The Tempo™ ht LC System: *High Quality, High Throughput Small Molecule Quantitation at Capillary Flow Rates*

Overview

The Tempo ht LC system is a new front-end capillary LC system designed for rapid, high-resolution HPLC applications The system provides significant improvements in sensitivity over conventional LC systems when coupled to MS/MS detection. With the Tempo ht LC system, significant peak compression is achieved as a result of the high precision rapid gradient, which leads to improved S/N and better detection limits in LC/MS/MS quantitative applications. Along with an increase in sensitivity, the overall throughput of an analysis is significantly increased with a dramatic reduction in solvent consumption. A typical quantitation example is shown to detail the high quality and high throughput achievable with the Tempo ht LC front end.

Introduction

Higher sample throughput and increasing the information collected per analysis are key drivers for LC/MS/MS applications, especially in quantitation. An important component of an LC/MS/MS system's throughput is the time spent separating analytes chromatographically prior to detection. The chromatographic separation, which is frequently the bottleneck in the process, involves the adjustment of columns, mobile phase composition and flow rates, in order to achieve the desired separation while keeping in mind speed of analysis and sensitivity. Many combinations of the above mentioned factors can be used to achieve resolution, speed and sensitivity, each with their own pros and cons, which translates into longer method development time prior to the analysis of real samples.

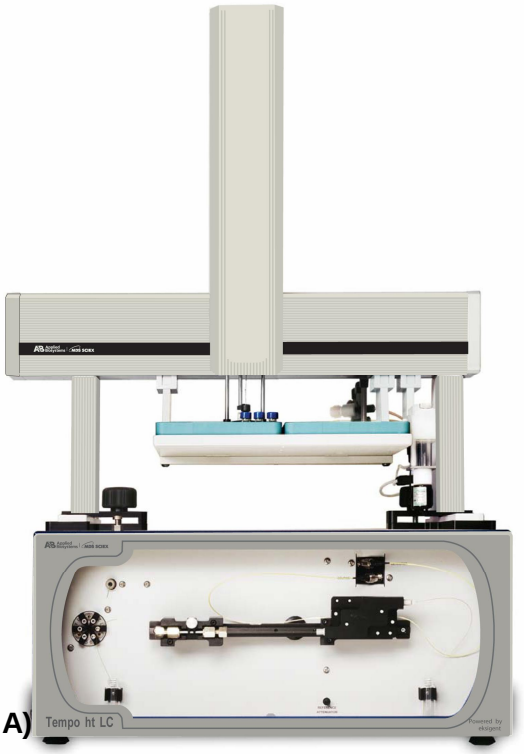
Historically, increases in sensitivity and separation capabilities have been demonstrated with nano-LC and capillary LC. These techniques are attractive for quantitative studies, especially when the sample volumes available for analysis are limited and increased sensitivity is required. However, both techniques are also viewed as requiring more sophisticated operation and show poor throughput capabilities due to long re-equilibration time of the column under gradient conditions. The flow regime required for nano-LC and capillary LC dictates that split-flow pumping principles are required for gradient delivery when conventional LC technology is used. This approach suffers from impaired gradient reproducibility and post-column band broadening and hence reduced separation efficiency and sensitivity

Here we describe a novel front-end capillary LC system, the Tempo ht LC, which combines advances in gradient delivery, flow control and sample injection for the quantitation of small molecules.

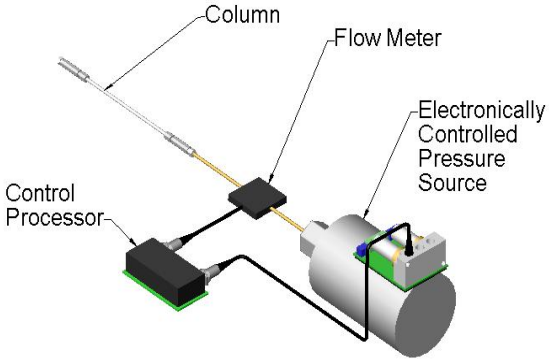
The Tempo ht LC system is used along with an API 3200™ mass spectrometer to demonstrate the ease of generating high quality, rapid separations for a multi-component analysis mixture of 8 benzodiazepines.

The Technology

Precise, accurate and reproducible gradients are critical for LC/MS/MS analyses, especially when comparing results from sample-to-sample, run-to-run, and lab-to-lab. The Tempo ht LC system (Figure 1) combines Microfluidic Flow Control (MFC) with continuous, independent flow rate feedback for each mobile phase to maintain precise flow rate control. This advanced flow control eliminates flow splitting and its inherent flow inaccuracies, resulting in more accurate and reproducible gradients and less solvent consumption. The entire line of Tempo LC systems use pressure modulated flow control as opposed to traditional reciprocating pumps, and require only one stroke per sample. There is no need for degassing or pulse damping.



A)



B)

Figure 1. A) The Tempo ht LC System is designed for rapid, high-resolution HPLC applications. The fully integrated system includes an autosampler (with a capacity of up to 4 plates), binary gradient pumps, injector, temperature controlled column compartment, and an array-based UV detection system. The system delivers stable gradient flows from 0.2 µL/min to 30 µL/min. **B)** Microfluidic Pump Control (MFC) technology. MFC works by continuously measuring the flow rate of each mobile phase. An embedded processor uses the flow rate measurements to control a rapid, electronically adjusted pressure source. Full gradients can be run as rapidly as 10 seconds with excellent reproducibility. MFC improves separation speed and flow stability.

Key Specifications

- Flow range: 200 nL/min to 30 µL/min
- Injection volume: 10 – 300 nL (standard). Higher injection volumes possible with larger sample loops

Materials and Methods

The test mixture consisted of eight benzodiazepines and a deuterated internal standard (see Table 1 for names and corresponding transitions monitored). Nine standards were prepared covering the concentration range from 5 pg/µL to 50 ng/µL as standard solutions. The LC/MS/MS system

consisted of the new Tempo ht LC system and an AB/MDS Sciex API 3200 mass spectrometer. Five replicate injections (0.3 µL) were made for each concentration level. Separation was achieved on an Eksigent Wakosil C18 (0.3 x 50 mm, 3µm) with an isocratic method (50:50 ACN:H_2O with 0.1% formic acid) at a flow rate of 30 µL/min and total run time of 1.0 minute. The analysis was performed with TurboIonSpray in positive ion mode. The Turbo V source incorporates a 30µm fused-silica transfer line from the Tempo ht LC system directly into a modified electrode of the TurboIonSpray probe (see Figure 2). For optimal performance, the distance from the end of the column to the mass spectrometer is minimized.

Analyte	MRM Transition
Alprazolam	309-281
Bromazepam	316-209
Clonazepam	316-270
Diazepam	285-193
Oxazepam	287-241
Flunitrazepam	314-268
Prazepam	325-271
Temazepam	301-255
ISTD	**MRM Transition**
Alprazolam-d5	314-286

Table 1. Test mixture of benzodiazepines and their corresponding MRM transitions monitored.

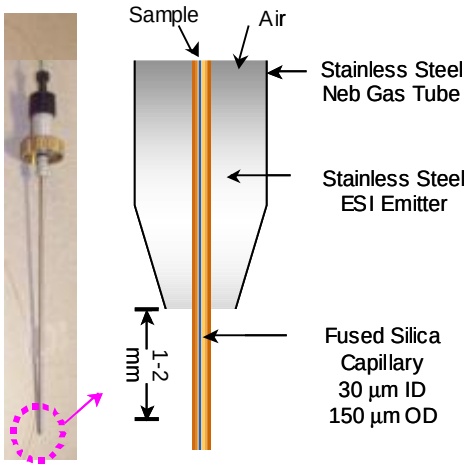


Figure 2. The Turbo V™ source incorporates a 30 µm fused-silica transfer line from the Tempo ht LC system directly into the TurboIonSpray® probe minimizing extra column volumes.

For comparison, conventional LC/MS/MS experiments at 250 µL/min through a BetaBasic C18, 2.1 x 50 mm, 3µm column and 2.0 mL/min through a Chromolith SpeedRod C18, 4.6 x 50 mm column were performed using a standard LC system.

Results

The Tempo ht LC system was evaluated on speed of sample analysis, chromatographic resolution (separation) and quality of data (sensitivity, reproducibility and linear dynamic range). Comparison against conventional chromatography showed several key advantages;

- ü shorter analysis times while maintaining resolution
- ü reduction in solvent consumption,
- ü better sensitivity (S:N)
- ü smaller sample consumption due to the smaller injection volumes possible.

Several examples of these are shown below.

Chromatographic Speed & Resolution

The benzodiazepine sample set was run under several conventional LC conditions, including 250 µL/min and 2.0 mL/min flow rates. Figure 3 shows the difference between the 250 µL/min flow rate method and that achieved with the new Tempo ht LC system at 30 µL/min.

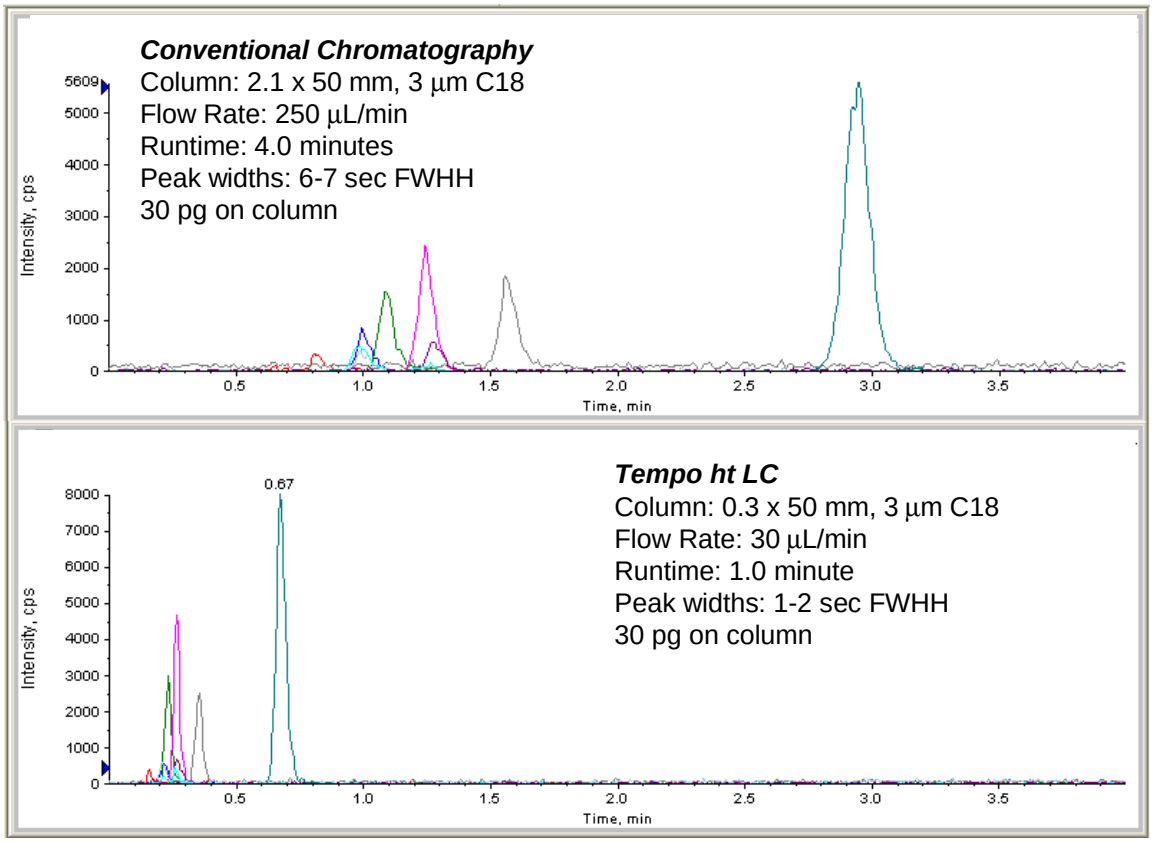


Figure 3. Conventional chromatographic separation techniques allowed a 4.0 minute runtime with sufficient separation from the void volume for the mixture of 8 benzodiazepines. Transferring this method to the Tempo ht LC system results in a 4x faster analysis time with better sensitivity, while maintaining chromatographic resolution and using >30x less solvent to do the separation.

Data Quality

Data quality is measured by a few, but important factors in quantitative LC/MS/MS applications; sensitivity of the technique, how reproducible the results are from injection-to-injection and the linear dynamic range of the analysis. As in most analyses, these factors are mainly a function of the front-end LC system employed to deliver the sample to the mass spectrometer. The LC system determines how fast the analysis can be done and how much solvent is required to complete the separation and delivery.

- ***Sensitivity and Reproducibility***

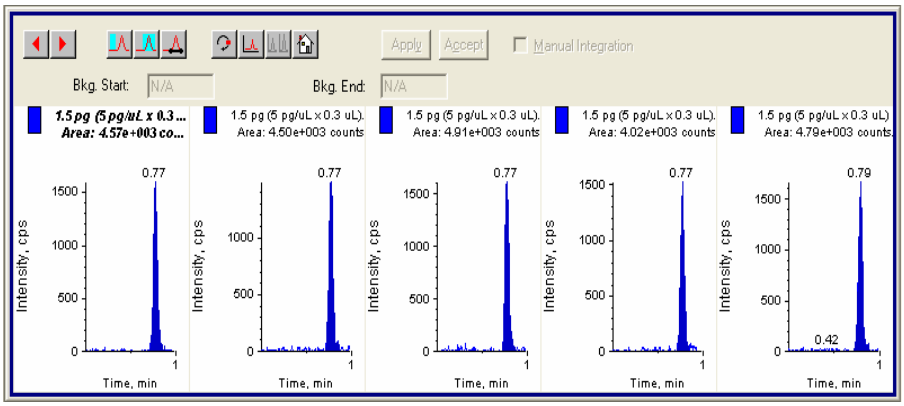


Figure 4. Five replicate injections of 1.5 pg on column of prazepam. The %CV for the five replicate injections is 10%. Based on this level, the estimated limit of detection (LOD) is between 250 and 500 fg on column.

- ***Linear Dynamic Range***

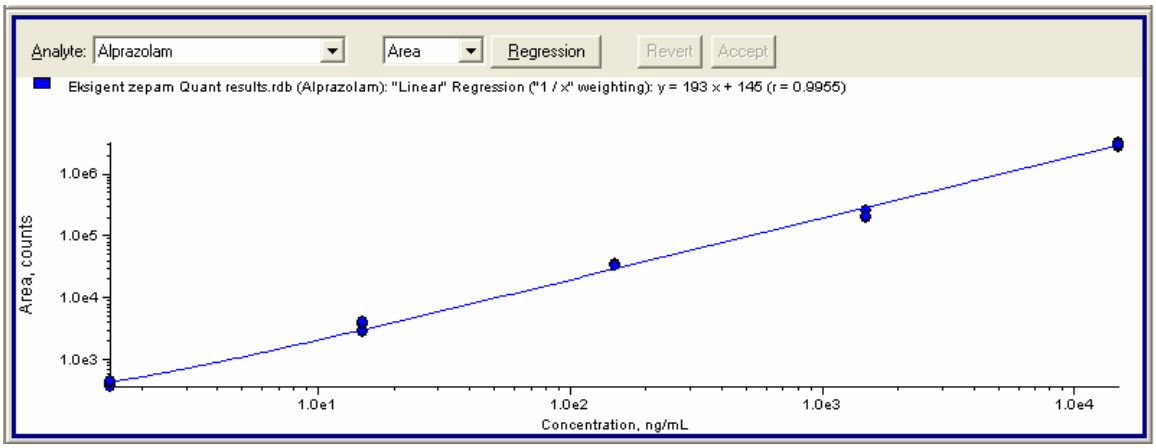


Figure 5. A sample calibration curve for alprazolam with 5 replicate injections at each concentration level. The linear dynamic range shown is 4 orders of magnitude on a log/log scale.

Table 2. Analysis time and consumables are significantly reduced using the Tempo ht LC system. For the high flow rate scenario, 2.0 mL/min were used with a monolithic column. While the runtime was cut in half compared to the 250 µL/min analysis, a significantly larger amount of solvent was used. The Tempo ht LC system exceeds the other two techniques in terms of analysis time and solvent usage.

	Tempo ht LC	Conventional LC	
		2.1 mm Column	*Monolithic Column*
Flow (µL/min)	30	250	2000
Analysis time (min)	1.0	4.0	2.0
Solvent consumption (µL)	30	1000	4000
# of Injection	35	35	35
Total solvent consumption (µL)	1050	35000	140000
Total solvent consumption (mL)	1.05	35	140
Total Analysis Time (min)	35	140	70

Conclusions

The Tempo ht LC system provides efficient, high-speed chromatography without the compromises associated with positive displacement pumps. Low volume, high efficiency gradient mixing and innovative flow control provide fast, accurate, and reproducible gradients to rapidly analyze multiple components in complex mixtures. Rapid, precise flow adjustment and minimum dead time increase analysis speed while decreasing solvent waste, significantly reducing running costs.

The Tempo ht LC system is ideal for fast, high-throughput quantitative LC/MS/MS applications.

- Maintains chromatographic resolution with high speed separation
- Excellent run-to-run reproducibility
- Better sensitivity through sharper analyte peaks (S:N) when compared to conventional separation techniques
- Extremely low solvent consumption
- Easy to set up and operate for high throughput quantitative studies