

AN ABSTRACT OF THE THESIS OF

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ABSTRACT

Quaternary Ammonium Compounds, Qams, are an important class of chemicals which present interesting challenges to analysts. This thesis reports results of investigations on uses of ion-pairing techniques for separations and detection of Qams. Special apparatus for post-column solvent extraction of liquid chromatographic eluates was developed as part of this work.

Analyses for Qams were developed using ion-pair extraction techniques with bromophenol blue, anthraquinone sulfate and naphthyl sulfate as ion-pair extraction reagents. Bulk extraction experiments were executed to evaluate the dependance of the ion-pair reagent concentration on the extraction efficiency of Qam ion pairs. The pH dependance on extraction efficiency for gallamine triethiodide and decamethonium bromide, bromophenol blue ion pairs was also evaluated. A solvent segmented flow injection analysis system was designed and assembled for the rapid evaluation of ion-pair extraction efficiency of several different Qams with bromophenol blue as the ion-pair extraction reagent.

Separations of Qams using ion-pair liquid chromatography were developed using reverse phase columns and both alkyl sulfonic acid and tetramethylammonium salts in the mobile phase as ion-pair liquid

chromatography reagents. A continuous post-column solvent extraction system was developed for the detection of Qam—bromophenol blue ion pairs with bromophenol blue being added directly to the mobile phase eluents. Both isochratic and gradient separations were developed for several Qams which have medical importance.

ION-PAIR HIGH PRESSURE LIQUID
CHROMATOGRAPHY TECHNIQUES FOR THE
DETERMINATION OF QUATERNARY AMMONIUM COMPOUNDS

by

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ION-PAIR HIGH PRESSURE LIQUID CHROMATOGRAPHY TECHNIQUES FOR THE DETERMINATION OF QUATERNARY AMMONIUM COMPOUNDS

INTRODUCTION

This thesis presents the results of experiments designed to develop a rapid, reliable liquid chromatography analysis technique for quaternary ammonium compounds (Qams). The techniques developed take advantage of the ionic nature of Qams and their ability to form extractable ion pairs with specific ionic substances. Special on-line, continuous, post-column solvent extraction apparatus was designed for the purpose of analyzing quaternary ammonium compound (Qam) ion pairs eluting off of an HPLC column. Table 4 in the Appendix lists the names, CAS registry numbers and structures of the ion-pair reagents used in this work to separate and extract quaternary ammonium compounds. The development of separation techniques for Qams using ion-pair high pressure liquid chromatography was a focus in this work and is also presented.

Qams are unique in chemistry and chemical applications. They are fully ionized organic substances whose ionization state is independent of pH. Qams are commercially obtained as salts of Cl^- , Br^- , I^- , ClO_4^- , CH_3SO_4^- or OH^- . Table 5 in the Appendix gives the structure and CAS registry numbers of the Qams discussed in this thesis.

For many years there has been intensifying interest in the study of these substances. The rapid increase in the frequency of literature citations concerning applications of Qams has spanned a wide range of chemical disciplines. Research and applications of Qams have been reported in biochemistry, chromatography, pharmacology, toxicology, electrochemistry, phase-transfer catalysis, polymers and solution chemistry as well as other areas. The release of Qams into the environment has also been brought under scrutiny as more information on their

chemistry has been elucidated. In addition to the experimental results and discussion, historical information on the chemistry, analysis and application of Qams will also be presented.

HISTORICAL

Chemistry of Quaternary Ammonium Compounds (Qams)

Properties and Physical Characteristics of Qams

Quaternary ammonium compounds (Qams) represent a group of substances which are distinguished by the presence of a nitrogen having four organic substituents bonded to it. Qams are classified according to two prominent characteristics; the quaternary nitrogen and the types of structures bonded to it. The former is the predominant factor promoting water solubility, and the latter modifies properties of Qams both in solution and on surfaces.

When Qams are dissolved in aqueous solution, changes occur in the bulk-solution properties (1). These changes are a result of solute-solvent interactions associated with their dissolution (2). One study on the properties of alkyl Qams in water found that for Qam concentrations ranging from 0.1 to 1 molal the solubility of the hydrocarbon gases methane, ethane, propane, and butane significantly increased (3). This ability is purportedly related to water-structure promoting abilities of the alkyl groups on the Qam (1). Water molecules surrounding the alkyl group aggregate in an ordered fashion distinguishing these Qams as "structure making" solutes. These hydrophobic regions are presumed to be responsible for the increased solubility of hydrocarbons in water in the presence of alkyl Qams (4).

The enhancement of hydrocarbon solubility by Qams diminishes rapidly when hydrophilic functional groups such as OH, C=O and NH₂ are added to the alkyl chain. Disruption of the solvent structure occurs

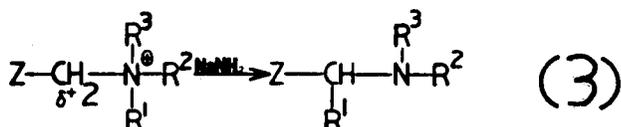
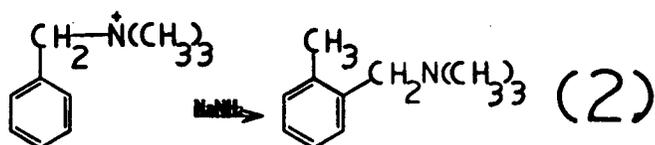
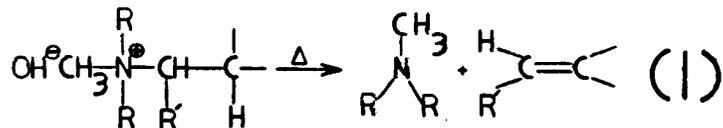
and a more random arrangement of water molecules results. Qams of this type are termed "structure breaking" (4). Thermodynamic studies of Qams have focused on the molar heat of solution for these substances. This thermodynamic value is given as a measure of the hydrophobic interaction for a particular Qam. The magnitude of the heat of solution has been related to the alkyl hydrocarbon solvating power of a particular Qam (4). In order to assess the contribution of H-bonding, this heat of solution for several Qam was measured in aprotic solvents such as N-dimethylformamide and N-methyl acetamide (5).

Coefficients of osmosis (6), diffusion (7) and activity (8) have also been established for several Qams.

Organic Chemistry of Qams

Qams are prepared in a straightforward manner by successive treatments of ammonia, primary, secondary, and tertiary amines with alkyl halides. This treatment is known as the Menshutkin process (9).

The reaction chemistry of Qams is perhaps most distinguished by the elimination processes first described by Hoffman (10). As shown below in equation 1, Qams are degraded to their tertiary amine analogs by the application of heat.



This decomposition has been used extensively in gas chromatographic analyses of Qams (11,12). Treatment of Qams with benzene thiolate also produces tertiary amine analogs with specific cleavage of the methyl group from the nitrogen nucleus (13,14). Demethylation reactions of Qams have been reviewed (15).

The Sommelet-Hauser rearrangement, equation 2, and Stevens rearrangement, equation 3, are competing reactions to Hoffman elimination in some cases. When a strong base, like sodium amide is reacted with a Qam, the two rearrangement reactions will occur with the Stevens rearrangement being favored at higher temperatures and the Sommelet-Hauser being favored at lower temperatures (16). Uses of these reactions in various synthetic applications has been reviewed (17).

Qams play an important role in another area of organic reaction chemistry which is Phase-Transfer Catalysis (PTC). PTC is a method by which two chemical substances that are not easily mixed are brought together in one phase via the assistance of a phase transfer agent (18).

The role of the Qam as a phase transfer agent is to shuttle reactive nucleophiles across a phase boundary. The selected substrate material then reacts with the nucleophile to produce the desired product. An example of PTC is the reaction of a phenoxide nucleophile with an alkyl halide. Phenol is added to an aqueous phase containing quaternary ammonium hydroxide where it is ionized and ion-paired with the Qam cation. A water immiscible organic solvent containing the alkyl halide is mixed with the aqueous solution. The phenoxide-Qam ion-pair distributes into the organic phase where the nucleophilic reaction between phenoxide and the alkyl halide occurs (19).

PTC reagents are usually Qams or crown ethers. Ion-pair formation and phase distribution are important phenomena in PTC and are discussed below. PTC reactions using Qams have been reviewed (20).

Biochemical and Clinical Aspects of Qams

Qams are important compounds in pharmacologic and biochemical studies. The functions of Qams in biological systems are essential for many life processes. The distinctive properties of the quaternary nitrogen, the accompanying organic functional groups, along with special molecular configurations of living systems have made Qams favored molecules for structure-activity relationship studies (21). These studies have helped reveal numerous complexities of biological interactions.

The roles of choline and acetylcholine, both Qams, are fundamental in neuromuscular synapse processes (22,23). Other Qams are also involved in the regulation and control of neural activity (24). Medical needs have spawned development of numerous important Qam drugs which arrest muscle activity. For example, in delicate surgical procedures it is sometimes necessary to immobilize skeletal muscles. Qams such as gallamine, pancuronium, succinyl choline, and tubocurarine are given intravenously for this purpose (25).

The skeletal muscle relaxation response to these Qams is dependent on the route used to introduce the drug. If administered orally, Qam drugs usually have almost no effect. However, if given subcutaneously, there is a dramatic increase in effect and maximum response occurs when these substances are administered intravenously.

These "curare-like" Qams have also been the focus of controversy both for legal reasons and in regard to their chemical analyses in cases

where their use was either suspected or proven in several murder cases (26). The crucial questions of stability, detectability and reliable analyses were central themes in those controversies.

Recent research has lead to the tremendously important development of specific Qam agents designed to shuttle therapeutic agents across the blood-brain barrier. These and other uses of Qams will continue to be important in the area of clinical medicine (27).

Industrial Uses and Practical Applications of Qams

Qams have many important uses for industry in a variety of ways. Their use as surfactants, disinfectants, coatings, detergents, corrosion inhibitors, catalysts, textile treatment compounds and polymers has established them as important substances in industrial processes (28-32).

Analytical Chemistry of Quaternary Ammonium Compounds (Qams)

Spectroscopy

A variety of methods have been applied to chemical analyses of Qams. Their usual lack of chromophoric functional groups has often precluded analyses by ultraviolet (UV) or visible spectroscopy. Qams such as gallamine, neostigmine, benzoyl choline, and tubocurarine can be determined by UV absorbance techniques. UV absorbance may not be capable of distinguishing these substances from one another or from the background but it may be useful in monitoring these types of Qams following a separation procedure.

Structural information relative to the chemical properties of Qams has been obtained using nuclear magnetic resonance (NMR) of ^{13}C and ^1H

nuclei present in curare-like alkaloids (33). These measurements may distinguish carbon atoms bonded to the quaternary nitrogen from others in the molecule.

Electrochemistry

Qams have been studied with electrochemical methods. The measurement techniques of potentiometry, voltammetry and conductometry have provided much of the information on the electrochemical behavior of Qams. Qams are also used as supporting electrolytes to assist in measurements of electroactive organic substances.

Potentiometric measurements have been popular for analyses of Qams. A variety of potentiometric electrode systems designed to monitor Qams have been characterized. These electrodes generally function on the basis that Qams can form ion-pairs with impregnated or otherwise immobilized anions of the electrode thereby generating a measurable potential at a membrane surface (34-37). Microelectrodes are proving to be reliable probes for determining *in vivo* activities of selected Qam ions. For example, both acetylcholine- and choline-selective microelectrodes based on ion-pair complexation with dipicryl amine have been reported for measuring these Qams in brain tissues (37).

Voltammetric measurements of Qams have also been studied primarily in exploring interactions of Qams with electrode surfaces. The interaction of Qams at a mercury surface was described by Southerland, et. al. (38). It was established that Qams were stable over a wide potential range and a mechanism for the reduction of Qams was presented. Oxidation of quaternary ammonium hydroxides in DMSO has been reported along

with a proposed electrochemical mechanism (39).

An important application of Qams is their use as supporting electrolytes for voltammetric studies of organic compounds (40). Their electrochemical stability and solubility properties have allowed studies on a wide range of electrochemically active organic substances.

Other studies of the voltammetry of Qams have emphasized transport of certain Qams between immiscible phases. For example, current vs. applied potential measurements have been used to investigate the transport of choline and acetylcholine across water/nitrobenzene interfaces (41).

Conductance measurements have been shown to be useful for studying the solution chemistry of Qams. From these measurements, inferences about ion-pair interactions have been made using classical approaches for evaluating these interactions (42).

Mass Spectrometry

The difficulty in detecting Qams by conventional spectroscopic methods has led to the development of mass spectrometric (MS) methods for measuring them. Early MS techniques depended on electron impact (EI) as the ionization mode. High energies of impact cause Qams to degrade by the Hoffman mechanism precluding the measurement of the molecular ion (43). Although a nuisance, the degradation is reproducible and demethylated fragments may be detected. Chemical ionization (CI) offers no significant improvement over EI; however, a number of biologically important Qams have been measured using CI-MS (44).

Recently, soft ionization methods have enhanced production of molecular-ions in mass fragmentograms for several Qams. Field desorption (FD) has been demonstrated to be a useful technique for mass spectrometry of thermally labile compounds such as Qams (45). FD, combined with collisional activation, has also been used as a tool to measure Qam molecular-ions (46). Secondary-ion mass-spectrometry (SIMS) has recently been applied to detection of Qams of biological interest (47,48).

Gas Chromatography

The gas chromatography (GC) of Qams has been examined by several researchers. The reported techniques generally have attempted to measure Qams at physiologic levels. The first Qams studied by GC were choline and acetylcholine via elution of their Hoffman degradation products produced by pyrolysis (49). The establishment of sensitive flame ionization detection methods for GC then led to improvements derivatization schemes for Qam analytes. Demethylation was used in an attempt to improve sampling specificity (50).

Interfacing a GC to a mass spectrometer provides another powerful method for detecting Qam eluates. For example, acetylcholine and choline have been separated by GC and then successfully determined by EI-MS (12). Similarly, CI has been used to ionize demethylated Qams eluted from a GC which resulted in more easily interpreted fragmentation patterns than those found with EI-MS (51).

As GC measurement techniques for Qams have become more selective and sensitive, they have been applied to increasingly complicated measurements. For example, experiments using radiolabeled compounds

along with MS detection have been used in attempts to delineate biosynthetic pathways for acetylcholine using a model precursor (52). The result was a myriad of radiolabeled products. Along these lines, difficulties from and limitations of using radiolabeled compounds in experiments in conjunction with both pyrolytic degradation and electron impact ionization of Qams have been reported (53,54). One successful application of these techniques correlated bull fertility with acetylcholine levels in spermatozoa (55) in that the higher the level, the more fertile the animal.

Ion-pair Extraction of Qams

Ion-pair extraction (IPE) is a valuable technique and is adaptable to many types of analyses. Many different reagents have been used for the extraction and subsequent determinations of low-molecular weight Qams. Sulfonophthalein indicators, bromophenol blue (BH_2) and bromocresol green were among the first ion-pairing agents to be used in IPE analyses of Qams (56). These methods have been used with minor revision (57) for many years and have been applied to analyses for several Qams (58). The stoichiometry of BH_2 extractions has been demonstrated to involve one BH_2 molecule for every two quaternary amine functionalities (58,59). However, data on potential interferences are lacking.

Recently, IPE of larger, more complicated Qams with these same indicator substances in the presence of quinine sulfate was reported (59). Sulfonic acids and other anionic substances have been demonstrated to be successful in Qam extractions (60). Picric acids and picrate salts have also been shown to be effective (60).

Liquid Chromatography Separations of Qams

Qams have been separated and determined by a variety of liquid chromatography (LC) techniques, with many of them employing some variation of ion-pair liquid chromatography (IPLC).

Thin layer chromatography (TLC) separations on silica gel treated with counter-ions on the surface or dissolved in the eluent have been reported for pancuronium bromide and its hydrolysis products (61), Qam drug substances (62), as well as a description of a general approach to separations of these substances (63). This latter work demonstrated that retention factors (R_f) varied with counter-ion such that $R_f(\text{Cl}^-) < R_f(\text{Br}^-) < R_f(\text{I}^-) < R_f(\text{ClO}_4^-)$ (63). The significance of this data is that as the ion pairing potential of a counter-ion increases the retention of the ionic sample also increases.

Qams have been successfully separated on normal phase chromatography columns. These normal phase separations have required that a counter ion be loaded onto the silica support phase. The counter ions employed for such a purpose included perchlorate (64), naphthalene sulfonate (65), and picrate (66). Elution of the Qam ion pairs was accomplished with relatively non-polar solvent eluents such as hexane and chloroform.

Reverse-phase separations have also been developed for analyses of Qams. These generally utilize polar eluents which are modified by addition of anionic organic salts. These applications have been primarily limited to Qam analytes which are easily detected by UV absorbance (67-70). These salts are usually sulfonate salts of the formula $\text{R-SO}_3^- \text{X}^+$. A Qam salt such as tetramethylammonium chloride (TMAC) may be added to

deactivate residual silanol sites of the stationary phase (70).

Ion-exchange liquid chromatography has also been used extensively for Qam analyses. Along with this separation technique, several interesting detection methods have been employed. For example, indirect photometric detection has been used for analyses of alkyl Qams (71) which are otherwise undetectable by usual techniques. Conductometric detection in conjunction with ion-exchange separations has also been used for Qam analyses (72). A unique ion-exchange membrane detection system has also been developed for determination of Qams (73).

Reaction Detectors

Improvements in separations technology has been accompanied by improvements in liquid chromatography detectors. Analysts now have a wide array of instruments to use as detectors for LC: mass fragmentometry, fluorescence, phosphorescence, UV-Vis absorbance spectroscopy, Fourier Transform infrared spectroscopy (FTIR) and electrochemical detection have all been successfully adapted to LC detection.

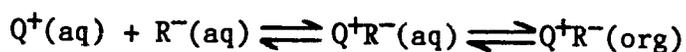
However, some substances such as Qams remain difficult to measure despite the extensive technological advancements which have been made. For some of these substances, additional measurement specificity can be introduced by chemical reaction of analytes with derivatization reagents which are highly selective for the analyte. Systems designed to chemically treat the eluting analytes to form detectable reaction products following chromatographic separation are called reaction detectors. Amino acids, sugars, alcohols, amines, and ketones can thus be selectively determined by measurement of reaction products. (74,75).

Reaction detectors which employ ion-pair extraction for analyte selectivity and enhancement of sensitivity are of particular interest. For example, the strong chromophore 9, 10 dimethoxyanthracene sulfonate has been used as a counter-ion to detect ion paired organic amines following separation on reverse phase packing and post-column extraction (76). The direct addition of this reagent to the mobile phase showed no effect on separation parameters (77). Similarly, nonchromophoric Qams have been measured by an LC-reaction detector system in which bromophenol blue was used as the counter-ion (78).

THEORY

Ion-Pair Extractions

Ion-pair extraction, IPE, is a widely used technique for the extraction of ionic analytes from aqueous matrices. The thermodynamics of these processes have been modeled by several authors (79-81). The general scheme



is described by an association and distribution constant. The entire process is represented by an extraction constant which is a product of the association and distribution constants. This extraction constant can be further refined to a conditional constant. Additionally the presence of interfering reactions gives rise to the equivalence constant which takes ion-pair concentration in the organic phase as the equivalent concentration of analyte Q_{am} and ion-pair extraction reagent (66).

In the empirical practice of IPE, there are many variables which affect extraction efficiency. The critical variables include extraction solvent, counter-ion, and pH. In IPE, the polar character of the ion-pair requires the use of a moderately polar extraction solvent. Typically, chlorinated solvents like methylene chloride, chloroform, dichloroethane, and trichloroethane are modified for IPE by addition of aliphatic alcohols. Modification of the solvent is done cautiously to avoid side reactions and losses in specificity for the ion-pair.

In performing an IPE, the choice of counter-ion will primarily determine the selectivity and solution conditions needed for the procedure. The counter-ion should have high selectivity for the quater-

nary amine and have few potential interferants. Counter-ions which have been utilized successfully for Qam analyses are bromophenol blue (56), bromocresol green (82,83), perchlorate ion (64), naphthalene 2-sulfonate (65), methylene blue (84) and several other acid-base indicator dyes (85). These reagents complex with quaternary amines forming neutral ion-paired complexes which extract into organic solvent phases.

An important consideration in using these reagents is the pH of the solution containing the sample. Many of the substances used as counter-ions are also pH indicators and their state of ionization is pH dependent. Tertiary amines constitute a potential interference if the pH is not sufficiently high. Consider the equilibria involved in the ion-pair extraction of a triquaternary ammonium compound like gallamine triethiodide with a diprotic indicator substance bromophenol blue. When the solution pH is held above 4.6 it may be assumed that bromophenol blue is completely deprotonated and that the only ion-pair species capable of distributing are those that consist of gallamine (G), iodine (I), and bromophenol blue (B) ions namely G_2I_4B , GIB, $G_2B_2I_2$, and G_2B_3 . It is uncertain as to how many of these species may actually exist in solution. An experiment could be designed which may answer the speciation question. It is none the less important to examine these speciation possibilities as they may occur under certain solution conditions.

Similarly, the case of the ion-pair extraction of decamethonium bromide (DBr_2), a diquaternary ammonium compound, with bromophenol blue poses a different complication. In addition to the neutral DB, D_2Br_2B species which are capable of distributing into an organic solvent, other ion-pair aggregates of the form D_nB_n and $D_nBr_nB_{(n-1)}$ might also be

present and constitute a potential interference. A study on the extraction of pancuronium bromide, a diquateryary "curare-like" drug, demonstrated a 1:1 stoichiometry between the analyte and bromophenol blue (58). Stoichiometry of 2:1 between other monoquateryary amine analytes and BH_2 have also been demonstrated (56,58,82). Control of pH is needed for these complexation reactions in order to ensure reproducibility.

Several researchers have presented models for the evaluation of extractability of ion pairs (80,86). Hansch and Anderson (86) developed a system for evaluating the effect of a particular functional group by measuring the partition coefficient of a control compound and an analog of the control which contained the functional group of interest. The difference between the log of the partition coefficient of the test compound and the log of the partition coefficient of the control compound was determined and listed as the constant for that functional group. These constants are summed with the relationship that the greater the sum the higher the partition coefficient. These empirical observations have been shown to be useful in predicting distribution properties (58).

Chromatographic Principles and Methodology

Advances in separations technology in recent years have produced an extensive array of new methods. Liquid chromatography has become particularly popular due to its versatility, efficiency and convenience of use.

The advent of bonded-phase packings has further extended this versatility especially for high pressure liquid chromatography (HPLC). A

very popular HPLC column packing consists of octadecyl silane groups chemically bonded to silica particles, producing what is commonly termed ODS reverse-phase packing. Reverse-phase here indicates that elution characteristics of solvents are in reverse order as compared to the eluotropic series used for normal phases such as silica or alumina. Other organic substances have also been bonded to silica with encouraging results, but straight chain alkyl substances remain the most popular among the available HPLC bonded phases.

The capacity factor, k' , is a measure of the affinity a sample has for a chromatography column under a given set of experimental conditions (87). k' is a measure of the mass distribution ratio, D_n , between the column packing and the sample. k' is directly calculated from the chromatogram by

$$k' = (t_R - t_0)t_0^{-1}.$$

Ion-Pair Liquid Chromatography

Ion-pair liquid chromatography, IPLC, is a technique applied to both normal- and reverse-phase systems. Mechanistic interpretations of IPLC recently have focused on the use of reverse-phase systems. Three general explanations have been presented in attempts to interpret retention characteristics of reverse-phase IPLC separations: ion-exchange (88), hatearic interaction (89), and ion-interaction (90). The ion-exchange model assumes that the reverse-phase surface is transformed to an ion exchange surface upon adsorption of an appropriate IPLC reagent. Analytes are thereby retained based on their ability to exchange counter-ions with the adsorbed IPLC reagent. The hatearic interaction

model presumes ion-pair formation in the mobile phase with the analyte separated by surface adsorption as an ion-pair complex. The ion-interaction model bridges these two interpretations with both ion-exchange and ion-pair formation contributing to analyte separation. A thermodynamic model of IPLC retention as also been formulated (91).

Figures 1-3 illustrate some possible orientations of a sample Qam with the HPLC packing and IPLC eluents. Figure 1 illustrates the case of unmodified ODS chromatography with a Qam as the analyte. The primary forces considered are the interactions of the hydrophobic portion of the analyte interacting with the hydrophobic bonded phase and the quaternary nitrogen interacting with the residual silanol groups. There exists little impetus for the ionic analyte to move from its adsorbed state with an aqueous eluent. Modification with an organic solvent like methanol may help solvate the adsorbed analyte but the result would be to drive the analyte to residual silanol groups resulting in tailing of the analyte peak.

Figure 2 illustrates the case where an IPLC reagent, $\text{RSO}_3^- \text{Y}^+$, has been added. The analyte could interact with the a counter ion which imparts a more neutral character to the ion-pair complex. The analyte is still capable of exchanging with residual silanol groups. The capacity of the column for the analyte would decrease but tailing from silanol interaction would persist.

Figure 3 illustrates the case where an IPLC reagent and a silanol deactivating Qam, Q^+ , has been added to the mobile phase. Separation of the ion-pair complex is enhanced by suppression of silanol interactions: The silica surface has been made less available to the analyte.

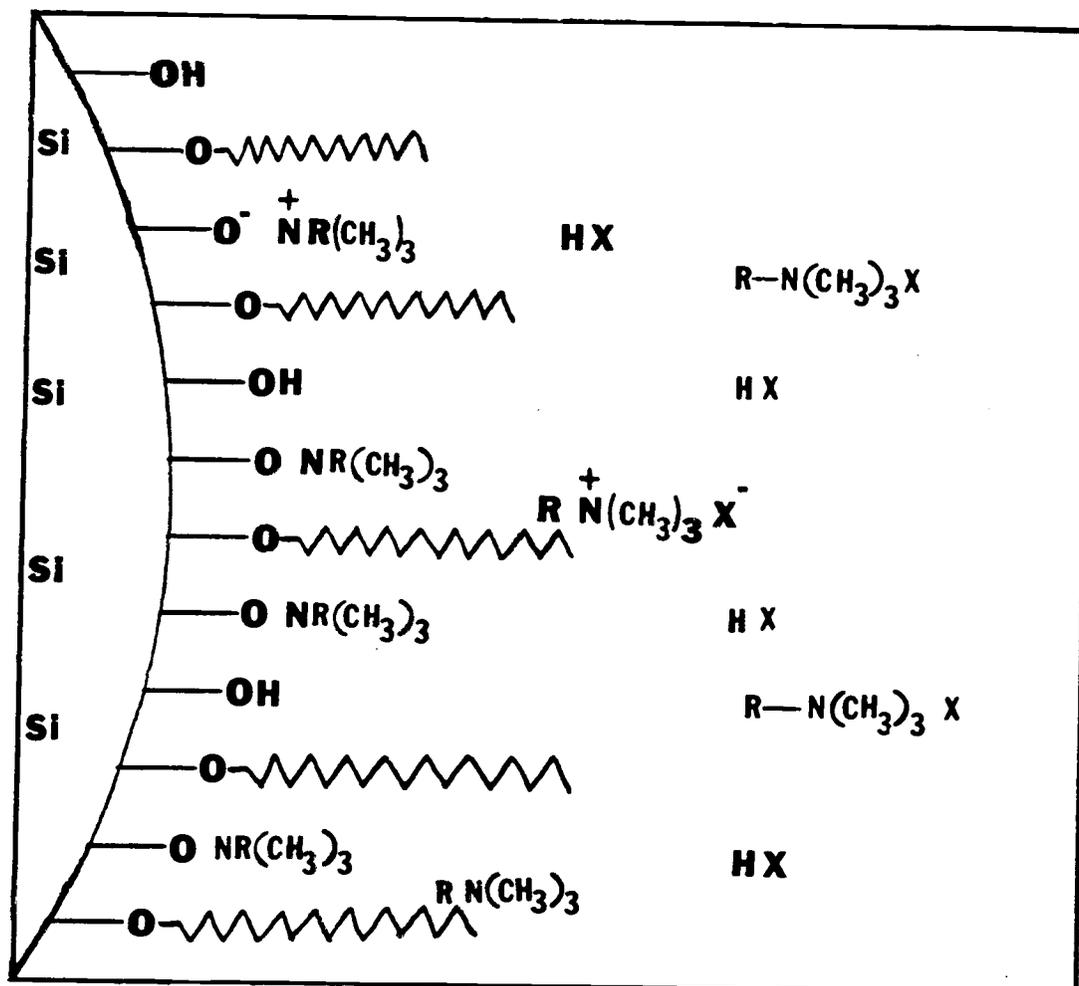


FIGURE 1

Orientation possibilities of a quaternary ammonium compound analyte on an ODS packing surface.

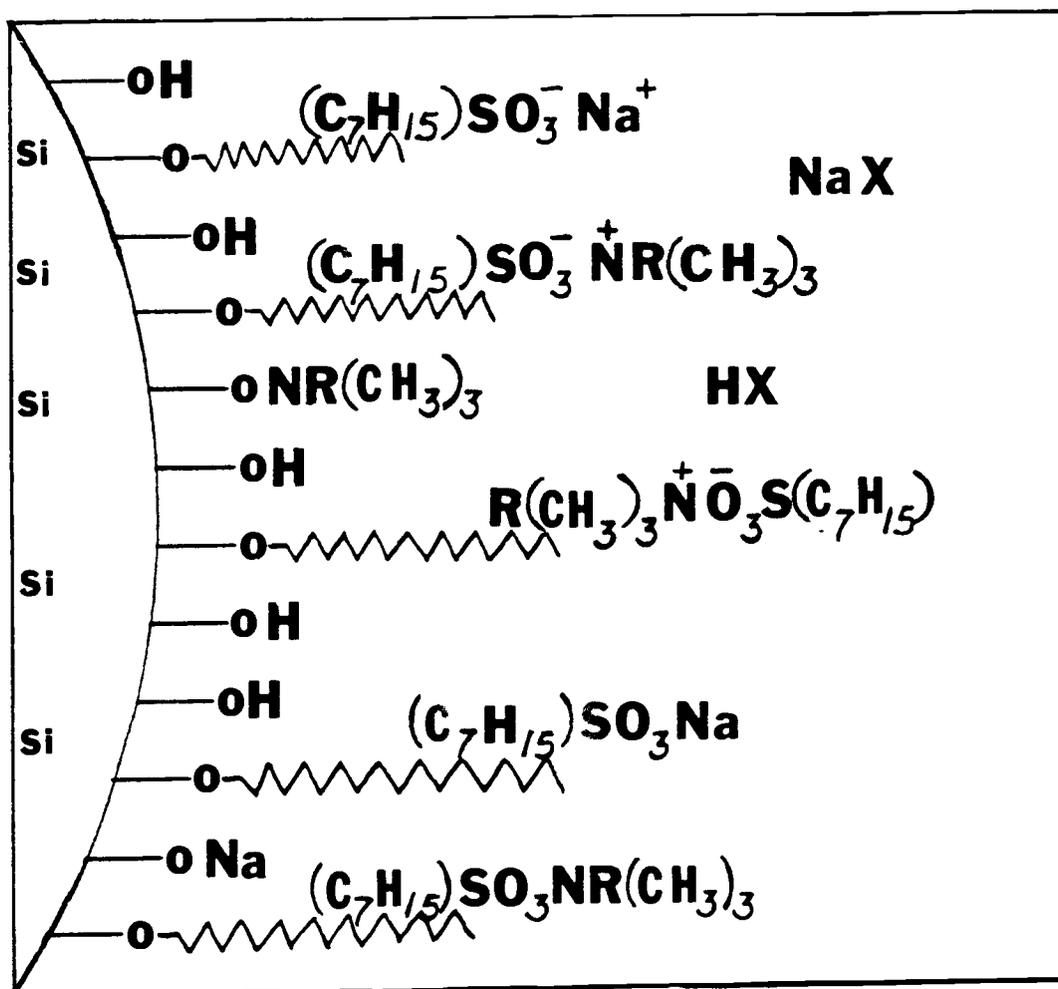


FIGURE 2

Orientation possibilities of a quaternary ammonium compound analyte on an ODS packing surface with an alkyl sulfonate salt added to the mobile phase.

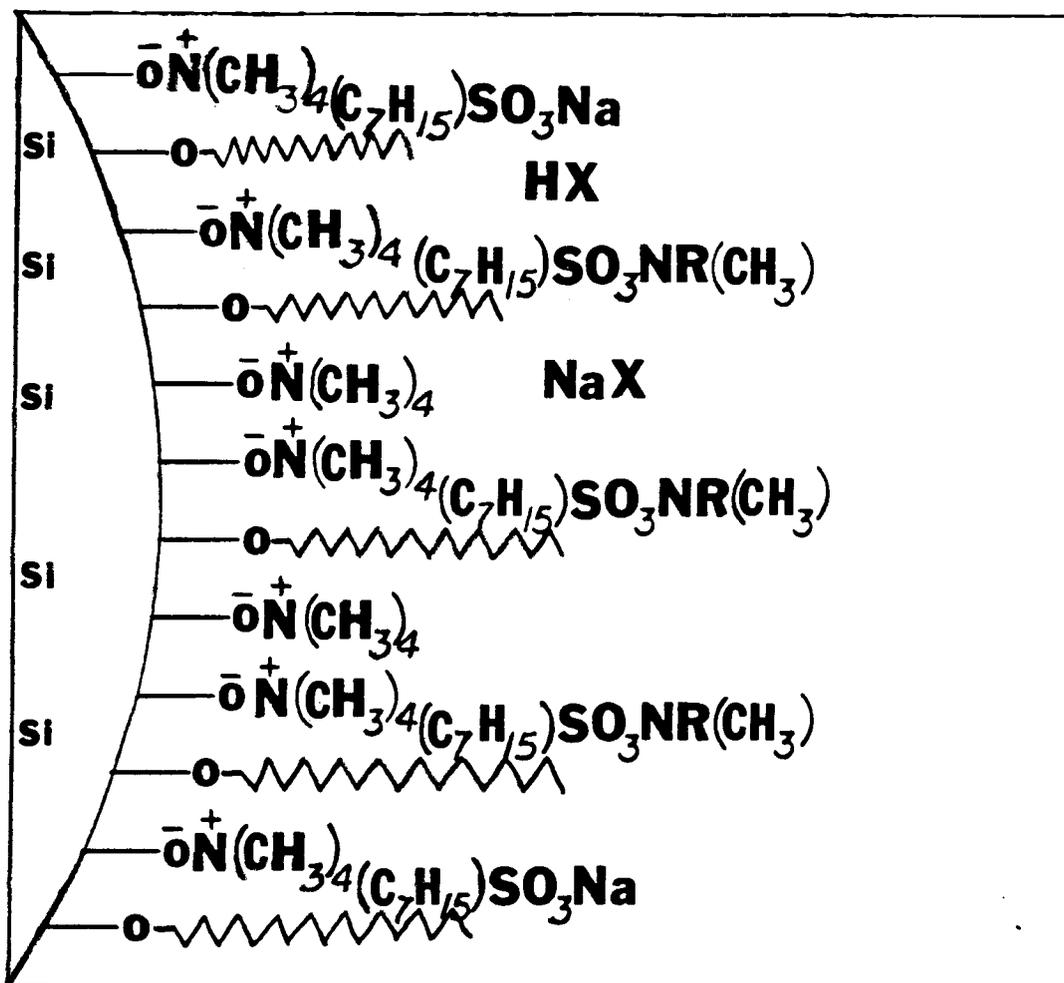


FIGURE 3

Orientation possibilities of a quaternary ammonium compound analyte on an ODS packing surface with an alkyl sulfonate salt and silanol deactivating quaternary ammonium salt added to the mobile phase.

Reaction Detector Performance

Reaction detectors are useful for monitoring analyte species which are difficult to detect using conventional instrumentation. As described earlier reaction detectors have been used for a variety of analytes. Sugars, amino acids, metals, and amines among others have been monitored using reaction detectors. Both LC and GC have employed reaction detectors for monitoring chromatographic eluents. It is possible to predict their performance by evaluation of equations which describe flow profiles. There are generally three types: straight open tubes, coiled open tubes, and bed reactors. Tubular types are further characterized as nonsegmented versus segmented. Segmented reactors involve either air segmentation or liquid segmentation. As the reactor systems become more complicated, so do the expressions which describe their performance.

Reaction detector performance is partially judged by the additional broadening introduced by the system. The total peak dispersion or variance may be modeled as a sum of the variances introduced by the system components,

$$\sigma^2_{\text{total}} = \sigma^2_{\text{inj}} + \sigma^2_{\text{column}} + \sigma^2_{\text{mixer}} + \sigma^2_{\text{reactor}} + \sigma^2_{\text{splitter}} + \sigma^2_{\text{det}} + \sigma^2_{\text{con}}$$

consisting of an injector (σ^2_{inj}), a separation column (σ^2_{column}), a solvent mixer (σ^2_{mixer}), a reactor ($\sigma^2_{\text{reactor}}$), a solvent splitter ($\sigma^2_{\text{splitter}}$), a detector (σ^2_{det}), and connecting tubes (σ^2_{con}). Each component has flow profiles which tend to broaden the sample zone. The mixing due to the injector and column components can be approximated using basic principles of chromatographic theory (87). The reactor sys-

tem can be evaluated using dispersion equations of fluid dynamics.

In a simple open tube, the dispersion of a sample zone flowing through it is given by (92).

$$\sigma^2 = 2D' t_v^3 L^{-2} + R^4 L \bar{Q} (24D_m)^{-1}$$

where D' is the diffusion coefficient (mm^2s^{-1}), t_v the residence time in the tube (s), L the tube length (mm), R the tube radius (mm^2), and \bar{Q} is the volumetric flow rate (mm^3s^{-1}). This expression is a sum of a diffusion term and a mass transfer term. The diffusion component can often be neglected since mass transfer effects generally overwhelm diffusion effects (92).

This expression above may be modified for the case of a coiled open tube by the inclusion of a geometrical factor, K , which modifies the mass transfer dispersion term. This factor is dependent on the solvent viscosity, tube diameter, coil diameter, solvent density and volumetric flow rate.

The models are greatly complicated when one considers gas segmented coiled reactors. Detailed descriptions of mathematical considerations for these systems have been published (83,93,94). Liquid segmentation reactors have other parameters which distinguish them. In considering an extraction detector, several aspects need to be described. For example, the flow rates of the two phases determine the segmentation frequency and length. A detailed study of dynamics of solvent segmentation has been published (95).

EXPERIMENTAL

Experimental Overview

The development of techniques for analyses of Qams was divided into four distinct areas: batch extraction of ion-pairs, continuous flow solvent extraction, chromatography separations for Qams, and the post-column solvent extraction system. The first three were investigated independent of each other with the last area resulting in an assemblage of the other three. Table 1 enumerates the experiments performed for this research in chronological order.

To execute the experiments, selected reagents, instruments, apparatus, and solutions were required. These are listed below in the following sections.

Reagents

All sample quaternary ammonium compounds and bromophenol blue were purchased from SIGMA Biochemical Co., St. Louis, MO. Ion-pairing reagents; heptane sulfonate, octane sulfonate, naphthyl sulfate, and anthraquinone sulfate were purchased from Eastman-Kodak Co.; J. T. Baker, Mallinkrodt, and Burdick and Jackson HPLC solvents were used throughout. For extractions, reagent grade chloroform and deionized water were used.

Apparatus

Eppendorf automatic pipets were employed for addition of volumes less than 1.0 mL. Class A volumetric glassware was used for all other transfers. Bulk extractions were made in 20 x 150 mm pyrex culture

Experiment Number	Experiment Description
1	Batch ion-pair extractions of Qams with BH ₂ and AQS
2	Evaluation of stoichiometry and pH effects on the ion-pair extraction of Qams with BH ₂
3	Evaluation of flow injection analysis ion-pair extraction system for analysis of Qams
4	Chromatographic separations of Qams using ion-pair liquid chromatography
5	Post-column solvent extraction of Qams separated by ion-pair liquid chromatography
6	Gradient separations of Qams with post-column solvent extraction
7	Calibration curves for Neostigmine and Decamethonium developed with isochiatic and gradient techniques, respectively.

TABLE 1. Experimental approach in the development of analysis techniques for the ion-pair extraction, ion-pair separation and post-column solvent extraction of Qams.

tubes with teflon lined caps. Spectrophotometric measurements were made with a Cary 118 UV-Vis spectrophotometer with quartz cells for UV wavelengths and glass cells for visible wavelengths.

Flow injection analysis experiments were performed with a Tracor 980A solvent programming system and a Tracor 955A HPLC. Samples were injected with a Rheodyne 7125 HPLC injector with 20, 50 and 100 L sample loops. The extraction solvent was delivered with a Milton Roy Instrument Co., Mini Pump.

A tee connection was assembled made from a Swagelock 1/16" tee-connector union specially modified to reduce post-column dead volume for the merging of the organic extraction solvent and the chromatography mobile phase. A diagram of the tee connector is shown in Figure 4.

At the tee connection where the extraction solvent and aqueous phase merge the hydrodynamic forces between the immiscible solvent streams act to cause segmentation of the resultant stream into organic and aqueous phase plugs (98). The teflon insert served to improve the segmentation pattern by decreasing the volume of aqueous phase introduced into the segmentor. Prior to this modification segments were irregular and not reproducible. The modification improved segmentation integrity. A flow rate ratio,

$$F_R = \frac{\text{Flow rate of organic solvent}}{\text{Flow rate of chromatography solvent}}$$

of near unity was found to give the good segmentation. Detailed studies of this phenomena were not pursued.

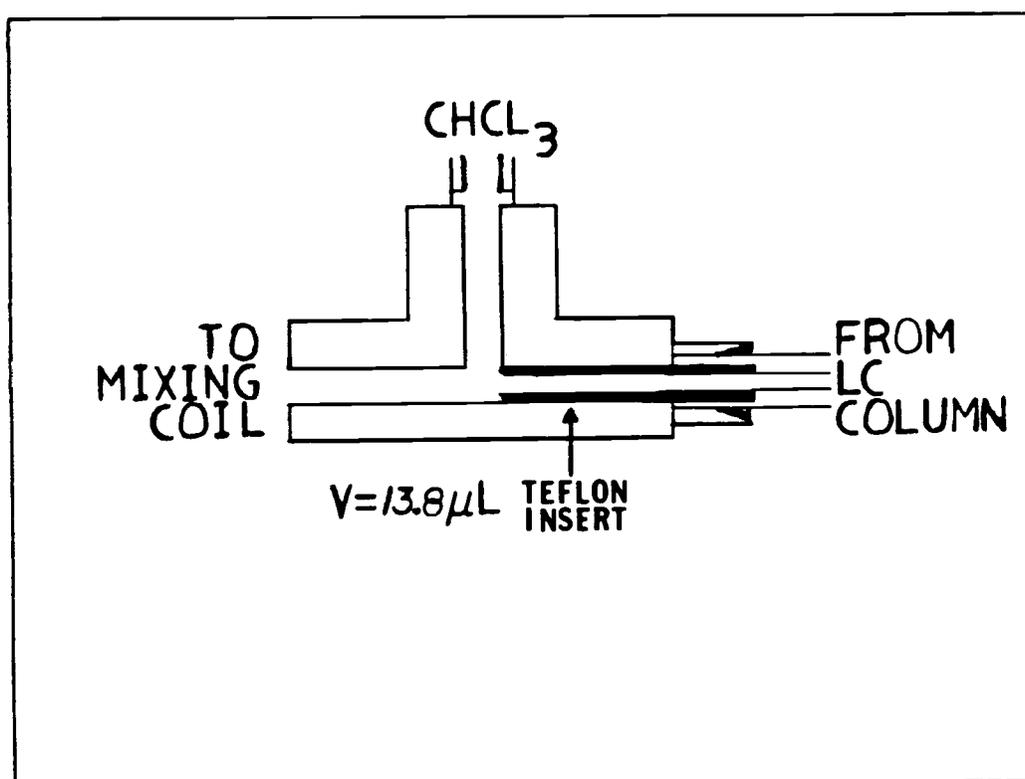


FIGURE 4

Solvent mixing unit for the mixing and segmentation of an aqueous and organic phase modified with a polytetrafluoroethylene insert.

The ion paired analytes were then extracted into chloroform with a mixing/extraction coil. Three types of coils were assembled and tested: glass, Teflon, and stainless steel. The specifications for each are listed in Table 2. For the glass and stainless steel extraction coils the aqueous phase preferentially wetted these surfaces resulting in the circumvallation of the organic phase by the aqueous phase. The Teflon coil was preferentially wetted by the organic phase and the aqueous phase is circumvallated. All three coils were tested but the glass and stainless steel coils were found to produce the least amount of peak broadening. The coiling of the extractor serves to decrease broadening due to edge effects by the presence of secondary flow profiles directed perpendicular to the flow direction (78). The solvent separator was constructed by a glass blower and fitted with PTFE inserts and cheminert fittings as shown in Figure 5. The inside of the solvent separator was treated with a fluorocarbon spray (Care Laboratories Inc., Collegeville, PA) to make the surface hydrophobic. The surface was therefore preferentially wetted by the organic phase which resulted in an easier desegmentation of the stream by forcing the aqueous plugs upward to the waste line, taking advantage of the natural solvent distribution. The backpressure adjustment was made to regulate the flow of solvent through the detector while keeping aqueous phase out.

Chromatographic separations were performed with an IBM Instruments (Danbury, CT) Model 9533 ternary gradient HPLC and a 4mm i.d. x 100mm length column packed with 10 um Lichrosorb RP-18 column packing material. Preliminary investigations were performed on 30-40 m Perisorb RP-18 packing. Column eluents were monitored with either a Laboratory

Coil	i.d. (mm)	o.d. (mm)	coil diameter (mm)	# of coils	length (mm)	volume (mL)
Glass	1.0	2.0	15.2	12	350	0.3
Teflon	0.7	1.6	15.2	16	250	0.1
Stainless Steel	0.5	1.6	15.2	12	250	0.05

TABLE 2. Specifications for reaction detector extraction coils.

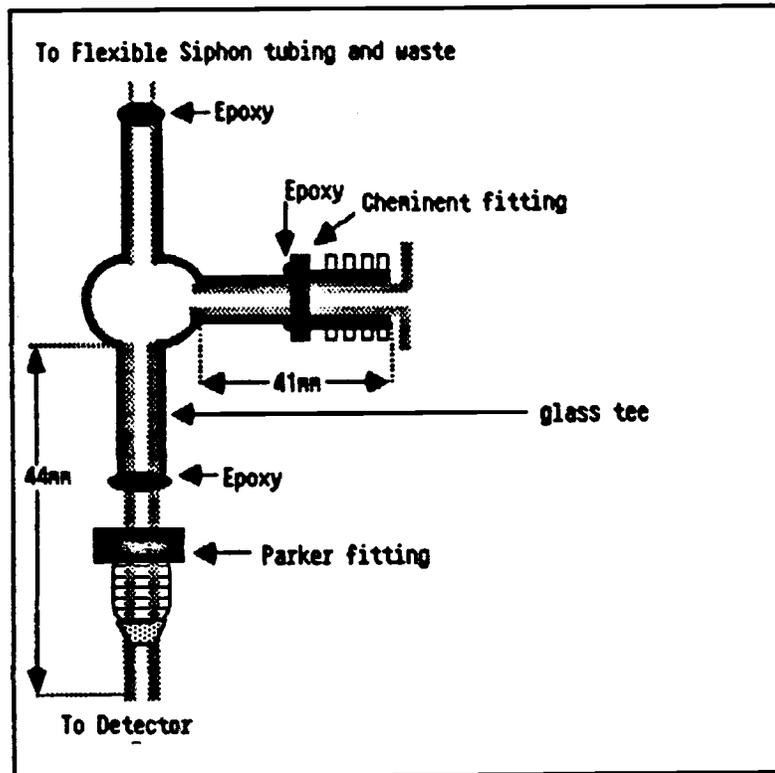


FIGURE 5

1/8" i.d. glass tee treated with a fluorocarbon spray and modified with a teflon insert tube for the desegmentation and splitting of the organic/aqueous stream.

Data Control 254 nm fixed wavelength detector or a Kratos (Westwood, NJ) Model SF770 UV-VIS variable wavelength HPLC detector. Chromatograms were recorded on Linear recorders, a Spectra Physics 4100 computing integrator, or an Hewlett Packard 3390A Integrator.

Solutions

Many different solutions were prepared in the course of this work. The list below gives the preparation procedures used for each solution made.

1. 0.1 M NaOH. Dissolved 1.00 g of NaOH pellets in 200 mL of deionized water and diluted to 250 mL.
2. 10% Na₂CO₃. Dissolved 25.0 g of solid Na₂CO₃ in 200 mL of deionized water and diluted to 250 mL.
3. 0.1 M phosphate buffer. Dissolved 3.00 g of Na₃PO₄ and 3.55 g of Na₂HPO₄ in 400 mL and diluted to 500 mL.
4. 0.1 M heptane sulfonate. Dissolved 5.506 g of C₇H₁₅SO₃Na·H₂O in 200 mL and diluted to 250 mL.
5. 0.1 M octane sulfonate. Dissolved 5.506 g of C₈H₁₇SO₃Na in 200 mL and diluted to 250 mL.
6. 0.1 M tetramethylammonium chloride. Dissolved 2.74 g of solid C₄H₁₂NCl in 200 mL and diluted to 250 mL.
7. 0.6 M bromophenol blue. Dissolved 100.0 mg of bromophenol blue solid in 200 mL with 0.25 mL of solution 1 and diluted to 250 mL.
8. 1.0 mM anthraquinone sulfate. Dissolved 0.3291 g of anthraquinone sulfate sodium salt in 90 mL and diluted to 100.0 mL.

9. 10.0 mM naphthyl sulfate. Dissolved 0.4670 mg of naphthyl sulfate sodium salt in 150 mL and diluted to 200 mL.
10. 10.0 mM tetrabromofluorescein. Dissolved 0.6938 g of tetrabromofluorescein in 90 mL and diluted to 100.0 mL.
11. Sample quaternary ammonium salts. Solutions of the various Qams were prepared by dissolving between 0.5 and 1.0 g of the sample Qam in 5.0 mL and diluting to 10.0 mL. Dilute sample solutions were prepared as necessary from these concentrated solutions.

Procedures

Batch Extractions with BH_2

These batch ion pair extractions were designed to evaluate the distribution properties of Qam with different counter ions. These experiments were also planned to determine the effect of pH on the distribution properties of Qam - bromophenol blue ion pairs. Particularly interesting was the stoichiometry of the ion-pair complex which was the first investigation made. What follows are the procedures.

Aliquots of the concentrated BH_2 solution ranging from 0 to 5 mL were added together with 1.0 mL of 1 mM gallamine triethiodide (GI_3), 1 mL of 0.1 M Na_2CO_3 and taken up to 10.0 mL with deionized water in a culture tube. An aliquot of 10.0 mL of $CHCl_3$ was also added to this solution. The tubes were shaken for 5 minutes, centrifuged at 1500 rpm for 15 minutes, and allowed to settle for 10 minutes. The organic phase was drawn off and its absorbance at 609 nm versus a solvent blank recorded. Reagent blanks were prepared and measured for each sample set.

To evaluate the pH dependence of the IPE of GI_3 with BH_2 , samples were prepared as above except that aliquot combinations of 0.1 M NaH_2PO_4 and 0.1 M Na_2HPO_4 totaling 1.0 mL were added to the extraction tube and the absorbance at 609 nm recorded. Similar procedures were followed to evaluate the pH effect on the extraction of decamethonium bromide.

Batch extractions with the other ion-pair reagents were performed in a similar fashion. The absorbance of the organic phase was recorded at 328 nm for AQS, 277 nm for NS, and 544 nm for TBF versus solvent blanks.

FIA Solvent Extractions

The instrument used in these studies for FIA solvent extraction of ion paired Qam is illustrated in Figure 6. A 10 mM BPB solution was degassed using He purging and pumped with the Tracor HPLC solvent delivery system. The pump was connected to a bypass valve and 20 L valve injector. Following the injector was a transfer line leading to the mixing tee where the extraction solvent was introduced from a second pump. The flow rate of the first pump was set to 1.0 mL/min and the extracting solvent pump to 0.5 mL/min. 20 L samples of Qam solutions were injected into the flowing stream. The extracted Qam ion pairs were then detected by monitoring characteristic absorbances for each IPE reagent employed.

Chromatography Procedures

Separations of sample Qams were done on reverse phase packings with the Tracor HPLC system at a flow rate of 2.0 mL/min. Repetitive injections of benzoyl choline 40 mg each, were made at one-minute intervals

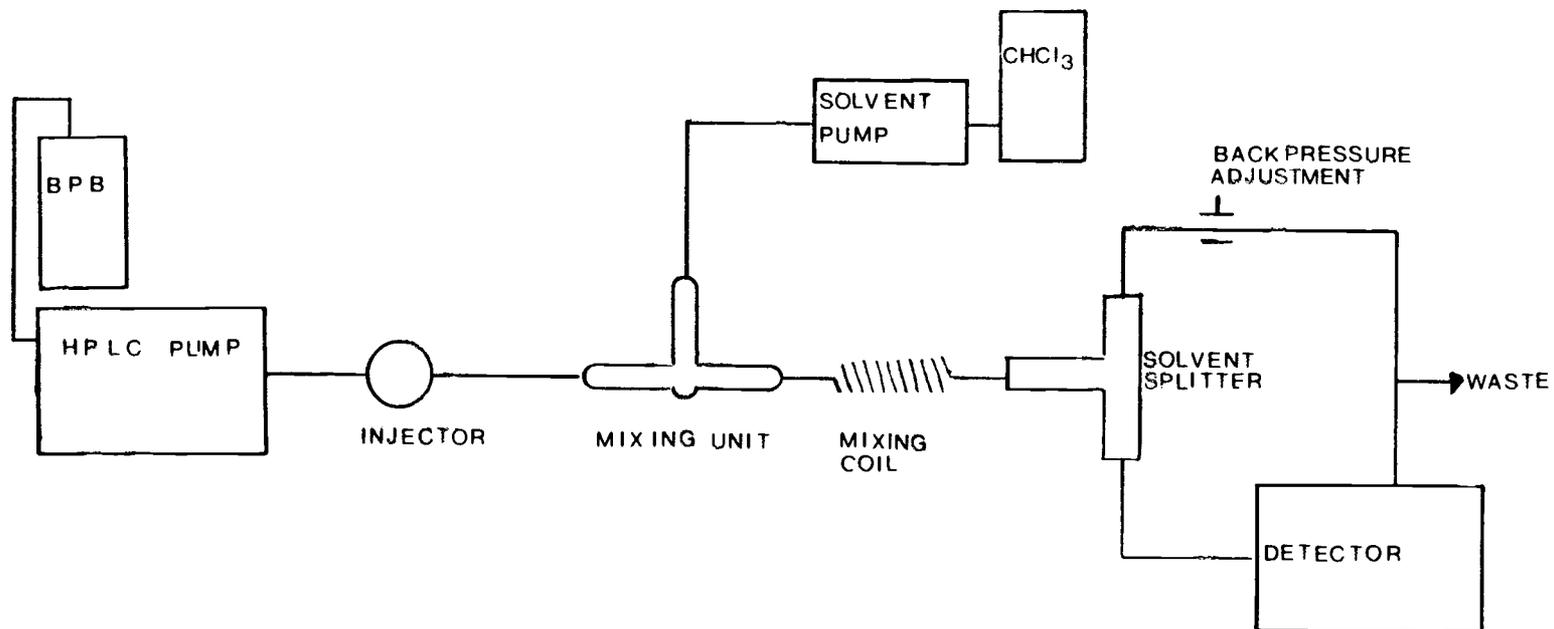


FIGURE 6

Instrumental configuration applied in the solvent segmented flow injection analysis determinations of quaternary ammonium compounds in aqueous solution.

onto a 10 cm length x 2 mm i.d. column packed with 30-40 μm ODS-18 reverse phase particles. These separations were performed with a deionized water eluent.

Ion-pair reagents heptane sulfonate and TMAC were then added to the eluent. The ion-pair reagent concentration was stepped from 0.01 mM to 10 mM in decade increments. Repetitive injections were made and the traces collected. As the ion-pair reagent concentration was increased, the representative peak became sharper with decreasing broadening. Peak shape reached an optimum at 10 mM ion-pair reagent concentration.

High efficiency separations were performed on a column of 10 μm reverse phase particles. To elute Qams with an ion paired eluent an organic modifier of methanol or acetonitrile was needed. Organic modifier percentages between 15 and 45 percent were tested. For methanol percentages of between 25 and 45 were found to give good peak shape in under fifteen minutes. For acetonitrile percentages of between 15 and 30 gave good peak shape in under fifteen minutes. The eluents were monitored at 225 nm for Qams with no phenyl rings and 254 nm for Qams with phenyl rings.

Post-Column Solvent Extraction

The FIA extraction apparatus was attached to the outlet end of the 10- μm octadecyl silica HPLC column. The organic modifier was limited to between 25 and 45 percent for methanol. The buffer component of the eluent consisted of 10.0 mM alkyl sulfonate salt, 10.0 mM TMAC and 1 mM BH_2 . The flow rate was 0.8 mL/min for the separation and from 0.5 to 0.9 mL/min for the extracting solvent, chloroform. The flow rate for the extracting solvent was adjusted to provide 3 mm long solvent plugs

in the solvent separator. The instrumental arrangement for the post-column solvent extraction of Qam analytes is shown in Figure 7.

A twenty minute equilibration time for the IPLC system was used for the ion-pair separations. The flow of solvent through the detector was established by adjustment of the back pressure regulator on the waste outlet of the separator system. The amount of back pressure necessary for adequate flow through the detector was dependent on the percentage of MeOH; as % MeOH increased so did the necessary back pressure.

Column Packing

In-house column packing was accomplished with the apparatus shown in Figure 8. Approximately 2.5 to 10 grams of packing material was measured into the chamber along with a stirring bar. The column was packed with methanol as the solvent. The column was secured to the fitting on the top and the pump connected to its fitting. Stirring was initiated. Methanol was pumped at a flow rate of 3 mL/min until the packing system was filled. Once this was verified, the flow rate was increased to 7 mL/min and the pressure was monitored until there was approximately a 5000 psi drop across the column and column packer. Once this pressure was reached, the flow rate was dropped to 5 mL/min and pumping continued for thirty minutes. After this time, the pressure drop across the column packing device was recorded and the pump was stopped. The column was capped with a suitable fitting and it was attached to the LC pump. Two gradients were run; first from 100% MeOH to 100% H₂O and then 100% H₂O and 100% MeOH. This was done twice. These gradient runs were made with the detector set to a low wavelength to observe any elution of serious column impurities. The column was then

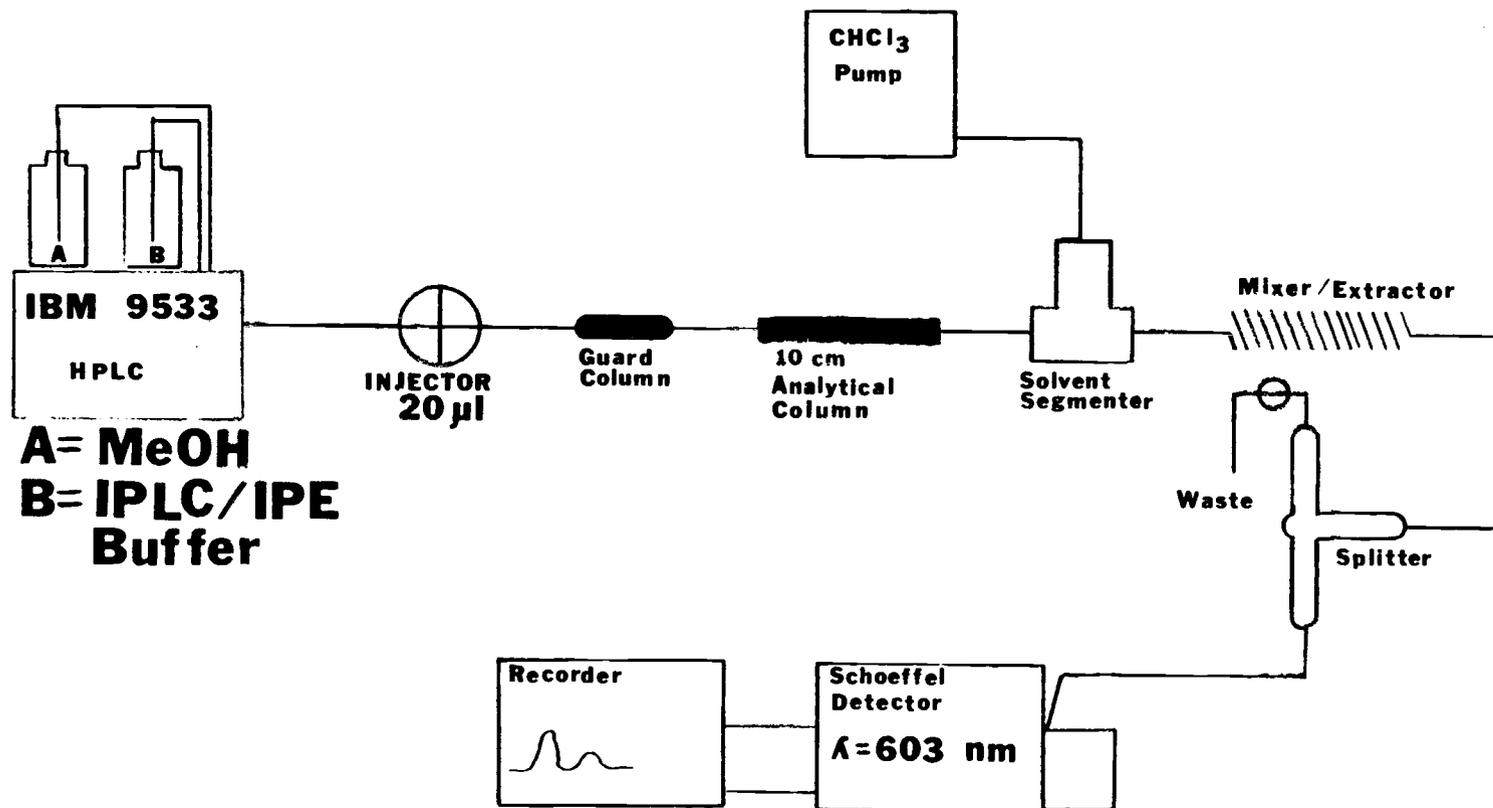


FIGURE 7

Instrumental configuration for the ion-pair liquid chromatography separation and post-column solvent extraction of quaternary ammonium compounds.

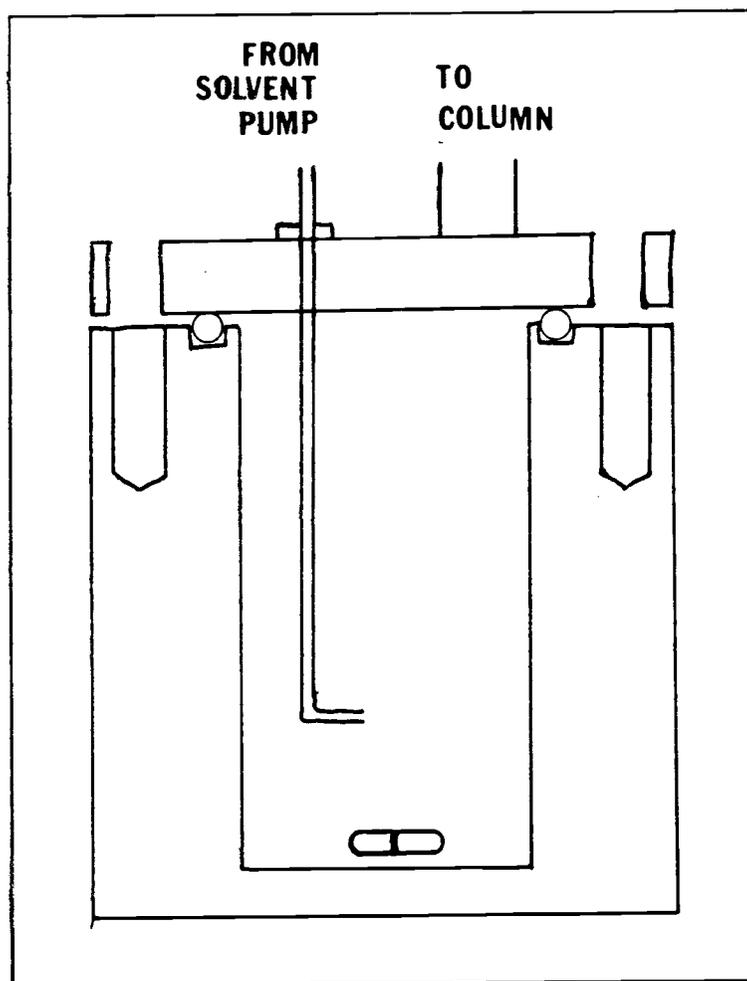


FIGURE 8

Apparatus for the packing of HPLC columns using the upflow technique.

tested using any available test mixture of known elution characteristics. The column efficiency was then computed according to accepted procedures (87).

RESULTS AND DISCUSSION

Overview

The presentation order of experimental results represents the progression of this research from the initial investigations of the ion-paired extraction of Qams to the assembly and testing of the final system for the separation and determination of Qams.

Ion-Pair Extractions

Batch Extractions

The distribution of gallamine triethiodide (GI_3) as an ion paired complex with bromophenol blue (BH_2) into $CHCl_3$ was measured as a function of the BH_2 concentration. The absorbance at 609 nm was plotted versus the volume of BH_2 added to the aqueous phase as shown in Figure 9. The purpose of this experiment was to determine the relationship between counter-ion concentration and GI_3 IPE efficiency.

A more insightful perspective was gained when the x-axis was relabeled to display the molar ratio of BH_2 to GI_3 . The data are plotted Figure 10 as the absorbance vs. the ratio of BH_2 to GI_3 . Linearity was assumed in the first three points and a plateau region assumed with the last two points. Lines were interpolated based on these two assumptions and a perpendicular dropped at their intersection. That point was determined to be 1.663 or approximately a 5 to 3 ratio. From this plot, it was concluded that for every 3 moles of GI_3 , 5 moles of BH_2 are needed for IPE.

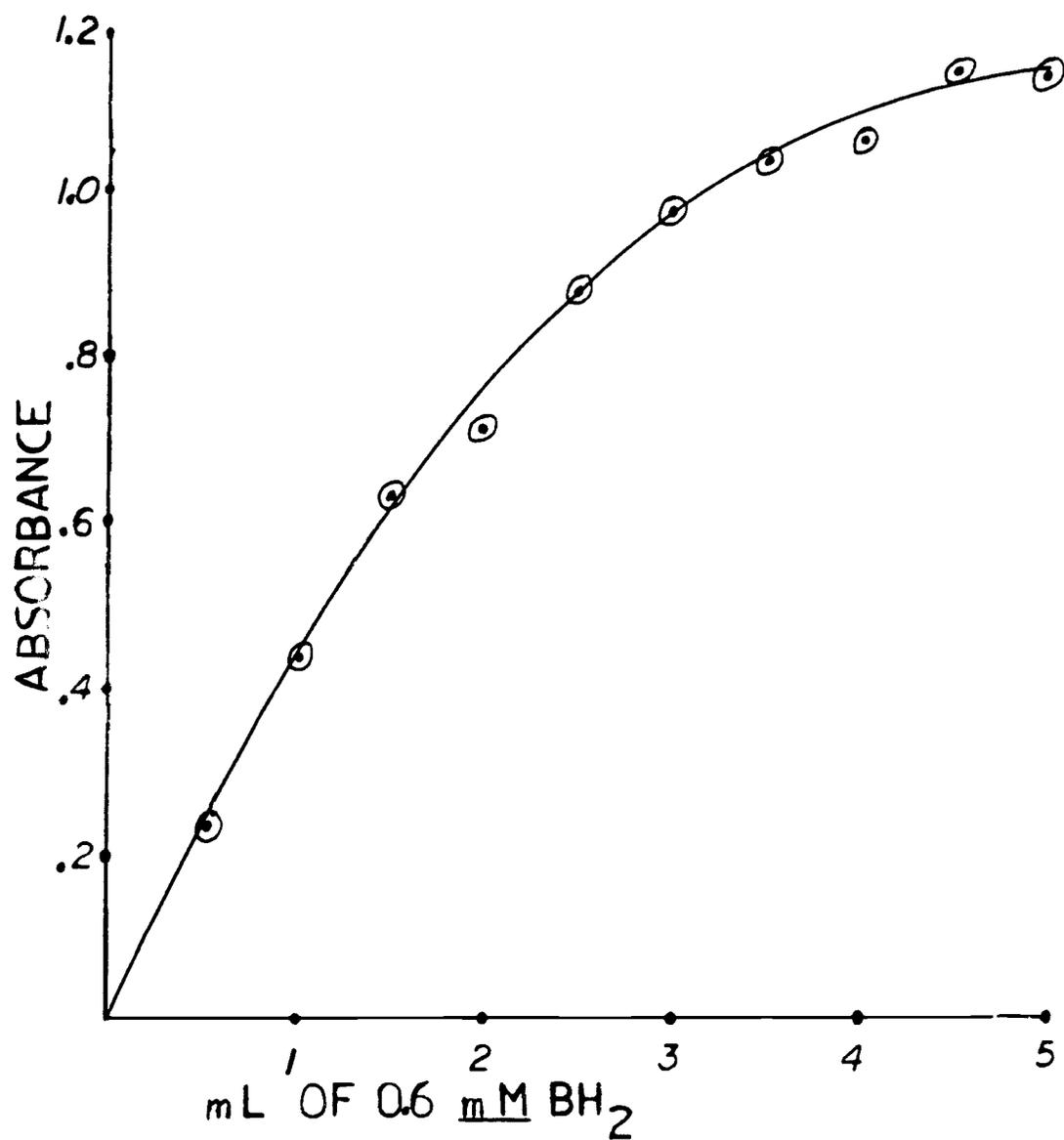


FIGURE 9

The absorbance of the CHCl₃ organic extraction solvent at 609 nm vs. the number of mL of 0.6 mM BH₂ added to a 1.0 mL aliquot of 1 mM GI₃, and 1.0 mL of 10% Na₂CO₃ in a total volume of 10.0 mL.

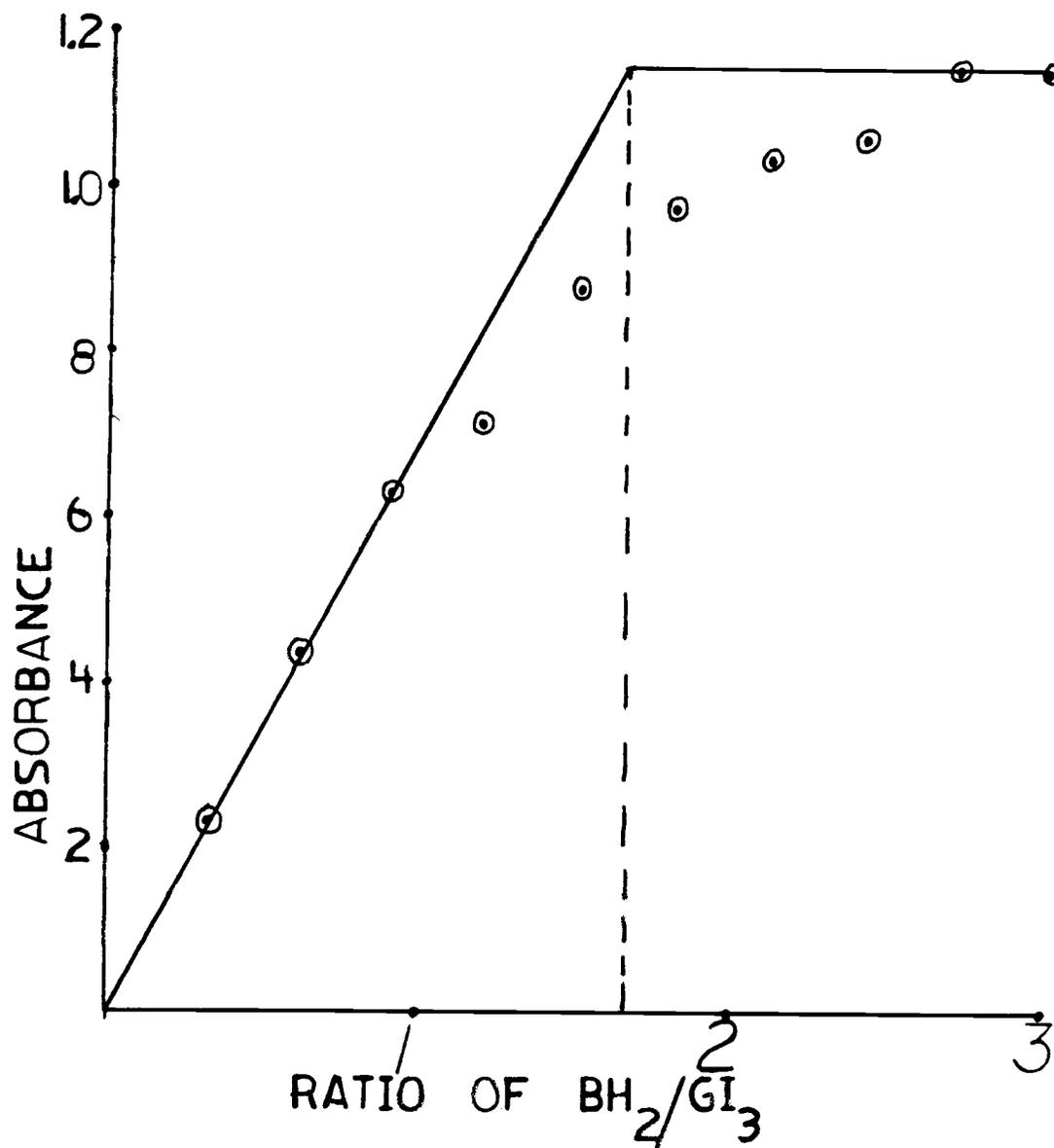


FIGURE 10

The absorbance of the CHCl_3 organic extraction solvent at 609 nm vs. the molar ratio of BH_2/GI_3 in solutions of 0.1 mM GI_3 , 1.0% Na_2CO_3 .

The stoichiometry based on a charge balance treatment predicts a 3 to 2 ratio between BH_2 and GI_3 . BH_2 is not stable in basic solution. Degradation is particularly pronounced in the presence of Qams (96). This would result in an artificially high value for the experimental equivalence point. Assay purity of GI_3 was stated at 95.0% minimum. This would result in an artificially low value for the experimental stoichiometry value. In the event that the 5 to 3 ratio is correct then 6 equivalents of an additional anionic counter-ion would be needed for charge balance. The most available being I^- . It is possible that I^- is participating in the ion-pair complex.

Measurement of the pH dependence on the IPE of GI_3 with BH_2 was also performed. The purpose of this experiment was to determine the effects of pH on the distribution of the $\text{GI}_3\text{-BH}_2$ ion-pair complex. Figure 11 illustrates the absorbance of the organic extraction phase vs. the aqueous pH value. This result points to an optimum pH of greater than 9.

A similar set of extractions were performed with decamethonium bromide (DBr_2) as the Qam analyte and BH_2 as the IPE reagent. The absorbance of the organic phase at 609 nm was plotted vs. the pH of the aqueous phase in Figure 12. The pH profile indicated that a pH of 5 or greater resulted in the highest absorbance. It is important to note that these experiments are not plotting the absorbtivity of BH_2 as a function of pH but the distribution of $\text{DBr}_2 - \text{BH}_2$ ion pairs as a function of pH.

The shape of the pH profile with a maximum point is not readily explained. There was no verification of this profile and a logical ex-

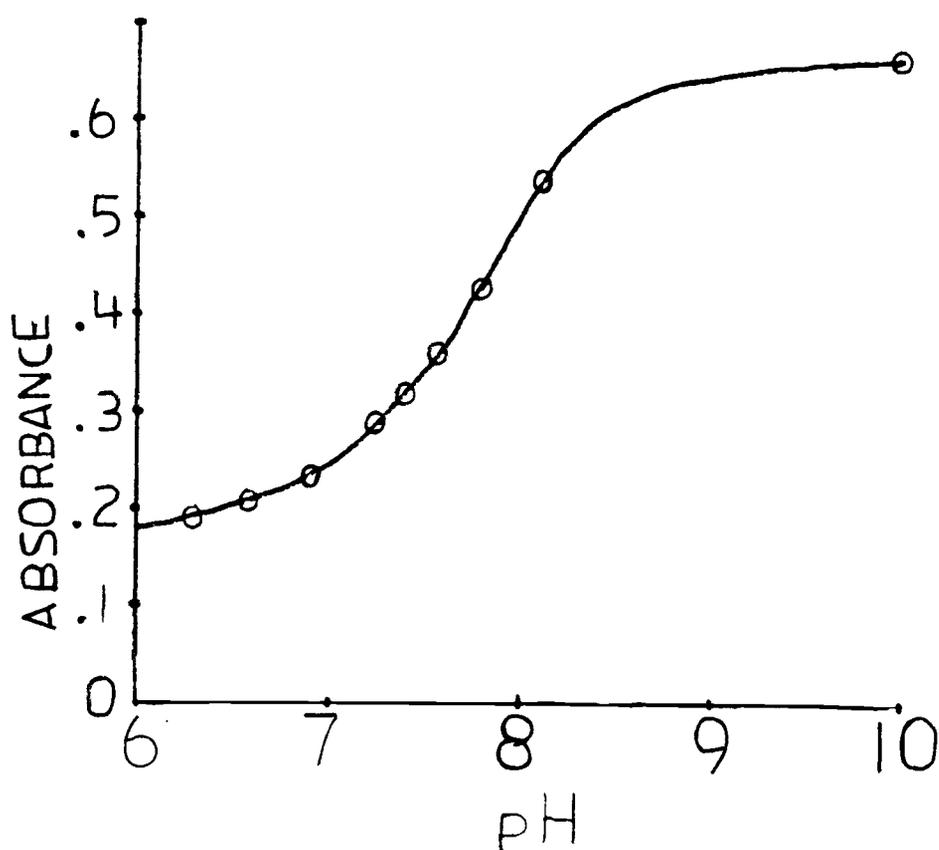


FIGURE 11

The absorbance of the CHCl_3 organic extraction solvent at 609 nm vs. the pH of the aqueous phase containing 0.1 mM GI_3 and 0.3 mM BH_2 . Variation of pH produced by the addition of different volume ratios of 0.1 M NaH_2PO_4 and 0.1 M Na_2HPO_4 .

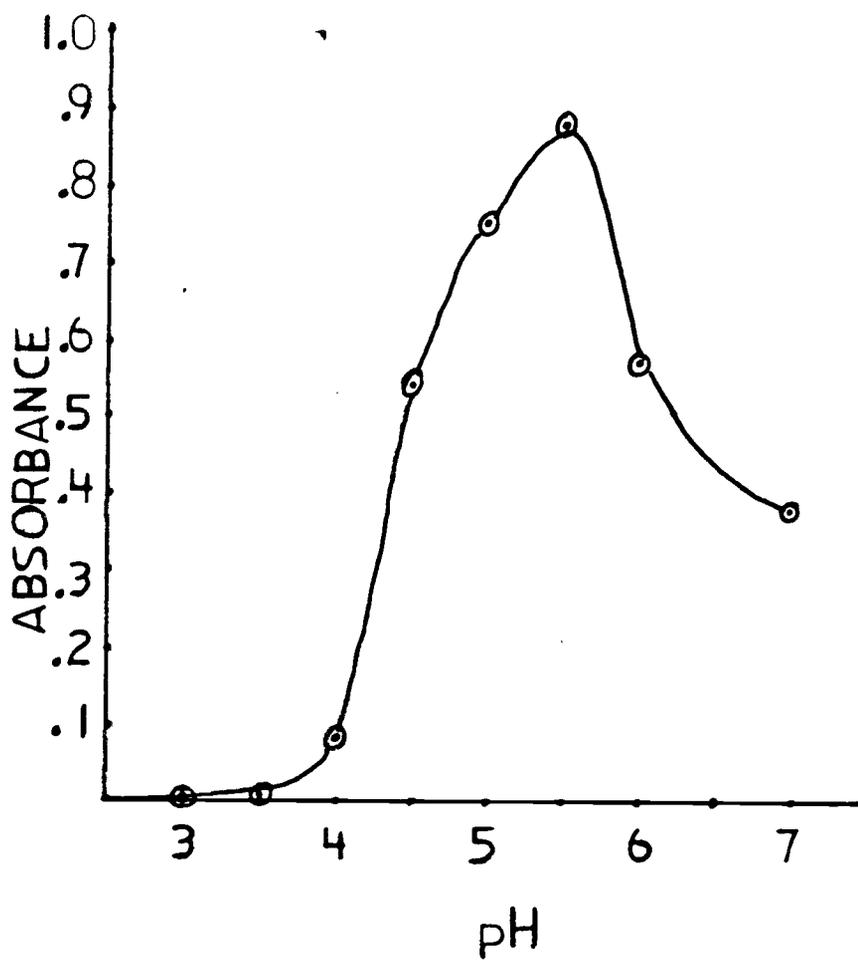


FIGURE 12

The absorbance of the CHCl_3 organic extraction solvent at 609 nm vs. the pH of the aqueous phase containing

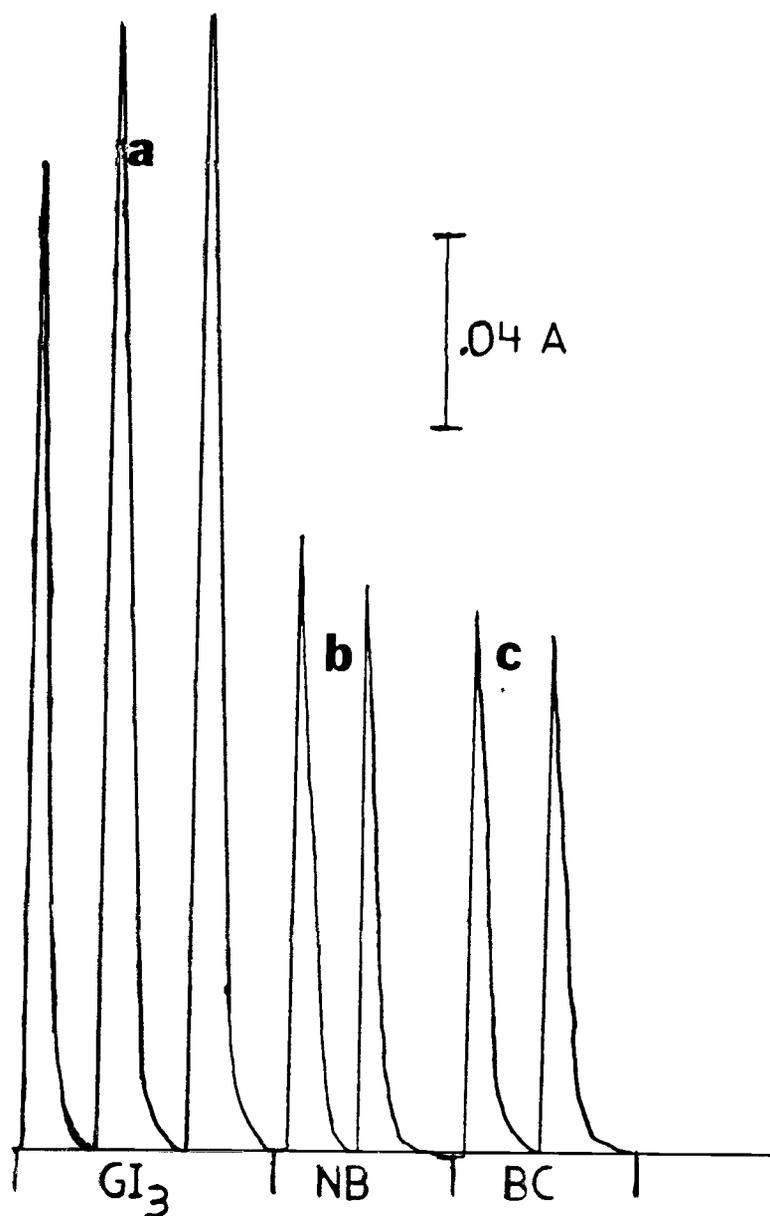
perimental resolution was not pursued. BH_2 degradation is one possible explanation. As BH_2 is lost, less BH_2 is available for ion pairing and saturation is achieved more rapidly.

For GI_3 the optimum pH was above 9 and for DBr_2 the optimum was above 5. These results could be explained by the nature of the counter ion paired to the analyte Qam. Iodide has greater nucleophilic character than bromide. Hence it was a greater affinity for the cationic quaternary nitrogen. A larger pH would be needed to displace iodide relative to bromide.

Flow Injection Analysis

Bulk ion-pair distribution extractions would be difficult and time consuming. To streamline the investigations, ion-pair extractions of Qams were performed on an in-house developed solvent segmented flow injection analysis extraction system using BH_2 as the ion-paired extraction reagent. The apparatus used for these analyses is diagrammed in Figure 6.

The experiments using the FIA system were designed to determine the extractability of several Qams. The peak absorbance of each Qam injected was divided by its concentration. This absorbativity quotient was then divided by the absorbativity quotient for GI_3 . The resultant number was termed the extraction ratio for the particular Qam sample relative to GI_3 . Figure 13 displays a series of three samples consisting of two injections each of gallamine triethiodide (556 mg), neostigmine bromide (667 mg), and benzoyl choline chloride (440 mg). Values of the extraction ratio for several Qams are given in Table 3.

**FIGURE 13**

Three 20 μ L injections of a) 31.2 mM GI_3 , b) two 20 μ L injections of 110 mM Neostigmine Bromide and c) two 20 μ L injections of 110 mM Benzoyl choline chloride into a 0.808 mM BH_2 to which 50 L of 0.1 M NaOH was added. The extraction solvent was $CHCl_3$ and the flow rates 1.6 mL/min aqueous, 0.37 mL/min $CHCl_3$.

Quaternary Amine	Extraction Ratio	# of Quaternary Nitrogens	k'
Acetylcholine	0.0041	2.23	0.8
Carbamylcholine	0.0048	.79	--
Succinylcholine	0.0052	2.23	1.1
Propionylcholine	0.0223	2.73	1.0
Butyrylcholine	0.0314	3.23	1.4
Acetyl-B-methylcholine	0.0672	2.73	0.9
Benzoylcholine	0.139	5.09	2.2
Neostigmine	0.148	3.22	1.1
S-butylthiocholine	0.206	—	2.1
Tubocurarine	0.245	14.34	6.4

TABLE 3. Extraction ratios, π constant summations divided by the number of quaternary nitrogens, and capacity factors on an ODS column for several Qams.

Table 3 also lists the values for the sum of the constants for each Qam tested on the FIA system. Comparison of the extraction ratio values and the constant summation revealed a correlation between predicted extractability and that observed in the experiment. Other interactions assist in the ion-pair formation and extraction such as steric bulk of functional groups and regio-chemical interactions. An example of this is the comparison of acetyl- β -methyl choline and propionyl choline. With the same molecular weight and π constant sum, it might be predicted that both would have the same extraction ratio. They are, however, significantly different. Their only difference is the distribution of functional groups attached to the molecular skeleton. The inclusion of an additional methyl group on the carbon adjacent to the acetyl group may disrupt the water structure surrounding the acetyl-methyl choline sample and increase the potential for ion pairing.

Similar batch extraction experiments were carried out using other ion pairing agents. Figure 14 illustrates the concentration dependence of the extraction of GI_3 with AQS where the blank corrected absorbance of the organic phase is plotted versus the amount of AQS present. In contrast to the BH_2 experiment, reagent blank correction was important when using AQS as the ion-paired extraction reagent. This was demonstrated in Figure 15 where the absorbance of the reagent blank organic phase is plotted vs. the amount of AQS as two absorbance maxima of AQS.

NS and TBF were also tested as ion-paired extraction reagents. Blank extraction and handling difficulties made the use of these reagents difficult. A blank extraction behavior similar to the AQS case

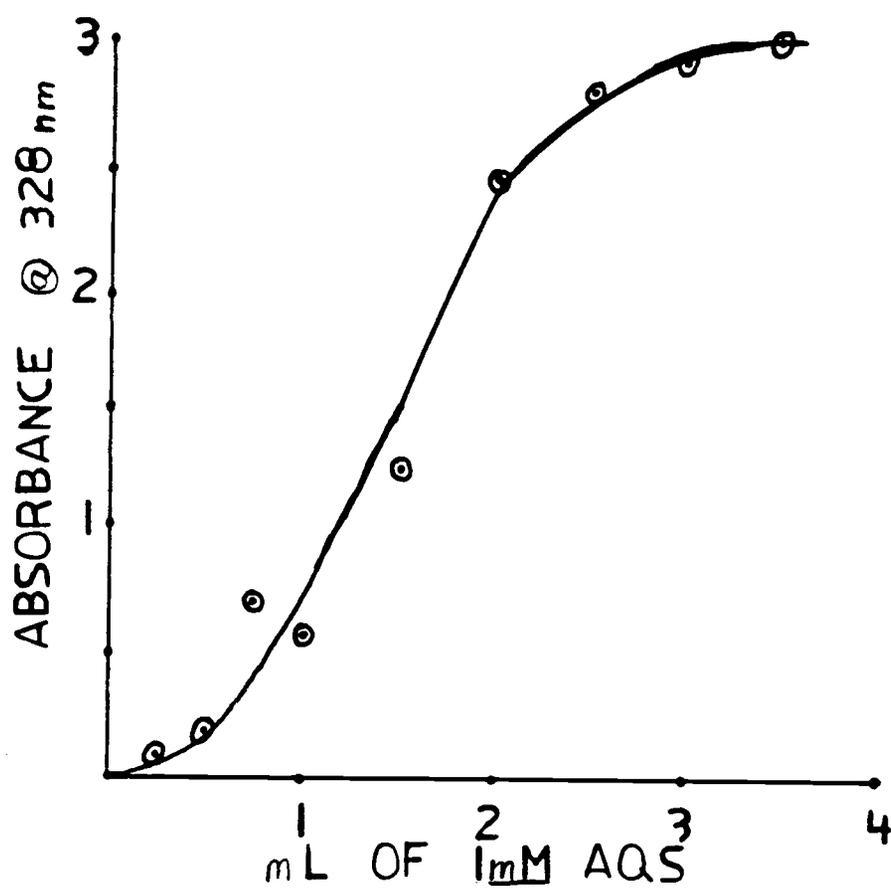
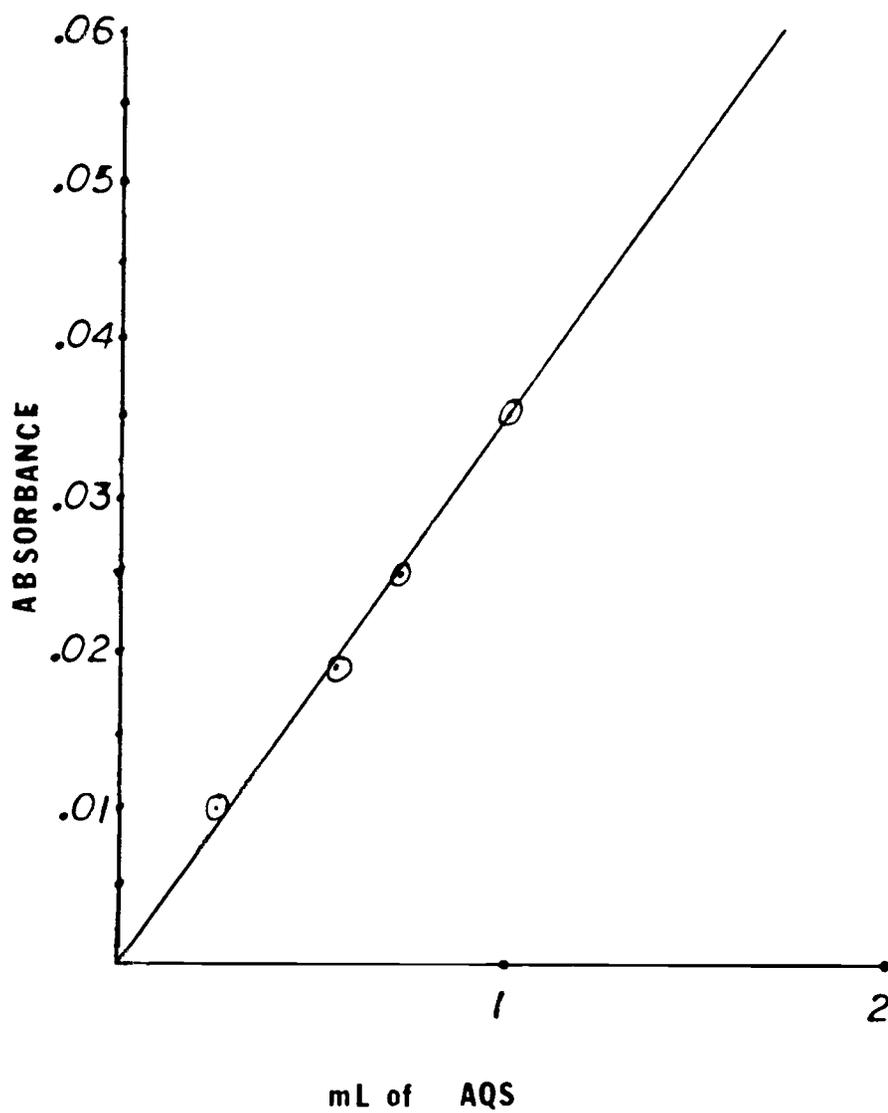


FIGURE 14

Absorbance of CHCl_3 organic extraction solvent at 254 nm vs. number of mL of 1 mM AQS added to a 0.1 mM GI_3 solution.

**FIGURE 15**

Absorbance of the CHCl₃ extraction solvent at 254 nm vs. the number of mL of 1 mM AQS added to a 10 mL total volume.

was observed with NS. This signal displayed a dependence on concentration. Detailed distribution studies were not performed. TBF, on the other hand, was difficult to use reproducibly due to its highly adsorbent nature and high staining potential. These difficulties precluded the further investigation of TBF as an ion-pair extraction reagent.

Chromatographic Development

Most modern liquid chromatography separations are performed on columns packed with silica particles onto which an octadecane alkyl group has been bonded. These columns exhibit high stability and efficiency for long periods of time. The eluents used for separations on these columns are readily available and easily disposed. Mobile phase eluents can be easily modified with a variety of reagents to produce a wide range of separation techniques for many different samples. For these reasons separations of Qams were developed on octadecylsilica (ODS) particles.

Initial separations of Qams were developed on a very low efficiency 40 μ m particle ODS column using an aqueous eluent. The elution profiles observed for benzoyl choline were extremely broad indicating very strong interactions between the analyte and the column. The benzoyl choline analyte was essentially irreversibly adsorbed onto the stationary phase.

To decrease the adsorptive activity of stationary phase, the aqueous eluent was modified with sequential additions of an alkyl sulfonate ranging in concentration from 0.01 to 10 mM. Injections of benzoyl choline were made and the chromatogram tracing was obtained. As the alkyl sulfonate concentration increased, the peak profile for an in-

dividual injection improved. Peak profiles sharpened, retention times decreased and peak widths decreased. Profile improvements ceased improving beyond the 10 mM concentration level. Peak tailing did however persist.

To decrease peak tailing, the aqueous phase was again modified with a silanol deactivating Qam namely tetramethyl ammonium chloride (TMAC) in progressively higher concentrations. Injections of benzoyl choline were made and the chromatogram tracing obtained. Peak tailing decreased as the concentration of TMAC increased. Again no further improvement was observed for TMAC concentrations above 10 mM.

Modern liquid chromatography separations are characterized by theoretical plate values of between 100 and 10,000 for most separations. Higher plate numbers are possible but for this work they remained modest. Thus the next step in the chromatography development was the use of a high efficiency column. With this step it became necessary to modify the eluent with an organic solvent like acetonitrile or methanol. Separations were performed with acetonitrile as the organic modifier and the 10 mM alkyl sulfonate TMAC IPLC buffer. Acetonitrile percentages were varied in 5 (% v/v) increments from 0 to 50 (% v/v). Organic modifier percentages of less than 10 (% v/v) produced broad peaks and long retention times and percentages of 50 (% v/v) and greater resulted with essentially no retention of the sample. An additional concern was the possibility of salt precipitation with increasing organic modifier percentage. No salt precipitation was observed at the 50 (% v/v) level but it was an element to be aware of.

Acetonitrile was used as the organic modifier for developing chromatograms for several Qams. Methanol could have also been used as the organic modifier but acetonitrile worked initially with good sharp peak profiles. As will be discussed later, acetonitrile was incompatible with BH_2 in the post column solvent extraction analyses. Generally samples elute faster when acetonitrile is used while elution order is retained. Sample selectivity could be more rapidly determined with acetonitrile. Acetonitrile organic modifier was used to develop chromatograms for several Qams. Samples of acetyl, propionyl, acetyl-methyl, succinyl, butyryl, benzoyl and S-butyryl thiocholine along with neostigmine and tubocurarine were separated using this eluent composition. Values for the capacity factors for these samples under these conditions are listed in Table 3. As with the ion-pair extraction of experiments, a comparison of the constant summations with the observed capacity factors revealed a correlation. the constant exercise was useful not only in prediction of extractability but also in prediction of relative retention.

An example of the chromatography was the analysis of succinyl choline. Figure 16 displays the trace obtained when two injections of succinyl choline were made onto the chromatography system. Three components and a hint of a fourth can be observed from these analyses. The early eluting peaks may be breakdown products of succinyl choline or simply impurities which naturally occur with the sample. There is a possibility that these early peaks are artifacts produced by reaction with acetonitrile and water in the mobile phase. None of these possibilities were investigated any further.

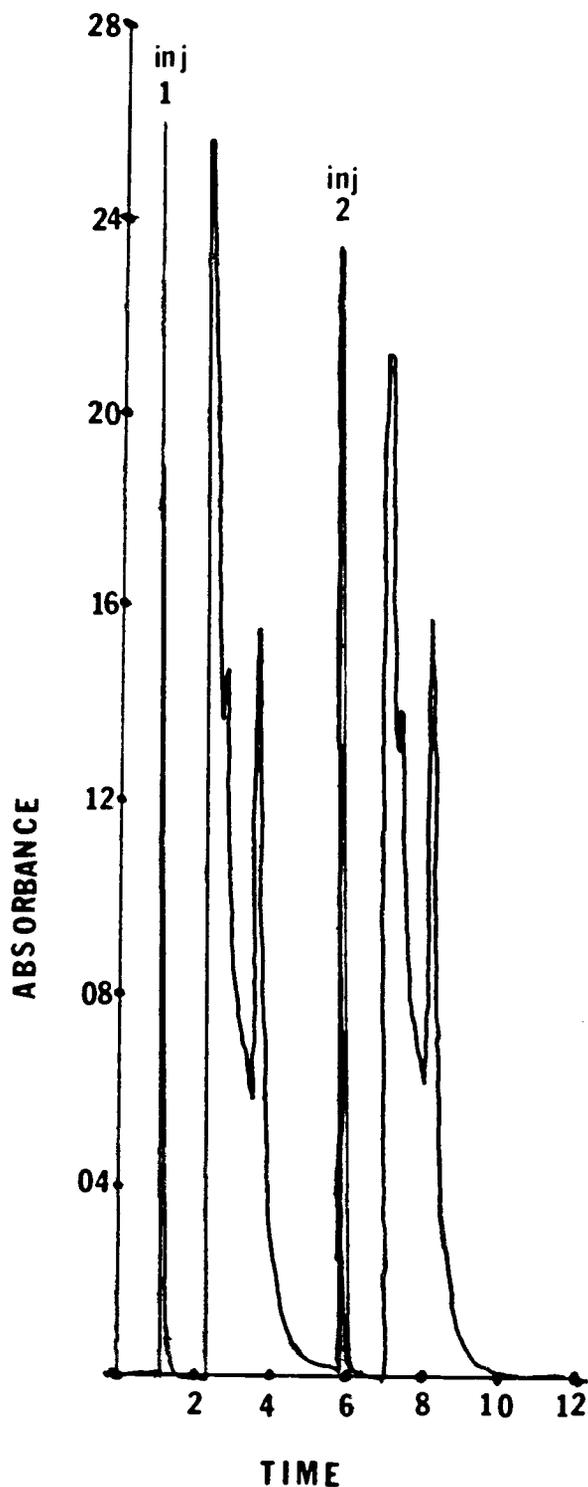


FIGURE 16

Chromatogram of two successive 20 μ L injections of 0.2 M succinyl choline (a) separated on a 10 cm Lichrosorb ODS-18 column with 30% CH_3CN and 70% 10 mM octane sulfonate sodium salt and 10 mM tetramethylammonium chloride as the eluent at 1.0 mL/min monitored at 225 nm. Impurities b and c not identified.

For the separation of Qams, the capacity factors, k' , generally increased as molecular weight increased. Similarly, and perhaps more descriptively, k' increased as the sum of constants increased. This is also a consequence of theory in that the greater the hydrophobicity of the groups attached to the Qam, the greater its tendency to form a stable, strong ion pairs. Consequently, the ion pair will be more strongly retained on the reverse phase surface of the column. The capacity factors observed for several Qams detected at 225 nm after eluting off of a 10 cm, 10 μ m octadecyl silica column with an eluent of 30% CH_3CN 70% 10 mM octane sulfonate and 10 mM TMAC at 0.8 mL/min were ranked as follows; acetyl choline < acetyl- ~~β~~ -methyl choline < propionyl choline < succinyl choline < neostigmine < butyryl choline < benzoyl choline < S-butylthiocholine < tubocurarine. This elution order was the same when methanol was used as the organic modifier.

Post Column Solvent Extraction

Following the investigation of the chromatography which separated Qams, the post column solvent extraction system was added to the system. The instrumental arrangement used for these analyses is diagrammed in Figure 7. Separations of Qams were developed previously with acetonitrile which is stronger than methanol as a solvent for reverse phase separations. The elution order for analytes separated with acetonitrile as the organic modifier remained the same when the separation was performed with methanol. The retention volume, thus k' , for a Qam analyte separated with acetonitrile was less than that for the same percentage of methanol. For this application, it was quickly determined that acetonitrile was incompatible with BH_2 for the post-column extrac-

tion of Qams. Upon the addition of even a very small amount of acetonitrile, the chloroform extraction solvent became discolored, perhaps due to extraction of BH_2 or some reaction product of acetonitrile and BH_2 . For this reason, separations were developed with methanol as the organic modifier: Methanol did not result in such interference.

For the post column solvent extraction of ion paired Qams, a new set of separation parameters using methanol was needed.

The separations developed with methanol as the organic modifier which was stepped in 10% increments from a starting point of 20%. As the concentration of methanol increased the retention time and peak half width decreased. Also as the concentration of methanol increased the baseline absorbance at 609 nm also increased at the rate of 0.002 Abs Units/% methanol increase. Additionally the concentration of the Qam- BH_2 ion-pair complex in the organic phase increased with methanol concentration. Peak height for neostigmine increased at the rate of 0.004 Abs. units/% Methanol increase. As an example a 50 μ L injection of neostigmine with 30% methanol showed a peak absorbance of 0.015. For 50% methanol modifier the observed peak absorbance was 0.076. Baseline noise also increased with methanol concentration as well as the occurrence of artifacts in the form of mobile phase corpuscles which contaminated the organic phase. This occurrence was particularly pronounced at methanol concentration greater than 40%. Concentrations of less than 25% methanol resulted in long analysis times and thus analyses were focused at methanol concentrations of between 25 and 40 (v/v %). An upper limit of 50 percent methanol was found to be the maximum allowable level of modifier. Above this level, the eluent and the

extraction solvent became miscible and high absorbances in the chloroform phase levels resulted in intolerable interferences.

Resolution, analysis time, sensitivity, and noise level were all dependent on the chromatography conditions. The following variables were critical in these separations; organic modifier concentration, anionic ion-pair reagent concentration, cationic ion-pair reagent concentration, IPE reagent concentration, pH, ionic strength, along with the variables affecting reaction detector performance which include, flow rate, materials of construction, and design specifications.

The system was then tested with a test solution consisting of propionyl choline, butyryl choline and S-butyryl thiocholine. The resulting chromatogram is shown in Figure 17. The mobile phase was modified with 40% methanol. The IPLC buffer was composed of 10 mM octane sulfonate, 10 mM TMAC, and 1 mM BH₂. The elution order was propionyl choline, followed by butyryl choline followed S-butyryl thiocholine. the resolution for this separation is adequate for identification but may not be sufficient for quantitation.

An experiment using choline chloride as the silanol deactivating agent was performed. The resulting chromatogram is given in Figure 18. Although only limited conclusions can be drawn from such an experiment, the use of a Qam modifier which more closely resembles the sample may enhance selectivity.

Next, the phase separation and detection systems were evaluated for reproducibility. Five consecutive injections of 180 μ g of neostigmine were done. The trace obtained for the five samples is shown in Figure 19. Peak area measurements were made and the % RSD was found to be

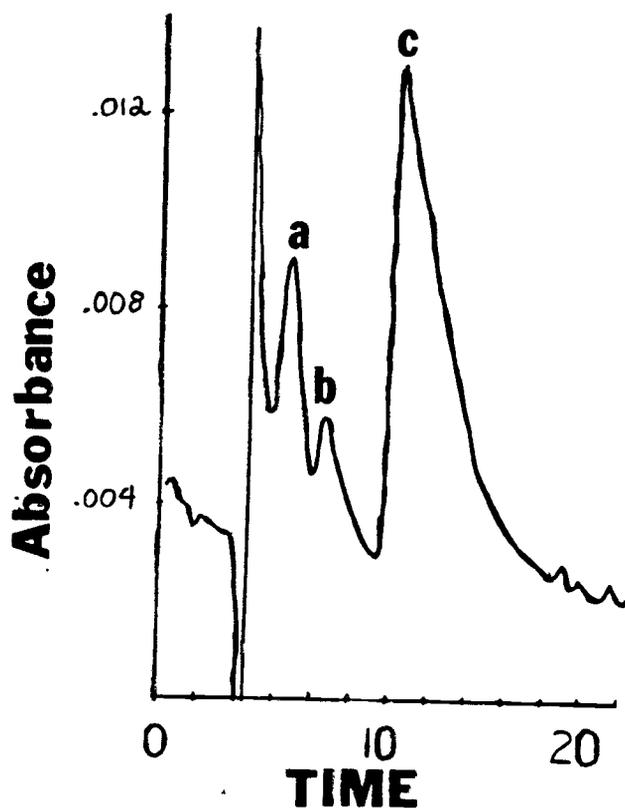


FIGURE 17

Chromatogram of a 20 μ L injection of a) 0.1065 M propionyl choline chloride, b) .0241 M benzoyl choline chloride and c) .0403 M S-butryl thiocholine chloride developed on a 10 cm Lichrosorb ODS-18 column with 40% CH_3OH and 60% 10 mM octane sulfonate sodium salt, 10 mM tetramethyl ammonium chloride, and 1 mM BH_2 as the eluent at 1.0 mL/min. CHCl_3 was added at 0.75 mL/min post column with the CHCl_3 monitored at 603 nm.

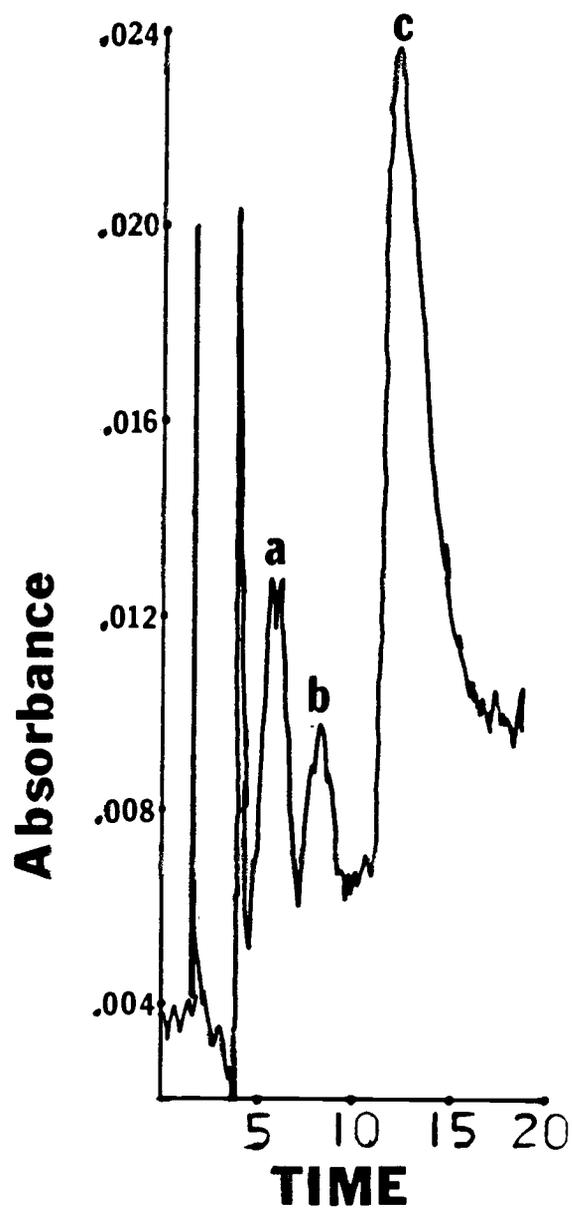


FIGURE 18

Separation of a) propionyl choline, b) butyryl choline and c) S-butyryl thio-choline as in Figure 17 except that 10 mM choline chloride was used instead of 10 mM TMAC in the eluent.

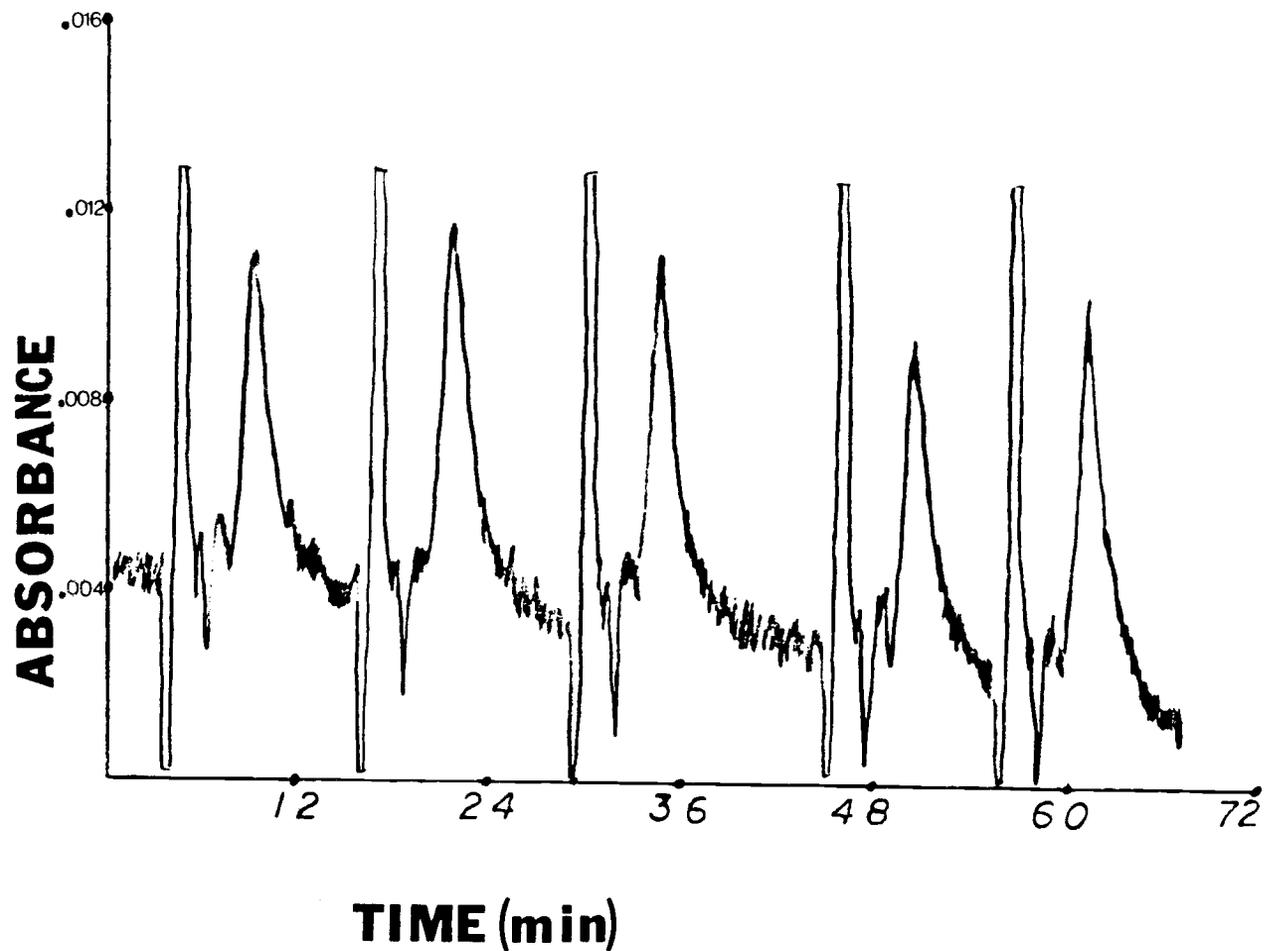


FIGURE 19

Recorder tracings of 5 consecutive 50 L injections of 11.9 mM Neostigmine solution separated with same conditions as in Figure 17.

6.5%. A detection limit for neostigmine was estimated to be 60 ug.

The broadened peaks observed in the chromatograms had essentially two components; a chromatographic component and a detector component. For neostigmine, the peak width at half height was observed to be 1.75 min. without the post column extraction system and 2.15 min. with the post column extraction system installed. Similarly, for benzoyl choline, the peak half width in the absence of the post column extraction system was observed to be 4.5 min. and 4.9 min. with the post column extraction system installed. Thus peak broadening due to the post-column solvent extraction phase separation system was approximated at between 0.4 and 0.6 minutes.

Band broadening in segmented post column systems is dependent on a number of factors. Segmentation frequency is directly related to peak variance (92). Broadening in a segmented reactor as a function of segmentation frequency shows Van Deemter-like behavior in that a minimum occurs between mass transfer and diffusion effect zones. Generally peak variance decreases with segmentation frequency until diffusion effects begin to predominate. It was soon discovered that segmentation frequency was also a function of organic modifier percentage. As organic modifier increased, segmentation frequency decreased. This was related to the change in surface tension and viscosity of the chromatography eluent relative to the extraction solvent. Obtaining reproducible segmentation frequency was somewhat difficult. The design of the mixer, the relative flow rates, relative surface tension of the two solutions, and back pressure conditions all contributed to the detectors performance.

It was also observed that band broadening was also related to the materials used in making the mixing coil. The teflon coil produced more corpuscle spikes in the detector than the glass coil. This was primarily due to the viscosity effects and the increased mixing seen by the extracted ion pair complex in the teflon coil relative to the glass coil. The glass coil had the disadvantage of being fragile and there were accidents. However fabrication of replacements was rapid and inexpensive.

The final component of the system was the phase separation device. It performed the function of splitting the organic extraction solvent away from the chromatography eluent. It too contributed dead volume to the system and thereby broadened peaks. Adjustment of the back pressure in the detector and/or waste end of the splitter permitted the control of flow into the detector keeping unwanted contaminant mobile phase excluded from the light path. The geometry employed in these analyses was but one of several possible designs (93).

Gradient HPLC is a useful way to obtain selectivity in a separation. For post column solvent extraction of a chromophoric ion pair, gradient changes alter the distribution properties of the complex. Drastic baseline shifts were also observed. Figure 20 shows a gradient separation of propionyl choline, butyryl choline and S-butyryl thiocholine. Selectivity was dramatically improved relative to the isochatic separation described above. Separation and resolution of propionyl choline and butyryl choline, which differ by only one carbon, was achieved. Neostigmine was also eluted by a gradient program. Retention time indicates neostigmine eluting just before S-butyryl

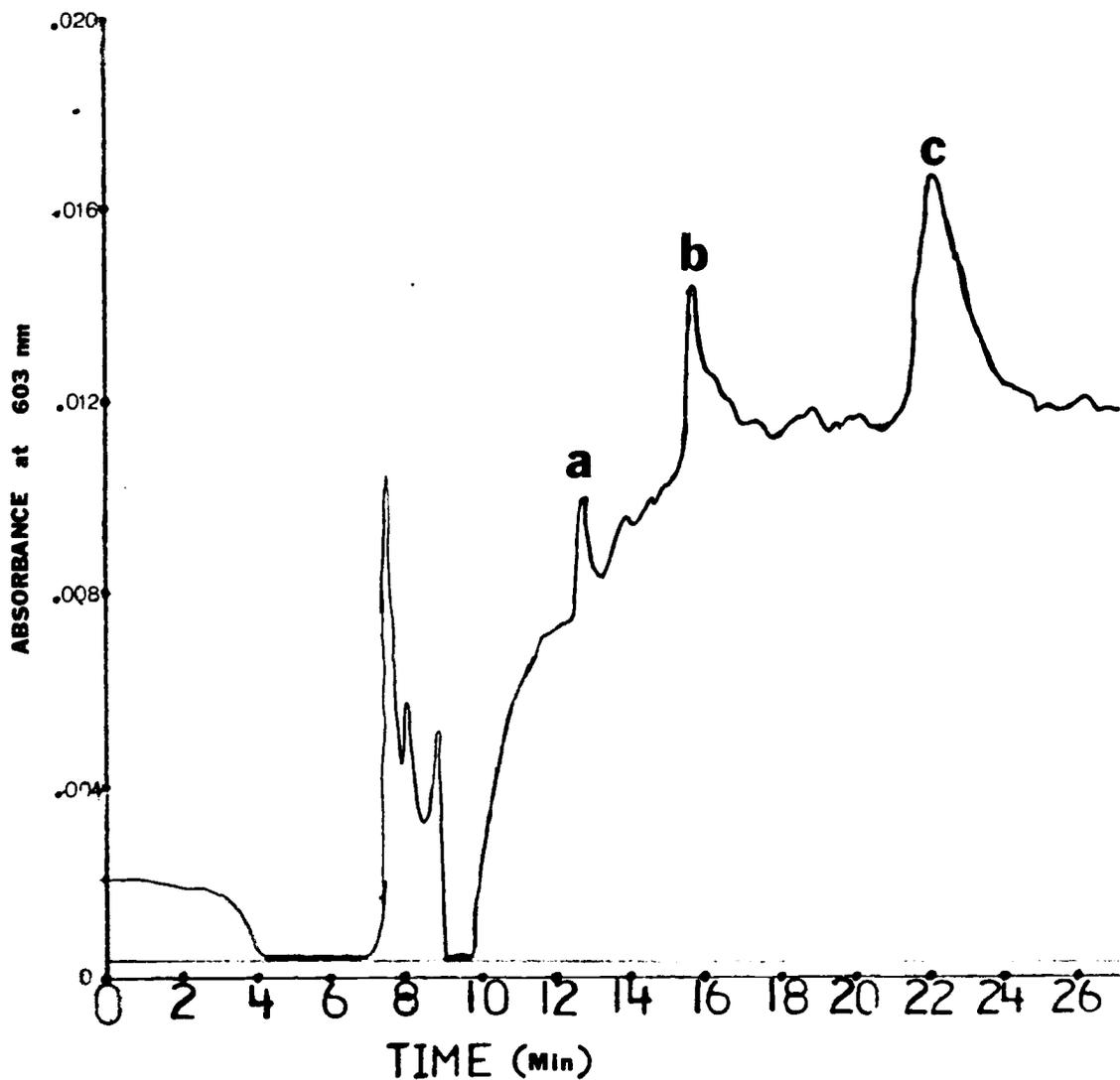


FIGURE 20

Chromatogram of a gradient separation using the PCSE apparatus to detect a) propionyl and b) butyryl choline and c) S-butyryl thiocholine. The aqueous eluent was the same as in Figure 17. The gradient used was 0% CH_3OH for 5 minutes which was increased to 40% CH_3OH in a linear fashion over 10 minutes and maintained for 15 minutes.

thiocholine. The gradient used was 0% MeOH for 5 minutes which was increased to 40% MeOH in a linear fashion over 10 minutes and maintained for 15 minutes.

The post column extraction system was then tested for its utility as a quantitative tool. A calibration curve for neostigmine was developed under isochatic conditions and is displayed in Figure 21. This plot demonstrates a linear response for neostigmine in the 1 to 130 ug/ml range. As mentioned earlier, blending of the isochatic solvent mixture prior to separation resulted in a smoother, less noisy baseline. This calibration curve was obtained using mechanical blending and thus performance suffered. However, linearity over two orders of magnitude concentration range was very encouraging. The concentration range, which brackets the calibration curve, permitted the analysis of a ophthalmic solution for neostigmine bromide. This was attempted on a 1% neostigmine sample solution but benzoyl alcohol, which is a solution preservative, interfered with the chromatography and determination was obtained.

A calibration curve was also prepared for gradient separation using decamethonium bromide was the analyte. The curve obtained is shown in Figure 22. This substance eluted at the peak of the gradient. Baseline assignment was somewhat difficult, but the calibration curve still demonstrated linear response over the concentration range.

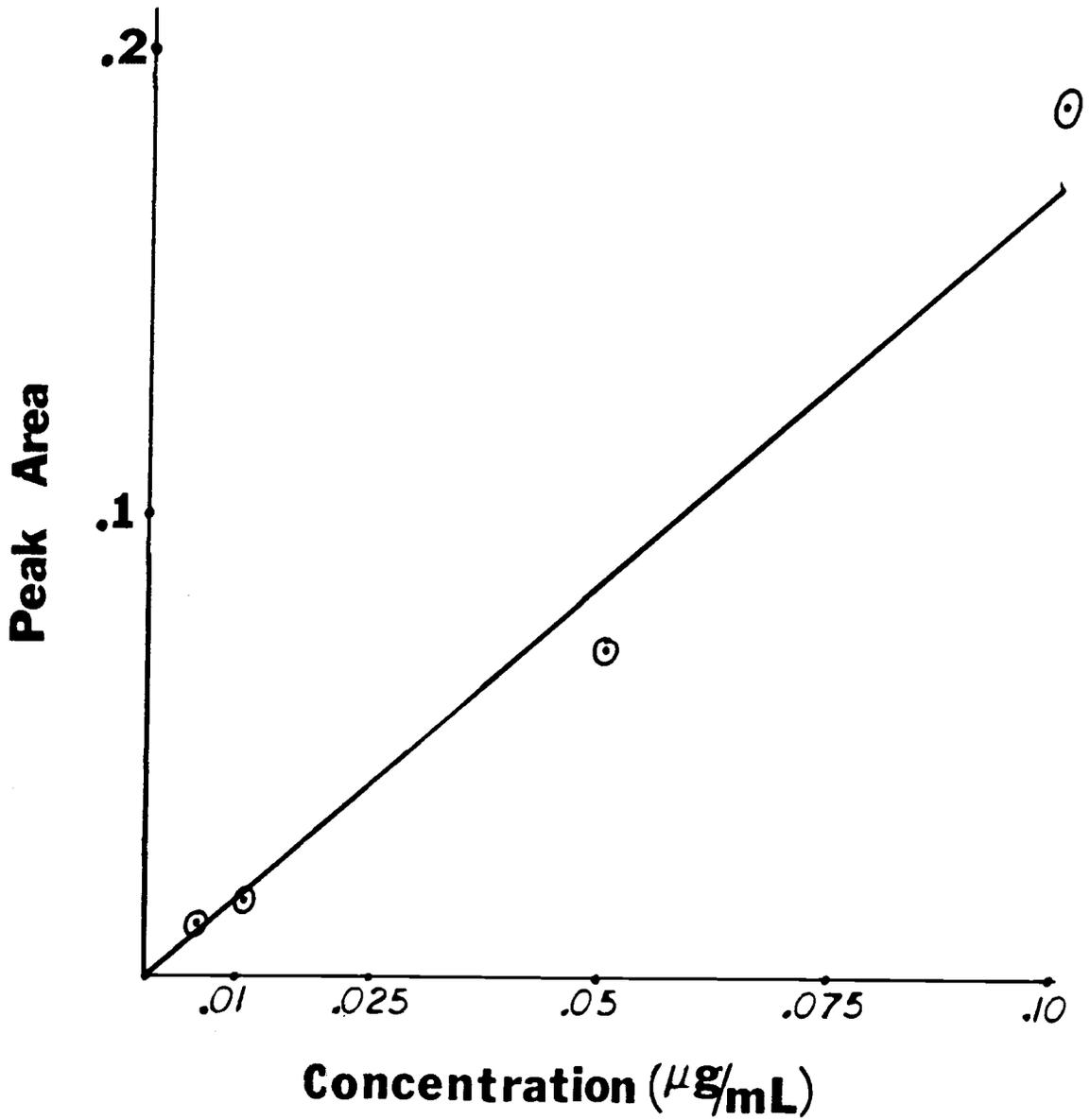


FIGURE 21
Chromatogram peak area vs. concentration of a series of neostigmine bromide solutions obtained with isochratic chromatography conditions of 35% CH_3OH , 65% 10 mM octane sulfonate, 10 mM TMAC, 1 mM BH_2 , CHCl_3 extraction solvent with 0.8 mL/min chromatography eluent, and 0.75 mL/min CHCl_3 . CHCl_3 solvent monitored at 603 nm.

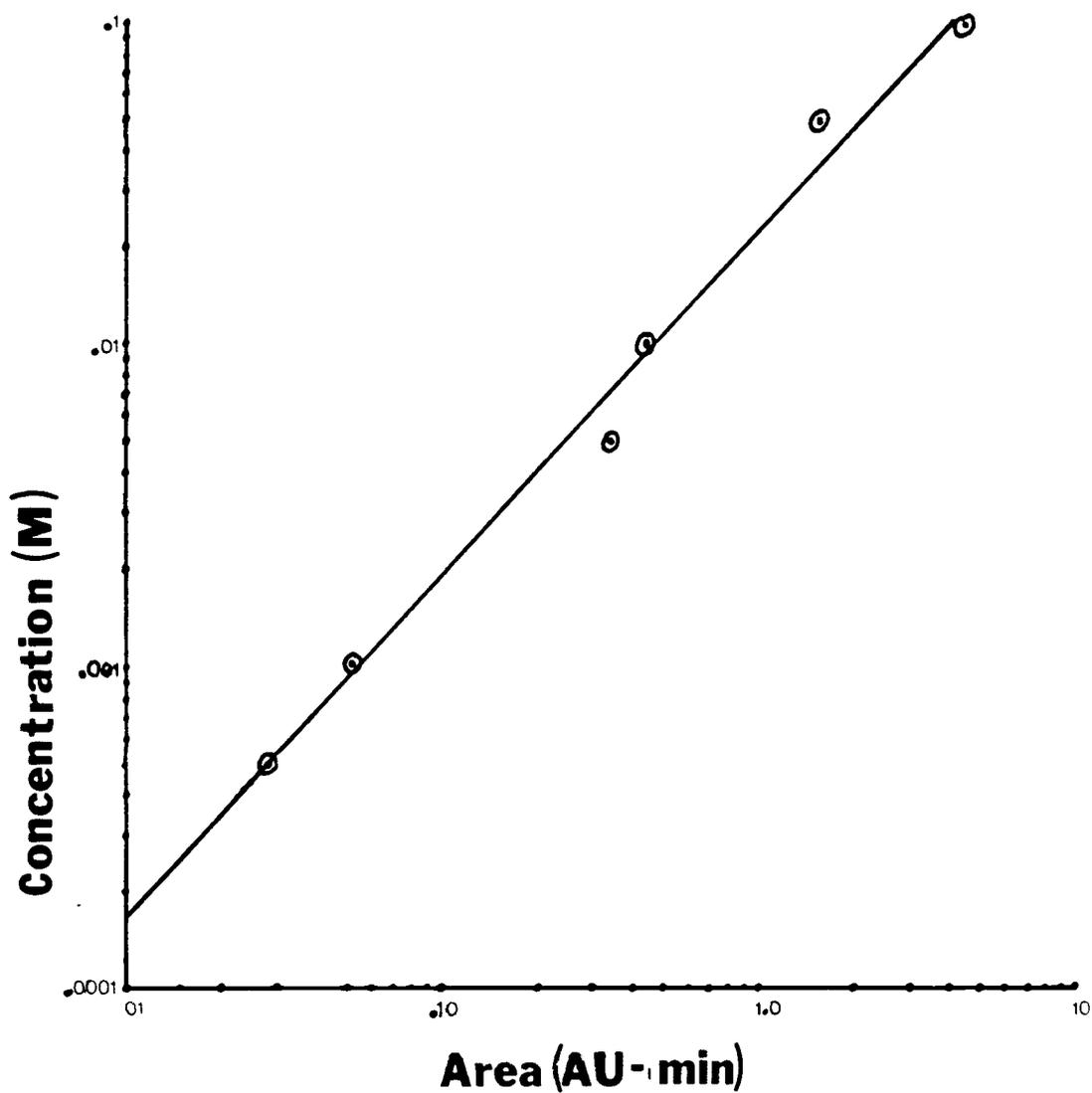


FIGURE 22

Log-log plot depicting the molar concentration of decamethonium bromide injected vs. the chromatogram peak area obtained with gradient chromatography. The gradient used was 30% CH₃OH for 5 minutes which was then increased to 50% in a linear fashion over 20 minutes and maintained for 10 minutes.

CONCLUSIONS

Qams are an important group of compounds which have many applications in a variety of chemical situations. It is important to develop reliable analytical techniques, which are capable of quantitating these substances.

Ion-pair extraction experiments in bulk solutions elucidated the solution conditions which resulted in efficient ion-pair complex distribution. A solvent segmented flow injection analysis ion-pair extraction system was assembled for the rapid evaluation of relative ion-pair extraction efficiencies. This system was then adapted to continuously extract Qams eluting of an ion-pair HPLC system. Reproducibility and selectivity for several Qams was demonstrated. Both gradient and isocratic separations were possible. Calibration curves using both types of separations were linear.

The use of post-column solvent extraction of ion-pair HPLC eluents for Qam analysis has been demonstrated to be a useful approach for the determination of Qams.

RECOMMENDATIONS FOR FUTURE RESEARCH

The research presented in this thesis represents a contribution to the general body of knowledge concerning measurements of Qams. This work could be enhanced and improved by the execution of additional experiments.

The use of alkyl sulfonic acids and quaternary ammonium hydroxide bases as ion-paired liquid chromatography reagents would decrease the ionic strength of the eluent and possibly improve the chromatography. The use of choline or betaine as silanol deactivators and cogeneric modifiers could enhance selectivity and sensitivity of the measurement. Decreasing the detector dead volume and segmentation optimization would enhance sensitivity and lower the detection limits.

Research directed towards preconcentration of Qams from real samples would be needed in order that these techniques could be applied. Development of an improved model of Qam extractability based on the functional groups which commonly occur and their regiochemistry would be important in further studies of the IPE of Qams.

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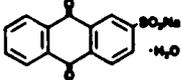
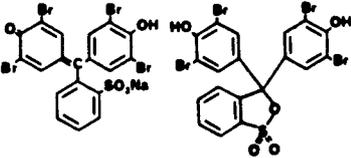
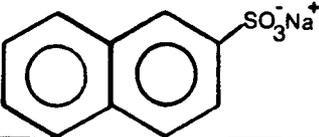
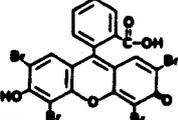
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APPENDIX

APPENDIX 1

TABLE 4. TABLE OF ION-PAIRING REAGENTS

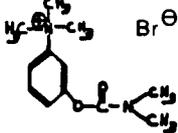
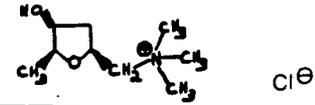
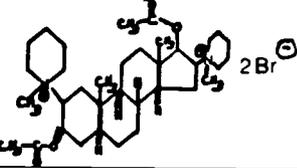
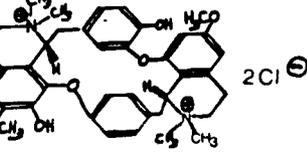
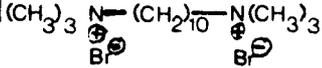
Name	CAS Registry Number	Structure
Anthraquinone - 2 - sulfonic acid, sodium salt	131-08-8	
Bromophenol Blue	115-39-9	
2-Naphthalene sulfonic acid, sodium salt	532-02-5	
Tetrabromofluorescein	548-26-5	
Heptane sulfonic acid, sodium salt	22767-50-6	
Octane sulfonic acid, sodium salt	5324-84-5	

APPENDIX 2

TABLE 5. TABLE OF QUATERNARY AMMONIUM COMPOUNDS

Name	CAS Registry Number	Structure
Acetyl choline chloride	60-31-1	$(\text{CH}_3)_3\text{N}^+\text{(CH}_2)_2\text{O}\overset{\text{O}}{\parallel}\text{CCH}_3$ Cl^-
Acetyl- β -methyl choline chloride	62-51-1	$(\text{CH}_3)_3\text{N}^+\text{CH}_2\underset{\text{CH}_3}{\text{CH}}\overset{\text{O}}{\parallel}\text{CCH}_3$ Cl^-
Acetyl- β -methyl thiocholine chloride		$(\text{CH}_3)_3\text{N}^+\text{CH}_2\text{CH}_2\overset{\text{O}}{\parallel}\text{CCH}_3$ Cl^-
Butyryl choline chloride	2963-78-2	$(\text{CH}_3)_3\text{N}^+\text{(CH}_2)_2\text{O}\overset{\text{O}}{\parallel}\text{CC}_3\text{H}_7$ Cl^-
Benzoyl choline chloride	2964-09-2	$(\text{CH}_3)_3\text{N}^+\text{(CH}_2)_2\text{O}\overset{\text{O}}{\parallel}\text{C}-\text{C}_6\text{H}_5$ Cl^-
S-butylthiocholine iodide	1866-16-6	$(\text{CH}_3)_3\text{N}^+\text{(CH}_2)_2\text{S}\overset{\text{O}}{\parallel}\text{CC}_3\text{H}_7$ I^-
Carbaryl choline chloride	51-83-2	$(\text{CH}_3)_3\text{N}^+\text{(CH}_2)_2\text{O}\overset{\text{O}}{\parallel}\text{CNH}_2$ Cl^-
Choline chloride	67-48-1	$(\text{CH}_3)_3\text{N}^+\text{(CH}_2)_2\text{OH}$ Cl^-
Gallamine triethiodide	65-29-2	$\left[\text{C}_6\text{H}_2(\text{OCH}_2\text{CH}_2\text{N}^+(\text{C}_2\text{H}_5)_3)_3 \right] \text{I}_3^-$

TABLE 5. TABLE OF QUATERNARY AMMONIUM COMPOUNDS (Continued)

Name	CAS Registry Number	Structure
Neostigmine Bromide	114-80-7	
Muscarine	300-54-9	
Pancuronium bromide	15500-66-0	
Succinyl choline chloride		
Tubocurarine chloride	57-94-3	
Decamethonium bromide	541-22-0	

APPENDIX 3

TABLE 6. LIST OF ABBREVIATIONS

AQS	- Anthraquinone Sulfate
B⁻²	- Bromophenol Blue Ion
BH₂	- Bromophenol Blue
cm	- Centimeter
DBr₂	- Decamethonium Bromide
FIA	- Flow Injection Analysis
g	- Gram
G⁺³	- Gallamine Ion
GC	- Gas Chromatography
GI₃	- Gallamine Triethiodide
HPLC	- High Performance Liquid Chromatography
i.d.	- Inside Diameter
IPE	- Ion Pair Extraction
IPLC	- Ion Pair Liquid Chromatography
L	- Liter
<u>M</u>	- Molar, mole/liter
min	- Minute
mg	- Milligram
mL	- Milliliter
<u>mM</u>	- Millimolar, 1×10^{-3} moles/liter
<u>mm</u>²	- Square millimeter
MS	- Mass Spectrometry
nm	- Nanometer, 1×10^{-9} meters
NS	- Naphthyl Sulfate

TABLE 6. LIST OF ABBREVIATIONS (Continued)

O.D. - Outside Diameter

PCSE - Post Column Solvent Extraction

psi - Pounds per square inch

Qam(s) - Quaternary Ammonium Compound(s)

RSD - Relative Standard Deviation

s - Second

TBF - Tetrabromofluorescein

UV-Vis - Ultraviolet - Visible Spectroscopy

v/v % - Volume/volume percent

m - Micrometer, 1×10^{-6} meters