A Gradient μLC/MS System Connected with a Glass Connector Cured by the Super-glue and Some Test Runs with It

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The earliest liquid chromatography/ mass spectrometry (LC/MS) appeared over twenty years ago.[1-3] Ultra-high vacuum systems of high capacity, physical concepts of separating solutes from the solvent, mechanical designs and low tempertature ionization techniques have been devised to construct commercially useful LC/MS systems. Thermospray,[4-6] particle beam,[7-9] fast atom bombardment,[10-20] atomospheric pressure ionization,[21] and electrospray[22-25] methods are the major techniques developed. Minimizing the LC flow rate is strongly recommended to reduce contamination of the MS system for long term maintenance even though some of the commercial MS systems are asserted to be directly coupled to a conventional LC with flow rates of 1mL/min or so. Use of a microcolumn for LC/MS is required in the practical case. Commercial packed silica capillary microcolumns are usually used for such purposes, but they are expensive, or hard to pack and handle since they are fragile.

We have been studying to make rather cheap and simple glass-lined stainless steel packed microcolumns and have recently constructed a gradient μLC system with them.[26-32] In this study, we have coupled the gradient μLC system to an electrospray mass spectrometer with a unique connection mechanism and have demonstrated our μLC/MS system involving a home-made glass-lined stainless steel microcolumn worked very well showing good sensitivity and selectivity.

Experimental Section

The Alltima C_{18} stationary phase (5 μm) from Alltech (Deerfield, IL, USA) was used as the packing material for the microcolumn. A 30 cm glass-lined stainless steel tubing (0.5 mm I.D.) from Alltech was chosen as the column material. The detailed procedure for column packing can be found elsewhere.[28-31] A piece of 5 cm×50 μm I.D. (400 μm O.D.) deactivated silica capillary from J&W (Folsom, CA, USA) was cut, treated to have a sintered-silica frit[28] at the tip, and placed in the column outlet union.

Two Shimadzu (Tokyo, Japan) 10AD pumps, a Shimadzu DGU-14A membrane degasser, a Tee union with a 1/16 inch I.D. stainless steel frit (as a micromixer), a Rheodyne (Cotati, CA, USA) 7520 injector with a 0.5 μL injection loop, and the home-made 0.5 mm I.D. microcolumn were combined to compose the LC part of the system.

A long piece of 80 cm×25 μm I.D. (400 μm O.D.) deactivated silica capillary from SGE (Sydney, Australia) was cut and coupled to the silica capillary of the column outlet through a glass connector (from Alltech). Some super-glue drops were applied to the internal surface of the glass connector around the juctions between the connector and the capillaries to prevent leaking. Curing was carried out overnight. Such connection of two capillaries minimizes the extracolumn void volume without causing random dispersed flows. The other end of the long silica capillary was introduced into the stainless steel capillary of the electrospray interface of the mass spectrometer. The connection scheme is briefly shown in Figure 1. The mass spectrometer was a VG Biotech (Manchester, UK) Quattro triple quadrupole MS system with a nitrogen-flow assisted electrospray interface. The electrospray voltage was set at 3.5 kV and the nitrogen gas flow rate, 0.3 L/min.

Polyethyleneglycol 300 and dyes were purchased from Alldrich (Milwakee, IL, USA), and, peptides from Sigma (St. Louis, MO, USA), and used as received. Methanol, acetonitrile, and water were of HPLC grade and obtained from Fisher (Pittsburg, PA, USA) and used without further purification.

Results and Discussion

The first sample we used to test the performances of our gradient μLC/MS system was a mixture of peptides. The mixture was composed of Thr-Tyr-Ser (m/z of molecular ion MH^+: 370.2), Pro-Phe-Gly-Lys (m/z of MH^+: 448.2), Val-Gly-Pro-Ile-Pro-Tyr (m/z of MH^+: 717.4), Angiotensin II (m/z of MH_2^{++}: 523.8, molecular weight: 1045.5), and Eledoisin (m/z of MH_2^{++}: 594.8, molecular weight: 1187.6). The

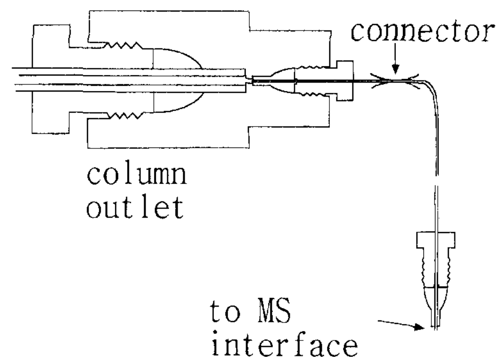

Figure 1. The connection scheme between the microcolumn and the MS interface.

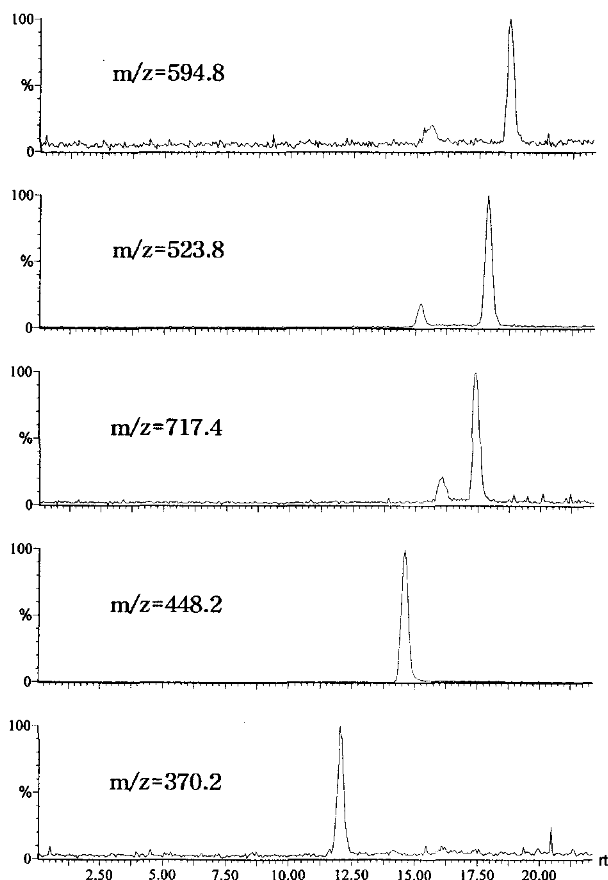

Figure 2. The chromatograms of peptides obtained in the SIM mode by the gradient μLC/MS with a glass-lined stainless steel Alltima C_{18} microcolumn. Thr-Tyr-Ser eluted first followed by Pro-Phe-Gly-Lys, Val-Gly-Pro-Ile-Pro-Tyr, Angiotensin II, and Eledoisin in sequence. See the text for the operational details.

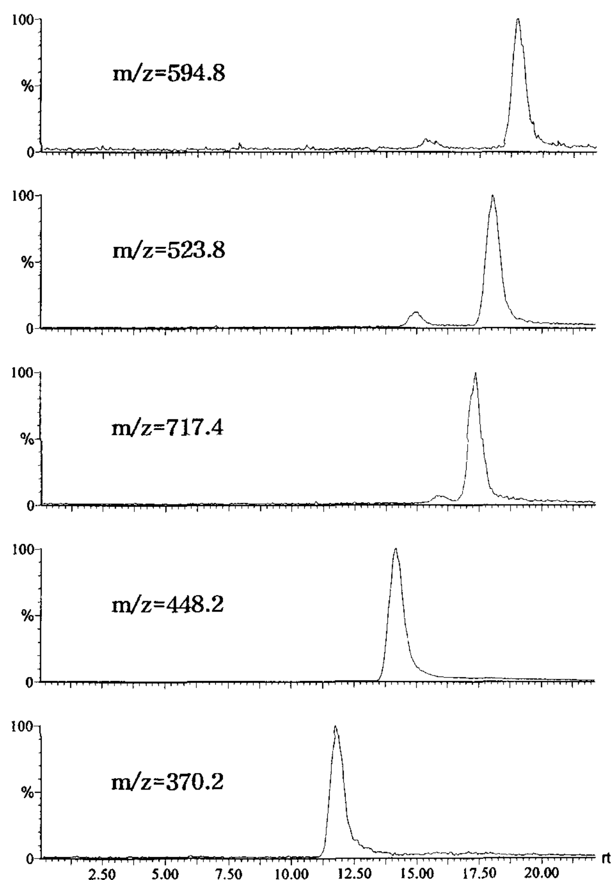

Figure 3. The chromatograms of peptides obtained as in Figure 1 except for use of a polymer tubing of 130 μm I.D. instead of a silica capillary of 25 μm I.D.

selective ion monitoring (SIM) chromatograms of the individual peptides are shown in Figure 2. The peaks were narrow and symmetrical. The Alltima C_{18} stationary phase has been known to be powerful for separation of polar retaining solutes such as peptides. The solvent A was 0.1% trifluoroacetic acid (TFA) in acetonitrile, and B, 0.1% TFA in water. The eluent composition was initially 10% A+ 90% B and was linearly varied to 90% A + 10% B in 20 min. The flow rate was 10 μL/min. The peptides eluted in the following sequence: Thr-Tyr-Ser, Pro-Phe-Gly-Lys, Val-Gly-Pro-Ile-Pro-Tyr, Angiotensin II, and Eledoisin.

The connection between the microcolumn outlet and the electrospray interface of the mass spectrometer was of crucial importance. As mentioned above, we used a deactivated silica capillary of 25 μm I.D. and a glass connector to minimize extracolumn void volume. We were able to obtain nice-looking solute peeks as shown in Figure 2 under such connection scheme. When we used a polymer PEEK tubing of 130 μm I.D. instead of the silica capillary leaving other chromatographic conditions unchanged, the solute peaks became much more broadened and tailed as shown in Figure 3. The void volume of the polymer tubing (130 μm I.D.) was estimated to be 11 μL, while that of the silica tubing (25 μm I.D.), less than 0.4 μL. Use of a silica capillary of a smaller I.D., however, was not acceptabale because silica capillaries of 10 or 5 μm I.D. were found easily clogged. The silica capillary of 25 μm I.D. was also found clogged with extended use. We found that silica capillaries of 50 μm I.D. were not clogged for prolonged use. The void volume of a 80 cm silica capillary of 50 μm I.D. was estimated 1.5 μL. Such a void volume was small enough not to impair column efficiencies when the flow rate was 10 μL/min or so. Thus we recommend deactivated silica capillaries of 50 μm I.D. for connecting the LC to the MS part for practical purposes.

The second sample we chose was PEG 300. It is a mixture of polyethyleneglycol oligomers with the average molecular weight of 300. The general expression of the molecular weight of a polyethyleneglycol oligomer is 18+44n (n = degree of polymerization), and the nominal mass number of its molecular ion, 19+44n, respectively. The SIM chromatograms of some oligomers and the total ion chromatogram are shown in Figure 4. All the individual oligomers were well separated. The solvent C was 0.1% formic acid in methanol, and D, 0.1% formic acid in water. The eluent composition was initially 30% C + 70% D, and was linearly changed to 50% C + 50% D in 5 min, and the composition, remained for 3 min, changed to 70% C + 30% D in 7 min, and finally changed to 100% C in 1 min.

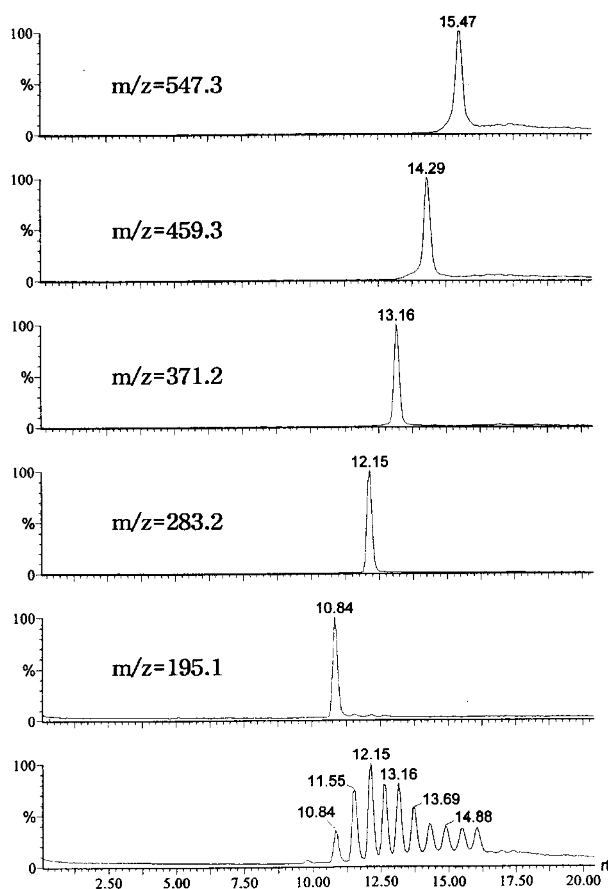

Figure 4. Separation of polyethylene glycol oligomers by the gradient μLC/MS. See the text for the operational details.

The last sample we examined was an artificial mixture of dyes whose molecular weights are close to or over 200. The motive of preparing this sample was to develop a convenient test mixture for LC/MS. We aimed to make a mixture of 5-6 components which have chemical stability and high mass spectromertic sensitivity and can be routinely used to check if a LC/MS system (both LC and MS) works properly. Using the solutes whose molecular weights are close to or over 200 was necessary to avoid mass spectrometric interferences from LC solvents. After some trials and errors, we came to select the following dyes: Methyl Orange (m/z of positive molecular ion: 306.1), Acridine Orange (m/z of molecular cation: 266.2), Crystal Violet (m/z of molecular cation: 372.2), 4-phenylazophenol (m/z of molecular ion, MH^+: 199.1), Sudan Orange G (m/z of MH^+: 215.1), and Oil Blue N (m/z of MH^+: 379.2). Their structures are given in Figure 5. The total ion chromatogram obtained in the mass scanning mode is shown in Figure 6. The elution sequence was as follows: Methyl Orange (6.92 min), Acridine Orange (8.01 min), Crystal Violet (8.57 min), 4-phenylazophenol (9.76 min), Sudan Orange (10.18 min), and Oil Blue N (18.30 min). The dye mixture sample was prepared by dissolving 1 mg of each dye in a 10 mL solvent composed of 2.5 mL water, 2.5 mL methanol, and 5 mL acetone. The injection volume was 0.5 μL, thus the amount of each solute injected

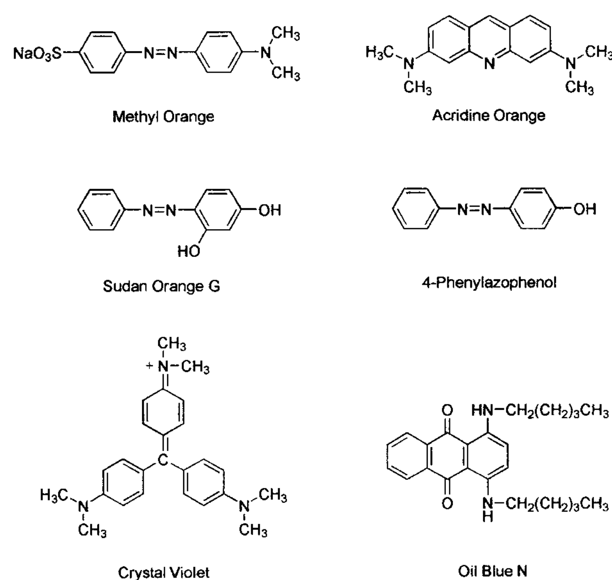

Figure 5. The dyes used for preparation of a LC/MS test standard.

was 50 ng. A very special elution program was employed to get such a chromatogram. The solvent gradient steps and the flow rate gradient steps were combined. The elution program included the following steps. The eluent composition was initially 70% E (0.1% TFA in methanol) + 30% F (0.1% TFA in water) and the initial flow rate was 10 μL/min. The initial eluent composition was maintained for 2 min, linearly changed to 100% E in 1 min, then the composition, maintained for 16 min, and finally linearly varied to 70% E in 1 min. The initial flow rate was kept for 10 min, linearly changed to 30 μL/min in 1 min, then the flow rate, kept for 8 min, and finally linearly changed to 10 μL/min in 6 min. Thus the initial eluent compostion and flow rate were recovered when the elapsed time hit 25 min. Additional 15 min later, the column was completely re-equilibrated with the initial condition of the elution program, and another injection of the dye sample gave reproducible chromtographic results. The reproducibility of the retention times for repetitive sample injections were better than 2%.

Such a program of flow rate variation was necessary to reduce the retention time of Oil Blue N (m/z: 379.2). The peak of Oil Blue N appeared far behind the other peaks even under the special elution program (Figure 5). Its retention time was more than 40 min when the initial flow rate was maintained throughout the run. We believed that it was necessary to check if a column ensures a kinetically rapid equilibrium of solute distribution between the mobile and stationary phases, thus we included such a long-retained dye in the test mixture on purpose. The peak at 14.24 min (m/z 319) seems to be caused by an unidentified impurity component. The peak of Methyl Orange (m/z: 306.1) was much weaker than the others. It is basically an anionic species and only a small portion of the anions will get two protons to become a cationic species in the eluent. The detection limits of the dyes in the SIM mode were found better than 5 pg (S/N ratio: 5) except for Methyl Orange and Sudan Orange G.

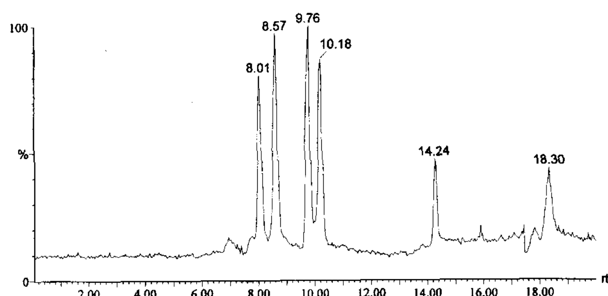

Figure 6. The total ion chromatogram of dyes (50 ng each injected) obtained in the mass scanning mode by the gradient μLC/MS. The flow rate gradient steps were included in the elution program in addition to the solvent gradient steps. Methyl Orange (6.92 min) eluted first, followed by Acridine Orange (8.01 min), Crystal Violet (8.57 min), 4-phenylazophenol (9.76 min), Sudan Orange (10.18 min), and Oil Blue N (18.30 min). See the text for operational details.

The SIM chromatograms and the total ion chromatogram for the four dyes obtained by injection of 5 pg each are shown in Figure 7. The dye mixture mother solution was consecutively diluted by factor 10. First, an 1 mL aliquot of the mother solution was taken in a small graduated cylinder and distilled water was added to the 10 mL mark. The final solution was again diluted in the same way and the procedure was repeated to give a dye concentration of 10 ng/mL. The volume of the sample loop was 0.5 μL, thus the amount of each solute injected by injection of the last solution was 5 pg. The signal reproducibility for repetitive sample injections was better than 10%. The detection limit of Methyl Orange was found *ca.* 1 ng, and that of Sudan Orange G, *ca.* 100 pg. The poor detection limit of Sudan Orange G was due to interference of solvent peaks (m/z 214, a cluster ion of aqueous methanol, and its satellites). We believe that this dye mixture should serve as a stable and convenient test standard for LC/MS. It should be noted, however, that the linearity of signal *vs.* concentration was poor for the dyes. The linear range was less than 10^1 order.

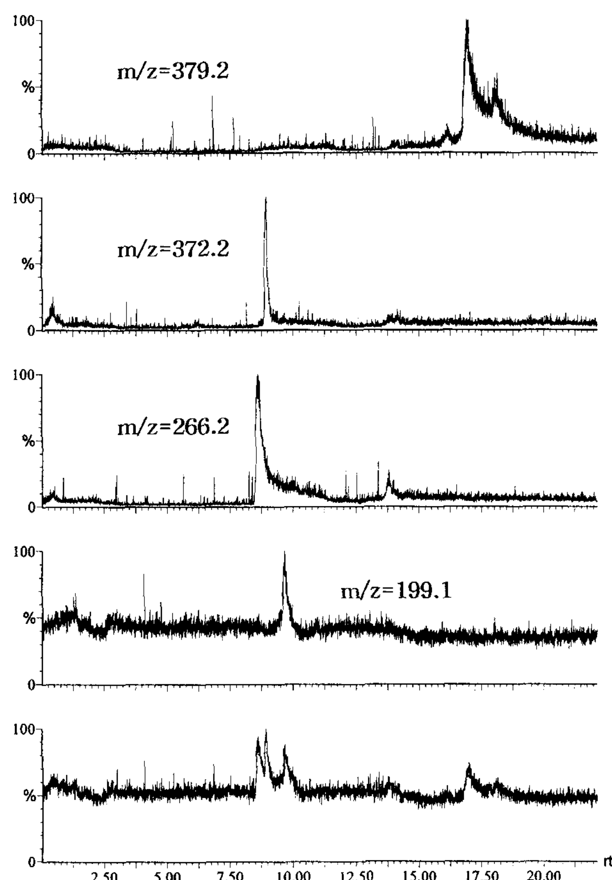

Figure 7. The SIM chromatograms of dyes obtained by injection of 5 pg each. From the top, Oil Blue N, Crystal Violet, Acridine Orange, 4-phenylazophenol. The bottom plot is the total ion chromatogram of the SIM ions.

Thus we have realized that our gradient μLC/MS system with a home-made cheap glass-lined stainless steel packed microcolumn and the Super-glue cured glass connector worked properly in view of selectivity and sensitivity after preliminary tests with some artificial samples. Future study will be focussed on application of this system for analysis of real samples such as urine and food.

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