THE PHARMACOKINETICS AND METABOLISM OF ALMITRINE BISMESYLATE

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by

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This work is Dedicated to my Father George

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CONTENTS

ABSTRACT

Almitrine Bismesylate (ABM) is a highly lipophillic compound of molecular weight 669.7, under development as a peripheral chemoreceptor stimulant in the treatment of chronic bronchitis and emphysema. Its pharmacokinetics and metabolism were investigated in animals and man.

Following oral administration of [^{14}C]-ABM, absorption of radioactivity is rapid and variable in man, rat, rabbit and dog. Plasma levels of radioactivity decline rapidly after both oral and intravenous administration, suggesting rapid uptake into tissues. A high affinity of almitrine for the carotid body of the urethane-anaesthetised rat confirms a prolonged and specific action of the compound.

Elimination of radioactivity is slow and mainly faecal and independent of administration route or species. Recovery of the adminstered radioactivity is incomplete in all other species except rat and in man, only 86 to 94% of the administered dose was recovered in 140 days. This slow elimination may be due to repeated gut secretion and reabsorption of almitrine.

Almitrine is metabolised by oxidation and N-dealkylation of the allyl side chain to form, respectively, the di- and tetrahydroxy almitrine and mono and di-deallyl almitrine with the corresponding dihydroxy monodeallyl almitrine. The major faecal metabolite in all species is tetrahydroxy almitrine (not found in plasma) and all species had qualitatively similar metabolic patterns to man both *in-vivo* and *in-vitro* from liver microsomes.

The pharmacokinetics of unchanged almitrine in man shows a rapid absorption from both solution and tablet formulations. Absorption is increased (48%) in the presence of food and bioavailability is high (74%). A large volume of distribution (3100 litres) and low clearance (64ml.min^{-1}) are consistent with a long half-life (614 to 1164h). Plasma protein binding is high (99%) in all species and is not affected by drugs likely to be coadministered with almitrine.

Clinically, there is a relationship between almitrine plasma concentration and changes in PaO_2 from baseline during repeated administration. Some patients with high plasma levels of almitrine (>300ng.ml^{-1}) experienced peripheral paresthesia which is reversible. Using simulations, dosage adjustments have shown that it is possible to maintain therapeutic levels of almitrine without side effects.

CHAPTER 1

INTRODUCTION

The Disease

Chronic airflow obstruction causes mismatching of ventillation to perfusion and areas of local alveolar hypoxia resulting in arterial hypoxemia. The presence of arterial hypoxia causes pulmonary vasoconstriction which is reversible in its early stages, although it eventually leads to narrowing of pulmonary arteries and raised pulmonary artery pressure, right ventricular hypertrophy and finally right ventricular failure (Renzetti *et al.*, 1966; Bishop, 1973; Summer, 1978; Moritz and Matthay, 1980; Matthay and Berger, 1981) and eventual death. Chronic airways disease occurs in a group of heterogeneous conditions, including chronic bronchitis and emphysema. These two conditions are the most important diseases encompassed in the term "Chronic Obstructive Airway Disease" or COAD. The diseases are characterised by recurrent productive cough, infective bronchitis and progressive breathlessness and a declining airway function when measured by spirometric tests, i.e. forced expiratory volume in one second (FEV_1), and forced vital capacity (FVC).

Chronic bronchitis is diagnosed by a history of cough for the previous two years, with sputum production during at least three months of each year. The many pathological changes which will be observed with this condition include increases in the number and size of the mucus-secreting cells in the mucosal layers of both large and small airways. As a consequence, there is excessive mucus production as well as a narrowing of the airways, the result of which is an increased resistance to airflow and airway obstruction results (Burki, 1986). Fletcher and Peto (1977) reported that cigarette smoking was the main cause of chronic bronchitis. They found that 40% of persons who smoked cigarettes over a period of 10 years developed airway mucus hypersecretion, of which 15 to 40%, depending on the number of cigarettes smoked daily, would develop airway obstruction. There is also some indication that coal dust inhalation for many years, e.g. from coal mines, may cause chronic bronchitis, but the pathological role of air pollution is not well defined. Several studies have suggested a higher incidence of chronic bronchitis in urban dwellers compared to rural inhabitants.

Emphysema is characterised by dilation and destruction of the gas-exchange units of the lungs, i.e., the lung parenchyma distal to the terminal bronchiole. Certain proteolytic enzymes are thought to be responsible for these destructions particularly

in panlobular emphysema in which the acinus distal to the respiratory bronchiole is affected. Emphysema can be either hereditary or cigarette related. The hereditary form is associated with panlobular emphysema and shows a deficiency in serum alpha1-antitrypsin. Cigarette smoking-related emphysema is more related to the centrilobular type which affects the respiratory bronchiole, sparing distal portions of the lobule.

Sufferers of COAD, particularly of chronic bronchitis, generally develop severe disabilities in advanced stages of the disease and this has been one of the major causes of health claims and loss of time from work over the last 30 years, particularly in the heavily industrialised regions. Flenley (1986) reported that COAD in 1982 caused some 30 million working days to be lost from British Industry, particularly as a cause of exacerbation of respiratory infection in the winter months. There is an increasing disability which initially limits the patient's efforts and thus preventing manual labour. Disability becomes so severe as to render the individual housebound. As the disease progresses in these patients, a proportion will develop chronic respiratory failure (FEV_1 falls below 2 litres) and this will be manifested by central cyanosis of the tongue and a disturbance of the arterial blood gases. A critical phase is reached when arterial oxygen tension at rest, breathing air, falls below 8 kPa (60mm Hg), a state of chronic hypoxia. Some patients will develop hypercapnia and recurrent exacerbations of oedema requiring frequent hospitalisation. This condition is called cor pulmonale. Survival becomes truncated at this point and if respiratory failure is not treated, some 80% of such patients will die within five years.

Therapy

Oxygen therapy has, by far, been the most effective treatment for COAD. Two studies in the 1970's, the Medical Research Council Study (Lancet, 1981), and the American Nocturnal Oxygen Therapy Trial (NOTTS, 1980), discovered that controlled low flow oxygen, given in the home in addition to conventional therapy, could improve survival by at least 50% when compared to control patients in the trial who received no oxygen therapy. Efficacy was defined as maintaining arterial oxygen tension at rest, above 8 kPa (60mm Hg) whilst breathing oxygen for at least 15 hours per day, that period including night-time therapy. Survival could improve somewhat greater if patients could tolerate taking oxygen for longer periods, e.g. 18 or even 24 hours a day.

Cooper *et al.* (1987) showed that supplementary oxygen does not arrest continuing decline of arterial oxygen or airway function. It temporarily relieves the consequences

of severe hypoxaemia in COAD and at best it improves survival up to 5 years. Ström and Boe (1991), in a larger study on long term domiciliary oxygen therapy (LTO_2) found that the Zubrod performance score, based on the degree of activity the patient could normally achieve, was the best predictor of survival. However, both age and sex influenced the level of survival. These studies, along with the studies of Fletcher and Peto (1977) and Weitzenblum *et al.,* (1981) showed that other predictors of survival in COAD were arterial oxygen tension (PaO_2), pulmonary hypertension, FEV_1 and FVC. Thus, drugs which influenced these parameters would be expected to improve hypoxia in COAD and therefore improve survival in these patients.

Bronchodilator therapy could probably be beneficial to these patients. However, the level of benefit is doubtful since bronchodilators generally act as expensive placebos and carry the risk of side effects. Bronchodilators fall into two broad classes: Xanthine derivatives and sympatomimetic agents. These are believed to act by increasing the level of cAMP in airway smooth muscle and facilitate bronchodilation. (Aubier*et al.,* 1981, Murciano *et al.*, 1984). The methyl xanthine, theophylline, increases ventillatory response to hypoxia and raises the volume of ventillation at any given PCO_2 during hypercarpnia (Dowell *et al.,* 1965, Saunders *et al.,* 1980). These products require close monitoring as there have been reports of levels of toxicity with theophylline which are unacceptably high.

Progestational agents such as medroxyprogesterone acetate (MPA) are known to stimulate breathing in normal and COAD subjects (Skatrud *et al.,* 1978). Although the mechanism of ventillatory stimulation is unknown, it is thought to occur either on the brainstem respiratory neurones or reflex receptor sites. The carbonic anhydrase reversible inhibitor acetazolamide causes an increase in respiratory ventillation and a parallel shift to the left ventillatory response to hypercarpnia (Fencl *et al.*, 1969). All of these agents appear to act centrally by affecting $PaCO_2$. However, improvement of hypoxia by oxygen therapy suggests that a greater response would be obtained by products which act on O_2 sensory centres, ie., the peripheral chemoreceptors.

The analeptic Doxapram improves both PaO_2 and decreases $PaCO_2$ by acting both centrally and peripherally (Kato and Buckley, 1964, Scott *et al.,* 1977). In low doses, doxapram has a respiratory stimulating effect mediated through a chemoreceptor effect on the carotid body as well as brainstem respiratory nuerones. At blood levels ranging from 1.6 to 3.0$\mu g.ml^{-1}$ doxapram increases the ventillatory response to both hypoxia and hypercarpnia in the absence of any alterations in oxygen consumption or CO_2 production (Calverley *et al.*, 1983). Doxapram, however, is rather limited since it

produces extreme restlessness and hallucinations particularly in alcoholics and others with abnormal liver function (Baxter, 1976).

The Carotid Body

The carotid body is a very vascular organ whose capillaries closely envelop clusters of specific glomus cells (McDonald and Haskell, 1984). Within these clusters are groups of Islands of glomus cells (type I) with numerous electron dense vesicles. They are wrapped around by the elongated processes of the Schwann like type II cells (McQueen and Pallot, 1983). McDonald and Mitchell (1975) showed that the carotid body was innervated by sensory fibres from the carotid sinus nerve, a branch of the glossopharyngeal nerve and sympathetic fibres mainly from the ganglioglomerula nerve and the sympathetic ganglion. Sympathetic activity and afferent fibres in the carotid sinus nerve depress sinus activity (Biscoe, 1971; Lahiri*et al.*, 1984 and O'Regan, 1977). Lahiri and Delaney (1975) showed that single afferent fibres conducted impulses when the carotid body receives either hypoxic-isocapnic or hypercapnic-isoxic blood. High levels of dopamine and noradrenaline are found in the carotid body and these are thought to be involved as neuromodulators in chemoreception. (Helström, 1977, Eyzaguirre and Zapata, 1984). Natural stimulation of chemoreceptors, namely hypoxia, hypercapnia and decreased pH, are able to alter dopamine dynamics in the carotid body (Brokaw *et al.*, 1985, Fidone *et al.*, 1982, Hanbauer and Hellstrom, 1978, Pallot and Al Neamy, 1983 and Rigual *et al.*, 1984). Dopamine, which inhibits ventillation through an action on the carotid body is probably present in the electron-dense vesicles of the type I cells (Barer *et al.*, 1986). McDonald and Mitchell (1975) hypothesised that afferent nerve endings on these type I cells are oxygen sensors and that impulses pass to the brain and also back via the reciprocal synapses to cause the type I cells to secrete dopamine. Acute hypoxia causes a reduction in dopamine but not noradrenaline content of the carotid body, whilst hypercapnia leads to an increased dopamine content.

Arias-Stella and Valcarcel (1973) showed that the human carotid body is enlarged in chronic hypoxia. This was first observed in high altitude dwellers where they reported an enlargement which increased with age and this was associated with hypoventillation. Heath and Williams (1981) found that some species at high altitude had larger carotid bodies compared to their counterparts living at sea level. Edwards *et al.*, (1971) also reported large carotid bodies in patients with cor pulmonale and emphysema and the weight of the two carotid bodies correlated with the weight of the right ventricles and this was later correlated with systemic hypertension. The enlarged

carotid body, which is an adaptation to a hypoxic state, will affect the function or efficiency of any drug which acts at its site.

Almitrine Bismesylate

The compound Almitrine (S 2620) N,N'-diallyl-6-{4-[bis(4-fluorophenyl)methyl] piperazinyl}-1,3,5-triazine-2-4-diamine (Fig. 1.1) is a weak base of molecular weight of 477.6 daltons. It is a white, tasteless and odourless crystalline powder, insoluble in water, but as the bismesylate salt (M.Wt. 669.7) it is sparingly soluble in water although both the salt and base are soluble in organic solvents (1:10, w/v methanol). Almitrine has two pKa's of 4.0 and 5.0 and an octanol/water partition coefficient of 2500, demonstrating a high lipid solubility.

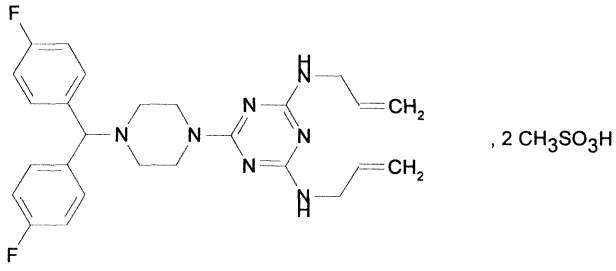


Figure 1.1: Structure of almitrine bismesylate; M.Wt. 669.7 (477.6 + 192.1)

Almitrine bismesylate is shown to increase ventillation through its action on the carotid body, i.e, stimulation of peripheral chemoreceptor rather than via a central action (Laubie and Schmitt, 1980). The site of action of almitrine was first demonstrated by Laubie and Diot (1972), who showed that the increase of ventillation in dogs was abolished by denervation of the carotid body and vagal section, and was enhanced by carotid artery infusion of almitrine. Bee *et al.*, (1983) demonstrated that the action of almitrine is restricted to the carotid body and pulmonary vasculature, the two sites where lack of oxygen has positive action, namely chemoreceptor stimulation and vasoconstriction. Almitrine (5mg.kg^{-1}, i.p) elicited a 34% decrease in dopamine content (4.6 ± 0.48pmol/CB) as early as 30 min after injection (6.97 ± 1.6pmol/CB) in the rat. In chronic almitrine treatment (15 days) dopamine levels were reduced by 55% relative to controls (Piquignot *et al.*, 1987). Pallot and Al Neamy (1983) also reported a fall in dopamine levels in rat carotid body within 30 min after single i.v infusion of almitrine (3mg.kg^{-1}). Noradrenline activity was less affected by almitrine.

Intravenous infusion of high doses of almitrine to anaesthetised dogs increases both ventillation and PaO_2 and diminishes $PaCO_2$ (Dhillon and Barer, 1982, Laubie and

Diot, 1972). Laubie (1982) showed that at lower doses (10-30µg.kg^{-1}), almitrine in similar experimental conditions increased PaO_2 but not ventillation.

In patients with COAD, almitrine induces a rise in PaO_2 which is greater than the corresponding fall in $PaCO_2$, with a consequent reduction in alveolar-arterial oxygen pressure difference (A-ao2). This implies an improvement of ventillation perfusion matching, possibly by redistribution of blood flow away from poorly ventillated areas (Castaign *et al.*, 1981, 1983 and 1986, Howard 1984 and Rigaud *et al.*, 1980). There was a dose dependent improvement in PaO_2 after oral (50-400mg) or iv (0.5mg.kg^{-1}.h^{-1}; total 1kg.kg^{-1}) administration with an increase of 6-18.7mm Hg in PaO_2 and a decrease of 3.7-8.2mmHg in $PaCO_2$ (Bury *et al.*, 1989, Riguad *et al.*, 1982, Yernault *et al.*, 1983, Neukirch *et al.,* 1974 and Damato *et al.*, 1988). In normoxic healthy man, iv infusion of almitrine (1mg.kg^{-1}.h^{-1}; total 25mg) improved PaO_2 from 87 ± 6 to 96 ± 5.2mm Hg (Gluskowski *et al.*, 1984); $PaCO_2$ was reduced from 40.5 ± 1.5mm Hg to 34.5 ± 3mm Hg whilst pulmonary artery pressure and pulmonary vascular pressure improved significantly. Similar improvements were seen in healthy man at high altitude whether natives (Villena *et al.*, 1985) or acclimatised lowlanders (Hackett *et al.*, 1987). Macnee *et al.*, (1986), compared the effects of almitrine with those of oxygen (given as 3 l.min^{-1} by nasal prongs) on PaO_2, mean pulmonary arterial pressure (Ppa) and right ventricular ejection fraction (RVEF) both at rest and during exercise in patients with chronic hypoxemia caused by chronic bronchitis and emphysema. They found a significant increase in PaO_2 with almitrine and oxygen both at rest and during exercise. Ppa increased both at rest and during exercise after almitrine but only during exercise whilst breathing oxygen. The increase in PaO_2 during exercise correlated with almitrine plasma concentration ($p < 0.05$) and was associated with a fall in RVEF from rest ($p < 0.01$).

The high lipophilicity and basic nature of almitrine should give rise to good absorption since, at the site of absorption, the compound would be expected to be mainly non-ionised in the basic environment of the duodenum. The allyl side chains and the presence of the piperazine ring combined with its high lipophilicity, makes almitrine amenable to extensive metabolism. Of particular interest is the fate of the benzhydryl moiety. The anti-emetics chlorocyclizine and cyclizine undergo N-demethylation respectively to norchlorocyclizine and norcyclizine (Kuntzman *et al.*, 1965). Norcyclizine N-oxide does not appear to be formed directly from norchlorocyclizine. However, Kuntzman *et al.*, (1967), found the N-oxide in the urine of rat and human after being treated with chlorocyclizine. Both N-1-butylchlorocyclizine and meclazine also underwent N-dealkylation to form norchlorocyclizine (Kamm *et al.*, 1972 and

Narrod *et al.*, 1965), whilst cinarizine was shown to form benzephenone as the major metabolite, although both norcyclizine and cinarizine-N-oxide were also present in rat faeces (Soudijn and Van-Wijngaarden, 1968). There was also a difference in the rate of elimination which was a function of the chloride atom, in that chlorocyclizine and norchlorocyclizine are metabolised much slower than cyclizine and norcyclizine, due to the more avid binding of the chloride molecule to plasma proteins (Kuntzman*et al.*, 1967).

Since almitrine is structurally similar to these molecules, N-dealkylation and subsequent formation of the N-oxide is highly probable. The two allyl side chains will probably undergo oxidation of the double bonds and/or N-dealkylation to produce the deallyl products. The presence of two fluorine molecules should increase the plasma protein binding in comparison to cyclizine and chlorocyclizine and this will influence the pharmacokinetics of the molecule.

Prior to its approval for marketing, a new chemical entity must be shown to be safe and therapeutically effective. This requires extensive investigative testing in both animals and human. The introduction of a drug from discovery to development (Preclinical and Clinical) requires metabolism studies in order to investigate the absorption, distribution, metabolism and elimination (ADME) of the compound in animals (usually the toxicological species). These studies are designed primarily to assist in the interpretation of toxicological results, to aid the extrapolation of animal safety data to man, to evaluate new dosage forms in animals and man and to relate pharmacological activity in both animals and man.

Aims of Thesis

The aim of this thesis is to :-

- compare the pharmacokinetics of almitrine in animals and man by (a) developing a specific but simple analytical method for the analysis of the intact drug in plasma and performing pharmacokinetic analysis on the data generated; (b) using the radiolabelled molecule, determine the plasma and blood kinetics of radioactivity and the route, rate and extent of elimination of drug derived material from excretion balance studies in selected animal species and man as part of the safety evaluation for development of the drug;
- relate plasma levels of drug and metabolite to activity by (a) investigating the uptake of [^{14}C]-almitrine and metabolites at the proposed site of action and comparing the levels in this organ to that observed in plasma and other important organs and (b) relating plasma levels determine during an International Multicentre Clinical Studies Programme to the dynamic measurements obtained during the clinical phase;
- identify major metabolites and establish the metabolic pathway of almitrine by developing and applying separation and isolation procedures based on thin layer chromatography and confirming the structures of these isolated products by mass spectrometry;
- investigate the pharmacokinetics in clinical studies and define the therapeutic dosage;
- relate pharmacokinetics to possible side effects by analysis of clinical plasma samples for almitrine and circulating metabolites as part of an International Multicentre Clinical Study Programme in Phase III of the development process and attempt to relate drug and/or metabolite levels to the magnitude of side effect.

CHAPTER 2

MATERIALS AND METHODS

This chapter deals with most of the methods and procedures used to generate analytical data for this thesis. The majority of procedures were developed and performed by me with the exception of the solvent system used for purification of radiolabel (this was supplied with the radiolabelled product), the procedures for location of radioactivity on TLC plates (standard laboratory procedures) and the microsomal studies described in section 2.2.5.1. A gas liquid chromatographic method for analysis of intact almitrine in plasma was developed based on an already existing but less manageable procedure. An existing HPLC method for the analysis of almitrine and metabolites in plasma was evaluated and validated in collaboration with other scientists within the Department.

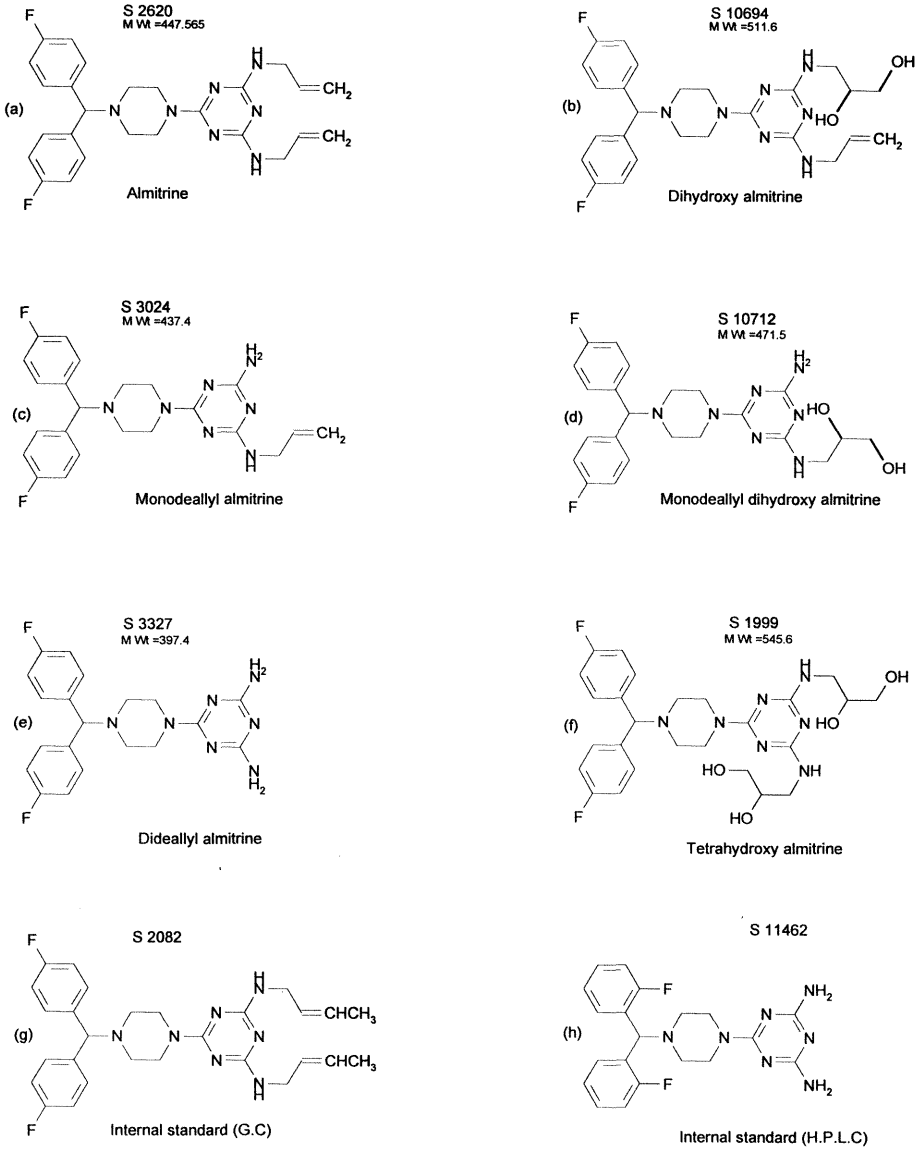
All metabolic, distribution and disposition studies involved the use of carbon-14 labelled almitrine bismesylate. An outline of radiochemical synthesis together with the radiochemical techniques used are presented here. Pharmacokinetic data on almitrine bismesylate were derived from analytical techniques designed for the separation and measurement of the parent compound and major metabolites. These procedures are also described here. This chapter contains the following sections:-

1. Materials
2. Radiochemical analytical methods
3. Non-radiochemical analytical methods
4. Pharmacokinetic analysis
5. Presentation of data

2.1 Materials

Radioactive almitrine bismesylate was supplied by CEA, Saclay, France. Almitrine, as the free base and the bismesylate salt, and related reference compounds (Fig. 2.1) were supplied by Les Laboratoires Servier, Suresnes and Gidy, France.

Figure 2.1 : Structure of Almitrine (a) and Reference Compounds including Internal Standards for GC (g) and HPLC (h) Analysis



Silica gel (G_{254}) was purchased from Merck Chemicals, Cambridge, UK.

The following reagents were obtained from Koch Light Laboratories, Colnbrook, UK: Triton X-100, D-L Malic acid (pure), Mannitol (pure) and methane sulphonic acid (pure). British Drug Houses Ltd., Poole, UK provided the following regents: Ethanolamine (Analar grade), Amberlite - XAD-2 resin (lab reagent grade), acetic acid (Analar grade), sodium acetate (Analar grade) and Polyethylene glycol 400 (laboratory grade).

The major components, 2,5-diphenyloxazole (P.P.O.) and Dimethyl-1,4-bis(5 phenyloxazol-2-yl)benzene (P.O.P.O.P.) used in scintillation cocktail mixture I and II were obtained from Packard Instruments Ltd., Reading, UK. These cocktails were made as follows:

Scintillation Counting Mixture 1

Toluene	666ml
Triton X-100	333ml
P.P.O.	100mg
Dimethyl P.O.P.O.P.	5.5g

This mixture was used for direct counting of solutions, urine and plasma.

Scintillation Counting Mixture II

Toluene	666ml
Methanol	333ml
P.P.O.	100mg
Dimethyl P.O.P.O.P.	5.5g

This mixture was used for combusted samples such as tissues, red cells and faeces.

All other reagents and the suppliers will be mentioned in subsequent sections. Other reagents were Analar grade and were supplied either by May and Baker Ltd., UK or by British Drug Houses Ltd., UK.

2.2 Radiochemical Analytical Methods

This section describes all radiochemical analytical procedures, including a summary of the synthesis of [^{14}C]-almitrine bismesylate. Chromatographic

procedures during purification and for metabolic studies, dose preparation procedures, *in-vitro* techniques for metabolism and protein binding studies and quantitation of radioactivity are described here.

Almitrine bismesylate was synthesised with carbon-14 in one of two positions, since metabolic breakdown was theoretically possible at both ends of the molecule. The benzhydryl position (*) (Fig. 2.2) was chosen as being the one most stable to metabolic decomposition based on the metabolism of structurally similar compounds described in Chapter 1. All disposition and metabolism studies described in this thesis have been conducted using this material.

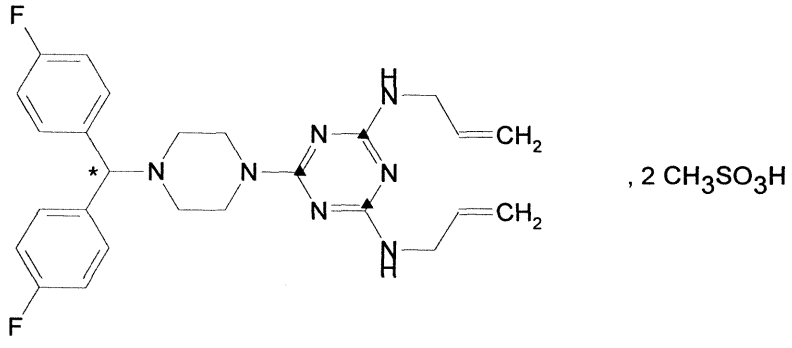


Figure 2.2 : [^{14}C]-almitrine bismesylate labelled in the benzhydryl (*) and triazine (▲) positions

Additionally, since it is possible for the molecule to be metabolically altered in the region of the triazine ring another molecule labelled in the triazine ring (▲) was synthesised in order to investigate the metabolic fate of this portion of the molecule which was also considered to possess the major activity of the drug.

2.2.1 Synthesis of [^{14}C]-benzhydryl labelled and [^{14}C]-triazine labelled almitrine bismesylate

[^{14}C]-Almitrine bismesylate labelled in the benzhydryl position was prepared by an eight stage process adapted from a procedure described in Journal of Labelled Compounds (1973) 9, 405. The final yield was 56%. This was supplied at 98% radiochemical purity with a specific activity of 1.25μCi.mg^{-1}.

[^{14}C]-Triazine labelled almitrine bismesylate was prepared by the three stage synthesis described by New England Nuclear, Boston, Mass, USA, starting with 2,4,6'trichloro-1,3-5 triazine (Cyanuric Chloride) uniformly labelled with [^{14}C]. The final yield of this product was 79%, with a radiochemical purity of 98% and specific activity of 9.14μCi.mg^{-1}.

2.2.1.1 Chemical purity

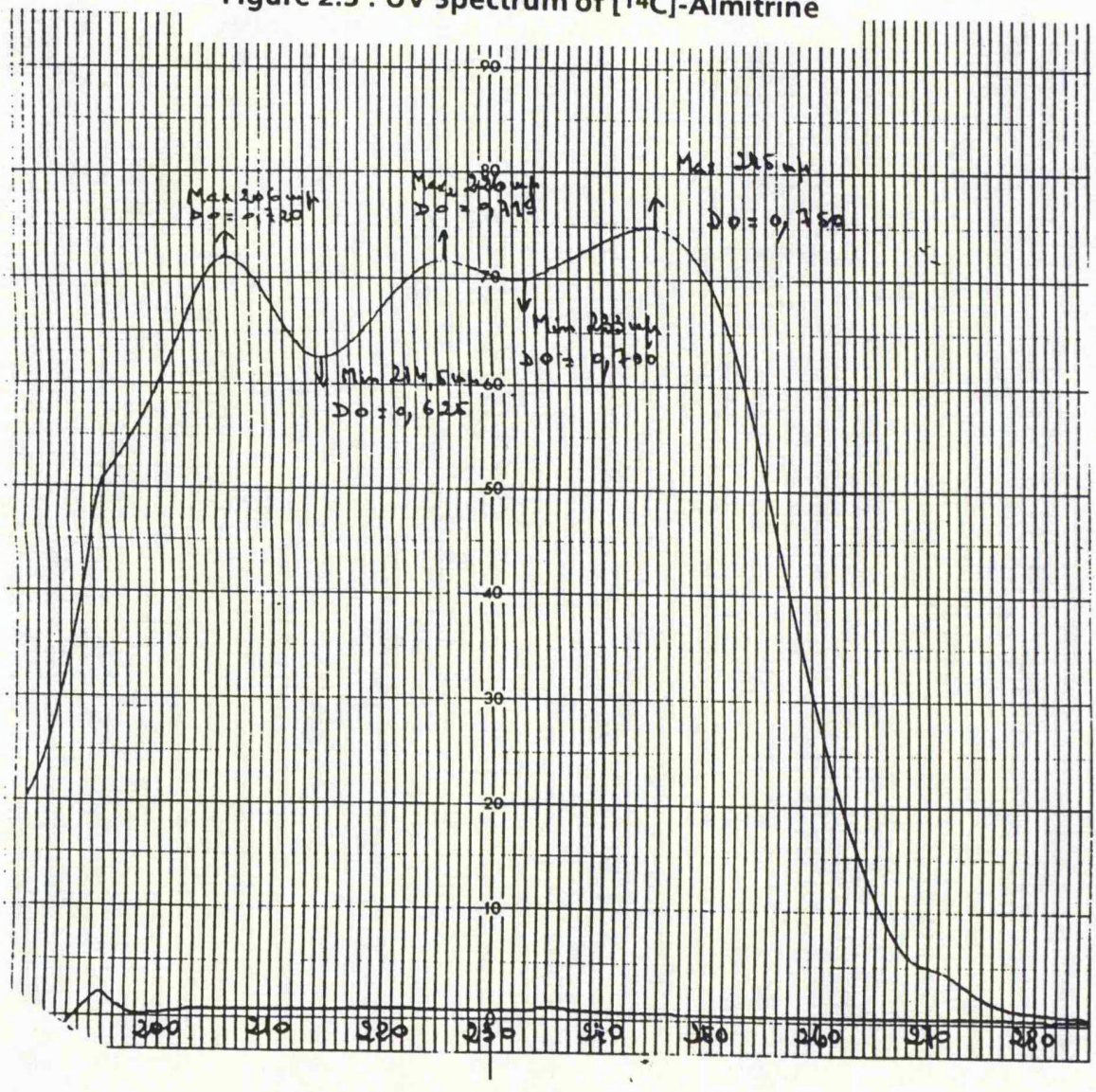
Chemical purity of the finished product was checked by u.v. spectrometry in methanol (80μg.ml^{-1}). The u.v. scan revealed the following:-

Max $_1$ = 206nm Min $_1$ = 214nm

Max $_2$ = 226.5nm Min $_2$ = 233nm

Max $_3$ = 245nm

Figure 2.3 : UV Spectrum of [^{14}C]-Almitrine



The u.v. absorption spectrum of the radiolabelled products was identical to that of a sample of unlabelled almitrine bismesylate, the purity of which had been varified previously by Technologie Servier (Fig. 2.3).

2.2.1.2 Radiopurity check, purification and quantitation of radioactive material

Before a radiochemical was used to formulate a dose or for *in-vitro* studies, the radiopurity of the [^{14}C]-material was checked by thin layer chromatography (TLC) using a solvent system of chloroform:methanol (90:10 v/v) followed by autoradiography and quantitation of the components by isolation and scintillation counting of the isolated silica adsorbent. Alternatively, radioactivity was analysed by quantitative radioscanning of the TLC plate. If the resultant purity was less than 97%, the material was subjected to further purification before use, since the impure product if used in a dose formulation can lead to erroneous interpretation of metabolic and pharmacokinetic radioactive data, as well as misinterpretation of *in-vitro* studies particularly method validation, *in-vitro* metabolism and protein binding studies (to be discussed later).

2.2.2 Purification of the radiolabelled material

The radiolabelled material was dissolved in a minimum volume of ethanol and streaked onto a 20 x 20cm thin layer chromatographic plate (silica gel G_{254}) approximately 2cm from the bottom of the plate. Next to the radioactive material was spotted an authentic sample (~25µg) of non-radiolabelled almitrine bismesylate as reference standard. The plate was developed in a solvent system of chloroform:methanol (90:10 v/v) to a distance of 15cm and the radioactive components located by autoradiography (to be detailed later) using a thirty minute exposure to Kodak X-ray film. Prior to exposure radioactive marker spots were placed on the top left hand side of the TLC plate. After processing, the autoradiograph was superimposed on the chromatogram by aligning the marker spots; the area of radioactivity corresponding to almitrine bismesylate was traced onto the silica in pencil. This region of silica was removed from the plate by careful scraping with a scalpel and the radiolabelled material eluted with three aliquots (3 x 1ml) of ethanol by vortex mixing for 5 min prior to centrifugation for 10 min. The radiochemical purity of the combined ethanolic extract was determined as previously described. If this was found to be greater than 97% the ethanolic solution was evaporated to a smaller volume (~200µl) and the radiochemical

purity checked once more prior to further use. If less than 97%, the material was subjected to a repurification.

2.2.2.1 Localisation and quantitation of radiolabelled components on TLC plates and whole body autoradiography

Autoradiography was used as a means of locating the radiolabelled components on the thin layer chromatograms. This technique was employed for all thin layer chromatographic procedures even when radioscanning was used for final quantitation.

Each chromatogram was carefully dried in a draught of air to remove every trace of the solvent and alignment marks, in the form of spots of radioactive ink, were applied to the plates. Working in total darkness, or with the aid of darkroom safety lights fitted with a red/green filter (Kodak 6B), the silica face of the chromatogram was placed in firm contact with a Kodak X-ray film. Adhesive tape was used to hold the film firmly to the plate if exposure was to be made for a considerable time and the plate and film placed into a light proof envelope. This was left undisturbed at room temperature for a suitable time. Storage time varied according to the amount of radioactivity on the plate and ranged from less than one hour for purification studies to weeks for metabolite profiling and whole body autoradiographic studies.

After the appropriate period of exposure, the X-ray film was developed under the safe light condition described earlier, using a 10% (v/v) solution of X-ray developer (200ml) for a period of 2-3 minutes, at a temperature of 35-40°C. The autoradiograph was then transferred to a fixing bath for 15 minutes. The fixer consisted of a 10% (v/v) solution of X-ray liquid fixer (200ml) to which was added X-ray liquid hardener (10ml) and the mixture maintained at 35-40°C. After fixing, the film was washed in water for 5 minutes, immersed in a 2% ammonia solution for two minutes and finally washed in running water for 15 minutes. Excess water was removed using a film scraper blade before the film was either hung to dry or placed in a hot air print dryer (Polysales, Surrey, UK).

When the film was dried, the individual radiolabelled components on the chromatographic plate were located by superimposing the autoradiograph on the chromatogram and aligning the marker spots, then tracing the areas in pencil onto the silica. If quantitation was required by scintillation counting the

located areas was scrapped off into scintillation vials. The remaining area of the chromatogram was then segmented into 1cm bands and each of these separate areas of silica were scraped into scintillation vials. All material was counted as a suspension in 4ml of water and 10ml of scintillation counting mixture I (described earlier).

For radiopurity studies, the radiopurity of the material was determined by expressing the amount of radioactivity associated with the area adjacent to the authentic reference sample as a percentage of the total radioactivity on the chromatogram. For metabolic studies (which will be described later) each radioactive component of interest was expressed as a percentage of the total radioactivity on the chromatogram.

2.2.2.2 Localisation and quantitation of radiolabelled components on TLC plates - radioscanning

Autoradiography in combination with liquid scintillation counting of TLC scraping or for metabolite profile studies can sometimes be time consuming, requiring long periods of exposure to X-ray film in order to locate the regions of radioactivity prior to quantitation.

The Berthold TLC radioscanner (Berthold Instrument (UK), Hertfordshire, UK) offered a quicker alternative to TLC quantitation of radioactivity. The plates were placed on an adjustable platform and the sensitivity, time constant and scan speed were set according to the level of radioactivity on the chromatogram. As the detector head travels along the chromatogram the radioactive areas are detected and integrated. A radiochromatograph is produced which can then be quantitated. The method of quantitation depends on the application. For metabolite profiling, each radioactive component is expressed as a percentage of the total chromatograph. The TLC plates can then be exposed to X-ray film for a defined period and the radioactive areas scraped and counted, if necessary, as described previously (2.2.2.1).

2.2.3 *In-vivo* studies

2.2.3.1 Human studies

Radiolabelled doses of [^{14}C]-almitrine bismesylate were prepared either from a lyophilised mixture dissolved in maleic acid or from a diluted solution of [^{14}C]-almitrine in methane sulphonic acid. The lyophilised dose preparation

was performed to mimic the intravenous non-radioactive dose of almitrine which was supplied as a lyophillate powder to be dissolved in 5ml maleic acid.

2.2.3.1.1 Preparation of radiolabelled doses from a lyophilised mixture

To a methanolic solution (50ml) of purified [^{14}C]-almitrine bismesylate (40-80µCi) was added non-radiolabelled almitrine bismesylate (29-250mg), methane sulphonic acid (85-100mg) and mannitol (20ml) dissolved in deionised water (100mg.ml^{-1}). The mixture was dissolved thoroughly and freeze dried overnight. The freeze-dried powder was then homogenised and aliquots accurately weighed to determine the specific activity of the radioactive mixture. This was performed by completely dissolving the weighed aliquots in methanol (100ml) and further aliquots diluted and counted for radioactivity. Dose purity and stability was also assessed as previously described.

For oral doses, a lyophilised mixture containing 100mg [^{14}C]-almitrine bismesylate (~40µCi ≡ 1.4mg.kg^{-1}) dissolved in maleic acid solution (80ml ≡ 1.25mg.ml^{-1}) was received by each volunteer.

For intravenous doses, the lyophilised mixture was dissolved in maleic acid (7ml ≡ 15mg.ml^{-1}), sterilised just prior to administration, and an aliquot taken for the determination of specific activity, dose purity and stability. The dose administered was approximately 20µCi, equivalent to 0.2mg.kg^{-1}. Intravenous dosage was administered over 12 minutes.

2.2.3.1.2 Preparation of radiolabelled doses as a standard dosing solution

To an ethanolic solution (1ml) of non-radiolabelled almitrine bismesylate (~250mg) was added an aliquot (200µl) of methane sulphonic acid and purified radiolabelled almitrine bismesylate (25µl ≡ 75µCi of a 3.0mCi.ml^{-1} ethanolic solution). The solution was gently mixed and deionised water slowly added until diluted to 50ml with gentle shaking. The solution was then sonicated for 10 minutes then aliquots taken for the final specific activity checks and to test for radiochemical purity. The dosing solution was sufficient for two oral doses each of 100mg ≡ 30µCi.

2.2.3.2 Animal studies

Before dosing animals with [^{14}C]-almitrine, certain points needed to be considered.

1. *The route of administration*

   Intravenous, subcutaneous and intraperitoneal routes in general required smaller volumes than oral routes. The dose composition and pH of the dosing matrix are also important factors. Oral doses, however, have fewer restrictions; larger volumes can be used and the composition and pH of the dosing matrix can be optimised to ensure solubility and stability and therefore homogeneity of the dose.

2. *Physical limitations*

   The size of the animal dictates the maximum volume of the dose and with the poor aqueous solubility of almitrine, the solubility of the drug for dosing small animals becomes an important factor in relation to the dose level and route to be administered.

2.2.3.2.1 Preparation of doses as a standard dosing solution

To an ethanolic solution of purified [^{14}C]-almitrine bismesylate was added a known quantity of non-radioactive almitrine bismesylate dissolved in methanol. This mixture was evaporated to dryness under a gentle stream of oxygen-free nitrogen then redissolved in a small volume (100µl) of ethanol and methane sulphonic acid (100µl); then slowly dilute with either a solution of mannitol (100mg.ml^{-1}) and made up to final volume with a solution of maleic acid dissolved in deionised water (15mg.ml^{-1}), or made to volume with gradual addition of deionised water with continuous sonication. The specific activity and radiochemical purity of the dose solution were then determined as described previously.

2.2.3.2.2 Preparation of doses using polyethylene glycol 400

For intravenous studies particularly in the rabbit, the aqueous formulations proved to be irritable to the animal due to the acidity of the dosing solution. Local necrosis was evident at the site of administration in rat studies and on administration to the more sensitive rabbits the animals reacted so violently (even from the placebo dose) that a non acidic formulation had to be investigated. Polyethylene glycol 400:water was found to be the most suitably tolerated formulation for these rabbits. An ethanolic solution

containing a mixture of purified [^{14}C]-almitrine bismesylate and non-radiolabelled almitrine bismesylate was evaporated to dryness then the residue redissolved in 0.2 to 1.0ml of hot ethanol (50°C). The solution was further diluted with a hot mixture (60°C) of polyethylene glycol 400:water (1:1 or 1:2 v/v) then after thorough mixing and allowing to cool to room temperature, the mixture was centrifuged and the supernatant retained for dosing. The specific activity and radiochemical purity were determined prior to dosing.

2.2.3.3 Dosing conditions and sample preparation and storage

Details of doses administered to various species are shown in Table 2.1.

For human studies, subjects were fasted for 12 hours prior to receiving the dose and 4 hours after dosing, although water was allowed *ad-libitum*. Control blood, urine and faeces were taken before dosing, then at certain times after dosing depending on the duration of the study. This ranged from 14 to 135 days. Blood was centrifuged to obtain plasma; the pH and volume of urine were measured where appropriate immediately after voiding. Faeces was collected into specially designed plastic bags. All samples were stored at -20°C until analysed.

Animals were in most cases fasted overnight and up to 3-4 hours after dosing, although water was allowed *ad-libitum*. Control blood, urine and faeces were taken before dosing, then at certain times after dosing. Wherever possible, with the exception of mice, all animals were individually housed in specially designed metabolic cages over defined periods to allow separate collection of urine and faeces. The cages were washed daily with a mixture of methanol:water (1:1) and these washes were retained for analysis. Where required, heparinised blood was centrifuged to obtain plasma. All sample were stored at -20°C until analysed.

2.2.4 Radioactive Analysis

2.2.4.1 Preparation of samples for liquid scintillation counting

Aqueous samples such as plasma, urine and cage washings (0.1 to 1.0ml) were counted directly in triplicate (if possible) in scintillation mixture I (10ml) dispensed into either 20ml capacity glass or polypropylene scintillation vials.

Table 2.1 : Summary of the Studies Undertaken of [^{14}C]-Almitrine

Species	Type	No./Sex/Strain	Dose	Route	Sample	Time (h)	Analysis
Rabbit	Balance Kinetics	4M/4F/NZW	0.5mg.kg^{-1} 0.8mg.kg^{-1}	I.V. P.O.	Plasma Urine Faeces	0-72 0-672	RA RA
Dog	Balance Kinetics	2M/2F/Beagle	0.5mg.kg^{-1} 5.0mg.kg^{-1}	I.V. P.O. I.V.	Plasma Urine Faeces	0-144 P.O. 0-336 I.V. 168	RA RA Non-RA
Rat	Balance Kinetics Tissue Distribution Metabolic	 2-3/M/Wistar 3/gp/M/Wistar 4M/Wistar	3.4mg.kg^{-1} 2.6mg.kg^{-1} 3mg.kg^{-1} 5mg.kg^{-1} 2mg.kg^{-1} 100mg.kg^{-1}	I.V. P.O. I.V. I.V. P.O. P.O.	Blood Blood Blood, Tissue Blood, Urine Faeces	0-72 0-72 0-16 0-120 0-48	RA RA, WBA RA, TLC RA, TLC MS
Human	Balance Kinetics Long Term Balance	1M/1F/C 2M/2F/C	0.02-0.19mg.kg^{-1} 1.5mg.kg^{-1} 1.5mg.kg^{-1}	I.V. P.O. P.O.	Plasma Urine, Faeces Plasma Urine Faeces	0-672 0-4 months	RA TLC, MS Non-RA RA, TLC

RA denotes Radioactivity
WBA denotes Whole Body Autoradiography
TLC denotes Thin Layer Chromatography
MS denotes Mass Spectrometry

Non-RA denotes Non Radiochemical Analysis
NZW denotes New Zealand White
M/F/C denotes Male/Female/Caucasian

Whole blood and red blood cells were homogenised with a spatula and, if possible, triplicate aliquots (100-500mg) were accurately weighed into combustor cones prior to combustion.

Smaller tissues such as adrenals, carotid artery, carotid body, superior cervical ganglion (SCG) and trachea were weighed directly into combustor cones prior to combustion.

Carcasses were homogenised in a bottom drive polytron homogeniser and triplicate aliquots (300-400mg) weighed accurately into combustor cones prior to combustion.

Faecal samples were homogenised, with a little water, using either a "Polytron" or a "Colworth Stomacher 400" sample homogeniser. Triplicate aliquots (300-500mg) were accurately weighed into combustor cones prior to combustion.

All other tissues such as liver, lungs, kidney, heart, brain, testes, small and large intestine and stomach were homogenised with a top drive Polytron homogeniser and triplicate aliquots (200-500mg) accurately weighed into combustor cones prior to combustion.

Combustion was performed on a Packard Model 306 sample oxidiser. The [^{14}C]-labelled carbon dioxide generated was trapped in 35% ethanolamine in methanol (8ml) and mixed with scintillation mixture II (12ml).

2.2.4.2 Determination of radioactivity

The radioactivity of all samples was determined by liquid scintillation spectrometry using a Packard Tricarb 3385 or 300 liquid scintillation spectrometer. The counting efficiency of each sample was determined by the automatic external standard ratio (AES) method by reference to a calibration curve produced with a series of sealed quenched standards supplied by Packard Instrument Co. Inc, covering an AES range of 0.16 - 0.96 (20-95% efficiency). The results were expressed as disintegrations per minute (dpm) and from these further converted to µg or ng equivalents per unit weight or volume or expressed as a percentage of the administered dose.

2.2.5 *In-vitro* Studies

2.2.5.1 Microsomal studies

The *in-vitro* metabolism of [^{14}C]-almitrine was investigated using liver microsomal preparations obtained from various species including man.

Prior to the full study, it was necessary to optimise the concentration of almitrine necessary for incubation such that a maximum rate of metabolism per total protein concentration could be achieved. The standard incubation mixture contained 2mg of microsomal protein in 0.5ml cofactor which comprised the following per 10ml.

Tris buffer (0.1M, pH 7.4)	9ml
$MgCl_2$ (0.15M)	1ml
Glucose-6-phosphate	12mg
Glucose-6-phosphate dehydrogenase	20 units
$NADP^+$	8mg

The volume of the incubation mixture was 1.5ml. To this was added 30µl (2%) of test solution containing [^{14}C]-almitrine bismesylate prepared at three different concentrations. The incubation mixture is summarised in Table 2.2.

Table 2.2 : Optimisation of the Incubation Concentration of [^{14}C]-Almitrine Bismesylate

Sample				Protein Content	Volume to give 2mg	Volume of Cofactor	Volume of Tris Buffer
Tube	Conc	Vol					
	(µg)	µl	µCi	(mg.ml^{-1})	(µl)	(µl)	(µl)
T_1	22.6	15*	0.48	12.68	158	500	812
T_2	45.2	30	0.96	12.68	158	500	812
T_3	90.4	30**	0.96	12.68	158	500	812
C_1	22.6	15*	0.48	-	-	500	970
C_2	45.2	30	0.96	-	-	500	970
C_3	90.4	30**	0.96	-	-	500	970
$T_{(0)}$	45.2	30	0.96	12.68	158	500	812

T1,2,3 = Test

C = Control

$T_{(0)}$ = Time, zero incubation

*15µl test solution + 15µl tris buffer

**0.96µCi + 45.2µg cold

All samples, except $T_{(O)}$ were incubated at 37°C in a shaking water bath for 2 hours. The incubation was terminated by the addition of 500µl acetonitrile. $T_{(O)}$ was terminated immediately the test material was added. After vortex mixing for 5 minutes, the samples were centrifuged at 3000rpm for 10 minutes, then aliquots (20µl) of the supernatant were counted to determine the recovery of radioactivity into the supernatant and the specific activity of these supernatant extracts. From the recovery and specific activity of the supernatant, the volume required to apply 15,000dpm onto a silica TLC plate was calculated (Table 2.3). The spots were developed in chloroform: methanol:ammonia (80:20:2 v/v) solvent system 1. Reference standards were chromatographed between samples. After development, the TLC plate was scanned on the Berthold TLC Scanner in order to establish the extent of metabolism of almitrine at the three concentrations.

Table 2.3 : Specific Activity and Recovery of Test Samples

Test Sample	Aliquot Counted (µl)	dpm	dpm.ml^{-1}	Mean dpm.ml^{-1}	Total in 2ml		% Recovery	µl Spotted
					dpm	µCi		
T_1	20	7239 7518	361936 375886	368911	737822	0.33	69	40
T_2	20	14976 15342	748775 767120	757948	1515895	0.68	71	20
T_3	20	14444 14691	722220 734560	728390	1456780	0.66	69	21
C_1	20	4692 4922	234606 246094	240350	480700	0.22	46	62
C_2	20	12912 13728	645580 686410	665995	1331990	0.60	63	23
C_3	20	10363 10392	518145 519595	518870	1037740	0.47	49	29
T_O	20	6270 7054	313487 352697	333092	666184	0.30	63	45

Figure 4.1 (Chapter 4) shows the low dose (test mixture 1) to be the most appropriate concentration for maximum metabolic conversion of almitrine.

2.2.5.2 Isolation procedures for metabolites

Many preparation procedures have been employed on plasma, urine, faeces and tissue samples for the separation and isolation of almitrine and its metabolites. The most commonly used were freeze-drying and XAD_2 extraction. These procedures and the extraction of small tissues are described in this section.

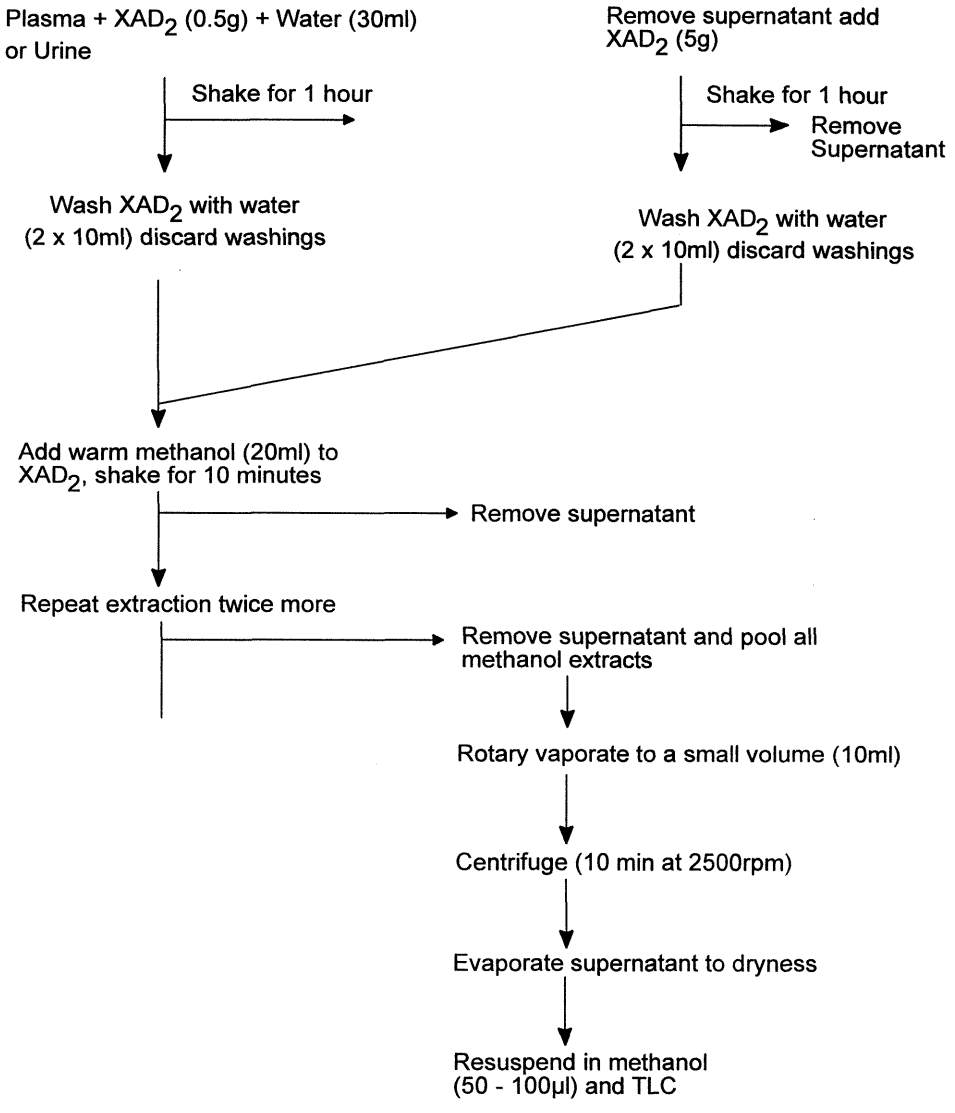
2.2.5.2.1 Extraction of almitrine and its metabolites from human and animal plasma and urine using Amberlite XAD_2

Amberlite XAD_2 was washed five times with distilled or deionised water to remove chloride ions. Then aliquots (0.5-5g) were added to diluted plasma or urine of known radioactivity held in conical flasks and the contents magnetically stirred for 1 hour. After allowing to settle, the supernatant was transferred to a second flask and further aliquots of XAD_2 were added and the mixture stirred for a further 1 hour. Each of the XAD_2 residues was washed twice with water and the washings discarded. Excess water was removed with the aid of a Pasteur pipette and the radioactivity extracted from XAD_2 with warm methanol (20ml) by magnetically stirring for 10 minutes. The supernatant was removed and the extraction procedure repeated twice more. An aliquot of the pooled methanolic extract was counted in order to determine the efficiency of extraction and the remaining methanol rotary evaporated to a small volume (5-10ml). After centrifugation for 10 minutes at 2500rpm the supernatant was evaporated to dryness under a gentle stream of oxygen-free nitrogen and the residue redissolved in a small volume (50-100µl) of warm methanol prior to application onto TLC plates. The extraction procedure is summarised in Figure 2.4.

2.2.5.2.2 Extraction of almitrine and its metabolites from plasma, urine, faeces and tissues by freeze drying or dried extraction

Plasma, urine, faecal and tissue homogenates were initially frozen then freeze dried for 2-16 hours, depending on the quantity of material, on a Modullo 50 freeze-drier. The lyophilised material was then exhaustively extracted into methanol (0.5 to 5ml) and the pooled methanol extracts rotary evaporated to a small (5ml) volume at room temperature. After centrifugation (15 minutes at 2500rpm) to remove debris the supernatant was evaporated to dryness under a gentle stream of oxygen-free nitrogen

Figure 2.4 : XAD_2 Extraction of Almitrine and Metabolites from Plasma and Urine
(Wash XAD_2 five times with water before use)



at 50°C and the residue redissolved in a small volume (100µl) of methanol prior to TLC.

Small tissues such as carotid body, carotid artery and superior cervical ganglion were gently ground in a test tube containing warm methanol (0.5ml) then sonicated for 20 minutes followed by mechanical shaking for a further 10 minutes. After centrifugation the supernatant was transferred to another tube and the residue extracted twice more with warm methanol (0.5ml). The pooled supernatant was evaporated to dryness under a gentle stream of oxygen-free nitrogen and the residue redissolved in a small volume of methanol (100µl) prior to TLC.

2.2.5.2.3 Hydrolysis of urine conjugates prior to extraction

For many drugs, the urinary excreted products are eliminated as conjugates mainly as the glucuronide or sulphate conjugate. Because of the polar environment, extraction of these conjugates from urine is difficult. Additionally chromatograhic separation for either profiling or purification purposes prior to structural identification is hindered because these highly polar conjugates tend to remain in the region of the origin on TLC plates where the vast majority of endogenous polar, non-migrating urinary component remain. Cleavage of the conjugate is therefore necessary to release the product of interest from the glucuronic acid or sulphate moiety. Enzyme hydrolysis is the most popular method of choice for hydrolysing these conjugates. These enzymes liberate the phase I products thus making isolation and purification for identification more manageable.

For almitrine, urine hydrolysis was performed on pooled composite (0-24 hour) aliquots (5-200ml). The urine pH was adjusted to pH 5.0 with glacial acetic acid and an aliquot (100µl - 1.0ml) of *Sac Helix pomatica* having both β-glucuronidase and sulphatase activity was added to the sample. The mixture was incubated at 37°C for 16 hours in a shaking water bath. Enzyme activity checks were performed by substituting urine with buffer containing phenolpthalein glucuronide. On addition of ammonia (2 drops) to the incubated mixture, a pink colouration indicated the release of free phenolpthalein from its glucuronide conjugate. Other control checks included a non-enzymatic urine sample and an enzyme inhibition check as outlined in Table 2.4.

Table 2.4 : Enzyme Hydrolysis Scheme

TUBE No.	*SAMPLE	β-glucuronidase	D-saccarine acid 1-4 lactose	Phenolphthalein Glucuronic Acid
1 - Control	Urine (5ml)	-	-	-
2 - Hydrolysis	Urine (5-200ml)	100µl - 1ml	-	-
3 - Inhibition check	Urine (5ml)	100µl	≈2mg	-
4 - Enzyme activity check	Buffer (5ml)	100µl	-	≈2mg

*Glacial acetic acid was used for urine to adjust to pH 5

After hydrolysis the samples were extracted either by XAD_2 or following freeze drying as described previously.

2.2.5.2.4 Efficiency of extraction procedures

The efficiency of extraction of radioactivity from plasma, urine, faeces and the larger organs following freeze-drying was greater than 90%. For plasma and unhydrolysed urine the efficiency of extraction by XAD_2 was 95%. The efficiency of extraction was 90% from hydrolysed urine.

2.2.5.3 Plasma protein binding

Of the many techniques available for the determination of the extent of binding of low molecular weight components to biopolymers such as plasma proteins, equilibrium dialysis has been found to be the most popular used. This technique has undergone several modifications since its early use by Davis (1943) and Klotz (1946).

All early studies on the plasma protein binding of almitrine were performed using the bag method in which visking tubing (molecular weight cutt off of 12,000 - 16,000 daltons) was soaked in running tap water for 1 hour, then soaked in Tyrode buffer. This buffer was prepared by dissolving the following in distilled water (100ml) and adjusting the pH to 7.4 using either HCl or NaOH (5N):- NaCl (800mg); KCl (20mg); anhydrous $CaCl_2$ (20mg); $MgCl_2$

(10mg); $NaHCO_3$ (100mg); dextrose (glucose) (100mg); $NaH_2PO_4.2H_2O$ (5mg).

Aliquots (2ml) of this buffer were transferred to 20ml test tubes and these were placed into a shaking water bath held at 37°C and allowed to equilibrate for at least 30 min. The prepared visking tubing was cut into 5 inch lengths and a double knot tied at one end to form a bag. Radiolabelled almitrine was added to freshly prepared human plasma and 2ml aliquots were added to these bags and the open ends were also sealed with a double knot, ensuring that no plasma flowed back whilst at the same time expelling as much air as possible. The knotted tubes or bags were immersed into the test tubes containing buffer with both ends held upwards and above the liquid. The tubes were stoppered and left in the shaking water bath for 24 hours after which they were removed and the contents transferred to sample tubes. Aliquots (200µl) of the dialysed plasma and of the buffer dialysate were counted for radioactivity and from these the plasma protein binding of almitrine was determined (see below).

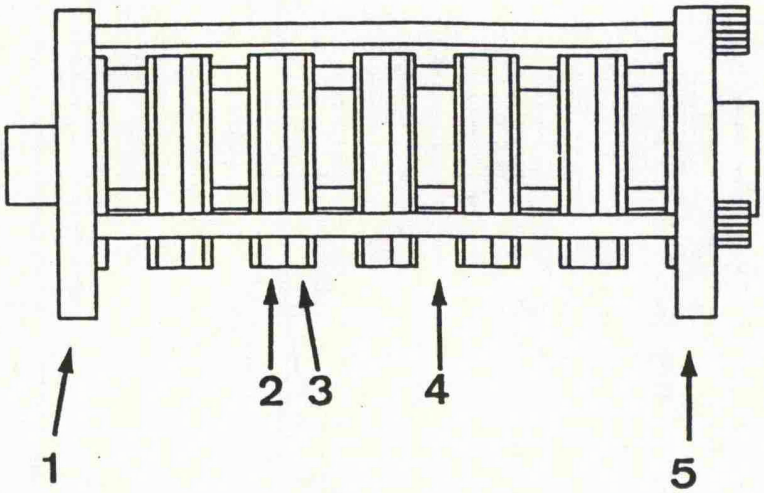
This method of determining plasma protein binding suffered many drawbacks.

1. Equilibrium was normally slow to be achieved and because of this, the time over which the study was conducted gave rise to tremendous volume changes in the bag due to osmosis. This increased volume varied from tube to tube and volumetric adjustments were erroneous.
2. Leakages during the tying of the open end of the tubes after addition of plasma gave rise to contamination of the dialysate and these resulted in erratic results. Erratic data could also have resulted in the long equilibrium time since bacterial degradation of the visking tubing could also give rise to leakage.
3. The recovery of radioactivity was low (losses greater than 70% were encountered) and variable due to binding of almitrine to the membrane and precipitation of the insoluble free material in the dialysate.

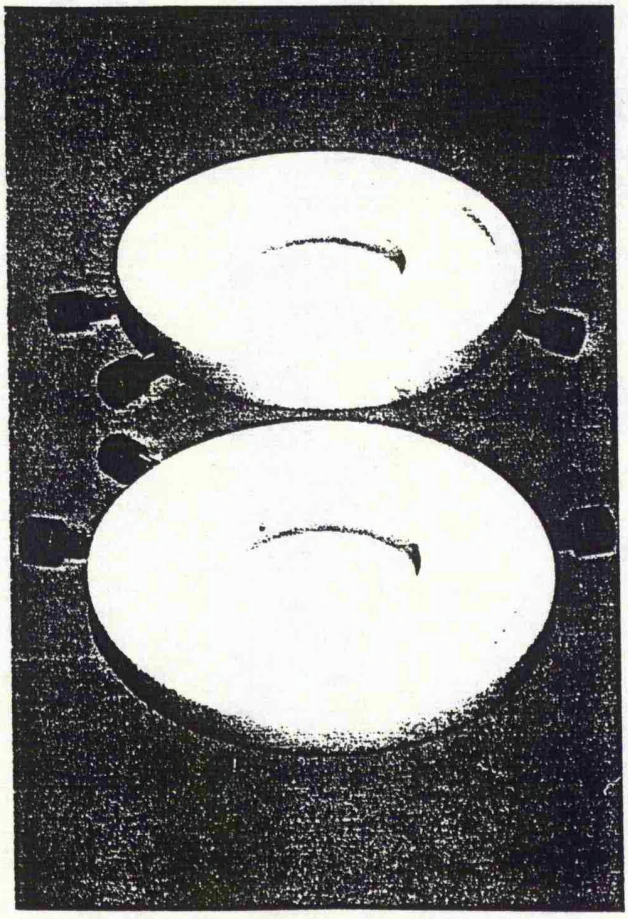
It was therefore necessary to try an alternative procedure which could eliminate some, if not all, of these drawbacks.

In the majority of studies reported, a twenty cell capacity Dianorm Equilibrium Dialyser was used (Fig 2.5).

Figure 2.5 : Diagram of Dianorm and Cell Units



1. Driving flange with guide rods
2. Teflon cell BASE) 5 pairs in complete stack
3. Teflon cell LID) 5 pairs in complete stack
4. Spring loaded cell spacers, 6 in complete stack
5. Bearing flange secured with 3 knurled nuts.



A disassembled cell with stoppers in position, showing the sealing ridge.

Each cell was made up of two halves of 2ml capacity. When assembled, each cell half was separated by a semipermeable dialysing membrane tubing (Spectrapor 2, MSE Scientific Instruments, Sussex), with a molecular weight cut off of 12,000 - 14,000 daltons. The dialysing membrane tubing had previously been prepared by soaking in distilled water (1 hour) then cut into half segments (6cm) before soaking and storing in isotonic phosphate buffer (pH 7.4) prior to use. Isotonic phosphate buffer (pH 7.4) was prepared as follows:- $Na_2HPO_4.12H_2O$ - 8.335g, $NaH_2PO_4.2H_2O$ - 0.876g, NaCl -5.58g. The volume was made up to 1 litre with distilled water and the pH adjusted to pH 7.4 with dropwise addition of either 10N HCl or 10N NaOH. Each assembled cell was mounted into a cell carrier and separated by spring loaded spacers (4), thus allowing free circulation of ligand in the temperature controlled environment. The top plate of the carrier was secured with three knurled nuts (5) to clamp the membrane in a leak-free manner.

After assembly, plasma (2ml) containing radioactive almitrine bismesylate was introduced into the "lid" half (3) of each cell and isotonic phosphate buffer (2ml) into the "base" half (2). The filled carriers were then mounted into a drive unit which had a variable speed motor, thus allowing the cell carriers to rotate slowly about its axis at a speed adjustable between 3 and 30rpm. Equilibrium dialysis was performed at 37°C for a predetermined time in either a Gallenkamp humidity oven or a variable speed thermostatically controlled water bath.

Following dialysis, the radioactivity in each cell compartment was determined and the extent of plasma protein binding of almitrine bismesylate at equilibrium was calculated as follows:-

$$\% \text{ bound} = \frac{\text{dpm plasma} - \text{dpm buffer}}{\text{dpm plasma}} \times 100$$

2.3 Non-Radiochemical Analytical Methods

Several methods have been described for the measurement of almitrine in biological fluids, and include radio-immuno-assay (RIA), gas liquid chromatography (GLC) and for its metabolites, high performance liquid chromatography (HPLC) employing both reverse phase chromatography and normal phase chromatography. The main characteristics of these methods are summarised in Table 2.5. Two of these methods were investigated

Table 2.5 : Main Characteristics of the Analytical Procedures for Almitrine

Author	Method	Biological Fluids	Sample Volume	Internal Standard	Quantitation Limit ($ng.ml^{-1}$)	Linearity Range	Intra-assay Precision (*)			
Bromet	RIA	Plasma	50µl		6	6-400				
Baune *et al*	GC	Plasma RBC Whole Blood	1ml	S2082	1	1-150	20.0(1)	8.5(20)	2.5(100)	
Gordon	GC	Plasma Blood	1ml 100µl	S2082 S2082	1 50	1-300 50-1000	2.5(5)	5.5(100)	4.9(250)	
Parkhurst *et al*	HPLC	Plasma	1ml	S2082	1	7-1000				
Aubert *et al*	HPLC	Plasma	1ml	S2721	5	5-1000	5(50)	3(250)	8(500)	
Jeanniot *et al*	HPLC	Plasma Urine Tissues Gastric Juice	1ml	S11462	Almitrine 5 dihydroxy almitrine 10 monodeallyl almitrine 5 tetrahydroxy almitrine 25 bisdeallyl almitrine 10 monodeallyl dihydroxy almitrine 10	5-1000 10-1000 5-1000 25-1000 10-1000 10-1000	1.9(25) 2.8(25) 2.4(25) 8.3(25) 4.0(25) 4.0(25)	0.9(100) 3.6(100) 0.5(100) 2.6(100) 2.1(100) 1.5(100)	1.1(250) 2.2(250) 1.3(250) 4.5(250) 2.0(250) 2.0(250)	1.3(500) 3.1(500) 0.9(500) 2.7(500) 1.2(500) 1.6(500)

* Results are expressed as % coefficient of variation (almitrine concentration in $ng.ml^{-1}$).

Structures of metabolites and internal standards are given in Figure 2.1

further (a) GLC for unchanged almitrine which was developed further and (b) HPLC for almitrine and its metabolites which was evaluated and validated.

2.3.1 Modifications to the method of Baune *et al.*

Baune *et al.*, (1981) described a GC method of analysis for almitrine in plasma employing liquid/liquid extraction at basic pH and detection by nitrogen phosphorus specific detection (NPD). The method, although sensitive to between 1 and 5ng.ml^{-1} and shown by mass spectrometry to be specific for almitrine, suffered the drawback of being a multiple extraction into cyclohexane with an "external standard" added later.

For routine analysis where large numbers of samples are to be analysed, as few manipulations as possible should be necessary for good precision of the assay. Furthermore, volumetric transfers will vary from sample to sample and from time to time. Spillages may occur which result in the loss of solvent. It is important, therefore, that a good analytical method has good precision, is robust and, particularly for pharmacokinetic studies, is accurate and sensitive enough to allow a good estimate of the terminal half-life of the compound. With multiple extraction of the sample and subsequent evaporation of the cyclohexane, the assay lent itself to being tedious, particularly dealing with cyclohexane which had to be kept under strict temperature conditions. The lack of an internal standard required strict, consistent volumetric transfer and therefore precision was always going to present problems in this assay, particularly for the inexperienced analyst. Furthermore, the occasional spillage would add to precision problems. With the aid of an internal standard, each sample would be extracted in a ratio concentration, and therefore the amount of solvent transferred was not always critical and certainly spillages would not affect the precision, since the ratio of the analytes would be constant and not dependant on the volume of remaining solvent. Furthermore, the addition of the external standard required the addition of an accurate volumetric transfer and consistant dissolution in order to get a reproducible result.

Surprisingly, the external standard S 2082 (Fig 2.1g) differed from almitrine by the presence of an additional methyl group on each of the allyl side chains. The compounds behaved similarly chromatographically; the internal standard eluting two minutes after almitrine under the conditions described for optimal chromatography, yet did not extract under the same conditions as almitrine,

i.e., under basic conditions when almitrine was supposed to be non-ionised and therefore extracted into the lipophillic organic phase. This was a perfect internal standard in its true sense since it was structurally similar to almitrine, and as a larger molecule would not in any way interfere with the analysis of almitrine or its metabolites since it would not be a potential metabolite. Furthermore, its retention time ensured good chromatographic reproducibility. It was therefore obvious that the two compounds had either markedly different pKa's, which would result in different extraction efficiencies at the same pH, or the S2082 internal standard was undergoing some structural modification such as cyclisation of the longer side chains to form a compound which was either then not extracted or chromatographed differently. The assay therefore required further investigation.

It was easier to approach the problem from a pKa point of view. The first approach was to investigate the efficiency of extraction of [^{14}C]-almitrine over the entire pH range. Radiolabelled almitrine was therefore spiked into plasma (100ng.ml^{-1}) and 1ml aliquots were adjusted to pH's between pH 2 and pH 11, using either HCl or NaOH. Each sample was extracted with cyclohexane and the efficiency of extraction at each pH was determined.

Table 2.6 : Extraction Efficiency of [^{14}C]-Almitrine (S 2620) from Cyclohexane over the pH range 2.5 to 11.2

pH	% Recovery
2.5	84.6
4.2	96.6
5.0	97.2
5.5	93.1
6.0	98.5
7.2	95.1
8.0	89.2
8.2	99.5
9.2	96.0
9.6	97.1
10.4	92.7
11.2	97.2

The efficiency of extraction of [^{14}C]-almitrine varied from 84.6% to 98.5% at pH 2.5 to 6.0 and between 89.2% and 99.5% at pH's greater than pH 7.0 (Table 2.6). The results suggested that almitrine is amphoteric and is extractable under all pH conditions. When the study was repeated using an internal standard prior to extraction the chromatographic results showed conclusively that under basic conditions the efficiency of extraction of the internal standard was extremely poor whereas almitrine behaved more amphoterically and had good extraction efficiencies at all pH's. The chromatograms also indicated that under acid conditions the internal standard was extractable but later running peaks from previous runs made it difficult to optimise conditions with cyclohexane extraction. Radioactive studies also showed that the recovery of almitrine into chloroform and ether from plasma or aqueous matrices at different pH's was greater than 80% between pH 1 and pH 14. However, the ratio of almitrine to internal standard increased relative to that of the unextracted standard as the pH increased from pH 2 to pH 7 and the internal standard was barely extractable beyond pH 7.4. Thus all efforts were concentrated on finding the optimal extraction pH and also the best extraction solvent which gave not only a good recovery but also least noise in terms of interference.

A low pH buffer was therefore the most appropriate starting point and a more volatile solvent such as diethyl ether for ease of transfer and evaporation was thought to be ideal. Other solvents such as hexane and diisopropyl ether were selected again because they were ideal as upper layer solvents for routine extractions and also were lypophillic enough to extract almitrine and the internal standard. Again, using a 100ng.ml^{-1} plasma standard, all three solvents were investigated with differing volumes of pH 1.3 KCl buffer (0.1 to 2ml) and comparing the peak heights of both almitrine and internal standard, and also the ratio almitrine to internal standard with that of the unextracted mixture of the same concentration dissolved in acetone. The plasma samples were extracted once into the solvent (1ml plasma + buffer + 7ml solvent) by mechanically shaking for 10 min, centrifuged then the organic solvent transferred to a tapered tube, evaporated to dryness under oxygen-free nitrogen, then the residue reconstituted in 100µl acetone, prior to injection of 3µl onto GC and analysed under the conditions described by Baune *et al.*, (1981). The best condition was found to be 1.5ml pH 1.3 buffer + 1ml plasma extracted in diisopropylether. Endogenous material gave rise to poor peak

shape and a small interference peak. It was therefore necessary to incorporate a further clean up procedure.

Since the compounds were extracted under acidic conditions, it was felt that the chromatographic problems encountered were due mainly to fatty acids or endogenous acidic components which were affecting the chromatography of the components of interest. Either a back extraction procedure or a solvent washing procedure would resolve this problem. Back extraction into acid and re-extraction under basic conditions gave a poor yield of almitrine and almost zero yield of the internal standard. Dissolving the extract in butylacetate then comparing the chromatograms before and after washing with NaOH showed not only did both compounds preferred to remain in the organic phase after the basic wash, but also the problem of poor peak shape was resolved since the acidic products were extracted into the basic environment, thus abolishing the tailing of peaks. The assay was therefore developed on the basis of an extraction with diisopropyl ether under acidic conditions, with an internal standard added prior to extraction.

2.3.1.1 Apparatus

A Hewlett-Packard, Model 5710A, gas chromatograph equipped with dual nitrogen-phosphorus detectors was used initially. The signals from the amplifier were recorded on a Perkin Elmer 56, single pen recorder with a 2mV f.s.d. and chart speed at 5mm.min^{-1}. Later analyses were performed on a Hewlett-Packard, Model 5880, gas chromatograph equipped with a single nitrogen-phosphorus detector and additionally either a flame ionisation or electron capture detector. The signals were recorded initially on a level 2 integrator from which peak heights were recorded (in cm) and later to a level 4, with the peaks integrated in the peak height mode.

Separation was achieved on a silanised glass column (1M x 2mm i.d.) packed with 3% OV1 on Gas Chrom Q (80-100mesh). Nitrogen (oxygen-free) carrier gas was maintained at a flow rate of 30ml.min^{-1}, with hydrogen and air flow through the detector at 3ml.min^{-1} and 60ml.min^{-1} respectively. Injector and detector temperatures were held at 300°C and the column oven temperature maintained at 270°C.

Samples were introduced by manual injection on the Hewlett Packard Model 5710A, using a 10µl Hamilton syringe. On the 5880 models, however,

sample introduction was via a Hewlett-Packard, Model 7672A autoinjector which had a capacity of 100 samples.

All glassware used for the preparation of standards and for the extraction from plasma were silanised before use in order to reduce the adsorption of drug onto the active sites of glass. A 5% solution of dichloromethylsilane in 1,1,1-trichloroethanol was used to fill the tubes and volumetric flasks which were allowed to stand for 5 minutes before decanting the solution. The glassware was allowed to dry by inverting in a drying basket then the HCl, formed as a result of the reaction of the excess dichloromethylsilane with atmospheric moisture, was washed off with deionised water and the glassware rinsed with methanol prior to drying before use.

2.3.1.2 Preparation of chromatographic stationary phase

The non-polar OV1 stationary phase was found to be the best phase for the separation of almitrine and internal standard from endogenous plasma material. Although chromosorb W has been used initially as support, better peak shape and resolution was found with Gas chrom Q, obtainable from Phase Separation Ltd.

Packing material was prepared by accurately weighing out 0.62g OV1 (Perkin Elmer Ltd) and dissolving in 75ml n-hexane for two hours on a hot plate (60°C) with a magnetic stirrer. The solution was added to 20g Gas Chrom Q (80-100 mesh A.W. DMCS) contained in a 1 litre round bottomed flask and the mixture held for 4 hours with occasional shaking. The hexane was slowly evaporated by a rotary evaporator with the flask immersed into a steam bath. Following evaporation, the coated support was transferred to a drying dish and was dried thoroughly for 16-24 hours in a desicator held in a drying oven set at 120°C. On cooling, the material was examined and any large aggregates were either broken into fine particles or removed completely since these were likely to affect the efficiency of packing into the glass column.

2.3.1.3 Packing and conditioning of analytical columns

In order to minimise adsorption of almitrine onto active sites of the chromatographic glass column, the empty column was filled with a 5% v/v dichloromethylsilane in toluene and left for approximately 30 minutes. The column was emptied, dried with oxygen-free nitrogen and rinsed with deionised water and methanol. After removal of the excess methanol by

blowing through oxygen-free nitrogen, the column was dried for 1 hour at 100°C. A silanised glass wool plug was inserted into the detector end of the column and this end was attached to a gentle vacuum. A small funnel was attached to the other end and the dried packing material introduced into the column with gentle tapping of the column or applying of a gentle vibrational force along the flow of the material into the column. The packing material was filled to the other end of the column and the open end plugged with more silanised glass wool. The column was connected onto the gas chromatograph with the detector end unattached and the injector port held at 250°C. An aliquot (75µl) of Silyl 8 (Pierce Chemicals) was injected onto the column. The oven temperature was held at 200°C for 2 hours then increased to 280°C at a rate of $0.1°C.min^{-1}$. This temperature was maintained for at least 6 hours, then the column was disconnected and a vacuum and vibration applied so that the packing material was properly bedded in the column, eliminating any air gaps formed during initial packing. Excess packing material was removed from the injector end to a length of approximately 3cm from the end in order to eliminate excessive column bleed. The column was then fully connected and the analytical conditions set up prior to injection (3µl) of a standard mixture ($1µg.ml^{-1}$) to establish retention times of almitrine and internal standard.

2.3.1.4 Preparation of standards

Calibration and validation or quality control standards were separately and independently prepared from separate stock solutions of almitrine base dissolved in acetone ($1mg.ml^{-1}$). Subsequent dilutions in acetone were prepared within the range $1µg.ml^{-1}$ to $10µg.ml^{-1}$ then aliquots (100µl) added to control plasma (10 to 20ml) to final concentrations ranging from $5ng.ml^{-1}$ to $1000ng.ml^{-1}$. Two assay ranges were employed, a macro and micro assay which will be described fully later. For the macro assay the calibration range used was 5 to $300ng.ml^{-1}$ and for the micro assay the concentration range was 50-$1000ng.ml^{-1}$ prepared in 20 and 10ml control plasma respectively. All volumetric glassware used for the preparation of standards were silanised as described previously.

2.3.1.5 Extraction of almitrine and internal standard from plasma

Two approaches were adopted for the analysis of almitrine in plasma. Single dose administration in which the plasma concentration ranged from $1ng.ml^{-1}$ to $300ng.ml^{-1}$ employed 1ml of plasma (Macro method). The second

approach (micro method) employed a smaller aliquot (100µl) of plasma and this was used mainly for small samples from animals and from clinical samples in which the patients received repeated doses of almitrine bismesylate. The plasma concentration of almitrine in these patients ranged from as low as 50ng.ml^{-1} to as high as 1000ng.ml^{-1}.

A 0.1M KCl buffer solution (pH 1.3) was prepared by dissolving 7.46g KCl in 500ml deionised water. An aliquot (250ml) of this solution was added to dilute HCl (542ml) prepared by diluting 17.2ml conc. HCl to 1 litre with deionised water. The volume of the mixture of KCl and diluted HCl was adjusted to 1 litre with deionised water and the pH of the buffer adjusted to pH 1.3 by the addition of 1 or 2 drops of either 5N HCl or 5N NaOH.

For the *macro method* an aliquot (1ml) of plasma was transferred to a 20ml silanised round bottomed tube, then internal standard (S2082 base 100µl) dissolved in acetone (2µg.ml^{-1}) was added and the mixture vortex mixed for 5 seconds. To this was added KCl buffer (1.5ml, to achieve a pH of 1.9 to 2.0) and di-isopropyl ether (7.5ml) then the mixture shaken mechanically for 15 minutes. After centrifugation at 3000rpm for 5 minutes to separate the phases, the upper organic phase was transferred to a 10ml silanised tapered tube and the solvent carefully evaporated to dryness at 50°C under a gentle stream of oxygen-free nitrogen. The residue was reconstituted in butyl acetate (100µl) and vortex mixed for 1 minute with an aliquot (100µl) of 1N NaOH. The tubes were centrifuged (3000rpm) for 5 minutes and the top butyl acetate phase was transferred to a silanised sample vial which was sealed prior to injection onto the gas chromatograph.

For the *micro method*, plasma (100µl) was transferred to a 10ml silanised tapered tube, spiked with internal standard (10µl ≡ 50ng) dissolved in acetone (5µg.ml^{-1}), then KCl buffer pH 1.3 (100µl) and butyl acetate (300µl) were added and the mixture vortex mixed for 15 minutes. After centrifugation, the upper organic phase was transferred to another silanised tapered tube containing 1N NaOH (100µl) and the mixture vortex mixed for 5 minutes. The tubes were centrifuged for 5 minutes (3000rpm) and the upper organic phase transferred to silanised micro vials which were sealed prior to injection onto the gas chromatograph.

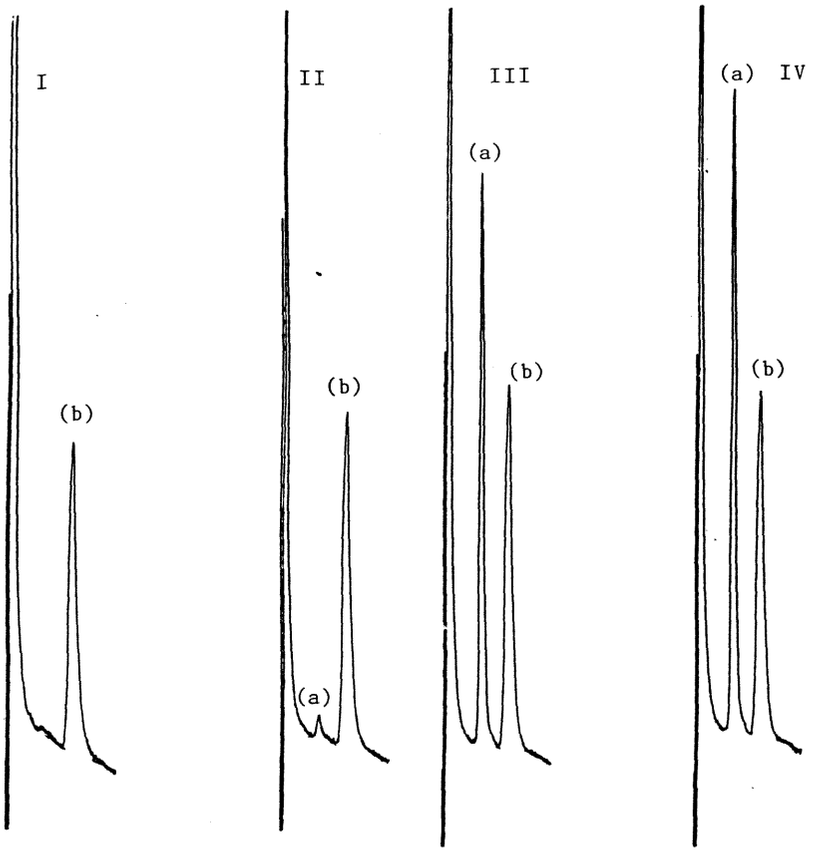
2.3.1.6 Results

The concentration of almitrine in plasma samples was determined from peak height ratios of drug to internal standard and reference to a calibration curve extracted with each analytical run. Additionally during routine use, quality control standards were randomly placed within each run at three concentrations within the range of the calibration curve and these served to monitor the performance of the assay during each analytical run. There were no interferences from endogenous plasma material (Figs 2.6 and 2.7) with almitrine and internal standard eluting at retention times of approximately 1.5 and 2.4 minutes respectively. Although the assay was linear within the range 0-1000ng.ml^{-1} the macro assay normally employed a calibration range of 0-250ng.ml^{-1} (Fig 2.8) and had a limit of quantitation of 1ng.ml^{-1}. The micro assay on the other hand used a calibration range of 50 to 1000ng.ml^{-1} (Fig. 2.9) and had a limit of quantitation of 50ng.ml^{-1}. The efficiency of extraction of a 100ng.ml^{-1} radiolabelled almitrine plasma sample was 91.5% for the macro assay and 86.0% for the micro assay.

The accuracy and precision of measurement for the macro assay was determined over the nominal concentration range of 20 to 200ng.ml^{-1} and also over the range 50 to 250ng.ml^{-1}. The mean precision over the range 20 to 200ng.ml^{-1} was 6.2% and this was 6.2% at 20ng.ml^{-1} and 4.4% at 200ng.ml^{-1}. The mean accuracy of measurement was within 2.8% of target value and this was +8.5% at 20ng.ml^{-1} and +0.9% at 200ng.ml^{-1} (Table 2.7). For the range 50 to 250ng.ml^{-1}, the mean precision was 4.8% and this was 8.2% at 50ng.ml^{-1} and 4.8% at 250ng.ml^{-1}. The mean accuracy as within 1.9% of target and under estimated by 3.3% at 50ng.ml^{-1} and 1.0% at 250ng.ml^{-1} (Table 2.8).

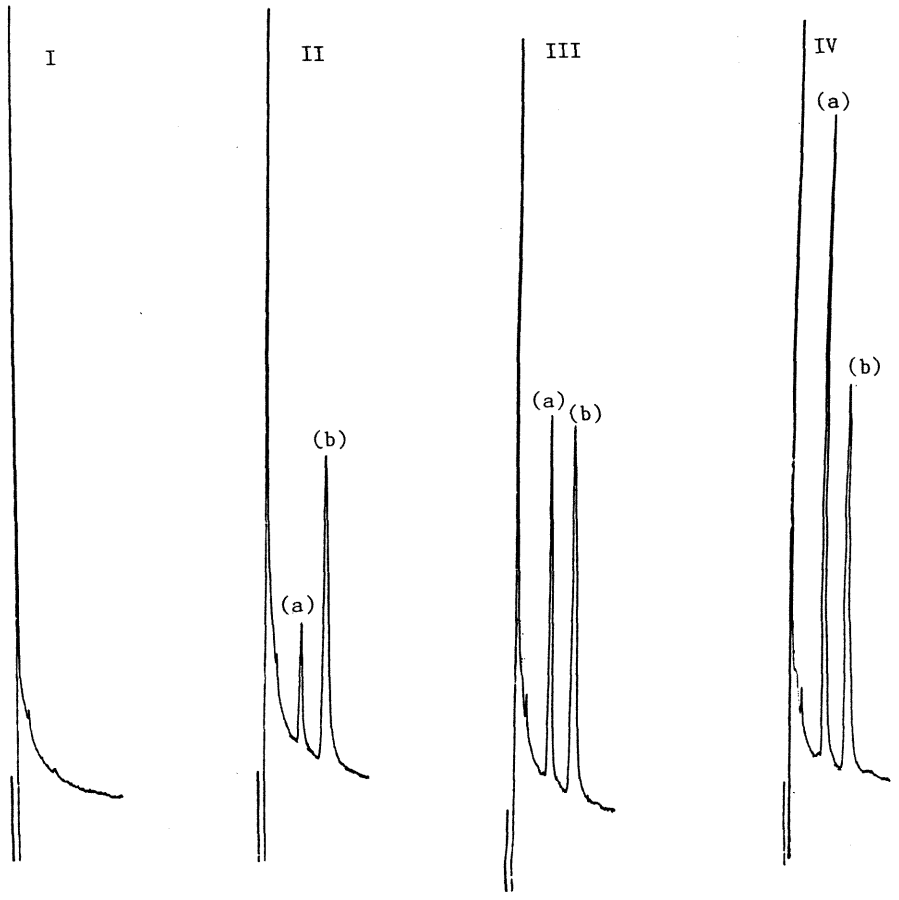
The micro assay was validated over the range 100 to 500ng.ml^{-1}. The mean precision of 6.9% varied from 12.1% at 100ng.ml^{-1} to 4.1% at 500ng.ml^{-1}. The mean accuracy which was within 1.7% of target value, underestimated by 1.9% at 100ng.ml^{-1} and overestimated by 3.1% at 500ng.ml^{-1} (Table 2.9).

Figure 2.6 : Representative Chromatograms of Almitrine and Internal Standard by the Macro Assay



I Blank plasma + Internal Standard (b)
II 5ng.ml^{-1} standard (a)
III 200ng.ml^{-1} standard
IV Subject sample containing almitrine (247ng.ml^{-1})

Figure 2.7 : Representative Chromatograms of Almitrine and Internal Standard by the Micro Method



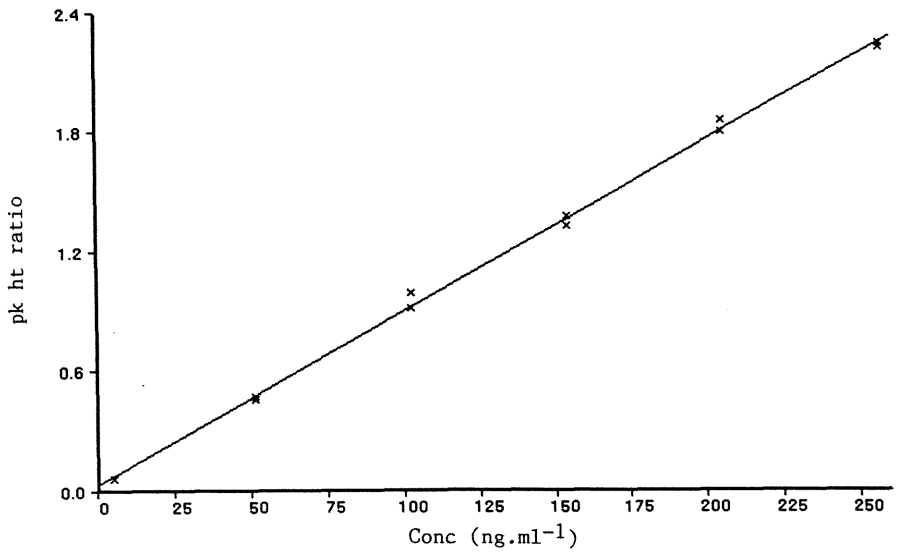
I Blank plasma

II Plasma spiked with almitrine (a) and Internal Standard at 100 and 500ng.ml^{-1} respectively

III 300ng.ml^{-1}

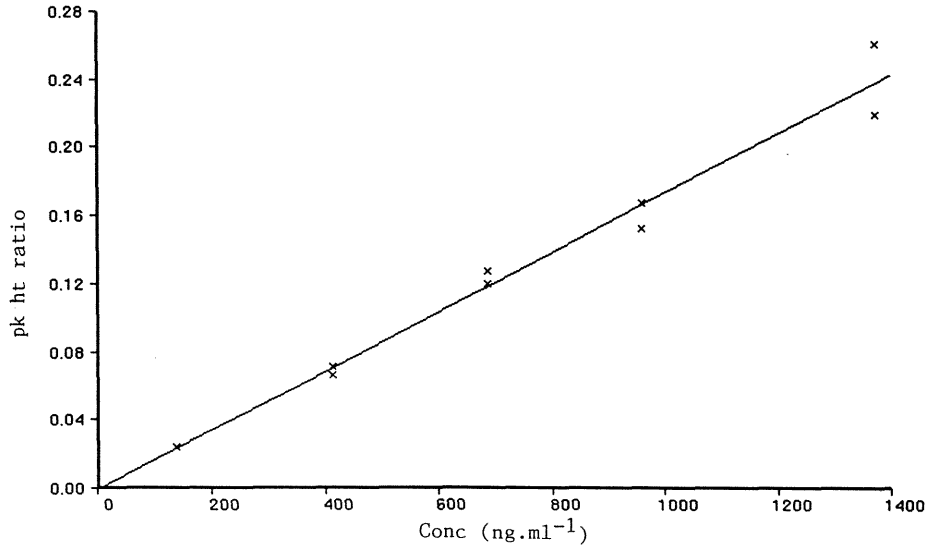
IV Subject sample containing almitrine at 430ng.ml^{-1}.

Figure 2.8 : Calibration Curve for Almitrine by the Macro (1ml) Method



R = 0.9993 S = 0.0086 I = 0.0319

Figure 2.9 : Calibration Curve for Almitrine for the Micro Assay



R = 0.9893 S = 0.000173 I = 0.00027

Table 2.7 : Intra Assay Validation of the Macro Assay for Almitrine at 20 to 200ng.ml^{-1}

REPLICATE	Conc (ng.ml^{-1})			
	20.1	50.4	100.8	201.5
1	20.9	53.2	99.3	211.7
2	20.1	48.1	100.2	192.1
3	23.9	53.6	98.5	193.5
4	21.5	42.1	98.3	207.4
5	22.8	48.2	105.7	212.6
6	22.0	53.2	111.3	202.8
Mean	21.9	49.7	102.2	203.4
±SD	1.4	4.5	5.2	8.9
% CV	6.2	9.1	5.1	4.4
% ACC	+8.5	-1.3	+1.5	+0.9

Mean % CV = 6.2 Mean % Acc = 2.8

Table 2.8 : Intra Assay Validation of the Macro Assay for Almitrine at 50 to 250ng.ml^{-1}

REPLICATE	Conc (ng.ml^{-1})				
	57.3	102.6	153.9	205.2	256.5
1	45.1	96.4	159.4	210.2	247.1
2	48.0	110.2	159.4	210.2	255.0
3	56.4	106.2	149.3	212.4	233.5
4	46.6	109.7	157.7	215.3	255.8
5	51.8	102.2	153.1	218.0	266.9
6	49.8	98.0	154.4	202.8	264.9
Mean	49.6	103.8	155.6	211.5	253.9
±SD	4.1	5.9	4.0	5.2	12.3
% CV	8.2	5.7	2.6	2.5	4.8
% ACC	-3.3	+1.2	+1.1	+3.1	-1.0

Mean % CV = 4.8 Mean % Acc = 1.9

Table 2.9 : Intra Assay Validation of the Micro Assay for Almitrine over the range 100 to 500ng.ml^{-1}

REPLICATE	Conc (ng.ml^{-1})		
	100	300	500
1	93.6	289.5	505.3
2	116.9	289.5	505.3
3	87.0	302.8	558.4
4	87.0	289.5	505.3
5	97.0	312.7	511.9
6	106.9	319.4	505.3
Mean	98.1	300.6	515.3
±SD	11.8	13.2	21.3
% CV	12.1	4.4	4.1
% ACC	-1.9	+0.2	+3.1

Mean % CV = 6.9 Mean % Acc = 1.7

The macro assay was used for pharmacokinetic studies whilst for clinical studies where almitrine was given repeatedly, the micro assay was used to measure plasma concentration of the unchanged drug.

2.3.2 The measurement of almitrine and its circulating plasma metabolites by high performance liquid chromatography

The gas liquid chromatographic methods described previously (2.3.1) although capable of analysing the mono-deallyl and dideallyl metabolites of almitrine, were more specific for almitrine. Several attempts have been made to develop high performance liquid chromatographic methods for the simultaneous determination of almitrine and its metabolites. Parkhurst *et al.* (1983) developed a reverse phase HPLC method to measure intact almitrine but had the problem of interference with endogenous compounds due to the simplicity of the extraction procedure.

More recently Jeanniot *et al.*, (1987) described a method, using normal phase chromatography, which was developed for the simultaneous measurement of almitrine and its 5 circulating plasma metabolites (Fig. 2.1). The specificity of the method has been confirmed by mass spectrometry and the limit of quantitation for almitrine has been shown to be comparable to that of the GC

procedures. This method is also applicable to whole blood, urine, gastric contents and tissues. An intra assay validation was performed over the concentration range 5-500ng.ml^{-1} for each of the postulated metabolites prior to its use in clinical studies.

2.3.2.1 Apparatus

A Hewlett Packard, Model 1090L, high performance liquid chromatograph equipped with a 101 capacity autoinjector was used for the analysis of almitrine and its metabolites. Detection was by U.V. using either the instruments variable wavelength filter photometric detector set at 230nm or a Kratos 783 ultra violet detector set at 230nm. The signal was recorded on a HP3392A integrator. Separation was achieved on a 5µm Ultraphase silica column (25cm x 4.6mm i.d.) assembled with a 5µm in-line filter and using a mobile phase of hexane:methanol:dichloromethane:70% perchloric acid (650:230:120:0.8 v/v/v/v) set at a flow rate of 1ml.min^{-1}.

2.3.2.2 Preparation of standards

Calibration and validation or quality control standards were separately and independently prepared from separate stock solutions containing a mixture of almitrine and its five metabolites dissolved in methanol (1mg.ml^{-1}). These stock solutions were further diluted with methanol to secondary solutions of concentrations ranging from 1µg.ml^{-1} to 50µg.ml^{-1}. From these, aliquots (100µl) were added to control plasma (20 or 50ml) to final concentrations ranging from 5ng.ml^{-1} to 500ng.ml^{-1}.

2.3.2.3 Extraction of almitrine and its metabolites from plasma

A 0.1M phosphate buffer pH 9.0 was prepared by adding 0.1M potassium dihydrogen orthophosphate (13.6g dissolved and diluted to 1 litre with deionised water) to 0.05M disodium tetraborate decahydrate (38.2g dissolved and diluted to 1 litre with deionised water) until a pH 9.0 was achieved.

A pH 7.0 buffer solution was prepared by adding 0.15M disodium hydrogen orthophosphate (10.65g dissolved and diluted to 500ml with deionised water) to 0.15M potassium dihydrogen orthophosphate (10.21g dissolved and diluted to 500ml with deionised water) until a pH of 7.0 was achieved.

For extraction, the thawed plasma sample was initially centrifuged to remove fibrin, formed as a result of freeze/thawing and an aliquot (1ml) was

transferred to a 20ml round bottomed tube. To this was added an aliquot (100µl) of internal standard, S11462 (Fig. 2.1(h)), dissolved in acetone (5µg.ml^{-1}), pH 9.0 buffer (20µl), then an aliquot (7ml) of a mixture of cyclohexane:diethylether (80:20 v/v). After mechanically shaking for 15 minutes the sample was centrifuged for 5 minutes at 3000rpm and the upper organic phase was transferred to a 10ml tapered tube and evaporated to dryness at 55°C under a gentle stream of oxygen-free nitrogen. The pH of the remaining aqueous phase was adjusted to pH 13 by the addition of an aliquot (125µl) of 1M sodium hydroxide and the mixture shaken as described previously with diethyl ether (7.0ml). After centrifugation, the organic phase was added to the tapered tube containing the residue of the first extract and this was evaporated to dryness at 25°C under oxygen-free nitrogen. The residue was redissolved in a mixture (150µl) of hexane:ethanol (70:30 v/v) and after centrifugation the supernatant was transferred to sample vials which were sealed prior to injection of an aliquot (25µl) onto the chromatograph.

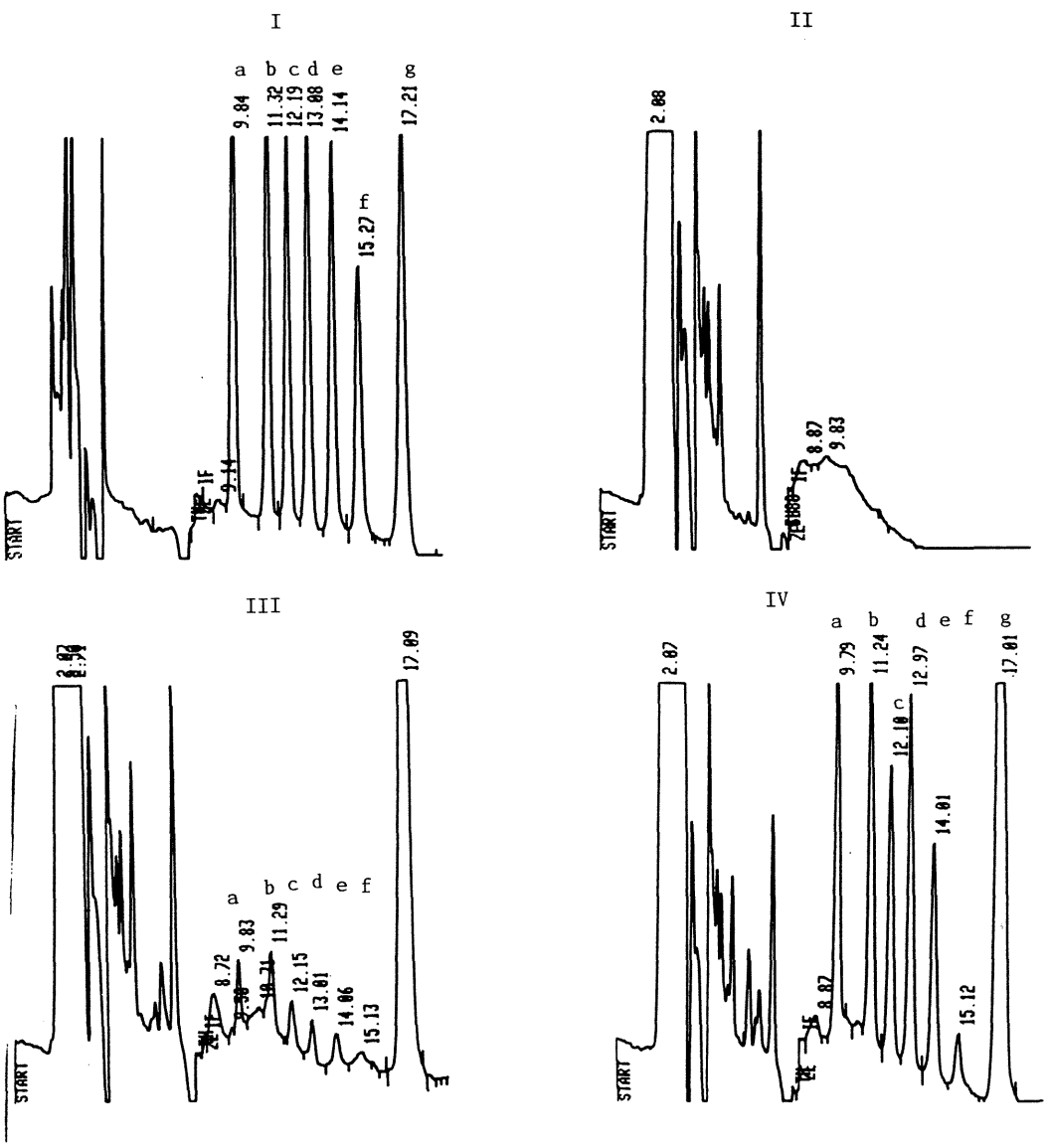
2.3.2.4 Results

The components of interest were well resolved from each other and from endogenous plasma material (Fig. 2.10). With a total chromatographic run time of approximately 25 minutes, the almitrine peak eluted first at approximately 9.5 minutes and the final peak of interest, S11462, eluting at approximately 19 minutes. With a much longer run time than the GC method described previously fewer samples will be analysed in a 24 hour period (approximately 60). However, far more data will be amassed from these fewer samples. The method is linear for all components (drug and metabolites) within the range 0-500ng.ml^{-1} (Fig. 2.11). Intra-assay validation of almitrine and its metabolites is summarised in Table 2.10.

The precision and accuracy of measurement for almitrine and its metabolites are summarised in Tables 2.11 and 2.12 respectively. With the exception of the 5ng.ml^{-1}, for all other concentrations of almitrine and metabolites the precision and accuracy of measurement were, on average, very good, the most variable being the highly polar tetrahydroxy-almitrine (S1999). Almitrine had a mean precision of 11.4% ranging from 37.2% at 5ng.ml^{-1} and improving to 3.4% at 500ng.ml^{-1}. The mean accuracy of measurement was within 4.7% of target value with good accuracy through the concentration range 10 to 500ng.ml^{-1}. The limit of quantitation for almitrine and that of the other major metabolites has been set at 10ng.ml^{-1} except for S1999 which has been set

at 25ng.ml^{-1} enabling the method to be used for clinical studies and animal studies where the compound has been repeatedly administered.

Figure 2.10 : Representative Chromatograms of Almitrine and Metabolites



(a) almitrine; (b) S3024; (c) S10694; (d) S3327; (e) S10712; (f) S1999; (g) Internal Standard

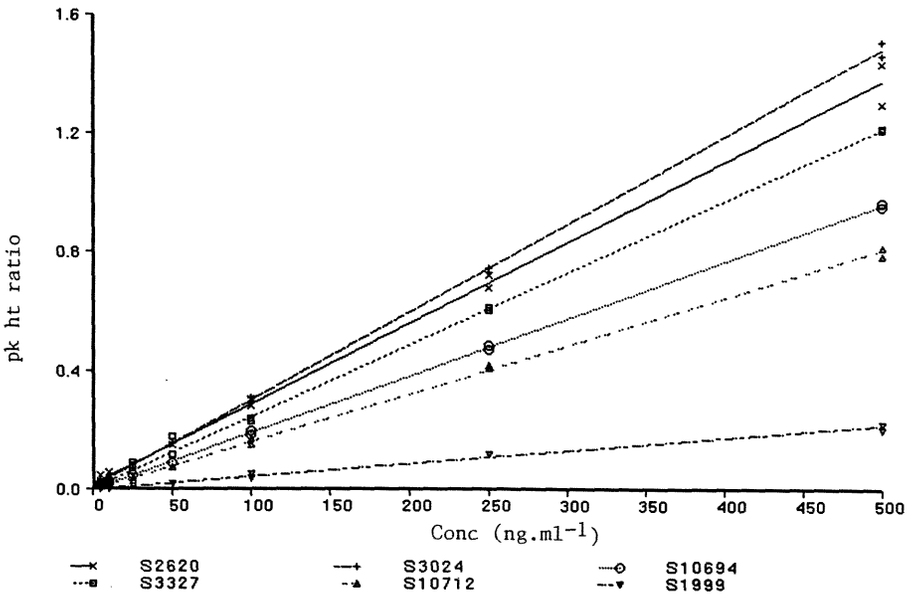
i Standard solution containing 100ng.ml^{-1}

II Blank plasma

III 5ng.ml^{-1} Standard extract

IV 100ng.ml^{-1} Standard extract

Figure 2.11 : Calibration Curves for Almitrine and its Metabolites



REGRESSION DATA

COMPOUND	CORRELATION COEFFICIENT	SLOPE	INTERCEPT
Almitrine (S2620)	0.99802	0.0027	0.0172
S3024	0.99974	0.0029	0.0093
S10694	0.99962	0.0019	0.0001
S3327	0.99918	0.0024	0.0067
S10712	0.99943	0.0016	0.0010
S1999	0.99551	0.0004	-0.0008

Table 2.10 : Intra Assay Variability of Measurement of S 2620 and its Metabolites in Human Plasma within the Range 0 - 500ng.ml^{-1}

COMPOUND		5ng.ml^{-1}	10ng.ml^{-1}	25ng.ml^{-1}	50ng.ml^{-1}	100ng.ml^{-1}	250ng.ml^{-1}	500ng.ml^{-1}
S2620	Mean	5.2	11.3	28.0	53.9	106.6	245.4	526.5
	S.D.	1.93	0.71	2.11	2.69	3.75	40.88	18.10
S3024	Mean	6.4	11.1	26.7	48.4	95.5	228.7	480.2
	S.D.	2.22	0.78	2.21	1.58	1.96	14.76	7.90
S10694	Mean	7.0	13.4	29.4	56.8	111.4	265.7	541.8
	S.D.	1.05	2.74	1.58	1.55	3.86	17.8	21.9
S3327	Mean	4.5	9.0	22.7	44.9	89.3	219.3	440.2
	S.D.	1.84	0.5	1.25	1.37	3.56	14.10	18.34
S10712	Mean	3.5	11.1	27.8	55.9	107.6	270.6	520.6
	S.D.	3.10	1.27	3.05	3.93	12.53	38.08	60.05
S1999	Mean	0	2.4	23.9	45.7	81.6	223.1	386.1
	S.D.	0	4.88	8.96	8.19	16.26	54.79	58.47

Table 2.11 : Precision of Measurement (%) of Almitrine and it's Metabolites within the Range 5 - 500ng.ml^{-1}

COMPOUND	NOMINAL Conc (ng.ml^{-1})							Mean
	5	10	25	50	100	250	500	(5-500ng.ml^{-1})
Almitrine	37.2	6.2	7.5	5.0	3.5	16.7	3.4	11.4
S3024	34.7	7.0	8.3	3.3	2.1	6.5	1.6	9.1
S10694	15.1	20.4	5.4	2.7	3.5	6.7	4.0	8.3
S3327	40.5	5.6	5.5	3.1	4.0	6.4	4.2	10.0
S10712	88.6	11.4	11.0	7.0	11.7	14.1	11.5	22.2
S1999	-	20.0	37.5	17.9	19.9	24.6	15.1	19.8

Table 2.12 : Accuracy of Measurement (%) of Almitrine and its Metabolites within the Range 5 - 500ng.ml^{-1}

COMPOUND	NOMINAL Conc (ng.ml^{-1})							Mean
	5	10	25	50	100	250	500	(5-500ng.ml^{-1})
Almitrine	0.0	+10.0	+8.5	+4.5	+3.3	-4.9	-2.0	4.7
S3024	+28.0	+12.2	+7.2	-2.6	-3.9	-8.0	-3.4	9.3
S10694	+34.6	+29.3	+13.5	+9.7	+7.6	+2.7	+4.7	14.6
S3327	-4.3	-3.2	-2.6	-3.6	-4.3	-6.0	-5.6	4.2
S10712	-30.0	+13.4	+12.6	+12.5	+8.4	+9.4	+5.2	13.1
S1999	-	+74.2	+0.8	-3.4	-13.7	-5.7	-18.4	19.3

*Accuracy expressed as % away from target value.

2.4 Pharmacokinetic Analysis

The complexity of the living system makes it difficult to develop a simple equation to describe all the processes describing the movement of a drug within and without the body. Numerous factors influence the blood concentration of drugs and include absorption, distribution (plasma and tissue protein binding), metabolism and excretion. These processes will occur at different rates dependent mainly on the lipid solubility of the molecule and pharmacokinetics has been used to quantitate the rates of the various processes described above. The calculation of these rates can assume that the drug behaves like some predefined model. However, the description and derivation of these models is given in numerous texts and reviews (Rowland and Tozer, 1980; Gibaldi and Perrier, 1982 and Benet*et al.*, 1984) and will not be a subject of this thesis. Instead, the programs and parameters used for the derivation of key pharmacokinetic parameters will be outlined in this section.

The pharmacokinetic parameters derived from blood and plasma concentration data for total radioactivity and intact almitrine were originally derived from the method of residuals (Gibaldi and Perrier, 1975) using a Wang Model 2200 micro computer. The half-lives of each phase were determined using sequential linear regression analysis of semi-logarithmic plots of the plasma or blood concentration versus time curve.

More recently, each of the plasma or blood concentration profiles was fitted to the sum of exponentials (equation 1) using a weighted iterative least squares program (Ings *et al.*, 1980) written for a Hewlett Packard series 1000A mini-computer. A reciprocal weighting factor was found optimal for most analyses although no weighting was sometimes used if indicated by the distribution of residuals.

$$Cp = \sum_{i=1}^{n} C_i e^{-\lambda_i t} \qquad \text{..... 1}$$

where Cp is the plasma concentration at time t, C_i is the coefficient and λ_i is the exponential constant to the ith term.

When the compound was administered orally, the term describing the input (absorption) function became negative thus:

$$Cp = C_1 e^{-\lambda_1 t} + C_2 e^{-\lambda_2 t} - C_3 e^{-\lambda_3 t} \quad \text{..... 2}$$

The half-life (t½) of each of the decline phases was obtained according to equation 3 and the area under the curve to the last measured time point (AUC_t) or to infinite time (AUC) were calculated either by direct integration (sum of exponentials) according to equation 4 or by the trapezoidal rule (Yeh and Kwan, 1978) according to equation 5.

$$t\,½ = \frac{0.693}{\lambda_i} \quad \text{..... 3}$$

$$AUC = \sum_{i=1}^{n} \frac{C_i}{\lambda_i} \quad \text{..... 4}$$

$$AUC = \sum \frac{C_{(n)} - C_{(n-1)}}{2} t_{(n)} - t_{(n-1)} + \frac{C_z}{\lambda_z} \quad \text{..... 5}$$

where C_n is the plasma concentration at time t_n,, C_{n-1} is the plasma concentration at the preceding time t_{n-1}, C_z is the last plasma concentration and λ_z is the slope of the terminal decline phase.

The contribution of each phase to the overall elimination of the compound following intravenous administration was also determined from the ratio of areas associated with each phase compared to the total area under the curve calculated by direct integration. The area under the curve to infinity following intravenous administration calculated by the trapezoidal rule was, in turn, used to calculate plasma clearance (CL) according to equation 6 after correcting for molecular weight differences between the administered bismesylate salt (M.Wt. 669.8) and the free base (M.Wt. 477.5) measured in plasma.

$$CL = \frac{Dose}{AUC} \quad \text{..... 6}$$

Plasma clearance was then used to determine the volume of distribution of almitrine based on area (V) using equation 7.

$$V_d = \frac{CL}{\lambda_z} \quad \text{..... 7}$$

where λ_z is the exponential constant for the terminal phase.

The initial distribution volume (V_i) was calculated from equation 8 and the volume of distribution at steady-state (V_{ss}) calculated from equation 9.

$$V_i = \frac{Dose}{\sum_{i=1}^{n} C_i} \quad \text{..... 8}$$

$$V_{ss} = \frac{Dose \sum_{i=1}^{n} C_i / \lambda_i^2}{(\sum_{i=1}^{n} C_i / \lambda_i)^2} \quad \text{..... 9}$$

After oral dosing, the maximum plasma level (C_{max}) and the time to achieve maximum plasma levels (t_{max}) were taken directly from the data. The absolute bioavailability of the oral dose of administration (F) was calculated from equation 10.

$$F(\%) = \frac{AUC_{po}}{AUC_{iv}} x \frac{Dose_{iv}}{Dose_{po}} x100 \quad \text{..... 10}$$

Finally the coefficients and exponentials were used for multiple dose simulations. These predictions were performed using equation 11 when there was a constant dose and dosing interval, or using the principle of superimposition when there was an unequal dose, with the programs SIMSUM and SIMDISC respectively.

$$Cp = \sum_{i=1}^{n} C_i \frac{1 - e^{-n\lambda_i t}}{1 - e^{-\lambda_i \tau}} e - \lambda_i t \quad \text{..... 11}$$

where τ is the dosing interval.

2.5 Presentation of Data

In order to simplify the presentation of results, the data will be summarised as tables, figures and appendices.

Plasma and blood concentrations, excretion balance results and quantitative tissue distribution data will be presented at the end of the thesis as appendices and where appropriate, represented within the text as figures. Pharmacokinetic and metabolism results will be summarised as tables within the text.

All appendices will be coded for the relevant section. For example, Appendix 3.1A will refer to Section 1 of Chapter 3 and Appendix 3.2A will represent data from Section 2 of Chapter 3.

CHAPTER 3

THE DISTRIBUTION AND DISPOSITION OF [^{14}C]-ALMITRINE BISMESYLATE IN ANIMAL AND MAN

This chapter describes the results of radioactive studies in animal and man which were designed and analysed by me. All doses were formulated and prepared by myself. All animal studies were performed at contract research organisations except for study 3.3.1 which was performed at the University of Leicester under the licence of Dr. D. Pallot. For human studies, dose administration was performed at the Lister Hospital, Hitchin and sample collection performed either at the Lister Hospital (3.1) or at the University of Leicester under the supervision of Dr. M. Prattern. All analyses were performed either by me or under my supervision.

The use of radiolabelled material is an important tool for following the course of a drug in the body after administration. The absorption, distribution, metabolism and elimination of almitrine bismesylate have been investigated in animals using [^{14}C]-almitrine bismesylate labelled principally in the benzhydryl position. The species used were those chosen for toxicological and pharmacological studies and include rat, rabbit and dog. Radiolabelled almitrine bismesylate was administered orally and intravenously (Table 3.1) at dose ranges of between 0.5 to 100mg.kg^{-1}. The disposition of [^{14}C]-almitrine was also studied in man following either intravenous or oral administration at doses ranging from 0.09 to 1.8mg.kg^{-1}. This chapter describes and compares the data from the different species and relates the results to man. Specific studies are also described in detail as these are considered important projects designed as part of the drug's development. The chapter will therefore head three sections. (1) Kinetics of radioactive almitrine following short term oral and intravenous studies, (2) The elimination of radioactivity into urine and faeces following short term (14 days) collection and (3) Special studies.

For convenience, pharmacokinetic data and figures are presented within the text. Large tables are added as appendices at the end of the thesis.

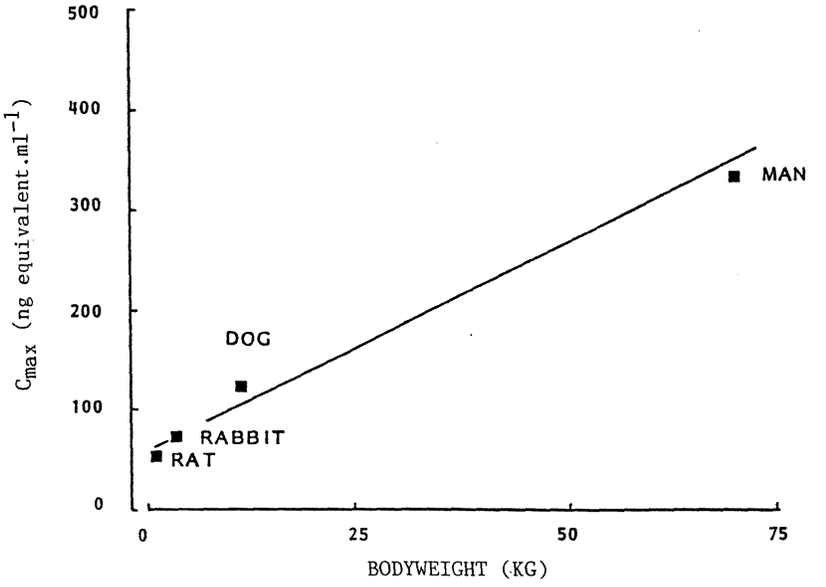
3.1 The Kinetics of Radioactivity Following Oral or Intravenous Administration of [^{14}C]-Almitrine Bismesylate

This section describes the plasma or blood kinetics of [^{14}C]-almitrine in rat, rabbit, dog and man following oral and intravenous administration of [^{14}C]-almitrine bismesylate. In order to make direct comparisons between species, the data have been normalised to a 1mg.kg^{-1} dose (Appendices 3.1A and 3.1B; Table 3.2).

3.1.1 Absorption

Following oral administration, radioactivity was relatively rapidly absorbed from the gastrointestinal tract in man, attaining peak plasma concentrations of 338 ± 184ng equivalents almitrine.ml^{-1} within 3.5 ± 0.7 hours (Table 3.2). In animals, however, absorption of radioactivity was much slower and reached maximum blood or plasma concentrations between 4.5 and 11.5 hours after administration (Table 3.2). The normalised peak concentration of radioactivity showed some interspecies variation which were directly related to the bodyweight, with man having the highest concentrations (338ng equivalents almitrine.ml^{-1}) whilst the rat had the lowest at 47.5ng equivalents almitrine.ml^{-1}. This is shown graphically in Figure 3.1.

Figure 3.1 : Relationship Between Bodyweight and Normalised Maximum Plasma/Blood Levels of Radioactivity

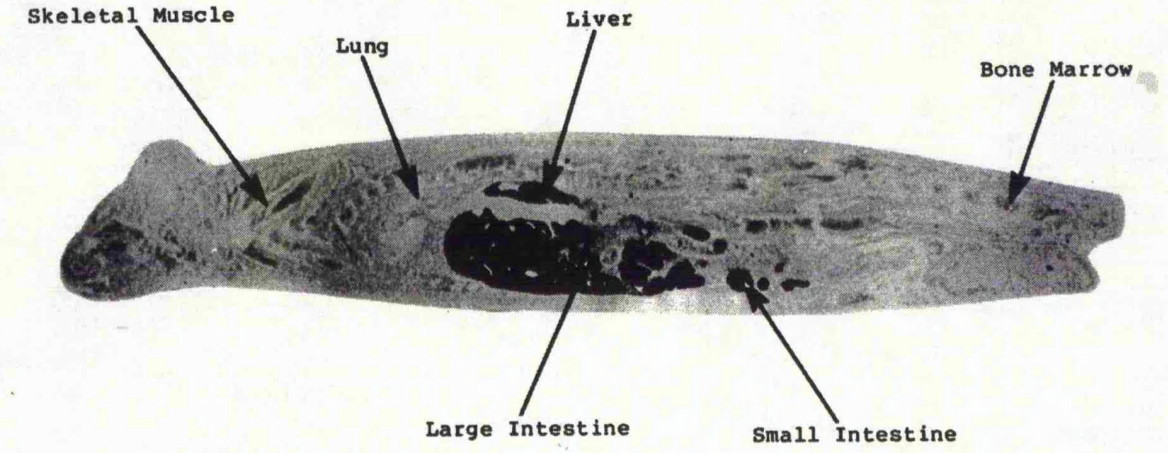


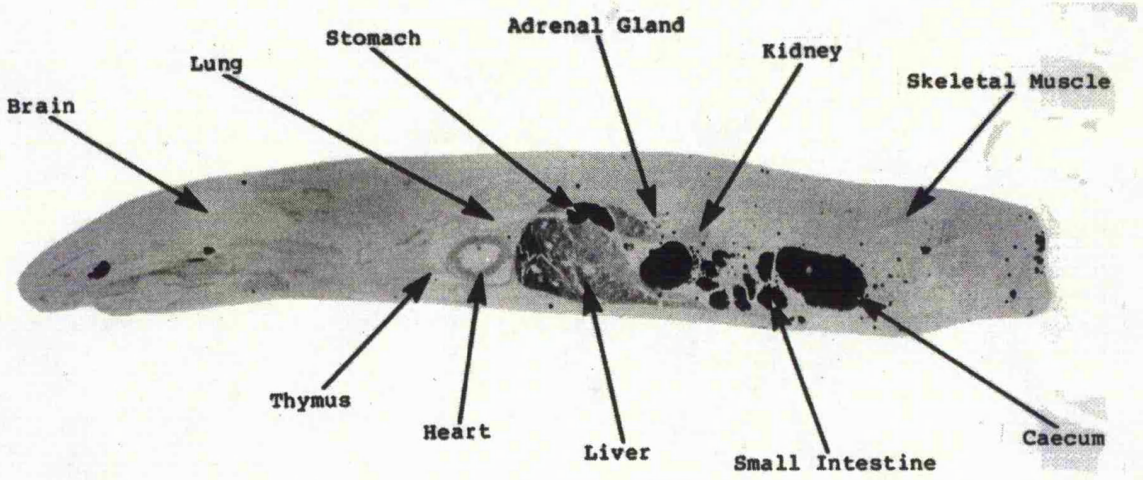
3.1.2 Distribution and elimination

The volume of distribution (based on area) after intravenous administration of [^{14}C]-almitrine bismesylate to various species was high and ranged from 17.8 l.kg^{-1} in the rat to 3.5 and 3.8 l.kg^{-1} in dog and man respectively. This large volume of distribution reflects the lipophilic nature of the compound and would give rise to extensive tissue uptake of radioactivity which includes almitrine and its metabolites. This tissue uptake, particularly in the rat, will be described in a more detailed study later (3.3.1). However, in an autoradiographic study in which a male Wistar rat was given an oral dose of [^{14}C]-almitrine bismesylate at 100mg.kg^{-1} then sacrificed 4 hours later, results of autoradiographic sections showed high concentrations of radioactivity along the gastro-intestinal tract (Figs 3.2a, b and c). This would initially suggest little or no absorption (although biliary excretion cannot be discounted); however, high levels of radioactivity were located in the liver and kidney, the two main excretory organs. Whilst most other major organs such as the brain, lungs, eyes, testes and spinal cord showed little or no radioactivity, the general musculature of the carcass showed extensive uptake of radioactivity which could have been attributed to the presence of radioactivity in the blood of this highly vasculated tissue. However, examination of the heart and lung showed that while there was little radioactivity in the lung at 4 hours, the heart muscle had higher levels of radioactivity relative to the central chamber containing blood (Figs 3.2b and 3.2c), confirming uptake of radioactivity into the tissue compartment.

Following oral absorption, blood and plasma radioactivity declined biphasically in all species (Figure 3.3a) with an initial elimination half-life of ~4 hours followed by a more prolonged half-life which ranged from 24 hours in the rat to 56 hours in man (Table 3.2). The elimination of radioactivity following intravenous administration was also biphasic (Fig. 3.3b) with an initial elimination phase of 2-3 hours and a terminal phase ranging from 25 hours in the rat to 39 hours in man. In all of these studies however, sampling time was relatively short (<5 days), and since in most species there was still a significant amount of radioactivity in the last plasma or blood sample, this terminal phase for radioactivity has obviously been underestimated, and does not reflect the true terminal half-life of radioactivity. A specific study in dog and man, which will be described in more detail later, will show the terminal half-life of radioactivity to be considerably longer.

Figure 3.2 : An Autoradiograph of a Male Wistar Rat Sacrificed 4h After Oral Dosing with [^{14}C]-Almitrine (100mg.kg^{-1})





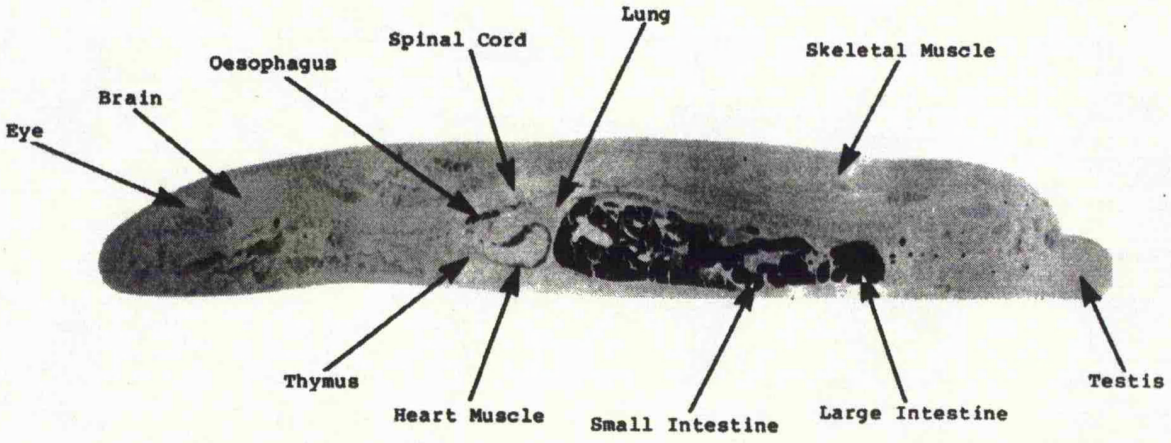


Table 3.1 : Summary of [^{14}C]-Almitrine Studies Undertaken in Animals and Man

SPECIES	STRAIN (Sex, No)	TYPE OF STUDY	DOSE (mg.kg^{-1})	ROUTE
RABBIT	New Zealand White 4 male, 4 Female	Kinetics and Balance	0.5 0.8	I.V., P.O.
DOG	Beagle 2 Male, 2 Female	Kinetics and Balance	0.5	I.V, P.O.
	Beagle - 2 Male	Kinetics and Balance	5.0	I.V.
RAT	Wistar Male - 2 per species	Kinetics and Balance	3.4 2.6	I.V. P.O.
	Wistar 4 Male, 10 Male	Metabolism	2 100	P.O.
	Wistar Male - 3 per group	Distribution + anaesthesia	3	I.V.
	Wistar	WBA	5	I.V.
HUMAN	Caucasian 1 Male, 1 Female	Kinetics and Balance	0.09 1.8	I.V. P.O.
	2 Males, 2 Females	Kinetics and Balance	1.8	P.O.

The clearance of radioactivity when normalised for body weight (Table 3.2) was similar for all species and ranged from approximately 1ml.min^{-1}.kg^{-1} in the dog, rat and man to 2.3ml.min^{-1}.kg^{-1} in the rabbit. These values are probably high for dog and man, and in studies in which longer sampling times were made, lower clearance values for radioactivity would be expected (see later study in dog).

Figure 3.3 : Mean Blood or Plasma Profiles of Radioactivity After Oral and Intravenous Administration of [^{14}C]-Almitrine Bismesylate in the Various Species (Data Normalised to a 1mg.kg^{-1} Dose)

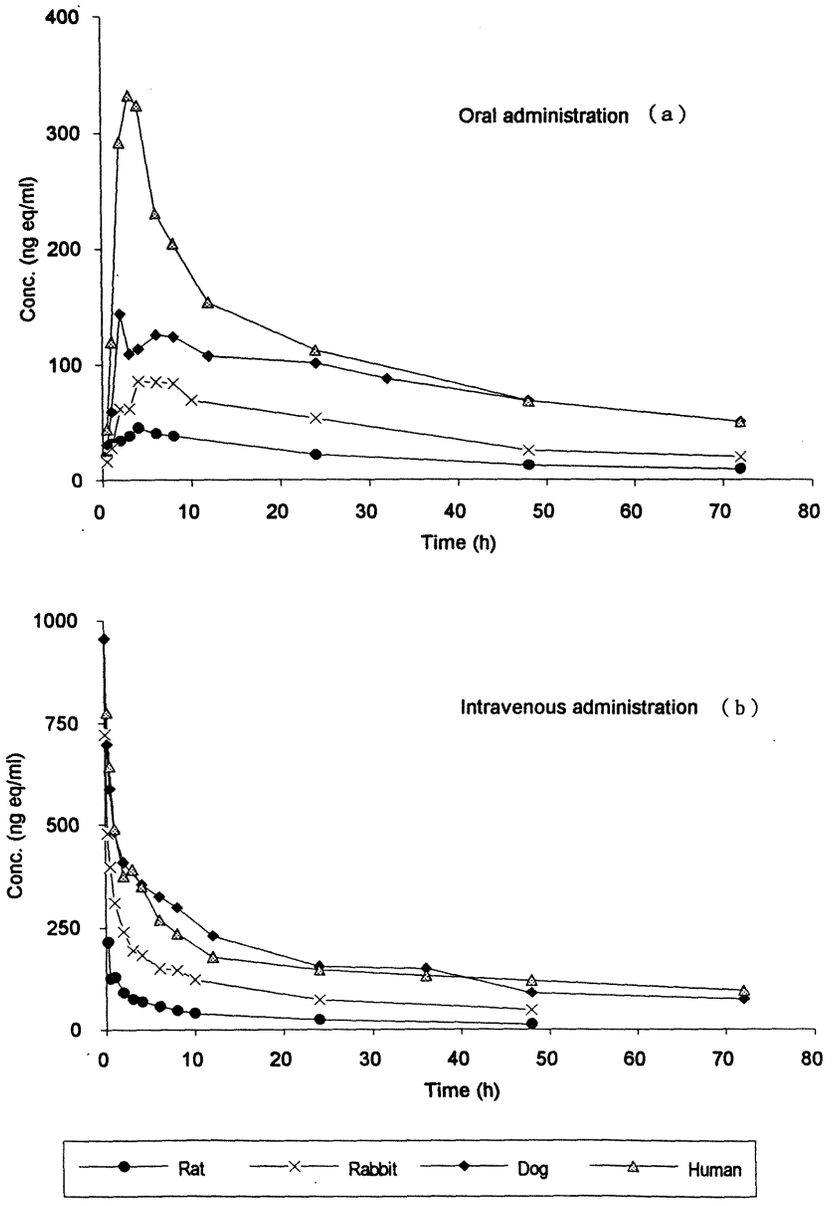


Table 3.2 : Summary of Pharmacokinetic Parameters of [^{14}C]-Almitrine in Various Species from Data Normalised to a 1mg.kg^{-1} Dose

SPECIES	ORAL					INTRAVENOUS				
	$C_{(max)}$ (ng.eq.ml^{-1})	$t_{(max}$ (h)	$t_{½}$ (h)			$t_{½}$ (h)			Vd (l.kg^{-1})	CL (ml.min^{-1}.kg^{-1})
			(1)	(2)	(z)	(1)	(2)	(z)		
RAT*	47.5 ± 6.9	4.6 ± 1.1	-	-	24.4 ± 8.5	0.3 ± 0.3	1.8 ± 0.1	25.0 ± 8.2	17.8 ± 8.5	1.4 ± 0.2
RABBIT	92.8 ± 25.7	5.4 ± 3.0	-	4.6 ± 4.1	32.8 ± 5.7	0.3 ± 0.1	2.1 ± 0.4	34.0 ± 12.4	6.2 ± 1.0	2.3 ± 0.7
DOG	127 ± 71.9	11.5 ± 8.4	-	-	50.5 ± 22.6	0.2 ± 0.1	3.1 ± 0.9	37.1 ± 7.6	3.5 ± 1.3	1.0 ± 0.2
HUMAN	338 ± 184	3.5 ± 0.7	0.7 ± 0.3	4.1 ± 0.9	56.4 ± 9.8	0.1 ± 0.0	2.1 ± 1.1	39.4 ± 9.4	3.8 ± 1.4	1.2 ± 0.6

* Denotes Blood

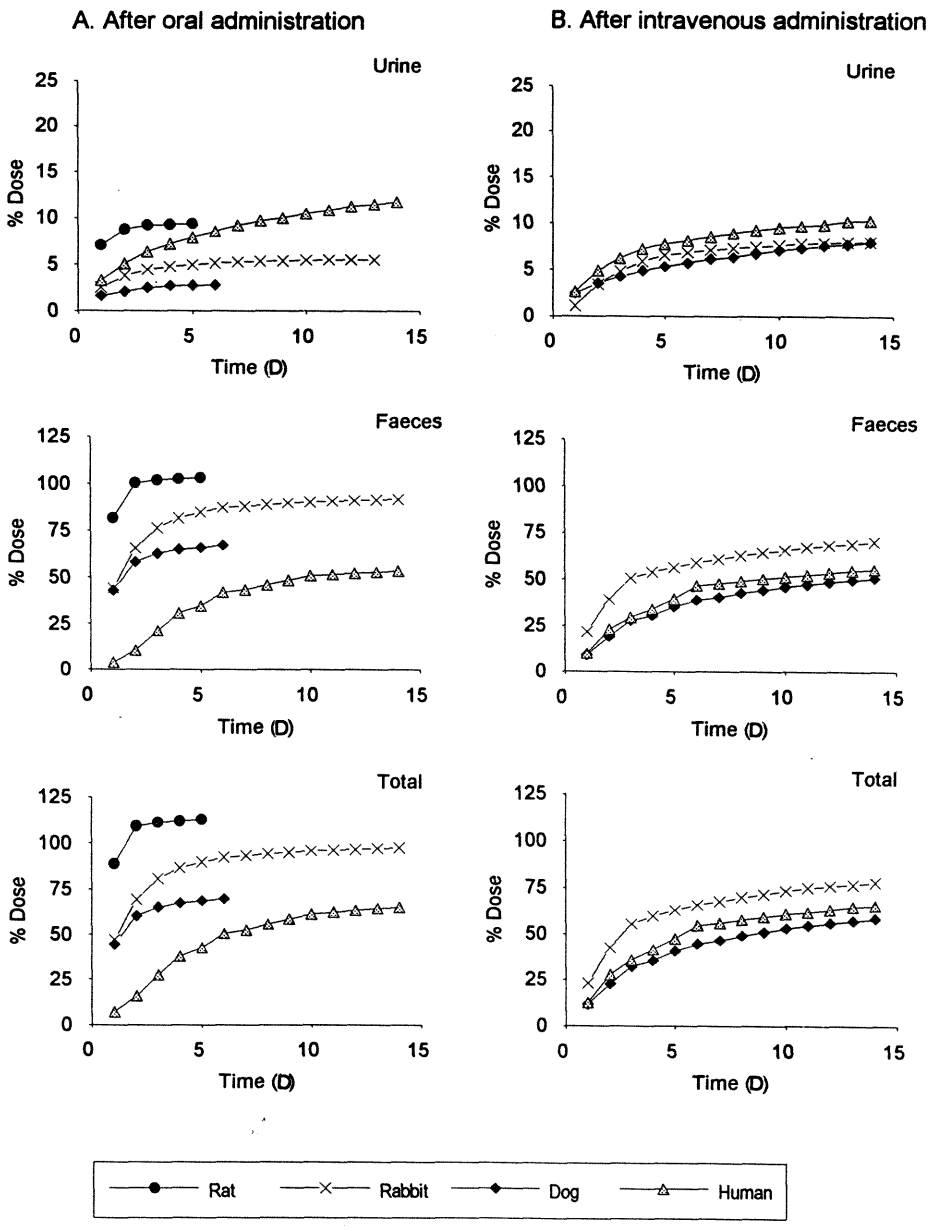
3.2 Elimination of Radioactivity into Urine and Faeces

The period over which excreta was collected varied with species and dose route and ranged from 5 and 6 days in the rat and dog respectively after oral administration, to 28 days in rabbit and man after intravenous administration. On average, urine and faeces were collected over 14 days and where possible comparisons will be made over this period between the species for both routes of administration.

3.2.1 Excretion after oral administration

The excretion of radioactivity into urine was slow and low for all species (Appendices 3.2A and 3.2B; Fig. 3.4). During the first 24 hours the amount of radioactivity excreted into urine ranged from 1.6% of the administered dose in the dog to 7.06% in the rat (1.60%, 2.51%, 3.30% and 7.06% dog, rabbit, man and rat respectively). The total radioactivity recovered in the urine of rat over the 5 day collection period was 9.39%. Over this period, the rabbit excretion 4.97%, dog 2.75% and man 7.94%. Thus in terms of excretion rate (% dose per day) the rat had the highest excretion rate ($1.88\%.d^{-1}$) whilst the dog, the lowest ($0.55\%.d^{-1}$). The dog excreted a total of 2.82% over a 6 day collection period with no indication of sex difference (2.81 and 2.83% male and female respectively) in the extent of excretion. A total of 5.54% of the dose was recovered in the rabbit over 13 days of urine collection with no overall sex difference (4.9% and 6.8% male and female respectively). In man, however, the total recovery of radioactivity over 14 days was 11.74% of the dose (urine collected over 14 days in male and 19 days in female subjects). The male subject however, excreted only 8.48% of the dose while in the female subject 15.01% of the administered dose was urinarily excreted, thus over the same period the female subject excreted approximately twice as much radioactivity into urine. Since only one male and one female subject was used, this difference may not be a gender difference but more reflects inter-individual differences in the rate of excretion.

Figure 3.4 : Mean Excretion Profiles of Radioactivity After Oral (A) and Intravenous (B) Administration of [^{14}C]-Almitrine Bismesylate in Urine and Faeces for the Various Species



In all species, radioactivity was excreted predominantly in the faeces and ranged from 103% in the rat over 5 days to 53% in man over 14 days (dog 70% in 6 days, rabbit 92% in 14 days, Appendix 3.2D). As with urine, except for man there was no indication of a sex difference in the extent of radioactivity faecally excreted. With the exception of the rat, faecal elimination was slow and the rate of elimination decreased with body weight (Appendices 3.2C and 3.2D; Fig. 3.4). The slow faecal elimination of radioactivity particularly during the early period (days 1-2) may be a function of gut transit time, particularly in man where only 10.68% of the dose was accounted for compared to 50-100% in the other species (Appendices 3.2C and 3.2D).

The total recovery of radioactivity varied from 65% in man over 14 days to 113% in the rat over 5 days (Appendix 3.2E; Fig 3.4). The dog excreted a total of 70% of the dose within 6 days after oral administration, whilst in the rabbit 98% of the administered dose was accounted for within 14 days. Although in man, excreta was collected for the female subject over 28 days, only 80% was accounted for with 65% recovered over 14 days. Thus although urinary excretion in the female subject was higher, the overall recovery (urine + faeces) was similar for both sexes. For man, in particular, daily excretion of radioactivity after 28 days was low with less than 1% daily excreted and as will be shown later, the elimination of radioactivity was prolonged.

3.2.2 Excretion after intravenous administration

As with oral dosing, the rate and extent of urinary excretion of radioactivity was low following intravenous administration (Appendices 3.2F and 3.2G; Fig. 3.4) and ranged from 7.92% and 7.98% in the dog and rabbit respectively over 14 days to 10.30% in man over a similar period. Again a subject difference was seen only in man in the extent of excretion of radioactivity into urine over this period (6.48 and 14.03% male and female respectively). For all species radioactivity was eliminated predominantly into faeces (Appendices 3.2H and 3.2I; Fig. 3.4) with 51%, 55% and 70% faecally excreted in the dog, man and rabbit respectively. After the first 7 days the daily rate of elimination of radioactivity into faeces was approximately similar for all species (Appendix 3.2H). The total cumulative excretion of radioactivity into urine and faeces after 14 days was 58%, 66% and 78% in dog, man and rabbit respectively (Appendix 3.2J; Fig. 3.4) and was lower than that observed after oral administration for the rabbit but similar in man.

Overall, the absorption of radioactivity after oral dosing was good with man and rabbit showing similar urinary activity after both oral and intravenous administration. However, the rate of elimination after intravenous administration was slower than after oral administration and this would suggest a greater extent of tissue uptake following intravenous administration than after oral. The route of elimination reflects both the lipophilic nature of the drug and the high molecular weight of the excreted products. These products will be described in a later chapter. However, direct comparison of urinary radioactivity between routes of administration is difficult since it is likely that metabolic patterns will be different.

3.3 Special Studies

This section deals with detailed studies designed to investigate specific aspects of drug development. The uptake and distribution of radioactivity into the tissues of rat is investigated. The kinetics of total radioactivity relative to the unchanged drug is compared in the dog following intravenous administration of [^{14}C]-almitrine bismesylate. Finally, in an attempt to investigate the prolonged excretion of almitrine and its metabolites, a disposition study has been performed in man, in which radioactivity was measured for as long as possible. The blood and plasma radioactivity kinetics is compared with that of the unchanged drug.

3.3.1 The kinetics of almitrine and its metabolites in the tissues of the rat

The rat has been selected as one of the pharmacological and toxicological models for the development of almitrine. Although the action of almitrine on the carotid body response to hypoxia has been established (Laubie and Schmitt, 1980; Pallot and Alneamy, 1983) the mechanism by which almitrine acts specifically on the carotid body, following administration of the drug under hypoxic and normoxic conditions is unknown. To more fully understand this specific pharmacological activity of almitrine, the uptake of almitrine into the carotid body in comparison with other tissues in the rat was investigated. The study also determined whether there was a rapid and specific uptake of almitrine into the carotid body and investigated whether there was a particular affinity for chemoreceptor tissue.

Since the majority of pharmacological experiments are performed under anaesthesia, the uptake of [^{14}C]-almitrine into the carotid body was initially

investigated in the anaesthetised rat. As pharmacological studies could only be maintained for a limited time (<2h) a second study was set up to look at the uptake of almitrine into unanaesthetised animals for longer periods since the normal physiology of the anaesthetised animal could be disturbed by the effect of anaesthesia.

3.3.1.1 Dosing and sampling

Male Wistar rats of weight range 150-200g were anaesthetised with urethane ($1.25g.kg^{-1}$ i.p.), then 10 minutes later administered [^{14}C]-almitrine bismesylate (nominal $3mg.kg^{-1}$, 3µCi) as a bolus injection into the exposed femoral vein. Groups of animals (n=3) were sacrificed at 5, 15 and 30 min, 1 and 2 hours after almitrine administration, by pentabarbitone injection ($100mg.kg^{-1}$), and a series of tissues (Appendix 3.3.1A) rapidly dissected out and stored in preweighed containers at -20°C.

A second group of male Wistar rats were similarly given an intravenous bolus of [^{14}C]-almitrine bismesylate ($3mg.kg^{-1}$, 3µCi) without anaesthesia. Groups of animals (n=3) were also sacrificed by pentabarbitone injection, but at the later times of 2, 4, 8 and 16 hours post almitrine administration. Fewer tissues were dissected out and only the more important tissues such as plasma, carotid body, carotid artery, superior cervical ganglion, liver, kidney, lung and heart were collected into preweighed containers and stored as previously described (Appendix 3.3.1B).

3.3.1.2 Quantitative tissue distribution

The larger tissues such as carcass, liver, lungs, kidneys, heart, gastro-intestines, testes and brain were homogenised prior to combustion as described in Chapter 2 (2.2.4.1). For tissues such as the carotid body and carotid artery which were used in metabolic studies, a small aliquot was weighed out into combusto-cones and the rest retained for metabolite profiling. An aliquot of plasma (100µl) was pipetted directly into combusto-cones whilst the remaining tissues such as superior cervical ganglion, bronchi, adrenals, red cells, white fat and muscle were weighed directly into combusto-cones. The skin was initially digested in methanolic KOH (2M) by refluxing overnight then the cooled digest neutralised with perchloric acid. After filtration of the neutralised digest, aliquots (1ml) were counted directly for radioactivity. The resultant radioactivity was calculated for each tissue and expressed either as a percentage or as µg equivalents of the administered

dose per gramme of tissue. Tissue to plasma ratios of radioactivity were also calculated as these gave a measure of the extent of uptake of radioactivity into the tissues from plasma.

3.3.1.3 Tissue distribution of metabolites

Based on the results of the distribution study, the tissues additional to the carotid body, selected for metabolic studies were liver, lung and plasma. Aliquots of these latter three tissues were freeze dried overnight then these and the carotid body exhaustively extracted into methanol (0.5-5ml) and the methanolic extracts evaporated to dryness under oxygen-free nitrogen. The residues were dissolved in a smaller volume (100µl) of methanol and applied to thin layer chromatographic plates prior to co-chromatography with almitrine and synthesised reference standards of metabolites in a solvent system of chloroform:methanol:0.880 ammonia (80:20:2 v/v).

The plates were then exposed to X-ray film (Kodak) for at least 6 weeks, developed and quantitated by either a Berthold Linear Analyser which provided both qualitative and quantitative profile, or scraped and the radioactivity determined as a gel suspension by liquid scintillation spectrometry. Each radioactive area along the chromatogram was quantitated and expressed as a percent of the complete chromatogram. This was then related to the total radioactivity data from the quantitative tissue distribution and the concentration of almitrine determined either as a percentage of the administered dose or µg per gramme of tissue.

3.3.1.4 Results

3.3.1.4.1 Tissue distribution

For the anaesthetised animals (Appendix 3.3.1A; Fig 3.5a) plasma levels of radioactivity were low as early as 5 minutes after an intravenous bolus administration of [^{14}C]-almitrine bismesylate (0.7% dose.g^{-1}) suggesting rapid uptake into tissues. High levels of radioactivity were observed particularly in highly perfused tissues such as lungs, bronchi and adrenals (12.26%, 10.62% and 10.66% dose.g^{-1} respectively) at this time. Radioactivity was also rapidly taken up by the carotid body (2.16% dose.g^{-1}) with levels approximately 3 times that of plasma (Appendix 3.3.1C). The cartoid artery on the other hand, which was used as a control tissue for the carotid body had levels 25% lower than the carotid body (1.7% dose.g^{-1}). However, the brain, gastrointestinal tract and less

perfused tissues such as muscle and trachea all had relatively low levels of radioactivity at this early time period.

By 15 and 30 minutes, levels had declined rapidly (2-6 fold) for the organs of high blood flow with the exception of the organs which eliminate the drug by metabolic transformation (liver) and urinary excretion (kidney) which showed a more gradual decline. There was a slow increase in levels (up to 30 minutes) into the slowly perfused tissues such as skin, white fat and trachea and those where diffusion occurred, such as the intestine and stomach as could be expected.

Radioactivity declined more rapidly in plasma ($t_{1/2}$ = 1 hour) and most other tissues up to 2 hours, with the exception of increasing levels into the gastrointestinal tract, which can be attributed to secretion and biliary excretion. There was an unexpected and complex distribution profile in the carotid body and carotid artery, where there was an initial fall in radioactivity similar to the decline in plasma, followed at 30 to 120 minutes, by a secondary rise of more than 50% in both these tissues. The same was seen in the superior cervical ganglion at 120 minutes.

In contrast, in the unanaesthetised animals used to investigate late time period, plasma radioactive levels declined gradually over the 2 to 16 hour period, with a half-life of approximately 15 hours, and the levels of most tissues fell in parallel (Appendix 3.3.1B; Fig 3.5b). There was a small apparent increase in carotid body levels after 16 hours, but the values lie within the experimental error of the assay at these low concentrations. Tissue levels were also considerably higher (5-20 times) in the liver, lung, kidney and heart compared to plasma. Carotid body levels of radioactivity were also higher (4 times) than plasma and carotid artery concentrations, suggesting a specific uptake into this tissue.

When the 2 hour levels in these unanaesthetised animals were compared to those measured at the same time in the anaesthetised rats, plasma and liver levels of radioactivity (expressed as % dose.g^{-1}) were found to be 2 to 3 times higher in the unanaesthetised group, whilst levels in the lung, kidney and heart were similar, but the carotid body, carotid artery and superior cervical ganglia were 2 to 8 times lower. However, a comparison of the 2 hour tissue:plasma ratios (Appendices 3.3.1C and 3.3.1D; Fig.

3.6) as a measure of the extent of uptake and equilibrium into tissues from plasma, showed that urethane anaesthesia increased the relative uptake of radioactivity into all tissues, with values ranging from a 13 and 22 fold increase in the carotid artery and superior cervical ganglion, 5-fold increase in the carotid body to only a 2% increase in the liver for the anaesthetised animals relative to the unanaesthetised group.

3.3.1.4.2 Metabolite profile

Thin layer chromatographic analysis of extracts of the tissues and organs of interest in the anaesthetised animals showed that at 15 minutes, metabolism was rapid in the liver, with the presence of at least 8 metabolites, whilst only 16% of the radioactivity remaining was associated with almitrine (Fig. 3.7). This was equivalent to 3.5$\mu g.g^{-1}$ tissue or 0.4% of dose per gram of tissue. There were a large number of circulating metabolites in plasma with almitrine (62%) being the major component (0.97$\mu g.g^{-1}$). However, with the exception of small amounts (approximately 16%) of the monodeallyl metabolite, these circulating metabolites were not detectable in the carotid body or lung. In the carotid body almitrine accounted for ~80% of the activity (8.0$\mu g.g^{-1}$ tissue) and the lung had the highest level per gram of tissue (106$\mu g.g^{-1}$ ≡ 90% of activity). By 2 hours, almitrine had been almost completely degraded by the liver, with approximately 10% remaining as the intact drug (1.1$\mu g.g^{-1}$) and similarly, more than 70% of the activity circulating in plasma was associated with metabolites. However, it would appear that in these animals, metabolites were not readily taken up by other tissues in comparison to almitrine, since more than 70% of the activity was associated with almitrine in tissues such as lung, and in the carotid body 82% of the activity remained as intact almitrine (11.7$\mu g.g^{-1}$).

At the same time, the 2 hour liver metabolite profile in the unanaesthetised animals showed a lower concentration of metabolites and thus lower circulatory levels of metabolites in plasma (Fig. 3.8). Conversely, there was a greater percentage (≈35-50%) of metabolites present in the carotid body and lung, with the carotid body displaying a qualitatively similar profile to that of plasma. Almitrine levels were much lower and represented only 1.7$\mu g.g^{-1}$ tissue although this was the major component (~65% of activity). By 8 hours, although all tissues displayed a large number of metabolites, the unchanged drug remained the largest single

component and whilst in the liver the unchanged drug represented approximately 40% of the total radioactivity on the chromatogram, the other tissues had slightly higher levels of almitrine (45-47%).

When comparing the extent of almitrine uptake at 2 hours (Figs. 3.7 and 3.8) the carotid body and lungs had 56 and 27 times higher levels of almitrine relative to plasma in the anaesthetised animals. The non-anaesthetised group had tissue levels only 3 fold higher than plasma.

3.3.1.5 Discussion

The highly lipophilic [^{14}C]-almitrine rapidly disappeared from plasma within 5 minutes of an intravenous administration to the anaesthetised rat with large quantities of radioactivity found particularly in the lung, adrenal, liver, kidney, heart and carotid body. Lower concentrations were found in the brain, red cells and the more poorly perfused tissues of fat and muscle, although considering the proportion of these latter tissues relative to the total bodyweight of the animal (20% and 40% body weight respectively) they would retain a relatively large proportion of the administered dose compared to other tissues. The initial rapid uptake and the decline observed in plasma is reflected in the carotid body and adrenal gland, both being small organs with high blood flow per gram of tissue. After 15 minutes, however, particularly for the carotid body, the increasing levels suggest either a slower specific uptake process independent of passive diffusion, or a specific binding where dissociation is slow. The carotid artery, examined as a control to the carotid body because of the difficulty of excision, showed a similar profile in anaesthetised animals, suggesting that the uptake into the carotid body, although complex, is not unique to this tissue.

In unanaesthetised animals, however, for both total radioactivity and unchanged almitrine (latter not presented), the carotid artery levels were lower (2-5 times) than the carotid body, suggesting in these animals at least, that a specific uptake could be occurring under non-anaesthetised or normal conditions. It is not known, however, if this process is active or passive. The pharmacological importance of the secondary rise in almitrine concentration under anaesthesia is difficult to ascertain. However, in studies where the action of almitrine on ventilation in anaesthetised rats has been observed for more than 30 minutes, there is a plateau of effect with urethane anaesthesia (Dhillon and Barer, 1982) and even an increase in activity at 1 hour with

chlorolose anaesthesia (Evrard, 1986 personal communication) despite apparent falls in plasma concentrations. This increasing ventilation towards the end of prolonged chlorolose anaesthesia could, however, also be due to the animals regaining some degree of consciousness, which would not occur with urethane.

One important finding from this study was that anaesthesia had a marked, but complex, effect on the tissue distribution of almitrine and its metabolites. The tissue:plasma ratio of carotid body radioactivity in the anaesthetised rats at 2 hours was 5 times higher compared to unanaesthetised animals at the same time. Almitrine tissue to plasma ratio for the same tissue was 17 fold higher under anaesthesia. There was also a 20% increase on the number of metabolites present in the carotid body of the unanaesthetised animals. A number of factors may contribute to the differences observed between these two groups of animals; these include (a) reduced clearance, (b) changes in blood flow, (c) displacement of almitrine and its metabolites from binding to plasma proteins or tissues, (d) urethane induced hypoxia and hypercapnia, (e) active uptake procedures (f) pH blood changes due to hyperventillation.

Although there are indications that different anaesthetics will alter vascular resistance and therefore blood flow (Amstrong *et al.,*1961; Zimpfer *et al.*, 1981), the effects of anaesthesia on cardiovascular response on the carotid body have not been extensively studied. Reduction in blood flow, induced by urethane, could alter the distribution of almitrine and its metabolites, but in the opposite direction to that observed in this study. Conversely, vasodilation could be a contributing factor. The clearance of almitrine, however, is low (Chapter 5) and changes in blood flow are unlikely to be important in the overall elimination of the drug.

Almitrine is highly bound to plasma proteins such as albumin (>99%, Chapter 5) and lipoproteins (Didey *et al.,* 1986 personal communication), and if displaced by urethane could result in lower plasma levels due to increased uptake into target tissues or in the liver with subsequent higher clearance and lower tissue metabolite levels, since these metabolites are likely to be less lipophilic and therefore less bound to plasma proteins. Suárez *et al.,* (1991) showed that patients who had received halothane anaesthesia had a significantly higher serum level of free diazepam compared to that observed before anaesthesia. Similarly, the halothane metabolite trifluoroacetic acid

significantly increased the free levels of diazepam, leading to the conclusion that halothane anaesthesia may potentiate the pharmacological effect of diazepam in the post operative period following anaesthetic procedures. Certainly a similar displacement of almitrine by urethane metabolites would lead to an increased uptake of almitrine into the carotid body but free almitrine levels were not measured to investigate this possibility. A further explanation is that urethane anaesthesia would depress ventilation, leading to hypoxia and hypercapnia, resulting in some degree of tissue acidosis. Almitrine is a weak base with a pKa of 5, and under these conditions would be more ionised in the tissues compared to plasma, assuming the pH partition hypothesis, therefore giving rise to higher tissue:plasma ratios. The metabolite, on the other hand, being more acidic compared to almitrine, would become less concentrated in tissues. Hepatic acidosis would similarly lead to greater almitrine uptake into the liver, and consequently increased metabolism of the drug.

One of the main questions that has been answered by these results is whether it is almitrine alone or its metabolites that stimulate the chemoreceptors. Certainly in the anaesthetised animals, almitrine was the major component in the carotid body, with only a small quantity ($\cong$ 10%) of the monodeallyl metabolite present. In the unanaesthetised animals, however, almitrine still remained the major component, but up to 10 metabolites were found to be also present. At 2 hours these metabolites represented 43% of the total activity in the carotid body and by 16 hours, this had increased to 65%. Pharmacological screening of the major identified metabolites (Evrard, 1986 personal communication) have shown that with the exception of the dihydroxy metabolite none of the other metabolites were found to increase ventilation when injected into anaesthetised rats. The activity of the dihydroxy almitrine was found to produce a transient increase in ventilation which lasted for only 15 minutes and was only 25% of that observed for almitrine.

In summary, this study has shown almitrine to be rapidly distributed into tissues, particularly highly perfused tissues, with a complex and sustained uptake into the carotid body of the anaesthetised animals and despite extensive metabolism, is the major active component. This sustained uptake may, in part, explain the prolonged activity observed in experimental animals. Tissue levels of unchanged almitrine are higher in urethane anaesthetised

animals than unanaesthetised animals whilst plasma levels are lower with greater hepatic metabolism. Both hypoxic tissue acidosis and plasma protein displacement due to urethane could provide an explanation for this finding, although the effect of anaesthesia on blood flow cannot be discounted, but further work would be necessary to confirm these hypotheses. It can be concluded that the pharmacological activity of almitrine is likely to be altered in animals which are anaesthetised, due to pharmacokinetic and metabolic considerations, and that this should be borne in mind when interpreting such data.

Figure 3.5 : Tissue Levels of Total Radioactivity Following Administration of [^{14}C]-Almitrine Bismesylate (3mg.kg^{-1}) to (A) Anaesthetised and (B) Unanaesthetised rats

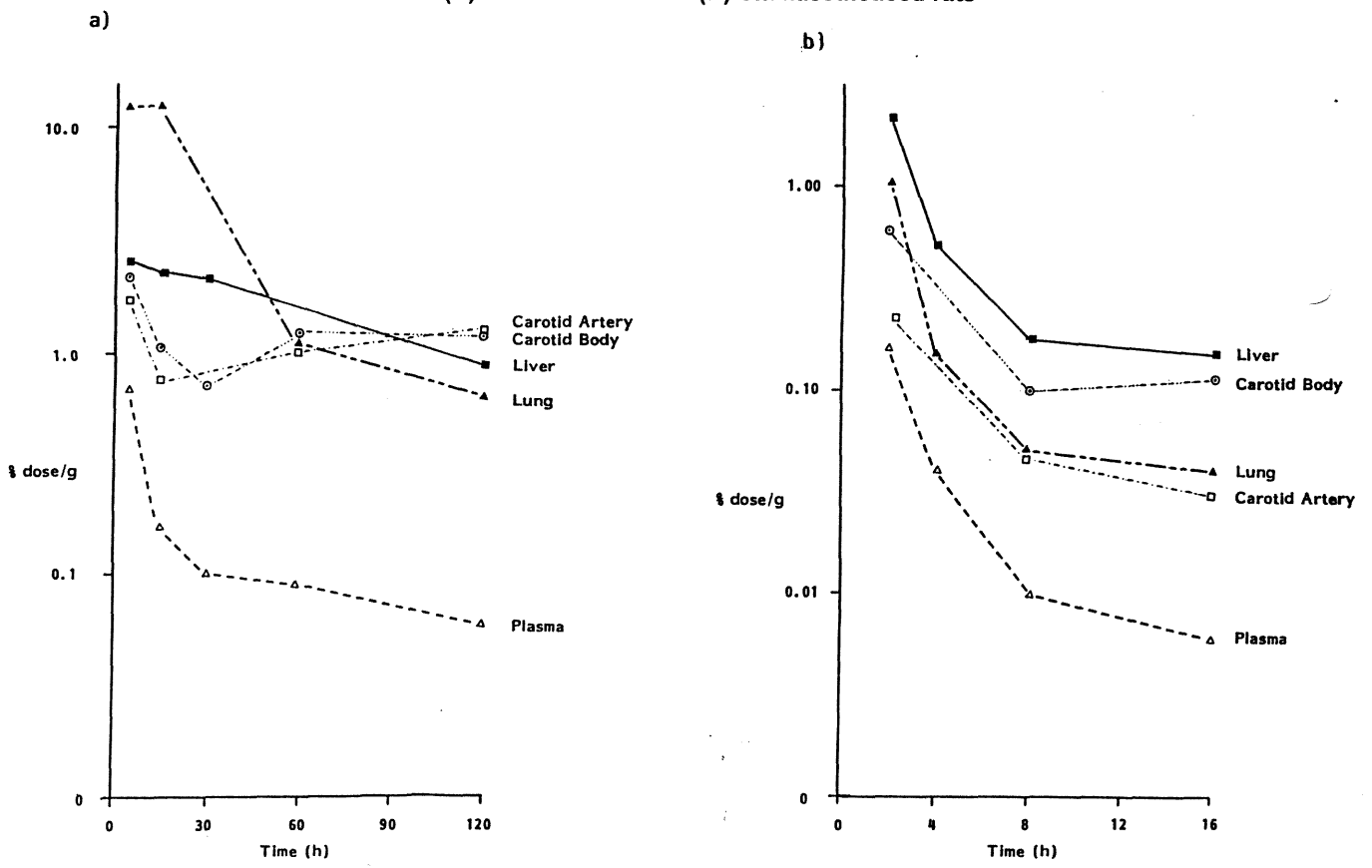


Figure 3.6 : The Relative Uptake of Radioactivity Into Tissues Expressed as Tissue:Plasma Ratios 2 Hours After an Intravenous Injection of [^{14}C]-Almitrine Bismesylate into (A) Anaesthetised (▨) and (B) Unanaesthetised (■) Rats

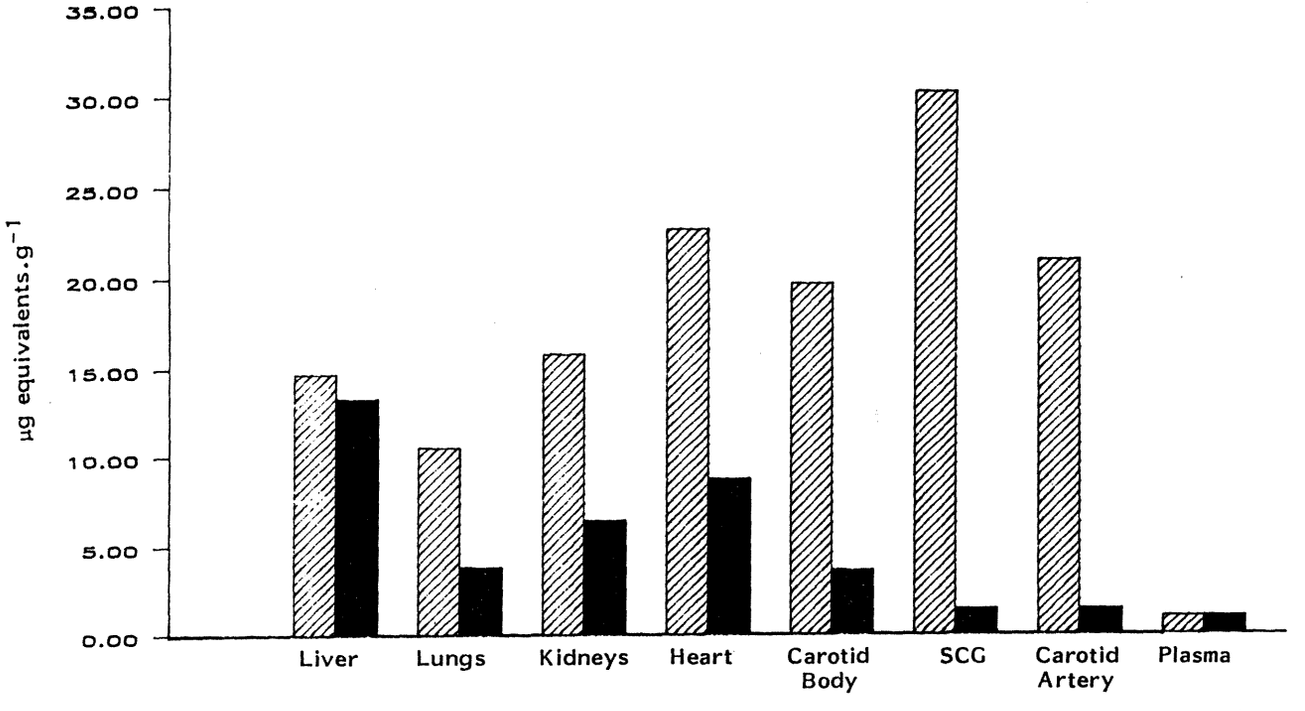


Figure 3.7 : The Relative Uptake of Almitrine and its Metabolites in the Liver, Plasma, Carotid Body and Lung in Anaesthetised Rats following Intravenous Administration of [^{14}C]-Almitrine Bismesylate

(a) Almitrine, (b) Monodeallyl, (c) Dihydroxy

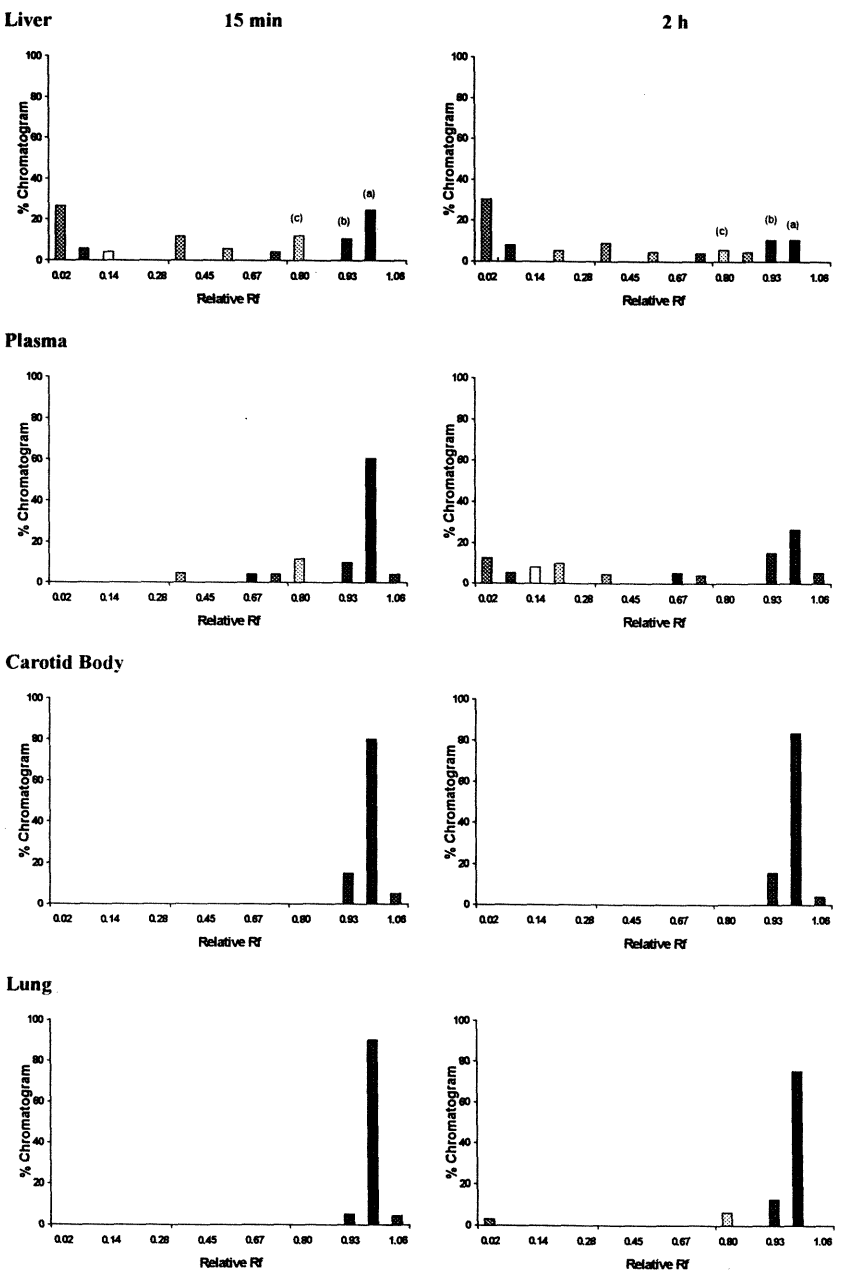
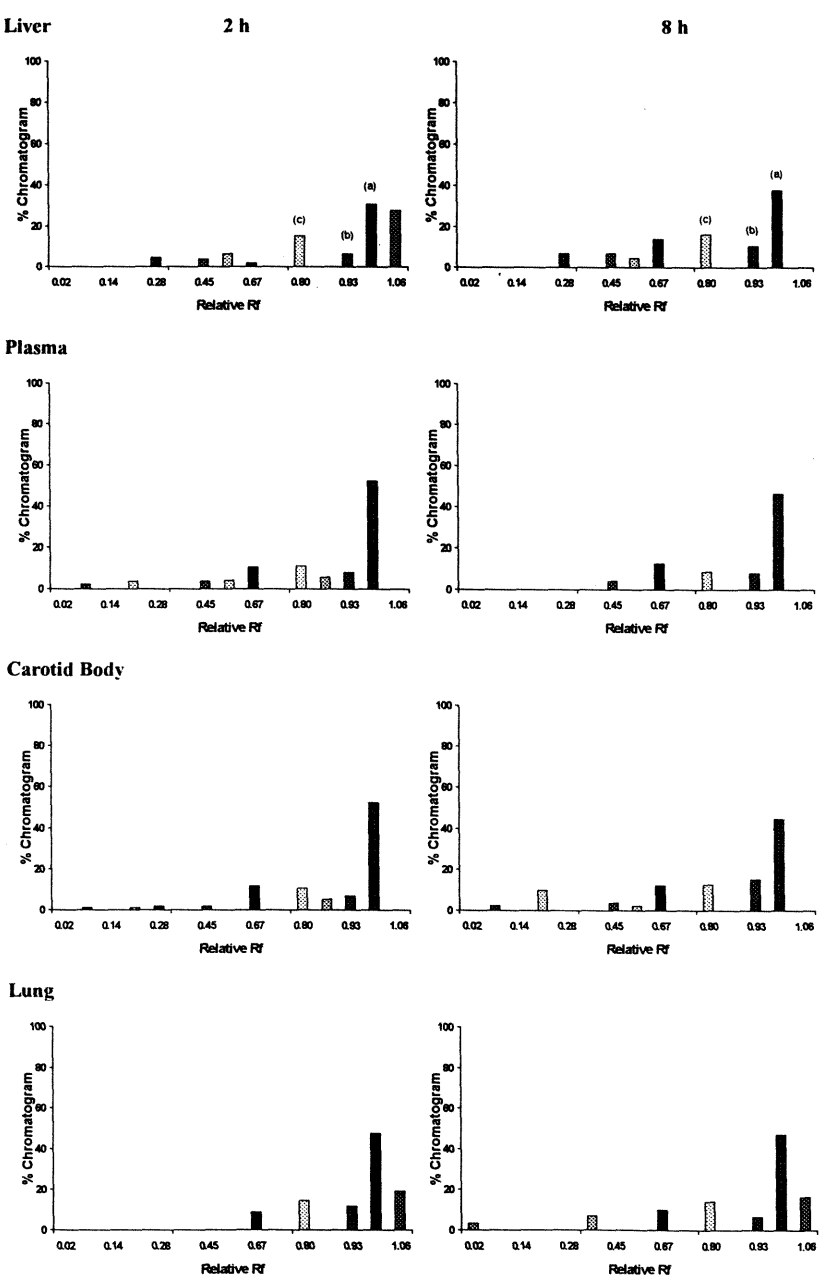


Figure 3.8 : The Relative Uptake of Almitrine and its Metabolites in the Plasma, Carotid Body, Lung and Liver in Unanaesthetised Rats following Intravenous Administration of [^{14}C]-Almitrine Bismesylate
(a) Almitrine, (b) Monodeallyl, (c) Dihydroxy



3.3.2 The pharmacokinetics of total radioactivity and unchanged almitrine in the dog following intravenous administration of [^{14}C]-almitrine bismesylate

In the studies described earlier in this chapter (3.1 and 3.2) the plasma profile of [^{14}C]-almitrine in the dog was monitored for only 72 hours whilst the excretion balance data indicated that only 66% of the administered dose was accounted for in the one week period over which radioactivity was monitored. The radioactive kinetic data suggested a low clearance and large volume of distribution with a terminal half-life which, although relatively long, would appear to be underestimated due to the short sampling time (72h). It was therefore necessary to perform a further study in which plasma could be monitored for a longer period and to try to establish how much of this long terminal half-life was attributed to the unchanged drug. If the specific assay was sensitive enough to allow the measurement of unchanged almitrine for the duration of the study, good pharmacokinetic data could be obtained for the compound in a large toxicological and pharmacological species. An intravenous study was therefore performed in which blood was monitored over 7 days and urine and faeces analysed over the same period in order to compare the data previously reported in this chapter.

Two male Beagle dogs (10 and 12kg dog 884 and 883 respectively) were given an intravenous bolus dose of [^{14}C]-almitrine bismesylate at a dose level of 5mg.kg^{-1} via the cephalic vein of one of the fore-legs. Blood (5ml) was taken predose and up to 168 hours after dosing via the other fore limb, into a lithium heparinised blood tube. A small aliquot (0.5ml) of each blood sample was retained for the determination of radioactivity, and the remainder centrifuged to obtain plasma which was aliquoted into two separate tubes for the determination of total radioactivity and the level of intact almitrine.

The animals were housed into separate metabolism cages and urine and faeces were collected separately predose and daily after dosing for 7 days and transferred into preweighed, suitably labelled containers. All samples were stored at -20°C until analysed for radioactivity or intact drug.

Plasma, blood, urine and faeces were analysed for radioactivity by liquid scintillation spectrophotometry as described in Chapter 2. For plasma and blood, the radioactivity was expressed as nanogram equivalents of free base per ml plasma or blood.

Plasma was analysed for almitrine free base by gas liquid chromatography with nitrogen phosphorus detection following liquid/liquid extraction. The plasma concentration of almitrine was expressed as ng free base per ml plasma. Pharmacokinetic analysis was performed as described in the Methods Section 4.

3.3.2.1 Results

3.3.2.1.1 Excretion of radioactivity

Radioactivity was excreted predominantly via the faeces with 44 and 48% of the dose eliminated into the faeces and 57 and 62% of the total dose recovered for dogs 884 and 883 respectively (Appendices 3.3.2A and 3.3.2B). For both animals just over 3% of the dose was eliminated into the urine during the 7 days (Fig. 3.9). These data were similar to that reported earlier.

3.3.2.1.2 Radioactive kinetics

Blood levels of radioactivity were measured at selected time points up to 72 hours and were much higher in dog 884 than dog 883, relative to dose (Appendix 3.3.2C). Following an initial distribution, blood radioactivity levels declined biphasically (Fig. 3.10) with an initial decline phase of half-life 6.64h and 4.31h in dogs 883 and 884 respectively. The half-life of the terminal phase was 62.8h and 72.3h for dogs 883 and 884 respectively (Table 3.3). The blood clearance of total radioactivity was low and varied between 11.9ml.min^{-1} in dog 884 and 15.6ml.min^{-1} in dog 883. Similarly, the volume of distribution based on area varied between 73.7 litres in dog 884 and 84.8 litres in dog 883.

Plasma radioactivity was measurable up to 168 hours and declined biphasically (Fig. 3.10) with an initial half-life of 2.7 to 9.9 hours respectively for dogs 884 and 883 and terminal half-lives in the same order as those seen in the blood radioactive profile, varying from 78.1 hours for dog 883 and 88.8 hours in dog 884. The plasma clearance of radioactivity was also low 12.2ml.min^{-1} and 5.3ml.min^{-1} for dogs 883 and 884 respectively and although the volume of distribution based on area for dog 883 was in the same order as that seen for blood (82.5 litres), dog 884 had smaller volume of distribution (40.7 litres) and was approximately half that seen in the blood. This was reflected in both the higher AUC and the lower clearance of radioactivity in plasma relative to blood, seen for this animal.

Figure 3.9 : Cumulative Excretion Profiles of Radioactivity in the Dog after a Single Intravenous Administration of [^{14}C]-Almitrine Bismesylate (5mg.kg^{-1})

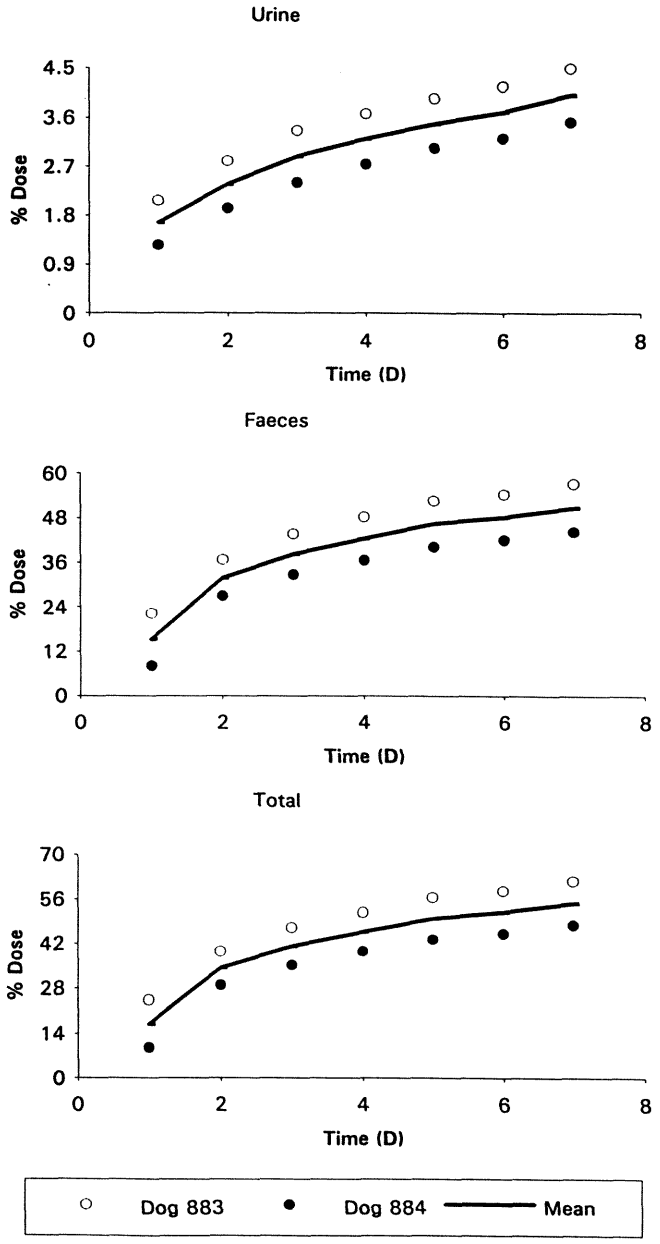
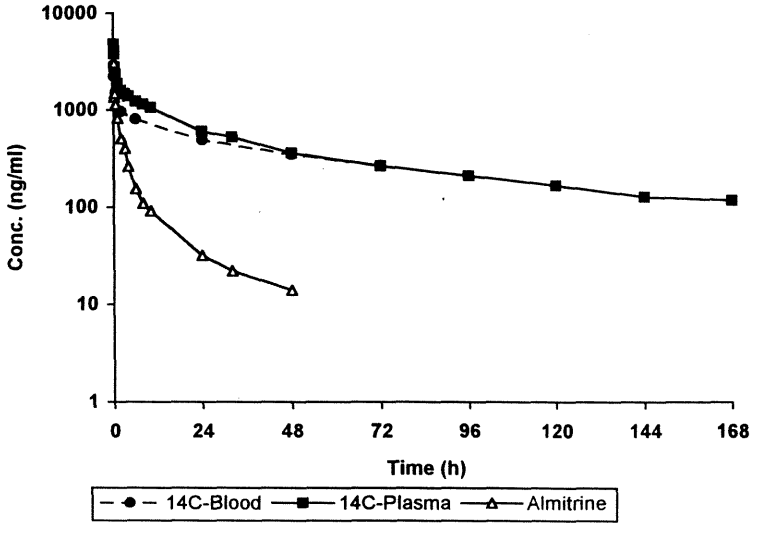


Figure 3.10 : Blood and Plasma Profiles of Radioactivity and Plasma Profile of Unchanged Almitrine in the Dog Following a Single Oral Dose of [^{14}C]-Almitrine Bismesylate (5mg.kg^{-1})

Dog 883



Dog 884

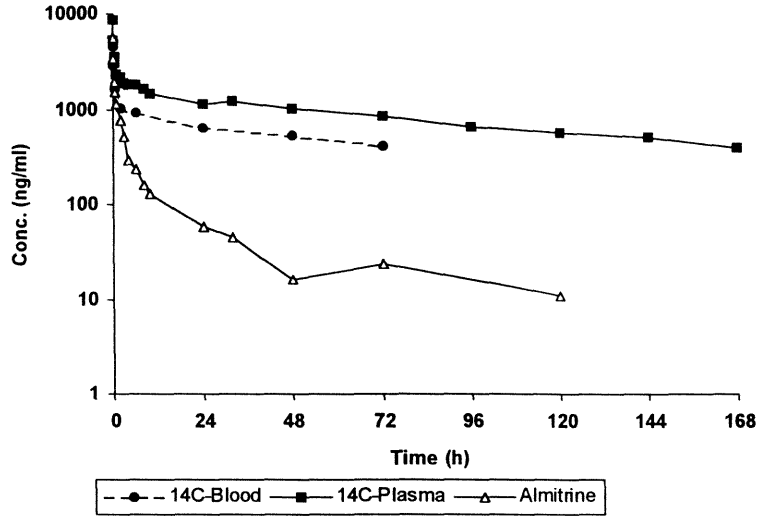


Table 3.3 : Summary of Pharmacokinetic Parameters for Radioactivity and Intact Almitrine in Dogs, Following Single Intravenous Administration of [^{14}C]-Almitrine Bismesylate at 5mg.kg^{-1}

	DOG 883			DOG 884		
	Whole Blood	PLASMA		Whole Blood	PLASMA	
	Radioactivity	Radioactivity	Intact Drug	Radioactivity	Radioactivity	Intact Drug
C_{max} (ng.ml^{-1})	2925	4807	3067	4545	8811	5734
t_{max} (h)	0.08	0.08	0.08	0.08	0.08	0.08
$t_{½}$ init (h)	6.6	9.9	3.3	4.3	2.7	7.4
$t_{½}$ z (h)	62.8	78.1	23.4	72.3	88.6	110
AUC (ng.ml^{-1}.h)	58738	74980	5327	88842	196500	10325
CL (ml.min^{-1})	15.6	12.2	172.0	11.9	5.3	101.7
Vd (l)	84.8	82.5	350.0	73.7	40.7	970.1

The blood to plasma ratio of radioactivity slowly increased with time for dog 883, ranging from 0.58 at 10 minutes to 1.01 at 72 hours, although over the first 6 hours the ratio was relatively constant and fluctuated between 0.58 and 0.66. For dog 884 the ratio remained relatively constant over the 72 hour period and fluctuated between 0.52 at 5 minutes after dosing to 0.48 at 72 hours post dose (Appendix 3.3.2C). In general, although the half-life of radioactivity was longer than previously reported (section 3.1.2) the clearance of radioactivity were similar.

3.3.2.1.3 Intact drug kinetics

For dog 883, due to analytical problems, intact drug could only be accurately measured for up to 48 hours after dosing. For dog 884, almitrine was measurable up to 120 hours (Appendix 3.3.2D). For both animals, plasma intact drug concentrations were much lower than those of total radioactivity even at the first time point (5 minutes) after bolus dosing. Following an initial distribution phase, plasma levels declined biphasically with initial half-lives of 3.3 hours and 7.4 hours respectively for dogs 883 and 884 and terminal half-lives of 23.4 hours and 110 hours for dogs 883 and 884 respectively. The plasma clearance of intact almitrine was 14 to 20 fold higher than that seen for total radioactivity and was 102ml.min^{-1} in dog 884 and 172ml.min^{-1} in dog 883.

3.3.2.2 Discussion

When the profiles and areas of total radioactivity and intact drug were compared for each animal, radioactivity levels in all cases were higher than that of the intact drug. Radioactive levels based on area under the curve were higher in plasma than blood by a factor of 1.3 and 2.2 in dogs 883 and 884 respectively. Although for dog 883 the area under the curve of the intact drug was underestimated due to analytical problems, the ratio area of radioactivity to intact drug was 14 fold greater. This compared well with the 19 fold ratio observed for dog 884. This would suggest, therefore, that the differences observed in the plasma levels of radioactivity and intact drug (Fig. 3.10) are due to extensive metabolism of almitrine and the clearance of these metabolites is lower and account for the long half-life of radioactivity in these animals. Since the blood and plasma radioactive levels were still high by 168 hours it is quite obvious that the radioactive half-life has been underestimated. When compared to previously reported data (section 3.2) in which radioactivity was collected for only 72 hours, a longer half-life (although

underestimated) of radioactivity was acheived in this study. This study showed a larger volume of distribution of radioactivity (4.1 to 6.9 $l.kg^{-1}$) and a lower clearance (0.53 to 1.0$ml.min^{-1}.kg^{-1}$) both hybrids of the determination of half-life. As with the previous study the recovery of radioactivity in excreta was only 80% after a one week collection. Extrapolation of the total radioactivity profile indicates a slow elimination of radioactivity which would achieve 100% over a long collection period.

In summary, the rate and extent of elimination of radioactivity after intravenous administration to the dog is similar to that reported previously, with the faecal route being the major route of elimination. The elimination half-life of intact almitrine is long with a low clearance and large volume of distribution. There is a lower clearance of radioactivity due mainly to circulating metabolites. Although these parameters appear to be underestimated (t½ and Vd), this study highlights the need to collect for as long as possible, data for compounds which show prolonged excretion.

3.3.3 The disposition, kinetics and metabolism of [^{14}C]-almitrine bismesylate in man, following oral administration

This section describes the results of a study performed in four healthy subjects (two male and two female), who had been given [^{14}C]-almitrine bismesylate. The main objectives of the study were:-

a) To determine the blood and plasma kinetics of radioactivity from [^{14}C]-almitrine bismesylate following oral administration and to compare them with the pharmacokinetics of intact drug and if possible metabolites.

b) To determine the rates of elimination of total radioactivity following oral administration, in urine and faeces.

c) To attempt to collect excreta until a complete balance of 100 ± 10% of the dose is obtained.

d) To separate, compare and quantify plasma, urinary and faecal metabolites.

3.3.3.1 Study design

Four healthy subjects, two male (3 and 4) and two female (1 and 2), were entered into the study. None of the subjects had a history of cardiac, hepatic or renal disfunction. All subjects had blood biochemical and haematological tests before commencing the study in order to ensure that they were suitable to be entered into the study. All subjects were free from medication, except oral contraceptives, where appropriate, for a period of one week prior to the start of the study. No alcohol was permitted 24 hours before and until 72 hours after receiving the dose of [^{14}C]-almitrine bismesylate. The subjects were fasted for a period of 12 hours before and for a further 4 hours after receiving the dose. The subjects were instructed to swallow their dose as quickly as possible and the vials rinsed twice with tap water and each rinse swallowed by the volunteer. Each subject had previously been instructed on the nature of the study in particular that they would be able and willing to collect urine and faeces for several months, and had given their signed, informed, consent prior to taking the dose.

Blood samples (20ml) were taken initially via an indwelling cannula, intermittently flushed with heparinised saline and thereafter by direct

venepuncture. Samples were taken predose then 0.5, 1, 2, 3, 4, 6, 8, 12, 24, 30, 48 hours and daily for 7 days. Thereafter twice weekly (Mondays and Fridays) for 2 weeks and once weekly (Wednesdays) for a further 3 weeks, or until unchanged almitrine was no longer quantifiable by GC analysis (limit of quantitation 1ng.ml^{-1}). A larger blood sample (50ml) was taken at 2, 4, 8 and 24 hours for metabolite profiling. For each blood sample, a small aliquot (1ml) was transferred to a small heparinised tube (1ml) and the remainder transferred into 10ml heparinised tubes for the preparation of plasma by centrifugation. All samples were stored at -20°C in the dark until analysed.

Urine was obtained predose then 3, 6, 12 and 24 hours and thereafter at 24 hour intervals, into preweighed plastic bottles, until the levels of radioactivity were no longer distinguishable from background for 3 consecutive samples. The weight of each sample was recorded and the total amounts for the first 48 hours retained; aliquots (20ml) thereafter were stored at -20°C in the dark until analysis.

A predose faecal sample, then daily total faeces were collected into preweighed containers until the levels of radioactivity were indistinguishable from background for 3 consecutive samples. It was the intention of the study that by this time the cumulated excretion into urine and faeces would be 100 ± 10%. All samples were stored frozen at -20°C until analysed.

3.3.3.2 Results

3.3.3.2.1 The kinetics of radioactivity

Weighed aliquots of blood samples were combusted before counting; plasma (weighed aliquots) was counted directly for radioactivity (Appendices 3.3.3A and 3.3.3B respectively). Plasma radioactivity was measurable up to 936 hours (39 days) in Subject 1, whilst blood radioactivity was detectable in Subject 4 up to 408 hours (17 days). The mean blood and plasma profiles of radioactivity and of intact almitrine, up to 120 hours are presented in Fig. 3.11. Individual profiles up to 168 and 408 hours are presented in Figure 3.12 for blood.

Radioactivity was rapidly absorbed in all subjects with a mean maximum blood concentration of radioactivity of 277 ± 113ng.eq.g^{-1} (Table 3.4) which ranged from 221ng.eq.g^{-1} in female subject 1 to 445ng.eq.g^{-1} in male subject 4 and which was achieved 1.75 ± 0.5 hours after

administration. Mean maximum plasma levels of radioactivity were higher (307 ± 101ng.eq.g^{-1}, Table 3.5) than blood and ranged from 220ng.eq.g^{-1} in female Subject 2 to 420ng.eq.g^{-1} in male Subject 3. The mean absorption half-life of radioactivity was 0.32 and 0.38 hours for blood and plasma respectively. Once maximum levels of radioactivity were achieved all subjects showed a biexponential decline (Figs. 3.12 and 3.13) with mean initial half-lives of 12.7 ± 7.0 and 15.3 ± 4.1 hours for blood and plasma respectively and mean terminal half-lives of 174 ± 81.7 and 204 ± 52.1 hours respectively for blood and plasma. The AUC for plasma (24003 ± 5618ng.eq.ml^{-1}.h) was higher than that of total blood (19033 ± 4231ng.eq.ml^{-1}.h) with a mean blood to plasma ratio of 0.75, again emphasising the fact that the radioactivity was mainly associated with plasma. The extrapolated AUC to infinity represented 27.8 ± 12.8 and 20.4 ± 8.9% of the total AUC for blood and plasma respectively. When normalised to a 1mg.kg^{-1} dose, plasma concentrations were within the ranges previously reported.

3.3.3.2.2 <u>The kinetics of intact almitrine in plasma</u>

The plasma concentration of intact almitrine, measured by the specific gas chromatographic method described in Chapter 2, are summarised in Appendix 3.3.3C. Plasma levels of unchanged almitrine were generally lower compared to total radioactivity. Unchanged almitrine was measurable for up to 480 hours (20 days) in Subject 2 and up to 2232 hours (93 days) in Subject 4. For Subjects 1 and 3 almitrine was measurable for up to 768 and 936h (32 and 39 days) respectively.

The pharmacokinetic parameters are summarised in Table 3.6. Almitrine was rapidly absorbed with a mean absorption half-life of 0.48 ± 0.19 hours which ranged from 0.26 hours in Subject 2 to 0.60 hours in Subject 1. It was not possible to obtain an absorption half-life for Subject 4. The mean maximum plasma concentration of almitrine (147 ± 69.9ng.ml^{-1}) was obtained at 2 hours after dosing. Following a maximum plasma concentration of between 64ng.ml^{-1} in Subject 2 and 222ng.ml^{-1} in Subject 3, there was in general triphasic decline in the plasma concentration of intact almitrine (Fig. 3.14). A distribution phase of half-life ranging from 1.59 hours in Subject 1 to 5.41 hours in Subject 4 (mean of 2.75 ± 1.79 hours) was followed by an initial elimination phase with a mean half-life of 34.4 ± 12 hours ranging 25.23 hours to 52.15 hours. The terminal half-life

of almitrine was extremely long and ranged from 407 hours (16.9 days) for Subject 2 whose plasma almitrine was measurable up to 480 hours (20 days) to 1785 hours (74.4 days) in Subject 4 whose plasma almitrine was measurable up to 2232 hours (93 days). The mean terminal half-life was 1163 ± 576 hours (48.5 ± 24 days).

The longer half-life of intact almitrine reflects the greater sensitivity of the specific method relative to radioactivity counting. The AUC for intact almitrine (7660 ± 3774ng.ml^{-1}.h) was only 32% of that of radioactive plasma AUC (24003 ± 5618ng.eq.g^{-1}.h), the remaining portion representing metabolites (Fig. 3.11). The extrapolated AUC to infinity represented 28 to 57% (mean 37.4 ± 13.6%) of the total AUC for intact almitrine. The mean comparative pharmacokinetic parameters of intact almitrine and radioactive blood and plasma are summarised in Table 3.7.

Table 3.4 : Pharmacokinetic Parameters of Total Radioactivity in Blood of Male and Female Subjects Following Oral Administration of [^{14}C]-Almitrine Bismesylate (~100mg ≅30μCi)

SUBJECT	1	2	3	4	MEAN ± SD
Dose (μCi)	29.99	29.96	29.23	40.59	32.44 ± 5.44
C_{max} (ng.eq.ml^{-1})	211	215	238	445	277 ± 113
t_{max} (h)	2.0	2.0	2.0	1.0	1.75 ± 0.50
$t_{½}$ (a) (h) [1]	0.41	0.39	0.42	0.06	0.32 ± 0.17
$t_{½}$ (1) (h) [2]	17.33	18.44	11.76	3.14	12.7 ± 7.0
$t_{½}$ (z) (h) [3]	138	202	272	82.4	174 ± 81.7
AUC_t (ng.eq.ml^{-1}.h)	10697	17593	11489	11636	12786 ± 3224
AUC (ng.eq.ml^{-1}.h)	16481	23140	19328	13181	18033 ± 4231
% Extrapolated AUC	35	24	41	12	27.8 ± 12.8

1. $t_{½}$a = absorption half-life
2. $t_{½}$(1) = distribution half-life
3. $t_{½}$ (z) = terminal half-life

Table 3.5 : Pharmacokinetic Parameters of Radioactivity in Plasma of Male and Female Subjects Following Oral Administration of [^{14}C]-Almitrine Bismesylate (~100mg ≅30μCi)

SUBJECT	1	2	3	4	MEAN ± SD
C_{max} (ng.eq.ml^{-1})	223	220	420	365	307 ± 101
t_{max} (h)	2.0	2.0	2.0	3.0	2.3 ± 0.5
$t_{½}$ (a) (h) [1]	0.37	0.30	0.43	0.41	0.38 ± 0.06
$t_{½}$ (1) (h) [2]	16.9	19.6	9.9	14.8	15.3 ± 14.2
$t_{½}$ (z) (h) [3]	260	187	228	140	204 ± 52.1
AUC_t (ng.eq.ml^{-1}.h)	21298	11828	24407	19511	19261 ± 535
AUC (ng.eq.ml^{-1}.h)	25916	17496	30666	21932	24003 ± 5618
% Extrapolated AUC	18	32	20	11	20.4 ± 8.9

1. $t_{½}$a = absorption half-life
2. $t_{½}$(1) = distribution half-life
3. $t_{½}$ (z) = terminal half-life

Table 3.6 : Pharmacokinetic Parameters of Intact Almitrine in Male and Female Subjects Following Oral Administration of [^{14}C]-Almitrine Bismesylate (~100mg ≅30µCi)

SUBJECT	1	2	3	4	MEAN ± SD
SEX	F	F	M	M	-
Dose (mg)	80.89	80.81	99.97	100.69	90.59 ± 11.25
C_{max} (ng.eq.ml^{-1})	119	64.0	222	184	147 ± 69.9
t_{max} (h)	2.0	2.0	2.0	2.0	2.0 ± 0.0
$t_{½}$ (a) (h) [1]	0.60	0.26	0.57	-	0.48 ± 0.19
$t_{½}$ (1) (h) [2]	1.59	2.11	1.89	5.41	2.75 ± 1.79
$t_{½}$ (2) (h) [3]	30.0	25.2	30.3	52.2	34.4 ± 12.0
$t_{½}$ (z) (h) [4]	1350	407	1109	1785	1163 ± 576
AUC_t (ng.eq.ml^{-1}.h)	3630	1542	6781	6720	4668 ± 2551
AUC (ng.eq.ml^{-1}.h)	8500	2128	10460	9552	7660 ± 3774
% Extrapolated AUC	57	28	35	30	37.4 ± 13.6

1. $t_{½}$a = absorption half-life
2. $t_{½}$(1) = distribution half-life
3. $t_{½}$ (2) = initial half-life
4. $t_{½}$ (z) = terminal half-life

Table 3.7 : Mean Pharmacokinetic Parameters of Intact Almitrine in Plasma and Total Radioactivity in Plasma and Whole Blood in Male and Female Subjects Following Oral Administration of [^{14}C]-Almitrine Bismesylate (~100mg≅30µCi)

MATRIX	PLASMA		BLOOD
	INTACT ALMITRINE	[^{14}C]-RADIOACTIVITY	[^{14}C]-Radioactivity
Dose (mg)	90.59 ± 11.25	90.59 ± 11.25	90.59 ± 11.25
Dose (µCi)	-	32.44 ± 5.44	32.44 ± 5.44
C_{max} (ng.eq.ml^{-1})	147 ± 69.9	307 ± 101	277 ± 12.5
t_{max} (h)	2.0 ± 0.0	2.3 ± 0.5	2.3 ± 1.3
$t_{½}$ (a) (h) [1]	0.48 ± 0.19	0.38 ± 0.06	0.32 ± 0.17
$t_{½}$ (1) (h) [2]	2.75 ± 1.79	15.3 ± 4.12	12.7 ± 6.99
$t_{½}$ (2) (h) [3]	34.4 ± 12.0	204 ± 52.1	174 ± 18.7
$t_{½}$ (z) (h) [4]	1163 ± 576	-	-
AUC_t (ng.eq.ml^{-1}.h)	4668 ± 2551	19261 ± 535	12786 ± 3224
AUC (ng.eq.ml^{-1}.h)	7660 ± 3774	24003 ± 5618	18033 ± 4231
% Extrapolated AUC	37.4 ± 13.6	20.4 ± 8.9	27.8 ± 12.8

1. $t_{½}$a = absorption half-life
2. $t_{½}$(1) = distribution half-life
3. $t_{½}$ (2) = initial half-life
4. $t_{½}$ (z) = terminal half-life

Figure 3.11 : Mean Profiles of Blood and Plasma Radioactivity and Plasma Unchanged Almitrine in 4 Subjects Following Single Oral Administration of [^{14}C]-Almitrine Bismesylate at 100mg.kg^{-1}

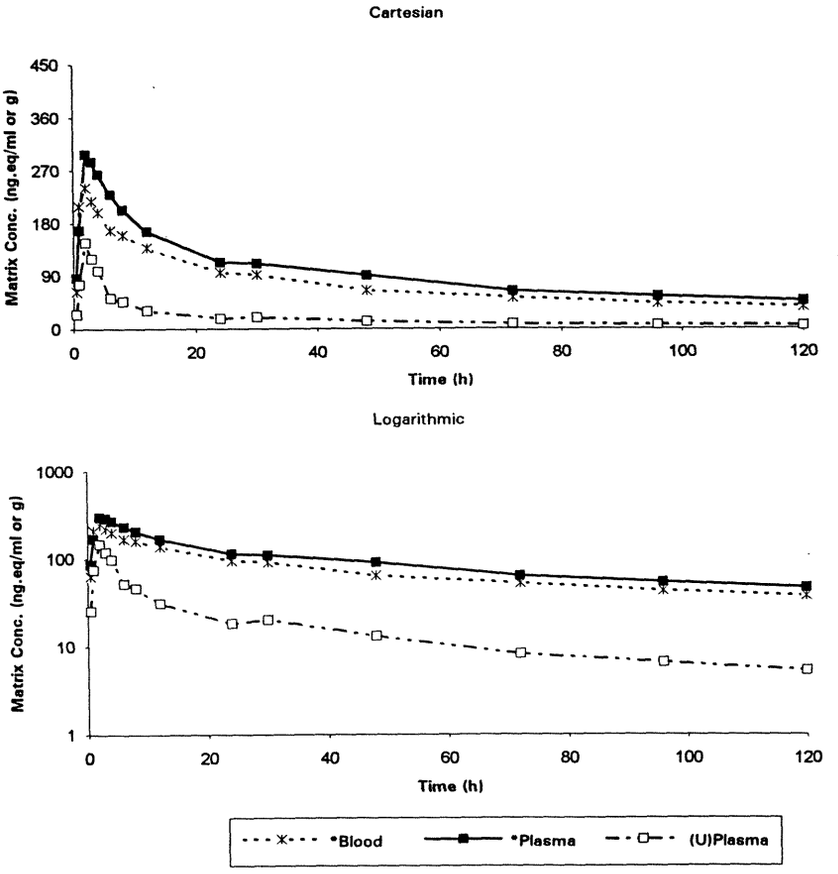


Figure 3.12 : Blood Profiles of Radioactivity in Subjects 1 to 4 Following Single Oral Administration of [^{14}C]-Almitrine Bismesylate at 100mg

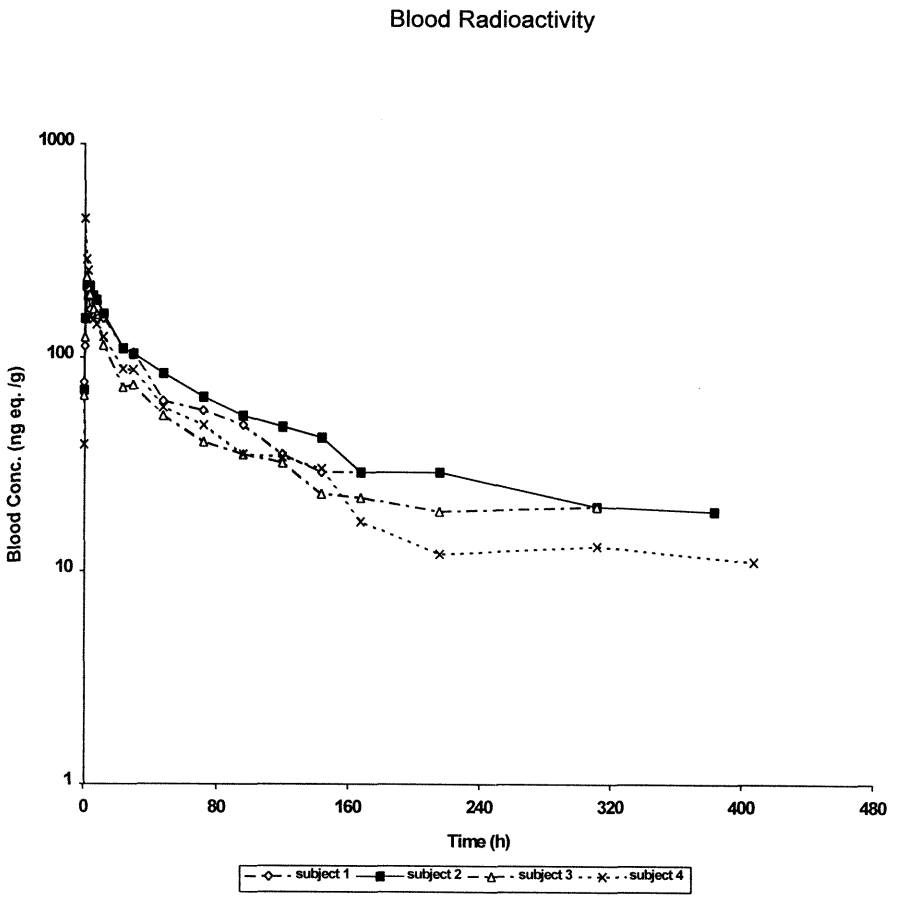


Figure 3.13 : Plasma Profiles of Radioactivity in Subjects 1 to 4 Following Single Oral Administration of [^{14}C]-Almitrine Bismesylate at 100mg

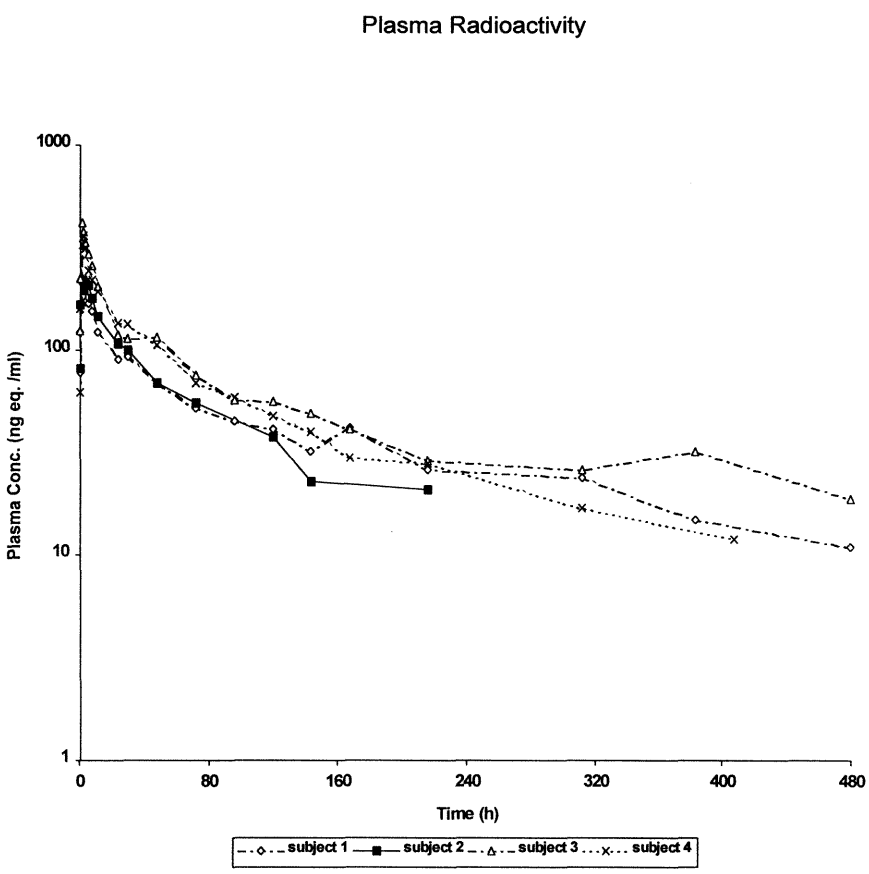
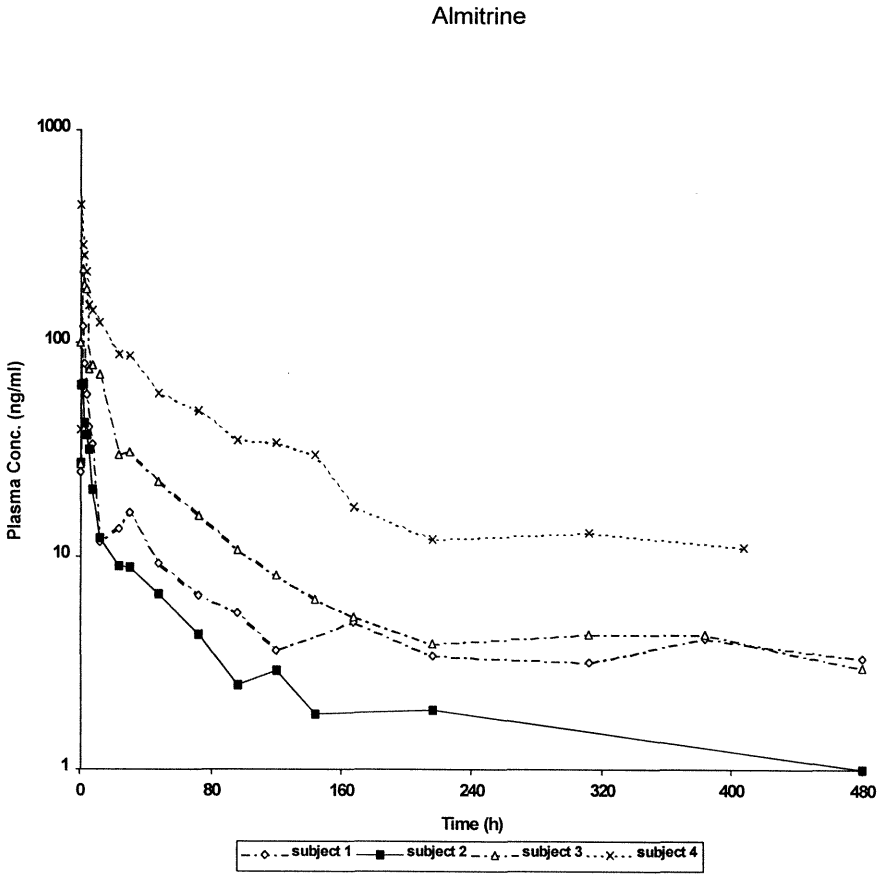


Figure 3.14 : Plasma Profiles of Intact Almitrine in Subjects 1 to 4 Following Single Oral Administration of [^{14}C]-Almitrine Bismesylate at 100mg



3.3.3.2.3 The elimination of radioactivity into urine and faeces

3.3.3.2.3.1 *Urinary elimination*

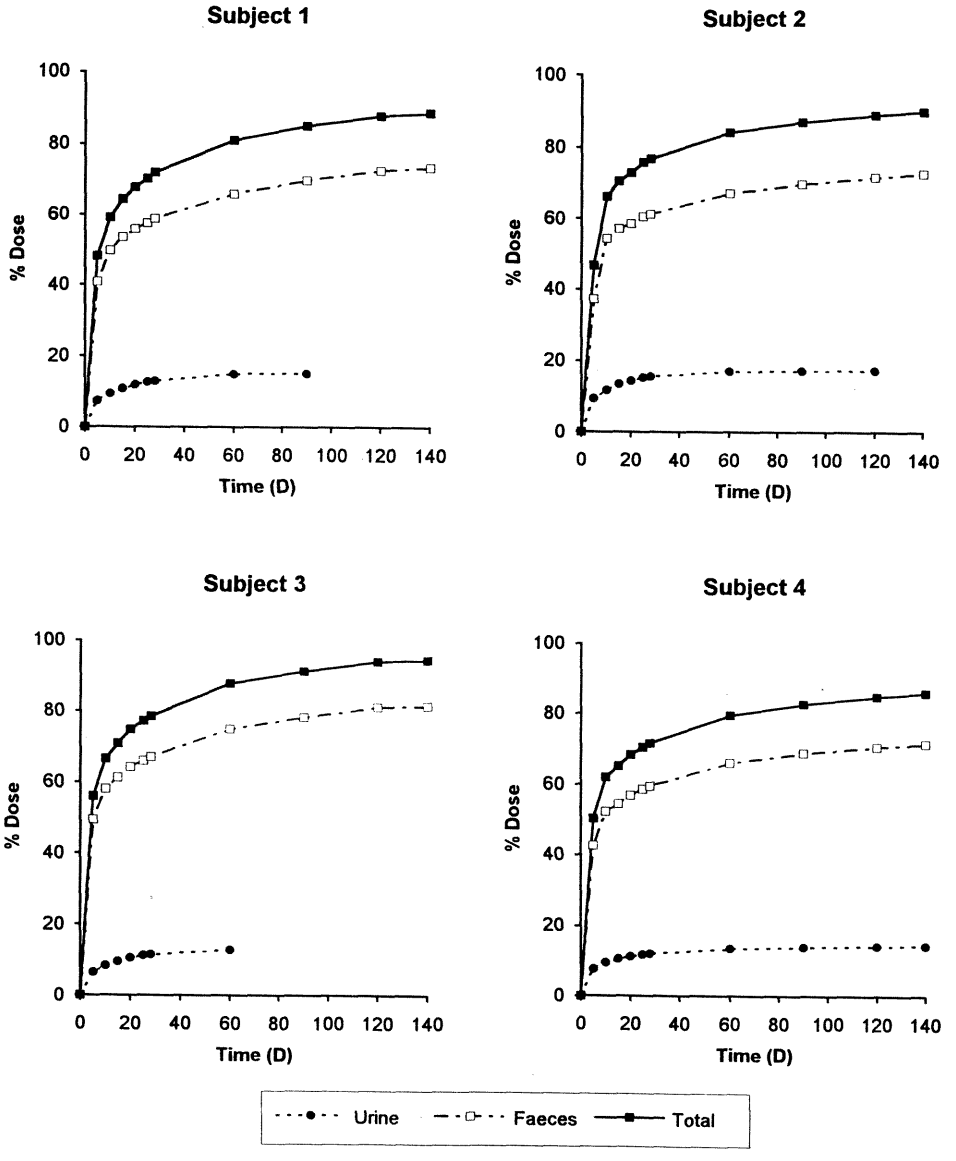
Following an initial 3.6 to 4.7% of the administered radioactivity being eliminated in the first 24 hours, daily excretion of radiolabelled material into urine was slow and low for all subjects (Appendix 3.3.3D). The rate and extent of elimination was similar to that reported earlier (Section 3.1, Appendix 3.2B) with 7 to 10% of the dose eliminated within 7 days and 9 to 13% after 14 days. Subject 1 eliminated 15.2% of the administered dose into urine in 69 days; Subject 2 accounted for 17.36% in 105 days; 12.93% of the dose was eliminated by Subject 3 in 63 days and 14.41% of the dose was accounted for in Subject 4's urine in 40 days (Fig. 3.15).

3.3.3.2.3.2 *Faecal elimination*

Faecal elimination was the major route of excretion of radioactivity with 71 to 81% of the administered dose accounted for over the period of collection (Appendix 3.3.3E). The highest single daily value recovered was 10.8%, 17.4%, 22.8% and 14.5% for Subjects 1-4 inclusive occuring on day 3 for Subjects 1 to 3 and day 1 for Subject 4 respectively. The gut mobility pattern would suggest that Subject 4 had used the wrong preweighed sample container on days 1 and 3 but this could not be confirmed. In the first seven days 47 to 55% of the administered dose was eliminated via faeces and by day fourteen, 52 to 61% of the dose was recovered in faeces. These levels were consistent with that reported earlier (Appendix 3.2D) in which 53% of the administered dose was faecally eliminated in the first 14 days. For Subject 1 a total of 73.4% of the administered dose was faecally eliminated over 133 days. Subject 2 eliminated 72.8% of the dose over 135 days and 81.5% and 71.5% were accounted for in Subjects 3 and 4 (Fig. 3.15) over 140 days respectively.

The total cumulative recovery of radioactivity was 88.60%, 90.14%, 94.39% and 85.88% in Subjects 1 to 4 respectively (Appendix 3.3.3F; Fig. 3.15) which considering the practical difficulties of such a drawn out study were exceptable recoveries. Overall, all subjects eliminated radioactivity very slowly suggesting tissue retention of drug derived material.

Figure 3.15 : Excretion Profile of Radioactivity in Male and Female Subjects Following Oral Administration of [^{14}C]-Almitrine Bismesylate (~100mg =30μCi)



The high elimination into faeces would suggest that the drug was poorly absorbed, however, the urinary elimination after oral and intravenous administration is similar and as will be shown in a later chapter, the extent of absorption is between 50 and 90%. The high molecular weight of almitrine and its high lipophilicity are probably the contributary factors for the faecel elimination of the compound and its metabolites through the bile. Gut secretion cannot, however, be discounted. Thus biliary excretion and gut secretion account for the major route of elimination of radioactivity.

3.3.3.2.4 Metabolite profiles

3.3.3.2.4.1 *Plasma*

The 2 hour plasma from Subjects 1 and 2 were extracted by liquid:liquid extraction according to the HPLC procedure and by XAD-2. The residues were subjected to TLC using a solvent system of chloroform:methanol:ammonia (80:20:2). The TLC profiles were similar for both extraction procedures and showed the presence of six radioactive components P1 to P6 (Fig. 3.16). The major bands, P4 and P6, corresponded to dideallyl almitrine and intact almitrine respectively. There was also evidence of monodeally almitrine (P5) and tetrahydroxy almitrine (P2). Traces of monodeallyl dihydroxy almitrine (P3) were also evident. At 2 hours the measured intact almitrine accounted for 53.4, 29.1, 52.9 and 56.3% of the total radioactivity for Subjects 1, 2, 3 and 4 respectively. This would indicate high first pass metabolism with the resultant deallylation of almitrine to the dideallyl product as the major metabolic component in plasma.

3.3.3.2.4.2 *Urine*

Pooled 0-24 hour composite urine samples of Subjects 1 and 2 and 0-12 hour of Subjects 3 and 4 were extracted on C_{18} 100mg Bond Elut cartridges which were previously solvated with methanol (1ml) and water (1ml) prior to loading an aliquot (2ml) of the composite urine sample. After washing with water (1ml), radioactivity was eluted with methanol (2ml) and the methanolic eluates were evaporated to dryness under oxygen-free nitrogen and the residue resuspended in a smaller volume (100µl) of methanol.

A larger aliquot (5ml) of each composite urine sample was hydroxylsed with β-glucuronic acid at 37°C for 16 hours prior to solid phase extraction on 200mg C_{18} Bond Elut cartridges as described above.

Table 3.8 : Human Urinary Metabolite Profiles Before and After Enzyme Hydrolysis Following Administration of [^{14}C]-Almitrine Bismesylate (100mg; 30μCi)

SUBJECT	U1	U2	U3	U4	U1H
1 (Control)	71 (2.6)	3 (0.1)	3 (0.1)	8 (0.3)	ND
1 (Hydrolysed)	5 (0.2)	<3	<3	<3	45 (1.6)
2 (Control)	76 (3.6)	4 (0.2)	<3	7 (0.3)	ND
2 (Hydrolysed)	7 (0.3)	3 (0.1)	<3	7 (0.3)	65 (3.1)
3 (Control)	73 (2.4)	4 (0.1)	<3	<3	ND
3 (Hydrolysed)	4 (0.1)	5 (0.2)	<3	<3	75 (2.5)
4 (Control)	81 (2.2)	5 (0.1)	<3	<3	ND
4 (Hydrolysed)	5 (0.1)	3 (0.1)	<3	<3	70 (1.9)

NB
Subjects 1 and 2 are 0-24 hour pools
Subjects 3 and 4 are 0-12 hour pools

ND denotes Not Detected
<3 denotes <3% of total activity on chromatogram
( ) represents % dose

Figure 3.17 shows a representative TLC (chloroform:methanol:ammonia 80:20:2 v/v/v) profile for urine before and after enzyme hydrolysis. Four metabolites (U1 to U4) were detected and quantified where possible, in the unhydrolysed urine of all subjects with U1 as the major component

representing 71 to 81% of the activity on the chromatogram and 2.2 to 3.6% of the administered dose (Table 3.8). Following enzyme hydrolysis, only U1 showed significant change to a new metabolite U1H representing between 45 and 75% of the radioactivity in the chromatogram with between 4 and 7% not hydrolysed by the β-glucuronidase. All other metabolites (U2 to U4) remained relatively unchanged, However, the hydrolysed product represented 1.6 to 3.1% of the administered dose. Because of the small amount of radioactivity applied to the plates after hydrolysis, there is some error in the absolute value of U1H for some subjects. It was evident from the chromatograms and autoradiograms, however, that this component was the predominant metabolite origination from U1 (see Chapter 4 for identification). There were trace amounts of what may be the tetrahydroxy almitrine metabolite (U3). None of the other products, including U1H ran at R_f's similar to the other reference compound. U1H had an R_f between that of the monodeallyl and dideallyl almitrine.

3.3.3.2.4.3 *Faeces*

Faeces from day 3 of Subjects 1-3 and day 1 of Subject 4 (the times of highest radioactivity elimination) were selected for metabolite profiling. Approximately 2g of faecal homogenate was freeze dried for 2 hours and the lyophillate extracted with methanol prior to thin layer chromatography. Day 7 samples were also extracted but the viscosity of these samples and that of Subject 3, day 3 was such that the chromatograms were not quantifiable.

Table 3.9 : Human Faecal Metabolite Profiles Following Administration of Almitrine Bismesylate (100mg; 30μCi)

	F1	F2	F3	F4	F5	F6	Almitrine F7
SUBJECT 1 DAY 3	<3	<3	8 (0.9)	<3	<3	3 (0.3)	80 (8.6)
SUBJECT 2 DAY 3	6 (1.0)	6 (1.0)	33 (5.7)	8 (1.4)	8 (1.4)	4 (0.7)	22 (3.8)
SUBJECT 4 DAY 1	8 (1.2)	13 (1.9)	38 (5.6)	6 (0.9)	6 (0.9)	3 (0.4)	15 (2.2)

<3 = less than 3% of total activity on chromatogram

( ) denotes % dose

For Subjects 1, 2 and 4 at least 7 quantifiable metabolites F1-F7 were present (Table 3.9; Fig. 3.16). Although the profiles were qualitatively similar, the major component for Subject 1 was F7 which had an R_f similar to almitrine, and represented 80% of the radioactivity on the chromatogram, equivalent to 8.6% of the administered dose. For Subjects 2 and 4 intact almitrine represented 22 and 15% respectively of the chromatogram and was equivalent to 3.8 and 2.2% respectively of the administered dose. The major faecal metabolite was F3, which co-eluted with tetrahydroxy almitrine and was the major faecal component for Subjects 2 and 4 representing 33 and 38% respectively of the chromatogram and this component was equivalent to 5.7 and 5.6% respectively of the administered dose. For Subject 1 tetrahydroxy almitrine (F3) was the second major component and represented only 8% of the chromatogram and equivalent to 0.9% of the administered dose. From Fig 3.16, the majority of the circulating metabolites (P2 to P6) had R_f's similar to those observed in faeces (F3 to F7). P6 and F7 had R_f's similar to intact almitrine whilst F3 and P2 represented tetrahydroxy almitrine. F6 and P5 co-chromatographed with monodeallyl almitrine whilst F5 and P4 were similar to dideallyl almitrine. Dihydroxy almitrine co-eluted with F4 and P3 but two other faecal metabolites F1 and F2 were not detectable in plasma. With the exception of F3 and F6 which represented 8 and 3% of the chromatogram in Subject 1, none of the other metabolites were quantifiable in this subject. For Subjects 2 and 4 these metabolites occurred in varying quantities and ranged from as low as 3% for F6 to 13% for F2. The products F1 and F2 ran close to the origin in this solvent system and are probably conjugates of the other metabolites which ran further up the chromatogram.

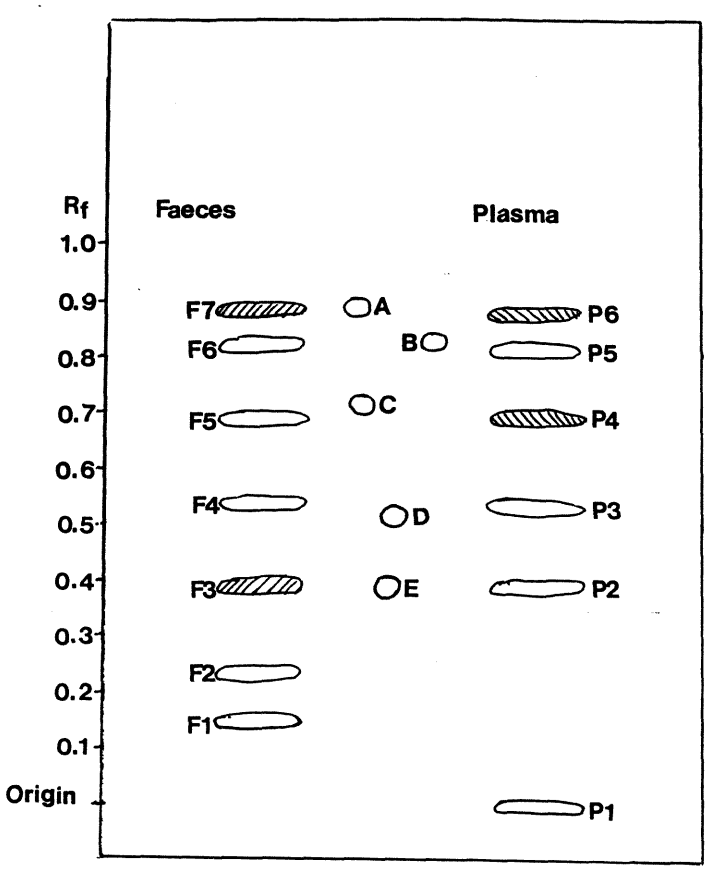
The product P1 which appears at the origin on the plasma chromatogram is probably U1, the major component in urine, but which is less important in faeces.

3.3.3.3 Discussion

The study has shown that the terminal half-lives of intact almitrine is long. Values of between 407 and 1785 hours (17 to 74 days) have been observed in this study and have been shown to be dependent on the ability to sample and measure for a long time. Certainly sensitive analytical techniques are

important for accurately defining the pharmacokinetics of compounds such as almitrine. Good study design with adequate sampling time has been shown to be also important for studying such compounds demonstrating long half-lives/low clearances. Even after collection for 140 days the total balance (89.75 ± 3.56%) was less than the originally anticipated 100 ± 10%. With a half-life of radioactivity of 1163 hours (48 days) on average, if it is assumed that it takes five half-lives for the administered product to be completely eliminated, sample collection would probably have to be continued up to 242 days after administration.

Figure 3.16 : Human Plasma and Faecal Metabolite Profile - A Diagrammatic Representation

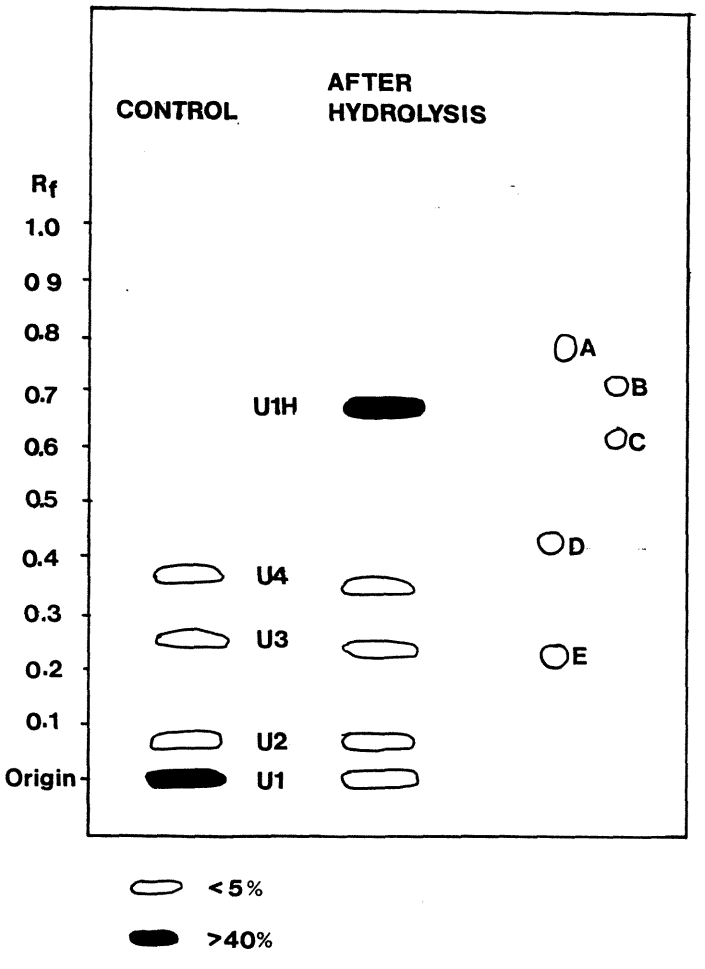


A = Almitrine B=Monodeallyl almitrine

C=Dideallyl almitrine D= Monodeallyl dihydroxy almitrine

E =Tetrahydroxy almitrine

Figure 3.17 : Human Urinary Metabolite Profiles Before and After Enzyme Hydrolysis - A Diagrammatic Representation



A=Almitrine B=Monodeallyl almitrine

C=Dideallyl almitrine D=Monodeallyl dihydroxy almitrine

E=Tetrahydroxy almitrine

In summary, following oral administration of [^{14}C]-almitrine bismesylate radioactivity is rapidly absorbed. The average blood to plasma ratio of radioactivity indicates that the circulating products remain predominantly in the plasma fraction and the pharmacokinetic parameters of both radioactivity and intact almitrine show a long half-life with a low clearance due possibly to a large volume of distribution. Faecal elimination is the major route of excretion of drug derived material with a maximum of 94% of radioactivity accounted for over a 135 day collection period. This study highlights the difficulties in undertaking balance studies with drugs with extremely long half-lives.

Almitrine is extensively metabolised with hydroxylation of the allyl side chain and subsequent deallylation as the possible route of metabolism. The large difference in AUC between intact almitrine and total radioactivity suggest a high first pass metabolism with a single major component eliminated into urine as a glucuronide conjugate, although this product represents less than 4% of the administered dose.

CHAPTER 4

THE METABOLISM OF ALMITRINE

This chapter summarises the results of a series of studies which investigated the *in-vivo* metabolism of almitrine after intravenous and/or oral administration to human, dog, rabbit, rat and mouse. The *in-vitro* metabolism of almitrine was also investigated using liver microsomal preparations from human, monkey, dog and rat to compare with *in-vivo* metabolism. The isolation and identification, using GC-MS analysis, of the major faecal components (almitrine and metabolites) in human and rat, the major plasma and urinary metabolite in human as well as the identity of the *in-vitro* metabolites using LC-MS-MS are outlined. The *in-vitro* study was performed with the assistance of the Metabolism Department. All *in-vivo* studies were performed by me with assistance in metabolite structural analysis from the Metabolism Department. Plasma analysis for almitrine and metabolites from clinical studies was performed under my supervision.

During the early development of almitrine, *in-vitro* techniques were less developed and all metabolic studies on the compound were performed using the conventional thin layer chromatographic separation of sample extracts obtained from *in-vivo* studies. Identification was then followed by GC-MS analysis of isolated material and this required large quantities of the products of interest. More recently, with the development and perfection of *in-vitro* techniques to study drug metabolism, coupled with the introduction of LC-MS and LC-MS-MS, *in-vitro* metabolism studies are performed much earlier in drug research and development. To fall in line with the modern approach, *in-vitro* metabolism is presented first and this is followed by the *in-vivo* metabolism of almitrine in the many species, including human.

4.1 *In-Vitro* Species Comparison of the Metabolism of [^{14}C]-Almitrine Bismesylate

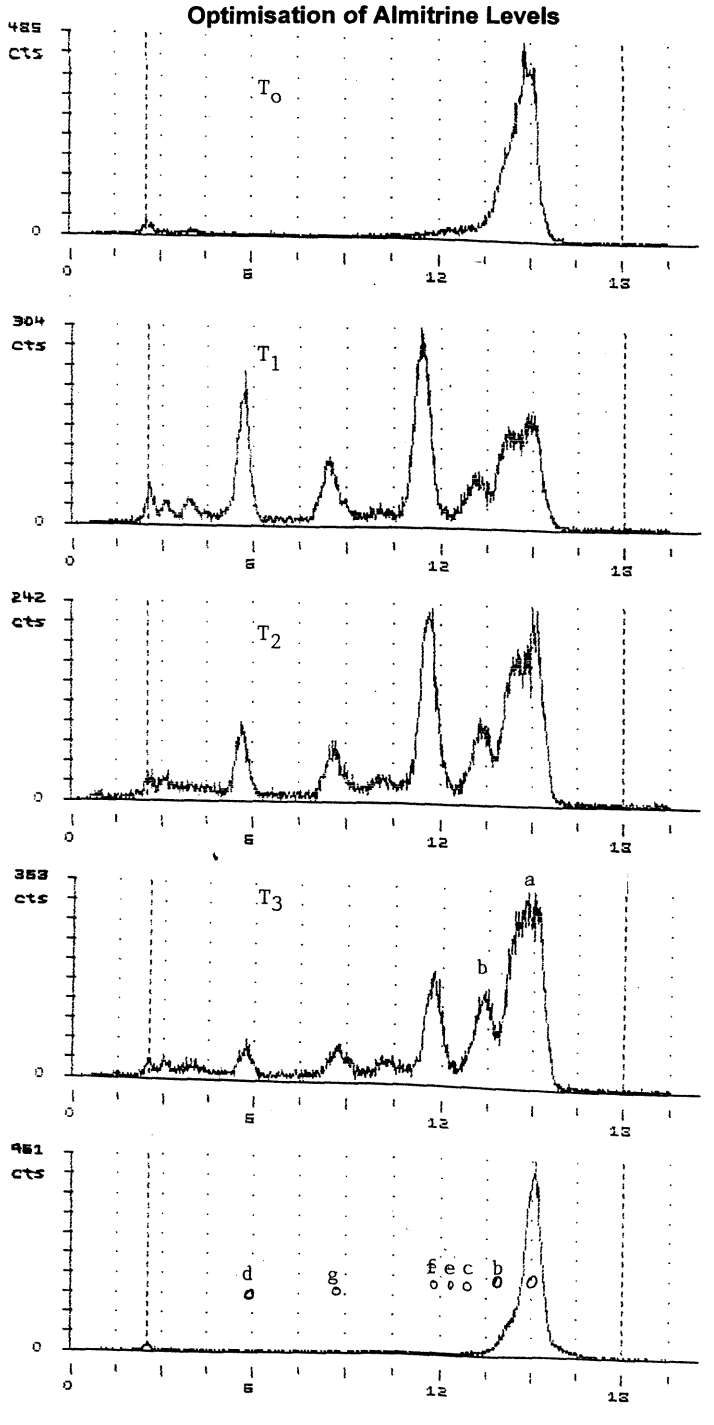
In-vitro metabolism was investigated in liver microsomal fractions of rat, dog, monkey (Cynomolgus) and man. Although no *in-vivo* metabolism of almitrine in the monkey was available, it was thought appropriate to use this opportunity to compare the metabolism of almitrine in man with that of another primate.

Following optimisation (Fig. 4.1) approximately 0.4μCi of [^{14}C]-almitrine bismesylate (equivalent to 0.02μmoles or 13.4μg) was incubated in a total

Table 4.1 : Interspecies Comparison of Metabolism of [^{14}C]-Almitrine Using Microsomes (Sample Details)

SAMPLE/ SPECIES	PROTEIN CONC ($mg.ml^{-1}$)	VOLUME FOR 2mg (µl)	DPM/40µl	$DPM.ml^{-1}$	TOTAL		
					DPM (2ml)	µCi REC	% REC
Rat Test 1	12.68	158	11266	281638	563276	0.23	62.5
Rat Test 2	12.68	158	9986	249647	499274	0.22	55.0
Rat Test 3	12.68	158	10196	254910	509820	0.23	57.5
Dog Test 1	6.70	299	8462	211554	423108	0.19	47.5
Dog Test 2	6.70	299	8481	212022	424044	0.19	47.5
Dog Test 3	6.70	299	10131	253283	506766	0.23	57.5
Monkey Test 1	11.03	181	10318	257958	515916	0.23	57.5
Monkey Test 2	11.03	181	11293	282335	564670	0.25	62.5
Monkey Test 3	11.03	181	9695	242383	484766	0.22	55.0
Human Test 1	20.00	100	9906	247653	495306	0.22	55.0
Human Test 2	20.00	100	10986	274655	549310	0.25	62.5
Human Test 3	20.00	100	10131	253285	506570	0.23	57.5

Figure 4.1 : Thin Layer Chromatograms of Microsomal Extracts for Optimisation of Almitrine Levels



T_o

Test 1
(Low Dose)

Test 2
(Medium Dose)

Test 3
(High Dose)

Control
(Medium Dose)

volume of 1.5ml of cofactor and tris buffer as previously described (Chapter 2). Incubation was performed for 3 hours using triplicate samples for each species. Incubation was stopped by the addition of 0.5ml acetonitrile and the recovery of radioactivity from the preparation estimated as described in Section 2.2.5 of Chapter 2, using a 40µl count. Table 4.1 summarises the details. The same quantity of radioactivity from the test sample of each species was applied to TLC and developed in solvent system 1 (chloroform:methanol:ammonia, 80:20:2 v/v/v).

4.1.1 Results

Figure 4.2 shows a comparative profile of almitrine metabolism by liver microsomes in the various species. Human and rat showed the most extensive metabolism with the monkey metabolising almitrine to a greater extent than the dog. Almitrine was almost completely metabolised in human and rat with quantitatively very little unchanged drug remaining after the 3 hour incubation. At least five major components were separated on TLC. Using the reference compounds detailed in Fig. 2.1, reference compounds 'a', 'b', 'd' and 'g' corresponding to almitrine, monodeallyl almitrine, tetrahydroxy almitrine and monodeallyl dihydroxy almitrine respectively were well resolved.

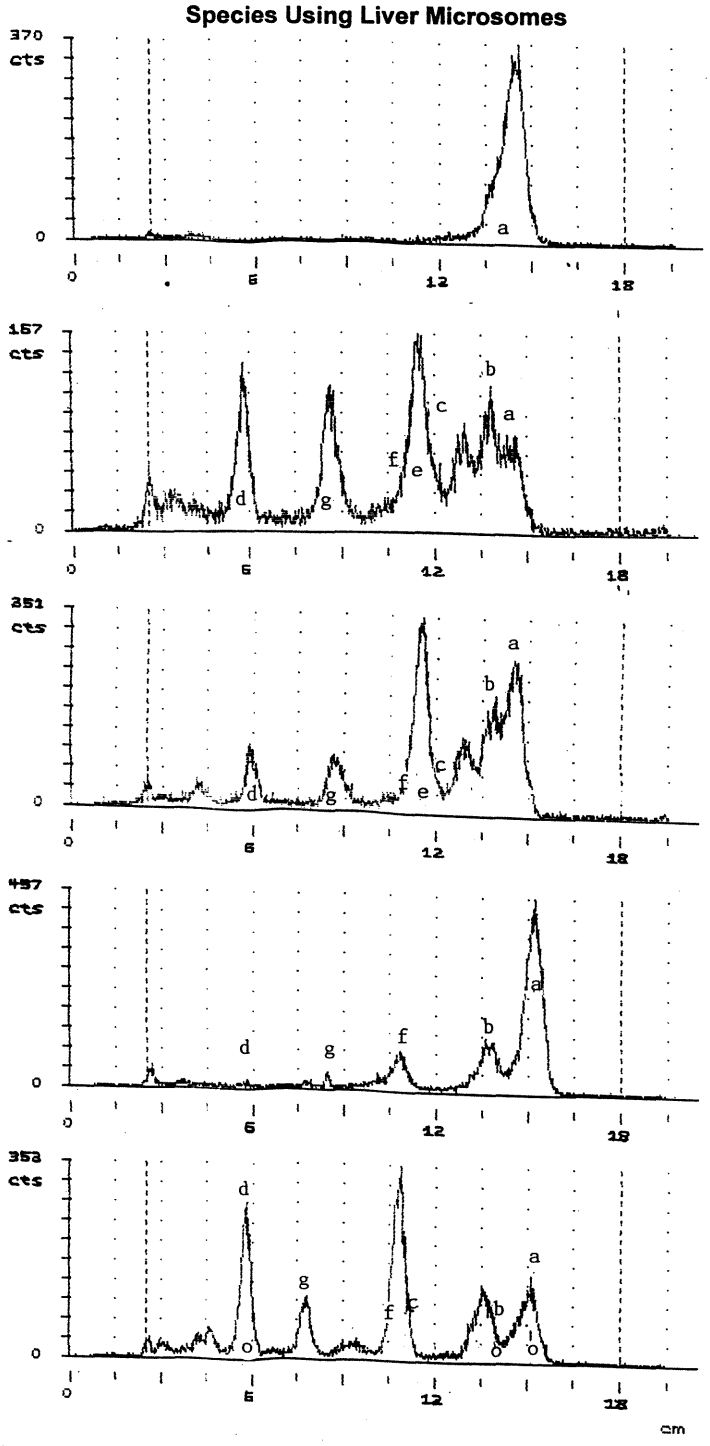
Reference compounds 'c', 'e' and 'f' (dideallyl almitrine, dihydroxy almitrine and detriazinyl almitrine respectively) ran very close and were almost unresolved.

In the dog, almitrine (a) was the predominant component with little metabolism. Monodeallyl almitrine (b) and trace amounts of the monodeallyl dihydroxy almitrine (g) were also evident and the only other detectable component was in the region in which the three unresolved reference compounds (c, e and f) co-eluted. The tetrahydroxy product (d) was not evident in this species.

The rat showed two major components (d and c/e/f) with three smaller compounds corresponding to 'a', 'b' and 'g' (almitrine, monodeallyl almitrine and monodeallyl dihydroxy almitrine).

The monkey and human profiles were qualitatively similar, with six major components. In the monkey, the major component was the c/e/f cluster, with almitrine (a), monodeallyl almitrine (b), monodeallyl dihydroxy almitrine (g) and tetrahydroxy almitrine (d) appearing in almost equal quantities, although almitrine may have been present at a higher level. There was an extra

Figure 4.2 : Comparative Profiles of the Metabolism of Almitrine in the Various Species Using Liver Microsomes



component between (b) and the c/e/f cluster which did not co-elute with any of the other reference components.

The same unknown component was seen in the human profile in which three major components corresponding to d, g and the c/e/f/ cluster predominated. Almitrine (a) was almost completely metabolised with component (b) appearing in slightly greater amounts. Overall all species, with the exception of dog, showed extensive metabolism of [^{14}C]-almitrine bismesylate in liver microsomal fractions.

4.2 *In-Vivo* Species Comparison of the Metabolism of Almitrine Following Single Intravenous and Oral Administration of [^{14}C]-Almitrine Bismesylate

In all species, the faecal route was shown to be the major route of elimination of radioactivity (Chapter 3) irrespective of the route of administration, with 60-90% of dose eliminated via this route.

Faeces

Faecal metabolite profiles were compared in man, dog, rabbit, rat and mouse in the 24 hour sample containing the highest daily elimination of radioactivity (Table 4.2). Faeces (1-5g) from each species (male) were freeze-dried and the radioactivity was extracted with hot methanol as described in Chapter 2. Rabbit faeces did not require freeze-drying and were finely ground prior to extraction. The extracts were applied to TLC plates and developed, along with reference compounds (Fig. 2.1) in Solvent System 1 (chloroform:methanol:ammonia 80:20:2 v/v/v). The plates were exposed to X-ray film for 3 weeks to locate the radioactive areas and these radioactive areas were quantitated by scraping and counting as described in Chapter 2.

In man, samples from day 1-7 and 8-14 were pooled and also extracted to observe any differences in the elimination pattern of radioactivity over these periods. Two dimentional chromatography of these and also the 24-48 hour sample was performed for comparison. (The plates were developed in Solvent System 1 then on drying turned 90° and redeveloped in Solvent System 2; ethyl acetate:methanol:acetic acid 80:20:2 v/v/v).

Table 4.2 : Details of the Faecal Samples which were used to study the Metabolism of [^{14}C]-Almitrine Bismesylate

SPECIES	I.V.			P.O.		
	Dosage ($mg.kg^{-1}$)	% (1)	Time (2) (h)	Dosage ($mg.kg^{-1}$)	% (1)	Time (2) (h)
HUMAN	0.19	8.0	24-48	1.33	13.6	24-48
DOG	0.66	23.6	0-24	0.54	65.3	0-24
RABBIT	0.53	29.2	0-24	0.83	58.0	0-24
RAT	2.71	42.8	0-24	2.09	47.4	0-24
MOUSE	3.80	8.6	0-48	-	-	-

(1) Percentage of administered drug which was extracted from faecal sample to examine metabolic pattern.

(2) Time over which this sample was collected. This represents the time of maximal elimination of radioactivity.

Plasma and Urine

Plasma metabolite profiles were established in human and rat after single oral administration of [^{14}C]-almitrine bismesylate at 200mg and 100mg.kg^{-1} respectively, as part of the study for the isolation and identification of the metabolites of almitrine (see Section 4.3). Samples were extracted using Amberlite XAD_2 and chromatographed in Solvent System 1.

For urine the metabolite profiles of the 0-24 hour samples were established before and after hydrolysis. Samples were extracted using Amberlite XAD_2.

For both species, a comparative profile of plasma, urine and faeces was produced (Figs. 4.8 (human) and 4.9 (rat)).

4.2.1 Results

4.2.1.1 Faeces

Intravenous administration

Figure 4.3 shows a representation of a one dimensional autoradiographic metabolic comparison between the species after intravenous administration at 0.19mg.kg^{-1} (human), 0.66mg.kg^{-1} (dog), 0.53mg.kg^{-1} (rabbit), 2.71mg.kg^{-1} (rat) and 3.8mg.kg^{-1} (mouse). Extensive metabolism was observed in all species.

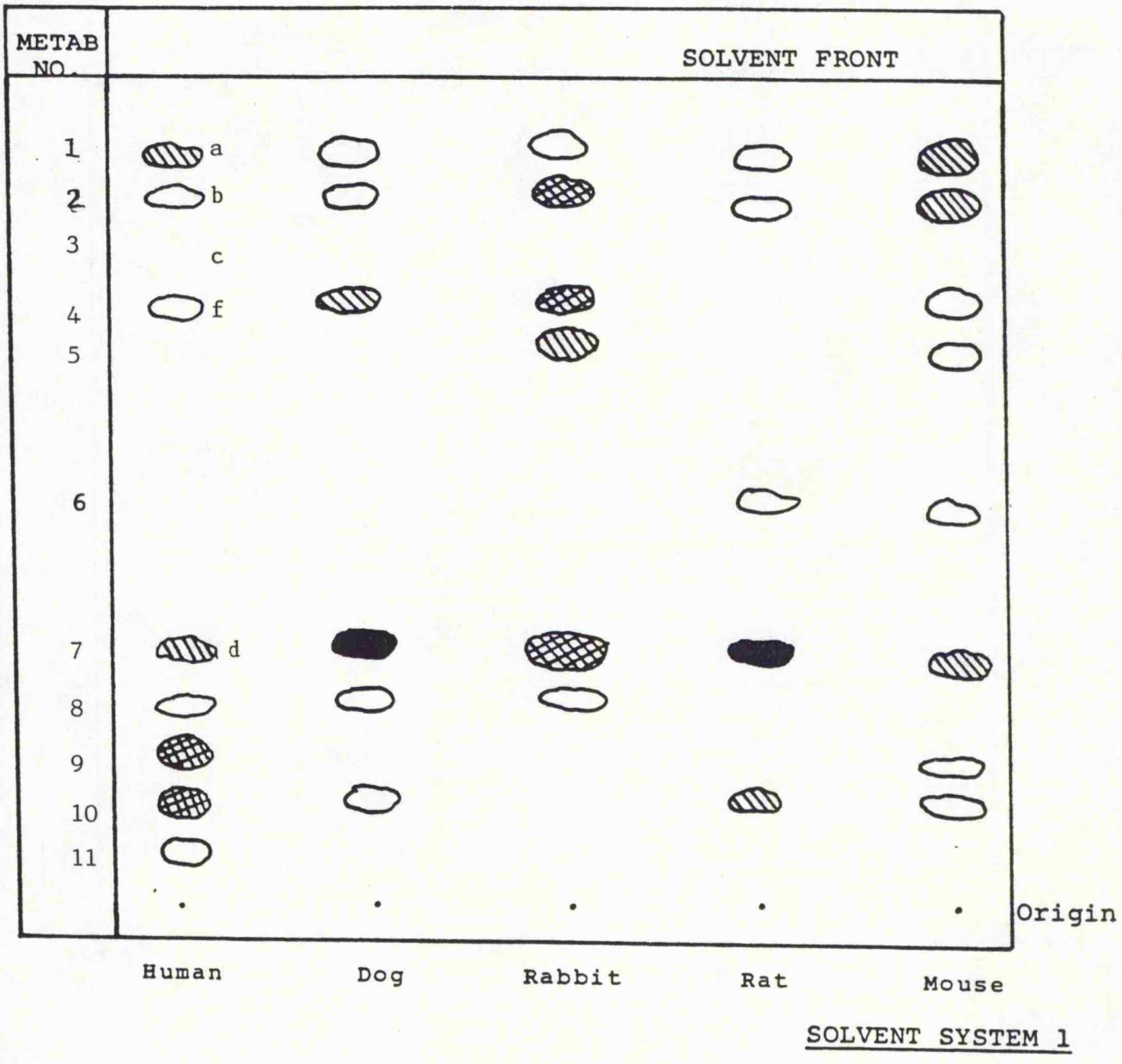
Table 4.3 gives the quantities of metabolites in faeces expressed as a percentage of the sample extracted.

For clarity, only metabolites representing greater than 5% of the total activity have been included. Several other metabolites were observed, but no single metabolite represented more than 2% or 3% of the total.

Figure 4.4 shows a histogram comparison of the metabolite patterns in the species.

Unchanged almitrine (reference compound 'a') was found in all species examined in approximately similar amounts, with levels ranging from 5% in the rat, to 13% in the mouse, with 7% in dog and rabbit and 11% in man. The major metabolite (No. 7), eliminated in the 0-24 hour sample of rat, dog, and rabbit and the 0-48 hour sample of the mouse, corresponded to

Figure 4.3 : Representation of Autoradiograms of Thin Layer Chromatography Showing Species Comparison of Faecal Metabolites (Intravenous Dosing)



KEY:

>30%	-	filled spots
15-30%	-	double cross hatched
10-15%	-	single cross hatched
5-10%	-	open spots

Table 4.3 : Species Comparison of the Percentages of each Metabolite Found in Faeces After Intravenous Administration of [^{14}C]- Almitrine Bismesylate

MET NO.	REF COMP	R_f	HUMAN	DOG	RABBIT	RAT	MOUSE
1	a*	0.90	11	7	7	5	13
2	b	0.86	5	6	16	8	11
3	c	0.76	-	-	-	-	-
4	f	0.70	8	12	16	-	6
5		0.58	-	-	10	-	5
6		0.45	-	-	-	7	8
7	d	0.28	10	31	22	37	14
8		0.24	5	5	6	-	-
9		0.16	21	-	-	-	8
10		0.10	25	5	-	13	9
11		0.04	6	-	-	-	-

Only discrete metabolites of greater than 5% of the total extracted are given.

Solvent System 1.

* = Almitrine

Figure 4.4 : Histogram Representations of the Major Metabolites of [^{14}C]-Almitrine Bismesylate (>5% of Activity in Sample) seen in a Methanolic Extract of Human, Dog, Rabbit, Rat and Mouse Faeces after Separation by Thin Layer Chromatography (Intravenous Dosing) - Chromatography in Solvent System 1.

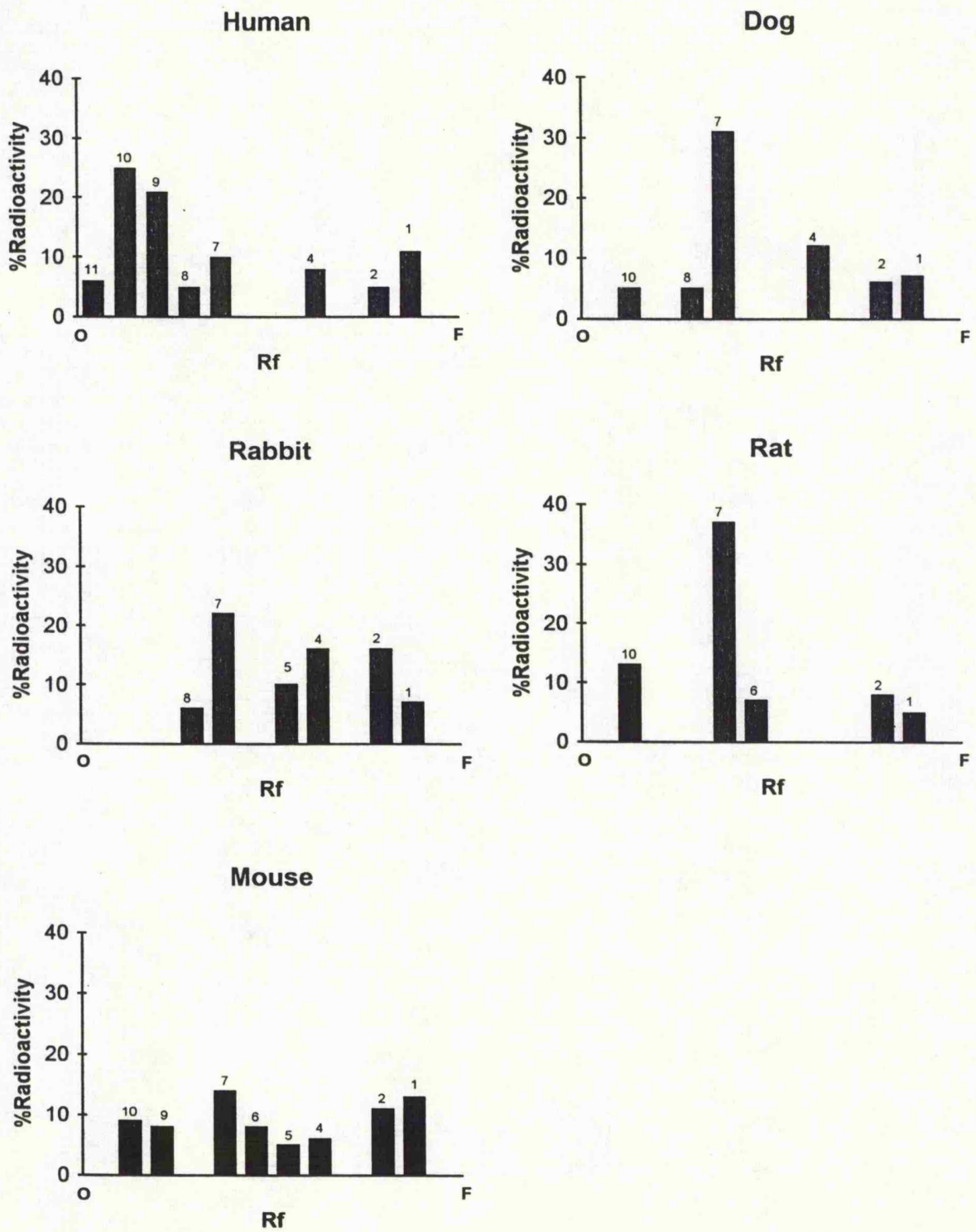
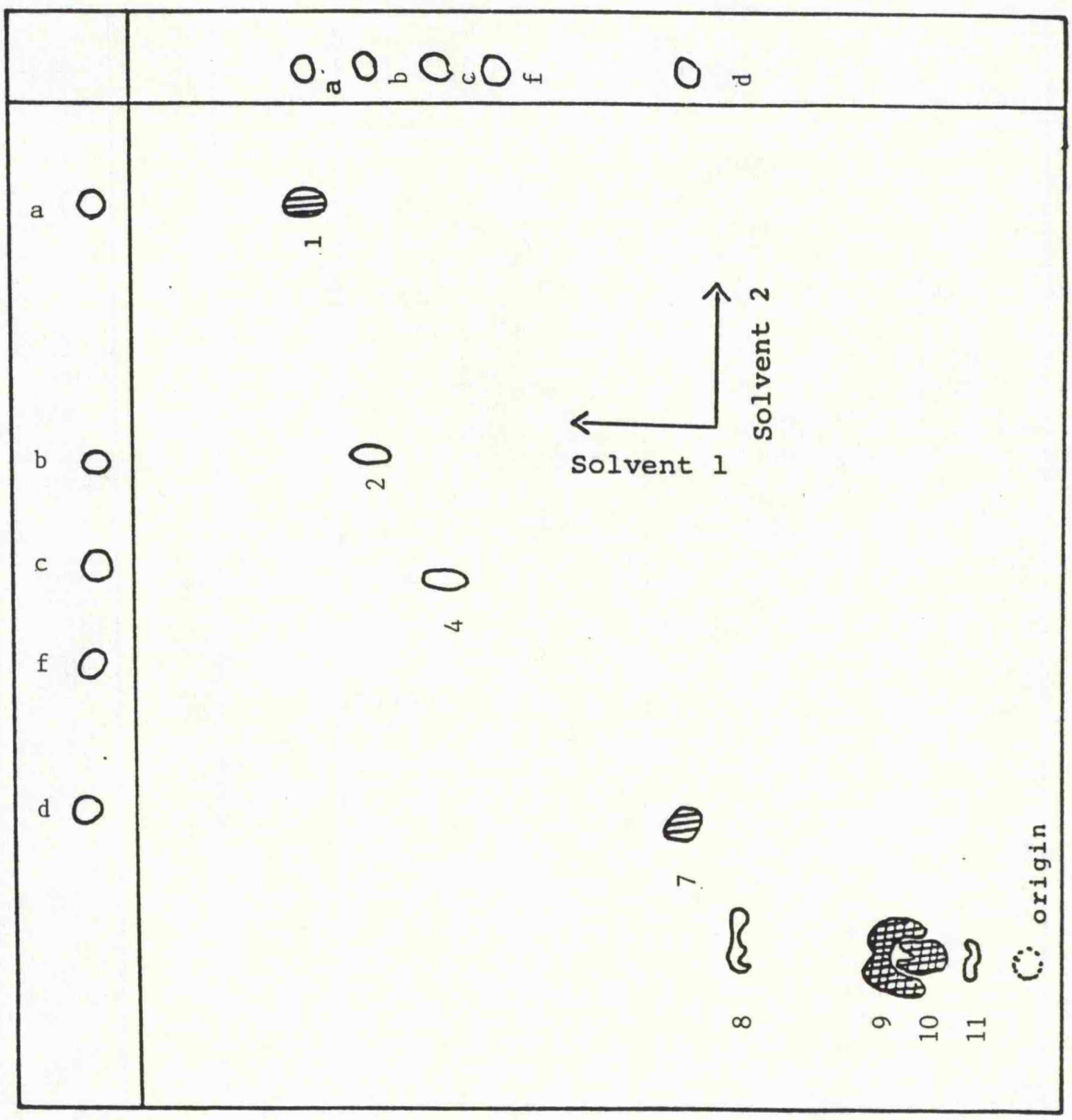


Figure 4.5 : An Autoradiograph of a Two Dimensional Chromatogram of a Methanolic Extract of Human Faeces (24-48 Hour Sample) Eluted With Solvent 1 Followed by Solvent 2 (Intravenous Dosing)



reference compound 'd', the tetrahydroxy derivative of almitrine, and represented 37%, 31%, 22% and 14% of total activity respectively. In man this metabolite was less important, representing only 10% of the 24-48 hour faecal sample. Two major metabolites in man (Nos 9 and 10), represented 21% and 25% respectively, appeared to be polar in nature since they did not run far from the origin. They did not co-run with any of the reference compounds used. One or both of these polar metabolites was found to a lesser extent in the other species except for the rabbit which did not eliminate either of these compounds.

Other important metabolites, Nos 4 and 2, were found in most of the species and co-ran either with reference compound 'b', the monodeallyl derivative, or 'f', the detriazinyl derivative. The rat appeared to be different from the other species, as it did not eliminate metabolite 'f', but instead eliminated larger quantities of the tetrahydroxy derivative metabolite 'd'.

Figure 4.5 shows a representation of an autoradiograph of a two dimensional chromatogram of the human faecal sample. It indicates that in solvent 1, all the spots seen including metabolites 9 and 10, were discrete metabolites and not a mixture, since they remained as single spots after chromatography in the second system. The profiles also indicated no instability on the TLC plate.

Oral administration

Figure 4.6 shows a representation of a one dimentional autoradiographic metabolic comparison after oral administration at 1.33mg.kg^{-1} (human), 0.54mg.kg^{-1} (dog), 0.83mg.kg^{-1} (rabbit) and 2.09mg.kg^{-1} (rat).

Table 4.4 gives a quantitative comparison of the metabolites eliminated in faeces following oral administration. The mouse was not studied using this route of administration since a very limited number of toxicology studies involving oral administration have been undertaken with this species.

Figure 4.7 shows a chromatographic histogram of the percentage of each metabolite eliminated in the faeces for each of the species.

Oral administration gave quantitatively different metabolic profiles to that observed after intravenous dosing. The most important difference can be seen in the rabbit and dog, which eliminated unchanged almitrine as the

Table 4.4 : Species Comparison of the Percentages of each Metabolite Found in the Faeces After Oral Administration of [^{14}C]-Almitrine Bismesylate

MET NO.	REF COMP	R_f	HUMAN	DOG	RABBIT	RAT
1	a*	0.90	16	35	75	21
2	b	0.86	-	6	5	7
3	c	0.76	-	5	-	8
4	f	0.70	7	-	5	-
5		0.58	-	-	-	-
6		0.45	-	-	-	5
7	d	0.28	21	27	5	23
8		0.24	-	-	-	-
9		0.16	10	-	-	5
10		0.10	9	-	-	10
11		0.04	7	-	-	-
12		0.93	10	-	-	6

Only discrete metabolites of greater than 5% of the total extracted are given.

Solvent System 1.

* = Almitrine

Figure 4.7 : Histogram Representations of the Major Metabolites of [^{14}C]-Almitrine Bismesylate (>5% of Activity in Sample) seen in a Methanolic Extract of Human, Dog, Rabbit and Rat Faeces After Separation by Thin Layer Chromatography (Oral Dosing) - Chromatography in Solvent System 1

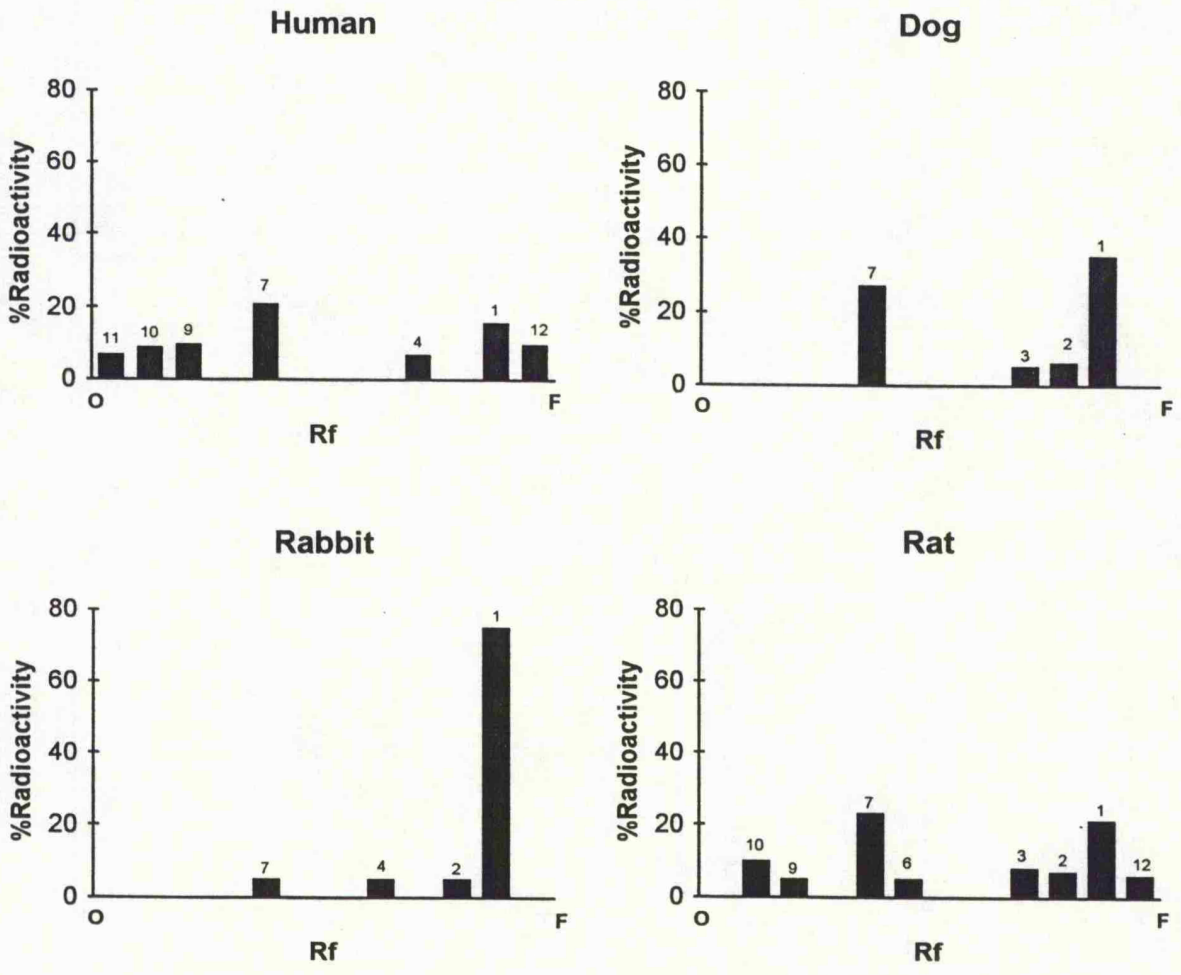
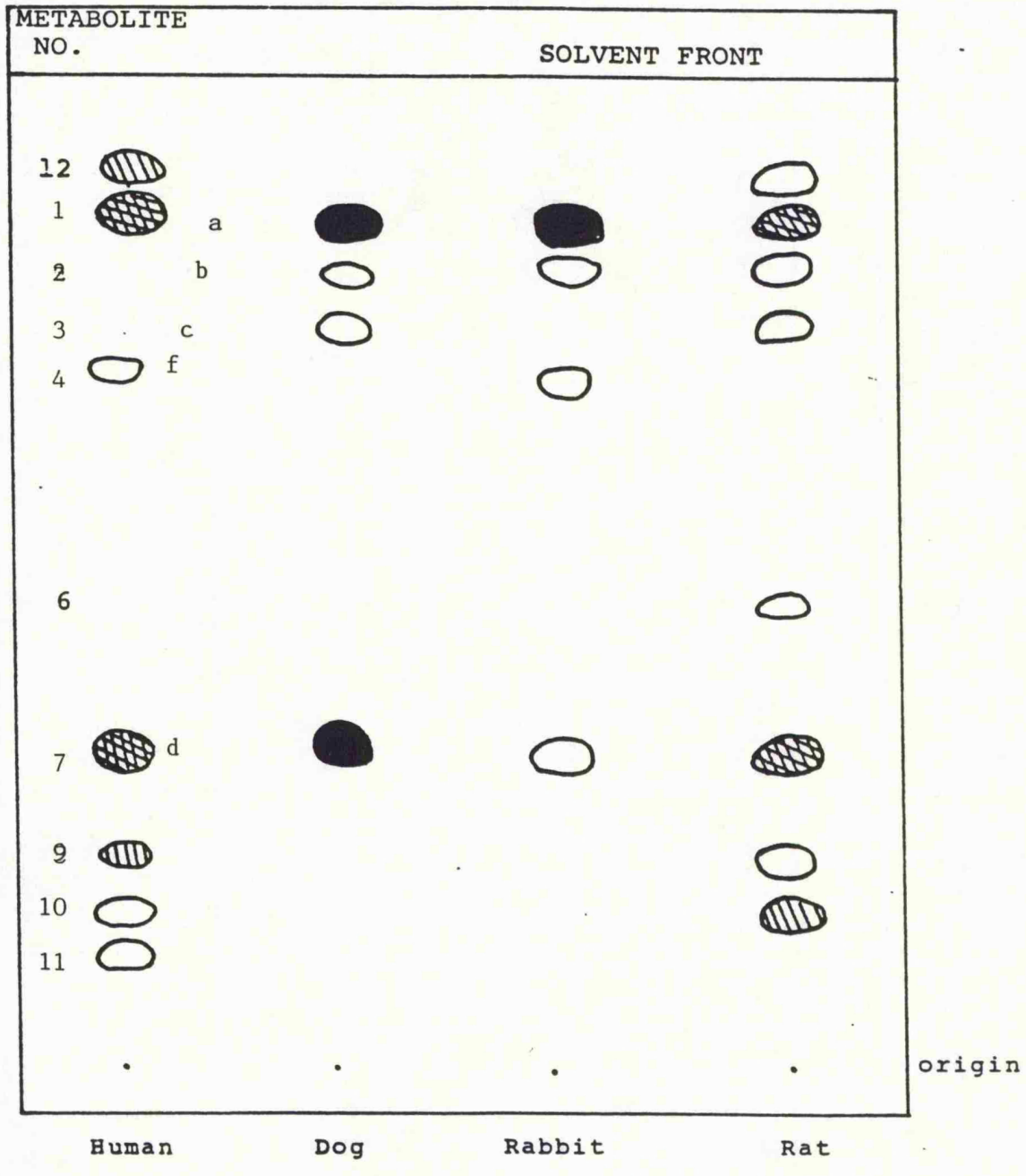


Figure 4.6 : Representations of Autoradiograms of Thin Layer Chromatography Showing Species Comparison of Faecal Metabolites (Oral Dosing) Solvent System 1



KEY:

>30%	-	filled spots
15-30%	-	double cross hatched
10-15%	-	single cross hatched
5-10%	-	open spots

major component, 75% and 35% respectively in the 0-24 hour sample; whilst the elimination of almitrine in the rat and human (0-24 hour and 24-48 hour respectively) was approximately the same as that found after intravenous administration.

The other difference between the two routes of administration was that the elimination of the tetrahydroxy derivative became more important in man (21%), slightly less in the rat and dog (27% and 23% respectively), and considerably less in the rabbit (only 5%).

Most of the other metabolites observed after intravenous administration were also detected, but to a lesser extent after oral dosing with the exception of the component in the region of the detriazinyl metabolite.

In rat and man an additional metabolite (No. 12) representing 10 and 6% respectively was found to run ahead of almitrine.

4.2.1.2 Plasma

Human

Three major components were seen in plasma after a single oral administration of 200mg. Metabolite P1 co-chromatographed with almitrine (reference compound 'a') and represented 70% of the radioactivity. Metabolite P2 co-chromatographed with monodeallyl almitrine (reference compound 'b'). Metabolite P3 chromatographed in the region of reference compound 'e', dihydroxy almitrine.

Rat

In the rat, six principle metabolites were present (Fig. 4.9).

Metabolites P4, P3 and P1 each represented approximately 25% of the total radioactivity in the 5 hour plasma samples. P1 corresponded to unchanged almitrine, P3 chromatographed in the region of reference compounds 'c', 'e' and 'f', (dideallyl, dihydroxy and detriazinyl almitrine) whilst P4 was similar to reference compound 'g' (monodeallyl dihydroxy almitrine) in its chromatographic behaviour. Metabolite P2, which represented about 10% of the radioactivity, chromatographed in a similar manner to reference compound 'b' (monodeallyl almitrine), whilst metabolite P5 (which accounts for 5-10% of the radioactivity) co-chromatographed with the tetrahydroxy

Figure 4.8 : Metabolite Patterns in Plasma, Faeces and Urine (Before (BH) and After (AH) Hydrolysis) of Humans Following an Oral Administration of [^{14}C]-Almitrine Bismesylate

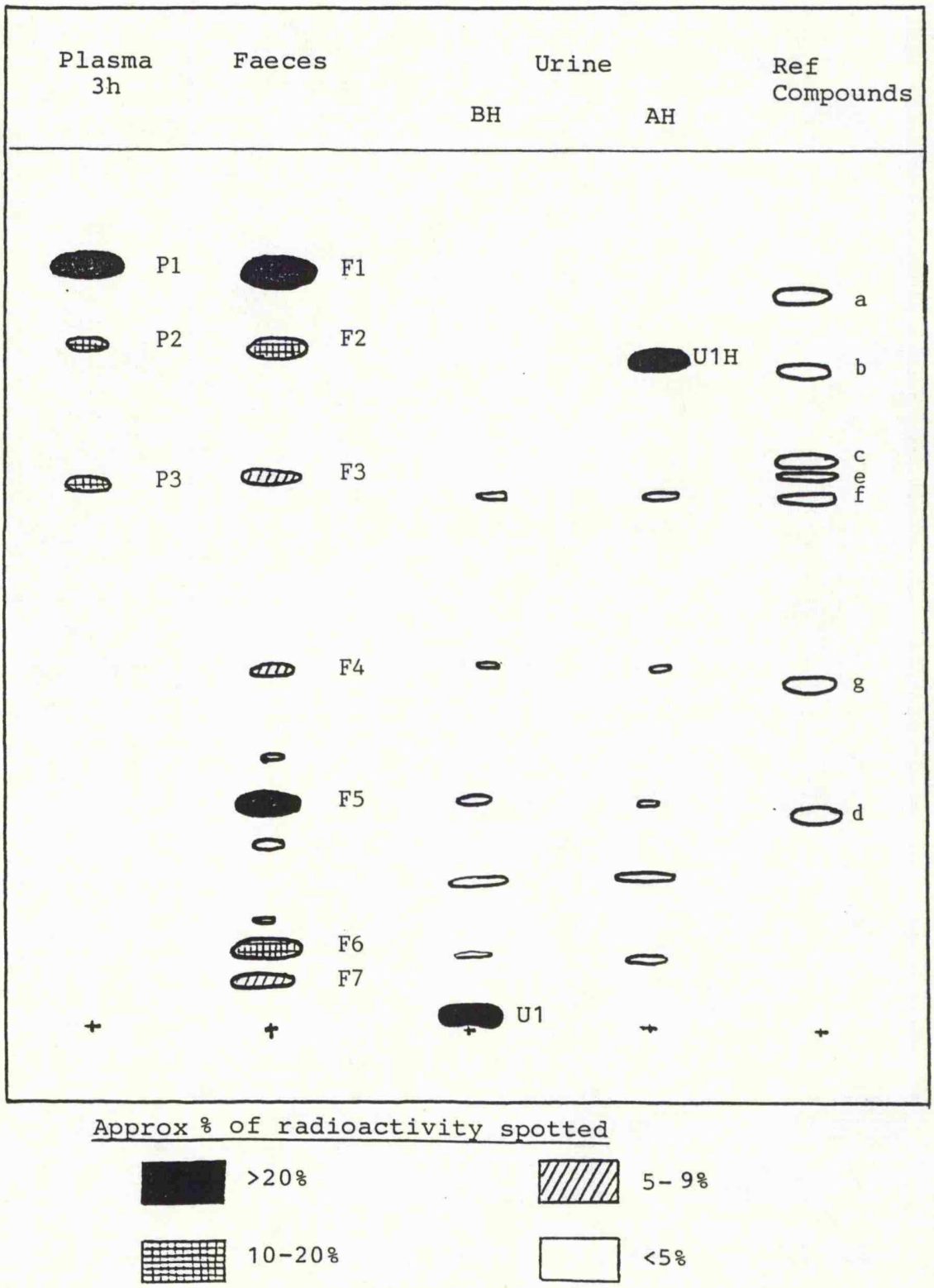
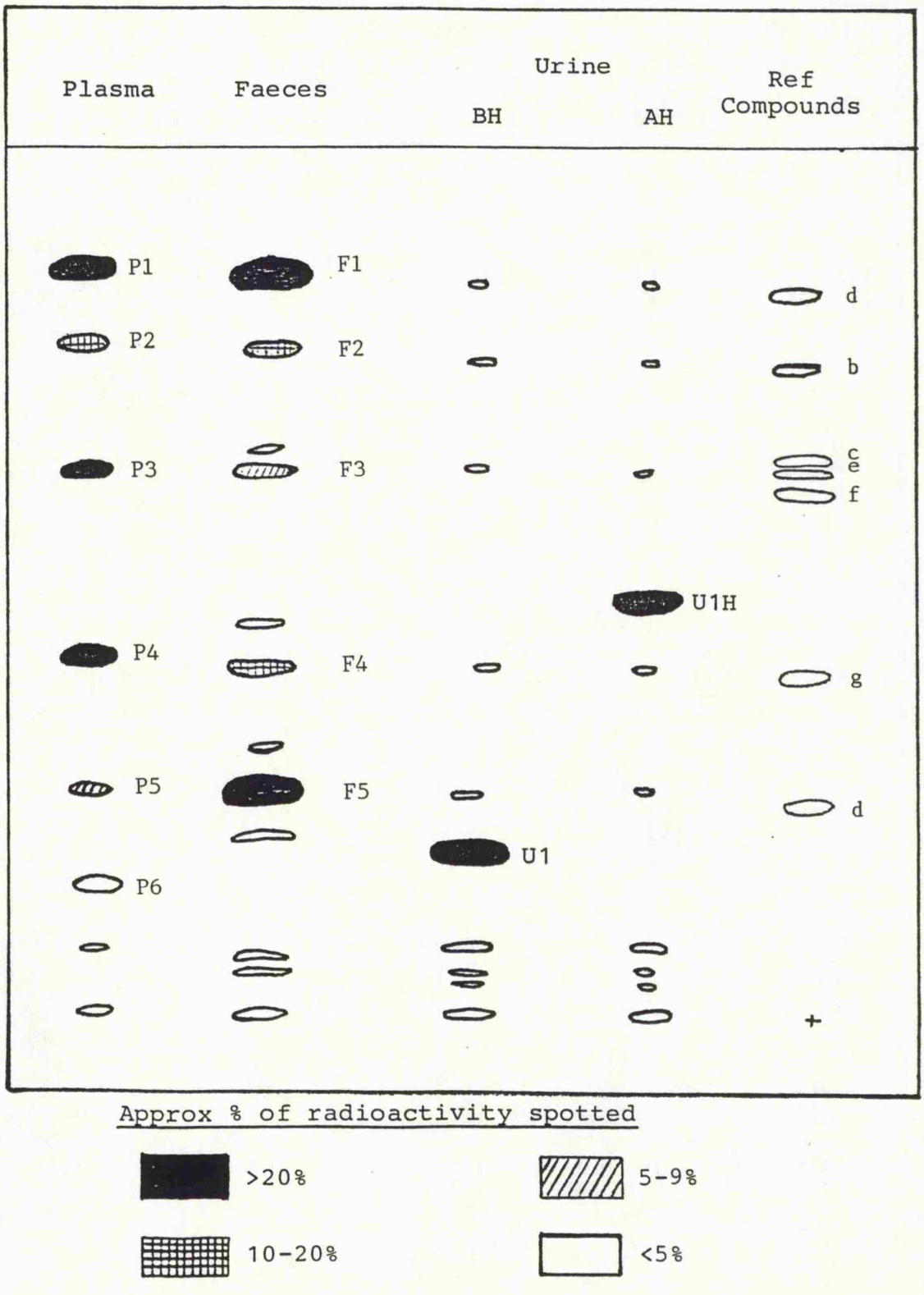


Figure 4.9 : Metabolite Patterns in Plasma, Faeces and Urine (Before (BH) and After (AH) Hydrolysis) of Wistar Rats Following a Single Oral Administration of [^{14}C]-Almitrine Bismesylate (100mg.kg^{-1})



compound (reference compound 'd'). Metabolite P6, representing a further 5% of the radioactivity, did not correspond with any of the available reference compounds.

4.2.1.3 Urine

Urinary metabolite patterns

The urinary metabolite profile was dominated by one major component (U1) for rat and human, which represented 60-70% and greater than 80% of the total urinary radioactivity in the two species respectively.

After enzyme hydrolysis, metabolite U1 was totally converted to a less polar compound (U1H). The human component co-chromatographed with monodeallyl almitrine (Reference compound 'b'), in the basic TLC system. However, when chromatographed in the acidic system it was clearly separated from all of the reference compounds available including monodeallyl almitrine. The rat component chromatographed in a region not related to any other reference products.

4.2.2 Discussion

4.2.2.1 Intravenous administration

All species showed extensive metabolism of almitrine. Although there were certain species differences, these were not important. Man excreted only low amounts (10%) of metabolite (7, corresponding to reference compound 'd', tetrahydroxy almitrine) after intravenous dosing whilst in all other species it was the major metabolite; in man, polar metabolites were eliminated but these were not apparent in the rabbit. The mouse however, eliminated both of the major polar metabolites observed in man whilst only one of these was found in both dog and rat.

Qualitatively, the dog was probably most similar to man in that all the metabolites eliminated in man were also found in the dog with the exception of metabolites 9 and 11, but these are quantitatively less important.

What is of interest is that in all the animals, unchanged drug (approximately 10%) was found in the faeces. This could either come from secretion into the stomach or, the more likely explanation, elimination through the bile. Theoretically, the latter is more probable since it has been shown that drugs

having a high molecular weight are eliminated in the bile (Millburn *et al.,* 1967). This is species-dependent and for the rat, the molecular weight threshold is approximately 300 and for man 500 (Hirom *et al.,* 1972; Millburn, 1970). It is not surprising then for almitrine, with a molecular weight of 477, to be found unchanged in human faeces after intravenous administration.

Other workers have shown that 50% of the dose is excreted into the bile of rats and perhaps a greater amount of unchanged drug would have been expected in rat faeces compared to human faeces than was actually found. The relatively low levels could be a consequence of more hepatic and/or gut metabolism in the rat.

4.2.2.2 After oral administration

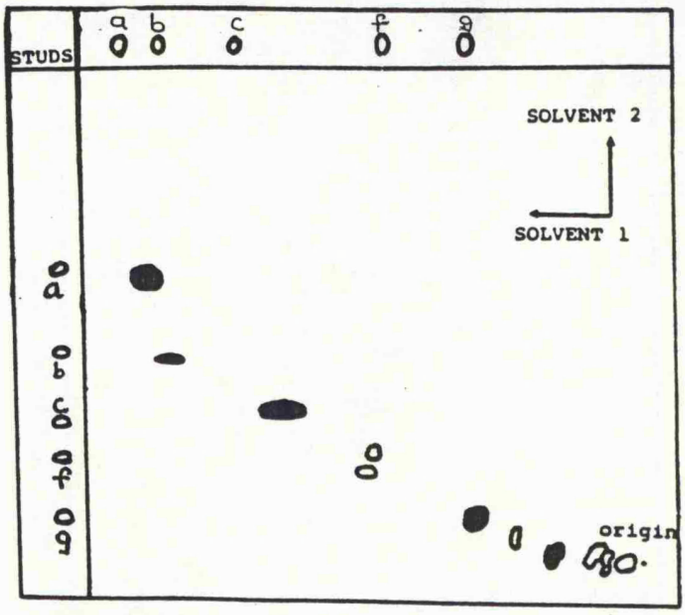
The pattern of metabolism was changed. Man eliminated more metabolite 7, the tetrahydroxy derivative, whilst the rat eliminated slightly less. The rabbit and the dog, however, eliminated large apparent quantities of unchanged drug, 75% and 35% respectively, compared to 10% after intravenous administration. It could be argued from these results that the drug was poorly absorbed in these species but this does not seem to be the case, since the comparison of areas under the plasma curve after intravenous and oral administration indicated a systemic availability of 65% and 80% for the rabbit and the dog respectively.

The animal most clearly similar to man, after oral administration of almitrine, was the rat. Both species had more polar components compared to dog and rabbit. Metabolite 12 was also found running above almitrine in both species. For this comparison, the 24 hour faecal samples with the largest percentage of activity were used. To rule out the possibility that this sample was not representative of the complete elimination, human p.o. composite samples on days 1 to 2, 1 to 7 and 8 to 14 were compared and, as can be seen in Fig. 4.10, there were no important differences indicating that, in man at least, a single sample was representative. It is interesting to note that the metabolite pattern was similar in all three samples, with almitrine present particularly on the 8-14 day composite. This would suggest a possible recirculation rather than distribution from tissues.

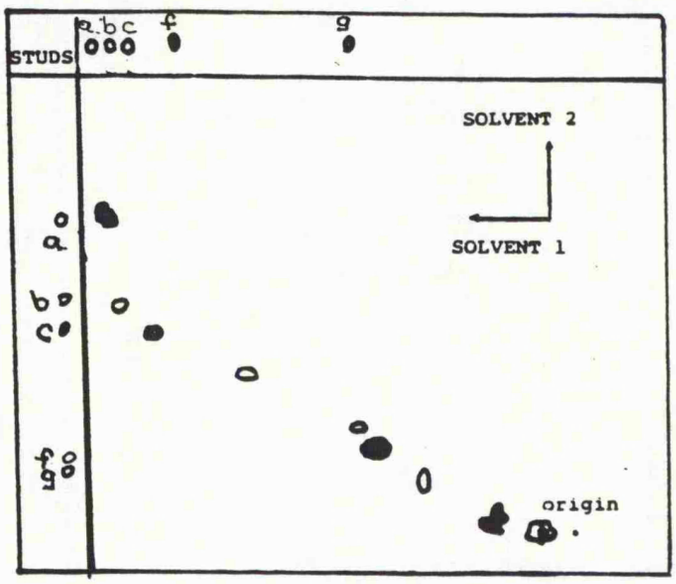
Other workers (Bromet - personal communication) have confirmed the identity of metabolite 7 in the dog as tetrahydroxy almitrine by mass

Figure 4.10 : Comparison of Human Faecal Metabolite Formation (A) 1-2, (B) 1-7 and (C) 8-14 Days After Administration of [^{14}C]-Almitrine Bismesylate

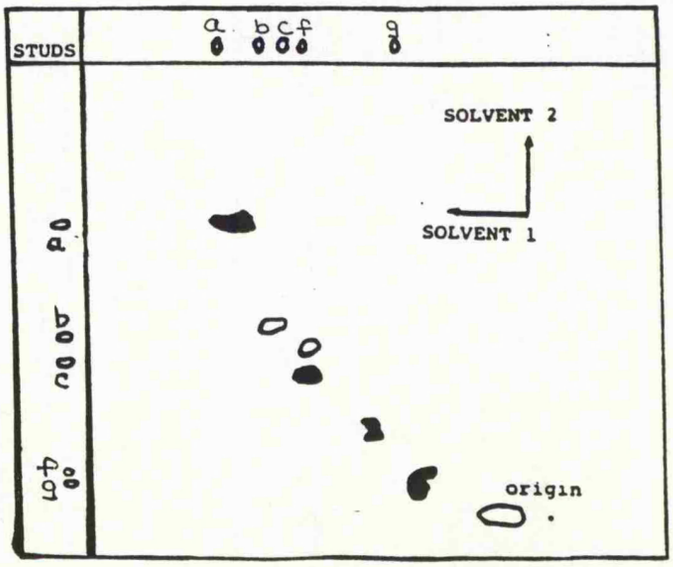
(a) Days 1-2



(b) Days 1-7



(c) Days 8-14



spectrometric comparison with the authentic synthesised material. This is the major metabolite in the dog, rat and rabbit and is also important in the other species. It does not appear to have any relevant pharmacological activity.

4.3 Identification of the Metabolites of Almitrine

The *in-vivo* metabolic profile of [^{14}C]-almitrine bismesylate investigated in man, dog, rat, mouse and rabbit has shown the drug to be extensively metabolised in each of these species but after oral administration it is the profile in the rat that appears most similar to that in man, when compared by chromatographic methods. Only tentative identifications can be made of all the metabolites of almitrine by the use of reference compounds co-chromatographed with the extracts. Positive identification of these metabolites requires the use of structural analysis techniques such as mass spectrophotometry and nuclear magnetic resonance. Mass spectral analysis is by far the easier mode of analysis, requiring lower levels of material than nuclear magnetic resonance. Furthermore, the final product does not require as much purification particularly for modern instruments incorporating liquid chromatography. Two approaches were made in the identification of almitrine and its metabolites. An identification of *in-vivo* metabolites in human and rat faeces and human plasma and urine using a Nermag R10-10C Mass Spectrometer. Identification of *in-vitro* metabolites in human, monkey, dog and rat microsomal fractions using a VG Quattro Mass Spectrometer.

4.3.1 Identification of *in-vivo* metabolites

At a normal therapeutic dose level of almitrine bismesylate (1-3mg.kg^{-1}) the quantities of metabolites excreted by the rat are too small to enable isolation and characterisation of individual components. Therefore, an initial comparative study was performed to ascertain whether, by increasing the dose to a level which would provide isolatable quantities of each metabolite, the pattern of metabolism would be significantly altered. From these results, a higher dose level was considered suitable and a study at 100mg.kg^{-1} was performed in the rat with the objective of isolating the major metabolites from urine and faeces.

In addition, the identity of the major urinary and faecal metabolites excreted by human volunteers and the presence of almitrine in plasma were investigated.

4.3.1.1 Study design and sample analysis

Rat study

Ten male Wistar rats were dosed orally by gavage, with [^{14}C]-almitrine bismesylate once daily for 4 days, at a dose level of 100mg.kg^{-1}.day^{-1} (2μCi.day^{-1}). The animals were housed in pairs in all-glass metabolism cages and food and water was available *ad-libitum*. Blood (0.5ml) was taken from each animal 5 hours after the first dose on day 1. Urine and faeces were collected daily until 48 hours after the last dose. After the final collection, the animals were exsanguinated and the blood separated into packed cells and plasma by centrifugation. All samples were stored at -20°C until analysed.

Human study

One healthy male subject (aged 39) received [^{14}C]-almitrine bismesylate (200mg, 35μCi) orally as an aqueous solution (50ml), following an overnight fast. No food was consumed until 4 hours after dosing, but non-alcoholic beverages were permitted in moderation.

A blood sample was taken by venepuncture, predose then larger samples (50ml) were also taken at 3, 8 and 24 hours after dosing to permit a more detailed investigation of the identity of the almitrine-related products present. An aliquot (1ml) of each sample was retained and stored in heparinised tubes at 4°C; the remainder was separated into packed cells and plasma by centrifugation and stored at -20°C until analysed.

Control samples of urine and faeces were collected on the day prior to dosing. After dosing, total voided urine was collected at 3, 6, 12 and 24 hours and then daily for 4 days. All faecal samples were collected for five days after dosing. All samples were stored at -20°C until analysed for radioactivity as previously described (Chapter 2).

Additionally, two healthy male volunteers (aged 30 and 39) each received non radioactive almitrine bismesylate (200mg) orally as an aqueous solution (50ml). A control urine sample was obtained from each subject prior to

dosing, then total voided urine was collected at 6, 12 and 24 hours after dosing. All samples were stored at -20°C until analysed.

Urine, faeces and plasma were analysed for radioactivity as described previously and aliquots extracted for thin layer chromatography in order to confirm that metabolite profiles were similar to those previously reported (Figs. 4.8 and 4.9). All remaining samples of highest radioactivity were used for the isolation of metabolites for mass spectral analysis.

4.3.1.2 Isolation of faecal metabolites

All the rats faecal samples (81% of dose) were pooled, homogenised and freeze dried. The 0-48 hour samples (50% of dose) from the human study were similarly prepared. Aliquots (10g) of the dried residues were eluted twice with warm (50°C) methanol and after centrifugation the eluate was evaporated to dryness under vacuum. The residue was redissolved in warm methanol (8ml), aliquots (0.5ml) streaked onto TLC plates (silica gel GF_{254} - 0.25mm layers) and developed in solvent system 1. The radioactive bands were located by autoradiography and removed by scraping off the relevant areas of silica. Corresponding bands from each of the plates were pooled, and eluted twice with warm (50°C) methanol (10ml). The eluates were centrifuged, concentrated to 1ml by gentle evaporation under nitrogen, then re-chromatographed on pre-washed silica plates using a solvent system of chloroform:methanol (90:10). The metabolites were located and eluted as before and after evaporation to dryness under nitrogen were submitted for mass spectral analysis.

4.3.1.3 Isolation of urinary metabolites

An aliquot of the radioactive 0-24 hour urine from Human Study 1 (7% of dose) was combined with the pooled non-radioactive 0-24 hour urine samples from Human Study II to serve as a radioactive tracer. The combined sample was then hydrolysed overnight at 37°C, pH 5.5, using the glucuronidase/sulphatase enzyme, and extracted using Amberlight XAD_2 resin until greater than 95% of the urinary radioactivity was extracted. The components were eluted from the resin with warm (50°C) methanol, the eluate evaporated to dryness and the residue redissolved in 2ml of methanol. This solution was then separated chromatographically and the major component isolated as described for the faecal metabolites.

4.3.1.4 Extraction of unchanged drug from plasma for mass fragmentography

Unchanged almitrine was extracted from the plasma samples from Human Study I using the procedures described for gas chromatographic analysis.

4.3.1.5 Identification of metabolites by mass spectrometry

The mass spectrometer used for all analyses was a Nermag R10-10C system, incorporating a solid sample Direct Insertion Probe with facilities for Desorption Chemical Ionization. Identification of the extracted metabolites was performed by mass spectrometry using two methods of ionisation.

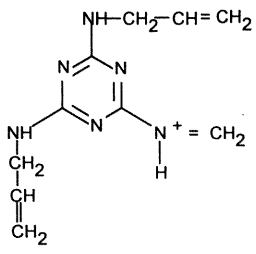
i) Desorption Chemical Ionisation (DCI) using ammonia as the reactant gas was used to establish the molecular weight of the compounds by detection of the quasi molecular ion at $(M+H)^+$.

ii) Electron Impact Ionisation (EI) was then used to yield structural information on the compounds by studying the fragmentation of the molecules.

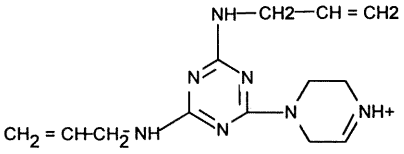
Human Plasma

The low concentrations of drug-related products in plasma and the high proportion of biological material, made the identification of these components impossible by the conventional mass spectrometric procedures already described. For this reason, plasma extracts were examined by the technique of mass fragmentography by focusing the instrument on two important and characteristic ions, which were specific to the parent compound, namely:

(i) $m/_e$ 219, corresponding to the fragment



(ii) and $m/_e$ 274, corresponding to the fragment



Chromatographic separation was achieved on a Carlo Erba 4160 gas chromatograph using a 6 metre SE52 fused silica capillary column, with an oven temperature programming from 20-250°C at 30°C.min^{-1} and a helium carrier gas pressure of 0.1 bar.

Mass fragmentography has confirmed the presence of unchanged almitrine in the plasma from component (P1) in Fig. 4.8. Figure 4.11 shows the fragmentograph of the 3 hour sample with peaks of mass 219 and 274 at a retention time of 6 min. 30 sec corresponding to almitrine (see fragmentation pattern Fig. 4.16). The peak at $m/_e$ 302 at 8 min. 30 sec corresponded to the internal standard used for the gas chromatographic assays (S. 2082). The levels of the two metabolites in plasma (Fig. 4.8) were very low so confirmation of their identity was extremely difficult.

Human and Rat faeces

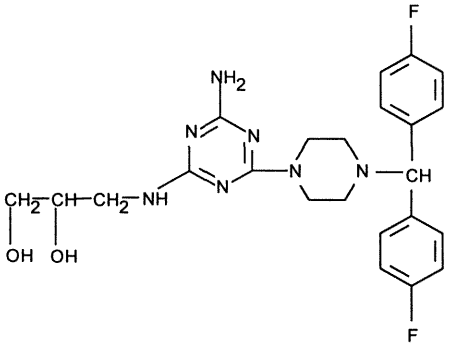
Rat and human faecal metabolite profiles were qualitatively similar. Metabolites F1 to F5 were quantitatively similar in both species. Two additional metabolites F6 and F7 were present in significant quantities in human but appeared to be less important in the rat, and as described earlier, these may be phase II metabolites by virtue of their R_f in this mobile phase. The five major components present in rat and human faeces (metabolites F1 - F5) were isolated by preparative thin layer chromatography and submitted for identification by mass spectral analysis.

Metabolite F5

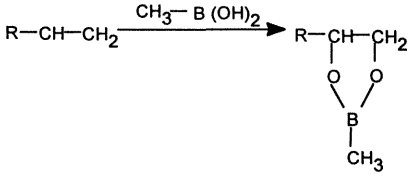
This metabolite co-chromatographed with reference compound 'd', tetrahydroxy almitrine. Chemical ionisation mass spectrometry confirmed the molecular weight to be 545 and electron impact ionisation yielded all the characteristic fragments (Fig. 4.12) found with the authentic reference compound.

Metabolite F4

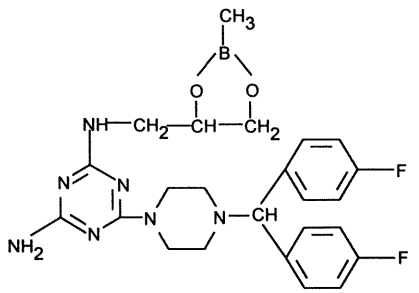
Chemical desorption mass spectrometry indicated a compound of molecular weight 471, and a fragmentation pattern obtained by electron impact ionisation (Fig. 4.13) was consistant with the structure:



To confirm the α-dihydroxy structure, the metabolite was derivatized by the technique of Blau and King (1977), to produce a cyclic methyl boronate derivative.



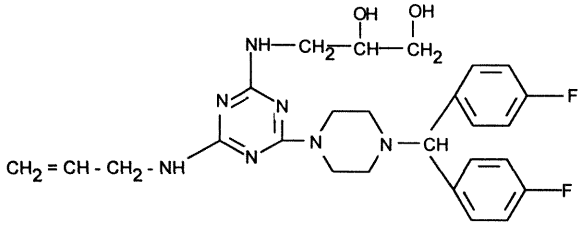
The mass spectrum of the derivative by chemical desorption showed the shift of the quasi molecular peak $(M+H)^+$ from $m/_e$ 472 to give the boron isotope doublet at $m/_e$ 495 and 496, thereby confirming the α-dihydroxy structure of metabolite F4.



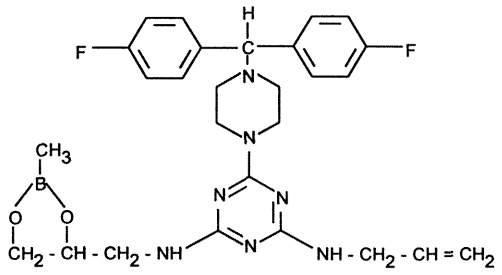
Monodeallyl dihydroxy almitrine was subsequently synthesised and co-chromatographed and confirmed it to be identical to metabolite F4 in human and rat.

Metabolite F3

The original chromatographic separation suggested that this component might be the same as reference compound 'c', dideallyl almitrine. The mass spectrum obtained after chemical desorption ionisation, however, suggested a product of molecular weight 511. Examination of the fragmentation by electron impact ionisation (Fig. 4.14) was consistant with the structure:



As for the previous metabolite, the α-glycol group was derivatised with methyl boronic acid, and in the mass spectrum the $(M+H)^+$ ion at $m/_e$ 512 was shifted to $m/_e$ 535 and 536, giving the appropriate 25-75 isotope ratio corresponding to the expected derivative:



Co-chromatography of the synthesised product has confirmed the identity of metabolite F3 to be dihydroxy almitrine.

Metabolite F2

This metabolite co-chromatographed with Reference Compound 'b', monodeallyl almitrine.

The mass spectrum using chemical ionisation produced a quasi-molecular ion $(M+H)^+$ at $m/_e$ 438, corresponding to the monodeallyl almitrine. Electron impact ionisation confirmed this by producing a fragmentation pattern (Fig. 4.15) identical to that of the reference compound.

Metabolite F1

This metabolite co-chromatographed with the unchanged drug (reference compound 'a') and mass spectrometry by chemical ionisation and electron impact (Fig. 4.16), confirmed the identity of this compound as almitrine.

Identification of human urinary metabolite U1H

Mass spectrometry of the hydrolysed human fraction using desorption chemical ionisation and electron impact ionisation, has identified this product as bis-(p-fluoro-phenyl) methanol. Comparison with an authentic reference sample (DLH 1305) by capillary GC-MS, before and after methylation of the hydroxyl group with Methelute (Pierce Chemical Co. Ltd., Illinois, USA) has confirmed this identification (see Fig. 4.21).

The rat urinary metabolite proved difficult to identify due to low levels of the product for GC-MS.

4.3.2 Identification of *in-vitro* metabolites

Incubation extracts from the *in-vitro* comparison described in Section 4.1 were subjected to mass spectral analysis using LC-MS in the electrospray ionisation (ESI) mode. A VG Quattro LC-MS was tuned to a capillary voltage of 3.8kV; the HV lens was set at 0.80 kV and the ESI focus at 55V. The instrument was set to scan over the mass range 100 to 650 atomic mass units (amu). An ABI microbore column (ODS, 280 x 2mm i.d.) was employed and the HPLC gradient solvent system used consisted of:

Solvent System A : 0.05M ammonium acetate:acetonitrile (95:5 v/v)

Solvent System B : Acetonitrile. The following gradient parameters were used:

Time (min)	%B
0	10
15	55
20	55
22	70
25	90

The flow rate was set at 200µl.min^{-1} and a sample injection volume of 10µl was introduced.

This more modern technique offers a tremendous advantage over GC-MS in that minimum sample pretreatment is required, no derivatisation is necessary to run even polar compounds and the protonated molecular ion (M+H) is monitored rather than a fragmentation pattern. Prior to analysis of the sample extracts, almitrine and reference metabolite standards were analysed in order to confirm the respective protonated molecular ions at the chromatographic intention times scanned. Figure 4.17(a) shows the total ion chromatogram (TIC) obtained from the injection of a mixture of almitrine (S2620) and reference metabolites and the electrospray scans in the regions of each component's retention time, with its corresponding protonated molecular ions. Figure 4.17(b) shows the spectra from the ion profile trace for each reference component.

Rat, monkey and human microsomal extracts showed the presence of almitrine (M+H = 478) and four metabolites, namely the three hydroxylated metabolites dihydroxy almitrine, (M+H = 512), monodeallyl dihydroxy almitrine (M+H = 472) and tetrahydroxy almitrine (M+H = 546). The fourth metabolite was monodeallyl almitrine (M+H = 438) (Figs. 4.18 and 4.19). The dog, however, revealed only three components namely, almitrine, dihydroxy almitrine and monodeallyl almitrine. These findings were consistant with the profiles described in Section 4.1.

4.4 Discussion

The metabolism of almitrine is potentially very complex since the molecule possesses many possible sites where metabolism can occur, giving a large combination of metabolites. Technically, metabolism studies with almitrine can be complicated by the fact that most of the drug-related products are eliminated in the faeces. Nevertheless, the preparation procedures for faecal and urinary extracts yielded material of sufficient quality and quantity for identification by GC-MS. The microsomal extracts were much cleaner and presented less problems for LC-MS.

For all species, metabolism of almitrine occurred mainly at the triazinyl end of the moelcule with very little or none occurring on either the piperazine ring or the two fluorophenyl rings. This would suggest that the presence of fluorine atoms structurally limits metabolism at this end of the almitrine molecule. Approximately 75% of the recovered metabolised dose in rat and man could be accounted for by hydroxylation of the double bond and/or N-dealkylation of one or both of the allyl side chains. There was some suggestion of conjugate formation of almitrine-related products. These represented up to 10% of the metabolites after oral administration (F6 and F7, Fig. 4.10) and 5 to 25% after intravenous administration (Fig. 4.3). The same products were seen in mouse faeces. Of the remaining metabolites in other species, only metabolites F6 and F7 in man, were unidentified.

None of the metabolites identified in faecal extracts involved a major reduction in the molecular weight of the almitrine-related products, with often the molecular weight increasing. As mentioned previously, it was not surprising, therefore, when considering that the molecular weight threshold for biliary excretion in rat is approximately 300 and in man is approximately 450, that the majority of these products are probably eliminated in bile. Moreover, it was interesting to note that the only important urinary metabolite in man, the bisfluoro-p-fluorophenyl methanol (representing 80-90% of the urinary excretion of radioactivity, although less than 10% of the administered dose of almitrine) has a much smaller molecular weight of only 220, although it was mainly excreted as a conjugate. A similar pattern is seen in rat urine, with a single conjugated metabolite representing 60-70% of the total radioactivity (about 5% of the administered dose). This compound, however, is not the same as that seen in man, but its appearance in urine again implies that it is a relatively small molecule.

The major products found in human plasma after a single dose were intact almitrine, (this being confirmed by mass fragmentography), the mono-deallyl and the dihydroxy metabolites. Although these two metabolites in plasma have not been positively confirmed by mass spectrometry, chromatographic evidence supports these identities. In a one year clinical study in hypoxaemic patients receiving almitrine bismesylate ($100mg.day^{-1}$) HPLC analysis, using the method described in Section 2.3.2, showed that the unchanged product was always the most important, accounting for 40-48% of all measured products in plasma. The four metabolites detected in

decreasing order of importance were, the dihydroxy (20-28%), the dideallyl (12-13%), the monodeallyl dihydroxy (10-11%) and the monodeallyl (7%) almitrine metabolites. The tetrahydroxy metabolite was, however, not detected (Fig. 4.20(a)).

Similarly, in a 80 week repeated dose toxicological study in the dog ($15mg.kg^{-1}.day^{-1}$), HPLC analysis also showed almitrine to be the major circulating product. The relative proportions of each metabolite at steady-state was found to be in decreasing order of importance, monodeallyl dihydroxy, dideallyl, dihydroxy, monodeallyl and tetrahydroxy almitrine (Fig. 4.20(b); courtesy of P. Klippert, Technologie Servier, Orléans, France). From the *in-vitro* data in the dog, it would appear that the lack of metabolite formation was more a function of the activity of the microsomes, rather than an inability to metabolise almitrine by this species. This is also supported by the *in-vivo* metabolism data after intravenous administration. In none of these studies was the detriazinyl product measured.

The apparent absence in plasma of some of the other products identified in faeces after single dose would suggest that these are rapidly eliminated following their production possibly by direct secretion into bile, with little, if any, entering the systemic circulation so they are present in plasma in very low concentrations ($<1ng.ml^{-1}$).

In rats, the plasma profile is qualitatively much more similar to that seen in the faeces, and all five major excretory products are present in significant amounts in the systemic circulation, where they account for about 90% of the total radioactivity in the peak level sample. These profiles compare well with the *in-vitro* results and confirm the liver as the major site of metabolism of almitrine.

The presence of unchanged almitrine in faeces following intravenous administration is probably through secretion into the gut rather than biliary excretion. This has been demonstrated in biliary excretion studies in both human and rats in which all the above metabolites and additional components have been tentatively identified by TLC, but no unchanged drug was present in the samples analysed (data from internal documents). The tetrahydroxy was the major component.

Finally, when considering the relationship between the toxicology, determined in experimental animals, and the situation in man, it was important to note that all the metabolites identified in man, accounting for >70% of the recovered almitrine dose, were also present in the rat and dog, these being the species used for long term toxicology. The mouse and rabbit which are used for reproductive studies show qualitatively and possibly quantitatively, similar metabolic profile to man. Thus the animal species used for toxicology and other safety evaluations were exposed to the same chemical entities arising from almitrine to those known to be produced in man. Although no *in-vivo* data is available in the monkey, the *in-vitro* results show this species to metabolise almitrine similarly to man and the other species. Thus the metabolic pathway of almitrine in these species is best summarised in Fig. 4.21.

In summary, almitrine is extensively metabolised in all species by two major pathways (a) hydroxylation of the allyl side chain and (b) N-dealkylation. Neither of these processes appear to be rapid, but the most important seems to be oxidation of the allyl side chains to form di and tetrahydroxy almitrine; the process involving microsomal enzymes. The high molecular weights and lipid solubility of these compounds preclude urinary excretion and they are eliminated by gut and biliary secretion into the faeces. N-dealkylation appears to be a less important pathway with lower levels of the metabolites found in the faeces compared to hydroxylated metabolites. The levels of the monodeallyl metabolite found in plasma from rat, man and dog suggest that once formed, it is easily metabolised to the hydroxylated product. The qualitative and quantitative similarities in the metabolism of almitrine in all species, compared to man, validate their use in preclinical development.

Figure 4.11 : Mass Fragmentograph of Human Plasma Component P1 (Unchanged Almitrine)

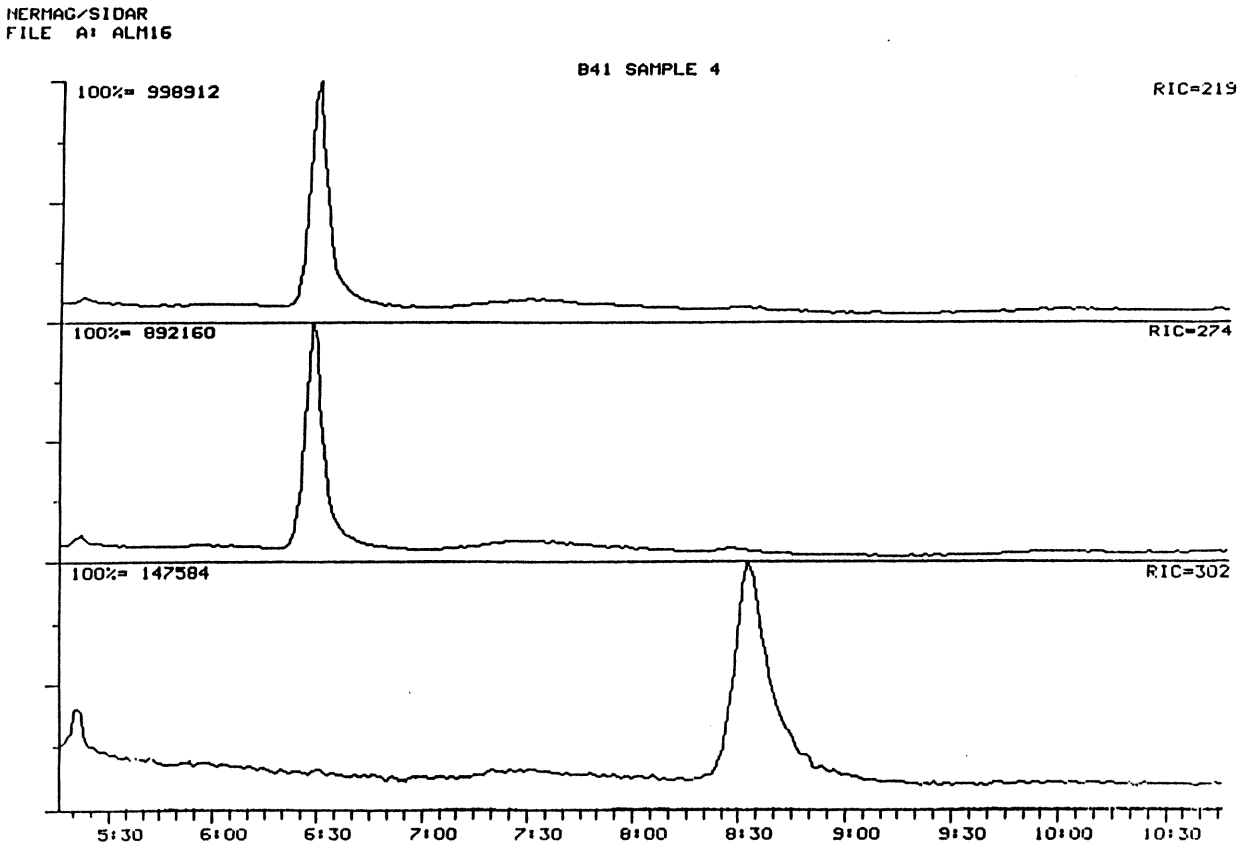


Figure 4.12 : Fragmentation Pattern of Human and Rat Metabolite F5 (Tetrahydroxy Almitrine) Using Electron Impact Ionisation

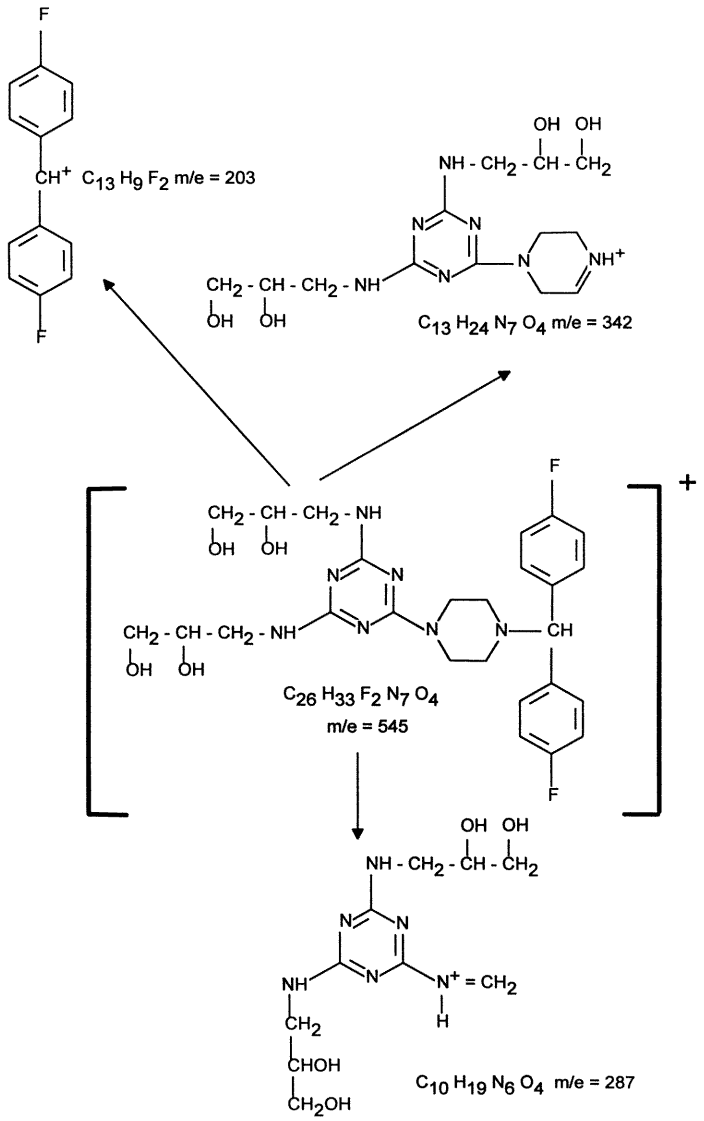


Figure 4.13 : Fragmentation Pattern of Rat Metabolite F4 (Monodeallyl Dihydroxy Almitrine) Using Electron Impact Ionisation

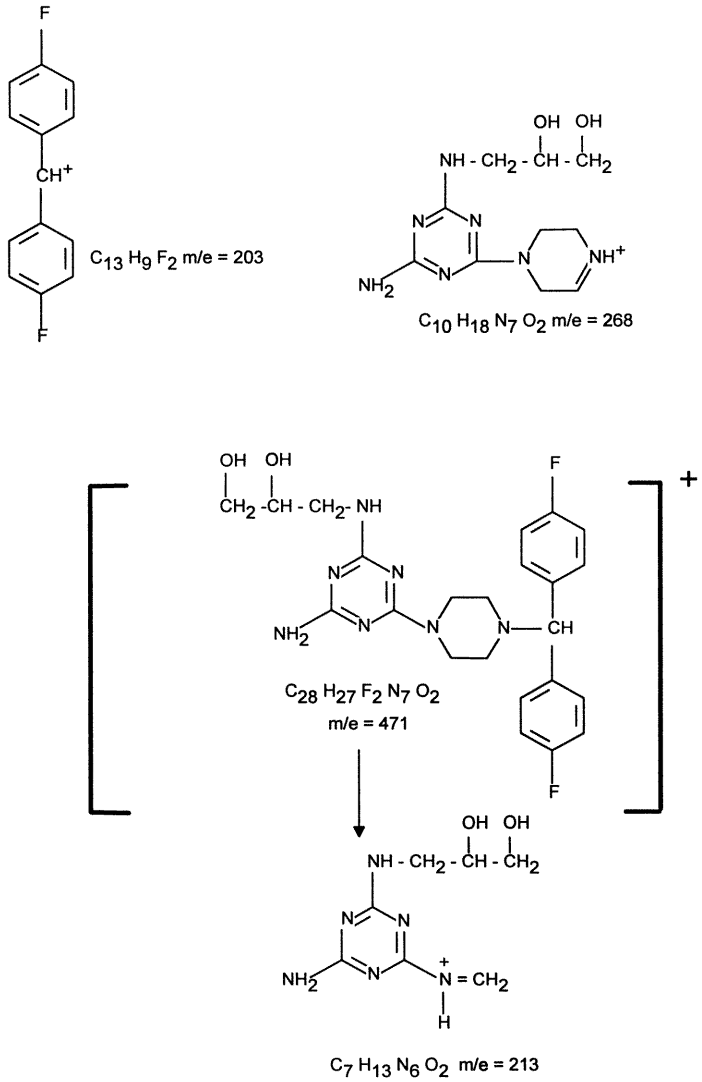


Figure 4.14 : Fragmentation Pattern of Rat Metabolite F3 (Dihydroxy Almitrine) Using Electron Impact Ionisation

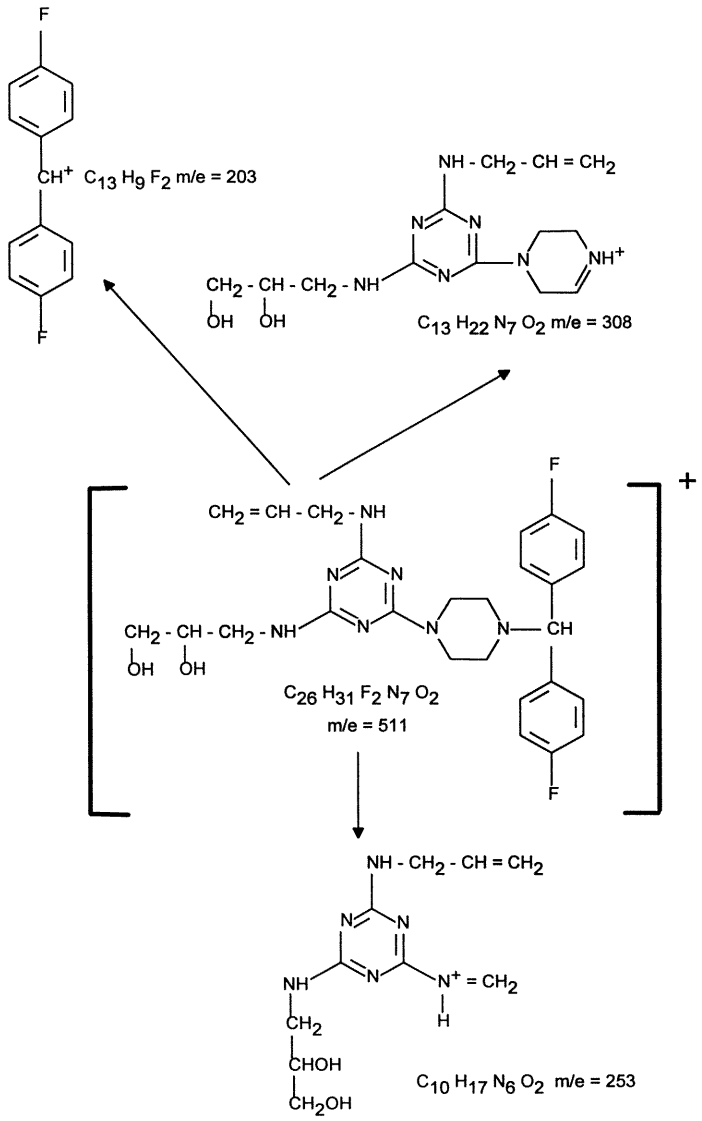


Figure 4.15 : Fragmentation Pattern of Human and Rat Metabolite F2 (Monodeallyl Almitrine) Using Electron Impact Ionisation

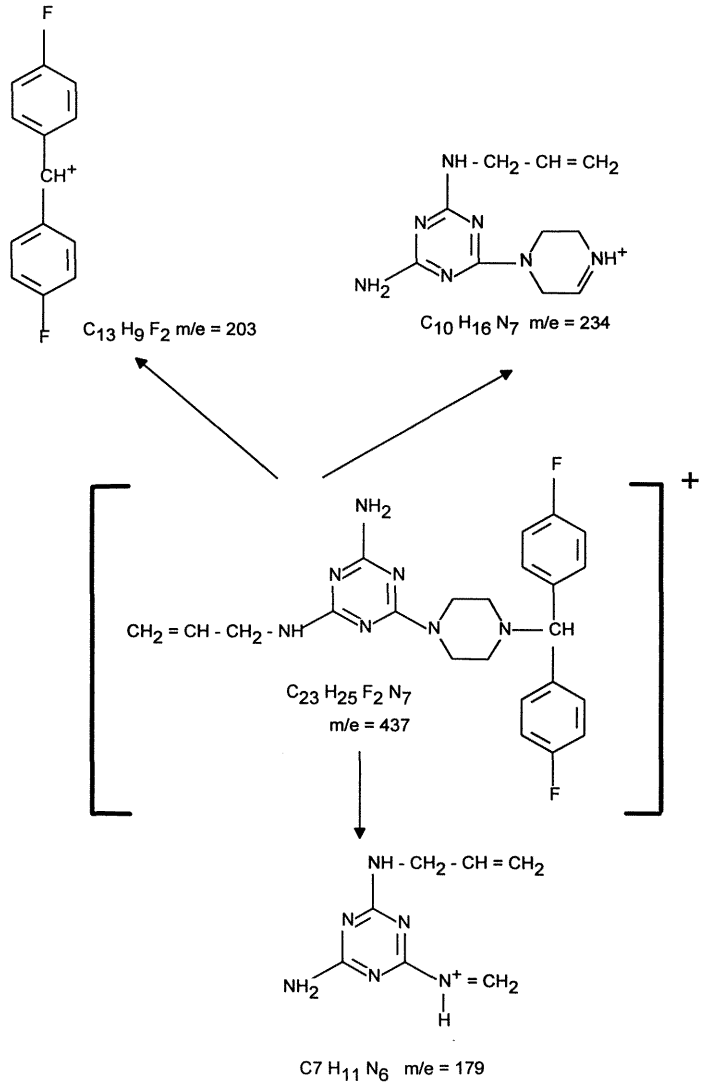


Figure 4.16 : Fragmentation Pattern of Rat and Human Metabolites F1 and P1 (Almitrine) Using Electron Impact Ionisation

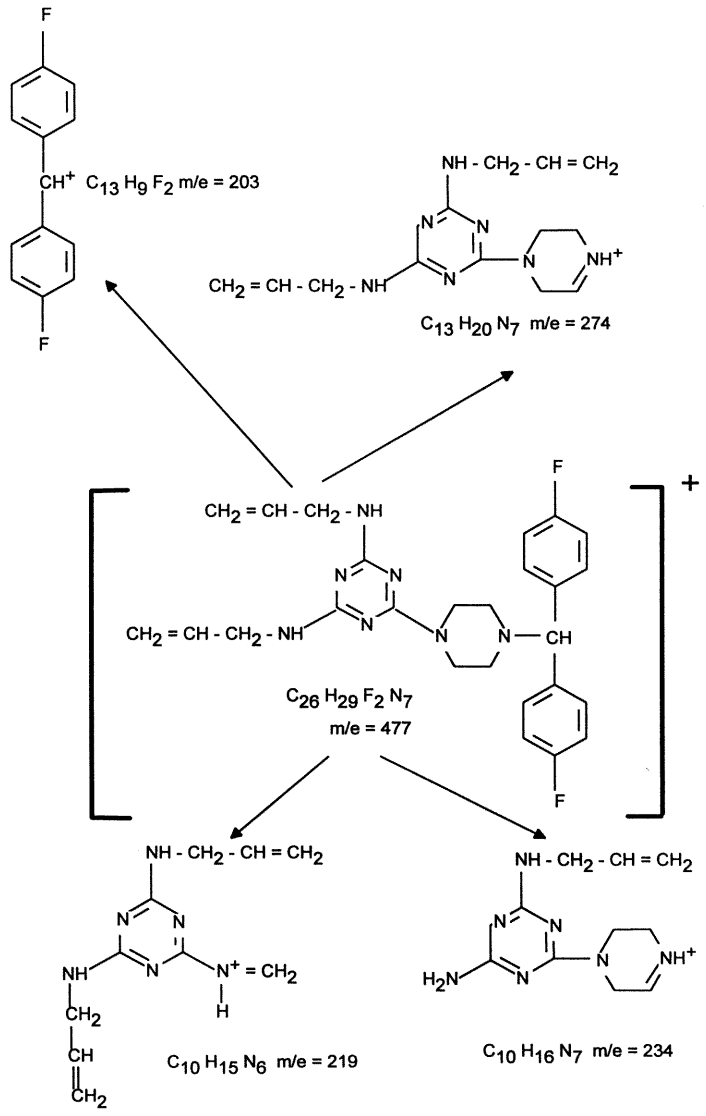
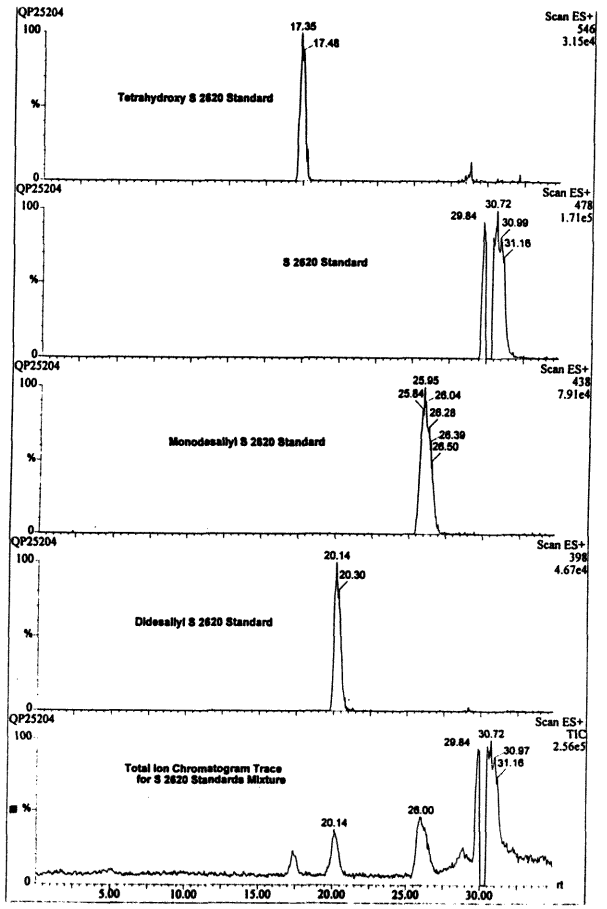


Figure 4.17 : (A) Total Ion and Electrochromatograms of Almitrine (2620) and its Reference Metabolites and (B) the Corresponding Molecular Ion Spectra for Each Component

(A) TIC AND ELECTROCHROMATOGRAMS



(B) SPECTRA OF IONIC PROFILES

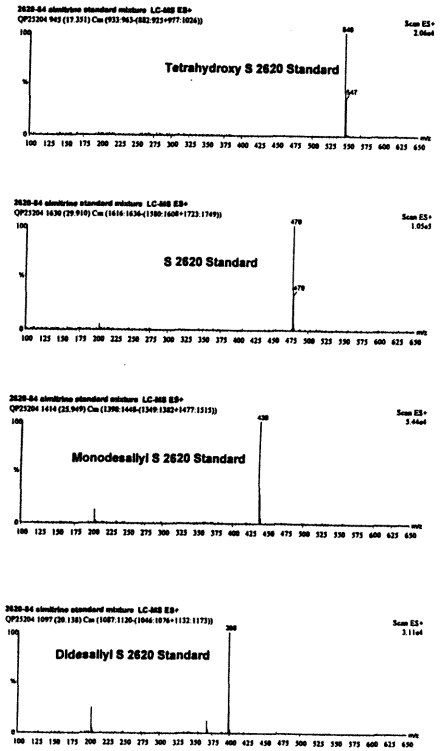
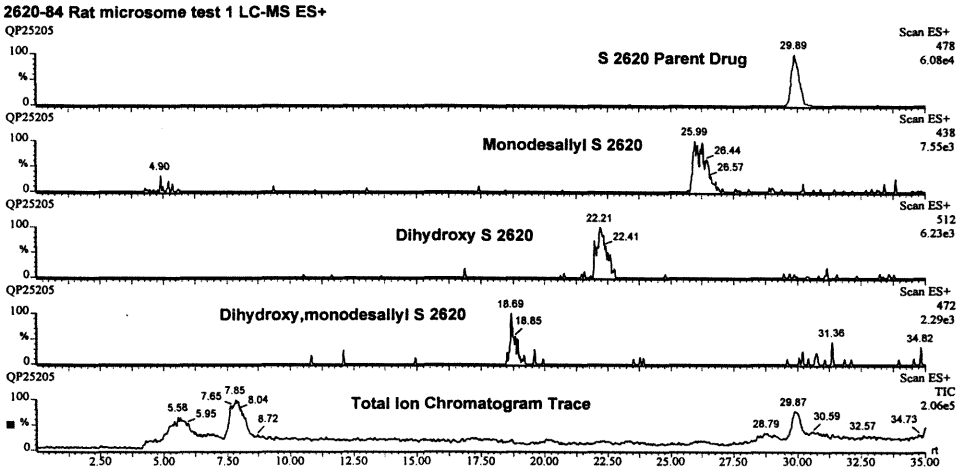


Figure 4.18 : Total Ion Chromatograms and the Protonated Molecular Ions of Almitrine and its Metabolites from Rat and Human Microsomal Extracts



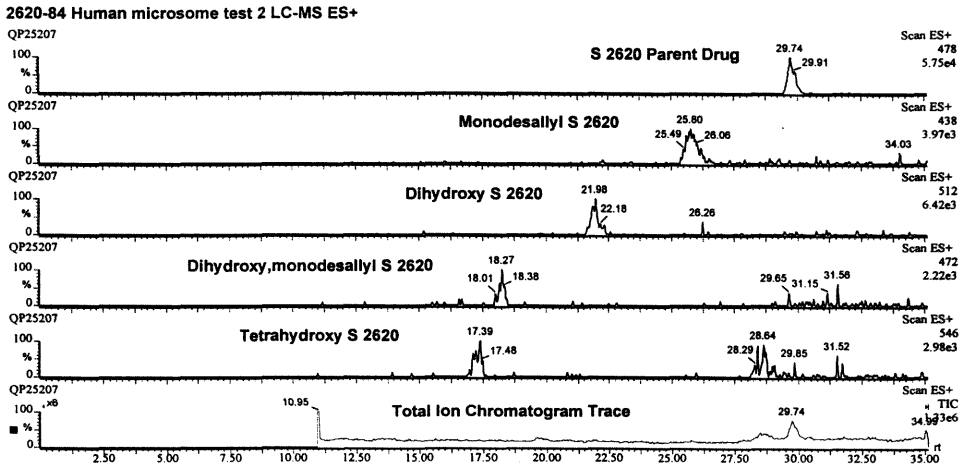
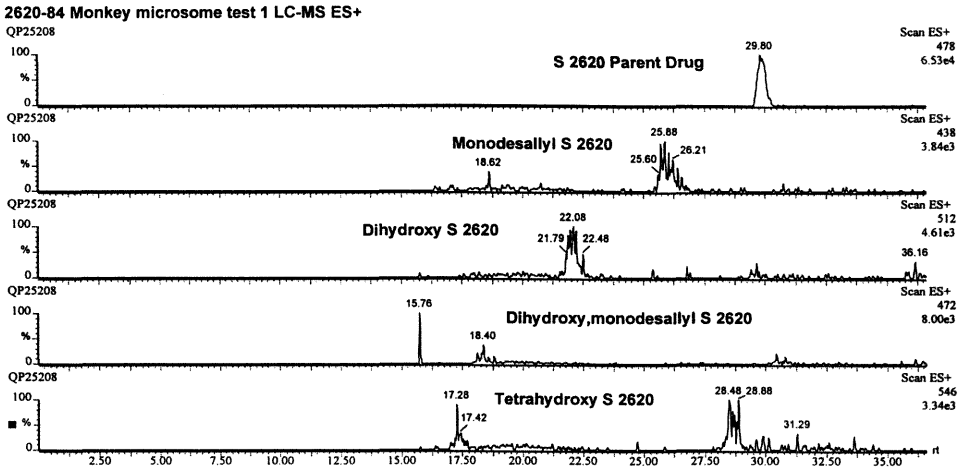


Figure 4.19 : Total Ion Chromatograms and the Protonated Molecular Ions of Almitrine and its Metabolites from Monkey and Dog Microsomes Extracts



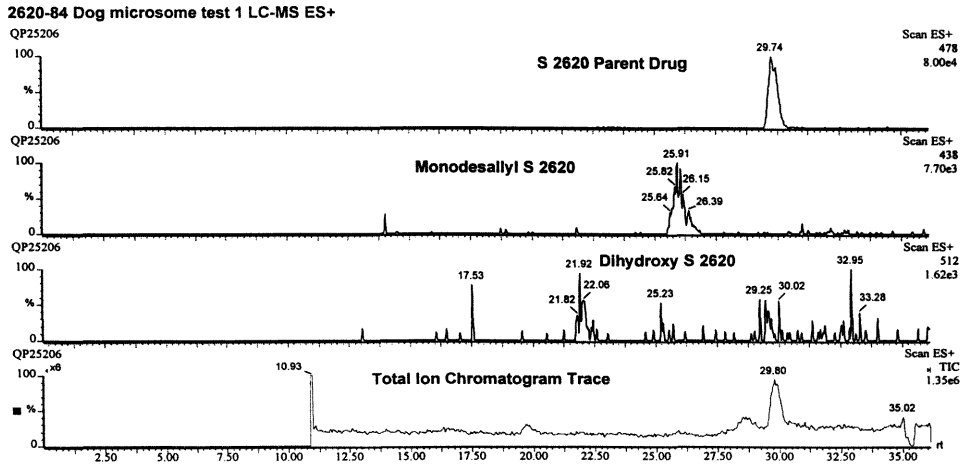
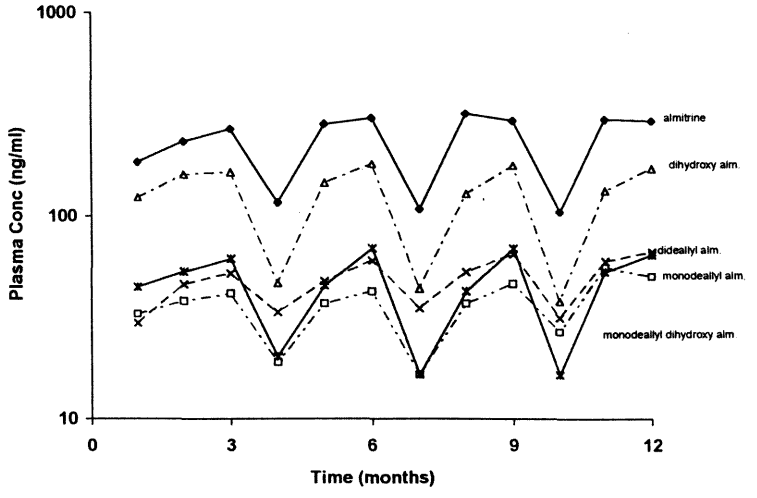


Figure 4.20 : Plasma Profile of Almitrine and its Major Circulating Metabolites in Human (a) and Dog (b) Following Repeated Oral Administration of Almitrine Bismesylate (100mg.kg^{-1}.d^{-1} and 15mg.kg^{-1}.d^{-1} Respectively)

(a) Human



(b) Dog

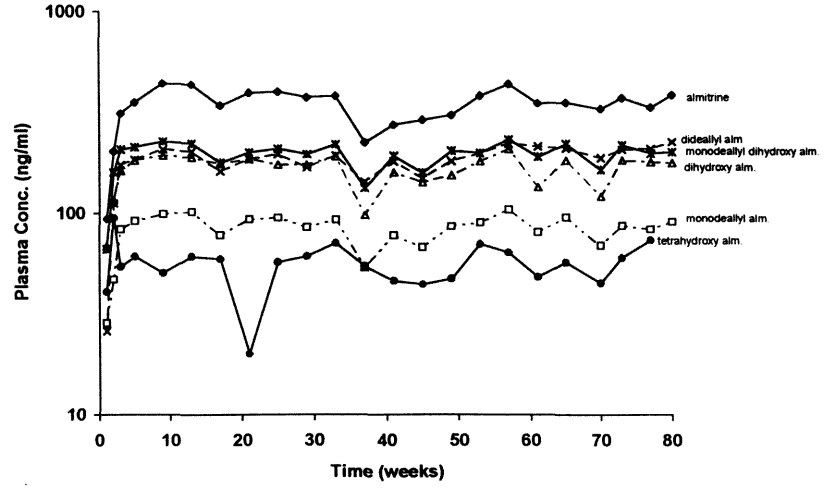
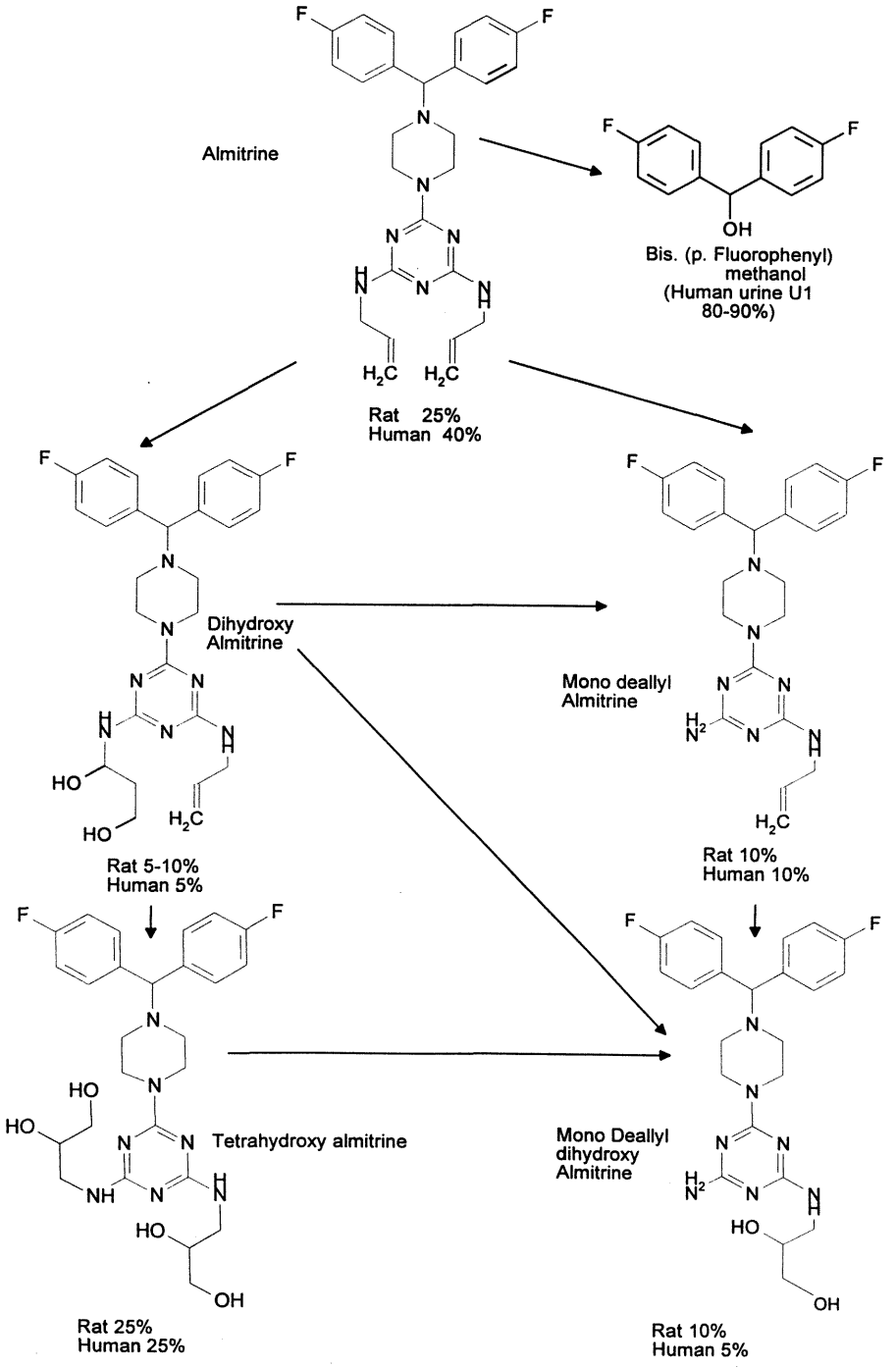


Figure 4.21 : Routes of Metabolism of Almitrine



CHAPTER 5

THE PHARMACOKINETICS OF ALMITRINE

This chapter describes the pharmacokinetics of almitrine from phase I and phase III clinical studies. All phase I studies (except the interaction study described in Section 5.4) was designed and organised by me with assistance from Dr. R. Richards, the Clinical Trials Co-ordinator. Oral dose solutions were formulated and prepared by me and dose administration and blood sampling were performed either at the Lister Hospital, Hitchin (study 1), or at the St. Bartholomews Hospital, London. All analyses were performed either by me or under my supervision for both phase I and phase III studies.

The pharmacokinetics of almitrine was investigated mainly in healthy volunteers in order to obtain basic pharmacokinetic parameters of the compound. These studies investigated the effect of different doses, food, routes of administration, formulation and co-administered drugs on the basic pharmacokinetics of almitrine. In the clinical situation steady-state plasma concentrations of almitrine were monitored by GC analysis and its major circulating metabolites by HPLC, as part of a series of multicentre studies. The basic pharmacokinetics of almitrine is presented and two studies relating plasma concentration to effect are briefly described.

The majority of the early pharmacokinetic studies in man, after both oral and intravenous administration, were performed over short sampling periods (48 to 72 hours) due to contraints on the protocol. Later studies allowed sampling to be performed for longer time periods in order to clearly define the pharmacokinetics of almitrine. Unless stated, all studies were performed on fasted subjects (fasted for at least 16 hours prior to dosing and 4 hours after dosing). Pharmacokinetic analyses were performed using either the method of residuals (Gibaldi and Perrier, 1975) or by fitting the data to the sum of exponentials using a weighted iterative least squares program (Ings *et al.*, 1980) as described in Chapter 2. (Section 2.4).

5.1 Absorption and Bioavailability

5.1.1 Fasting

In a preliminary study investigating the bioavailability of almitrine from a solution formulation (Study No. 1), four male subjects received an oral dose of

almitrine bismesylate (100mg) and this was compared to an intravenous dose (15mg) administered over 12 minutes. Absorption was rapid with maximum plasma levels (C_{max}) of almitrine varying between 182 and 378ng.ml^{-1} (mean 241 ± 93ng.ml^{-1}) which was achieved 2 hours after administration (Appendix 5.1, and Table 5.1; Fig. 5.1a). When corrected to the intravenous dose, this showed the bioavailability of almitrine from the oral solution to be greater than 100% (132 ± 19%).

In a second study however, (Study No. 2), using a lower dose, eight subjects (4 male and 4 female) received an oral dose of almitrine bismesylate (30mg) in solution and a similar intravenous dose (30mg) infused over 12 minutes in a balanced crossover design, with a two week washout between each dose. C_{max} varied from 35 to 91ng.ml^{-1} (mean 59 ± 24ng.ml^{-1}) and occurred earlier than the previous study (mean t_{max} 1.6 ± 0.7h). This lower solution had a mean bioavailability of 74 ± 23%, ranging from 45 to 108% (Appendix 5.2a and Table 5.2; Fig. 5.1b). These results would suggest that there was a saturation of elimination from the 100mg oral solution giving rise to an over estimation of bioavailability. However, the areas under the plasma concentration versus time curves, AUC's, for the oral formulations were proportional, and this would indicate that the intravenous dose was underestimated for the first study.

When a 50mg film-coated tablet formulation of almitrine bismesylate (Study No. 3) was compared to a solution formulation (2 x 50mg tablet vs 100mg solution) in 12 healthy male subjects using a cross-over design (14 day washout between each dose) absorption of the tablet formulation was rapid but variable (Appendix 5.3a, and Table 5.3; Fig. 5.1c) with C_{max} varing between 72 and 387ng.ml^{-1} (mean 176 ± 82ng.ml^{-1}) achieved between 1 and 4 hours (mean t_{max} 2.7 ± 0.98h). C_{max} for the solution (Appendix 5.3b and Table 5.3; Fig. 5.1c) varied between 98 and 378ng.ml^{-1} (mean 192 ± 85ng.ml^{-1}) and these were achieved between 2 and 4 hours after dosing (mean t_{max} 2.3 ± 0.62h). The bioavailability of the tablet formulation, relative to the solution was 89% but varied between 56 and 192%. An analysis of variance of AUC according to the procedures of Wagner (1975) and Armitage (1971) confirmed significant subject variability ($p<0.05$) but there was no siginificant difference between the two formulations. A 26% confidence interval was determined for the spread of the data according to the methods of Westlake (1972) and Shirley (1976) and this showed the bioavailability of the tablet formulation to lie between 74-116% with respect to the solution (Table 5.4).

Table 5.1 : Pharmacokinetic Parameters of Almitrine in Healthy Male Human Volunteers Following Either Oral (100mg) or Intravenous (15mg) Administration of Almitrine Bismesylate as a Solution (Study No. 1)

PARAMETER	INTRAVENOUS					ORAL				
	SUBJECTS				MEAN	SUBJECTS				MEAN
	1	2	3	4	±SD	1	2	3	4	±SD
C_{max} (ng.ml^{-1})	222	293	218	578	398 ± 170	182	184	219	378	241 ± 93
t_{max} (h)	0.03	0.03	0.03	0.03	0.03 ± 0.00	2.0	2.0	2.0	2.0	2.0 ± 0.0
$t_{½(1)}$ (h)	0.11	0.01	0.09	0.10	0.08 ± 0.05	-	-	-	-	-
$t_{½(2)}$ (h)	1.2	0.50	1.01	0.86	0.90 ± 0.30	3.23	2.77	2.90	2.43	2.83 ± 0.33
$t_{½(z)}$ (h)	23	17	29	19	22 ± 5	45	27	27	33	33 ± 8.5
AUC_t (ng.ml^{-1}h)	328	151	278	490	312 ± 140	2378	1725	2911	3208	2556 ± 665
AUC (ng.ml^{-1}.h)	396	219	449	578	411 ± 149	4004	2078	3689	4256	3506 ± 980
F (%)	NA	NA	NA	NA	NA	152	142	123	110	132 ± 19
CL (ml.min^{-1})	449	810	397	308	491 ± 220	NA	NA	NA	NA	NA
$V_{(area)}$ (l)	893	1189	1009	518	902 ± 284	NA	NA	NA	NA	NA
V_{ss} (l)	1019	1232	1486	574	1078 ± 386					

NA denotes Not Applicable

Table 5.2a : Pharmacokinetic Parameters of Almitrine in Healthy Volunteers given the Bismesylate Salt (30mg) Orally as a Solution (Study No. 2)

PARAMETER	SUBJECT								MEAN
	1	2	3	4	5	6	7	8	± SD
Age (yrs)	43	44	26	26	24	27	26	23	30 ± 815
Height (cm)	180	161	183	180	180	168	175	160	173 ± 9.2
Body Weight (kg)	76	58	80	71	79	59	70	50	68 ± 11
Sex	M	F	M	F	M	F	M	F	-
C_{max} ($ng.ml^{-1}$)	91	86	44	52	42	35	84	36	59 ± 24
t_{max} (h)	1.0	3.0	2.0	2.0	1.0	1.0	1.5	1.5	1.6 ± 0.7
$t_{½(1)}$ (h)	1.4	2.5	1.8	-	1.0	0.9	1.2	1.1	1.4 ± 0.6
$t_{½(z)}$ (h)	40	43	49	71	59	28	43	33	46 ± 14
AUC_t ($ng.ml^{-1}h$)	1014	860	607	1481	968	491	898	494	852 ± 329
AUC ($ng.ml^{-1}h$)	1421	1044	888	3335	1225	570	1144	638	1283 ± 877
F (%)	108	103	60	81	71	45	73	49	74 ± 23

Table 5.2b : Curve Fitting Parameters of Almitrine in Healthy Volunteers given the Bismesylate Salt (30mg) Orally as a Solution (Study No. 2)

PARAMETER	SUBJECT							
	1	2	3	4	5	6	7	8
C_1 ($ng.ml^{-1}$)	-1144	-255.5	-223.2	-43.14	-730.1	-359.2	-469.3	-256.0
λ_1 (h^{-1})	4.930	1.830	0.5081	2.422	0.7485	1.000	0.960	0.9796
C_2 ($ng.ml^{-1}$)	111.0	125.1	234.9	-	714.4	313.3	412.9	212.4
λ_2 (h^{-1})	0.4895	0.2748	0.3829	-	0.6671	0.7390	0.5813	0.6530
C_3 ($ng.ml^{-1}$)	21.20	11.55	9.997	29.20	17.32	12.38	15.76	11.39
λ_3 (h^{-1})	0.01722	0.01631	0.01421	0.009711	0.01167	0.02521	0.01629	0.02081

Table 5.2c: Pharmacokinetic Parameters of Almitrine in Healthy Volunteers given the Bismesylate Salt (30mg) Intravenously as a Bolus (Study No. 2)

PARAMETER	SUBJECT								MEAN
	1	2	3	4	5	6	7	8	± SD
$t_{½(1)}$ (h)	0.07	0.07	0.04	0.34	0.04	0.06	0.05	0.05	0.09 ± 0.10
$t_{½(2)}$ (h)	1.1	1.1	0.4	13.9	1.1	1.2	0.9	1.1	2.6 ± 4.6
$t_{½(z)}$ (h)	35	32	25	114	46	43	41	39	47 ± 28
AUC_t ($ng.ml^{-1}h$)	1067	834	1350	1163	1064	956	1105	1033	1072 ± 150
AUC ($ng.ml^{-1}h$)	1321	1018	1491	4125	1733	1267	1575	1311	1730 ± 991
AUC λ_1 (%)	2.0	7.0	1.2	1.0	0.5	3.4	1.8	3.3	2.5 ± 2.1
AUC λ_2 (%)	9.6	12.7	3.2	5.6	5.7	13.9	7.7	13.7	9.0 ± 4.1
AUC λ_3 (%)	88.4	80.3	95.6	93.4	93.9	82.7	90.5	83.0	88.5 ± 5.8
CL ($ml.min^{-1}$)	270	350	239	86	206	281	226	272	241 ± 76
V_i (l)	61	28	46	241	109	36	42	31	74 ± 72
$V_{(area)}$ (l)	822	968	507	849	827	1052	797	908	841 ± 160
$V_{(ss)}$ (l)	729	845	496	1055	857	884	752	757	797 ± 160

Table 5.2d: Curve Fitting Parameters of Almitrine in Healthy Volunteers given the Bismesylate Salt (30mg) Intravenously as a Bolus (Study No. 2)

PARAMETER	SUBJECT							
	1	2	3	4	5	6	7	8
C_1 ($ng.ml^{-1}$)	253	704.8	352.4	61.97	113.8	484.6	402.3	553.2
λ_1 (h^{-1})	9.337	10.66	19.52	2.027	15.80	11.39	14.18	12.69
C_2 ($ng.ml^{-1}$)	77.63	81.01	77.83	8.871	60.00	100.3	87.47	114.7
λ_2 (h^{-1})	0.6133	0.6774	1.696	0.0500	0.6723	0.5762	0.7538	0.6399
C_3 ($ng.ml^{-1}$)	23.02	16.44	39.32	17.82	22.01	16.61	23.35	19.56
λ_3 (h^{-1})	0.0197	0.0217	0.02829	0.006076	0.01494	0.01609	0.01702	0.01798

Table 5.3 : Pharmacokinetic Parameters of Almitrine in Healthy Male Subjects after being dosed Orally with Almitrine Bismesylate as either a Solution (100mg) or Tablet (2 x 50mg)

(Study No. 3)

PARAMETER	SUBJECT												MEAN
	1*	2*	3*	4*	5*	6*	7+	8+	9+	10+	11+	12+	± SD
SOLUTION													
C_{max} (ng.ml^{-1})	182	184	219	378	98	186	160	114	108	118	282	278	192 ± 85
t_{max} (h)	2	2	2	2	3	4	2	2	2	2	2	2	2.3 ± 0.62
$t_{½(1)}$ (h)	-	-	-	-	1.6	1.1	0.4	0.4	0.7	0.5	0.7	0.4	0.73 ± 0.43
$t_{½(2)}$ (h)	1.4	1.1	0.9	1.1	1.3	0.9	1.0	1.1	1.6	1.0	1.4	0.9	1.1 ± 0.23
$t_{½(z)}$ (h)	23	14	19	18	24	15	7	22	17	23	15	21	18 ± 4.9
AUC (ng.ml^{-1}.h)	3344	2206	3926	4442	2304	2957	1273	2470	1763	1992	3802	4944	2951 ± 1143
TABLET													
C_{max} (ng.ml^{-1})	72	146	167	276	151	153	150	138	182	116	177	387	176 ± 82
t_{max} (h)	2	3	1	2	3	3	2	2	4	4	4	2	2.7 ± 0.98
$t_{½(1)}$ (h)	0.8	0.8	-	0.4	0.7	0.6	0.5	0.2	1.0	1.4	1.4	0.7	0.77 ± 0.38
$t_{½(2)}$ (h)	1.2	0.8	2.5	1.1	2.9	3.7	1.0	1.7	2.7	0.9	1.3	1.1	1.7 ± 1.0
$t_{½(z)}$ (h)	29	23	17	12	13	11	16	23	32	14	20	18	18 ± 5.4
AUC (ng.ml^{-1}.h)	2041	3000	2554	2466	2173	2238	1695	2675	3391	1949	3341	4386	2659 ± 761
Bioequivalence (%)	61	136	65	56	94	76	133	108	192	98	88	89	89 ± 44

*Solution followed by tablet +Tablet followed by solution

$t_{½(1)}$ Absorption half-life (- signifies insufficient points to calculate $t_{½}$)

$t_{½(2)}$ Fast disposition phase half-life

$t_{½(z)}$ Terminal half-life

Table 5.4 : Statistical Analysis of the Area Under the Curve ($ng.ml^{-1}.h$) of Almitrine from Two Different Formulations in 12 Healthy Subjects

ANALYSIS OF VARIANCE
VARIABLE : AUC

Source of Variation	Degress of Freedom	Mean Square	F Value	Sig
Dose	1	514508	1.26	NS
Subject	11	1385901	3.41	*
Order	1	1450417	3.57	NS
Reduce	10	405785		

NS denotes no significant difference
* denotes significant at the 5% level

As reported earlier (Chapter 3) in four healthy subjects (2 male and 2 female) receiving a radioactive dose (100mg) of almitrine as a solution, mean C_{max} was 147 ± 70$ng.ml^{-1}$ and this occurred 2.0 hours after dosing. In all of these studies there was a large, intersubject variation in peak plasma levels of almitrine (CV ~40%) and considering that the first pass effect is negligible (see later) this large variation could well be due to differences in absorption.

5.1.2 Food

The rate and extent of absorption of almitrine was investigated in ten healthy male volunteers in the presence and absence of food (Study No. 4). Subjects received almitrine formulated as a 100mg tablet either after an overnight fast or following ingestion of a standardised breakfast according to Melander (1978). This consisted of low fat milk (150ml), orange juice (100ml), one boiled egg, five pieces of crispbread (Ry King 70% wholemeal rye crispbread), orange marmalade (20g), cheddar cheese (20mg) and non-sweetened black coffee or tea (100ml) eaten over 30 minutes. Almitrine bismesylate was taken immediately after breakfast.

Table 5.5 : Pharmacokinetic Parameters of Almitrine in Healthy Male Volunteers Following Oral Administration of Almitrine Bismesylate Tablets (2 x 50mg) in either a Fasting State or After a Standardised Breakfast (Study No. 4)

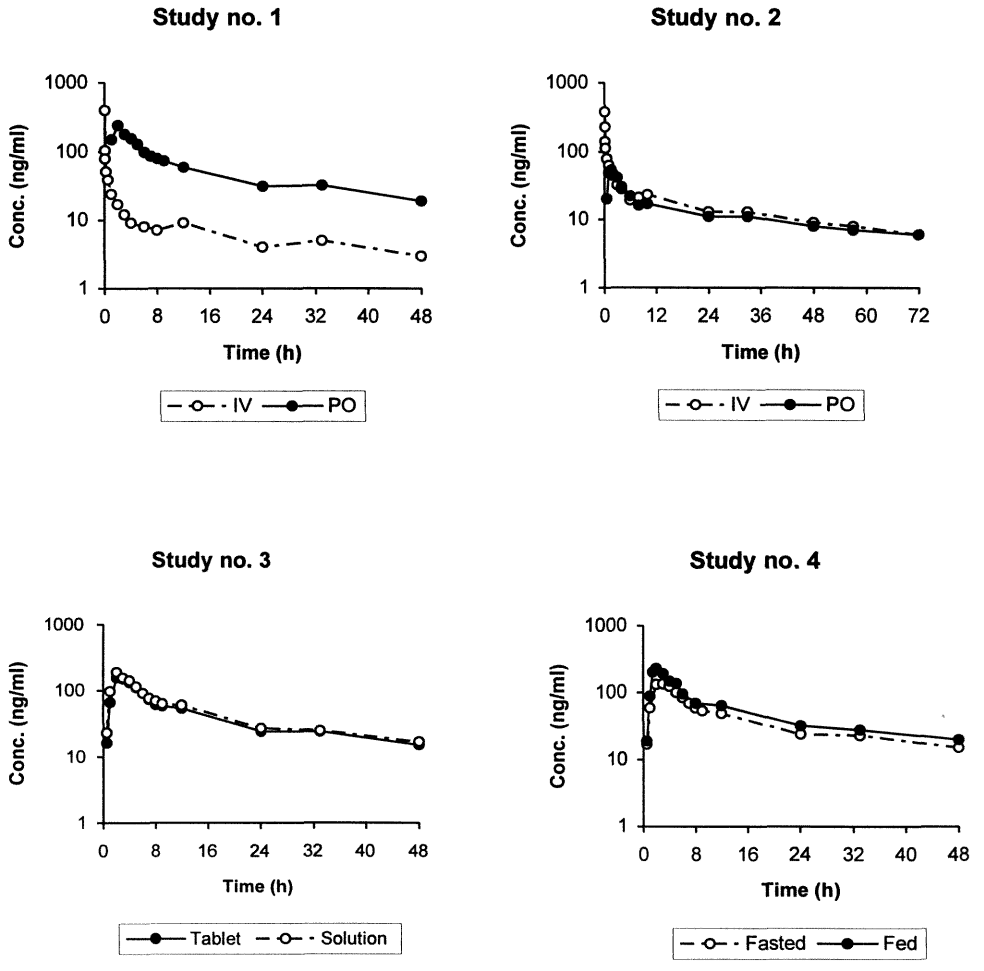
PARAMETER	SUBJECT										MEAN
	1	2	3	4	5	6	7	8	9	10	± SD
STANDARDISED BREAKFAST											
C_{max} (ng.ml^{-1})	329	183	380	143	191	215	230	255	262	298	249 ± 72**
t_{max} (h)	2.0	1.5	2.0	2.0	2.0	1.0	2.0	3.0	1.5	1.5	1.9 ± 0.53
$t_{½\,(1)}$ (h)	0.94	0.25	0.32	0.81	0.20	-	0.23	0.52	0.78	0.57	0.51 ± 0.25
$t_{½\,(2)}$ (h)	4.8	2.1	0.96	1.9	2.3	2.2	1.5	1.9	3.2	2.2	2.30 ± 1.1
$t_{½\,(z)}$ (h)	45	14	13	78	14	26	24	37	48	67	37 ± 23
AUC (ng.ml^{-1}.h)	4752	2016	2603	6630	1785	1928	3266	3855	5258	6734	3883 ± 1887*
FASTING											
C_{max} (ng.ml^{-1})	158	153	276	72	138	116	150	182	177	146	157 ± 52
t_{max} (h)	1	3	2	2	2	4	2	4	4	3	2.70 ± 1.06
$t_{½\,(1)}$ (h)	0.18	0.59	0.47	0.60	0.22	0.71	0.33	0.60	0.61	0.31	0.46 ± 0.19
$t_{½\,(2)}$ (h)	3.14	2.14	0.91	2.85	1.90	1.53	0.64	0.92	2.41	3.20	1.96 ± 0.95
$t_{½\,(z)}$ (h)	34	13	13	60	26	25	17	27	40	53	31 ± 16
AUC (ng.ml^{-1}.h)	2913	2221	2239	2813	2678	1457	1624	3101	3563	3597	2621 ± 734

$t_{½\,(1)}$ Absorption half-life (- signifies insufficient points to calculate $t_{½}$)
$t_{½\,(2)}$ Fast disposition phase half-life
$t_{½\,(z)}$ Terminal half-life
AUC calculated according to the trapezoidal rule

** = Statistical significant difference $p<0.01$
* = Statistical significant difference $p<0.05$

Figure 5.1 : Comparative Plasma Profiles of Almitrine in Healthy Human Subjects

Study 1 : 15mg IV, 100mg PO (solution formulations)
Study 2 : 30mg IV and PO (solution formulations)
Study 3 : Tablet and Solution (100mg)
Study 4 : Effect of Food (100mg Tablet)



In the presence of food, the absorption of almitrine was more rapid, with mean t_{max} reduced from 2.7 ± 1.1h (1 to 4h) to 1.9 ± 0.53h (1.5 to 3h). There was a significant increase ($p<0.01$) in C_{max} after food (mean 249 ± 72ng.ml^{-1}, range 143 to 380ng.ml^{-1}) compared to the fasted condition (mean 157 ± 52ng.ml^{-1}; range 72 to 182ng.ml^{-1} Appendix 5.4a and b,and Table 5.5; Fig. 5.1d). The area under the plasma concentration time curve to infinite time increased by 48% ($p<0.05$) after food suggesting an increased bioavailability in the presence of food, possibly due to an increased absorption from the gut.

5.2 Distribution

5.2.1 Plasma Protein Binding

The *in-vitro* plasma protein binding of [^{14}C]-almitrine bismesylate was performed in control plasma obtained from 8 healthy subjects, 4 hours after a meal. Mean plasma protein binding at 1µg.ml^{-1} was 99.5 ± 0.34% and ranged from 99.3 ± 0.3% to 99.7 ± 0.2% (Table 5.6).

Table 5.6 : *In-Vitro* Plasma Protein Binding of [^{14}C]-Almitrine Bismesylate in Healthy Human Subjects (1µg.ml^{-1})

Subject No.	1	2	3	4	5	6	7	8	Mean
% Bound	99.4	99.3	99.6	99.6	99.3	99.4	99.7	99.7	99.5
±SD	0.29	0.28	0.02	0.09	0.60	0.44	0.00	0.24	0.34

No difference in binding at the same concentration was observed in control plasma obtained after fasting and in the same matrix containing a range of other drugs likely to be co-administered with almitrine to patients suffering from chronic obstructive lung disease (Table 5.7). The binding which ranged from 99.3% in combination with ephedrine hydrochloride and isoprenaline sulphate to 99.7% in combination with codeine, aminophylline and phenylbutazone suggest that none of these drugs were likely to displace almitrine from plasma proteins when co-administered.

Table 5.7 : The Effect of the Addition of Various Drugs to a [^{14}C]-Almitrine/Plasma Protein System on the Binding of [^{14}C]-Almitrine Bismesylate (Almitrine and Drug Spiked at 1μg.ml^{-1})

DRUG	% BINDING OF ALMITRINE
Almitrine only	99.5 ± 0.18
Sulbutamol Sulphate	99.6 ± 0.06
Ephedrine HCl	99.3 ± 0.14
Carbocisteine	99.5 ± 0.06
Sodium Warfarin	99.6 ± 0.02
Isoprenalin Sulphate	99.3 ± 0.27
Codeine	99.7 ± 0.00
Aminophylline	99.7 ± 0.21
Phenylbutazone	99.7 ± 0.03

When compared to other species (Table 5.8), the extent of plasma protein binding of almitrine was similar in human (99.5 ± 0.21%), dog (99.6 ± 0.09%), rabbit (99.6 ± 0.06%) and rat (99.4 ± 0.14%) at 1μg.ml^{-1}. At 10μg.ml^{-1}, plasma protein binding was significantly reduced in human plasma (98.7 ± 0.67%) leading to a doubling of free drug levels, but not in rabbit (99.2 ± 0.41%), rat (99.2 ± 0.14%) or dog plasma (99.6 ± 0.20%).

Table 5.8 : A Species Comparison of the Binding of [^{14}C]-Almitrine Bismesylate to Plasma Protein at Two Different Concentrations

SPECIES	% BINDING	
	1μg.ml^{-1}	10μg.ml^{-1}
HUMAN	99.5 ± 0.21	98.7 ± 0.67
DOG	99.6 ± 0.09	99.6 ± 0.20
RABBIT	99.6 ± 0.06	99.2 ± 0.41
RAT	99.4 ± 0.14	99.2 ± 0.14

5.2.2 Tissue

Despite the high plasma protein binding, almitrine rapidly disappears from blood into tissue after intravenous administration. Following a 12 minute infusion of almitrine bismesylate mean plasma levels fell from 398 ±

170ng.ml^{-1} two minutes after the end of infusion of a 15mg dose to 51 ± 18ng.ml^{-1} 15 minutes later (Study No. 1). The same pattern was seen for a 30mg dose in which mean plasma levels fell from 378 ± 140ng.ml^{-1} two minutes after the end of infusion to 113 ± 37ng.ml^{-1} 15 minutes later (Appendices 5.1 and 5.3b respectively). The initial distribution half-lives ($t_{½(1)}$) were 0.08 ± 0.05 and 0.09 ± 1.0h respectively. The volume of distribution based on area (902 ± 284 litres and 841 ± 160 litres respectively) and on steady-state (1078 ± 386 litres and 797 ± 160 litres respectively) were large (Tables 5.1 and 5.10). The volume of distribution is, however, indirectly a function of the terminal half-life and in these early studies performed only up to 72 hours, almitrine plasma levels at this last time point were well above the limit of quantitation (1ng.ml^{-1}) for the assay. The terminal half-lives (22-24h) obtained from these studies were therefore grossly underestimated. This in turn gave only estimates of other parameters such as volume of distribution and clearance which are calculated using the terminal half-life. It was therefore necessary to perform a study in which almitrine could be infused to a safe steady-state level and plasma collected over a sufficiently long period to allow a more accurate estimation of the terminal half-life of the compound. Using data from early studies, it was possible to calculate an infusion rate to give a plasma concentration which achieved C_{max} approximating that of a 100mg oral dose.

Calculation of the intravenous infusion rate for almitrine to give maximum plasma levels resembling those obtained after a 100mg oral dose

For this calculation it is assumed almitrine has an average clearance (CL) of 241ml.min^{-1}, calculated from bolus intravenous data (Study No. 2), and it peaks at a concentration of 170ng.ml^{-1} (C_{max}) at 2 hours (t_{max}) after oral dosing of 100mg (Study No. 4).

At steady-state: rate in = rate out
= CL x C_{ss}
(ml/min x ng/ml) = ng.min^{-1}
let C_{ss} = C_{max} after oral dosing
rate in = 241 x 170ng.min^{-1}
= 40970ng.min^{-1}
= 40.97µg.min^{-1}

$= 2458.2\mu g.h^{-1}$
$= 2.46mg.h^{-1}$

Taking the longest mean terminal half-life ($t_{½\,z}$) of almitrine as approx 47 hours (IV data from Study No. 2), this steady-state plasma will not be achieved until approx 5 x $t_{½\,z}$ (235h).

In order to calculate the infusion rate to give plasma levels of 170ng at 2 hours, the assumption of a mono-or biphasic decline has to be made:

Monophasic
During the infusion:

$Cp = C_{ss}(1-e^{-\lambda t})$
where $C_{ss} = 170ng.ml^{-1}$

$\lambda = \frac{0.693}{t_{½}} = \frac{0.693}{47} = 0.0148h^{-1}$

time = 2 hours

substituting into the equation:

$Cp = 170(1-e^{-0.0148x2})$
$= 4.96ng.ml^{-1}$

Therefore, adjusting the infusion rate to bring the plasma levels at 2 hours from $4.96ng.ml^{-1}$ to $170ng.ml^{-1}$ the following is obtained:

Final infusion rate $= \frac{2.46}{4.96} \times 170mg.h^{-1}$
$= 84.3mg.h^{-1}$
$= 168.6mg$ infused total

This is obviously considerably higher than the 100mg oral dose which achieves the same maximum plasma levels at the same time. This indicates, therefore, that the assumption that the kinetics approximates to a mono-exponential decline is wrong. An examination of the oral profiles of almitrine does, in fact, show a pronounced 'nose'.

Biexponential Decline

This is more complicated and requires a compartmental analysis of the data. For convenience, mean plasma level data is used for this estimation. The equation used for the calculation is:

$$Cp = \frac{R_o}{V_i}\left[\frac{C_1^1(1-e^{\lambda_i t})}{\lambda_i} + \frac{C_2^1(1-e^{\lambda_2 t})}{\lambda_2}\right]$$

where R_o is the infusion rate ($2.46mg.h^{-1}$), V_i is the initial distribution volume (177 litre), λ_1 is the first exponential constant ($0.0204h^{-1}$), λ_2 is the second exponential constant ($1.132h^{-1}$), $C1_1$ is the first dose normalised coefficient (0.15), $C1_2$ is the second dose normalised coefficient (0.85) and t is time (2h).

The dose normalised coefficients ($C1_i$) are obtained by:

$$C_1^1 = \frac{C_i}{\sum C_i}$$

For the almitrine mean intravenous bolus data this equation is:

$$Cp = 143.45e^{-1.132t} + 25.80e^{-0.0204t}$$

therefore $$C_i^1 = \frac{25.80}{(143.45 + 25.80)} = 0.15$$

$$C_2^1 = \frac{143.45}{(143.45 + 25.80)} = 0.85$$

substituting the figures into the original equation:

$$Cp = \frac{(2.46x1000x1000)}{(177x1000)}$$

$$\frac{0.15}{0.0204}(1-e^{-0.0204x2}) + \frac{0.85}{1.132}(1-e^{-1.132x2})$$

$$= 13.4ng.ml^{-1}$$

Thus at 2 hours after an infusion rate of 2.46mg.h^{-1}, plasma levels of 13.4ng.ml^{-1} would be expected. Thus adjusting the infusion rate to bring the plasma levels at 2 hours to 170ng.ml^{-1}.

$$\text{Final infusion rate} = \frac{2.46}{13.4} \times 170\text{mg.h}^{-1}$$

$$= 31.2\text{mg.h}^{-1}$$

or 62.4mg over the 2 hour period

This would seem more realistic and is consistant with the known absolute bioavailability of almitrine. It was therefore recommended that an infusion rate of 30mg.h^{-1} over a 2 hour period is used.

Based on these calculations, three healthy male subjects received an intravenous infusion dose of almitrine bismesylate at an infusion rate of 30mg.h^{-1} over 2 hours (Study No. 5). Blood samples were obtained for as long as conveniently possible (up to 912 hours, 38 days) after dosing and plasma was analysed for unchanged almitrine by GC/NPD (Appendix 5.5).

The mean plasma concentration after the end of infusion was 327± 87ng.ml^{-1} (242-415ng.ml^{-1}) and a mean C_{max} of 353± 92ng.ml^{-1} was achieved at 1.5 ± 0.5h after the start of infusion. Both C_{2h} and C_{max} were higher than that predicted for the infusion rate used. Once infusion had stopped plasma levels of almitrine declined rapidly in a triphasic manner (Fig. 5.2b). There was a mean initial distribution half-life of 1.0± 0.55h and an initial elimination half-life of 39 ± 13h which was similar to the terminal half-lives reported for the short term studies. A larger volume of distribution was obtained for all subjects and this varied between 3108 ± 245 litres based on area and 3128 ± 1200 litres based on steady-state (Table 5.9a). These large volumes of distribution show extensive distribution of the drug out of plasma into tissues and reflect the high lipophillic nature of the compound.

An interesting observation from the plasma levels was secondary peaks associated with food, particularly on the day after dosing (Fig. 5.2a). It can be postulated that these are caused by the food stimulating the emptying of bile, containing biliary excreted almitrine, from the gall bladder into the gastro-intestinal tract, and the subsequent absorption of the almitrine which has

Table 5.9a : Pharmacokinetic Parameters Of Intravenously Infused Almitrine Bismesylate In Healthy Volunteers (Study No. 5)

PARAMETER	SUBJECT			MEAN ± SD
	1	2	3	
Age (yrs)	41	35	31	36 ± 5.0
Body Weight (kg)	60	76	66	67 ± 8.1
Total Dose (mg)	60.65	61.56	61.98	61.40 ± 0.68
C_{2h} ($ng.ml^{-1}$)	415	242	323	327 ± 87
C_{max} ($ng.ml^{-1}$)	456	280	323	353 ± 92
t_{max} (h)	1.50	1.00	2.00	1.50 ± 0.50
$t_{½(1)}$ (h)	0.66	0.71	1.63	1.00 ± 0.55
$t_{½(2)}$ (h)	29	35	55	39 ± 13
$t_{½(z)}$ (h)	721	398	724	614 ± 187
Trapezoidal AUC_t ($ng.ml^{-1}.h$)	8551	6579	10964	8698 ± 2196
Trapezoidal AUC ($ng.ml^{-1}.h$)	13355	7854	16164	12458 ± 4227
Integrated AUC_t ($ng.ml^{-1}.h$)	8462	6448	10635	8515 ± 2094
Integrated AUC ($ng.ml^{-1}.h$)	13767	7820	16288	12625 ± 4348
$AUC_{\lambda 1}$ (%)*	4.76	8.18	3.52	5.49 ± 2.41
$AUC_{\lambda 2}$ (%)*	12.76	22.16	13.40	16.11 ± 5.25
$AUC_{\lambda z}$ (%)*	82.48	69.67	83.09	78.41 ± 7.58
CL ($ml.min^{-1}$)	52.35	93.55	45.22	63.71 ± 26.09
$V_{(d)}$ (l)	3272	3226	2826	3108 ± 245
$V_{(ss)}$ (l)	2729	4477	2178	3128 ± 1200

* Area under the curve associated with each decline phase.

Figure 5.2 : Plasma Profiles of Almitrine in Healthy Human Subjects Following Intravenous Infusion of Almitrine Bismesylate (60mg) Over 2 Hours

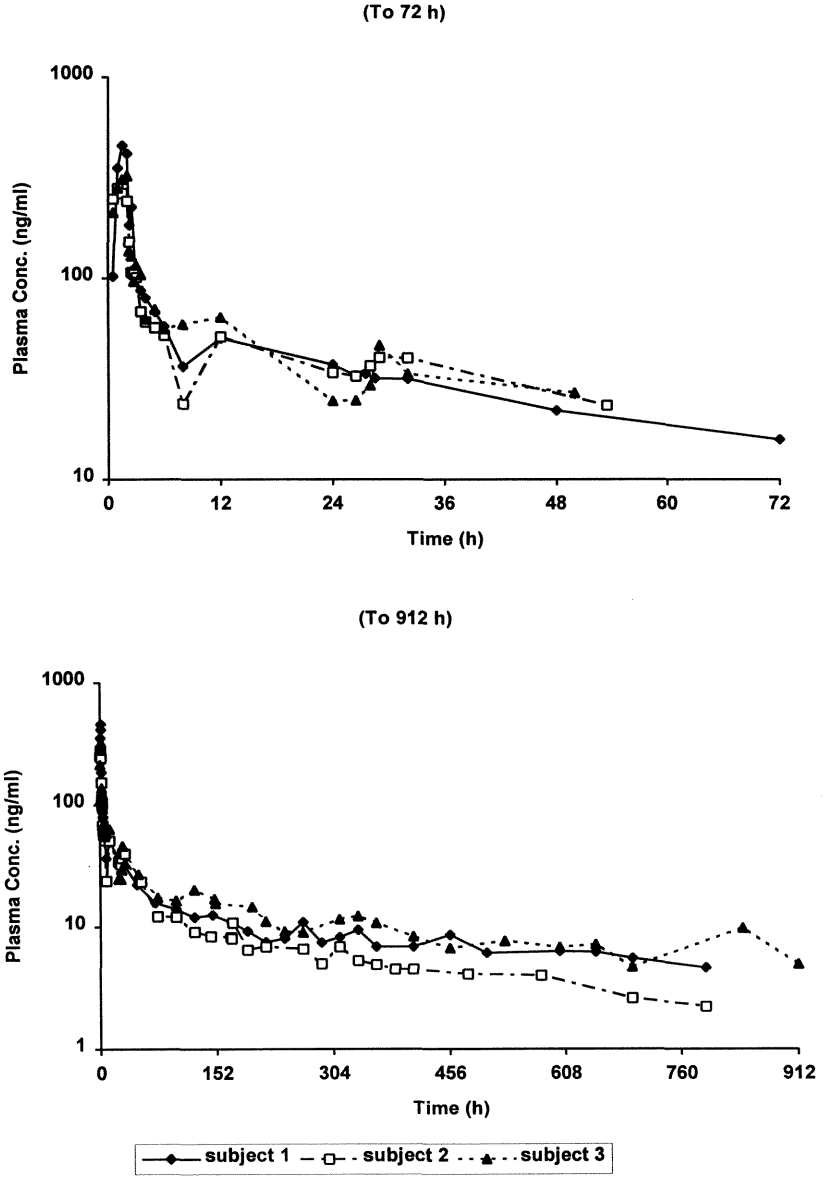


Table 5.9b: Curve Fitting Parameters for Almitrine Bismesylate Given to Healthy Volunteers as a Constant Rate Infusion (Approx 30mg.h^{-1}) (Study No. 5)

The Fits assume a zero order input

PARAMETER	SUBJECT		
	1	2	3
C_1 (ng.ml^{-1})	1742.75	1023.94	246.71
λ_1 (h^{-1})	1.04938	0.98150	0.42438
C_2 (ng.ml^{-1})	42.60	35.11	26.81
λ_2 (h^{-1})	0.02379	0.02006	0.01268
C_3 (ng.ml^{-1})	10.91	9.50	12.58
λ_3 (h^{-1})	0.00096	0.00174	0.00096
Correlation coefficient (r)	0.977	0.985	0.939

entered the intestinal tract (ie enterohepatic circulation). However, unpublished work has shown that in both biliary cannulated rats and patients given a T-cannula, [^{14}C]-almitrine was not eliminated into the bile after I.V. administration. Examination of the data from this study shows that for two of the subjects (2 and 3), these secondary peaks are large whereas for the third (1), they are very small. It is noteworthy that both subjects 2 and 3 had a large meal with a normal fat content whereas subject 1 had a light meal composed of coffee and fruit. Both types of meal should be sufficient to promote the emptying of the contents of the gall bladder into the gastro-intestinal tract and hence cause secondary peaks of almitrine. It seems more probable, therefore, that another mechanism causes these secondary peaks. As discussed previously (Chapter 3), almitrine is directly secreted into the gut and although a direct effect on the absorption of almitrine cannot be excluded at the early times after dosing, the most likely mechanism is a redistribution phenomenon such as that caused by competition for plasma protein binding sites between almitrine and free fatty acids and the subsequent redistribution of almitrine from tissues into plasma. This phenomenon is not uncommon for drugs which distribute extensively into tissues and show a high affinity to albumin.

5.3 Elimination

The parameters relating to the elimination of almitrine appear to be dependent on the length of the sampling time (Table 5.10). When sampled for up to 72 hours, the terminal half-life ($t_{½z}$) ranged from 7 hours (Table 5.3b) to 114 hours (Table 5.4c). In the 2 hour infusion study (Study No. 5) in which sampling continued for up to 912 hours (792-912h), the mean $t_{½(z)}$ of 614 ± 187h varied between 398 and 724 hours. As a consequence, the plasma clearance, which is a function of $t_{½(z)}$ varied from 45ml.min^{-1} to 94ml.min^{-1} (mean 64 ± 26ml.min^{-1}, Table 5.9a). This low clearance varied markedly from those estimated in the short collection studies with mean clearances of 241 ± 76ml.min^{-1} for sample collection terminated after 72 hours and 491 ± 220ml.min^{-1} where a 48 hour sampling procedure was employed. A better estimation of the true terminal half-life of almitrine was achieved from the long collection study described in Chapter 3. A mean $t_{½(z)}$ of 1163 ± 576 was calculated following oral administration of a 100mg dose in solution and sample collection for up to 2232 hours (93 days). It is important to note that even 38 days after the 2 hour infusion, and 93 days after an oral solution, almitrine was still measurable. To adequately define a log-linear phase, blood

Table 5.10 : Summary of the Pharmacokinetic Parameters of Almitrine in Man

Formulation Study No	Solution Study 1		Solution Study 2		Tablet/Solution Study 3		Tablet (PO) Study 4		Solution Study 5	Solution Study 6
Route	PO	IV(12 min)	PO	IV (12 min)	PO (Sol)	PO (Tab)	Fasted	Fed	IV (2h)	PO*
n	4	4	8	8	12	12	10	10	3	4
Duration (h)	48	48	72	72	48	48	48	48	792-912	480-2232
Dose (mg)	100	15	30	30	100	100	100	100	60	100
C_{max} ($ng.ml^{-1}$)	241±93	398±170	59±24	378±140	192±85	176±82	157±52	249±72	353±92	147±70
t_{max} (h)	2.0±0.0	0.03±0.0	1.6±0.70	0.03±0.0	2.3±0.62	2.7±0.98	2.7±1.1	1.9±0.53	1.5±0.50	2.0±0.0
$t_{½(1)}$ (h)	-	0.08±0.05	-	0.09±1.0	0.73±0.43	0.77±0.38	0.46±0.19	0.51±0.28	1.0±0.55	2.75±1.79
$t_{½(2)}$ (h)	2.8±0.33	0.90±0.30	1.4±0.60	2.6±4.6	1.1±0.23	1.7±1.0	2.0±0.95	2.3±1.1	34±12	39±13
$t_{½(z)}$ (h)	33±8.5	22±5.4	46±14	47±28	18±4.9	18±5.4	31±16	37±23	614±187	1163±576
AUC_t ($ng.ml^{-1}.h$)	2556±652	312±140	852±329	1072±150	-	-	-	-	8698±2196	4668±2251
AUC ($ng.ml^{-1}.h$)	3506±980	411±149	1283±877	1730±991	2951±1143	2659±761	2621±734	3883±1887	12858±4227	7660±3774
F (%)	132±19	-	74±23	-	-	89±44**	-	-	-	-
CL ($ml.min^{-1}$)	-	491±220	-	241±76	-	-	-	-	64±26	-
$V_{(d)}$ (l)	-	902± 284	-	841±160	-	-	-	-	3108±245	-
$V_{(ss)}$ (l)	-	1078±386	-	797±160	-	-	-	-	3128±1200	-

* From Radioactive Study

** Bioequivalence

least 3 half-lives and as can be seen from these two studies, the duration was probably still insufficient to adequately define the terminal half-life of almitrine. Taking the 1163 hour value, a sampling time of at least 145 days (3489 hours) would be required if the terminal half-life does not increase further.

Despite the probable error on the terminal half-life, it is readily apparent that the clearance of almitrine (64 ± 26ml.min^{-1}) is considerably lower than the short collection studies had earlier indicated. However, clearance tends to be relatively insensitive to error compared with some of the other pharmacokinetic parameters, and even if the true half-life is considerably longer (eg 60 days or 1440 hours), the clearance would only be marginally less (25% or ~50ml.min^{-1}).

5.4 Interactions

Sufferers from chronic obstructive airway disease (COAD) invariably develop infections which require antibiotic therapy. Because of the tremendous strain on the heart, cardiac problems will be encountered by these patients, and these will require drug treatment. The possible pharmacokinetic interaction of repeated oral dosing of almitrine bismesylate (50mg b.i.d.) on the oral antibiotic, erythromycin (500mg) and the cardiac glycoside, digoxin (0.25mg) two products used in COAD was examined in six healthy male volunteers. In the same study, since almitrine was administered repeatedly, antipyrine (500mg) was used to determine whether almitrine induced or inhibited the metabolic clearance of antipyrine (i.e. does almitrine cause its own induction or inhibition of metabolism on repeated administration).

Antipyrine (500mg) was first given after an overnight fast and saliva samples (5ml) were collected, up to 33 hours after dosing, for analysis of antipyrine by GC (based on the method described by van Boxtel *et al.*, (1976); LOQ 1.0µg.ml^{-1}). Forty eight hours after antipyrine administration, the same subjects received a tablet (500mg) of erythromycin and blood samples (15ml) were collected, up to 10 hours after dosing, for analysis of erythromycin by a microbiological assay according to the method described by Illiopoulou*et al.*, (1981); (LOQ 0.05µg.ml^{-1}). Each subject then received a tablet (0.25mg) of digoxin 48 hours following erythromycin administration and blood (0.5ml) was collected, up to 72 hours after dosing, for analysis of digoxin by radioimmunoassay (Serono Diagnostics, Woking, UK); (LOQ 0.5ng.ml^{-1}).

Seven days after digoxin administration, each subject started a dosage regimen of almitrine bismesylate (50mg b.i.d.) with a tablet taken at 9am and 9pm respectively for 25 days. Blood samples (5ml) were taken 2 hours after the first dose of almitrine on certain days. On day 14 of repeated dosing six further blood samples were taken throughout the day and on day 15 the antipyrine, erythromycin and digoxin regimen and sampling were repeated. Finally, after the last almitrine dose, blood samples were taken at selected times up to 72 hours after the dose. All blood samples were centrifuged to obtain plasma for drug analysis (erythromycin, digoxin and almitrine).

All of these products were well tolerated by the subjects with no reports of major side effects or missed doses. Minor observations during repeated almitrine dosage included increased sweating, especially with alcohol (2 subjects), a feeling of being hot at night (3 subjects) and a sensation of light headiness after carbonated drinks (1 subject).

Following repeated administration of almitrine bismesylate at 2 x 50mg b.i.d., mean 2 hour plasma levels of almitrine were 69 ± 47ng.ml^{-1} on day 1 and 205 ± 41ng.ml^{-1} on day 29 of the dosing regimen (Fig. 5.3). Plasma levels were variable between and within subjects and ranged from as low as 10ng.ml^{-1} for one subject on day 1 to as high as 473ng.ml^{-1} for another subject on day 19 (Appendix 5.6). These levels on repeated dosing fell within the range of those seen in patients receiving a similar dosage regimen. On day 15 of almitrine treatment, when samples were collected over a 24 hour period, mean C_{max} levels of 230 ± 60ng.ml^{-1} were obtained at 3.2 ± 2.4h after the respective almitrine dose, whereas after the final dose of almitrine bismesylate, C_{max} levels of the drug were similar at 236 ± 37ng.ml^{-1}, occurring later at 4.3 ± 2.9 hours after the final dose (Table 5.11). Half-life determination of almitirne measured over a 72 hour period after the final dose was not performed since mean plasma levels at 72 hours were 105 ± 14ng.ml^{-1} (81 - 123ng.ml^{-1}).

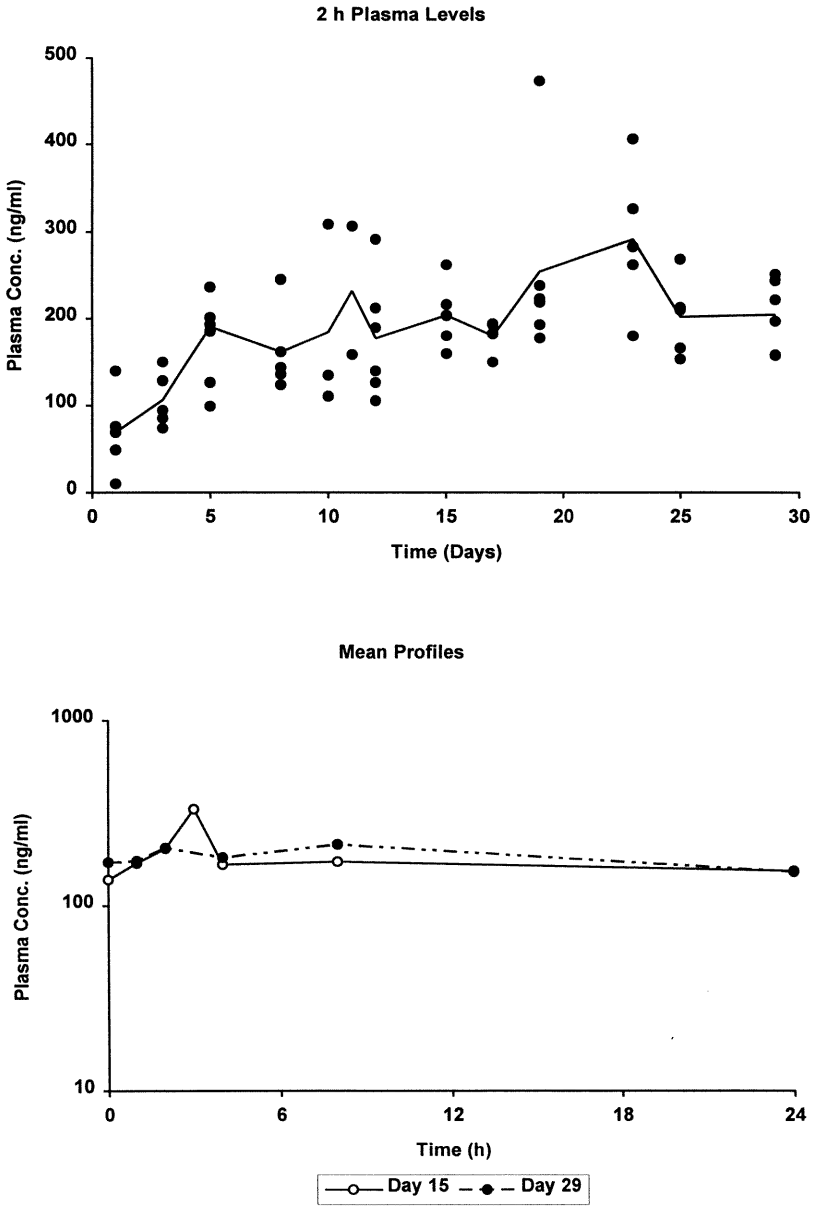
The first saliva sample for antipyrine determination was collected 3 hours after the antipyrine dose to avoid any possible contamination of the sample from that dose. As a consequence, combined with the rapid absorption of antipyrine, C_{max} of the drug always occurred with the initial (3 hour) sample with mean values of 17.9 ± 1.6 and 18.4 ± 4.0µg.ml^{-1} before and during almitrine dosing respectively (Appendix 5.7 and Table 5.12; Fig. 5.4). With no absorption data,

Table 5.11 : Summary of the Pharmacokinetic Parameters of Almitrine in Subjects given the Drug Repetitively (50mg b.i.d.)

SUBJECT	C_{max} ($ng.ml^{-1}$)		t_{max} (h)		AUC_{24} ($ng.ml^{-1}.h$)	
	D15	D29	D15	D29	D15	D29
1	159	222	2	2	3451*	4487*
2	262	251	2	2	4454	4518
3	204	244	2	2	3552	4609
4	203	256	2	8	3683	5287
5	332	169	3	4	4200	3361
6	216	273	8	8	4756	4490
MEAN	230	236	3.2	4.3	4015	4459
± SD	60	37	2.4	2.9	531	62

* AUC_{24} estimated

Figure 5.3 : 2 Hour Plasma Levels of Almitrine and Mean Profiles (Day 15 and Day 29) of Almitrine in Healthy Subjects Receiving Daily Doses (50mg b.i.d.) for Almitrine Bismesylate



a zero order (constant rate) was used as the input function followed by a single exponential to describe the decline kinetics (Fig. 5.4). This gave a half-life of 12 ± 2.9 hours during almitrine treatment which compared to 11 ± 3.2 hours before almitrine. Similar AUCs of 253 ± 21 and 266 ± 52μg.ml^{-1}.h were obtained before and during almitrine treatment. Analysis of variance showed no significant overall difference between any of these parameters before and during almitrine treatment.

For erythromycin, C_{max} of 1.3 ± 0.7μg.ml^{-1} before almitrine and 1.1 ± 0.6μg.ml^{-1} during almitrine treatment were achieved at 1.3 ± 0.61 and 1.5 ± 0.45h respectively (Appendix 5.8 and Table 5.12). In all cases, once C_{max} levels were reached they declined monoexponentially (Fig. 5.4) with mean half-lives of 1.65 ± 0.26h before almitrine treatment and 2.16 ± 0.68h during almitrine treatment. This relatively short half-life meant that the elimination of erythromycin was virtually complete over the duration of the sampling period used so that AUC_t of 5.2 ± 3.9 and 4.0 ± 1.9μg.ml^{-1}.h respectively only increased minimally to 5.4 ± 4.0 and 4.3 ± 1.9μg.ml^{-1}.h respectively when extrapolated to infinity (Table 5.12). Again ANOVA showed no significant differences between any of the measured pharmacokinetic parameters for erythromycin before and during almitrine treatment.

The absorption of digoxin was rapid with C_{max} of 2.5 ± 0.8 and 2.9 ± 1.1ng.ml-^{1} before and during almitrine treatment respectively occurring 1.33 ± 0.61 and 1.25 ± 0.5h respectively (Appendix 5.9 and Table 5.12). There was a biphasic decline after C_{max} with an initial rapid phase before and during almitrine of 1.06 ± 0.37 and 1.08 ± 0.23 hours respectively and terminal half-lives of 57 ± 20 and 50 ± 12h respectively (Fig. 5.4). AUCs of 48 ± 20 and 37 ± 7.7μg.ml^{-1}.h respectively increased by approximately 50% from the AUC_t (28 ± 5.1 and 25 ± 3.1μg.ml^{-1}.h) respectively. Again, none of these parameters were significantly different when analysed by ANOVA.

The potential for drug-drug interactions in normal clinical use is extremely high since polypharmacy is a common medical practice, especially with the more seriously ill patients. Such a group of patients is those likely to be receiving almitrine bismesylate since this drug is used for the treatment of chronic bronchitis and emphysema. Thus, these patients will probably also be receiving concomitant treatment for complications associated with chronic respiratory disease such as respiratory infections and/or cardiovascular

Table 5.12 : Summary of Pharmacokinetics of Three Compounds which have been Administered Before (A) and During (B) Almitrine Repeated Dosing

ANTIPYRINE

SUBJECT	C_{max} (µg.ml^{-1})		$t_{½}$ (h)		AUC_t (µg.ml^{-1}.h)		AUC (µg.ml^{-1}.h)	
	A	B	A	B	A	B	A	B
1	19.0	16.3	8.7	7.7	253	213	275	234
2	16.5	14.0	15.5	12.7	292	235	386	281
3	16.7	22.1	9.4	9.1	233	246	256	271
4	19.9	20.1	10.2	14.1	252	343	282	432
5	19.1	23.3	9.4	10.4	252	319	290	363
6	16.2	14.4	15.6	15.1	236	239	308	307
MEAN	17.9	18.4	11.5	11.5	253	266	300	313
SD	1.6	4.0	3.2	2.9	21	52	46	74
ANOVA	NS	NS	NS	NS	NS	NS	NS	NS

ERYTHROMYCIN

SUBJECT	C_{max} (µg.ml^{-1})		t_{max} (h)		$t_{½}$ (h)		AUC_t (µg.ml^{-1}.h)		AUC (µg.ml^{-1}.h)	
	A	B	A	B	A	B	A	B	A	B
1	0.7	0.3	1.0	2.0	1.9	1.6	2.1	1.6	2.2	1.8
2	1.2	1.3	1.0	1.5	1.6	1.9	4.1	4.6	4.3	4.8
3	0.6	0.8	2.0	1.5	1.4	3.2	1.4	2.9	1.5	3.7
4	2.0	1.3	0.5	1.0	2.1	2.8	10.5	6.0	11.0	6.4
5	1.3	0.9	1.5	1.0	1.6	1.7	3.3	2.5	3.5	2.6
6	2.2	2.2	2.0	2.0	1.5	1.7	9.5	6.3	9.9	6.4
MEAN	1.3	1.1	1.3	1.5	1.7	2.2	5.2	4.0	5.4	4.3
SD	0.7	0.6	0.61	0.45	0.26	0.68	3.9	1.9	4.0	1.9
ANOVA	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

NS denotes Not Significant at the 5% level

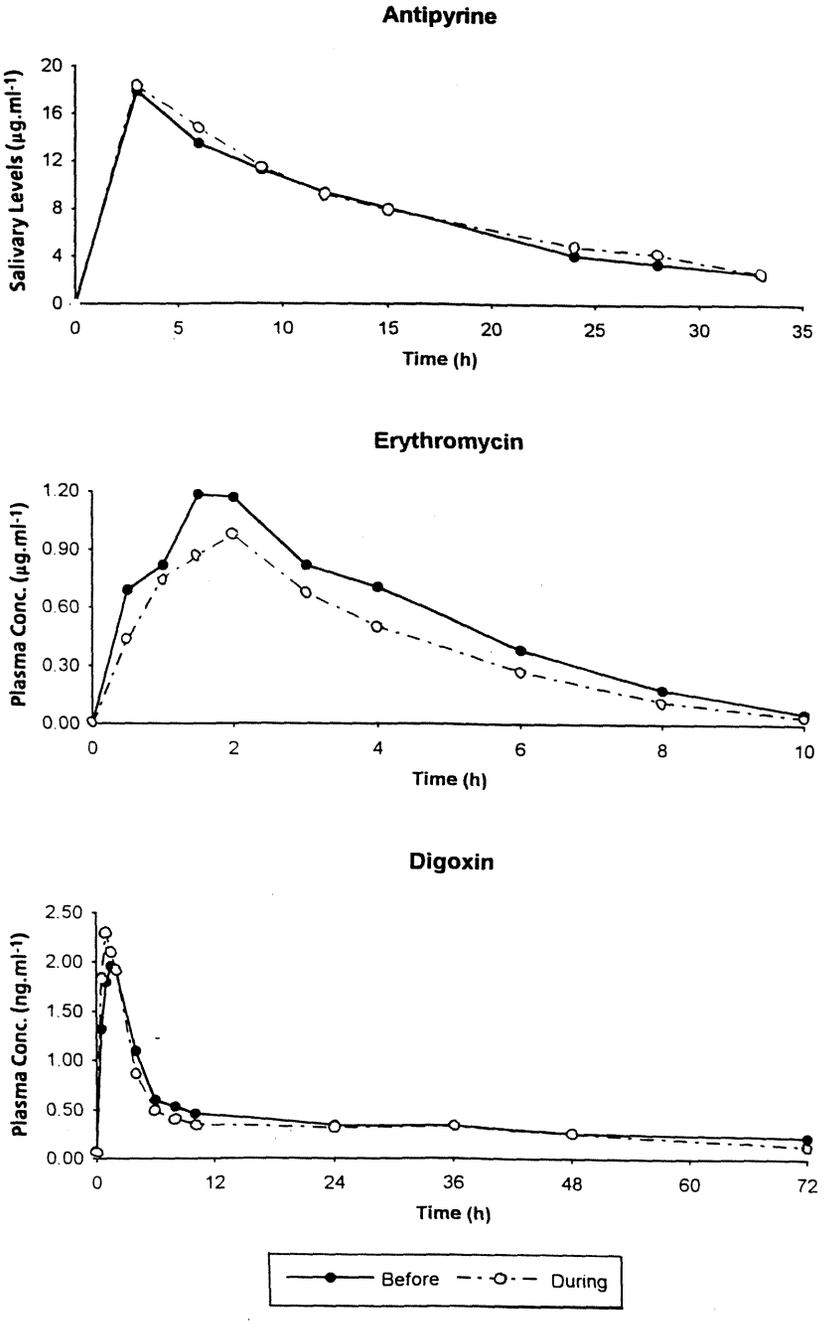
Table 5.12 (Cont) : Summary of Pharmacokinetics of Three Compounds which have been Administered Before (A) and During (B) Almitrine Repeated Dosing

DIGOXIN

SUBJECT	C_{max} ($\mu g.ml^{-1}$)		t_{max} (h)		$t_{½}$ (h)		AUC_t ($\mu g.ml^{-1}.h$)		AUC ($\mu g.ml^{-1}.h$)	
	A	B	A	B	A	B	A	B	A	B
1	2.5	2.6	0.5	1.5	60	54	21	22	43	43
2	2.2	2.5	2.0	1.0	32	38	28	24	37	30
3	4.1	4.0	1.5	1.0	86	44	32	29	68	38
4	2.0	1.5	1.0	2.0	54	47	35	23	56	31
5	2.0	2.4	2.0	1.5	70	46	24	25	40	32
6	2.4	4.4	1.0	0.5	40	74	28	29	41	50
MEAN	2.5	2.9	1.3	1.3	57	51	28	25	48	37
SD	0.8	1.1	0.61	0.52	20	12	5.1	3.1	12	7.7
ANOVA	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

NS denotes not significant at the 5% level

Figure 5.4 : Mean Plasma Profiles of Antipyrine, Erythromycin and Digoxin in Healthy Subjects Before and During Repeated Administration of Almitrine Bismesylate (50mg b.i.d.)



disorders. Two of the most common drugs likely to be co-prescribed with almitrine are the antibiotic, erythromycin and the cardiac glycoside, digoxin, both of which are known to have a narrow therapeutic index due to the narrow range of plasma levels where the drugs are effective without producing undesirable side effects. The risk of digoxin toxicity in particular is known to be increased by hypoxia (Morrison and Killip,1971). As a consequence, these two drugs have been extensively studied and the relationship between their respective toxicities and plasma levels has been well defined so it is now possible to use the pharmacokinetics of erythromycin and digoxin to predict possible drug-drug interactions which could be of clinical significance.

Pharmacokinetic drug interactions can occur at many different sites including the gastrointestinal tract with possible alterations in gastric emptying time, gastrointestinal motility, pH, gut flora (especially with an antibiotic such as erythromycin) and splanchnic blood flow, as well as complexation between two drugs. In addition, distributional changes can occur with alterations in tissue and/or plasma protein binding. Elimination of one drug can also be altered by the action of another drug either by changing the renal, biliary or metabolic clearance. Since the mean steady-state plasma levels of a drug on a repeated dosage regimen, such as used with erythromycin and digoxin, is a function of their rate of input and clearance, the most important drug interactions, clinically, tend to be those which affect either absorption or the elimination processes. Consequently, this present study was designed to determine the effect, if any, of almitrine upon the pharmacokinetics, especially with respect to the absorption and elimination, of erythromycin and digoxin when almitrine was administered in its normal dosage regimen (50mg b.i.d.) and when the plasma levels of the drug were within the normal therapeutic range. Also, whilst repeat doses of almitrine bismesylate were being given, the opportunity was taken to investigate whether chronic almitrine treatment could cause either induction or inhibition of oxidative metabolism of other drugs by examining the pharmacokinetics of antipyrine before and during the dosage regimen of almitrine. It was noteworthy, however, although not a primary objective of the study since almitrine has quite a wide therapeutic index, that none of the drugs tested had any detectable influence on the plasma levels of almitrine.

Antipyrine is probably the most widely used drug for the determination of the *in-vivo* drug hydroxylation status since it is exclusively eliminated by oxidative metabolism (Branch *et al.*, 1973; Homeida *et al.,* 1979; Branch, 1982) and has a very low protein binding (<10%). Thus changes in half-life due to changes in volume of distribution are minimal and any alteration of antipyrine clearance as a result of induction/inhibition by another drug is directly reflected by a corresponding change in its half-life. In this present study, determining salivary levels of antipyrine, which measures the unbound concentration of the drug, there were no statistically significant differences in the maximum saliva concentration, half-life or area under the curve showing that repeated doses of almitrine are neither an inducer or inhibitor of drug hydroxylation reactions. Thus, it can be concluded that almitrine will not affect the elimination of those drugs which are primarily cleared by oxidative metabolism such as warfarin and theophylline.

Theophylline is known to interact with erythromycin (Renton *et al.,* 1981) and although the mechanism of the interaction still has to be fully elucidated, it is thought to be by an action on hepatic blood flow. In this present study, no such interaction could be seen between almitrine and erythromycin. This finding was also of interest because both almitrine metabolites and intact erythromycin are extensively cleared in the bile and the lack of interaction between these two drugs shows that there is minimal or no competition for the biliary elimination processes. Also, since erythromycin is acid-labile, the lack of interaction between almitrine and the antibiotic would suggest that almitrine has little or no effect on gastric emptying which could have important implications when other acid labile drugs may be prescribed.

Digoxin, unlike antipyrine and erythromycin, is primarily cleared via the kidneys (60-80% Reuning *et al.*, 1973), although a significant proportion is also cleared non-renally, probably via the bile. Its distribution and clearance is known to be altered by quinidine (Hager *et al.*, 1979; Doering *et al.*, 1982). Again, because of the high biliary clearance of almitrine metabolites, combined with the very narrow therapeutic index of digoxin, it was thought desirable to determine the effect of almitrine upon the pharmacokinetics of this cardiac glycoside. As before, no effect could be seen on the maximum plasma levels, time to maximum plasma levels, area under the curve and half-life of digoxin showing

that almitrine has no effect either the absorption, distribution or elimination of digoxin.

In summary, almitrine bismesylate when administered in the same dosage regimen as used clinically and given plasma levels within the therapeutic range, had no effect on the pharmacokinetics of antiyprine, erythromycin and digoxin. It can be concluded, therefore, that, not only is there no pharmacokinetic interaction between almitrine and these drugs, but that almitrine is neither an inducer or inhibitor of drug hydroxylation reactions.

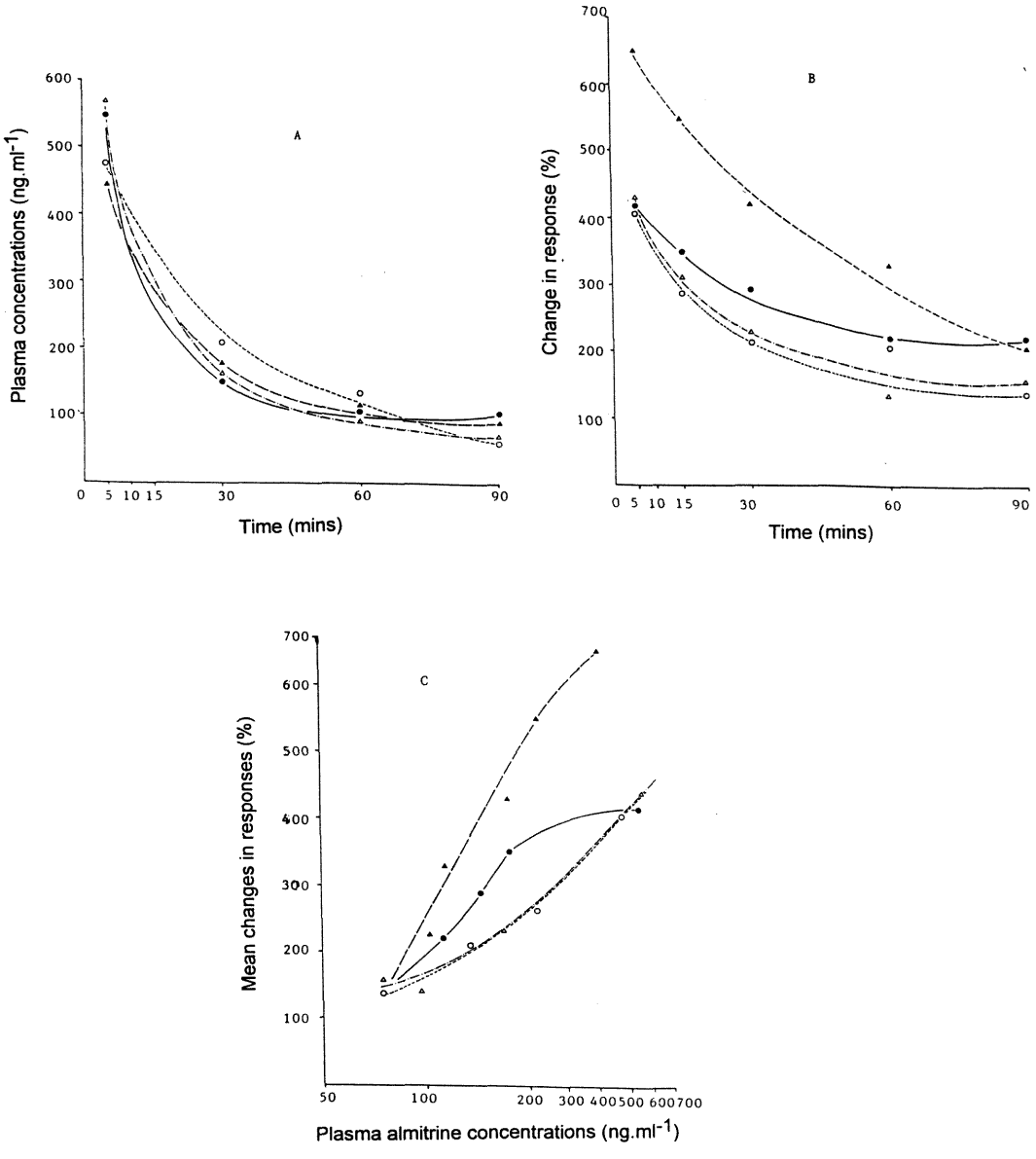
Overall, the absorption of almitrine, given either as a tablet or solution, is rapid with large intersubject variability in absorption. Co-administration of food increases both the rate and extent of absorption of almitrine. The plasma protein binding is high with a large volume of distribution and long elimination half-life which is consistant with a low total clearance of the compound. There is no interaction between almitrine and drugs such as digoxin and erythromycin, both of which are known to have a narrow therapeutic index. Repeated administration of almitrine does not lead to induction or inhibition of its own metabolism.

5.5 Pharmacokinetic and Pharmacodynamic Interactions

5.5.1 Animal studies

The effect of a bolus unjection of almitrine bismesylate (0.5mg.kg^{-1}, IV) on chemoreceptor discharge in cats anaesthetised with pentobarbitone (42mg.kg^{-1}, IP) and artificially ventillated was investigated (Campbell *et al.*, 1988; McQueen *et al.*, 1989). Carotid discharge in response to almitrine was greater and lasted longer when the ganglioglomerular nerves were cut, compared to the intact preparation. This difference in chemoreceoptor discharge was not due to difference in plasma almitrine concentration, since average plasma concentrations of almitrine in the GGN intact nerve (552 ± 48ng.ml^{-1}) were similar to that measured in the cut preparation (446 ± 76ng.ml^{-1}) although carotid discharge increased 363 ± 41% in the intact preparation 5 minutes after almitrine injection (0.5mg.kg^{-1}). In the cut preparation, almitrine produced a significantly higher discharge (805 ± 113%, $p<0.01$) after a similar administration. Both preparations showed a correlation between increased chemoreceptor discharge and plasma concentration of

Figure 5.5 : A: Mean Plasma Levels of Almitrine Following Single IV Injections of Almitrine Bismesylate (0.5mg.kg^{-1}) in Animals; B : Mean Percentage Change in Carotid Chemoreceptor Response; C: Response Curves Relating Drug Levels and Chemoreceptor Response



Control (n=5) o GGN cut (n=4) ▲ contralateral carotid nerve cut (n=4) ●
and naloxone (5mg.kg^{-1}, i.v.) 5 min before almitrine (n=2) Δ

almitrine (Fig. 5.5). This shows, for the first time, a non-competitive inhibitory feedback regulation of breathing through the ganglioglomerular nerve.

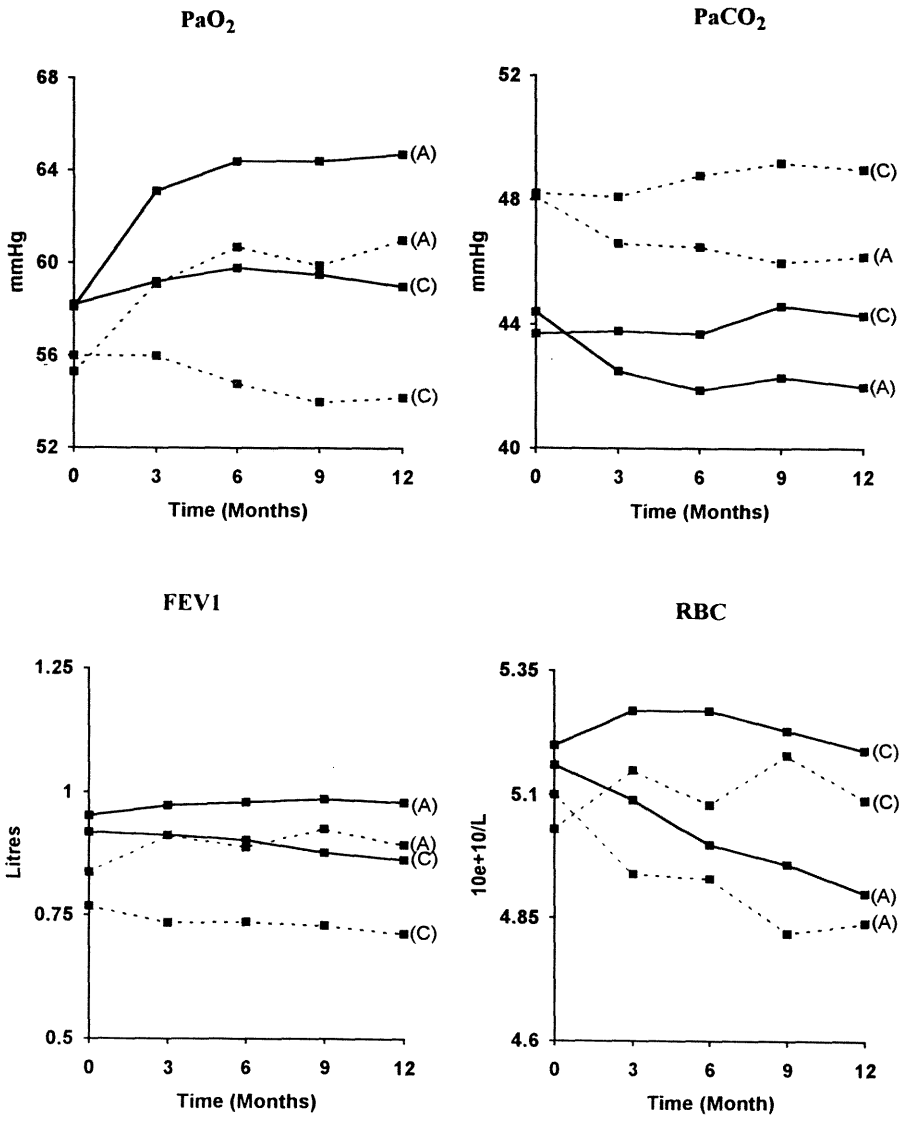
5.5.2 Phase III Clinical study

In a one-year, placebo controlled, double-blind Multi-Centre International Clinical trial involving 701 COAD patients, one group (n=344 patients) received almitrine bismesylate (100 - 200mg/day) whilst the second group (n=357 patients) received no almitrine. Both groups contained a subgroup which received long term oxygen therapy (LTO_2). Blood gases (PaO_2 and $PaCO_2$), haematological and other relevant clinical parameters were measured (Ansquer *et al.*, 1985).

PaO_2 improved significantly in the almitrine treated groups with and without LTO_2 therapy, when compared to the control group ($p<0.0001$); PaO_2 improved from 58mmHg on entry to 64mm Hg after 1 year of treatment (Table 5.13, Figure 5.6). PaO_2, when breathing air was stable in the control group without LTO_2 but worsened in the control group receiving LTO_2, showing that oxygen therapy alone did not prevent more aggravation of the more severe cases. On the other hand, both almitrine subgroups improved their PaO_2 levels by ~ +7mm Hg in patients without LTO_2 and 6mm Hg in patients with concomitant oxygen therapy when breathing air. A 2mm Hg reduction in $PaCO_2$ was also demonstrated in the almitrine group. Other improvements in the almitrine treated group included an improvement in FEV_1 which correlated well with PaO_2 changes; a reduction in polycythemia and red cell counts (5 to 10%), the latter possible due to a feedback process caused by an improved PaO_2.

Significant adverse effects were observed due to almitrine and these included both CNS and respiratory effects ($p<0.01$). However, a more significant side effect was peripheral neuropathy (paresthesia) in which subjects experienced "pins and needles" and less commonly, pain in the lower limbs which were approximately 5 times higher in the almitrine group. These were observed in the treatment group, on average, after 7 months treatment and examination of the overall plasma concentrations of almitrine (Table 5.14) showed that patients most likely to develop peripheral neuropathy had much higher plasma levels and these exceeded 300ng.ml^{-1} after 3 months of treatment and levels continued to increase with time (Figure 5.7). The investigators concluded that

Figure 5.6 : Graphical summary of the clinical observations in two groups of patients entered into a one year study. (Group 1 (A) received almitrine 100 to 200mg/d, with or without concomitant oxygen therapy, Group 2 (C), a control group receiving no almitrine but also with or without oxygen therapy).



patients susceptible to developing peripheral neuropathy, 1) had severe respiratory problems as judged by their clinical history; 2) received more treatments such as, theophylline, corticosteroids and/or other diuretics and 3) possibly had more severe subclinical electrophysiological abnormalities.

Table 5.13: Summary of the Clinical Observations in two groups of patients entered into a one year study . (Group 1 received almitrine 100 to 200mg.d^{-1}, with or without concomitant oxygen therapy, Group 2, a control group receiving no almitrine but also with and without oxygen therapy).

Parameter	Group	n	T0	T3	T6	T9	T12	p
PaO_2 (mm Hg)	Almitrine	196	57.5±60	62.1±9.3	63.5±9.5	63.3±10.1	63.8±9.8	<0.0001
	Control	256	57.7±5.9	58.7±8.2	58.8±8.9	58.3±8.5	58.0±8.6	
$PaCO_2$ (mm Hg)	Almitrine	199	45.3±6.3	43.5±6.9	43.1±6.4	43.2±6.6	43.0±7.0	<0.05
	Control	257	44.7±6.6	44.7±7.4	44.8±7.1	45.6±7.2	45.3±7.2	
FEV1 (L)	Almitrine	186	0.926± 0.373	0.959± 0.403	0.959± 0.380	0.973± 0.378	0.961± 0.371	<0.001
	Control	240	0.891± 0.346	0.880± 0.340	0.873± 0.357	0.851± 0.345	0.837± 0.335	
RBC ($10^{12}.L^{-1}$)	Almitrine	171	5.14±0.55	5.05±0.51	4.99±0.49	4.93±0.46	4.89±0.49	<0.0001
	Control	219	5.16±0.55	5.25±0.55	5.23±0.56	5.22±0.54	5.17±0.52	

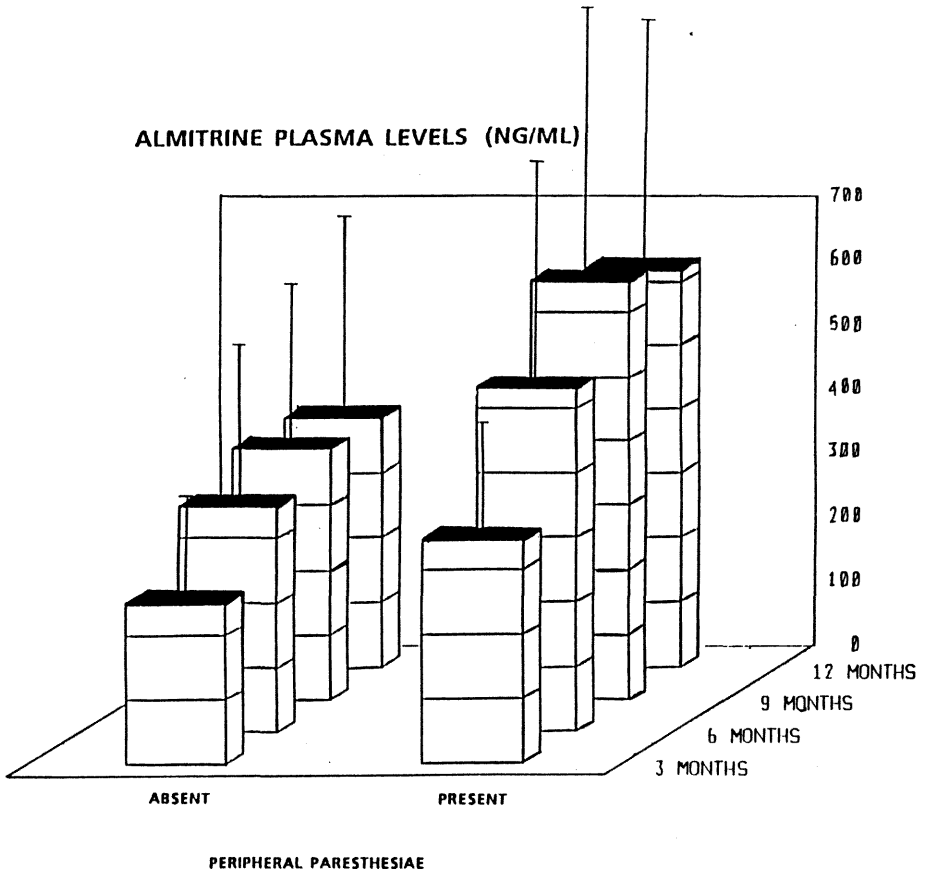
There was a relationship between PaO_2 response and almitrine plasma levels, this relationship being more evident with almitrine plasma levels in the range 200 to 300ng.ml^{-1}. Increasing the daily dose in order to improve the response did not alter the results of non-responsive patients, but rather led to higher risks of side effects due to higher plasma levels.

As with the Phase 1 study, there was considerable inter-subject variability in plasma levels of almitrine. Such inter-subject variability has also been reported by other workers (Bromet *et al.*, 1983; MacLeod *et al.*, 1983; Campbell *et al.*, 1985). Herchuelz *et al.*, (1991) used the phenotyped volunteer panel approach to examine whether a genetic polymorphism in oxidation of the debrisoquine type (Eichelbum *et al.*, 1979; Mahgoub *et al.*, 1977) contributed to this large inter-subject variability and whether this could in part explain the incidence of peripheral neuropathy. There were no differences in the pharmacokinetics of almitrine or its major circulating metabolite (dihydroxy almitrine) between poor and extensive metabolisers of debrisoquine. These results were consistent with a similar study (Belec *et al.*, 1989) involving 15 patients with almitrine peripheral neuropathy. Although these patients were extensive metabolisers (as determined by the dextromethorpham model), no clear relationship could be found between the incidence of peripheral neuropathy and lack of metabolism of almitrine. The mechanism of peripheral neuropathy and the association with almitrine needs further investigation.

Table 5.14 : Comparison of Plasma Levels of Almitrine ($ng.ml^{-1}$) in Patients showing symptoms of Peripheral Paresthesia and those without symptoms during Almitrine Treatment over 12 Months

	T3	T6	T9	T12
No peripheral paresthesiae	248.8 ± 169.5 n = 213	348.6 ± 246.3 n = 187	388.8 ± 257.5 n = 163	387.0 ± 307.0 n = 151
Peripheral paresthesia	344.4 ± 176.2 n = 48	533.2 ± 353.7 n = 44	649.9 ± 419.6 n = 37	616.5 ± 396.0 n = 28

Figure 5.7 : Comparative Levels of Almitrine in Patients showing signs of Peripheral Paresthesia during Almitrine Treatment over 12 Months



CHAPTER 6

DISCUSSION AND CONCLUSION

The determination of plasma/serum concentration of drug and/or metabolite(s) is important for the understanding of how the body handles the drug (pharmacokinetics and metabolism) and to relate a particular matrix concentration to an effect, be it desirable (pharmacological/clinical) or undesirable (toxicological).

Radio-labelled studies form an important part of the drug development programme for observing the distribution of drug-derived material in the body and establishing the rate, route and extent of elimination of drug and metabolites from the body. This helps to relate particular toxicological effects to target tissue concentrations following repeated administration and from a pharmacological point of view, confirmation of the presence of drug-derived material at the site of the target tissue/organ. More importantly, the fate of drug/metabolite, be it tissue exposure, elimination or metabolism, can be extrapolated from animal to man.

Almitrine and its metabolites were shown to be slowly eliminated from the body after both oral and intravenous administration using the [^{14}C]-labelled product. Faecal elimination was the major route of excretion and this was neither species nor administration route dependent due to gut secretion. Early studies in man and dog after oral administration and in man, dog and rabbit after intravenous administration of [^{14}C]-almitrine showed a slow and low recovery of radioactivity over the normal collection period. This was more evident after intravenous administration in which 58 to 78% of the dose was accounted for in 14 days. This would suggest a distribution out of plasma and retention into tissue compartment or reabsorption of drug and metabolite through a process of gut and/or biliary secretion. The presence of radioactive almitrine in the 8-14 day composite faecal metabolite profile after oral administration, described in chapter 4 (Table 4.10), supports the view that reabsorption of almitrine through gut secretion is the most likely cause of this prolonged excretion of radioactivity, since no unchanged almitrine had been identified in bile from other studies after oral and intravenous administration.

The opportunity to perform the long-term disposition study in four healthy human subjects allowed the observation of this recirculation process and highlighted the

importance of good study design for these types of studies with compounds which have a prolonged excretion.

Another important area for the use of radio-labelled drugs is to observe the distribution of drug-derived material throughout the body. The distribution of almitrine and its metabolites is shown to be different between urethane anaesthetised and non-anaesthetised animals. Tissue uptake of radioactivity 2h after IV administration was far greater in the anaesthetised animal compared to the non-anaesthetised, with a 5 fold higher tissue:plasma ratio and 17 fold higher almitrine level in the carotid body of the anaesthetised rat compared to the non-anaesthetised animal. Of the many factors which have been postulated for the differences between the two groups, reduced clearance due to changes in blood flow appears to be the most plausible. Gumbleton *et. al.* (1990) demonstrated significant differences in renal and hepatosplanchnic haemodynamics with different anaesthetics in rats. Assuming a cardiac output of 325ml.min^{-1}.kg^{-1}, urethane reduced cardiac output by approximately 50% to 174ml.min^{-1}.kg^{-1}, with hepatosphlanchnic blood flow reduced to 33.4ml.min.$^{-1}$.kg^{-1} (normally 80ml.min^{-1}.kg^{-1} in the conscious rat). Although almitrine is eliminated metabolically and appears to have a low hepatic extraction, urethane may affect the compound through changes in the rate of hepatic oxygen delivery as demonstrated for antipyrine, a compound with a low hepatic extraction ratio (Brouwer and Vore, 1985). Under the experimental conditions used in the standard pharmacological studies for almitrine, this study had for the first time, demonstrated that almitrine was the major component in the carotid body and had validated the data generated on the action of almitrine at this site of action.

The effect of anaesthesia however, on the pharmacokinetics and pharmacodynamics (PaO_2) of almitrine merits further investigation since a large proportion of the pharmacological studies performed in the rat, dog and cat were carried out under urethane anaesthesia. These studies demonstrated a prolonged action of almitrine on increasing PaO_2 (Dhillon and Barer, 1982) and an increase in activity after 1h under chlorose anaesthesia (Evrard, 1986; personal communication). These findings highlight the need to determine the effect of anaesthesia on the pharmacokinetics of compounds where pharmacological studies are required to be performed under anaesthesia.

The metabolism of almitrine involves a series of n-dealkylation and oxidation reactions, all of which occur in the allyl side chain of the triazine moiety. Oxidation appears to be the predominant route with the formation of the tetrahydroxy product as

the major metabolite. N-oxidation of the piperazine ring has not been observed, neither is the n-dealkylation between the piperazine-triazine linkage proven. N-dealkylation between the piperazine and benzhydryl moiety has occurred to give the ketone as the main urinary metabolite in man and the hydroxy product in rat urine. Thus although almitrine appears to have the same characteristics as the other halogenated benzhydrylpiperazines in that it has a high plasma protein binding and a low clearance, it does not appear to share the same metabolic fate as these compounds. It is possible that the allyl side chains protect the site of dealkylation and N-oxidation which these other benzhydryl piperazines do not possess.

The data generated from these studies have validated the selection of the dog as the large species for toxicology since this animal showed a similar *in-vivo* metabolite profile to man. The *in-vitro* metabolite profile however showed less metabolism in the dog compared to man and the other species. This may well have been a function of the batch of microsomes used in this limited study, rather than the inability for dog microsomes to metabolise almitrine. A more detailed assessment of the microsomal metabolising capacity for each species should have been attempted. this, however, was not possible due to the limited supply of material at the time. The good correlation between *in-vivo* and *in-vitro* data demonstrates the use of *in-vitro* studies as an alternative for predicting drug metabolism. this technique is continuing to gain favour in drug discovery and drug development.

The early Phase 1 pharmacokinetic studies on almitrine were performed over short sampling periods due mainly to the constraints of the study design. However, as the kinetics of the compound were reviewed through its development, it became apparent that the overall pharmacokinetic parameters of almitrine needed further evaluation since, clearance appeared to be over-estimated and both volume of distribution and terminal half-life to be under-estimated. The two hour intravenous infusion study, although performed in a limited number of subjects, confirmed for the first time that the pharmacokinetics of almitrine were indeed underestimated and that the earlier reported half lives of 7 to 114h were somewhere between the 26 days reported in this study and the 72 days observed in the long term excretion study reported in Chapter 4. These findings demonstrated the importance of good study design in establishing the pharmacokinetics of a compound exhibiting a low clearance and a long half life. The data generated from these two studies have been used successfully to determine new dosage regimen for almitrine in the long term treatment of COAD patients with a view to maintaining a therapeutic level of the compound and a reduced incidence of side effects, namely peripheral neuropathy. Ings and Thomas, (1987) used these

newly calculated half lives of almitrine to predictively simulate the plasma levels of the drug on repeated administration with different dosage regimen. Their data showed that the average daily dosage could be achieved by a dosage regimen of 50mg b.i.d for 3 month periods composed of 1 month treatment interruptions and 2 months treatments at 50mg b.i d (Figure 6.1).

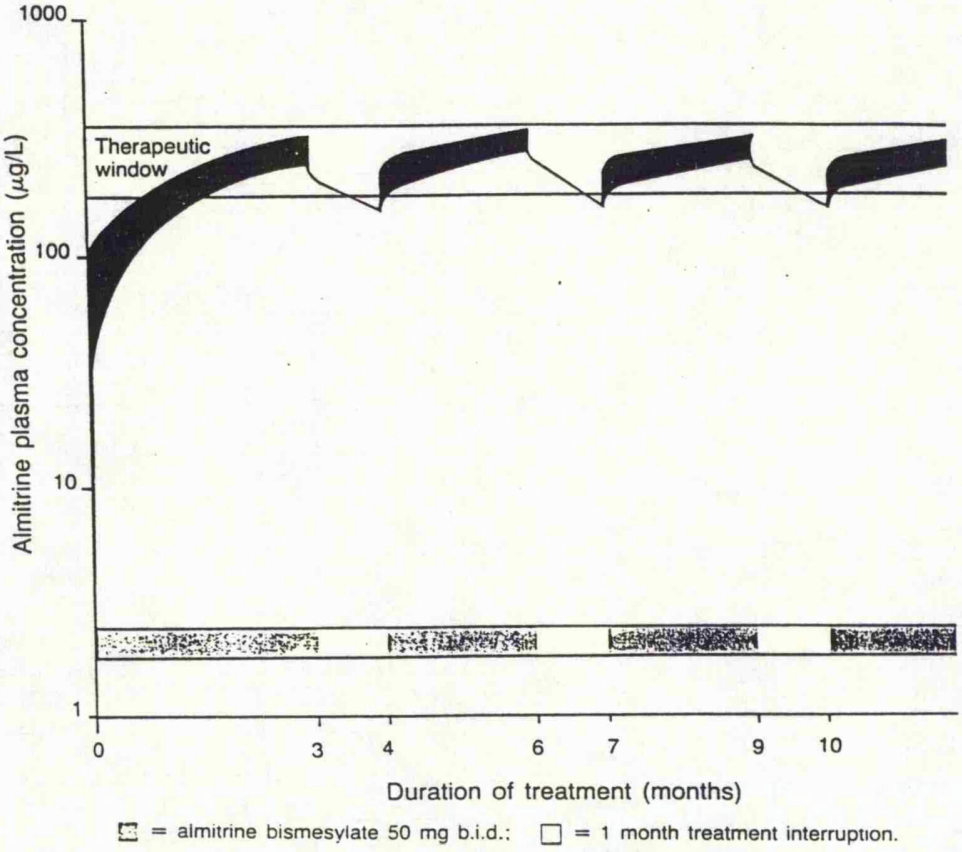


Fig 6.1 : A simulation of almitrine plasma concentrations following the recommended dosage regimen, i.e. 50mg b.i.d. for 3 months, followed by 50mg b.i.d. for 2 months out of 3.
(From Thomas and Ings, 1987)

The effect of COAD on the pharmacokinetics of almitrine has not been extensively investigated. The majority of studies performed on the pharmacokinetics of almitrine in COAD patients have involved short term sampling and therefore, incomplete parameters have been obtained. The long half life established in healthy human subjects has been established in COAD patients receiving almitrine bismesylate at 75mg b.i.d for 6 months (Agard *et. al.,* 1987). A mean terminal half life of 55 ± 16h was reported. Evans *et. al,* (1986) estimated a half life of 23.3 days, from a long term oxygen/almitrine study, by virtue of the time taken for plasma concentrations of

almitrine to fall to half its concentration after almitrine therapy had stopped. Johanson *et. al.* (1986) also indirectly demonstrated a long half-life in COAD patients in a 56 day repeated dose regimen (2 x 50mg or 2 x 100mg per day). PaO_2 had significantly increased from baseline after 14 days treatment and had not returned to baseline 14 days after almitrine treatment had ceased, demonstrating a long half-life of the compound. Bury *et. al.* (1989) demonstrated a linear relationship between mean C_{max} and AUC and dose in patients receiving a single dose of almitrine bismesylate over the range 50 to 150mg. A half-life of 116 to 140h was reported but plasma levels were only monitored over 72h.

The metabolism of almitrine in COAD patients was not demonstrated. However, although the liver is commonly recognised as the major organ of drug metabolism by virtue of the high levels of drug metabolising enzymes compared to other organs, Many organs possess the ability to metabolise xenobiotics although their capacity is lower than that of the liver (Roth 1984). The lung contains enzymes capable of metabolising therapeutic drugs such as chlorpromazine (Ohmiya and Mehendale, 1980) and pentobarbital (Law *et. al.,* 1974), environmental toxicants such as benzo[*a*]pyrene (Vahakangas *et. al.,* 1977a & b) and endogenous agents such as biogenic amines (Junod, 1972, Albaster and Bahkle, 1970 and Wiersma and Roth, 1980). Diseases of the lung that lead to alveolar hypoxia may also result in altered clearance of circulating substances. Hypoxia causes a decreased rate of absorption of hexobarbital following I.P administration. In addition, hepatic metabolism of the drug was reduced (Roth and Rubin 1976a). Hepatic clearance of hexobarbital is blood-flow dependant in the rat and hypoxia causes a marked reduction in hepatic blood flow (Roth and Rubin 1976b). It would seem therefore that alveolar hypoxia not only causes a decrease in oxygen content in blood perfusing the liver, but also decreases liver blood flow (i.e. drug delivery) to the organ. Kirby *et. al.* (1976) suggested a significant redistribution of blood flow away from splanchnic regions in chronically hypoxic human patients. In some species including human, chronic hypoxia also results in substantial increases in cardiac output. Thus the contribution of the lung to total body clearance might be increased for certain drugs for which pulmonary and/or hepatic clearance is flow dependant. This may not be the case for almitrine since it has a low clearance and high plasma protein binding. However, increased α_1-acid glycoprotein associated with chronic hypoxia (du Souich *et. al.,* 1978) could increase the binding of almitrine and therefore the total circulating plasma concentration of the compound. Consequently, since the product is metabolically cleared, the extent of tissue uptake can be significantly increased if clearance is reduced due to reduced hepatic blood flow caused by chronic hypoxia. This, in turn

could result in peripheral paresthesia which correlates well with high circulating levels of almitrine.

Chronic Obstructive Airway disease continues to be a poorly treated disease in the Western world as continued heavy cigarette smoking and industrial pollutants play a major role in the prognosis of this disease. Whilst long term oxygen therapy (LTO_2) continues to be the only non-pharmaceutical treatment, other drugs such as β-agonists, corticosteroids and methylxanthines, although successful against other pulmonary diseases such as asthma, have proved to be unsuccessful in COAD. Almitrine remains the only alternative to LTO_2 despite its major side effect which is reversible and controllable through correct dosage regimen.

In conclusion, this work was set out to achieve the main objectives set out in Chapter 1 (Introduction) with a view to establishing the pharmacokinetics and metabolism of the compound for its eventual use in man. However, due to its high plasma protein binding, large volume of distribution, low clearance and slow but extensive metabolism, the project provided a model to investigate an unusual pharmacokinetic and metabolic profile. Having obtained incomplete but useful results by following the normal drug development program for drug metabolism and pharmacokinetics, it became necessary to have good study designs in order to obtain better information for such a compound with a long terminal half life, so that pharmacokinetic/pharmacodynamic modelling could be applied to establish the correct dosage regimen for therapeutic efficacy with minimal adverse effects. Perhaps if these problems were known during the early development of the compound, a different approach would have been adopted and those problems would have been overcome. Certainly the metabolism of almitrine in the healthy subjects and COAD patients would have been compared and the full pharmacokinetics of the compound in these patients would also have been established. Nevertheless, the data generated have provided a better understanding of the compound and have helped to provide models which can be used to provide a safe dosage regimen in clinical practice.

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ABBREVIATIONS

COAD		Chronic obstructive airway disease
FEV_1	l	Forced expiry volume in one second
FVC	l	Forced vital capacity
mmHg		Millimetre of mercury (unit of pressure)
kPa		kilo Pascals (unit of pressure)
NOTTS		Nocturnal oxygen therapy trial
LTO_2		Long term oxygen therapy
cAMP		cyclic Adenosine mono phosphate
PaO_2	mmHg	Arterial oxygen pressure
PCO_2	mmHg	Partial pressure of carbon dioxide
MPA		Medroxyprogesterone acetate
ABM		Almitrine bismesylate
M. Wt.	Daltons	Molecular weight
mg		Milligram
kg		Kilogram
ip		Intraperitoneal
mmol		Millimol.
pmol		Picomol
CB		Carotid body
µg		microgram
ng		Nanogram
$A\text{-}aO_2$	Δ	Alveolar-arterial oxygen pressure difference
IV		Intravenous
h		Hour
l		Litre
min		Minute, minimum
Ppa	mmHg	Mean pulmonary arterial presure
RVEF		Right ventricular ejector fraction
ADME		Absorption, distribution, metabolism and elimination
GC/GLC		Gas liquid chromatography
LC/HPLC		High performance liquid chromatography
P.O.P.O.P		1,4-bis(5 phenyloxazol-2-yl) benzene
P.P.O		2,5-diphenyloxazol
Ci		Curie
M		Metre
mm		Millimetre
nm		Nanometre
µm		Micrometre
TLC		Thin layer chromatography
cm		Centimetre
v	ml, l	Volume
ml		Millilitre
°C		Degrees centrigrade
PO		Oral
RA		Radioactivity
WBA		Whole body autoradioagraphy

MS		Mass spectroscopy
NZW		New Zealand white
M		Male
F		Female
C		Caucasian
SCG		Superior cervical ganglion
AES		Automatic external standard
dpm		Disintegration per minute
wt.	ng; µg; mg.g; kg	Weight
rpm		Revolution per minute
NADP		Nicotinamide adenine dinucleotide phosphate
RIA		Radioimmuno assay
RBC		Red blood cells
i.d	mm	Internal diameter
A.W. DMCS		Acid washed dimethylchlorosilane
CV	%	Coefficient of variation
Acc	%	Accuracy
Conc.	ng; µg; $mg.ml^{-1}$	Concentration
M		Molar
SD		Standard deviation
Cp	ng; $\mu g.ml^{-1}$	Plasma concentration
t	sec. min. h. d	Time
λi	h^{-1}	The exponential constant (slope) obtained from linear regression of the semi-logarithmic plots of blood or plasma concentration versus time curve
AUC_t	$ng.ml^{-1}.h$ $\mu g.ml^{-1}.h$	Area under the blood concentration versus time curve up to the last measurable concentration at time t after the dose
AUC	$ng.ml^{-1}.h$ $\mu g.ml^{-1}.h$	Area under the blood concentration versus time curve extrapolated to infinity
CL	$ml.min.^{-1}.kg^{-1}$ $ml.min^{-1}$	Clearance
$t_{1/2}$	h	Half-life
$t_{1/2a}$	h	Absorption half-life
$t_{1/2z}$	h	Terminal half-life
Vd	l	Volume of distribution based on area
V_{ss}	l	Volume of distribution at steady-state
Vi	l	Initial volume of distribution
τ	h	Tau, dosing interval
F	%	Bioavailability, (solvent) front
%		Percent
C_{max}	$ng.ml^{-1}$ $\mu g.ml^{-1}$	Maximum plasma (blood) concentration
t_{max}	h	Time to achieve C_{max}
d		Day
eq.		Equivalent

ND		Not determined
NS		No sample, Not statistically significant
BLQ		Below limit of quantitation
REC	%	Recovery
O		Origin
Rf		Retention factor
DCI		Desorption chemical ionisation
EI		Electron impact ionisation
TIC		Total ion chromotogram
ES		Electrospray
NA		Not applicable
Yrs		Years
Sig		Statistically significantly different
C_{ss}	$ng.ml^{-1}$	Steady state concentration
NPD		Nitrogen-phosphorus-detection
UV		Ultraviolet
LOQ	$ng.ml^{-1}$	Limit of quantitation
AUC_{24}	$ng.ml^{-1}.h$	Area under the plasma concentration versus time
	$\mu g.ml^{-1}.h$	curve up to the 24h time point after dose
ANOVA		Analysis of variance
b.i.d		Twice daily
amu		Atomic mass units
f.s.d		Full scale deflection

APPENDICES TO CHAPTER 3

Appendix 3.1A : Comparison of Blood or Plasma Levels of [^{14}C]-Almitrine (ng.eq.ml^{-1}) in Various Species Normalised to a 1mg.kg^{-1} Oral Dose of [^{14}C]-Almitrine Bismesylate

TIME (H)	*RAT (n=3)	RABBIT (n=4)	DOG (n=3)	HUMAN (n=2)
0.5	21.3 ± 8.5	16.4 ± 11.8	31.6 ± 23.1	45.4 ± 23.5
1.0	31.8 ± 16.3	28.8 ± 21.5	60.0 ± 26.1	119.0 ± 15.4
2.0	35.1 ± 12.2	62.6 ± 29.7	104.0 ± 60.7	292.5 ± 102.3
3.0	39.4 ± 7.4	62.4 ± 27.7	109.2 ± 67.7	331.8 ± 193.2
4.0	46.5 ± 6.7	85.8 ± 22.2	113.6 ± 75.6	323.2 ± 162.9
6.0	41.2 ± 11.3	85.6 ± 27.4	125.4 ± 79.9	230.4 ± 90.5
8.0	39.4 ± 13.5	84.4 ± 22.7	124.0 ± 78.3	204.5 ± 93.7
10.0	NS	69.9 ± 18.8	NS	NS
12.0	NS	NS	107.4 ± 68.7	153.9 ± 42.1
24.0	22.7 ± 4.4	54.1 ± 26.9	101.4 ± 54.1	112.3 ± 46.6
32.0	NS	NS	87.8 ± 68.0	NS
48.0	12.8 ± 6.7	26.3 ± 12.9	71.6 ± 46.7	69.4 ± 20.4
72.0	9.4 ± 3.9	20.5 ± 8.6	55.2 ± 32.8	51.5 ± 17.6

*Blood

NS denotes No Sample taken

Appendix 3.1B : Comparison of Blood or Plasma Levels of [^{14}C]-Almitrine (ng.eq.ml^{-1}) in Various Species Normalised to a 1mg.kg^{-1} Intravenous Dose of [^{14}C]-Almitrine Bismesylate

TIME (H)	*RAT (n=3)	RABBIT (n=4)	DOG (n=6)	HUMAN (n=2)
0.083	ND	722.2 ± 136.1	956.2 ± 244.3	NS
0.25	216.3 ± 75.2	478.9 ± 102.3	697.4 ± 145.6	776.0 ± 442.0
0.5	127.6 ± 20.0	399.3 ± 36.7	587.3 ± 152.1	643.3 ± 354.2
1.0	131.3 ± 44.7	311.0 ± 18.3	483.8 ± 114.9	489.7 ± 335.0
2.0	94.3 ± 26.0	240.9 ± 18.7	409.4 ± 118.1	377.3 ± 284.0
3.0	78.2 ± 16.2	196.1 ± 9.4	NS	392.3 ± 223.2
4.0	71.2 ± 20.4	184.6 ± 27.3	356.0 ± 90.6	350.7 ± 194.2
6.0	60.0 ± 21.6	152.6 ± 8.1	325.2 ± 90.6	269.3 ± 210.4
8.0	49.1 ± 17.1	148.8 ± 12.0	299.3 ± 58.2	236.3 ± 188.6
10.0	41.8 ± 16.2	125.0 ± 5.5	NS	NS
12.0	NS	NS	229.8 ± 37.2	179.7 ± 163.2
24.0	24.7 ± 6.7	74.4 ± 8.8	156.9 ± 21.0	149.0 ± 116.3
36.0	NS	NS	150.5 ± 22.7	134.0 ± 100.4
48.0	12.9 ± 0.4	49.1 ± 15.8	92.2 ± 14.6	122.0 ± 97.8
72.0	NS	NS	76.0 ± 14.6	97.5 ± 62.9

*Blood

NS denotes No Sample taken

Appendix 3.2A : The Daily Excretion of Radioactivity (Expressed as % Dose) into Urine of Rat, Rabbit, Dog and Man Following Oral Administration of [^{14}C]-Almitrine Bismesylate

TIME (DAYS)	RAT	RABBIT	DOG	MAN
1	7.06	2.51	1.60	3.30
2	1.69	1.26	0.50	1.74
3	0.42	0.66	0.37	0.34
4	0.11	0.32	0.24	0.83
5	0.11	0.22	0.07	0.73
6	NS	0.21	0.07	0.60
7	NS	0.08	NS	0.67
8	NS	0.09	NS	0.45
9	NS	0.06	NS	0.37
10	NS	0.05	NS	0.48
11	NS	0.04	NS	0.32
12	NS	0.02	NS	0.42
13	NS	0.02	NS	0.25
14	NS	NS	NS	0.24

NS denotes No sample collected for analysis

Appendix 3.2B : The Cumulative Excretion of Radioactivity (Expressed as % Dose) into Urine of Rat, Rabbit, Dog and Man Following Oral Administration of [^{14}C]-Almitrine Bismesylate

TIME (DAYS)	RAT	RABBIT	DOG	MAN
1	7.06	2.51	1.60	3.30
2	8.75	3.77	2.10	5.04
3	9.17	4.43	2.47	6.38
4	9.28	4.75	2.68	7.21
5	9.39	4.97	2.75	7.94
6	NS	5.18	2.82	8.54
7	NS	5.26	NS	9.21
8	NS	5.35	NS	9.66
9	NS	5.41	NS	10.03
10	NS	5.46	NS	10.51
11	NS	5.50	NS	10.83
12	NS	5.52	NS	11.25
13	NS	5.54	NS	11.50
14	NS	NS	NS	11.74

NS denotes No sample collected for analysis

Appendix 3.2C : The Daily Excretion of Radioactivity (Expressed as % Dose) into Faeces of Rat, Rabbit, Dog and Man Following Oral Administration of [^{14}C]-Almitrine Bismesylate

TIME (DAYS)	RAT	RABBIT	DOG	MAN
1	81.76	44.27	42.94	3.88
2	18.56	21.30	15.24	6.80
3	1.69	10.80	4.40	10.64
4	0.67	5.44	2.09	9.57
5	0.71	2.91	1.21	3.99
6	NS	2.54	1.08	7.15
7	NS	0.90	NS	1.29
8	NS	0.81	NS	2.66
9	NS	0.84	NS	2.62
10	NS	0.60	NS	2.32
11	NS	0.45	NS	0.62
12	NS	0.45	NS	0.84
13	NS	0.39	NS	0.53
14	NS	0.28	NS	0.31

NS denotes No sample collected for analysis

Appendix 3.2D : The Cumulative Excretion of Radioactivity (Expressed as % Dose) into Faeces of Rat, Rabbit, Dog and Man Foilowing Oral Administration of [^{14}C]-Almitrine Bismesylate

TIME (DAYS)	RAT	RABBIT	DOG	MAN
1	81.76	44.27	42.94	3.88
2	100.32	65.57	58.18	10.68
3	102.01	76.37	62.58	21.32
4	102.68	81.81	64.67	30.89
5	103.39	84.72	65.88	34.88
6	NS	87.26	66.96	42.03
7	NS	88.16	NS	43.32
8	NS	88.97	NS	45.98
9	NS	89.81	NS	48.60
10	NS	90.41	NS	50.92
11	NS	90.86	NS	51.54
12	NS	91.31	NS	52.38
13	NS	91.70	NS	52.91
14	NS	91.98	NS	53.22

NS denotes No sample collected for analysis

Appendix 3.2E: The Cumulative Excretion of Total Radioactivity (Expressed as % Dose) into Urine and Faeces of Rat, Rabbit, Dog and Man Following Oral Administration of [^{14}C]-Almitrine Bismesylate

TIME (DAYS)	RAT	RABBIT	DOG	MAN
1	88.82	46.78	44.54	7.18
2	109.07	69.34	60.28	15.72
3	111.18	80.80	65.05	27.70
4	111.96	86.56	67.35	38.10
5	112.78	89.69	68.63	42.82
6	NS	92.44	69.78	50.57
7	NS	93.42	NS	52.53
8	NS	94.32	NS	55.64
9	NS	95.22	NS	58.63
10	NS	95.87	NS	61.43
11	NS	96.36	NS	62.37
12	NS	96.83	NS	63.63
13	NS	97.24	NS	64.41
14	NS	97.52	NS	64.96

NS denotes No sample collected for analysis

Appendix 3.2F: The Daily Excretion of Radioactivity (Expressed as % Dose) into Urine of Rabbit, Dog and Man Following Intravenous Administration of [^{14}C]-Almitrine Bismesylate

TIME (DAYS)	RABBIT	DOG	MAN
1	1.13	2.46	2.67
2	2.31	1.06	2.20
3	1.37	0.72	1.38
4	1.05	0.61	1.01
5	0.70	0.45	0.53
6	0.30	0.43	0.37
7	0.23	0.42	0.36
8	0.20	0.22	0.40
9	0.19	0.39	0.30
10	0.17	0.36	0.30
11	0.16	0.19	0.15
12	0.07	0.25	0.18
13	0.05	0.18	0.32
14	0.05	0.18	0.13

Appendix 3.2G: The Cumulative Excretion of Radioactivity (Expressed as % Dose) into Urine of Rabbit, Dog and Man Following Intravenous Administration of [^{14}C]-Almitrine Bismesylate

TIME (DAYS)	RABBIT	DOG	MAN
1	1.13	2.46	2.67
2	3.44	3.52	4.87
3	4.81	4.24	6.25
4	5.86	4.85	7.26
5	6.56	5.30	7.79
6	6.86	5.73	8.16
7	7.09	6.15	8.52
8	7.29	6.37	8.92
9	7.48	6.76	9.22
10	7.65	7.12	9.52
11	7.81	7.31	9.67
12	7.88	7.56	9.85
13	7.93	7.74	10.17
14	7.98	7.92	10.30

Appendix 3.2H: The Daily Excretion of Radioactivity (Expressed as % Dose) into Faeces of Rabbit, Dog and Man Following Intravenous Administration of [^{14}C]-Almitrine Bismesylate

TIME (DAYS)	RABBIT	DOG	MAN
1	21.75	9.09	9.82
2	17.30	10.01	12.93
3	11.54	8.84	6.87
4	3.13	2.56	4.47
5	2.77	4.85	5.55
6	2.32	3.39	6.90
7	1.99	1.70	1.09
8	1.84	2.15	1.36
9	1.49	1.65	1.16
10	1.50	1.63	1.24
11	1.40	1.46	1.00
12	1.10	1.12	0.94
13	0.66	1.02	1.27
14	1.32	1.09	0.70

Appendix 3.2l: The Cumulative Excretion of Radioactivity (Expressed as % Dose) into Faeces of Rabbit, Dog and Man Following Intravenous Administration of [^{14}C]-Almitrine Bismesylate

TIME (DAYS)	RABBIT	DOG	MAN
1	21.75	9.09	9.82
2	39.05	19.10	22.75
3	50.59	27.94	29.62
4	53.72	30.50	34.09
5	56.49	35.35	39.64
6	58.81	38.74	46.54
7	60.80	40.44	47.63
8	62.64	42.59	48.99
9	64.13	44.24	50.15
10	65.63	45.87	51.39
11	67.03	47.33	52.39
12	68.13	48.45	53.33
13	68.79	49.47	54.60
14	70.11	50.56	55.30

Appendix 3.2J: The Cumulative Excretion of Total Radioactivity (Expressed as % Dose) into Urine and Faeces of Rabbit, Dog and Man Following Intravenous Administration of [^{14}C]-Almitrine Bismesylate

TIME (DAYS)	RABBIT	DOG	MAN
1	22.88	11.55	12.49
2	42.49	22.62	27.62
3	55.40	32.18	35.87
4	59.58	35.35	41.35
5	63.05	40.65	47.43
6	65.67	44.47	54.70
7	67.89	46.59	56.15
8	69.93	48.96	57.91
9	71.61	51.00	59.37
10	73.28	52.99	60.91
11	74.84	54.64	62.06
12	76.01	56.01	63.18
13	76.72	57.21	64.77
14	78.09	58.48	65.60

Appendix 3.3.1A : Mean Tissue Levels of Radioactivity Expressed as % Dose Per Gram and µg.eq. Per Gram Following an Intravenous Dose of [^{14}C]-Almitrine Bismesylate (3mg.kg^{-1}) to Anaesthetised Rats

TISSUE	5 min		15 min		30 min		60 min		120 min	
	% dose. g^{-1}	µg.eq. g^{-1}	% dose. g^{-1}	µg.eq. g^{-1}	% dose. g^{-1}	µg.eq. g^{-1}	% dose. g^{-1}	µg.eq. g^{-1}	% dose. g^{-1}	µg.eq. g^{-1}
Liver	2.55	23.92	2.28	21.72	2.13	19.69	1.54	13.39	0.88	10.76
Lungs	12.26	107.95	12.30	117.48	5.46	50.44	1.00	9.74	0.63	7.68
Trachea	0.36	4.67	0.13	1.61	0.11	1.60	0.45	4.84	0.12	2.33
Kidneys	1.58	14.43	1.27	12.10	1.45	13.98	1.21	10.60	0.95	11.60
Heart	2.73	24.67	2.13	20.40	1.63	15.07	1.75	15.22	1.36	16.73
S. Intestine	0.44	3.96	0.56	5.37	0.85	7.90	0.69	5.97	2.02	24.59
L. Intestine	0.32	2.90	0.13	1.26	0.12	1.15	0.23	2.01	0.15	1.81
Stomach	0.21	1.88	0.25	2.37	1.61	14.82	2.73	24.25	0.87	10.57
Testes	0.08	0.76	0.09	0.87	0.07	0.62	0.09	0.75	0.07	0.88
Brain	0.43	3.79	0.31	2.99	0.20	1.83	0.16	1.38	0.09	1.10
Muscle	0.26	2.76	0.25	2.70	0.31	3.11	0.31	2.99	0.19	3.86
Carcass	0.16	1.45	0.22	2.15	0.26	2.44	0.30	2.57	0.25	3.11
Skin	0.00	0.00	0.08	0.77	0.12	1.11	0.16	1.36	0.09	1.07
White Fat	0.05	0.60	0.02	0.28	0.34	3.20	0.17	1.58	0.12	1.59
Plasma	0.69	6.00	0.16	1.56	0.10	1.02	0.09	0.84	0.06	0.78
Red Cell	0.27	2.37	0.07	0.68	0.05	0.47	0.03	0.31	0.02	0.31
Bronchi	10.62	93.03	7.21	68.86	3.70	34.14	0.89	7.88	0.69	7.78
Carotid Body	2.16	18.31	1.06	10.04	0.71	6.56	1.22	10.20	1.18	14.23
Sup Cer Gang	0.26	2.46	1.48	14.19	1.04	9.62	1.64	13.64	1.81	21.83
Carotid Artery	1.70	15.91	0.75	7.17	0.71	6.61	0.99	8.25	1.26	15.32
Adrenals	10.66	66.93	6.00	50.30	4.8	39.30	2.58	20.21	2.00	17.56

Appendix 3.3.1B : Mean Tissue Levels of Radioactivity Following an Intravenous Dose of [^{14}C]-Almitrine Bismesylate (3mg.kg^{-1}) to Unanaesthetised Rats

TISSUE	2 HOURS		4 HOURS		8 HOURS		16 HOURS	
	% dose. g^{-1}	µg.eq. g^{-1}	% dose. g^{-1}	µg.eq. g^{-1}	% dose. g^{-1}	µg.eq. g^{-1}	% dose. g^{-1}	µg.eq. g^{-1}
Liver	2.51	9.58	1.54	6.97	0.55	2.50	0.45	2.08
Lung	0.61	2.76	0.45	2.05	0.16	0.71	0.12	0.53
Kidney	1.02	4.60	0.67	3.04	0.27	1.20	0.23	0.92
Heart	1.37	6.19	1.01	4.57	0.34	1.56	0.27	1.26
Plasma	0.16	0.72	0.11	0.52	0.04	0.18	0.02	0.10
Carotid Body	0.58	2.63	0.88	4.00	0.26	1.16	0.36	1.64
Sup Cer Gang	0.22	0.96	1.39	6.27	0.27	1.22	0.59	2.73
Carotid Artery	0.23	1.05	0.06	0.25	0.16	0.71	0.08	0.37

Appendix 3.3.1C : Tissue:Plasma Ratio of Radioactivity Following an Intravenous Dose of [^{14}C]-Almitrine Bismesylate (3mg.kg^{-1}) To Anaesthetised Rats

TISSUE	5 MIN	15 MIN	30 MIN	60 MIN	120 MIN
Liver	4.0	13.9	19.3	15.9	13.8
Lungs	18.0	75.3	49.5	11.6	9.8
Trachea	0.8	1.0	1.7	5.8	3.0
Kidneys	2.4	7.8	13.7	12.6	14.9
Heart	4.1	13.1	14.8	18.1	21.4
S. Intestine	0.7	3.4	7.7	7.1	31.5
L. Intestine	0.5	0.8	1.1	2.4	2.3
Stomach	0.3	1.5	14.5	28.9	13.6
Testes	0.1	0.6	0.6	0.9	1.1
Brain	0.6	1.9	1.8	1.6	1.4
Muscle	0.5	1.7	3.0	3.6	4.9
Carcass	0.2	1.4	2.4	3.1	4.0
Skin	0.0	0.5	1.1	1.6	1.4
White Fat	0.1	0.2	3.1	1.9	2.0
Plasma	1.0	1.0	1.0	1.0	1.0
Red Cells	0.4	0.4	0.5	0.4	0.4
Bronchi	15.5	44.1	33.5	9.4	10.0
Carotid Body	3.1	6.4	6.4	12.1	18.2
Sup Cerv Gang	0.4	9.1	9.4	16.2	28.0
Carotid Artery	2.7	4.6	6.5	9.8	19.6
Adrenals	11.2	32.2	38.5	24.1	22.5

Calculated from µg. equivalents per gram.

Appendix 3.3.1D : Tissue:Plasma Ratio of Radioactivity Following an Intravenous Administration of [^{14}C]-Almitrine Bismesylate (3mg.kg^{-1}) to Unanaesthetised Rats

TISSUE	2 HOURS	4 HOURS	8 HOURS	16 HOURS
Liver	13.3	13.4	13.9	20.8
Lung	3.8	3.9	3.9	5.3
Kidney	6.4	5.8	6.7	9.2
Heart	8.6	8.8	8.7	12.6
Plasma	1.0	1.0	1.0	1.0
Carotid Body	3.7	7.7	6.4	16.4
Sup Cer Gang	1.3	12.1	6.8	27.3
Carotid Artery	1.5	0.5	3.9	3.7

Appendix 3.3.2A : Percentage of Radioactive Dose Recovered in Dog Number 883 Following a Single Intravenous Administration of [^{14}C]-Almitrine Bismesylate at 5.0mg.kg^{-1}

TIME (H)	PERCENTAGE DOSE		
	URINE	FAECES	TOTAL
24	2.07	22.33	24.40
48	0.73	14.38	15.11
72	0.56	6.93	7.49
96	0.30	4.59	4.89
120	0.27	4.44	4.71
144	0.23	1.76	1.99
168	0.33	2.77	3.10
TOTAL	**4.49**	**57.20**	**61.69**

Appendix 3.3.2B : Percentage of Radioactive Dose Recovered in Dog Number 884 Following a Single Intravenous Administration of [^{14}C]-Almitrine Bismesylate at 5.0mg.kg^{-1}

TIME (H)	PERCENTAGE DOSE		
	URINE	FAECES	TOTAL
24	1.25	8.19	9.44
48	0.68	18.88	19.56
72	0.47	5.74	6.21
96	0.33	3.87	4.20
120	0.30	3.61	3.91
144	0.19	1.76	1.95
168	0.30	2.26	2.56
TOTAL	**3.52**	**44.31**	**47.83**

Appendix 3.3.2C : Blood Levels of Radioactivity and Blood:Plasma Ratio of [^{14}C]-Almitrine in the Dog Following a Single Intravenous Administration of [^{14}C]-Almitrine Bismesylate at 5mg.kg^{-1}

TIME	RADIOACTIVE CONC (ng.eq.ml^{-1})		BLOOD:PLASMA RATIO	
(H)	DOG 883	DOG 884	DOG 883	DOG 884
0.08	2925	4545	0.61	0.52
0.16	2207	2824	0.58	0.55
0.5	1491	1614	0.64	0.52
2	968	1012	0.61	0.47
6	807	931	0.66	0.52
24	495	642	0.82	0.56
48	348	528	0.96	0.51
72	267	411	1.01	0.48

Appendix 3.3.2D : Plasma Levels of Radioactivity Expressed as ng.eq.ml^{-1} and Intact Almitrine in ng.ml^{-1} in the Dog Following A Single Intravenous Administration Of [^{14}C]-Almitrine Bismesylate at 5mg.kg^{-1}

TIME	DOG 883		DOG 884	
(H)	[^{14}C] ng.eq.ml^{-1}	Cold ng.ml^{-1}	[^{14}C] ng.eq.ml^{-1}	Cold ng.ml^{-1}
0.08	4807	3067	8811	5734
0.16	3796	1348	5172	3393
0.33	2773	1475	3561	1921
0.50	2339	1146	3083	1485
1	1864	825	2314	1135
2	1588	514	2172	767
3	1465	403	1843	524
4	1383	263	1775	293
6	1222	154	1791	234
8	1147	109	1637	160
10	1054	91	1442	128
24	605	32	1138	58
32	528	22	1204	45
48	363	14	1028	16
72	264	NR	860	24
96	208	NR	657	BLQ
120	166	NR	572	11
144	126	NR	516	BLQ
168	117	NR	405	BLQ

BLQ denotes Below Limit of Quantitation
NR denotes No Results

Appendix 3.3.3A : Blood Levels of Radioactivity (ng.eq.g^{-1}) in Male (3 and 4) and Female (1 and 2) Subjects Following Oral Administration of [^{14}C]-Almitrine Bismesylate (~100mg ≅30μCi)

TIME (H)	1	2	3	4	MEAN ± SD
0	BLQ	BLQ	BLQ	BLQ	-
0.5	76	70	66	39	62.8 ± 16.4
1	113	152	124	445	208.5 ± 58.5
2	221	215	238	288	240.5 ± 33.1
3	192	212	213	256	218.3 ± 27.0
4	167	215	197	217	199.0 ± 23.2
6	155	195	169	151	167.5 ± 19.9
8	151	186	159	143	159.8 ± 18.7
12	152	160	114	125	137.8 ± 21.8
24	110	110	72	88	95.0 ± 18.5
30	105	104	74	87	92.5 ± 14.8
48	62	84	53	58	64.3 ± 13.7
72	56	65	40	48	52.3 ± 10.7
96	48	53	35	35	42.8 ± 9.2
120	35	47	32	34	37.0 ± 6.8
144	29	42	23	30	31.0 ± 8.0
168	29	29	22	17	24.3 ± 5.9
216	BLQ	29	19	12	15.0 ± 12.2
240	NS	NS	NS	NS	-
264	NS	NS	NS	NS	-
288	NS	NS	NS	NS	-
312	BLQ	20	20	13	13.2 ± 9.4
384	BLQ	19	BLQ	NS	6.3 ± 11
408	NS	NS	NS	11	11
480	BLQ	BLQ	BLQ	BLQ	-

BLQ denotes Below Limit of Quantitation; BLQ = 0 for calculation of mean
NS denotes No Sample

Appendix 3.3.3B : Plasma Levels of Radioactivity (ng.eq.g^{-1}) in Male (3 and 4) and Female (1 and 2) Subjects Following Oral Administration of [^{14}C]-Almitrine Bismesylate (~100mg ≅30µCi)

TIME (H)	1	2	3	4	MEAN ± SD
0	BLQ	BLQ	BLQ	BLQ	-
0.5	77	81	124	62	86.0 ± 26.6
1	123	166	222	159	167.5 ± 40.9
2	223	220	420	327	297.5 ± 95.6
3	200	197	378	365	285.0 ± 100.0
4	172	213	338	311	263.5 ± 83.7
6	169	206	297	245	229.3 ± 54.8
8	155	178	259	221	203.3 ± 46.1
12	123	145	203	192	165.8 ± 38.0
24	90	107	119	136	113.0 ± 19.4
30	92	100	113	133	109.5 ± 17.9
48	69	69	115	105	89.5 ± 20.0
72	52	55	75	69	62.8 ± 11.0
96	45	NS	57	59	53.7 ± 7.6
120	41	38	56	48	45.8 ± 8.0
144	32	23	49	40	36.0 ± 11.1
168	42	NS	41	30	37.7 ± 6.7
216	26	21	29	28	26.0 ± 3.6
240	NS	NS	NS	NS	-
264	NS	NS	NS	NS	-
288	NS	NS	NS	NS	-
312	24	BLQ	26	17	16.8 ± 11.8
384	15	BLQ	32	NS	15.7 ± 16.0
408	NS	NS	NS	12	12
480	11	BLQ	19	NS	10.0 ± 9.5
600	12	BLQ	NS	NS	6
768	8	BLQ	NS	NS	4
936	4	BLQ	NS	NS	2

BLQ denotes Below Limit of Quantitation; BLQ = 0 for calculation of mean
(Minimum of 3 for calculation of SD)
NS denotes No Sample

Appendix 3.3.3C : Plasma Levels of Unchanged Almitrine (ng.ml^{-1}) in Male (3 and 4) and Female (1 and 2) Subjects Following Oral Administration of [^{14}C]-Almitrine Bismesylate (~100mg ≅30μCi)

TIME (H)	1	2	3	4	MEAN ± SD
0	BLQ	BLQ	BLQ	BLQ	-
0.5	24.9	27.2	26.9	19.2	24.6 ± 3.7
1	64.2	63.0	101.1	72.1	75.1 ± 17.8
2	118.9	64.0	221.9	183.8	147.2 ± 69.9
3	79.4	42.3	186.8	168.4	119.2 ± 69.5
4	56.9	37.2	179.5	119.0	98.2 ± 64.5
6	40.7	31.5	75.6	61.1	52.2 ± 19.9
8	33.7	20.4	79.0	49.6	45.7 ± 25.2
12	11.7	12.2	70.6	29.0	30.9 ± 27.7
24	13.4	9.0	29.9	21.0	18.3 ± 9.2
30	16.0	8.9	30.7	24.8	20.1 ± 9.6
48	9.3	6.6	22.2	12.0	12.5 ± 6.8
72	6.5	4.3	15.6	6.2	8.2 ± 5.1
96	5.4	2.5	10.7	7.8	6.6 ± 3.5
120	3.6	2.9	8.1	6.6	5.3 ± 2.5
144	NR	1.8	6.3	4.9	4.3 ± 2.3
168	4.9	BLQ	5.2	4.3	3.6 ± 2.4
216	3.4	1.9	3.9	4.1	3.3 ± 1.0
312	3.2	BLQ	4.3	3.0	2.6 ± 1.6
384	4.1	BLQ	4.3	NS	2.8 ± 2.4
408	NS	NS	NS	2.4	2.4
480	3.3	1.0	3.0	NS	2.4 ± 1.3
600	BLQ	BLQ	4.5	NS	1.5 ± 2.6
768	2.5	BLQ	3.7	NS	21 ± 1.9
936	BLQ	BLQ	3.3	NS	1.1 ± 1.9
1560	NS	NS	NS	1.8	1.8
1726	NS	NS	NS	1.7	1.7
2232	NS	NS	NS	1.1	1.1

BLQ denotes Below Limit of Quantitation

NS denotes No Sample

Appendix 3.3.3D : Excretion of Radioactivity into Urine of Male and Female Subjects Following Oral Administration of [^{14}C]-Almitrine Bismesylate (~100mg ≅30μCi)

Subject No. / Time (Day)	1		2		3		4	
	% Dose	Cumu lative %Dose	% Dose	Cumu lative %Dose	% Dose	Cumu lative %Dose	% Dose	Cumu lative %Dose
1	3.65	3.65	4.74	4.74	3.98	3.98	3.86	3.86
2	1.31	4.96	1.53	6.27	0.85	4.83	1.32	5.18
3	0.97	5.93	1.07	7.34	0.93	5.76	1.07	6.25
4	0.71	6.64	0.94	8.28	0.32	6.08	0.80	7.05
5	0.66	7.30	1.07	9.35	0.46	6.54	0.68	7.73
6	0.52	7.82	0.81	10.16	0.48	7.02	0.5	8.24
7	0.52	8.34	0.63	10.79	0.44	7.46	0.44	8.68
8	0.46	8.80	0.30	11.09	0.36	7.82	0.35	9.03
9	0.36	9.16	0.32	11.41	0.32	8.14	0.29	9.32
10	0.33	9.49	0.40	11.81	0.28	8.42	0.27	9.59
11	0.30	9.79	0.49	12.30	0.34	8.76	0.22	9.81
12	0.35	10.14	0.42	12.72	0.24	9.00	0.21	10.02
13	0.19	10.33	0.40	13.12	0.24	9.24	0.21	10.23
14	0.31	10.64	0.24	13.36	0.23	9.47	0.20	10.43
15	0.21	10.85	0.17	13.56	0.09	9.56	0.18	10.61
16	0.22	11.07	0.22	13.75	0.23	9.79	0.15	10.76
17	0.21	11.28	0.22	13.97	0.18	9.97	0.14	10.90
18	0.22	11.50	0.18	14.15	0.16	10.13	0.15	11.05
19	0.21	11.71	0.17	14.32	0.23	10.36	0.13	11.18
20	0.20	11.91	0.15	14.47	0.26	10.62	0.13	11.31
21	0.13	12.04	0.19	14.66	0.17	10.79	0.11	11.42
22	0.17	12.21	0.21	14.87	0.12	10.91	0.11	11.53
23	0.17	12.38	0.15	15.02	0.10	11.01	0.10	11.63
24	0.16	12.54	0.18	15.20	0.13	11.14	0.09	11.72
25	0.15	12.69	0.15	15.35	0.15	11.29	0.08	11.80
26	0.15	12.84	0.12	15.47	0.09	11.38	0.08	11.88
27	0.11	12.95	0.10	15.57	0.06	11.44	0.09	11.97
28	0.12	13.07	0.08	15.65	0.08	11.52	0.08	12.05
29-60	1.99	15.06	1.43*	17.08	1.41*	12.93	1.44	13.49
61-90	0.14*	15.20	0.18**	17.26	-	-	0.47	13.96
91-120	-	-	0.1***	17.36	-	-	0.30	14.26
121-140	-	-	-	-	-	-	0.15	14.41

*60-69

*29-49
**72-90
***92-105

*29-63

Appendix 3.3.3E: Excretion of Radioactivity into Faeces of Male and Female Subjects Following Oral Administration of [^{14}C]-Almitrine Bismesylate (~100mg ≅30μCi)

Time (Day) \ Subject No.	1		2		3		4	
	% Dose	Cumulative %Dose	% Dose	Cumulative %Dose	% Dose	Cumulative %Dose	% Dose	Cumulative %Dose
1	NS	NS	0.13	0.13	0.40	0.40	14.45	14.45
2	1.07	1.07	6.93	7.06	18.81	19.21	9.95	24.40
3	10.81	11.88	17.41	24.47	22.76	41.97	0.48	24.88
4	NS	11.88	9.43	33.90	4.73	46.70	13.51	38.39
5	28.97	40.85	3.46	37.36	2.91	49.61	4.29	42.68
6	3.33	44.18	7.40	44.76	2.48	52.09	2.01	44.69
7	3.29	47.47	4.70	49.46	2.44	54.53	4.38	49.07
8	2.25	49.72	NS	49.46	1.28	55.81	1.21	50.28
9	NS	49.72	4.33	53.79	1.27	57.08	1.37	51.65
10	NS	49.72	0.58	54.37	1.01	58.09	0.74	52.39
11	1.17	50.89	NS	54.37	0.59	58.68	0.68	53.07
12	0.49	51.38	1.00	55.37	1.29	59.97	0.67	53.74
13	NS	51.38	0.50	55.87	0.55	60.52	0.21	53.95
14	0.97	52.35	0.14	56.01	NS	60.52	0.21	54.16
15	1.20	53.55	1.07	57.08	0.82	61.34	0.35	54.51
16	NS	53.55	0.24	57.32	0.84	62.18	1.10	55.61
17	0.86	54.41	0.24	57.56	0.48	62.66	0.33	55.94
18	0.87	55.28	0.31	57.87	0.47	63.13	0.27	56.21
19	0.26	55.54	0.60	58.47	0.49	63.62	0.45	56.66
20	0.29	55.83	0.09	58.56	0.58	64.20	0.28	56.94
21	NS	55.83	0.50	59.06	0.31	64.51	0.61	57.55
22	0.81	56.64	0.24	59.30	0.37	64.88	0.33	57.88
23	NS	56.64	0.24	59.57	0.49	65.37	0.29	58.17
24	0.54	57.18	0.60	60.17	0.29	65.66	0.31	58.48
25	0.47	57.65	0.34	60.51	0.20	65.86	0.19	58.67
26	0.61	58.26	0.20	60.71	0.57	66.43	0.52	59.19
27	NS	58.26	0.15	60.86	0.28	66.71	0.28	59.47
28	0.65	58.91	0.36	61.22	0.33	67.04	NS	59.47
29-60	7.03*	65.94	5.94	67.16	7.92	74.96	6.59	66.06
61-90	3.85**	69.79	2.64*	69.80	3.32	78.28	2.74	68.80
91-120	2.82	72.61	1.97	71.77	2.90	81.18	1.83	70.63
121-140	0.79	73.40	1.01**	72.78	0.28*	81.46	0.84	71.47

*29-59
**Including estimated 1.21% calculated over 8 day non collection
***121-133

*Including estimated 0.40% calculated over 8 day non collection
**121-135

*134-140

Appendix 3.3.3F : Cumulative Total Excretion of Radioactivity into Urine and Faeces of Human Subjects Following Oral Administration of [^{14}C]-Almitrine Bismesylate (~100mg ≅30μCi)

Subject No. / Time (Days)	1	2	3	4	MEAN ± SD
1	3.65	4.87	4.38	18.31	7.80 ± 7.02
2	6.03	13.33	24.04	29.58	18.25 ± 10.57
3	17.81	31.81	47.73	31.13	32.12 ± 12.24
4	18.52	42.18	52.78	45.44	39.73 ± 14.82
5	48.15	46.71	56.15	50.41	50.36 ± 4.15
6	52.00	54.92	59.11	52.93	54.74 ± 3.16
7	55.81	60.25	61.99	57.75	58.95 ± 2.72
8	58.52	60.55	63.63	59.31	60.50 ± 2.25
9	58.88	65.20	65.22	60.97	62.57 ± 3.17
10	59.21	66.18	66.51	61.98	63.47 ± 3.51
11	60.68	66.67	67.44	62.88	64.42 ± 3.19
12	61.52	68.09	68.97	63.76	65.59 ± 3.54
13	61.71	68.99	69.76	64.18	66.16 ± 3.86
14	62.99	69.37	69.99	64.59	66.74 ± 3.47
15	64.40	70.61	70.90	65.12	67.76 ± 3.48
16	64.62	71.07	71.97	66.37	68.51 ± 3.57
17	65.69	71.53	72.63	66.84	69.17 ± 3.42
18	66.78	72.02	73.26	67.26	69.83 ± 3.29
19	67.25	72.99	73.98	67.84	70.47 ± 3.42
20	67.74	73.03	74.82	68.25	70.96 ± 3.51
21	67.87	73.72	75.30	68.97	71.47 ± 3.60
22	68.85	74.17	75.79	69.41	72.06 ± 3.45
23	69.02	74.59	76.38	69.80	72.45 ± 3.60
24	69.72	75.37	76.80	70.20	73.02 ± 3.59
25	70.34	75.86	77.15	70.47	73.46 ± 3.56
26	71.10	76.18	77.81	71.07	74.04 ± 3.48
27	71.21	76.43	78.15	71.44	74.31 ± 3.52
28	71.98	76.87	78.56	71.52	74.73 ± 3.52
29-60	81.00	84.24	87.84	79.55	83.16 ± 3.69
61-90	84.99	87.06	91.21	82.76	86.51 ± 3.59
91-120	87.81	89.13	94.11	84.89	88.99 ± 3.85
121-140	88.60	90.14	94.39	85.88	89.75 ± 3.56
TOTAL (DAYS)	**133**	**135**	**140**	**140**	**137 ± 3.56**

APPENDICES TO CHAPTER 5

Appendix 5.1 : Plasma Levels of Almitrine (ng.ml^{-1}) in Healthy Male Human Volunteers Following Either Oral (100mg) or Intravenous (15mg) Administration of Almitrine Bismesylate as a Solution

TIME (H)	SUBJECTS				MEAN	±SD
	1	2	3	4		
Body Weight (kg)	78	75	65	61	71	7.9
Age (yrs)	30	35	27	30	31	3.3
ORAL						
1.00	80	167	115	240	151	70
2.00	182	184	219	378	241	93
3.00	128	141	181	265	179	62
4.00	119	127	163	215	156	44
5.00	104	100	144	169	129	33
6.00	81	74	109	132	99	27
7.00	84	64	93	104	86	17
8.00	80	58	91	85	79	14
9.00	75	60	81	80	74	10
12.00	56	35	75	71	59	18
24.00	36	16	34	36	31	10
33.00	32	16	47	32	32	13
48.00	25	9	20	22	19	7
INTRAVENOUS						
0.03	222	293	218	578	398	170
0.08	NS	95	71	150	105	41
0.16	70	65	54	125	79	32
0.25	44	34	51	75	51	18
0.50	22	34	34	65	39	18
1.00	17	18	22	37	24	9
2.00	13	17	17	20	17	3
3.00	12	7	7	21	12	7
4.00	9	5	9	13	9	3
6.00	9	4	7	10	8	3
8.00	6	4	7	11	7	3
12.00	9	4	5	18	9	6
24.00	5	2	3	4	4	1
33.00	5	NS	4	5	5	1
48.00	2	NS	4	3	3	1

NS denotes No Sample

Appendix 5.2a : Plasma Levels of Almitrine ($ng.ml^{-1}$) in Male and Female Subjects Following Oral Administration of Almitrine Bismesylate Solution (30mg)

(Study No. 2)

TIME (H)	SUBJECTS								MEAN ± SD
	1	2	3	4	5	6	7	8	
0.50	11	19	30	18	27	11	35	8	20 ± 10
1.00	91	67	39	20	43	35	65	23	48 ± 24
1.50	63	76	35	35	42	33	84	36	51 ± 21
2.00	57	56	44	52	42	32	66	28	47 ± 13
3.00	47	86	30	25	29	24	56	28	41 ± 22
4.00	32	41	30	16	32	22	47	21	30 ± 10
6.00	31	37	23	8	20	14	26	13	22 ± 10
8.00	18	21	17	19	17	9	17	11	16 ± 4
10.00	20	20	12	32	15	11	15	8	17 ± 7
24.00	12	8	6	17	12	8	11	10	11 ± 3
33.00	14	7	9	25	16	4	8	4	11 ± 7
48.00	7	4	4	18	18	4	8	4	8 ± 6
57.00	8	6	4	17	4	3	7	3	7 ± 5
72.00	7	3	4	18	3	2	4	3	6 ± 5

Appendix 5.2b : Plasma Levels of Almitrine (ng.ml^{-1}) in Male and Female Subjects Following Intravenous Administration of Almitrine Bismesylate (30mg)
(Study No. 2)

TIME	SUBJECTS								MEAN
(H)	1	2	3	4	5	6	7	8	± SD
0.03	336	536	298	ND	150	472	328	525	378 ± 140
0.08	164	455	177	83	107	270	268	294	227 ± 120
0.16	135	186	110	86	95	198	133	176	140 ± 43
0.25	128	130	92	ND	56	128	89	168	113 ± 37
0.50	85	88	77	46	64	82	95	87	78 ± 16
1.00	62	44	46	ND	65	79	59	84	63 ± 15
1.25	ND	ND	ND	37	ND	ND	ND	ND	ND
1.50	55	52	45	ND	44	53	58	70	54 ± 9
2.00	43	ND	43	ND	38	55	42	42	44 ± 6
3.00	32	27	40	ND	20	35	32	35	32 ± 6
4.00	31	21	37	27	28	23	24	30	28 ± 5
5.00	ND	ND	ND	23	ND	ND	ND	ND	ND
6.00	22	16	24	16	17	17	17	22	19 ± 3
8.00	20	9	32	21	22	15	28	ND	21 ± 8
9.00	ND	ND	ND	ND	ND	ND	ND	16	ND
10.00	19	17	33	38	26	16	18	15	23 ± 9
24.00	ND	9	11	17	14	12	15	10	13 ± 3
33.00	12	11	24	11	10	10	13	16	13 ± 5
48.00	8	3	ND	18	9	7	9	8	9 ± 5
57.00	9	5	8	14	ND	7	9	6	8 ± 2
72.00	5	4	4	ND	10	5	8	5	6 ± 2

ND denotes Not Determined

Appendix 5.3a : Plasma Levels of Almitrine (ng.ml^{-1}) in 12 Male Subjects Following Oral Administration of Almitrine Bismesylate Tablets (2 x 50mg)

(Study No. 3)

TIME	SUBJECT												MEAN
(H)	1	2	3	4	5	6	7	8	9	10	11	12	± SD
0.50	17	20	37	11	15	6	14	1	ND	12	ND	24	16 ± 10
1.00	42	38	167	90	26	48	67	18	20	77	22	175	66 ± 54
2.00	72	133	158	276	145	150	150	138	96	105	56	387	156 ± 91
3.00	62	146	143	194	151	153	117	96	161	104	166	302	150 ± 60
4.00	44	98	109	167	132	127	83	126	182	116	177	226	132 ± 49
5.00	45	84	98	135	139	118	65	115	123	111	106	175	110 ± 34
6.00	52	82	96	109	104	124	50	69	64	57	138	146	91 ± 34
7.00	44	69	86	66	91	107	41	67	80	43	85	105	74 ± 23
8.00	38	61	83	62	ND	97	35	51	62	31	67	86	61 ± 22
9.00	37	57	70	49	71	67	34	55	59	33	78	89	58 ± 18
12.00	28	49	44	62	49	ND	39	48	56	41	78	96	54 ± 19
24.00	20	28	17	21	14	27	18	37	30	9	29	36	24 ± 8
33.00	19	30	32	16	27	17	16	28	25	12	33	32	24 ± 7
48.00	18	21	17	7	10	9	7	18	25	5	20	23	15 ± 7

Appendix 5.3b : Plasma Levels of Almitrine (ng.ml^{-1}) in 12 Male Subjects Following Oral Administration of Almitrine Bismesylate Solution (100mg)

(Study No. 3)

TIME	SUBJECT												MEAN
(H)	1	2	3	4	5	6	7	8	9	10	11	12	± SD
0.50	ND	ND	ND	ND	27	14	14	25	19	9	12	65	23 ± 18
1.00	80	167	115	240	79	54	49	76	67	68	77	94	97± 55
2.00	182	184	219	378	95	136	160	114	108	118	282	278	188 ± 87
3.00	128	141	181	265	98	156	101	95	102	115	250	233	155 ± 63
4.00	119	127	163	215	85	186	101	72	79	107	236	207	141 ± 58
5.00	104	100	144	169	82	123	74	85	56	58	217	170	115 ± 50
6.00	81	74	109	132	64	121	52	75	76	47	155	121	92 ± 34
7.00	84	64	93	104	55	89	40	63	45	41	133	113	77 ± 30
8.00	80	58	91	85	53	78	36	56	39	37	114	100	69 ± 26
9.00	75	60	81	80	44	71	29	49	50	28	84	100	63 ± 23
12.00	56	35	75	71	38	ND	49	44	41	38	92	120	60 ± 27
24.00	36	16	34	36	23	24	6	29	18	22	36	44	27 ± 11
32.00	32	16	47	32	22	18	2	26	17	14	25	43	25 ± 13
48.00	25	9	20	22	18	14	1	18	11	13	22	32	17 ± 8

ND denotes Not Determined

Appendix 5.4a : Plasma Levels of Almitrine (ng.ml^{-1}) in Healthy Male Subjects Following Oral Administration of Almitrine Bismesylate Tablets (2 x 50mg) in either A Fasting State or After a Standardised Breakfast (Study No. 4)

Fasted

TIME	SUBJECT										MEAN
(H)	1	2	3	4	5	6	7	8	9	10	± SD
0.50	37	6	11	17	BLQ	12	14	BLQ	BLQ	20	17 ± 10
1.00	167	48	90	42	18	77	67	20	22	38	59 ± 45
2.00	158	150	276	72	138	105	150	96	56	133	133 ± 61
3.00	143	153	194	62	96	104	117	161	166	146	134 ± 39
4.00	109	127	167	44	126	116	83	182	177	98	123 ± 44
5.00	98	118	135	45	115	111	65	123	106	84	100 ± 28
6.00	96	124	109	52	69	57	50	64	138	82	84 ± 31
7.00	86	107	66	44	67	43	41	80	85	69	69 ± 22
8.00	83	97	62	38	51	31	35	62	67	61	59 ± 21
9.00	70	67	49	37	55	33	34	59	78	57	54 ± 16
12.00	44	NS	62	28	48	41	39	56	78	49	49 ± 14
24.00	17	27	21	20	37	9	18	30	29	28	24 ± 8
33.00	32	17	16	19	28	12	16	25	33	30	23 ± 8
48.00	17	9	7	18	18	5	7	25	20	21	15 ± 7

NS denotes No Sample
BLQ denotes Below Limit of Quantitation

Appendix 5.4b : Plasma Levels of Almitrine (ng.ml^{-1}) in Healthy Male Subjects Following Oral Administration of Almitrine Bismesylate Tablets (2 x 50mg) in either A Fasting State or After a Standardised Breakfast (Study No. 4)

Standard Breakfast

TIME	SUBJECT										MEAN
(H)	1	2	3	4	5	6	7	8	9	10	± SD
0.50	5	9	BLQ	43	BLQ	39	BLQ	BLQ	11	9	19 ± 17
1.00	27	94	119	54	85	215	20	70	92	114	89 ± 55
1.50	155	183	372	84	174	180	156	153	241	298	200 ± 83
2.00	329	180	380	143	191	152	230	213	207	263	229 ± 76
3.00	299	167	242	137	150	131	175	255	NS	152	190 ± 60
4.00	219	114	157	76	123	99	153	152	262	121	148 ± 56
5.00	243	144	143	83	95	80	127	141	215	131	137 ± 54
6.00	132	78	78	65	86	62	87	111	166	96	96 ± 32
8.00	70	71	70	67	52	45	61	79	108	81	70 ± 17
12.00	110	44	51	50	59	44	78	64	85	60	64 ± 21
24.00	33	21	37	45	12	14	41	33	40	41	32 ± 12
32.00	31	21	13	46	11	17	29	36	34	39	28 ± 12
48.00	23	7	9	37	4	8	20	22	28	40	20 ± 13

NS denotes No Sample
BLQ denotes Below Limit of Quantitation

Appendix 5.5 : Plasma Levels of Almitrine ($ng.ml^{-1}$) in Male Subjects Given the Drug (Approx 60mg) as a Constant Rate Infusion Over a 2 Hour Period (Study No. 5)

TIME (H)	SUBJECT		
	1	2	3
0.5	102	249	213
1	352	280	281
1.5	456	264	310
2	415	242	323
2.25	183	152	137
2.5	225	107	129
2.75	NS	105	96.5
3	98.1	101	116
3.5	86.8	68.1	104
4	79.3	60.4	62.4
5	67.5	56.6	69.9
6	57.1	51.7	55.7
8	36.4	23.7	58.5
12	50.0	51.1	63.7
24	37.0	33.9	24.6
26.5	32.8	32.6	24.7
27.5	33.5	NS	NS
28	NS	36.8	29.4
28.5	31.8	NS	NS
29	NS	40.2	46.2
32	31.6	40.1	33.5
48	22.0	NS	NS
50	NS	NS	27.0
53.5	NS	23.4	NS
72	15.7	NS	NS
74.5	NS	NS	17.4
75	NS	12.2	NS
99	13.9	12.1	16.3
123	12.0	9.1	20.1

NS denotes No Sample

Appendix 5.5 (cont) : Plasma Levels of Almitrine ($ng.ml^{-1}$) in Male Subjects Given the Drug (Approx 60mg) as a Constant Rate Infusion Over a 2 Hour Period
(Study No. 5)

TIME (H)	SUBJECT		
	1	2	3
144	NS	8.4	NS
146.5	12.5	NS	NS
149	NS	NS	16.9
150	NS	NS	15.6
170.5	NS	8.2	NS
171.5	10.8	8.0	NS
172.5	NS	10.8	NS
192	9.2	6.5	NS
198	NS	NS	14.5
216	7.5	6.9	11.1
240	8.0	NS	9.2
264	10.9	6.6	9.0
288	7.4	5.0	NS
312	8.2	6.9	11.6
336	9.4	5.3	12.3
360	6.9	4.9	10.8
384	NS	4.5	NS
408	6.9	4.5	8.4
456	8.6	NS	6.7
480	NS	4.1	NS
504	6.1	NS	NS
528	NS	NS	7.7
576	NS	4.0	NS
600	6.3	NS	6.8
648	6.2	NS	7.2
696	5.5	2.6	4.7
792	4.6	2.2	NS
840	NS	NS	9.8
912	NS	NS	5.0

NS denotes No Sample

Appendix 5.6 : Plasma Levels of Almitrine (ng.ml^{-1}) in Subjects Given the Drug Repetitively (50mg b.i.d.)

Study Day / SUBJECT	DAYS ON ALMITRINE										
	1	3	5	8	10	11	12	17	19	23	25
	15	17	19	22	24	25	26	31	33	37	39
1	-	128	126	245	110	-	126	182	177	-	-
2	49	74	99	123	134	-	105	194	223	406	213
3	139	149	185	161	-	306	212	149	238	262	166
4	10	94	193	143	-	-	139	193	193	180	268
5	69	-	236	135	-	158	189	-	473	326	210
6	76	85	201	-	308	-	291	183	219	283	153
MEAN	69	106	190	161	184	232	177	180	254	291	202
±SD	47	31	73	49	108	105	69	18	110	83	45

TIME (Hours) / SUBJECT	DAY 29 (DAY 15 ON ALMITRINE)							DAY 43 (DAY 29 ON ALMITRINE)									
	0	1	2	3	4	8	24	0	1	2	4	8	24	26	30	48	72
1	106	137	159	-	143	150	-	139	149	222	196	203	-	79	130	109	81
2	123	210	262	-	220	186	160	199	211	251	211	-	151	-	188	139	123
3	-	160	204	-	122	153	141	167	197	244	213	198	168	-	173	108	106
4	142	124	203	-	173	154	141	222	150	197	251	256	177	-	170	170	109
5	166	180	180	332	184	179	147	154	162	157	169	140	123	-	138	124	106
6	153	202	216	-	162	218	187	142	-	158	50	273	144	-	142	123	105
MEAN	138	169	204	332	167	173	155	171	174	205	182	214	153	79	157	139	105
±SD	24	35	35	-	34	27	19	33	28	41	70	53	21	-	28	23	14

Appendix 5.7 : Salivary Levels of Antipyrine (μg.ml^{-1}) After an Oral Dose of the Drug (500mg) Before and During Repeated Dosage Regimen (50mg b.i.d.) of Almitrine Bismesylate

TIME	BEFORE ALMITRINE							DURING ALMITRINE						
	DAY 2							DAY 30 (16 DAYS ON ALMITRINE)						
(H)	1	2	3	4	5	6	Mean ±SD	1	2	3	4	5	6	Mean ± SD
0.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
3.0	19.0	16.5	16.7	19.9	19.1	16.2	17.9 ± 1.6	16.3	14.0	22.1	20.1	23.3	14.4	18.4 ± 4.0
6.0	15.3	13.9	11.4	14.2	15.6	10.3	13.5 ± 2.1	14.0	13.0	15.6	16.9	17.9	11.5	14.8 ± 2.4
9.0	11.9	12.2	11.4	10.0	12.1	9.6	11.2 ± 1.1	10.8	10.0	10.5	15.0	12.7	10.0	11.5 ± 2.0
12.0	9.1	10.9	9.2	9.3	8.7	9.0	9.4 ± 0.8	6.7	8.2	7.9	12.1	11.9	8.4	9.2 ± 2.2
15.0	8.1	9.7	8.1	7.6	7.6	7.3	8.1 ± 0.9	5.9	7.0	6.6	11.1	9.5	7.6	8.0± 2.0
24.0	3.5	6.1	3.2	4.1	3.4	4.6	4.2 ± 1.1	2.8	4.8	3.3	6.9	6.4	5.2	4.9 ± 1.6
28.0	2.6	5.2	2.9	3.1	2.5	4.3	3.4 ± 1.1	2.9	4.4	NS	6.2	3.5	4.3	4.3 ± 1.2
33.0	1.8	4.2	1.7	2.1	2.8	3.2	2.6 ± 1.0	1.0	2.5	1.9	4.4	2.9	3.1	2.6 ± 1.2

NS denotes No Sample

Appendix 5.8 : Plasma Levels of Erythromycin ($\mu g.ml^{-1}$) After an Oral Dose of the Drug (500mg) Before and During a Repeated Dosage Regimen (50mg b.i.d.) of Almitrine Bismesylate

TIME	BEFORE ALMITRINE						DURING ALMITRINE					
	DAY 5						DAY 32 (18 DAYS ON ALMITRINE)					
(H)	1	2	3	4	5	6	1	2	3	4	5	6
0.0	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
0.5	0.37	0.84	0.14	2.02	ND	0.07	0.09	0.13	0.14	1.08	0.30	0.88
1.0	0.68	1.17	0.15	1.92	0.88	0.11	0.29	1.18	0.39	1.27	0.88	0.79
1.5	0.55	1.14	0.57	1.88	1.29	1.66	0.17	1.34	0.82	0.57	0.70	1.61
2.0	0.50	0.93	0.57	1.97	0.85	2.18	0.33	1.19	0.59	0.97	0.59	2.19
3.0	0.26	0.64	0.22	1.47	0.46	1.84	0.30	0.75	0.45	0.99	0.41	1.15
4.0	0.24	0.53	0.15	1.39	0.35	1.56	0.27	0.55	0.36	0.95	0.24	0.65
6.0	0.11	0.18	0.06	0.90	0.17	0.89	0.15	0.26	0.33	0.49	0.15	0.26
8.0	0.01	0.08	ND	0.29	0.06	0.41	0.07	0.11	0.16	0.18	0.05	0.13
10.0	<0.05	<0.05	<0.05	0.17	<0.05	0.17	<0.05	0.07	<0.05	0.09	<0.05	0.07

ND denotes Not Determined

Appendix 5.9 : Plasma Levels of Digoxin ($ng.ml^{-1}$) After an Oral Dose of the Drug (0.25mg) Before and During a Repeated Dosage Regimen (50mg b.i.d.) of Almitrine Bismesylate

TIME	BEFORE ALMITRINE						DURING ALMITRINE					
	DAY 8						DAY 36 (DAY 22 ON ALMITRINE)					
(H)	1	2	3	4	5	6	1	2	3	4	5	6
0.0	0.05	0.09	0.04	0.07	0.03	-	0.05	0.04	0.13	0.00	0.04	0.07
0.5	2.46	0.08	-	1.53	0.63	1.88	1.14	2.26	1.35	0.36	1.55	4.36
1.0	2.32	1.08	1.76	1.95	1.24	2.42	2.12	2.50	4.02	0.99	2.31	1.84
1.5	1.52	2.00	4.07	1.43	1.04	1.68	2.58	1.59	3.33	1.18	2.44	1.52
2.0	1.16	2.17	3.18	1.69	2.01	1.34	2.03	1.31	3.35	1.55	1.79	1.47
4.0	0.78	0.96	1.30	1.71	1.06	0.75	1.12	0.70	0.90	0.86	0.79	0.86
6.0	0.62	0.53	0.55	0.74	0.49	0.70	0.56	0.53	0.51	0.50	0.41	0.44
8.0	0.51	0.44	0.56	0.59	0.41	0.73	0.53	0.41	0.42	0.39	0.31	0.43
10.0	0.39	0.50	0.43	0.69	0.30	0.49	0.44	0.30	0.43	0.35	0.29	0.33
24.0	0.33	0.35	0.32	0.40	0.34	0.32	0.32	0.29	0.33	0.27	0.37	0.35
36.0	0.33	0.35	0.31	0.51	0.23	0.32	0.28	-	0.26	0.31	0.34	0.49
48.0	0.25	0.25	0.29	0.26	0.27	0.26	0.26	0.30	0.24	0.23	0.25	0.28
72.0	-	0.20	0.29	0.26	0.16	0.22	-	0.10	0.15	0.13	0.11	0.19