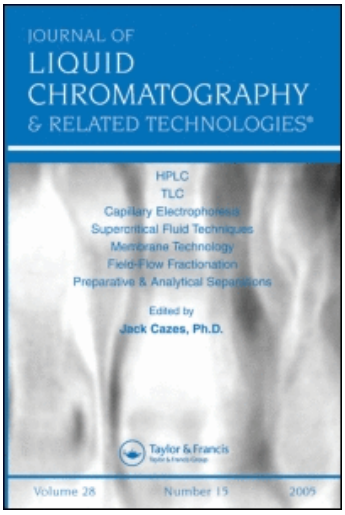

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HIGH SPEED LIQUID CHROMATOGRAPHY: A REVIEW

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INTRODUCTION

Modern high performance liquid chromatography (HPLC) emerged in the late 1960's. The characteristics which make HPLC such a powerful technique, that is, versatility, selectivity, and sensitivity, are rooted in the chromatographic theory that evolved from Martin and Synge's work of the 1940's (1). Presently there are several HPLC techniques being developed and refined in analytical and instrumental laboratories. The goal of making HPLC separations faster, more

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efficient, and more selective is being achieved by the development of sensitive detectors and improvements in overall instrumentation. In addition, significant changes and advances have taken place in the design and manufacturing of chromatographic columns.

Currently column technology is moving in two directions. First, towards the reduction in column diameter and second, towards reduction in column length. Conventional HPLC columns have internal diameters of 3-5 mm. Knox and others have thoroughly treated the theoretical and experimental aspects of narrow bore or"micro" liquid chromatography (2-9). Microbore columns, typically 1 mm i.d. and packed with 10-20 um particles offer several advantages over conventional (4.6mm i.d.) HPLC columns. These advantages include low solvent consumption, a greater sensitivity when sample volume is limited, and a potential gain in analysis time and efficiency. Scott and co-workers have investigated the performance characteristics and applicability of these columns for use in the analytical laboratory (10-13).

The simultaneous reduction in column length and particle size is the other direction of interest in current HPLC column technology. Conventional HPLC columns are typically 25-30 cm in length and are packed with 10 um particles. More recently, columns of 5-15cm and smaller, packed with 3-5um materials

have become commercially available. These short columns of conventional internal diameter packed with small particles have become known as "high speed" liquid chromatographic (HSLC) columns.

The question to be addressed in this review is why has it taken almost 50 years for high speed liquid chromatography to become a viable tool for the separation scientist?

The answer to the question may be two-fold.

First, the literature abounds in experimentally sound and proven separation methods. HPLC methods which utilize conventional HPLC columns, are now routinely being used in pharmaceutical, clinical, quality control, and environmental laboratories. The scientists using these methods on a daily basis are not likely to change over to high speed analyses until HSLC becomes employed more frequently by researchers and cited in the literature. Only when HSLC becomes the method of choice for a given chromatographic separation will the impact be felt. Column manufacturers have only recently begun actively marketing short columns with small diameter packing materials. The availability of these columns from several manufacturers indicates that HSLC will become an essential and integral part of the chromatography laboratory.

The second and more defined drawback to the use of these high speed columns has been the slow evolution of instrumentation design necessary for high speed separations.

Since components of an instrumental set-up must be optimized to perform efficient high speed separations, each component will be discussed in this review.

INSTRUMENTATION

Pumps

Pulse-free pumps must deliver precise flow rates and be capable of the high pressures necessary to perform HSLC. In gradient analyses, the pump system must have a reduced delay volume or delay time. This reduced delay volume should allow for adequate mixing of the mobile phase components and thereby achieve the fast gradient changes required. DiCesare and co-workers found that delay volumes of 0.5 ml maintains adequate mixing (14). Typical delay volumes in conventional HPLC systems are 2-4 ml.

Injectors

HPLC injectors must be capable of injecting accurate, small amounts of sample to the chromatographic column, thereby minimizing band broadening effects. Band broadening due to sample injection volume has been discussed in detail by Kirkland and others (15-18). Typical injection volumes of up to 6ul minimize band broadening effects. These volumes are also compatible with commercially available autosampling devices.

In addition to the small sample volumes (1-6ul) the injector should have short valve-switching times (less than 0.1sec) (19). It has been shown that long valve-switching times are responsible for some column deterioration due to the pressure surges encountered in the valve-switching procedure (20,21). This problem has been overcome by the use of injectors with built-in flow bypasses which eliminate the detrimental pressure surges. These injectors are now commercially available.

Detectors

The efficient use of high speed liquid chromatographic columns has forced the dramatic improvement of HPLC detector capabilities. The engineering difficulties in proper cell design and decreasing cell volumes have hampered the progress of high speed LC. Essential changes which have taken place are in detector flow cell volumes, detector response time and detector sensitivity.

Conventional HPLC detector flow cells have total volumes of 8-10ul. Mellor found that using a 5cm, 3um column of conventional diameter with a uv detector equipped with an 8 ul flow cell, only 90% of the potential column efficiency is achieved (22). He concluded that conventional detector flow cell volumes are too large and add in extra column dispersion (band broadening) when coupled with high speed columns.

Poile and coworkers (23) designed a 2.4ul flow cell for a uv detector. This smaller flow cell reduced extra column band broadening by a factor of five over a conventional 8ul cell (24,25). The influence of the extra column band broadening on chromatographic performance is illustrated in Figure 1. Katz and Scott report that with a 1.4ul cell, extra column dispersion was not significant (26). More recently, 3cm, 3um columns have been found to provide similar efficiency when used with 2.4ul and 12ul uv detector cells, however lower efficiencies were found when 3 X 3 columns were used in a system equipped with a 10ul cell (27). Thus it appears that the effects of a detector cell on 3x3 column efficiency are determined by the design of the cell as well as the volume.

The requirement for rapid detector response times or time constants for conventional HPLC has been studied in detail. Higgins noted that HETP values and peak symmetry were dependent on the time constant of the detector (28). Low and Haddad recommend that column efficiencies be quoted at 0 time constant in order to remove the variability in plate count introduced by the varying time constant (29). They also recommend that the detector time constant should not exceed one hundredth of the peak width for peaks used in calculation of plate numbers.

HSLC places even further restraints on the response times of detectors in that the time corresponding to the peak

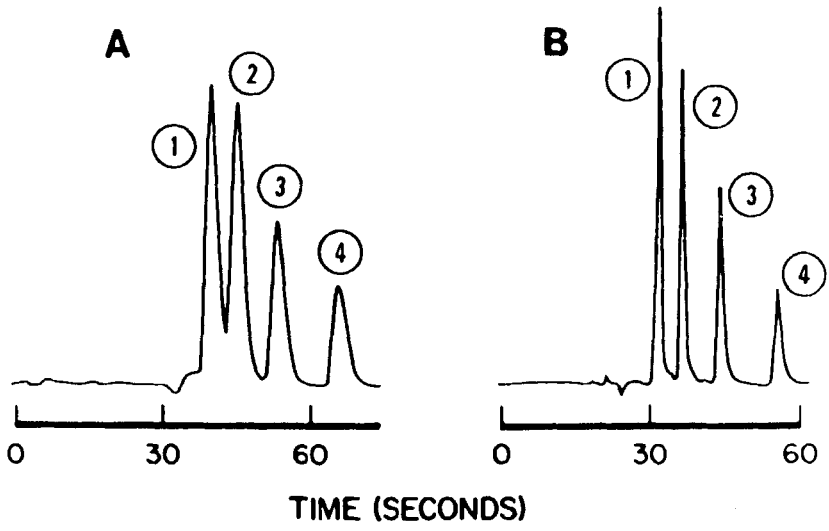

FIGURE 1.

Analysis of parabens using different detector flowcells.
(A) 8ul flowcell
(B) 2.4ul flowcell
Column: 100x4.6mm i.d., 3um C18.
Mobile Phase: Acetonitrile-water (65:35) at 2.3 ml/min.
Ambient Temperature
UV detector at 254nm.
Peaks: (1) methyl paraben, (2) ethyl paraben, (3) n-propyl paraben,
(4) n-butyl paraben.

(Reproduced from reference 25).

width is smaller. Rapid response times are required so that peaks are not distorted. The effect of detector response time is most pronounced for early eluting compounds. Figure 2 illustrates the influence of detector response time constant on band broadening especially for early eluting peaks. Response times should be reduced as the flow rate is

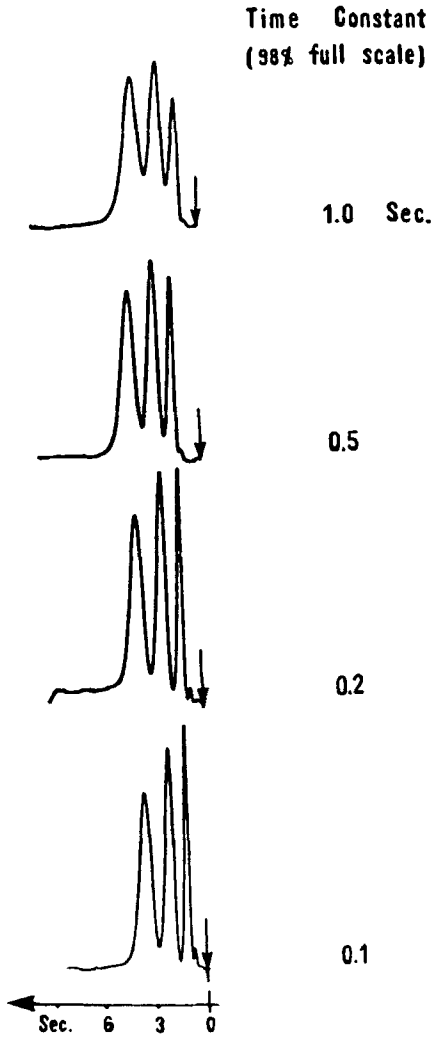

FIGURE 2.

Influence of detector response time constants on chromatographic resolution. (Taken from reference 28).

increased, because the sample components spend less time in the detector cell. Typical detector response times for HSLC are in the 20-100 msec range.

One of the major advantages of using high speed columns is the gain in mass sensitivity. This gain is achieved by the higher efficiency, faster flow rates and lower void volumes associated with short high speed columns. The end result is a chromatogram with reduced peak width and increased peak height, as illustrated in Figure 3. Therefore much smaller amounts of solutes will give peaks of the same height as the height obtained from a larger amount eluting from a conventional length column. As a result, detection limits are lower in spite of reduced sample size.

The significant factor in maintaining detector sensitivity is that long path lengths (~6mm) must be used (30).

The major difficulties in establishing good detector performance for HSLC have been the engineering problems associated with designing flow cells of low volume while maintaining a path length long enough to enhance detector sensitivity.

Data Handling Devices

The final instrumental drawback is the need for rapid data collection, recording and handling devices. Data collection, including integration of peaks, calculation of

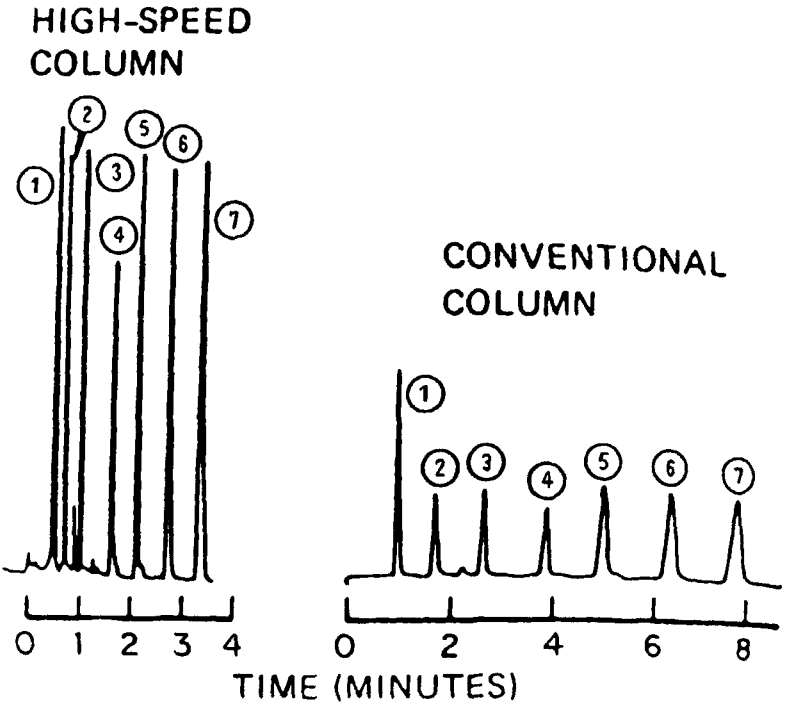

FIGURE 3.

Analysis of reverse phase test mixture on C18 bonded phase packings.
High speed column: 100x4.6mm i.d., 3um particles.
Conventional column: 250x4.6mm i.d., 10um particles.
Mobile phase: 65:35 Acetonitrile-water at 2.3ml/min.
Ambient temperature
Sample Volume: 6ul in both cases.
UV detector at 254nm, same attenuation, cell volume 2.4ul.
Peaks: (1)uracil, (2) phenol, (3) nitrobenzene, (4)toluene, (5)ethylbenzene, (6)isopropylbenzene, (7)tert-butylbenzene.

(Reproduced from reference 24).

amounts and preparation of reports must be performed within seconds. It is impratical to have a 10 second separation if the data system takes 20 seconds to interpret the chromatogram. Therefore, with extremely rapid analyses, faster recording devices are necessary.

APPLICATIONS

To date, separation methods which utilize high speed liquid chromatography are becoming more frequent in the literature. Since 1980, the use of high speed liquid chromatography has been demonstrated in environmental, clinical, pharmaceutical, biological, food and plastics separations.

In 1981 DiCesare and coworkers demonstrated the use of coupled high speed columns for improved resolution and relatively fast analysis of polynuclear aromatic hydrocarbons (PAH) and other pollutants (24,31).

Since 1981 several HSLC applications pertinent to the clinical and pharmaceutical fields have appeared in the literature. Kabra and Marton reported an LC analysis for serum theophylline in less than 70 seconds (32). Sample chromatograms are shown in Figure 4. A 12.5cm, 5um C18 column and UV detection at 273nm were used for this sensitive and specific chromatographic procedure. Sample preparation procedures required less than 100ul of serum. The savings in

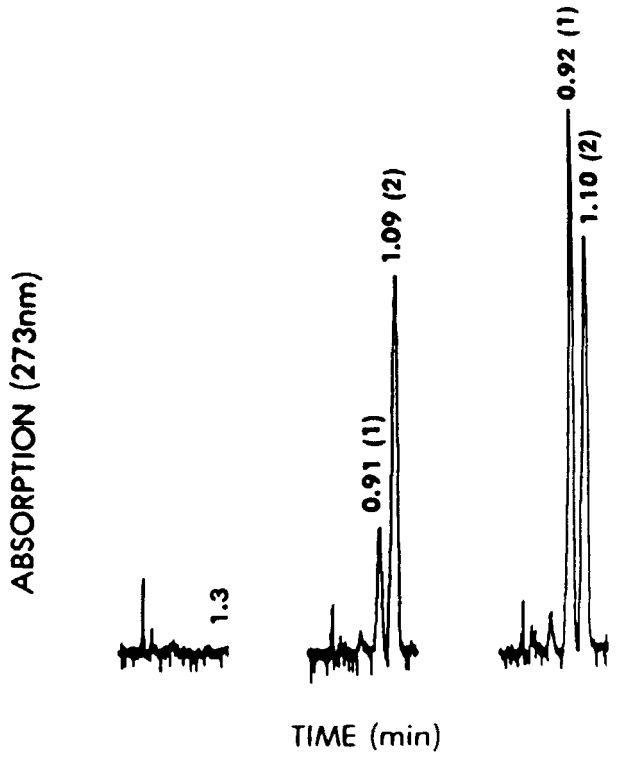

FIGURE 4.

Chromatograms of (left) theophylline-free serum; (middle) a patient's serum with 5.6mg of theophylline per liter; (right) a patient's serum with 21.5mg of theophylline per liter.
Column: 125x4.6mm i.d., 5um C18.
Mobile phase: Acetonitrile:phosphate buffer (9.5:90.5) at 4.5 ml/min.
UV detector at 273nm, 2.4ul flow cell.
Peaks: (1) theophylline, (2) B-hydroxyethyl theophylline (internal standard).

(Reproduced from reference 32).

reagent cost and analysis time makes the method competitive with the traditional EMIT^{R} enzyme immunoassay technique (33). Dong and Gant reported a 45 second theophylline assay on a 3.3cm, 3um C18 column in 1984 (34).

Kinberger and Holmen analyzed serum for the anticonvulsant drugs carbamazepine and phenytoin (35). These compounds are the two most frequently used in the treatment of epileptic patients. Isocratic elution at a flow rate of 1.5ml/min with a 10.0cm, 3um C18 column permitted a chromatographic analysis completed within 2 minutes. Conventional methods of HPLC analysis often require more than 10 minutes to obtain similar resolution (36).

The HSLC analysis of commonly abused drugs such as-barbiturates and tricyclic antidepressants were performed in 1982 (37). Dong and DiCesare used 10.0cm, 3um and 12.5cm, 5um C18 columns and achieved a 2-4 fold reduction in analysis time. Five tricyclic antidepressants are separated on the 3um column in one minute and in 3 minutes on the 5um column. Eight common barbiturates are separated and resolved in a little over 3 minutes on the 3um column. The chromatographic analysis of anticonvulsants frequently required for therapeutic drug monitoring typically takes 10-15 minutes. Using the 5um high speed column, the separation was completed in under 3 minutes. A toxicological screening of 18 commonly abused drugs was performed by gradient elution on the high

speed 5um column in 6 minutes. Precision in retention times and peak area reproducibilty for these high speed analyses were found to be 1-2%. In 1984, an 80 second separation of eight common barbiturates was reported using a 3cm, 3um reverse phase column (34).

The HSLC analysis of analgesic drugs, available over the counter, was performed by DiCesare and coworkers (24). The separation of the four major components of these drugs, acetylsalicylic acid, caffeine, phenacetin and salicylamide is achieved in 3-4 minutes with isocratic elution at 2.2ml/min and a 10.0cm, 3um C18 column. In 1984 this separation was performed on a 3.0cm, 3um C18 column in less than 2 minutes (34). Gfeller et.al. separated and quantified similar pharmaceutical preparations within 40 seconds on a 10cm, 5um C18 column (38).

Other applications of clinical significance include the isocratic separation of cardiac agents, including procainamide, in 3 minutes on a 12.5cm, 5um C18 column, and the 7 minute isocratic analysis of water soluble vitamins such as ascorbic acid, niacin, and thiamine also on a 12.5 cm, 5um C8 column (14).

In 1983, Lin and Blank used 3um reverse phase columns for the determination of biogenic amines (39). Separation time was less than 3 minutes per sample. However using high flow rates, they experienced a loss in column efficiency. Fast, reproducible analysis of 6 different catecholamines was

reported in 1984 (40). HSLC coupled with amperometric detection affords a signal of 500pA/pmol at 0.7 Volts oxidizing potential and a flow rate of 1ml/min. The separation took less than 8 minutes using a 5cm column packed with 3um material and is illustrated in Figure 5. Earlier methods which achieved similar resolution took one hour for complete analysis (41).

DiCesare and coworkers reported the separation of 17 phenylthio-hydantoin (PTH) derivatized amino acids (14). They utilized step gradients and a 10.0cm, C18, 3um column for an analysis which was complete in less than 6 minutes.

Dong and Gant separated 12 nucleosides and bases in 70 seconds on a 3cm, C18, 3um column. Applied to deproteinized human serum, the separation was complete in less than 2 minutes (34).

Applications in biochemistry are now appearing in the literature; thus indicating that HSLC is becoming a more common tool in clinical, pharmaceutical, environmental and industrial laboratories. HSLC has wide applicability in routine analyses and is well suited for methods development where optimum conditions for a new analysis can often be determined in a matter of minutes.

The advantages of faster analysis times, increased mass sensitivity, and higher efficiencies make HSLC an attractive alternative for separation and quantitation of complex mixtures of components.

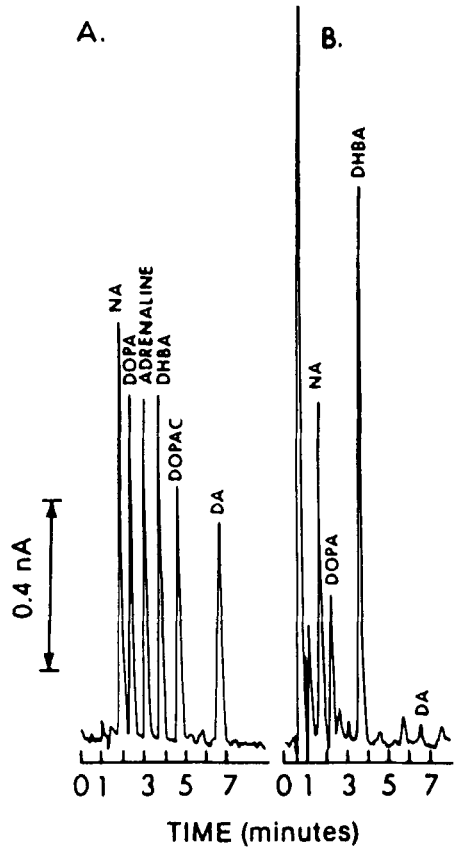

FIGURE 5.

Chromatograms of catechols. (A)Standards (2pmol each); (B)10ul of 75ul
alumina eluate of rat brain prefrontal cortex from an animal which had
been injected with an inhibitor of aromatic amino acid decarboxylase
(m-hydroxylbenzylhydrazine 0.72 mmole/kg i.p.) 30 minutes earlier.
Column: 5x0.5cm, 3um C18.
Mobile phase: 0.1 M monochloroacetic acid, pH 3.0, w/ 0.3mM Na^+
octylsulfate, 0.1mM EDTA, and 1% v:v acetonitrile at 1.0ml/min.
Amperometric detector: 0.70 Volts.

(Reproduced from reference 40).

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