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AMPEROMETRIC DETECTION OF REDUCING CARBOHYDRATES IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING AN AMINO-BONDED COLUMN AND ACETONITRILE–WATER AS THE ELUENT

NORIYUKI WATANABE
Department of Industrial Chemistry, Faculty of Engineering, University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113 (Japan)
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SUMMARY

Amperometric detection of reducing carbohydrates using copper bis(phenanthroline) as the mediator in the post-column reaction was applied to the partition mode of high-performance liquid chromatography using an amino-bonded column with acetonitrile–water as the eluent. The proposed method appears to be the most sensitive means of detection in the partition separation of carbohydrates. Glucose can be determined at levels down to 5 pmol (1 ng injection). Excellent selectivity was demonstrated in examples of chromatograms obtained with fruit juices and human urine.

INTRODUCTION

The amperometric detection of reducing carbohydrates in high-performance liquid chromatography (HPLC) has recently been developed, in which the reducing ability of carbohydrates is coupled with the redox reaction of copper bis(phenanthroline) (CBP) as mediator[1]. CBP, reduced by carbohydrates in alkaline solution at high temperature in a post-column reaction, is re-oxidized with the amperometric detector.

This method has several advantages. First it is highly sensitive. A 1-pmol amount of glucose was detectable by using a cation-exchange column and water as the eluent[1]. This detection limit was roughly one order of magnitude lower than that attained by the most sensitive fluorimetric method so far[2]. Second, the selectivity is excellent. The applied potential of the working electrode was +40 mV, which was low enough for the electrochemical oxidation of most organic substances and moderately high for the electrochemical reduction of dissolved oxygen. The chemical conditions optimized for post-column reaction were an additional factor for improving the selectivity. The limitations of both the electrochemical potential and the chemical conditions made this method highly selective. In fact, surprisingly simple chromatograms with minimal interference were obtained for samples of human serum and urine pre-treated by simple deproteinization[3]. Third, the method is easy to per-

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form. The post-column reagent solution containing CBP was stable for long-term operation. The reaction conditions were not as severe as those involved in existing methods. Finally, the method is universal, that is, applicable to all of the separation modes for carbohydrates. It was stated recently that our method is not applicable to HPLC using organic solvents[4]; this misconception should be corrected as soon as possible, as indicated in this paper.

EXPERIMENTAL

Materials

All chemicals were commercially available materials of analytical-reagent grade unless stated otherwise. CBP was prepared as described previously[3]. The reagent solution was prepared by dissolving CBP in 0.1 *M* Na_2HPO_4 as supporting electrolyte and the pH was adjusted to the desired value by addition of 2 *M* NaOH solution.

Urine and fruit juices were deproteinized by mixing with an equal volume of acetonitrile followed by centrifugation for 1 min at 4500 *g*, then diluted with distilled water. Finally, the solvent composition of all samples for injection was made the same as that of the eluent (*i.e.*, acetonitrile–water, 7:3).

High-performance liquid chromatography

The HPLC apparatus was equipped with a Constametric IIG constant-flow pump (Milton Roy, Philadelphia, PA, U.S.A.), used at a flow-rate of 0.35 ml/min, an amino-bonded silica (NH_2-silica) column (TSK gel NH_2-60, 5 μm; 25 cm × 4.6 mm I.D.; Toyo Soda, Tokyo, Japan), a sample injector with a 20-μl loop (Model 7120; Rheodyne, U.S.A.) and an amperometric detector (LC-4B; Bioanalytical Systems, West Lafayette, IN, U.S.A.). The reagent solution containing CBP was delivered at a flow-rate of 0.8 ml/min by a Tri Rotar-II constant-flow pump (Japan Spectroscopic, Tokyo, Japan) and mixed with the column eluate by means of a stainless-steel tee-piece. PTFE tubing (6 m × 0.5 mm I.D.) was used as the reaction coil, which was immersed in a three-necked flask. The temperature of the reaction flask was controlled at 98.5 ± 0.1°C under reflux. The reaction mixture was then passed through a cooling coil (30 cm × 0.25 mm I.D.) immersed in a mixture of water and ice before reaching the detector.

The mobile phase was acetonitrile–water (7:3). The reagent solution contained 1 m*M* CBP in 0.1 *M* Na_2HPO_4. The potential applied to the working glassy carbon electrode was fixed at +40 mV *vs.* Ag–AgCl.

The mobile phase was freed from oxygen by repeated introduction and evacuation of nitrogen gas into and from the bottle containing the mobile phase. Exclusion of oxygen from the reagent solution was effected by passing the reagent solution through a PTFE tube (10 m × 0.46 mm I.D., 0.92 mm O.D.) closed in a small cylinder kept under vacuum by a rotary pump.

RESULTS AND DISCUSSION

The rate of reduction of CBP by carbohydrates depends significantly on pH and temperature[1]. As the rate increased monotonously and rapidly with increasing

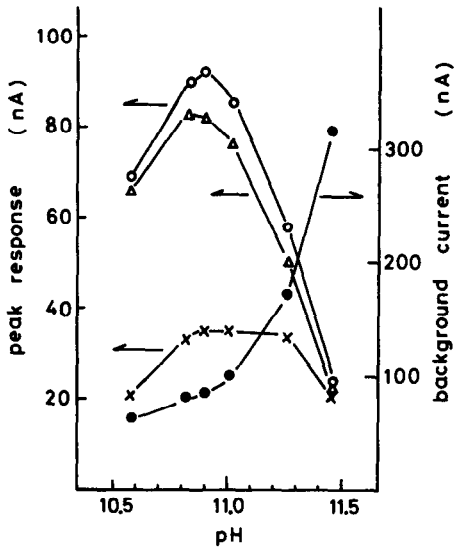

Fig. 1. Peak responses and background current *vs.* pH of reagent solution. Eluent: acetonitrile–water (7:3), 0.35 ml/min. Reagent solution: 1 m*M* CBP in 0.1 *M* Na_2HPO_4. Reaction coil: PTFE tube, 6 m × 0.5 mm I.D., 98.5°C. ○, Glucose; △, xylose; ×, rhamnose; 6 ppm each (120 ng injection). ●, Background current.

temperature, the temperature of the reaction bath was desired to be as high as possible. The reaction bath consisted of a three-necked flask, to one of which a condenser was fitted for refluxing. The flask was heated by a mantle heater, the input power to which heater was regulated. Mixing of the medium, water, was carried out by boiling under reflux. By this means, the temperature of the bath was maintained at 98.5°C to within ±0.1°C. Fluctuations in the detection due to evolution of gas even at such high temperatures did not occur. As it was not necessary to replenish the water in the bath, routine operation was considerably simplified with this reaction bath.

Fig. 1 shows the dependences of the peak responses and background current on the pH of the reagent solution. Optimum responses were obtained at pH 10.9 for three carbohydrates studied. The optimal pH was considerably lower than that when water was used as the eluent, in which the maximum response was attained at pH 11.20. The background current increased rapidly when the pH was increased above 11.2. The background current was almost constant or changed only slightly over the pH range 10.0–12.4 in previous work using water or a buffer solution as the eluent[1,3,5]. The magnituide of the background current at pH 11.0 was about four times higher than those observed in previous work. There was some experimental evidence that the background current was caused by reducing contaminants in the reagent solution and eluents when water or borate buffer was used as the eluent. However, with acetonitrile–water as the eluent there appears to be another cause, although its origin is uncertain at present.

Fig. 2 shows the dependence of the peak response on the length of the reaction coil (PTFE tube, 0.5 mm I.D.) soaked in the reaction bath. Maximal responses were obtained with a 6-m coil. The background current increased rapidly with lengths

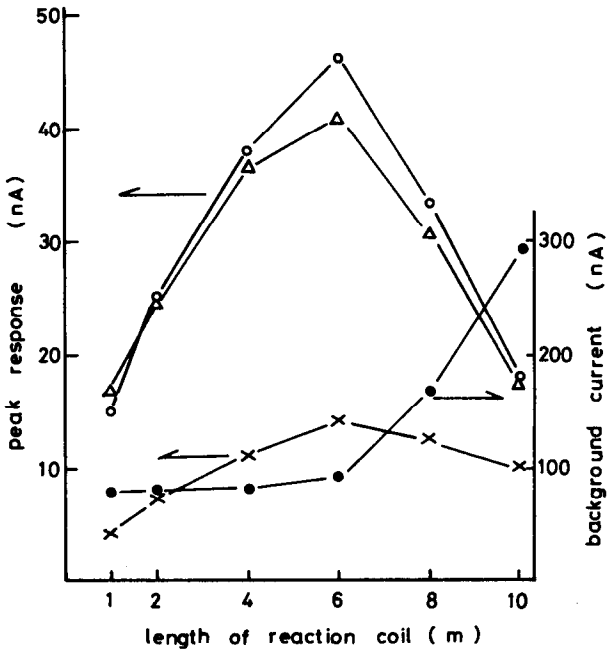

Fig. 2. Peak responses and background current *vs.* length of reaction coil. Reagent solution: 1 m*M* CBP in 0.1 *M* Na_2HPO_4, pH 10.9. ○, Glucose; △, xylose; ×, rhamnose; 3 ppm each (60 ng injection). ●, Background current.

greater than 8 m. The significant decline of the response with longer coils might be attributed to a decrease in the effective concentration of CBP, resulting from consumption as the background current.

The coefficients of variation ($n = 5$) for the determination of rhamnose, xylose and glucose were 6.2, 4.4 and 3.3%, respectively, for 100-pmol injections. The detection limit was 5 pmol for glucose (0.05 ppm concentration, 1 ng injection) at a signal-to-noise ratio of 3.

The refractive index (RI) detector is the most popular method of detection of carbohydrates in partition chromatography using organic solvents as eluents (in general, acetonitrile–water). An advantage of RI detection is that it is directly applicable without the need for derivatization. However, the RI detector is not sensitive enough to be able to detect sub-microgram levels of sugars[6]. Moreover, the selectivity of the RI detector is so low that its application to complex samples such as biological fluids is almost impossible. D'Amboise *et al.*[7] applied post-column derivatization with tetrazolium blue and visible light absorption detection to the HPLC of anion-exchange resins with acetonitrile–water as the eluent. The detection limits were reported to be about 10 ng of monosaccharides. Honda *et al.*[4] described electrochemical detection after post-column derivatization with 2-cyanoacetamide in which 4 ng of glucose could be detected in the partition mode using an NH_2-bonded column and acetonitrile–water as the eluent. Hence amperometric detection using CBP in the post-column reaction provides the most sensitive method for detection in the partition mode of HPLC with an amino-bonded column and acetonitrile–water as the

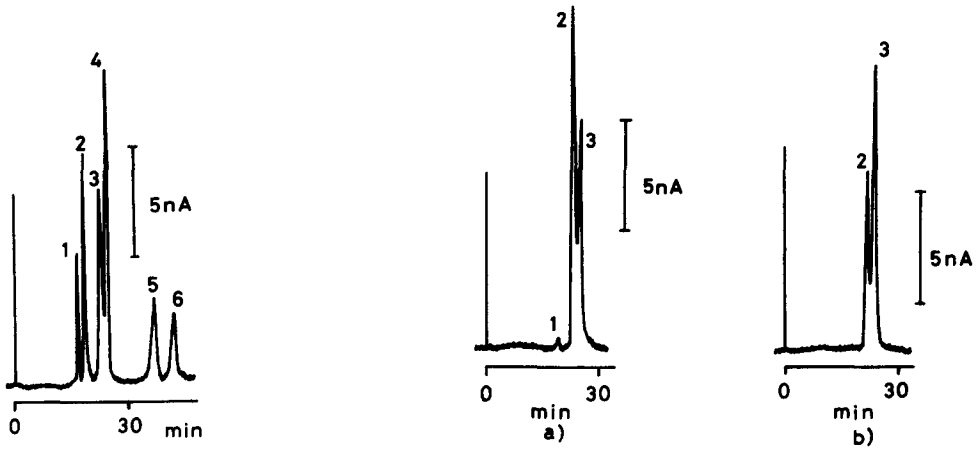

Fig. 3. Chromatogram of an authentic mixture of carbohydrate. Eluent: acetonitrile–water (7:3), 0.35 ml/min. Reagent solution: 1 m*M* CBP in 0.1 *M* Na_2HPO_4, pH 10.9. Reaction coil: PTFE tube, 6 m × 0.5 mm I.D., 98.5°C. Peaks: 1 = rhamnose; 2 = xylose; 3 = fructose; 4 = glucose; 5 = maltose; 6 = lactose; 1 ppm each (20 ng injection).

Fig. 4. Chromatograms of fruit juices. HPLC conditions as in Fig. 3. Samples of the originally squeezed juices were diluted 1:20 000. Peaks: 1 = xylose; 2 = fructose; 3 = glucose. Juice: (a) apple; (b) hattusaku (a Chinese citrus).

eluent. The dynamic range of the linear response for glucose extended from injections of 5 pmol to 2 nmol. Linearity over the same range was also observed with other carbohydrates.

Fig. 3 shows the chromatogram obtained for an authentic mixture of six carbohydrates (rhamnose, xylose, fructose, glucose, maltose and lactose) with a 100-pmol injection (1 ppm, 20 ng of each). Ribose, fucose, arabinose, mannose, galactose, sorbose, cellobiose, glucosamine and galactosamine showed similar relative response. Sucrose did not respond at all, as expected from its non-reducibility.

Fig. 4 shows the chromatograms obtained for 20 000-fold dilution of fruit juices. Fructose and glucose were always observed as major components, although trace amounts of xylose were often identified in apple juice. In general, almost the same amount of sucrose as glucose or fructose is present in fruits. However, sucrose completely escaped detection, reflecting its non-reducing ability.

Fig. 5 shows the chromatogram obtained for urine from a healthy male. Again, a simple chromatogram was observed. It is surprising that such a simple chromatogram was obtained, considering the complex matrix (urine) and the poor specificity involved in the separation mode using an amino-bonded column and acetonitrile–water as the eluent compared with that using an anion-exchange column and borate buffer. The urine was deproteinized by mixing with an equal volume of acetonitrile followed by centrifugation and dilution. The final concentration in acetonitrile–water (7:3) on injection corresponded to one fiftieth of the original urine. The first, very large, peak in Fig. 5 is due to an unknown component. Uric acid was also detectable at about 75 min, although not shown here. Other peaks coincided with ribose, xylose, arabinose, fructose, glucose and lactose, in order of elution. The amounts of xylose

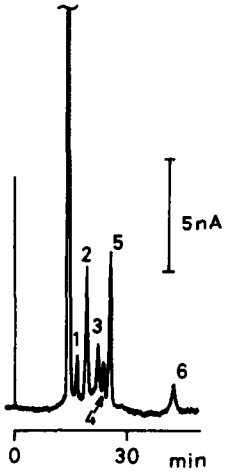

Fig. 5. Chromatogram of human urine. HPLC conditions as in Fig. 3. The original urine was diluted 1:50. Peaks: 1 = ribose; 2 = xylose; 3 = arabinose; 4 = fructose; 5 = glucose; 6 = lactose.

and glucose in urine were calculated to be approximately 150 and 130 nmol/ml, respectively. These values were consistent with those reported elsewhere[7,8].

It can be seen from Figs. 4 and 5 that the proposed method has excellent selectivity. It is noteworthy that the concentration of 1 m*M* CBP in the reagent solution employed corresponded to roughly 0.05% (w/w), which is fairly dilute. At such a low concentration of CBP, the sensitivity increases linearly with CBP content[1]. Hence some further improvement in sensitivity is still expected even after allowing for the increase in background current.

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