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Chromatographic developments in low flow delivery for liquid chromatography–mass spectrometry

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ABSTRACT

A method has been demonstrated for producing accurate, reproducible gradients at low microliter per minute flow-rates suitable for liquid chromatographic—mass spectrometric (LC–MS) applications using capillary chromatography columns. The technique employs a low-cost, well characterized balance-column flow-splitter which can be simply added to a high-performance LC–MS solvent delivery system. Performance of the pump for LC–MS techniques at higher flow-rates is preserved.

INTRODUCTION

Liquid chromatographic–mass spectrometric (LC–MS) techniques such as electrospray and continuous-flow fast atom bombardment (FAB) require solvent delivery as low as 1–3 μl/min, defined by the nebulizing or liquid handling capacity of the interface design. The flow demands are similar to those required for capillary column and microbore high-performance liquid chromatography (HPLC) [1,2]. Using a capillary column for high-sensitivity gradient work demands a solvent delivery system capable of efficient low-volume eluent blending and accurate flow delivery.

With syringe-driven pumps, solvent compressibility, system compliance and solvent compositional accuracy have not been accommodated with the same success as with systems designed for higher flow-rates [3]. Over the years, methods of adapting existing liquid chromatographs to the requirements of low flow-rate gradients for capillary LC have met with some success [4,5] and others have made use of splitting techniques for low flow-rate LC–MS [6–8].

Our balance-column flow-splitting configuration (Fig. 1) uses inexpensive, readily available parts, is conveniently housed within the instrument and is designed for quick removal for conversion to higher flow-rates. Convenience and reproducible long-term performance were also considered in the design and choice of materials. The design makes use of the studies that developed the Waters 600-MS LC–MS flow feedback software and hardware. A successful μl/min delivery system accommodates the effects of compressibility and varying internal pressures produced during gradient delivery, which are of greater signif-

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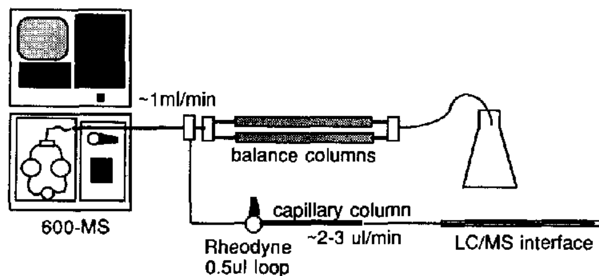

Fig. 1. Balance-column splitter for LC–MS with a capillary column.

icance at lower flow-rates. The reproducibility of gradient formation with this system was investigated by making repetitive injections of a tryptic digest.

EXPERIMENTAL

Chromatography

A Waters 600-MS solvent delivery system, specifically designed for LC–MS, was used for all chromatography. The splitter used two Waters microBondapak™ 150 mm × 3.9 mm I.D. columns plumbed in parallel to provide the suitable back-pressure differential at the split for flow-rates suited to electrospray or flow-FAB (2–5 μl/min). This scheme allows the HPLC pump to be programmed to operate in the 0.5–1 ml/min range where gradient delay is minimal. Pressure traces were made with a strip-chart recorder, measuring directly from the pressure transducer of the pump.

Plumbing to a capillary column used 229 μm (0.009 inch) I.D. stainless-steel tubing of the shortest possible length and a low-volume Rheodyne 0.5-μl injector (Berkeley, CA, U.S.A.). The column was plumbed directly into the back of the injector. From the column outlet to the probe, tubing depends on the interface. The peptide maps were carried out on a 150 mm × 0.32 mm I. D. Fusica C_{18} column (LC Packings, San Francisco, CA, U.S.A.). The eluents were 0.1% trifluoroacetic acid (TFA) in water (A) and 0.1% TFA in acetonitrile–water (55:45, v/v) (B); a gradient was run from 100% A to 100% B over 60 min at a flow-rate of 3 μl/min.

Post-column make-up flow of water was added through a low-volume T-fitting, so that a standard UV flow cell could be used. Flow was delivered from a Waters 590-MS isocratic pump at 0.3 ml/min. Peptides were detected at 214 nm with a Waters 441 UV detector.

Reagents

Acetonitrile and methanol were HPLC grade. Water was purified from a Millipore Milli-Q system (Bedford, MA, U.S.A.). A standard of β-lactoglobulin tryptic digest was purchased from Applied Biosystems (Foster City, CA, U.S.A.).

Eluents for the peptide maps were made up with Sequanal-grade TFA in 1-ml ampoules (Pierce, Rockford, IL, U.S.A.).

RESULTS AND DISCUSSION

Balanced-column flow-splitting

Current LC systems require flow-splitting as the primary means of achieving high-chromatographic-quality delivery below 10 μl/min [3]. Normally an HPLC pump is considered as a closed system, with solvent pumped to waste, and the system pressure is dependent on the flow-rate and viscosity of the solvent. With flow-splitting, a restrictor determines the system pressure, causing a small proportion of the delivered flow to be diverted to the capillary column and LC–MS interface. The splitter creates an open system, with the diverted flow-rate depending on the pressure and viscosity of the solvent.

Several methods of creating an appropriate restriction are available, all having inherent limitations. A pre-determined length of capillary silica gas chromatographic tubing forms a restriction, but is easily clogged by particulates, which will alter the split ratio. Mechanical valve restrictors, particularly when used to generate high split ratios, can easily fall out of adjustment, resulting in large changes in the split ratio. Both devices respond to the aqueous–organic exchange produced by a gradient differently to a packed chromatography column. This may have repercussions on both flow-rate and gradient integrity.

The balance-column method of flow-splitting has several potential advantages.

(i) Viscosity changes during the gradient are emulated by the balance column, the split ratio is maintained (in Fig. 2 the pressure profiles of a capillary restrictor

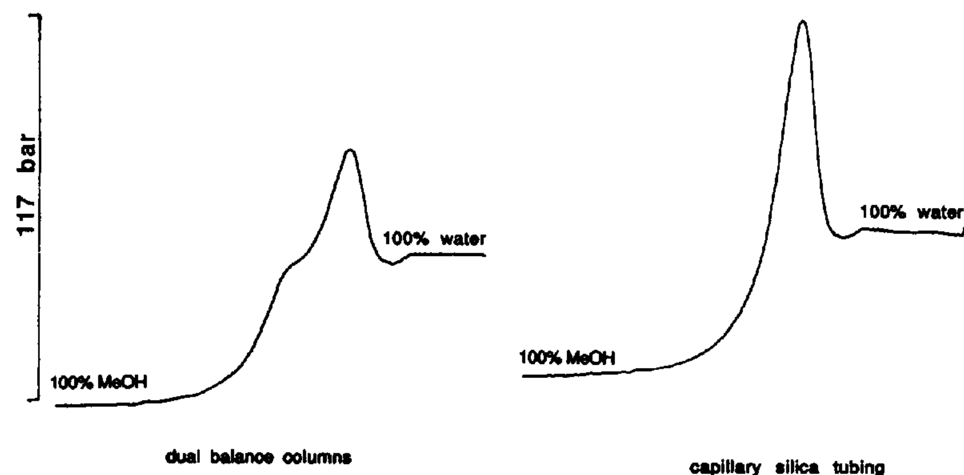

Fig. 2. Pressure profile of a step gradient with different flow-splitters.

and balance-column splitter show the differences in response when running a step gradient).

(ii) Using similar column chemistries on both sides of the split, pressure differentials caused by solvent/packing interactions are minimized.

(iii) Even if isocratic liquid delivery is the primary goal, the benefits of using chromatographic packed columns are a high degree of control over the delivered flow-rate and a long-term stability of the system. The resistance is distributed over the cross-sectional area and length of the column, so that minor particulate contamination or degradation of the packing material does not affect the operating pressure.

A dual-column system diminishes variations in back-pressure between purchased columns and allows conversion from 1 μl/min capability to 50 μl/min 1 mm I.D. column capability by removing a single column to adjust the split preference.

Measurement of flow and pressure

The relationships between pump flow-rate, system pressure, solvent viscosity and delivered flow were investigated with the system. Flow-rates were obtained by collecting solvent for several minutes and measuring the volumes with a calibrated micropipettor. A plot of μl/min delivered *versus* pump pressure (Fig. 3) is

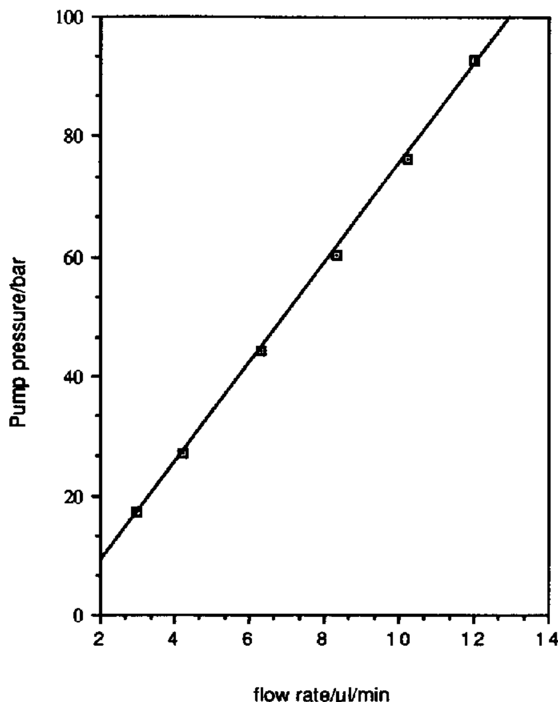

Fig. 3. Delivered flow-rate to the interface as a function of system pressure.

linear over a wide flow-rate range for any particular solvent mixture. For any selection of capillary and balance columns and solvents, once a calibration line is established, the flow-rate to the interface can be set by adjusting the pump flow-rate to produce the required pressure. The system is tolerant of day-to-day changes. System back-pressure held within 14 bar produced flow-rate changes of less than 0.2 μl/min.

During a gradient, however, the solvent viscosity varies, so the effect of the associated pressure changes on the delivered flow-rate were investigated. Pressure varies from 34 to 103 bar during the course of a typical reversed-phase separation using a gradient from 0 to 60% methanol (Fig. 4). With acetonitrile, the viscosity changes are less dramatic, but still produce a two-fold change across the gradient. In this situation, the delivered flow-rate will stay within the range 4.7–5.4 μl/min as shown in Fig. 5. Providing that chromatography is reproducible, the LC–MS interface will tolerate these changes in flow.

Capillary column chromatography

The reproducibility of gradient formation with this system was investigated by making repetitive injections of 25 pmol of a known standard such as the β-lac-

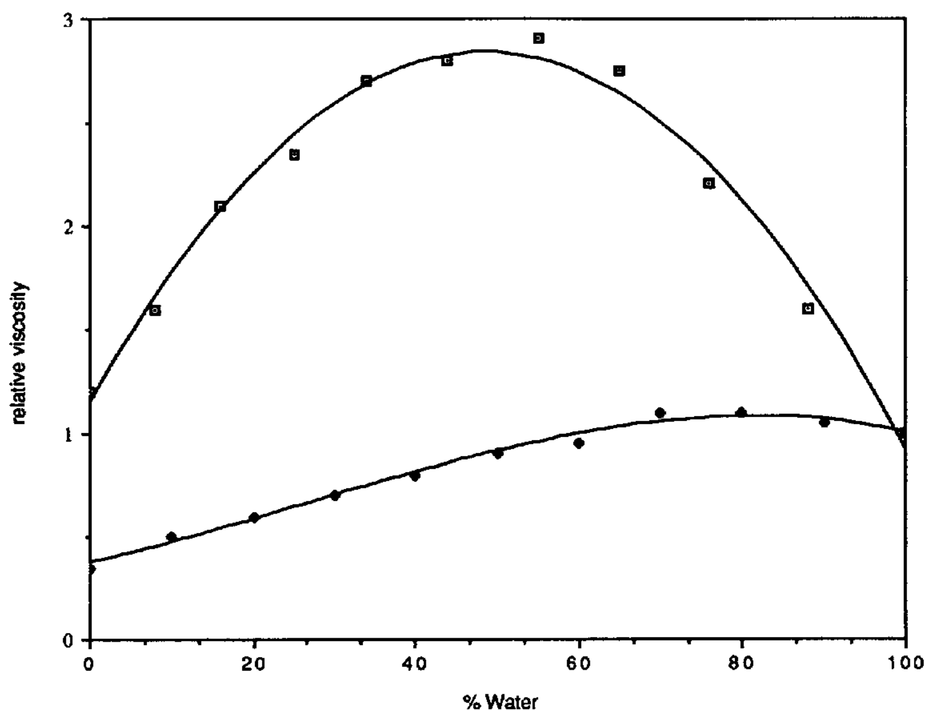

Fig. 4. Effect of solvent composition on the viscosity of methanol–water (□) and acetonitrile–water (◆) solutions.

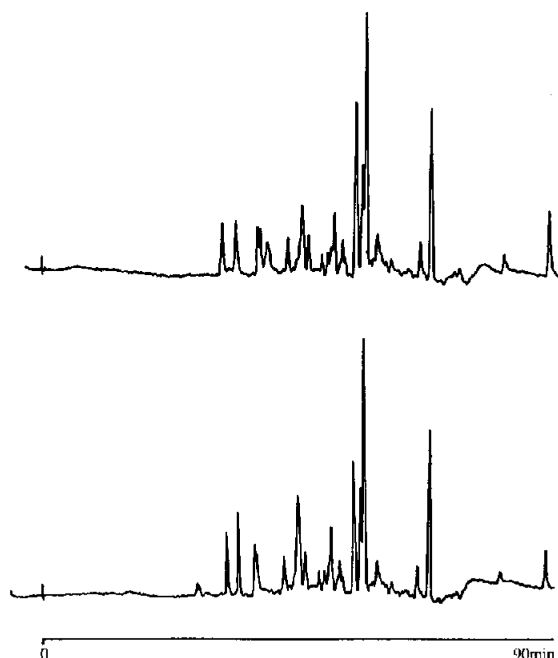

Fig. 5. Chromatograms 1 and 42 from repetitive capillary column peptide maps of 25 pmol of β-lactoglobulin tryptic digest, showing gradient reproducibility.

toglobulin tryptic digest shown in the example in Fig. 5. Since peptides elute at widely different organic concentrations, a digest provides a sensitive test for gradient reproducibility. The separation of the peptide map was run at 3 μl/min. Post-column addition of water at 0.3 ml/min allowed for detection of the peptides at 214 nm with a standard analytical cell in the UV detector. After 40 injections the character and retention times of the separation were the same, showing both the reproducibility of the gradient formation and the stability of the capillary column.

CONCLUSION

Practitioners of LC–MS, who may use several different interfaces, need a chromatographic system capable of operating from 1 μl/min through typical HPLC flow-rates, with smooth flow delivery and accurate gradient formation over this range.

A balance-column splitting method using two columns plumbed in parallel gives the correct back-pressure to provide an appropriate split ratio when a HPLC solvent delivery system is pumping solvent in the 0.5–1 ml/min range, where gradient delay is minimal. Removing one of the balance columns and

running the system with approximately the same settings will produce an appropriate flow for a 1 mm I.D. column (50 μl/min).

The flow-splitting method gives a well characterized flow delivery that has been demonstrated for a demanding peptide mapping application. Capillary columns show high resolution and can be used for reproducible chromatography. Further work is continuing in the application of the technique to flow FAB and electrospray LC–MS analysis of complex mixtures.

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