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Collection tubes containing citrate stabiliser over-estimate plasma glucose, when compared to other samples undergoing immediate plasma separation

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ABSTRACT

Objectives: Blood collection tubes containing citrate lower pH, thereby inhibiting glycolysis. When compared to other additives, they introduce an over-estimation in measured glucose. This study explored this over-estimation across a range of glucose values. Blood samples collected into lithium-heparin tubes then cooled prior to immediate refrigerated plasma separation, were used as the primary comparator.

Design and methods: Venous blood from individuals with and without diabetes was collected into tubes containing lithium-heparin, or fluoride, or fluoride-citrate (Terumo™ Venosafe). Plasma was separated at time intervals of zero, 2 and 24 h. Preparation of the 'time zero' lithium-heparin and fluoride samples was optimised by processing these samples under cooled conditions. The remaining samples were prepared at room temperature. Plasma was analysed in the routine clinical laboratory using the hexokinase method.

Results: Median plasma glucose for the 50 participants was 7.1 mmol/L (range 3.1–21.5). At 'time zero', fluoride-citrate glucose was 0.37 mmol/L (95% CI 0.26–0.48) higher than lithium-heparin glucose and 0.29 mmol/L (95% CI 0.21–0.36) higher than glucose from fluoride tubes. Following delayed plasma separation at 24 h, glucose loss from the lithium heparin tubes averaged 0.2 $mmol \cdot L^{-1} \cdot hr^{-1}$. In contrast, the fluoride-citrate tubes showed minimal glucose loss over 24 h.

Conclusions: Acid stabilises glycolysis but causes an over-estimation in glucose, across a range of plasma glucose values, when compared to blood collected into conventional tubes under cooled conditions. The magnitude of the over-estimation seen with the fluoride-citrate tubes is unlikely to be due solely to the differential glucose stabilisation rates of acid, compared to cooling.

1. Introduction

Plasma glucose measurement is affected by ex vivo glycolysis. Traditionally, glycolysis is inhibited using one or more of the following methods: Separation of plasma from the cellular components of blood, or by cooling [1,2], or by using a glucose stabiliser such as sodium fluoride [2,3]. In contrast to sodium fluoride, which inhibits an enzyme lower in the glycolytic pathway, the reduction of blood pH below 7 with a citrate stabiliser has an early, more immediate effect on pathway inhibition [4]. Multiple studies using a range of methodologies have examined the effect of citrate stabilisers on the measurement of plasma glucose [5–17]. These studies have however tended to use blood samples across a narrow range of glucose values, with samples collected predominantly from healthy volunteers. Some but not all studies of citrate tubes show a small increase in measured plasma glucose, when compared to traditional methods of glucose stabilisation [10,13,17]. There are however no studies that compare citrate stabilisation with optimal stabilisation of glucose using both rapid cooling and near-immediate separation of plasma, which also actively recruit participants with diabetes. There is therefore is a paucity of information about the efficacy of citrate stabilisation across a wide range of glucose values.

In the current study we aimed to include participants with and without diabetes, so that samples provided a wide range of glucose values. The study's primary aim was to compare glucose results by collection tube type (lithium-heparin-PST™, NaF-K-oxalate and NaF-EDTA-citrate, using Terumo™ Venosafe collect tubes), following early plasma separation ('time zero' samples). The secondary aims were to compare loss of glucose from the three collection tubes, following separation of

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plasma at 2 and 24 h. In addition, the study also explored whether glucose loss is best expressed as an absolute, rather than relative value, when considering a wide range of baseline glucose values.

2. Methods

2.1. Participants

Participants were recruited either by advertisement or through the local diabetes clinic, aiming for a minimum of 15 participants in each of the following subgroups; healthy volunteers, type 1 diabetes, type 2 diabetes. Exclusion criteria were age <18 years, pregnancy, known haematological conditions and a history of difficulty with venesection. Written consent was obtained prior to participation.

2.2. Blood collection tubes, sample collection and sample processing

Three types of blood collection tubes were used; lithium-heparin-PST™ (plasma separator tubes), BD (Becton Dickinson) Vacutainer®, reference 367375, draw volume 4.5 mL; NaF-K-oxalate (sodium fluoride potassium oxalate), BD (Becton Dickinson) Vacutainer®, reference 367935, draw volume 4 mL; NaF-EDTA-citrate (sodium fluoride ethylenediaminetetraacetic acid citrate), Terumo™ Vensosafe™, reference 367935, draw volume 2 mL. The Terumo™ NaF-EDTA-citrate tubes contain lyophilised rather than liquid additives, thus they do not require a dilutional correction factor. Lithium-heparin-PST™ tubes were included because they do not contain any glycolytic inhibitor which might have a direct or indirect effect on glucose measurement. Fluoride collection tubes were included in the study as they are commonly used for the routine measurement of plasma glucose.

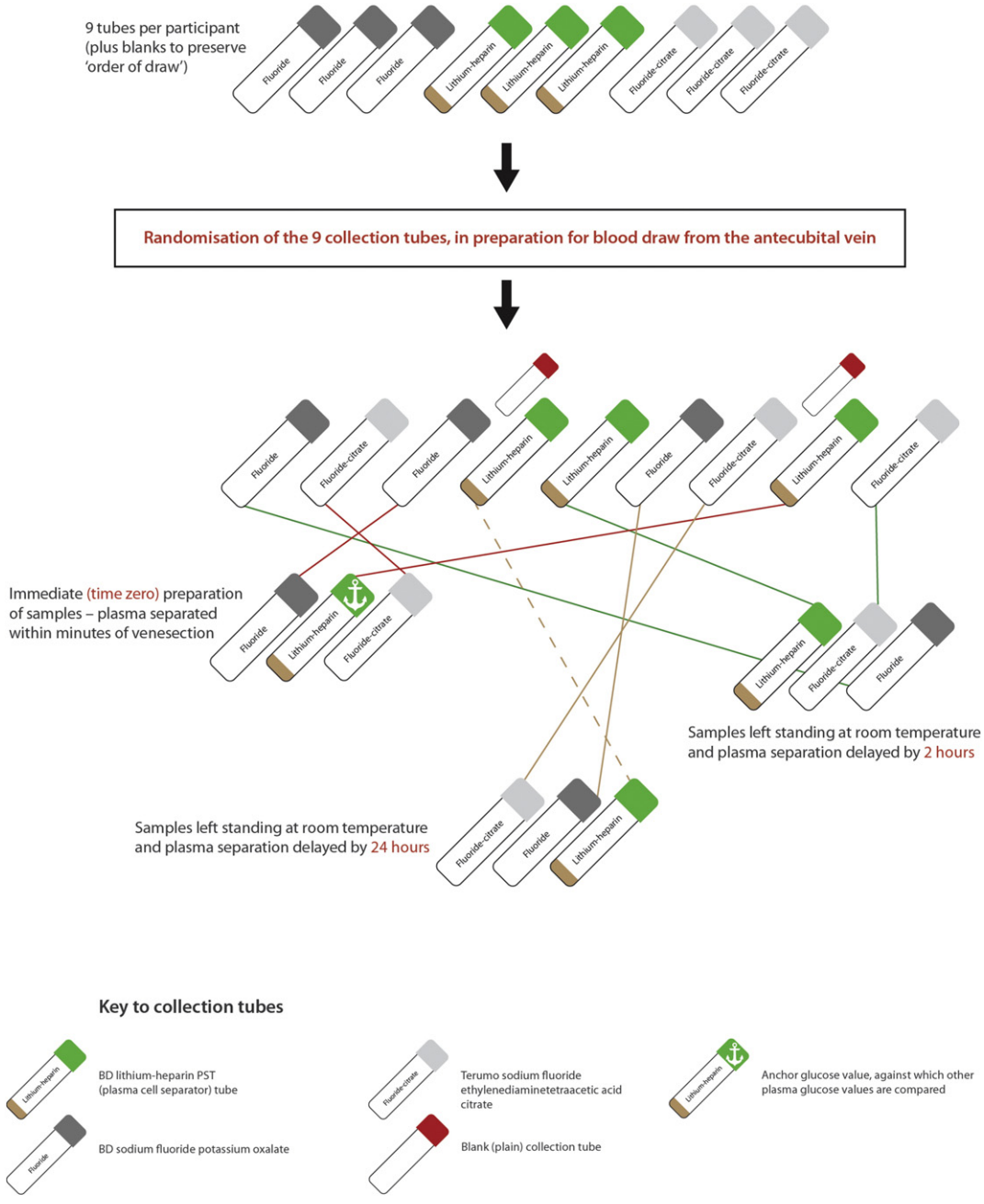

Fig. 1. Overview of study methodology.

For brevity, we subsequently describe the collection tubes as lithium-heparin (for lithium-heparin-PST™), fluoride (for NaF-K-oxalate) and fluoride-citrate (for NaF-EDTA-citrate). Fig. 1 summarises sample collection and processing procedures. The order in which tubes were used for antecubital fossa venous blood draw from individual participants was randomised. This ensured that the inevitable slight time delay between drawing blood and centrifugation of 'time zero' samples was randomly distributed across all three collection tube types. Order of draw was preserved following randomisation by use of blank collection tubes (see Fig. 1).

Manufacturers' instructions for handling of blood collection tubes were followed. Particular care was taken to avoid tube under-filling. A minimum of 9 collection tubes of blood was obtained from each participant. The three different types of tubes studied were processed in parallel; that is plasma was separated at the same time points, for each of the three tube types. The lithium-heparin and fluoride collection tubes allocated for 'time zero' sample preparation were placed immediately onto an ice slurry in the clinical area. The aim was to commence refrigerated centrifugation at +4 °C for 15 min, in the adjacent laboratory processing area within 5 min of venesection. Glycolysis in these 'time zero' tubes was therefore minimised by both rapid cooling and also early plasma separation. These tubes might therefore be considered to be undergoing optimal pre-analytical preparation. The fluoride-citrate tubes allocated for 'time zero' preparation were kept at room temperature, thereby replicating intended usage. The remaining blood samples rested upright at room temperature for either 2 or 24 h before undergoing centrifugation, which was followed immediately by plasma separation. The 2 and 24 h centrifugation and plasma separation steps were undertaken at room temperature. Further methodological details about sampling and centrifugation are outlined in a previous study [18].

2.3. Laboratory analysis

Analytical imprecision was minimised as follows: At each sample separation time point (i.e. zero, 2 and 24 h), one tube of each type was batched so that these samples were analysed in the same 'run', in triplicate (see Fig. 1 for details of triplicate batching). In addition to this batching process, every individual glucose sample was analysed twice and plasma glucose was reported as the mean of these duplicate measures. Analysis was undertaken in an accredited laboratory during routine 'runs' in the clinical biochemistry department, using the hexokinase method (Abbott C16000 analyser, Abbott Diagnostics, North Chicago, IL, USA). High levels of haemolysis lower the measured plasma glucose [19]. The HI (haemolysis index) was therefore measured using the same analyser, at the same time as plasma glucose was measured.

No attempt was made to include a cellular count within this study, as we have shown previously in a similar study population, that there is no relationship between cellular count and pre-analytical glucose loss from lithium heparin and fluoride tubes, up to two hours after venesection [18].

2.4. Statistical methods

Parametric and non-parametric methods were used as appropriate. Parametric comparisons using ANOVA and paired t tests were undertaken using SPSS version 22. Difference plots were prepared using MedCalc, version 12.2.1.0.

As fluoride tubes increase pre-analytical haemolysis when compared to other additives, the cooled 'time zero' lithium-heparin tube, rather than the fluoride tube, was therefore considered to reflect most accurately, the true plasma glucose. Thus when a single baseline result was needed for descriptive purposes or for statistical comparisons, the 'time zero' lithium-heparin tube was used as the 'anchor' or reference plasma glucose value.

A previous study which focused on pre-analytical factors across a range of glucose values showed that glucose loss was best described as an absolute loss (i.e. mmol/L), rather than as a relative or proportional loss (i.e. %) of glucose [18]. The primary calculations undertaken in this study therefore used absolute, rather than percentage glucose differences.

This study was registered with the Australian New Zealand Clinical Trials Registry, trial ID ACTRN 12615000004561. Ethical approval was granted by the New Zealand Health and Disability Ethics Committee, reference 14/STH/220.

3. Results

3.1. Participant characteristics

Characteristics of the 50 participants are outlined in Table 1. The majority (90%) were of New Zealand European ethnicity. No participant had any known pre-existing haematological condition.

3.2. Sample processing and analysis

For the time zero lithium-heparin and fluoride tubes, placement onto an ice slurry occurred immediately after venesection. There was however a small but inevitable time delay averaging 4 min, between venesection and commencing refrigerated centrifugation within the relevant processing area of the research clinic. Room temperature during the study ranged from 19 to 24 °C. Within run precision for duplicate glucose measures showed a coefficient of variation of 0.7%.

3.3. Comparison of results from healthy volunteers and participants with diabetes

Results from participants by subgroup (healthy volunteers, type 1 and type 2 diabetes), are shown in the Supplementary Materials (Supplementary Tables 1 and 2). When considering results from each blood collection system separately, there was no difference in glucose loss between participant subgroups across any of the time points studied (ANOVA, $p > 0.16$). Results from all three participant subgroups (healthy volunteers, type 1 and type 2 diabetes) have therefore been combined in subsequent analyses.

3.4. Baseline differences across different types of collection tubes

At 'time zero', mean (mmol/L ± SD) plasma glucose for the 50 participants was: lithium-heparin 7.72 ± 3.30, fluoride 7.80 ± 3.43 and fluoride-citrate 8.09 ± 3.39, with the plasma glucose values obtained from the fluoride-citrate tubes being significantly higher than those obtained from the other two tubes ($p < 0.001$). Comparison of results from the 'time zero' lithium-heparin and fluoride tubes showed that they were similar ($p = 0.15$). This similarity was observed despite the fluoride tubes showing a higher HI than the other tubes (see below). Visual inspection of the glucose difference plots between the 'time zero' lithium-heparin and fluoride-citrate tubes showed an overestimation of the fluoride-citrate tubes relative to the lithium heparin tubes but no other obvious relationship was observed from these plots (see Supplementary Fig. 1). We therefore report both absolute and also percentage differences for these baseline measurements: Mean absolute difference was 0.37 mmol/L (95% CI 0.26 to 0.48), see also

Table 1
Participant characteristics.

	Healthy volunteers	Type 1 diabetes	Type 2 diabetes
Number (M:F)	17 (5:12)	18 (6:12)	15 (7:8)
Mean age (years, range)	39 (22–77)	52 (20–76)	67 (39–82)
Mean (SD) plasma glucose (mmol/L^{-1})[a]	5.59 ± 0.99	7.94 ± 3.04	9.88 ± 3.91

[a] Plasma glucose obtained from 'anchor' glucose value (see Methods section).

Supplementary Fig. 1a. Mean percentage difference was 5.1% (95% CI 3.7 to 6.6), see also Supplementary Fig. 1b. The fluoride-citrate overestimation extended to the comparison with the fluoride tubes at time zero: Mean absolute glucose baseline difference for the fluoride tubes compared to the fluoride-citrate tubes was 0.29 (0.21–0.42) mmol/L.

3.4.1. Glucose loss over time, following delayed plasma separation

As predicted from earlier studies [1,3–18], following delayed plasma separation, there was measureable loss of glucose. For fluoride tubes, loss became attenuated after 2 h. For the fluoride-citrate tubes, loss was minimal over the 24 h of study. These results are summarised in Fig. 2.

Table 2 summarises the absolute difference between baseline ('time zero') 'anchor' glucose and other glucose results, by collection tube type and time to plasma separation.

3.4.2. Absolute versus relative loss of glucose

In addition to recording absolute loss of glucose (mmol/L), Table 2 also includes the slope of the absolute glucose difference (y axis) against absolute glucose (x axis). If the slope is parallel to the x axis (i.e. the gradient is zero) and the associated CIs (confidence intervals) are narrow and *p* values large, this implies that the absolute glucose differences are independent of baseline glucose concentration. Thus within the context of the study and its wide baseline glucose values, glucose loss might best be expressed as an absolute, rather than as a percentage [20]. Without the presence of a glucose stabiliser, glucose loss over a 24 h time period shows best fit with a model of absolute loss. (See Table 2, glucose loss from lithium-heparin tubes at 2 and 24 h). This is supported by the findings in Supplementary Fig. 2, which shows the relationship between baseline glucose and glucose loss for the lithium-heparin tubes at 24 h, both an absolute and also a proportional (percentage) difference. As glucose concentration increases, absolute loss remains constant but percentage loss becomes smaller, also the 95% CI for the slope of the percentage loss regression line does not cross zero.

Further support for expressing glucose loss from collection tubes without glucose stabiliser as an absolute rather than a relative value, can be obtained by comparing percentage glucose loss at 24 h across the three participant subgroups, each of which had differing baseline glucose values. For healthy volunteers, 24 h loss was 70% and the corresponding values for type 1 and type 2 diabetic participants were 51% and 40% respectively. In contrast, there was no difference in absolute glucose loss (mmol/L) across the same participant subgroups (see Supplementary Table 2; differences non-significant using ANOVA). Considering all these results together, they confirm previous findings that, in the absence of a glucose stabiliser and when considering a wide range of glucose values, pre-analytical glucose loss is best fits a model of absolute (mmol/L), rather than a percentage or proportional value. Absolute glucose loss at 24 h was 3.98 mmol/L (see Table 2), equating to an hourly loss of around 0.2 $mmol \cdot L^{-1} \cdot hr^{-1}$.

In contrast to the lithium heparin collection system, the fluoride and fluoride-citrate collection systems did not show any consistent pattern of glucose loss across time points that might lead to favouring the expression of glucose loss as either an absolute or a percentage loss.

3.5. Haemolysis index

Details of the HI by time and tube collection type are show in Supplementary Table 3. Median HI at 'time zero' for the lithium-heparin, fluoride and fluoride-citrate tubes were 5.2, 15.9 and 8.9 mg/dL respectively. For clinical purposes, a HI of 36–100 is regarded as slight haemolysis and only one sample showed a HI > 100 at 'time zero', thus baseline haemolysis was minimal. For the fluoride and fluoride-citrate tubes, as anticipated, HI increased over time for tubes that were left standing before plasma was separated. In contrast, for the lithium-heparin-PST™ tubes, the haemolysis index was lower at times 2 and 24 h than it was at 'time zero'.

4. Discussion

The current study showed that baseline ('time zero') plasma glucose from fluoride-citrate tubes reads higher than the 'anchor' glucose value from lithium-heparin tubes by 0.37 mmol/L. This finding was unexpected, as several other studies using Terumo™ citrate tubes show minimal or no difference in plasma glucose when compared to samples collected into lithium-heparin or fluoride tubes, undergoing rapid pre-analytical processing [5,6,15]. Juricic has recently contrasted findings obtained from lyophilised citrate with liquid citrate tubes, both from her own studies and those of others. She reports that liquid citrate additives tend to over-estimate glucose compared to plasma glucose measured from both fluoride and lithium heparin tubes [16]. Three additional papers have been published following submission of Juricic's recent paper. Bonetti, in two separate papers [14,15], used GlucoEXACT citrate tubes (liquid citrate) and Terumo tubes (lyophylised citrate) and showed no difference in results, when compared to collection into lithium heparin tubes undergoing immediate cooling and rapid plasma separation. Gupta used a liquid citrate additive, prepared 'in house' and samples also underwent rapid plasma separation. Gupta's study showed citrate glucose results that were 6.1 mg/dL (0.34 mmol/L)

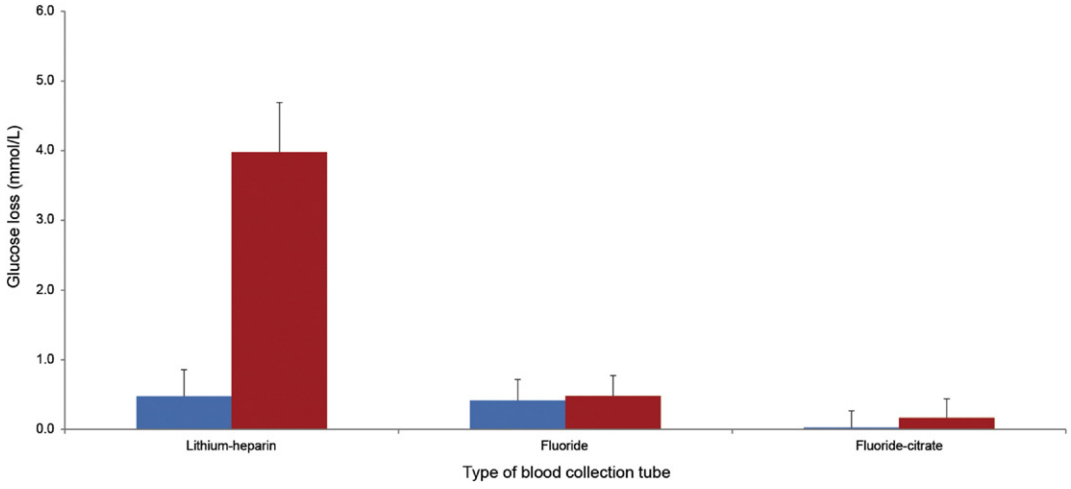

Fig. 2. Glucose loss over 2 and 24 h Glucose loss after 2 h is shown in the blue columns. Glucose loss after 24 h is shown in the red columns. Bars represent standard deviations. 'Time zero' samples from each different type of blood collection tubes were used as the baseline comparator.

Table 2
Analyses of absolute differences in measured glucose.

	'Time zero'[a]	Time 2 h	Time 24 h
Lithium-heparin-PST™ (lithium-heparin) tubes			
Absolute difference mmol/L (95% CI)	–	−0.47 (−0.58 to −0.37)	−3.98 (−4.18 to −3.78)
Slope[b] (95% CI)	–	0.02 (−0.02 to 0.05) $p = 0.36$	−0.03 (−0.09 to 0.03) $p = 0.29$
NaF-K-oxalate (fluoride) tubes			
Absolute difference mmol/L (95% CI)	0.08 (−0.03 to 0.19)	−0.34 (−0.44 to −0.23)	−0.40 (−0.51 to −0.29)
Slope[b] (95% CI)	0.03 (−0.002 to 0.06) $p = 0.07$	0.04 (0.01 to 0.07) $p = 0.02$	0.02 (−0.01 to 0.05) $p = 0.24$
NaF-EDTA-citrate (fluoride-citrate) tubes			
Absolute difference mmol/L (95% CI)	0.37 (0.26 to 0.48)	0.34 (0.23 to 0.45)	0.20 (0.10 to 0.31)
Slope[b] (95% CI)	0.02 (−0.01 to 0.05) $p = 0.22$	0.02 (−0.007 to 0.06) $p = 0.13$	0.03 (−0.006 to 0.06) $p = 0.12$

[a] The 'time zero' lithium-heparin-PST™ collection tube was placed on ice immediately after venesection and then underwent refrigerated centrifugation.
[b] The slope describes the comparison of absolute glucose difference (y axis) against 'time zero' lithium-heparin glucose (x axis).

higher at zero hours, compared to glucose from a fluoride collection tube [17].

There are inevitably several slight methodological differences between the various studies comparing plasma glucose from tubes containing citrate additive, with other types of collection tube. For example many earlier studies followed American Diabetes Association (ADA) and National Academy of Clinical Biochemistry guidelines for the preparation of plasma glucose samples, which state that blood may be placed on an ice slurry and plasma separated within 30 min of collection, rather than immediately after venesection [2]. Most previous studies focused on glucose concentrations at or near the normal range and recruited different study populations compared to the current study. It has also been hypothesised that differences in study findings between lyophilised and liquid citrate additives may be due to imprecise dilutional correction factors, but additional factors such as differences in sample pH, ionic strength, protein configuration and membrane transport mechanisms have also been considered [16]. Another point of difference between liquid and lyophilised citrate additives is that a liquid additive mixes quickly with blood and is therefore likely to induce rapid glycolytic inhibition, compared to a lyophilised additive. The extent to which these methodological differences account for the observed differences between studies, is unclear.

As others have suggested, even small differences in plasma glucose measurement are likely to change the prevalence of diabetes diagnosed by oral glucose tolerance test [13,21]. The current study and similar studies highlight the difficulty of defining 'true' plasma glucose, as there is no agreed, practical primary reference method for glucose that is available for use within the routine clinical biochemistry laboratory.

A small loss of glucose within the lithium-heparin tube (and also the fluoride tube) is likely to have occurred in the first several minutes after blood collection, as cooling (ice slurry) will not fully inhibit glycolysis until the sample's temperature drops from body temperature to just above freezing [2,12]. In contrast, the effect of acid on glucose stabilisation is thought to be virtually immediate [12], thus there is a differential rate of the glucose stabilisation effect for acid, compared to cooling. The temperature dependent glycolysis effect that occurs as the collection tube cools over several minutes causes an approximately 0.05 to 0.1 mmol/L lowering of glucose [12], so is likely to explain only a some of the observed 0.37 mmol/L glucose difference (95% CI 0.26–0.48), between the fluoride-citrate tubes and 'anchor' lithium-heparin tubes.

Although haemolysis in the study was modest (see Supplementary Table 3), the potential impact of haemolysis on results was nevertheless explored. At 'time zero', the higher HI seen with the fluoride tubes was not sufficient to have a significant impact on measured glucose, as glucose results from fluoride and the lithium-heparin-PST™ tubes were comparable. Since the baseline HI for fluoride-citrate tubes was intermediate between that of lithium-heparin-PST™ and fluoride tubes, it therefore follows that haemolysis is unlikely to have had an impact on measured glucose for the fluoride-citrate tubes. The increase in HI over time from sample collection for the fluoride and fluoride-citrate tubes mirrors findings from earlier studies. A reduction in HI for lithium-heparin-PST™ samples following delayed plasma separation is most likely due to uptake of haem, which contains hydrophilic components, by the PST™ gel [22].

Loss of glucose continued over the 24 h of study for all collection tubes, but was attenuated after 2 h for fluoride tubes and was minimal for the fluoride-citrate tubes. This pattern is similar to that described in other studies, [4–17], thus the current study extends previous observations across a wide range of glucose values.

The current study showed that, in the absence of a glycolytic inhibitor, the rate of glucose loss is easiest to describe as being independent of glucose concentration (i.e. it approximates zero order kinetics). Participant recruitment was designed to focus on glucose values in the normo- and hyperglycaemic range. Study design did not focus on frequent sampling time points, over a prolonged time period. Several previous studies have used the alternative approach of fixing the concentration of glucose (that is, samples were from healthy volunteers and were within a narrow glucose range), focussing instead on recording glucose loss over a prolonged time period [3,23]. These previous studies found that glucose loss is best described as a percentage loss (first order kinetics). It can therefore be hypothesised that the kinetics describing the rate of glycolysis within a closed in vitro system (blood collection tubes) are likely to be both complex and also dependent on substrate availability and on time. Describing kinetics in more detail would require a study over many hours, with recordings taken from a wide range of glucose concentrations, across multiple time points.

This study has several additional limitations. Firstly, there was no exploration of the mechanism(s) responsible for the high baseline ('time zero') glucose values seen with the fluoride-citrate tubes. Secondly, the range of glucose values for the participants with diabetes showed no extreme glucose values, thus the range was typical of outpatient based study volunteers. It is therefore unknown whether pre-analytical glucose loss in patients with acute metabolic perturbations such as severe hypoglycaemia would follow the same pattern as that seen in the current study. Thirdly, we are aware of three commercially available brands of fluoride-citrate tube, all of which lower pH to around 5.5, however only one brand of collection tube (Terumo™) was assessed in the current study and as discussed above, liquid citrate collection tubes show a greater tendency to over read plasma glucose, compared to tubes using a lyophilised additive.

5. Conclusions

Plasma glucose collected into lyophilised fluoride-citrate tubes 'reads high', when compared to samples collected into lithium-heparin or fluoride tubes, undergoing rapid cooling and early separation of plasma. This finding is seen across a range of glucose values. Knowing that fluoride-citrate tubes may 'read high' across a range of glucose values, albeit by only a small margin, is likely to help with interpretation

of results from this collection system. There are many everyday situations where delayed separation of plasma from the cellular component of blood is inevitable, for example when collecting clinical or research samples in geographically remote settings. In these situations, the benefits of fluoride-citrate induced glucose stabilisation are likely to outweigh the disadvantage of a small over-estimation in measured plasma glucose, when compared to other collection tube systems.

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Declarations

HL is on the Sanofi (NZ) speakers' bureau and has presented on behalf of Eli Lilly. CMF has received honoraria from BioRad and Merck Sharp and Dohme.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.clinbiochem.2016.05.017.

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