A Novel Capillary LC/MS/MS System

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ABSTRACT

We describe a unique capillary LC/MS/MS system designed to take full advantage of low flow rate ESI sensitivity in a robust manner compatible with a variety of application areas. The system is comprised of a novel fluid delivery module, a new nanoflow optimized atmospheric pressure MS interface, and a modified hybrid triple quadrupole/ linear ion trap mass spectrometer based on the API 4000 design.

INTRODUCTION

The broad acceptance of capillary LC/MS into a variety of application areas has been limited by several factors. One of the primary limitations has been the precision, accuracy, and reliability of the fluid delivery systems. Routine and reliable chromatographic systems have in the past been primarily relegated to conventional flow HPLC. Secondly, electrospray ion sources designed to operate with conventional chromatographic flows have also proven to be more robust and reliable than those optimized for the nanoliter to low microliter per minute range.

A new fluid delivery system based on direct pumping and microfluidic flow control is described that provides precise and robust nanoscale gradient chromatography. In addition this system is capable of near instantaneous flow breaking (peak parking) to enhance MS/MS signal-to-noise (S/N) with signal averaging techniques. This LC system is coupled to a mass spectrometer via an new atmosphere to vacuum interface that is optimized to both stabilize low flow electrospray emitters across the solvent composition range of typical gradients and to consume and vaporize a greater percentage of the charged droplets. This interface also sorts charged residues from ions and clusters to improve the long term stability of the MS.

The above described elements are coupled to a new hybrid triple quadrupole/ion trap mass spectrometer designed for application versatility. This instrument combines the quantitative MRM sensitivity available from high end triple quadrupoles with the high full scan sensitivity and MS^3 capability from linear ion traps. In addition to the obvious utility for protein sequencing and characterization this combination of instrumental elements for capillary LC should be very applicable to general pharmaceutical quantitative and qualitative needs in drug discovery and development.

MATERIALS AND METHODS

Nanoscale Pumping system.

Nanoscale chromatography was performed with a NanoLC-1D Proteomics system, which uses microfluidic flow control technology to deliver binary gradients in the flow rate range of 20-500 nL/min without the use of flow splitting. Microfabricated flow meters continuously measure the flow rate of each mobile phase by measuring a differential pressure across a known flow resistance. These measurements are used for active feedback control of an electronically controlled pressure source to precisely control the flow rate during a run. Flow rates are unaffected by viscosity changes due to mobile phase mixing or downstream pressure fluctuations. Flow rate precision of the NanoLC system is <0.5% RSD at 500 nL/min

MS Interface

Fused silica capillaries drawn to 10micron apertures were used as the electrospray emitters (New Objective, Cambridge, MA). A Microlon Spray assembly was used to mount the emitters and allow a flow of 0.3L/min of air to cool and stabilize the spray. A countercurrent flow of 1 L/min curtain gas was used to keep the entrance region free of neutral particles with 1 kV voltage on this plate. The removable chamber was held at 140°C and 500 volts. The orifice plate was not directly heated and held at 90 volts.

Mass Spectrometer

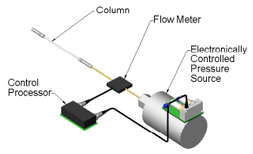
The mass spectrometer is an API 4000 triple quadrupole (mass range 2800 Daltons) with modified electronics to support linear ion trapping in quadrupole 3. The main Rf drive frequency is 816 kHz (8 kV p-p) where the amplitude is scanned during mass analysis for both Rf/DC and linear ion trap modes. The auxiliary Rf frequency is fixed at 312 kHz (5 v p-p) with ejection at a q value of 0.84. For MS^3 operation, after ejection of unwanted ions with the appropriate Rf/DC voltages, excitation is achieved with an auxiliary Rf of 69 kHz (200mV p-p) at a q value of 0.235.
Ion peak widths are tuned to .2-.4 amu peak width half height in the trapping mode.

RESULTS

Figure 1. NanoLC-1D Instrument

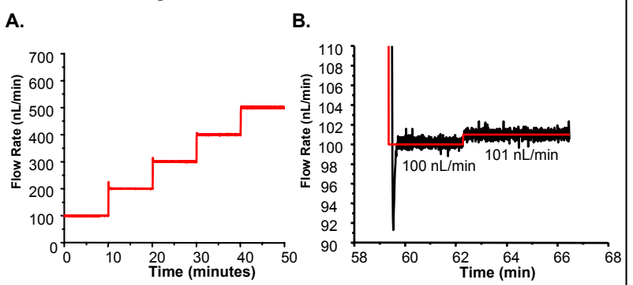
The NanoLC-1D instrument is a direct pumping, binary gradient system optimized for a flow rate range of 20-500 nL/min without the use of flow splitting. Mobile phases are stored in pressurized loops within the fluidic system.

Figure 2. Microfluidic Flow Control Technology



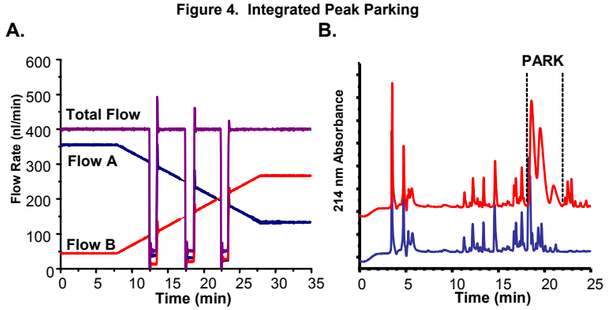
Microfluidic flow control technology consists of an electronically controlled pressure source, an in-line flow meter, and a control processor. The NanoLC-1D system contains 2 of these modules for binary gradient delivery. The flow meters continuously measure the actual flow rate of each mobile phase. This flow rate information is passed to an embedded processor that controls a rapid, electronically adjusted variable pressure source.

Figure 3. Precise control of flow rates



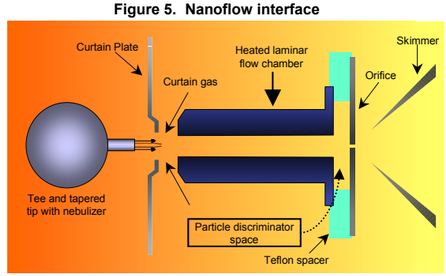
A. Stepped increase in flow rate from 100 nL/min to 500 nL/min. Response time of the system for each change in flow rate is <1 sec. **B**. Response of the NanoLC system to a 1 nL/min change in flow rate when the setpoint is changed from 100 nL/min to 101 nL/min, is demonstrated. Displayed is the flow rate setpoint (red) and the actual measured flow rate (black).

Figure 4. Integrated Peak Parking



A. Flow profiles for total flow (purple), mobile phase A (blue) and mobile phase B (red) are shown for a gradient run at 400 nL/min (10-60%B in 20 min; A=0.1% TFA, B=90% ACN/0.08% TFA). At 3 separate timepoints during the gradient 'peak parking" was implemented in which the flow rate was rapidly dropped to 50 nL/min. Restoration of the flow rate to 400 nL/min after each parked phase shows that no interruption of the gradient profile occurs. **B**. Gradient separations of a cytochrome c tryptic digest on a 15 cm, 100 micron i.d. column packed with 3 micron C18 particles, at a constant flow rate of 300 nL/min (blue) and with peak parking at 50 nL/min implemented during the time indicated (red) are shown. Peak parking extends the detection time for the peptide peaks eluting during this period, with no loss of resolution of the peaks eluting after the flow rate was restored to 300 nL/min.

Figure 5. Nanoflow interface



The interface is comprised of 5 elements each serving a different purpose and held at different electrical potentials:

-The electrospray emitters are typically pulled fused silica floated at the appropriate voltage. Typically a co-axial flow of gas (not shown) is used to cool the emitters and aid in the stabilization of the spray. The emitters are positioned such that the vacuum drag generated by the mass analyzer inhales the majority of the plume.

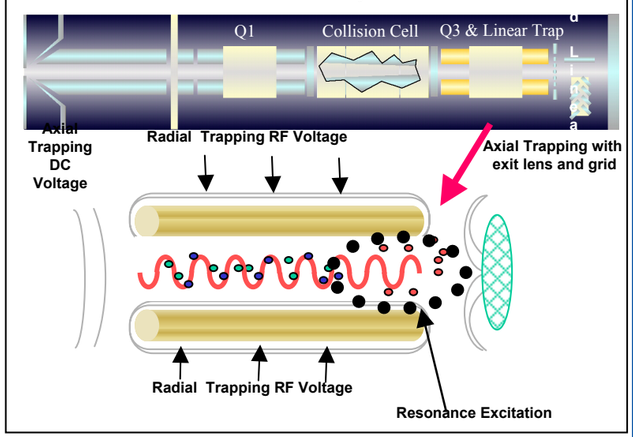
-The Curtain Plate directs the counter flow of gas to aid in desolvation and evacuate large neutral particles.

-The Chamber establishes laminar flow conditions and aids desolvation with elevated temperatures in this region.

-The Gap between the Chamber and orifice creates a region of partial turbulence. The different potentials between the chamber and orifice sort the large charged particles from the ions in the gas stream, the particles depositing in a region away from the orifice and instrument vacuum system.

-The Orifice plate contains the aperture that restricts the gas flow into the vacuum chamber. The voltage on this element controls vacuum declustering.

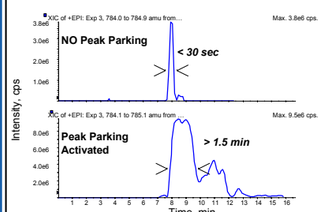
Figure 6. Hybrid Triple Quad/Linear Ion Trap 4000 Q TRAP™ System



Operation mode 1: The hybrid instrument operates both mass analyzers in classical Rf/DC mode for MRM data acquisition. In this fashion the highest possible sensitivity and precision measurements can be made for quantitative measurements.

Operation mode 2: The second mass analyzer is operated in linear ion trap mode. Ions are bottled in the quadrupole with entrance and exit DC voltages. Both the main drive Rf and an auxiliary Rf is applied to these rods. When the main drive Rf amplitude is scanned the ions are sequentially brought into resonance. When a particular m/z is in resonance and it approaches the rods it picks up sufficient axial energy from the rod fringe fields to surmount the gridded trapping barrier and be detected. In this fashion the duty cycle is greatly improved over an Rf/DC scan and the highest possible sensitivity and speed can be made for full scan spectra acquisition. Also MS^3 spectra can be obtained.

Figure 7. Detection of α-casein phosphopeptide using peak parking



A fast gradient separation of an equimolar mixture of tryptic digests of α-casein, β-casein, and BSA was performed at a flow rate of 500 nL/min. A neutral loss scan was used to detect the phosphopeptide of α-Casein (976.3). Peak parking at 50 nL/min was triggered automatically by the mass spectrometer using Information Dependent Acquisition. The extracted ion chromatogram (XIC) of its fragment ion (784) clearly shows that the acquisition was extended by 5x by activating peak parking, thus ensuring sufficient data collection in MSMS mode.

Figure 8. Combination of specific scan with peak parking to increase phosphopeptide detection

	Survey Scan Peak Park m/z	MS Mascot Search	Neutral Loss OFF Score	Neutral Loss ON Score
YPQLEIVPNSAEER + Phospho (S7)	830.7	nid	nid	32
YKVPQLEIVPNSAEER + Phospho (S7)	976.3	nid	nid	45
FFVAPFPEVFGK	662.8	43 (17)	nid	47
EPMGVNQELAYFYPELFR	772.6	50	nid	31
HQGLPQEVLNENLLR	880.8	115	nid	nid
alpha-Casein (overall score)		208	nid	151
YQKFPQYLQYLYQGPIVLNPWDQVKR + Phospho (S7)	1030.3	113	86	101
beta-Casein (overall score)		113	65	101
KVPQYSTPTLVEVSR	820.3	41	nid	nid
DAFLGSFLYEYSR	784.5	nid	14	nid
TVNENFVAFVDK	700.3	nid	73	18
IPQYSTPTLVEVSR	756.2	73	nid	61
Bovine Serum Albumin (BSA)		114	67	79

Single MS and fast chromatography still provide reasonable detection of protein with significant scores. However, combination of a more specific scan, such as neutral loss, in combination with peak parking, ensures that peptides of interest are detected and confirmed. In this case, all phosphopeptides of α-casein and β-casein were detected with a neutral loss scan of 49 and confirmed in EPI scan while peak parking was activated.

CONCLUSIONS

This work describes an instrumental system where three critical elements are design optimized for routine operation in the 50 to 500 nL/min chromatographic flow regime. A new fluid delivery system is presented that directly delivers, without flow splitting, the full flow range with nanoliter per minute resolution.

An atmosphere to vacuum interface is described that creates conditions more conducive to robust unattended operation of nanoflow ESI chromatography.

And a highly versatile mass analyzer based on the API 4000 triple quadrupole with modified electronics to allow linear ion trapping in quadrupole 3 is described. With this mass analyzer the system described should be applicable to a broad variety of application areas including proteomics, drug discovery, and drug development work.

REFERENCES

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