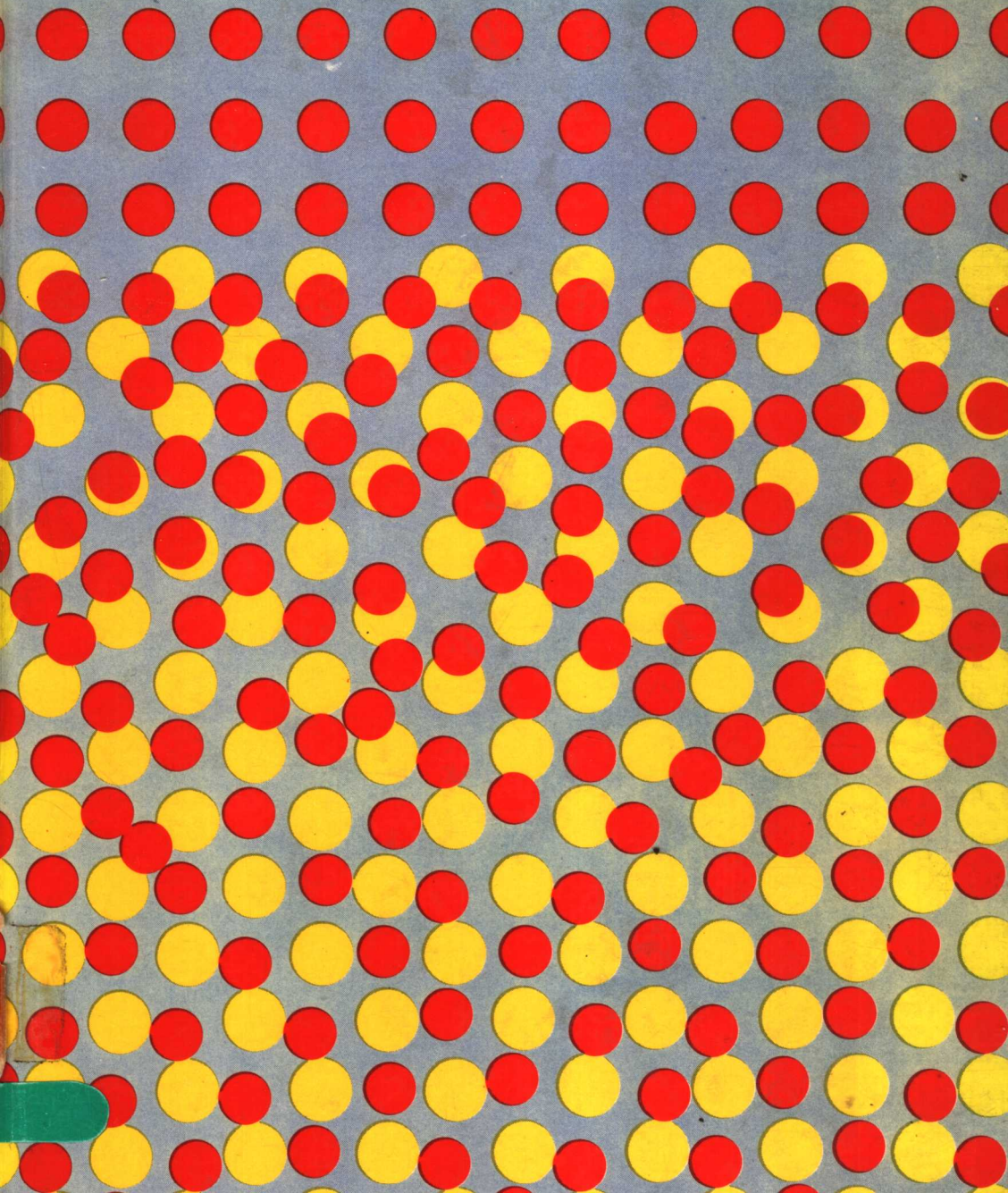
A. Pryde and M.T. Gilbert

# APPLICATIONS OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY



# Applications of high performance liquid chromatography

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# Preface

The rapidly increasing number of publications which are appearing on HPLC at this time makes it opportune to review progress in the applications field. In the first part of the book we have confined our discussion of chromatographic theory to those areas which have a direct bearing on practical results; HPLC equipment and the various modes of chromatography are discussed at more length and where possible we have tried to offer tips for avoiding some of the pitfalls of HPLC. In the succeeding sections, HPLC applications are divided into broad areas of interest (Pharmaceutical, Biochemical and Environmental analysis) and, within each section, treated according to compound class. Clearly, some of the material could have been classed in more than one section (this is especially true of some of the Pharmaceutical and Biochemical sub-sections) and we trust we have arrived at a satisfactory arrangement of the material.

We have tried to cover the literature up to the Spring of 1977. Completely comprehensive coverage of HPLC applications is becoming an increasingly difficult task since; as the technique has gained rapidly in popularity over the last few years, more and more applications are to be found in publications of a more general nature. Hopefully, we have covered each section in sufficient depth to provide an idea of the most promising HPLC systems in that area. In discussing individual applications, we have usually tried to give sufficient details of the chromatographic conditions used (e.g. the column dimensions, packing material and eluent, as well as the detection system and detection limits where appropriate) to convey an idea of the potential of the method.

In summary, we hope to have succeeded in our aim of providing a straightforward and practically-oriented text on HPLC applications which will help those who actually have to solve problems by HPLC to find at least one approach suitable to their problem.

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Finally, our thanks are in no small measure due to our secretary, Mrs Marjory Duncan.

# Abbreviations and symbols

Throughout this book the pressure conversion factors used are:

$$14.7 \text{ psi} = 1 \text{ bar} = 10^5 \text{ Nm}^{-2} = 10^5 \text{ Pa} = 10^6 \text{ dyne cm}^{-2} = 0.987 \text{ std atm.}$$

**Abbreviations**

| | |
|---|---|
| AUFS | absorbance units full scale |
| BOP | $\beta,\beta'$-oxydipropionitrile |
| DNP | dinitrophenylhydrazine |
| EDTA | ethylenediaminetetraacetic acid |
| GLC | gas-liquid chromatography |
| H.E.T.P. | height equivalent to a theoretical plate |
| HPLC | high performance liquid chromatography |
| IR | infrared |
| MS | mass spectrometry |
| NMR | nuclear magnetic resonance |
| ODS | octadecylsilyl |
| PEG | polyethylene glycol |
| RI | refractive index |
| TLC | thin layer chromatography |
| UV | ultraviolet |

**Symbols**

| | |
|---|---|
| $W$ | base width of peak |
| $k'$ | column capacity ratio |
| $d_c$ | column diameter |
| $L$ | column length |
| $k$ | column permeability |
| $\phi'$ | column resistance parameter |
| $A,B,C$ | constants in Knox equation |
| $D_m$ | diffusion coefficient |
| $\psi$ | eluent association factor |
| $u$ | eluent linear velocity |
| $M_{\text{eluent}}$ | eluent molecular weight |
| $\nu$ | eluent reduced velocity |
| $\epsilon$ | molar extinction coefficient |

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| | |
|---|---|
| $N$ | number of theoretical plates |
| $d_p$ | particle diameter |
| $H$ | plate height |
| $\Delta P$ | pressure drop down column |
| $h$ | reduced plate height |
| $R_s$ | resolution |
| $t_1, t_2$ | retention distances for two peaks |
| $R_f$ | retention rate in TLC |
| $t_r$ | retention time of retained peak |
| $t_0$ | retention time of unretained (solvent) peak |
| $\alpha$ | selectivity |
| $V_{solute}$ | solute molar volume |
| $\epsilon^0$ | solvent strength parameter |
| $\sigma$ | standard deviation |
| $T$ | temperature |
| $\eta$ | viscosity |
| $W_{HH}$ | width of peak at half height |

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# Part I
## Theory and practice of HPLC

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# 1

# Liquid chromatography: history and state of the art

The technique of high performance liquid chromatography (variously called high speed and high pressure liquid chromatography) although only around ten years old, has already made a significant contribution to pharmaceutical, biochemical, clinical and environmental analysis. The earliest example of a liquid chromatographic separation is credited to the Russian botanist Tswett who, in 1903, separated plant pigments by adsorption chromatography [1]. The technique was not followed up until the 1930s when Kuhn and Lederer [2] and Reichstein and Van Euw [3] again used adsorption chromatography for the separation of natural products. In 1941, Martin and Synge, who were subsequently awarded a Nobel Prize, described the discovery of liquid–liquid partition chromatography [4], and in the same paper laid the foundation of gas–liquid chromatography (GLC), and high performance liquid chromatography (HPLC). Martin and Synge introduced the concept of the 'height equivalent to a theoretical plate', which has since been adopted as the measure of chromatographic efficiency. They also pointed out that improved performance would be achieved in liquid chromatography by the use of smaller particles and higher pressures, and that the liquid mobile phase could be replaced by a gas. Around ten years later, in 1952, James and Martin described the first use of GLC [5] and the technique was rapidly developed during the ensuing decade. Liquid chromatography remained relatively neglected during this period although Hamilton *et al.* reported on the improvement in efficiency with reduction in particle size in the ion exchange chromatography of amino acids [6] and Snyder made a significant contribution to the understanding of adsorption chromatography [7].

In the early 1960s, Giddings showed that the theoretical framework developed for GLC applied equally well to liquid chromatography [8] and between 1967–1969, Kirkland [9], Huber [10] and Horvath, Preiss and Lipsky [11] described the first high performance liquid chromatographs. By operating at high pressure (up to 5000 psi; 34 MPa) these instruments overcame the effect of higher liquid viscosities relative to gas viscosities and gave analysis times comparable with GLC. In the early instruments, single or dual wavelength ultraviolet detectors allowed detection of nanogram amounts of suitably UV

absorbing compounds, but were insensitive to compounds with little or no UV absorbance. Since then, variable wavelength UV spectrophotometers have greatly increased the scope of the UV detector; refractive index and transport detectors have been developed as more universal detectors; selective detection systems such as spectrofluorimeters and electrochemical systems are becoming common, and HPLC/mass spectrometric interfaces are now being developed.

The key to increased efficiency and improved analysis times in HPLC lay in the development of suitable support materials in which the intraparticle diffusion rates were improved by reducing the distance over which the solutes had to diffuse. Thus, Horvath *et al.* [11] coated impervious glass beads with a layer of ion exchange resin, and Kirkland prepared a very efficient pellicular support for liquid–liquid partition chromatography by coating glass beads with a thin layer (1–2 $\mu$m) of silica microspheres [9]. Alternatively, fully porous particles of much smaller diameter (40 $\mu$m) than those used in GLC were used as these had higher capacity than the pellicular materials. The early HPLC materials could be packed reproducibly into chromatographic columns by dry packing techniques.

Further increases in efficiency have been obtained by reducing the particle size to its present value of around 5 $\mu$m. This size represents a good compromise between efficiency, pressure drop, analysis time and reproducibility of packing. For the small particles ($< 15$–$20$ $\mu$m) dry packing techniques cannot be used and slurry packing techniques have been developed.

The range of applicability of HPLC was extended by the preparation of chemically bonded stationary phases in which the nature of the adsorbent was modified by bonding organic groups to the adsorbent surface. The first bonded phases for HPLC were prepared by Halasz *et al.* who reacted silica with alcohols [12] and amines [13]. Subsequently, materials with greater hydrolytic stability were prepared by bonding organosilanes to the surface of silica and a wide range of such phases is now commercially available. The chemically modified adsorbents can be used for reversed phase and ion exchange chromatography.

Compared with classical column chromatography where the columns are gravity fed and a separation can take hours or even days, HPLC can offer analysis times of 5–30 min, times which are comparable with GLC. HPLC is particularly suited to the analysis of those compounds which are not readily handled by GLC. For example, thermally labile compounds can be analyzed at ambient temperatures by HPLC, highly polar compounds can be chromatographed without prior derivatization and polymeric samples can also be analyzed. Sample clean-up is usually much less of a problem with HPLC than GLC and biological fluids can often be directly injected onto an HPLC column. Much sample pretreatment is also avoided since aqueous solvents can be used in HPLC. A combination of these factors has meant that in the decade since its inception, HPLC has already made a significant impact in pharmaceutical, clinical, forensic and environmental analysis, and is an ideal complementary technique to GLC. Preparative HPLC is also beginning to make an impact and is

likely to be more successful than preparative GLC methods with their attendant problems of volatilization and thermal instability. Already gram quantities of materials have been purified by preparative HPLC techniques. On the analytical scale, selective fluorescence and electron capture detectors capable of monitoring $10^{-10}$ g of an injected compound have been developed. Automatic sample injection devices allow many samples to be analyzed without operator intervention and these will clearly be useful in clinical and quality control laboratories.

To summarize, the advantages which HPLC can offer in the analysis of pharmaceutical products, body fluid samples and environmental residue samples guarantee that the wide interest generated by the technique over the last decade will be maintained during the next one.

# 2

# Chromatographic parameters

## 2.1 Column capacity ratio

The column capacity ratio, $k'$, or simply the $k'$ value of a solute is the usual method of indicating solute retention. As shown in Fig. 2.1, $k'$ values are obtained from the elution chromatogram by

$$k' = \frac{t_r - t_0}{t_0} \tag{2.1}$$

where $t_r$ is the retention time of the given peak and $t_0$ is the retention time of the unretained (or solvent) peak. (Strictly speaking retention volumes should be used in chromatographic calculations rather than retention times. However, if constant flow rates can be assumed, retention times are more convenient to use and are obtained directly from the elution chromatogram). The use of $k'$ values is preferred to simply quoting retention times, since the latter can vary with flow rate variations from day to day, whereas $k'$ values remain constant.

The $k'$ value in HPLC is related to the $R_f$ value in thin layer chromatography (TLC) by

$$k' = \frac{1}{R_f} - 1 \tag{2.2}$$

or

$$R_f = \frac{1}{1 + k'}. \tag{2.3}$$

The $R_f$ value in TLC is the ratio of the distance travelled by the solute to the distance travelled by the solvent front. The $k'$ value measures the ratio of the time spent by the solute in the stationary phase to the time spent in the mobile phase.

## 2.2 Number of theoretical plates

The efficiency of a chromatographic column is measured by the number of theoretical plates, $N$, to which the column is equivalent. This parameter is

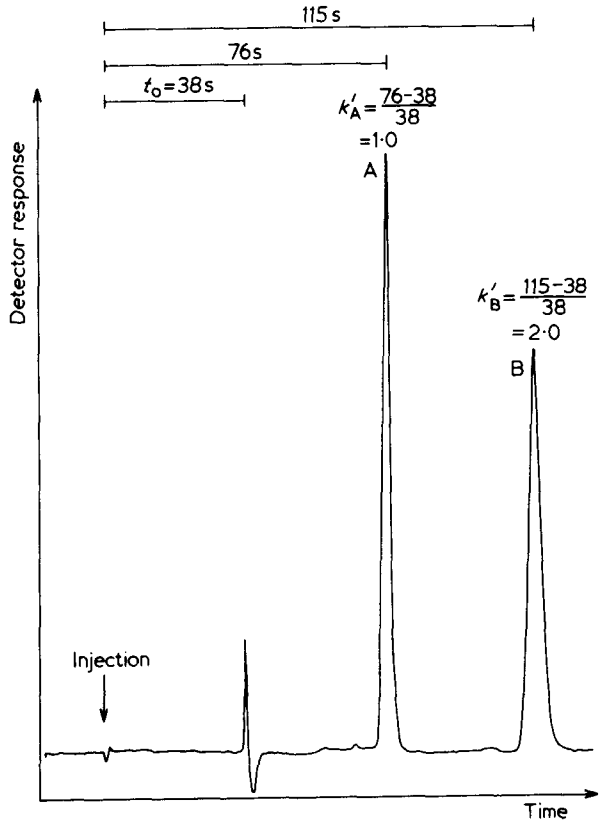


*Fig. 2.1* Measurement of the column capacity ratio, $k'$. (The unretained peak is a refractive index effect caused by injecting the samples in pentane, with hexane as eluent.)

calculated from

$$N = 16\left(\frac{t_r}{W}\right)^2 \tag{2.4}$$

where $t_r$ is the retention time of the peak and $W$ is the base width of the peak measured in the same units (mm, ml, s, etc.) and is obtained by extrapolation of tangents at the points of inflection to the baseline, as shown in Fig. 2.2. The plate number of a column is a measure of the amount of spreading of a solute band as it travels down the column, and efficient systems are characterized by high values of $N$. For a Gaussian peak, $W$ represents 4 standard deviations (*i.e.* $4\sigma$). It is also possible to calculate $N$ from the width of the peak at half height, $W_{HH}$, using

$$N = 5.54\left(\frac{t_r}{W_{HH}}\right)^2. \tag{2.5}$$

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