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USE OF ETHYLENEDIAMINE SULPHATE FOR POST-COLUMN DERIVATIZATION OF REDUCING CARBOHYDRATES TO ELECTROCHEMICALLY OXIDIZABLE COMPOUNDS IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A method for post-column derivatization of carbohydrates to electrochemically oxidizable compounds has been proposed. This method is based on reaction of carbohydrates with ethylenediamine in a weakly alkaline medium at elevated temperature, and provides an effective means for sensitive monitoring of carbohydrates in high-performance liquid chromatography in various separation modes. The lower limit of detection for aldoses was *ca.* 1 pmol, and good linearity was observed in the range 5–10 nmol with high reproducibility. This method gave smaller variation of sensitivity for carbohydrates, compared with the 2-cyanoacetamide method.

INTRODUCTION

Carbohydrates have no chromophores to permit direct photometric monitoring in high-performance liquid chromatography (HPLC) in ordinary ultraviolet and visible regions. Although they are sometimes detected in the near-ultraviolet region, interference by accompanying substances is very serious. Refractometry is the most popular method for physical detection and has recently been much improved, but the lower limit of detection is ordinarily as high as 10–100 nmol. Under these circumstances chemical derivatization has played an important role for the detection of carbohydrates in HPLC. Of the two modes of chemical derivatization, pre- and post-column, the latter is superior because it does not require tedious procedures for derivatization and is easily automated. From this viewpoint a number of methods have been developed for photometric (*e.g.*, refs. 1–5) and fluorimetric (*e.g.*, refs. 6–10) post-column labelling. The lowest detection limit achieved by these labellings is at best the 100 pmol level. On the other hand, electrochemical detection (ED) after post-column derivatization with a copper(II)–bisphenanthroline reagent[11] attained the most sensitive detection (at the 1 pmol level) of reducing carbohydrates. However, the redox reaction used in this chemical derivatization is not selective to reducing carbohydrates, and the established conditions were not suited, without special con-

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siderations, to separation systems involving elution with organic or aqueous organic solvents. Microanalysis is also possible with pulsed amperometry[12], but it is applicable only in an anion-exchange system with strong alkali as eluent. Such systems allow separation of only limited members of carbohydrates, though the high sensitivity to alditols and aldoses should be investigated.

In our previous paper[13] we proposed a method for post-column derivatization suitable for ED, which is based on transformation of reducing carbohydrates to easily oxidizable dienols by reaction with 2-cyanoacetamide. This method is highly selective to reducing carbohydrates, and subsequent ED permitted carbohydrates to be monitored at the 10 pmol level, separated by various modes including anion-exchange as borate complexes, gel permeation, ligand-exchange and reversed-phase partition. Nevertheless, it has a shortcoming in that the sensitivity varies rather widely between the carbohydrate species, as exemplified by a high value (*ca.* 5) for the molar response for galactose relative to glucose. Since the reaction of reducing carbohydrates with 2-cyanoacetamide yielded fluorescent products[10], in addition to electrochemically oxidizable derivatives, we compared the oxidizabilities of the reaction mixtures obtained with other fluorogenic reagents. The results indicated that fluorogenic reagents such as ethylenediamine[6], 2-aminopropionitrile[7], ethanolamine[8], arginine[9] and malonamide[14] also gave oxidizable products, with the ease of oxidizability in this order under the conditions established for fluorimetric detection. It was remarkable that the ethylenediamine method, which gave the most readily oxidizable product, showed a relatively low variation of oxidizability for various carbohydrate species. Therefore, we have made optimization studies and demonstrated the usefulness of this method for sensitive detection in HPLC.

EXPERIMENTAL

Chemicals

A reagent-grade sample of ethylenediamine sulphate was obtained from Tokyo Kasei Kogyo (Nihonbashi, Chuo-ku, Tokyo) and used without further purification. Other chemicals and carbohydrate samples were of the highest grade commercially available. Double-distilled water was used for all solutions and eluents.

Apparatus

A Hitachi 638 high-performance liquid chromatograph was used for pumping the carrier (0.01% ethylenediaminetetraacetic acid) or the eluent. Samples were introduced via a Rheodyne injector with a 20-μl loop. A reagent solution and a buffer for derivatization were supplied by Pharmacia P-500 pumps at the same flow-rate (0.25 ml/min) and mixed in a Y-shaped connector. The resultant solution was led into a stream of carrier or eluate via another Y-shaped connector. The derivatization reaction was conducted by passing the mixed effluent through a PTFE coil (30 m $\times$ 0.5 mm I.D.) set in a thermostated oven, and the reaction solution was introduced to an Irika E-502 amperometric detector via a cooling coil (10 m $\times$ 0.2 mm I.D.) immersed in a water-bath, through which water was circulated. The working and reference electrodes of the detector were made of glassy carbon and silver chloride, respectively.

Columns of Hitachi 2633 (8 cm $\times$ 8 mm I.D.), Merck LiChrosorb NH_2 (25

cm × 4 mm I.D., 7 μm) and Shodex SC-1821 (30 cm × 8 mm I.D.) were used in the anion-exchange mode as borate complexes, the partition mode and the ligand-exchange mode, respectively.

RESULTS AND DISCUSSION

Optimization

Conditions were optimized in the flow-injection mode by changing one parameter at a time while keeping the others constant. The solid line in Fig. 1 indicates the relationship between the peak response and the applied potential, obtained with glucose as the model sugar, and the dashed line shows the change of background current. This indicates that the peak response increased rapidly up to *ca.* 350 mV, but more slowly thereafter. On the other hand, the background current increased slowly until *ca.* 400 mV but rapidly after this point. Therefore, a potential of 350 mV was the best.

The results of the optimization study of chemical derivatization are summarized in Fig. 2. Glucose and fructose gave similar curves of pH dependence, with a maximal response at pH 9.0 (Fig. 2a). The pH dependence of glucosamine was considerably different, with a maximum at *ca.* pH 8.0. However, the response at pH 9.0 was more than 90% of that at pH 8.0. The change in the background current was similar to the peak response of glucose, but the variable range was narrow.

The peak responses of glucose, fructose and glucosamine increased with increasing borate concentrations to maxima at 0.6 *M*, 0.7 *M* and 0.4 *M* borate, respectively (Fig. 2b). The responses of fructose and glucosamine at 0.7 *M* (the optimum concentration for glucose) were 94% and 69% of the respective maximal values.

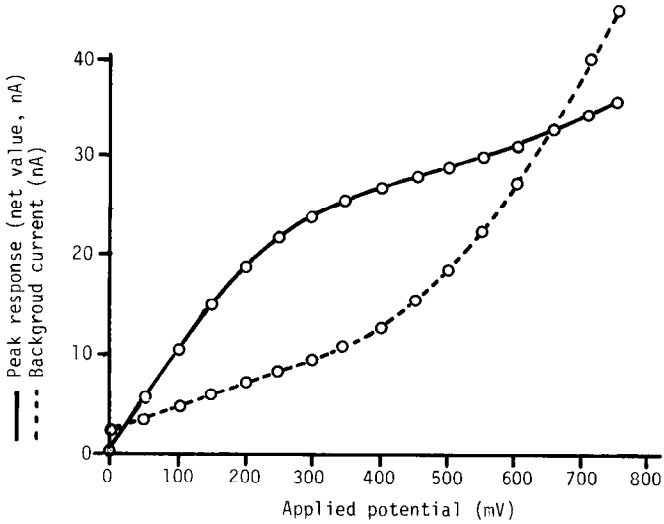

Fig. 1. Effect of applied potential on peak response of glucose (solid line) and background current (dashed line). Carrier, 0.01% ethylenediaminetetraacetic acid at 0.50 ml/min; reagent solution, 0.10 *M* ethylenediamine sulphate at 0.25 ml/min; reagent buffer, 0.70 *M* borate buffer (pH 9.0) at 0.25 ml/min; reaction temperature, 150°C; sample scale, 1 nmol.

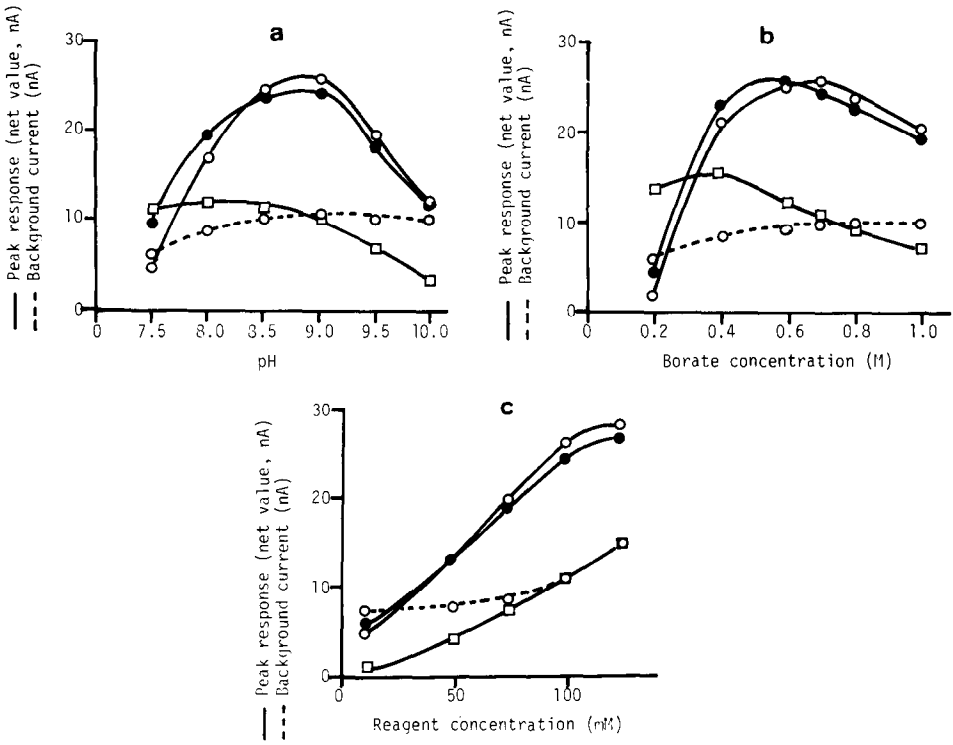

Fig. 2. Effects of pH (a) and salt concentration (b) of reagent buffer, and concentration of reagent solution (c), on peak response of glucose (○—○), fructose (●—●) and glucosamine (□—□). The dashed line indicates change of background current. The above factors were varied, while the other factors were fixed as in Fig. 1. Applied potential, 350 mV; sample scale, 1 nmol each.

The change in the background current was similar to that observed for pH dependence.

The relationship between the peak response and the concentration of the reagent solution was simple for glucosamine: the response continued to increase almost linearly up to 125 mV (Fig. 2c). For glucose and fructose the rate increased less rapidly above 100 mV to give convex curves. The background current also continued to increase, but only slowly.

The effect of changing the reaction temperature is shown in Fig. 3. For glucose, the peak response below 100°C was very small, but above 120°C it increased rapidly to reach a maximum at 150°C. The response at 150°C was about two and five times as great as those at 130°C and 120°C, respectively.

Calibration curves

On the basis of the optimization study, a calibration curve of glucose was obtained by using 0.1 *M* ethylenediamine sulphate and 0.7 *M* borate buffer (pH 9.0) at 150°C, with monitoring at an applied potential of 350 mV. Excellent linearity was observed for the logarithmic plots of applied potential *vs.* sample amount at least in the range from 5 pmol to 10 nmol. Similar results were obtained for other aldoses.

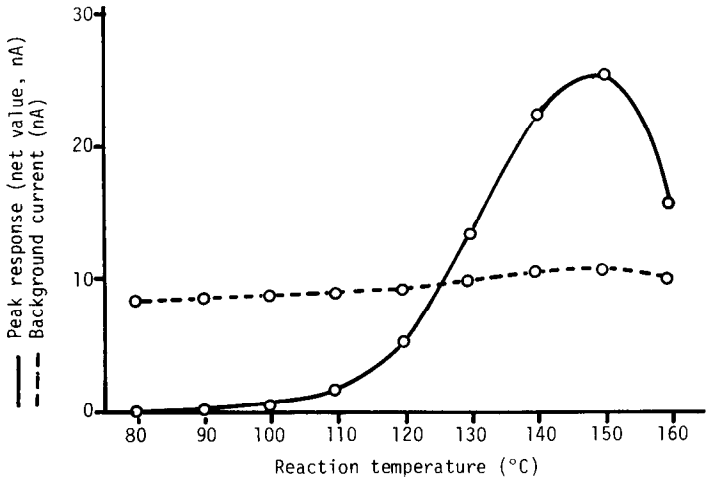

Fig. 3. Effect of reaction temperature on peak response of glucose (solid line) and background current (dashed line). The analytical conditions were the same as those in Fig. 1, except that the pH value of the reagent buffer and the applied potential were fixed at 9.0 and 350 mV, respectively, and the reaction temperature was varied. Sample scale, 1 nmol.

Limit of detection

The lower limit of detection for aldoses was *ca.* 1 pmol at a signal-to-noise ratio of 2. This is comparable with higher values (100–400 pmol at a signal-to-noise ratio of 3) for fluorimetric detection, obtained by Mopper *et al.*[6] with this reagent, though it should be taken into account that these values were for a chromatographic system. It is noticeable that the background current was as low as *ca.* 10 nA, in contrast to those of other post-column derivatizations [the copper(II)–bisphenanthroline method[11], *ca.* 25 nA; the 2-cyanoacetamide method[13], *ca.* 60 nA]. Such a low level of background current is considered to have played a role in compensating for baseline fluctuations due to the instability of the pressure at high reaction temperatures, and in backing to high sensitivity.

Reproducibility

The coefficients of variation for ten determinations of glucose at the 10 pmol, 200 pmol and 5 nmol levels were 4.5%, 2.0% and 1.0%, respectively, under the aforementioned conditions.

Relative sensitivities of carbohydrates

Table I summarizes the relative molar responses of aldopentoses, aldohexoses and 6-deoxyaldohexoses, as referred to glucose. They varied between 0.91 and 1.53, which is a much narrower range than that obtained for the 2-cyanoacetamide method (1.00–5.13). Notice that both ketoses (fructose and sorbose) gave values within this range (1.00 and 1.03, respectively) in contrast to the low values obtained by the 2-cyanoacetamide method (0.08 and 0.005, respectively), presumably because they were transformed into more reactive aldose species during derivatization at high temperature. It is striking that 2-deoxyglucose gave a high relative molar response (0.89), compared with the low value (0.09) in the 2-cyanoacetamide method. The latter

TABLE I

RELATIVE SENSITIVITIES OF VARIOUS CARBOHYDRATES

Carbohydrate	*Relative molar response*		*Carbohydrate*	*Relative molar response*	
	*Ethylenediamine method**	*2-Cyanoacetamide method***		*Ethylenediamine method**	*2-Cyanoacetamide method***
Glyceraldehyde	0.25	2.07	Galactosamine hydrochloride	0.87	1.82
Arabinose	1.28	4.96	Glucosamine hydrochloride	0.52	1.81
Lyxose	1.34	2.56	N-Acetylgalactosamine	0.80	2.08
Ribose	1.30	2.05	N-Acetylglucosamine	0.75	1.64
Xylose	1.16	1.87	N-Acetylmannosamine	0.70	1.63
Galactose	1.53	5.13	N-Acetylneuraminic	0.20	0.03
Glucose	1	1	acid		
Mannose	0.95	1.91	Galacturonic acid	1.13	3.14
Fucose	1.44	1.64	Glucuronic acid	0.95	1.64
Rhamnose	0.91	2.18	Maltose	1.76	0.73
Fructose	1.03	0.08	Cellobiose	1.97	0.81
Sorbose	1.06	0.05	Lactose	1.94	0.91
2-Deoxyglucose	0.89	0.09	Gentiobiose	1.64	0.78

* The analytical conditions were the same as those in Fig. 3, except that reaction temperature was 150°C. Sample scale, 1 nmol each.

** Ref. 13.

method gave high values for hexosamines (*ca.* 1.8) and N-acetylhexosamines (1.6–2.1), whereas the present method afforded values less than unity for these compounds. N-Acetylneuraminic acid gave a moderate value of 0.20. The sensitivities for uronic acids were around unity, comparatively lower than those in the 2-cyanoaetamide method (3.14 for galacturonic acid, 1.64 for glucuronic acid). For reducing disaccharides situation was reversed: the relative molar responses obtained by the present method (1.64–1.97) were much greater than those of reducing monosaccharides, contrary to those obtained by the 2-cyanoacetamide method. Non-reducing carbohydrates such as aldonic acids, saccharic acids, alditols and non-reducing oligosaccharides, as well as neutral polysaccharides, were almost negative, as in the 2-cyanoacetamide method. For non-reducing oligosaccharides, combination of the method with catalytic hydrolysis to reducing saccharides, as described by Vrántný *et al.*[4]. will increase the sensitivity.

Application to HPLC

The established conditions were applied to the detection of reducing carbohydrates separated by various modes of HPLC. The first example (Fig. 4) was obtained for ligand-exchange chromatography on a column of a sulphonated styrene–divinylbenzene copolymer. Since the eluent was pure water in this case, application was simple and four aldoses at the 50 pmol level were easily detected. The second example was an application to partition chromatography on a column of aminopropylated silica gel, with aqueous acetonitrile as eluent. The organic solvent hampered application to a considerable extent, but detection at the 200 pmol level was successful, as shown in Fig. 5. In this case a ketose (fructose) was also detected with approximately the same sensitivity as those of the aldose (rhamnose) and the reducing disaccharide (maltose). The last example, which involves application to anion-exchange chromatography as borate complexes (Fig. 6), also led to sensitive detection of aldoses at the 100 pmol level, in spite of the use of a rather high concentration of borate buffer.

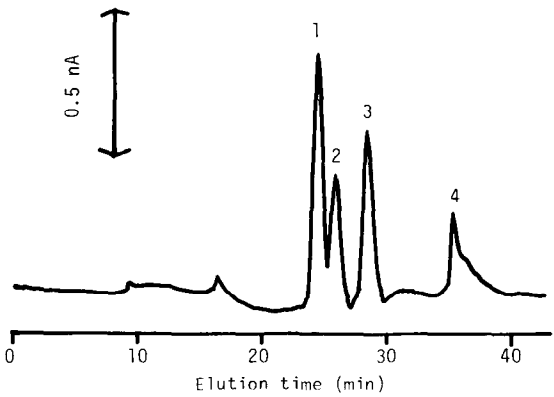

Fig. 4. Detection of aldoses and a reducing disaccharide separated in the ligand-exchange mode. Column, Shodex SC-1821 (30 cm × 8 mm I.D.); column temperature, 80°C; eluents, water; flow-rate, 0.50 ml/min. Detection was performed under the same conditions as those described in Fig. 1, except that the pH of the reagent buffer and the applied potential were fixed at 9.0 and 350 mV, respectively. Sample scale, 50 pmol each. Peaks: 1 = maltose; 2 = glucose; 3 = arabinose; 4 = ribose.

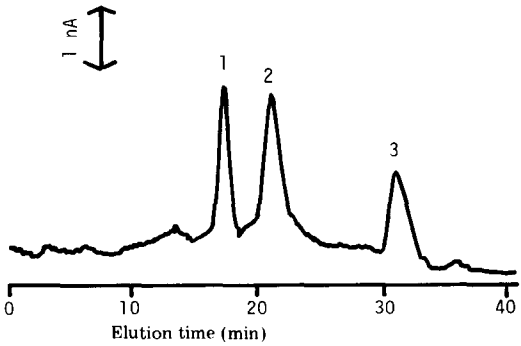

Fig. 5. Detection of mono- and disaccharides separated in the partition mode. Column, LiChrosorb NH_2 (25 cm × 4 mm I.D.); column temperature, ambient; eluent, acetonitrile–water (3:1, by volumes); flow-rate, 0.50 ml/min. Detection was performed under the same conditions as those described in Fig. 1, except that the pH value of the reagent buffer, the reaction temperature and the applied potential were fixed at 9.0, 140°C and 350 mV, respectively. Sample scale, 200 pmol each. Peaks: 1 = rhamnose; 2 = fructose; 3 = maltose.

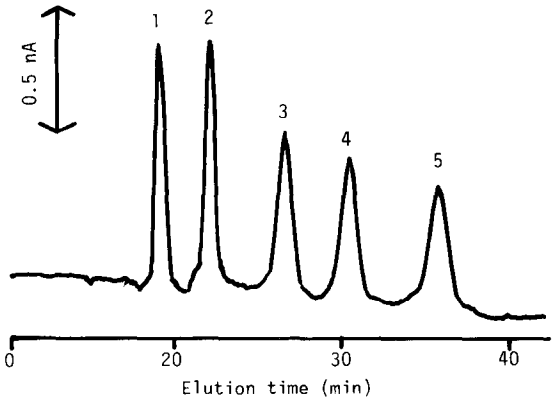

Fig. 6. Detection of aldoses separated in the anion-exchange mode as borate complexes. Column, Hitaschi 2633 (8 cm × 3 mm I.D.); column temperature, 60°C; eluent, 0.70 *M* borate buffer (pH 8.5) containing 0.01% ethylenediaminetetraacetic acid; flow-rate, 0.70 ml/min. Detection was performed under the same conditions as those described in Fig. 1, except that the pH value of the reagent buffer, the reaction temperature and the applied potential were fixed at 9.0, 140°C and 350 mV, respectively. Sample scale, 100 pmol each. Peaks: 1 = rhamnose; 2 = mannose; 3 = galactose; 4 = xylose; 5 = glucose.

Thus, the ethylenediamine method was applicable to all major separation modes currently employed in HPLC of carbohydrates.

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