PHARMACOKINETICS OF NEOSTIGMINE AND PYRIDOSTIGMINE IN MAN

Thesis

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by

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ABSTRACT

Pharmacokinetics of Neostigmine and Pyridostigmine in Man, by Akbar Dehghan

Most methods used for the extraction of quaternary amines in biological fluids have been based on ion-pair extraction techniques. In this study, a sensitive and selective chromatographic procedure is described to measure the concentration of pyridostigmine, neostigmine and their major metabolites in the plasma and urine. The methods involve a preliminary selective ion-pair extraction of the unchanged drugs and their metabolites into dichloromethane and dichloromethane-acetone mixture respectively. Quantitation is possible down to 3 ng/ml for parent drugs and 50 ng/ml for the metabolites. The present work also described a modified procedure to measure neostigmine and pyridostigmine in plasma simultaneously, using a specially synthesized pyridostigmine analogue as a common internal marker.

The plasma concentration of pyridostigmine was measured after three different doses of the quaternary amine (36.2 μg/kg, 72.4 μg/kg and 144.8 μg/kg) were given intravenously to twenty five surgical patients during anaesthesia. The relation between the plasma concentration of pyridostigmine and time was invariably expressed as a bi-exponential equation, and the data was interpreted in terms of a two compartment model. The slow disposition half-life of the drug was progressively prolonged as the dose of the drug was raised from 36.2 μg/kg to 144.8 μg/kg. A similar increase in the half-life was observed in cross-over studies. The clearance of the quaternary amine was enhanced at intermediate dose (72.4 μg/kg) but significantly reduced after high dose (144.8 μg/kg). It was suggested that these results may partially account for the observed differences in the duration of action of neostigmine and pyridostigmine in man. The dose of pyridostigmine used to reverse non-depolarizing neuromuscular block is 4-5 times greater than neostigmine.

After intramuscular administration of neostigmine to five myasthenic patients, the plasma concentration of the drug declined monoexponentially from 21 ± 2 ng/ml to 9 ± 1 ng/ml between 30 and 120 minutes and data was interpreted in terms of a one-compartment model. Estimates of the plasma half-life varied from 56.9 - 100.1 minutes.

The oral administration of pyridostigmine alone and of both neostigmine and pyridostigmine in two different groups of myasthenic patients, were also studied. There was a direct linear relation between area under the plasma concentration - time curve and total daily dose of pyridostigmine in the first group of patients ($r = 0.95$), but no such observation was noticed in either all patients (two groups; $r = 0.15$) or in the second group who were treated with both drugs ($r = -0.08$). It was suggested that there might be a drug-drug interaction between pyridostigmine and neostigmine during oral absorption.

The relation between plasma levels of pyridostigmine to clinical evaluation of muscle power was examined in nine myasthenic patients during treatment with pyridostigmine in doses of 60 to 1080 mg per day. Five of the nine subjects demonstrated a trend towards a positive correlation and in two of them was this significant at $p < 0.05$. In addition, the presence or absence of a possible correlation between muscle power and plasma concentration was not related to the duration of disease, additional prednisolone therapy or thymectomy.

CONTENTS

PREFACE

This thesis, comprising of 7 Chapters, presents the development of the techniques available to measure the concentration of neostigmine and pyridostigmine in human biological fluids and also a study of the pharmacokinetics of the drugs in man. In addition, clinical studies were carried out to investigate the relation of plasma levels of pyridostigmine to clinical effects in patients with myasthenia gravis.

In the first Chapter, the known pharmacological properties and clinical use of quaternary ammonium compounds are discussed. An introduction to the mathematical expressions of pharmacokinetics and a review on previous methods available for the determination of quaternary ammonium compounds is described in Chapter 2. Chapter 3 includes the development of the techniques used in the present investigations. The studies of pharmacokinetics of pyridostigmine in surgical patients are presented in Chapter 4. In Chapter 5 the study of pharmacokinetics of neostigmine after intramuscular administration to myasthenic patients is described. Chapter 6 includes the study of pharmacokinetics and pharmacologic effects of pyridostigmine and neostigmine after oral administration in patients with myasthenia gravis. The main findings and conclusions of this thesis are summarized in the final chapter. The appendices expand some experimental details and tabulated results not included elsewhere in the thesis.

The investigation was carried out in the Pharmaceutical Chemistry section of the School of Pharmacy, Liverpool Polytechnic. I wish to express my appreciation to Dr. K. Chan for the consistent

advice and encouragement he has given me whilst supervising this work. I express my thanks also to Dr. T.N. Calvey for his advice and co-operation in providing human blood samples to carry out the studies in Chapter 4 and 5. I am indebted to Dr. D.M. Freeman and Dr. M. Hayward for permitting the study of patients admitted under their care and to the patients for their ready co-operation.

I am grateful to Dr. Susan C. Davison and Dr. N. Hyman of the Radcliffe Infirmary, Oxford, for their joint effort in the study on clinical response and plasma pyridostigmine (Chapter 6). Finally, I would like to thank Dr. P. Deravi for his assistance in computer programming.

CHAPTER 1

GENERAL INTRODUCTION

CHAPTER ONE

ANTICHOLINESTERASE AGENTS

HISTORICAL BACKGROUND

Drugs that inhibit or inactivate acetylcholinesterases (AChE) are called anticholinesterase (anti-ChE) agents and thus are potentially capable of producing effects equivalent to continuous stimulation of cholinergic fibres throughout the central and peripheral nervous system. In view of the widespread distribution of cholinergic neurons, it is not surprising that the anti-ChE agents as a group have assumed more extensive practical application as toxic agents, in the form of agricultural insecticides and potential chemical warefare "nerve gases" than as drugs, nevertheless, there are certain representatives that are clinically useful. (See Table 1-1).

Prior to the Second World War, only "reversible" anti-ChE agents were generally known, of which physostigmine (eserine) is the outstanding example. Shortly before and during World War II a comparatively new class of highly toxic chemicals, the organophosphorus was developed, chiefly by Schrader, of I.G. Farbenindustrie. These were first used as agricultural insecticides and later as potential chemical warfare agents (Roelle 1975). The extreme toxicity of these compounds was found to be due to their "irreversible" inactivation of AChE, thereby exerting their effects for considerably longer periods than do the classical reversible inhibitors. Since the pharmacological actions of both classes of anti-ChE agents are qualitatively similar, they will be discussed as a group, and the important special features of the individual classes or compounds will be pointed out.

Physostigmine, the first known anti-ChE agent, is an alkaloid obtained from the Calabar or ordeal bean of Physostigma Venonosum Balfour, which is a perennial woody climber growing in tropical West Africa. The calabar bean, also known as Esere nut, chop nut, or bean Etu Esere, was once used by native tribes of West Africa as an ordeal poison in trials for witchcraft. It was brought to England in 1840 by Daniell who was a British Medical Officer stationed in Calabar. Early investigations of its pharmacological properties were induced by Christieson (1825), Fraser (1863), and Argyll-Robertson (1863). The pure alkaloid of physostigmine was isolated by Jobts and Hesse in 1864, and named physostigmine and the following year Vee and Leven (Goldman, 1975) obtained the same alkaloid which they named as eserine. The alkaloid was used first as a therapeutic agent in 1877 by Laqueurs in the treatment of glaucoma. Polonovski and Polonovski (1923) and Stedman and Bargar (1925) elucidated the chemical structure (see Table 1.1) of physostigmine, and its synthesis was accomplished by Julian and Pikl, (1935). Interesting accounts of the history of physostigmine have been presented by Rodin (1947), Karczmar (1970) and Holmstedt (1972).

As a result of the basic research of Stedman and associates, (1929a, 1929b) in elucidating the chemical basis of the activity of physostigmine, Aeschlimannand Reinard (1931) systematically investigated a series of substituted phenyl esters of alkyl carbamic acids. Neostigmine (see Table 1.1) a member of this series, was introduced into therapeutics in 1931 for its stimulant action on the intestinal tract. It was reported independently by Remen (1932), and Walker (1935) to be

effective in the symptomatic therapy of myasthenia gravis. Additional anti-ChE agents that bear a general structural resemblance to neostigmine were introduced for the treatment of myasthenia gravis. Among these are pyridostigmine and ambenonium. Edrophonium, a drug of simpler structure and with an extremely brief duration of action, is used in the diagnosis and evaluation of the therapy of the same disease.

It is interesting to note that the first account of the synthesis of a highly potent compound of the organophosphorus anti-ChE series, tetraethylpyrophosphate (TEPP), was published by Clerment in 1854, ten years prior to the isolation of physostigmine. More remarkable still as Holmstedt (1963) pointed out, is the fact that the investigator survived to report on the compound's taste as a few drops of the pure compound placed on the tongue would be expected to prove rapidly fatal. During the synthesis and investigation of approximately 2,000 compounds, Schrader (1952) defined the structural requirements for insecticidal (and as learned subsequently, for anti-ChE) activity of compounds in this early series, parathion became the most widely employed insecticide of this class. The synthesis of several compounds of much greater toxicity than parathion such as Sarin, Soman and Tabun resulted (Schrader 1952), and these were used as nerve gases in chemical warfare.

THE MECHANISM OF ACTION OF ANTI-ChE AGENTS

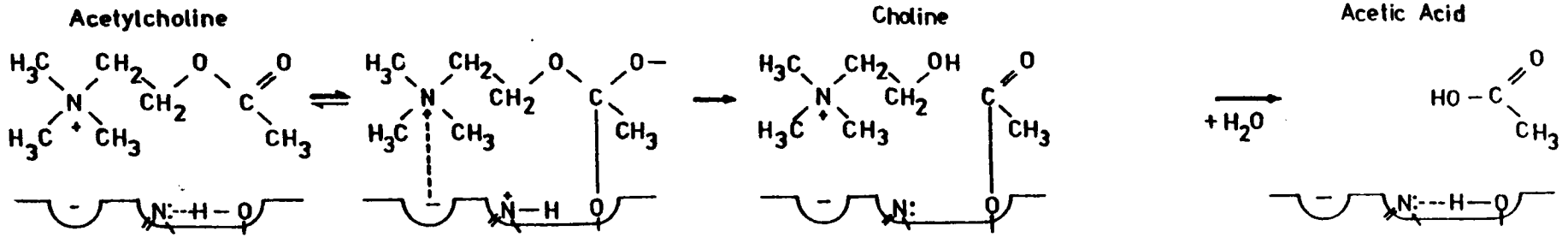
The anti-ChE agents are among the relatively few drugs for which the mechanism of action can presently be described in precise molecular terms. This can be related directly to their

overall pharmacological effects and has led to the development of useful compounds capable of reversing the actions of anti-ChE agents. The interactions between most of the anti-ChE agents and the enzyme AChE differ primarily in quantitative respects from the reaction between AChE and its normal substrate acetylcholine (ACh).

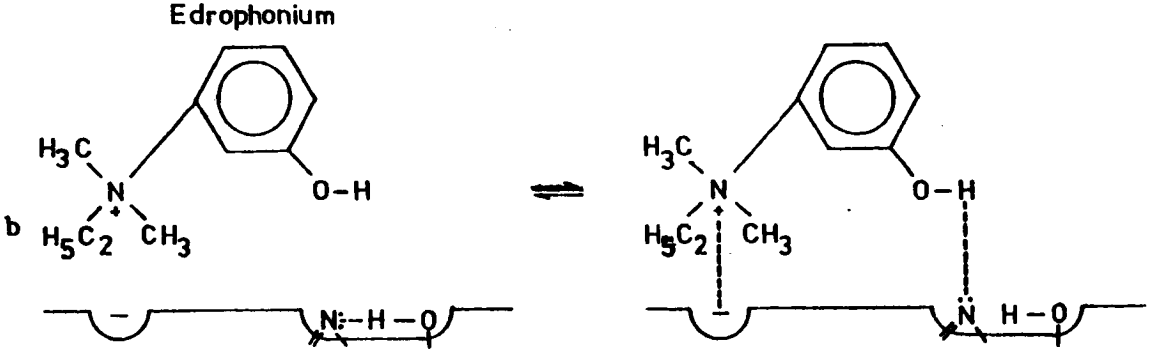
From studies initiated by Wilson and Bergmann (1950) and extended by Wilson and others, it has been established that the active surface of the enzyme unit consists of two sites, an anionic and an esteratic site (see Figure 1.1). At the anionic site (a negative charge probably of the free ionised carboxyl group of a dicarboxylic amino acid), the postively charged quaternary N atom of ACh is attracted by electrostatic forces, and bonding is enhanced by hydrophobic forces exerted on the N-methyl groups.

The esteratic site consists of essentially two components located 2.5 and 5Å respectively from the anionic site; a potentially acidic function (the hydroxy group of serine) and a nucleophilic group (the basic N of an imidazole group of histidine). The imidazole group by hydrogen bonding (see Figure 1.1) enhances the nucleophilic activity of the serine hydroxyl group, thus enabling it to inter act with the electron deficient carbonyl C atom of ACh. A covalent bond is formed with the production of an acetylated enzyme intermediate and the release of choline (Figure 1.1a.). The electron deficient C atom of the acetyl group then undergoes nucleophilic attack by the oxygen atom of a water molecule. The acetyl enzyme complex is thus hydrolyzed and the products are the regenerated enzyme and acetic acid.

a


Acetylcholine
Choline
Acetic Acid
+ H_2O
Anionic Site
Histidine
Serine
Esteratic Site
Acetylcholinesterase
Enzyme-Substrate Complex
Acetylated Enzyme
Regenerated Enzyme

b


Edrophonium
Enzyme-Inhibitor Complex

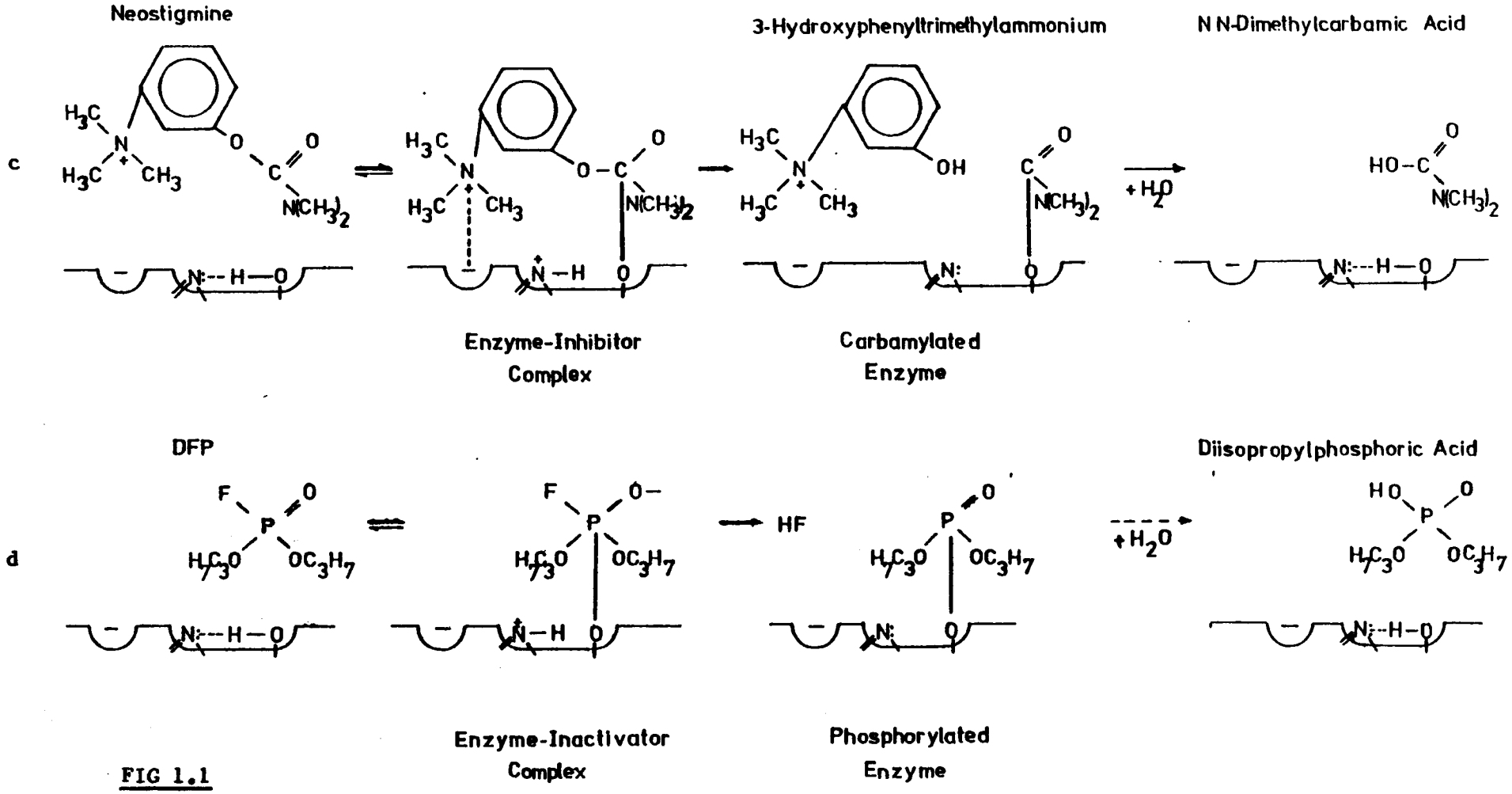


FIG 1.1

Steps involved in the hydrolysis of acetyl (Ach) by acetylcholinesterase (a), and the inhibition of AchE by reversible (b), carbamylester (c), and organophosphorus (d) agent.
Heavy, light, and dashed arrows represent extremely rapid, intermediate and extremely slow or insignificant reaction velocities, respectively. (Wilson, 1954, 1967; Fioed and Wilson, 1971; Kitz 1933).

The rate constants in this reaction sequence are extremely high, thus the time required for the complete hydrolysis of one ACh molecule (the turnover time), is only 80 micro seconds (Wilson et al., 1960).

The mechanisms of action of compounds that typify the three classes of anti-ChE agents are shown in Figure 1.1(b,c,d). Simple quaternary compounds, such as tetraethylammonium ion, inhibit the enzyme reversibly by combining with it only at the anionic site, and thus blocking attachment of the substrate. Much more potent reversible inhibitors, such as edrophonium, combine in addition with the imidazole nitrogen atom of the esteratic site by hydrogen bonding through the phenolic OH group (Figure 1.1b). In all such cases, inhibition is rapidly reversible and such drugs have a very brief duration of action following systemic administration.

It was at one time generally assumed that physostigmine, neostigmine and related potent inhibitors that possess a carbamyl ester linkage or urethane structure, in addition to a tertiary amino or quaternary ammonium group, inhibit the enzyme in the same reversible fashion. However, careful kinetic studies have since shown that only a negligible amount of an inhibitor of this type is released reversibly from the enzyme; actually, physostigmine and neostigmine are hydrolyzed extremely slowly by cholinesterases (Goldstein and Hamlish, 1952; Myers, 1952, 1956; Wilson et al., 1960). It is likely that inhibitors of this class form complexes in which the inhibitor is attached to the enzyme at both the anionic and esteratic sites, following which their hydrolysis proceeds in a manner

analogous to that of ACh (Figure1.1c), the alcoholic moiety is split off, leaving a carbamoylated enzyme that reacts with water to release a substituted carbamic acid and regenerated enzyme. The main difference between the hydrolysis of the natural substrate, ACh, and the inhibitor, neostigmine, is the velocity of the final step, the half-life of dimethylcarbamoyl-AChE, formed by the reaction with neostigmine, is more than 40 million times that of the acetylated enzyme (30 minutes and 42 micro-seconds respectively, Wilson and Harrison 1961). Thus, these drugs and newer carbamate insecticides are called "competitive substrates" or "acid transferring inhibitors" and can be distinguished from the simply reversible inhibitors described above.

The reaction between AChE and most organophosphorous inhibitors such as di-isopropylphosphorofluoridate (DFP) occurs only at the estratic site, but proceeds in a comparable fashion (Figure1.1d). Here the resultant phosphorylated enzyme is extremely stable; if the attached alkyl groups are methyl or ethyl, significant regeneration of the enzyme by hydrolytic cleavage requires several hours; with isopropyl groups, as in the example given, virtually no hydrolysis occurs and the return of AChE activity is dependent upon synthesis of new enzymes, a process requiring days to months.

From the foregoing account it is apparent that the terms "reversible" and "irreversible" as applied to the carbamoyl ester and organophosphorous anti-ChE agents respectively, reflect only quantitatively differences and that both classes of drugs react with the enzyme in essentially the same manner as does ACh.

THE CHEMISTRY AND STRUCTURE-ACTIVITY RELATIONSHIP OF SOME REVERSIBLE CARBAMATE INHIBITORS.

Drugs of this class that are of therapeutic interest are shown in Table 1-1. After the structure of physostigmine was established, Stedman (1929a, 1929b) undertook a systematic investigation of a number of related synthetic compounds. They concluded that the essential moiety of the physostigmine molecule was the methyl carbamate of a basically substituted simple phenol (Table 1-1). The quaternary ammonium derivative, neostigmine, is a compound of greater stability and equal or greater potency. The simple analogues of neostigmine that lack the carbamoyl group, such as edrophonium, are less potent and much shorter acting anti-ChE agents, since they produce a truly reversible inhibition as described above.

Retention of the dimethylcarbamate side chain in the meta position of phenol with the incorporation of the quaternay N atom within the ring to form a pyridinium nucleus results in compounds with anti-ChE and pharmalogical properties similar to those of neostigmine. Pyridostigmine, a drug of this class, is used in the treatment of myasthenia gravis.

A marked increase in anti-ChE potency and duration of action can result from linking of two quaternary ammonium nuclei by a chain of appropriate structure and length. One such example is the miotic agent demecarium, which consists of two neostigmine molecules connected at their carbamate nitrogen atoms by a series of ten methylene groups. It is likely that this compound combines with the anionic and esteratic sites of two adjacent AChE units within the same tetramer just as the bis-quaternary

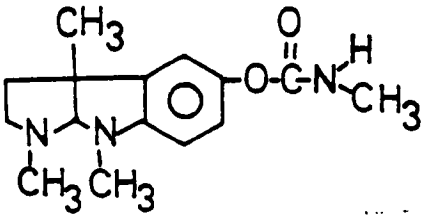
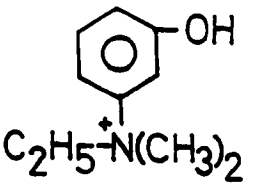
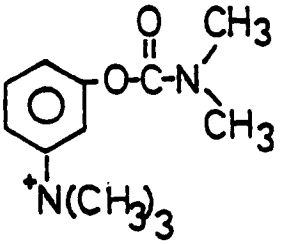
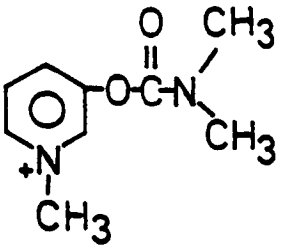
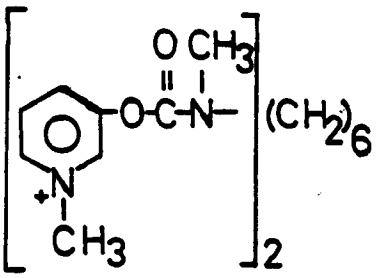
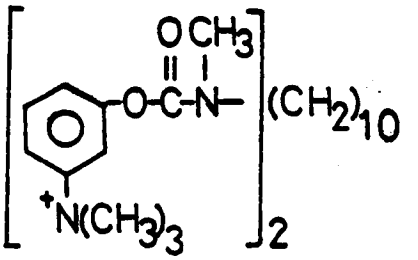
COMPOUND	STRUCTURAL FORMULA
(a) TERTIARY AMINE	
Physostigmine (eserine)	
(b) MONOQUATERNARY AMINE	
Edrophonium 'Tensilon'	
Neostigmine 'Prostigmin'	
Pyridostigmine 'Mestinon'	
(c) BISQUATERNARY AMINE	
Distigmin 'Ubretid'	
Demecarium 'Rosmilen'	

TABLE 1.1

Representative "reversible" anti-ChE agents employed clinically.

neuromuscular blocking agent, decamethonium, is presumed to act at two adjacent cholinoreceptors. Another class of bis-quaternary compounds is represented by the dioxamide, ambenenium, used in the treatment of myasthenia gravis. In addition to its potent anti-ChE activity, this drug has a variety of actions at both the prejunctional and postjunctional membranes of the skeletal muscle motor end-plate (Karczmar 1967). The other bis-quaternary amine, distigmine (Ubretid), is a long acting reversible anti-ChE agent with a much longer duration of action than neostigmine and pyridostigmine. It is 2.7times more powerful in the invivo inhibition of non-specific ChE in the whole blood of the rat (Chan 1980). Clinically, it is used in the prevention and management of post-operative urinary retention, intestinal atony and paralytic ileus in surgical and gynaecological patients. Occasionally it is also used as an adjuvant in the treatment of myasthenia gravis (Chan 1980).

PHARMACOLOGICAL AND TOXIC PROPERTIES OF ANTI-ChE AGENTS

The pharmacologic effects of anti-ChE agents are due primarily to the fact that the acetylcholine (ACh) released in the course of normal physiological activity is not destroyed. The released ACh, therefore, accumulates in the body where it exerts both muscarinic and nicotinic actions. The effects of individual anti-ChE agents may be modified by their other properties; some anti-ChE agents have an ACh like action in their own right, while others may cause neuromuscular blockade. The effects of some compounds are complicated by the production in vivo of other toxic substances. Lipid soluble substances, such as organophosphorous derivatives, penetrate easily into the

central nervous system and their central effects are, therefore, more pronounced than those produced by anti-ChE agents of lower lipid solubility. Finally, some of the anti-ChE agents have additional actions which arise, neither from their relationship to ACh or ChE, nor to their metabolic transformation into toxic substances. While these possible sources of variation in pharmacological activity should be borne in mind, the major actions of all the anti-ChE can nevertheless be summarized by Table 1-2. All muscarinic effects of anti-ChE can be attenuated or abolished by atropine. The central stimulation effects are antagonized by atropine although, not as completely as the muscarinic effects at autonomic effector sites.

TABLE 1-2

PHARMACOLOGIC AND TOXIC EFFECTS OF ANTI-ChE AGENTS

SITES OF ACTION	EFFECTS
MUSCARINIC EFFECTS	
Smooth muscles	Increased peristalsis, miosis, relaxed sphincters, brocho-constriction, vasodilation and cycloplegia.
Cardiac muscles	Bradycardia and vasodilatation, hypotension.
Exocrine glands	Lacrimation, salivation and sweating.
NICOTINIC EFFECTS	
Skeletal muscles	Generalised muscle twitching followed by muscle weakness, muscle cramps.
Ganglia	Stimulation and modification of muscarinic effects.
CENTRAL EFFECTS *	
General	Restlessness, dizziness, tremor, insomnia, aphasia, disorientation, hallucination.
Poisoning	Coma, convulsions and death.

* Not all AChE agents penetrate into the central nervous system.

THE EFFECTS OF THE QUATERNARYANTI-ChE AGENTS ON NEUROMUSCULAR TRANSMISSION

The quaternary ammonium anti-ChE drugs such as neostigmine, pyridostigmine and edrophonium are known to have a facilitatory action on neuromuscular transmission (Randall and Jampolski 1953). The increased tension developed in the muscle due to the anti-ChE agents is thought to result from the production of a series of repeated discharges in the muscle fibres in response to a single supramaximal nerve stimulation (Wescoe and Riker 1951). The precise mode of action of these drugs has not yet been fully established, but several mechanisms have been suggested. It was originally proposed that their facilitatory action was due to the inhibition of AChE at the end-plate resulting in the persistance of transmitter at the junctional region. Many authors still adhere to this original supposition (Hobbiger 1952; Smith, Cohen, Pelikan and Unna, 1954; Katz and Thesleff 1957; Ferry and Marshal 1971; Whittaker 1975). However, in recent years it has been questioned whether these anti-ChE drugs produce facilitation of neuromuscular transmission only by the inhibition of AChE at the motor end-plate. The possibility that the facilitatory action of these drugs may be attributed to the depolarizing action of the quaternary ammonium group has been proposed on the basis of a direct stimulating action on mammalian skeletal muscle. For example, the inter-arterial injection of neostigmine into chronically denervated muscle, or into normally innervated muscle in which essentially all the AChE has been inactivated by a prior dose of DFP (Di-isopropylphosphorofluoridate), evokes an

immediate contraction, whereas physostigmine does not (Riker and Wescoe 1946).

It has also been suggested that the anti-ChE compounds induce antidromic discharges in the pre-synaptic nerve terminals which are then disseminated by local axon reflexes causing the additional release of transmitter (Riker, Roberts, Standert and Fujimort 1957; Werner and Kuperman 1963; Blaber and Christ 1967). Thus, the mode of action of these drugs may be due to one or a combination of any of these suggested mechanisms.

THE USE OF ANTI-ChE DRUGS IN MYASTHENIA GRAVIS

The major therapeutic uses of the quaternary ammonium anti-ChE drugs are in the stimulation of the smooth muscles of the intestinal tract and the urinary bladder, the reduction of high intra-ocular pressure in glaucoma, the reversal of drug induced neuromuscular blockade after surgical anaesthesia and the diagnosis and treatment of myasthenia gravis. Myasthenia gravis is characterised by weakness and rapid fatiguability of skeletal muscle. The close resemblance of myasthenia gravis to curare poisoning reported by Jolly in 1895, and the success with treatment of myasthenia gravis in the 1930's (Walker 1934, 1935), have long indicated the involvement of a defect in neuromuscular transmission. The difficulty has been the exact location of this defect. There are three major hypotheses concerning the pathogenesis of this disease: Firstly, a presynaptic defect in the synthesis or release of ACh, resulting in the failure of an appropriate amount of ACh to reach the receptor sites of the sub-neural apparatus following rapid repetitive nerve stimulation. Secondly, a circulating competitive blocking

agent interfering with the normal interaction between ACh and the post-synaptic receptor. Finally, a deficiency in post-synaptic receptors or a generalised decrease in the sensitivity of the receptors to ACh in consequence of an auto-immune reaction towards the nicotinic receptors at the motor end-plate.

The sugestion that myasthenia gravis may be due to impaired release of ACh from motor nerve endings (Desmedt 1959), resulted from the resemblance between the post-tetanic exhaustion of myasthenic muscle and of normal muscle treated with Hemicholinium which inhibits the synthesis of ACh. Elmquist, Hoffman, Kugelberg and Quastel (1964) found that miniature end-plate potentials in myasthenic patients were normal in frequency, but decreased in amplitude. They tentatively interpreted this observation as being due to a decrease in the amount of ACh released from the nerve terminal. However, this decreased amplitude might equally be due to a decreased number or the decreased sensitivity of a normal number of post-synaptic receptors.

Reports have been made of the demonstration in the blood of myasthenic patients of a substance capable of producing neuromuscular block in experimental animals (Wilson and Stoner 1944). It has been shown that thymopoetin which is the thymic hormone, released and responsible for inducing the differentiation of T-cells, in thymic diseases, causes a neostigmine responsive neuromuscular block in mice which resembles that of myasthenia gravis. Such evidence would lend support to the empirically proven treatment in a high proportion of patients

with myasthenia gravis by early total thymectomy (Keynes 1954). The poor results of thymectomy in some cases have been attributed to residual or ectopic thymictissue (Harvey 1948), or to irreversible damage caused by long standing disease (Wolf 1966).

Myasthenia gravis may also be due to an auto-immune response in which an antibody to the end-plate receptor protein is produced by the reticulo-endothelial system (Simpson 1960). This suggestion was supported by the finding of antibodies to the skeletal muscle which also react with the myo-epithelial cells of the thymus in the serum of 50% of patients with myasthenia gravis (Downes, Greenwood and Wray 1966). However, in patients with observable thymoma the incidence was almost 100% (Oosterhuis, Van Der Geld and Feltkamp 1967). In addition, studies utilizing labelled α-bungarotoxin, a snake venom that irreversibly binds to ACh receptors, has shown that the number of receptors at the motor end-plate is considerably reduced in myasthenia gravis (Farnborough, Drachman and Satymurti 1973).

In myasthenia gravis there is, therefore, evidence of immunological reactions to the ACh receptor protein which could reduce the number and possibly the sensitivity of the ACh receptors at the motor end-plate and thereby produce the pathological and pharmacological symptoms associated with this disease.

Noestigmine, pyridostigmine and ambenonium are the standard anti-ChE drugs used in the symptomatic treatment of myasthenia gravis. All can increase the response of myasthenic muscle to repetitive nerve impulses, probably primarily by the preservation of endogenous ACh and secondarily by their direct

cholinomimetic action. When the diagnosis of myasthenia gravis has been established, treatment can be instituted with any of these drugs, then change if optimal improvement in strength is not achieved initially or at any time subsequently.

In the majority of patients, especially in those with predominantly bulbar involvement, best results can be obtained with pyridostigmine, which is available only in certain countries, as time-span tablets containing a total of 180mg of which 60mg is released immediately and 120mg over several hours. This preparation is of particular value in maintaining patients overnight.

When the muscles of the extremities are most affected, ambenonium or neostigmine may be the drug of choice. Not infrequently better results can be obtained by alternating equivalent doses (See Table 1-3) of different anti-ChE or by combining fractional doses of two agents.

TABLE 1-3

APPROXIMATE DOSES OF COMMONLY USED ANTI-ChE

DRUGS	ORAL DOSE	INTRAVENOUS DOSE	INTRAMUSCULAR DOSE
Neostigmine "Prostigmine"	15mg	0.5mg	1.0 - 1.5mg
Pyridostigmine "Mestinon"	60mg	2.0mg	2.0mg
Pyridostigmine "Time-span"	180mg	-	-
Ambenonium "Mytelase"	5 - 7.5mg	none available	none available

Patients with generalised myasthenia gravis maintained on ambenonium often need supplementation with pyridostigmine 45 - 60minutes before meals to facilitate mastication and deglutition. Parenteral administration of the standard anti-ChE agents is sometimes requested in desparately ill myasthenic patients who do not respond adequately to oral medication, the subcutaneous, intramuscular or intravenous route may be used. Atropine sulphate 0.4 - 0.6mg or more, intravenously should be given immediately if a severe reaction (cholinergic crisis) ensues.

CHAPTER II

PHARMACOKINETICS

CHAPTER TWO

PHARMACOKINETICS

INTRODUCTION

The word pharmacokinetics was introduced by Dost in 1953 to mean the application of kinetic studies to drugs and poisons (Greek Pharmakon). The term incorporates the kinetic aspects of in vivo absorption, distribution, metabolism and excretion of drugs, poisons and some endogenous substances. Pharmacokinetic studies use data obtained from concentration curves of drugs or their metabolites in biological fluids; this data is processed by means of mathematical equations. The relationship between pharmacological response and concentration of drugs or their metabolites in body fluids is also included within the scope of pharmacokinetics.

The study of pharmacokinetics was originated over 40 years ago. Widmark (1922) dealt exhaustively with the pharmacokinetics of ethyl alcohol and his mathematical treatment is still used. Equations describing first-order processes for absorption and elimination were derived by Gehlen (1933) and Beccari (1938). Tarsten Teorell (1937 a,b) first developed the theory of compartments and this work laid the foundation for much of modern pharmacokinetics. Teorell assumed that the drug and/or its metabolites were equitably dispersed in one of several tissues of the body and that the drug in these tissues acted as kinetically homogenous. Such a tissue, which acts on isotropicfluid, is termed a compartment. Compartments are separated by barriers that inhibit free diffusion from one compartment to another. The barriers are kinetically definable

in that the rate of transport of drug (or metabolites) across the membrane between the compartments is a function of the amounts of drugs in these compartments.

Progress in pharmacokinetics was initially slow, due to widespread criticism of the compartment theory. However, the ideas of Teorell were reborn in the work of Druckery and Kupfmuller (1949) and Dost (1953); both groups of workers utilized data obtained from blood. The use of data obtained from urine for pharmacokinetic analysis was extended by Wiegand and Taylor (1960). The compartment theory is now generally accepted and coupled with the development of more precise means of measuring the required experimental data pharmacokinetics has made dramatic advances in the last decade.

CONCEPTS OF PHARMACOKINETIC MODELLING

Wagner (1968) described reasons and advantages of pharmacokinetic modelling to interpret quantitatively the fate of a drug in the body. These are; to summarise observed data in terms of an equation or equations which may help to describe better the fate of a drug than a plasma concentration-time profile of the drug; to increase understanding of the disposition process (absorption, distribution and elimination) involved, to be able to predict dosage regime for better quality of therapy, to compare several drugs with similar pharmacologic action for screening of new drugs, and to quantitatively relate biological activity with pharmacokinetic data for better control of drug action.

The advance of analytical techniques for the determination of drugs in biological fluids has allowed more accurate and precise analysis to describe the fate of drugs in the body. The term 'compartment' should not be restricted to the well-known body fluid compartments or organs; a pharmacokinetic compartment is referred to as a hypothetical region in the body where the drug concentration is everywhere the same, and is not related to any particular anatomical compartment. But it is helpful to conceptualize the central pharmacokinetic compartment as consisting of such physiological units as the extracellular space and well-perfused organs (e.g., kidney and liver), and the peripheral compartment as consisting of poorly perfused organs and tissues (e.g., muscle or fat). Drug elimination (via renal, biliary and other routes of excretion and metabolism) is usually assumed to occur from the central compartments.

It is assumed that the processes of absorption, distribution and elimination associated with each model can be described by first-order rate kinetics, where the rate of transfer of a given drug is proportional to the amount or concentration of the drug. Kinetic linearity was defined by Kruger-Thiemer (1968) as direct proportionality of transfer rates or concentration of differences of drugs. Many authors have derived equations to interpret data for linear pharmacokinetics (Benet and Turi 1977; Benet 1979; Thron 1974).

A modern view of pharmacokinetics was emphasised by Wagner (1973) to include both linear and nonlinear systems. He reviewed over 160 examples of nonlinearities in pharmacokinetics. The cause of nonlinear pharmacokinetics is

due to a deviation from first-order rate kinetics. Nonlinearities can result from incomplete or abnormal drug absorption, in binding reactions to limited numbers of binding sites on plasma proteins or in tissues, in active transport processes with limited available carrier, in active tubular secretion and reabsorption in the kidney, in biliary excretion of drugs and their metabolites, and in enzymatic transformations obeying Michaelis-Menten kinetics which is dose dependent kinetics. Enzyme stimulation and inhibition may also cause nonlinearities. It is important to recognize nonlinearity deriving a pharmacokinetic model. Sometimes this deviation becomes less obvious if data are obtained from carefully designed experiments. Beckett, Salmon and Mitchard (1969), by maintaining an acidic urinary PH, demonstrated that the urinary excretion rate of amphetamine follows first-order kinetics, whilst formerly it was not possible to investigate the pharmacokinetics of amphetamine in man (Beckett and Rowland 1964). Although Wagner (1973) emphasised that most drugs show nonlinear pharmacokinetics charactierstics, the concept of linear pharmacokinetic theory can be based just as easily on the simple assumption of linearity, with no assumptions regarding pharmacokinetic details. A theory resting on this foundation is much less vulnerable than one based on a possible erroneous specific pharmacokinetic model.

PHARMACOKINETIC MODELS.

After a drug reaches the general circulation, it can undergo a variety of processes, diffusion and binding in extravascular fluids and tissues, metabolism, nonenzymatic

chemical reaction and excretion. Excretion may take place in several pathways, the most common one is through the kidneys and bile, other routes include the skin, lungs and gastrointestinal tract. An enterohepatic cycle may develop where biliary excretion and intestinal absorption continue until most of the drug is eliminated through other pathways.

ONE COMPARTMENT MODEL

Often the rate of concentration changes for a drug in blood, plasma, or serum can be described as a single first-order process after an initial rapid distributive phase (Fig 2.1). That is, after a rapid intravenous injection the logarithm of the plasma drug concentration decreases linearly as a function of time as shown in Figure 2.2.

The Figure 2.1 illustrates this one compartment rate constant for elimination K of the drug quantity in the body X, is equal to the sum of the apparent rate constant for metabolism, K_m and excretion K_e:-

$$K = K_m + K_e \qquad \text{Equation (1)}$$

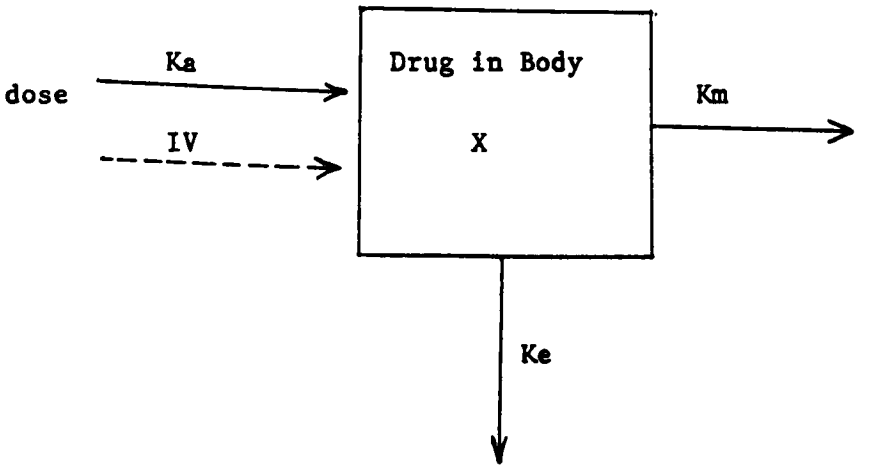
For most drugs the rate of elimination is proportional to the amount of drug in the body (X) at any time t:

$$\frac{dX}{dt} = -KX \qquad \text{Equation (2)}$$

The negative sign indicates that drug is being lost from the body. Integration of equation (2) produces:

FIG 2.1

A typical one compartment model of a drug, where Ka, Ke, Km are absorbtion, excretion and metabolism rate constants respectively X is amount of drug in the body.



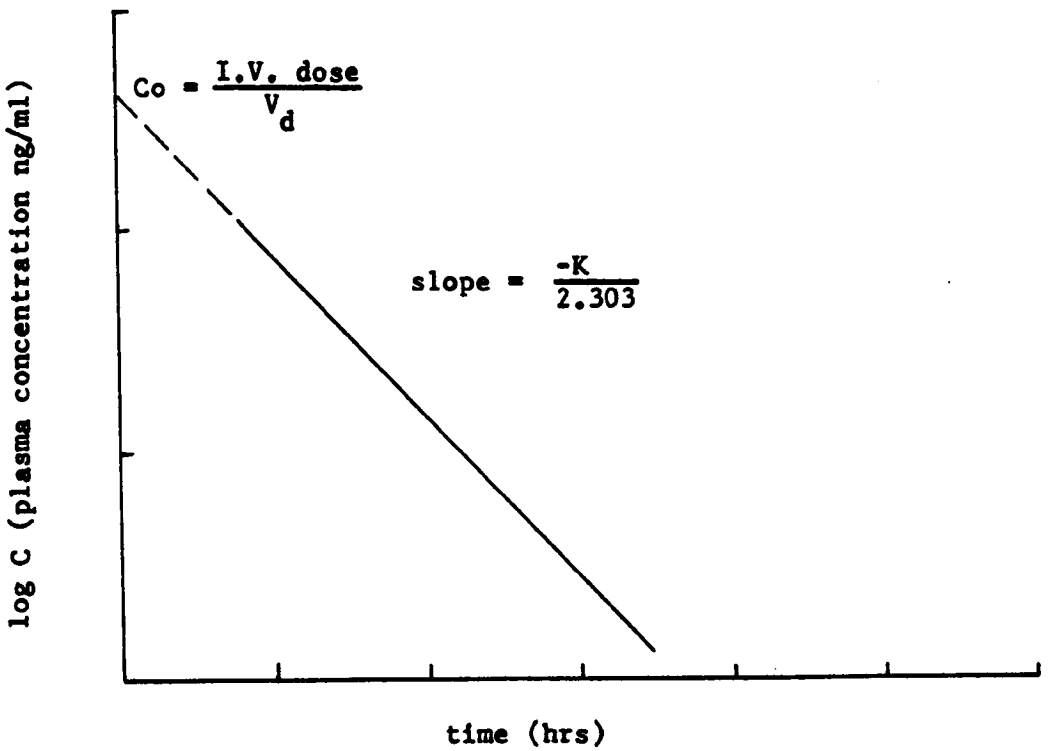


FIG 2.2

Log C versus time, drug plasma level elimination according to Model 1.

$$X = X_o e^{-Kt} \qquad \text{Equation (3)}$$

Where X_o is the amount injected (i.e., dose) and e represents the base of the natural logarithm. Taking the natural logarithm of both sides of equation (3) and then converting to common logarithms (base 10, log), gives:

$$\log X = \log X_o - \frac{Kt}{2.303} \qquad \text{Equation (4)}$$

The body is obviously not homogenous even if plasma concentration and urinary excretion data can be described by representing the body as a one-compartment model. Drug concentration in the liver, kidneys, heart, muscle, fat and other tissues will usually differ from one another as well as from the concentration in the plasma. If the relative binding of a drug to compartments of these tissues and fluids is essentially independent of a drug concentration, then the ratio of drug concentration in the various tissues and fluids is constant. Consequently, there will exist a constant relationship between drug concentration in the plasma, C, and the amount of drug in the body:

$$X = V_d C \qquad \text{Equation (5)}$$

The proportionality constant V_d in this equation happens to have the units of volume and is known as the apparent volume of distribution. Despite its name, this constant usually has no direct physiologic meaning and does not refer a real volume.

The relationship between plasma concentration and the amount of drug in the body, as expressed by equation (5), enables the conversion of equation (4) from an amount-time to concentration-time relationship which can be expressed as

$$\log C = \log C_o - \frac{Kt}{2.303} \qquad \text{Equation (6)}$$

Where C_o is the plasma drug concentration immediately after injection (t = o). Equation (6) shows that a plot of log C versus t will be linear under the stated conditions (Fig 2.2). C_o may be obtained by extrapolation of the log C versus t plot to time zero. This intercept, C_o, may be used in the calculation of the apparent volume of distribution. Since X_o equals the amount of drug injected intravenously i.e., the intravenous (I.V.) dose, V_d may be estimated from the relationship:

$$V_d = \frac{\text{I.V. dose}}{C_o} \qquad \text{Equation (7)}$$

It follows from equation (6), that if plasma concentrations are plotted on a logarithimic scale against time, a straight line should result. The slope of such a plot is -K/2.303 and related to the biological half-life ($t_{\frac{1}{2}}$) of drug through equation (8)

$$K = \frac{0.693}{t_{\frac{1}{2}}} \qquad \text{Equation (8)}$$

Nelson and O'Reilly (1960) derived an equation which describes the amount of unchanged drug excreted at any time during the post-absorptive phase after distribution equilibrium has been established, equation (9)

$$U = U^{\infty} \ (1 - e^{-Kt}) = \frac{KeXo}{K} (1 - e^{-Kt}) \qquad \text{Equation (9)}$$

The terms U and U^{∞} represent the amount of unchanged drug excreted up to time t and infinite time, respectively. Differentiating equation (9) and taking logarithms yield equation (10)

$$\log \frac{dU}{dt} = \log KU^{\infty} - \frac{Kt}{2.303} \qquad \text{Equation (10)}$$

$$= \log KeX_o - \frac{Kt}{2.303}$$

It follows from equation (10), that if the logarithm of the rate of excretion is plotted against time, a straight line should result. The slope of this line will also be -K/2.303. Equation (8) can be used for the determination of the biological half-life of the drug.

Data obtained from urine has been processed in the above manner in order to obtain K and the $t_{\frac{1}{2}}$ for drugs such as sulphonamides (Swintosky 1957; Nelson and Schaldemes 1959; Nelson and O'Reilly 1960); digitoxin (Swintosky 1957); amphetamines (Chapman et al., 1959; Beckett and Rowland 1965b) paracetamol (Cummings et al., 1967) and chloropropamide (Taylor 1972).

Urine data can also be employed to estimate the individual rate constants which are included in the term Kel. The excretion rate constant (K_e) can be calculated from equation (11):

$$\frac{K_e}{K} = \frac{U^{\infty}}{X_o} \qquad \text{Equation (11)}$$

Where X_o is the amount of drug absorbed. If the drug is completely absorbed X_o will be equivalent to the dose of drug administered. Substituting the calculated value of K_e in equation (1), K_m may be determined. The later value may be a hybrid rate constant, since it can combine the rate constants of further biotransformations, as indicated in equation (12).

$$K_m = (K_{m1} + K_{m2} + K_{m3} \ldots\ K_{mn}) \qquad \text{Equation (12)}$$

Plots of log rate of excretion or plasma level against time do not always yield a straight line. The influence of slow distribution of the drug throughout the body on this relationship has been discussed by Nelson (1964), who also observed that if the results of several individuals are averaged a deviation from the straight line relationship occurs. Plasma protein binding of drugs also has an influence on this curve (Kruger-Thimer 1966c; Kruger-Thimer et al., 1969; Diller 1964 and Martin 1965a,b;and Wagner and Northam 1967) have discussed the introduction of a second body compartment to explain these deviations.

In this simplest form of the one compartment model, after a rapid intravenous (I.V.) injection, the drug is placed into the single compartment and consequently there will be no absorption phenomena (see Fig 1.1). Alternatively, an I.V. infusion can be employed and the pharmacokinetic parameters can be assessed from post-infusion blood curves.

TWO-COMPARTMENT MODEL

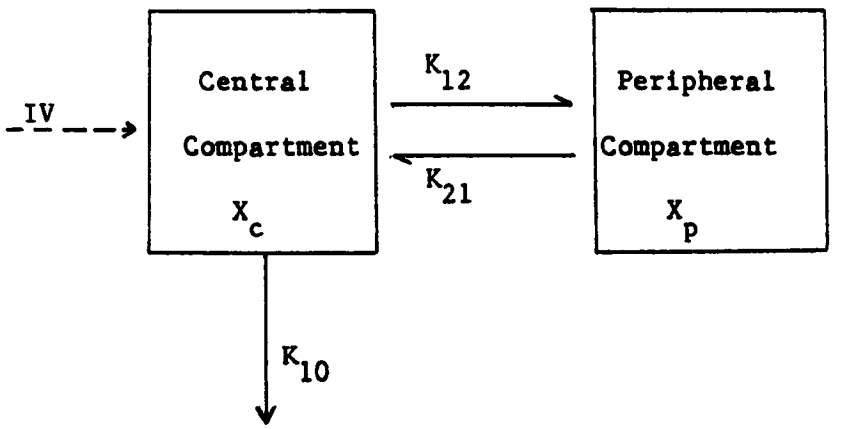
There is often an initial rapid fall of the drug concentration in the blood (and urine) when the drug is administered intravenously (I.V.), and then its first-order rate of elimination is established (Fig 2.4). Garrett (1964) and Garrett and Alway (1963) have shown that this type of data is best analysed by a two-compartment open model. This model was first related to drug kinetics by Teorell (1937a,b). It presumes that a drug administered I.V. rapidly equilibrates between two-compartments, a central compartment and a peripheral compartment (Fig 2.3).

Usually elimination (metabolism and excretion) is considered to occur at a rate proportional to the drug concentration in the plasma. However, metabolism could occur in the tissues and this possibility was investigated for acetylsalycylic acid by Rowland et al., (1970).

If the model shown in Fig 2.3 is assumed to be correct, then there is a rapid initial drop in blood concentration of drug (Fig 2.4), until a pseudo-steady state is established between the drug in the central (or blood) compartment. Changes in the concentration in the blood can then be related to changes

FIG 2.3

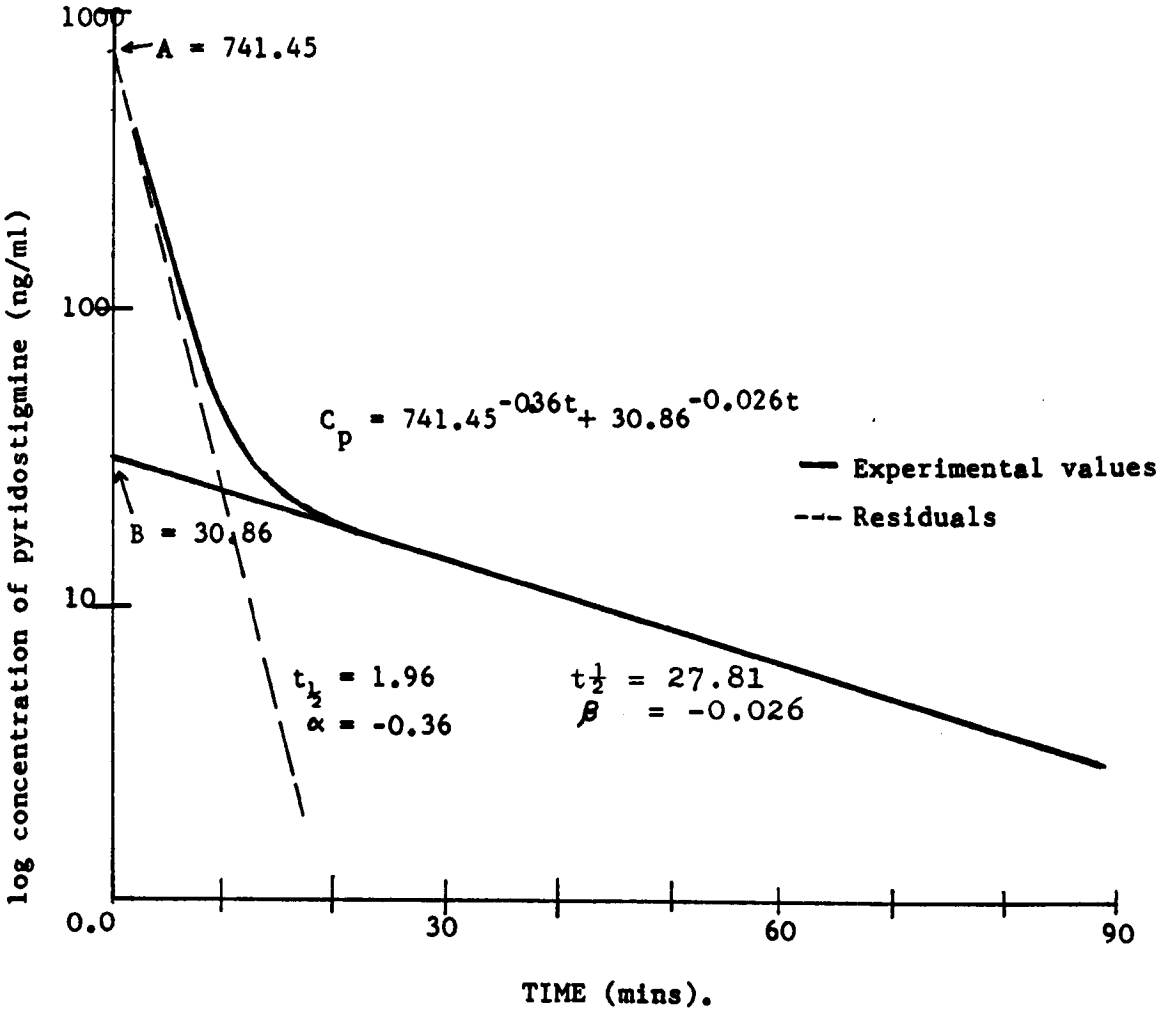
Schematic representation of a two-compartment system consisting of a central compartment and a peripheral (tissue) compartment.



where X_c and X_p are the amount of drug in the central and peripheral compartments respectively, K_{21} and K_{12} are the apparent first-order intercompartmental rate constants and K_{10} is the apparent first-order elimination rate constant from the central compartment.

FIG 2.4

A semilog plot of the plasma level of a drug which obeys the kinetics of a two-compartment model of I.V. administration.



Experimental values and calculated curve in man after I.V. administration of 72.4mg/kg of pyridostigmine bromide. The experimental values are the mean of six observations (see Chapter IV).

in the concentration of the tissue compartment through the equilibrium or partition constant (K_{21}/K_{12}).

DRUG LEVEL IN THE CENTRAL COMPARTMENT

Following rapid I.V. injection of a drug that distributes in the body according to a two-compartment model with elimination occuring from the central compartment, the rate of change in the amount of drug in the central compartment Xc can be described by the following differential equation:

$$\frac{dXc}{dt} = K_{21} X_p - K_{12} X_c - K_{10} X_c \qquad \text{Equation (13)}$$

Where X_p is the amount of drug in the peripheral compartment, K_{21} and K_{12} are the apparent first-order intercompartmental distribution rate constants, and K_{10} is the apparent first-order elimination rate constant from the central compartment (see Fig 2.3). Taking the Laplace transform of equation (13) yields equation (14) and after partial fraction analysis of equations (14) and (15), the amount of drug in the central compartment Xc is given by equation (16):

$$\alpha+\beta = K_{12} + K_{21} + K_{10} \qquad \text{Equation (14)}$$

$$\alpha \cdot \beta = K_{21}K_{12} \qquad \text{Equation (15)}$$

$$Xc = \frac{Xo\ (\alpha - K_{21})}{\alpha - \beta} e^{-\alpha t} + \frac{Xo\ (K_{21} - \beta)}{\alpha - \beta} e^{-\beta t} \qquad \text{Equation (16)}$$

Where α and β are complex constants which simply serve to replace other constants as in equations (14) and (15); X_o is the intravenous dose.

Although the central compartment is obviously not homogeneous by assuming that the ratio of drug concentrations in the various tissues and fluids of the central compartment is constant (i.e., there is a very rapid distribution between the plasma and fluids and tissues of the central compartment), a linear relationship exists between the drug concentration in plasma C and the amount of drug in central compartment, that is:

$$X_c = V_c \cdot C \qquad \text{Equation (17)}$$

Where V_c is the apparent volume of central compartment. This relationship enables the conversion of equation (16) from an amount-time to a concentration-time equation, which can be expressed as:

$$C = \frac{X_o(\alpha - K_{21})}{V_c(\alpha - \beta)} e^{-\alpha t} + \frac{X_o(K_{21} - \beta)}{V_c(\alpha - \beta)} e^{-\beta t} \qquad \text{Equation (18)}$$

Or

$$C = Ae^{-\alpha t} + Be^{-\beta t} \qquad \text{Equation (19)}$$

where

$$A = \frac{X_o(\alpha - K_{21})}{V_c(\alpha - \beta)} \qquad \text{Equation (20)}$$

and

$$B = \frac{X_o (K_{21} - \beta)}{V_c (\alpha - \beta)} \qquad \text{Equation (21)}$$

A plot of the logarithm of drug plasma concentration versus time according to equation (19) will yield a bioexponential curve (Fig 2.4). The constant α is by definition larger than β, and therefore at some time the term $Ae^{-\alpha t}$ will approach zero while $Be^{-\beta t}$ will still have a finite value. Equation (19) will then reduce to:

$$C = Be^{-\beta t} \qquad \text{Equation (22)}$$

which in common logarithms is:

$$\log C = \log B - \frac{\beta t}{2.303} \qquad \text{Equation (23)}$$

Hence, an estimate of β can be obtained from the slope - $\beta/2.303$ of the terminal exponential phase, and biological $t_{\frac{1}{2}}$ can be determined employing the following relationship:

$$t_{\frac{1}{2}} = \frac{0.693}{\beta} \qquad \text{Equation (24)}$$

The zero-time intercept obtained by extrapolation of the terminal linear phase to t = o is B. Application of 'the method of residuals' yields a second linear segment with a slope equal to - $\alpha/2.303$ and a zero-time intercept A.

The constants A,B, α and β may be obtained graphically as shown in Fig 2.4, or with the aid of a digital computer.

The first approach is to fit the entire plasma level-time curve by means of a digital computer program which provides a nonlinear regression analysis of the curve. Once these experimental constants are obtained, the pharmacokinetic parameters V_c, K_{12}, K_{21} and K_{10} can be generated by considering the following relationship.

At time t = o, equation (19) becomes

$$C_o = A + B \qquad \text{Equation (25)}$$

Where C_o is the zero-time plasma concentration, substituting the values of A and B from equation (20) and equation (21), respectively into equation (25), and then simplify it yields:

$$C_o = \frac{Xo}{Vc} \qquad \text{Equation (26)}$$

According to equation (25), yields:

$$Vc = \frac{Xo}{A+B} \qquad \text{Equation (27)}$$

Where X_o is intravenous dose.

Rearrangement of equation (27) and substitution A+B in equation (21) yields:

$$B = \frac{(A+B)(K_{21} - \beta)}{\alpha - \beta} \qquad \text{Equation (28)}$$

$$K_{21} = \frac{A\beta + B\alpha}{A+B}$$ Equation (29)

$$K_{10} = \frac{\alpha\beta}{K_{21}} = \frac{\alpha\beta(A+B)}{A\beta + B\alpha}$$ Equation (30)

Determination of these rate constants permits an assessment of the relative contribution of distribution and elimination processes to the drug concentration versus time profile of a drug. It may also aid in elucidating the mechanism of drug interactions and of effects of disease, age, genetic influence, etc., on drug disposition.

One useful additional equation is the equation for the total volume of distribution of drug in the body:

$$V_d = \frac{V_c K_{10}}{\beta}$$ Equation (31)

Where V_d can be defined as the apparent volume of distribution of drug in the body (Gibaldi 1975). The parameter V_d will relate the amount of drug in the body to the plasma concentration at any time during the postdistributive phase, that is

$$V_d = \frac{X_o}{\beta \int_o^\infty C dt}$$ Equation (32)

which is a useful relationship for estimating V_d.

DRUG LEVELS IN THE TISSUE (PERIPHERAL COMPARTMENT)

The differential equation describing the rate of change in the amount of drug in the peripheral compartment is:

$$\frac{dXp}{dt} = K_{12}X_c - K_{21}X_p \qquad \text{Equation (33)}$$

Where X_c and X_p are the amount of drug in the central and peripheral compartment respectively. After the Laplace transformation and partial fraction analysis of equation (33) yield equation (34)

$$X_p = \frac{K_{12}X_o}{\beta-\alpha} e^{-\alpha t} + \frac{K_{12}X_o}{\alpha-\beta} e^{-\beta t} \qquad \text{Equation (34)}$$

or

$$X_p = \frac{K_{12}X_o}{\alpha-\beta} (e^{-\beta t} - e^{-\alpha t}) \qquad \text{Equation (35)}$$

Equation 35 describes the time-course of the amount of drug in the peripheral compartment following intravenous administration. It is obvious from equation (35) that after a sufficiently long period of time the term $e^{-\alpha t}$ approaches zero and equation (35) reduces to

$$X_p = \frac{K_{12}X_o}{\alpha-\beta} e^{-\beta t} \qquad \text{Equation (36)}$$

Hence, the slope of the terminal exponential phase equals to $-\beta/2.303$. Therefore, in the postdistributive phase plasma and peripheral compartment levels decline in parallel.

Equation (36) may be useful in determining the relationship between pharmacologic effect and tissue levels of a drug. It must be kept in mind, however, that such relationships are only approximations. The hypothetical tissue levels may not accurately reflect the concentration of drug at the site of action, even though the site of action may be a part of the peripheral compartment (Gibaldi 1975).

SOME APPLICATION OF PHARMACOKINETIC PARAMETERS.

(a) PLASMA OR BLOOD LEVELS OF DRUG AND PLASMA $t_{\frac{1}{2}}$

Measuring plasma or blood concentrations of drugs and deducing the plasma half lives, $t_{\frac{1}{2}}$, from such data has often been used to monitor drug therapy. Plasma level determination is unnecessary if the patient responds satisfactorily to a carefully chosen dosage schedule, but can clarify the situation when the desired therapeutic effect is not achieved or when toxic effects are suspected or when the patient has not taken the drug. However the interpretation of drug plasma or blood levels becomes difficult when there is an intersubject variation in the plasma or blood profile of the drug. Factors which are responsible for their inter-individual variations have been discussed by Vesell (1973). There are genetic variations and variation in the absorption, distribution and elimination of the drug among individuals which may be due to environment or disease states. When all these factors are operating in severely ill patients, drug plasma or blood are difficult to interpret. Therefore, utilisation of plasma or blood concentration of drug or its plasma $t_{\frac{1}{2}}$ as a therapeutic guide alone may be limited (Perrier and Gibaldi, 1974).

(b) THE APPLICATION OF AREA UNDER PLASMA OR BLOOD CONCENTRATION TIME CURVE (AUC) OF DRUGS

Frequently, the technique of area analyais has been applied to assess the absorption and bioavailability of a drug. This is because the area under a blood level curve is proportional to the drug available from a dose (Wagner and Nelson, 1963; 1964; Loo and Riegelman, 1968). However, it

is observed that there is a reduction in the AUC following oral drug administration as compared with that obtained after intravenous drug administration. This observation led to the assumption that orally administered drugs are either incompletely absorbed or metabolised in the gastro-intestinal tract. Subsequently, it is demonstrated that there is also a comparable reduction in the AUC after drug infusion via the portal vein.

The total AUC of a drug can be easily calculated using Equation (37), which results from integration of equation (19):

$$AUC = \frac{A}{\alpha} + \frac{B}{\beta} \qquad \text{Equation (37)}$$

From equations (27), (30), and (37) Equation (38) can be obtained, which shows that AUC is directly related to the dose X_o and inversely to the elimination constant K_{10} and the volume of central compartment V_c.

$$AUC = \frac{X_o}{V_c K_{10}} \qquad \text{Equation (38)}$$

The AUC of a drug can also be determined according to equation (39):

$$AUC = \int_0^\infty X_c dt = F\left(\frac{X_o}{VK}\right) \qquad \text{Equation (39)}$$

Hence, AUC is dependent upon the elimination rate constant, K, the volume of distribution V and the total quantity of available drug FX_o. For a drug where the total orally administered dose

is absorbed, F = 1, in a one compartment model. Similarly, the AUC is identical to that for the two compartment system except for the different definitions of distributive volume ($K = \beta$ and $V = V_B$). Wagner (1967a) applied equation (40) to twelve separate studies with tetracycline and novobiocin

$$\frac{F}{0.693V} = \frac{AUC}{X_o t_{\frac{1}{2}}} \qquad \text{Equation (40)}$$

combinations from 167 sets of serum tetracycline curves. Both children and adults were used in the studies. Similar relative tetracycline availability were evident from the average area/dose times half-life ratios, which ranged from 1.09 to 1.23. Absolute availabilities can easily be assessed by a comparison of areas with the use of equation (41), where the AUC per unit dose by any route of administration is expressed as a percent of the intravenous AUC per unit dose. The total quantities of intact drug excreted after any administrative route can also be compared to the intravenous data.

$$\text{Percent of absolute availability} = \frac{(AUC)_{oral} \times Dose_{I.V.} \times 100}{(AUC)_{I.V.} \times Dose_{oral}} \qquad \text{Equation (41)}$$

Obviously, the percentage of absorption can only be obtained with equation (41) if no significant metabolism occurs before the drug reaches the general circulation. This was the case with the compounds studied by Dost (1963) and Gladtke (1964, 1968). However, with oral administration, if the drug is

metabolized prior to, or during absorption, or during the 'first pass' through the portal system, then the oral AUC will be less than that obtained after intravenous administration, even if absorption is completed. This was discussed by Harris and Riegelman (1969) with reference to a rapidly metabolized drug, aspirin, using data obtained in dog experiments after oral dosage, vena – cava and hepatic portal vein infusion. Gibaldi et al. (1971) described such observation as the 'first-pass' effect, which indicates that drugs are exposed to metabolism in the liver before reaching the bloodstream after oral administration.

THE AIMS OF THE THESIS

Clinical laboratories are in need of a reliable procedure to analyze quaternary ammonium drugs because this class of compound includes the neurotransmitter acetylcholine as well as the anticholinesterase, neostigmine and pyridostigmine, which are important in the diagnosis and management of myasthenia gravis. Individual differences in the dosage requirement of the drugs are commonly attributed to varying severity in the course of disease. The role of inter-individual differences in drug metabolism and excretion is a matter of conjecture since analytical methods that can be applied to the routine measurement of pyridostigmine and neostigmine concentrations in man have not been developed.

The aims of the experimental work are to develop some sensitive and selective analytical methods to measure the concentration of the drug and their metabolites and also to investigate the pharmacokinetics of pyridostigmine and neostigmine after IV and IM and oral administration. It is hoped that the clinical findings will help to elucidate further the mechanisms of action of these drugs, permit a more systematic and controlled investigation of a similar nature and ultimately, help to identify the contributing factors in the difficult management of patients under going anticholinesterase therapy.

CHAPTER III

PART ONE

A REVIEW ON ANALYTICAL TECHNIQUES AVAILABLE FOR STUDIES OF ANTI-CHE DRUGS.

PART TWO

DEVELOPMENT OF METHODOLOGY FOR THE DETERMINATION OF NEOSTIGMINE, PYRIDOSTIGMINE AND THEIR METABOLITES IN HUMAN PLASMA AND URINE .

CHAPTER THREE

A REVIEW ON ANALYTICAL TECHNIQUES AVAILABLE FOR DISPOSITION STUDIES OF ANTI-ChE DRUGS

Early techniques for the determination of quaternary Anti-ChE drugs and their metabolites in body fluids were based on spectrophotometry. After ion-pair extraction of the compounds into organic solvents, the ion-pair is subsequently dissociated with dilute hydrochloric acid and the quaternary amines can then be assayed by standard spectrophotometric techniques. These methods have been applied to the measurement of pyridostigmine in plasma (Coper, et al., 1974). The quaternary amine was extracted as an iodide complex into dichloromethane and its absorption was measured at various wave lengths. The limit of sensitivity of this assay procedure is 0.1 - 0.2 μg/ml, and interference by other drugs and endogeneous substance may occur. Kornfeld, et al. (1970) and Somani et al. (1972) have had little success with this procedure which does not appear to separate pyridostigmine from its main metabolites; apparently, the major metabolite, 3-hydroxy-N-methylpyridinium, gives maxima at 250, 288 and 320nm (Somani et al., 1972). A method for the estimation of neostigmine and pyridostigmine in urine was described using ion exchange treatment and colorimetric estimation of the blue complex produced when either of the drugs was complexed with bromophenol blue (Nowell et al., 1962). However, the procedure suffers from lack of sensitivity (lowest limit for detection is 2 to 3 μg/ml) and selectivity as the assay could not be used to distinguish between neostigmine and pyridostigmine.

Radioisotopic techniques have been used to study the disposition of edrophonium in the rat using (^{14}C)-edrophonium (Back and Calvey 1974). The disposition and metabolism of neostigmine in the hen and rat were studied using ^{14}C-labelled neostigmine (Roberts et al., 1965). Similarly, metabolism studies of pyridostigmine in rat (Birtley et al., 1966) and in man (Kornfeld et al., 1970) were carried out using ^{14}C-labelled pyridostigmine. These investigations are expensive and involve the administration of large doses of radioactivity and are not suitable for human studies. Radioisotopic techniques are often non-specific and cannot distinguish the parent drug from its metabolites. Electrophoretic and chromatographic techniques are often involved in the isolation and separation of parent drugs and metabolites when radioisotopes of the parent drugs are used.

Enzymatic procedures have been used to assay reversible anti-ChE agents, such as edrophonium, in plasma (Barber et al, 1976). These methods have been used to study the correlation between the plasma concentration of the drug and pharmacological effects (Calvey et al., 1976). Although these assay procedures are sensitive, they are time-consuming and exacting and can only be used for truly reversible anti-ChE agents.

Analyses based on gas-liquid chromatographic (GLC) techniques have been used for the quantification of some quaternary amines. For instance, acetylcholine was demethylated into dimethyl-aminoacetate by N-demethylation using benzenethiolate in butanone solution (Jenden et al., 1968); the

subsequent dequaternized dimethylaminoacetate was assayed by a GLC procedure. We have had limited success with this procedure for measuring neostigmine and pyridostigmine. Apparently the methylation of the quaternary nitrogen by benzenthiolate would also cause the hydrolysis of the carbonate ester and we did not obtain reproducible demethylation (Chan 1980).

Methods based on thermal dequaternization have been reported for a number of quaternary compounds; including acetylcholine, acetyl-β-methyl- choline, butyrylcholine and choline (Szilagyi, 1968 and Schmidt 1975), thiazinolnium (Jonkman et al., 1975), neostigmine and pyridostigmine (Vidie, 1972; Chohan 1976). Vicie(1972) and Chohan (1976) in their methods used the methyliodide (part of the dequaternarnized products, see Chan 1980) as a measure of the quaternary amines in the plasma. However, this procedure suffers from lack of selectivity as other quaternary compounds may also be ion-pair extracted and similarly assayed.

Recently, Chan et al. (1976) developed a sensitive and selective procedure based on GLC analysis, for the determination of nostigmine and pyridostigmine in human plasma. The procedure involved preliminary ion-pair extraction of the drugs using potassium iodine-glycine buffer, into dichloro-methane, followed by concentration and analysis of the ion-pair complex using GLC system fitted with a nitrogen-sensitive detector. Using peak area ratio techniques, pyridostigmine bromide was used as the internal marker for the determination

of neostigmine in plasma; neostigmine bromide was used as the internal standard for the quantitation of pyridostigmine. The method depends on the dequaternization of the quaternary nitrogen to form the tertiary analogue which is resolved by the GLC system. Chan et al. (1976) reported that gas chromatography-mass spectrometry (GC-MS) of a plasma extract containing neostigmine and pyridostigmine as iodide complex gave a one compound whose mass spectrum showed a molecular ion at m/e 166, and second compound with a molecular ion at m/e 208, corresponding to pyridostigmine and neostigmine bromide or the appropriate tertiary analogues of these drugs. Apparently, both Anti-ChE drugs, either as the bromide salt or as the iodide complex, were thermally demethylated to their corresponding tertiary analogues which were then resolved by the GLC system. Other authors have used similar methods to measure quaternary amines in plasma. The plasma and urine level of thiazinamium methylsulphate, an anticholinergic agent for treatment of bronchial asthma,was determined by a similar ion pair extraction and GLC procedure (Jonkman et al., 1975). Acetylcholine and its related analogues have been measured by an assay based on pyrolysis gas chromatography and pyrolytic products of these compounds are the corresponding 2-dimethyl-amine esters and methyl halides (Szilagyi,1968; Schmidt,1975). Aquilonius et al., (1979) reported an identical extraction procedure using deuterated d_6- neostigmine as the internal standard and mass spectrometer as the detector for the determination of neostigmine in plasma. The limit of quantitation of this method was about 1ng/ml, but the use of

GC-MS system is not always available in every laboratory due to the high cost of such a system.

Electron capture gas chromatography method for determination of quaternary ammonium compounds has been reported by Pohlman and Cohan (1977). The principle of this analytical method is to isolate the quaternary ammonium compound as its iodide from biological fluids. The iodide extract is then thermally degraded to release methyl iodide upon its injection onto the chromatographic column and measured by the electron capture detector. This method has a few potential problems, including choline which is found in serum may also be demethylated at the temperature employed (Pohlman and Cohan 1977). Although these assay procedures are sensitive, they are time-consuming (over 48 hours) and non-specific.

High performance liquid chromatography technique has been reported for the quantitation of pyridostigmine in plasma by Yakatan and Tien (1979). Perchlorate was used as the counter ion in the extraction of pyridostigmine and measured at a wave length of 269nm using a UV detector. The limit of sensitivity of this assay procedure is 20ng/ml by utilizing 1 ml plasma. Mean recovery from plasma was 63.9% (Yakatan and Tien 1979).

RATIONAL FOR MONITORING PLASMA LEVEL OF ANTI-ChE DRUGS

The influence of plasma concentrations of a drug by pharmacokinetic processes such as disposition and elimination, is often considered to be responsible to a greater extent for the variation in individual response to the drug than are the

differences in target organ sensitivity among individuals (Smith et al., 1973). Thus, the monitoring of plasma levels of drugs in pharmacotherapy may be of adverse side effects or reactions due to the drug or combination of drugs (Mucklow 1973) and provide more efficient pharmacotherapy.

At present, the regulation of the oral anti-ChE drugs for patients with myasthenia gravis is empirical, depending on the patients' subjective responses and on patients' co-operation when testing motor strength. Edrophonium has been used as an adjunct in regulation drug dosage (Osserman et al., 1966), but results are difficult to interpret and may not be helpful to the physician in deciding whether medication is necessary when the patient complains of motor weakness, dyspnea or diplopia. However, if a relationship between the plasma level of these anti-ChE drugs and clinical responses of the myasthenic patient could be demonstrated, it would be possible to regulate the dosage regimen by measurement of the plasma concentration of the drug to determine if the patient with an unsatisfactory clinical response to the anti-ChE drug treatment requires more medication or is manifesting symptoms of cholinergic crisis, due to drug toxicity from over medication.

Recently, Chan and Calvey (1977) demonstrated that the plasma concentration of pyridostigmine was invariably positively correlated with the improvement in neuromuscular transmission in patients with typical electromyographic decrement in the adductor pollices. The plasma concentration to normal varied over a 5-fold range (27.8 - 125.7 ng/ml), reflecting the variable

severity of the disease. In a patient with purely ocular symptoms, there was a significant correlation between the plasma concentration of the drug and diameter of palpebral fissure. A separate study on the plasma level of pyridostigmine in myasthenic patients has shown considerable intersubject variation in the bio-availability of the quaternary amine (Calvey and Chan 1977); the six patients on an oral pyridostigmine bromide were stablized on widely different doses of the drug (60 - 660mg/day). Therefore, the role of individual differences in drug disposition may no longer be a matter of conjecture and the monitoring of plasma levels of anti-ChE drugs should be of particular value in the clinical assessment of patients with myasthenia gravis. A method to calculate the optimal daily doses of pyridostigmine in individual myasthenic patients is described by Chan and Calvey (1977). For instance, the optimum plasma level of the drug for each myasthenic patient can be defined by relating the plasma concentration of pyridostigmine to determine the volume of distribution in the affected muscle groups. Subsequently, a test dose of oral and intravenous pyridostigmine could be used to determine the volume of distribution, the elimination half-life and the fraction of the dose of the drug required to maintain the steady-state plasma level can be calculated for each myasthenic patient, according to the following equation:-

$$\text{Dose mg/Kg/24hr} = \frac{\text{required steady-state plasma concentration } \mu g/ml \times \text{volume of distribution (ml/Kg)}}{\text{fraction of dose absorbed} \times \text{half-life (min)}}$$

The pharmacokinetics of neostigmine after intravenous administration to surgical patients during the reversal of neuromuscular block have recently been investigated (Aquilonius 1979; Smith 1973). We have done similar IV studies for pyridostigmine (see chapter 4). It is possible to utilize these pharmacokinetic parameters for the design of a dosage regimen as outlined in the above equation (Chan and Calvey 1977). Such parameters may also help to select which one of the two anti-ChE drugs is better for treatment. Baker et al., (1978) have also studied the plasma clearance of neostigmine and pyridostigmine in conscious dogs by the use of a cross-over design. Results suggest that the longer duration of action of pyridostigmine is related to the differential clearance of the two quaternary amines from plasma, as the slow disposition half-life of pyridostigmine is approximately three times longer than that of neostigmine.

CONCLUSION

It is evident that previous methods for the study of the metabolism and disposition of anti-ChE drugs are not selective and sensitive. Over the last twenty years the most important progress observed in the research of fundamental biology has been in the technical means (Dreux 1979), such as electronic microscopy, gas-liquid chromatography, high performance liquid chromography, gas chromatography - mass spectroscopy, methods of structural determination for proteins and the use of immunological methods (enzymatic-multiple immunological

techniques and radio-immunoassay). With these techniques it is possible to measure minute quantities of drugs up to the molecular level 10^{-12}g. The determination of anti-ChE drugs and their metabolites offers better assessment of anti-ChE therapy in myasthenia gravis and in the reversal of neuromuscular blockade after surgical operation.

PART TWO

Development of methodology for the determination of neostigmine , pyridostigmine & their metabolites in human plasma & urine.

INTRODUCTION

Sensitive and specific methods for the analysis of drugs in biological fluids are essential for pharmacokinetic studies, particularly when plasma concentrations of drugs which have a low post-absorption concentration or rapid elimination are studied after administration of small doses. Assay methods reported for the determination of anti-ChE drugs; as mentioned earlier (Part One page 53) are difficult to use, or suffer from low sensitivity or lead to interference by other compounds.

In this part, the latest development of the analytical methods and their application to the determination of anti-ChE drugs in human plasma and urine is described. These developed methods are based on the preliminary ion-pair extraction of neostigmine, or pyridostigmine and their major metabolites as well as appropriate internal standards into dichloromethane or dichloromethane:acetone(90:10), followed by concentration and analysis of the ion-pair complex, using a GLC system.

These developed procedures will be described in terms of the following section.

Method 1 was used for the isolation and determination of neostigmine or pyridostigmine and their metabolites. To measure unchanged drugs, pyridostigmine bromide was used as an internal marker to measure neostigmine; and neostigmine bromide was the internal marker for the quantititation of pyridostigmine. To measure the metabolites of pyridostigmine and neostigmine in urine, one metabolite was used as the internal standard for the other and vice versa.

Method 2, developed for use in the simultaneous determination of the unchanged drugs using a common internal marker.

METHOD 1.

APPARATUS AND CONDITIONING

A Perkin-Elmer model F17 gas chromatography all-glass system, fitted with a phosphorus-nitrogen detector and linked to a Perkin-Elmer chart paper Model 56 (Perkin-Elmer, Beaconsfield, U.K.) and to an autolab minigrator (Spectra Physics) was used.

For the determination of parent drugs, this instrument was operated with coiled glass columns (2m x 6.5mm OD) silanized with Hexamethyldisilanize(HMDS) and packed with 10% OV-17 or 3% OV-17 on chromosorb W-AW (100-120mesh); for the determination of metabolites, it was operated with 10% OV-17 on chromosorb W-AW (100-120 mesh). Gas flow-rates were: nitrogen (carrier), 30ml/min; hydrogen, 2ml/min; and air, 100ml/min. The temperatures were: 300^{0}C for the injector and the detector, and column temperature at 200 - 220^{o}C for parent drugs; and 190-200^{o}C for metabolites. All columns were conditioned at 20^{o}C below the maximum recommended temperature for the relevant stationary phases for 24 hours. Each column was then silanized twice in situ with samples of HMDS (Phase Separation LTD, Great Britain) before use.

Other apparatus used were: 10ml capacity centrifuge tubes with well-fitting screw caps (Sovirel, Levallois-Perret, France); 15 ml stoppered evaporating tubes with finely tapered bases (Beckett, 1966); 10µl-S.G.E. microsyringe (Chromatography Services,

Wirral, Great Britain); Pipetman microsyringes (Anachem, Bedford U.K.); thin layer chromatography (TLC), TLC plates covered with polyamide; 0.15mm thick (Schleicher and Schull, W. Germany); Automatic shaker (Hecto, Denmark).

MATERIALS

The following materials were used.

Acetone, dichloromethane, diethylether, ethanol and methanol, all of analar grade and freshly redistilled; Analar anhydrous sodium sulphate; ether-washed sodium hydroxide solution (5N and 0.1N); 0.1M glycine buffer which is made up of the following:-

Analar glycine	7.505g
Analar sodium chloride	5.185g
Distilled water to	1000ml

Potassiumiodide-glycine buffer which is made up of:-

Analar potassium iodide	12.8g
0.1N Sodium hydroxide solution	4ml
0.1M glycine buffer	6ml

Potassium triiodide solution which consists of:-

Iodine	1g
Potassium Iodide	2g
Distilled water	20ml

Neostigmine bromide (Koch-Light, Colnbrook, U.K.); pyridostigmine bromide, U.S.P; 3-Hydroxy-pyridine and 3-dimethylaminophenol (Aldrich Chemical Company Limited, Gillingham, Dorset, U.K); 3-hydroxy-N-methylpyridiniumbromide,3-OHNMP (Roche,Welwyn Garden, U.K); 3-hydroxyphenyltri-methylammonium bromide,

3-OHPTMA (Koch-Light, Colnbrook, U.K.); ^{14}C-3-OHNMP and ^{3}H-3-OHNMP (The Radiochemical Centre, Amersham, U.K).

TREATMENT OF GLASSWARES

It was found that all glassware should be cleaned and silanized before use. Accordingly the following procedure, in order to increase the sensitivity of measurement of the drugs, was used. A considerable quantity of the drug might be lost due to adsorption onto the walls of glassware. Evaporating tubes and screw capped centrifuge tubes were cleaned by soaking in a 2.5% solution of RBS (Chemical concentrates -RBS - Limited, London) in water overnight, then rinsed with methanol (Commercial Grade) and hot tap water, followed by distilled water. The tubes were then dried at 105°C. After cooling at room temperature, all the tubes were silanized by rinsing with a 3% solution of HMDS in chloroform. They were then dried at 250°C overnight. Silanization of tubes should be repeated, when necessary, at monthly intervals.

DETERMINATION OF NEOSTIGMINE IN PLASMA

Blood samples were obtained by venous puncture and collected in heparinized polythene tubes. After centrifugation (5000g for 10mins) the plasma samples (1.0 - 3.0ml) in a 10-ml glass centrifuge tube was made alkaline (pH = 10-12) with 20µl 5N sodium hydroxide solution and the marker solution (60µl; equivalent to 50ng base of pyridostigmine bromide in methanol) was added. The alkaline solution was extracted with freshly redistilled diethyl ether (2 x 6ml) by shaking vigorously by hand for 2min. then centrifuged

at 3000g for 5min. to break the emulsion and the ethereal extracts were discarded. The remaining traces of ether were removed by purging nitrogen gas over the aqueous phase. Potassium iodide-glycine buffer (1ml) was added to the ether-washed plasma and the resultant iodide-glycine drug complexes were extracted into freshly redistilled dichloromethane (2 x 6ml) using an automatic shaker.

After centrifugation, the plasma layer was discarded and the bulked dichloromethane extracts were dried by shaking with a quantity of anhydrous sodium sulphate (Ca. 2g). The water-free extract was transferred carefully into an evaporating tube and evaporated to dryness by a gentle stream of nitrogen gas. Redistilled methanol (20µl) was added, using a Pipetman microsyringe, to redissolvethe dried extract by vortexing (5secs.). A sample (2-5µl) of the methanolic solution was injected onto the gas chromatographic column with a 10µl S.G.E. syringe. The concentration of neostigmine present in a sample was determined from the ratio of the integrated peak area for neostigmine to that for the internal marker. A calibration graph was prepared by adding known amounts of neostigmine bromide to 3ml of drug-free plasma, covering the concentration range equivalent to 5.0 to 100ng/ml neostigmine base (Fig 3.1).

DETERMINATION OF PYRIDOSTIGMINE IN HUMAN PLASMA

A procedure similar to the one described above was used for the determination of pyridostigmine in human plasma using neostigmine bromide as the internal marker. A calibration graph was prepared,by adding known amounts of pyridostigmine bromide to

3ml of drug-free plasma, covering the concentration range equivalent to 5.0 to 100ng/ml pyridostigmine base.

DETERMINATION OF ANTI-ChE AGENTS AND THEIR METABOLITES IN HUMAN URINE

Neostigmine and its major metabolite 3-hydroxyphenyltrimethyl-ammonium bromide (3-OHPTMA) as well as pyridostigmine and its major metabolite 3-hydroxy-N-methyl-pyridinium (3-OHNMP) could be separated by a TLC system consisting of a glass TLC plate (polyamide, 0.15mm thick) & a solvent system of methanol, strong ammonia solution & butan-2-one in the ratio of 15:20:60. Visualization of the TLC spots was carried out using Dragendorff reagent (Clarke 1969) and under UV light (Chan and Black unpublished observation 1977). This system was used to identify the parent drugs and metabolites extracted from the urine samples using the procedure described in the scheme (Fig 3.1).
Subsequently it was found that the TLC procedure was not sensitive for the quantification of the drugs. A GLC system was then developed using a 2m glass column packed with 10% OV-17 on chromasorb W-AW(100-120 mesh).

Extracts from step 2 of the scheme (Fig 3.1) were analysed for the parent drug and the corresponding internal standard and extracts from step 3 of the scheme were analysed for the metabolites using GLC system (10% OV-17 on chromosorb W-AW 100-120mesh). To measure the metabolites, 3-OHPTMA (a major neostigmine metabolite)was used as the internal standard for the quantification of 3-OHNMP(a major pyridostigmine metabolite) and 3-OHNMP was used as the internal standard for the determination of 3-OHPTMA.

RESULTS

1. ION-PAIR EXTRACTION OF PARENT DRUGS AND METABOLITES

It was demonstrated that the mean recoveries of neostigmine and pyridostigmine from plasma were 85% and 89% respectively, using potassium iodide-glycine buffer at pH10 (Chan et al., 1976). In the present method the recoveries were improved to 95% and 94% (at 60ng per ml level) respectively for neostigmine and pyridostigmine when all relevant glassware was silanized in the present modified procedure. This procedure invariably increased the sensitivity of detection at a low concentration level.

Fig 3.2 shows that the ion-pair extraction of the neostigmine metabolite (3-OHPTMA) and the pyridostigmine metabolite (3-OHNMP) from the urine sample spiked with known concentrations of the parent drugs (neostigmine and pyridostigmine) and their corresponding metabolites was proved to be specific. There were no TLC spots corresponding to the metabolites from concentrate of the Step 2 (Fig 3.1) extraction in the general scheme nor were there TLC spots corresponding to the parent drugs extracted by Step 3 in the general scheme (Fig 3.1).

The mean recoveries of 3-OHPTMA and 3-OHNMP, using potassium tri-iodide as counterions at pH4 were 80% at 1.6nmol level using a previous procedure. Similar value of recoveries were obtained when radioactive samples were used (Wareing and Calvey, personal communication).

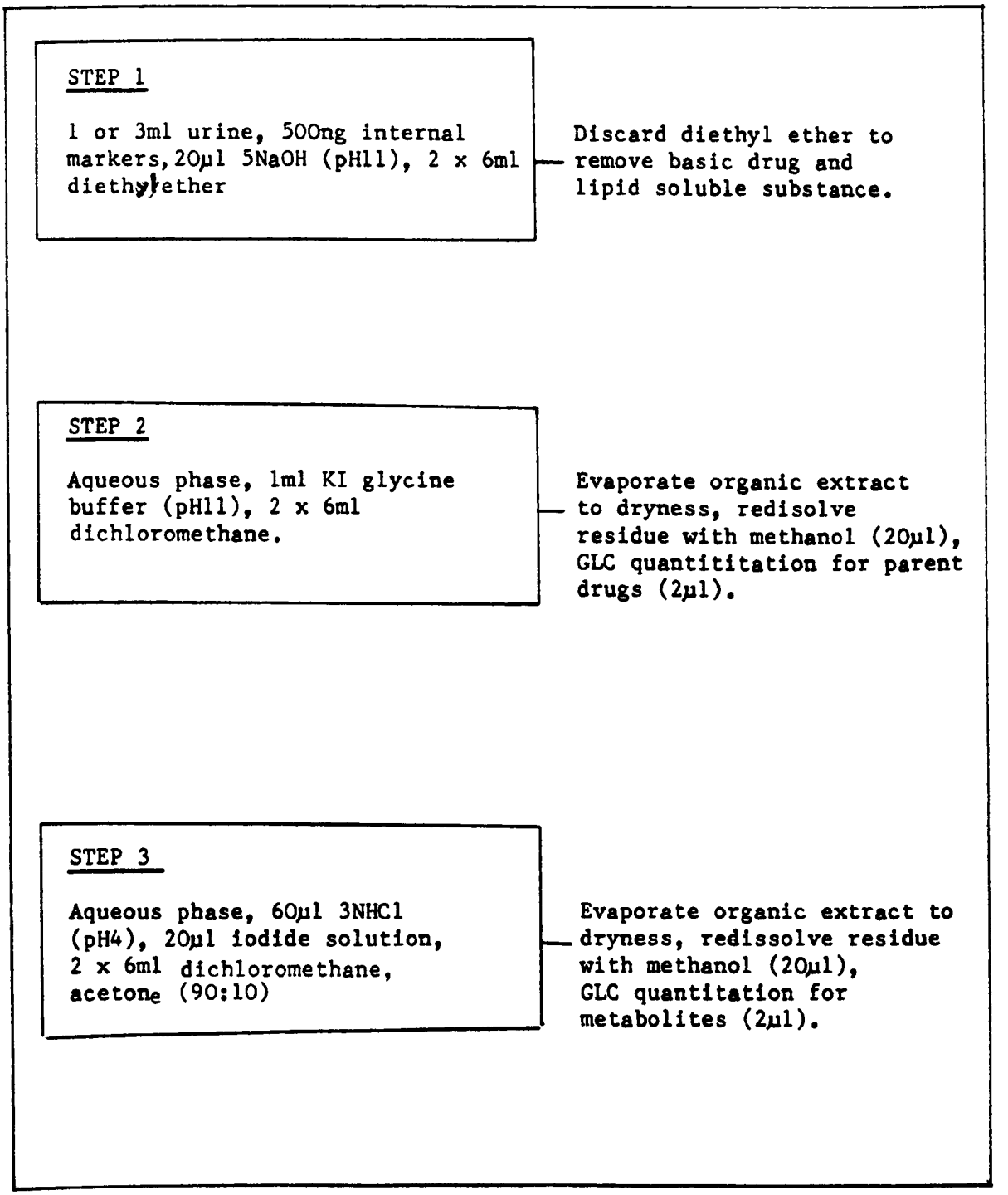


FIG 3.1

SCHEME FOR THE DETERMINATION OF NEOSTIGMINE, PYRIDOSTIGMINE AND THEIR METABOLITES IN URINE.

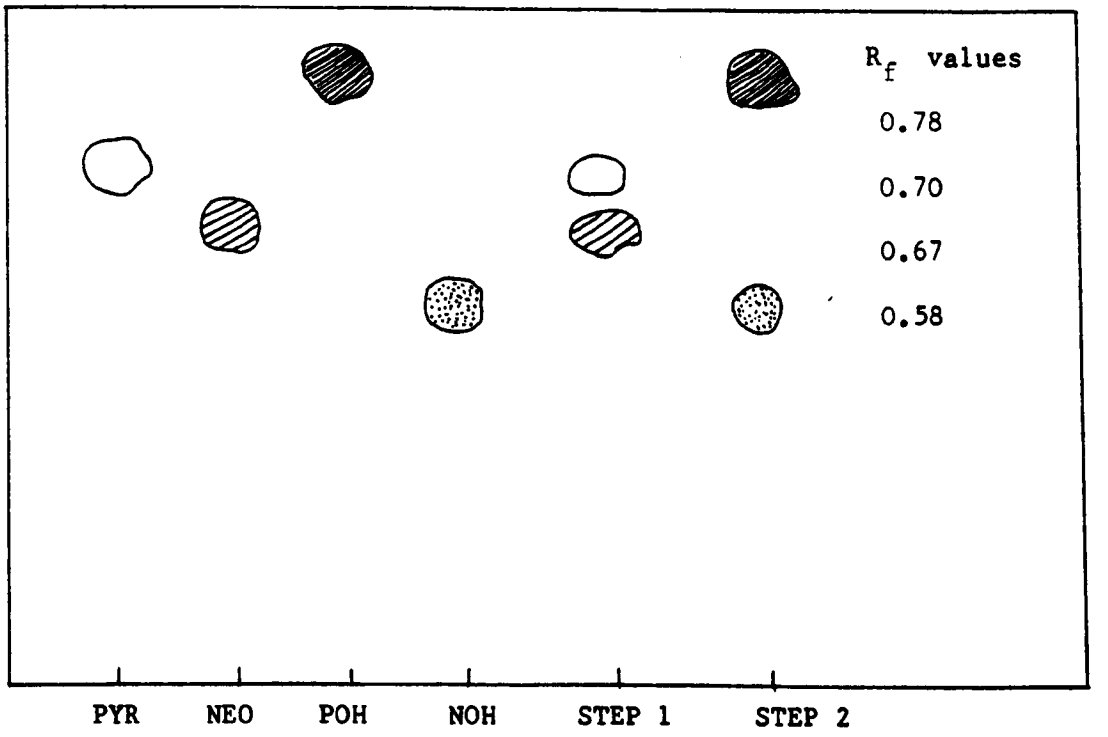


FIG 3.2

TLC of ion-pair extracts of pyridostigmine (PYR), neostigmine (NEO), 3-OHNMP(POH) and 3-OHPTMA (NOH) in urine containing spiked concentration of 5µg each. TLC plates: glassplate (polyamide, 0.15mm thick), solvent system: strong ammonia solution: butan-2-one: methanol (20:60:15). Developing spray: Dragendorff reagent, Temperature: 20 $\pm$ 2°C.

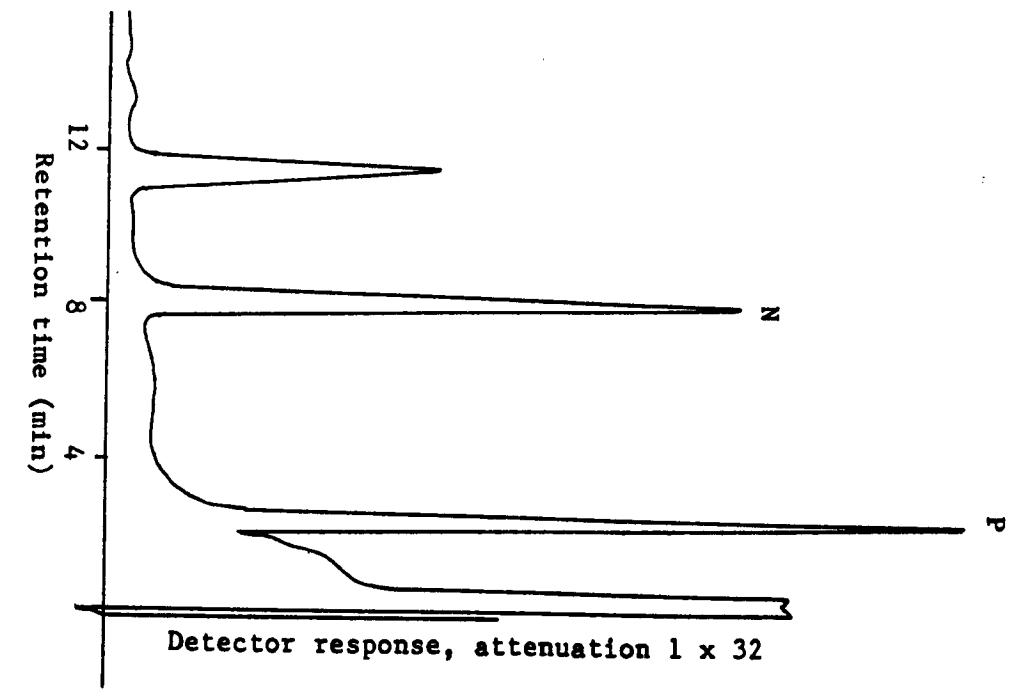


FIG 3.3 Chromatogram of plasma extracts containing 5 ng/ml of pyridostigmine(P) and neostigmine (N), using 10% OV-17 on chromosorb W.

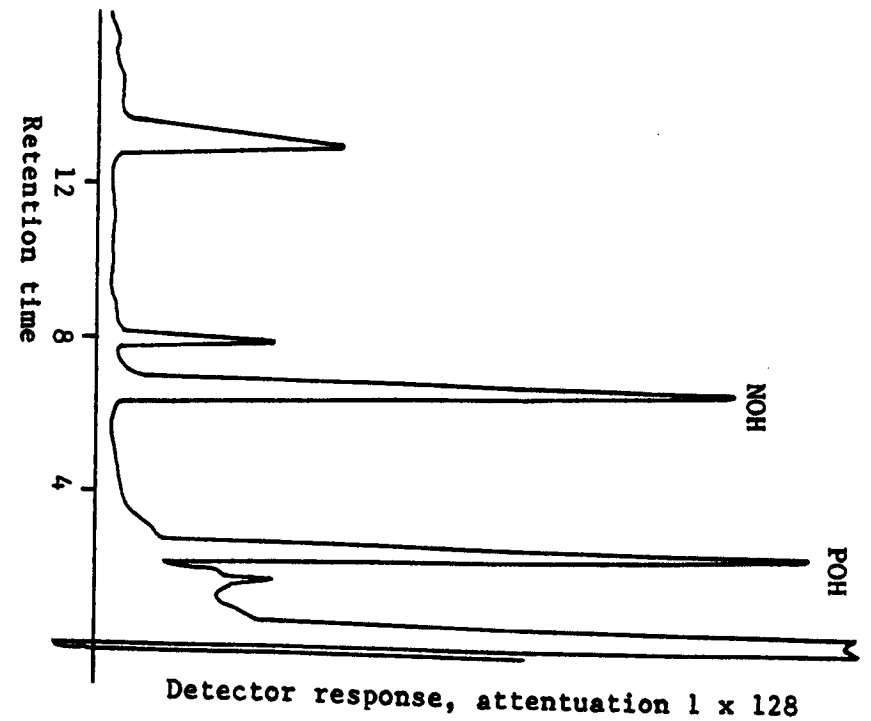


FIG 3.4 Gas chromatogram of urine extracts containing 50 ng/ml of 3-OHNMP (POH) and 3-OHPTMA (NOH) using 10% OV17 on chromosorb W.

TABLE 3.1

PERFORMANCE OF GLC SYSTEMS (10% OV-17 ON CHROMOSORB W-AW, 100 - 120 MESH)[a]

COMPOUND IN METHANOL	RETENTION TIME (MIN)	SYMETRY FACTOR[b] (0.95 - 1.05)	RESOLUTION BETWEEN MARKER[b] (>1.0)
3-OHPTMA bromide	6.3	1.0	3.5
3-OHPTMA-iodide complex			
TLC spot corresponding to 3-OHPTMA			
3-dimethylamino phenol			
3-OHNMP bromide	2.2	0.9	-
3-OHNMP-iodide complex			
TLC spot corresponding to 3-OHNMP			
3-hydroxypyridine			

(a) Temperature: injection, 300°C; oven, 200°C; detector, 300°C. Gas flow rates: nitrogen (carrier), 30ml/min; hydrogen 2ml/min ; air 100ml/min.

(b) British Pharmacopeia (1968) specification for GLc analysis.

2. GLC ANALYAIS OF PARENT DRUGS AND METABOLITES

The respective retention times, symmetry factors and resolution of the parent drugs (neostigmine and pyridostigmine) were described by Chan et al. (1976). Fig 3.3 shows the chromatogram of neostigmine and pyridostigmine at 5ng/ml level.

Table 3.1 and Fig 3.4 summarize the performance of GLC system for the analysis of the metabolites (3-OHPTMA and 3-OHNMP). The retention times of these compounds indicated that 3-OHPTMA and 3-OHNMP (either as the bromide salts, or as the extract from TLC spots, or as the iodide ion complexes) were thermally decomposed at the injection part to their corresponding tertiary analogues, 3-dimethylaminophenol and 3-hydroxypyridine respectively, which were resolved by the GLC system. The mechanism of thermal dequaternization of the parent quaternary amines has been discussed in detail by Chan et al., (1976) and Chan (1980).

The reproducibility of dequaternization at the injection port, where a glass liner was inserted, indicated that the iodide complexes of neostigmine and pyridostigmine yielded tertiary amines which gave linear peak area ratios with their corresponding internal markers over the range of 5 - 100ng/ml (Fig 3.5). Similarly, results were obtained for the analysis of the metabolites over the range of 50 -1000ng/ml (Fig 3.6). These graphs were found to be reproducible when repeated five times during the studies.

APPLICATION

The procedure has been used to measure the concentration of pyridostigmine in human plasma after a single intravenous injection

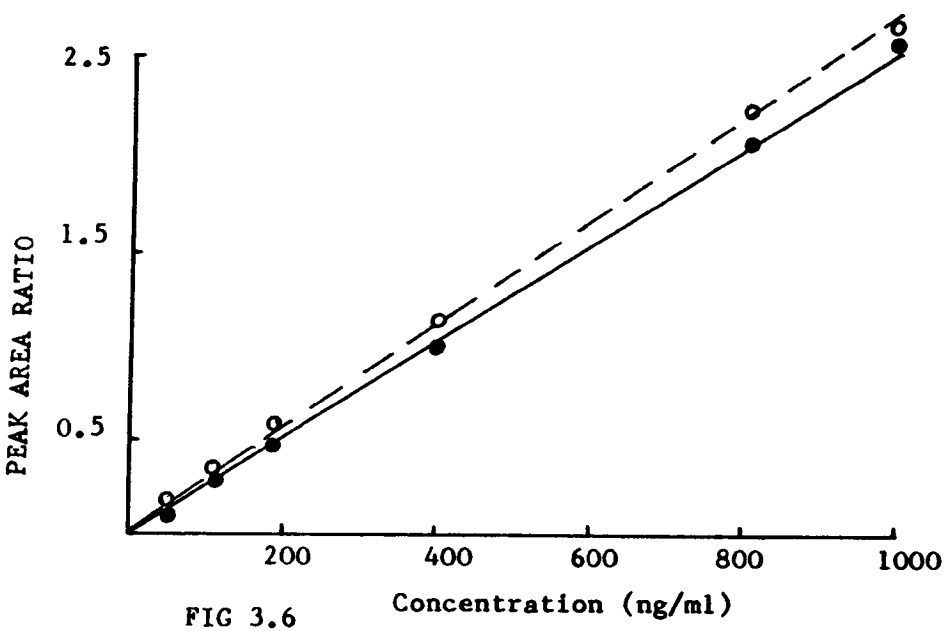


FIG 3.6

Calibration graphs of 3-OHPTMA using 3-OHNMP as the internal marker (O), methanolic solution; (●), urine extracts of iodide complexes of them. Each point represents the mean of five experiments.

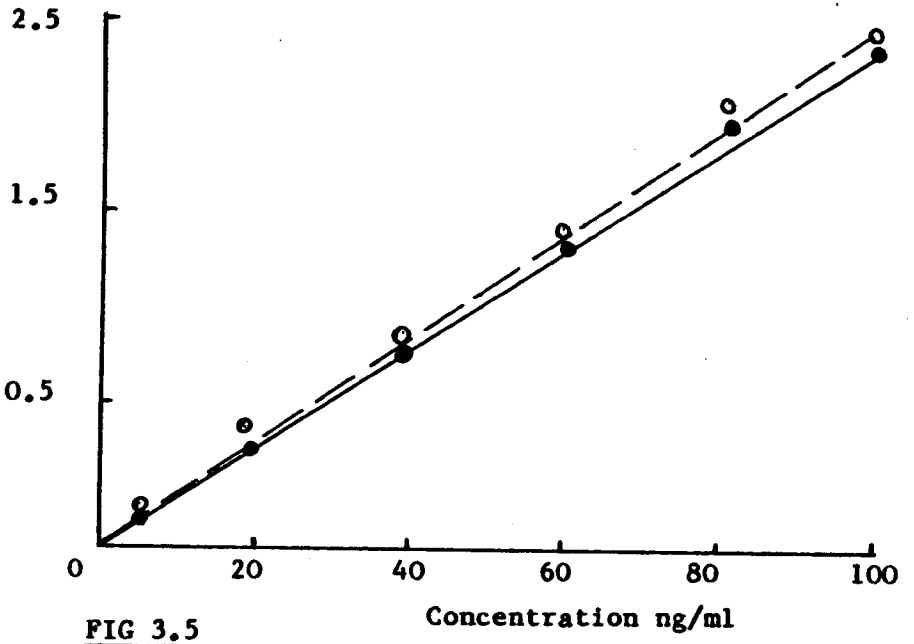


FIG 3.5

Calibration of neostigmine using pyridostigmine as the internal marker. (O), methanolic solution; (●), plasma extracts of iodide complex of them, each point represents the mean of five experiments.

of the anti-ChE to twenty-five patients, that is described in detail in Chapter 4.

DISCUSSION

This method (Method 1) is a selective and sensitive procedure for the isolation and subsequent determination of some clinically useful anti-ChE and their major metabolites. Most previously published methods, based on spectrophotometric detection, were not sensitive enough to measure these quaternary amines. Methods based on thermal dequaternization have been reported for a number of quaternary compounds, including acetylcholine (Szilazyi et al., 1972), thiazinamium (Jonkman et al., 1974) and neostigmine and pyridostigmine (Chan et al., 1976; Pohlmann and Cohan, 1977). Pohlmann and Cohan (1977) in their report used methyiodide (part of dequaternized products) as a measure of quaternary amines in the plasma. However, this method suffers from the lack of selectivity as other quaternary compounds may also be ion-pair extracted and similarly assayed. The procedure described above measures apparently, specifically the dequaternized tertiary analogues.

During the separation of major metabolites from their parent compounds, the phenolic OH groups of the 3-hydroxy metabolites tend to ionize at alkaline pH. Thus, Step 2 in the general Scheme (Fig 3.1) preferentially extracts the parent drugs. Barber et al., (1972) demonstrated that 3-OHPTMA, the major metabolite of neostigmine, was not extracted at pH10 using tetraphenyl boron sodium as the counter ion. They were able to separate neostigmine and 3-OHPTMA at two different pH ranges.

The treatment of all glassware with silylating agent was found to be essential to increase recoveries of drug. This is a generally accepted procedure for working with drugs at low concentration.

This method was subsequently published (Chan and Dehghan 1978).

METHOD 2

THE SIMULTANEOUS DETERMINATION OF NEOSTIGMINE AND PYRIDOSTIGMINE IN HUMAN PLASMA AND URINE

INTRODUCTION

The anticholinestrase agents, neostigmine and pyridostigmine, are the most widely used compounds in the treatment of myasthenia gravis. A recent review has indicated that the monitoring of plasma levels of the anti-ChE agents may have considerable value in the pharmacotherapy of myasthenia patients (Chan 1980). Previously published GLC methods (including Method 1 described in page 64) for the determination of these drugs can only measure one drug at a time while the other is used as an internal marker (Chan et al., 1976 and Chan and Dehghan 1978).

Recently, Aquilonius et al., (1979) reported an identical extraction procedure using deuterated (d6) neostigmine as the internal standard and mass spectrometer (MS) as the detector for the determination of neostigmine in plasma. But the use of a GC-MS system is not always available in every laboratory, due to the high cost of such a system. The method (Method 2)

describes the syntheses of a series of pyridostigmine analogues and the development of one for use as a common internal standard for assaying neostigmine and pyridostigmine in plasma simultaneously.

MATERIALS AND METHODS

The method and materials described for the determination of anticholinesterase drugs (Method 1) in plasma was used with the following modification. The injector and detector temperature was increased from 300 to 350°C, and column temperature increased from 200 - 220°C to 225°C. Other materials used were: TLC plate coated with silica gel 0.15mm thick (Schleicher and Schull, Dassel, W.G.) and analogues of pyridostigmine (synthesized in own laboratory).

SYNTHESIS AND CHARACTERIZATION OF PYRIDOSTIGMINE ANALOGUES

Various analogues of pyridostigmine bromide were synthesized in three stages.

1. The synthesis of acid chlorides ($ClCONR_2$) was carried out according to Werner (1919). Dibutylamine, diethylamine or dipropylamine, equimolar with pyridine, was added to phosogene (12% in toluene i.e., in excess) at 0°C. The reaction by products were removed as follows: Pyridine hydrochloride by filtration and phosogene, toluene and pyridine by evaporation. The residual yellow acid was micro-distilled under vacuum to give the carbonyl chlorides (about 60% yield) and the corresponding ureas were distilled at a high temperature.

The boiling points at 4mm Hg of diethyl, dipropyl and dibutyl carbonyl chlorides were 55°C, 88°C and 104°C respectively.

2. The carbamates of 3-hydroxypyridine were synthesized according to Wurst and Sakel (1951). The 3-hydroxypyridine was dissolved in warmed toluene by stirring. The appropriate acid chloride was added in 1.25fold excess. The reaction was started by adding triethylamine, equimolar with the acid chloride and the mixture was stirred under reflex for four hours. At the end of the reaction, the triethylamine hydro chloride was removed by filtration and the toluene by evaporation and the colourless residual oil was micro-distilled under vacuum to yield the appropriate 3-pyridyl-n,n-dialkyl carbamates.

The characterization of the tertiary analogues are summarized in Table 3.2.

3. The quaternization of the tertiary analogues from Stage 2 (see Table 3.2) was carried out by reacting bromomethane (in excess) to toluene with the 3-pyridyl-n,n-dialkyl carbamates at room temperature. The quaternary bromide was partitioned into water. Toluene and water were initially removed by evaporation under vacuum and final products were dried over phosphorous pentoxide. White deliquescent crystals were produced, which were crystallized from dry ether/ethanol (1:1) mixture and stored in dissicator before use.

The products at each of the three synthesis stages were characterized by the measurement of boiling points at 4mmHg infrared spectrometry (IR), gas chromatography (GC) or thin-

layer chromatograph (TLC). The characteristics of the products of Stage 3 are summarized in Table 3.3.

From these analogues, N,N-dipropylcarbamoyloxy-1-methylpyridinium bromide was considered the most suitable compound for use as a common internal marker for the simultaneous determination of neostigmine and pyridostigmine in human plasma.

SIMULTANEOUS DETERMINATION OF NEOSTIGMINE AND PYRIDOSTIGMINE

A procedure similar to the one described earlier (determination of neostigmine in page 66) was carried out for measuring of neostigmine and pyridostigmine simultaneously, using the bromide salt of N,N-dipropylcarbamoyloxy-1-methylpyridinium as the internal marker.

The concentration of both drugs present in a sample was determined from the ratio of the integrated peak area to that for the internal marker. A corresponding calibration graph was prepared by adding a known amount of the drug to drug-free plasma (3ml) covering the range of concentration between 5.0ng to 100.0ng/ml base, using 50ng/ml base of the internal marker (Fig.3.9). The whole procedure of the assay is summarized in Scheme (Fig 3.7).

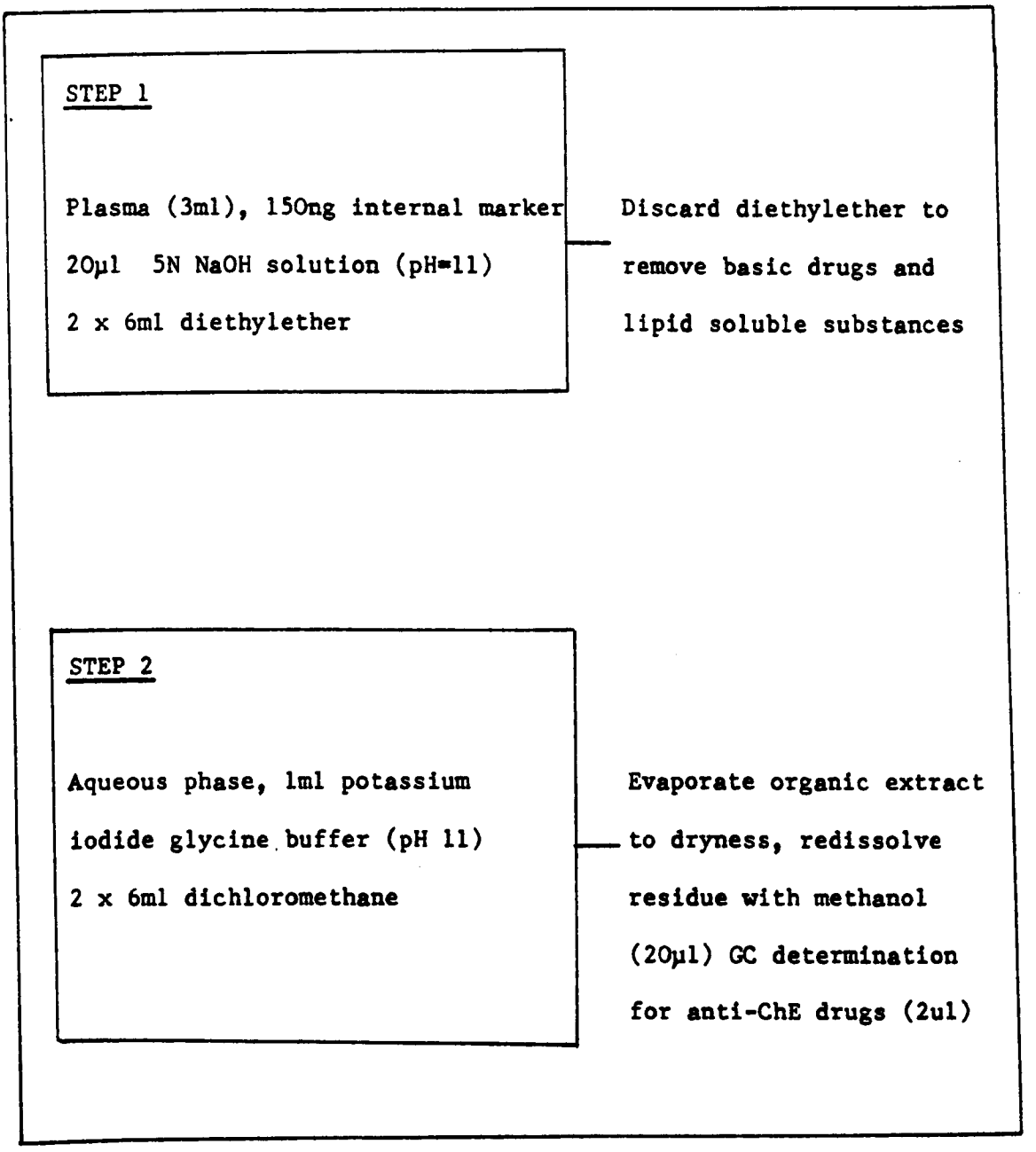
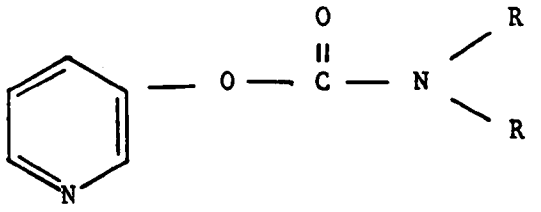


FIG 3.7

A SUMMARY OF THE ASSAY PROCEDURE FOR NEOSTIGMINE AND PYRIDOSTIGMINE IN PLASMA

TABLE 3.2

CHARACTERIZATION OF 3-PYRIDYL-N,N-DIALKYL CARBAMATES



COMPOUND	BOILING POINT (AT 4mmgHg)	IR (CARBONYL RESONANCE)	GC RETENTION TIME (min)	TLC R_f* VALUES
A. R=C_2H_5	136°C	1720	2.8	0.86
B. R=C_3H_7	144°C	1725	4.8	0.89
C. R=C_4H_9	168°C	1725	7.8	0.91

* TLC Silica gel plates, developing system consisting of methanol: strong ammonia solution: butan-2-one (15:20:60).

RESULTS

CHARACTERIZATION OF PYRIDOSTIGMINE ANALOGUES

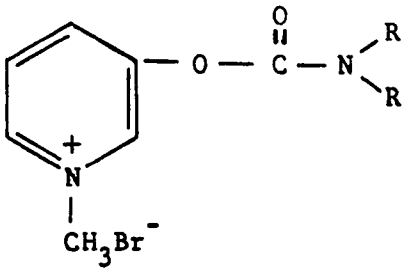
Table 3.2 summarizes the boiling points (at 4mm Hg), the IR characteristics (carbonyl resonance), GC retention times and TLC R_f values of the tertiary bases which were subsequently quaternized to yield the corresponding pyridostigmine analogues. It is interesting to note that when these tertiary analogues were run on a non-polar TLC plate (polyamide) their R_f values (0.97) were indistinguishable. Table 3.3 illustrates the GC and TLC characteristics of neostigmine (E), pyridostigmine (A) and the three synthesized pyridostigmine analogues (B,C and D). Thus, Compound C, the dipropylcarbamoyloxy analogue was chosen as the common internal marker for the simultaneous determination of neostigmine and pyridostigmine.

SIMULTANEOUS GC ASSAY OF NEOSTIGMINE AND PYRIDOSTIGMINE IN PLASMA

The respective retention times, symmetry factors and resolution of neostigmine, pyridostigmine and the chosen internal marker are summarized in Table 3.4 Figure 3.8 shows the chromatogram obtained in the analysis of plasma samples containing 100ng/ml of the drugs. The mean recoveries of neostigmine and pyridostigmine were 94% and 93% (at 60ng/ml level) respectively. The reproducibility of dequaternization at the injection port , where a glass liner was inserted, indicated that bromides or iodide complexes of neostigmine , pyridostigmine and internal marker yielded their corresponding tertiary amines, which gave linear peak area ratios

TABLE 3.3

GC and TLC CHARACTERISTICS OF PYRIDOSTIGMINE ANALOGUES



COMPOUND		GC RETENTION TIME (Minutes)	TLC R_f VALUES*
A.	R = CH_3 (pyridostigmine)	2.2	0.83
B.	R = C_2H_5 (diethylcarbamoyloxy analogue)	2.8	0.88
C.	R = C_3H_7 (dipropylcarbamoyloxy analogue)	4.4	0.90
D.	R = C_4H_9 (dibutylcarbamoyloxy analogue)	7.8	0.93
E.	Neostigmine (3-dimethylcarbamoyloxyphenyl trimethyl ammonium bromide)	8.0	0.86

* TLC - Polyamide plates, developing solvent system consisting of methanol: strong ammonium solution: butan-2-one (15:20:60).

TABLE 3.4

PERFORMANCE OF THE GC SYSTEM (10% OV-17 ON CHROMOSORB W-AW 100-120 MESH)[a]

COMPOUND IN METHANOL	RETENTION TIME (min.)	SYMMETRY FACTORY[b] (0.95-1.05)	RESOLUTION BETWEEN MARKER[b] (>1.0)
Pyridostigmine bromide or iodide-complex	2.2	0.96	2.5
Dipropyl analogue bromide or iodide complex	4.4	1.0	
Neostigmine bromide or iodide complex	8.0	1.0	3.75

(a) Temperature: injection port 350°C; oven 225; detector 350°C; gas flow rates: nitrogen (carrier gas), 30ml/min; hydrogen, 2ml/min; air 100ml/min.

(b) British pharmacopeia (1973) specification for GC analysis.

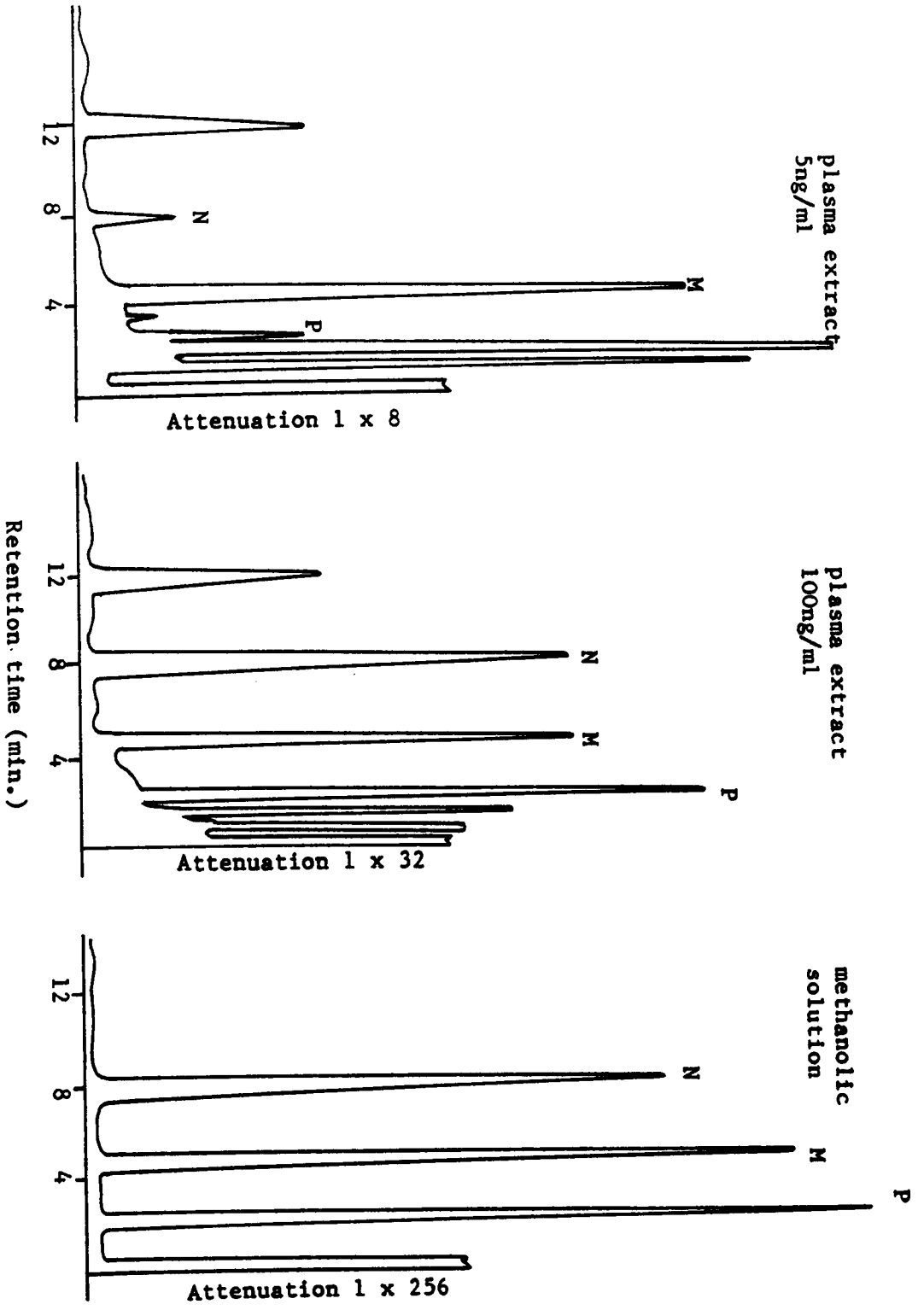


FIG 3.8

Typical chromatogram of plasma extract containing pyridostigmine (P), internal marker (M) and neostigmine (N).

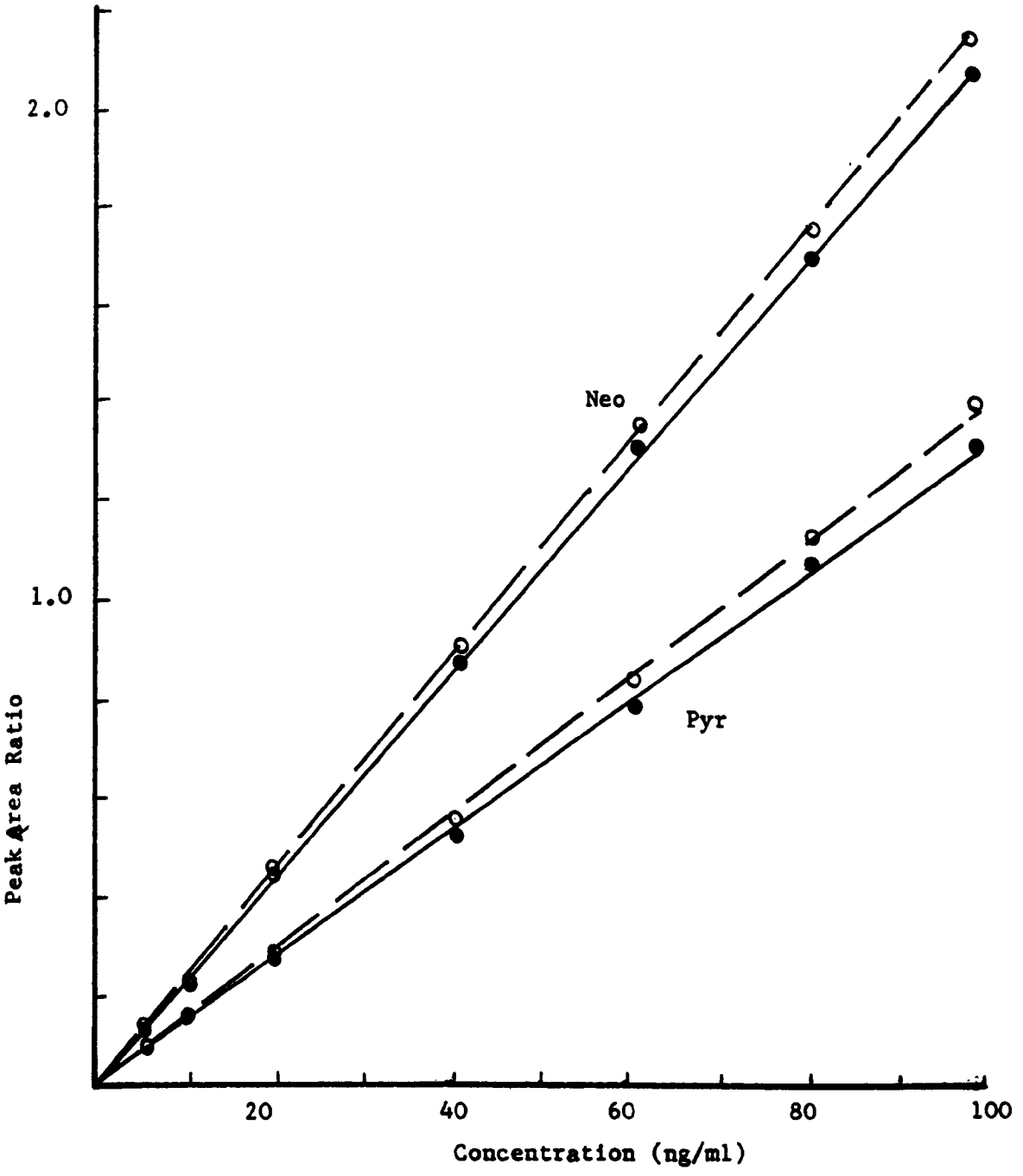


FIG 3.9

Calibration graphs of neostigmine (neo) and pyridostigmine (pyr) using a common internal marker; (O), methanolic solutions of the drugs; (●) plasma extracts of the drug-iodide complexes. Each point represents the mean of six experiments.

over the concentration range between 5 to 100ng/ml (Fig 3.9). These graphs were found to be reproducible when repeated six times.

DISCUSSION

The determination of plasma concentrations of neostigmine and pyridostigmine simultaneously using a common internal marker (N,N-dipropylcarbamyloxy-1-meth ylpyridinium bromide) was based on the ion-pair extraction of the quaternary ammonium compounds into dichloromethane. The organic extract was analyzed by a gas chromatographic procedure. The GC quantitation depends on complete dequaternization of the quaternary amines, either as the bromide or as the iodide-complexes, to their corresponding tertiary analogues, which are then resolved by the GC system.

This was achieved by applying a high temperature (350°C) in the injection port to ensure complete pyrolysis of the quaternary moieties. A mechanism for this thermal decomposition of this group of quaternary compounds was suggested in a review by Chan (1980).

The present assay procedure allows the simultaneous monitoring of the plasma levels of neostigmine and pyridostigmine when both of these anti-ChE agents are used for pharmacotherapy of myasthenia gravis. The technique can detect as low as 1ng/ml of the drug in a 3ml plasma or urine sample, but accurate measurement is achieved within the range of 5 to 100ng/ml or higher.

The evidence of intersubject variations in the absorption of neostigmine (Aquilonius et al., 1979) and pyridostigmine (Calvey et al., 1977) suggests that the simultaneous monitoring of both of the anti-ChE agents in plasma should be important, as there is a lack of information concerning the effect of combined treatment with neostigmine and pyridostigmine in myasthenia gravis.

This procedure has been used to measure the concentration of neostigmine and pyridostigmine in the plasma of seventeen myasthenic patients, who were treated with both drugs (combined dose). For more detail see Chapter 6 (page 119-128).

Part of this work was presented at the British Pharmacological Society Meeting (1979) and later on published (Davison et al., 1980).

CHAPTER IV

DOSE DEPENDENT KINETICS OF INTRAVENOUS PYRIDOSTIGMINE IN SURGICAL PATIENTS.

CHAPTER IV

INTRODUCTION

In anaesthetic practice, anti-ChE drugs are widely used to reverse non-depolarising neuromuscular blockade. In Great Britain, neostigmine methylsulphate (Prostigmine) is most commonly employed for this purpose. Occasionally pyridostigmine bromide is used, since its duration of action is approximately 30% longer than neostigmine (Miller et al., 1974), and it has been suggested that it is less likely to induce significant bradycardia Katz (1967).

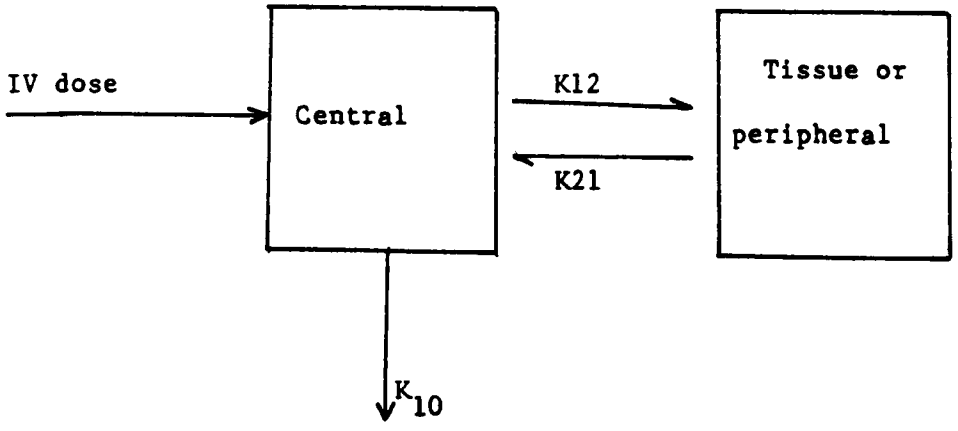
The plasma concentration of pyridostigmine was measured after three different doses of the quaternary amine (36.2µg/kg, 72.4µg/kg and 144.8µg/kg) were given to 23 surgical patients during general anaesthesia. The relation between the plasma concentration of pyridostigmine and time was invariably expressed as a bi-exponential equation, and the data was interpreted in terms of a two compartment mammillary pharmokinetic model (Fig 4.1).

PATIENTS AND METHODS

Studies were carried out in twenty-three patients during the plastic surgical procedure. The subjects were 16 - 64 years old and their body weight ranged from 49.9 - 93.4kg (see Appendix 4.1). None of the patients had any clinical evidence of pre-existing hepatic or renal disease and the results of pre-operative haematological and biochemical assessment (i.e., haemoglobin and full blood count, plasma electrolytes, urea, creatinine, uric acid, inorganic phosphate, calcium, iron, bilirubin, albumin, globulin and aminotransferase) were usually within normal limits (Approximately 10% of the

FIGURE 4.1

THE TWO-COMPARTMENT OPEN MODEL



The drug is administered into the central compartment by rapid intravenous injection. K_{12} and K_{21} are the first-order distribution rate constants and K_{10} is the first-order elimination rate constant.

results of biochemical assessment were slightly outside the normal range and the significance of these results is a matter of conjecture.) One subject (Patient 2) was receiving concurrent treatment with anticonvulsant drugs (phenytoin sodium 300mg/day and phenobarbitone 90mg/day). All the patients were premedicated with nitrazepam (10mg orally given on the previous night).

In studies using low or medium doses of pyridostigmine i.e., 36.2 µg/kg (200n mol/kg) or 72.4µg/kg (400n mol/kg), anaesthesia was induced by thiopentone sodium (200 - 400mg); suxamethonium bromide (100mg) with tubocurarine chloride (15mg) was used for intubation. Anaesthesia was maintained with nitrous oxide (60 - 70%) and halOtane (1 - 2%) in oxygen, using intermittent positive pressure ventilation. During the course of the operation, a polyethylene cannula was placed in a superficial vein, attached to a three-way tap, and kept patent by the infusion of saline. Pyridostigmine (36.2 µg/kg or 78.4 µg/kg) was injected intravenously as the bromide salt over a 15 second period. Blood samples approximately 8ml were removed at 2, 3, 4, 5, 7, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, 100, 120 and 140min., and kept in lithium heparin blood tubes.

In other studies, higher doses of pyridostigmine 144.8µg (800n mol/kg) were used to antagonise non-depolarizing neuromuscular block. In these conditions, anaesthesia was induced by thiopentone sodium (200 - 400mg), followed by tubocurarine chloride (45mg), and maintained with nitrous oxide (60 - 70%) and halothane (0.5%) in oxygen. At the end

of the operation, a polyethylene cannula was placed in a superficial vein as described above, and neuromuscular block was reversed by pyridostigmine (144.8 µg/kg) administered as the bromide salt and atropine sulphate (1.2mg). Blood samples approximately 8ml were usually removed at 2,3,4,5,7,10,15,20, 40,60,80,100,120,140,160 and 180 min., and kept in lithium heparin.

In all experiments plasma was removed from blood samples as soon as possible by centrifugation and stored at -20°C before analysis.

CALIBRATION GRAPHS AND DETERMINATION

Two calibration graphs were prepared (using method 1 page 64), by adding known amounts of pyridostigmine bromide to 3ml of drug-free plasma, covering the concentration range 5.0 to 100.0ng/ml base, and 100.0 to 1000.0ng/ml base, using neostigmine bromide (50ng/ml base and 200ng/ml base respectively) as the internal standard (Fig 4.2a,b). Pyridostigmine was extracted from plasma of surgical patients as an iodide complex, and its concentration for three different doses (200, 400 and 800nmol/kg or 36.2, 72.4 and 144.8µg/kg respectively) was determined by GLC method 1(page 64) and the results are summarized in Appendix 4.2; 4.3; and 4.4; respectively.

RESULTS

After intravenous injection of lower doses of pyridostigmine (36.2µg/kg or 72.4µg/kg), the unchanged drug was rapidly

eliminated from plasma (Fig. 4.3; 4.4; 4.6). In these studies, only trace amounts of pyridostigmine were detected after 90 minutes (patients 8,12 and 16; Appendix 4.2; 4.3). By contrast, after larger doses of pyridostigmine (144.8µg/kg) significant concentrations of the drug (16 - 21ng/ml) were still detectable in plasma after three hours (Fig 4.4; Fig 4.5; Appendix 4.4).

In all experiments, there was an initial rapid fall in the concentration of the unchanged drug, followed by a slower decline. The relation between the plasma concentration of pyridostigmine and time was invariably expressed as a biexponential function equation of the form

$$C_p = Ae^{-\alpha t} + Be^{-\beta t} \qquad \text{Equation (19)}$$

using non-linear least-square regression analysis (Appendix 4.5; 4.6; and 4.7) where C_p is the plasma concentration at time t, and A,B, α and β are constant. Values for the fast disposition half-life ($T_{\frac{1}{2}}\alpha$) and the slow disposition half-life ($T_{\frac{1}{2}}\beta$) were derived from each biexperiential equation and interpreted in terms of a two compartment mammillary pharmacokinetic model (Fig 4.1). The parameters of the model were determined by standard method described in Chapter II and summarized in Appendix 4.8; 4.9; 4.10.

After intravenous injection of pyridostigmine (36.2µg/kg), the fast disposition half-life of the drug was 1.00 $\pm$ 0.12 min. (mean $\pm$ SE), and its terminal half-life was 22.79 $\pm$ 2.09 min. (Table 4.1). The volume of distribution (V_d) of

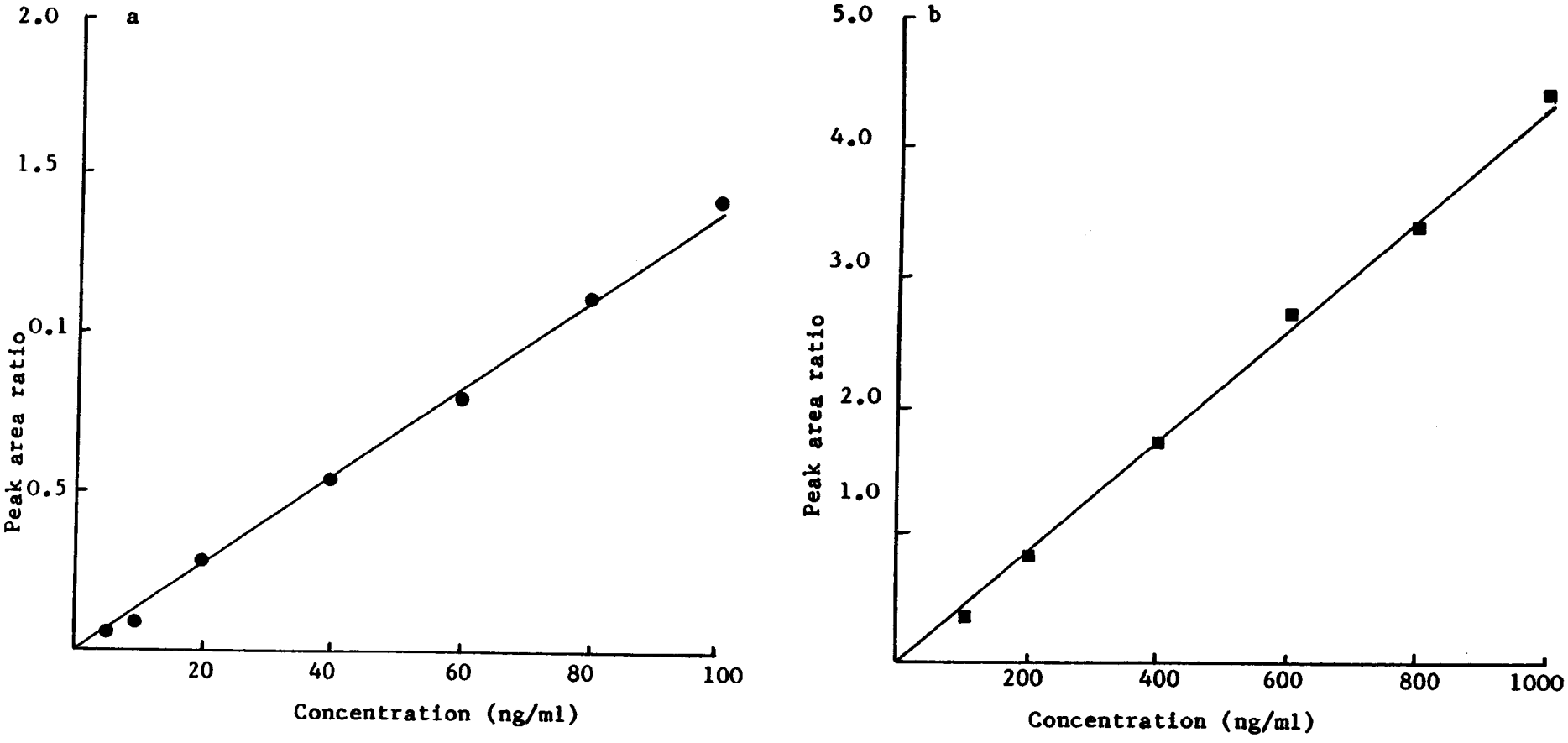


Fig 4.2

a: Calibration graph of pyridostigmine, range 5.0 to 100 ng/ml base, using neostigmine as the internal marker (50ng/ml base); slope = 72.5.

b: Calibration graph of pyridostigmine, range 100.0 - 1000.0 ng/ml base, using neostigmine as the internal marker (200ng/ml base); slope = 230.

Each point represents the mean of six eperiments.

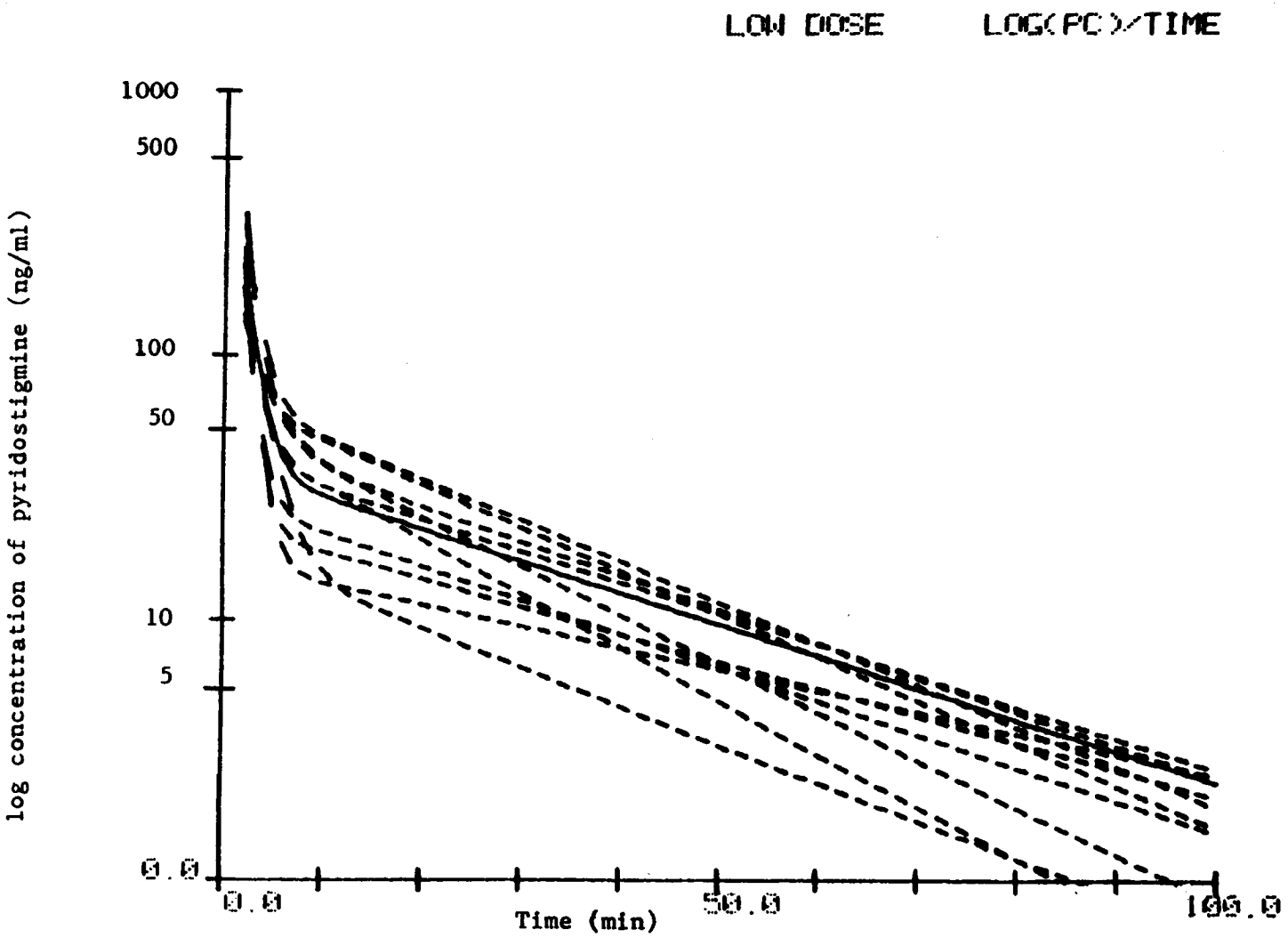


FIG 4.3

Plasma concentration of pyridostigmine following an IV injection (36.2 ug /kg) in ten surgical patients. The dashed lines (calculated curves from the biexponential function and ploted by computer) represent each subject; solid line represents the mean of ten observations.

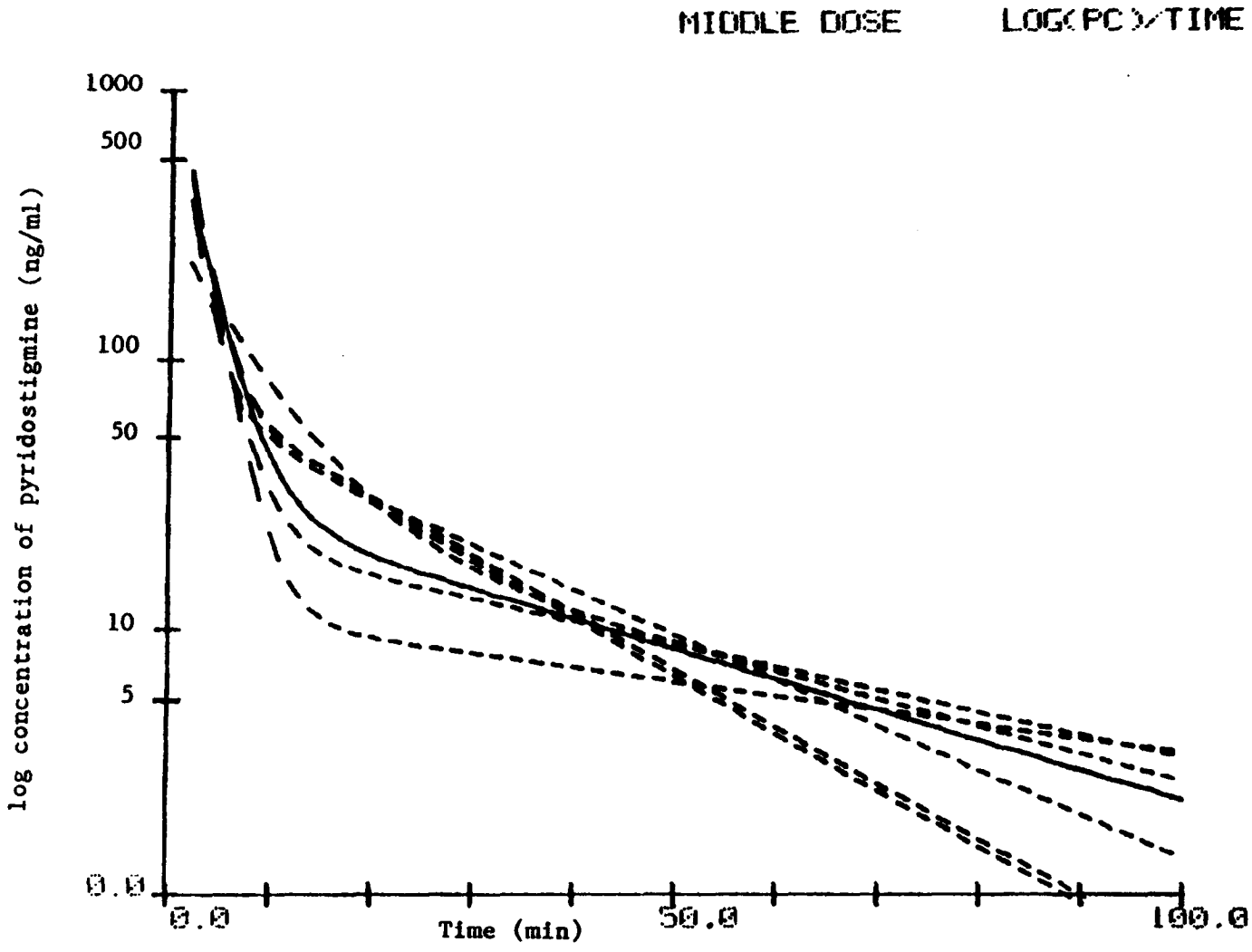


FIG 4.4

Plasma concentration of pyridostigmine, following an intravenous injection (72.4µg/kg) in six surgical patients. The dashed lines (calculated curves from the biexponential function and ploted by computer) represent each subject; solid line represents the mean of the six observations.

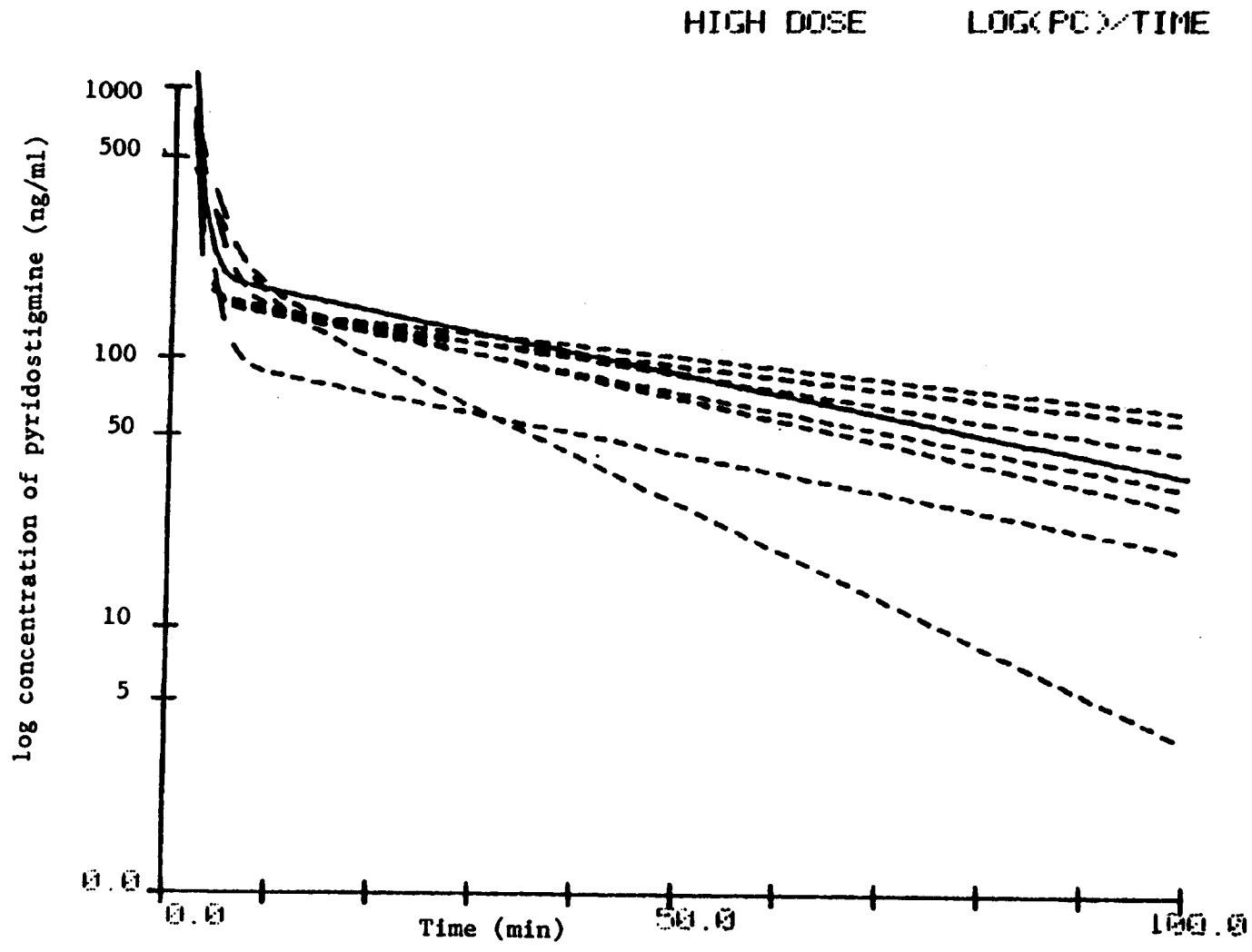


FIG 4.5

Plasma concentration of pyridostigmine after IV injection (144.8 µg/kg) in seven surgical patients. The dashed lines (calculated curves from the biexponential function and plotted by computer) represent each subject; solid line represents the mean of seven observations.

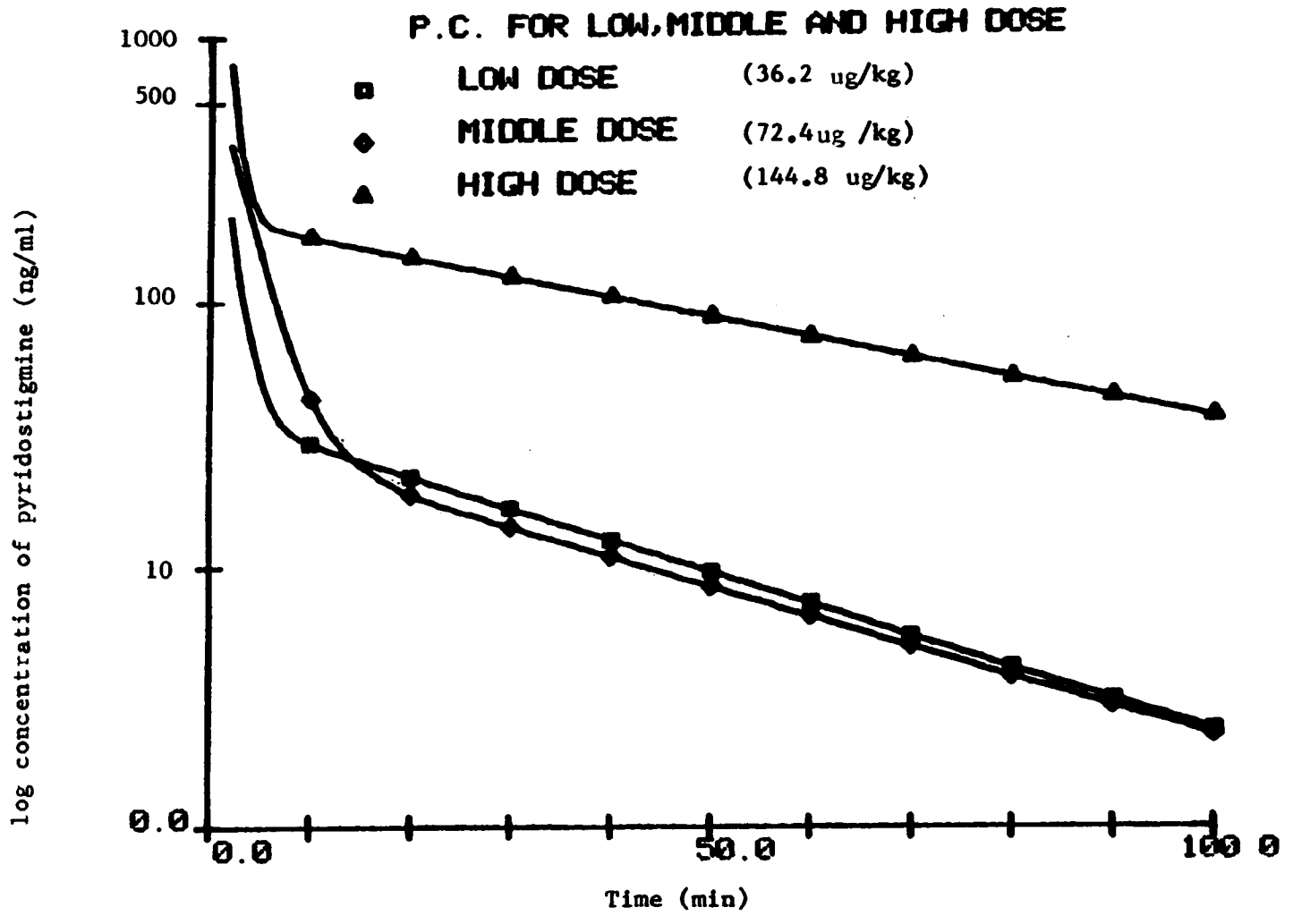


FIG 4.6

Decline of plasma concentration of pyridostigmine after an IV injection. (■) low dose, (◆) middle dose, and (▲) high dose, each line represents the mean of 10, 6 and 7 observations respectively.

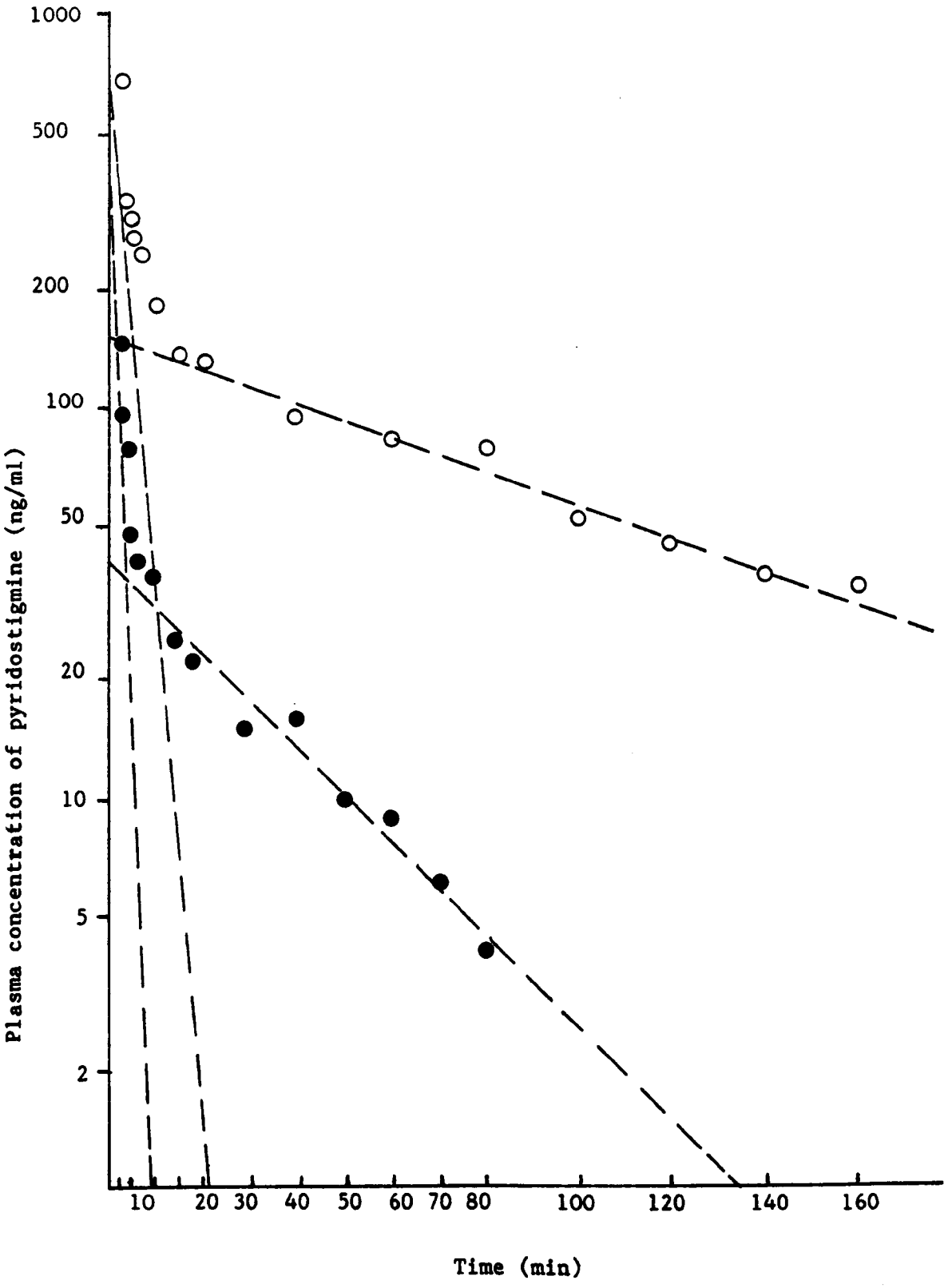


FIG 4.7

Plasma concentration in one patient (6 or 18) who received different doses of pyridostigmine (● ,36.2ug/kg; ○ , 144.8ug/kg) on separate occasions. The dotted lines correspond to the exponential components in each experiment.

pyridostigmine (530.845 ± 62.044 ml/kg) was generally greater than presumptive values for extracellular fluid volume and the clearance (C) was 16.252 ± 1.550 ml/min/kg (Table 4.1). In the one subject (Patient 2) studied, these kinetic parameters were not apparently affected by concurrent therapy with phenytoin and phenobarbitone.

After higher doses of pyridostigmine (72.4µg/kg), the fast disposition half-life almost doubled to 1.96 ± 0.44 min. and the clearance increased to 21.324 ± 0.820 ml/min/kg. Differences between the other kinetic parameters were not statistically significant (Table 4.1). By contrast, after the highest dose of pyridostigmine (144.8µg/kg), different results were obtained. The fast disposition half-life (1.01 ± 0.30 min.) was similar to the values after the lowest dose of pyridostigmine, although the terminal half-life (46.41 ± 6.50min.) was significantly greater (Table 4.1). In two patients (Patients 6 and 10 or 18 and 17 see Appendix 4.1) different doses of pyridostigmine (i.e., 36.2µg/kg and 144.8µg/kg) were given to the same subject on separate occasions. In both instances the slow disposition half-life was clearly prolonged after the larger dose of pyridostigmine (Fig 4.7). In addition, the total body clearance was significantly decreased (to 8.744 ± 1.499ml/min/kg) although the other kinetic parameters were not significantly affected (Table 4.1).

TABLE 4.1

KINETIC PARAMETER (mean ± SE) AFTER I.V. ADMINISTRATION OF DIFFERENT DOSES OF PYRIDOSTIGMINE (36.2µg/kg, 72.4µg/kg, and 144.8µg/kg).

DOSE	36.2µg/kg	72.4µg/kg	144.8µg/kg
$T_{\frac{1}{2}\alpha}$ (min)	1.00 ± 0.12	1.96 ± 0.44*	1.01 ± 0.30
$T_{\frac{1}{2}\beta}$ (min)	22.79 ± 2.09	27.83 ± 5.75	46.41 ± 6.50**
V_d (ml/kg)	530.845 ± 62.044	859.238 ± 175.068	536.057 ± 79.691
C (ml/min/kg)	16.252 ± 1.550	21.234 ± 0.820*	8.744 ± 1.499**
K_{10} (min^{-1})	0.406 ± 0.067	0.269 ± 0.36	0.572 ± 0.184

$T_{\frac{1}{2}\alpha}$ = rapid disposition half-life of pyridostigmine

$T_{\frac{1}{2}\beta}$ = Slow disposition half-life of pyridostigmine

V_d = Total apparent volume of distribution of pyridostigmine

C = Total plasma clearance of pyridostigmine

K_{10} = Elimination rate constant

* = Significantly different ($0.05 > P > 0.02$) from the mean values after 36.2µg/kg (paired t test).

** = Significantly different ($0.01 > P > 0.002$) from the mean values after 36.2µg/kg (paired t test).

DISCUSSION

If pyridostigmine is distributed and eliminated by first-order processes, the half-life of the drug should be independent of the intravenous dose. However, in the present study the slow disposition half-life of pyridostigmine was progressively prolonged (from 22.79 ± 2.09 min. to 46.41 ± 6.50min) as the dose of the drug was raised from 36.2µg/kg to 144.8µg/kg. The increase in the terminal half-life of pyridostigmine was also observed in crossover studies in which different doses of the drug were administered to the same patient on separate occasions. The explanation for this phenomenon is obscure. The slow disposition half-life of pyridostigmine is dependent on the volume of distribution and the total plasma clearance of the drug, and it is difficult to relate the progressive increase in the half-life of the quaternary amine to corresponding changes in these primary kinetic parameters. Thus, there was no significant change in the volume of distribution of pyridostigmine as the dose of the drug was increased; by contrast, the clearance of the quaternary amine was enhanced at intermediate dose levels (i.e., 72.4µg/kg) but significantly reduced at high doses (144.8µg/kg). Cronnelly et al. (1980) have recently studied the kinetics of a two fold higher dose of pyridostigmine (350µg/kg), administered over a two minute period in patients with normal and impaired renal function. Values for the slow disposition and fast disposition half-lives of the quaternary amine in patients with normal renal function were longer than those observed in the present study, although the plasma clearance was similar. In both studies, the volume of

distribution was greater than that presumptive values for plasma or extracellular fluid volume.

Although the interpretation of the results is difficult, and their significance is a matter of conjecture, they may partially account for the observed differences in the duration of action of neostigmine and pyridostigmine in man (Miller et al., 1974). These differences are difficult to explain on pharmacodynamic grounds, since both quaternary amines are closely related chemically and inhibit junctional acetylcholinesterase in an idential manner (i.e., by carbamylation of the esteratic site on the enzyme). Thus, neostigmine and pyridostigmine produce the same inhibited enzymes with a half-life of recovery of approximately 30minutes (Wilson I.B., et al., 1961; Wilson I.B., 1966; Kitz, 1964). When the kinetics of the lower dose of pyridostigmine (200nmol/kg) were compared with the results of equimolar doses of neostigmine (i.e., 206 - 308 nmol/kg) during the reversal of neuromuscular block (Calvey, et al., 1979), it is clear that there are two important differences in the kinetics of the quaternary amines.
In the first place, the volume of distribution of pyridostigmine 530.845 $\pm$ 69.044 ml/kg; mean $\pm$ SE) is greater than neostigmine (122.21 $\pm$ 46.62 ml/kg mean $\pm$ SE), reflecting its more extensive distribution and enhanced tissue up-take.
Secondly, the clearance of pyridostigmine (16.252 $\pm$ 1.550 ml/min/kg; mean $\pm$ SE) is at least five times greater than neostigmine (3.15 $\pm$ 0.808 ml/min/kg; mean $\pm$ SE); this difference is presumably related to the differential renal elimination of the two quaternary amines, since pyridostigmine is less extensively

metabolised than neostigmine (Somani et al., 1972). Similar results have been observed in experimental animals (Baker et al., 1978), and probably reflect differential steric and ionic properties of the two quaternary amines. In consequence, the elimination half-lives of pyridostigmine (22.79 ± 2.09 min; mean ± SE) and neostigmine (25.46 ± 2.74 min; mean ± SE) were similar and the difference between the mean values was not statistically significant ($t = 0.777$; $0.50 > P > 0.40$). However, when these drugs are used in anaesthetic practice to reverse non-depolarizing neuromuscular block, the dose of pyridostigmine is normally 4 to 5 times greater than neostigmine, due to differences in the affinity of their quaternary groups for the anionic site on acetylcholinesterase. In these conditions, the slow disposition half-life of pyridostigmine may be prolonged, allowing significant recarbamylation of acetylcholinesterase to occur, and thus increasing the duration of action of the drug.

CHAPTER V

KINETICS OF INTRAMUSCULAR NEOSTIGMINE IN MYASTHENIA GRAVIS

INTRODUCTION

Neostigmine methylsulphate has been used in the treatment of myasthenia gravis for more than forty years (see Chapter I page 19 and Chapter 3 page 51). In addition, the drug is widely used in clinical anaesthesia to antagonise the effects of muscle relaxants after operative surgery. In these conditions, the clearance of the drug from plasma has been studied using a novel gas-liquid chromatographic technique (see page 68). After rapid intravenous injection, neostigmine was eliminated in a biexponential manner, the fast disposition half-life ($t_{\frac{1}{2}}\alpha$) of the drug was invariably less than one minute and the slow disposition half-life ($t_{\frac{1}{2}}\beta$) ranged from 15.4 - 31.7 minutes (Calvey et al., 1979).

In general, these results have been confirmed by the subsequent work of others. For instance, an identical gas-liquid chromatographic procedure (using a mass spectrometer as a detector) has confirmed that neostigmine is rapidly eliminated from plasma after intravenous injection, although slightly different values for the slow disposition half-life were obtained (Aquilonius et al., 1979).

Both of these studies were based on the measurement of neostigmine in plasma during the antagonism of neuromuscular block. In the present work we have studied the kinetics and metabolism of the drug in plasma after its intramuscular administration to patients with myasthenia gravis. Intramuscular neostigmine methylsulphate is frequently used during diagnosis or initial therapy of myasthenic patients, and

knowledge of its kinetics in these circumstances may well be of clinical significance.

METHODS AND PATIENTS

Studies were carried out in five myasthenic patients who had a classical history of voluntary muscle fatiguability for periods of two months to two years. In most subjects, there was characteristic electromyographic decrement in response to the indirect stimulation of the affected muscle, and a positive response to edrophonium chloride ('Tensilon') was obtained. None of the patients was concurrently taking other compounds or was on anticholinesterase drugs.

EXPERIMENTAL PROCEDURE

In five patients, neostigmine methylsulphate (2.0mg) and atropine sulphate (0.6mg) were given simultaneously by intramuscular injection. Samples of blood were usually removed by venepuncture at 15, 30, 60, 90, 120, 150, 180 and 240 min.

Plasma was obtained by centrifugation and stored at -20°C before analysis. The concentration of neostigmine in plasma was measured by gas-liquid chromatography using a nitrogen sensitive detector and pyridostigmine bromide (50.0ng/ml base) as the internal marker (see Chapter III page 68 ; and Figure 3.5).

RESULTS

In two myasthenic patients, neostigmine was detected in plasma within 15 minutes of intramuscular injection (Figure 5.1;

Table 5.1). The drug was not present until 30 minutes after administration in the other three subjects, and the plasma concentration declined from 21 $\pm$ 2 ng/ml (mean S.E.M.) to 9 $\pm$ 1 ng/ml mean $\pm$ S.E.M.) between 30 minutes and 120 minutes. In three patients the neostigmine level was low at 150 minutes; in one of these subjects, the drug was still detectable at 180 minutes. No quaternary amine could be detected in plasma at 240 minutes (Table 5.1).

The decline in the plasma concentration of neostigmine after intramuscular injection was interpreted in terms of a one compartment model (Gibaldi, 1975). Estimates of the half-life ($t_{\frac{1}{2}}$) of the drug were obtained from a semilogarithmic plot relating the plasma concentration of neostigmine to time between 30 and 150 minutes (The results obtained at 15 and 180 minutes were disregarded, since they were only obtained in a minority of the patients studied and were usually inconsistent with the trend of the other data points.) In the five patients studied, the half-life of neostigmine ranged from 56.9 - 100.1 minutes, corresponding to an apparent first-order elimination rate constant (K) of 0.0122 min^{-1} to 0.0069 min^{-1} (Table 5.2). The extrapolated concentration of neostigmine at zero time (C_o) was used to determine the total apparent volume of distribution V_d, from equation (7) in Chapter II (page33).

$$V = \frac{\text{dose (as neostigmine base)}}{C_o}$$

Values for the total apparent volume of distribution varied from 35.2 - 63.0 litres. Corresponding values for total body clearance as calculated from the product of V_d and K, ranged from

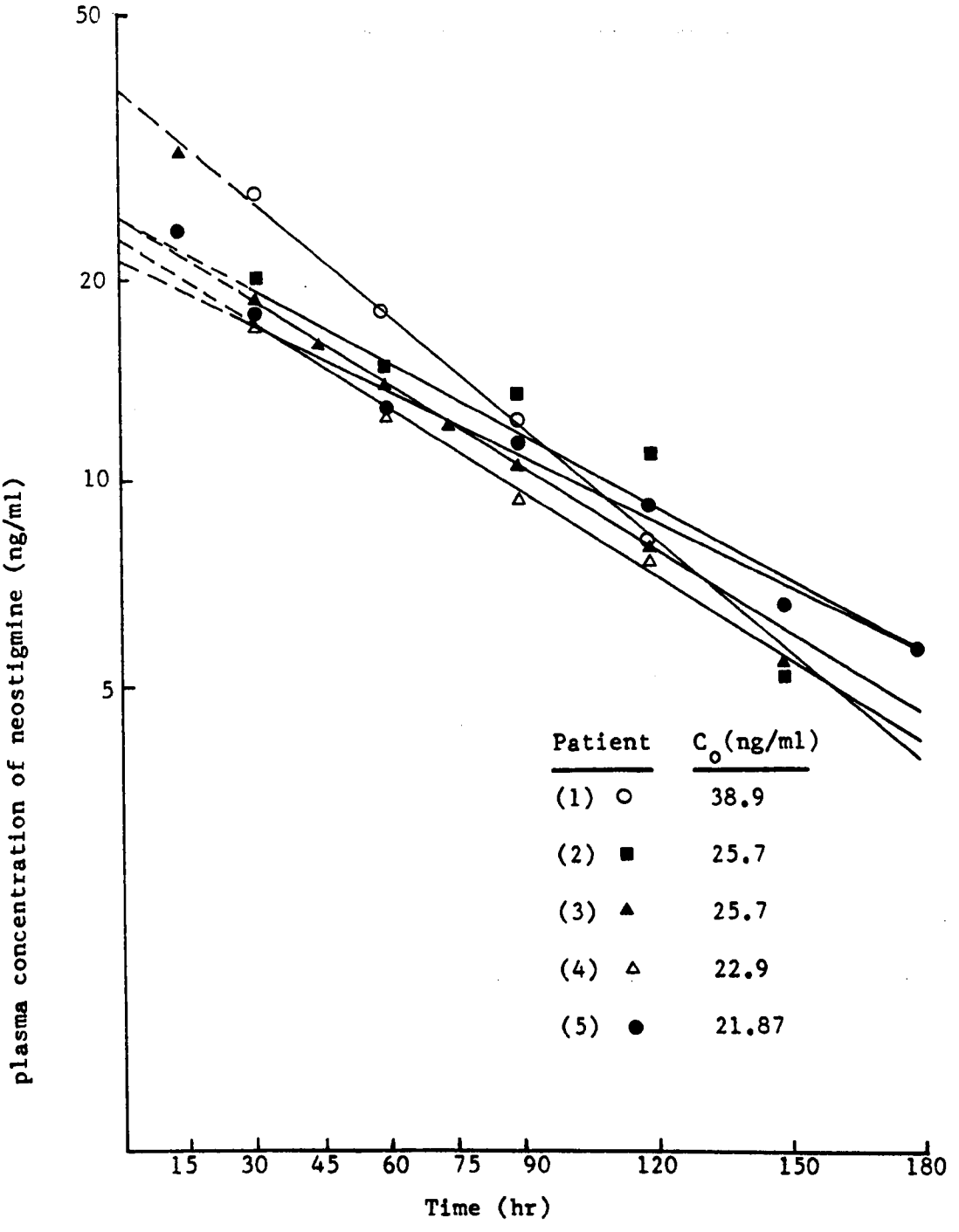


FIGURE 5.1

The plasma concentration of neostigmine in five myasthenic patients after intramuscular administration of the drug. Each symbol (● , △ , ▲ , ○ and ■) represents the results obtained in five different subjects between 30 and 150min. The dotted lines indicate the extrapolation to zerotime with C_o as concentration in mg/ml.

TABLE 5.1

Plasma concentration of neostigmine (ng/ml) in five myasthenic patients following an IM dose of 2mg of neostigmine methylsulphate.

TIME	SUBJECTS				
	1	2	3	4	5
0					
15		NS	32	NS	24
30	26.9	20	19.2	18	18.5
45			16.2		
60	17.9	15	14.1	12	13.5
75			12.5		NS
90	12.9	14.1	10.8	9.2	11.2
120	8.6	11.1	8.4	7.5	9.3
150	ND	5.2	NS	NS	6.9
180	NS	NS	NS	NS	5.7
240	NS	NS	NS	NS	NS

ND = non detected

NS = non sample

TABLE 5.2

Kinetic parameter of neostigmine after intramuscular administration.

PATIENT	$t_{\frac{1}{2}}$ (min)	K (min^{-1})	V_d (L)	$V_d \times K$ (ml/min)
1	56.9	0.0122	35.2	429
2	92.5	0.0075	53.2	399
3	73.1	0.0095	53.2	505
4	66.0	0.0105	59.3	623
5	100.1	0.0096	63.0	435
MEAN ±	77.7 ±	0.0093 ±	52.8 ±	478 ±
S.E.M.	8.1	0.0010	4.8	4.0

$t_{\frac{1}{2}}$ biological half-life

K elimination rate constant

V_d total apparent volume of distribution

$V_d \times K$ total body clearance

399 - 623 ml/min (Table 5.2).

DISCUSSION.

The present experiments show that neostigmine is rapidly absorbed and eliminated after intramuscular administration to patients with myasthenia gravis. In all the subjects studied, maximal concentrations (18 - 32ng/ml) were present in plasma between 15 and 30 minutes, although only small or undetectable concentrations were present after 120 minutes. Thus, the presence of detectable concentrations of the drug in plasma approximately corresponds to its duration of action in myasthenic patients. In the present experiments as well as in previous studies of Calvey et al. (1979) and Chan et al. (1976), the analytical method has been shown to detect neostigmine at concentrations of 5 - 7 ng/ml in the plasma, and can be used for the assessment of anticholinesterase therapy in man. Other authors (Aquilonius, 1979) suggested that the method only allows accurate measurement of neostigmine at concentrations above 50 ng/ml, but this has not been shown in the present study.

After intramuscular administration of neostigmine to five myasthenic patients, there was a monoexponential decline in the concentration of the drug in plasma between 30 and 120 minutes. When the results were interpreted in terms of a one compartment model, the half-life of the drug ranged from 56.9 - 100.1 minutes, the apparent volume of distribution from 35.2 - 63.0 litres. The half-life and intercept of the slow disposition phase was observed after intravenous injection of

neostigmine (Calvey, 1979). By contrast, there may be a forty-fold difference in plasma concentration in patients receiving oral neostigmine (Aquilonius 1979). Since this variability is not observed after intramuscular or intravenous neostigmine, it is presumably related to inter-individual differences in drug absorption and/or variation in the first-pass effect. As neostigmine is extensively metabolised in man the first-pass effect may be of considerable importance.

The volume of distribution of neostigmine was approximately equal to total body water. Distribution studies in experimental animal suggests that the drug is extensively localized in extracellular fluid, liver and kidney, but not in other tissues (Somani, 1975). The results of the present study are not inconsistent with this interpretation. Total body clearance of the drug ranged from 399 - 623 ml/min; these values are greater than the glomerular filtration rate, and suggest the occurrance of tubular secretion or extensive metabolism of neostigmine.

CHAPTER VI

PART ONE

PLASMA LEVELS OF NEOSTIGMINE AND PYRIDOSTIGMINE IN MYASTHENIC PATIENTS AFTER ORAL ADMINISTRATION.

PART TWO

THE RELATIONSHIP OF PLASMA LEVELS OF PYRIDOSTIGMINE TO CLINICAL EFFECT IN PATIENTS WITH MYASTHENIA GRAVIS.

CHAPTER SIX

PART ONE

PLASMA LEVELS OF NEOSTIGMINE AND PYRIDOSTIGMINE IN MYSTHENIC PATIENTS AFTER ORAL ADMINISTRATION.

INTRODUCTION

Over the past forty years, anticholinesterase (Anti-ChE) agents such as neostigmine and pyridostigmine have been used as the first line of treatment for most patients with myasthenia gravis and pyridostigmine is the most widely used drug. However, only until recently disposition and pharmacokinetic studies of these agents have been carried out in man due to recent advance of analytical techniques.

The metabolism of pyridostigmine in man was studied by Kornfeld et al., (1971) using ^{14}-C-labelled analogue and by Somani and others (1972) using mass spectrometry. Cohen and colleagues (1976) monitored plasma concentration of pyridostigmine in myasthenic patients and demonstrated that there was a possible relationship between plasma levels of the drug and clinical response. Recently, Calvey and Chan (1977) have shown that there was a considerable variation in the bioavailability of pyridostigmine among six myasthenic patients and that the plasma concentration of the drug was invariably positively correlated with the improvement in neuromuscular transmission in patients with typical electromyographic decrement using the adductor pollicis.

The pharmacokinetics of neostigmine after intravenous administration to surgical patients during the reversal of neuromuscular block have recently been investigated (Aquilonius et al., 1979; Calvey et al., 1979). Both studies suggested that neostigmine was eliminated from plasma biexponentially. In a separate study on intramuscular

neostigmine in myasthenic patients, the kinetics were best described by a one compartment model (Somani et al., 1980). However, there is no information on the kinetics of the two anti-ChE-agents when both neostigmine and pyridostigmine are used in severe cases of myasthenia gravis. Recently, we have developed a gas-liquid chromatographic method which can determine simultaneously the plasma concentrations of neostigmine and pyridostigmine using a synthesised pyridostigmine analogue, n,n-dipropylcarbamoyloxy-N-methyl pyridinium bromide, as a common internal marker (see Chapter III part two). This part of the work is concerned with the concentration of these two anti-ChE drugs in the plasma of seventeen myasthenic patients who were stabilized either by pyridostigmine only, or on both neostigmine and pyridostigmine therapy.

PATIENTS AND METHODS

SUBJECTS

Studies were carried out on eight myasthenic patients who were stabilized on varying amounts of oral pyridostigmine bromide only (appendix 6.1), and nine patients who were maintained by the oral administration of neostigmine and pyridostigmine (Appendix 6.2 and 6.3). In conjunction with the anti-ChE treatment, some patients were also given prednisolone and azathioprine supporting therapy.

PROCEDURE

The dosage schedule of each patient was not modified during the study. Blood samples (usually 10 ml) were removed by

venupuncture at two hourly intervals after the initial morning dose (Appendix 6.4), during the waking day (8.00am to 8.00pm). The samples were kept in blood tubes containing lithium heparin and plasma was promptly obtained by centrifugation and stored at -20°C before analysis.

DETERMINATION OF PLASMA CONCENTRATION OF THE ANTI-ChE DRUGS.

The plasma concentrations of neostigmine and pyridostigmine were determined by the gas-liquid chromatographic technique described in Chapter III (page 80) using a common internal standard n-n-dipropylcarbamoyloxy-N-methyl pyridinium bromide.

A corresponding calibration graph was prepared by adding known amounts of the drug to drug-free plasma (3ml), covering the range of concentrations between 5.0ng to 100.0ng/ml base using 50ng/ml base of the internal marker (Fig. 3.9 page 87).

RESULTS

PLASMA PYRIDOSTIGMINE

Plasma levels of pyridostigmine were monitored from 8.00am to 8.00pm in eight myasthenic patients, who were stabilized on intermittent dosage from 60 to 540 mg orally. In these patients the maximum plasma concentration throughout the whole waking day ranged from 12.4 to 64.5ng/ml, and the minimum from 1.3 to 25.0ng/ml (Appendix 6.5). Typical plasma profiles of pyridostigmine for patient 1 (lowest daily dose) and patient 5 (highest daily dose) are shown in Fig 6.1. The area under plasma concentration-time curves (AUC) of pyridostigmine in these patients ranged from 587.5 to 4560.0ng/ml^{-1}hour, over the twelve hour period (Fig 6.2).

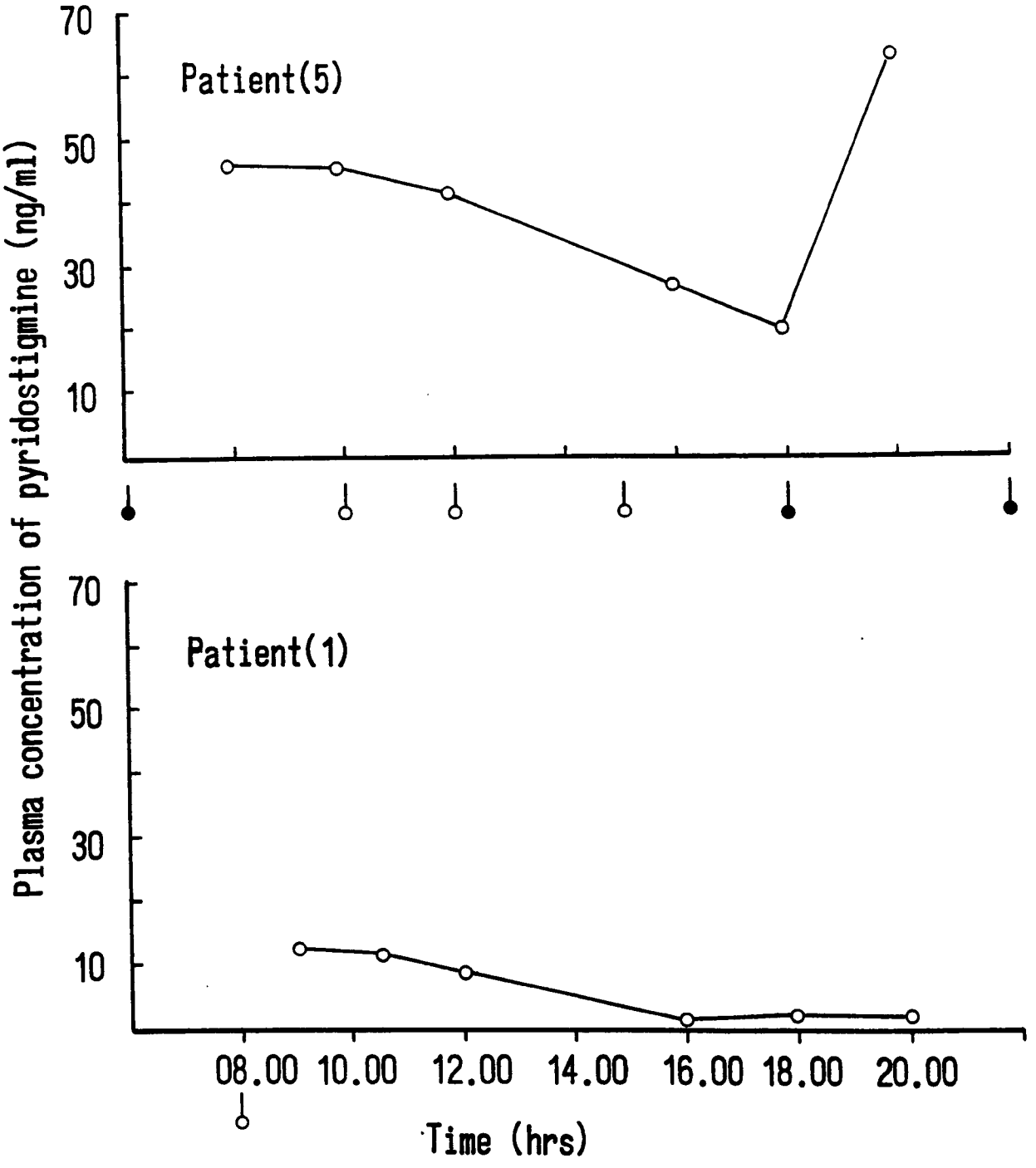


FIG 6.1

Typical plasma profile of pyridostigmine for patient 1 (lowest drug dose 60mg/day) and patient 5 (highest dose 540mg/day) after oral administration, (↓), (↓) represent oral doses of pyridostigmine 60mg and 120mg respectively.

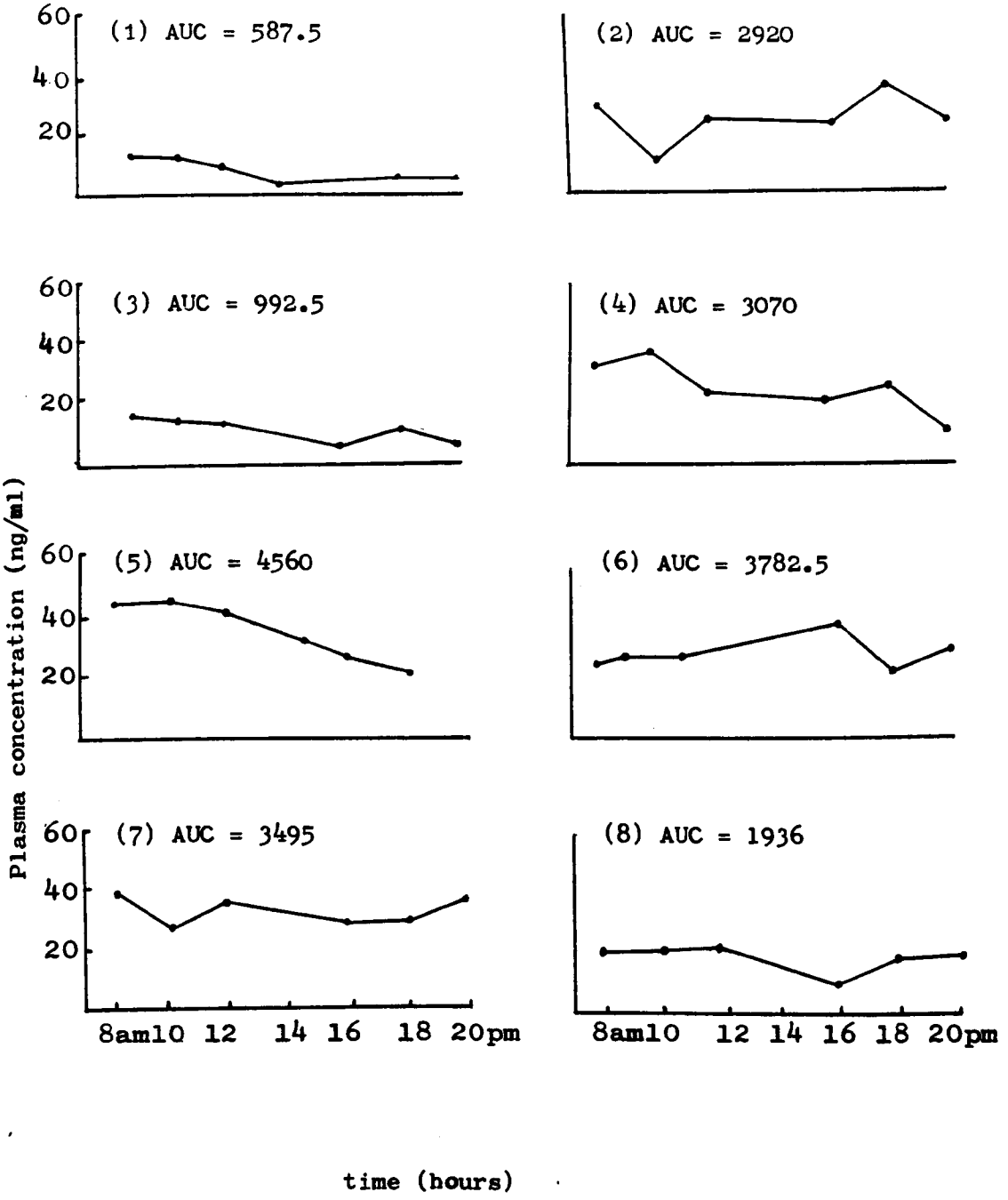


Fig.6.2

The area under plasma concentration-time curve (AUC) of pyridostigmine in 8 patients (ranged from 587.5 to 4560.0 ng/ml^{-1} hour) over 12 hour period.

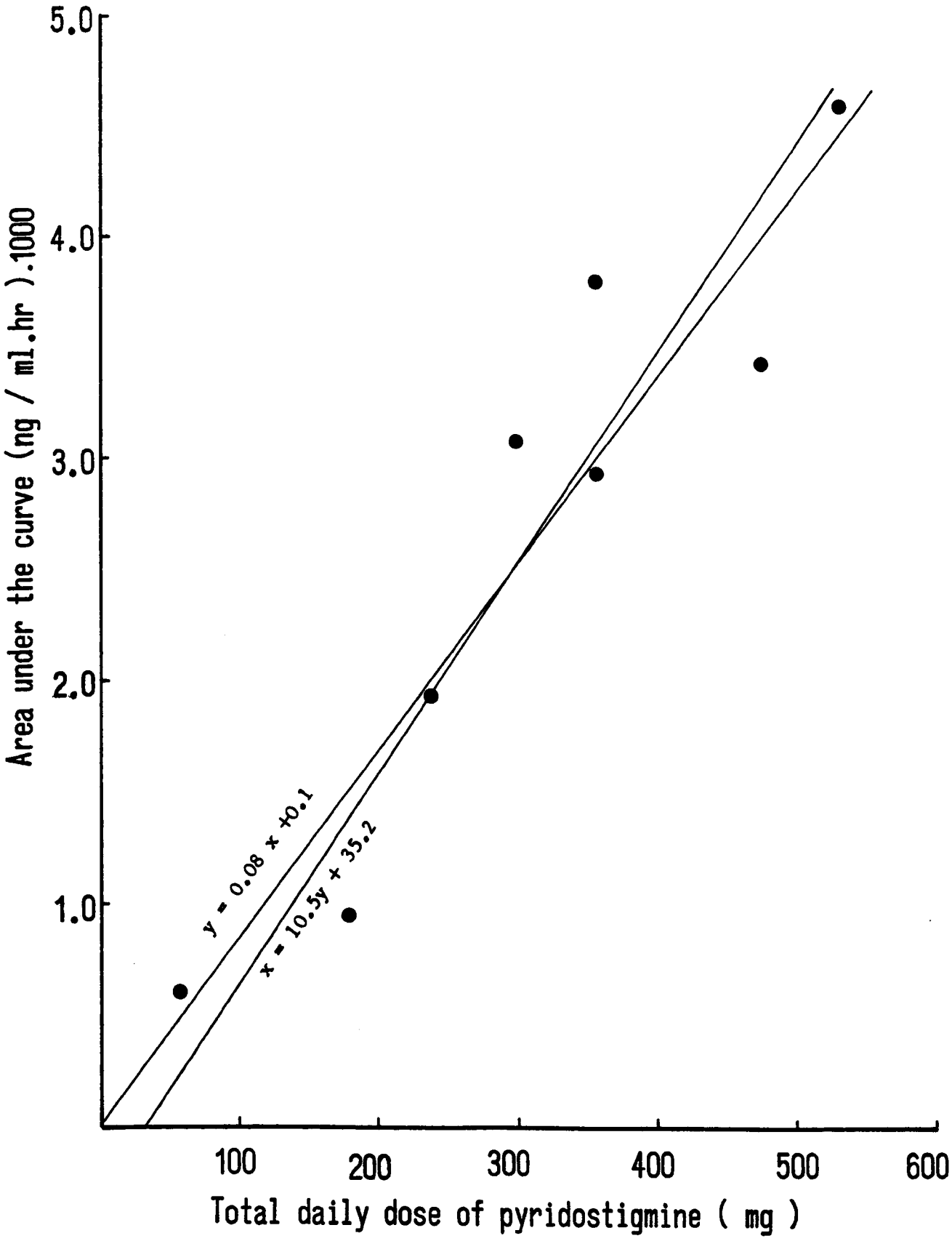


FIG 6.3

Direct linear relationship between the area under plasma concentration-time curves (AUC) and total daily dose in the first group of myasthenic patients (r = 0.95.)

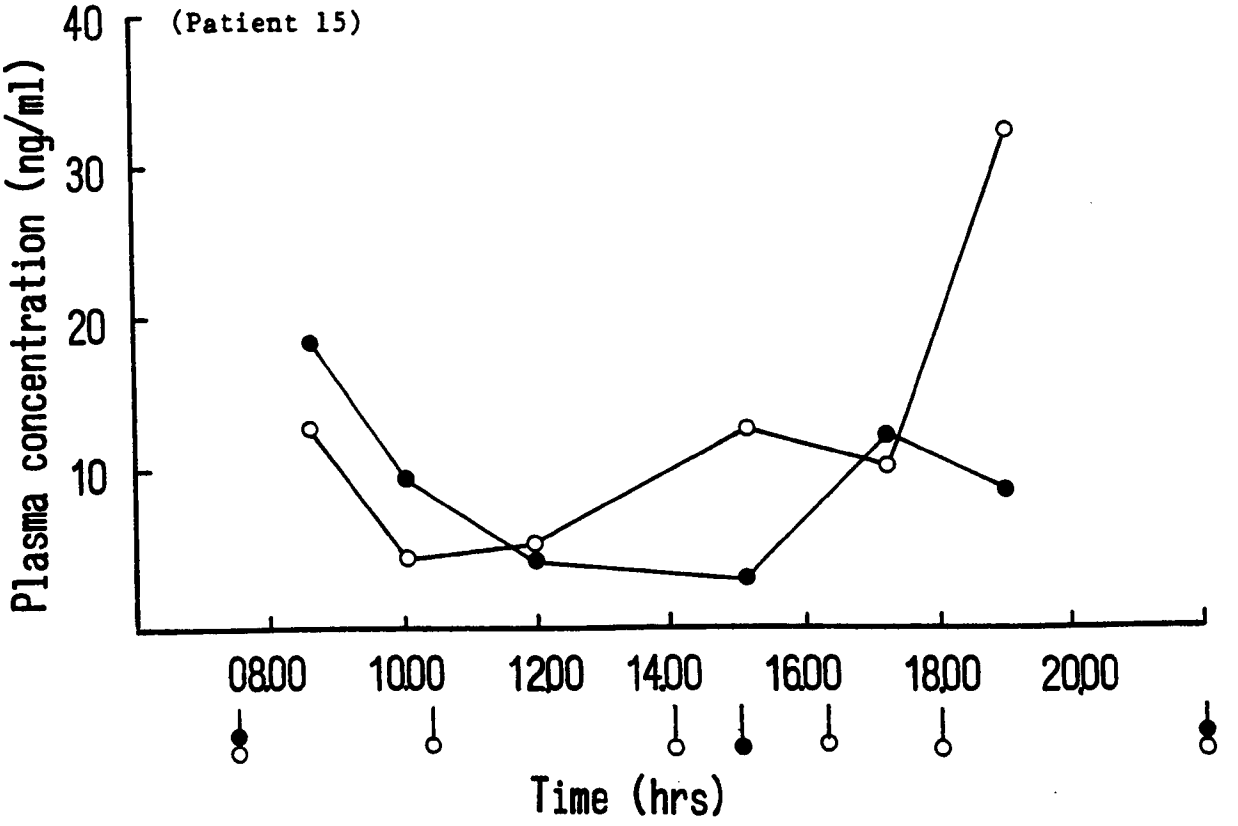


FIG 6.4

Plasma concentration of neostigmine (O—O) and pyridostigmine (●—●) in a myasthenic patient (Patient 15) on an ordinary dose schedule O, ● represents oral doses of neostigmine (30mg) plus pyridostigmine (60mg) at indicated intervals, respectively.

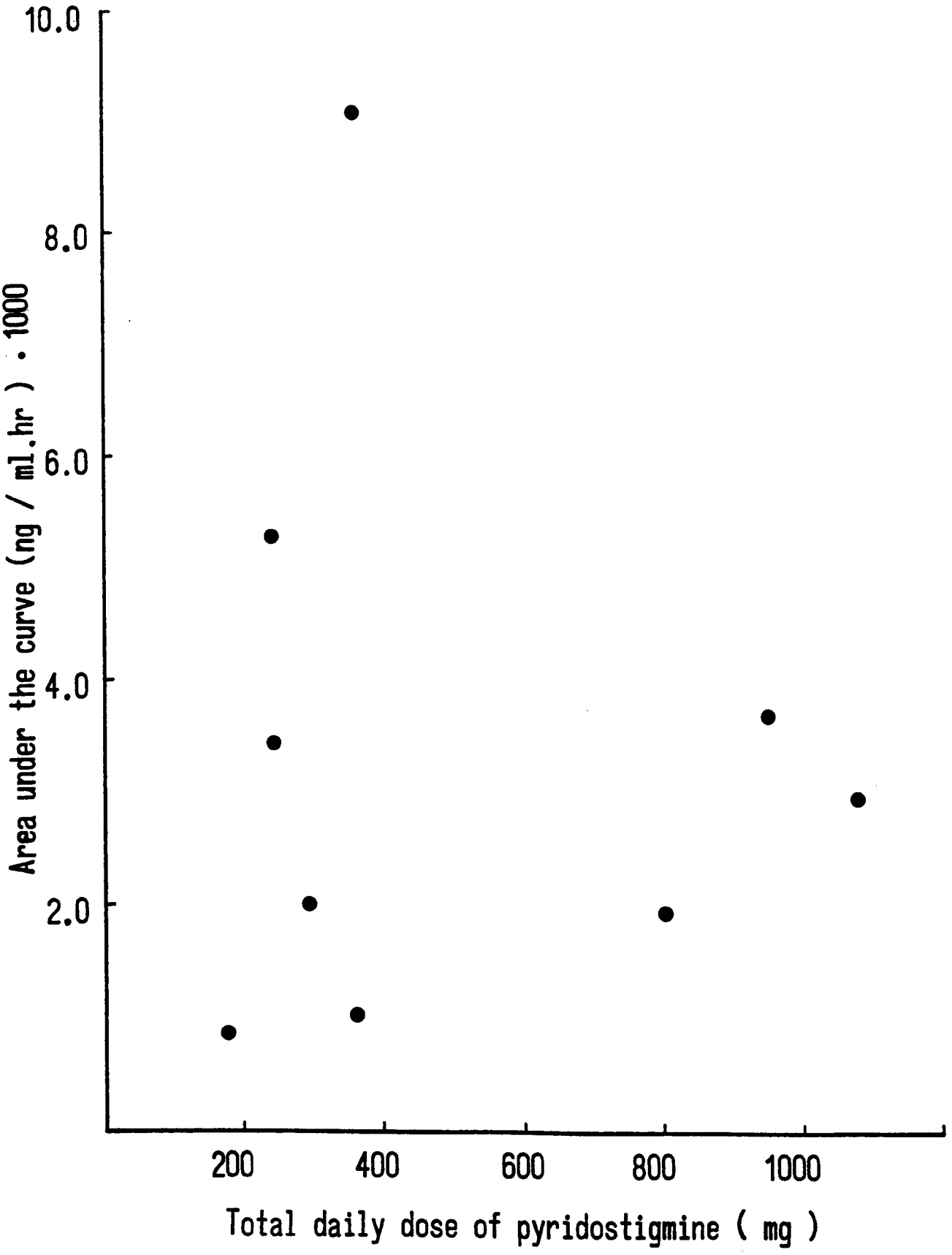


FIG 6.5

There is no linear correlation between AUC of pyridostigmine and total daily dose in this group of patients who received both neostigmine and pyridostigmine therapy (r = - 0.08).

TABLE 6.1

Plasma concentrations and bioavailability of pyridostigmine between 8.00am and 8.00pm after oral administration of intermittent doses of pyridostigmine only.

PATIENT	DAILY DOSE OF PYRIDOSTIGMINE BROMIDE mg	MIN. and MAX. CONCENTRATION (mg per ml)	AREA UNDER PLASMA CONCENTRATION-TIME CURVE ($ng\ ml^{-1}\ hr$)
1	60	1.3 - 12.4	587.5
2	360	9.0 - 36.0	2920.0
3	180	5.0 - 14.0	992.5
4	300	11.0 - 40.0	3070.0
5	540	20.5 - 64.5	4560.0
6	360	25.0 - 40.0	3782.5
7	480	24.0 - 39.0	3495.0
8	240	6.2 - 23.0	1936.0

TABLE 6.2

Plasma concentrations and bioavailability of pyridostigmine between 8.00am to 8.00pm after oral administration of intermittent doses of neostigmine and pyridostigmine.

PATIENT	DAILY DOSE OF		MIN. & MAX. CONCENTRATION of		AREA UNDER PLASMA CONCENTRATION-TIME CURVE (pyridostigmine)
	NEOSTIGMINE BROMIDE mg	PYRIDOSTIGMINE BROMIDE mg	NEOSTIGMINE (ng per ml)	PYRIDOSTIGMINE (ng per ml)	(ng/ml^{-1} hr)
9	150	240	ND	12.3 - 61.5	5276
10	120	300	ND	8.5 - 33.0	2070
11	180	360	ND	5.4 - 15.3	1034
12	60	360	ND	50.0 -144.0	9140
13	60	240	ND	5.5 - 60.0	3433
14	90	360	ND	9.3 - 25.5	2137
15	180	180	4.7 - 33.0	2.7 - 18.6	864
16	15	1080	ND	11.0 - 36.5	3022
17	480	810	ND	5.3 - 28.0	1956

ND - not detected

When the AUC was plotted against the total daily dose of pyridostigmine for all eight patients a linear relationship was observed (Fig 6.3).

PLASMA PYRIDOSTIGMINE AND NEOSTIGMINE

In only one of the nine patients (patient 15) both neostigmine and pyridostigmine were detected in the plasma (Fig 6.4). In this group of patients, the plasma concentrations of pyridostigmine were higher and more diversified; the minimum range was 5.4 to 60.0ng per ml and the maximum range was 15.3 to 144.0ng per ml (Table 6.2). No linear correlation between AUC of pyridostigmine and total daily dose was observed in this group of patients who received both neostigmine and pyridostigmine therapy (Fig 6.5).

DISCUSSION

In the present investigation, the eight myasthenic patients were stabilized on widely different daily dose of pyridostigmine (Appendix 6.3 and Table 6.1). Although the concentration of pyridostigmine in plasma varied between 12.4 to 64.5ng/ml (maximum range) and 1.3 to 25.0ng per ml (minimum range) there was a direct relationship between the AUC and total daily dose of the drug in these eight patients over the twelve hour period of investigation. In an investigation on six myasthenic patients, the peak plasma concentration of the drug ranged from 44.2 to 84.0ng per ml; and in most cases the level was maintained between 20 to 60ng per ml in a period of eight hours, when the dose of pyridostigmine was between 60 to 660 mg per day (Calvey and Chan 1977). These observations

suggest that the widely different dosage requirement of pyridostigmine in myasthenic patients may be partly related to intersubject variation in drug absorption and disposition and to varying severity of the disease.

The presence and significance of intersubject variation in the oral absorption of pyridostigmine is difficult to demonstrate and assess. In a previous study, the AUC of pyridostigmine in 3 myasthenic patients was maintained in a narrow range four hours after the same oral dose of 60mg (Calvey et al., 1977) suggesting that absorption of the drug from the gut is relatively constant. In the present study, the plasma level of pyridostigmine was monitored over a longer period of time (up to twelve hours after the first oral dose), and there is a positively direct relationship between the AUC of pyridostigmine and total daily dose during this period, $r = 0.95$ (Fig. 6.3). These observations are in direct contrast to that for one myasthenic patient, whose bioavailability of the drug was increased six fold by doubling the dose of pyridostigmine bromide from 30 to 60mg (Calvey et al., 1977). Perhaps there is also an intra-subject variation in the absorption of the drug.

In general, quaternary amines cannot readily penetrate lipophilic cellular membranes in the gut, due to their complete ionization at all pH. Some quaternary amines are known to form ion-pair complexes with intestinal components such as mucin or bile salt, which are readily extracted into organic solvents (Levine and Clark 1957; Schanker, 1963). Thus, in the present experiments, there were frequent fluctuations in

the plasma concentration of pyridostigmine which were not apparently related to dosing intervals. These fluctuations were not observed after intravenous injection of the drug (see Chapter IV). In patient 1 (Fig. 6.1), there was a secondary rise in plasma concentration after a single dose of pyridostigmine bromide (60mg). This observations is consistent with previous observations (Calvey et al., 1977). The secondary rise may be related to the formation and absorption of lipophilic ion-pair complex although the actual mechanism of absorption of pyridostigmine is unknown.

The processes of drug absorption and disposition may be influenced by the presence of other drugs. The renal clearance of pyridostigmine was reported to be reduced by concurrent administration of some tertiary amines which are partially present as cations at physiological pH and may compete with pyridostigmine for renal tubular transport (Chan and Calvey, 1978). Of the nine myasthenic patients (patients 9 - 17, Appendix 6.3 and Table 6.2), who were maintained by both oral neostigmine and pyridostigmine therapy, only in patient 15 both drugs were detected in the plasma. Two possible explanations may be considered for this observation. Firstly, the bioavailability of neostigmine was estimated to be 1 - 2% of the ingested dose and considerable interindividual differences in the absorption of neostigmine after oral ingestion of the drug were observed Aquilonius et al., (1979). The low bioavailability of oral neostigmine is probably due to its extensive metabolism by the liver after 'first-pass effect'(Somani et al., 1972). The

sampling procedure in the present study might have missed the peak concentration or the assay procedure was not sensitive enough to detect concentration approaching picogramme range. Secondly, the possibility of drug-drug intraction between neostigmine and pyridostigmine at the absorption phase should not be ruled out. There was no direct correlation between the AUC of pyridostigmine and total daily dose in these patients (9 - 17) who were treated with both anticholinesterase drugs (Fig. 6.5), compared with pyridostigmine only (Fig. 6.3). The highest and lowest concentration ranges of pyridostigmine in patients 9 to 17 were higher and more diversified than those of patients 1 to 8 (Table 6.1 and 6.2). When the AUCs of pyridostigmine of all seventeen patients were plotted against total daily dose of the drug, there was no correlation ($r = 0.15$). Thus, pyridostigmine appears to compete for oral absorption with neostigmine. Patient 15 was receiving the lowest daily dose of pyridostigmine (180mg) and in whom neostigmine was detected in the plasma (Fig 6.4, Table 6.2). These observations suggest that if there is a correlation between clinical response and plasma level of anticholinesterase drug in myasthenia gravis the concurrent administration of oral neostigmine and pyridostigmine may not benefit the patients. A combination of intramuscular and oral routes of these two quaternary amines may be more advantageous. However, further studies should be carried out to substantiate the present observations of interaction between neostigmine and pyridostigmine and to elucidate the mechanism of absorption of these drugs in the gut.

PART TWO

The relationship of plasma levels of pyridostigmine to clinical effect in patients with myasthenia gravis.

ACKNOWLEDGEMENT:

The clinical studies of global assessment of Muscle Power and fatigueability in myasthenic patients and the determination of antibodies in these patients plasma were carried out by Dr. Susan C. Davison in The Radcliffe Infirmary, Oxford.

INTRODUCTION

Pyridostigmine bromide (mestinon) is one of the main anticholinesterase drugs used in the treatment of myasthenia gravis (Drachman, 1978). Very little is known of the effective plasma concentration of the drug in myasthenic patients, although the daily dosage requirement may vary from 30 to 2000 mg per day (Greene 1969). There was a possible relationship between plasma pyridostigmine levels and clinical response in myasthenia gravis (Cohen et al., 1976). Recently, Chan and Calvey (1978) showed that in four patients with typical electromyographic decrement in the adductor pollicis, the plasma pyridostigmine levels were directly related to the effect on neuromuscular transmission.

The present study was undertaken to examine the relation between plasma levels of pyridostigmine and a global clinical evaluation of muscle power and fatigueability such as could be carried out during out-patient consultation. The discovery of a positive correlation between a global assessment of muscle power and plasma levels of pyridostigmine would be a valuable aid in optimising dosage schedules in individual patients.

PATIENTS AND METHODS

Two male and seven female patients between the ages of 19 and 58 years with histories of myasthenia gravis of two to twenty-two years duration were studied; five of them had had thymectomies and three were being treated with prednisolone on alternate days. The total daily dose of

pyridostigmine ranged from 60 to 1080 mg (Table 6.3). A diagnosis of myasthenia gravis was made when two of the following criteria were fulfilled:-

(a) A characteristic electromyographic response (Greene 1969).

(b) A positive edrophonium test (Greene , 1969), and

(c) An excess of auto antibody directed against human muscle acetylcholine receptors (Greene , 1969).

None of the subjects was severely disabled by the disease and informed consent was obtained.

Patients were admitted to hospital for a period of twenty-four hours and maintained on their normal drug regime during that time. All subjects rehearsed a routine of timed exercises in order to test a variety of different muscle groups for susceptibility to fatigue before starting the experiment. Details of the routine are summarized in Table 6.4 together with the end points. An individual test took a maximum of twenty minutes to complete, being terminated after two minutes if no fatigue occurred. The test was performed immediately before each blood sample was taken. Between the hours of 08.00 and 20.00 (Appendix 6.5) venous blood samples (10ml) were taken at roughly two hours intervals, the sampling times occurring at one to four hours after an oral dose of pyridostigmine. Immediately after the blood sample was taken it was mixed with lithiumheparin in the blood tube and the plasma was separated by centrifugation and stored at -20°C before analysis. The concentration of

pyridostigmine was subsequently measured in duplicate by gas liquid chromatography (see Chapter VI, Part One). A small quantity of plasma set aside to measure the concentration of acetylcholine receptor antibody using acetylcholine receptor prepared from human calf muscle, labelled with ^{125}I-bungarotoxin as the antigen.

RESULTS

For each subject, each separate exercise testing a particular function was evaluated across all six trials in two ways; the trials were ranked 0 to 5 from worst to best performance and they were also rated on a continuous scale with best and worst set 10 and 0 respectively. The global assessment for each trial was simply expressed as the average rank or scale score calculated from the eight different tests. These were then plotted against plasma concentration of pyridostigmine in ng/ml (Fig. 6.6 and 6.7).

Regression analysis demonstrated (using a computer program, Appendix 6.6) a trend towards a positive correlation between a global assessment of muscle powers and plasma concentration in five of the nine subjects (Fig 6.6), but in only two of them was showed significance at $p<0.05$ level (Patients 8 and 16). Of the remaining four, one showed a negative correlation at $p<0.1$. The findings are summarized in Table 6.5. No single test of function, e.g., vital capacity, correlated more closely with plasma drug level than the global assessment.

Diurnal variation in plasma levels of pyridostigmine and global scores for muscle strength were observed in most subjects,

TABLE 6.3

Case history of myasthenic patients in the study.

SUBJECT	SEX	AGE YEAR	DURATION OF DISEASE YEARS	HISTOLOGY OF THYMUS	TOTAL DAILY DOSE OF PYRIDOSTIGMINE mg	AUTO ANTIBODY TO MOTOR ENDPLATE Mol/L b
1[p]	F	19	2	Hyperplasia[a]	60	685×10^{-10}
2[p]	F	34	7	-	360	770×10^{-10}
3	F	50	22	-	180	4690×10^{-10}
4	F	38	3	Hyperplasia[a]	300	316×10^{-10}
5	F	58	7	Thymoma[a]	540	4370×10^{-10}
6[c]	F	58	13	-	360	40×10^{-10}
7	F	31	7	Hyperplasia[a]	48o	1645×10^{-10}
8	M	19	3	-	240	10×10^{-10}
16[pd]	M	33	11	Hyperplasia[a]	1080	2620×10^{-10}

p alternate day prednisolone
a thymectomy
b excess of antibody over normal control
c concurrent treatment with amitriptyline
d concurrent treatment with neostigmine.

TABLE 6.4

THE ROUTINE OF TIMES EXERCISES AND MEASURES USED TO ASSESS SUSCEPTIBILITY TO MUSCLE FATIGUE

INSTRUCTION	END POINT
Look straight ahead and fix on an object at eye level	Widest part of the palpebral fissure measured using a transparent rule calibrated in millimeters.
While facing forward, fix eyes a point above your head.	Diploplia or loss of fixation.
Hold arms outstretched, horizontally from the shoulders.	When arms drop below the horizontal.
Grip with each hand in turn the inflated sphigmomanometer cuff as tightly as possible.	When the pressure registered on the manometer drops to one half the maximum pressure.
Vital Capacity	Best of three tries.
While lying supine raise each leg in turn and hold it at 45^{o} to the horizontal for as long as possible.	When the heel touches the bed.
While lying supine, raise the head off the bed and hold it there unsupported for as long as possible.	When the occiput touches the bed.
Walk a distance of 75 yards, as fast as possible.	Crossing the finishing line.

TABLE 6.5

The correlation of plasma levels of pyridostigmine with performance in the test routine scored both by ranking and by using a scale.

SUBJECT	PLASMA CONCENTRATION ng/ml		CORRELATION COEFFICIENTS		SIGNIFICANCE
	MINIMUM	MAXIMUM	(Rank Score) r	(Scale Score) r	P, for scale score
1	1.3	12.4	0.76	0.80	0.025>P
2	9.0	36.0	0.35	0.34	0.35 >P
3	5.0	14.0	0.27	0.24	0.354>P
4	11.0	40.0	0.35	0.41	0.25 >P
5	20.5	64.5	0.64	0.61	0.1 >P
6	25.0	40.0	-0.78	-0.62	0.1 >P
7	24.0	39.0	0.54	0.58	0.15 >P
8	6.2	23.0	0.91	0.72	0.05 >P
16	11	36.5	0.40	0.55	0.05 >P

TABLE 6.6

The diurnal variation in plasma concentration of pyridostigmine and performance in the test routine, the average of the three measures before and after noon are compared.

SUBJECT	BEFORE NOON		AFTER NOON	
	PLASMA LEVEL	SCALE SCORE	PLASMA LEVEL	SCALE SCORE
1	10.7ng/ml	6.7	1.5ng/ml	3.9
2	21	5.8	28	3.3
3	12	6.8	7	6.0
4	33.7	5.3	19.7	3.3
5	44.6	5.7	37.5	4.4
6	28.2	5.7	31.8	4.4
7	33.2	5.3	29.7	5.1
8	17.5	5.2	31.2	6.5
16	20	5.5	16.6	3.4

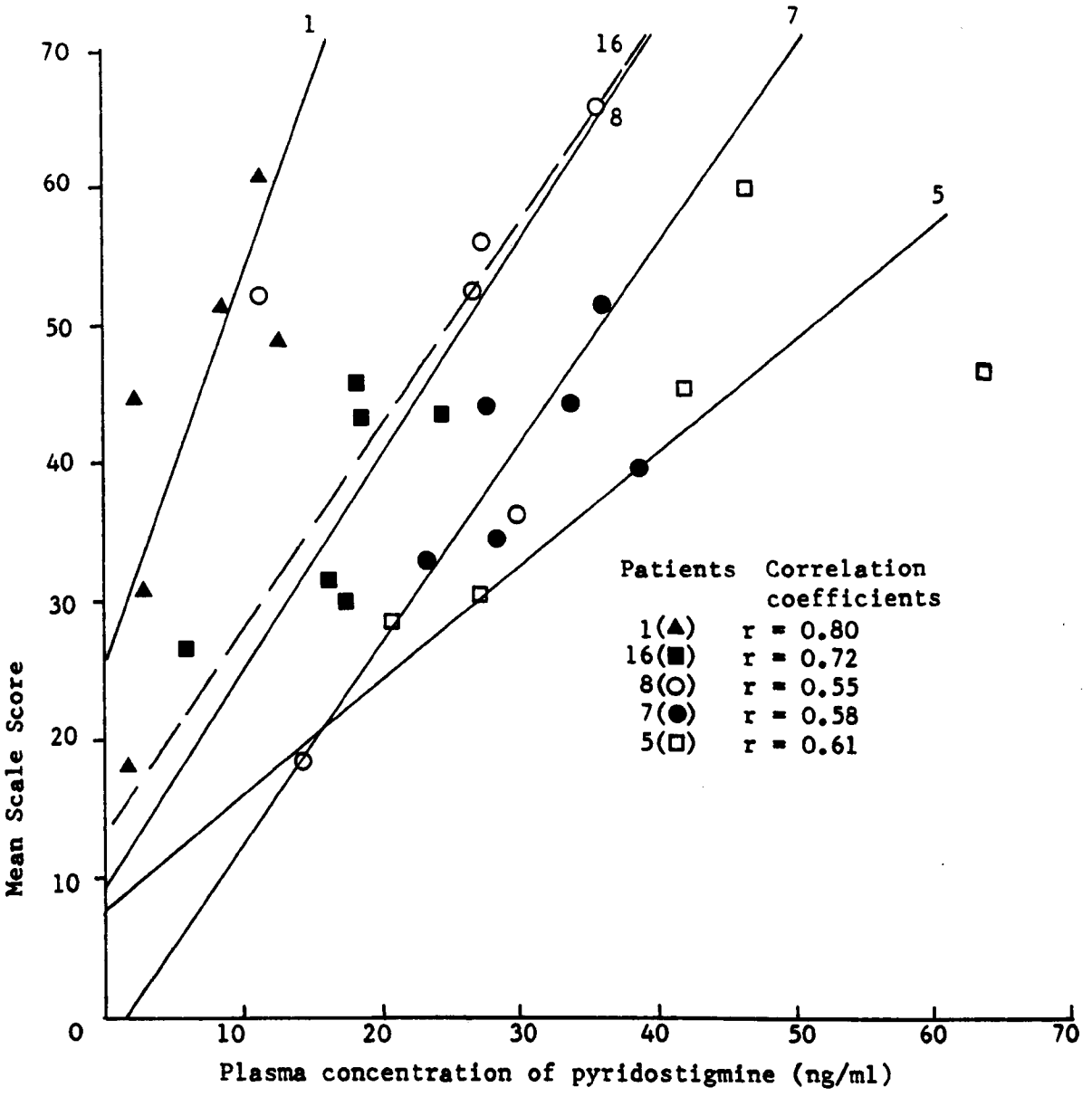


FIG 6.6

Five patients showing trend towards more actual positive correlation between plasma levels of pyridostigmine and assessment of muscle strength.

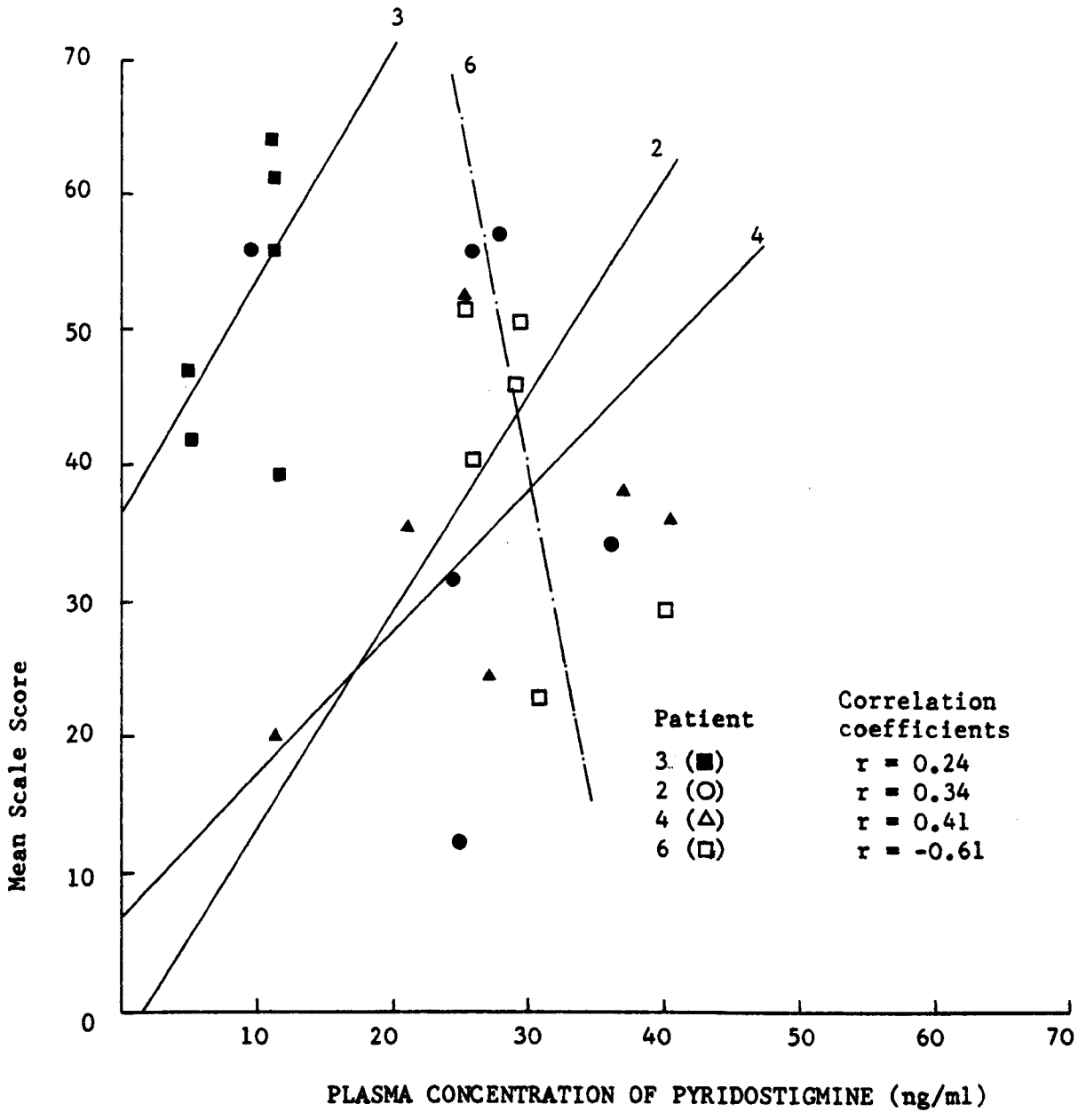


FIG 6.7

Four patients showing low or negative correlation between plasma levels of pyridostigmine and global assessment of muscle strength.

both muscle strength and drug level being higher in the morning than in the afternoon (Table 6.6).

DISCUSSION

Studies of plasma levels of anti-ChE agents in myasthenia gravis are required since alteration of doses of oral anti-ChE drugs depend largely on the patient's own subjective response. The edrophonium test (Green, 1969) to determine optimal dosage is frequently difficult to interpret because the effect varies at different times in the same day and on other occasions it may improve function of some muscles, but either does not alter or worsen the strength in others. It might, therefore, be predicted that similar difficulties would be encountered with the interpretation of serum levels when correlated to clinical signs. In addition, the pharmacokinetics of pyridostigmine are poorly understood and there is no good reason to predict that such a correlation should exist. Nevertheless, some previous studies suggest that a relationship between serum level and response may exist. In one such trial (Cohen, et al., 1976), nine myasthenic patients were examined at thirty minute intervals for six hours after drug administration, and improved motor strength subjectively was apparently parallelled by serum pyridostigmine concentrations, but the authors felt that the clinical response was not quantifiable. In another study (Calvey et al., 1977) wide variations in the oral dosage (60 to 660mg/day), nevertheless produced a relatively narrow plasma pyridostigmine range (20 to 60mg/ml). In an earlier study (Chan and Calvey, 1977), four patients with

unsatisfactory control had lower serum levels than those who were well controlled. The strength of these patients improved when the serum pyridostigmine increased and they demonstrated that the original low levels were due to malabsorption.
A further type of correlation was made on four patients with typical electromyographic decrements in adductor pollicis consistent with myasthenia gravis (Chan and Calvey, 1977). There was a positive correlation between the concentration of pyridostigmine plasma and the effect of the drug on neuromuscular transmission. In the present series of nine patients, a wide variety of exercises each testing particular functions was thought important, due to possible variations in the strength of different muscle groups in the same patient.
In addition, the clinical responses were quantified satisfactorily. In only two subjects was there a significant correlation between muscle power and plasma concentration; three other patients showed apparent positive correlation. No single test of motor function correlated more closely with plasma drug level than the global assessment. In addition, the presence or absence of a possible correlation between muscle power and plasma concentration was not apparently related to the duration of the disease, addition prednisolone therapy or thymectomy.

CHAPTER VII

FINAL DISCUSSION

DETERMINATION OF ANTI-ChE DRUGS AND METABOLITES IN BIOLOGICAL FLUIDS

Quaternary amines in general, are strong bases which have pK_a values approaching fourteen and are completely ionized in all biological fluids. Absorption from the gastrointestinal tract is incomplete and even neglible for certain quaternary ammonium compounds such as d-tubocurarine, pancuronium and related neuromuscular blocking agents.

Distribution in the body is usually restricted to the extracellular fluids as they traverse cell membranes with difficulty (Golstein 1974) and elimination is mainly by excretion of the unchanged drug in the urine or in the bile and also by metabolism in the liver (Dal Santo 1969). Thus, the chemical and distribution-elimination characteristics of quaternary amines will influence their extraction procedure from biological fluids.

In general the measurement of drugs in plasma or urine samples after therapeutic doses involves a preliminary extraction of the drug into a suitable organic solvent. A subsequent concentration of the organic extract and the final measurement of the drug in the concentrate by a sensitive and selective instrumental technique.

Most previously published methods, based on spectrophotometric detection, radio isotopic techniques and enzymatic procedures were not sensitive enough or selective and these investigations are expensive or time-consuming. Analysis based on gas-liquid chromatographic techniques have been used for the quantitation of pyridostigmine and neostigmine (Chan 1976). In the development of the method, treatment of all glassware with silylating agent was found to be essential to increase

recoveries of drug to 95% and 94% (at 60ng/ml level) for neostigmine and pyridostigmine respectively (see Chapter III).

The ion-pair extraction of neostigmine, pyridostigmine and their metabolites was proved to be specific using TLC and GLC methods. The extraction of parent drugs has been achieved at pH = 11, and the limit of quantitation is 1ng/ml using 10%OV17 on chromosorb W-AW. The extraction of the metabolites of neostigmine and pyridostigmine has been done using potassium tri-iodide as counter-ions at pH=4, and the quantitation is possible down to 50 ng/ml in urine using 3ml samples.

The synthesis of a series of pyridostigmine analogues and the development of one (N,N-dipropylcarbomoyloxy-1-methyl pyridinium bromide) for use as a common internal standard for assaying neostigmine and pyridostigmine in plasma simultaneously, has also been achieved (Chapter III). The assay involved a preliminary ion-pair extraction of the drugs and the internal marker from plasma using potassium-iodide glycine buffer. The assay procedure allows the simultaneous monitoring of the plasma levels of neostigmine and pyridostigmine when both of these anti-ChE agents are used for pharmacotherapy of myasthenia gravis. The evidence of intersubject variations in the absorption of neostigmine (Aquilonius, 1979) and pyridostigmine (Calvey and Chan, 1977) suggests that the simultaneous monitoring of both of the Anti-ChE agents in plasma should be important, as there is a lack of information concerning the effect of combined treatment with neostigmine and pyridostigmine in myasthenia gravis. The methods developed in the present study

represented a significant improvement over those systems previously available.

KINETICS OF IV PYRIDOSTIGMINE IN SURGICAL PATIENTS

Neostigmine methylsulphate (prostigmin) is one of the Anti-ChE drugs which is widely used to reverse non-depolarising neuromuscular blocks in anaesthetic practice. In the study of IV pyridostigmine (200 nmol/kg) compared with the kinetics of approximately equimolar doses of neostigmine (i.e., 206 - 308 n mol/kg, Calvey et al., 1979), it is clear that there are two important differences in the kinetics of the quaternary amines; firstly, the volume of distribution of pyridostigmine (530.845 $\pm$ 62.0 mean $\pm$ SE) is greater than neostigmine (122.21 $\pm$ 40.62, mean $\pm$ SE) reflecting its more extensive distribution and enhanced tissue uptake. Secondly, the clearance of pyridostigmine (16.25 $\pm$ 1.5, mean $\pm$ SE) is at least five times greater than neostigmine (3.15 $\pm$ 0.80 mean $\pm$ SE); this difference presumably related to the differential renal elimination of the two quaternary amines. In consequence, the elimination half-lives of pyridostigmine (22.79 $\pm$ 2.09, mean $\pm$ SE) and neostigmine (25.46 $\pm$ 2.74, mean $\pm$ SE) were similar. However, when these drugs are used in anaesthetic practice to reverse non-depolarizing neuromuscular block, the dose of pyridostigmine is normally 4 - 5 times greater than neostigmine, perhaps due to differences in the affinity of their quaternary groups for the anionic site on acetylcholinesterase.

The slow disposition half-life of pyridostigmine was progressively prolonged (from 22.79 $\pm$ 2.09 min. to 46.41 $\pm$

6.5 min) as the dose of the drug was raised from 36.2 μg/kg to 144.8 μg/kg. The increase in the terminal half-life of pyridostigmine was also observed in crossover studies in which different doses of the drug were administered to the same patients (see Chapter IV) on separate occasions. In addition, the total body clearance was significantly decreased (to 8.75 ± 1.5 ml/min/kg) although the other kinetic parameters were not significantly affected.

KINETICS OF INTRAMUSCULAR NEOSTIGMINE

Neostigmine was rapidly absorbed and eliminated after IM administration, maximal levels were reached in plasma at 30 minutes but only small or undetectable amounts were present after 120 minutes (see Chapter V). The kinetic parameters obtained are based entirely on experimental data during this period. When the results were interpreted in terms of a 1-compartment model, the elimination $t_{\frac{1}{2}}$ ranged from 56.9 to 100.1 minutes and V_d from 35.2 to 63.0L., and the clearance from 399 to 623 ml/min. In this study the neostigmine $t_{\frac{1}{2}}$ (77.7 ± 6.4 mean ± SE) was 2-3 times as long as the slow disposition $t_{\frac{1}{2}}$ (25.45 ± 6.7 mean ± SE) after intravenous injection (Calvey 1979), and the volume of distribution reported in the IV study was at least three times that after intramuscular injection in the present study.

The volume of distribution of neostigmine was approximately equal to total body water. Distribution studies in experimental animals suggests that the drug is localized in extracellular fluid (Somani, 1975). The results of IM study are

consistent with this interpretation. Total body clearance of the drug was greater than glomerular filtration rate, and suggests tubular secretion or extensive metabolism (see also Chapter VI). Studies on the elimination of radioactivity in urine after intramuscular ^{14}C-neostigmine (Somani, et al., 1980) confirmed that the drug is partially metabolized in man. The proportion of the dose eliminated as unchanged neostigmine and its metabolites increased from approximately 44% at one hour to 82% at 24 hours. Neostigmine was eliminated predominantly during the first two hours only a further 4% of the dose was excreted as unchanged drug between two and twenty-four hours. Their results suggest that metabolism and biliary excretion of the drug may play significant roles in its elimination in man and that neostigmine may be preferable to pyridostigmine (or other cholinesterase inhibitors) when renal function is impaired.

ORAL NEOSTIGMINE AND PYRIDOSTIGMINE, AND THE CORRELATION OF CLINICAL RESPONSE TO PLASMA PYRIDOSTIGMINE IN MYASTHENIA GRAVIS.

When plasma levels of pyridostigmine were monitored in eight myasthenic patients who were stabilized on oral pyridostigmine bromide only, there was a widely different daily dosage regime (see Chapter VI). Although the concentration of pyridostigmine in plasma varied, there was a direct relationship between the AUC and total daily dose of the drug ($r = 0.95$). These observations are in direct contrast to that for one myasthenic patients whose bioavailability of the drug was increased six-fold by doubling the dose of pyridostigmine bromide (Calvey et al., 1977).

Perhaps there is also an intrasubject variation in the absorption of the drug. No such observation was noticed in the nine patients who were treated with both oral pyridostigmine and neostigmine ($r = -0.080$). Neostigmine was only detected in one of the nine patients (second group). It was suggested that there might be a drug-drug interaction between pyridostigmine and neostigmine during oral absorption, so the concurrent administration of oral pyridostigmine and neostigmine may not benefit the patients. A combination of IM and oral routes of these two quaternary amines may be more advantageous in the treatment of severe cases of myasthenia gravis.

The pharmacokinetics of pyridostigmine are not entirely understood and there is no good reason to predict that a relationship between serum levels and clinical responses should exist. Previous studies by Chan and Calvey (1977) indicate that in four patients with typical electromyographic decrement in the adductor pollicis, there was a positive correlation between the concentration of pyridostigmine in plasma and the effect on neuromuscular transmission. The plasma concentration of pyridostigmine required to restore transmission to normal varied over a five-fold range, reflecting the variable severity of the disease. In another myasthenic patient with purely ocular symptoms they reported that there was a significant correlation between the plasma concentration of the drug and the diameter of the palpebral fissure.

The present studies on plasma pyridostigmine concentration and global assessment of nine patients (Chapter VI) show

that there is a trend towards a positive correlation between global assessment of muscle power and plasma concentrations in five patients, of the remaining four patients, three showed correlation approaching significance at $p < 0.35$, while one showed a negative correlation (Chapter VI page 151). In these studies, a wide variety of exercises (each testing particular muscle functions) was used to account for the possible variations in strength of different muscle groups in the patients. Such global assessment, together with clinical improvement of the patients could be quantified more rationally than most of those reported previously. From the present findings it is probable that the differential dosage requirement of pyridostigmine in myasthenia gravis were related to both inter-subject variation in drug disposition and different severity of the disease in eight of the nine patients studied. However, it is not possible to explain the negative correlation in the patient (patient six) who was also treated with amitriptyline for depression.

Further work on the kinetics of neostigmine and pyridostigmine should be designed to elucidate whether the intersubject variation in plasma levels and the drugs is due to (a) a difference in the actual oral absorption of the Anti-ChE agents or (b) a difference in the elimination (i.e., metabolism or excretion) of the drugs. From a pharmacokinetic point of view, neostigmine may offer no better advantage over pyridostigmine in the long term pharmacotherapy of myasthenia gravis. This can be explained in terms of low bioavailability

(Chapter VI, Part I, page 130) and the shorter plasma half-life (Chapters IV and V) of neostigmine. These findings are also supported by other published work (Aquilonius et al., 1979; 1980). A combination therapy of neostigmine and pyridostigmine may not be of advantageous as proven by the findings in Chapter Six (Part I). However, further study on this interaction between these two Anti-ChE agents at the absorption phase should also be of interest.

APPENDICES

APPENDIX 4.1

PATIENT	SEX	AGE (YR)	WEIGHT (KG)	IV DOSE OF PYRIDOSTIGMINE BROMIDE (mg)	OTHER DRUGS CONCURRENTLY ADMINISTERED
1	F	22	64.42	3.362(36.2µg/kg)	
2	M	36	53.08	2.771 "	phenytoin
3	M	34	76.66	4.0 "	sodium (300
4	M	20	82.10	4.29 "	mg/day) and
5	F	41	79.83	4.17 "	phenobarbitone
6	M	45	84.37	3.10 "	(9mg/day)
7	F	16	59.42	3.84 "	
8	M	50	73.48	4.88 "	
9	M	64	93.44	2.60 "	
10	M	23	49.9	2.32 "	
11	M	34	80.74	8.429(72.4µg/kg)	
12	M	29	67.59	7.056 "	
13	F	23	63.96	6.677 "	
14	F	19	62.14	6.488 "	
15	F	59	70.08	7.316 "	
16	M	55	71.44	7.458 "	
17	M	23	49.9	10.41(144.8µg/kg)	
18	M	45	84.37	17.6 "	
19	M	20	58.06	12.123 "	
20	M	31	87.09	18.181 "	
21	M	60	75.30	15.723 "	
22	F	56	75.75	15.817 "	
23	F	26	57.15	11.93 "	

Intravenous injection (IV) doses of pyridostigmine bromide and other data in 23 myasthenic patients.

APPENDIX 4.2

BLOOD SAMPLING TIME (min)	PLASMA CONCENTRATION OF PYRIDOSTIGMINE (ng/ml) OF TEN PATIENTS										MEAN ± SE CONCENTRATION OF TEN PATIENTS
	1	2	3	4	5	6	7	8	9	10	
0	0	0	0	0	0	0	0	0	0	0	0 ± 0
2	189	150	227	237	325	146	206	132	360	146	221.8 ± 23.7
3	115	109	101	106	174	92	100	97	159	98	115.1 ± 8.8
4	49	54	49	53	101	77	65	80	94	83	70.5 ± 6.9
5	26	31	27	39	87	47	-	70	72	65	51.6 ± 7.1
7	14	25	23	31	61	40	43	59	53	40	36.3 ± 5.5
10	14	20	15	17	49	37	34	39	53	38	31.6 ± 4.4
15	13.9	19	-	9	38	26	24	35	45	32	26.9 ± 3.6
20	10.3	16	13	8	31	23	19	26	27	23	19.6 ± 2.4
30	-	14	12	7	23	15	10	19	19	17	15.1 ± 1.5
40	9	8	11	5	18	16	8	15	15	10	11.5 ± 1.3
50	6	-	6	-	-	10	6	10	12	8	8.3 ± 0.9
60	-	-	-	-	-	9	3	7	-	4	5.8 ± 3.9
70	-	-	-	-	-	6	-	6	-	-	6.0 ± 0.0
80	-	-	-	-	-	4	-	6	-	-	5.0 ± 0.9
90	-	-	-	-	-	-	-	3	-	-	3.0 ± 0.0
100											

Plasma concentration of pyridostigmine (ng/ml) in ten surgical patients following an IV dose of 36.2µg/kg (200n mol/kg) as the bromide salt, over a period of time (2 to 90 min.).

APPENDIX 4.3

BLOOD SAMPLING TIME (min.)	CONCENTRATION OF PYRIDOSTIGMINE (ng/ml) of SIX PATIENTS						MEAN ± SE CONCENTRATION OF SIX PATIENTS	
	11	12	13	14	15	16		
0	0	0	0	0	0	0	0.0 ±	0.0
2	221	196	213	448	102	482	227.16 ±	62.3
3	-	119	114	376	500	279	227.60 ±	74.5
4	30	114	88	180	389	130	155.2 ±	50.6
5	34	97	75	155	205	112	113.0 ±	24.6
7	15	47	52	113	103	72	67.0 ±	15.0
10	15	17	36	47	7.7	70	43.6 ±	10.6
15	ND *	9	19	35	35	56	30.8 ±	0.84
20	ND	6	14	30	21	34	21.0 ±	5.0
30	ND	ND	9	-	10	9	9.3 ±	0.33
40	ND	ND	ND	4.6	4.3	12	6.9 ±	2.5
50	ND	ND	ND	ND	ND	9	9.0 ±	0.0
60	ND	4	ND	ND	ND	ND	4.0 ±	0.0
80	ND	3.6	ND	ND	ND	3	3.3 ±	
100	ND	4	ND	ND	ND	ND	4.0 ±	0.29
120	-	ND	ND	ND	ND	ND	-	-
140	-	ND	ND	-	ND	1.5	1.5 ±	0.0
160		ND						

*ND non-detectable

Plasma concentration of pyridostigmine (ng/ml) in six surgical patients following an IV dose of 72.4µg (400 nmol/kg) as the bromide salt, over a period of time (2 to 140min.).

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APPENDIX 4.4

BLOOD SAMPLING TIME (min.)	CONCENTRATION OF PYRIDOSTIGMINE (ng/ml)							MEAN ± SE CONCENTRATION OF SEVEN PATIENTS (ng/ml)
	17	18	19	20	21	22	23	
0	0	0	0	0	0	0	0	
2	743	683	859	31	259	1034	218	546.7 ± 376.1
3	235	326	276	-	177	369	-	276.6 ± 33.6
4	207	307	184	61	93	155	138	163.6 ± 30.5
5	179	273	178	62	71	116	-	146.5 ± 32.5
6	-	-	-	-	-	-	74	- - -
7	163	247	176	102	64	-	-	150.4 ± 31.5
8	-	-	-	-	-	-	40	- -
10	156	180	169	40	38	-	28	101.8 ± 29.9
12	-	-	-	-	-	-	15.5	- -
14	-	-	-	-	-	-	16	- -
15	-	139	146	34	29	21	-	73.8 ± 28.1
16	-	-	-	-	-	-	13.2	- -
18	-	-	-	-	-	-	12.5	- -
20	-	129	112	31	20	20	5.6	52.9 ± 21.7
30	84	-	88	-	-	2.3	-	58.1 ± 27.9
40	70	96	80	19	19	1.4	-	47.5 ± 15.9
60	47	83	70	10	11	ND	-	44.2 ± 13.5
80	-	77	63	-	1.5	ND	-	47.1 ± 23.0
100	35	51	52	3.2	1.3	ND	-	28.5 ± 11.1
120	-	44	38	ND	ND	ND	-	38.0 ± 1.6
140	-	38	-	-	-	ND	-	38 ± 0
160	-	37	-	-	-	ND	-	37 ± 0

Plasma concentration of pyridostigmine (ng/ml) in seven surgical patients following an IV dose of 144.8 µg/kg (800 nmol/kg) as the bromide salt, over a period of time (2 - 160mins.).

APPENDIX 4.5

36.2µg/kg (200 nmol/kg)

PATIENT	A	α	B	β
1	1355.30260502	-0.944996917	16.69443210	-0.01872773
2	955.30379702	-0.93786065	28.79343498	-0.02929981
3	2226.61023006	-1.13701322	23.02670883	-0.02380174
4	423.71959618	-0.51397795	18.41024429	-0.03390703
5	1102.20438655	-0.76440487	69.98901569	-0.03584905
6	1109.59149759	-0.98375852	51.57408111	-0.04681768
7	344.13689680	-0.60538857	41.37857085	-0.02726522
8	191.02627973	-0.38852012	48.39894485	-0.02971152
9	2074.7113419	-1.00370201	69.12596296	-0.03760846
10	249.01705324	-0.51343527	57.16288831	-0.04240521
MEAN	783.68778601	-0.75847727	38.24626591	-0.02761647

The constants A,B, α and β after an IV dose of 36.2µg/kg pyridostigmine bromide in ten surgical patients. The relation between the plasma level of the drug and time was expressed as a biexponential equation, using non-linear least-squares regression analysis (Appendix 4.11).

APPENDIX 4.6

72.4µg/kg (400 nmol/kg)

PATIENT	A	α	B	β
11	271.59072273	-0.15210356	28.59039765	-0.02356911
12	1001.25029035	-0.58995577	79.82283586	-0.04948122
13	1221.47011207	-0.45890156	11.76888473	-0.01217860
14	997.18416539	-0.45831706	65.95525678	-0.03867220
15	1135.67892945	-0.5159774	82.62542153	-0.04904937
16	793.46534621	-0.40728614	23.20797875	-0.01940935
MEAN	741.45710403	-0.36122138	30.86143323	-0.02595166

The constants A, B, α and β after an IV dose of 72.4 µg/kg of pyridostigmine bromide in six surgical patients. The relation between the plasma level of the drug and time was express as a biexponential equation, using non-linear least-squares regression analysis (Appendix 4.11).

APPENDIX 4.7

144.8 µg/kg (800n mol/kg)

PATIENT	A	α	B	β
17	44962.95924472	-2.18512759	181.02599474	-0.0189862
18	590.20000457	-0.27303476	150.29374238	- .00978790
19	19258.27158164	-1.68219653	168.84184758	-0.01348325
20	30712.42343735	-1.73100019	168.42750114	- .01648719
21	1759.60980943	-0.57259128	160.76348009	-0.00952932
22	4707.61671567	-0.93709249	102.84040331	-0.01686908
23	7401.22105981	-0.46058482	220.89231301	-0.04061196
MEAN	6780.93715310	-1.2875357	208.43052515	-0.01755862

The constants A, B, α and β following an IV dose of 144.8 µg/kg of pyridostigmine bromide in seven surgical patients. The relation between the plasma level of the drug and time was expressed as a biexponential equation, using non-linear least-squares regression analysis (Appendix 4.11).

APPENDIX 4.8

PATIENT	K_{10} (min^{-1})	$t_{\frac{1}{2}} \alpha$ (min)	$t_{\frac{1}{2}} \beta$ (min)	V_c (ml/kg)	C (ml/min/kg)	V_d (ml/kg)
1	0.592	0.73	37.01	26.385	15.616	833.852
2 *	0.492	0.74	23.66	35.348	17.711	604.463
3	0.769	0.61	29.12	16.091	12.337	519.834
4	0.323	1.35	20.44	81.876	26.474	781.796
5	0.345	0.91	19.34	30.882	10.665	297.501
6	0.521	0.70	14.81	31.176	16.237	347.809
7	0.185	1.14	25.42	93.900	17.353	636.454
8	0.113	1.78	23.33	151.195	17.070	574.536
9	0.549	0.69	18.43	16.886	9.270	246.485
10	0.167	1.35	16.35	118.231	19.749	465.718
MEAN ± SE	0.406 ± 0.067	1.00 ± 0.12	22.79 ± 2.09	60.197 ± 19.039	16.252 ± 1.550	530.845 ± 62.044

Kinetic parameters after IV administration of pyridostigmine bromide (36.2 µg/kg) in ten surgical patients.

* Patient 2, concurrently treated with phenytoin sodium(300mg/day) and phenobarbitone (90mg/day).

APPENDIX 4.9

PATIENT	K_{10} (min^{-1})	$t_{\frac{1}{2}} \alpha$ (min)	$t_{\frac{1}{2}} \beta$ (min)	V_c (ml/kg)	C (ml/min/kg)	V_d (ml/kg)
11	0.100	4.56	29.409	231.145	24.145	1024.414
12	0.327	1.17	14.01	66.883	21.871	442.001
13	0.340	1.51	56.92	58.691	19.955	1638.566
14	0.274	1.51	14.13	68.080	18.654	482.373
15	0.314	1.34	35.71	59.401	18.652	380.268
16	0.260	1.70	26.71	88.573	23.029	1186.480
Mean ±	0.269 ±	1.96 ±	27.83 ±	97.128 ±	21.234 ±	859.238 ±
SE	0.031	0.44	5.75	26.97	0.820	175.068

Kinetic parameters after IV administration of pyridostigmine (72.4µg/kg) in 6 surgical patients.

K_{10}	elimination rate constant	(using equation (30) Chapter II)
$t_{\frac{1}{2}} \alpha$	rapid disposition half-life	(" (24) ")
$T_{\frac{1}{2}} \beta$	Slow disposition half-life	(" (24) ")
V_c	Volume of central distribution	(" (27) ")
C	Total plasma clearance	(" (39) ")
V_d	Total apparent volume of distribution	(" (31) ")

APPENDIX 4.10

PATIENT	K_{10} (min^{-1})	$t_{\frac{1}{2}} \alpha$ (min)	$t_{\frac{1}{2}} \beta$ (min)	V_c (ml/kg)	C (ml/kg/min)	V_d (ml/kg)
17	1.497	0.32	36.66	3.208	4.803	253.988
18	0.042	2.54	70.82	196.808	8.266	844.533
19	0.810	0.41	51.41	7.458	6.041	448.017
20	1.105	0.40	42.04	4.686	5.179	314.132
21	0.096	1.21	72.74	75.636	7.261	761.914
22	0.433	0.74	41.09	30.354	13.022	771.918
23	0.191	1.50	17.07	89.38	17.073	420.387
MEAN ± SE	0.572 ± 0.184	1.01 ± 0.30	46.41 ± 6.5	58.218 ± 26.6	8.744 ± 1.499	536.057 ± 79.691

Kinetic parameters after IV administration of pyridostigmine bromide(144.8 µg/kg) in seven surgical patients.

APPENDIX 4.11 /Continued....

```
17/52/53            04/01/83           COMPILED BY XALT MK. 5B
STATEMENT
      0                 'LIST' (LP)
      0                 'SEND TO'(ED,ICLA-DEFAULT,.AXXX)
      0                 'WORK' (ED,ICLF-DEFAULT)
      0            'PROGRAM' (VSAK)
      0             'INPUT' 0,3=CR0
      0             'OUTPUT' 0,2=LP0
      0            'BEGIN'
      1            'INTEGER' S,L,Z,D;
      1            'REAL''ARRAY'LNPLASMA,PLASMA,TIME[1:100];
      2            L:=READ; S:=0;
      5            HER: D:=READ;
      6            'FOR' Z:=1'STEP'1'UNTIL'D'DO' 'BEGIN'
      7            PLASMA[Z]:=READ;
      9            TIME[Z]:=READ;
     10            LNPLASMA[Z]:=LN(PLASMA[Z]);
     11            'END';
     12            'BEGIN'
     12            'REAL' B, YZERO, XBAR, A, A1, B1, X1BAR, Y1ZERO, SUMSQDEV, MIN, MA,
     12            MAL, MB, MBB;
     12            'INTEGER' I, M, N;
     13            'REAL''ARRAY'LNCPA,LNCPB,CPB,CPA,LNCPCALC,CPCALC,SQDEV[1:100];
     14            'PROCEDURE'REGRESSION(M,N,P,T,A,B,XBAR,YZERO,I);
     16            'VALUE' M,N;
     17            'REAL'A,B,YZERO,XBAR;
     18            'INTEGER' M,N,I;
     19            'ARRAY' P,T;
     20            'BEGIN'
     20            'REAL' X,XSQ,Y,YSQ,XY,BEE;
     20            'INTEGER' Q;
     21            Q:=0; X:=XSQ:=Y:=YSQ:=XY:=0;
     24            'FOR'I:=M'STEP'1'UNTIL'N'DO'
     25            'BEGIN'
     25            Y:=Y+P[I]; 'IF'P[I]=0'THEN''GOTO'GAB;
     28            X:=X+T[I]; XSQ:=XSQ+T[I]+2;YSQ:=YSQ+P[I]+2;
     31            XY:=XY+(T[I]*P[I]); Q:=Q+1;
     33            GAB:'END';
     34            'IF'Q'LE'1'THEN''GOTO'OUT;
     35            A:=Y/Q;XBAR:=X/Q;B:=(XY-(X*Y/Q))/(XSQ-((X)+2/Q));
     38            BEE:=A-(B*X/Q);
     39             YZERO:=EXP(BEE);
     40            'GO TO' IN;
     41            OUT:
     41            YZERO:=B:=0;
     42            IN:
     42            'END';
     43            WRITETEXT('('C'('P3C')' B' EXPONENTIAL*LEAST*SQUARE*FIT'('2C')'')');
     44            WRITETEXT('('C'('C2S')' A'('21S')'ALPHA'('25S')'B'('21S')'BETA'('19S')
     44            LOG*SUM*OF*SQUARES'('2C')'')');
     45            N:=2;M:=1;
     47            ONE:NEWLINE(1);REGRESSION(M,N,LNPLASMA,TIME,A,B,XBAR,YZERO,I);
     49            'FOR'Z:=N+1'STEP'1'UNTIL'D'DO'
     50            'BEGIN'
     50            CPB[Z]:=YZERO*EXP(-ABS(B)*TIME[Z]);
     52            CPA[Z]:=PLASMA[Z]-CPB[Z];
     53            'IF'CPA[Z]<0'THEN''GOTO'TWO;
     54            LNCPA[Z]:=LN(CPA[Z]);
     55            'GOTO'SIX;
```

APPENDIX 4.11 /Continued....

```
TWO:LNCPA[Z]:=0;
SIX:'END';
REGRESSION(N+1,D,LNCPA,TIME,A1,B1,X1BAR,Y1ZERO,I);
SUMSQDEV:=0;
'FOR'Z:=1'STEP'1'UNTIL'N'DO'
'BEGIN'
CPCALC[Z]:=Y1ZERO*EXP(-ABS(B1)*TIME[Z])+YZERO*EXP(-ABS
(B)*TIME[Z]);
LNCPCALC[Z]:=LN(CPCALC[Z]);
SQDEV[Z]:=(LNCPCALC[Z]-LNPLASMA[Z])+2;
SUMSQDEV:=SUMSQDEV+SQDEV[Z];
'END';
'IF'N=2'THEN'
'BEGIN'
MIN:=SUMSQDEV;MA:=Y1ZERO;MAL:=B1;MB:=YZERO;MBE:=B;
'END';
'IF'SUMSQDEV<MIN'THEN'
'BEGIN'
MIN:=SUMSQDEV;MA:=Y1ZERO;MAL:=B1;MB:=YZERO;MBE:=B;
'END';
SPACE(5);PRINT(Y1ZERO,8,8);SPACE(5);PRINT(B1,2,8);
SPACE(5);PRINT(YZERO,8,8);SPACE(5);PRINT(B,2,8);
SPACE(5);PRINT(SUMSQDEV,8,8);
N:=N+1;
'IF'N=D-1'THEN''GOTO'KELVIN'ELSE''GOTO'ONE;
KELVIN:
NEWLINE(1);
WRITETEXT('('('('6C')'MINIMUM*FIT*FOR*BIEXPONENTIAL'('2C')')')');
SPACE(5);PRINT(MA,8,8);SPACE(5);PRINT(MAL,2,8);SPACE(5);
PRINT(MB,8,8);SPACE(5);PRINT(MBE,2,8);SPACE(5);PRINT(MIN,8,8);
NEWLINE(1);
'END';
'BEGIN'
'REAL'B,YZERO,XBAR,A,A1,B1,X1BAR,Y1ZERO,SUMSQDEV,A2,B2,X2BAR,Y2ZERO
,MA,MAL,MB,MBE,MC,MGA,MIN;
'INTEGER'YE,CANT,EN,I, M,N;
'REAL''ARRAY'LNCPA,LNCPB,CPB,CPA,CPCALC,CPC,CPT,LNCPCALC,
LNCPT,SQDEV[1:100];
'PROCEDURE'REGRESSION(M,N,P,T,A,B,XBAR,YZERO,I);
'VALUE'M,N;
'REAL'A,B,YZERO,XBAR;
'INTEGER'M,N,I;
'ARRAY'P,T;
'BEGIN'
'REAL'X,XSQ,Y,YSQ,XY,BEE;
'INTEGER'Q;
Q:=0;X:=XSQ:=Y:=YSQ:=XY:=0;
'FOR'I:=M'STEP'1'UNTIL'N'DO'
'BEGIN'
Y:=Y+P[I]; 'IF'P[I]=0'THEN''GOTO'BAG;
X:=X+T[I]; XSQ:=XSQ+T[I]+2; YSQ:=YSQ+P[I]+2;
XY:=XY+(T[I]*P[I]);Q:=Q+1;
BAG:'END';
'IF'Q'LE'1'THEN''GOTO'OOUT;
A:=Y/Q;XBAR:=X/Q;B:=(XY-(X*Y/Q))/(XSQ-((X)+2/Q));
BEE:=A-(B*X/Q);YZERO:=EXP(BEE);'GOTO'INN;
OOUT:YZERO:=B:=0;
INN:
'END':
WRITETEXT('('('P3C')'TRI*EXPONENTIAL*LEAST*SQUARE*FIT'('3C12S')'
```

APPENDIX 4.11 /Continued....

```
A1('('ASI')'ALPHA'('155')'B'('135')'BETA'('165')'C'('155')'GAMA'('145')'
SUM*OF*SQUARES'('20')'')');
N:=2;M:=1;EN:=3;XE:=4;CANT:=0;
DONE:
NEWLINE(1);REGRESSION(M,N,LNPLASMA,TIME,A,B,XBAR,YZERO,I);
'FOR'Z:=N+1'STEP'1'UNTIL'D'DO'
'BEGIN'CPB[Z]:=YZERO*EXP(-ABS(B)*TIME[Z]);
CPA[Z]:=PLASMA[Z]-CPB[Z];
'IF'CPA[Z]'LE'0'THEN''GOTO'THREE;
LNCPA[Z]:=LN(CPA[Z]);
'GOTO'SWIM;
THREE:LNCPA[Z]:=0;
SWIM:'END';
SING:REGRESSION(EN,XE,LNCPA,TIME,A1,B1,X1BAR,Y1ZERO,I);
'FOR'Z:=XE+1'STEP'1'UNTIL'D'DO'
'BEGIN'
CPC[Z]:=Y1ZERO*EXP(-ABS(B1)*TIME[Z]);
CPT[Z]:=CPA[Z]-CPC[Z];
'IF'CPT[Z]'LE'0'THEN''GOTO'TTWO;
LNCPT[Z]:=LN(CPT[Z]);
'GOTO'SIX;
TTWO:LNCPT[Z]:=0;
SIX:'END';
REGRESSION(XE+1,D,LNCPT,TIME,A2,B2,X2BAR,Y2ZERO,I);
SUMSQDEV:=0;
'FOR'Z:=1'STEP'1'UNTIL'D'DO'
'BEGIN'
CPCALC[Z]:=Y2ZERO*EXP(-ABS(B2)*TIME[Z])+Y1ZERO*EXP(-ABS(B1)
*TIME[Z])+YZERO*EXP(-ABS(B)*TIME[Z]);
LNCPCALC[Z]:=LN(CPCALC[Z]);
SQDEV[Z]:=(LNCPCALC[Z]-LNPLASMA[Z])**2;
SUMSQDEV:=SUMSQDEV+SQDEV[Z];
'END';
'IF'CANT>0'THEN''GOTO'GEOFF;
'BEGIN'MIN:=SUMSQDEV;MA:=Y2ZERO;MAL:=B2;MB:=Y1ZERO;MBE:=B1;MC:=YZERO;
MGA:=B;
'END';
'IF'CANT=0'THEN''GOTO'L1;
GEOFF:
'IF'SUMSQDEV<MIN'THEN'
'BEGIN'
MIN:=SUMSQDEV;MA:=Y2ZERO;MAL:=B2;MB:=Y1ZERO;MBE:=B1;MC:=YZERO;MGA:=B;
'END';
L1:CANT:=CANT+1;
PRINT(Y2ZERO,4,6);PRINT(B2,2,6);PRINT(Y1ZERO,4,6);
PRINT(B1,2,6);PRINT(YZERO,4,6);PRINT(B,2,6);PRINT(SUMSQDEV,8,8);
NEWLINE(1);
XE:=XE+1;
'IF'XE=D-1'THEN''GOTO'SUN'ELSE''GOTO'SING;
SUN:
N:=N+1;EN:=EN+1;XE:=EN+1;
'IF'N=D-3'THEN''GOTO'SYLVIA'ELSE''GOTO'DONE;
SYLVIA:
NEWLINE(1);
WRITETEXT('('('6C')'MINIMUM*FIT*TO*TRIEXPONENTIAL'('12C')'')');
PRINT(MA,4,6);PRINT(MAL,2,6);PRINT(MB,4,6);PRINT(MBE,2,6);
PRINT(MC,4,6);PRINT(MGA,2,6);PRINT(MIN,8,8);
NEWLINE(1);
'END';
S:=S+1;
```

APPENDIX 4.11

Digital computer programe for bi-tri-exponential analysis of the plasma profile of IV pyridostigmine.

```
        ??1          'IF'S=L'THEN''GOTO'GRB;
        ???          'GOTO'HER;
        ??3          GRB;
        ??3          'END'
SEGMENT VSAK        .     LENGTH  1716
NO OF BUCKETS USED      36
COMPILED    #VSAK              EC


DOCUMENT *VSAKRA      , NORM(*VSAKRA      ; LP00 ON 04/01/80 AT 11.53


CONSOLIDATED BY XPCK 12X     DATE    04/01/80   TIME 11/53/25

PROGRAM VSAK
EXTENDED DATA (22AM)
COMPACT PROGRAM (DBM)
CORE                 8064

                  CLV      ALGOL60
                  SEG      WRITETEXT
                  SEG      EXP
                  SEG      PRINT
                  SEG      ABS
                  SEG      NEWLINE
                  SEG      LN
                  SEG      READ
```

APPENDIX 6.1

The times pyridostigmine (pyr.) and other drugs were taken.

PATIENT NUMBER		
1	Pyr. 60mg	08.00
	prednisolone, 40mg	11.00
	Slow potassium 600mg	08.00
	600mg	18.00
2	Pyr. 60mg	07.00, 11.00, 15.00, 19.00, 22.30
	Prednisolone 25mg	alternate days.
3	Pyr. 60mg	07.30, noon, 17.00
4.	Pyr. 120mg	06.00
	60mg	10.00, 15.30, 18.00
5	Pyr. 120mg	06.00, 18.00, 22.00
	60mg	10.00, noon, 15.00
6.	Pyr. 120mg	06.00, 10.15
	60mg	14.00, 18.00
7.	Pyr. 120mg	07.50, noon, 17.00, 22.00
8.	Pyr. 60mg	07.00, noon, 17.00, 22.00

APPENDIX 6.2

The times pyridostigmine (pyr.), neostigmine (Neo.), and other drugs were taken.

PATIENT			
9	Pyr.	60mg	10.00, 14.00, 18.00, 22.00
	Neo.	30mg	07.00, 10.00, 14.00, 18.00, 22.00
10	Pyr.	60mg	06.30, 12.30, 15.30, 18.30, 23.00
	Neo.	30mg	06.30, 09.30, 15.30
	Neo.	15mg	12.30, 18.30, 21.00
11	Pyr.	120mg	06.00, noon
		60mg	18.00, 21.00
	Neo.	45mg	06.00
	Neo	30mg	09.00, noon, 15.15, 18.00
	Neo.	15mg	21.00
12	Pyr.	60mg	07.00, 10.00, noon, 18.00, 22.00
	Neo.	15mg	0700, noon, 16.00, 22.00
13	Pyr.	360mg	(total daily dose)
	Neo.	30mg	three hourly
14	Pyr.	360mg	(total dose)
	Neo.	30mg	three hourly
15	Pyr	60mg	07.30, 15.00, 22.00
	Neo.	30mg	07.30, 10.30, 14.00, 16.00, 19.00, 22.00
16	Pyr.	120mg	0700, 09.00, 11.00, 13.00, 15.00, 17.00 19.00, 21.00, 23.00
	Neo.	15mg	07.00
	Prednisolone	20mg	alternate days
17	Pyr.	90mg	06.30, 09.00, 11.30, 14.00, 15.45, 17.45 19.45, 21.30, 22.30
	Neo.	60mg	06.30, 11.30, 14.00, 15.45, 17.45, 19.45, 21.30.

APPENDIX 6.3

Daily dose of Anti-ChE agents and other drugs used for treatment of 17 myasthenic patients.

PATIENT	AGE YEARS	SEX	DURATION OF DISEASE DISEASE (YR)	HISTORY OF THYMECTOMY	ORAL DOSE OF PYRIDOSTIGMINE Br.(mg day^{-1})	ANTI-ChE DRUGS NEOSTIGMINE Br.(mg day^{-1})	CONCURRENT DRUGS ADMINISTERED
1	19	F	2	hyperplasia	60	-	prednisolone 40mg. Slow K 600mg b.d.
2	34	F	7	-	360	-	prednisolone 7mg a.d.
3	50	F	22	-	180	-	-
4	31	F	3	hyperplasia	300	-	-
5	58	F	7	thymoma	540	-	-
6	58	F	13	-	360	-	amitriptyline 25mg nocte
7	31	F	7	hyperplasia	480	-	-
8	19	M	3	-	240	-	-
9	24	F	5	hyperplasia	240	150	-
10	29	M	1	-	300	120	-
11	36	F	8	-	360	180	-
12	30	F	10	-	360	60	-
13	68	M	12	thymectomy	240	60	-
14	34	F	6	-	360	90	prednisolone 10 o.d. azathiaprine 125mg o.d.
15	29	F	10	hyperplasia	180	180	-
16	33	M	11	-	1080	15	prednisolone 20mg a.d.
17	36	F	13	thymectomy	810	480	-

APPENDIX 6.4

Blood sample times and concentration of pyridostigmine and neostigmine, throughout the waking day (8.00am to 8.00pm). Numbers in brackets indicate the patient's number.

(1)

TIME (hr)	PYR. (ng/ml)
16.15	1.3
18.00	2.2
20.00	2.1
09.00	12.4
10.00	11.3
12.00	8.3

(2)

TIME (hr)	PYR. (ng/ml)
16.00	24
18.00	36
20.00	24
08.00	28
10.00	9
12.00	28

(3)

TIME (hr)	PYR. (ng/ml)
16.00	5.0
18.00	11.0
20.00	5.0
09.00	14.0
10.00	11.0
12.00	11.0

(4)

TIME (hr)	PYR. (ng/ml)
16.00	21.0
18.00	27.0
20.00	11.0
08.00	36.0
10.00	40.0
12.00	25.0

(5)

TIME (hr)	PYR. (ng/ml)
16.00	27.5
18.00	20.5
20.00	64.5
08.00	46.0
10.00	46.0
12.00	42.0

(6)

TIME (hr)	PYR. (ng/ml)
16.00	40
18.00	25
20.00	30.5
08.00	26.5
09.00	29.0
11.00	29.0

APPENDIX 6.4 /Continued....

(7)

TIME (hr)	PYR. (ng/ml)
16.00	24.0
18.00	28.5
20.00	36.5
08.00	39.0
10.00	27.5
12.00	33.0

(8)

TIME (hr)	PYR. (ng/ml)
16.00	6.2
18.00	17.2
20.00	16.5
08.00	18.6
10.00	18.5
20.00	23.0

(9)

TIME (hr)	PYR. (ng/ml)	NEO. (ng/ml)
16.10	55	-
18.00	43	-
20.00	54	-
08.00	14	-
10.00	12.3	-
12.00	61.5	-

(10)

TIME (hr)	PYR. (ng/ml)	NEO. (ng/ml)
16.15	5	-
18.00	11	-
20.00	5	-
09.00	14	-
10.30	11	-
12.00	11	-

(11)

TIME (hr)	PYR. (ng/ml)	NEO. (ng/ml)
18.00	8.0	-
20.00	8.0	-
08.00	?	-
20.00	7	-
12.00	5.4	-
14.00	15.3	-

(12)

TIME (hr)	PYR. (ng/ml)	NEO. (ng/ml)
15.15	66	-
17.15	144	-
19.00	59	-
08.40	69	-
10.10	67.5	-
12.00	50	-

(13)

TIME (hr)	PYR. (ng/ml)	NEO. (ng/ml)
16.00	-	-
18.00	16.2	-
20.00	11.4	-
08.00	5.5	-
10.00	60.0	-
12.00	35.0	-

(14)

TIME (hr)	PYR. (ng/ml)	NEO. (ng/ml)
16.00	23	-
18.00	26.5	-
20.00	9.3	-
08.00	13.4	-
10.00	13.10	-
12.00	14.0	-

APPENDIX 6.4 /Continued....

(15)

TIME (hr)	PYR. (ng/ml)	NEO. (ng/ml)
15.15	2.7	12.7
17.15	12.2	10
19.00	9.4	33
08.40	18.6	13
10.05	9.5	4.7
12.00	4.5	5.5

(16)

TIME (hr)	PYR. (ng/ml)	NEO. (ng/ml)
16.00	27	-
18.00	36.5	-
20.00	30.0	-
08.00	14.0	-
10.00	17.0	-
11.50	27.4	-

(17)

TIME (hr)	PYR. (ng/ml)	NEO. (ng/ml)
15.50	5.3	-
18.00	5.0	-
20.00	22	-
08.00	28	-
10.00	28.5	-
11.50	23.5	-

APPENDIX 6.5

Plasma concentration of pyridostigmine and performance in the test routine.

PATIENT	TIME (hr)	PLASMA PYR. (ng/ml)	RANK SCORE	SCALE SCORE
1	16.15	1.3	7.5	18.4
	18.00	2.2	20.5	44.6
	20.00	2.1	14.0	31.2
	09.00	12.4	21.0	48.8
	10.00	11.3	31.0	61.2
	12.00	8.3	26	51.2
MEAN ± SD		6.28 ± 5.021	20 ± 8.37	42.57 ± 15.36
2	16.00	24	19.5	31.9
	18.00	36	18.5	34.1
	20.00	24	7.5	12.5
	08.00	28	30.0	57.9
	10.00	9	13.0	25.5
	12.00	28	31.0	55.5
MEAN ± SD		25.5±8.80	20 ± 9.186	36.23 ± 17.56
3	16.00	5.0	14.5	41.4
	18.00	11.0	23.0	56.1
	20.00	5.0	17.0	47.8
	09.00	14.0	13.0	38.9
	10.00	11.0	28.0	64.3
	12.00	11.0	24.0	61.1
MEAN ± SD		9.5 ± 3.67	20 ± 6.09	51.6 ± 10.5
4	16.00	21.0	21.0	35.24
	18.00	27.0	14.5	24.73
	20.00	11.0	14.0	19.85
	08.00	36.0	20.5	38.5
	10.00	40.0	21.5	35.76
	12.00	25.0	28.5	52.76
MEAN ± SD		26.67 ± 10.45	20 ± 5.33	34.48 ± 11.50

APPENDIX 6.5/Continued....

PATIENT	TIME (hr)	PLASMA PYR. (ng/ml)	RANK SCORE	SCALE SCORE
5	16.00	27.5	14.5	39.9
	18.00	20.5	15.0	28.6
	20.00	64.5	23.0	40.8
	08.00	46.0	16.5	31.4
	10.00	46.0	28.0	59.7
	12.00	42.0	23.0	45.3
MEAN ± SD		41.08 ±15.53	20 ± 5.47	40.45 ± 12.23
6	16.00	40	14.0	29.7
	18.00	25	27.0	52.5
	20.00	30.5	14.0	23.8
	08.00	26.5	21.5	40.3
	09.00	29.0	20.5	46.4
	11.00	29.0	23.0	51.2
MEAN ± SD		30 ± 5.28	20 ± 5.15	40.65 ± 11.74
7	16.00	24	14.5	32.5
	18.00	28.5	16.5	34.4
	20.00	26.5	25	56.2
	08.00	39	18.5	39.7
	10.00	27.5	21.5	43.9
	12.00	33	24	43.4
MEAN ± SD		31.41 ± 5.74	20 ± 4.19	41.68 ± 8.5
8	16.00	6.2	14.5	26.7
	18.00	17.2	17.5	29.2
	20.00	16.5	19.0	30.3
	08.00	18.6	21.0	43.7
	10.00	18.5	22.0	45.5
	20.00	23.0	25.5	43.4
MEAN ± SD		16.67± 5.6	20 ± 3.87	36.47 ± 8.58
16	16.00	27.0	20	52.5
	18.00	36.5	26.5	66.5
	20.00	30.0	15.0	37.3
	08.00	14.0	8.5	17.8
	10.00	11.0	23.5	51.9
	11.00	27.4	26.5	55.6
MEAN ± SD		24.32±9.81	20 ± 7.13	46.93 ± 17.06

APPENDIX 6.6

Digital computer program for regression analysis between global assessment of muscle power and plasma concentration of pyridostigmine, after oral administration.

```
20/19/35        15/04/80        COMPILED BY XALT MK.  5A
STATEMENT
        C          'LIST' (LP)
        C          'SEND_TO'(ED,TCLA=DEFAULT,.AXXX)
        C          'WORK' (ED,ICIF=DEFAULT)
        C       'PROGRAM' (VSAK)
        C       'INPUT' 0,3=CR0
        C       'OUTPUT' 0,2=LP0
        C       'TRACE' 2
        C       'BEGIN' 'REAL' 'ARRAY' D[1:50];
        1        'INTEGER' I,N,P,Q; 'REAL' S1,S2,VAR,SD,SE;
        3        WRITE TEXT('('4C14S')'SUM'('11S')'MEAN'('11S')'VARIANCE'('11S')'SD
        4        '('11S')'SE'('C')'')');
        5       P:=READ; 'FOR' Q:=1 'STEP' 1 'UNTIL' P 'DO'
        7        'BEGIN' N:=READ;
        9        'FOR' I:=1 'STEP' 1 'UNTIL' N 'DO' D[I]:=READ;
       11        S1:=0; S2:=0; 'FOR' I:=1 'STEP' 1 'UNTIL' N 'DO'
       14        'BEGIN' S1:=S1+D[I]; S2:=S2+D[I]↑2; 'END';
       18        VAR:=(S2-(S1↑2/          N))/(N-1);
       16        SD:=SQRT(VAR); SE:=SD/SQRT(N);
       21        NEWLINE(5); SPACE(5); PRINT(S1,4,4); PRINT(S1/N,          4,4);
       25        PRINT(VAR,4,8); PRINT(SD,4,8); PRINT(SE,5,8); NEWLINE(1);
       29        'END'; 'END'
SEGMENT VSAK          LENGTH   239
NO OF BUCKETS USED     10
COMPILED   #VSAK
```

APPENDIX 6.6 /Continued....

```
DOCUMENT *VSAKRA     , NORM[*VSAKRA     ; LPOO ON 15/04/80 AT 20.19

CONSOLIDATED BY XPCK 12K    DATE   15/04/80   TIME 20/19/49

PROGRAM VSAK
EXTENDED DATA (22AM)
COMPACT PROGRAM (DBM)
CORE              6848

                  CIV     ALGOL60
                  SEG     WRITETEXT
                  SEG     PRINT
                  SEG     NEWLINE
                  SEG     SORT
                  SEG     READ
```

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