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Major Professor

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MICROSCALE BIOSAMPLING FOR OPEN CAPILLARY LIQUID CHROMATOGRAPHY

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Michael John Capacci

December 1986

DEDICATION

The author would like to dedicate this work to both his parents. First to John J. Capacci, who taught me that the most important thing in this world is a good education, and did everything in his power to be sure that I got one; and to my mother, Elizabeth S. Capacci, who has believed in me from the start and has never stopped believing in me.

Thanks Ma . . . wish you were here Dad.

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Tony B., Chuck K., Ed B., Randy P., Bruce T., Don A., Don B., Rick B., Jeff Q., Angie A., Sam R., and last but certainly not least . . . Dave C., for their unending support through the times when I needed it most, and their help in making this possible;

My parents, John J. and Elizabeth S. Capacci, for their love and support of me throughout my entire existence.

ABSTRACT

Conventional means of sample introduction are generally not compatible with open capillary liquid chromatography (OCLC). Two new methods of sample introduction into capillary columns are described with which biological fluids can be directly injected into an OCLC column. This assures a close correlation between the concentration of the analyte in the living system and that of the extracted (in this case injected) sample.

This thesis contains descriptions of the construction and use of these biological sampling devices used in both injection techniques, as well as optimization procedures followed to determine how they could be used to their fullest potential. Reproducibility results and test procedures are also included.

A chromatographic system is also described which is well suited for the types of analysis that would be encountered when using these injection techniques for bioanalysis. This system, which involves the use of dynamically modified stationary phases with OCLC columns, shows particular promise for the separation of sample components of biological origin.

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CHAPTER 1

INTRODUCTION

Open capillary liquid chromatography (OCLC) has been known to be compatible with biological analysis (1). The small sample volume permitted by OCLC facilitates its use in chemical systems, such as biological samples, in which the available sample volume may be limited. It has been found that stationary phases can be chemically bonded directly to the inner wall of a glass capillary column (2). While this technique has already been described, it is not without problems. The major problem inherent in this technique is column occlusion. In many instances a column may not even reach the final step of preparation before it must be discarded due to occlusion. Takeuchi et al. (3) have reported the use of dynamically modified stationary phases as an alternative to this bonding procedure. Dynamically modified columns lack a true bonded stationary phase. Instead, the mobile phase includes a surfactant, which electrostatically attaches to the inner surface of the glass column. The surfactant used in this work was cetyltrimethylammoniumbromide, (cetrimide). This addition creates an equilibrium in which a portion of the total surfactant concentration electrostatically bonds to the "free" silanol groups at the glass surface, thus creating a reversed-phase type of stationary

phase. Separations that can be readily achieved on chemically bonded columns can be performed on a dynamically modified column with similar results. This procedure for preparing OCLC columns has reduced occlusion problems which plagued earlier work, by reducing the number of steps involved in the preparation. Moreover, the resulting system permits the separation of a wide variety of compounds, particularly those of biological interest.

Biological samples often contain both charged and neutral compounds. The ion pair capability of the surfactant present in the "dynamic" mobile phase permits the partitioning of otherwise charged solutes into lipophilic stationary phase. This facilitates the analysis of samples which contain components that vary in ionic state.

An investigation to solve the problem of sample introduction had to be performed. Two separate methods were evaluated. I will refer to them as the "heating method", and the "sample tube method".

When OCLC columns are used, care must be taken to ensure that favorable injection, connection, and detection systems are employed, due to the very low total volumes used. The basic relationship to determine the observed peak variance (σ_{obs}) in a liquid chromatographic system is shown by equation 1. The peak varience is described as the dispersion of an injected band as it passes through a chromatographic

column. Equation 1 illustrates that the square of the observed peak varience is a sum of the squares of the individual contributions from the injector, detector, column, and the connections between these parts. When employing the systems discussed here, many of the terms in this equation are eliminated. With the on-column detection scheme used in this work, there is no contribution from the detector to be considered. As will be discussed later, with the heating method there is no contribution from connecting tubing and the contribution from the injection process is very small, provided smaller injections are involved (4). The "sample tube" method has very little connecting tubing and its contribution can be minimized if care is taken in connecting the tube to the column. Furthermore, if the filling of the sample tube is done carefully very little contribution from the injection process is noted. The construction and use of these devices will be discussed later in this thesis. With these terms eliminated from the equation, the only source for peak variance is the column, and since a narrow-bore open capillary column is employed in the work, the contribution from it is lower than with packed columns (5).

$$(\sigma_{obs})^2 = (\sigma_{in})^2 + (\sigma_{con})^2 + (\sigma_{col})^2 + (\sigma_{det})^2, \qquad (1)$$

where

(σ_{obs}) = peak varience observed at detector,

(σ_{in}) = contribution to peak varience from injection system,

(σ_{con}) = contribution to peak varience from connecting tubing,

(σ_{col}) = contribution to peak varience from column, and

(σ_{det}) = contribution to peak varience from detector employed.

The usual methods of injection are not compatible with OCLC because these techniques involve large sample sizes and large injector related dead volumes. A sample loop injection system, for example, might cause so much band broadening that the usefulness of the analysis would be lost.

Traditional sampling procedures in a biological chromatographic analysis do not generally assure a close correlation between the concentration of the substance in the extracted sample and the chemical of interest in the actual living system. When an analysis is performed on a biological sample, it usually involves some type of sample removal from the organism prior to analysis. Many times the sample is taken assuming that the sampled quantity was a true representation of the actual living system sampled. The

sample may then go through preparation steps to prior to analysis. A technique is needed in which small amounts of sample are taken directly from the organism being studied and analized directly. Elimination of sample preparation steps also reduces the possibility for external contamination of the sample, which would give false results.

A typical situation in which this sampling technique is useful is the determination of the antitumor drug doxorubicin and two of its metabolites (Figure 1). If sampling can be done at the site of the tumor, and at varying time intervals, the accurate pharmacokinetic behavior of the drug and its metabolites may be determined. Multisite sampling could also provide biodistribution data. This is possible because the sample is introduced in vivo into the OCLC column and quickly analyzed to determine if any of metabolites B & C (see Figure 1) have been formed. This makes the technique more selective than previous in vivo measurement devices which tend to give just one signal for the drug and its metabolites (6). It is this advantage that makes this technique a valuable and useful tool for the analytical or biological chemist.

This research involved the development and characterization of dynamically modified OCLC columns. In addition I have devised two injection techniques for use with this form of chromatography. Human serum was used in several of the

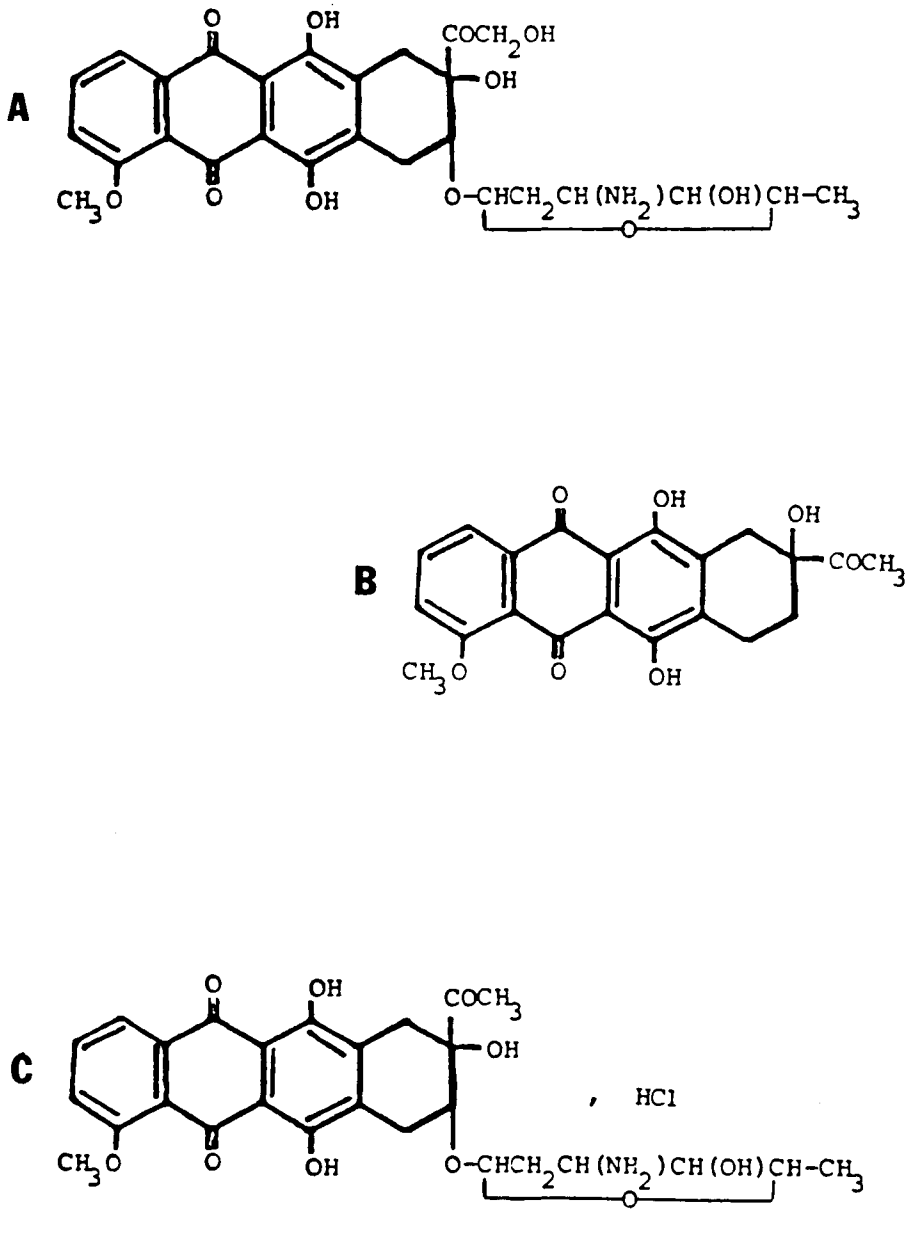


Figure 1. Antitumor Drug Structures.
A: Adriamycin; B: Daunorubicin aglycon;
C: Daunorubicin, Hydrochloride.

experiments to demonstrate the utility of this technique when adapted to a biological matrix. Also included are design and optimization procedures for the sampling devices.

CHAPTER 2

EXPERIMENTAL

The apparatus used in this work consisted of two different components: the chromatographic system, and the actual sampling device. The following is a description of the manner in which each of these was constructed and the method of connection.

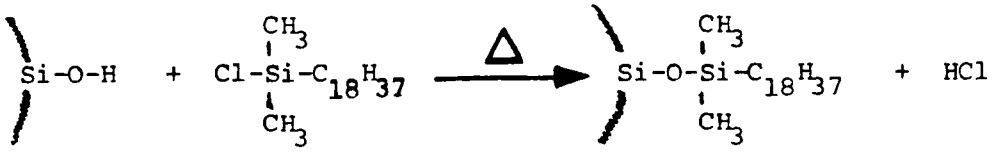
The chromatographic columns used here were both chemically bonded and dynamically modified, with most of the work involving the latter. The method of preparation for each type of column is as follows:

Bonded Stationary Phase

The columns were drawn from glass capillary tubing using a model RSL glass drawing machine obtained from Alltech Associates. These columns were then electroetched as described by Jorgensen (7). Subsequently, the column was rinsed in a variety of solvents, the sequence of which are as follows: 10% chlorodimethyloctadecysilane (ODS) in toluene @ 80°C for 3 hours, toluene for 1 hour, CH_2Cl_2 for 1 hour, acetonitrile for 1 hour, and finally a 1 hour rinse with methanol. The bonding process involving the ODS is completed as shown in Figure 2. The column must be equilibrated with mobile phase before it may be used in an analysis.

PREPARATION OF BONDED AND DYNAMICALLY MODIFIED STATIONARY PHASES

Bonded ODS Columns



Dynamically Modified Cetrimide Columns

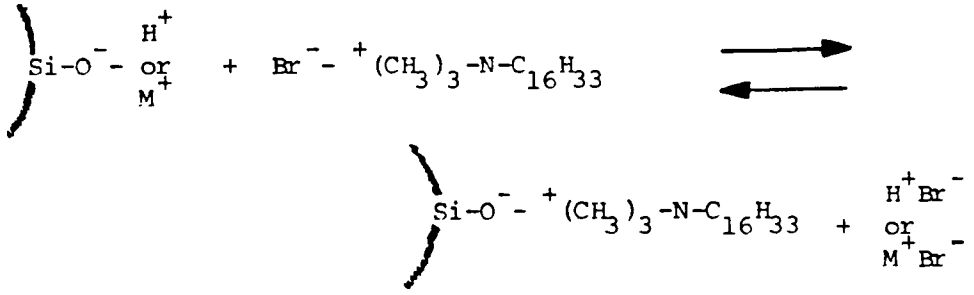


Figure 2. Attachment of the Stationary Phase in Bonded Phase and Dynamic Chromatography.

Dynamic Stationary Phase

The columns are prepared in the same manner as the bonded phase columns up to and including the electroetching step.

After the column is prepared and treated as described earlier, it are placed in the experimental apparatus. A block diagram of the apparatus is shown in Figure 3. An on-column, laser-based detection system is employed which is identical to the system described by Sepaniak et al. (8). This on-column detection was achieved using the 488 nm output line of a Model 270 Spectra Physics Argon Ion Laser, for the excitation of NBD-amines, which were used as a test sample, (emission wavelength = 540 nm), and antitumor drugs (emission wavelength = 585 nm). Fluorescence emission signals were collected using a Model H-10 monochromater (Instruments SA) and detected with a RCA Model 1P28 photomultiplier. Photo currents were processed with a Model 126 photometer (Pacific Precision Instruments). The resulting chromatograms were recorded on a Model BD40 strip chart recorder (Kipp and Zonen).

The mobile phase used throughout this work consisted of 65% pH 6.86 phosphate buffer; 25% acetonitrile; 10% methanol (all Fisher Scientific). The mobile phase for all dynamic separations contained 0.1% cetrimide (w/v) in addition to the other components. The biological matrix used was human

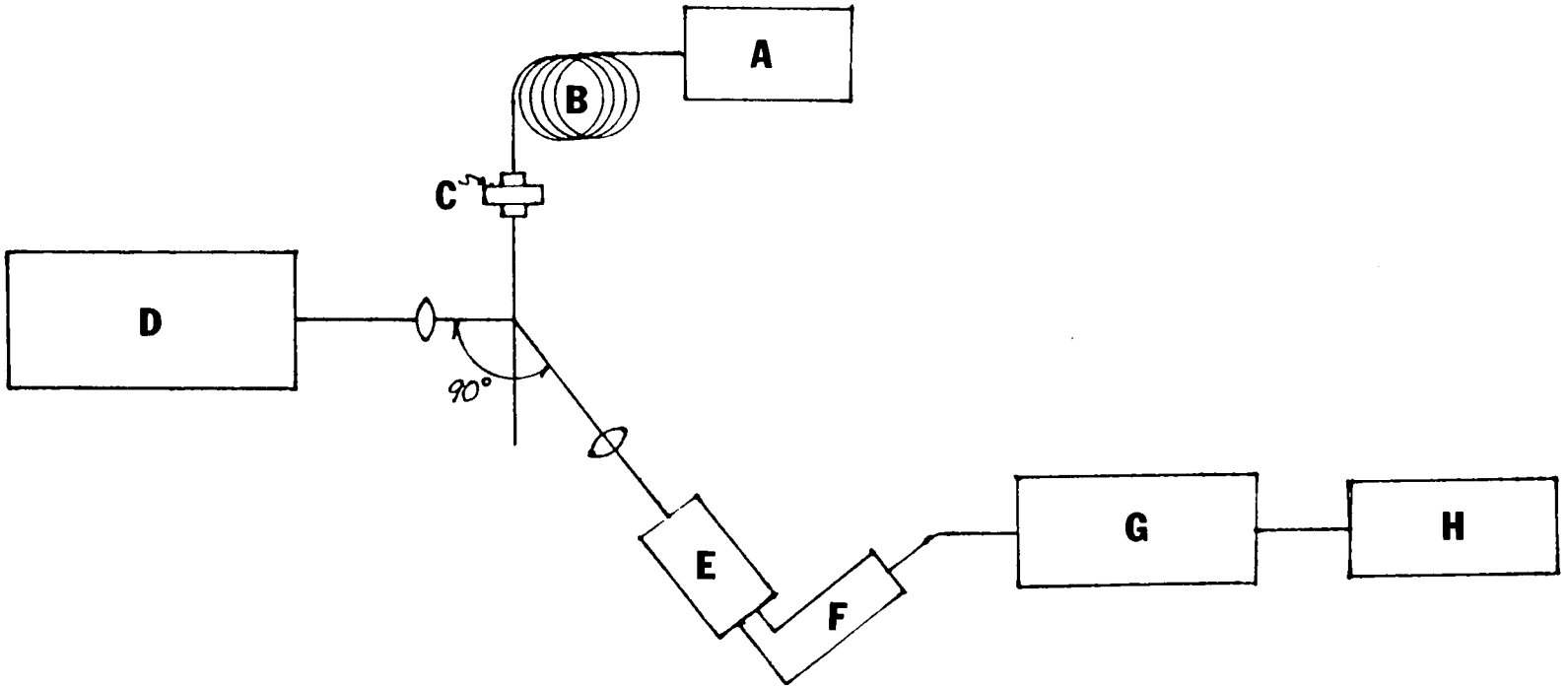


Figure 3. Apparatus Used for OCLC with Laser-Based Fluorescence Detection.
A: Pump; B: OCLC Column; C: Fiber Optic Positioner;
D: Argon Ion Laser; E: Monochromater; F: Photomultiplier Tube;
G: Photometer; H: Chart Recorder.

serum (Fisher Scientific). The amines used in this work were derivatized with NBD-Cl using the process described by Murray et al. (9).

Two methods of injection were developed in this work. They are described here as the "heating method" and the "sample tube method". The heating method involves the controlled and reproducible heating of a small portion of the column near the inlet end of the chromatographic column which causes the liquid mobile phase to volitalize and to evacuate a short section of the column. The inlet end of the column is then placed in the sample and the heating stopped. The sample is then injected as the column cools.

The final design of the heating apparatus, and its relation to the remainder of the experimental apparatus, is illustrated in Figure 4. The apparatus consists of a large forceps clamp anchored to a base. Affixed to each side of the tip of the forceps is a split glass tube with a single transformer wire running through its length. The transformer wire is connected to the inner glass surface by electrically conductive paint. Figure 5 is a side and top view of this tube. The two sections of glass are positioned across from each other creating a hollow tube which fits around the open capillary column.

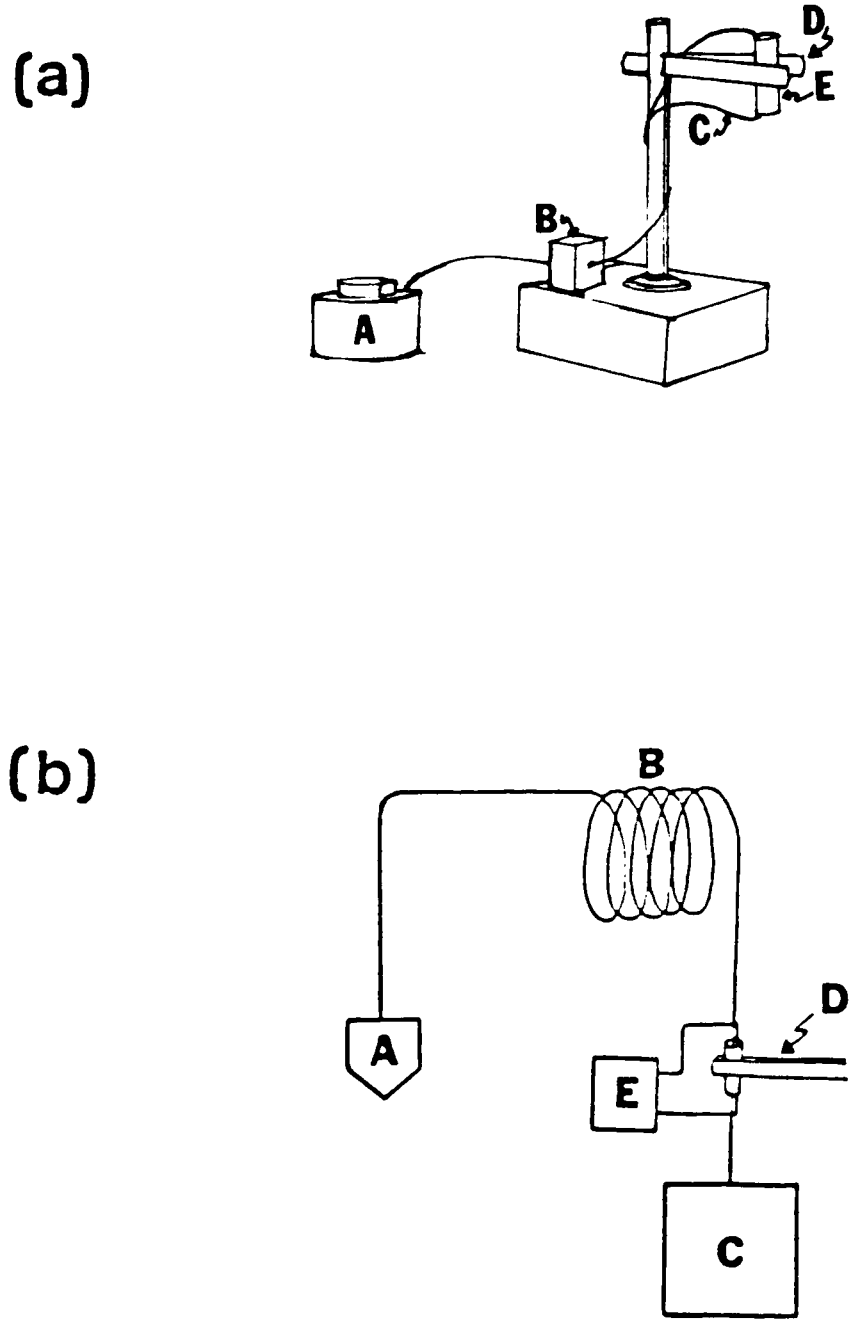


Figure 4. Apparatus for "Heating" System.
(a) "Heating Apparatus"
A: Variable power source; B: Transformer;
C: Transformer Wire; D: Forcep Clamp;
E: "Heating Tube".
(b) Apparatus for "Heating" System
A: Detection System; B: OCLC Column; C: Pump;
D: Forcep Clamp; E: Variable Power Source and Transformer.

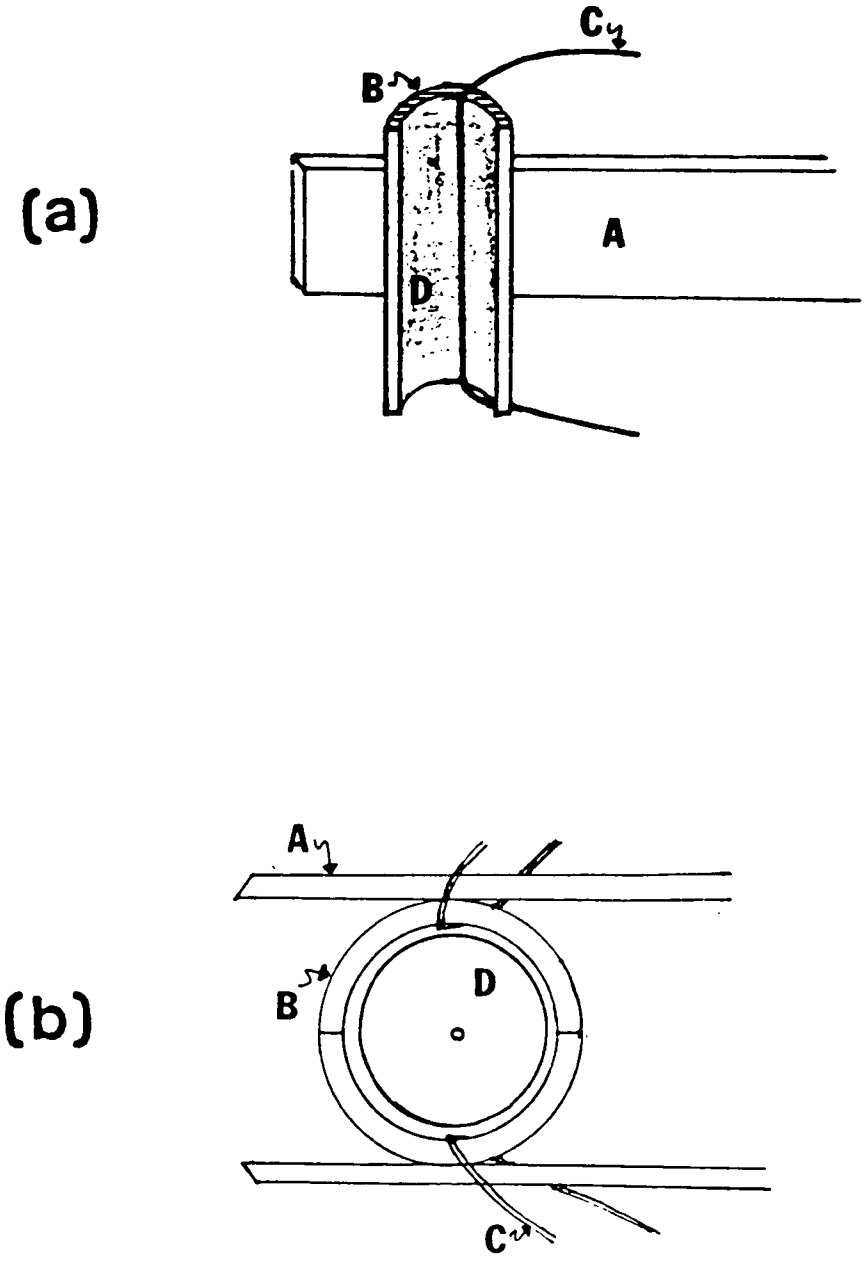


Figure 5. Detail Diagram of Heating Tube.
(a) Side View
A: Forcep Clamp; B: Split Glass Tube;
C: Transformer Wire; D: Electrically Conductive Paint.
(b) Top View
A: Forcep Clamp; B: Split Glass Tube;
C: Transformer Wire; D: OCLC Column.

This tube constitutes the heating element portion of the device. The wires are connected through a transformer and a variable voltage regulator which permits control of the resistive heating occurring at the hollow tube. In order to use this device, the column must first be disconnected from the pumping source, then the heating element must be closed around the inlet end of the OCLC column. Thereafter, the power supply is activated for a predetermined amount of time and then the inlet of the column is placed in the sample. The power is turned off to the device, and the column is then allowed to cool while in the sample. After the device is removed from the column, the column is once again connected to the pump and the separation is accomplished. The column is equipped with removable fittings which enable direct body sampling by simply placing the inlet end of the column into a needle which has been inserted into the appropriate region of the body. It has been demonstrated previously that sampling of nanoliter volumes of interstial fluids from tumors can be accomplished using a capillary tube (6).

The sample tube injection technique involves aspirating a sample into a small section of narrow-bore glass tubing (the sample tube). The sampling tubes used in this work

were 4 cm lengths of the drawn capillary used for the column. Once filled with sample, the sampling tube is connected to the pump via a conventional union and to the column via a specially prepared zero dead-volume union (see Figure 6). The apparatus used for the sample tube method of injection is shown in Figure 7. The sample tube-column union is a modification of a connection device (union) used to couple a detector to a capillary LC column (10). The connection between the tube and the column is accomplished by using a 1/16" stainless steel union which is drilled out to an i.d. of 0.082". A piece of teflon tubing is then placed through the inner hole of the union to create a tight fit. Shrinkable PTFE tubing is then placed around the end of the column and the end of the sample tube. Care must be taken to ensure that the end of the column and the tube are flush with the end of the tubing placed over them (see Figure 8). With the ends of the column and the sample tube treated in this way, they fit tightly inside the union. Vespel ferrules are then placed around the sample tube and column and the connection process is completed by carefully tightening the nuts of the union (11). Theoretically, the entire sample tube should be constructed so that it could be placed inside a hypodermic needle and be introduced into the

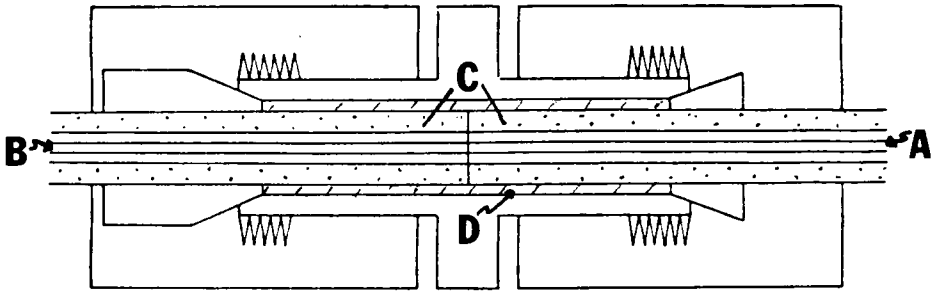


Figure 6. Enlarged View of Modified Union for Coupling. A: OCLC Column; B: Sample Tube; C: Shrinkable PTFE Tubing; D: PTFE Tubing.

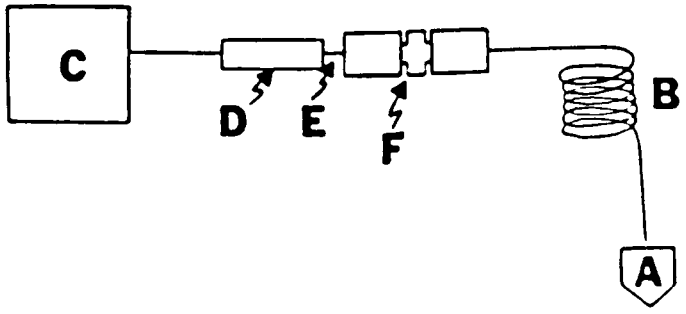


Figure 7. Sample Tube Apparatus System.
A: Detection System; B: OCLC Column; C: Pump;
D: Zero Dead Volume Union; E: Sample Tube;
F: Modified Union (see Figure 6).

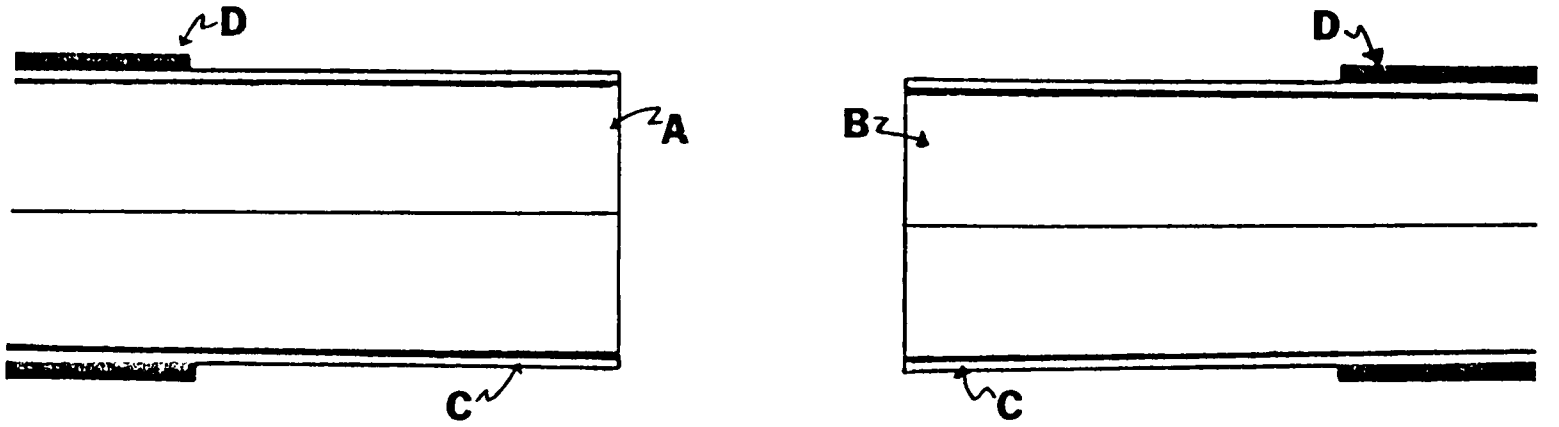


Figure 8. Enlargement of Modifications on Ends of the Column and Sample Tube.
A: Sample Tube; B: OCLC Column; C: PTFE Tubing;
D: Stainless Steel Tubing.

organism being studied. However, living organisms were not used in this work. Instead sampling was performed in vitro using human serum as a biological sample matrix.

CHAPTER 3

RESULTS AND DISCUSSION

When attempting to summarize the advantages of the dynamic technique over the bonded phase technique, one has many facts to consider. The dual function of the surfactant which can serve as both a stationary phase and ion-pairing reagent, is an important advantage. The cationic cetrimide will form lipophillic ion pairs with the anions and permit partitioning with the dynamic stationary phase. Hanson and coworkers have demonstrated this phenomenon clearly in the separation of benzoate ions in benzene on a dynamically modified packed silica column (12). My coworkers and I have also done work in this area, achieving an ion-pairing separation of a mixture of neutral and charged dansylated amino acids on a dynamically modified OC column (1). A chromatogram of this separation is shown in Figure 9. This point is of particular interest when dealing with a biological sample where other methods, such as pH adjustment to suppress ionization and permit efficient chromatographic separation are not always effective or possible.

Since the amount of sample necessary to obtain an analysis is on the order of the low nanoliter range, this technique is well suited for those situations where sample volume is limited. Such situations are quite common when

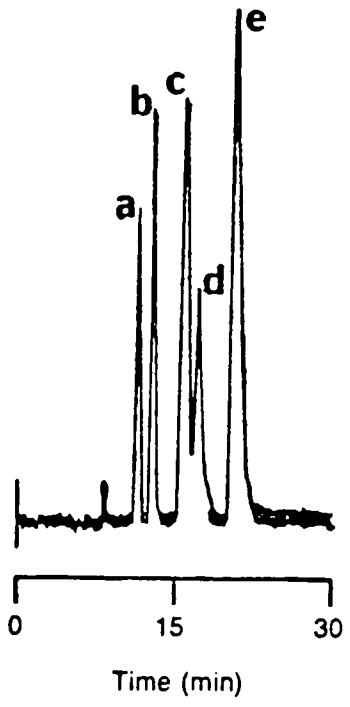


Figure 9. Separation of DNS-Amino Acids.
Peak Identification: a: Threonine; b: Glycine; c: Valine; d: Glutamic acid; e: Leucine.

dealing with biological samples. Thus minute volumes of fluids such as those found in the interstitial spaces of soft tissue (e.g., tumors in cancer patients) can be directly sampled to provide clinical data for pharmacokinetic and biodistribution studies of drugs such as the antitumor agents mentioned earlier. Using a fiberoptic-based sensor designed for in vivo fluorescence measurements, sampling of interstitial fluids was accomplished, via capillary action, into a glass capillary tube which encased the fiberoptic probe (6). The sensor was used to monitor doxorubicin present in human tumors which had been transplanted into laboratory mice. Unfortunately, it was not possible to discriminate between the signals arising from the drug and those arising from its metabolites. By sampling (injecting) body fluids directly into an OCLC column, the separatory power of the OCLC column can be exploited to improve selectivity, while maintaining some of the clinical advantages afforded by in vivo measurements.

Injection in OCLC columns in our laboratories were initially accomplished by passing a match or butane lighter near the injection end of the column. This rapid temperature change caused the evacuation of the mobile phase inside the heated section of column and the sample was introduced in much the same manner as described earlier for the heating technique. This method was effective, but not reproducible.

It required much skill and practice to obtain reproducible injection volumes. Futhermore, the open flame would tend to blacken the column, making it difficult to perform subsequent injections.

The next logical step was an attempt to attach some kind of heating element to the injection end of the column. This led to a single wire coiled around the injection end of the column (Figure 10). Studies were done to determine the number of coils necessary, and also the appropriate amount of current flowing through those coils to produce the desired heating. The major disadvantage with this method seemed to be that the wire was permanently affixed to the column and could only be used with that one column. The heating tube has shown much more durability and versatility than this single wire setup (11).

The heating method is a true "on-column" type of injection and allows less chance for solute band dispersion to occur than conventional modes of injection, because there are no fittings in the system to contribute to band dispersion.

A study was done to determine the appropriate voltage and amount of time this voltage should be applied to the column for this heating technique to be effective. The plot shown in Figure 11 illustrates the findings of this study. The plot relates the peak height of methylamine-NBD to the

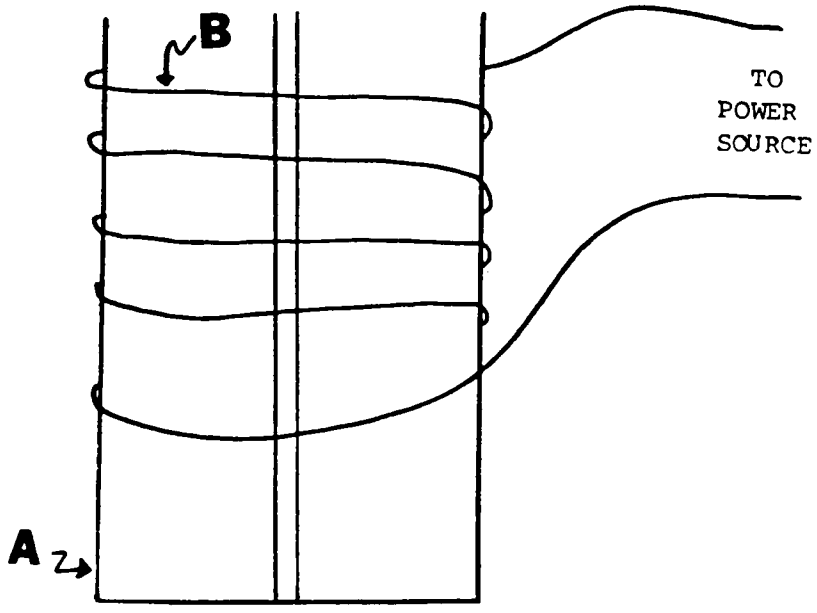


Figure 10. Single Wire Apparatus for Sampling.
A: Inlet End of OCLC Column; B: Transformer Wire.

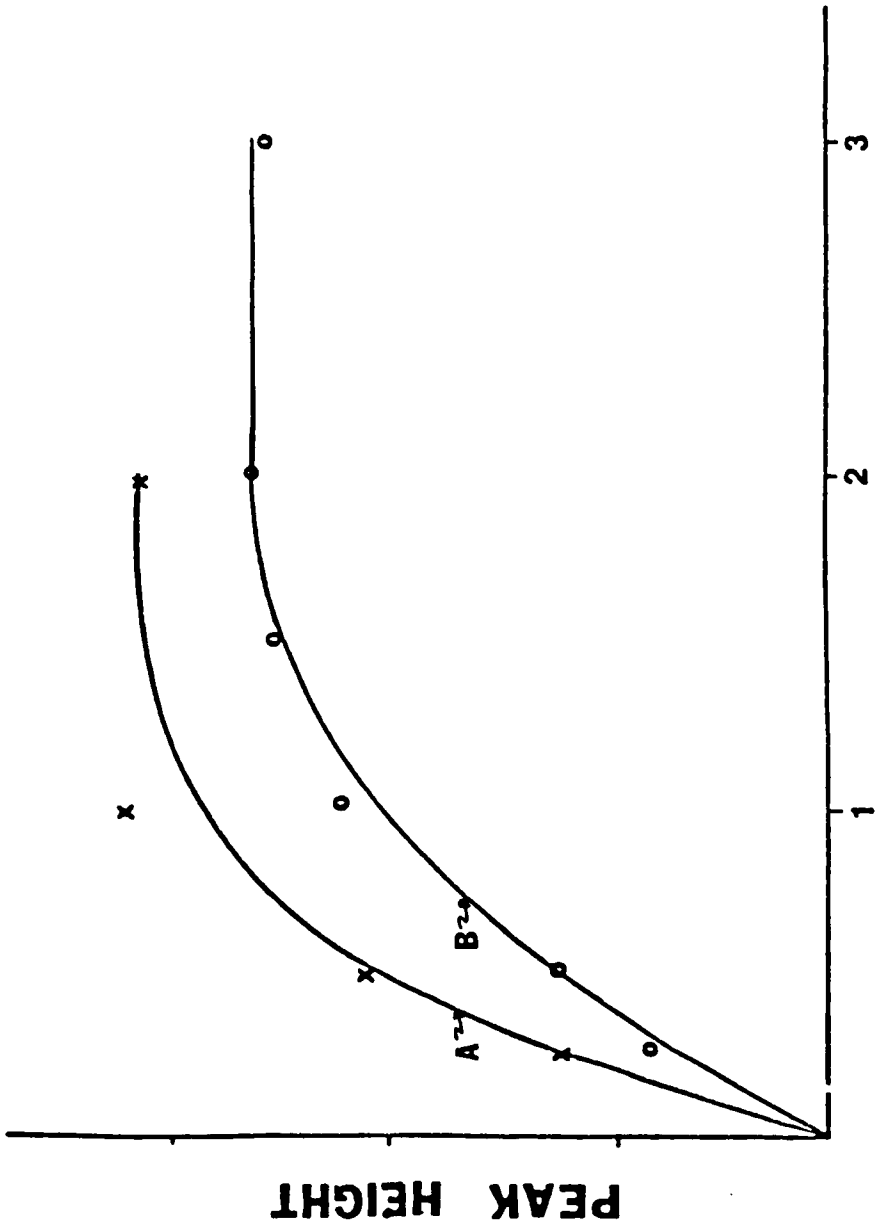


Figure 11. Plot of Heating Time vs. Peak Height for Two Different Applied Voltages.
A: 1.20 Volts
B: 1.00 Volts

amount of time the heating apparatus is subjected to voltage. Two different voltages were used for the trials. It was found that after a certain length of time, the injection volume became constant. The heat generated in the heating tube is conducted along the column, resulting in an evacuated area which is larger than the actual area covered by the heating tube. This area was found to be approximately 8 cm in length as opposed to a heating tube length of 2 cm, for an applied voltage of 1.2 volts for two minutes. This volume corresponds to an injection volume of 40 nanoliters. It is also necessary to note that each time this apparatus is used on a different column, it must be calibrated for use with that column. Since many glass OCLC columns are drawn in the laboratory, variations in column thickness or other parameters may exist and effect the injection.

The reproducibility of this technique was evaluated using voltage and time conditions which corresponded to a flat portion of the plots in Figure 11. Ten to 12 injections of samples containing NBD-derivatized methylamine were performed. The relative standard deviation in the peak heights obtained was 12% using either the mobile phase or human serum as the sample solvents. This percentage may seem to be slightly high, but if it is considered that this is the only means available to introduce the sample directly

into the column, it seems that the relative standard deviation is well within the limits of acceptance.

In order to test this heating technique on a real sample, a mixture containing the antitumor drug doxorubicin and two of its metabolites (daunorubicin hydrochloride and daunorubicin aglycon) was injected into a 40 μm i.d. dynamically modified capillary column. The resulting chromatogram is shown in Figure 12.

The second injection technique to be discussed is the sample tube technique. This technique involves aspirating the sample into a small section of narrow bore glass tubing (the sample tube). It is very important that the size of the sample tube match the size of the capillary column, otherwise band dispersion can be problematic. Moreover, if the connection between the sample tube and the column is not made properly, the sample will not be transferred in a "pluglike" manner, resulting in a poor injection. It is for this reason that the sample tube was constructed from a short section of the same capillary column that was actually used for the separation. If these two sections cannot be lined up correctly (i.e., have their internal channels in line), the technique would be ineffective.

The sample tube technique has the advantage of permitting sampling at a rapid rate at a site away from the separation experiment. These repetitive samples can be taken,

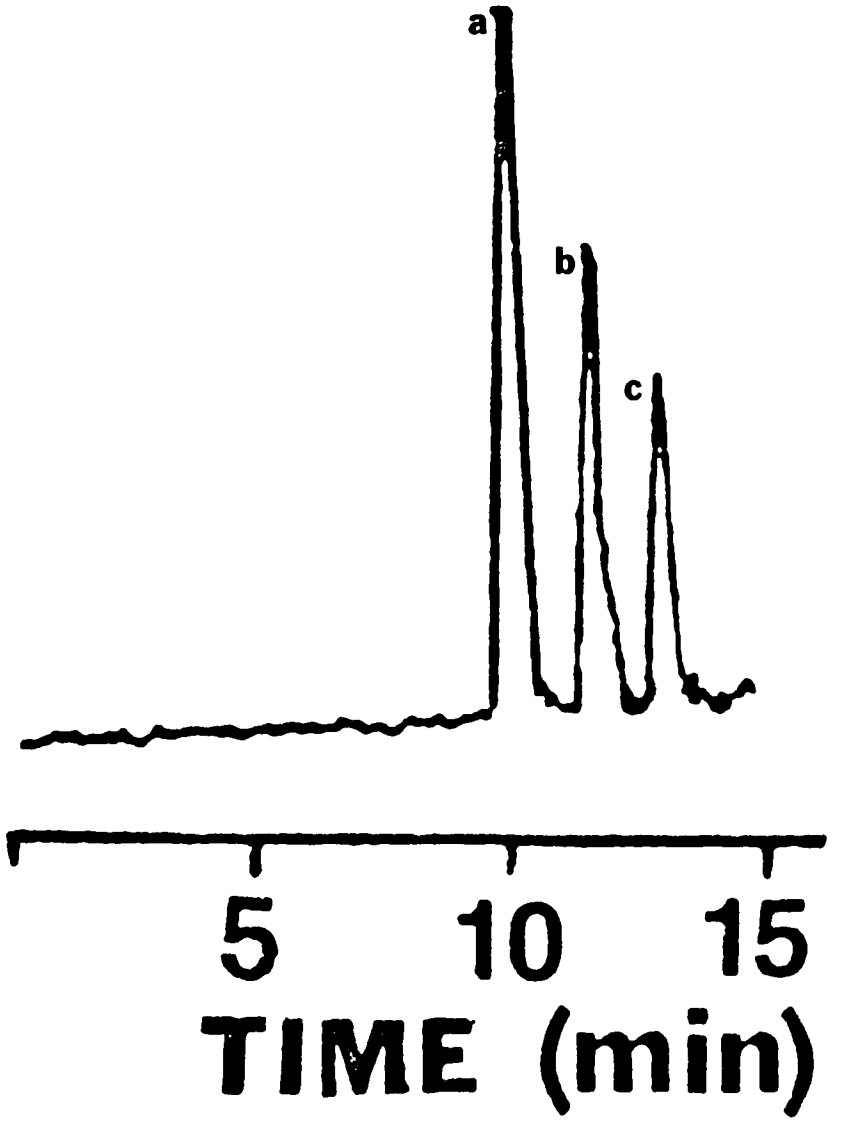


Figure 12. Separation of Antitumor Drugs.
Peak Identification: a: Daunorubicin aglycon;
b: Adriamycin; c: Daunorubicin, hydrochloride.

stored directly in the sample tubes (perhaps by freezing), then analyzed sequentially using a single OCLC column. This sampling method could be very useful for in vivo pharmacokinetic studies. Care must be taken, however, to ensure that no evaporation or dehydration of the sample in the tube between the time the sample is taken and the time the separation is performed.

The volume to be injected can be varied in this technique by simply changing the length of the sample tube employed. This makes for an easily adaptable system for many different types of analysis.

The sample tube injection method was also tested for reproducibility using both mobile phase and a human serum matrix. Three tubes were used, and each tube was tested for a total of eight injections, four in each matrix. The relative standard deviations for this means of sampling ranged between 5-8% for each individual tube, when using the same test sample as was used to evaluate the heating technique. Although the dimensions of the sampling tubes were nearly identical, the intersample tube reproducibility was rather poor. This may be due to the differences in the permeability of these very narrow-bore tubes, which make it difficult to completely fill each tube by simple aspiration. It could also be caused by differences in the ends of the sample tubes themselves. An effective connection requires that

both ends of the sample tube, along with the end of the column, fit as flush as possible to each other. If part of the sample becomes trapped in the small portions of "dead space" between the two portions of capillary, it will broaden the injection length causing poor results at the detector. This is graphically depicted in Figure 13. It was necessary to lightly file the ends of the column and the sample tube in order to assure an even fit. Care must be taken to avoid any small fragments of glass or other material from entering the column and possibly causing an occlusion problem. The mobile phase solvent in the above tests yielded a 1-2% better relative standard deviation than did the biological matrix, but the biological usefulness of the sample tube technique is still intact.

One minor problem which may be encountered when attempting to use this sample tube is its fragility. Since the tube is approximately 600 μm in diameter, it cannot withstand much pressure before breaking. One possible solution to this problem may be to encase the tube inside a small section of stainless steel tubing. If this is done, one must ensure that the tubing does not extend too far down the ends of the tube. If this occurs, the purpose of the described construction will be defeated, and the exact matching of the ends of the tube and the column would not occur. The outer diameter of the tubing should also be

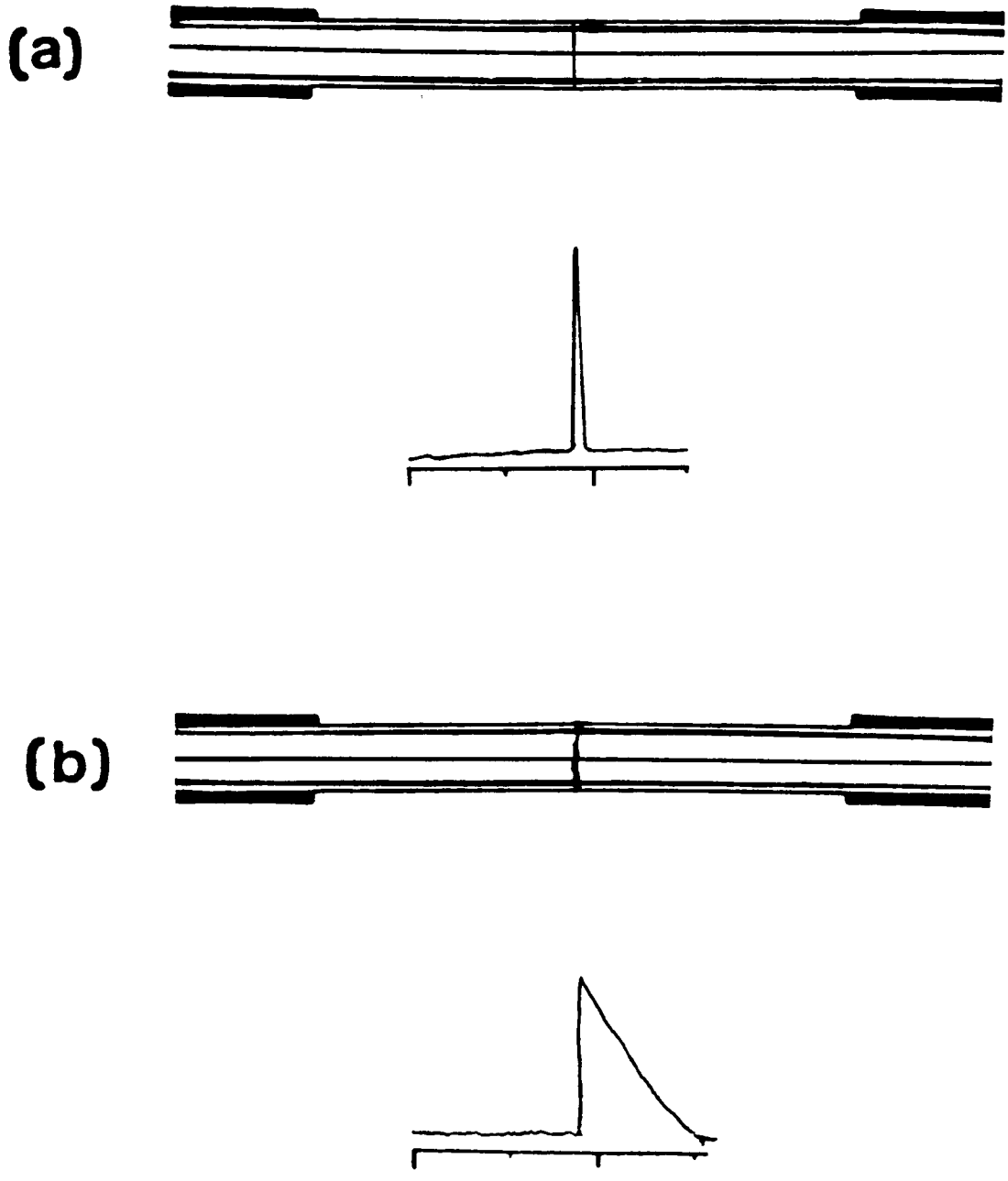


Figure 13. Comparison of Different Connections on Peak Shape.
a: Shows good connection.
b: Shows poor connection.

considered. If it is to large it may hamper the ability of the tube to be placed in vivo, as too large a tube may disrupt the area from which sample is obtained and cause the technique to be unreliable.

A comparison of the column efficiencies for the heating and sample tube techniques for injections of an NBD-derivatized methyl amine solution, yielded a 9% lower plate height (3000 versus 3250 plates) for the former injection technique. It is expected that the injection related band dispersion would be even more severe for the sample tube injection procedure if extreme care had not been taken in preparing, filling, and connecting the sample tube.

CHAPTER 4

CONCLUSIONS

The methods of chromatography and sampling described herein have many advantages over those techniques used in the past. First, some of the advantages of dynamic modification in open capillary chromatography will be summarized:

1. Small sample volume requirement.
2. Easy to prepare columns.
3. The ability to use on-column fluorescence detection.
4. The ability to separate positive and negative charged ions along with neutral species without major modifications of the mobile phase.

Next, the advantages of direct body sampling into a capillary chromatographic column, or a capillary sampling tube for subsequent chromatographic analysis are summarized below:

1. Sample is taken directly from the host environment.
2. Analysis is started immediately after sampling.
3. Small sample volumes are required.
4. Repetitive sampling is much easier and less time consuming.

5. Individual sample components can be chromatographically resolved. Sample components can also be resolved from interferences.
6. Possible shorter analysis times.

Although no actual in vivo measurements were made, it is expected that the systems described here would be applicable to such measurements. The ability to distinguish different components of the sample is a great advantage over previous in vivo measurement devices, such as the fiberoptic probe mentioned earlier.

In light of the information put forth in this thesis, along with the experimentation to support it, I feel that in vivo injections should be possible using either of the techniques mentioned in this work. Thus, these injection techniques should make it easier to provide useful bioanalytical information and should be considered when performing biological analysis.

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