Ristocetin and the thrombin clotting time

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SUMMARY The addition of the antibiotic ristocetin to plasma accelerated the thrombin clotting time (TCT) in 20 out of 22 subjects. Prior incubation of ristocetin with thrombin or plasma did not alter its effect on the TCT. Ristocetin accelerated clotting greatly at low but not at high levels of thrombin. A simple linear correlation between heparin concentrations and the TCT was demonstrated when ristocetin at 2.5 mg per ml was added to plasma containing between 0.05 and 0.5 unit of heparin per ml. There are implications for assay procedures involving heparin and the TCT.

The thrombin clotting time (TCT) is a simple, rapid, and clinically useful test (Merskey, 1972). It is prolonged in hypofibrinogenaemia and in the presence of heparin and fibrinogen/fibrin degradation products. It is also prolonged in congenital dysfibrinogenaemia, and occasionally in liver disease and myelomatosis (Dacie and Lewis, 1975). The test has been used to monitor thrombolytic therapy and forms the basis for assays of heparin and heparin-neutralising activity. The use of the TCT for definitive assays is complicated because the test is extremely sensitive to heparin and heparin-like substances. The range of heparin concentrations spanning a normal TCT and an incoagulable TCT is narrow.

The antibiotic ristocetin is well known for its platelet-aggregating effect (Howard and Firkin, 1971). Less well known is its ability to precipitate fibrinogen, particularly at low temperatures (Goh and Firkin, 1976). Some workers claim that it shortens the TCT (Ts'ao *et al.*, 1975), while others say that it does not (Jenkins *et al.*, 1974). We have studied the effect of ristocetin on the TCT with and without added heparin.

Material and methods

BOVINE TROPICAL THROMBIN (Parke-Davis, Michigan, USA)

A fresh solution of 1000 units per ml in 0.15M NaCl

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was made up at the start of each working day and kept at 4°C. Working solutions were diluted in 0.15M NaCl as required and were replaced by fresh solutions every hour. A concentration of 10 units per ml was used in all experiments except where otherwise stated.

HEPARIN (MTC Pharmaceuticals, Ontario, Canada)

This was diluted in 0.15M NaCl as required.

RISTOCETIN SULPHATE (H. Lundbeck and Co, Copenhagen, Denmark)

This was dissolved in 0.15M NaCl to give the required final concentrations. A fresh solution was made up at the start of each working day. All concentrations mentioned in the text are final concentrations except when the actual concentration is stated. A concentration of 2.5 mg per ml was used in all experiments except where otherwise stated.

FIBRINOGEN (Grade L, Kabi, Stockholm, Sweden)

One gram was dissolved in 100 ml of distilled water at 30°C and was twice absorbed with aluminium hydroxide (moist gel, BDH, Toronto, Ontario; 20% w/w moist gel in saline, 1 ml per 10 ml fibrinogen solution). The solution was clarified by centrifugation at 20 000 g for 30 minutes and dialysed at room temperature against 8 volumes of 0.15M NaCl in an Amicon diafiltration cell (UM 30 membrane; MW cut-off 30 000). The concentration determined by absorbance at 280 nm ($E_{1\%}^{1\text{cm}} = 13.6$) (Sober, 1968) was 6.4 g/litre. The solution was aliquoted and stored at -70°C.

BOVINE SERUM ALBUMIN (BSA Fraction V, Sigma, St. Louis, USA)

A stock 6% solution was made up in 0.15M NaCl at the start of each working day.

COLLECTION OF BLOOD

Samples were obtained from healthy volunteers and from a group of patients with various forms of renal disease. Blood from the latter group was used only in the experiment which compared the length of the TCT with and without ristocetin. In all other experiments blood from healthy volunteers was pooled. All blood was collected in plastic syringes and immediately mixed with a one-ninth volume of 0.1M sodium citrate in plastic tubes.

Platelet-poor plasma (PPP) was obtained by centrifuging samples for 20 minutes at 1000 g. The samples were tested immediately or stored at -20°C .

The basic assay system was essentially a thrombin clotting time (TCT). 0.1 ml of the plasma or fibrinogen to be tested was placed in a previously warmed glass tube in a 37°C waterbath. 0.05 ml 0.15M NaCl was added, and the contents were mixed. 0.05 ml of thrombin was then added and the clotting time was recorded. The end-point of the reaction was taken as the first appearance of fibrin strands or granularity.

Ristocetin and/or heparin was added to the system in 0.025 ml aliquots, replacing similar quantities of normal saline.

EFFECT OF RISTOCETIN ON TCT

Samples of PPP from 22 subjects were studied. Ten were normal volunteers and the remainder had various forms of renal disease. In each case a basic TCT was done and then repeated immediately with added ristocetin (final concentration 2.5 mg per ml). The visible precipitate of fibrinogen that invariably formed was easily redissolved by agitation of the tube briefly at 37°C before the addition of the thrombin.

VISIBLE EFFECTS OF RISTOCETIN ON PPP OR FIBRINOGEN

A range of dilutions of ristocetin giving final concentrations of 0.8, 1.6, 2.4, 3.2, and 4.0 mg per ml was added to 0.1 ml of either PPP or fibrinogen solutions in 0.025 ml aliquots. Observations were made at 37°C . The fibrinogen solutions were diluted in 0.15M NaCl or in equal quantities of 6% albumin.

EXCLUSION OF POSSIBLE CONTAMINANT PROTEOLYTIC ACTIVITY

Ristocetin was reconstituted in 0.15M NaCl and boiled at 100°C in a waterbath for three hours. Aliquots were removed at 30, 60, 90, and 180

minutes, and their effect on the TCT of a normal plasma pool was tested.

INCUBATION OF RISTOCETIN WITH THROMBIN

One part of ristocetin was added to one part of 0.15M NaCl and two parts of thrombin. After one and three minutes 0.1 ml of this mixture was added to 0.1 ml of PPP in a waterbath at 37°C , and the clotting time was recorded.

INCUBATION OF RISTOCETIN WITH PPP

One part of ristocetin was incubated with four parts of PPP and one part of 0.15M NaCl at 37°C . At 1, 2, 3, and 4 minutes aliquots of 0.15 ml were subsampled into glass tubes in a 37°C waterbath. 0.05 ml thrombin was then added and the clotting time was recorded. As a time control, PPP was similarly incubated with 0.15M NaCl alone.

VARYING THE CONCENTRATIONS OF RISTOCETIN AND THROMBIN

Ristocetin was diluted in 0.15M NaCl to give final volumes of 0.5, 1.0, 1.5, 2.0, and 2.5 mg per ml. Thrombin was diluted in 0.15M NaCl to 1, 2, 4, 8, and 10 units per ml. Each concentration of thrombin was then used in the TCT against the range of ristocetin concentrations.

VARYING THE CONCENTRATION OF FIBRINOGEN

Aliquots of fibrinogen were thawed and diluted in 0.15M NaCl to give concentrations of 1.28, 2.56, 3.84, 5.12, and 6.40 g/l. Each was then diluted with equal amounts of 6% bovine serum albumin, thus halving the fibrinogen concentrations. TCTs were carried out on each dilution, with ristocetin added at 1.5 mg per ml.

EFFECT OF HEPARIN

A range of heparin dilutions was made in 0.15M NaCl. Increasing concentrations were added to PPP pools until the TCT became unrecordable. The PPP was tested without added ristocetin and with ristocetin added at 1.0 mg per ml and at 2.5 mg per ml.

Results

EFFECT OF RISTOCETIN ON TCT

The TCTs with and without added ristocetin are shown in Figure 1. In 20 out of 22 samples tested with ristocetin the TCT was shortened by more than two seconds, and in two it remained within one second of the original TCT. The mean value in seconds for the TCTs without ristocetin was 14.22 ± 2.45 ($\pm$ SD). The mean value with added ristocetin was 9.69 ± 1.11 ($\pm$ SD). A paired Students' *t* test

Fig. 1 Thrombin clotting time with (B) or without (A) added ristocetin. Final concentration of ristocetin 2.5 mg per ml. The horizontal lines indicate the mean values.

showed that this shortening of the TCT was highly significant ($t = 8.06$, $P < 0.0005$).

VISIBLE EFFECT OF RISTOCETIN ON PPP

A cloudy white precipitate appeared immediately after the addition of 0.025 ml of ristocetin to 0.1 ml of PPP at what would have been final concentrations for ristocetin of 1.5, 2.0, and 2.5 mg per ml had the whole TCT been carried out. At the time of observation the concentrations were in fact 2.4, 3.2, and 4.0 mg per ml. These precipitates could be rapidly dissolved by agitating the mixture at 37°C. No precipitate was visible at lower concentrations.

When a pure fibrinogen solution was used all actual concentrations of ristocetin tested (0.8, 1.6, 2.4, 3.2, and 4.0 mg per ml) caused visible precipitation; only the precipitation caused by a true concentration of 0.8 mg per ml ristocetin was reversible. Dilution with albumin gave visible precipitation from 1.6 mg per ml actual concentration of ristocetin upwards. Only the precipitate caused by the actual concentration of 4.0 mg per ml of ristocetin was irreversible.

EXCLUSION OF CONTAMINANT PROTEOLYTIC ACTIVITY

There was no apparent diminution of the ability of

ristocetin to shorten the TCT after boiling for up to three hours.

INCUBATION OF RISTOCETIN WITH THROMBIN

The incubation of thrombin with ristocetin for three minutes as opposed to one minute did not enhance or impair the ability of thrombin to clot fibrinogen.

INCUBATION OF RISTOCETIN WITH PPP

As shown in Table 1, over a period of four minutes there was no significant temporal impairment or enhancement of fibrinogen-fibrin conversion after the addition of thrombin.

Table 1 Effect on TCT (TCT_R) of incubating ristocetin with platelet-poor plasma

Incubation time (min)	TCT_R	TCT_0^*
1	10.5	13.8
2	9.1	14.5
3	9.8	14.0
4	10.1	13.1

*PPP was also incubated with 0.15 M NaCl alone (TCT_0).

VARYING THE CONCENTRATION OF RISTOCETIN AND THROMBIN

The results are given in Table 2. At all concentrations of ristocetin clotting was accelerated greatly at low but not at high levels of thrombin.

Table 2 Effect of varying concentrations of ristocetin on TCT of platelet-poor plasma at varying thrombin concentrations: means of three separate experiments

Ristocetin conc. (mg/ml)	Thrombin concentration (U/ml)				
	1	2	4	8	10
0	90.3	42	21	13.5	10.8
0.5	54.5	28.4	15.9	10.1	9.0
1.0	42.8	23.3	14.3	9.4	9.0
1.5	35.7	22.1	14.4	9.9	7.8
2.0	28.5	18.7	13.1	8.1	8.0
2.5	25.0	16.5	11.9	10.0	9.7

EFFECT OF HEPARIN

The results are shown in Figure 2. The addition of more than 0.1 unit of heparin per ml of plasma rendered PPP incoagulable. If ristocetin at a final concentration of 1.0 mg per ml was added, more than 0.15 unit of heparin per ml was needed to produce the same effect. When the concentration of ristocetin was increased to 2.5 mg per ml final concentration, the dosage of heparin could be increased to over 0.5 unit per ml of plasma before it became incoagulable. The point of incoagulability

Fig. 2 Effect of adding varying concentrations of heparin to the thrombin clotting time with and without added ristocetin. Thrombin strength was held at 10 u/ml; ristocetin concentration was 1.0 mg/ml in B and 2.5 mg/ml in C. No ristocetin was added in A.

lay somewhere between 0.5 and 1.0 unit of heparin per ml of plasma for this concentration of ristocetin. There was a simple linear relationship between heparin concentration and TCT when the heparin concentration lay between 0.05 and 0.5 unit per ml of plasma.

Discussion

In this study we have confirmed the observation of Ts'ao and colleagues (1975) that albumin inhibits fibrinogen precipitation due to ristocetin. We have also shown that it facilitates resolubilisation at 37°C. With the addition of 6% albumin to fibrinogen we were able to redissolve precipitated fibrinogen at an actual concentration of ristocetin up to 3.2 mg per ml. When ristocetin was added to PPP we found no difficulty in redissolving the precipitates caused by actual ristocetin concentrations of up to 4.0 mg per ml.

Proteolytic contamination of ristocetin was first noted by Morgan *et al.* in 1974. Subsequent to that time the antibiotic was allegedly free from such contamination (Goh and Firkin, 1976). Nevertheless we felt it prudent to establish that any shortening effect ristocetin might have on the TCT was still present after boiling for three hours, a manoeuvre which should inactivate proteolytic enzymes.

The TCT in samples from 20 out of 22 subjects was shortened by the addition of ristocetin (2.5 mg per ml) to the test system, and the difference between the TCTs with and without ristocetin was highly significant (Fig. 1). In other experiments reported in this paper, we have found shortening of the TCT at ristocetin concentrations as low as 0.5 mg per ml and

have thus clearly established that ristocetin can shorten the TCT. Ristocetin did not appear to alter the effectiveness of thrombin when the two substances were incubated for up to three minutes at 37°C. Furthermore, incubation of ristocetin with PPP for up to four minutes did not alter its temporal effect on the TCT (Table 1). The cause of the acceleration of clotting by ristocetin is uncertain. Because the action of ristocetin is most efficient at low thrombin concentrations it might be suggested that its action is applied at the proteolytic stage of fibrinogen/fibrin conversion. It is possible that ristocetin shortens the thrombin clotting time by aggregating fibrinogen, thereby increasing the local concentration of fibrinogen during thrombin proteolysis and polymerisation of the fibrin monomer. Further experiments are needed to elucidate the mechanism of action.

When ristocetin (2.5 mg per ml) was added to PPP in the presence of heparin the range of the TCT was markedly increased. Furthermore, a linear relationship between the TCT and heparin concentration was obtained when the heparin concentration lay between 0.05 and 0.5 unit per ml of plasma (Fig. 2). This practically encompasses the range of heparin concentrations used in the PF₄ assay (13) (Harada and Zucker, 1971), and may make the use of a single dilution of heparin more acceptable in this assay. 0.5 unit of heparin per ml of plasma represents only some 1500 units of circulating heparin in a 70-kg man with a normal packed cell volume, and, therefore, although there are implications for a simple heparin assay the usefulness of this test in the control of clinical anticoagulation is probably limited.

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References

- Dacie, J. V., and Lewis, S. M. (1975). Investigation of the haemorrhagic disorders. In *Practical Haematology*, 5th edition, pp. 314-403. Churchill Livingstone, Edinburgh and London.
- Goh, A. T. W., and Firkin, B. G. (1976). Further studies on the interaction between ristocetin and fibrinogen. *Haemostasis*, **5**, 57-65.
- Harada, K., and Zucker, M. B. (1971). Simultaneous development of PF_4 activity and release of ^{14}C -serotonin. *Thrombosis et Diathesis Haemorrhagica*, **25**, 41-45.
- Howard, M. A., and Firkin, B. G. (1971). Ristocetin—A new tool in the investigation of platelet aggregation. *Thrombosis et Diathesis Haemorrhagica*, **26**, 362-369.
- Jenkins, C. S. P., Meyer, D., Dreyfus, M. D., and Larrieu, M. J. (1974). Willebrand factor and ristocetin. I. Mechanism of ristocetin-induced platelet aggregation. *British Journal of Haematology*, **28**, 561-578.
- Merskey, C. (1972). Defibrination syndrome. In *Human Blood Coagulation, Haemostasis and Thrombosis*, edited by R. Biggs, pp. 444-475. Blackwell Scientific Publications, Oxford.
- Morgan, F. J., Goh, A., and Firkin, B. G. (1974). Proteolytic activity in ristocetin preparations. *Thrombosis Research*, **5**, 565-569.
- Sober, H. A., Ed. (1968). *Handbook of Biochemistry*, p. C-76. Chemical Rubber Co., Cleveland, Ohio.
- Ts'ao, C., Green, D., and Rossi, E. C. (1975). Some factors affecting fibrinogen precipitation by ristocetin ultrastructure of precipitates. *Blood*, **45**, 621-629.

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