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〔原　　著〕

Effects of Added Substances in Injections on Blood Coagulation, Fibrinolysis and Platelet Aggregation

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Summary

Effects of six kinds of additives, i.e. benzyl alcohol, surfactant HCO-60, macrogol 4000, urethane, sodium bisulfite and sodium sulfite, on blood coagulation, fibrinolysis and platelet aggregation were studied. First, these additives were separately investigated with following results: benzyl alcohol depressed platelet aggregation, and sodium bisulfite and sodium sulfite inhibited both blood coagulation and fibrinolysis within the concentration range of additives commercially available. It was found that the inhibition of sodium bisulfite on blood coagulation and fibrinolysis might be primarily ascribed to low pH of its solution and that of sodium sulfite to SO_3^{2-} ion. Next, the combination of these additives were discussed in taking into consideration the fact that many injections which contain more than one additive were currently produced. The results demonstrate that some combinations can make severe effect on blood functions than the simple summation of the effects of each additive. Especially, the combined preparation of benzyl alcohol with either sodium bisulfite or sodium sulfite showed drastic inhibition of fibrinolysis and platelet aggregation.

Keywords　additives in injections; HCO-60; benzyl alcohol; urethane; macrogol 4000; sodium bisulfite; sodium sulfite; coagulation; fibrinolysis; platelet aggregation.

Introduction

The injections commercially available contain several kinds of materials for the purpose of solubilization, dispersion, painlessness, antioxidation, antisepsis and so on. It is very important for the evaluation of products and the design of the dosage form to investigate the effects of the additives on the blood functions because additives as well as the active ingredients come in direct contact with the blood. Sometimes, additives can make severe effect on blood system, such as, the inhibition of platelet aggregation by vitamin E (1) is

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accelerated by the additive, benzyl alcohol.(2) However, the effects of these additives have been little reported in detail. The present study was undertaken to examine the effects of the following six kinds of injection additives on blood coagulation, fibrinolysis and platelet aggregation: benzyl alcohol widely used for the solubilization and painlessness; urethane prohibited to be used because of its possible teratogenicity; HCO-60 a surfactant most widely used in injections for the solubilization of oil soluble substances; macrogol 4000 added to the globulin preparations; sodium bisulfite and sodium sulfite used as the antioxidant. Our experiments were also extended to allow for the effects of the combinations of the above additives because many injections commercially produced contain more than one kind of additive.

Experimentals

Materials

1. Additives: Benzyl alcohol and urethane were the reagent grade from Nakarai Chemical Co. (Kyoto), HCO-60 (polyoxyethylene hydrogenated caster oil derivative) was obtained from Nikko Chemicals Co. (Tokyo), macrogol 4000 was JP grade from Hoei Pharmaceutical Co. (Osaka) and sodium bisulfite and sodium sulfite were reagent grade from Wako Pure Chemical Industries (Tokyo). All the additives were diluted with distilled water to prepare the sample solutions.

2. Reagents: Ci-TROL I (citrated control plasma) was used as the human control plasma, Actin (cephalin extracted from dehydrated rabbit brain in 1.0×10^{-4}M ellagic acid, 2 ml/vial) as the phospholipid, 0.02M $CaCl_2$ solution (5 ml/vial) as a Ca^{2+} solution, for the blood coagulation test and Tromboplastin. C(acetondehydrated rabbit brain thromboplastin with 1.16×10^{-2}M Ca^{2+}, 4 ml/vial) for the prothrombin time measurements. These reagents were commercially obtained from Dade Diagnostics Inc. (USA), and used with or without reconstitution with distilled water.

Human fibrinogen (1 g/vial), Human thrombin (500 units/vial) and urokinase for injections (6000 IU/vial) were used for the fibrinolytic activity measurements. These reagents were obtained from Green Cross Co. (Osaka) and diluted with 1/10 M phosphate buffer (pH 7.4) or physiological saline when in use. As the urokinase solution was particularly unstable, it was stored under the frozen condition at −30°C and thawed when in use. Bosmin injection solution (Dai-ichi Pharmaceutical Co., Tokyo, 1 mg/ml), Collagen reagent (Hormon-Chemie Co., West Germany, 1 mg/ml, pH 2.7) and adenosin-5′-diphosphate (Sigma Co., USA) were diluted with 0.05 M Tris-HCl buffer solution (pH 6.8) to prepare 10^{-4} M epinephrine, 10 μg/ml collagen and 10^{-4} M ADP solutions, respectively, for the platelet aggregationg activity measurement.

3. Buffer solutions: Acetate-veronal buffer solution was prepared according to the method of Michaelis (3).

Apparatuses

Auto-Fi Coagulation Instrument (Dade Diagnostics Inc.) was used for measuring the activated partial thromboplastin time (aPTT) and prothrombin time (PT). For the measurement of the platelet aggregating activity, Automatic Platelet Counter (PL-100, Toa Medical

Electronics Co., Tokyo) and 4-channel Automatic Platelet Aggregometer (Hema-Tracer 1, Model PAT-4A, NKK Co., Tokyo) were used.

Assay Methods

1. Measurement of aPTT : The moment when 0.1 ml of the sample solution was put in the sample pan of the Auto-Fi, 0.1 ml of each of Ci-TROL, Actin and 0.02 M $CaCl_2$ solution were added automatically, and the clotting time was recorded.
2. Measurement of PT : 0.1 ml of Ci-TROL and 0.2 ml of Tromboplastin. C were added to 0.1 ml of the sample solution in the sample pan of the Auto-Fi, and the clotting time was automatically recorded.
3. Measurement of the fibrinolytic activity by fibrin-plate method : 4 ml of 0.6% fibrinogen solution, 4 ml of 1/10 M phosphate buffer and 0.1 ml of 10 units/ml thrombin were poured, in this sequence, into a plastic laboratoly dish (86 mm) on a horizontal plated, then the dish was gently swung to mix the solution for a short time and allowed to stand at room temperature for 30 min, resulting in the formation of the fibrin-plate with uniform thinkness. Thereafter 0.03 ml of sample solution with 400 IU/ml urokinase was applied on the surface of the fibrin-plate in the shape of hemisphere with a pipet. It was incubated at 37°C for 6 hr and the lysed area was measured as the indicator of the fibrinolytic activity.
4. Measurement of the platelet aggregating activity : The platelet aggregating activity was measured according to the methods of Born (4) or O' Brien (5) as follows : One volume of 3.8% sodium citrate was mixed with 9 volumes of the freshly collected human blood, and then the mixture was centrifuged for 10 min at 1000 mpm to obtain the platelet rich plasma (PRP) in the supernatant. The precipitate layer including the blood cells was centrifuged again for 20 min at 3000 rpm, and the platelet poor plasma (PRP) was obtained in the supernatant. The number of the platelet contained in PRP was counted and PPP was added to the PRP to adjust the number of the platelet in the plasma to be $3\times10^5/\mu l$. Then 0.2 ml of the plasma and 50 μl of the sample solution were put into the cylindrical cuvett (7 mm $\phi\times50$ mm) on the platelet aggregometer, and stirred. Thereafter, 20 μl of the inducer of the aggregation, ADP, epinephrine or collagen, was added to the test solution, and the aggregation behavior was observed.

Results

1. Effects of the additives on blood coagulation system

In Fig. 1 and 2, aPTT and PT were plotted against the original concentrations of the additives added to the cuvette. The final concentrations of the additives reduced to 1/4 by dilution with the other reagents.

The figures showed that all of the six additives induced prolongation of the clotting times in both aPTT and PT reasurements, which were dependent on their concentrations. Especially, benzyl alcohol, sodium bisulfite and sodium sulfite strongly affected blood coagulation system. Benzyl alcohol markedly lengthened aPTT at 2% of concentration which was curently used in injections. Sodium bisulfite and sodium sulfite gave the strongest inhibitory effect on aPTT and PT of all additives examined. Even an addition of small amount of them made remarkable prolongation in clotting time until the aPTT and PT could not be

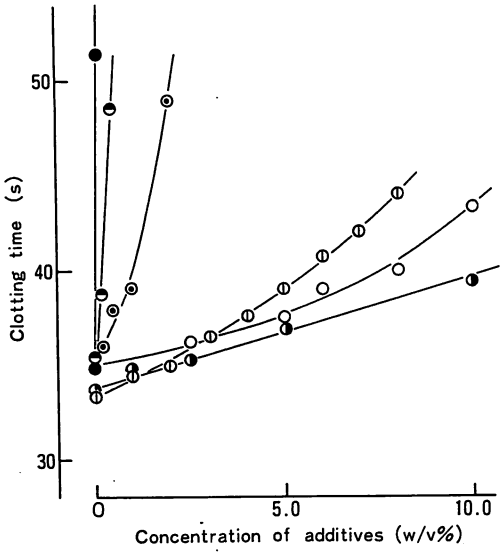

Fig. 1 Relation between Additive's Concentration and Activated Partial Thromboplastin Time (aPTT)
◉ : benzyl alcohol ◑ : HCO-60
○ : urethane ⊕ : macrogol 4000
● : NaHSO3 ◐ : Na_2SO_3

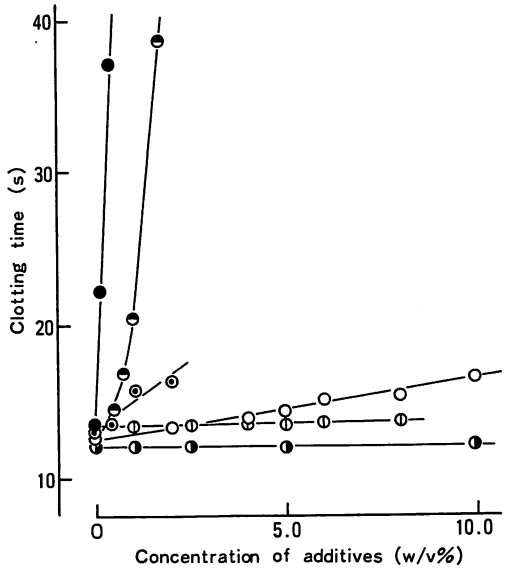

Fig. 2. Relation between Additive's Concentration and Prothrombin Time (PT)
◉ : benzyl alcohol ◑ : HCO-60
○ : urethane ⊕ : macrogol 4000
● : $NaHSO_3$ ◐ : Na_2SO_3

measured. First we investigated the relation between the ionic strength of the sample solutions and clotting time. The result was shown in Fig. 3. It was found that either $NaHSO_3$ or Na_2SO_3 induced the prolongation of aPTT and PT more strongly than NaCl whose solution had the same ionic strength as that of $NaHSO_3$ or Na_2SO_3. These results suggest that SO_3^{2-} must inhibit the blood coagulation. But $NaHSO_3$ showed stronger

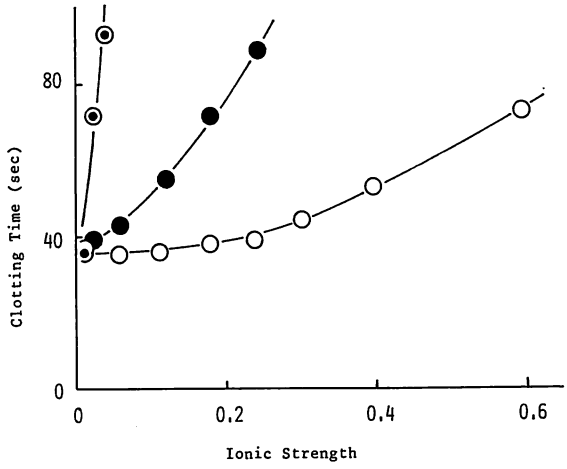

Fig. 3. Relation between Ionic Strength of Additives and Clotting Time (aPTT)
○ : NaCl ●Na_2SO_3 ◉ : $NaHSO_3$

activity of inhibition than Na_2SO_3. This finding can not be accounted by the inhibitory effect of SO_3^{2-} only. While Na_2SO_3 does not make any significant pH change in the experimental concentration region of the solution, $NaHSO_3$ lowers pH noticeable. Next the relation between the clotting time and pH was examined. The standard curves of pH dependencies of aPTT and PT were made by using acetate-veronal buffer. As shown in Fig. 4, the abrupt prolongation of the clotting time in both aPTT and PT was brought about by the change of pH from neutral to acid. The plots of the relation between the prolongation of clotting time by $NaHSO_3$ and pH of the solution almost coincided with the standard curves. This finding shows that the effect of $NaHSO_3$ on the prolongation of aPTT and PT is primarily due to its pH lowering. In another word, the strong effect of the pH change on clotting time seems to overshadow the effect of SO_3^{2-} itself for $NaHSO_3$ sample.

2. Effects of the additives on fibrinolysis system

As a measure of the acceleration or the inhibition of fibrinolysis, the effects of the additives on urokinase activity were examined on the fibrin-plate method. As shown in Fig. 5, HCO-60, urethane and benzyl alcohol induced almost neither inhibition nor acceleration of fibrinolysis in the concentration range examined. On the other hand, $NaHSO_3$ inhibited strongly the lysis activity of urokinase even at its low concentration. Thus inhibiting activity of $NaHSO_3$ is presumably due to low pH of the solution. Na_2SO_3 and macrogol 4000 also inhibited urokinase activity but the power was not so remarkable as that of $NaHSO_3$.

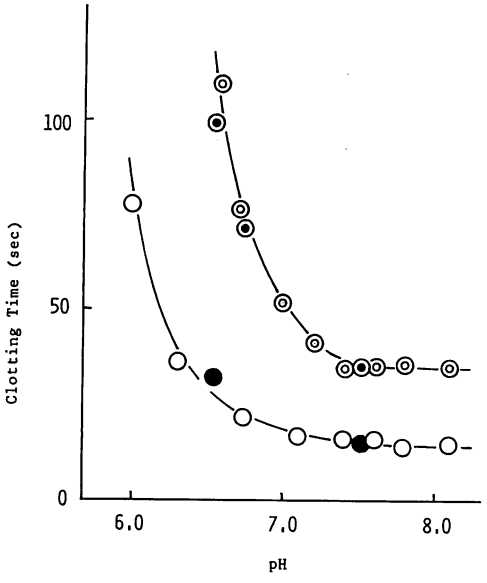

Fig. 4. pH Dependencies of Clotting Time
◎ : aPTT, standard
◉ : aPTT, $NaHSO_3$
○ : PT, standard
● : PT, $NaHSO_3$
○ : PT, standard
● : PT, $NaHSO_3$

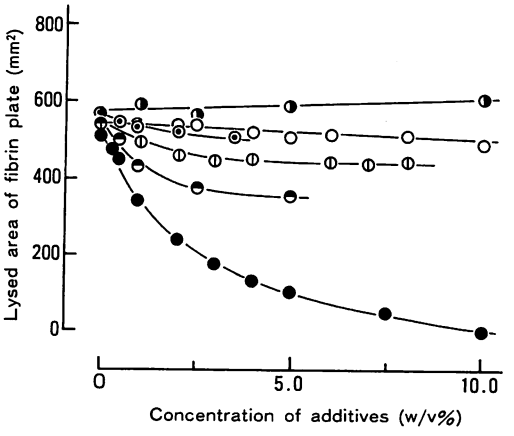

Fig. 5. Relation between Additive's Concentration and Lysing Activities of Urokinase Assessed by Fibrin-plate Method
◉ : benzyl alcohol ◑ : HCO-60
○ : urethane ⦶ : macrogol 4000
○ : $NaHSO_3$ ◐ : Na_2SO_3

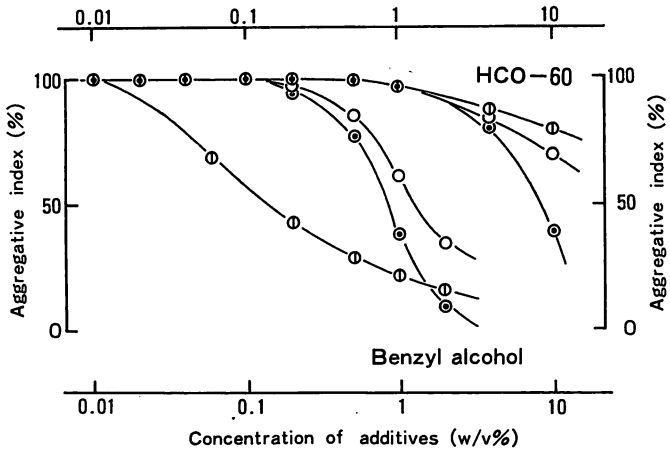

Fig. 6. Relation between Inhibitory Behaviors of Platelet Aggregation and Additive's Concentration
◉: collagen ○: ADP ⓘ: epinephrine

3. Effects of the additives on the platelet aggregation

The results of benzyl alcohol and HCO-60 were shown in Fig. 6. The abscissa axis indicates the concentration of the additives in logarithmic scale and the ordinate indicates the per cent inhibition of platelet aggregation. The per cent inhibition was defined as the ratio at per cent scale of the light transmission of the platelet dispersion to the control. The maximal concentration of the additives were settled as those contained in the corresponding injections commercially available. These sample solutions were placed on dilution to 18.5% of the original concentrations by other reagents in cuvettes when measured. Both benzyl alcohol and HCO-60 showed distinct inhibitory effect on platelet aggregation at the concentrations used in commercial injections with slight variations depending on the inducers. $NaHSO_3$, Na_2SO_3, urethane and macrogol 4000 showed no inhibitions on the platelet aggregation in all the tested concentrations.

4. Effects of the conbinations of two kinds of additives on blood functions

Two kinds of additive solutions from benzyl alcohol, $NaHSO_3$, Na_2SO_3 and macrogol 4000 with several concentrations were mixed at the same volume. The effects of these combined preparations on blood coagulation, fibrinolysis and platelet agregation were investigated in the same procedure mentioned above. The typical results are presented by the three-dimentional maps in Fig. 7-9. The abcsissa in the figures indicates the original concentrations of the additives which were added to the reaction mixtures. The final concentrations of the additives would be reduced to 1/8 by dilution with the other reagents in blood coagulation system (Fig. 7), to 1/2 in fibrinolysis system (Fig. 8, 9) and 1/11 in platelet aggregation reaction (Fig. 10). Fig. 7, 8, 9 and 10 showed that the combinations of these additives potentiated the inhibitory effect on blood coagulation, fibrinolysis and platelet aggregation. Especially, the combination of benzyl alcohol and either Na_2SO_3 or $NaHSO_3$ showed remarkable effects on fibrinolysis. Take the conbination of benzyl alcohol and Na_2SO_3 for instance. At zero concentration of benzyl alcohol, the effect of Na_2SO_3 was rather small, while in the presence of benzyl alcohol, it showed strong inhibitory effect on

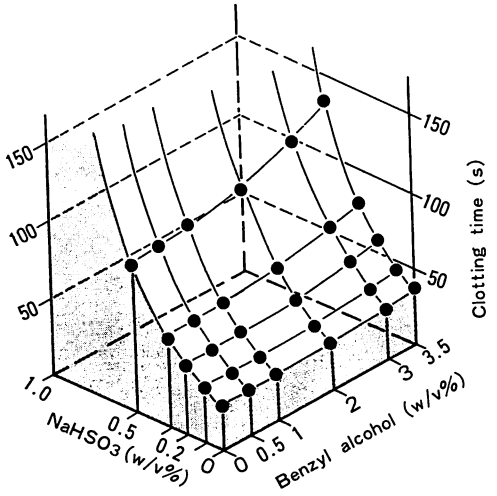

Fig. 7. Relation between Concentrations of Benzyl Alcohol and $NaHSO_3$ and Activated Partial Thromboplastin Time (aPTT)

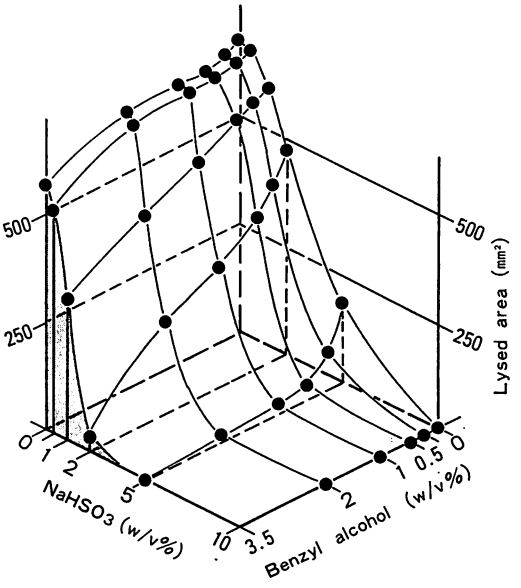

Fig. 8. Relation between Concentrations of Benzyl alcohol and $NaHSO_3$ and Lysing Activities of Urokinase Assessed by Fibrin-plate Method

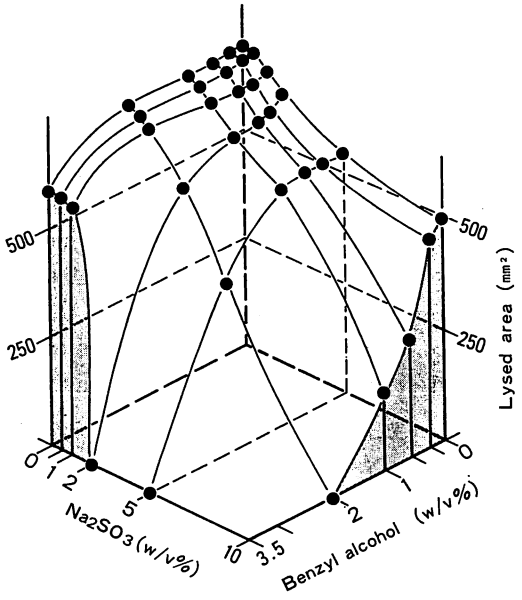

Fig. 9. Relation between Concentrations of Benzyl Alcohol and Na_2SO_3 and Lysing Activities of Urokinase Assessed by Fibrin-plate Method

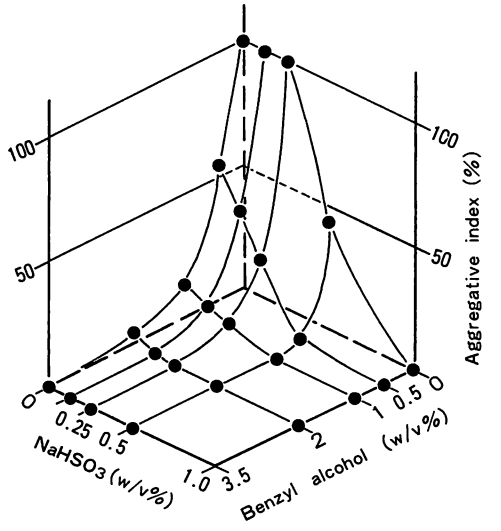

Fig. 10. Relation between Concentrations of Benzyl alcohol and Inhibitory Behaviors of Platelet Aggregation Induced by Collagen

fibrinolysis. Also at zero concentration of Na_2SO_3, benzyl alcohol had practically no effect on fibrinolysis system, while in the presence of Na_2SO_3, it showed significant inhibitory effect. Any combinations with macrogol 4000 showed little potentiation to the effects on blood functions examined.

Discussion

Urethane and macrogol 4000 were considered to be relatively safe additives from the view point of hematology, such as blood coagulation, fibrinolysis and platelet aggregation. HCO-60 showed the inhibition of platelet aggregation around 10% of concentration. This inhibiting action cannt be regarded as the characteristics of HCO-60, because many substances will show the similar behaviors in such a high concentration as 10%. Benzyl alcohol, $NaHSO_3$ showed remarkable inhibition on coagulation, fibrinolysis and platelet aggregation. The combined preparations, in some case, such as the conbination of benzyl alcohol with sodium bisulfite or sodium sulfite, had the strong inhibitory effects on blood coagulation, fibrinolysis and platelet aggregation, which could be considered as the potentiation rather than the simple addition of these additives. In in vivo experiments using rabbits according to Rodriguez-Erdmann's method (6), no acute adverse reactions were observed (data not shown). It may be ascribed to dilution of the additive by much amount of blood of the body. But in the experiments the "local actions" of the additives before dilution and long term administration could not be evaluated. Recently, it has been reported that multiple injections of heparin preparations containing benzyl alcohol to immature infants caused functional neurosis, severe metabolic acidosis, thrombocytopenia etc, to drive death (7, 8) or several immature infants died of a syndrome which might be caused by benzyl alcohol poisoning or its metabolites (9). At present, many injections containing benzyl alcohol, sodium sulfite or sodium bisulfite, or in some case, containing both of them, are commercially available. This kind of injections has possible adverse reaction to depress and/or inhibit the fundmental functions concerning homeostasis in the blood such as coagulation, fibrinolysis and platelet aggregation. Therfore, more caution for the adverse reactions should be directed to the additives contained in injections as well as the main component, especially, when the treatment with the injections continues for a long time and/or gives to an immature infant.

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