



Title	Time And temperature affect glycolysis in blood samples regardless of fluoride-based preservatives: a potential underestimation of diabetes
Authors(s)	Stapleton, Mary, Daly, Niamh, O'Kelly, Ruth, Turner, Michael
Publication date	2017-11-01
Publication information	Stapleton, Mary, Niamh Daly, Ruth O'Kelly, and Michael Turner. "Time And Temperature Affect Glycolysis in Blood Samples regardless of Fluoride-Based Preservatives: A Potential Underestimation of Diabetes" 54, no. 6 (November 1, 2017).
Publisher	Sage
Item record/more information	http://hdl.handle.net/10197/9368
Publisher's statement	Stapleton, M., Daly, Niamh., O' Kelly, R., Turner, M.J. Time and temperature affect glycolysis in blood samples regardless of fluoride-based preservatives: a potential underestimation of diabetes, Annals of Clinical Biochemistry: International Journal of Laboratory Medicine (54,6) pp. 871-676. Copyright © 2017 (Sage). Reprinted by permission of SAGE Publications
Publisher's version (DOI)	10.1177/0004563216682978

Downloaded 2023-10-05T14:16:07Z

The UCD community has made this article openly available. Please share how this access benefits you. Your story matters! (@ucd_oa)



© Some rights reserved. For more information

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Title:

Time and temperature affect glycolysis in blood samples regardless of fluoride-based preservatives: a potential underestimation of diabetes.

Authors: Mary Stapleton¹, Niamh Daly², Ruth O’Kelly¹ and Michael J Turner²

Address 1 Biochemistry Laboratory,
Coombe Women and Infants University Hospital,
Dublin, Ireland.

Address 2 UCD Centre for Human Reproduction,
Coombe Women and Infants University Hospital,
Dublin, Ireland.

Corresponding Author: Mary Stapleton,
Biochemistry Laboratory,
Coombe Women and Infants University Hospital,
Dublin, Ireland

Email: stapleton.mary@yahoo.co.uk

Conflict of Interest: The authors have no conflict of interest to report.

Funding: *This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.*

Ethical Approval: The ethics committee of the Coombe Women and Infants University Hospital approved the study (Study No 26-2011).

Contributorship: MS and ROK researched literature, conceived the study. ND and MT obtained ethical approval. ND recruited patients. MS wrote the first draft of the manuscript. All authors reviewed the manuscript and approved the final version.

Guarantor: ROK

Acknowledgements: The authors wish to acknowledge the support of staff in the biochemistry laboratory throughout this study.

Abstract word count: 245

Article word count: 2,093

Abstract**Background:**

The inhibition of glycolysis prior to glucose measurement is an important consideration when interpreting glucose tolerance tests. This is particularly important in gestational diabetes mellitus where prompt diagnosis and treatment is essential. A study was planned to investigate the effect of preservatives and temperature on glycolysis.

Methods:

Blood samples for glucose were obtained from consented women. Lithium heparin and fluoride-EDTA samples transported rapidly in ice slurry to the laboratory were analysed for glucose concentration and then held either in ice slurry or at room temperature for varying time intervals. Paired fluoride-citrate samples were received at room temperature and held at room temperature, with analysis at similar time intervals.

Results:

No significant difference was noted between mean glucose concentrations when comparing different sample types received in ice slurry. The mean glucose concentrations decreased significantly for both sets of samples when held at room temperature (0.4mmol/L) and in ice slurry (0.2mmol/L).

1
2
3
4
5
6
7
8
9 A review of patient GTTs reported in our hospital indicated that 17.8%
10 exceeded the recommended diagnostic criteria for GDM. It was predicted that if
11 the results of fasting samples were revised to reflect the effect of glycolysis at
12 room temperature, the adjusted diagnostic rate could increase to 35.3%.

13
14
15
16
17 **Conclusion:**

18
19 Pre-analytical handling of blood samples for glucose analysis is vital. Fluoride-
20 EDTA is an imperfect antiglycolytic, even when samples are transported and
21 analysed rapidly provides such optimal conditions. The use of fluoride-citrate
22 tubes may offer a viable alternative in the diagnosis of diabetes mellitus.
23
24
25
26
27
28
29

30
31 **Introduction:**

32
33 Accurate diagnosis of gestational diabetes mellitus (GDM) remains dependant
34 on the results of an oral glucose tolerance test (OGTT) using limits
35 recommended by international bodies, such as the International Association of
36 Diabetes and Pregnancy Study Groups¹ (IADPSG). Guidance from the IADPSG
37 is based on data obtained from the Hyperglycaemia and Adverse Pregnancy
38 Outcomes (HAPO) study.²
39
40
41
42
43
44
45
46
47

48 The HAPO study used standardised sample collection criteria - fluoride oxalate
49 in ice slurry - across its many study centres. The OGTT comprises 3 glucose
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6
7
8
9 samples taken at 0, 1 and 2 hours after a 75g glucose load. It is reported that
10 despite the use of fluoride as an anti-glycolytic agent, glycolysis continues in
11 blood samples after collection.³ If samples are held together as a set prior to
12 analysis, a delay in receipt and analysis may affect the reported results of the
13 first sample more than the last.
14
15
16
17
18
19

20
21 Accurate GDM diagnosis and risk stratification requires the use of accurate
22 measurement of properly stabilised samples for the measurement of glucose.^{4,5}
23
24 This is particularly important in antenatal and postnatal clinics, where there is a
25 limited timeframe in which GDM is diagnosed and treatment initiated.
26
27
28
29
30
31

32
33 The National Academy of Clinical Biochemistry (NACB) guidelines⁶ recognises
34 the importance of preventing *in vitro* glycolysis due to its effects on diagnosis.
35
36 The guidance recommends either transport of the sample in ice and separation
37 from blood cells (within 30 minutes) or the use of an anti-glycolytic agent such
38 as citrate buffer.
39
40
41
42
43
44

45
46 The aim of the study is to investigate the effect of preservatives and ice as
47 glycolytic inhibitors over the time period normally experienced in the hospital
48 setting, as well as its effect of on the diagnosis of GDM.
49
50
51
52
53
54
55
56
57
58
59
60

Materials and Methods:

Venous blood for glucose analysis was obtained from 200 pregnant women with consent. The study was approved by the Hospital Research Ethics Committee.

Blood was drawn from each subject at intervals during their pregnancy (12 weeks, 24-28 weeks and 36 weeks) and 6 weeks postnatal into a fluoride-EDTA tube (Sarstedt 05.1073.001; Sarstedt, Numbrecht, Germany). All fluoride-EDTA samples were placed in an ice-water slurry for transport to the laboratory.

Blood samples were drawn from a random subgroup of this cohort into Lithium Heparin tubes (Sarstedt 05.1553.001), and held either in ice-water slurry or at room temperature during transport to the laboratory. These samples were paired to the fluoride-EDTA samples transported in ice-water slurry.

Blood samples were drawn from a random subgroup of this cohort into citrate-EDTA tubes (Sarstedt 05.1074.001) and paired to the fluoride-EDTA samples which were being transported in ice-water slurry. The citrate-EDTA samples were transported to the laboratory at room temperature.

Glucose concentration measurements were performed in a laboratory, accredited to ISO 15189, on a Beckman Coulter AU640 (Beckman Coulter Inc, Brea, CA, USA) automated chemistry analyser. Analytical imprecision (standard deviation) was 1.64% (0.09mmol/L) at glucose concentration 5.46mmol/L.

1
2
3
4
5
6
7
8
9 Statistical analysis, using Bland-Altman analysis to compare paired results, was
10 performed using Analyse-It software for Microsoft Excel (Analyse-It Software
11 Ltd, Leeds UK).
12
13
14
15

16
17 *Study Design:*

18
19 The time of receipt of samples to the central laboratory was measured.

20
21 The results of two cohorts of patients were examined, and the diagnostic rates
22 of GDM was calculated using these results against the HAPO criteria.
23
24

25 Paired samples were received, and the transport time from phlebotomy was
26 recorded for each sample. Paired samples received within 30 minutes were
27 separated and analysed after aliquots were removed. The aliquots were held for
28 specified times and temperatures before separation and analysis (Figure 1).
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

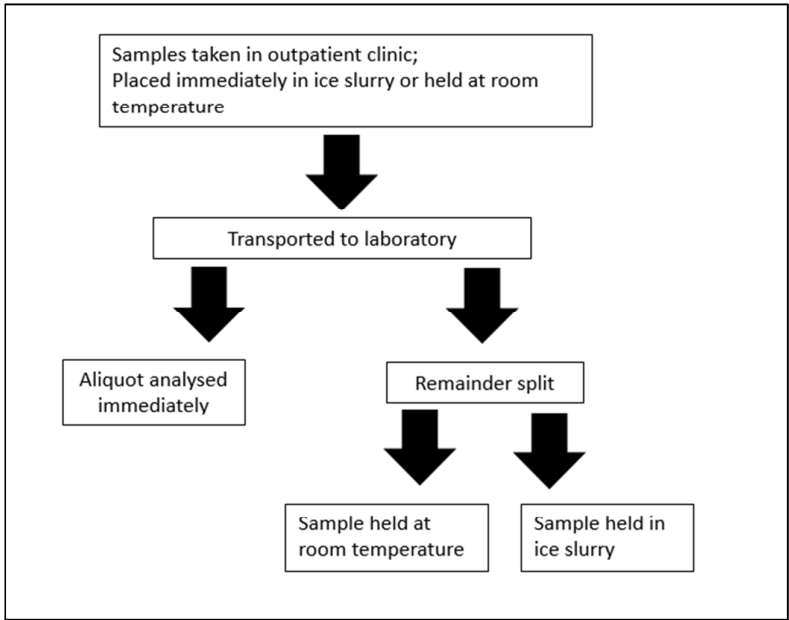


Figure 1. Flow chart for sample analysis; fluoride-EDTA and lithium heparin samples

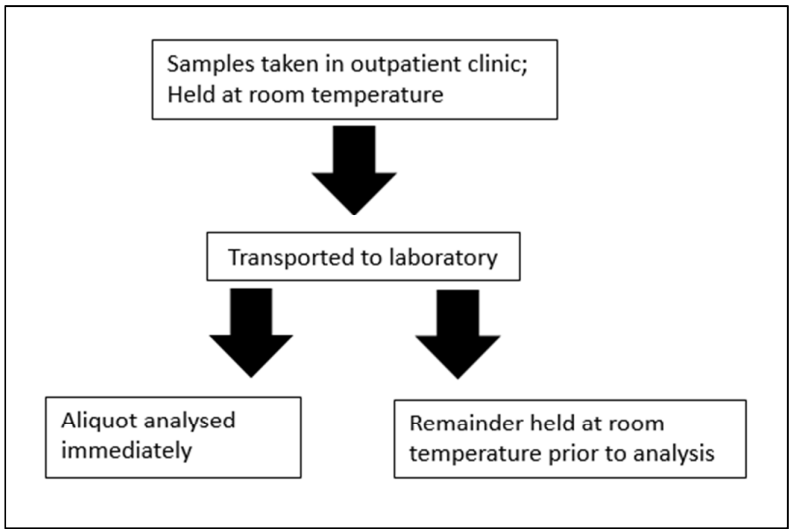


Figure 2: Flow chart for sample analysis. Fluoride-citrate samples

1
2
3
4
5
6
7
8
9 The difference in glucose concentration between samples held on ice and room
10 temperature for different time intervals was calculated.
11

12
13
14
15 The standard against which other preservatives and conditions were compared
16 was the glucose concentration reported in samples of fluoride-EDTA
17 preservative which were received on ice.
18
19

20
21
22 The difference in mean glucose concentrations were analysed using Student's t
23 test.
24
25
26

27
28 To investigate the effect of temperature and/or preservative on glycolysis, the
29 glucose concentration at time of receipt was compared for paired samples.
30
31

- 32 1. Fluoride-EDTA on ice; Lithium Heparin on ice
- 33 2. Fluoride-EDTA on ice; Lithium Heparin received at room temperature.
- 34 3. Fluoride-EDTA on ice; Citrate-EDTA received at room temperature.
- 35
- 36
- 37
- 38
- 39
- 40

41
42 To investigate the effectiveness of preservatives on glucose over time, the
43 glucose concentration was measured at various time intervals within the time
44 period determined by the findings of transport time to the laboratory.
45
46
47

- 48 1. Samples (Fluoride-EDTA and Lithium heparin) were held on ice or room
49 temperature for 2.5 hours before separation and glucose measurement.
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

2. Samples (Fluoride-EDTA and Lithium Heparin) were held on ice or room temperature for up to 90 minutes with separation and glucose measurement of aliquots at 15 minute intervals.
3. Samples were held on ice (Fluoride-EDTA) or room temperature (Citrate-EDTA) for up to 90 minutes with separation and glucose measurement of aliquots at 15 minute intervals.

Results:

The mean time taken for samples from GTT series to be received into the laboratory was 154 minutes (range 123-190 minutes). Based on these findings, the time used to delay the analysis of samples was chosen to be 150 minutes (2.5 hours).

There was no significant difference between mean glucose concentrations in the fluoride-EDTA and lithium heparin samples received in ice slurry within 30 minutes of sampling. There was a significant difference between the glucose concentrations from fluoride-EDTA samples received in ice slurry and lithium heparin samples received at room temperature (Table 1).

There was a significant difference between mean glucose concentrations when received in either fluoride-EDTA or citrate-EDTA preservative type. Mean glucose concentration in the citrate-EDTA set was greater than that seen in the fluoride-EDTA samples (Table 1).

Table 1.

Difference in glucose concentration at time of receipt between different preservatives transported at differing temperatures.

Difference between Fluoride-EDTA (on ice) at time of receipt and:			
	Lithium Heparin		Citrate-EDTA
Transport conditions	Ice slurry	Room temperature	Room temperature
Time to receipt	15±6 minutes	15±5 minutes	17±8 minutes
n	60	36	24
Mean difference [Cl] mmol/L	-0.01 mmol/L [-0.06, 0.03]	-0.08 mmol/L [-0.13, -0.03]	+0.06 mmol/L [-0.02, 0.14]
Percentage difference	-0.21%	-1.63%	+0.96%
P value	0.631	0.001	0.14

Aliquots of paired fluoride-EDTA and lithium heparin samples were incubated for 2.5 hours at two different temperatures; room temperature and ice slurry.

The mean glucose concentration decreased significantly for samples preserved in lithium heparin or fluoride-EDTA when held at either room temperature or in ice slurry (Table 2). Mean glucose concentration remained stable over the 2.5 hours for samples in fluoride-citrate preservative.

Table 2:

Difference in glucose concentration when held for 2.5 hours. Three preservative types compared.

Incubation Temperature	Room Temperature			Ice Slurry	
	Li-Heparin	FI-EDTA	Fluoride-citrate	Li-Heparin	FI-EDTA
Time to receipt	15±6 min	14±6 min	17±8 min	15±6 min	14±6 min

(minutes)					
n	46	192	22	49	169
Mean difference [CI] mmol/L	-0.76 mmol/L [-0.83, -0.69]	-0.43 mmol/L [-0.46, 0.41]	-0.02 mmol/L [-0.04, 0.13]	-0.22 mmol/L [-0.27, -0.16]	-0.18 mmol/L [-0.20, -0.15]
Percentage difference	-14.5%	-7.6%	-0.34%	-4.1%	-3.1%
P value	P < 0.05	P < 0.05	0.248	P<0.05	P<0.05

The rates of glycolysis within each preservative type at either room temperature or in ice slurry was compared for paired samples. Differences in glucose concentrations measured at the time of receipt and time points chosen for analysis were compared (Figures 3 and 4).

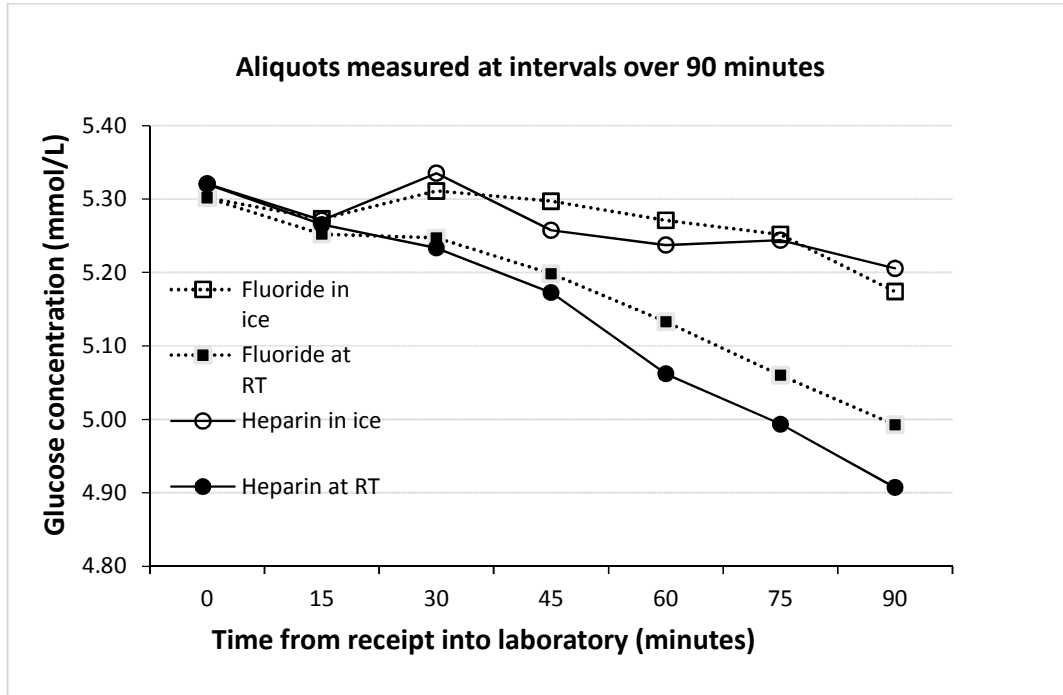


Figure 3. Samples analysed at 15 minute intervals: Fluoride-EDTA and Lithium Heparin.

Paired fluoride-EDTA and Lithium heparin samples (n=23);

The differences in glucose concentration between t=0 and samples held at room temperature were statistically significant at every time point.

The differences in glucose concentration between t=0 and samples held in ice slurry were statistically significant at every time point for lithium heparin samples.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

The differences in glucose concentration between t=0 and samples held in ice slurry were statistically significant for time points after 30 minutes for fluoride-EDTA samples.

The change in glucose concentration between preservative types at the same environmental conditions was also compared. The rate of change was similar between sample types if held at the same temperature. However, there was a significant difference between rates after an hour for samples held at room temperature.

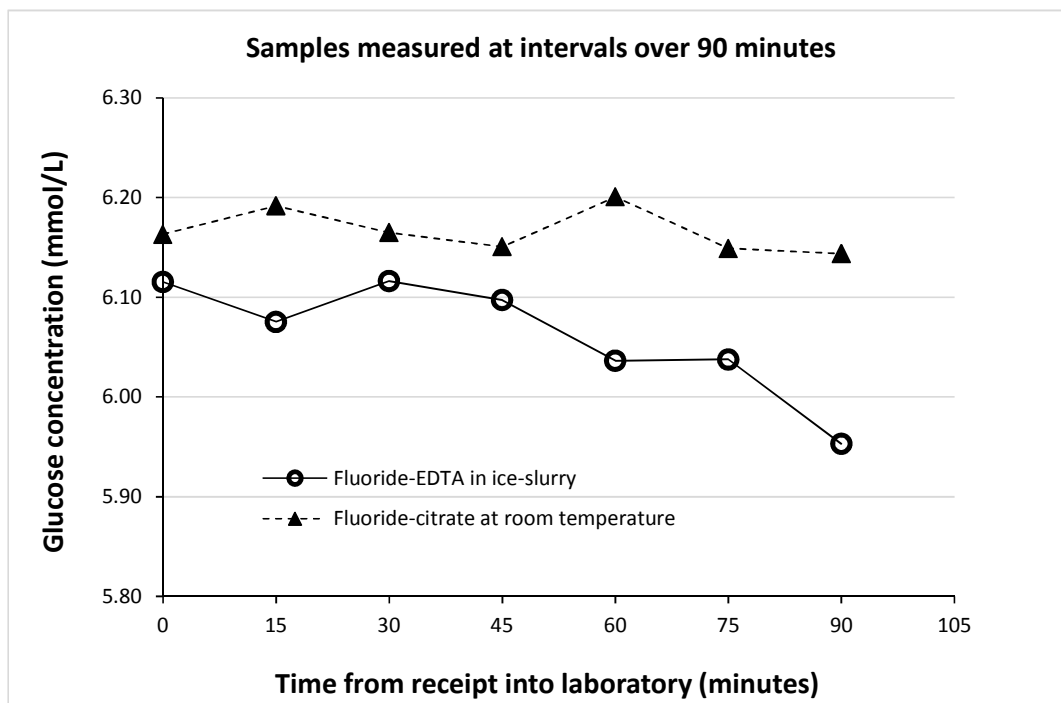


Figure 4: Samples analysed at 15 minute intervals: Fluoride-EDTA and Fluoride-citrate.

1
2
3
4
5
6
7
8
9
10
11 The changes in mean glucose concentration between paired samples of
12 fluoride-EDTA held on ice and fluoride-citrate samples held at room
13 temperature were examined (Figure 4). A difference in mean glucose
14 concentration is noticeable, with a significant difference in fluoride-EDTA
15 samples noted at 15 minutes post receipt. Reviewing data from the fluoride-
16 citrate tubes, it was noted that there was no significant difference between
17 mean glucose concentration at time of receipt and at any of the time points
18 investigated.
19
20
21
22
23
24
25
26
27
28
29

30
31 A review of 400 patient glucose tolerance tests reported in an outpatient clinic in
32 our hospital indicated that 17.8% were reported as fulfilling the recommended
33 diagnostic criteria for GDM in one or more samples. Of a sample of 113 patient
34 glucose tolerance tests sampled at a clinic located 30km from the hospital
35 (external clinic), 10.6% were reported as exceeding the recommended
36 diagnostic criteria for GDM in one or more samples.
37
38
39
40
41
42
43

44 It was predicted that if the results of fasting samples were reviewed to include or
45 incorporate the difference due to glycolysis in fluoride tubes at room
46 temperatures as calculated in this study (0.4mmol/L), the revised diagnostic rate
47 would be 35.3% of the women chosen for selective screening in the outpatient
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6
7
8
9 clinic. For samples taken in the external clinic, this value could be added to all
10 patient results, due to the increased delay in sample receipt. It is predicted the
11 revised diagnostic rate would be 38.1% of these women chosen for selective
12 screening.
13
14
15
16
17
18

19 **Discussion**

20
21 The diagnostic limits derived from the HAPO study were based on samples
22 collected and transported in ice-slurry, and separated within one hour. To obtain
23 accurate diagnosis using these limits, methods of glycolysis inhibition when
24 analysing glucose samples in antenatal OGTTs ought to mirror those used in
25 the study.⁷
26
27
28
29
30
31

32 It is shown that if there is rapid transport of samples prior to separation and
33 analysis, there is little difference in glucose concentrations reported when
34 paired samples are transported on ice, even in the absence of fluoride-EDTA
35 preservative.
36
37
38
39
40

41 When comparing average glucose concentrations of samples received and
42 analysed rapidly (within 30 minutes) in fluoride-EDTA to fluoride-citrate
43 preservatives, a positive bias of mean glucose concentration is observed for
44 fluoride-citrate samples. This bias may be due to ongoing glycolysis in the
45 fluoride-EDTA tubes due to delayed cooling of the blood in the tubes.⁸
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6
7
8
9 Alternatively, it has been proposed that the conversion factor used for liquid
10 preservative may require revision.⁹

11
12 When investigating a delay of 2.5 hours for samples held at room temperature -
13 the time taken for samples to reach the laboratory - it can be seen that the 7.6%
14 (0.4mmol/L) decrease seen in the fluoride-EDTA samples is consistent with
15 previous reports, while that of the lithium heparin nears 15% (0.8mmol/L). The
16 difference in glucose concentration calculated from samples of citrate
17 preservative is negligible over the 2.5 hours.
18
19

20
21 In the unlikely event that samples would be held for extended periods of time in
22 ice slurry, it is shown that a significant difference is seen at 2.5 hours for
23 samples containing the fluoride-EDTA preservative. At a 3% (0.2mmol/L)
24 decrease from the original analysis, this decrease is similar to that seen in the
25 lithium heparin samples (4%; 0.2mmol/L). The ice therefore appears to have an
26 incomplete effect in inhibiting the glycolytic process. This is in contrast with the
27 consistency in mean glucose concentration noted in the fluoride-citrate samples
28 over the same time period. Indeed, over a longer time period - 4 hours - there is
29 negligible difference (0.28%) in mean glucose concentration measured in
30 fluoride-citrate tubes.
31
32

33
34 When results from delayed analysis using shorter timeframes are examined, it
35 may be seen that even at a short an interval as 15 minutes, there is a significant
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6
7
8
9 change in glucose concentration for paired samples held either at room
10 temperature or for samples held on ice. This consistent change is not seen in
11 samples preserved using fluoride-citrate.
12

13
14
15 Although the rates of change are similar across time points, there is a slight
16 variability in average glucose concentration at each time point. This may be due
17 to the small sample size of the study.
18
19

20
21
22 Transporting every glucose sample individually on ice prior to analysis is not a
23 viable option for hospitals and clinics. A possible alternative is the use of
24 fluoride-citrate preservative for glucose analysis.^{10, 11}
25
26

27
28
29 The recommendation of citrate as an antiglycolytic agent has been popularised
30 recently, due it's reemergence as a viable glycolytic inhibitor.^{6, 10, 12, 13}
31

32
33 Sample tubes containing either powdered or liquid preservative have been
34 studied and results compared against traditional fluoride glucose tubes.¹⁴
35
36

37
38 Due to the dilution of the sample by the liquid preservative, a correction factor is
39 required for samples, the published values of which have come under scrutiny.
40

41
42 The difference between mean glucose concentrations reported in fluoride when
43 held on ice and those reported in citrate tubes using a liquid preservative have
44 been sufficiently similar to allow their introduction. However, this consistent
45 difference was investigated and has been reported as due to the manufacturer's
46 recommended correction factor.⁹
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Conclusion

This study investigated the effect of fluoride, citrate and ice as glycolytic inhibitors over the time period normally experienced in the hospital setting

The importance of an early and accurate diabetes diagnosis, including GDM by the stringent HAPO criteria, is such that health professionals cannot overlook the impact of glycolysis. By diagnosing using HAPO criteria, and not adhering to the collection practices in the study, it is likely that there is consistent underdiagnosis of GDM in our patient population.

Optimal pre-analytical standardised conditions are critical for the accurate classification of diabetes, particularly GDM, where the window for diagnosis is limited and the treatment is immediate. Where the findings in our paper are important in all investigations of patients for diabetes mellitus and impaired glucose tolerance, the lack of diagnostic utility of HBA1c in the setting of GDM, makes defining the evidence base for pre-analytical criteria for the GTT critical.

References

1. International association of Diabetes and Pregnancy Study Groups Consensus Panel. International association of diabetes and pregnancy

- 1
2
3
4
5
6
7
8 study groups recommendations on the diagnosis and classification of
9 hyperglycaemia in pregnancy. *Diabetes Care* 2010; 33(3); 676-82
10
11
12
13 2. HAPO Consultative Study Group, Hyperglycaemia and Adverse
14 Pregnancy Outcomes. *N Eng J Med* 2008; 358(19); 1991-2002
15
16
17 3. Mikesh LM, Bruns DE. Stabilisation of glucose in blood specimens:
18 Mechanism of delay in fluoride inhibition of glycolysis., *Clin Chem*
19 2008;54(5):930-1.
20
21
22
23 4. Bruns DE, Knowler WC. Stabilisation of Glucose in Blood Samples: Why
24 it matters, *Clin Chem* 2009; 55(5): 850-852
25
26
27
28 5. Gambino R, Bruns DE. Stabilization of Glucose in Blood Samples: out
29 with the old, in with the new, *Clin Chem Lab Med* 2013; 51(10): 1883-5
30
31
32
33 6. Guidelines and Recommendations for Laboratory Analysis in the
34 Diagnosis and Management of Diabetes Mellitus. The National Academy
35 of Clinical Biochemistry Laboratory Medicine Practice Guidelines. Ed.
36 Sacks DB.
37
38
39
40
41
42 7. Daly N, Stapleton M, O'Kelly R, et al. The role of preanalytical glycolysis
43 in the diagnosis of gestational diabetes in obese women., *Am J Obs*
44 *Gynae* 2015; 213(1): 84 e1-5.
45
46
47
48 8. van den Berg SAA, Thelen MHM, Salden LPW, et al. It takes acid, rather
49 than ice, to freeze glucose. *Sci Rep.* 2015 Mar 9;5: 8875
50
51
52
53
54
55
56
57
58
59
60

- 1
2
3
4
5
6
7
8
9 9. van der Hagen EAE, Kleefman AMD, Thelen MHM, et al. Normalisation
10 issues in glucose measurements using phlebotomy tubes with liquid
11 additives. *Clin Chem Lab Med*. 2016 ePub ahead of print Jun 30 2016.
12
13
14
15 10. Gambino R, Piscitelli J, Ackattupathil TA, et al. Acidification of blood is
16 superior to sodium fluoride alone as an inhibitor of glycolysis, *Clin Chem*
17 (2009);55(5):1019-21.
18
19
20
21 11. Daly N, Flynn I, Carroll C, et al. Comparison of Citrate-Fluoride-EDTA
22 with Fluoride-EDTA Additives to Stabilize Plasma Glucose
23 Measurements in Women Being Screened during Pregnancy with an
24 Oral Glucose Tolerance Test: A Prospective Observational Study. *Clin*
25 *Chem* 2016 Jun; 62(6):886-7
26
27
28
29
30
31
32 12. del Pino IG, Constanso I, Mourín LV, et al. Citric/citrate buffer: an
33 effective antiglycolytic agent. *Clin Chem Lab Med*. 2013 Oct;
34 51(10):1943-9
35
36
37
38
39 13. Norman M, Jones I. The shift from fluoride/oxalate to acid citrate/fluoride
40 blood collection tubes for glucose testing - the impact upon patient
41 results. *Clin Biochem* 2014 May; 47(7-8):683-5
42
43
44
45
46 14. Yagmur E, van Helden J, Koch A. et al. Effective inhibition of glycolysis in
47 venous whole blood and plasma samples. *J Lab Med* 2012; 36(3): 169-
48 177
49
50
51
52
53
54
55
56
57
58
59
60