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# Study of Zn(II)-coumarin complex by electrochemical method with increased anticoagulant potency

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## Abstract

The formation of complexes of coumarin and Zn(II) was studied in aqueous media at pH 8.2±0.1 by polarography and spectroscopy. The polarogram indicated formation of complexes between coumarin and Zn(II). Coumarin produces a well-defined direct current polarogram and differential pulse polarogram in 1 M ammonium tartrate (supporting electrolyte) at pH 8.2±0.1. The stoichiometry of the Zn(II)-coumarin complex is 1:1. Anticoagulant studies on the drug and its metal complex have been performed in albino mice, revealing the complex to be more potent in anticoagulation activity compared to the parent drug.

**Keywords:** coumarin; differential pulse polarography (DPP); direct current polarography (DCP); prothrombin time; zinc complex.

## Introduction

Coumarin is the lactone of *o*-hydroxycinnamic acid, it occurs as colorless, prismatic crystals and has a characteristic fragrance bitter taste, and aromatic, burning (Dharmaratne et al. 1998, Floc'h et al. 2002, Gwynne 2002, Tornoe et al. 2002). It is soluble in alcohol.

Coumarin has a widespread occurrence in natural products, generally being liberated from the corresponding glycoside (melilotoside) on drying coumarin containing herbal material. Dicoumarol is a microbiological biotransformation product in spoiled melilotus clover and other hay products and its presence in fodder at >10 ppm is a cause for concern, as it is responsible for fatalities by hemorrhaging in cattle. This is because dicoumarol interferes with vitamin K reductase in the liver and the liver is unable to reactivate vitamin K, which leads to a decrease in vitamin K-dependent clotting proteins. Coumarin can be synthesized readily (Wang 2001). Coumarin is rather widely distributed in nature and in addition to its occurrence in tonka beans (Feuer 1974, Katz and Skalka 1994, Vlietinck et al. 1998, Lake 1999, Dharmaratne et al. 2002), it has been isolated from sweet vernal grass (Born et al. 1997, Lee and Morris-Natschke 1999, Xu et al. 2000) (*Anthoxanthum odoratum* Linn, Family Gramineae), *Cynosurus cristatus* (Crested Dog's-tail), *Anthoxanthum odoratum* L. and L. *Narcissus* spp., Clary sage *Salvia sclarea* L. (Quick 1966, Vocanson et al. 2006) sweet clover (*Melilotus albus Medicus* and *Melilotus officinalis* Lamarck, Family Leguminosae). Coumarin finds its use as an anticoagulant and because of its easy availability from plants and in synthetic form it has now become a popular drug among physicians (Vocanson et al. 2006). Because of the easy availability of the drug, we have attempted to modify the coumarin molecule to improve its anticoagulation potency. The results of which have been discussed in the present paper.

## Materials and methods

### Instruments

**Polarography** All polarograms were recorded on a Microprocessor (μp) polarographic analyzer model CL-362. An Elico digital pH meter model 335 (Elico Ltd, Hydrabad, India) was used for pH measurement. The polarographic cell consisted of a three-electrode assembly with a saturated calomel electrode (reference electrode) as working electrode.

**Spectroscopy** The IR spectrum of solid complex was recorded using KBr pellets on a model 8400s IR-spectrophotometer (Shimadzu, Japan).

### Chemicals

Coumarin chemical used for the present study was of CDH grade, and ammonium tartrate from Himedia grade was used. Stock solutions of the reagents were prepared in requisite amount of distilled water.

### Preparation of complex

Qualitative and quantitative studies on coumarin were carried out using direct current polarography (DCP) and differential pulse polarography (DPP). The pH of the test solution was adjusted to 8.2±0.1 to avoid a matrix effect for electrochemical behavior of coumarin.

Coumarin (0.230 g) was dissolved in 100 ml ethanol and a set of solution containing varying concentrations of coumarin was prepared in 0.01 M overall concentration of ammonium tartrate at pH 8.2±0.1.

For the study of stoichiometry and formation of the complex, Lingane's polarographic method was used,

which is a simple method over the entire range of ligand concentration.

Experimental solutions were prepared by keeping overall zinc (metal ion) and ammonium tartrate concentration fixed at 0.2 M and 1 M, respectively, while varying the ligand concentration from 0 to 15 mM. The pH value was adjusted to 8.2±0.1, and the solution was deaerated with purified $H_2$ gas. Polarogram was recorded keeping the initial potential set to -1100 mV.

### Synthesis of solid complex

A white solid was synthesized by refluxing 1:1 aqueous solution of ferrous ammonium sulfate and coumarin in water and ethanol (55:45 v/v) for approximately 4 h. The complexation was marked by precipitation after reducing the volume to a quarter of the original volume. The product was filtered, washed, dried over $P_4O_{10}$ and stored.

### *In vivo* study of Zn(II)-coumarin complex

**Pharmacological study (prothrombin time)** Pharmacological screening of coumarin and its complex was done *in vivo* through the study of anticoagulation activity to check efficacy and safety of the prepared complex of coumarin. Because coumarin is an indirect acting anticoagulant which inhibits the clotting of blood *in vivo*, thus the mode of screening of the new complex of coumarin was screened through the same pharmacological test, i.e., average plasma prothrombin time determination. The *in vivo* experiments were done using albino mice animals. Approval was obtained for the ethical treatment of animals used.

Coumarin anticoagulant acts by inhibiting the synthesis of vitamin K-dependent clotting factors, which include factors II, VII, IX, and X. Vitamin K is an essential cofactor for the post-ribosomal synthesis of vitamin K-dependent clotting factors. The vitamin promotes the biosynthesis of carboxyglutamic acid residues in the proteins which are essential for biological activity. The anticoagulation effect generally occurs within 24 h after drug administration. However, peak anticoagulation effect could be delayed for 72 to 96 h. Anticoagulants have no direct effect on an established thrombus, nor do they reverse ischemic tissue damage. The plasma prothrombin time is a test to assess hemostatic function of blood coagulation mechanism and screening of patients suffering bleeding disorders or who have undergone anticoagulation therapy. The plasma prothrombin time tests for prothrombin activity in addition to factors VII and X which are sensitive to the presence of coumarin in blood. Thus, we screened anticoagulation activity of coumarin and its complex through average plasma prothrombin time by Quick's method (Quick 1966) in different plasma samples. The time required for coagulation of citrated plasma after addition of thromboplastin calcium mixture is known as prothrombin time. The clotting of citrated plasma involves several coagulation factors such as factors II, VII, IX, and X in the presence of thromboplastin and calcium.

**Quick's method** One stage plasma prothrombin time was calculated by Quick's method. In this regard, 2 ml of fresh citrated plasma was obtained as mentioned in the experimental section. Then, 0.1 ml of this citrated plasma was mixed with 0.1 ml of thromboplastin in a glass tube or in a dish placed in a water bath at 37°C. After a delay of 2 min, 0.1 ml of prewarmed 0.025 M $CaCl_2$ solution was added to this mixture carefully and mixed well. A stopwatch was started and the tube was held with its lower end submerged in a water bath at 37°C. The tube was continuously but gently inclined from the vertical to just short of the horizontal so that its contents could be observed for the first sign of clotting and time was noted. The test was repeated three times with the same citrated plasma sample and average prothrombin time was calculated.

***In vivo* (albino mice plasma)** Coumarin dose shows a relationship between anticoagulation effect and antithrombotic efficacy. The anticoagulation activity of coumarin and complex was screened in albino mice animals for *in vivo* effect. In this regard, a standard procedure was adopted for dose of drugs under study and for *in vitro* studies the dose was designed as 1 mg/100 kg. Each drug was administrated orally, on a set of four healthy animals according to their weight (albino mice). Then, 2 ml of venous blood samples of the mice were collected through orbital puncture after 24 h of dose. Without delaying, blood samples were mixed with sodium citrate in a requisite amount and citrated plasma was obtained from each blood sample. This citrated plasma obtained from albino mice for each newly designed coumarin molecule was used to determine average prothrombin time in seconds by Quick's method.

## Results and discussion

The DCP and DPP of the authentic sample solution of coumarin in ammonium tartrate (0.2 M) at pH 8.2±0.1 produced a well-defined polarographic wave/peak with $E_{1/2}/E_p$=-1.60 V/-1.60 V vs. SCE. Coumarin is polarographically active in both acidic and basic environments (Figures 1 and 2).

### Polarographic study of M:L complexation equilibrium

Both Zn(II) and its complex with coumarin produce a reversible two-electron reduction wave in 0.2 M ammonium tartrate at pH 8.2±0.1. Complex formation between Zn(II) and coumarin (Supplementary Material) was revealed by the shift in half-wave potential and peak potential to a more negative value and decrease in the height of the diffusion current with gradual increase of the coumarin concentration. Plots of $\Delta E_{1/2}$ (shift in the half wave potential). $\Delta E_{1/2}=(E_{1/2})_c-(E_{1/2})_s$ against log Cx (logarithm of the concentration of the ligand) resulted in a linear plot (Figure 3), showing formation of a single complex in solution. Lingane treatment of the observed polarographic data revealed 1:1 Fe(II)-coumarin complex with formation constant log $\beta_1$=5.85.

**IR spectral analysis of Zn-coumarin complex** On comparing the IR spectra of coumarin and its Zn(II) complex, it was observed that the band at 1725 $cm^{-1}$ due to C=O group

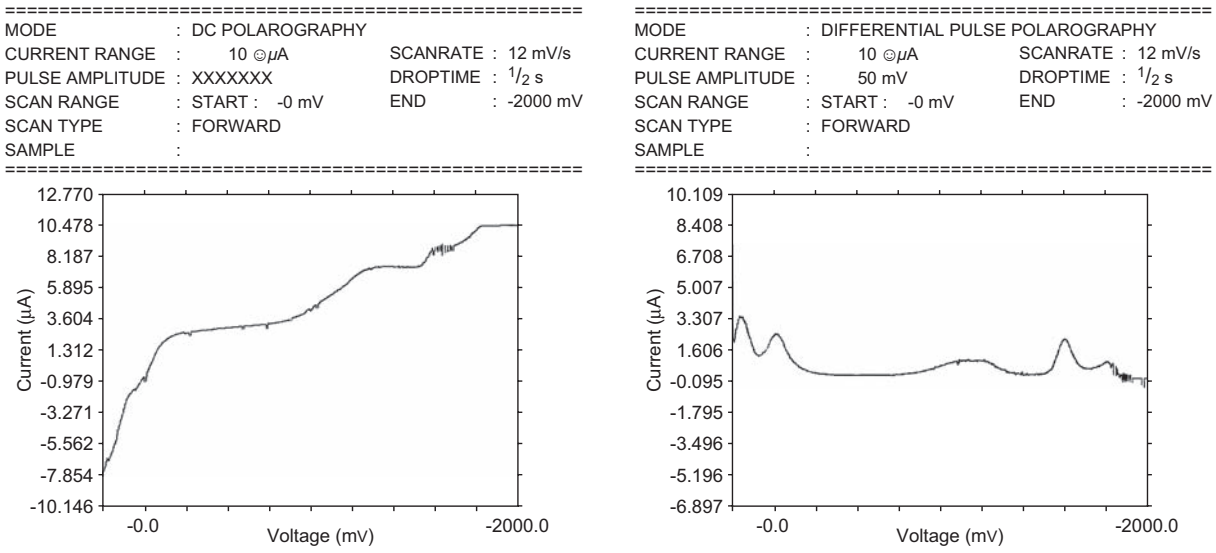



**Figure 1** Direct current polarogram and differential pulse polarogram of coumarin (0.2 mM) in ammonium tartrate (0.2 M) pH 8.2±0.1.

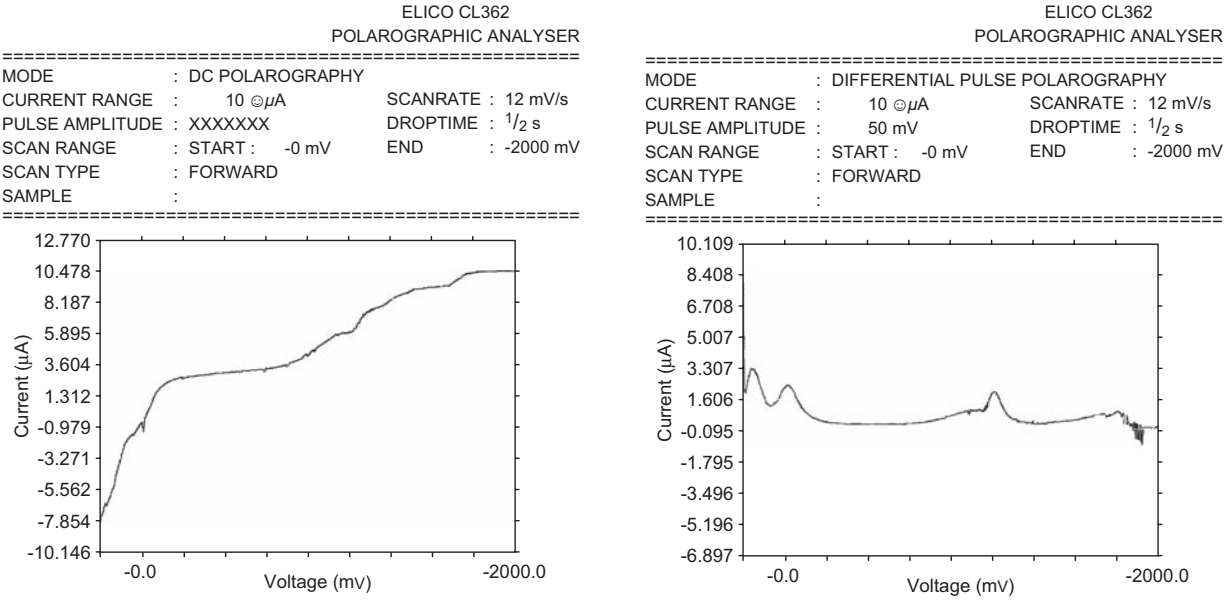



**Figure 2** Direct current polarogram and differential pulse polarogram of Zn(II)-coumarin complex in ammonium tartrate (0.2 M) pH 8.2±0.1.

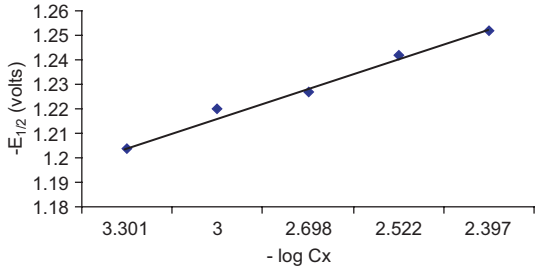



**Figure 3** Zn(II)-coumarin complex.

**Table 1** Normal anticoagulation action without any drug in albino mice.

| Serial no. | Body weight of animal (g) | Plasma prothrombin time (s) | | | Average prothrombin time (s) |
|---|---|---|---|---|---|
| | | a | b | c | (a+b+c)/3 |
| 1. | 42.20 | 11.92 | 11.96 | 11.99 | 11.95 |
| 2. | 35.32 | 11.22 | 11.65 | 11.86 | 11.57 |
| 3. | 40.28 | 11.90 | 11.92 | 11.95 | 11.92 |
| 4. | 42.36 | 11.98 | 11.99 | 11.96 | 11.97 |
| 5. | Mean average prothrombin time | | | | 11.85 |

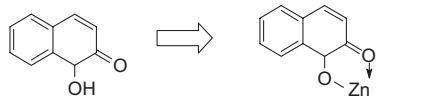
**Table 2** *In vivo* anticoagulation action of coumarin in albino mice.

| Serial no. | Body weight of animal (g) | Dose administered (ml) | Plasma prothrombin time (s) | | | Average prothrombin time (s) |
|---|---|---|---|---|---|---|
| | | | a | b | c | (a+b+c)/3 |
| 1. | 48.44 | 0.48 | 20.52 | 20.66 | 20.78 | 20.65 |
| 2. | 45.52 | 0.45 | 20.92 | 20.75 | 20.88 | 20.85 |
| 3. | 42.12 | 0.42 | 20.75 | 20.72 | 20.75 | 20.75 |
| 4. | 40.11 | 0.40 | 20.70 | 20.72 | 20.72 | 20.72 |
| 5. | Mean average prothrombin time | | | | | 20.74 |

**Table 3** *In vivo* anticoagulation action of Zn(II)-coumarin complex in albino mice.

| Serial no. | Body weight of animal (g) | Dose administered (ml) | Plasma prothrombin time (s) | | | Average prothrombin time (s) |
|---|---|---|---|---|---|---|
| | | | a | b | c | (a+b+c)/3 |
| 1. | 40.44 | 0.40 | 21.02 | 21.16 | 21.16 | 21.16 |
| 2. | 43.52 | 0.43 | 21.22 | 21.22 | 23.78 | 21.22 |
| 3. | 42.12 | 0.42 | 21.35 | 21.32 | 21.35 | 21.35 |
| 4. | 40.11 | 0.40 | 21.20 | 21.23 | 21.23 | 21.23 |
| 5. | Mean average prothrombin time | | | | | 21.24 |

in the spectrum of pure drug disappeared in the spectrum of its Zn(II) complex. The sharp -OH signal at 3570 $cm^{-1}$ is observed in coumarin. This band is shifted in the spectrum of Zn(II)-coumarin complex, which confirms involvement of C=O and -OH in the complexation of Zn(II). Thus, on the basis of polarographic and IR studies a tentative structure to 1:1 Zn(II)-coumarin complex could be as follows.




Coumarin Zn(II)-coumarin complex

### Pharmacological experiments (average prothrombin time)

***In vivo* (albino mice) anticoagulation action** The *in vivo* anticoagulation action of coumarin and its complex was screened on albino mice. The results of anticoagulation activity obtained for normal and each test sample species of drugs are shown in Tables 1–3. The average prothrombin time as observed using complex of coumarin is depicted in the bar diagram (Figure 4). It is rather clear from the bar diagram that although the mean average prothrombin time using coumarin as anticoagulant increases to 20.74 s as compared to that observed with pure blood plasma of the animal which is 11.94 s (without drug dose), the coumarin complex showed increased anticoagulation activity to 21.24 s.

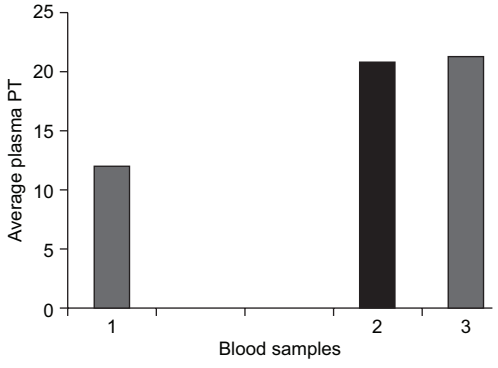



**Figure 4** Bar diagram of plasma prothrombin time (s). Blood samples: 1, normal blood plasma; 2, blood plasma sample of coumarin; 3, blood plasma sample of Zn-coumarin.

## Conclusion

The data show stoichiometric ratio of 1:1 for the Zn(II)-coumarin complex. The polarographic method is used for qualitative and quantitative analysis of coumarin and is recommended for quality control in the drug industry. The increased potency of the complex could allow its use as a potent anticancer drug.

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