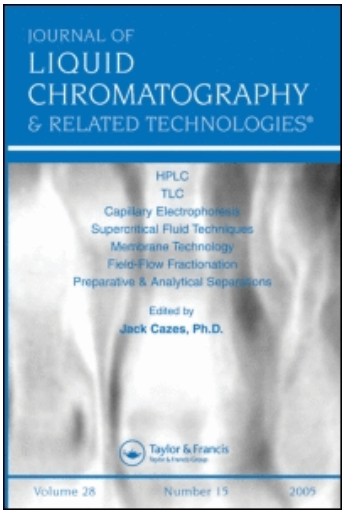

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A MINIATURIZED PRECOLUMN FOR ON-LINE TRACE ENRICHMENT AND DIRECT PLASMA INJECTION IN NARROW-BORE LIQUID CHROMATOGRAPHY

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ABSTRACT

A precolumn for on-line trace enrichment in narrow-bore liquid chromatography has been designed and some critical parameters like precolumn length, inlet capillary I.D. and flow-rate have been studied. The precolumn allows direct injection of plasma and serum samples without any previous clean-up. Due to the trace enrichment, concentration sensitivity is improved 50-fold when 100 µl samples are applied as compared to standard micro-loop injections of 0.5 µl. The applicability of the system has been demonstrated by the analysis of the anti-cancer drug etoposide (VP-16) in human plasma with comparable or even better results than those obtained with conventional-size systems.

INTRODUCTION

Narrow-bore liquid chromatography (LC) - featuring separation columns of about 1 mm I.D. - offers advantages such as

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relatively low solvent consumption and high mass sensitivity. However, due to the limited injection volume of about 1 µl and the diminished pathlength in, e.g., UV absorption detector flow-cells, concentration sensitivity is often rather bad. Poppe and Kraak (1) concluded that narrow-bore LC often is not the correct choice when doing trace analysis. To solve this problem, several authors describe trace-enrichment techniques prior to the actual analysis. Ishii et al. (2) used a micro-precolumn which is loaded off-line and then placed on the top of the separation column; automation is impossible. Krejci (3) and Apffel et al. (4) used on-column preconcentration applying the peak-compression principle but column lifetime was shortened considerably when real samples were analyzed. Kok et al. (5) designed a micro-precolumn for an on-line system which performed quite well; the main disadvantage was the impossibility of a continuous flow through the separation column. A relatively long narrow-bore precolumn was used by Scott and Kucera (6) in combination with gradient elution which seems to be essential there for a good overall performance. Obviously, despite all these attempts, there still is a need for a simple micro-precolumn which can be packed manually and used in on-line (automated) systems.

In biomedical analysis, the available sample volume is much higher than 1 µl and generally is in the 100-1000 µl range. This means that narrow-bore LC with on-line trace enrichment is especially suitable for biomedical applications. Enrichment factors of about 100 should be obtainable when 100 µl samples are used and desorption is efficient. In recent years, direct injection of biomedical samples on a conventional-size precolumn has become more and more popular (7-11). In several studies, relatively large precolumns (30 x 4.6 mm I.D.) were used containing 30 µm C-18 particles. Various applications with (semi-)automated systems have appeared in the literature. Recently, Juergens (12) used short (5 x 4.6 mm I.D.) precolumns for direct injection of serum samples. Thanks to the short length, backflush desorption was not necessary any more and the forward desorption used re-

duced the precolumn clogging problems often caused by constituents of serum samples.

This paper describes the design of a micro-precolumn for on-line trace-enrichment in narrow-bore LC which is compatible with direct plasma injection without any sample pretreatment. The system has been applied to the analysis of the anti-cancer drug etoposide (VP-16) in plasma samples.

GENERAL CONSIDERATIONS

In our group, various types of precolumns have been designed for conventional-size systems (4.6 mm I.D.) and they have been routinely applied in serveral research projects (13). Recently, we developed a 2 mm I.D. precolumn based on the same design and used it with 3 mm I.D. LC systems (14); today, it is also coupled to 2 mm I.D. separation columns for on-line LC-mass spectrometry (15). However, when further decreasing the (pre)column diameter, i.e., to 1 mm I.D., several problems arise. From a construction point of view, further reduction of the previous design (13) is almost impossible and, besides, the design does not meet the requirements of narrow-bore LC such as, e.g., negligible band broadening. In other words, one has to develop a completely different type of precolumn for narrow-bore work. The following considerations have to be taken into account.

A micro-precolumn should be used efficiently, because of the small amount of packing material present. That is, there should be a flow-spreading device on the top of the precolumn, which effects spreading of the sample over the total diameter of the precolumn. Usually, the top of the precolumn is sealed with a porous frit, but with direct plasma injections metal sieves (screens) are preferred to prevent clogging of the precolumn by protein fragments (11). The use of screens instead of porous frits also reduces band broadening. However, screens will give no flow-spreading which means that the inner diameter of the inlet capillary and the flow-rate during the loading step may

play an important role with regard to the efficiency of the precolumn and the reproducibility of its performance.

Direct plasma injection without any clean-up requires wide-bore connecting capillaries (11). For long precolumns (30 mm) this holds true for the inlet side of the precolumn only, but for short micro-precolumns where protein fragments are expected to pass through the precolumn, wide-bore capillaries are needed on both the inlet and the outlet side of the precolumn. However, this is incompatible with the requirements of high-efficiency narrow-bore LC where band broadening should be as low as possible. That is, combining direct plasma injection with narrow-bore LC will unavoidably lead to a compromise between sensitivity enhancement by trace enrichment and sensitivity loss by extra-column band broadening.

EXPERIMENTAL

Apparatus

A Kontron (Zürich, Switzerland) Model 410 pump and a Uvikon 720 LC variable wavelength UV detector were used in combination with a Gilson (Villers Le Bel, France) Model 302 pump and two modified Valco (Houston, TX, USA) six-port valves. A Perkin Elmer (Norwalk, CT, USA) LS-4 fluorescence detector equipped with a 3 µl flowcell and operated at 230 nm excitation and 328 nm emission wavelength was used for etoposide studies. For band broadening studies a home-made 0.5 µl injection valve modified from an early Jasco valve (16) and a Kratos (Ramsey, NJ, USA) Model 773 variable-wavelength detector with a 0.5 µl flowcell, operated at 230 nm, were used. Chromatograms were recorded on a Kipp & Zonen (Delft, The Netherlands) BD 40 recorder. For peak area measurements, an Anacomp (Kontron) computing integrator was used.

Chemicals

HPLC-grade water and methanol were obtained from Baker (Deventer, The Netherlands). HPLC-grade acetonitrile was obtained from Promochem (Wesel, FRG). The test solutes, phenol,

4-chlorophenol and 3,5-dichlorophenol, were purchased from Fluka (Buchs, Switzerland). The anti-cancer drug etoposide (VP-16) was a gift from the Free University Academic Hospital (Amsterdam, The Netherlands).

Stationary phases and columns

The analytical column was a 20 cm x 1 mm I.D. glass-lined stainless-steel column home-packed with 5 µm LiChrosorb RP-18 (Merck, Darmstadt, FRG). Precolumns were packed with 40 µm C-18 (Baker) or 40 µm C-8 (Analytichem, Harbor City, CA, USA) bonded silica. Metal sieves (screens) were obtained from Chrompack (Middelburg, The Netherlands) (5 µm), Paul GmbH (Steinau, FRG) (13 µm) and Dinxperlo BV (Dinxperlo, The Netherlands) (36 µm). Fiberglass discs were from Knauer (Bad Homburg, FRG).

Design of the miniaturized precolumn

The final precolumn which was successfully used for direct plasma injection in narrow-bore LC, was made from a stainless--steel body which resembles a connector (Figure 1).
The packing material is held between two screens which are fitted in between the 'connector' body and the 1/16 inch O.D. connecting capillaries. Various inner diameters of these capillaries were studied (see below). Four precolumns, with inner bed lengths of 2, 3, 4 and 5 mm were constructed with the same inner diameter of 1 mm. The precolumns could be packed manually by first replacing a screen and a connecting capillary by a syringe adapter, as shown in the top half of Figure 1. Next, a syringe filled with a thin slurry of the packing material in methanol was fitted to the adapter and the precolumn was packed. The precolumn was emptied by using a syringe filled with methanol after the other screen had also been removed.

Methods

THe influence of the precolumn length and the inner diameter of the inlet capillary on the retention of the test solute 3,5--dichlorophenol (dissolved in HPLC-grade water) on 40 µm C-18

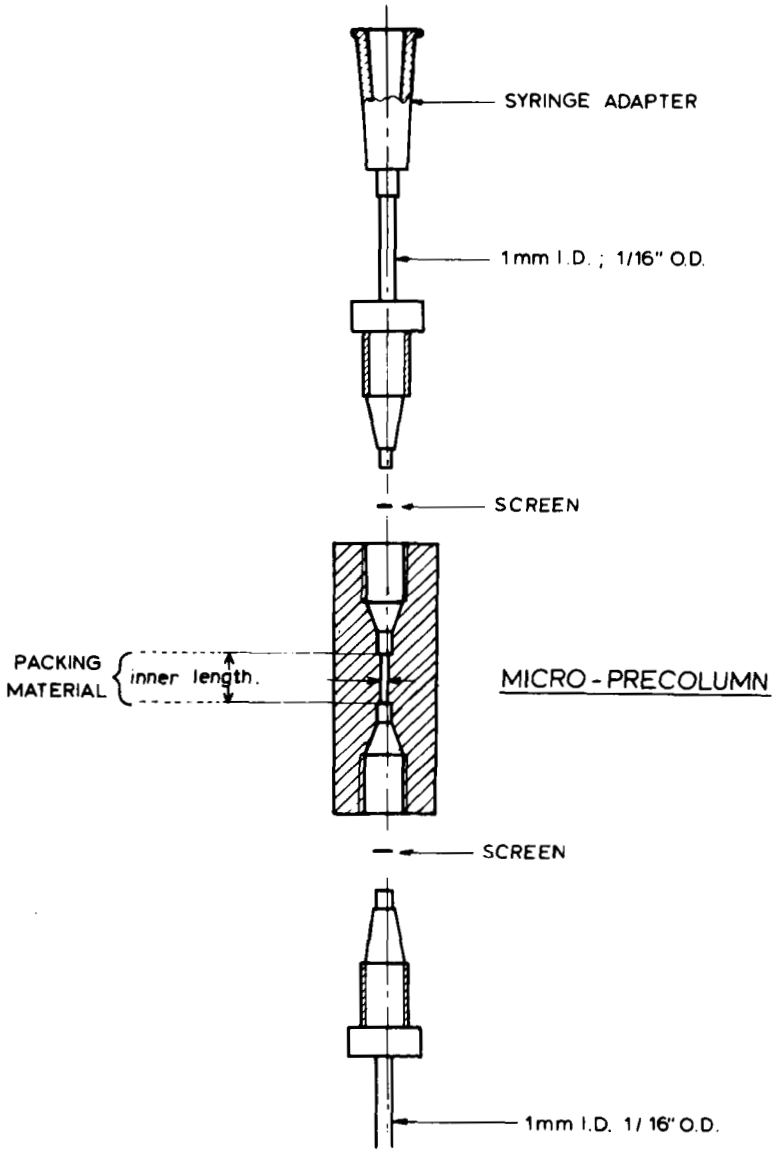

FIGURE 1. Micro-precolumn with syringe adapter for hand-packing and 'field-sampling'.

bonded silica, was studied by determining breakthrough volumes according to the procedure of ref. 17. Precolumns with an inner bed length of 2-5 mm were used and the inner diameter of the inlet capillary was varied between 0.13 and 1.1 mm. Breakthrough curves were recorded at 100 µl/min and the breakthrough volume was determined.

The recovery was calculated by comparing the results of a series of 300 µl on-line trace enrichment experiments on a 5 x 1 mm I.D. precolumn with those of enrichment of the same sample volume on the analytical column. The additional band broadening caused by the micro-precolumn and the connecting capillaries was determined by comparing bandwidth (at 0.6 of the peak height) obtained in 100 µl trace enrichments with that from 0.5 µl loop injections (without trace enrichment).

The experiments involving direct plasma injection were done using the set-up of Figure 2. Fresh human plasma was centrifuged and spiked with etoposide by adding 1 ml of a 400 ppb standard solution to 1 ml plasma. 100 µl samples were directly injected into the loop and transferred to the micro-precolumn with a forward-flush step of 3000 µl water at 200 µl/min. Desorption was done in the forward-flush mode during 2 min with 40% acetonitrile in water at 100 µl/min. Then the precolumn was reconditioned in forward flush with 2000 µl water (at 200 µl/min) prior to the next sample injection.

RESULTS AND DISCUSSION

Design of the miniaturized precolumn

In principle, screens of different pore sizes can be used in the precolumn. However, their structure may play an important role. For example, with the 13 µm screen material, no leak-tight connection could be made. The other screens (5 and 36 µm) performed well in this respect. Using a precolumn equipped with the 5 µm screens, both 5 µm and 40 µm particles could be packed, either manually or under high pressure (110-160 bar). For short precolumns (2 x 1 mm I.D.) there was no difference in performance

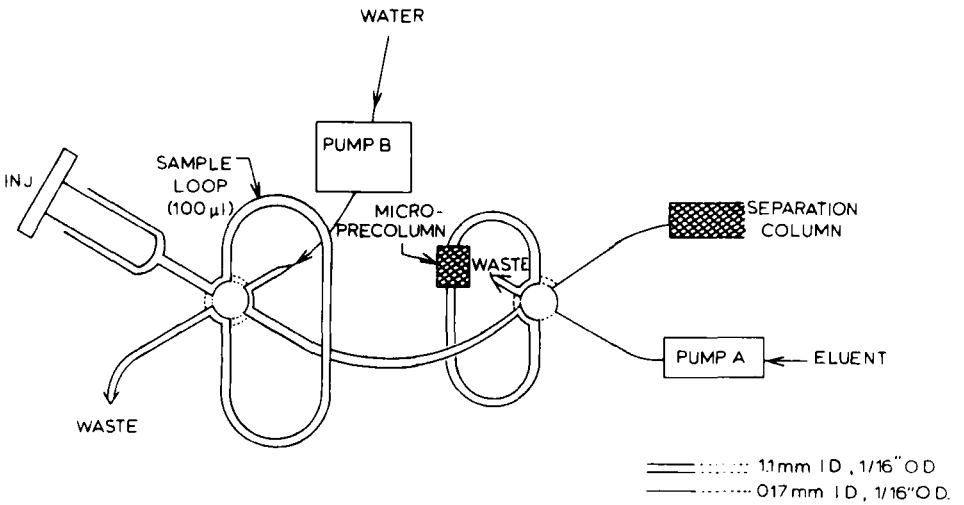

FIGURE 2. Experimental set-up for direct plasma injection and on-line trace enrichment of etoposide. Precolumn 5 x 1 mm I.D. Analytical column, 20 cm x 1 mm I.D. packed with 5 µm LiChrosorb RP-18. Eluent, 40% acetonitrile at 100 µl/min. Pump B, 200 µl/min. Detection by fluorescence at 230 (ex.)/328 (em.) nm.

- expressed as breakthrough volume of 4-chlorophenol on 5 µm C-18 bonded silica - between hand-packing using the syringe method and high-pressure packing of the precolumn. For longer precolumns (5 x 1 mm I.D.) a difference was observed in the case of the 5 µm material (345 µl ± 26% versus 470 µl ± 21%; n = 3) but it is not statistically significant. When 40 µm C-18 bonded silica was used, no difference was found between hand-packing (475 µl ± 10%) and high-pressure packing (425 µl ± 23%) for the 5 x 1 mm I.D. precolumn. We used the 40 µm particles in all further experiments in order to prevent clogging of the precolumn in the plasma studies.

As was mentioned above, because of the intended use of the precolumn (blood samples), we used wide-bore (1.1 mm I.D.) inlet and outlet capillaries. It is evident that, with cleaner samples, a narrow-bore outlet capillary can be used, which will help to reduce extra-column band broadening. The precolumn design allows

both forward-flush and backflush elution. Although the latter technique will cause decreased band broadening during the desorption step, we agree with Juergens (12) that forward-flush desorption should be preferred because backflush desorption can lead to clogging of the separation column.

Precolumn length and inlet capillary I.D.

For each precolumn length and at each I.D. of the inlet capillary three precolumns were packed and on each precolumn the breakthrough of 3,5-dichlorophenol was measured in triplicate. Breakthrough volumes were read from the breakthrough curves and corrected for the dead volume of the system by measuring the breakthrough for phenol which may be considered to have negligible retention in this system.

From Figures 3 and 4 one can read that the breakthrough volume per unit bed length is between 300 and 400 µl/mm, provided one uses 3-5 mm bed lengths and 0.50 mm or wider-bore capillaries. That is, trace enrichment can be carried out satisfactorily, even for relatively polar compounds such as dichlorophenol on micro--precolumns. With very short precolumns, and especially when using a 0.13 mm I.D. inlet capillary, the situation is rather critical (see the results for the inter-column comparison, Figure 4) probably due to a lack of efficient flow-spreading over the entire precolumn diameter. This is seen even more clearly from the rel. S.D. data in Figure 5.

Some further remarks should be made regarding the performance of the very short precolumns. (1) The breakthrough volumes for 3,5-dichlorophenol per mm bed length generally are rather low; in the case of the 2 mm precolumn, this is even observed with the 1.1 mm I.D. inlet capillary. This phenomenon is due to a small distortion of the top of the precolumn bed which will be of more significance at shorter length. (2) The rel. S.D. of the 2 mm length/0.13 mm I.D. precolumn (see Figure 5) was further increased when the flow through the precolumn was higher than 0.5 ml/min. Obviously, at such a flow-rate which is extremely high for narrow-bore LC, mechanical distortion of the precolumn

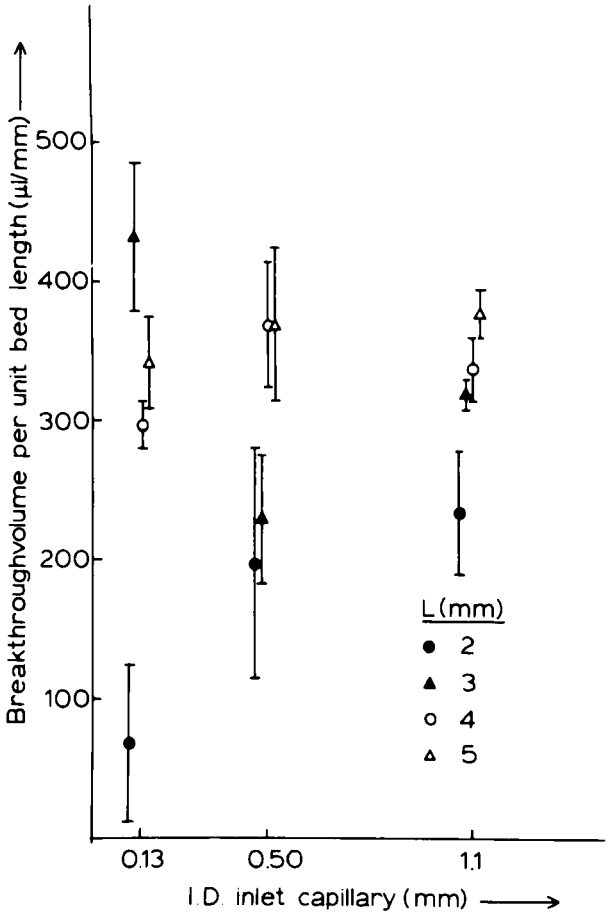

FIGURE 3. Dependence of breakthrough volume per unit bed length of the precolumn (µl/mm) on the inner diameter of the inlet capillary within one precolumn (n = 3). Conditions: Precolumns of 2, 3, 4 and 5 mm bed length, and all having 1 mm I.D., packed with 40 µm C-18 bonded silica and equipped with 5 µm screens. Test solute, 3,5-dichlorophenol in distilled water (338 ng/ml). Flow-rate, 100 µl/min. UV detection at 211 nm.

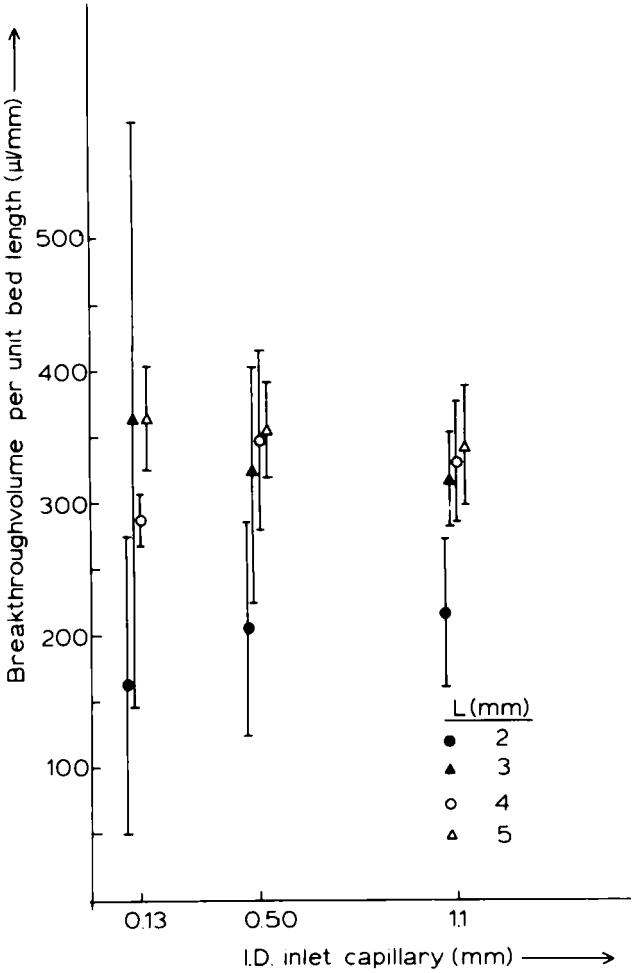

FIGURE 4. Dependence of breakthrough volume per unit bed length of the precolumn (µl/mm) on the inner diameter of the inlet capillary for three different precolumns. Conditions as in Fig. 3.

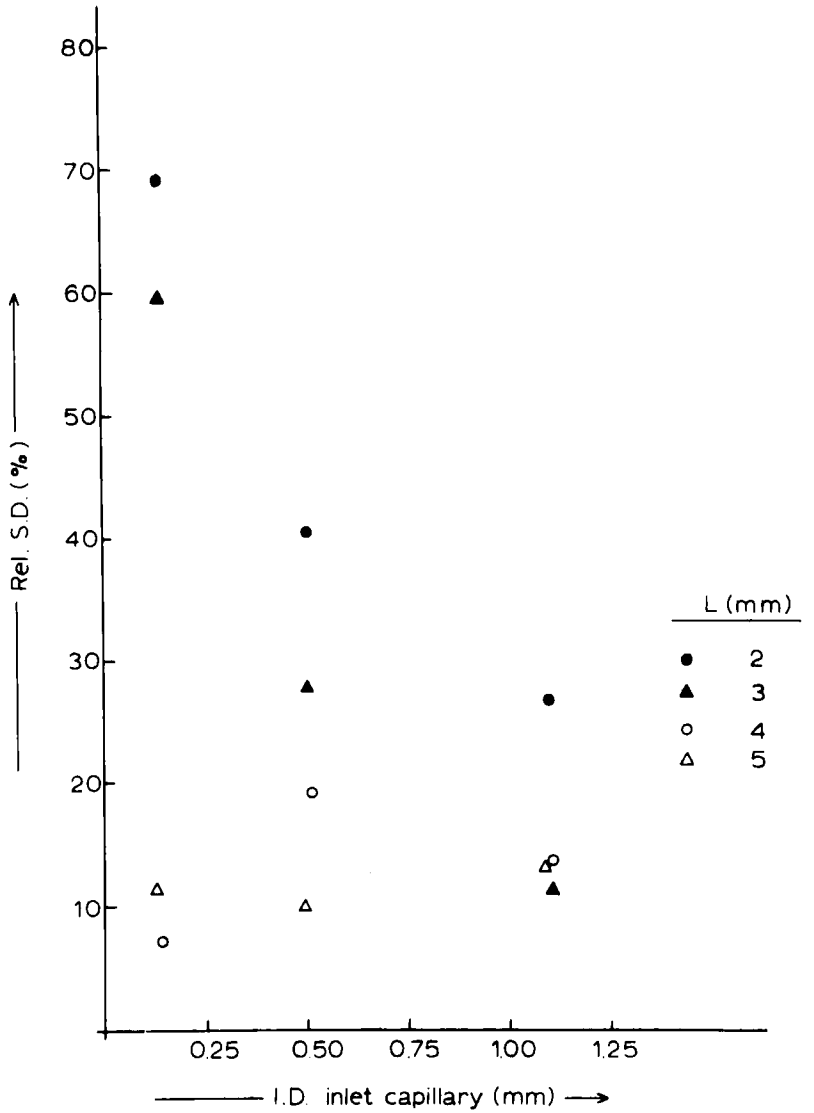

FIGURE 5. Relative standard deviation (%) of the mean breakthrough volume as a function of precolumn length and inlet capillary I.D. Calculations based on the data in Figs. 3 and 4.

bed occurs. (3) When fiberglass discs were applied instead of the 5 µm screens, the rel. S.D. of the 2 mm length/0.13 mm I.D. decreased from 69% to 13% because of the better flow-profile inside the precolumn. However, the discs had insufficient mechanical strength when combined with 1.1 mm I.D. capillaries which have smaller contact areas. Another disadvantage is the thickness of the fiberglass discs which will cause relatively much extra-column band broadening in high-efficiency applications.

In summary, for easy and reliable trace-enrichment experiments, one should use precolumns of 4-5 mm bed length, with a 0.5-1.0 mm I.D. inlet capillary, and provided with screens.

Application to etoposide

The analytical applicability of the micro-precolumn was studied with etoposide (VP-16) as model compound. A 5 x 1 mm I.D. precolumn with 1.1 mm I.D. capillaries was used, with 36 µm screens and packed with 40 µm C-8 bonded silica.

Recovery: 300 µl of a standard solution of etoposide in water were concentrated on the analytical column (in triplicate) using the peak-compression technique and detected by fluorescence. The mean peak area so obtained was compared with that in on-line trace enrichment on the precolumn, at a loading speed of 200 µl/min. The subsequent forward-flush step was varied from 700 to 1200 µl water (6 data points). The breakthrough volume under these conditions was sufficiently high (4500 µl) to prevent breakthrough in these experiments. The recovery calculated via peak area measurement was 81 $\pm$ 1%. This percentage was constant within one precolumn and in between three different precolumns over a period of several weeks.

The excellent reproducibility of the recovery does not contradict the results of the breakthrough studies for 3,5-dichlorophenol because of the large safety margin between the breakthrough volume of etoposide and the actually applied flush volume (4500 versus 1200 µl). The 20% loss in recovery cannot be

explained by insufficient sampling (from loop to precolumn) or breakthrough during the flush step because varying the flush volume from 700 to 1200 µl did not influence the recovery.

Band broadening caused by the micro-precolumn: With etoposide as the test compound (k' = 1.5), the band width, σ_t, for a 10,000 plate C-18 analytical column, was increased by a factor of 3.5 when on-line trace enrichment of a 100 µl sample was compared with 0.5 µl micro-loop injection. This means that the actual enrichment factor was 200/3.5 = 55 instead of 200, which still is an improvement in terms of sensitivity (expressed in units of concentration) of more than 50-fold. In principle, additional band broadening will be less dominant for peaks with higher k' values, and the enrichment factor will be further increased.

Analysis of blood samples: The micro-precolumn was used for the determination of etoposide in plasma and serum samples. 100 µl plasma and serum samples, containing 200 ng/ml of etoposide, were injected and concentrated without any previous filtration or clean-up on the 5 x 1 mm I.D. precolumn packed with 40 µm C-8 bonded silica. A relatively large flush volume of 3000 µl water was used to prevent clogging problems during the desorption and to remove polar interferences. On-line desorption in the forward-flush mode was done with only 200 µl eluent in order to retain the less polar contaminants on the precolumn. This procedure resulted in a sufficiently clean chromatogram (Figure 6) and eliminated the need for post-column extraction for further clean-up (18). The detection limit of etoposide was 500 pg (signal-to-noise ratio, 3:1) or 5 ng/ml in plasma and is comparable to, or better than, the results obtained in conventional systems (18,19).

The repeatability of the method was tested by injecting a spiked plasma sample ten times on the same precolumn. Each time the precolumn was regenerated on-line with 2000 µl of water before being used again for the next analysis. Peak height

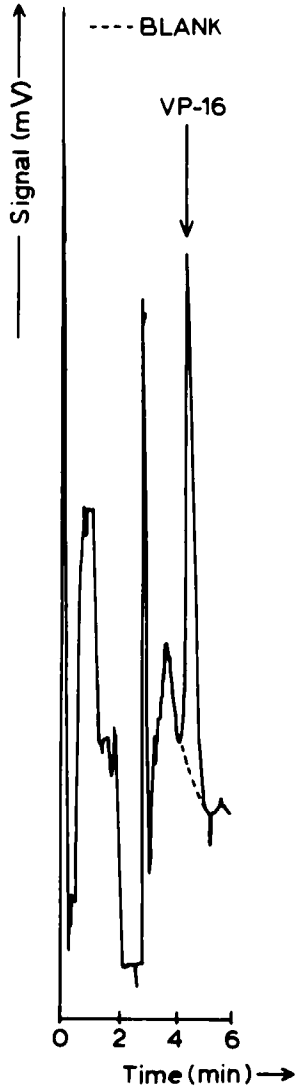

FIGURE 6. Trace enrichment of etoposide (200 ng/ml) from 100 µl of a known plasma sample on a 5 x 1 mm I.D. precolumn, packed with 40 µm C-8 bonded silica. Other conditions as in Fig. 2.

measurements showed the rel. S.D. to be ± 5.4% (n = 10). The back-pressure in the system did not noticeably increase during these experiments.

The recovery of etoposide from spiked human plasma as compared to preconcentration of a standard solution on the same precolumn was 70%; for spiked calf serum the recovery was 90%.

CONCLUSION

The sample capacity in narrow-bore LC can be successfully improved by on-line trace enrichment on a small precolumn. The precolumn developed in the present study allows hand-packing by means of a syringe. The same principle can be applied for field sampling; for example, small blood samples can be loaded on such (low-cost) precolumns in the hospital or in a doctors office and analysis can afterwards be done in the laboratory. For reproducible breakthrough volumes, precolumns with inner lengths of 4-5 mm are recommended in combination with 0.5-1.1 mm I.D. inlet capillaries, and provided with stainless-steel screens.

The additional band broadening caused by the precolumn is relatively large because wide-bore inlet and outlet capillaries have to be used to allow injection of plasma samples without any pretreatment. In spite of this band broadening, enrichment factors of more than 50 are easily obtained with real samples (100 µl trace enrichment vs. 0.5 µl loop injection). This has been demonstrated for plasma samples spiked with low ppb levels of etoposide.

Further reduction of band broadening and automation of sample handling in narrow-bore LC are currently under investigation.

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