Comparison of micro-enzymatic and high performance anion exchange chromatography methods for the analysis of glucose in human serum

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Dedicated to Professor Dr Rosa M. de Lederkremer

Abstract

In this study we have performed a comparison of the most used spectroscopic glucose-oxidase assay for routine clinical monitoring of serum glucose, with the high-performance anion-exchange chromatographic method using pulsed amperometric detection (HPAEC-PAD). The linearity, repeatability and internal reproducibility of the chromatographic method were determined. The correlation by linear regression and difference plot was analyzed. It is known that the enzymatic method leads to unreliable results when glucose must be determined in samples containing high concentration of bilirubin or lipids. On the contrary, the accuracy of the HPAEC-PAD method with these complex matrix sera resulted similar to normal samples showing no interferences. Another advantage was the minimal amount of human serum needed: one microliter was largely sufficient to perform the whole analysis. This fact would make the HPAEC-PAD method applicable when samples of preterm infants or new born babies of low weight must be analyzed.

Keywords: Glucose determination, HPAEC-PAD, human serum analysis, glucose-oxidase method

Introduction

Glucose is the most important carbohydrate in the medical clinic. Depending on the analytical method used and because of the relatively large intra individual biological variability, glucose normal levels in sera are in the range of 70-110 mg/dl. Abnormal high levels of glucose are found more frequently in people with diabetes mellitus, pancreatitis, with liver or some

endocrine diseases. Lower than normal levels may indicate diseases such as hypopituitarism or hypothyroidism. Early diagnosis may prevent ketoacidosis and long term complications rising from hyperglucemia by means of an adequate treatment. The particular physiological and pathological conditions of each patient must be taken into account, as there are a lot of factors causing hyper or hypoglucemia.

Several colorimetric methods have been described for the assay of glucose in biological fluids. The first analysis for glucose in sera was based on the oxidation of glucose in the presence of Cu^{+2} to give Cu_2O detected by different methods (Foli-Wu, Somogyi-Nelson). Although these methods were widely used, nowadays they have become unsuitable because of their low specificity[1]. The reaction of glucose with o-toluidine or other aromatic amines at low pH has also been very popular. This method is accurate and precise. Nevertheless it carries as main disadvantage the toxicity of the reactives as well as a non-specific reaction with urea and other hexoses, specially mannose and galactose [2-3].

More recently, procedures for glucose analysis in sera involving enzymes, have been developed. Reactions with hexokinases and glucose-oxidase (GOD) have been automatized reaching high specificity and accuracy. In the first case, glucose reacts with ATP in the presence of hexokinase forming ADP and glucose-6-phosphate. In the presence of NAD the enzyme glucose-6-phosphate dehydrogenase, oxidases glucose-6-phosphate to 6-phosphogluconate. The increasing NADH concentration is directly proportional to the glucose concentration and can be measured at 340 nm [4-6].

On the other hand, the Trinder method is the most frequently method used [7-8]. It is based on two sequential enzymatic reactions, the first one involves the oxidation of glucose to gluconic acid and H_2O_2. As the enzyme is very specific for β-D-glucose, most preparations also contain mutarrotase to catalyze the conversion of α-D-glucose to the β-anomer. Then, the H_2O_2 produced is quantified by a chromogenic reaction with peroxidase (POD) and 4-aminophenazone (4-AP) developing a red dye that is measured at 505 nm. Other oxygen acceptors such as 3-metil-2-benzothiazolinone hydrazone, N,N-dimethylaniline may also be used. These glucose-oxidase methods have been adapted to a lot of automatized instruments including dry reactives in films and strips. However, these methods present as main disadvantage that sera with visible or intense haemolysis must be deproteinised and bilirubine above 200 mg/l, ascorbic acid above 75 mg/ml and uric acid above 200 mg/ml may also interfere. In addition the oxidation of several compounds present in sera by the hydrogen peroxide produced in the reaction may yield a negative deviation. On the other hand, several compounds can oxidase the dye indicator, giving positive deviations. It must be taken into account that results obtained with these methods depends on the specific reactives and instrument used, so each laboratory must fix their own reference range [9].

Traditional and historical methods, for carbohydrate determination by chromatography have been hampered by two factors: the lack of suitable high performance separation method and the inability to detect the carbohydrates at low levels[9]. The gas-liquid chromatography (GLC) methods require intensive sample preparation stages which involve solvent extraction and/or ion-

chromatography, followed by a derivatization stage to form volatile products prior to injection[10-15].

Although these methods have been shown to be precise, they are time consuming and unsuitable for the assay of large numbers of samples in routine clinical practice. High Performance Liquid Chromatography (HPLC) methods required post-column derivatization to provide a sensitive detection system and are, at present, almost neglected [15-17]. Huges and Johnson introduced pulse amperometric detection (PAD) and the triple pulse amperometry in 1981[18-19]. The weak acidic properties of carbohydrates allow ionization and chromatographic separation in alkaline solutions and potential separation by high anion-exchange chromatography (HPAEC)[20]. The combination of HPAEC with PAD allows a highly selective separation and detection of non-derivatized carbohydrates at low picomolar concentrations[21].

The aim of the present work was to compare the performance and suitability for routine clinical use of a very sensitive and simple method based on HPAEC-PAD, with an established technique, the enzymatic Trinder method, for glucose determination in serum. For this purpose we performed a statistical approach to asses the degree of agreement which includes the linearity and correlation between both methods. The applicability has been demonstrated by analysis of 24 relevant samples, an hemolysed sera, two highly lipemic sera and two samples containing high concentrations of bilirubine. The scope of the method is based on the lack of interference from most non-carbohydrate matrix components and the minimal amount of human serum needed, making this analysis minimal invasive. This fact would be very profitable, for example when samples of preterm infants or new born babies of very low weight, must be analyzed.

Results and Discussion

A mixture of the pure standards in water was injected. Both components were well resolved with short retention times, the 2-deoxy-D-glucose peak (tr = 6.66 min) and D- glucose peak (tr = 10.22 min) corresponds to 1.83 nmoles and 2.29 nmoles, respectively. A chromatogram of one serum sample is also shown (Fig. 1).

Concentration values were obtained by calculation of the peak area of glucose with respect to the peak area corresponding to a known amount of 2-deoxyglucose added to each sample (Internal standard method). Results were the average of three injections.

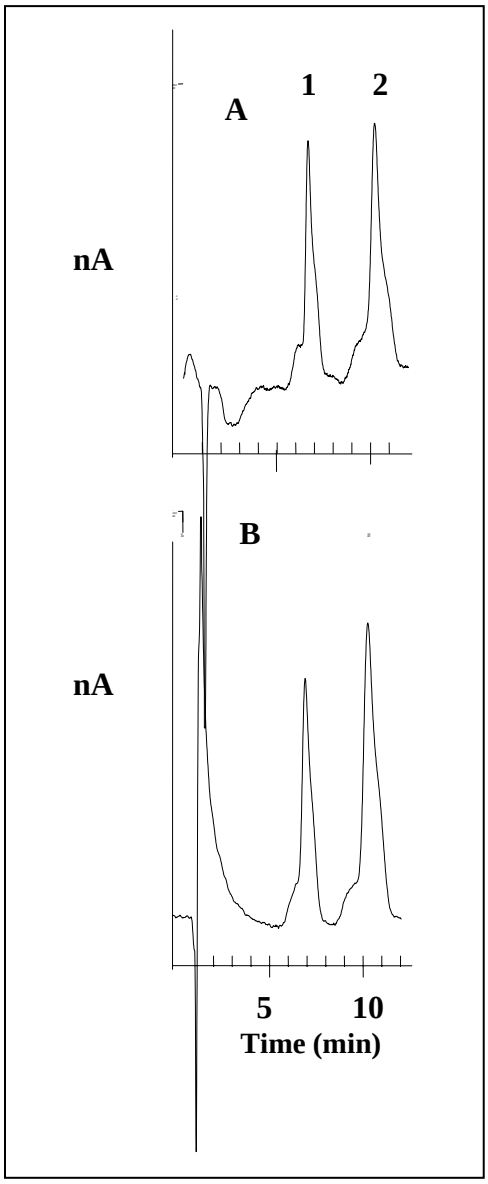


Figure 1. Representative chromatograms of **A:** pure standards of 2-deoxyglucose (1) and Glucose (2) in water **B:** serum sample, with 2-deoxy-D-Glc added as internal standard.

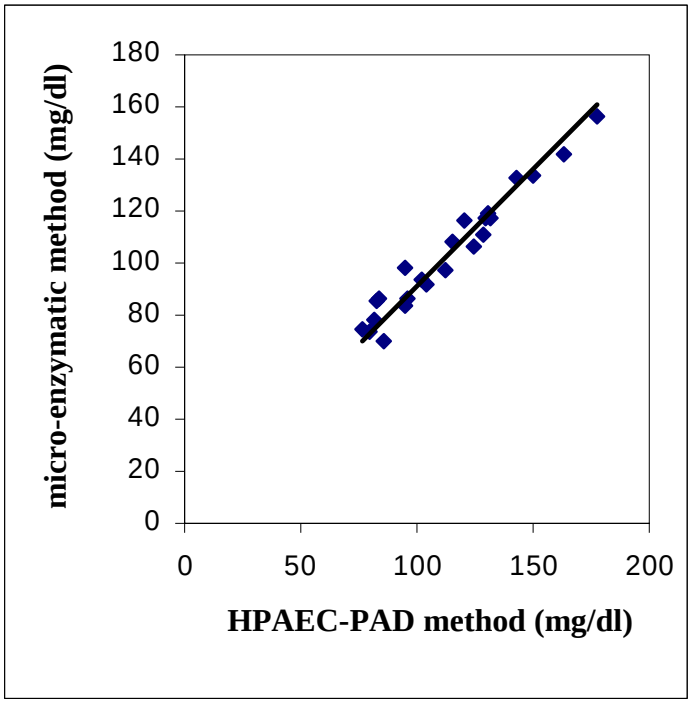
Linearity

The linearity on a six point calibration curve was checked ranging from 80 to 600 ng of glucose This range covers with the dilutions of sera used, concentrations between 24 to 200 mg/dl ($r = 0.995$). The limit of detection defined as the concentration corresponding to three times the level of baseline noise was 50 ng for glucose.

Measuring Agreement

Samples collected from normal subjects were analysed by the microenzymatic method and by HPAEC-PAD[23]. The data were assessed by using a scatter plot and difference plot as shown in Fig. 2 and Fig. 3.

Figure 2. Glucose determination with microenzymatic method and HPAEC-PAD method, with line equality.



Comparison by least squares regression shows good correlation between the methods (r = 0.970). However this type of analysis for good comparison is considered not appropiate, since a good linear correlation does not indicate that the two methods agree[23].

A plot of the difference between the two methods against the mean was considered more informative. As we do not know the true value, the mean of the two measurements was considered the best estimation. In this case the mean difference (microenzymatic – HPAEC-PAD) is 10.13 mg/dl and standard deviation of the differences (SD) is 7.24 mg/dl. The value of the mean difference shows that the microenzymatic method as it is a spectroscopic method, presents higher values than the chromatographic method. The limits of agreement (mean $\pm$ 2SD of the difference) are - 4.36 and 24.62 mg/dl and as shown in figure 3, all the measurements are distributed within these values. These results indicate that, although the two methods may not be interchanged, with the determination of new reference ranges, the chromatographic method may be useful.

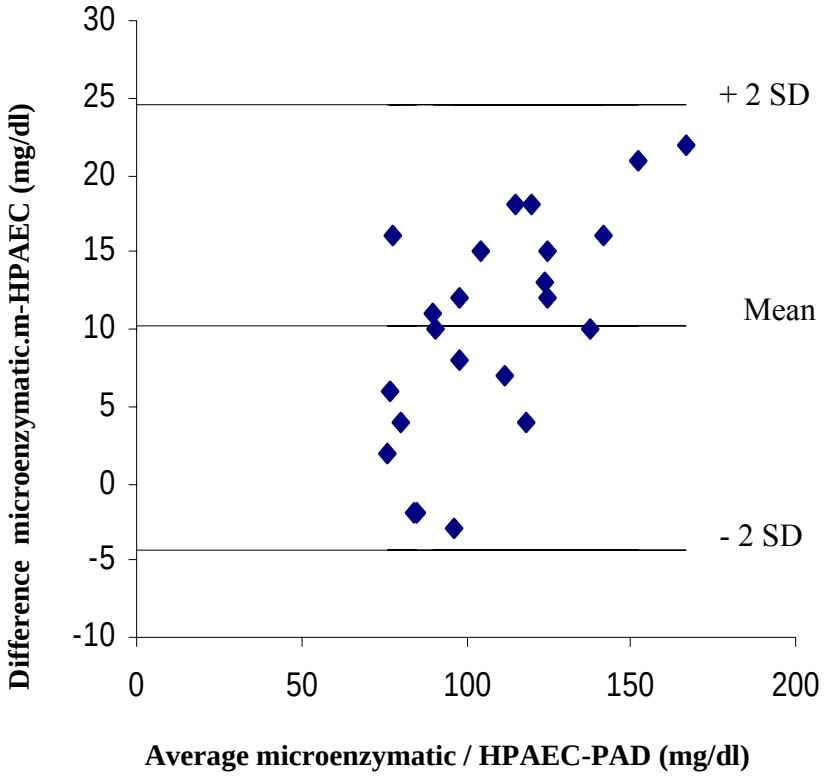


Figure 3. Difference against mean values for glucose determination.

Repeatability and internal reproducibility

The method was validated with respect to the repeatability (within-day precision) and internal reproducibility (day- to- day precision). Table 1 shows the repeatability calculated on six repeated injections for two different sera samples.

Table 1. Repeatability: within-day precision

	Glucose	
Mean (mg/dl)	97	57
SD (mg/dl)	1.92	1.00
RSD (%)	1.97	1.75

Table 2 shows the internal reproducibility during the analysis of two different sera. The samples were analyzed in duplicates on 2 different days.

Table 2. Internal reproducibility: day to day precision

	Glucose	
Mean (mg/dl)	96	59
SD (mg/dl)	1.15	0.21
RSD (%)	1.20	0.35

Taking into account that glucose present in hamolysed sera, in the presence of high concentrations of bilirubin or in sera containing a high lipidic concentration are difficult to be determined by the microenzymatic method, we tested those sera by the HPAEC-PAD method. In all cases, no interferences in the normal sera pattern were observed. To determine the accuracy of the method, recovery was measured in three different samples by spiked analysis by adding a known amount of glucose. A normal sample (A), a sample containing a high concentration of bilirubine (B) and a hamolysed sera (H) were chosen. The accuracy was expressed as percentage error [(found concentration- spiked concentration)/spiked concentration] x 100(%) (Table 3).

Table 3. Accuracy

Sample	Relative error (%)
A	1.7
B	2.9
H	2.7

In conclusion, the linearity, repeatability and internal reproducibility of the HPAEC-PAD method to analyse glucose in human sera have been determined with satisfactory results. A comparison with the most popular microenzymatic method was performed, finding that despite a good agreement by linear regression was found, the assay methods did not demonstrate satisfactory agreement when using the difference plot. However, although these methods are not interchangeable, the chromatographic method will be useful if new reference ranges are fixed.

The accuracy of the HPAEC-PAD method of sera containing high concentrations of bilirubin or with lipemic sera resulted similar to normal sera showing that there was no interference with the matrix. Therefore, the chromatographic method would be suitable for the analysis of complex matrix samples as the microenzymatic method leads to unreliable results.

Moreover, as only one microliter of sera is largely sufficient for the whole chromatographic analysis, this method would be specially considered in the context of preterm infants or new born babies of low weight. In these cases the blood taken in a glass capillary would be largely enough for the whole determination.

Experimental Section

Materials. All reagents were analytical grade. Standards agents D-glucose and 2-deoxy-D-glucose were purchased from Sigma. Sodium hydroxide (50%) was purchased from Merck. Enzymatic glycemia AA, Wiener Lab, Cod. 1400106 was used.

Samples. Blood samples were obtained for glucose determination by venipuncture between August and November 2004 in a public hospital from the Gobierno de la Ciudad Autónoma de Buenos Aires, Argentina. The clinical conditions of patients were unknown. Samples were centrifuged within two hours after extraction and the supernatants were transferred to plastic vials to be processed up to four hours later. Samples that could not be analyzed in this period were stored at -20ºC until analyzed.

Enzymatic method. Glucose was quantitated at 37 °C by the Trinder method[8] at 505 nm.

Preparation of the reactive. A vial containing the enzymes was resuspended with an aliquot of buffer. The solution was mixed and transferred to the buffer container (250 ml) and mixed until complete dissolution. This solution is stable for 60 days at 2-10 º C. Final concentrations were: GOD $\geq$ 10 kU/l; POD $\geq$ 1 kU/l; 4-AP 0.5mM; 100mM phosphate buffer pH= 7; hydroxibenzoate 12 mM.

The final reactive solution may develop a slight pink colour. This solution must be discarded when the blank absorbance is > 0.250 O.D.

Instrument. Determinations were performed in an automatic analyzer Technicon RA-XT, Bayer S.A. Accuracy and precision were in accordance with internal (Qualitrol HS N Merck, Germany, Cod. 1.10932, lote 441) performed once a day and external (C.E.M.I.C.) controls twice a month. A 1: 100 sample: reactive relationship was used. A glucose standard (100 mg/dl) and a blank were performed in parallel once a day.

The reaction is lineal up to 450 mg/dl of glucose. Sera samples with higher concentrations were diluted with water and re-analyzed (manual dilution).

The variance changed from 1.26 and 1.05 % with glucose concentration (89 mg/dl and 180 mg/dl respectively).

High performance anion exchange chromatography method

Instrument. A Dionex DX-300 high-performance liquid chromatograph (Sunnyvale, CA, USA) equipped with a manual injector with a 20 μl fixed loop and a pulse amperometric detector fitted with a gold working electrode and solid-state silver reference electrode was used. Separation was achieved using Carbopack PA-10 guard (4 x 50 mm) and analytical (4 x 250 mm) columns, both purchased from Dionex (Sunnyvale, CA, USA).

The mobile phase consisted in 0.2M NaOH (sv A) and water (sv B). Prior to use, the mobile phase was degassed. An isocratic method with 12 % A and 88% B, flow rate 1 ml/min at room temperature was used. The column was conditioned with 100% sv A for ten minutes each 20

injections. Three injections of standard solution were run daily to check the resolution and the suitability of the chromatographic system.

Standard stock solutions were prepared by dissolving sugars (1 μg/μl) in water and stored at -20 ºC. Standard working solutions were obtained by making appropriate dilutions of the stock solutions with water. In general 3 μl taken to 100 μl.

Sera samples were diluted 1/60 prior to injection.

Calculations. Concentration values were obtained by measuring the peak area of glucose with respect to the peak area corresponding to a known amount of 2-deoxyglucose added to each sample (Internal standard method).

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