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# THE LIMITS OF SPEED IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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## SUMMARY

Routine applications of high-performance liquid chromatography (HPLC) are the main beneficiaries of increased efficiency in separation per unit time. Separation speed also plays an important role in method development. Very high speed and super speed liquid chromatography with retention times of unretained peaks of less than 1 sec generate more than 500 plates per second. Isocratic and gradient elution separations can be completed in less than 10 sec.

The limits of speed in HPLC are discussed from the following viewpoints: chromatographic theory — the roles of columns design, packing materials, temperature, solvent and solute; instrumentation — requirements for future HPLC instrument components; and practical needs — requirements of typical users in research, method development and quality control.

## INTRODUCTION

Since the introduction of modern liquid chromatography with high-performance column packing materials (HPLC), improvements in separation power have been an important goal of method development. However, the resolution of two components can be achieved not only by using a large number of plates but also by having a better separation selectivity. Given adequate selectivity, many routine problems can be solved with fewer than 1000 theoretical plates.

Only in the last few years has the impact of separation speed been investigated[1–3]. Separation power is measured by the number of plates, $N$. The separation performance, $P$, is defined as

$$P\ (\mathrm{sec}^{-1}) = \frac{\mathrm{d}N}{\mathrm{d}t} \tag{1}$$

Any practical application of HPLC must consider the time that is required. Millions of plates would have little practical value if they took months to generate. Fast HPLC plays an increasingly important role in routine applications. The separation speed significantly influences the number of analyses per instrument per day, and therefore the price per analysis.

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TABLE I

DEFINITIONS OF FAST HPLC

| *Type of HPLC* | *Dead-time* ($t_0$) | *Typical analysis time* (*up to* $k' = 10$) |
|---|---|---|
| Conventional | *ca.* 1 min | *ca.* 10 min |
| Very high speed | *ca.* 10 sec | *ca.* 100 sec. |
| Super speed | 1 sec | *ca.* 10 sec |

Definitions of different modes of fast HPLC are compiled in Table I. The differentiation of very high speed and super speed LC is justified by the more stringent instrumental requirements of super speed LC.

Because of the important economic impact of fast HPLC, the trends and limits of speed were investigated, not with the intention of convincing workers to throw away the old HPLC apparatus but in order to set standards and requirements for new instruments and replacement of HPLC components.

The limits of speed in HPLC can be discussed from the following viewpoints: chromatographic theory (the roles of column design, packing materials, temperature, solvent and solute), instrumentation (requirements for future HPLC instrument components), and practical needs (requirements of typical users in research, method development and quality control).

## THEORETICAL LIMITS OF SPEED IN HPLC

There is no need for new theories for the interpretation of speed and separation performance in HPLC. Giddings[4], Knox[5] and Guiochon[6] have laid down the framework of theory for such discussions of chromatographic processes.

The Knox equation:

$$h = \frac{2\gamma}{v} + A\,v^{1/3} + Cv \tag{2}$$

where $h = H/d_p$ is the reduced plate height, $v = u\,d_p/D_M$ is the reduced velocity, $d_p$ is the particle size, $u$ is the mobile phase velocity, $D_M$ is the diffusion coefficient and $H$ is the height equivalent to a theoretical plate, shows that for high mobile-phase velocity the last term, which is linear, dominates the plate height and separation power of a system.

Eqn. 2 suggests that $C$ must be as small as possible for fast separations. It has been known for years that packing materials with small particle size give smaller linear terms of the $H/u$ function. Giddings[4] has shown that the $C$-term contributions to the plate height $H$ is

$$C = \frac{C_{sm}\,d_p^2}{D_m} + \frac{C_s\,d_f^2}{D_s} \tag{3}$$

(mobile phase) (stationary phase)

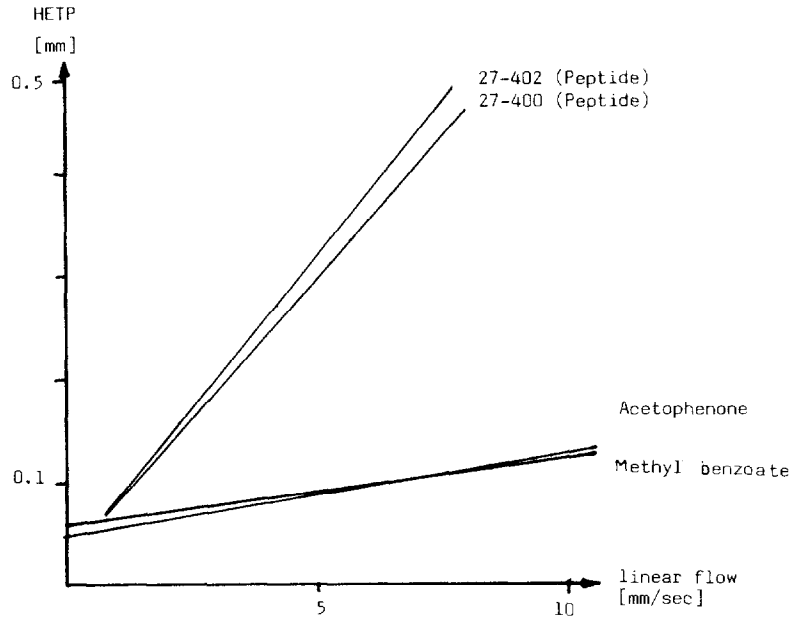


Fig. 1. Different slopes of $H$ *vs.* $u$-curves of normal compounds (acetophenone and methyl benzoate) and two peptides (27-400 and 27-402) with molecular weights of *ca.* 1200. HPLC conditions: column, RP-8, 10 $\mu$m, 100 × 4 mm I.D.; mobile phases, methanol–water, 100:40 and 50:50.

where $D_m$ is the diffusion coefficient of the solute in the mobile phase, $C$, $C_s$ and $C_{sm}$ are the plate-height coefficients, $d_f$ is the thickness of the stationary phase and $D_s$ is the diffusion coefficient of the solute in the stationary phase.

Eqn. 3 shows that the mobile-phase contribution increases with the square of the particle diameter and is inversely proportional to the diffusion coefficient. Fig. 1 shows practical examples of the effect of diffusion on separation efficiency.

Especially for biologically important high-molecular-weight compounds, the effect of diffusion can significantly influence the plate number at high speed. Fig. 2 shows the effect of the mobile phase viscosity on the HETP *versus* linear velocity function. The propanol system, having a high viscosity, gives significantly higher plate heights than the acetonitrile system, which has a lower viscosity. The effect of temperature can be attributed mainly to its effect of reducing the viscosity.

In order to improve the separating performance, $P = dN/dt$, it is always possible to use a longer column and to increase the flow-rate of the mobile phase. Guiochon[6] has shown that for very high flow-rates and pressures $dN/dt$ approaches a limit:

$$\left(\frac{dN}{dt}\right)_{limit} = \frac{1}{C} \cdot \frac{D_m}{(1 + k')\, d_p^2} \tag{4}$$

It is interesting to compare eqn. 4 with the equation for the limit of plate numbers[6]:

$$N_{limit} = \frac{k_0}{\eta D_m} \cdot \frac{d_p^2}{2\gamma} \cdot \Delta P \tag{5}$$

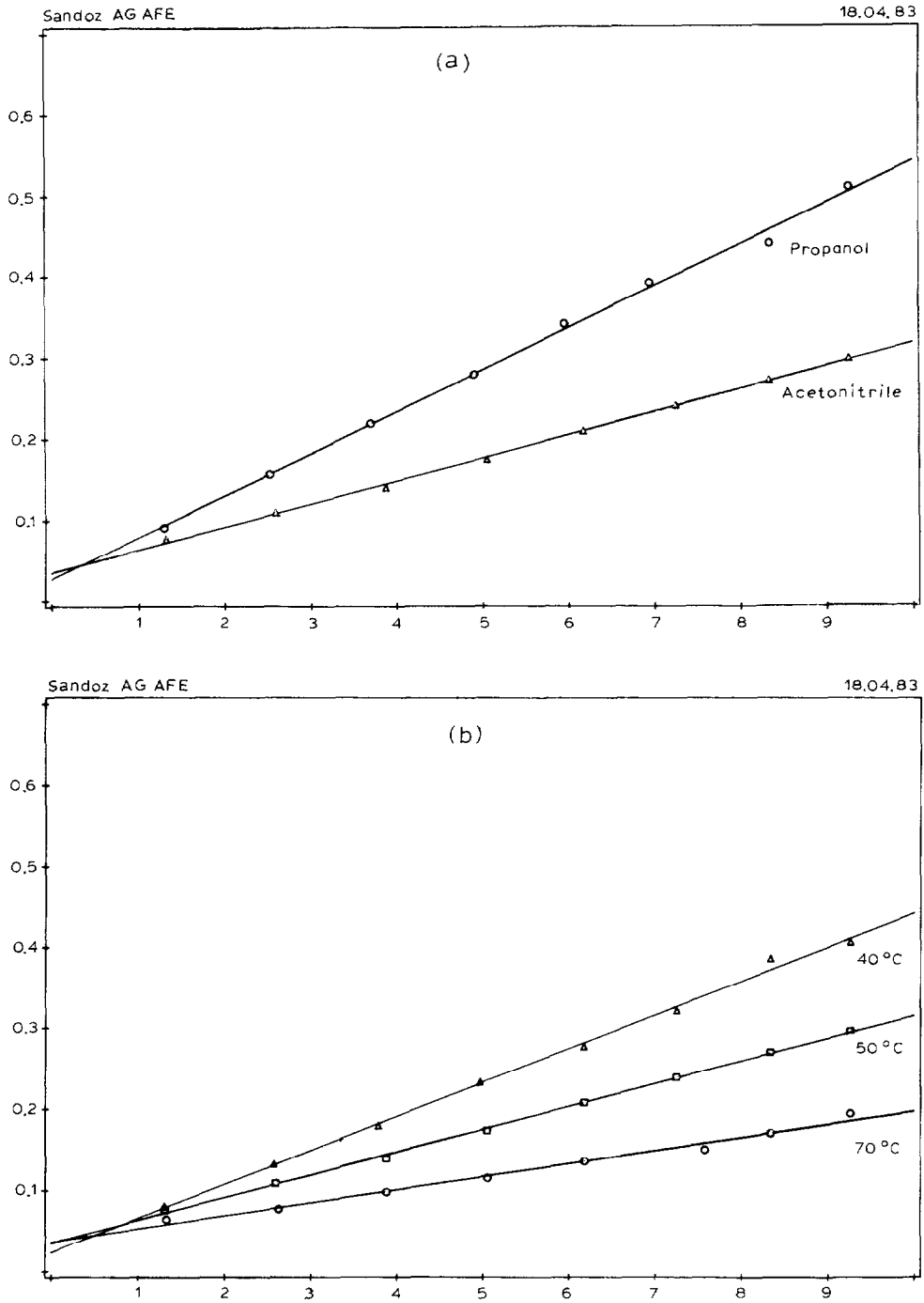


Fig. 2. (a) Influence of mobile phase viscosity on the $H$ *vs.* $u$ curve of a peptide with propanol or acetonitrile as organic modifiers (column temperature, 50°C); (b) Influence of column temperature on the $H$ *vs.* $u$ curve of a peptide with acetonitrile as organic modifier.

where $k_0$ is the specific permeability, $\eta$ is the viscosity and $\Delta P$ is the pressure drop.

In order to obtain the optimum plate number, $N$, with a given pressure, a very long column with large particles and a mobile phase with a low diffusion coefficient of the solute are required. In order to obtain the optimal performance, $P$, the inverse is required: small particles and a mobile phase with a high diffusion coefficient.

Eqn. 4 does not involve the pressure of the separation system. The question concerning the fastest possible separation with a given number of plates, $N$, and a given pressure, $p$, was answered by Guiochon[6]. It can be shown that the fastest separations are achieved by operating columns at the minimum of the $H$ *vs.* $u$ curve. For a given pressure, $\Delta p$, and plate number, $N$, the optimal particle diameter, $d_p$, is

$$d_p = \sqrt{\frac{D_m N h}{k_0 \Delta p}} \tag{6}$$

and the column length, $L$, is

$$L = N h d_p \tag{7}$$

The retention time, $t_R$, for a solute with a capacity factor $k'$ will then be

$$t_R = N \cdot \frac{(1 + k')}{D_m} \cdot \frac{h}{\gamma} \cdot d_p^2 \tag{8}$$

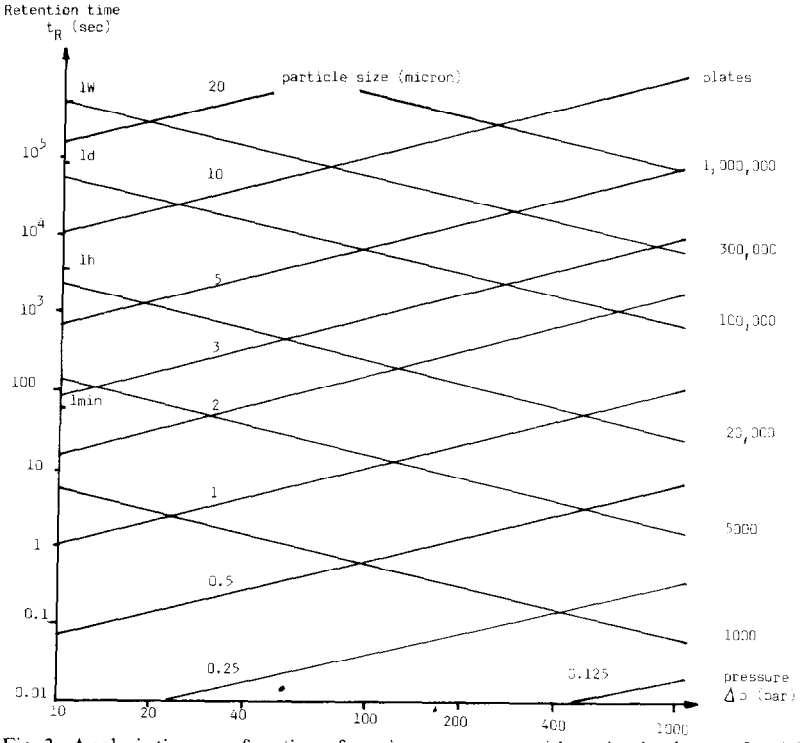


Fig. 3. Analysis time as a function of maximum pressure with optimal columns. Particle diameter, column length and retention time according to eqns. 6, 7 and 8 with other typical conditions as follows: $k' = 3$; $\nu = 3$; $D_M = 10^{-5}$ cm$^2$/sec; $\eta = 0.4$ cP; $k_0 = 10^{-3}$.

Fig. 3 shows a graphical representation of eqns. 6, 7 and 8. It clearly demonstrates the trend towards small particles for fast HPLC. Under the conditions given in Fig. 3, a future optimal LC instrument may be expected to have a column 0.5 mm long with a particle size of 0.25 $\mu$m, operated at 400 bar and yielding 1000 theoretical plates and retention times of the order of 0.1 sec.

The above equations were developed for packed columns. Knox[7] has shown that for open-tubular microcolumns similar separation speeds may be expected. When we compare these theoretical limits with what is available at present, we can conclude that we are still far away from what is possible in terms of speed in HPLC.

## INSTRUMENTAL LIMITS

The three main limitations of HPLC instruments today are (1) the time constant of the detection, sample handling, recording and data handling system, (2) the instrumental band broadening or flow limits and (3) the maximum available column inlet pressure. Fig. 3 shows that for fast separations the trend is definitely towards stationary phases with small particle size. Eqn. 8 indicates that the retention time increases with the square of $d_p$. Surprisingly, Fig. 3 shows that, with the currently available column material of 3 $\mu$m or more, pressure is really only a limiting factor under optimal conditions for separations, requiring more than 100,000 plates. Routine separations with only 1000–5000 plates can be accomplished on 3-$\mu$m columns at less than 20 bar in about 1 min. The problem is that only few HPLC instruments can actually handle really good columns.

Fig. 4 shows the time resolution of HPLC detection systems necessary for the detection of fast peaks. Only very few commercial instruments can match these re-

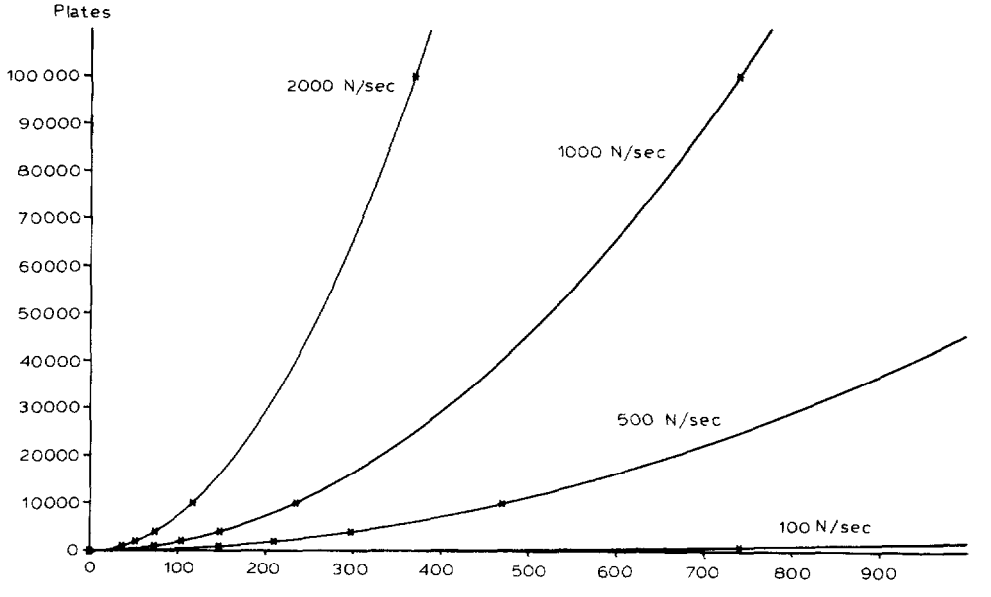


Fig. 4. Performance of a separation system as a function of the minimum detectable peak width and column plate number.

quirements for more than 1000 plates per second. Additionally, there are other time constant problems with instrument components related to fast HPLC. If separations can be completed in seconds, there must also be automatic injectors that can handle samples in the same time. At present, only specially modified samplers manage to inject a sample every 10 sec. At the other end of the chromatographic system, the data handling system must also be able to integrate the peaks, calculate amounts and prepare reports within seconds. It makes no sense in practical applications to obtain a chromatogram in 10 sec, only to wait 20 sec afterwards until the data system is ready for the next analysis and another minute to see the report printed on a slow printer. There is a need for sophisticated multi-task data systems and paperless documentation to keep up with the speed of chromatography.

Another severe limitation to speed is instrumental band broadening. In principle, the column inner diameter can be chosen to match the given instrumental bandwidth. However, there are three restrictions to this: (1) the volume flow is proportional to the square of the column diameter; (2) heat transfer at high speed leads to a radial temperature profile in the column that influences the separation; and (3) optimum packing of the columns becomes more complicated as the ratio of column length to column diameter decreases.

At present, most HPLC pumps cannot deliver more than 10 ml/min at maximum pressure. For large linear velocities and short analysis times it is therefore necessary to use small-bore columns to cope with normal flow-rates of up to 5–10 ml/min. Obviously, heat transfer is less of a problem with small-bore columns, which can be packed to give excellent plate counts[8]. Additionally, the mass sensitivity is increased in proportion to the square of the column diameter. On the other hand, the external peak broadening causes a problem. Very short, very good small-bore columns with small particles have very small elution volumes and peak volumes (see Table II). Present commercially available detection systems can rarely cope with the requirements of a short, efficient 3-$\mu$m microbore column.

TABLE II

TYPICAL DEAD VOLUMES AND PEAK WIDTHS OF REVERSED-PHASE RP-18 COLUMNS

| *Particle size ($\mu$m)* | *Column length (cm)* | *Inner diameter (mm)* | *Dead volume ($\mu$l)* | *Peak width at half-height of an unretarded peak ($\mu$l)* |
|---|---|---|---|---|
| 10 | 10 | 4.6 | 1000 | 50 |
| | | 2.1 | 210 | 10 |
| | 3 | 4.6 | 300 | 22 |
| | | 2.1 | 65 | 5 |
| 5 | 10 | 4.6 | 1000 | 30 |
| | | 2.1 | 210 | 7 |
| | 3 | 4.6 | 300 | 15 |
| | | 2.1 | 65 | 3 |
| 3 | 10 | 4.6 | 900 | 21 |
| | | 2.1 | 200 | 4 |
| | 3 | 4.6 | 280 | 12 |
| | | 2.1 | 60 | 2 |

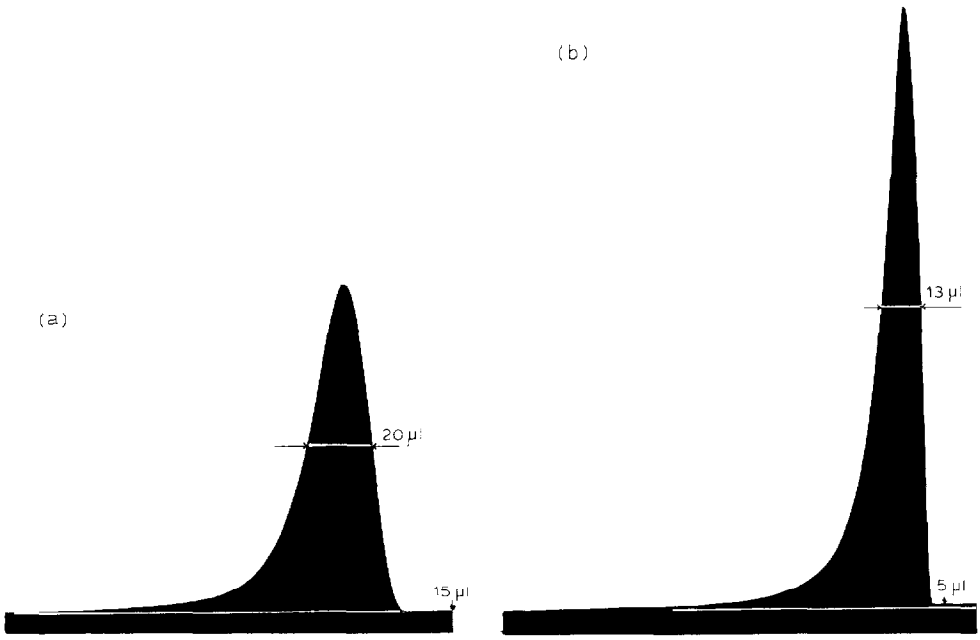


Fig. 5. (a) Detector peak broadening with a 2.8-$\mu$l flow cell. Conditions: time constant, 0.1 sec (98% full scale); flow-rate, 0.5 ml/min; injection volume, 3 $\mu$l; "dummy" column with 0.1 mm I.D. capillary. (b) Detector peak broadening with improved connections according to Fig. 6 [conditions as in (a)].

It is not only the nominal cell volume of a UV detector that contributes to instrumental band broadening but also the flow characteristics of the system. Fig. 5 compares the same 2.8-$\mu$l UV flow cell with two different modes of connections: Fig. 5a shows the flow profile of an injection to a "dummy" column of 1.2-$\mu$l internal volume with conventional tubing and connections; Fig. 5b shows the flow profile with a optimized instrumental setup for injector, column and detector cell (see Fig. 6). When the elution volumes of peaks in Table II are compared with the delay volume in the tubing, it is obvious that the instrumental setup in Fig. 6 shows adequately small delay volumes. Even if it is true that band broadening is very small in tubing of small inner diameter, it must be realised that the delay volume of many "compact instruments" have the same order of magnitude as the column volume of short microbore columns. Few workers compensate for the delay volume when they calculate retention parameters. When it comes to gradient elution, the small column volumes must also be taken into account[9]. The dead-volume of the mixing chamber and the delay volume of the gradient delivery system must be smaller than the column void volume. It seems to be nearly impossible to meet the strict standards of fast microbore columns with low-pressure side gradient mixing systems having delay volumes of up to 5 ml. With high-pressure gradient mixing, super speed gradient elution HPLC is possible under routine conditions, as shown in Fig. 7.

Another problem related to the small volume between pump and column, the high flow-rate and the low compressibility of solvents is the pressure jump during the injection of a sample due to the short interruption of flow during the actuation of

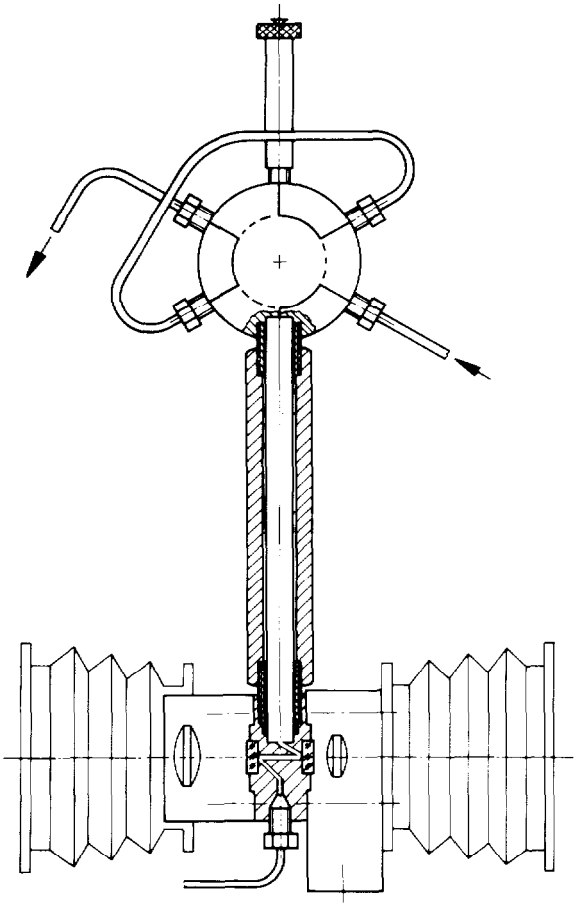

Fig. 6. Schematic diagram of the optimized setup of injector, column and detector flow cell (Kontron, Zurich, Switzerland). All connections are omitted.

the injection valve. This pressure jump can be more than 150% of the normal operating pressure and shortens the column life significantly. DiCesare *et al.*[10] proposed the use of an injector bypass to overcome this problem. However, the bypass contributes to additional sample dilution during injection and may adversely affect the column performance. By partially plugging the loop with particles of the sample solution or grindings from the seal, the bypass may cause irreproducible results. Experiments have shown (see Fig. 8) that by increasing the air flow through the valve of the pneumatic actuator the pressure jump during injection can be diminished to acceptable levels without the dilution and other potential problems of a bypass. Experience has shown that very good column lifetimes can be maintained with this system.

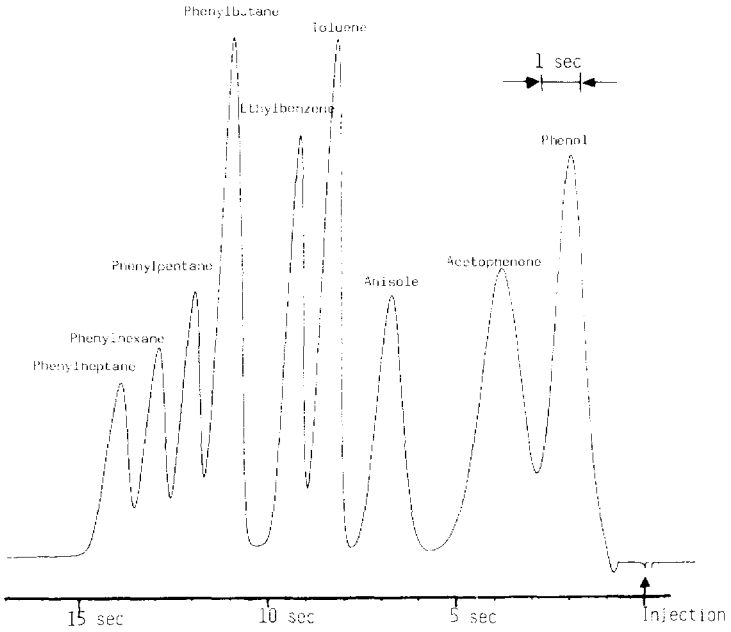


Fig. 7. Super speed gradient elution HPLC of a test mixture. Column, 30 × 2.1 mm I.D., RP-18, 5 μm; flow-rate, 7 ml/min; steps of 30–70% acetonitrile in water.

## PRACTICAL NEEDS

Typical applications of fast HPLC in the pharmaceutical industry are stability tests, dissolution rates, content uniformity, method development, kinetics, and process control. The last two examples are extensions of HPLC to new fields where speed is a condition of its use as an analytical technique. In the other examples the reasons for using fast LC are faster results, more analyses per unit time, more analyses per instrument and reduced cost per analysis.

Reducing the analysis time with fast HPLC from 100 min to *e.g.*, 0.6 sec has significantly different practical and economic relevance. To illustrate the situation, two typical standard HPLC applications are discussed (see Fig. 9). In case A chromatography contributes only 50% to the total price of an analysis. The other 50% is due to time-consuming sample preparation and other fixed costs, such as solvent and column materials. By increasing the speed of HPLC, a sharp decrease in the analysis cost is obtained, but the decrease is only 50%, even at infinite speed. This situation is typical of the analysis of complicated samples, *e.g.*, biological materials requiring time-consuming manual sample preparation. In case B, where no sample pre-treatment is necessary, HPLC analysis contributes 95% to the total cost. Fast HPLC can bring the analysis cost down to approximately 5%. Case B is typical of simple analyses, such as dissolution rate testing or assay of liquids without sample cleanup. However, even in the best case (Fig. 9), the price reductions are levelling off. For practical applications it is therefore sensible to reduce the analysis time from 10 to 1 min or maybe 6 sec. Any further reduction entailing increased instrumental costs may not be cost effective.

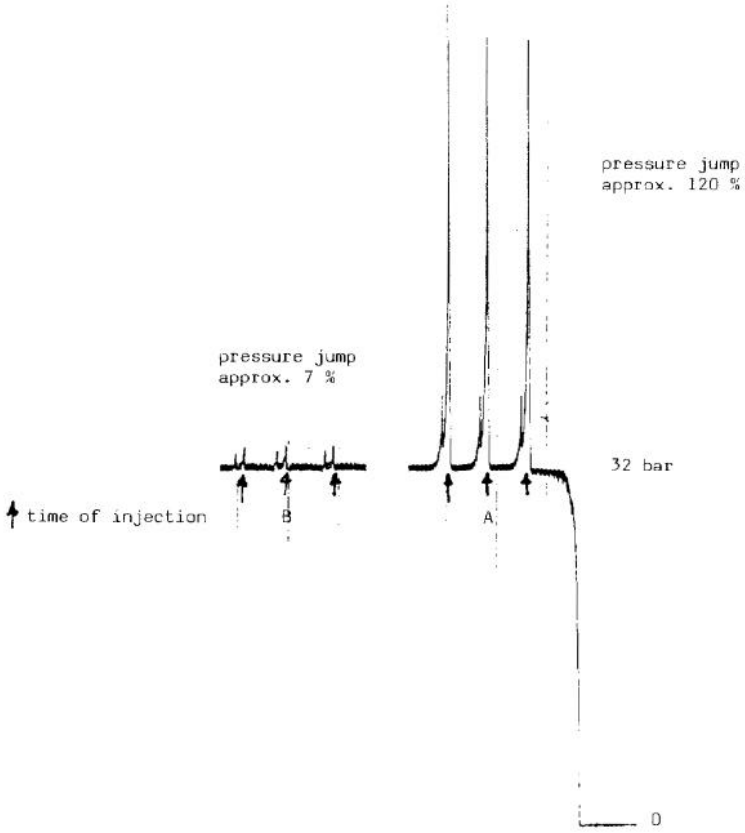


Fig. 8. Pressure jump during injection. (A) Normal pneumatic valve (1/8 in.) for pneumatic control; (B) 1/4 in. pneumatic valve for improved air flow in fast pneumatic actuation of the injection valve.

TABLE III

NUMBER OF EXPERIMENTS IN METHOD OPTIMIZATION BY A MIXED APPROACH OF SIMPLEX OPTIMIZATION AND FACTORIAL DESIGN

| *Parameter* | *Number of experiments* | *Cumulated number of experiments* |
|---|---|---|
| Four solvents, *e.g.*, water, methanol, acetonitrile, tetrahydrofuran | 7 | 7 |
| Four column types, *e.g.*, RP-18, RP-8, RP-2, Diol | 4 | 28 |
| pH (5, 7, 9) | 3 | 84 |
| Temperature (30, 50, 70°C) | 3 | 252 |
| Identification of four known components | 4 | 1004 |

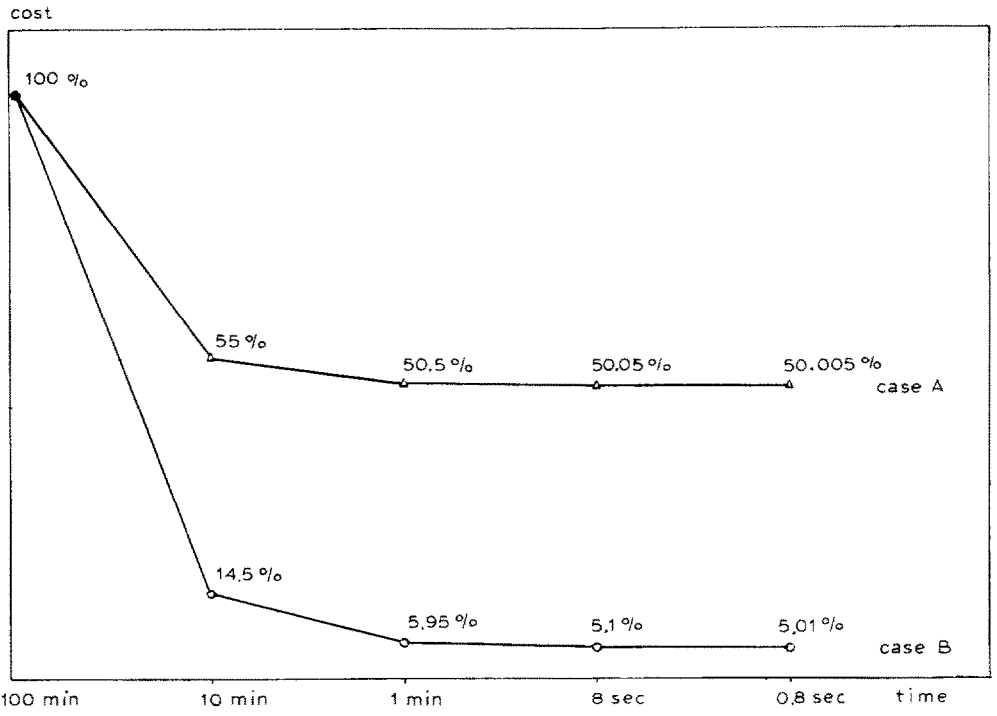


Fig. 9. Reduction of costs by fast HPLC. Case A, 50% fixed costs (time-consuming sample preparation); case B, 5% fixed costs (no sample preparation).

Fast HPLC seems to have a promising future in automatic optimization of the selectivity by solvent selection. Glajch and Kirkland[11] proposed a mixed approach of factorial design and simplex optimization. It is possible to design an automatic system that performs all these experiments with different solvent systems. As shown in Table III, the number of experiments increases very rapidly in reversed-phase chromatography, because there are so many independent parameters to optimise. Table IV shows the number of experiments that an instrument can perform in a day if the analysis time can be decreased. It is obvious that fast HPLC can contribute to

TABLE IV

NUMBER OF ANALYSES PER DAY WITH VARYING ANALYSIS TIME

| *Type of HPLC* | *Analysis time* | *Number of analyses per day* |
|---|---|---|
| Conventional | 1 h | 24 |
| | 30 min | 48 |
| | 10 min | 144 |
| High speed | 1 min | 1440 |
| Super speed | 10 sec | 8640 |
| | 1 sec | 86,400 |

the handling of the enormous amount of work involved in automatic method optimization. For manual optimization, a fast response to changes in parameters is even more important for better and faster method development.

## CONCLUSIONS

Theoretical aspects indicate that the trend towards smaller particles for column packing will continue and finally lead to extremely fast HPLC, even at moderate pressure. The real limits of speed in HPLC are on the instrumental side. The time constant of the detector and the instrumental bandwidth are the most important instrumental requirements for fast HPLC. Practical aspects of the routine application of fast HPLC lead to the conclusion that it can reduce significantly the cost of analysis, especially for simple assays. There are new fields of application, such as process control and the investigation of fast kinetics, where fast analyses are essential. Fast HPLC also contributes to method development and makes new automatic approaches involving hundreds of experiments feasible.

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