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Review

Ligand-exchange chromatography of carbohydrates and glycoconjugates

Morgan Stefansson[a], Douglas Westerlund[b,*]

[a] *Institute of Analytical Chemistry, Uppsala University, P.O. Box 531, 751 21, Uppsala, Sweden*

[b] *Analytical Pharmaceutical Chemistry, Uppsala University Biomedical Centre, P.O. Box 574, 751 23 Uppsala, Sweden*

Abstract

The current status of ligand-exchange chromatography (LEC) is reviewed in the light of recent developments, especially regarding mobile phase conditions and choice of metal ions. Further, parameters governing selectivity are emphasized. The paper is divided into two parts: LEC at acidic/neutral pH and at alkaline pH. The general characteristics of each part are outlined and illustrated by appropriate applications, including bioanalysis of carbohydrates in complex mixtures. In particular, the exceptionally strong complexation between carbohydrates and certain metal ions at alkaline pH appears promising for enrichment and clean-up possibilities owing to the high degree of inherent selectivity. Finally, future directions are discussed with regard to the intricate isolation and separation problems associated with glycotechnology. Further advances within this field will depend on the development of analytical methodologies for minute amounts (femtomoles) of complex carbohydrate mixtures present on proteins, receptors and cell surfaces and inside the cells.

Contents

* Corresponding author.

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1. Introduction

There are three major classes of biopolymers present in nature: polynucleotides, proteins and carbohydrates. Nucleic acids, carriers of genetic information, and proteins have received enormous attention, and until the late 1960s carbohydrates were merely regarded to serve as energy sources. The tedious work and, occasionally, lack of analytical methodologies discouraged further progress in carbohydrate chemistry. The type of monosaccharide unit, linkage and the presence and number of branching points that constitute a certain carbohydrate make them excellent carriers of information owing to the vast number of combinations possible. Four different monosaccharides can assemble 35560 unique tetrasaccharides. For nucleotides the number is 24 [1]. Later work has shown that carbohydrates are involved in a number of biochemically important functions, such as cell–cell interaction and communication, attachment for infectious bacteria, viruses, toxins and hormones, to mention just a few [1].

Ligand-exchange chromatography (LEC) on cation exchangers in metal ion forms has been used extensively for the separation of mono-/oligosaccharides and polyols, and several reviews on the vast topic of carbohydrate separation have been published [2–4].

The exceptional physico-chemical properties of saccharides put very high demands on the analytical methodology applied. Often, additional precautions regarding each individual step during the analysis must be taken, including sampling, sample treatments, separation and detection principles. Carbohydrates are highly polar compounds, although an amphiphilic nature for certain polysaccharides does exist [5]. They are generally water soluble and for many of the larger polymers complex equilibria, such as gel and helix formation, adsorption of uncharged and charged components, phase separation [6], etc., take place. Further, they are slightly acidic with pK_a values of the order of 12–14 [7].

The complexation between neutral sugars and metal ions co-ordinated to cation resins was first demonstrated by Jones et al. [8] in 1960. The separation mechanism involved in LEC has been described as a combination of ligand exchange, steric exclusion, partitioning, hydrophobic adsorption and electrostatic attraction or repulsion [9–12].

The aim of this review is to outline the general characteristics of LEC of carbohydrates and glycoconjugates with emphasis on the use of alkaline mobile phases. Illustrative examples of recent advances in LEC will be surveyed and, concurrently, new possibilities for appropriate applications.

2. LEC at acidic or neutral pH

2.1. Theory

The set-up of a LEC system consists of metal ions electrostatically immobilized on a strong cation exchanger (polystyrene–divinylbenzene resin with 4–8% cross-linking) using water as the mobile phase. Besides the complex formation between polyols and metal ions, the separations are governed by size-exclusion mechanisms and oligomers elute in order of decreasing molecular mass [9]. In order to avoid contamination on the resin from other metal ions present in the sample or the chromatographic system, Brunt [13] included 50 ppm of Ca-EDTA in the mobile phase. The selectivity is determined largely by the nature of the cation employed, Ca^{2+}, Ag^{+} and Pb^{2+} being the most commonly used.

Depending on the coordination number, a certain number of water molecules bind to the metal ion. When a carbohydrate mixture is introduced into the mobile phase, the sample components will displace some of the bound water molecules, forming a donor–acceptor complex [14]. The stability constant for the complex formation depends on a number of parameters, such as carbohydrate structure, type of metal ion, temperature and the presence of mobile phase additives which, hence, determine the separation. The magnitude of the stability constants is relatively small [15] with the exception of sugars containing amino or carboxyl groups. As a result, relatively low capacity factors are

obtained, severely limiting the operational range for optimization procedures regarding the separation selectivity. However, suitable selectivity for a given carbohydrate mixture can usually be obtained by appropriate manipulation of the chromatographic conditions and column length.

To form a bidentate or tridentate complex via two or three hydroxyl groups, the cation must have an octahedral structure [15]. The stability constants are of the order of 0.1–10 mol^{-1}, respectively. Maximum stability of the complexes is obtained with a cationic radius of ca. 1 Å and a tridentate complex with the hydroxyl groups in an axial–equatorial–axial (a–e–a) sequence on a pyranose ring [16], in *cis–cis* on a furanose ring [16] and on an M–P sequence on an open chain where the first and second carbons are *gauche* clockwise and the second and third carbons are *gauche* anticlockwise or vice versa [17]. The stability of the complexes decreases in the order *threo–threo* (t–t) > a pair of *threo* adjacent to a primary hydroxyl (w–t) > *erythro–threo* (e–t) > a pair of *erythro* adjacent to a primary hydroxyl (w–e) [17].

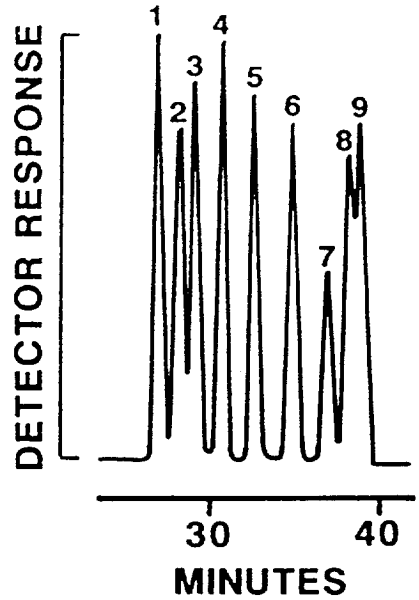

Fig. 1. LEC of mono- and disaccharides on two Pb^{2+} columns (60 cm, SP 1010) from Shodex. Eluent, water at 0.6 ml/min; temperature, detection, 80°C; refractive index; sugar concentrations, 0.4% each. Peaks: 1 = sucrose; 2 = maltose; 3 = lactose; 4 = glucose; 5 = xylose; 6 = galactose; 7 = arabinose; 8 = mannose; 9 = fructose. From Showa Denko application data on Shodex sugar series.

2.2. Type of metal ion and selectivity

Separations of hexoses, pentoses and the corresponding sugar alcohols have been reported [18] using seven different cations, Ca^{2+}, Sr^{2+}, Pb^{2+}, Ba^{2+}, Y^{3+}, La^{3+} and Pr^{3+}. All polyols eluted after the sugars, probably owing to their ability to form tridentate complexes, and the retention was highest with the rare earth metals. However, the sugars were less retained on these resins, presumably owing to exclusion effects. Ca^{2+} seemed to be the most efficient cation for sugar–sugar separations whereas La^{3+} gave the highest selectivity for polyols. For Pb^{2+}, the sugars had a much higher retention owing to the partially vacant 5d orbital, hence facilitating the complexation with the soft acid nature of hydroxyl groups. Lead also accelerates the speed of mutarotation [19]. A separation of mono- and disaccharides is shown in Fig. 1.

A Pb^{2+}-loaded resin has also been used in the bioanalysis of carbohydrates in fermentation substrates and broths [20]. The analyses of two lignocellulose hydrolysates, spent sulfite liquor (SSL) and an enzymatic hydrolysate of steam-pre-treated *Salix caprea* (EH), are presented in Fig. 2. The sample clean-up and pretreatment involved centrifugation, filtration and solid-phase extraction by stirring the sample with a polymeric anion- and cation-exchange support (Amberlite). Detection was by refractive index monitoring. The chromatographic isolation of ^{14}C-labelled glucooligosaccharides, monosaccharides and sugar degradation products has also been reported [21] using strong cation exchangers in different metal forms and water as the mobile phase.

In another study [22], alditols were found to form reasonably strong complexes with lanthanide (La^{3+} and Y^{3+}) and alkaline earth metal ions (Ca^{2+} and Sr^{2+}). Fe^{3+} was the only 3d transition metal ion to interact markedly. In

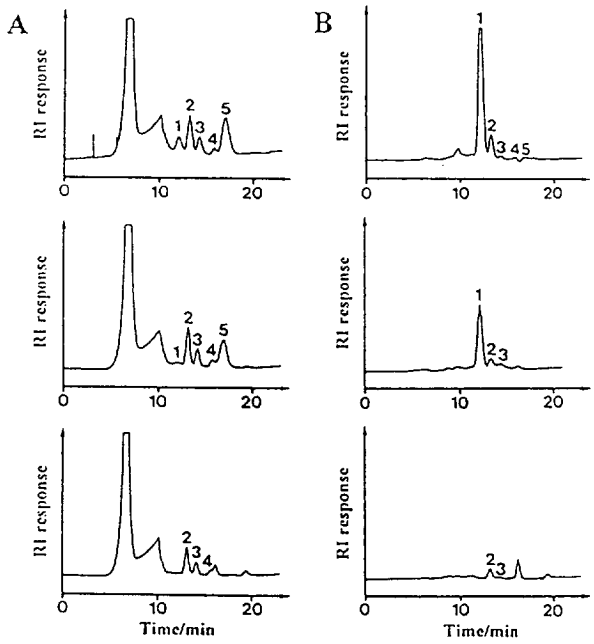

Fig. 2. Chromatograms of samples taken after (from top to bottom) 0, 1 and 24 h of fermentation in (A) SSL and (B) EH. The analytical column (300 × 7.8 mm I.D.) was a ligand-exchange column in the Pb^{2+} form (Aminex HPX-87P) at 85°C using Milli-Q-purified water as the mobile phase and refractive index detection [20]. Peaks: 1 = glucose; 2 = xylose; 3 = galactose; 4 = aribinose; 5 = mannose.

support of a previous finding [23], complexation of alditols with copper(II) acetate was noticed, but not with copper(II) sulfate. The presence of dimeric $[Cu_2(OH)_2]^{2+}$ as the active species has been proposed [24]. In an evaluation of thirteen resin cation forms, 28 sugars and related compounds were investigated [25] in order to generate a database on sugar elution behaviour. Generally, the retention of oligosaccharides varied over a comparatively narrow range, whereas ribose, tagatose and xylitol appeared to be very sensitive to column cation form. Group IA elements produced small deviations in elution times for all sugars while lead, calcium and strontium produced higher deviations. For some pairs of sugars that are poorly separated on the commercially available columns, strontium exhibited the highest selectivity. Arabinose and galactose were readily separated on rubidium or caesium-form columns. In the case of an especially hard to separate mixtures, columns in different metal forms might be connected in series to improve the separation selectivity, exemplified by a separation of carbohydrates and degradation products from hydrolysed wood samples [26] using Ca^{2+} and Ag^{+} columns. Owing to the not fully resolved peaks, quantitative measurements are limited for components present at low levels. Ag^{+} columns used in series were able to resolve oligosaccharides with a degree of polymerization (DP) up to 20 [27].

2.3. Temperature

When a reducing monosaccharide (e.g. D-glucose) is dissolved in an aqueous solution, at least six different dynamic equilibria prevail, including α- and β-anomers, pyranose and furanose conformations and open forms. The relative amount of the separate forms differs between different sugar molecules and, further, is influenced by ionic strength, pH, temperature, additives, etc. The mutarotation between α- and β-anomers is a slow process on a chromatographic time-scale and the systems are most often run at elevated temperatures in order to avoid broad or double peaks owing to resolution of the individual anomers [14]. Further, the slow diffusion-controlled partitioning processes are accelerated, enhancing peak performance [28].

2.4. Particle size

Optimization of the separation of two industrial sugar alcohol mixtures using polynomial correlation procedures was recently reported [29]. The parameters investigated were reticulation, particle size and resolution. An increase in cross-linking was found to increase the capacity and improve the chromatographic behaviour of the resin owing to less swelling. This was counteracted, however, by restrictions in intra-particle diffusion and no systematic correlation could be determined. Specific interactions between the analytes and a polystyrene resin have been reported [13,30] where elution times between cyclic and linear maltooligosaccharides with the same number of carbons were found to

differ greatly. Combinations of size exclusion, hydrophobic interaction ligand-exchange and partitioning are mechanisms likely to affect the overall retention.

3. LEC at alkaline pH

3.1. Principle and retention model

One of the properties of carbohydrates is the weak acidity [7]. The pK_a values are of the order of 12–14 and with increasing hydroxide concentration several of the hydroxyl groups become ionized, the C-2 being the most acidic. Owing to inductive effects, the β-form is twice as acidic as the α-form for glucosides. Further, at millimolar concentrations of hydroxide the mutarotation of sugars is very rapid and the individual anomeric forms are no longer resolved which, basically, is an advantage.

At highly alkaline pH, carbohydrates and glycoconjugates have been shown to form exceedingly strong complexes with resins in rare earth metal ion, uranyl [31] and for saccharides even iron(III) forms [32]. As the complexation readily occurs with the ionized sugar unit, there is a strong increase in retention (k') at pH > 11. When the solute is fully ionized, depending on the pK_a of the sugar, the retention decreases with further increase in hydroxide concentration owing to competition for complex formation. A general retention model for a fully ionized solute is given by [31]

$$k' = qK_C^*[\text{resin} \cdot \text{Tb(OH)}_2]/[\text{OH}^-]^x \quad (1)$$

where q is the phase ratio in the column, K_C^* is the apparent thermodynamic exchange constant, $K_C^* = K_C\,[1/(1 + 10^{pK_a - pH})]$, and x is the number of hydroxide molecules displaced by the solute. The columns were prepared as follows: the cation exchanger in the H^+-form was converted into the metal form through injections of concentrated metal salts. The excess was removed by washing and the resin was finally converted into the metal hydroxide form by introduction of sodium hydroxide. The metal complex was shown, through titrations [31], to be resin–$SO_3^-M^{3+}(OH^-)_2$.

Acccording to the retention model, rectilinear relationships are obtained by plotting ln k' versus ln $[OH^-]$, as demonstrated in Fig. 3 for sorbitol, glucuronic acid and glucose on a column in the europium(III) form. Distribution isotherms have been investigated for both glycoconjugates and carbohydrates on different columns [31,32] and one example, including the isotherm plotted according to the retention model, is presented in Fig. 4A and B for phenyl galactoside on a terbium(III)-modified column. The deviation from the straight line in Fig. 4B was due to saturation of binding sides.

If a rapid elution of an injected sample components is desired, introduction of an acidic mobile phase can be performed, e.g., 0.3 *M* morpholinoethanesulfonate (MES), pH 5. The MES buffer has no affinity for the cations and keeps the resin intact, thus preventing metal stripping.

3.2. Retention characteristics

The influence of analyte structure on the complex formation and measurements of the distribution isotherms have been investigated by frontal analysis using different metal ions. The breakthrough curve is characterized by pro-

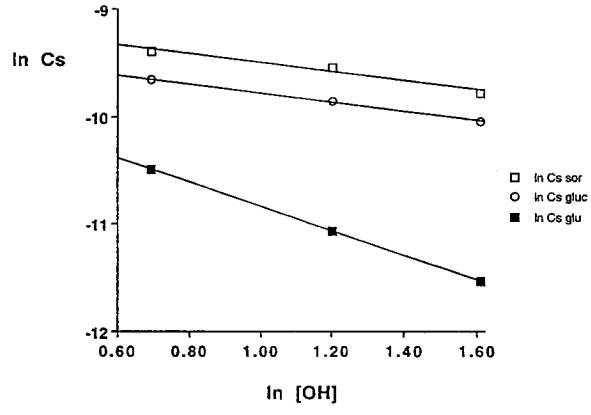

Fig. 3. Hydroxide competition on complex formation for (□) sorbitol, (○) glucuronic acid and (■) glucose on a europium(III)-loaded column and plotted according to the retention model [32].

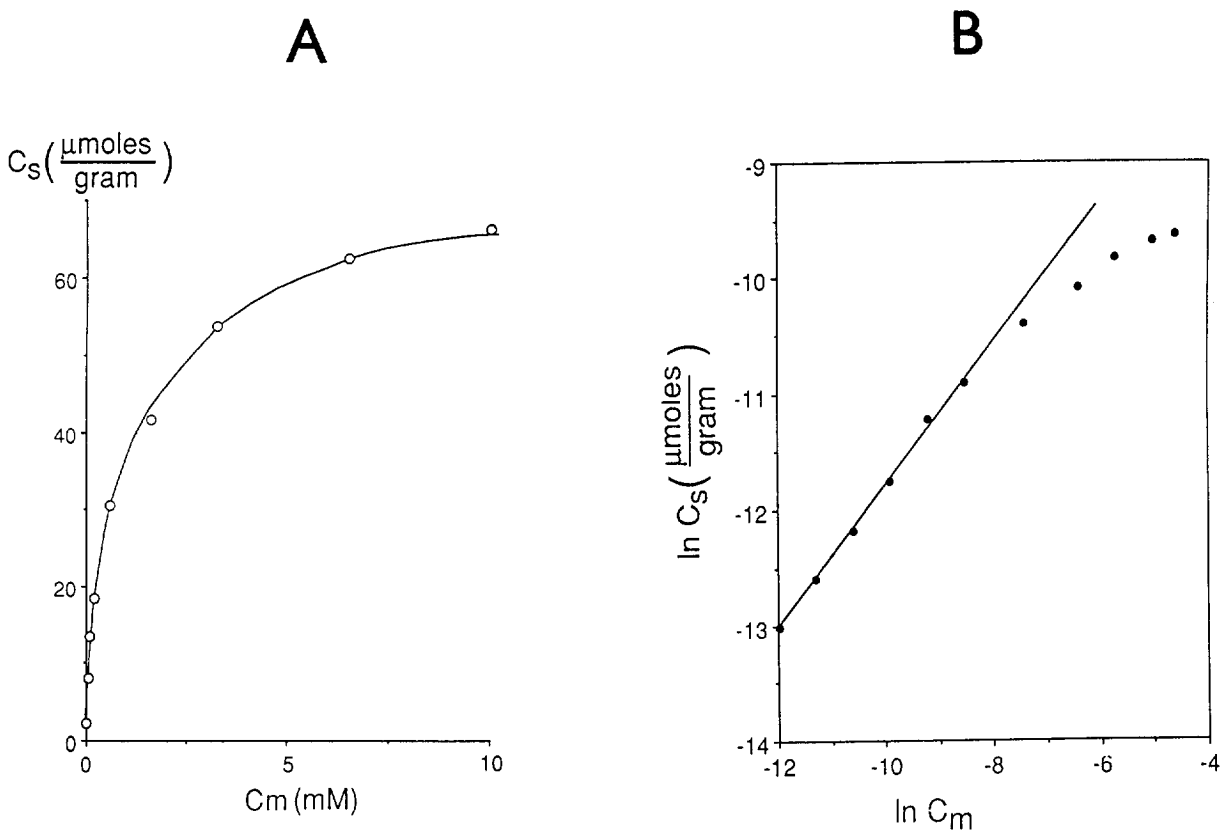

Fig. 4. (A) Distribution isotherm and (B) isotherm plotted according to retention model for phenyl-β-D-galactoside [31].

nounced tailing and the time- and concentration-dependent distributions have been shown to involve second-order kinetics.

3.2.1. Glycoconjugates

High capacity ratios were obtained for different furanose derivatives [31] containing *cis*-oriented hydroxyl groups, e.g. ribose-containing compounds. The stability increased with increasing substitution of the 5′-hydroxyl for phosphate groups whereas 3′-phosphate, 2′-deoxy or 5′-iodo derivatives were unretained, indicating requirements of tridentate chelation. For pyranosides, β-glycosides were more retained than the corresponding α-form, which was inconsistent with the acidity of the compounds (see above). Glycoconjugates containing axially oriented hydroxyls, i.e. mannoside, rhamnoside, fucoside and galactoside, gave stronger complexes, as did solutes containing an acidic group. Solutes lacking the C-6 hydroxyl group were less retained. Compounds with acidic groups gave more stable complexes owing to inductive effects, as did disaccharides, probably caused by a higher acidity, as shown by electrophoretic measurements for homologous series of sugars oligomers with increasing degree of polymerization [33]. Alcohols, phenols, catechols, hydroxy acids and amino acids were poorly retained, thus demonstrating the high selectivity of the systems. The retention power of the rare earth metal ions decreases in the order Eu > Tb > Y, which was mainly due to their basicity, i.e., hydroxide formation.

3.2.2. Carbohydrates

The distribution of saccharides and polyols to a cation exchanger in the Eu(III) form are

displayed in Table 1. Monosaccharides were more retained than the corresponding oligosaccharides [32]. With increasing size of the sugar molecule, a constant magnitude of the distribution was obtained, probably indicating an exclusion effect from the pores. Sugar alcohols exhibited differences in complex formation compared with their corresponding sugars and no relationship between the number of hydroxyl groups and retention could be obtained. In spite of their higher pK_a values (about ten times less acidic) they were retained to a similar extent as the monosaccharides. This is believed to be due to the open-chain structures and their ability to rotate the carbon–carbon bonds in order to orient the hydroxyl groups towards the metal ion and, hence, facilitating the complexation. Owing to the outstanding complexation reactions of the sugar alcohols, even iron(III) ions could be utilized. Because of the pronounced hydroxide competition on the Fe^{3+} resin, very few classes of compounds are retained, resulting in a high degree of selectivity.

3.3. Mobile phase additives

3.3.1. Organic modifiers

Addition of components to the mobile phase can either change the apparent stability constant for the complexation or compete for binding itself. When different organic and uncharged solvents were included in the mobile phase [34], an exponential increase in distribution with the fraction of modifier added was obtained. The order between different solvents was acetonitrile, 2-propanol and methanol, using phenyl

Table 1
Influence of structure on retention in the Eu(III) system

Solute	C_s (μmol/g)	Solute	C_s (μmol/g)
Monosaccharides		*Tetrasaccharide*	
Ribose	158	Stachyose	30.2
Tagatose	119		
Fructose	99.8	*Sugar acids*	
Sorbose	79.4	Galacturonic acid	77.7
Lyxose	78.9	Glucuronic acid	70.4
Galactose	74.2		
Mannose	66.4	*Amino sugars*	
Xylose	55.2	Galactosamine	18.9
Arabinose	46.9	Glucosamine	7.9
Glucose	45.8		
Fucose	40.8		
		Sugar alcohols	
Disaccharides		Xylitol	110
Sucrose	38.4	Sorbitol (glucitol)	104
Gentiobiose	37.4	Galactitol	94.8
Maltose	30.4	Arabitol	80.2
Melibiose	30.4	Mannitol	74.8
Lactose	28.9	Ribitol (adonitol)	41.1
Cellobiose	23.8		
		Miscellaneous	
Trisaccharide		Myoi-Inositol	43.9
Raffinose	30.2	β-Cyclodextrin	15.6

Column: Hitachi 3011-S, 21 × 4.6 mm I.D. in Eu(III) form. Mobile phase: 0.1 *M* NaOH and 1.0 m*M* saccharide. C_s (μmol/g), the concentration of solute on the solid phase, was measured at the inflection point with the baseline using frontal analysis.

glucuronide as the solute. However, if a solvent has affinity for the metal ions, e.g., 1,2-ethanediol, the complexation is reduced or even cancelled.

3.3.2. Displacement

Citrate, phosphate, mannitol and lactose were investigated as displacing agents [34] owing to their complexation with metal ions. The procedure was a follows: first 4-nitrophenyl glucuronide was injected into the column, then an alkaline mobile phase containing the displacer was introduced. Finally, an acidic MES buffer was introduced for elution of any remaining solute. For all the agents examined, only partial desorption at alkaline pH could be obtained, indicating that the solute was retained in several kinds of complexes, all of which were not subject to exchange with the displacers.

3.4. Temperature

Evidence for more than one complexation mechanism was also obtained in temperature studies on inosine-5′-monophosphate (IMP) and phenyl-β-D-galactoside (PG) distribution. In van't Hoff plots, both solutes had identical slopes in the interval 10–30°C, indicating similarities in their complexation behaviour. However, above 30°C the distribution of IMP to the solid phase increased and at 69°C there was a pronounced decrease in the distribution for PG, causing a reversal in the strength of the apparent stability constants. Additionally, the influence of hydroxide concentration (competition) on the IMP distribution at 60°C revealed differences in complexation mechanisms compared with 20°C. No concentration dependences for the complexation could be observed, however, in the interval 12.5–1000 μM.

3.5. Applications and future directions

Contrary to LEC at acidic or neutral pH, strong complexation between carbohydrates and suitable metal ions is obtained at alkaline pH. This provides the means for manipulation of the systems due to the operational space brought forth by the high capacity ratios. Concurrently, as common chelating ligands such as amino acids, hydroxy acids and amines stay unbound, a high degree of selectivity is obtained. The preconcentration capabilities are obvious and a 10 μM solution of glucose was enriched by a factor of 3500 using a terbium-loaded column [32]. This would permit equivalent improvements regarding the detection sensitivity in many of the currently used systems.

The methodology has potential for both analytical and preparative work and can be employed on- or off-line depending on the application. One example on off-line use is the separation of paracetamol glucuronide (Fig. 5). After direct injection and clean-up of a urine sample on a terbium-loaded column, the glucuronide was desorbed with acidic MES buffer and a fraction of the eluate was then injected on a C_{18} column and separated by ion-pair LC [31]. The stability of the ligand-exchange column was excellent, 90% of the metal ions remaining after 90 ml of urine had been pumped over the resin. Owing to the "over-capacity" of the column, when used under analytical conditions, this corresponds to several thousand injections.

An optional utilization would be preparative work-up of synthetic products from components present in a solution after the reaction. This

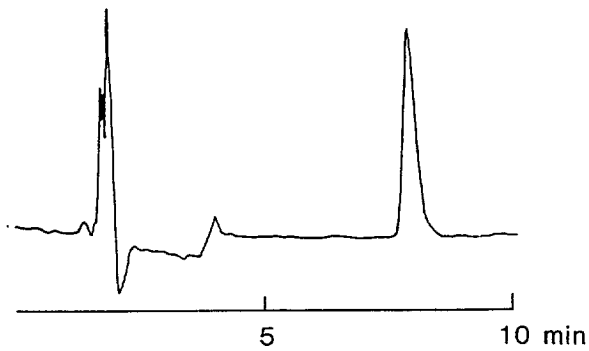

Fig. 5. Chromatogram of paracetamol-β-D-glucuronide in urine (retention time 8 min) collected 1.5–2.5 h after oral administration of 1 g of paracetamol. The analytical column (150 × 4.6 mm I.D.) was Nucleosil 100-5 C_{18} and 50% methanol, 0.05 M glycine (pH 3.0), containing 15 mM dodecylamine · HCl as a counter ion for the anionic paracetamol glucuronide, was used as the mobile phase. UV detection at 254 nm [31].

could be especially valuable for very polar compounds not soluble in organic solvents and, hence, incompatible with normal-phase LC systems.

By connecting the ligand-exchange column on-line in a coupled column separation system, transfer and recovery are optimized. Further, it is possible to automate such an arrangement. An on-line analysis of the diastereomeric glucuronides of almokalant, an antiarrhythmic drug, from urine [35] is displayed in Fig. 6, using porous graphitic carbon as the analytical separation column. Any background due to endogenous material was very low, even though UV detection at 248 nm was employed. Additional selectivity for the analytes was obtained during elution from the ligand-exchange column due to hydrophobic interaction with the polystyrene–divinylbenzene backbone. Almokalant has two chiral centers, resulting in four glucuronides. The, to some extent, broadened peaks originate from the partial resolution of the two remaining diastereomers, which could be resolved in an alternative system, also demonstrated in the paper [35].

Studies on the determination of absolute recoveries have not been performed for this kind of system. However, the calibration graphs were linear with very small intercepts permitting the assay of femtomole levels of the analytes. The highly alkaline phases used in the systems may present problems for saccharides sensitive for epimerisation and degradation reactions. Complexation with metal ions may, however, improve the stability, but it is essential to penetrate this problem closer in future studies.

The concept of using LEC for preconcentration and clean-up purposes has distinct potential in the isolation and analysis of carbohydrates and related compounds, e.g., glycopeptides, oligosaccharides and monosaccharides, released through chemical or enzymatic treatment of glycoproteins, cell surfaces, receptors, etc. Because of their exceptional hydrophiliticity, they are often present in complex mixtures and at low concentrations together with other water-soluble material and effective means for enrichment, separation, detection and structure elucidation are necessary. Further progress and gain in knowledge in the exponentially growing field of glycotechnology will depend on advances in the development of improved analytical methodologies.

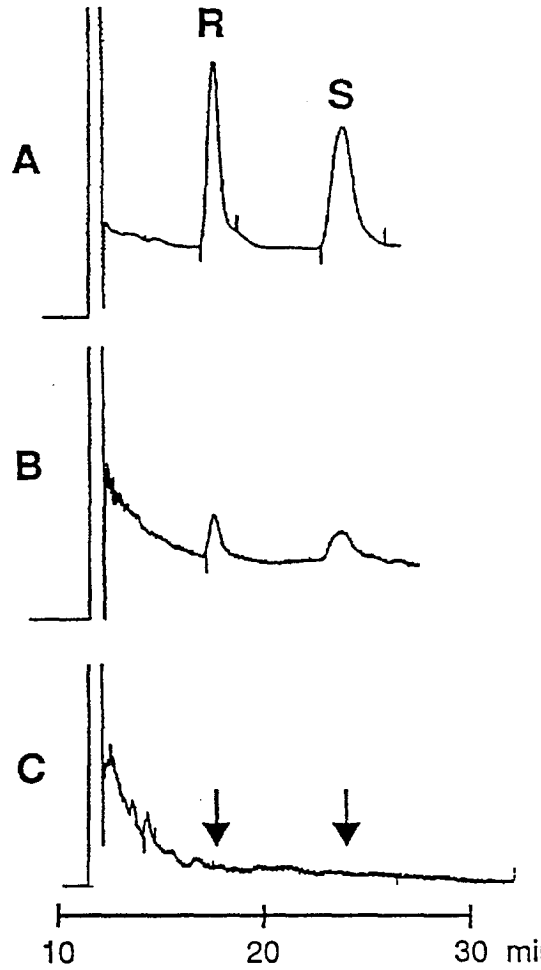

Fig. 6. Separation of diastereomeric almokalant glucuronides from direct injection of 50 μl of urine. (A) 30 μg/ml; (B) 0.5 μg/ml; (C) urine blank. Porous graphitic carbon (Hypercarb) was used as the separation column (100 × 4.6 mm I.D.) and 30% acetonitrile containing 0.1 *M* acetic acid as the mobile phase [35].

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