Age and sex dependent reference intervals for random plasma/serum glucose concentrations related to different sampling devices and determined by an indirect procedure with data mining

Urgent plea for studying the diagnostic efficiency of various concepts proposed to improve the pre-examination phase for determining blood glucose concentrations

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Abstract: The glucose concentration in plasma or serum is one of the most often requested analytical values in laboratory medicine. Whereas the analytical part of the glucose determination is well standardised, the standardisation of the pre-examination part (pre-analytical phase) is not sufficiently solved, yet. In view of the present controversial discussion regarding the most efficient prevention of pre-analytical glycolysis, the question arises whether the economical and logistic expenses for inhibiting glycolysis determining random glucose concentrations are justified. In hospitals with adequate logistics (e.g. pneumatic tube systems for blood tubes) to guarantee a blood sample transport time of about 1 – 2 h, plasma or serum without prevention of glycolysis can be applied for random glucose concentrations if the reference limits are estimated by the laboratory. If such logistics are not available, especially in primary care services, either plasma or serum samples or whole blood in special tubes with anti-glycolytic additives may be sent to the laboratory.

Keywords: age partitioning; data mining; indirect reference limits; random plasma glucose.

Introduction

Glucose concentration in serum or plasma is one of the most often requested analyses in laboratory medicine. In hospitals, it is required for detecting hypoglycaemia or hyperglycaemia which may occur due to several well-known reasons. Observational studies have shown that hyperglycaemic patients with or without prior diagnosis of diabetes have an increased risk of complications and mortality, a longer hospital stay, and a higher admission rate to the intensive care unit [1, 2].

Glucose concentration is usually determined from blood samples sent to the laboratory to perform a whole array of analyses. These, called random (casual) glucose concentrations [3], are not considered to be suited for the diagnosis of diabetes mellitus, unless special pre-examination precautions are met. Whereas the reliability of the analytical
(examination) part is well standardized, the pre-analytical (pre-examination) phase is still a matter of debate and of unsolved problems as recently summarized by Orth et al. [2]. However, for asymptomatic persons, a hyperglycaemic value may be suspicious and may require further diagnostic procedures to confirm or rule out a possible diabetes mellitus [2].

Preanalytical factors for glucose measurement are challenging and both the patient preparation (fasting, physical activity, smoking, emotional stress, etc.) as well as the sample stability after blood drawing before and after centrifugation can impact glucose results in vitro and, therefore, the patient classification based on glucose testing results [2, 4].

The problem of glycolysis caused by the cellular blood components is not sufficiently solved, yet. None of the several proposals for preventing glycolysis is generally accepted because each has its specific disadvantage.

The ideal solution is the immediate centrifugation of heparinised blood after blood aspiration at the patient within 2 min by a high-speed centrifuge or with a cooled centrifuge followed by separation of plasma from the cellular components. Then, the glucose concentration only drops about 0.1%/h [5]. In most cases, this is not possible under routine conditions. The present expert recommendation is the storage of blood in an ice bath for immediate transport to the laboratory [6]. However, most health care systems consider this as impractical. As an alternative, capillary blood may be transferred in hemorrhising or deproteinizing solutions [7–9]. Further alternatives are to draw blood in special containers with glycolysis inhibitors (sodium fluoride (NaF), citrate, oxalate). Even with NaF, the glucose concentration drops by about 7% during the first 4 h [10]. Combining citrate, NaF and EDTA (CFE tubes) reduces the glucose concentration by less than 0.5% during the first 2 h and by less than 1% during 24 h [10, 11]. CFE tubes either contain liquid reagents (prone to errors due to incomplete filling, dilution factors vary between 1.05 and 1.16), or dry reagents (no dilution factors, but 10 times inverting of the tubes required). Lippi et al. [12] recommended CFE tubes after having performed a literature overview because they were more efficient in preventing glycolysis and less prone to haemolysis compared to NaF blood tubes. However, Orth et al. [2] found that CFE tubes often do not inhibit glycolysis sufficiently and pointed out that with CFE tubes, too many diabetic hyperglycaemias occur if the current internationally recommended decision limits are applied.

Under the present controversial discussion about the most efficient prevention of pre-analytical glycolysis, it may be questioned whether the economical and logistic expenses are justified to use special sample tubes to prevent glycolysis for determining random glucose concentrations. This might be especially questioned in hospital logistics (e.g. pneumatic tube system) which offer a fast transportation to the laboratory with small effects on glucose concentrations [13]. Thus, we investigated the hypothesis, that special sample tubes are not required if intralaboratory reference limits are applied.

Methods

Glucose concentrations were determined in 3 university hospitals, laboratory 1 (Bochum), laboratory 2 (Kiel), laboratory 3 (Cologne). Additionally, data from 2 laboratories of the primary care section, laboratory 4 (Karlsruhe) and laboratory 5 (Hannover) were included.

All laboratories used Cobas analysers applying the hexokinase method and the corresponding reagents from Roche Diagnostics (Basel, Switzerland) as indicated in Table 1. The laboratories which provided the measurement values were accredited according to ISO 15189 and followed the Guidelines of the German Medical Association on Quality Assurance in Medical Laboratory Examinations (RiliBAEK) [14].

Glycolysis was inhibited as indicated in Table 2 by using tubes containing NaF or citrate, fluoride and EDTA (CFE tubes). Laboratory 5 used CFE tubes containing the inhibitors either in liquid form (GlucoEXACT, Sarstedt AG, Nümbrecht, Germany) or in dry form (GLUCOMEDICS, Greiner Bio-One GmbH, Frickenhausen, Germany). Gel separator tubes were purchased from Sarstedt AG (Nümbrecht, Germany).

TMC approach

The TMC approach is an indirect method for the determination of reference intervals (RIs) of routine laboratory data (data mining). Such data usually containing

| Laboratory | Serum/plasma | Gel separator | Analytical system |
|------------|--------------|---------------|-------------------|
| No. 1 (Bochum) | Serum | Yes | Cobas 600, c501 |
| No. 2 (Kiel) | Heparin plasma | No | Cobas 8000, c702 |
| No. 3 (Cologne) | Heparin plasma | Yes | Cobas 8000, c702 |
| No. 4 (Karlsruhe) | Serum | No | Cobas 8000, c702 |
| No. 5 (Hannover) | Heparin plasma | No | Cobas 8000, c702 |
Table 2: The prevalence of hyperglycaemic glucose concentrations (upper part: mg/dL; lower part: mmol/L) in NaF and CHE containers.

| Laboratory no. | Women, 18–49 years | Women, 50–90 years | Men, 18–49 years | Men, 50–90 years |
|----------------|---------------------|---------------------|------------------|-----------------|
|                | IRL\(^a\) uRL\(^b\) | n\(^c\) | lp\(^d\) | up\(^e\) | IRL\(^a\) uRL\(^b\) | n\(^c\) | lp\(^d\) | up\(^e\) | IRL\(^a\) uRL\(^b\) | n\(^c\) | lp\(^d\) | up\(^e\) | IRL\(^a\) uRL\(^b\) | n\(^c\) | lp\(^d\) | up\(^e\) |
| 1 (no inhibitor) | 71.5 117.8 8541 0.02 0.117 | 75.6 127.1 32900 0.01 0.264 | 72.4 124.4 11031 0.01 0.105 | 75.7 132.3 29368 0.01 0.267 |
| 2 (no inhibitor) | 64.3 117.3 68396 <0.01 0.074 | 69.2 142.5 162888 <0.01 0.093 | 68.1 132.6 60834 <0.01 0.046 | 71.7 142.4 179972 <0.01 0.133 |
| 3 (no inhibitor) | 69.8 112.4 29084 0.04 0.142 | 71.6 126.8 63152 0.02 0.234 | 72.3 115.9 27885 0.04 0.173 | 72.8 133.5 84181 0.02 0.230 |
| 4 (CFE) | 80.4 110.5 4958 <0.01 0.093 | 83.3 129.4 8357 <0.01 0.163 | 84.3 116.1 3758 <0.01 0.162 | 84.3 116.1 3758 <0.01 0.142 |
| 4 (NaF) | 69.9 98 78440 0.02 0.098 | 72.3 109.2 104271 0.00 0.211 | 72.1 103.1 64343 0.03 0.109 | 73.7 115.7 90228 <0.01 0.226 |
| 4 (no inhibitor) | 70.1 105.1 84057 0.04 0.067 | 73.5 115.3 161549 0.02 0.202 | 72.0 110.8 66927 0.04 0.077 | 72.3 124.9 135598 <0.01 0.174 |
| 5 (CFE.Glucoact) | 74.3 127.4 28222 <0.01 0.250 | 84.0 137.3 9820 <0.01 0.193 | 83.5 122.6 2305 <0.01 0.129 | 86.9 146.9 7408 <0.01 0.250 |
| 5 (CFE.Glucomedics) | 79.1 122.7 2789 <0.01 0.135 | 84.9 137.9 3349 <0.01 0.161 | 85.8 119.5 1280 <0.02 0.217 | 89.5 136.1 3389 <0.01 0.296 |
| 5 (NaF) | 69.5 109.1 20851 0.01 0.218 | 74.9 122.7 20851 0.00 0.230 | 75.3 110.9 20851 0.02 0.141 | 77.2 130.6 20851 <0.01 0.255 |
| 5 (no inhibitor) | 61.3 103.1 199 | 63.9 116.4 1264 | 63.7 117.8 191 | 65.4 134.3 1248 |

NaF, sodium fluoride; CFE, citrate fluoride EDTA. \(^a\)Lower reference limit. \(^b\)Upper reference limit. \(^c\)Number of observations. \(^d\)Lower prevalence. \(^e\)Upper prevalence.
measurements from non-diseased as well as from diseased patients. The data must therefore be considered as a mixture of values produced by 2 distributions, namely the distribution of values from non-diseased patients and the distribution of values from diseased patients. Mixture components use to overlap to some extent.

The TMC method was recently described in detail [15]. The method first identifies an interval containing essentially non-diseased patients, the so-called truncation interval. Then, a power normal distribution (PND) is fitted to this interval by an iterative minimum χ²-approach. This approach accounts for the fact that only a part of all values from non-diseased patients is used for parameter estimation. Rls are calculated from the estimated PND parameters.

The present study uses an advanced version of the procedure described in ref. [15]. Now, the size of the truncation interval is automatically determined on the basis of fit criteria. The truncation interval size was formerly fixed at 70% of all values and is now automatically selected from the range 60 to 85%. The selection criteria for truncation intervals are (i) the p value for the goodness of fit must be >0.01 and (ii) the estimated prevalence must be ≥0. If these conditions hold for several truncations interval candidates, the largest interval is used for parameter estimation. To ensure condition (ii), a corresponding penalty term was added to the estimation procedure as an additional constraint.

A script for performing the TMC analysis, written in the R programming language, can be requested from the authors. Reference limits (RLs) are estimated from the fitted PND, together with their confidence intervals. If the user supplies data on the patients’ sex and age together with an age grouping, the analysis is automatically stratified by the resulting sex/age groups. If more than 4 age groups are defined, a spline function is used to generate a continuous relation between patient age and the Rls. The spline function provides numerical RL predictions for all ages in the age interval covered by the data. Their graphical presentation has no artificial “jumps” between age groups. Typically, 10 years intervals are used for adults, as in this paper. Using 5 years intervals led to very similar spline functions, but confidence intervals of estimated Rls were slightly larger, as expected.

Differences between Rls were considered relevant if the critical difference Dc was surpassed. Dc was estimated on the basis of the permissible imprecision as recently suggested [16]. It can be calculated by a software tool available free of charge on the home page of the DGKL [17]. The critical difference for an upper RL of 6.4 mmol/L (115 mg/dL) is 0.3 mmol/L (5.4 mg/dL), for 7.2 mmol/L (130 mg/dL) it is 0.34 mmol/L (6.2 mg/dL) and 7.8 for mmol/L (140 mg/dL) is 0.39 mmol/L (7.1 mg/dL).

Data selection and sources

The laboratory results of patients were selected as described earlier [18]: only first values were used if several results from the same patient were obtained during a hospital stay or during the data selection time. First values were chosen to avoid ties (correlated observations from the same patient, which is against the assumptions of both direct and indirect methods).

The new proposal was used with real data obtained by routine laboratories, either of the primary health care sector (private laboratories serving mainly general practitioners) or from large university hospitals (tertiary care services). Laboratory 4 and laboratory 5 received less than 5% samples from hospitalised patients. Laboratory 2 excluded hospitalised patients from special wards (e.g. intensive care units and gynaecological units to minimize the number of pregnant women in the data analysed). Subpopulations of the primary health care sector usually have lower prevalence (lower percentage of diseased subjects) than hospitalised patients. Therefore, no subjects were excluded from primary health care data, in contrast to data from hospitalised patients.

The time period for collecting the data (data acquisition time) was either one or several years. The stability of the analytical procedure during the data acquisition time was verified by plotting the monthly medians with confidence limits as described previously [18].

Results

Age and sex dependency of reference intervals

The upper reference limits (uRLs) slightly increased with age (Figure 1) as already reported by others [19, 20] in both sexes due to reduced glucose metabolism [19]. The Rls for women were slightly lower than those for men in the younger age groups. The lower Rls were almost not affected by increasing age. Because of the relatively slight increase of the uRLs with age, it may be justified to consider only 2 age groups for adults, one from 18 to 49 years, and another one from 50 to 90 years. The differences between the age groups and both sexes were relevant in most cases.
In laboratory 1, 2 and 3 (tertiary care), the RLs for serum/plasma (no glycolysis inhibitor) in the age group 18–49 years for women were 6.54, 6.51 and 6.24 mmol/L (Table 2), and 6.90, 7.36 and 6.43 mmol/L for men. The uRLs of the age group 50–90 years were 6.98, 7.91 and 7.04 mmol/L for women and 7.34, 7.90 and 7.41 mmol/L for men (Table 2). In the 2 primary care laboratories (laboratory 4 and 5), the uRLs were lower than in laboratory 1-3 in the absence of glycolysis prevention, but were approximately similar-for CFE plasma. The uRLs using NaF or without any glycolysis inhibitors were lower as compared to CFE. The RLs obtained with CFE tubes were slightly higher when the glycolysis inhibitors were supplied in liquid form in laboratory 4.

The differences between the RLs of women aged 18–49 years were not relevant, but they were relevant in all other age groups because they surpassed the critical differences mentioned above. The differences between primary and tertiary care laboratories may be explained by the transport times of the blood samples, which probably was shorter in the hospital environment (about 1–2 h) than in the primary care laboratories.

The diagnostic efficiency of the glycolysis prevention could not be studied from the available data sets. However, the prevalence of hypoglycaemia (glucose values below the lower reference limit) and for hyperglycaemia (glucose values above the upper reference limit) could be calculated by the TMC procedure as recently described [15]. The mean prevalences of hyperglycaemia were 0.10 for men and women in the age group 18–49 and 0.20 in the age group 50–90 years. If glycolysis was inhibited, the mean prevalences were 0.14 in the younger group and 0.21 in the older group (Table 2). The prevalences were based on uRLs derived from the particular population subsets.

The lower RLs do not depend on age nor on sex (Table 2). The prevalences of hypoglycaemia were much lower as compared to hyperglycaemia and did not differ between the various stratification variables.

**Discussion**

The present study was focused on random plasma/serum glucose concentrations. In the case that the presence of diabetes mellitus is suspected, further diagnostic procedures should follow according to international recommendations [3, 4]. Independent of these recommendations, reevaluation of the decision limits for diabetes mellitus is postulated because the pre-analytic phase was not adequately considered when these limits were established [2]. Furthermore, it has been proposed [21–23] to lower the internationally recommended diagnostic decision limits [3, 4].

All proposals for pre-analytical preventing glycolysis are prone to errors made by personnel which is not under the supervision of the laboratory and, therefore, are hard to be controlled. Orth et al. [2] pointed out that switching to CFE tubes would increase the percentage of patients with diagnoses of impaired fasting glucose and diabetes when using the established decision limits (high false-positive rates). In some patients, however, the long-term stability of glucose in CFE tubes was poor which contradicts changing the decision limits, to avoid an increased rate of false-negative results in diabetes screening. Decision limits for fasting blood glucose and for hyperglycaemia were derived from studies before commercial tubes with additional inhibition of glycolysis using citrate as additive were available [2].

Usually, the rate of these errors is unknown under practical conditions. Outcome studies are required which would determine which pre-examination method leads to the highest diagnostic efficiency under practical conditions occurring in the various health care systems. Due to the various limitations associated with all methods of glycolysis inhibition, it can be speculated that the overall diagnostic efficiency (as the sum of all correctly positive and correctly negative results) is not inferior without any glycolysis inhibition, at least in hospitals where the transport time may not exceed 2 h and reference intervals.
have been determined by the laboratory. In the latter case, the reference intervals can be stratified for age and sex, and analytical errors can be neglected.

The prevalences of hypoglycaemia and hyperglycaemia were used as a surrogate for the diagnostic efficiency. If the application of a glycolysis inhibitor would increase the rate of true positive results, the prevalence should rise. This was not the case in the 2 primary care laboratories. The use of CFE tubes led to the same prevalences as of using the NaF tubes, although the RLs were higher. Similar RLs and prevalences were observed in the absence of anti-glycolytic additives and in the presence of NaF. Laboratories are encouraged to estimate their own reference limits under the pre-analytical conditions presently applied by them. Thus, anti-glycolytic additives did not have a diagnostic advantage when measuring random plasma/serum blood glucose concentrations.

Limitations of the study: no standardising patient's posture during phlebotomy as requested by Lippi et al. [24] was imposed. Furthermore, daytime of blood collection has been neglected. However, these variables have also usually been neglected in most other studies on reference and decision limits for blood glucose concentrations. Furthermore, imposing this would be difficult under routine conditions and can only be considered in research studies.

Conclusions

(1) Random glucose values may only serve to detect distinct hypo- or hyper-glycaemia and to indicate a possible diabetic metabolic disorder. In the case of suspicious diabetic glucose values, the exact diagnosis should follow current international recommendations [3, 4, 20].

(2) Random glucose values may be determined in plasma or serum with and without glycolysis prevention. However, the RLs should be determined for each sample system individually and stratified at least for sex and age. Then, the rate of „diseased cases“ is almost identical for samples with and without inhibition of glycolysis.

(3) In hospitals with appropriate logistics (e.g. pneumatic tube systems for blood tubes) to ensure a blood sample transport time of about 1 – 2 h, plasma or serum can be applied for measuring random glucose concentrations if the RLs are estimated intra-laboratorially. If such a logistic is not available, especially in the primary care sector, either plasma or serum samples (separated immediately after blood collection) may be sent to the laboratory or whole blood in special tubes with anti-glycolytic additives.

(4) Age and sex dependency should be considered instead of a fixed decision limit for both genders and the entire age range. Although the age dependency is continuous over the entire range between 18 and 90 years, only 2 decision limits appear sufficient (e.g. 18–49 and 50–90 years) due to the slight increase. There is no age and sex dependency of the lower reference limit to be considered.

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