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Synthesis and Characterization and Anticoagulant Properties of Diethyl Citrate

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Anticoagulation is an essential for the success of hemodialysis. However, sodium citrate (Na_3Cit), a major anticoagulant in current clinical application, causes hypocalcemia due to its strong binding with Ca^{2+} ion and the slow Ca^{2+} release rate from the chelate formed. We have synthesized a novel anticoagulant: diethyl citrate (Et_2Cit). The compound was charcterized by its elemental analysis, infrared spectroscopy, mass spectrometry and nuclear magnetic resonance. The purity was established by thin layer chromatography and acid titration. The mass fraction of Et_2Cit was 99.27 %. The *in vitro* anticoagulatory effect of Et_2Cit was investigated by measuring the whole blood activated clotting time. Since the introduction of two ethyl groups to citric acid enhanced the steric hindrance for Ca^{2+} chelation, it led to a reduced Ca^{2+} chelation and accelerated Ca^{2+} release from Et_2Cit in comparison to Na_3Cit. Acute toxicity test in mice shows that Et_2Cit has no serious risk of acute poisoning. Therefore, Et_2Cit can be a potential and an ideal anticoagulant.

Key Words: Anticoagulant, NMR, Mass spectrometry, Diethyl citrate, Acute toxicity test.

INTRODUCTION

Hemodialysis is routinely used for toxin clearance in various acute or chronic renal insufficiency. During hemodialysis, anticoagulant agent is required to prevent clot formation in dialyzer and dialysis pipeline and resulting in failure of hemodialysis. However, there is no perfect and simple anticoagulation method in clinical appplication as yet.

Nowadays, heparin is a widely used anticoagulant[1,2], but excessive heparin treatment during dialysis inhibits platelet aggregation, which enhances bleeding tendency with a secondary hemorrhage incidence as high as 10-30 %. In addition, it causes several other side effects, such as osteoporosis. Therefore, how to choose appropriate anticoagulant(s) for blood purification therapy is one of the major clinical problems.

The alternative approaches for heparin treatment include low molecular weight heparin dialysis, no heparin dialysis, low dose heparin dialysis, regional protamine-heparin neutralization anticoagulation dialysis, prostacyclin anticoagulation dialysis, recombinant hirudin anticoagulation dialysis and sodium citrate (Na_3Cit) anticoagulation dialysis.

However, all these methods exhibit limitations[3-7]. For example, in regional Na_3Cit anticoagulation dialysis, Na_3Cit can bind calcium ion (Ca^{2+}) to form calcium citrate, which is difficult to dissociate but water-soluble and thus, the concentration of free Ca^{2+} ions in plasma is reduced and *in vitro* anticoagulation is achieved[5,8,9]. This solves the problem of secondary hemorrhage and has attracted a lot of attention in recent years[5,8-12]. However, this method can cause hypocalcemia, citric acid poisoning, *etc.*[10]. The limitations of Na_3Cit anticoagulation are mainly attributed to its strong chelating capability with Ca^{2+} ion and the resulting slow Ca^{2+} dissociation rate, which restricts its clinical application.

Based on the above knowledge, we aim to synthesize and characterize a new anticoagulant with the advantages of Na_3Cit but reducing Ca^{2+} chelating capability.

That is, by substituting two hydroxyl atoms in -COOH in citric acid with oxyethyl groups ($-OC_2H_5$), a new anticoagulant, diethyl citrate (Et_2Cit), was synthesized. Diethyl citrate can chelate with Ca^{2+} ion to reduce the plasma calcium concentration to promote anticoagulation. At the same time, the alkyl around the carboxyl oxygen atoms produces steric hindrance, which reduced the chelation of these ester groups with Ca^{2+}, *i.e.*, the chelate formed by Ca^{2+} ions and citrate ester should exhibit higher Ca^{2+} dissociation rate than calcium citrate. Thus, diethyl citrate can potentially be a novel anticoagulant.

EXPERIMENTAL

All reagents used in experiments were in analytical grade, including citric acid, anhydrous ethanol, benzene, petroleum ether (b.p. 60-90 ºC), ethyl acetate and sodium carbonate.

Instruments used in experiments include CHN-O-Rapid elemental analyzer (Foss-Heraeus Company), Bruker AM 500 NMR instrument (with $CDCl_3$ as solvent and TMS as internal standard), FT-IR spectrometer (Nicolet-170 SX-type), high resolution mass spectrometer (MAT95XP, Finnigan, USA) and UV-VIS-NIR UV-visible spectrophotometer (UV-3100 type, Shimadzu Corporation).

Synthesis and purification of diethyl citrate

Synthesis: Diethyl citrate (Et_2Cit) was prepared from citric acid and anhydrous alcohol. The reaction scheme is shown in Fig. 1. In a 250 mL three-neck bottle installed with a condenser and a constant pressure dropping funnel filled with anhydrous magnesium sulfate particles, 21 g (0.1 mol) citric acid monohydrate and 0.2 g catalyst *p*-toluene sulfonic acid (PTS) were dissolved in 42 mL (0.7 mol) anhydrous ethanol under magnetic stirring at room temperature. 70 mL benzene was added to the bottle as a water carrier and the reaction was carried out at 90 ºC for 10 h. Due to the formation of ethanol-benzene azeotrope, the temperature within bottle is actually maintained at *ca.* 72 ºC. After the benzene and the remaining ethanol were removed by reduced-pressure distillation, a viscous, light-yellow coloured liquid was finally obtained.

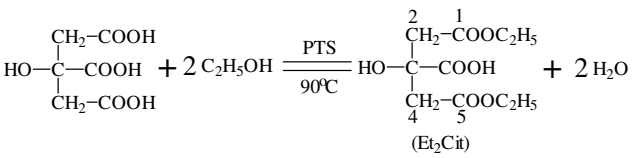


Fig. 1. Synthesis of diethyl citrate (Et_2Cit)

Purification: To remove triethyl citrate, the viscous liquid was diluted with 20 mL distilled water, adjusted to pH 8 with Na_2CO_3 and then extracted with 3 mL × 50 mL petroleum ether and 1 mL × 50 mL ethyl acetate, respectively. The carboxyl groups (-COOH) in the remaining citric acid and some by-products (monoethyl citrate and diethyl citrate) were neutralized by Na_2CO_3 to form sodium salt (-COONa) and left in aqueous solution, while the triethyl citrate (Et_3Cit) was extracted to the organic layer and removed.

To remove citric acid and monoethyl citrate, the above system was adjusted to pH 4 with hydrochloric acid, which allowed citric acid, its monoethyl ester and the catalyst *p*-toluene sulfonic acid to be dissolved in water phase, while Et_2Cit and the sodium salt residues were extracted to organic phase by ethyl acetate. Then ethyl acetate was distilled in rotary evaporator to obtain the crude Et_2Cit. The carboxylic acid sodium salt (-COONa) in crude product was converted to carboxylic acid (-COOH) by adjusting to pH 1 with hydrochloric acid and then the solution was extracted with 3 mL × 50 mL ethyl acetate. Water in the organic extract was removed by anhydrous sodium sulfate and the solvent was distilled in a rotary evaporator. About *ca.* 16.2 g Et_2Cit was obtained and the yield was 65.3 %. The product was analyzed by elemental analysis, infrared spectroscopy, mass spectrometry, nuclear magnetic resonance and thin-layer chromatography and the purity was determined by acid titration.

Primary spectral data of Et_2Cit: Elemental analysis ($C_{10}H_{16}O_7$, M = 248.2): calculated values: C 48.35, H 6.50; experimental values: C 47.82, H 6.80. FT-IR (KBr, ν_{max}, cm^{-1}): 3469 (-OH), 2986-2940 (-CH_2, -CH_3), 1736 (RO-C=O). ^1H NMR ($CDCl_3$): δ_1 (ppm) 1.24-1.33 (6H, -CH_3), δ_3 2.75-3.00 (4H, -OCH_2-), δ_4 4.05-4.35 (4H, -CH_2-). ^{13}C NMR ($CDCl_3$): δ (ppm) 14.0-14.2 (-CH_3), 43.0-43.4 (-CH_2 in -C-CH_2-COO-), 61.1-62.6 (-CH_2 in -OCH_2CH_3), 73.1-77.1 (C-OH), 173.4-173.9 (-COO in -CH_2COOR), 176.5-176.7 (R-COOH). UV-VIS (aqueous solution): 220 nm.

Purity analysis by acid titration: Because the product might contain a small amount of monoethyl citrate, mass fraction of Et_2Cit in the final product was determined by acid titration. Certain amount of product was accurately weighed and diluted in 20 mL distilled water in a 100 mL flask. 3-5 drops of phenolphthalein was added and the solution was titrated with 0.1 mol/L standard KOH solution, whose concentration was calibrated by potassium hydrogen phthalate. The mass fraction of Et_2Cit was calculated based on the consumption of KOH solution.

Anticoagulatory analysis of diethyl citrate: The efficacy of *in vitro* anticoagulation for Et_2Cit was investigated by measuring activated coagulation time (ACT). 12 mg commercial silica was pre-warmed in a 1-cm diameter glass test tube in 37 ºC water bath and then 1 mL venous blood from the experimental dogs was added immediately after extraction. After supplementing anticoagulant as shown in Table-1, the test tube was quickly plugged with a rubber plug and the solution was mixed upside down for three times. The tube was placed into 37 ºC water bath. After 1 min, the blood clotting was monitored every 5 s by tilting the tube. The time when the first blood clot(s) appeared was recorded as activated coagulation time.

TABLE-1
WHOLE BLOOD ACTIVATED CLOTTING TIME (ACT) FOR CANINE VENOUS BLOOD SAMPLES IN THE PRESENCE OF 10.9 mmol/L VARIOUS ANTICOAGULANTS

Anticoagulant	ACT/s
Control group (group I)	115 ± 10
Na_3Cit group (group II)	> 14400
Diethyl citrate group (group III)	141 ± 15
Triethyl citrate group (group IV)	122 ± 13

Twenty whole blood samples from selected experimental dogs were divided into four groups, each group using the following anticoagulant:

Group I was the negative control group: 0.9 % physiological saline.

Group II was the positive control group: 10.9 mmol/L of sodium citrate solution.

Group III was experimental group 1:10.9 mmol/L of diethyl citrate solution.

Group IV was experimental group 2:10.9 mmol/L of triethyl citrate solution.

Acute toxicity test in animals: Animal test was processed by an up-and-down procedure. Each time drug was administered to one animal. If the animal survived, higher dose was administered to the second animal; if the first animal died or nearly died, lower dose was administered to the second animal.

Method of drug delivery: An intraperitoneal injection was used for drug delivery. The details sdfd as follow: seven healthy female mice were chosen, each weighing 18-22 g. All

the mice were provided by Medical School Animal Experiment Center, Xi'an Jiaotong University.

The mice were randomly divided into 7 groups and each group contained one mouse. The control group only received physiological saline. The other six groups received various amounts of Et_2Cit as indicated in Table-2. After Et_2Cit injection, each mouse was carefully examined and recorded two times for the first day and then one time per day. This observation continued to the 14th day. The following content was monitored: the time that toxic effects appeared and disappeared for each animal, the breathing, autonomic activity and behaviour of the central nervous system, *etc*. Mice were weighed 1 week before and after drug administration. All animals were analyzed by autopsy and the abnormal organ was examined histo-pathology.

RESULTS AND DISCUSSION

Common physical and chemical properties of Et_2Cit: The final product of diethyl citrate (Et_2Cit) is a colourless and transparent liquid with ester scent. Its boiling point is 355 ºC, relative density (25 ºC) is 1.28 g/cm^3 and refractive index (25 ºC) is 1.477. Diethyl citrate can dissolve in water, alcohol, ether and ethyl acetate. The solubility in water is 15.7 g/100 mL (25 ºC). Et_2Cit is light-, heat- and oxygen-stable. The pH value of 0.1 mol/L Et_2Cit aqueous solution at 25 ºC is 2.22, which is higher than 0.1 mol/L citric acid (pH 2.04) but lower than 0.1 mol/L acetic acid (pH 2.84).

Purity analysis of diethyl citrate

Purity analysis by acid titration: After synthesis reaction, the Et_2Cit contained certain by-products, including monoethyl citrate, triethyl citrate and the unreacted citric acid. During the purification process, triethyl citrate was completely removed by extraction with petroleum ether and ethyl acetate owing to its low solubility in water (S = 6.5 g). Citric acid could barely exist in the final product because of its high solubility (S = 60 g). The solubility of monoethyl citrate in water (S = 31 g) was higher than that of diethyl citrate (S = 15.7 g), so did the acidity and thus, most monoethyl citrate was removed by the pH 4 extraction step. In the final product, the major possible impurity is a small amount of monoethyl citrate. The purity of Et_2Cit was determined by acid titration.

Three Et_2Cit samples, weighing 0.2368, 0.2236 and 0.2535 g, was titrated with 0.09231 mol/L standard KOH solution and the consumed KOH volume was 10.53, 9.78 and 11.10 mL, respectively. The mass fractions of Et_2Cit were 98.19, 99.86 and 99.70 %, respectively, with an average value of 99.27 %. When calculating Et_2Cit mass fraction, monoethyl citrate was regarded as the sole remainder, *i.e*., 1 mol Et_2cit consumed 1 mol KOH, whereas 1 mol monoethyl citrate consumed 2 mol KOH.

Thin layer chromatography (TLC): To further confirm the purity of Et_2Cit product. TLC analysis was performed using silica gel G plates, with the developing agent of butyl alcohol, acetic acid and water (4:1:5, v/v). After development, silica gel G plates were removed and left in air for 2 h to volatilize acetic acid and then stained by bromocresol green. The product showed accumulated spots, with a RT of 3.6, which was significantly higher than that of citric acid (3.0), indicating high purity of Et_2Cit product. It is worth noting that the acetic acid on silica gel G plates needs to be completely volatilized before staining; otherwise, bromocresol green would react with acetic acid and make the whole plate yellow, which seriously interfered with the observation.

Spectral analyses of diethyl citrate

FT-IR spectrum of diethyl citrate: Because Et_2Cit was a liquid sample, its FT-IR spectrum was recorded by liquid membrane method. Two thin slices of KBr were prepared. A small amount of Et_2Cit was evenly spread on one KBr slice and then covered by the other. FT-IR spectrum was recorded using the conventional approach and the results were shown in Fig. 2.

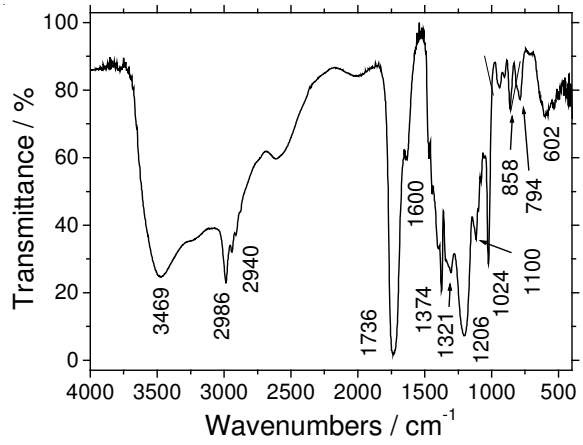


Fig. 2. FT-IR spectrum of diethyl citrate

The stretching vibration absorption peak for carboxyl (-COOH) of Et_2Cit appeared at 3469 cm^{-1}, which exhibited *ca.* 150 cm^{-1} redshift compared with free -COOH, indicating the existence of hydrogen bond in carboxyl group.

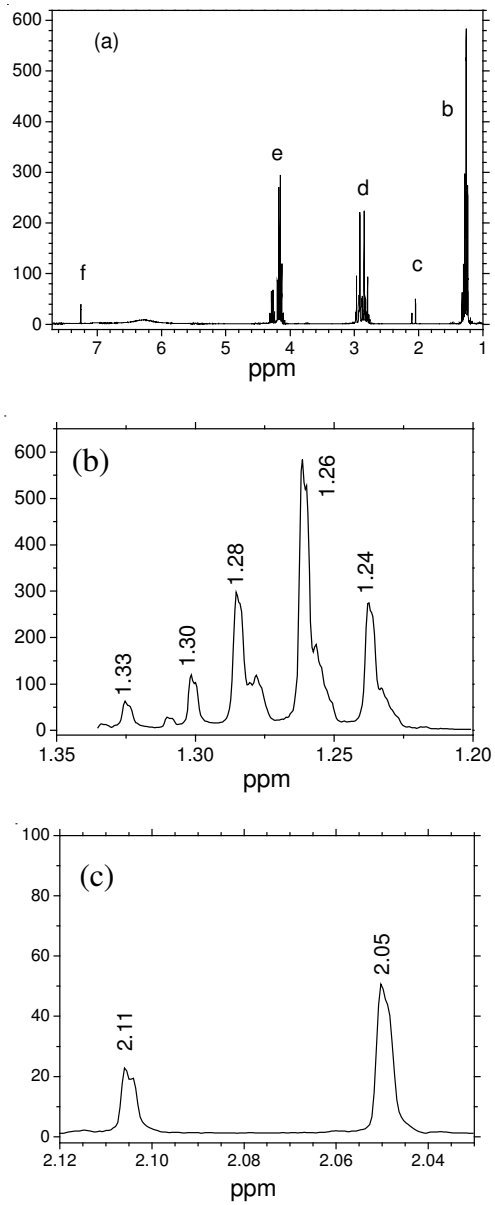
TABLE-2
RESULTS OF Et_2Cit TOXICITY TEST (n = 1)

Group	Time of drug administration	Et_2Cit dose (mg)	Et_2cit dose/mouse body weight (g/kg)	Mouse reaction
1 (control group)	2011.4.23	0	0	Normal activities, no death
2	2011.4.23	94	4.9	No significant reduction of activity; no appearance of unsteady gait or slowness; no slack or shortness of breath and jump; no death
3	2011.4.24	129	5.9	Ibid
4	2011.4.25	161	6.9	Ibid
5	2011.4.26	190	7.9	Activity reduction, accompanied by unsteady gait, slowness, sluggish, shortness of breath and jump; no death
6	2011.4.27	208	8.9	Ibid
7	2011.4.28	258	9.9	Ibid

There were a pair of vibration absorptions at 1374 and 1321 cm^{-1}, which result from the coupling of ν(OH) and ν(C=O) in interior surface. However, these two absorption peaks were weak because two of the three -COOH groups in citric acid turned into ester groups (-$COOC_2H_5$) and only one carboxyl left. The absorption peaks of methyl and methylene appeared at 2986 and 2940 cm^{-1}.

The characteristic absorption peak of carbonyl [ν(C=O)] in ester appeared at 1736 cm^{-1}, which was consistent with the published value for free fatty ester[13,14]. In addition, absorption peaks of esters for asymmetric [(ν_{as}(C-O-C)] and symmetric stretching vibration [ν_s(C-O-C)] were observed at 1206 and 1100 cm^{-1}, respectively and the intensity of as C-O-C absorption peak was stronger than that of ν_s(C-O-C). The simultaneous appearance of as C-O-C and ν_s(C-O-C) absorption peaks is a significant characteristic for esters in comparison to other carbonyl compounds (such as carboxylic acid).

^{1}H NMR spectra of Et_2Cit: The ^{1}H NMR spectra of Et_2Cit were shown in Fig. 3.



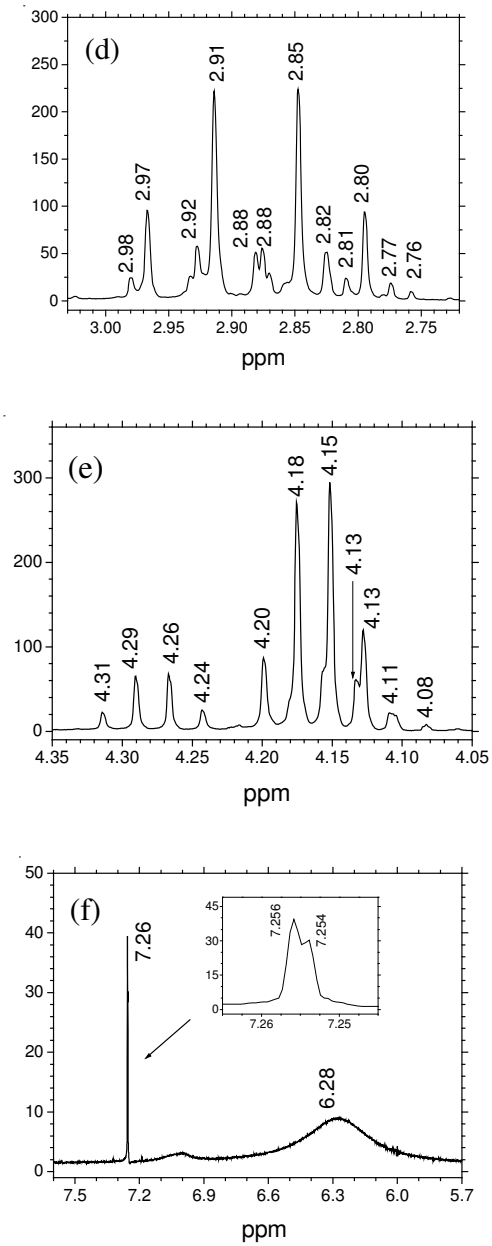


Fig. 3. ^{1}H NMR spectra of diethyl citrate. (a) Whole spectrum; (b) δ = 1.24-1.33; (c) δ = 2.02-2.12; (d) δ = 2.75-3.00; (e) δ = 4.05-4.35; (f) δ = 5.70-7.50

A group of peaks appeared at δ = 1.24-1.33 ppm (Fig. 3b, the relative integral peak area is 44.42), indicating the presence of hydrogen in methyl (-CH_3). This methyl is connected with rich charged atoms or groups since the peak position is slightly higher than that of -CH_3 in a straight-chain alkane (*ca.* 0.9 ppm). The molecular structure of Et_2Cit (Fig. 1) revealed that the rich charged group was -OCH_2-.

A series of peaks (Fig. 3e, with the relative peak area of 29.97) appeared at δ = 4.05-4.35 ppm, indicating the existence of hydrogen in methylene (-CH_2-). These hydrogen atoms were potentially located on the carbons that were connected with the charge-rich atoms or groups. It is concluded that it was a -$COOCH_2CH_3$ group because typical -OCH_2CH_3 group has a triplet peak at *ca.* 1.25 ppm with the integral number of 3 and a quartet peak at *ca.* 4.0 ppm with the integral number of 2.

According to the coupling and splitting as shown in Fig. 3e, the peak series include two groups of quartet peaks: one is larger (δ = 4.22-4.34 ppm) and the other is smaller (δ = 4.06-4.22 ppm). Since the peaks at δ = 1.24-1.33 ppm are not triplet (Fig. 3b), there may be two types of $-OCH_2CH_3$ groups in different chemical environments: 1,3-diethyl citrate and 1,5-diethyl citrate. However, further HLPC-MS analysis is required to depict the ratio of these two compounds.

A series of peaks (Fig. 3d, with the relative peak area of 30.61) at δ = 2.75-3.00 ppm was attributed to the hydrogen atoms of two methylene groups (on C_2 and C_4, Fig. 1) in citric acid. It included multiple peaks with irregular shapes, indicating the presence of hydrogen atoms of methylene groups in various chemical environments.

When comparing the relative peak areas and choosing the peak number at δ = 2.75-3.00 ppm (relative peak area 30.61) as 4, the peak number at δ = 1.24-1.33 (area 44.42) is 4 × 44.42/30.61 = 5.80 ≈ 6 and the peak number at δ = 4.05-4.35 (area 29.97) is 4 × 29.97/30.61 = 3.92 ≈ 4. Therefore, this product is Et_2Cit.

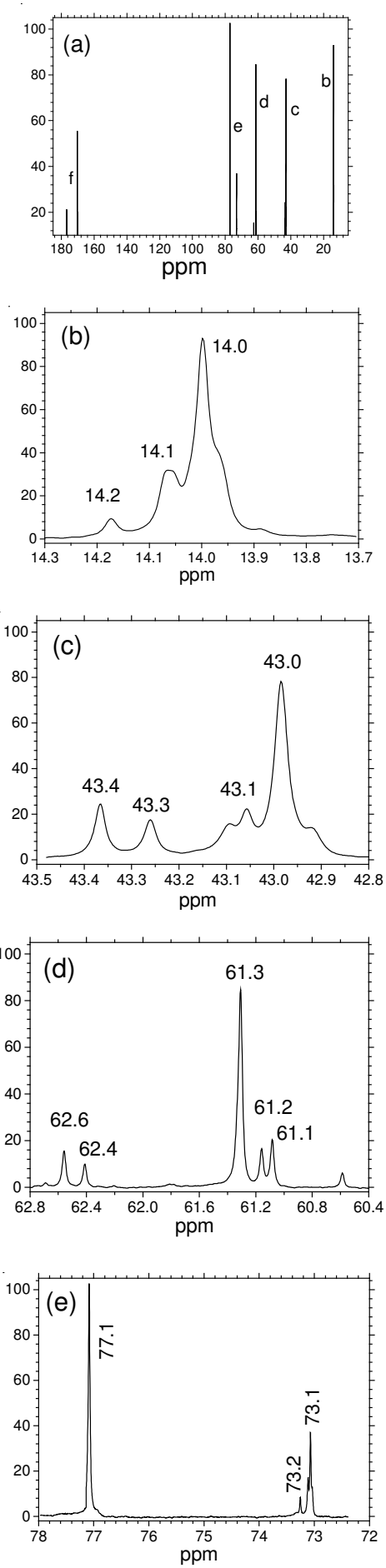
In the ^1H NMR spectra of Et_2Cit, no normal signal for hydrogen atoms of -COOH or -OH group was detected, except a weak signal at 7.256 ppm (Fig. 3f). This is because the carboxyl hydrogen atom is active and easily dissociated in polar solvent and they are easily deuterated in $CDCl_3$ solvent. Relative area of this peak is *ca.* 0.8, only one tenth of a normal proton signal, indicating that most hydrogen atoms in -COOH and -OH groups were deuterated during spectrum measurement.

In addition, a proton signal with peak area of 1.44 appeared at δ = 2.02-2.12 ppm. It is not a sharp and narrow peak like other proton spectra, but a wide and short "slope" (Fig. 3c), which should be due to the presence of small amount of water molecule.

It is worth noting that, despite Et_2Cit is a mixture of two isomers, they share similar physical and chemical properties and the anticoagulatory potential. Therefore, there is no need to further separate them.

^{13}C NMR spectra of Et_2Cit: The ^{13}C NMR spectra of Et_2Cit were shown in Fig. 4. (1) A group of peaks appearing at δ = 14.0, 14.1 and 14.2 ppm (Fig. 4b) indicate the presence of carbon atom in methyl ($-CH_3$). (2) There are two groups of peaks in the range of δ (ppm) = 42-44 (Fig. 4c). A series of peaks appeared at δ = 43.0, 43.1, 43.3 and 43.4), indicating the existence of carbon of $-CH_2-$ connecting to carbonyl (-CO) in $-C-CH_2-CO-$. The strongest peak at δ (ppm) = 43.0 was attributed to the carbon of $-CH_2-$ in two symmetric $-C-CH_2-COOR$ in 1,5-diethyl citrate (Fig. 5a). For the two $-CH_2-$ groups connecting to carbonyl in the 1,3-diethyl citrate, one was ester carbonyl ($-C-CH_2-COOR$), the other was the carboxyl carbonyl ($-C-CH_2-COOH$), therefore, the δ (ppm) appears at 43.3 and 43.4, respectively.

The multiple peaks appearing in δ (ppm) = 61.1, 61.2, 61.3, 62.4 and 62.6 were attributed to the carbon in methylene ($-CH_2-$) in $-OCH_2CH_3$ (Fig. 4d). The strongest peak at δ (ppm) = 61.3 was the carbon in $-CH_2-$ in the two symmetry $-OCH_2CH_3$ in 1,5-diethyl citrate (Fig. 5a). In the 1,3-diethyl citrate, the chemical environment of the two $-OCH_2CH_3$ is not exactly the same, therefore, one appears in δ (ppm) = 61.1 and 61.2 and the other appears in δ (ppm) = 62.4 and 62.6.



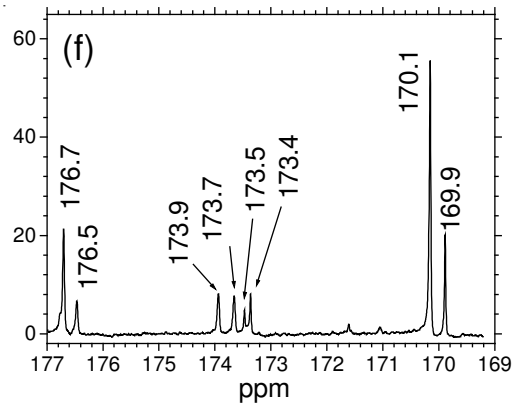
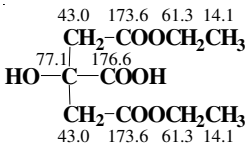
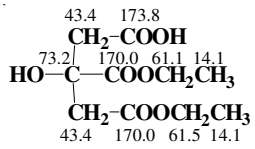


Fig. 4. ^{13}C NMR spectra of diethyl citrate. (a) Whole spectrum; (b) δ = 13-15; (c) δ = 42-44; (d) δ = 60-63; (e) δ = 72-78; (f) δ = 169-177 ppm



(a)



(b)

Fig. 5. Two isomers of diethyl citrate. (a) 1,5-diethyl citrate; (b) 1,3-diethyl citrate. The figures are the d (ppm) values of corresponding C atoms

From this discussion, it is suggested that there is -$CH_2COOCH_2CH_3$ in the molecule being detected. Because there are two groups of peaks, one group is relatively strong and the other group is relatively weak, it indicates the existence of two different chemical environments of -$CH_2COOCH_2CH_3$ group. The strong peaks are attributed to the two symmetrical -$CH_2COOCH_2CH_3$ groups in 1,5-diethyl citrate and the weaker peaks are attributed to two asymmetric -$CH_2COOCH_2CH_3$ groups in 1,3-diethyl citrate.

As -OH group is an electron-withdrawing group, the carbon peak in HO-C(s) (s, showing quaternary carbon atom) shifts to low field. Because of the existence of two different chemical environments of OH-C(s) groups, there have been two groups of peaks in Fig. 4e. The peaks at δ (ppm) = 73.1 and 73.2 are attributed to the carbon in HO-C of 1,3-diethyl citrate; and peaks at δ (ppm) = 77.1 is attributed to the carbon in HO-C of 1,5-diethyl citrate.

The peaks at chemical shift δ (ppm) in the range of 169-177 are typical carbonyl carbon atoms (-CO-) peaks (Fig. 4f). The two peaks at δ (ppm) = 176.5 and 176.7 are the carbon peaks of carboxyl (R-COOH, R is a saturated group). The four peaks appeared at δ (ppm) = 173.4, 173.5, 173.7 and 173.9 (average 173.6) are attributed to the carbons of esters in the two symmetrical -CH_2COOR. While the peaks appeared at δ (ppm) = 169.9 and 170.1 are attributed to the carbons of esters in the two asymmetrical -CH_2COOR in 1,3-diethyl citrate.

In summary, ^{13}C NMR spectra also showed that the synthesized product is the target product Et_2Cit. Furthermore, there are two kinds of ester with different chemical environments, that is, 1,3-diethyl citrate and 1,5-diethyl citrate. However, the proportion between them is still difficult to be judged.

Mass spectra of diethyl citrate: The mass spectra of Et_2Cit were shown in Fig. 6, according to which the molecular fragments were shown in Fig. 7. In Fig. 7a, the M/z peaks of 203, 185, 156, 114 and 70 represented the molecular fragments of 1,3-diethyl citrate. In Fig. 7b, the M/z peaks of 203, 158, 141, 116 and 74 represented the molecular fragments of 1,5-diethyl citrate. Because the molecular fragment of MW (molecular weight) 277 did not appear in the spectrum, the sample does not contain triethyl citrate. Because the molecular fragment of MW 175 also did not appear in the spectrum, the sample does not contain monoethyl citrate.

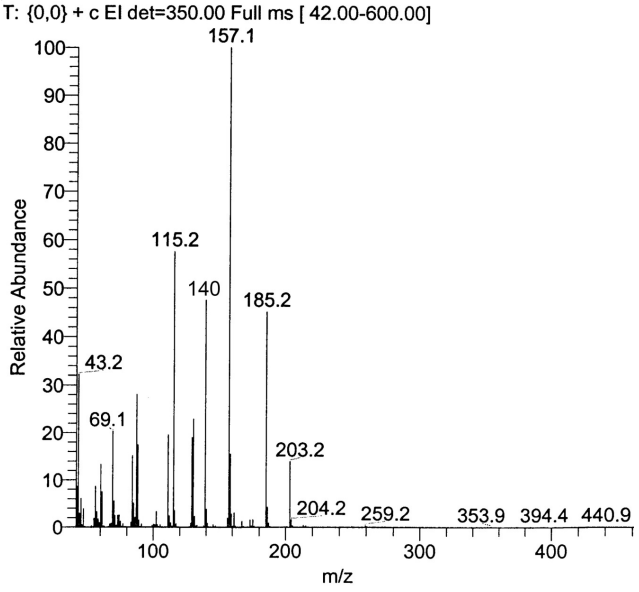


Fig. 6. Mass spectrum of diethyl citrate

Based on the results of elemental analysis, mass spectrometry, ^1H NMR, FT-IR and chromatography, it can be concluded that the product was Et_2Cit as expected.

Anticoagulation analysis of Et_2Cit: Reduction of plasma Ca^{2+} concentration (generally to 0.4 mmol/L) is essential for successful anticoagulation. However, hypocalcemia occurs when serum Ca^{2+} concentration is below 0.8 mmol/L.

When using Na_3Cit as anticoagulant, the strong chelating capability of Na_3Cit with Ca^{2+} leads to dramatic reduction of plasma Ca^{2+} concentration and causes hypocalcemia, which requires timely calcium supplement. In addition, after dialysis, the slowly release of Ca^{2+} ion from calcium citrate and the supplemented calcium results in hypercalcemia in patients.

After substituting two carboxyl groups (-COOH) in citric acid with ester groups (-$COOC_2H_5$), the new anticoagulant Et_2Cit displays the following advantages: Et_2Cit still has oxygen atoms (in ester and hydroxyl, as shown in Fig. 1) that can chelate with Ca^{2+} ion, leading to reduced plasma calcium concentration and achieving anticoagulation. During to the introduction of two ethyl groups in citric acid, steric hindrance augmented for Ca^{2+} chelation, which leads to reduced chelating ability of Et_2Cit in comparison to Na_3Cit. However, it is easier for Ca^{2+} ion to release from the chelate formed by Et_2Cit and Ca^{2+} than

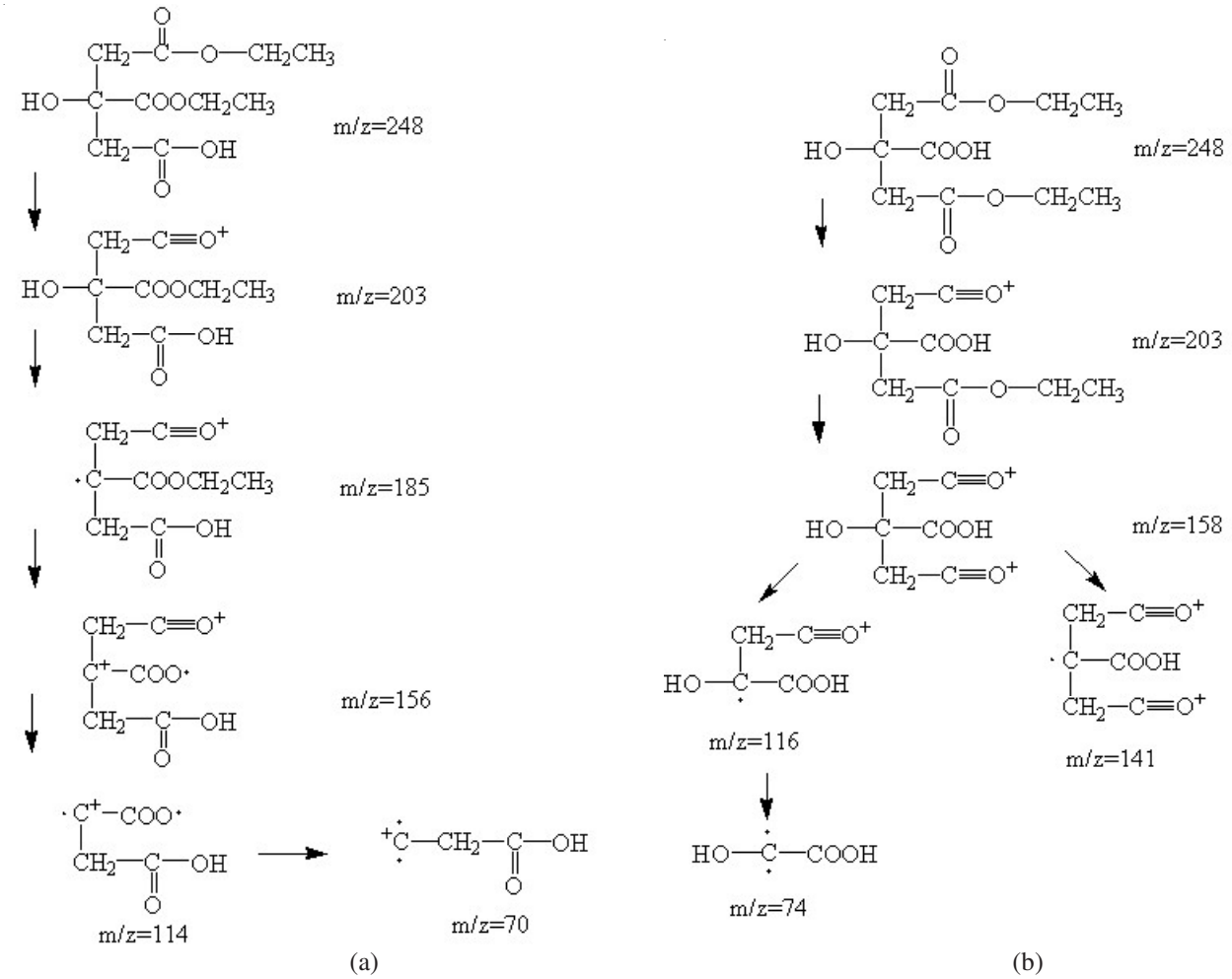


Fig. 7. Schemes of fragment peaks for two diethyl citrate isomers. (a) 1,3-diethyl citrate; (b) 1,5-diethyl citrate

from calcium citrate. This helps to overcome the Na_3Cit-caused hypocalcemia and other problems. The appropriate ethyl size add little molecular weight after substitution of two H in citric acid and Et_2Cit can easily go through the dialysis membrane. Therefore, Et_2Cit is a better anticoagulant

Table-1 showed the whole blood activated clotting time (ACT) for canine venous blood samples in the presence of various anticoagulants. Twenty canine blood samples were divided into four groups, each containing five. The ACT for Et_2Cit group was 141 ± 15 s, which was significantly higher than the control group (115 ± 10 s) and triethyl citrate group (122 ± 13 s), but markedly lower than the Na_3Cit group, in which blood did not coagulate within 4 h (14400 s). These data suggest that the chelating capability of Et_2Cit is weaker than Na_3Cit, but stronger than triethyl citrate. Therefore, Et_2Cit may potentially be an ideal anticoagulant due to the efficient anticoagulation and rapid calcium dissociation.

The experimental data was analyzed by inter-group t test between group I and group III ($P_1 = 0.001$), group I and group IV ($P_2 = 0.115$), group II and group III ($P_3 = 0.001$), group III and group IV ($P_4 = 0.001$), respectively.

Acute toxicity test in mice: Acute toxicity test in mice was carried out to observe the safety of Et_2Cit. The experimental results for each group were shown in Table-2. No significant adverse effect was observed in control mice, which had normal activity and no mortality.

For experimental mice, no significant reduction of activities or death occurred when Et_2Cit was less than 6.9 g/kg. When the dose of Et_2Cit increased 9.9 g/kg, activity reduction, ataxia, slowness and sluggish and other adverse effects immediately occurred, but still no animal died. These side effects can gradually disappear in 1 h.

There were no significant difference of female mice weight between experimental animals and control animals. No significant lesions of the body tissues or organs were observed by macroscopic analysis at the end of experiment.

According to British Toxicology Society Working Party on Toxicity (1984)[15], when LD50 (Lethal Dose, 50 %) value > 2.0 g/kg, it indicate this drug has no serious risk of acute poisoning. Therefore, Et_2Cit has no serious risk of acute poisoning according to the data in Table-1.

Conclusion

By substituting two hydroxyl atoms in -COOH in citric acid with oxyethyl groups ($-OC_2H_5$), a new anticoagulant, diethyl citrate (Et_2Cit), was synthesized and charcterized by its elemental analysis, infrared (IR) spectroscopy, mass spectrometry (MS) and nuclear magnetic resonance (NMR). Its purity was established by thin layer chromatography (TLC) and acid titration. Since the introduction of two ethyl groups to citric acid enhanced the steric hindrance for Ca^{2+} chelation, this led to a reduced Ca^{2+} chelation and accelerated Ca^{2+} release from

Et_2Cit in comparison to Na_3Cit, which overcomes Na_3Cit-caused hypocalcemia and other problems. Acute toxicity test in mice showed Et_2cit has no serious risk of acute poisoning. Therefore, diethyl citrate can be a potential and an ideal anticoagulant.

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