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High-pH ion-exchange separation and electrochemical detection of alditols, carbohydrates and acidic sugars

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SUMMARY

Recent advances in electrochemical detection of carbohydrates and related compounds have produced a need for chromatographic procedures which can effectively carry out the separation of these compounds under strongly alkaline conditions. Accordingly, the retention properties of commercially available, high-pH anion-exchange and ligand-exchange columns were evaluated for alditols, carbohydrates and aldonic, uronic and aldaric acids. It was shown that anion-exchange techniques were able to retain and resolve these groups from one another in a straightforward fashion by managing the mobile phase ionic strength. Separations involving mixtures of compounds belonging to any one family were difficult only for the alditols, which are only weakly retained under these conditions. For this specific case, ligand-exchange columns were found to be able to resolve most mixtures.

INTRODUCTION

In recent years, several electrochemically based approaches have been reported for the detection and quantitation of carbohydrates following liquid chromatography[1–5]. The most widely used of these to date has undoubtedly been the pulsed amperometric detection (PAD) scheme developed by Johnson[1,2]. This approach, which makes use of the adsorption of carbohydrate compounds onto platinum or gold electrodes to enable their oxidation at very low potentials, has evolved to a sufficient extent that instrumentation capable of generating the required sequence of applied potentials and current measurements is now commercially available. Very recently, a catalytic electrode system utilizing a copper-containing film deposited onto glassy carbon was shown to permit constant-potential detection of carbohydrates using conventional liquid chromatography (LC)–electrochemical detection (ED) equipment[5]. This approach is of interest because, by avoiding PAD's need to expose the platinum or gold electrode to regular cleaning and regeneration potentials, the copper-based chemically modified electrode's operation is simpler and appears to be slightly more sensitive. For example, glucose oxidation at the modified electrode gave an optimum detection limit of only 0.2 ng at +0.50 V *vs.* Ag/AgCl and was sufficient-

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ly stable that 80–100 injections were reproducible to a relative standard deviation of 1–5%[5,6]. In both cases, the electrochemical methodologies are extremely attractive because of the capability that they afford for direct monitoring of trace quantities of these compounds without the time-consuming or inefficient derivatization steps routinely required for the usual UV–visible absorption or fluorescence techniques to be applicable.

The principal drawback of all of the recommended electrode systems is that they uniformly require strongly alkaline conditions in order for optimum carbohydrate oxidation (and detection) to occur. Thus, LC–ED applications based on these electrode systems have typically employed mobile phases containing 0.001 *M* to 0.15 *M* OH^-. Fortunately, this detector requirement, which drastically limits the stationary phase options available to carry out the carbohydrate separation, has not presented an insurmountable problem to the chromatographer. High-performance ion-exchange column packings which are stable at high pH conditions have conveniently become available within the last few years. This development, coupled with the fact that simple sugars possess pK_a values between 12 and 13 (ref. 7), has made anion-exchange chromatography in high pH solutions a viable choice for cabrohydrate separation prior to ED[3–5, 8–10].

Very recently, we have shown that the copper-based chemically modified electrode (Cu-CME) initially used for oxidation and detection of simple carbohydrates[5] exhibits similar activity toward a wide range of related compounds including, most notably, alditols and aldonic, uronic and aldaric acids[11]. In order to realize the potential of electrochemical detection for these important classes of natural products, it is necessary to develop compatible chromatographic procedures for their separation. Previous liquid chromatography approaches for the acidic sugars have involved anion-exchange separations which are often coupled with a pre- or post-column derivatization step to enhance detectability[12–14]. Alditols, which are ionized only in quite strong base, have been separated directly with aminoalkyl bonded phase columns[15] and as their borate complexes with anion-exchange systems[16]. These and other chromatographic approaches have been reviewed by Honda[17] and by Shaw[18]. However, in no cases of which we are aware have the strongly alkaline conditions required for efficient ED been employed. Thus, there is a need to develop high-pH chromatographic procedures suitable for the separation of alditols and acidic sugars. In this work, we have tried to meet this need by taking advantage of the compatibility of the new high-pH ion-exchange chromatography columns with the high-pH detection requirements of the Cu-CME in order to formulate direct and highly sensitive analysis procedures for these compounds.

EXPERIMENTAL

Reagents

Stock solutions of all alditols, carbohydrates and acidic sugars (purchased from Sigma and Aldrich) were prepared fresh daily in deionized water. Just prior to use, the stock solutions were adjusted to the desired concentration and pH by addition of the appropriate hydroxide-containing diluent. Mobile phases used for liquid chromatography were prepared from carbonate-free sodium hydroxide and thoroughly degassed deionized water. A.C.S. reagent grade sodium sulphate and sodium nitrate were obtained from Baker.

Electrodes

Electrode modification procedures were similar to those described previously[5,11]. A freshly polished thin-layer glassy carbon electrode (Bioanalytical Systems, West Lafayette, IN, U.S.A.) was immersed at open circuit for 5 min in a 0.050 *M* $CuCl_2$ solution. At this point, a white deposit appeared on the glassy carbon surface; and the CME was ready for use. During exposure to the $CuCl_2$, it was necessary to immerse the entire electrode assembly, including the metallic leads, in order for the catalytically active deposit to develop. Subsequent investigations, to be reported separately[6], have indicated the most likely source of the deposit to be the galvanic formation of a $CuCl_2$ species. The activity of the modified electrode could be restored to that of the original glassy carbon by polishing with alumina.

Apparatus

Liquid chromatography experiments were carried out with either a Waters Model M-45 or a Beckman Model 110B pump, a Rheodyne (Berkeley, CA, U.S.A.) Model 7125 injector with a 20-μl sample loop, an SSI Model LP-21 pulse dampener and a Bioanalytical Systems Model LC-4B electrochemical detector maintained at +0.50 V *vs.* Ag/AgCl. The chromatographic columns employed were either a 25 cm × 4 mm I.D. Dionex (Sunnyvale, CA, U.S.A.) CarboPac PA1 column or a 30 cm × 4.6 mm I.D. Supelco (Bellefonte, PA, U.S.A.) C611-SP ion-exchange column. The mobile phase flow-rate was always 0.1 ml/min.

RESULTS AND DISCUSSION

Electrochemical detection

The construction and electrocatalytic response of the Cu-CME toward carbohydrates, alditols and acidic sugars has been described at length in our earlier reports[5,11]. Briefly, the CME, which was produced by deposition of a Cu/Cl-containing film onto an ordinary glassy carbon surface, exhibited broad anodic waves for these compounds in both cyclic voltammetry and flow injection analysis experiments. These oxidations, which were centered at +0.5 V *vs.* Ag/AgCl and occurred to an appreciable extent only at hydroxide concentrations of 10^{-3} *M* or higher, were well suited for use in LC–ED and provided detection limits in the nanomole-to-picomole range in the applications considered. In principle, the chromatographic work described here could have been carried out with Au or Pt electrodes using the PAD approach[1,2] or, in fact, with any of the other electrode systems previously recommended for carbohydrate detection. However, because of its ease of operation and its somewhat greater tolerance for lower hydroxide concentrations, the Cu-CME was utilized for all experiments that form the basis of this work.

Liquid chromatography

In view of the very weak acidities of simple carbohydrates, it is expected that anion-exchange chromatography in strongly basic solution might present a reasonable separations approach for sugar-containing samples. In fact, several successful high pH anion-exchange procedures have been developed and reported for mono- and oligosaccharides[8–10]. Analogous procedures for alditols, the pK_a values of which fall approximately from 13 to 14 (ref. 19) and acidic sugars, the pK_a values of which

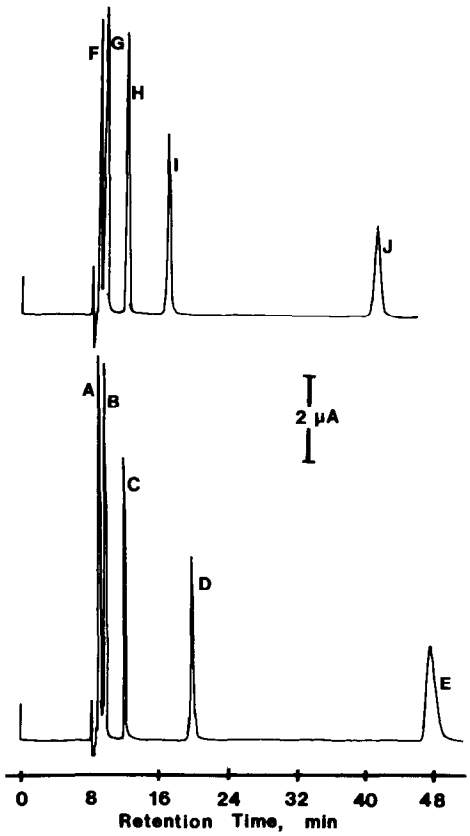

Fig. 1. Chromatograms of glucose and galactose families. Stationary phase, Dionex Carbopac PA1; mobile phase, 0.020 *M* sodium sulfate–0.134 *M* sodium hydroxide. Labeled peaks correspond to glucitol (A); glucose (B), gluconic acid (C, $8 \cdot 10^{-6}$ *M*); glucuronic acid (D); glucaric acid (E); galactitol (F); galactose (G), galactonic acid (H, $2.5 \cdot 10^{-5}$ *M*); galacturonic acid (I); and galactaric acid (J); all concentrations were $4 \cdot 10^{-5}$ *M* unless indicated otherwise.

are generally between 3 and 4 (ref. 7), should also be feasible under the appropriate mobile phase conditions. To our knowledge, however, such separations have not been reported previously.

TABLE I

CAPACITY FACTORS FOR GLUCOSE AND GALACTOSE DERIVATIVES

Chromatographic conditions as in Fig. 1.

Compound	*k'*	*Compound*	*k'*
Glucitol	0.09	Galactitol	0.08
Glucose	0.17	Galactose	0.17
Gluconic acid	0.45	Galactonic acid	0.45
Glucuronic acid	1.4	Galacturonic acid	1.0
Glucaric acid	4.8	Galactaric acid	3.9

The chromatograms shown in Fig. 1 for sample mixtures containing glucose and galactose as well as their alditol and acidic sugar derivatives illustrate the nature of the separations that can be achieved by means of the high pH anion-exchange approach. The isocratic mobile phase employed consisted of a mixture of 0.13 *M* sodium hydroxide and 0.02 *M* sodium sulfate. The former was present in order to ensure optimum electrode performance while the latter was used as the principal means of adjusting solvent strength. In both cases, the order of elution observed (alditol, aldose, aldonic acid, uronic acid, aldaric acid) was exactly that expected on the basis of the compounds' pK_a values and ionic character. Under the conditions shown, the retention of the column varied widely for the different analytes as reflected in the capacity factors (k') which, as shown in Table I, varied from 0.1 for the alditols to 4–5 for the aldaric acids.

Close comparison of the two chromatograms in Fig. 1 supports our general observation that different members of the same carbohydrate families exhibit roughly similar retention properties. Thus, it is relatively easy to resolve complex samples on the basis of functional group or family, *e.g.*, alditols from aldonic acids and so on. Furthermore, our work here, as well as that reported earlier[20], indicated that, for the more highly retained acidic sugars, resolution of individual members of each group can usually be accomplished quite easily by simply adjusting concentration of sodium sulfate (or other salt) to an optimum level for retention of that group. It is apparent from Fig. 1, for example, that glucuronic and galacturonic acids and glucaric and galactaric acids would be baseline-resolved by the mobile phase composition already in effect. The most challenging separation problems appear to be those involving the carbohydrates themselves and the alditols, both of which are practically unretained under the mobile phase conditions in effect in the Fig. Several studies[3–5,8–10] have shown that acceptable carbohydrate separations can be achieved largely by using mobile phases containing sodium hydroxide only. By so doing, the solvent strength is decreased sufficiently that enough retention of the carbohydrates occurs that careful adjustment of the sodium hydroxide concentration can produce a reasonable degree of selectivity.

TABLE II

EFFECT OF HYDROXIDE CONCENTRATION ON CAPACITY FACTORS OF ALDITOLS IN ANION-EXCHANGE CHROMATOGRAPHY

Stationary phase, Dionex Carbopac PA1 column; mobile phase, sodium hydroxide concentration indicated; other conditions as in Fig. 2.

$[OH^-]$ (*M*)	*Glycerol*	*Threitol*	*Erythritol*	*Ribitol*	*Galactitol*	*Glucitol*	*Mannitol*	*Inositol*
0.50	0.20	0.28	0.30	0.47	0.46	0.50	0.58	0.17
0.45	0.20	0.27	0.27	0.51	0.48	0.55	0.60	0.20
0.40	0.24	0.28	0.23	0.55	0.55	0.58	0.65	0.18
0.30	0.21	0.30	0.29	0.62	0.62	0.63	0.72	0.22
0.15	0.25	0.33	0.31	0.72	0.72	0.72	0.93	0.25
0.075	0.25	0.33	0.31	0.75	0.75	0.75	1.0	0.29
0.010	0.24	0.32	0.32	0.82	0.75	0.82	1.1	0.32
0.005	0.27	0.38	0.29	0.90	0.90	0.98	1.3	0.29
0.001	0.27	0.40	0.29	0.97	1.6	1.6	1.6	0.29

By far the most difficult separation to achieve is that involving the alditols. For these compounds, decreases in solvent strength produced by elimination of the sodium sulfate employed above were not able by themselves to allow the alditols to be retained strongly enough for separation to be practical. Thus, further reductions in solvent strength could only be accomplished by decreasing the concentration of sodium hydroxide in the mobile phase. However, given the extremely weak acidity of the alditols and the changes in ionization likely to accompany a substantial OH^- decrease, it was unlikely that the resulting effects on retention would be straightforward. As shown in Table II, this was in fact the case. In general, increases in k' were observed as the sodium hydroxide concentration was decreased. But for many of the alditols examined (in particular, glycerol, threitol, erythritol and inositol) the changes in k' were quite minor even over a 500-fold sodium hydroxide concentration range. The best separations able to be achieved by this approach occurred at the lowest OH^- concentrations and for mixtures of different size alditols. For example, there was a nearly baseline separation (see Fig. 2) of a homologous series including glycerol, threitol, ribitol and glucitol for a 0.0010 *M* sodium hydroxide mobile phase.

An alternative approach for carbohydrate separations involves the use of a "fixed-cation" or "ligand-exchange" stationary phase in place of the direct anion-exchange column used above[18]. Ligand-exchange columns usually contain strongly cationic sulfonic acid type resins which have been permanently loaded with one or more cationic counterions such as Ca^{2+}, Ag^+, Pb^{2+} or H^+. The retention mechanism for carbohydrates on these columns is based on a chelation process in which the hydroxyl groups of the carbohydrate displace water molecules from the cation coordination sphere and form a complex with it. The selectivity of the resulting column is determined largely by the nature of the cation employed, with Ca^{2+}-loaded columns showing particular affinity for alditols as well as carbohydrates. Ordinarily, the carbohydrate separations with these columns have employed water or simple water–organic mobile phases at near neutral pH values. However, as such ligand-exchange systems have become commercially available in forms stable in relatively strong base, it seemed reasonable to investigate their utility for LC–ED under these conditions.

Initial experiments with such a column attempted to characterize the dependence of alditol retention on factors such as mobile phase pH and ionic strength. This was done by examining the effects that such changes exercised on the retention of

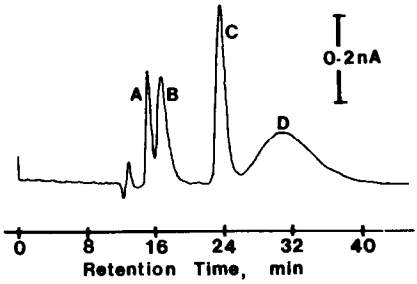

Fig. 2. Chromatogram of alditol test mixture on anion-exchange column. Stationary phase, Dionex Carbopac PA1; mobile phase, 0.0010 *M* sodium hydroxide. Labeled peaks correspond to glycerol (A, $6 \cdot 10^{-5}$ *M*), threitol (B, $6 \cdot 10^{-5}$ *M*), ribitol (C, $5 \cdot 10^{-5}$ *M*) and glucitol (D, $5 \cdot 10^{-5}$ *M*).

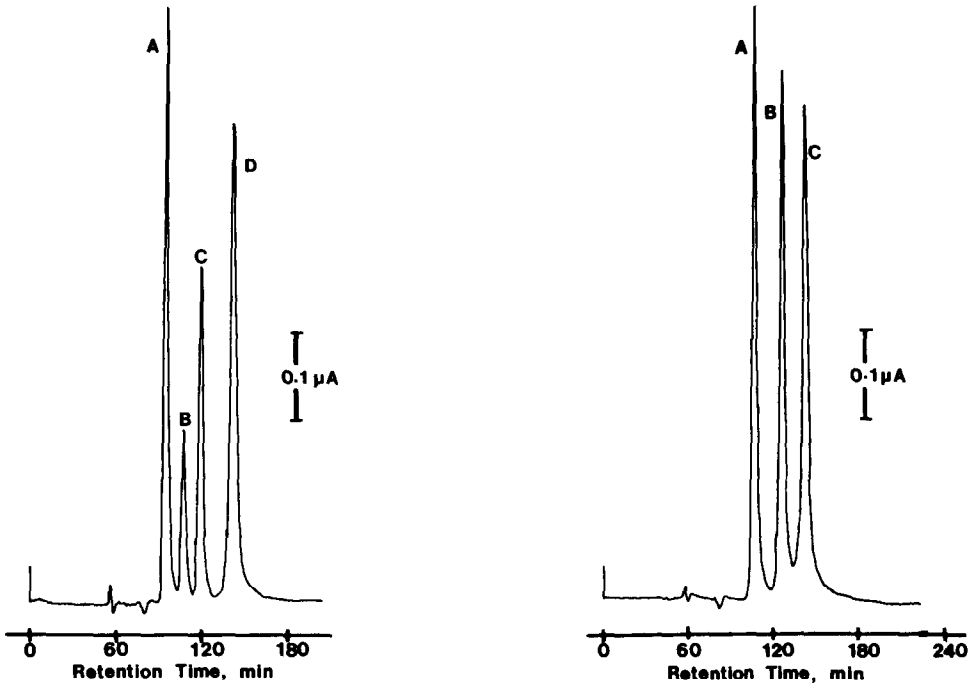

Fig. 3. Chromatogram of alditol test mixture on ligand exchange column. Stationary phase, Supelco C611-SP; mobile phase, 0.040 *M* sodium nitrate–0.0020 *M* sodium hydroxide. Labeled peaks correspond to ribitol (A, $1 \cdot 10^{-5}$ *M*), glycerol (B, $2 \cdot 10^{-5}$ *M*), threitol (C, $1 \cdot 10^{-5}$ *M*) and glucitol (D, $1 \cdot 10^{-5}$ *M*).

Fig. 4. Chromatogram of hexitol test mixture on ligand-exchange column. Stationary phase, Supelco C611-SP; mobile phase, 0.040 *M* sodium nitrate–0.0020 *M* sodium hydroxide. Labeled peaks correspond to mannitol (A), galactitol (B) and glucitol (C); all concentrations were $1 \cdot 10^{-5}$ *M*.

glycerol, threitol, ribitol and glucitol. The effect of pH changes was determined by systematically varying the sodium hydroxide concentration while maintaining ionic strength constant at 0.01 by adjusting the sodium nitrate concentration. Unfortunately, with the particular column used here, the pH could be usefully varied only from 10.5 to 11.6. At more acidic conditions, the response of the Cu-CME was far out of its optimum range while the ligand-exchange column was unstable and showed signs of deterioration at higher OH^- levels. Over the narrow pH range examined, however, the retention properties of the alditols were practically constant.

Fortunately, variations in ionic strength proved to be much more chromatographically useful. Such changes, achieved here by varying the salt concentration while holding the pH constant, produced the expected changes in retention: decreases in ionic strength always resulted in increased retention. Although retention of the alditols was never extremely strong (*i.e.*, $k' < 2$), acceptable separations, illustrated in Fig. 3 for the four test compounds and in Fig. 4 for the hexitols mannitol, galactitol and glucitol, were usually able to be obtained. Typical capacity factors, corresponding to various sodium nitrate concentrations at pH 11.2, are shown in Table III for eight alditols. Clearly, some individual pairs of compounds (*e.g.*, erythritol–inositol and glycerol–mannitol) would be extremely difficult to resolve by this approach; but suffi-

TABLE III

EFFECT OF SODIUM NITRATE CONCENTRATION ON CAPACITY FACTORS OF ALDITOLS IN LIGAND-EXCHANGE CHROMATOGRAPHY

Stationary phase, Supelco C611-SP column; mobile phase, 0.002 *M* sodium hydroxide plus the sodium nitrate concentration indicated; other conditions as in Figs. 3 and 4.

$NaNO_3$ (M)	*Glycerol*	*Threitol*	*Erythritol*	*Ribitol*	*Galactitol*	*Glucitol*	*Mannitol*	*Inositol*
0.001	1.3	1.5	1.2	1.0	1.6	2.0	1.2	1.2
0.003	1.2	1.4	1.1	0.96	1.5	1.9	1.2	1.1
0.005	1.2	1.4	1.0	0.91	1.5	1.9	1.1	1.0
0.007	1.1	1.4	1.0	0.89	1.5	1.8	1.1	1.0
0.010	1.1	1.4	0.97	0.88	1.5	1.8	1.1	0.97
0.015	1.1	1.3	0.95	0.85	1.4	1.8	1.0	0.95
0.020	1.1	1.3	0.94	0.81	1.4	1.8	1.0	0.94
0.030	0.96	1.2	0.89	0.76	1.3	1.6	0.95	0.89
0.040	0.88	1.1	0.77	0.66	1.2	1.5	0.86	0.77

cient differences in retention exist that most mixtures could be reasonable well resolved.

The most troublesome aspect of the separations carried out on the ligand-exchange columns was undoubtedly the very long times often required. These times, which exceeded two hours for the mixtures in Figs. 3 and 4, were dictated by the very low flow-rates used in order to obtain good retention and resolution. For more favorable cases involving some of the more easily resolved alditols, much higher flow-rates and shorter retention times could certainly be achieved. Additional improvements might also be expected to accrue from operation of the column at higher temperature or under gradient elution conditions; if successful, this would likely produce greater resolution and allow the use of higher mobile phase flow-rates as well. Furthermore, as a greater variety of ligand-exchange columns becomes available, marked changes in the retention properties of the alditols might well be possible.

CONCLUSIONS

The separation and quantitation of carbohydrates and related compounds remains a challenging analytical problem for which the combination of high-pH ion-exchange chromatography and ED offers interesting and useful possibilities. In particular, anion-exchange techniques compatible with most suggested electrochemical monitoring systems are able to retain and resolve carbohydrates, alditols, aldonic acids, uronic acids and aldaric acids in a straightforward fashion by managing the mobile phase ionic strength. Separations involving mixtures of compounds belonging to any one family are difficult only for the alditols which are only weakly retained on simple anion-exchange columns. For this specific case, ligand-exchange columns provide an attractive ancillary approach. Thus, LC–ED clearly can be considered to offer not only extremely sensitive direct detection for carbohydrate-related compounds but also versatile and effective separations capabilities as well.

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