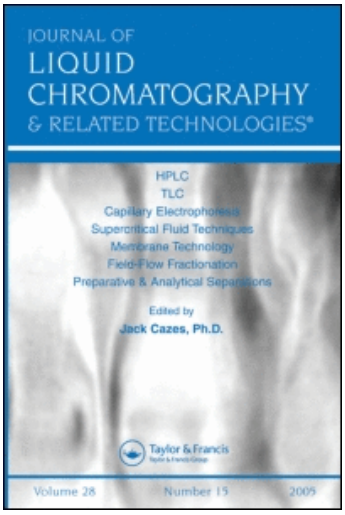

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High-Performance Liquid Chromatographic Assay of D-Glucose in Erythrocytes

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY OF D-GLUCOSE IN ERYTHROCYTES

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ABSTRACT

A procedure is presented after several attempts with different modes of chromatography for measuring high concentrations of d-glucose in erythrocytes. The procedure utilizes rapid deproteinization of hemolysate by mixing with acetonitrile. The supernatant is analyzed by strong cation exchange chromatography, using an Organic Analysis Column. Separation conditions are: eluent = 0.01 N H_2SO_4, flow rate = 0.6 ml/min, detection = 195nm at 0.05 AUFS, sample size = 20 µl and temperature = ambient. The coefficients of variation for 5 mg/ml samples were (within-run) 6.7%, and (day-to-day) 7.1%. This study shows the presence of a high concentration (1900 mg/dl) of d-glucose within the erythrocytes as a result of a high external d-glucose concentration (2000 mg/dl) in plasma, and suggests that d-glucose is rapidly transported into the cell.

INTRODUCTION

An increase in electronic mean corpuscular volume (MCV) induced by marked hyperglycemia was recently reported by Morse, et al. (1) A patient showed a high glucose serum concentration of 2250 mg/dl (22.5 mg/ml) with MCV of 128 μ^3. Postulations

included the displacement of Na^+ and Cl^- ions as a result of the association of glucose with the cell membrane, or the volume change within the cell membrane caused by glucose induced osmotic effect. The same report noted that sucrose did not induce any volume changes. In order to determine the validity of the latter postulation, intra-erythrocytic monosaccharide concentration measurements might be useful.

Measurement of carbohydrates in blood may be accomplished by a wide variety of methods. These methods include chemical testing (2) (3), enzymatic methods (4) (5), thin-layer chromatography (6) (7) (8), and gas-liquid chromatography (9) (10). More recently, high-performance liquid chromatographic (HPLC) columns have been available to measure carbohydrates (11) (12) (13). For our investigation, it would be desirable to measure high concentration of glucose (20 mg/ml) in erythrocyte with minimum sample preparations. For these purposes, GLC assays which require extensive sample preparation were not attempted while HPLC was chosen for this study so that a simple deproteinization step was used for sample clean-up, followed by HPLC separation of d-glucose from endogenous interference peaks.

Separation of carbohydrate by HPLC is based upon three different kinds of mechanism as recently reviewed by Schwarzenbach (14). These are ionic exchange chromatography, partition chromatography on ion exchange resin and partition chromatography on chemically bonded phases. The present study outlines several

attempts to measure endogenous d-glucose in the red blood cell as well as exogenous d-glucose using one of the above three mechanisms.

EXPERIMENTAL

Reagents

D-glucose, 'Baker-Analyzed' reagent was purchased from J. T. Baker Chemical Co., (Phillipsburg, N. J. 08865). Acetonitrile, UV grade, was obtained from Burdick and Jackson Laboratories (Muskegon, Mich. 49442). Water was double distilled. Tetra-ethylene-pentyl amine (TEPA) was purchased from Aldrich Chemical Co., (Milwaukee, Wis. 53233).

High-Performance Liquid Chromatographs

Several HPLC were used in this study. Preliminary studies with a Radial-pak B column, consisting of silica gel, were performed by using a M6000A pump with a Model 450 variable wavelength detector from Waters Assoc., (Milford, Mass.). The separation was carried out by using a mixture of acetonitrile/water 75:25, TEPA (0.01%) as eluent at a flow rate of 2 ml/min, and at room temperature. The detector was set at 195 nm with an attenuation of 0.02 AUFS. The other preliminary studies with a carbohydrate column from Bio-Rad Laboratories, (Richmond, Calif.), were carried out by using a Model 5000 HPLC from Varian Associates, (Walnut Creek, Calif.), with a Rheodyne 7125 injector equipped with a 20 µl loop. The detector was a Vari-Chrom variable wavelength detector from

Varian Associates, set at 195 nm, 0.1 or 0.05 AUFS attenuation. The eluent was water. The separation was carried out at a flow rate of 0.3 ml/min and at a temperature of 85°C.

Analyses of normal and high concentration erythrocytic d-glucose were performed by using a Laboratory Data Control Constametric III Pump with a Spectro-Monitor III variable wavelength detector from Laboratory Data Control (Riveria Beach, Florida). The injector was a Rheodyne 7125 injector with a 100 µl loop. The column was an Organic-acid analysis column from Bio-Rad Laboratory (Richmond, Calif.). Column packing was made from strong cation ion exchange resin with proton as the counter ion. The guard column was a size-exclusion HPX-87 cartridge from Bio-Rad. The eluent was 0.005N H_2SO_4. Analyses were carried out at a flow rate of 0.3 ml/min at room temperature. Detector was set at 195 nm and at an attenuation of 0.01 AUFS. The established procedures, including calibration, precision and high d-glucose concentration studies, were carried out by using a Model 5000 HPLC from Varian Associates as described previously. The eluent was 0.01N H_2SO_4. Flow rate was 0.6 ml/min and the separation temperature was ambient.

Sample Preparation

The preliminary studies using the silica-gel, carbohydrate and organic acid analysis columns involved protein precipitation of normal and high d-glucose concentration samples by mixing with

acetonitrile (1:1 or 1:2) and spun for ten minutes. Aliquots of the clear supernatant were injected into the HPLC.

For the calibration and precision studies, the following protocol was followed. EDTA blood was spun at 4000 revolutions per minute for 5 minutes. Plasma and upper layer of cells were removed. One ml of packed red blood cells was transferred to a marked test tube, and 9 ml of distilled water was added. To this hemolysate preparation, d-glucose was added to yield a concentration of 20 mg/ml. By serial dilution with normal hemolysate, the following d-glucose hemolysate calibration samples were prepared: 0, 2, 8, 10 and 15 mg/ml. Five 5 mg/ml d-glucose hemolysate replicates were also included for the precision study. Preparation of the high external d-glucose concentration hemolysate involved incubation of d-glucose/whole blood (20mg/ml) at room temperature for one hour. Then, the hemolysate was prepared from this sample as described previously.

From the above hemolysate preparations, 200 µl was taken from each sample and mixed with 400 µl of acetonitrile. These mixtures were vortexed, followed by spinning at 2000 RPM for 10 minutes. Twenty µl of the clear supernatant was injected for HPLC analysis. Peak heights were plotted against d-glucose hemolysate concentrations. Linear regression analysis was carried out to determine the correlation coefficient, slope and intercept. From the peak heights of the precision studies (5mg/ml) samples

and the high concentration hemolysate, the d-glucose hemolysate concentrations were estimated from these plots.

Results and Discussions

Measurement of d-glucose in erythrocytes consisted of two parts: the preparation of the hemolysates from the red blood cells, and the analyses of the hemolysates by HPLC. In attempting to optimize the hemolysate preparation, the procedure was kept as simple as possible so that any enzymatic activity would be mini-

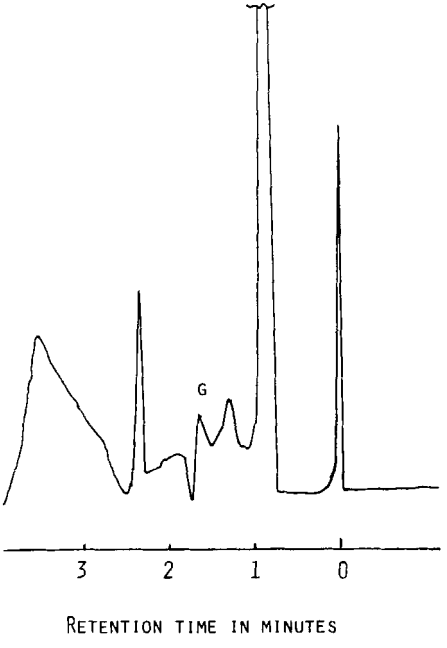


Figure 1: Chromatogram of a normal hemolysate sample. Column: RP-B, (Silica gel) Waters Associates, mobile phase: acetonitrile/water (75:25) with 0.01% TEPA, flow rate 2 ml/min, detection at 195 nm, 0.02 AUFS, injection volume 40 µl, and G = d-glucose.

mized. This was accomplished by protein precipitation by acetonitrile. Figure 1 shows the chromatogram of a normal hemolysate. The separation was carried out by using a Radial-Pak B column consisting of silica-gel packing, and the eluent was acetonitrile/water (75:25), TEPA (0.01%). D-glucose eluted at 1.5 minutes with retention volume (V_R) of 3 ml. The normal hemolysate chromatogram showed a peak with the same retention time as glucose. Endogenous peaks eluted before and after this glucose peak. Due to the small V_R = 3 ml and the negative peak following the d-glucose peak which resulted in inaccurate quantitation, this assay was not further developed. In addition, the many advantages of ion exchange resin over silica-gel packing, such as long column life and wide useable pH range (a.) 0 to 14 for cation exchange and (b.) 0 to 12 for anion exchange, led the investigation to ion exchange mode for assaying erythrocytic d-glucose.

Figures 2A and 2B show the chromatograms of a normal and high concentration (2 mg/ml) hemolysates. The column was a strong cation exchange column with Mg^{+2} as the counter ion. Mobile phase was water. Separation was carried out at 85°C and the detector was set at 195 nm. D-glucose eluted on the shoulder of an endogenous peak with retention time of 4.6 minutes, and V_R = 1.4 ml. The incomplete separation and small V_R indicated that these chromatographic conditions were not ideal to identify d-glucose in hemolysate. Thus, another form of ion exchange column packing was used.

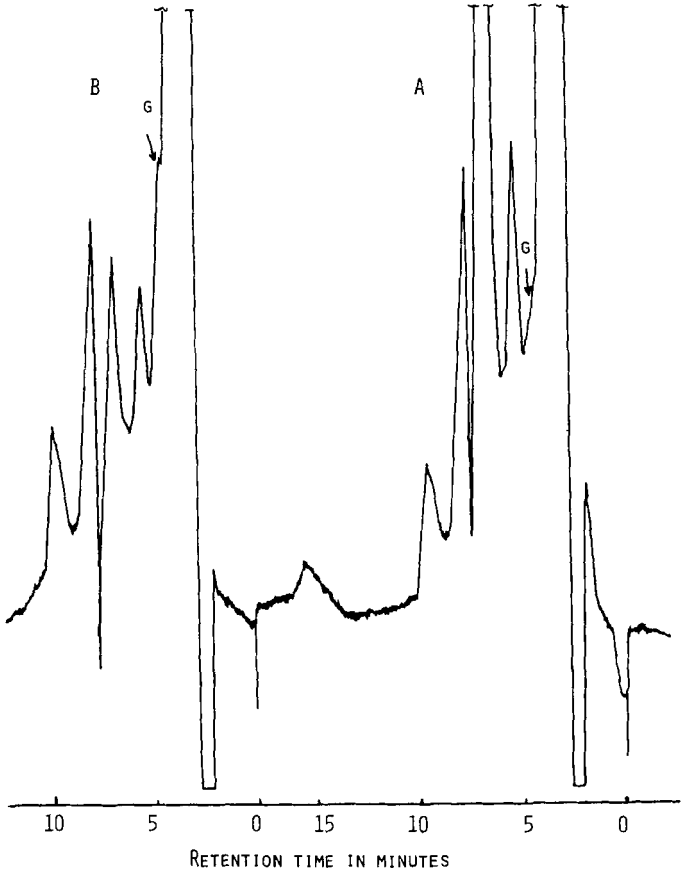
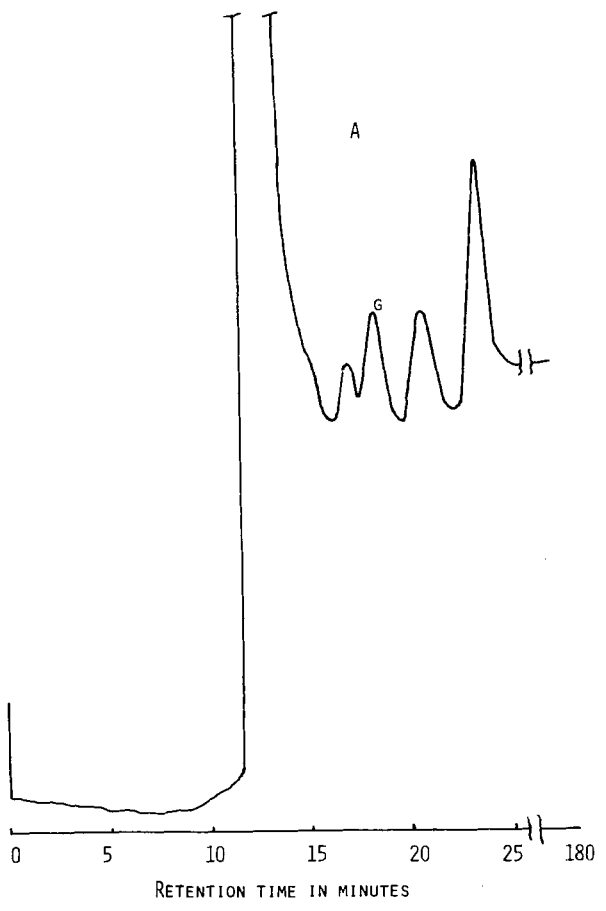


Figure 2A: and 2B: CHromatograms of a normal and high d-glucose hemolysate sample. Carbohydrate column (Bio-Rad Laboratories), mobile phase: water, flow rate 0.3 ml/min, detection at 195 nm, at 0.02 AUFS, temperature = 85°C, injection volume = 4 μl and G = d-glucose.

The column of choice was a newly available organic acid column with a strong cation exchange packing and a counter ion of proton. Figures 3A and 3B show the chromatograms of normal and high concentration (3 mg/ml above normal) hemolysate prepared by mixing plain or glucose-spiked red cells with an equal volume of acetoni-



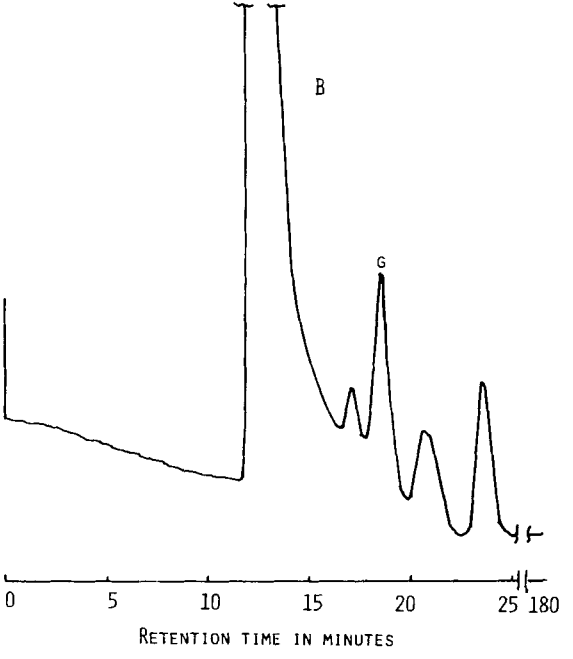


Figure 3A:
and 3B:

Chromatograms of a normal and high d-glucose hemolysate sample. Organic Acid Analysis Column (Bio-Rad Laboratories), mobile phase: 0.005 N H_2SO_4, flow rate 0.3 ml/min, detection at 195 nm 0.01 AUFS, injection volume 5 µl, and G = d-glucose.

trile for protein precipitation. The eluent was 0.005N H_2SO_4 at a flow rate of 0.3 ml/min with detection at 195 nm. The normal hemolysate showed the presence of a glucose peak at 19 minutes, V_R = 5.7 ml, while the high concentration hemolysate showed a higher peak at the same retention time. These two chromatograms suggested the presence of d-glucose in normal hemolysate. The glucose peak was followed by peaks eluting with retention time up to two and a half to three hours. These peaks were probably due to

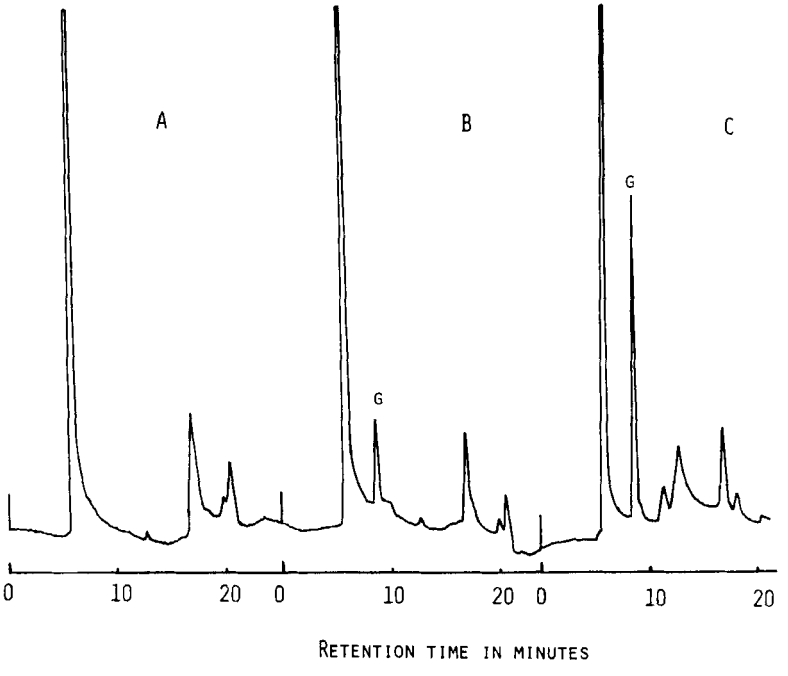


Figure 4A: Chromatograms of a normal, 2 mg/ml and 10 mg/ml d-
4B: glucose hemolysate sample. Organic Acid Analysis
and 4C: Column, (Bio-Rad Laboratories) mobile phase: 0.01 N H_2SO_4, flow rate 0.6 ml/min, detection at 195 nm, 0.05 AUFS, injection volume 20 µl, and G = d-glucose.

endogenous substances such as amino acids and nucleosides. Since the chromatographic separation time was lengthy before the column was completely regenerated for the next sample analysis, this method was not used for multiple samples analysis needed to assay d-glucose concentration in erythrocytes. Thus, chromatographic conditions were optimized to analyze the hemolysates within 30 minutes.

Figure 4 shows the chromatograms of normal hemolysate, 2 mg/ml and 10 mg/ml spiked hemolysates. The hemolysate samples were prepared as outlined in the experimental section. Figure 4A shows the chromatogram of a normal sample. D-glucose was not found due to the dilution factor of thirty fold . Normal glucose concentration in whole blood is 65-110 mg/dl, i.e. 0.7 to 1.1 mg/ml. The maximum amount of d-glucose introduced for analysis was 0.6 μg, not detectable with the present detector and its appropriate attenuation.

Figures 4B and 4C show the chromatograms of hemolysate samples with 2 mg and 10 mg per ml of hemolysates respectively. Retention time of d-glucose was 9 minutes, with V_R = 5.4 ml. The d-glucose peak was well resolved from the endogenous peak at 6 minutes. Multiple peaks eluted after d-glucose. Analysis time for each sample was between 20 to 30 minutes.

Figure 5 shows that the plot of peak height of d-glucose versus d-glucose/hemolysate concentration was linear with correlation coefficient = 0.9977, slope = 0.878 and intercept = -0.020.

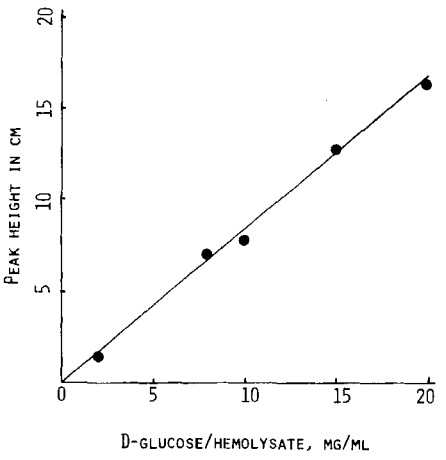


Figure 5: Calibration curve for d-glucose/hemolysate concentration versus d-glucose peak heights.

Precision studies showed that the within-run coefficient of variation (CV) for the 5 mg/ml samples was 6.7% (mean = 4.91, S.D. = 0.33 and number of samples = 5), and the day-to-day CV was 7.1% (mean = 5.04, S.D. = 0.36 and number of samples = 40). This assay was used to measure erythrocytic d-glucose with high d-glucose whole blood concentration. D-glucose was added to the whole blood so that the concentration was 2000 mg/dl above normal concentration. Figure 6 shows the chromatogram of the hemolysate of such a sample. D-glucose peak height was 1.6 cm, corresponding to 1.9 mg of d-glucose per ml of hemolysate, prepared by ten fold dilution of erythrocytes. This was equivalent to 19 mg/ml, or 1900 mg/dl of erythrocytes. This d-glucose concentration probably represented d-glucose inside the red blood cells as well as the d-glucose in the plasma trapping. Plasma trapping occurred as a

result of the separation of plasma from red blood cells by centrifugation. The amount of plasma trapped between the red blood cells was estimated to be about three to five percent.Thus the majority of the 1900 mg/dl d-glucose detected was due to the erythrocytic d-glucose.

The present study shows a method of simple measurement of d-glucose in hemolysate using a single deproteinization step, followed by strong cation exchange HPLC using an organic acid analysis column. The experience from three months of precision studies indicated that this column was stable. The absence of d-glucose response of normal hemolysate was due to the dilution

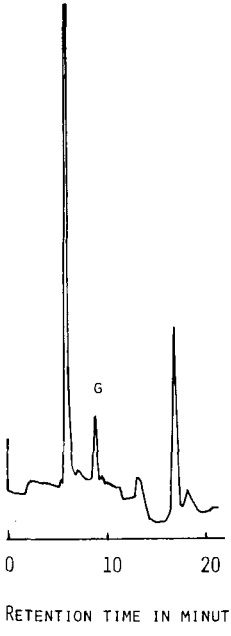


Figure 6: Chromatogram of a high external d-glucose hemolysate sample. Conditions same as in figure 4A.

factor. The assay utilized the UV detection of carbohydrate at 195 nm instead of a refractive index detector. This assay may be developed to measure metabolite of d-glucose such as sorbitol. The present study represents a new liquid chromatographic method to measure directly high d-glucose concentration inside the red blood cells.

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