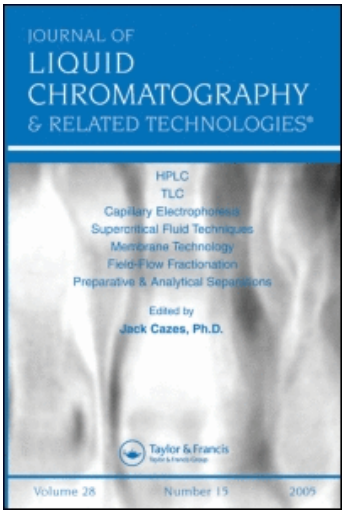

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USE OF BENZOYL HYDRAZINE REAGENT FOR MONOSACCHARIDE DETERMINATION BY HIGH PERFORMANCE CAPILLARY ELECTROPHORESIS

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ABSTRACT

Benzoyl hydrazine derivatives of reducing carbohydrates were analyzed by high performance capillary electrophoresis with on-tube ultraviolet detection. The derivatives of 9 reducing monosaccharides were completely separated in *ca.* 30 min. using a capillary tube (50 μm i.d., 62 cm) and 200 mM borate buffer, pH 10.8, as carrier. On-column UV detection at 220 nm was able to achieve sensitive detection down to 30~40 femtomole levels. The experimental parameters for optimum precolumn are discussed along with derivatization and separation. It was demonstrated that this method allows quantification of reducing carbohydrates with high accuracy and reproducibility.

INTRODUCTION

The carbohydrate moieties of glycoproteins or glycolipids perform a number of functions in a wide variety organisms, including protection from proteolysis, receptors for bacteria and viruses, and other functions related to immunological specificity and cellular differentiation.[1] Compositional analysis of the complex carbohydrates is of fundamental importance in structural studies of these compounds. A variety of modern chromatographic techniques have been applied to this task, including gas chromatography,[2] supercritical fluid chromatography,[3] and liquid chromatography.[4] More recently, high performance capillary electrophoresis (HPCE) has shown promise as a new analytical method for carbohydrate analysis because of rapid and quite high-resolution.[5,6] In many cases, HPCE has demonstrated separation far superior to that of other chromatographic methods. Carbohydrates are usually neutral and do not possess chromophores; they, therefore, pose problems for CE separation and optical detection methods. One common strategy to realize separation and detectablility is to perform derivatization to introduce charged groups and chromophores. Various precolumn derivatizations have already been used in capillary zone electrophoresis (CZE),[5,7-12] for example, reducing monosaccharide were derivatized to N-(2-pyridyl)glycamines which formed charged borate complexes with the borate in the buffer solution.[5] Another method for the analysis of carbohydrate with CE is based on fluorogenic reagents, such as 3-(4-carboxybenzoyl)2-quinolinecarboxaldehyde or 3-benzoyl-2-naphthaldehyde.[6] They are based on the reaction of the primary amine group of the derivatization reagents with the carbonyl group of the sugar or the primary amine group of the sugars with the carbonyl group of the derivatization reagents. Hydrazine reagents have been used as carbonyl group reagents for the determination of sugars because of rapid, specific, and high derivative yield.[13,14]

In this paper, benzoyl hydrazine was adapted as a derivatization reagent for monosaccharide analysis. The derivatization, separation and quantitation of sugars were investigated.

MATERIALS AND METHODS

Benzoyl hydrazine was purchased from Serva Co. (Germany) and used without further purification. All carbohydrate samples were of the highest grade commercially available from Packard-Becker. B. V., Netherlands. Ethanol and acetic acid, used as reaction medium for precolumn derivatization, were reagent grade from the Beijing Chemical Factory (Beijing, China).

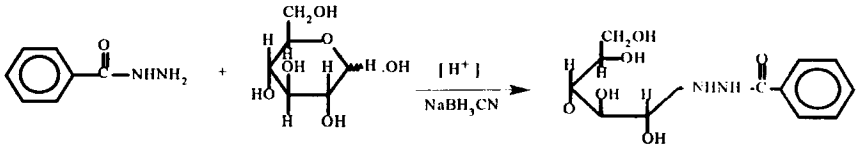

Figure 1. Reaction of benzoyl hydrazine with carbohydrate.

Sodium cyanoborohydride was purchased from Fluka (Switzerland) and calf serum fetuin from Sigma. Redistilled, deionized water was used to prepare the buffer. Borate solutions were prepared by dissolving pellets of boric acid in redistilled water and adjusting the pH to the indicated value with NaOH. Saccharide standards were dissolved in 75% (v/v) ethanol to give 5 mM solutions; they were stored at 4 °C.

Apparatus

HPCE was carried out with the Spectra PHORESIS 1000™ system with PC1000 vision 3.0 software from Thermo Separation Products, Inc., Fremont, CA. Their breadboard included the scanning focus 1000 detector, a 0 to 30 kv DC power supply, an air thermostatic capillary compartment, and a vacuum sample injector. Fused silica capillary of 50 μm i.d. and 375 μm o.d. was purchased from Yongnian Photoconductive Fiber Factory (Hebei, China). All experiments were carried out with uncoated fused silica capillaries. A new capillary was first flushed with 1 M sodium hydroxide, followed by water and then the running buffer. The running buffer was renewed after 5-6 runs, and the capillary column was flushed with fresh buffer before each injection in order to ensure reproducible separations.

Derivatization Procedure with Benzoyl Hydrazine (see Fig. 1)

All procedures were performed in a 0.5 mL polypropylene tube with a screw cap. To 20 μL of an ethanol solution, containing 100 nmol of reducing sugars, was added 90 μL of ethanol containing 1~2 % acetic acid and 1% sodium cyanoborohybride followed, with mixing, by 10 μL of a specific concentration of benzoyl hydrazine in 75% ethanol, such that the range of molar ratios of saccharide to benzoyl hydrazine was between 1:2.5 to 1:20 for

sugar standard and 1:200 for hydrolysis sample of glycoproteins. The mixture was heated at 60 °C for 300 min. in a water bath and then cooled to room temperature. The derivatized sugars were directly injected into the capillary for separation.

Hydrolysis of Glycoprotein

According to the method described previously,[14] acid hydrolysis of calf serum fetuin was carried out as follows. Samples were dissolved with 200 μL of water in screw-cap Teflon tubes and 200 μL of 8 M TFA was added. The samples were hydrolyzed in a boiling-water bath for 6 h. The tubes were cooled, and the samples were dried by nitrogen, dissolved in ethanol, and subjected to derivatization as described above.

RESULTS AND DISCUSSION

Optimization of Derivatization Conditions

The reaction of hydrazine with aldehyde groups is a well known, specific acid-catalyzed nucleophilic addition,[15] which yields hydrazone products. When the reaction is conducted in the presence of a reducing agent, sodium cyanoborohydride ($NaBH_3CN$), the hydrazone amine was alkylated as shown in Figure 1. To achieve optimum detection sensitivity and reproducibility, it was necessary to optimize pre-column derivatization conditions with respect to acid concentration, reaction time and the molar ratios of the hydrazine to sugar. The three most common monosaccharides (mannose, glucose and galactose) were examined for determination of the optimum conditions. Figure 2 illustrates the relationship between the peak areas of the three sugar derivatives and the concentration of acetic acid over the range 0.1-10 % (v/v). It shows that the peak areas increased markedly at acid concentrations greater than 2.5%. However, the mannose derivative showed a decreased peak area at high acid concentration (>5%, v/v). In addition, much higher concentration of acid resulted in alteration of carrier pH, which destroyed the peak reproducibility. Therefore, the acetic concentration was fixed, usually, at 1~2% (v/v).

To determine the proper ratio of benzoyl hydrazine reagent to sugar, the molar ratio of benzoyl hydrazine to sugar was varied from 2.5:1 to 20:1 at 3h, 60 °C. As demonstrated in Figure 3, onsets of curvatures were observed.

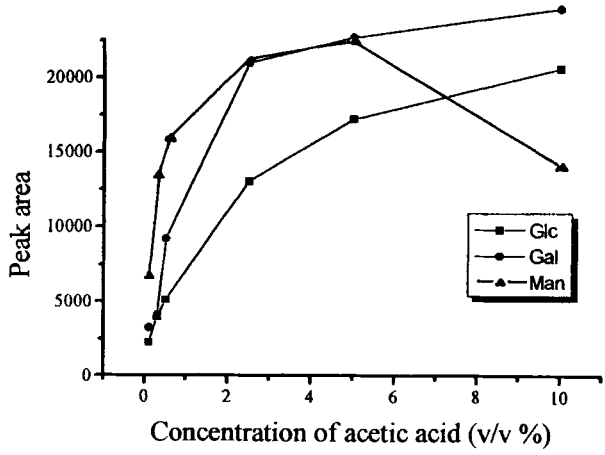

Figure 2. Effect of acetic acid concentration on the peak areas of precolumn sugar derivatives. Derivatization performed at 50 °C for 3 h. Reaction products electrophoretic conditions: applied voltage, 16 kv; capillary, 44 cm total length, 36 cm to the detector, 50 μm i.d.; buffer, 150 mM borate, pH10.5; ambient temperature, 30 °C.

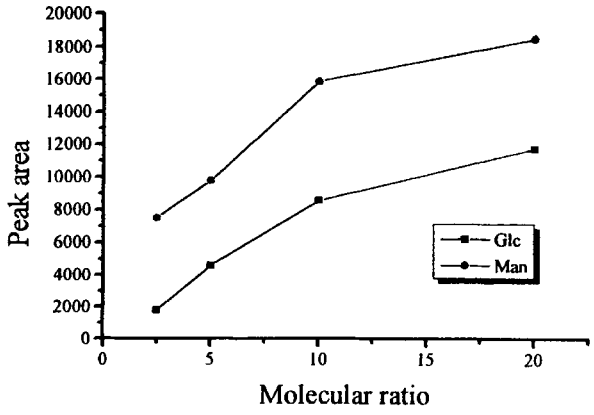

Figure 3. Effect of molecular ratio of saccharide to benzoyl hydrazine on derivatization reaction at 60 °C for 3 h. Electrophoretic conditions as in Figure 2.

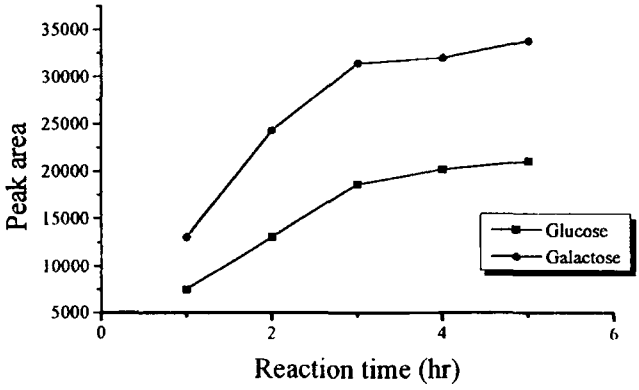

Figure 4. Effect of reaction time on precolumn derivatization reaction at 60 °C, Electrophoretic conditions as in figure 2.

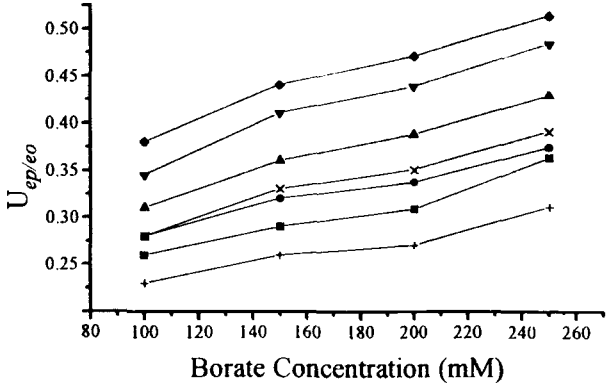

Figure 5. Plots of relative mobility *vs.* concentration of borate buffer at pH10.2. Conditions: fused silica capillary, 70 cm × 50 μm i.d.; voltage, 18 kV; detection wavelength, 220 nm; ambient temperature, 25°C. ■ Rhamnose, ● Xylose, Δ Glucose, ▽ Fucose, ◇Galactose, + N-acetylgalactosamine, × Ribose.

Although the benzoyl hydrazine was eluted at the electroosmotic flow without interfering with the sugar derivative separation, a large amount of excess reagent resulted in current waving, which decreases the experimental reproducibility. Thus, the optimum molecular ratio was usually twenty-fold.

The optimum temperature for hydrazone formation was found to be 60 °C; at lower temperatures, the reaction was slow while, at higher temperature, it was very difficult to maintain a constant acid concentration. The reaction time was varied to study its effects on reaction efficiency at 60 °C, acetic concentration 1.5 % (v/v). Here again, convex curves were observed (Figure 4). This result is different from that with Dns-hydrazine, used for labeling sugars, where the derivatization recovery decreased with extended the reaction times after reaching the maximum yield.[16] In the interest of time economy, three hours was adopted. At this reaction time, the relative peak area was about 91% of that at 5 h for all these carbohydrate derivatives.

Optimization of the Electrophoretic Analysis Condition

Precision of relative mobility

Separation in CZE is achieved via the distinct migration velocities of analytes under the influence of an electric field. An analyte is typically identified by its migration time (t_R) in the electropherogram or electrophoretic mobility (μ_{ep}). They are both dependent upon electrophoretic conditions with respect to applied voltage, capillary length and column temperature. Therefore, the migration time precision and reproducibility are usually poor.[17] In our experiment, a relative mobility parameter ($\mu_{ep/eo}$) was adopted to describe the analyte migration, as defined below:

$$\mu_{ep/eo} = \frac{\mu_{ep}}{\mu_{eo}} \tag{1}$$

where μ_{ep} is the electrophoretic mobility of the analyte, μ_{eo} is electroosmotic mobility of the carrying solution. When contributions from the relaxation effect are neglected, they both can be expressed by following equation (17):

$$\mu_{ep} = \frac{2\varepsilon}{3\eta}\zeta_a f(\kappa a) \tag{2}$$

$$\mu_{eo} = \frac{\varepsilon}{\eta}\zeta_c \tag{3}$$

where ε is the permitivity of carrying solution, η the viscosity of the carrying solution, ζ_a the potential of the analyte, k the thickness of diffusion layer, a is

the "radius" of the analyte, f(κa) is a function dependent upon the shape and κa of the analyte in the buffer and ζ_c is the Zetta potential between the capillary and carrying solution.

Combination of eqn. (1), (2), and (3) leads to an expression for $\mu_{ep/eo}$:

$$\mu_{ep/eo} = \frac{2\zeta_a}{3\zeta_c} f(\kappa a) \tag{4}$$

From equation (4), it can be seen that the relative mobility is dependent upon ζ_a in a giving buffer, but not ε and η. Therefore, the variation in temperature and the accidental error was decreased. In our study, the electrophoretic mobility of the analyte and electroosmotic mobility of the carrying solution can be calculated by following equations, respectively.

$$\mu_{ep} = \frac{lL}{V}\left(\frac{1}{t_R} - \frac{1}{t_0}\right) \tag{5}$$

$$\mu_{eo} = \frac{lL}{V}\frac{1}{t_0} \tag{6}$$

where l is the distance between the inlet of the capillary tube and the detector, L the total length of the capillary tube, t_0 the retention time of the neutral marker, t_R the migration time of the analyte and V is the applied voltage.

Thus, the relative mobility of the analyte can be determined by combining equations (5) and (6) to give:

$$\mu_{ep/eo} = \frac{t_0 - t_R}{t_R} \tag{7}$$

From equation (7), it can be seen that the relative mobility of analyte is independent of the length of capillary and the applied voltage, which the variation in the set of experimental conditions was decreased. Therefore, quantitative precision and good reproducibility can be obtained. A reproducibility comparison of t_R, μ_{ep} and $\mu_{ep/eo}$ obtained in the same capillary is shown in Table 1.

Table 1

Comparison of Precision of t_R, μ_{ep} and $\mu_{ep/eo}$

	----- R.S.D. % (n = 6) -----		
Sugars	t_R	μ_{ep}	$\mu_{ep/eo}$
Rhamnose	0.98	0.68	0.17
Xylose	1.0	0.68	0.23
Glucose	0.96	0.84	0.28
Fucose	1.1	0.63	0.30
Galactose	1.1	0.70	0.22

Conditions: applied voltage, 16.0 kv; capillary length, 36 cm to the detector; i.d., 50 μm; buffer, 200mM borate, pH 10.8; ambient temperature, 30°C.

As shown in Table 1, the precision of each of the parameters, as indicated by RSD's is impressive. However, the precision of $\mu_{ep/eo}$ is clearly superior to that of t_R and μ_{ep}.. An explanation for this might be the thermal effect (Joule heat) in the experimental conditions. Both t_R and μ_{ep} are rather temperature-sensitive, but not $\mu_{ep/eo}$., In the following experiments, the relative mobility was used to specify the analytes.

Retention behavior

Since monosaccharides are usually neutral, CZE is not directly applicable. Using borate as the buffer, they can be converted to anionic borate complexes by complexation of hydroxyl groups with borate ion.[5] Solute migration in this investigation is dependent on a number of factors, the most important of which is probably the anionic borate complex formation. To achieve optimum separation, it was necessary to optimize complexation conditions with respect to borate concentration, pH and column temperature.

Effect of concentration of the buffer solution

The dependence of relative mobility of sugar derivatives on borate concentration was examined at pH 10.2 and shown in Figure 5. It is observed that increasing the borate concentration resulted in an increase of the relative

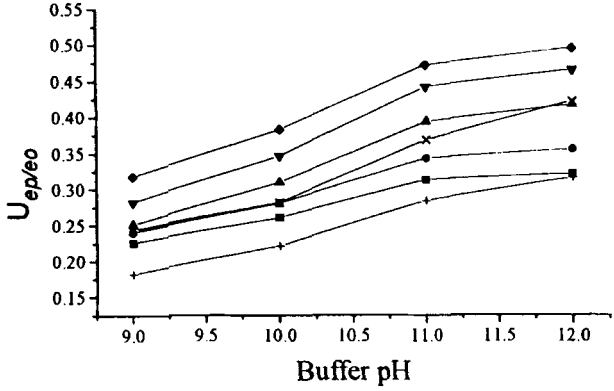

Figure 6. Dependence of the derivative relative mobility on pH with 150mM borate as carrying electrolyte. Conditions: applied voltage, 18 kv; capillary, 70cm total length, 62cm to the detector, 50 μm i.d., ambient temperature, 30°C. ■ Rhamnose, ● Xylose, ▲ Glucose, ▼ Fucose, ◆ Galactose, + N-acetylgalactosamine, × Ribose.

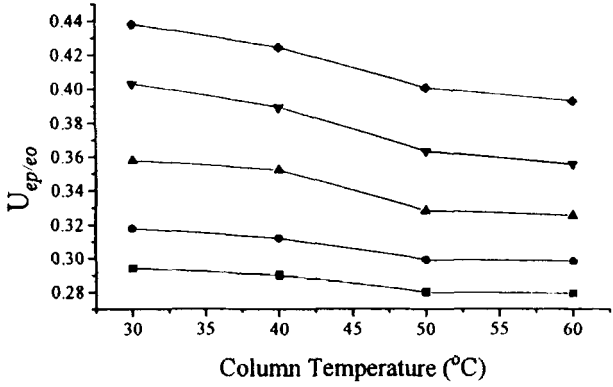

Figure 7. Effect of column terperature on the derivative relative mobility. Conditions: applied voltage, 18 kv; capillary, 70cm total length, 62cm to the detector, 50 μm i.d.., buffer, 150mM borate, pH 10.2; ambient temperature, 30°C. ■ Rhamnose, ● Xylose, ▲ Glucose, ▼ Fucose, ◆ Galactose.

mobility. Increasing the concentration of carrying electrolyte decreases the thickness of the diffusion layer, consequently decreasing the Zetta potential (ζ_c), thus increasing the relative electrophoretic mobility according to Equation 4.

The plot shows that high borate concentration provides optimum selectivity by magnifying small steric differences between closely related isomers. However, satisfactory separation of sugars could not be achieved by varying the borate concentration alone, since baseline noise became significant above 250mM.[5]

Effect of pH of the buffer solution

Formation of a borate complex is facilitated with alkali. Figure 6 shows the effects of buffer pH on the relative mobilities of sugars. The general trend observed is that as the pH increased, the relative mobility increased. This can be attributed to the increase of potential of the analyte (ζ_a). Higher pH values benefit formation of borate-sugar complexes, and result in the analyte potential increase.

Effect of operating temperature on the separation

The effects of varying column temperature on the separation of the five sugars is depicted in Figure 7. It was observed that, in general, as the separation column temperature increased, the relative migration mobility of sugars decreased. This can be attributed to an increase in the Zeta potential. When temperature increased, the diffusion coefficient D increased and, hence, the Zeta potential increased.

The plot shows that slightly lower temperature provides the optimum selectivity. This result is contrary to the result that high temperature gives better selectivity with underivatized monosaccharides.[18]

Figure 8 depicts the electropherogram of the reaction mixture of 9 reducing monosaccharides, obtained under optimized conditions. Separation was completed in about 30 min. and large, high resolution, numbers of theoretical plates were readily achieved for monosaccharides. The values ranged from 100,000 to 400,000 theoretical plates per meter.

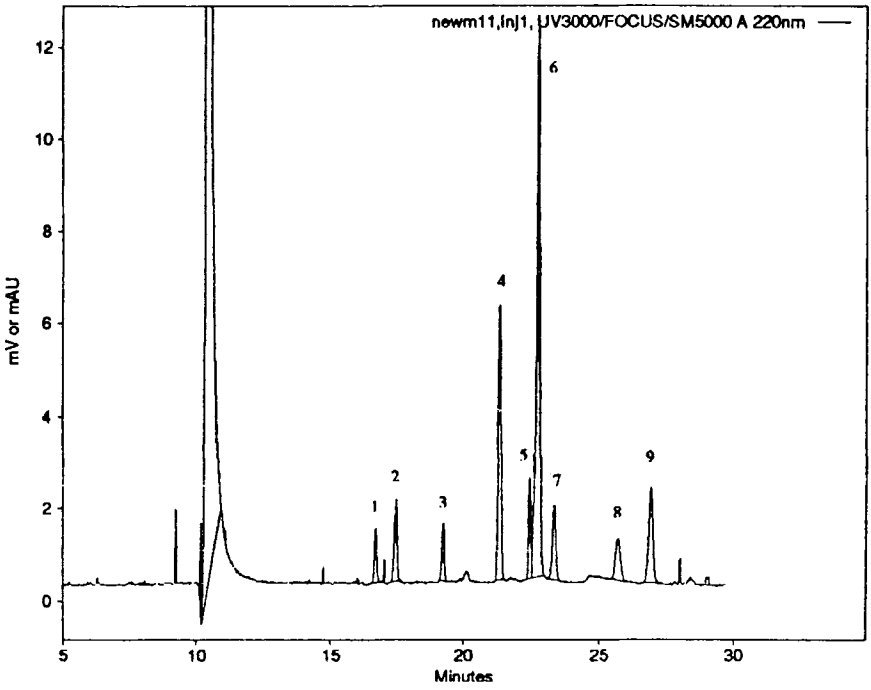

Figure 8. Electroopherogram of 9 standard reducing monosaccharide derivatives. Conditions: injection, vaccum 8 sec.; temperature, 30 °C; capillary length 70 cm (62 cm to detector); i.d. 50 μm; applied voltage, 20 kv; buffer, 200 mM borate, pH10.8; detection, 220 nm. 1- N-acetylgalactosamine, 2- Rhamnose, 3-Lyxose, 4-Xylose, 5-Manose, 6-Glucose, 7-Arabose, 8-Fucose, 9-Galactose.

Reproducibility of Quantitative Analysis

Sensitivity and linearity of response

The sugar derivatives possess intense ultraviolet absorption. The optimum wavelength is 220 nm. The detection limits of sugars were determined with a signal-to-noise ratio of 3, as shown in Table 2. The low detection limit is attributed to the high selectivity and low nanoliter sample injection volumes. The linearity of the detector response was investigated by injection of progressively diluted samples of sugar derivatives.

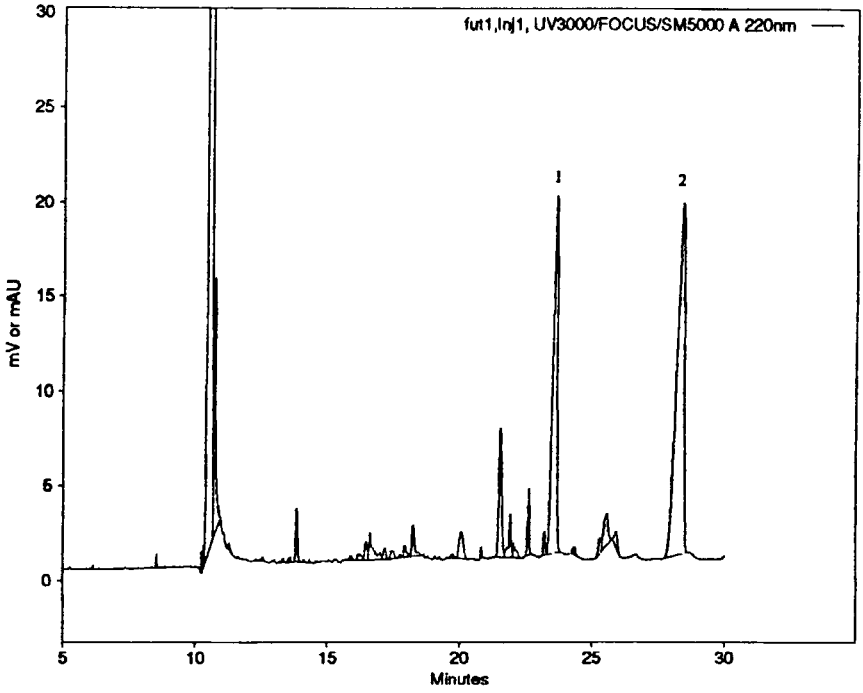

Figure 9. Analysis of the constituent monosaccharides of calf fetuin by CZE. The analysis condition as in Fig 8. 1 - Manose, 2 - Galactose.

Using β-indoleacetic acid as the internal standard, the response for the standard monosaccharides concentration to the relative peak area was linear over the range 10-700 ppm with satisfactory correlation coeffiecy R^2 =0.998 and determination was reproducible.

Analysis of the Monosaccharide Components of Glycoproteins

The well characterized glycoprotein fetuin was hydrolyzed and the released sugars were derivatized with benzoyl-hydrazine to test the accuracy and the sensitivity of the analytical method. Figure 9 shows the electropherogram for a hydrolysate of fetuin in 4M THF.

The value obtained for the molar proportion, liberated by heating for 6 h. in 4M trifluoroacetic acid at 100 °C, was mannose:galactose, 0.64:1, which is in excellent agreement with the previously reported values (18,19), demonstrating the reliability of this method.

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