# Potency and Coagulation Factor Effects of 2,3,5,6-Tetrachloro-4-pyridinol Compared to Warfarin and Its Antagonism by Vitamin K (36242)

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It was previous reported that 2,3,5, 6-tetrachloro-4-pyridinol (TCP) increased prothrombin time following administration to rats (1). TCP denotes a new structure for this type of pharmacologic activity. This report deals with the comparison of the anticoagulant potency of TCP relative to warfarin using the 1-stage prothrombin time as the response. The effects of these compounds are also compared with regard to 2-stage prothrombin time, and the activities of factors V; VII + X; X; IX; and fibrinogen. In addition, the effects of menadione sodium bisulfite and of phytonadione were determined on the hypoprothrombinemic response produced by TCP.

*Materials and Methods.* Sixty-four male, Sprague-Dawley rats (Sprague-Dawley Farms, Madison, WI) were used to estimate the potency of TCP relative to warfarin. TCP was dissolved in dilute sodium hydroxide solution; the final pH was 7.0. Four doses of warfarin sodium (Abbott Laboratories, North Chicago, IL; 0.3, 0.6, 0.9, and 1.2 mg/kg) and 4 doses of TCP (18, 36, 54 and 72 mg/kg) were administered intragastrically (ig) to different groups of rats containing 8 rats/group. An additional group of 7 rats received nothing and served as controls. The peak activity of TCP was previously found to be approximately 48 hr post drug (1). Pilot experiments indicated that warfarin sodium had approximately the same peak activity time. Therefore, 1-stage prothrombin times were performed at 48 hr after dosing.

Blood was obtained by cardiac puncture after the rats were lightly anesthetized with ether. Platelet-poor plasma was prepared by centrifugation after mixing blood with a 10% vol of 0.1 *M* oxalate solution. Solutions of plasma (25%) were prepared for prothrombin time determinations with 0.85% sodium chloride solution, and kept at 4° until used. One-stage prothrombin time determinations were performed in duplicate using a Mechrolab clot timer. Simplastin suspension (0.2 ml) (General Diagnostics) was placed in the cup and 0.1 ml of diluted plasma was placed on the rotor.

One animal, which received 72 mg/kg of TCP, died 48 hr after dosing. Three animals in this same group showed prothrombin times greater than 350 sec. Four animals in the 54 mg/kg group and 2 animals in the 36 mg/kg group also showed prothrombin times greater than 350 sec. Similarly, in the groups receiving warfarin sodium, 4 animals in the 1.2 mg/kg and 2 animals in the 0.9 mg/kg groups showed prothrombin times greater than 350 sec. These data were not used in the statistical analysis. Data were analyzed according to the method outlined by Finney (2) for unsymmetrical bioassays. The metameters used were log of dose and log of prothrombin time (sec).

Five pure-bred beagle, male dogs (American Animal Industries, Indianapolis, IN) 8 to 9 months old and weighing approx 15 kg were used in the study of the effects of TCP and warfarin on coagulation factors. All blood determinations were performed in duplicate. Plasma fibrinogen was determined according to the method of Goodwin (3). Two-stage prothrombin time, which specifically measures prothrombin, was done according to the method of Ware and Seegers (4). The method of Borchrevink (5) was used to estimate factor V activity. The assay method of Hougie (6) was used to estimate the activity of factor X. The activities of factors VII and

TABLE I. Determination of the Potency of 2,3,5,6-Tetrachloro-4-pyridinol (TCP) Relative to Warfarin Sodium Using 1-Stage Prothrombin Time.

| | Prothrombin time (sec) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | TCP (mg/kg, ig) | | | | Warfarin sodium (mg/kg, ig) | | | |
| | Control | 72 | 54 | 36 | 18 | 1.2 | 0.9 | 0.6 | 0.3 |
| $n$ | 7 | 4 | 4 | 6 | 8 | 4 | 6 | 8 | 8 |
| $\bar{X} \pm$ SD | 22.2 ±2.0 | 170.7 ±40.9 | 93.8 ±28.7 | 68.1 ±44.3 | 14.2 ±10.7 | 154.1 ±63.8 | 88.9 ±32.7 | 64.2 ±67.0 | 30.8 ±10.3 |

X together were estimated exactly as in the assay for factor X with the exception that Russel viper venom was omitted from the clotting mixture. The measurement of the activity of these two factors together was necessary because of the lack of a substrate plasma suitable for the measurement of factor VII specifically. Factor IX was measured by a modification of the procedure of Langdell *et al.* (7) for factor VIII assay, incorporating the kaolin activation principle of Hardisty and MacPherson (8). Factor IX-deficient plasma (Hyland Laboratories, Glendale, CA) was used as the substrate in this test. Blood was drawn from a cephalic vein and mixed with a 10% vol of 3.2% sodium citrate solution, centrifuged; and the platelet-poor plasma was drawn off immediately for storage at 4° until used. Control values were established for the 5 dogs for the above coagulation factors. The sodium salts of TCP and of warfarin were administered as single, oral doses in gelatin capsules 7 hr after the control samples were drawn. Two dogs received 40 mg/kg of TCP sodium, while 2 dogs received 4 mg/kg of warfarin sodium. One dog received nothing and served as an additional control. Blood samples were drawn at 17, 41, 89, and 161 hr following the administration of drugs for assay of coagulation factors. Aliquots of plasma, obtained from the control blood samples drawn from the five dogs, were pooled and several dilutions were made. Clotting times were determined in the various assays using these dilutions; and standard curves were constructed by plotting the times against plasma concentration. These standard curves were used throughout the experiment to convert clotting times in the assays to percentage of control activity. The dilutions used of the post drug plasmas were according to the requirements for each assay.

Seventy male rats (Wistar Strain, Harlan Industries, Cumberland, IN) were used to determine the effects of menadione sodium bisulfite (Vitamin $K_3$, Mann Research Laboratories, New York) and of phytonadione (Vitamin $K_1$, AquaMephyton, Merck, Sharp and Dohme, West Point, PA) on the hypoprothrombinemic effect of TCP. Seven groups of 10 animals each were formed. Three groups received 30 mg/kg of the sodium salt of TCP intraperitoneally. Of these 3 groups, one received 3 mg/kg of menadione sodium bisulfite subcutaneously, and one received 3 mg/kg of phytonadione subcutaneously. Similarly, another 3 groups received 60 mg/kg of the sodium salt of TCP and one each of these received either menadione sodium bisulfite or phytonadione. The seventh group received 0.9% sodium chloride solution intraperitoneally as a control. One-stage prothrombin times were determined at 48 hr following the administration of drugs, using the same procedure as previously described with the exception that the prothrombin times were performed manually. Plasma (25%) was used in all cases except for the group receiving 60 mg/kg of TCP sodium alone, in which case, 100% plasma was utilized. This was necessary because the diluted plasmas were found to be unclottable. Aliquots of plasma samples from the group receiving sodium chloride were pooled; and a standard curve was constructed by plotting plasma concentration against prothrombin time. Prothrombin times of all rats were converted

TABLE II. TCP and Warfarin Sodium on Various Coagulation Factors Following a Single Oral Dose.

| Post drug (hr) | | (mg/kg) | Dog no. | (% Control activity) Prothrombin time; stage: 1 | 2 | Factor VII + X | X | IX | V | Plasma fibrinogen (mg/100 ml) |
|---|---|---|---|---|---|---|---|---|---|---|
| 0 | Warfarin sodium | 4 | 1 | 82 | 95 | >100 | 92 | 93 | >100 | 305 |
| | | | 2 | 80 | 97 | >100 | 94 | 100 | >100 | 377 |
| | TCP sodium | 40 | 3 | 87 | 72 | >100 | >100 | 100 | >100 | 378 |
| | | | 4 | 87 | 70 | >100 | >100 | 100 | >100 | 341 |
| | Control | | 5 | 32 | 57 | >100 | >100 | >100 | >100 | 372 |
| 17 | Warfarin sodium | 4 | 1 | 36 | 59 | >100 | 78 | 79 | >100 | 341 |
| | | | 2 | 32 | 50 | 94 | 70 | 83 | 95 | 390 |
| | TCP sodium | 40 | 3 | 59 | 91 | 87 | >100 | 100 | >100 | 363 |
| | | | 4 | 41 | 75 | >100 | 91 | 84 | >100 | 360 |
| | Control | | 5 | — | — | — | — | — | — | — |
| 41 | Warfarin sodium | 4 | 1 | 16 | 15 | 67 | 84 | 34 | >100 | 305 |
| | | | 2 | 13 | 18 | 64 | 66 | 24 | >100 | 380 |
| | TCP sodium | 40 | 3 | 24 | 27 | 83 | 89 | 58 | >100 | 361 |
| | | | 4 | 27 | 28 | 77 | 90 | 63 | >100 | 305 |
| | Control | | 5 | 36 | 61 | >100 | 100 | >100 | >100 | 372 |
| 89 | Warfarin sodium | 4 | 1 | 11 | 12 | 100 | 100 | 17 | >100 | 299 |
| | | | 2 | 9 | 10 | 86 | 78 | 17 | >100 | 341 |
| | TCP sodium | 40 | 3 | 30 | 15 | >100 | >100 | 33 | >100 | 351 |
| | | | 4 | 65 | 38 | >100 | 86 | 63 | >100 | 335 |
| | Control | | 5 | 33 | 65 | >100 | 100 | >100 | >100 | 354 |
| 161 | Warfarin sodium | 4 | 1 | 90 | >100 | >100 | >100 | >100 | >100 | 323 |
| | | | 2 | 76 | >100 | >100 | 88 | 95 | 100 | 341 |
| | TCP sodium | 40 | 3 | 82 | 68 | >100 | 100 | 60 | >100 | 351 |
| | | | 4 | 90 | 95 | >100 | >100 | 93 | >100 | 348 |
| | Control | | 5 | 37 | >100 | >100 | >100 | >100 | >100 | 354 |

TABLE III. The Effect of Menadione Sodium Bisulfite and Phytonadione on the Hypoprothrombinemic Response to TCP Sodium.

| Group | No. of animals | (% Control activity; $\bar{X} \pm$ SE) |
|---|---|---|
| 0.25 ml 0.9% NaCl, ip | 10 | 102.4 ± 32.3 |
| TCP Na, 30 mg/kg, ip | 10 | 19.4 ± 5.6 |
| TCP Na, 30 mg/kg, ip + menadione Na bisulfite, 3 mg/kg, sc | 10 | 34.5 ± 16.4 |
| TCP Na, 30 mg/kg, ip + phytonadione, 3 mg/kg, sc | 9 | 88.8 ± 10.8 |
| TCP Na, 60 mg/kg, ip | 10 | 2.4 ± 0.6 |
| TCP Na, 60 mg/kg, ip + menadione Na bisulfite, 3 mg/kg, sc | 10 | 8.2 ± 2.6 |
| TCP Na, 60 mg/kg, ip + phytonadione, 3 mg/kg, sc | 9 | 54.3 ± 9.2 |

into percentage of control activity by the use of this curve.

The effect of TCP in the presence and absence of menadione and phytonadione on 1-stage prothrombin activity was compared using the 4 point, parallel line bioassay technique described by Finney (2). Two rats in the experiment died from an overdose of anesthetic and blood was unobtainable. Since this statistical model does not allow for unbalanced observations in dose groups, an analysis for missing entries was conducted, which is also outlined by Finney.

*Results and discussion.* Statistical analysis indicates that when a value of 100 is assigned to the hypoprothrombinemic potency of warfarin sodium, TCP has 2.25% the potency of warfarin sodium on a weight basis with a 95% confidence interval of 2.20 to 2.30%. Thus, TCP possesses low potency relative to warfarin sodium. However, the compounds are of equal potency in terms of maximum effect since either compound when given at a specific dose can produce unclottable plasmas in the 1-stage prothrombin time test. The mean prothrombin times for the various groups ± standard deviation are shown in Table I.

Table II shows the effects of warfarin sodium and TCP sodium on 1-stage prothrombin activity and on the activity of various coagulation factors. Dogs 1 and 2 received 4 mg/kg of warfarin sodium; and dogs 3 and 4 received 40 mg/kg of TCP sodium orally in a gelatin capsule 7 hr after time 0 blood samples were drawn. Dog 5 received nothing and served as an additional control throughout the experiment. As shown, a reduction in 1-stage prothrombin activity for animals receiving drugs appeared at 17 hr post drug. Peak reduction in 1-stage prothrombin activity occurred in the 41 hr post drug blood sample for TCP sodium and at 89 hr post drug blood sample for warfarin sodium. A return to approximately pre-drug values for both drugs was seen at 161 hr post drug. Although the control dog showed the lowest 1-stage prothrombin activity, the values did not appear to change significantly throughout the experiment.

The 2-stage prothrombin activity reflects changes in prothrombin specifically. The responses post drug for warfarin and TCP sodium-treated dogs parallel those seen in the 1-stage prothrombin test. With respect to the combined activities of factors VII and X, a reduction in activity was seen in one each of the dogs receiving warfarin and TCP sodium at 17 hr post drug. Peak reductions were noted for dogs receiving either drug at 41 hr post drug. Activity returned to pre-drug or near pre-drug values for all treated dogs at 89 hr post drug.

Reductions in factor X activity were seen with all treated dogs with little consistency as to the time of peak reduction in activity. One dog, which had received warfarin, had

not returned to the pre-drug value at the end of 161 hr post drug. At 41 hr post drug, there was a consistently lower activity for the treated dogs in factor VII + X activity than in the activity of factor X measured alone. This is interpreted to indicate that for both drugs there was some reduction in the activity of factor VII at this time.

Factor IX activity was reduced as a result of the administration of warfarin or TCP sodium. As in the previous assays, the dose of TCP employed showed less activity than for the dose of warfarin employed.

Table II also shows that the activity of factor V is not significantly influenced by either warfarin or TCP administration. Similarly, plasma fibrinogen levels also did not appear to be affected.

Table III identifies the groups of animals used in the experiment in which the effect of menadione and phytonadione was determined on the hypoprothrombinemic response to TCP sodium. Table III also shows the mean percentage of control 1-stage prothrombin activity along with standard errors. Statistical analysis of the data indicate that the menadione sodium bisulfite-treated groups (3 mg/kg) responded with 72% of the activity shown by the groups receiving TCP sodium alone. Another interpretation, which may be placed upon these data is that menadione sodium bisulfite produced a 28% antagonism of TCP sodium. Similarly, phytonadione (3 mg/kg) produced an 80% antagonism of TCP sodium. According to these data, phytonadione is approximately 52% more potent than menadione sodium bisulfite in antagonizing the effect of TCP sodium on 1-stage prothrombin activity.

*Summary.* The potency of 2,3,5,6-tetrachloro-4-pyridinol (TCP) was compared to that of warfarin sodium in which the drugs were administered intragastrically to rats and 1-stage prothrombin times were determined 48 hr later. TCP was found to have 2.25% the potency of warfarin sodium. Both compounds were found to produce unclottable plasmas in the 1-stage prothrombin time test when larger doses were administered.

Studies in beagle dogs, using single oral doses of TCP and warfarin sodium, were done to determine the effects of the drugs on various coagulation factors. Both compounds produced a lowering in the activities of prothrombin, factors VII + X, factor X, and factor IX. No significant alterations in factor V activity or in plasma fibrinogen concentration were found. The peak effects were usually reflected in the 41 hr post drug blood samples and pre-drug or near pre-drug values were approached at 161 hr post drug.

Menadione sodium bisulfite (vitamin $K_3$) and phytonadione (vitamin $K_1$) were found to be effective antagonists to the effects produced on 1-stage prothrombin time when TCP sodium was administered to rats. Phytonadione was found to be 52% more effective than menadione sodium bisulfite in this activity.

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