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Short communication

Capillary high-performance liquid chromatography/electrospray ion trap time-of-flight mass spectrometry using a novel nanoflow gradient generator☆

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Abstract

A type of high-performance liquid chromatography (HPLC) based on a novel nanoflow gradient generator (Asymptotic-Trace-10-Port-Valve (AT10PV) nanoGR generator) was developed and coupled with an electrospray ion trap time-of-flight mass spectrometer (ESI-IT-TOF MS). Stability of the nanoflow GR HPLC system was tested at flow rates of 20 and 50 nL/min by using a nanoflow meter. Average flow rates in a 2-h run were 51.2 nL/min with RSD 0.7% and 21.0 nL/min with RSD 1.8%. Repeatability of analysis of the nanoHPLC/ESI-IT-TOF MS system was also tested by injecting 1.0 μL of trypsin digested bovine serum albumin (BSA) (100 fmol) into a monolithic silica-ODS column (30 μm i.d., 150 mm in length) through a packed silica-ODS trapping column (particle size 5 μm, 150 μm i.d., 10 mm in length). At a flow rate of 50 nL/min, the result demonstrated a reasonably good repeatability of peak retention times (RSD: 0.32–1.1%) and base-ion peak areas (RSD: 4.4–6.6%).

Keywords: Nanoflow gradient elution; Monolithic capillary column; Mass spectrometry; BSA peptides

1. Introduction

A capillary high-performance liquid chromatography (HPLC) system using a small-bore column with an internal diameter (i.d.) of less than 1 mm was initiated by several research groups during the late 1970s [1–7]. Since then, combining the powerful, versatile separation capability of capillary HPLC with very informative mass spectrometry, such as capillary gas chromatography/mass spectrometry (capillary GC/MS) became an important topic in analytical chemistry. Many types of ionization interfaces have been developed and modified to realize this hyphenated instrument (capillary HPLC/MS). At present, electrospray (ESI) developed by Yamashita and Fenn [8–10] and Aleksandrov et al. [11] is becoming a standard ionization interface in capillary HPLC/MS [12]. The low-flow ESI techniques, such as microESI developed by Emmett and Caprioli [13,14] and nanoESI developed by Wilm and Mann [15,16] opened a new phase of ESI–MS. It demands a true nanoflow capillary HPLC system, especially a stable, reliable nanoflow gradient generator. To meet this new demand, a number of methods have been revived or newly developed, such as (1) flow splitting [17–24], (2) miniaturization of high-pressure gradient pumps [25,26], (3) exponential (sigmoidal)-gradient formation [27–31], and (4) off-line formation and storage of

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gradient solvents in injection loop(s) or preformed-gradient loop(s) [32–34].

We also have recently proposed a new method based on "digital" flow splitting (in contrast to conventional "analog" splitting) and the off-line (but real-time) formation of gradient solvent [35]. The method simply consists of a conventional semi-microflow gradient pump, an isocratic syringe pump, and a 10-port switching valve with two injection loops installed; it was called an "Asymptotic-Trace-10-Port-Valve (AT10PV)" nanoGR generator [35]. An important aspect of this new method is that any nanoflow gradient profile can be easily and effortlessly generated by simply shortening a switching period of the 10-port switching valve digitally without the problem of mixing solvent at nanoflow rates. In previous reports [35,36], the repeatability of peak retention times of chromatograms was tested by using a prototype of the AT10PV nanoGR generator and a UV or a MS detector in the range of flow rates of 200 and 500 nL/min and reasonably good results were obtained. Based on the promising results obtained, we developed a product based on the AT10PV nanoGR generator and a new capillary HPLC/MS system coupled with an ESI ion trap time-of-flight mass spectrometer (ESI-IT-TOF MS) which was also developed at the same time and already reported [37]. This study reports the performance of this capillary HPLC/ESI-IT-TOF MS system at flow rates of 50 and 20 nL/min, by injecting 1.0 μL of trypsin digested BSA (100 fmol) into a monolithic silica-ODS column (30 μm i.d., 150 mm in length) through a packed silica-ODS trapping column (particle size 5 μm, 150 μm i.d., 10 mm in length). Repeatability (RSD%) of peak retention times at a flow rate of 50 nL/min is 0.32–1.1% and the repeatability (RSD%) of peak areas estimated from mass chromatograms is 4.4–6.6%.

2. Experimental

2.1. Instrumentation

The capillary HPLC/MS used is a NanoFrontier system (Hitachi High-Technologies, Tokyo, Japan) is composed of a capillary HPLC system based on the AT10PV nanoGR generator and an ESI-IT-TOF MS. As the MS part has already been reported [37], Fig. 1 illustrates the capillary HPLC part of the NanoFrontier system, which consists of two conventional semi-microflow gradient pumps (Hitachi L-2100 pumps) with a low-pressure (maximum four solvents) gradient capability, a newly developed isocratic nanoflow pump, a 10-port valve (10-PV) (Rheodyne, Rohnert Park, CA) with two 1-μL injection loops, two 6-port values (Rheodyne) for drain (DV) and sample trapping (TV), and a conventional auto-sample injector (Hitachi L-2200 autosampler). Although the nanoflow gradient generation and solvent delivery are, in principle, the same as those described in the previous report [35], the isocratic nanoflow pump and valves were compacted in a unit (indicated by the broken line in Fig. 1). Several types of fused-silica capillary tubes (10, 20, 50 μm i.d. and 0.36 mm o.d.) obtained from GL Science (Tokyo, Japan) were used for connecting each unit in the system. Connecting unions and in-line filters were purchased from Upchurch Scientific (Oak Harbor, WA). A Senshu SSC-5430 UV detector with a flow-cell volume of 31 nL (Senshu Scientific, Tokyo, Japan) was

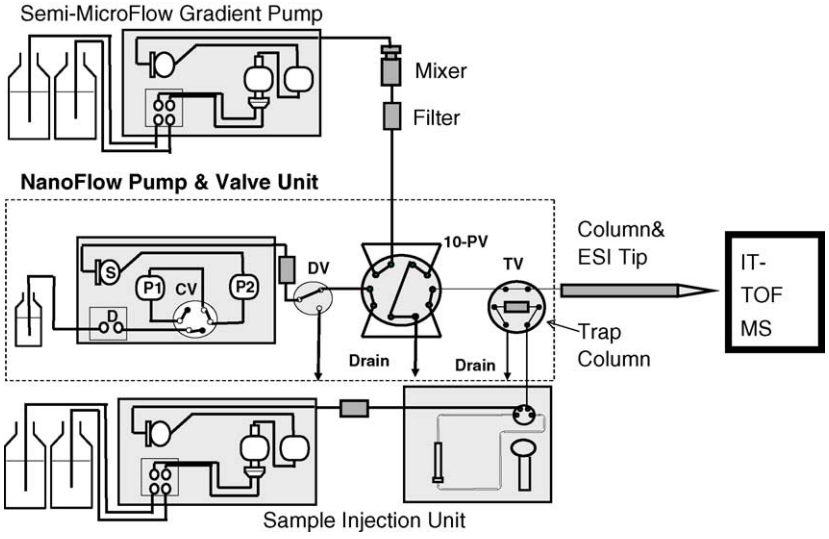

Fig. 1. Schematic diagram of NanoFrontier (capillary HPLC/ESI-IT-TOF MS) system. The HPLC part consists of a conventional semi-microflow gradient pump, an isocratic nanoflow pump, a 10-port valve (10-PV) with two 1.0-μL injection loops, two 6-port values for drain (DV) and sample trapping (TV), and a sample injection unit (i.e., a conventional semi-microflow gradient pump and an auto-sampler). The AT10PV nanoGR generator consists of a semi-microflow gradient pump including a mixer and a filter, and a unit consisting of an isocratic nanoflow pump and valves indicated by the dotted line. The newly developed isocratic nanoflow pump is described in the text in detail.

used only for a performance test of gradient profiles. A liquid mass flow meter SLG 1430 (Sensirion, Zurich, Switzerland) was also used for measuring variations in flow rate.

2.2. Chemicals, sample, and columns

HPLC-grade methanol, acetonitrile, water, acetone, trifluoroacetic acid (TFA), formic acid, ammonium bicarbonate, and trypsin were purchased from Wako Chemical (Tokyo, Japan). Bovine serum albumin (BSA) was obtained from Tokyo Chemical Industry (Tokyo, Japan). BSA (1 mg) was dissolved in 1 mL of 100 mM ammonium bicarbonate buffer (pH 8.0) and kept at 100 °C for 10 min. Tryptic digestion was performed at 37 °C for 24 h by 0.01 mg trypsin. The monolithic silica-ODS capillary column (30 μm i.d. and 150 mm in length) obtained from Kyoto Monotec (Kyoto, Japan) was combined with a SilicaTip (tip diameter 10 μm) (New Objective, Woburn, MA).

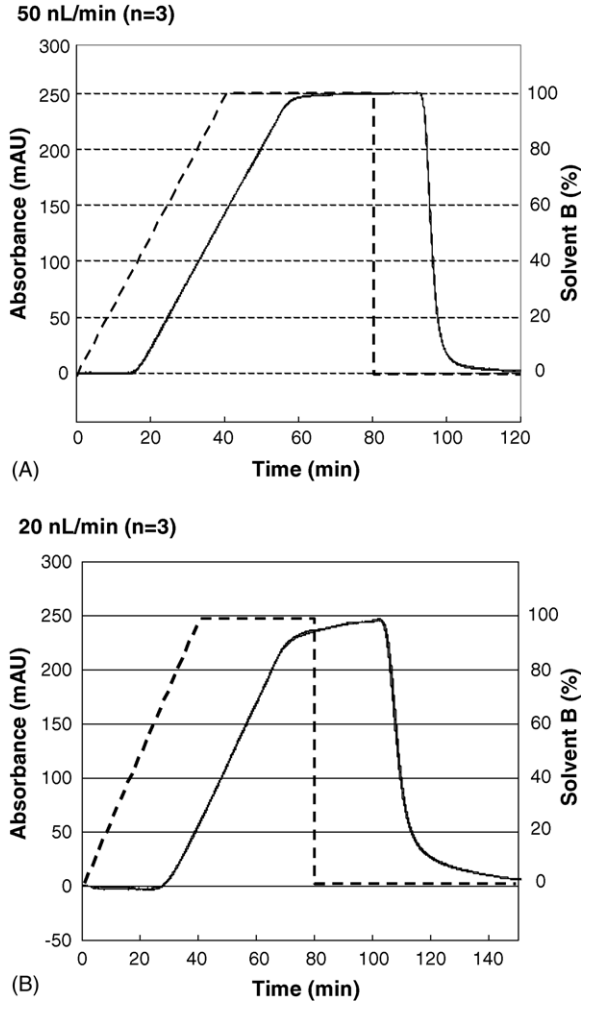

Fig. 2. Repeatability of gradient profiles of three sequential runs at flow rates of 50 nL/min (A) and 20 nL/min (B). Solvents A: water and B: 50 ppm caffeine; gradient program of solvent B: 10% (0 min) → 100% (40 min) → 100% (80 min) → 10% (80.1 min) → 10% (150 min). A fused-silica capillary tube (10 μm i.d., 1500 mm in length) was used as a dummy column. The UV wavelength was 273 nm and the 10-port valve was switched at 1-min intervals.

2.3. Analytical conditions

The flow rate of the semi-microflow gradient pump delivering solvents A (0.1% formic acid in 2% acetonitrile) and B (0.1% formic acid in 98% acetonitrile) was set at 100 μL/min. The gradient profile used was as follows: solvent B composition was linearly increased from 10% at 0.0 min to 40% at 70.0 min. A flow rate of the isocratic nanoflow pump was set to 50 or 20 nL/min and the 10-port switching valve was switched at 1-min intervals unless stated otherwise. The sample injection volume was 1.0 μL (i.e., 100 fmol) of BSA peptides. The ESI-IT-TOF MS conditions were as follows: the ESI voltage was 1.6 kV; curtain (nitrogen) gas was used at a flow rate of 1.0 L/min without heating; the scan mass range (*m*/*z*) was 300–2000; and the mass accuracy and resolution were within ±50 ppm and more than 8000 FWHM, respectively.

The repeatability of the gradient profiles was tested at flow rates of 50 and 20 nL/min using the Senshu SSC-5430 UV detector and using a fused-silica capillary tube (10 μm i.d., 1500 mm in length) as a dummy column. Solvents A (water) and B (50 ppm caffeine) were used to make the gradient profile (gradient program of solvent B: 10% (0 min) → 100% (40 min) → 100% (80 min) → 10% (80.1 min) → 10% (150 min). The wavelength of the UV detector was 273 nm. Under similar conditions, variations of

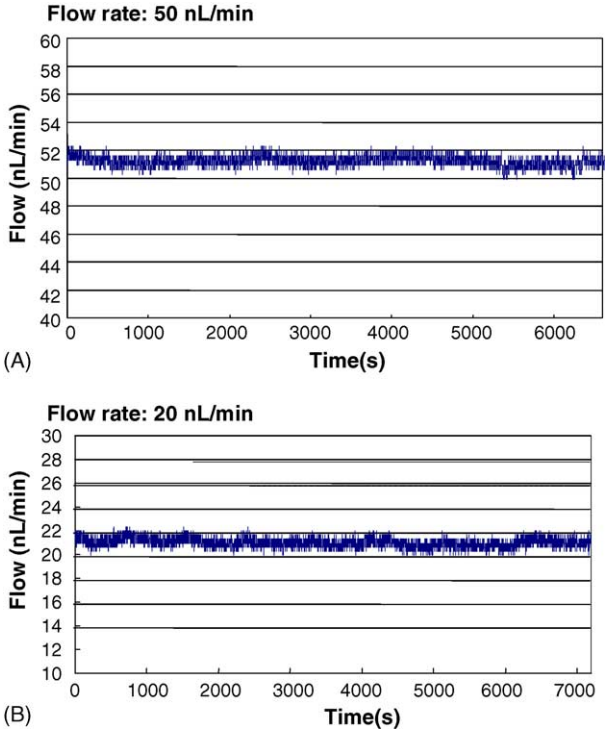

Fig. 3. Flow stability results monitored by a mass flow meter (the unit of time: second). The experimental conditions are the same as those of Fig. 2.

the flow rate were also recorded by using a liquid mass flow meter SLG 1430 (Sensirion).

3. Results and discussion

The method used to generate and deliver the nanoflow gradient solution was described in a previous report [35]. Here, we first briefly describe the isocratic nanoflow pump newly developed for the NanoFrontier system (see Fig. 1). This isocratic pump is a type of tandem double plunger pump as well as a semi-microflow gradient pump and has two features. First, the two plungers (the solvent-supply plunger P1 with a syringe volume of 100 μL and the constant-flow plunger P2 with a syringe volume of 25 μL) are independently motor-driven. The role of plunger P1 is to suck the solvent into the P1 syringe through the vacuum degasser (D) and to supply the P2 syringe after compressing the solvent monitoring pressure detected by a sensor (S) in real time. In addition, during the drawing period of plunger P2, plunger P1 delivers the solvent instead of plunger P2. However, such the sucking and drawing operations are usually performed between the end of one analysis run and the start of the next analysis run. During data acquisition, only the plunger P2 precisely delivers the solvent. Second, instead of the inlet and outlet check valves normally used in a conventional HPLC pump, a 4-port valve (CV) is used. This system is more reliable (resistant) against solvent leakage than that of the conventional check valves. When plunger P1 starts sucking the solvent, the 4-port valve (CV) is switched to open the inlet side. After the P1 syringe is filled with solvent, the valve is switched again to close the inlet side and open the outlet side. According to the two features described, the new isocratic nanoflow pump achieves a very precise, continuous solvent delivery in the range of nanoflow rates during an analysis run.

Next, we report on the repeatability of gradient profiles. Fig. 2 overlays the gradient profiles of the flow rates of 50 nL/min (A) and 20 nL/min (B) that were monitored by the UV detector. The details of the experimental conditions are described in Section 2, above. The repeatability of the gradient profiles obtained from three continuous runs is very good in both cases. At the flow rate of 20 nL/min (B), however,

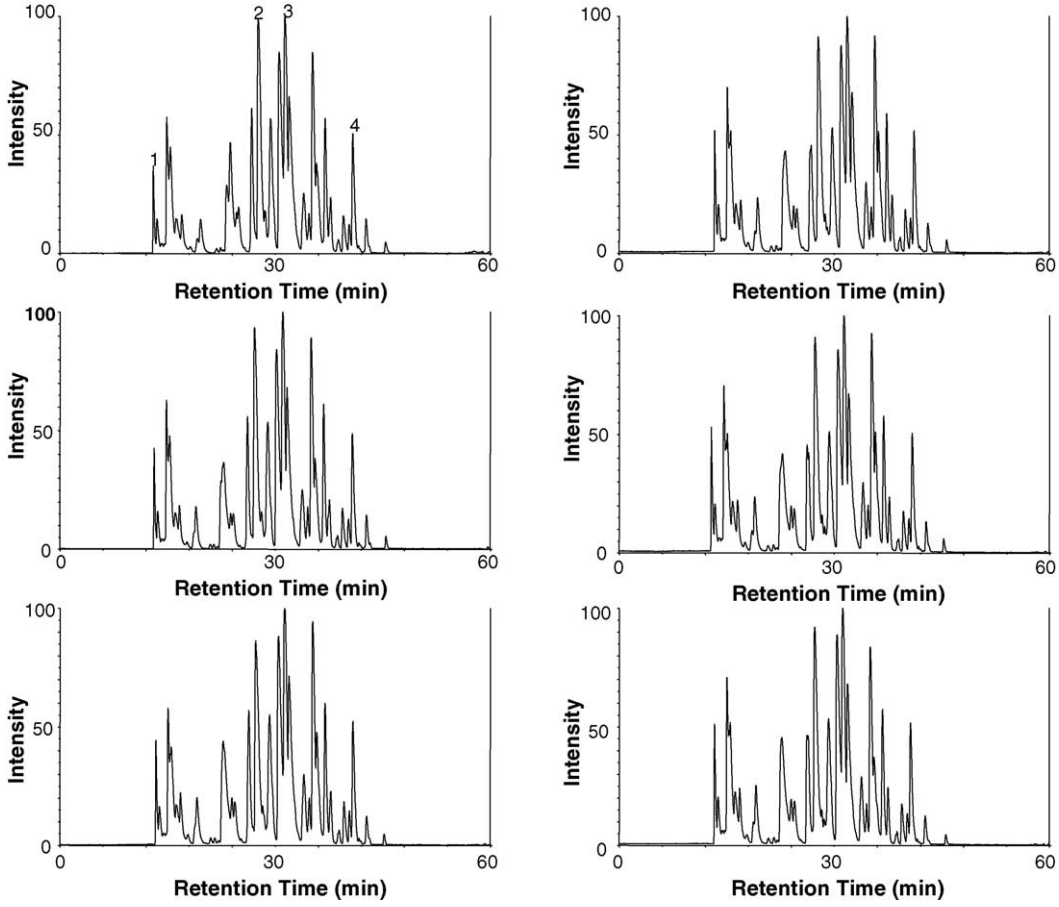

Fig. 4. Total ion-intensity chromatograms (TIC) of BSA (100 fmol) peptides of six sequential runs at a flow rate of 50 nL/min. Solvents A: 0.1% formic acid in 2% acetonitrile and B: 0.1% formic acid in 98% acetonitrile. The composition of solvent B was linearly increased from 10% at 0.0 min to 40% at 70.0 min. Period of 10-port valve switching: 1 min. BSA peptide sample 1.0 μL (i.e., 100 fmol) was injected into a monolithic silica-ODS column (30 μm i.d., 150 mm in length) through a packed silica-ODS trapping column (particle size 5 μm, 150 μm i.d., 10 mm in length). The ESI voltage was 1.6 kV; curtain (nitrogen) gas was used at a flow rate of 1.0 L/min; the scan mass range (*m*/*z*) was 300–2000.

Table 1
Repeatability of peak retention times (RT) and areas estimated from chromatograms in Fig. 3

	Peak 1 *m/z* 570.7 (2+)		Peak 2 *m/z* 547.3 (3+)		Peak 3 *m/z* 628.2 (3+)		Peak 4 *m/z* 499.3 (2+)	
	RT	Area	RT	Area	RT	Area	RT	Area
Experiment 1	15.4	38229	27.7	104405	31.4	69653	40.9	19297
Experiment 2	15.3	36964	27.2	91740	31.1	69849	40.8	19937
Experiment 3	15.4	35256	27.3	98039	31.3	68100	40.9	22568
Experiment 4	15.6	41914	27.8	96660	31.8	64922	41.1	21686
Experiment 5	15.1	41275	27.3	93684	31.4	63807	40.9	21957
Experiment 6	15.5	39969	27.3	94291	31.2	63504	40.7	22158
Mean	15.4	38935	27.4	96470	31.4	66639	40.9	21267
SD	0.172	2582.185	0.250	4479.781	0.242	2909.458	0.133	1325.798
RSD (%)	1.120	6.632	0.913	4.644	0.772	4.366	0.325	6.234

the dwell time of the gradient profile becomes large and the gradient profile becomes dull, especially after 70 min. This is caused by the dead volumes inside the capillary tubes, the connecting unions, and the valves. This clearly shows that further minimization of such dead volumes is required in such a lower range of nanoflow rates. Similarly, Fig. 3 shows the results obtained by the liquid mass flow meter SLG 1430 at flow rates of 50 nL/min (A) and 20 nL/min (B). The average flow rates in a 2-h runs were 51.2 nL/min with RSD 0.7% and 21.0 nL/min with RSD 1.8%.

Fig. 4 shows total ion-intensity chromatograms (TIC) of the BSA peptides (100 fmol) separated with the monolithic silica ODS column (30 μm i.d., 150 mm in length) at a flow rate of 50 nL/min after 1.0 μL of trypsin digested BSA sample (i.e., 100 fmol) was injected through a packed silica-ODS trapping column (particle size 5 μm, 150 μm i.d., 10 mm in length). The BSA sample was measured and injected by using the auto-sampler and delivered to the trap column at a flow rate of 10 μL/min. After the trapping and desalting on the trapping column for 2 min, the BSA peptides were separated by the monolithic silica ODS column. Table 1 summarizes the repeatability results of the peak retention times (RT) and areas obtained from six sequential runs. RSD (%) of the retention times are 0.32–1.1%, which gradually improve as RT increases. The peptide peak areas were estimated from each mass chromatogram (MC), because a peak on a TIC in Fig. 4 usually contains several overlapping peptides. The obtained result is summarized in Table 1. RSD (%) of the base-ion peak areas are 4.4–6.6%, which seem to be reasonably good as well.

4. Conclusion

The repeatability of chromatograms obtained by the capillary HPLC/ESI-IT-TOF MS system, which was newly developed based on the AT10PV nanoflow GR generator, was investigated. It was shown that the repeatability of peak retention times and base-ion peak areas are at almost the same level as the conventional HPLC analysis even at a flow rate of 50 nL/min. However, it was shown that at a flow rate of 20 nL/min, we must further minimize all dead volumes inside the 6- and 10-port switching valves, the connection tubes and unions, and the ESI tip in order to avoid a large dwell time and a dullness of the gradient profile. Although the present experiments were performed in a relatively well temperature-controlled laboratory, more precise temperature control is necessary for experiments of low nanoflow rates. This will be the next challenge for the nanoHPLC/MS system.

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