At the end of the 1970s, HPLC reached a first level of maturity as a method for instrumental analysis. Breakthroughs at the base of this progress were the availability of columns with stable microparticulate packings of the reversed-phase type, and the development second- and third-generation pumps, autosamplers, and spectrophotometric detectors. These modules were much more robust and rugged than the first-generation instrumentation that appeared in the early part of that decade. At that time, the optimal internal diameter for an HPLC column was regarded to be \( \geq 4.0 \text{ mm} \) (normal-bore columns). A number of papers in 1980\(^1\) gave comprehensive reviews of the state of the art in HPLC.

In a landmark paper,\(^2\) Knox outlined the performance characteristics of packed and open-tubular columns with much smaller internal diameters.\(^2\) He also described instrumental requirements with regard to band spreading, sample injection, and detection volume. The main conclusion of his work was that, provided the dispersion requirements in the system are met, HPLC columns narrower than 1 mm i.d. will be as efficient as columns with \( \geq 4.0 \text{ mm} \) i.d. In addition, Knox concluded that the permeability of very narrow columns is higher when the ratio of tube-to-particle diameter decreases. In more recent work, Knox has expanded his findings.\(^3\)

In addition to these theoretical considerations, narrow- and therefore low-volume columns have advantages because of minimized solvent usage, more sensitive detection in sample limited cases, and less demanding coupling requirements and higher sensitivity with mass spectrometric detectors. However, for practitioners of HPLC in the 1980s and ‘90s, it became clear that commercial instrumentation was compromised by constraints in physics (especially optics) and manufacturing technology. Therefore, in general, separation efficiency and concentration sensitivity were less when narrow-bore columns (3 mm i.d.) were used than with larger-diameter columns. Cost, labor, and analysis time benefits were marginal or not relevant (e.g., solvent usage). Most importantly, narrow columns established an impression for lack of robustness and ruggedness, were more prone to fouling, and in general were more difficult to operate. Therefore, the use of narrow-bore columns was not established as a routine methodology and has found application only in some important niche areas.

In this article, the authors describe a capillary HPLC system that offsets the aforementioned shortcomings of older systems. Properties of capillary HPLC columns for this system are discussed, and applications in particular in combination with MS are given.

### Nomenclature for HPLC columns

The ambiguous description of HPLC columns with regard to their diameter confuses practitioners of HPLC. Terms such as “microbore” and “capillary columns” are used without reference to a general norm. Therefore, the designation of an HPLC column according to its diameter and consequently type of chromatography as used in this article is given in Table 1. It would be beneficial for communication among chromatographers if this ambiguity could be removed by adhering to this (or similar) proposal as a de facto standard.

In Table 1, the internal diameter and typical flow rate range for operation of a particular column type

<table>
<thead>
<tr>
<th>Description</th>
<th>Diameter</th>
<th>Typical flow rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Open-tubular liquid chromatography</td>
<td>( \leq 20 \mu \text{m i.d.} )</td>
<td>(&lt;50 \text{ nL/min})</td>
</tr>
<tr>
<td>Packed capillary liquid chromatography</td>
<td>( \geq 50 \mu \text{m} &lt; 1.0 \text{ mm i.d.} )</td>
<td>(0.2-100 \mu \text{L/min})</td>
</tr>
<tr>
<td>Microbore column liquid chromatography</td>
<td>( \geq 1.0 \mu \text{m} \leq 2.1 \text{ mm i.d.} )</td>
<td>(100-500 \mu \text{L/min})</td>
</tr>
<tr>
<td>Small (narrow)-bore column liquid chromatography</td>
<td>( \geq 2.1 \mu \text{m} &lt; 3.9 \text{ mm i.d.} )</td>
<td>(500-1500 \mu \text{L/min})</td>
</tr>
<tr>
<td>Normal-bore column liquid chromatography</td>
<td>( \geq 3.9 \leq 5 \text{ mm i.d.} )</td>
<td>(1.5-5 \text{ mL/min})</td>
</tr>
<tr>
<td>Semipreparative column liquid chromatography</td>
<td>( &gt;5 \text{ mm i.d.} )</td>
<td>(&gt;5 \text{ mL/min})</td>
</tr>
</tbody>
</table>
are given. The system described in this paper falls under the category of “Capillary column liquid chromatography,” and consequently will be designated as capillary HPLC system.

**Requirements for a capillary HPLC system**

The first step in defining the instrumental requirements for an HPLC system is the selection of the major field of application of the system and the corresponding column. Addressing recent trends in the HPLC market and/or customer needs is also paramount.

There is no doubt that the current pressure on the pharmaceutical industry is to reduce time and costs for the development of new drugs and therapies. This has spurred progress in the development of high-throughput instrumentation for (parallel) synthesis, screening, and analysis. The improvements can be achieved only with a simultaneous significant reduction in scale of operation. The value chain in the (bio)pharmaceutical industry—unraveling the molecular base of disease, new drug discovery, drug development, clinical trials, and manufacturing—is characterized by the necessity to handle increasing numbers of smaller samples for screening and analysis (e.g., drug metabolism pharmacokinetic [DMPK] studies).

Furthermore, the postgenomic era has begun. Proteomics involves the comprehensive, quantitative measurement of the expressed protein complement in a cell or tissue under different conditions (e.g., normal versus tumor cell). Automated handling and analysis of minute amounts of proteins obtained from a 2-D polyacrylamide gel electrophoresis (PAGE) plate will be required.

These developments have such common requirements as handling of very small samples, high sensitivity, high throughput (96- or 384-well plate compatibility), and seamless coupling with mass spectrometric detection.

With these requirements and the general considerations in mind, it was obvious to opt for an HPLC system able to operate packed capillary columns (0.3–1.0 mm i.d.). From that selection, dimensions of such a system with regard to abilities of pumps, injection device, column thermostat, and detector can be derived. The real challenge for development, however, was to take a major step forward in ruggedness and robustness of such a system without compromising overall performance to change the negative perception of capillary HPLC systems indicated above.

Therefore, the objective was set to develop an HPLC system on a modular basis that is able to run HPLC columns of 0.3–1.0 mm i.d. with similar functionality, performance characteristics, robustness, and ruggedness, i.e., one that a practitioner in the field expects from a system that runs normal-bore HPLC columns. The system should be easy to set up, use, validate, maintain, diagnose, and repair, with high day-to-day reproducibility, high reliability, uptime, and high sample throughput running packed capillary columns that have uncompromised performance and longevity.

The resulting system is shown in Figure 1. Individual requirements for the modules and their realization are detailed in the following paragraphs.

**Capillary HPLC pumping system**

With the diameter of the columns for the capillary HPLC system fixed, one can calculate the volumetric flow rates required to operate these columns at the mobile phase velocities typical for HPLC columns (1–6 mm/sec). In practice, the maximum velocity is constrained by the maximum pressure at which the pump can deliver the solvent viz., 400 bar. The result of this calculation is given in Figure 2.
The calculations were done with basic chromatographic equations for pressure drop as a function of velocity. In Figure 2, the horizontal arrows show the volumetric flow rate range for a particular column diameter with the assumptions given in the figure caption. Thus, for example, for a column with 0.3 mm i.d., a typical flow rate range of 3–18 µL/min is needed. The overall flow rate range for packed capillary columns according to Table 1 will typically be 1–100 µL/min, with the capability to extend this range below 1 µL/min and above 100 µL/min, also allowing the occasional usage of wider i.d. columns. For the practitioner, it is desirable that this flow rate range (1–100 µL) be well controlled and independent of the column backpressure (unlike in passive split-flow arrangements). Delay volume/time in a capillary pump must be low and allow effective mixing so that solvent gradients are delivered to the column quickly, accurately, and precisely. These goals are achieved with an 1100 series high-pressure binary gradient pump equipped with electronic flow control (Agilent Technologies), as illustrated in Figure 3.

**Figure 3** Schematic representation of the electronic flow control in the 1100 series capillary HPLC system.

An electromagnetic purge valve (EMPV) splits the solvent delivered by the high-pressure pump in a controlled manner. This valve has an outlet (waste) with an adjustable flow restrictor and an outlet that comprises the microflow path, containing a flow sensor that measures the flow rate. In practice, two separate sensors are used for two different capillary flow rate ranges—one for 1–20 µL/min and one for 10–100 µL/min. A feedback control loop regulates the flow resistance in the waste outlet in order to maintain a constant microflow rate. For example, when the pressure decreases on the column, flow rate increases, which is counteracted by a reduction in the flow resistance in the waste path. In this way, the capillary pump will be insensitive to pressure variations induced by solvent change such as in gradient elution or by a gradually plugging column.

Since the flow measurement sensor response depends on the solvent composition, highly sophisticated instrument control is needed. Time delays from the mixing point of the high-pressure gradient pump to the sensor need to be known exactly to account for the response change when the solvent composition is varied in this pump.

The flow rate before the EMPV is at normal values of 0.2–1.0 mL/min (primary flow rate). Therefore, the delay time for a new solvent composition to the flow sensor and the capillary column is low, despite the relatively high delay volume (0.5–0.8 mL) in this part of the pump. The total dead volume from the sensor to the head of the column is 5 µL for the 1–20 µL/min flow rate sensor. With the 10–100 µL/min flow sensor, the volume is 14 µL (because slightly wider connection capillaries are used in this case).

The pumping performance of the pump is given in Figure 4. Typical values for the precision of retention times at a flow rate of 4 µL/min is <0.2–0.3%. After run 24, the backpressure was increased by 80 bar by placing a restriction capillary after the detector. As can be seen, there is barely a significant change in the absolute retention time and precision under the simulated higher backpressure conditions.

With this system, depending on the actual flow sensor installed, one can operate between 1 and 20 µL/min or 10 and 100 µL/min with a controlled rate. It is this flow rate control that warrants the required robustness and ruggedness of the pump, and gives confidence that the microflow rate specified is actually delivered independent of the backpressure. If one wants to operate at higher flow rates, which are actually within the specified range of the 1100 series binary high-pressure gradient pump, the flow sensor can be bypassed and normal mode selected to allow a flow rate up to 2.5 mL/min.

The pump is equipped with a two-way selection valve for each pump channel and a seal wash option. Moreover, the system is supplied with an on-line degasser that has a low volume (1 mL). Through the use of new, more effective gas exchange membranes, the degassing efficiency is the same as that found with larger-volume solvent degassers.

**Figure 4** Flow rate precision during repetitive injection at different column backpressures. Column: Hypersil ODS (Thermo Hypersil Ltd., Runcorn, Cheshire, U.K.), 5 µm, 150 × 0.3 mm; flow rate: 4 µL/min; solvent: water/acetoneitrile; gradient: 5–85% acetoneitrile column; sample: isocratic checkout sample; injection volume: 0.1 µL; temperature: 25 °C; detection wavelength: 250 nm. Pressure increased from 80 to 160 bar after run 24.
Table 2  Overview of capillary HPLC pump system

<table>
<thead>
<tr>
<th>Requirement</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precise gradient delivery of 1-99%</td>
<td>Solvent delivery at higher primary flows; split with flow measurement</td>
</tr>
<tr>
<td>backpressure</td>
<td>control of column flow through feedback control loop</td>
</tr>
<tr>
<td>Accurate microflow rate delivery</td>
<td>Flow measurement and feedback control</td>
</tr>
<tr>
<td>independent of solvent mixing</td>
<td></td>
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<tr>
<td>contraction and backpressure</td>
<td></td>
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<tr>
<td>Small delay volume/time for fast</td>
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<tr>
<td>gradients and uncompromised gradient</td>
<td></td>
</tr>
<tr>
<td>profile</td>
<td></td>
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<tr>
<td>Full pressure range (0–400 bar)</td>
<td></td>
</tr>
<tr>
<td>available for microflow delivery</td>
<td></td>
</tr>
<tr>
<td>Robustness and ruggedness comparable</td>
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<tr>
<td>to conventional HPLC systems;</td>
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<tr>
<td>user-friendly handling of</td>
<td></td>
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<tr>
<td>connections, minimal plugging,</td>
<td></td>
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<tr>
<td>and diagnosis of microleaks</td>
<td></td>
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</tbody>
</table>

The requirements and features of the 1100 series capillary HPLC pump are summarized in Table 2.

**Capillary HPLC system aspects**

In order to maintain the column’s separation efficiency, a reduction in column diameter mandates a reduction in the sample injection volume and amount of solute applied to the column. The reduction factor equals the ratio of the squares of column diameters. For example, a 5-µL injection on a 4.6-mm-i.d. column is equal to a 60-nL injection volume on a 0.5-mm-i.d. column. Therefore, an autosampler for a capillary HPLC system will need to be able to inject samples down to the nanoliter scale with good precision.

Conversely, the gain in detection sensitivity in capillary HPLC is achieved when the few available microliters of sample is injected on a very low-volume column, sacrificing efficiency for gain in detection sensitivity (Figure 5). In the figure, 0.1 µL of a sample containing 200 ppm biphenyl is injected on columns with decreasing i.d., illustrating a limited sample volume case. It is obvious that the highest sensitivity is obtained with the narrowest i.d. column.

This is of particular importance in many life science and biopharmaceutical applications, since only microliter-sized samples are available. Therefore, it is also a requirement for a capillary HPLC autosampler to inject a relatively large sample volume (up to several microliters) from small-volume containers with high recovery. In combining these requirements, the 1100 series capillary HPLC autosampler allows injection of sample volumes of a range of 3 orders of magnitude, i.e., from 30 nL to 40 µL.

Moreover, reduced volumes and optimized flow geometries of all connections and capillaries are required. This will be discussed in the next section. The sample injection valve for the capillary injector has been re-worked to minimize dead volume and optimize flow paths. Wear resistance of the rotor/stator assembly has been significantly improved for high sample throughput. Even more importantly, though, is the ability to handle multiple 96-(384-) well plates, which can be thermostated. Since such plates provide very high sample numbers, the ability to overlap the sampling cycle with the separation cycle is indispensable.

A typical example that reflects the precision achievable with sample injection at a 0.1 µL sample injection volume level is given in Figure 6.

On the detection side, one is again confronted with the demand to minimize detection flow cell volume and maximize pathlength for highest solute band integrity and response. Moreover, spectral integrity must be maintained with diode array spectrophotometric detection. This demands the use of flat detection cell windows. With these considerations in mind, a square, Z-shaped detector flow cell (0.7 × 0.7 × 10 mm) was developed similar to the high-sensitivity detection flow cell for the Agilent Technologies capillary electrophoresis system. Despite the cell volume (0.5 µL), it provides favorable flow characteristics, conserving the peak integrity of packed capillary columns.

**Connection capillaries**

The connection capillaries are the main contributors to extra-column band spreading in an HPLC system. Especially in a modularly built instrument, governed by spatial and physical requirements of the individual modules, a relatively long length of connection tubing is required. This is contrary to the necessity to minimize the dead volume in a system that operates with packed capillary HPLC columns. Fortunately, hydrodynamics helps to cope with this contradiction.
The volume variance is then given by:

\[ \sigma_{v,tot}^2 = \sigma_{v,col}^2 + \sigma_{v,ext}^2 \]  

where \( \sigma_{v,col}^2 \) is the total volume variance of the solute band, \( \sigma_{v,col} \) is the variance of the column itself, and \( \sigma_{v,ext}^2 \) is the variance of zone broadening in the extra-column volume.

Zone dispersion in an HPLC column is described by the well-known van Deemter equation, given in its simplified form in Eq. (2). \( H \) is the height equivalent to a theoretical plate; \( u \) is the solvent velocity; and \( A, B, \) and \( C \) are regression coefficients.

\[ H (u) = A + \frac{B}{u} + Cu \]  

The coefficients in the van Deemter equation are independent of the column diameter (assuming that the bed structure is independent of the column diameter). If the column diameter is reduced and the solvent velocity is kept constant by reduction of the volumetric flow rate by the square of the column diameter ratio, the same plate height is obtained in the narrower column. The volume variance is then given by:

\[ \sigma_{v,col}^2 = \frac{V_b}{N} \frac{\varepsilon_i V_{col} (1 + k' H (u))}{L_{col}} = constant_1 \cdot d_i^4 \cdot \frac{H (u)}{L_{col}} \]  

where \( V_b \) is the retention volume of the solute, \( N \) is the plate number, \( \varepsilon_i \) is the total porosity of the column, \( V_{col} \) is the geometric volume of the column, \( L_{col} \) is the column length, and \( k' \) is the capacity ratio. The dispersion in the connection capillary is given by the well-known Aris-Taylor equation. Neglecting the static diffusion contribution in this equation and supposing that only the connection capillaries contribute to external band spreading, the volume variance due to extra-column band spreading can be described by:

\[ \sigma_{v,ext}^2 = \frac{\pi \cdot d_m^4 \cdot F \cdot L_{cap}}{96D_m} = constant_2 \cdot d_{cap}^4 \cdot L_{cap} \cdot u_{cap} \]  

where \( d_m \) is the diameter of the connection capillary, \( F \) is the volumetric flow rate, \( L_{cap} \) is the length of the connection capillary, \( D_m \) is the diffusion coefficient of the solute, and \( u_{cap} \) is the solvent velocity through the capillary.

Suppose one is comparing two cases: a column with 4.6 mm i.d. with a total length of connection capillaries of \( L_{cap} \) and a column with 0.5 mm i.d. with the same length of connection capillary. To maintain the same linear velocity through the column, the flow rate will be reduced by the square ratio of the column diameter, i.e., by a factor of 84. In that case, the volume variance of the solute band decreases by the same factor (Eq. [3]). Suppose the diameter of the connection capillary is proportionally reduced by the ratio of the column diameters, i.e., from 0.25 to 0.027 mm, which leads to the same velocity, \( u_{cap} \) at the 84 \times lower flow rate in the capillary than the variance of the band spreading (Eq. [4]) reduces with a factor of almost 7200. Thus, the effect of external band spreading on overall band spreading in Eq. [1] is negligible in this case.

With these conditions in mind, it becomes clear that the internal diameter of the connection capillaries can be reduced less than the column diameter is reduced i.e., by half the ratio of the column diameter reduction. In this practical case, the connection capillary i.d. is 0.05 mm. Now, the band spreading contribution by the capillaries reduces by a factor of 625 compared to a peak variance reduction of 84.

There are additional advantages. First, the solvent velocity in the capillary is lower, leading to lower variance of extra-column band spreading (Eq. [4]). Second, a 2 \times lower pressure drop over the same length of connection capillary under comparable conditions is obtained.

However, these considerations are not very practical when stainless steel connection (SST) capillaries are used. Due to fabrication limitations, very narrow-i.d. SST capillaries have high wall roughness and are prone to plugging. Plastic capillaries have limitations with concentricity of the tubing and will not withstand high pressure with organic solvent, especially at high temperatures. Fused silica would be the material of choice, but does not have good robustness. In the authors’ approach, the best properties of fused silica and plastic tubing were combined by heat shrinking a PEEK outside tube onto fused-silica capillaries with the required internal diameter. The heat shrinking process generates an intimate connection to the poly-
imide outside coating of fused-silica tubing, avoiding formation of small annulus space between the tubes or movement of the inside tube. In this way, the capillaries (0.8 mm o.d.) combine the robustness and ruggedness of plastic capillaries with the favorable properties of fused silica (very smooth inner wall and therefore very low tendency to plugging). The availability of these capillaries is essential to the ability of this system to run packed capillary HPLC columns that are uncompromised by external band spreading, while providing good robustness and ruggedness.

Columns for capillary HPLC systems

Stationary phases

At the beginning of the 1970s, microparticulate reversed-phase-type stationary phases became available and were a major factor in establishing HPLC as a routine analysis method. Nevertheless, these initial stationary phases were far from ideal in handling important compound classes such as basic molecules and biologically important high-molecular-weight substances like peptides, proteins, oligonucleotides, and nucleic acids. Workarounds were conceived, but through the years, especially in the late 1980s and early ’90s, a more fundamental approach in stationary phase development began. This has led to largely improved properties of column packings such as highly uniform and homogeneous surface silanol structure, low metal ion content, wider pore structure, and more stable (wider pH range) stationary phase bonding. In this respect, the ZORBAX range of stationary phases has been expanded to comprise a series of stationary phases for optimal usage at a particular solvent pH to match the chromatographic properties of the solutes. The basis for the enhanced stability of these newer stationary phases is based in the type of bonding that has been applied. An overview of the bonding chemistry of the phases is given in Figure 7. The optimal working pH range for the phases is shown in Table 3.

The StableBond series has good stability at low pH since two bulky isopropyl or isobutyl groups on the lateral silicon atom sterically protect the siloxane bond against hydrolysis and degradation. This approach is also used in the Bonus RP series where, in addition, a polar group (PG) is embedded in the alkyl side chain. This provides good wetting of the stationary phase at very low or no organic solvent content in the mobile phase. In addition, the embedded group provides for good peak asymmetry with highly polar and ionizable solutes by minimizing unwanted interaction with unreacted acidic silanol groups. The Eclipse XDB series has an exhaustively endcapped silica surface that gives solid protection of the silica backbone against basic hydrolysis. Therefore, the operating pH range is shifted up higher than the StableBond series. The best stability at high pH is provided by the bidentate silane modification used by the ZORBAX Extend series. The ring-like structure of the reacted bidentate silane, with a hydrophobic carbon chain between the silanizing atoms, effectively shields the silica surface from hydrolytic attack. With this approach, the operating pH range can be extended from 2.0 to 11.5. Working at high pH has particular advantages for basic compound and peptide separations, as will be shown later.

Recently, Kirkland et al. reported results, with a composite particle shown in Figure 8. This superficially porous particle, POROSHELL, has an impervious core. On the outside of the particle, a 0.25-µm

Table 3

<table>
<thead>
<tr>
<th>ZORBAX phases</th>
<th>Recommended pH range</th>
</tr>
</thead>
<tbody>
<tr>
<td>StableBond</td>
<td>C18, C8, C5, CN, phenyl</td>
</tr>
<tr>
<td>Bonus RP</td>
<td>Polar alkyl</td>
</tr>
<tr>
<td>Eclipse XDB</td>
<td>C18, C8, phenyl</td>
</tr>
<tr>
<td>Extend</td>
<td>C18</td>
</tr>
</tbody>
</table>

Figure 7 Chemical structure of StableBond, Eclipse XDB, Bonus RP, and Extend binding of alkylsilanes to silica surface.
porous layer is constructed by deposition of colloidal silica particles on the surface. Bonding chemistries, as described above, can be applied to this porous layer. In this way, particles are obtained that have an interparticle space of a 5-µm particle but a very short intraparticle diffusion path. Such particles are preferably operated at very high mobile phase velocity with macromolecules. Columns with POROSHELL have the same backpressure as columns packed with 5-µm particles, but separation efficiency like columns packed with very small particles. An example of a separation with a packed capillary column with POROSHELL will be given later.

**Column hardware**

The surface finish of the inner wall of normal- and small-bore column tubing has to be very smooth. In very narrow tubes such a surface finish with stainless steel tubing is difficult to obtain. Therefore, glass-lined, steel tubing (0.3 and 0.5 mm i.d.) was selected for the capillary HPLC columns. Frits are troublesome in very narrow columns. The columns used by the authors are terminated with metal sieves (2 µm). In order to meet dead volume requirements in the fittings, standard zero dead volume unions are used. The overall O.D. of the column is ¼ in. to provide robustness to the tube.

Many combinations of stationary phase type (StableBond, Eclipse XDB, Bonus RP, Extend, and Hypersil), type of bonding (C18, C8, phenyl, and cyano), particle size, diameter, and length can be conceived.7 Efficiency of packed capillary HPLC columns

To illustrate the efficiency that can be achieved with capillary HPLC columns, a height equivalent to a theoretical plate (HETP) versus mobile phase velocity curve was recorded. The result is given in Figure 9 and Table 4. The particular column used in the test was a ZORBAX Eclipse XDB C18, 3.5 µm with 150 mm length and 0.5 mm i.d. The regression coefficients were calculated with Origin™ (Originlab Corporation, Northampton, MA). A few striking conclusions can be drawn from these results. This column had equal, or better, efficiency than a corresponding 4.6 mm i.d. with the same phase. The calculated value of minimum in the H-u curve was about 7 µm or a reduced plate height of 2. This result was also obtained for the last retained peak, showing that in the

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7 From the plot of velocity versus pressure, a column resistance factor of approx. 350 was calculated.
capillary HPLC system, external band broadening is negligible. In particular, this finding underlines Knox's prediction from 1980 that column efficiency is not dependent on column i.d. Overall, the results illustrate that packed capillary HPLC columns with 0.5 mm i.d. can be used without compromise in this instrument.

A second interesting observation can be made. The column resistance factor, which is the chromatographic permeability of the column, normalized for the particle size and calculated from the pressure/velocity plot, is approximately a factor of two lower than for normal-bore columns. Again, this is exactly in line with predictions by Knox. (This is actually a great benefit because one needs a factor of two lower pressure to drive the solvent at a particular velocity in a packed capillary column compared with a normal-bore column.) With this finding in mind, one can prepare a plot of total pressure (column + connecting capillaries) versus flow rate (Figure 11). This plot reveals that even at the highest velocity that would make sense chromatographically, a pressure of less than 400 bar is required to drive the solvent through both the column and the connection capillaries. The combination of the new pumping concept, the relatively wider i.d. of the connection capillaries, and the higher permeability of packed capillary columns together allow the full pressure range of the pump to be exploited for solvent delivery.

Application examples

As a first example (Figure 10), a very short packed capillary column was used to demonstrate speed and reproducibility at a very low flow rate. The column was a 35 × 0.3 mm column, packed with 3 µm Hypersil ODS. The flow rate is 20 µL/min, which equals 6.7 min/sec. Twelve repetitive injections are shown of 0.1 µL of a sample containing seven homologous parahydroxybenzoic acid alkyl esters (n = 1–7) and thiourea as a dead time marker. The gradient was 50–90% in 2 min with a 0.5-min hold and 1.5-min column reequilibration time. Sample injection was in the overlap mode (i.e., the next sample is taken in the injection needle while the previous sample is still eluting). An overall cycle time of 4 min is possible in this case. Precision of retention time of all peaks under these conditions is 0.1–0.4% RSD and of raw peak areas is 2–5% RSD. This is an illustrative example and does not represent the ultimate limit in speed of analysis with this system.

In the second example (Figure 12), repetitive injections of a tryptic digest of myoglobin are shown. The column used in this case was 250 × 0.3 mm and packed with ZORBAX 300SB-C18, 5 µm. This stationary phase is specifically designed for the separation of peptides with TFA containing solvent gradients. The main trace shows the overlay of 10 repetitive injections. The RSD of the retention time over these 10 injections is designated on top of the peaks. Since the time variations are so minor, the 40–41 min region has been expanded to illustrate the actual span of retention time for these peaks (0.16 min).

In Figure 13a, an example of the benefit of running a peptide separation at high pH is compared with the same separation attempted under acidic conditions (Figure 13b). The ZORBAX Extend bonding chemistry provides good resistance of the column at high solvent pH. Therefore, ammonia-containing mobile phases can be used. At the high pH, the charge state of a peptide changes radically, leading to different chromatographic selectivity. A column with a stationary phase that tolerates both low and high pH allows orthogonal separations to be carried out without changing the column. Moreover, ammonia is advantageous in coupling with...
mass spectrometry. As a volatile additive, it does not pose any problem to be removed in the MS interface.

The data in Figure 13 were obtained on a 2.1-mm-i.d. column packed with ZORBAX 300 Extend C18. In Figure 13a, the separation is shown with an acidic TFA containing mobile phase. In Figure 13b, the same separation is shown at high solvent pH. The difference in selectivity is remarkable and plausible. Angiotensin II and III differ in just one amino acid—aspartic acid (see Table 5). Thus, at low pH, the overall charge in angiotensin II and III will be about the same and (accidentally) the retention is the same. At high pH, however, the additional, aspartic acid moiety produces a higher charge on the molecule than angiotensin I and III and thus is less retained.

The use of ammonia containing mobile phases significantly increases the signal response and reduces the background signal and noise partly because of the low molecular weight of the additive ammonia. However, the complete basis for the advantage of ammonia-based mobile phases in LC-MS studies has not yet been determined.

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The sample was a mixture of angiotensin I, II, and III. In Figure 13a, the separation is shown with an acidic TFA containing mobile phase. In Figure 13b, the same separation is shown at high solvent pH. The difference in selectivity is remarkable and plausible. Angiotensin II and III differ in just one amino acid—aspartic acid (see Table 5). Thus, at low pH, the overall charge in angiotensin II and III will be about the same and (accidentally) the retention is the same. At high pH, however, the additional, aspartic acid moiety produces a higher charge on the molecule than angiotensin I and III and thus is less retained.

The use of ammonia containing mobile phases significantly increases the signal response and reduces the background signal and noise partly because of the low molecular weight of the additive ammonia. However, the complete basis for the advantage of ammonia-based mobile phases in LC-MS studies has not yet been determined.

In Figure 14, the separation of a peptide mixture is shown by packed capillary HPLC. Column: ZORBAX Poroshell 300SB-C18, 150 × 0.5 mm; solvent: 0.1% TFA in water/ 0.1% TFA in acetonitrile; gradient: 5–65% in 15'; flow rate: 20 µL/min; sample: peptide mixture, about 20 ppm; injection volume: 0.1 µL; detection wavelength: 214 nm; temperature: 25 °C. Peaks identified in figure.

Table 5

<table>
<thead>
<tr>
<th>Structures of angiotensin I, II, and III</th>
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<td>Angiotensin I</td>
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<td>Angiotensin II</td>
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<td>Angiotensin III</td>
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</table>

Figure 12 Separation of a tryptic digest of myoglobin. Column: 0.3 × 250 mm ZORBAX 300SB-C18, 5 µm; flow rate: 5.5 µL/min; solvent A: 0.05% trifluoroacetic acid (TFA) in water, solvent B: 0.045% TFA in acetonitrile; gradient: 1–61% 0.5% B/min; sample myoglobin tryptic digest: 7.5 pmol/µL; injection volume: 1.3 µL; temperature: 25 °C; detection wavelength: 214 nm.

Figure 13 Comparison of peptide separation at high and low pH. Column: ZORBAX Extend C18, 150 × 2.1 mm; solvent: 0.05% trifluoroacetic acid (TFA) in water, solvent B: 0.045% TFA in acetonitrile; gradient: 1–61% 0.5% B/min; sample angiotensin I, II, and III, 50 pmol each; injection volume: 2.5 µL; flow rate: 0.2 mL/min; temperature: 35 °C; MS detection: positive ion electrospray ionization (ESI); fragmentor voltage: 70 V; capillary voltage: 4.5 kV; nebulizing gas: N2, 35 psi, 12 L/min; tip temperature: 325 °C; top acidic elution: 0.1% TFA in water/0.085% TFA in 80% acetonitrile; bottom basic elution: 10 mM NH4OH in water/10 mM NH4OH in 80% acetonitrile.

Figure 14 Fast peptide separation by packed capillary HPLC. Column: ZORBAX Poroshell 300SB-C18, 150 × 0.5 mm; solvent: 0.1% TFA in water/ 0.1% TFA in acetonitrile; gradient: 5–65% in 15'; flow rate: 20 µL/min; sample: peptide mixture, about 20 ppm; injection volume: 0.1 µL; detection wavelength: 214 nm; temperature: 25 °C. Peaks identified in figure.
porous layer particles have a higher surface area than nonporous particles; therefore, higher sample loading is permitted. By virtue of their structure, such particles are very strong and can be made in principle in a variety of particle sizes, porosities, and thicknesses of the layer and bonding chemistry. With this particular stationary phase, “300” refers to the average pore diameter of the silica layer; “SB-C18” refers to the StableBond type of binding of the C18-silane to the surface.

In this case, the flow rate used was 20 µL/min, which is regarded as low flow velocity (2.4 mm/sec) for this column. The separation of the five peptides has a much improved efficiency compared with the separation of the mixture on a normal ZORBAX 300SB column. The potential of the POROSHELL stationary phases has yet to be defined.

In a final example given in Figure 15, the separation of a HAEIII restriction enzyme digest of pBR322 plasmid DNA is given. In this case, a prototype column, 75 x 0.5 mm, was prepared with the ZORBAX Eclipse dsDNA stationary phase. Typical conditions for this separation are the use of a triethylammonium acetate/water/acetonitrile gradient. The separation obtained is again comparable in all aspects with this separation when executed with a 2.1- or 4.6-mm-i.d. column.

Conclusions

This paper demonstrates that capillary HPLC at a flow rate from 1 to 100 µL/min with columns of 0.3 and 0.5 mm i.d. meets the requirements for a routine analytical separations method. An innovative concept allows the solvent in this flow rate range to be delivered in a controlled manner, making the solvent delivery system rugged and robust. A significant reduction in the diameter of the connection capillaries was achieved with PEEK-covered fused-silica capillaries that are far less prone to plugging. The autosampler has been devised to deliver samples down to a volume of 30 nL. An optimized flow path and low-volume detection cell provided the best compromise between sensitivity and band spreading.

Column technology of ZORBAX phases has advanced in a comparable fashion. Consequent considerations of the bonding chemistry have expanded the working pH range of silica-based stationary phases to 1–11.5. The advantage of working at the high pH range with ammonia as a mobile phase additive is significant with respect to the orthogonal selectivity obtained when working at low pH. Porous layer-type silica modified by the same new bonding chemistries, POROSHELL is very attractive for the high-efficiency, high-speed separation of peptides and proteins.

Finally, a number of examples have demonstrated that noncompromised capillary HPLC systems are feasible, addressing specifically those analyses in which very low sample amounts/volumes are inherent to the problem.

References


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