ANALYTICAL AND METABOLIC STUDIES OF THE TRYPANOCIDAL DIAMIDINES

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

by

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My parents and family back in Ghana are gratefully acknowledged for their prayers and support throughout my studies in Glasgow.

Knowledge becomes useful only when it is applied to solve problems in life.

Wisdom is the application of knowledge to solve specific problems and therefore:

"If any of you lack wisdom, let him ask of God, that giveth to all men liberally and upbraideth not; and it shall be given him".

James 1:5 (K.J.V)

CONTENTS

ABSTRACT

Following the expiry of patent protection of the innovator product Berenil®, there has been an influx of substandard generic substitutes of the veterinary trypanocide in international commerce, which have been implicated as being a major contributor to the emergence of drug resistance. This situation has necessitated the development of analytical techniques, which would help safeguard the quality and efficacy of generic formulations of diminazene. A selective, accurate, precise and simple reverse-phase isocratic HPLC method for the simultaneous assay of diminazene aceturate and antipyrine (excipient) in pharmaceutical formulations has been developed and validated. The degradation and manufacturing impurities of diminazene have been identified by electrospray ionization mass spectrometry and characterized by NMR spectroscopy of the synthetic compounds. The developed method has been applied for the quality evaluation of over one hundred generic samples of diminazene obtained from Sub-Saharan Africa. The results give an indication that the quality of generic formulations of diminazene on the African market is compromised.

Changes in the metabolism of a drug can lead to altered pharmacokinetics, resulting in an increase or decrease in drug plasma concentration, leading to toxification or therapeutic failure. The metabolism of diminazene and pentamidine in isolated rat and pig hepatocytes have been investigated. Diminazene was not metabolized in either rat or pig hepatocytes. While there were no obvious qualitative differences in the metabolic profiles of pentamidine in either of the animals, the rate of metabolism in rats appeared to be faster. Pretreatment of rats with either 3-methylcholanthrene (3-MC), phenobarbitone (PB) or deltamethrin (DM) caused inhibition of pentamidine metabolism to different extents. There were significant differences between the profiles of the three major metabolites of pentamidine in DM and 3-MC pretreated rats compared to the control group, whereas pretreatment with PB did not result in any significant changes in profiles of metabolites.

GLOSSARY

HPLC	High performance liquid chromatography
ESI-MS	Electrospray ionization mass spectrometry
min	Minute(s)
h	Hour
C_8, C_{18}	Octyl-, Octadecylsilane
NMR	Nuclear magnetic resonance
COSY	Correlation spectroscopy
HMBC	Hetero molecular bonded correlation
R_f	Retention factor = distance traveled by solute from origin/ distance traveled by solvent front
m/z	Mass to charge ratio
SEM	Standard error of mean
RSD	Relative standard deviation
cDNA	Complementary deoxyribonucleic acid
UV	Ultraviolet
RT	Retention time
ppm	Parts per million
CD_3OD	Deuterated methanol
FTIR	Fourier transform infrared spectroscopy
LC-MS	Liquid chromatography mass spectrometry
GC-MS	Gas chromatography mass spectrometry
pKa	Acid dissociation constant
kDNA	Kinetoplastic DNA
DM	Deltamethrin
3-MC	3-Methylcholanthrene
PB	Phenoberbitone
DDT	Dichlorodiphenyltrichloroethane
CID	Collision induced dissociation
SPE	Solid phase extraction

PEEK	Polyetheretherketone
API	Atmospheric pressure ionization
AhR	Aryl hydrocarbon receptor
TLC	Thin layer chromatography
APCI	Atmospheric pressure chemical ionization
SIM	Selected ion monitoring
MRM	Mixed reaction monitoring
HAT	Human African trypanosomosis
PCR	Polymerase chain reaction
CATT	Card agglutination test for trypanosomes
ICH	International conference on harmonization
VICH	Veterinary international conference on harmonization

CHAPTER 1

INTRODUCTION

1.1 General Introduction

Over hundred years since the discovery by David Bruce (1895) of the trypanosome as the causal agent of a disease in cattle *(nagana)* transmitted by the tsetse fly, trypanosomosis (*formerly referred to as trypanosomiasis*) continues to ravage many parts of Africa, despite advances in the knowledge of the unique biochemistry and life cycle of the parasites. Although a number of health organisations and academic institutions have dedicated substantial resources to tackle the disease at both national and international levels, there has been a resurgence of the disease in recent times (Smith *et al.*, 1998). Several factors have contributed to the current status of the disease in Africa:

- Political upheavals and the attendant state of insecurity leading to a disruption of surveillance and monitoring activities which form a vital part of disease control.
- The re-direction of scarce resources to deal with other serious communicable diseases such as AIDS/HIV, malaria and tuberculosis (TB) has resulted in a lack of interest and de-prioritization of sleeping sickness in public health terms.
- The absence of a sustained tsetse fly eradication campaign, which would ensure that areas previously cleared of tsetse flies are not re-infested.
- Poor prospects for new drug development, a situation that has been complicated by the emergence of drug resistant parasites (trypanosomes).

Unlike diseases such as smallpox which have been eradicated through extensive vaccination campaigns, trypanosomosis has not been amenable to control by vaccination due to the antigenic variability of the surface glycoprotein (VSG) found in trypanosomes. However, recent advances in the field of proteomics and genetic engineering are being exploited to provide viable options for eradication of the disease (El-Sayed *et al.*, 1995). Peptide mapping and sequencing of the genome of trypanosomes could facilitate the identification of the unique proteins and specific

genes involved in such important functions as drug resistance, pathogenesis and antigenic variations. It would also be a major resource for molecular epidemiologists and parasitologists engaged in the study of trypanosomosis. Unfortunately, application of proteomics and genomics to this research is still in its infancy, and in the absence of a vaccine, vector control and chemotherapy remain the only major options to curtail human and animal trypanosomosis in affected areas worldwide.

Vector control, as effective as it may be, cannot make an immediate impact on people already affected by the disease. Consequently, there is an even greater reliance on drug treatment as a short-term control measure in both human and animal trypanosomosis. However, with the few trypanocides which have been used continuously for at least 35 years, there is growing evidence to implicate drug resistance in treatment failures of infections caused by trypanosomes (Peregrine, 1994). Such desperate situations call for desperate measures, which will help sustain the efficacy of available trypanocides by delaying the emergence of drug resistance.

To this end, the work presented in this thesis has been committed to help solve part of the problem of trypanocidal drug resistance that threatens to undermine efforts aimed at eradication of trypanosomosis in humans and animals in developing countries.

1.1.1 Life cycle of the trypanosome

The interactions between trypanosomes and their invertebrate vector and vertebrate (mammalian) hosts is of considerable importance in the epidemiology of trypanosomosis. Understanding the detailed life cycle of the trypanosome is essential for identification of potential targets that could lead to the rational design of lead compounds for eradication of the disease. African trypanosomosis in sub-Saharan Africa is transmitted by the blood feeding tsetse fly. The parasite undergoes significant biochemical changes as it moves from the blood of the mammalian host to the gut of the tsetse fly, a process that is necessitated by the requirement for adaptations to the different environmental conditions encountered in the two very

different hosts (Matthews, 1999). Vickerman (1985) has observed that the most prominent changes in the life cycle of trypanosomes (*T. brucei*) occur in the mitochondrial system in relation to switches in the pathways of energy metabolism, (from a non-Krebs cycle respiratory pathway in the mammal to a Krebs cycle pathway in tsetse midgut) and in the trypanosome surface glycoprotein in relation to evasion of the mammalian host's immune response through antigenic variation.

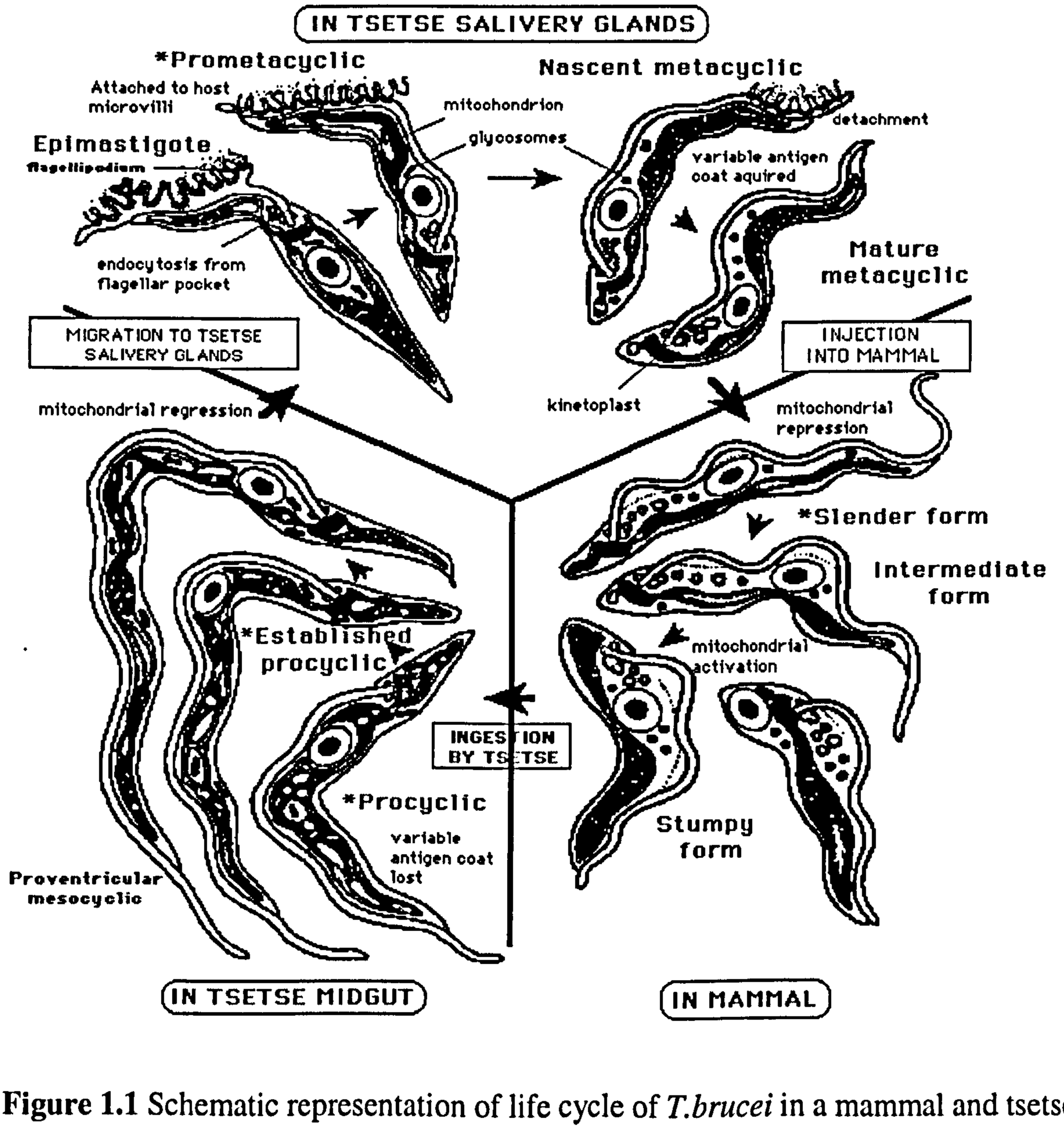


Figure 1.1 Schematic representation of life cycle of *T.brucei* in a mammal and tsetse fly vector. [Adapted from Vickerman, 1985]

After a blood meal, the bloodform trypanosomes called *trypomastigotes* transform and develop in the insect's midgut (figure 1.1), first as procyclic insect stages. They then migrate to the proventriculus, where they transform to *epimastigotes* and from where they infect the salivary glands of the tsetse fly. Once in the salivary glands they attach themselves to the gland microvilli and multiply as attached *epimastogotes*. They are subsequently transformed to metacyclic trypomastogotes, which are infectious to the mammalian host. In the mammal, they develop as long slender trypomastogotes with a free flagellum at the anterior end and then to a short stumpy form with no flagellum (figure 1.1). There is evidence, that should infected blood be ingested by a tsetse fly, it is mainly the stumpy form rather than the slender forms which develop in the midgut of the fly to initiate the cycle in the fly (Vickerman, 1965).

1.1.2 Antigenic variability

The survival of African trypanosomes in the mammalian blood stream is attributed to a highly sophisticated strategy involving switching between the transcription of one of over thousand Variant Surface Glycoprotein (VSG) genes (Wang, 1995). As they multiply in the mammalian blood, the host mounts an effective immune response against trypanosomes with a given variant of VSG coat. This removes all parasites with the old VSG coat, leaving behind those that have switched to a new (and temporarily unrecognisable) VSG coat. These new antigenic variants form the next wave of infection resulting in irregular parasitaemia. Antigenic variation of the surface coat allows the trypanosome to maintain chronic infections, which can last for years (Hide *et al.*, 1997).

1.2 Human trypanosomosis

1.2.1 Epidemiology

Human African Trypanosomiasis (HAT) (sleeping sickness) is caused by protozoan hemoflagellates of the genus *Trypanosoma*, subgenus *Trypanosoon* and *brucei* species which classically include three subspecies: *Trypanosoma brucei brucei (T.b.brucei)*, *T.b. gambiense* and *T.b. rhodesiense*. Trypanosomes are transmitted through the bite of tsetse flies (*Glossina* genus) and two forms of the disease are recognised in humans.

- A chronic form occurring in West and Central Africa is caused by *T.b.gambiense* and can last for years. It presents initially as an unrecognised illness with episodes of fever and malaise, which is followed by a progressively fatal meningo-encephalitis.
- An acute and fatal form caused by *T.b. rhodesiense* is observed in Eastern and Southern Africa. It is characterized by a rapid progress to meningo-encephalitis followed by coma and death within a few months. However, *T.b.brucei* is not considered to be pathogenic in humans (Smith *et al.*, 1995).

1.2.1.1 Distribution

In West and Central Africa, sleeping sickness caused by *T.b.gambiense* is transmitted by riverine species of tsetse fly (*palpalis* sub-genus), which prefer humid and dense riverine habitats. Consequently, man-fly contact is highest in foci where the life style and occupation of the people brings them close to the habitat of the fly. For instance, in some foci of the Ivory Coast, higher prevalence of *gambiense* infections have been found in people who fetch water than in other inhabitants (Laveissiere *et al.*, 1986) while people living in coffee and cocoa plantation settlements have been shown to be five times more likely to develop the disease than their counterparts in open villages (Meda *et al.* 1993). Humans are known to provide the main reservoir for infections of *T.b.gambiense* although wild and domestic animals may play a minor role in specific foci. In Southern Africa, *T.b.rhodesiense* transmission is effected by savannah species of tsetse fly (*morsitans* group), which are adapted to survival in dry savannas, and open areas of woodland. The tsetse feed preferentially on game and

domestic animals, which provide a reservoir of infection *(zoonotic infection).* As a result, human infections occur in individuals (poachers, hunters, tourist and honey harvesters) who come in contact with the zoonotic cycle. Transmission of *T.b.rhodesiense* infections in Eastern Africa is by a riverine species of tsetse fly (*Glossina fuscipes),* with domestic cattle as the main reservoir. Thus cattle movement is important in the incidence and distribution of sleeping sickness in this region. In Uganda, a country with high endemicity, both *T.b.gambiense* and *T.b.rhodesiense* infections are found, the former in the northwest of the country close to the Sudanese border and the latter in the southeast of the country (Table 1.1).

Thus HAT is prevalent mainly in rural areas where health systems are least effective or non-existent. It is estimated that, about 60 million of the 400 million people inhabiting 36 sub-Saharan African countries where the disease is endemic are at risk (WHO-1998).

Table 1.1 Number of treated cases of sleeping sickness per country over five years

Sleeping Sickness	**Country**	**1995**	**1996**	**1997**	**1998**	**1999**	**2000**
T.b. gambiense	Angola		6,786	8,275	7,373	5,351	4,546
	D.R. Congo	18,158	19,342	25,200	25,044		
	Sudan		157	737	1,800	1,500	2,000
	N.W.Uganda	1,062	980	1,069	967	1,020	
T.b.rhodesiense	S.E.Uganda	497		271	287	299	
	Tanzania		400	531	421	588	627

Source: Report of the Scientific Working Group meeting on African Trypanosomasis, June 2001

As a result of financial limitations and inadequate logistics, only 3 to 4 million of the population under surveillance benefit from comprehensive control programmes. In recent times, there has been a significant resurgence of the disease notably in endemic countries such as Tanzania, Sudan, Uganda and the Democratic Republic of

Congo (DRC) (table 1.1). However due to recent civil wars in some affected areas (e.g. Sudan, Angola) the extent of HAT is not known. Though not in epidemic proportions, the current statistics (table 1.1) indicate that the disease is still uncontrolled.

1.2.1.2 Diagnosis

The successful treatment of HAT depends on an accurate clinical diagnosis of the stage of the disease and the identification of the subspecies of trypanosomes implicated. Failure to make an early diagnosis of sleeping sickness may be a contributory factor in the resurgence of the disease since the differentiation of sleeping sickness from the wide range of other febrile diseases like malaria, bacterial meningitis and HIV can be difficult in the absence of effective diagnostic tools. Two stages of the disease are recognised in humans.

- Stage (I) or early-stage of the disease is characterized by the presence of trypanosomes in the lymph fluid or blood of sufferers (also referred to as haemolymphatic stage).
- Stage (II) or late-stage of the disease, involves the invasion of the central nervous system (CNS) by the parasites leading to encephalopathy.

Serological diagnostic techniques currently used include techniques based on antibody detection like the Card Agglutination Test for Trypanosomes (CATT) and antigen detection methods such as the Card Indirect Agglutination Test for Trypanosomes (CIATT) (Magnus *et al.*, 1978; Komba, 1992; Nantulya, 1997). Though useful for mass screening purposes, serological techniques do not discriminate between past and present infections. As a result a more sensitive and specific method based on the polymerase chain reaction (PCR) has been recently developed for identification of trypanosomes (Kyambadda *et al.*, 2000). However PCR is both complicated and expensive for application as a routine diagnostic test and thus its use is limited to research laboratories and referral hospitals.

1.2.2 Control of African sleeping sickness

Two major approaches employed for the control of sleeping sickness are:

- vector control and
- chemotherapy

Vector control

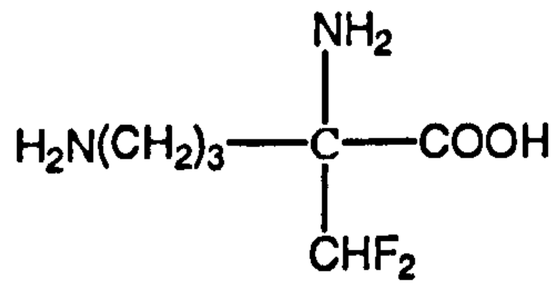
Though vector control does not make any impact on people already infected with trypanosomes, it reduces the frequency of human-tsetse fly encounters by suppressing tsetse fly populations. Techniques employed for vector control five decades ago included the shooting of game animals, which serve as reservoirs, vegetation clearance to destroy fly habitat and the aerial spraying with organochlorine insecticides such as dichlorodiphenyltrichloroethane (DDT) and dieldrin. However, environmental concerns have led to their replacement with environmentally friendly tsetse control techniques including sterile insect release techniques, the use of insecticide impregnated traps, screens or bait technology; and the application of non-residual synthetic pyrethroids such as deltamethrin and permethrin for ground and aerial spraying. The sustainability of vector control in endemic countries has been shown to be dependent on the cost effectiveness of applied methods of control (Barrett, 1994). Pilot projects in Congo (Gouteux *et al.* 1990) and Ivory Coast (Laveissiere *et al.*, 1994) have demonstrated the feasibility and efficiency of cheap traps and screens used with the active participation of rural communities. Consequently the choice of tsetse control technique must be made in the context of the prevailing epidemiological conditions and available local resources.

Chemotherapy

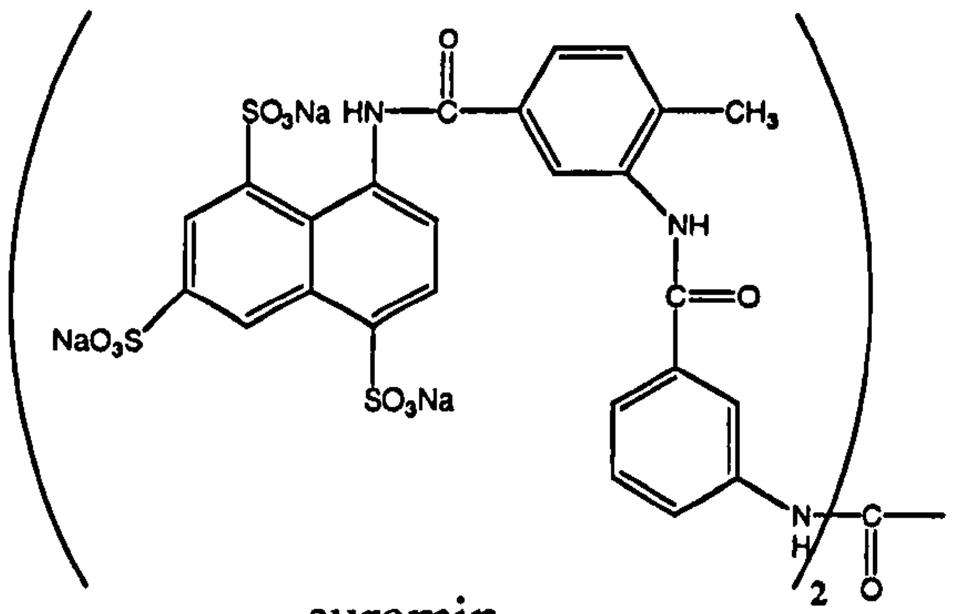
Drugs used for treatment of sleeping sickness (Table 1.2) share the common features of limited availability, high cost, toxicity and a requirement for patient hospitalization during treatment. Moreover, most of the available drugs have been in use for over five decades and drug resistance is curtailing their efficacy. Successful

chemotherapy depends on accurate diagnosis of the stage of disease as well as the subspecies of trypanosomes involved. Chemical structures of drugs that are currently used for treatment of human African trypanosomosis are shown in figure 1.2.

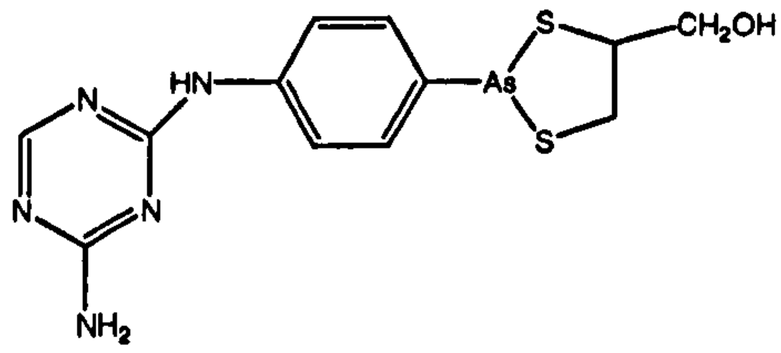
pentamidine



eflornithine



suramin



melarsoprol

Figure 1.2 Chemical structures of the common trypanocides used in the chemotherapy of human African sleeping sickness

The chemotherapeutic armoury for sleeping sickness is very poor and the only novel drug available to treat the late-stage of the disease since the introduction of the trivalent arsenical melarsoprol [Mel B®] in 1949 (Friedheim, 1949) is eflornithine [Ornidyl®] (figure 1.2). Eflornithine is however ineffective against *T.b.rhodisiense* infections. Nifurtimox (Lampit®, Bayer) (Table 1.2), introduced originally for treatment of Chagas' disease in 1965, is not licensed for treatment of sleeping sickness but is currently being tried on a compassionate basis for the treatment of melarsoprol refractory cases.

At present, the World Health Organization (WHO) acts as a focal point for the supply of drugs like melarsoprol, pentamidine and eflornithine in affected countries. Finance for the supply of these drugs is often via appeals to donors or through emergency or humanitarian assistance programmes, a situation that potentially creates shortages and could inevitably lead to resurgence of the disease.

Table 1.2 Chemotherapy of African sleeping sickness

Drug	Pentamidine	Suramin	Melarsoprol	Eflornithine	*Nifurtimox
Indications	Stage (I) *T.b.gambiense*	Stage(I) *T.b.gambiense* *T.b.rhodesiense*	Stage(I) and (II) *T.b.gambiense* *T.b.rhodesiense*	Stage(I) and (II) *T.b.gambiense*	
Mode of application	Intra-muscular	intravenous	intravenous	intravenous	Oral
Dosage	4mg/kg body wt. once daily for 7 days	5mg/kg body wt. on day 1, then 20mg/kg body wt. on days 3, 10, 17, 24 and 31	3-4 daily injections of 3.6mg/kg body wt. separated by intervening rest periods of 7-10 days	400mg/kg body wt./day and 100mg every 6h for 14 days	15-20mg/kg body wt./day in 3 divided doses for 30-60 days
Adverse effects	Hypertension Hypo-and hyper-glycaemia, renal & hepatic toxicity	Nausea, vomiting, skin reactions renal failure jaundice	Skin reactions, renal & hepatic toxicity, frequent fatal reactive encephalopathy	Few reported 7 day course lead to unacceptably high relapse	Relapse with low doses reported. Coma & death with high dose

* Drug under clinical trial

1.3 Animal trypanosomosis

1.3.1 Epidemiology

The current situation of African animal trypanosomosis is no better than that of human sleeping sickness. Due to political instability, inadequate logistics and poor surveillance, there is a lack of accurate information about the prevailing status of the disease and its impact on agriculture in endemic countries like Sudan, Angola, Somalia and the Democratic Republic of Congo.

The incidence of tsetse transmitted African animal trypanosomosis in ruminants (cattle, sheep and goats) is characterized by three species of trypanosomes: *T. congolense*, *T. vivax*, and *T. brucei.brucei.* Pathogenic trypanosomes in ruminants are classified into two groups: (i) vascular and (ii) extra-vascular groups (Losos and Ikede, 1972). The vascular or haematic trypanosomes include *T. congolense* and *T.vivax* which are regarded as the most pathogenic species in ruminants while the extra-vascular trypanosomes are represented by the *T. brucei*-group which are considered to be less pathogenic than the vascular trypanosomes (Ikede and Losos, 1975). However animal trypanosomosis is not limited to Africa. Trypanosome species such as *T. evansi* affect camels in India and Russia, *T. equiperdum* is transmitted venerally between horses in Kazakhstan, while *T. vivax* occurs in South America (Peregrine and Mamman, 1993).

1.3.2 Impact of animal trypanosomosis on agriculture

Animal trypanosomosis constrains agricultural productivity in sub-Saharan African. Compared with animals in trypanosomosis free areas, animals kept in areas of moderate risk of trypanosomosis are known to experience lower calving rates, lower milk yields, higher rates of calf mortality and require more frequent treatment with preventive and curative doses of trypanocides (Ahmedin and Hugh-Jones, 1995). In areas where animal traction is employed for crop production, the disease reduces the work efficiency of oxen used for cultivation. The investment of scarce financial resources for the purchase of prophylactic and curative trypanocides has had a negative impact on agriculture.

It has been estimated that an average of $35 million per year is spent on trypanocides in Africa, most of which are likely to be used on cattle (Geerts and Holmes, 1997). Put together, the cost of tsetse control and chemotherapy of infected livestock could adversely affect the overall cost of agricultural productivity.

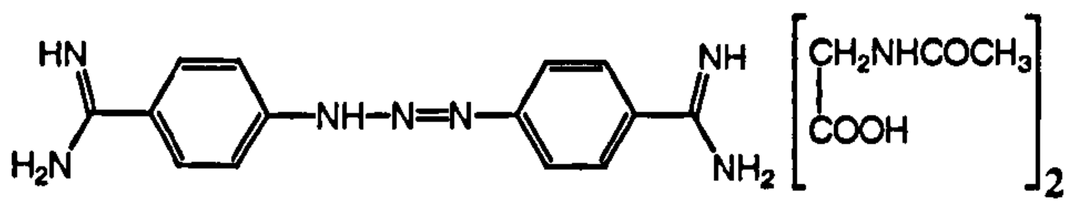
1.3.3 Control strategies of animal trypanosomosis

In addition to chemotherapy and vector control, two other major strategies employed in the control of animal trypanosomosis are the use of trypanotolerant breeds of cattle and the direct application of insecticides on livestock (cattle dipping). Trypanotolerance has been defined as the innate capacity of an animal to resist the development of parasites and to limit their pathological effects such as anaemia (Murray *et al.*, 1984). There is evidence that in areas of low to moderate trypanosomosis challenge, the productivity of trypanotolerant cattle (N'dama) is more favorable than other breeds (Feron *et al.*, 1987). However when the merits of using trypanotolerant breeds is weighed against the demerits of lower yields of meat and milk production due to the smaller size of the breed, then trypanotolerance may not necessarily be a very profitable option. A cost-effective approach aimed at reducing animal-tsetse fly contact has been the direct treatment of livestock with insecticides as a pour-on or dip treatment. Although this technique confers transient protection to livestock against tsetse flies and other tick born diseases, the effects of long term application of insecticides on cattle is yet to be established.

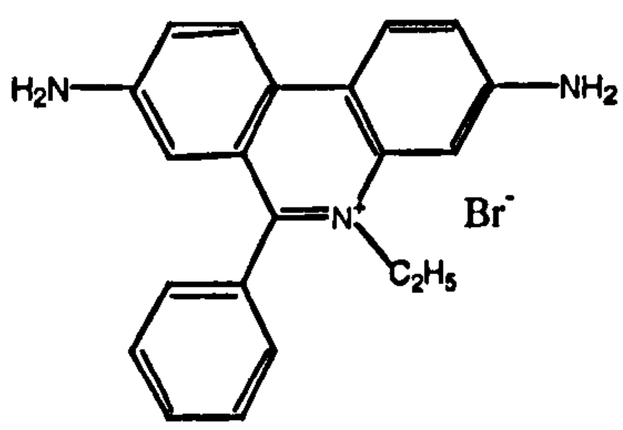
1.3.4 Chemotherapy

Trypanocidal drugs remain the principal method of control of animal trypanosomosis in affected areas worldwide. Currently, chemotherapy relies on only three major veterinary trypanocides, the aromatic diamidines, diminazene aceturate (Berenil®), and the

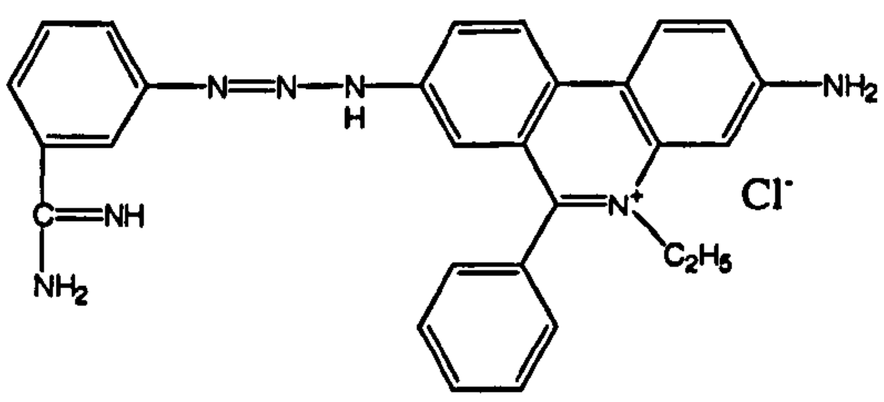
phenanthridines, isometamidium chloride (Samorin®) and homidium bromide (Ethidium) (figure 1.3).



diminazene aceturate



homidium bromide



isometamidium chloride

Figure 1.3 Chemical structures of currently available veterinary trypanocides

Isometamidium (ISM) and homidium are employed for both curative therapy and prophylaxis while diminazene is used mainly for curative purposes. Although their toxicity and mutagenic activity have been known for a long time, the phenanthridines, ISM and especially ethidium continue to be widely used (Fukunaga *et al.*, 1980; Singer *et al.*, 1999).

Diminazene on the other hand, is relatively less toxic and has been successfully used for the treatment of early stage cases of human sleeping sickness (Abaru *et al.*, 1984). A recent survey has shown that in areas where the three trypanocides are available, farmers prefer to use diminazene to the phenanthridines (Van den Bossche *et al.*, 2000). Sadly, the repeated use of all of these trypanocides for over 35 years and the lack of comprehensive quality assurance policies in affected countries have resulted in widespread drug resistance (Peregrine, 1994).

1.4 Drug resistance

The ability of pathogens to adapt and survive in the lethal environment of a chemotherapeutic agent (drug) or chemical (pesticide) is generally termed drug resistance. In recent times, the emergence of drug resistance in pathogens associated with major diseases like malaria, tuberculosis and bacterial infections has become a subject of great concern among scientists and health authorities world wide.

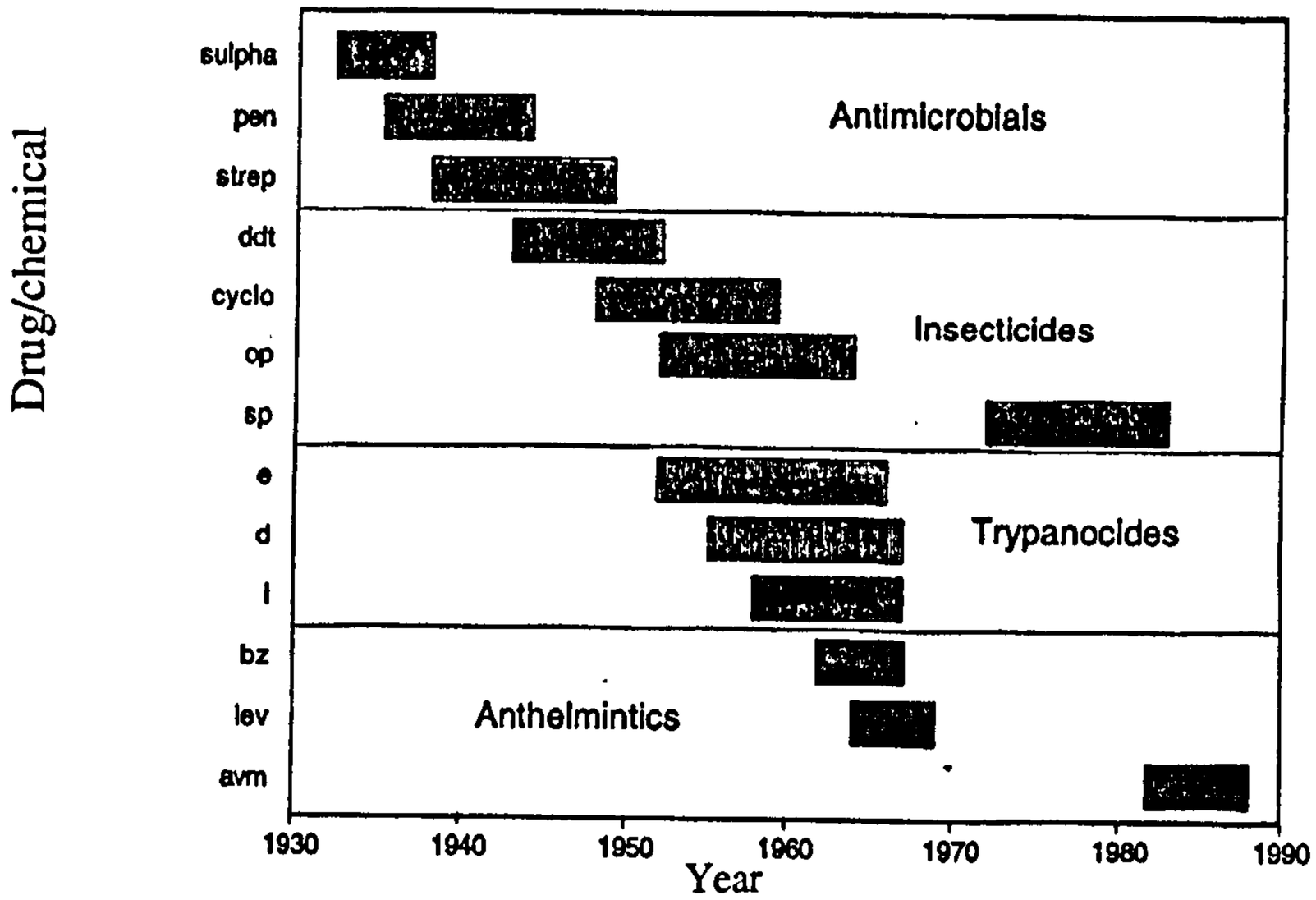


Figure 1.4. Evolution of resistance: year of commercial release of drug or chemicals and first appearance of resistance in target organisms

[*Source:* Adapted from Waller, 1994]

Key:

Antimicrobials:

Sulpha = sulphonamides

Pen = penicillin

Strep = streptomycin

Insecticides:

ddt = dicophane

cyclo = cyclodienes

op = organophosphates

sp = synthetic pyrethroids

Trypanocides:

e = ethidium

d = diminazene

i = isometamidium

Antihelminthics:

bz = benzimidazoles

lev = levimasole

avm = avermectins

Waller (1994), has observed that drug resistance, systematically occurred within approximately ten years following the introduction of antimicrobials, insecticides, trypanocides and antihelmintics to the market (Figure 1.4).

Extensive studies of the underlying mechanisms of drug resistance have shown that the following mechanisms can be involved:

a) Alterations to the drug target (Carter *et al.*, 1995)

(i) Elimination of target (e.g. induction of alternative pathway)

(ii) Change of targets' affinity for the drug

b) Exclusion of drug

(i) Loss of drug accumulation mechanism (Sutherland *et al.*, 1991)

(ii) Increased drug elimination mechanism (Sutherland and Holmes, 1993)

c) Drug metabolism (Tschida *et al.*, 1995)

(i) Deactivation of drug (by host or target organism)

(ii) Inability to activate pro-drug in the host

1.4.1 Trypanocidal drug resistance

1.4.1.1.Current situation

The first reported cases of acquired resistance to veterinary trypanocides were published during the 1960s (Finelle and Yvore, 1962; Jones-Davies and Folkers, 1966; Na'Isa, 1967). Since then, a significant number of reported cases have been documented (Lemonade, 1979; Peregrine, 1994; Joshua *et al.*, 1995). In the case of human trypanosomosis, recent reports indicate that there is an increase in the number of treatment failures with late-stage drugs like melarsoprol (Legros *et al.* 1999; Bacchi, 1993). However, most of the currently available information on drug resistance is derived from a limited number of case reports, which may not give an indication of the prevailing situation. Furthermore, there is considerable variation in the protocols that have been used to diagnose drug resistance.

1.4.1.2 Non-biological causes of trypanocide drug resistance

The exposure of parasites to sub-therapeutic drug concentrations (owing to under dosing) has been considered as the most important factor for the development of trypanocidal drug resistance (Boyt, 1986). Thus, it is possible to take drug-sensitive clones of trypanosomes and induce drug resistance rapidly by repeated under dosing. Osman and colleagues (1992) were able to produce a 80-fold increase in diminazene resistance of a cloned population of *T. evansi* over a nine week period by sub-curatively treating immunosuppressed mice. The administration of sub-therapeutic drug concentrations can exert a strong selective pressure for the emergence of resistance clones that pre-exist in trypanosome populations.

Drug under-dosing commonly occurs when farmers or unskilled persons underestimate the weight of animals during treatment (Besier and Hopkins, 1988) or over-dilution of drugs due to financial constraints or the administration of generic products with compromised efficacy as has been shown in the field of antihelmintics (Van Wyk *et al.*, 1997). The lack of comprehensive drug regulation and enforcement policies in some developing countries facilitates trading in counterfeit and substandard drugs. For economic reasons, drugs destined for use in the public sector are usually purchased on open tenders and contracts offered to local or foreign pharmaceutical companies based on a bidding system without reference to the drug regulatory authority. This favours the use of generic drugs in the public health sector, which provides health care for the majority of patients with sleeping sickness. Generic drugs are required to satisfy criteria of therapeutic equivalence to the innovative product with respect to;

(a) Manufacturing and quality control, (b) product characteristics (stability/excipients/labelling) and (c) biological/chemical equivalence. Consequently, the registration and licensing of generic trypanocides by regulatory bodies is necessary for their quality assurance. However, this cannot be achieved without pharmacopoeial specification, laboratory services and appropriate analytical techniques, which are pivotal to any system of drug control.

1.4.1.3 Biological causes of trypanocide drug resistance

Although there are reports in the literature of extensive studies to unravel the molecular and genetic basis of trypanocidal drug resistance, very little is known about the effects and impact of drug metabolism. The biotransformation of a drug in the body can result in (i) deactivation of the parent drug (ii) activation of parent drug to a metabolite that is pharmacologically more potent or different and (iii) the formation of toxic metabolites (Gibson and Skett, 2001). However, in order to achieve the desired therapeutic objectives, the concentration of a drug in the plasma and at the site of action needs to be carefully maintained. The induction or inhibition of (hepatic) metabolizing enzymes as a result of concomitant exposure to other apparently non-related xenobiotics (eg pesticides) could affect the circulating plasma levels of a drug (Tarrus *et al.*, 1987), which could have pharmacological or toxicological outcomes. Consequently blood parasites may be exposed to sub-lethal drug concentrations which can select resistant clones of trypanosomes. Tettey *et al.* (1999), have demonstrated that the hepatic metabolism of ethidium bromide by rat hepatocytes is significantly altered following the induction of hepatic enzymes by pre-treatment with classic cytochrome P450 inducers. Alternatively, there is a possibility that some resistant trypanosomes may have evolved mechanisms that enable them to deactivate trypanocides by metabolism and this has been demonstrated in *T. brucei* by Boibessot *et al.* (2002).

From the foregoing discussions, it is evident that any attempt aimed at solving the problems of trypanocidal drug resistance will have to address questions related to:

(i) Quality and therapeutic equivalence of generic trypanocides

(ii) The metabolic fate of trypanocides in different mammalian species

(iii) The possible interaction of environmental chemicals with trypanocide metabolism

1.5 Aims and objectives

The work in this thesis is aimed at:

1. The development and validation of a selective, precise and accurate liquid chromatographic method for the quantification of diminazene aceturate in the bulk drug substance and pharmaceutical formulations. Identification and structural characterization of major manufacturing impurities and degradation products that may be present in formulations of diminazene will be performed.

2. Application of the developed chromatographic method for the quality evaluation of generic formulations of diminazene sourced from different locations in affected regions of Africa.

3. The use of *p*-aminobenzamidine as a model aromatic amidine to define the metabolic fate of the amidino functionality using freshly isolated rat and pig hepatocyte suspensions. This will facilitate studies of the metabolism of the two-trypanocidal diamidines; diminazene aceturate and pentamidine isethionate in rat and pig hepatocytes. The effect of classic inducing agents (phenobarbitone and 3-methylcholanthrene), and a pyrethroid insecticide (deltamethrin) on the metabolism of the two trypanocidal diamidines in rat will be investigated.

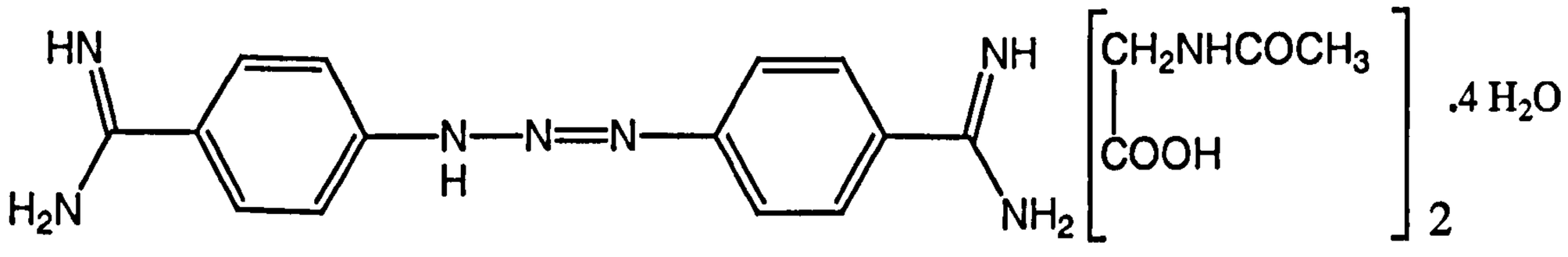
CHAPTER 2

DETERMINATION OF DIMINAZENE ACETURATE IN PHARMACEUTICAL FORMULATIONS AND THE IDENTIFICATION OF RELATED SUBSTANCES

DETERMINATION OF DIMINAZENE ACETURATE IN PHARMACEUTICAL FORMULATIONS AND IDENTIFICATON OF RELATED SUBSTANCES

2.1 Introduction

The aromatic diamidine, diminazene aceturate (4,4'-diamidino diazo -aminobenzene diaceturate tetrahydrate) (Figure 2.1) was first introduced as a trypanocide and babesiacide for domestic livestock in 1955 (Jensch, 1955) and patented to Farbwerke Hoechst GmbH, Germany (1954) *[Patent No. US 2,673,197]*. Marketed under the trade name Berenil®, the use of diminazene as a trypanocide rapidly increased with the number of administered doses to cattle in Kenya rising from 2000 in 1957 to 190,000 in 1961 (Fairclough, 1962). Diminazene has become the most commonly used therapeutic agent for treating trypanosomosis in domestic livestock because it has a higher therapeutic index compared to other trypanocides (Fairclough, 1963; Williamson, 1970) and it is active against trypanosomes that are resistant to other trypanocides used in cattle (Williamson, 1970). Recent surveys to investigate the preference and strategy of usage of veterinary trypanocides among local farmers in the Eastern province of Zambia, has shown that the majority of cattle owners preferred to use diminazene aceturate rather than isometamidium chloride (Van den Bossche *et al.*, 2000).



[$C_{22}H_{29}N_9O_6.4H_2O$, Molecular weight (M.wt).=587.6; Base, M.wt.=281.2]

Figure 2.1 Chemical structure of diminazene diaceturate tetrahydrate

2.1.1 Therapeutic activity

Diminazene aceturate has been reported to be effective against a wide range of trypanosome species including *T. congolense, T. vivax* and *T. b. brucei* in domestic livestock and *T. equiperdum* in horses (Peregrine and Mamman, 1993). However, it is recommended only for use as a therapeutic agent since it is thought to be rapidly excreted and may have little prophylactic activity (Bauer, 1958). Diminazene aceturate is typically administered intramuscularly, at doses of 3.5mg/kg body weight (b.w) or 7.0mg/kg b.w to maintain breeds of cattle that are trypano-susceptible, in areas of low to medium trypanosome challenge (Wilson *et al.,* 1975). Though not registered for use in humans, diminazene has been demonstrated to be effective in the treatment of early stage cases of sleeping sickness caused by *T.b. gambiense* and *T. b. rhodesiense* infections (Hutchinson and Watson, 1962; Temu, 1975; Abaru and Motavu, 1981; Abaru *et al.,* 1984). In addition to its trypanocidal activity, diminazene aceturate has been used for treatment of bovine, canine, porcine and human babesiosis (Kuttler, 1988), and for visceral and cutaneous leishmaniasis in man (Rees *et al.,* 1985; Lynen and Van Damme, 1992).

2.1.2 Mechanism(s) of action

Diminazene has been shown to bind to trypanosomal kDNA to cause the selective fragmentation of the kinetoplast (MacAdam and Williamson, 1972). The binding which is known to be non-intercalative (Bénard and Riou, 1980), is specific via interactions with sites rich in adenine-thymine (A-T) base pairs (Newton 1972; Brack and Delain, 1975). Non-intercalative binding of diminazene to DNA, with a strong affinity for A-T base pair regions, has been well demonstrated *in vitro*, using DNA obtained from different sources (Baguley, 1982). Through this specific binding interaction in trypanosomes, diminazene inhibits synthesis of RNA primers and thereby inhibits kDNA replication (Newton, 1972; Brack and Delain, 1975). It has also been established by Shapiro and Englund (1990), that diminazene specifically inhibits mitochondrial type II topoisomerase in viable trypanosomes.

2.1.3 Multi-source pharmaceutical formulations

Following the expiry of patent protection of the innovator product (Berenil®), there has been an influx onto the market of several multi-source (generic) formulations of diminazene aceturate. For economic reasons, the use of generic substitution is increasingly being supported by health authorities world-wide which may cause potential problems for drugs with a narrow therapeutic window if their quality control and/or bioequivalence is not optimal (Meredith, 1996). Nominally equivalent generic pharmaceutical products may not usually contain the same amount of active ingredients as the innovator. Consequently, their clinical interchangeability may be in doubt (WHO, 1997). The concept of interchangeability applies not only to the dosage form of a generic product but also to the instructions for use and packaging specifications, which may be critical to the stability and shelf-life of the drug (WHO, 1997).

In economies where the manufacture, supply and distribution of medicines are not well regulated, generic substitution creates the ideal conditions for the influx of substandard formulations (Shakoor *et al.*, 1997). Several factors have facilitated the proliferation of counterfeit drugs in developing countries (WHO, 1992) and include the following;

- Weak drug regulatory control and enforcement
- Scarcity and/or erratic supply of basic medicines
- Existence of unregulated markets and distribution chains, in both developing and developed countries
- Price differentials which potentially create an incentive for drug diversion within and between established channels
- Lack of effective intellectual property protection
- Poor quality assurance

As a result, regulatory measures are necessary to ensure that safety, quality and efficacy are not sacrificed for low priced generic medicines. For drug regulation to be effective, analytical techniques are essential for monitoring specific ingredients in pharmaceutical products.

2.1.4 Methods of analysis for diminazene

The determination of diminazene in biological matrices for the generation of pharmacokinetic data in different animal species has been performed by the use of various analytical techniques. A colorimetric method was used to investigate the pharmacokinetics of diminazene in rats and monkeys (Raether *et al.*, 1972; Peregrine and Mamman, 1993), cattle (Klatt and Hajdu, 1976) and dogs (Onyeyili and Anika, 1991). Colorimetic methods are mainly based on the spectrophotometric measurement of coloured complexes formed by the reaction of a reagent with the analyte of interest and are generally characterized by poor specificity and low sensitivity. In an attempt to increase the sensitivity of diminazene assays, radiometric methods were developed by Gilbert (1983) and Kellner *et al.* (1985). Although radiometric assays can detect diminazene in concentrations as low as 28 ng/ml of plasma, they are deficient in specificity, since they measure total radioactivity and may not distinguish the parent drug from the modified form of the drug. Fouda (1978), employed HPLC and gas chromatography-chemical ionization mass spectrometry (GC-MS) for the analysis of diminazene in plasma. Although sensitive, the assay required tedious and protracted sample preparation steps, involving the reduction of diminazene to 2 moles of 4-aminobenzamidine (figure 2.2), prior to extraction, derivatization and analysis.

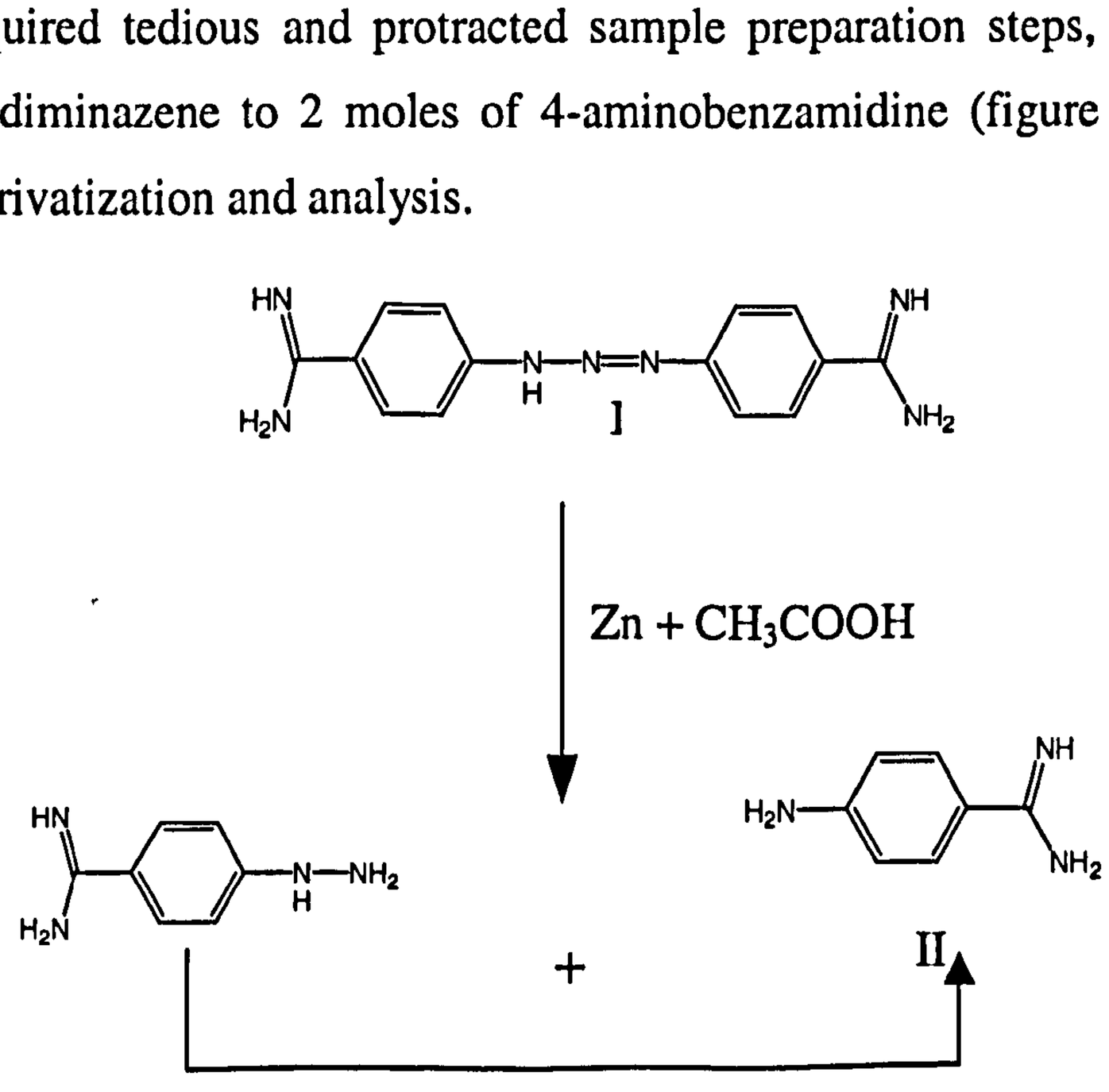


Figure 2.2 Reduction of diminazene (I) to 4-aminobenzamidine (II)

Thin layer chromatographic (TLC) methods involving fluorodensitometric analysis of fluorescent diamidines in fluid and tissue sample extracts have also been described (Gluth *et al.*, 1986). HPLC techniques have been proven to be more sensitive and specific for quantification of diminazene in biological matrices (Aliu and Ødegaard, 1983). However, the presence of two highly basic amidino groups (pKa~11.6) in diminazene (figure 2.1), makes it highly susceptible to residual interactions with silanol groups of standard silica-based reverse phase stationary phases. Consequently, the reported liquid chromatographic methods for diminazene, have been associated with poor peak shape (tailing peaks), the use of complex mobile phases and poor recoveries (Fouda, 1977, Gummow *et al.*, 1995).

2.1.5 Manufacturing impurities and related substances

The safety of a drug is dependent not only on the toxicological properties of the active drug substance, but also on those of the impurities or related substances that may be present. Related impurities may arise during the manufacturing process and/or storage of the drug substance and may include;

- Starting Materials
- By-Products
- Intermediates
- Degradation Products

Owing to safety and toxicological implications, drug impurities have received considerable attention from both manufacturers and regulatory agencies (Berridge, 1995). The International Conference on Harmonization (ICH) is a tripartite organisation which provides guidelines on impurities in drug substances and products for adoption by the regulatory bodies of the European Union, Japan and United States of America. The guidelines define manufacturers goal for the purity criteria of their products. The ICH (1995) guidelines groups impurities into three major classes namely; residual solvents, organic and inorganic impurities. A key component of the guideline is Qualification. Qualification is defined as "the process of acquiring and evaluating data which establishes the biological safety of the

individual impurity or a given impurity profile at specified level(s)". The ICH (1995) guideline recommended that any organic impurity present at a level more than 0.1% (of parent compound) needs to be qualified. The Veterinary International Conference on Harmonisation VICH (1999) consensus guidelines sets a threshold for qualification of organic impurities at 0.5% for the majority of compounds. However lower qualification threshold limits are to be applied when there is concern about the safety of a impurity in a specific drug substance. The guidelines (ICH, 1995) also make provisions for the qualification of degradation products (threshold~ 0.1%). However, establishing threshold limits of impurities in drug substances can present considerable analytical challenges and this justifies the development of specific, selective and sensitive analytical techniques. Changes in the impurity profile of a drug substance can occur when generic versions of an innovator product are produced through modifications/scale up of the synthetic process of the original manufacturer or when an entirely different route of chemical synthesis is used.

The synthesis of diminazene (as described in the patent application of 1954 by Farbwerke Hoechst: *patent No.US 2,673,197*) is typically carried out by the diazotization of *p*-aminobenzamidine hydrochloride in a mixture containing sodium nitrite and concentrated hydrochloric acid. The resultant diazonium salt then couples with the unreacted amine in a weakly acidic solution to form the triazene product-diminazene (figure 2.3). It is evident from the reaction scheme of diminazene that the purity of the starting material (4-aminobenzamidine) is an important determinant of any process impurities that may be formed in the end product. Moreover, diminazene is known to have a short duration of stability in aqueous solutions (2-3 days) (Fairclough, 1962) and as a result it is marketered in combination with the stabilizer phenyldimethyl pyrazolone (antipyrine) (figure 2.4).

Antipyrine (phenazone) has antipyretic and analgesic properties and has been traditionally used in a similar manner to paracetamol. Ear drops containing approximately 5% of antipyrine are used for the relief of painful ear conditions (Codex, 1979). Although the mechanisms underlying its effects as a stabilizer of diminazene are not clear, it is probable that antipyrine helps to control the fevers associated with onset of animal trypanosomosis.

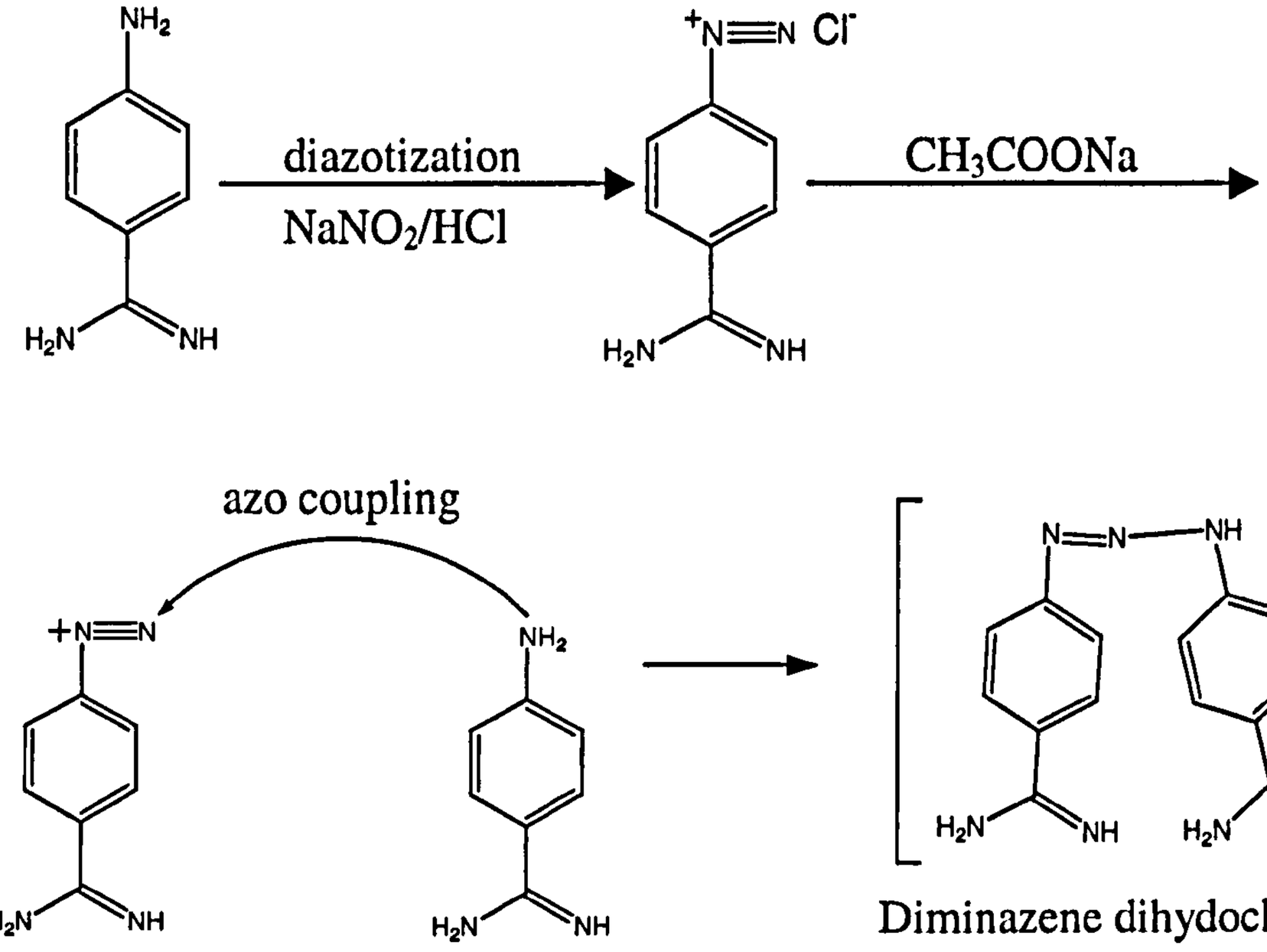


Figure 2 3 Reaction scheme for the synthesis of diminazene

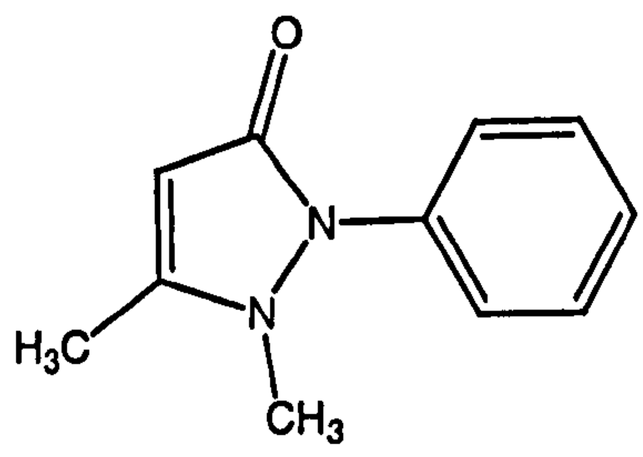


Figure 2.4 Chemical structure of antipyrine (1,5-Dimethyl-2-phenyl-1,2-dihydro-pyrazol-3-one) [pH of 5% solution = 5.8-7.0]

2.2 Materials and Methods

2.2.1 Materials

HPLC-grade methanol, acetonitrile and analytical reagent grade formic acid (98% v/v), toluene, n-propanol and ammonia solution (35% v/v) were obtained from BDH Laboratory Supplies (Poole, UK). Ammonium formate (97%), hydroxylamine monohydrochloride (99%) and *p*-aminobenzamidine (98%) were purchased from Sigma-Aldrich Chemicals (Dorset, U.K). Diminazene aceturate (4,4′-diamidinodiazoaminobenzene diaceturate tetrahydrate) (99.6%) was a gift from Intervet, Germany and antipyrine (phenazone) (1,5-dimethyl-2-phenyl-pyrazolidin-3-one) (>99%) was a gift from the Medicines Testing Laboratory (Edinburgh U.K). Preparative thin layer chromatography plates (PK6F silica Gel60, 1000μm thickness) were purchased from Whatman lab. Division (Clifton, USA).

2.4.2 Measurement of U.V and fluorescence spectral properties of diminazene

In order to determine a suitable wavelength for simultaneous monitoring of diminazene and antipyrine, the U.V spectra of separate solutions (20μg/ml) of diminazene and antipyrine in distilled water were acquired by scanning (200-500nm) with a double beam UNICAM UV4-100 UV/visible spectrophotometer (*v*1.30), set at a bandwidth of 2.0nm and normal chart speed. From the superimposed spectra (figure 2.5), 254nm was considered as a suitable wavelength that would allow the simultaneous monitoring and quantification of the diminazene and antipyrine. Setting the monitoring wavelength at 370nm (λ_{max} of diminazene) could have improved the limit of detection of diminazene but would not have allowed for the detection of antipyrine which does not show any absorbance above 300nm.

A dilute solution of diminazene aceturate (2.5μg/ml) was prepared in distilled water to examine the possible fluorescent properties of diminazene, with a fluorescence spectrophotometer (Model RF-530, Shimadzu Ltd, Kyoto, Japan) with instrument settings at predetermined excitation and emission wavelengths (λex = 370nm, λem = 420nm). The results indicated that diminazene did not possess fluorescent properties.

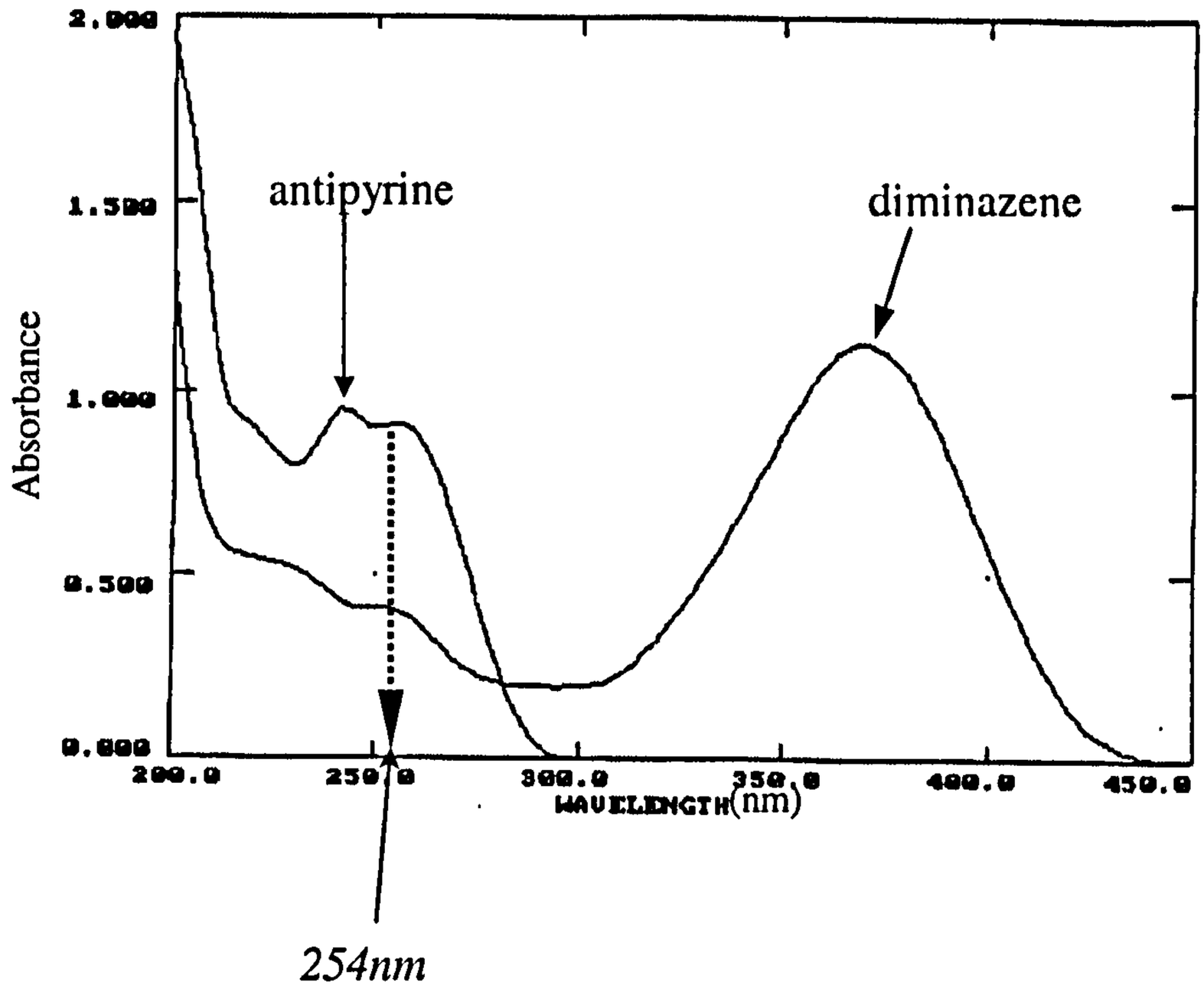


Figure 2.5 Superimposed UV-visible spectra of solutions (20μg/ml) of diminazene aceturate and antipyrine

2.2.3 HPLC analyses

The analysis of diminazene aceturate was performed with an HPLC system consisting of a Spectra-SYSTEM P2000 pump (Thermoseparations) equipped with an online autosampler Spectra SYSTEM AS1000 (Thermoseparations) and 20µl fixed injection steel loop. Analytes were monitored at 254nm with a Spectra System 6000LP-photodiode-array (PDA) detector (Thermoseparations) and spectral data collected over a range of 200-500nm. Chromquest chromatography workstation software (*Version 2.51*, 1999) was used for instrument control and data acquisition. Chromatographic separations were performed at ambient temperature on a Lichrospher® 60 RP-select B (C8) column (125 x 4mm i.d.), 5µm particle size, (Hewlett Packard, Germany,) with a 20 x 2 mm i.d. guard column (packed with Lichrosorb C8, 5µm particle size; E. Merck, Darmstadt, Germany). The mobile phase (acetonitrile-methanol-ammonium formate buffer) (20mM, pH 4.0) (10:10:80 v/v/v) was delivered at a flow rate of 0.7 ml/min. Ammonium formate buffer (20mM, pH 4) was prepared by dissolving 1.2612g ammonium formate in a 1000ml

volumetric flask with distilled water. The pH of the resulting solution was adjusted to 4.0 with formic acid and made up to the mark with distilled water.

2.2.4 Impurity profiling of diminazene aceturate

A solution of diminazene aceturate at a concentration of 300µg/ml was prepared by dissolving 30mg of the substance in 100ml of water. The injection volume of 20µl of the solution resulted in 6µg of diminazene aceturate being placed on the column for HPLC and LC-MS analyses. The HPLC chromatogram of a representative injection is shown in figure 2.6.

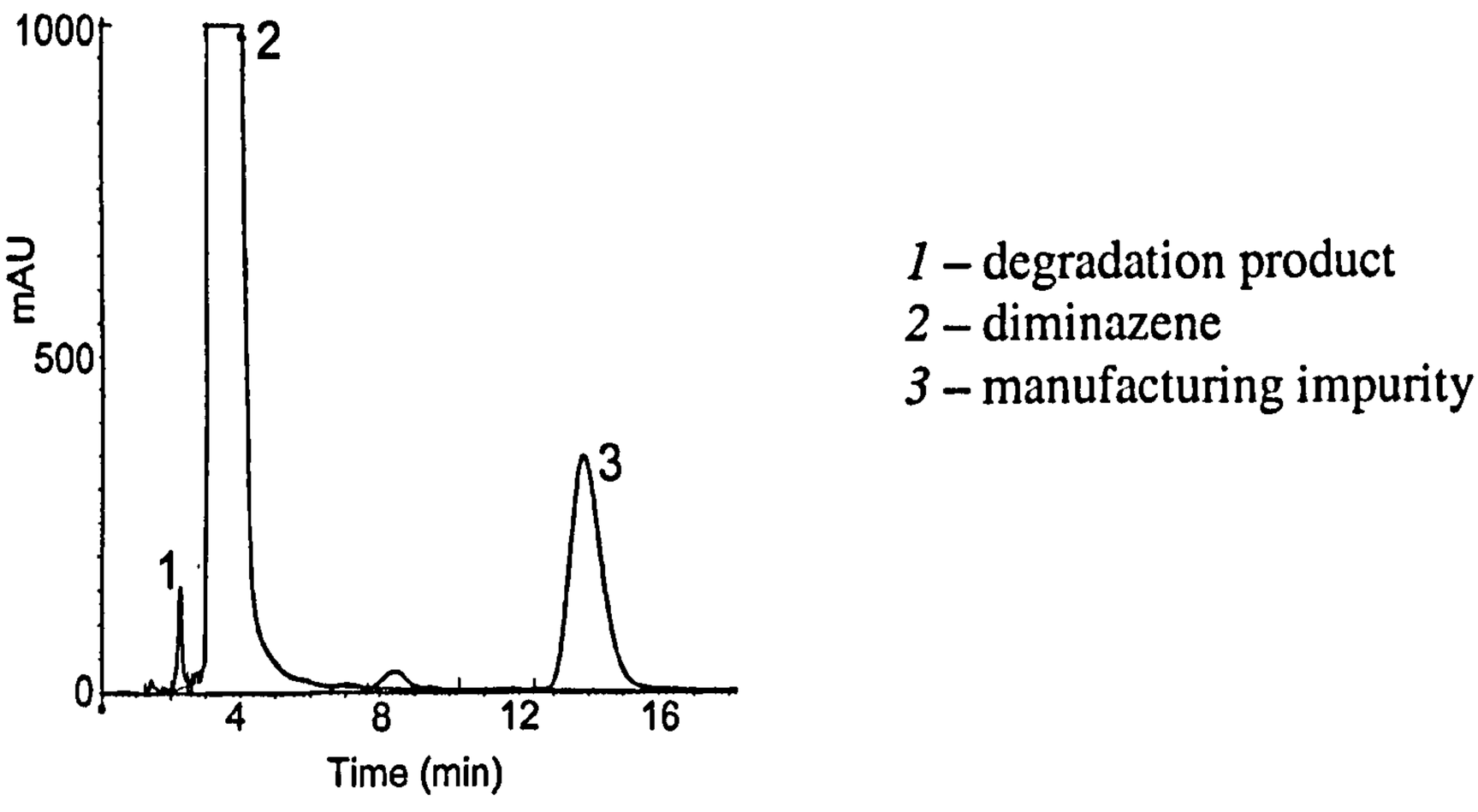


Figure 2.6 HPLC–UV chromatogram of a concentrated solution (300µg/ml) of diminazene showing the peaks due to the manufacturing impurity and degradation product; Mobile phase: Acetonitrile (MeCN): methanol (MeOH): Ammonium formate buffer (pH4.0, 20mM) (10:10:80 v/v/v) at a flow rate of 0.7mlmin^{-1} and UV detection at 254nm

2.2.4.1 Identification of related substances of diminazene

Electrospray ionization mass spectrometry

Mass spectral identification of diminazene and its related substances was carried out with a Thermoquest Automass LC/MS instrument equipped with an electrospray ionization interface and a quadrupole mass analyzer. The mobile phase consisted of a mixture of acetonitrile-methanol-ammonium formate buffer (20mM, pH 4.0), 10:10:80 v/v/v, which was delivered at 0.7ml/min, and nebulized by nitrogen gas at 480°C into an electrospray mass analyzer operated in the positive ion mode with a cone voltage of 18V and corona needle voltage at 2180 V. Thermoquest software, X-calibur was used for data acquisition.

2.2.4.2 Synthesis of major manufacturing impurity of diminazene.

1- (4´-Amidino phenyl) 3-(4-carbamoyl phenyl)-triazene

The major manufacturing impurity of diminazene, 1- (4 amidino phenyl) 3-(4 carbamoyl phenyl)-triazene was synthesized by refluxing a mixture of diminazene diaceturate tetrahydrate (300mg) and anhydrous sodium carbonate (50mg) in 50ml of a mixture of methanol/water 1:1 (v/v) for 2 hours over a steam water bath. The product (impurity) was isolated by preparative thin layer chromatography (mobile phase: n-propanol-toluene-ammonia solution, 3:1:1 v/v/v) (diminazene R_f *0.0*; impurity R_f *0.31*). The silica in the region of the product in the chromatogram (R_f 0.31) was removed and extracted with methanol. The suspension was then centrifuged at 3500*g* (10min) to sediment the silica. The supernatant was removed and evaporated to dryness in a stream of nitrogen gas to yield the product.

The structural identity of the product (figure 2.7) was established by infrared (IR) and nuclear magnetic resonance (NMR) spectroscopy.

Instrumentation: FTIR spectra were obtained with a MATTSON 5000 FTIR spectrophotometer and ^{1}H and ^{13}C-NMR spectra with a Bruker AMX 400 spectrometer.

NMR data

^{1}H-NMR (400MHz, CD_3OD, δ Hz) (Appendix 1A) Assignment of chemical shifts were; 7.94 (2H, m, J=8.8 Hz: H-3,5), 7.58 (2H brd, J=8.5 Hz: H-2,6), 7.86 (2H, dt, J=8.9 Hz: H-3',5'), 7.63 (2H, brd, J=8.6 Hz: H-2',6'); ^{13}C-NMR (100MHz, CD_3OD, δ *ppm*-exact assignment from HMBC spectrum) 171.1 (C=O, C-7), 167.9 (C=NH, C-7'), 151.6 (C–NH–N, C-1'), 150.8 (C–N=N, C-1), 132.3 (C-4), 124.5 (C-4'), 130.3 (C-3, 5), 130.8 (C-3', 5'), 117.9 (C-2', 6'), 119.5 (C-2,6).

LC/+ESI-MS data: m/z 283 $[M+H]^+$ base peak (Figure 2.10D), m/z 255 $[M+H-N_2]^+$, m/z 324 $[M+H+CH_3CN]^+$ acetonitrile adduct.

Conditions: Positive electrospray ionisation mode with a cone voltage of 18V

FTIR spectroscopy

Comparison of the KBr spectra of diminazene and the impurity (appendix 1B) shows the distinctive C=O stretching band at 1650cm^{-1} of the impurity.

(KBr): υ = 3600-2800 (NH), 1650 (C=O) cm^{-1}

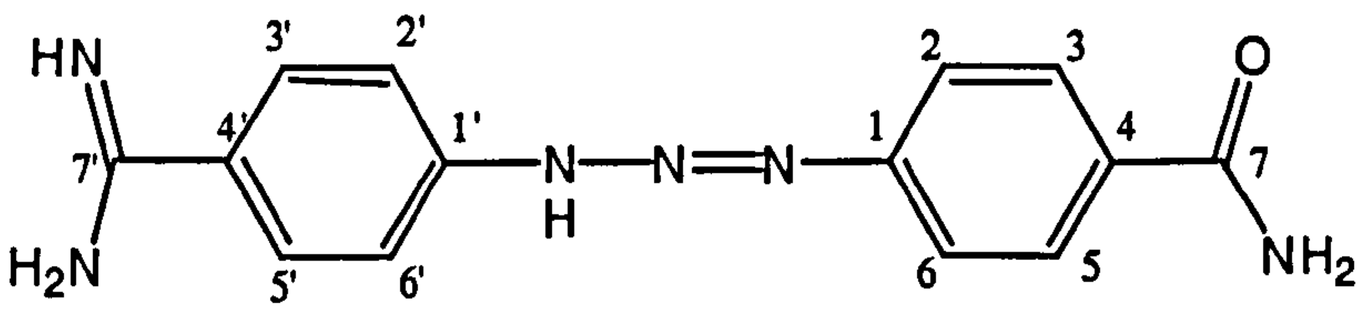


Figure 2.7 Chemical structure of the major impurity {1- (4′-amidino phenyl)-3-(4-carbamoyl phenyl)-triazene} of diminazene

2.2.4.3 Co-elution of related substances of diminazene with spiked reference standards

A simple means of peak identification is to analyse a sample spiked with a reference standard. For identification to be confirmed, the spiked peak area is expected to be greater, but not wider at half height than the original peak. This technique was employed to confirm the identity of the related substances of diminazene (figure 2.8).

The deduced structure of the major manufacturing impurity of diminazene in this study is consistent and analogous to that of pentamidine published in the monograph of the British Pharmacopoeia (B.P. 2000)

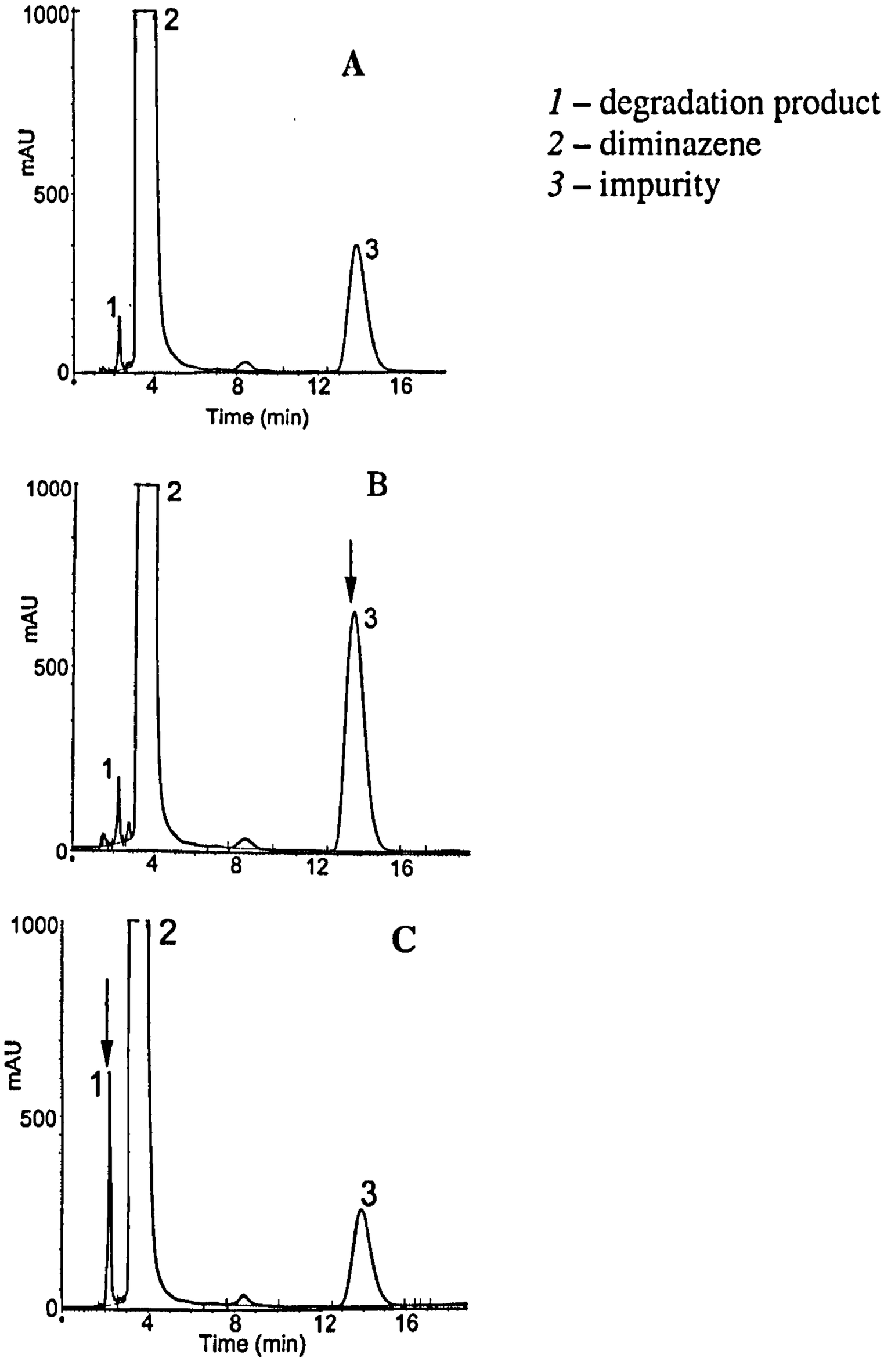


Figure 2.8 HPLC chromatograms of a spiked solution of diminazene A) and after the addition of the synthetic reference impurity B), the reference degradation product C); Mobile phase: (MeCN): (MeOH): $HCOONH_4$ buffer (pH4.0, 20mM) (10:10:80 v/v/v) at a flow rate of 0.7mlmin^{-1} with UV detection at 254nm

Spiking a solution of diminazene aceturate (300µg/ml) (figure 2.8A) with a solution of the synthesized impurity (0.5mg/ml) resulted in an increase in the peak height of the peak due to the corresponding impurity in the solution of diminazene (figure 2.8B). Similarly, the reference standard of the degradant product (*p*-amino benzamidine) (0.2mg/ml), coeluted with the degradation present in the solution of diminazene (Figure 2.8C).

2.2.5 Preparation of standard calibration solutions

Exactly 50mg of diminazene aceturate and antipyrine were accurately weighed, and transferred together into a 100ml volumetric flask and made up to the mark with water to produce the stock solution S_0 containing equal concentrations (500µg/ml) of both antipyrine and diminazene aceturate. Volumes (4ml and 20ml) of the stock solution (S_0, 500µg/ml) were transferred with glass bulb pipettes into volumetric flasks, 20ml and 50ml respectively and made up to the mark with distilled water to produce solutions S_1 (100µg/ml) and S_2 (200µg/ml). A separate volume (4ml) of solution S_1 was pipetted into a volumetric flask (20ml) and diluted to the mark with distilled water to produce a calibration solution (20µg/ml). Similarly, specific volumes (4, 6 and 8ml) of solution S_2 were carefully transferred into three separate volumetric flasks (20ml) and diluted to the mark with water to produce calibration solutions 40, 60 and 80µg/ml. Aliquots of these standard calibration solutions (20, 40,60, 80 and 100µg/ml) were transferred into glass vials and analysed with an on-line HPLC autosampler.

2.2.6 Method validation

2.2.6.1 Stability of solutions of diminazene and antipyrine

In order to demonstrate the stability of solutions of diminazene and antipyrine during analysis, a standard solution consisting of a mixture of diminazene aceturate (50µg/ml) and antipyrine (50µg/ml) was prepared in water and aliquots analyzed at specific time intervals: 2, 4, 6, 8, 24 and 48 hours at room temperature (22°C).

2.2.6.2 Specificity and robustness

The specificity of the method was confirmed by comparing the peak purity index values of the chromatographic peaks due to diminazene and antipyrine obtained from injections of standard solutions with those obtained from injections of generic samples of diminazene aceturate. Standard solutions of diminazene aceturate and antipyrine (50µg/ml, see section 2.2.5) were also spiked with solutions of the degradation product and the synthesized impurity of diminazene and the peak purity index values compared. This was to ensure that the peaks due to the analyte were homogenous and that there was no interference from degradation products, impurities of diminazene and any other components. The peak purity index was determined by using the online Chromquest workstation software (version 2.51, 1999). Robustness was assessed by determining the retention times of diminazene and antipyrine when two slightly modified mobile phases were used. Modifications of mobile phases were the following:

A: MeCN: MeOH: $HCOONH_4$ buffer (pH4, 20mM) (8: 8: 84 v/v/v)

B: MeCN: MeOH: $HCOONH_4$ buffer (pH4, 20mM) (12: 12: 76 v/v/v)

2.2.6.3 Precision and linearity of method

Precision of the method was determined by the analysis of five replicates (n=5) of standard solution mixtures of diminazene and antipyrine at three concentrations: 20, 50 and 100µg/ml. To determine intra-day precision of the method, replicates (n=5) of the three standard solutions were analyzed and for inter-day precision, replicates (n=5) of freshly prepared standard solutions were analysed on three different days. The relative standard deviations of the analyte peak areas were determined.

The linearity of detector response for diminazene and antipyrine was determined by evaluation of regression statistics of five calibration curves acquired from replicate (n=3) analysis of five different sets of calibration solutions of analytes prepared at concentrations of 20, 40, 60, 80 and 100µg/ml.

2.2.6.4 Limits of detection and quantification

The limit of detection was established by determining the concentration of a dilute solution of diminazene that gave a signal to noise ratio of 3:1. The limit of quantification was determined as the lowest concentration of diminazene which had an imprecision value of not more than 3% (RSD) for triplicate (n=3) injections.

2.3 Results and Discussion

Successful resolution of diminazene, antipyrine (excipient) and its related substances (Figure 2.6 and 2.9) was achieved with a base deactivated stationary phase column, Lichrospher®60 RP (C8) select-B. The presence of "free silanol" groups in conventional silica based reversed phase columns, which occurs mainly as a result of incomplete end-capping, can give rise to residual interactions with highly basic analytes in chromatographic separations. This may result in tailed peaks, which could markedly impair quantification.

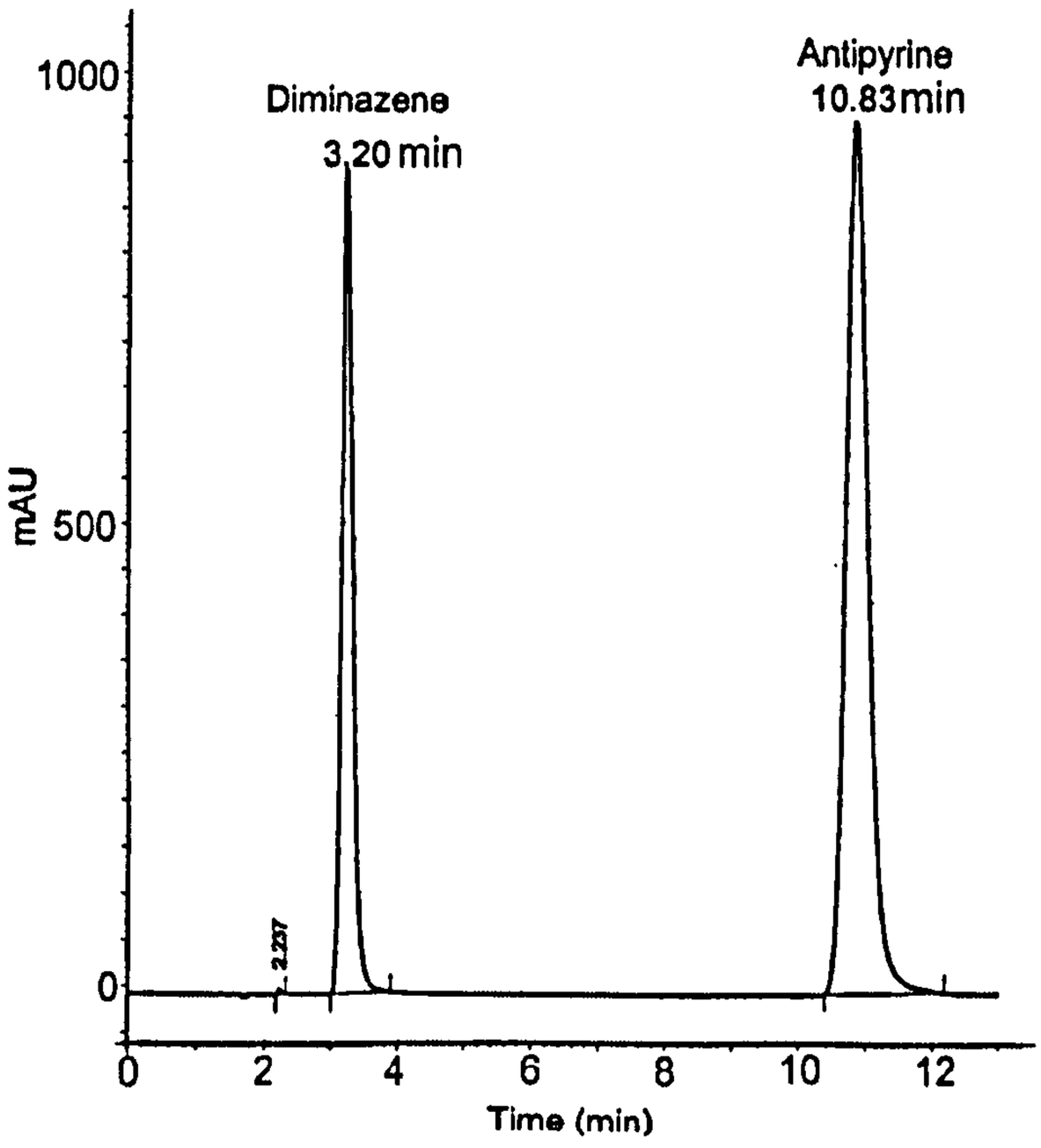


Figure 2.9 HPLC chromatogram of diminazene aceturate and antipyrine monitored at 254nm; Mobile phase: MeCN: MeOH: $HCOONH_4$ buffer (pH4, 20mM) (10: 10: 80 v/v/v); flow rate: 0.7mlmin^{-1}.

However, the pretreatment of stationary phase silica (base deactivation) reduces the number of active silanol groups producing relatively sharper peaks for basic analytes. Lichrospher®-60 RP C_8 (5μ particle size), which was used in this study gave a good peak shape for diminazene aceturate and antipyrine (Figure 2.9) (Asymmetry factor at one-tenth peak height ≅ 1.10 for diminazene). The pH of the mobile phase was maintained at 4.0 in order to further suppress silanol group ionization and reduce residual interactions with the basic amidino moieties of diminazene. In order to

arrive at an optimum mobile phase composition for separation, a series of trial injections of standard samples were carried out with three different solvent mixtures (MeOH:MeCN:$HCOONH_4$); (i) 30:25:45v/v/v; (ii) 20:15:65v/v/v; (iii) 10:5:85v/v/v. The mobile phase composition was subsequently adjusted until the least retained peak (degradation product of diminazene) eluted with a retention time >2.0 min. The presence of ammonium formate buffer in the mobile phase provided competing ammonium ions that helped to improve the peak shape of diminazene. The average retention times of antipyrine, diminazene and its related substances is shown in Table 2.1.

Table 2.1 Retention times of antipyrine, diminazene and related substances

Compound	Degradation product	diminazene	antipyrine	Manufacturing impurity
*Retention time (min)	2.26	3.30	10.62	12.74

** Values are an average of ten determinations (n = 10)*

2.3.1 LC-MS identification of related substances of diminazene

Electrospray ionization (ESI) is a soft ionization technique providing a sensitive means of analysis of a wide range of polar molecules. However, it generates multiply charged ions and can result in the formation of adducts with components of the mobile phase. The selected ion chromatogram (fig. 2.10A) of a concentrated solution of diminazene (300µg/ml), shows three peaks of ions monitored at *m/z* 136, 282 and 283 due to quasi molecular ions $[M+H]^+$ of a degradation product (RT: 2.32 min), diminazene (RT:3.54 min) and an impurity (RT:13.28 min) respectively. The product ion spectrum of the degradation product (Figure 2.10B) has peaks at *m/z* 177 and *m/z* 136 due to the formation of an acetonitrile adduct ion ($[M+H+CH_3CN]^+$ and a protonated molecular ion $[M+H]^+$ respectively. The product ion spectra of diminazene and its impurity (Figure 2.10C and 2.10D) has quasi-molecular ions (base peak) at *m/z* 282 and 283 for diminazene and its major impurity respectively. The distinctive ions at *m/z* 182 and 183 in the ion spectrum of diminazene (Figure 2.10C) are due to the formation of acetonitrile adducts of the doubly charged ions of

diminazene $[(M)^{2+}+CH_3CN]$ and $[(M)^{2+}+H+CH_3CN]$. The absence of an ion at *m/z* 184, in the ion spectrum of the impurity is an indication of the absence of doubly charged ions and is consistent with the structure of the impurity being 1- (4 amidino phenyl) 3-(4 carbamoyl phenyl)-triazene (Figure 2.7).

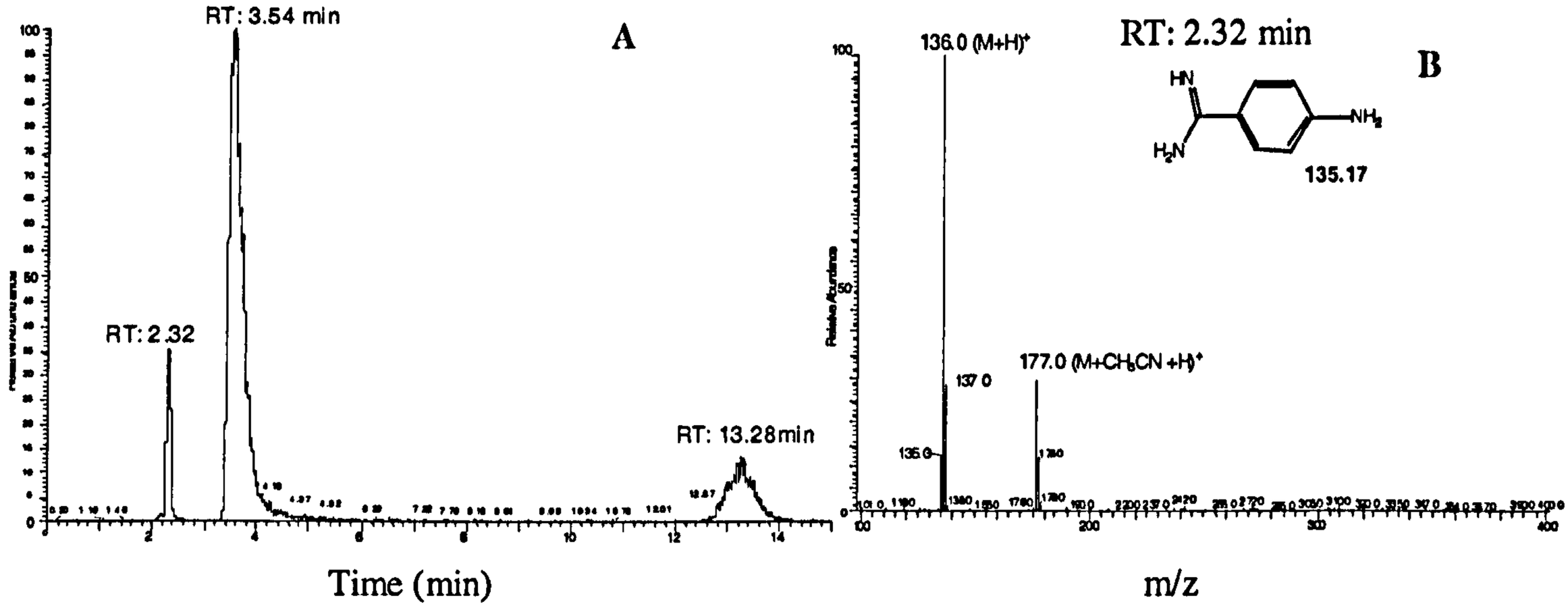


Figure 2.10 Selected ion mass chromatogram of a solution (300μg/ml) of diminazene aceturate monitored at m/z 136, 182, 283 A) and B) product ion spectra of the degradation product, C) diminazene and D) impurity. [Mass spectrometer operated in +ESI mode with cone voltage of 18V and N_2 gas temperature of 480°C]

2.3.2 Stability of diminazene during analysis

The stability of diminazene and antipyrine in solution during analysis (section 2.2.6.1) was determined in order to evaluate the stability indication of the assay. The results (table 2.2) indicate that diminazene is stable during analysis and that degradation in solution is marked after 24 hours storage at room temperature (22°C). Cleavage of the triazene bridge of the diminazene molecule in acidic solution readily occurs and this may be due to an attack on a triazene nitrogen by hydronium ions (H_3O^+) producing *p*-aminobenzamidine as the major degradation product (Figure 2.10B).

Table 2.2 Stability of diminazene and antipyrine in solution.

Time (Hours)	Diminazene	Antipyrine
	50µg/ml (n = 3)[a]	50µg/ml (n = 3)[a]
0	100	100
2	99.2 ± 0.44	99.98 ± 0.03
4	100.1 ± 0.58	99.98 ± 0.16
6	98.29 ± 0.52	100.04 ± 0.13
8	97.98 ± 0.65	100.13 ± 0.22
24	94.55 ± 0.60	100.10 ± 0.25
48	91.28 ± 1.65	99.79 ± 0.12

[a] *Values are % of initial concentration (mean ± S.D) (100% at t = 0)*

2.3.3 Specificity and robustness

Peak purity analysis is designed to detect the presence of an impurity peak that is coeluting with the analyte peak. A photodiode array detector is used to determine peak purity by determining spectral homogeneity across a given peak. Algorithms are used to analyze each spectrum at several points across a peak and compared to the apex spectrum which is used as an internal reference point. If there are spectral

differences then it implies that there are two or more compounds producing that chromatographic peak. A peak purity index value of 1.00 is indicative of the highest spectral homogeneity. The peak purity index values of diminazene and antipyrine peaks in chromatograms of all the standard and generic sample solutions were above 0.997 indicating that there was no interference from impurities, degradation products or excipients thus providing evidence of the selectivity of the HPLC method. The chromatographic peaks of the degradation product and manufacturing impurity of diminazene (figure 2.6) were clearly resolved from diminazene. When compared to retention times of analytes in the developed method (diminazene RT: 3.3 ± 0.89, antipyrine RT: 10.62 ± 0.75; mean ± S.D., n=5), small changes in the mobile phase composition (section 2.2.6.2) did not produce any significant changes in retention times of analytes (Mobile phase A: diminazene RT: 3.61 ± 0.67, antipyrine RT: 10.9 ± 0.48; Mobile phase B: diminazene RT: 2.9 ± 0.77, antipyrine RT: 10.1 ± 0.45; mean ± S.D., n=5), thus demonstrating the robustness of the method.

2.3.4 Linearity and precision

There was a rectilinear relationship between detector response and concentration over a concentration range of 0 to 100 µg/ml for diminazene aceturate and antipyrine (Table 2.3), which encompassed a nominal sample assay value for both analytes. This would ensure that determination of analytes in formulation products of diminazene can be performed by using a single reference standard.

Table 2.3 Regression statistics for diminazene and antipyrine

Analyte	Range (µg/ml)	n	Slope	Intercept	Correlation coefficient (r^2)
Diminazene	0 – 100	5	196025 ± 986	454924 ± 88128	0.9996 ± 0.001
Antipyrine	0 - 100	5	439045 ± 482	317556 ± 41565	0.9999 ± 0.001

Slope, Intercept: mean ± S.E.; Correlation coefficient (r^2): mean ± S.D

Values for the imprecision of the assay, calculated as relative standard deviation (RSD) of five replicate measurements were within acceptable limits (Table 2.4) for

intra-day and inter-day assay variability and therefore no internal standards are required for assays. This demonstrated the ruggedness of the method, and its suitability for use in routine analysis (quality control) of diminazene aceturate in the bulk substance as well as in generic formulations.

Table 2.4. Intra- and inter-day assay imprecision for diminazene aceturate

Concentration (µg/ml)	Imprecision (%RSD)	
	Intra-day	Inter-day
20	1.6	2.3
50	0.4	1.1
100	0.2	1.2

RSD: relative standard deviation

2.3.5 Limits of detection and quantitation

The limit of detection (LOD) of diminazene (based on three times the average noise level) was 50ng/ml, while the limit of quantification (LOQ) was 10µg/ml (RSD < 3%, n = 3 injections). At LOQ values less than 10µg/ml, the RSD of injections were > 5% , which was outside the expected limits of deviation. Consequently, the high value of LOQ relative to LOD was based on experimental determination to allow for accurate determination of diminazene at low concentrations.

2.4 Summary

The developed method has been demonstrated to be specific, precise and accurate for the determination of diminazene aceturate in the presence of its related substances and excipient (antipyrine). The major manufacturing impurity [1- (4′-amidino phenyl)-3-(4-carbamoyl phenyl)-triazene] and degradation product (*p*-aminobenzamidine) of diminazene have been separated by HPLC and identified by electrospray ionization mass spectrometry.

CHAPTER 3

QUALITY ASSESSMENT OF GENERIC FORMULATIONS OF DIMINAZENE OBTAINED FROM SUB-SAHARAN AFRICA

QUALITY ASSESSMENT OF GENERIC FORMULATIONS OF DIMINAZENE IN SUB-SAHARAN AFRICA

3.1 Introduction

3.1.1 Quality assurance of pharmaceutical products in Sub-Saharan Africa

A comprehensive system of quality assurance is founded on a reliable structure for licensing, independent analysis of finished product as well as an assurance that all manufacturing operations are carried out in conformity with accepted norms referred to as good manufacturing practice (GMP) (WHO, 1997). In issuing pharmaceutical product licenses, national drug regulatory authorities aim to establish the detailed composition and formulation of the product, the pharmacopoeial or other officially recognized specifications of its ingredients and its clinical interchangeability in the case of multisource (generic) products. To be effective, a mandatory system of registration and licensing of products, manufacturers, importing agents and distributors must be put in place. However the achievement of these objectives is subject to the availability of independent quality control laboratories, operating to standards that will ensure credibility of their evaluation results. The capacity of a laboratory to undertake complete analyses of pharmaceutical products (raw materials and dosage forms) is dependent on the availability of modern analytical equipment and qualified manpower with the necessary technical competence. Unfortunately, several countries in Africa lack the technical, financial and human resources required for the operation of independent laboratories. Consequently such countries are particularly vulnerable to the influx of substandard drugs. Counterfeit drugs are those that mimic the authentic drugs; Substandard drugs are those produced with little or no attention to GMP.

Although the extent of the proliferation of counterfeit and substandard drugs in developing countries is not known, there are documented cases of such fraudulent activities. It has been reported (WHO drug information, 1995) that during the meningitis epidemic in Niger (February to May 1995), the country received a donation of Pasteur Merieux and SmithKline Beecham vaccines from Nigeria. A

Medicines Sans Frontieres (MSF) team working with local health authorities noticed, that the vaccines from Nigeria had an unusual appearance with black filaments in the solution. Investigations revealed that the batch numbers and expiry dates did not correspond to the manufacturing records of the purported supplier, Pasteur Merieux. Tests carried out found no traces of active product, confirming they were false. Some of the false vaccines were subsequently located and destroyed. Such production would have necessitated an industrial-scale production facility, which indicates the possible existence of organized illegal circuits set up to manufacture "copies" with known trademarks. In another recent study, which assessed the quality of different preparations of antimalarial and antibacterial drugs obtained from retail outlets in Nigeria and Thailand, investigators reported that an appreciable proportion (36.5%) of the samples were substandard with respect to pharmacopoeial limits (Shakoor et al., 1997). The trade in fake and substandard drugs is also reported to be a common phenomenon in India, which is rated as the worlds` largest producer (35%) of all fake drugs (Chatterjee, 2002). The fake medicines are reportedly made in the northern states of the country and are circulated beyond the borders of India. There is evidence that fake analgesics are being manufactured in northern India, with falsely marked packaging to portray they were made in Russia (Chatterjee, 2002).

In places where adequate laboratory facilities are available, the necessary pharmacopoeial or official specifications may be non existent as is the case for a veterinary trypanocide like isometamidium hydrochloride (Samorin® and Veridium®). Over three decades since it was first introduced on the market, there are no pharmacopoeial specifications for isometamidium in official compendia. The work of Tettey *et al* (1999) demonstrated for the first time, that the quality of isometamidium available in international commerce is compromised. It is however not immediately clear if this problem extends to all other available trypanocides (i.e. human and veterinary). For diminazene, the official specifications outlined in the British Veterinary Codex (1965) has not been reviewed for over three decades. In developing countries, the proliferation of unregulated drug distribution channels (importers, retailers, chemical and pharmacy shops) accompanied by price

differentials due to scarcity, creates an incentive for drug diversion within and between established channels. There is however inadequate information about the scale of trypanocide counterfeiting in international commerce.

Diminazene aceturate (Berenil®), has been selected as a paradigm for investigating the incidence of substandard trypanocides because of it being widely used as a veterinary trypanocide. Moreover, the substitution of the innovator product (Berenil®) with several generic versions as well as the incidence of drug resistance to veterinary trypanocides (Peregrine, 1994) justifies the need for the quality assessment of generics of diminazene on a wider scale.

3.1.2 Experimental design

The study was designed to investigate the validity of manufacturers' label claims on the content of diminazene and antipyrine in generic formulations. The influence of supply and distribution channels such as private pharmacies, veterinary clinics, unregulated open market and government supply systems on the quality of diminazene products has been investigated. The area covered for the sampling of diminazene was designed to provide data representative of sub-Saharan Africa.

Figure 3.1 Geographical distribution of eleven participating countries in the study

3.1.3 Collection of samples

Samples of diminazene formulations from eleven participating countries in Africa (Figure 3.1) were dispatched to the animal production and health division of the Food and Agricultural Orgnisation (FAO) of the United Nations, Rome, Italy via the FAO regional office in Accra, Ghana. They were subsequently forwarded to the University of Strathclyde (Glasgow) for analysis. On arrival, the samples were weighed, transferred from sachets into bottles and assigned code numbers by laboratory technicians. This was to ensure that the analyses were performed as blind assays.

3.2 Methods

3.2.1 Sample preparation and analyses

3.2.1.1 Reference standards

Diminazene aceturate (99.6% purity) obtained from Intervet GmbH, Germany and antipyrine (99.8%) from the Medicines Testing Laboratory in Edinburgh U.K., were used as external standards.

3.2.2 Preparation of solutions

3.2.2.1 Standard solutions

The standard solutions were prepared as previously described in section 2.2.5. Diminazene aceturate (50mg) and antipyrine (50mg) were accurately weighed and transferred together to a 100ml volumetric flask and made to the mark with distilled water. This stock solution was serially diluted (using glass bulb pipettes) in water to give calibration standards containing 20, 40, 50, 60 and 80μg/ml of both diminazene and antipyrine. Portions of these solutions were subsequently transferred into autosampler vials and loaded into the liquid chromatographic system.

3.2.2.2 Preparation of generic samples

A weight (112mg) of the contents of a sachet (stated content of diminazene aceturate and antipyrine 1.05g/2.36g granules and 1.31g/2.36g granules respectively) was weighed accurately into a volumetric flask (class *A*) and made up to the mark with water. An aliquot (5ml) of the sample solution was diluted to 50ml with water to produce a solution containing a nominal concentration of 50.0μg/ml diminazene aceturate and 62.2 μg/ml antipyrine. A sample of this solution was transferred into vials and injected into the LC system by an autosampler.

3.2.3 HPLC analyses

The analyses of the generic samples were performed using a liquid chromatographic system fitted with a photodiode array detector and a Spectra System AS1000 autosampler (ThermoSeparations Inc., CA, U.S.A). Analytes were separated on a Lichrospher®-60 RP-select B analytical column (125 x 4mm i.d., 5μ Hewlett Packard, Germany) with a mobile phase composed of acetonitrile-methanol-ammonium formate buffer (20mM, pH 4) (10:10:80v/v/v) at a flow rate of 0.7ml/min as described previously in section 2.2.3.

3.3 Results and Discussion

The survey revealed the presence of at least seventeen different generic products of diminazene in sub-Saharan Africa (Table 3.1).

Table 3.1 Multisource formulations of diminazene showing manufacturing source and availability in eleven participating countries.

Product	Manufacturer	Country of manufacture	Availability	No. of samples
Berenil	Hoechst	Germany, Ireland, India	8/11	20
Diamyl	Meprodex Inc.	Canada	4/11	10
Diminal	Eagle chemical Co.	Nigeria	1/11	1
Diminaphen	Phenix Pharmaceuticals	Belgium	3/11	4
Diminasan	Alfasan	Holland	3/11	10
Diminaveto	VMD nv/sa	Belgium	3/11	3
Diminaze	Pantex Holland	Holland	1/11	1
Diminazen	Farvet Bladel	Holland	3/11	4
Diminazene	EAF	France	1/11	1
Diminazene	Kyron Laboratories (PTY)	Not stated	1/11	2
Nortryp	Norbrook laboratories	U.K. (NI)	1/11	1
Nozomill	Kepro BV	Holland	1/11	1
Samorenil	Alfasan BV	Holland	1/11	1
Sangavet	Vetoquinol Veterinary Pharm.	France	4/11	10
Trypamyl	ALM International	France	2/11	6
Trypan	Champharma Arznemeittel	Germany	1/11	1
Trypazen	Virbac Laboratories	France	2/11	6
Veriben	Sanofi Sante Animale/ Ceva Sante Animale	France	7/11	19
Veriben solution	Ceva Sante Animale	France	1/11	2

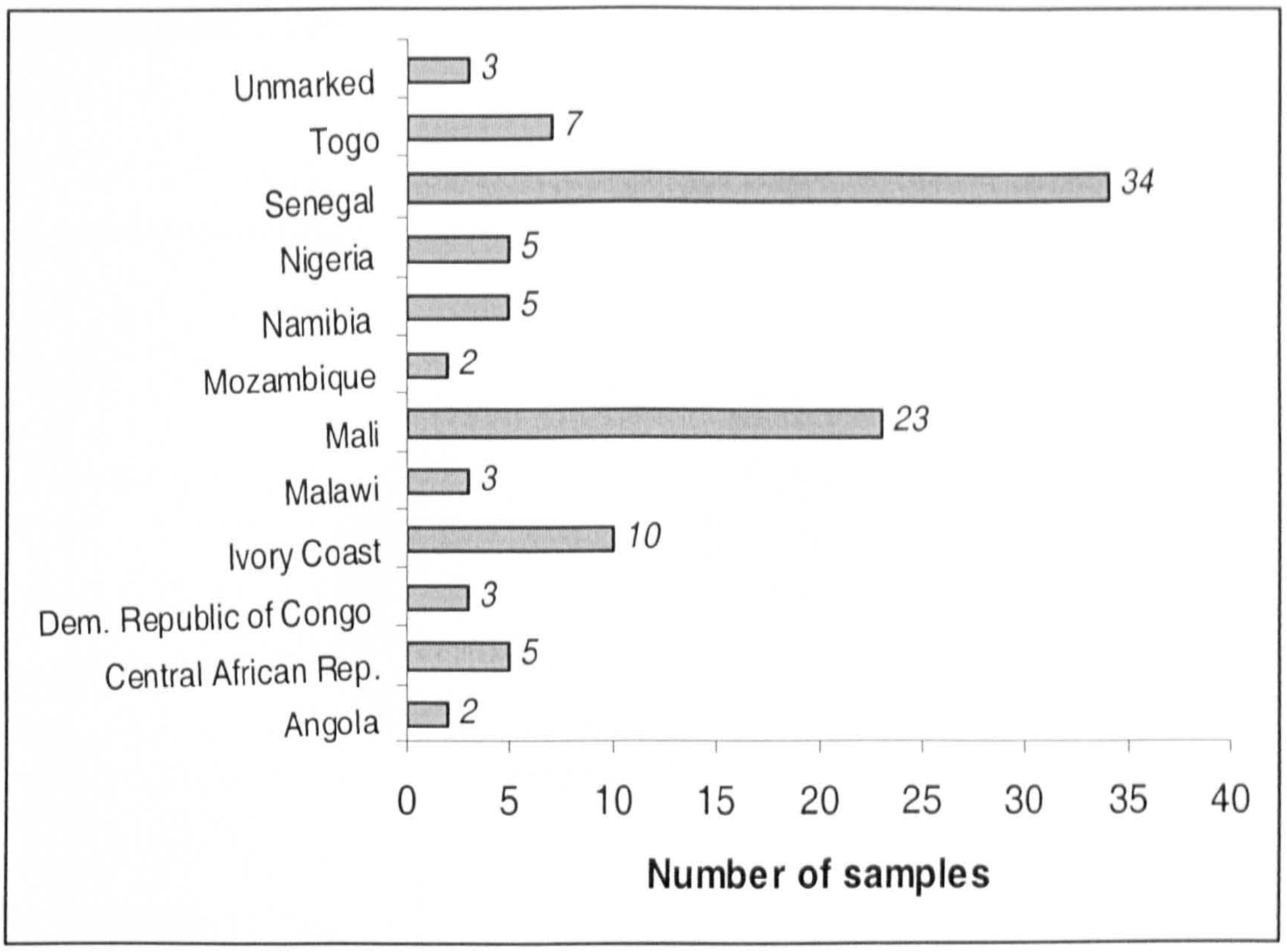


Figure 3.2 Distribution of samples provided by participating countries

The sampling in Mali, the second highest in the study (figure 3.2) was done at three different levels and this allowed an assessment of the influence of supply sources such as private pharmacies, veterinary clinics, unregulated open markets and governmental supply systems (e.g ministry of agriculture) on the quality of the diminazene products.

3.3.1 Quality of diminazene preparations

A total of 102 samples of diminazene preparations were analysed and the content of diminazene aceturate and antipyrine was expressed as a percentage of the label claim (Appendix II). The pass criterion was set at ±10% of the label claim in accordance with specifications for diminazene in the British Veterinary Codex (1965).

The date of expiry was not indicated on the individual sachets of five products.

The results obtained from the quality analysis show that approximately one out of every three samples of diminazene (24%) fell outside the ±10% of the manufacturers' label claim (figure 3.3). Of greater concern is the fact that 75% of

samples outside the ±10% tolerance window contained less than 90% of the label claim.

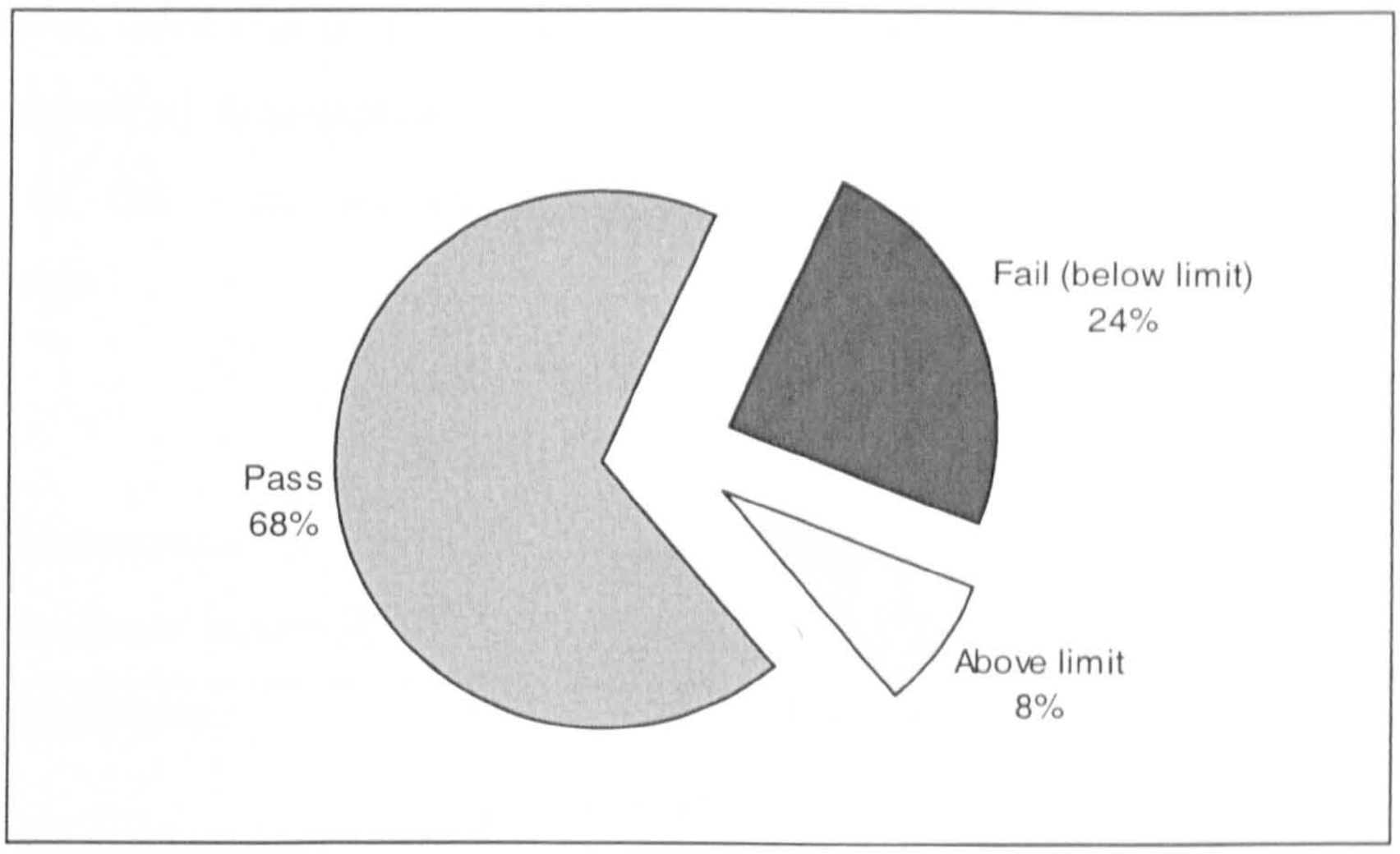


Figure 3.3 Analysis of multisource formulations of diminazene aceturate showing the percentage of samples within (pass), below (fail) and above the tolerance limits (90-110%).

Since diminazene is usually administered as a single dose (3.5mg or 7.0mg/kg body weight), treatment of animals with poor quality formulations could result in therapeutic failure and may have implications on the emergence of diminazene resistant trypanosomes. When the content of diminazene in formulations from the different supply channels were compared (table 3.2), there were no statistically significant differences ($P > 0.05$, Mann-Whitney non-parametric test). Arguably, these results may not provide a true representation of the situation in some affected countries due to the relatively small number of samples provided for the study. Besides, it takes much effort and perseverance on the part of surveillance officers to locate and obtain products from the "black markets" in some affected countries.

The levels of the major manufacturing impurity of diminazene, estimated as percentage of peak area ratio of impurity peak and diminazene peak area, was less than 0.45% in generic samples showing detectable peaks of impurity. Since the

maximum level of impurity in the analysed samples was below 0.5%, their contents in individual samples were not reported. Representative HPLC-UV profiles showing differences in the levels of impurity in three samples is shown in figure 3.4. There were differences in the physical appearance of analysed samples as shown in figure 3.5. However, there was no correlation between the physical appearance of granules and the content of diminazene aceturate or antipyrine. These observations provide evidence of the lack of regard to good manufacturing practice by some manufacturers.

Table 3.2 Comparison of quality of products of diminazene analysed based on the source of material

Source of products	**Content of diminazene (% of label claim) ***	**Number of samples (n)**
Govermental sources	99 ± 8	18
Private Veterinary clinics	95 ± 10	12
Pharmacy/Chemical shops	98 ± 9	56
Market	96 ± 8	10

* Values are (mean ± S.D) of *n* samples

In order to effectively control the quality of diminazene preparations and trypanocides in general, the following key issues have to be addressed;

- There is a need for the establishment of effective national licensing/registration of all veterinary trypanocides as well as manufacturers and distributors in the affected countries.

- Access to independent laboratory facilities is necessary for the validation of certificate of analysis of veterinary pharmaceutical products, issued by manufacturers in the exporting countries and locally.

• Pharmaceutical inspectorate departments in affected countries should be provided with adequate logistics, technical training as well as having the legal powers to "flush" out substandard drugs from unregulated distribution channels and markets.

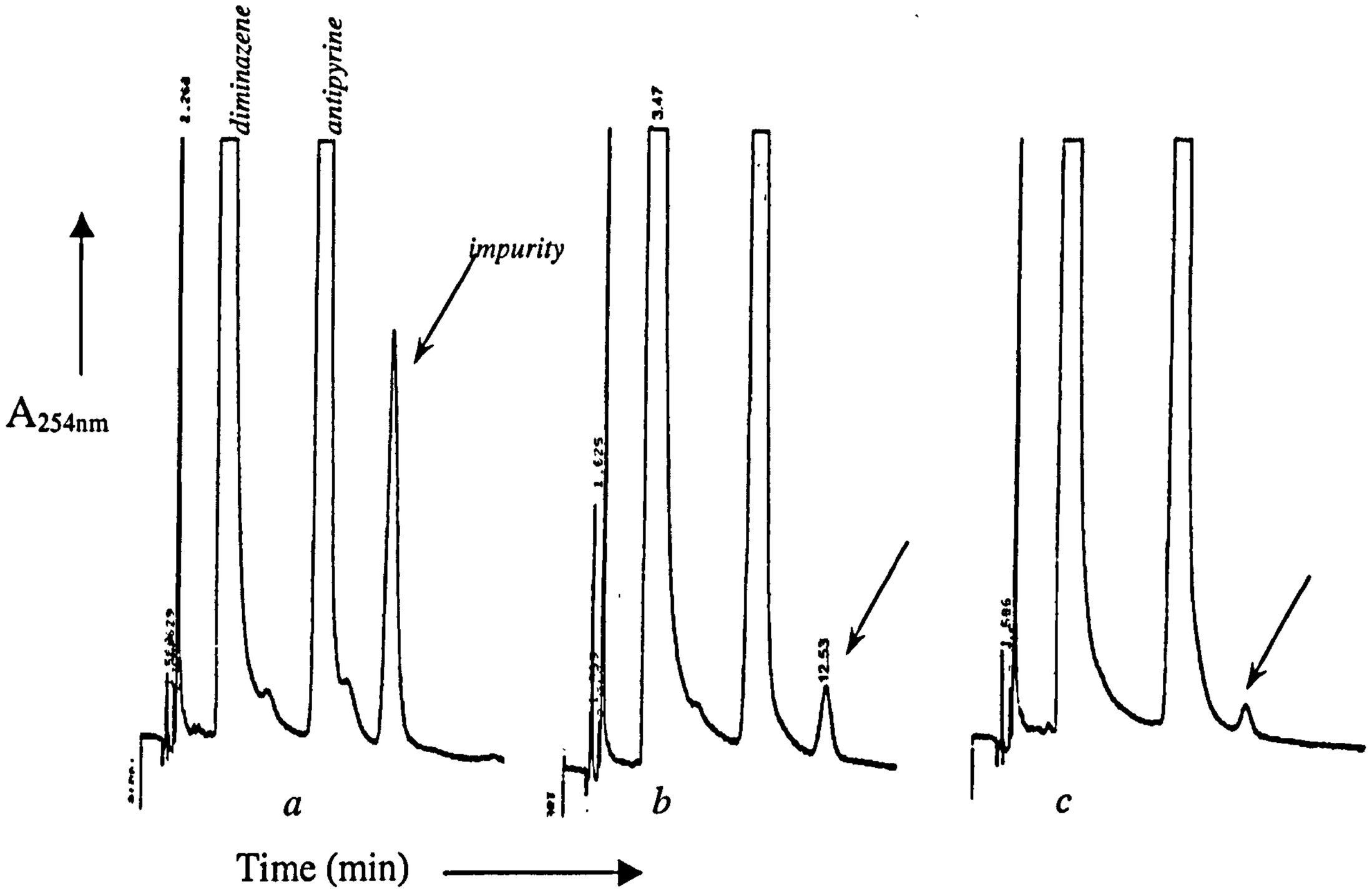


Figure 3.4 HPLC-UV profiles of three randomly selected samples of diminazene formulations (a,b,c) integrated at a low attenuation showing the difference in the levels of impurity present [1-(4-amidinophenyl)-3-(4´-carbamoyl phenyl)-triazene)]. Mobile phase; MeCN: MeOH: $COONH_4$ buffer (pH4.0, 20mM) (10:10:80 v/v/v) at a flow rate of 0.7mlmin^{-1} with UV detection at 254nm.

Figure 3.5 Three formulations of diminazene aceturate [showing differences in appearance of the dosage forms]

3.4 Summary

Nominally equivalent generic pharmaceutical products are required by international pharmaceutical regulations to contain the same amount of the same therapeutically active ingredients in similar dosage forms as the innovator product. Regrettably, the results obtained from the quality evaluation of generic formulations of diminazene aceturate in the current study clearly shows that the quality of veterinary trypanocides in international commerce may be compromised and this may have implications for the emergence of drug resistant trypanosomes.

CHAPTER 4

METABOLISM OF TRYPANOCIDAL DIAMIDINES

4.1 Introduction

In order to achieve its therapeutic objective, the concentration of a drug at the site(s) of action needs to be maintained within a narrow range (therapeutic window) for the duration of therapy. Consequently, any factor(s) that effectively alters the concentration of a drug at the site of action can result in a change of the pharmacological response to the drug (Gibson and Skett, 2001). One of the major contributors to the changes in plasma drug concentrations is drug metabolism. The main purpose of drug metabolism is that of detoxification, involving the conversion of lipophilic foreign compounds (xenobiotics) to more polar and readily excretable products (Adrien, 1987). Drug metabolism can however alter the pharmacological properties of a drug either by pharmacological deactivation or activation (toxification). A typical example of a deactivation by biotransformation is the sulfation of paracetamol to form the inactive sulphate conjugate (figure 4.1A). On the contrary, some drugs require metabolic activation in order to exert their pharmacological action and a classic example is the azo reduction of prontosil (inactive azo dye) to form the active (bacteriostatic) metabolite, sulphanilamide (figure 4.1B).

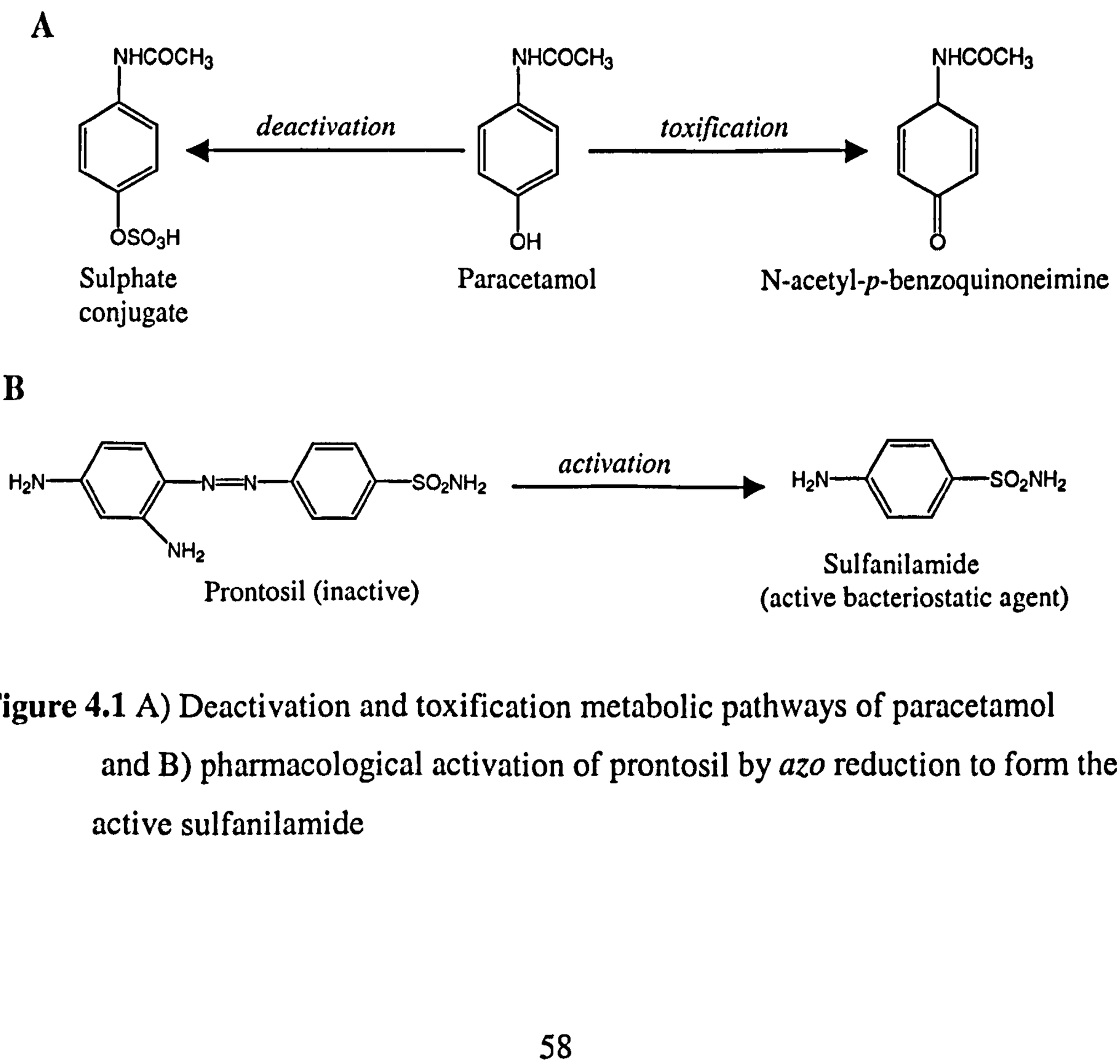


Figure 4.1 A) Deactivation and toxification metabolic pathways of paracetamol and B) pharmacological activation of prontosil by *azo* reduction to form the active sulfanilamide

Biotransformation of a drug can also lead to the formation of toxic metabolites (toxicological activation) as exemplified by the biotransformation of paracetamol to the toxic *N*-acetyl-p-benzoquinone imine (fig. 4.1A).

The metabolism of xenobiotics involves a diversity of pathways normally divided into two distinct phases; phase I (functionalization) and phase II (conjugation) reactions.

4.1.1 Phase I metabolism

Phase I reactions are functionalization reactions which include oxidation, hydrolysis and reduction. The reactions are mediated by several enzyme systems for example the microsomal cytochrome P450 dependent mixed function monooxygenases.

Cytochrome P450s are comprised of a gene superfamily (isoenzymes) with broad substrate specificities for both endogenous and exogenous substances (Gonzalez, 1988). They are terminal electron acceptors in a membrane-bound electron transport chain with reducing equivalents derived from NADPH, via the flavo protein NADPH-cytochrome P450 reductase. The reducing equivalents are used to reduce molecular oxygen, an atom of which is incorporated into the substrate as illustrated by the following reaction:

$$NADPH + H^+ + O_2 + RH \longrightarrow NADP^+ + H_2O + ROH$$

[Where RH represents an oxidizable drug substrate and ROH the hydroxylated metabolite].

The cytochrome P450s catalyse a broad variety of oxidation reactions of carcinogens and protoxicants and in many cases can be responsible for the formation of many toxic metabolites (Guengerich *et al.*, 1988), which may subsequently become substrates for phase II conjugation reactions. Cytochrome P450 proteins that exhibit at least 40% amino acid sequence identity are classified within the same gene family (families identified by Arabic numerals: 1, 2, 3, etc.), while P450s that are more than 55-60% similar are grouped within the same subfamily (subfamilies identified by capital letters: A, B, C, etc.) (Waxman and Azaroff, 1992). Individual P450 isoforms within a subfamily are numbered sequencially, e.g. Cytochrome P450 2B1, 2B2, 1A1

(abbreviated as CYP 2B1, 2B2 etc.) An important aspect of the P450 dependent monooxygenase system is its inducibility by wide range of environmental chemicals (Dubois et al., 1996; Okey, 1990). For example, the expression of CYP1A1 can be elevated 100-fold or more in liver and many extrahepatic tissues following exposure of laboratory animals to dioxin, 3-methylcholanthrene and other polycyclic aromatic hydrocarbons (Whitlock, 1990).

4.1.2 Phase II metabolism

Phase II reactions are usually considered as detoxifying (chemoprotective) reactions and involve the conjugation of electrophilic xenobiotics with endogenous co-substrates to form excretable water soluble metabolites. In the absence of conjugative reactions, electrophilic intermediate compounds (formed from phase I reactions), can react with nucleophilic groups present in DNA leading to point mutations and genetic lesions such as those that characterize carcinogenesis (Miller and Miller, 1985). Conjugative pathways include glucuronidation, sulphation, acetylation and glutathione conjugation reactions catalysed by UDP-glucuronosyltransferases, sulfotransferases, acetyltransferase and glutathione-S-transferases (GSTs) respectively (figure 4.2).

Glutathione conjugation is recognised as a major detoxification pathway because of the sequestration of reactive electrophiles in the mammalian liver. GSTs are a multi-gene family found predominantly in the cytosolic fraction of liver cells (and other tissues) where they catalyse the conjugation of the tripeptide glutathione (γ-glutamylcysteinlyglycine) to a variety of electrophilic compounds (figure 4.2B) (Waxman, 1990). Glutathione conjugates may be excreted directly in urine, or bile or further metabolised with the subsequent removal of the glutamate and glycine moieties to yield the cysteine conjugate. Their formation is thus an important biomarker of cellular toxicity.

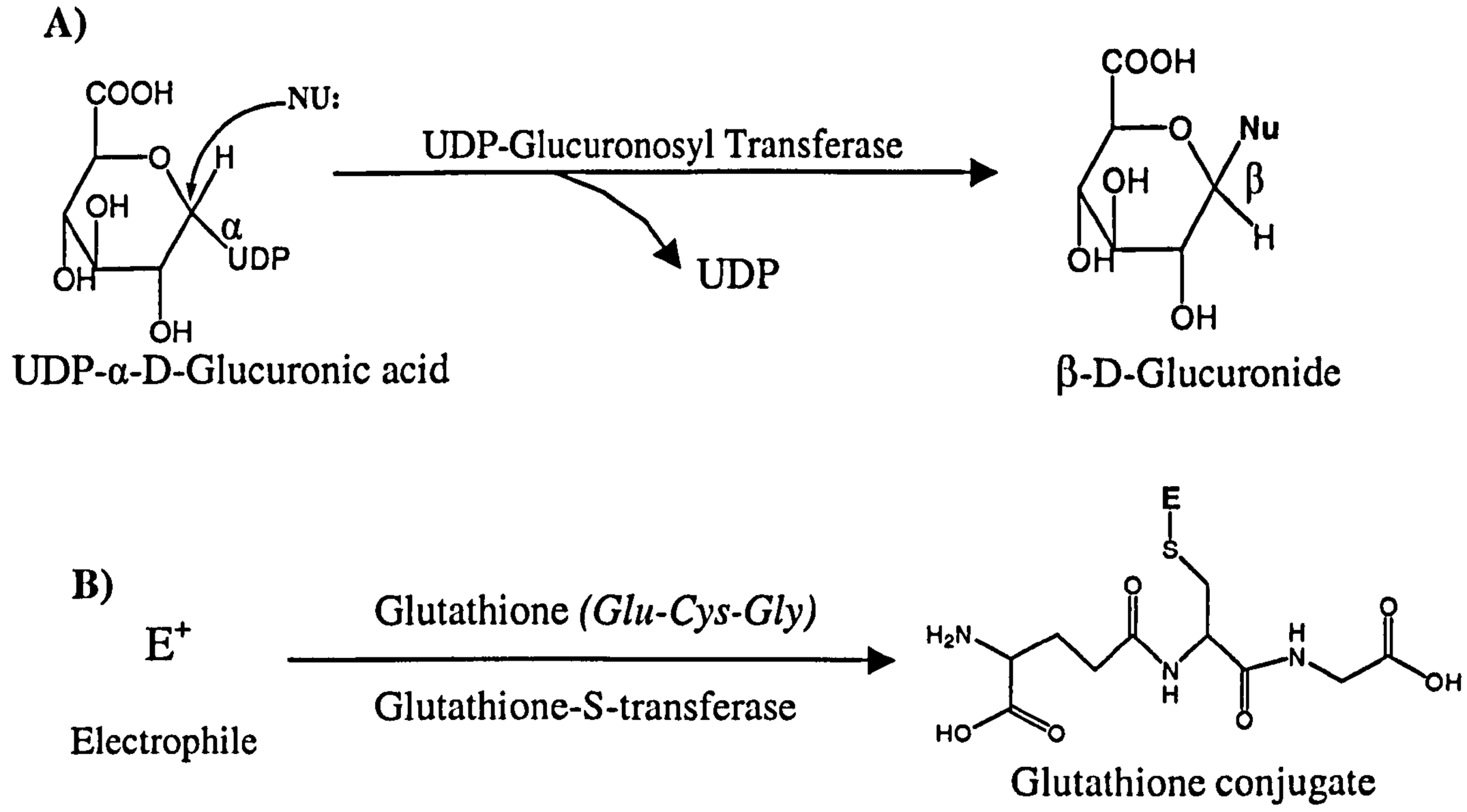


Figure 4.2 Generalized representations of A) a glucuronidation reaction and B) glutathione conjugation reaction.

Where: Nu -represents a nucleophile; E^+ represents an electrophile

4.1.3 Induction and inhibition of drug metabolism

Induction

Alteration of the concentrations of drug metabolizing enzymes in humans and animals as a result of exposure to environmental chemicals can have implications for the pharmacological activity of drugs (Gibson and Skett, 2001). The activity of microsomal and cytosolic drug metabolizing enzymes can be increased by exposure to a wide range of chemicals (inducing agents) resulting in a stimulation (increase) of enzyme activity, referred to as enzyme induction (Dubois *et al.*, 1996; Williams *et al.*, 2000). In recent times, studies on the inducibility of drug metabolising enzymes have centred on hepatic cytochrome P450 monooxygenase catalysed reactions (Porter and Coon, 1991). Induction of hepatic cytochrome P450 enzymes results in an increase of the overall size of the liver (Williams *et al.*, 2000) and there is evidence that a majority of cytochrome P450s are induced at the level of transcriptional activation (table 4.1) (Gibson and Skett, 2001). Induction of CYP1A1

and 1A2 by polycyclic aromatic hydrocarbons such as 3-methylcholanthrene (3-MC) is known to be mediated by a specific cytosolic receptor, termed the Aryl Hydrocarbon Receptor-Ah receptor *(AhR)*. The receptor is thought to combine with 3-MC and the complex formed is then translocated into the nucleus of the cell, where it subsequently interacts with the promoter region of the cytochrome P450 1A1 gene leading to elevation in the levels of the mRNA for the enzyme (Kimura *et al.*, 1986 Frotschl *et al.*, 1998). In addition to transcriptional gene activation, the post transcriptional stabilization of the mRNA appears to play a significant role in induction of CYP1A1/2 (Kimura *et al.*, 1986).

Phenobarbitone (PB) administration causes induction of CYP2B1, 2B2 and 2C6 (Waxman and Azaroff, 1992). PB-induced increase in P4502B1 and 2B2 mRNA levels is primarily due to increased transcription of the corresponding CYP2B genes. This transcriptional activation is known to be rapid and can reach a level of 20 to 50-fold higher than the basal transcription rate (Hardwick *et al.*, 1983).

Deltamethrin, a synthetic pyrethroid and other polychlorinated biphenyls (PCBs) are known to act as PB-type inducers of cytochrome P450 (Dayal *et al.*, 1999; Okey, 1990).

Table 4.1 Differences in induction mechanisms for cytochrome P450 enzymes*

P450	Inducer	Induction mechanism
1A1	Dioxin	Transcriptional activation by ligand activated Ah receptor
1A1/1A2	3-Methylcholanthrene	Transcriptional activation and mRNA stabilisation
2B1/ 2B2	Phenobarbitone	Transcriptional gene activation
2E1	Ethanol, acetone, isoniazid	Protein stabilisation (in part)
3A1	Dexamethasone	Transcriptional gene activation

* Modified from Waxman and Azaroff (1992)

Cytosolic enzymes such as GSTs are also known to be inducible (Puri and Kohli, 1995). Lindane, a widely used chlorinated insecticide, has been shown to cause a biphasic induction of cytochrome P450 and GST activity in rats (Dwivedi and Kumar, 1989; Puri and Kohli, 1995). The chemoprotective agent, oltipraz and other dithiolethiones protect against the acute toxicities of many xenobiotics and are effective inhibitors of experimental carcinogenesis. These protective effects are mediated, in part, through induction of GSTs and UDP-glucuronosyltransferase activities in the liver and other target tissues in humans (Egner et al., 1994). When induction of phase I enzymes (that produce electrophilic intermediates) is not matched by similar increases in the phase II enzymes (e.g GSTs) responsible for sequestering electrophiles, then increased toxicity may result (Williams *et al.*, 2000).

Inhibition

The inhibition of drug metabolising enzymes as a result of drug-drug interactions or exposure to xenobiotics is a well-recognised phenomenon (Langouet *et al*, 2000; Simard *et al.*, 2001; Tarrus *et al.*, 1987). Compared to enzyme induction, enzyme inhibition may pose a more serious problem since its onset is rapid (Guengerich, 1996). At the molecular level, inhibition of drug metabolism is known to occur in several ways including the destruction of pre-existing enzymes, inhibition of enzyme synthesis or by inactivation of the enzyme through the formation of complexes with the competing substrate inhibitor (Gibson and Skett, 2001). For example, a common non-specific cytochrome P450 inhibitor, SKF 525A (diethyl aminoethanol ester of diphenylpropyl acetic acid), is believed to operate by acting as an alternative substrate i.e. a competitive inhibitor (Williams *et al.*, 2000). Inhibition of drug metabolism in man may result in elevated drug plasma levels leading to toxicity and therapeutic failure. Concomitant administration of cisapride (used to increase gastro-intestinal motility) and simvastatin (for treatment of hypercholesterolaemia) in humans has been shown to cause an increased plasma concentration of cisapride with potential risk of toxicity (Simard *et al.*, 2001).

4.1.4 Effects of pesticides on drug metabolising enzymes

The term pesticides encompasses a group of chemical compounds used for the control or elimination of pests and are grouped into various classes (insecticides, fungicides, herbicides, rodenticides) based on the target organism. Insecticides include a broad class of chemical compounds including organochlorines, (DDT, lindane, aldrin, dieldrin, mirex), organophosphates (malathion, parathion, diazinon), carbamates (carbaryl, zectran, pyrolan) and synthetic pyrethroids (permethrin, deltamethrin, allethrin). Due to their lipophilic nature and slow chemical/biological degradation, pesticides, especially organochlorines, tend to be taken up by biological membranes and tissues and progress up the food chain (Ahlborg *et al.*, 1992; Safe, 1986). There is continuous debate concerning their role in various chronic effects in animals and humans which include neuro-, cyto- and geno-toxicity and enzyme induction (Chambers and Carr, 1995).

Pesticides are known to function as substrates, inhibitors and inducers of drug-metabolizing enzymes, with the same compound frequently acting in more than one of these roles (Hodgson *et al.*, 1995). The interactions of pesticides with phase I and phase II enzymes is well documented. Lindane has been implicated as a bifunctional inducer of cytochrome P450s and GSTs in rat hepatocytes (Puri and Kohli, 1995). Delescluse and collaborators (1998) have demonstrated the induction of CYP1A1 in rat hepatocytes following oral administration of carbaryl and cypermethrin (among other insecticides). Interest in CYP1A activity lies in its ability to catalyse the activation of polycyclic aromatic hydrocarbons to reactive metabolites that can interact with DNA resulting in chemical carcinogenesis. Although cypermethrin and carbaryl appeared to exhibit a 3-MC type of induction (CYP1A), there was no evidence of involvement of the specific cytosolic Ah receptor *(AhR)* in the enzyme induction. The organophosphate insecticide, fenitrothion, has been reported to alter estradiol and testosterone metabolism by inhibition of cytochrome P450 isoenzymes (Berger and Sultatos, 1997; Clos *et al.*, 1994). Inhibition of cytochrome P450 dependent metabolism of endogenous steroid hormones could lead to profound disturbances of the biochemical systems regulated by such hormones.

Synthetic pyrethroids are the newest major class of insecticides and by 1991 they accounted for 21% of worldwide insecticide use (Smith, 1991). Deltamethrin (S-α-cyano-3-phenoxybenzyl(1R,3R)-3-(2,2-dibromovinyl)-2,2-dimethyl-cyclopropane- carboxylate) is one of the most widely used pyrethroids (Smith, 1991). It has been shown to act on multiple sites in the central nervous system (Ray and Cremer, 1979), and is rapidly metabolised after oral administration before it enters the central and peripheral nervous system (Anadon et al., 1996). Its marked neurotoxicity in laboratory animals following acute and chronic exposure (Rickard and Brodie, 1985) has been attributed to its interference with nerve membrane sodium channels (Lund and Narahashi, 1983; Eells *et al.*, 1992). Marginal induction of the activity of cytochrome P450 enzymes in laboratory animals has been reported following oral administration of deltamethrin and other synthetic pyrethroids (Dayal *et al.*, 1999).

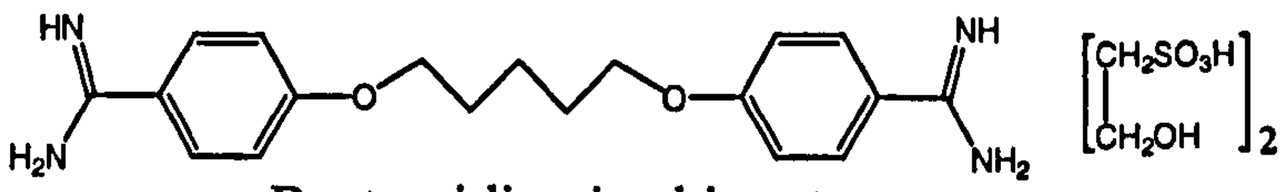
Exposure of man and animals to pesticides has been classified as being by direct and indirect routes (Matsumura and Madhukar, 1980). Direct exposure to pesticides at low or high doses can occur via oral, dermal or respiratory routes. The direct application of insecticides on cattle and livestock as pour-on or vat dipping formulations (Holmes, 1997) for the control of animal trypanosomosis provides a good illustration of the dermal route of exposure to insecticides. Indirect exposure to pesticides occurs through ingestion of pesticide residues in foods and drinking water as a result of insecticides leaching from the soil into water bodies and uptake by plants, which form the primary trophic level in the food chain (Matsumura, 1975). It has been documented that, organochlorine insecticides (DDT, aldrin, dieldrin) are readily incorporated into the milk and fat of ruminants. Wilson and Cook (1972) compared the proportion of dieldrin excreted in milk to that in faeces and urine following oral administration to cows over 6 weeks. Altogether, 42.77% of administered dieldrin was recovered; 31.6% in faeces, 8.1% in fat and 3.1% in milk. From the foregoing discussions, it is discernible that extensive use of pesticides in tsetse and mosquito eradication campaigns in endemic areas of Africa could have pharmacological and toxicological implications for drug therapy.

4.1.5 Metabolism of trypanocidal aromatic diamidines

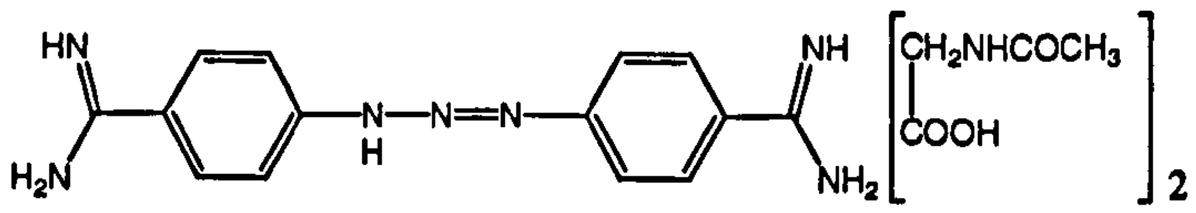
Strongly basic amidine functional groups are components of numerous active compounds (Clement, 1989a). The aromatic diamidines, pentamidine and diminazene, represent an important class of compounds, which were developed principally because of their activity towards trypanosomes (Clement, 1989b).

Pentamidine (figure 4.3), synthesized in 1939, has been used to treat haemolymphatic stage cases (stage I) of human African trypanosomosis and visceral leishmaniasis for over 50 years (Goa and Campoli-Richards, 1987; Sands *et al.*, 1985). More recently, pentamidine (isethionate salt) has become a drug of choice for the treatment of *Pneumocyctis carinii* pneumonia (PCP), one of the very frequently occurring secondary infections in patients with acquired immunodeficiency syndrome (AIDS) (Masur, 1992). Furthermore, over 45% of patients that receive the drug are known to suffer severe adverse reactions including hypo- and hyper-glycaemia (Murdock and Keystone, 1983) with possible progression to insulin dependent diabetes mellitus and both renal and hepatic toxicity (Goa and Campoli-Richards, 1987).

Diminazene aceturate (Berenil®), developed in 1955, has been used (as the aceturate salt) (figure 4.3) primarily for the treatment of veterinary trypanosomosis and babesiosis in affected areas world wide (see section 2.1) (Bauer, 1958). It has been employed successfully for treatment of early stage cases of human African sleeping sickness (Abaru and Motavu, 1981).



Pentamidine isethionate

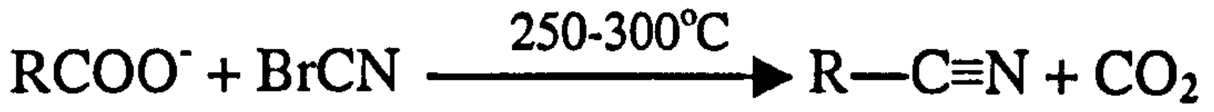


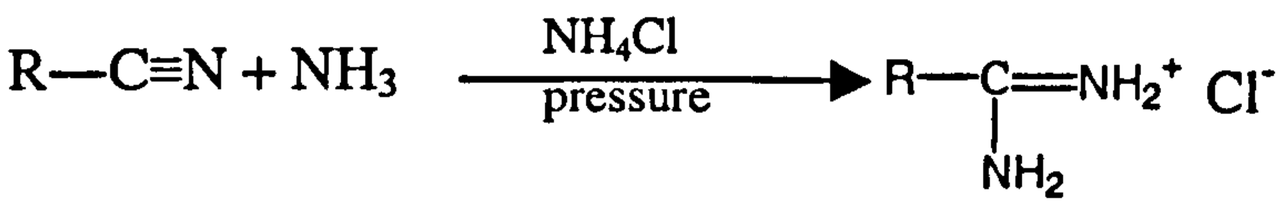
Diminazene aceturate

Figure 4.3 Chemical structures of the trypanocidal diamidines; pentamidine isethionate and diminazene aceturate

4.1.6 Chemical properties of amidines

Amidines are formally derived from carboxylic acids by substitution of the two oxygen atoms for nitrogen. Unsubstituted amidines can be prepared by the addition of ammonia or amines to nitriles as shown in the reaction equation below (March,1992);





Amidine HCl

Amidines are highly basic compounds, possessing high pKa values as a consequence of the formation of highly mesomerically stabilized cations; the pKa of benzamidine is 11.6 (Albert *et al.*, 1984). They are generally more basic than aliphatic amines and it is remarkable that though they are known to exist exclusively in the protonated form under physiological conditions, they undergo biotransformation (Clement, 1983).

4.1.7 Biotransformations of aromatic amidines

Based on earlier work by Launoy *et al* (1960), pentamidine was thought to be metabolically inert. The authors' conclusion was mainly based on the observation that no radiolabelled carbons were detectable in CO_2 gas produced by mice and rats following administration of [^{14}C] pentamidine. However, Clement (1983), used the 9000*g* supernatant of rat liver homogenate to demonstrate the conversion of benzamidine to *N*-hydroxybenzamidine (figure 4.4) following TLC-MS analysis of incubates.

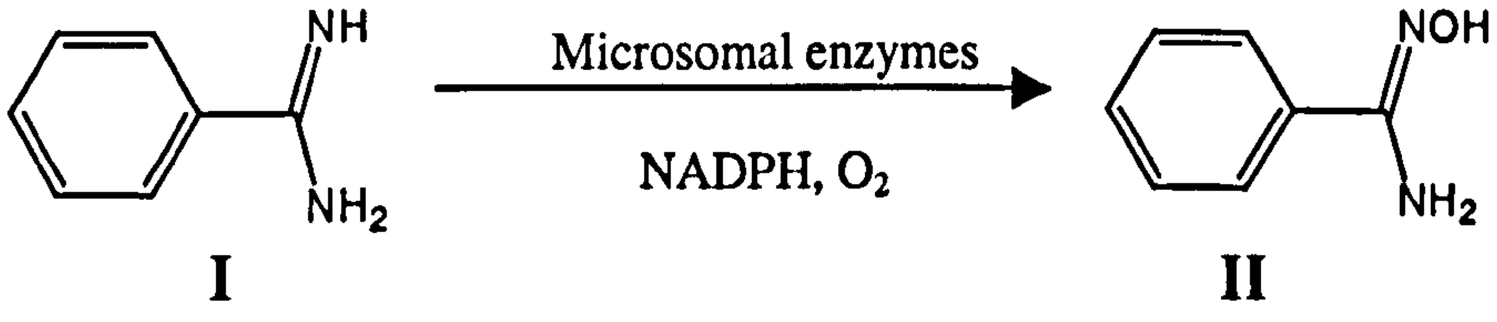


Figure 4.4 *N*-hydroxylation of *N,N'*-unsubstituted benzamidines(I) to benzamidoxime(II)

4.1.7.1 Metabolism of pentamidine

As a result of the recent development of sensitive HPLC analytical techniques, the metabolism of pentamidine has been studied by several authors and it is now a well established fact that pentamidine is extensively metabolised in hepatic microsomal preparations from rats (Berger *et al.*, 1990, 1991,1992), rabbits and humans (Clement and Jung, 1994). At least seven phase I metabolites of pentamidine have been identified in rat microsomal incubations, while two phase II metabolites of pentamidine have been recovered from bile secretions of a perfused rat liver system by Berger and collaborators (1992) (figure 4.5). Of the recovered metabolites in rat liver microsomes, compounds I and II (figure 4.5), 1,5-bis(4`-amidinophenoxy)-2-pentanol and 1,5-bis(4`-amidinophenoxy)-3-pentanol, respectively, were reported as the two major metabolites of pentamidine (Berger et al, 1992). However, *N*-hydroxypentamidine (compound III, figure 4.5) was found to be the major phase I metabolite of pentamidine in human liver microsomes (Clement and Jung, 1994), thus demonstrating species difference in the metabolism of pentamidine.

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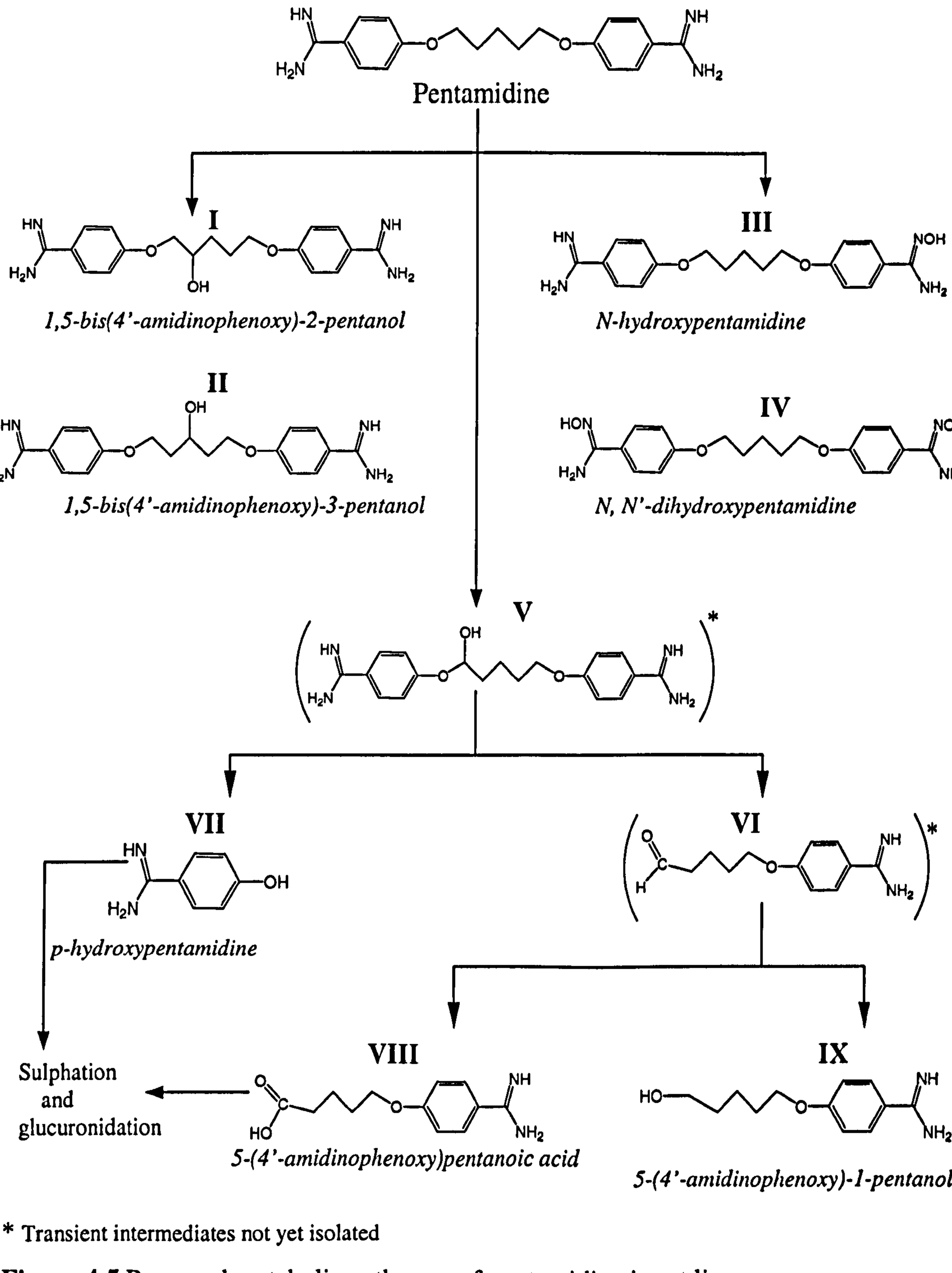


* Transient intermediates not yet isolated

Figure 4.5 Proposed metabolic pathways of pentamidine in rat liver (Berger *et al.*, 1992)

4.1.7.2 Metabolism of diminazene

Paradoxically, diminazene is not known to be metabolised to the same degree as pentamidine. Kellner and coworkers (1985), studied the pharmacokinetics and *in vivo* metabolism of [^{14}C] diminazene aceturate in two male calves. In their study, they used TLC for the detection of metabolites in urine samples and liver homogenates following intramuscular administration of radiolabelled diminazene aceturate. The homogenised livers from the calves previously dosed *in vivo* with diminazene were centrifuged (4000rpm, 15min), followed by extraction of the pellet with 50ml of methanol containing 0.2ml acetic acid. The extract was then concentrated under nitrogen gas and chromatographed using Kieselgel 60 F_{254} TLC plates. However, the authors failed to detect any metabolites of diminazene in the liver extracts or in urine samples. Although they detected traces of p-aminobenzamidine in the urine sample of one calf during the course of the study, the evidence was not convincing and could possibly be due to formation of artefacts. The fact that diminazene is unstable in aqueous solutions and degrades to form *p*-aminobenzamidine (see section 2.6.1) can present problems for studies of its metabolism. Moreover, since diminazene (like other amidines) is strongly adsorbed to biological material, the use of methanol-acetic acid mixture for its extraction from liver homogenates can result in poor recoveries. To date, only one reported study by Clement *et al.* (1992) has demonstrated the metabolic *N*-hydroxylation of diminazene which was observed in supernatants of rabbit liver homogenate (figure 4.6). The authors used *TLC-MS* analysis for identification of the metabolites (amidoximes) of diminazene following incubation of diminazene (5µmol) with the 1200*g* supernatant from rabbit liver homogenates. The freeze-dried incubation mixtures were extracted with methanol and acetone, followed by TLC-MS analysis of the reconstituted evaporated residue. One way of improving recovery of highly basic compounds in biological matrices is the introduction of a competing-ion (cation) in the extraction solvent. The extraction methodologies used by previous authors for metabolism studies of diminazene failed to address this problem.

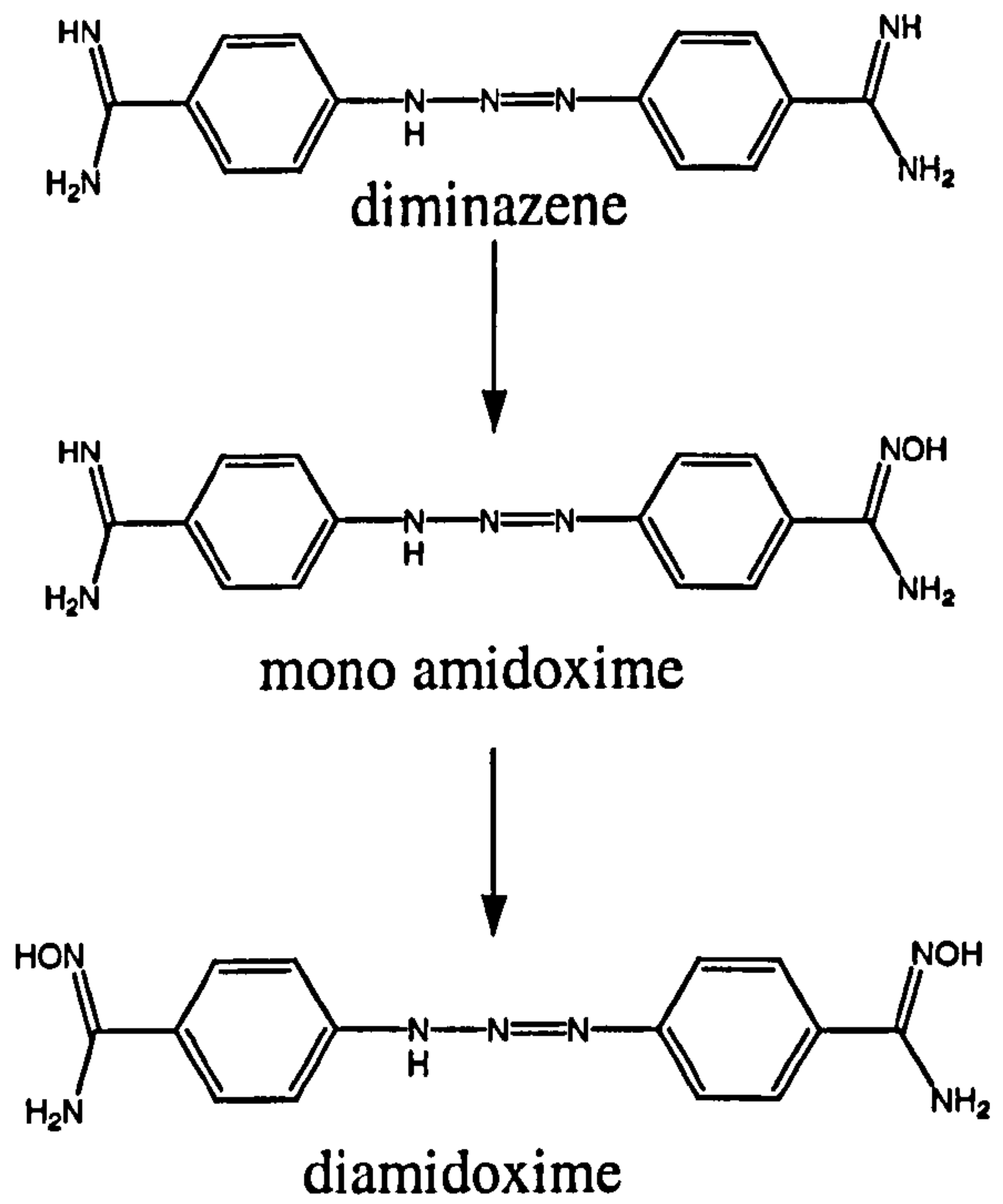


Figure 4.6 Biotransformation of diminazene *in vitro* to form the mono- and diamidoximes in supernatants of rabbit liver homogenates (Clement *et al.*, 1992)

4.1.8 Pharmacological and toxicological properties of N-hydroxylated metabolites of aromatic diamidines

It is known that several aromatic amines and certain aromatic amidines need to be metabolised by N-hydroxylation before they exhibit toxic and carcinogenic properties (Nelson, 1985) and for this reason, the pharmacological activity of *N*-hydroxylated metabolites of the trypanocidal diamidines has been of interest to researchers. The trypanocidal and leishmanicidal activities of the amidoximes of pentamidine (compounds III &IV, figure 4.5) have been investigated (Clement and Raether, 1985). The antiprotozoal activity of the synthetic analogue *of N,N'*-dihydroxypentamidine (compound IV) was tested against various trypanosome and leishmania donovani species in infected mice and golden hamsters respectively. In that study, it was established that the antiprotozoal activity (trypanocidal and Leishmanicidal potency) of *N,N'*-dihydroxypentamidine was less than that of the

parent drug (pentamidine). Similar experiments performed in infected mice indicated that the trypanocidal activity of the diamidoxime metabolite of diminazene was inferior to that of diminazene (Clement and Raether, 1992). Consequently, there has been lack of interest in the further development of amidoximes into potential trypanocides.

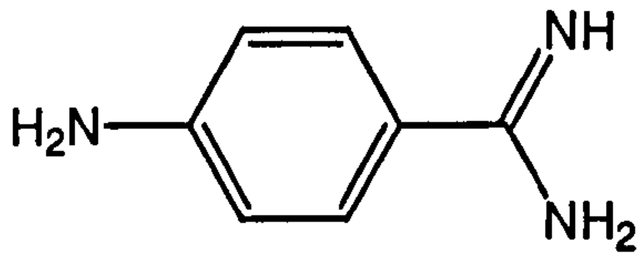
Berger and colleagues (1990), determined the relative mutagenicities of *N*-hydroxypentamidine, *N,N*'-dihydroxypentamidine and pentamidine by using the Marion and Ames test. Neither pentamidine nor the two pentamidoximes were found to be highly mutagenic. On account of the fact that benzamidoxime has been shown to be only weakly mutagenic (Clement *et al.*, 1988), it is conceivable that the oximes of diminazene may not show any mutagenicity.

4.1.9 Cytochrome P450 isoenzymes involved in metabolism of diamidines

The participation of the hepatic microsomal cytochrome P450 enzyme system in the biotransformation of amidines was demonstrated by Clement (1983). Following incubations of benzamidine with rabbit liver microsomes, in the presence of typical cytochrome P450 inhibitors such as SKF 525-A and CO, relatively small amounts of benzamidoxime was recovered. Since the microsomal biotransformation of benzamidine to benzamidoxime was not inducible by 3-MC, PB, ethanol or benzamidine itself (Clement and Zimmermann, 1987) the authors suggested that the P450 isoenzymes inducible by these inducers did not participate in the reaction. Similar investigations carried out with pentamidine and rat liver microsomes in the presence of other classical cytochrome P450 inducers, failed to induce pentamidine metabolism, although there was previous evidence that pentamidine metabolism was cytochrome P450 dependent (Berger *et al.*, 1990). Recent studies using purified enzymes, have identified the non-inducible rabbit liver cytochrome P-4502C3 to be involved in the *N*-hydroxylation of benzamidine and pentamidine (Clement *et al*, 1992; Clement and Jung, 1994). However, not all the cytochrome P450 isoenzymes involved in the biotransformation of pentamidine have been defined.

4.1.10 Para-aminobenzamidine as a model aromatic amidine in drug metabolism studies

p-Aminobenzamidine (figure 4.7) is a simple aromatic amidine that has been demonstrated to inhibit the growth of tumors in several rodent models (Jankun *et al.*, 1997). As a result of its antiproteinase activity, it has been indicated as a potential agent for overcoming the enzymatic barrier to oral administration of therapeutic peptides and proteins (Bernkop-Schnurch, 1998). There is however no information in the literature about its metabolism (either *in vivo* or *in vitro*) and since several groups of diamidine drugs possess amidine functional groups bearing an aromatic moiety, the study of *in vitro* metabolism of p-aminobenzamidine will be of interest.



p-aminobenzamidine

Figure 4.7 Chemical structure of *p*-aminobenzamidine

4.1.11 *In vitro* models for drug metabolism studies

The liver is the predominant organ of xenobiotic biotransformation and since the efficacy or toxicity of a given drug is dependent on its metabolic pathway, drug metabolism studies have centred on liver/hepatic tissue from animals and human sources (Gibson and Skett, 2001). The preferential and extensive use of *in vitro* rather than *in vivo* models in drug discovery is due to several factors including;

- Ethical issues associated with the use of whole animals and dose limitations when testing highly toxic compounds
- The demand for high throughput screening systems to cope with the huge number of drug candidates generated by combinatorial chemistry

- The high cost involved in the maintenance and use of the "whole animal" for investigation of minor metabolism issues, and
- The fact that the analysis of urine, faeces and plasma samples of the whole animal alone may fail to detect transient metabolites, which could have pharmacological as well as toxicological effects.

Though *in vitro* systems may not provide the complete *in vivo* scenario, their proper use can give reliable insight into the problems related to *in vivo* drug metabolism (Guengerich, 1996). There is a wide range of available *in vitro* models for drug metabolism studies and the choice of any model is dependent on the metabolism issues to be addressed. *In vitro* models that are commonly used in drug metabolism studies include; purified or single-enzyme systems; microsomal fractions; perfused (whole) liver; liver slices and isolated hepatocytes (Rossi and Sinz, 2002).

Single-enzyme systems

Single-enzyme systems are typically obtained from cDNA expression systems and provide an effective technique for the study of individual enzyme reactions, particularly the cytochrome P450 isoenzymes. However there are caveats about the stability of the enzyme during purification and what the overall contribution of the enzyme may be to the metabolism of the drug in the cells of the liver (Guengerich, 1996). Highly purified variants of cytochrome P4502C3 isolated from rabbit liver and expressed by recombinant *E-coli* have been identified as the microsomal pentamidine *N*-hydroxylase (Clement, 1994).

Microsomes

Microsomes are widely used subcellular fractions and they contain the majority of the oxidative (phase I) drug-metabolizing enzymes, such as the cytochrome P450 monooxygenases (Rossi and Sinz, 2002). However, they lack the regulatory controls of cofactor availability in whole cells, the natural relationship between phase I and II enzymes and the membrane regulated transport of xenobiotics observed in intact cells (Shull *et al.*, 1987).

Liver slices

Liver slices provide an *in vitro* system of higher architecture, with intact cell-cell junctions and bile canuliculi. However, they are unsatisfactory due to problems of the non-uniformity of slices and substrate/nutrient access throughout the slices (Gibson and Skett, 2001).

Perfused livers

Perfused livers have the advantage of complete preservation of organ architecture such as vasculature and bile ducts. The limitations inherent in their use lie in the fact that only a single experiment (involving a single variable) can be performed at a time (no control experiments with same liver) and the viability of the cells composing the liver is not easily determined (Rossi and Sinz, 2002).

Isolated hepatocytes

Isolated hepatocytes (cell suspensions) are a complete drug-metabolizing unit, with intact cell membranes, preserved intracellular environment (organelles and protein synthesis) and intracellular concentrations of cofactors as well as phase I and II drug metabolizing enzymes. They play an important role in xenobiotic metabolism (Begue *et al.*, 1983; Lavrijsen *et al.*, 1992, Guillouzo *et al*, 1993) and toxicity (Foxworthy *et al.*, 1990; Swales and Caldwell, 1992). They offer an ideal model for examining a wide range of experimental variables using cell preparations from only one liver and the determination of cell viability is less complicated compared to other physiological *in vitro* models (liver slices and perfused liver) (Berry and Hall, 1992).

Early attempts to isolate hepatocytes employed mechanical force and subsequently, perfusion of the liver with Ca^{2+} chelators, which produced viable cells in low yield. Since the first successful isolation of viable hepatocytes in high yield from rats by collagenase perfusion of the liver was reported by Berry and Friend (1969), the technique has been widely applied for preparation of hepatocytes from different animal species.

Isolated hepatocytes have been described as superior *in vitro* models for the prediction of *in vivo* drug clearance (Houston and Carlile, 1997) and they have many applications in drug discovery and development.

Most of the reported studies on the metabolism of trypanocidal diamidines in the literature have been obtained mainly using microsomes, with only one experiment involving metabolism studies of [^{14}C] pentamidine in a perfused rat liver system (Berger *et al,* 1992). Metabolism of diamidines in isolated rat hepatocytes have not been previously studied and since they have advantages over other *in vitro* models as outlined above, their use in the present study can be regarded as a novel approach.

4.1.12 Application of LC-MS to drug metabolism studies

The need for rapid, sensitive methodologies for the identification and quantification of drugs and their metabolites to accelerate drug discovery and development has given atmospheric pressure ionization liquid chromatography-mass spectrometry (API LC-MS) its central position in the drug metabolism field (Rossi and Sinz, 2002).

The development of atmospheric pressure ionization (API) interfaces has largely overcome the initial problems involved in coupling two seemingly incompatible techniques; a dynamic liquid chromatographic system and mass spectrometry. Undoubtedly, API is responsible for the dramatic growth and application of LC-MS in pharmaceutical analysis, proteomics, genomics and drug metabolism (Lees and Kerns, 1999; Oliveira and Watson, 2000).

4.1.12.1 Atmospheric pressure ionisation interfaces

The two main API interfaces are electrospray ionization (ESI) and atmospheric pressure chemical ionisation (APCI). In an API source, analyte ionization competes with the background matrix from the chromatographic effluent, buffer additives and sample matrix. Thus ionization of analytes is not totally efficient and results in production of a mixture of ions and neutrals (figure 4.8).

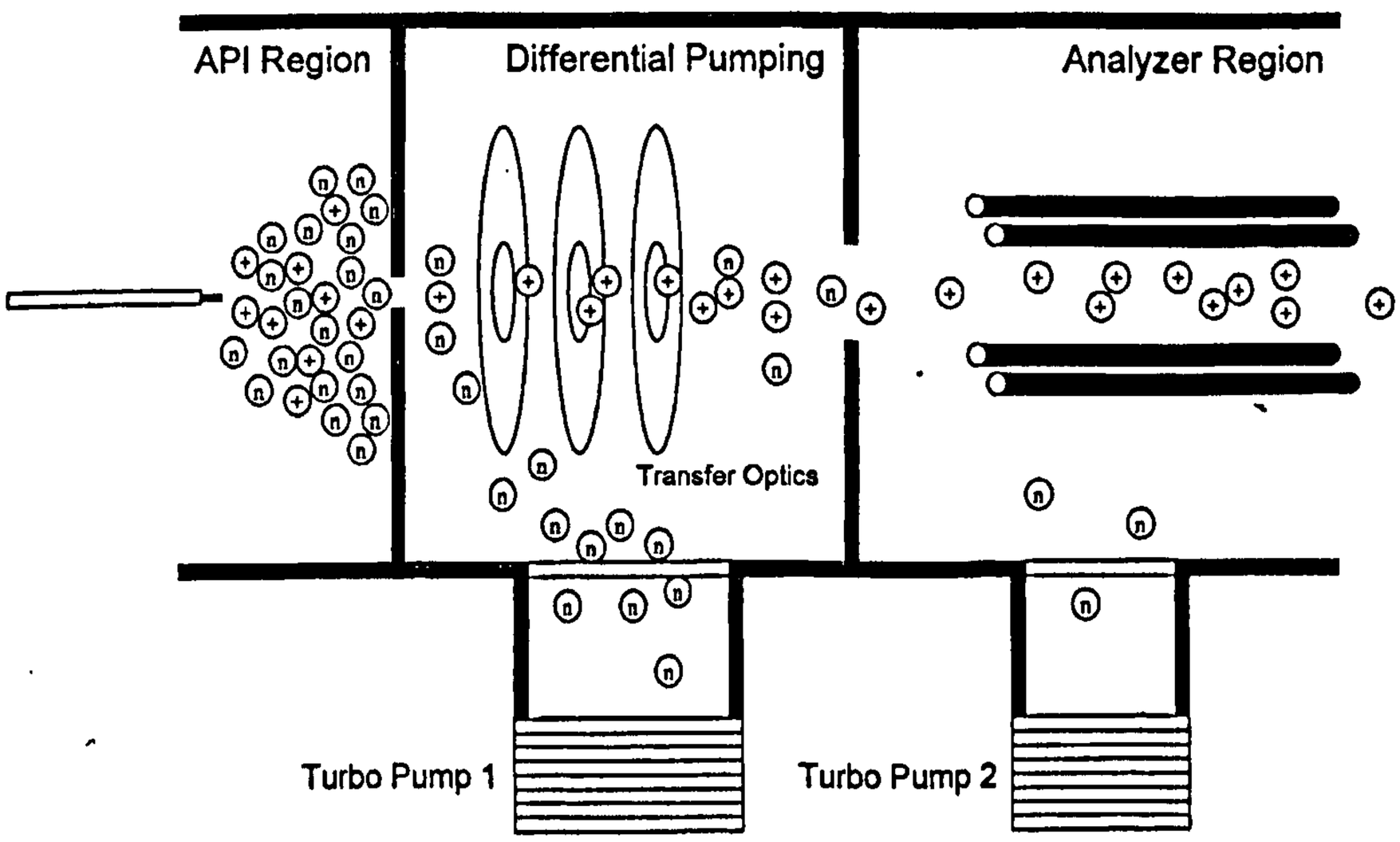


Figure 4.8 Schematic representation of a generic atmospheric pressure ionisation source showing a differential pumping system and mass analyzer,

Once ions and neutrals are introduced into the first pumping stage, ion enrichment is achieved by differential pumping and by the electronic field of the tuning lenses. The ions finally reach the quadrupole mass filter (analyzer) where they are separated according to mass to charge ratios *(m/z)*.

Electrospray ionisation

ESI is regarded as a soft ionization technique providing a sensitive means of analyzing a wide range of polar molecules. It operates by the process of emission of ions from a droplet into the gas phase, a process known as Ion Evaporation. The ion evaporation process is assisted by a concentric flow of heated nitrogen gas. A solvent

is pumped through a stainless steel insert capillary which carries a high potential, typically 3 to 5kV (figure 4.9).

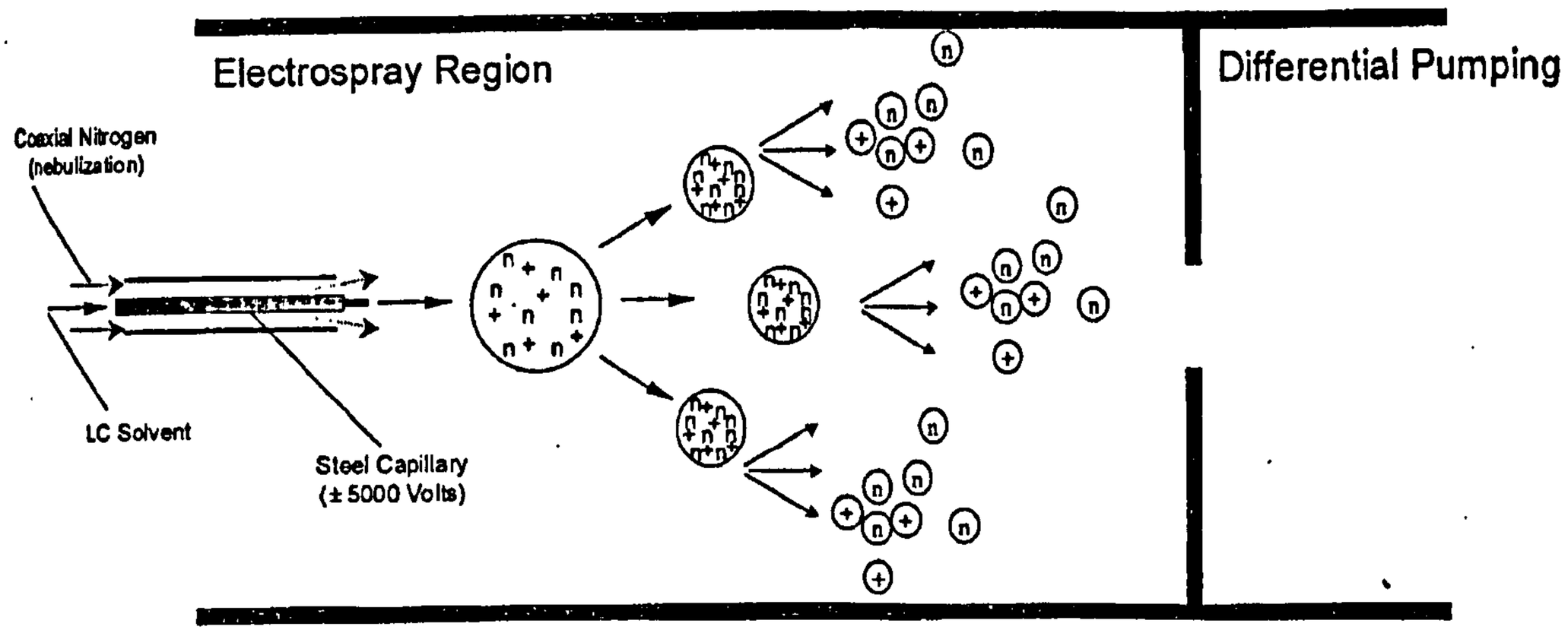


Figure 4.9 Diagram of a generic electrospray ionization source

The strong electric field generated by this potential causes the solvent to be sprayed from the end of the insert capillary as a result of electrostatic repulsion (hence electrospray) and highly charged droplets are produced. As the droplets evaporate, the ions within it undergo further desolvation assisted by electrostatic repulsion. LC flow rates for most commercial nebulization-assisted ESI sources range from 1 to 1000μl/min. A common characteristic of electrospray is the generation of multiply charged ions and the formation of adducts with components of the mobile phase. Common adducts are formed with ammonium ions NH_4^+ $[M+18]^+$ and sodium ions Na^+ $[M+23]^+$.

Atmospheric pressure chemical ionisation

APCI is a gas-phase ionization technique in which ionization of the analyte takes place by means of a chemical ionization process. The solvent vapour is initially ionized by the discharge (corona pin) (figure 4.10) and then efficiently ionizes sample molecules.

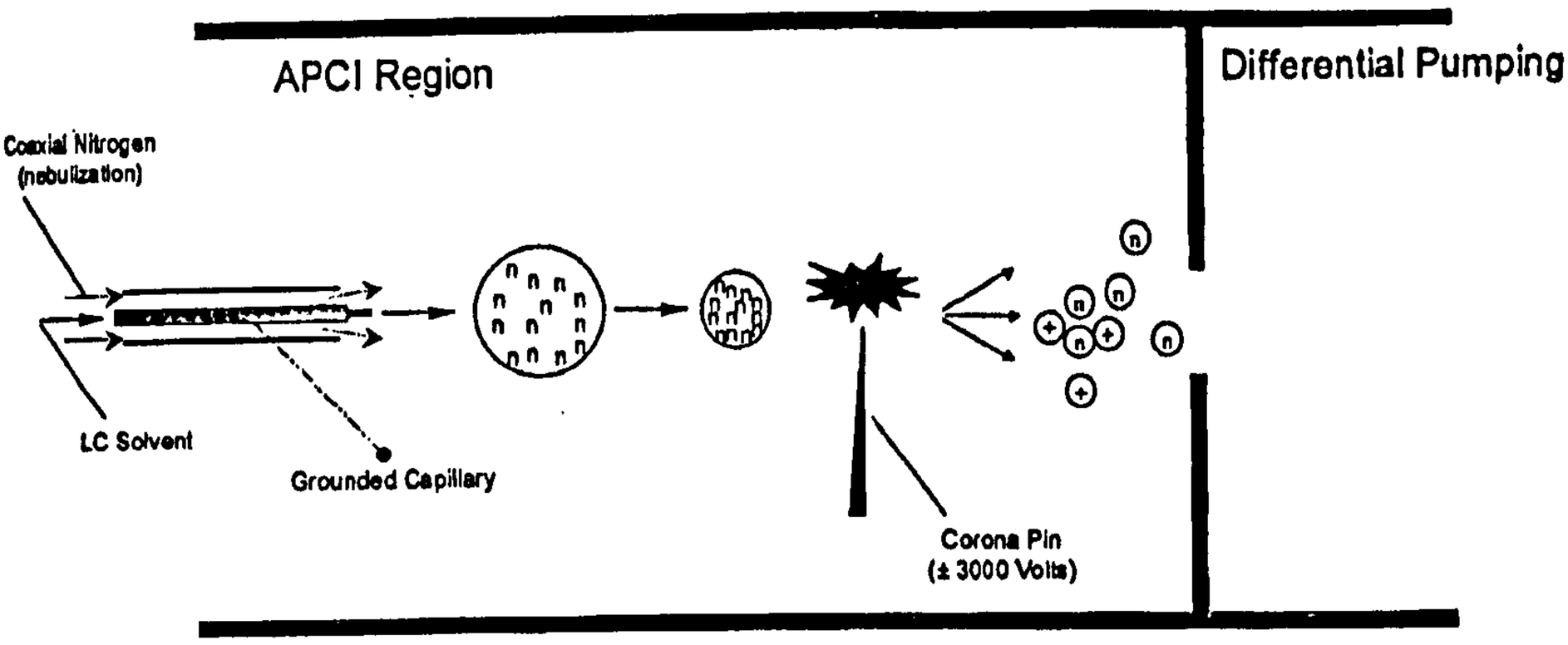


Figure 4.10 Diagrammatic representation of a generic atmospheric pressure chemical ionization source

Proton transfer from species such as $(H_3O)^+$ occurs in the positive-ion mode while reaction with solvated O_2 is predominant in the negative-ion mode. A stream of nitrogen drying gas removes most clustering solvent molecules from the analyte ions and prevents neutral solvent molecules from entering the mass analyser (Rossi and Sinz, 2002).

4.1.12.2 Detection modes

The application of mass spectrometry in drug metabolism studies is divided into two distinct areas; quantitative measurement of the amount of a metabolite formed during a specific time interval; and qualitative experiments to elucidate the structure of a metabolite. However, obtaining meaningful qualitative and quantitative information requires the application of different sets of detection techniques using the mass spectrometer. Since API interfaces generally provide soft ionisation with less fragmentation of the molecular ion, they do not provide enough structural information. The use of tandem mass spectrometry (LC-MS/MS) provides a means of inducing fragmentation in the analyte ion by the introduction of a collision cell (figure 4.11).

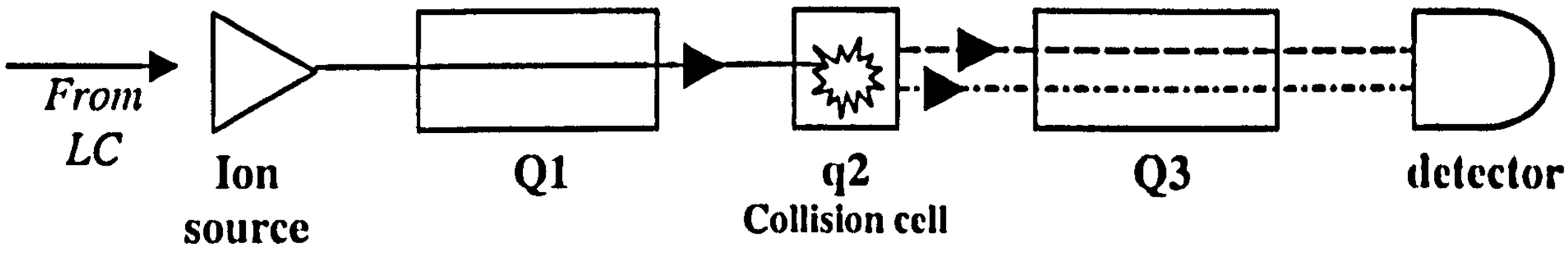


Figure 4.11 Schematic representation of a triple quadrupole MS instrument (LC- MS/MS) showing a collision cell *(q2)* between two quadrupole mass filters *(Q1, Q3)*

There are five major ion detection modes of operation for drug metabolism studies with a triple quadrupole mass analyzer;

i) Full Scan Mode

In full-scan mode of ion detection, the first quadrupole (Q1) (fig. 4.11) is operated in a linear (radio frequency-direct current RF/DC) scan mode, where a range of *m/z* values encompassing the analytes of interest is constantly monitored. In this configuration, the collision cell is evacuated while the third quadrupole (Q3) operates in an RF-only mode to provide uninhibited transmission. The quadrupole mass filter is generally inefficient at detecting ions in the full scan mode and detection limits are improved mainly by operating Q1 within a narrow scan *(m/z)* window.

ii) Selected Ion Monitoring (SIM)

SIM is a fundamental quantitative configuration. By setting the quadrupole (Q1) to pass only ions of interest through to the detector, a selective detection system is created. This reduces chemical noise leading to higher mass spectral sensitivity and selectivity. The tandem mass spectrometry analog to SIM experiments is known as multiple reaction monitoring (MRM).

iii) Product (or Daughter) Ion Scanning

Product ion scanning is a qualitative mode that can provide structural information of a given compound. The first quadrupole (Q1) acts as a mass filter to selectively

isolate a single mass/charge ion from the ion source. The selected ion undergoes fragmentations in the collision cell and is directed to the third quadrupole (Q3), which operates in a linear scanning mode to produce a mass spectrum of the fragment ions.

iv) Parent (Precursor) ion scanning

Parent ion scanning can be considered the opposite of product ion scanning. With Q1 set to scan in a linear mode, fragmentation is induced and the daughter ions are directed to Q3, which operates in SIM mode to filter out only one specific product ion for each precursor ion. The use of this detection strategy helps to ascertain the origin of a particular product ion which may be a common moiety of several parent ions. For example, given three compounds (AX, AY, AZ) with different molecular weights each of which have a common charged moiety A^+ in a Q1 scan, then by configuring Q3 as a static mass filter for ion A+, only parent ions, which produce A^+ upon fragmentation in the collision cell will be detected (table 4.2).

Table 4.2 Possible fragmentation pathways of a hypothetical homologous series of three compounds

Parent ion scanning-common product ion formed from each compound			Constant neutral loss-unique ion formed from each compound		
Precursor ion	Product ion	Neutral	Precursor ion	Product ion	Neutral
AX^+	A^+	X	AX^+	X^+	A
AY^+	A^+	Y	AY^+	Y^+	A
AZ^+	A^+	Z	AZ^+	Z^+	A

v) Constant neutral loss scanning

In constant neutral loss scanning mode, Q1 is operated in a linear scan mode within a mass range that covers the *m/z* ratios of the target analytes. Upon undergoing fragmentation in the collision cell, the ions from Q1 are directed into Q3 which

operates in a scan mode but offset from Q1 by a value that corresponds to the mass of a common neutral fragment "A" (table 4.2). In this mode all components of the sample reaching the detector share the same fragmentation pattern and it is well suited for samples where several metabolites sharing the same metabolic pathway are expected. This is particularly useful in determination of glucuronidation pathways of drug substances. If the glucuronidation of a parent drug (M^+) along with other transformations is expected, then the transition at *m/z* M^+-177 (corresponding to a loss of glucuronic acid moiety) can be monitored to identify all metabolites of this type.

4.1.13 Sample preparation

One major problem associated with the application of LC-MS techniques to analysis of drugs and their metabolites is interference from artefacts and background noise from cofactors, adducts and buffer salts that may be present in complex sample matrices (Henion and Brewer, 1998). Thus the sample preparation steps employed prior to bioanalysis by LC-MS will determine how clean the samples are and they can affect the recovery of analytes (Henion and Brewer, 1998).

Two commonly used sample preparation techniques are;

- Protein precipitation
- Solid phase extraction

4.1.13.1 Protein precipitation

Protein precipitation is essentially a phase separation process involving the addition of a protein denaturing solvent (often two parts of denaturant to one part of sample matrix, volume by volume) to a crude sample matrix containing proteins and (or) cellular components (e.g. incubation mixture of drug and cells/microsomes) (Rossi and Sinz, 2002). Protein denaturation is facilitated by vortexing and centrifugation, allowing collection of denatured protein as a pellet at the bottom of the vessel. Protein denaturing solvents commonly used include simple organic solvents such as methanol, ethanol, acetone and acetonitrile. A denaturing solvent mixture made up of

$ZnSO_4$: acetonitrile (1:1 v/v) has been reported to be effective at providing very clean supernatants (Lam and Boseli, 1986) Although protein precipitation is simple and useful as a sample preparation tool, it is ineffective at removing salts and lipids from biological matrices and these eventually end up on the HPLC column. The choice of a protein precipitating solvent system has to be weighed against the possibility of ion suppression of the analytes in the ion source of the LC-MS instrument. Acidic solvents such as trifluoroacetic acid and trichloroacetic acid are effective at denaturing and removing proteins from samples but are incompatible with LC/MS due to ion suppression (Rossi and Sinz, 2002).

Ion suppression is a phenomenon in which the extent of ionization of an analyte is decreased due to competition between analyte and matrix components within the atmospheric ion source (Constantopoulos *et al.*, 1999). Studies have shown that there is a consistent loss of analyte molecular ion signal with increased amount of added salt (Constantopoulos *et al.*, 1999). Moreover, since injection of strong solvents onto silica-based reversed phase columns can impair chromatographic separation (peak tailing and band broadening), protein precipitation may not always be appropriate.

4.1.13.2 Solid phase extraction

Solid phase extraction (SPE) is a popular and extensively used sample clean up technique (Scheurer and Moore, 1992; Fedenuik and Shand, 1998; Franke and Zeeuw, 1998). The demand for high throughput applications (combinatorial chemistry, drug screening, coupling with LC-MS) has been at the origin of the development of small volume SPE with a packed bed of 50-100mg. One of the newest formats is the 96-well SPE disc format described by Simpson and colleagues (1998). Whether off-line or on-line, SPE method development is related to the properties of the analytes of interest, the nature of matrix and the type of chromatography involved in the separation step. Understanding the nature of interactions between the analyte, matrix and sorbent is the key to the development of an efficient process with high recovery. The initial step in SPE is conditioning of the

sorbent bed. For a reversed-phase sorbent such as octadecylsilane (ODS) this is typically done with a small volume of methanol or acetonitrile followed by water or buffer. The sample may be buffered in order to control the ionization state of the analyte which partitions with the sorbent after loading. A series of sorbent wash steps are performed to in order to remove salts and matrix components while leaving the analyte unaffected. Finally an elution step using a strong solvent is applied to remove the analyte from the sorbent. This is usually followed by solvent evaporation (concentration) in a gentle stream of nitrogen and reconstitution of analyte in an appropriate buffer. The use of SPE for samples in metabolism studies can present significant challenges, particularly when the metabolites have widely different physico-chemical properties from the parent drug. It is therefore desirable to minimize the sample preparation steps when the aim is to detect metabolites (Nordin *et al*, 1997). SPE has been used for sample preparation in metabolism and bioanalytical studies of pentamidine (Yeh *et al*, 1993; Nordin *et al*, 1997). Nordin and colleagues (1997) used LC-MS for the identification of metabolites of pentamidine in rat urine. They compared the effectiveness of direct urine injection to that of injection of SPE urine samples. While the direct injection of urine led to contamination of the heated capillary in the API housing and to matrix effects (due to interference from other compounds in urine), the major problem associated with the SPE samples was that of non-reproducible recoveries of metabolites. Yeh and colleagues (1993), described a SPE method for bioanalysis of pentamidine in plasma. They demonstrated that the recovery of pentamidine from SPE-C8 columns using ammonium acetate buffer varied with the pH value of the buffer, recovery at pH 3, 4 and 5 was 90, 88 and 78% respectively.

4.2 Materials and methods

4.2.1 Materials

Diminazene aceturate (4,4`-diamidinodiazoaminobenzene diaceturate tetrahydrate) (99.6%, M.wt.: 587.6)) was a gift from Intervet, GmbH Germany. p-Aminobenzamidine HCl (98%, Molecular weight (M. wt.) = 208.09), pentamidine [1,5-di(4´-amidinophenoxy) pentane] (97%, F. wt. = 592.7) as its isethionate salt, olive oil, 3-methylcholanthrene, HEPES [N-(2-hydroxyethyl)piperazine-N´-[2-ethane sulfonic acid], heparin sodium, potassium dihydrogen phosphate, potassium chloride, phosphate buffered saline (PBS, pH 7.4), Trypan Blue in PBS (0.4%w/v) and collagenase type IV were obtained from Sigma-Aldrich Company Ltd. (Poole, Dorset, UK). Phenobarbitone sodium (BP Vet) was purchased from Rhone Merieux Ltd (Essex, England). Deltamethrin was obtained from Riedel-de Haen laborchemicalien (GmbH & Co. Germany).

HPLC grade methanol and acetonitrile were purchased form Merck Ltd., (Lutterworth, Leicester, UK). Analar grade ammonium formate ($HCOONH_4$, 97% assay), ammonium chloride (98%) and formic acid (98%) were obtained from Fischer Scientific (Loughborough, Leicestershire, UK). All other reagents were of analytical grade.

4.2.2 Methods

4.2.2.1 Preparation of solutions and buffers

p-Aminobenzamidine HCl solution (500μM)

p-Aminobenzamidine (10.4mg) was weighed into a 100ml volumetric flask, dissolved and made up to the volume with Krebs-Hepes buffer, pH 7.4, to give a 500μM stock solution. This stock solution was diluted five-fold to obtain a final incubation concentration of 100μM in rat hepatocyte suspensions.

Diminazene aceturate (500μM)

Exactly 29.36mg of diminazene aceturate was weighed into a 100ml volumetric flask, dissolved and made up to the mark with Krebs-Hepes buffer pH 7.4. The resulting stock solution (500μM) was diluted five-fold in order to obtain a concentration of 100μM diminazene aceturate in the final hepatocyte incubation mixture.

Pentamidine isethionate (500μM)

Pentamidine isethionate (29.64mg) was accurately weighed and transferred into a 100ml volumetric flask, dissolved and made up to the mark with Krebs-Hepes buffer pH 7.4 to produce a stock solution (500μM). In order to obtain a 100μM final incubation concentration, the stock solution was diluted five-fold.

3-Methylcholanthrene(3-MC) in olive oil (2.5%w/v in olive oil)

3-MC (25mg) was weighed into a vial and dissolved (by vortex) in 1ml olive oil to produce a 2.5 %w/v suspension (25mg/ml).

Deltamethrin (DM) in olive oil (2.5%w/v in olive oil)

To a vial containing 50mg deltamethrin, 2ml olive oil was added and vortexed to produce a 2.5%w/v suspension (25mg/ml)

Phenobarbitone solution (0.1%w/v)

Phenobarbitone sodium (0.5g) was accurately weighed, transferred into a volumetric flask (500ml), dissolved and made up to the mark with water.

Ammonium formate buffer (pH3, 20mM)

Ammonium formate (1.2612g) was weighed into a one litre graduated beaker and dissolved with water (~700ml). The pH of the solution was then adjusted to 3.0 with a pH meter with (few drops) a concentrated solution of formic acid (35M) while stirring with a magnetic stirrer. The resultant solution was then transferred back into a 1000ml volumetric flask and made up to the mark with distilled water.

Ammonium chloride (0.2%w/v in methanol)

To a 200ml volumetric flask containing 0.4g ammonium chloride, methanol was added to volume and the solution sonicated for 5 minutes in order to completely dissolve all crystals of NH_4Cl.

Hank`s Buffer (10x)

The following salts were weighed and dissolved together with distilled water in a 1000ml volumetric flask and stored at 4°C for later use.

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

Krebs-Henseleit Buffer (2x)

The following salt solutions were added in the order shown below into a 2 litre amber coloured bottle.

Distilled water	785ml
16.09% w/v NaCl	200ml
1.01% w/v KCl	150ml
0.22M KH_2PO_4	25ml
2.74% w/v $MgSO_4.7H_2O$	50ml
0.12M $CaCl_2.6H_2O$	100ml

This mixture of salts was bubbled with 95%O_2/ 5%CO_2 for 10min.

Carbogen gas (95%O_2/5%CO_2) was bubbled through a solution of $NaHCO_3$ (9.71g/litre distilled water) for 10minutes, and then added to the salt solution. This Krebs-Henseleit Buffer was stored at 4°C for later use.

Perfusion Solutions

Hank I Buffer (500ml)

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

$NaHCO_3$	1.05g
HEPES	1.50g
BSA (fraction V)	3.33g
EGTA [Ethanedioxy-bis(ethylamine)tetraacetate]	114mg
Distilled water	450ml
Hank's Buffer (10x)	50ml

Hank II Buffer (500ml)

The following compounds were added into a 500ml beaker and the pH of the resultant 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
Hank's Buffer (10x)	50ml

Krèbs-Albumin Buffer (KA)

The following components were mixed 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 components below were mixed 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

All four perfusion solutions were sterile filtered through a 0.22μ filter before storage at 4°C.

4.2.2.2 Trypan Blue exclusion test

The test for cell viability was based on the principle of dye exclusion by undamaged cell membranes. Hepatocytes with damaged cell membranes (dead cells) are readily stained by Trypan blue, while viable cells appear refractile under a light microscope. Trypan Blue (990μl, 0.1%w/v in phosphate buffered saline, pH 7.4) was added to the cell suspension (10μl) (100-fold dilution) and gently mixed. An aliquot of this mixture was applied onto a haemocytometer which was mounted under a light microscope Diaphot-TMD (Nippon Kogaku KK, Japan) with x10 objective lens. The

cells in nine segments of the haemocytometer were counted and the viability calculated as;

$$\text{Viability (\%)} = \frac{\text{Number of cells excluding Trypan Blue}}{\text{Total no. of Cells}} \times 100$$

The viable cell density (viable cells /ml) was determined using the following equation;

$$\text{Viable cells/ml} = \frac{\text{Number of cells excluding Trypan Blue}}{\text{Number of segments counted on haemocytometer}} \times 10^4 \times 10^2$$

[Where 10^4 is a conversion factor from haemocytometer chamber count per $0.1mm^3$ to cm^3 and 10^2 is the dilution factor].

4.2.2.3 Pre-treatment of rats for enzyme induction experiments

Pretreatment of rats with phenobarbitone sodium

Male Sprague-Dawley rats rats (180-220g, n=3) received phenobarbitone sodium solution (0.1%w/v) in place of the normal drinking water for 3 days prior to the isolation of hepatocytes.

Pretreatment of rats with 3-MC

Male Sprague-Dawley rats (180-220g, n=3) were administered with a single intra-peritoneal injection of 3-MC (25mg/ml) in olive oil (at a dose of 50mg/kg body-weight in 2ml/kg olive oil) 3 days prior to isolation of hepatocytes. Control animal experiments consisted of rats (180-220g, n=3), which had received a single intra-peritoneal injection of olive oil (at a dose of 2ml/kg body-weight) 3 days prior to isolation of hepatocytes.

Pretreatment of rats with deltamethrin

Male Sprague-Dawley rats (180-250g, n=3) were administered with a single intra-peritoneal injection of deltamethrin (25mg/ml) in olive oil (at a dose of 50mg/kg body-weight in 2ml/kg olive oil) 3 days prior to isolation of hepatocytes.

4.2.2.4 Preparation of rat hepatocytes

Isolation of rat hepatocytes was carried out by collagenase perfusion of the liver following preperfusion with a solution containing a Ca^{2+} chelating agent as described by Moldeus *et al.*, (1978).

Perfusion system

The perfusion setup is as shown in figure 4.12. The set up consisted of three plastic beakers (200ml) seated in a thermostatted water bath (37°C), a peristaltic pump (Watson Marlow, UK) to circulate perfusion buffers from the beakers to a clamped reservoir and a pressure guage to monitor the flow of carbogen gas (5%CO_2/ 95% O_2). Perfusion was performed by gravity, with a perfusion pressure of 20-22cm water.

Isolation of hepatocytes

Male Sprague-Dawley rats (180-250g) were anaesthesised by an intra-peritoneal injection of sodium pentobarbitone (Sagatal® 60mg/ml, Rhone-Poulenc Rorer, Dagenham) at a dose of 60mg/kg body weight. The peritoneal cavity was opened by a midtransversal incision and heparin sodium (0.1ml (1000U/ml in phosphate buffer saline) was injected into the inferior vena cava. The portal vein was cannulated (Steel cannula: Internal/external diameter = 1.75mm/2.5mm), the liver dissected and perfused *ex vivo* without recirculation for 2-3 min, followed by recirculating perfusion for 7-8 min with Hank I buffer (150ml), which was Ca^{2+} free and contained 0.6mM EGTA. This was followed by perfusion for 10-15min with Hank II buffer containing 2mM Ca^{2+} and 0.02 %w/v collagenase (type IV from Sigma equivalent to 92 units/ml collagen digesting activity). During the perfusion period, all solutions were bubbled with 5%CO_2/ 95%O_2 and maintained at 37°C with a perfusate flow rate of about 6ml/min. When the liver felt very soft and the cells inside the liver sac had dissociated, it was dispersed using two forceps into 100ml Krebs-Albumin buffer that contained 1% w/v BSA. The cell suspension was filtered through a sterile cotton gauze mesh to remove remaining connective tissues and clumps of cells.

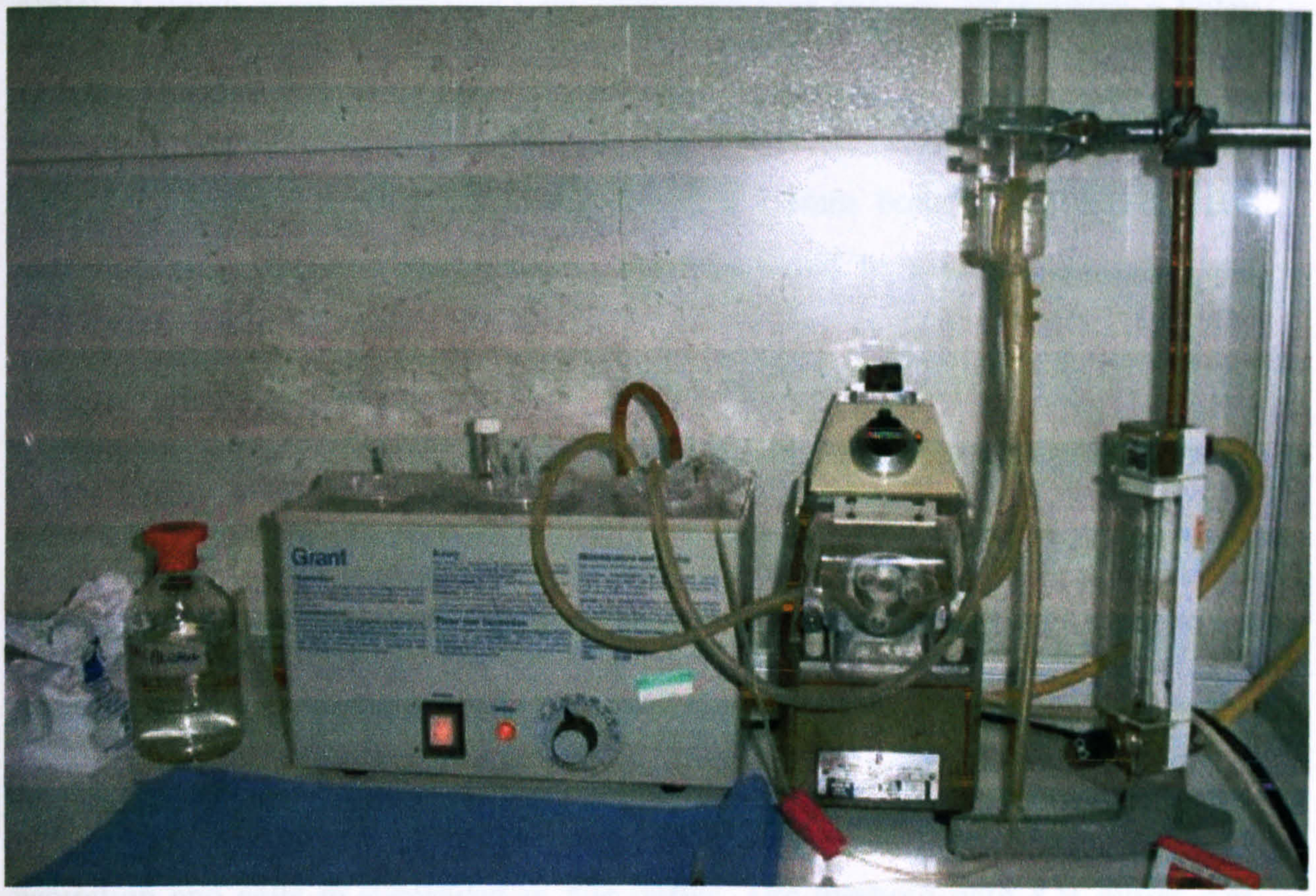


Figure 4.12 Photographic representation of the liver perfusion system used in preparation of isolated rat hepatocytes

The filtrate was collected in a 100ml bottle and the cells allowed to settle by gravity. The supernatant was removed by aspiration and the cells were washed (twice) with 50ml KII buffer, by allowing the cells to settle by gravity after each wash. The cells were then counted and viability and viable density determined by Trypan Blue exclusion (as described in section 4.2.2.2). The average viability of cells used for incubation experiments in this study was $\geq$ 75%.

4.2.2.5 Pig hepatocytes

Pig hepatocytes were obtained from The Department of Medicine (Edinburgh, Royal Infirmary). Hepatocytes were isolated from Great White pigs (15-25kg) by a collagenase perfusion technique adapted from a published method (Gerlach *et al.*,

1994). Animals were euthanased with phenobarbitone sodium, after which a Foley catheter was inserted into the portal vein via the vena cava followed by perfusion with phosphate buffered saline (PBS, pH 7.4). Perfusion was performed by gravity, with a hydrostatic head of 50cm water, via a 1.5mm bore tubing. During this perfusion stage the liver was excised and transferred to a sterile container and sequentially perfused with:

a) Hank's I buffer without recirculation at 70ml/min

b) Hank's II buffer containing 450mg collagenase with re-circulation at 70ml/min for 30-40min.

c) KA buffer (600ml) without recirculation

The liver tissue was disrupted with forceps in KA buffer and the resultant suspension filtered through three stainless steel meshes of decreasing porosity (1000μm, 600μm and 180μm). The filtrate was sedimented under gravity, the supernatant removed by aspiration and cells re-suspended in KH buffer. The washing with KH buffer was performed twice. The viability of the cells was determined by Trypan-Blue exclusion method as described in section 4.2.2.2 and the viability of pig hepatocytes used in this study ranged between 65-78%.

4.2.2.6 Incubation procedures for metabolism studies.

Incubations of the aromatic amidines (100μM) (*p*-aminobenzamidine, diminazene aceturate and pentamidine isethionate) with isolated hepatocytes (5 x 10^6 viable cells/ml), were performed for both rat and pig hepatocytes in a thermostatted water bath (37°C). Incubations were carried out in round bottom flasks (50ml), attached to a Bucchi Rotavapor (Bucchi Laboratory Technik Ag Switzerland) via a four-way adapter (figure 4.13). To ensure adequate oxygenation during the incubation, carbogen gas was applied continuously to the surface of the incubation medium through the central vacuum exit of the evaporator.

Figure 4.13 Photographic representation of incubation apparatus showing incubation flasks containing isolated rat hepatocytes attached to a rotavapor connected to carbogen ($5\%CO_2/95\%O_2$) gas supply.

A volume (2ml) of the stock solution of test drug (500μM) (either p-aminobenzamidine or diminazene or pentamidine) was placed in a flask and diluted with the appropriate volume of KH buffer such that, addition of the isolated hepatocyte suspension (*Xml*, equivalent to 5×10^6 cells/ml) made up a total volume of 10ml. In this way, a consistent drug concentration (100μM) was obtained in all test incubations. Control incubations used in these studies were; hepatocytes in KH buffer (without added drug) and a solution of the drug in KH buffer (100μM, without hepatocytes). Aliquots (0.5ml) of incubation media were taken at specific time intervals (t = 0, 30, 60, 90, 120 and 180min) and the reaction terminated by addition of an equal volume of 0.2%w/v methanolic ammonium chloride and the sample vial placed on ice. In order to ensure that the incubation drug concentration (100μM) was suitable, a time course viability study was performed to compare viability of cells in test incubations containing drug (100μM) and in control incubation of hepatocytes

only (without added drug). The cell viability data were compared by a Students' t-test at a 5% significance level.

Table 4.3 Hepatocyte incubation protocols

Drug/Compound	Untreated	PB	3-MC	DM
Pentamidine	n = 3	n = 4	n = 3	n = 3
	rat & pig	rat	rat	rat
Diminazene	n = 6 rat	n = 5	n = 3	n = 3
	n = 3 pig	rat	rat	rat
p-Aminobenzamidine	n=2	-	-	-
	rat & pig			

Incubations which were carried out with hepatocytes from rats, pigs and with cells from inducer treated rats are shown in table 4.3.

4.2.7 Sample preparations

Incubation mixtures (contained in vials) were sonicated (15min) in order to rupture cell membranes and release the cellular contents, vortexed (20sec) and centrifuged (9000*g*, 10min) to sediment cellular debris as pellets. The supernatant was aspirated with a Gilson pipette and samples were stored (-20°C) for subsequent analysis.

Solid phase extraction (SPE)

In order to allow for the detection of trace metabolites, incubates were preconcentrated by SPE. The supernatant obtained after centrifugation of the incubates (containing 50% methanol), was evaporated to about half of the original volume under a gentle stream of N_2, in order to reduce the methanol content to below 10%. This was to allow adequate partitioning of metabolites with the C_{18} sorbent (3ml/500mg Isolute®-Crawford Scientific, Lanarkshire-UK). The SPE catridges were preconditioned with methanol (3ml) followed by distilled water (3ml), loaded with the incubation samples, washed with distilled water (2ml) and eluted with 0.2% w/v NH_4Cl in methanol (3ml) under vacuum. The eluate was evaporated and

reconstituted in water (250μl). Samples were stored (-20°C) for subsequent analysis by LC/MS.

4.2.2.8 Recovery of diamidines from hepatocyte suspensions

In order to determine the recovery of diminazene or pentamidine from hepatocyte suspensions, the freshly isolated cells were stored in the refrigerator (~4°C) over 48h to render them unviable (confirmed by the Trypan-Blue exclusion test). The dead cells were incubated for 60min with diminazene (100μM) or pentamidine (100μM) in a similar manner to that described in section 4.2.2.6. Control incubations contained only diminazene or pentamidine in KH buffer. Aliquots (0.5ml) of incubation mixture were added to an equal volume of 0.2% w/v methanolic NH_4Cl in a vial, sonicated, vortexed, centrifuged (9000*g*) and the supernatants were stored (-20°C) for subsequent analysis by HPLC-UV.

The recovery was calculated as;

$$\frac{\text{Average detector response of compound in dead hepatocyte incubations}}{\text{Average detector response for control incubations}} \times 100$$

The recoveries of pentamidine isethionate and diminazene aceturate were determined as 81.0 ± 1.2% and 70.8 ± 3.1(mean ± S.D., n=5) respectively.

4.2.2.9 Determination of cytochrome P450 content of rat hepatocytes

Measurement of cytochrome P-450 content of hepatocytes (from pretreated and untreated rats) was carried out by a modification (Watts et al., 1995) of the method of Omaru and Sato (1964). Freshly isolated rat hepatocytes (5 x 10^6cells/ml) were homogenised in 0.1M sodium phophate buffer (2ml), pH 7.6 containing 1mM dithiotreitol, 1mM ethylene glycol-bis (beta-amino-ethyl ether) EGTA, 20%v/v glycerol and 0.02%v/v Nonidet P-40 (Sigma Chem. Co) (P450 buffer) and placed immediately on ice. Isolated hepatocytes were homogenised using a motor driven

teflon-glass homogenizer (Potler-Eljehem homogenizer) and stored at –70°C. Carbon-monoxide (CO) treated homogenates were compared to CO-treated and sodium dithionite-reduced homogenates with a Shimadzu UV-2101PC two channel spectrophotometer, using 1ml quartz cuvettes. The differences in absorbance between 450 and 469nm were used to determine the cytochrome P450 content. The cytochrome P-450 extinction coefficient of $91mM^{-1}cm^{-1}$ was used in calculations for the estimation of cytochrome P-450 content:

Absorbance difference (450-469nm) = Z

Extinction coefficient at 450nm = $91mM^{-1}cm^{-1}$

By applying Beer's law,

Cytochrome P450 concentration = $\frac{(Z \times 1000)}{91}$ nmol/ml [in the diluted sample]

4.2.2.10 HPLC analyses

The HPLC system comprised of a Spectra-SYSTEM P2000 pump, photo-diode array detector (Spectra-SYSTEM 6000LP) and on line autosampler (Spectra-SYSTEM AS1000) with a fixed 20μl steel injection loop.

Pentamidine and its metabolites were resolved at ambient temperature on a Hypersil BDS (3μm particle size) (100 x 4.6mm i.d) C-18 column with two solvent systems; *Solvent A:* 20mM ammonium formate buffer (pH 3) in distilled water; *Solvent B:* Acetonitrile. Analytes were separated with a linear gradient from 10 to 65%B (acetonitrile) for 14 min at a flow rate of 0.6ml/min. Quantification of pentamidine and its 3 major metabolites was performed using a discrete UV channel at 254nm and PDA spectra were also collected from 200-480nm.

Incubation samples containing diminazene and p-aminobenzamidine were analysed using a Hypersil BDS (3μm particle size) (100 x 4.6mm i.d) C-18 column using a linear gradient mobile phase; *Solvent A:* ammonium formate buffer (pH4, 20mM); *Solvent B:* Acetonitrile. Analytes were separated with a linear gradient from 10-65% solvent B at a flow rate of 0.6ml/min for 12 minutes at a discrete wavelength of 290nm.

Using these HPLC methods, a rectilinear relationship was established between the detector (UV @ 254nm) response and the concentration of pentamidine solutions over a dynamic range of 10-80μM ($r^2 > 0.972$, n = 3) (figure 4.14).

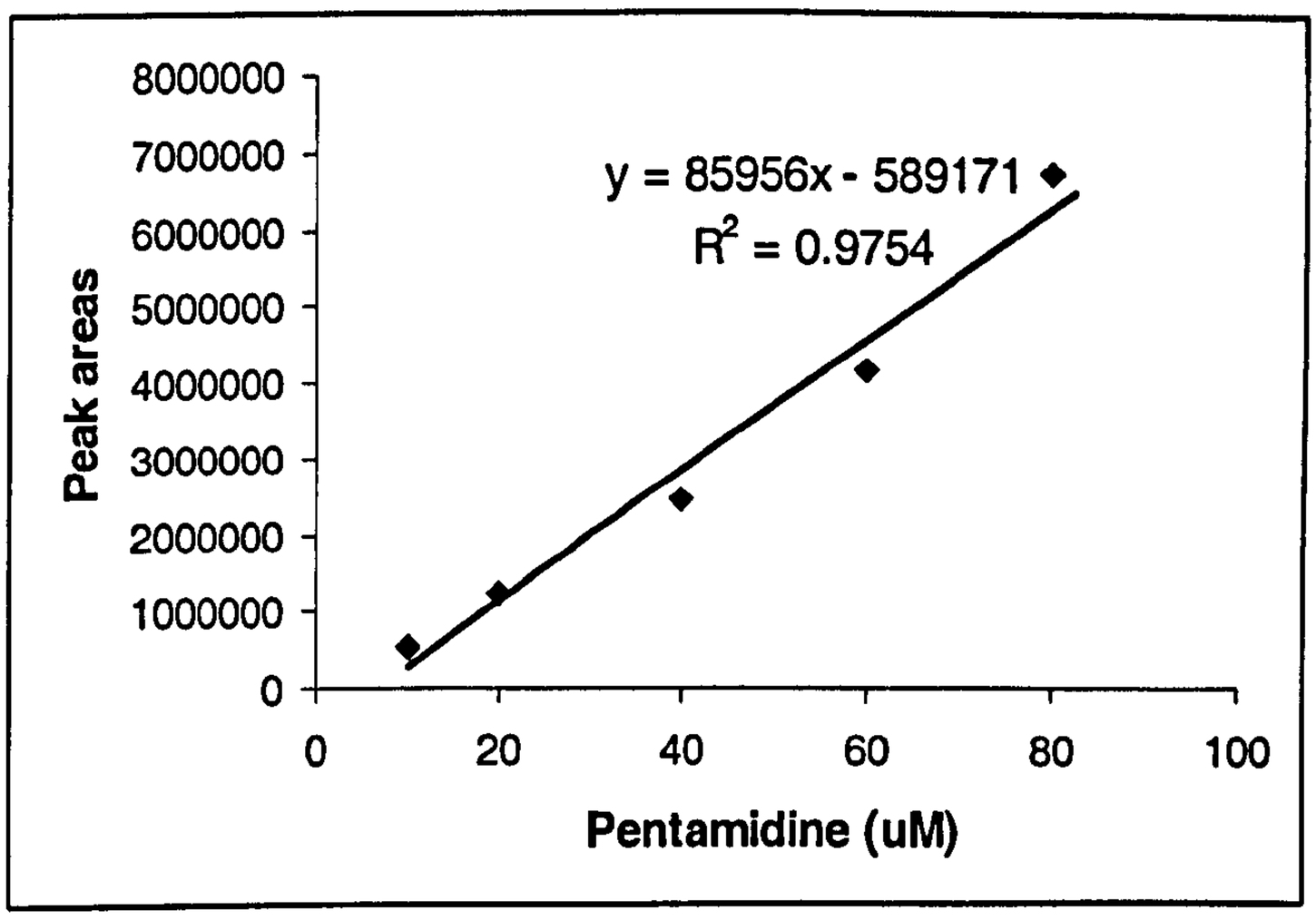


Figure 4.14 Linear relationship between concentration of pentamidine (10-80μM) and detector response

4.2.2.11 LC-MS/MS analyses

Identification of metabolites was performed by using a Finnigan MAT *TSQ-7000* triple quadrupole MS system operated in positive ESI mode. The ESI source was operated at a capillary voltage of 4.5kV and temperature of 300°C. The eluate from the LC system was nebulized into the electrospray chamber with sheath and auxiliary N_2 gas pressures of 80 and 20 *psi*, respectively. A collision cell energy setting of 30eV and CID gas pressure of 0.6mT was used for MS/MS scans of pentamidine and its metabolites.

4.2.2.13 Statistical Tests

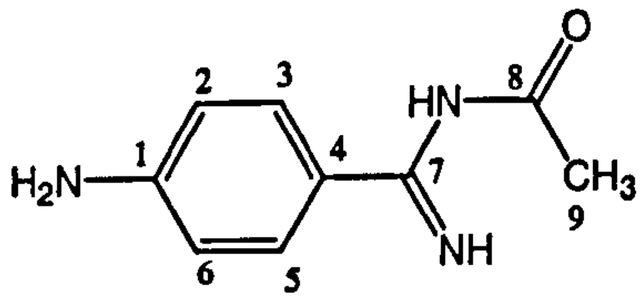
Statistical analyses used in this chapter namely, regression analysis, one-way analysis of variance (ANOVA), two-sample t-test and descriptive statistics (mean, standard error of mean, standard deviation) were performed with Microsoft® excel 2000 (Microsoft Corporation, USA) software unless otherwise stated.

4.2.2.13 Synthesis of *p*-amino-*N*-acetylbenzamidine

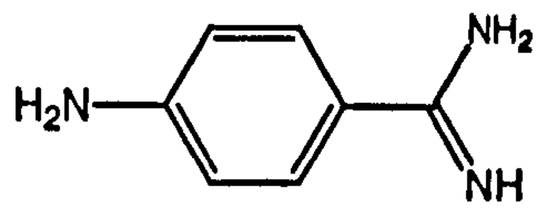
N-Acetyl-p-aminobenzamidine was synthesised by reacting acetic anhydride with p-aminobenzamidine hydrochloride in dry ethanol. Ethanol (98%) was dried by the addition of anhydrous $MgSO_4$ (500mg) to ethanol (100ml) in a stoppered conical flask. The suspension was mixed by shaking, allowed to stand over night (24h) and filtered. To the dried ethanol (20ml), p-aminobenzamidine hydrochloride (136mg) was added, followed by the addition of acetic anhydride (10ml). The mixture was refluxed over a steam bath (100°C) for 45min. The formation of the product (N-acetyl-p-aminobenzamidine) was monitored by UV-HPLC analysis of aliquots of diluted reaction mixture by the method described in section 4.6.8. The solution was then cooled and allowed to stand in cold water for about 30*h*. The white crystalline precipitate which was formed (N-acetyl-p-aminobenzamidine) was filtered off and subjected to structural confimation by LC/UV-MS and NMR spectroscopy.

NMR spectroscopy (Bruker AMX 400)

^{1}H-NMR and ^{13}C-NMR (400MHz, DMSO-d_6) chemical shifts (δHz) of p-aminobenzamidine and the synthesised compound (N-acetyl-p-aminobenzamidine) assignments from HMBC spectra are tabulated in table 4.3.



N-acetyl-p-aminobenzamidine



p-aminobenzamidine

Table 4.3 ^{1}H-NMR and ^{13}C-NMR chemical shifts (δHz) of p-aminobenzamidine and N-acetyl-p-aminobenzamidine

Position	N-acetyl-p-aminobenzamidine		p-aminobenzamidine	
	^{1}H shifts (δHz)	*^{13}C shifts (δHz)*	*^{1}H (δHz) shifts*	*^{13}C shifts (δHz)*
1	—	144.6	—	152.9
2/6	6.73, *brs* (4H)	119.9	6.74, d (4H)	113.7
3/5	7.72, *brs* (4H)	129.9	7.62, d (4H)	130
4	—	122.7	—	122.7
7	—	165.8	—	164.6
8	—	171.2	—	—
9	2.07, s (3H)	24.7	—	—
	8.70, *brs* (N<u>H</u>COCH$_3$)	—		
	10.45, *s* (=N<u>H</u>)	—	10.45, *s* (=N<u>H</u>)	

Where *brs* = broad singlet; *d*=doublet; *s* = singlet

Mass spectrometry

LC+ESI-MS: *m/z* 218 $[M + CH_3CN]^+$ as base peak, 177 $[M]^+$, 163 $[M+H-NH_2]^+$

4.3 Results and Discussion

4.3.1 Effect of diamidines on the viability of isolated rat and pig hepatocytes.

Following a 3h incubation period, there was no significant difference ($p > 0.05$) between the viability of rat hepatocytes in control cells (56.5 ± 3.5 %, n=3) and cells incubated with either diminazene aceturate (100μM) (50.4 ± 2.1%, n=3) or pentamidine isethionate (100μM) (46.0 ± 5.7%, n=3) (figure 4.15).

In a similar manner, there was no significant difference ($p > 0.05$) between the viability of control pig hepatocytes (49.4 ± 10.3%, n=2) and cells incubated with either diminazene (μM100) (42 ± 4.2 %, n=2) or pentamidine (μM100) (45.5 ± 10.6 %, n=2) (figure 4.16).

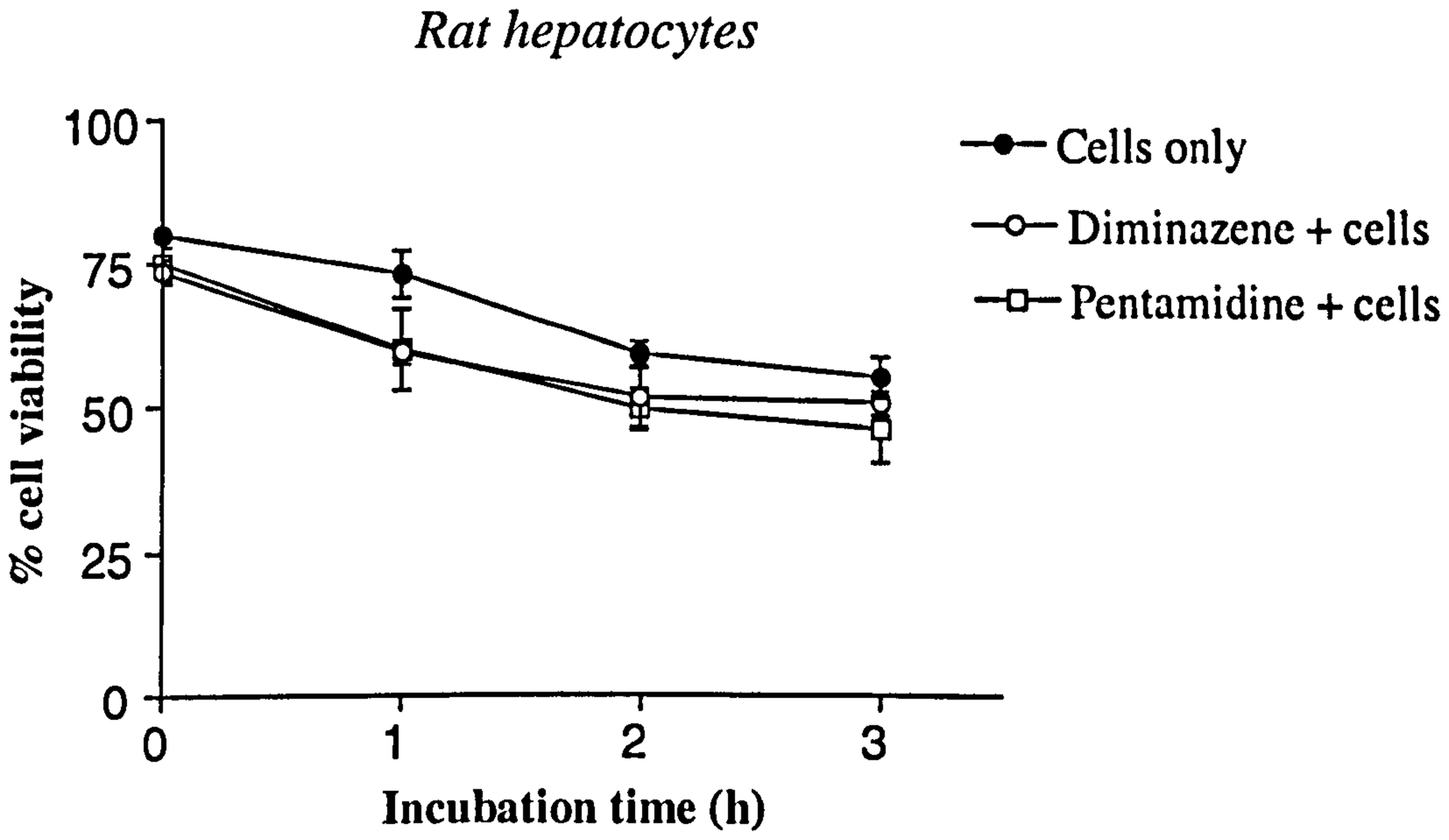


Figure 4.15 Effect of diminazene (100μM) and pentamidine (100μM) on the viability of rat hepatocytes (as determined by Trypan Blue exclusion) over a 3h incubation period (mean ± S.D., n=3)

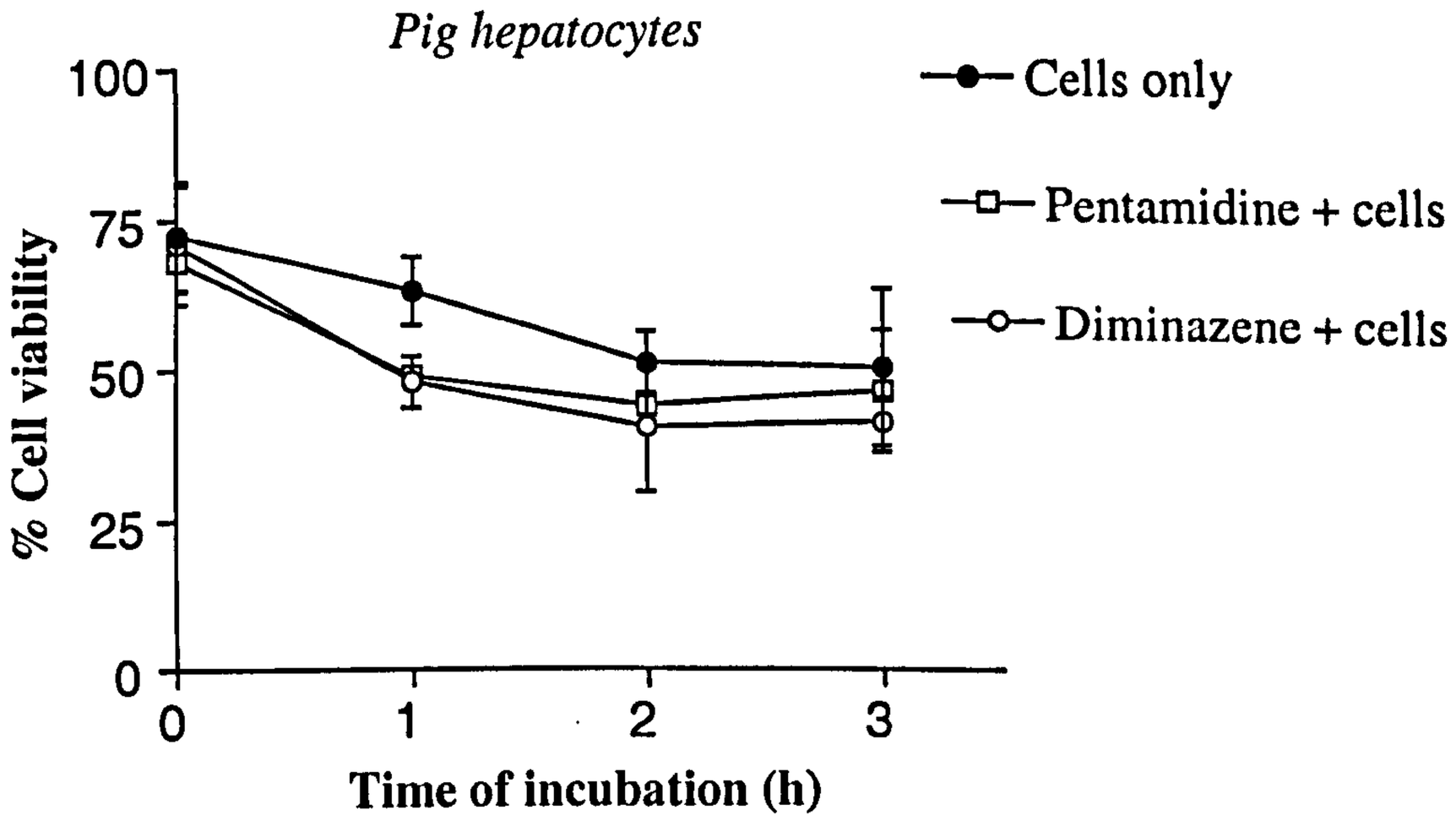


Figure 4.16 Effect of diminazene and pentamidine (100μM) on the viability of pig hepatocytes (as determined by Trypan Blue exclusion) over a 3h incubation period (mean ± S.D., n=3)

Since the incubation of either diminazene or pentamidine (100μM) with rat or pig hepatocytes did not result in a significant reduction in the viability of cells isolated from either species, a drug concentration of 100μM was used in all subsequent incubation experiments.

4.3.2 Metabolism of *p*-aminobenzamidine by rat and pig hepatocytes

4.3.2.1 Metabolism by rat hepatocytes

Following a 3h incubation of *p*-aminobenzamidine (p-AB)(100μM) and rat hepatocytes, the HPLC-UV chromatograms of the preconcentrated incubation samples (SPE) showed the presence of a new peak designated M_1 (figure 4.17A), which was absent in the corresponding control incubation samples (figure 4.17B, C). The peak (RT: 4.17min) thought to be a metabolite was chromatographically well resolved from the peak of the parent compound (*p*-aminobenzamidine) (RT: 3.23min). ESI mass spectra of incubation

samples are shown in figure 4.18. The extracted mass chromatograms of the test incubation sample *m/z* 135, 177 (figure 4.18A) produced two distinct peaks, consistent with the HPLC-UV profiles observed in figure 4.17A. The product ion spectrum of the metabolite peak (RT: 4.25min) (fig.4.18C), shows the presence of distinct ions at *m/z* 218 $[M+CH_3CN]^+$, 177 $[M]^+$ and 163 $[M+H-NH_2]^+$ which suggests the formation of the *N*-acetylated metabolite (*p*-amino-*N*-acetylbenzamidine) of p-AB. Furthermore, the ion spectrum of pAB (RT:3.23min) (figure 4.18B), shows the formation of the acetonitrile adduct ion at *m/z* 176 $[M+CH_3CN]^+$ which is 42 mass units ($-COCH_3$) less than that of the corresponding adduct ion formed by the metabolite at *m/z* 218 $[M+CH_3CN]^+$. The structural identity of the metabolite was confirmed by the synthesis of the putative metabolite and unequivocal characterization by NMR spectroscopy and mass spectroscopy (section 4.2.2.13).

The synthesized compound (*p*-amino-*N*-acetylbenzamidine), co-eluted with the metabolite peak (M_1) in the incubation samples (figure 4.19). The photodiode array-UV spectra of the metabolite and the synthesized reference compound gave similar absorption maxima (267-268nm) (figure 4.20), which confirmed that the identity of the metabolite was *p*-amino-*N*-acetylbenzamidine [see appendix III for NMR spectra of synthesized compound].

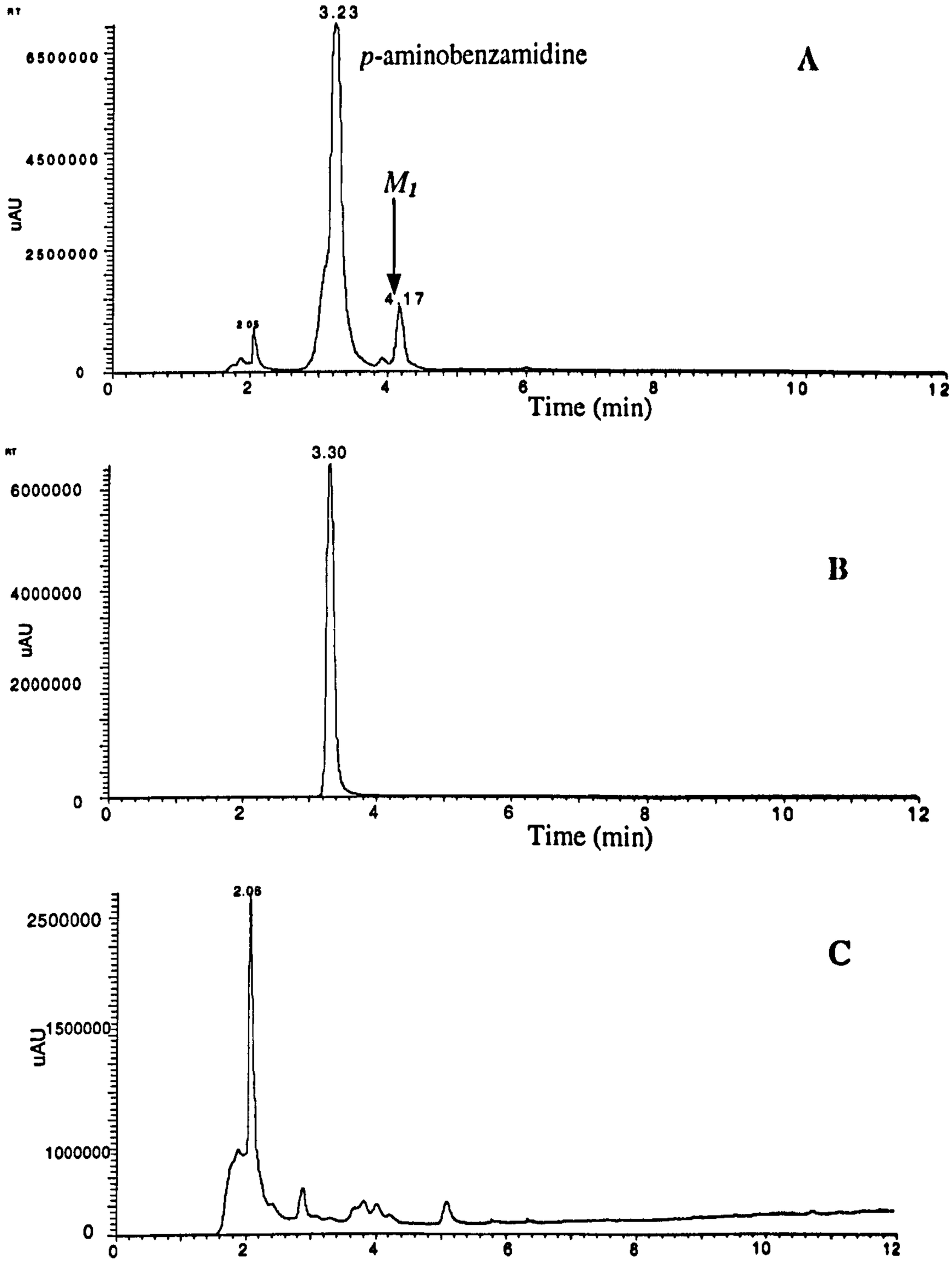


Figure 4.17 HPLC chromatograms of 3h incubation of A) p-aminobenzamidine (100μM) and rat hepatocytes ($5x10^6$/ml) and the control incubations of B) 100μM *p*-aminobenzamidine only in buffer and C) cells ($5x10^6$/ml) in buffer only; [M_1 = metabolite peak].

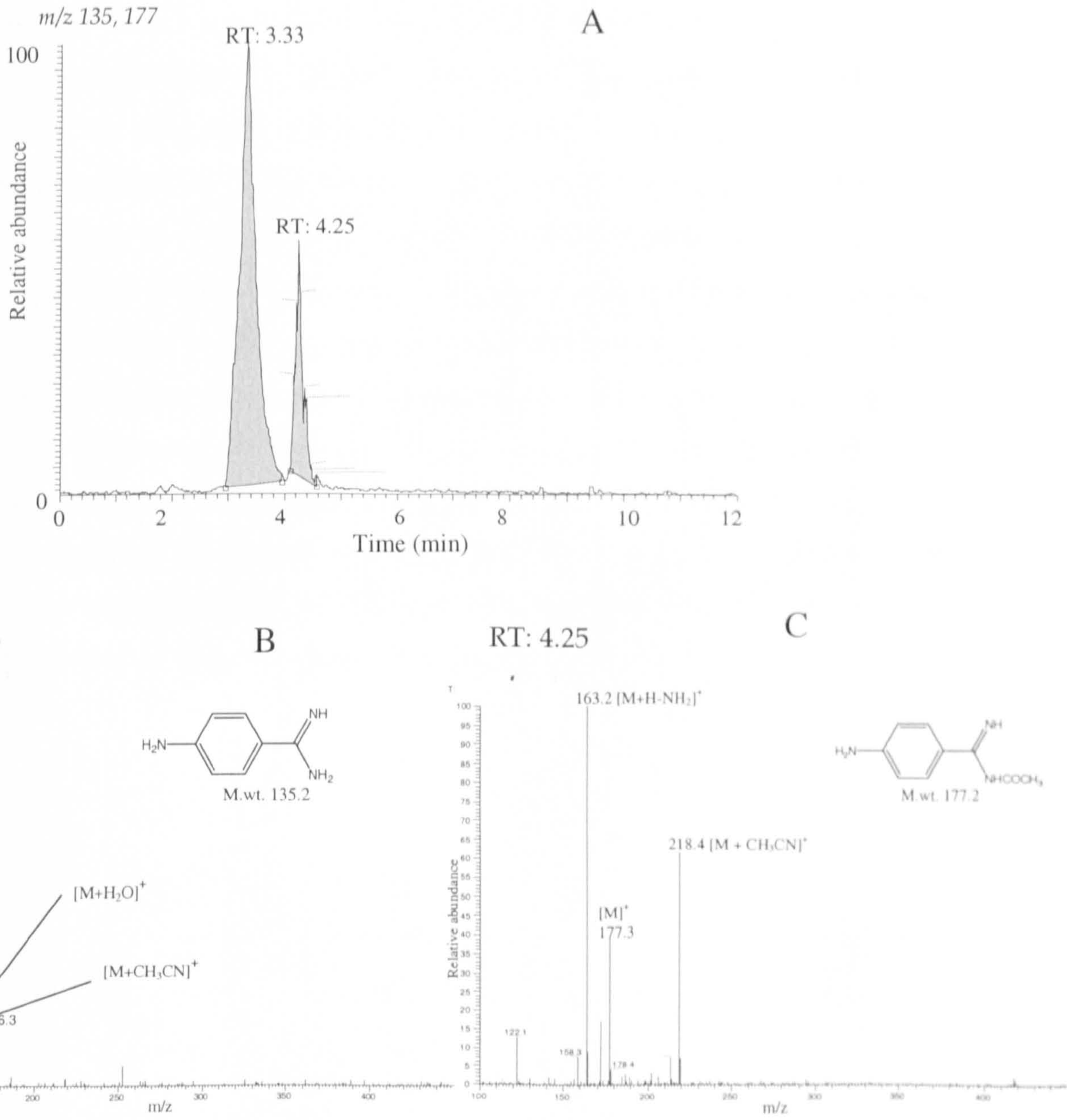


Figure 4.18 Mass chromatogram of 3h incubation sample of p-amino-benzamidine (100μM) and rat hepatocytes $5x10^6$/ml showing A) a selected ion mass spectrum (*m/z* 135,177) and ion spectra of B) *p*-aminobenzamidine (RT:3.33min) and C) metabolite peak (RT: 4.25min) [RT represents retention time (min)]

4.3.2.2 Metabolism of p-aminobenzamidine (p-AB) by pig hepatocytes

Similar incubations of p-AB (100μM) with isolated pig hepatocytes resulted in the formation of the *N*-acetylated metabolite of p-AB. A representative extracted ion chromatogram (m/z 135, 177) is shown in figure 4.21A. For comparison, the extracted ion chromatogram of a 3h incubation mixture of p-AB (100μM) with rat hepatocytes is also shown (figure 4.21B). Unfortunately, a time course study for the disappearance of p-AB in rat or pig hepatocytes was not performed. This would have allowed a quantitative comparison of the rates of metabolism of p-AB in the two animal species. However, by expressing the peak area of the metabolite formed after a 3h incubation period as a percentage of the peak area of the initial amount of p-AB added (i.e time zero), it was possible to compare the relative amounts of *p*-amino-*N*-acetylbenzamidine formed in the both animal species. This was however based on the assumption that, the metabolite formed is stable and does not undergo further metabolism.

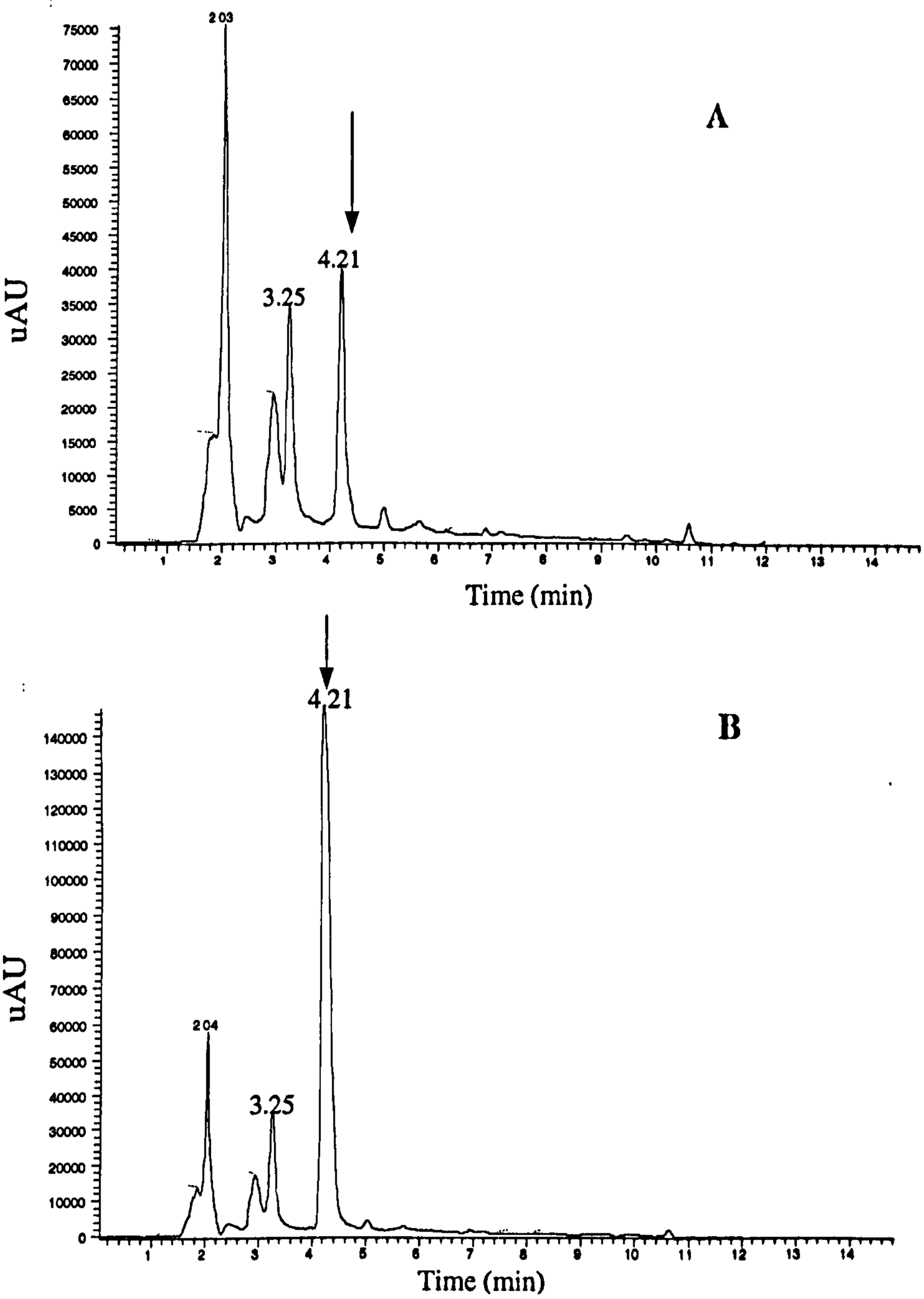


Figure 4.19 HPLC-UV chromatograms of A) 3h incubation mixture of p-amino-benzamidine(100μM) and rat hepatocytes ($5x10^6$cells/ml) and B) spiked incubation mixture of sample chromatogram A) with authentic sample of *p*-amino-*N*-acetylbenzamidine.

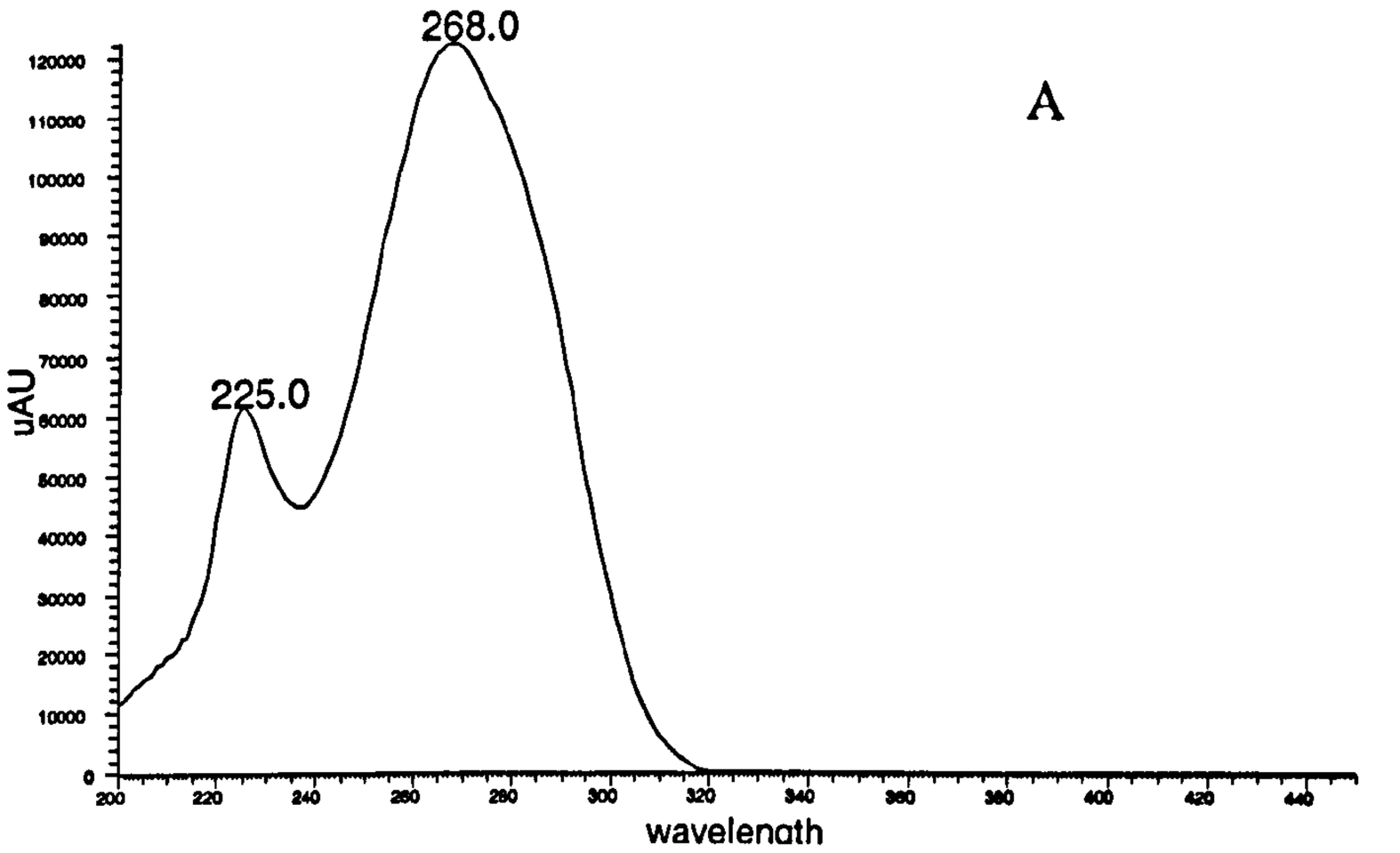


Figure 4.20 Photodiode array spectra of A) recovered metabolite (*N*-acetyl-*p*-aminobenzamidine) in rat hepatocyte incubations and B) synthesized analogue of metabolite.

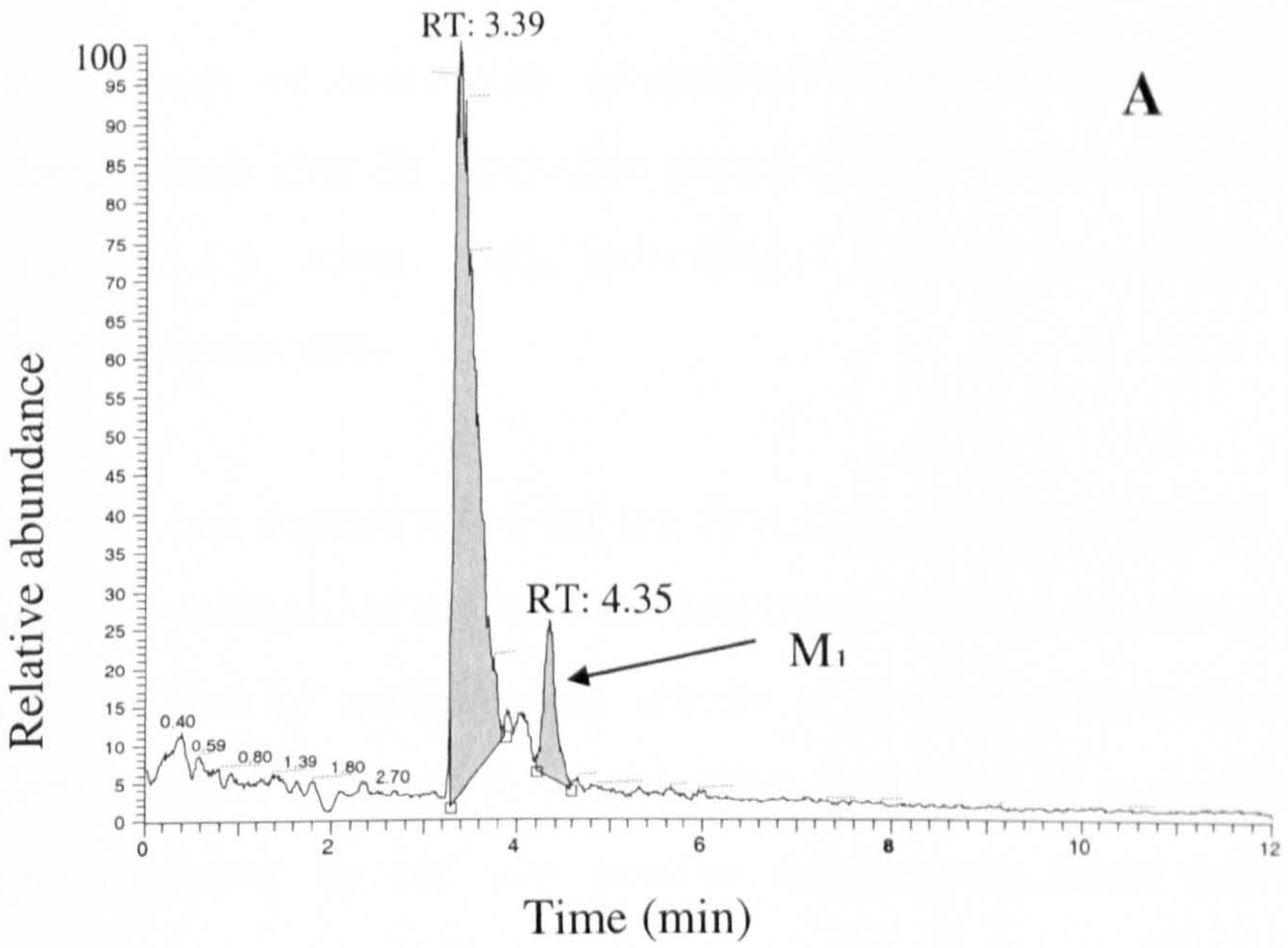


Figure 4.21 Extracted ion chromatograms (*m/z* 135, 177) of 3h incubation mixtures of *p*-aminobenzamidine (100μM) with A) pig hepatocytes ($5x10^6$cells/ml) and B) with rat hepatocytes ($5x10^6$cells/ml).

The relative amount of metabolite (*p*-amino-*N*-acetylbenzamidine) formed in pig hepatocyte incubations after 3h incubation period (5.6%, mean; n=2) was less than that formed in rats (15.8%, mean; n=2), indicating a 2.8-fold greater production of the metabolite by rat hepatocytes.

This study has demonstrated for the first time the *N*-acetylation of an aromatic amidine. Since the presence of the *N,N'*-unsubstituted amidino functional group has been shown to be essential for antiprotozoal activity of aromatic diamidines (Clement and Raether, 1985; Buddhu *et al.*, 1991), the biotransformation of *p*-aminobenzamidine to the *N*-acetyl derivative by rat and porcine hepatocytes could be regarded as a deactivation pathway and may be of pharmacological significance. Acetylation polymorphism, has been observed in man and other mammalian species (rat and rabbit) (Timbrell, 1991) and the acetylator phenotypes, named 'slow' and 'fast' acetylators are related to toxicity of xenobiotics (e.g. slow and fast acetylator phenotypes of isoniazid) (Gibson and Skett, 2001).

4.3.3 Metabolism of pentamidine in isolated rat hepatocytes

A typical HPLC chromatogram of a 60min incubation mixture of pentamidine and uninduced rat hepatocytes is shown in figure 4.22A. Comparison with the chromatograms of the corresponding control incubations (figure 4.22B, & C) reveals seven new chromatographic peaks designated 1 to 7 (figure 4.22A), which represent the putative metabolites of pentamidine (peak *P*).

While previously published HPLC methods employed for the bioanalyses of pentamidine and its metabolites have involved the use of complex mobile phases with analysis times of over 30 minutes (Bronner *et al.*, 1995; Berger et al., 1992), the present newly developed method gave baseline resolution of pentamidine and its metabolites within *14* minutes (fig. 4.22A) on a *Hypersil* BDS 3µ particle size, C-18 (100 x 4.6mm id) reversed phase column. Initial attempts to separate pentamidine and its metabolites with a Lichrospher®60 C8, 5µ particle size (125 x 4.6mm) column (Hewlett Packard, Germany) failed to provide base line resolution of all the metabolites and resulted in broad peaks. The use of a *3µ* particle size, C18 column (Hypersil) with a higher column efficiency (smaller theoretical plate height) helped to markedly improve the chromatography i.e. separation. Unlike previously published methods that utilized modifiers like heptane sulfonate, tetramethyl ammonium chloride and triethylamine (Berger *et al*, 1992, Bronner *et al.*, 1995), the mobile phase employed (ammonium formate/acetonitrile) in this work was relatively simple and compatible with ESI-MS.

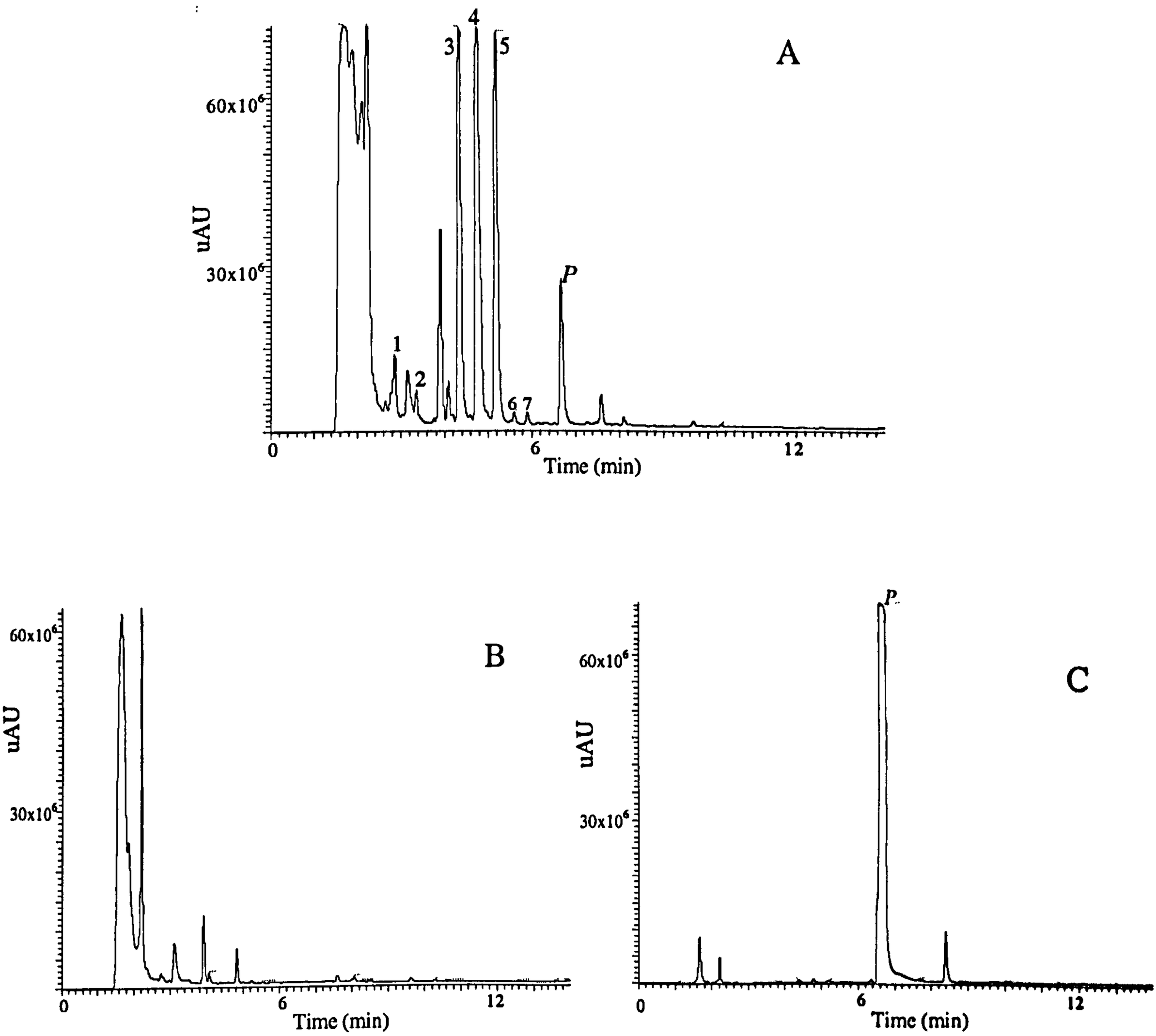


Figure 4.22. Metabolism of pentamidine (100μM in Krebs-Hepes buffer, pH 7.4) by isolated rat hepatocytes ($5x10^6$ cells ml^{-1}) for 1h showing HPLC-UV chromatograms of: A) experimental incubation of pentamidine and isolated hepatocyte suspension; B) control incubation of hepatocytes alone in buffer without added drug; C) control incubation of pentamidine in buffer.

Three major metabolite peaks (*3,4* and *5*, figure. 4.22A) were observed in chromatograms of rat hepatocyte incubations and this is in contrast to results obtained in earlier studies where only two major metabolites [(1,5-bis(4-amidinophenoxy)-2-pentanol and 1,5-bis(4-amidinophenoxy)-3-pentanol)] (figure 4.5, section 4.1.7.1) were recovered in incubations of pentamidine with Sprague-Dawley and Fisher rat liver microsomes (Berger *et al.*, 1990; Tuttle *et al.*, 1997). The metabolites producing peaks 1, 2, 6 and 7 (Figure 4.22) could not be quantified due to the fact that the amounts formed over the 3h incubation period were below the limits of quantification (LOQ) and could not be integrated accurately by either LC-UV or LC-MS. It appears that these metabolites (1,2,6 and 7) were formed as minor metabolites of pentamidine in rat hepatocytes.

4.3.3.1 Identification of metabolites

Identities of metabolites was established by LC-MS/MS. However, due to the lack of reference standards, their structures could not be absolutely confirmed. The partial identification of the metabolites was performed with pre-concentrated incubation samples obtained by SPE (see section 4.2.2.7). This helped to improve the chromatographic signal-to-noise ratios of all metabolites, especially the minor ones (*1*, *2*, *6*, and *7*, figure 4.22A). The total ion chromatogram (figure 4.24) was obtained following injection of 100µl of a SPE (2h) incubation mixture into the LC-MS system. The extracted ion chromatograms and the corresponding product ion spectra of the putative metabolites are shown in figures 4.25 to 4.29. The extracted ion chromatogram at *m/z* 136 generated more than four other related peaks (figure 4.25), which was a result of the fact that, the ion at *m/z* 136 was formed as a stable product ion of pentamidine and its other metabolites. Thus, by operating the instrument in tandem MS mode at CID gas pressure of 1.2mT and collision energy of 35*eV*, fragmentation was induced in the analytes, with the ions at *m/z* 119, 136 being formed as the predominant (stable) product ions of pentamidine and its three major metabolites (table 4.4). The tentative structure of the ion at *m/z* 119 is shown below in figure 4.23.

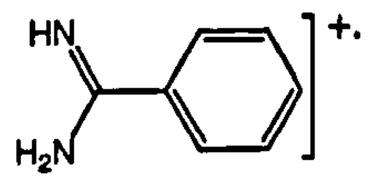


Fig. 4.23 Tentative structure of the product ion at *m/z* 119

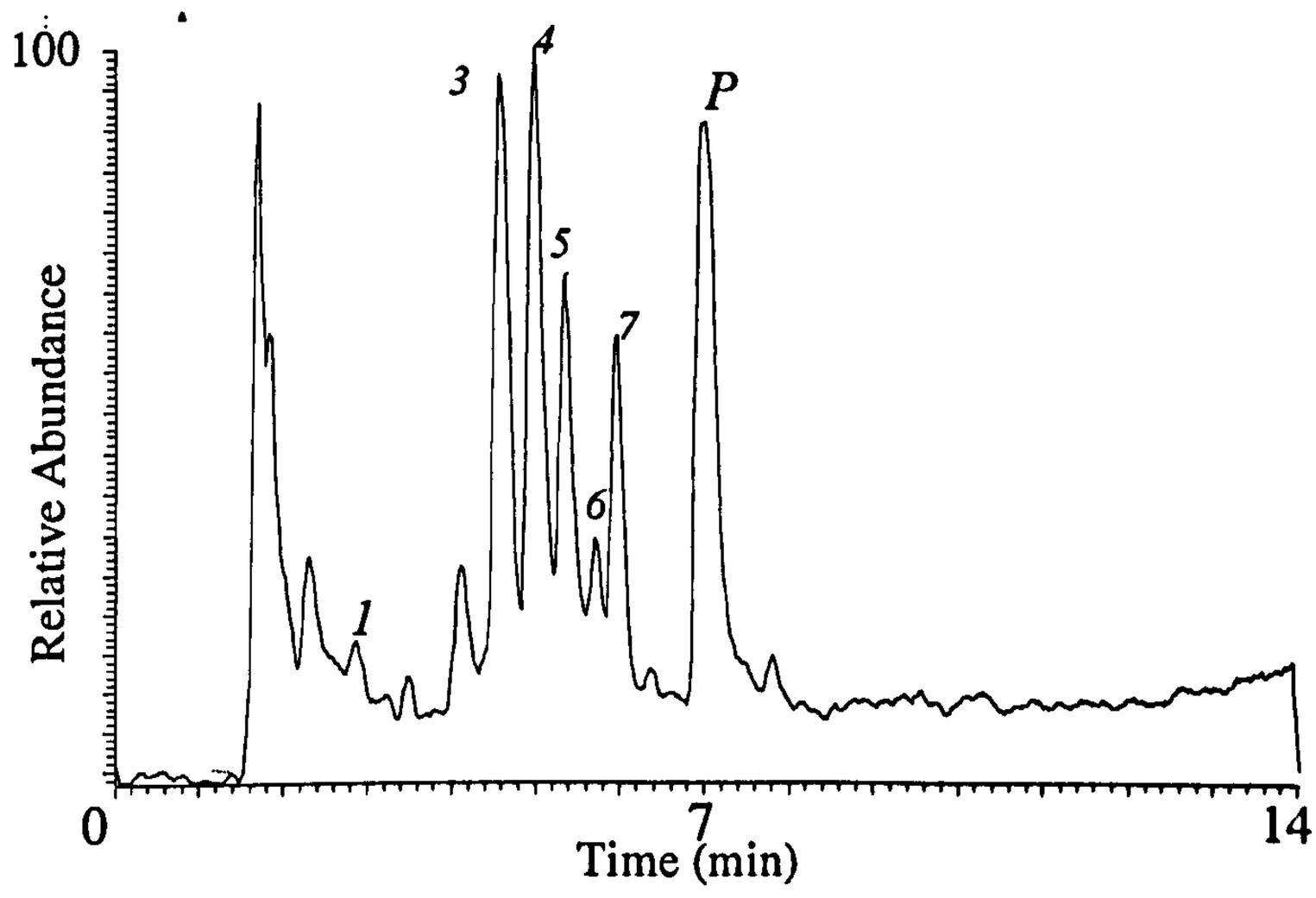


Figure 4.24 Total ion chromatogram of a 2h incubation mixture of pentamidine (100μM) and rat hepatocytes ($5x10^6$cells/ml) obtained from 100μl injection of the pre-concentrated sample by SPE [P-represents pentamidine]

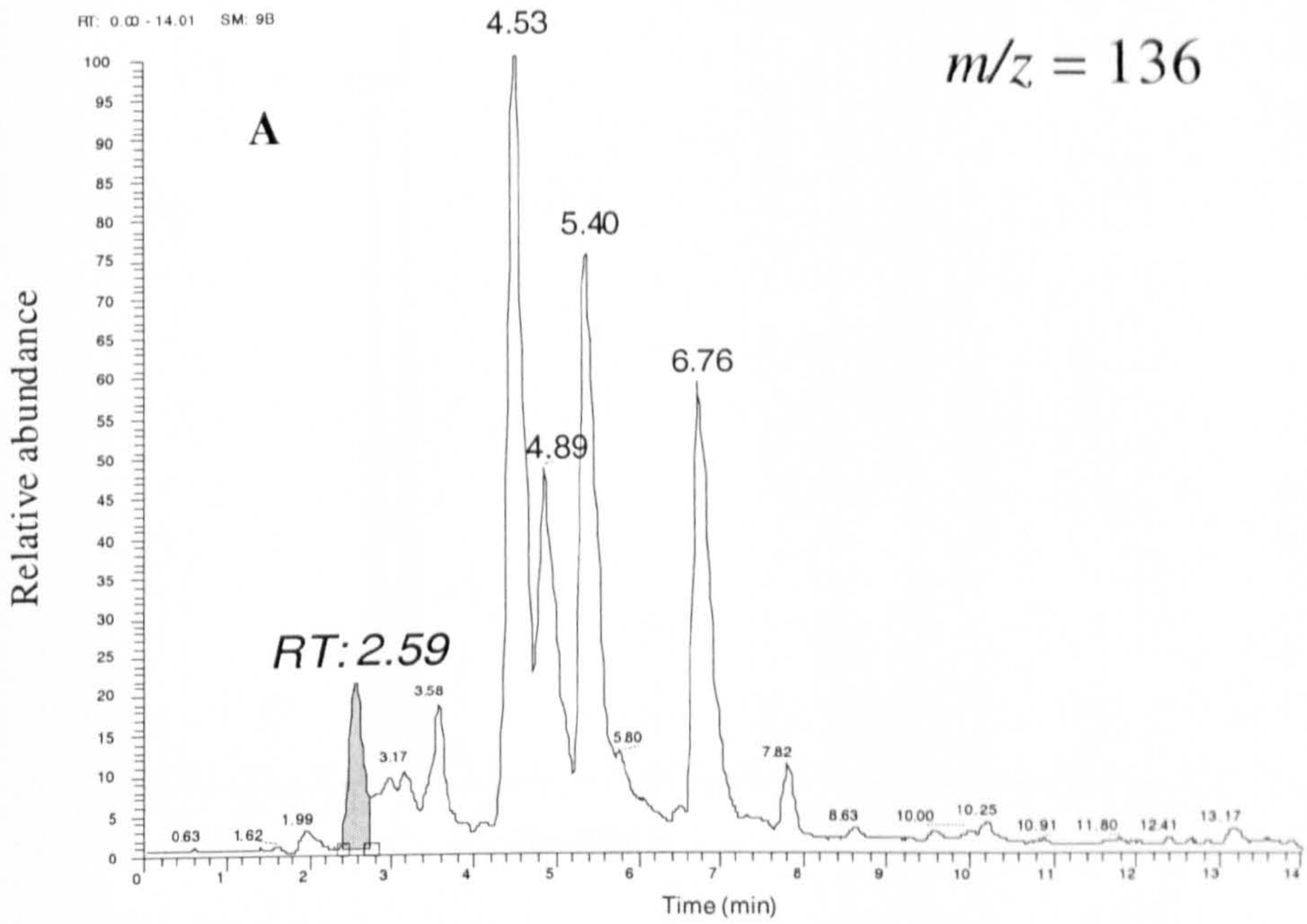


Figure 4.25 Extracted ion (*m/z* 136) chromatogram A) and product ion spectrum B) of proposed metabolite (*p*-hydroxybenzamidine) with retention time (RT) 2.59min.

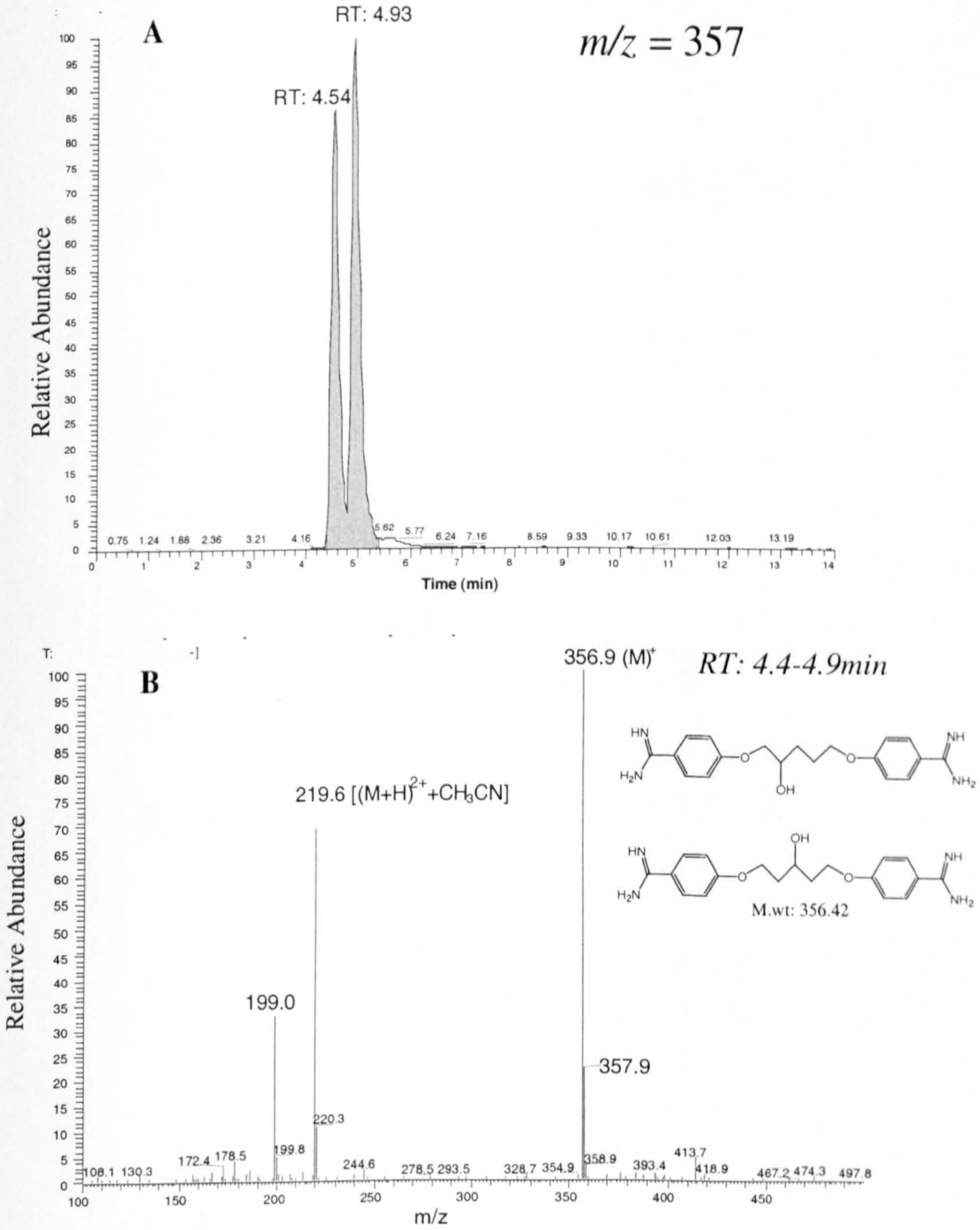


Figure 4.26 Extracted ion (*m/z* 357) chromatogram A) and product ion spectrum B) of isomeric metabolites (2-hydroxy- and 3-hydroxypentamidine) at retention times (RT) of 4.54 and 4.93min.

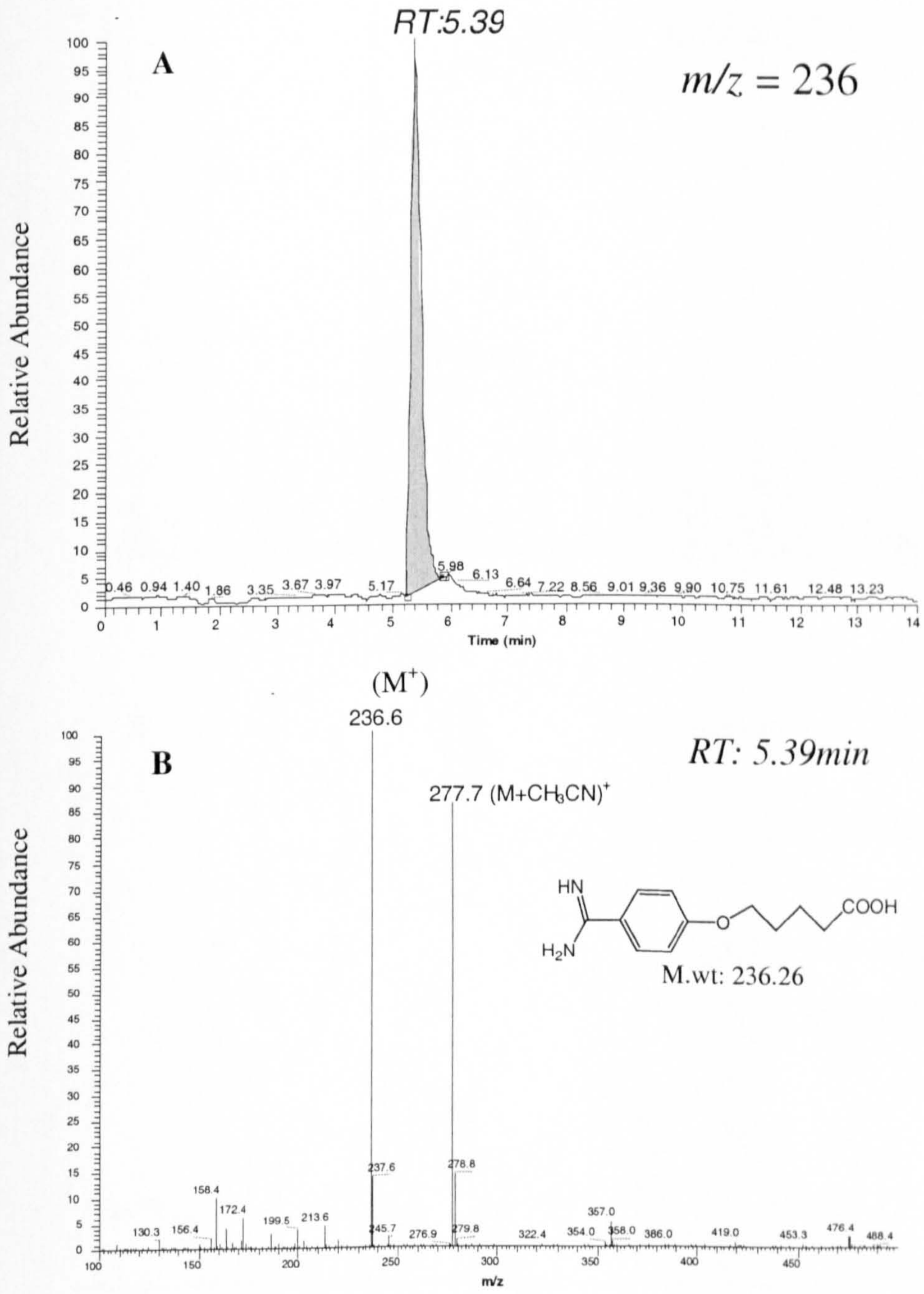


Figure 4.27 Extracted ion (*m/z* 236) chromatogram A) and product ion spectrum B) of the proposed metabolite (5-(4'-amidinophenoxypentanoic acid)) with retention time (RT) 5.39 min.

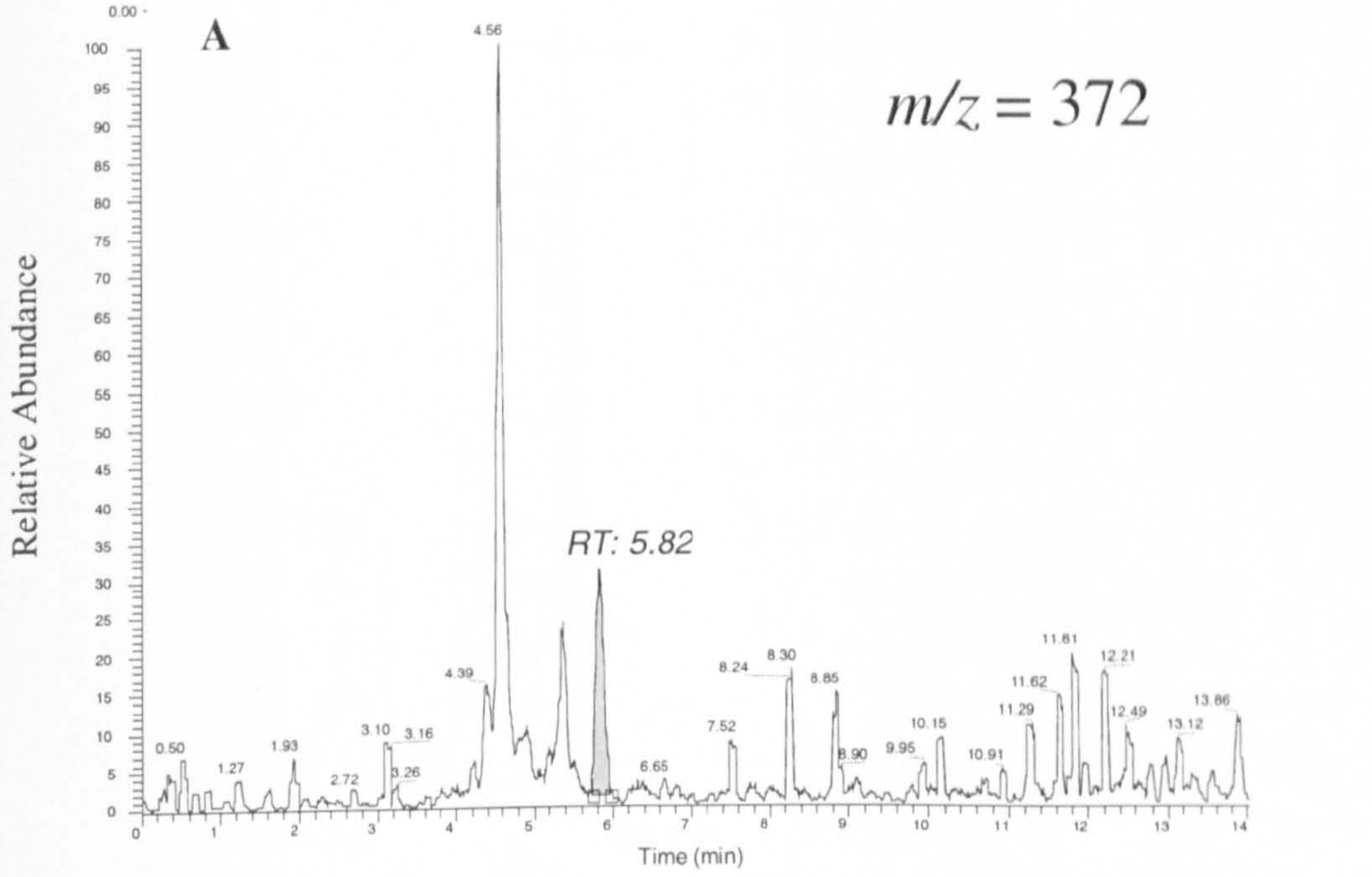
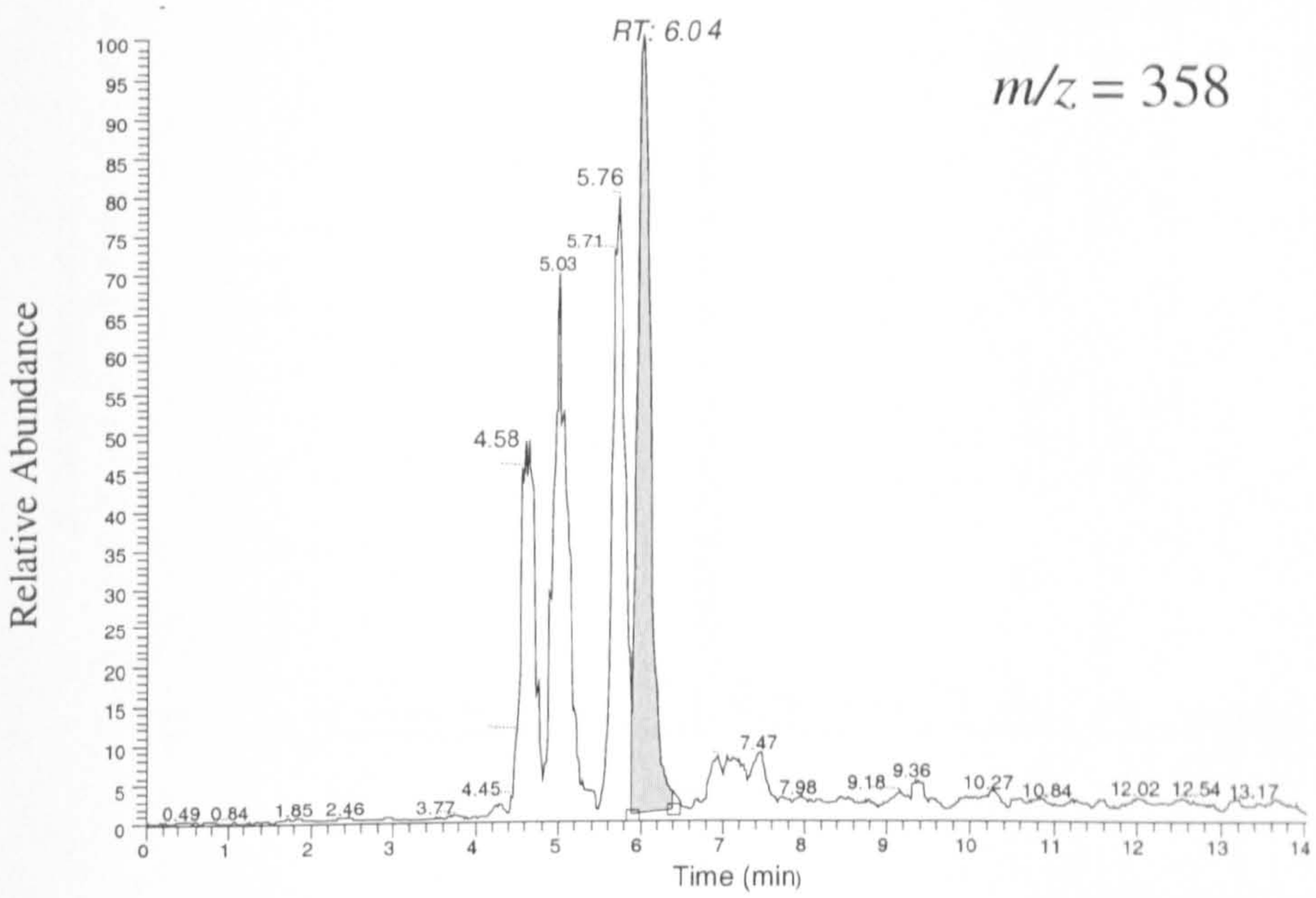


Figure 4.28 Extracted ion (*m/z* 372) chromatogram A) and product ion spectrum B) of the proposed metabolite (*N,N´*-dihydroxypentamidine) at retention time 5.82min



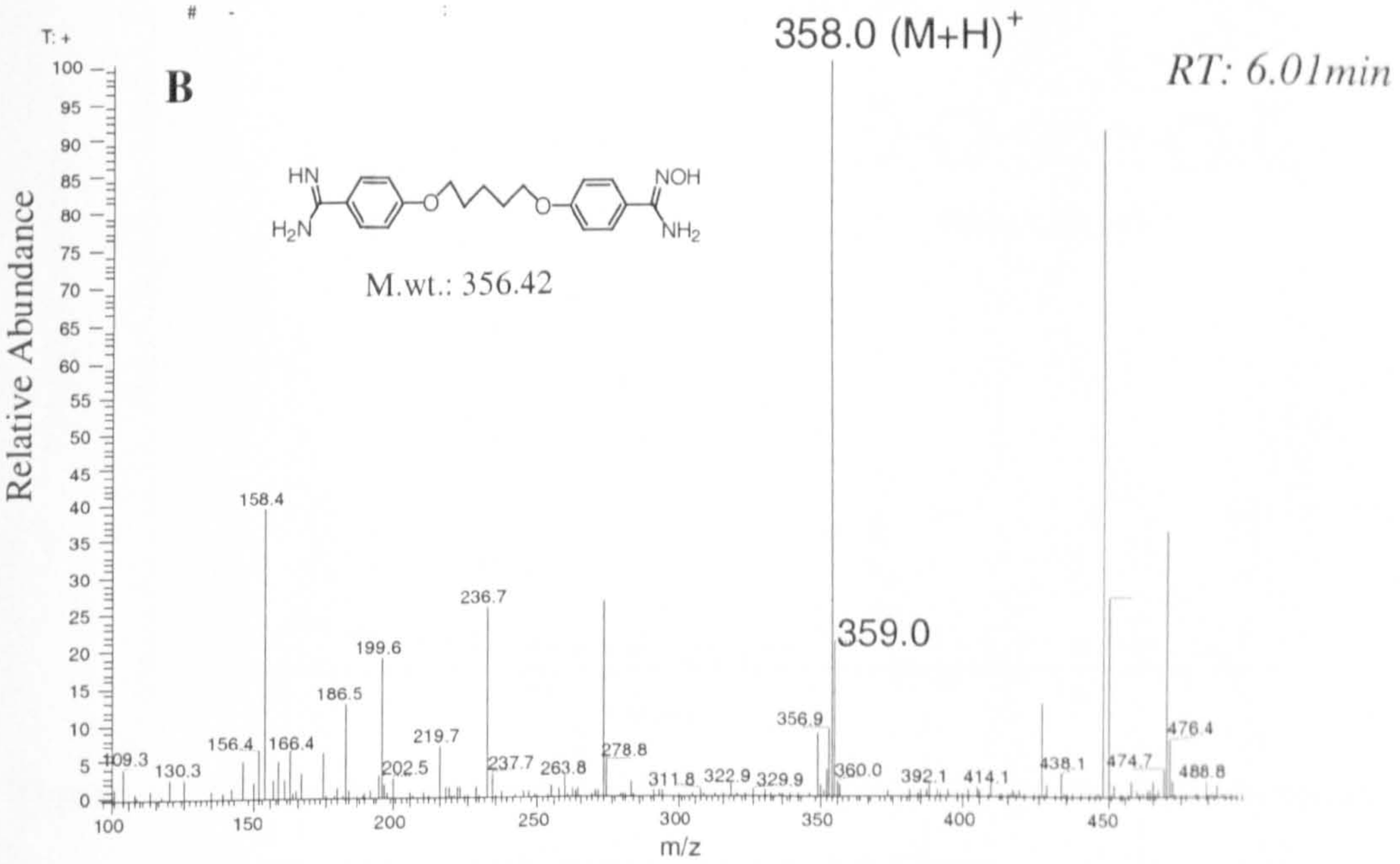


Figure 4.28 Extracted ion (*m/z* 358) chromatogram A) and product ion spectrum B) of the proposed metabolite (N-hydroxypentamidine) with retention time 6.01min

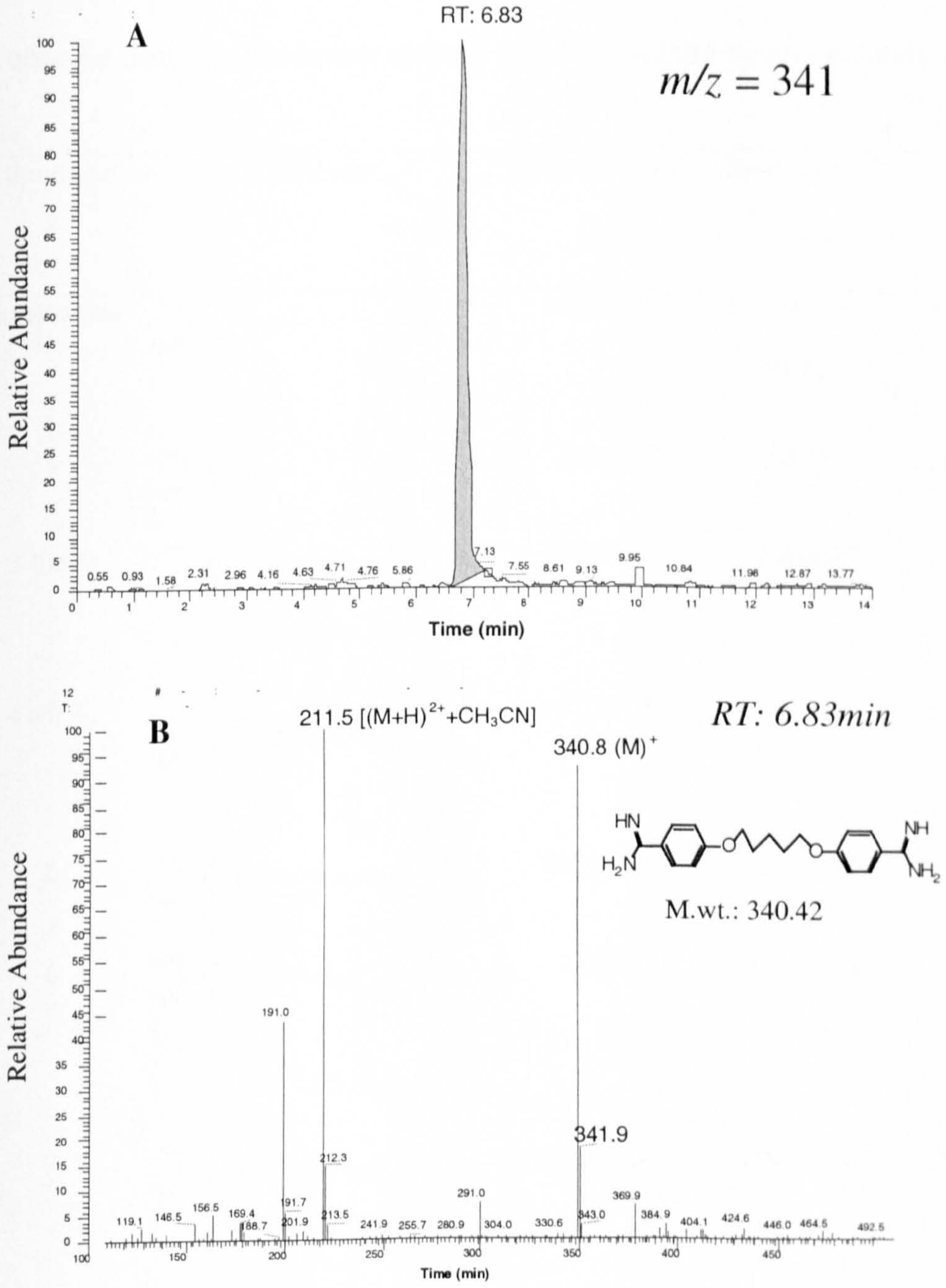
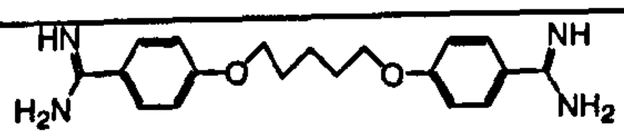
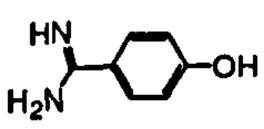
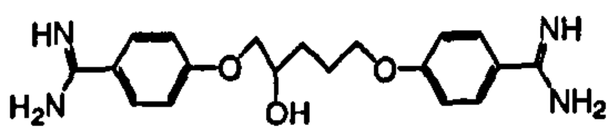
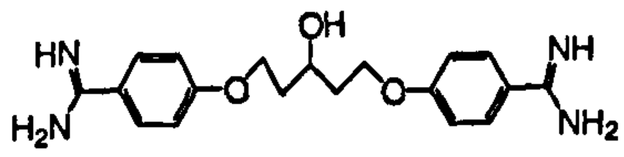
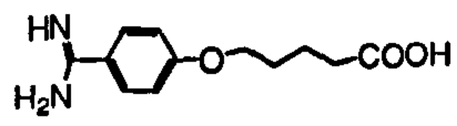
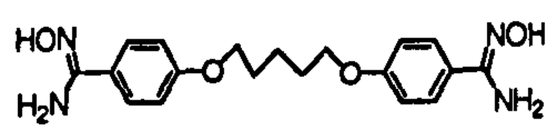
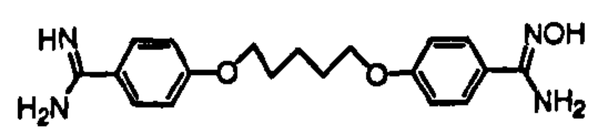


Figure 4.29 Extracted ion (*m/z* 341) chromatogram A) and product ion spectrum B) of pentamidine with retention time at 6.83min

Table 4.4 Structural and mass spectral data of pentamidine and putative metabolites

Compound	Structure	Molecular ion m/z	Base peak m/z	Fragment ions m/z
Pentamidine		341[M+H]$^+$	212[(M+H)$^{2+}$ + CH_3CN]	119,136,160,209,324
1		136 [M]$^+$	136 [M]$^+$	104,119
3 (or 4)		357[M+H]$^+$	178[M]$^{2+}$	119,136,148,160,340
4 (or 3)		357[M+H]$^+$	178[M]$^{2+}$	119,136,160,308,340
5		237[M+H]$^+$	236[M]$^{+\cdot}$	119,136,160
6		372[M]$^{+\cdot}$	358[M+H -OH]$^+$	131,152,178,209,262
7		358[M+2H]$^+$	358[M+2H]$^+$	131,152,187,209,237

The extracted ion chromatogram at *m/z* 357 (figure 4.28) produced two ion peaks with similar product ions at *m/z* 219 $(M+CH_3CN)^+$ and 178 $(M)^{2+}$, which appears to be consistent with the structures of the metabolites being the 2-hydroxypentanol and 3-hydroxypentanol metabolites of pentamidine. However, induced fragmentation failed to produce any diagnostic ions (table 4.4) that might help to discriminate between the two positional isomers. The extracted ion at *m/z* 358 (figure 4.28) produced four peaks which is not unexpected since metabolites *3*, *4*, *7* (table 4.4) have similar molecular weights (357) and metabolite *6*, having a molecular ion at *m/z* 372 is also a parent ion of *m/z* 358. One metabolite peak, designated *2* (figure 4.20) could not be identified in the ion chromatograms. The retention times of pentamidine and metabolite peaks are presented in table 4.5. The differences in the rentention times recorded in the LC-UV chromatograms and mass chromatograms is due to the dead volume introduced by the polyetheretherketone *(PEEK)* tubing that connects the HPLC system to the API ion source of the mass spectrometer.

Table 4.5 Retention times of pentamidine and metabolites by LC-UV and mass spectral detection

Compound	*Retention time (min)	
	UV chromatogram	Mass chromatogram
Pentamidine	6.75	6.86
1	2.49 *(n=2)*	2.50 *(n=2)*
2	3.40	Not detected
3	4.39	4.55
4	4.78	4.94
5	5.23	5.37
6	5.65	5.73
7	5.96	6.03

** Values are averages of 5 determinations (n = 5)*

The metabolites identified in this work have extended the findings from previous studies of the *in vitro* metabolism of pentamidine in rat microsomes (Berger *et al*, 1992). However, while previous reports indicated the formation of only two major metabolites as compounds 3 and 4 (table 4.4), the current study has shown the formation of three major metabolites of pentamidine in rat hepatocytes. In view of a recent report that the oxidative cleavage metabolite of pentamidine, 5-(4-amidinophenoxy)pentanoic acid (compound *5*, table 4.4) can cause kidney necrosis in rats (Zhang *et al.*, 1996), the results of this study have provided important evidence that may help in understanding the toxicity associated with the use of pentamidine.

4.3.4 Metabolism of pentamidine in pig hepatocytes

Qualitatively, there appeared to be no obvious differences in the metabolism of pentamidine by either rat or pig hepatocytes. Five of the seven putative metabolites of pentamidine identified in rat hepatocytes, were observed in pig cells (figure 4.30), which is indicative of the similarities that may exist in metabolic pathways of pentamidine in the two animals. Comparison of HPLC chromatograms of 3h incubation mixtures of pentamidine in the pig and rat hepatocytes (figure 4.31), clearly illustrates the quantitative differences in the metabolic profiles. Whereas metabolite *5* [5-(4'-amidinophenoxy)-1-pentanol] was formed as one of the major metabolites in rat hepatocytes (figure 4.31B), relatively smaller amounts were detected in pig hepatocytes (figure 4.32A). Since relatively small amounts of metabolites were formed in pig hepatocytes (< limit of quantification, LOQ), it was not possible to monitor their rate of formation by LC-UV or LC-MS. Compared to the half-life in rat hepatocytes ($t_{½}$=79.6min), the half-life of pentamidine in pig hepatocytes was much longer ($t_{½}$=693.0min) (appendix IV), showing that the metabolism of pentamidine in rats occurs at a faster rate than in pigs (figure 4.32). The amount of pentamidine remaining after a 3h incubation period with pig hepatocytes (68.56 ± 5.02%) (mean ± S.D., n = 3), was

significantly different ($p < 0.001$, one-way ANOVA) from the amount remaining in rat hepatocytes (3.2 ± 2.3%) (mean ± S.D., n = 3). The results clearly demonstrate the interspecies differences that exist in the metabolism of pentamidine in the two animals.

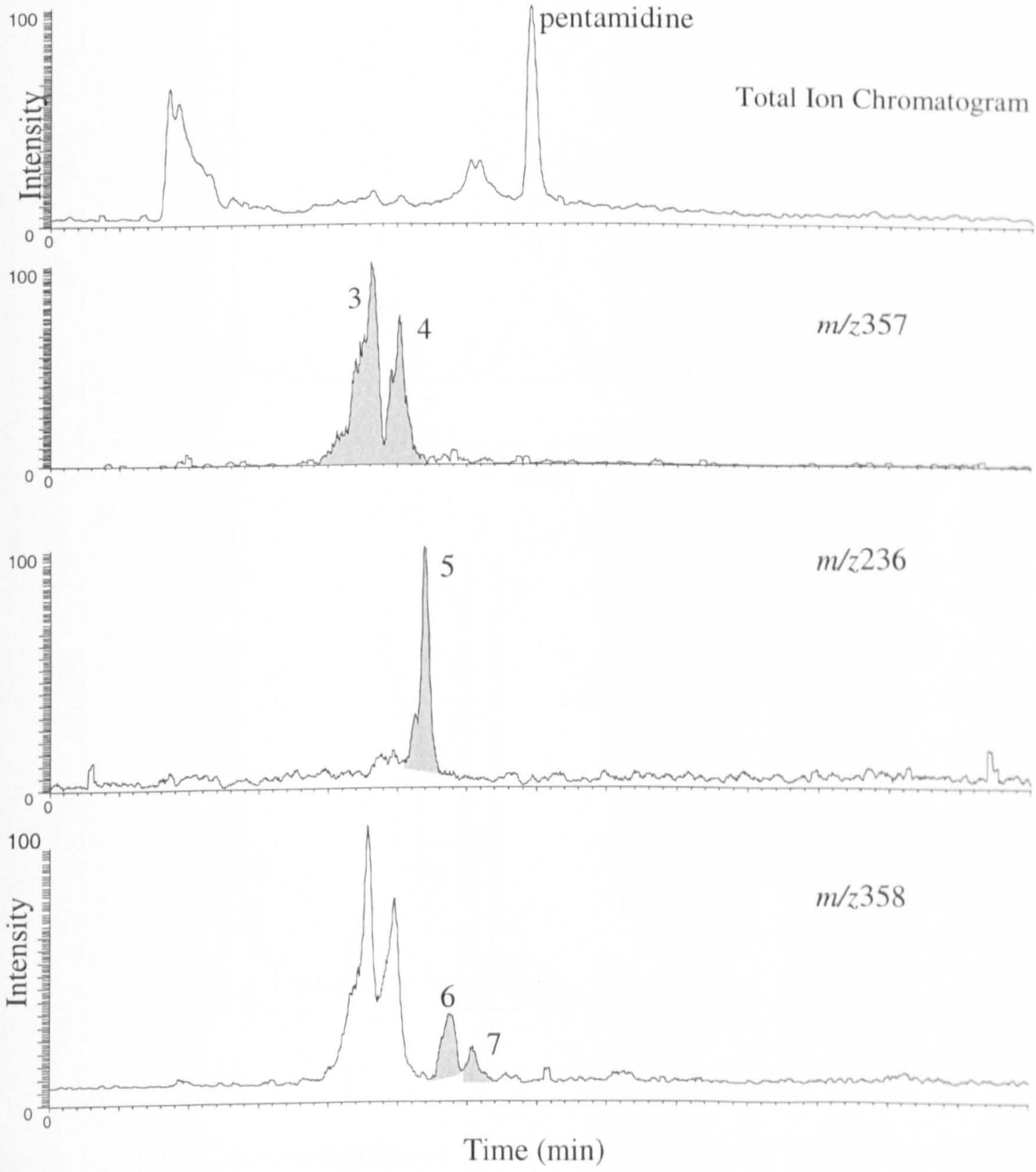


Figure 4.30 Mass chromatograms of a 3h incubation mixture of pentamidine (100μM) with pig hepatocytes ($5x10^6$/ml) showing the total ion chromatogram and the extracted ion chromatograms of metabolites designated 3 to 7 at *m/z* 357, 236 and 358.

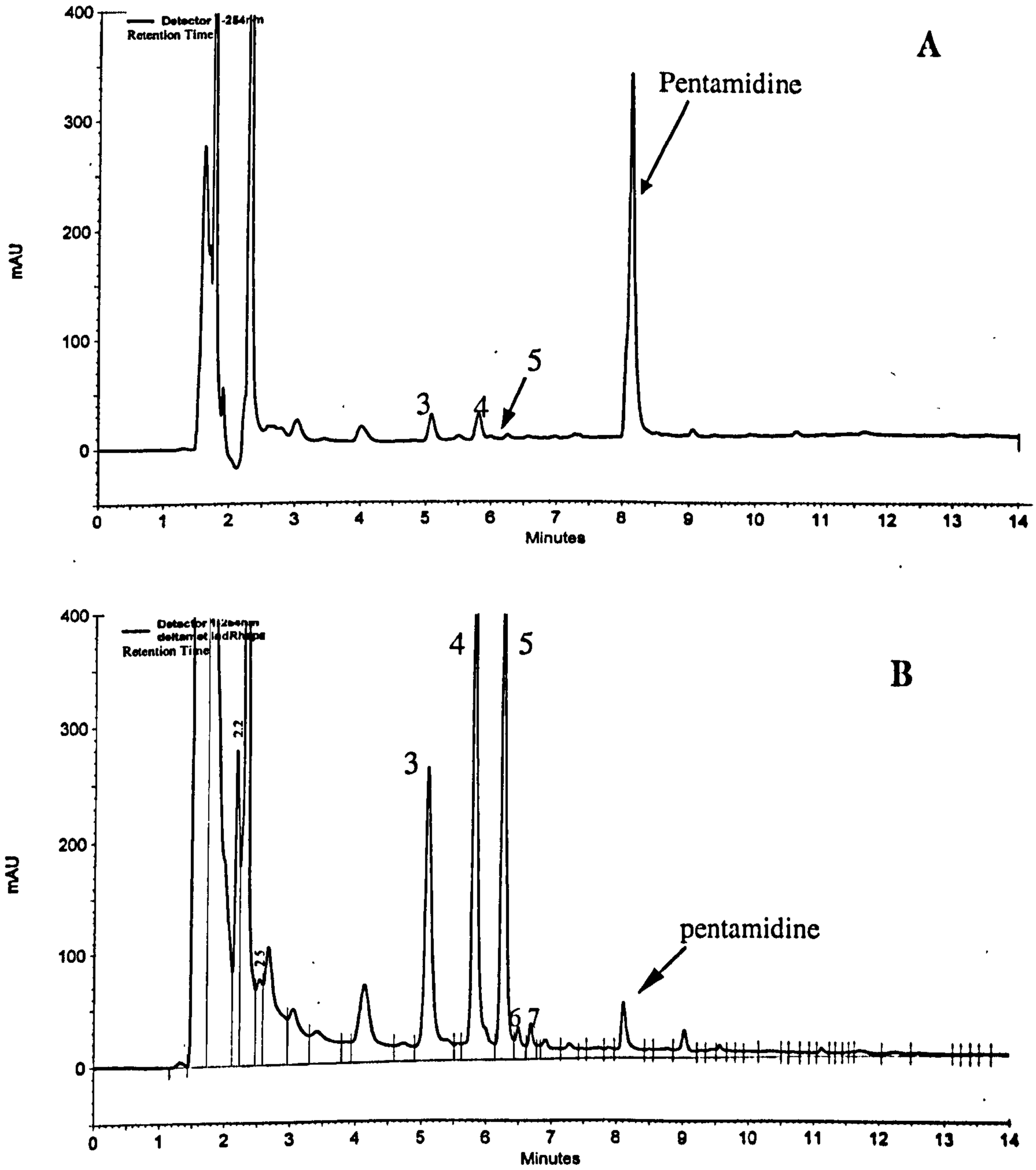


Figure 4.31 HPLC chromatograms of 3h incubation mixtures of pentamidine (100μM) with, A) pig hepatocytes ($5x10^6$/ml) and B) rat hepatocytes ($5x10^6$/ml) showing metabolite peaks designated 3 to 7.

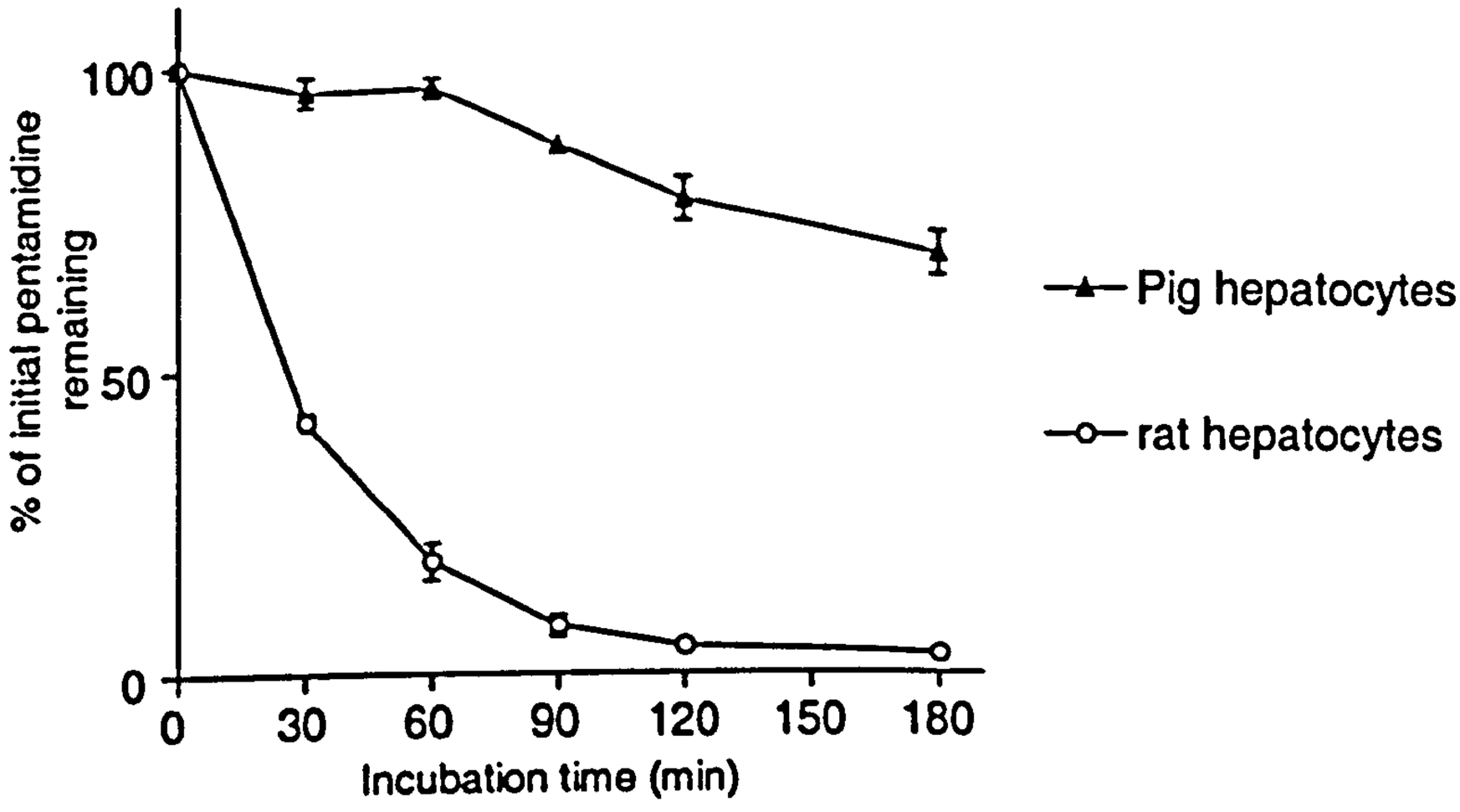


Figure 4.32 Time course of the metabolism of pentamidine (100μM) by pig and rat hepatocytes (5×10^6 viable cells/ml) determined by HPLC.

[Results are mean ± SEM of three determinations (animals)]

Species differences in drug/xenobiotic metabolism have been known for many years but have become topical due to the necessity to relate the metabolism of drugs in animal systems to that in man during routine drug discovery. The results demonstrate that extrapolation of metabolism data from one animal species to another, or to man, may lead to pharmacological problems due to differences in metabolism between different species.

4.3.5 Effects of inducing agents on the metabolism of pentamidine in isolated rat hepatocytes

4.3.5.1 Induction of cytochrome P450 enzymes in isolated rat hepatocytes

The cytochrome P450 content of hepatocytes from control rats was 0.08 ± 0.02 nmol/10^6 cells (mean ± S.D, n=3). This was significantly different ($P<0.05$) from that in cells from PB pretreated (0.67 ± 0.05nmol/10^6 cells) and from 3-MC pretreated animals (0.21 ± 0.01 nmol/10^6 cells) (mean ± S.D, n=3) (Figure 4.33). There was however, no significant difference ($p=0.25$) between the cytochrome P450 content of hepatocytes from DM pretreated rats (0.16 ± 0.08 nmol/10^6 cells) (mean ± S.D, n=3) and that of the control group.

The observed enlargement of livers in inducer treated (PB, 3-MC) rats and the significant increases in hepatocyte cytochrome P450 content gives an indication of successful enzyme induction in the animals used for this study. The absence of significant induction by DM in this study is also consistent with previous studies indicating that pyrethroids are generally weak inducers of rat hepatic microsomal enzymes (Krechniak and Wrzesniowska, 1991). Dayal and collaborators (1999) have reported the absence of significant increases in hepatic P450 content in rat livers following the intraperitoneal administration of deltamethrin at low doses (5mg/kg) over 7 days. However significant increases in P450 content were observed after intraperitoneal injection of consistently high doses (10-15mg/kg) of DM over the same period of time (7 days). Rats used in our induction studies were pretreated with a single intraperitoneal dose of deltamethrin (50mg/kg) before isolation of the livers.

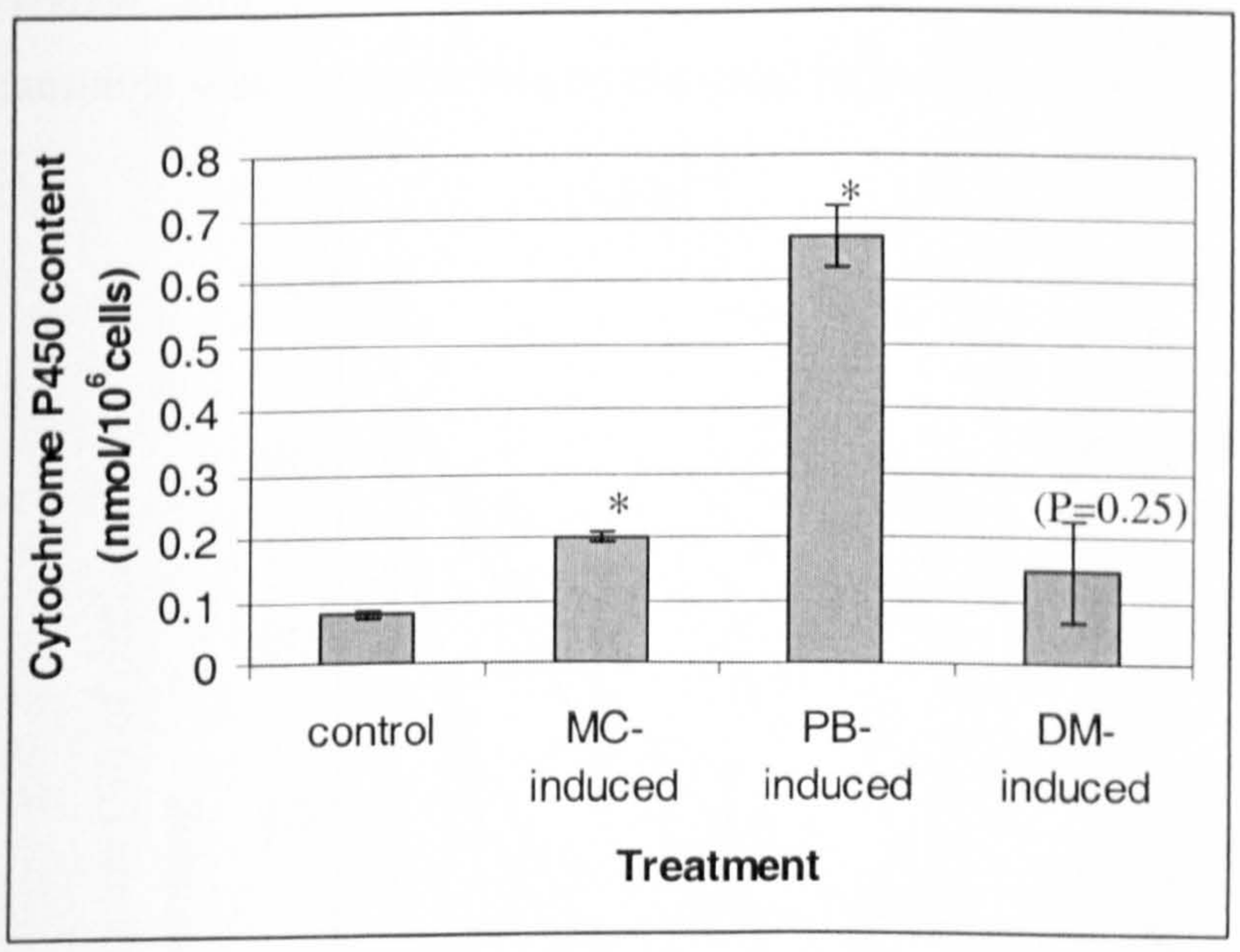


Figure 4.33 Effects of inducing agents (3-MC, PB and DM) on the content of cytochrome P450 in rat hepatocytes isolated from pretreated animals. [mean ± S.D., n=3]. * $p<0.05$, compared with control, by one-way ANOVA.

4.3.5.2 Effects of induction on pentamidine metabolism

There were significant differences in the rate of metabolism of pentamidine in hepatocytes from the inducer treated rats compared with that in hepatocytes from the control animals (figure 4.34). The percentage of initial amount of pentamidine remaining after a 3h incubation with hepatocytes from control animals was 3.2 ± 2.3% (mean ± S.E.M., n=3 animals). This was significantly different ($P< 0.05$, one-way ANOVA) compared with that remaining after 3h incubation with cells from rats pretreated with 3-MC (25.41 ± 1.6%), PB (15.5 ± 3.1%) and DM (7.1 ± 1.2%) (mean ± *S.E.M., n=3*). 3-MC caused the highest overall inhibitory effect on pentamidine metabolism (judged by a comparison of the percentage of parent pentamidine remaining after 3h) of 8-fold, PB

4.8-fold and DM 2.2-fold. The above results appear to confirm earlier observations made by Berger and coworkers (1992), indicating that the microsomal metabolism of pentamidine was not inducible by classical inducing agents.

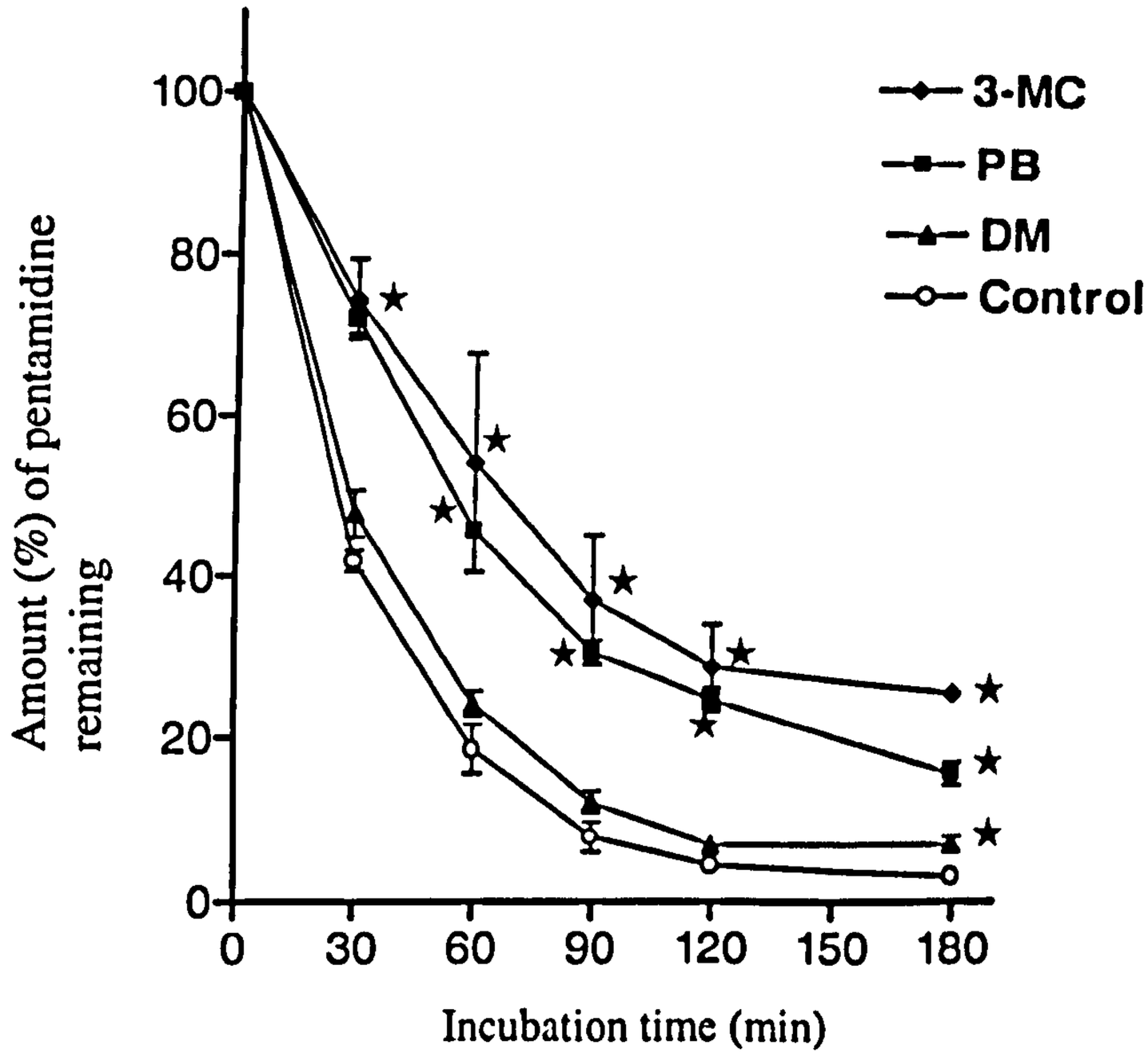


Figure 4.34 Time course of the disappearance of pentamidine (100μM in Krebs-Hepes buffer, pH 7.4) over a 3h incubation period in hepatocyte incubations ($5x10^6$ cells ml^{-1}) from control (n=3), PB-(n=4), 3-MC-(n=3) and DM-(n=3)pretreated rats [Results are mean ± S.E.M]. *P<0.05, on-way ANOVA.

These observations raise a number of unanswered questions as follows:

- If the metabolism of pentamidine in rat liver microsomes has been shown to be cytochrome P450 dependent (Berger, 1990), why does the induction of cytochrome P450 (by PB and 3-MC, figure 4.33) result in the inhibition of pentamidine metabolism (figure 4.34)?
- What are the toxicological and/or pharmacological implications of these results for patients that use pentamidine?

In order to find answer(s) for the observed inhibition of pentamidine metabolism in inducer treated animals, there is a need to examine the specific P450 isoenzymes that are inducible by PB, 3-MC or DM, and to find out whether or not the induction of these specific isoenzymes can exert a negative (down) regulation of pentamidine metabolism. PB administration in rat is known to cause major increases in CYP2B1, 2B2 and 2C6, while 3-MC causes induction of CYP1A1 and 1A2 isoenzymes (Waxman and Azaroff, 1992; Whitelock, 1986). Deltamethrin has been shown to be a mixed-type of inducer the effects of which are reminiscent of a PB-type inducer (Dayal et al, 1999). Although DM has also been shown to induce CYP1A1/1A2 in rat hepatocytes, it is not known to act at the Ah-receptor and has effects more similar to those of PB at high doses ((Dayal et al, 1999). Although the specific isoenzymes involved in the metabolism of pentamidine are not fully defined, the results in this study clearly show that pentamidine is not a substrate of the isoenzymes inducible by either PB, DM or 3-MC. This is supported by the work of previous authors (Clement and Jung, 1994) who have identified the non-inducible rabbit liver cytochrome P450-2C3 to be a microsomal *N*-hydroxylase found in humans and rats. It is also likely that the induction of a particular set of P450s is leading to down regulation of the isoenzyme(s) responsible for the metabolism of pentamidine.

The results in this study may have important toxicological implications for the clinical use of pentamidine for treatment of African sleeping sickness and *pneumocyctis carinii* pneumonia in HIV patients. The use of multiple drug therapy in management of diseases such as AIDS can result in drug-drug interactions, which could mimic the inhibitory effects of the inducers used in this study. This might result in apparent increases in the half-life of pentamidine in plasma leading to toxic effects.

4.3.5.3 Effects of induction on the metabolite profiles of pentamidine

When compared to animals in the control group, pretreatment of rats with either PB, 3-MC or DM did not result in any qualitative differences in metabolic profiles of pentamidine. However, there were quantitative differences in the formation rates of the three major metabolites of pentamidine (figure 4.35). By assuming equal molar extinction coefficients of pentamidine and metabolites, the formation of each of the 3 major metabolites (compounds *3*, *4* and *5*, table 4.4) was expressed as a percentage of the initial concentration of pentamidine. When compared to the control group, pretreatment of rats with PB did not result in any major differences in the profiles of the 3 major metabolites of pentamidine (figure 4.35). The overall formation profiles of metabolites M3 and M4 (2-hydroxy and 3-hydroxy pentamidine) in the four groups of rats appeared to be similar over the 3h incubation period (figure 4.35). However, there were significant differences ($p < 0.05$, one-way ANOVA) between the amounts of metabolites (M3, M4) formed in 3-MC pretreated animals and that of the control group, between 1h to 3h incubation period. There was a 3.3- and 3.4-fold increase in the relative amounts of metabolites M3 and M4 (38.93 ± 2.0% and 51.48 ± 2.1%; mean ± S.E.M., n=3) formed by hepatocytes from 3-MC pretreated rats compared with that formed by the cells from the control rats (11.9 ± 2.2% and 14.79 ± 2.7%; mean ± S.E.M., n=3) after 3h, whereas no major differences were observed between the control and either PB or DM treated groups. There was a 5.1-fold decrease in the relative amount of M5 [5-(4-amidinophenoxy) pentanoic acid)] formed by cells from 3-MC pre-treated rats (4.36 ± 0.2% of initial amount of pentamidine, mean ± S.E.M., n=3) compared with that formed in hepatocytes from control rats (22.0 ± 2.9% of initial amount of pentamidine, mean ± S.E.M., n=3) ($p<0.05$) (figure 4.35) after a 3h incubation period. Pretreatment of rats with DM resulted in a 1.5-fold increase in the amount of M5 formed by hepatocytes after a 3h incubation period (32.5 ± 2.1%) compared with that in the cells from control animals (22.0 ± 2.9%; mean ± S.E.M., n=3).

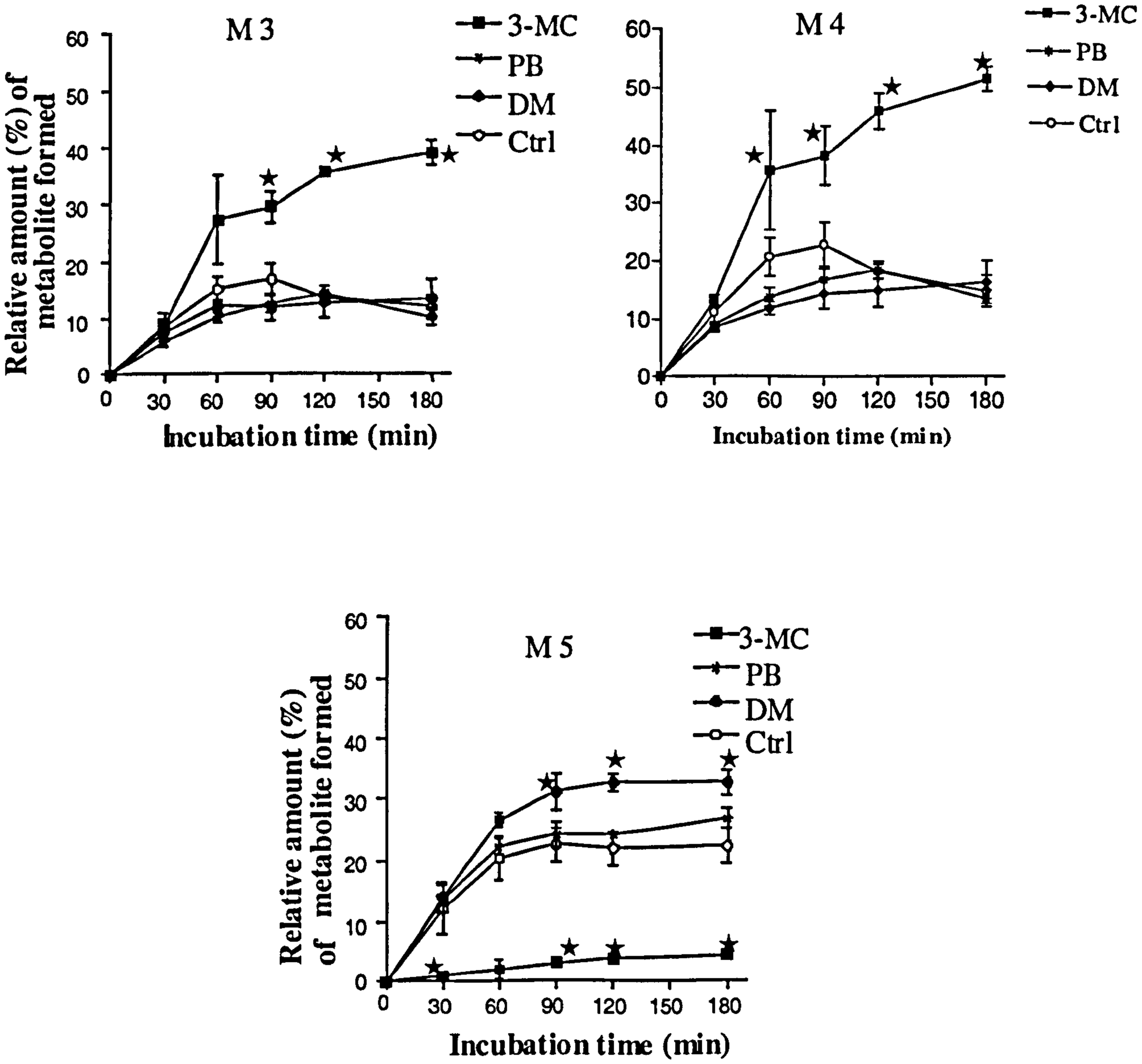


Figure 4.35 Differences in the relative formation rates of the three major metabolites of pentamidine; M3, M4 (2-hydroxy and 3-hydroxy pentamidine) and M5 [5-(4-amidinophenoxy)-pentanoic acid] in 3h incubations of pentamidine (100μM) with the four groups of rats hepatocytes used for incubation experiments [Ctrl = control].

Knowledge of the pharmacological and/or toxicological properties of the three major metabolites of pentamidine underlines the relevance of the results in this study. It has been recently reported (Zhang *et al.*, 1996) that metabolite M5 [compound 5, table 4.4], one of the major metabolites of pentamidine recovered in this study, causes kidney necrosis in rats. The fact that the present study has demonstrated significant differences in the profiles of this metabolite in DM and 3-MC pretreated rats (fig. 4.35), implies that pentamidine metabolism with the concomitant exposure to pyrethroid insecticides such as deltamethrin could lead to toxification. This is of clinical relevance especially in trypanosomosis endemic countries of Africa, where pyrethroid insecticides are employed for the control of insect vectors such as tsetse flies and mosquitoes (malaria).

4.3.6 Metabolism of diminazene by rat and pig hepatocytes

Unlike *p*-aminobenzamidine and pentamidine, diminazene was not metabolized by either rat or pig hepatocytes. Metabolic investigations with diminazene gave rise to a number of challenges. For comparative purposes, there was the need to synthesize the potential metabolites (*N*-oxygenation derivatives) of diminazene, which could be formed with hepatocyte incubations. The synthesized reference compounds could then be used in preliminary experiments for HPLC method development and to confirm their stabilities under the conditions prevailing during the metabolic experiments and sample work up. The *N*-hydroxylated derivatives of diminazene were synthesized by the method of Clement *et al.* (1992) and identified by LC-MS analyses of the crude mixture (figure 4.37). However, attempts to isolate and purify the individual synthetic compounds by preparative HPLC was unsuccessful as a result of co-eluting products and the degradation of products in the acidic mobile phase prior to evaporation. Attempts to separate the components of the crude mixture by TLC failed as a result of the difficulty encountered in separating the strongly adsorped analytes from the silica gel.

In order to improve the chances for metabolite detection and identification, a larger incubation volume (10ml) was pooled from separate incubation experiments carried out between 1 to 3h, and pre-concentrated by SPE as described in section 4.2.2.7. Injection of 50-100µl of the reconstituted (250µl water) incubation samples were made onto the LC-MS system and the representative chromatograms are shown in figure 4.38.

Examination of the HPLC chromatograms did not reveal any new peaks which could be assigned to metabolites. Another alternative to facilitate detection was the operation of the LC-MS instrument in a highly sensitive mode; *i.e* selected ion monitoring mode (SIM) with the mass filter set to monitor the molecular ions of putative metabolites of diminazene (*m/z* 297, 313, 151,177, 323; figure 4.39).

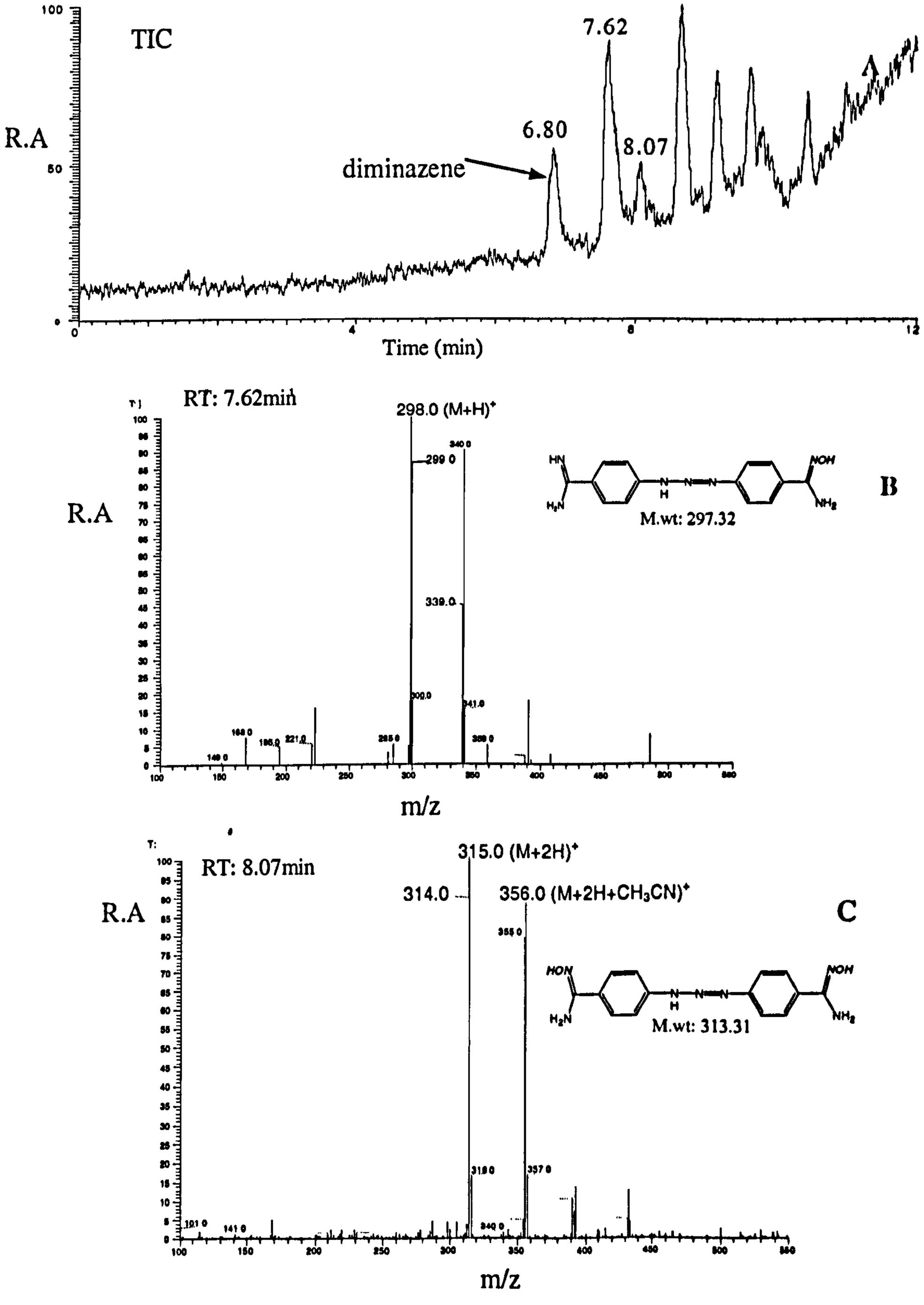
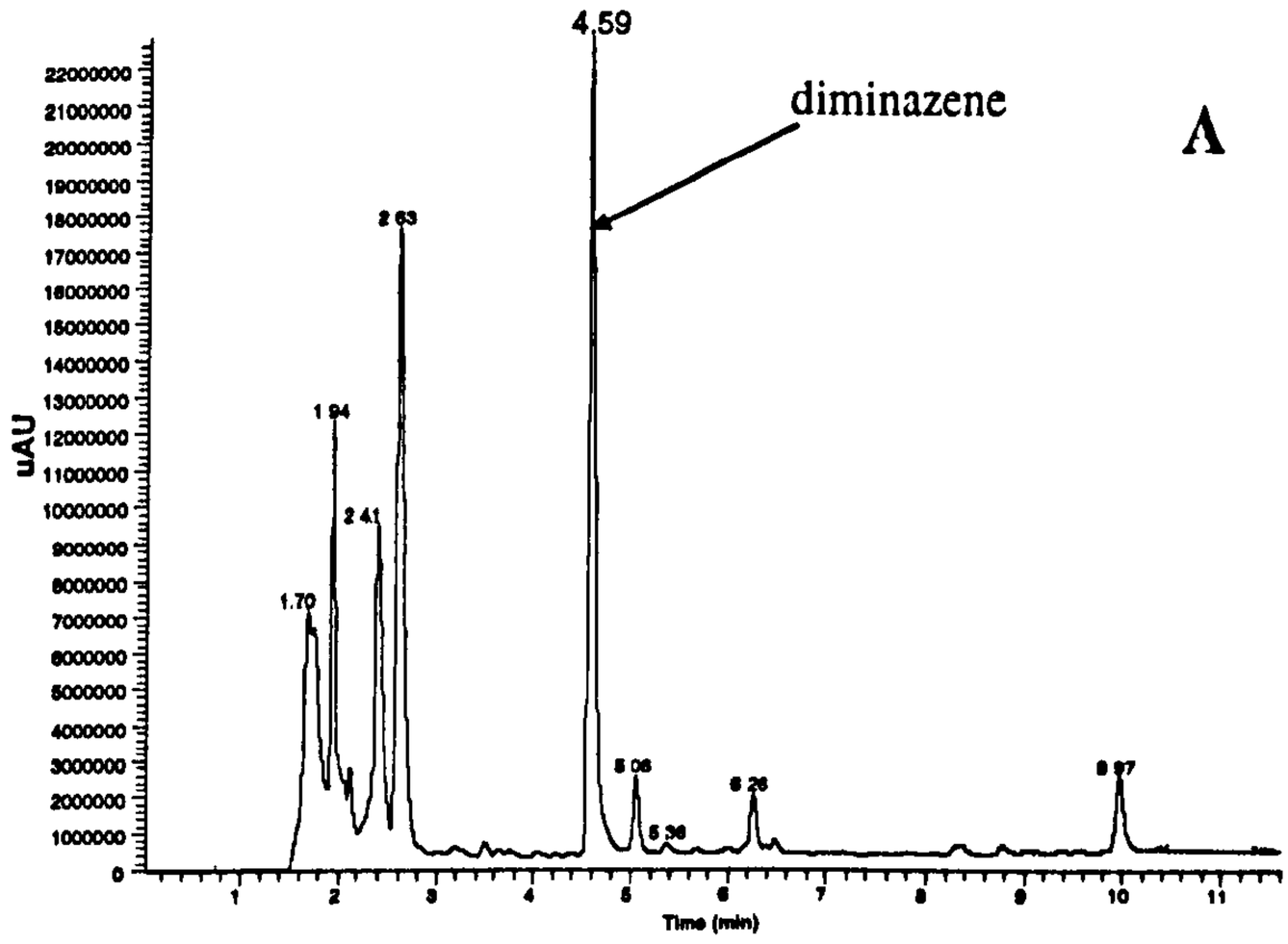
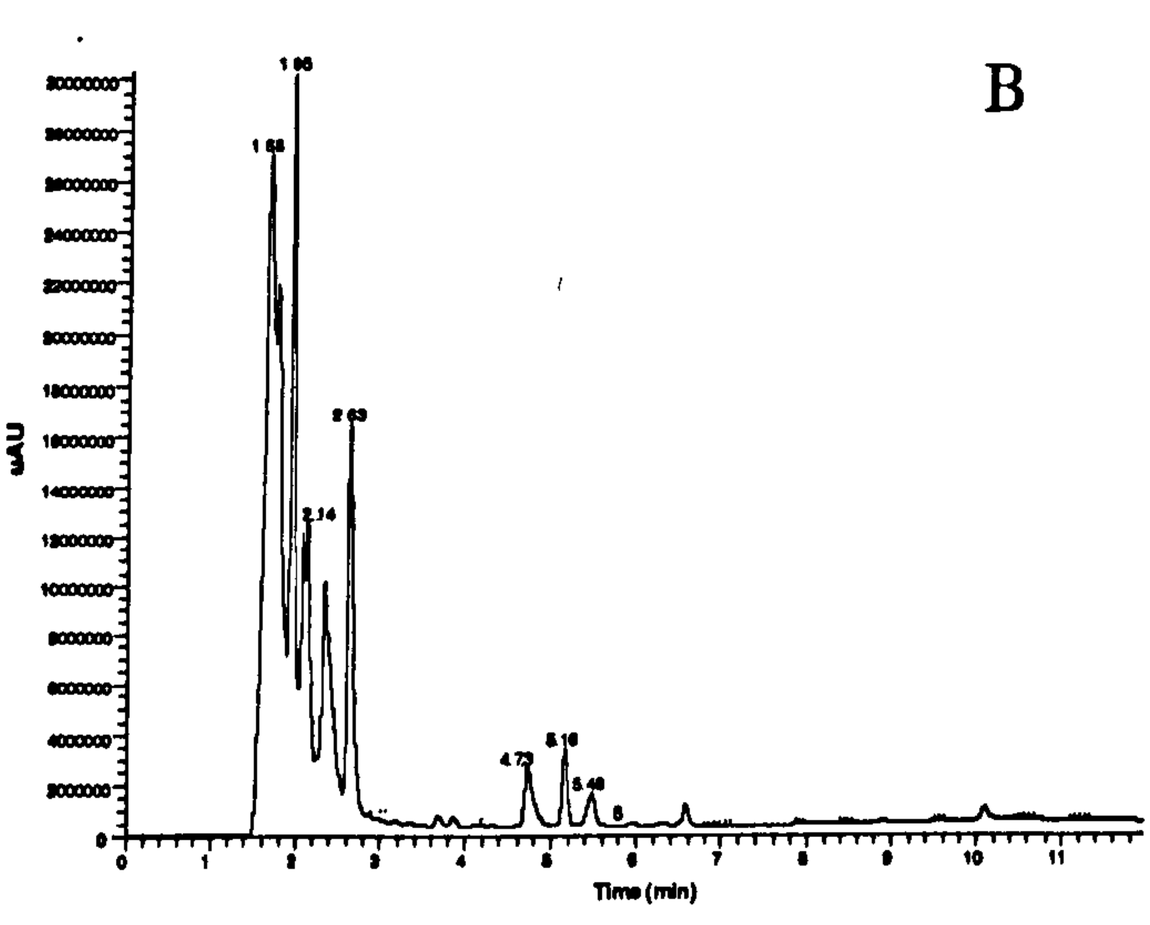


Figure 4.37 A) Total ion chromatogram (TIC) of a crude synthetic mixture of diminazene and *N*-oxygenated products (mono- and dioximes), and product ion spectra of B) monoxime (RT: 7.62min) and the C) dioxime (RT: 8.07min).

In view of the fact that the biotransformation of *p*-aminobenzamidine to *p*-amino-*N*-acetyl-benzamidine had earlier been demonstrated in this study with isolated rat and pig hepatocytes, it was perceived that *N*-acetylation of diminazene could be a possible metabolic pathway in the two animal species. However, attempts to extract the molecular ions of all the possible metabolites (fig. 4.39) in mass chromatograms of respective incubation mixtures of diminazene with rat and pig hepatocytes failed to uncover any metabolite peaks.





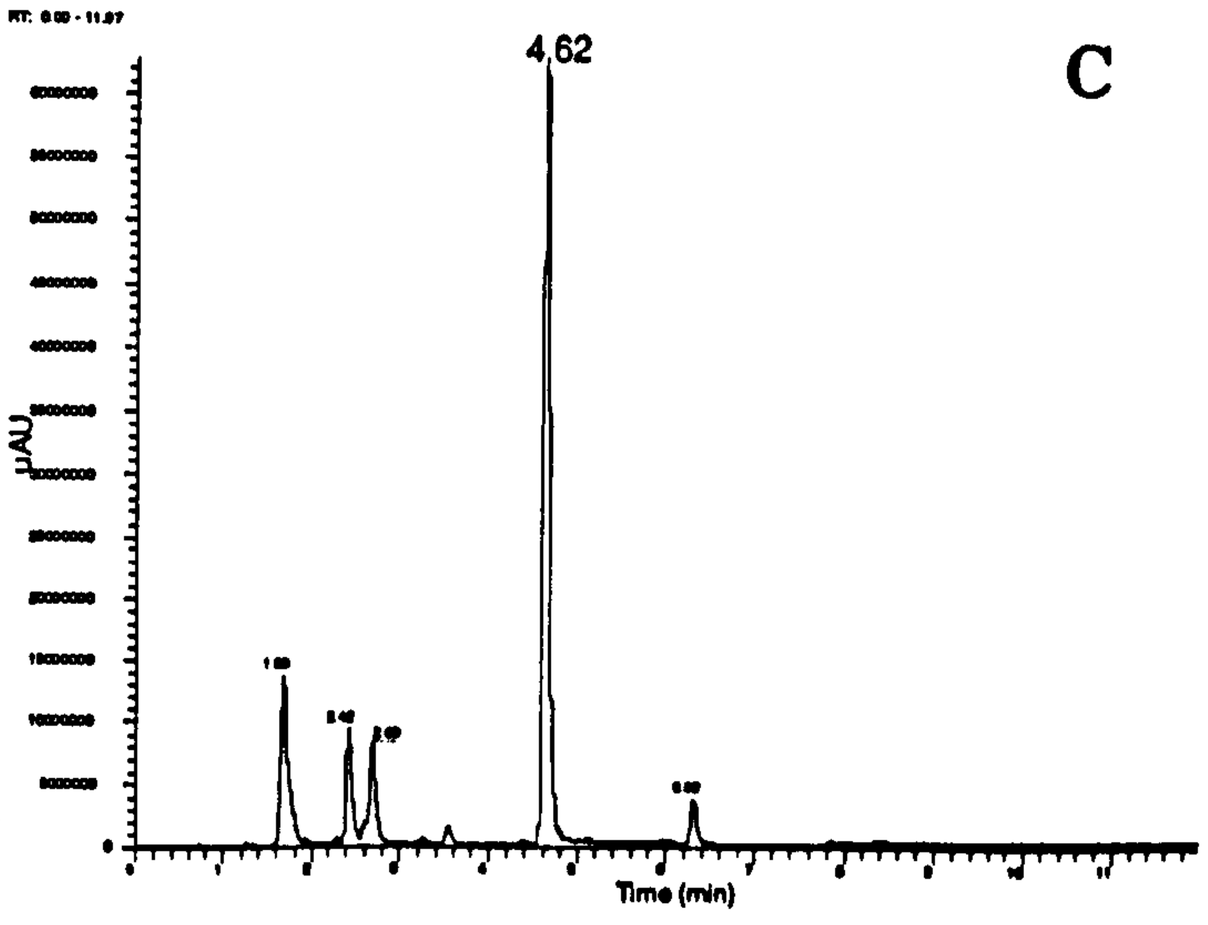
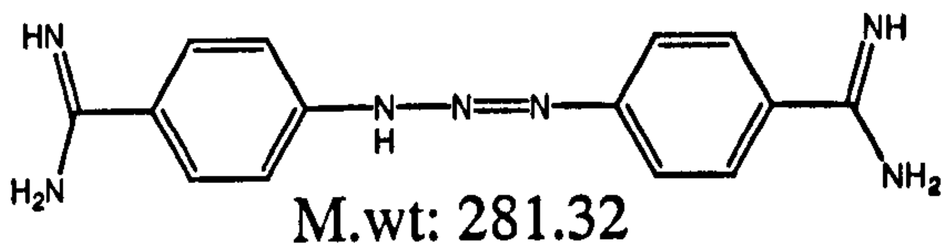
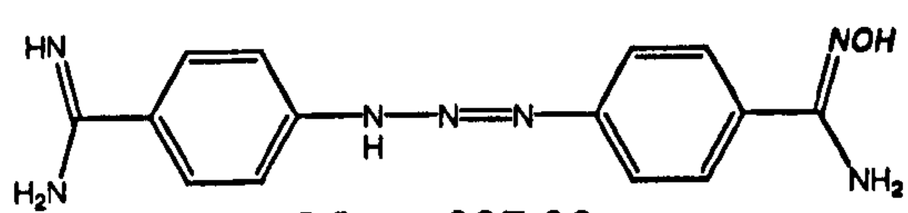


Figure 4.38 HPLC-UV chromatograms of: A) a 3h incubation of diminazene (100µM) and rat hepatocytes ($5x10^6$cells/ml); B) cell control incubation (without added drug) and C) drug control (without added cells)

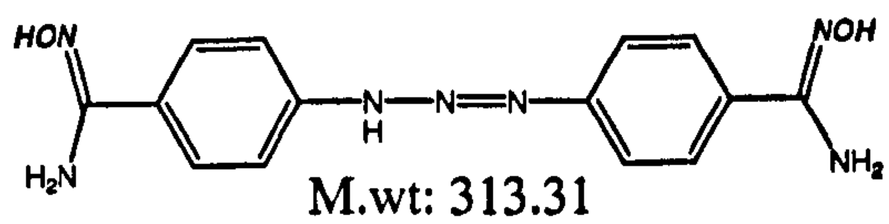


M.wt: 281.32

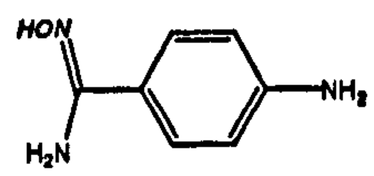
diminazene



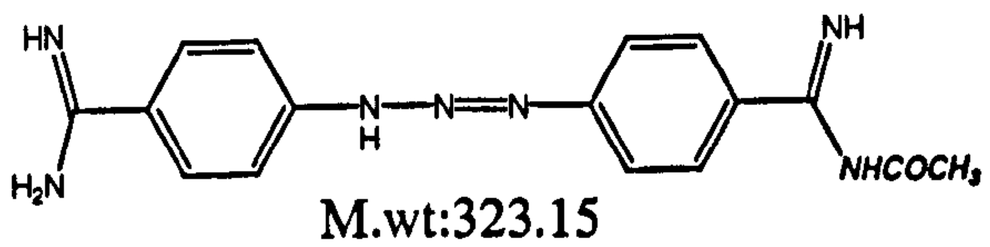
M.wt: 297.32



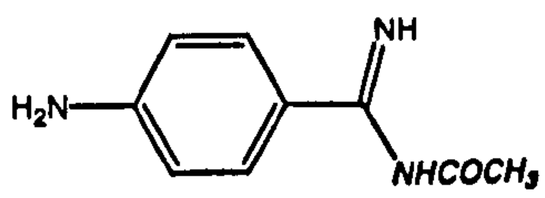
M.wt: 313.31



M.wt: 151.07



M.wt:323.15



M.wt:177.20

Figure 4.39 Possible metabolites of diminazene

4.3.6.1 Effect of induction on metabolism of diminazene in rat hepatocytes

No metabolites of diminazene were detected in incubations with rat hepatocytes isolated from any of the inducer treated animals. The inability to observe metabolism of diminazene in the current study raises a number of important questions:

- If isolated rat and pig hepatocytes are capable of *N*-hydroxylation of pentamidine, and N-acetylation of *p*-aminobenzamidine, (as demonstrated in this study), why is the same not true for diminazene, which also has amidino functionality?
- Is diminazene inert to metabolism in other animal species, especially cattle and other ruminants that are treated with the drug?

Understanding the structure-activity relationships and mechanisms that underlie the microsomal N-hydroxylation of aromatic amidines, would help finding an answer to the first question above.

4.3.6.2 Structure-activity relationships and mechanisms of N-oxidative metabolism of amidines

Since Clement (1983) first reported the microsomal *N*-hydroxylation of benzamidines to benzamidoximes, this biotransformation has been the subject of continued investigations. In attempts to elucidate the mechanism of microsomal *N*-hydroxylation of *N,N*-unsubstituted benzamidines (figure 4.40), Clement and Zimmermann (1988b) investigated the structure-activity relationships of a number of *para*-substituted benzamidines (table 4.6), by measuring the formation rates of the respective benzamidoximes in the 12,000*g* supernatant fraction of rabbit liver homogenates. They observed that the presence of electro- donating substituents (methyl and methoxy groups) appeared to increase the reaction velocity (V_{max}) i.e the formation rates of the benzamidoximes, whereas the presence of electron-accepting substituents (halides-Cl, -Br and nitriles) decreased the rate of N-hydroxylations (table 4.6). However, no metabolite (benzamidoxime) was formed in incubations with *para*-nitrobenzamidine.

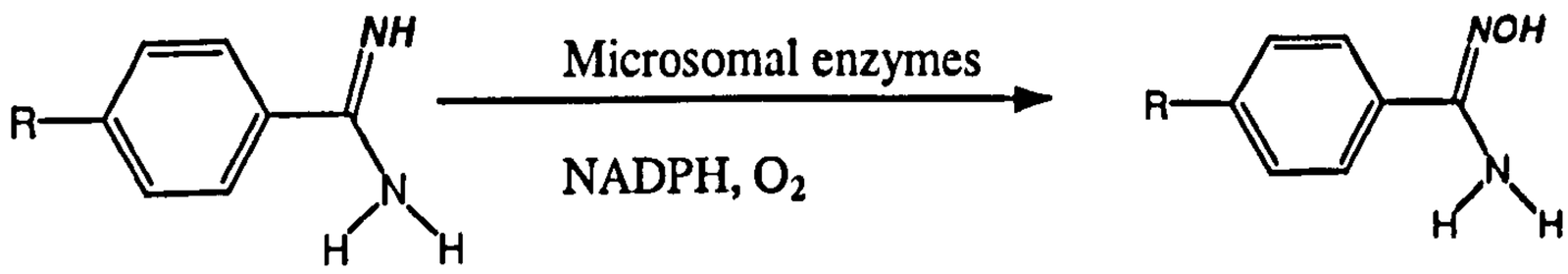
The authors obtained a significant correlation between the reaction velocity (V_{max}) and the Hammett σ_{para} constants of para-substituents (table 4.6) for a reaction constant of $\rho = -0.88$.

Hammett Constant (σ)

For a substituted aromatic compound meta- or para- XC_6H_4Y, where Y is the site of reaction and X-is a variable substituent, the σ values are numbers that sum up the total electrical effects (resonance plus field) of a group X when attached to a benzene ring and it is derived from the *Hammett equation;* $\log k/k_o = \sigma\rho$

Where k_o is the rate constant for X= H, k is the rate constant for the group X, ρ is a constant for a given reaction under a given set of conditions and σ is a constant characteristic of the group X (March, 1992; Exner, 1988). The value of ρ measures the susceptibility of the reaction to electrical field effects and a negative value indicates that the reaction is facilitated by electron-donating groups and vice versa. A positive value of σ indicates an electron-withdrawing group and a negative value is indicative of an electron-donating group.

From their results (table 4.6), the authors proposed that the presence of electron-donating groups facilitated the attack of the electrophilic enzyme (cytochrome P450) on the amidine system whereas the presence of acceptors hinders it. It is interesting to note that, no benzamidoxime was formed for the *para*-substituted nitrobenzamidine, which not surprisingly has a positive σ value: +0.81 (electron-withdrawing effect) (table 4.6). A search of tables of Hammett constants indicated that that the azo-pheny group (N=NPh) has a σ value: +0.34, whereas the NH_2 group has a negative σ value : -0.57 (electron-donating effect).



N,N-unsubstituted benzamidine — *N-hydroxybenzamidine*

Where *R* = H, *para*-CH_3, *p*-OCH_3, *p*-Cl, *p*-Br, *p*-CN, *p*-NO_2

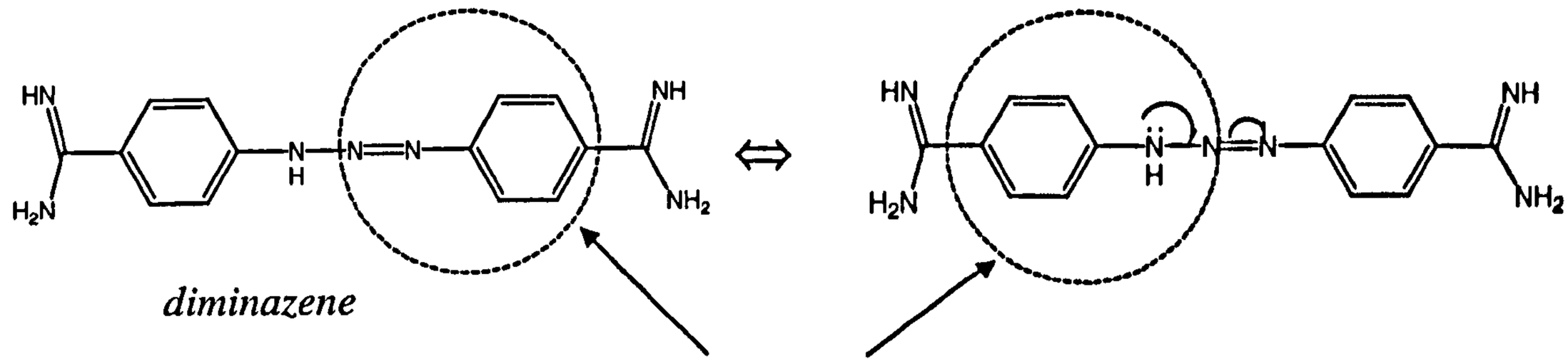
Figure 4.40 *N*-Hydroxylation of *N,N*-unsubstituted benzamidines to benzamidoximes

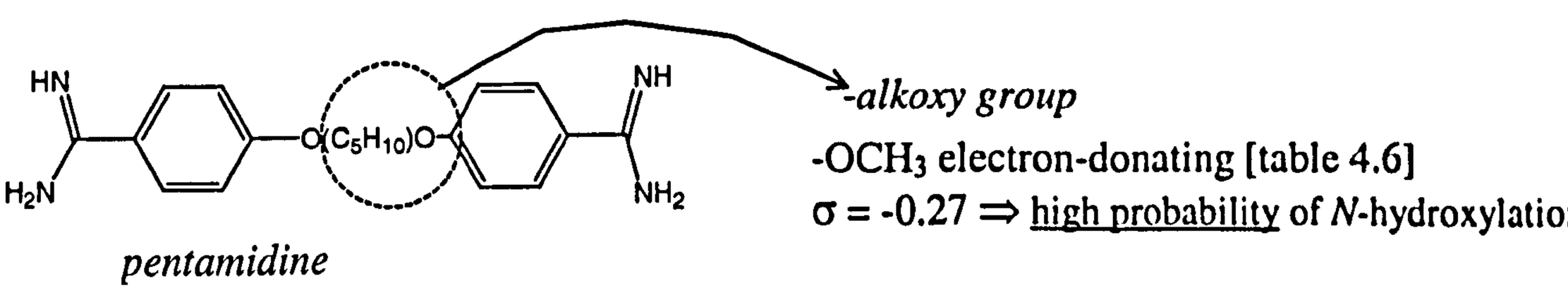
Table 4.6 Structure activity relationships for the N-hydroxylations of parasubstituted benzamidines to benzamidoximes (Clement and Zimmerman, 1988b)

X (= substituent R)	$V_{max}X$ (nmol/min/mg protein)	Hammett constant (σ_{para})
CH_3	1.03 ± 0.14	-0.17
OCH_3	0.93 ± 0.17	-0.27
H	0.56 ±0.19	0
Cl	0.42 ± 0.08	+0.23
Br	0.41 ± 0.11	+0.23
CN	0.15 ± 0.03	+0.66
NO_2	No detected metabolite	*+0.81**
N=N*Ph**	-	*+0.34*
NH_2*	-	*-0.57*

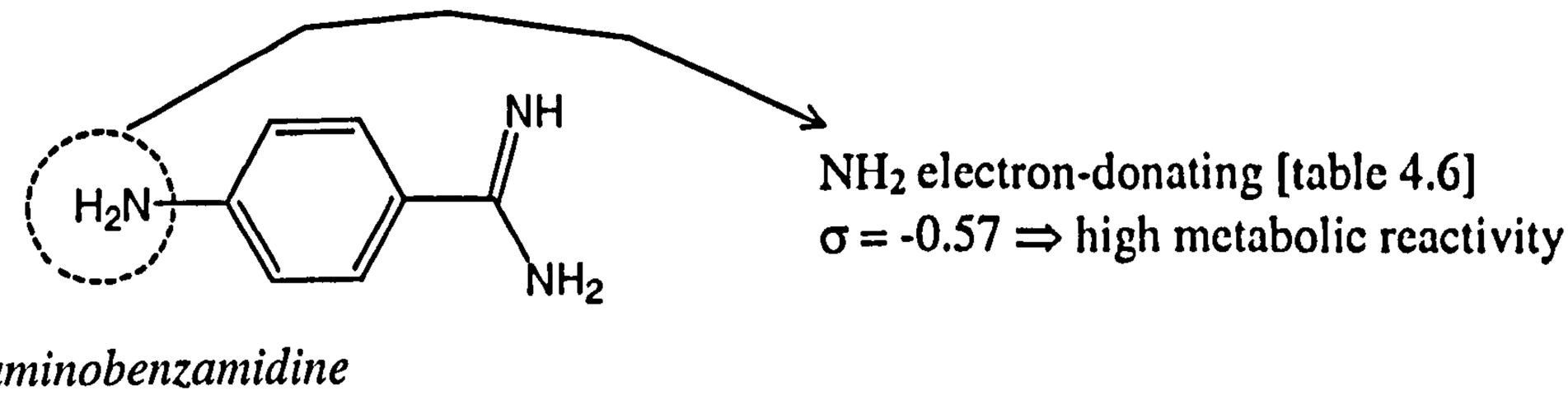
* *Hammett constants obtained from tables* (March, 1992) [Ph-Phenyl]

Application of the above principles to the aromatic amidines used in this study (figure 4.41) appears to provide good reasons for the metabolic inertness of diminazene compared with pentamidine and *p*-aminobenzamidine which are readily metabolized in rat and pig hepatocytes.





pentamidine



p-aminobenzamidine

Figure 4.41 Structure-activity relationships that may determine the metabolic reactivity of diminazene, pentamidine and *p*-aminobenzamidine

The contribution of metabolically reversible reactions of oximes cannot be overlooked. Since Clement and Jung (1994) have demonstrated the retroreduction of *N*-hydroxypentamidine to pentamidine by microsomal fractions from rabbit liver, it is plausible that the *N*-hydroxylated metabolites of diminazene, once formed in hepatocytes are reduced back to diminazene.

Although diminazene has been extensively applied for treatment of veterinary trypanosomosis in affected areas world wide, there is very little information in the literature, about its metabolism in ruminant animals such as goat, sheep and cattle. Even though the pretreatment of rats with cytochrome P450 inducers (PB, 3-MC) and DM did not appear to change the metabolic inertness of diminazene, as demonstrated in this study, the effects of such inducers in other animals (particularly the African species) may produce entirely different results. Furthermore, there are increasing reports of the use of diminazene aceturate for successful treatment of human trypanosomiasis (caused by *T.b.gambiense*), without the major adverse reactions associated with the use of pentamidine (Abaru and Motavu, 1984). Consequently, knowledge of the metabolism of diminazene in humans and other animal species (livestock) could have an important role in appraisal of the current scope of diminazene usage and the generation of relevant data leading to a better understanding of the problem of diminazene resistance.

CHAPTER 5

SUMMARY AND CONCLUSION

Summary and conclusion

Trypanosomosis constitutes a serious health threat to both man and livestock across Sub-Saharan Africa and control of the disease continues to rely heavily on chemotherapy. However, chemotherapy is dependent on a small armoury of trypanocides, which suffers from several limitations including toxicity and drug resistance. Increasing incidence of resistance to currently used drugs is a major problem in the field of anti-microbial chemotherapy (Sefton, 2002) and anti-protozoal chemotherapy such as African trypanosomosis (Peregrine, 1994). In the case of African trypanosomosis, the relative high cost of drugs coupled with poverty and proliferation of substandard drugs makes the problem of drug resistance an acute one. The "handful" of trypanocides available for treatment of the disease in humans (pentamidine, suramin, melarsoprol) and animals (diminazene, isometamidium and ethidium) have been used for more than four decades and their use is generally associated with severe side effects (Peregrine, 1994). Moreover, the reluctance of pharmaceutical companies to invest resources for development of new drug therapies destined solely for "poor markets", casts a shadow on prospects for drug development and makes the situation more desperate. To this end, the work presented in this thesis has been focussed on issues related to identification of the causes of trypanocidal drug resistance, and more specifically, resistance to the diamidine trypanocides (diminazene and pentamidine).

The goal of the first part of this study was to investigate the quality of generic formulations of diminazene aceturate available in international commerce. Following the expiry of patent protection of the innovator product (Berenil®), there has been an influx of generics onto the African market, where drug regulation and enforcement is generally more relaxed, thereby creating a conducive environment for proliferation of counterfeit and substandard trypanocides. The approach has been to develop and validate a rapid and sensitive analytical method (HPLC) for the determination of diminazene aceturate in pharmaceutical formulations and then apply the developed method for quality evaluations of generic formulations randomly sampled from affected areas in Africa. This would provide a "snapshot" of the prevailing situation of quality of generics on the African market.

The results of these investigations are as follows:

- A specific, precise and accurate chromatographic method (HPLC), has been developed and validated for the determination of diminazene aceturate in pharmaceutical formulations. The major manufacturing impurity and a degradation product of diminazene have been identified by mass spectrometry and structural elucidation of the impurity perfomed by unequivocal synthesis and NMR spectroscopy of the synthesized product.
- The developed HPLC method has been applied for quality evaluation of over one hundred generic samples of diminazene sourced from at least eleven countries in affected areas in sub-Saharan Africa, through the FAO liaison offices in the respective countries.
- About one-third (24%) of the total samples analyzed were below the ±10% tolerance limits of manufacturers' label claim, while 8% were found to contain diminazene above the tolerance limits.

The evidence uncovered in this study confirms a previous parallel study with isometamidium performed in our laboratory (Tettey *et al,* 1999), which revealed that the quality of generic formulations of phenanthridine trypanocides (veterinary trypanocides) in international commerce are compromised and that this could be a major contributory factor for re-emergence of trypanocidal drug resistance.

The second part of this study has investigated one of the possible biological causes of drug resistance i.e. drug metabolism. Environmental chemicals such as pesticides and polycyclic aromatic hydrocarbons (PAH) are known to affect hepatic drug metabolizing enzymes. Since pesticides are commonly employed for insect vector (*tsetse fly*) control in the management of animal and human trypanosomosis, it was necessary to investigate the possible interactions of environmental xenobiotics (insecticides) with the metabolism of trypanocidal diamidines. We have used *in vitro* animal models (rat and pig hepatocytes) to investigate the effects of classical inducing agents PB and 3-MC as well as DM, a pyrethroid insecticide, on the metabolism of diminazene and pentamidine.

p-Aminobenzamidine was used as a model aromatic amidine to investigate the metabolic fate of *N,N*-unsubstituted aromatic amidines in hepatocytes from control rats and pigs.

In summary the results of these investigations are the following;

- In hepatocytes isolated from both animal species, *p*-aminobenzamidine was biotransformed to *p*-amino-*N*-acetylbenzamidine. However the rate of metabolism in the rat appeared to be faster (4.5-fold increase in metabolite formation) than that in the pig. This is the first report of the phase II metabolism of the aromatic amidine; p-aminobenzamidine in rats and pigs.
- The metabolism of pentamidine in isolated rat and pig hepatocytes has been described. At least *six* primary metabolites of pentamidine, which had been previously reported in rat microsomes (Berger *et al.*, 1992) have been identified in both animal species using a newly developed LC-MS method of analysis. Qualitatively, there appeared to be no differences in metabolic profiles between the two animal models, suggesting that the metabolic pathways of pentamidine in rats and pigs are similar. However there were quantitative differences in the rates of metabolism of pentamidine. There was a 21.4-fold increase in the disappearance of pentamidine in rat hepatocytes compared to that in the pig.
- Diminazene was not metabolised in hepatocytes isolated from either control or inducer treated rats, or pigs.
- The cytochrome P450 content of hepatocytes isolated from inducer treated rats (PB, 3-MC) was significantly increased ($p<0.05$), whereas pretreatment of rats with deltamethrin failed to induce any significant increases ($p=0.25$) in P450 content. The results seem to confirm previous reports, which indicate that pyrethroid insecticides are relatively poor inducers of cytochrome P450 enzymes (Dayal *et al.*,1999).
- The metabolism of pentamidine by hepatocytes isolated from inducer treated rats was significantly inhibited compared to that in the incubations with cells from control rats, with 3-MC causing the highest inhibition of 8-fold, PB 4.8-fold and

DM 2.2-fold (measured in terms of disappearance of pentamidine from incubations).

There were quantitative differences in the profiles of the 3 major metabolites of pentamidine formed in hepatocytes from DM and 3-MC pretreated rats compared to the control group. There was a 5.1-fold decrease in the relative amount of metabolite-*5* [5-(4-amidinophenoxy) pentanoic acid] formed by cells from 3-MC pretreated rats and a concomitant 3.3- and 3.4-fold increase in the relative amounts of metabolites-*3* and -*4* (2- and 3-hydroxypentamidine). Pretreatment of rats with DM resulted in a 1.5-fold increase in the amount of metabolite *5* formed after 3h incubation period. When compared with the control group, there were no significant differences in metabolite profiles in the PB pretreated animals.

The inhibition of metabolism of pentamidine by concomitant exposure to inducing agents could have toxicological implications for patients that receive pentamidine in addition to other drugs (common with AIDS patients being treated for pneumonia). This scenario is reminiscent in Africa where other febrile diseases such as malaria and typhoid fever are common and may present with clinical symptoms similar to sleeping sickness. As a result simultaneous treatment of trypanosomosis and other common tropical diseases may be inevitable leading to drug-drug interactions.

In most rural communities in Africa where trypanosomosis is endemic, the burning of fossil wood is a major means of food preparation and land clearing for farming activities. Such activities can generate smoke which when inhaled consistently can mimic the inducing effects of polycyclic aromatic hydrocarbons (3-MC and pyrolysis products). The excessive application of pyrethroid insecticides (such as deltamethrin) in testse fly infested areas could serve as potential sources of environmental pollutants, which can accumulate in humans via the food chain (vegetables and root crops) and affect drug metabolising enzymes in patients undergoing pentamidine therapy. Although the results of the current study may not appear to implicate metabolism as a cause of trypanocidal (pentamidine or diminazene) drug resistance, previous parallel studies in our laboratory have shown that the concomitant exposure of rats to inducing agents (PB, 3-MC) leads to rapid hepatic metabolism of ethidium bromide (Tettey *et al.*, 1999). Further investigations

with hepatocytes from humans or other animals could provide evidence leading to a better understanding of trypanocidal drug resistance.

5.1 Suggestions for future work

There is the need for further quality control studies of the generic formulations of diminazene aceturate in international commerce. This will help provide a clear picture of the quality status of diminazene aceturate on the African market.

Although diminazene was not licenced for use in humans, there are reports indicating its uses for the successful treatment of early stage cases of human sleeping sickness (Abaru and Motavu, 1984), without causing the severe side effects commonly associated with the uses of pentamidine. In the light of this report, it would be of interest to perform comparative toxicity testing of diminazene and pentamidine in isolated hepatocytes (from humans and different animal models) by monitoring the depletion of reduced glutathione. This would generate the necessary data in support of appraising the scope of diminazene use in humans.

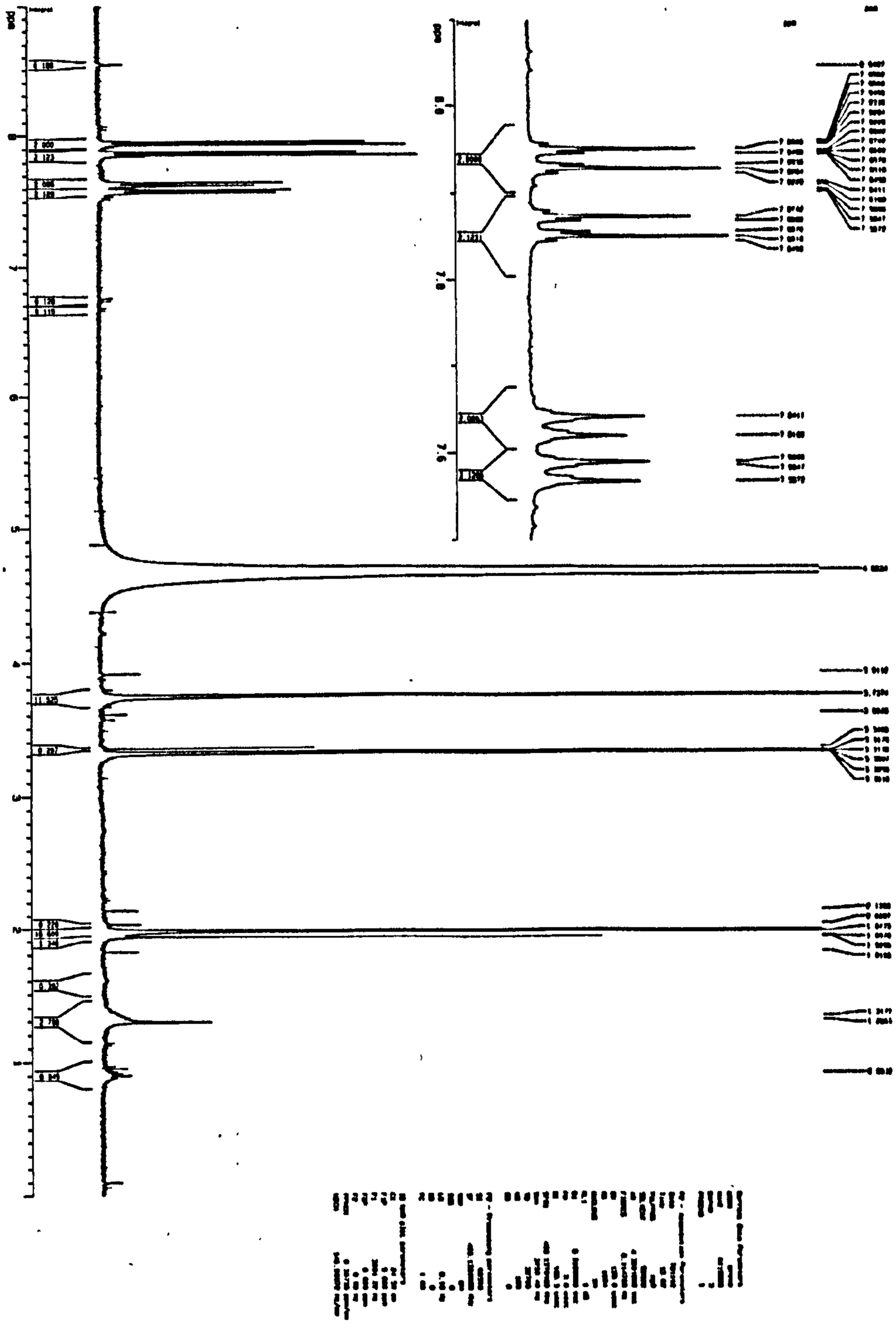
The direct application of cattle and livestock with insecticides as pour-on formulations (vat dipping) in trypanosome endemic areas by farmers is a well known phenomenon (Holmes, 1997) which could have effects on drug metabolising enzymes in such animals. It would be of relevance to establish the effects of pesticides on the hepatic metabolism of diminazene as well as other veterinary trypanocides in ruminant animal models such as goats, sheep or cattle.

One important question that has remained unanswered is whether resistant strains of trypanosomes are capable of metabolization of trypanocides? Recent investigations involving collaborative studies between our laboratory and Glasgow University has uncovered evidence which suggests that ethidium bromide could be metabolised in *T. brucei* species. Could it be possible that diminazene or pentamidine is metabolised in certain resistant strains of trypanosomes? Re-infestation of drug treated animals

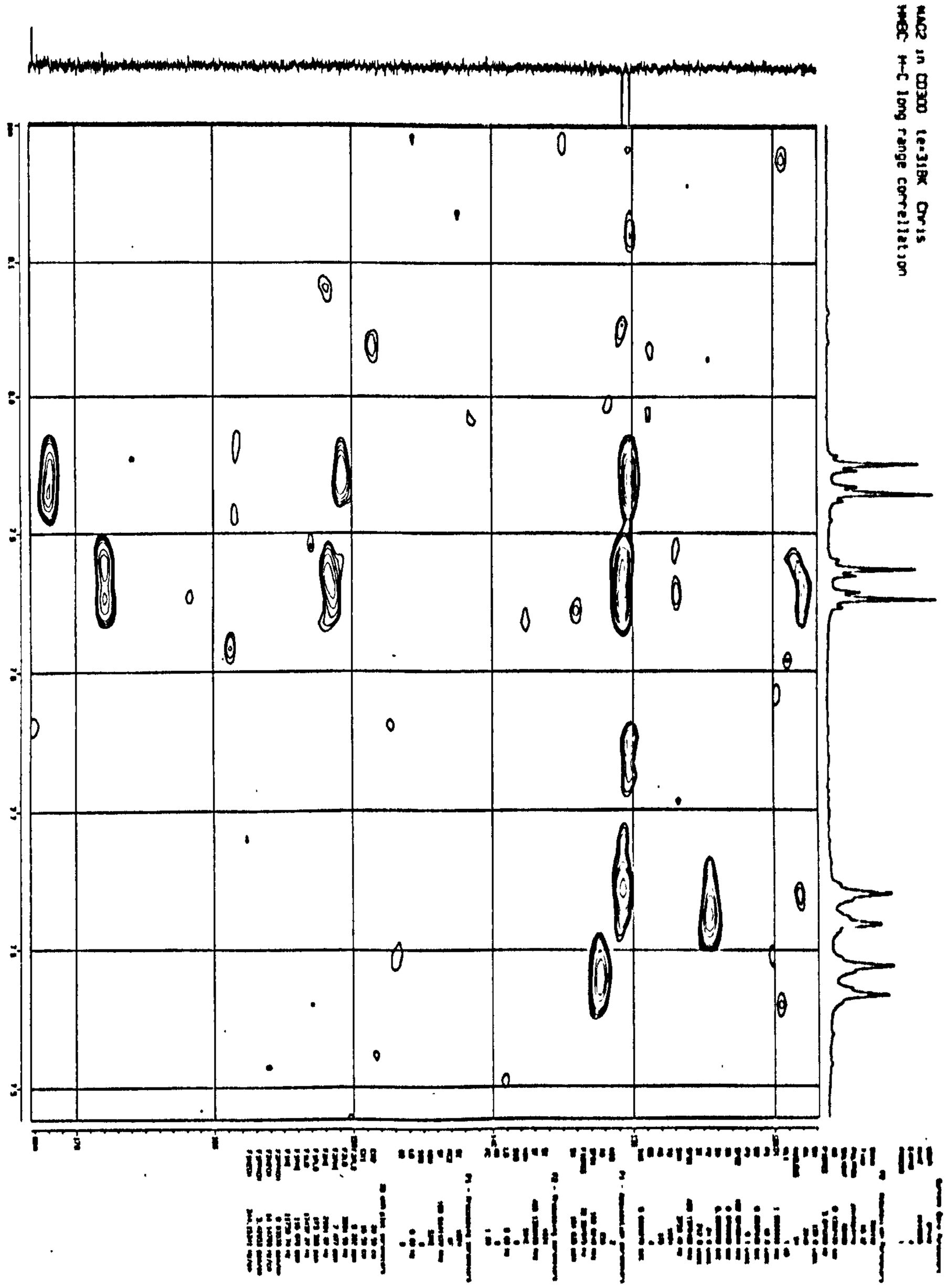
with trypanosomes during the elimination phase of trypanocides could create excellent sub-lethal plasma drug concentrations, which could exert selective pressure for emergence resistant strains. Consequently, it would be of relevance to establish whether such resistant trypanosomes are capable of evading the lethal effects of trypanocides by biotransformation.

APPENDIX I

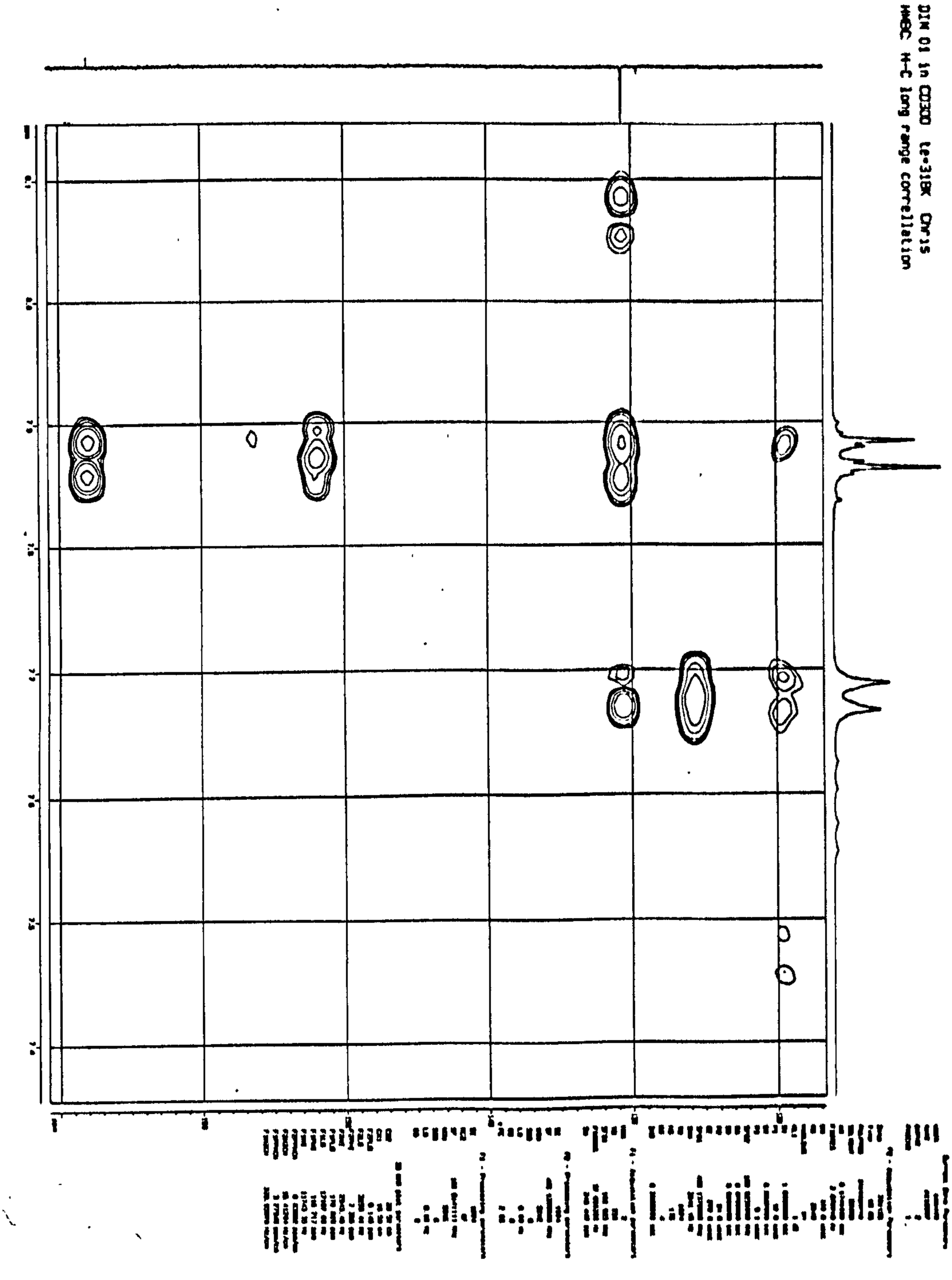
Appendix IA: ^{1}H-NMR spectrum of manufacturing impurity of diminazene



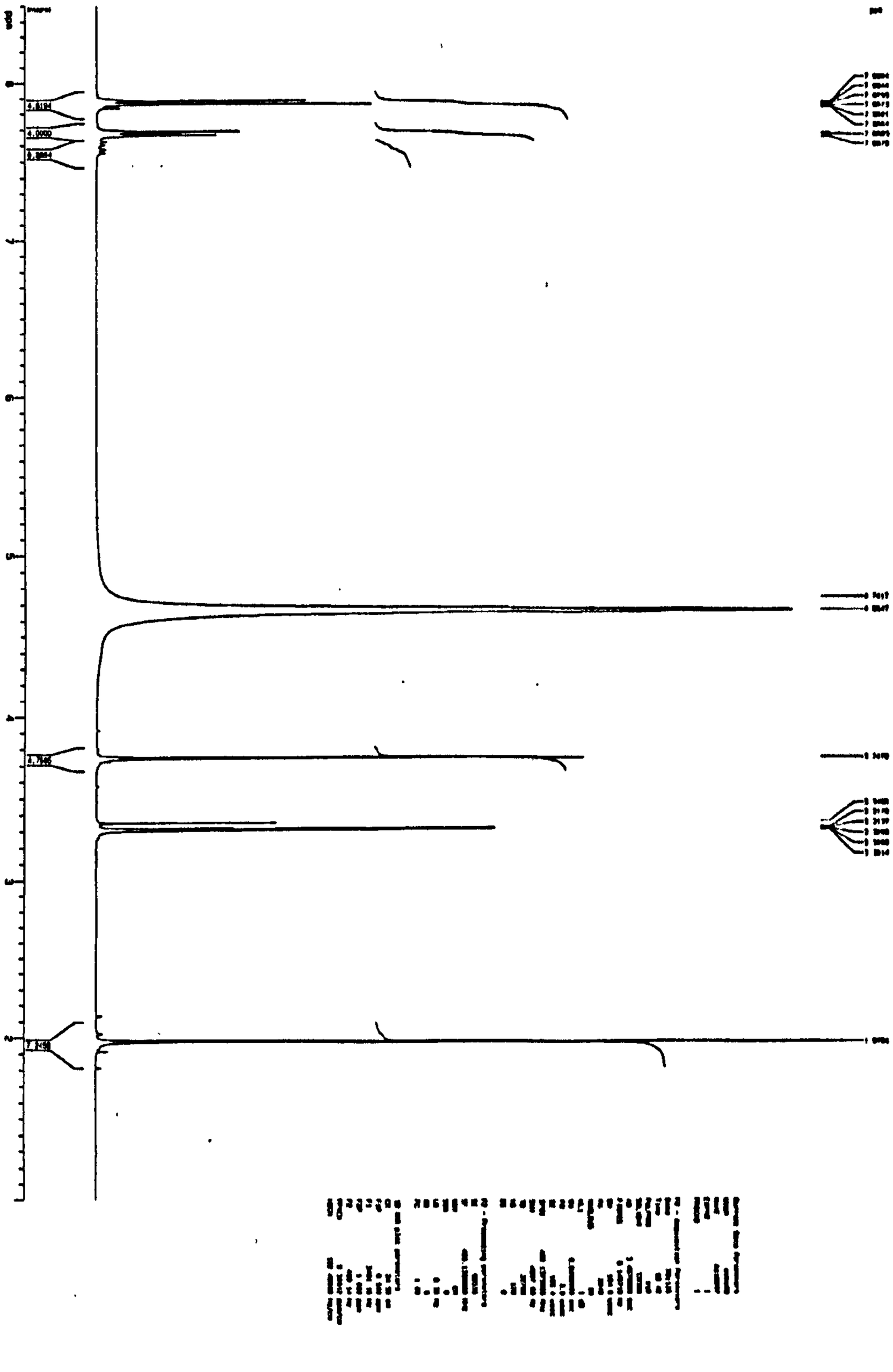
HMBC Spectrum of manufacturing impurity of diminazene



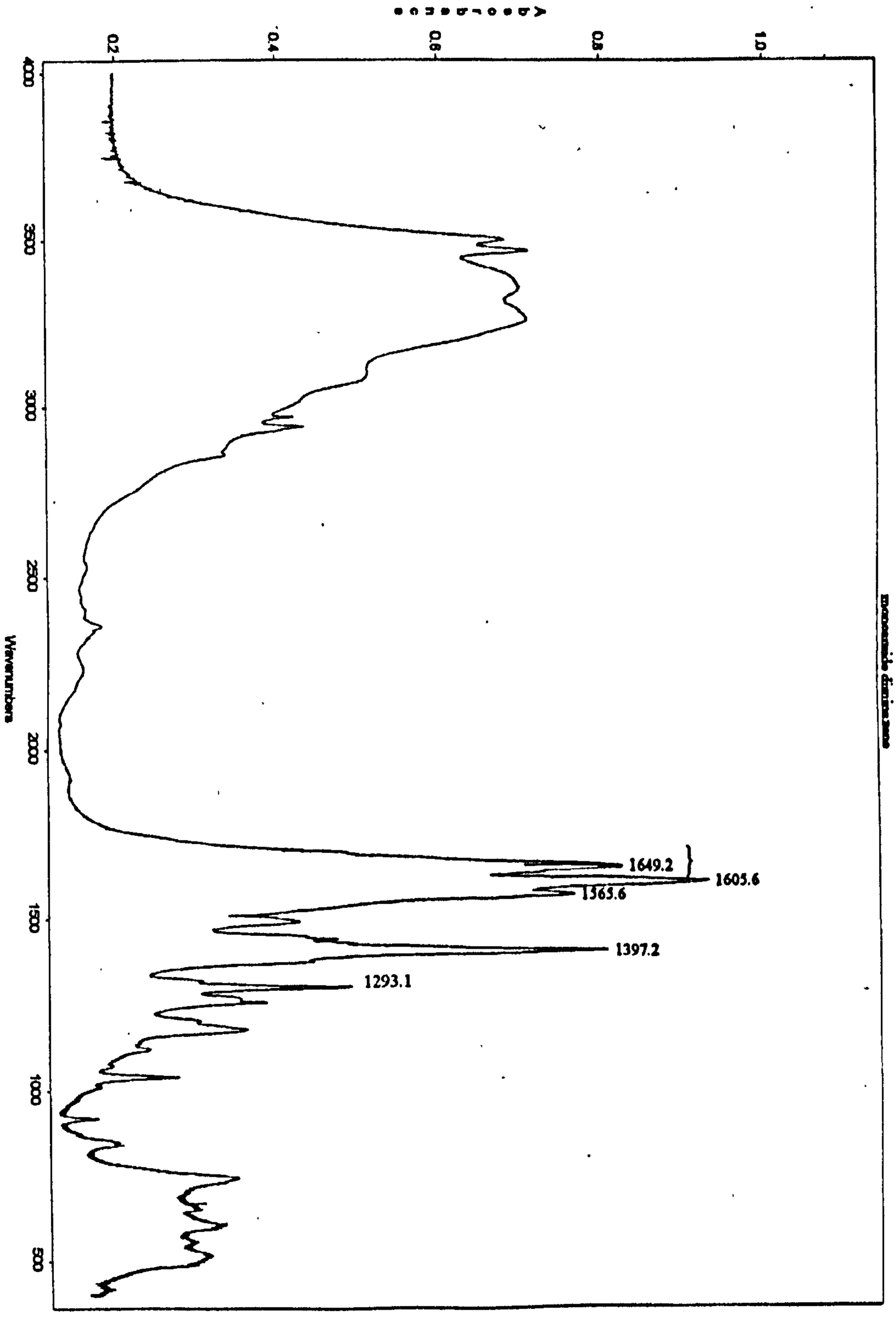
HMBC Spectrum of diminazene



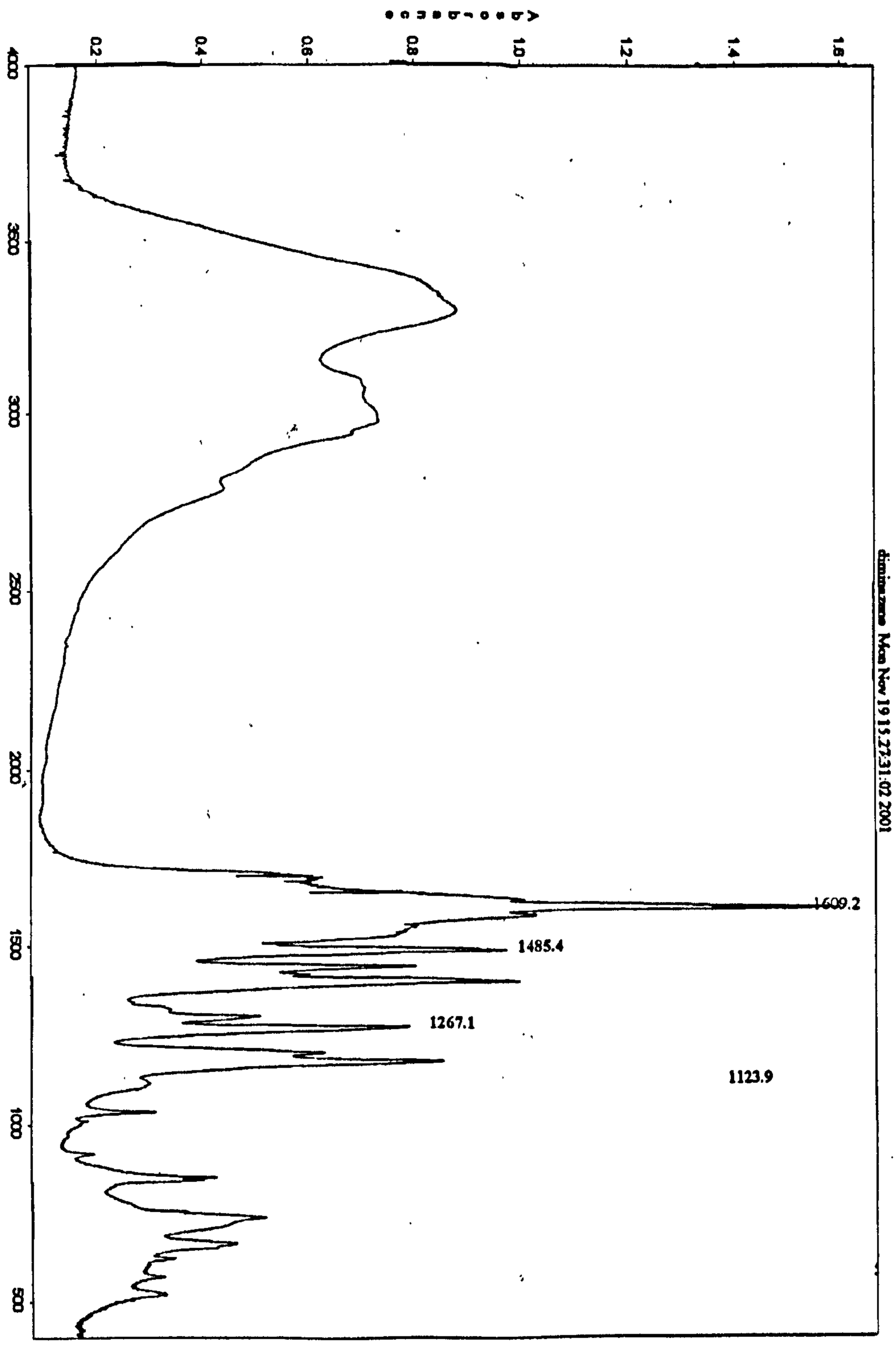
1H-NMR spectrum of diminazene aceturate



Appendix IB: FTIR spectrum (KBr) of impurity of diminazene



Appendix IB: FTIR spectrum (KBr) of diminazene aceturate



APPENDIX II

RESULTS OF ANALYSES OF GENERIC SAMPLES OF DIMINAZENE ACETURATE FROM SUB-SAHARAN AFRICA

APPENDIX II: Analysis results of content of diminazene aceturate and antipyrine (phenazone) in generic samples

Source[Country]	Sample Code	Diminazene % of label claim	Antipyrine % of label claim
Angola	USG-FAO-08	92.4	96.8
Angola	USG-FAO-21	89.4	101.1
Central Afr. Rep.	USG-CAR-01	102.8	89.3
Central Afr. Rep.	USG-CAR-02	88.6	102.8
Central Afr. Rep.	USG-CAR-03	98.6	91.1
Central Afr. Rep.	USG-CAR-04	92.3	100.7
Central Afr. Rep.	USG-CAR-05	94.9	89.6
Dem. Rep. Of Congo	USG-FAO-54	102.3	102.7
Dem. Rep. Of Congo	USG-FAO-63	88.0	103.4
Dem. Rep. Of Congo	USG-FAO-87	87.0	103.5
Ivory Coast	USG-FAO-12	114.2	86.5
Ivory Coast	USG-FAO-33	101.6	101.0
Ivory Coast	USG-FAO-09	101.5	96.7
Ivory Coast	USG-FAO-15	85.9	102.7
Ivory Coast	USG-FAO-23	88.4	94.5
Ivory Coast	USG-FAO-32	97.3	107.0
Ivory Coast	USG-FAO-36	84.7	102.9
Ivory Coast	USG-FAO-24	108.1	85.8
Ivory Coast	USG-FAO-25	82.1	111.5
Ivory Coast	USG-FAO-40	95.7	103.5
Malawi	USG-FAO-55	101.4	100.5
Malawi	USG-FAO-64	103.8	101.4
Malawi	USG-FAO-94	94.9	99.7
Mali	USG-FAO-66	101.5	90.8
Mali	USG-FAO-77	87.5	104.1
Mali	USG-FAO-88	84.1	104.6
Mali	USG-FAO-91	91.2	91.2
Mali	USG-FAO-95	92.4	100.0
Mali	USG-FAO-69	95.3	101.2
Mali	USG-FAO-81	95.8	100.1
Mali	USG-FAO-82	112.0	91.5
Mali	USG-FAO-75	106.0	88.7
Mali	USG-FAO-73	90.5	102.1
Mali	USG-FAO-86	95.8	102.2
Mali	USG-FAO-70	96.6	97.7
Mali	USG-FAO-67	91.2	101.2
Mali	USG-FAO-79	81.2	103.5
Mali	USG-FAO-76	86.9	103.3

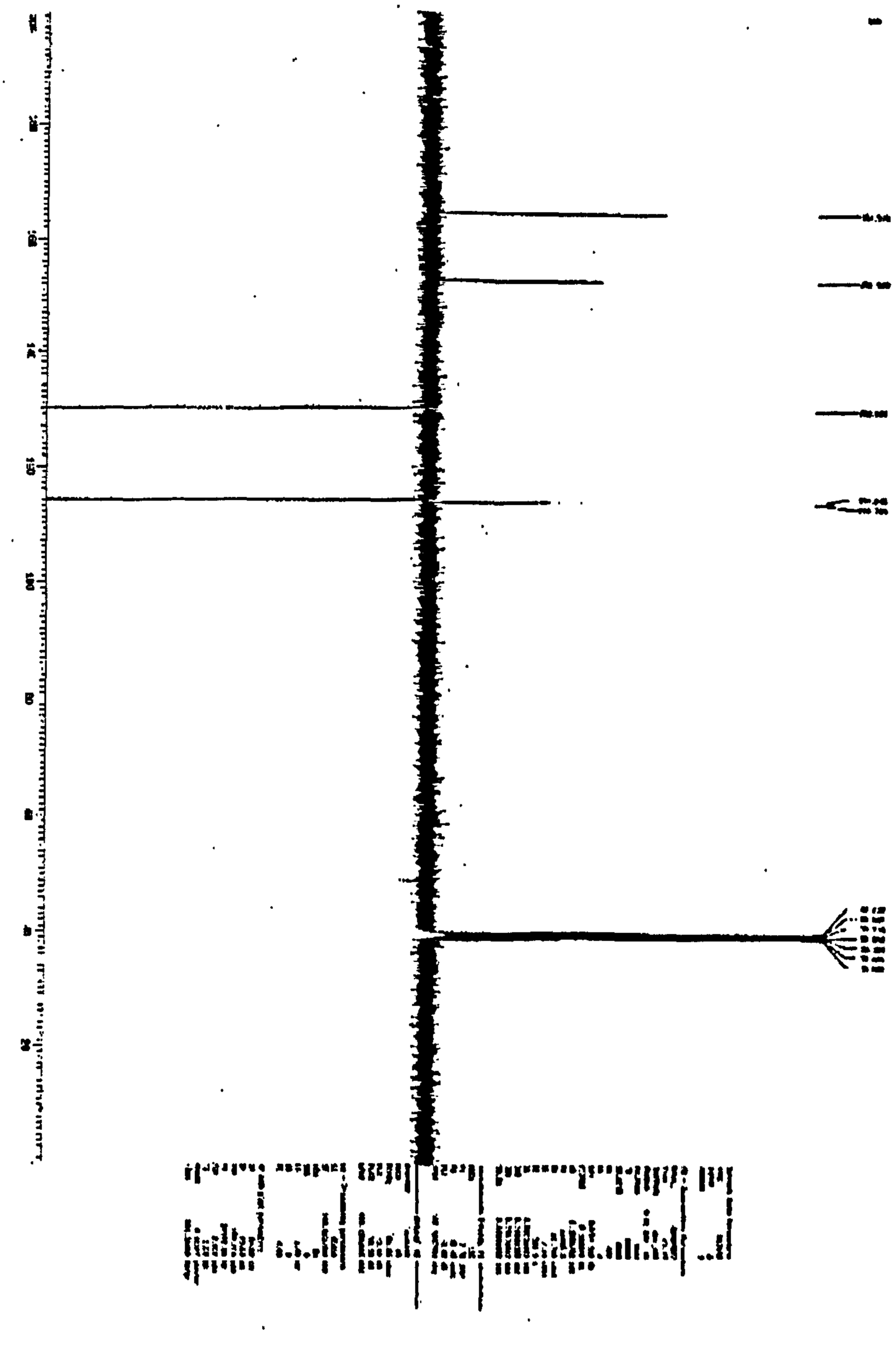
Source[Country]	Sample Code	Diminazene % of label claim	Antipyrine % of label claim
Mali	USG-FAO-78	96.2	102.2
Mali	USG-FAO-85	90.9	102.2
Mali	USG-FAO-71	84.5	102.1
Mali	USG-FAO-68	90.0	103.3
Mali	USG-FAO-72	105.2	88.6
Mali	USG-FAO-89	107.4	88.8
Mali	USG-FAO-74	83.9	102.0
Mali	USG-FAO-83	93.2	102.7
Mozambique	USG-FAO-53	107.5	90.2
Mozambique	USG-FAO-90	90.6	99.2
Namibia	USG-FAO-56	103.0	92.2
Namibia	USG-FAO-58	103.5	90.8
Namibia	USG-FAO-80	99.9	90.7
Namibia	USG-FAO-84	100.5	90.3
Namibia	USG-FAO-93	106.7	106.7
Nigeria	USG-FAO-92	97.1	101.5
Nigeria	USG-FAO-96	86.7	102.6
Nigeria	USG-FAO-97	93.6	103.4
Nigeria	USG-FAO-98	89.3	98.8
Nigeria	USG-FAO-99	88.4	100.4
Senegal	USG-FAO-02	94.0	96.9
Senegal	USG-FAO-11	104.1	99.1
Senegal	USG-FAO-16	91.4	99.8
Senegal	USG-FAO-17	96.6	101.0
Senegal	USG-FAO-31	99.9	101.8
Senegal	USG-FAO-07	104.6	86.0
Senegal	USG-FAO-20	101.9	90.2
Senegal	USG-FAO-38	98.3	101.9
Senegal	USG-FAO-22	106.2	91.7
Senegal	USG-FAO-27	100.1	91.4
Senegal	USG-FAO-29	100.0	89.7
Senegal	USG-FAO-03	92.0	97.5
Senegal	USG-FAO-05	87.4	98.3
Senegal	USG-FAO-19	86.0	103.2
Senegal	USG-FAO-28	89.9	105.2
Senegal	USG-FAO-34	98.4	102.8
Senegal	USG-FAO-45	97.0	104.5
Senegal	USG-FAO-06	86.0	93.4
Senegal	USG-FAO-14	94.3	99.0
Senegal	USG-FAO-18	84.7	97.6

Source[Country]	Sample Code	Diminazene % of label claim	Antipyrine % of label claim
Senegal	USG-FAO-30	93.8	100.1
Senegal	USG-FAO-35	93.0	100.0
Senegal	USG-FAO-01	106.2	80.4
Senegal	USG-FAO-13	110.7	83.3
Senegal	USG-FAO-37	106.9	83.9
Senegal	USG-FAO-41	110.5	84.4
Senegal	USG-FAO-44	113.4	84.6
Senegal	USG-FAO-04	104.2	86.9
Senegal	USG-FAO-10	109.1	88.1
Senegal	USG-FAO-26	102.5	91.7
Senegal	USG-FAO-39	111.4	90.6
Senegal	USG-FAO-42	109.9	89.3
Senegal	USG-FAO-*52(SOLN)*	107.5	
Senegal	USG-FAO-62(SOLN)	105.6	
Togo	USG-FAO-43	109.6	88.6
Togo	USG-FAO-50	99.3	101.3
Togo	USG-FAO-65	101.5	89.9
Togo	USG-FAO-61	86.4	103.4
Togo	USG-FAO-47	95.6	99.1
Togo	USG-FAO-46	115.0	88.3
Togo	USG-FAO-48	110.8	85.6
Unmarked	USG-FAO-51	90.7	102.8
Unmarked	USG-FAO-60	88.4	105.4
Unmarked	USG-FAO-59	96.7	96.9
Unmarked	USG-FAO-57	103.0	91.4

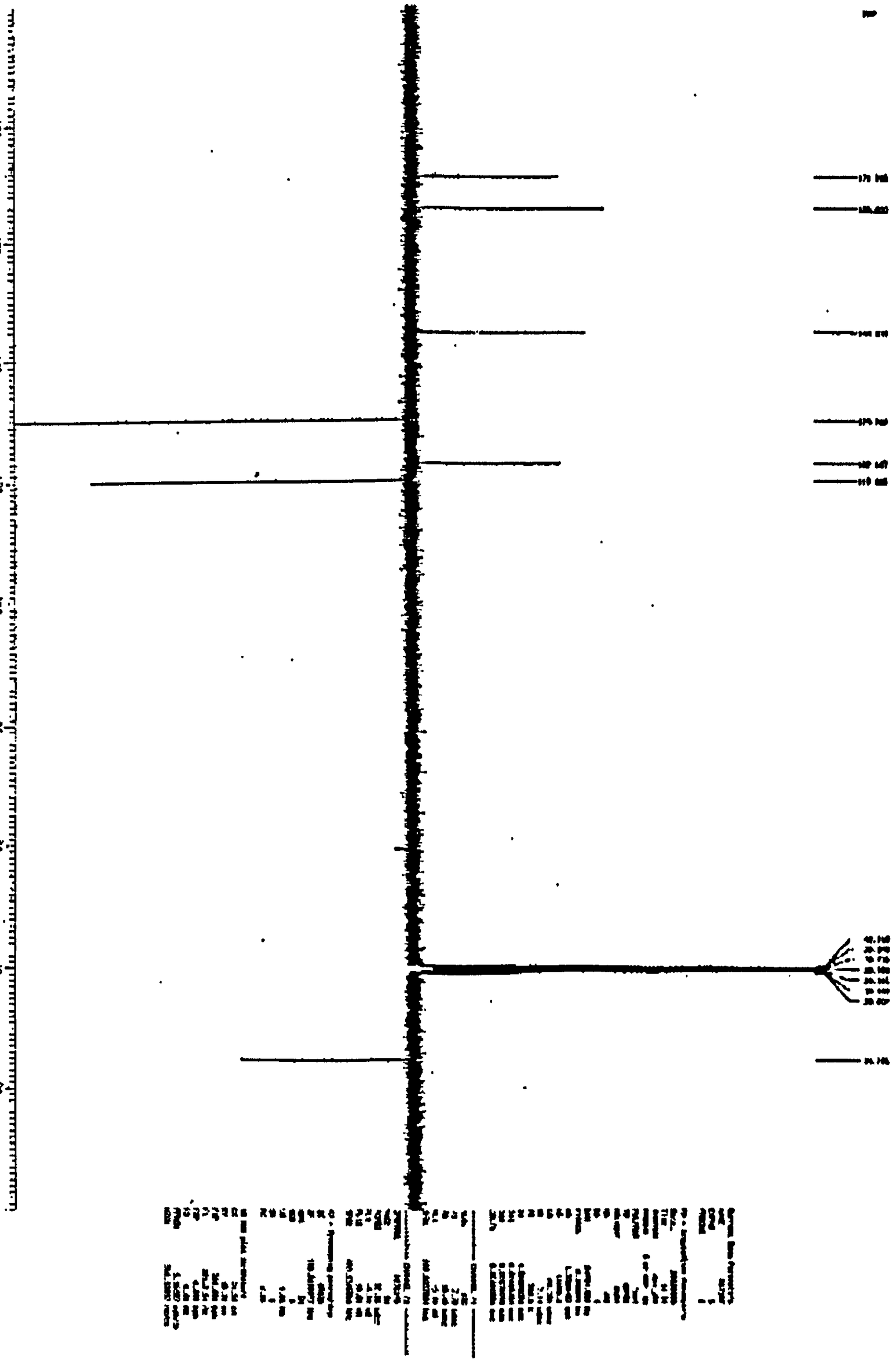
APPENDIX III

NMR SPECTRA

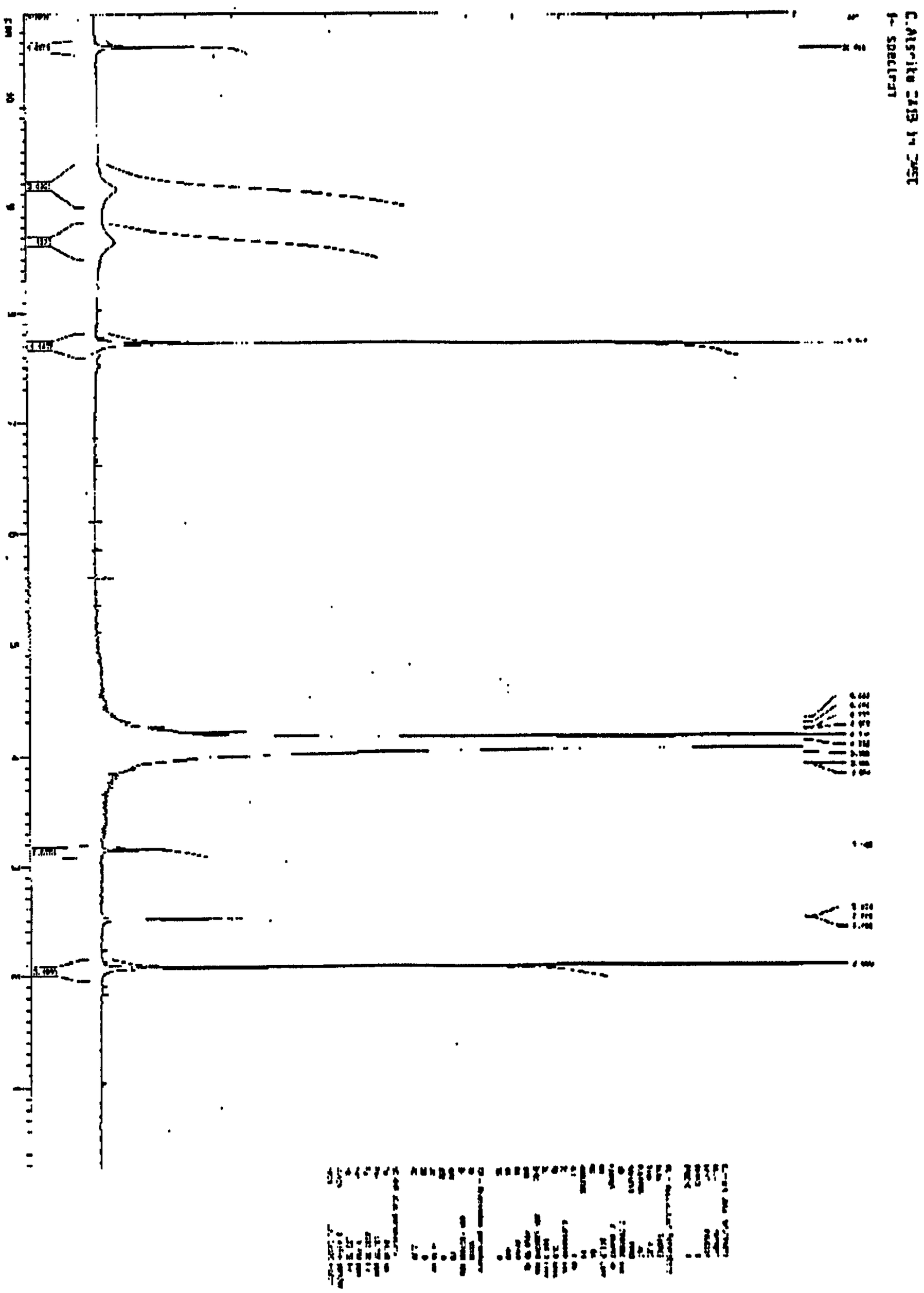
Appendix III: ^{13}C NMR spectrum of *p*-aminobenzamidine HCl



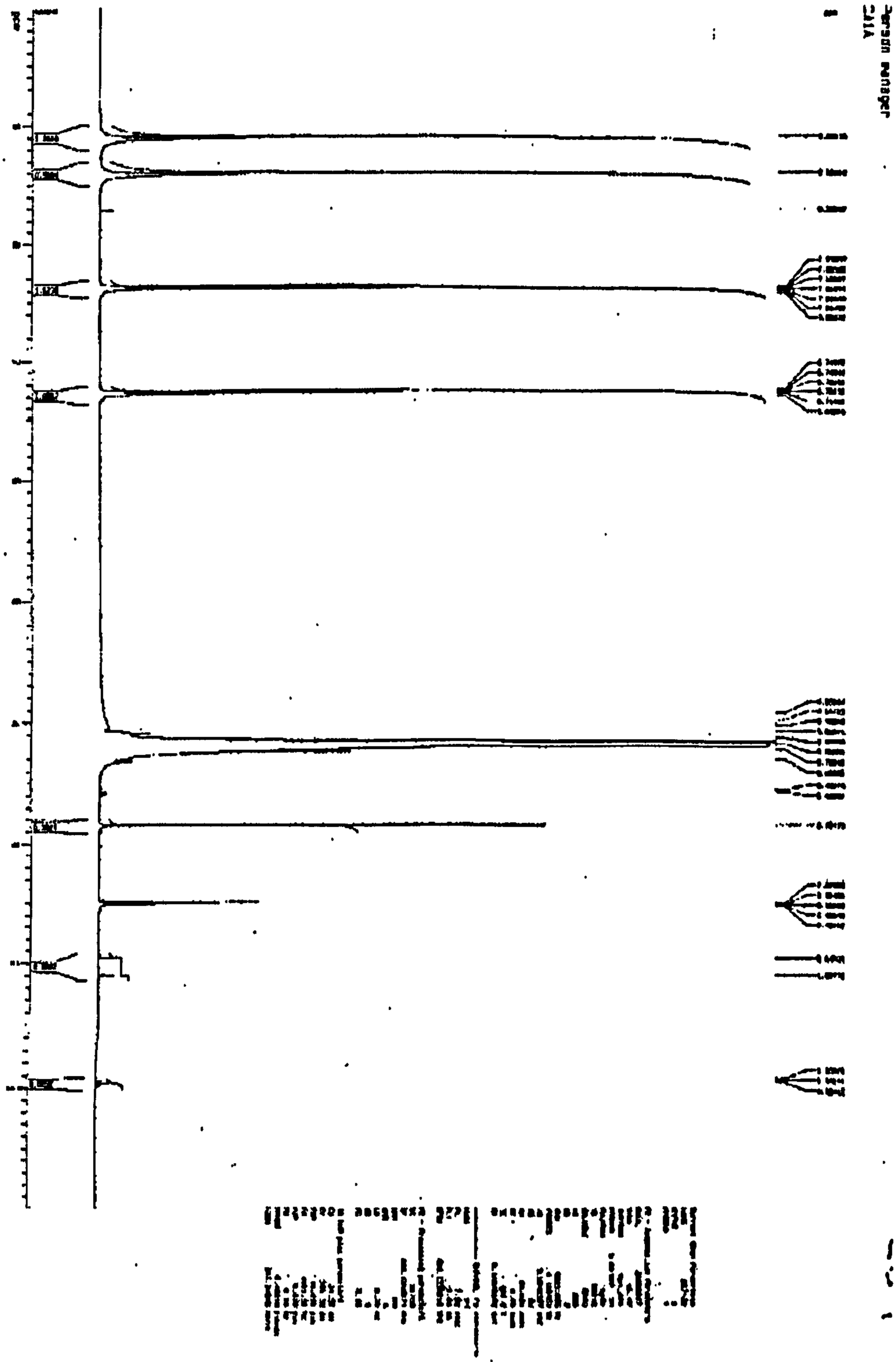
^{13}C NMR spectrum of *N*-acetyl-*p*-aminobenzamidine (synthetic reference)



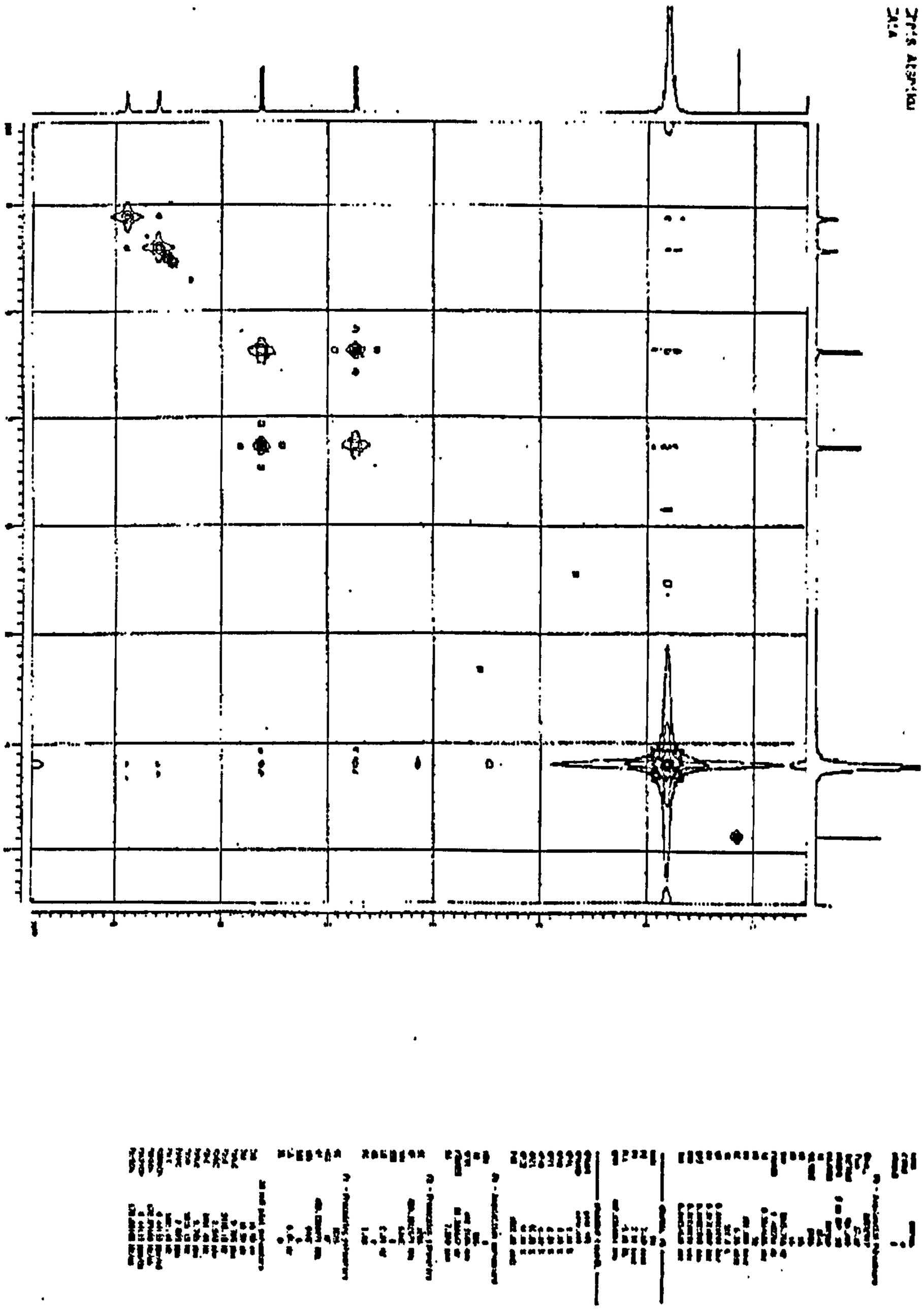
^{1}H NMR spectrum of *N*-acetyl-*p*-aminobenzamidine



^{1}H NMR spectrum of *p*-aminobenzamidineHCl



HMBC spectrum of *N*-acetyl-*p*-aminobenzamidine



APPENDIX IV

Appendix IV

Determination of hepatic elimination half-life ($t_{½}$) of pentamidine in rat and pig hepatocytes

Calculations:

By assuming a first order elimination of pentamidine

$$C = C_0.e^{-kt}$$

Where C = concentration of pentamidine remaining after incubation time (t min)

C_0 = Initial incubation concentration of pentamidine

t = incubation time

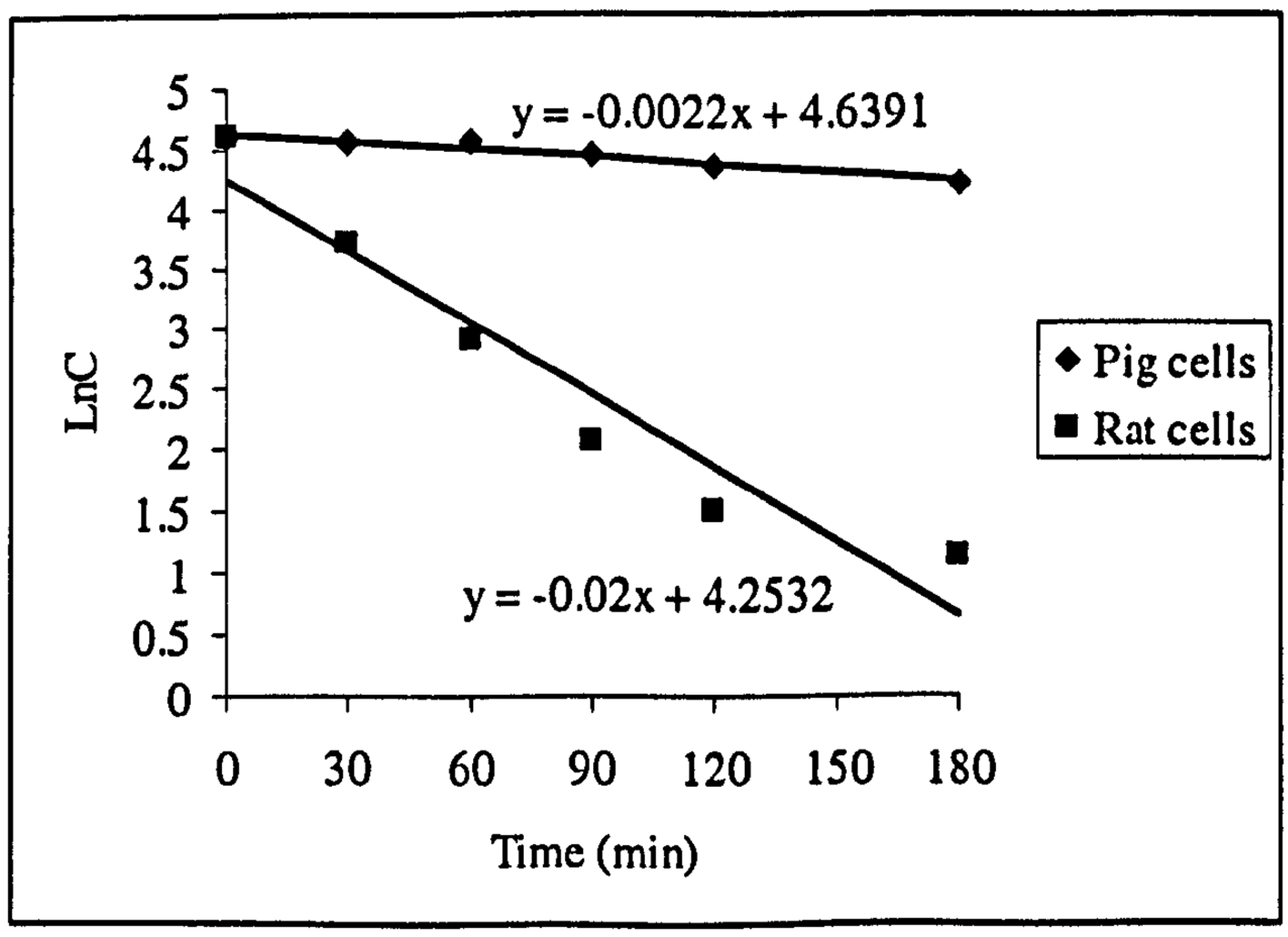
k = metabolic rate constant

$$\Rightarrow \ln C = \ln C_0 - kt \text{ (where k = slope)}$$

$$\text{when } C = ½C_0$$

$$\Rightarrow \ln 2 = kt_{½}$$

$$0.693 = kt_{½} \Leftrightarrow t_{½} = 0.693/k$$



In rat hepatocytes; k = 0.02 $\Rightarrow t_{½}$ = 0.693/0.02 = 34.65 min

In pig hepatocytes; k = 0.002 $\Rightarrow t_{½}$ = 0.693/0.002 = 346.5 min

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