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New techniques for liquid chromatography in open-tubular columns*

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Abstract: Methods for sample introduction, capillary column fabrication, and on-column detection are discussed. A new cross-linked polymer stationary phase coating (based on similar coatings for capillary gas chromatography) is described. Results of separations of urinary components using 16-μm open-tubular columns and electrochemical detection and laser-induced fluorescence detection are shown.

Keywords: *Open-tubular liquid chromatography; capillary liquid chromatography; electrochemical detection; laser-induced fluorescence.*

Introduction

Open-tubular columns offer the greatest potential for achieving high separation efficiencies in liquid chromatography. It can be projected that an open-tubular column of 2 μm i.d. and 2 m in length should produce separation efficiencies of one million theoretical plates for solutes with a capacity factor of ten [1]. The utility of such high-efficiency separations for the analysis of complex mixtures is obvious. However, these columns present their own special difficulties. The injection and detection volumes for such columns should be in the low picolitre range [2]. Clearly, conventional techniques of sample injection and post-column detection will not be applicable to these columns and new approaches will be required. In particular, on-column detection, where eluting solutes are detected while still physically in the column, is desirable as it avoids any post-column band broadening or make-up flows which dilute the solutes of interest. Fabrication of columns is also a difficult problem. Methods must be found for depositing significant amounts of stable stationary phase on the walls of the capillary. This paper will describe some of the authors' efforts in developing realistic open-tubular liquid chromatography systems.

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Experimental

Sample introduction

Introduction of sample into the capillary column is accomplished using the 'static splitting' system previously described [1]. In principle this system permits injection of virtually any volume of sample into any diameter capillary column. The volume of sample injected is simply the product of the volumetric flow rate through the column and the length of time of the injection prior to the rinse.

Columns

Capillary columns were fabricated by one of two methods. The first column (referred to hereafter as "ODS") was made by the etching procedure followed by bonding of octadecylsilane, as previously described [1]. This column was constructed from a 250 cm by 16-μm i.d. capillary of Pyrex type 7740 borosilicate glass. This first column was used in conjunction with electrochemical detection.

The second column was also made from a 250 cm by 16-μm i.d. Pyrex type 7740 borosilicate capillary. This capillary was rinsed with methanol, water, rinsed for 1 h with 3.0 M HCl, then rinsed again with water, methanol, and finally dried with helium flow. The column was then coated with a polymer stationary phase which was subsequently crosslinked following (in general) the method of Wright *et al.* [3]. Using positive pressure the column was filled with a solution containing 3% m/v of OV-17 (modified with 2% vinyl groups; stock No. 08277, Alltech Associates, Deerfield, IL, USA) and 0.3% m/v benzoyl peroxide (Aldrich Chem. Co., Milwaukee, WI, USA) dissolved in methylene chloride. The solvent was removed from the column by sealing one end in silicone rubber sealant (Dow Corning, Midland, MI, USA) while connecting the other end to a vacuum. During this procedure the column was immersed in a 60°C oil bath in order to speed the evaporation process. Following complete evaporation of the solvent, the column was flushed with helium and then placed in a 170°C oven for 16 h. Under these conditions, free-radical crosslinking of the stationary phase takes place producing a generally non-extractable polymer stationary phase. The column was finally washed with methylene chloride to remove small amounts of extractable polymer. At this point the column was ready for use. This second column was used in conjunction with laser-induced fluorescence detection, and will be referred to as the 'polymer' column.

Mobile phase

In the case of electrochemical detection the mobile phase was a 0.1 M phosphate buffer pH 4.3 with $1 \cdot 10^{-4}$ M EDTA. For fluorescence detection the mobile phase was a 0.1 M phosphate buffer pH 7.

Detection

Two modes of on-column detection were employed. The first was on-column amperometric detection using a single 9-μm carbon fiber as the working electrode [4]. This fiber is inserted into the outlet end of the capillary using a micropositioner. This detection scheme was arranged as previously described with three modifications. First, a very stable applied potential was generated using a mercury cell and voltage divider. Second, currents were measured with a Kiethley model 610 C electrometer (Kiethley Instruments Inc., Cleveland, OH, USA). Third, the entire column and electrode system was enclosed in a copper mesh Faraday cage to reduce extraneous electrical interference.

These modifications permitted improved detection limits over those reported previously [4]. The carbon fiber was operated at +950 mV vs a silver–silver chloride reference electrode.

The second detector was an on-column fluorescence detector. This detector used a helium–cadmium laser as the excitation source as described previously [5]. The laser can deliver approximately 4 mW of 442 nm blue radiation to a 5-μm focussed spot in the capillary. The emission filter was a 500 nm cut-off long pass filter (No. 5220, Oriel Corporation, Stratford, CT, USA).

Sample preparation

Two urine samples were used in this work. The first, a 24-h urine, was acidified to pH 1.0 with HCl, heated to 80°C for 30 min, cooled, and adjusted to pH 3.5 with NaOH. Prior to injection into the capillary, the urine was filtered to remove any precipitates. This urine was separated on the ODS column and detected electrochemically.

The second urine sample was from a single elimination. Three millilitres of this urine was mixed with 15 ml of 0.1 M carbonate–bicarbonate buffer pH 10, and 2 ml of 'FITC' solution (FITC solution is prepared by dissolving 4.0 mg of fluorescein isothiocyanate into 20 ml of 0.1 M phosphate buffer pH 7). The urine was allowed to react at room temperature for 24 h. Under these conditions, FITC reacts with primary and secondary amines to form highly fluorescent labelled products [6]. A 5 ml sample of this labelled urine was then diluted with 10 ml of 0.4 M phosphate buffer pH 7. The labelled sample was filtered and separated on the polymer column using fluorescence detection.

Results and Discussion

Separation of the first urine sample on the ODS capillary using oxidative electrochemical detection is shown in Fig. 1. In the lower half of this figure the effect of reduced column inlet pressure and hence lower flow rate is apparent in improved resolution at the expense of increased analysis times. The improvements in the electrochemical detector design described in the experimental section have reduced detection limits to concentrations of *ca* 5×10^{-9} M for a two-electron oxidation of catechol.

Separation of the second urine sample as fluorescein-labelled amines was performed on the polymer column with laser-induced fluorescence detection as shown in Fig. 2. The fluorescein-labelled compounds proved to be excellent fluorophores when excited with the 442 nm helium–cadmium laser light. Detection limits for these compounds are approximately $1 \cdot 10^{-9}$ M.

It is too early to give a detailed comparison of the performance of the ODS versus the polymer columns, as the authors have only preliminary experience with the polymer columns. However, initial results with the polymer-coated capillaries look promising, with column efficiencies comparable to those of the ODS columns. Polymer-coated columns appear to offer at least three distinct advantages over the ODS columns. First, the total glass surface area is much lower in the polymer column so that as expected less peak tailing has been observed with these columns. Second, it is expected that the polymer columns should have a longer working life. The ODS columns suffer from a gradual loss of retention over a period of months, presumably due to hydrolysis of the ODS groups from the column surface. The authors' expectations are that the polymer columns will not suffer so greatly from this kind of problem and hence will remain useful over a longer period of time. Third, without requiring an etching step to increase surface

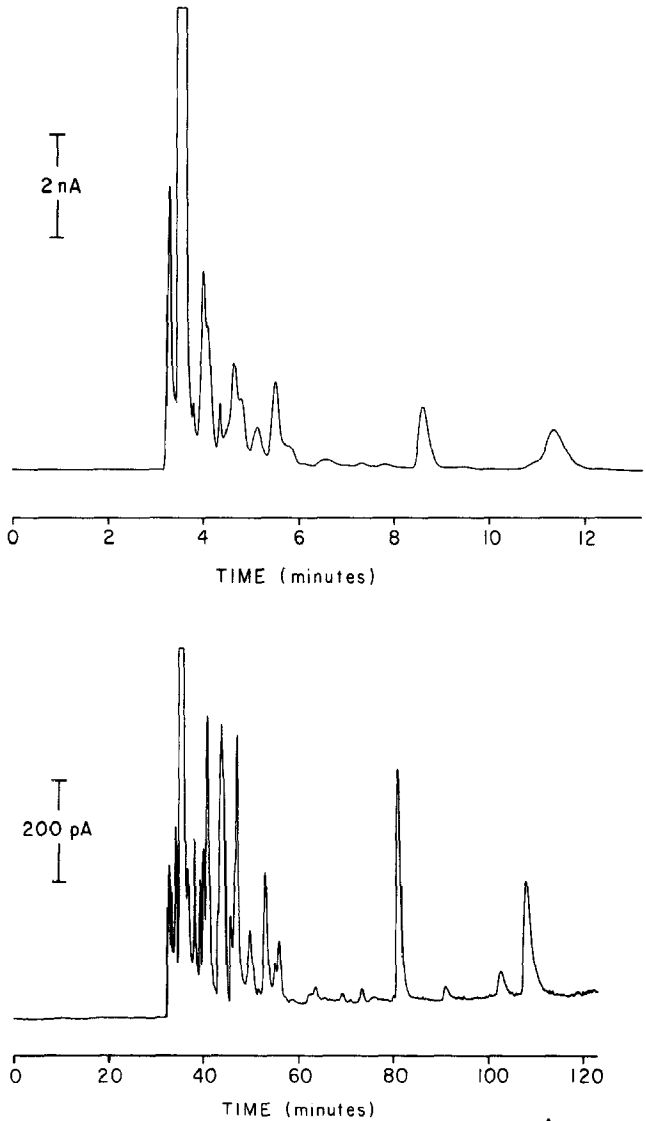

Figure 1
Separation of urine constituents on an ODS column with oxidative electrochemical detection at an electrode potential of +950 mV vs a silver–silver chloride reference electrode. Upper panel: mobile phase head pressure, 400 PSIG; injection time, 5 s. Lower panel: mobile phase head pressure, 40 PSIG; injection time, 15 s.

area, polymer columns can be fabricated from fused silica capillaries. Not only is flexible fused silica more convenient to work with, it also yields a much lower fluorescence background and thus permits lower detection limits in on-column fluorescence detection.

Following further refinement of column fabrication techniques, the authors' research will be directed towards three main goals. First, reduction in column diameters below 16 μm will be pursued. Second, some system permitting mobile phase gradient elution, such as the split-flow system of Van der Wal and Yang [7], must be developed. Third, development of additional detection modes including photoionization, chemiluminescence, and combined LC–MS will be necessary. Improvements in these areas will be necessary to make open-tubular LC a realistic method of analysis.

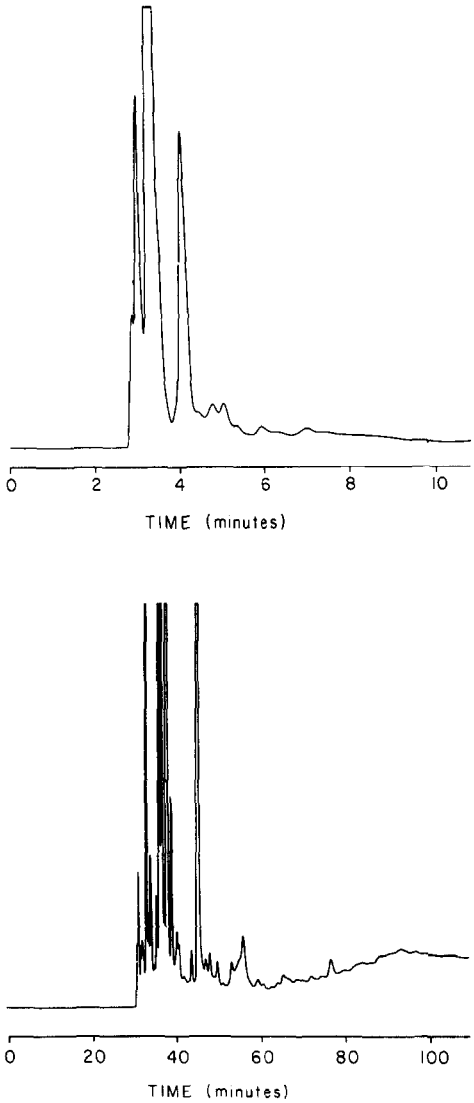

Figure 2
Separation of fluorescein-labelled urinary amines on a polymer column with laser-induced fluorescence detection. Upper panel: mobile phase head pressure, 400 PSIG; injection time, 5 s. Lower panel: mobile phase head pressure, 40 PSIG; injection time, 5 s.

Certainly one goal of open-tubular LC is the achievement of high column efficiencies. However, the requirement for extremely small sample volumes is also of potential interest. As an example, it is conceivable that by using a microcapillary pipette a sample could be withdrawn from a single cell and injected (with the aid of a micropositioner) into a capillary column. Once in the capillary, high resolution separation and analysis is easily carried out. The potential for monitoring hundreds of organic constituents from a single cell creates interesting possibilities for research in biochemistry, neurosciences and pharmacology.

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