HPLC of macromolecules

a practical approach

Edited by

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Preface

This text is a companion volume to *HPLC of Small Molecules: A Practical Approach*, edited by Dr C.K.Lim. The aim of the present text is to present in a concise, experimentally explicit manner, examples of high-performance liquid chromatographic separations of large biological molecules (molecules having a molecular mass greater than 1000). Because of the differences in size of the molecules under consideration, chapters are included on the application of the techniques of size-exclusion HPLC and also of high-performance affinity chromatography in addition to the more common HPLC techniques presented in its sister volume. There are also chapters on HPLC column support materials and on HPLC instrumentation since these subjects were not included in the previous volume.

It is hoped that this text will encourage more workers to attempt to purify and analyse intact large biological molecules by HPLC rather than degrading these and then purifying the resultant small fragment molecules. It is advisable for the researcher in biological materials to gain experience in HPLC techniques as applied to macromolecules because once the initial analytical separations have been achieved these provide the basis for subsequent preparative scale separation. This means that the recent developments in recombinant DNA technology which have led to the ready production of mixtures of polypeptides and proteins in large quantities, many of which are of considerable therapeutic and industrial importance, may be exploited since these mixtures may then be purified. It is probably true to state that most reported analytical and small scale purification of such mixtures contain at least one HPLC step.

Reversed-phase (RP)-HPLC is probably still the most widely used mode of HPLC separation for such mixtures in spite of the fact that significant losses of protein may occur. Thus a wide-pore C_{18}-column has been used to purify calcitonin, a 32-amino acid peptide hormone, which had been produced in *Escherichia coli* as a fusion product of chloramphenicol acetyltransferase to calcitonin–glycine. A cation-exchange HPLC method was used to separate the polypeptide hormone urogastrone produced in *E.coli* by recombinant DNA methods. Size-exclusion HPLC has been employed to separate the large hepatitis B immunoglobulin aggregates whilst affinity HPLC has been used to purify so many hundreds of enzymes that it would be inviduous to pick out any one for special mention here. For the separation of very complex mixtures obviously either specific sample pre-treatment, e.g. solvent extraction, followed by a single HPLC technique may be employed and examples of this are to be found in this text. Alternatively, combinations of the various modes of HPLC can and have been employed, for example in the purification of membrane proteins.

The range of biological macromolecules covered in the chapters on specific applications of HPLC includes polypeptides and proteins, enzymes, oligonucleotides and glycopeptides. The presentation and discussion of the HPLC separation of such a wide range of materials could only have been done by enlisting the experience of a number of researchers, both academic and industrial, who are expert in these various fields. The editor would therefore like to thank all of the invited authors for giving

their time and energy to write their chapters in the first place and for their patience, understanding and co-operation which has led to its final production in the style of the Practical Approach series. Finally the editor would also like to thank all of the Instrument Companies which responded so promptly to his enquiries and especially to Messrs. Kontron for providing excellent photographs of parts of equipment not normally seen!

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Abbreviations

AC	affinity chromatography
ADH	alcohol dehydrogenase
p-APM	*p*-aminophenyl-α-D-mannopyranoside
AUFS	absorbance units full scale
BSA	bovine serum albumin
CAGG	*N*-chloroacetylglycylglycine
CM	carboxymethyl
CDI	carbonyldiimidazole
Con A	concanavalin A
DEAE	diethylaminoethyl
DMF	dimethylformamide
DMSO	dimethylsulphoxide
ELISA	enzyme-linked immunosorbent assay
E-PHA	phytohaemagglutinin-erythrocytes
GalNAc	*N*-acetylgalactosamine
GlcNAc	*N*-acetylglucosamine
HA	hydroxyapatite chromatography
HFBA	heptafluorobutyric acid
HIC	hydrophobic interaction chromatography
HK	hexokinase
HPAC	high-performance affinity chromatography
HPIEC	high-performance ion-exchange chromatography
HPLC	high-performance liquid chromatography
HSA	human serum albumin
IEC	ion-exchange chromatography
L-PHA	phytohaemagglutinin-lymphocytes
LCA	lentil lectin (*Lens culinaris*) agglutinin
LDH	L-lactate dehydrogenase
α-mm	α-methyl mannoside
NeuAc	sialic acid (*N*-acetyl-neuraminic acid)
OPD	orthophenylene diamine dihydrochloride
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
PEI	polyethyleneimine
PGK	3-phosphoglycerate kinase
PNA	peanut agglutinin
QEA	quaternary amine
RCA	*Ricinius communis* agglutinin
RP-HPLC	reversed-phase HPLC
RPC	reversed-phase chromatography
SAX	strong anion-exchange
SDS	sodium dodecyl sulphate
SE	size-exclusion
SEC	size-exclusion chromatography
SE-HPLC	size-exclusion HPLC
TAPA	trimethyl (*p*-aminophenyl) ammonium chloride

TCA	trichloroacetic acid
TEAP	triethylammonium phosphate
TFA	trifluoroacetic acid
TSH	thyrotropin
WGA	wheat germ agglutinin

Pressure units

1 bar ≡ 14.5 p.s.i. ≡ 0.1 MPa.

Contents

CHAPTER 1

Columns for HPLC separation of macromolecules

DENISE JOHNS

1. INTRODUCTION

The application of high-performance liquid chromatography (HPLC) to the separation of macromolecules is a fairly recent development. Traditionally, protein chemists have used semi-rigid or non-rigid gels which have a relatively low mechanical strength, limiting the chromatographer to low flow-rates and large particle sizes, and resulting in poor resolution with long separation times. Alternative techniques, such as electrophoresis consequently prevailed.

HPLC was limited by the slow development of suitable alternative stationary phases, but as a result of the last few years progress, the chromatographer is now presented with a bewildering choice of both techniques and stationary phases. This chapter will try to unfold the seemingly complex nature of modern HPLC support materials by explaining the reasons for their development and the differences between the major phases available today. Because different types of biological macromolecules utilize varying types of chromatographic technique, proteins and peptides, oligonucleotides, immunoglobulins and oligosaccharides will be discussed separately.

2. COLUMNS AND PACKING MATERIALS FOR PROTEIN AND PEPTIDE HPLC

What is required for the separation of proteins and peptides is a column support material that will withstand high pressures, allowing eluants to be pumped through the column at high velocities, and one which avoids the polar, highly absorptive surface associated with many untreated silica based HPLC phases.

In summary, a support for the separation of these biopolymers ideally needs to be:

(i) chemically stable, but not unreactive such that chemical modification is difficult;
(ii) macroporous, to allow high-molecular-weight proteins to permeate and make contact with the media surface, and yet rigid enough to withstand the operating pressures encountered in HPLC;
(iii) well endowed in surface area, for best possible resolution characteristics;
(iv) weakly hydrophilic, to allow wetting of the surface, but not soluble in water;
(v) if the surface of the support requires modifying, or bonding, in order to achieve these criteria, such bonding needs to be highly selective so as to minimize non-specific adsorption.

Several approaches have been made to solve these seemingly insurmountable criteria and many are highly successful. The fact that proteins vary enormously in their behaviour

can be utilized for their separation in HPLC. The diverse biological and biochemical functions of proteins are largely due to the different amino acid side chains which convey different properties onto the protein, providing a guide to possible methods of separation.

(i) Interaction between the side chains of a protein or peptide results in a specific (tertiary) structure for the protein and hence differences in size can be utilized for HPLC [size-exclusion chromatography (SEC), or SE-HPLC].

(ii) Amino acid side chains may be ionized, either as anions or cations and this ionization will change with pH (making ion-exchange chromatography a useful mode to use).

(iii) Amino acid side chains may also differ in their polarity: reversed-phase chromatography is therefore a popular technique.

(iv) Because of their different conformational states, proteins will bind specifically to certain molecules, for example enzymes will bind to substrates, co-enzymes, inhibitors and activators. If these are immobilized on an HPLC column, separation may be achieved (affinity chromatography).

The availability of column supports which utilize each of these four HPLC modes for the separation of proteins and peptides is discussed in Sections 2.1–2.4.

2.1 **Size-exclusion HPLC**

Although high-performance columns for size-exclusion have been available for some time, the significant development for protein chemists has been the recent introduction of rigid aqueous compatible SEC materials. These allow the separation of biopolymers by molecular size, the largest molecules eluting from the column first.

2.1.1 *Choice of SEC column*

Stationary phases for SEC achieve separation by simply depending on the size of the molecules affecting the degree of penetration into the pores. The pore size is therefore chosen such that small molecules pass in and out of the pores essentially without hindrance, whilst large molecules which do not penetrate the pores, elute from the column first at the void volume. Molecules of intermediate size, about the size of the pore, pass through the column with difficulty, resulting in differing retention times. Some columns, notably silica gel columns (1,2), have a high enough resolution to separate molecules which have small size differences, even for molecular weights below 1000.

The initial task in using these columns is to choose the appropriate pore size for the separation. In practice, all manufacturers quote a molecular weight exclusion limit, above which a protein molecule will be totally excluded, and they may also provide a calibration curve which plots (i) the logarithm of molecular weight against elution volume (*Figure 1*) or (ii) the logarithm of molecular weight against the distribution coefficient K_D (*Figure 2*). K_D is defined as the ratio

$$\frac{V_e - V_o}{V_t - V_0}$$

where V_e = elution volume of a macromolecule, V_o = void volume of the column,

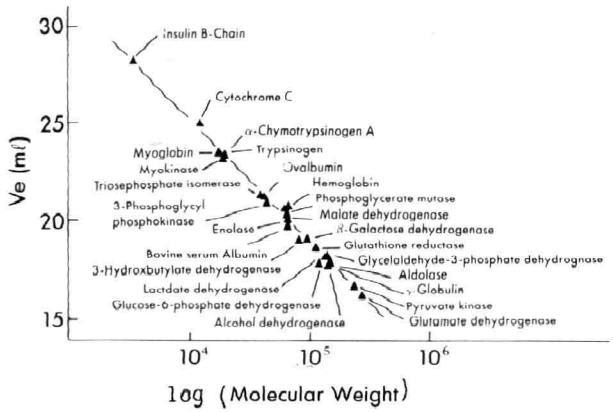

Figure 1. Calibration curve for proteins on TSK 3000SW columns. Column, TSKgel G3000SW (7.5 mm i.d. × 60 cm); eluant, 50 mM phosphate buffer, pH 7.5 + 0.2 M NaCl; flow-rate, 0.3 ml/min.

V_t = elution volume of small molecule. The standards used to derive these calibration curves are frequently dextrans or sulphonated polystyrenes, although this tends to (incorrectly) assume a linear structure for the biological macromolecule. The behaviour of proteins and peptides on SEC columns is more accurately represented by the use of globular proteins as calibration standards. Anderson and Stoddart observed (3) that in the middle of the K_D range ($K_D = 0.15-0.80$) these theoretical plots are essentially linear (see *Figure 2*), and they can therefore assist in the choice of SEC column. It is consequently generally best (4) to choose a column on which the molecules of interest elute near the middle of the calibration curve, where this linearity occurs. Occasionally, two or more columns may be used in series. When connected in series, columns of the same pore size will increase resolution, whilst columns of different pore size will broaden the range of molecular sizes separated.

The choice of SEC column must also take into account whether the protein is to be denatured before analysis. It is common to employ denaturing mobile phases, such as those containing detergents or organics, particularly when using silica gels (which are very stable in the presence of denaturants). The use of sodium dodecyl sulphate, urea, guanidinium chloride, or organic solvents such as acetonitrile, will denature the protein, preventing self-association, and increasing solubility. This can, however, greatly decrease the radius of the protein, and their exclusion range will move to lower molecular weights. *Table 1* shows reported exclusion limits for SEC columns of different pore sizes; denatured proteins will exhibit calibration curves more closely resembling linear molecules than those of globular proteins.

One final criteria for the choice of SEC column is that there should be no interaction between the solute and the surface of the stationary phase so that there is nothing eluting after the smallest molecule, that is separation occurs exclusively by size-exclusion. The problem of excessive interaction of the protein with the stationary phase, especially common with silica based materials, is solved by bonding the surface with a modifier. However, bonding on silica is often incomplete for steric reasons, so these materials

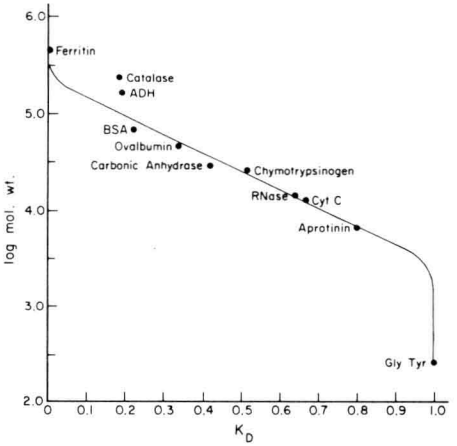

Figure 2. Calibration curve for proteins on SynChropak 100 (5 μm).

Table 1. Reported molecular weight exclusion limits for linear and globular macromolecules on differing pore size SEC supports manufactured by SynChrom Inc. (SynChropak).

Pore size of SEC support (nm)	*Molecular weight*			
	Linear molecule		*Globular molecule*	
	Excluded	*Included*	*Excluded*	*Included*
6	20 000	300	30 000	300
10	60 000	2000	300 000	3000
30	200 000	4000	1 500 000	6000
50	300 000	9000	10 000 000	20 000
100	400 000	15 000	50 000 000	25 000
400	10 000 000	20 000	Not available	

often exhibit residual ion-exchange properties. The effects of the slightly negative charge on residual silanols can be compensated for by adding salts to the aqueous mobile phase. Generally, a 0.05–0.2 M solution of buffer (e.g. potassium phosphate) is effective, although care should be taken not to use too high a salt concentration since this increases hydrophobic interactions. If these occur, an organic modifier, such as methanol, can be added, or an alternative mobile phase could be used (e.g. 0.1% v/v trifluoroacetic acid for the elution of peptides).

2.1.2 *Commercially available SEC materials*

Columns containing support materials having pore sizes from 5 to 400 nm are readily available. *Table 2* lists some of those available commercially and includes both silica based and crosslinked polystyrene (polymeric) supports. The manufacture of SEC materials having a carefully controlled pore size and pore size distribution is accomplished in several ways. For polymeric materials, there are basically two methods of manufacture. By carefully controlling the crosslinking in a polystyrene–divinyl