

Performance advantages using micro-LC columns packed with < 3 μm particles in LC/MS/MS

David Neyer, Remco van Soest, and Steve Hobbs
Eksigent Technologies, 5875 Arnold Road, Dublin, CA 94568



Introduction

The predominant analytical approach for quantitative DMPK studies is to utilize LC with mass spectrometry. However, the growing number of samples that are being analyzed in today's drug discovery and development labs are pushing the limits of current LC/MS system performance and throughput. Increasing pressure ratings on commercially available pumps have allowed the historic trend toward ever smaller particle diameters to continue. However, limited pump performance at low flow rates has slowed the progress toward smaller column diameters. We will describe the performance of a low flow, ultra-high pressure LC system for use with mass spectrometers. We will demonstrate that the use of sub-3 μm particles and 0.5-1 mm ID columns reduces cycle times and solvent usage.

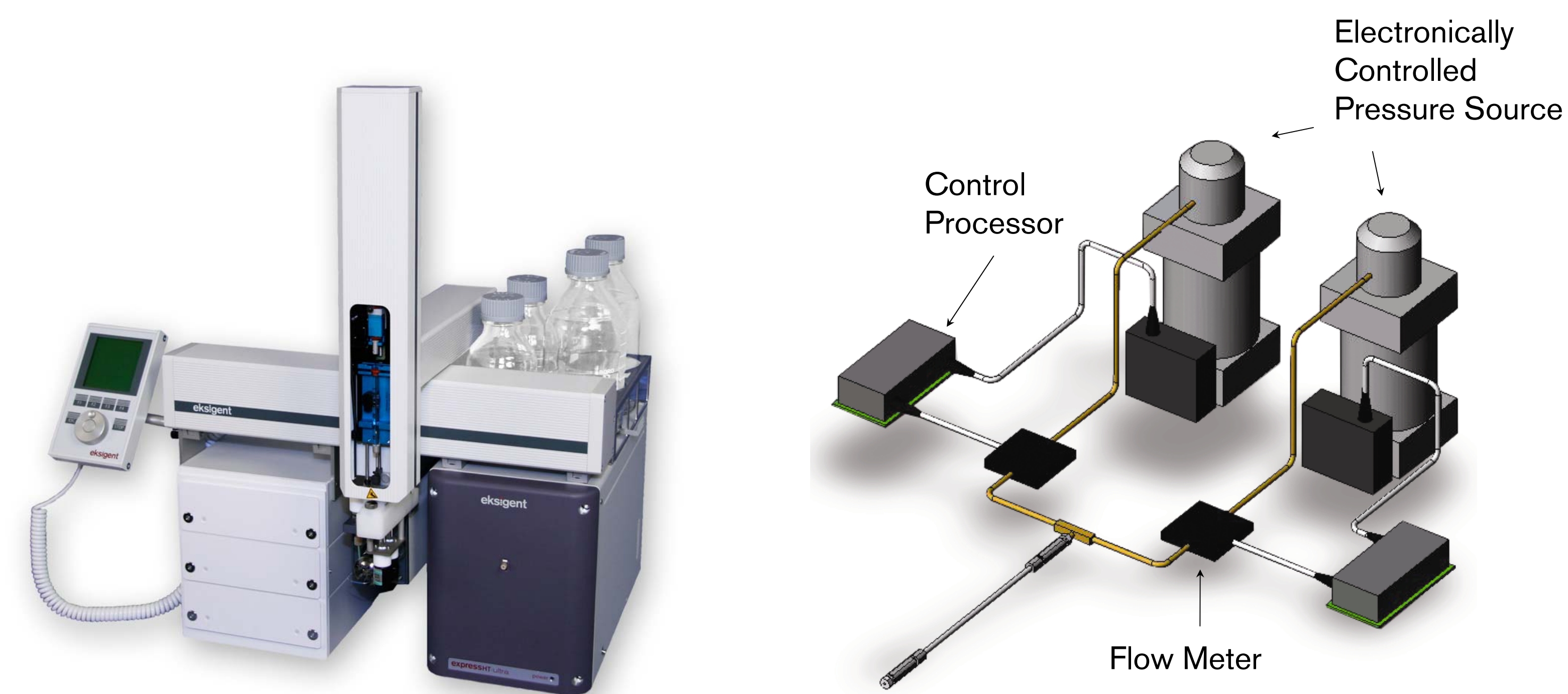
Methods

An HPLC system, the ExpressHT-Ultra, was developed that consists of (1) a binary gradient pump capable of delivering 20-200 μL/min flows at 10,000 psi (2) and injection valve capable of 0.1 to 10 μL injection volumes (3) a two-channel wash system that delivers solvents for rapid washing of the injector and syringe (4) an industry standard CTC autosampler and (5) a six-position CTC cooled plate holder (6) and a column oven for temperatures up to 80C. HPLC columns of 0.5 to 1 mm ID, 3-5 cm long and 2.7 μm diameter particles were packed in-house.

Microfluidic Flow Control at ultra high pressures

The ExpressHT-Ultra's solvent delivery system is based on a binary gradient pumping system designed for HPLC columns with diameters of 1 mm or smaller. A benefit of small diameter columns is a reduction of the required mixing volume. Less volume means shorter gradient delays and faster separations. Column and system re-equilibration also take much less time. The result is a system with cycle times as short as 60 seconds for high analytical throughput. The entire system is designed to operate at pressures up to 10,000 psi. This allows the use of columns with small diameter stationary phase particles (1.5 to 3 μm) delivering fast, high resolution separations

Eksigent's Microfluidic Flow Control ensures precise and stable flow rates. With MFC technology, direct pumping is used to deliver accurate, repeatable gradients. Each pressure source delivers fluid with the actual flow rate monitored using the flow modules in each channel. A feedback loop makes real-time adjustments to the flow rate. By continuously monitoring the flow from each of the binary system's pumps, the flow rate can be adjusted many times per second. The result is retention times with an RSD typically below 0.3%.



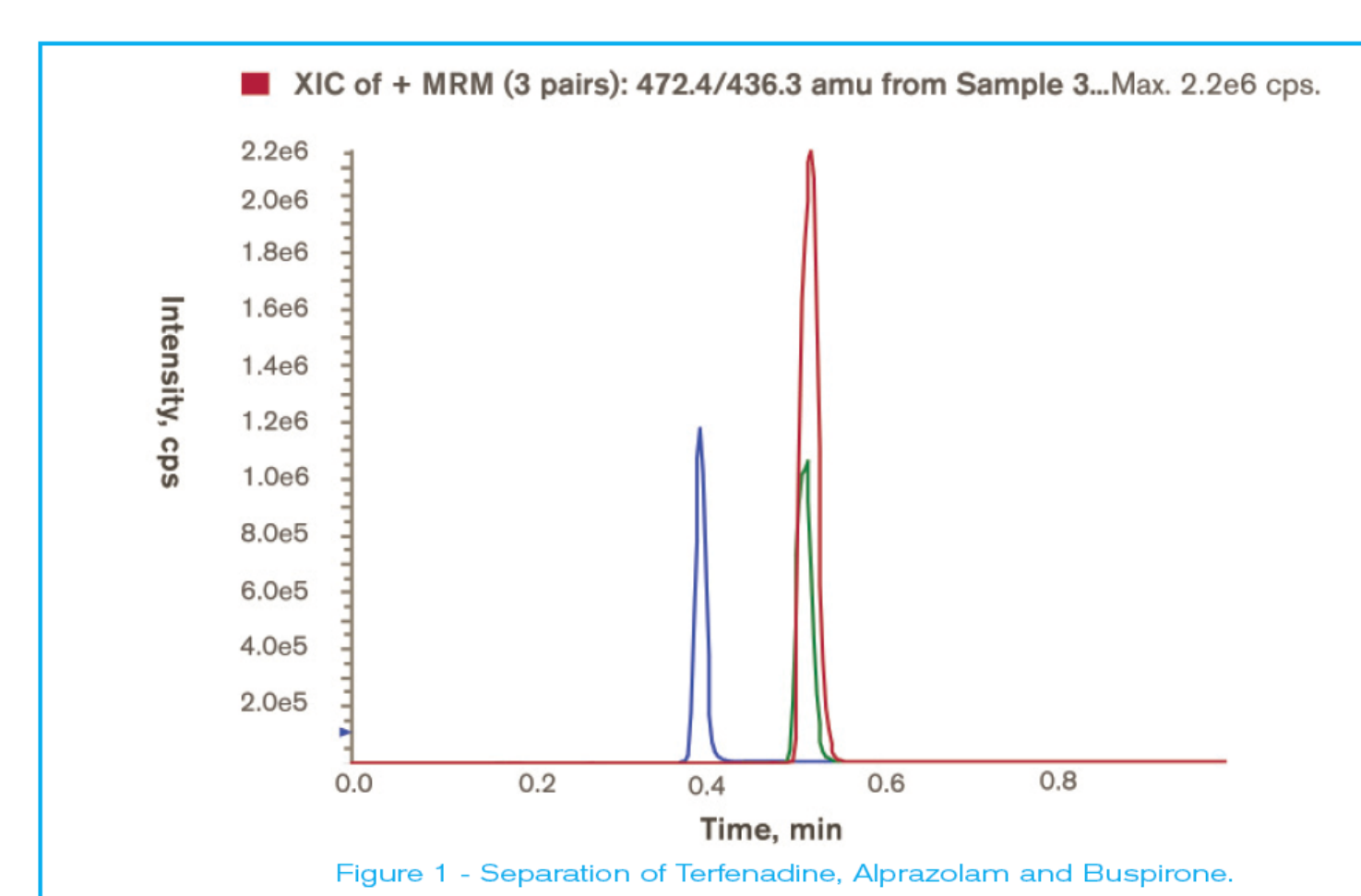
Schematic of the Microfluidic Flow Control (MFC) setup. In-line flow meters provide high speed measurements of the delivered flow rate to a built-in control processor. The processor performs real-time feedback control of the electronically controlled pressure sources to provide accurate flow for microflow LC.

The ExpressHT-Ultra system for ultra high pressure LC/MS.

High performance chromatography with rapid cycle times allows fast LC/MS/MS for bioanalysis

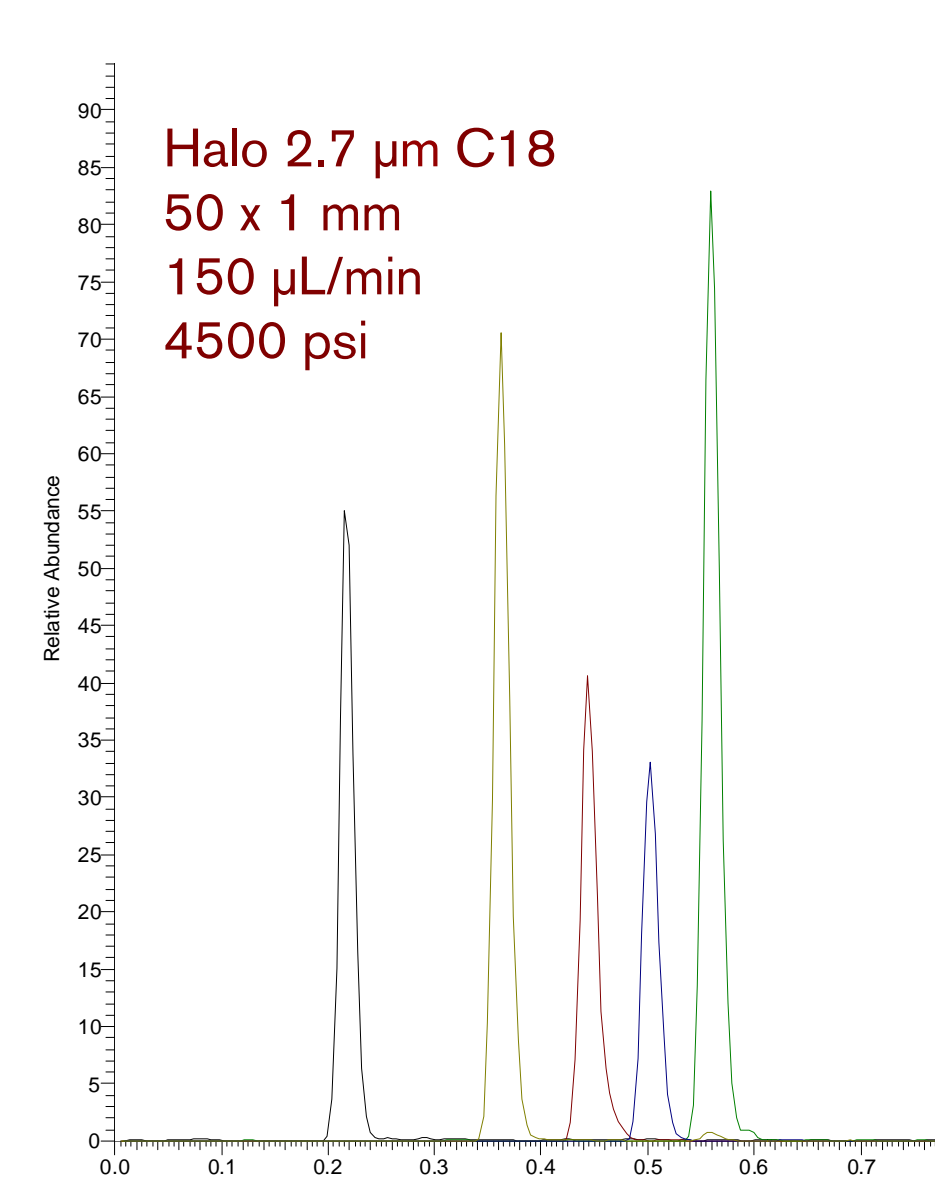
Fast LC/MS/MS using triple quadrupole mass spectrometers is used for many bioanalytical methods monitoring potential drug compounds. MRM monitoring of compounds in biological matrices is conducted in both discovery, preclinical, and clinical evaluation of compounds. Fast separations and cycle times allow for increased throughput to address an increasing sample load.

A typical measurement of three different compounds in precipitated rat plasma is shown below. Separations were conducted with a 1 mm x 50 mm column packed with 2.7 μm diameter HALO particles.



Analysis of terfenadine, alprazolam, buspirone in rat plasma

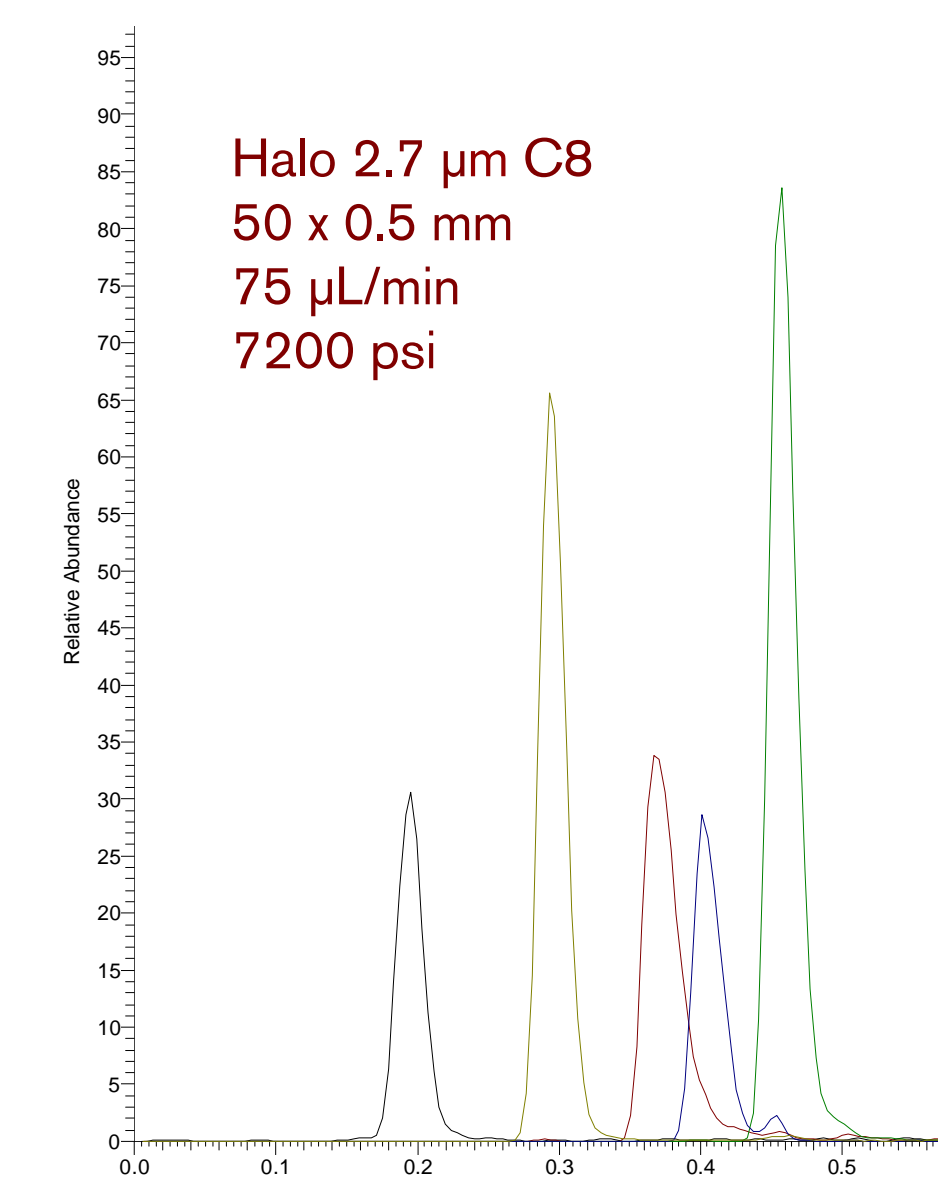
50 x 1 mm Halo C18 column
Water/ACN 0.1% FA gradient:
5% to 90% in 0.6 min
Flow rate = 150 μL/min; 2 μL injection
API 4000 Triple Quadrupole Mass Spec



Fast analysis of peptide mixtures
TSQ Quantum Access
5 μL injection of peptide mix
Column as shown
Water/ACN/0.1% formic acid

min	% B
0	10
0.9	50
1	90
1.5	90

Gly-Tyr: 239 > 136
Val-Tyr-Val: 380 > 136 / 235
Methionine Enkephalin: 574.5 > 277
Leucine Enkephalin: 556 > 397
Angiotensin II: 524 > 263 / 785



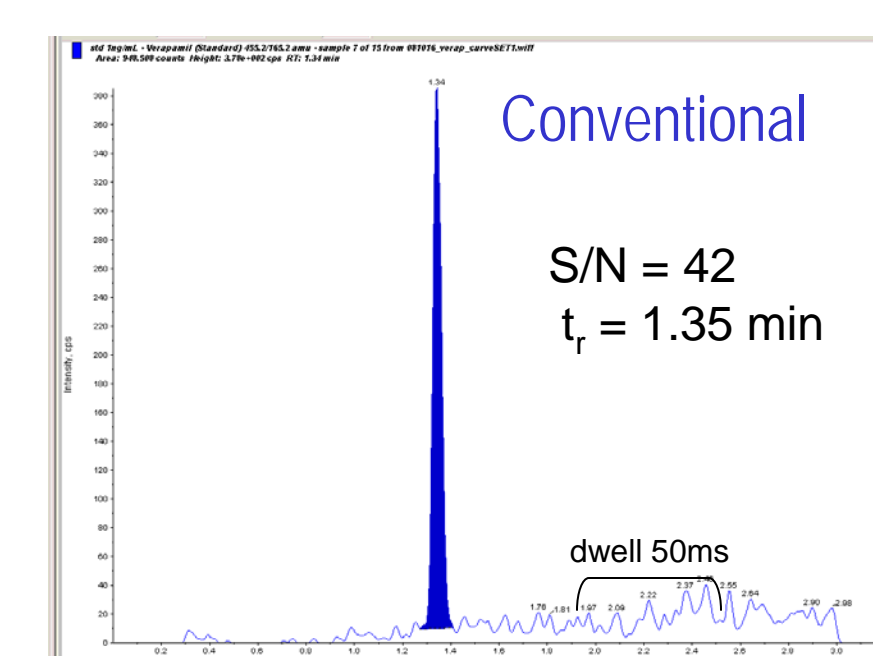
Halo 2.7 μm C8
50 x 0.5 mm
75 μL/min
7200 psi

Baseline separation of five peptides in just over 0.5 minutes demonstrating high chromatographic speed. On the left, the separation was conducted on a 1 mm ID Halo C18 column. On the right, the separation was conducted on a 0.5 mm ID Halo C8 column at ultra high pressures. A newly developed, high pressure active wash system is used to wash the injection valve, sample loop, and autosampler syringe. This allows one to maintain low carryover with cycle times less than 60 seconds.

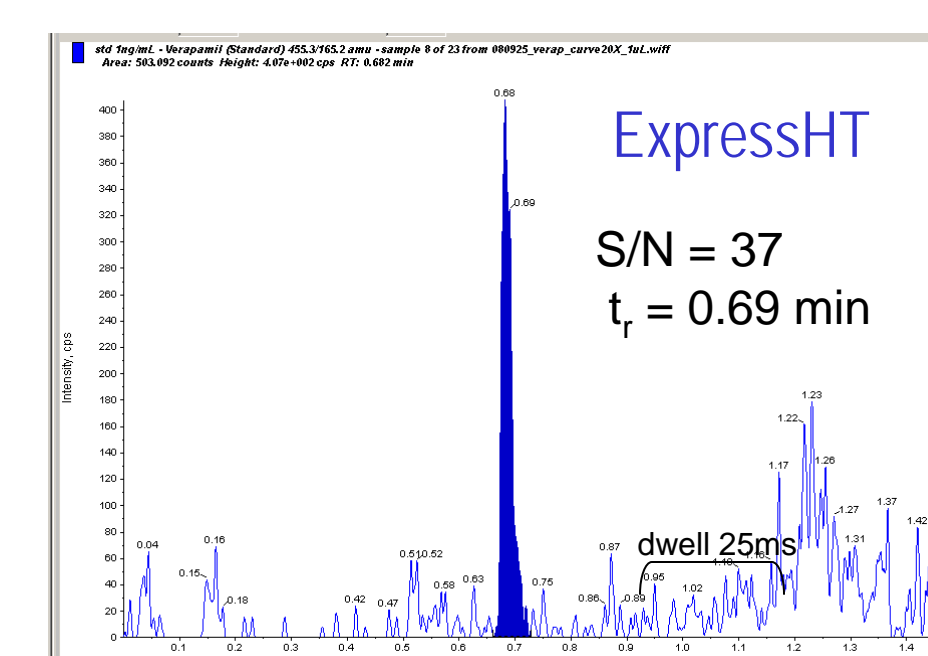
High performance and robust microLC for bioanalysis

	Haloperidol Retention Time (min)	Buspirone Retention Time (min)	Haloperidol Peak Area	Buspirone Peak Area
Mean	0.668	0.599	179905	7445
Std Dev	0.0007	0.0007	5205	342
% RSD	0.10	0.12	2.89	4.59

Retention times and peak areas from LC-MS-MS chromatograms for 250 injections of a two-component mixture extracted from rat plasma—haloperidol standard at 200 ng/mL and buspirone internal standard at 5 ng/mL. Both compounds were monitored by MRM.*



Flow: 500 μL/min; Injection Volume: 10 μL
Column: Halo RP C18, 2.7 μm; 50x2 mm



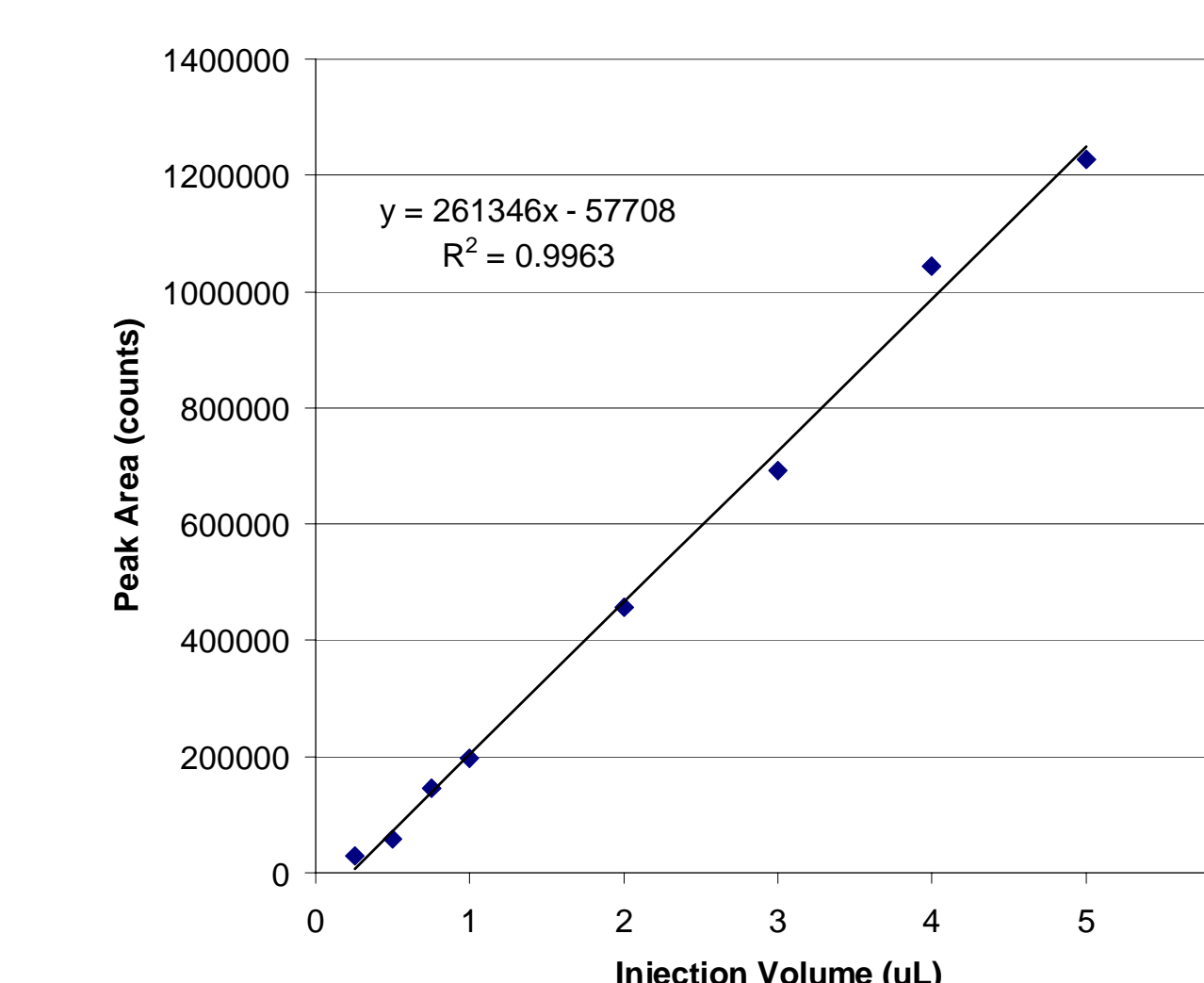
Flow: 150 μL/min; Injection Volume: 1 μL
Column: Halo RP C18, 2.7 μm; 50x1 mm

Comparable S/N ratios are measured comparing a more conventional separation using 2.1 mm columns and the ExpressHT-Ultra operating with a 1 mm diameter column. The separation on the ExpressHT-Ultra is conducted in half the time, with 1/10 of the sample volume, and >3x lower flow rates and solvent consumption. MS detection was conducted on an API 4000 equipped with a TurboV ESI interface. For the ExpressHT measurements, a newly developed low-volume ESI electrode assembly was used.*

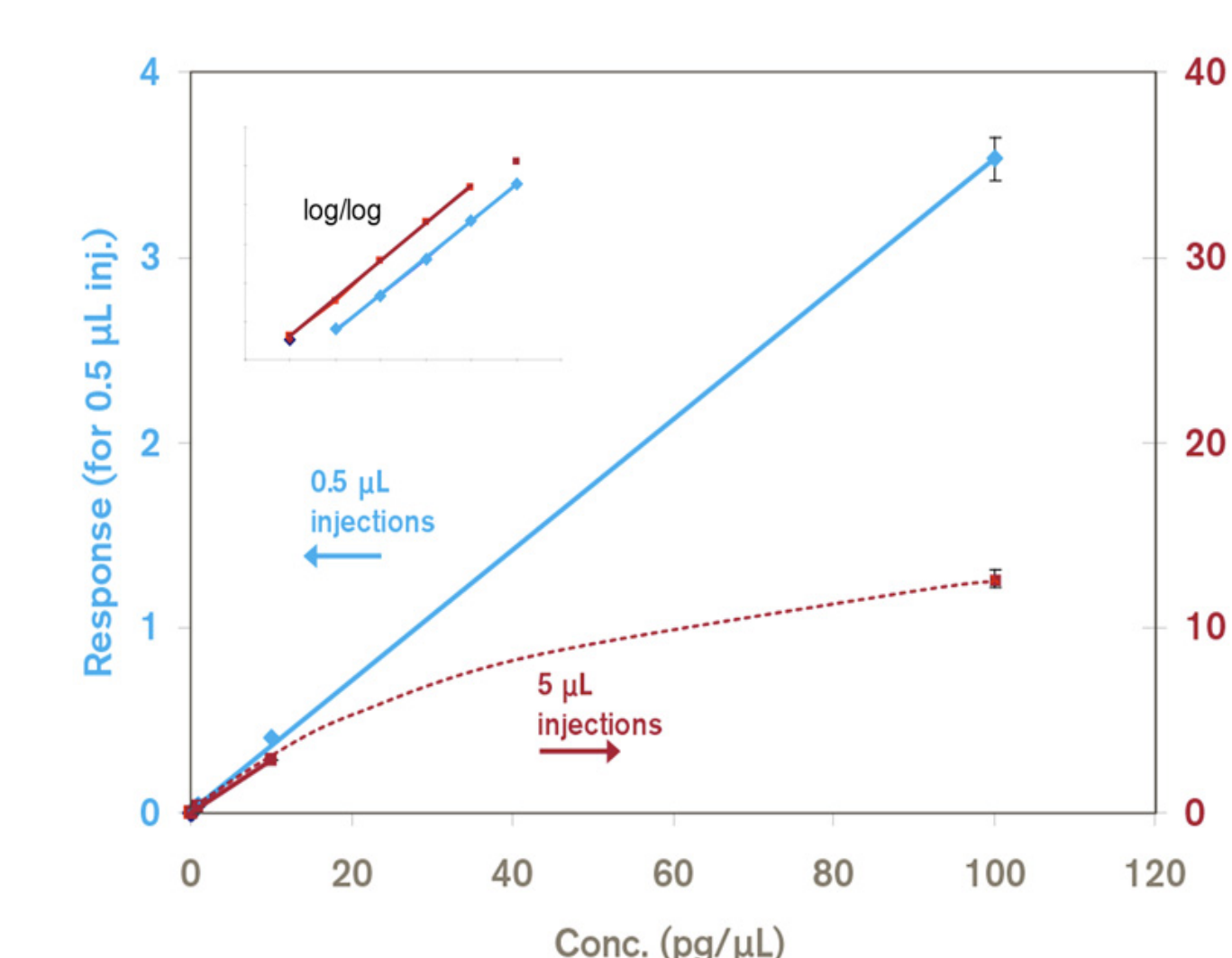
Variable injection volumes to extend quantitation

Precise and accurate pump flow control, in combination with a high speed injection valve, allow for programmable metered injections with volumes as low as 100 nL. The filled injection loop is switched to the inject position for a pre-determined length of time to displace the programmed amount of sample. This feature can be extremely useful in cases where the upper dynamic range of a linear calibration curve is insufficient to cover samples of unexpected high concentration. Instead of diluting the entire series of samples and re-analyzing them, one can simply re-inject the same samples using a smaller injection volume.

The data on the left shows the peak area as a function of injection volume using the metered injection approach. As illustrated at right, by reducing the injected volume from 5 μL to 0.5 μL, the upper linear dynamic range is effectively extended by an order of magnitude. There is no need for additional sample dilution steps or exchange of the injection loop.



Peak areas as a function of injection volume. The injection volume was changed using the metered injection approach described above. Data is shown for reserpine monitored by SRM transition (609.5 > 195.1). 15 μL of sample was used to load a 10 μL sample loop on the injector. Injections from 0.250 to 5 μL are shown. The peak areas are linear with injection volume over this range with an R² value of >0.99.



Calibration curves for haloperidol in 80/20 water/acetonitrile with verapamil as the internal standard. An ExpressHT-Ultra was used in conjunction with an ABI API 5000 triple quadrupole mass spectrometer. MRM transitions were m/z 376 > 165 and 455 > 165 for haloperidol and verapamil, respectively. The sample concentration range is from 1 fg/μL to 100 pg/μL, and the internal standard concentration is 3 fg/mL. Inset is a log/log plot of the data showing the linearity over the concentration range.

Conclusion

An ultra high throughput micro LC system for LC/MS analysis has been developed. The small mixing and delay volumes of the micro LC system provide fast separations and fast cycle times while affording significant savings in mobile phase and solvent usage. Excellent performance for bioanalytical methods using samples extracted from biological matrix (plasma) has been demonstrated, providing sensitivity, robustness, and reliability while using less than 10% of the solvent consumed by a conventional system. Variable injections using metered injections over >10x range of volumes have been demonstrated and provide opportunities for increased quantitation ranges without additional sample preparation.

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* Data provided by H. Skor, D. Gale, and R. Rahavendran, Pfizer Global Research & Development-La Jolla, CA