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Note

Quantitative method for the detection of glucose in body fluids by high-performance liquid chromatography with fluorescence detection

D.A.S. FINDEN, R.R. FYSH and P.C. WHITE*

The Metropolitan Police Forensic Science Laboratory, 109, Lambeth Road, London, SE1 7LP (U.K.)

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Diabetes mellitus is a metabolic disorder which occurs when glucose accumulates in the blood and exceeds the normal level of 0.65—1.2 mg ml^{-1} [1]. Oral therapy with hypoglycaemic drugs, dieting and injections of insulin are prescribed methods used to reduce and control the glucose levels.

A lowering of blood glucose levels to below normal (hypoglycaemia) can arise if diabetics suffer an overdose of insulin and/or fail to maintain carbohydrate levels via dietary control. Symptoms of hypoglycaemia include drowsiness, lack of judgement and self-control, and if subjects attempt to drive a vehicle whilst impaired they are liable to prosecution under the Road Traffic Act (1972). Apart from the requirement to measure glucose levels in these cases, Forensic Laboratories are requested to analyse post-mortem samples as an aid to the pathologist to determine if a cause of death is related to an insulin overdose or due to very high glucose levels (hyperglycaemia).

Since relatively few cases require quantitative analysis for glucose in this Laboratory it was uneconomic to purchase a commercial clinical glucose analyser. Enzyme assay kits, specifically those using hexokinase and glucose oxidase were designed mainly for use with plasma samples, and have proved to be too expensive due to the short shelf-life of the enzymes. Furthermore, poor reproducibilities were recorded with these kits [2] for the whole blood samples encountered, which were often several days old and contained preservatives.

Recently several sensitive high-performance liquid chromatographic (HPLC) methods incorporating fluorescence derivatization procedures have been reported for the analysis of sugars. Post-column derivatization with 2-cyanoacetamide [3, 4], ethanolamine [5] or ethylenediamine [6] offers excellent

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sensitivity but requires additional instrumentation. Dns hydrazine (N-dimethylaminonaphthalene-5-sulphonic acid) has been used as a fluorescence labelling reagent for the analysis of reducing sugars by thin-layer chromatography [7]. Pre-column HPLC methods utilising this derivatization procedure with both reversed-phase [8] and normal-phase [9] separations have been developed, but neither was applied to the analysis of glucose in small volumes of body fluids.

In this paper we describe the development of an HPLC method using Dns hydrazine for the analysis of glucose in small volumes of blood, cerebrospinal fluid (CSF) and vitreous humour. The results from samples analysed by this procedure were compared with those obtained with a clinical glucose analyser. The effects of storage on glucose levels in preserved whole blood samples at various temperatures are also reported.

EXPERIMENTAL

Materials

Dns hydrazine (Grade II), D-(+)-glucose and D-(+)-xylose were purchased from Sigma (St. Louis, MO, U.S.A.). All other chemicals were of the highest grade commercially available.

Reagent solutions

Dns hydrazine solution (1%, w/v): a 50-mg sample of Dns hydrazine was dissolved in ethanol. The solution was stored at −10°C until required for use.

Trichloroacetic acid (TCA) solution (3%, w/v): a 750-mg sample of trichloroacetic acid was dissolved in 25 ml of distilled water.

Sugar solutions: freshly prepared standards of glucose (0.1%, w/v) and xylose (0.2%, w/v) were prepared in distilled water when required.

Chromatographic conditions

Analyses were performed on a 25 cm × 4.9 mm I.D. stainless-steel column slurry-packed with ODS Hypersil (5 μm particle size, Shandon Southern Products, Cheshire, U.K.). An ACS pump (Model 400, Applied Chromatography Systems, Luton, U.K.) was used to deliver the eluent, acetonitrile—water (22:78), at a flow-rate of 1.7 ml min^{-1}. The eluate was monitored with a fluorimeter (Fluoromonitor III, Laboratory Data Control, Riviera Beach, FL, U.S.A.) that was fitted with a zinc lamp. An excitation wavelength of 214 nm and emission wavelength of 418—700 nm were selected with the appropriate filters. Injections were made under continuous flow conditions via an injection valve (Negretti and Zambra, Southampton, U.K.), fitted with a 25-μl volume injection loop.

Derivatization procedure

Solutions of the internal standard, xylose (60 μl), and trichloroacetic acid (900 μl) were added to the sample (100 μl of aqueous glucose standard or body fluid) and the mixture was vortex-mixed and allowed to stand for 5 min to precipitate any proteins. After centrifuging at 2 000 *g* for 7 min an aliquot of the supernatant (100 μl) was transferred to a screw-capped test tube together with distilled water (50 μl) and Dns hydrazine solution (200 μl). Following

incubation of the sealed tube at 50°C for 1 h the reaction mixture was washed with toluene (2 ml twice) by vortex-mixing for 30 sec. A portion of the remaining aqueous phase (50 μl) was diluted with HPLC eluent (200 μl) and aliquots were analysed by HPLC.

RESULTS AND DISCUSSION

Choice of chromatographic conditions

As was stated earlier, reversed-phase and normal-phase chromatographic systems were used to separate the Dns derivatives of sugars, but the former was preferred for this study because of the polar nature of the components associated with body fluids. From results reported by Alpenfels [8], the best efficiencies were obtained with an octadecyl-modified silica packing material, and therefore initial trials were performed on a Hypersil ODS column. With the reported eluent the glucose derivative was found to be strongly retained under these conditions, and to reduce the analysis time the flow-rate was increased from 1.0 to 1.7 ml min^{-1}. A range of sugars was Dns-derivatised and chromatographed under these modified conditions and xylose was chosen as the internal standard since it was resolved from glucose with retention times of 12.6 and 9.0 min, respectively. No further alterations of the chromatographic conditions were required since the components of interest were completely resolved from any interferences.

Optimal derivatization conditions

Preliminary derivatization studies were performed according to the method of Alpenfels [8], but the sample clean-up procedure was omitted. Dns derivatives of sugars in aqueous solutions were produced, but no glucose Dns hydrazone was detected when 100-μl samples of preserved whole blood were taken through the reaction scheme. Addition of TCA solution to the blood samples caused coagulation, and it was postulated that incomplete protein precipitation was preventing the derivatization of glucose. This problem was overcome by increasing the amount of TCA added from 100 μl of a 4% (w/v) solution to 900 μl of a 3% (w/v) solution, and derivatization with this increased amount of TCA produced the glucose derivative.

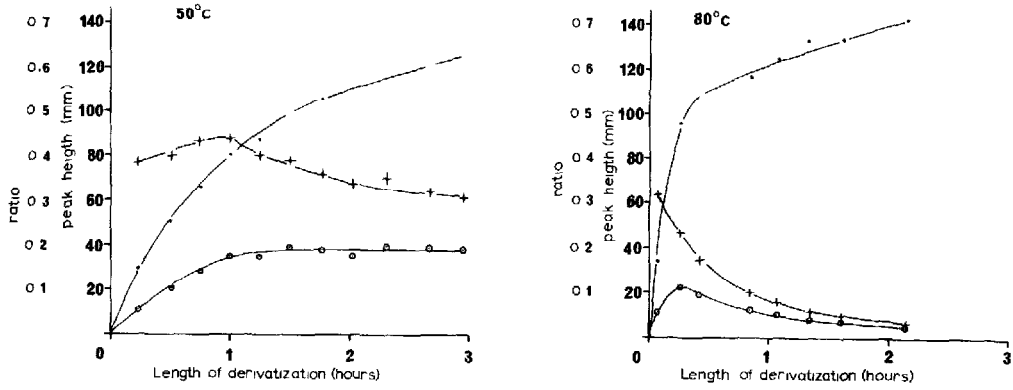

Fig. 1. Effect of temperature (50°C and 80°C) on the Dns derivatization. (○) Glucose peak height; (+) xylose peak height; (•—•—) glucose: xylose peak height ratio.

A batch of ten aqueous glucose/xylose standards analysed under these slightly modified conditions yielded a coefficient of variance of 10%. This result was unacceptable. Further investigations indicated that after incubation for the prescribed period of 10 min at 80°C the derivatization was incomplete and still continued even when the reaction mixtures were stored at 4°C. Glucose and xylose were also found to Dns derivatise at slightly different rates.

It was evident that the optimum reaction conditions had not been established and further kinetic studies were necessary. Takeda et al. [9] had shown previously that an increase in Dns hydrazine concentration produced higher yields of glucose derivative, but solubility problems arose when concentrations exceeded 1% (w/v). A solution strength of 1% (w/v) was used therefore throughout this investigation. To assess the effects of time and temperature on the reaction, temperatures were maintained either at 50°C or 80°C and aliquots were withdrawn from the mixtures at various intervals. The results of these investigations are given in Fig. 1.

Although these studies were performed with an increased amount of TCA, the results are similar to those previously reported [9]. They indicated that at elevated temperatures degradation of the derivatives occurred rapidly and a decision was made to carry out the reactions at 50°C over a period of 1 h.

These studies also confirmed that with temperatures of 80°C and reaction times of only 10 min, any error in the time of sampling would produce irreproducible results.

Sample clean-up procedure

The major reaction by-products are the reagent Dns hydrazine and Dns sulphonic acid. With the chosen excitation and emission wavelengths these compounds fluoresced strongly and were detected within the solvent front and at a retention time of 30 min, respectively. Although no sample clean-up

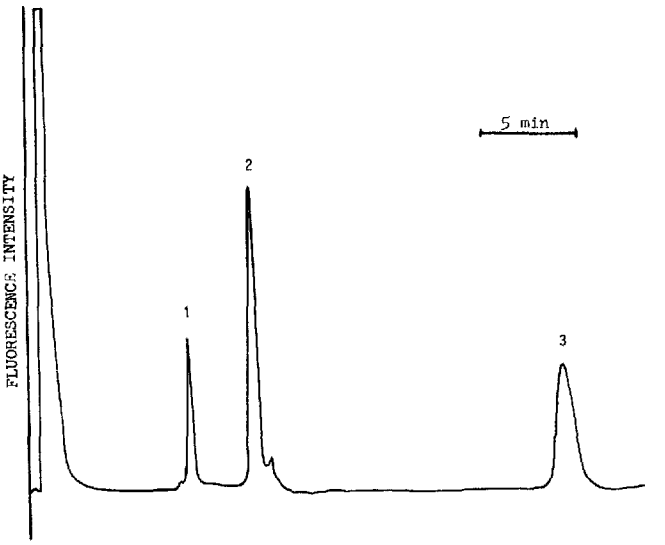

Fig. 2. Typical result obtained from the analysis of a blood sample after derivatization and sample clean-up procedure. The chromatographic conditions are as described in the text. Peaks: 1 = glucose; 2 = xylose (internal standard), 3 = Dns sulphonic acid.

procedure was used initially, it was considered that by incorporating such a process, reduced response from the sample background and shorter analysis times could be achieved. Alpenfels [8] had recommended the use of C_{18} Sep-Pak cartridges (Waters Assoc., Milford, MA, U.S.A.) for purifying Dns derivatization reaction mixtures. However, when some samples were treated as described, analyses indicated that this method was not entirely successful.

It has been reported that with amino acid derivatizations the reagent Dns chloride and the reaction by-products were removed by washing with either *n*-heptane or toluene [10]. Similar treatment was applied to some reaction mixtures and of these two solvents toluene was found to be the most successful. After extraction with 2 ml of toluene, considerable quantities of Dns hydrazine and Dns sulphonic acid were removed without the loss of the glucose or xylose derivatives. A second extraction produced similar results, but further extractions removed only small amounts of the by-products at the expense of significant losses of the sugar derivatives. A clean-up procedure involving two extractions with toluene was considered to be the most favourable, although traces of Dns sulphonic acid still existed, and therefore the overall analysis time could not be reduced. However, the rapidity of this method meant that reactions were stopped almost immediately after the addition of toluene. Typical results obtained from the analysis of a blood sample after derivatization and sample clean-up as described are shown in Fig. 2.

Calibration and reproducibility of the method

For the determination of glucose levels in body fluids aqueous standards have to be used. Calibration plots of peak height ratios (glucose:xylose) versus glucose concentrations were obtained from standards after derivatization and sample clean-up. Linearity was maintained over a concentration range of 0—1.5 mg ml^{-1}, and a correlation coefficient of 0.99 was recorded. Further investigations have shown that the calibrations were linear up to a concentration of 5.0 mg/ml^{-1}, and the method was suitable for analysing samples from patients suffering from hyperglycaemia.

Reproducibility studies have indicated that the best results were obtained when the toluene clean-up procedure was performed immediately after the incubation period. For a set of ten 0.5 mg ml^{-1} aqueous standards analysed in this fashion a coefficient of variation of 2.73% was obtained. With a group of ten blood samples a value of 2.79% was recorded.

Blood sample storage trials

Whole blood samples are submitted to this Laboratory generally in vials which contain an anticoagulant (potassium oxalate) and a bacteriostat (sodium fluoride). The amount of fluoride added to these vials will produce a concentration of greater than 1% (w/v) and therefore glycolysis of glucose to lactic acid is arrested. However, glycolytic rates are reported to be temperature-dependent [11], and because blood samples prior to receipt may have been subjected to a range of temperatures, storage trials at 4°C, room temperature (22°C) and 37°C were performed to determine if temperature affected the glucose levels.

Blood samples were taken by venepuncture from a healthy subject, and

aliquots of 2—2.5 ml were placed and sealed in three vials that contained preservatives. The glucose level of one sample was determined immediately after which the vials were stored at the temperatures stated previously. Glucose levels in each vial were measured periodically thereafter. No discernible changes in the glucose concentrations of the samples stored at 4°C or room temperature were detected over a period of a month, but at 37°C the concentration decreased by approximately 20%.

Validation of the method

Clinical glucose analysers are required to meet agreed specifications for the detection and measurement of glucose, and to check the validity of this HPLC method a number of samples were analysed by both techniques. A batch of 39 blood and CSF samples previously analysed in a clinical laboratory with a glucose analyser (YSI Model 23A, Yellow Springs Instrument, OH, U.S.A.) were analysed within seven days by HPLC. A scatter diagram depicting the correlation between results obtained from the two methods in shown in Fig. 3. A linear least-squares fit for the two sets of data gave a line with a slope of 0.98. The correlation coefficient was calculated as 0.99.

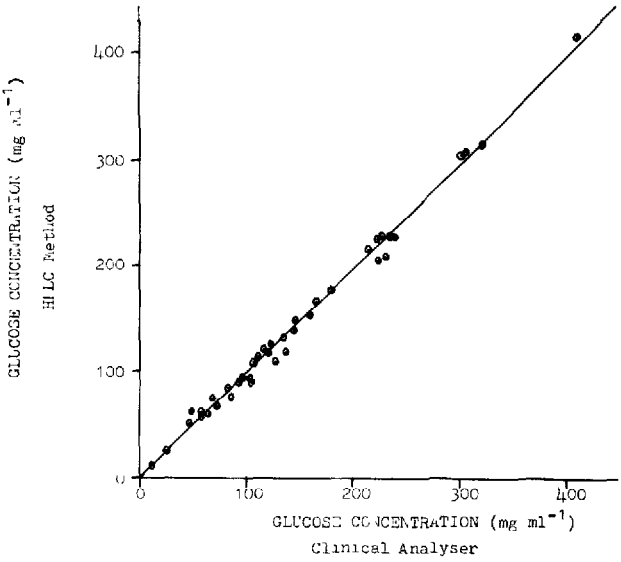

Fig. 3. Scatter diagram depicting the correlation between results obtained by the HPLC method and the YSI model 23A glucose analyser for a batch of blood and CSF samples.

Applications of the method

The two main applications of this method within this Laboratory have been the determination of hypo- and hyperglycaemic levels of glucose in blood samples originating from Road Traffic Act cases and glucose concentrations in preserved vitreous humour from post-mortem samples.

The interpretation of glucose levels in post-mortem samples must be treated with caution because of the rapid glycolysis and glycogenolysis which occurs after death. Results from the analysis of blood or urine samples are of limited value, but it has been reported [12] that results from the analyses of CSF

samples collected by puncture of the cisterna magna within 6 h of death are more reliable in diagnosing the diabetic state of a subject.

Samples of vitreous humour are reported to be far less susceptible to rapid chemical changes and offer an isolated pool of post-mortem material suitable for analysis. Although glycolysis occurs in this fluid and lactic acid is produced, it has been demonstrated that the diagnosis of fatal diabetes mellitus can be confirmed by measurement of the combined glucose and lactate levels [13, 14]. This approach was used for the analysis of post-mortem samples, and the glucose levels were obtained with the HPLC method and lactate concentrations were determined by isotachophoresis [15]. In this example the glucose and lactate levels were determined to be 4.1 and 3.6 mg ml^{-1}, respectively. The combined level of 7.7 mg ml^{-1} was found to exceed any case previously reported [14].

CONCLUSIONS

It has been established that pre-column derivatization with Dns hydrazine and subsequent analysis by HPLC offers a valid method for the determination of glucose levels in samples of blood, CSF and vitreous humour. With a simple and rapid clean-up procedure, results are reproducible over a wide concentration range, thus allowing both hypo- and hyperglycaemic levels to be determined. At room temperature and 4°C glucose levels in preserved whole blood samples remained constant over a period of a month.

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