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Determination of carbohydrates as their *p*-sulfophenylhydrazones by capillary zone electrophoresis☆

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Abstract

p-Hydrazinobenzenesulfonic acid was explored as an ultraviolet labeling reagent for capillary electrophoresis of mono-, di- and trisaccharides. The labeling reaction that produces *p*-sulfophenylhydrazines took less than 8 min, and introduced both chromphore and charged groups into the carbohydrate molecules. The derivatives of nine mono- and disaccharides were completely separated in 9 min using a 100 mM borate buffer at pH 10.24. On-column UV detection at 200 nm allowed the detection of glucose with a mass detection limit of 17.6 fmol or a concentration limit of 3.6 μM. Reproducible quantification of carbohydrates was achieved in the concentration range of 0.1–9.1 mM in reaction solution. The method was applied successfully to determine the monosaccharide composition of laminaran.

Keywords: *p*-Hydrazinobenzenesulfonic acid; Carbohydrate; Capillary electrophoresis; Precolumn derivatization

1. Introduction

Carbohydrates are among the key biological substances involved in many life processes such as cell recognition and the immune response. Since the function of carbohydrates depends on their unique structures, their analysis and identification have become increasingly important.

Capillary zone electrophoresis (CZE) has been successfully applied to carbohydrate analysis. The main features that CZE offers are high efficiency, high speed, and the requirement of only a minute amount of sample. However, carbohydrates are in general difficult to separate and detect due to their lack of readily ionizable functional groups and chromophores.[2] Various approaches have been worked out to accommodate CZE analysis, and these have been reviewed in detail by El Rassi.[3–5] Precolumn derivatization is the most widely used means, and most of the methods are based on reductive amination.[6–9] Although this method gives excellent results, it is generally time-consuming (requiring 1–4 h) and may result in the cleavage of some important sugar residues, such as sialic acid.

Hydrazine-containing reagents such as dabsylhydrazine, FMOC-hydrazine, and dansylhydrazine have been applied to TLC[10,11] and HPLC procedures[11–13] with success. Pérez and

Abbreviations: BHZ, *p*-hydrazinobenzenesulfonic acid; Man, mannose; Gal, galactose; GlcNAc, *N*-acetylglucosamine; Glc, glucose; Fuc, fucose; Ara, arabinose.

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Colón[14] reported the CZE of dansylhydrazine-labeled sugars using laser-induced fluorescence (LIF) detection. The most appealing feature of it was that it took only 15 min for derivatization. The detection limits of the derivatized mono- and disaccharides reached 100 attomoles. Moreover, such reagents react specifically with ketone and aldehyde groups and can be used directly on biological samples. FMOC-hydrazine and dansylhydrazine have been employed for the determination of sugars in plasma[15] and tear fluid,[14] respectively. However, these reagents introduced no charge to the sugar molecules, which is necessary for CZE.

It would be most convenient for the CZE of carbohydrates if the derivatized sugars carried charges over a wide pH range. This is especially desirable in oligosaccharide separations.[16] *p*-Hydrazinobenzenesulfonic acid (BHZ) was thus selected, and the results showed that the labeling process could be carried out within 10 min.[1] In this work the derivatization and CZE separation of sugars were studied in detail.

2. Results and discussion

Derivatization. derivative configuration.—The possible products of the hydrazine-containing reagent labeled sugar are imide, hydrazone and osazone. Our results showed that some sugars such as GlcNAc and some di- and trisaccharides yield two electrophoretic peaks. This phenomenon also occurred with some other hydrazine-containing reagents.[14,17,18] Mopper[17] and Pérez[14] postulated that some equilibration existed between the hydrazone and osazone derivatives. However, Bendiak has demonstrated that both the acyclic and cyclic hydrazones are produced from an acetohydrazide-derivatized sugar by ^{1}H NMR spectroscopy.[18] To confirm the structure of the products of the derivatization, in this paper, FABMS (negative-ion detection) was performed with labeled GlcNAc. The MS data showed a peak at m/z 390 that corresponds to the GlcNAc hydrazone $[M-H]^-$. No imide or osazone peak was detected at m/z 408 or 519, which precludes the possibility of the osazone and hydrazone coexisting. Therefore the products were the mixtures of the isomeric compounds, perhaps either acyclic and ring (pyranosyl or furanosyl) forms of the hydrazones such as those of acetohydrazine[18] or a pair of syn and trans isomers of an open-chain hydrazone. We conducted ^{1}H NMR spectroscopy in an effort to identify the derivatives, but further experiments are still needed to definitely ascertain the structure of the isomers.

According to experimental results, isomer B of the GlcNAc derivative seems to be thermodynamically preferred over isomer A in this case. The ratio of isomer B can thus increased through increasing the reaction time, temperature and/or the ratio of BHZ to sugar (Fig. 1). Isomer B of the GlcNAc derivative is the predominant component under the optimum reaction conditions. Moreover, the same trend was not observed for some of the di- and trisaccharides, e.g., maltose and melitose, which were found yield two reaction products. For maltose and melitose, the ratio of the two isomers remained almost constant as the reaction time varied.

pH of reaction solution.—The pH of the reaction solution plays an important role in derivatization. As shown in Fig. 1(a), the optimum pH ranges for GlcNAc-b, Fuc, Gal were pH $\sim$ 3–4, $\sim$3–5 and $\sim$2–4, respectively. Outside these ranges, the yields decreased. This is apparently caused by the dual effects of H^+ on the derivatization: H^+ will protonate both the aldehyde group of sugars and the amino group of BHZ. The former reaction increases the reaction activity, while the latter reduces the nucleophilic properties of BHZ. Moreover, the pH of the reaction mixture may not be maintained if strong acids such as HCl are used as catalysts. The situation becomes worse if the BHZ concentration is increased, because its sodium salt is a mild base. A NH_4Ac–HCl buffer was finally chosen to control the pH of the reaction mixture. To suit most of the sugars, it is suggested that the solution pH be maintained in the range of pH $\sim$ 3–4.

The molar ratio of BHZ to sugar.—Fig. 1(b) shows that the peak heights of monosaccharides increase sharply with the concentra-

tion of BHZ at the beginning of the reaction and level off over the ratio 10:1. Excessive reagent caused precipitation and interfered with the subsequent CZE separations. The best ratio was ~10:1–20:1.

Temperature and time.—Fig. 1(c) shows that the optimum reaction temperatures were about 70, 60 and 50 °C, for GlcNAc-b, Fuc and Gal, respectively. Interestingly, GlcNAc-b was sensitive to temperature variations while the other two sugars were not. For fast labeling, higher temperatures were preferred. It is suggested that the derivatization be routinely carried out at 70 °C. At this temperature the reaction will be completed in about 10 min (see Fig. 1(d)).

Separation of BHZ hydrazones.—In an alkaline borate buffer, the BHZ hydrazones are negatively charged, due to both the sulfonate and the borate complex. Their electrophoretic behavior has been studied as detailed in the following paragraphs.

Buffer pH and concentration.—In order to be reproducible and to minimize errors from temperature variation and other factors,[19] relative mobility, μ_{ep}/μ_{eo} was adopted as the criterion in this study:

$$\mu_{ep}/\mu_{eo} = 1 - \frac{t_0}{t_R}$$

where t_0 is the rentention time of dimethyl sulfoxide and t_R the migration time of the test substance. Fig. 2 shows the effects of pH and concentration of borate buffer on the relative mobility of the BHZ derivatives. It is evident that with increasing pH or borate concentration the relative mobilities of the derivatives became greater due to the facilitation of the formation of borate complexes. According to Fig. 2(a), optimum borate concentration range

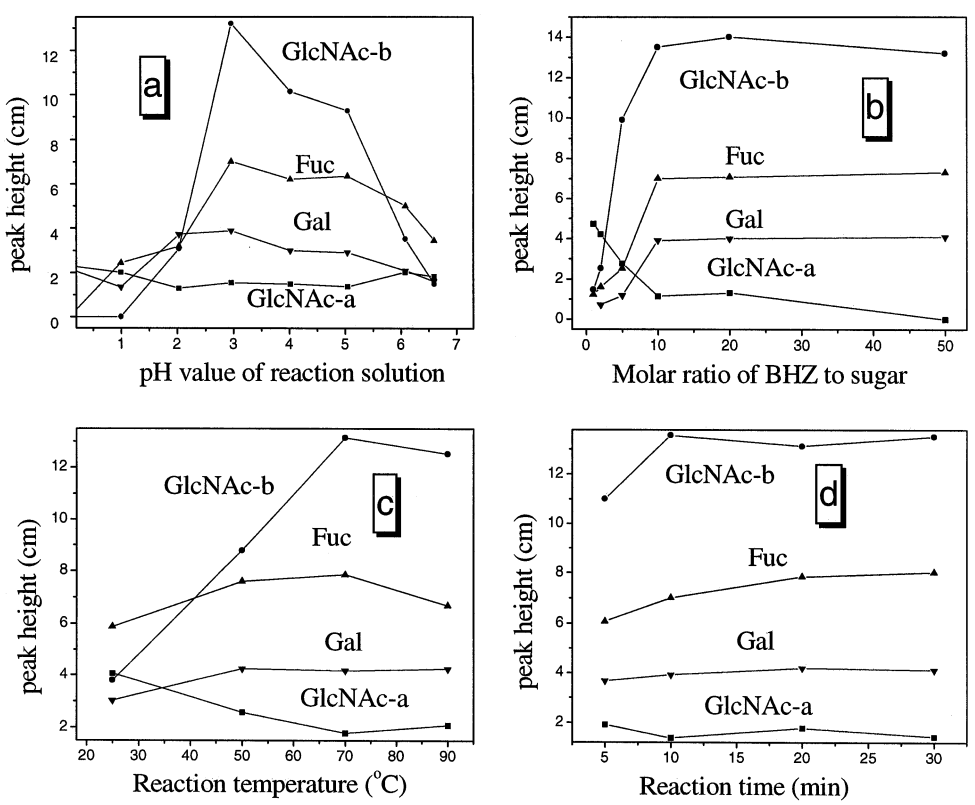

Fig. 1. Effects of various factors on precolumn derivatization of selecting sugar to BHZ. (a) pH value of reaction solution. The concentration of BHZ was 0.2 M. Reaction temperature, 70 °C; reaction time, 20 min. (b) The molar ratio of BHZ to sugar. The pH value of reaction solution was fixed at pH 3.0. Reaction temperature, 70 °C; reaction time 20 min. (c) Reaction temperature. The pH value of reaction solution was pH 3.0. The concentration of BHZ was 0.2 M. Reaction time 20 min. (d) Time course of derivatization. The pH value was 3.0. The concentration of BHZ was 0.2 M. The reaction temperature was 70 °C. In all experiments the reaction products were analyzed by using 100 mM borate buffer, pH 10.4, 300 V/cm field intensity. On-column detection was carried out at 206 nm. The concentration of carbohydrates in the sample solution was 5 mM.

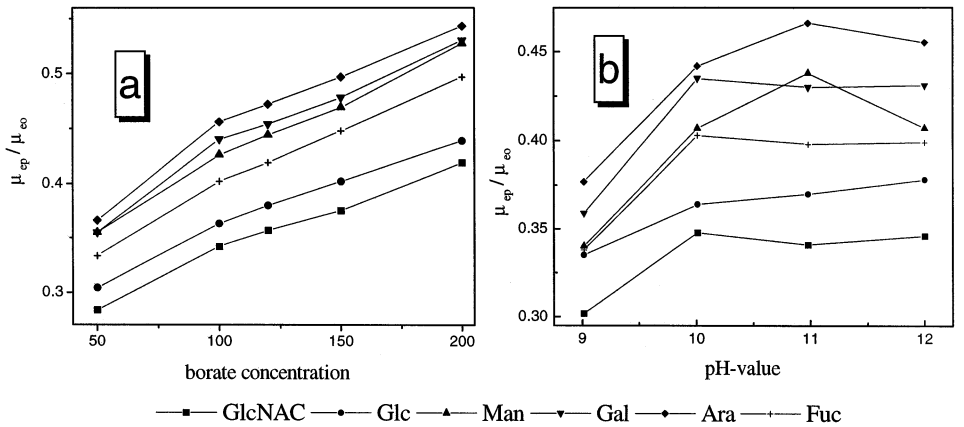

Fig. 2. Effects of pH and borate concentration of buffer on the separation of BHZ-sugars. The borate concentration in (a) and pH in (b) were fixed at pH 10.5 and 100 mM, respectively. The CZE conditions followed those in Fig. 1.

was 100–150 mM. In order to avoid Joule heating and to shorten the separation time, a lower concentration of borate was found to be beneficial. Fig. 2(b) shows the relationship between pH and the relative mobility at a constant borate concentration of 100 mM. Between pH 10.2 and 10.8, most monosaccharides showed the best resolution. From the results sketched in Fig. 2(a and b), a pH ~ 10.2–10.8 and a borate concentration of 100 mM were chosen for the subsequent analyses.

Capillary zone electrophoresis (CZE).—Fig. 3 depicts the electrophorogram of the derivatized mixture of nine reducing mono- and disaccharides together with Me_2SO as the internal standard. The reagent migrated slower than sugar derivatives, and the separation was completed in 9 min. The efficiencies obtained were between 110,000 and 290,000 theoretical plates per meter for all the nine sugars. Both maltose and melitose had two peaks, and the minor peaks could not be suppressed significantly by varying the reaction conditions.

Quantitative analysis and sensitivity.—Sugar mixtures with differing concentrations were derivatived and separated by CZE, with UV detection at 200 nm. Although the yields of the BHZ derivatives were not uniform, they were reproducible enough to allow reliable quantification. The calibration curves for the test sugars showed quite acceptable linearity over a concentration range of 0.1–9.1 mM. The respective equations of the line and the regression analysis results are listed in Table 1. A coefficient of variation below 4.2% was obtained with five repeated runs. The mass detection limit of glucose was 17.6 fmol or 3.6 μM in terms of concentration, at a signal-to-noise ratio of 3. This indicated that the

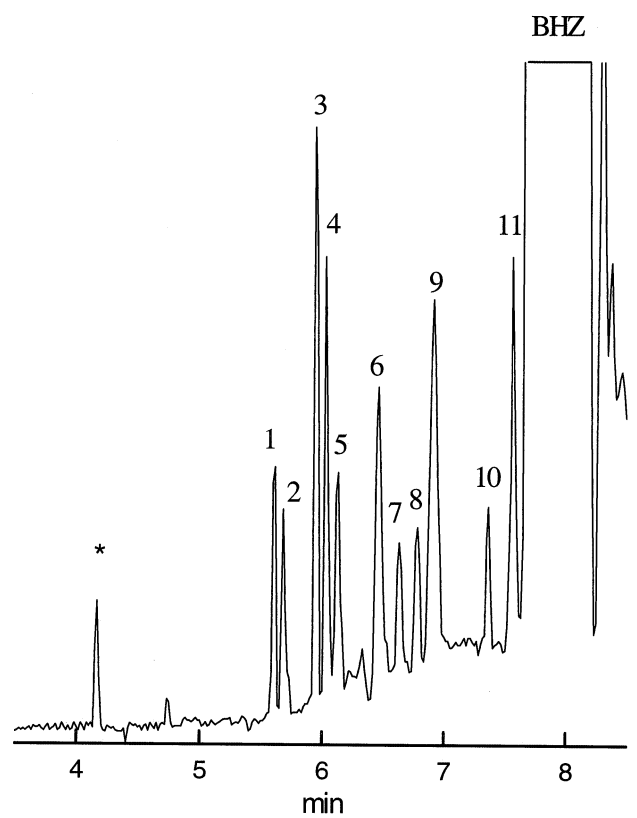

Fig. 3. Separation of nine sugar-BHZ derivatives: *,Me_2SO; 1, cellobiose; 2, maltose (a); 3, *N*-acetylglucosamine; 4, melitose (a); 5, glucose; 6, fucose; 7, mannose; 8, galactose; 9, arabinose; 10, maltose (b); 11, melitose (b). Concentration of each sugar was 0.2 mM. Separation conditions: Beckman P/ACE 2050, 200 nm detection; fused silica capillary, 55/62 cm; voltage, 18.6 kV; separation carrier, 100 mmol/L boric acid (pH 10.24); pressure injection, 3.44 kPa, 3 s.

Table 1
Regression equation and correlation coefficients for monosaccharides

Monosaccharide	Regression equation	Correlation coefficient (R)	RSD ($n = 5$)
Arabinose	$Y = -0.0607 + 216.3037X$	0.996	0.042
Fucose	$Y = -0.0062 + 82.8772X$	0.995	0.016
Glucose	$Y = -0.0295 + 286.5016X$	0.997	0.023
Galactose	$Y = 0.0065 + 17.31372X$	0.994	0.004
Mannose	$Y = 0.0172 + 92.9369X$	0.999	0.002
N-acetylglucosamine (b)	$Y = 0.0289 + 48.05302X$	0.999	0.016
Maltose (a)	$Y = 0.0064 + 17.99916X$	0.999	0.035
Maltose (b)	$Y = 0.0084 + 31.95348X$	0.995	0.005

method was applicable, considering that the results were obtained by directly derivatizing the sugar solution of different concentrations. It is noticeable that the sugars, e.g., maltose, which produces two peaks, can also be quantitatively determined because either one or both of their peaks could be used for calibration purposes. The two-peak phenomenon did not diminish the accuracy of the quantitative analysis.

Application to component analysis.—The applicability of this method was demonstrated by analyzing the monosaccharide components of laminaran extracted from kelp. The resulting CZE peaks were identified by spiking standard sugar-BHZ derivatives. Galactose, mannose, fucose and glucose were determined in laminaran hydrolysate with a molar ratio of 0.67:0.092:1:0.124, which were comparable with those in the literature.[20]

Precolumn derivatization of carbohydrates with *p*-hydrazinobenzenesulfonic acid is a rapid, simple and reproducible method that is suitable for quantitative analysis. Although a few sugars formed two hydrazone products after derivatization, the method serves well for quantitative analysis, as the derivatization is quite reproducible. The two-peak phenomenon might also be useful in component identification.

3. Experimental

Apparatus.—Quantitative analysis was performed on a Beckman CZE instrument model P/ACE 2050 (Fullerton, USA) using the SYSTEM GOLD software. Other experiments were performed on a homemade CZE system. The detection wavelengths were set at 200 nm for P/ACE or 206 nm for the homemade system. Separations were carried out in fused-silica capillaries (Yongnian Optical Fiber Factory, Hebei, China) of 50 μm i.d. and 375 μm o.d. The effective length was 55 cm, and the total length was 62 cm for P/ACE or 75 cm for the homemade system. The applied electrical field strength was 300 V/cm. Prior to each injection, the capillaries were sequentially flushed with 0.1 M NaOH, 0.1 M HNO_3, water and separation buffer for 2 min for reproduciblility. The capillary tubing was filled with 0.1 M NaOH for overnight storage. The separation buffer was boric acid adjusted to the desired pH by adding KOH pellets. All the water used was double-distilled water. The sample was introduced to the anode end of the tubing by gravity injection for 20 s at a 10 cm height on the homemade CZE system or by pressure injection for 2 s at 3.44 kPa on P/ACE. ^{1}H NMR experiments were conducted on a Bruker ARX 400 MHz instrument. Mass spectrometry was performed in a Kyky-ZHP-5 instrument (The Center of Science Instrument, CAS; Beijing, China) using FAB as the ion source and glycerol as the matrix.

Chemicals.—All sugars were purchased from Sigma Chemical Co. (St. Louis, USA). *p*-Aminobenzenesulfonic acid was a product of Beijing Chemical Factory (Beijing, China).

Preparation of p-*hydrazinobenzensulfonic acid (BHZ).*—BHZ was synthesized according to the literature procedure,[21] with modifications described hereafter. *p*-Aminobezenesulfonic acid (19 g) was dissolved in 25 mL of 20% NaOH, then 100 mL of 3.5% (w/v) $NaNO_2$ was added. After cooling, the solution was treated with 50 mL of 1.5 M H_2SO_4 by

stirring at −5 °C for 20 min. The mixture was filtered, the precipitate was suspended in 20 mL of concd HCl, and 32% $NaHSO_3$ was added dropwise until the reaction solution became clear. The solution was then acidified with 20 mL of concd HCl, boiled and filtered to yield approximately 9 g of *p*-hydrazinobezenesulfonic acid. The product was isolated as crystalline needles. The crude product was purified by recrystallization until the electrophorogram showed a single peak. Fresh BHZ solution for derivatization was prepared daily by dissolving in an equivalent amount of Na_2CO_3 solution.

Derivatization of sugars with BHZ.—An aliquot of 10 μL of 0.3–10 mM sugar solution in a 2 M ammonium acetate solution of pH ~ 3.0–5.0 (adjusted by concentrated HCl) was placed in an Eppendorf tube and mixed with 10 μL of 0.2 M BHZ. The mixture was allowed to react at 70 °C for 10 min and then quenched by freezing at −20 °C. The reaction product was stored in the dark at −20 °C and analyzed within 7 days. For ^1H NMR, this procedure was modified. *N*-Acetylglucosamine or glucose (50 μmol) was dissolved in 500 μL of 0.01 M BHZ solution in D_2O and added to 50 μL of 0.2 M HCl (D_2O). The solution was then heated at 70 °C for 20 min.

Purification of GlcNAc derivatives.—The reaction of GlcNAc and BHZ was followed by TLC (5 × 10 cm HPTLC Silica Gel 60 F_{254}, E. Merck, Germany) using 15:4:1 CH_3CN–water–HOAC as eluant. An alternative method was to separate the mixture with Sephadex G-15 (Pharmacia, Sweden). The separated sample was lyophilized to dryness for MS.

Hydrolysis of polysaccharides.—Laminaran (2 mg, containing 80% saccharides) was hydrolyzed by adding 100 μL of 2.0 M trifluoroacetic acid in a screw-capped vial and heating to 100 °C for 4 h. After cooling, the product was neutralized by adding 100 μL of 1.0 M Na_2CO_3, and then it was diluted to 1 mL with water. The hydrolytic products were derivatized as described above.

Acknowledgements

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