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Two dimensional mapping of cancer cell extracts by liquid chromatography–capillary electrophoresis with ultraviolet absorbance detection

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Abstract

A two-dimensional liquid chromatography/capillary electrophoresis technique was developed for rapid and comprehensive mapping of cell extracts. The cell extracts were first separated by reversed-phase HPLC based on hydrophobicity. Fractions of the effluent from the HPLC system were collected into 96-well microtiter plates and dried under vacuum. The fractions were reconstituted with deionized water, separated by capillary array electrophoresis based on charge-to-size ratio, and detected by UV absorption at 214 nm. Prior to analysis by multiplexed capillary electrophoresis, the reconstituted fractions were concentrated on-column using large volume sample stacking with polarity switching. In this way, high-resolution analysis of even the minor components in the complicated mixture was possible.

Keywords: Two-dimensional mapping; Cancer cell extracts

1. Introduction

In the last few years there has been an increased effort in the separation, quantification and identification of all components of a cell or tissue. This field of study presents a much bigger challenge to analytical chemists than that of genomics. The genome of a multicellular organism is constant over years and almost the same among different cell types, while the proteome and the metabolome are highly variable with cell types, environment and cell history [1]. In addition, an ordinary mammalian cell may contain up to 50 000 different proteins with concentrations spanning several orders of magnitude [2].

In recent years, a number of multidimensional separation techniques [3–6] have been developed in analogy to 2-D PAGE [7–10] for analysis of complex protein samples. Moore and Jorgenson [3] used on-line reversed-phase high-performance liquid chromatography/capillary electrophoresis (RP HPLC/CE) to resolve a complex mixture of peptides. The approach is facilitated by the automation of different steps of the whole analytical process. However, it places stringent requirements on the equipment since the second dimension (CE) must be run much faster than the first dimension (HPLC). The columns used in HPLC and CE are further restricted in their relative dimensions, which would limit the sample size. Smith et al. [4] used off-line size-exclusion chromatography (SEC)/capillary isoelectric focusing (CIEF) to separate complex *E. coli* cytosolic extracts. The procedure is simple and easy to perform

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using commercially available equipment. However, it is very time consuming as many runs are required to analyze multiple HPLC fractions by CE using a single capillary for separation. Issaq et al. [5] used off-line RP-HPLC/capillary array electrophoresis (CAE) for the separation of a mixture of protein digests, whereby HPLC fractions were collected automatically with the aid of a fraction collector into polypropylene 96-well microtiter plates and analyzed by 96-array CE with fluorescence detection. The technique has the advantages of short analysis time and high detection sensitivity. However, fluorescence labeling of proteins changes their physicochemical properties. Moreover, it is almost impossible to find a labeling reagent which is universally applicable to all species in diversified cell environments.

In this work, we developed a straightforward and universal approach for rapid two-dimensional separation of complex biological samples by combining RP-HPLC with multiplex CE/UV. As most organic compounds, including proteins, have UV absorption, this method does not require fluorescent labeling or silver staining. The applicability of the technique was demonstrated in the separation of cancer cell extracts.

2. Experimental

2.1. Apparatus

Large volume sample stacking of HPLC fractions was optimized on an ISCO (Lincoln, NE) model 3140 Electropherograph System before the multidimensional multiplexed CE runs. Bare fused-silica capillaries (Polymicro Technologies, Phoenix, AZ) with 35-cm effective lengths and 55-cm total lengths (75 μm I.D. and 360 μm O.D.) were used. The experimental CE setup for 12-capillary array electrophoresis is similar to the 96-capillary array system described in Ref. [11]. Fused-silica capillaries were packed side by side at the detection window and clamped between two flat surfaces of a plastic mount. At the outlet, 12 capillaries were bundled together and immersed in the separation buffer in the outlet vial. Nitrogen gas from a gas cylinder connected to the cap of the outlet vial is used to drive NaOH (0.2 *M*), deionized water and separation buffer in the outlet vials to simultaneously condition the 12 capillaries between runs. At the injection end, the 12 capillaries were spread and mounted along a row on a copper plate with dimensions that fit into a 96-well microtiter plate for sample introduction. Gold-coated pins (MillMax Mfg. Corp., Oyster Bay, NY) were mounted on the copper plate near each capillary tip to serve as individual electrodes, with capillary tips slightly extended (~0.5 mm) beyond the electrodes to guarantee contact with small-volume samples. A positive high-voltage power supply (Glassman High Voltage Inc., Whitehorse Station, NJ) was used to drive the electrophoresis.

The light source, interference filter, capillary array holder, and detector were all contained in a light-tight metal box attached to an optical table. A 213.9-nm zinc lamp (model ZN-2138, Cole-Parmer, Vernon Hills, IL) was used as the light source for UV absorption detection. The transmitted light from the capillary array passed through an interference filter (Oriel, Stratford, CT) and a quartz lens (Nikon, focal length =105 mm, f/4.5). An inverted image of the capillary array at a nominal magnification factor of 1.2 was created by the quartz lens on the face of the PDA. The PDA (Hamamatsu model S5964, Hamamatsu, Japan) incorporated a linear image sensor chip (1024 diodes, 25 μm in width, 2.5 mm in height), a driver/amplifier circuit, and a temperature controller. The built-in driver/amplifier circuit was interfaced to an IBM-compatible computer via a National Instrument PCI E Series multifunction 16-bit data acquisition board. All codes used to operate the PDA and to acquire the data were written in-house using Labview 5.0 software (National Instruments, Austin, TX). The raw data sets were converted into single-diode electropherograms by another in-house Labview program. Data treatment and analysis were performed using Microsoft Excel 97, Grams/32 5.05 (Galactic Industries, Salem, NH) and Labview.

2.2. Chemicals

Acetonitrile was purchased from J.T. Baker (Phillipsburg, NJ). Sodium hydroxide was from Sigma (St. Louis, MO, USA). Trisma-base (tris[hydroxymethyl]aminomethane) and sodium tetraborate were from Fisher Scientific (Fairlawn, NJ). The water

used to prepare buffer and reaction solutions was purified by a Milli-Q water purification system (Millipore, Worcester, MA).

2.3. Procedures

Frozen ovarian cancer cells (containing ~10^7 cells) were reconstituted in 300 μl of D.I. water (in a 500-μl micro centrifuge tube). The suspension was placed in an ultrasonic bath for 20 min to lyse the cells. Then the suspension was spun down and 100 μl of the supernatant was injected for HPLC separation.

The HPLC separations were carried out on an HPLC instrument model 1090 from Hewlett-Packard (Rockville, MD) equipped with a data station and a Vydac C-4 column, 250×4.6 mm, packed with 5-μm spherical silica particles from Resolution Systems (Holland, MI). Proteins from the ovarian cancer cells were dissolved, after extraction, in 300 μl of D.I. water. The injection volume was 100 μl and the flow-rate was 1 ml/min. Buffer A was 0.1% TFA in water and Buffer B 0.1% in acetonitrile. The gradient was a two-step gradient; 15–30% B in 15 min followed by 30–70% B in 105 min. The column effluent was collected into 200-μl volume, 96-well microtiter plates every minute between 10 and 106 min with the aid of a fraction collector, model Foxy Jr. from Isco (Lincoln, NE). After collection, the microtiter plates were placed in the SpeedVac (Savant, Bethesda, MD) and dried at room temperature under vacuum. The sample in each well was reconstituted before CE analysis with 10 μl deionized water.

The 12 samples in the first row (A1–A12) were transferred to a 12-tube strip, and injected into the 12-capillary array using gravity injection. The injection volume in each capillary was about 1.6 μl. It should be noted that the inlet tips of the capillaries and adjacent electrodes were dipped in deionized water for a few seconds before sample injection. The operation was to ensure that the separation buffer left on the capillary tip and the electrodes was not introduced into the sample solution where the conductivity was very low. The positive voltage was applied across the capillary with the anode at the outlet and the cathode at the inlet. The electroosmotic flow (EOF) backs the sample plug out of the capillary while anionic analytes move toward the outlet and stack at the interface with the background electrolyte (BGE). The initial total current was 420 μA. The electrophoretic current, which was an indicator of the fraction of the capillary filled with the sample plug, was monitored using a multimeter until it reached approximately 380 μA (90% of its original value). At that point the polarity was switched with the anode at the inlet and the cathode at the outlet. Separation of the concentrated sample then occurs. The above injection, stacking, switching and separation process was repeated for the remaining rows of samples, from B1 to H12. Between runs, the capillary array was rinsed with 1 ml, 0.2 *M* NaOH for 4 min, 1 ml deionized water for 2 min, and 1 ml BGE for about 4 min.

3. Results and discussion

3.1. HPLC of cell extracts

Reversed-phase HPLC is an established method for the separation of relatively complex mixtures, such as cell extracts and protein digests. With the use of trifluoroacetic acid (TFA) to ion-pair with proteins or peptides, separation is achieved based on the hydrophobicity of the complexes. Fig. 1 shows the liquid chromatogram of cancer cell extracts. Despite the use of gradient elution, many peaks overlapped

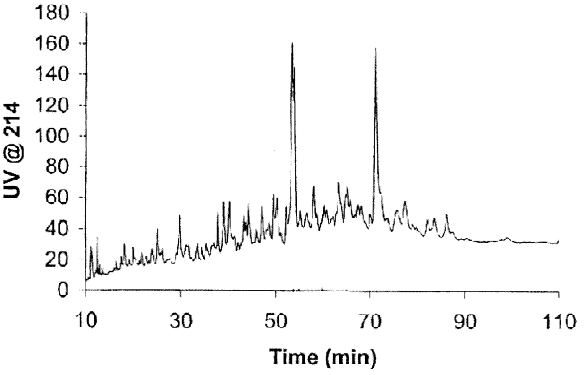

Fig. 1. Liquid chromatogram of cancer cell extracts. Conditions: column: 250 mm×4.6 mm, C4 column from Vydac. Eluent A: 0.1% TFA in water; Eluent B: 0.1% TFA in acetonitrile. Gradient: 15–30% B (from 0 to 15 min); 30–70% B (from 15 to 120 min); flow: 1 ml/min.

one another, indicating the presence of a complex mixture that was not fully resolved into its individual components. Therefore, a second dimension of separation by CE is required for further characterization of the HPLC fractions, most of which likely contained more than one component.

3.2. Separation and detection of HPLC fractions of cell extracts by capillary zone electrophoresis

Capillary zone electrophoresis (CZE) is an appropriate separation technique for the further separation of HPLC fractions, as the separation based on charge-to-size ratio is essentially orthogonal to that of HPLC based on hydrophobicity. However, CZE inherently suffers from the low concentration sensitivity resulting from the extremely low sample volume (several nanoliters) that can be injected [12,13]. In our initial experiments, the HPLC fractions of cell extracts were reconstituted with deionized water. They were then injected into the CE capillary using normal injection mode with an injection volume of about 10 nl. It was found that the electropherograms of all test fractions only showed three small peaks (Fig. 2). These peaks were components or impurities of the mobile phase used in HPLC rather than components of the cell extracts. The absence of peaks for cellular components in the electropherograms indicates that CZE with normal injection mode does not provide adequate sensitivity for detection of proteins in the HPLC fractions.

For sensitivity enhancement, preconcentration by normal field amplification and extremely large volume sample stacking (LVSS) was investigated. The mobile phase used in HPLC was a mixture of acetonitrile, water and TFA. As these are all volatile, they are completely removed after evaporation in microtiter wells under vacuum. The removal of TFA made it possible that the conductivities of the sample solutions remained low after they were reconstituted with deionized water. It was observed that the conductivities of the reconstituted HPLC fractions were between 1/100 and 1/200 of that of the background electrolyte (23 m*M* borate buffer). The low conductivity of the sample solution facilitates preconcentration of dilute samples by field-amplified

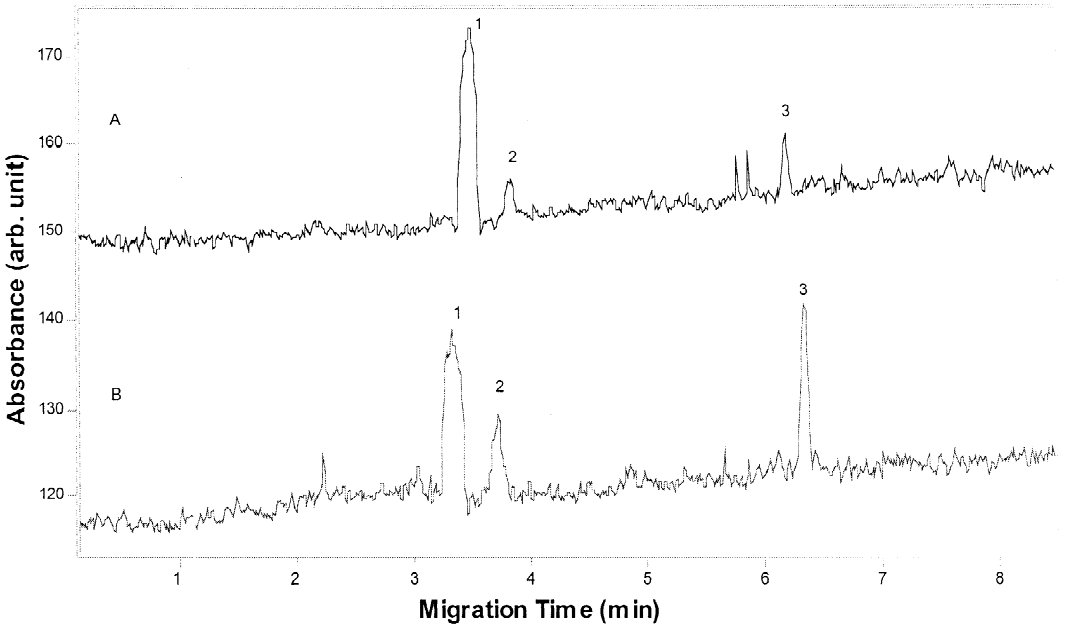

Fig. 2. Electropherograms of two HPLC fractions obtained with normal injection mode. Conditions: capillary, 60 cm (effective length, 35 cm), 75 μm I.D.; buffer, 23 m*M* borate, pH 9.2; applied voltage, 12 kV; gravity injection, 10 cm for 10 s. Peaks are numbered to aid comparison. (A) A4 fraction, (B) D6 fraction.

sample stacking (FASS) without polarity switching or LVSS with polarity switching.

FASS [14,15] is based on the idea that ions electrophoretically migrating through a low-conductivity solution into a high-conductivity solution slow down dramatically and thus stack at the boundary of the two buffers. When FASS without polarity switching is used to concentrate the HPLC fractions, the long plug of sample matrix is not opposed by EOF, but is moved toward the detection window assisted by EOF. The cations stack up in the front of the sample plug, the anions in the rear, and the neutral species are left in the sample zone and co-elute with it. However, the maximum sample volume that can be injected is limited by the reverse hydrodynamic flow, decreasing the effective separation length and reducing resolution. Fig. 3 shows the electropherogram of a HPLC fraction after FASS-CZE. The injection volume is about 100 nl with a corresponding length of sample plug of 2.2 cm. Compared to that in Fig. 2a, peak 3 in Fig. 3 increased substantially in height, but moved much closer to the two peaks in the front. In addition, the migration time was significantly decreased due to increased EOF and reduced effective separation length because of the long water plug that remained in the capillary during separation.

To further increase the amount of sample injected, LVSS with polarity switching was investigated. LVSS is a technique demonstrated by Chien and Burgi [16] that is performed by dissolving the sample in water and hydrodynamically filling a large part of, or even the whole, capillary with the sample. Reverse polarity is applied with the BGE at the detection end of the capillary. As a result, EOF opposes the migration of the sample plug while anionic analytes move toward the detection end and stack at the interface with the BGE. The electrophoretic current is monitored as a measure of the extent of replacement of the sample plug by BGE until it reaches approximately 95% percent of its original value. At that point the polarity is returned to normal, and separation occurs in the usual fashion. This method has been applied to the trace analysis of drugs, metal in hair, chelates, and chemicals of environmental concern [17–19]. In this work, LVSS was applied to the preconcentration of trace components in the HPLC fractions of cell extracts. As

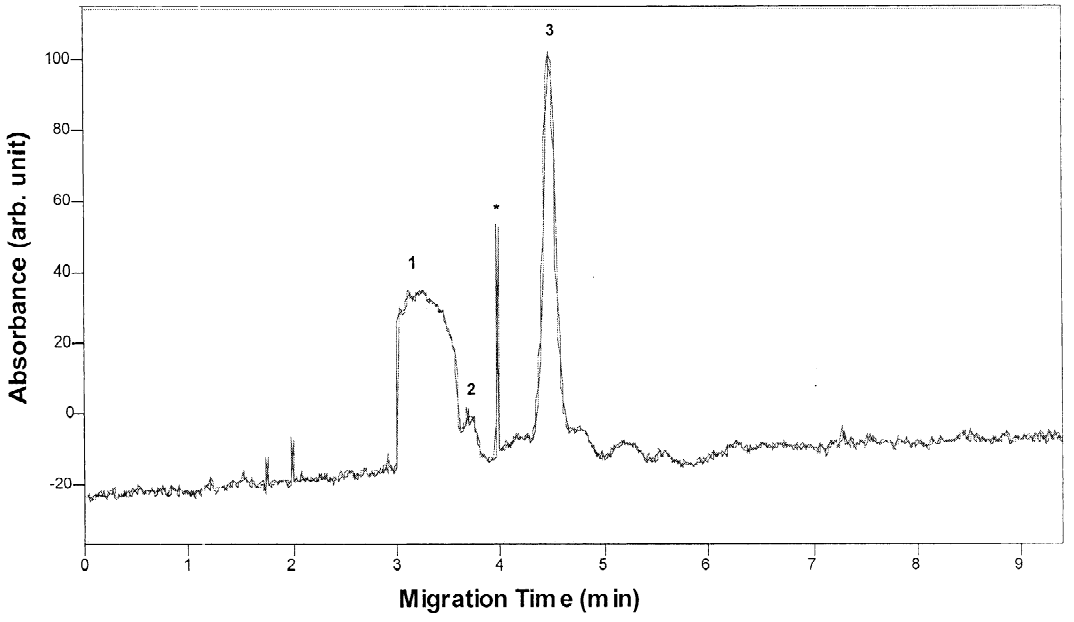

Fig. 3. Electropherogram of the A4 HPLC fraction obtained with FASS. Injection, 10 cm for 100 s. Other conditions are the same as in Fig. 2.

large as 1.7 μl of sample with plug length of 37 cm was hydrodynamically injected into the capillary. The injection and stacking process was monitored, as shown in Fig. 4a. The injection process was stopped after the sample matrix passed the detection window as indicated by the baseline ramp at about 7 min. The reverse polarity was then applied with EOF driving the boundary of the sample matrix and the BGE back to the detection window. Since the pH of the BGE is 9.3, most proteins were negatively charged and moved against the EOF to stack at the boundary between the sample matrix and BGE. The initial stacking (portion of the sample that went past the detection window on injection) was indicated by the sharp peak at about 10 min in Fig. 4a. During the stacking process, no separation occurred as most of the voltage was dropped across the sample plug. Fig. 4b shows the electropherogram of the A12 fraction concentrated by LVSS. As compared to peak 3 in Fig. 2A, nearly 100-fold increase in height was achieved by using LVSS. In addition, a number of small peaks not detected in Figs. 2 and 3 became evident in Fig. 4B.

Fig. 5 illustrates the reproducibility of LVSS–CZE for one HPLC fraction. The two electropherograms gave very similar peak patterns in terms of migration times. However, the heights of some peaks vary to a certain extent from run-to-run due to the manual stacking operation. Different HPLC fractions were tested by LVSS–CZE with some of the results shown in Fig. 6. The different peak patterns indicated in the electropherograms demonstrate the capability of LVSS–CZE to resolve and detect dilute cell lysate samples from HPLC fractions.

3.3. Multiplexed LVSS–CZE for fast analysis of multiple HPLC fractions

LVSS–CZE using single-capillary CE is time consuming when it is used for the analysis of 96 HPLC fractions. It would take over 1 week (8 h per day) to analyze all 96 HPLC fractions using a single-capillary CE instrument. In addition, extended storage of the collected samples will cause some changes in sample composition. Thus, rapid sample processing and analysis is crucial for obtaining reliable mapping of cell extracts.

We reported earlier the use of multiplexed CE/UV

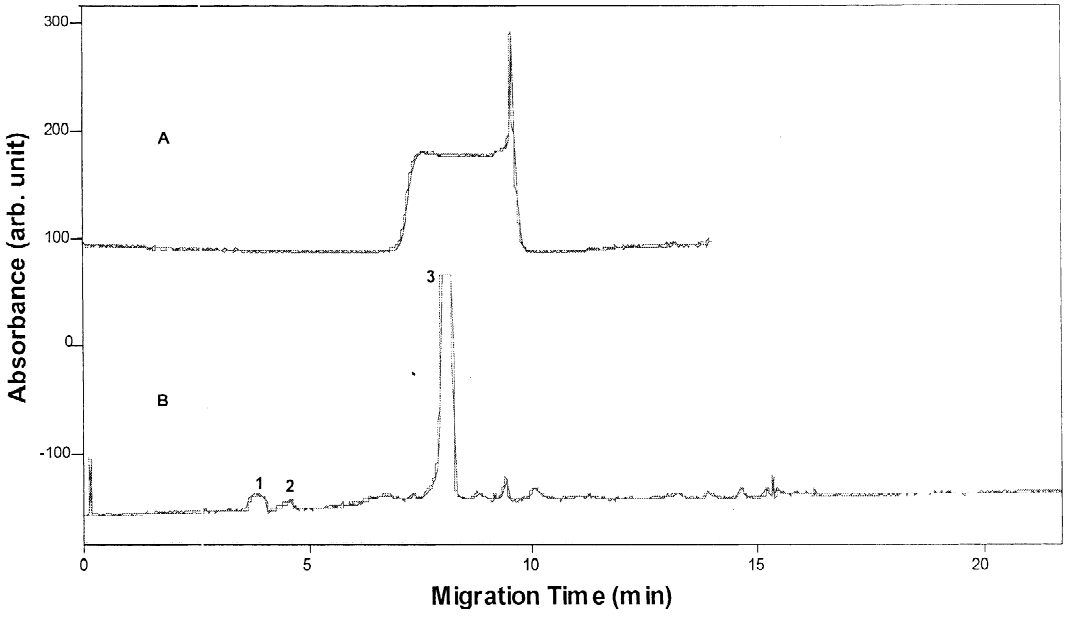

Fig. 4. Electropherograms showing the operation of large volume injection, sample stacking, and separation. (A) large volume injection and sample stacking, (B) separation of stacked sample. Gravity injection, 20 cm for 7.5 min. Original current, 34 μA. The reverse polarity is switched back to normal when the current approaches 32 μA. Other conditions are the same as in Fig. 2.

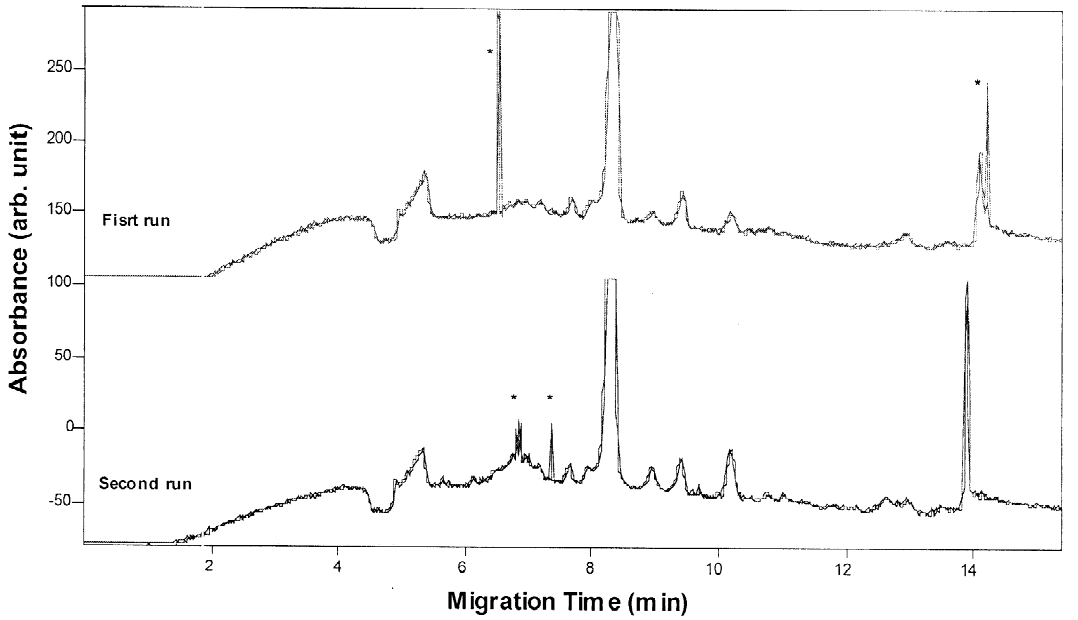

Fig. 5. Electropherograms showing the reproducibility of LVSS–CZE for one HPLC fraction. Other conditions are the same as in Fig. 4.

for high-throughput DNA and protein analysis [20,21]. LVSS has never been used before following HPLC separation. Here, multiplexed CE/UV was used together with LVSS for fast analysis of multiple HPLC fractions. Simultaneous LVSS of multiple samples using capillary array electrophoresis pre-

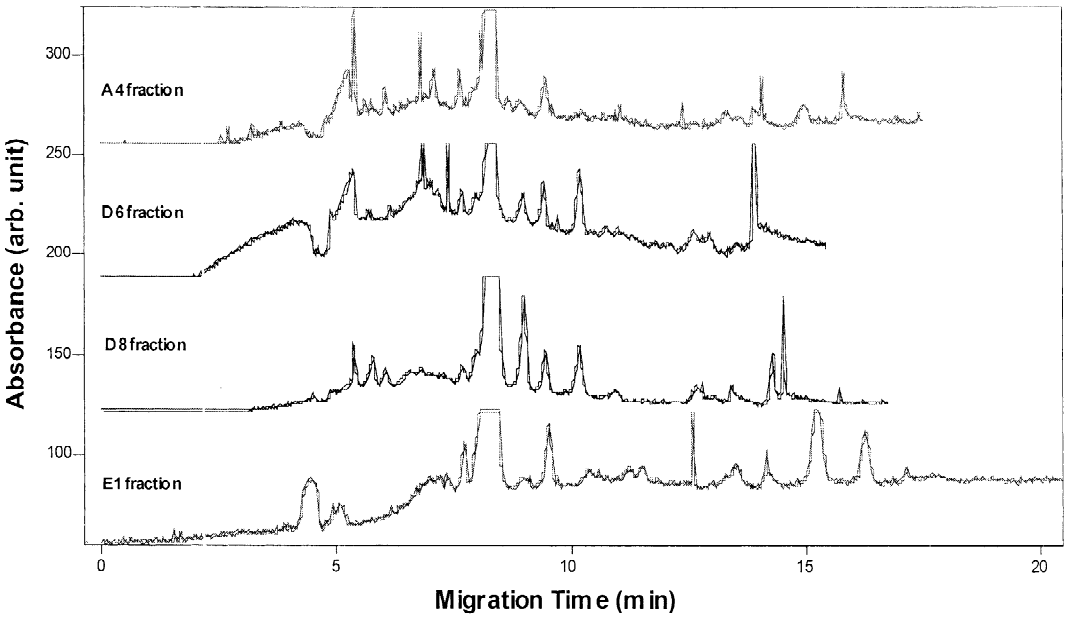

Fig. 6. LVSS–CZE analysis of four HPLC fractions of cancer cell extracts. The conditions are the same as in Fig. 4.

sents certain problems for optimizing polarity switching based on the variation of total current during the stacking process. Optimal polarity switching requires that all capillaries have exactly the same total length, completely free of plugging (same I.D.), and good current stability. For example, partial clogging of even one capillary will affect the total current of the entire capillary array, and thus the timing for polarity switching. In our experiments, it was found that a compromise between high throughput and high reliability can be achieved. Reverse polarity is switched to normal when the total current approaches 90% (vs. 95% for LVSS in a single capillary) of its original value to eliminate the chance of the stacked samples being pushed out of some of the capillaries. The stacking efficiency was only slightly lower than before. By using a capillary array, HPLC fractions were simultaneously concentrated and separated by multiplexed LVSS–CZE within 30 min. Fig. 7 shows the electropherograms of all 96 HPLC fractions. It should be noted that the brightest line there represents peak 3 in Fig. 2. This component was present in all fractions and can be conveniently used as an inherent internal standard to normalize migration times and perhaps even peak areas in different capillaries. Even more spots will be seen if we use microcolumn HPLC followed by LVSS–CZE.

4. Conclusions

The combination of RP-HPLC and multiplexed LVSS–CZE provides a straightforward approach for

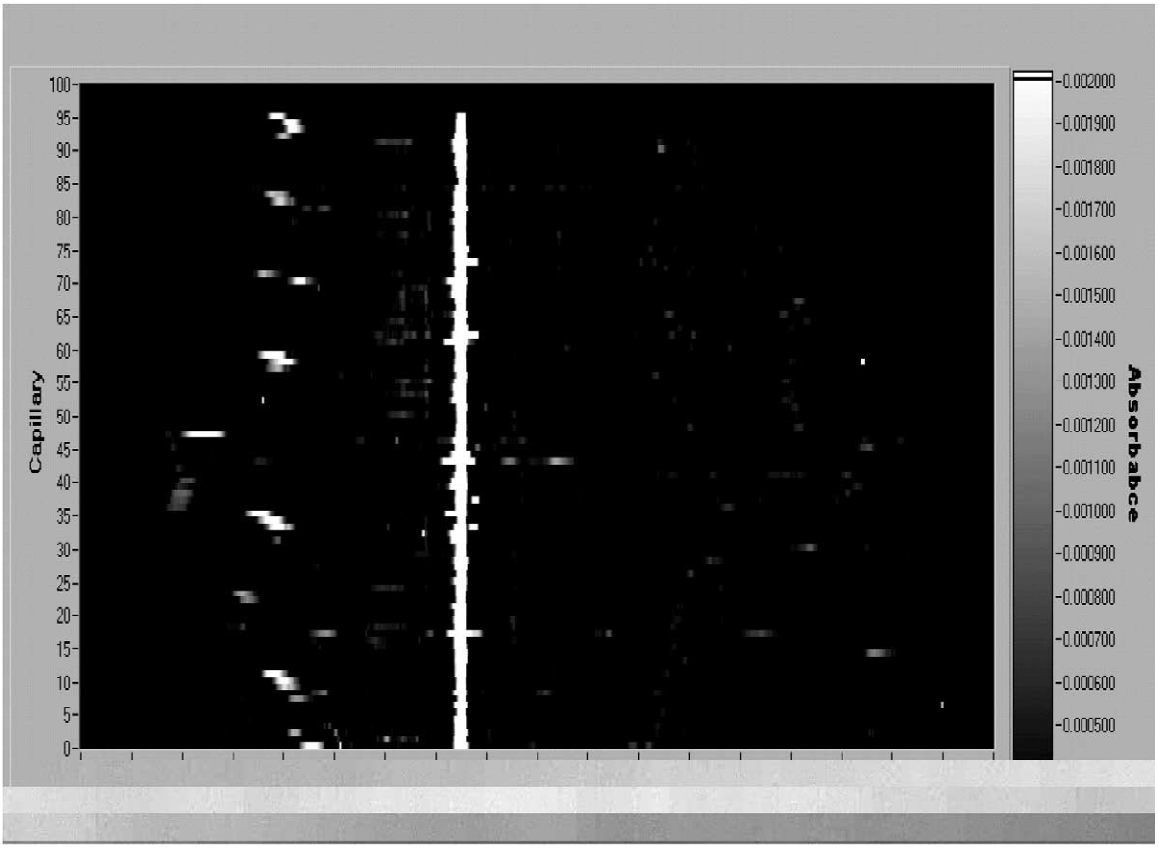

Fig. 7. Multiplexed LVSS–CZE analysis of all 96 HPLC fractions. This gray-scale plot is obtained by assembling all electropherograms collected as in Fig. 6.

rapid two-dimensional mapping of cell extracts. Direct CE coupled to LC will not work with this type of sample because of the very low concentrations. This is the first time such high sensitivity has been achieved by using UV detection. It does not require special equipment or methodologies. Even though this is a two-step (HPLC, CE) procedure, the total separation time is only 1.5 h, or 96 times faster than sequential analysis by CE. Transfer by fraction collection can be fully automated. The collected volumes are also compatible for proper LVSS–CZE. In contrast to on-column switching [3], the high concentration factor and high separation efficiency of normal CE is preserved. No fluorescence labeling (in CAE-LIF described in Ref. [5]) or silver staining (in 2-D PAGE) is involved here due to UV detection. Besides, what we observe here are not just the proteins but also all the small molecules (amino acids, metabolites). This is very different from 2-D protein gel electrophoresis. Mass spectrometry (MS) can be used for analysis, but unfortunately MS is currently only sequential and cannot be implemented with present technology. Our technique should also be applicable to the separation of other complex mixtures, such as unlabeled protein digests. Although a 12-capillary array was used here, scaling to a commercial 96-capillary instrument [22] would increase the throughput by another 8-fold.

Acknowledgements

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References

[1] H.J. Issaq, Electrophoresis 22 (2001) 3629.
[2] G.L. Vuong, S.M. Weiss, W. Kammer, M. Priemer, M. Vingron, A. Nordheim, M.A. Cahill, Electrophoresis 21 (2000) 2594.
[3] A.V. Moore, J.W. Jorgenson, Anal. Chem. 67 (1995) 3448.
[4] Y. Shen, J.B. Scott, R.D. Smith, J. Chromatogr. A 914 (2001) 257.
[5] H.J. Issaq, K.C. Chan, C. Liu, Q. Li, Electrophoresis 22 (2001) 1133.
[6] K. Wagner, T. Miliotis, G. Marko-Varga, R. Bischoff, K. Unger, Anal. Chem. 74 (2002) 809.
[7] F.C. Neidhardt, D.B. Appleby, P. Sankar, M.E. Hutton, T.A. Phillips, Electrophoresis 10 (1989) 116.
[8] N.L. Anderson, R. Esquer-Blasco, J.P. Hofmann, N.G. Anderson, Electrophoresis 12 (1991) 907.
[9] S.D. Patterson, Electrophoresis 16 (1995) 1104.
[10] P.H. O'Farrell, J. Biol. Chem. 250 (1975) 4007.
[11] X. Gong, E.S. Yeung, Anal. Chem. 71 (1999) 4989.
[12] J.L. Beckers, P. Bocek, Electrophoresis 21 (2000) 2747.
[13] D.M. Osbourn, D.J. Weiss, C.E. Lunte, Electrophoresis 21 (2000) 2768.
[14] F.E.P. Mikkers, F.M. Everaerts, T.P.E.M. Verheggen, J. Chromatogr. 169 (1979) 11.
[15] D.S. Burgi, R.L. Chien, Anal. Chem. 63 (1991) 2042.
[16] D.S. Burgi, R.L. Chien, Anal. Chem. 64 (1992) 1046.
[17] G. McGrath, W.F. Smyth, J. Chromatogr. B 681 (1996) 125.
[18] W.F. Smyth, G.B. Harland, S. McClean, G. McGrath, D. Oxspring, J. Chromatogr. A 772 (1997) 161.
[19] F. Hissner, B. Daus, J. Mattusch, K. Heinig, J. Chromatogr. A 853 (1999) 497.
[20] Y. Zhang, Y. He, E.S. Yeung, Electrophoresis 22 (2001) 2296.
[21] S.H. Kang, X. Gong, E.S. Yeung, Anal. Chem. 72 (2000) 3014.
[22] www.combisep.com