative screen for the purity of bulk preparations of ISM and for the identification of the known related substances. A combination of this HPTLC method and HPLC (Section 2.3.3) would provide a definitive test for the presence of related substances in commercial preparations. Figure 2.7 shows a densitogram of the analysis of **II** in a typical manufacturing batch of Samorin® (Lot P517).

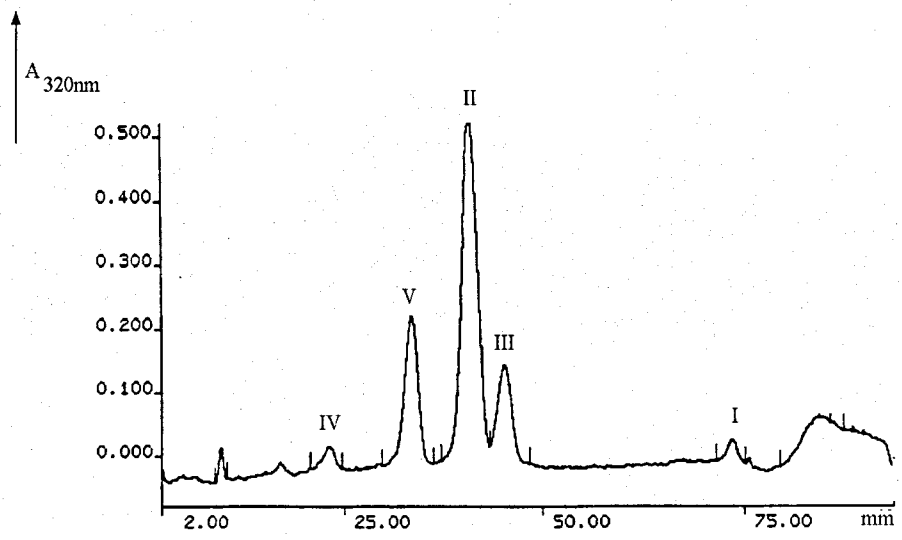


Figure 2.7 Densitometric scan of a high performance thin-layer chromatogram of ISM (II) and its related substances. Eluent system: pyridine-acetonitrile-butanol-formic acid (6:6:4:1 v/v/v/v). Other chromatographic conditions were as described in Section 2.2.3.2.

2.3.3 HPLC

The phenanthridines were separated successfully on a base-deactivated silica-based HPLC stationary phase (Lichrospher-60 RP-select B). When the pH of the mobile phase was 4.5 or greater, the strong residual interactions between the basic sites of the phenanthridine

compounds and silanols resulted in badly tailing peaks. The pH of the mobile phase was maintained at a value of 3.0 to suppress these interactions. Consequently, the stationary phase produced low peak tailing for **II** (asymmetry factor at one-tenth peak height ≅ 1.10 at pH 3.0) at a concentration of 0.0005%w/v. Optimum separation of all the constituents of Samorin® (Lot P517) was obtained with a mobile phase composed of acetonitrile-KH_2PO_4 (pH 3.0, 20mM)(22.5:77.5, v/v), with a $R_{S\ min}$ of 2.9 for the two isomers (**II** and **III**) and a k_{max} = 13 (compound **I**, t_0 = retention time of unretained acetonitrile). Figure 2.8 shows the separation of ISM and related substances in a typical manufacturing batch of Samorin® (Lot P517). Peaks were identified by comparison of their retention times with those of authentic standards (**I, II, III, IV** and **V**).

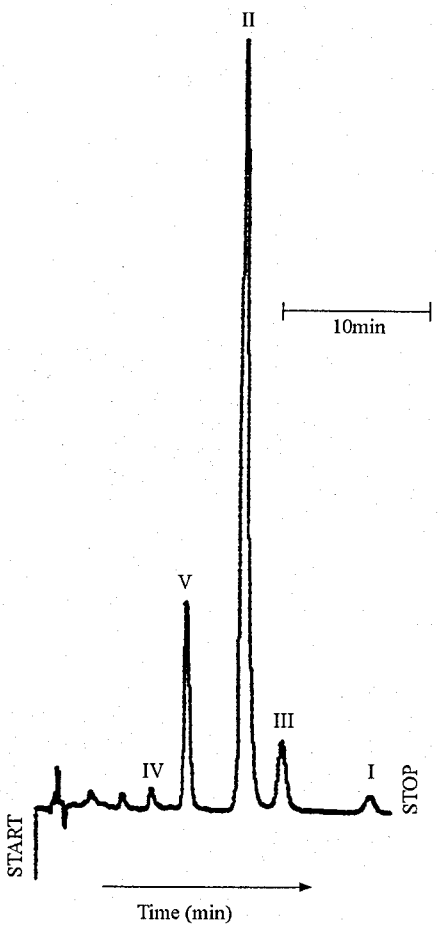


Figure 2.8 High performance liquid chromatogram of ISM (**II**) and related substances in Samorin® (Lot P517) using the conditions described in Section 2.2.3.3 with UV detection at 320nm. Mobile phase: KH_2PO_4 (pH 3.0, 20mM) (22.5:77.5, v/v). Retention times (min): **IV**, 7.7; **V**, 10.1; **II**, 13.6; **III**, 16.2; **I**, 21.9.

It was evident that when the content of acetonitrile in the mobile phase was below 22.5%v/v, the increase in analysis time was not paralleled by a marked improvement in the resolution between the two true positional isomers **II** and **III** (Figure 2.9). However, the separation between **II** and **III** was fairly robust and adequate for quantitative analysis.

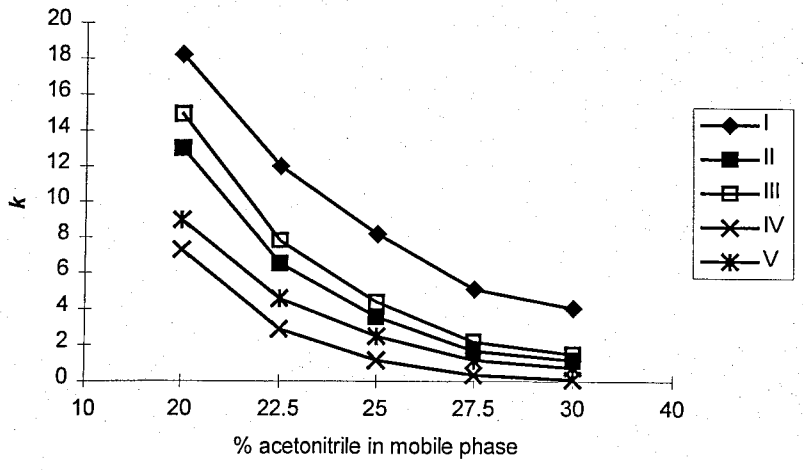


Figure 2.9 Effect of percentage of organic modifier in the mobile phase on ***k*** (capacity factor) for ISM and related substances.

The fluorescence parameters (λex = 385nm, λem = 590nm) used in previous studies (Zilberstein *et al.*, 1993, Wilkes *et al.*, 1995, Kinabo and Bogan 1988b) for the determination of ISM were evaluated for selectivity in the presence of the known related substances in Samorin®. The separated components of a solution of Samorin® (0.002%w/v) were monitored with UV (320nm, Figure 2.10a) and fluorescence detection (λex = 385nm, λem = 590nm, Figure 2.10b). Figure 2.10b was obtained with an increased integrator sensitivity (integrator attenuation = 1) compared with Figure 2.10a (integrator attenuation = 5). The peak areas of **II** and **III** using the different methods of detection are shown in Table 2.3.

Table 2.3 Comparison of peak area (arbitrary units) of **II** and **III** in the same solution of Samorin (Lot P517) measured by UV detection and by fluorescence detection.

	Fluorescence detection (λex = 385nm, λem = 590nm)	UV detection (λ = 320nm)
ISM (**II**)	209911	9061024
M&B 38897 (**III**)	414229	2110402

It was apparent when comparing the chromatograms (Figure 2.10) and the peak area measurements (Table 2.3) that the fluorescence detection conditions of λex = 385nm, λem = 590nm (Philips *et al*, 1967; Kinabo and Bogan, 1988b; Sutherland *et al*, 1991; Zilberstein *et al*., 1993; Wilkes *et al*, 1995) were not specific for **II**; that related substance **III** possessed a greater quantum yield of fluorescence (ϕ) than **II** and could contribute significantly in the analysis. Compound **IV** was detected at these fluorescence settings.

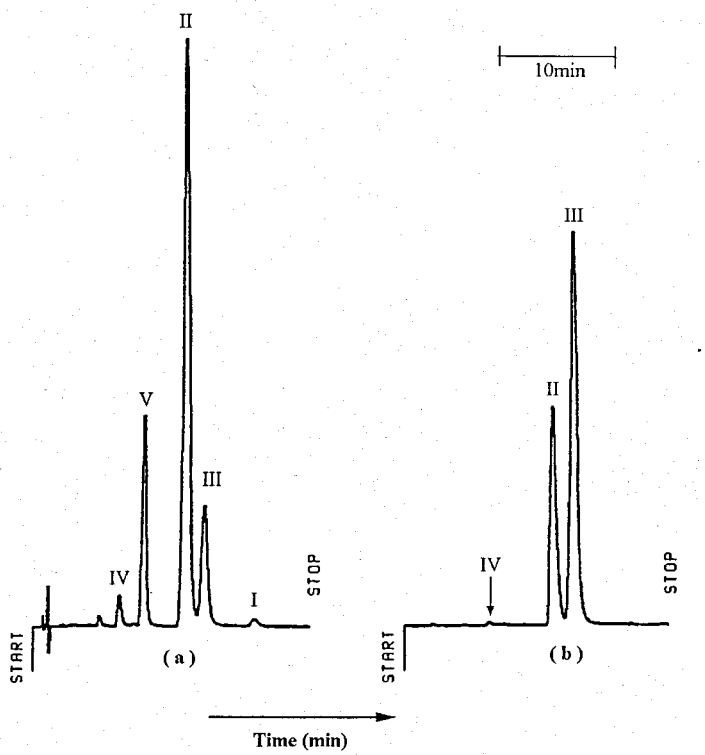


Figure 2.10 High performance liquid chromatogram of ISM (**II**) and related substances in the same sample solution of Samorin® (0.002%w/v, Lot P517) with (a) UV detection at 320nm, at an integrator attenuation of 5, and (b) fluorescence detection at an integrator attenuation of 1.

The superior sensitivity afforded by UV detection relative to fluorescence detection is consistent with the structure of **II**. The rotational freedom of the *m*-amidinobenzenediazo substituent increases the probability that excited molecules will decay to the ground state by rotational and vibrational transfer of energy, leading to a decrease in the probability of photon emission. This is supported by the observed increase in fluorescence emission of **II** in viscous solutions and after intercalation with DNA (Wilkes *et al.*, 1995). It is consistent also with reduced transfer of absorbed UV energy to emitted fluorescence in dilute solutions of **II**; due to considerable internal conversion processes, like free rotation of the substituent. Presumably, the participation of the lone pair of electrons on the NH group of the triazene in a resonance configuration with the phenyl ring of the amidinobenzene moiety (Figure 2.11) and also with the quaternary nitrogen (Figure 2.2) would result in a more rigid triazene bond in **III**. This would reduce the likelihood of free rotation, lower internal conversion processes and result in maximal conversion of absorbed UV radiation to emitted fluorescence.

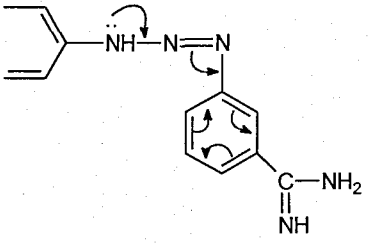


Figure 2.11 Participation of the lone pair of electrons on the NH group of the triazene in a resonance configuration with the phenyl ring of the *m*-amidinobenzene moiety.

Replicate injections of solutions of **II** using a sample injection loop made of stainless steel produced low precision (RSD > 10%, n = 10). An increase in loop-flush volume with solutions of **II**, below and up to the rated loop capacity (20µl), produced a corresponding linear increase in detector response (Figure 2.12).

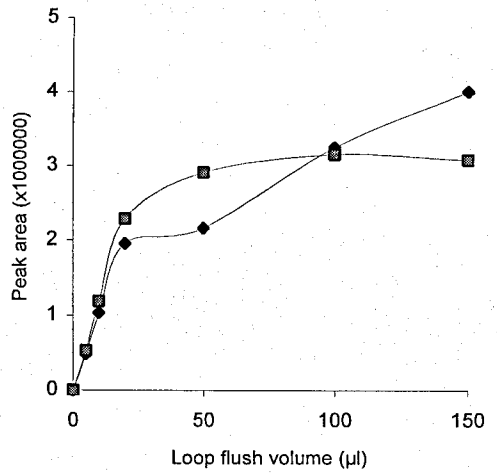


Figure 2.12 Effect of increasing loop flush volume of a solution of ISM (0.0003%w/v) in 30%v/v acetonitrile through injector and a 20µl loop made of stainless steel (◆) and PEEK (■). Mobile phase: acetonitrile-KH_2PO_4 (pH 3.0, 20mM) (25:75, v/v), flow rate 1.0ml/min. Chromatographic conditions are as described in Section 2.2.3.3.

However, with sample loop-flush volumes above the rated loop capacity, a further increase in detector response was observed; a feature characteristic of adsorption of the sample on loop surfaces (Dolan 1996, Lough *et al.* 1996, MacLeod *et al.* 1990). Peak symmetry and chromatographic resolution were not affected. The use of an injector loop made of polyetheretherketone (PEEK) improved the precision of replicate injections of solutions of **II** (RSD = 0.51%, n = 10). The effect of increasing sample loop-flush volumes up to, and above, the rated loop volume exhibited characteristics typical of an ideal non-

adsorbing loop with PEEK (Figure 2.12). Calibration solutions of **II** prepared within a range of 0.0001 to 0.0005% w/v and encompassing the nominal assay value of 0.00025%w/v were linear (r^2 = 0.997 ± 0.002 (mean ± SD), n = 5) with small standard residuals randomly distributed about the regression line (Figure 2.13). Inter- and intra-day assay variation were calculated as 0.87 and 1.30% respectively (RSD, n = 4). The limit of detection (three times the average noise level) of **II** was 45ng/ml. The limit of detection of the potential inteferent **III** in the HPLC analysis was determined as 25ng/ml.

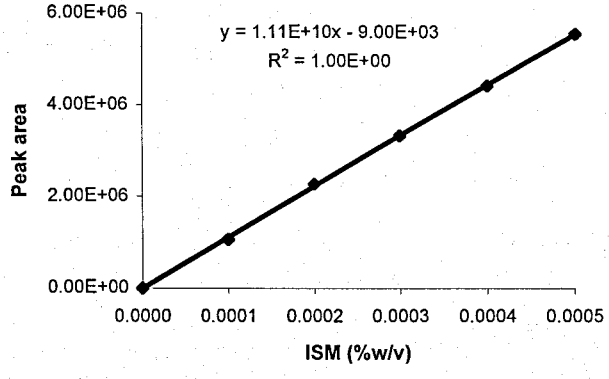


Figure 2.13 Relationship between the concentration of ISM and detector response (peak area) at 290nm.

2.3.4 Determination of ammonium chloride

The UV-Visible spectrum of the blue-green indophenol dye formed from sodium salicylate and ammonium chloride in the presence of sodium hypochlorite exhibited a broad absorption band with a maximum at 670nm (Figure 2.14), which was in agreement with the reported literature value of 667nm (Searle, 1984).

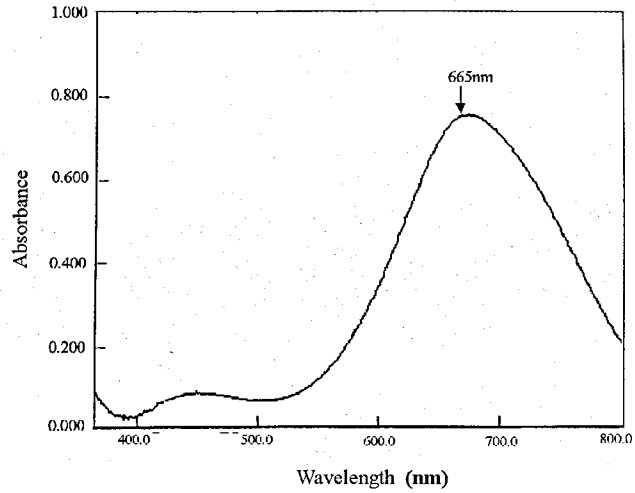


Figure 2.14 UV-Vis spectrum of the indophenol dye formed from sodium salicylate and ammonium chloride in the presence of sodium hypochlorite.

A rectilinear relationship ($R^2 = 1.000$, n = 2) was obtained between the absorbance of the indophenol and the concentration of ammonium chloride over the range 0 to 8.0µg/ml (Figure 2.15).

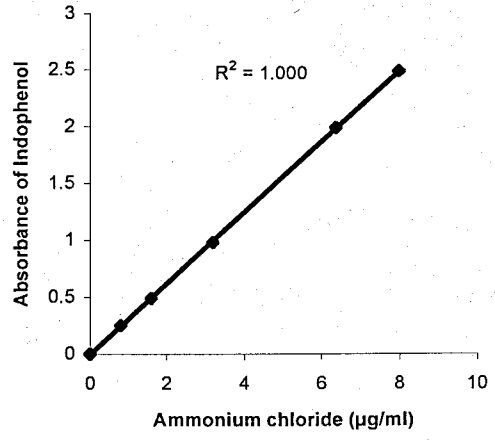


Figure 2.15 Relationship between the concentration of ammonium chloride and the absorbance (at 665nm) of the formed indophenol.

The possibility of interference from the other (i.e. organic) constituents of Samorin® in the colorimetric determination of ammonium chloride was examined by obtaining a visible (400 – 800nm) spectrum of a solution of Samorin® (Lot P517) with the same concentration used in the assay (0.0008%w/v). At a wavelength of 665nm, the absorbance due to Samorin® was shown to be negligible (Figure 2.16).

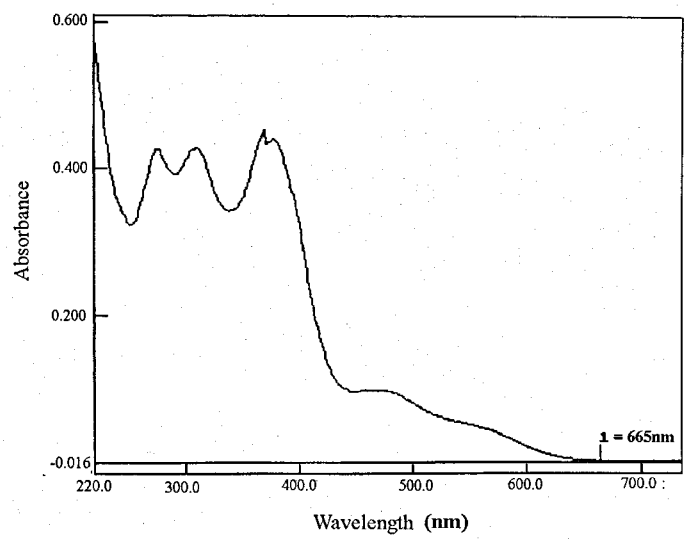


Figure 2.16 UV-Visible spectrum of Samorin® solution (0.0008%w/v), illustrating the negligible absorbance at 665nm.

A rectilinear relationship (r^2 = 1.000, n = 2) between absorbance readings at 665nm and the content of ammonium chloride (in spiked samples of the bulk preparation) within the range 0 to 40%w/w was obtained. Low magnitudes of residuals were observed about the regression line. Recoveries of ammonium chloride at concentrations of 1, 2, 3, 4, 5, 10, and 30%w/w in spiked samples of Samorin® (batch P517) (corrected for matrix effect) were in all cases more than 92% (Table 2.4). The precision of replicate determinations at a low concentration of ammonium chloride (0.2%w/w) in Samorin® (batch P517) was illustrated by a low spread of values (0.21 ± 0.03 (mean ± SD, n = 5)).

Table 2.4 Recovery of ammonium chloride from spiked samples of Samorin® (batch P517)

Ammonium chloride added (%w/w)	Ammonium chloride recovered (%w/w)
1.00	0.92
1.99	1.89
2.95	2.97
3.98	3.91
4.98	4.98
9.83	9.63
30.39	30.52

2.3.5 Analyses of batches of Samorin® and Veridium®

The methods developed for the analysis of ISM (Section 2.3.3) and the determination of ammonium chloride (Section 2.3.4) were applied to different batches of Samorin® and Veridium®. Data concerning the content of ISM and that of ammonium chloride in the batches of Samorin® and Veridium® are given in Table 2.5. The results are the average of two determinations.

Table 2.5 Content of ISM (**II**) and ammonium chloride in different batches of Veridium® and Samorin®.

Sample	ISM (%w/w)	NH_4Cl (%w/w)
Samorin Batch M157971	59.0	0.1
Samorin Batch M158971	60.4	0.1
Samorin Batch M159971	59.0	0.2
Samorin Batch M160971	61.7	0.2
Veridium Lot 1A2	74.5	0.9
Veridium Lot 2A1	53.4	25.8
Veridium Lot 3A1	53.6	27.1
Veridium Lot 4A1	38.6	38.9

Samorin® demonstrated a batch to batch consistency with respect to the content of ISM of 60.1 ± 1.3%w/w (mean ± SD, n = 4). In contrast, Veridium® demonstrated considerable batch to batch variations with the content of ISM ranging from 38.6 to 74.5%w/w (55.0 ± 14.8% (mean ± SD, n = 4)). The wide spread of values observed for the batches of Veridium® (relative standard deviation of 26.8%) compared with the batches of Samorin® (relative standard deviation of 2.2%) may illustrate the difficulty of controlling the manufacturing process and the concomitant implications for the composition of the product.

Representative chromatograms obtained for solutions of samples (0.002%w/v) of batches of Samorin® and Veridium® (in 30%w/v acetonitrile in water) are shown in Figure 2.17.

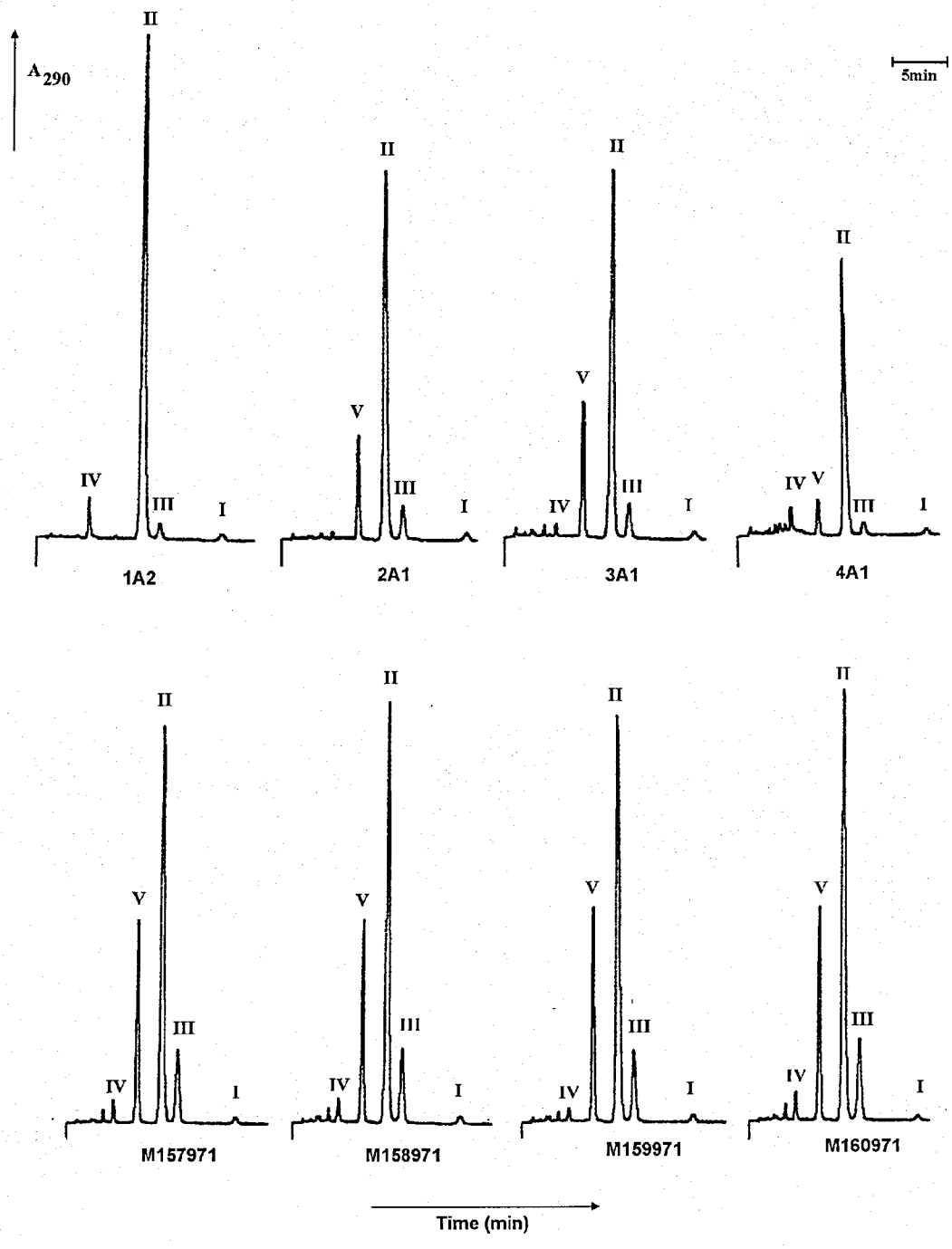


Figure 2.17 HPLC profiling of ISM (**II**) and related substances (**I**, **III**, **IV** and **V**) in different batches of Samorin® and Veridium® using a 125 x 4mm Lichrospher Select-B column (mobile phase: 25%v/v acetonitrile in 20mM phosphate buffer, adjusted to pH 3 with orthophosphoric acid). The upper and lower panels show chromatograms obtained with 20µl injections of 0.002%w/v solutions of Veridium® (Lot 1A2, 2A1, 3A1 and 4A1) and Samorin® (Batch M157971, M158971, M159971 and M160971) respectively. Chromatograms were acquired at the same attenuation.

The response factors of the three major related substances (**III**, **IV** and **V**) in commercial samples were determined relative to that of ISM (**II**). A summary of relative response factors (mean ± SD, n = 4) for the three major related substances in each proprietary brand of isometamidium is presented in Table 2.6.

Table 2.6 Relative response factors of ISM-related substances in Veridium® and Samorin®.

	Relative response factors (%) of related substances		
Sample	M&B38897 (**III**)	M&B4596 (**IV**)	M&B4250 (**V**)
Samorin® Batch M157971	20.1	2.2	36.9
Samorin® Batch M158971	20.2	3.9	32.4
Samorin® Batch M159971	20.7	3.7	36.6
Samorin® Batch M160971	19.4	4.1	35.0
Veridium® Lot 1A2	3.4	4.7	0.0
Veridium® Lot 2A1	9.7	0.0	19.0
Veridium® Lot 3A1	9.5	2.1	26.3
Veridium® Lot 4A1	5.2	6.3	9.0

The relative response factors of **III**, **IV** and **V** were consistent in all the different batches (n = 4) of Samorin® analysed with relative standard deviations of 2.7%, 24.8% and 5.8% respectively. In contrast, there was a significant inter-batch variation in the relative response factors between the four batches of Veridium® with relative standard deviations of 45.4%, 85.5% and 84.7% for **III**, **IV** and **V** respectively.

In Veridium Lot 1A2, whilst the content of **II** was high (74.5%w/w), **V** could not be detected. However **V** was present in varying amounts in the other three batches. These differences probably reflect the consequences of poor control of the reaction conditions such as pH, which determines the relative ratios of **II** and **V** (Berg, 1963a, 1963b). It is abundantly clear on examination of the chromatographic profiles (Figure 2.17) of the four

batches of Samorin® and of Veridium® that there are significant differences between the compositions of the batches of products made by the two manufacturers. One of the basic tenets in rational chemotherapy is to achieve optimum concentrations of the therapeutic agent at the desired site of action. Therefore, the use of substandard (relative to an innovator product of proven efficacy) drugs in anti-parasitic chemotherapy may lead to the development of drug-resistant strains, since the parasites are only exposed to sub-lethal doses as a result of an alteration in the pharmacokinetic profile of the drug.

The content of ammonium chloride in four batches of Samorin® and of Veridium® is given also in Table 2.5. It was consistent and less than 0.30%w/w (maximum of 0.21%w/w) in the four batches of Samorin® analysed. However, the samples of Veridium® showed a wide range (0.9 - 38.9%w/w) for the content of ammonium chloride.

2.4 Conclusions

Since its synthesis about four decades ago, the potential for the manufacturing process of ISM to result in the formation of other related substances has been investigated (Berg 1960, 1963a and 1963b, Brown *et al.*, 1961, Berg *et al.*, 1963). However, the lack of methods capable of determining ISM in the presence of its known related substances was a source of concern. With the introduction of generic forms of the innovator product, Samorin®, and the rising incidence of resistance to all the available trypanocides, it is vital to maintain the therapeutic efficacy of ISM preparations. Consequently, it is vital to ensure that formulations of ISM from different manufacturers (and, in extreme cases from the same manufacturer) are chemically equivalent.

This chapter describes the development of an HPLC method for the determination of ISM, in the presence of its known related substances. A mobile phase consisting of

acetonitrile and phosphate buffer with a Lichrospher-60 Select-B column separated ISM (**II**) and its process-related and degradation impurities (**I**, **III**, **IV** and **V**). The method described is simple, selective, precise, and suitable for quality control of the bulk drug substance. The fluorescence parameters (λex = 385nm, λem = 590nm) which have been employed by previous workers for the analysis of ISM, are not selective for ISM in the presence of related substances. The work presented in this thesis has shown that chromatographic separation of the components of ISM prior to UV or fluorescence detection provides selectivity in the analysis of ISM. It has been shown also, that UV detection is more sensitive than fluorescence detection in the determination of ISM in solution.

An HPTLC method using ultraviolet detection and a pyridine-based solvent system has been described for the separation of ISM and related substances. The method provides a rapid screen for the identification of related substances in commercial preparations of ISM.

The Berthelot reaction has been adapted and validated for the determination of the inorganic impurity, ammonium chloride, in commercial samples of ISM. The method is accurate and not subject to matrix interference.

The HPLC method for the determination of ISM and the modified Berthelot reaction for the determination of ammonium chloride have been amalgamated into a protocol for establishing the chemical equivalence of preparations containing ISM. The use of this protocol in the chromatographic profiling, analysis of the principal component (**II**) and determination of ammonium chloride in different batches (n = 4) of Veridium® and Samorin® has illustrated the implications of the complex nature of the manufacture of **II** and the difficulties in its control. The innovator product, Samorin®, which has been on the market for over three decades, shows consistency (quantitatively and qualitatively) of the components in the batches analysed (n = 4). In contrast, there are batch-to-batch (n = 4)

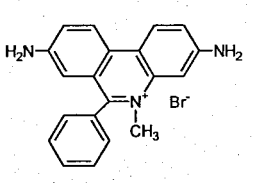
variations in the nature, and the relative response factors of related substances, the content of **II** and ammonium chloride in Veridium®. In the light of these results, this protocol should be of value in making policy decisions over sources of supply of this valuable product. The HPLC method also provides a basis for the development of specific and selective bioanalytical methods for ISM, which will facilitate the interpretation of experimental pharmacological and biological data.

CHAPTER 3: METABOLISM OF THE TRYPANOCIDAL PHENANTHRIDINES

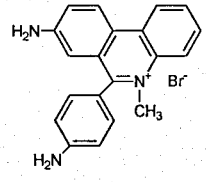
3. METABOLISM OF THE TRYPANOCIDAL PHENANTHRIDINES

3.1 Introduction

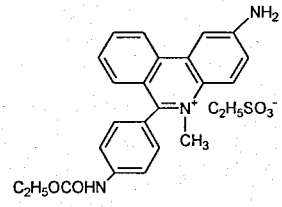
The trypanocidal activity of the phenanthridinium compounds was first discovered by Browning *et al.* (1938). It was found that two compounds, phenidium (8-amino-5-methyl-6-(p-aminophenyl)-phenanthridinium bromide, Figure 3.1) and dimidium (3,8-diamino-5-methyl-6-phenylphenanthridine bromide, Figure 3.1), were valuable for veterinary practice; but treatment with dimidium was often followed by delayed toxic effects (which became manifest 2-3 months later) and proved a serious disadvantage (Hawking 1965).



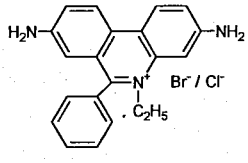
Dimidium (Browning *et al.* 1938)



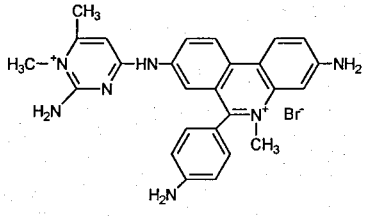
Phenidium (Browning *et al.* 1938)



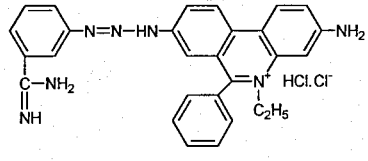
Carbidium (Goodwin et al. 1950)



Ethidium (Watkins 1952)



Prothidium (Watkins and Woolfe 1956)



Isometamidium (Wragg 1958, Berg 1960)

Figure 3.1 The trypanocidal phenanthridines

Watkins (1952) introduced ethidium bromide (EBr) as a substitute for dimidium. In this compound the methyl group on the quaternary nitrogen of dimidium had been replaced by an ethyl group and the change seemed to be effective in retaining activity and preventing the delayed toxic effects (Watkins & Woolfe 1952). More active members of the series have

been prepared by attaching a structural unit typical of part of some other trypanocidal compound to one of the amino groups (Dann 1975) - thus isometamidium (ISM) has a substituent corresponding to a substantial part of the diminazene molecule (Figure 1.2).

EBr and ISM remain the only aromatic phenanthridinium compounds currently used for the treatment of animal trypanosomiasis in affected areas worldwide (Wang 1995). These compounds have been used extensively since the 1960s, but their usefulness has been greatly reduced owing to widespread trypanosomal resistance. EBr is used routinely in research laboratories for staining nucleic acids because of its fluorescence and nucleic acid-intercalating properties (Waring 1966), yet its mechanism of trypanocidal action is not well understood (Wang 1995). Similarly, the mechanism of trypanocidal action of ISM is not understood; although the mechanism of resistance has been associated recently with reduced accumulation of the drug in trypanosomes (Sutherland *et al.* 1991). As yet it is not known whether or not the metabolism of these compounds by animals (and/or by the trypanosome) contributes to the trypanocidal activity or to the development of resistance.

3.1.1 Metabolic fate of the phenanthridines

The earliest reported investigation into the metabolic fate of the phenanthridinium salts was on the distribution and excretion of carbidium (2-amino-6-p-carbethoxyaminophenyl-5-methylphenanthridinium ethanesulphonate, Figure 3.1) in rats and rabbits (Goodwin *et al.* 1950). Carbidium was assayed in terms of diazotizable amino groups and no effort was made to determine whether or not metabolites were present. The assay procedure (which involved heating bile and tissue samples in 1M HCl at 80°C for 5 minutes) would have hydrolyzed possible metabolites (e.g. amino conjugates). Attempts were made to detect metabolic products of prothidium (Figure 3.1) in various tissues and

fluids (Taylor 1960), but the practice of homogenizing tissue samples in 1M sulphuric acid and using the ultra-violet absorption spectrum of prothidium (λmax = 315nm in 0.5M sulphuric acid) as a criterion that the substance present in extracts of the biological matrices was identical to prothidium would have hydrolysed any amino conjugates.

Kandanswamy and Henderson (1963) investigated the metabolic fate of EBr in mice and Ehrlich ascites carcinoma cells using [^{14}C-6]-labelled EBr. After intraperitoneal administration of EBr, ethanolic extracts of tumour, liver and urine were analysed by descending paper chromatography on Whatman No.1 paper with isopropanol-HCl, isopropanol-NH_3 and sodium acetate-HCl systems. Radioactivity measurements failed to detect any metabolites and they concluded that all of the recovered compound(s) could be accounted for as unmodified EBr. Philips *et al.* (1967) investigated the physiological disposition and cellular localisation of ISM in rats, dogs and monkeys. In this study, no metabolites of ISM were detected in serum or plasma except after oral administration to rats, where the drug produced ethidium due to cleavage of the acid-labile triazeno group.

On the basis of the studies above, it was assumed that the trypanocidal phenanthridinium compounds were not metabolised in mammals and that their pharmacological actions were due to the parent compounds. MacGregor and Clarkson (1971) investigated the nature of the biliary metabolites of three phenanthridinium salts (3,8-diamino-6-p-aminophenyl-5-methylphenanthridinium chloride, EBr and carbidium (Figure 3.1)) in rats with ligated renal pedicules. Of the 15mg/kg dose of EBr administered, 50 - 55% was recovered in the bile in 16 - 18 hours and the parent compound accounted for 20 - 25% of the compounds recovered. Two metabolites were found and it was proposed that these were monoacetyl conjugates of ethidium, since they could be diazotized and coupled with *N*-(1-naphthyl)ethylenediamine hydrochloride by the method of Bratton and

Marshall (1939) without prior acidic hydrolysis. The two metabolites accounted for 65 - 70% and 10% of the compounds recovered. Therefore, it was proposed that *N*-acetylation of the available amino groups of the phenanthridines was the principal route of metabolism. This study by MacGregor and Clarkson (1971) completely changed the concept of previous investigators that phenanthridinium salts were not biotransformed in mammalian systems.

To date, the most comprehensive study performed on the metabolism of a phenanthridinium compound concerned the metabolism of EBr in rats (Fraire *et al.* 1981). In this study, an attempt was made to purify and identify the biliary metabolites of the drug in control and pre-treated rats, in order to account for metabolism by the hepatic cytochrome P450 dependent mixed-function oxidases (MFO), and to specify unequivocally the position of acetylation. The biliary excretion products recovered after intravenous administration of EBr to rats consisted of the unmodified drug, 8-acetylethidium and an *O*-glucuronide (which only occurred in rats treated with Aroclor-1254 and 3-methylcholanthrene (3-MC)). It was established by spectroscopic analysis that the aglycone of the *O*-glucuronide was 2-hydroxy-8-acetylethidium (Gaugain *et al.* 1981). This study specifically excluded the possibility of the formation of 3-acetylethidium and 3,8-diacetylethidium which, in view of the previous study by MacGregor and Clarkson (1971), raised uncertainties as to the extent and nature of the metabolism of EBr in rats, both *in vivo* and *in vitro*. A possible pathway for biochemical transformation was proposed (Figure 3.2).

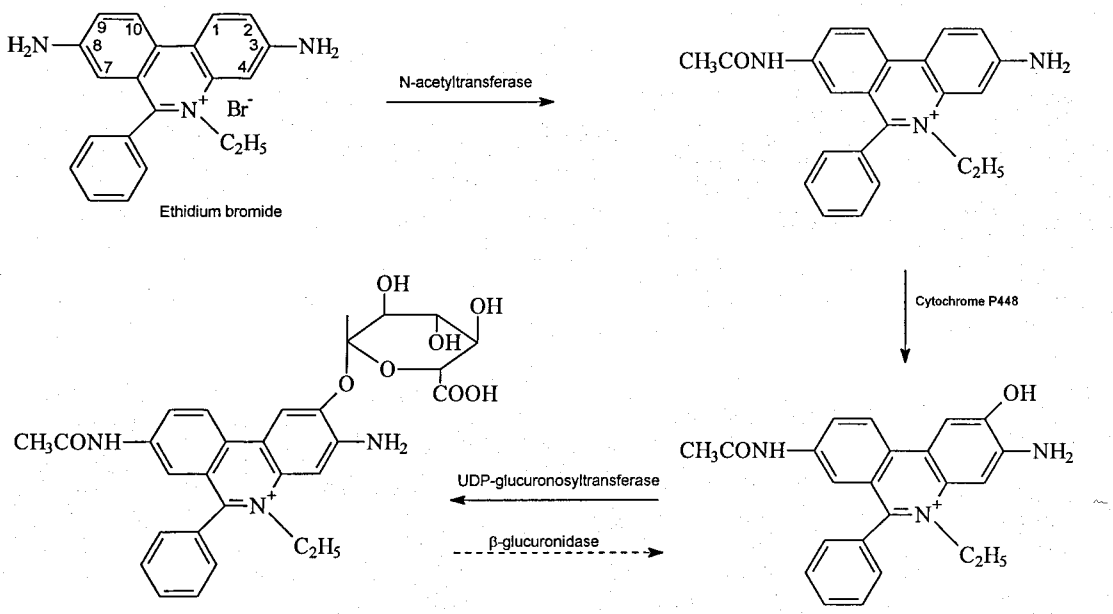


Figure 3.2 Proposed pathway for the metabolism of ethidium bromide in rats (Gaugain *et al.* 1981).

It has been shown that EBr and other phenanthridinium compounds undergo *in vitro* metabolic activation to one or more reactive species, which were mutagenic in *Salmonella typhimurium* (MacGregor and Johnson 1977, Lecointe *et al.* 1981). Lecointe *et al.* (1981) confirmed an earlier report by MacGregor and Johnson (1977) concerning the requirement for NADP(H) in the formation of reactive species and further demonstrated that hepatic microsomal metabolism of EBr required the activity of the MFO system, which is induced by polycyclic aromatic hydrocarbons, like Aroclor-1254. To date the specie(s) responsible for the mutagenicity of ethidium after metabolic activation has/have not been identified.

Following the reports of the acetylation of EBr in rats (MacGregor and Clarkson 1971, Fraire *et al.* 1981), Gilbert and Newton (1982) investigated the metabolic fate of EBr in rabbits and calves. Gel chromatography and TLC of urine from both species and of bile from rabbits demonstrated the presence of two acid-labile non-acetylated derivatives of EBr (which accounted for 38% and 8% of the eluted radioactivity) following intramuscular

administration of [^{14}C-6]-EBr (1mg/kg). The study specifically excluded the presence of acetyl derivatives of EBr in rabbits and calves and this was confirmed by Murilla *et al.* (1996a) also in the faeces of cattle. These investigations highlight the difficulties brought about by extrapolation of results from one species of animal to another. The non-acetylated metabolites of EBr reported by Gilbert and Newton (1982) have not been further investigated.

Following the parenteral administration of ISM to cattle (Kinabo and Bogan 1988d, Mdachi *et al.* 1991, Murilla *et al.* 1996b) and rats (Philips *et al.* 1967), metabolites could not be detected in plasma or serum. After administration of ISM to rats by intragastric intubation, the drug itself was not present in urine or in the liver (Philips *et al.* 1967). However, the total amount of ISM recovered in the faeces was equivalent to 30-38% of the administered dose and the remainder was due to ethidium. The conversion of ISM to ethidium after intragastric intubation was explained by cleavage of the acid-labile triazene bond due to the acidic environment of the stomach. This explanation was supported by the observation that ISM (which is unchanged in phosphate buffer pH 7.5) is rapidly converted to ethidium in acetic acid (0.1M) (Philips *et al.* 1967). To date, no evidence has been provided to suggest that there is metabolic cleavage of the triazene bond in ISM to form ethidium.

The previous investigations into the metabolic fate of the phenanthridines, especially EBr, has brought to light many unresolved issues. These include the extent of *N*-acetylation of EBr (MacGregor and Clarkson 1971, Fraire et al. 1981), the nature of the non-acetylated acid-labile metabolites described by Gilbert and Newton (1982) and the participation of the MFO system in the metabolism of the phenanthridines (MacGregor and Johnson 1977,

Lecointe *et al.* 1981). Questions that remain unanswered as a result of the previous studies include;

1. Are there inter- and intraspecies differences in the acetylation of EBr?
2. Are the two "non-acetylated, acid-labile" metabolites described by Gilbert and Newton (1982) indicative of unstable amino-conjugates (which are likely to be glucuronides)?
3. Is ISM converted to ethidium by the liver?

Therefore, there is a need to investigate the metabolic fate of the aromatic phenanthridines, particularly EBr, using an appropriate model system for metabolism and suitable modern analytical techniques.

3.1.2 Selection of an *in vitro* model system to investigate metabolism

While the whole animal approach (*in vivo* studies) in large food animals can provide essential metabolic data, it suffers from a number of setbacks. The usual analysis of plasma, urine, milk or faeces may fail to detect a metabolite(s) of pharmacological or toxicological significance and this problem is exacerbated when testing highly toxic compounds (Shull *et al.* 1987). EBr and ISM possess toxicity (with LD50 (mg/g) of 0.07 and 0.30, respectively) in mice when administered by the subcutaneous route (Wragg *et al.* 1958). Since the administered dose must be curtailed to avoid intoxication of the host, metabolites of pharmacological and toxicological significance may be below the limit of detection of most methods.

The various alternatives to the whole animal approach in metabolism include; organ perfusion, subcellular fractions and isolated cells. The isolated liver perfusion approach is as non-flexible as that using the whole animal in that only one set of variables can be studied in a single liver (Shull *et al.* 1987). The most common method for studying xenobiotic

metabolism in any species *in vitro* is by the use of subcellular fractions (homogenates, S-9, and microsomes). Subcellular fractions provide valuable information concerning the biochemical, physiological and pharmacological mechanisms regulating xenobiotic metabolism. Methodologically, it is a simple, inexpensive and versatile approach requiring small quantities of tissue. However, subcellular fractions may represent an unacceptable model for the prediction of biotransformations *in vivo*. The most serious limitations arise from the loss of intracellular compartmentalisation, control mechanisms and essential inter-relationships between various subcellular organelles and between cytosolic and microsomal enzyme systems (Shull *et al.* 1987). Subcellular organisation may play an important role in the regulation of xenobiotic metabolism in the intact animal. Mitochondrial control over levels of cofactors which, in turn, affects xenobiotic metabolism in the endoplasmic reticulum, is inoperative in subfractions (Shull *et al.* 1987). It is thought that the metabolism of xenobiotics *in vivo* is both limited by and regulated by the supply of cofactors and also by competing reactions. In investigations using subcellular fractions, an excess of cofactors is added normally to achieve optimum activity and generally these do not correspond with the natural physiological levels. This may result in the formation of metabolites which may be of minor importance *in vivo*. Other limitations include loss of the natural relationships between phase I and phase II enzymes and the regulatory influence of xenobiotic translocation into and out of intact cells.

The use of isolated hepatocytes has become an increasingly valuable method for the assessment of xenobiotic metabolism and toxicity *in vitro* (Berry and Halls 1992). Some of the characteristics which make isolated hepatocytes an excellent approach for studying xenobiotic metabolism *in vitro*, are:

- Maintenance of complete cellular organisation, including the various metabolic enzymes, cofactors, membranes and organelles.
- Control of hormonal or homeostatic mechanisms by manipulation of the environment occupied by the cells.
- Study of both phase I and II metabolism, including the normal sequence of reactions, inter-relationships of multiple pathways or substrates, rate-limiting steps, or relationships with other biochemical processes (such as those involved in energy and the biosynthesis of cofactors).
- Assessment of the relationship between metabolism and toxic manifestations by measuring effects on viability, functions or morphology of the cells.
- Possibility of examining a wide array of experimental variables using cells prepared from one liver.

Since the first successful isolation of viable hepatocytes from rat liver was reported (Berry and Friend 1969), high yield preparations from various species have been achieved. In studies comparing biotransformation of xenobiotics in different species, the isolated hepatocyte appears to provide a rapid, efficient and low-cost means of predicting species differences in metabolism (van't Klooster 1992). Previous pharmacokinetic studies on the phenanthridines (Philips *et al.* 1967, Gilbert and Newton 1982, Kinabo and Bogan 1988d, Murilla *et al.* 1996a, Murilla *et al.* 1996b) in different species identified the liver as the principal repository for administered doses. Therefore, the use of isolated hepatocytes presents an appropriate model for estimation of the metabolic fate of a large proportion of the administered dose of the phenanthridinium trypanocides.

3.1.3 Potential pathways for the biotransformation of the trypanocidal phenanthridines

The pathways of the metabolism of xenobiotics are conventionally divided into two distinct groups; phase-I (functionalisation or non-synthetic) and phase-II (conjugation or synthetic) reactions. Phase-I metabolism reactions result in the exposure or introduction of hydrophilic groups and are mediated principally by oxidation (including aromatic ring hydroxylation, *N*- and *O*-dealkylation, sulphoxide formation), and to a lower extent reduction, hydrolysis and hydration. The phase-II reactions principally involve conjugation with glucuronic acid but acetylation, methylation, sulphation, condensation and conjugation with glutathione, fatty acids and amino acids occur also. Generally, phase-II reactions remove or mask functional groups (e.g. amino, carboxyl, hydroxyl, and sulphydryl) on the drug or phase-I metabolite by the addition of an endogenous substrate (Tilstone and Stead 1986).

The chemical structure of the prototype trypanocidal phenanthridine, EBr, presents a unique potential for a wide array of biotransformation pathways which include; phase-I reactions (e.g. hydroxylation of the aromatic phenanthridine ring, de-ethylation of the quaternary nitrogen) and phase-II conjugation reactions (e.g. glucuronidation, acetylation, sulphation and methylation of the aromatic amino groups) (Gibson and Skett 1994).

Aromatic ring hydroxylation

Hydroxylation is a common phase-I reaction for drugs and xenobiotics containing an aromatic ring. This is exemplified by the conversion of lignocaine to its 3-hydroxy derivative (Gibson and Skett 1994) and, perhaps more appropriately, the extensive biotransformation of phenanthridine to 1-hydroxy-, 2-hydroxy-, 10-hydroxy-, 1,2-

dihydroxy-, and 9,10-dihydroxyphenanthridine in rats (Lavoie *et al.* 1985). The cytochrome P450 catalysed mixed-function oxidase (MFO) system (found primarily in the endoplasmic reticulum of liver and also in the kidney, lung and intestine) is responsible for these hydroxylation reactions, which proceed according to the following stoichiometry

$$NADPH + H^+ + O_2 + RH \xrightarrow{\text{Cytochrome P-450}} NADP^+ + H_2O + ROH$$

where RH represents the oxidisable drug substrate and ROH the hydroxylated metabolite; the overall reaction being catalysed by the enzyme, cytochrome P450.

The cytochrome P450s exist as multiple forms or isoenzymes. These isoenzymes are classified into gene families and gene sub-families on the basis of their amino acid sequence homology. Cytochrome P450s belonging to one gene family exhibit more than 40% amino acid sequence similarity. The amino acid sequences of members of a gene sub-family are more than 70% identical (Gibson and Skett 1994). The existence of multiple forms of cytochrome P450s provides an explanation for the substantial differences in drug metabolism observed as a function of sex and species variability, ontogeny, health status and dietary factors. Enzyme inducers have been used to classify the cytochrome P450 enzymes into several groups, named after a prototype inducer, such as 3-MC and phenobarbitone (Table 3.1).

Table 3.1 Classification of cytochrome P450s based on prototype inducers

Enzyme inducer	Cytochrome P450 species induced
Polycyclic aromatic hydrocarbons (e.g. 3-MC)	CYP 1 family (1A1, 1A2) and CYP 2A subfamily
Phenobarbitone	CYP 2B, 2A, 3A and 2C subfamilies
Ethanol	CYP 2E subfamily
Peroxisome-proliferator (e.g. clofibrate)	CYP 4A subfamily (e.g. 4A1, 4A2, 4A3)
Glucocorticoids (e.g. pregnenolone)	CYP 3A subfamily

Adapted from van't Klooster 1992, Gibson and Skett 1994.

Glucuronidation

Glucuronidation is an important phase-II reaction in drug metabolism which involves the transfer of the glucuronic acid group from uridine diphosphate (UDP) glucuronic acid (UDPGlucA) to a nucleophilic acceptor group on a substrate. Substrates susceptible to glucuronidation contain hydroxyl (alcohols, phenols, hydroxylamines), carboxyl (aromatic, arylalkyl), amino (aromatic amines, sulphonamides) and sulphydryl (thiols) functional groups. The reaction is catalysed by the UDP-glucuronosyl transferases (UDPGTs; EC 24.1.17) which exist as a multigene family, resulting in a range of isoenzymes, each possessing different, but closely related, physical and catalytic properties. The UDPGTs are classified into two broad families, UGT1 and UGT2, on the basis of similarities in their amino acid sequences, analogous to the classification of cytochrome P450 enzymes. Within a family, the UDPGT protein sequences exhibit greater than 55% homology (Clarke and Burchell 1994). Although substrate specificity allows some discrimination, it is not unequivocal because of the wide overlap between the forms of UDPGTs (Mulder 1992). At least eleven isoenzymes of each of the two subfamilies, UGT1 and UGT2, have been purified to homogeneity in man (Burchell *et al.* 1995). UDPGTs are mainly localised in the membrane of hepatic endoplasmic reticulum and also occur in other tissues such as the kidney, small intestine, lung, skin, adrenals and spleen.

Induction of UDPGT activity toward different groups of substrates by either phenobarbitone or 3-MC indicates that there are distinct differences between the activities of the UDPGT forms concerned. Generally in the rat, polycyclic aromatic hydrocarbons (e.g. 3-MC and β-naphthoflavone) induce UDPGT activity with respect to simple flat planar substrates or phenolic compounds (e.g. 1-naphthol, *N*-hydroxy-2-naphthylamine, or 3-

hydroxy-benzo[α]pyrene), the process being dependent on the aromatic hydrocarbon (Ah) receptor as the analogous induction process for the cytochrome P450 1A subfamily. Phenobarbitone-type compounds increase UDPGT activity with respect to a number of structurally diverse compounds, which include morphine, chloramphenicol, testosterone and bilirubin. The steroidal compound, pregnenolone-16α-carbonitrile, induces UDPGT activity with respect to digitoxigenin monodigitoxoside (Clarke and Burchell 1994).

The mechanism of the reaction catalysed by UDPGTs is a SN_2 reaction; the acceptor group of the substrate attacking *C*-1 of the pyranose ring of UDPGA, which results in the formation of a glucuronide (Figure 3.3).

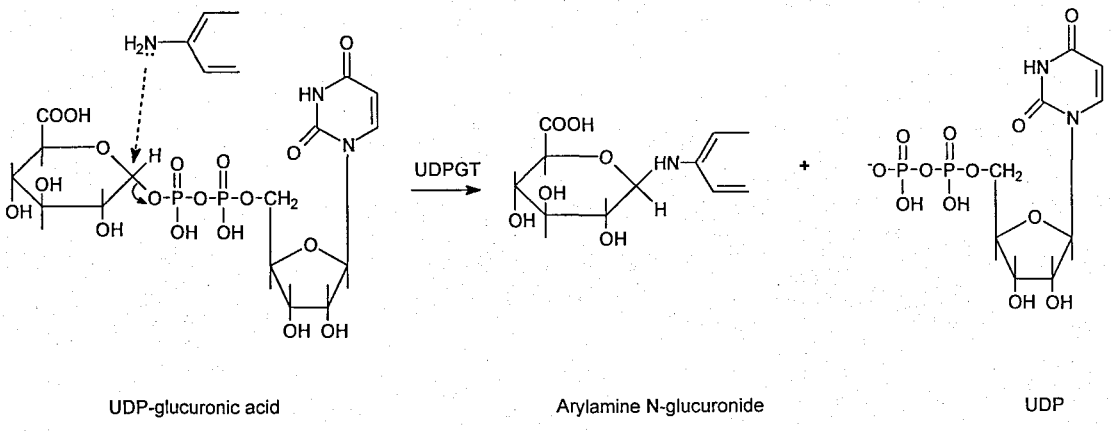


Figure 3.3 Glucuronidation of an arylamine catalysed by uridine diphosphate (UDP) glucuronosyl transferase (UDPGT).

Generally the resultant glucuronide is more water-soluble, has reduced biological activity and is more easily excreted than the parent compound. Glucuronidation of drugs can lead also to enhanced biological activity; as reported for morphine-6-glucuronide (which is about 800 times more active than morphine when administered intrathecally) and for the

glucuronidation of steroids at the 17β position (which produces cholestatic conjugates of endogenous estradiol and ethinylestradiol) (Mulder 1992).

Acetylation

Acetylation, another phase-II reaction, of the primary amino group of arylamines (e.g. aniline), sulphonamides (e.g. sulfanilamide) and hydrazines (e.g. isoniazid) is a major route of biotransformation of these substances. Acetylation in humans and animals involves the enzymic transfer of the acetyl group from endogenous acetyl coenzyme A (AcCoA) to molecules which contain a primary amine (Figure 3.4), a hydroxyl or a sulfhydryl group (Weber *et al.* 1990). The acetylation of choline and coenzyme A are examples of endogenous substances which undergo transfer of an acetyl moiety to a hydroxyl and sulfhydryl group respectively.

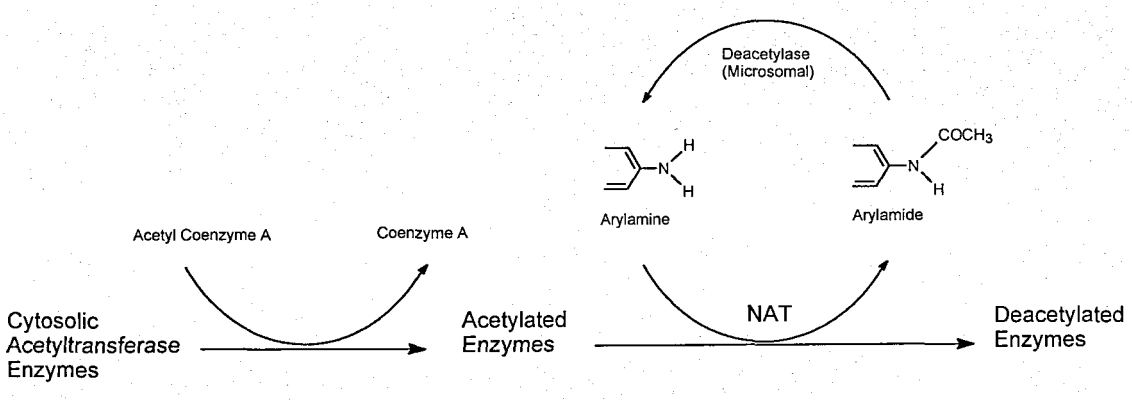


Figure 3.4 Acetylation of an arylamine catalysed by *N*-acetyltransferase (NAT).

Genetic polymorphism occurs with the acetylation of xenobiotics. This has led to the designation of 'slow' and 'fast' acetylator phenotypes in mammals including man, rabbit, rat and hamster (Timbrell 1991). Normally the anti-tuberculosis drug, isoniazid, is

biotransformed and eliminated from the body by *N*-acetylation. With the 'slow acetylator' phenotype, the build up of the unchanged drug after the administration of otherwise therapeutic doses leads to side effects of central stimulation and peripheral neuritis. However, 'fast' acetylators are more susceptible to drug-induced hepatic damage (Gibson and Skett 1994). It has been shown that induction effects on *N*-acetyltransferases do not occur with the classical inducers (phenobarbitone and 3-MC). However, it has been reported that glucocorticoids such as hydrocortisone, and immunostimulants, such as zymosan have an inducing effect on *N*-acetyltransferase activity (Weber *et al.* 1990).

The phenanthridines may undergo other metabolic reactions also (such as *N*-de-ethylation, *N*-methylation and *N*-sulphation), but these may represent minor pathways (Gibson and Skett 1994).

3.1.4 Pesticides as potential inducing agents in the metabolism of the phenanthridines

It is well established that inducing agents of the 3-MC or phenobarbitone type cause a selective increase in the activity of distinct forms of microsomal cytochrome P450 and thus stimulate different monooxygenase activities. The differential induction of isoenzymes of UDPGTs by 3-MC and phenobarbitone is well documented also (Clarke and Burchell 1994) and, therefore, these two agents (3-MC and phenobarbitone) are classified as bi-functional inducers. The major inductive effect of phenobarbitone on cytochrome P450 and UDPGT is to increase specific mRNA levels by augmenting transcription, rather than stabilising pre-existing levels of protein precursors or increased translational efficiency, which leads to an increased synthesis of the protein (Clarke and Burchell 1994, Gibson and Skett 1994). Specific cytoplasmic or nuclear receptors for phenobarbitone have not been identified to date. In contrast to induction of drug-metabolising enzymes by phenobarbitone, it is thought

that induction by polycyclic aromatic hydrocarbons (e.g. 3-MC, dioxin) is associated with a specific cytosolic receptor, termed the aromatic hydrocarbon (Ah) receptor. The inducer-receptor complex is translocated to the nucleus of the hepatocyte, induction-specific mRNA is transcribed from DNA and this is followed by increased *de novo* protein synthesis (Park *et al.* 1996). It has been reported that environmentally applied chemicals, including pesticides (such as DDT and its analogs (e.g. methoxychlor) and dieldrin) possess phenobarbitone-type-inducing properties (Lilienblum *et al.* 1982, Gibson and Skett 1994, Li *et al.* 1995, de Sousa *et al.* 1997). Pesticides (such as diflubenzuron (benzoylurea), tetrachlorvinfos (organaphosphorous), cypermethrin (pyrethroid) and carbaryl (carbamate)) possess 3-MC-type-inducing properties as a result of a dose-dependent increased accumulation of mRNA encoding for the CYP1A enzymes.

Pesticides are intended to kill pest populations by toxic and other deleterious mechanisms. In order to control the spread of trypanosomiasis in affected areas the insect vector, *Glossina spp.*, has been treated with pesticides such as DDT (2,2-bis[p-chlorophenyl]-1,1,1-trichloroethane), dieldrin (1,2,3,4,10,10-hexachloro-exo-6,7-epoxy-1,4,4a,5,6,7,8a-octahydro-1,4-endo,exo-5,8-dimethanonaphthalene), deltamethrin® and endosulfan (Thiodan®, 6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6-9-methano-2,4,3-benzodiaxathiepin 3-oxide) (Williams *et al.* 1993, Barrett 1997, Holmes 1997). Ironically, the beneficial effect of pesticides in eliminating the insect vector is accompanied by substantial detrimental effects to humans, food animals and the environment (Matsumura *et al.* 1984). Generally, exposure of man and animals to pesticides is classified as direct or indirect.

1. *Direct exposure* to pesticides occurs via the oral, respiratory or dermal routes. It may involve high or low doses and the effects may be acute or chronic. The use of pesticides

in the control of trypanosomiasis by persistent aerial and ground spraying (Williams *et al.* 1993, Barrett 1997) and by direct application to cattle (Holmes 1997) represents instances of *direct exposure*.

2. *Indirect exposure* to pesticides occurs as a result of residues of pesticides in food, air and drinking water. The effects are normally chronic in nature. The quantities of pesticides involved may be small (e.g. soil residues of 0.75-2.03ppm of DDT (Matsumura *et al.* 1984)). However, the duration of exposure makes the impact of *indirect exposure* significant.

Although the concentration of pesticides to which humans and animals are directly or indirectly exposed may be low, lipophilic substances such as DDT, dieldrin, aldrin and endrin accumulate in adipose tissue. Subsequently, these substances are released into the systemic circulation over a long period of time because of their lengthy biological half-lives e.g. $t_{0.5}$ of 115days and 1year for DDT and dieldrin respectively (Hathway 1984). This produces a prolonged state of enhanced drug metabolism in humans and animals and may affect the pharmacological or toxicological fate of administered drugs. The half-life of DDT in the adipose tissue of a human subject exposed to the pesticide was 3.7 years (Hathway 1984) and this exemplifies the enormous pharmacological and toxicological implications of exposure to these biologically active lipophilic substances.

Lake Victoria in East Africa (one of the regions which has been exposed to huge deposits of pesticides), which is the world's second largest fresh water lake, spans a land area of approximately 69,482 sq. km (source, Microsoft Encarta® 96). This lake is drained by the River Nile, the longest river in the world, with a river basin of 3,349,000 sq. km. Together, Lake Victoria, the River Nile and minor sources (like the Congo and Zambezi Rivers) flow through several countries from Zambia through Ethiopia to Egypt. Therefore,

the possible environmental impact of massive pesticide contamination in Central Africa may be experienced in quite remote areas (as far as the Nile basin) and illustrates a typical instance of *indirect exposure* to pesticides in drinking water. Increased metabolism of trypanocides by animals exposed to pesticides may result in low circulating concentrations of the agent due to rapid elimination. Therefore, the trypanosomes would be exposed to sub-lethal doses, which may lead to the development of resistant strains of the parasite. Interestingly, it has been reported that trypanosomes which are resistant to the available trypanocides are present in a region from Central Africa (Peregine 1991) to the Ghibe Valley in Ethiopia (Codjia *et al.* 1993); regions which could be classified as having experienced *direct* or *indirect* exposure to pesticides. Although a link between the use of pesticides and development of resistance by the parasite may be speculative, it is essential to investigate the effect of inducers on the metabolism of phenanthridinium trypanocides.

3.1.5 Localisation of phenanthridines in hepatocytes; use of confocal laser scanning microscopy (CLSM)

The phenanthridines possess an intense intrinsic fluorescence because of their planar structure. This property has been exploited in image analysis to study the accumulation of ISM in trypanosomes (Sutherland *et al.* 1991) and intracellular localisation in different tissues obtained from rat (Philips *et al.* 1967). The combined use of isolated hepatocytes and confocal laser scanning microscopy (CLSM) is an attractive method for investigating the differences and similarities in the intracellular localisation of the phenanthridines.

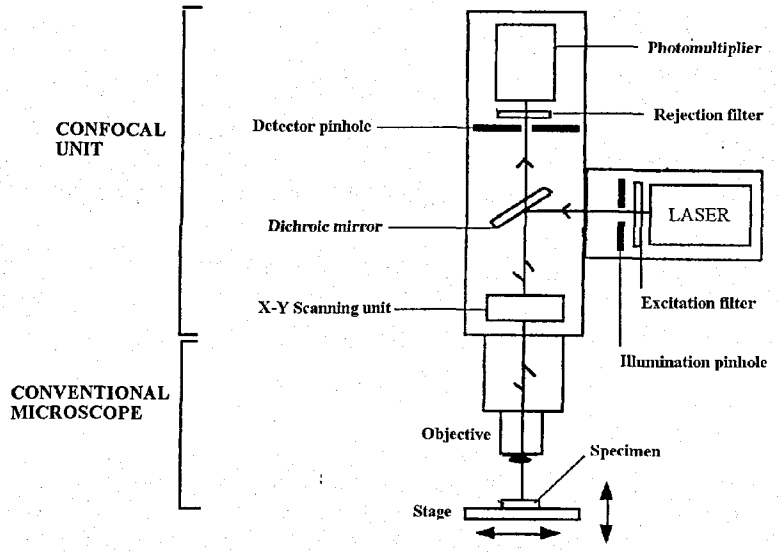


Figure 3.5 A typical CLSM system

In CLSM (Figure 3.5), a laser light beam is converted into a scanning beam by an X-Y deflection mechanism and focused to a small spot by an objective lens onto a fluorescent specimen. The mixture of reflected light and emitted fluorescent light is captured by the same objective and (after conversion into a static beam by the same X-Y scanner device) is focused onto a photodetector via a dichroic mirror. The dichroic mirror deflects the reflected light while the emitted fluorescent light passes through in the direction of the photomultiplier. A confocal aperture (pinhole) is placed in front of the photodetector such that the fluorescent light from points on the specimen (that are not within the focal plane where the laser beam was focused) are largely obstructed. In this way, out-of-focus information, above and below the focal plane, is greatly reduced. This is important when dealing with thick specimens. The spot that is focused on the centre of the confocal aperture is referred to as the "confocal spot". A two-dimensional image of a small partial volume of the specimen centred around the focal plane (referred to as an *optical section*) is generated

by performing a raster sweep of the specimen at that focal plane. As the laser scans across the specimen, the analog light signal detected by the photomultiplier, is converted into a digital signal, contributing to a pixel-based image displayed on a computer monitor attached to the CLSM. The relative intensity of the fluorescent light (emitted from the laser-hit point) corresponds to the intensity of the resulting pixel in the image and therefore allows quantitation of fluorescence. A three-dimensional image of a specimen can be reconstructed by stacking two-dimensional sections collected in series. The advantages of CLSM over conventional fluorescence microscopy include; a high signal-to-noise ratio, high resolution and acquisition of well-resolved images from specimens at various depths because of the rejection of out-of-focus fluorescence information. This technique should help in obtaining more reliable information on the intracellular localisation of the phenanthridines.

3.1.6 Aims and objectives

The previous sections have highlighted the paucity of understanding of the metabolism of the phenanthridine trypanocides. The potential for environmental chemicals, such as pesticides, to alter the metabolic fate of these therapeutically important compounds has been defined. Therefore, the present study proposes to;

1. Investigate the use of CLSM in identifying differences and similarities in the intracellular localisation of EBr and ISM in rat hepatocytes. This should provide a suitable analytical model for future studies on the intracellular localisation of these agents in resistant and non-resistant trypanosomes.
2. Define the metabolism of the prototype phenanthridine, EBr, by isolated rat hepatocytes. The use of authentic synthetic standards of the acetyl-derivatives of ethidium should help to resolve the impasse resulting from the studies of MacGregor

and Clarkson (1971) and Fraire *et al.* (1981) on the extent of acetylation of the compound.

3. Evaluate the adequacy of the rat model of metabolism by investigating the metabolism of EBr by hepatocytes obtained from pigs and sheep. This should highlight the interspecies differences and similarities in the metabolism of EBr.
4. Determine the effect of classical inducers (e.g. phenobarbitone and 3-MC) of xenobiotic metabolism on the metabolism of EBr using rat hepatocytes. Particular attention would be given to metabolic pathways relevant in target animals (such as pigs and sheep). This should be a good indicator of the possible effects of pesticides on the metabolism of EBr by ruminants.
5. Investigate whether or not there are structural requirements (e.g. presence of a free 8-amino group) for the metabolism of the phenanthridines. The metabolism of ISM (mol. wt. 460, with a substituted 8-amino group) and that of the structurally similar isomer M&B4250 (mol. wt. 460, with a free 8-amino group) will be examined. This should give further insight into the reported lack of metabolism of ISM.

3.2 Materials and Methods

3.2.1 Materials

Standard compounds

Ethidium Bromide

Ethidium bromide (3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide, EBr) was a gift from Laprovet, France. The purity was determined by TLC (Gilbert and Newton 1982, butanol-acetic acid-water (4:1:1 v/v/v), single red spot with R_f = 0.21) and HPLC (Section 3.2.2.11). There was no secondary spot or peak upon TLC and HPLC analysis respectively. The structure was confirmed by FAB-MS (*m/z* E^+ = 314, Appendix II) and 400MHz ^{1}H-nmr spectroscopy (Firth *et al.* 1983, Section 2.3.1).

Isometamidium chloride hydrochloride (ISM)

Isometamidium chloride hydrochloride (Batch GHS3331, 90%w/w) was a gift from Rhône Poulenc Rorer, Dagenham, England. The purity was determined by HPLC and HPTLC (Sections 2.3.2 and 2.3.3). There was no secondary spot or peak upon HPTLC and HPLC analysis respectively. The structure was confirmed by 400MHz ^{1}H-nmr spectroscopy (Section 2.3.1).

M&B4250

M&B4250 (7-(*m*-amidinophenylazo)-3,8-diamino-5-ethyl-6-phenylphenanthridinium chloride hydrochloride) was a gift from Rhône-Poulenc Rorer, Dagenham, England. The presence of the *m*-amidinophenylazo moiety at the *C*-7 position was confirmed by 400MHz

^{1}H-nmr spectroscopy (downfield shift and change in multiplicity of the signal corresponding to the 9H proton of the phenanthridinium ring [δ (DMSO) = 7.88ppm, doublet] compared to those of EBr [δ (DMSO) = 6.26ppm, doublet of doublets], Table 3.2, Appendix I). The 9H proton in ethidium showed an *ortho*-coupling to the 10H proton (J = 9.2) and a small *meta*-coupling to the 7H proton (J = 2.0). In comparison, the 9H proton in M&B4250 showed an *ortho*-coupling to the 10H proton (J = 9.4) in M&B4250 and *meta*-coupling was absent, confirming substitution at the C-7 position.

3,8-Diacetylethidium bromide

3,8-Diacetylethidium bromide was synthesised according to the published method of MacGregor and Clarkson (1971). EBr (100mg) was dissolved in 5ml of water and the solution was warmed to 40°C. Pyridine (250µl) was added, followed by 20ml of acetic anhydride, and the solution maintained at 40°C for 30min, when it was allowed to cool and the yellow precipitate collected and dried at 60°C. It was dissolved in methanol (HPLC grade, 2 x 50ml) and the solution evaporated to dryness at 40°C under reduced pressure to give 110mg (90%) of yellow crystals (m.p 270°C with decomposition). Diacetylation of EBr was confirmed by FAB-MS (*m/z* [E^+ - 2H] + 2(CH_3CO) = 398, Appendix II) and 400MHz ^{1}H-nmr spectroscopy (Appendix I). Diacetylation of the 3- and 8-amino groups of EBr resulted in a downfield shift of the signals due to the 2H, 4H, 7H and 9H protons and of the 3- and 8-NH protons relative to those in EBr (Table 3.2).

Table 3.2 ^{1}H NMR chemical shifts (δ) for 3,8-diacetylethidium, 8-acetylethidium, M&B4250 and ethidium in DMSO-d_6 solution

R	3,8-Diacetyl-ethidium	8-Acetyl-ethidium	M&B4250	Ethidium bromide
CH_3	1.52 (t)	1.44 (t)	1.40 (s)	1.41 (t)
CH_2	4.65 (q)	4.48 (q)	4.40 (q)	4.46 (q)
H_1	9.10 (d)	8.78 (d)	8.78 (d)	8.66 (d)
H_2	8.19 (dd)	7.40 (dd)	7.44 (dd)	7.34 (dd)
H_4	9.03 (d)	7.43 (d)	7.44 (d)	7.37 (d)
H_7	7.90 (d)	7.82 (d)		6.26 (d)
H_9	8.44 (dd)	8.30 (dd)	7.88 (d)	7.52 (dd)
H_{10}	9.08 (d)	8.81 (d)	8.81 (dd)	8.61 (d)
CH_3CO (3-NH)	2.22 (s)			
NH_2 (3)	10.83 (s)	6.57 (s)		6.38 (s)
CH_3CO (8-NH)	2.03 (s)	2.00 (s)		
NH_2 (8)	10.50 (s)	10.35 (s)		5.94 (s)

Where d = doublet, dd = doublet of doublet, t = triplet, s = singlet, q = quartet and m = multiplet.

8-Acetylethidium bromide

8-Acetylethidium bromide was synthesised according to the published method of Walls (1956). A solution of EBr (1.0g) in hot water (10ml) was shaken with acetic anhydride (0.4ml). Red needle-like crystals of 8-acetylethidium began to appear immediately and the reaction was heated for 30min at 100°C, cooled and the red precipitate collected and dried at 60°C. The crystals were dissolved in methanol (HPLC grade, 2 x 50ml) and the solution evaporated to dryness at 40°C under reduced pressure to give 0.8g (72%) of red crystals (m.p. 280°C with decomposition).

Monoacetylation was confirmed by FAB-MS (m/z [E^+ - H] + CH_3CO = 356, Appendix II). The preferential acetylation of the 8-amino group in the latter compound was confirmed by ^{1}H-nmr spectroscopy (downfield shift of the signals corresponding to the 7H and 9H protons of the phenanthridine ring [δ (DMSO) = 7.82 and 8.30ppm respectively, Table 3.2, Appendix I] compared to those of EBr [δ (DMSO) = 6.26 and 7.52ppm respectively, Figure 2.5].

3-Acetylethidium bromide

3-Acetylethidium occurred in admixture with synthesised 8-acetylethdium with a characteristic chemical shift of the protons of the CH_3CO- substituent at the 3-amino position of δ (DMSO) = 2.19ppm compared to those of the CH_3CO- substituent at the 8-amino position in 8-acetylethidium [δ (DMSO) = 2.00ppm] (Appendix I, proton NMR spectrum of 8-acetyl-ethidium).

Solvents and reagents

HPLC grade methanol and acetonitrile were obtained from Merck Ltd., Lutterworth, England. Analytical reagent grade acetone and absolute ethanol were obtained from the University of Strathclyde Chemistry Stores, Glasgow, Scotland. Carbogen (95% O_2, 5% CO_2) gas was obtained from BOC Speciality Gases, Surrey, England. Chee's medium (serum-free) was obtained from Gibco-Life Technologies, Paisley, Scotland. The following chemicals (which were of ACS grade or better) were obtained from Sigma-Aldrich Co. Ltd., Dorset, England;

3-Methylcholanthrene (20-methylcholanthrene)

Acetic anhydride

Ammonium acetate

Bovine serum albumin (BSA) fraction V

Butanol

Calcium chloride

Collagenase Type-IV (Clostripeptidase-A from Clostridium histolyticum)

Collagenase Type-VIII (Clostripeptidase-A from Clostridium histolyticum)

Disodium hydrogen orthophosphate

DMSO-d_6

Dulbecco's modified Eagle's medium (DMEM 10X)

Ethyleneglycol-bis(β-aminoethylether)-*N*,*N*,*N*',*N*'-tetraacetic-acid (EGTA)

Ethylenediamine tetraacetic acid (EDTA)

Glacial acetic acid

Glucose

Heparin sodium

HEPES (*N*-[2-hydroxyethyl]piperazine-*N*'-[butanesulfonic acid])

Magnesium sulphate heptahydrate

Olive oil

Phenobarbitone sodium

Phosphate buffered saline (PBS, pH 7.4)

Potassium chloride

Potassium dihydrogen phosphate

Pyridine

Sodium chloride

Sodium hydrogen carbonate

Trypan Blue in PBS (0.4%w/v)

Trypsin inhibitor Type II-S (from porcine pancreas)

University of Wisconsin solution

3.2.2 Methods

3.2.2.1 Preparation of solutions and buffers

Ethidium bromide solution (100μM)

EBr (*ca.* 3.9mg) was weighed accurately and placed in a 100ml volumetric flask, dissolved in and made up to volume with Krebs-Hepes buffer pH 7.4. For the determination of the linearity of detector response to different concentrations of EBr, this solution was diluted with water to obtain solutions of concentrations of EBr of 10, 20, 30, 40 and 50μM.

ISM solution (100μM)

ISM (*ca.* 5.6mg) was weighed accurately and placed in a 100ml volumetric flask, dissolved in and made up to volume with Krebs-Hepes buffer pH 7.4.

M&B 4250 solution (50μM)

M&B4250 (*ca.* 2.8mg) was weighed accurately and placed in a 100ml volumetric flask, dissolved in and made up to volume with Krebs-Hepes buffer pH 7.4

Ammonium acetate buffer (20mM, pH 3.5)

Ammonium acetate (1.54g) was placed in a 1000ml volumetric flask and dissolved in water (*ca.* 900ml). A volume of 7M HCl (2.34ml) was added and the pH of the resultant solution adjusted to a value of 3.5 with 2M HCl. The resultant solution was made up to volume with water.

3-Methylcholanthrene solution (2.5%w/v in olive oil (for enzyme induction studies))

A volume (4ml) of olive oil was transferred to a pre-weighed vial containing 3-methylcholanthrene (100mg) to produce a 2.5%w/v suspension (25mg/ml).

Phenobarbitone solution (0.1%w/v (for enzyme induction studies))

Phenobarbitone sodium (0.5g) was weighed accurately and transferred into a 500ml volumetric flask after which it was dissolved in, and made up to volume with tap water.

10X Hank's Buffer

The following compounds were weighed accurately and placed in a 1000ml flask, dissolved in and made up to volume with water. The resultant solution was stored at 4°C until required.

NaCl	80.0g
KCl	4.0g
$MgSO_4.7H_2O$	2.0g
$Na_2HPO_4.2H_2O$	0.6g
KH_2PO_4	0.6g

2X Krebs-Henseleit Buffer Stock Solution

The following solutions were added in the order shown below into a 2000ml amber coloured bottle, after which a gas comprising 95%O_2/5%CO_2 was passed through the resulting solution for 10min.

Distilled water	785ml
16.09% NaCl	200ml
1.10% KCl	150ml
0.22M KH_2PO_4	25ml

2.74% $MgSO_4.7H_2O$	50ml
0.12M $CaCl_2.6H_2O$	100ml

A solution of $NaHCO_3$ (9.71g/l of distilled H_2O) was sparged with 95%O_2/5%CO_2 for 10min and added to the salt solution. This Krebs-Henseleit buffer stock solution was stored at 4°C until required.

Hank I Buffer (Hank's I)

The following compounds were placed in a 500ml beaker and the pH of the resulting solution was adjusted to a value of 7.4 with 5M NaOH.

$NaHCO_3$	1.05g
HEPES	1.50g
BSA (fraction V)	3.33g
EGTA	114mg
Distilled water	450ml
10X Hank's buffer stock solution	50ml

Hank II Buffer (Hank's II)

The following compounds were placed in a 500ml beaker and the pH of the resulting solution was adjusted to a value of 7.4 with 5M NaOH.

$NaHCO_3$	1.05g
HEPES	1.50g
$CaCl_2.2H_2O$	147mg
Distilled water	450ml
10X Hank's buffer stock solution	50ml

Krebs Albumin Buffer (KA)

The following compounds were placed in a 500ml beaker and the pH of the resulting solution was adjusted to a value of 7.4 with 5M NaOH.

HEPES	1.5g
BSA	5g
Distilled water	250ml
2X Krebs-Henseleit Buffer	250ml

Krebs-Hepes Buffer (KH)

The following compounds were placed in a 500ml beaker and the pH of the resulting solution was adjusted to a value of 7.4 with 5M NaOH.

HEPES	1.5g
Distilled water	250ml
2X Krebs-Henseleit Buffer	250ml

Krebs medium (for preparation of sheep hepatocytes)

A stream of 95%O_2/5%CO_2 was bubbled for 20min through a solution (1000ml) containing the following compounds at the stated concentrations.

NaCl	118mM
KH_2PO_4	1.2mM
$MgSO_4.7H_2O$	1.2mM
KCl	4.75mM
$NaHCO_3$	25mM
Glucose	12mM

Then an aliquot (10ml) of HEPES buffer (0.5M) was added and the pH of the resultant solution adjusted to a value of 7.4 with 5M NaOH.

Dulbecco's Modified Eagle's medium (DMEM, 0.1X)

DMEM (50ml, 10X) was dissolved in and made up to 5000ml with distilled water.

The buffers (Hank's I, Hank's II, KA and KH) used for the perfusion of the livers were stored at 4°C after sterile filtration through a Nalgene® 0.2μm cellulose acetate filter / storage system (Fisher Scientific, Loughborough, UK).

3.2.2.2 Treatment of dialysis tubing for preparation of rat-tail collagen

Dialysis tubing (Visking®, size 5, 24/32 inches, Medicell International Ltd., London, England) was treated, before storage at 4°C in distilled water, by sequential simmering of 50cm lengths for 1h periods in 50%v/v ethanol in water, two changes of 10mM $NaHCO_3$ containing 1mM EDTA, followed by two changes of distilled water. When required for dialysis, the tubing was rinsed in distilled water followed by DMEM (0.1X).

3.2.2.3 Preparation of rat-tail collagen

Collagen was prepared from rat-tail tendons by an acid-extraction technique adapted from the method of Elsdale and Bard (1972). Tendons were pulled out from rat-tails, weighed, and added to 0.5M acetic acid to produce a 1%w/v solution. The resultant solution was stirred for 48h at 4°C and then filtered through sterile gauze. The filtrate was transferred to dialysis tubing and dialysed twice against DMEM (0.1X) at 4°C for 24h. The dialysate was sterilised by centrifugation at 1 x 10^4rpm for 2h at 4°C. The concentration of collagen (mg/ml) was calculated by determining the dry-weight at 37°C of a known volume of the supernatant (1 x 10^4rpm, 2h). Cell culture vessels (for CLSM, Section 3.2.2.7) were

coated with collagen after diluting the stock solution to a concentration of 0.5mg/ml in sterile water.

3.2.2.4 Trypan Blue exclusion test

Viability was based on the principle of dye exclusion (Trypan Blue) by undamaged cell membranes. Trypan Blue (950µl, 0.1%w/v in phosphate buffered saline, pH 7.4) was added to the cell suspension (50µl) and an aliquot of the resultant suspension was loaded onto a haemocytometer. The haemocytometer chamber was examined using a Nikon Diaphot-TMD (Nippon Kogaku KK, Japan) light-microscope with a x10 objective lens. The cells in at least six sections of the haemocytometer were counted and the viability determined as;

(No. of cells excluding Trypan Blue ÷ Total No. of cells) X 100%

The number of viable cells/ml (viable cell concentration) of hepatocyte suspension was calculated as;

(No. of cells excluding Trypan Blue ÷ Total No. of cells) $\times 10^4 \times 20$

Where 20 is the dilution factor and 10^4 the conversion from haemocytometer chamber count per $0.1mm^3$ to cm^3.

3.2.2.5 Treatment of rats for enzyme induction studies

Treatment of rats with phenobarbitone sodium.

Male Sprague-Dawley rats (180 – 200g, n = 3) received a phenobarbitone sodium solution (0.1%w/v) in place of the normal drinking water for 3days prior to the removal of livers for the preparation of hepatocytes.

Treatment of rats with 3-MC.

Male Sprague-Dawley rats (180 – 200g, n = 3) received a single intra-peritoneal injection of 3-MC (25mg/ml) in olive oil (at a dose of 50mg/kg body-weight) 3days prior to the removal of livers for the preparation of hepatocytes. Control animals were rats (180 - 200g, n = 2) which had received a single intra-peritoneal injection of olive oil (at a dose of 2ml/kg body-weight) 3days prior to preparation of the hepatocytes.

3.2.2.6 Preparation of isolated hepatocytes

Rat hepatocytes

Hepatocytes were obtained from male Sprague-Dawley rat (180 – 220g) livers by the collagenase perfusion technique adapted from a published method (Moldeus *et al.* 1978). Rats were anaesthetised by an intra-peritoneal injection of sodium pentobarbitone (Sagatal® 60mg ml^{-1}, (Rhône-Poulenc Rorer, Dagenham)) at a dose of 60mg kg^{-1} body weight. Then the peritoneal cavity was opened by a mid-transversal incision and heparin sodium (0.1ml, 1000 IU ml^{-1} in phosphate buffered saline, pH 7.4) was injected into the inferior vena cava above the renal vein branch. The portal vein was cannulated, the liver dissected from the body and perfused *ex vivo* without recirculation for 2-3mins, followed by recirculating perfusion for 10 to 12min with Hank's I buffer. Then it was perfused for 15min (recirculating) with Hank's II buffer containing 0.04%w/v collagenase type IV (equivalent to 160units/ml of collagen digestion activity). During the perfusion period, all of the buffer solutions (Hank's I, Hank's II and KA) were sparged with 95%O_2/5%CO_2 and maintained at 37°C. The perfusate flow rate was approximately 5ml min^{-1}. When the liver felt very soft (and the cells inside the liver sac had dissociated) it was dispersed (using sterile forceps)

into KA buffer (100ml) containing 1% w/v bovine serum albumin (fraction V). The cell suspension was filtered through sterile cotton gauze to remove remaining connective tissue and clumps of cells, the filtrate was collected in a 100ml bottle and the cells allowed to sediment under gravity. The supernatant was removed and the cells were washed with two volumes (50ml) of KH buffer, allowed to settle again and the excess buffer removed by aspiration. Cells were counted and the viability and viable cell concentration determined by Trypan Blue exclusion (Section 3.2.2.4). The viability of rat cells used in this study was typically 75% or higher.

Sheep hepatocytes

Hepatocytes were prepared from Finn x Dorset horn crossbred ewes (3-4 year old, Hannah Research Institute, Ayr, Scotland) by a previously described collagenase perfusion technique (Emmison *et al.*, 1991). Sheep were anaesthetised with an intravenous injection of sodium pentobarbitone (*q.s.*) before excision of the caudate lobe of the liver. The portal vein and the hepatic artery were cannulated and the caudate lobe of the liver was cleared of blood, immediately upon removal, by perfusion with calcium-free Krebs medium (500ml) containing heparin sodium (0.1mg/ml) and EGTA (0.5mM), whilst being transferred to the perfusion equipment. Then the perfusion with calcium-free Krebs medium was followed by a re-circulating collagenase perfusion with Krebs medium (500ml) containing $CaCl_2.6H_2O$ (2.5mM), collagenase (60mg, Type V-III) and trypsin inhibitor (5mg, Type II-S Soybean). Digestion continued for various periods (30 – 70min), dependent on the size of the caudate lobe, until a suitable consistency was obtained. The sac was then ruptured and the cells dispersed into Krebs medium. Initially the suspension of hepatocytes was filtered sequentially through two stainless steel meshes of decreasing porosity (250μm and 120μm)

and the cells were sedimented from the filtrate by intermittent centrifugation at 1400rpm. The supernatant was aspirated and the resultant pellet was re-suspended with Krebs medium with intermittent centrifugation at 1400rpm. The viability of the cell preparation was determined by Trypan Blue exclusion (Section 3.2.2.4). The viability of sheep cells used in this study was typically 90% or higher.

Pig hepatocytes

Hepatocytes were isolated from female Great White pigs (15 - 25kg, Roslin Institute, Edinburgh, Scotland) by a collagenase perfusion technique adapted from a published method (Gerlach *et al.*, 1994). Animals were euthanased with an intravenous phenobarbitone sodium overdose, after which a Foley catheter was inserted into the portal vein via the vena cava. The liver was perfused with 1500ml of University of Wisconsin (UW) solution. Perfusion was performed by gravity, with a hydrostatic head of 50cm, via a 1.5mm bore tubing. During this perfusion stage, the liver was excised and transferred to a sterile bowl containing UW solution. Then the liver was sequentially perfused with:

1. Hank's I buffer (1.6l) without re-circulation at 70ml min^{-1}.
2. Hank's II buffer (1.6l) containing 450mg collagenase with re-circulation at 70ml min^{-1} for 30-40min.
3. KA buffer (600ml) without re-circulation.

Then the liver tissue was manually disrupted in KA buffer. The resultant cell suspension was filtered sequentially through three stainless steel meshes of decreasing porosity (1000μm, 600μm and 180μm). The filtrate was then left to stand to allow the cells to sediment under gravity, the supernatant was removed by aspiration and the cells re-suspended in KH buffer. The cells were washed twice with KH buffer and excess

supernatant removed after sedimentation of the cells. The viability of the cell preparation was determined by Trypan-blue exclusion (Section 3.2.2.4). The viability of pig cells used in this study was typically 50% or higher.

3.2.2.7 Investigation of the intracellular localisation of EBr and ISM in rat hepatocytes

Treatment of rat hepatocytes with EBr and ISM

Rat hepatocytes were cultured on Lab-Tek 2-chamber Permanox slides (Nunc Inc., Illinois, USA) coated with a solution of collagen type 1 (0.03mg/cm^2, section 3.2.2.3). Each chamber was filled with 2ml of hepatocyte suspension prepared in serum-free Chee's medium at a seeding density of 4 x 10^5 viable cells/ml. The culture dish was incubated at 37°C for 2h in an atmosphere of air / 5% CO_2. Chee's medium was washed off with Krebs-Henseleit buffer, pH 7.4, containing 12.5mM HEPES and the cells examined with a Nikon Diaphot-TMD light microscope (x10) to confirm the formation of a cell mono-layer. EBr (2ml, 100μM in Krebs-Hepes buffer) was added to each chamber of the culture dish prior to incubation at 37°C for 5, 10, 15, 30, 60 and 90min. The chambers of the culture dish were rinsed with the Krebs-Hepes buffer after the incubation period to remove excess EBr, and the mono-layer fixed with a mixture of acetone: ethanol (1:1 v/v) for 30min. The fixative was washed off with distilled water and the cell monolayer incubated further in distilled water for 30min at 37°C, after which the water was aspirated, the culture dish covered in glycerol and sealed. Prior to examination by CLSM, the glycerol was drained off and the walls of the chambers detached. Cover slips were attached and sealed with cyanoacrylate. The resulting slides were examined within 24h of preparation.

The intracellular localisation of ISM (100μM) was examined using the same procedures and conditions described above for EBr.

Examination of cells by CLSM

Cells were examined with a Leitz Aristoplan CLSM (Leica Lasertechnik GmbH, Germany) using a 488nm-excitation beam from an argon-ion laser. A 510nm dichroic mirror and a 590nm long-pass filter were used respectively to exclude reflected light and to allow the native fluorescence of EBr and ISM to enter the photomultiplier. The photomultiplier settings of the CLSM (laser power; 36,000units) were kept constant (PMT_1=500V, PMT_2= 200V) to facilitate direct comparison of fluorescence. Images were obtained with an oil immersion lens (x100/1.30) from single optical sections (0.4μm thick) of individual cells. Measurements of fluorescence in the cytoplasm and nucleus were obtained for different isolated cells (n = 8) with an average of three determinations per cell. The recorded cellular fluorescence excludes the dull green diffuse auto-fluorescence seen in hepatocytes from control incubations not containing EBr or ISM.

3.2.2.8 Recovery of phenanthridines from hepatocytes

Suspensions of isolated hepatocytes used for the determination of the efficiency of recovery of EBr or ISM were rendered incapable of metabolism by freezing them at –4°C, thawing at ambient temperature and ultra-sonication for 5min. To each of ten test tubes containing EBr or ISM (100μM, 5ml) was added *X*ml (equivalent to 12 x 10^6 cells) of a solution of suspended hepatocytes. Two test tubes were set up as controls by using *X*ml of Krebs-Hepes buffer in place of the hepatocyte suspension. The contents of the test tubes were swirl-mixed, kept at 37°C for 30min and then ultra-sonicated for 5min. An aliquot

(1ml) of the contents of each test-tube was transferred into a sample tube containing an equal volume of a solution of NaCl (0.2%w/v) in methanol. The resultant suspension was ultra-sonicated (5min) and the 7500rpm (5min) supernatant was analysed directly by HPLC without further processing (Section 3.2.2.11).

The recovery of M&B4250 from rat hepatocytes was determined by the same procedure described for EBr and ISM using a 50μM solution of M&B4250 in Krebs-Hepes buffer.

The recovery efficiency was calculated as;

$$\frac{\text{(Average detector response for samples)} \times 100}{\text{(Average detector response for controls)}}$$

In this thesis, it has been assumed that a linear relationship exists between the detector response and the concentration (amount) of EBr recovered from hepatocytes for the purposes of quantitative analysis. This assumption was tested by investigating the relationship between detector response and the amount of EBr recovered from hepatocytes (from rat, pig and sheep) at different concentrations of the compound (25, 50, 75 and 100μM) using the same procedure as described in this section. A regression coefficient (R^2) of 0.99 or better was set as the criterion for linearity.

3.2.2.9 Incubation conditions and sampling protocols for measuring the metabolism of EBr

Isolated hepatocytes (2.4 x 10^6 viable cells/ml) from the following preparations were incubated with EBr:

Untreated rats (n = 4)	Phenobarbitone treated rats (n = 3)
3-MC treated rats (n = 3)	Olive oil (vehicle control for 3-MC) treated rats (n = 2)
Pigs (n = 3)	Sheep (n = 3)

The cells were incubated with EBr (100μM in Krebs-Hepes buffer pH 7.4, containing 12.5mM HEPES in rotating 50ml round-bottomed flasks, for specific times (between 5min and 3h), at 37°C in an atmosphere of 95%O_2/5%CO_2. The round-bottomed flasks were rotated (approx. 60rpm) by attachment to a Bucchi Rotavapor-R (Bucchi Laboratory Technik Ag. Switzerland) using a four-way connector ("pig"). The times of incubation stated in the text refer to the length of time between the introduction of EBr into the suspension of hepatocytes and the removal of 0.5ml samples of the incubate. An equal volume of a solution of NaCl (0.2% w/v) in methanol was added to the samples immediately to stop the reaction. The resultant suspension was ultra-sonicated (5min) using a Decon FS100 ultrasonic bath (Decon Ultrasonics Ltd., Sussex, England), centrifuged (7500rpm, 5min) using a MSE Centaur 2 centrifuge (MSE Ltd., England) and then the supernatant was analysed directly by HPLC (Section 3.2.2.11) without further processing. Controls used in these studies were; hepatocytes in KH buffer (without EBr) and a solution of EBr in KH buffer (100μM, without hepatocytes).

The possibility of formation of the diacetyl derivative of EBr was investigated by using a suspension containing 4.8 x 10^6 viable cells/ml (instead of 2.4 x 10^6 viable cells/ml)

in incubations. The HPLC chromatograms obtained from extracts of hepatocytes were compared with those of standards of the acetyl derivatives of EBr.

3.2.2.10 Incubation conditions and sampling protocols for measuring the metabolism of ISM and M&B4250

Selection of suitable concentration of ISM for metabolism studies

The principle was based on the Trypan Blue exclusion test (Section 3.2.2.4) used in the determination of the viability of cells. Isolated rat hepatocytes were incubated in solutions of ISM of concentrations 100μM and 200μM using the incubation conditions described for EBr (Section 3.2.2.9). The viability of the cells was determined at the start of the incubation (t = 0h) and subsequently at 1, 2 and 3h. Control incubations were set up without ISM. The cell viability data for 100μM and 200μM were compared separately with that of the control by a two-sample paired test using a 5% significance level.

Metabolism of ISM

Isolated hepatocytes (2.4 x 10^6 viable cells/ml) from untreated rats (n = 3) were incubated with ISM (100μM in Krebs-Hepes buffer pH 7.4, containing 12.5mM HEPES) using the incubation conditions and sample treatment procedures described for EBr (Section 3.2.2.9). Controls used in these studies were; hepatocytes in KH buffer (without ISM) and a solution of ISM in KH buffer (100μM, without hepatocytes). Samples were analysed using the HPLC conditions described in Section 3.2.2.11.

Metabolism of M&B4250

Isolated hepatocytes (2.4 x 10^6 viable cells/ml) from untreated rats (n = 3) were incubated with M&B4250 (50μM in Krebs-Hepes buffer pH 7.4, containing 12.5mM HEPES) using the conditions and sample treatment procedures described for EBr (Section 3.2.2.9). Controls used in these studies were; hepatocytes in KH buffer (without M&B4250) and a solution of M&B4250 in KH buffer (50μM, without hepatocytes). Samples were analysed using the HPLC and LC-MS conditions described in Sections 3.2.2.11 and 3.2.2.12 respectively.

3.2.2.11 HPLC analyses

Analysis of EBr and its metabolites

EBr and its metabolites were separated at ambient temperature on a Lichrospher® 60 RP-select B column (Hewlett Packard, Germany, 125 x 4mm ID, 5μm particle size) with a 20 x 2mm ID guard column (packed with Lichrosorb® C8, 5μm particle size (E. Merck, Darmstadt, Germany)). The mobile phase (22.5% v/v acetonitrile in 20mM phosphate buffer, adjusted to pH 3.0 with orthophosphoric acid) was delivered at a flow rate of 1ml/min with a Spectra-Physics P100 isocratic pump equipped with a Spectra-Physics SP8450 UV-Visible detector (Thermo Separations Inc., California, USA) set at 290nm. Chromatograms were acquired and analysed with a HP 3395 reporting integrator (Hewlett Packard, Waldbronn, Germany). UV diode-array spectra (200 - 350nm) of the eluted compounds were obtained with a Waters 991 photodiode-array detector with a peak resolution setting of 2nm. Detector responses at 290nm were normalised assuming equal molar extinction coefficients for all eluted metabolites. It is known that there are minor

differences between the molar extinction coefficients of EBr and its *N*-substituted analogs at 290nm (Firth *et al.*, 1983).

Analysis of isometamidium (ISM)

ISM was analysed using the chromatographic conditions described for EBr. The HPLC mobile phase was composed of 22.5%v/v acetonitrile in 20mM phosphate buffer (adjusted to pH 3.0 with orthophosphoric acid). The organic modifier content was adjusted when required to alter analyte retention times (see Figure 2.9).

Analysis of M&B4250 and metabolites

The blue isomer of ISM (M&B4250) and its metabolites were separated using the chromatographic conditions described for EBr with a modified composition of the mobile phase of 17.5%v/v acetonitrile in 20mM phosphate buffer (adjusted to pH 3.0 with orthophosphoric acid).

Micro-bore HPLC analysis of metabolites of EBr and M&B4250

EBr and its metabolites were separated on a 15cm x 1.0mm Lichrospher RP-Select B column (E. Merck, Darmstadt, Germany) using a mobile phase composition of 20%v/v acetonitrile in ammonium acetate buffer (20mM, pH 3.5, Section 3.2.2.1). The mobile phase was delivered at a flow rate of 60µl/min using a Constametric Model-3200 isocratic pump (ThermoSeparations Inc., California, USA). The compounds eluting from the column were monitored at 290nm with a Spectra-Physics UV100 detector (ThermoSeparations Inc., California, USA) equipped with a low dispersion flow cell of dimensions 1.2µl x 3.0mm (volume x pathlength).

M&B 4250 and its metabolites were separated using the chromatographic conditions described for EBr with a modified composition of the mobile phase of 17.5%v/v acetonitrile in ammonium acetate buffer (20mM, pH 3.5).

3.2.2.12 LC-MS Analyses

LC-MS analyses were carried out on a Shimadzu LC-10 HPLC system (Shimadzu Ltd., Kyoto, Japan) connected to a VG Platform single-quadruple mass spectrometer (Fisons Instruments, Loughborough, England) via an electrospray interface. Aliquots (1μl) of hepatocyte extracts were injected onto a 15cm x 1.0mm Lichrospher RP-Select B column. The metabolites of EBr were separated with a mobile phase of 20% v/v acetonitrile in ammonium acetate buffer (20mM, pH 3.5) at a flow rate of 60μl/min. The eluent from the column was split 1:1 and passed to the mass spectrometer and a UV detector (290nm) configured in parallel to facilitate peak assignment. Continuum mass spectra were collected in the range 150-700 Daltons (Da) with a sweep time and interscan delay of 2.0s and 0.1s respectively, using VG MassLynx software. Single ion chromatograms were obtained using the MassLynx software with a 1Da window.

M&B4250 and its metabolites were separated using the same LC-MS conditions with a mobile phase of 17.5%v/v acetonitrile in ammonium acetate buffer (20mM, pH 3.5).

3.2.2.13 Statistical Tests

Statistical tests used in this thesis, namely; regression analysis, one-way analysis of variance (ANOVA), two-sample t-test, two-sample paired t-test and descriptive statistics (mean, standard error of the mean, standard deviation) were performed with Microsoft®

Excel 97 software (Microsoft Corporation, USA). Unless otherwise stated, all statistical tests were conducted at the 95% confidence interval.

3.3 Results and discussion

3.3.1 Cellular uptake and localisation of the phenanthridines

Ethidium Bromide

The incubation of rat hepatocytes with EBr was characterised by a visible, rapid loss of the red colour of the medium (due to the presence of EBr) and a concomitant appearance of the red colour in the hepatocytes. This phenomenon was investigated by CLSM in order to define the intracellular localisation of EBr in hepatocytes. The influx of EBr into hepatocytes as observed by CLSM (Section 3.2.2.7) was extremely rapid. The fluorescence associated with the phenanthridine was localised in the nucleolus and nuclear membrane after 5min (Figure 3.6a). The drug and/or its metabolites displayed a high affinity for the nuclear membrane, nucleolus and endoplasmic reticulum. The intracellular fluorescence was more diffuse after 10min (Figure 3.6b) and was localised non-specifically throughout the cytoplasm.

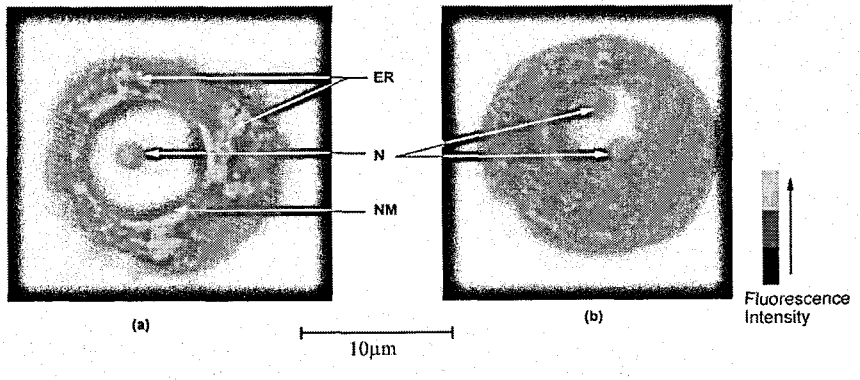


Figure 3.6 CLSM images of isolated rat hepatocytes exposed to EBr (100μM) for **a**) 5min and **b**) 10min, showing fluorescence associated with the nucleolus (N), endoplasmic reticulum (ER) and nuclear membrane (NM)

Representative CLSM images of hepatocytes at the specified time points after exposure to EBr (5, 10, 15, 30, 60 and 90min) are shown in Appendix III.

Quantitative measurements of the fluorescence of the cytosol and nucleus were obtained by CLSM (Section 3.2.2.7) for different isolated cells (n = 8) with an average of three determinations per cell. The measured cellular fluorescence due to EBr and/or its metabolites increased substantially over a 90min period (Figure 3.7).

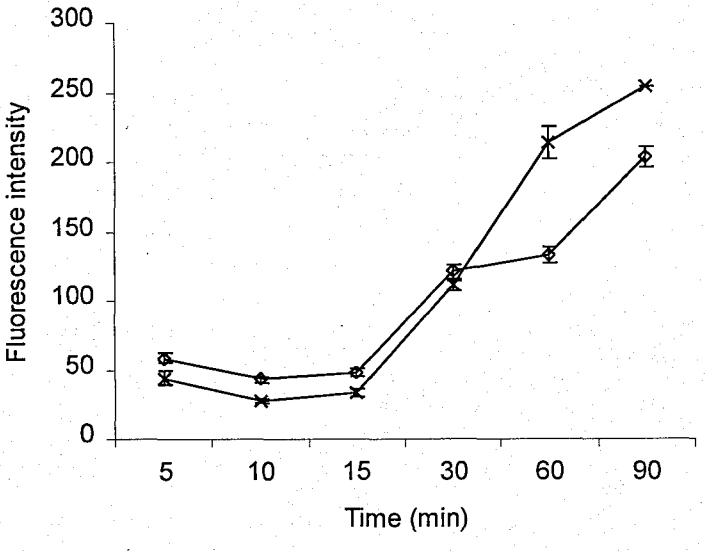


Figure 3.7 Intensity of fluorescence at 590nm in the nucleus (×) and cytosol (◇) of isolated rat hepatocytes with time (min) after exposure to EBr (100μM) as measured by CLSM. Results are mean ± s.e. mean (n = 8).

The initial rapid uptake of EBr (as measured by the intensity of intracellular fluorescence) was followed by a steady redistribution phase in both the nucleus and cytosol, up to 15min after the start of the study. The uptake of EBr then displayed non-saturable kinetics up to 90min. This finding supports an earlier report on the pharmacokinetics of EBr (Murilla *et al.* 1996a), which showed that there was non-attainment of equilibrium at all

concentrations between the free drug in plasma and tissue. Statistical analysis of the fluorescence measured in the cytosol and nucleus over the 90min period showed that there were no significant differences between the two compartments (Two-sample paired t-test, $p = 0.48$). At low resolution settings of the microscope, it was observed that some of the isolated cells exposed to EBr (100μM, without fixation) lost the bright red fluorescence after 2h and this may coincide with the formation of metabolites with fluorescence emission wavelengths outside the confocal window.

The most characteristic constituents of the nucleus in mammalian cells are the nucleic acids, DNA and RNA. The nucleolus, which is present in the nucleus, contains high concentrations of RNA and proteins and plays a major role in ribosomal synthesis and assembly. Studies of the nucleolus under the electron microscope show that it does not have an outer limiting membrane and because it contains most of the RNA of a resting nucleus, it stains deeply with basic dyes (Alberts *et al.* 1989). Binding of EBr to nucleic acids and cell macromolecules may explain the specific localisation seen on exposure of hepatocytes to the drug for 5min. The high concentrations of nucleic acids at these sites and the well-known affinity of EBr for DNA and RNA seem a plausible reason for this distribution (Waring 1966, Borst 1972, Kinabo and Bogan 1987). However, binding to nucleic acids and other cell macromolecules does not offer a satisfactory explanation for the increased uptake of EBr with time, resulting in a diffuse non-specific intracellular localisation as shown by CLSM. The uptake characteristics shown for EBr parallel those reported for the Trypanosomatid flagellates, *Crithidia fasciculata* and *Leptosomonas seymouri* (Coolbear and Midgley 1986). In these organisms, the uptake of EBr into cells was characterised by a rapid external binding phase and a prolonged energy-dependent transport phase. This may signify the presence of an active transport mechanism which, in turn, may explain the high

levels of EBr found in the liver after administration of therapeutic doses. The absence of the characteristic red fluorescence observed in some isolated cells after prolonged exposure (after 2h) to EBr may be due to the formation of 3,8-diacetyl-EBr (excitation wavelength 488nm, emission wavelength 516nm), which shows a greenish-yellow fluorescence on ultraviolet radiation.

Intracellular localisation of ISM

The incubation of isolated rat hepatocytes with a solution of ISM (100μM) was characterised by a phenomenon similar to that observed for EBr. Visible staining of the hepatocytes paralleled the rapid loss of the red colour of ISM from the medium. Examination of the hepatocytes by CLSM at specified times (5, 10, 15, 30, 60 and 90min, Appendix III) after exposure to ISM showed the presence of the phenanthridine in the nuclear region at 5min. In parallel with the observations for EBr, ISM and/or its metabolites displayed a high affinity for the nucleolus, nuclear membrane and the endoplasmic reticulum.

The measurement of fluorescence in the cytosol and nucleus of hepatocytes exposed to ISM (Figure 3.8) showed a definite (albeit an irregular) increase with a marked difference between the intensity measured in the nucleus and the cytosol over the 90min period. Statistical analysis of measured fluorescence in the cytosol and nucleus over the 90min period showed that there was a significant difference between the two cellular compartments (Two-sample paired t-test, $p = 0.02$). At 90min, the fluorescence in the nuclear region was four times as intense as that of the cytosol.

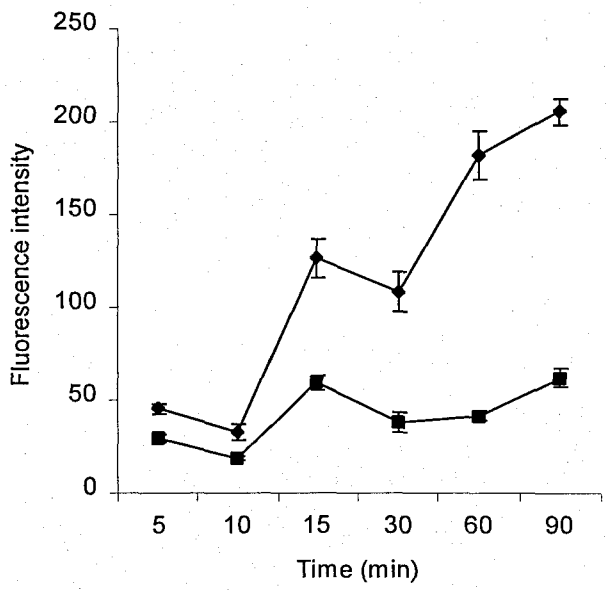


Figure 3.8 Intensity of fluorescence at 590nm in the nucleus (◆) and cytosol (■) of isolated rat hepatocytes with time (min) after exposure to ISM (100μM) as measured by CLSM. Results are mean ± SEM (n = 8).

The relative binding of ISM and EBr to calf thymus DNA has been reported (Kinabo and Bogan 1987). The K_d (apparent dissociation constant) of ISM was determined as 1.7 x 10^{-7}M, which is four times less than the corresponding value (of 7 x 10^{-7}M) for the binding of EBr to calf thymus DNA under identical conditions (Waring 1966). Therefore, ISM seems to exhibit a higher affinity than EBr for DNA which might explain the differences in their relative localisations in the cytosol and the nucleus. The differences in relative binding to nucleic acids and cell macromolecules may also explain why ISM is a more effective prophylactic agent than EBr. It has been reported that the duration of prophylaxis provided by ISM and EBr (each at a dose of 1m/kg bodyweight in cattle (n = 30)) was of the order of 7.5 ± 1.9weeks and 4.6 ± 2.1weeks respectively (Stevenson *et al.* 1995).

The rapid increase in the cellular fluorescence due to ISM may be further evidence for the 'ISM-specific' active transport mechanism, which was postulated by Philips *et al.* (1967) for rat hepatocytes. It has been reported that the concentrations of ISM in the liver of cattle 1week after intramuscular administration (1mg/kg body weight) were approximately 250 times higher than the mean maximum concentration measured in serum (Eisler 1996). Therefore, the existence of an active transport mechanism seems reasonable in view of the high equilibrium concentration gradient between hepatocytes and serum.

The uptake of ISM into trypanosomes has been investigated previously using fluorescence microscopy and radio-analysis of ^{14}C –labelled Samorin® (Zilberstein *et al.* 1993, Wilkes *et al.* 1995) and it was reported that the correlation between data from the two techniques was good. Trypanosomes accumulated the drug in a time-dependent manner, conforming to a first-order rate process. The accumulation of Samorin® by blood stream forms of trypanosomes was inhibited by *N*-ethylmaleimide, which implicated the existence of a specific transport protein in the accumulation (Wilkes *et al.* 1995). In a previous study using trypanosomes, Sutherland *et al* (1992) reported that the uptake of ^{14}C-ISM was by a specific, energy-dependent, receptor-mediated process that was sensitive to inhibition.

Significantly, the studies above (Sutherland *et al.* 1992, Zilberstein *et al.* 1993, and Wilkes *et al.* 1995) involved the use of the commercial samples of ISM in the form of Samorin®. Evaluation of the composition of Samorin® in this study has shown that, in addition to ISM, significant amounts of structurally related substances may be present (Chapter 2). Attempts to synthesise ^{14}C -labelled ISM from ^{14}C-6 labelled EBr will result, not only in the desired product, but inevitably in the formation of other structurally related radiolabelled isomers of ISM. The investigation of the specificity of fluorescence analysis of ISM in the presence of M&B38897 (Section 2.3.3) has shown also that the latter

contributes significantly to the observed fluorescence at 590nm. Therefore, these quality issues make the interpretation of data from the previous studies on the transport of ISM into trypanosomes difficult, if not impossible. The possibility of using HPLC and HPTLC to screen products to ensure that they are composed of pure ISM (Sections 2.3.2 and 2.3.3), coupled with the CLSM analysis, should facilitate a meaningful comparison of the uptake events in mammalian cells (hepatocytes) and trypanosomes, and between resistant and non-resistant trypanosomes.

3.3.2 Metabolism of EBr

Using the previously described HPLC conditions (section 3.2.2.11), the peak due to EBr eluted at a retention time of approximately 27min. A rectilinear relationship was obtained between the HPLC UV detector response (peak area) at 290nm and the concentration of EBr solutions over the range 0 to 50μM ($R^2 > 0.995$, n =3, Figure 3.9).

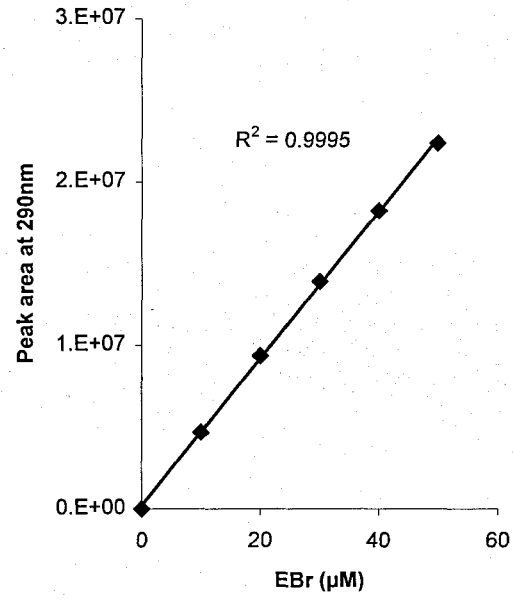


Figure 3.9 Rectilinear relationship between detector response at 290nm and the concentration of EBr solutions over the range 0 – 50μM.

The precision (RSD) of replicate injections of a solution of EBr (30μM) was calculated as 0.50% (n = 20). EBr (100μM) was recovered quantitatively from hepatocytes; with efficiencies of 99.8 ± 0.7% (mean ± sd., n = 10), 101.1 ± 0.6% (mean ± sd., n = 5) and 89.9 ± 1.6% (mean ± sd., n =5) for rat, sheep and pig respectively. A rectilinear relationship was obtained between the detector response and the amount of EBr recovered from spiked samples (rat, sheep and pig hepatocytes), over the concentration range 0 to 100μM (R^2 > 0.995, n = 3). Figure 3.10 shows representative chromatograms of HPLC analyses of extracts from the incubation medium following a 3h incubation of EBr with rat hepatocytes (2.4 x 10^6 viable cells/ml), together with the appropriate controls (Figures 3.10a and 3.10b).

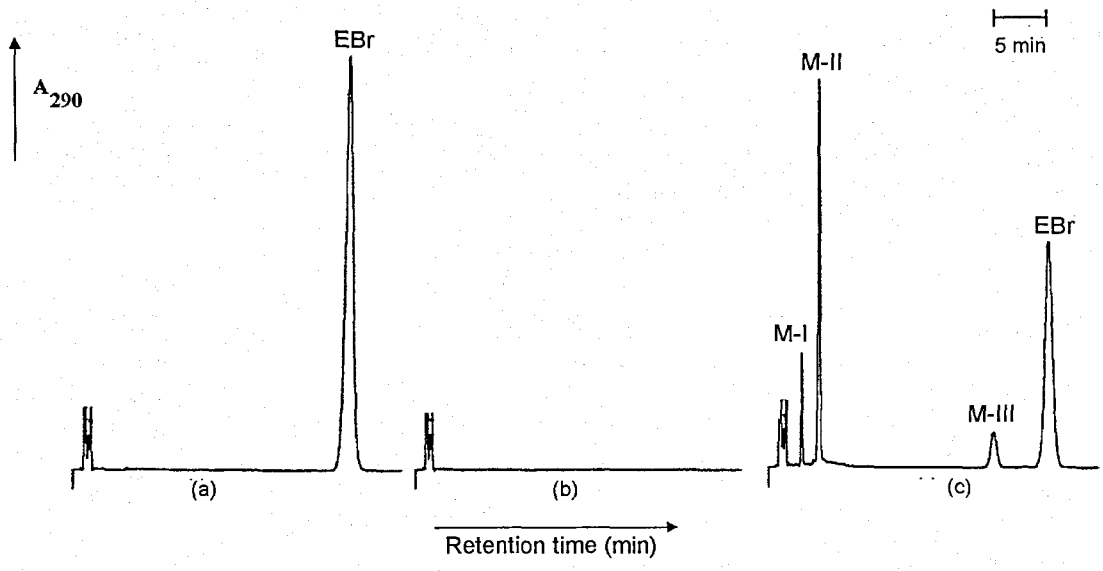


Figure 3.10 HPLC chromatograms of extracts from the incubation medium following 3h incubations of **a)** EBr (100μM) with Krebs-Hepes buffer, pH 7.4 **b)** rat hepatocytes (2.4 x 10^6 viable cells /ml) with Krebs-Hepes-buffer, pH 7.4 and **c)** rat hepatocytes (2.4 x 10^6 viable cells /ml) with EBr (100μM).

Solutions of EBr in KH buffer (pH 7.4) were stable with the incubation conditions used (37°C, 3h), and this was illustrated by the absence of secondary peaks in the chromatogram for the EBr control (Figure 3.10a). The ratio of the areas of the EBr peak after incubation for 0h and 3h was approximately one, thus confirming its stability with the incubation conditions. The result of chromatographic analysis of extracts from hepatocytes in KH buffer (Figure 3.10b) demonstrated that there was no matrix interference in analysis. HPLC analysis of a complete reaction mixture at various time intervals over a 3h period showed a loss of 36.7 ± 6.3% (mean ± SEM, n = 4, Figure 3.11) of the parent compound in 3h (EBr, t_R (retention time) = 27.3min).

With the disappearance of EBr, there was a concomitant appearance of three metabolites (t_R = M-I, 3.2min; M-II, 4.8 min; M-III, 21.9 min, Figures 3.10c and 3.12). These metabolites (M-1, M-II and M-III) represented 6.4 ± 0.7%, 19.5 ± 1.2% and 10.7 ±

2.3% respectively of the total drug recovered after 3h. Metabolite M-III exhibited chromatographic and spectral characteristics (t_R, 21.9 min; absorbance maxima = 216, 290nm, see Appendix IV) identical to those of authentic 8-acetyl-EBr (section 3.2.1, Appendix IV). Its formation was rapid; with the corresponding chromatographic peak detectable after 5min of incubation.

M-I and M-II were formed in detectable quantities after 30 and 15min respectively; the latter being the major metabolite after 60min. After 150min, there was no significant change in the quantities of M-I and M-III produced. However, the formation of M-II was approximately linear over the 180min incubation period. Metabolites M-I and M-II exhibited identical diode-array absorbance spectra (absorbance maxima at 220, 290nm, see Appendix IV).

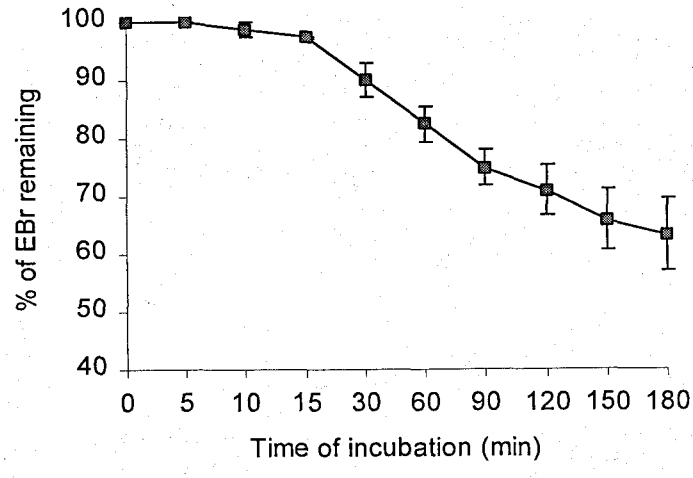


Figure 3.11 Time course of the metabolism of EBr by rat hepatocytes as determined by HPLC. Results are mean ± SEM of four determinations

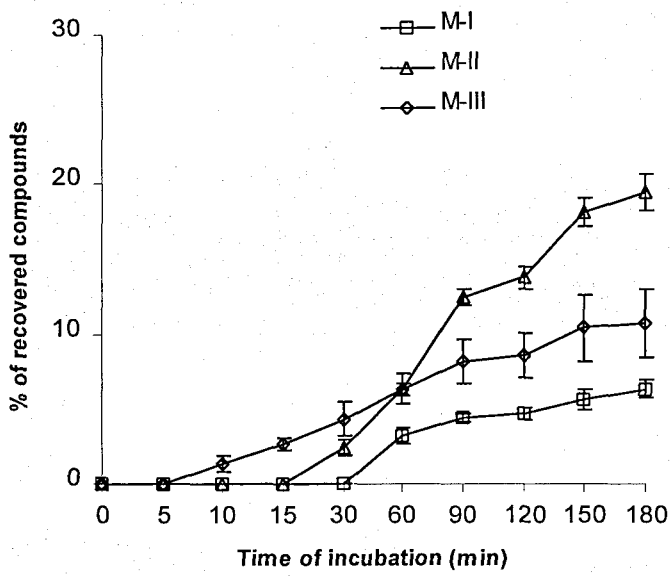


Figure 3.12 Time course of the metabolism of EBr showing the formation of the major metabolites M-I, M-II and M-III as determined by HPLC. Results are mean ± SEM of four determinations.

The retention times of EBr and its major metabolites (M-I, M-II and M-III), formed by rat hepatocytes, are summarised in Table 3.3.

Table 3.3 HPLC retention times of EBr and its major metabolites formed by rat hepatocytes

Compound	Retention time (min)
M-I	3.2
M-II	4.8
M-III	21.9
EBr	27.3

3.3.3 LC-MS method development

HPLC has been used routinely for the separation of components of biological matrices for many years, and today its use in conjunction with electrospray ionisation mass spectrometry (ESI-MS) is widespread (Pitt 1998). HPLC-ESI-MS is ideal for the analysis of highly charged polar compounds (like the phenanthridines), which have the potential to form labile acetyl or glucuronide conjugates. The problems associated with the coupling of HPLC to the mass spectrometer are associated mainly with the high solvent flow rates and the use of non-volatile buffers, which reduce the sensitivity of detection. Ammonium acetate was used as the mobile phase buffer salt (because of its volatility) to facilitate the use of LC-ESI-MS for the identification of the metabolites of EBr. EBr and its major metabolites were separated with a mobile phase composition of 20%v/v acetonitrile in ammonium acetate buffer (20mM, pH 3.5) at a flow-rate of 1ml/min (Figure 3.13, t_R; M-I, 3.0min; M-II, 4.5min; M-III, 25.2min; EBr, 32.6min). The detector flow cell capacity and pathlength were 15µl and 10mm respectively.

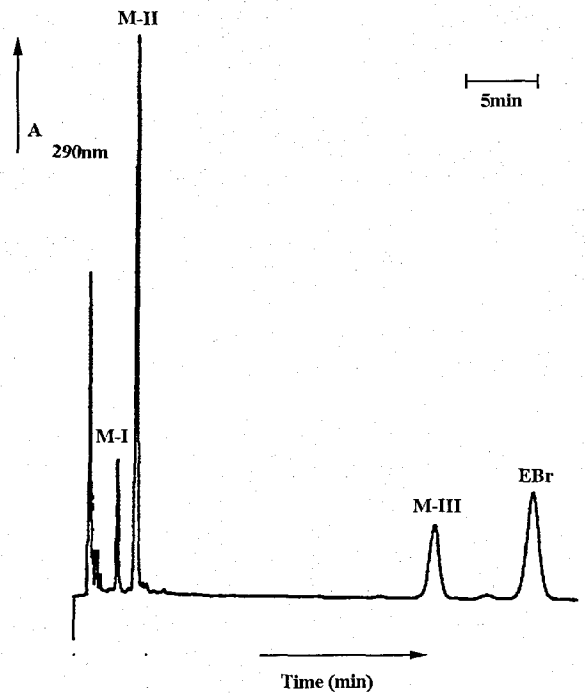


Figure 3.13 HPLC separation of EBr and its major metabolites (M-I, M-II and M-III) on a Lichrospher RP-Select B (125 x 4mm) column using a mobile phase composition of 20% v/v acetonitrile in ammonium acetate buffer (20mM, pH 3.5) at a flow rate of 1ml min^{-1}

The separation obtained with this new mobile phase composition on the Lichrospher® Select-B (125 x 4mm) column was also obtained on a Lichrospher® Select-B (150 x 1mm, micro-bore) column. A detector flow cell with reduced dimensions (capacity 1.2µl, pathlength 3.0mm) was used to compensate for the reduced peak volume obtained with micro-bore HPLC. EBr and its metabolites were separated successfully using a mobile phase composition of 20% v/v acetonitrile in ammonium acetate buffer (20mM, pH 3.0) delivered at a flow-rate of 0.06ml/min on the micro-bore column (Figure 3.14).

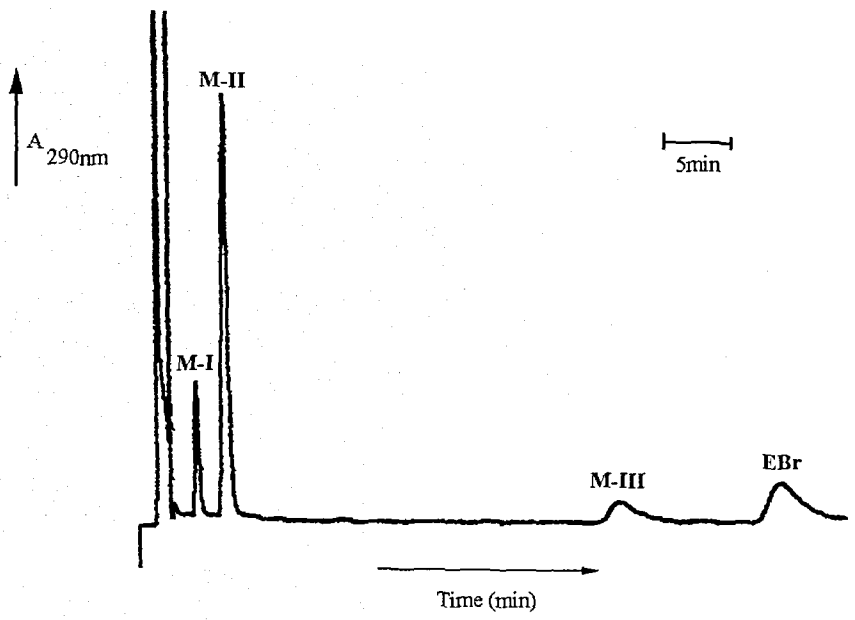


Figure 3.14 HPLC separation of EBr and its major metabolites (M-I, M-II and M-III) on a Lichrospher RP-Select B (150 x 1mm) column using a mobile phase composition of 20% v/v acetonitrile in ammonium acetate buffer (20mM, pH 3.5) at a flow rate of 60 μl min^{-1}.

The peaks due to M-III and EBr (t_R = 33min and 44min respectively) showed that these were subject to reduced detector responses (Figure 3.14) compared with the corresponding peaks obtained with the analytical column (Figure 3.13). This is a result of the inverse second-order dependence of micro-bore HPLC on retention volumes, which results in the loss of sensitivity due to peak broadening (Scott 1984). However, the compounds of interest, M-I and M-II (t_R = 4min and 6min respectively), eluted as sharp peaks with low peak volumes (approximately 20μl and 30μl).

Then the micro-bore HPLC system was coupled with a quadrupole mass-spectrometer using the conditions described in Section 3.2.2.12. The column eluent was split 1:1 and passed to the mass spectrometer and a UV detector (290nm) configured in parallel to facilitate peak assignments. Electrospray-mass spectrometry of the eluate from the

chromatographic column showed that both M-I and M-II (as shown on the single ion chromatogram (Figure 3.15a)) yielded ions with *m/z* values of 490 amu. Figure 3.15b confirms the identity of M-III as a mono-acetyl derivative (*m/z* = 356 ($[M_E^+$ - H] + CH_3CO^-)) of EBr (Figure 3.15c, *m/z* M_E^+ = 314). It is highly likely that this is the 8-acetyl analogue, since it is well known that the amino group at position 8 is much more susceptible to electrophilic attack than that at position 3. The ion of *m/z* of 490amu ($[M_E^+$ - H] + 177Da) produced by metabolites M-I and M-II is consistent with mono (*N*-) glucuronide formation at either the 3- or 8-amino groups of EBr.

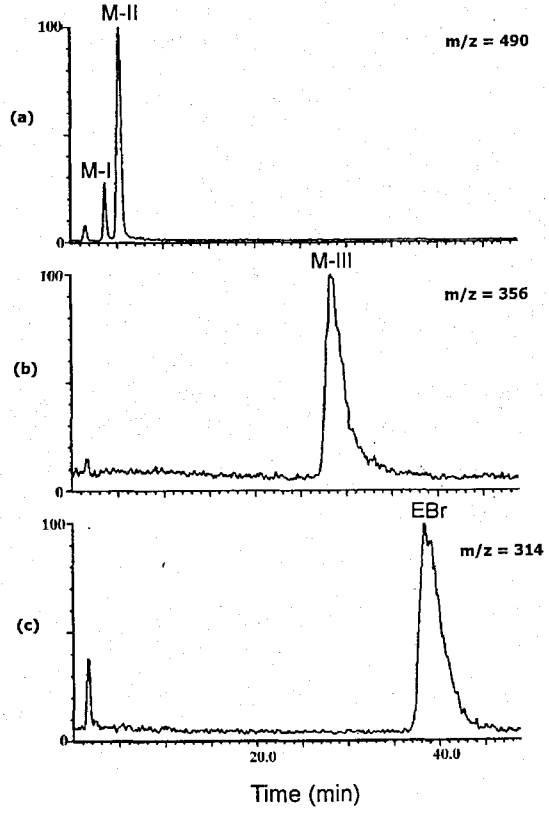


Figure 3.15 LC-ESI-MS single-ion chromatograms of extracts of 3h incubations of 100μM EBr with 2.4 x 10^6 viable cells /ml showing **a)** M-I and M-II (*m/z* = 490), **b)** M-III (*m/z* = 356) and **c)** E^+ (*m/z* = 314).

The existence of *N*-glucuronides of EBr has not been reported previously. The time-course study (Figure 3.12) showed that *N*-glucuronidation represents a major pathway for the metabolism of EBr in rats; with the formation of M-II being predominant (accounting for 19.5 ± 1.2% of total recovered drug in 3h). These *N*-glucuronides of EBr are remarkably stable in aqueous solution at ambient temperature. Consequently it is likely that they will be stable *in vivo* and then may act as transport forms of the parent compound, leading to its liberation at sites of pharmacological and toxicological significance, remote from the site of metabolism. Therefore, this pathway could form a major contribution to the *in vivo* distribution of the drug. By analogy with the argument for greater reactivity of the 8-amino group, metabolites M-I and M-II have been assigned as the 3-*N*-glucuronide and 8-*N*-glucuronide derivatives of EBr respectively. The time-course of the metabolism of EBr, which shows that M-II appears earlier (15min) than M-I (30min) (as well as in greater amounts) supports the structural assignments. These *N*-glucuronides may represent the two unidentified 'acid-labile, non-acetylated' metabolites of EBr, which were reported previously in the urine of rabbits and calves (Gilbert and Newton 1982).

Generally, glucuronidation changes the biological effects of xenobiotics as a result of the addition of a large hydrophilic group, so that the metabolite will not easily fit into its receptor binding sites (Mulder 1992). The increased water solubility of the glucuronide compared to that of the parent compound facilitates its excretion from the body. In the rat, glucuronides of molecular weight greater than 400Da are generally excreted predominantly in the bile (Gibson and Skett 1994). Therefore, the glucuronides of EBr (490Da) will serve as ideal substrates for biliary excretion. The presence of significant amounts of the enzyme β-glucuronidase, an enzyme that catalyses the hydrolysis of most glucuronide conjugates, in the intestine may result in the formation of the free drug (aglycone) from the glucuronide.

The aglycone may be reabsorbed, transported to the liver, re-conjugated and re-excreted in a process known as hepatic recirculation. This may prolong the half-life and consequently, the effects of the drug in the body. Although this study has indicated that EBr is rapidly metabolised by glucuronidation, a previous study (Murilla *et al.* 1996a) in cattle found that low levels of the drug remained in circulation up to four weeks after intravenous administration. It was speculated that EBr might be bound to plasma proteins. However, in view of our present findings, it would be prudent to assume that hepatic recirculation also may be a factor in the persistence of EBr *in vivo* for long periods after administration.

An illustration of the possible fate of the glucuronides of EBr is shown in Figure 3.16. Normally arylamine *N*-glucuronides are labile and may be hydrolysed easily to the parent amine under weakly acidic conditions, which can exist in the urinary bladder. It is known that, in 2-naphthylamine, the *N*-glucuronide decomposes in slightly acidic urine to the hydroxylamine and an extremely reactive nitrenium ion, which may be an ultimate carcinogen (Miller and Miller 1981).

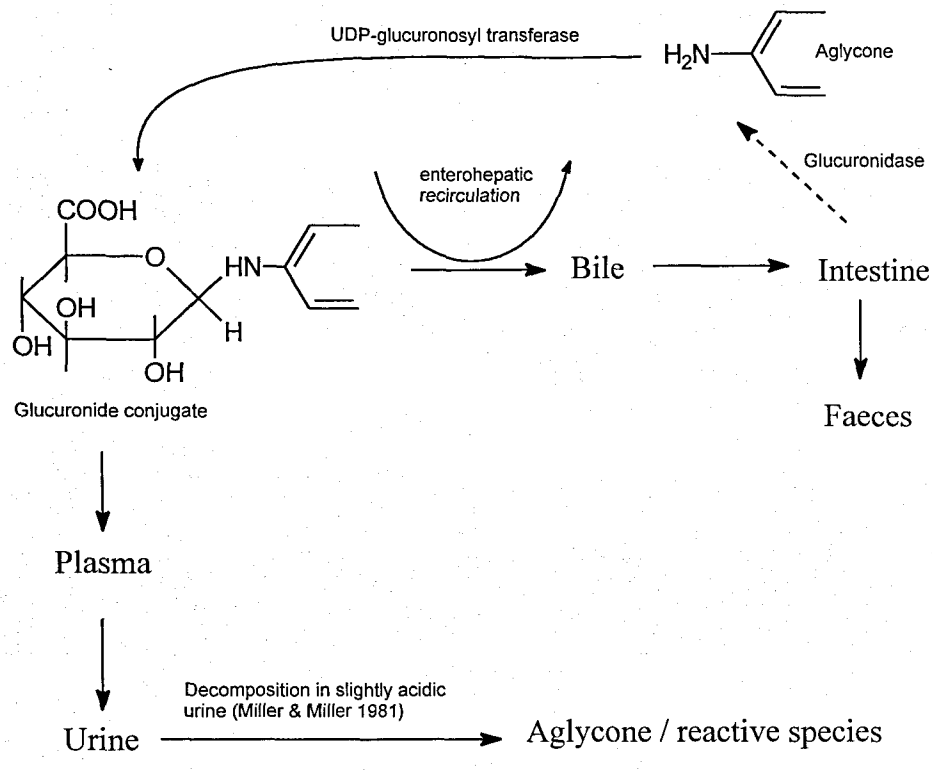


Figure 3.16 Possible fate of the glucuronides of arylamines (Adapted from Gibson and Skett 1994).

Although glucuronidation of xenobiotics is considered generally to be a significant detoxification step, certain glucuronides which are present in the circulating blood, such as the glucuronides of retinoic acid or 1,15-dihydroxy vitamin D, may retain biological activity (Mulder *et al.* 1990). The biotransformation of morphine to morphine-6-glucuronide, which enhances the binding of the latter to the δ-receptor with a resultant potentiation of the analgesic effect, is a classical example of a glucuronidation-enhanced activity of a therapeutic agent (Tephly and Burchell 1990, Mulder *et al.* 1990). Thus there is the possibility that the glucuronides of EBr may possess pharmacological activity. The stability of these *N*-glucuronides in solution, coupled with the probability of enterohepatic recirculation after biliary excretion, then would make them possible transport forms of EBr activity *in vivo*.

3.3.4 Extent of N-acetylation of EBr

The possibility of the formation of 3-acetyl and 3,8-diacetyl-EBr was investigated by increasing the number of cells/ml used in the incubation solutions (4.8 x 10^6 viable cells/ml of 100μM EBr solution [as opposed to 2.4 x 10^6 viable cells/ml]).

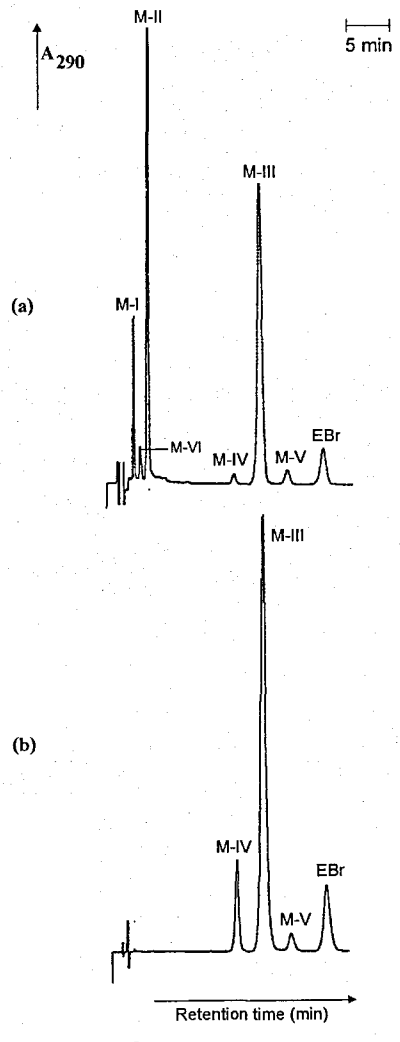


Figure 3.17 HPLC chromatograms of **a)** an extract from 3h incubations of 100μM EBr with 4.8 x 10^6 viable cells /ml and **b)** a mixture of authentic acetyl derivatives of EBr. M-III, M-IV and M-V represent peaks for 8-acetyl-EBr, 3,8-diacetyl-EBr and 3-acetyl-EBr respectively.

Comparison of a chromatogram of a solution of the two authentic monoacetyl and the diacetyl derivatives of EBr with that of an extract from the incubation mixture containing the increased cell concentration (Figure 3.17) with the same analytical conditions identified peaks M-IV (t_R = 12.5 min) and M-V (t_R = 18.2 min) as 3,8-diacetyl and 3-acetyl-EBr respectively. The ratio of formation of 3,8-diacetyl-EBr: 3-acetyl-EBr: 8-acetyl-EBr was 1:2:40 after incubation for 3h (as determined by relative chromatographic peak areas). Another compound, M-VI (t_R = 4.1 min), was detected using the modified incubation conditions and this accounted for less than 1% of the total compounds recovered. The elution characteristics of M-VI in reversed-phase HPLC suggested an increase in polarity compared to that of EBr and its structure has been established as an *O*-glucuronide of EBr (Section 3.3.5).

This study has shown that EBr is metabolised rapidly by rat hepatocytes, with the formation of at least six metabolites. A previous study of the metabolism of EBr *in vitro* and *in vivo*, using rats pre-treated with Aroclor, 3-MC and phenobarbitone suggested that only two metabolites (8-acetyl-EBr and an 8-acetylated hydroxylated compound) were formed (Fraire *et al.* 1981). Although the authors excluded the possibility that 3-acetyl-EBr and 3,8-diacetyl-EBr might be formed, analytical evidence was not provided to substantiate that the methods used could detect these compounds, had they been present. The current study has demonstrated that both of these acetylated analogues are formed, albeit as minor metabolites (< 1% of total recovered drug). Although *N*-hydroxylated metabolites were not detected, the possibility of the formation of these compounds in small amounts cannot be excluded since they are known to be unstable (Timbrell 1991).

EBr is highly toxic and so doses are curtailed to prevent intoxication of the host (in *in vivo* studies) and the possibility of tissue damage (in *in vitro* systems). This may result in the production of some metabolites, which may be of toxicological or pharmacological significance, in such small quantities that they are not detectable. The selection of the isolated hepatocyte as the *in vitro* model system in this study, coupled with the sensitivity and selectivity of analytical procedures employed, facilitated the detection of the 3-acetyl and 3,8-diacetyl derivatives of EBr. The chemistry of the functional groups of EBr would predict that the 8-amino group is relatively more reactive than that at position 3 and this would account for the favoured 8-acetylation. This is the result of resonance stabilisation involving the distribution of the positive charge between the quaternary nitrogen and the 3-amino group (Berg 1963b, Firth *et al.* 1983, see Figure 2.2). Therefore, the formation of the less favoured 3-acetyl derivative would be limited by the cellular concentrations of acetyl-CoA. Acetylation of the 3-amino group was observed with higher cell densities, demonstrating the requirement for higher quantities of acetyl-CoA to produce detectable quantities of the metabolite.

Acetylation of the aromatic amino groups in trypanocidal phenanthridines has immense implications, since a structure-activity study suggested that acetylation of either of the aromatic amino groups resulted in decreased activity against *Trypanosoma spp.* (Walls 1945). Acetylation is catalysed by acetyltransferases, which show polymorphism (Weber *et al.* 1990, Vatsis and Weber 1994). Inter- and intra-species differences in the capacity for acetylation would exert significant effects on individual responses to EBr with rapid acetylators more likely to show reduced blood and tissue concentrations of the administered drug. Therefore, the therapeutic effect will tend to be greater in slow acetylators, since the target micro-organism will be exposed to higher concentrations of the drug. Hence, if *N*-

acetylation were prominent in the host it would result in a decreased therapeutic effect with the development of resistant parasites as a logical consequence. Acetylation polymorphism, which has been reported extensively in the human population, has been observed also in other mammalian species including rabbit, rat and hamster (Timbrell 1991). In addition to influencing the efficacy of treatment with several acetylatable drugs, the acetylator phenotype has been implicated as a factor in a number of toxic effects due to foreign compounds, including the carcinogenicity of aromatic amines (Timbrell 1991). Rapid acetylators encounter less risk, probably because of the protection of the amino group from metabolic activation to reactive species. Lecointe *et al.* (1981) reported that acetylation of the 3- and 8-amino groups in EBr resulted in the complete loss of mutagenicity in the Ames' *Salmonella* assay.

A proposed pathway for the metabolism of EBr by rat hepatocytes is shown in Figure 3.18.

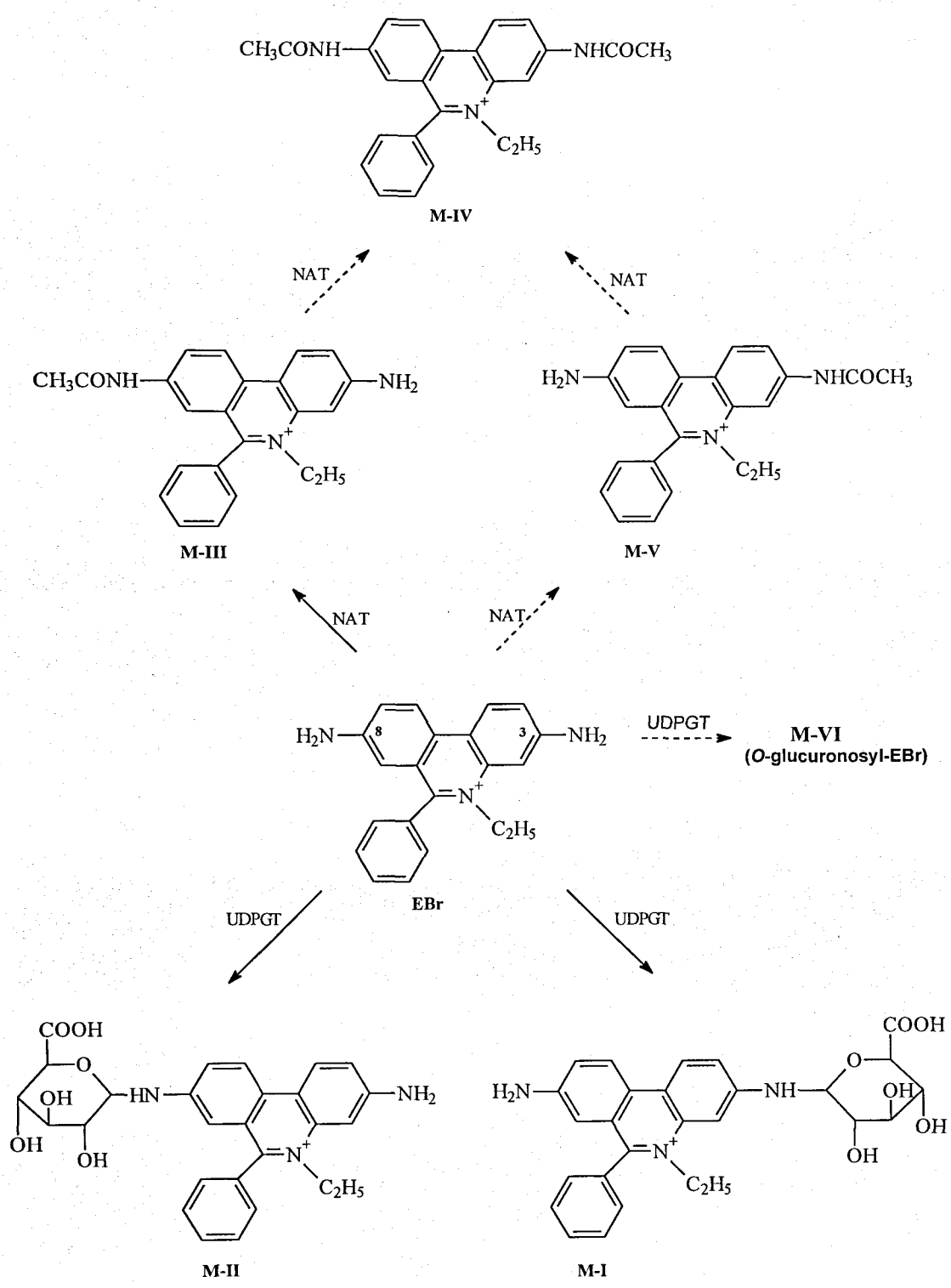


Figure 3.18 Proposed pathway for the metabolism of EBr in rat hepatocytes showing the major (solid arrows) and minor (broken arrows) routes mediated by N-acetyltransferase (NAT) and UDP-glucuronosyl transferase (UDPGT).

3.3.5 Induction of the metabolism of EBr

Treatment with phenobarbitone

Hepatocytes obtained from rats pre-treated with phenobarbitone sodium (Section 3.2.2.5) were incubated in a solution of EBr (100μM) for 3h as described previously (Section 3.2.2.9). Representative HPLC chromatograms of a 3h incubation of EBr with hepatocytes isolated from phenobarbitone-treated rats, together with those form the appropriate control, are shown in Figure 3.19. The chromatographic nature of metabolites was similar to that of those produced by hepatocytes obtained from untreated rats, as shown by the formation of M-I (t_R = 3.2 min), M-II (t_R = 4.8 min) and M-III (t_R = 21.7 min) (Figures 3.10 and 3.19). Provisional assignments of chromatographic peaks were made using chromatographic (retention times, Table 3.3) and spectral (UV photodiode-array) data (Appendix IV).

LC-ESI-MS analysis of extracts of the incubation medium, following a 3h incubation of EBr with hepatocytes obtained from phenobarbitone-treated rats, confirmed that M-I and M-II (*m/z* = 490) were *N*-glucuronides of EBr, and M-III (*m/z* = 356) was an *N*-acetyl derivative of EBr, consistent with 8-acetyl-ethidium. There was no evidence for the formation of a di-glucuronide (*m/z* = 666) analogous to that of the formation of diacetylethidium (Section 3.3.4).

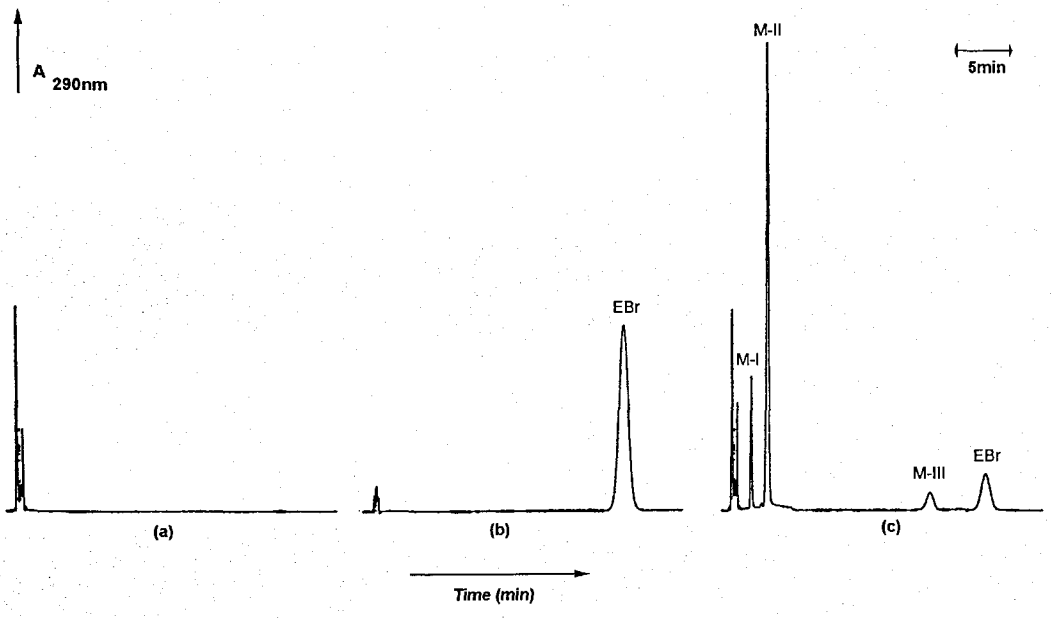


Figure 3.19 HPLC chromatograms of extracts of 3h incubation mixture of **a**) phenobarbitone treated-rat hepatocytes (2.4 x 10^6 viable cells /ml) with Krebs-Hepes-buffer, pH 7.4 **b**) EBr (100μM) with Krebs-Hepes buffer, pH 7.4 and **c**) phenobarbitone treated-rat hepatocytes (2.4 x 10^6 viable cells /ml) with EBr (100μM).

There were quantitative differences in the rates of metabolism of EBr (Figure 3.20) and the rates of formation of these metabolites (Figure 3.21), compared with the results (Figures 3.11 and 3.12) in untreated rats.

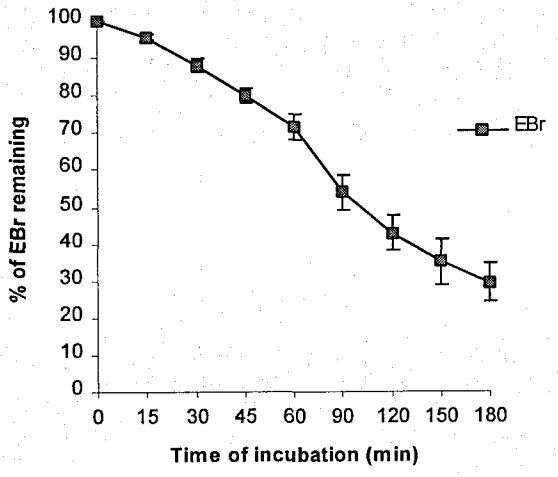


Figure 3.20 Time course of the metabolism of EBr by phenobarbitone-treated rat hepatocytes as determined by HPLC. Results are mean ± SEM of three determinations

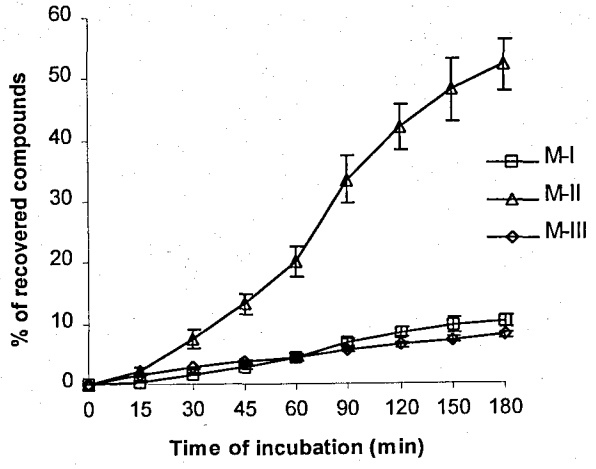


Figure 3.21 Time course of the formation of metabolites of EBr by phenobarbitone-treated rat hepatocytes as determined by HPLC. Results are mean ± SEM of three determinations.

The treatment of rats with phenobarbitone resulted in a loss of EBr of 70.4 ± 5.2% over 3h (mean ± SEM, n = 3), compared with 36.7 ± 6.3% (mean ± SEM, n = 4) in untreated rats. The analysis of variance (one-way ANOVA, 95% C.I.) of the quantities of EBr remaining after 3h in untreated and phenobarbitone-treated rats showed a statistically significant difference (p = 0.002). Compound M-II was the predominant metabolite in both instances and showed an approximately 2.5-fold increase in phenobarbitone-treated rats (52.2 ± 4.2%, mean ± SEM, n = 3) compared with untreated rats (19.5 ± 1.2%, mean ± SEM, n = 3). Metabolites M-I and M-III accounted for 10.3 ± 0.9% and 7.9 ± 0.5% respectively of the compounds recovered after 3h and did not differ markedly from the results in control rats (see Figure 3.25).

Treatment with 3-MC

Rats were treated with 3-MC (Section 3.2.2.5) and the isolated hepatocytes were incubated in a solution of EBr (100μM) for 3h as described previously (Section 3.2.2.9). Differences were observed in both the chromatographic profile and the amounts of metabolites of EBr formed in hepatocytes from rats (n = 3) on pre-treatment with 3-MC. Representative HPLC chromatograms of 45min and 3hr incubation mixtures of EBr with hepatocytes isolated from 3-MC-treated rats, with the appropriate controls (control results were obtained using hepatocytes isolated from rats treated with olive oil, see Section 3.2.2.5) are shown in Figure 3.22. Provisional assignments were made using chromatographic (retention times, Table 3.3) and spectral (UV photodiode-array) data (Appendix IV).

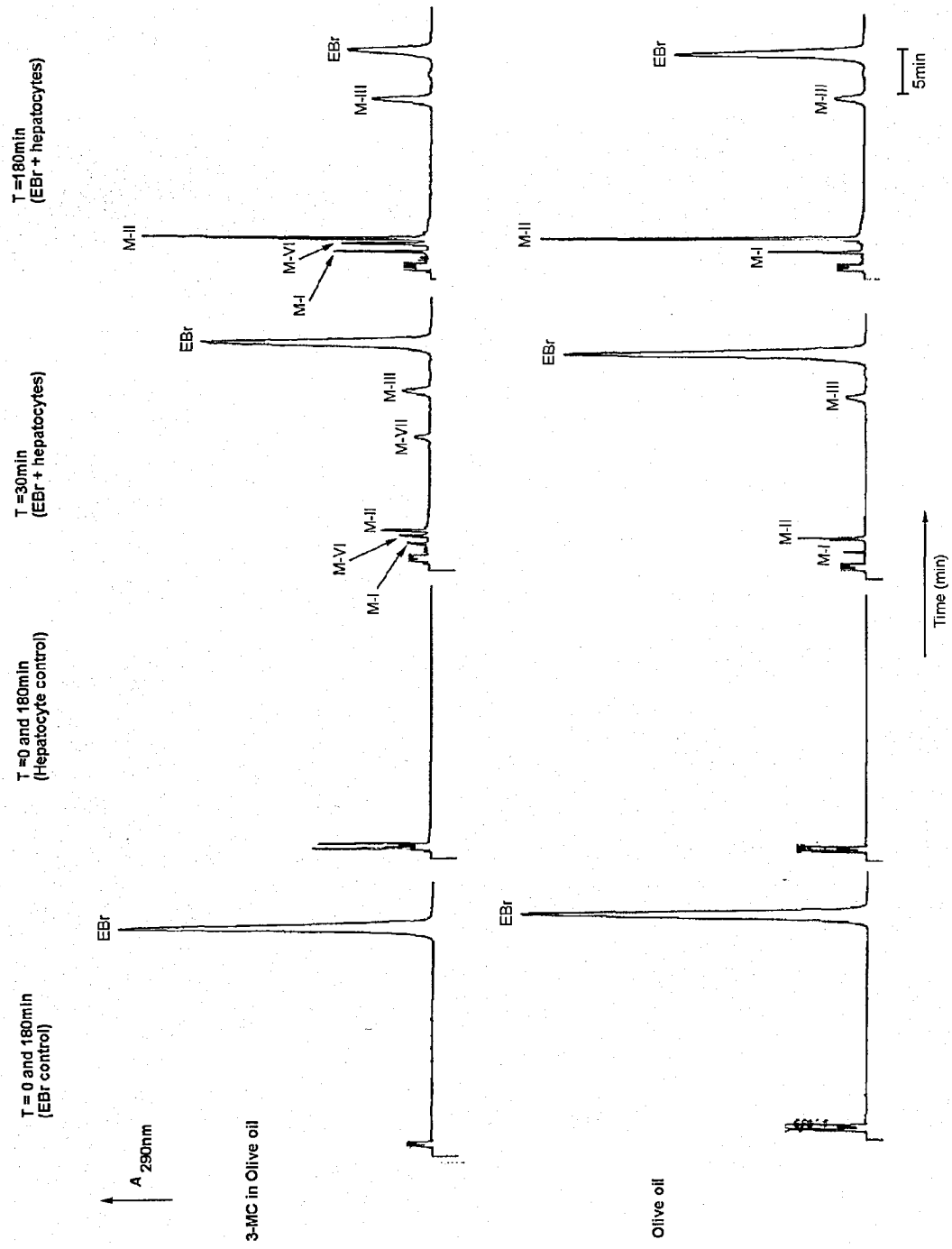


Figure 3.22 Comaparative HPLC chromatograms of the metabolism of EBr by hepatocytes from 3-MC-treated rats and olive oil-treated rats. Chromatograms were acquired at the same attenuation.

The time course of the disappearance of EBr (Figure 3.23) showed a loss of 58.2 ± 4.2% (mean ± SEM, n = 3) of the initial amount of EBr in 3h. In addition to the peaks due to M-I (t_R = 3.2min), M-II (t_R = 4.8 min) and M-III (t_R = 21.6min), two other peaks [M-VI (t_R = 4.1min) and M-VII (t_R = 16.1min)] were observed in the HPLC chromatogram (Figure 3.22).

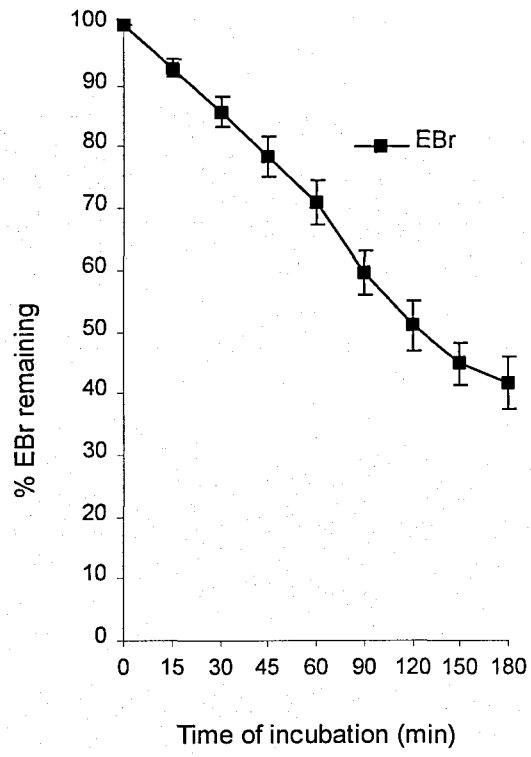


Figure 3.23 Time course of the metabolism of EBr by 3-MC treated rat hepatocytes as determined by HPLC. Results are mean ± SEM of three determinations.

The metabolite designated M-VI represented 6.9 ± 0.8% (mean ± SEM, n = 3) of the total recovered drug after 3h and eluted with the same retention time (4.1min) as the minor metabolite (M-VI) observed when the concentration of hepatocytes used in the incubation mixture was increased (Section 3.2.2.9, Figure 3.17). The metabolite M-VII was a transient

species (Figure 3.24); attaining a maximum concentration at approximately 15min (3.2 ± 0.6% of recovered compounds, mean ± SEM, n = 3) and disappearing completely from the incubation medium at 3h.

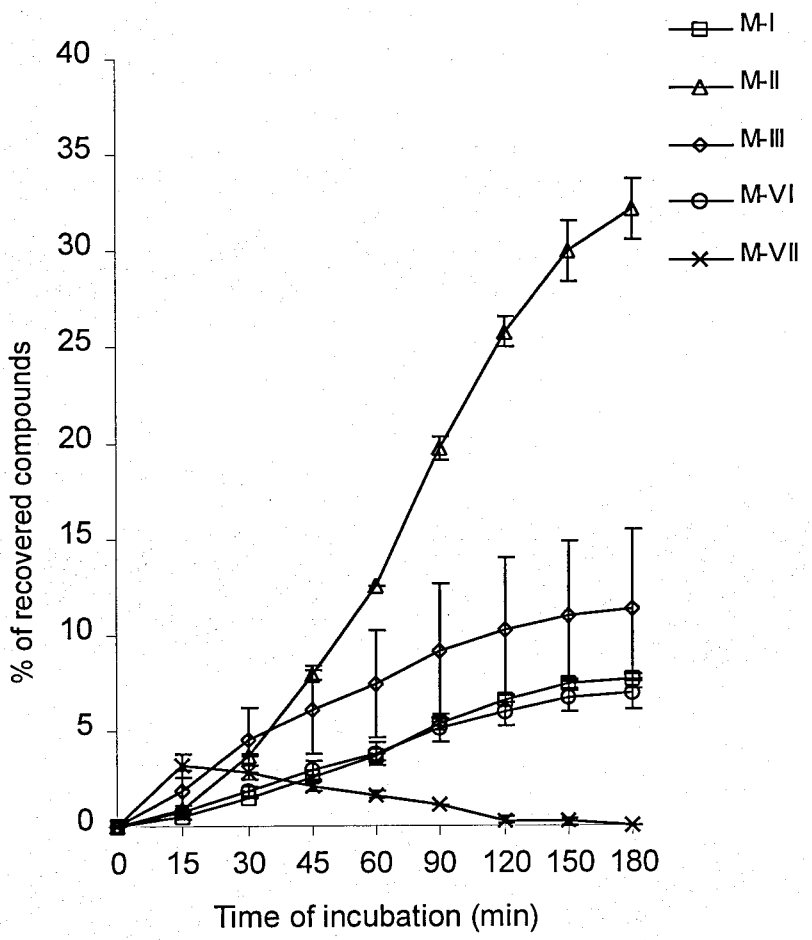


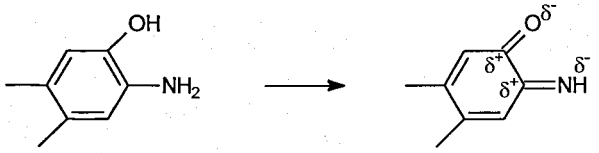
Figure 3.24 Time course of the formation of metabolites of EBr by 3-MC-treated rat hepatocytes, as determined by HPLC. Results are mean ± SEM of three determinations

LC-ESI-MS analysis of extracts of the incubation medium, following a 3h incubation of EBr with hepatocytes obtained from 3-MC-treated rats, confirmed the assignment of peaks M-I (m/z = 490) and M-II (m/z 490) as *N*-glucuronides, and M-III (m/z = 356) as a mono-acetyl derivative of EBr (8-acetyl-ethidium). Metabolite M-VI produced an ion of *m/z* value 506, which is consistent with the formation of an *O*-glucuronide ([M_E+ - H] + 16 + 177]). This could arise from cytochrome P450-mediated ring hydroxylation (Lavoie *et al.* 1985) followed by a 3-MC-specific UDPGT-mediated glucuronidation. The transient nature of the formation of M-VII is consistent with its being the ring-hydroxylated intermediate compound. However, because of the small amounts of M-VII formed, its *m/z* value could not be measured. There was no evidence for the formation of a diglucuronide (*m/z* = 666).

Metabolite M-VI corresponds, in terms of retention times, to the previously observed minor peak (M-VI) that was detected when the concentration of cells used in the incubation was doubled (see Section 3.3.4, Figure 3.17). Therefore, treatment of the rats with 3-MC leads to an increase in the formation of M-VI from a relative amount of less than 1% in control rats to 6.9 ± 0.8% (mean ± SEM, n = 3).

Hydroxylation of xenobiotics involves the participation of NADPH and it is known that EBr can undergo metabolic activation which involves the participation of NADPH (MacGregor and Johnson 1977, Lecointe et al. 1981). The species responsible for the metabolic activation and resultant toxicity are not known. This novel metabolite (M-VI) may represent the formation of the metabolite that is dependent on NADPH, which has eluded detection over the years (MacGregor and Johnson 1971, Lecointe *et al.* 1981). Perhaps the transient nature of its existence, the low maximal concentration in untreated animals and the probability of chromatographic co-elution with the other polar metabolites (M-I and M-III) are the reasons why it has not been reported by previous investigators.

The hydroxylation of the phenanthridine ring at positions ortho- to the amino moieties may result in the formation of quinone amines which are known to be toxic and reactive species (Timbrell 1991).



quinone imine

The treatment of rats with 3-MC produced a greater loss of EBr (58.2 ± 4.2%, mean ± SEM, n = 3) compared with that in untreated rats (36.7 ± 6.3%, mean ± SEM, n = 4). The analysis of variance (one-way ANOVA, 95% C.I.) of the quantities of EBr remaining after 3h in untreated and 3-MC-treated rats showed a statistically significant difference (p = 0.004). Although M-II was the predominant metabolite in both instances, approximately 1.5 times more of it was formed in hepatocytes from 3-MC-treated rats (32.2 ± 1.6%, mean ± SEM, n = 3, compared with 19.5 ± 1.2%, mean ± SEM, n = 4, in untreated rats).

A graphical summary of the relative proportions of EBr and its metabolites at 3h on incubation with hepatocytes obtained from untreated, phenobarbitone-treated and 3-MC-treated rats is shown in Figure 3.25.

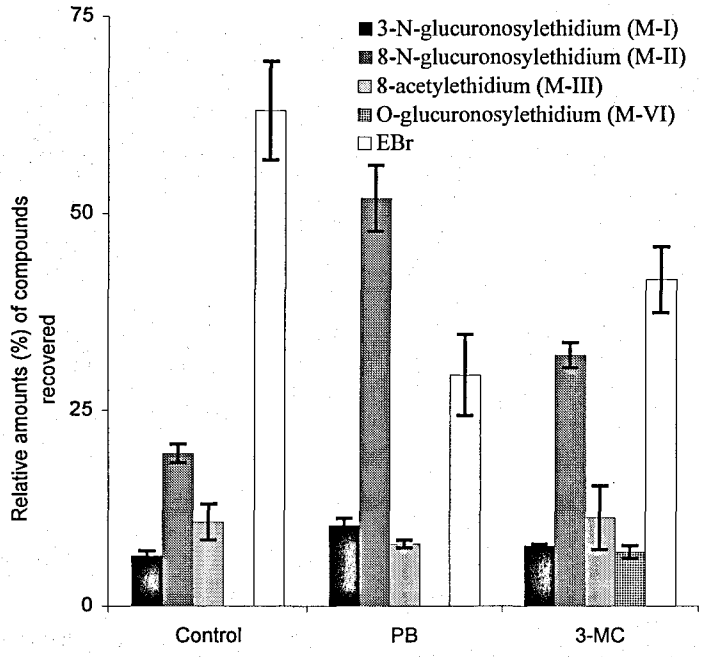


Figure 3.25 Relative amounts (%) of EBr and its metabolites formed by untreated (control, n = 4) and treated (phenobarbitone (PB) and 3-MC, n = 3) rat hepatocytes. Values are mean ± SEM.

It has been demonstrated that the treatment of rats with 3-MC and phenobarbitone results in a significant enhancement of metabolism of EBr via the *N*-glucuronidation pathway. This observed induction of *N*-glucuronidation has immense pharmacological and toxicological implications. It has been known for almost three decades that phenanthridinium compounds are transformed into reactive species in the presence of an *in vitro* rat liver activation system from Aroclor-1254 treated rats (MacGregor *et al.* 1977, Lecointe *et al.* 1981). Aroclor-1254 is a mixture of polychlorinated biphenyls with both phenobarbitone- and 3-MC-inducer properties (Lilienblum *et al.* 1982, Gibson and Skett 1994). It has been demonstrated that Metabolite M-II is under the regulatory control of UDPGTs inducible by both phenobarbitone and 3-MC. Consequently, it is probable that the formation of M-II will be influenced significantly by the treatment of rats with Aroclor-

1254. Figure 3.26 shows the glucuronosylation pathways in the metabolism of EBr which are altered by phenobarbitone (PB) and 3-MC.

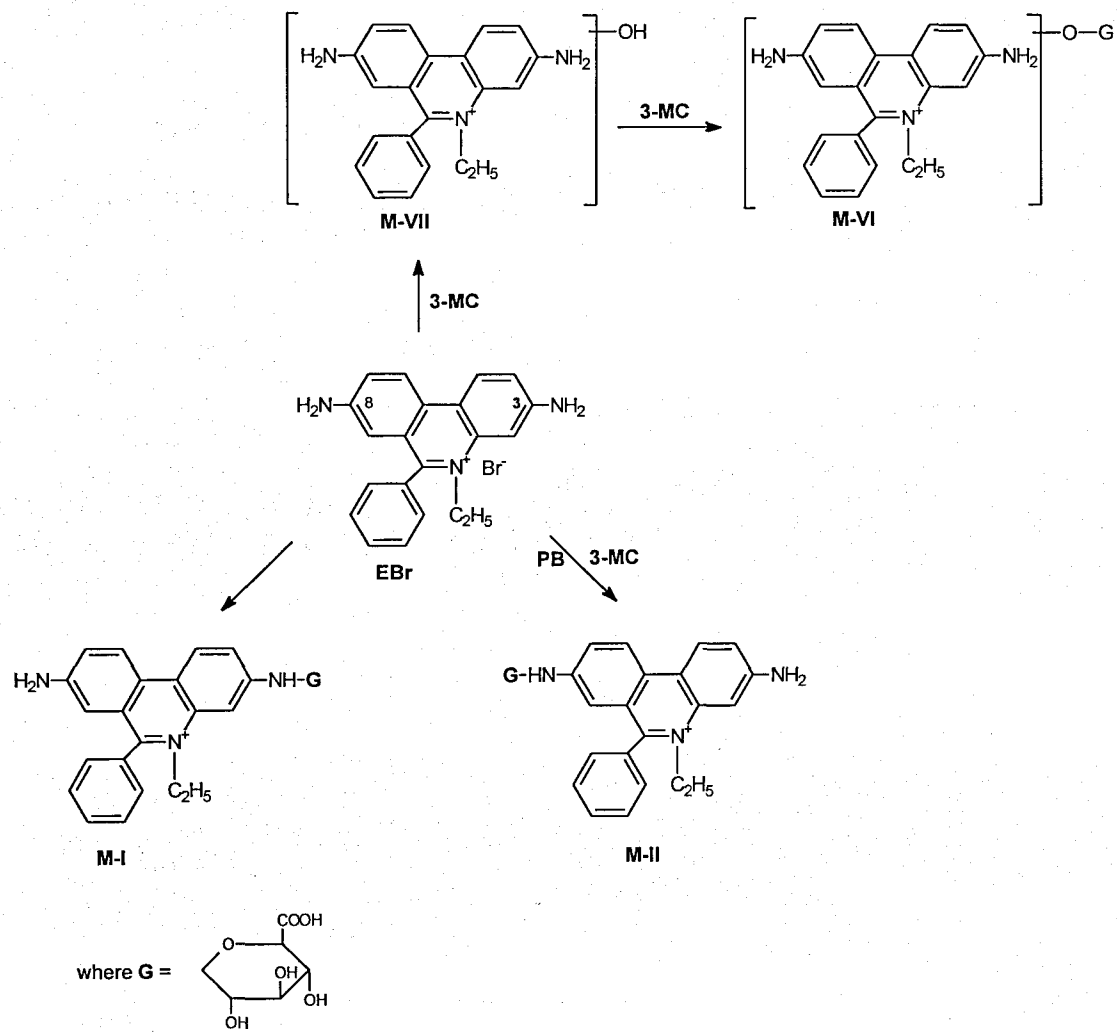


Figure 3.26 Glucuronosylation of EBr by rat hepatocytes showing the pathways influenced by 3-MC and phenobarbitone (PB).

The formation of metabolite M-VI (*O*-glucuronosyl-hydroxyethidium) in rats is induced by 3-MC, but is unaffected by induction with phenobarbitone. The presence of the 3-MC-inducible metabolite (M-VII) which may represent the hydroxyethidium intermediate

required for the formation of M-VI, suggests that there has been increased transcription of the CYP1A gene (Table 3.1). The formation of the acetyl derivatives of EBr (M-III. M-IV, M-V) were not affected by the pre-treatment of rats with either phenobarbitone or 3-MC and this is consistent with the fact that *N*-acetyltransferase is not induced by these agents. There was no marked increase in the formation of M-I after treatment of rats with either of the two inducing agents.

Glucuronidation of xenobiotics is considered generally as a significant step in their detoxification and elimination from the body. However, it is known that active metabolites, such as morphine-6-glucuronide and the 17β-glucuronides of estradiol and ethinylestradiol, exist (Tephly and Burchell 1990, Mulder 1992). Pharmacologically, an increase in the biotransformation of EBr to the possibly 'inactivated glucuronide' as a result of enzyme induction would result in an increased clearance of the drug from the host. Subsequently, parasites (i.e. trypanosomes) would be exposed to sub-lethal doses, which may result in the development of resistance to otherwise therapeutic doses of ethidium. The earliest reports of resistance of the parasite to EBr were in the 1960's (Leach *et al.* 1981) and occurred after large scale operations involving aerial applications of organochlorine pesticides (such as DDT and dieldrin) were undertaken in East Africa and South Africa in the period 1945 to 1951 (Barrett 1997). Whilst the co-occurrence of these events may be purely coincidental, the present study has provided a link between the use of pesticides capable of phenobarbitone- and 3-MC-type-induction with an increase in the rate of glucuronidation of EBr. However, it should be borne in mind that the induction of *N*-glucuronidation of aromatic amines (arylamines) does not always lead to deactivation or detoxification. An increase in toxicity may be observed which may be due to several factors; for example, enhanced bioactivation, decreased detoxification or decreased bioinactivation (Park *et al.*

1996). It is known that, in the case of 2-naphthylamine, the *N*-glucuronide decomposes in slightly acidic urine to the hydroxylamine and an extremely reactive nitrenium ion which may be an ultimate carcinogen (Miller and Miller 1981).

3.3.6 Interspecies differences in the metabolism of EBr

From an evolutionary point of view, all mammals are similar, because they originate from a common ancestor; yet they differentiate because of their dissimilar environmental adaptations (Lin 1995). Thus, significant differences occur between different species and sometimes within species. This presents difficulties in extrapolation of metabolic data from one species to another. In an article entitled "Of mice, microsomes and men", Brodie (1964) wrote:

> "Nature has raised an enormous barrier to drug development by assigning the drug-metabolism enzymes to various species in astonishingly diverse amounts. So great are the differences that it is often a matter of pure luck that animal experiments lead to clinically useful drugs".

The metabolism of EBr was investigated in sheep and pig hepatocytes using the procedures described for rat hepatocytes (Section 3.2.2.9). Sheep hepatocytes metabolised EBr to two compounds over an incubation period of 3h, as detected by HPLC (Figure 3.27), with t_R values of 3.1min and 4.7min. The retention times and UV photodiode array spectra of these peaks were consistent with those of M-I and M-II (Table 3.3, Appendix IV).

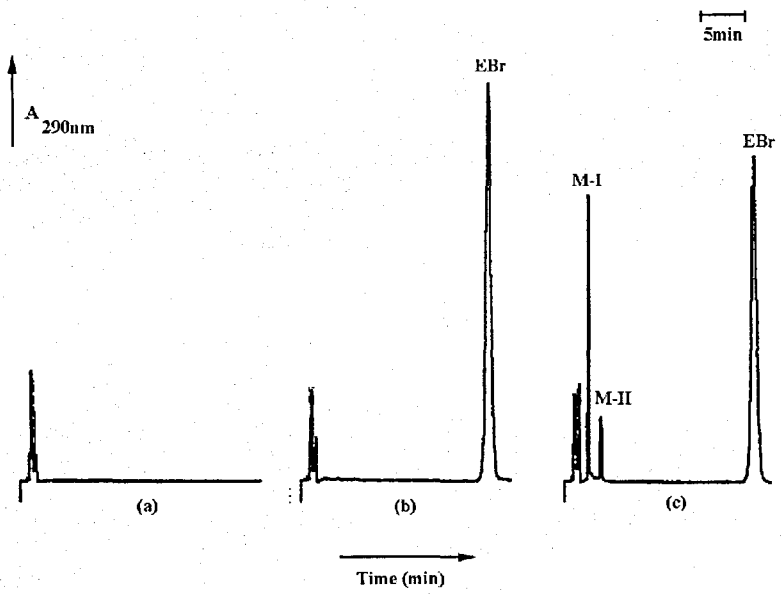


Figure 3.27 HPLC chromatograms of extracts of a 3h incubation mixture of **a**) sheep hepatocytes (2.4 x 10^6 viable cells /ml) with Krebs-Hepes-buffer, pH 7.4 **b**) EBr (100μM) with Krebs-Hepes buffer, pH 7.4 and **c**) sheep hepatocytes (2.4 x 10^6 viable cells /ml) with EBr (100μM)

The time course of the disappearance of EBr (Figure 3.28) showed a loss of 14.4 ± 3.2% of the parent compound after 3h with the concomitant formation of M-I and M-II (Figure 3.29). These two metabolites (M-I and M-II) accounted for 10.3 ± 2.2% and 4.1 ± 1.0% respectively of the compounds recovered after 3h. Contrary to the observation with rat hepatocytes (Figure 3.10, where the amount of M-II was determined to be greater than that of M-I), sheep hepatocytes formed more of M-I than M-II. However, there was no evidence for the *N*-acetylation of EBr by sheep hepatocytes.

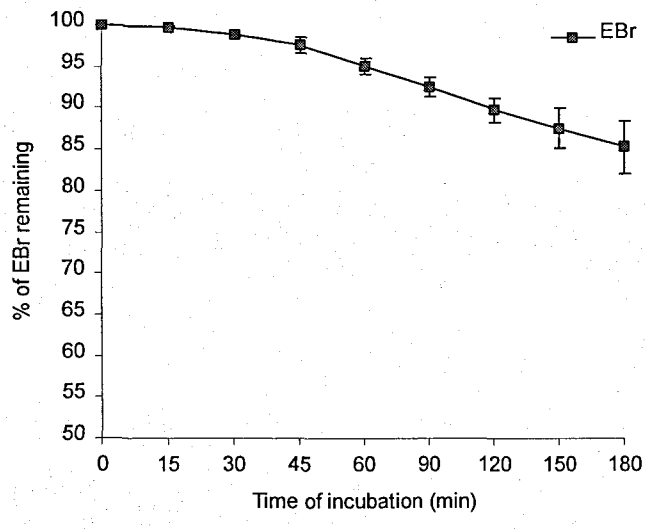


Figure 3.28 Time course of the metabolism of EBr by sheep hepatocytes as determined by HPLC. Results are mean ± SEM of three determinations

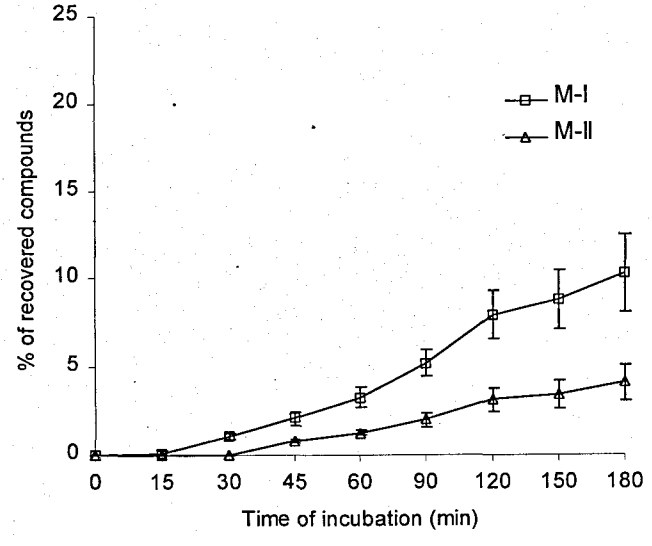


Figure 3.29 Time course of the metabolism of EBr by sheep hepatocytes showing the formation of the metabolites M-I and M-II, as determined by HPLC. Results are mean ± SEM of three determinations

The metabolism of EBr by pig hepatocytes was found to be negligible compared with results obtained for rat and sheep hepatocytes. The only metabolite present after 3h (Figure 3.30) accounted for 1.5 ± 0.3% (mean ± SEM, n = 3) of the total recovered compounds. The retention time (t_R = 4.7) and the UV photodiode array spectrum of this peak was consistent with those of M-II. There was no evidence for *N*-acetylation of EBr by pig hepatocytes

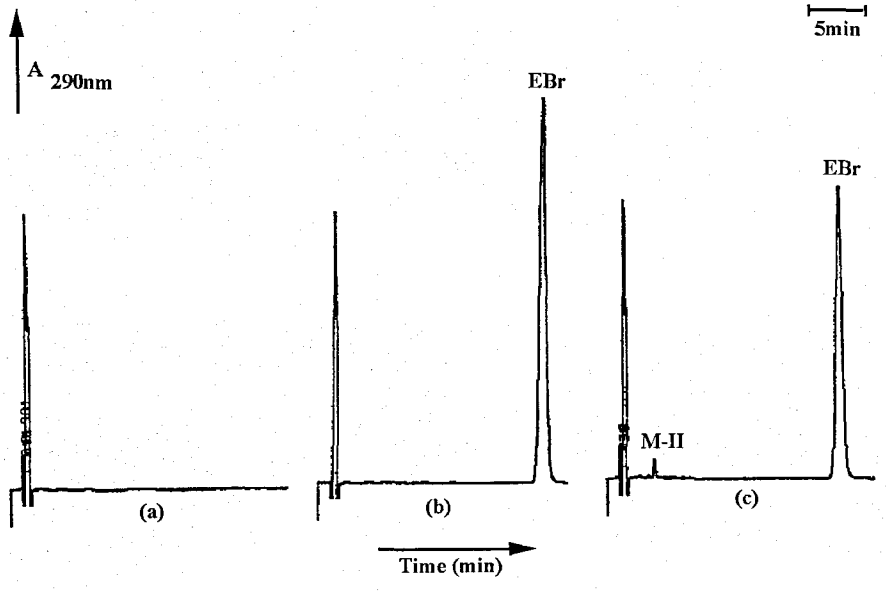


Figure 3.30 HPLC chromatograms of extracts of 3h incubations of **a**) pig hepatocytes (2.4 x 10^6 viable cells /ml) with Krebs-Hepes-buffer, pH 7.4 **b**) EBr (100μM) with Krebs-Hepes buffer, pH 7.4 and **c**) pig hepatocytes (2.4 x 10^6 viable cells /ml) with EBr (100μM).

As a result of the relative amounts of metabolites M-I and M-II formed by rat hepatocytes, these compounds were provisionally assigned as 3-*N*-glucuronosylethidium and 8-*N*-glucuronosylethidium respectively on the basis of the relative chemical reactivities of the amino groups of the phenanthridines. However, the subsequent study with sheep

hepatocytes showed a reversal in the relative amounts of M-I and M-II which indicates that, in addition to the chemistry of the phenanthridine ring, the nature and availability of the UDPGT isoenzymes responsible for these biotransformations may be different in the two species. Ideally, collision induced fragmentation by MS/MS analysis of these *N*-glucuronosylethidium metabolites of identical *m/z* value (490Da) should yield daughter ions (Figure 3.31) of different *m/z* values and allow unambiguous assignment of their structures.

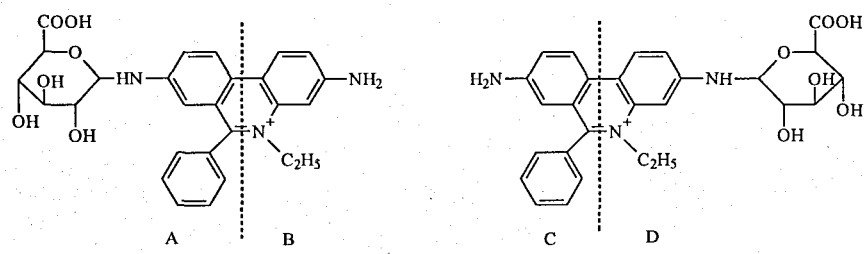


Figure 3.31 Chemical structures of the two *N*-glucuronosylethidium metabolites showing the ideal positions (indicated by broken lines) of collision induced fragmentation which would allow the formation of daughter ions (A, B, C and D) of different *m/z* values.

However, because of the rigidity of the planar phenanthridine ring, attempts to perform collision induced fragmentation of the two metabolites resulted in the labile glucuronide moiety being lost before the cleavage of the ring structure. Therefore, unambiguous assignment of the positions of *N*-glucuronidation may require enzymatic synthesis using purified UDPGT isoenzymes or the newer technique of on-line LC-MS-NMR.

Perhaps the best-known instances of species variation in metabolism are the inability of the cat to form glucuronides of certain foreign compounds (Lin 1995) and the reduced capacity for acetylation in the dog (Weber *et al.* 1990). Originally it was thought that UDP-glucuronosyltransferase and *N*-acetyltransferases were deficient genetically in the cat and

dog respectively, but it is now clear that the species defect is substrate-dependent (Lin 1995). It has also been reported (van't Klooster 1992), that the reduced capacity of the sheep, pig and goat, relative to the rat and rabbit, to acetylate sulphadimidine is due to a higher deacetylase activity. It is important to note that, although the rabbit has a high *N*-acetylating capacity (Weber 1990), a previous study (Gilbert and Newton 1982) demonstrated that acetylated derivatives of EBr were absent in this specie. The inability of pig and sheep hepatocytes used in this study to form acetylated derivatives of EBr agrees with data generated for cattle *in vivo* (Gilbert and Newton 1982, Murilla 1996a). It is clear from results obtained in this, and previous, studies (MacGregor and Clarkson 1971, Fraire *et al.* 1981, Gilbert and Newton 1982, Murilla 1996a) that the extrapolation of data on the metabolism of EBr from one species to another may not be appropriate and that the rat model may have some limitations.

Significantly, this study has been able to demonstrate that the novel *N*-glucuronosylation pathway for the metabolism of EBr is relevant in ruminant species and it has been shown that this pathway is the major route of metabolism in the rat, sheep and pig. The findings that the rat and sheep show different ratios of M-I and M-II, and pigs form only M-II, may be a good indication of the involvement of different UDPGT isoenzymes with different substrate specificities (Mulder *et al.* 1990).

Although these results have demonstrated a species difference in the metabolism of EBr, caution must be exercised in its interpretation because of the lack of information on the P450 levels and of hepatocyte functions of the individual preparations from sheep and pigs. Nevertheless, data available from our laboratory indicate that these preparations from large animals provide good quality functional hepatocytes which compare well with rat cells (Watts *et al.* 1995, Dr. M.H. Grant, *personal communication*).

3.3.7 Structural requirements for the metabolism of the phenanthridines: application to the metabolism of ISM and M&B4250

In the absence of data on the metabolism *in vitro* of ISM and M&B4250, a preliminary toxicological screen was used in the selection of a suitable working concentration of the phenanthridines. The principle was based on the Trypan Blue exclusion test (section 3.2.2.4) used in the determination of the viability of cells. Isolated hepatocytes were incubated in solutions of ISM of concentrations 100μM and 200μM. The viability of the cells were determined at the start of the incubation (t = 0h) and subsequently at 1, 2 and 3h. The data were compared with those from control incubations conducted in the absence of ISM (Table 3.4).

The cell viability data obtained at concentrations of the drug of 100μM and 200μM were compared with those of the control by a two-sample paired test using a 5% significance level. In both comparisons, the computed p-values were greater than 0.05 ($p > 0.05$), indicating that the result of the two-sample paired test was not significant at the 5% level (i.e. the means of the two populations (control and treated) in each comparison were not statistically different). Therefore, studies of the metabolism of ISM were performed with 100μM solutions in KH buffer.

The same toxicological screen was not feasible with M&B4250 because of its purple colour, which would have masked the presence of Trypan Blue in the cells. Therefore, M&B4250 was used as at a concentration of 50μM since it present in Samorin® at approximately half the content of ISM (section 2.3.5).

Table 3.4 Comparison of the viability of control and ISM-treated hepatocytes.

	% Viability		
Time of incubation (h)	Control	100μm ISM	200μM ISM
0	73	73	73
1	72	76	74
2	79	77	78
3	74	72	76

The efficiency of recovery of ISM (100μM) from rat hepatocytes using the procedure previously described (section 3.2.2.8) was 81.3 ± 0.8% (mean ± S.D, n = 10). This was lower than that of EBr (99.8 ± 0.7%) and might arise from the stronger binding of ISM to cell macromolecules and nucleic acids (Kinabo and Bogan 1987). A control incubation of EBr (100μM) was set up to confirm that the hepatocyte preparations used in the metabolism of ISM were viable metabolically. The cell preparations used (n = 3) converted EBr to its known metabolites.

There were no secondary peaks in the HPLC chromatogram of ISM (t_R = 9.5min) in KH buffer alone after 3h incubation (Figure 3.32b), indicating that the parent phenanthridine was stable under these conditions. HPLC analysis of an extract of hepatocytes incubated with ISM did not show any secondary peaks (Figure 3.32c). At the termination of incubation (3h), the percentage of ISM (recovered relative to the starting concentration) was approximately 89.1 ± 2.6% (mean ± SEM., n = 3).

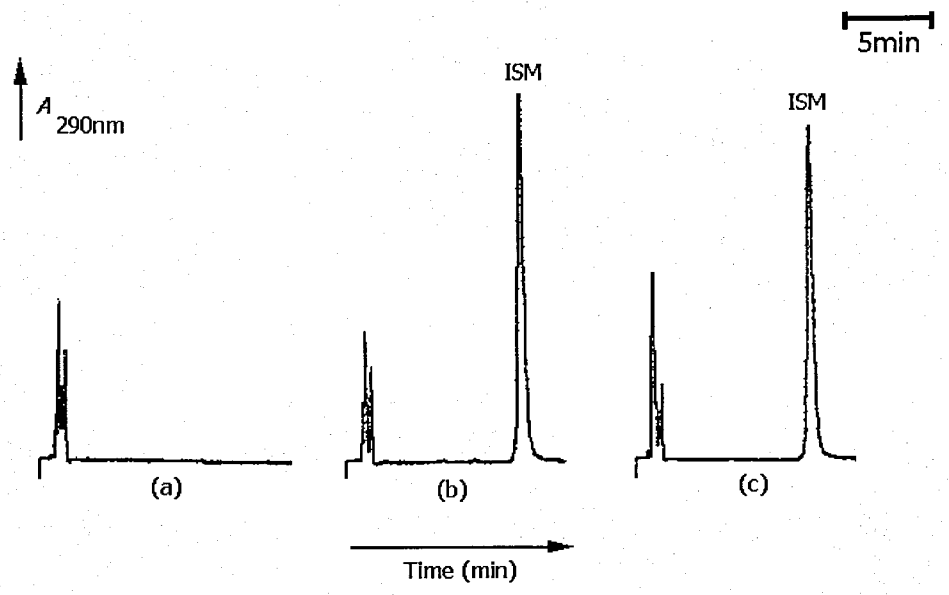


Figure 3.32 HPLC chromatograms of extracts of 3h incubations of **a**) rat hepatocytes (2.4 x 10^6 viable cells /ml) with Krebs-Hepes-buffer, pH 7.4 **b**) ISM (100μM) with Krebs-Hepes buffer, pH 7.4 and **c**) rat hepatocytes (2.4 x 10^6 viable cells /ml) with ISM (100μM).

This study has confirmed the findings from previous studies of the metabolism of ISM in rats *in vivo* (Philips *et al.* 1967) and cattle (Kinabo and Bogan 1988d, Mdachi *et al.* 1991, Murilla 1996b). Based on the metabolism of EBr, in which both amino groups are conjugated with acetyl and glucuronide moieties, it would have seemed reasonable to anticipate that the 3-amino group of ISM would be 'available' for such reactions. It has been reported that compounds (such as diminazene (Figure 1.2) and pentamidine) bearing chemical groups structurally similar to the benzamidine moiety of ISM are extensively metabolised by rats via *N*-hydroxylation to the corresponding amide oximes (Berger *et al.* 1990, Berger *et al.* 1991, Clement *et al*, 1992). The identification of these amide oximes metabolites was based on their behaviour in HPLC (Berger *et al.* 1990, Berger *et al.* 1991) and TLC (Clement *et al*, 1992) and on comparison of the mass spectral data for the metabolites with those for synthesised standards. Therefore, the inability to detect

metabolites of ISM does not necessarily eliminate the possibility of their occurrence, but may rather reflect the limitations of current analytical methodologies. However, it is obvious that the metabolism of ISM is far less extensive than that of EBr, since 89.1% of the former parent compound was recovered, compared with 63.3% of EBr after 3h incubation.

The metabolism of the structurally related compound M&B4250, which possesses 3- and 8-amino groups (i.e. it is similar to EBr) was investigated using rat hepatocytes. The efficiency of recovery of M&B4250 (50μM) from rat hepatocytes using the procedure previously described (section 3.2.2.8) was 75.3 ± 2.4% (mean ± S.D, n = 5) and at least two metabolites (X (t_R = 12.9min) and Y (t_R = 27.1min), Figure 3.33c; *cf.* M&B4250 (t_R = 31.5min)) were detected by HPLC (section 3.2.2.11). The percentage of the amount of M&B4250 recovered relative to the amount at the start of the incubation was 31.4 ± 10.7% (mean ± SEM, n = 3) and this demonstrates a large variability in the metabolism.

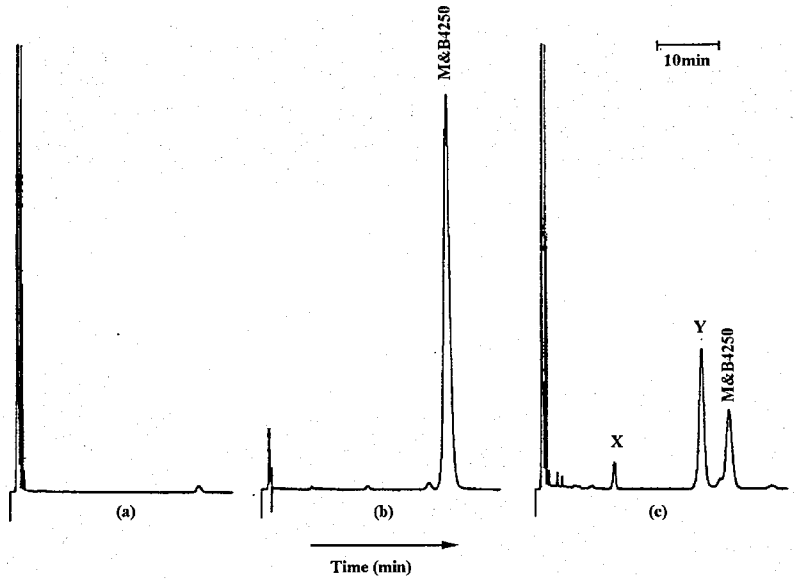


Figure 3.33 HPLC chromatograms of extracts of 3h incubations of **a**) rat hepatocytes (2.4 x 10^6 viable cells /ml) with Krebs-Hepes-buffer, pH 7.4 **b**) M&B4250 (50μM) with Krebs-Hepes buffer, pH 7.4 and **c**) rat hepatocytes (2.4 x 10^6 viable cells /ml) with M&B4250 (50μM).

HPLC-ESI-MS analysis of extracts of 3h incubations of rat hepatocytes with M&B4250 showed that compounds X an Y produced ions of *m/z* values of 453 and 679 respectively (Figure 3.34). A third metabolite (Z), which was present in a small amount and co-eluted with the peak for M&B4250, produced an ion of *m/z* 679. The *m/z* value of 679 is consistent with the formation of *N'*-acetyl-*N''*-glucuronosyl-M&B4250 ($[M_{M\&B4250}^{+} - H] + CH_3CO^- +$ glucuronide (177Da)). Therefore, the detection of two peaks of *m/z* value 679 indicates that there may be the two possible positional isomers; 3-*N'*-acetyl-8-*N''*-glucuronosyl-M&B4250 and 8-*N'*-acetyl-3-*N''*-glucuronosyl-M&B4250 (Figure 3.35). The photodiode-array spectrum (Appendix IV) of X showed a typical phenanthridine absorbance at 290nm. However, the *m/z* of 453 (loss of 7Da from the molecular ion of M&B4250)

could not be rationalised as a single step transformation of M&B4250 and may be the result of structural rearrangement.

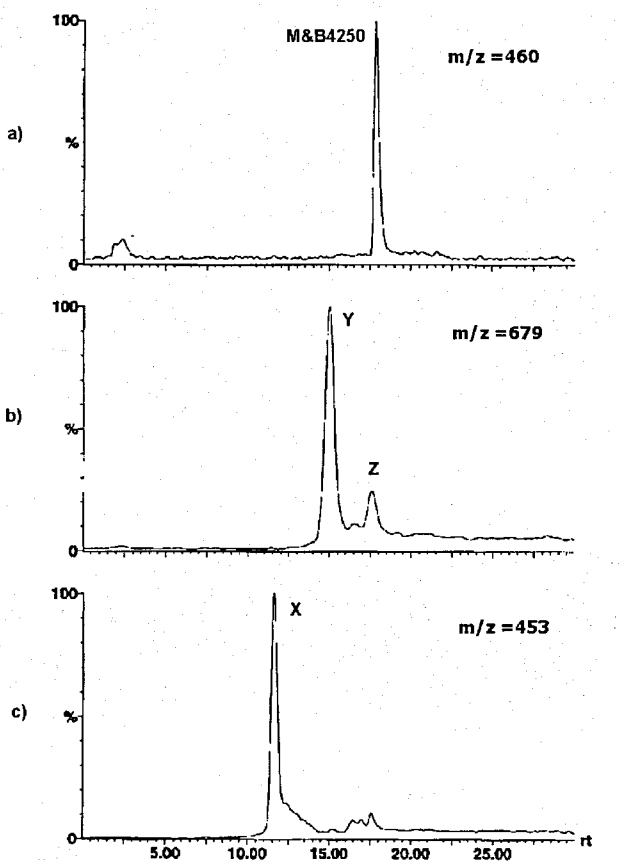


Figure 3.34 LC-ES-MS single-ion chromatograms of extracts of 3h incubations of 50μM M&B 4250 with 2.4 x 10^6 viable cells /ml showing **a)** M&B 4250 (*m/z* = 460), **b)** Y and Z (*m/z* = 679) and **c)** X (*m/z* = 453).

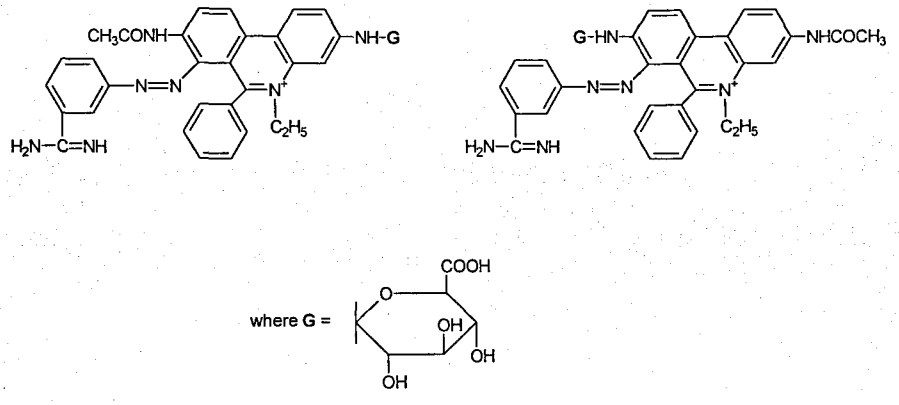


Figure 3.35 Possible chemical structures of the M&B4250 metabolites **Y** and **Z**.

A comparison of the structures of ISM, M&B 4250 and EBr (Figure 3.36) suggests that the 8-amino group should not be substituted if metabolism of the phenanthridine is to take place. Chemically, the 8-amino group in the phenanthridine structure shows greater reactivity to electrophiles than the 3-amino group, since the latter participates in a resonance configuration with the quaternary nitrogen (Figure 2.2).

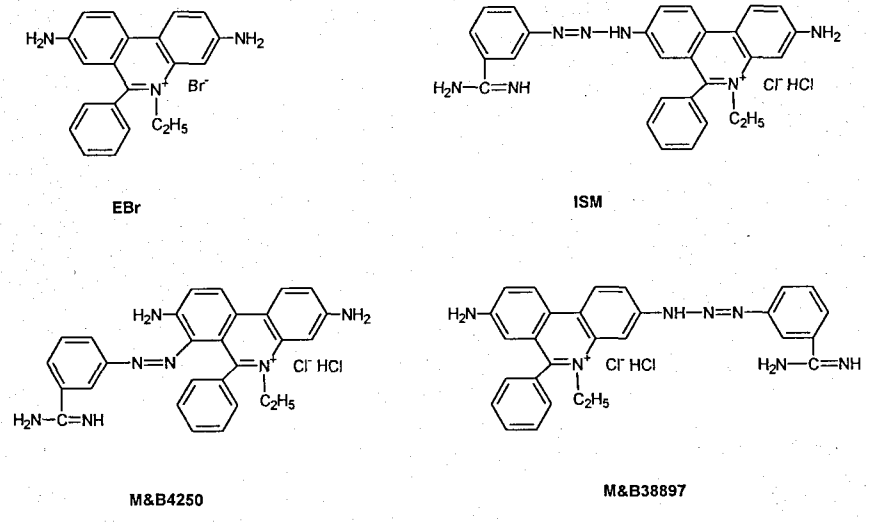


Figure 3.36 Chemical structures of ISM and its analogues with available 8-amino groups (EBr, M&B4250 and M&B38897).

Significantly, the amino group at position-8 of the major related substances of ISM (M&B4250 and M&B38897, together with EBr, Figure 3.36) in Samorin® is not substituted and this may result in extensive metabolism of administered doses of the product.

3.4 Conclusions

The use of CLSM has shown that similarities and differences exist between the localisation of EBr and ISM in isolated rat hepatocytes. The rapid influx of the phenanthridines into hepatocytes may confirm that there is as active transport mechanism for the uptake of ISM into cells as postulated by Philips *et al.* (1967). Qualitatively, both compounds show affinity for the nucleolus, cell membrane and endoplasmic reticulum. Quantitatively, a significant difference was observed between the amount of ISM in cytosol and nucleus. On the contrary, the relative amounts of EBr in the two different cellular compartments were not significantly different. These differences may explain the longer prophylactic periods obtainable with ISM. The ability to screen samples of ISM by HPLC and HPTLC for purity (chapter 2), combined with the possibility of studying the uptake of ISM into hepatocytes (and possibly trypanosomes) by CLSM should facilitate a better understanding of the transport of this agent into resistant and non-resistant trypanosomes.

Ever since MacGregor and Clarkson (1971) demonstrated that the aromatic phenanthridines could be metabolised, the emphasis has been on the formation of acetyl-amides. Although subsequent studies (Gilbert and Newton 1982, Murilla *et al.* 1996a) suggested that acetylation of the phenanthridines may not be a relevant pathway in ruminants, these observations were not further investigated. Perhaps the concept that *N*-acetylation (generally a non-inducible metabolic pathway (Weber *et al.* 1990)) was the only route for the metabolism of the phenanthridines contributed to the lack of interest in investigating the effect of environmental chemicals on the metabolism of the phenanthridines. The study reported in this thesis has demonstrated that the prototype trypanocidal phenanthridine, EBr, is rapidly metabolised by hepatocytes from untreated rats to at least six compounds and that the formation of two novel *N*-glucuronosylethidium

compounds is the principal route of metabolism. A third novel metabolite, *O*-glucuronosylethidium occurs as a minor metabolite in untreated rats. This study has provided strong evidence for the presence of 8-acetylethidium, 3-acetylethidium and 3,8-diacetyl ethidium, which has resolved the impasse on the extent of acetylation that was generated by previous studies (MacGregor and Clarkson 1971, Fraire *et al.* 1981).

Investigation of the metabolism of EBr using hepatocytes obtained from rats, sheep and pigs has revealed species differences and similarities. *N*-acetylation of EBr was not evident with pig and sheep hepatocytes. Significantly, the novel *N*-glucuronosylation pathway (which appertains in rats) was present in ruminants but the relative abundance of the two *N*-glucuronosylethidium metabolites in the sheep was different to that in rats. Pigs afforded only one of the two *N*-glucuronosylethidium metabolites, albeit in minute quantities. There was no evidence of the formation of *O*-glucuronosylethidium in pigs and sheep.

The current study has demonstrated also that inducing agents (such as phenobarbitone and 3-MC) significantly increase the metabolism of EBr by rats by the UDP-glucuronosyltransferase pathway. *N*-glucuronosylethidium metabolites are inducible by treatment of rats with phenobarbitone and 3-MC but the *O*-glucuronosylethidium metabolite is induced only after treatment with 3-MC. This inducibility of the metabolism of EBr has provided indirect evidence that pesticides and other environmental chemicals, which display inducing properties similar to those of phenobarbitone and 3-MC, can potentially alter its metabolism in ruminants.

The apparent metabolic inertness of ISM reported in previous studies (Philips *et al.* 1967, Kinabo and Bogan 1988d, Murilla *et al.* 1996b) has been confirmed and metabolites of ISM were not detected in studies with rat hepatocytes. On the contrary, the positional

isomer of ISM, M&B4250, was readily metabolised by both *N*-acetyltransferase and UDP-glucuronosyltransferase pathways. This suggests that the 8-amino group of the phenanthridine must not be substituted if the compound is to be prone to metabolism and this is supported by the results obtained for EBr.

CHAPTER 4: SUMMARY AND SUGGESTIONS FOR FUTURE WORK

4. Summary and suggestions for future work

4.1 Summary

Currently, the therapeutic efficacy of the few drugs available for the treatment of African animal trypanosomiasis, such as ethidium bromide (EBr), isometamidium chloride hydrochloride (ISM) and diminazene aceturate, is threatened by a high incidence of resistance of the parasite (Peregrine *et al.* 1991). When this is linked with the reluctance of the pharmaceutical industry to invest in the development of new and more effective drugs since a viable return on a substantial investment is unlikely, the continued use of the few available compounds is inevitable. Consequently, the study in this thesis was initiated to investigate the possible contributions of the quality of trypanocides in international commerce and the metabolism of the trypanocidal phenanthridines to the phenomenon of high parasitic resistance. Although the results from this study will not result in the reversal of parasitic resistance *per se*, it raises awareness of the possible causes of resistance and emphasises the importance of the rational use of these valuable trypanocides.

One of the basic tenets in rational chemotherapy is to achieve optimum concentrations of the therapeutic agent at the desired site of action. Therefore, the use of substandard drugs in anti-parasitic chemotherapy may lead to the development of drug-resistant strains, since the parasites are only exposed to sub-lethal doses. The commercial formulations of ISM (Samorin®/Trypamidium® and Veridium®) contain an array of process-related and degradation impurities (Figure 2.1). The amount and nature of these impurities are critically influenced by small variations in the conditions of synthesis such as pH and temperature (Berg 1963). Consequently, it is a matter of concern that, prior to the present work, there were no suitable methods in the public domain for the quality control of commercial preparations which contain ISM in the presence of these substances.

The study reported here has described a method for the determination of ISM in the presence of four process-related and degradation impurities by RP-HPLC using a Lichrospher-60 RP Select B column with a mobile phase composition of acetonitrile-KH_2PO_4 (pH 3.0, 20mM)(22.5:77.5, v/v) with UV detection at 320nm. The method is selective, reproducible and precise with a limit of detection of 45ng/ml for ISM. A HPTLC system (Kieselgel 60 F_{254}, pyridine-acetonitrile-butanol-formic acid (6:6:4:1, v/v/v/v), with UV densitometric evaluation at 320nm) suitable for the resolution of ISM and the related substances is reported also. A procedure for the evaluation of the chemical equivalence (or otherwise) of proprietary formulations of ISM is described. This combines the determination of the principal component (ISM) by the aforementioned HPLC method, the identification of the related substances and the quantitative determination of the inorganic impurity, ammonium chloride, using a modification of the Berthelot (Indophenol) reaction. Application of these procedures to analyses of commercially available sachets from four different batches of Samorin®/Trypamidium® and four different batches of Veridium® has demonstrated that there are marked qualitative and quantitative differences between batches from these two sources. Whilst the former showed inter-batch consistency of composition, there was considerable inter-batch variation between the samples of Veridium®. The content of ISM in samples of Veridium® ranged from 38.6 to 74.5%w/w (n = 4) with Samorin®/Trypamidium® showing a narrower range (59.0 to 61.7%w/w, n = 4). The content of ammonium chloride in the four batches of Samorin®/Trypamidium® was consistent and less than 0.30%w/w (maximum of 0.21%w/w). However, the samples of Veridium® showed a wide range (0.9 – 38.9%w/w, n = 4) for the content of ammonium chloride. The examination of the chromatographic profiles of the four batches of Samorin®/Trypamidium® and of Veridium® showed that there are significant differences in the composition of the batches of products made by the two manufactures.

The existence of trypanocides (particularly those with claims to 'essential similarity' or chemical equivalence) of variable quality raises serious concerns in the light of increasing reports on the development of drug-resistant strains. Obviously, the administration of substandard products containing sub-lethal doses will render parasites eventually resistant to therapeutic doses. A high incidence of adulteration of trypanocides, especially ISM, has been reported in the field (Professor Peter Holmes, *personal communication*) and this has been a major problem for regulatory bodies in developing countries with limited resources for analytical development and post-market surveillance. This study has demonstrated that even with recognised sources of supply, the quality of preparations containing ISM may be severely compromised. Consequently, the analytical procedures which have been developed in this study will facilitate the characterisation of commercial preparations containing ISM in international commerce and aid in policy decisions over sources of supply.

It is known that the trypanocidal phenanthridines, EBr and ISM, accumulate in the liver of treated animals (Murilla *et al.* 1996a, Murilla *et al.* 1996b). Generally, a longer period of prophylaxis (8weeks) against trypanosomal infections is obtained after treatment of the animals with ISM compared with that of EBr (5weeks), although the mechanisms involved in the relative differences in prophylaxis are not well understood. Exploitation of the intrinsic fluorescence of EBr and ISM in CLSM in the current study has demonstrated that both compounds are taken up rapidly into the nucleoli and nuclear membranes of isolated hepatocytes. ISM demonstrated a higher affinity than EBr for the nuclear region of the hepatocyte and this may explain the longer prophylactic periods obtained after treatment with ISM. The release of the bound ISM may then be a function of the turnover of the cell. This technique may be adaptable to enable the future investigation of potential differences in the uptake of the fluorescent trypanocides by resistant and non-resistant trypanosomes.

Environmental chemicals, such as insecticides and pesticides, influence the metabolism of drugs. In addition to achieving optimum concentrations of therapeutic agents at the desired site of action, the duration of action of a drug is an important facet of rational chemotherapy. Therefore, any alteration of the metabolism of an administered drug which may result in a decrease in the circulating concentration, will affect the expected therapeutic response. EBr was used as the model compound in this study and the effect of classical inducers, such as phenobarbitone and 3-MC, on its metabolism was investigated.

EBr was biotransformed extensively by isolated hepatocytes from untreated rats and at least six metabolites were detected by HPLC. Two novel metabolites, 3- and 8-*N*-glucuronosylethidium, were identified by HPLC-ESI-MS and together represent the major pathway of metabolism, accounting for 6.4 ± 0.7 and 19.5 ± 1.2 % (mean ± SEM, n = 4) respectively of total recovered drug after incubation. Two other new metabolites; 3,8-diacetylethidium and an *O*-glucuronosyl derivative of EBr, were formed in trace quantities (< 1%) in untreated rats. The other two metabolites detected, 3-acetylethidium (< 1%) and 8-acetylethidium (10.7 ± 2.3%), have been reported previously (McGregor and Clarkson 1971, Fraire *et al.* 1981).

Treatment of rats with 3-MC resulted in the selective induction of the formation of the *O*-glucuronosylethidium, with a relative amount of 6.9 ± 0.8% after 3h. When the rats were pre-treated with phenobarbitone and 3-MC, there was a 2.5-fold and 1.5-fold increase respectively (compared with control rats) in the amounts of the 8-*N*-glucuronosylethidium. Statistical analysis of the amount of EBr remaining after 3h showed a significant difference ($p < 0.05$, one-way ANOVA) between control and treated (both phenobarbitone and 3-MC) rats. This study has demonstrated that environmental chemicals, such as pesticides with inducing properties similar to those of phenobarbitone and 3-MC, may alter the metabolism of EBr and, therefore, the pharmacological effect.

Major interspecies variability was observed in the nature and extent of the metabolism of EBr by rat, sheep and pig hepatocytes. Mono- and di-acetyl conjugates were absent in sheep and pigs. Sheep hepatocytes metabolised EBr to the two *N*-glucuronides, 3-*N*-glucuronosylethidium and 8-*N*-glucuronosylethidium, with the formation of the former being the major pathway. In rats, the 8-*N*-glucuronosylethidium metabolite was the major pathway and this was the only metabolite formed in pigs.

The study reported in this thesis has confirmed the results of a previous investigation (Philips *et al.* 1967) that ISM is not biotransformed by the rat. Comparison of the metabolism of the two structurally similar phenanthridines, ISM and M&B4250, has indicated that the absence of an unsubstituted and reactive 8-amino group in ISM may account for it being metabolically inert. In support of this, M&B4250 and EBr, each of which possesses an unsubstituted 8-amino group, are metabolised by *N*-acetylation and *N*-glucuronidation.

In conclusion, a number of the aims and objectives of this project (see page 16) have been addressed successfully. It has been shown that;

- The quality of trypanocides in international commerce, especially that of preparations containing ISM, may be severely compromised and this may contribute to the development of resistant strains of trypanosomes.
- The differences observed in localisation of ISM and EBr in hepatocytes may explain the differences in length of the prophylactic periods afforded by these agents.
- The trypanocidal phenanthridine (EBr) is metabolised extensively by rats; principally by *N*-glucuronidation and, to a lesser extent, by *N*-acetylation.
- Species differences exist in the metabolism of the phenanthridines, as shown by the absence of *N*-acetylation in sheep and pigs. The *N*-glucuronidation pathway operates in sheep and pigs.

- The metabolism of EBr may be altered (quantitatively and qualitatively) by environmental chemicals with inducing properties similar to those of 3-MC and phenobarbitone.
- The presence of an unsubstituted 8-amino group in the phenanthridines appears to be a requirement for their metabolism.

4.2 Future directions

The development of suitable methods for the determination of ISM in biological fluids has been limited by the low circulating concentrations of the drug, which are observed shortly after administration (Kinabo and Bogan 1988b). Therefore, enzyme linked immunosorbent assays (ELISA, with sensitivities in the sub-nanogram range) have been used for the analysis of ISM (Eisler *et al.* 1993, Eisler *et al.* 1996). However, the possibility of cross-reactivity of these ELISAs to the whole gamut of related substances in commercial preparations of ISM has not been eliminated. The HPLC method developed in this thesis forms the basis for the development of a more specific and sensitive method for the determination of ISM in biological fluids. The rapid improvements in HPLC column technology, such as microbore and capillary columns, offers an opportunity for extending the sensitivity of the method described in this thesis into the sub-nanogram range. The use of more specific methods for the determination of ISM in biological fluids will provide more reliable data on the pharmacokinetics and allow better interpretation of biological data.

The use of CLSM in studying the uptake and intracellular localisation of the phenanthridines can be adapted in future studies to provide answers to the following questions;

1. Does resistance in trypanosomes alter the uptake of ISM and its trypanocidal related substances?
2. Are there species differences in the uptake and localisation of the phenanthridines in subcellular organelles of hepatocytes?

The metabolism of EBr by hepatocytes prepared from rats, sheep and pigs has been investigated and the results presented in this thesis. However, cattle are the most economically significant victims of animal trypanosomiasis (Williams *et al.* 1993) and it will

be worthwhile (if not imperative) to expand future studies to include this species. It will be useful also, to obtain both *in-vitro* and *in-vivo* data on clearance of the phenanthridines in rats as this will facilitate the prediction of *in-vivo* clearance of these agents in sheep, pigs and cattle from *in-vitro* data.

In the current study, provisional structures have been assigned to the *N*-glucuronides of EBr on the basis of well-documented relative reactivities of the 3- and 8-amino groups in the phenanthridinium molecule (Berg 1963b). This was due to the difficulties in producing suitable daughter-ions of these glucuronides by LC-MS to aid in the confirmation of the position of glucuronosylation. Unambiguous assignment of the positions of glucuronosylation will be facilitated by the enzymic synthesis of the two possible *N*-glucuronides of EBr. The successful and unequivocal synthesis of the glucuronosyl metabolites will help to establish whether or not the activities/toxicities of the phenanthridines are due to the metabolites or to the parent compounds. The possible contribution of the related substances (and their putative metabolites) in Samorin®/Trypamidium® to the activity/toxicity of the product also requires investigation.

This study has provided (albeit indirect) evidence for the alteration of metabolism of the phenanthridines by pesticides with inducing properties similar to those of phenobarbitone and 3-MC. It will be useful to confirm this inference by comparing the metabolism of these agents using hepatocytes from control animals and from animals suitably exposed to levels of pesticides in the range of those in their habitat.

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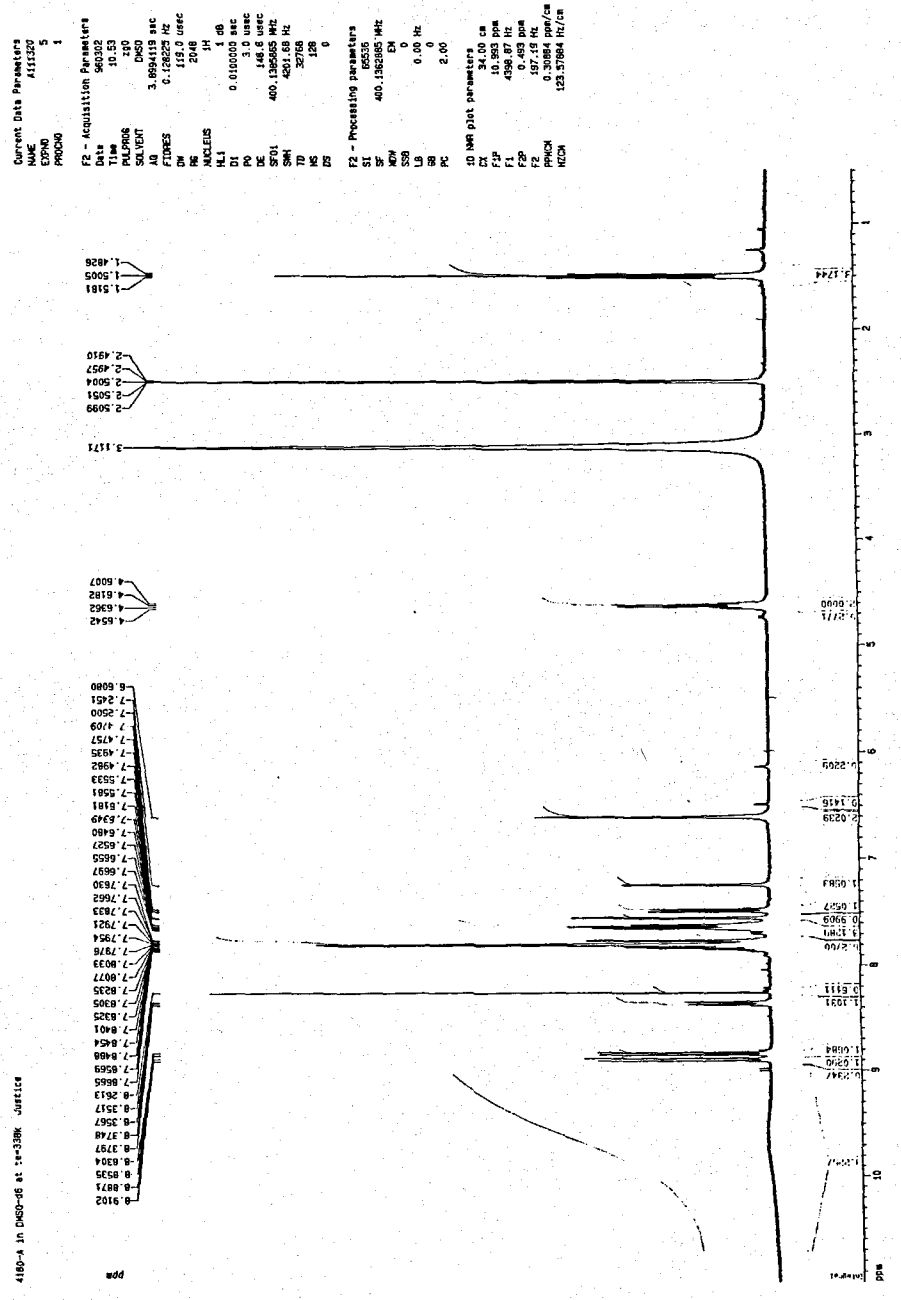
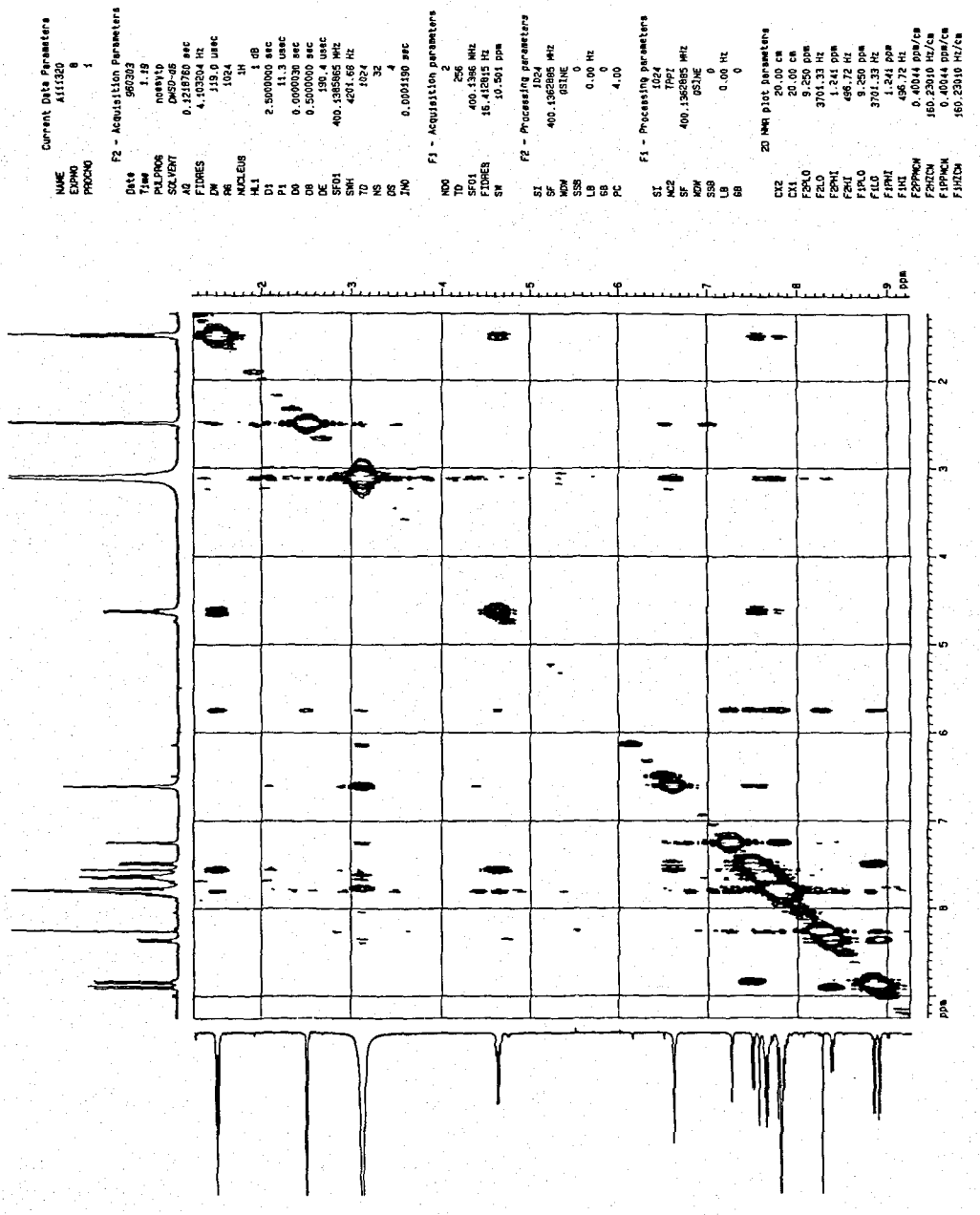
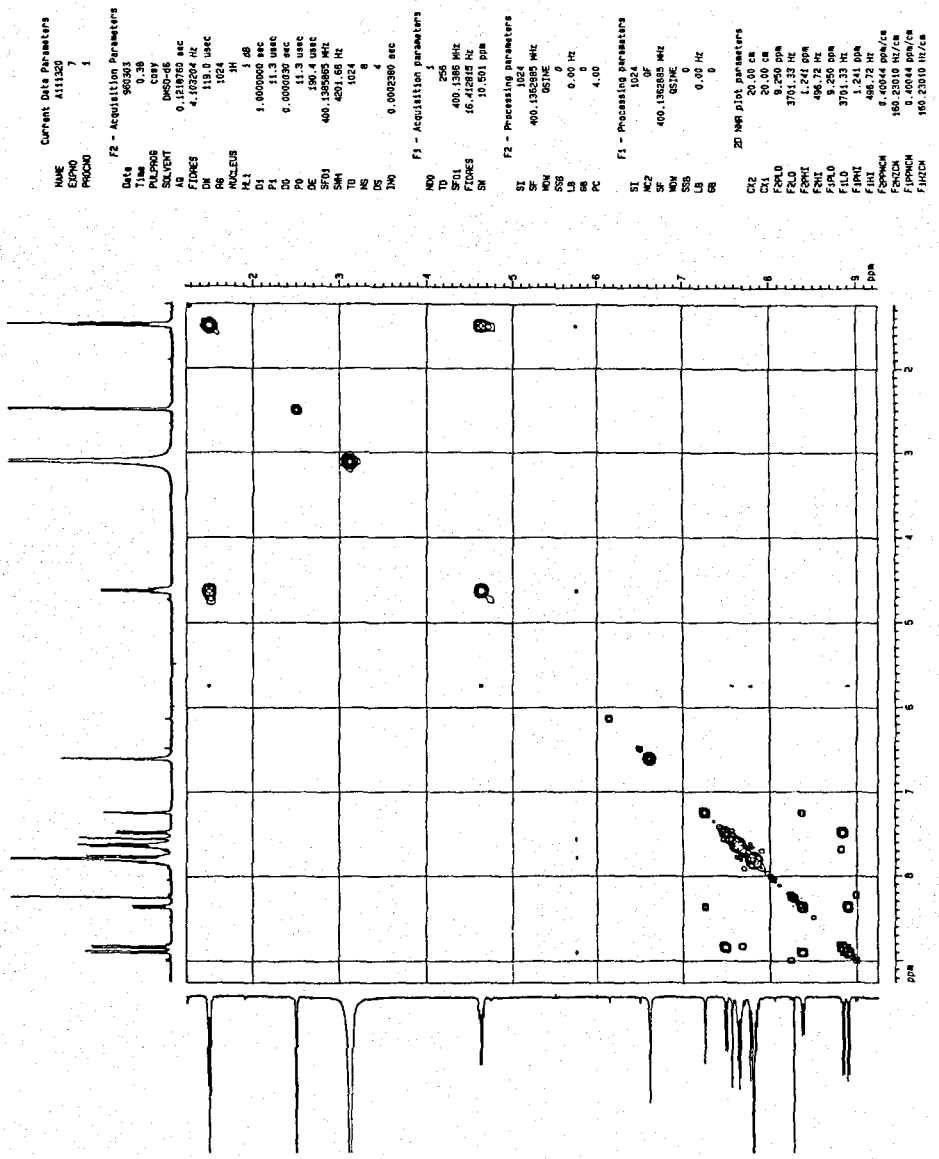
APPENDIX I: NMR SPECTRA



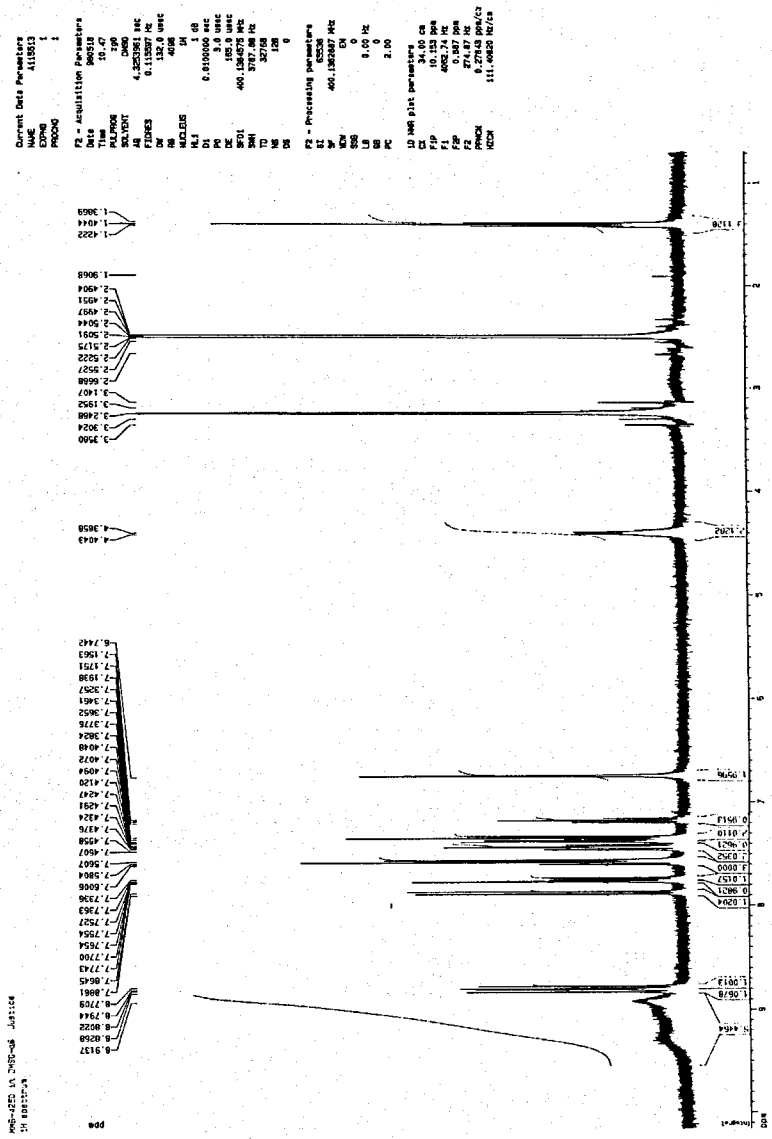
400MHZ Proton-NMR spectrum of isometamidium (ISM) in DMSO-d_6



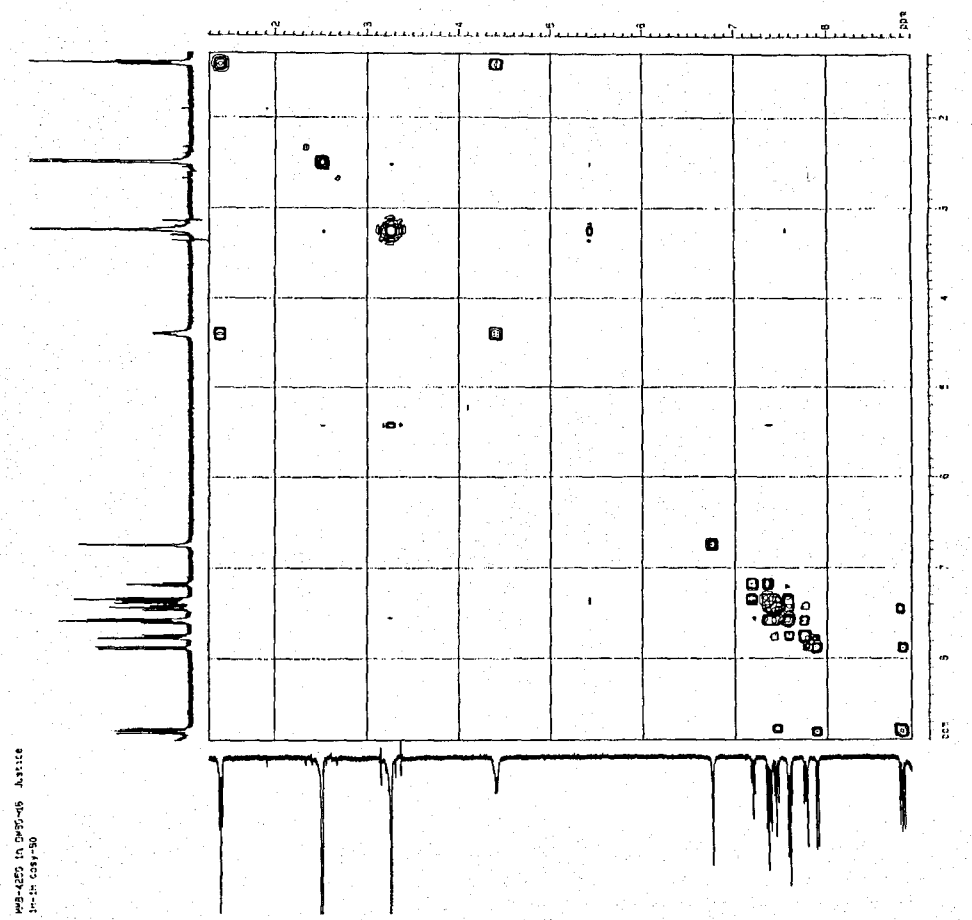
^{1}H-^{1}H NOESY *Spectrum of isometamidium* (ISM) in DMSO-d_6



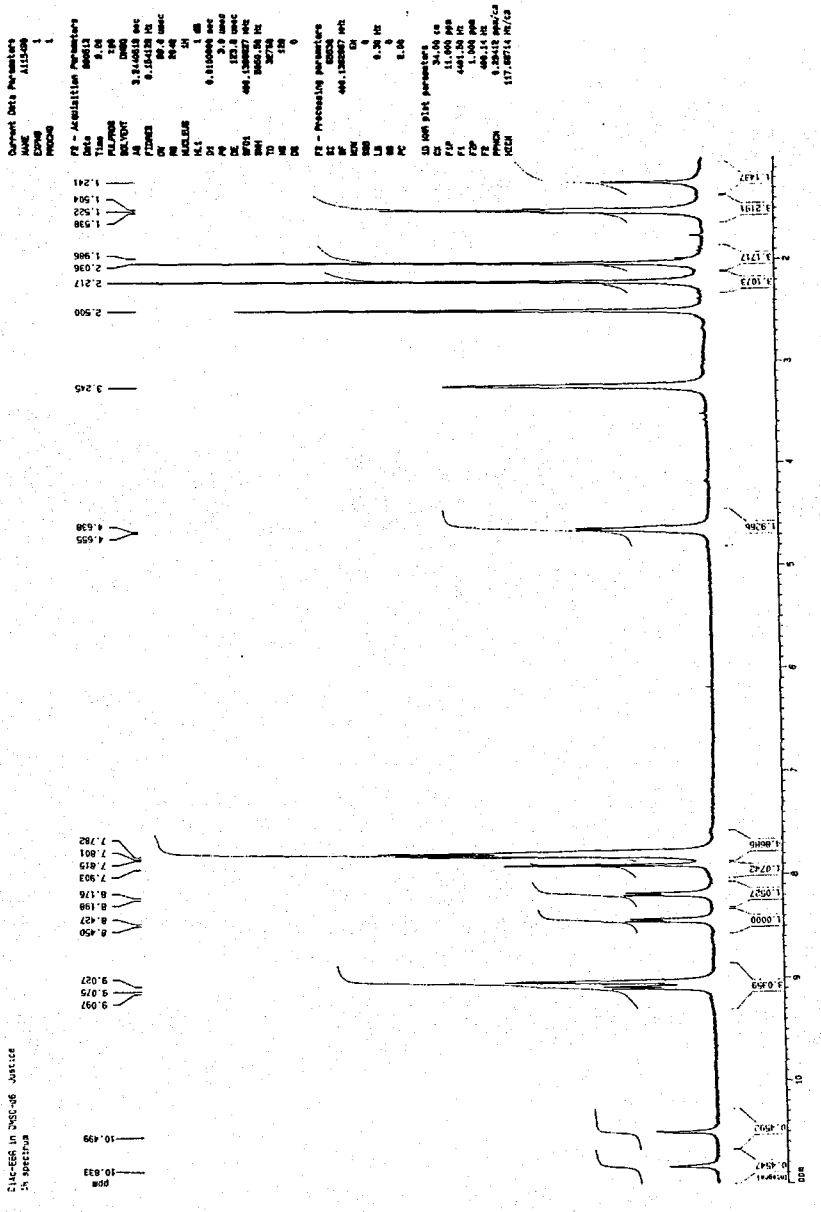
^{1}H-^{1}H COSY spectrum of isometamidium (ISM) in DMSO-d_6



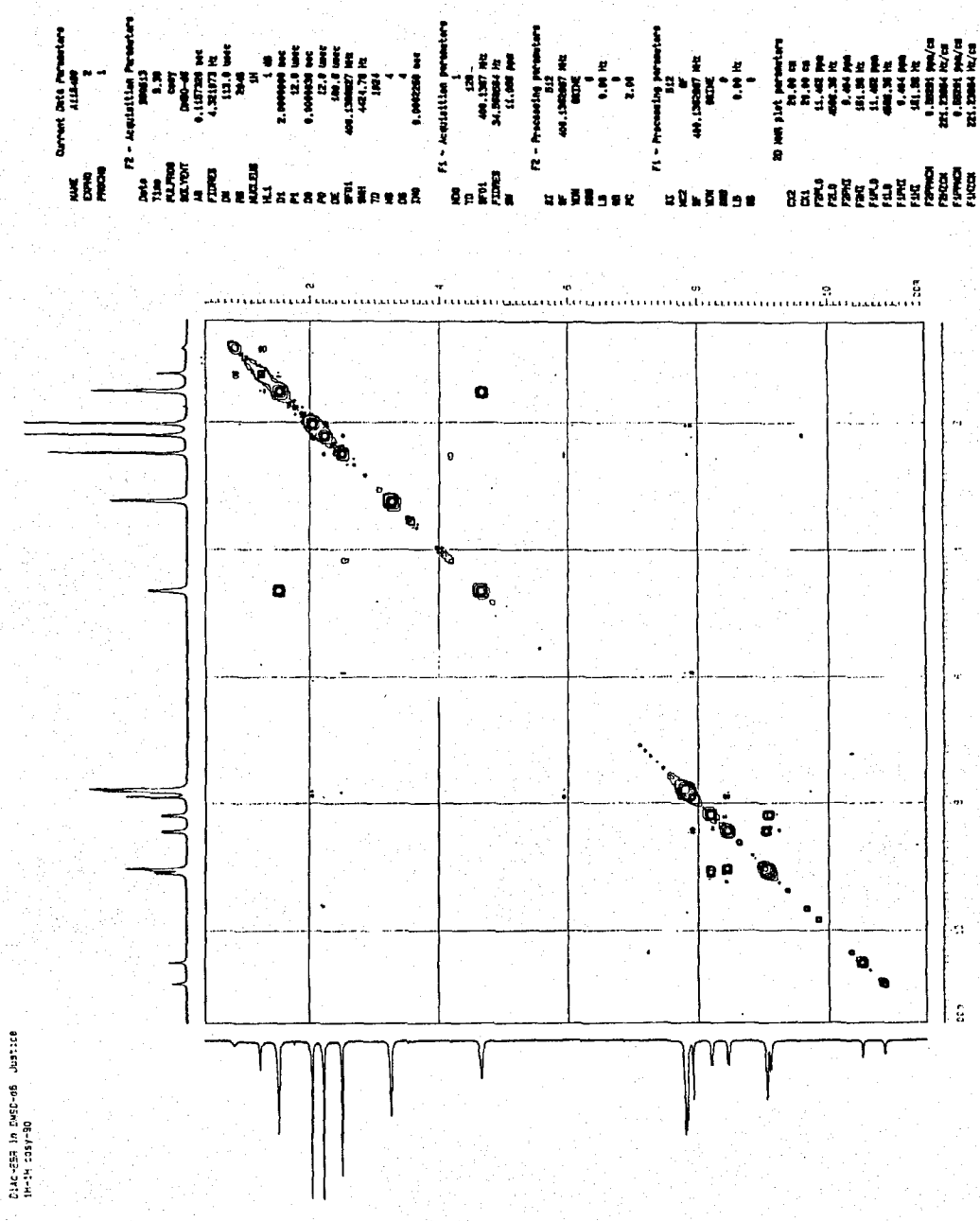
400MHZ proton-NMR spectrum of M&B4250 in DMSO-d_6



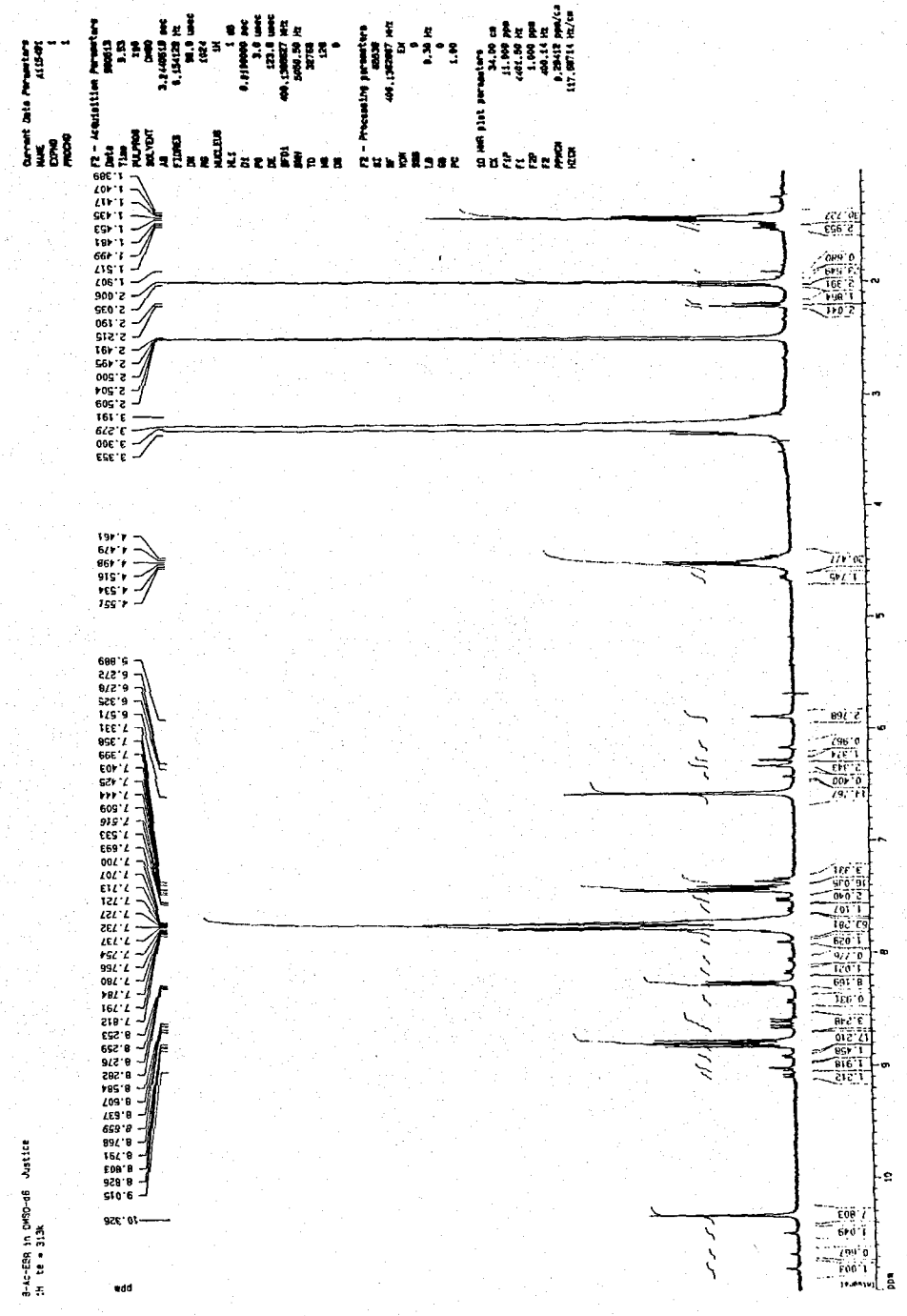
^{1}H-^{1}H COSY spectrum of M&B4250 in DMSO-d_6



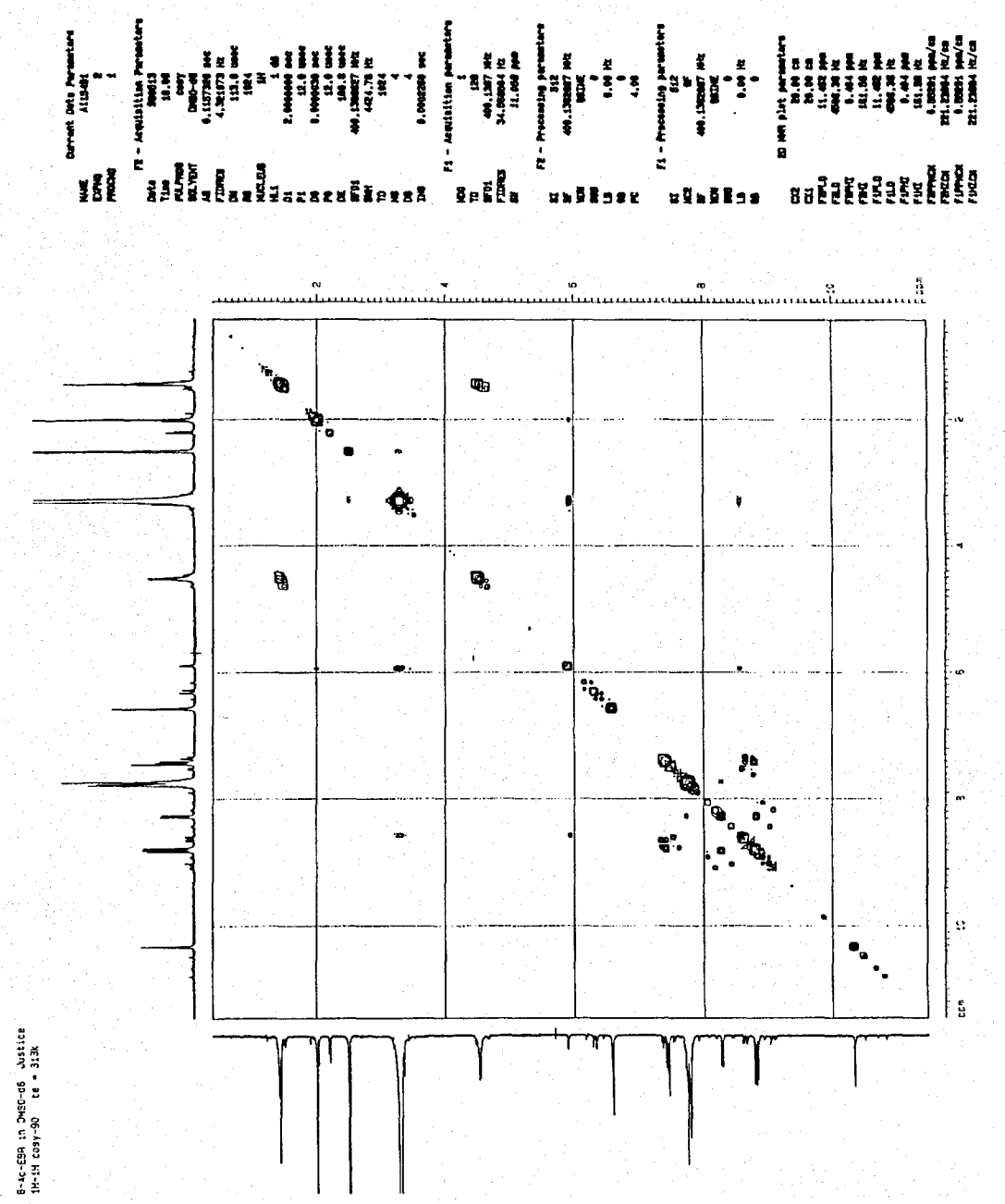
400MHZ proton-NMR spectrum of 3,8-diacetyl-ethidium in DMSO-d_6



^{1}H-^{1}H COSY spectrum of 3,8-diacetyl-ethidium in DMSO-d_6

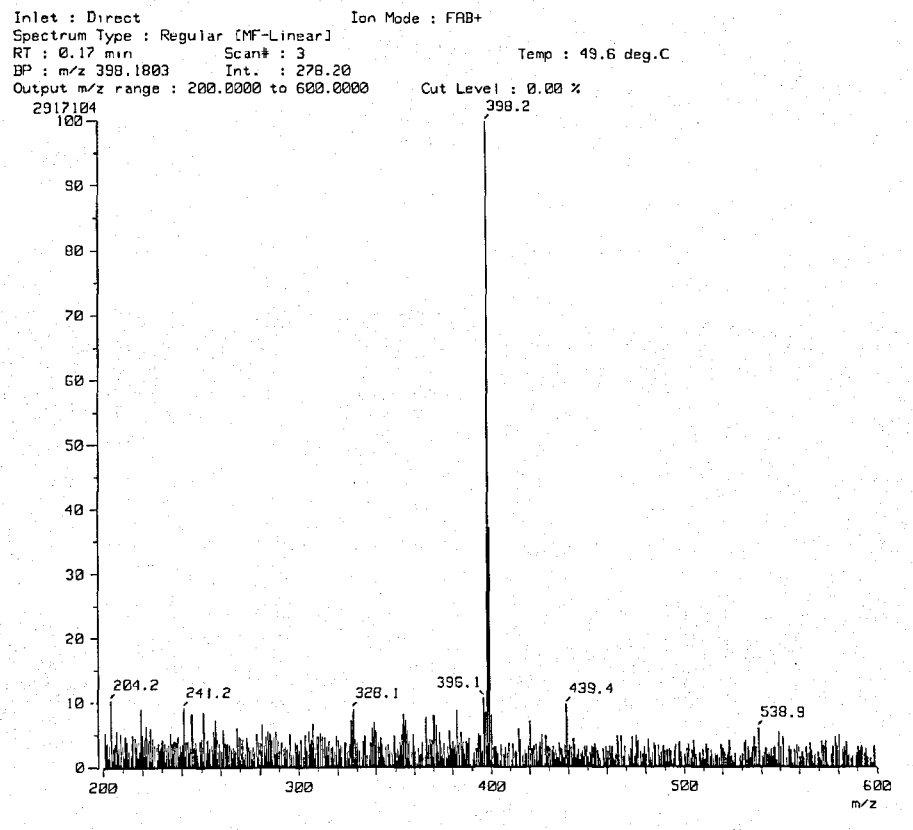


400MHZ proton-NMR spectrum of 8-acetyl-ethidium in DMSO-d_6

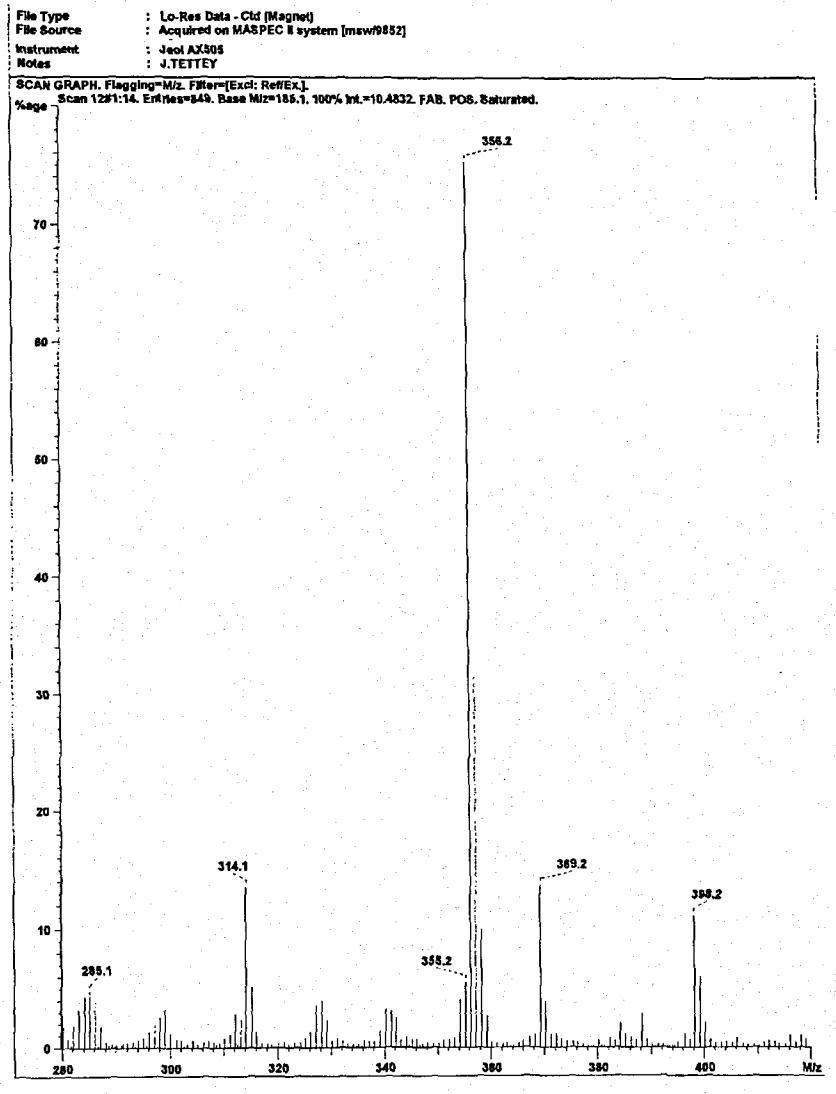


^{1}H-^{1}H COSY spectrum of 8-acetyl-ethidium in DMSO-d_6

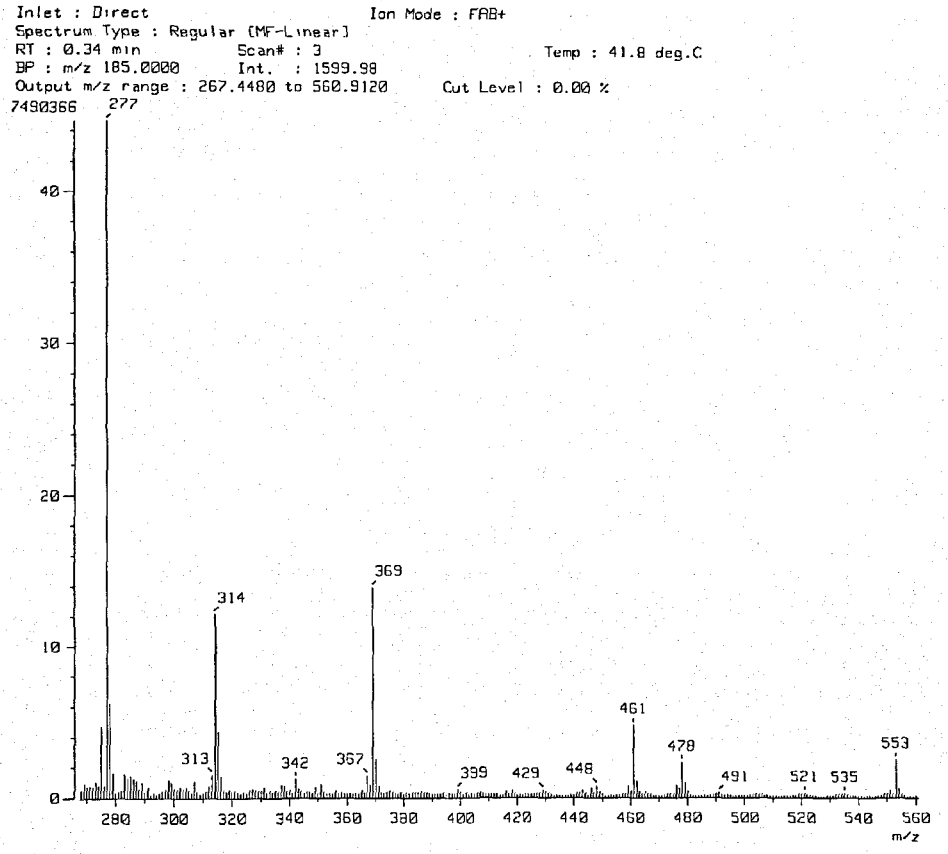
APPENDIX II: MASS SPECTRA



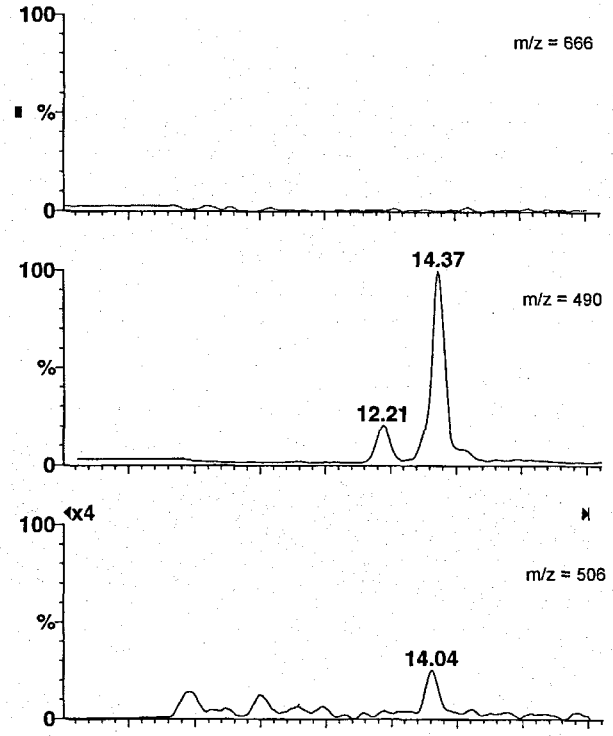
FAB mass spectrum of 3,8-diacetyl-ethidium (m/z = 398.2)



FAB mass spectrum of 8-acetyl-ethidium (m/z = 356.2)



FAB mass spectrum of ethidium (m/z = 314) in a matrix of glycerol (m/z = 277, 369, 461 and 553 [92n + 1, where n = positive integer]).



Single ion chromatograms from ESI-MS analysis of an extract of 3h incubations of ethidium with hepatocytes from 3-MC treated rats. The LC conditions were modified to shift the peaks of interest away from the solvent front.

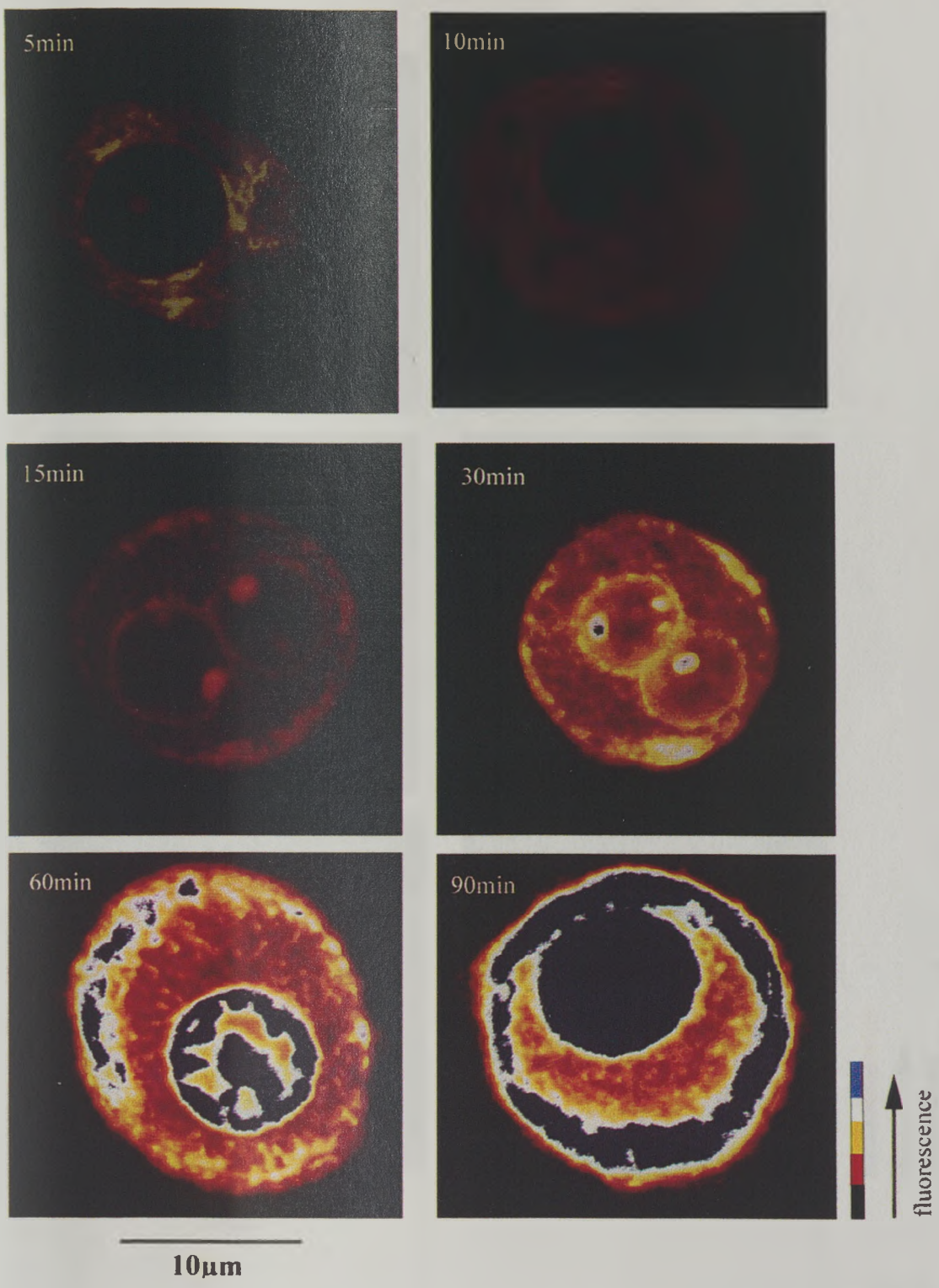
Key

M-I t_R = 12.21min m/z = 490

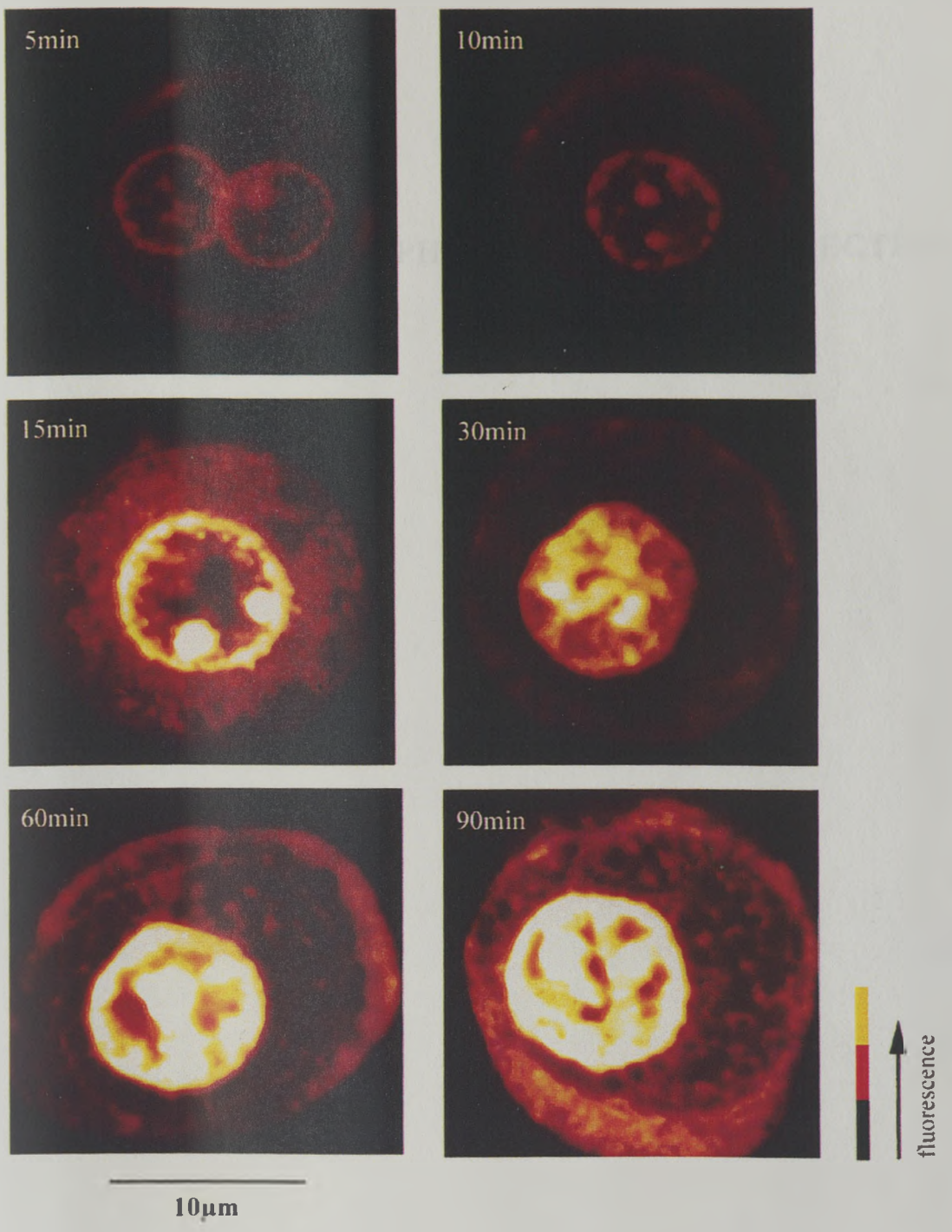
M-II t_R = 14.37min m/z = 490

M-VI t_R = 14.04min m/z = 506

APPENDIX III: CLSM IMAGES

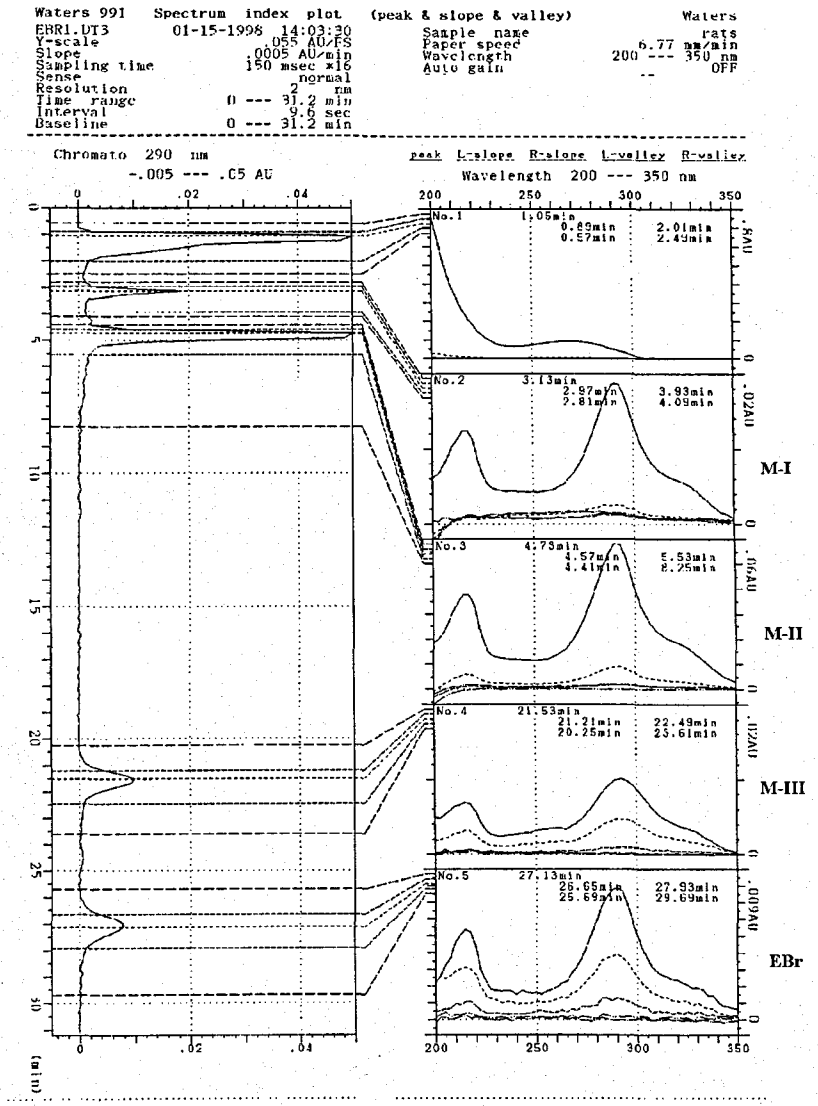


CLSM images of isolated rat hepatocytes exposed to EBr (100µM) for specified time periods (shown on the images)

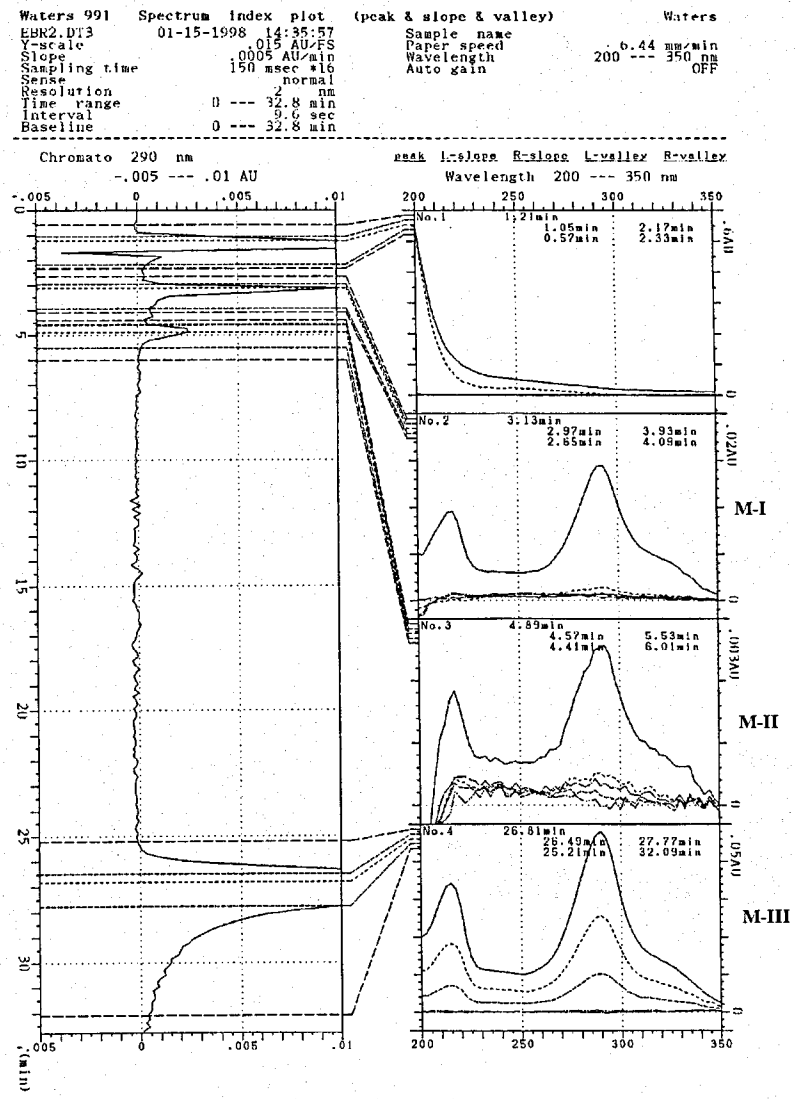


CLSM images of isolated rat hepatocytes exposed to ISM (100µM) for specified time periods (shown on the images)

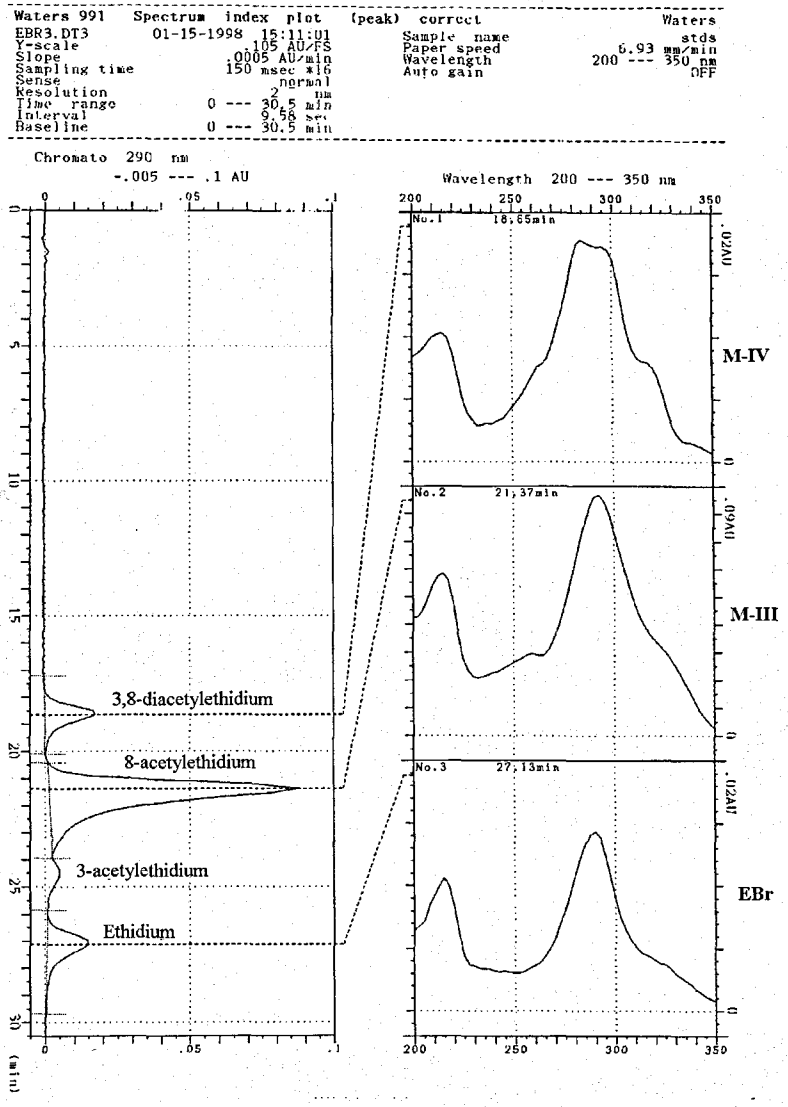
APPENDIX IV: UV PHOTODIODE ARRAY SPECTRA



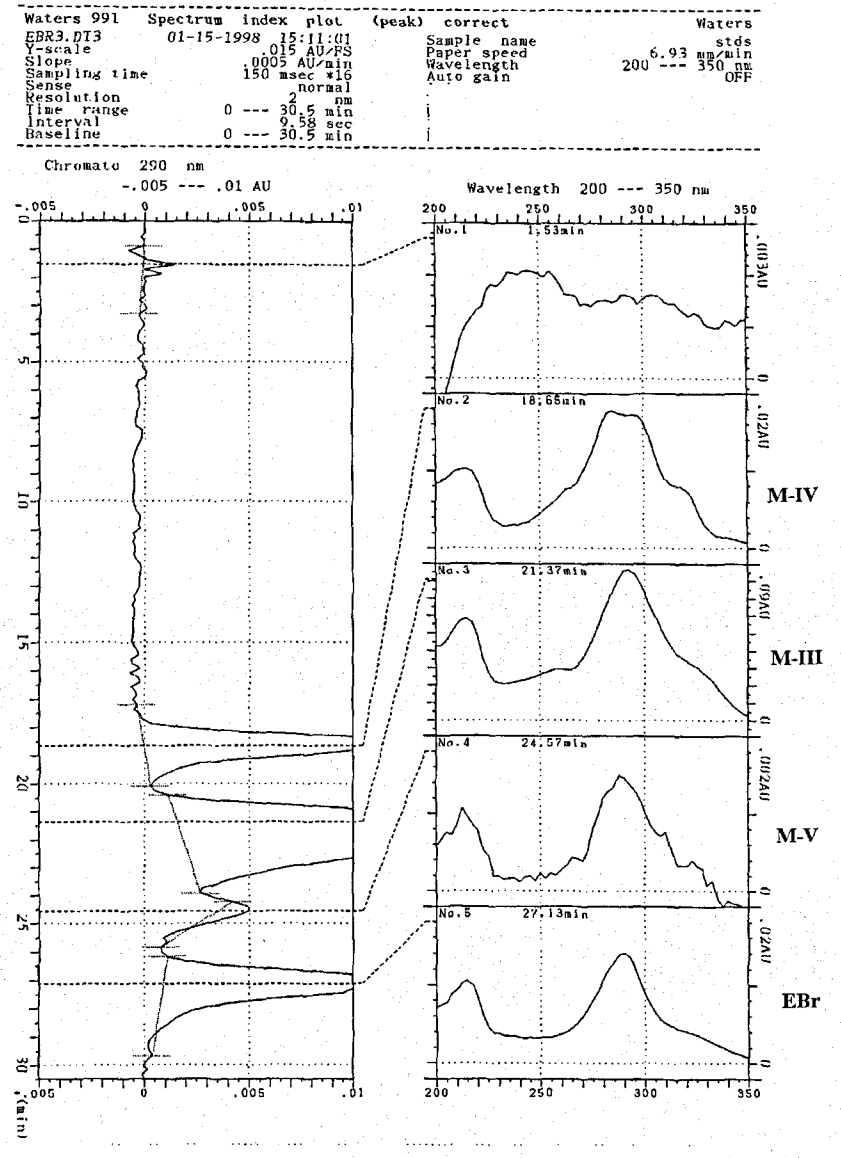
Analysis of extracts from incubation of EBr with hepatocytes from untreated rats (*The spectra obtained for an extract from hepatocytes from phenobarbitone-treated rats was essentially similar*).



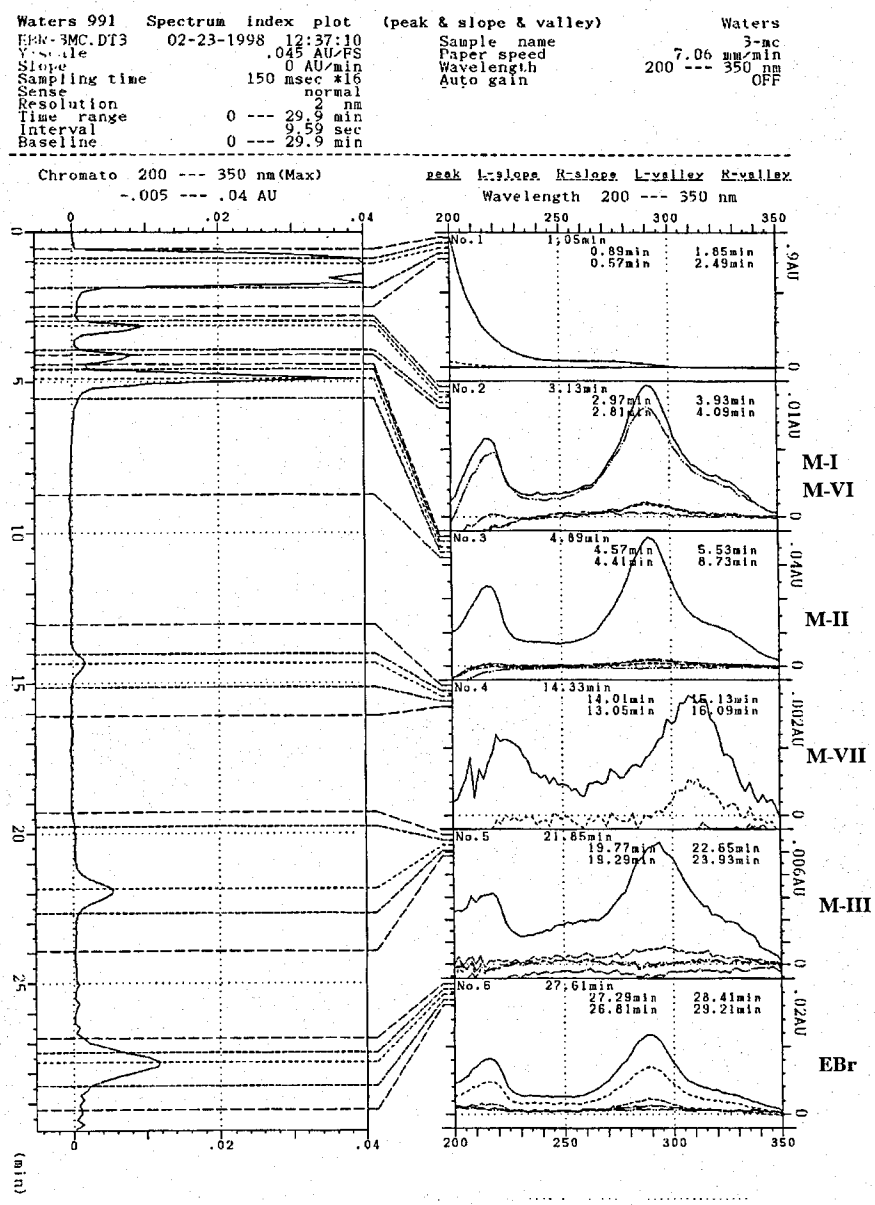
Analysis of extracts from incubation of EBr with hepatocytes from sheep



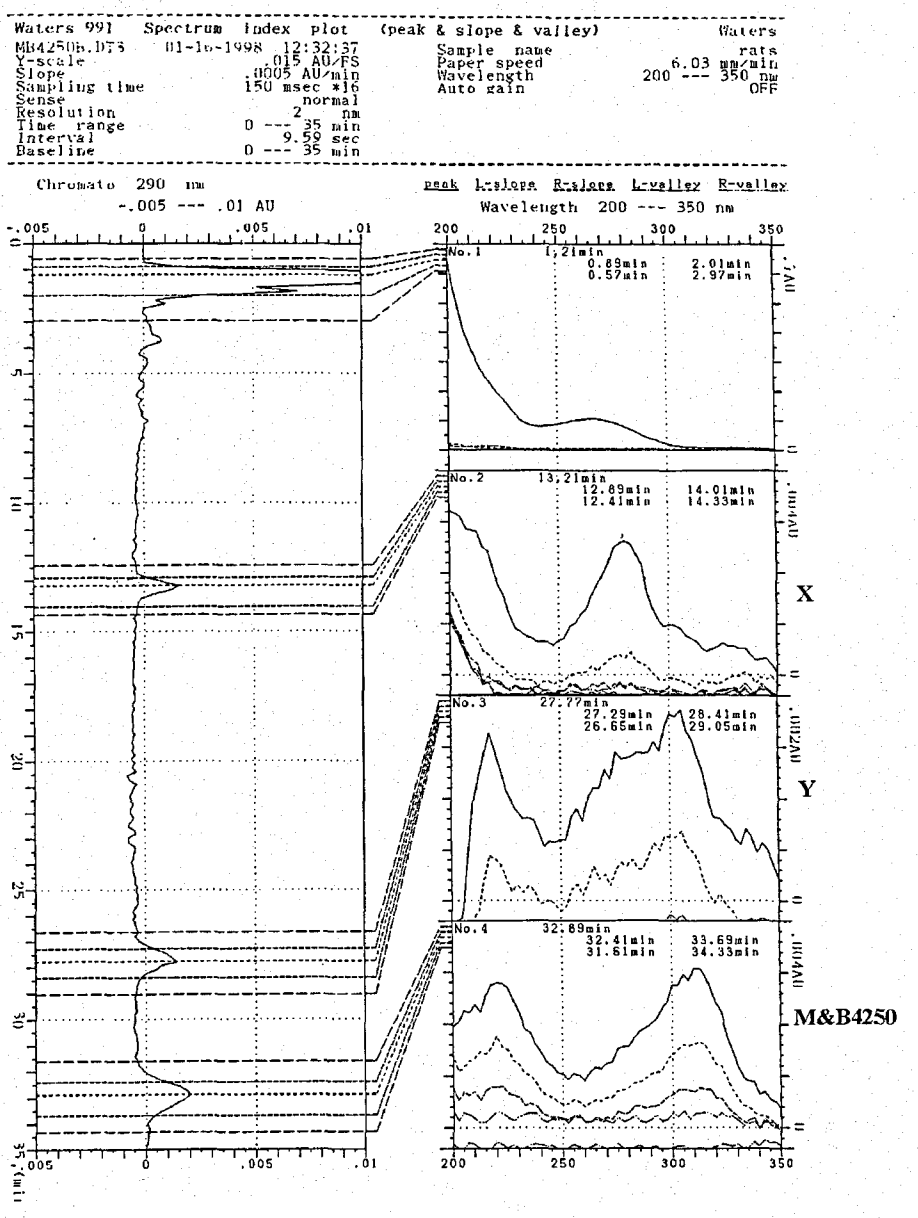
Analysis of synthesised standards of the acetyl derivatives of EBr



Analysis of synthesised standards of the acetyl derivatives of EBr



Analysis of extracts from incubation of EBr with hepatocytes from 3-MC treated rats



Analysis of extracts from incubation of M&B4250 with hepatocytes from untreated rats

APPENDIX V: PUBLICATIONS AND CONFERENCE PROCEEDINGS

PUBLICATIONS

Full papers

Tettey JNA, Skellern GG, Midgley JM, Grant MH (1998). HP-TLC and HPLC determination of isometamidium in the presence of its manufacturing and degradation impurities. J. Pharm. Biomed. Anal. **17**, 713-718.

Tettey JNA, Skellern GG, Midgley JM, Grant MH, Wilkinson R, Pitt AR (1998) Intracellular localisation and metabolism of the phenanthridinium trypanocide, ethidium bromide, by isolated rat hepatocytes. Xenobiotica (*in press*).

Tettey JNA, Skellern GG, Grant MH, Midgley JM (1998). Investigation of the chemical equivalence of the trypanocidal products, Samorin® and Veridium®. J. Pharm. Biomed. Anal. (*submitted*).

Abstracts

Tettey JNA, Skellern GG, Midgley JM, Grant MH (1998) Effect of inducing agents on the metabolism of ethidium bromide by rat hepatocytes. Human Exp. Toxicol. (*in press*).

Tettey JNA, Skellern GG, Midgley JM, Grant MH, Wilkinson R (1997). Uptake and metabolism of ethidium bromide by rat hepatocytes. Br. J. Clin. Pharmacol., 43 (2) 215.

CONFERENCE PROCEEDINGS

Tettey JNA et al. (1998) Effect of inducing agents on the metabolism of ethidium bromide by rat hepatocytes. Proceedings of the British Toxicology Society meeting (September 1998, York, England).

Tettey JNA et al. (1998) Intracellular localisation and metabolism of ethidium bromide. Pfizer Advances in Drug Metabolism poster symposium (September 1998, Canterbury, England).

Tettey JNA et al. (1998) Species differences in the metabolism of the trypanocidal phenanthridines. Scottish & Newcastle drug metabolism group meeting (July 1998, Glasgow, Scotland).

Tettey JNA et al. (1997). *In vitro* metabolism of the trypanocidal phenanthridines. Proceedings of the British Pharmaceutical Conference (Medicinal Chemistry Group, September 1996, Glasgow, Scotland).

Tettey JNA et al. (1996). Uptake and metabolism of ethidium bromide by rat hepatocytes. Proceedings of the British Pharmacological Society (September 1996, Dundee, Scotland).