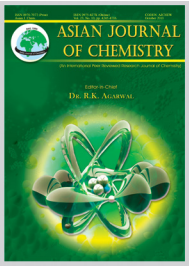
Asian Journal of Chemistry; Vol. 24, No. 10 (2012), 4717-4722

ASIAN JOURNAL OF CHEMISTRY

www.asianjournalofchemistry.co.in



Thermodynamic Features of Diethyl Citrate Calcium Complexes and Factors Affecting the Complex Stability

YAN OU[1], BIN CHEN[2], HUA PENG[2], BAO-SONG GUI[1,*], XIU-QIONG YAO[2] and JIAN-MING OUYANG[2,*]

[1]Department of Nephrology, The Second Hospital of Xi'an Jiaotong University, Xi'an 710004, P.R. China
[2]Institute of Biomineralization and Lithiasis Research, Jinan University, Guangzhou 510632, P.R. China

*Corresponding authors: E-mail: guibsdoctor@sina.com; toyjm@jnu.edu.cn

(*Received*: 1 September 2011; *Accepted*: 12 May 2012) AJC-11476

Diethyl citrate (Et_2Cit) is a promising new anticoagulant. In this study, the stoichiometry of the Et_2Cit complex with Ca^{2+} ions and the stability constant (K_s) were determined. The effect of pH and temperature on K_s were then discussed. Thermodynamic functions (ΔG, ΔH, ΔS) of the complex were also calculated. These results were compared with those of sodium citrate (Na_3Cit), a current clinical anticoagulant. Both Et_2Cit and Na_3Cit formed complexes with Ca^{2+} ion at 1:1 ratio. The K_s measured for calcium diethyl citrate ($CaEt_2Cit$) and calcium citrate (CaCit) was 231 and 1988, respectively, at pH 7.4, 37 ºC. It is indicated that CaCit was more stable than $CaEt_2Cit$. Increasing pH or solution temperature favoured the formation of both complexes. Increasing temperature from 15 to 40 ºC led to a shift of K_s from 58 up to 327 for $CaEt_2Cit$ and 327 up to 2327 for CaCit, as a result of their endothermic reactions. Animal testing results on rabbits showed that the recovery speed of blood calcium concentration with Et_2Cit as anticoagulant was more rapid than that with Na_3Cit. Et_2Cit is expected to circumvent the problems of hypocalcemia and hypercalcemia which are usually encountered when using Na_3Cit for anticoagulation. Therefore, Et_2Cit has shown great potential as a new anticoagulant.

Key Words: Stability constant, Anticoagulant, Diethyl citrate, Ion-selective electrode, Complex, Sodium citrate.

INTRODUCTION

Up to date, sodium citrate (Na_3Cit) is a major anticoagulant for clinical applications. Its mechanism for anti-coagulantion is based on the fact that Na_3Cit can bind calcium ion (Ca^{2+}) to form a calcium citrate complex (CaCit), which is difficult to dissociate. This chelating effect of Na_3Cit helps reduce the concentration of free Ca^{2+} in plasma ($c(Ca^{2+})$) and thus largely slows down the blood-clotting process to achieve anticoagulation[1]. However, it can cause hypocalcemia and hypercalcemia when using Na_3Cit as anticoagulant[2], due to its excessively strong chelating capability with Ca^{2+} and therefore low Ca^{2+} dissociation rate. It requires 0.5 h or so for the body to completely metabolize CaCit and release calcium ions.

Considering the drawbacks of Na_3Cit, we have proposed using the derivative diethyl citrate (Et_2Cit) of Na_3Cit as a novel anticoagulant[3]. In contrast to calcium citrate (CaCit), the large steric effect of Et_2Cit is expected to lower the stability of the complex formed between Ca^{2+} and Et_2Cit (CaEtCit), which would increase the dissociation rate of Ca^{2+} from CaEtCit in the body. Therefore we assume using Et_2Cit as anticoagulant may reduce the occurrence of hypocalcemia and hypercalcemia.

Previous studies[3] have shown that Et_2Cit reduces the concentration (Ca^{2+}) in the blood, suggesting its anticoagulant effect. Ca^{2+} was found to dissociate from Et_2Cit significantly faster than from Na_3Cit 10 min post-injection, with the largest difference appearing at 1 min post-injection. In addition, the recovery rate of blood calcium concentration when using Et_2Cit was higher than using Na_3Cit. These findings strongly indicate that Et_2Cit is effective in preventing hypocalcemia.

There has been no mechanistic studies on formation of the complex of Ca^{2+} with Et_2Cit. None of the thermodynamic features of the complex including stoichiometry, stability constant (K_s) and thermodynamic functions have been reported. It is also unclear how specific factors affect these parameters. K_s is a widely-used indicator of the capability of a ligand for coordinating with metal ions. Therefore, characterization of K_s and thermodynamic functions of the Ca^{2+}-Et_2Cit complex would further our understanding of the anti-coagulation mechanism of Et_2Cit and ultimatley benefit its clinical application.

Two methods are commonly used for determining K_s of complexes. One is to directly determine changes of the concentration of the germplasm point in coordination reactions, using potentiometry (including pH potentiometry), ion-selective

electrode, polarography, solvent extraction, ion exchange, *etc.*[4]. The other method indirectly determines K_s by measuring changes of certain parameters such as conductivity or kinetic properties in the process of complex formation[5].

In this study, the stoichiometry and K_s of the complex of diethyl citrate (Et_2Cit) or sodium citrate (Na_3Cit) with Ca^{2+} ions were determined using an ion-selective electrode[6], the effects of pH and temperature on K_s measurement were studied and the thermodynamic functions of the complexes were also calculated.

EXPERIMENTAL

Electrode potential was measured by a PHS-3C pH meter; pH was measured by an E-201-C pH electrode assembly. Both instruments were purchased from Shanghai Precision & Scientific Instrument Co., Ltd.

Et_2Cit was prepared in our laboratory (99.3 % purity). The detailed procedure will be published elsewhere. All the other chemicals were analytical-grade and purchased from Sigma or Fluka or Shanghai Chemicals Co.

Ten clean and healthy male rabbits, weighing between 1.5 and 1.7 kg, were obtained from the animal experiment center of the medical college of Xi'an Jiaotong University and used in the present study.

Methods

Electrode standard curves and linear range: Two series of standard solution of Ca^{2+} ions were prepared in the concentration range of 0.01-100 mmol/L in presence of 0.50 mol/L KCl or saline (0.15 mol/L NaCl) solution for ionic strength adjustment. The equilibrium electrode potentials were determined on 20 mL of these standard solutions at constant temperature (37 ºC) and under stirring condition.

Effect of pH on electrode potential: The pH of the solutions was adjusted to 4, 5, 6, 7, 8, 9, 10, 11 and 12, respectively, using diluted HCl (10 and 1 mmol/L) and diluted NaOH. The equilibrium electrode potentials of these solutions were determined at constant temperature (37 ºC) and under stirring condition.

Determination of stoichiometry of complexes: 10 mL 10 mmol/L $CaCl_2$ solution was added to a set of beakers, then 1.0, 2.50, 5.00, 7.25, 10.0, 15.0, 20.0, 25.0 mL 10 mmol/L Et_2Cit solution was added to the beakers to prepare working solutions containing complexes with varying C_L/C_M ratios (M represents Ca^{2+}, L represents ligand). The volume was bought up to 45 mL with saline. Then their pH was adjusted to 7.4. The final volume of all these solutions was adjusted to 50 mL. The electrode potentials of these solutions were determined. A diagram was plotted for C_M–$c(Ca^{2+})$ against C_L/C_M and the C_L/C_M value at the linear inflection point of the plot was the stoichiometric ratio of the complex.

The stoichiometry of the Ca^{2+}-Na_3Cit complex was determined using the same method as described above.

Determination of stability constants of complexes: The stability constant (K_s) was determined for the complexes of known stoichiometry according to the following procedure. After mixing 2 mmol/L $CaCl_2$ solution with 2 mmol/L Na_3Cit or Et_2Cit solution, the electrode potentials of the mixed solutions were determined at constant temperature (37 ºC).

The concentration of Ca^{2+} ions, that is $c(Ca^{2+})$, was calculated according to the electrode potential and the regression equation. Because the stoichiometric ratio of complexes were 1:1 and $CaCl_2$ was mixed with Et_2Cit or Na_3Cit at a ratio of 1:1, the concentration of the ligand (Et_2Cit or Na_3Cit) was equal to that of Ca^{2+}.

If using C_M to represent the initial concentration of $CaCl_2$, C_M–$c(Ca^{2+})$ refers to the actual concentration of the complex, The stability constants (K_s) of $CaEt_2Cit$ and CaCit can be defined as follows:

$$CaEt_2Cit: K_s = \frac{c(CaEt_2Cit)}{c(Ca^{2+})c(Et_2Cit)} = \frac{[C_M - c(Ca^{2+})]}{c(Ca^{2+})^2} \quad (1)$$

$$CaCit: K_s = \frac{c(CaCit)}{c(Ca^{2+})c(Cit^{3-})} = \frac{[C_M - c(Ca^{2+})]}{c(Ca^{2+})^2} \quad (2)$$

Effect of pH on stability of complexes: After the pH of 2 mmol/L $CaCl_2$ solution and 2 mmol/L Na_3Cit or Et_2Cit solution was adjusted to 4.0, 5.0, 6.0, 6.5, 7.0, 7.5, 8.0, respectively, the $CaCl_2$ solution was mixed with the Na_3Cit or Et_2Cit solution with the same pH and the electrode potentials of the mixed solutions were determined. The concentrations of Ca^{2+} ions [$c(Ca^{2+})$] and the complexes [$c(CaEt_2Cit)$ or c(CaCit)] at specific pH levels were calculated according to the electrode standard curves.

Effect of temperature on the stability of complexes: The electrode potentials of the mixed solutions of $CaCl_2$ with Et_2Cit or Na_3Cit were determined at different temperature (15, 20, 25, 30, 37 or 40 ºC) in accordance with the above described procedure. The final concentrations of $CaCl_2$, Na_3Cit and Et_2Cit were 1 mmol/L. The values of $c(Ca^{2+})$, $c(CaEt_2Cit)$ and c(CaCit) were calculated according to the electrode standard curves and finally the stability constants of specific complexes at different temperature can be calculated using eqns. 1 and 2.

***In vitro* anticoagulant experimentation: Activated coagulation time test on blood of rabbits:** The *in vitro* anticoagulant effects of Et_2Cit were observed by measuring whole blood activated coagulation time (ACT). Twelve milligrams of commercially available silica was placed inside a small glass test tube with a 1 cm diameter, then the activated coagulation time test tubes were obtained. The tubes were placed in a water bath at 37 ºC to be pre-warmed. Extracted 12 mL arterial blood from carotid artery of the rabbits. The blood was quickly added to six activated coagulation time test tubes, each tube by adding 1.8 mL, then 0.2 mL of anticoagulants was also added to the tube. The concentration of anticoagulant was 0, 21.8, 54.5, 76.3, 87.2 and 109 mmol/L for Et_2Cit and 0, 2.18, 5.45, 7.63, 8.72 and 10.9 mmol/L for Na_3Cit, respectively. These tubes were plugged with a rubber stopper and rapidly reversed three times. A stopwatch was started at the same time. Blood clotting was observed in the water bath at 37 ºC by tilting the test tube once every 5 s, which began at 60 s. The activated coagulation time value is the time displayed on the stopwatch when the first blood clot appeared. The test was repeated three times and the average value was used.

The concentrations of free calcium ions in blood $c(Ca^{2+})$, in the presence of various concentrations of anticoagulants, was also determined.

RESULTS AND DISCUSSION

Calcium electrode standard curve and its linear range: The standard curves for measurement with the ion-selective electrode in 0.50 mol/L KCl and saline (0.15 mol/L NaCl) were shown in Fig. 1. The linear ranges of both curves fell between 0.1-100 mmol/L. When the concentration was lower than 0.1 mmol/L, large deviation was observed.

The linear regression equation for the sample series in 0.50 mol/L KCl was: y = 30.4x + 84.5 (y represents the electrode potential (E), x represents -pCa). The correlation coefficient was 0.9993 and the electrode slope was 30.4 mV/pCa (37 ºC).

The regression equation for the sample series in saline was: y = 30.0x + 83.0. The correlation coefficient was 0.9985 and the electrode slope was 30.0 mV/pCa (37 ºC).

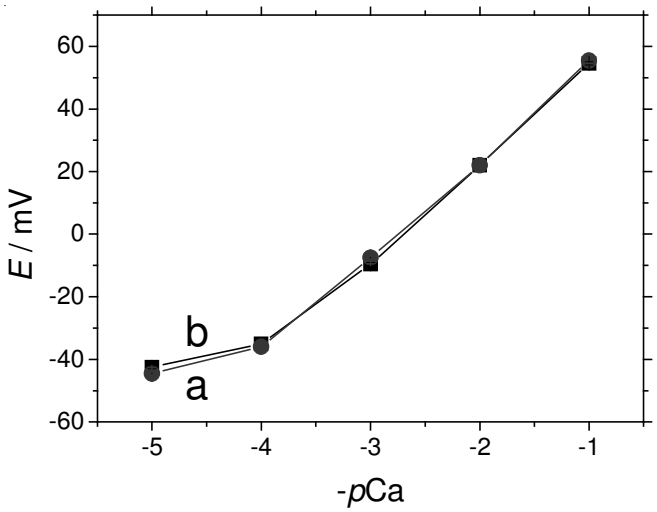


Fig. 1. Nernst response curves of the calcium ion-electrode in different solution for ionic strength adjustment (a) 0.50 mol/L KCl, (b) saline solution (0.15 mol/L NaCl)

Effect of pH on the electrode potential: The normal pH range of human blood is between 7.35-7.45. However, the pH may go beyond this range in acid-base balance disorders such as acidosis or alkalosis. Therefore, the effect of changing pH on electrode potential was measured (Fig. 2). The electrode potential turned out to be stable when the pH was within the range of 5 to 9 for 1 mmol/L $CaCl_2$ solution. Therefore, regular pH fluctuation in the blood will not affect measurement of the electrode potential.

However, we observed the curve bended when pH < 5 or pH > 9. This was due to the effect of H^+ or OH^- ions on the sensitive membrane of the electrode, causing the changes of the electrode potential. When pH < 5, the concentration of H^+ significantly interrupted the selectivity of the calcium ion-selective electrode. When pH > 9, the OH^- might combine with Ca^{2+} and led to the formation of $Ca(OH)_2$ precipitate.

Determination the stoichiometry of complexes: When the electrode potentials of a series of working solutions with different C_L/C_M ratios were determined, the concentrations of Ca^{2+} ions were calculated according to the regression equation derived from the standard curve (Fig. 1b). The concentration of the complex is defined by C_M–$c(Ca^{2+})$, where C_M represents

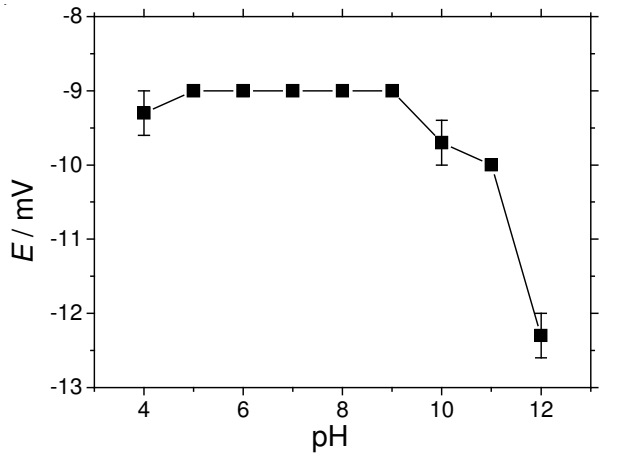


Fig. 2. Effect of pH on measurement of electrode potentials of the calcium ion-electrode in saline solution. $c(Ca^{2+})$ = 1 mmol/L

the initial Ca^{2+} concentration which was 2 mmol/L. Fig. 3 shows the plot of C_M–$c(Ca^{2+})$ as a function of C_L/C_M. In this plot, the C_L/C_M ratio at the inflection point is the stoichiometric ratio of the complex.

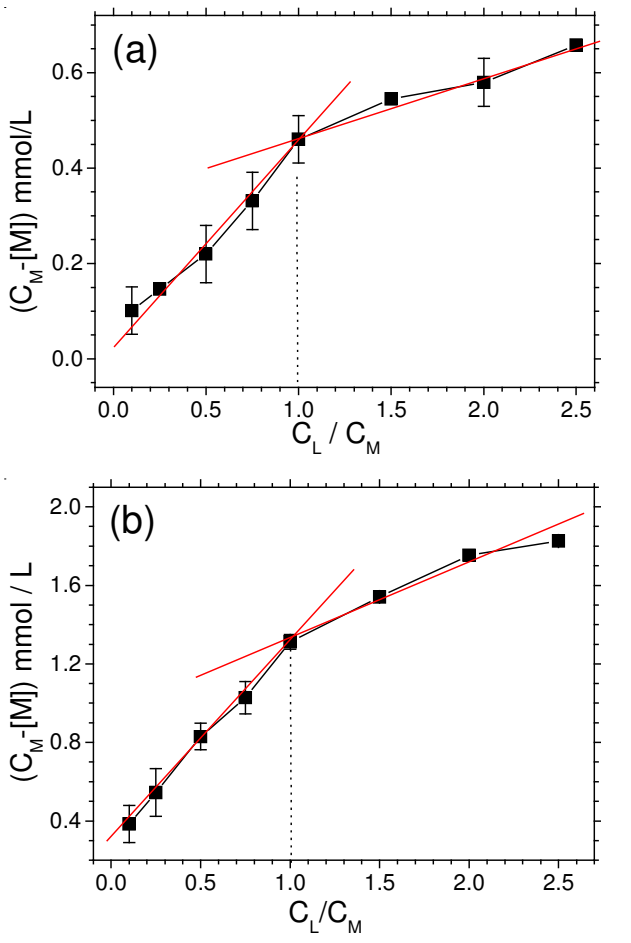


Fig. 3. Electrode potentials of the solutions of $CaCl_2$ mixed with Et_2Cit (a) and Na_3Cit (b) at varying molar ratios (37 ºC, saline solution)

Because the C_L/C_M ratios at the inflection points of the two curves for Et_2Cit and Na_3Cit complexes were both found to be 1, it is concluded that 1:1 complexes were formed between Ca^{2+} and Et_2Cit or Na_3Cit at pH 7.4 and 37 ºC.

Determination of the stability constants of complexes: With the known stoichiometry of the two complexes (1:1), we were able to determine their specific stability constants (K_s). We first mixed Et_2Cit with $CaCl_2$ at the same concentration (1 mmol/L) and measured the electrode potentials of three experimental replicates to be -9, -9 and -10 mV, with an average value of -9.3 mV. According to the regression equation, the concentration of Ca^{2+} was calculated to be 0.838 mmol/L, which was then used to determine the concentration of the complex $CaEt_2Cit$ as: $c(CaEt_2Cit) = C_M\text{-}c(Ca^{2+}) = (1.0\text{-}0.838) = 0.162$ mmol/L.

Similarly, the measured electrode potentials of three replicates of mixing Na_3Cit with $CaCl_2$ were -15, -17 and -16 mV, respectively. The average value was -16 mV. Thus the concentration of Ca^{2+} was calculated to be 0.501 mmol/L and the concentration of the complex CaCit was determined as: $c(CaCit) = C_M\text{-}c(Ca^{2+}) = (1.0\text{-}0.501) = 0.499$ mmol/L.

The stability constants of the CaCit and $CaEt_2Cit$ complexes formed at pH 7.4 and 37 ºC in the saline solution can be calculated by substituting these values into eqns. 1 and 2, as shown below:

Calcium diethyl citrate ($CaEt_2Cit$):

$$K_s = \frac{c(CaEt_2Cit)}{c(Ca^{2+})c(Et_2Cit)} = \frac{1.62\times10^{-4}}{(8.38\times10^{-4})^2} = 231$$

Calcium citrate (CaCit):

$$K_s = \frac{c(CaCit)}{c(Ca^{2+})c(Cit^{3-})} = \frac{4.99\times10^{-4}}{(5.01\times10^{-4})^2} = 1988$$

Obviously, the stability constant of the complex $CaEt_2Cit$ (K_s = 231) was significantly lower than that of CaCit (K_s = 1988), which indicates much lower stability of $CaEt_2Cit$ than CaCit. It should be noted that the stability constants of CaCit measured in this study are in accordance with the values reported in previous literature, which are listed in Table-1.

TABLE-1
COMPARISON OF THE STABILITY CONSTANTS OF THE COMPLEX CaCit REPORTED IN PREVIOUS LITERATURE

Method	Temp. (ºC)	Ionic strength	K_s	Ref.
Solubility	37	0.15 mol L^{-1} NaCl	2040	7
Solubility	37	0.15 mol L^{-1} NaCl	1940	8
Ca electrode	37	0.15 mol L^{-1} NaCl	1850	8
Ca electrode	37	0.15 mol L^{-1} NaCl	2138	9
Ca electrode	37	0.15 mol L^{-1} NaCl	1988	This test

The use of Na_3Cit as anticoagulating agent was mainly based on the fact that Na_3Cit can bind to calcium ion (Ca^{2+}) to form a water-soluble complex calcium citrate (CaCit) that is difficult to dissociate. Generation of CaCit reduces the concentration of free Ca^{2+} in plasma and thus anticoagulation is achieved. However, due to the strong coordination ability of Na_3Cit, it can cause hypocalcemia by chelating the Ca^{2+} ion in the blood. As a result, patients are usually required to replenish calcium after injecting Na_3Cit for treatment. Furthermore, the CaCit complex formed in the process of dialysis will be decomposed eventually in the body. These free Ca^{2+} ions released from CaCit decomposition, together with the aforementioned supplement of calcium, cause patients to be hypercalcemia.

By contrast, Et_2Cit can chelate Ca^{2+} faster than Na_3Cit to form a less stable complex, therefore, we would expect Et_2Cit, not only reduces the concentration of free calcium to prevent coagulantion, but also lowers the chances of hypocalcemia and hyperlipidemia.

Effect of pH on the stability of complexes: The stability constants of the complexes $CaEt_2Cit$ and CaCit determined at 37 ºC and varying pH from 4 to 8 were listed in Table-2.

As the pH increased from 4 to 8, the concentrations of Ca^{2+} after reacting Et_2Cit with $CaCl_2$, decreased from 0.906 mmol/L to 0.794 mmol/L, but the concentrations of $CaEt_2Cit$ increased from 0.094 mmol/L to 0.206 mmol/L. Under the same condition, the concentrations of Ca^{2+}, after reacting Na_3Cit with $CaCl_2$, decreased from 0.719 mmol/L to 0.465 mmol/L, but the concentrations of CaCit increased from 0.281 mmol/L to 0.535 mmol/L.

Present results first suggested that the increase of pH facilitates formation of the complexes. Secondly, the ability of Na_3Cit to chelate Ca^{2+} is significantly higher than that of Et_2Cit, given that the Ca^{2+} concentration in the solution of Na_3Cit and $CaCl_2$ was largely reduced compared with that in the solution of Et_2Cit and $CaCl_2$ at the same pH. It indicated that Na_3Cit more readily causes hypocalcemia than Et_2Cit when used as anticoagulant during dialysis.

Effect of temperature on stability constants: The stability constants of the complexes $CaEt_2Cit$ and CaCit were determined at different temperature (15, 20, 25, 30, 37 and 40 ºC) at pH 7.4. The plots of K_s against temperature for each complex were shown in Fig. 4.

As the temperature increased from 15 to 40 ºC, the K_s of $CaEt_2Cit$ and CaCit rose from 58 to 327 and from 327 to 2327, respectively. It indicates that the increase of temperature in

TABLE-2
ELECTRODE POTENTIALS AND CONCENTRATIONS OF Ca^{2+} AND THE COMPLEXES AFTER REACTING $CaCl_2$ WITH EQUIVALENT MOLAR OF Et_2Cit OR Na_3Cit AT DIFFERENT pH

	pH						
	4.0	5.0	6.0	6.5	7.0	7.5	8.0
$Et_2Cit\text{-}CaCl_2$ system							
$c(Ca^{2+})$ (mmol/L)	0.906	0.877	0.857	0.838	0.838	0.794	0.794
$C_M\text{-}c(Ca^{2+})$ (mmol/L)	0.094	0.123	0.143	0.162	0.162	0.206	0.206
K_s	114	160	195	231	231	327	327
$Na_3Cit\text{-}CaCl_2$ system							
$c(Ca^{2+})$ (mmol/L)	0.719	0.528	0.501	0.501	0.475	0.490	0.465
$C_M\text{-}c(Ca^{2+})$ (mmol/L)	0.281	0.472	0.499	0.499	0.525	0.510	0.535
K_s	543	1693	1988	1988	2327	2124	2474

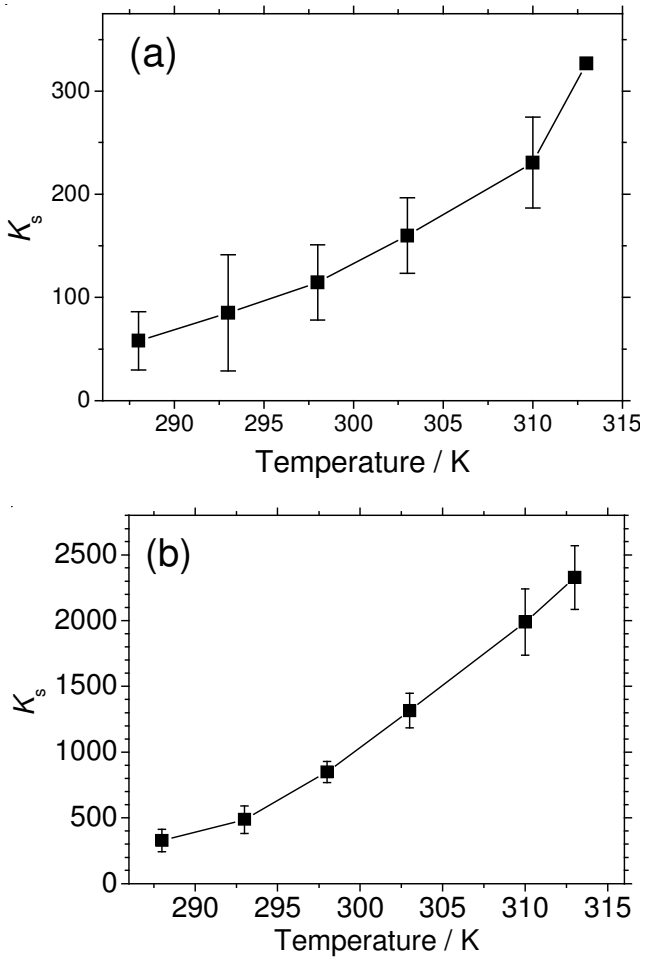


Fig. 4. Effect of temperature on the stability constants of complexes (a) $CaEt_2Cit$; (b) CaCit

the range of 15-40 ºC (288-313 K) is in favour of formation of the complex.

Calculation of thermodynamic functions: The Gibbs free energy ΔG of the complex formed at different temperature can be calculated according the following eqn. 3:

$$\Delta G = -RT \ln K \quad (3)$$

Assuming the change of enthalpy with temperature was little and ΔH was approximated as a constant, by integrating the Gibbs-Helmholtz equation $[\frac{\partial(\frac{\Delta G}{T})}{\partial T}]_p = -\frac{\Delta H}{T^2}$ on both sides of eqn. 4, we obtain:

$$\Delta H = (\frac{\Delta G_2}{T_2} - \frac{\Delta G_1}{T_1}) / (\frac{1}{T_2} - \frac{1}{T_1}) \quad (4)$$

The ΔS at temperature T can be calculated by eqn. 5:

$$\Delta S = \frac{\Delta H - \Delta G}{T} \quad (5)$$

The thermodynamic functions of the complexes were calculated based on the eqns. 3, 4 and 5 and the results were shown in Table-3. Our data indicated:

(1) Both the coordination reactions of Ca^{2+} with Et_2Cit or Na_3Cit were endothermic because their changes of enthalpy (ΔH) were above zero. The high absolute value of ΔH, further suggested a large impact of temperature on the coordination reactions.

(2) Both coordination reactions were spontaneous reactions because the changes of their Gibbs free energy (ΔG) were below zero. As the ΔG of reacting Na_3Cit with Ca^{2+} was more negative than that of reacting Et_2Cit with Ca^{2+}, it is indicated that the coordination reaction between Na_3Cit with Ca^{2+} was a more spontaneous process.

(3) It can be inferred from the slope of the T-K_s diagram (Fig. 4) that the K_s of $CaEt_2Cit$ and CaCit complexes increased significantly with rising temperature, further indicating that higher temperature in the range of 15-40 ºC was correlated to formation of more stable complexes.

(4) The ΔS of both coordination reactions were above zero, which seemingly contradicted the fact that formation of complexes is a process of reducing the micro-state number. However, it can be explained by entropy compensation principle. Ca^{2+} ions in the aqueous solution possessed hydration shells, that is, Ca^{2+} ions were in the actual form of $Ca(H_2O)_4^{2+}$. For Cit^{3-} and Et_2Cit, they were also trapped in a similar hydrated shell of orderly-arranged water molecules. When Ca^{2+} and Cit^{3-} or Et_2Cit broke their hydration shells and coordination reactions occurred, the water molecules originally in ordered arrangement restored their regular iceberg structure, leading to reduced order. Consequently, this was an entropy-increasing process. In other words, the change of entropy ΔS measured in the experiment was the sum of ΔS for both cocmbining ions ($\Delta S_1 < 0$) and breaking the hydration shells ($\Delta S_2 > 0$ and $\Delta S_1 + \Delta S_2 > 0$). Indeed, it was the entropy increase of the solvent water that compensated the entropy reduction in the process of ion combination to form complexes, which drove the coordination reaction to occur spontaneously[10].

TABLE-3
THERMODYNAMIC FUNCTIONS OF CaCit AND $CaEt_2Cit$ AT DIFFERENT TEMPERATURE

	Temperature (ºC)					
	15	20	25	30	37	40
CaCit						
K_s	327	487	848	1316	1988	2327
ΔG (kJ mol^{-1})	-13.9	-15.1	-16.7	-18.1	-19.6	-20.2
ΔH (kJ mol^{-1})	58.9	58.9	58.9	58.9	58.9	58.9
ΔS (J mol^{-1} K^{-1})	252	252	254	254	253	252
$CaEt_2Cit$						
K_s	58	85	114	160	231	327
ΔG (kJ mol^{-1})	-9.7	-10.8	-11.8	-12.8	-14.0	-15.1
ΔH (kJ mol^{-1})	51.9	51.9	51.9	51.9	51.9	51.9
ΔS (J mol^{-1} K^{-1})	214	214	214	213	213	214

***In vitro* anticoagulant experimentation:** Fig. 5 showed the effect of concentration of anticoagulants on whole blood activated coagulation time (ACT) for arterial blood samples of rabbits. The activated coagulation time values increased from 152 ± 21 s to 428 ± 62 and 847 ± 138 s, respectively and the concentrations of free calcium ions in blood c(Ca^{2+}) decreased from 1.32 ± 0.04 mmol/L to 1.01 ± 0.05 and 0.58 ± 0.02 mmol/L, respectively, when the Et_2Cit concentration was increased from 21.8 mmol/L to 54.5 and 76.3 mmol/L. When the Et_2Cit concentration was greater than 7.2 mmol/L, blood coagulation did not occur within 1200 s and c(Ca^{2+}) was lower than 0.2 mmol/L.

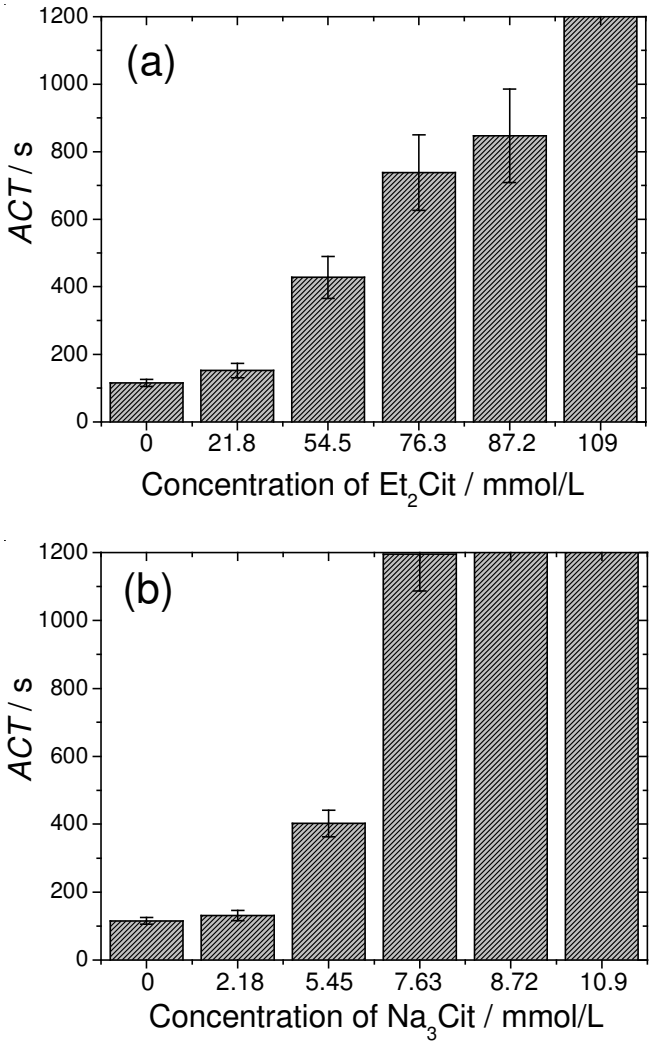


Fig. 5. Effect of concentration of anticoagulants on whole blood activated coagulation time (ACT) for arterial blood samples of rabbits (a) Et_2Cit; (b) Na_3Cit

In comparison, when Na_3Cit with a concentration equivalent to one-tenth of Et_2Cit was used, the activated coagulation time values increased from 131 ± 15 s to 402 ± 39 and 1195 ± 108 s and c(Ca^{2+}) decreased from 1.29 ± 0.03 mmol/L to1.03 ± 0.01 and 0.50 ± 0.03 mmol/L when the Na_3Cit concentration was increased from 2.18 mmol/L to 5.45 and 7.63 mmol/L, respectively. Blood coagulation was not observed within 1200 s when the Na_3Cit concentration was greater than 8.72 mmol/L and the blood Ca^{2+} concentration was lower than 0.2 mmol/L.

Conclusion

Calcium ions can form complexes with Et_2Cit or Na_3Cit at a ratio of 1:1 and the stability constants of $CaEt_2Cit$ and CaCit measured at 37 ºC and pH 7.4 are 231 and 1988, respectively. It is indicated that CaCit is much more stable than $CaEt_2Cit$. The increase of pH and temperature is in favour of formation of the two complexes. The thermodynamic properties (ΔG, ΔH, ΔS) reveales that the two coordination reactions are endothermic and spontaneous reactions. Activated coagulation time testing results on animal rabbits showed that activated coagulation time exceeded 1200 s and c(Ca^{2+}) was lower than 0.2 mmol/L when the concentrations of Et_2Cit and Na_3Cit were greater than 87.2 and 8.72 mmol/L, respectively. The recovery speed of blood calcium concentration with Et_2Cit as anticoagulant was more rapid than that with Na_3Cit. Since Et_2Cit can chelate Ca^{2+} ions to form a complex of much lower stability than CaCit, we envision Et_2Cit could be a more effective anticoagulant by avoiding the problems of hypocalcemia and hypercalcemia usually caused by using Na_3Cit. Therefore, Et_2Cit offers new promise for anticoagulantion treatment. The future direction would be an in-depth study of the pharmacodynamics, pharmacokinetics and safety issue of this new agent.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (30871164).

REFERENCES

1. H.M. Oudemans-van Straaten, R.J. Bosman, M. Koopmans, P.H. van der Voort, J.P. Wester, J.I. van der Spoel, L.M. Dijksman and D.F. Zandstra, *Crit. Care Med.*, **37**, 545 (2009).
2. J. Kozik-Jaromin, V. Nier, U. Heemann, B. Kreymann and J. Bohler, *Nephrol. Dial. Transplant.*, **24**, 2244 (2009).
3. Z. Chen, B. Chen, X.-Q. Yao, B.-S. Gui, J. Han and J.-M. Ouyang, *Blood Purif.*, **33**, 30 (2012).
4. K. Uselova-Vcelakova, I. Zuskova and B. Gas, *Electrophoresis*, **28**, 2145 (2007).
5. Yuan-Pei, Xia Z-N, Liu-Yong, *J. Instrum. Anal.*, **20**, 79 (2001).
6. F. Jing, X.-J. Shen and J.-J. Wang, *Electroanalysis*, **13**, 1115 (2001).
7. A.B. Hastings, F.C. McLean, L. Elchelberger, J.L. Hall and E. Dacosta, *Biol. Chem.*, **107**, 351 (1934).
8. R.P. Singh, Y.D. Yeboah, E.R. Pambld and P. Debayle, *J. Chem. Eng.*, **36**, 52 (1991).
9. A.K. Covington and E.Y. Danish, *J. Solution Chem.*, **38**, 1449 (2009).
10. K. Sharp, *Protein Sci.*, **10**, 661 (2001).