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Capillary electrochromatography–laser-induced fluorescence method for separation and detection of dansylated dialkylamine tags in encoded combinatorial libraries

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Abstract

LC–fluorescence and LC–MS methods have been previously reported for use in decoding bead-based combinatorial libraries. We present the use of capillary electrochromatography (CEC) for highly selective decoding in combination with laser-induced fluorescence (LIF) detection for high sensitivity. The results are compared to prior data obtained using HPLC with fluorescence detection. The use of CEC shows promise for miniaturization and multiplexing for future applications, and the use of LIF detection can allow for detection at sub-pmol amounts.

Keywords: Electrochromatography; Combinatorial chemistry; Alkylamines; Amines

1. Introduction

Combinatorial libraries have become important new tools for modern drug discovery and compound optimization. Libraries prepared on beaded resins by the split-pool approach combine the advantages of split-pool synthesis with those associated with the screening of discrete compounds, since individual beads (therefore single compounds) may be assayed for biological activity. Because the beads are randomized in each pooling operation, tracking the reagents and building blocks to which each bead is exposed becomes troublesome, and the identity of the chemical ligand on an active bead may be hard to determine.

A general solution to this problem is provided by encoding, whereby a surrogate analyte or ''tag'' is attached to the beads to allow determination of the reaction history of a given bead. A variety of encoding strategies [1] has now been proposed; they share the critical property that the code is much easier to analyze than the associated compounds. Technology required to encode such libraries has evolved continuously in our organization leading to the invention of polyamide ''hard tags'' [2,3], which have been shown to be compatible with a wide range of synthetic chemistries, and of demonstrable utility in drug discovery [4–6].

Fig. 1 shows the general structure of a compound

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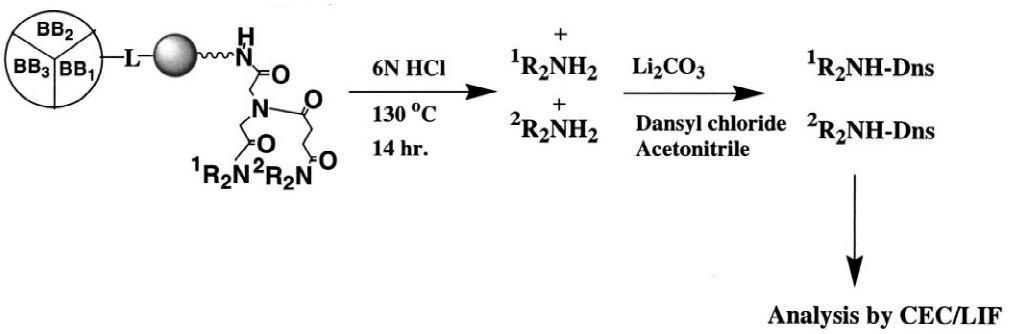

Fig. 1. General structure of an encoded compound prepared on a polymer bead.

prepared on a polymer bead using polyamide tags. It shows the construct for a three-step synthesis where the first two steps are encoded; the differentiated bead (shaded), the ligand building blocks (BB_1, BB_2, BB_3) and the code for a construct with N^1R_2 and N^2R_2. A cleavable ligand linker (labeled ''L'') allows for a photo- or acid-cleavage for screening; the tag will stay with the bead until mineral acid hydrolysis frees it for structure determination. Building blocks are encoded by mixtures of secondary amines, HNR_1R_2. Decoding entails release of the tags, determination of the tags, and (with reference to a coding table) determination of the structure of the ligand which was carried by the bead. Several analytical methods, such as liquid chromatography (LC)–fluorescence, LC–mass spectrometry (MS) and capillary electrochromatography (CEC)–MS–MS have been developed recently for reading the codes after the initial description of the Affymax encoding and decoding technologies [7,8]. CEC–laser-induced fluorescence (LIF) is presented in this paper as a new and alternative analytical method for identification of the codes.

CEC is a hybrid of micro-high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) where a capillary is packed with HPLC packing materials. A voltage is applied across the packed capillary that generates an electroosmotic flow (EOF) driving solutes along the capillary towards the detector. LIF is playing an important

Table 1
Tag set

Tag No.	Name	M_r	No. of Carbons
1	2,2,2-Trideuteroethylbutylamine	104	6
2	2,2,2-Trideuteroethylpentylamine	118	7
3	2,2,2-Trideuteroethylhexylamine	132	8
4	2,2,2-Trideuteroethylheptylamine	146	9
5	2,2,2-Trideuteroethyloctylamine	160	10
6	2,2,2-Trideuteroethylnonylamine	174	11
7	2,2,2-Trideuteroethyldecylamine	188	12
8	Pentyloctylamine	199	13
9	Diheptylamine	213	14
10	2,2,2-Trideuteroethyldodecylamine	216	14
11	Heptyloctylamine	227	15
12	Dioctylamine	241	16
13	Pentyldodecylamine	255	17
14	Hexyldodecylamine	269	18
15	Heptyldodecylamine	283	19
16	Didecylamine	297	20

role in the detection of trace levels of analytes in analytical chemistry [9–12], and can be a suitable alternative to mass spectrometric detection when high sensitivity is required. The combination of LIF with the bubble-cell detection window on the CEC capillary can increase the concentration sensitivity from micro- to pico-molarity.

The combination of CEC with LIF offers high efficiency and sensitivity along with the benefits of small sample requirements for injection and fast speed. CEC shows promise for miniaturization and multiplexing; thus the combination of CEC–LIF could increase the throughput for reading the codes of combinatorial libraries.

The tags in current use are shown in Table 1.

The codes are a series of alkylamines that are added to the beads by formation of amide bond during library synthesis. The encoding amines released by acid hydrolysis of the bead after screening are derivatized with dansyl chloride (Fig. 1) to give the corresponding fluorescent dansyl derivatives.

The decoding amines were identified by both HPLC–fluorescence detection and CEC–LIF.

2. Materials and methods

2.1. Chemicals

Trisma base was obtained from Sigma (St. Louis, MO, USA). Hydrochloric acid and acetonitrile were obtained from J.T. Baker (Phillipsburg, NJ, USA). Dansyl chloride was obtained from Pierce (Rockford, IL, USA). A mixture of 16 amine tags was prepared as previously described [1].

2.2. CEC with LIF

The CE system used was a Hewlett-Packard ^{3D}CE system (Agilent Technologies, Palo Alto, CA, USA) coupled to both a diode array detector (Agilent Technologies) and an LIF detector (Picometrics, Ramonville, France) equipped with a 325 nm He–Cd laser system (Melles Griot, Carlsbad, CA, USA). Data acquisition and handling were performed using HP Chemstation software.

The mobile phase used in the method was acetonitrile–Tris buffer (50 m*M*, pH 8.0) (9:1). The buffer was prepared by dissolving the appropriate amount of Tris in water and adjusting the pH to 8.0 with 30% hydrochloric acid. The solution was made to volume after pH adjustment and filtered through a 0.2-μm filter.

Two different CEC capillaries were used. Capillary 1 was a 40 cm×75 μm I.D. specially packed CEC capillary (Agilent Technologies, Waldbronn, Germany) with 3 μm Hypersil-C_{18} as the stationary phase and a bubble cell window. Capillary 2 was a 25 cm×100 μm I.D. packed CEC capillary (Agilent Technologies, Waldbronn, Germany) which also contained 3 μm Hypersil, but had a standard cell window.

Samples were injected by electrokinetic injection at 20 kV for 5 s. A voltage of 30 kV was applied, and the temperature of the capillary in the cassette was kept at 30°C. An external pressure was applied to the inlet of the capillary to accelerate the separation.

2.3. HPLC–fluorescence (FL) system conditions

The HPLC system used was a Hewlett-Packard 1100 binary pump and autosampler (Agilent Technologies, Palo Alto, CA, USA) coupled with a Jasco FP-920 intelligent fluorescence detector (Jasco, Tokyo, Japan). The column was a Zorbax SB-CN (Agilent Technologies, Palo Alto, CA, USA) 3.5 μm, 50×4.6 mm with a pre-column filter (0.5 μm) operating at room temperature. The mobile phase consisted of acetonitrile–water with gradient conditions of 50 to 70% acetonitrile in 3.5 min, followed by 70 to 75% acetonitrile to 4.5 min. The flow-rate was 2.0 ml/min and an injection volume of 25 μl. was used. Fluorescence detection was set at an excitation wavelength of 325 nm and an emission wavelength of 510 nm.

3. Results and discussion

3.1. High efficiency and resolution

An electrochromatogram of baseline separation for 16 amine tags by CEC–LIF is shown in Fig. 2, and a chromatogram of the same mixture separated by HPLC–fluorescence is shown in Fig. 3.

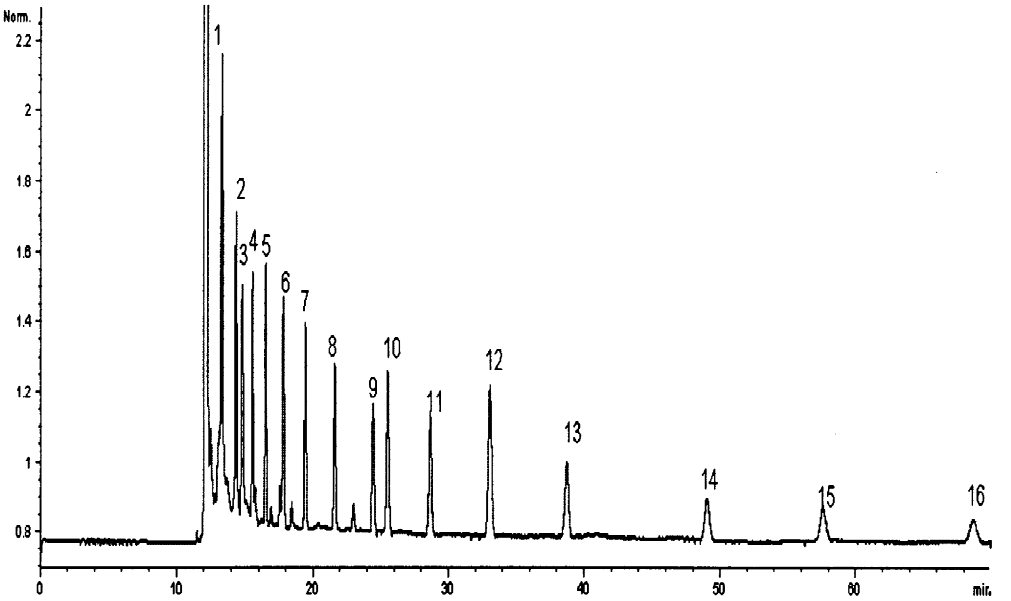

Fig. 2. CEC–LIF separation of a 16-tag mixture. Capillary: Hypersil-C_{18}, 3 μm, 40 cm×75 μm I.D. with a bubble cell window. Mobile phase: acetonitrile–Tris buffer (50 m*M*, pH 8) (9:1). Injection: 20 kV for 5 s. Inlet pressure: 12 bar. Temperature: 30°C. Sample: dansylated 16-tag mixture diluted with acetonitrile–water (2:1) in 1:5. Numbered peaks correspond to the numbered compounds listed in Table 1.

Resolution (R_s) was calculated using the halfwidth method defined as:

$$R_s = \frac{t_{R,2} - t_{R,1}}{0.5 \cdot (w_1 + w_2)} \quad (1)$$

Plate counts (N) were calculated using the halfwidth method defined as:

$$N = 5.54 \cdot (t_R / w_{1/2})^2 \quad (2)$$

where t_R is the retention time for two adjacent peaks and w is the peak width at the base of a peak for the corresponding peaks.

The values for efficiencies (62 000–112 000 plate count) and resolution (2–13) obtained by CEC–LIF is much higher than those obtained by HPLC–fluorescence (2000–11 000 and 0.6–2, respectively) shown in Table 2. Diheptylamine and 2,2,2-trideuteroethyldodecylamine, both of which have 14 carbons were easily separated by CEC (resolution: 3.59) while they were poorly separated by HPLC (resolution: 0.55). In addition, the peak symmetries by CEC were also better compared to HPLC.

3.2. High sensitivity

For the CEC–LIF separation, electrokinetic injection conditions were used for each of the 16 tags (20 kV for 5 s), and was roughly equivalent to 2–8 fmol (15–30 nl) being injected. The limit of detection (LOD) for CEC under this condition was estimated (as 2 S/N) at 58 amol. Compared to CEC–LIF, the LOD was estimated to be 58 fmol by HPLC–FL.

We observed that the patterns of the peak heights in CEC (Fig. 2) and HPLC (Fig. 3) are different for the same mixture. This is due to the electrokinetic injection in CEC separation in which the number of moles of analyte injected onto the capillary depends on the EOF and electrophoretic mobility of the analyte [13]. The electrokinetic injection volume is estimated by the following two equations:

$$V = \frac{E_{inject}}{E_{separate}} \cdot \frac{t_{inject}}{t_{retention}} \cdot V_{capillary} \quad (3)$$

$$V_{capillary} = \frac{\pi d^2 L}{4} \quad (4)$$

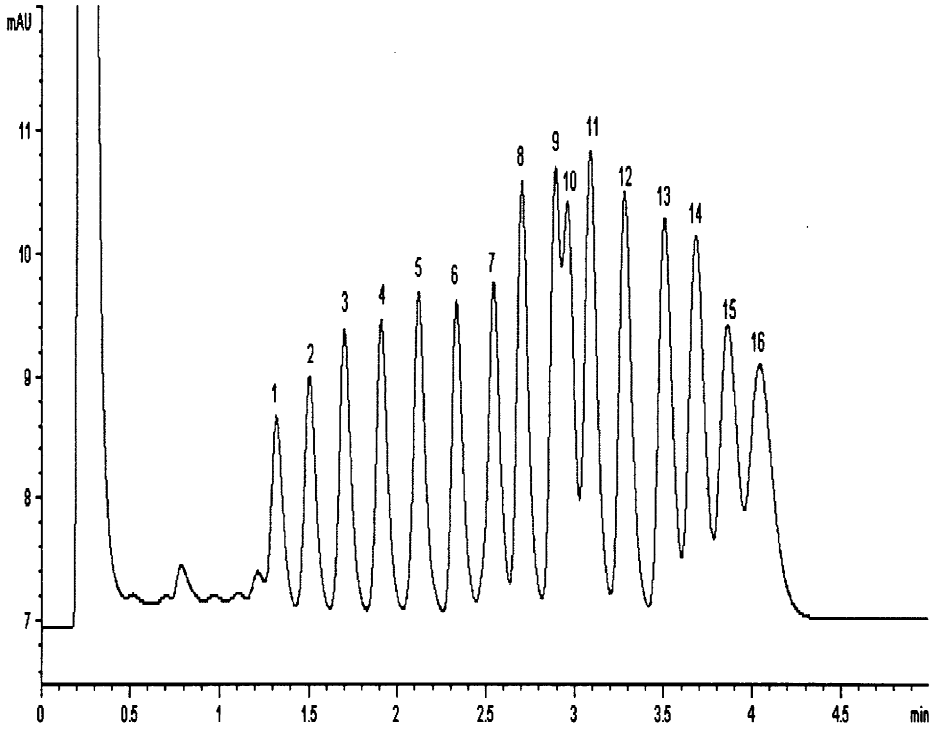

Fig. 3. HPLC–FL separation of a 16-tag mixture. Column: Zorbax SB-CN 3.5 μm, 50×4.6 mm. Injection volume: 25 μl. Temperature: ambient. Excitation wavelength 325 nm, emission wavelength 510 nm. Mobile phase: gradient: 50/70/75/50% acetonitrile in 0/3.5/4.5/6 min. Flow-rate: 2.0 ml/min. Sample: dansylated 16-tag mixture diluted with acetonitrile–water (2:1) in 2:10. Numbered peaks correspond to the numbered compounds listed in Table 1.

Table 2
Comparison of chromatographic properties for CEC and HPLC: plate counts, resolution and symmetry

Peak No.[a]	Plate count		Resolution		Symmetry	
	CEC	HPLC	CEC	HPLC	CEC	HPLC
1	61 758	2142	–	–	0.91	0.80
2	81 288	2865	5.04	1.63	0.98	0.75
3	74 473	3616	2.15	1.74	0.63	0.74
4	99 558	4625	3.60	1.83	0.80	0.72
5	112 285	5705	4.88	1.88	0.97	0.74
6	112 111	7150	6.30	1.91	1.46	0.69
7	109 032	7598	7.25	1.85	0.87	0.86
8	107 357	8891	8.69	1.36	0.99	0.71
9	106 780	10 766	10.07	1.68	0.90	1.10
10	112 197	8785	3.59	0.55	0.90	0.62
11	96 773	9718	9.32	1.02	0.97	0.75
12	80 433	10 297	10.53	1.51	0.98	0.70
13	92 107	9615	11.56	1.65	1.00	0.72
14	76 322	8256	16.66	1.16	0.96	0.76
15	92 380	6300	11.66	1.01	0.91	0.81
16	75 690	3337	12.62	0.76	1.13	0.68

[a] Numbered peaks correspond to the numbered compounds listed in Table 1.

where E_{inject} is the injection potential, $E_{separate}$ is the separation potential, t_{inject} is the time of injection, $t_{retention}$ is the retention time for the analyte, $V_{capillary}$ is the volume of a capillary, d is the diameter of the capillary and L is the length of the capillary [13].

3.3. Speed

The CEC separation has the potential to be designed into a miniaturized and multiplexed system that can allow for high throughput decoding. Unlike HPLC, there is no back pressure with EOF, so longer columns and small particles (1–3 μm) can be used to improve separation efficiencies and resolution. Unfortunately, a longer capillary was necessary for our CEC–LIF evaluation due to the constraints of the instrumental set-up. The minimum capillary length of 33 cm is necessary to fit into the cartridges for the

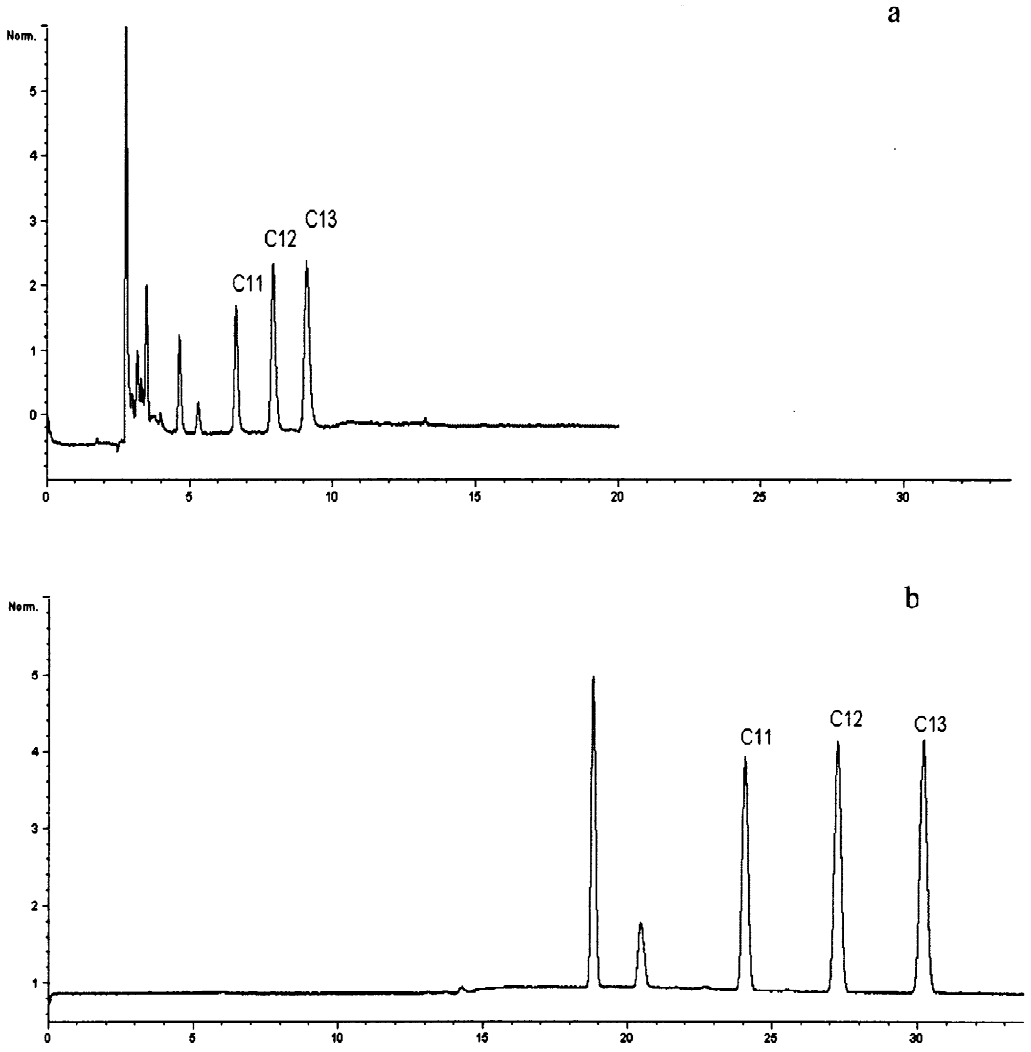

Fig. 4. Separation of three tags on different length CEC C_{18} capillaries. Sample: mixture of 2,2,2-trideuteroethylnonylamine (C_{11}), 2,2,2-trideuteroethyl decylamine (C_{12}) and propyldecylamine (C_{13}). Voltage: 28 kV. Temperature: 20°C. Mobile phase: acetonitrile–Tris buffer (50 m*M*, pH 8) (9:1). (a) By CEC–UV: capillary: Hypersil-C_{18}, 3 μm, 25 cm×100 μm I.D. with standard cell window. Injection: 3 kV, 3 s. UV wavelength 273 nm. (b) By CEC–LIF: capillary: Hypersil-C_{18}, 3 μm, 40 cm×75 μm I.D. with bubble cell window. Injection: 20 kV, 5 s.

CE system. The detector requires the use of the CEC–mass spectrometer interface, which needs 25 cm for the total length. Finally, the attachment to the detector requires an additional 35 cm length.

The effect of a short capillary on the migration time can be easily seen in Fig. 4 with 25 cm (a) and 72 cm (b) effective lengths. The migration times for 2,2,2-trideuteroethylnonylamine (C_{11}), 2,2,2-trideuteroethyldecylamine (C_{12}) and propyldecylamine (C_{13}) are 6.6, 7.9 and 9.1 min, respectively, with resolutions of 5.23, 3.96 and 4.61 for the shorter capillary while 24.1, 27.3 and 30.2 min with resolutions of 7.31, 6.31 and 6.77 for the longer one under similar conditions. It is believed that much shorter columns and smaller particles in a miniaturized CEC format can be used to achieve much faster speed and higher efficiency.

4. Conclusions

We have shown the feasibility of using CEC with LIF detection as a sensitive method to read the codes for combinatorial libraries. This method offers high chromatographic efficiency, resolution and sensitivity.

Although this method has many attractive advantages, current instrumentation needs to accommodate the use of shorter capillaries.

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