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Brief Communications

Acidification of Blood Is Superior to Sodium Fluoride Alone as an Inhibitor of Glycolysis

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BACKGROUND: Sodium fluoride is the preferred agent to inhibit glycolysis. Its action is not immediate, however, and complete inhibition is delayed for up to 4 hours. A more effective method is needed. Acidification of blood combined with the addition of NaF and EDTA appears to be such a method. We studied whether acidification was indeed more effective than NaF.

METHODS: We conducted 6 independent studies over a 10-month period at 3 Quest Diagnostics laboratory sites. In each study, we drew venous blood from 6–24 nonfasting employee volunteers into 3 or 4 different serum- or plasma-collection tubes, which were stored under different conditions and aliquoted at different times. We analyzed the aliquots in duplicate by means of a hexokinase-based enzymatic method.

RESULTS: The mean glucose concentration decreased by 0.3% at 2 h and by 1.2% at 24 h when blood was drawn into tubes containing citrate buffer, NaF, and EDTA. In contrast, the mean glucose concentration decreased by 4.6% at 2 h and by 7.0% at 24 h when blood was drawn into tubes containing NaF and sodium oxalate.

CONCLUSIONS: Acidification should replace NaF alone as the recommended method for obtaining an accurate glucose concentration. Diagnostic cut points based on blood samples collected into tubes containing NaF as the only inhibitor of glycolysis are likely to be too low.

The most recent guidelines for laboratory analysis in the diagnosis and management of diabetes mellitus recommend that glucose concentrations be measured in plasma samples separated from cells within 60 min. If that is not possible, the guidelines recommend the use of NaF to inhibit glycolysis *(1)*.

Although convenient, NaF is not an effective agent to prevent glycolysis *(2)*. Fluoride inhibits enolase, which is far downstream in the glycolytic pathway *(3)*. Enzymes upstream of enolase remain active and continue to metabolize glucose until substrates are exhausted. Thus, the antiglycolytic action of fluoride is delayed for up to 4 h *(2)*. Stahl et al. confirmed that NaF alone is not an effective antiglycolytic agent *(4)*. They also found that time and temperature were critical variables and that icing a specimen could lengthen the allowable time between collection and separation before clinically significant glycolysis occurred.

It is not reasonable, however, to expect that every sample be cooled and separated soon after collection. A simple and effective replacement for NaF is needed. We undertook an extensive Internet-based search to find if such a method had already been developed. We found a relevant US patent (no. 4 780 419), which was applied for in 1986 and issued in 1988 to K. Uchida, S. Okuda, and K. Tanaka, and assigned to Terumo Corporation, Tokyo, Japan *(5)*. A further literature search revealed that after filing the US patent, Uchida et al. published a detailed study of how acidification quickly inhibits glycolysis *(6)*. Acidification inhibits hexokinase and phosphofructokinase, enzymes that act early in the Embden–Meyerhof pathway. Glycolysis is instantly inhibited in erythrocytes, leukocytes, and platelets when the blood pH is maintained between 5.3 and 5.9 with a citrate buffer. The inhibitory effect of acidification is sustainable for approximately 10 h at 25 °C. Uchida et al. therefore added a small amount of NaF to maintain the inhibitory effect for a longer time and added EDTA to chelate magnesium to further inhibit enzyme activity. The final composition of their inhibitory reagent was a granular mixture of citric acid, trisodium citrate, disodium EDTA, and NaF in a gravimetric ratio of 3.4:1.6:4.8:0.2. Ten milligrams of this mixture was added to each milliliter of whole blood.

Terumo Medical Corporation manufactures a blood-collection tube (Venosafe® Glycaemia) that contains the ingredients identified in the patent. These ingredients are a granular mixture of a citrate buffer, NaF, and disodium EDTA with a combined mass of 7.5 g/L of blood. Terumo does not specify the ratios of the ingredients. Moreover, we found no published study of this acidified Terumo blood-collection tube for measuring blood glucose concentrations. This tube is available in Europe but not in the US. We were able to obtain samples of the tube containing the acidification reagents, for experimental use only, through our laboratory in the UK.

Our study was designed to address several questions:

- Is acidification, as defined in the Terumo patent, an effective inhibitor of glycolysis?
- Is NaF alone an effective inhibitor of glycolysis?

Table 1. Effect of collection tube type and additives on stability of glucose.

Sample type, postdraw storage	Comparator, postdraw storage	Mean delta, mmol/L[a]		
		Delta (%)	95% CI	*P* (n)[b]
Citric acid plasma, 2 h at 37 °C	Heparin plasma, 30 min at 0 °C	6.393 − 6.414 = −0.021 (0.3)	−0.07–0.02	0.33 (30)
Citric acid plasma, 24 h at 37 °C	Heparin plasma, 30 min at 0 °C	6.393 − 6.316 = 0.077 (1.2)	−0.002–0.06	0.05 (30)
Fluoride plasma, 2 h at 37 °C	Heparin plasma, 30 min at 0 °C	6.393 − 6.099 = 0.294 (4.6)	0.23–0.35	<0.001 (30)
Fluoride plasma, 24 h at 37 °C	Heparin plasma, 30 min at 0 °C	6.393 − 5.943 = 0.450 (7.0)	0.37–0.53	<0.001 (30)
Plasma, 30 min, ambient	Serum, 30 min, ambient	5.589 − 5.638 = −0.049 (0.9)	0.021–0.077	<0.001 (90)
Barrier serum, 24 h at 37 °C	Barrier serum, 30 min, ambient	5.826 − 5.819 = 0.007 (0.1)	−0.011–0.025	0.45 (66)

[a] Delta values represent mean comparator values minus mean sample-type values.
[b] Paired *t*-test; n = number of pairs.

- Are glucose concentrations different in paired samples of serum and plasma when blood samples are held at the same temperature and separated from cells at the same elapsed time following venipuncture?
- How stable is glucose in serum that overlays a barrier separating the serum from the cells?

To this end, we conducted 6 small experiments at 3 sites over a 10-month period. In each experiment, we assembled 6–24 nonfasting employee volunteers and drew venous blood from the antecubital fossa of each participant into 3 or 4 of the following 4 types of collection tubes: (*a*) BD Medical Systems Na Heparin 68 USP units, 4.0-mL draw; (*b*) BD Medical Systems Na Fluoride/K Oxalate 10 mg/8 mg, 4.0-mL draw; (*c*) BD Medical Systems SST Plus (serum separator tube), 7.5-mL draw; and (*d*) Terumo Venosafe Glycaemia, 3.0-mL draw. In each experiment, we rotated the draw sequence from individual to individual such that the sequencing of tube order was 1-2-3-4 for individual 1, 2-3-4-1 for individual 2, and so on. The collection tubes were then aliquoted at different times and processed under different conditions for the subsequent duplicate measurements of glucose. To amplify the effect of time on glycolysis, we stored most prealiquoted samples at 37 °C. The reference concentration of glucose was defined as the glucose concentration in heparinized plasma obtained from a blood sample immersed immediately after collection into a slurry of ice and water, centrifuged, and aliquoted at ambient temperature within 30 min of collection. All aliquots were stored fully immersed in ice water in a refrigerator before analysis, and all aliquots were assayed at the same time. We used a hexokinase-based reagent system to measure glucose concentrations on Olympus 5400 analyzers with reagents and calibrators from Olympus Diagnostic Systems (Olympus America). The CVs of the glucose assay, as defined by duplicate assays 1 h apart, were 0.83%–1.2% in 12 independent data sets.

Table 1 summarizes the results of the 6 comparison studies. For each comparison, the table identifies the samples used, the storage time and temperature before aliquoting, and, if applicable, the storage time and temperature after aliquoting.

When blood-collection tubes were stored for 2 h at 37 °C before plasma was separated from cells, the mean glucose concentration in blood collected in Terumo tubes containing acidification reagents had decreased by only 0.3% ($P = 0.33$). After 24 h at 37 °C, the mean glucose concentration had decreased by 1.2% ($P = 0.05$). In contrast, when NaF was the sole inhibiting agent and the tubes were stored at 37 °C, the mean glucose concentration decreased by 4.5% after 2 h ($P < 0.001$) and by 7.0% after 24 h ($P < 0.001$).

Contrary to a published statement, "Glucose concentrations in heparinized plasma are reported to be 5% lower than in serum . . ." *(1)*, we found that plasma glucose concentrations were 0.9% higher ($P < 0.001$) than serum glucose concentrations in 90 paired samples when the paired blood samples for serum and plasma were collected and stored at the same ambient temperature and centrifuged at the same elapsed time after venipuncture. The slightly higher glucose concentration in plasma is unexpected, however, because the lower water content of plasma compared with serum should produce lower plasma glucose results. This finding suggests that clotting consumes glucose.

Finally, glucose is very stable when a gel barrier separates serum from cells; however, separating serum from cells within 30 min of collection is not a practical solution to the glycolysis problem in practice because the elapsed time between collection and centrifugation is variable and uncontrolled.

The acidification of blood combined with the addition of NaF and EDTA may replace the use of NaF

alone as the recommended method for measuring the concentration of plasma glucose. We believe this step will lead to more accurate and consistent results. Current diagnostic cut points based on blood samples collected into tubes with NaF alone are likely to be too low *(7, 8)*. To what degree the cut points should be raised will remain unknown until they are reevaluated with samples that are either iced or collected into acidified tubes.

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