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Short three-micron columns

Applications in high-speed liquid chromatography

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Columns for liquid chromatography are becoming smaller, that is, both shorter in length and packed with smaller particles. In the early 1970s, meter-long pellicular columns were rendered obsolete seemingly overnight by the emergence of 10-μm microparticulate columns. For almost a decade, 25–30 cm columns packed with 10-μm particles dominated the LC market. By the late 1970s, however, as a result of continuing developments in column technology, the more efficient 5-μm microparticulate column made its debut. Packed in lengths from 12.5 cm to 25 cm, the 5-μm column generated 10,000–20,000 theoretical plates, which contributed to its rapid rise in popularity. The transition from 10-μm to 5-μm columns, however, was less complete. The maturing LC market, based almost entirely on the standard 10-μm packings, was more reluctant to change.

With the advent of 3-μm microparticulate silica in 1980, the disparity in column performance became substantial. The inherently higher efficiency of 3-μm particles (150,000 plates/m for 3-μm versus 40,000 plates/m for 10-μm) permits the use of much shorter columns (3–5 cm), which are as efficient as the standard 10-μm columns and have analysis speeds that are as much as an order of magnitude faster. Many practicing chromatographers are impressed with the possibilities of increased laboratory productivity using these columns; however, many remain skeptical about their practicality with respect to precision, stability, column lifetime, and instrument compatibility. In this article, the authors address some of these important factors by reviewing the past developments and present status of high-speed LC.

HIGH-SPEED LC: AN HISTORICAL PERSPECTIVE

The concept of performing fast chromatography using columns packed with very small particles is not new. In 1941, A.J.P. Martin and R.L. Synge postulated that "the smallest HETP should be obtained by using very small particles and a high pressure difference across the length of the column" (1). In the following decades, this concept of increasing LC performance by reducing particle size was expanded and refined through theoretical treatments by Halász (2,3), Guiochon (4), and Knox (5,6). By the mid-1970s, it became increasingly evident that the practical optimum particle diameter (d_p) for high-speed LC was less than 5 μm (2–6). The actual commercial implementation of particles smaller than 10 μm was slow, however, because of considerable technical problems. Nevertheless, reports on experimental 3-μm particles by Halász (2), Kirkland (7), Unger (8), and Cooke (9) in the late 1970s demonstrated that it was feasible to produce these particles — a possibility which generated substantial interest. In 1980, one of the first commercially available 3-μm columns was introduced, which demonstrated an efficiency of 160,000 plates/m (10). Other research groups have since reported studies on various aspects of these columns (11–17).

By 1980, the deleterious effects of extracolumn dispersion had become quite obvious. Until this time, experimentalists had to design and implement modifications for their existing LC systems to minimize extracolumn dispersion. The successful use of 3-μm particles had finally pressured instrument manufacturers into redesigning their instruments based upon bandwidth considerations. With increased availability of this improved equipment, further reductions in column length to increase analysis speed could now be accomplished. In two recent articles, Majors observed that more than 13 companies had introduced short 3-μm particle columns (3.3–7.5 cm long) by the time of the 1984 Pittsburgh Conference (18,19).

THREE-MICRON PARTICLES FOR HIGH-SPEED LC

The 3-μm particle is the smallest high-quality packing material currently available in sufficient quantities for commercial use. Theory dictates that, for the same efficiency at optimum flow rates, the analysis speed is inversely proportional to the square of the particle diameter (20); therefore, even smaller particles are of interest. When using particle diameters of less than 3 μm, however, adverse effects such as viscous heating (21) and column permeability (22) might become more of a problem. Further studies in these areas are required to fully understand the nature of the problems associated with these particles.

One way to compare the chromatographic performance of packings of different particle size is by calculating the number of plates/m and plates/sec (11). Table I lists these comparative data for typical commercial prepacked columns.

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TABLE I: PERFORMANCE LEVELS OF 10-μm, 5-μm, AND 3-μm COLUMNS

Particle Diameter, d_p (μm)	Typical Lengths (cm)	Plates/Col	Plates/Meter	Plates/Sec*
10	25–30	10,000–12,000	40,000	50–100
5	12.5–25	10,000–20,000	80,000	200–300
3	3.3–15	4,950–22,500	150,000	300–600

*For peaks at low k' using a low-dispersion instrument (11).

The reason that 3-μm particles are more useful for high-speed applications is also quite evident from examining the curves of height equivalent of a theoretical plate (HETP) versus flow rate for representative 3-, 5-, and 10-μm d_p columns (Figure 1). Several points become obvious from the interpretation of these curves:

- smaller particle diameter yields lower HETP; thus, there are more plates per unit column length
- the optimum flow rate is higher for smaller particles; this observation is interpreted as a reduction in analysis time
- the slope of the van Deemter curves (the C terms) decreases as particle diameter decreases; thus, there is less loss of efficiency when operating above the optimum flow rates with smaller particles.

These factors illustrate that small particles are better for fast analyses (provided that particles with the proper chromatographic properties can be manufactured reproducibly in a narrow size range).

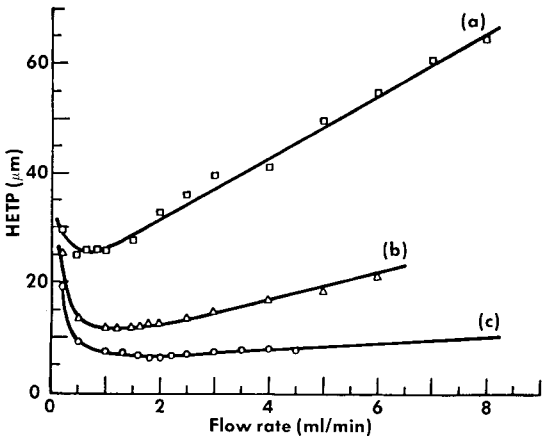

FIGURE 1: HETP versus flow rates for representative (a) 10-μm, (b) 5-μm, and (c) 3-μm columns. Columns: (a) 250 mm x 4.6 mm, 10-μm P-E Anal C18; (b) 125 mm x 4.6 mm, 5-μm P-E HS/5 C18; (c) 100 mm x 4.6 mm, 3-μm P-E HS/3 C18; mobile phase: 65% CH_3CN/H_2O; temperature: 22 °C; sample: *t*-butylbenzene.

APPLICATION OF HIGH-SPEED COLUMNS

Experimental: The column used in this study was a 33 mm x 4.6 mm column (P-E 3x3; Perkin-Elmer Corp., Norwalk, Connecticut) packed with 3-μm C18 bonded-phase support. The average efficiency of this column is greater than 5000 theoretical plates (corresponding to 150,000 plates/m or a reduced plate height of about 2.1 d_p). The support material was bonded using octadecyldimethylchlorosilane and was fully endcapped. It had a carbon loading of 11%, a surface area of 100 m^2/g, and an average pore diameter of 100 Å.

The LC system consisted of a liquid chromatograph (P-E Series 4) equipped with either a model 7125S injection valve or a Rheodyne 7510 injection valve, a spectrophotometric detector (P-E LC-85B), and a laboratory computing integrator (P-E LCI-100). The detector was

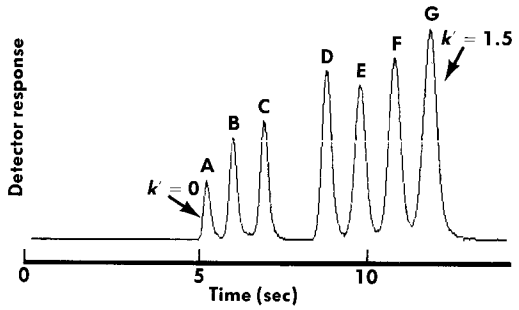

FIGURE 2: An example of high-speed LC under mild flow conditions. Column: P-E 3x3 C18; mobile phase: 90% CH_3CN/H_2O; flow rate: 3.5 ml/min; pressure: 1800 psi; detection: UV 254 nm. Peaks: A = uracil (*N* = 2980), B = phenol (*N* = 3411), C = nitrobenzene (*N* = 3555), D = toluene (*N* = 3609), E = ethylbenzene (*N* = 3663), F = *n*-propylbenzene (*N* = 3874), G = *t*-butylbenzene (*N* = 3959).

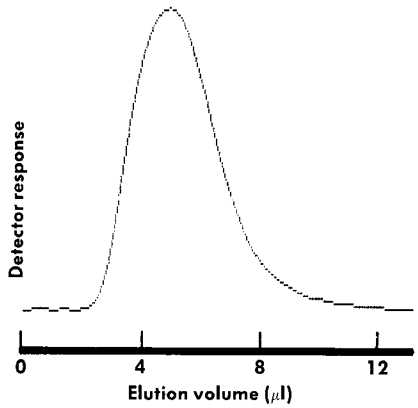

FIGURE 3: A trace of IBW for a modern LC system. Chromatographic conditions described in text, except detector had a 1.4-μl flow cell and data acquisition rate was 200 points/sec.

used with either a 1.4-μl or an 8-μl flow-cell assembly, each having a 6-mm path length. For applications with fast gradient analyses, a P-E Series 10 LC system was used with a 0.2-ml high-speed mixer. For automated sample injections, a P-E LC-600 autosampler was used. For instrumental bandwidth measurements using the central moments method, data were digitized through a P-E chromatographic interface followed by statistical calculation using a BASIC program in a P-E Model 3600 data station.

Chromatography in seconds using short columns: A chromatographic analysis of seven alkylbenzenes performed on a high-speed LC system is shown in Figure 2. This chromatogram illustrates the performance levels achieved with short columns. Note the following points:

- all seven components are well resolved in just 13 sec under fairly mild flow rate (3.5 ml/min) and pressure conditions (1700 psi or 12 MPa)
- using the first peak (uracil) as a column void marker, all peaks elute between k' 0–1.5
- about 4000 plates were observed at k' of 1.5 at about twice the optimum flow rate
- about 600 plates/sec were generated for the first peak.

Obviously, this type of fast LC analysis is very desirable to both analytical chemists and laboratory managers. It provides a means of addressing the ever-increasing sample load common to many analytical facilities; however, analysis time is not the only important criterion. Fast analysis is practical only if other desirable characteristics such as precision, flexibility, column lifetime, and sensitivity can be maintained.

Criteria of instrumental design to minimize extracolumn dispersion: Short columns packed with 3-μm particles inherently generate sharp and fast-eluting peaks. The first peak in Figure 2 (uracil), for instance, has a peak width of 0.5 sec or a peak volume of about 30 μl. Most liquid chromatographs in use today have instrumental bandwidths of 75–150 μl (12) and are not designed with these small-volume peaks in mind.

Instrumental bandwidth (IBW) of an LC system can be measured by replacing the column with a zero dead-volume union and recording the peak base width of a small-volume injection (11). Dispersive effects in the sample flow path (the injector, connection tubes, and detector flow cell) contribute to extracolumn dispersion.

Instrumental bandwidth can be reduced by miniaturizing these components and improving the flow characteristics. The contribution of each component in the LC system to the total IBW has been studied in depth (7,13). In general, the dispersive effect of the detector flow cell is the most important factor. By redesigning the injector and the flow cell, further reductions in IBW can be effected. Figure 3 illustrates the IBW trace of such an LC system with a total bandwidth (4 σ calculated by the second moments method) of 5.8 μl at a flow rate of 2.0 ml/min.

Figure 4 illustrates the detrimental effect of IBW on the observed column efficiency. The chromatogram on the right, however, was obtained by replacing the 1.4-μl detector flow-cell assembly with an 8-μl flow-cell assembly. This substitution increased the total IBW from 6 μl to 83 μl. Consequently, degradation of efficiency and peak heights was observed for early-eluting peaks; for example, the observed plate count of the first peak (uracil) degraded from 2000 to 300 plates. The later-eluting peaks, however, suffered less deleterious effects because of much larger peak volumes; for example, the plate count of the last peak, *t*-butylbenzene (k' = 9) was decreased from 5300 to 4800 plates. Because the same analysis time is maintained in both cases, Figure 4 shows that even on a nonoptimized system, the short column might, in many cases, yield fast and adequate separation.

Typical instrument characteristics necessary for high-speed LC using very short columns are summarized below. Compromises are made between bandwidth requirements and practical chromatographic considerations such as convenience and sensitivity (11) (based on an LC system capable of maintaining 80% of the efficiency of a well-packed 33 mm x 4.6 mm 3-μm column at k' = 3).

- LC system: IBW < 20 μl
- Pump: pulse-free, low delay volume for fast gradients
- Injector: low bandwidth, flow bypass to eliminate pressure surges
- Tubing: short lengths of 0.005–0.007 in. i.d.
- Detector: low bandwidth flow cell, response time <200 msec; long path lengths to maintain sensitivity
- Data handling: data acquisition rates >10 pt/sec.

Future column geometries might require different and more stringent specifications for instrumentation.

In order to circumvent the problems associated with extracolumn band broadening so that 3-μm columns can be made more compatible with existing instrumentation, some column manufacturers advocate the use of a wider

bore high-speed column with an inner diameter of 6 mm or larger (23). Although the idea is plausible, several important benefits that result from the smaller column void volumes, such as solvent savings and mass sensitivity, are not realized.

HIGH-SPEED LC: BENEFITS

Fast analysis speed: The consequence of using short columns packed with 3-μm particles and operated at moderate flow rates (2–4 ml/min) is fast analysis, typically 15–120 sec for isocratic analyses. Figure 5 shows several examples of this fast isocratic analysis capability in the clinical and pharmaceutical areas. Figure 5a illustrates the separation of eight common barbiturates in 80 sec; an analgesic tablet is separated in 100 sec in Figure 5b; and Figure 5c illustrates a theophylline analysis in 45 sec.

Another advantage of short columns is the ability to increase peak resolution by going to very high capacity factors (k') using low solvent-strength conditions. Using a mobile phase of 50% CH_3CN/H_2O, four antimicrobials (methylparaben to butylparaben) are baseline-resolved in about 20 sec (Figure 6a). When the mobile phase composition is decreased to 20% CH_3CN/H_2O, butylparaben elutes at a k' value of 134 in 10.5 min. Note that peak shape and column efficiency were well maintained at these very high k' values. The resolution between propylparaben and butylparaben in Figure 6b, however, is increased from 3.4 to 10.8. This approach of decreasing solvent strength is often an effective means of increasing resolution (20). The short column makes this approach attractive by allowing the attainment of very high k' values in a reasonable time and with adequate detectability.

The low void volume of short columns also facilitates solvent changeover during gradient analysis or methods development. Assuming, for example, that a mobile phase volume of five times the column void volume is required to equilibrate the column, then a 3.3-cm column with a void volume of 0.3 ml can be equilibrated in 30 sec at 3 ml/min. (In practice, this is true only if the delay volume of the LC pumping system is negligible.)

Several examples that illustrate the feasibility of this approach are shown in Figures 7 and 8. Figure 7a shows the separation of an eleven-component test mix of hydrocarbon solutes with wide polarities using a 1-min gradient from 15% to 100% CH_3CN/H_2O. Figure 7b shows the gradient separation of 16 polynuclear aromatic hydrocarbon (PAH) priority pollutants in 70 sec. A similar separation according to EPA Method 610 of the *Federal Register* takes more than 40 min (24). Twelve nucleosides and bases are separated in 70 sec in Figure 8a. The chromatographic profile of a sample of deproteinized human serum illustrates the separation of nucleosides and other UV-absorbing materials and was obtained in less than two min (Figure 8b). This type of serum profile analysis might be important for disease diagnostic purposes (25). These examples illustrate the usefulness of short columns for diversified samples and applications.

Rapid methods development: Figure 9 shows an example of rapid methods development for analysis of a pharmaceutical preparation. The sample was injected in a mobile phase of 100% CH_3CN and reinjected at successively lower CH_3CN concentrations (80%, 65%, . . .) with a 2-min equilibration time after each solvent composition change. At 50% CH_3CN/H_2O, the analyte peak is partially resolved, and at 40% CH_3CN/H_2O the analyte peak is completely resolved from other components. The total methods development time was 15 min (similar to an analysis on a conventional 10-μm column). This benefit is important for any research organization in which scientists expend considerable time and resources on analytical methods development.

Because 5000 theoretical plates are generated in 1–2 min with the column used in this study (or similar columns), one can address the majority of the LC applications in use today (4). Some situations will, of course, be encountered in which more resolution is needed. With the ability to manipulate mobile phase selectivities (conveniently generated by modern multisolvent pumps), and a better understanding of multisolvent optimization schemes (26,27), the need for higher efficiency, however, is usually limited to very complex samples.

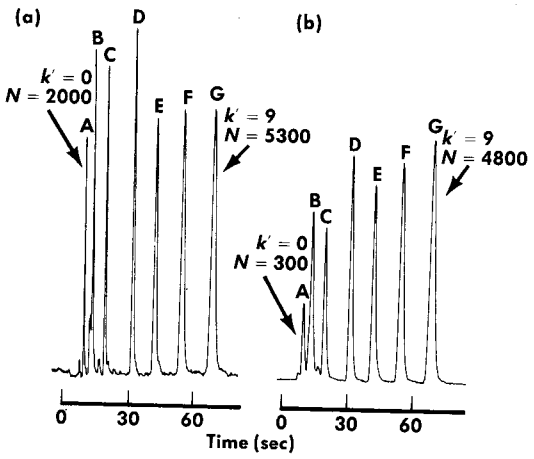

FIGURE 4: The effect of extracolumn band broadening on peak efficiencies. (a) Detector had 1.4-μl flow cell, (b) detector had 8-μl flow cell. Experimental conditions same as in Figure 2 except mobile phase: 60% CH_3CN/H_2O; flow rate: 2.5 ml/min. Peaks same as in Figure 2.

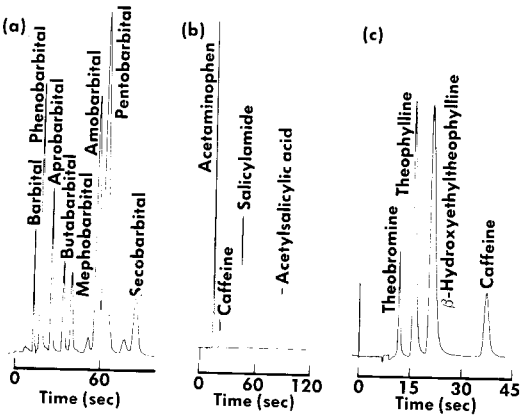

FIGURE 5: Examples of pharmaceutical analyses using fast LC. (a) Common barbiturates; column: 3x3 C18; mobile phase: MeOH/0.015 *M* $(NH_4)H_2PO_4/H_2O$ (55:20:25); flow rate: 2.5 ml/min; pressure: 3000 psi; detection: UV 200 nm. (b) Analgesic tablet; column: 3x3 C18; mobile phase: CH_3CN/0.1% H_3PO_4 (15:85); flow rate: 2.5 ml/min; detection: UV 240 nm. (c) Theophylline; column: 3x3 C18; mobile phase: CH_3CN/H_2O (11:89); flow rate: 3 ml/min, detection: UV 273 nm.

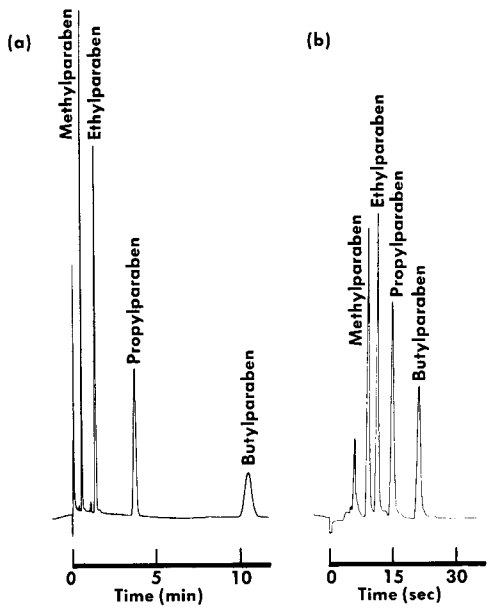

FIGURE 6: Separation of four parabens. Column: 3x3 C18; mobile phase: (a) 50% or (b) 20% CH_3CN/H_2O; flow rate: 4 ml/min; pressure: 2300 psi; detection: UV 254 nm.

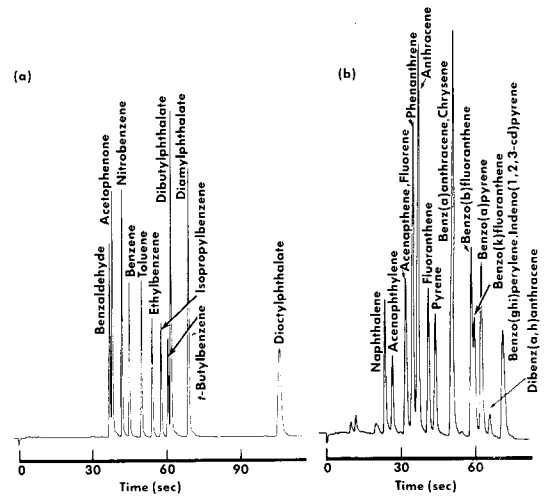

FIGURE 7: Fast gradient analyses of hydrocarbons. (a) Eleven component hydrocarbon mixture; column: 3x3 C18; mobile phase: 15% to 100% CH_3CN/H_2O linear program in 1 min; flow rate: 3.5 ml/min; pressure: 3000 psi; detection: UV 254 nm. (b) 16 PAH priority pollutants; column: 3x3 C18; mobile phase: 60% to 100% CH_3CN/H_2O in 1 min; flow rate: 3 ml/min; pressure: 1600 psi; detection: UV 254 nm.

High mass sensitivity: Figure 10 illustrates a comparison of mass sensitivities for a standard 10-μm column and a short 3-μm column. Chromatographic conditions and equipment were identical in both cases. A sixfold increase in peak height or sensitivity can be attributed to the lower void volume of the short column (2.5 ml for the standard column versus 0.3 ml for the short column) (28). The enhancement in mass sensitivity means that a smaller sample size is required for analysis, which is an important advantage when limited amounts of sample are available. It should be mentioned, however, that Beer's law dictates that the longest path length must be used for maximum detection sensitivity, which often conflicts with bandwidth requirements for a smaller flow cell. In this case, practical compromises in flow-cell design are important in achieving an acceptable balance between these requirements (11).

Economical advantages: Lower column void volumes, faster analysis, and shorter equilibration times all contribute to a reduction in mobile phase consumption. It is estimated that an 85% savings in solvent use can be effected by switching from a standard 25-cm column to a short column. Additional savings should be realized from corresponding reductions in the disposal of smaller volumes of waste solvents.

The faster analysis time leads to an increase of an order of magnitude in sample throughput per instrument for routine assays. Short columns can thus decrease sample turnaround times and reduce the cost per analysis.

Excellent analytical precision: The reproducibility of data obtained from replicate injections onto 3-μm columns has been previously demonstrated (11,17). An autosampler was used to make 12 replicate injections of a 5-μl sample of methyl and ethyl paraben with a mobile phase of 55% CH_3CN/H_2O. The relative standard deviations of retention times (±0.1 min), peak areas (±1%), and peak heights (±0.6%) are all consistent with those of a standard column.

Column lifetime: One of the major concerns about any new column is reliability and lifetime. If filtered and clean samples are used, the lifetime of a well-packed 3-μm short column should be similar to that of the standard 10-μm column (29,30,31). Figure 11 illustrates that one of these columns can undergo several thousand injection cycles without any appreciable efficiency loss (30). The use of an injector with a flow bypass is mandatory to prevent severe pressure surges during the injection process (29). Also, because the small-particle columns are inherently more prone to plugging problems, only well-filtered mobile phases and samples should be used. In practice, it was found that the installation of a short precolumn (such as a 3-cm C18 column packed with 10-μm particles) as a filter between the pump and the injector was an effective means for preventing plugging problems. In addition, the use of a silica presaturator column to prevent silica dissolution in the analysis column is also recommended for maximum column life under isocratic analysis conditions (32).

POTENTIAL PROBLEMS AND PRACTICAL PRECAUTIONS

The discussion of any new development is not complete without addressing the problem areas. Table II lists for 3-μm columns potential problems, causal factors, and recommended solutions. Some of these problem areas are discussed in the text below.

Because the short 3-μm column is a packed silica-based column as is a standard 10-μm LC column, care and maintenance precautions such as those suggested by Rabel (33) should be followed. The 3-μm C18 bonded-phase columns appear to have an upper temperature limit of

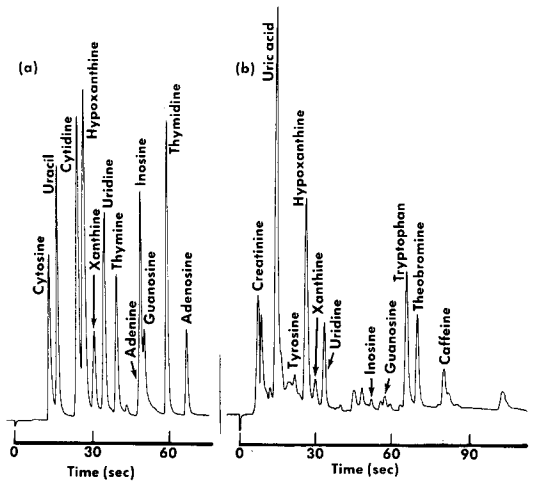

FIGURE 8: Fast gradient analyses of nucleosides. (a) Twelve nucleosides and bases; column: 3x3 C18; mobile phase: A =MeOH/H_2O (4:6), B =50 m*M* NaH_2PO_4, pH 5.5; linear program from 1% to 60% A in 1 min; flow rate: 2.5 ml/min; pressure: 2400 psi; detection: UV 254 nm. (b) Chromatographic profile of a deproteinized human serum sample; conditions same as in Figure 8a.

50 °C; above 50 °C an irreversible loss in efficiency can occur (34).

The use of low-bandwidth LC systems with small injection loops and very narrow-bore tubing (0.005–0.007 in. i.d.) also means that additional care is required in using filtered samples and solvents to prevent plugging. Connection tubing should be square cut and butted against the bottom of the fitting to prevent the formation of dead volume. Commercial precut tubes are recommended. Also, the instrumental bandwidth should be measured periodically to ensure system integrity. A well designed high-speed LC system that uses very short columns should work and operate like a conventional HPLC system and have the same reliability.

Small guard columns (for example, 33 mm x 2.6 mm) dry-packed with pellicular bonded phase materials can be used quite effectively. Under isocratic conditions, some efficiency loss for peaks at low k' can occur; however, at higher k' values (greater than 5), there is little loss of efficiency. Under gradient conditions, little discernible degradation in efficiency can be observed. More research is certainly needed to develop a compatible guard column that has an adequate capacity and does not cause a loss in resolution.

The need for concern about the effects of viscous heating and the formation of temperature gradients within the column remains somewhat disputed (3,12,15,31,35,36). The general consensus among practicing chromatographers appears to be that, under normal operating conditions of moderate flow rates (2–4 ml/min) and ambient temperature, viscous heating is not a major factor affecting performance (12,31).

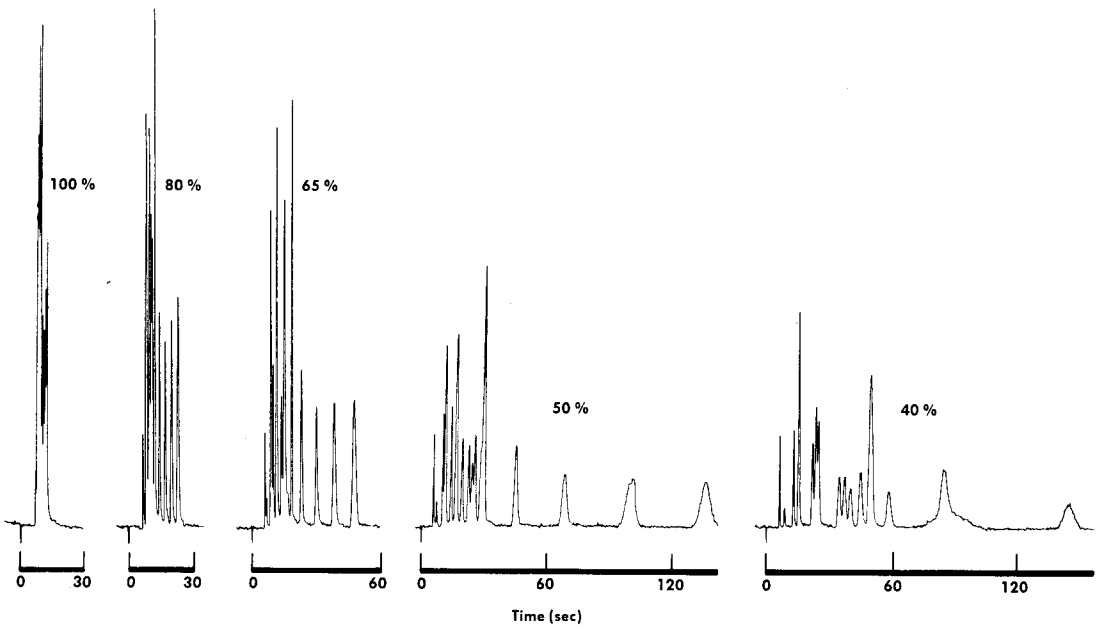

FIGURE 9: Rapid methods development. Column: 3x3 C18; mobile phase: CH_3CN/H_2O; flow rate: 2.5 ml/min; detection: UV 254 nm.

TABLE II: POTENTIAL PROBLEM AREAS AND SOLUTIONS

Potential Problems	Causal Factors	Resolved?	Solutions
Column lifetime	Injection pulses	Yes	Use injectors with flow bypass
	Plugging	Yes	Use filtered solvents and samples; install solvent filters between pump and injector; use guard column
	Column temperature	Partial	Keep column temperature below 50 °C
	Silica dissolution	Yes	Use silica saturator column
Compatible LC system	Injector	Yes	Use low-bandwidth injector with small loop and flow bypass
	Connecting tubing	Yes	Use short lengths of 0.005–0.007 in. i.d. tubes
	Detector	Yes	Use compatible detector of low bandwidth and fast response
	Guard column	Partial	Use small pellicular guard column
	Cost	Yes	Slight incremental cost versus conventional system
Loss of resolution caused by viscous heating	Frictional heating caused by small particle packings	Partial	Not a practical problem under normal operating conditions

CONCLUSIONS AND FUTURE TRENDS

A short 3-μm column operates like any packed bonded-phase column and is therefore capable, at least in theory, of addressing the entire spectrum of applications for C18 bonded-phase columns. Because of the moderate plate count, however, extremely complex samples and components with very small selectivity (α) will be better resolved by concatenation of several short columns (7,9,14) or by using longer columns packed with 3-μm (or larger) particles.

Applications of high-speed LC have been published in several areas of analytical chemistry, including the analysis of commonly abused drugs (37), priority pollutants (38), food substances (39), and plastics materials (40). Although most of the data presented in this article and in the literature cited were obtained with a commercially available 3.3-cm column, the conclusions apply to very short columns in general, and references to articles on longer small-particle columns are included (41–45).

The short small-particle column, along with new developments in microbore (46,47), packed capillary (48,49), and open tubular capillary LC columns (50,51), represents the major trend in LC columns today. The short column offers significant increases in analysis speed, sensitivity, and economy in addition to retaining the other desirable characteristics of conventional HPLC, such as convenience, gradient capability, mobile phase selectivity, and equipment reliability.

The actual column dimensions will always be dictated by the instrumental bandwidth of the LC system. Systems with lower IBW will allow the use of shorter, narrower columns (52,53) packed with even smaller particles. Encouraging results on 2-μm particles have already been reported by Verzele et al. (54). New problems will certainly emerge as our present injectors, data-handling devices, and sample preparation procedures are found to be unable to keep up with the speed and performance of further-improved columns. These developments should be viewed as progress and a new challenge in the ever-evolving world of liquid chromatography.

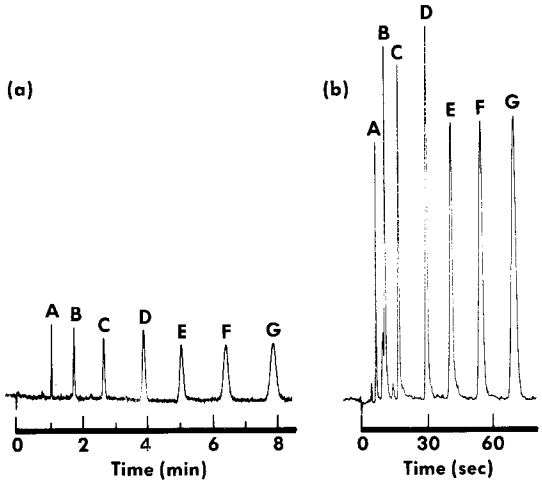

FIGURE 10: A comparison of mass sensitivity between standard 10-μm and 3x3 columns. Columns: (a) 250 mm x 4.6 mm, 10-μm C18. (b) 33 mm x 4.6 mm, 3-μm C18; mobile phase: CH_3CN/H_2O (60:40); flow rate: 2.5 ml/min; detection: UV 254 nm. Peaks same as in Figure 2.

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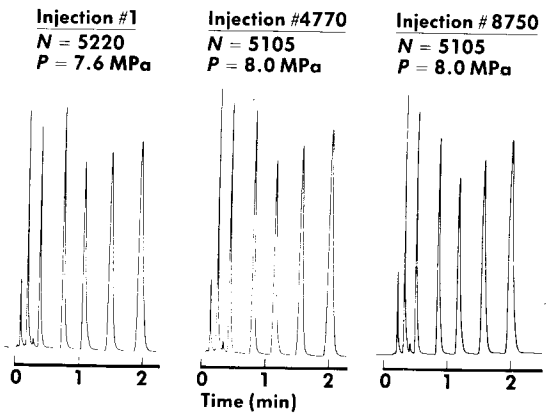

FIGURE 11: Column lifetime testing. Column: 3x3 C18; mobile phase: CH_3CN/H_2O (60:40); flow rate: 1.5 ml/min.

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