Comparative effects of the human protein C activator, Protac, on the activated partial thromboplastin clotting times of plasmas, with special reference to the dog

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Abstract

The commercial snake venom extract, Protac, is a specific activator of the anticoagulant zymogen, protein C (PC) in human plasma. This specific action has led to its use in developing coagulation-based and amidolytic-based assays for the diagnosis of quantitative and/or qualitative PC deficiency states in human beings. The purpose of the present study was to compare the effects of Protac on the activated partial thromboplastin times (APTT) of human, bovine, equine, and canine plasmas in order to determine the potential value of this venom extract as an activator in functional PC assays in these domestic animal species. As expected, Protac significantly prolonged the APTT of normal human plasma, but had no effect on plasma known to be devoid of PC. Clotting times were prolonged by 34%–214% with concentrations of venom activator ranging from 0.1–1.0 U/mL. Under identical conditions, Protac prolonged the APTT of equine plasma by 11%–98% over control times. Even more dramatic was the inhibitory effect of Protac on the clotting of bovine plasma, extending the APTT more than 3-fold at a venom concentration of 0.1 U/mL. At higher venom concentrations, most bovine plasmas remained unclotted after 300 s (control time 34.1 s). Under similar conditions, the canine APTT was unaffected by Protac, even when the venom concentration was increased to 3 U/mL. In order to determine the reason for the lack in response of canine plasma, the concentration of the APTT reagent was altered (decreased), exposure time of the plasma to the Protac was increased from 2 min to 9 min, and the plasma was diluted to assess for the potential existence of plasma PC inhibitors. Protac caused an unexpected shortening of the APTT when the contact activator reagent was diluted. Increasing the exposure time had no effect. Although a slight prolongation of the canine APTT was detected when the plasma was diluted, the presence of strong plasma PC inhibition was considered an unlikely cause of the lack of significant anticoagulant action. The failure of Protac to exert a strong inhibitory effect on the canine APTT, as well as to generate amidolytic activity, suggests that this venom extract does not stimulate the production of activated PC activity in canine plasma. This may result from molecular differences in the canine PC molecule that prevent the formation of the stoichiometric complex of venom extract, APTT reagent, and canine protein, a complex thought to be essential for the PC-activating function of Protac. Protac may be suitable as an activator of PC in bovine and equine plasmas; however, it appears ineffective in generating anticoagulant activity in canine plasma.

Résumé

Un extrait commercial de venin de serpent, le Protac, est un activateur spécifique de la protéine C (PC) laquelle est un zymogène anticoagulant retrouvé dans le plasma humain. Cette action spécifique a entraîné son utilisation dans le développement d'épreuves de coagulation et amidolytiques afin de diagnostiquer quantitativement et qualitativement chez l'humain des déficiences de PC. Afin d'évaluer chez différentes espèce animales le potentiel d'utilisation de cet extrait de venin comme activateur fonctionnel dans des épreuves mesurant la PC, les effets du Protac sur les temps de thromboplastine partielle activée (APTT) de plasma humain, bovin, et équin furent comparés. Tel qu'attendu, le Protac a prolongé de façon significative l'APTT du plasma humain normal, mais n'a eu aucun effet sur les plasmas ne contenant pas de PC. Une augmentation de 34 % à 214 % du temps de coagulation fut observé avec des concentrations de venin variant de 0,1 à 1,0 U/mL. Dans des conditions identiques, le Protac a prolongé l'APTT du plasma équin de 11 % à 98 % par rapport aux temps témoins. L'effet inhibiteur du Protac sur la coagulation du plasma bovin fut encore plus marqué, alors qu'une concentration de 0,1 U/mL a multiplié par trois l'APTT. À des concentrations de venin plus élevées et après 300 sec, la plupart des plasmas de bovin sont demeurés non coagulés (temps témoin : 34,1 sec). Dans des conditions similaires, l'APTT d'échantillons canins ne furent pas affectés par le Protac, même à des concentrations de venin de 3 U/mL. Afin de déterminer la raison de l 'absence de réponse du plasma canin, la concentration du réactif de l'APTT

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fut diminuée, le temps d'exposition du plasma au Protac fut augmenté de 2 à 9 min, et le plasma fut dilué pour évaluer l'existence possible d'inhibiteurs de PC plasmatique. Une réduction de l'APTT par le Protac fut notée lorsque le réactif de l'activateur fut dilué. Aucun effet ne fut observé suite à une augmentation du temps d'exposition. Chez le chien, bien qu'une légère prolongation de l'APTT fut observée lorsque le plasma était dilué, la présence d'inhibiteurs de PC n'était pas considérée comme une cause significative de l'action anticoagulante. L'échec du Protac à avoir inhiber fortement l'APTT canin, de même que son manque d'activité amidolytique, laisse à croire que cet extrait de venin ne stimule pas la production de PC activé dans le plasma de chien. Ceci peut être du à des différences moléculaires dans la molécule de PC d'origine canine qui empêcherait la formation du complexe entre l'extrait de venin, le réactif de l'APTT, et la protéine canine, ce complexe étant jugé essentiel à la fonction d'activation de PC du Protac. Le Protac serait adéquat comme activateur de PC du plasma bovin et équin; toutefois, il semble inefficace à générer une activité anticoagulante dans le plasma de chien.

(Traduit par docteur Serge Messier)

Introduction

The commercial product Protac is a single-chained glycoprotein with a molecular weight of 36 kD that is derived from the venom of the southern copperhead snake, *Agkistrodon contortrix contortrix*. This specific serine protease has found broad application in blood coagulation research and diagnosis as an activator of the anticoagulant protein C (PC) in human plasma (1,2). Through this activation, Protac causes a marked prolongation of the activated partial thromboplastin time (APTT) of human plasma (3,4). This inhibition of the intrinsic clotting pathway appears to involve complex interactions between Protac, plasma, and the phospholipid/contact activator components of the APTT reagents forming a stoichiometric complex that exerts PC activity. It has been suggested that although the prolonging effect of Protac on the human APTT is PC-dependent, the snake venom extract actually interferes with the contact mechanism of the intrinsic clotting pathway (2,3,5,6). The biochemical mechanisms of this interference in human plasma remain unknown (5).

Although the anticoagulant effect of Protac on human plasma is well documented, there is little information available regarding the effect of Protac on the intrinsic clotting mechanism of plasmas from non-human species, particularly the dog.

At sites of vascular injury, the generation of thrombin serves to activate platelets and clot fibrinogen, producing a stabilized hemostatic plug. Excess thrombin is washed downstream where it interacts with thrombomodulin (TM), a proteoglycan in intact endothelium (7). The complexing of thrombin with TM neutralizes thrombin's procoagulant and platelet-stimulating activities. This attenuation of thrombin's prothrombogenic actions is also facilitated by antithrombin III (ATIII) and vascular heparin proteoglycans (4). The thrombin-thrombomodulin complex (TTC) plays a major role in the activation of the vitamin K-dependent anticoagulant, protein C (PC). The activated form of PC (aPC) inhibits the action of activated Factor V and Factor VIII, delaying fibrin formation. The action of aPC in cleaving and inhibiting Factor V and Factor VIII is catalyzed by the cofactor, protein S (PS), which is located on cell surfaces (8). In addition, aPC can stimulate fibrinolysis, and appears to inhibit the inflammatory response (8–10). Although PC activation can be initiated slowly by thrombin alone, the process is greatly accelerated when thrombin complexes with TM.

The PC-PS-TTC anticoagulant system is now considered to be a key physiological regulator of blood coagulation and is thought to be critical in "sensing" the degree of injury to a blood vessel by applying an appropriate negative feedback response to prevent unnecessary extension of a blood clot (4,10).

Deficiencies in the PC-PS-TTC system cause predisposition to thrombotic disease (8,10). Assessment of the functional integrity of this anticoagulant system is, therefore, important in the detection and characterization of such disorders. Both quantitative and qualitative deficiencies of PC and PS have been described in man (2,8,10). A common cause of failure of the human PC-PS-TTC anticoagulant system is aPC resistance, a qualitative deficiency recently attributed to a genetic defect in the FV molecule (FV Leiden) which prevents aPC from binding to, and inactivating, Factor V (7,13,14). Quantitative deficiencies of PC have also been described in dogs with nephrotic syndrome and/or disseminated intravascular coagulation (15,16), and in horses (17) with thrombotic disease.

Like most hemostatic factors, PC can be assessed by both immunological and functional assays. Although there is normally good correlation between these 2 methods of evaluation, the relationship between protein concentration (PC antigen) and protein function may be lacking in diseased states (13,18,19). It is likely that the in vivo activity of the protein is more accurately reflected by functional assays than by immunological assays (3,18). Functional tests require the activation of PC to aPC, with the latter being recognized either by its ability to inhibit clot formation in a coagulation assay (anticoagulant effect), or by its action on a synthetic chromagen (amidolytic action) (9). A major problem in developing qualitative human PC assays has been to induce rapid activation of PC in order to minimize the effects of naturally occurring plasma PC inhibitors. With slow activators such as thrombin, pre-treatment of plasma was required to remove this inhibitory effect (3,6,15). Attempts to increase the rate of activation of human PC using TTM complexes have been unsuccessful (3,20). The identification of the snake venom PC activator, however, has dramatically increased the reliability of the human PC assay, as this agent rapidly and specifically activates human PC, therefore minimizing any influence of natural plasma PC inhibitors (2,3,21).

The purpose of the present study was to examine the anticoagulant effect of Protac on the plasmas of several domestic animal species, with the specific aim of ultimately developing an APTT-based

assay for the assessment of PC activity in animal plasmas, in particular, canine plasma.

Materials and methods

Plasma samples

Plasma samples were obtained from citrated blood (1:9, 3.8% citrate:blood) collected from clinically normal adult dogs, horses, and cows. The dogs (a variety of breeds), horses (standardbred or thoroughbred horses) and cows (Holsteins) were housed at the University of Guelph. The blood collection was performed in accordance with the guidelines set by the Canadian Council on Animal Care, and was approved by a committee concerned with ethical review at the University of Guelph. The human plasmas were lyophilized, normal reference plasmas obtained from several different companies (Organon Teknika Corp, Durham, North Carolina, USA; Baxter Dade, Miami, Florida, USA; Bio/Data Corp, Hatboro, Pennsylvania, USA; Sigma Diagnostics, St. Louis, Missouri, USA; Diagnostica Stago, Asnières, France). Human protein C deficient plasma was obtained from Sigma Diagnostics. In accordance with the manufacturers' instructions, human plasmas were reconstituted in distilled water. Plasmas were stored in multiple aliquots at −20°C or −60°C for up 2 mo or 10 mo, respectively, prior to use. The plasmas were thawed rapidly at 37°C immediately before use, and were kept on ice.

Reagents and equipment

The snake venom PC activator, Protac (American Diagnostica Inc., Greenwich, Connecticut, USA) was reconstituted in distilled water at a stock concentration of 1 U/mL, and was stored at −20°C. Dilutions of the stock solution were made in distilled water.

The cephaloplastin/contact activator used for the activated partial thromboplastin time (APTT) assays was a rabbit brain preparation containing ellagic acid as the soluble contact activator (Dade Actin; Dade Behring Inc, Newark, Delaware, USA). This APTT reagent was either used undiluted, or diluted in Tris-buffered saline (TBS), pH = 7.4 (diluted APTT reagent).

All clotting tests were carried out in duplicate at 37°C by using a coagulometer (Coag-a-Mate XM; Organon Teknika).

Effect of Protac on the APTT assay in different species

To determine Protac's effects on the intrinsic coagulation mechanism (APTT) in different species, 6 human, 7 equine, 7 bovine, and 7 canine plasmas were assessed. The APTT assay was performed as follows: 75 μL of plasma was mixed with 75 μL of APTT reagent in a prewarmed coagulometer cuvette. After 1 min, 75 μL of Protac (1.0 U/mL, 0.5 U/mL, or 0.1 U/mL concentrations), or 75 μL of distilled water (untreated control plasma), was added. Following a further 2-minute incubation at 37°C, clotting was triggered by the addition of 75 μL $CaCl_2$. All plasmas were assayed in duplicate and the clotting times were recorded to a maximum of 300 s. The effect of Protac on the APTT was expressed as the ratio of the clotting time for the Protac-treated plasma to that for the untreated control plasma (Protac/control ratio).

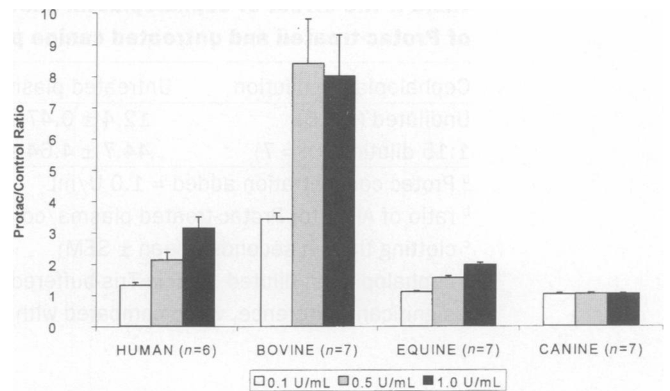

Figure 1. The ratio of the activated partial thromboplastin time of Protac-treated plasma to that of untreated plasma (Protac/control ratio) in man, horse, cow, and dog. Results are shown as mean ± standard error of the mean. The Protac concentration shown is the concentration added to equal volumes of cephaloplastin (undiluted) and plasma, 2 min prior to recalcification with $CaCl_2$.

Effect of cephaloplastin/contact activator concentration on the action of Protac on canine plasma

To ascertain whether or not the concentration of cephaloplastin/contact activator (APTT reagent) used in the assay influenced the interaction of Protac with canine plasma, 7 plasma samples were assayed as above, except that the APTT reagent was diluted 1:15 in TBS before use (diluted APTT reagent). The Protac concentrations used were 1.0 U/mL, 0.5 U/mL, and 0.1 U/mL, with a 2-minute Protac-plasma incubation period.

Four dog plasmas were incubated with Protac before being exposed to the diluted APTT reagent, in order to determine if pre-exposure of plasma to the cephaloplastin/contact activator affected the interaction of Protac with canine PC. In this modification of the APTT, 75 μL of canine plasma and 75 μL of Protac (1.0 U/mL) were pre-incubated for 2 min at 37°C before the addition of 75 μL of the 1:15 cephaloplastin/contact activator solution. After a further 3-minute incubation, 75 μL of $CaCl_2$ was added to initiate clotting.

Effect of increased Protac exposure time on the APTT of canine plasma

Four canine plasma samples were subjected to an increase in the exposure time to Protac in order to determine the effect on the APTT. The assay was performed as above except that the diluted APTT reagent/plasma mixture was incubated with Protac (or distilled water) for 9 min, instead of 2 min, prior to recalcification. Protac concentrations of 1.0 U/mL and 0.5 U/mL were used.

Effect of plasma dilution

In order to determine whether or not PC inhibitory activity was present in canine plasma, 8 samples were assayed as described above, but using 75 μL of plasma diluted 1:8 in TBS. The APTT results were compared in Protac-treated (1.0 U/mL) and untreated canine plasmas.

All data were expressed as mean ± standard error of the mean. Statistical significance between Protac-treated and untreated groups

Table I. The effect of cephaloplastin dilution on the activated partial thromboplastin time of Protac-treated and untreated canine plasma

Cephaloplastin dilution	Untreated plasma	Protac-treated plasma[a]	P/C ratio[b]
Undiluted ($n = 6$)	12.4 ± 0.47 s[c]	12.7 ± 0.26 s	1.03 ± 0.029
1:15 dilution[d] ($n = 7$)	44.7 ± 4.64 s	39.7 ± 3.43 s[e]	0.90 ± 0.043

[a] Protac concentration added = 1.0 U/mL
[b] ratio of APTT for Protac-treated plasma/control (untreated) plasma
[c] clotting time in seconds (mean ± SEM)
[d] cephaloplastin diluted 1:15 in Tris-buffered saline
[e] significant difference, when compared with clotting time of untreated plasma ($P < 0.05$)

was determined using the Student's *t*-test for paired data. Differences were considered significant when $P < 0.05$.

Results

Comparative effects of Protac on the APTT assay

The effect of Protac on the APTT of normal human plasma compared to its effect on the APTT of plasmas from the domestic animal species is shown in Figure 1. The APTT of human plasma was significantly prolonged from a mean of 48.5 s to means of 63.8 s, 96.6 s, and 147.3 s, with Protac concentrations of 0.1, 0.5, and 1.0 U/mL, respectively. The ratio of the clotting time in the Protac-treated plasma to that for the untreated plasma (Protac/control ratio) ranged from 1.34 with the lowest concentration of Protac, to 3.14 with the highest concentration. The APTT of human PC-deficient plasma was 39.2 s in Protac-treated (1.0 U/mL) plasma, compared to 39.6 s in untreated plasma.

The APTT of equine plasma was consistently prolonged following exposure to Protac. The mean clotting time was significantly prolonged compared to the untreated control, with even the lowest Protac dose ($P = 0.0004$). The Protac/control ratios for equine plasmas increased from a mean of 1.11 to a mean of 1.98 as the Protac concentration was increased (Figure 1).

The APTT of bovine plasma was even more dramatically prolonged by Protac. At a concentration of 0.1 U/mL, Protac significantly increased the average bovine APTT from 34.1 s to 117.2 s (Figure 1). At the higher Protac concentrations, the majority of the bovine plasmas failed to clot in less than 300 s, and Protac/control ratios in individual bovine samples ranged from 2.36 to 14.63. Because of this pronounced inhibitory effect of Protac on the bovine APTT, a dose of 0.01 U/mL was also used. Even at this low concentration, Protac still prolonged the clotting time by 27%, extending the average APTT from 34.1 s to 44.2 s ($P = 0.002$).

The APTT of canine plasma was not significantly altered by exposure to any of the Protac concentrations used. The mean clotting times for canine plasmas exposed to Protac concentrations of 0.1, 0.5, and 1.0 U/mL were 12.6 s, 12.7 s, and 12.7 s, respectively (compared with 12.4 s for the untreated dog plasma). The Protac/control ratios for these 3 snake venom concentrations were essentially the same (1.03, 1.04, and 1.03, respectively) (Figure 1). Since Protac did not appear to affect the APTT of canine plasma, the experiments were repeated using a higher dose (3.0 U/mL). Even at this concentration, the resultant Protac/control ratio was only 0.97, indicating no significant prolongation (anticoagulant effect).

Effects of cephaloplastin/contact activator concentration on Protac's effect on the canine APTT

As shown in Table I, decreasing the concentration of APTT reagent increased the mean APTT of untreated canine plasma from 12.4 s to 44.7 s. Diluted cephaloplastin caused an unexpected shortening of the APTT in the Protac-treated plasma (1.0 U/mL) compared with the untreated plasma, with this difference being statistically significant ($P = 0.042$). Pre-incubating the canine plasmas with Protac prior to exposure to the diluted APTT reagent had no effect on the APTT of Protac-treated canine plasma. The mean APTT of the Protac-treated plasma was 22.3 s, compared with 21.8 s for the untreated plasma.

Effects of Protac exposure time on the APTT of canine plasma

The effect of increasing the exposure time of canine plasma to Protac, from 2 min to 9 min, is shown in Table II. Although the prolonged incubation time reduced the APTT, the difference in the clotting times between the Protac-treated and untreated canine plasmas was not significantly different at either of the Protac concentrations used.

Effects of plasma dilution

Diluting the canine plasmas 1:8 with TBS caused a slight, but statistically significant prolongation ($P = 0.021$), of the Protac-treated plasma when compared to the untreated plasma (Table III). The Protac/control ratio for diluted plasma was 1.09 compared with a ratio of 0.91 for the undiluted plasma ($P = 0.0005$).

Discussion

In the present study we confirmed that Protac, the commercial snake venom activator of human protein C, prolonged the APTT of normal human plasma in a dose-dependent manner over the range of concentrations used, and that this anticoagulant effect was absent in human plasma known to be devoid of protein C.

The effects of Protac on the intrinsic clotting times of plasmas from the domestic animal species investigated differed significantly. The coagulation times of bovine plasmas were markedly prolonged following exposure to Protac. Regardless of the concentration used,

Table II. The effects of increased exposure time to Protac on the activated partial thromboplastin times of canine plasma

		Protac-treated plasma	
Exposure time	Untreated plasma	1.0 U/mL[a]	0.5 U/mL
2 min	44.7 ± 4.64 s[b]	39.7 ± 3.43 s	41.5 ± 4.42 s
9 min	18.6 ± 2.26 s	20.0 ± 3.49 s	18.3 ± 3.2 s

[a] concentration of Protac added

[b] clotting time in seconds (mean ± SEM)

Table III. The effect of plasma dilution on Protac-induced changes in the activated partial thromboplastin times of canine plasma (n = 8)

	Untreated plasma	Protac-treated plasma[a]	P/C ratio[b]
Undiluted plasma	39.6 ± 1.42 s[c]	35.6 ± 0.79 s	0.91 ± 0.041
1:8 plasma dilution[d]	32.5 ± 1.31 s	35.5 ± 1.69[e]	1.09 ± 0.035

[a] Protac concentration added = 1.0 U/mL

[b] ratio of APTT for Protac-treated plasma/control (untreated) plasma

[c] clotting time in seconds (mean ± SEM)

[d] plasma diluted in tris buffered saline

[e] significantly different, compared with clotting time of untreated plasma ($P < 0.05$)

the venom extract exerted a much greater anticoagulant affect on bovine plasma than on human plasma. This high sensitivity of bovine plasma to the anticoagulant effect of Protac was also observed in a bovine plasma sample examined by Stocker et al (3,4). In the present study, the degree of prolongation of the equine APTT induced by Protac was quantitatively more comparable to that observed with human plasma than to that seen with bovine plasma. Stocker et al (4), however, reported the failure of a similar concentration of Protac to prolong the APTT of a single equine plasma. This contradiction in the effect of Protac on equine plasma may be due to differences in population size studied or to differences in the method of blood collection. In the present study, there was consistent prolongation of the APTT in all horse plasmas studied. These plasmas were derived from freshly collected blood samples obtained by clean venipuncture from healthy adult donors, as compared to blood collected from a slaughtered animal as described by Stocker et al (4). Barton et al (22) and Prasse et al (23) have reported the successful use of Protac as an activator in an amidolytic assay for equine PC, supporting our conclusion that Protac can, in fact, generate aPC-like activity in horse plasma.

The results of the present study indicate that, in contrast to its effects on human, bovine, and equine plasmas, Protac does not exert any detectable anticoagulant effect on canine plasma tested under similar conditions. This lack of effect did not appear to be related to decreased sensitivity to the venom extract, as tripling the concentration of the venom or prolonging the exposure time of the plasma to the venom had no effect. Diluting the concentration of contact activator used in the canine APTT assay resulted in Protac actually causing an unexpected shortening of the clotting time. The reason for this shortening of the canine APTT, in the presence of reduced contact activator, is unclear; however, it could reflect other (procoagulant) properties of the venom. Venom-induced activation of prothrombin has been described in human plasma after exposure for 15–30 min (9). The shortened APTT clotting time observed with a diluted APTT reagent might also be related to enhancement of the contact mechanism by Protac (5).

When both the APTT reagent and plasma were diluted, this enhancement of intrinsic clotting by Protac was lost, and a slight, but statistically significant, prolongation of the canine APTT was observed. Although the degree of prolongation of the APTT was minimal, this observation could suggest that by diluting plasma we are removing the effects of weak canine plasma PC inhibitors. Stocker et al (4) reported that Protac also failed to prolong the intrinsic clotting time in porcine plasma, and suggested that this lack of anticoagulant effect reflected the presence of high concentrations of protein C inhibitors or anti-activators, since Protac did generate PC-related amidolytic activity when pig plasma was diluted. The fact that dilution of canine plasma caused only a slight prolongation of the canine APTT, and the determination that Protac failed to generate significant PC-like amidolytic activity in canine plasma (unpublished data), suggests that it is unlikely that a plasma PC inhibitor is the primary cause of the failure of the venom extract to prolong the canine APTT. Stocker et al (3,4) have suggested that the interaction between Protac, the APTT reagents, and plasma to form a stoichiometric complex with PC activity is a species-specific reaction. The present results may reflect the effect of changes in APTT contact activation conditions and a resulting failure of the venom extract, APTT reagents, and canine plasma to interact in a manner that produces a stoichiometric complex with strong aPC activity.

Protac has been described as a site-specific molecule whose action relies on the specificity of the interaction between its activation site and that of the PC molecule (1,4). Although Leeb et al (24) reported that canine PC was approximately 72% identical to the human protein, the genomic variations that do exist may account for the apparent differences in the ability of Protac to activate the human and canine proteins. Additional studies are needed to define the molecular interactions between Protac, APTT reagents, and canine plasma.

Coagulation-based PC assays have an obvious advantage over amidolytic PC assays in that the former rely on the natural substrates (Factor Va and/or Factor VIIIa), and can, therefore, potentially detect variant forms of PC deficiency that may exhibit normal amidolytic activity (25,26).

A major objective of this study was to assess the potential value of Protac as an activator in an APTT-based coagulation assay for PC activity. Protac appears to offer potential as an activator in equine and bovine coagulation-based PC assays, as it induces anticoagulant activity in plasmas from these species. The apparent failure of Protac to prolong the canine APTT and to generate significant amidolytic activity in canine plasma, however, indicates that this specific venom extract is unlikely to be useful in either coagulation-based or amidolytic assays for canine PC.

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