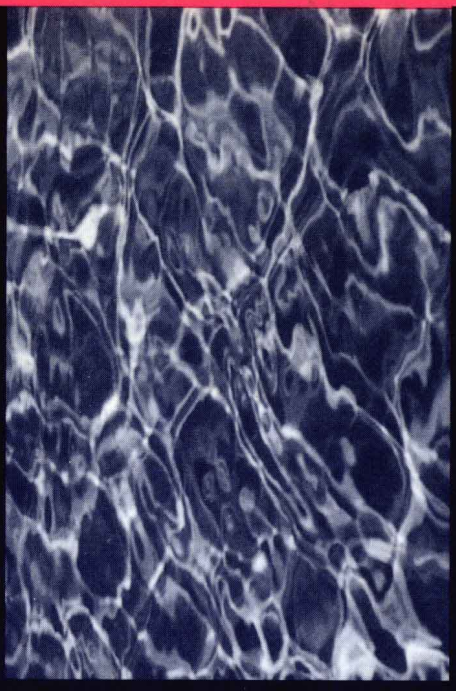

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

FUNDAMENTAL PRINCIPLES AND PRACTICE

Edited by W. J. Lough and I. W. Wainer

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High Performance Liquid Chromatography

Fundamental Principles and Practice

Edited by

W.J. LOUGH
School of Health Sciences
University of Sunderland

I.W. WAINER
Department of Oncology
McGill University
Montreal

江苏工业学院图书馆
藏书章

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Preface

Since its infancy in the late 1960s and early 1970s, the use of high performance liquid chromatography (HPLC, or LC as it is increasingly becoming known) has expanded rapidly. Today, it is a highly popular analytical technique which is extensively used in many fields of activity. Not surprisingly, it has been the inspiration for much scientific literature, including a significant number of books. These books have taken various forms, such as practical handbooks, modified short-term manuals, reports on selected topics, theoretical treatises and even attempts at comprehensive works.

In almost every way, HPLC may be regarded as a mature technique. However, there is one sense in which it is still developing. Only recently has its importance been acknowledged by accepting it as a subject worthy of inclusion in undergraduate science courses. The aim of this book is therefore to support the newly acquired status of HPLC by providing a presentation of the technique primarily aimed at undergraduate students on courses containing a significant component of analytical science. The intention has been to come up with a different slant to that already available, characterised by an emphasis on understanding. Why is HPLC so useful? When should it be used? What is the reason for certain practices? Most importantly, how does HPLC work? The responsibility to deliver a thorough coverage of theory has not been shirked.

While the book is, as the editors intended, for the teaching of undergraduate analytical chemistry, another aim is to make the book as versatile as possible. HPLC is taught not only on chemistry-based courses, but also on non-chemistry applied science courses. This is particularly the case for taught Masters courses. Students who have taken a more traditional science first degree often enhance their job prospects by proceeding to an applied science Masters degree, many of which have a strong analytical content. Versatility has therefore been sought by making this a contributed rather than a single author text. This is particularly important for the applications chapters, since an expert in each application area is best able to identify those aspects of HPLC which are important in that application area. Hence the analytical student will obtain an accurate view of the role played by HPLC in, say, environmental science, and the environmental science student will be directed to the most relevant parts of the book. By allowing a certain amount of overlap between contributors, versatility should also arise, because each chapter is more able to stand on its own and therefore be more accessible.

We wish to thank our colleagues and associates who have taken part in this project and trust that our readers will find the book fulfils the rationale described above.

W.J.L.
I.W.W.

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Contributors

P. Hambleton School of Health Sciences, University of Sunderland, Fleming Building, Chester Road, Sunderland SR1 3SD, UK

A.J. Handley ICI Chemicals and Polymers Ltd, PO Box 14, The Heath, Runcorn, Cheshire WA7 4QF, UK

D.K. Lloyd Department of Oncology, McGill University, 3626 St Urbain Street, Montreal, Quebec H2X 2P2, Canada

W.J. Lough School of Health Sciences, University of Sunderland, Fleming Building, Chester Road, Sunderland SR1 3SD, UK

T. Noctor Department of Biopharmaceutical Analysis, Hazleton Europe, Otley Road, Harrogate, North Yorkshire HG3 1PY, UK

D. Perrett Department of Medicine, St Bartholomew's Hospital Medical College, West Smithfield, London EC1A 7BE, UK

P.J. Rennie Yorkshire Environmental LabServices, Templeborough House, Mill Close, Rotherham, South Yorkshire S60 1BZ, UK

C.M. Riley Du Pont Merck Pharmaceutical Company, PO Box 80400, Wilmington, DE 19880–0400, USA

D. Stevenson Robens Institute of Health and Safety, University of Surrey, Guildford, Surrey GU2 5XH, UK

I.W. Wainer Department of Oncology, McGill University, Montreal General Hospital, 1650 Cedar Avenue, Montreal, Quebec, H3G 1A4, Canada

P. White Forensic Science Unit, University of Strathclyde, Royal College, 204 George Street, Glasgow G1 1XW, UK

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1 Introduction

W.J. LOUGH and I.W. WAINER

1.1 Analysis and chromatography

Before embarking on a study of high-performance liquid chromatography (HPLC), it is instructive to take a look at how this analytical technique emerged and to consider its relative importance in the field of analytical chemistry. Although HPLC is used to determine what is in a sample (qualitative analysis), its primary application is as a quantitative analytical tool and as such it plays a key role in analytical methods, since the analytical technique is only one part of the overall analytical method. An analytical method may be thought of as consisting of five distinct parts:

- defining the problem
- taking the sample
- sample pre-treatment
- measurement
- calculating, assessing and reporting the results

While great importance is attached to the measurement step, the other parts of an analytical method are by no means trivial. For example, all the subsequent steps depend on defining the problem. In choosing the appropriate subsequent steps in the method it must be borne in mind exactly what it is that it is wished to find out about the sample. For example, it is very likely that the steps taken to confirm that the content of a formulated drug substance was within specification (i.e. the amount of drug present was within acceptable limits as stated on the container label) would differ from those taken to determine a minor degradation product. Similarly, there are large differences in the sampling of liquids, gases and solids. Liquids and gases are homogenous and sampling often only amounts to ensuring that a large enough sample has been taken. Solid samples on the other hand can be non-homogenous and it is very important that great care is taken to ensure that a representative sample is taken by sampling from different parts of the whole. 'Sample pre-treatment' also may be a simple and straightforward task amounting only to ensuring that the analyte is in a phase compatible with the measurement technique and present in an appropriate concentration. However, as will be seen elsewhere in this book, sample pre-treatment is often a very complex and important operation. The importance of 'calculating and

reporting the results' is self-evident and obviously cannot be emphasised enough. The assessment of the results particularly by the extensive use of statistical methods is also an important and ever-increasing feature of the work of an analyst.

To understand why HPLC is frequently used in quantitative analytical methods, it is useful to assess whether or not an analytical method is suitable for its intended purpose and, in doing so, consider the deficiencies in methods employing classical measurement steps. Analytical method validation is the process of assessing the fitness for purpose of an analytical method; in choosing an analytical method issues such as cost, simplicity, operator experience, availability etc. are of secondary importance to the actual validity of the method under consideration. In the validation procedure, tests are typically carried out for the following properties:

Specificity. An assay is specific if the 'analytical response' (i.e. that which is measured) arises from the analyte of interest only and cannot arise from any other compound likely to be present in the sample.

Robustness. A robust or rugged method is one which is not greatly affected by minor changes in experimental variables that might reasonably be expected during the course of an assay.

Linearity. A method is linear if there is a linear relationship between the 'analytical response' and concentration of analyte in the sample solution over a specified range of concentrations of the analyte (e.g. 20–120% of the anticipated levels of analyte concentration in the sample solutions). It would also be anticipated that the plot of 'analytical response' versus analyte concentration would have a negligible intercept.

Precision. The precision of a method is the degree of scatter of the results and is usually reported as a percentage relative standard deviation. It is often subdivided into repeatability (precision on replicate measurements of the same solution) and reproducibility (precision of the results from measurements of different solutions, i.e. of the complete method).

Accuracy. An assay is accurate if the mean result from the assay is the same as the true value (i.e. there is no bias (systematic error)). Assessment is notoriously difficult since often the true value is not known but good accuracy is generally a consequence of the other validation parameters being within acceptable limits.

Limit of detection. The limit of detection is the amount of analyte which can be reliably detected under the stated experimental conditions. Often a

statistical approach may be adopted in defining what is 'reliable' and what is not.

Limit of quantitation. The limit of quantitation is the amount of analyte which can be reliably quantified under the stated experimental conditions. Often a statistical approach may be adopted in defining what is 'reliable' and what is not.

Stability in solution. It is necessary to study stability in solution in order to be able to make a statement such as, e.g. "the analyte was sufficiently stable in solution in the solvent used for preparing sample solutions for reliable analysis to be carried out."

1.2 HPLC versus other analytical methods

The subject of analytical method validation is very complex but the brief explanation given above of the tests that must be carried out should be sufficient to enable an evaluation of the relatively simple techniques that were used prior to the development of chromatographic techniques such as HPLC.

1.2.1 Volumetric analysis

Volumetric analysis using titration methods is very cheap and simple. In recent times it has even been possible to automate such methods thereby reducing operator errors and increasing sample throughput. However, although such methods may be adapted to give reasonably low limits of detection and quantitation they are normally used in the determination of millimolar to high micromolar quantities in fairly concentrated solutions (e.g. 0.05 M). Precision, accuracy and robustness are often problems in volumetric analysis but only if there has been an inappropriate choice of indicator. Stability in solution is related to the analyte and the solvent in which it is dissolved and is not dependent on the measurement technique being used. Probably the most important validation test is that for specificity and unfortunately it is here that volumetric analysis falls down. This is particularly the case for determining the purity of complex molecules such as drugs. Such molecules are generally prepared by a multi-stage synthesis and the drug sample may contain water, solvents, trace catalysts, inorganics, and structurally related synthetic by-products, i.e. intermediates, isomers, degradation products and products of side reactions. For a basic compound it is highly likely that all or almost all of these structurally related impurities will also be basic. Therefore titration against an acid would not give purity but would give some measure of the

total amount of drug, structurally related impurities and other basic impurities. Thinking particularly of this example of drugs, this is obviously not an acceptable situation, since it is usually most important to determine the structurally related impurities since it is these that are most likely to give rise to toxicological problems.

1.2.2 Ultraviolet spectrophotometry

Similar difficulties arise when using ultraviolet (UV) spectrophotometry, in which analyte concentrations are determined by measuring the absorbance of UV light by the sample solution. For this common, simple technique, detectability (down to low nanogram levels) is good and there is a wide linear range. However, that all-important feature, specificity, is again a problem. For structurally related impurities of a UV-absorbing compound it is highly likely that they will contain a very similar chromophore (i.e. the part of the molecule which actually absorbs the UV radiation) or part of the chromophore to that present in the compound. Therefore incident radiation at any particular wavelength of the UV spectrum of the compound would almost certainly be absorbed by structurally related compounds as well as the compound itself.

1.2.3 Using chromatography to improve specificity

There are two approaches to solve this critical problem of lack of specificity in the relatively simple analytical techniques discussed above. One is to measure a physical or chemical property of the analyte that is unique. This is a tall order since not just acidity, basicity and UV spectrum but almost all the properties of compounds are similar to those of their structurally related impurities. The property that perhaps comes closest to being unique is the mass spectrum of a compound (i.e. the pattern obtained when a compound is ionised under high energy conditions and the resultant ions traverse a magnetic field) which gives characteristic information on molecular mass and the mass of fragments of the molecule. However, mass spectrometry (MS) requires expensive instrumentation and therefore only under very challenging circumstances would it be considered an appropriate option for routine quantitative analysis (also it can fail to distinguish between isomers).

The second approach to specificity in analysis is fortunately more readily achievable. It involves separating all the components of a mixture from one another (or at least from the analyte) before measurement takes place. To a certain extent this may be done in the sample pre-treatment step of an analytical method which, as well as ensuring the analyte is in a suitable phase in a suitable concentration for measurement, typically involves some form of sample 'clean-up'. By using simple separation pro-

cesses such as dissolution and filtration, liquid–liquid extraction, etc. in the sample pre-treatment step of the analytical method, it is possible to rid the sample of gross interferences. To use a pharmaceutical illustration again, in determining drugs in biological fluids, it is possible to remove large and small polar molecules endogenous to the biological fluid leaving behind primarily the drug and its metabolites. However, such sample pre-treatment will rarely isolate the analyte from structurally related compounds present in the sample. It is therefore necessary to use more powerful separation processes, i.e. chromatographic techniques.

Chromatography is a differential migration process where sample components are distributed between a stationary and a mobile phase. Depending on the affinity of a component for either or both of these phases, the rate of migration varies from zero to the velocity of the mobile phase. The distribution coefficient (D) for an analyte between the two phases is

$$D = A_s / A_m$$

where A_s is the activity (often approximates to concentration) of the analyte in the stationary phase and A_m is the activity of analyte in the mobile phase. The larger the value for D the greater the affinity of the analyte for the stationary phase. If two components of a mixture have even a very small difference in their D values, they may be separated, since the chromatographic process of separation may be repeated a large number of times and this serves to amplify that difference as migration proceeds.

Since chromatographic techniques are separation processes they could be regarded as very powerful means for 'sample pre-treatment'. However, since the term, chromatography, is generally regarded as incorporating the 'measurement' step (i.e. detection subsequent to the separation), it is more accurate to view it as a combination of 'sample pre-treatment' and 'measurement'. Also it is frequently not the complete 'sample pre-treatment' since very often a more rudimentary form of sample pre-treatment will be required prior to chromatography.

HPLC is but one of several chromatographic techniques. Having looked at the role of chromatographic techniques in analysis in order to appreciate their importance, it now remains to look at how HPLC emerged as a logical progression out of the historical development of chromatography.

1.3 Historical development of chromatography

There are a number of examples of work carried out prior to the twentieth century in which experiments were conducted which are recognisable in retrospect as early forms of chromatography. However, it was the work

of Russian botanist Michael Tswett, first publicly reported in 1903, that constituted the first systematic study of what would today be recognised as chromatography. He used column liquid chromatography in which the stationary phase was a solid adsorbent packed into a glass column and the mobile phase was a liquid. He carried out experiments on chlorophyll extracts in petroleum spirit with over 100 adsorbents. Although most of these adsorbents are now no longer important, it is interesting to note in the list the inclusion of materials such as silica, alumina, charcoal, calcium carbonate, magnesia and sucrose which are still in use. He also confirmed the identities of the fractions obtained by spectrophotometry at various wavelengths thus anticipating the commonest mode of detection in liquid chromatography. In a paper published in 1906 he first used the term 'chromatography' (from the Greek *khroma, -atos* colour; *graphos* writing). In 1910 Tswett obtained a Russian doctorate (having received his earlier education in Switzerland) and his doctoral thesis was published as a monograph again showing further development and refinement of his ideas. This monograph marked the end of his chromatographic work. This was not surprising since he was a botanist and chromatography was merely a means to an end.

Despite the fact that Tswett travelled widely in Germany, France and Holland and must have discussed his ideas on chromatography with leading scientists of those countries, chromatographic methods were largely ignored until the early 1930s. One of the few exceptions was the work of an American, L.S. Palmer, who from 1913 onwards published a number of papers describing the use of column liquid chromatography for the separation of the pigments in plants and dairy products. This culminated in a book published in 1922 summarising his work and in which the earlier work of Tswett was acknowledged. There are various reasons for the lack of interest in chromatography at the time, the main one being that it was a relatively small-scale physical method and the scientific community was mainly orientated towards large-scale synthetic organic chemistry.

In 1930 in Germany, Edgar Lederer drew upon the work of Tswett and Palmer in using chromatography in an investigation into the pigments in egg yolk. Because of the relative speed of the technique it was possible to avoid the degradation of the carotene molecules. Thereafter there was steady success including the development of forms of chromatography other than column liquid chromatography and of instrumental methods of analysis (e.g. infrared spectroscopy and mass spectrometry) which would much later be incorporated into instrumental chromatography. In 1938 Eastern European workers carried out planar chromatography in which the powder was spread on a glass plate. Thin-layer chromatography had its origins in this work but at this time the plate had to be horizontal otherwise the layer of powder would be displaced.

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