Abstract

In this technical note, the performance of PharmaFluidics µPAC™ capLC columns is compared to the current state-of-the-art in commercial capillary flow LC columns. The advantages for capillary flow reversed phase liquid chromatography analyses is discussed based on experimentally derived chromatographic parameters such as column pressure, retention factor and efficiency in both isocratic and gradient separation mode. Owing to their superior permeability and theoretical plate numbers, µPAC™ capLC columns are shown to outperform commercial capillary flow HPLC columns over the entire range between 1 to 10 µl/min.

Introduction

During the past decades, LC column technology has experienced significant technical progression with a clear trend towards the use of smaller packing particle sizes, decreasing column diameters and lower flow rates. Whereas decreasing the packing particle size mainly serves to increase separation efficiency, a variety of factors drive the trend to decrease the column diameter. Fundamental benefits when working with smaller diameter columns include reduced solvent consumption, reduced sample injection volumes, improved compatibility with electrospray ionization techniques and increased detection sensitivity as analytes elute at higher relative concentrations [1]. For this reason, LC-MS analyses that require high sensitivity with limited sample amounts are preferentially conducted within the nanoflow LC regime. Even though most technical hurdles in the routine implementation of nano LC have been tackled, capillary and microflow LC-MS solutions are recently gaining interest as the demand for large quantitative studies that require increased throughput and robustness is growing and the sensitivity of MS instrumentation has increased significantly [2]. By working at higher flow rates, the impact of gradient delay and sample loading volumes on the total analysis time can be reduced and the problems related to the low flow rate or small column dimensions (such as electrospray instability, column clogging or the presence of void volumes in the analytical flow path) can be minimized.

The term capillary (flow) liquid chromatography relates to the bore size of the columns that are used in this segment, with typical inner diameters of 150 to 300 µm. As such, these columns can typically be operated at flow rates ranging from 1 to 15 µl/min. As an alternative to the commercially available nanoflow LC micro pillar array column formats, the present technical note introduces a micro pillar array (µPAC™) based-solution for capillary flow LC-MS. In contrast to conventional LC columns that contain randomly packed beads as their stationary phase, micro-chip based pillar array chromatography columns have a separation bed of perfectly ordered and freestanding pillars obtained by lithographic etching of a silicon wafer. The regular mobile phase flow pattern through these micro-chip pillar array columns adds very little dispersion to the overall separation, resulting in better peak resolution, sharper elution and increased sensitivity [3]. The freedom of design also leads to much lower back pressure build-up and makes it possible to operate longer columns [4].
Experimental

The separation performance of 3 state-of-the-art packed bed (PB capillary flow LC columns (150 x 0.3 mm, obtained from 3 different vendors) and a 50 cm long µPAC™ capillary flow column were compared with a sample containing uracil as a non-retained marker and a mixture of alkylphenones. For every column, the separation performance was evaluated in both isocratic and gradient mode using gradient times between 5 and 60 min, and this in triplicate as illustrated. Column properties are listed in Table 1.

Table 1. Capillary flow HPLC column properties

<table>
<thead>
<tr>
<th>Column Flow column</th>
<th>Column diameter (mm)</th>
<th>Column Length (mm)</th>
<th>Column Volume (µL)</th>
<th>Particle/Pillar size (µm)</th>
<th>Particle type</th>
<th>Chemistry</th>
<th>Pore size (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 µm CS C18</td>
<td>0.3</td>
<td>150</td>
<td>10</td>
<td>3</td>
<td>Core-shell</td>
<td>C18</td>
<td>100</td>
</tr>
<tr>
<td>2 µm C18</td>
<td>0.3</td>
<td>150</td>
<td>10</td>
<td>2</td>
<td>Fully porous</td>
<td>C18</td>
<td>130</td>
</tr>
<tr>
<td>1.7 µm C18</td>
<td>0.3</td>
<td>150</td>
<td>9</td>
<td>1.7</td>
<td>Fully porous</td>
<td>C18</td>
<td>100</td>
</tr>
<tr>
<td>µPAC™ capLC C18</td>
<td>0.19*</td>
<td>500</td>
<td>11</td>
<td>5</td>
<td>Core-shell</td>
<td>C18</td>
<td>100-300</td>
</tr>
</tbody>
</table>

* Equivalent diameter was calculated from rectangular bed dimensions

Experimental set-up

All columns were installed in the column compartment of a Thermo Scientific™ Ultimate 3000 RSLC system and maintained at 35°C during the entire experiment. The capillary flow LC system was configured to perform direct injection of 4 nL sample plugs onto the column using an external 4 nL injection valve (VICI C4N-4004-004EUHA). All columns were operated at flow rates ranging from 0.5 to 10 µL/min. For the isocratic performance experiments mobile phases consisting 88.0, 79.9, 70.3 and 53.5% solvent B were used for the 2.0 µm, 1.7 µm, 3.0 µm and µPAC™ capLC column respectively. For gradient performance evaluation, linear solvent gradients from 45, 40, 40 and 2.5% solvent B to 97.5% solvent B were used respectively. Solvent A: 2% Acetonitrile, 98% Water, 0.1% Formic acid. Solvent B: 98% Acetonitrile, 2% Water, 0.1% Formic acid. UV detection was performed at 260 nm wavelength using a 3 nL flow cell for the isocratic measurements and a 45 nL flow cell for the gradient measurements.

Results

Column Backpressure Comparison

The pressure drop across an LC column is directly proportional to the column length, flow rate and mobile phase viscosity and is inversely proportional to the square of the particle size diameter and the square of the column internal diameter. For classical packed bed column formats with spherical particles, column manufacturers can roughly play around with three variables to define columns. These variables are, column cross-section, particle diameter and column length. As particle sizes are reduced, chromatographic resolution is increased at the cost of increased column backpressure. Figure 1 shows how the column backpressure of a µPAC™ capLC column clearly produces a smaller backpressure than the packed bed capillary flow LC columns. In accordance with the Kozeny-Carman equation, the pressure needed to operate a column with 1.7 µm diameter particles is roughly three times higher compared to that needed to operate a column with the same internal diameter and length, but packed with 3 µm diameter particles. Even though the capillary flow micro Pillar Array Column has a smaller cross section (equivalent to 190 µm diameter columns) and is 3.33 times longer, it can be operated at LC pump pressures that are considerably lower. This can be explained on the one hand by differences in interstitial porosity between both column formats and on the other hand by the fact that in a pillar bed the pillars never touch each other, thus leaving maximal space for an unobstructed flow. Unlike the slurry packing method used to manufacture packed bed columns, silicon microfabrication technology allows truly designing the stationary phase support morphology. The result is a 50 cm long pillar array column with an internal volume that is comparable to 15 cm long packed bed competitors but can be operated at significantly lower pump pressures.
Capacity factor

The retention for octanophenone has been determined experimentally at different mobile phase compositions and was plotted as a function of the percentage organic modifier (Acetonitrile) in the mobile phase (Figure 2). Retention was found to be the highest for the column packed with 2 µm diameter fully porous particles, followed by the 1.7 µm diameter fully porous particle column. For core shell type of stationary phase support materials (3 µm packed bed column and PharmaFluidics capLC µPAC™), only the outer layer of the particles or pillars is porous which results in lower retention capacity. It is important to note the lines in Fig. 2 run perfectly parallel, showing selectivities in the µPAC™ will be similar to that in the packed bed columns.

Isocratic performance

In order to compare the isocratic performance that can be obtained with different column formats, column dispersion measurements have been conducted at a fixed retention factor value. The mobile phase composition corresponding to a retention factor of 2.3 for octanophenone was used for all columns. UV chromatograms obtained for the separation of uracil, propriophenone, hexanophenone and octanophenone are shown in Figure 3.
Analyses have been performed over a wide range of flow rates (0.25–10 µL/min) and corresponding plate counts have been plotted as a function of the linear mobile phase flow rate for all columns and analytes (Figure 4). Plate height was calculated by dividing the column length by the amount of theoretical plates. The number of theoretical plates was calculated according to the European Pharmacopeia, which takes the peak width at half height and the retention time into account.

As a result of the reduced internal diameter (190 µm i.d.–equivalent compared to 300 µm), analyte bands will experience a much higher linear velocity for a given flow rate when injected onto the µPAC™ column. Elution profiles do however look similar as the total column volume is approximately the same. For the µPAC™ column, optimal chromatographic performance is achieved for all analytes at a linear mobile phase velocity of 1 mm/s, which corresponds to a flow rate of 1 µL/min. For the packed bed columns, there is a clear contrast in the performance that can be achieved for different analytes. For analytes that elute close to the void volume time of the column, considerably higher plate heights and relatively poor peak shapes are observed (Figure 3 chromatograms, peak 1,2,3). When comparing the performance that can be achieved for octanophenone, a clear trend towards lower plate heights and higher optimal linear velocities is observed for smaller packing particles. Because octanophenone elutes at a retention factor of 2.3, it is less affected by pre column band dispersion. For the 2 and 3 µm particle columns, optimal performance is achieved around 1.5 mm/s or 5 µL/min and for the 1.7 µm particle column, optimal performance is achieved around 2 mm/s or 6 µL/min. An entirely different perspective is created by plotting the actual number of theoretical plates as a function of the flow rate. Unlike the plate height, this value is representative for the actual peak width in the chromatograms. Up to 5 µL/min, significantly more theoretical plates (and thus sharper peaks) are obtained when working with the µPAC™ column. Going down to 2 or 1 µL/min, plate numbers as high as 100,000 actually can be achieved, whereas the maximum plate count that can be achieved for the packed bed column types does not exceed 30,000.

Figure 4. Column performance expressed as plate count (N) as a function of the flow rate for uracil, propriophenone, hexanophenone and octanophenone.
Gradient performance

In gradient elution mode, chromatographic performance is commonly expressed as the peak capacity $n_C$, a measure that takes both the elution window or gradient duration and the average peak width of the analytes into account [5]. Peak capacity represents the amount of peaks or analytes that can theoretically be separated within a certain time frame. The peak capacity that can be achieved for a mixture of analytes depends largely on the dispersion generated by the LC column, but is also affected by the gradient duration and the flow rate that is applied. A mixture of uracil and 8 alkylphenones was injected onto all column types and separated using linear solvent gradients ranging from 5 to 60 min. UV chromatograms obtained for a 40 min gradient separation at 5 different flow rates are shown in figure 5. Peak capacity curves for each flow rate are shown next to the chromatograms.

Figure 5. Left: Chromatograms obtained for gradient separation of uracil and 8 alkylphenones (each 250 ppm). UV detection using a 45 nL flow cell at 260 nm wavelength. Right: Peak capacity plotted as a function of the elution windows for all flow rates tested (2-4-6-8-10 µL/min).
Over the entire flow rate range and for all gradient lengths tested, the µPAC™ capLC clearly outperforms the classical packed bed alternatives. Even though the highest flow rate of 10 µL/min results in lower isocratic performance for the µPAC™ column, peak capacity values over 200 could be obtained for the longest gradients. For the packed bed alternatives, maximum peak capacity at this flow rate was between 135 and 170, with the highest value for the column packed with smallest particles. Whereas pump pressures close to 700 bar were needed to operate the 1.7 µm particle column, only 200 bar was needed for the µPAC™ capLC column. By reducing the flow rate systematically with steps of 2 µL/min, a steady increase in peak capacity up to 250 could be obtained for the µPAC™ column. On the other hand, this did not have a positive effect on peak capacity for any of the packed bed alternatives. On the contrary, peak capacity drops below 150 when going below 5 µL/min.

Figure 6. Peak capacity obtained for the separation of uracil and 8 alkylphenones at different flow rates with the µPAC™ capLC column. Top: Plotted as a function of the effective elution window. Bottom: Plotted as a function of the relative elution to void time (tE/t0).

When aiming for the highest peak capacity within a given time frame, each column has an ideal flow rate and gradient length combination. This is clearly illustrated in figure 6, where the peak capacity obtained with the µPAC™ column is plotted for all flow rates that were tested. When high throughput is needed with very short analysis times, flow rates above 8 µL are advised. Medium flow rates (8-4 µL/min) will give the highest peak capacity for separations that generate an elution window between 15 and 45 min and lower end capillary flow (≤ 2 µL/min) can be used to get maximum peak capacity for long gradient separations with elution windows beyond 45 min.

Conclusions

As an extension to the successful nanoLC µPAC™ formats, a capillary flow µPAC™ column is presented that offers unprecedented performance over a wide range of capillary LC flow rates (1–10 µL/min), and that given its low pressure-drop can even be used to flow rates up to 15 µL/min. Compared to packed bed alternatives that are designed to be operated in the same flow rate range and have similar column volumes, significantly lower pump pressures are needed. Theoretical plate heights as low as 5 µm can be achieved with this column, resulting in approximately 100,000 effective theoretical plates at a flow rate of 1 µL/min. The maximum number of theoretical plates that could be obtained with state-of-the-art packed bed columns was close to 30,000. When performing capillary flow gradient separations, superior peak capacity was observed for all flow rates that were tested (2–4–6–8–10 µL/min), and this for all gradient durations (5–60 min). Maximum absolute peak capacity can be obtained at low flow rates (≤ 2 µL/min) with long solvent gradients (≥ 45 min). However, when maximum peak capacity for a given analysis time is pursued, it is advised to match flow rate and elution window for optimal chromatographic performance.
**Key features of the µPAC™ capLC column**

**Flow rate flexibility:**
1 to 15 µL/min — corresponding column backpressures of respectively 19 and 300 bar — maximum operating pressure is 350 bar.

**Column robustness:**
Each column has been manufactured by etching channels out of a solid piece of silicon, the column is perfectly bidirectional and contains no particles nor frits.

**Column to column reproducibility:**
Each column is manufactured using the same lithographic mask, making every column identical.

**Separation performance:**
Peak capacity values above 200 can be obtained with short (30-90 min) gradient separations.

**References**


μPAC™ driven separations – Better by Design

Conventionally LC columns are fabricated by stacking (packed beds) or depositing (monoliths) material into a capillary. PharmaFluidics’ μPAC™ technology (micro Pillar Array Column) is unique in its kind as it is built upon the precise micromachining of designed chromatographic separation beds into silicon. This approach brings along three crucial and unique characteristics:

**Perfect Order.**
μPAC™ beds are designed with a high degree of order, eliminating heterogeneous flow paths otherwise present in conventional columns (so called Eddy dispersion). Flow through μPAC™ columns adds very little dispersion to the overall separation. As a result, peaks remain sharper and sensitivity is increased.

**High Permeability.**
μPAC™s operate at moderate pressures, typically lower than 300 bar. Separation channels with exceptional length (50 cm to 200 cm) are therefore possible. These are folded onto a small footprint by a interconnecting concatenating bed segments.

**Solid Backbone.**
The micromachined backbone of the separation bed forms a rigid structure that is not influenced by pressure. There are no obstructions by touching surfaces, and there is no risk for perturbations by pressure fluctuations.