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Review

Recent progress in reversed-phase and hydrophobic interaction chromatography of carbohydrate species☆

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Abstract

This review article is aimed at (i) providing a brief description of the fundamentals of reversed-phase liquid chromatography (RPLC) and hydrophobic interaction chromatography (HIC), (ii) describing the most useful chromatographic systems for RPLC, ion-pair RPLC and HIC of carbohydrate species, (iii) discussing the operating conditions as applied to the area of mono- and oligosaccharides, glycopeptides and glycoproteins and (iv) describing recent applications.

Contents

1. Introduction

Reversed-phase liquid chromatography (RPLC) and to a lesser extent hydrophobic

☆ Dedicated to Professor Csaba Horváth on the occasion of his 65th birthday.

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interaction chromatography (HIC) are currently widely used in the separation and determination of carbohydrate species. While HIC has been exclusively used for the separation of proteins including glycoproteins, RPLC has been applied successfully to the separation of both small and large carbohydrate molecules. In RPLC and HIC, solute retention has the same intrinsic physicochemical basis: the hydrophobic effect, which is essentially a solvent effect. In both RPLC and HIC, the non-polar moieties of the solute and the stationary phase undergo non-covalent association in hydro-organic media or aqueous solutions of neutral salts, respectively. In contrast, the operation conditions as well as the nature of the mobile and stationary phases used in HIC and RPLC are very different. In RPLC, the stationary phase is a highly non-polar surface consisting mainly of long alkyl chains covalently bound to the solid support. In HIC, the stationary phase consists of a highly hydrophillic organic layer to which widely spaced, short alkyl or small aryl functions are attached. These considerable differences in the nature of the stationary phases employed in RPLC and HIC, mandate the use of different mobile phases and operating conditions to bring about elution and separation of the solutes.

This article is to complement a recent and extensive review by the author on the topic of RPLC and HIC of carbohydrates and glycoconjugates [1]. Therefore, besides providing a summary of earlier and relevant work, an emphasis will be placed on describing the advances made in the methodology and application of HIC and RPLC in the carbohydrate area over the last two years. Of course, in overviewing the fundamentals of HIC and RPLC, original work from prior years will be discussed.

2. Basic theoretical concepts

In this section, the underlying principles for retention and separation in HIC, RPLC and ion-pair RPLC are outlined in simple terms for clarity of presentation. For more rigorous and detailed treatments, the interested reader may consult the referenced literature.

The retention process in HIC and RPLC involves an interaction between two non-polar groupings: the hydrophobic moiety of the solute and the non-polar ligand bonded to the surface of the solid support. According to the thermodynamic model of Horváth and co-workers [2–7], which is based on the solvophobic theory of Sinanoglu [8], the free-energy change upon solute binding is related to the process of the solvation of the solute, the stationary-phase ligands and the complex of the two. The solvation process of the solute involves two distinct steps. First, a cavity in the solvent is created of the same shape and size as the incoming solute. Second, the solute enters the cavity and subsequently interact with its surroundings through electrostatic and Van der Waals forces. The free energy for cavity formation is of particular importance since it is mainly responsible, together with the Van der Waals interaction, for the hydrophobic effect.

In chromatography, solutes undergo a series of sorption–desorption steps while moving down the column. According to the hydrophobic theory [9], the net free-energy related to the reduction of the cavity size as a result of solute binding or sorption, ΔG_c^0, increases with the contact area between the solute and the stationary-phase ligand, ΔA, and with the microthermodynamic surface tension of the mobile phase. In general, ΔG_c^0 can be expressed as [7]

$$\Delta G_c^0 = -N\gamma\Delta A - N\gamma A_s(\kappa^e - 1) \quad (1)$$

where N is the Avogadro number, A_s is the surface area of the solvent, γ is the surface tension of the solvent and κ^e is a correction factor for surface tension for solute of radius r in solvent. In HIC, Eq. 1 takes the following approximate form [6]

$$\Delta G_c^0 = -\Delta A\sigma m + \text{constant} \quad (2)$$

which reflects the direct proportionality of ΔG_c^0 on the salt molality m and the molal surface tension increment σ. ΔA is given by [7]

$$\Delta A = (A_l + A_x) - A_{xl} \quad (3)$$

where A_l is the surface area of the stationary phase ligand, A_x the surface area of the cavity (i.e., surface area of the solute) and A_{xl} is the surface area of the solute–stationary-phase complex. Thus, ΔA represents the molecular surface area by which the total cavity area is reduced upon solute binding to the stationary phase. In other words, it is the surface area no longer accessible to the solvent when the complex formation occurs, i.e., the non-polar contact area between the two species in the complex.

According to Eq. 1, the higher the value of γ, the greater the retention. For hydro-organic solvent mixtures that are used as eluents in RPLC, the surface tension decreases almost exponentially with increasing the amount of the organic solvent in the mobile phase, while in HIC with aqueous buffer solutions containing neutral salts, γ increases linearly with increasing the molal concentration, m, of the salt in the eluent as follows [2,6]

$$\gamma = \gamma^0 + \sigma m \tag{4}$$

where γ^0 is the surface tension of pure water and σ is the molal surface tension increment of the salt.

Therefore, the capacity factor k' decreases with increasing organic solvent concentration and decreasing salt concentration in the eluent in RPLC and HIC, respectively. Solutes are most strongly retained on RPLC sorbents when plain water is the eluent since it has the highest surface tension among all common solvents, whereas in HIC, where the hydrophobic character of the stationary phase is much weaker than in RPLC, plain water is usually a strong eluent. In RPLC, the effect of the organic modifier is such that the retention decreases, to a good approximation, exponentially with increasing the organic solvent concentration in the eluent. In fact, plots of log k' against percent (v/v) of organic modifier are quasi-linear [10]. The slope of such plots is negative and roughly proportional to the size of the solute molecule. Thus, the retention of large molecules such as proteins is more sensitive to small changes in organic modifier concentration than small molecules and the former are better separated by gradient elution.

The retention behavior of proteins in HIC as a function of the salt concentrations in the eluent has been shown to follow the trends predicted by the solvophobic theory [6,10,11]. The capacity factor, k', first decreases with increasing salt concentration because the electrostatic effect of the salt weakens solute binding. With further increase in the salt concentration, hydrophobic interaction becomes predominant so that k' increases, and the dependence of log k' on salt molality becomes linear at sufficiently high salt concentration according to the following relationship [6]

$$\log k' = \lambda m + C \tag{5}$$

where C is an amalgam of the salt-independent terms and λ is the limiting slope of the plot of log k' versus salt molality m. The limiting slope λ, termed the hydrophobic interaction parameter, is given in the simplest cases by

$$\lambda = \sigma \Delta A \tag{6}$$

This slope was shown to correlate with the molecular mass of the proteins, when a given column was used, and with the hydrophobic character of the stationary phase when a given set of proteins was used under otherwise identical conditions [11]. In other words, λ was strongly influenced by ΔA. Also, λ was largely affected by σ, and the slopes of the lines (i.e., plots of log k' versus m) for sodium sulfate were greater than for ammonium sulfate for various model proteins [12]. With certain salts such as $MgCl_2$ and $CaCl_2$, due to specific salt binding by the protein [13], the observed retention behavior is different from that predicted by the surface-tension argument [14].

The effect of salt on protein adsorption in HIC has also been treated by applying Wyman's thermodynamic theory of linked functions for equilibrium reactions [15,16]. The linked-functions approach [17] allowed the correlation of HIC adsorption to preferential interactions of the salts with the stationary phase and the protein analyte [18,19]. For further details, the interested reader is advised to consult Ref. [20].

In both HIC and RPLC, in addition to Van der

Waals interactions with both the stationary and the mobile phases, the solute undergoes polar interaction (e.g., electrostatic interaction) with the mobile phase. Thus, under a given set of conditions, the introduction of polar groups into the solute molecule usually results in decreasing retention. Also, the ionization of the solute with mobile-phase pH strongly affects solute retention. Usually, solute retention decreases with increasing ionization due to increasing polar interactions with the mobile phase. Therefore, for a sufficiently small sample, if the solute is in rapid equilibrium between ionized (i-form) and non-ionized forms (j-form), a single elution band will be observed and its k' value will be given by

$$k' = f_i k'_i + f_j k'_j \quad (7)$$

where f_i and f_j represent the fraction of solute in ionized and non-ionized form, respectively, at any time, and k'_i and k'_j refer to the k' values for species i and j. Thus, the control of pH of the eluent is an important means to adjust the retention of the analytes.

In RPLC, the retention of solutes invariably decreases with increasing temperature, unless the solute undergoes conformational changes in the temperature range employed such as in the case of proteins. Proteins may unfold as the temperature is varied, and consequently retention may change in several different ways. For small saccharides and glycoconjugates, temperature-induced conformational changes may not be significant, and therefore, plots of log k' versus the reciprocal of temperature are linear. From the slopes of these plots, known as Van't Hoff plots, the retention enthalpy can be readily extracted [21].

Conversely, in HIC increasing temperature is normally accompanied by an increase in retention [22]. This is because HIC is known to be an entropy-driven process. Since HIC employs weakly hydrophobic stationary phases and aqueous eluents, both the protein and the stationary-phase ligands are associated with structured water molecules. Upon solute binding, the surfaces of the solute molecule and the stationary-phase ligands release the structured water and, consequently, the entropy of the bulk solution increases. In addition, since Van der Waals forces are the major non-polar interaction forces and the contact area of the protein with the surface is small, ΔH^0 may also be a small positive or negative value. According to the equation

$$\Delta G^0 = \Delta H^0 - T\Delta S^0 \quad (6)$$

the Gibbs free energy, ΔG^0, is controlled by a large positive entropy change and therefore increases with temperature. According to the equation

$$\ln k' = \ln \varphi - (\Delta G^0 / RT) \quad (7)$$

where R is the gas constant, φ is the phase ratio and T is the absolute temperature, the capacity factor would increase with increasing temperature. This may be regarded as an advantage for proteins in the sense that subambient temperature can be used to achieve weaker interaction and bring about elution and separation of proteins under mild conditions with little or no denaturation of the analytes.

Many glycoconjugates as well as acidic carbohydrates are better separated by ion-pair RPLC. Also, some of the mobile-phase additives normally used in RPLC, e.g., trifluoroacetic acid or phosphoric acid, may undergo ion-pair formation with glycoproteins and their peptide and glycopeptide fragments, thus altering the retention of the separated analytes. Therefore, a brief description of the basic principles of ion-pair RPLC is in order.

In ion-pair RPLC, the column packing is usually the same as in RPLC, and the mobile phase is a buffered hydro-organic eluent containing an ion-pairing agent. For instance, the ion-pairing agent will be positively charged (P^+) for the retention and separation of sugar anions (S^-). Typical examples of cationic ion-pairing agents are tetrabutylammonium or alkyltrimethylammonium salts. The basis of retention in ion-pair RPLC is still controversial. Two different processes are possible [23,24]: (i) adsorption of ion pairs to the hydrophobic sorbent or (ii) formation of an in situ ion-exchanger column (dynamic ion-exchange). In the former

retention process, an ion-pair (S^+P^-) is formed in the mobile phase and is then retained by the stationary phase. The latter ion-pair RPLC retention process involves initial retention of the ion-pairing agent followed by ion-exchange between the charged solute and the mobile phase counterions.

Although the two ion-pair retention processes are quite different, they lead to quite similar prediction of retention as a function of experimental conditions. In both retention models, increasing the concentration of the ion-pairing agent in the mobile phase leads to an increase in solute retention. Also, increasing the concentration of the organic modifier in the mobile phase at constant ion-pairing concentration leads to decreasing solute retention since the organic solvent lowers either the ion-pair adsorption to the stationary phase or lowers the amount of dynamically adsorbed surfactants to the surface of the stationary phase.

For other recent treatments of the fundamentals of RPLC and HIC, the reader may consult Refs. [1,20,25,26].

3. Separation approaches and applications

As stated in the introduction, the significant differences in the nature and composition of the stationary phases used in HIC and RPLC mandate the use of different types of mobile phases and operating conditions for the elution and separation of analytes. Thus, the understanding of the structure and composition of the HIC and RPLC sorbents is essential for the design of separations in both methods.

3.1. Reversed-phase liquid chromatography

3.1.1. Stationary phase

RPLC employs non-polar stationary phases, which traditionally consist of a microparticulate silica support with covalently bound alkyl or aryl functions at the surface. Among the alkylated silica-based stationary phases, octadecyl- (C_{18}), octyl- (C_8) and butyl-coated (C_4) sorbents are the most widely used. While C_{18}-silica has been the most useful in the RPLC of carbohydrates, C_4- and to a lesser extent C_8-silica are used in glycoprotein RPLC because the C_4- and C_8-silica are less retentive toward the biomacromolecule than C_{18} sorbents, thus allowing higher protein recovery. Phenyl-silica stationary phases have found use in protein RPLC, and afforded different selectivity due to their aromatic character.

Alkyl-bonded silica-based stationary phases are formed by reacting silica gels with alkyl monochloro-, dichloro- or trichlorosilane. With monofunctional silanizing agents (i.e., alkyldimethylchlorosilane), the surface of the silica support is covered by a molecular "fur" or "brush", and the resulting stationary phases are termed "monomeric". With trifunctional silanizing agents (i.e., alkyltrichlorosilane), a cross-linked layer of alkylpolysiloxane is formed at the surface of silica, and the stationary phases thus obtained are termed "polymeric". While monomeric stationary phases offer the highest separation efficiency, polymeric stationary phases are more stable and resist hydrolytic degradation when in contact with aqueous mobile phases due to their cross-linked network. Since the silanol groups at the surface of silica cannot be reacted completely, the unreacted silanols on the surface of monomeric stationary phases may give rise to heterogeneous surface, and as a result a mixed retention mechanism involving hydrophobic and silanophilic interactions may be present [27]. To reduce silanophilic interaction, "endcapping" of the surface silanols is normally carried out by treatment with trimethylchlorosilane [28]. End-capped, non-polar bonded silica columns are preferred for the separation of glycoproteins and glycopeptides in terms of separation efficiencies and analyte recovery.

The nature, alkyl chain length and density of the non-polar ligands at the surface of the stationary phase affect retention, the loading capacity and the selectivity of the column [7]. While the chain length of the bonded alkyl ligand strongly influences retention and selectivity of small molecules including small carbohydrates, it does not seem to strongly affect retention and selectivity of proteins [29]. However, the extent of protein recovery from non-polar

sorbents is an inverse function of the chain length of the alkyl ligand [29]. The chemical nature of the hydrophobic ligand seems to influence the selectivity of the RPLC system. Phenyl and cyano columns offer different selectivity than alkyl-coated sorbents [30].

In RPLC of carbohydrates, the percentage of the bonded alkyl chains, i.e., density of the non-polar ligands, seems to have a profound effect on resolution [31]. It has been demonstrated that a lower figure of only 12% against the normal 17–18% for C_{18}-silica worked the best [31]. Also, a commercial RPLC column under the trade name of Dextropak having a C_{18} coverage of 10% and a pore diameter of 120 Å has been shown to afford superior resolution in RPLC of carbohydrates with plain water as the eluent [32]. More recently, several C_{18} columns were evaluated in RPLC of carbohydrates, including silicone encapsulated silica with C_{18} moieties, porous gel-based C_{18} column and end-capped monomeric C_{18} silica based columns [33]. Among these columns, the monomeric and end-capped silica-based stationary phase showed the best performance. The results of these studies [31–33] may mean that the presence of some unreacted silanol groups plays a role in the chromatographic process. However, a partially derivatized phase having a very high silanol-groups concentration does not separate or even retain sugars [31]. Thus, the retention of sugars on RPLC columns with water as eluent must be complex, with hydrophobic interaction also playing a role.

Silica has been the most widely used support because of (i) its availability in several particle and pore sizes, (ii) the ease with which the silanol groups at the surface can be derivatized with a wide variety of functional groups via siloxane bridges and (iii) its high mechanical strength and chemical stability at low pH. However, silica-based stationary phases undergo rapid degradation at high pH. This hydrolytic instability is a major problem when it comes to utilize silica-based stationary phases in process-scale purification of pharmaceutical proteins most of which are glycosylated. The column cannot be cleaned with sodium hydroxide solution which has proved to be the most effective in desorbing proteins from the surface of the stationary phase before reusing the same column for the purification of another sample of protein. Several approaches have been described to improve the stability of silica-based stationary phases:

(i) The use of "bidentate" silanes having two silicon atoms to obtain higher surface coverage than is achievable with monodentate silane [34].
(ii) The use of silanizing agent with bulky side groups to produce hydrolytically stable monomeric phase [35,36].
(iii) The coating of silica with a polysiloxane or a polybutadiene (i.e., "encapsulation") layer in order to yield silica surface that is sealed and protected by a polymeric film that also serves as the non-polar phase [37,38].
(iv) The bonding via Si–C bonds to the surface instead of the Si–O–Si bonds by the chlorination of the surface followed by alkylation with a Grignard reagent, all under strict anhydrous conditions [39].
(v) The cladding of the silica surface with zirconia, which afforded a limited success [40]. Such stationary phases can be used with mobile phases of pH below 10.
(vi) The method of monolayer self-assembly by horizontal polymerization which involves the use of mixed trifunctional silanizing agents was reported very recently for potentially achieving hydrolytic stability for silica-based stationary phases [41]. The method requires anhydrous conditions, except for a monolayer of water on silica. This leads to the so-called horizontal polymerization and is characterized by higher density at the silica surface boundary.

In search for rigid stationary phases having the mechanical strength of silica gels but affording a wider pH range stability for use in high-performance liquid chromatography (HPLC), rigid microparticulate carbon black [42], polystyrene-divinylbenzene (PSDVB) [43] and other resinous

phases [44] have been developed. Several reviews on polymer-based packing for RPLC and other modes of HPLC have recently appeared [45–49].

Recently, there has been an increasing interest in inorganic sorbents that combine the mechanical strength of silica with the chemical stability of polymeric-based stationary phases. These phases include polymer encapsulated alumina [50,51], alkyl bonded alumina [52], octadecyl-titania [53], polybutadiene coated-zirconia [54,55], zirconia sorbents having vapor-deposited carbons [56], polybutadiene–carbon composite zirconia sorbents [57] and octadecyl-zirconia [53,58]. These inorganic-based stationary phases have proved very stable at alkaline pH. For recent reviews on zirconia- and alumina-based stationary phases, see Refs [59,60].

Generally speaking, an ideal column matrix that is free of all undesirable properties seems to be unrealistic. While silica is known for its residual adsorptivities, alumina and zirconia based stationary phases have also shown affinity toward species with phosphate or carboxylate functional groups even though the stationary phases were coated with thick polymeric layer [55]. Thus, it can be anticipated that both organic- and inorganic-based sorbents will continue to coexist and complement each other.

Besides brief exploration of the utility of octadecyl-zirconia in the RPLC separation of derivatized oligosaccharides [58] (see Fig. 1), all other inorganic sorbents have not been yet tested in RPLC of carbohydrates. This may be due to the fact that only recently some of these inorganic-based RPLC columns have become commercially available, and some others are still in the stage of characterization.

The use of carbonaceous non-polar stationary phases (e.g., pyrocarbon) in RPLC is not new [42,61]. These early studies, which were aimed at exploring the potential of carbon and gaining an understanding of the RPLC process yielded separation efficiencies far inferior to those obtained on silica-based octyl or octadecyl columns. This may be attributed to the less favorable pore structure of the porous carbon, and to the highly active surface of carbon sorbent. More

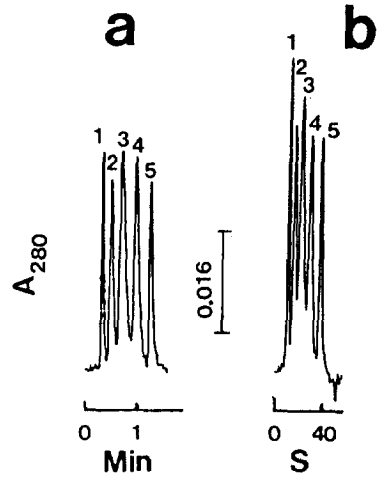

Fig. 1. Chromatograms of *p*-nitrophenyl maltooligosaccharides. Column, "polymeric" octadecyl-zirconia, 3.0 × 0.46 cm I.D.; linear gradient in 1 min (a) and 0.5 min (b) from 0 to 20% (v/v) acetonitrile in water at 0.05% (v/v) trifluoroacetic acid; flow-rates, 1.0 ml/min (a) and 4.0 ml/min (b). Solutes: *p*-nitrophenyl-α-D derivatives of 1 = glucose, 2 = maltose, 3 = maltotriose, 4 = maltotetraose, 5 = maltopentaose. (Reproduced with permission from Ref. [58].)

recently, porous graphitized carbon (PGC), which was originally developed at the University of Edinburgh by Knox and co-workers [62,63], has become commercially available under the trade name of Hypercarb since over eight years. PGC, which has proved very useful in many types of separations [64], has only recently been evaluated in HPLC of carbohydrates and glycoconjugates [65–67]. While the column cannot be used for monosaccharides because they were weakly retained and eluted with plain water [67], PGC has been shown useful for the separation of oligosaccharides released from fetuin and asialofetuin, as shown in Fig. 2.

Besides the chemical and mechanical stabilities of the column packing, other factors are also important. The pore size of the support must be much larger than the molecular size of the analyte. Whereas for small carbohydrates and glycoconjugates pore sizes of 100 to 150 Å are adequate, pores larger than 300 Å are needed for high-molecular-mass proteins, glycoproteins and polysaccharides.

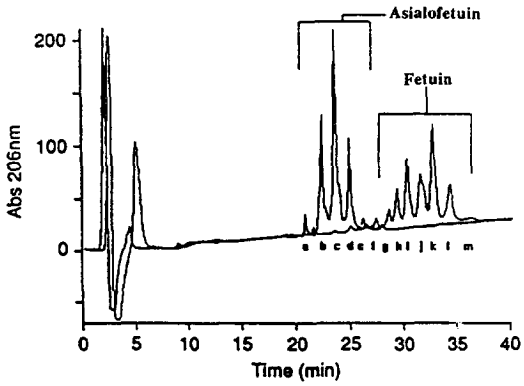

Fig. 2. Superimposed chromatograms of oligosaccharides released from asialofetuin and fetuin by PNGase F on a PGC column (100 mm × 6.4 mm). Linear gradient in 35 min from water at 0.05% TFA to water–acetonitrile (60:40) at 0.05% TFA. (Reproduced with permission from Ref. [66].)

There have been two major approaches for providing adequate sorbents for the separation of high-molecular-mass substances. Recently, PSDVB support containing a combination of very large 8 000 Å transecting pores and smaller 100–1500 Å interconnecting pores, has been introduced [68]. These RPLC packings allow the mobile phase to flow or perfuse through the support matrix thus permitting the transport of the solutes to the interior of the sorbent particle much more rapidly than by diffusion, and consequently enabling separations to be achieved in a minute or less. In a second approach, rigid non-porous stationary phases based on silica and polymeric supports have been developed for the separation of proteins. These packings are characterized by a thin retentive film on the outer surface of the particles which is readily accessible to the separated analytes. Due to the lack of internal porosity, intraparticle diffusion resistances are absent, thus favoring rapid separation of slowly diffusing biopolymers. Silica microspheres having 1.5 μm particle diameter were covalently bound with *n*-octyl functions, and the short columns provided rapid separation of proteins with a sample capacity of ca. 1 mg of protein per column volume, without a loss of resolution [69,70]. Conversely to porous stationary phases, when the chain length of bonded ligand was varied, the retention of a set of standard proteins in gradient elution followed the ligand sequence $C_{18} > C_8 \approx C_4 \approx$ phenyl $> C_2$ under otherwise identical conditions [71]. Silica microparticles (2 μm) which were modified with *n*-octyldimethylchlorosilane allowed the separation of five proteins in less than 20 s [72]. Also, a polymeric "non-porous" sorbent based on cross-linked polystyrene particles having a mean particle diameter of 3 μm and a rugulose surface was introduced for the RPLC of peptides and proteins [73]. Although the polymeric support did swell slightly in organic solvent, the estimated 5 to 8% change in particle diameter did not adversely affect the efficiency of the column. As expected, the column was stable at extreme pH for several months [73]. Also, non-porous microspherical resins of 2.5 μm in diameter have become commercially available as packed columns of TSK gel octadecyl-NPR for RPLC [74,75]. This resin permitted the rapid separations of proteins and peptides with sample loads in the order of 500 ng. High-resolution separation of seven different standard proteins was realized in less than 7 min. This rapid separation could be shortened to 8 s on a silica-bonded C_{18} stationary phase [76], because silica has a higher mechanical strength thus enabling higher flow velocities.

3.1.2. Mobile phase

In RPLC, the mobile phase is more polar than the stationary phase, and consists of a buffered or unbuffered hydro-organic solvent mixture containing methanol, isopropanol, *n*-propanol, acetonitrile or tetrahydrofuran as the organic modifier. In choosing the eluent composition, one should consider the eluting strength, the solubility of the solute and compatibility with the detector (e.g., optical transparency). The eluting strength of the organic modifiers commonly used in RPLC follows the order methanol $\leqslant$ acetonitrile $<$ ethanol $\approx$ acetone $\approx$ dioxane $<$ isopropanol $\leqslant$ tetrahydrofuran [77]. The viscosity of the mobile phase is another property that must be considered in choosing the organic modifier. Besides determining the pressure drop across the column, it also affects separation efficiency through its effect on diffusion rates.

An in-depth treatment of the effects of the various properties of the hydro-organic eluents can be found in Ref. [7].

The separations of glycoproteins and glycopeptides are generally performed by gradient elution at increasing organic solvent concentration in the eluent. Also, most derivatized saccharides are better separated by gradient elution. For RPLC of saccharides, the mobile-phase composition must be selected to provide an adequate resolution and rapid analysis time. For RPLC of proteins and glycoproteins, in addition to achieving high resolution and rapid analysis, the composition of the mobile phase must be adequately selected to minimize protein denaturation during the chromatographic run and to maximize protein recovery from the RPLC column. The pH, the nature of the organic solvent and other additives all have an effect on the quality of protein separation and purification.

Protein separation in RPLC is most commonly achieved at low pH in the presence of small amounts of trifluoroacetic acid (TFA) or phosphoric acid in the eluent [78]. Under these conditions, the surface silanols are not ionized and the protein is thought to form an ion-pair with the acid, thus becoming almost a neutral analyte. Hydrochloric acid, despite its corrosivity, has also found use in RPLC. This is because HCl is volatile, as TFA, and in addition is characterized by an excellent UV transparency [79]. Despite its relatively low volatility, phosphoric acid is used whenever higher efficiency and recovery of proteins and peptides are required [80]. In the case of very hydrophobic proteins such as membrane proteins and glycoproteins, formic acid at high concentration has been found suitable for such applications [81]. The addition of chaotropic salts to the eluent can also result in increased peak sharpness and weaker retention [82].

The separation of underivatized carbohydrates is better achieved when plain water is used as the mobile phase. In some instances, addition of neutral inorganic salts increases the capacity factors considerably and allows good resolution of some compounds poorly resolved in water alone [32]. However, when water (with or without neutral salts) is used as the eluent, pairs of peaks corresponding to the α and β anomers of the reducing sugars are normally observed, which complicate the chromatogram and the quantitation of the analytes. As will be discussed below, several approaches have been introduced to avoid the formation of doublets [32,83,84]. The retention of underivatized saccharides can be regulated by the inclusion of surfactants, tetramethyl urea and organic solvents in the eluent. These additives cause faster elution, and allow the separation of larger oligomers in an acceptable time [83,84].

Other mobile-phase additives include ion-pairing agents, since ionized carbohydrates are most often separated as ion-pairs with mobile phase containing tetraalkylammonium salts, such as tetrabutylammonium salts, dodecyltrimethylammonium chloride, etc., see section below on ion-pair RPLC.

3.1.3. Some general aspects of RPLC of carbohydrates and selected applications

3.1.3.1. Underivatized saccharides

As mentioned above, underivatized saccharides are usually chromatographed using plain water as the mobile phase [85]. This is because carbohydrates are polar compounds requiring high surface tension mobile phases to bring about their retention and separation. In general, monosaccharides elute first and are not resolved from one another [84]. Pairs of peaks corresponding to the α and β anomers are observed for reducing saccharides from a degree of polymerization (dp) ≥ 3. Several ways were introduced to alleviate or eliminate the problem of doublets:

(i) Reducing the sugar sample with sodium borohydride. In this approach, each pair of peaks is then replaced by a single peak corresponding to the sugar alditol. The reduced forms are eluted before the unmodified sugars (e.g., cellobitol before cellobiose) [85,86]. This effect becomes less significant with increasing dp as the relative

effect of one alditol residue becomes smaller [84].

(ii) Increasing column temperature. This accelerates the rate of interconversion between the α and β anomers of saccharides, thus eliminating unwanted double peaks [21,67,83], but the retention is markedly decreased with increasing column temperature [21], which also leads to some reduction in column selectivity.

(iii) The inclusion of detergents, such as Triton X-100, in the eluent [32] usually eliminates double peaks and reduces retention.

(iv) A fourth approach has been by base catalysis. It has been shown that the addition of 10 m*M* triethylamine (pH 10) to the eluent reduced the retention times and eliminated anomeric doublets [83]. However, such approach is harmful for silica-based stationary phases, which undergo hydrolytic degradation at high pH. To solve the anomerization problem without worrying about column stability by going to basic pH, porous polymer gel-based RPLC columns that consists of vinyl alcohol copolymer gel with bonded C_{18} moieties have been used successfully for the separation of gluco-oligomers (dp 2–23) [87].

The retention behavior of underivatized sugars on RPLC packing is rather complex. However, a few guidelines can be formulated for the design and prediction of separations. In general, and as predicted by the solvophobic theory, the elution volume of free monosaccharide is lower than that of the corresponding glycoside [85]. The same is observed in the separation of xylose from methylated pentoses, e.g., rhamnose and fucose [85]. Thiooligosaccharides are less polar than neutral oligosaccharides and their elution volumes are increased [85]. Also, for naturally acetylated sugars such as chitin oligosaccharides, the addition of 3% (v/v) acetonitrile decreased the retention into a convenient range [86]. In general, closely related isomers such as N-acetylgalactosamine (GalNAc) and N-acetylglucosamine (GlcNAc) are not resolved [86]. With water as the eluent, the elution order of oligomers is that of increasing molecular mass [86].

Besides the importance of the molecular mass in influencing RPLC retention, structure is also an important factor that must be taken into account. In fact, there are several exceptions to the generalization that the higher the molecular mass, the longer the retention time of structurally similar oligosaccharides [88]. For instance, stachyose (a tetrasaccharide) eluted before raffinose (a trisaccharide), and β-1$\rightarrow$4 linked-D-mannose oligomers are not resolved. This means that structure predominates over molecular mass in the interactions with C_{18} stationary phases, and especially when going from linear to branched oligosaccharides. In this case it seems that linear molecules interact most with the C_{18}. In fact, the elution times of branched isomaltodextrins were shorter than the linear isomaltodextrins of the same degree of polymerization. McGinnis et al. [89] evaluated the behavior of unsubstituted carbohydrates in RPLC with water as the eluent. The retention time of the individual oligosaccharides was found to be dependent on the molecular mass of the analyte and the type of the anomeric linkage.

Despite some difficulties, RPLC with plain water was useful for the separation of various underivatized carbohydrates including O-linked neutral oligosaccharides from human meconium glycoproteins [90], fucose-containing pentasaccharides of goat's milk [91], cyclic (1$\rightarrow$2)-β-D-glucans (cyclosophoraoses) of high dp ($>$24) [92], the isolation of the carbohydrate chains of the respiratory-mucus glycoproteins which were released by alkaline borohydride treatment of a pronase digest [93], and the separation of a phosphocholine β-(1,3);(1,6)macrocyclic glucan from *Bradyrhizobioum japonicum* USDA 110 by taking advantage of the relatively hydrophobic nature of the material [94].

In addition, RPLC with pure water as eluent was shown useful for the separation of fructo-oligosaccharides from red squill (*Urginea maritima*) [95]. As a typical example, two trisaccharides that differed only in the position of the sugar residues were well resolved. These were 1-kestose [α-D-Glcp-(1$\rightarrow$2)-β-D-Fruc*f*-(1$\rightarrow$2)-

β-D-Fruf] and neoketose [β-D-Frucf-(1→2)α-D-Glcp-(1→2)-β-D-Fruf]. An important application of RPLC in the area of underivatized carbohydrates has been the determination of ascorbic acid in a multi-component elemental diet using electrochemical detection with an applied voltage of 70 mV versus Ag/AgCl [96]. This determination was performed on a C_{18} column with 100 m*M* KH_2PO_4, pH 3.0, containing 1 m*M* EDTA disodium salt, and the linear range extended from 0–1.0 μg.

Very recently, native and reduced α-glucan oligosaccharides were successfully separated on a C_{18} column for up to a dp > 40 (see Fig. 3) using a gradient elution with acetonitrile and a post-column reactor with co-immobilized glucoamylase and glucose oxidase for the detection after RPLC separation [97]. In this process, oligomers eluting from the column were cleaved into monomers which were oxidized with the production of hydrogen peroxide, which was measured amperometrically at a platinum electrode. Detection of α-glucans proved to be highly selective and sensitive, and the enzyme reactor was stable under the conditions used. Linear calibration graphs were observed over three decades, and the detection limit for various oligosaccharides is in the lower nanogram range [97]. The use of a post-column enzyme reactor for sugar detection after HPLC separation was recently reviewed [98].

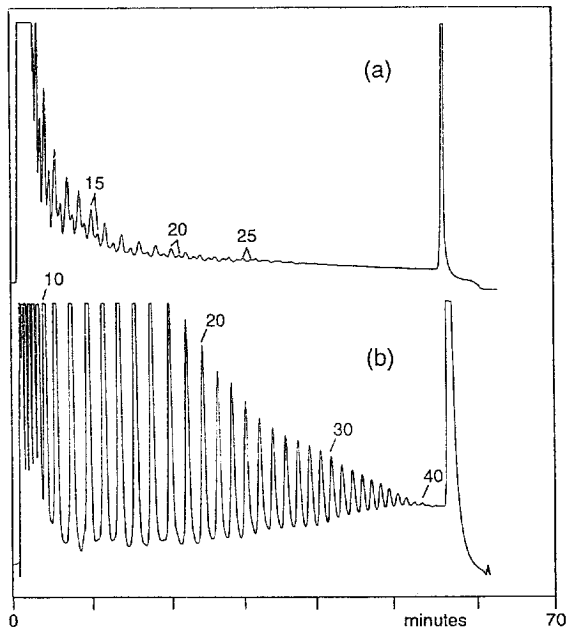

Fig. 3. Chromatogram of maltodextrins (a) and reduced maltodextrins (b) obtained on a Bio-Sil C_{18} HL 90S-3 column (spherical 3-μm particles, 100 mm length × 4.6 mm I.D.). Peak numbers refer to degree of polymerization. Chromatographic conditions: detection by a co-immobilized glucoamylase/glucose oxidase reactor (50 mm × 2.1 mm I.D.) in series with an electrochemical detector; flow-rate, 1 ml/min; eluent A: 0.1 *M* NaH_2PO_4, eluent B: 10% acetonitrile in A. Gradient: from 0 to 20% B in 55 min, 100% B at 60 min. (Reproduced with permission from Ref. [97].)

Houben and Brunt [99] described a relatively simple method for the determination of the glycoalkaloids α-chaconine and α-solanine in potato tubers, which involved the preconcentration of glycoalkaloids from potato by solid-phase extraction with a disposable C_{18} cartridge followed by RPLC on a C_{18} column having reduced amount of residual silanol groups to minimize solute–silanol interaction. The mobile phase was water–acetonitrile, a condition that afforded a longer life time for the column. The determination of pectins in raspberries as galacturonic acid by RPLC using C_{18}-silica column and acidified water as the eluent was reported recently [100]. In this work, the limit of detection was 0.04 mg of galacturonic acid per ml at 200 nm using forward optical scanning detection.

An important development in the area of carbohydrate HPLC has been the recent use of graphitized-carbon stationary phases for the separation of carbohydrates [66,67,101–103]. As with octadecyl-silica, monosaccharides were weakly retained on a porous graphitized carbon (PGC) column (Hypercarb column), and were eluted with plain water [67]. Also, each peak of xylose, glucose, galactose and fucose was split into α- and β-anomer peaks. Unlike octadecyl-silica, gluconic and glucuronic acids were retained too strongly for practical chromatography [67]. On the other hand, disaccharides were eluted with 15:85 (v/v) methanol–water or 4:96 (v/v) acetonitrile–water, and again each peak of the individual saccharides showed two anomeric peaks. These peaks each coalesced into a single peak upon the addition of sodium hydroxide to

the eluent. The basic mobile phase allowed the use of a pulsed amperometric detector (PAD) for the detection of the disaccharides. Another difference from octadecyl-silica is that with cyclodextrins, the PGC column necessitates the inclusion of higher organic-solvent concentrations in the eluent as compared to C_{18}-silica, indicating that Hypercarb is more hydrophobic than regular C_{18} column. The retention tends to decrease with increasing the organic modifier, like in RPLC, however, the elution order is different. Conversely to RPLC with silica-based columns, the retention increases with increasing temperature and the peaks become sharper. Furthermore, positional isomers of glucosyl-inositol and of dimaltosyl-β-cyclodextrin, neither of which could be separated on conventional bonded phases, were well resolved on this column [67].

The retention behavior of oligosaccharide alditols was evaluated on a Hypercarb PGC column [66]. The oligosaccharides of dp = 1 to 6 were substantially retained and could be eluted in a gradient of acetonitrile from 0 to 25% (v/v) in 0.05% aqueous trifluoroacetic acid. In this study, the separation of oligosaccharide alditol isomers differing only by a (1→3) or (1→4) linkage has shown that PGC is a useful adjunct to C_{18}-silica stationary phases. However, fucosylated pentasaccharide isomers which were successfully separated by C_{18}-silica column, were not resolved with the PGC column. In another contribution from the same laboratory [101], the PGC column was shown to give good separation of oligosaccharides and oligosaccharide alditols released from protein by enzymes (N-linked chains) or base-borohydride degradation (O-linked chains), with the advantage that reducing oligosaccharides, sialo-oligosaccharides and oligosaccharide alditols can be chromatographed under the same conditions with volatile mobile phases so that they can be readily recovered by evaporation for sensitive liquid secondary-ion mass spectrometry (MS) analysis.

Very recently, Okada et al. [102] used PGC and C_{18} columns for the separation and characterization of five positional isomers of trimaltosyl-cyclomaltoheptaose (trimaltosyl-β-cyclodextrin). Finally, Fan et al. [103] showed that oligosaccharides which normally cannot be retained on C_{18} column even under high-salt and low-pH conditions, could be effectively retained and separated on a PGC column.

3.1.3.2. Derivatized saccharides

Pre-column detection-oriented derivatization schemes are currently widely exploited in the RPLC analysis of carbohydrates. The topic of pre-column derivatization of carbohydrates has been reviewed very recently by Hase [104], and the chromatographic behavior of various sugar derivatives in RPLC has been very recently discussed by the author elsewhere [1].

With a few exceptions, pre-column derivatization reactions can be classified into two major categories. One category is based on the introduction of chromophores via the hydroxyl groups of the sugar solute [105–107], a process that gives anomeric mixtures from reducing carbohydrates, and the second category encompasses the reactions occurring at the carbonyl group which involve coupling to amines or to tritium. In the second category, the carbonyl groups of carbohydrates are coupled to amines to form (i) imines (Schiff bases) or glycosylamines [108,109] or (ii) amines by reductive amination [110–117]. The carbonyl groups of carbohydrates are stoichiometrically reduced with sodium [^{3}H]borohydride in an alkaline solution to alditols with the incorporation of tritium [118]. This approach allows the detection of the radio-labeled sugars by a liquid scintillation counter or a flow-through counter. Carbonyl derivatization into imines or glycosylamines yields derivatives that are somewhat unstable. Since the parent sugars can be regenerated for further use, the unstability of glycosylamines can be advantageous if the derivatives are stable enough to allow their separations by HPLC. However, the derivatization of sugars to imines usually leads to the formation of syn/anti-isomers and anomeric mixtures. These problems can be overcome by reductive amination (i.e., reduction of an imine into an amine), which eliminates the problems associated with anomerization, by means of sodium cyanoborohydride or borane · dimethylamine complex.

Thus, pre-column derivatizations by reductive amination are advantageous in this regard and are currently the most widely used in HPLC and capillary electrophoresis [119].

There are several useful miscellaneous pre-column derivatization reactions for carbohydrates. The reducing end of sugars can be tagged with 1-phenyl-3-methyl-5-pyrazolone (PMP) [120] or 1-(*p*-methoxy)phenyl-3-methyl-5-pyrazolone (PMPMP) [120–122]. Amino groups of amino sugars and amino sugar alcohols were derivatized with *o*-phthaldehyde [123] in the same way as amino acids or with phenyl isothiocyanate (PITC) to produce phenyl isothiocarbamyl derivatives [124,125]. Hexosamines were derivatized with toluene sulfonyl chloride or 5-dimethylaminonaphthalene-1-sulphonyl chloride [126]. Finally, selective pre-column derivatization for sialic acids was introduced which involved the reaction with 1,2-diamino-4,5-methylenedioxybenzene [127–129].

In general, a pre-column derivatization is aimed at enhancing the sensitivity of detection of the parent sugars. In some other instances, the derivatization, e.g., peralkylation, is carried out for structural determination of oligo- and polysaccharides [130–132]. Pre-column alkylation may inevitably occur, such as during the degradation of polymeric carbohydrates by methanolysis, a process in which the degraded carbohydrates become methylated [133,134]. In any event, pre-column derivatizations with an organic tag provide the sugar solutes with the hydrophobicity necessary for their retention by RPLC.

As mentioned above, pre-column derivatization by reductive amination seems to be the most attractive. Recently, Kown and Kim [135] reported the RPLC separation of monosaccharide components of glycoproteins which were pre-column derivatized with *p*-aminobenzoic acid ethyl ester (ABEE), a reaction that was first introduced by Wang et al. [136], see Fig. 4. In this report, after acid hydrolysis of glycoproteins with 2 *M* trifluoroacetic acid, the sugars were reductively aminated in the presence of sodium cyanoborohydride with ABEE, which strongly absorbs UV light at 254 nm. The RPLC analysis

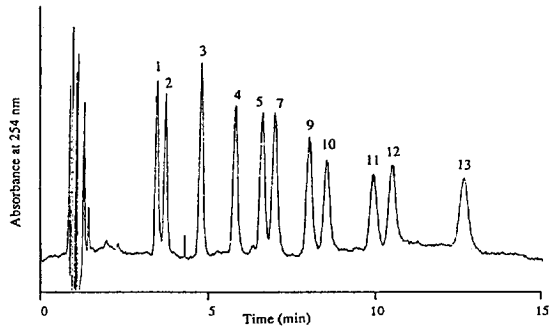

Fig. 4. RPLC separation in the pmol range of ABEE-sugar derivatives. Chromatography of 50 pmol of each component using the following conditions: column, Pico·Tag (150 × 3.9 mm I.D.), temperature, 45°C; isocratic elution at 1.2 ml/min using a ratio of solvent A to solvent B of 75:25 (v/v); solvent A, 50 m*M* sodium acetate buffer, pH 4.5; solvent B, solvent A–acetonitrile–methanol = 40:40:20 (v/v). Sugar derivatives: 1 = glucosamine; 2 = galactosamine; 3 = lactose; 4 = maltose; 5 = galactose; 6 = glucose; 7 = mannose; 8 = ribose; 9 = xylose; 10 = N-acetylglucosamine; 11 = N-acetylgalactosamine; 12 = fucose; 13 = 2-deoxyglucose. (Reproduced with permission from Ref. [135].)

was performed on a C_{18} column in an isocratic mode using ternary mixtures of aqueous sodium acetate containing methanol and acetonitrile as the mobile phases and 45°C as the column temperature. Monosaccharide composition and contents determined on glycoproteins were very comparable to those previously reported by other techniques.

The pre-column derivatization with 2-aminopyridine (2-AP) by reductive amination, i.e. reductive pyridylamination, is still enjoying popularity. The chemical structures of the sugar chains of α_1-antitrypsin (AAT) from patients with hepatocellular carcinoma (HCC) and from healthy individuals with a different affinity for *Lens culinaris* agglutinin were examined by pyridylamination of their oligosaccharides and stepwise exoglycosidase digestion in combination with RPLC and size-exclusion chromatography [137]. The report reached the conclusion that the carbohydrate chains of AAT from patients with HCC have an increment in focusylation. Along the same lines, pyridylamination has been used in assessing the alteration of asparagine-linked glycosylation in serum transferrin of patients with HCC [138]. The sugar chains released with

N-glycanase were derivatized by reductive pyridylamination and then analyzed by RPLC and normal-phase chromatography. The HPLC analysis in combination with exoglycosidase digestion revealed an increase of a biantennary complex type sugar chain with a fucosylated trimannosyl core in seven of 13 cancer patients and an increase in a sugar chain with a fucosylated trimannosyl core and bisecting N-acetylglucosamine in one of the 13 cancer patients. This fucosylated alteration of the sugar chain was detected also in α_1-antitrypsin, hemopexin, α_1-acid glycoprotein and α_2-HS glycoprotein from one of the patients with increased fucosylated transferrin.

Two-dimensional HPLC mapping involving RPLC and size fractionation in combination with exoglycosidase digestions afforded the determination of the primary structures of the asparagine-linked carbohydrate chains derivatized with 2-AP which were isolated from the membrane glycoproteins of the three insect-cell lines *Mamestra brassicae* (Mb-0503), *Bombyx mori* (Bm-N) and *Spodoptera frugiperda* (Sf-21) [139].

Suzuki-Sawada et al. [140] reported the analysis of oligosaccharides by LC–MS. In this study, the oligosaccharides derived from human immunoglobulin G were labeled with 2-AP and then analyzed by RPLC and detected on-line by MS. The observed molecular masses were close or identical to those expected from the structures, which were estimated from the elution position by HPLC. The described method is rapid and simple as the mass spectrometer can give the accurate molecular mass of each PA-oligosaccharide (i.e., pyridylamino-oligosaccharide) in a single chromatographic run even if the HPLC separation is incomplete. Other recent applications of RPLC involved the analysis of sugar chains tagged with 2-AP which were derived from porcine plasma vitronectin [141] and from hen egg yolk riboflavin-binding protein [142].

Very recently, an elegant modification of the tagging with 2-AP has been reported [143]. This study has expanded the scope of fluorescent tagging of free oligosaccharides by reductive amination. The authors have synthesized a fluorescent reagent, 2-amino(6-amidobiotinyl)pyridine (BAP), which has been shown to tag oligosaccharides via reductive amination under non-degradative conditions, resulting in adducts that exhibited suitable behavior in RPLC with sensitive detection in the picomole range. The biotinyl group can be used to recover the sugar chains from reaction mixtures through the high-affinity interaction of the biotinyl group with multivalent avidin or streptavidin. In addition, this binding can be used to create the functional equivalent of neoglycoproteins carrying multiple copies of oligosaccharides of defined structures. They can also harness the power of (strept)avidin-biotin technology for the detection and isolation of oligosaccharide-specific receptors from native sources of recombinant libraries.

A method for determining the glycosidic linkage position to the pyridylamino-reducing end residue of pyridylamino-oligosaccharides (PA-oligosaccharides) was developed using disaccharides as model compounds [144]. The method involved Smith degradation and RPLC analysis.

Kaheki and Honda reported an overview of the analysis of O-linked and N-linked carbohydrate chains of various glycoprotein which were derivatized with 2-AP or 1-phenyl-3-methyl-5-pyrazolone (PMP) [145].

Strydom [146] applied the pre-column derivatization with PMP, which was previously introduced by Honda et al. [120], to the analysis of hydrolyzates containing neutral, basic and acidic reducing monosaccharides. In particular, glucuronic and galacturonic acids, glucosamine and galactosamine are separated completely both from one another and from the aldoses of glycoproteins commonly found (see Fig. 5). The analysis of carbohydrates as their PMP derivatives is attractive owing to its high sensitivity of detection and the generation of single derivatives of each aldose molecule.

The amino sugars normally found in synthetic glycopeptides were analyzed by RPLC as 4-dimethylaminoazobenzene-4′-sulfonyl chloride [147] using C_{18} column and a ternary solvent gradient consisting of aqueous sodium citrate, N,N-dimethylformamide and acetonitrile at pH 6.50–6.52.

Neutral and amino sugars occurring in glyco-

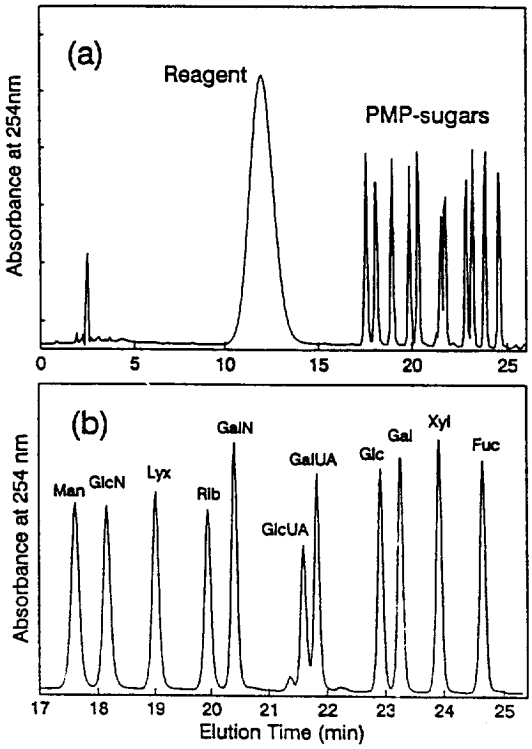

Fig. 5. Chromatography of PMP derivatives of common reducing monosaccharides. (a) Separation of the remaining reagent (broad peak) from the PMP derivatives (sharp peaks). (b) An expanded region of the chromatogram in (a): the PMP derivatives of the common neutral, basic and acidic reducing monosaccharides. Man = mannose; GlcN = glucosamine; Lyx = lyxose; Rib = ribose; GalN = galactosamine; GlcUA = glucuronic acid; GalUA = galacturonic acid; Glc = glucose; Gal = galactose; Xyl = xylose; Fuc = fucose. (Reproduced with permission from Ref. [146].)

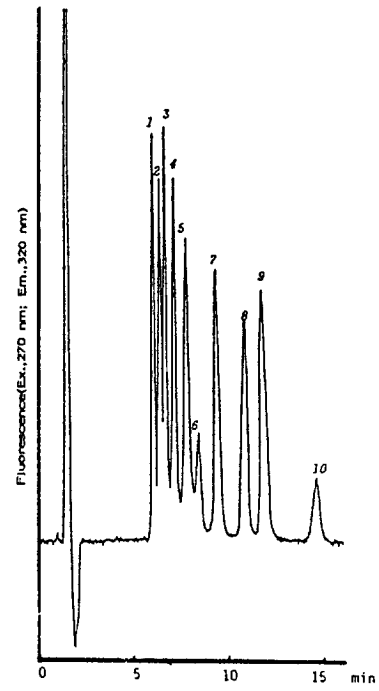

Fig. 6. RPLC of sugar Fmoc-hydrazones. Chromatographic conditions: column, Cosmosil 5 C_{18} (150 × 4.6 mm I.D.), particle size, 5 μm; mobile phase, 30% (v/v) aq. acetonitrile; fluorescence detection. Peaks: 1 = N-acetylgalactosamine; 2 = N-acetylglucosamine; 3 = mannose; 4 = galactose; 5 = glucose; 6 = sorbose; 7 = fructose; 8 = ribose; 9 = xylose; 10 = fucose; column efficiency (N) = 62 000 plates/m for xylose. (Reproduced with permission from Ref. [148].)

proteins were determined by RPLC as 9-fluorenylmethoxycarbonyl (Fmoc) hydrazide and 9-fluorenylmethyl chloroformate (Fmoc-Cl) derivatives using isocratic elution conditions and either fluorescence or ultraviolet detection in the picomole or sub-picomole range [148]. Fig. 6 is a typical example of RPLC of sugar Fmoc-hydrazones.

A simple, rapid and sensitive method for determining N-acetyl- and N-glycolylneuraminic acids in serum and in submandibular, sublingual and parotid glands using RPLC and fluorometric detection was described. It entailed the release of neuraminic acids in the presence of 2 M acetic acid by means of microwave hydrolysis for 10 min and the subsequent conversion of the sugars into highly fluorescent derivatives by labeling the neuraminic acid with 1,2-diamino-4,5-methylenedioxybenzene, a fluorescent tag for α-keto-acids [129]. The derivatives were separated on RPLC column by isocratic elution using water (pH 3.0)–methanol–acetonitrile (86:6:8, v/v) as the mobile phase. Fluorescence was performed at an excitation wavelength of 373 nm and an emission of 448 nm. This same pre-column derivatization was also applied to the determination of N-acetylneuraminic acid in several body fluids such as serum, cerebrospinal fluid,

saliva, urine, amniotic fluid and milk using RPLC with fluorometric detection [149].

The N-linked oligosaccharides from bovine fetuin were first released form tryptic glycopeptides with N-glycosidase F and then desialylated with neuraminidase, which resulted in the formation of monosialooligosaccharide and asialooligosaccharides [150]. The reducing ends of these oligosaccharides were converted first to glycosylamine and then reacted with N-hydroxysuccinimide ester of Boc-tyrosine. The tyrosinated oligosaccharides were resolved into individual peaks on RPLC column and could be detected at 280 nm, and had sufficient hydrophobicity to be separated by RPLC. One virtue of this derivatization is that the group can be deprotected by removal of Boc to reveal a primary amine suitable for further derivatization and can be also radio-ionidated for tracking during biological experiments.

Karamanos [151] described an isocratic system for RPLC analysis of xylitol and hexoaminitols present in the reduced terminal of glycosaminoglycans. The sugars were separated as the per-O-benzoylated derivatives on a C_{18} column using acetonitrile-rich eluents and the eluted derivatives were detected at 231 nm. Under these conditions, the detection limit was 10 pmol for xylitol and 6 pmol for hexosaminitols. The reaction of per-O-benzoylation was shown to give linear calibration graphs up to 150 nmol (30 μg).

3.1.3.3. Glycopeptides

The behavior of glycopeptides in RPLC is very similar to that of non-glycosylated peptides [152–154]. Glycopeptides are eluted slightly earlier than non-glycopeptides, and the major factor governing retention is hydrophobicity arising from the peptide backbone, even when 50% of the molecular mass of the glycopeptide is made up of carbohydrates [153]. However, even though the effect of the carbohydrate is considerably smaller than expected, it is still possible to separate by RPLC two peptides that have the same polypeptide structure but differ by the presence or absence of carbohydrates. It has been shown that there is a slight increase in the retention of deglycosylated peptides when compared to the parent glycopeptides in the RPLC peptide maps before and after digestion of the glycoprotein with PNGase F, an enzyme that cleaves N-linked oligosaccharides from glycoproteins or glycopeptides [155].

Recent studies with synthetic glycopeptides yielded results that were in agreement with the above observations [156]. O- and N-glycosylation reduced the retention times of the peptides similarly, but this reduction is not a linear function of the number of carbohydrate moieties. Incorporation of the first sugar reduced the retention time much more than a subsequently added sugar moiety. Glycosylation of the asparagine residue of a pentapeptide with a trisaccharide (chitotriose) almost did not decrease the elution time further than glycosylation with a disaccharide (chitobiose) [156]. Also, glycosylation of the same dodecapeptide with two different disaccharides, either chitobiose or cellobiose, yielded identical retention times for the two glycopeptides.

Conversely, glycopeptides with short peptide backbone behave differently. This is the case of glycopeptides generated by exhaustive treatments of glycoproteins with pronase, which usually leads to the formation of glycopeptides containing essentially very short peptide portions, most probably a single amino acid residue, i.e., the glycosylation site. Under these circumstances, the magnitude of retention is largely influenced by the carbohydrate portion of the glycopeptide. In fact, glycopeptides derived from ovalbumin by pronase digestion could be separated by RPLC on the basis of stereochemical differences of the carbohydrate moieties [157].

Very recently, PGC stationary phase, which can be viewed as a reversed-phase column with charge transfer property due to the delocalized electron clouds on the graphitized carbon, has been found useful in the separation of peptides and glycopeptides [66]. The dual character of the PGC column afforded the longest retention for the glycopeptides having acidic amino acids and sialic acids in their peptide backbone and sugar moieties, respectively.

The products of pronase digestion of the crystalline surface layer (S-layer) glycoproteins

of *Thermoanaerobacter thermohydrosulfuricus* strains L111-69 and L110-69 where isolated by various chromatographic techniques including RPLC. Four compounds were obtained by RPLC, and were subsequently analyzed by monosaccharide analysis, one- and two-dimensional 500 and 600 MHz ^{1}H and ^{13}C NMR, methylation analysis, gas–liquid chromatography–mass spectrometry, and matrix-assisted laser desorption ionization mass spectrometry [158].

Microsequence information was obtained from low picomole amounts of non-specific cross-reacting antigen (NCA) 160, a granulocyte membrane glycoprotein. The tryptic digest of this glycoprotein was mapped by capillary RPLC. The various peptide fragments were collected and further analyzed by Edman degradation microsequencing and mass spectrometry [159]. Also, RPLC was shown as a major step in the identification, quantification and characterization of glycopeptide fragments of 1.6 nmol of bovine fetuin tryptic digest [160]. The high resolving power of RPLC was exploited in the purification of a glycopeptide derived from yeast invertase that acted as an elicitor to tomato cells as well as to microsomal membranes derived from these cells [161]. Glycopeptides derived from the tryptic digest of intact recombinant HIV-1 gp120 (rgp 120) of the HIV-1 BH8 produced by a baculovirus expression system or of cyanogen bromide-generated fragments of rgp 120 were first isolated on concanavalin A-Sepharose column and then purified by RPLC [162]. The purified glycopeptides were treated with PNGase F to release the carbohydrate chains, which were isolated by high-pH anion-exchange chromatography and later analyzed by ^{1}H NMR. RPLC mapping of recombinant glycoprotein (e.g., recombinant tissue plasminogen activator, rt-PA) continues to be an important step in the characterization of these proteins [163].

RPLC was shown useful in assessing the extent of periodate oxidation and subsequent coupling of dansylethylenediamine to monosialylated biantennary glycopeptides derived from bovine fibrinogen. It is well established that the side chain of sialic acid can be oxidized with periodate to generate aldehyde functions which can be coupled to amines or hydrazine for a variety of purposes including the sensitive detection of glycoconjugates [164].

3.1.3.4. Glycoproteins

Since the polypeptide chain of glycoproteins is much larger than that of glycopeptides, the effect of glycosylation on RPLC retention of the glycoproteins is even much smaller. Usually, protein glycosylation occurs at more than one position in the amino acid sequence, and the sugar chains at even a single position may be heterogeneous or may be missing from some molecules of a given glycoprotein. This leads to populations of glycosylated variants of a single protein, usually referred to as glycoforms. Glycoproteins having multiple attachment sites for oligosaccharide (i.e., glycoforms) eluted from an RPLC column within a relatively narrow range, despite the heterogeneity of the carbohydrate chains [154]. This led to the formation of peaks that are not symmetrical Gaussian curves but have irregular shapes containing multiple components (i.e., glycoforms), indicating that carbohydrate microheterogeneities contribute to some extent to the retention in RPLC.

RPLC was shown to be useful for the characterization of α-L-arabinofuranoside in conjunction with denaturing gel electrophoresis and size-exclusion chromatography [165]. Also, RPLC proved suitable for the analysis of various glycoforms of recombinant human granulocyte colony stimulating factor produced in Chinese hamster ovary cells [166]. Furthermore, RPLC was used to assess the hydrophobicity of the high-molecular-mass, glycosylated subunits 2, 7, 8 and 12 of glutenin from Chinese Spring and TAM 105 wheats [167]. The various subunits eluted at 45% (v/v) acetonitrile within a 3-min range. Each pair of identically numbered subunits (e.g., 2 for Chinese Spring and 2 for TAM 105) exhibited similar, but not identical, elution times, i.e., hydrophobicities. According to this study, the HPLC peak for a purified protein consistently was found within a 0.05-min range for a series of injections over a period of one month or more [167]. A wide-pore C_4-bonded silica stationary

phase proved useful for RPLC determination of the glycoprotein hormones from human pituitary glands and of placental origin (e.g., thyroid-stimulating hormone, luteinizing hormone and chorionic gonadotropin) and for the isolation of pure chorionic gonadotropin and its subunits, using eluents of neutral pH [168]. Also, a wide-pore C_4-bonded silica column was utilized for the purification of biglycan associated colony stimulating factor with a molecular mass of 100 000, which did not carry an immunological motif of macrophage colony stimulating factor, but predominantly stimulated the proliferation and differentiation of monocytic lineage cells from bone marrow cells, non-adherant thymic cells and peritoneal exudate cells.

3.2. Ion-pair reversed-phase liquid chromatography

Ion-pair RPLC with its unique selectivity has been an attractive alternative to ion-exchange chromatography for the separation of ionic species including ionic carbohydrates. The most widely used ion-pairing agents are the tetrabutylammonium salts and alkyltrimethylammonium salts. Usually, retention increases with increasing concentration of ion-pairing agent (in the low concentration range) in the eluent. The retention factor k' decreases with increasing the ionic strength of the mobile phase or the organic modifier concentration. The pH is also used to manipulate retention.

Ion-pair RPLC has been used for the separation of a large number of acidic saccharides including oligosaccharides generated from various glycosaminoglycans (e.g., heparin, heparan sulfate, chondroitin sulfates, dermatan sulfate and keratan sulfate) by nitrous acid cleavage [169] or enzymatic cleavage [170], the quantitative determination of hyaluronic acid in biological tissue and fluids [171], unsaturated oligogalacturonic acids obtained by the degradation of pectic acid by pectic acid lyase [172,173], and oligouronic acids such as oligogalacturonic, oligoguluronic and oligomannuronic acids [174].

Exploiting the fact that carbohydrates are weakly acidic and partly present as anions at $pH \geq 12$, Stefansson and Westerlund [175] and Stefansson and Lu [176] introduced an HPLC method for the separation of carbohydrates as ion pairs in strongly alkaline mobile phases containing hydrophobic quaternary ammonium counterions and using polymeric stationary phases or Hypercarb PGC columns. The ion-pairing agents, dodecylethyldimethylammonium bromide, hexadecyltrimethylammonium bromide and nonyltrimethylammonium bromide were converted into the hydroxide forms by shaking with silver oxide and extraction with dichloromethane. Sugars, sugar acids, amino sugars and oligosaccharides were separated using these ion-pairing agents [176] and polystyrene divinyl-based column and Hypercarb PGC column [176. Important parameters for regulation of retention and selectivity are nature and concentration of the counterion, pH (hydroxide concentration) and temperature. Oligosaccharides are highly retained in these chromatographic systems. The addition of organic modifiers to the mobile phase for elution of the solutes was found to interfere with the pulsed-amperometric detection. Anions added to the mobile phase decreased solute retention due to their competing effect for ion-pair formation, and phosphate could be used for this purpose in the separation of maltooligomers ($dp = 2$ to 10) from corn syrup.

Recently, mass spectrometry was interfaced to an HPLC system using pneumatically assisted electrospray interface to determine oligosaccharides from enzymatic digestion of heparin with heparinase in the presence of tetrapropylammonium bromide as the ion-pairing agent and using a hexyl reversed-phase column [177].

Very recently, a simple and sensitive method for the determination of 4′-epimeric uridine diphosphate (UDP) sugars by ion-pair RPLC was described [178]. As expected, the ion-pair RPLC was advantageous over ion-exchange chromatography in terms of sensitivity and separation efficiency as well as the stability of the column. The method described in this study utilized octadecyltrimethylammonium bromide (OCTA), hexadecyltrimethylammonium bromide (HEXA) or tetradecylammonium bromide

(TETRA) as the ion-pairing agent in the presence of borate. The separation is influenced by the ability of borate ions to react with *cis*-diols resulting in the formation of UDP-sugar–borate complexes with different charges. To obtain a similar retention (i.e., k'), the following order of concentration was required: OCTA < HEXA < TETRA. In the range of concentration of ion-pairing agents tested, an increase in OCTA or HEXA concentration gave rise to linear increase in k' of the UDP-sugars, whereas in the case of TETRA a hyperbolic curve was obtained. Good resolution and rapid separation of all 4'-epimeric UDP-sugars was readily achieved, and the method was shown suitable for the determination of the UDP-2-deoxyglucose and UDP-2-deoxygalactose formed in yeast upon incubation in the presence of 2-deoxygalactose.

3.3. Hydrophobic interaction chromatography

3.3.1. Stationary phase

In HIC, the stationary phase is much less hydrophobic than in RPLC. The surface of the rigid support is usually covered with a hydrophillic coating which becomes highly hydrated when in contact with aqueous mobile phases. The hydrophobic ligands, usually widely spaced short alkyl or aryl functions, are attached to the strongly hydrophillic surface layer. This sublayer, i.e., hydrophillic surface layer, also serves as an inert spacer so that the incoming solute would interact with the hydrophobic ligands and not with the support proper. This mimics the inertness and highly hydrated nature of polysaccharide gels which are used as the support in traditional low-pressure HIC. Along this strategy, several rigid microparticulate HIC stationary phases based on organic or inorganic supports have been introduced. For instance, silica and polymeric supports having surface-bound hydrophillic layers, which were originally designed for size-exclusion chromatography of biopolymers, were further derivatized through their hydroxyl groups with butyl, phenyl [179] or polyether functions [180]. Weakly hydrophobic polyamide ligands were attached to silica [181,182] to produce HIC sorbents. Also, silica-based stationary phases containing methyl, hydroxypropyl, propyl, benzyl, or pentyl were introduced [183]. Ether ligands of the structure–Si–$(CH_2)_3$–O–$(CH_2CH_2–O)_n$–R, where n is 1, 2 or 3 and R methyl, ethyl or butyl function, were covalently attached to microparticulate silica supports [184]. Polyethylene glycol of M_r 400 was coupled to an epoxy activated silica gel and yielded a column that afforded 90% recovery of proteins [185]. A series of poly(alkylaspartamide)-silica stationary phases is also available for HIC of proteins [186]. Silica gels with surface-bound polyvinyl alcohol or oligoaminoalcohol ligands [11], or amide functions [187] were shown to be useful for HIC of proteins. Hjertén et al. [188] introduced rigid microparticulate highly cross-linked agarose, which may be used in HIC of proteins under HPLC conditions. Separon HEMA materials, which are copolymers of 2-hydroxyethyl methacrylate (HEMA) and ethylene dimethacrylate, are rigid materials that can be used under HPLC conditions without any modification as HIC sorbents [189]. Under this condition, only the hydrophobic sites on the surface of proteins can interact with hydrophobic areas on the support. This has been shown to yield low efficiency. To overcome this drawback, simple modification of Separon HEMA with benzoyl chloride and especially with 1,2-epoxy-3-phenoxypropane yielded increased efficiency and selectivity towards proteins [190]. The utility of the derivatized Separon HEMA was demonstrated in the HIC of glycoproteins [190]. In another approach, RPLC columns were transformed dynamically to HIC columns by coating the RPLC column with non-ionic surfactant of polyoxyethylene type [191].

As in the case of RPLC, several non-porous HIC column materials have been introduced for the rapid separation of proteins on the time scale of a few seconds and minutes. Monodisperse non-porous silica columns with surface-bound amide or ether [192] were introduced for rapid HIC. As expected, differences in selectivity were observed between the amide and the ether phase. Hjertén and Liao [193] and Hjertén et al. [194] converted macroporous agarose beads into non-porous particles by shrinkage and cross-link-

ing in organic solvents, and showed that these beads could be used for high-performance HIC without derivatization, because the 1,4-butanediol diglycidyl ether, used as cross-linker, gives relatively hydrophobic bridges. These particles have the advantage over silica-based materials of being stable up to pH 14. Kato et al. [195] modified a non-porous hydrophillic resin of 2.5-μm particle diameter into an HIC sorbent by covalently attaching butyl functions to the surface of the rigid particles, and the resulting packing is commercially available under the trade name TSKgel Butyl-NPR. Recently, Kalghatgi [76] introduced a silica-based HIC column by modifying 2.0-μm particles with polyether functions. A mixture of four proteins could be separated in less than 2.0 min.

Among the stationary phases available for HIC, the more hydrophobic are those bearing short alkyl chains at the surface of the support, followed by phenyl, polyether and glycol phases. On polyether stationary phases, the retention was found to increase with no significant change in selectivity when the alkyl end group was varied from methyl to ethyl. Stationary phases with butyl end group adsorbed irreversibly proteins, which were not eluted even with a dilute buffer [184]. On the other hand, in the case of poly(alkylaspartamide)-silica, not only did changing the length of the ligand from ethyl to propyl increase protein retention, but also the selectivity of the separation was affected significantly [186].

3.3.2. Mobile phase

In HIC, the sample is first loaded onto a column that is equilibrated with a mobile phase of relatively high salt concentration. Subsequently, the adsorbed proteins are eluted by stepwise or gradient elution at decreasing salt concentration in the eluent. In this adsorption–desorption process, the nature and concentration of the neutral salt play an important role in determining the magnitude of retention and selectivity.

Generally, and in the absence of specific interactions between the salt and the protein, the effectiveness of the salt in promoting hydrophobic interaction follows the Hofmeister series, which is based on the ability of the salt to cause precipitation of proteins from aqueous solutions. Protein retention is stronger with kosmotropic salts (also called antichaotropic salts, i.e., water-structure makers) that increase the surface tension of aqueous solutions (e.g., phosphates, sulfates and citrates) than with chaotropic salts (water-structure breakers) such as perchlorates and thiocyanates.

To be effective in HIC, the neutral salt must be highly soluble (solubility > 1.0 M) so that it can be added in relatively high concentration to the eluent to induce hydrophobic interaction. Other important factors for choosing the neutral salt are (i) viscosity, (ii) UV transparency, and (iii) stability at alkaline pH. Among the useful salts, ammonium sulfate is the least viscous, whereas sodium sulfate and sodium phosphate are characterized by a higher molal surface tension increment. On this basis, sodium sulfate and sodium phosphate exhibited higher retention while ammonium sulfate provided a better separation efficiency and sharper peaks [196] under otherwise identical conditions. Although ammonium sulfate has a very high solubility and is available in high purity, the chromatography at alkaline pH is accompanied by decomposition of the salt and liberation of ammonia. In this case, monosodium glutamate or sodium sulfate is an alternative salt [197].

The choice of the salt and its concentration in the eluent is dictated by the hydrophobic character of the proteins and that of the stationary phase. Hydrophobic proteins are better chromatographed with mildly hydrophobic stationary phases such as polyether phases and with salts of relatively low molal surface tension increment such as sodium chloride, whereas hydrophillic proteins would require stationary phases with stronger hydrophobic character such as butyl or phenyl and salts of high surface tension such as citrates or sulfates at high concentration, to bring about retention and separation of the proteins. When the protein and/or the stationary phase are relatively hydrophobic, the inclusion of a small amount of organic solvent (ethanol or propanol) is recommended to bring about better mass recovery of the protein [22]. For hydrophobic and insoluble proteins, the addition of ionic or non-ionic surfactants at concentrations

below their critical micelle concentration should be considered if mild conditions do not suffice to elute the proteins. In some instances, a detergent is added to solubilize the solutes such as membrane proteins and to keep the biomolecules from forming molecular aggregates in the eluent [198]. Also, the addition of denaturing agents such as urea and guanidine chloride may be useful in eluting strongly bound proteins [199].

Recently, a novel approach has been introduced for the elution and separation of proteins by HIC [200]. It involved the use of binary and ternary salt gradients with mixtures of chaotropic and antichaotropic salts as well as organic salts. At constant eluent surface tension, gradient with two or three aqueous salt solutions was found to be superior to single salt gradient in modulating HIC retention and selectivity. The effect was attributed to the competitive salt-specific binding to the protein molecule and/or the stationary phase. In general, with chaotropic–antichaotropic salt gradient systems, the retention of basic protein increased while that of acidic proteins either decreased or remained unchanged with the use of chaotropic salts. At the same surface tension of the eluent, KSCN and $KClO_4$ yielded different selectivity. The addition of organic salts, such as tetrabutylammonium bromide, was found to be suitable for the separation of proteins having a wide range of isoelectric points [200].

3.3.3. Some general aspects of HIC of glycoproteins and selected applications

Unlike RPLC, HIC is virtually not denaturing for proteins because organic solvent is not required for elution, and the stationary phases, which are mildly hydrophobic, would not cause any substantial change in the conformation of the protein molecule. As a typical example, ceruloplasmin, which was denatured in RPLC, was recovered from an HIC column in its native structure [154]. Also, β-2-glycoprotein I, which binds lipid because it is a constituent of lipoprotein, showed two distinct peaks in HIC versus a single peak in RPLC. This behavior was explained by the fact that some of the molecules are associated with lipids which can be washed by the organic solvent used in RPLC, thus yielding a single peak [154].

Although rigid microparticulate stationary phases are now commercially available from several column manufacturers for high-performance HIC, most of the applications are still carried out by classical, low-pressure HIC using aryl- or alkyl-agarose gel as the sorbents. Some of the applications which utilized rigid microparticulate HIC columns are summarized below.

High-performance HIC was recently applied to the purification of glycoprotein hormones, e.g., chorionic gonadotropin CG and thyroid-stimulating hormone (TSH), on phenyl-modified polymeric microparticulate support (10 μm Separon HEMA BIO 1000 Phenyl) and a descending gradient of ammonium sulfate [168]. The CG and TSH exhibited relatively strong hydrophobic behavior and necessitated the addition of 10% ethanol to the gradient former. The loading capacity of the Separon column was relatively high, permitting the isolation of 100 mg of hormone per column. Human luteinizing hormone (hLH), which is a heterogeneous glycoprotein, was analyzed by HIC to assess heterogeneity other than that based on charge [201].

Rat sperm maturation glycoprotein (SMemG) was purified on a propylaspartamide HIC column using a decreasing salt gradient of ammonium sulfate at pH 6.0. The apparent molecular mass of the purified SMemG as measured by SDS-PAGE was found to be 26 000 [202].

The isolation of a protein from a given sample often requires the use of more than one chromatographic technique. HIC is usually used in combination with other chromatographic techniques to carry out a given purification. For instance, the various entities present in a crude phosvitin/phosvette were resolved by a combination of size exclusion, anion exchange and HIC. This system allowed the identification of four major entities (phosvitins 1 and 2 and phosvettes 1 and 2) and the microheterogeneity was apparent particularly by HIC. Phosvitins are highly acidic phosphorylated glycoproteins found in vertebrate egg yolk. Smaller phosvitin-like proteins, termed phosvettes, have been found in the eggs of chicken. In HIC, at least 14 peaks were discerned with a decreasing salt gradient of

ammonium sulfate on a TSK phenyl-5-PW HIC column [203]. Recombinant human granulocyte-macrophage colony stimulating factor (rhGM-CSF) was purified to homogeneity by a three-step chromatographic procedure in which Phenyl-Sepharose fast-flow column was used [204]. Also, HIC on fast-flow Phenyl-Sepharose was used in combination with thiophilic interaction chromatography in an attempt to obtain enriched human immunoglobulin G (IgG) subclasses from a therapeutic immunoglobulin preparation [205].

A purified murine monoclonal anti-granulocyte IgG_1, designated as IMMU-MN3, which yielded two heavy chain bands of unequal intensity and only one light chain band by SDS-PAGE, gave two resolved peaks by HIC, indicating the presence of two populations of the antibody. Based on subsequent concanavalin A affinity chromatography and digestion with endoglycosidase F and carbohydrate analysis, it was found that the heterogeneity observed with SDS-PAGE and HIC was due to differences in glycosylation [206]. The potential of HIC was also demonstrated in the purification of β_2-microglobulin and monoclonal antibody against prolactin on Separon HEMA 1000 H phenyl column [190].

Besides its major use in separation, HIC retention data have been shown useful in determining the location of hydrophobic functional groups in the protein molecule, e.g., leucine-rich glycoprotein (LRG). The highly glycosylated LRG, which is highly soluble in water (i.e., highly hydrophillic), showed excessive retention in HIC, indicating that the hydrophobic patches (i.e., clustering of the hydrophobic leucine) is probably exposed outside of the molecule, and the carbohydrate did not have much effect on the interaction between the protein and the HIC stationary phase [154].

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