
Neutralization of Heparin Using a Protamine Titration Assay and the Activated Clotting Time Test

Frank LaDuca, Dennis Mills, Steven Thompson, and Karen Larson

Minneapolis Heart Institute
Minneapolis, MN
John Hopkins Hospital
Baltimore, MD
International Technidyne Corporation
Edison, NJ

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Abstract

(*J. Extra-Corpor. Technol.* 19 [3] p. 358–364 Fall 1987, 16 ref.) Many of the clinical protocols currently used to calculate the protamine (Pr) dosage for heparin neutralization following cardiopulmonary bypass (CPB) utilize an empirical Pr to heparin ratio. Pr titration, though a proven method of dosage calculation, is inherently tedious and thus not routinely used in the operating room (OR). In the present study Pr titration was performed by preparation of diatomaceous earth-activated clotting time (ACT) test tubes containing known amounts of lyophilized Pr. The ACT determined in these test tubes was called the P-ACT. The Pr titration curve was constructed using a status (heparinized) ACT (S-ACT) and P-ACT at Pr concentrations of 10, 15, 20 and 30 mcg/ml. The ACT was performed using an automated technique (Hemochron). The intercept of the linear regression dose-response neutralization curve (ACT vs Pr concentration) with the baseline ACT was the calculated Pr concentration required for neutralization. Addition of calculated Pr to in vitro heparinized donor blood normalized the ACT and non-activated clotting time (NACT). A two-point dose-response curve (S-ACT and P-ACT, 15 mcg/ml) produced equivalent results to the linear regression dose-response calculation ($r = .95$). In 30 CPB patients infusion of the calculated Pr (based on the two-point assay and the patient's blood volume, adjusted for changes during CPB) normalized the ACT and

produced clinical hemostasis. This assay provides a method of Pr quantification in the OR reducing the likelihood of over- or under-infusion of protamine and the adverse consequences associated with either.

Introduction

Protamine sulfate is used to neutralize the heparin infused during cardiovascular surgery procedures requiring cardiopulmonary bypass (CPB). The amount of heparin neutralized by protamine is in direct proportion to the amount of protamine present in the blood. Thus, titration methods can be accurately used to quantify the amount of protamine required to neutralize a given quantity of circulating heparin.

Clinically useful protamine titration methods were originally described by Allen et al.¹ and later modified by Perkins et al.² and Hurt et al.³ These protocols involved in vitro addition of varying amounts of liquid protamine to heparinized blood and determination of the amount of protamine required to normalize the whole blood clotting time. Recently Dutton et al.⁴ described a similar protamine titration methodology, using the Activated Clotting Time (ACT) test as the means to determine the amount of protamine necessary to neutralize heparin and normalize the ACT.

The ACT is the preferential method of heparin monitoring during CPB.^{5,6} Yet the clinical use of a protamine titration assay based on ACT technology has been limited due to the lack of a convenient and accurate assay. Many surgical teams continue to use one of several modifications of the Heparin Dose-Response Curve described by Bull et al.⁷ In this procedure, the patient's heparin concentration at the end of cardiopulmonary bypass is calculated based on an ACT

Direct communications to: Dr. Frank LaDuca, Research Division, International Technidyne Corporation, 23 Nevsky St. Edison, NJ 08820

determination. By converting this ACT into a heparin concentration, the protamine dose required to neutralize this heparin load is calculated using an empirical protamine to heparin ratio. In many clinical settings the ACT value is not used in determining protamine dose. Rather, protamine dosage is calculated based on a ratio of protamine to total heparin infused during the procedure.⁸⁻¹⁰ Another method of dose calculation used is an automated heparin-protamine titration assay which is independent of the ACT methodology and incorporates an empirical protamine to heparin ratio.⁶ Because of the pharmacology of protamine, however, it is important to quantify protamine dose and avoid the possible morbidity associated with under- or over-infusion of protamine.¹⁰⁻¹⁴ For these reasons, and since more accurate quantification of protamine dose can be achieved by using titration assays than by using empirical data, protamine titration is a preferred method of dose calculation.

This paper describes a convenient and accurate method for protamine titration using the principle of dose-response heparin neutralization as monitored by the automated ACT.

Materials and Methods

Protamine Titration Assay. Stock solution of protamine sulfate was prepared in 0.9% NaCl to a final concentration of 1 mg/ml. Protamine was added in varying amounts (10-160 mcl) to Hemochron^a brand test tubes (#H106) and lyophilized in a vacuum dryer. Diatomaceous earth, 12 mg (celite^b), was added to each tube, the tubes were evacuated (to draw 2.5 mls), capped, and stored at room temperature. Test tubes containing protamine and diatomaceous earth were designated P-ACT test tubes. The titration assay was performed by addition of exactly two mls of blood to each tube resulting in final protamine concentrations of 5 to 80 mcg/ml. Titration assays using liquid protamine were performed by addition of varying amounts of protamine (10-160 mcl) to plastic tubes. Blood was added in an appropriate volume producing final protamine concentrations of 5-80 mcl/ml. After mixing, 2.0 ml was transferred to a Hemochron ACT test tube (#CA510) and the ACT¹⁵ determined using standard Hemochron procedure. In some studies the non-activated clotting time was performed using #S412 test tubes.

For in vitro studies, blood was obtained by venipuncture from a normal donor. One aliquot was immediately transferred to a #CA510 test tube for determination of the baseline ACT, and a second ali-

quot immediately added to a plastic tube containing heparin (derived from either porcine intestine^c or beef lung^d) in final concentration of 1-5 units/ml. Following mixing, 2.0 ml was transferred to a #CA510 test tube, for determination of the status ACT (S-ACT), and to each P-ACT test tube previously prepared. The ACT and P-ACT values were determined using Hemochron Models 400 or 800. These values were graphed on standard graph paper (y-axis) versus the corresponding protamine concentration (x-axis).

The dose-response neutralization curve was constructed from the S-ACT to the baseline ACT by plotting the linear regression line of best fit among the P-ACT values falling within this range. The protamine value at the point of intercept of the neutralization curve with the baseline ACT represented the unit protamine concentration (UPC) required to normalize the ACT.

Protamine Index Factor: The heparin neutralizing capacity of the protamines used clinically were quantified against the protamine used in the titration assay. This was done by simultaneous determination of the dose-response neutralization curve of a heparinized blood specimen using both protamine preparations. For each protamine the UPC value was determined and the Index Factor (I_{PS}) calculated by the formula:

$$I_{PS} = \frac{\text{UPC (clinical protamine)}}{\text{UPC (reference protamine)}}$$

Clinical Studies: Patients ranged in age from 7 months to 74 years. The surgical procedures requiring CPB included: coronary artery bypass graft (CABG), heart valve replacement, combination CABG and valve replacement, repair of congenital septal defect and heart transplant.

Following determination of the baseline ACT, the patient was given a bolus heparin dose of 3 mg/kg (body weight). Heparin used was either porcine intestinal or beef lung. The ACT value was determined five minutes following the heparin infusion and the Heparin Dose-Response Curve⁷ constructed. The patient's blood volume (BV) was estimated from the height and weight.¹⁶ The adjusted blood volume (ABV) was calculated with the formula:

$$\text{ABV} = \text{BV} + \text{pump prime volume} + \text{cardioplegic volume.}$$

The Final ABV was calculated at the end of the procedure by the formula:

$$\text{Final ABV} = \text{ABV} - \text{residual volume left in pump.}$$

a International Technidyne Corp., Edison, NJ 08820
b Johns Manville Corp., Denver, CO 80201

c Eli Lilly and Co., Indianapolis, IN 46285
d The Upjohn Co., Kalamazoo, MI 49003

While on CPB the patient was infused with heparin to maintain the ACT at 480 seconds or greater.

Protamine titration was performed 20 minutes prior to patient removal from CPB (approximately 10 minutes after the start of patient rewarming). A 4.5 ml blood sample was obtained from a previously flushed, post-oxygenator, access port and 2.0 ml was added simultaneously to an ACT test tube (#CA510) and a P-ACT test tube. If the maintenance ACTs during CPB were 700 seconds or greater the P-ACT test tube used contained 20 mcg/ml protamine; if the maintenance ACTs were 699 seconds or fewer the P-ACT tube used contained 15 mcg/ml protamine. The ACT and P-ACT values were plotted and the dose-response neutralization curve constructed by drawing a line through the two data points to the intercept of the baseline ACT. The protamine concentration at the point of intercept was the UPC value. Protamine dose was calculated by the formula:

$$\text{Dose} = \text{UPC} \times \text{Final ABV} \times I_{PS}$$

As standard procedure this protamine value was compared with that value prescribed by the protocol normally used by the respective clinical team. Three different clinical protocols were used: A) Heparin Dose-Response,⁷ B) Empirical 1:1 ratio, C) Empirical 1.5:1 ratio.

Immediately following patient removal from CPB the calculated protamine dose was infused. Five minutes following the completion of the infusion a second 4.5 ml blood sample was obtained for verification of heparin neutralization by simultaneously performing an ACT and P-ACT test using a tube containing 5 mcg/ml protamine.

Results

Protamine Titration: In Vitro Studies

Dose-Response Neutralization Curve: The prolonged ACT of an in vitro heparinized blood specimen was shortened in a manner proportional to the amount of protamine added to the blood (Figure 1). The linear first phase of this dose-response neutralization curve was characterized by a shortening of the heparinized ACT value proportionally to the final protamine concentration. A protamine concentration was eventually obtained at which the P-ACT approximated the initial preheparinized baseline ACT. Further increases of protamine from this concentration resulted in a plateau of protamine-heparin inactivity characterized by little deviation of the P-ACT from the baseline value. At higher protamine concentrations a slight prolongation of the P-ACT was evident. The rate of heparin neutralization, proportional to the increase of prot-

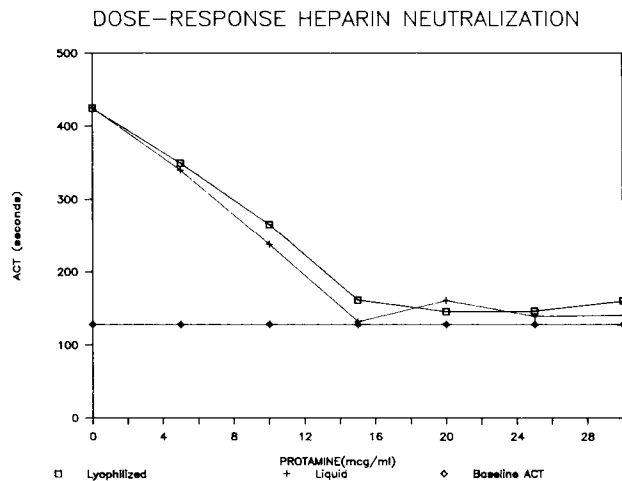


Figure 1: The neutralization of heparin is proportional to the amount of protamine added to the blood. Heparin neutralization is measured by the degree of shortening of the ACT from the prolonged value of the heparinized specimen. The Dose-Response Neutralization Curve is similar using either liquid or lyophilized protamine sulfate.

amine concentration was dependent on the source of donor blood and on the source of heparin (i.e., derived from beef lung or porcine intestine). The neutralization curve was similar using either liquid or lyophilized protamine titration procedures.

Extension of the linear first phase of the neutralization curve to the intercept of the baseline ACT value predicted the protamine concentration required to completely normalize the ACT. This protamine concentration, expressed in mg/ml, defined as the Unit Protamine Concentration (UPC), represented that amount required for heparin neutralization. The addition of this UPC to an aliquot of the heparinized blood resulted in normalization of the ACT and NACT.

The reproducibility of the linear first phase of the neutralization curve was evaluated by analysis of the variability of the UPC value determined by (A), a linear regression analysis of the dose-response neutralization curve using the heparinized ACT value and multiple P-ACT values (10, 15, 20, 30 mcg/ml) and (B) a regression analysis using only two data points (the heparinized ACT and a single P-ACT). Comparison of these values demonstrated similar UPC determinations by the two methods $r = .95$ (Figure 2).

Protamine Potency: Protamine solutions prepared from different manufacturer sources demonstrated a variable degree of heparin neutralization capacity as determined by the reduction of the prolonged ACT of an in vitro heparinized blood specimen. A dose-response neutralization curve was generated for each and the calculated UPC expressed as the fractional

ratio of the reference protamine (i.e., that protamine used for the titration assay). Among the protamines evaluated an Index Factor of 0.8 to 1.7 was determined. A ratio less than 1.0 indicates a protamine more potent than the control, an index greater than 1.0 indicates a lesser potent protamine.

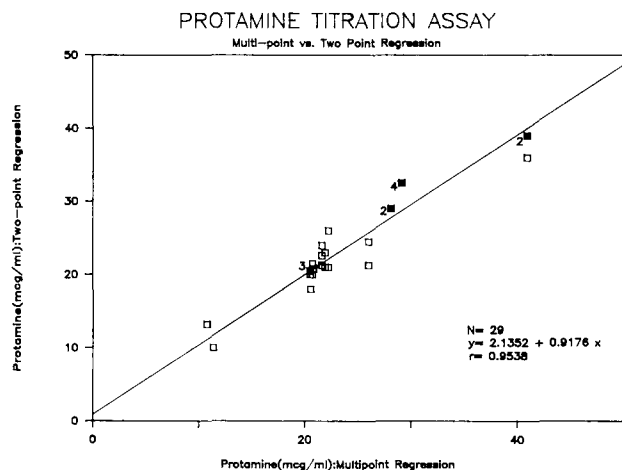


Figure 2: For individual heparinized blood specimens a neutralization curve was constructed. The UPC value was determined by two techniques: A. Linear regression analysis using the Status ACT and all P-ACT values, and, B. Regression analysis using the Status ACT and a single P-ACT. Each open symbol represents a single data point, the solid symbols represent multiple points, the corresponding Arabic numbers denote the number of data points. As determined from the coefficient of correlation, there was no difference of value determined by these two methods.

Clinical Studies

The titration assay was evaluated in 30 patients requiring CPB. The infusion dose was quantified by the formula described earlier in the text. In these patients the mean UPC (\pm standard deviation) was 0.023 ± 0.006 mg/ml of blood with a range of .013 to 0.04 mg/ml. The mean calculated protamine infusion dose (\pm standard deviation) was 210 ± 78 mg. The actual infusion dose was 203 ± 79 mg, reflecting the fact that 11 patients received from 2–9% less protamine than that calculated by the assay. Five minutes following completion of the total protamine infusion, the ACT was normalized in all patients. The mean post-infusion ACT was decreased 20 seconds from the baseline ACT. Clinical hemostasis was observed in the operative field.

In two patients the total protamine infusion dose calculated by the assay was administered in incremental infusions. Following each infusion, an ACT and P-ACT were performed to evaluate the need for

additional protamine. In these studies, with each infusion, the ACT shortened progressively from the pre-infusion value. At each blood sampling point an additional dose-response neutralization curve was constructed and the additional protamine quantified (Table 1).

The protamine dose quantified by the titration assay was less than that determined using either a Heparin Dose-Response Curve or an empirical protamine to heparin ratio (Figure 3). In individual patients the protamine titration dose was expressed as a percentage of the protamine dose calculated by these conventional methods (Table 2). The wide range of dosage percentage among this patient population indicated that the required protamine infusion dose as calculated by the titration assay was not directly proportional to infusion doses calculated by these other methods.

Discussion

An accurate protamine titration assay is described in which lyophilized protamine in standard amounts is added to Activated Clotting Time (ACT) test tubes containing a diatomaceous earth blood coagulation activator. The addition of blood at a fixed final volume serves to both rehydrate the lyophilized protamine and initiate celite activation of the clotting time test which is determined using an automated procedure (Hemochron). The ACT value determined in protamine containing tubes, called the P-ACT, is directly proportional to the extent of heparin neutralization in the patient's blood. In this manner the protamine concentration, expressed in mg per ml of blood, required to normalize the patient's prolonged heparinized ACT to a pre-established baseline ACT, is quantifiable. This unit protamine concentration (UPC) when multiplied by the patient's adjusted blood volume calculates the precise protamine dose required to neutralize the patient's heparin load. The adjusted blood volume represents the total system volume (patient plus extracorporeal circuitry) at the time of protamine dose quantification. Adequate protamine dose for neutralization includes that volume of the heparinized extracorporeal circuitry ultimately infused to the patient.

Complete heparin neutralization following protamine infusion is verified by normalization of the ACT value. A prolonged ACT following infusion indicates either inadequate heparin neutralization or surgically acquired coagulopathy (due to hemodilution¹⁵ or platelet destruction¹³). Differentiation of these physiologic states is achieved by determination of a second P-ACT following infusion. If this P-ACT is shortened from the simultaneously performed ACT value, addi-

tional protamine is indicated, if this P-ACT value is unchanged from the ACT a possible coagulopathy exists.

The protamine infusion dose calculated by this assay was less than that indicated by other conventional methods. Of utmost importance, however, is the observation that the protamine infusion dose is not universally proportional to these other methods. Thus it is not sufficient to attempt to neutralize all patients with 75% of the dose calculated by the Heparin Dose-Response Curve⁷ (Table 2) as individual patients may require from 32–108% of that dose. A finding of equal importance is that depending on the manufacturing source protamine may vary in heparin neutralizing potency. The full physiologic implications of this finding remain to be delineated but for the purposes of

this titration assay, the Index Factor (of potency) proved an important variable.

Our finding that approximately one-third of the patients required 2–9% less protamine than calculated is most likely due to a slight over-estimation of the adjusted blood volume. However, this minimal added protamine is insignificant in comparison to the calculated dose, and furthermore is still less than that calculated by other conventional methods.

The clinical importance of the application of this assay is that patient individualized protamine dose may be more accurately quantified and stringently controlled. By this method, the consequences of under- or over-infusion of protamine may be avoided. Insufficient infusion results in continued heparin-related bleeding.¹⁰ Excess protamine may result in bleeding,

Table 1
Sequential Protamine Titration Following Partial Dose Infusion

Case A—Female

Height 1.7 m Weight 112 Kg BV 5800 ml
 ABV 7300 ml Protamine Index 1.5
 Baseline ACT 166 Post heparin (3 mg/kg) ACT 340

Patient Status

**DOSE-RESPONSE
NEUTRALIZATION**

Protamine	S-ACT	P-ACT (mcg/ml)	UPC	Dose Calculation
Post CPB	707	458 (20)	.04	438 mg
250 mg	315	217 (5)	.013	142 mg
50 mg	221	173 (5)	.007	77 mg
100 mg	162			
Infusion Dose = 400 mg			Initial Calculated Dose = 438 mg	

Case B—Male

Height 1.8 m Weight 53 Kg BV 4800 ml
 ABV 7300 ml Protamine Index 1.0
 Baseline ACT 135 Post heparin (3 mg/kg) ACT 588

Patient Status

**DOSE-RESPONSE
NEUTRALIZATION**

Protamine	S-ACT	P-ACT (mcg/ml)	UPC	Dose Calculation
Post CPB	519	289 (15)	.025	182 mg
120 mg	219	152 (10)	.011	80 mg
60 mg	111			
Infusion Dose = 180 mg			Initial Calculated Dose = 182 mg	

Protamine infusion dose was calculated for two patients (Case A and Case B) by the standard technique. The initial calculated dose (indicated by Post CPB on the Table) was infused in partial increments, following which the Status ACT (S-ACT) and P-ACT was evaluated. With each successive infusion the Status ACT value demonstrated a greater degree of normalization. A new protamine dose was calculated from the dose-response curves constructed. As the total infusion dose approximated the initial calculated dose, the Status ACT was at or below the baseline value.

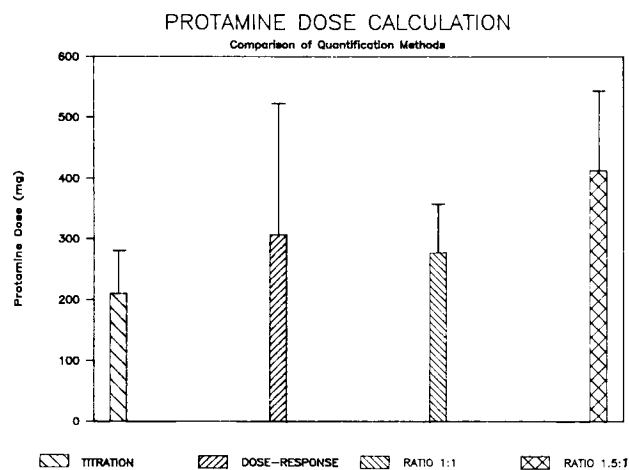


Figure 3: Protamine infusion calculated for 30 CPB patients using the protamine titration assay was compared to the infusion dose calculated by conventional methods. These included the Heparin Dose-Response Curve⁷ (Dose-Response), an empirical protamine to heparin ratio of 1:1 (Ratio 1:1), and an empirical ratio of 1.5:1 (Ratio 1.5:1).

Table 2

Comparison of the Protamine Titration Assay Dose Calculation and Conventional Protamine Dose Calculation Methods

	Conventional Method		
	Dose-Response	Ratio 1:1	Ratio 1.5:1
Mean ± SD	76 ± 22	76 ± 20	52 ± 15
Range	32 – 108	39 – 119	26 – 79

The protamine infusion dose for 30 CPB patients, calculated by the titration assay, is expressed as a percentage of the dose calculated by the Heparin Dose-Response Curve⁷ (Dose-Response) and by empirical protamine to heparin ratios (Ratio 1:1, Ratio 1.5:1).

severe hypotension, anaphylactic shock, cardiac decompensation, or in severe cases, death.¹¹⁻¹⁴ A further clinical consideration is that despite adequate heparin neutralization in the OR the CPB patient requires continuous coagulation monitoring postoperatively.^{5,6}

In summary, the protamine dose calculation assay described in this report represents a convenient and accurate method of protamine quantification. The assay is readily performed using the ACT technology available with the Hemochron instrument. Protamine dose calculation provides a means to individualize prot-

amine infusion and help avoid the adverse consequences associated with insufficient or excess protamine infusion.

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Question from the Audience

Question: Al Adams, Daphne, AL: Maybe I misunderstood you. But did you say you calculated the blood volume on the solution in the pump and the cardioplegic solution? Have you missed something here? How about all the flush solution that the anesthesiologists use? How about all the fluid that is added up at the table? And how about all the urinary output? Did you take those into consideration?

Response: We did initially. We studied all our patients and we did just that. There are a lot of perfusionists that are angry that it took all the time to do that. But we measured anesthesia input, urine output, blood losses, everything that was added. What it came down to is if we simplified the format to this formula we got a dose which was efficacious and we got a volume which was nearly equitable in all those instances. In other words you could ignore all these other things and come up with a reasonable solution. And what we are working on now is to eliminate even the cardioplegia addition to that.