MICRO-ANALYSIS OF NEOSTIGMINE BROMIDE FROM PLASMA IN SURGICAL CONCENTRATIONS

by

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ABSTRACT

Neostigmine, a potent anticholinesterase agent, has been empirically used (1.0-5.0 mg total dose) in surgery for the past forty years to competitively inhibit the transmitter blockade produced by Curare type compounds. The metabolism and excretion kinetics of neostigmine in humans can only be postulated from radioactive disposition studies on rats, dogs, and chickens. Current analytical techniques did not provide the necessary sensitivity to detect even the maximum suspected plasma concentration of 0.5 μg/ml after surgical dosing. A gas-liquid chromatographic determination from plasma was developed based upon the quantitative demethylation of neostigmine's quaternary amine by lithium n-propyl mercaptide in hexamethylphosphoramide. Neostigmine's detectable limit with the new analytical procedure was 0.1 μg/ml of plasma. A tenfold extension to neostigmine's sensitivity is possible by using ten-milliliter aliquots of plasma. The new analytical procedure proves to be a valuable tool for further experimental work on neostigmine. The procedure's applicability does not limit it to neostigmine alone; with minor modifications this analysis could be used for most quaternary amine salts. In addition, modifications can be made to also accommodate urine samples and tissue extracts.

INTRODUCTION

Uses and Mode of Action

Neostigmine (Figure 1) is a potent anticholinesterase agent and has been used in surgery and clinical therapeutics for the past forty years (Osserman, 1958; Beckman, 1961). Neostigmine is a substituted phenyl ester of an alkyl carbamic acid and was introduced in 1931. Therapeutically, neostigmine is used as an active antagonist to non-depolarizing type myoneural blockades, as a stimulant to peristalsis and micturation in patients with paralytic ileus, as a depressant of cardiac rate in certain arrhythmias, and as an antagonist in acute congestive glaucoma by reducing the intraocular pressure around the trabecular space (Goodman and Gilman, 1970).

The use of curare type agents depends upon their binding to cholinoceptive sites at the postjunctional membrane to produce a blockade at the motor endplate and thereby blocking the transmitter action of acetylcholine and any further transmission of nerve impulses to muscle fibers (Goodman and Gilman, 1970). This transmitter blockade produces a skeletal muscle relaxation and provides a good surgical field during an operation. This neuromuscular blockade must be reversed at the conclusion of surgery. Neostigmine competitively inhibits the curare type action and restores neuromuscular transmission by its inhibition of acetylcholinesterase (Randall and Lehmann, 1950).

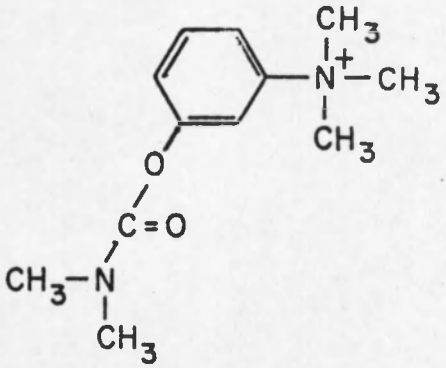

Figure 1. Structure of Neostigmine (3-dimethylcarbamoyloxyphenyl) trimethyl ammonium salt.

While curare agents competitively block acetylcholine receptors, neostigmine inhibits the destruction of acetylcholine by inhibiting the acetylcholinesterase enzyme. The consequent increase of available acetylcholine displaces the curare agent at the motor endplate by competitive inhibition (Aeschlimann and Reinert, 1931; Blaber, 1963). Additional effects include a direct excitatory action of neostigmine on the motor endplate region (DiPalma, 1971), and propagation of a repetitive discharge by axon reflex into the remaining fibers of a muscle unit. The displaced curare is removed from the vicinity of the neuromuscular junction by the normal processes of redistribution and finally eliminated from the body.

Distribution, Metabolism, and Excretion Kinetics

The onset of action is governed by the rate at which neostigmine and acetylcholine concentrations increase at the endplate region of a

neuron (McIntyre, 1964). Following a single intravenous injection, neostigmine is distributed throughout the plasma and extravascular fluid. Therefore, the levels of neostigmine in the plasma may not reflect its true activity since its concentration at the neuron probably determines its binding to the enzyme.

The blood concentration of neostigmine depends on (1) route of administration, usually oral or intravenous, (2) the amount bound to acetylcholinesterase sites, (3) the amount bound to plasma cholinesterase sites, (4) destruction by hydrolysis at the sites of actions, (5) liver metabolism, and (6) excretion rate.

At present, distribution studies of neostigmine have not been attempted in humans. Distribution studies have been completed on rats, hens, and dogs but only through the use of radioactive carbon-14 labeled neostigmine. Thirty minutes after an intramuscular injection in rats the highest radioactivity detected was in the liver and intestinal contents, much lower amounts detected in the heart, kidney, and lung, and little or no activity in skeletal muscle and brain (Husain et al. (1969). Roberts, Thomas and Wilson (1965a), Figure 2, also noted that the concentration in the liver of rats rapidly reaches its peak in thirty minutes (x) and slowly decreases to a negligible level in twenty-four hours, whereas the peak activity in blood occurs ten minutes (o) after an intramuscular injection and rapidly declines within thirty minutes.

Christensen, Broen, and Helleberg (1974) reported that following an intravenous injection of carbon-14 neostigmine in rats the radioactivity rapidly declined in the plasma during the first two hours.

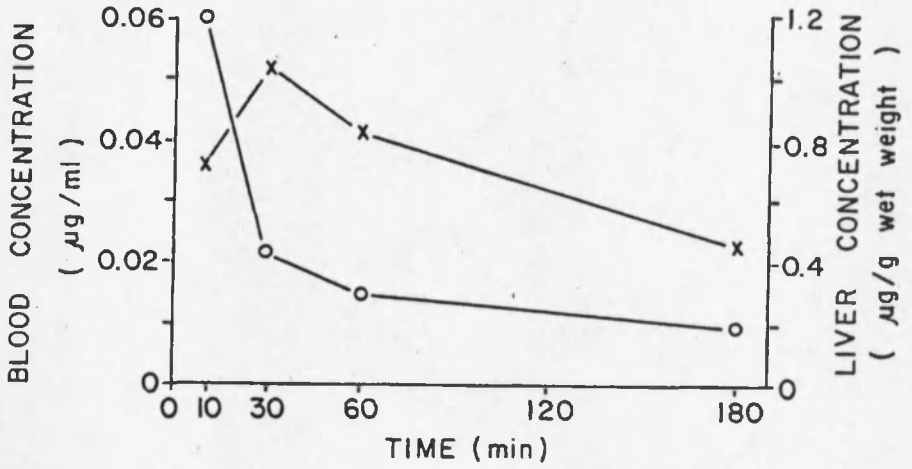

Figure 2. Mean concentration of radioactivity in rat blood (o) and liver (x) after intramuscular injection of carbon-14 labeled neostigmine (25 µg). (Roberts et al., 1965a).

The radioactivity remaining represented only one percent of the original dose in the plasma. This in vivo study established that the concentration of carbon-14 neostigmine in the femoral quadriceps, the sternomastoid, and the intercostal muscles to be 1/3 to 1/2 the plasma concentration, in contrast to the diaphragm which contained twice the plasma concentration after the two-hour period. Christensen's in vitro study confirmed the uptake of neostigmine by the diaphragm and indicated a possible membrane transport system. The uptake was partially energy-dependent and saturable, which suggested a carrier-mediated transport system.

Metabolism studies using carbon-14 labeled neostigmine have been carried out in animals by Roberts, Thomas and Wilson (1965b) and Husain et al. (1969). Neostigmine metabolism by plasma and tissue

cholinesterase are minimal when compared with that found in the liver (Nowell and Scott, 1962). When the urine from the rats given repeated doses of neostigmine was investigated by paper chromatography, the presence of five metabolites was indicated (Somani et al., 1970). The separation of neostigmine from its metabolites was complicated by the fact that the parent drug and several of its metabolites have the same net charge on the quaternary nitrogen. Three of the five metabolites were identified but the other two (M4 and M5) were detected but not identified. Somani et al. (1970) proposed that neostigmine was first oxidatively metabolized to 3-hydroxyphenyltrimethyl-ammonium ion (3-OH PTMA), which in turn forms a glucuronide. The primary metabolite, 3-OH PTMA, also undergoes N-demethylation to 3-hydroxyphenyldimethyl-ammonium ion (3-OH PDMA) which is probably also conjugated with glucuronic acid (M4), Figure 3, (Somani et al. 1970).

Distribution studies by Roberts et al. (1965a, 1965b), Calvey (1966), and Husain et al. (1969) emphasized the importance of the liver in the clearance of neostigmine. The most prominent excretion products other than neostigmine are 3-OH PTMA and its glucuronic acid conjugate. Roberts et al. (1965a) discovered an initial rapid excretion of neostigmine during the first hour when approximately 43% of the intramuscular injection was excreted unchanged. Further experimentation concluded that neostigmine was being secreted by the renal tubules and this mechanism could be inhibited by Cyanine 863, which blocks the tubular transport mechanism of quaternary nitrogen type compounds.

After the first hour very little unchanged neostigmine is excreted; thereafter increasing amounts of free 3-OH PTMA and its

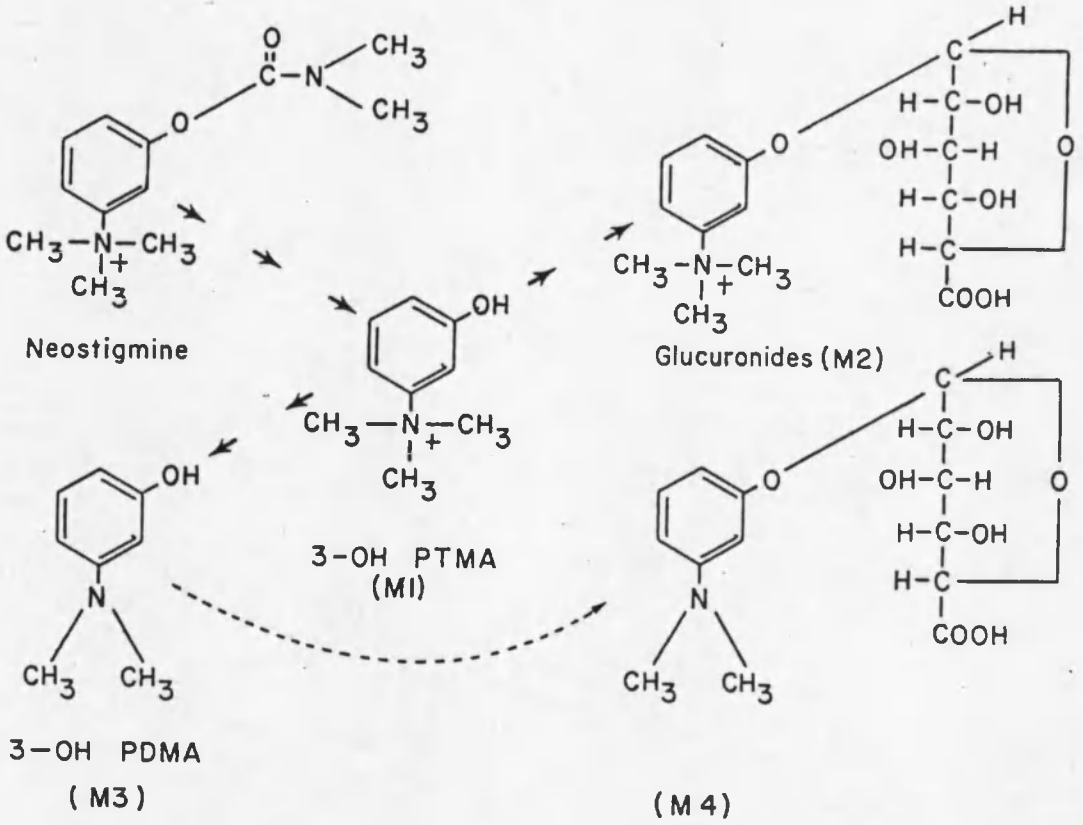

Figure 3. Metabolism products of neostigmine.

glucuronide conjugate are excreted (Figure 4). The delay in glucuronide formation and excretion are related to the sequence of events involved in the metabolic conversion of neostigmine to 3-OH PTMA. MacFarlane et al. (1950) have demonstrated that 3-OH PTMA, like neostigmine, is effective in alleviating the muscular weakness of a myasthenic patient but is of shorter duration of action than neostigmine.

The major biliary excretion products are unchanged neostigmine and 3-OH PTMA. Both Calvey (1966) and Roberts et al. (1965a) reported that only 6% of the administered dose is excreted in the bile as neostigmine and its metabolite and therefore biliary secretion plays only a minor role in the elimination process of neostigmine.

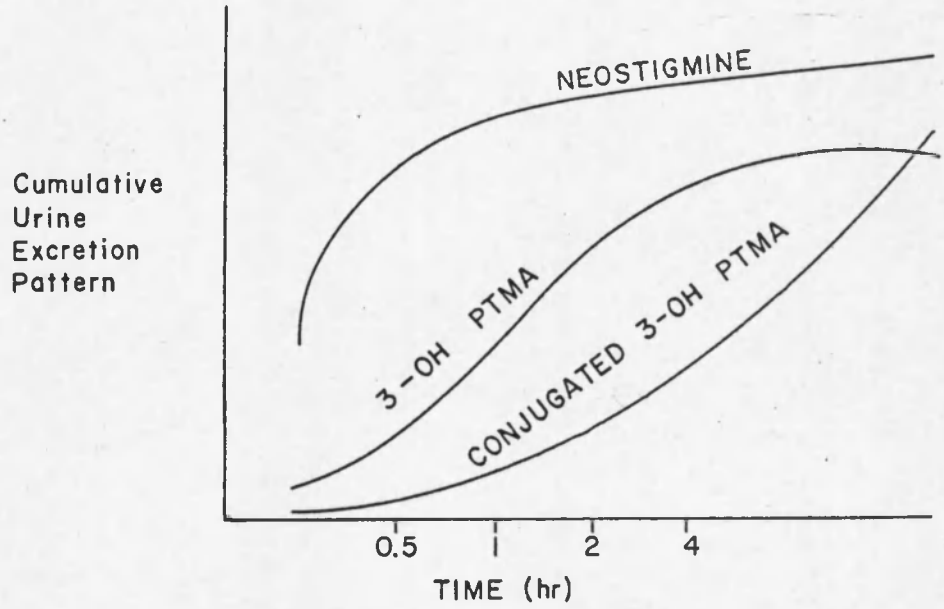

Figure 4. Excretion of neostigmine, 3-OH PTMA, and conjugated 3-OH PTMA in rat urine after intramuscular injection of carbon-14 labeled neostigmine (Husain et al. 1969).

It is often assumed that drugs are eliminated by first order kinetics where the biological half life and composition of excretion products are independent of dose. Deviations from the apparent first order drug elimination kinetics can be detected by changes in fractional composition of excretion products as the dose is changed (Levy, 1968). Gibaldi and Feldman (1972) found that after rapid administration of neostigmine in rats by intravenous injection via the jugular vein, there was no change in fractional composition of excretion products with dose, but over the same dose range changes in fractional composition of excretion products with dose were observed following portal vein administration (which is equivalent to the route used for therapy of myasthenia gravis or intestinal problems- oral route). Thus, Gibaldi and Feldman (1972) suggested a dose-dependent elimination kinetics.

The fraction of the dose eliminated as neostigmine was greatly reduced and the metabolite fraction increased after portal vein administration when compared to jugular vein administration of the same dose. Thus, administration by portal vein was subject to extensive metabolism during the first pass through the liver. This same "first pass phenomena" was also observed for 3-OH PTMA after portal vein administration; therefore 3-OH PTMA excretion is also dose dependent.

For drugs which are eliminated by first order kinetics after repeated dosing, the drugs attain a plateau level that is proportional to the dose administered, but when drugs are eliminated by nonlinear kinetics the magnitude of the plateau is not directly proportional to the dose and may change in a manner other than proportionately (Tsuchiya and Levy, 1972). The results indicate that neostigmine is eliminated by nonlinear mechanisms after portal vein administration (Barber and Bourne, 1974). Thus, administration by oral ingestion follows a dose-dependent relationship whereas parenteral administration of neostigmine follows first order elimination kinetics.

Current Analytical Methods for Neostigmine

The accepted dose range of neostigmine for surgical application is 1.0 - 5.0 milligrams. Bridenbaugh and Churchill-Davison (1968) stated that when a severe paresis had been achieved, a dose of 2.5 milligrams of neostigmine was insufficient to restore normal neuromuscular transmission. However, when this dose was increased to the range of 4.0 to 5.0 milligrams, full muscle activity was restored and there did not appear to be any justification for exceeding the total dose of 5.0 milligrams of neostigmine.

Current dosages have not deviated from those first administered. With the immediate distribution of neostigmine throughout the plasma and extravascular fluid, the maximum dosage of 5.0 milligrams would have a concentration in plasma of 0.5 μg/ml prior to any metabolic or excretory processes. This maximal blood level is lower than current analytical procedures' sensitivity limits.

In 1933 the first identification procedure by Amelink (1932) listed $PtCl_4$ + $HgCl_2$, $K_4Fe(CN)_6$, picric acid, HgI_2 + KI, and Bouchardat's reagent as giving crystal precipitates with neostigmine. In 1945 a collaborative study on tablets of neostigmine yielded a determination of neostigmine based on an analysis of its hydrolysis product after treatment with hot alkali. The $(CH_3)_2NH$ formed was distilled and collected in a measured amount of standard acid (McNall, 1945).

Mitchell and Clark (1952) developed an assay for quaternary ammonium compounds by adding the colored anion, bromphenol blue, to the cationic salt in the original biological media (serum or urine). The ion pair complex formed was quantitatively extracted into ethylene dichloride without any interference from unreacted dye still present in the biological media. The lower limit of sensitivity for neostigmine from the colorimetric analysis was 2-3 micrograms per sample from either serum or urine. This assay was used to analyze neostigmine and pyridostigmine in the urine of patients with myasthenia gravis (Nowell, Scott Wilson, 1962).

In 1970 Hoffman-LaRoche introduced another colorimetric determination for neostigmine. The yellow picric acid complex formed at pH 10.4 was selectively extracted into 1,2-dichloroethane and the

absorbance measured at 375 nanometers. The lower limit of sensitivity from blood is 2.5 µg/ml using four milliliters of serum and only 6 µg/ml using one milliliter of serum.

Kling (1971) was the first to introduce an ultraviolet spectrophotometric assay for neostigmine. The absorbance of neostigmine's alkaline hydrolysis product, 3-dimethylaminophenol, was measured in solutions used for intravenous injection. The method calls for an extraction of phenol and/or paraben preservatives with $CHCl_3$ from an acidified solution. The preservative free acid solution was made basic and then hydrolyzed on a steam bath. The resulting hydrolysis product was then analyzed by ultraviolet spectrophotometry. No mention was made of confirming the structure of the hydrolysis product or how sensitive the assay was for neostigmine. Injection samples and simulated injectable samples analyzed were in the concentration range of 0.2 to 1.0 mg/ml.

In 1971 Tsubouchi published a colorimetric analysis for neostigmine which used the indicator tetrabromophenolphthalein as the anionic counterpart forming an ion pair complex which was measured spectrophotometrically at 615 nanometers. Biological analyses were not attempted but the analysis was used to determine concentrations of neostigmine in injectables and tablets. The lowest concentration range tested was 0.2 to 0.6 mg/ml with no information as to the limit of sensitivity of the analysis.

Kling (1973, 1974b) developed another separation technique for removing preservatives in tablets and ophthalmic solutions. A chromatographic column containing Celite and a pH 5.8 buffer was prepared.

After an ether-chloroform wash to remove organic soluble contaminants and other drug excipients, an ion pair complex between the quaternary ammonium compound and bis (2-ethylhexyl) hydrogen phosphate (DEHP) in chloroform was formed. The ion pair complex was eluted from the column with 0.1N H_2SO_4 and the ion pair dissociated at this low pH. The solution was made basic and then hydrolyzed on a steam bath. The resulting hydrolysis product was analyzed by ultraviolet spectrophotometry at 293.5 nanometers. The tablets and ophthalmic solutions contained 15-50 milligrams of neostigmine; no biological studies were attempted. As previously stated, no confirmation of structure of the hydrolysis product had been done and it was suggested by Ehrlen (1946) that the alkaline hydrolysis product of neostigmine was the quaternary phenol, 3-trimethylaminophenol. Kling (1974a), using ultraviolet, infrared, and nuclear magnetic resonance spectra of the alkaline hydrolysis product of neostigmine, confirmed that the product was the m-hydroxyphenyl-trimethylammonium salt as mentioned in earlier literature.

Pyridostigmine and physostigmine (Figure 5), like neostigmine, are reversible anticholinesterase agents employed clinically.

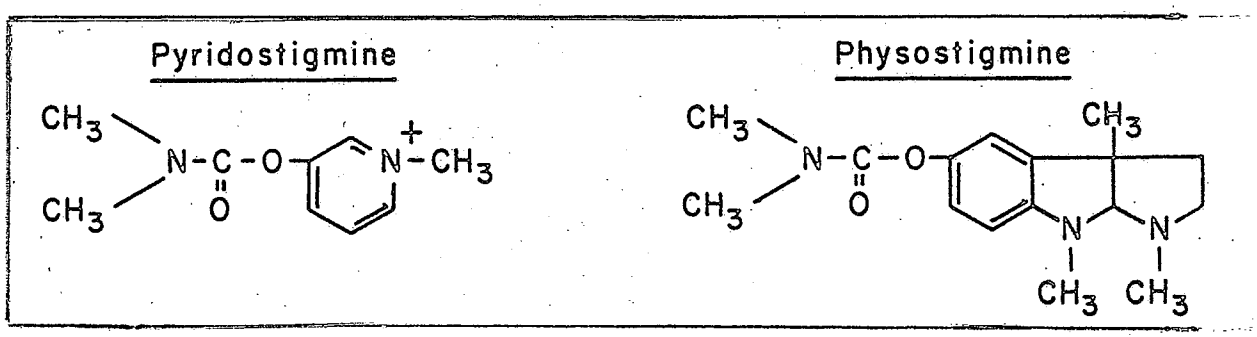

Figure 5. Structures of pyridostigmine and physostigmine.

Structurally, these compounds are similar to neostigmine due to their quaternary nitrogens and/or carbamic acid side chains. Analysis of these anticholinesterase compounds, like neostigmine, are based on the same functional groups.

Coper, Deyhle and Dross (1974) reported a spectrophotometric method for the determination of pyridostigmine in plasma. Pyridostigmine was transferred to an organic solvent from plasma as an ion pair with the aid of hexanitrodiphenylamine (dipicrylamine). The ion pair was dissociated and a new iodine complex formed after the addition of KI_3. This complex was extracted with dichloromethane, and determined spectrophotometrically at 293, 329, and 365 nanometers. The lower limit for the determination of pyridostigmine was 0.3 µg/3ml plasma.

Teare and Taylor (1967) and Khalil and Patterson (1971) presented quantitative determinations for physostigmine in pharmaceutical preparations. Udenfriend et al. (1957) reported a spectrophotofluorometric study on organic compounds of pharmacological interest, which included physostigmine. The sensitivity attained for physostigmine was 0.04 µg/ml. Neostigmine was also examined but did not fluoresce in any media at concentrations up to ten micrograms per milliliter. Taylor (1967) reported two different fluorometric methods for the determination of physostigmine in tissue samples. The first method utilizes physostigmine's natural fluorescence and can detect 0.1 microgram per gram of tissue sample. The second method, a more sensitive fluorimetric technique for the estimation of physostigmine, involves condensation with ethylenediamine and subsequent extraction of the fluorescent product with isobutanol. This method can detect 0.002 µg/ml physostigmine.

In tissue samples, catecholamines will seriously interfere, which restricts the application of this reaction to pure solutions and tissue extracts containing little or no catecholamines.

Statement of Problem and Demethylation Reactions

The analytical procedures described in the preceding section were the only literature articles available for the determination of neostigmine. The lowest limit of sensitivity reached by these procedures was two to three micrograms per sample. Under ideal conditions with the maximum neostigmine dosage of five milligrams, the plasma concentration after distribution would be approximately 0.5 µg/ml and declines rapidly during the first thirty minutes as was described earlier (Figure 2). A further decrease in plasma concentration occurs due to liver metabolism, hydrolysis of neostigmine by plasma and tissue cholinesterases, and the excretion of these metabolic products by the kidney and intestines. Thus, the need arises for an analytical procedure applicable in biological studies for the determination of neostigmine in submicrogram quantities.

Previously mentioned distribution, metabolism, excretion, and kinetic studies of neostigmine have all been done using the rat, hen, and dog. Radioactive carbon-14 labeled neostigmine has to be used to analyze minute concentrations of the metabolites in urine and feces. The main metabolite, 3-OH PTMA, from therapeutic amounts of neostigmine was found in the urine of patients with myasthenia gravis. Correlation studies of distribution, metabolism, excretion, and kinetics in humans have not been attempted. Since nonradioactive procedures have not been

sensitive enough, other types of response tests have been used to estimate whether the muscle response has been completely restored. It is not known whether the correlation of mechanism of action, metabolism, and excretion in animals directly corresponds to those in related human studies; onset, adequacy of spontaneous respiration, grip strength, vital capacity, nerve stimulation, and twitch force are the major response tests used to determine pharmacodynamic properties in humans at the present time.

Inadequate analytical procedures for neostigmine prompted investigations into the possibility of using gas-liquid chromatography because of its extremely high boiling point and very low vapor pressure; even at the temperature limits of the column. Tertiary amines, which have much lower boiling points and appreciable vapor pressures, on the other hand, are easily analyzed by this technique. Therefore, demethylation of the quaternary ammonium salts to a tertiary amine was adopted as a feasible method to develop a viable quantitative assay. Enzymatically, the liver demethylates quaternary amines; organically, demethylation reactions with specificity have been known and reported as early as 1875 by Meyer. Meyer and Lecco (1875) pyrolyzed the chloride salt of the amine and produced the amine base and methylene chloride at temperatures exceeding 200° C. This demethylation method is not feasible for microanalysis techniques and could lead to extensive decomposition of samples.

House et al. (1963) demethylated quaternary ammonium salts utilizing the iodide ion from lithium iodide as the nucleophile and then

refluxed the mixture in decanol at 230° C. This procedure is designed for large quantities of the quaternary amines.

Adaptation of this method for a microanalysis to maintain quantitative results would be extremely difficult. Another demethylation procedure using large amounts of the quaternary salts reported by Grovenstein et al. (1966) reacted sodium metal with the salt in liquid ammonia; this reaction has a specificity for allyltrimethylammonium compounds.

Wilson and Joule (1968) reported a specific demethylation reaction using the acetate anion (Figure 6) as the nucleophile. Using an Amberlite I.R.A. 400 column an anion exchange is done on the quaternary amine with the acetate ion. Heating the newly formed quaternary salt causes the nucleophilic displacement of the methyl group, and the solvent is evaporated under reduced pressure at which time the highly hydroscopic crystalline methyl acetate forms. The process is irreversible under the conditions employed since the methyl acetate formed does not react with tertiary amines.

Analogs of trimethylaniline were the primary compounds tested with yields of tertiary amines exceeding 90% but the procedure dictated large concentrations of the quaternary salts. Predicted losses of micro-quantities would far exceed those being tested.

Cope et al. (1960) found that excess lithium aluminum hydride (Figure 7) could be used in a tetrahydrofuran solvent with a quaternary ammonium iodide salt such as neostigmine to give demethylation by an Sn2 displacement with the hydride ion nucleophile. Again, this procedure was discarded as the refluxing and steam distillation techniques

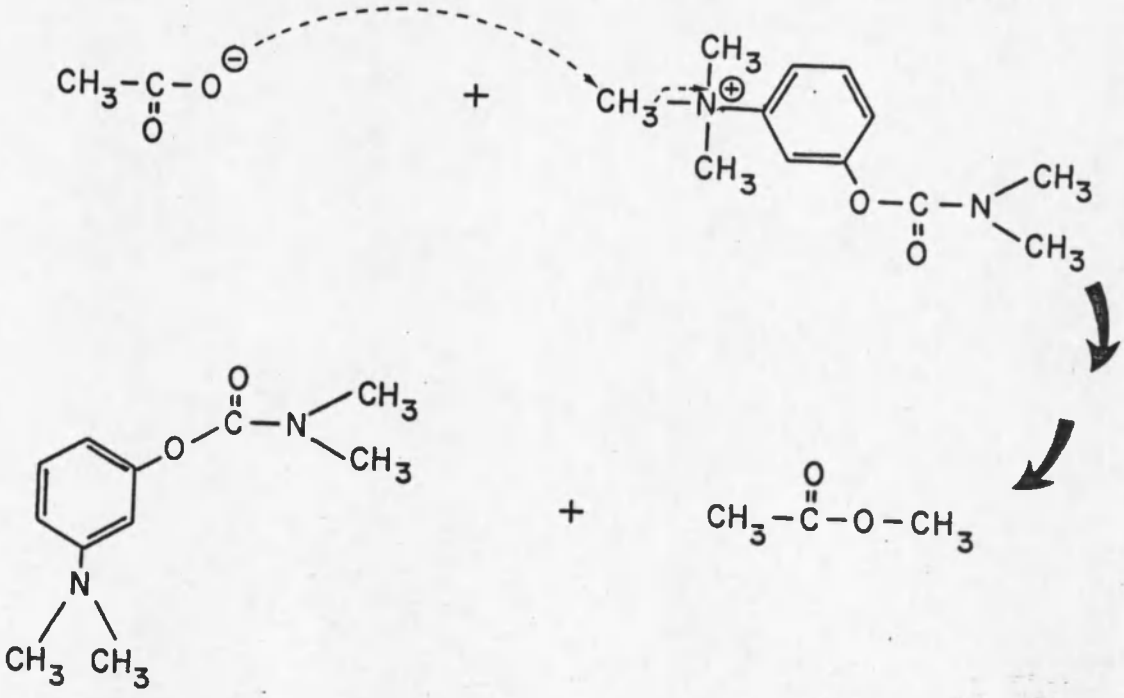

Figure 6. Acetate ion nucleophilic demethylation of neostigmine.

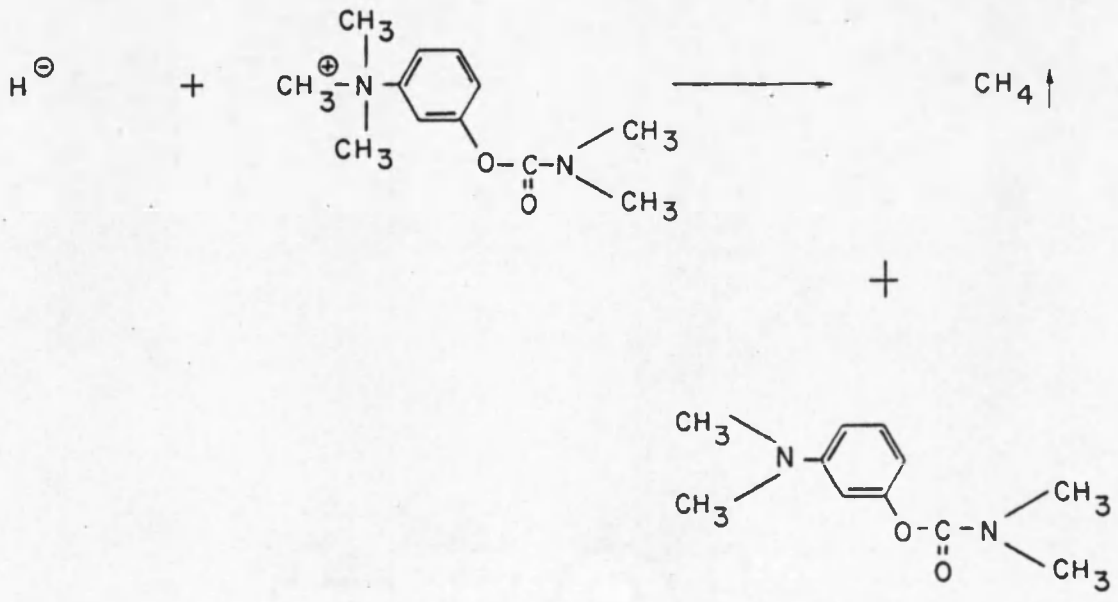

Figure 7. Hydride ion nucleophilic demethylation of neostigmine.

necessary for separation and purification would not be acceptable for a quantitative microanalysis.

Hunig and Baron (1957) reported a simple "one test tube" demethylation reaction of a quaternary ammonium salt in a fity mole excess of ethanolamine when the mixture was heated at 100°-150° C. for thirty minutes. Ethanolamine is the solvent and also the receiver of the transferred methyl group. The reaction was quenched by adding water and the tertiary amine extracted into an organic solvent.

Hanin and Jenden (1969) introduced a new species of nucleophile (Figure 8) for demethylation reactions and successfully determined the microgram quantities of acetylcholine in tissue extracts. The mercaptide nucleophile, sodium benzenethiolate, demethylates aliphatic or aromatic quaternary ammonium salts. Further development of Hanin's and Jenden's (1969) original method has been done by Cranmer (1968), Hanin and associates (1972, 1973a, 1973b) and Jenden, with his various associates. Choline and acetylcholine were demethylated and quantitatively determined from tissue extracts by gas chromatography with a lower limit of 100 picomoles (six nanograms) and 50 picomoles (seven and one half nanograms), respectively. The reaction is a nucleophilic displacement of an N-methyl group on the tertiary amine by the benzenethiolate ion.

Jenden et al. (1968) and Hanin and Jenden (1969) found that N-demethylation proceeded quantitatively on choline esters when vigorous anhydrous conditions were maintained using 2-butanone as the solvent under a nitrogen atmosphere at 80° C. The procedure was carried out in air-tight equipment that had been flushed with a dry stream of nitrogen. If this precaution is not taken, recoveries are lower and more variable,

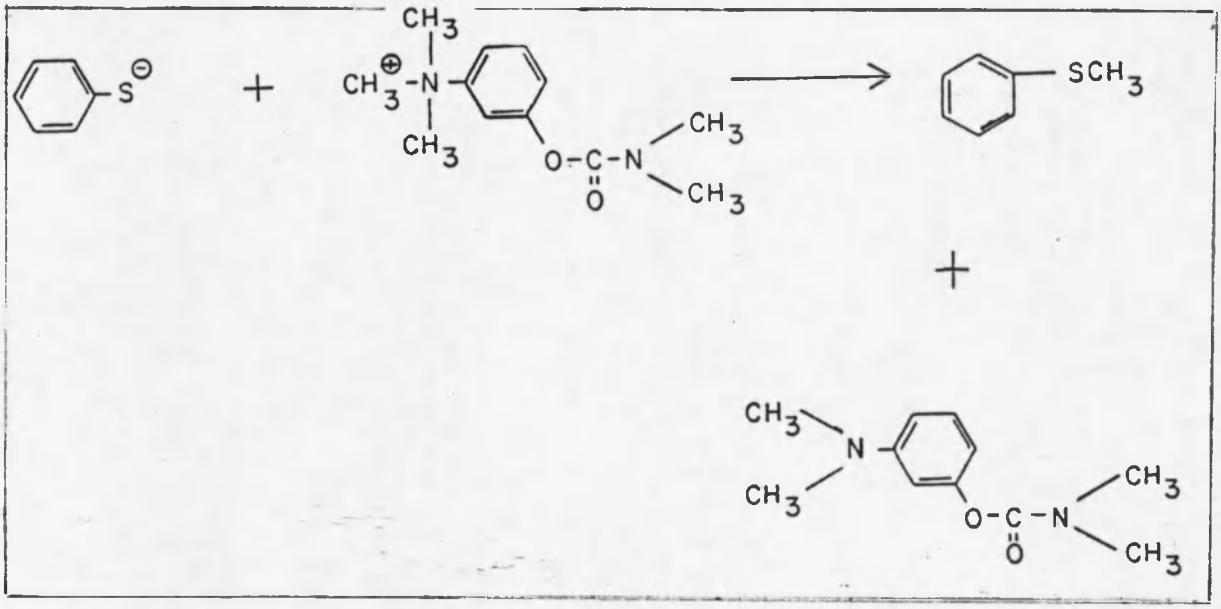

Figure 8. Benzenethiolate ion nucleophilic demethylation of neostigmine.

probably because of water formation (Figure 9) by oxidation of benzenethiol.

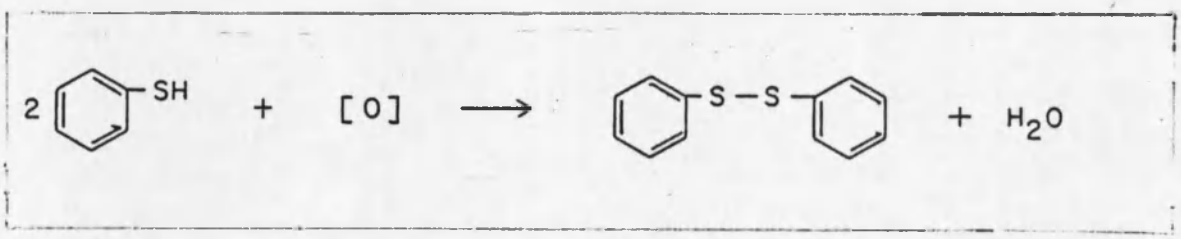

Figure 9. Benzenethiol depletion by oxygen.

This method of demethylation was adapted to fit the model of an aromatic quaternary amine salt.

Posner and Ting (1974) reported a selective demethylation of N-methyl quaternary ammonium salts using the nucleophile, cuprous phenylmercaptide, whose displacement reaction does not cause side reactions with ester functions. This procedure requires refluxing the methiodide salt with excess cuprous phenylmercaptide in anhydrous pyridine under a nitrogen atmosphere for 5-10 hours. Two disadvantages

of this method are the moderate yields of tertiary amines and the lack of a completely specific attack by cuprous phenylmercaptide at the methyl groups of most N-methyl quaternary ammonium salts.

Hutchins and Dux (1973) reported that the nucleophile lithium n-propylmercaptide (Figure 10) in hexamethylphosphoramide provides a mild, rapid, and convenient system for demethylation of quaternary ammonium salts in excellent yields with a high selectivity for methyl group removal. These facts coupled with the minimal amount of equipment and simple adaptation to a micro-determination made this method an excellent choice to research for the demethylation of neostigmine. Demethylation with lithium n-propylmercaptide requires strict anhydrous conditions and oxygen-free hexamethylphosphoramide under an inert atmosphere. All solvents and reagents must be carefully distilled, dried, and flushed with an inert gas just prior to analysis.

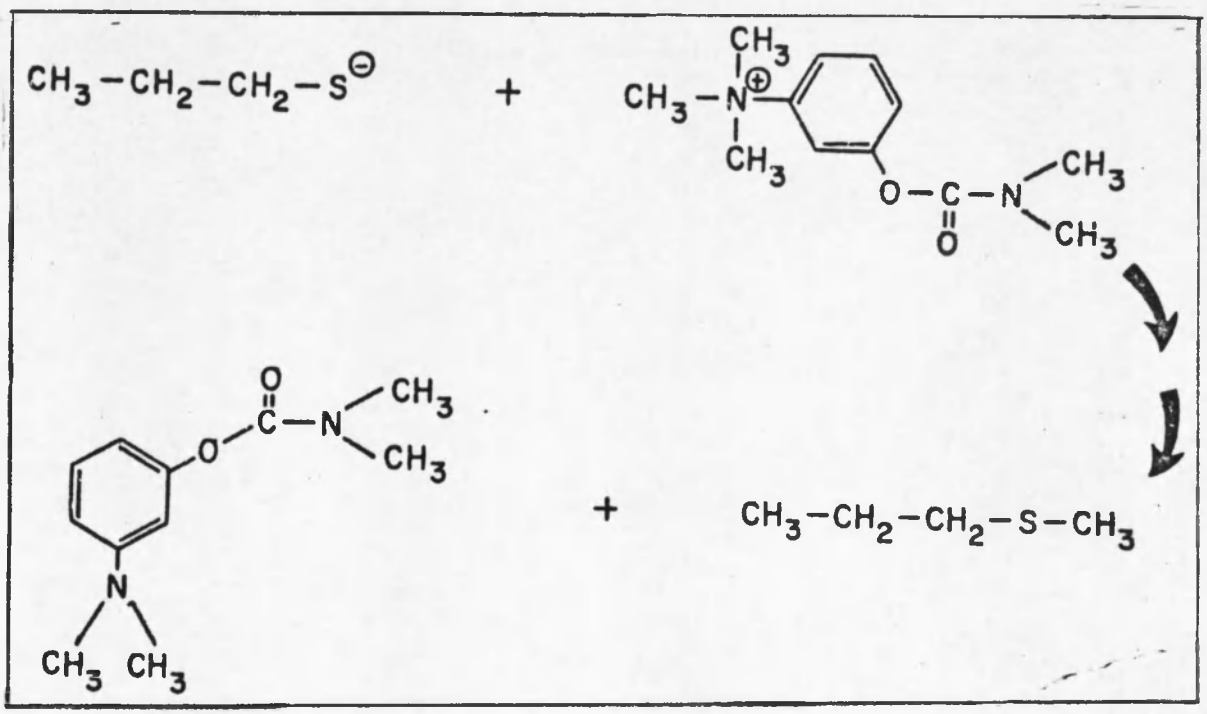

Figure 10. n-propylmercaptide nucleophilic demethylation of neostigmine.

The latest demethylation reaction was published by Cooke and Parlman (1975). Lithium triethylborohydride was reported to have a remarkable nucleophilic hydride ion as demonstrated by its ability to reduce organic halides susceptible to Sn2 displacement. Aromatic trialkylammonium salts readily react with lithium triethylborohydride to give tertiary amines resulting from the Sn2 displacement reaction. The mechanism for the nucleophilic hydride ion attack on quaternary ammonium salts is the same as stated earlier for lithium aluminum hydride. Reaction times are under an hour and displacement takes place at room temperature. The reaction solvent was tetrahydrofuran rather than hexamethylphosphoramide. This procedure does not have the selectivity that lithium n-propylmercaptide possesses but does demethylate substituted aromatic trimethylammonium compounds with cleavage of side chains containing ester functions.

EXPERIMENTAL

Demethylation Procedures

Lithium Aluminum Hydride Nucleophile

A three-neck flask was fitted with a reflux condenser containing a drying tube and a magnetic stirrer and heated by a hot plate. Tetrahydrofuran (250 milliliters) was distilled with sodium metal to remove residual water. Lithium aluminum hydride (13.3 grams) was added and refluxed for one hour (bubbles indicate water still present). Five grams of trimethylaniline iodide was added and refluxed another hour. A 25% sodium hydroxide solution was added and the demethylation product, dimethylaniline, was steam distilled. The distillate was acidified with concentrated HCl and extracted three times with ten milliliters of pentane. The aqueous fraction was made basic by adding NaOH pellets until a second layer appeared. This mixture was then extracted twice with 25 milliliters of dichloromethane and a one microliter aliquot was injected into the gas chromatograph.

Ethanolamine Nucleophile

Ten micrograms of trimethylaniline iodide or neostigmine bromide was added to five milliliters of ethanolamine and heated in an oil bath at 150° C. for one hour, cooled, and five milliliters of water added. The reaction mixture was extracted twice with ten milliliters of ether. An aliquot of one microliter from the combined ether fraction was injected into the gas chromatograph.

Benzenethiolate Nucleophile

Standard microgram samples of trimethylaniline iodide and neostigmine bromide were prepared. The demethylation reagent (0.5 milliliters), sodium benzenethiolate in butanone, was added to each centrifuge tube and the air displaced from the tube with a dry nitrogen stream. The tubes were capped tightly and placed in a water bath at 80° C. for 45 minutes. The tubes were cooled and 100 microliters of 0.5M citric acid was added. The tubes were mixed and two microliters of pentane added, mixed thoroughly, and the organic phase discarded. Fifty microliters of dichloromethane and one hundred microliters of the ammonium citrate buffer (pH 10.5) were added, mixed vigorously, and centrifuged at 2000 RPM for 30 minutes. A one microliter aliquot was withdrawn and injected into the gas chromatograph.

Column Preparation

A new liquid phase, 4-dodecyldiethylenetriamine succinamide (DDTS), was introduced by Jenden, Booth, and Roch (1972) for the separation of amines and alkaloids. A column with 4% DDTS on Porapak P was prepared according to Jenden, Booth, and Roch (1972) and used for all preliminary analysis with the above mentioned demethylation procedures. This column's retention time for the demethylation products of trimethylaniline and neostigmine were 15 and 35 minutes, respectively. This column was later replaced by a column of 3% OV-17 on Gas Chrom Q (discussed later).

Neostigmine Determination

General Considerations

Quantitative results for this analysis are dependent upon the demethylation reaction and its yield of the tertiary amine. Preparation of equipment and materials for this analysis have to be meticulously stringent. Strict attention must be paid to all steps prior to the demethylation to provide a rigorously anhydrous and oxygen-free reaction mixture. Side reactions with oxygen and water (Figure 11) deplete both the mercaptan and the lithium hydride salt essential for demethylation.

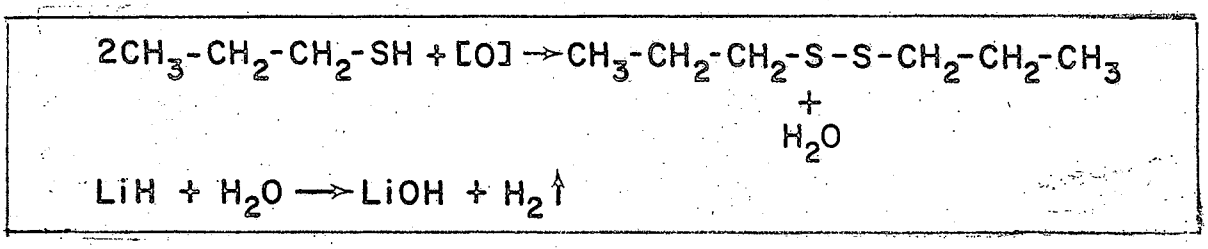

Figure 11. Depletion of the demethylating nucleophile components.

Water, inherent within the system or as the product of the first reaction, will deplete lithium hydride used to form the demethylating nucleophile.

Materials and Preparation

Unless otherwise stated, all organic and inorganic chemicals mentioned are Reagent Grade. Any further preparation of these chemicals, if needed, will be covered in continuing sections.

Glassware. All culture and conical centrifuge tubes, including their airtight caps, pipets, solvent containers, distillation apparatus and extraneous glassware were rigorously cleaned of residual organics that might interfere with the demethylation reaction or that might have

a retention time close to that of the tertiary amine product. The glassware was washed with soap and water; rinsed with distilled water; treated with potassium dichromate cleaning solution; rinsed with distilled, deionized, and permanganate distilled deionized water; and dried in an oven at 120° C.

Ammonium Reineckate Solution. Two hundred milligrams of the Reinecke salt (NH_4 $Cr(NH_3)_2$ $(SCN)_4$ H_2O), ammonium tetrathiocyanodiammonochromate (Fisher Scientific), was stirred intermittently with 10 milliliters of ice-cold distilled water for 45 minutes. The solution was filtered using Whatman #42 and must be used immediately. Fresh Reineckate solution was prepared each time the analysis is run.

Silver p-Toluenesulfonate Solution. Silver p-toluenesulfonate (139 milligrams, 5 millimolar) (Eastman, #7912) was dissolved in 100 milliliters of acetonitrile (Matheson, Coleman, and Bell; Spectroquality, AZ 145). This solution was kept well stoppered at room temperature in a brown bottle.

Ammonium Citrate (2 molar)/Ammonium Hydroxide (7.5 molar) Solution. Ammonium citrate, $(NH_4)_2HC_6H_5O_7$, (42.5 grams) was dissolved in 20 milliliters of specially prepared distilled water. To this was added 50 milliliters of 15 Normal ammonium hydroxide slowly with stirring. Subsequently, the solution was diluted to 100 milliliters with specially prepared distilled water. The buffer was stored at room temperature in a tightly stoppered bottle.

Hydrochloric Acid/Potassium Chloride Buffer. The HCl/KCl (pH 1.5) buffer was prepared, as directed in the Handbook of Chemistry and Physics, 56th edition; a reprint from the Butterworth's Scientific

Publication called "Electrolyte Solutions." If the container remained tightly stoppered, it was not necessary to remake this buffer before each run.

Water. Organic free water, needed in the preparation of solutions and for cleaning glassware, was prepared by taking distilled water, passing it through a commercial ion exchange column, and then distilling the deionized water from potassium permanganate.

n-Propyl Mercaptan. n-Propyl mercaptan (Aldrich) was freshly distilled once a week and stored in a brown bottle with a rubber medical seal cap for easy syringe access. The mercaptan was always flushed with argon to remove any residual oxygen present.

Dichloromethane. Dichloromethane, the solvent used to extract the tertiary amine, was distilled twice for purity, stored in a glass stoppered bottle, and kept in a cool location prior to use.

Hexamethylphosphoramide (HMPA). HMPA ($[(CH_3)_2N]_3PgO$) was distilled at 6mm Hg at 100° C. over calcium hydride and stored under vacuum in a cool location. HMPA, solvent for the demethylation reaction, was flushed with argon for 15 minutes prior to its use. Extreme caution must be taken when using HMPA due to a recently discovered nasal squamous cell carcinoma in rats by DuPont and published by Chemical and Engineering News on September 25, 1975.

Demethylation Product. Neostigmine's pure demethylated tertiary amine ([3-dimethylcarbamoyloxyphenyl] dimethylamine) was donated, compliments of Hoffman-LaRoche.

Analytical Procedure

Analysis of neostigmine is not trivial and strict attention to detail is imperative to obtain quantitative results. Depending upon how proficient one becomes at preparation of materials during the analytical procedure, the analysis may run anywhere from seven to ten hours in length. This time does not include preparation of the instrument or its operation. If it is not feasible to run the entire analysis in one day, several breaking points can be utilized to facilitate handling and operation. Breaking points occur between F and G (Figure 12), between I, J and K, and between L and M. Blank samples were always used with each set of samples to help identify any extraneous peaks due to a particular batch of samples.

Blood Preparation. (Steps A and B, Figure 12). Whole blood samples were collected in heparinized tubes to prevent coagulation. Precipitation and separation of the quaternary amine from plasma should be completed prior to 72 hours after obtaining whole blood samples due to possible interference from fibrin. Whole blood samples were dosed with the appropriate range of neostigmine for standards. Whole blood was centrifuged at room temperature in a Sorvall RC2-B at 3000 RPM for 15 minutes. An 1.0 milliliter aliquot was taken from the plasma supernatent for the analysis. In future human studies, 10 milliliter aliquots can be used to increase the sensitivity limit ten-fold from plasma.

Separation Procedure. (Steps C through F, Figure 12). To each dosed plasma aliquot, one milliliter of ammonium reineckate solution per milliliter of sample was added and mixed (Vortex mixer) thoroughly after the addition. These samples were placed in a 0° C. ice bath for 45

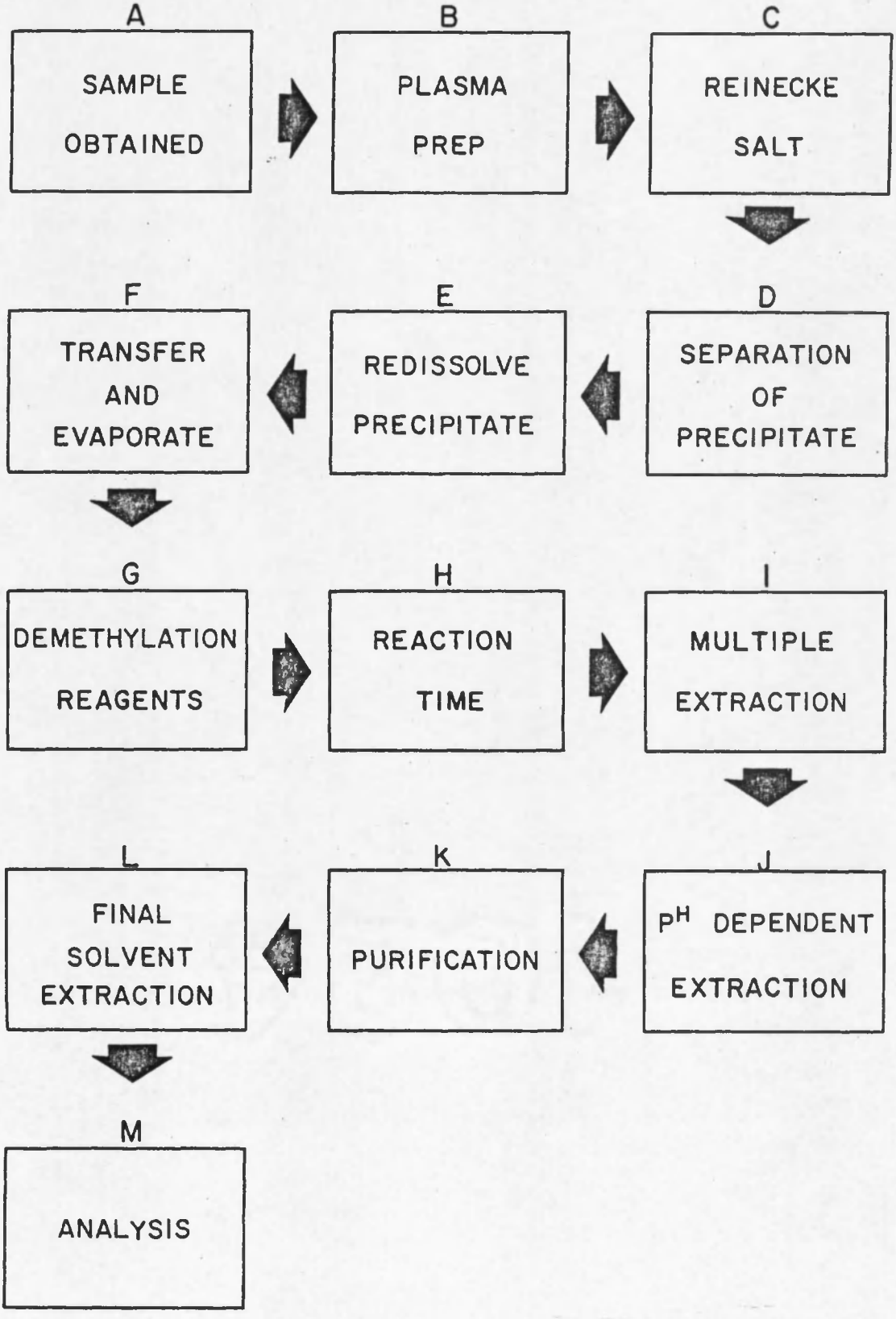

Figure 12. Procedure flow chart.

minutes and then centrifuged for 10 minutes at 2000 RPM in a refrigerated centrifuge (Sorvall RC2-B) using polycarbonate centrifuge tubes (Damon, IEC, 10 ml, #2067). The supernatant was discarded and the reineckate-quaternary amine precipitate was dried in a desiccator under high vacuum. To the dried precipitate one milliliter (five millimolar) of silver p-toluenesulfonate in acetonitrile was added and the precipitate broken up. The solution was mixed thoroughly for two minutes and centrifuged two minutes at 2000 RPM. The quaternary amine redissolved and the silver reineckate salt precipitated. The supernatant containing the quaternary amine was transferred to a clean culture tube and the solution was evaporated to dryness in a vacuum desiccator; the procedure should take at least 10 minutes.

Demethylation Procedure. (Steps G and H, Figure 12). To each culture tube was added 1 milliliter of HMPA (hexamethylphosphoramide) and 21 milligrams of lithium hydride (Matheson, Coleman, and Bell). The excess lithium hydride ensured that the HMPA was completely anhydrous (check for bubbles). A 1/4-inch magnetic stirring bar was added, the tube flushed with argon, and the solution mixed on a magnetic stirrer for 5-10 minutes. The culture tube was reopened and 0.1 milliliters n-propyl mercaptan added with a syringe and again the culture tube was flushed with argon.

The reaction mixture was checked for bubbling to ensure that the demethylating nucleophile (Figure 13) was forming. All culture tubes were placed on a magnetic stirrer for a half-hour reaction period.

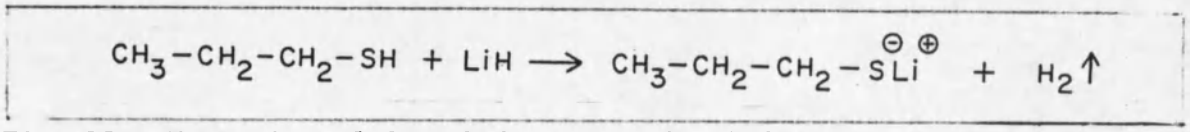

Fig. 13. Formation of demethylating nucleophile.

Extraction and Purification. (Steps I through L, Figure 12). The culture tubes were placed in an ice bath and five milliliters of water was added slowly to the reaction mixture (Figure 14) to quench the reaction and decrease the solubility of the tertiary amine in HMPA. Two milliliters of cyclohexane were added, mixed for one minute, shaken for 10 minutes, and centrifuged (Damon, IEC Ultracentrifuge) for 10 minutes at 2000 RPM. This procedure was repeated three times, and the aliquots of cyclohexane were removed and placed in a conical centrifuge tube.

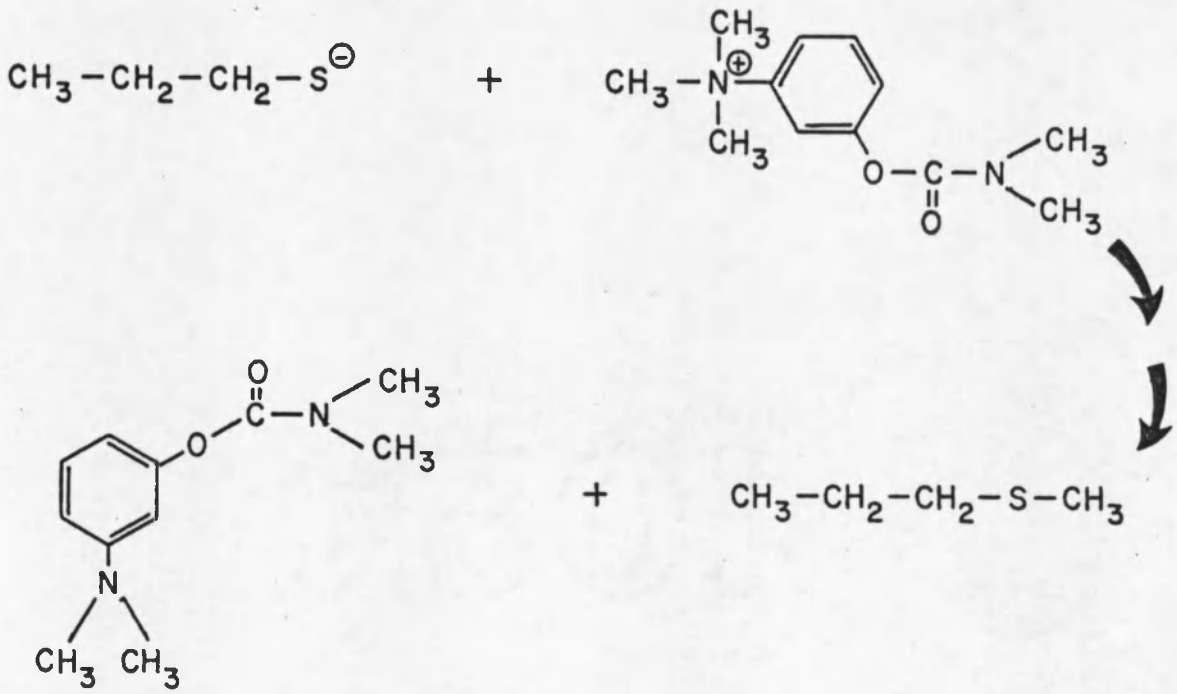

Figure 14. Demethylation of neostigmine using n-propylmercaptide nucleophile.

The cyclohexane extracts were combined and 0.4 milliliters of an HCl/KCl buffer (pH 1.5) were added and mixed for one minute, shaken for a half hour, and centrifuged for 10 minutes. The tertiary amine moved to the aqueous phase. The cyclohexane (upper phase) was aspirated off and the aqueous phase washed with approximately two milliliters of pentane to remove any unwanted residual organic substances. The tubes were again mixed for one minute, centrifuged for five minutes, and the pentane layer aspirated off. This was repeated twice and any residual pentane removed by blowing a stream of dry nitrogen gas on the aqueous layer. One-half milliliter of citrate buffer (pH=9.5-10.0) and 50 microliters of dichloromethane were added to the HCl/KCl buffer mixture. This final mixture was thoroughly mixed for two minutes, shaken for 15 minutes, and centrifuged for a half hour at 2000 RPM. The dichloromethane phase (bottom of tube) contained the tertiary amine; the buffered aqueous phase should remain at a pH greater than 9.5. If the analysis were delayed until a more convenient time, the last mixing and centrifuging should be repeated.

Operational and Analysis Dependent Factors

Instrumentation. The analysis was run on a Varian Aerograph Series 2100 gas chromatograph with a Varian Aerograph Model 20 recorder. The flame ionization detector (FID) was thoroughly cleaned according to the Varian 2100 Service Manual. After each series of samples the septum was changed, the column reconditioned at 185° C. overnight and the detector temperature raised to "bake off" any formed residue.

Operational Conditions.

Injector temperature	205° C.
Oven temperature (Isothermal)	160° C.
Detector temperature	205° C.
Hydrogen	45 ml/min
Air	280 ml/min
Carrier Gas (N_2)	25 ml/min

Column Preparation. The column used was a 3% OV-17 (Varian) on Gas Chrom Q (Applied Sciences Laboratory, Inc.; silanized with dimethyldichlorosilane, 100/120 mesh). Three percent (by weight of Gas Chrom Q) OV-17 was dissolved in enough acetone to make a slurry when the Gas Chrom Q was added. The slurry was shaken for several minutes and blown dry with a stream of nitrogen. The glass U-tube column was washed with dilute acid, dilute base, several organic solvents, including those used during column operation. The column was blown dry with a stream of nitrogen. The U-tube was tightly packed with the prepared Gas Chrom Q and silanized glass wool was used to plug the ends of the column to prevent the packing from being blown out under the positive nitrogen (carrier gas) pressure. The column was conditioned for at least 24 hours prior to use and again after each set of runs to maintain good column stability during operation. The column used was a 6 ft. x 1/4-inch U-tube. A four-hour warm-up was needed to achieve baseline stability at the operational range one and attenuation four. A 10 µl Hamilton syringe was used for injections between one and two microliters.

Detector Response -- Standard Curve. The linearity of the detector response for the demethylated neostigmine was analyzed by using

the pure tertiary amine (compliments of Hoffman-LaRoche). Weighed amounts of the pure tertiary amine were dissolved in dichloromethane and injected into the gas chromatograph. The values of the peak areas (discussed later) were plotted against the known weight injected. The resultant calibration curve (Figure 15) was linear and did pass through the origin (no sample, no response). The standard calibration curve furnished a tool to determine the results of unknowns and to check reaction yields of the tertiary amine.

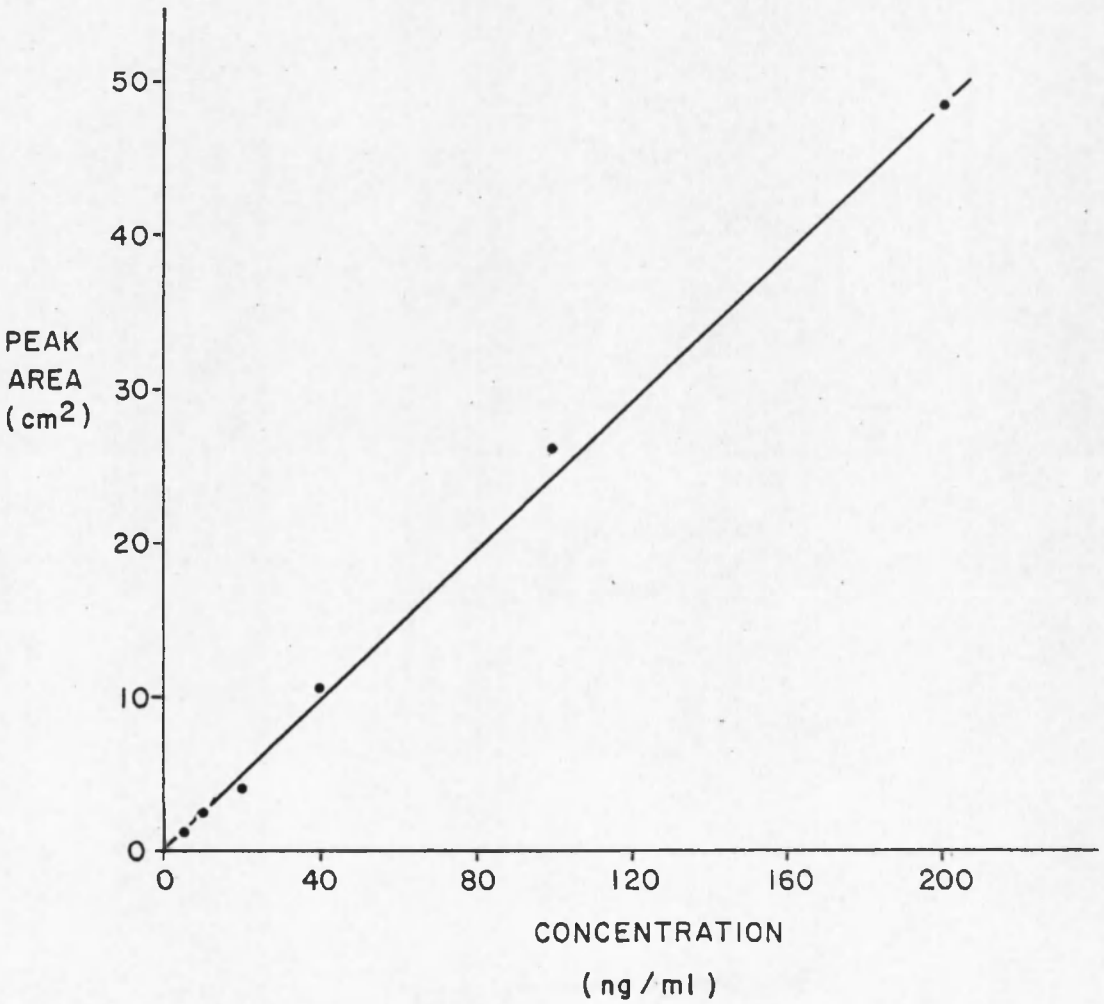

Figure 15. Standards for demethylation product.

Peak Identification. The demethylation product (the tertiary amine) was identified using peak retention times (Figure 16). Due to the careful elimination of extraneous organic compounds and the purification steps in the procedure, the chromatogram of neostigmine was free of unwanted peaks.

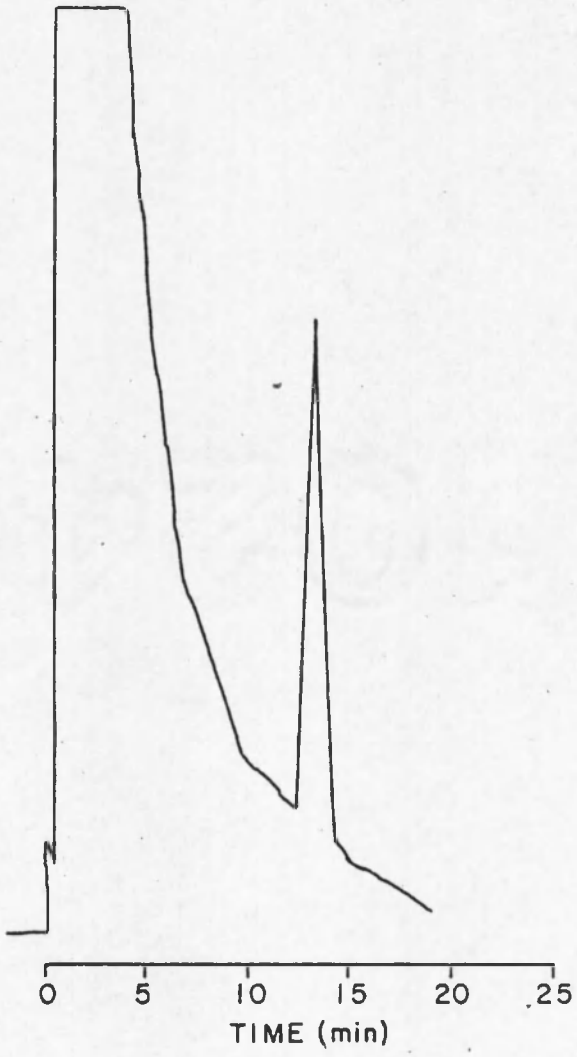

Figure 16. Chromatogram of neostigmine's demethylation product (3-DMCP-DMA).

pH Dependent Extraction. In Hanin's and Jenden's (1969) micro-determination of acetylcholine, an aqueous citric acid solution (pH = 3.5) was used to extract acetylcholine from the reaction mixture. The demethylation product extraction was found to be pH dependent. Buffers were made from citric, oxalic, acetic, and tartaric acid and HCl/KCl in the pH range from 1.50 to 3.5. All pH measurements were made on a Beckman digital pH meter. A standard curve was prepared for neostigmine's tertiary amine by measuring the absorbance (Beckman, Model 25 Spectrophotometer) of several standard concentrations (by weight) prior to absorbance measurements on the pH dependent extraction. A cyclohexane extract (Step 1) was prepared containing a concentration of 5 µg/ml of the tertiary amine. The assorted aqueous acid buffers were individually added, shaken, and centrifuged. Aliquots of the cyclohexane (top phase) were removed and the absorbance measured on a UV spectrophotometer (Beckman, Model 25) to detect quantities of the tertiary amine not extracted into the acidic phase.

Calculations. During the pH dependent extraction study absorbances were measured (Beckman, Model 25 UV Spectrophotometer) on the amount of tertiary amine present after the extraction. Using

$$A = abc \qquad (1)$$

the value of the absorptivity, (a), was calculated from the standard curve knowing b, the light path length through the cuvette; c, the concentration being tested; and A, the absorbance measured. To calculate the percentage of the tertiary amine extracted the absorbance was measured of the cyclohexane phase after the extraction and using

equation (1) the concentration of the remaining tertiary amine was determined. Using

$$100\% - \frac{\text{concentration remaining}}{\text{original concentration}} \times 100 = \% \text{ extracted} \qquad (2)$$

the percentage of the tertiary amine extracted was calculated.

The quantitative calculation of the tertiary amine by gas chromatography was based on the determination of the area under the peak which has a proportionality to the concentration of the compound. The peak areas were calculated using

$$\text{Peak Area} = \text{Peak Height} \times \text{Peak Width at Half-Height} \qquad (3)$$

as discussed by Purnell (1962). Many other factors enter into the calculation of the Actual Peak Area. Using

$$\text{Actual Peak Area} = \frac{R \times A \times PH \times PW}{ACF \times ICF} \qquad (4)$$

where,

R = Range

A = Attenuation

PH = Peak Height

PW = Peak Width at Half-Height

ACF = Aliquot Correction Factor

ICF = Injection Correction Factor

The Aliquot Correction Factor was a volume ratio as shown below:

$$\text{Aliquot Correction Factor} = \frac{\text{Total Aliquot Removed}}{\text{Total Cyclohexane Added}} \qquad (5)$$

The Injection Correction Factor normalized all peaks measured by correcting the volume injected to one microliter, thus standardizing all concentrations.

Once the Actual Peak Area of the unknown had been determined a simple ratio was used to correlate its concentration providing a standard concentration and its peak area were also known.

$$\frac{C_a}{A_a} = \frac{C_u}{A_u}$$

where,

C_a = Concentration of Standard

A_a = Peak Area of Standard

C_u = Concentration of Unknown

A_u = Peak Area of Unknown

RESULTS

The results section for the micro-determination of neostigmine will include the following: (1) Demethylation Procedures, (2) Detector Response-Standard Curve, (3) Optimum Reaction Time, (4) Distribution Coefficient for Cyclohexane Extraction, and (5) pH Dependent Extraction.

Demethylation Procedures

Lithium Aluminum Hydride Nucleophile

The demethylation reaction (Figure 7) described by Cope et al. (1960) was tested using the quaternary ammonium salt, trimethylaniline iodide. The method's demethylation reaction product, dimethylaniline, produced a single peak whose retention time on the gas chromatograph matched the standards for dimethylaniline (DMA). The method was rejected because the yield of dimethylaniline was only 36.3%, and the refluxing and steam distillation techniques necessary for separation and purification were not adaptable to a micro-analysis.

Ethanolamine Nucleophile

The demethylation reaction, described by Hunig and Baron (1957), used ethanolamine both as the solvent and the demethylating nucleophile. It was not possible to find a solvent immiscible with ethanolamine whose distribution coefficient was favorable for the extraction of the demethylated quaternary amine without also extracting substantial quantities of ethanolamine and its methylated counterpart. At microgram

concentrations of trimethylaniline and neostigmine, the yields of dimethylaniline and (3-dimethylcarbamoyloxyphenyl) dimethylaniline (3-DMCP-DMA) were poor and variable. Tertiary amine yields for neostigmine were lower than for trimethylaniline.

Table 1. Ethanolamine demethylation reaction yields

CONCENTRATION RANGE	DIMETHYLANILINE YIELDS	3-DMCP-DMA YIELDS
10.0 µg/ml	4.73%	1.67%
5.0 µg/ml	3.25%	1.56%
2.5 µg/ml	3.38%	2.11%
1.0 µg/ml	4.08%	1.27%
0.5 µg/ml	4.19%	0.98%

Benzenethiolate Ion Nucleophile

The demethylation reaction, described by Jenden et al. (1968) and Hanin and Jenden (1969) in the micro-determination for choline and acetylcholine, was repeated until the demethylation product, dimethylaminoethylacetate (for acetylcholine), could be identified between 1-10 micrograms (Figure 17). This method of demethylation was adapted to fit the model of the aromatic quaternary amine salts--trimethylaniline iodide and neostigmine bromide (Figure 8). Experimental work showed the demethylation yield of the tertiary amine, dimethylaniline, from trimethylaniline (concentration range 0.1-0.5 µg/ml) to be 65-70% (Table 1). Repeated attempts failed to increase the yield of neostigmine's demethylation product above 2-5% for the same concentration range.

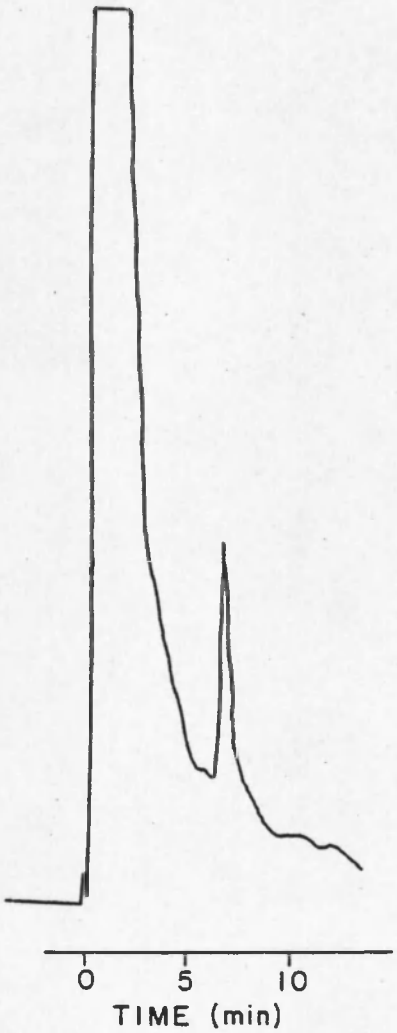

Figure 17. Chromatogram of dimethylaminoethylacetate.

Neostigmine's Determination

The modified demethylation procedure, Hutchins and Dux (1973), is described in detail in the experimental section. Figure 18 shows the standards for dimethylaniline. Figures 19 and 20 show how the concentration of the trimethylaniline (TMA) and neostigmine standards, respectively, before demethylation is related to their corresponding

demethylation products' peak area. Tables 2 and 3 show the same concentration range from Figures 18 and 19 and the percentage yield of the demethylated product.

Table 2. Benzenethiolate demethylated trimethylaniline and neostigmine yields

CONCENTRATION RANGE	DIMETHYLANILINE YIELDS	3-DMCP-DMA YIELDS
0.5 µg/ml	64.9%	2.67%
0.4 µg/ml	65.3%	2.87%
0.3 µg/ml	67.2%	3.41%
0.2 µg/ml	68.7%	3.80%
0.1 µg/ml	69.4%	4.12%

Table 3. Demethylation product yields of trimethylaniline

STANDARD CONCENTRATIONS	DIMETHYLANILINE YIELDS
10.0 µg/ml	87.4%
5.0 µg/ml	87.1%
2.0 µg/ml	88.1%
1.0 µg/ml	88.7%
0.5 µg/ml	89.5%
0.1 µg/ml	97.2%

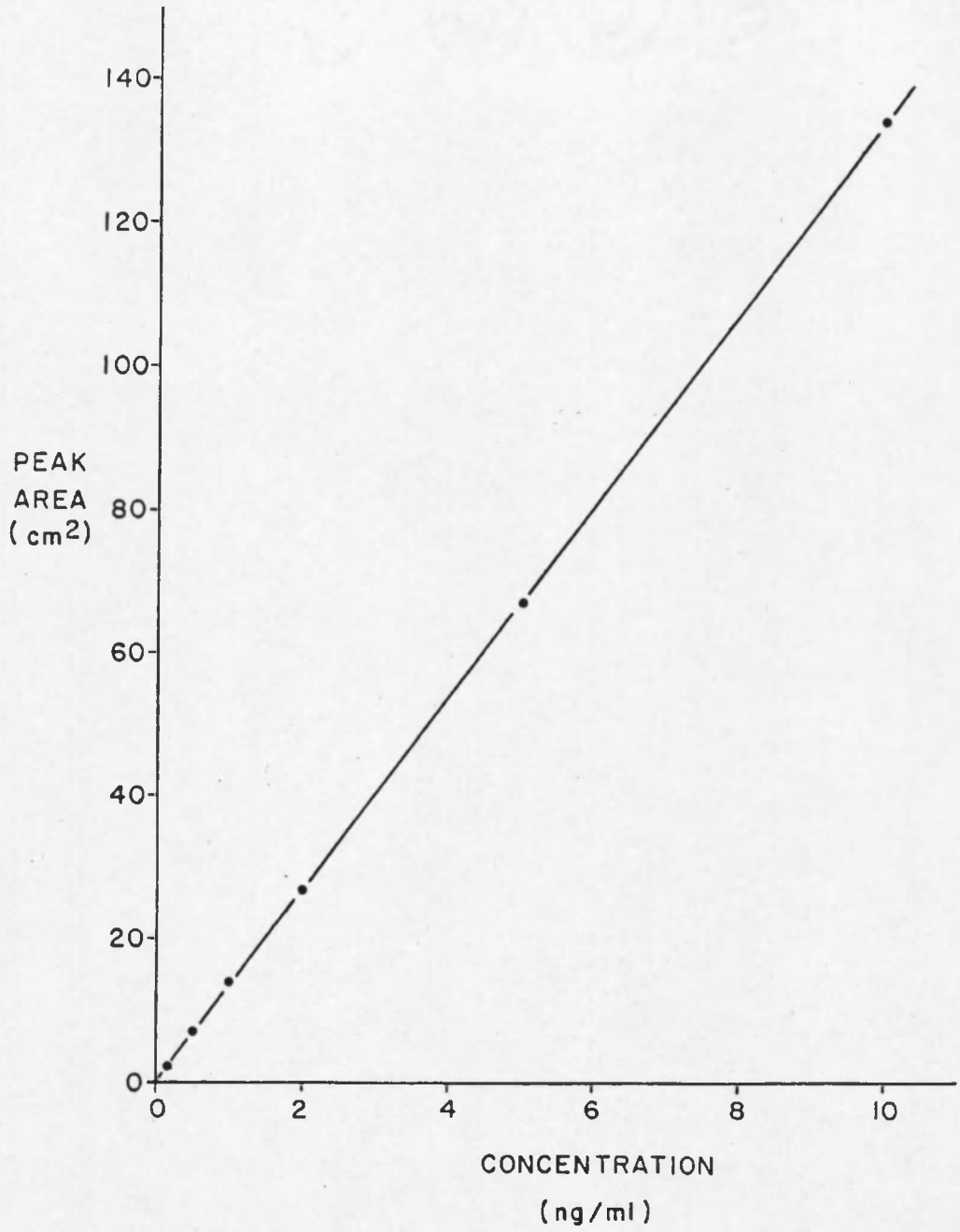

Figure 18. Standard curve for dimethylaniline.

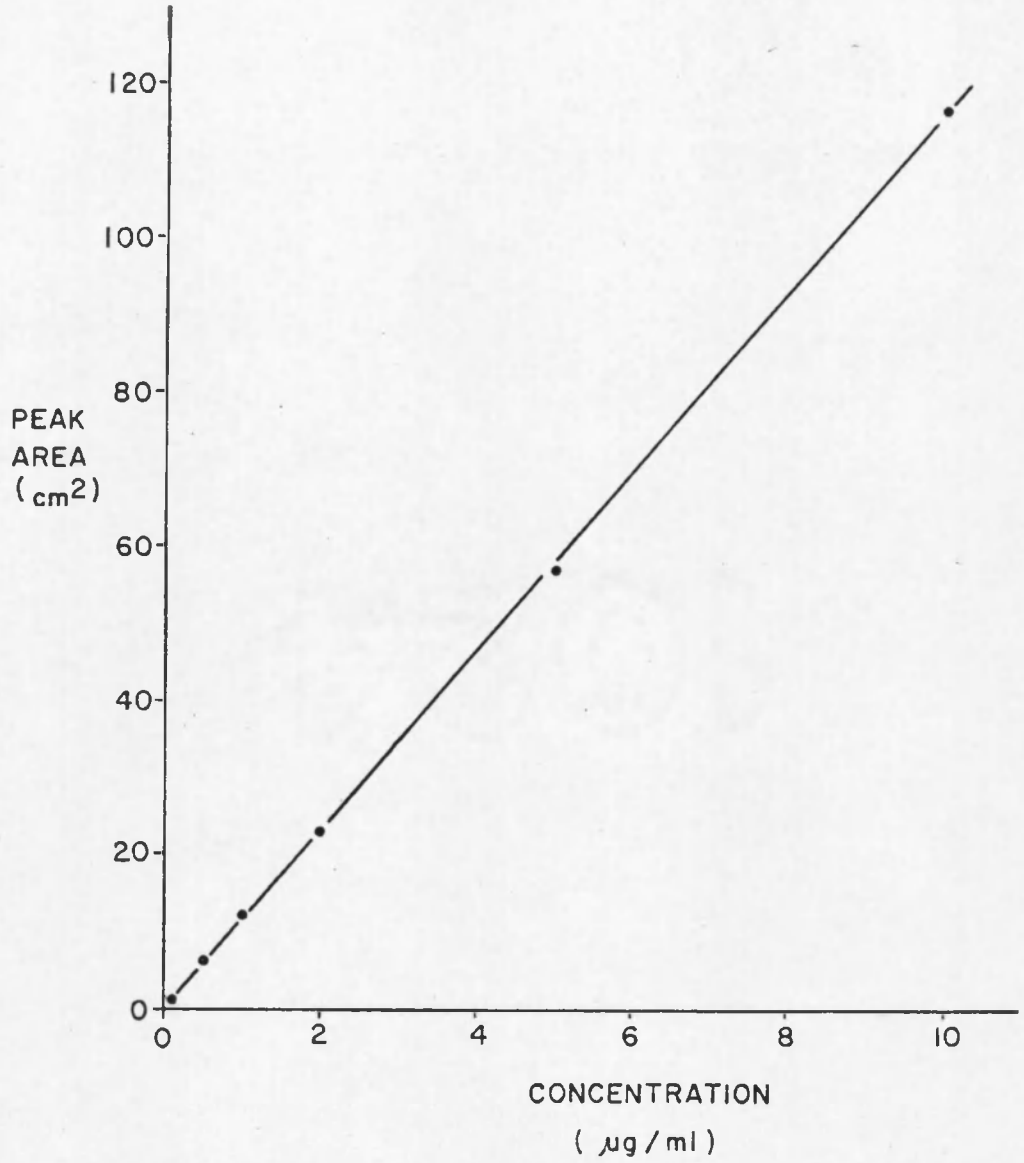

Figure 19. Demethylation standard curve for trimethylaniline.

Figure 20 shows a standard curve for neostigmine. Plasma was dosed with known amounts of neostigmine (Table 4) and the entire procedure was carried out as described in the experimental section. Table 5 shows the same standards as in Figure 21, with their corresponding percentage yield of 3-DMCP-DMA, and the percentage recovery of neostigmine after precipitation with the Reinecke salt.

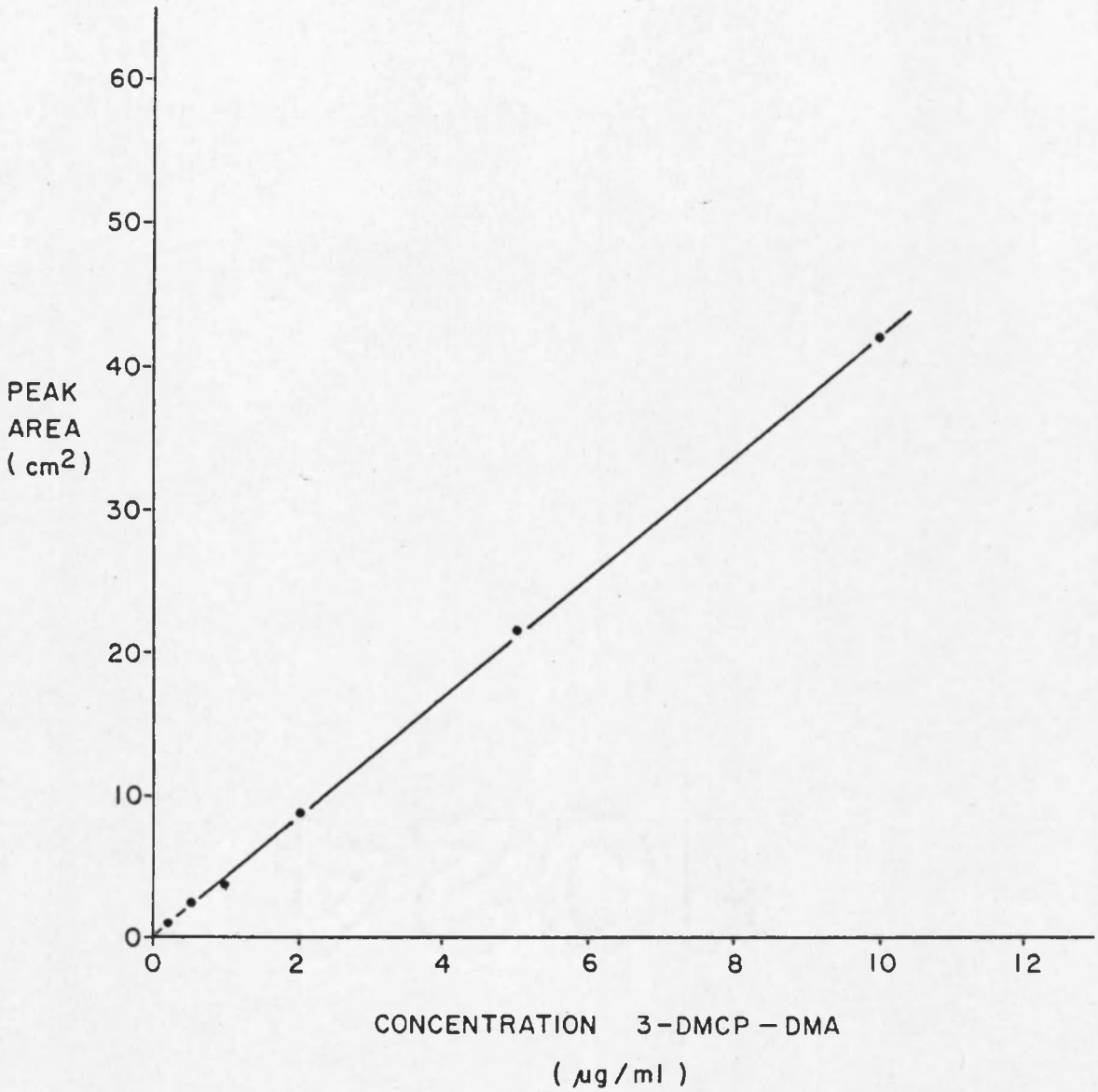

Figure 20. Standard curve for demethylation product of neostigmine.

Table 4. Demethylation product yields of neostigmine

STANDARD CONCENTRATIONS	PERCENTAGE YIELD
10.0 µg/ml	83.9%
5.0 µg/ml	83.4%
2.0 µg/ml	84.1%
1.0 µg/ml	84.9%
0.5 µg/ml	87.3%
0.1 µg/ml	100.0%

Table 5. Demethylation product yields of neostigmine in plasma.

STANDARD CONCENTRATIONS	PERCENTAGE YIELD	REINECKE PRECIPITATION YIELD
10.0 µg/ml	77.9%	93.5%
5.0 µg/ml	78.9%	93.4%
2.0 µg/ml	78.5%	92.8%
1.0 µg/ml	78.1%	92.9%
0.5 µg/ml	81.2%	92.1%
0.1 µg/ml	89.4%	90.6%

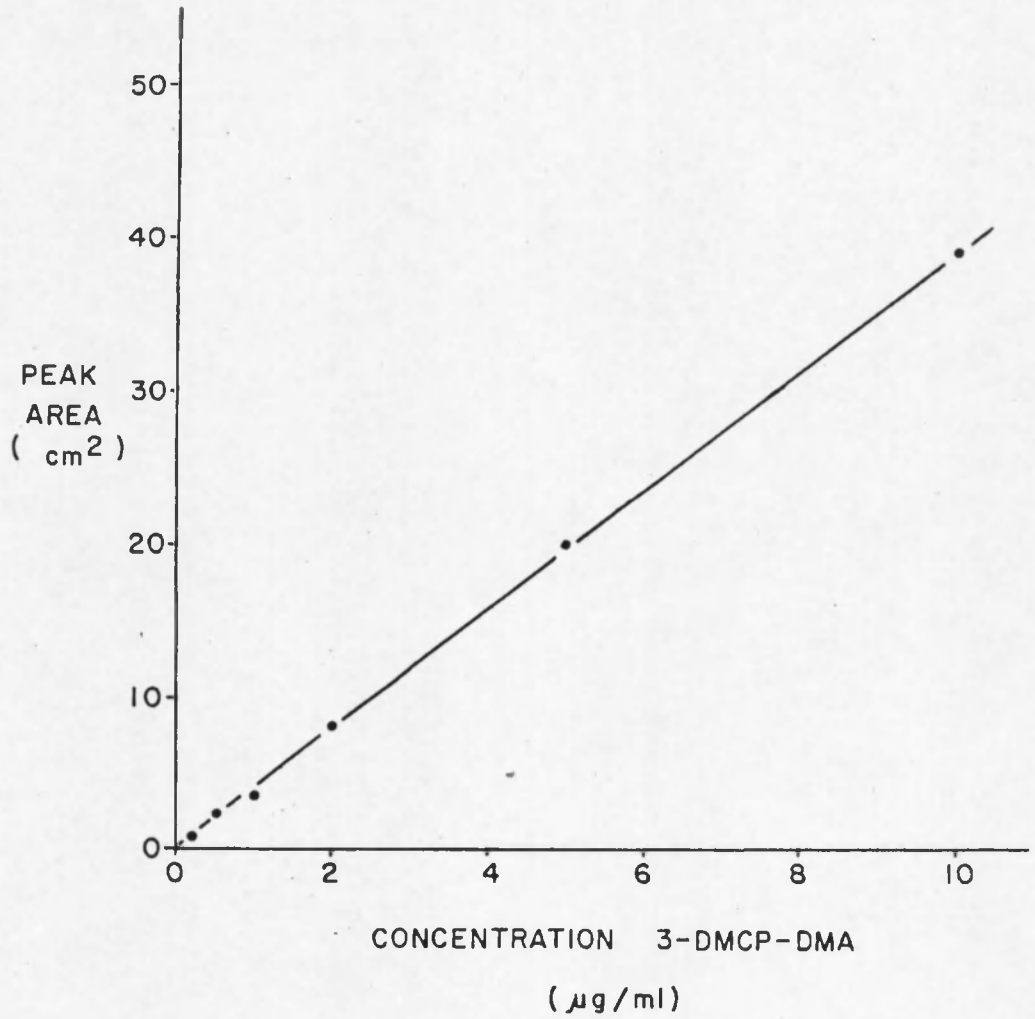

Figure 21. Neostigmine demethylation product standards using plasma.

Detector Response and Standard Curve

Known weights of pure 3 -DMCP-DMA, neostigmine's demethylation product, were injected and the peak areas measured. The detector response in the desired concentration range (0.1-10.0 µg/ml) was linear as shown in Figure 22.

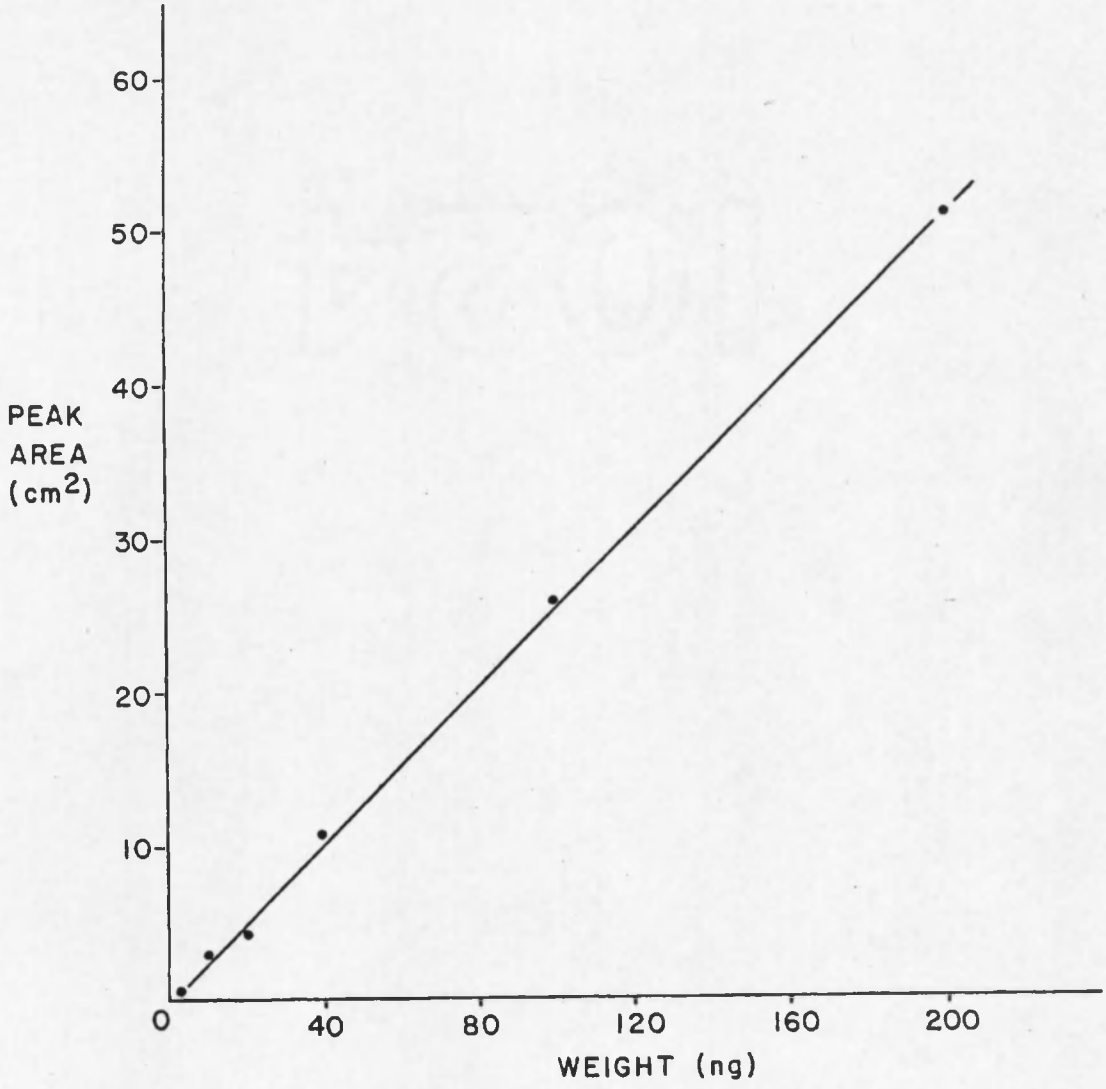

Figure 22. Detector response curve.

Optimum Reaction Time and Temperature

The optimum temperature for the demethylation of neostigmine was 23° C., room temperature. Figure 23 shows the optimum reaction time for the demethylation of neostigmine as thirty minutes.

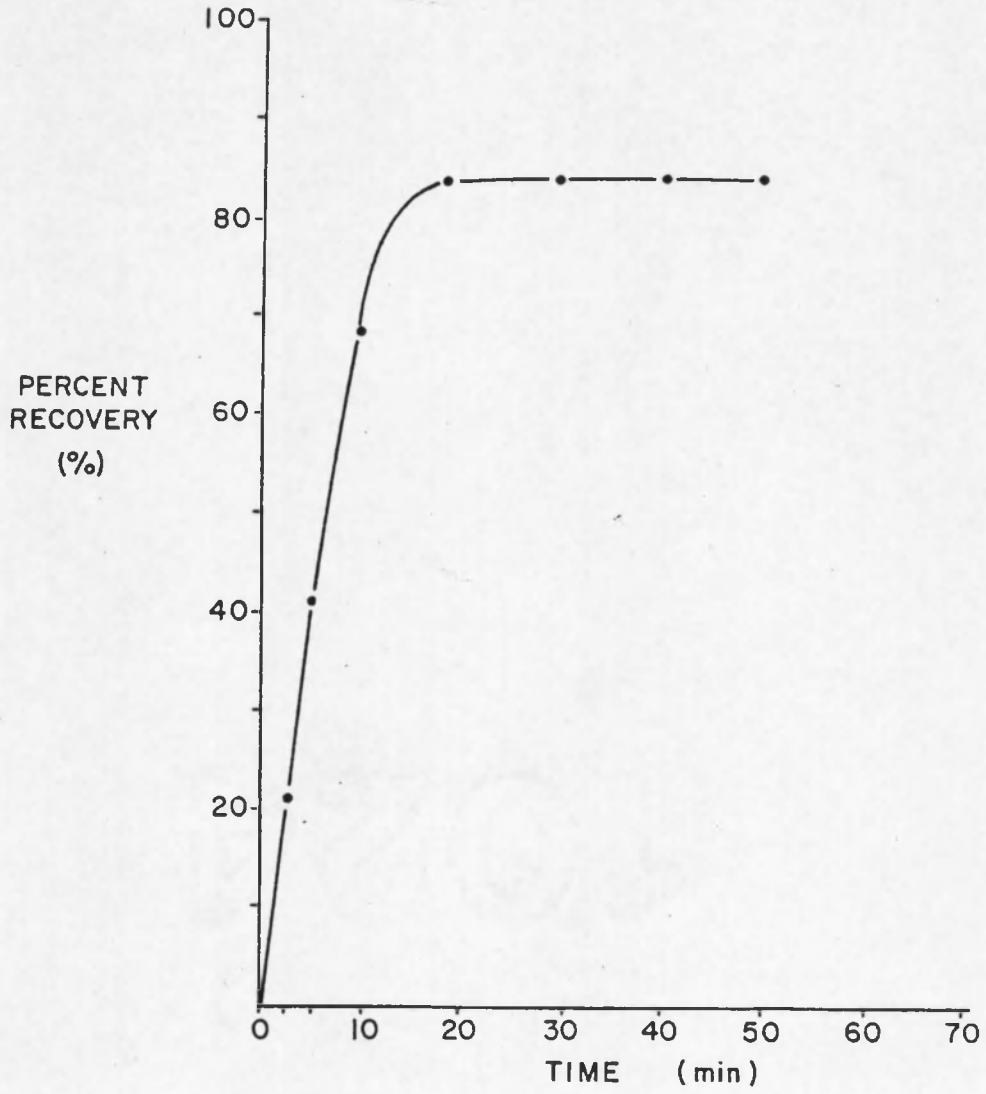

Figure 23. Optimum reaction time.

Distribution Coefficient for Cyclohexane Extraction

The distribution coefficient for the extraction of 3-DMCP-DMA from the reaction mixture into cyclohexane was determined by injecting a one microliter aliquot of the cyclohexane layer onto the OV-17 column. The distribution coefficient for the demethylation product is 2.70 or 73% extraction. Three extractions from the reaction mixture yielded 98% of the demethylation product in the combined cyclohexane fraction.

pH Dependent Extraction

Using an HCl/KCl buffer the demethylation product was extracted completely into this aqueous phase from cyclohexane at pH 1.5. Figure 24 shows the pH dependence of the extraction of the demethylation product of neostigmine, 3-DMCP-DMA.

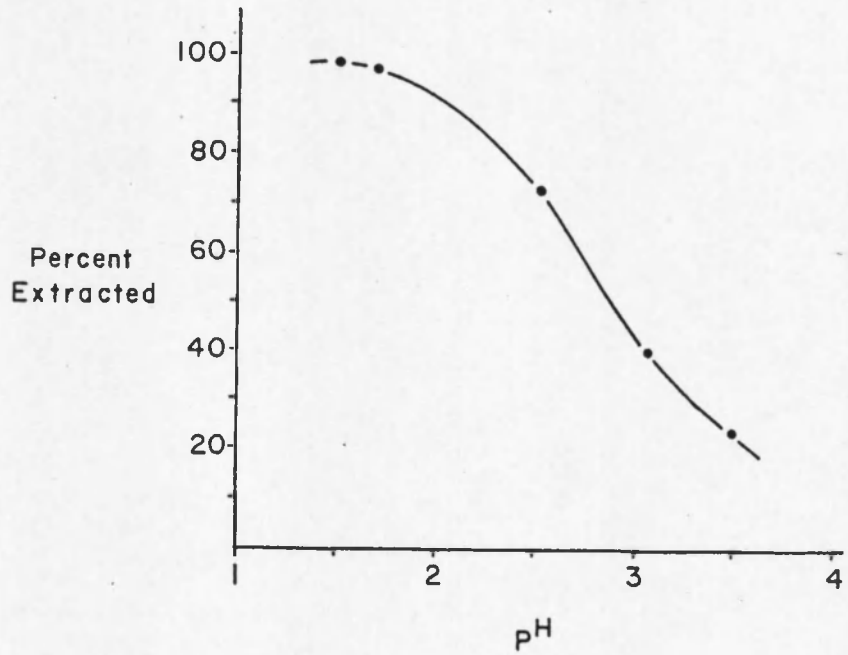

Figure 24. pH dependent extraction.

DISCUSSION AND CONCLUSION

Comparison of Demethylation Reactions

Most of the demethylation reactions already discussed were discarded due to their nonadaptability to a microanalysis. The demethylation nucleophiles considered were the acetate ion (Wilson and Joule, 1968), lithium aluminum hydride (Cope et al., 1960), ethanolamine (Hunig and Baron, 1957), sodium benzenethiolate (Hanin and Jenden, 1969), and lithium n-propylmercaptide (Hutchins and Dux, 1973). Based on the demethylation yields, reaction time, and reaction temperature for the above reactions, the lithium n-propylmercaptide is the strongest nucleophile (base).

When using the demethylating nucleophiles, lithium aluminum hydride and ethanolamine, the possibility existed that side reactions could interfere with the expected reaction (Figure 7) and produce even lower yields of the demethylated product. Low yields were observed with these nucleophiles but it was not established if side reactions were the cause.

The nucleophilic strength of the benzenethiolate ion was sufficient to demethylate the aromatic quaternary amine trimethylaniline with a yield of 65-70%. The reaction conditions (seven hours at 80° C.) were more stringent than for the propylmercaptide and would add considerable time to the entire analysis. In addition, the demethylation yields for neostigmine, even with hexamethylphosphoramide as the solvent, were lower than ten percent under the conditions used.

Experimental Discussion

The lithium n-propylmercaptide nucleophile in hexamethylphosphoramide at room temperature proved to be a mild, rapid, and a convenient system for demethylating neostigmine in excellent yields with a high selectivity for the methyl group removal. The author (Hutchins and Dux, 1973) warned that due to the high nucleophilicity of the reagent system, compounds must be devoid of ester groups which could be cleaved easier as the temperature is raised above room temperature. At room temperature, there was no evidence (additional peaks) that hydrolysis of the ring bound ester function (carbamic acid) of neostigmine occurred.

In preparation for the demethylation reaction, strict precautions must be observed and solvents and reagents must be monitored. After the precipitation and separation of the quaternary amine from plasma, there is a crucial drying step in a vacuum desiccator which should take at least ten minutes. If these preparatory precautions are taken, unwanted side reactions (Figure 11) will not deplete the reagents needed in making the demethylating nucleophile and product yields will be maximal.

Table 4 shows the chosen concentration range for neostigmine which should be the approximate plasma concentrations after surgical application. The demethylation product peak areas (Figure 20) have a linear relationship with the neostigmine concentrations because of the constant percentage yield from the demethylation reaction over the one hundred-fold concentration range. After the neostigmine standards were determined in plasma, the calculated percentage yields from the

demethylation procedure were lower. This was due to loss of neostigmine during the precipitation with the Reinecke salt. There was approximately a 7-8% loss of neostigmine in the precipitation step but the percentage yield of the demethylation product was still constant, thus producing a linear concentration verus peak area relationship (Figure 21).

The linearity of the demethylation yields provides a useful tool for the measurement of neostigmine in the biological concentrations used in surgery. A tenfold increase in the concentration range can be obtained merely by taking ten milliliter aliquots of plasma, thus extending the sensitivity limit of the assay from 0.1 to 0.01 μg/ml (10.0 ng/ml) with the flame ionization detector.

Detector response was monitored before each set of determinations to make certain that all results could be compared with the neostigmine standards already established. When the detector response did not correspond to the established neostigmine standards, the temperature was raised for three to four hours or until the response from the detector exactly matched that of the standards. This, normally, was not necessary and the samples could be injected without a further time delay.

The optimum reaction time was established by a set of time-dependent runs on the same concentration of neostigmine (10.0 μg/ml). The percent recovery of the demethylation product was measured versus the reaction time at room temperature. The reaction time taken as the standard was thirty minutes. Experimentation was not conducted to find a reaction temperature, other than room temperature, because of the possibility of cleavage of the carbamic acid side chain.

After the demethylation reaction, the cyclohexane extraction was found to be a key step in the determination of percentage yield of the demethylated product. The distribution coefficient of the demethylation product into cyclohexane was found experimentally to be 2.70. With multiple extractions, the yield of the demethylation product of neostigmine increased substantially and there was less variability in the yield as the concentration varied.

The pH dependent extraction is also a key step in the determination of the final yield of the demethylation product. The pH experimentation found an HCl/KCl buffer extraction at pH 1.50 completely removed the demethylation product from the cyclohexane phase (Figure 24).

Conclusion

The purpose of this experimental work was to find a method for the analysis of neostigmine in blood that was applicable in the concentrations used in surgery. The proposed demethylation procedure exceeds the sensitivity limit of all existing methods of analysis for neostigmine by one hundredfold. The procedure's applicability does not limit it to neostigmine alone; with minor modifications this analysis could be used for most quaternary amine salts. In addition, modifications can be made to accommodate samples of urine and tissue extracts.

Further experimental work could yield a procedure more sensitive than the proposed demethylation reaction. After the hydrolysis of the carbamic acid from neostigmine and the subsequent synthesis of a side chain abundant with halogen molecules, an electron capture detector

could increase the sensitivity limit one thousandfold. Another promising procedure is an ion pair fluorimetric analysis. Neostigmine could be extracted from a plasma medium by precipitation or ion-pair extraction and purified. After forming another ion pair with a large fluorescent molecule, the fluorimetric analysis could be completed with an approximate one hundredfold increase in the sensitivity limit.

Great potential exists with the proposed demethylation procedure in reference to human studies and the discovery of many factors still unknown to researchers because of the previously inadequate analysis methods.

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