REVIEW ARTICLE

Protamine – antagonist to heparin

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Summary: Protamine is used for titration of heparin *in vitro* for diagnosis of hemorrhagic states and for neutralization of heparin *in vivo* to terminate heparinization. The protamine equivalent varies with the heparin preparation, conditions of testing and, *in vivo,* with the amount of heparin present in the circulation. The latter depends on time after administration and the hemodynamic and metabolic state of the patient. Protamine, when injected rapidly, will release histamine and agglutinate platelets. Bleeding (spontaneous hemorrhage) demonstrates a multiple breakdown of hemostatic mechanisms due to surgical stress, drugs, exposure of the blood to foreign surfaces, etc. Simple rules for the amount of protamine required for an individual patient based on clinical judgement will be satisfactory in most cases. When hemostasis is not achieved, it must be appreciated that heparin and protamine are only part of a complex deteriorating situation.

Protamine has long been used in conjunction with heparin therapy. At the suggestion of Professor C. H. Best, in 1937 I undertook a study on the practical use of protamine as a heparin antagonist, after the demonstrations by A. Fischer[1] that basic proteins could hinder the anticoagulant activity of heparin and by Chargaff and Olson[2] that protamine was particularly effective. Our studies demonstrated two uses for this property of protamine. The first[3] was neutralization of the anticoagulant action of heparin in blood samples, both to demonstrate the presence of heparin in blood and to measure the amount of heparin in the sample. It was known as *protamine titration* and has since been widely used by hematologists and others. Since an excess of protamine shows anticoagulant properties when added to blood samples, the minimal amount of protamine required to bring the clotting time to a normal value provides a measure of the amount of heparin present in the blood sample. The second use of protamine, as demonstrated by Jaques, Charles and Best,[4] was to reduce hypocoagulability due to heparin by its injection into the circulation, known as *protamine neutralization.*

In order to titrate heparin with protamine *in vitro* or to neutralize heparin *in vivo,* it is necessary to know the equivalence of protamine and heparin. However, we originally observed that the value of the factor for this equivalence can be changed both *in vitro* and *in vivo* and this is confirmed by the recent report of Lowary *et al*[5] of a study of neutralization *in vitro* and *in vivo* which demonstrated "a significant difference in the neutralization of different types of sodium heparin by protamine sulfate".

In vitro equivalence of protamine and heparin

Our early studies demonstrated that *in vitro* the number of milligrams of protamine required to neutralize exactly 1 mg. of heparin depended upon the particular protamine preparation (source, method of extraction, accompanying materials, etc.), the heparin preparation, and the actual coagulation system used for the testing. No constant relationship could be established between the quantities of protamine required for neutralization of a given number of units of heparin. This appeared to be owing to differences in accompanying materials in both protamine and heparin preparations and to the fact that the ability of protamine to combine with heparin was not related specifically to those groups in heparin responsible for its anticoagulant activity.[6]

Today it is still difficult to establish a relationship between milligrams of protamine and units of heparin, essentially for the same reasons, but in particular because of the considerable variability found in heparin preparations commercially available and the difficulties in standardizing them in terms of international units. In recent studies[7-9] we have conducted chemical analyses on a number of commercial heparin preparations and have found a great variation in many chemical parameters. Furthermore, no correlations were found between activity on biological potency tests *in vitro* or *in vivo* and any chemical parameter. It is therefore not surprising that Lowary *et al* found a similar variation in protamine neutralization. To discuss the equivalence of protamine to heparin in terms of milligrams per mg. as compared to units per mg. requires a value for units per mg. of heparin. The crystalline hydrated barium salt has a well documented value of 100 units/mg. Removal of water and barium results in a value of 156 units/mg. Current heparin preparations show a wide range of values, from 60 to 180 units/mg. Heparin is supplied for clinical use in bottled solutions containing a specific number of units as determined by biological assay (several different official assay methods exist). The actual weight of heparin used will vary with manufacturer, batch, assay procedure, etc. To consider 100 units as equivalent to 1 mg. of heparin is convenient, but the figure so obtained will not give the actual weight of heparin used.

The considerable variability in heparin preparations is in part related to difficulties in standardization because of problems in biological assay of heparin. It has been

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widely assumed that comparison of the anticoagulant activity of one heparin preparation with a standard heparin on blood or plasma is sufficient. However, Jaques and Charles[10] showed that the relative activity of heparins from different sources, even when prepared in the same manner as the crystalline barium salts, varied with different coagulation assay systems. Evidently this is because heparin preparations have different activities with respect to various stages of blood coagulation. The importance of this phenomenon has been emphasized recently by the international collaborative study of the assay of heparin. Thirteen laboratories studied coded heparin preparations supplied by the WHO Laboratory for International Standards.[11] This study demonstrated that while all the assay methods give very similar values for any heparin preparation when tested blind against itself, large discrepancies appear when two heparin preparations are compared blind. This is because the preparations exhibit different relative activities with changes in substrate plasma, etc. These discrepancies can be as great as 40% with the same assay procedure (e.g. the USP assay) when different batches of substrate (in this case sheep plasma) are used. The US Pharmacopoeia describes procedures for standardizing protamine for use with insulin and with heparin. However, with such variation in relative activity of heparin preparations in different test systems, a figure for the neutralization equivalent in mg. of protamine thus standardized per 100 units of heparin must be considered with some reserve, particularly when USP heparin standard, which contains a large amount of diluent, is used.

The protamine titration for heparin

Shortening of a prolonged clotting time on addition of protamine is usually an adequate demonstration of the presence of heparin in the blood sample. However, since *in vitro* it is easy to add amounts of protamine sufficiently large to increase the clotting time, it is necessary to determine the minimal amount of protamine required to shorten the clotting time to normal values. To be able to express this as equivalent heparin, it is necessary to titrate in normal blood a known heparin preparation with the protamine to be used. Further, the test is only valid with respect to the patient's plasma if normal levels of the different components of blood coagulation are present and if the plasma does not contain protamine or abnormal amounts of mucopolysaccharides. Protamine combines with mucopolysaccharides, nucleic acids and fibrinogen. Fibrinogen split products, identified as anticoagulants[12] occurring in blood in the intravascular coagulation syndrome, may contribute to a titration value for protamine.

Distribution and fate of heparin *in vivo*

When protamine is used to neutralize heparin in the circulation, the immediate question is how much heparin is present. This requires a knowledge of how much heparin has been injected, the period of time since the injection, the distribution of heparin in body compartments, and the functional status of the kidneys, reticulo-endothelial system and other organs involved in removing heparin from the circulation. To assume that the amount of heparin present is the amount injected divided by the total blood volume may give a figure in error by several orders of magnitude.

As indicated in Table I, heparin in the circulation is disposed of in various ways, depending on whether it is associated with plasma protein or is "free". At concentrations up to 2 units/ml. blood, the heparin appears to be attached to plasma proteins in connection with the coagulation system.[13] In agreement with this is Estes' calculation[14] that the relative volume of distribution of heparin in man for median doses of 2 mg./kg. is 57 mg./kg. or close to the plasma volume. With higher concentrations in the blood (larger single intravenous doses), some heparin is not bound and passes into tissue spaces and appears in the lymph. Some is taken up by macrophages in the reticulo-endothelial system and some by endothelium. At blood concentrations above 7 units/ml., unchanged heparin is excreted by the kidney. Partially degraded heparin (uroheparin) is also found in the urine. However, for heparin attached to plasma protein, destruction involves removal of sulfate and its excretion as inorganic sulfate. Hence removal of heparin from the circulation is not effected by a single route; this is an added safety factor in its use. Estes found a plasma half-life of 1.5 hours for heparin in man with a dose of 2 mg./kg. Olsson, Lagergen and Ek[15] found that the plasma half-life of heparin increases with increasing dosage. Further, Solandt and Best[16] demonstrated that the effect of heparin on platelets persisted longer than the hypocoagulability but that this effect can also be reversed with protamine. Estes has similarly found that the plasma half-life for heparin is not the same when measured by different coagulation tests.

The amount of heparin present in the circulation can be determined by various tests — clotting time (increased), protamine titration, partial thromboplastin time (increased), thrombin clotting time, and extraction followed by measurement in colorimetric or anticoagulant tests. Tests based on blood coagulation are, in general, speedy but they require that the coagulation system be essentially normal. Hence, while such tests are very successful in the control of long-term intravenous heparin therapy,[17] they are less successful in surgical patients suddenly developing a complex hemostatic defect.

Table I
Relation of heparin concentration in the blood and mode of removal

Concentration of heparin in blood	State in body fluids	Metabolic route	Renal excretion
0 — 2 u./ml.	Associated with plasma proteins	Desulfated	Inorganic SO_4
> 2 u./ml.	Not associated with plasma proteins. Enters tissue spaces, lymph.	Uroheparin	Uroheparin
> 7 u./ml.	Part taken up by macrophages, endothelium, etc.		Unchanged heparin

Adapted from references 10 and 11

Heparin and hemostasis

While we were developing heparin for clinical use in 1934 we were told by colleagues that this was a waste of time since patients (certainly surgical patients) who received heparin would bleed to death. The universal administration of heparin to patients without the occurrence of spontaneous hemorrhage, even during major operations, is evidence that this is not the case. Hemorrhage associated with the administration of heparin means that hemostasis has been deranged by several factors simultaneously.[13] Extensive experimental and clinical observations are summarized in Table II.

Spontaneous hemorrhage has multiple causation. A single agent producing a single disruption in hemostasis does not result in spontaneous hemorrhage. Blood coagulation has been suspended in normal subjects by excessive doses of anticoagulants in combination without spontaneous hemorrhage. Spontaneous hemorrhage occurs when, in addition to suspension of coagulation, there is interference with platelet function or with the vascular component of hemostasis, either by the presence of disease or by stress. Since spontaneous hemorrhage is the result of combined defects of hemostasis, identification of its causes is not simple. Cessation of hemorrhage by a treatment does not identify the cause of the hemorrhage, since the direct action of the treatment (e.g. protamine in a heparinized patient) may have only suspended the effect of the immediate hemorrhagic agent (stress) by neutralizing an accessory variable (heparin).

Another useful way of picturing hemostasis is as the algebraic sum of the effectiveness of hemostatic mechanisms — blood coagulation, platelets, vascular response — in relation to hemodynamic forces — flow and blood pressure, i.e. the hemostatic balance.

$$\text{Hemostatic efficiency} = \frac{\text{Coagulation} + \text{Platelets} + \text{Vessel wall}}{\text{Blood pressure}}$$

If the mechanisms in the numerator are all impaired, even though not sufficient singly to cause bleeding, hemostasis is seriously impaired. Thus, in a patient with stress and some degree of thrombocytopenia and/or other impairment of platelet function, bleeding can occur with a small amount of heparin in the circulation and cease with its removal.

Table II
Occurrence of spontaneous hemorrhage

Components of hemostasis:	(1) blood coagulation (2) platelets (3) vascular wall	
Treatments and conditions lowering hemostatic efficiency of components		
Group 1:	(a) anticoagulants (dicumarol, phenylindanedione, heparin)	
	(b) deficiencies of coagulation factors as in hemophilia, disseminated intravascular coagulation, etc.	
Group 2:	(a) thrombocytopenia	
	(b) drugs affecting platelet function, such as acetylsalicylic acid, reserpine, etc.	
Group 3:	(a) stress: frost-bite, hypertonic intraperitoneal saline, operation, restraint, subcutaneous physiological saline daily, electroshock, insulin convulsions, LSD, anesthetics and depressants	
	(b) changes in the adrenopituitary axis: ACTH, STH, salicylates, histamine, adrenalin, desoxycorticosterone, adrenalectomy	
	(c) local pathology: inflammation, bacterial toxins, tumours, etc.	

Spontaneous hemorrhage results (+) from combinations of treatments

		Treatment Group 1	Group 2	Group 3
Treatment	Group 1	−	+	+
	Group 2	+	−	+
	Group 3	+	+	−

Spontaneous hemorrhage does not result from two treatments which produce a single defect in hemostasis. It results from a combination of treatments, producing multiple defects.

Spontaneous hemorrhage (breakdown of hemostasis) has multiple causes

Toxicity of protamine

In 1949 I reported studies on the toxicity of intravenous protamine in animals.[18] In confirmation of the report of Thompson,[19] I found that protamine causes a pronounced fall in blood pressure in the anesthetized dog and causes cardiovascular collapse in other animal species. Many of the actions observed were histamine-like. These effects could be reduced by injecting the protamine slowly. Protamine toxicity in part appears to be due to its action as a histamine-liberator and possibly in part as a histamine simulator. Protamine in combining with heparin in mast cells will displace histamine and/or serotonin. Experience with relatively large doses of protamine after overdosage with heparin suggests that protamine first combines with exogenous heparin in the circulation and that only when there is protamine in excess of the amount required for combination are the mast cells affected. This is a further reason for limiting protamine dosage to that equivalent to the heparin in the circulation. Ellison, Ominsky and Wollman[20] reported that intravenous injections in eight volunteers of multiple doses of protamine totalling 800 mg./70 kg. produced the typical symptoms of histamine release — itching/flushing, fatigue/malaise, nausea/vomiting, headache, hyperventilation and temperature elevation. All exhibited itching/flushing, while only one showed hyperventilation and temperature elevation. The intensity of symptoms increased as the dose was increased. Adverse effects seen on the electrocardiogram appear related to histamine release. Gourin, Streisan and Stuckey[21] observed in dogs an increase in coronary sinus blood flow and decrease in peripheral resistance.

As the difficulties with protamine are largely those associated with defective hemostasis, it is surprising that little attention has been paid to the observation[18] that protamine caused marked platelet clumping with disappearance of platelets both *in vivo* and *ex vivo* and that the hypotension produced in dogs appeared to be related to the production of thrombocytopenia. Thrombocytopenia produced by protamine can contribute to the difficulties in controlling bleeding at operation,

while the release of serotonin, etc. by the disintegrating platelet masses in the pulmonary microcirculation will embarrass the pulmonary circulation.

Occasionally, by mistake, heparin is given in gross overdosage. There is a tendency to administer immediately a corresponding overdosage of protamine. However, while suspension of blood coagulability (in the absence of any other hemostatic defect) is relatively harmless, this is certainly not the case for protamine. The same hypotension caused by rapid injection of protamine in dogs has been produced in man by too zealous use of protamine as an antidote. Hence protamine should be used in this situation only when there are specific symptoms to indicate that the heparin overdosage must be counteracted and then with great care. The demonstration by Gore *et al*[22] of pulmonary arteritis produced in rabbits by the injection of protamine suggests that such injections may also be responsible for prolonged pathological change.

Protamine dosage for heparin neutralization *in vivo*

According to Berger, Ramaswany and Ryan,[23] excessive bleeding is one of the unsolved problems of open-heart surgery. Because a sufficiently large excess of protamine can be added to blood *in vitro* to exert an anticoagulant effect and cause the protamine test for heparin to appear negative despite its presence, it has been assumed this is also the case when protamine is used for neutralization of heparin *in vivo*. The minimum amount of protamine required *in vitro* to increase the clotting time is approximately 0.02 mg./ml. of blood and five times this (0.1 mg./ml. requiring a dose of 700 mg./70 kg. body weight) is required to produce a marked prolongation.[13] Ellison, Ominsky and Wollman[20] have examined the effects of overdoses of protamine in both patients and volunteers. Repeated doses in volunteers of 200 mg./70 kg. (up to 800 mg. total) caused only a transitory increase of the Lee-White coagulation time from 6.7 to 9.4 minutes with no change in the partial thromboplastin time. Hence difficulties due to bleeding after the injection of protamine must usually involve more than the minimal prolongation of clotting produced by the protamine. Raby and Servelle[24] reported three cases in which hypocoagulability and difficulty in producing surgical hemostasis were due to excess protamine; coagulability and hemostasis were normalized after the injection of heparin.

On the other hand, it has been observed that after neutralization of heparin in the circulation by protamine following the institution of extracorporeal circulation for open-heart surgery, later blood samples are incoagulable. Frick and Brögli[25] have reproduced this phenomenon by neutralizing heparin with protamine in normal individuals, in blood from the heart-lung machine, and in decalcified plasma. They find it is due to the protaminase known to be present in blood and also that this effect is not seen if protamine chloride is used instead of protamine sulfate.

There has been wide variation in the amount of protamine recommended for clinical neutralization of heparin. A ratio of 1 or 2 mg. of protamine sulfate for each 100 USP units of exogenous sodium heparin is widely accepted and a commonly used figure is 1.3 mg. of protamine for each 100 units of heparin. Osborn[26] suggested, when using 300 units of heparin/kg., giving 5 mg. protamine/kg. of body weight in 50 ml. of 5% glucose over 10 minutes, followed in one hour by a further 1 to 2 mg./kg. Castaneda[27] advised that instead of using protamine, reliance be placed on spontaneous return to a normal coagulation mechanism. Berger, Ramaswany and Ryan[23] reported improved hemostasis with lower doses of protamine; a single dose of 0.5 to 0.66 mg./kg. was used for each 100 USP units of heparin, with additional small amounts (25 to 50 mg.) if required.

Since, as indicated previously, there are many variables that will affect the length of time heparin remains in the circulation, it would seem preferable to determine the amount of protamine required for the individual patient by a protamine titration. As adapted by Perkins *et al*,[28] the procedure can be conducted in the operating room. Hawksley[29] reported that in 27 cases

in which this was done, the amount of protamine required was less than would be given by rule-of-thumb dosages. However, one limitation is that, for technical reasons, the protamine titration does not detect traces of heparin. Yet traces of heparin (as indicated in discussing the hemostatic balance) will have an unfavourable effect in the presence of a lowering of other hemostatic parameters. Rothnie and Kinmonth[30] found such traces were responsible for the postoperative oozing both in the experimental animal and in man after open-heart surgery. To detect traces of heparin, they used the thrombin clotting time with toluidine blue as heparin antagonist and injected additional protamine as indicated.

Conclusions

Protamine is still the chief agent available for neutralizing heparin. Applied to the titration of the anticoagulant action of heparin *in vitro,* it provides a useful means of diagnosis of hemorrhagic states. The amount of heparin present in the circulation is related to total dosage and the time interval from its administration, but depends markedly on the absolute concentrations reached in the blood and the hemodynamic and metabolic condition of the patient. Direct determination *in vitro* in the operating room of the amount of protamine required to neutralize the heparin present in the patient's blood can provide an equivalence figure applicable to the patient. (In addition, it may demonstrate the presence of such problems as rapid fibrinolysis.) Because of the variability in heparin preparations (probably related to difficulties of standardization), figures for the heparin equivalent of protamine preparations are of limited value. Therefore, it is wiser, when a titration on the blood is not done, to depend on a rule-of-thumb dosage such as not more than 1.0 mg./100 USP units of heparin. This is to be injected slowly in divided doses, stopping the injection when hemostasis is achieved. The amount should be reduced in proportion to the time which has elapsed since the last heparin administration (by about 1 mg./min. for the average patient). In using protamine to neutralize heparin after extensive and prolonged operations, no simple rule of protamine-heparin equivalence will suffice to ensure that hemostasis will always be instantly achieved. When hemostasis is not effected it is essential to appreciate the many contributing factors and assess their significance for the patient.

Résumé

La protamine, antagoniste de l'héparine

La protamine est employée pour titrer l'héparine *in vitro* dans le diagnostic des diathèses hémorragiques et pour neutraliser l'héparine *in vivo,* en vue de mettre fin à l'héparinisation. La dose de protamine varie avec la préparation d'héparine utilisée, les conditions particulières de l'essai et *in vivo,* avec la quantité d'héparine présente dans le sang circulant. Ce dernier facteur est fonction du laps de temps écoulé depuis l'administration de l'héparine et de l'état hémodynamique et métabolique du malade. Injectée rapidement, la protamine libère de l'histamine et agglutine les plaquettes. Le saignement (hémorragie spontanée) signe une altération des mécanismes de l'hémostase provoquée par une agression chirurgicale, la prise de médicaments, le contact du sang avec des corps étrangers, etc. Dans la majorité des cas, des règles très simples, basées sur le jugement clinique, permettent de connaître la dose de protamine nécessaire à un malade donné. Si l'hémostase n'est pas réalisée, il faudra songer que l'héparine et son antagoniste la protamine peuvent n'être, dans le cas en question, qu'une partie d'une situation pathologique complexe.

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