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EFFECT OF THE POSITION OF THE LASER BEAM FOCAL POINT ON A CAPILLARY FLOW-THROUGH CELL ON THE SIGNAL-TO-NOISE RATIO FOR A FLUORIMETRIC DETECTOR IN CAPILLARY COLUMN LIQUID CHROMATOGRAPHY

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SUMMARY

The use of a high-sensitivity laser-induced fluorescence detector with a capillary flow-through cell (100 μm I.D.) in capillary column liquid chromatography was studied. To reduce the background noise from scattering of the laser light from the cell walls by reflection and refraction, the geometric position of the focal point of the laser beam on the capillary cell is very important. By moving the focusing lens of the laser beam, the best position of the laser on the capillary cell was examined. The use of a video camera was also helpful in setting the laser beam on the capillary cell. With the optimum geometric arrangement, 1.3 fmol of the 4-bromomethyl-7-methoxycoumarine derivative of caproid acid ($k' = 3$) was detected at a signal-to-noise ratio of 5.

INTRODUCTION

Micro-column liquid chromatography (LC) has the advantages of a higher resolution and a smaller amount of sample than those in conventional column LC[1–6]. Micro-column LC has a greater potential for the analysis of complex samples in biological and medical areas where the amount of sample available may be limited[1,4]. As the amount of sample injected in micro-LC is very small owing to the low loading capacity of the micro-column, suitable detection techniques of high sensitivity are necessary. Several novel devices, such as laser-based[5–12] and electrochemical[13,14] detectors, have been studied.

Fluorescence is one of the most sensitive detection techniques available in LC. The use of lasers as excitation sources[5–12,15] is very suitable for a micro-cell, because the beam can be focused on to a very small area with high intensity. Although laser fluorescence detectors have been well studied, there have been few reports concerning the position of the focused area of the laser beam in or on the very small cell. Folestad *et al.*[6] discussed the relationship between the relative fluorescence intensity and the distance between the lens and the cell, and Dovichi *et al.*[7] examined the fluorescence

intensity as a function of laser-induced position in the cell. Lyons and Faulker[15] examined the position of the collection lens and the effect of the shapes of cells for conventional LC.

In this work we examined the focused position of the laser beam on a capillary flow-through cell. We found that the position of the spot of the laser beam is important for obtaining a good signal-to-noise (S/N) ratio. After finding the optimum position of the laser beam spot, we applied the present system to the separation of fluorescent derivatives of carboxylic acids.

EXPERIMENTAL

Chromatographic system

A schematic diagram of the analytical system is shown in Fig. 1. A 25 cm $\times$ 320 μm I.D. fused-silica capillary tube (Quadrex, supplied from Tokyo Kasei Kogyo, Tokyo, Japan) was slurry packed with Shim-pack CLC-ODS, 5 μm (Shimadzu, Kyoto, Japan). The slurry solvent was carbon tetrachloride–liquid paraffin (1:1)[16]. A Model LC-6A pump (Shimadzu) was used at constant pressure or constant flow-rate. A Shim-pack SBC-ODS column (15 cm $\times$ 2.5 mm I.D.) was used as in a resistance in the split-flow line. A 0.06-μl internal loop injector (Model C-14W; Valco Europe, Schenkon, Switzerland) was used. The column head was inserted into the injector. Just after a PTFE frit (Kusano Kagaku Kogyo, Tokyo, Japan) at the end of the slurry-packed capillary column, a length of fused-silica capillary (50 or 100 μm I.D.) was used for connection to a flow-through capillary cell.

Laser fluorescence detector

The 325 nm output of a helium–cadmium laser (Model 4240 NB, 10 mW) (Liconix, Sunnyvale, CA, U.S.A.) passed through a short-pass filter (Type UV D 36C; Toshiba, Tokyo, Japan), which cut out wavelengths of more than 360 nm, then the beam passed through a focussing convex lens (synthesized quartz, focus 25 mm;
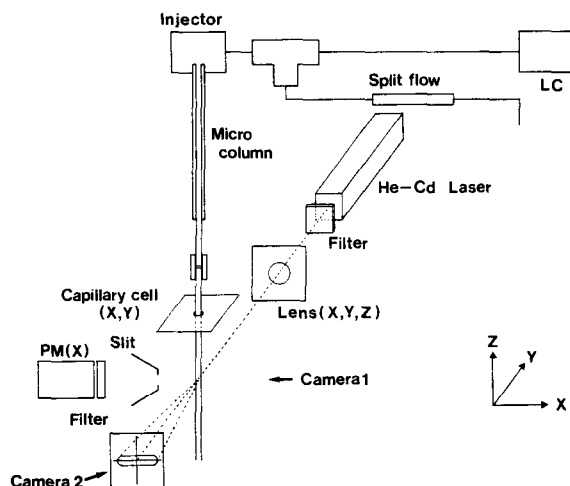

Fig. 1. Schematic diagram of laser fluorescence detector device for micro-LC.

Sigma Kohki, Irima-gun, Saitama, Japan). The focusing lens was moved in the x and z directions by a positioner with help of synchronous motors (225 s per rotation per 0.5 mm) and in the y direction manually by a positioner, as shown in Fig. 1. By adjusting the positioner on the y axis, the laser was focused on the capillary flow-through cell. The spot of the laser beam on the capillary was transferred according to the position of the focusing lens (x and z axes). Fluorescence, scattered light and refracted light from the cell were received by a photomultiplier tube (PM) (Type R374; Hamamatsu Photonix, Shizuoka, Japan) after passing through a slit of 1 mm I.D., and a long-pass filter which cut the wavelength down to 380 nm (Type SCP-38L; Shigma). A high-voltage supply (Model C665) and a pre-amplifier (Model C1556) (Hamamatsu Photonix) were used. The amplified output of the PM was recorded with a data-handling instrument (C-R5A; Shimadzu). The positions of the long-pass filter and the PM were adjustable on the x axis. The PM was arranged perpendicularly to the laser beam. A video camera–TV screen system (Model GX-N4CH; Japan-Victor, Tokyo, Japan) was used.

Detector cell

As a capillary flow-through cell, a fused-silica capillary tube (100 μm I.D. and 160–180 μm O.D.) was used. The polyimide coating of the capillary tube had been removed for a length of about 10 mm in the region on which laser beam was focused. The capillary cell was moved by positioners in the x and y directions. The capillary column and the capillary cell were connected with PTFE tubing (*ca.* 10 mm × 0.1 mm I.D.) as described by others[5].

Reagent

Carboxylic acids were derivatized with the fluorescence reagent 4-bromomethyl-7-methoxycoumarin (Br-Mmc) (Tokyo Kasei Kogyo) according to the procedure of Dunges[17]. All reagents used were of guaranteed grade.

RESULTS AND DISCUSSION

Because of the coherence properties of a laser, it can deliver to a target a highly collimated beam of light with high power levels. The 325-nm (10mW) laser beam used was focused as a small, round spot of diameter 30 μm. The area of the spot was observed with the video camera and its diameter was measured by using a 10-μm slit. In capillary column LC, the capillary cell has a very small (sub-nanolitre) volume to avoid band broadening in the extra column parts. Therefore, light for excitation should be focused accurately on a very small area of the capillary cell, and this can be achieved without much difficulty with a laser beam. Although a strong excitation beam is desirable for obtaining strong fluorescence, it is also necessary to reduce the background interference and to collect as much fluorescent light from the cell as possible for high-sensitivity detection. This background interference arises from (a) scattering of the laser light from the cell walls by reflection and refraction (RR), (b) elastic (Rayleigh) scattering of the light, (c) inelastic (Raman) scattering of the laser light and (d) fluorescence from other interfering species[15]. Raman scattering is usually excluded by a suitable combination of excitation and detection wavelengths. The fluorescence from other interfering species comes mostly from the eluent itself, its

impurities and the LC system, especially impurities from the column. Our concern is therefore with processes (a) and (b) above.

Even though a long-pass filter is used, shown in Fig. 1, for eliminating the scattered light produced by processes (a) and (b), the detection system cannot separate light at the detection wavelength perfectly from light scattered at the excitation wavelength. To obtain a higher sensitivity of detection, it is very important to find a means of decreasing the background interference. For this purpose, we searched for the optimum geometric position of the spot of the laser beam by scanning the position of the spot on the cell in the x, y and z directions. In this experiment, fluorescence and scattered light were received by the PM just after passing a long-pass filter.

Visual arrangement of the line of the capillary cell and laser beam

Using video camera 1 and a TV screen, as shown in Fig. 1, we could see roughly the position of the laser beam on the capillary cell by magnifying the image up to 100-fold on the TV screen (1 mm equal to 10 cm on the screen). By adjusting the position of the focusing lens in the x, y and z directions, we could obtain a round spot of laser light as small as 30 μm in diameter on the capillary cell. After the laser light had been scattered by the capillary cell, it gave several different pictures at the back of the capillary cell. We placed a sheet of paper containing fluorescence material at its back position, and observed the image on the paper with video camera 2, as shown in Fig. 1. Sketches of the images on the TV screen are shown in Fig. 2, and the assumed part of the capillary cell through which light was passed and the light loci are shown in Fig. 3a and b, respectively. For image A in Fig. 2 the laser beam was focused well at the centre of the capillary cell along the X_a line (position x_c in Fig. 3a). For C and E the laser beam was focused on the two edges of the capillary cell, that is, positions x_a and x_b, respectively, in Fig. 3a. Images B and D were obtained between x_a and x_c and between x_c and x_b, respectively. It is possible to establish the location of the focused position of the laser beam from the observation of the scattered light images, and to adjust the position of the laser at the centre of the capillary cell by using the positioners.

Adjustment of the distance between capillary cell and slit by observing the response of the PM

The slit in Fig. 3a, through which scattered light and fluorescence were passed to the PM, had a hole of 1 mm diameter. The capillary cell was set at the middle of the hole by the y positioner of the cell by observation with camera 1. Then the cell was set at different position on the X_a line with the positioner. Laser light was moved from the slit position ($x = 0$) to the cell by moving the focussing lens along the x axis with a motor. As the focus of the lens is always on the X_a line in Fig. 3a, the laser is also always focused on the X_a line. The laser mostly crosses the X_a line perpendicularly.
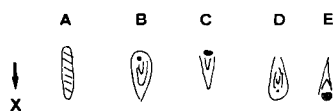

Fig. 2. Sketches of scattered patterns of the laser beam at the back of the capillary cell.
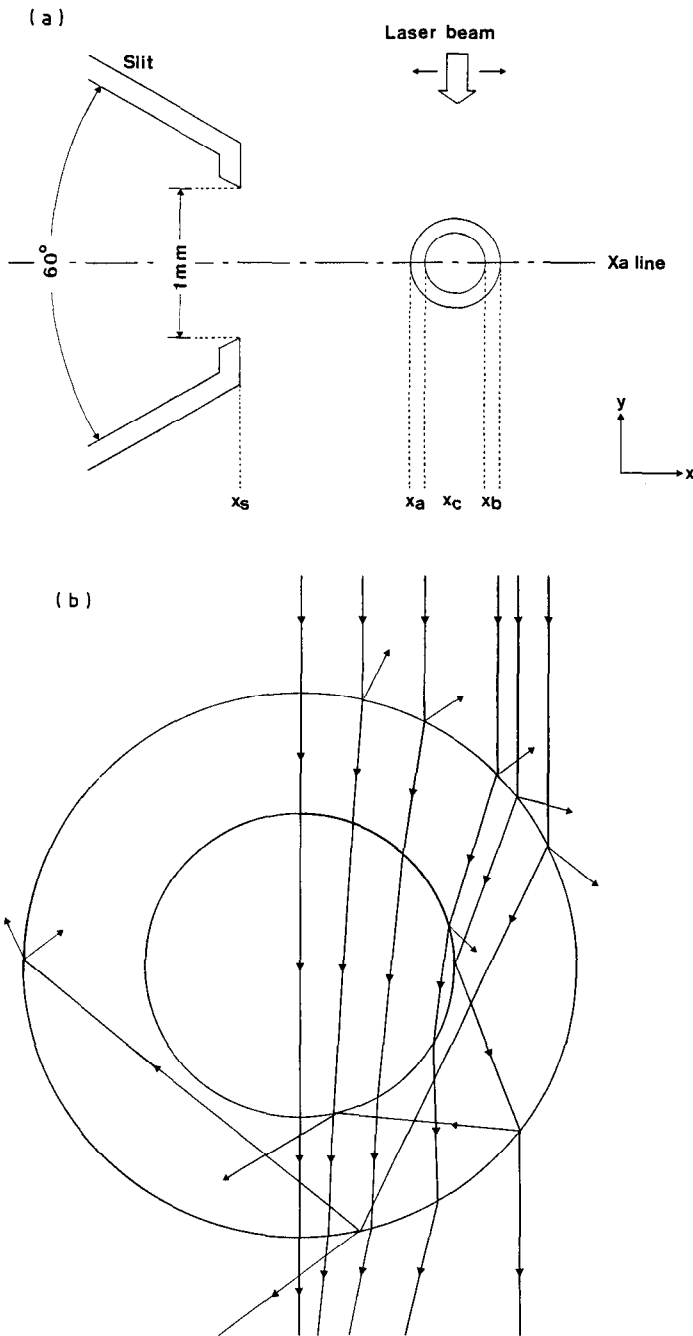
Fig. 3. (a) Sectional view of capillary cell and slit. (b) Assumed light loci at capillary cell filled with acetonitrile. Capillary cell: fused-silica tubing, 100 μm I.D. and 180 μm O.D. (thickness of the tube wall, 40 μm). The refractive indices of quartz and acetonitrile are assumed to be 1.458 and 1.344, respectively. The critical angles of the former and the latter are 43.3° and 67.2°, respectively.

The maximum deflection of the laser from the perpendicular to the X_a line would be 2.3°, because the focal length of the lens was 25 mm and the total transferred distance of the lens was 2 mm.

In Fig. 4, the applied voltage to the PM was set at 450 V and the response of the PM was recorded with movement of the lens. The first large peak ($x = 0$) came from direct reflection of the laser beam at the edge of the slit (laser position at x_s in Fig. 3a). The second (a) and third peaks (b) correspond to the laser at positions x_a and x_b, respectively, in Fig. 3a. In these positions, the laser beam was reflected on the surface and also passed through the tube wall with complex loci, showed in Fig. 3b. However, at position x_c, the laser beam passed through the inside of the capillary cell, and minor RR might have occurred.

There are narrow regions of low noise level between peaks a and peak b in 1–3 in Fig. 4. However, there are relatively larger regions of low noise level in 4–7 in Fig. 4; here not much light due to RR would reach the PM, because the distances between the slit and the capillary cell are relatively large compared with the former. The noise levels in the region between a and b in 4–7 in Fig. 4 are also smaller than those in 1–3. It is important that the focused position of the laser beam on the capillary corresponds to the region of this lower noise level. The position of the laser spot on the z axis was also examined; this result will be discussed elsewhere.
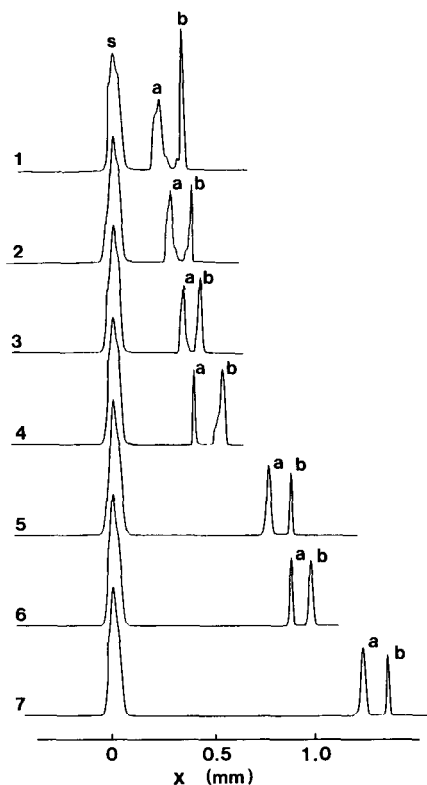

Fig. 4. Background of scattered light at different cell distances from the slit. Peaks s, a and b come from the slit, x_a and x_b in Fig. 3a, respectively.
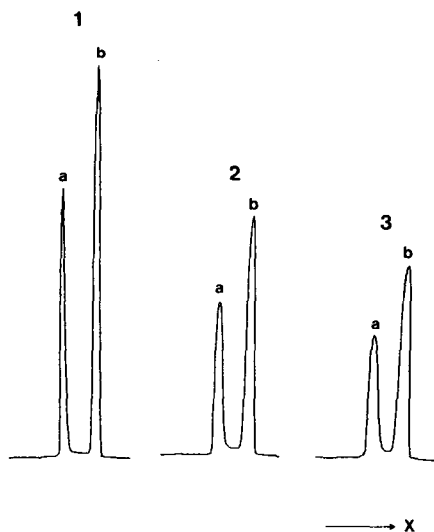
Fig. 5. Effect of focusing on the capillary cell. The optimum focusing is 1; 2 and 3 were not well focused.

Effect focusing on capillary cell

The laser beam should be focused on the capillary cell, otherwise the spot area of the laser beam becomes wider than that with optimum focusing. Three typical examples of the effect of focusing are shown in Fig. 5. We measured the noise levels; the response in Fig. 5 is equal to the noise level. In Fig. 5, the numbers 1, 2 and 3 are set at the best focused position f_a (the distance between the focusing lens and the capillary cell was assumed to be f_a mm), $f_a = 1.25$ mm and $f_a = 1.75$ mm, respectively. In the last two instances, the laser beam was not well focused on the capillary cell; as the spot areas on the capillary cell were wider here than that in case 1, larger RR was observed. Also, the background noise levels between a and b in cases of 2 and 3 were higher than that in case 1. These results indicate that focussing is very important for obtaining low background noise.

Fluorescence with continuous flow of reagent

When the fluorescent reagent (Br-Mmc derivative of caproic acid) was flowed continuously through the capillary cell, the response of the PM along the X_a line was as shown in Fig. 6 (1 and 2 represent experiments with and without a flow of Br-Mmc derivative). The capillary cell was fixed at the optimum position. The response in 1 in Fig. 6 is a combination of fluorescence and RR. Although some fluorescence was obtained even at the two edges of the capillary cell (peaks a and b), the S/N ratios at these positions were not good. From 1 in Fig. 6, the best S/N ratio is considered to be obtained near the middle between the two edges of the capillary cell.

We recommend the following procedure for adjusting the geometric position of the capillary cell to obtain low background noise: (1) place the capillary cell at the centre of the slit; (2) set the position of the capillary cell 0.5 mm from the slit along the X_a line' (3) focus the laser beam well on the capillary cell by adjusting the position of the focusing lens; and (4) adjust the position of laser spot at the centre of the capillary
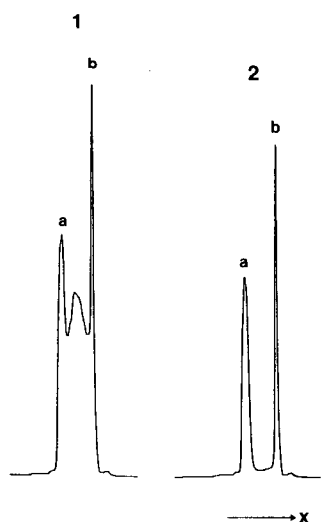
Fig. 6. Flurorescence and scattered light under conditions of continuous flow, (1) with and (2) without fluorescent reagent. Flow-rate, 4 μl/min; fluorescent solution, 5 μM Br-MMc derivatives of caproic acid in acetonitrile. Eluents for 1 and 2 were fluorescent solution and pure acetonitrile, respectively.

cell in the x and z directions so as to obtain the lowest output level of background interference.

After above adjustments, we separated Br-Mmc derivatives of carboxylic acids in a test mixture. A typical example is shown in Fig. 7. The peak of 8 fmol of the Br-Mmc derivative of caproic acid is eluted at 14 min with a peak height of 900 μV.

min
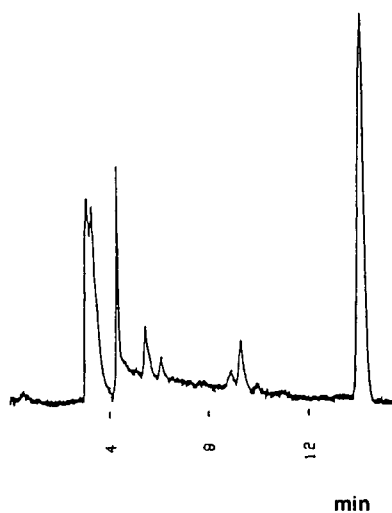
Fig. 7. Chromatogram of 8 fmol of Br-Mmc derivative of caproic acid. Slurry-packed fused-silica ODS (5 μm) capillary column (25 cm × 320 μm I.D.). Eluent, water–acetonitrile (1:9), 4 μl/min. Br-MMc derivative of caproic acid was eluted at 14 min.

At S/N = 5, the minimum detectable amount of the Br-Mmc derivative of caproic acid would be *ca.* 1.3 fmol. The calibration graph for fluorescence derivatives is linear over three orders of magnitude of concentration.

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