FAST ANALYSIS OF BIOLOGICAL COMPOUNDS BY GRADIENT LIQUID CHROMATOGRAPHY USING PILLAR ARRAY COLUMN

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ABSTRACT

Here we describe fast analysis of biomolecules by newly developed pressure-driven liquid chromatography (LC) on a chip with integration of a gradient elution system. We have reported the fabrication of a gradient elution system using a cross-Tesla mixer on a chip with pillar array columns [1]. In this study, we applied the chip to the analysis of biological compounds, and showed that 10 times faster separation was achieved under gradient elution than under isocratic elution. The retention times under gradient elution conditions were corresponded to the values estimated by semi-empirical equation, which showed that the gradient elution system worked validly.

KEYWORDS

Pressure driven, Peak width, NBD-F, Aliphatic amines, Fluorescence

INTRODUCTION

Recently, more research was focused on the pressure-driven LC with pillar array columns containing perfect structures for faster separation [2]. In order to improve the separation efficiency, we utilized a low-dispersion turn to make a longer pillar array column on the microchip [3]. However, to analyze the biological samples containing components with quite different polarity properties, a gradient elution system which accelerates the elution of strongly retained solutes is necessary. In our previous research, the separation efficiencies of two coumarin dyes were greatly improved by utilizing the newly developed gradient elution system [1]. However, the availability for the analysis of complex compounds has not been confirmed. In this study, the developed chip was applied to analyze aliphatic amines, since they are involved in many biological functions. Furthermore, the retention times were compared with the data estimated by an semi-empirical equation to confirm that gradient elution worked well.

EXPERIMENT

A cross-Tesla mixer, a separation channel (58 mm long) with pillar array, and a sample channel were fabricated on a 20 × 20 mm silicon chip (Figure 1). 4-Fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) was used as a fluorescent derivatization reagent for aliphatic amines (pentylamine, hexylamine, heptylamine, and octylamine).

Four NBD-aliphatic amines were separated under isocratic and gradient elution system. Under isocratic elution, water/acetonitrile/TFA (90/10/0.12) was used as the mobile phase. Under gradient elution, the mobile phase was changed from water/acetonitrile/TFA (90/10/0.12) to water/acetonitrile/TFA (10/90/0.12) within 10, 5, and 1.5 min. The experiment was performed at the flow rate of 1 μL/min.

The retention times of all the compounds under different gradient elution conditions were estimated by using a semi-empirical equation [4].

$$V_R = \frac{1}{mB}\log\left\{2.31mB[V_m 10^{(a-mA)}]+1\right\}+V_m$$

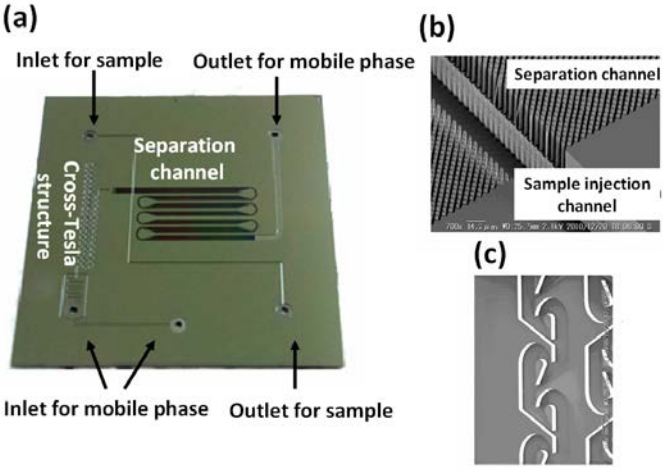

Figure 1. (a) Overview of the fabricated microchip. (b) pillar array column and sample injection channel fabricated on a chip, and (c) cross-Tesla mixer.

In the equation, parameters of a and m could be determined by the linear regression relationship of the isocratic log$k$ and φ, which can be expressed as the equation log$k$=a-$m\varphi$. k is the retention factor, φ is the concentration of the organic solvent in the mobile phase. A is the starting concentration of the organic solvent, B is the slope of the gradient elution. V_m is the hold up volume of the column.

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RESULTS AND DISCUSSION

First, four NBD-aliphatic amines were separated under isocratic elution. As shown in Figure 2(a), NBD-pentylamine, -hexylamine, and -heptylamine could be eluted in 25 min under isocratic elution, while the peak of NBD-heptylamine was very broad and NBD-octylamine could not be eluted. This indicated that isocratic elution was not suitable for the analysis of NBD-aliphatic amines. Under gradient elution, the retention times became shortened with shorter gradient time (Figure 2 (b), (c), and (d)). With the gradient time of 1.5 min, all the compounds could be separated within 110 sec, which was only one tenth of that under the isocratic elution conditions. As shown in Table 1, the peak width of NBD-heptylamine and -octylamine became much smaller under gradient elution conditions. Under isocratic elution, the peak width of NBD-heptylamine was about 190 sec. With a gradient time of 10 min, its peak width was much shortened to 4.4 sec. With a gradient time of 1.5 min, the peak width was 1.3 sec. Hence, compared with isocratic elution, a much faster analysis could be achieved by gradient elution.

Based on the retention times of NBD-aliphatic amines under different elution conditions, the linear regression between the logarithm of the solute retention factor (log$k$) and the volume fraction of the organic modifier in the mobile phase (φ) was established. Calibration curves were linear over the range of φ from 10 to 40 % with correlation coefficients of 0.9992 or better for each NBD-aliphatic amines. The equations for the four NBD-aliphatic amines were $y = -1.74x + 0.92$, $y = -2.26x + 1.44$, $y = -2.62x + 1.98$, and $y = -3.10x + 2.61$. Accordingly, the parameters of a and m could be obtained from the equation of $\log k = a - m\varphi$. Then, the estimation of retention times of these NBD-aliphatic amines was calculated on the semi-empirical equation. The parameter of A, B, and V_m could be calculated by the experimental condition. A was 0.1, and B was 0.080, 0.16, and 0.53 when the gradient time was 10, 5 and 1.5 min, respectively. As shown in Table 2, the retention times under the different elution conditions corresponded to the values which were estimated by the equation. The accuracies which were calculated as the experimental retention time divided by the estimated retention time were ranged from 93.0 to 114.8 %, which indicated that the present gradient elution system worked validly.

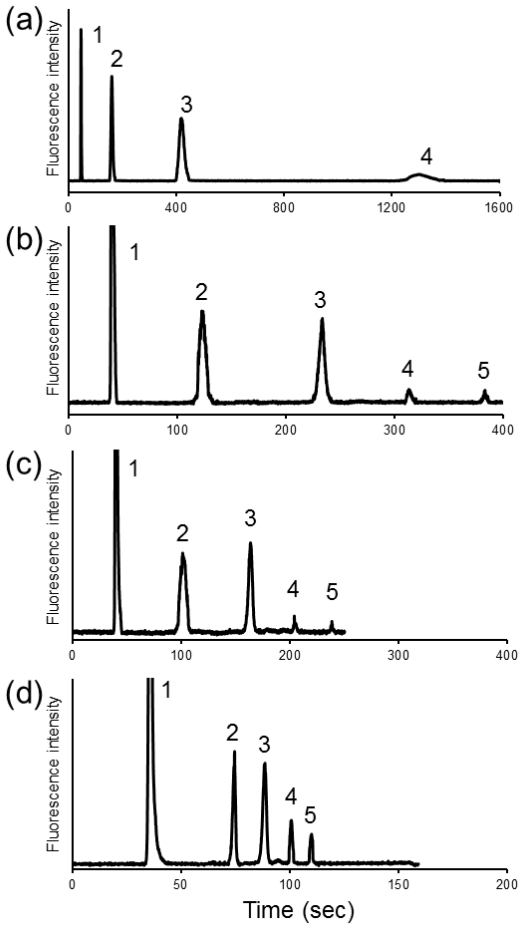

Figure 2. Chromatograms of separation of NBD-OH (peak 1) and NBD-aliphatic amines (peak 2, NBD-pentylamine; 3, NBD-hexylamine; 4, NBD-heptylamine; and 5, NBD-octylamine) obtained under isocratic elution (a, mobile phase: water/acetonitrile/TFA (90/10/0.12); flow rate: 1 µL/min) and gradient elution (mobile phase: water/acetonitrile/TFA (0 sec, 90/10/0.12; end, 10/90/0.12); gradient time: b, 10 min; c, 5 min; and d, 1.5 min. flow rate: 1 µL/min.

Table 1. The peak width of NBD-heptylamine and NBD-octylamine under isocratic and gradient elution.

Peak width (sec)	isocratic elution	gradient time 10 min	gradient time 1.5 min
NBD-heptylamine	190	4.4	1.3
NBD-octylamine	ND*	3.1	0.9

*ND, not detected

Table 2. Comparison of the retention times of NBD-aliphatic amines obtained by experiments and semi-empirical equation.

Gradient time		Retention time (sec)			
		NBD-pentylamine	NBD-hexylamine	NBD-heptylamine	NBD-octylamine
10 min	Exp*	123	233	314	385
	Est*	117	203	313	418
5 min	Exp	102	145	205	239
	Est	101	152	208	256
1.5 min	Exp	67	88	100	110
	Est	70	84	100	111

Exp*, retention time obtained by experiments
Est*, retention time obtained by estimation

In conclusion, fast analysis of biological compounds was performed by using a gradient elution system with a pillar array column. Four NBD-aliphatic amines could be separated within 110 sec, which was much faster than under an isocratic elution condition. The retention times of NBD-aliphatic amines showed good agreement with the estimated ones calculated by a semi-empirical equation, which showed that the gradient elution system worked efficiently. This chip should be useful for more efficient analysis in metabolomics study.

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