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ELECTROCHEMICAL DETECTION OF REDUCING CARBOHYDRATES IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AFTER POST-COLUMN DERIVATIZATION WITH 2-CYANOACETAMIDE

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SUMMARY

Reducing carbohydrates were derivatized post-column with 2-cyanoacetamide to readily oxidizable compounds. Electrochemical detection allowed sensitive and reproducible monitoring of glucose with a detection limit and a linear range of 20 pmol and 50 pmol–2 nmol, respectively. Similar results were obtained with other reducing carbohydrates. This method was universally applicable to high-performance liquid chromatography in gel-permeation, ion-exchange and partition modes.

INTRODUCTION

Among many methods for the detection of carbohydrates in liquid chromatography, the method based on refractivity measurement is the most popular, but it is insensitive. Direct measurement of the absorption in the near-ultraviolet region provides a more sensitive method, but it is non-selective. In contrast, methods involving post-column derivatization are excellent with respect to both sensitivity and selectivity. Therefore, we have made a series of investigations on post-column derivatization, and have succeeded in developing a few methods[1–3]. Among them, the 2-cyanoacetamide method[1] is especially important, because it is widely applicable to reducing carbohydrates. The derivatization proceeds under mild conditions, and the products not only fluoresce intensely (*e.g.*, ref. 4), but also absorb ultraviolet light at *ca.* 280 nm, where commercial mercury lamps emit most abundantly (*e.g.*, ref. 5).

In a course of studies on the mechanism of this derivatization reaction, we found that the products were also readily oxidized on a glassy carbon electrode. This paper is concerned with studies on the optimization of electrochemical detection based on this derivatization, and with its application to high-performance liquid chromatography (HPLC).

EXPERIMENTAL

Materials

A reagent-grade sample of 2-cyanoacetamide was purchased from Kanto Ka-

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gaku (Nihonbashi, Chuo-ku, Tokyo, Japan), and purified by passing it through a column of charcoal, followed by recrystallization from methanol. All other chemicals and carbohydrate samples were of the highest grade commercially available. Water was double distilled in Pyrex glassware. All eluents, reagent solutions and buffers were filtered through Millipore membranes having a pore size of 0.45 μm, and degassed before use.

Instrumentation

Optimization studies were performed in the flow injection mode without columns, by using a double-plunger piston pump of a Hitachi 638 high-performance liquid chromatograph, a Rheodyne sample injector carrying a 20-μl loop and an Irika E-502 electrochemical detector equipped with a glassy carbon working electrode and a silver–silver chloride reference electrode. Samples were introduced into the carrier (water) provided by the piston pump. The reagent solution and the supporting electrolyte were supplied by two Pharmacia P-500 syringe pumps and mixed by using a Y-shaped connector. The resultant solution was led into the carrier stream via another Y-shaped connector. The proportions of the flow-rates of the carrier, the reagent solution and the supporting electrolyte were maintained at 2:1:1 throughout the work. PTFE tubes with an inner diameter of 0.5 mm were used as reaction coils, which were immersed in a reaction bath filled with glycerol. The reaction mixture was passed through a PTFE cooling coil (1 m × 0.5 mm I.D.) in a water-bath before being introduced into the detector.

When this system was applied to HPLC, columns were connected between the sample injector and the two syringe pumps for reagent delivery, but the other conditions were the same as those in the optimization studies. A Gelko C-610 (30 cm × 10.7 mm I.D.), a Hitachi 2617 (8 cm × 8 mm I.D.) and a Shodex RSpak DC-613 (25 cm × 4 mm I.D.) columns were used for gel-permeation, ion-exchange and partition chromatography, respectively. These columns were supplied by Hitachi Kasei (Hitachi-shi, Ibaragi, Japan), Nissei Sangyo (Shiba-Nishikubo, Minato-ku, Tokyo, Japan) and Showa Denko (Shiba-daimon, Minato-ku, Tokyo, Japan), respectively.

RESULTS AND DISCUSSION

Optimization

The conditions for electrochemical detection were optimized by using 1 nmol of glucose as a model sugar. In Fig. 1 the detector response is plotted as a function of applied potential. The net response increased almost linearly from 0.25 V *vs.* Ag–AgCl to reach a plateau at 0.4 V *vs.* Ag–AgCl. The background current increased only slightly in this range, although the rate of increase became higher above 0.4 V.

Fig. 2a shows the dependence of the detector response on total flow rate. The maximal response was observed at 0.6 ml/min. It is considered that the low response at lower flow-rates was due to peak broadening, whereas the decrease in response at higher flow-rates was caused by incompleteness of both derivatization and electrode reactions. However, the rate giving the maximal response, *i.e.*, 0.6 ml/min, was too slow to afford a constant flow. The carrier stream was especially liable to fluctuate at flow-rates slower than 0.5 ml/min, because a piston pump was used. For this reason, the total flow-rate was raised to 1.0 ml/min, although it slightly deviated from

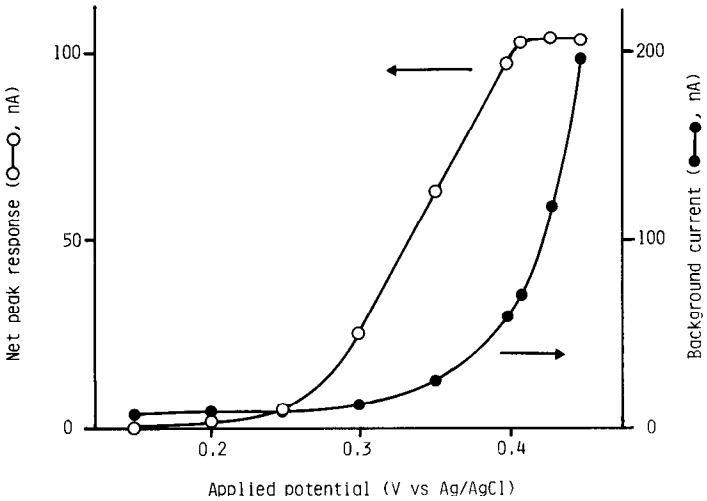

Fig. 1. Dependence of peak response (○) and background current (●) on applied potential. Applied potential, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40, 0.41, 0.43 and 0.45 V *vs.* Ag–AgCl; flow-rate of the carrier (water), 0.50 ml/min; reagent solution, aqueous 1.5% 2-cyanoacetamide (0.25 ml/min); supporting electrolyte, 0.20 *M* borate buffer (pH 9.5, 0.25 ml/min); reaction temperature, 100°C; length of reaction coil, 10 m; size of sample (glucose), 1 nmol.

the maximum point. As a result, the flow-rates of the carrier, the reagent solution and the supporting electrolyte were 0.5, 0.25 and 0.25 ml/min, respectively.

As can be seen from Fig. 2b, the optimal pH of the supporting electrolyte was 9.5. On the other hand, the curves in Fig. 2c and d indicate that the minimal concentration of the borate salt in the supporting electrolyte and 2-cyanoacetamide in the reagent solution that gave the maximal responses were 0.2 *M* and 1.5%, respectively.

Fig. 2e shows the effect of reaction temperature on peak response. The response increased rapidly with elevation of temperature, but temperatures higher than the boiling point of water were disadvantageous for baseline stabilization owing to bubbling. The peak response also increased as the reaction coil became longer (Fig. 2f), but it reached a plateau at 10 m.

On the basis of these results, the derivatization was performed in a PTFE coil (10 m × 0.5 mm I.D.) at 100°C by using 1.5% 2-cyanoacetamide and 0.20 *M* borate buffer (pH 9.5) at the same flow-rate (0.25 ml/min). Samples were delivered at 0.50 ml/min, and the products were detected at 0.40 V *vs.* Ag–AgCl.

Sensitivity

Under the optimal conditions the calibration graph for glucose was linear for amounts ranging from 50 pmol to 2 nmol. The detection limit was about 20 pmol for glucose at the signal-to-noise ratio of 2. Linearity was also observed for approximately the same range with other reducing carbohydrates. The oldest method for electrochemical detection of carbohydrates, described by Takata and Muto[6], based on electrode oxidation of a hexacyanoferrate(II) salt, formed by reduction of a hexacyanoferrate(III) salt with carbohydrates, could monitor aldoses and ketoses at levels

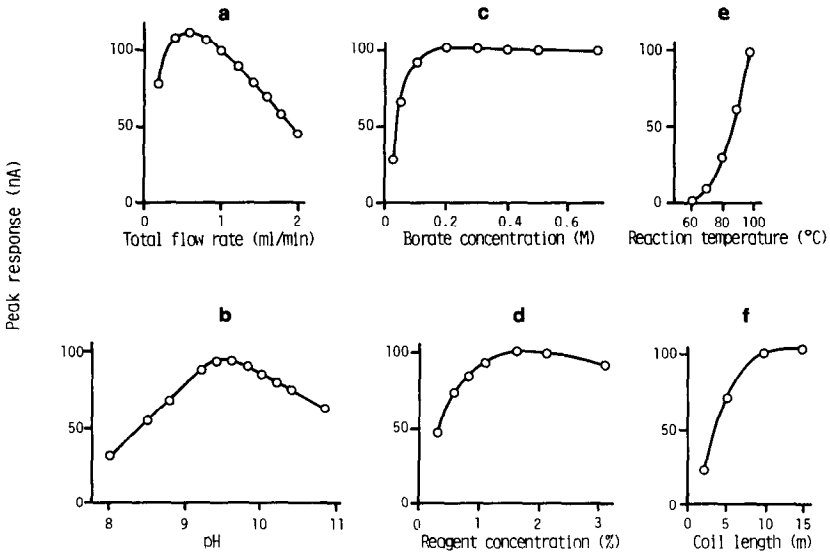

Fig. 2. Optimization of the conditions for electrochemical detection. (a) Effect of flow-rate. Total flow-rate, 0.20, 0.40, 0.60, 0.80, 1.0, 1.2, 1.4, 1.6, 1.8 and 2.0 ml/min. The proportions of the flow-rates of the carrier (water), the reagent solution and the supporting electrolyte were constant (2:1:1). Applied potential, 0.40 V *vs.* Ag–AgCl. Other conditions as in Fig. 1. (b) pH dependence. pH of supporting electrolyte, 8.0, 8.5, 8.8, 9.2, 9.4, 9.6, 9.8, 10.0, 10.2, 10.4 and 10.8; applied potential, 0.40 V *vs.* Ag–AgCl. Other conditions as in Fig. 1. (c) Effect of borate concentration in the supporting electrolyte. Concentration of the borate ion, 0.025, 0.05, 0.10, 0.20, 0.30, 0.40, 0.50 and 0.70 *M*; applied potential, 0.40 V *vs.* Ag–AgCl. Other conditions as in Fig. 1. (d) Effect of concentration of reagent. Concentration of 2-cyanoacetamide, 0.25, 0.50, 0.75, 1.00, 1.50, 2.00 and 3.00%; applied potential, 0.40 V *vs.* Ag–AgCl. Other conditions as in Fig. 1. (e) Temperature dependence. Reaction temperature, 60, 70, 80, 90 and 100°C; applied potential, 0.40 V *vs.* Ag–AgCl. Other conditions as in Fig. 1. (f) Effect of coil length. Length of reaction coil, 2.0, 5.0, 10 and 15 m; applied potential, 0.40 V *vs.* Ag–AgCl. Other conditions as in Fig. 1.

above 100 nmol. Hence the sensitivity of the present method is approximately three orders of magnitude higher than that of the hexacyanoferrate(II) method. The electrochemical detection in the present method also has a sensitivity one or two orders of magnitude higher than those of fluorimetric (*e.g.*, ref. 4) and photometric (*e.g.*, ref. 5) detection, respectively, which were based on the same derivatization under similar conditions. The coefficients of variation ($n = 10$) for the determination of glucose were 3.1, 1.8 and 0.8% at the 75 pmol, 250 pmol and 2 nmol levels, respectively.

Comparison of the sensitivities to various carbohydrates

Table I summarizes the relative molar responses of various carbohydrates, referred to glucose. It is noteworthy that all reducing carbohydrates except 2-deoxyglucose gave strong responses, whereas non-reducing carbohydrates, such as alditols, aldonic acids, aldaric acids and non-reducing oligosaccharides, were negative. Ketoses and N-accetylneuraminic acid gave only slight responses. The relative molar responses to reducing carbohydrates varied in a wider range than in fluorimetric and photometric detection.

TABLE I

RELATIVE MOLAR RESPONSES OF VARIOUS CARBOHYDRATES*

Carbohydrate	*Relative molar response*	*Carbohydrate*	*Relative molar response*
Glyceraldehyde	207	Galacturonic acid	314
Arabinose	496	Glucuronic acid	164
Lyxose	256	2-Deoxyglucose	9
Ribose	205	Gluconic acid	2
Xylose	187	Glucaric acid	0
Galactose	513	N-Acetylneuraminic acid	3
Glucose	100	Erythritol	0
Mannose	191	Xylitol	0
Fucose	164	Galactitol	0
Rhamnose	218	Glucitol	1
Fructosse	8	Sucrose	5
Sorbose	5	Raffinose	0
Galactosamine · HCl	182	Maltose	73
Glucosamine · HCl	181	Cellobiose	81
N-Acetylgalactosamine	208	Lactose	91
N-Acetylglucosamine	164	Gentiobiose	78
N-Acetylmannosamine	163	Dextran	0

* The analytical conditions were the same as those described in Fig. 1, except that the applied potential was 0.40 V *vs.* Ag–AgCl.

Nature of the oxidizable products

Studies of the mechanism of the reaction between 2-cyanoacetamide and reducing carbohydrates[7] suggested the intermediate formation of conjugated diene–monool compounds, which absorb strongly at 280 nm but do not fluoresce. As the optimum reaction conditions for electrochemical detection were more similar to those for photometric detection than to those for fluorimetric detection, the diene–monool compounds are considered to be the substances electrochemically oxidized. Actually isolated intermediate compounds were readily oxidized under the same conditions as those for electrochemical detection, whereas the final fluorescent products were not oxidized. Further mechanistic studies are in progress.

Application to HPLC

The established conditions, as mentioned above, were applied to the detection of reducing carbohydrates in HPLC eluates. Separation was performed in various modes, including gel permeation, ion exchange and partition. Fig. 3a and b show examples of application to gel permeation. They were obtained by using a column packed with Gelko C-610, a sulphonated styrene–divinylbenzene copolymer, and water as eluent. Several hundred picomole amounts of (a) malto- and (b) isomaltodextrins were analysed without problems. The cation-exchange chromatographic separation of hexosamines as borate complexes was performed by using a Hitachi 2617 column according to the previous paper[2], and the result is shown in Fig. 4. The reaction mixture contained a high concentration of a borate salt, but electrochemical detection was not affected. The third example, shown in Fig. 5, was obtained by partition chromatography. In this instance the applied potential was slightly reduced to 0.30 V *vs.* Ag–AgCl, because the background current was increased rapidly from

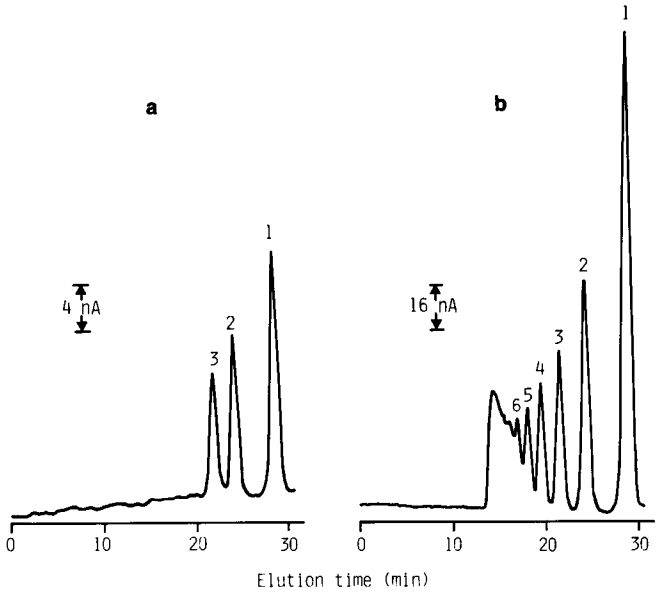

Fig. 3. Analysis of (a) malto- and (b) isomaltodextrins, as detected by electrochemical oxidation. Column, Gelko C-610 (30 cm × 10.7 mm I.D.); eluent, water; flow-rate, 0.60 ml/min; applied potential, 0.40 V *vs.* Ag–AgCl; sample size, (a) 500 pmol each and (b) 10 μg as dextran (starting material). Other conditions for detection as in Fig. 1. Peak numbers indicate the degree of polymerization.

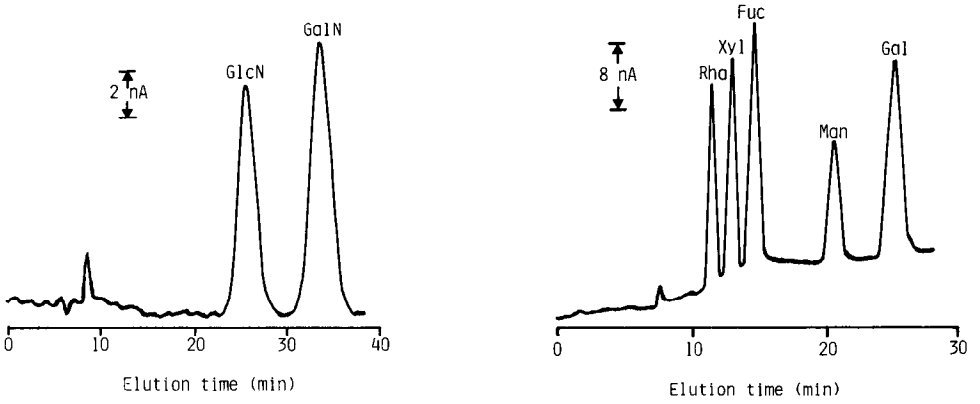

Fig. 4. Analysis of hexosamines, as detected by electrochemical oxidation. Column, Hitachi 2617 (8 cm × 8 mm I.D.); eluent, 0.16 *M* borate buffer (pH 7.5); flow-rate, 0.50 ml/min; applied potential, 0.40 V *vs.* Ag–AgCl; sample size, 500 pmol each. Other conditions for detection as in Fig. 1. GlcN = glucosamine; GalN = galactosamine.

Fig. 5. Analysis of aldoses, as detected by electrochemical oxidation. Column, Shodex RSpak DC-613 (H^+) (25 cm × 4 mm I.D.); eluent, acetonitrile–water (9:1); flow-rate, 0.60 ml/min; applied potential, 0.30 V *vs.* Ag–AgCl; sample size, 400 pmol each. Other conditions as in Fig. 1. Rha = rhamnose; Xyl = xylose; Fuc = fucose; Man = mannose; Gal = galactose.

0.35 V *vs.* Ag–AgCl due to fluctuations caused by acetonitrile, an organic solvent having a low boiling point. However, a mixture of 400 pmol each of aldoses was completely separated and monitored effectively.

Recently, a more sensitive method for the electrochemical detection of reducing carbohydrates was devised by Watanabe and Inoue[8], which involves post-column transformation of a copper(I) salt, resulting from reduction of a copper(II) salt, to the bis(phenanthroline) complex, but their method was not applicable to HPLC using organic solvents. Another method based on the pulsed potential technique, described by Rocklin and Pohl[9], is as sensitive as the copper(I) complex method, but its application is similarly restricted to anion-exchange chromatography with aqueous sodium hydroxide as eluent. In contrast, the present method may be evaluated as a universal method applicable to all types of HPLC currently used for the analysis of carbohydrates.

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