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Research Paper

Evaluation of citrate consumption by microorganisms in anticoagulant bags of sodium citrate in case of contamination

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Abstract

Stability in the amount of sodium citrate in anticoagulant bags is very important. Ensuring that this substance is not reduced or degraded in all manufacturing processes up to storage is a requirement of pharmaceutical companies producing anticoagulants containing sodium citrate. Many citrate-consuming microorganisms can reduce the amount of sodium citrate present in the product, which doubles the importance of checking for the absence of these microorganisms before final sterilization. In the present study, Sodium citrate 4% (w/v) bags were inoculated with half McFarland suspension of 4 bacterial strains *Pseudomonas aeruginosa*, *Serratia marcescens*, *Klebsiella Pneumoniae*, *Enterococcus Faecalis* separately and incubated at 35 ° C for 120 hours until citrate was consumed. Citrate levels were measured at the pre-inoculation stage and then up to 120 hours at regular intervals. The results showed that the amount of citrate remaining in the sample inoculated with *K. Pneumoniae* was significantly ($p< 0.05$) the lowest and in the samples inoculated with *P. aeruginosa* and *E. Faecalis* was significantly ($p< 0.05$) the highest.

Keywords: Anticoagulants, Citrate consuming bacteria, Sodium citrate, potentiometer.

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1. Introduction

The first attempts at transfusing blood from animals to patients were made in 1667 with disastrous results. The first successful transfusions of human blood were performed by Dr James Blundellin 1818; 5 of his 10 attempts proved beneficial. The first successful whole blood transfusion for hemophilia was carried out by Dr Samuel Armstrong Lane in 1840. Dr, Karl Landsteiner, recipient of the 1930 Nobel Prize for Physiology or Medicine, and his colleagues' identification of the human blood groups in 1901 and the finding by Dr Richard Lewison at the Mount Sinai Hospital in New York in 1914 that sodium citrate would prevent blood from clotting made effective whole blood transfusions feasible and safer. Dr Bernard Fantus in 1937 in Chicago at Cook County Hospital opened the first blood bank in the United States after the Russians had set up more than 500 blood centers in that country. Transfusions became much more common with World War II. The addition of glucose and other compounds to anticoagulated blood made it possible to store refrigerated blood for up to 42 days, and if properly prepared, for up to 10 years if frozen (1, 7).

Today the United States utilises approximately 14 million units of whole blood, drawn from about 8 million volunteer donors to make products that are transfused into more than 3.5 million Americans. Some of this blood, and an

additional 12 million units of source plasma, is further processed into products referred to as derivatives. Improvements in the safety of the blood supply and the increasing costs associated with transfusion therapies have led to a re-evaluation of the clinical practices of blood transfusion and blood conservation (2).

During the past decade, therapeutic plasma exchange (TPE) has been used for many indications, including autoimmune and rheumatologic diseases, liver failure, sepsis, hematologic disorders, renal and neurologic diseases, drug removal, and organ transplantation. Anticoagulation is of major importance during TPE to prevent clotting of the extracorporeal circuit. Frequent coagulation in circuits not only shortens the effective treatment time of patients, but also increases the cost of treatment and causes greater blood loss. However, excessive anticoagulation can lead to bleeding and endanger patients' lives. Therefore, choosing safe and effective anticoagulants is very important (3). Continuous renal replacement therapies (CRRT) are the most commonly used renal replacement therapies in critically ill patients with acute renal failure (4,5). During continuous renal replacement therapy (CRRT), anticoagulation is required to preserve filter performance, avoid filter clotting and prevent blood loss due to circuit clotting. As anticoagulation is optimized, the risk of haemorrhage is also heightened (6).

Anticoagulants are additives that inhibit the clotting of blood and/or plasma, thereby ensuring that the concentration of the substance to be measured is changed as little as possible before the analytical process. (7). The type of anticoagulant present in the collection tube is as relevant as its concentration in the blood sample after specimen extraction. Anticoagulant pH and concentration should be within an appropriate range; otherwise, undesirable alterations in blood cells affecting their counting, size, morphology, cell activity and maybe even their antigenic characterization could occur (8).

The mechanism of action of these anticoagulants falls into two basic categories: thrombin neutralizers (heparin) and calcium binders (oxalate, ethylenediaminetetraacetic acid and citrate) that bind the main cofactor of clotting reactions. The mechanism of action of chelating substances is reversible and can be overcome by supplementing an excess of divalent cations back into the blood (8). Whole blood is collected into a primary blood bag containing a citrate based anticoagulant (CPD, CPDA-1 or CP2D) and adequate mixing is ensured. The anticoagulant solution may contain nutrients such as glucose and adenine. Citrate is the most common anticoagulant used in apheresis blood collection. Sodium citrate (4%) is used for plasma apheresis and ACD-A is used for platelet apheresis (2).

Sodium citrate functions as an anticoagulant via the chelation of ionized calcium in the blood and tissues by the citrate ion, which prevents activation of calcium-dependent pro-coagulants. In April 2003, our incentre haemodialysis unit converted to locking all central venous haemodialysis catheters with sodium citrate 4% instead of heparin 10000 U/ml. A retrospective analysis was conducted to evaluate whether replacing heparin with sodium citrate 4% would ensure cost-effective, long-term interdialytic anticoagulation and satisfactory catheter function without exposing patients to systemic heparinization (9).

Sodium Citrate is a urine alkalinizing agent (8) and is a component of acid citrate dextrose (ACD) and citrate theophylline adenosine dipyrridamole (CTAD) anticoagulants (12). After absorption it is metabolized to produce bicarbonate. It can be used to treat metabolic acidosis, where the generated bicarbonate buffers excess hydrogen ions in the blood, raising its pH (11). Citrate is abundant in nature because it is a normal constituent of all living cells, Because of its ubiquitous distribution, it is not surprising, that a large variety of bacterial species can utilize citrate as a carbon source for growth (13).

Examining the protection and stability of sodium citrate in the product can be the main concern of companies producing blood anticoagulants using sodium citrate. Degradation of this active substance can be caused by various processes such as physical conditions and improper storage, chemical and biological activities.

In this study, the degradation of sodium citrate in the consumption of *Pseudomonas aeruginosa, Serratia marcescens*, *Klebsiella Pneumoniae*, *Enterococcus Faecalis* has been investigated. Injectable pharmaceutical products are generally sterile and non-pyrogenic, and until final sterilization, these products may be exposed to citrate-degrading agents, so the absence of citrate consuming microorganisms in the products is very important before and after sterilization.

2. Materials and methods

2.1. Materials

Tryptic Soy Broth and Tryptic Soy Agar (Himedia, India) were used to enrich the bacterial strains. For potentiometric test, Sodium citrate 4% bags made by Goya innovative Biotech company (Inoclon), 0.1 N perchloric acid and glacial acetic acid of Merck Germany were used**.**

2.2. Culture Conditions of Bacterial Strains

Four bacterial strains *Pseudomonas aeruginosa*, *Serratia marcescens*, *Klebsiella Pneumoniae*, *Enterococcus Faecalis* were purchased from the Goya innovative Biotech company (Karaj, Iran). The strains have already been purified and stored for long term storage at -80 C°. The contents of the cryovial were then transferred to a test tube containing Tryptic Soy Broth culture medium and incubated at 35±1 ° C for 24 hours. After the incubation period, 100 µl of the inoculated culture medium containing the grown microorganisms was transferred to the Tryptic Soy Agar plate and cultured in several steps until single clone was obtained. After the incubation period, the plates were examined for the single colony and biochemically confirmed by differential tests (14). In parallel with differential tests, half McFarland solution was prepared by physiological serum from the colonies obtained. For half McFarland solution (OD= 0.08 - 0.13) preparation spectrophotometer at 625 nm was used (15).

2.3. Inoculation of Sodium citrate 4% (w/v) bags with target strains

Two Sodium citrate 4% (w/v) bags with O1319018 and O1319019 batch numbers which produced in Goya innovative Biotech company were chosen, pooled and after measuring the amount of citrate at time=0, poured in sterile bags and transferred to a microbial laboratory. Then 1 ml of each of the suspensions prepared in section 2-2, was inoculated into each 4 treatment bags separately and these bags with 2 sterile control bags (without inoculation) incubated at 35 ° C. All steps were performed under laminar hood under sterile conditions to minimize the possibility of any microbial contamination.

2.4. Measurement of citrate content changes in inoculated sodium citrate bags

A potentiometer was used to measure changes in citrate contents which the mechanism was based on titration. To do this, 2 ml of the sodium citrate sample was poured into a beaker which heated on a heater and allowed to dry. Then, in the same beaker, 25 ml of citric acid was added and the beaker was placed on the potentiometer until completely stirred. All crystals were dissolved in citric acid and then the electrode of the potentiometer was placed in beaker and titration was performed with Perchloric Acid ($HClO_4$). After titration, the amount of sodium citrate was evaluated. This operation was performed again at 48, 72, 96 and 120 hours to investigate changes in sodium citrate intake by long-term inoculated microorganisms.

3. Results and Discussion

3.1. Investigation of citrate changes in Sodium citrate 4% (w/v) bags

The results of citrate measurements in controlled and inoculated Sodium citrate 4% (w/v) bags at times up to 120 hours are shown in Table 3-1.

Table 3.1. The amount of citrate changes measured in inoculated and controlled Sodium citrate 4% (w/v) bags within 120 hours

Strain	Time (Hours)				
	0	48	72	96	120
Control 1	100.25 ± 00^{Aa}	100.25 ± 00^{Aa}	100.25 ± 00^{Aa}	100.25 ± 00^{Aa}	100.25 ± 00^{Aa}
Control 2	100.25 ± 00^{Aa}	100.25 ± 00^{Aa}	100.25 ± 00^{Aa}	100.25 ± 00^{Aa}	100.25 ± 00^{Aa}
P. aeruginosa	100.25 ± 00^{Aa}	98.26 ± 0.23^{Bb}	94.95 ± 0.22^{Cc}	92.94 ± 0.81^{Dc}	89.32 ± 0.20^{Ecd}
S. marcescens	100.25 ± 00^{Aa}	99.65 ± 0.22^{Aa}	97.48 ± 0.96^{Bc}	95.8 ± 0.23^{Bc}	92.22 ± 0.16^{Ccd}
K. Pneumoniae	100.25 ± 00^{Aa}	94.33 ± 0.90^{Bc}	93.13 ± 0.35^{Cd}	91.47 ± 0.96^{CDd}	89.8 ± 0.52^{Dd}
E. Faecalis	100.25 ± 00^{Aa}	99.47 ± 0.56^{Aab}	96.66 ± 0.86^{Bc}	94.5 ± 0.85^{Cc}	90.81 ± 0.65^{Dc}

The data represented are the mean of three independent experiments and the standard deviations are noted. Lowercase letters show significant differences at the level of $p< 0.05$ per **column** and uppercase letters show significant differences at the level of $p< 0.05$ per **row**.

As can be seen in Table 3-1, in the control treatments, the amount of sodium citrate remained constant at all hours of measurement, while in the other treatments inoculated with bacterial strains, the amount of sodium citrate decreased. For all samples inoculated with bacterial strains, the highest amount of citrate was measured at time 0 and the lowest amount of citrate was at 120 h, indicating that citrate was consumed by these strains. The lower the amount of citrate measured, causes the blood to clot.

Also, the lowest amount of citrate measured in all days except time 0 of measurement, was related to the treatment inoculated with *K. Pneumoniae* strain, which had the highest amount of citrate consumption.

Klebsiella pneumoniae belongs to the family Enterobacteriaceae, which includes the well-known genera Salmonella and Escherichia. Several species of enterobacteria, but not *Escherichia coli*, are able to use citrate as sole carbon and energy source, both aerobically and anaerobically. The one that has been studied most extensively with respect to citrate metabolism is *Klebsiella pneumoniae*. *K. pneumoniae* has long been recognized as an agent of disease (first described as a cause of pneumonia by Carl Friedländer in 1882), and remains among the world's most common nosocomial pathogens (16, 17, 18).

During aerobic growth, citrate is taken up in symport with protons and then metabolized via the tricarboxylic acid cycle. The transport is catalysed by a protein encoded by the citH gene, which is apparently expressed constitutively in the presence of oxygen. During anaerobic growth, uptake of citrate is a Na^+-dependent process (15). The fermentation process involves uptake of citrate by a Na+ - dependent citrate carrier, cleavage into oxaloacetate and acetate by citrate lyase, and decarboxylation of oxaloacetate to pyruvate by oxaloacetate decarboxylase. Finally, pyruvate can be converted to acetate, formate and carbon dioxide by means of anaerobic pyruvate catabolism (19).

The highest amount of citrate measured except in control treatments was related to inoculated treatments with E. Faecalis and P. aeruginosa strains.

Enterococcus group is one of the most controversial lactic acid bacteria (LAB), and they are ubiquitously disseminated in a diverse niche (soil, plant, water, vegetable, food, and gastrointestinal tract of animals and insects) (20). Citrate metabolism plays an important role in many food fermentations involving lactic acid bacteria, since it occurs in many natural substrates, such as milk, vegetables, and fruits (21). The behavior of lactic acid bacteria may differ from one species to another, and not all lactic acid bacteria are able to metabolize citrate (22). The ability to metabolize citrate is invariably linked to endogenous plasmids that contain the gene encoding the transporter which is responsible. Since citrate is a highly oxidized substrate, no reducing equivalents, such as NADH, are produced during its degradation, which results in the formation of metabolic end products other than lactic acid. Some of these end products, such as diacetyl, acetaldehyde, and acetoin, have very distinct aroma properties and significantly influence the quality of fermented foods. For instance, diacetyl determines the aromatic properties of fresh cheese, fermented milk, cream, and butter but is considered the most important off-flavor compound in the brewing process and in the wine industry. The breakdown of citrate also results in the production of carbon dioxide, which can contribute to the texture of some fermented dairy products (23).

Sarantinopoulos *et al.,* (23) reported that the increasing the citrate concentration enhanced the growth rate of *E. faecalis FAIR-E 229* in media containing both glucose and citrate and this strain is able to use citrate as a sole carbon source for growth and energy production. In all media containing both glucose and citrate, the pH declined during growth, but the final pH increased as the initial citrate concentration increased. This may be attributed mainly to the buffering capacity of the non-catabolized citrate.

Pseudomonas aeruginosa is the most frequent cause of infection among non-fermenting Gram-negative bacteria, predominantly affecting immunocompromised patients, but its pathogenic role should not be disregarded in immunocompetent patients. These pathogens present a concerning therapeutic challenge to clinicians, both in community and in hospital settings, due to their increasing prevalence of resistance, and this may lead to prolonged therapy, sequelae, and excess mortality in the affected patient population (24). *P. aeruginosa* can be identified biochemically as having indophenol oxidase-positive, citrate-positive, and l-arginine dehydrolase-positive activity (25).

The results show that contamination before and after sterilization is very important in sodium citrate bags and any microbial growth can change the concentration of citrate in the bags. Griesbach (1891) stated that ammonium citrate interferes with clotting, but he did not understand the mechanism. Pekelharing (1892b), who found that blood (90 cc) mixed with citrate (10 cc of a 5% solution) remained fluid, realized that the effect was due to the affinity of calcium for citric acid (26).

The study in 2013, compared the growth of *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, and *Haemophilus influenzae* in blood culture bottles containing anticoagulants, sodium polyanethol sulfonate (SPS) and sodium citrate. One hundred and fifty colony forming units of bacterial species were inoculated into standard aerobic (SA) and standard anaerobic (SN) bottles and were combined with 5 mL of human blood in solution with SPS or sodium citrate. Time to detection (TTD) was then monitored. Compared to the bacteria-only controls, cultures containing S. aureus, E. coli, P. aeruginosa, and S. pneumoniae plus SPS blood or citrated blood trended toward reduced TTD in both SA and SN bottles; however,

there was no significant difference in TTD between SPS and sodium citrate anticoagulant. Although *H. influenzae* showed a remarkable difference in TTD between SPS (SA 14.8 h, SN 15.0 h) and sodium citrate (SA 23.5 h, SN 18.3 h), this difference was not statistically significant (P=0.10). Addition of blood enhanced growth of bacteria. All experimental bacteria except *H. influenzae* showed similar TTD in SPS blood and citrated blood. These results support the usefulness of sodium citrate anticoagulant for artificial inoculation in blood culture bottles (27).

In a study, Several patients with heparin intolerance were dialysed with tri-sodium citrate as anticoagulant without acute clinical problems (good tolerance). After some weeks however problems arose. In all patients an alkalosis developed: the pre dialysis bicarbonate level rose progressively from 27 mmol/l to 40 mmol/l. This could be tempered by lowering the dialysis fluid bicarbonate concentration from 37 mmol/l to 25 mmol/l. A second problem was a progressive rise in pre dialysis sodium level from a mean of 136 mmol/l to 150 mmol/l. Adapting the dialysis fluid sodium concentration from 140 mmol/l towards 132 mmol/l could solve this. The third problem was a progressive rise in serum aluminium level in patients from 3 microg/l to 38 microg/l. After excluding water, concentrate, dialysis fluid, drug intake, etc… as possible sources, we controlled the aluminium level in the glass bottle containing tri-sodium citrate. We noted the very high value of 35,300 microg/l. After replacing the glass bottles with polyvinylchloride bags with a negligible aluminium content, the serum aluminium levels returned back to normal. It is known that citrate chelates the aluminium present in the glass of bottles or vials (28).

In a study reported by Fernández *et al*., (29), A retrospective comparative cohort study based on a prospective observational registry was conducted including critically ill children undergoing CRRT. Efficacy, measured as circuit survival, and secondary effects of heparin and citrate were compared. 12 patients on CRRT with citrate anticoagulation and 24 patients with heparin anticoagulation were analyzed. Median citrate dose was 2.6 mmol/L. Median calcium dose was 0.16 mEq/kg/h. Median heparin dose was 15 UI/kg/h. Median circuit survival was 48 hours with citrate and 31 hours with heparin (P = 0.028). 66.6% of patients treated with citrate developed mild metabolic alkalosis, which was directly related to citrate dose. There were no cases of citrate intoxication: median total calcium/ionic calcium index (CaT/I) of 2.16 and a maximum CaT/I of 2.33, without metabolic acidosis. In the citrate group, 45.5% of patients developed hypochloremia and 27.3% hypomagnesemia. In the heparin group, 27.8% developed hypophosphatemia. Three patients were moved from heparin to citrate to control post operatory bleeding. In conclusion citrate is a safe and effective anticoagulation method for CRRT in children and it achieves longer circuit survival than heparin.

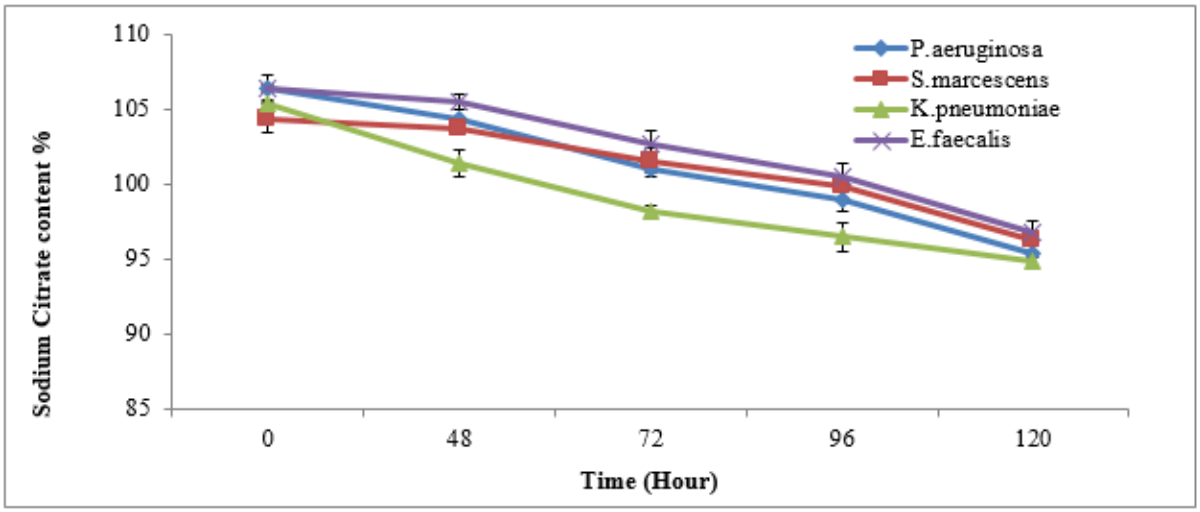
In a study the effect of sodium citrate on the properties and biological activity of plasma rich in growth factors (PRGF) investigated. PRGF was obtained from trisodium citrate and plain extraction tubes. Hematological parameters, growth factors' release kinetics from both PRGF clots and their releases' biological effect on human bone cells were evaluated. The results showed that the platelet enrichment factor, the growth factors' content and the release kinetic of PRGF were similar for both groups. The proliferation, collagen type I synthesis and tissue-nonspecific alkaline phosphatase activity of human osteoblasts showed no statistically significant differences. The use of sodium citrate does not influence the composition, the growth factors' release kinetics or the biological effect of PRGF, but it increases its clinical versatility (30).

In another study Yuan *et al.,* (3) compared different anticoagulants in mTPE and observe the effectiveness, safety, and advantages of RCA. Both regional citrate anticoagulation (RCA) and heparin are used as anticoagulants during membrane therapeutic plasma exchange (mTPE). The peripheral blood platelet count, prothrombin time (PT), activated partial thromboplastin time (APTT), and electrolytes were detected before and after treatment in all patients. The results showed that in the RCA group, the patients did not experience hypocalcemia or hypercalcemia, and no separator clotting occurred. RCA is safe, feasible, and effective in mTPE, especially for patients with bleeding tendency and frequent monitoring is needed. It is worth widely developing and applying it in clinical practice**.**

3.2. The rate of citrate reduction in in Sodium citrate 4% (w/v) bags

The rate of change of sodium citrate is shown in Figure 3.1.

Figure 3.1. Evaluation of the rate of Sodium citrate changes in Sodium citrate 4% (w/v) bags inoculated with bacterial strains.



Data represented here is the mean of three independent experiments and the standard deviations are noted as error bars.

As can be seen in Figure 3-1, the highest rate of citrate utilization and lowest citrate remaining is related to K. pneumoniae strain, which was explained in Section 3.1.

4. Conclusion

The results showed that the strains used in this article were citrate positive and had the ability to use citrate as an energy source. In all inoculated Sodium citrate samples, the amount of citrate was reduced. Therefore, microbial contamination before and after sterilization may use citrate and reduce its concentration. Decreased citrate concentration in bags can cause clots during medical and laboratory operations. In some medical or laboratory procedures, sodium citrate products or tubes are used to prevent blood clots. In plasma separation and dialysis operations where the absence of blood clots is of great importance and the return of clotted blood to the patient's vein can lead to the death of the patient or plasma donor. Therefore, this product is a vital and great care must be taken in its production and maintenance.

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