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Chemical and microbiological stability, anticoagulant efficacy and toxicity of 35 and 90 mM trisodium citrate solutions stored in plastic syringes

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Abstract

Objectives: Trisodium citrate represents an interesting alternative to heparin for the prevention of circuit clotting during extracorporeal procedures, but some protocols require not commercially available citrate concentrations. There is very little information available in the literature about the stability of diluted citrate solutions. This study is aimed to evaluate the long-term stability, efficacy and toxicity of 35 mM and 90 mM trisodium citrate solutions prepared by diluting commercially available sterile solution, stored in plastic syringes, and used as anticoagulant during the citrate bag changes in coupled plasma filtration adsorption (CPFA) technique in the COMPACT-2 clinical trial.

Methods: The chemical stability of trisodium citrate solutions was evaluated by HPLC after 7, 14, 21 and 28 days of storage. The sterility tests were performed both immediately after preparation and after 28 days of storage.

Results: After 28 days of storage, the concentration of trisodium citrate do not change in comparison to day 1, and both the solutions passed the sterility test. A preliminary test indicated that 35 mM solution is not enough to ensure an effective anticoagulant action on extracorporeal circuit, but the 90 mM solution was successfully used during a total of 7 CPFA treatments in 2 patients without clinical signs of toxicity.

Conclusions: Both the 35 mM and 90 mM solutions are chemically and microbiologically stable for 28 days when stored at room temperature in 50 mL syringes protected by light. The 90 mM solution is effective and safe as regional anticoagulant in the CPFA protocol.

INTRODUCTION

The hemodialysis procedure requires the maintenance of an adequate blood flow into the extracorporeal circuit, so the use of an anticoagulant is essential to avoid the intraluminal thrombus formation that could decrease the hemofiltration efficiency. Heparin is a systemic anticoagulant commonly used as thrombolytic agent in different pathological conditions and in the prevention of thrombus formation during a number of extracorporeal medical procedures.[1] Heparin exerts its anticoagulant activity through inhibition of factor Xa and thrombine II, leading to the block of coagulation cascade before the activation of fibrine from fibrinogen.[1] One of the most important drawbacks in the use of heparin as anticoagulant during hemodialysis, is its long-lasting action. The biological half-life of heparin, in fact, is in the range of hours, and this feature exposes the patients to potential systemic anticoagulant action, with increased risk of thrombocytopenia, bleeding and haemorrhage.[2] This risk is particularly relevant in uremic, septic or critically ill patients, as they have an increased likelihood of bleeding due to recent surgery, trauma, mucosal lesions or coagulopathy.[3-5] Moreover, binding of endothelial antithrombin to administrated heparin inhibits its anti-inflammatory actions and prevents local prostacyclin formation that, in turn, may even aggravate sepsis-mediated disorders.[3 6]

Trisodium citrate represent an interesting alternative to heparin when anticoagulant action is required only locally (e.g. central venous catheter or extracorporeal circuit during hemofiltration or hemodialysis).[3-5 7] The regional anticoagulant action of trisodium citrate is due to its ability to chelate the free ionized calcium in the blood, with the consequence of the blockage of calcium-dependent clotting formation in the coagulation cascade. By chelating calcium, citrate acts regionally as an anticoagulant when administered pre-filter, and thereby reduces the risk of bleeding compared to systemic anticoagulation.[4] During continuous renal replacement therapy (CRRT), an optimal regional anticoagulant activity occurs when the ionized calcium concentration in the extracorporeal circuit is below 0.35 mmol/L.[8] The remaining citrate, free and calcium bound, is partially removed by hemofiltration (from 20% to 80%, depending on the blood and effluent flow rate, and CRRT modality), and partially enters the systemic circulation. When citrate reach the bloodstream, its anticoagulant action is rapidly reverted because the calcium citrate chelates are efficiently metabolized by liver and muscles, and free calcium ions become again available in the blood.[3 9 10] Furthermore, the normal patient's systemic calcium level is commonly restored by post-hemofilter replacement infusion of calcium solution.[11]

Recent reviews on clinical studies comparing the regional citrate anticoagulation versus systemic heparinisation in critically ill patients treated with CRRT, indicate that citrate is superior in terms of safety, efficacy and costs, and allows better filter survival times with higher delivered CRRT doses.[3-5 7 8 10 11] Consequently, the most recent Clinical Practice Guidelines for Acute Kidney Injury recommend the use of citrate as anticoagulant in patients requiring CRRT.[12]

Different methods of regional citrate anticoagulation have been described in the literature: in some protocols, citrate is administered in the extracorporeal circuit as a separate solution, whereas, in other protocols, citrate is added to a calcium-free predilution replacement fluid.[7 8 10 11 13 14]

The administration of citrate may cause serious adverse effects, such as metabolic alkalosis and acidosis, hyper- and hyponatremia, and hypocalcemia. Thus, it is important to carefully design citrate anticoagulation protocols, and it is preferred to incorporate an appropriate citrate module in the CRRT device, in order to continuously monitor the citrate concentration in the prefilter. Finally, the patient's level of electrolytes and calcium, and the acid–base status should be frequently monitored, in order to minimize the risk of toxicity.[15 16]

Septic shock is a systemic imbalance of proinflammatory and anti-inflammatory response that can be induced by the endotoxin of Gram-negative bacteria. Therefore, the treatment strategies should pursue a twofold objective: to counteract the infection and to rebalance the immune response. While the first objective is achieved by the administration of antibiotics, the second could be accomplished by various blood purification techniques, among which the coupled plasma filtration adsorption (CPFA), that is able to unselectively remove the majority of soluble inflammatory mediators involved in sepsis pathogenesis.[17 18] The efficacy of CPFA in the treatment of critically ill patients with septic shock has been compared with standard care in the COMPACT multicentre randomised controlled clinical trial (NCT00332371), but the trial was prematurely terminated on the grounds of the high number of protocol violations in the CPFA arm in terms of low volume of plasma treated per day.[19] The main reason for not reaching the prescribed volume of plasma treated, was the clotting of the circuit, in spite of the use of heparin for anticoagulation. In an attempt to overcome this problem, the extracorporeal circuit has been modified, in order to support regional anticoagulation with citrate, and a new multicentre randomised controlled clinical trial, COMPACT-2 (NCT01639664) has been started.

The aim of this study is to evaluate the long-term stability, efficacy and toxicity of two different concentrations of trisodium citrate (35 and 90 mM) stored in plastic syringes, suitable for the use in CPFA procedure. There is very

little information available in the literature about the chemical stability of diluted citrate solutions, and published papers reported only the data on the 136 mM solution.[20] [21] In addition, no information about the sterility maintenance of these kinds of solution during storage are reported.

The solutions were prepared by diluting commercially available sterile trisodium citrate concentrate with sterile 0.9% sodium chloride solution under sterile conditions, and were stored in 50 mL syringes for up to 28 days at room temperature. The solutions were then used as regional anticoagulant during CPFA therapy in the COMPACT-2 clinical trial. The immediate availability of citrate syringes ready-to-use for Nephrology and Intensive Care Unit (ICU) is very important in the treatment of patients with acute kidney failure or septic shock, as the prompt start of CRRT or CPFA procedures is critical for the chances of a successful treatment.[22] [23]

METHODS

Chemicals, reagents and materials

Commercially available trisodium citrate sterile concentrate solution (46.7%) was purchased from Monico (Mestre/Venice, Italy), sterile 0.9% sodium chloride solution was provided by Baxter (Rome, Italy), sterile 50 mL polypropylene (PP) syringes UV-protected with luer lock caps were purchased from Artsana (Como, Italy). Monobasic potassium phosphate, phosphoric acid, standard trisodium citrate, sodium hydroxide, hydrochloric acid, 30% hydrogen peroxide, and methanol HPLC-grade were provided by Sigma-Aldrich (Milan, Italy).

Preparation of trisodium citrate solutions

Two different concentrations of trisodium citrate were prepared: 35 mM (1.029%) and 90 mM (2.65%). Trisodium citrate solutions were prepared by diluting commercially available sterile concentrate (1.59 M, 46.7%) with sterile 0.9% sodium chloride solution under sterile conditions, in accordance with the European Pharmacopoeia[24]. The procedure was identical to that usually employed in our hospital pharmacy for the preparation of similar solutions for parenteral administration. This standard procedure has been validated and is regularly monitored. In order to assure aseptic filling of the syringes, the whole procedure were performed in a glove box isolator (Isoclean® Healthcare Platform Isolator HPI-G3, manifactured by Esco Pharma,

Rotherham, UK), avoiding the direct exposition of solutions and syringes to the room environment or to compounding personnel. The filled syringes were then capped with a sterile luer lock tip, delivered to Nephrology unit and ICU, and stored at room temperature (21 ± 2 °C) protected from direct light.

HPLC analysis

Trisodium citrate concentration was determined by isocratic reverse-phase HPLC by a method adapted from the official method 986.13 of the Association of Official Analytical Chemists.[25] HPLC system consisted of a YL9300 HPLC (Young Lin Instruments, Anyang, Korea) equipped with a vacuum degasser, a quaternary pump, a manual injector with a 200 µL loop, and an UV-Vis detector set at 200 and 215 nm. The analytical column was a Symmetry C18 (250 × 4.6 mm i.d., particle size 5 µm), equipped with a Symmetry C18 guard column, supplied by Waters (Vimodrone, Milan, Italy). The mobile phase consisted of a mixture of 0.08 M potassium dihydrogen phosphate buffer (adjusted to pH 2.6 with phosphoric acid) and methanol (98:2 v/v), and was delivered at 1 mL/min at room temperature (21 ± 2°C). Peak heights and areas were recorded on PC and processed with YL-Clarity software (Young Lin Instruments, Anyang, Korea). In these conditions, the retention time of trisodium citrate was about 8 min. Standard curve was calculated by least-squares linear regression from six trisodium citrate standard concentrations ranging from 10 to 500 µg/mL (10, 20, 50, 100, 250, and 500 µg/mL), with each point consisting of six independent measurements. The concentrations of 20, 100 and 250 µg/mL were used to determine accuracy and precision. Accuracy was reported by calculating the bias expressed as (measured concentration−nominal concentration)/(nominal concentration))×100, and precision by the coefficient of variation (CV) expressed as (S.D./mean)×100.

Trisodium citrate concentrations of solution stored in the syringes were determined from the peak area ratios *versus* the standard curve.

Forced degradation of trisodium citrate

In order to verify the specificity of HPLC method of analysis, the degradation of citrate was forced by exposing citrate solution to acidic, alkaline or oxidizing condition. For the acidic or alkaline degradation, concentrated hydrochloric acid or 6N sodium hydroxide was added to 50 mL aliquots of 35 mM or 90 mM citrate solution in a 100 mL flask, in order to adjust the pH to about 1 or 12 respectively. For the oxidizing condition, 25 mL of 30%

hydrogen peroxide was added to 50 mL of 35 mM or 90 mM citrate solution in a 100 mL flask. The samples were incubated at 50°C for 24 hours, and then each flask was made up to volume with deionised water. Three aliquots (0.5 mL) of each flask were centrifuged at 15000g for 5 minutes in order to precipitate any insoluble material eventually formed. Samples were then analysed by HPLC after a 1:25 v/v (for 35 mM) or 1:50 v/v (for 90 mM) dilution in deionised water, and the chromatograms were compared to that of fresh trisodium citrate solution at the same concentration.

Stability study

Syringes containing trisodium citrate solutions were stored at room temperature and kept away from direct exposure to light. For each concentration, three syringes were randomly taken for the determination of trisodium citrate amount on days 1, 7, 14, and 28. Samples from selected syringes were diluted with deionised water (1:50 v/v for 35 mM solution, or 1:100 v/v for 90 mM solution), and analysed in triplicate by HPLC.

Three syringes containing 35 or 90 mM trisodium citrate solutions were stored at 4°C, and the trisodium citrate amount was evaluated after 28 days of storage.

Sterility test

The sterility of the solutions was tested by the Chemical Laboratory of CCIA (Turin Chamber of Commerce) according to the European Pharmacopoeia essays.[24] The laboratory is certified ISO 9001, and accredited in accordance with the recognized International Standard of ISO/IEC 17025.

For each concentration, the sterility tests were done on 4 syringes, from a batch of 40, both immediately after the preparation, and after 28 days of storage at room temperature.

Use of trisodium citrate solutions as anticoagulant agent in the CRRT procedure

The trisodium citrate solutions were used as regional anticoagulant during CPFA therapy in the COMPACT-2 clinical trial (NCT01639664). CPFA was performed with the use of a four-pump modular treatment (AMPLYA, Bellco, Mirandola, Italy) consisting of a plasma filter (0.45 m^2 polyethersulfone) and a following absorption on an unselective hydrophobic resin cartridge (140 mL for 70 g, with a surface of about 700 m^2/g) and a final passage of the reconstituted blood through a high-permeability 1.4 m^2 polyethersulfone hemofilter, in which convective

exchanges may be applied in a postdilution mode. The 10/2 citrate solution is administered through a fifth pump in predilution 25%. During the citrate bag changes, the fifth pump is stopped for 3-7 minutes, in order to avoid air entrance into the CPFA circuit, but this procedure increases the risk of circuit clotting. For this reason, the circuit has been modified by adding an alternative citrate inlet through which a citrate solution, stored in a 50 mL external syringe, is pumped at 300 mL/h, so ensuring the right citrate concentration in the CPFA circuit.

According to the available clinical evidence, CPFA was to be repeated daily, lasting at least 10 h each time, so that an average of 0.15 L/kg predicted body weight (PBW) of plasma should have been treated per day.[19]

RESULTS

Validation of HPLC assay

The method was found to be linear over the range of 10 to 500 μg/mL, with an $r^2 \geq 0.999$ and a mean absolute percentage deviation of standards from their nominal concentration <4%. Intra-day accuracy ranged from −1.06 to 3.11% and inter-day accuracy ranged from −1.45 to 4.05%. Intra-day precision was <4%, while inter-day precision was <5%. The limit of quantification (LOQ) of the method was 10 μg/mL.

Forced degradation of trisodium citrate solutions

The results of forced degradation of trisodium citrate solutions showed that the incubation in oxidizing conditions for 24 h cause the formation of almost two new peaks, with a retention time of about 4.5 and 6.2 minutes, with only 89.7% of citrate remaining unchanged (Figure 1). The solutions exposed to acidic or alkaline condition for 24 h showed no sign of degradation, with a mean amount of 98.8% and 99.2% of unmodified trisodium citrate respectively. There was no difference in trisodium citrate degradation between 35 or 90 mM concentrations.

Stability study

During a storage period of 28 days at room temperature in 50 mL PP syringes, the trisodium citrate solutions remained clear, colourless, and without the formation of any precipitate. The concentrations of citrate, determined by HPLC after 7, 14, 21 and 28 days of storage, do not change in comparison to that of day 1, resulting in the

range of 100 ± 10% of the initial concentration (Table 1). Similar results were obtained from syringes stored for 28 days at 4° C, with a mean percentage of day 1 concentration residual of 99.6 for 35 mM and 102.4 for 90 mM solutions.

Table 1. Stability of 35 mM and 90 mM trisodium citrate solutions stored at room temperature. Data are the mean of three different samples, each analysed in triplicate.

	Days of storage				
Trisodium citrate solution	1	7	14	21	28
35 mM % of day 1 concentration residual	100.0	103.7 ± 3.2	99.7 ± 2.2	106.3 ± 1.9	105.7 ± 2.1
90 mM % of day 1 concentration residual	100.0	96.9 ± 3.5	95.4 ± 2.2	98.6 ± 2.6	95.9 ± 2.7

Mean ± standard deviation

Sterility test

Both trisodium citrate solutions (35 mM and 90 mM), passed the sterility test both immediately after the preparation and after the storage period of 28 days at room temperature in 50 mL PP syringes.

Efficacy of trisodium citrate solutions as anticoagulant in the CRRT procedure

The use of the external syringe prepared with 35 mM citrate solution was abandoned early because a preliminary test indicated that this concentration is not enough to ensure an effective anticoagulant action during the change of the citrate-bags. Conversely, the external syringe containing 90 mM citrate solution was used during seven complete CPFA treatments in two patients admitted in ICU with septic-shock diagnosis (Table 2). Three sessions of CPFA was administered to patient number 1 and four sessions was administered to patient number 2. Both patients were treated with a mean of over 0.22L/kg PBW per session. In both patients it was possible to overcome 10 hours of CPFA treatment without clotting (Table 2). Moreover, after the CPFA session CRRT continued without the need of circuit substitution before its normal duration.

Both patients do not develop clinical signs of citrate toxicity during CPFA/CRRT treatments.

Table 2. Clinical characteristics of patients at ICU admission and characteristics of CPFA sessions.

Characteristics	Patient 1	Patient 2
Clinical characteristics of patients		
Sex	male	female
Age (years)	64	71
Predicted body weight (Kg)	63	46
SAPS 2	54	68
Systolic blood pressure (mmHg)	95	105
Lactate (mmol/L)	5.8	3
Cardiac frequency (bpm)	113	118
Systemic vascular resistance index (dines s/cm^5)	110	1400
Cardiac index (L/min/m^2)	4	2.8
Temperature (°C)	35.8	36.3
Central saturation O_2 (%)	71	66
Platelet (x1000/mL)	237000	186000
White blood cells (x1000/L)	13500	18000
Bilirubin (mg/dL)	1.1	0.5
Creatinine (mg/dL)	1	1.3
Procalcitonin (ng/mL)	59	44
pH	7.43	7.29
PT INR	1.4	2.5
Haematocrit (%)	37	31
Noradrenaline dosage (µg/Kg/min)	0.2	0.3
Characteristics of CPFA sessions		
Mean treated plasma (L)	14	11
Target plasma treated (L)	13	9
Mean treated plasma/kg PBW (L/Kg)	0.22	0.24
CPFA Sessions number	3	4
Mean session duration (h)	12	12

ICU: Intensive Care Unit; CPFA: Coupled Plasma Filtration Adsorption; SAPS: Simplified Acute Physiology Score; PT: Prothrombin Time; INR: International Normalized Ratio; PBW: Predicted Body Weight

DISCUSSION

The administration of trisodium citrate solution in the extracorporeal circuit proved to be an effective means of regional anticoagulation in patients treated with CPFA, but the commercially available solutions are not suited for this application. The aim of this study was to evaluate the chemical and microbiological stability of two different concentrations of trisodium citrate solutions, 35 mM and 90 mM, prepared in a hospital pharmacy and stored at room temperature in 50 mL syringes "ready-to-use" in ICU.

The chemical stability was evaluated by HPLC, and the specificity of the method was proved by the separation of degradation products from unmodified trisodium citrate in the analysis of sample treated by oxidizing conditions. Both the 35 mM and 90 mM trisodium citrate solutions are chemically stable for 28 days when stored at room temperature in 50 mL syringes protected by light, as demonstrated by the high percentage of citrate still present (at least 95%) in comparison to day 1.

Results of sterility test demonstrated the microbiological stability of solutions during the storage.

A preliminary test indicated that 35 mM trisodium citrate concentration is not enough to ensure an effective anticoagulant action in the extracorporeal circuit during the change of the citrate bag, for this reason only the 90 mM concentration was used for CPFA/CRRT treatment. The efficacy of 90 mM trisodium citrate solution as regional anticoagulant was demonstrated by the absence of circuit clotting, premature hemofilter change or loss in plasma treated in a total of seven sessions administered to two different patients. Moreover, no signs of citrate toxicity were observed during treatments, so demonstrating the safety of this anticoagulation procedure.

In conclusion, our results demonstrate that both the 35 mM and 90 mM trisodium citrate solutions are chemically and microbiologically stable for 28 days when stored at room temperature in 50 mL PP syringes protected by light. The 90 mM solution prove to be effective and safe as regional anticoagulant during the citrate-bags changes in the CPFA protocol, and will be used for the treatment of all patients enrolled in the COMPACT-2 clinical trial.

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Conflicts of Interest

Nothing to report.

Key messages

What is already known on this subject

- Recent reviews on clinical studies in critically ill patients treated with CRRT, indicate that regional citrate anticoagulation is superior to systemic heparinisation in terms of safety, efficacy and costs, and allows better filter survival times with higher delivered CRRT doses.
- The clinical trial COMPACT, aimed to compare the efficacy of coupled plasma filtration adsorption (CPFA) with standard care in the treatment of critically ill patients with septic shock, was prematurely terminated because of the high number of protocol violations, mainly due to the clotting of the circuit in spite of the use of heparin for anticoagulation. For this reason, the extracorporeal circuit has been modified in order to support regional anticoagulation with citrate, and a new clinical trial (COMPACT-2) has been started.
- Some protocols require not commercially available citrate concentrations, and very little information is reported in the literature regarding the stability of diluted citrate solutions. In addition, advance preparation and storage in Nephrology and ICU is very important, as the prompt start of CRRT or CPFA procedures is critical for the treatment of patients with acute kidney failure or septic shock.

What this study adds

- Syringes “ready-to-use” containing 50 mL of 35 mM or 90 mM sterile solutions of trisodium citrate have a shelf life of almost 1 month if stored at room temperature protected by light. Solutions at suitable concentration may be prepared by the hospital pharmacist and stored in Nephrology and ICU in order to be immediately available at any time.
- The use of 90 mM trisodium citrate as regional anticoagulant during the citrate bag changes in CPFA is safe and more effective than heparin for the prevention of circuit clotting.

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Legends of figures

Figure 1. Chromatograms of a trisodium citrate sample (35 mM, λ= 215 nm) treated by forced degradation at oxidizing condition (a) and control (b). Arrows indicates presumed degradation products. I = injection; T = trisodium citrate.