

A capillary-scale liquid chromatography system that improves the practical sensitivity of HPLC-MS(MS) analysis

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Today's drug discovery scientists are asked to obtain increasing amounts of information on samples that would have been considered absurdly small only a few years ago. As samples, and the concentrations of target analytes, become smaller, the scale of the separation methodology must shrink as well in order to obtain quality information from such small samples.

This article describes a gradient capillary-scale liquid chromatography (LC) system for sample-limited proteomic and preclinical drug research: the CapLC™ System (Waters Corp., Milford, MA). Applications are shown for characterizing enzymatically derived peptides at extremely low concentrations, and small molecules using fast gradients.

The mother of invention: The need for sensitivity

To understand the need for high sensitivity in drug research, it helps to understand the way molecules, such as proteins, have been

traditionally analyzed. Protein mixtures obtained from cells and tissues contain numerous components. Today, the most common method for separating such complex samples is two-dimensional polyacrylamide gel electrophoresis (2-DE). This technique separates protein mixtures first by isoelectric focusing (IEF), which separates proteins by charge, followed by a size-based separation that further separates them by their molecular weight. This orthogonal separation is capable of extremely high resolution, with the capacity to resolve more than 1000 proteins in a single analysis.

Individual spots can be extracted for further identification or structural analysis either directly from the gel or by transfer-

tions can now be performed, have begun to change the way high-sensitivity protein and small-molecule drug analysis is conducted.

Matters of size

LC has long been a favorite of pharmaceutical scientists. It is a proven technique, one whose mechanisms have been well understood for decades. But discovery chemists with very unique needs are pushing the limits of the technology.

In the discovery chemist's world, samples are "vanishingly small," confined to perhaps a few picomoles. Discovery chemists are typically focused on synthesizing,

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ring the target proteins to a membrane surface. This stage of the investigation is where complications can occur because of the low amount of material (50–1000 ng) present in a typical spot. New and sensitive methods for studying intact proteins or fragments (peptides) from the protein parent have been the focus of much research and development. Improvements in high-performance liquid chromatography (HPLC) and mass spectrometry (MS), notably the scale at which separa-

elucidating the structure of proteins, and screening them for bioactivity. They must also measure drugs and their metabolites in small amounts of biofluids, typically derived from expensive laboratory animals.

The well-documented advantages of MS have popularized its use with LC, changing the way LC is perceived. Many discovery chemists now regard LC as a sample inlet method for MS. In these laboratories, precision and accuracy take a back seat to spectral

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sensitivity and peak capacity (or the total number of chromatographic peaks capable of being baseline resolved during a separation).

Today's state of the art: Capillary LC

Previous attempts to achieve greater mass sensitivity by converting analytical LC systems into capillary LC systems have been dogged by difficulty. The problems can be grouped into four cat-

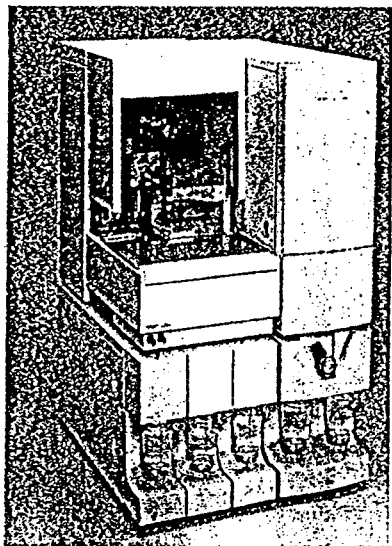


Figure 1 CapLC/MS System with ZMD ES/APCI (electrospray/atmospheric pressure chemical ionization) mass detector.

egories: detector signal to noise, gradient formation, flow splitting, and piecemeal solutions.

More than just scale

The immediate result of attempts to scale down separations is a lack of detector sensitivity. Analytical detector flow cells introduce excessive band spreading and low peak heights, drastically reducing peak capacity and making peak integration all but impossible. Simply passing the light beam across the capillary at the end of the column minimizes band spreading, but the short pathlength yields very low absorbance.

Other attempts at capturing a

greater signal have led to the design of shaped flow cells. These cells attempt to increase pathlength by transmitting light between two bends in the capillary tubing, several millimeters apart. Unfortunately, the bends make poor optical surfaces and light transmission is low. Of the light that is transmitted, a significant portion passes through the silica walls rather than the sample, resulting in reduced sensitivity and a strong, nonlinear response. Although the shaped capillaries are an improvement over a cross-capillary detection cell, they fall well short of the performance one might expect on the basis of their nominal pathlength.

Metering solvents at extremely low flow rates

Another problem lies with forming accurate gradients under such low-flow conditions. For gradients, one solvent must be accurately metered at only fractions of the total flow (e.g., perhaps only 50 nL/min at 1% when the total flow is 5 μ L/min).

Flow splitting

One solution for metering low microliter-per-minute flow rates is to employ a flow splitter. In these systems, the flow is split based on the relative backpressure in two

flow paths: the sample path and a path to waste equipped with a restrictor. As long as the ratio of these values remains constant, consistent results can be achieved. Over time, however, backpressure on the column will ultimately change the ratio and, thus, the net flow to the column. Measuring the flow change at 3–5 μ L/min can be time consuming and laborious. This change in column backpressure can occur more rapidly, especially when analyzing real samples where small amounts of particulates or precipitated protein immediately increase backpressure and compromise chromatographic reproducibility.

Excessive band broadening caused by poor component integration

Scientists must exercise great care in piecing together a modular instrument. System volume and band spreading must be kept to acceptable levels. Tubing and fittings must be carefully assembled. Poorly assembled connections (unions) are a hidden source of peak broadening.

Capillary-scale HPLC system

The CapLC capillary-scale HPLC system (Figure 1) overcomes the fundamental limitations that have kept capillary LC out of more laborato-

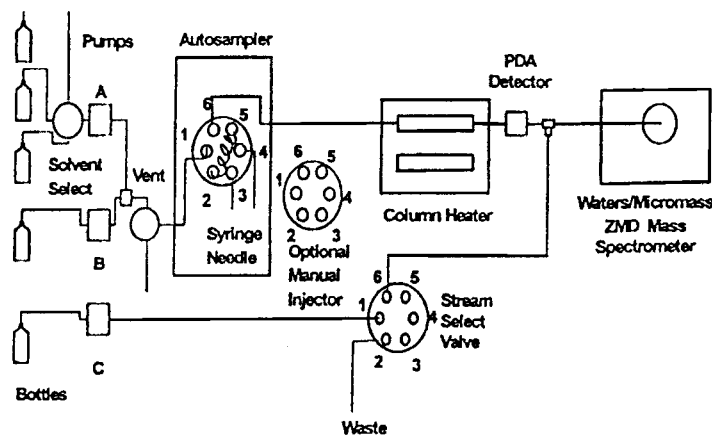


Figure 2 CapLC System configured for binary gradient with postcolumn addition. The system is well suited for direct coupling to an electrospray mass detector. This LC-MS configuration supports pre- and postcolumn reagent addition and gives the scientist structural information on proteins, peptides, and small molecules.

Comparison of Capillary to Standard Chromatography

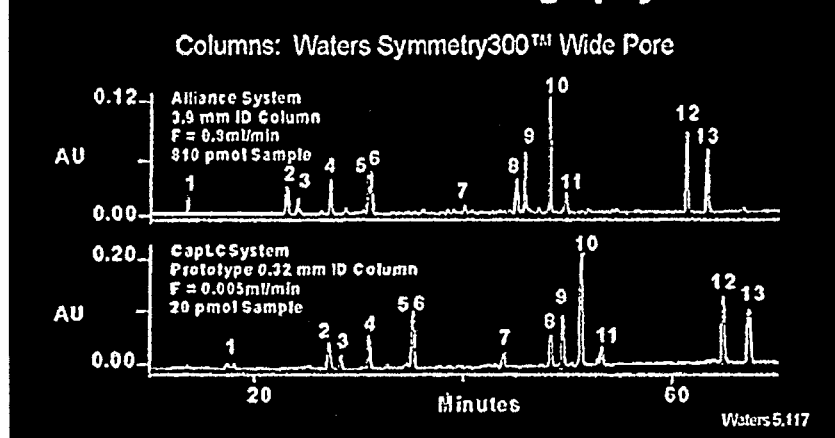


Figure 3 Comparison of capillary LC results to those of analytical LC. With 40x less sample, the CapLC System generated a 2x greater peak height response for an 80-fold enhancement in mass sensitivity. Conditions—mobile phase: 0–40% B in 80 min, A = 0.1% trifluoroacetic acid (TFA) in H₂O and B = 0.085% in MeCN; flow rate: 800 μ L/min (analytical LC system) and 5 μ L/min (CapLC system); detection: 214 nm; column: Symmetry C₁₈ Wide Pore, 3.9 \times 150 mm (analytical LC) and 0.32 \times 150 mm (CapLC); sample: cytochrome C tryptic digest.

ries. The system features patented solvent delivery and detector technology that allows sample-limited chemists to obtain quality UV spectra and measure subpicomole analyte concentrations with resolution and reproducibility that are comparable to analytical HPLC. The system includes an integrated auto-sampler and built-in column heater. It is entirely under MassLynx™ software control (Micromass Ltd., Manchester, U.K.). MassLynx acquires UV and MS data simultaneously, and provides complete post-run analysis capability for both data types. The system is plumbed and performance-tested before being shipped. All software is Y2K compliant.

Integrated design

The automated, binary gradient (ternary optional) LC system (Figure 2) is easily interfaced and optimized for benchtop or research-grade MS systems supplied by Waters or Micromass Ltd. The integrated system design is essential for minimizing band spreading. Typical total system variance (σ) is less than 0.3 μ L. Strict control of system band spreading and volume allows one to match the separation efficiency of typical analytical-scale HPLC separations (Figure 3).

PDA detection sensitivity

A patented low-volume flow cell¹ (Figure 4) combines long pathlength and high light throughput. The CapLC photodiode array (PDA) detector cell pathlength is 5 mm, and volume is 250 nL. It guides light like an optical fiber. The cell is constructed of Teflon® (DuPont, Wilmington, DE) AF, a unique amorphous fluoropolymer that combines very good UV and visible transparency, chemical inertness, and a refractive index less than that of water. The Teflon AF is analogous to the low-index

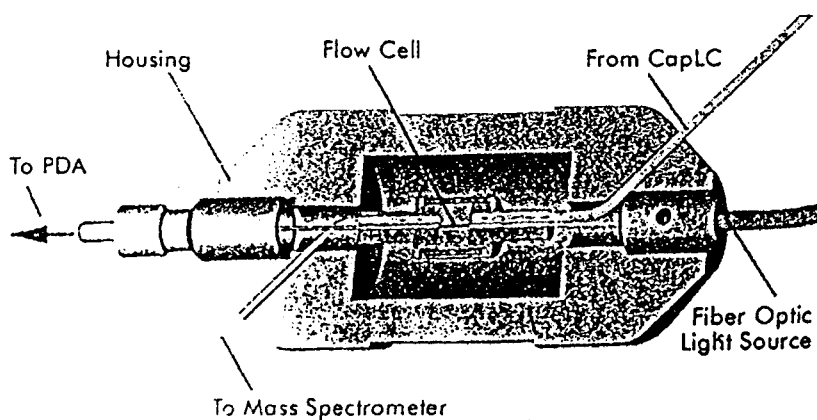


Figure 4 The system's patented light-guided flow cell focuses and guides light from a deuterium lamp source along the length of the flow cell. The light-guiding properties of the flow cell permit the CapLC cell to transmit as much light as a conventional 8- μ L PDA flow cell.

cladding of an optical fiber and the flowing sample stream is analogous to the core. The light-guiding properties of the flow cell permit the CapLC cell to transmit as much light as a conventional 8- μ L PDA flow cell.

The resulting low noise, combined with the small volume and long path, dramatically lower the limits of detection—in the femtomole range for peptides—to make them comparable to MS. Since all the light passes through the sample, linearity is preserved. Excellent peak shape is maintained by connecting the capillary column to the flow cell with 50- μ m-i.d. tubing.

MS interface

To maintain chromatographic resolution, a capillary LC-compatible interface has been developed for the Waters ZMD single quadrupole mass detector. With this modification, the sharp bands eluting from the PDA cell experience little increase in volume, thus preserving the separation efficiency. Peak width is typically >40% narrower with the new interface as compared to the unmodified interface.

Solvent delivery

The solvent delivery system for the CapLC was designed with gradient LC-MS (MS) applications in

Fast Gradient Reproducibility (N = 8)

Eluent System: Formic Acid/MeCN

Gradient: 5-95% B in 5 min

Flow: 10 μ l/min

Standard Deviation Range: 0.002 - 0.003

%RSD Range: 0.04 - 0.20%



Figure 5 A rapid gradient separation with a 12-component test mix illustrates typical system reproducibility for gradient analysis. In this study of eight consecutive injections, the SDs for retention time were all less than 1 sec. Conditions—mobile phase: 5–95% B in 5 min, A = 0.1% aqueous formic acid and B = 0.07% formic acid in MeCN; flow rate: 10 μ L/min; detection: 254 nm; column: Symmetry C₁₈, 0.32 \times 150 mm; sample: 12-component small-molecule test mix.

mind such as peptide mapping and fast small-molecule analysis. The syringe-style, positive-displacement, continuous delivery design (patents pending) delivers solvent at the low-microliter flow rates that are optimal for electrospray MS. The system performs gradient separations in the 1–40 μ L/min flow rate range. Each syringe is motor driven and software controlled so that crossover-related flow phenomena are eliminated. Gradients are mixed under high pressure to minimize the gradient delay and overall system volume (as little as 16 μ L). The syringe pumps are self priming and self purging. A high-pressure switching valve allows scientists to perform ternary gradients as well as on-column concentration or postcolumn reagent delivery.

Automated sample introduction

The integrated autosampler can access samples from 2-mL vials, PCR (Hoffmann LaRoche, Inc., Nutley, NJ) and microcen-

trifuge tubes, and 96 (shallow and deep well) and 384 multiple-well plates. As little as 20 nL of sample can be injected at a time. The autosampler will inject larger volumes (5 μ L or more) for concentrating dilute samples containing well-retained molecules such as peptides, proteins, or hydrophobic drugs. This can be highly advantageous for high-sensitivity analysis via tandem MS (MS-MS).

Column chemistries

Separations with the system are performed on 320 μ m \times 15 cm fused-silica capillary columns packed with 5 μ m Symmetry[®] C₁₈ material (Waters Corp.). For small molecules and peptides, 100 Å and 300 Å pore size packing materials are used, respectively. Reproducible gradient performance is illustrated with a small-molecule mixture of 12 components (Figure 5).


Conclusion

LC combined with MS has given chemists a powerful new way to fully analyze a multitude of products. Few technologies are as sensitive as MS or as demanding on LC systems.

Sample-limited chemists enjoying the benefits of LC-MS(MS) are offered a capillary-scale LC that offers significantly more mass sensitivity than an analytical LC system and is better equipped at delivering the optimal flow required of MS and MS-MS.

The introduction of the capillary-scale LC system brings to the laboratory a new tool for micro-scale LC. Combined with MS systems, the CapLC System offers research chemists an integrated solution to the problem of obtaining quality UV and mass spectral data in a single injection from trace sample amounts.

Reference

1. U.S. Patent #5,184,192. Light Guiding Flow Cell Formed of an Amorphous Fluoropolymer. February 2, 1993. 

20th Annual Analytical Chemistry Starter Grant Award

The Society for Analytical Chemists of Pittsburgh will award one grant of \$20,000 to an assistant professor in the field of analytical chemistry. The purpose of this grant is to encourage high-quality, innovative research by a new analytical chemistry professor and to promote the training and development of graduate students in this field. Assistant professors who have accepted a United States college or university appointment since December 31, 1995, are eligible. Contact Art Byers, Starter Grant Committee, Society for Analytical Chemists of Pittsburgh, 300 Penn Center Blvd., Ste. 332, Pittsburgh, PA 15235; tel.: 800-825-3221; fax: 412-825-3224. Completed applications must be received by March 31, 1999.