

Master of Medicine in Anaesthesiology
Research Submission



A comparative study of ROTEM- EXTEM results obtained from EDTA-treated whole blood samples and Sodium Citrate- treated whole blood samples in healthy volunteers.

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Submitted to: Faculty of Health Sciences, University of Cape Town

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It has been submitted for the degree of Master of Medicine (Anaesthesiology) and is based upon research conducted in the Department of Anaesthesia of the Groote Schuur Hospital, Anzio Road, Observatory, Cape Town.

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31 October 2016

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List of abbreviations

aPTT	Activated partial thromboplastin time
α	Alpha-angle
CFT	Clot formation time
CT	Clotting time
CI	Confidence interval
EDTA	Ethylenediamine tetraacetic acid
INR	International normalized ratio
Ca^{2+}	Ionized calcium
MCF	Maximum clot firmness
PTT	Prothrombin time
ROTEM	Rotational thromboelastometry
SD	Standard deviation
TF	Tissue factor
VET	Viscoelastic test
MPC	Mean platelet component
TEG	Thromboelastography

Abstract

Background

A number of anticoagulants are available in clinical use to preserve blood samples in liquid form until a suitable time for laboratory testing.

Rotational thromboelastography is usually performed on a blood sample that has been anticoagulated with sodium citrate and then recalcified immediately prior to testing.

In our institution we have had shortages of citrated Vacutainer® sample tubes. The use of a single in vitro anticoagulant promises to cut costs, simplify laboratory processes as well as limit the amount of blood drawn from patients.

This together with the known problems with using citrate as an anticoagulant for viscoelastic testing (VET) prompted us to investigate the suitability of EDTA as anticoagulant for VET.

Method

Blood samples from 20 healthy volunteers were divided into citrated and EDTA Vacutainer® tubes. A ROTEM EXTEM® assay was performed on each sample in both groups following the manufacturer's guidelines. Clotting time (CT), clot formation time (CFT), alpha angle (α -angle) and maximum clot firmness (MCF) results were compared. Ionised calcium concentrations were measured on each sample before and after recalcification with CaCl_2 to determine if there was a significant difference in post - recalcification ionised calcium concentrations between the groups.

Results

The results from the two groups were treated by Bland-Altman analysis. Apart from MCF values there was significant bias between all parameters measured in the two groups. The limits of agreement for all parameters apart from MCF were unacceptable.

Conclusion

We found that ROTEM EXTEM® results from EDTA samples were not comparable to or interchangeable with those from citrated samples.

The difference in results is not due to differences in ionised calcium concentration levels in the samples post-recalcification as the ionised calcium concentrations in both groups post-recalcification were adequate for coagulation.

EDTA samples did show superior consistency in all parameters and may be a suitable alternative for sample preservation for VET if reference ranges can be established.

Introduction

Rotational thromboelastometry (ROTEM®) is a whole blood coagulation test where clotting is activated by a biochemical agonist in a reproducible way. It is one of a few point-of-care devices used for viscoelastic testing. Specific reagents are available that standardize the in vitro coagulation process and also speed up the analysis, an advantage over conventional laboratory based coagulation screens. (Macey, Azam et al. 2002, Ganter and Hofer 2008) Viscoelastic tests (VET) assess the viscoelastic properties of blood samples under low shear conditions. It provides information about coagulation initiation, clot propagation kinetics, clot firmness, fibrin-platelet interaction and fibrinolysis.

It plays an increasingly important role in the point-of-care management of bleeding patients in the acute perioperative period. (Ganter and Hofer 2008, Bolliger, Seeberger et al. 2012, Espinosa and Seghatchian 2014, Karon 2014, Whiting and DiNardo 2014)

In the ROTEM® EXTEM assay a 300µl whole blood sample is transferred to a plastic cup with a precision pipette. The cup is pre-warmed to 37°C and preloaded with tissue factor and calcium chloride. The reagent activates the tissue factor coagulation pathway and shortens the test duration. (Macey, Azam et al. 2002, Lang 2005, Ganter and Hofer 2008) This is similar to the prothrombin time (PT) test. The calcium chloride is necessary to replenish the ionized calcium in the sample that has been removed by the chelating agent.

Conventionally, blood samples for laboratory coagulation testing such as prothrombin time and activated partial thromboplastin time (aPTT) and ROTEM® analysis are drawn into a Vacutainer® tube containing sodium citrate. (Horsti 2001, Macey, Azam et al. 2002) The sodium citrate inhibits clotting of the specimen via the action of the citrate ion to mildly chelate free ionized calcium, making ex vivo calcium unavailable to the coagulation system. (Whitten and Greilich 2000, James and Roche 2004, Lippi, Salvagno et al. 2010)

Ethylenediamine tetraacetic acid (EDTA) is a polyprotic acid containing four carboxylic acid groups and two amine groups with lone pair electrons that strongly chelates calcium and several other metal ions in 1:1 metal

EDTA-complexes. It is not routinely used for coagulation testing. (Horsti 2000, Banfi, Salvagno et al. 2007, Lippi, Salvagno et al. 2010)

Although there is literature that shows that conventional laboratory coagulation testing can be done on EDTA-treated blood samples (Horsti 2001), citrate remains the anticoagulant of choice as the results obtained are more consistent. (Lippi, Salvagno et al. 2010)

In our institution we have stock issues from time to time and availability of citrated Vacutainer® tubes have been problematic. A universal sample would solve this problem as well as reduce the volume of blood collected from patients and reduce the number of venepunctures performed.

No literature is available to ascertain if ROTEM® EXTEM (Rotational thromboelastography; TEM International, Munich, Germany) analysis results on EDTA-treated whole blood samples is compatible with those obtained from citrated samples. The aim of this study is to establish this and also to evaluate the ionized calcium levels in EDTA and citrate samples when recalcified as per ROTEM® protocol. A study by Horsti et al found that total calcium levels in sample reagent mixtures for prothrombin testing are very similar after recalcification, resulting in comparable PT/INR results on EDTA and citrate-treated treated samples (with a correction factor). (Horsti 2000, Horsti 2001) A study published by James et al in 2004 showed that the minimum ionised calcium concentration for initiation of coagulation in vitro is 0.33 mmol/L and that in samples with Ca^{2+} levels above 0.56 mmol/L clotting was normal in almost all whole blood samples. (James and Roche 2004) Both EDTA and citrate chelate calcium albeit with different affinities. This should not matter if samples are flooded with calcium prior to coagulation testing as is the norm with thromboelastography.

If there is a significant difference in the ionized calcium levels after recalcification as per ROTEM® protocol, this might be the key factor in any differences in clotting parameters obtained

Our hypothesis was that ROTEM® EXTEM parameters obtained from EDTA treated whole blood samples should be comparable to those obtained from citrated whole blood samples.

Structured Literature review

Objectives of the literature review:

The main objective of this literature review was to evaluate the current published literature on the listed topics/points. There is no published data on the use of EDTA (Ethylenediamine tetraacetic acid) treated blood samples in Rotational Thromboelastometry (ROTEM®) specifically, so a comprehensive review was made of the methodology of thromboelastography and rotational thromboelastometry as well as factors that may influence results obtained. The two in vitro anticoagulants compared in my study were reviewed and specifically their influences on ionised calcium levels in the samples as this was believed to be the key factor in any potential differences in results.

Method of literature review

A PubMed literature search was performed using the free text and MeSH thesaurus terms listed below in different combinations.

- 'Anticoagulant'
- 'Edetic acid'
- 'EDTA'
- 'Ethylenediaminetetraacetic acid'
- 'Pharmacology'
- 'Pre-analytical factors'
- 'Rotational thromboelastometry'
- 'ROTEM'
- 'Sample'
- 'Sodium citrate'
- 'Standard plasma coagulation test'
- 'TEG'

- 'Thromboelastography'
- 'Thromboelastometry'
- 'Vacutainer'
- 'Viscoelastic test'
- 'Viscoelastic'

A Google Scholar search was performed using the same terminology.

Relevant research papers were identified by their abstracts. An author-date referencing method was used.

Further appropriate papers were identified by searching reference lists of relevant published papers.

Device manuals and manufacturer's guidelines were obtained via Google search.

Blood sample preservation for laboratory testing

An anticoagulant is often added to a blood sample or specimen to maintain blood in a fluid state for diagnostic laboratory testing. There are a number available commercially and suited for different types of laboratory analysis.

Properties of the ideal anticoagulant:

Enable laboratory to obtain suitable plasma from the sample for clinical chemistry analyses and coagulation studies

Not expensive

A universal coagulant will optimise laboratory workflow, sample volume and turnaround time. (Banfi, Salvagno et al. 2007)

Preserve activation status of platelets until samples are analysed (Macey, Azam et al. 2002)

Does not affect staining properties.

Halt enzyme processes completely until reactivation by another reagent.

As EDTA and Sodium Citrate are the main anticoagulants in use for in vitro testing and the anticoagulants compared in this study, a comprehensive review was done on their indications, methods of action and limitations.

Sodium Citrate as in vitro anticoagulant

Sodium citrate is primarily used as an anticoagulant for coagulation studies. It has been used for over a century in coagulation testing. In fact many studies showed that samples other than citrated plasma are unsuitable for coagulation testing as the results were unreliable and misleading.(Lippi, Salvagno et al. 2010)

Commercially there are two solutions of sodium citrated evacuated tubes available namely 3.2% and 3.8%. There is contradicting evidence as to what degree the solution used influences results.

Sodium citrate causes less spontaneous activation of platelets in vitro than EDTA. Citrate causes no initial change in volume or shape of platelets when blood is collected, but it causes platelets to become spherical and increase in size over time.(Macey, Azam et al. 2002)

Citrate binds calcium more avidly than magnesium and this may result in a relative increase in magnesium concentration in the citrated blood sample.(James and Roche 2004)

A previous study by James et al showed that ionised magnesium had a negligible effect on clotting as measured by thromboelastography.(James and Roche 2004)

Citrated plasma contains significantly reduced levels of potassium, chloride, calcium and magnesium, but sodium is markedly increased compared with native plasma.(Lippi, Salvagno et al. 2010)

Lippi et al found that the mean total calcium level in citrated blood samples was 1.98 ± 0.02 mmol/l compared to our data where the ionised calcium level was undetectable prior to recalcification. The mean total calcium level in untreated serum in their study was 2.37 ± 0.003 mmol/l. The reason for this is that only ionised calcium is chelated by citrate. The free ionised calcium is also only mildly chelated by citrate according to this author. (Lippi, Salvagno et al. 2010) However it is only the ionised fraction that is relevant to coagulation.

The difference between this study by Lippi and the one we are currently conducting is that the electrolytes were tested on the plasma component of blood only. Total calcium was evaluated as opposed to ionised calcium

and was assayed using a Roche/Hitachi Modular System P as opposed to a blood gas analyser as in our study.(Lippi, Salvagno et al. 2010)

EDTA as in vitro anticoagulant.

EDTA is the anticoagulant recommended for full blood counts and white blood cell differential analysis by the National Committee for Clinical Laboratory Standards. It has excellent cell preservation properties.(Macey, Azam et al. 2002, Stokol and Erb 2007)

EDTA is a polyprotic acid that contains 4 carboxylic acid groups and two amine groups with lone pair electrons that bind to and chelate calcium and other metal ions in a 1:1metal –EDTA complex. It results in a strong bond with these metal ions like calcium that are cofactors for enzymatic processes and thus prevents some enzyme reactions. As calcium is an important cofactor for clotting enzymatic reactions (see discussion on calcium's role in coagulation) it prevents the clotting of blood in the specimen tube which then remains in fluid state suitable for laboratory testing. EDTA results in the optimal extended stability of cells and particles in blood.(Banfi, Salvagno et al. 2007)

Three different formulations of EDTA are available. In Europe and Japan the dipotassium salt of EDTA (K_2EDTA) is mostly used for cytometry as recommended by the International Council for Standardization in Haematology. In the United States and the United Kingdom the tripotassium salt (K_3EDTA) is mainly used. (Macey, Azam et al. 2002, Banfi, Salvagno et al. 2007)

K_2EDTA does not result in dilution of blood samples as it is spray dried on the sides of the tube. This is important especially with small sample volumes.

EDTA salts are hyperosmolar and can lead to a small degree of shrinkage of cells. K_2EDTA has a smaller osmotic effect on cells than K_3EDTA . This effect is not clinically important.

Blood samples are stable in EDTA for up to 24 hrs and longer if stored at 4° Celsius. (Banfi, Salvagno et al. 2007)

EDTA does not affect staining of smears with stains such as May-Grunwald Giemsa. (Banfi, Salvagno et al. 2007)

It is used for the measurement of cytokines, protein and peptide and cardiac markers. (Banfi, Salvagno et al. 2007)

The cytokine levels measured in plasma might be altered by EDTA if the stored sample is kept too long before separating the plasma. (Banfi, Salvagno et al. 2007)

EDTA plasma is one of the better agents for proteomics studies. It has a strong stabilising effect on several labile molecules and cause no plasma dilution that may influence the protein profile. (Banfi, Salvagno et al. 2007)

EDTA is also used for measurements of certain drugs, cardiac markers, molecular biology, virology and infectious diseases. (Banfi, Salvagno et al. 2007)

A limitation of EDTA is that it does not completely stabilise platelets. It may lead to changes in measured platelet count, mean platelet volume, mean platelet component, platelet activation and platelet aggregation.

Platelets quickly becomes spherical when EDTA is added to a blood specimen. Initially they remain the same volume and then increase in size before decreasing again. (Macey, Azam et al. 2002)

EDTA, especially K_2EDTA is not recommended for tests of platelet adhesion as it may result in platelet activation immediately after blood drawing. This is more prevalent with K_2EDTA and heparin than with citrate. (Banfi, Salvagno et al. 2007)

EDTA may cause an increase in CD62P expression of the platelet and this combined with a decrease in MPC (mean platelet component) is an indicator of platelet activation. These changes are temperature dependant. (Macey, Azam et al. 2002)

EDTA can cause in vitro platelet aggregation (clumping) and a pseudothrombocytopenia. (Stokol and Erb 2007) The prevalence of this phenomenon is around 0.1% and more prevalent in samples from thrombocytopenic patients. This might influence platelet count in laboratory testing but does not usually signify platelet dysfunction or abnormalities. EDTA may cause structural changes in platelets that leads to the expression of GPIIb/IIIa on the cell surface. This complex exposed on the cell surface may trigger a reaction with IgM autoantibodies. This reaction is not induced or mediated by the calcium chelation effect of EDTA, but by other mechanisms. (Macey, Azam et al. 2002, Banfi, Salvagno et al. 2007, Stokol and Erb 2007)

No anticoagulant currently in clinical use can prevent platelet activation but the degree depends on anticoagulant used. (Macey, Azam et al. 2002)

EDTA has been used in studies to evaluate platelet status and function and if the effects of EDTA are taken in consideration the data could still be clinically useful, but its use in measuring platelet activation remains questionable (Macey, Azam et al. 2002)

Calcium, iron and magnesium levels cannot be measured in EDTA samples due to chelation of these metal ions. K_3EDTA contains markedly

increased potassium levels. Na_2EDTA contains markedly elevated levels of sodium. Chloride levels are decreased in EDTA plasma. (Lippi, Salvagno et al. 2010)

Thus, sodium and potassium levels cannot be measured correctly in a sample when they are used as a salt in the formulation.

With newer automated and innovative tests the role of EDTA may need to be reviewed in haematology as EDTA may have influences on these tests that are unknown.

The use of EDTA in coagulation testing

A literature review was done to establish prior use of EDTA in coagulation tests.

Prothrombin time (PT) is widely used to measure the effect of oral anticoagulant therapy. PT is usually measured on blood collected into a collection tube and diluted or mixed with different preparations of sodium citrate. (Horsti 2001). The International Normalized Ratio (INR) was established to eliminate dependence on sensitivity of different reagents.

Horsti et al tried to establish the similarity of INR and PT results in seconds between citrate and EDTA samples using the Owren method in patients on oral anticoagulation therapy. They used citrate based calibrators and a correlation equation to calculate INR and results in seconds for EDTA samples. They found a strong correlation between results obtained from the same patients, but a correlation equation needed to be used as the results differed numerically. (Horsti 2000, Horsti 2001)

They found that the difference in results depended mainly on sample dilution by citrate solution and when the PT test was calibrated with normal EDTA plasma the differences disappeared. There were no publications found on VET done on EDTA samples on humans.

Crist et al also evaluated the use of EDTA in standard plasma coagulation testing and found the biggest differences between EDTA and citrate treated plasma was for prothrombin time, partial thromboplastin time as well as in functional assays for factors V, VIII, protein C and protein S. (Crist, Gibbs et al. 2009) They advise against the use of EDTA for plasma coagulation testing.

Role of Ionised calcium in blood coagulation

Calcium plays a vital role in in vivo coagulation. It is a co-factor for clotting factors V, VII, VIII, IX, X and XIII. It is also necessary for pro-thrombin to thrombin conversion and the formation of fibrin from fibrinogen. (James and Roche 2004)

The physiological ionised calcium concentration in blood is 1.1-1.4 mmol/L, which is well above the minimum levels of ionised calcium necessary for reactions in the coagulation cascade.

Variation of ionised calcium concentrations should not have any significance on specific coagulation factor activation as long as it is above a certain threshold value. The aim of a study done by James et al in 2004 was to obtain this threshold value by using VET on whole blood samples. The samples were citrated and then recalcified with varying amounts of 10% calcium chloride. After recalcification the ionised calcium concentration in each sample was measured with a blood gas analyser and TEG® analysis was performed on the samples simultaneously. (James and Roche 2004)

They found the threshold value for initiation of clotting was between 0.33 and 0.39 mmol/L. An ionised calcium concentration of 0.56 mmol/L was necessary for a normal r-, k-time and α –angles as per manufacturer's normal values. An ionised calcium concentration of greater than 0.41 mmol/L was adequate for normal maximal amplitude levels. They found no correlation between ionised calcium concentration and clot lysis in this study. They also found no correlation between increasing ionised calcium concentrations within the clinical range and any TEG® parameters obtained. (James and Roche 2004)

Ataullakhanov et al defined a lower limit of 0.25 mmol/L Ca^{2+} for thrombin generation and an upper limit of 0.5 mmol/L Ca^{2+} above which thrombin generation was not further enhanced. This study only looked at thrombin generation and not at other parameters of coagulation. (Ataullakhanov, Pohilko et al. 1994)

The threshold of ionised calcium concentration necessary for platelet aggregation is 2×10^{-4} mmol/L which is much less than that required for fibrin formation. (James and Roche 2004)

Calcium chelation does not inhibit contact activation in the specimen tube. This could explain why an accelerated trace is seen in stored citrated samples as the contact activation pathway up to the activation of factor IX continues in the presence of a calcium chelator and the activated precursors are "ready" when the sample is recalcified. (Roche and James 2003) Overall, the literature suggests that VET results from citrated blood

samples are relatively unstable unless the sample is stored for at least 30 minutes.

Ionised calcium levels in blood samples for coagulation testing

There is a paucity in the literature regarding ionised calcium concentrations in samples and the effect it has on coagulation results. Apart from the study by James already discussed, Horsti et al also found that in PT/INR testing using the Owren method the coagulation reagent contained sufficient ionized calcium to allow coagulation with either sample type (EDTA or Citrate). Ca^{2+} in their reaction mixtures after recalcification was 1.525 mmol/L for citrate and 1.708 mmol/L for EDTA. These measurements was done on the plasma component of blood only.(Horsti 2000)

Lippi et al evaluated total calcium concentrations in native, citrated and EDTA plasma and found the values to be 2.37 mmol/L, 1.98 mmol/L and 0.00 mmol/L respectively. The authors concluded that the total calcium concentration remained high in the citrated plasma due to the fact that citrate only binds ionised calcium.

Principles of viscoelastic testing (VET)

VET has become a popular monitoring mode for haemostasis and transfusion management in trauma, major surgery and haemophilic patients.

It can be used as a point-of-care coagulation device in the operating theatre and emergency room reducing the time to obtain information regarding haemostasis to few minutes.(Bolliger, Seeberger et al. 2012)

VET assesses clot formation, platelet function, fibrinogen levels, thrombin generation and clot breakdown in real time. It is thus used to assess hyper- and hypo-coagulable states and to guide therapeutic interventions such as transfusion of platelets, fresh frozen plasma and coagulation factor concentrates.(Lang 2005, Bolliger, Seeberger et al. 2012, Whiting and DiNardo 2014)

VET assesses the viscoelastic properties of clot formation under low shear conditions in whole blood. This is done after adding a specific coagulation activator such as tissue factor extracted from rabbit brain in

the case of the ROTEM EXTEM® assay. The interaction between activated platelet glycoprotein IIb/IIIa receptors and polymerising fibrin during thrombin generation results in a tensile or viscoelastic force between the cup of the device and an immersed pin. The tensile force is reduced by fibrin degradation by fibrinolysis. (Bolliger, Seeberger et al. 2012, Whiting and DiNardo 2014)

Two devices are commercially available namely TEG® (Thromboelastograph – Haemoscope/Haemonetics, Niles, Ill) and ROTEM® (Rotational thromboelastography; TEM International, Munich, Germany). (Ganter and Hofer 2008, Bolliger, Seeberger et al. 2012) They provide essentially the same information, but because of differences in operating characteristics the results are not interchangeable. They use different nomenclature to describe the same parameters. (Whiting and DiNardo 2014)

A variety of activators and inhibitors are available to accelerate the test times on these devices and to obtain differential diagnostic information regarding a patient's haemostatic status. (Lang 2005) Different activators and inhibitors isolate parts of the coagulation cascade that are then better evaluated.

In the TEG® apparatus a metal pin that is suspended by a torsion wire is immersed in a metal or plastic cup containing whole blood. The disposable cup oscillates through a 4.75° angle around the fixed immersed pin. The torque generated between the pin and cup by clotting of the blood is detected and transmitted electronically to a computer processing unit. As the clot later dissolves the torque is diminished. It is then presented as a tracing of clot formation and dissolution over time. (Bolliger, Seeberger et al. 2012, Whiting and DiNardo 2014) Clot formation is initiated by recalcification and (usually) stimulation of Factor XII by kaolin or celite. As a result, since activation of FXII is not inhibited by chelation of calcium, citrated samples may show variable rates of activation unless stabilised for 30 minutes prior to testing (see below).

The ROTEM® apparatus differs in that it consists of a plastic pin that rotates in a fixed cup made from polymethylmethacrylate through an angle of 4.75°. The torque generated is transmitted optically and not electronically. (Ganter and Hofer 2008)

The ROTEM® instrument has four measurement channels that can be simultaneously and independently used compared with the TEG® system's two channels. (Lang 2005, Bolliger, Seeberger et al. 2012)

The ROTEM® system contains an automated pipetting system that allows mixing to be performed in a standardized way. (Lang 2005)

It is less sensitive to vibration than the TEG® apparatus.(Whiting and DiNardo 2014)

The whole blood sample required for ROTEM® analysis is usually collected in a Vacutainer® tube containing sodium citrate. The sample is then recalcified when the blood sample is pipetted into the sample cup.

Assays available on ROTEM®:

EXTEM: Contains tissue factor as activator of the extrinsic pathway.

INTEM: Ellagic acid and phospholipids. Evaluation of intrinsic pathway.

HEPTEM: INTEM plus heparin inhibitor. Compared to INTEM analysis to evaluate heparin effect.

FIBTEM: Cytochalasin D inhibits platelet function after tissue factor (TF) activation. When compared to EXTEM analysis allows qualitative analysis of fibrinogen contribution to clot strength.

APTEM: Aprotinin to inhibit plasmin and fibrinolysis and EXTEM reagent. Compared with EXTEM analysis to evaluate fibrinolysis. (Lang 2005, Bolliger, Seeberger et al. 2012, Whiting and DiNardo 2014)

Results obtained from native bloods samples cannot be directly compared to those from citrated samples that has been recalcified.

Compared with the TEG® device intra- and inter- individual operator variability was superior with ROTEM®.(T.Haas 2014)

ROTEM® Parameters:

As our study was done using the ROTEM® device an overview of ROTEM® parameters are included.

CT: Clotting time (equivalent to TEG® R-time)

CFT: Clot formation time (equivalent to TEG® K-time)

α- angle: Alpha angle (equivalent to TEG®)

MCF: Maximal clot firmness (equivalent to TEG® MA)

A5 –A30: Amplitude at 5-30 minutes

CLI30-CLI60: Clot Lysis Index

(Whiting and DiNardo 2014)

The most important parameters obtained from ROTEM® are the amplitude or clot firmness which represent the viscoelasticity of the sample (MCF).

Additional parameters are obtained from the time course of viscoelastic changes. It depicts the rate and stability of clot formation e.g. CT, CFT, α (angle at 2 mm amplitude) and Lysis times. (Bolliger, Seeberger et al. 2012)

The clotting time (CT) reflects initial fibrin formation after thrombin generation. It is defined by the computerised trace reaching an amplitude of 2 mm. (Lang 2005, Ganter and Hofer 2008, T.Haas 2014)

Clot formation time (CFT) is the time it takes for amplitude to increase from 2 to 20 mm and alpha angle (α) is the tangent of the slope during this period. (Lang 2005, T.Haas 2014)

Maximum clot firmness (MCF) is the maximal amplitude of the curve and can be reliably predicted from A5 and A10 values. It signifies the maximum firmness the clot achieves. Platelet activation and aggregation, fibrin polymerisation and cross-linking by factor XIII all play a role in MCF. (T.Haas 2014)

A5 – A30 values signify the clot strength after the respective time intervals. (Lang 2005, T.Haas 2014)

The clot lysis index describes degree of fibrinolysis at the specific time.

Reference ranges for ROTEM EXTEM® assay:

CT: 42-77 seconds

CFT: 46 – 148 seconds

α - angle: 63 – 81 °

A10: 43 -65 mm

MCF: 49 – 71 mm

These reference ranges were obtained from a multi-centre investigation on reference ranges for ROTEM® done by Lang et al in 2004. The ROTEM® user manual refers to this study by Lang for reference ranges. (Lang 2005, Pentapharm 2007, Bolliger, Seeberger et al. 2012)

TEG® parameters and reference ranges will not be discussed as ROTEM® was the device used in our study.

Preanalytical factors playing a role in VET

Reference ranges in VET depend to some degree on pre-analytical factors. Multiple studies have been done on both the TEG® and ROTEM® systems to evaluate the influence of these factors on reference ranges and reproducibility.

Threshold values for haemostatic interventions or clinical outcomes should be locally evaluated for each system and available components/reagents used. (Bolliger, Seeberger et al. 2012)

Method of sample acquisition

Platelet activation and tissue factor contamination during venepuncture will affect VET results. It is important to minimize tissue trauma during venepuncture. In animal studies the R-time (TEG®) was shortened with poor venepuncture technique. The use of a discard tube (where first few millilitres of blood obtained are discarded) mitigates the effect of a traumatic venepuncture and is good practice. (Flatland, Koenigshof et al. 2014)

Any large vessel with free flowing blood can be used for sampling.

The use of 21g or larger needles is recommended as well as the use of evacuated tubes for collection of blood. If blood is taken from a central line contamination with heparin should be considered. The method for collection and storage of blood in an institution should be standardised for clinical studies and to evaluate serial sampling in a patient in a clinical scenario to eliminate the contribution of preanalytical factors. Method specific reference ranges should be used if they are available (Flatland, Koenigshof et al. 2014)

A recent review paper found no studies comparing results from arterial and venous blood. (Flatland, Koenigshof et al. 2014)

The use of an anticoagulant

There are quite a few studies on the effect of citrate and specifically the time the citrated sample were stored on results obtained in VET.

James et al found that recalcified citrated samples has shortened R –and K- times and an enhanced α -angle when compared to native whole blood samples when evaluated with TEG®. This was prevalent in samples

stored at room temperature as well as those that were refrigerated. The maximal amplitudes of the samples remained unaffected. This result of relative hypercoagulability found in citrated samples were also obtained by Camenzind et al. (Camenzind, Bombeli et al. 2000, Roche and James 2003)

This was ascribed to the incomplete inhibition of the activation of the coagulation cascade in the sample while it was stored. (Zambruni, Thalheimer et al. 2004)

The concentration of the anticoagulant will also play a role.

Transport of sample

There is some evidence that transport of the sample in a pneumatic tube system may alter results, but this is of no relevance to the present study.(Martin, Schuster et al. 2012, Espinosa and Seghatchian 2014, Flatland, Koenigshof et al. 2014)

Sample storage time and temperature

Animal studies showed that for tissue factor activated TEG®, samples that has been stored for a prolonged time appeared hypercoagulable when compared to samples stored for a shorter period.

Strong coagulation activators might eliminate the effects of prolonged sample storage.(Flatland, Koenigshof et al. 2014)

Camenzind et al showed stability in samples stored for up to 8 hours when a potent activator of coagulation (Celite) was used.(Camenzind, Bombeli et al. 2000, Zambruni, Thalheimer et al. 2004)

Animal studies found that in refrigerated samples (4°C) CT, MCF was significantly higher and α -angle significantly lower when compared to room temperature samples. There does not appear to be any major difference in samples stored at room temperature and those kept at 37°C for up to 30 minute storage times.(Flatland, Koenigshof et al. 2014).

Roche and James found a hypercoagulable trace in refrigerated samples as well that they ascribed to cold activation of factor VII and platelets.(Roche and James 2003)

Martin et al found a prolonged ROTEM EXTEM® CT after 30 minutes of storage of citrated samples. (Martin, Schuster et al. 2012)]

Theusinger et al found no differences in EXTEM®, INTEM® and FIBTEM® assays over 2 hours of storage.(Martin, Schuster et al. 2012)

Bohner and von Pape found that for ROTEM® analysis citrated samples could be stored for up to 4 hours without clinically significant changes in the results.(Bohner and von Pape 2003, Lang 2005)

Repeated sampling

There is a trend towards hypercoagulability when repeated sampling is done on the same specimen. Repeated sampling leads to reduced R-times, K-times as well as increased α - angle. The contact activated coagulation pathway is probably activated by repeated sampling. (Zambruni, Thalheimer et al. 2004)

System and activators used

The use of a potent activator significantly reduces changes observed during sample storage compared to non-activated tests.(Lang 2005, deLaforcade, Goggs et al. 2014)

CT and CFT are strongly dependant on the type and concentration of activator.

There is more variation in results with kaolin based tests such as TEG®. Variation is less with tissue factor activated tests such as EXTEM.

Variation is least for MA/CFT, independent of reagent used.(Bolliger, Seeberger et al. 2012)

Haematocrit of sample

There are studies that show hypercoagulable TEG® tracings in samples that were haemodiluted to haematocrits of 10-30% (Bolliger, Seeberger et al. 2012)

Haematocrit values below 25% results in an increased FIBTEM® MCF. This is due to an increased plasma fraction in the sample. The correlation between plasma fibrinogen levels and FIBTEM® MCF levels are increased with a low haematocrit.

Usefulness of VET in clinical settings

The management of a bleeding patient is quite complex. Therapeutic interventions range from the transfusion of whole blood or allogeneic blood components to specific procoagulant drugs and purified clotting factors or a combination of the above.(T.Haas 2014)

The therapeutic intervention may be empiric, based on standard plasma coagulation tests or from the results of point-of-care devices.(T.Haas 2014, Whiting and DiNardo 2014)

The goals of intervention are to stop bleeding, minimize risk of thrombotic events and to reduce the transfusion of allogenic blood products.(Whiting and DiNardo 2014)

There is some evidence that VET-based algorithms reduce transfusion requirements and blood loss in massive trauma, cardiac surgery and liver transplantation.(Kang, Martin et al. 1985, Bolliger, Seeberger et al. 2012, Karon 2014, Whiting and DiNardo 2014) (Espinosa and Seghatchian 2014)

The liberal transfusion of blood products has been associated with complications such as increased mortality, transmitted infections and organ dysfunction.(Bolliger, Seeberger et al. 2012)

Algorithms to guide transfusions based on VET have been implemented successfully. VET-based transfusion algorithms in paediatric cardiac surgery leads to reduced post-operative blood loss, reduced need for transfusion and a shorter intensive care unit stay. (Nakayama, Nakajima et al. 2014)

It should be noted however, that the cut-off values for transfusion are not well validated at this time. Serial VET measurements are useful in guiding haemostatic interventions, but should be used in conjunction with other clinical and laboratory parameters. The patient's body temperature, haematocrit and acid-base status should be taken in consideration. A normal VET may indicate that further surgical exploration is warranted in a bleeding patient.(Whitten and Greilich 2000, Bolliger, Seeberger et al. 2012)

It is important to understand the limitations and working principles of VET when interpreting results and using it to guide haemostasis therapy. Using it correctly may result in optimizing goal directed transfusion therapy and thus avoiding empirically administering large volumes of multiple components and the related morbidity and mortality.(Bolliger, Seeberger et al. 2012, Whiting and DiNardo 2014)

Limitations of VET

The global nonspecific nature of the VET measurements may be both its greatest weakness and strength. Although specific coagulation assays does not necessarily correlate with blood loss, they do provide specific diagnoses that respond to specific treatments.(Whitten and Greilich 2000)

TEG® and ROTEM® are both moderately complex devices to operate requiring trained personnel. They require frequent stringent quality control procedures.(Ganter and Hofer 2008, T.Haas 2014)

If situated in a centralized laboratory it requires a rapid transport system as well as a computer and network for online display of results to reduce turnaround times.(Bolliger, Seeberger et al. 2012). The procedures for transport should be standardised otherwise it may influence reproducibility of results.

Pin slippage or vibration of the testing platform may result in artefacts and aberrant results. (Karon 2014)

A variety of other preanalytical factors may influence VET results. These factors were discussed previously.

VET is performed under low shear stress / no flow conditions and in the absence of endothelial cells.(Ganter and Hofer 2008, Espinosa and Seghatchian 2014, T.Haas 2014)

The positive predicted value of ROTEM® in elective surgery to predict bleeding is limited. As with standard plasma coagulation tests the patient's clinical condition and evidence of active bleeding should be considered before any therapeutic intervention.

The negative predictive value of ROTEM® is high, thus ongoing bleeding with a normal ROTEM® result is indicative of surgical bleeding.(Haas, Fries et al. 2014)

ROTEM® is not very sensitive in detecting platelet function disorders or the effect of anti-platelet drugs.(T.Haas 2014). It is not possible to distinguish bleeding as a result from thrombocytopenia and dilutional coagulopathy when using a single ROTEM® assay activated with kaolin.

Parallel EXTEM® and FIBTEM® analysis is necessary to distinguish hypofibrinogaemia from thrombocytopenia. The Claus determination of fibrinogen levels remain a superior functional fibrinogen assessment.(Whiting and DiNardo 2014)

There is still limited conclusive evidence and a lack of adequately powered outcome studies on the success of VET in predicting bleeding and changing perioperative clinical outcomes.

This together with a lack of the quality-assurance methodologies required by regulatory agencies such as the Food and Drug Administration (FDA), limits the acceptance of point-of-care viscoelastic tests.(Whitten and Greilich 2000)

Methodology

Ethical approval was obtained from University of Cape Town Human Research Ethics Committee.

This was a volunteer study with blood samples taken from individuals giving written informed consent for the procedure. Eligible volunteers included adults between the ages of 18-65 years of age without detectable intercurrent disease. Excluded was any individual currently treated with any potentially coagulation-altering agent or with an intrinsic coagulation abnormality. Pregnant individuals were also excluded.

Ten ml of venous whole blood was obtained from twenty healthy volunteers by venipuncture from an antecubital vein. A tourniquet was lightly applied to the upper arm. An 18G butterfly needle was used and the blood collected with the Vacutainer® system and sampling tubes. The first Vacutainer® tube containing 3ml of blood drawn was discarded.

Four ml of obtained blood was drawn into a Vacutainer® tube containing 7.2 mg freeze – dried K₃EDTA. The tube was inverted 3-4 times to ensure adequate mixing of the specimen. A further 2.7 ml of obtained blood was immediately transferred into a silicon- coated glass Vacutainer® tube containing 0.5 ml of buffered sodium citrate. This results in a 0.109 M (3.2%) solution containing 1 part citrate to 9 parts blood. The tube was inverted 3-4 times to ensure adequate mixing of the specimen.

In a previous study by Bohner et al. it was shown that the citrated samples used for ROTEM® can be stored up to 4 hours at room temperature without any clinically significant changes to the results obtained. (Bohner and von Pape 2003)

All samples were stored for 15 minutes and warmed to 37 degrees Celsius prior to testing. Although waiting 30 minutes may have resulted in more comparable results between the two types of anti-coagulant, such a delay would obviate one of the main advantages of a point-of-care test, namely fast availability of results

The samples were processed as per ROTEM®-EXTEM protocol: 20 µl of the EXTEM reagent (recombinant tissue factor and phospholipids, heparin inhibitor, preservatives and buffer) and 20µl of the recalcification agent (0.2 mol/l CaCl₂ in HEPEs buffer pH 7.4 and 0.1% sodium acid) were added into the pre-warmed cup of the ROTEM® analyzer. Then 300 µl of blood was pipetted from the sample obtained using the provided

electronic ROTEM® precision pipette. Rotation thromboelastometry by ROTEM®-EXTEM system was performed simultaneously on the 2 different samples from the same participant. The tests were performed on all samples by the same trained technologist.

The test ran until conclusion and the following parameters were obtained:

Clotting time (CT) – period to 2 mm amplitude

Clot formation time (CFT) – period from 2 to 20 mm amplitude

α -angle (α) – clot strengthening - slope of tangent at 2 mm amplitude

Maximum clot firmness (MCF) - maximum amplitude

One ml of the remaining blood in each specimen tube was drawn into a heparin-coated syringe and the Ca^{2+} concentration measured by the calcium electrode of an ADL 800 BASIC blood gas analyzer.

Another ml of the remaining blood in each specimen tube was recalcified to the same concentration as that of the sample processed by ROTEM® in a clean, non-coated polypropylene/plastic tube. Please see addendum A for the calculation. This recalcified sample was drawn immediately into a heparin-coated syringe and the Ca^{2+} measured by the calcium electrode of an ADL 800 BASIC Blood gas analyzer. The analyzer was calibrated against standardized calcium solutions daily.

The data obtained was exported and tabulated using Microsoft Excel software.

The two groups of ROTEM® results from each type of sample were treated by Altman and Bland analysis to establish bias and to examine the limits of agreement and to determine whether or not these are in an acceptable range.(Martin Bland and Altman 1986) The median values, standard error and 95% CI were obtained using the statistical program Statistica Version 12 and were compared to the respective reference ranges provided by the manufacturer.(Lang 2005)

There are no studies on coagulation differences between citrated and EDTA-treated samples to allow for a meaningful power calculation.

However, Crist et al compared citrate and EDTA- treated samples across a variety of coagulation measures (including PT and PTT, but not ROTEM) and found significant differences between them in 10 healthy volunteers.(Crist, Gibbs et al. 2009) Thus, for the primary outcome

variable, coagulation, a minimum of 10 samples ought to suffice for a meaningful comparison.

For the ionized calcium measurements, the Ca^{2+} concentration prior to and after recalcification were compared between the two groups using paired t-tests. From the work of Lippi et al, it is likely that the calcium concentration in the citrated samples would be at least 10 times that in the EDTA treated samples with a SD of 0.1 mmol/L. (Lippi, Salvagno et al. 2010) On this basis, only 6 samples would be required for a 90% power at an alpha value of 0.05. Similarly, a power calculation based on the work of Horsti et al suggests that, after recalcification, the difference in calcium concentration between EDTA- treated and citrate-treated samples was 1.52 versus 1.71 mmol/L with a SD of 0.07.(Horsti 2000) Based on these numbers, even allowing for an increased variability and a SD of 0.1, 6 samples would be sufficient to establish differences in calcium concentrations at an alpha level of 0.05 and a power of 90%.

Thus, 20 samples in each treatment group (obtained by splitting the 10ml blood samples taken from 20 participants) were considered to provide sufficient information for a meaningful Altman and Bland analysis and would provide more than enough statistical power to explore differences in coagulation measures and ionized calcium concentration between the treatment groups.

Results

There were substantial differences between the results obtained from the citrated and EDTA samples.

In the Altman and Bland analysis of CT, there was a considerable bias of 20.5 seconds, with the EDTA samples showing a significantly longer CT. The limits of agreement (3.82 to -44.7 sec) were also unacceptably wide. The mean values for CT were significantly different (see table 1). The Altman and Bland analysis together with the box and whisker plots of these results are shown in figures 1(a) and 1(b).

CFT also showed a substantial bias between the samples, but for this measure, the EDTA samples showed a faster clot formation time. The limits of agreement were also wide, demonstrating that the two sample types are not interchangeable. There was a statistically significant difference in CFT between the two groups. These results are summarised in figures 2(a) and 2(b) and in table 1.

The α -angle showed minimal bias between the samples and acceptable limits of agreement. Although these results were statistically significantly different, there was no clinically important difference between them. However, the α -angle was larger for the EDTA sample group. These results are summarised in figures 3(a) and 3(b) and in table 1.

MCF was similar between the groups with no statistically significant differences. The Bland and Altman plot showed minimal bias and acceptable limits of agreement, indicating that the final clot strength in the two sample groups was interchangeable. These results are summarised in figures 4(a) and 4(b) and in table 1.

The ionised calcium (Ca^{2+}) concentrations showed substantial differences between the two groups that was highly significant (Table 1).

We did not compare Lysis parameters as previous work indicated that ionised calcium concentration has no effect on fibrinolysis. (James and Roche 2004)

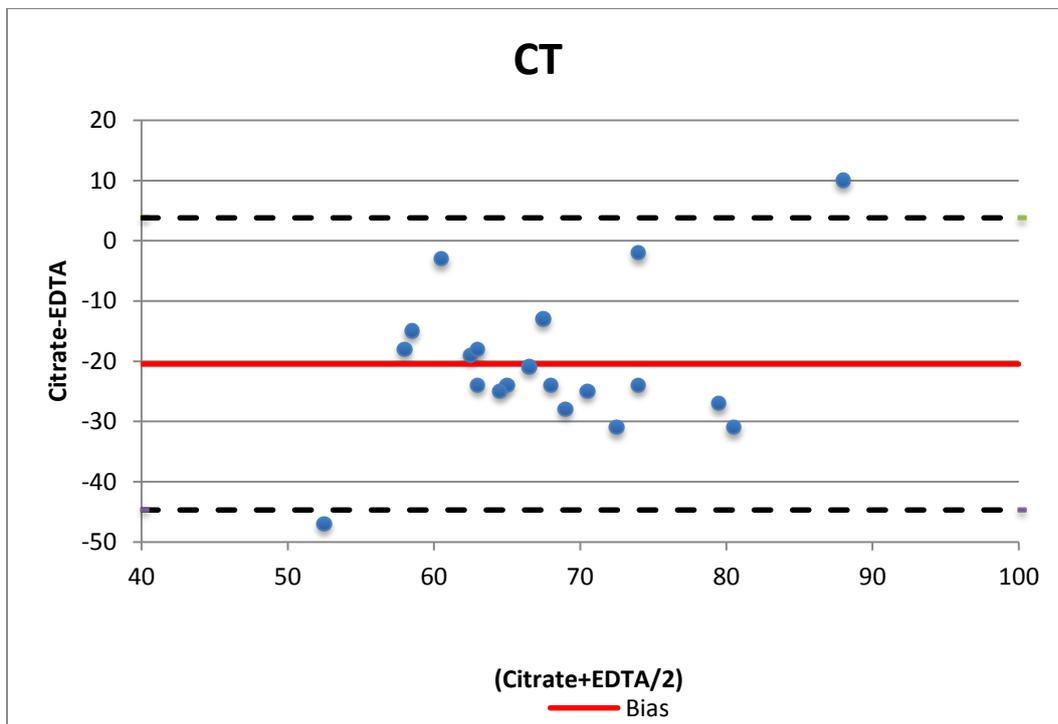
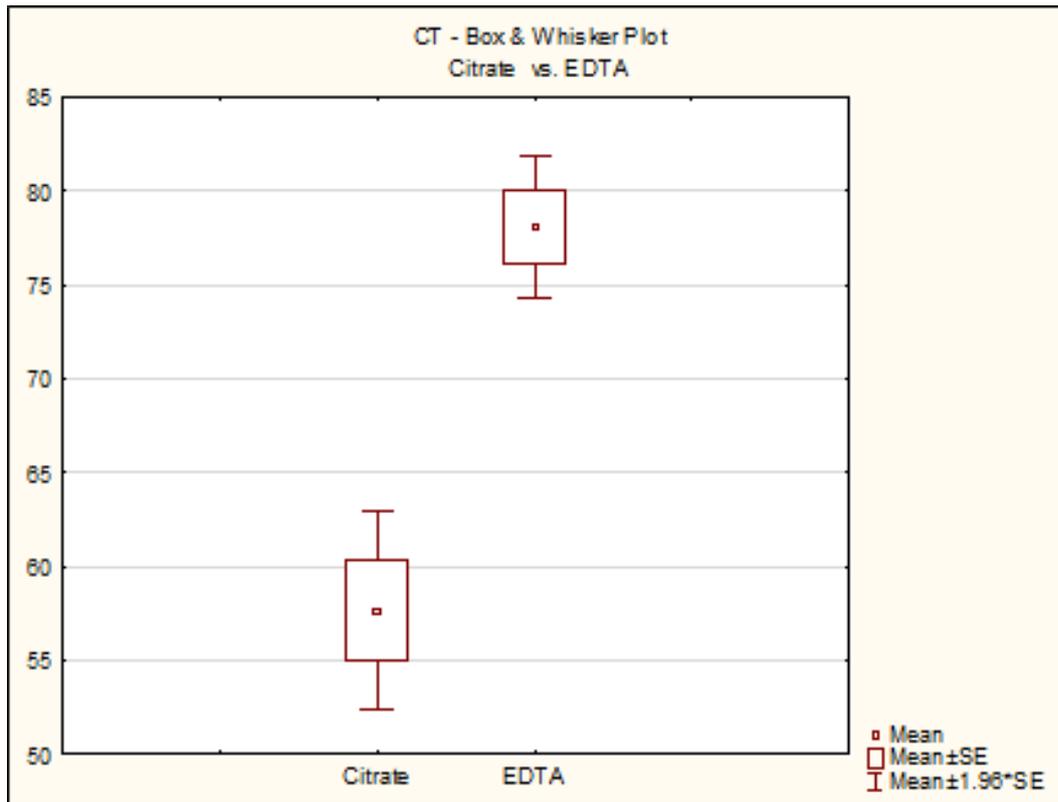


Figure 1a and b. Box and whisker plot of CT results for each specimen (a) together with Bland-Altman plots of the results (b).

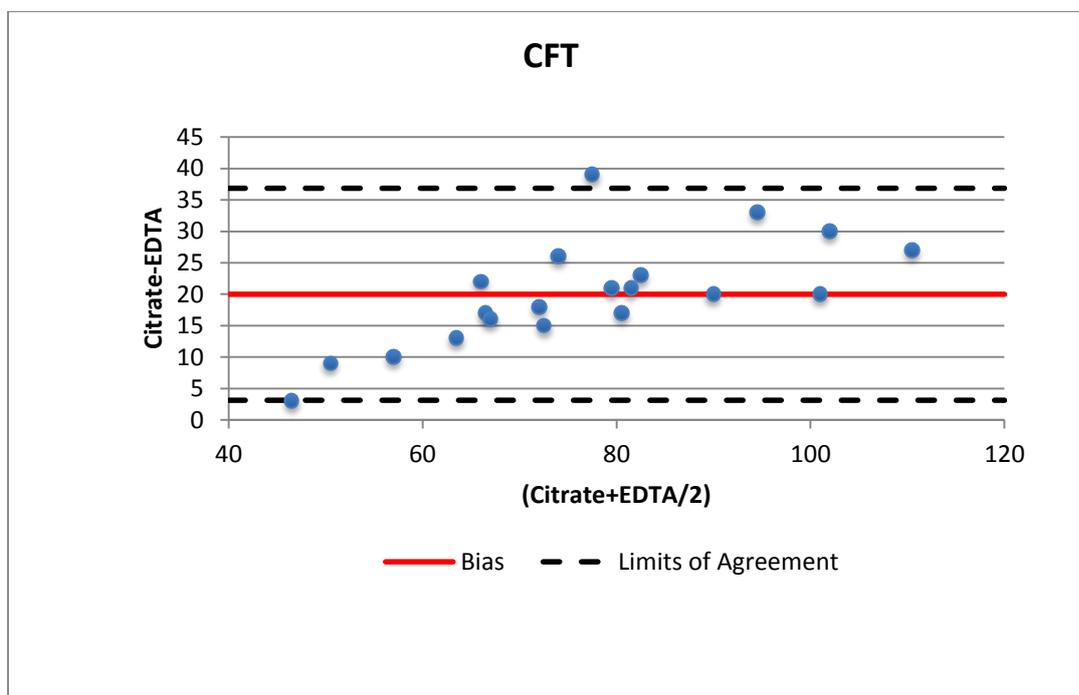
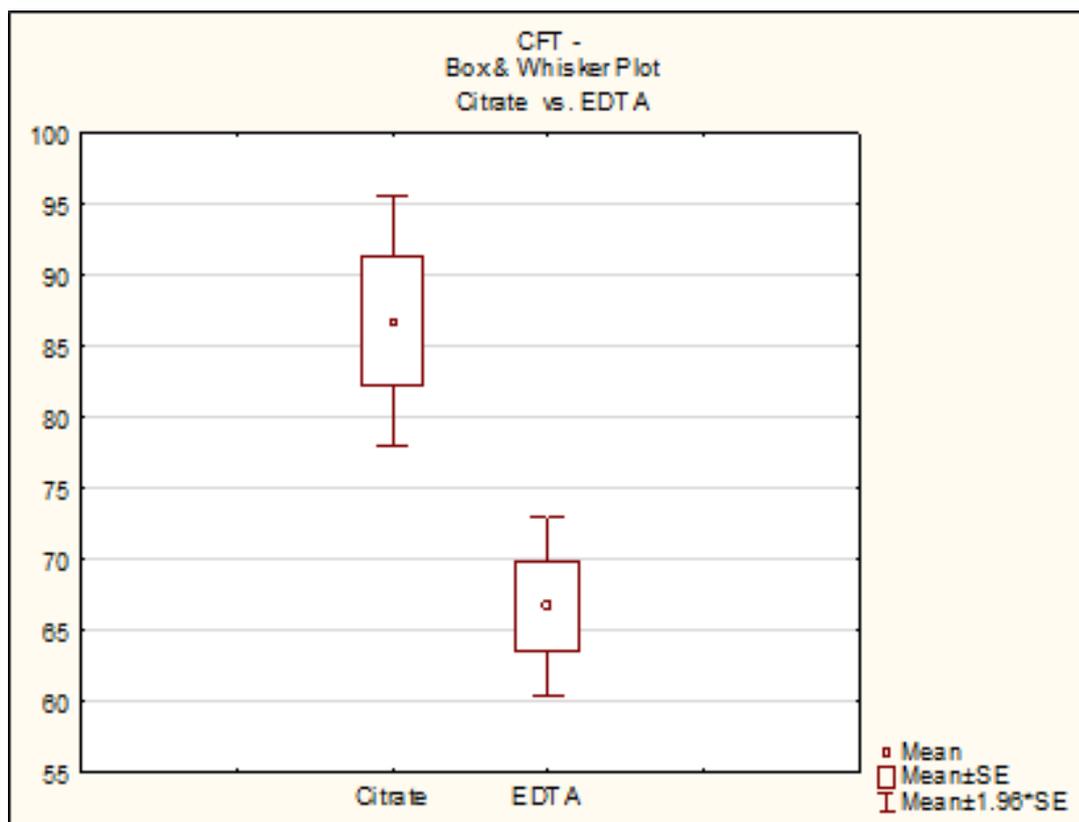


Figure 2a and b. Box and whisker plot of CT results for each specimen (a) together with Bland-Altman plots of the results (b).

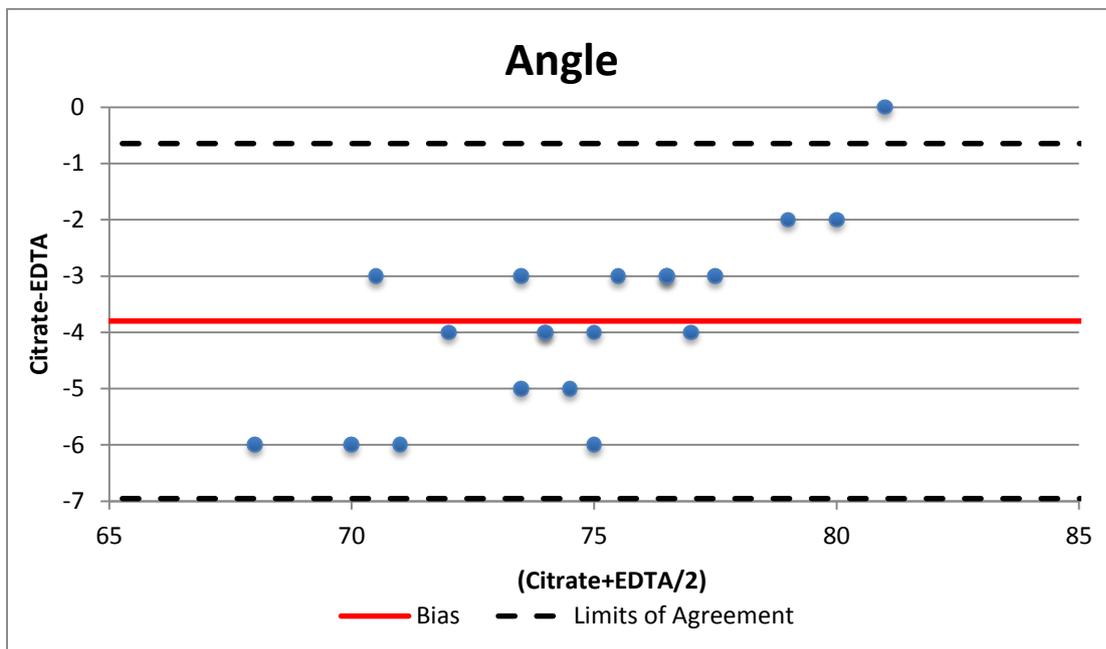
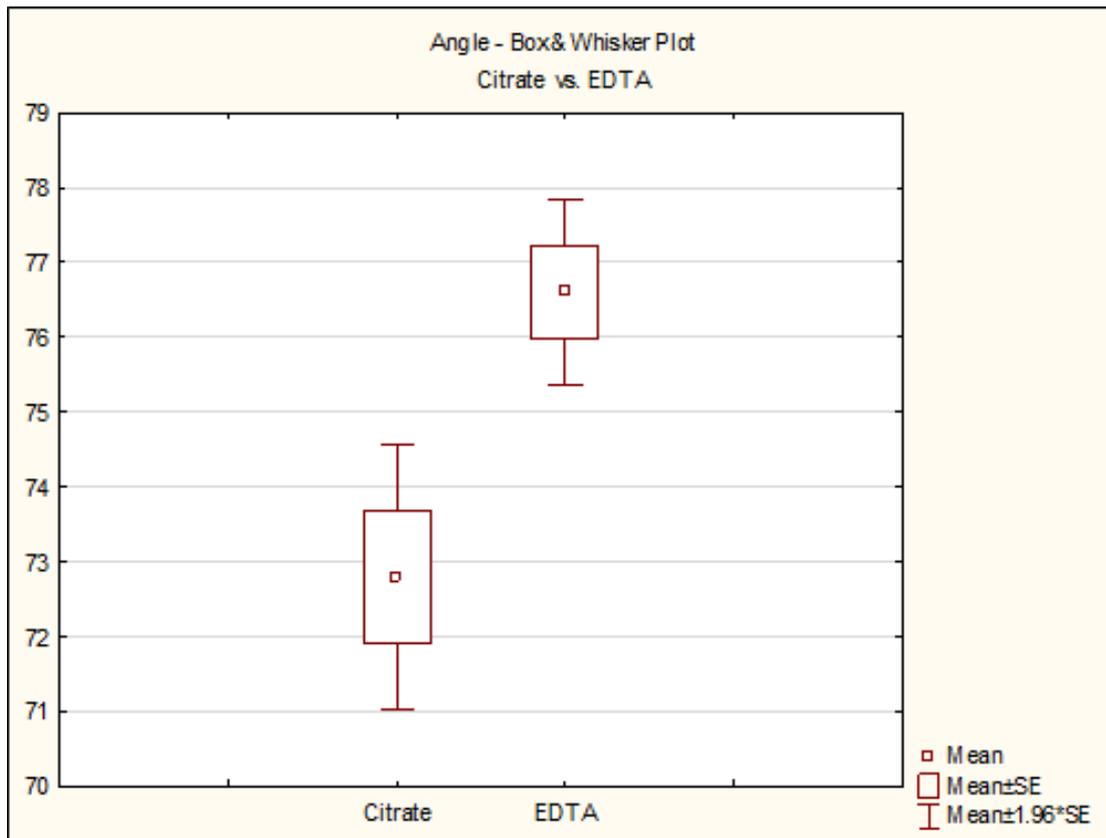


Figure 3a and b. Box and whisker plot of CT results for each specimen (a) together with Bland-Altman plots of the results (b).

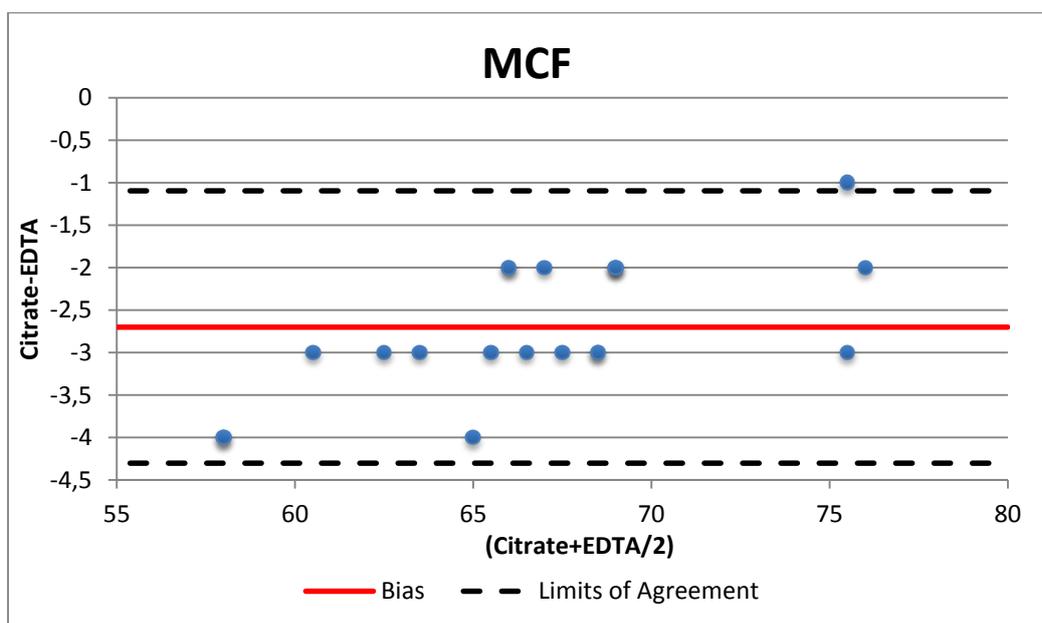
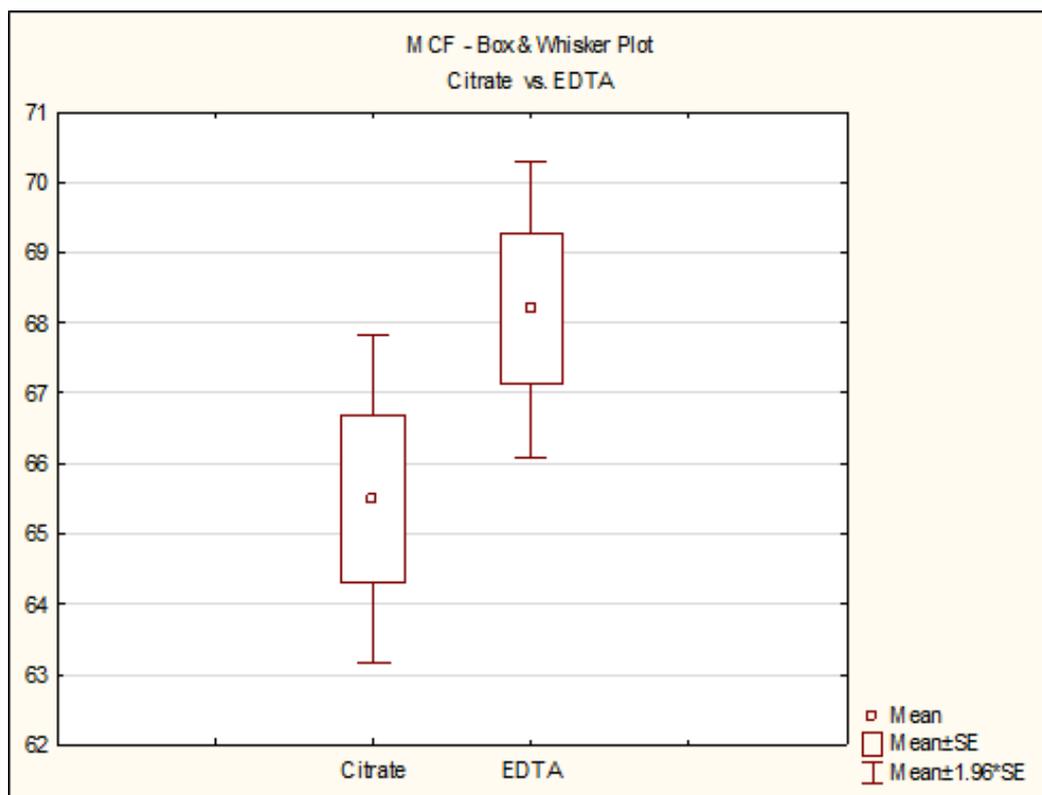


Figure 4a and b. Box and whisker plot of CT results for each specimen (a) together with Bland-Altman plots of the results (b).

Measure	Group	Mean	Range	95%% CI	SD	p	Ref
CT (sec)	Citrate	57.6	29-93	52.0-63.3	11.91	<0.0001	42-77
	EDTA	78.1	62-96	74.1-82.2	8.7		
CFT (sec)	Citrate	86.7	48-124	77.3-96.2	20.1	<0.001	46-148
	EDTA	66.7	45-97	60.0-73.5	14.3		
α -angle (°)	Citrate	72.8	65-81	70.1-74.7	4.0	0.001	63-81
	EDTA	76.6	71-81	75.3-77.9	2.8		
MCF (mm)	Citrate	65.5	56-75	63.0-68.0	5.3	0.1	49-71
	EDTA	68.2	60-77	65.9-70.5	4.8		
Ca ²⁺ (mmol/L)	Citrate	3.8	3.2-4.2	3.6-3.9	0.3	<0.0001	
	EDTA	7.6	6.1-8.6	7.3-8.0	0.7		

Table 1. EXTEM and calcium results of the two groups. Ca²⁺ - ionised calcium concentrations. Ref- manufacturer's reference ranges

Discussion

As expected, ionised calcium levels were undetectable in all samples prior to recalcification in our study. The mean Ca^{2+} concentrations in both the EDTA and citrated groups post-recalcification were well above the minimal value for coagulation as established by James et al in 2004. The mean value in the EDTA group was 7.62 mmol/L and 3.77 mmol/L in the citrate group.

It is interesting that the mean ionised calcium is much higher in the EDTA treated group than in the citrated group. Previous studies alluded to the fact that EDTA binds calcium irreversibly and to a higher degree than does citrate and that is the reason for EDTA not being a suitable anticoagulant for in vitro coagulation testing. (Lippi, Salvagno et al. 2010). Our results do not reflect this enhanced calcium binding with EDTA and the reasons for this are not clear. Lippi et al evaluated total calcium concentrations in native, citrated and EDTA plasma and found the values to be 2.37 mmol/L, 1.98 mmol/L and 0.00 mmol/L respectively. The fact that the total calcium concentration remained high in their citrated plasma could be due to the fact that citrate only binds ionised calcium and they measured total plasma calcium.

It seems that recalcifying of the ROTEM® samples leads to supra-physiological levels of ionised calcium in both groups. There is no positive correlation between Ca^{2+} concentration and coagulation as measured by the TEG® system once a threshold of 0.56 mmol/L is reached. James et al found that there is no inhibition of clotting as a result of hypercalcaemia, apart from a weak negative correlation between MA and ionised calcium concentrations, but the Ca^{2+} concentrations we obtained in this study are well above the levels they investigated. (James and Roche 2004)

As discussed in the literature review, VET results from citrated samples are influenced by the duration of sample storage. It is possible that citrate takes longer to completely chelate calcium and that this results in a shortened CT compared to EDTA. As our samples were only stored for 15 minutes before VET was performed, there might have not been enough time for the citrate to completely chelate the calcium in the specimen tubes. (Martin, Schuster et al. 2012). Although ionised calcium concentrations were zero in all samples in both groups prior to recalcification, the ionised calcium concentrations were not measured immediately after the ROTEM analyses were performed.

Although waiting 30 minutes may have resulted in more comparable results between the two types of anti-coagulant, such a delay would obviate one of the main advantages of a point-of-care test, namely fast availability of results.

It would be interesting to evaluate the effect that the time of storage has on EDTA preserved samples. If EDTA binds ionised calcium faster than citrate, more consistent results over different storage times might be obtained.

Mann et al suggested the collection of blood into citrate results in alterations that are not reversible by Ca^{2+} replacement. It seems that sodium citrate has an influence on the dynamics of thrombin generation after tissue factor activation that is independent of chelation. (Mann and Whelihan 2007)

As the differences in clotting parameters are not fully explained by differences in ionised calcium concentrations between the two groups, there must be another unmeasured effect of the anticoagulant on the sample.

We know that calcium chelation by citrate does not inhibit contact activation in the specimen tube. This could explain why an accelerated trace is seen in stored citrated samples as the contact activation pathway up to the activation of factor IX continues in the presence of a calcium chelator and the activated precursors are “ready” when the sample is recalcified. (Roche and James 2003) It is unclear if EDTA inhibits contact activation to a higher degree than citrate.

It has been shown that the electrolyte composition of samples after anticoagulation with citrate and EDTA differs vastly depending on the specific formulation used. This is why EDTA and citrate samples are not suitable for chemistry testing (Banfi, Salvagno et al. 2007, Lippi, Salvagno et al. 2010, Lima-Oliveira, Salvagno et al. 2014). It is not known if the high potassium levels might play a role in the prolonged initiation of clotting as seen with the EDTA group.

The parameters indicative of clot formation kinetics and maximal clot strength show that the EDTA samples appear hypercoagulable compared to the citrate group, despite the relatively delayed onset of initial clot formation.

The slightly greater alpha angle in the EDTA-treated samples might reflect better preserved platelet function, but the similar CFT results argue against this.

Although statistically different values were obtained for CT, CFT and α -angle parameters for EDTA and citrated samples, the range, 95% CI and

standard deviation (SD) for EDTA for all these parameters were significantly smaller than those obtained with citrate (Table 1).

These measures suggest that results from the EDTA samples are more consistent and show smaller variability (if not numerically similar) compared to those obtained from citrates samples. EDTA might be a good alternative if a reference range for EDTA samples can be established. The reason for EDTA having narrower confidence intervals is unclear.

Conclusion

We found that ROTEM EXTEM® results obtained from EDTA samples were not comparable to or interchangeable with those from citrated samples.

The difference in results is not due to differences in ionised calcium concentration levels in the samples post-recalcification, as the ionised calcium concentrations in both groups post-recalcification were adequate for coagulation.

EDTA samples did show superior consistency in all parameters and may be a suitable alternative for sample preservation for VET if reference ranges can be established.

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Addendum A

Calculation

The STARTEM® reagent supplied by the manufacturer of ROTEM® provides 0.2 mol/l or 0.2 mmol/ml CaCl₂.

For the ROTEM EXTEM® assay 20µl STARTEM® reagent and 20µl EXTEM® reagent are added to 300 µl of blood. A total sample volume of 340µl is added to the cup of the device.

20 µl of the 0.2 mmol/ml STARTEM® reagent contains 0.004 mmol CaCl₂ (0.02ml x 0.2 mmol/ml)

The concentration of CaCl₂ in the 340 µl recalcified ROTEM® sample is thus **0.01176471 mmol/ml**

$$(0.004 \text{ mmol} \times \frac{1 \text{ ml}}{0.34 \text{ ml}} = 0.01176471 \text{ mmol/ml})$$

This is the concentration of CaCl₂ we aimed to obtain in our blood samples for ionized calcium testing after recalcification so that it is the same as that of the ROTEM® samples prior to testing.

A total sample volume of greater than 1.5 ml is required for the ADL 800 BASIC Blood gas analyzer we used to measure ionized calcium concentration s in our samples.

The CaCl₂ concentration available at our institution is 6.8 mmol in 10 ml (0.68 mmol/ml)

To get to a concentration of CaCl₂ similar to that of the ROTEM samples after recalcification (0.01176471 mmol/ml) we added 26.427µl CaCl₂ to 1500µl of blood taken from each type of Vacutainer® tube providing a sample of 1526.427 µl in total which was then added to a Pico® heparinised blood gas analysis syringe. Electronic precision pipettes were used.

To simplify the volumes for pipetting the calculation was made as follows:

$$\frac{0.01176471 \text{ mmol/ml}}{0.68 \text{ mmol/ml}} \times 1 \text{ ml} = \frac{0.01176471 \text{ mmol/ml}}{0.68 \text{ mmol/ml}} \times 1000 \text{ ul} = 17.30104 \text{ µl}$$

1000 µl sample - 17.30104 µl CaCl₂ of 0.68 mmol/l CaCl₂ = 982.69896 µl blood

Thus 17.30104 µl CaCl₂ added to 982.69 ml of blood will give us the desired concentration of 0.117 mmol/l CaCl₂ in a total sample of 1ml

To calculate the volume CaCl_2 needed to be added to 1.5ml of blood as we planned to do:

$$17.301\mu\text{l} \times \frac{1500\mu\text{l}}{982\mu\text{l}} = 26.427 \mu\text{l CaCl}_2$$

Thus we had a sample of 1526.427 μl in total (1500 μl blood + 26.427 μl CaCl_2) in the Pico® syringes after recalcification.

To test our calculation:

$$26.427 \mu\text{l} (0.026427 \text{ ml}) \times 0.68 \text{ mmol/ml} = 0.01797036 \text{ mmol CaCl}_2$$

$$\frac{0.01797036 \text{ mmol}}{1.526 \text{ ml}} = \mathbf{0.117 \text{ mmol/l}}$$
 which is the desired concentration