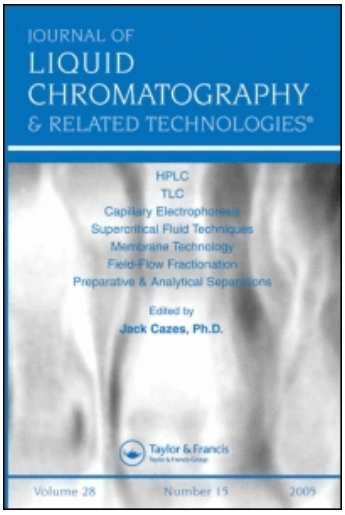

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BIOLOGICAL SAMPLE INJECTION FOR OPEN TUBULAR LIQUID CHROMATOGRAPHY

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ABSTRACT

Two injection procedures for open tubular liquid chromatography have been evaluated for their ability to directly sample biological components in minute volumes of body fluids. The techniques provided R.S.D.s in peak heights of less than 12% for injections of low nL volumes of human serum spiked with a test compound. A dynamically modified open tubular column was employed to separate the anti-tumor drug doxorubicin hydrochloride and two of its metabolites.

INTRODUCTION

One of the advantages of capillary liquid chromatography is the very small sample volumes needed for analysis. This is particularly true for open tubular columns, which generally have i.d.s less than 50 μm and injection volumes in the low nL range(1). This characteristic of open tubular liquid chromatography (OTLC) lends itself well to the bioanalysis of minute volumes of body fluids

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(e.g., the interstitial fluids in soft tissue). Many attempts have been made to obtain reproducible and reliable methods for sample injection in capillary liquid chromatography, which do not degrade the chromatographic efficiency of the capillary column. These methods include using a heat gun(2), methods of split injection(3), a "heart cut" injecting process(4), and the use of an internal-loop valve injection device(5). These techniques have been successful to some extent, but are not generally applicable to direct biological sampling. In this paper, we will describe and compare two methods of sample injection for OTLC which should be useful for direct (in vivo) biosampling. The open tubular column used in this work was an electroetched borosilicate glass capillary, which was dynamically modified by incorporating the surfactant cetyltrimethyl ammonium bromide (centrimide) into the mobile phase(6). The surfactant electrostatically attaches to the inner surface of the open tubular column, thereby forming a thin layer of lipophilic stationary phase. The ion pairing capabilities of this mobile phase make it well-suited for the separation of biological samples, which often contain components that vary in ionic state and polarity(6).

METHODS AND MATERIALS

Column preparation

The column used was drawn from borosilicate glass capillary tubing (Kimble Glass Co.), using a Model RSL glass drawing machine obtained from Alltech Associates, to dimensions of 24 μm i.d. by 6 m in length. Electro-etching of the column was performed as described by Jorgenson(7).

Chromatography

The mobile phase used for this work was: acetonitrile: methanol: pH 6.86 phosphate buffer solution (25:10:65, respectively), which was also 2.5 mM in cetramide. On-column fluorescence detection was performed using a Spectra Physics Model 171 argon ion laser, 488 nm, for the excitation of NBD-amines (λ emission = 540 nm) and anti-tumor drugs (λ emission = 585 nm). Fluorescence emission signals were isolated with a Model H-10 monochromator from Instraments SA and detected with a RCA Model 1P28 photomultiplier tube. Photocurrents were processed with a Model 126 photometer from Pacific Precision Instruments. Chromatograms were recorded on a strip chart recorder. This on-column detection scheme was reported previously(8).

Chemicals

The antitumor drugs, doxorubicin hydrochloride, and its metabolites daunorubicin hydrochloride and daunorubicin aglycon, were supplied by Rhone-Poulenc (Paris, France). Methyl amine (Fisher Scientific) was derivatized with NBD-chloride (Regis Chemical Co.) using a procedure given by the chemical supplier. HPLC grade solvents were used in the preparation of the mobile phase.

"Heating" injection techniques

The "heating" injection device is shown in figure 1a. Injection using this device involves controlled heating of a small portion of the inlet end of the column to force the mobile phase inside that portion of the column out and, upon cooling, allow for introduction of the sample solution into the column. The device

consists of a large forcep clamp anchored to a base. Affixed to each side of the tip of the forceps is a split glass rod with a single wire running through its length which is connected to the inner glass surface by electrically conductive paint. The two sections of glass are positioned across from each other creating a hollow tube which fits snuggly around the open tubular column.

This tube constitutes the heating element portion of the device. The wires are connected through a transformer to a variable voltage regulator which permits control of the resistive heating occuring at the hollow tube. In order to use this device, you must first disconnect the column from the pumping source, then close the hollow glass tube around the inlet end of the OTLC column, turn on the power supply for a predetermined amount of time, place the tip of the column in the sample you wish to introduce into the column, allow the column to cool while in the sample, remove the device from the column, and finally reconnect the column to the pump so the separation can be performed. The column is equipped with removeable fittings which would enable direct body sampling by placing the inlet end of the column into a needle which has been inserted into the appropriate region of the body. We have previously demonstrated the sampling of nL volumes of interstitial fluids from tumors using a capillary tube(9).

"Sample tube" injection technique

This technique for sample introduction involves aspirating 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

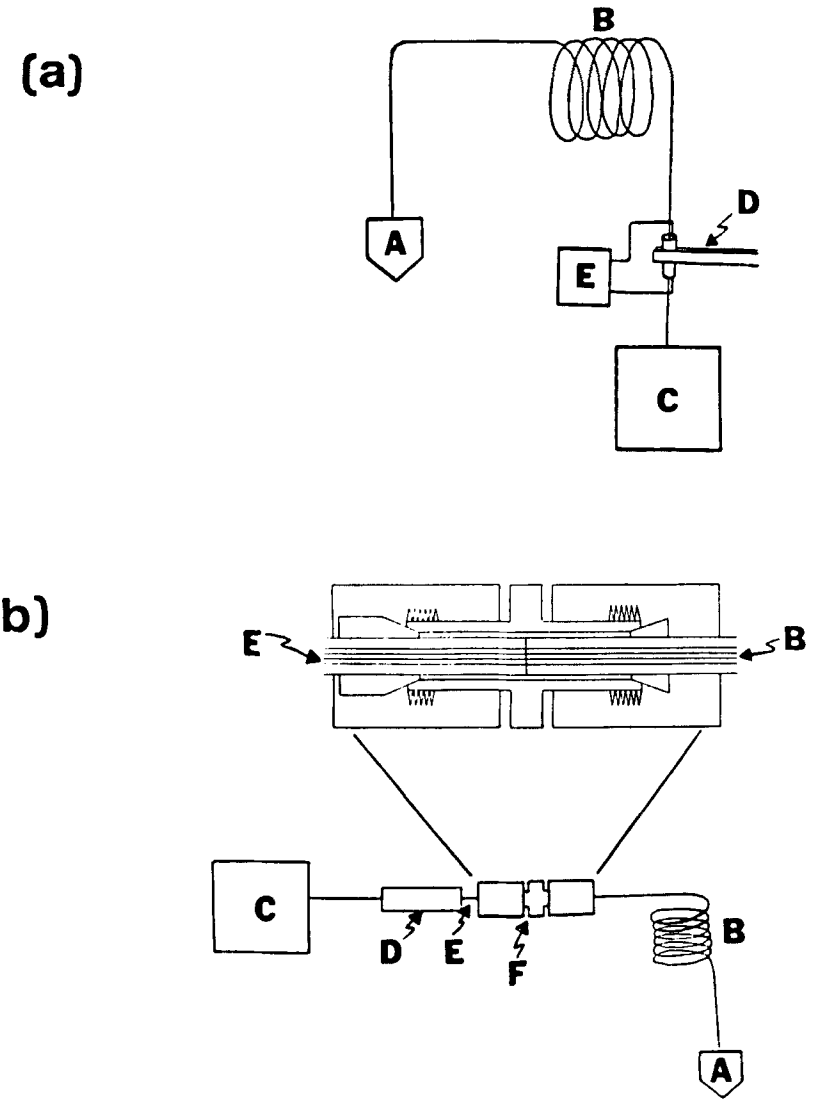

Figure 1 (a) "Heating" injection apparatus; A, detector; B, OTLC column; C, pump; D, clamp; E, transformer and power supply.

(b) "Sampling tube" injection apparatus: A, detector; B, OTLC column; C, pump; D, conventional union; E, sample tube; F, coupler.

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 1b). The sample tube-column union is a modification of a connection device 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 be sure that the end of the column and tube are flush with the end of the tubing placed over it (see insert in figure 1b). With the ends of the column and sample tube treated in this way, they fit tightly inside the teflon tubing inside the union. Vespel ferrules are then put around the sample tube and column and the connection process is completed by carefully tightening the nuts of the union.

RESULTS AND DISCUSSION

Both techniques are well-suited for sampling biological systems. Since extremely small sample volumes are required for analysis, samples can be taken frequently without harm to the organism being studied. However, each technique has its advantages and disadvantages. Since the "heating" method involves true "on-column" injection, there is less chance for solute band dispersion to occur, because there are no fittings or other coupling methods in the system to contribute to band dispersion. A

comparison of the column efficiencies for the "heating" and "sample tube" injection techniques, for injections of a NBD-dervatized methyl amine solution, yielded a 9% lower plate height 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.

The plot shown in figure 2 illustrates the effects of heating time and applied voltage on injection volume for the heating technique. The plot relates the peak height of methyl amine-NBD to the amount of time the heating apparatus is subjected to voltage. Two separate voltages were used for the trials. Both curves reach a constant value after a length of time which depends on the voltage applied. The heat generated in the heating tube is conducted along the column, resulting in an "injection length" (L_I) of sample within the column which can be greater than the tube length. In this work the heating tube was approximately 2 cm in length and the value of L_I (as observed with a microscope) was about 8 cm, when a voltage of 1.2 volts was applied for two minutes. This corresponds to an injection volume of 40 nL.

There are many parameters and conditions which influence L_I. It is important to adjust and control conditions, such as the thermodynamic properties of the mobile phase, heating tube position on the column, air currents near the injection device, and heating time, so that the value of L_I is both reproducible and not so large as to cause injection related band despersion.

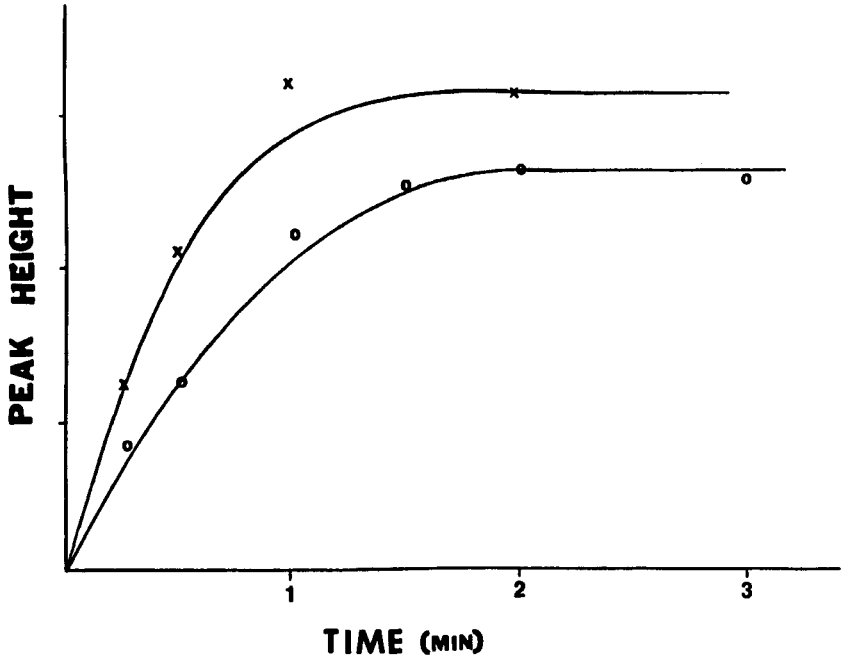

Figure 2. Plot of peak height vs. heating time for "heating" injection technique. The upper and lower plots are for applied voltages of 1.2 and 1.0 volts, respectively.

Equation 1 relates the plate height caused by the injection process (H_I) to the column length (L) and L_I(11).

$$H_I = \frac{L_I^2}{12\ L} \qquad (1)$$

The calculated value of H_I for our conditions was 0.09 mm. The contribution of H_I to the total plate height was sufficiently small so as to provide for baseline resolution of the antitumor drug doxorubicin hydrochloride and two of its metabolites (see

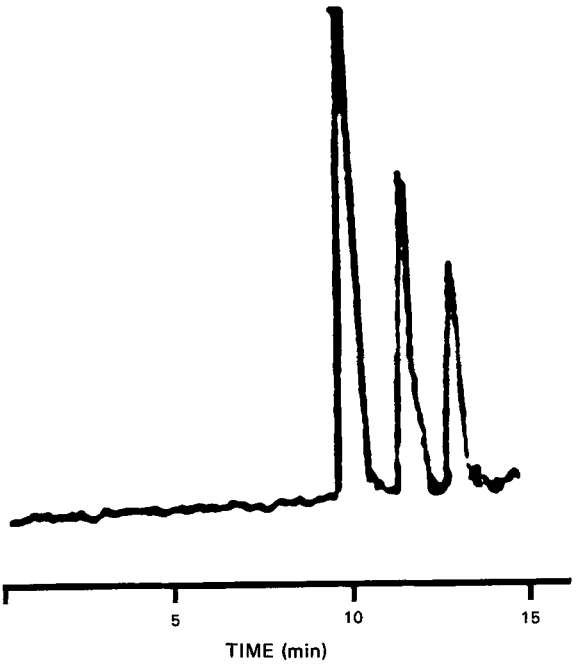

Figure 3. Separation of anti-tumor drugs. Injection was done with the "heating" technique. The compounds in order of elution are: daunorubicin aglycon, doxorubicin hydrochloride, and daunorubicin hydrochloride.

figure 3). The utility of this technique for direct bioanalysis is largely dependent on the sensitivity of the detector employed. Using laser-based fluorescence detection for capillary LC, we have routinely obtained minimum detectable injection quantities in the low pg to high fg range(8,12). A detection limit of one pg corresponds to a concentration in a 40 nL sample of approximately 25 ppb. The therapeutic levels for doxorubicin hydrocloride in plasma are in the 20-200 ppb range, indicating the potential usefulness of

this technique for the direct bioanalysis of the anti-tumor drug and its metabolites.

The reproducibility of the "heating" injection technique was evaluated using a heating time which corresponds to the flat portion of the plots in figure 2. Eight injections of samples containing NBD-derivatized methyl amine were performed. The relative standard deviations in the peak heights obtained were 12% using either the mobile phase or human serum as the sample solvents.

The main disadvantage of the "heating" injection technique is the inconvenience of having to take the sample at the site of the separation, or having to transport the column from the sampling site to the separationlaboratory, then reconnect the column to the pump and realign the detection optics. In any event, rapid, repetitive analysis is not possible with the "heating" injection technique. The "sample tube" injection technique has the advantage of being able to take samples at a rapid rate at a site away from the separation experiment. OTLC using the "sample tube" injection technique should prove useful for pharmokinetic and biodistribution studies. Repetitive samples can be taken, stored directly in the sample tubes (perhaps with freezing), then analyzed sequentially using a single OTLC column. Care must be taken, however, to insure that there is no evaporation or degradation of the sample in the tube between the time the sample is taken and the separation is performed. The volume to be injected can be varied in this injection technique by simple changing the length of the tube employed. This makes for an easily adaptable system of analysis.

The "sample tube" injection technique was also tested for reproducibility using both mobile phase and human serum solvents. Three sample 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 and 8% for individual tubes, when using the same test sample as was used to evaluate the reproducibility of the other injection technique. Although the dimensions of the three tubes were almost identical, the inter-sample tube reproducibility was rather poor. This is possibly due to differences in the permeability of these very narrow-bore tubes, which makes it difficult to completely fill each tube by simple aspiration. The mobile phase solvent yielded a 1-2% better relative standard deviation than did the biological matrix, but the biological usefulness of the "sample tube" injection technique is still intact.

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