Journal of Chromatography, 638 (1993) 343–348
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 871

Capillary electrophoresis of *o*-phenylenediamine derivatives (quinoxalines) of dicarbonyl sugars

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ABSTRACT

Dicarbonyl sugars were separated by capillary electrophoresis as *o*-phenylenediamine derivatives (quinoxalines). Tetrabutylammonium bromide was used as a modifier; D-*arabino*-2-hexosulose (D-glucosone), D-*lyxo*-2-hexosulose (D-galactosone), 7-deoxy-L-*galacto*-2-hexosulose and 5-hydroxy-2,3-dioxohexanal served as standards. The separation procedure was optimized with respect to pH, buffer and solute concentrations. In most instances optimum separations were obtained in 75 m*M* borate buffer (pH 11.0); 0.04 mg/ml was found to be the optimum sample concentration. The detection limit was *ca.* 0.1 pmol. Analyses of a mixture of fermentation products or Maillard reaction products show the applicability of the method.

INTRODUCTION

The role of dicarbonyl monosaccharides in a number of metabolic pathways seems to be well established; some of these compounds are capable of inhibiting the proliferation of malignant cells and others appear to be involved in diabetes or rat hypoglycaemia. Of particular importance is D-*arabino*-2-hexosulose, the metabolism of which is best known: this sugar is involved in lignin biodegradation and in some fungi it is dehydrated with the formation of an antimicrobial antibiotic, cortalcerone. Other dicarbonyl monosaccharides have been shown to be present in other antibiotics also (for a review, see ref. 1). Dicarbonyl sugars arise also in vertebrate tissues as a result of monosaccharide autoxidation in the presence of the free amino groups. The interest in this respect is stimulated particularly by the fact that dicarbonyl sugars appear to be involved in tissue ageing and in a number of pathological processes. Dioxo compounds (*e.g.*, 3-deoxy-2-hexosulose) were reported to represent the key point in the Maillard (browning) reaction [2,3], which stresses further the need for a reliable separation procedure, preferably complementary to chromatography.

In spite of considerable efforts to separate dicarbonyl sugars in the underivatized form, no completely satisfactory method is available. There are several reasons for this situation: thin-layer chromatography (TLC) is used mostly for screening purposes with limited possibilities of quantification; in high-performance liquid chromatographic (HPLC) procedures (nitrile-bonded

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0021-9673/93/$06.00

phases), dicarbonyl sugars tend to be irreversibly bound to the sorbent; with gas chromatography (GC) the difficulty lies in the instability of the trimethylsilyl derivatives under the analysis conditions. Detection represents another problem. As underivatized sugars are devoid of UV absorbance, their spectrophotometric detection is difficult. It is possible to use short-wavelength UV (typically 185 nm) or IR detection. In the latter instance no satisfactory detection limits serving biomedical purposes can be achieved and in the former the profiles are frequently obscured by the presence of accompanying compounds that absorbing in the same UV region [1].

o-Phenylenediamine was introduced some time ago as a suitable derivatization reagent for dicarbonyl sugars. In this derivatization reaction dioxo compounds are converted in the corresponding quinoxalines:

This reaction was successfully applied to the isolation of dicarbonyl sugars from mycelial macerates [4] and for the characterization of various homoglucans [5]. Chromatographic techniques such as ion-exchange chromatography [6], TLC, HPLC [7,8] and GC–MS [5,9,10] have been applied for the separation of *o*-phenylenediamine derivatives of carbohydrates with limited success. All these procedures are fairly insensitive. *o*-Phenylenediamine has also been used as a spray reagent for revealing sugar acids on thin layers [11].

In this paper we report the separation of dicarbonyl sugars by capillary electrophoresis (CE).

EXPERIMENTAL

Chemicals

o-Phenylenediamine was obtained from Sigma (St. Louis, MO, USA). D-*arabino*-2-Hexosulose (D-glucosone), D-*lyxo*-2-hexosulose (D-galactosone) and 7-deoxy-L-*galacto*-2-hexosulose were prepared enzymatically [12] from corresponding aldoses. 5-Hydroxy-2,3-dioxohexanal was prepared enzymatically from 6-deoxy-D-glucose [13]. Tetrabutylammonium bromide and D-(+)-glucose were from obtained Lachema (Brno, Czech Republic) and L-lysine from Serva (Heidelberg, Germany).

Quinoxaline formation and preparation of the standards

Equimolar amounts of *o*-phenylenediamine were added to the solution of a dicarbonyl compound in citrate buffer (pH 4.00). After vigorous stirring for 2 h (40°C) the resulting quinoxalines were extracted with *n*-butanol and recrystallized from ethanol. The structure of the quinoxalines was confirmed with NMR and mass spectral measurements.

Capillary electrophoresis

Separations were carried out with a laboratory-made capillary electrophoresis apparatus [14] with an untreated fused-silica capillary (50 μm I.D., 70 cm to the detector) at 15 kV; the detection wavelength was set at 220 nm. Calibration was done with an SP 4290 integrator (Spectra-Physics, San Jose, CA, USA).

All measurements were carried out in boric acid of appropriate concentration with 8 mmol/l tetrabutylammonium bromide titrated to the desired pH by 1 mol/l NaOH. This buffer was passed through a 0.5-μm filter (Waters, Milford, MA, USA) before application. Standards were dissolved in methanol (glucosone, galactosone and 7-deoxy-L-*galacto*-2-hexosulose at 0.5 mg/ml, *o*-phenylenediamine at 2 mg/ml and 5-hydroxy-2,3-dioxohexanal at 3 mg/ml). Aliquots of these stock solutions were evaporated to dryness and the residue was dissolved in separation buffer. The optimum running concentrations of the standards for measurement were 0.04 mg/ml for glucosone, galactosone and 7-deoxy-L-*galacto*-2-hexosulose, 0.08 mg/ml for *o*-phenylenediamine and 0.05 mg/ml for 5-hydroxy-2,3-dioxohexanal.

Additional procedures

The Maillard reaction products were analysed according to the following protocol. A mixture of lysine (2 mmol), glucose (2 mmol) and

o-phenylenediamine (1 mmol) was incubated in 10 ml of 0.2 mol/l phosphate buffer (pH 7.4) at 60°C for 20 h. After incubation the sample volume was adjusted to 5 ml. The quinoxalines formed precipitated during the incubation period and were collected by centrifugation (4000 *g*, 15 min). The supernatant was discharged and the pellet was dissolved in methanol (2 mg/ml).

The Maillard reaction products were purified as follows. A mixture of Maillard reaction products was applied to the Silufol UV 254 silica gel thin-layer plates (15 × 15 cm) (Kavalier, Votice, Czech Republic) and developed with chloroform–methanol (22:8, v/v) (20 × 20 × 6 cm chamber, saturated for 0.5 h). The compounds were detected as quenching spots under UV light at 254 nm. The prominent spot occurring at $R_F = 0.33$ was extracted with ethanol and further analysed.

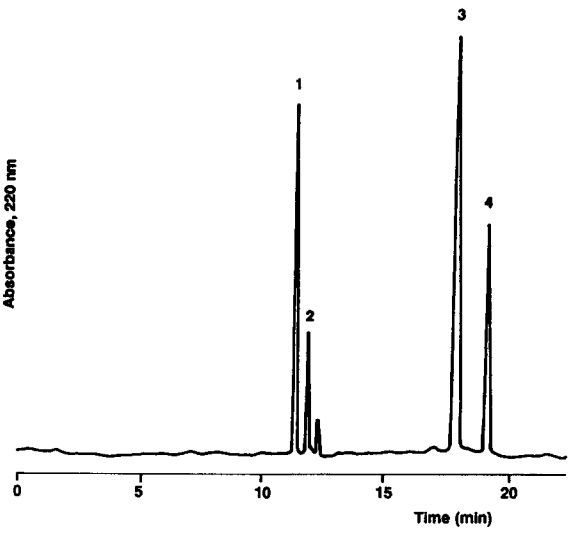

Fig. 1. Electrophoretic profile of standards under optimum condition (75 mmol/l boric acid, pH 11.0). Peaks: 1 = *o*-phenylenediamine; 2 = quinoxaline from 5-hydroxy-2,3-dioxohexanal; 3 = quinoxalines from D-*lyxo*-2-hexosulose (D-galactosone) and 7-deoxy-L-*galacto*-2-hexosulose; 4 = quinoxaline from D-*arabino*-2-hexosulose (D-glucosone).

RESULTS AND DISCUSSION

Condensation products of D-*arabino*-2-hexosulose (D-glucosone), D-*lyxo*-2-hexosulose (D-galactosone), 7-deoxy-L-*galacto*-2-hexosulose and 5-hydroxy-2,3-dioxohexanal with *o*-phenylenediamine exhibit a strong UV absorbance at 315 nm and, consequently, represent suitable derivatives for the separation of dioxo sugars with UV detection. As the chromatographic techniques used previously for *o*-phenylenediamine dioxo sugar derivatives give relatively high detection limits, capillary electrophoresis appeared to be the method of choice. In preliminary experiments disappointing results were obtained with sodium dodecyl sulphate as micelle-forming reagent; on the other hand, tetrabutylammonium bromide when used as a modifier showed good results. The main separation mechanism seems to be borate complex formation with *cis*-diols. Ion-pair formation of the borate complex with the ammonium salt improves the selectivity. The solute strongly coupled with the borate will migrate slowly to the negative electrode. The separation procedure itself was optimized with respect to pH, buffer and solute concentrations. In most instances optimum separations were obtained with 75 m*M* borate buffer (pH 11.0) and a sample concentration of 0.04 mg/ml. The detection limit is *ca.* 0.1 pmol.

The separation conditions were optimized by using a standard mixture as specified under Experimental. A typical separation of standards is shown in Fig. 1. Here the first peak corresponds to the derivatization reagent (*o*-phenylenediamine) followed by quinoxalines with 5-hydroxy-2,3-dioxohexanal, D-*lyxo*-2-hexosulose (D-galactosone) and 7-deoxy-L-*galacto*-2-hexosulose and D-*arabino*-2-hexosulose (D-glucosone). In preliminary experiments optimum pH conditions and the optimum buffer concentration were established (Figs. 2 and 3). Fig. 2 summarizes the mobility changes relative to the derivatization reagent between pH 9.5 and 11.5. At pH 9.5–10.0 5-hydroxy-2,3-dioxohexanal is not separated from the reagent peak and therefore the higher pH value is advisable for separation. On the other hand, an extremely alkaline pH should be avoided because of solubility problems with tetrabutylammonium bromide. In fact this is not much of a problem because the separation between pH 10.5 and 11.5 is almost equally good. Consequently, pH 11.0 of the

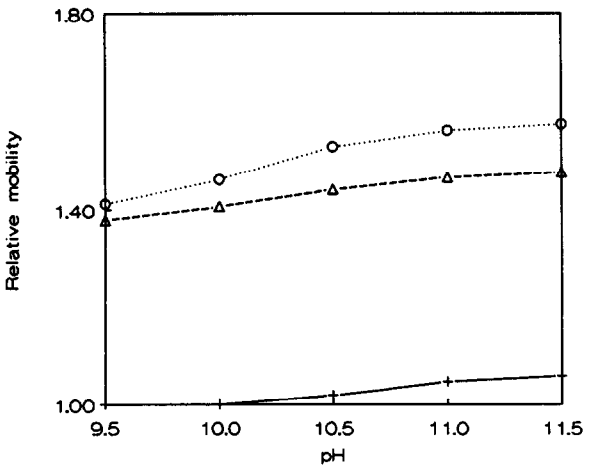

Fig. 2. Influence of pH on the relative mobility (ratio of migration times, *o*-phenylenediamine = 1.00). Measurements were made in 50 mmol/l boric acid. + = 5-Hydroxy-2,3-dioxohexanal; Δ = D-galactosone and 7-deoxy-L-*galacto*-2-hexosulose; ○ = D-glucosone.

running buffer appeared to be an acceptable compromise (R.S.D. = 4.8%, $n = 6$).

The optimum boric acid concentration was sought at pH 11.0 in a separate set of experiments. As shown in Fig. 3, with increasing boric acid concentration the relative retention of all components increased and optimum separations were obtained using 75–100 mmol/l boric acid.

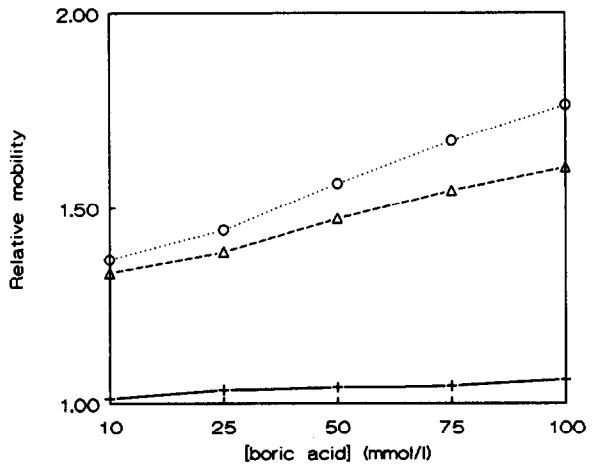

Fig. 3. Influence of boric acid concentration on the relative mobility (ratio of migration times, *o*-phenylenediamine = 1.00). Measurements were made at pH 11.0. + = 5-Hydroxy-2,3-dioxohexanal; Δ = D-galactosone and 7-deoxy-L-*galacto*-2-hexosulose; ○ = D-glucosone.

Whereas at a low concentration of boric acid poor separations were obtained, concentrations above 100 mmol/l precluded by excessive heating of the capillary (over 50 μA). Therefore, 75 mmol/l boric acid appeared to be the optimum concentration as it offered baseline separation of the standards and good peak shapes and no cooling of the capillary was necessary. The following calibration graphs were obtained (y = amount in pmol, x = peak area):

for D-glucosone,

$y = 0.595x - 0.026 \quad r = 0.984$

linearity range = 0.5–8 pmol; detection limit = 0.05 pmol;

for D-galactosone,

$y = 0.855x - 0.127 \quad r = 0.990$

linearity range = 0.5–8 pmol; detection limit = 0.05 pmol;

for 7-deoxy-L-*galacto*-2-hexosulose,

$y = 0.653x - 0.215 \quad r = 0.994$

linearity range = 0.5–8 pmol; detection limit = 0.05 pmol;

for 5-hydroxy-2,3-dioxohexanal,

$y = 3.233x - 0.325 \quad r = 0.984$

linearity range = 5–40 pmol; detection limit = 0.15 pmol.

Two practical applications of the method are shown in Fig. 4. In Fig 4A a profile of a crude D-glucosone solution (fermentation broth) is presented. This profile shows the conversion of glucose to glucosone by a mixture of enzymes from the basidiomycete *Phanerochaete chrysosporium* in the presence of *o*-phenylenediamine. The major peak in this profile corresponds to D-glucosone; the smaller peak with a shorter migration time represents an unknown impurity.

Fig. 4B shows the profile of dicarbonyl sugars present in a mixture of Maillard reaction products; the double peak No. 4 (and also other components of the mixture) can be preseparated by TLC and isolates tested for purity by CE. As shown in Fig. 4D, homogenous products can be obtained in this way. By combining TLC with CE it is possible to show the presence of

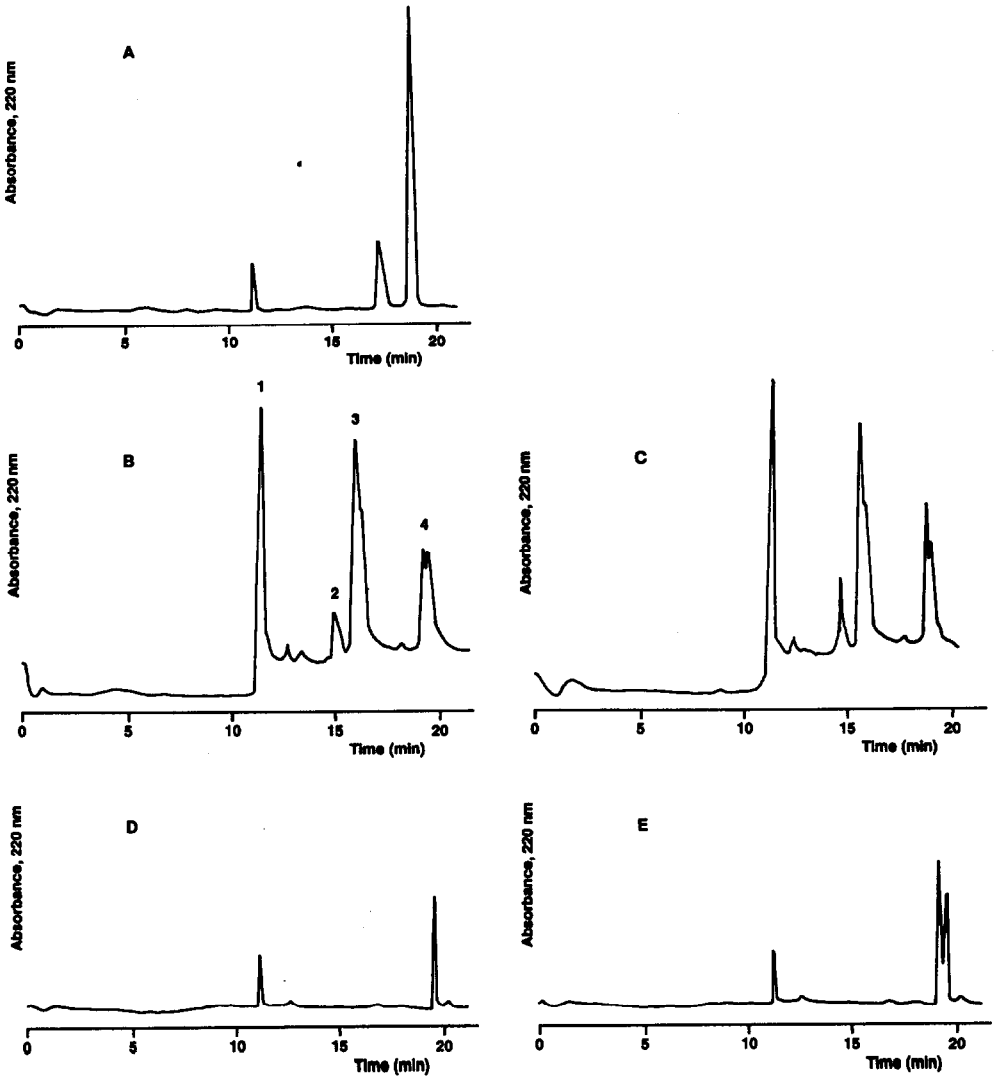

Fig. 4. Application of CE separation of *o*-phenylenediamine derivatives of dicarbonyl sugars. (A) Profile of crude solution of D-glucosone before purification procedures; the smaller peak represents an unidentified impurity; (B) profiles of quinoxalines of Maillard reaction products; (C) same as (B) but spiked with D-glucosone; (D) profile of purified product of Maillard reaction; (E) same as (D) but spiked with D-glucosone. The major peaks in (B) are numbered in the order in which they pass the detection window to facilitate reference in the text (see Results and Discussion).

D-glucosone in this reaction mixture (data not presented) or to isolate, *e.g.*, the accompanying compound in peak No. 4, as shown in Fig. 4E.

ACKNOWLEDGEMENT

This work was supported by the Czechoslovak Academy of Sciences (Grant No. 71129).

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