Chapter 5 -

Comparative analysis of the concentration of certain coagulation factors between controls, aspartame treated rabbits and humans

RESEARCH QUESTION 3:

Is there a difference between the blood clotting time, coagulation profile and different coagulation factors of the control and aspartame treated groups and how does these values compare to that of humans?

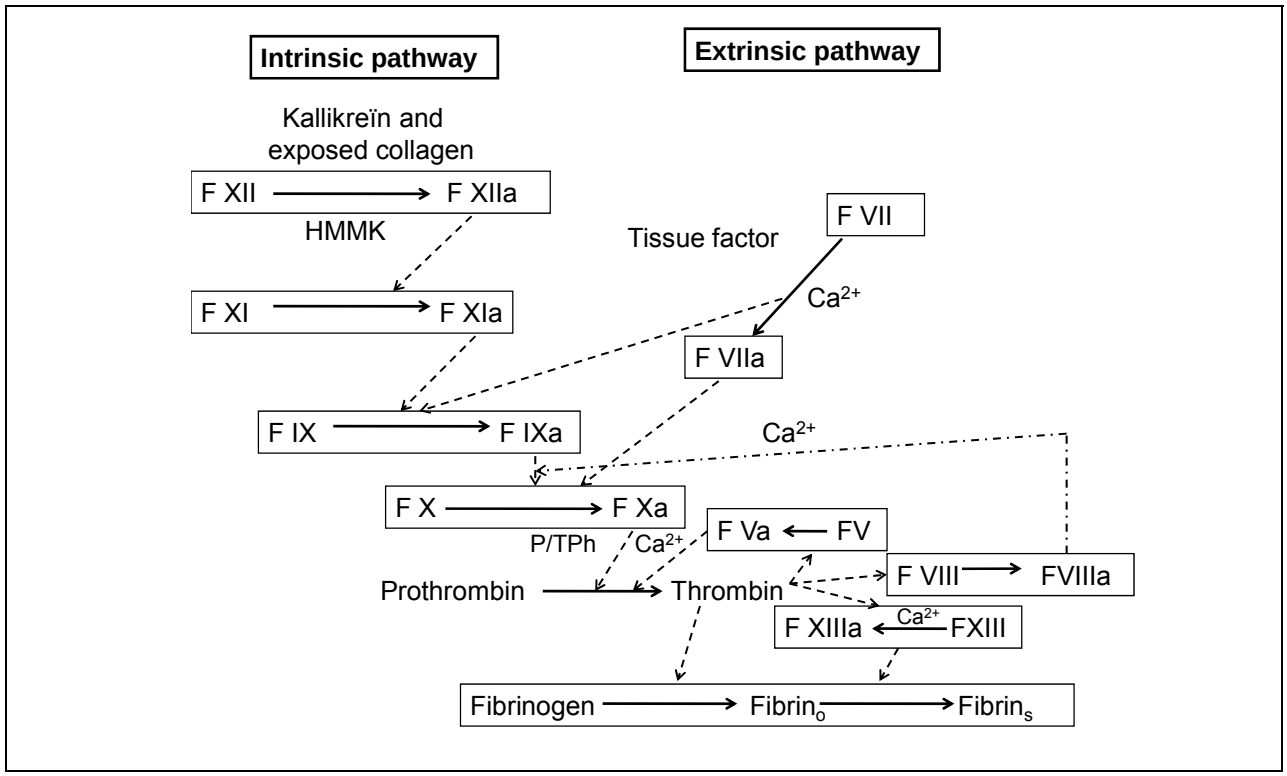
It was determined previously that the rabbit could indeed be used as a successful experimental animal model. This chapter will focus on techniques whereby coagulation factors were studied by measuring the change of coagulation factors due to the intake of aspartame, also defined as metabolomics. Metabolomics aims at measuring all metabolites present in an organism qualitatively and quantitatively; in studies of pathogenesis analysing materials such as urine and serum, this is referred to as metabonomics (Verpoorte *et al*., 2005). However, to obtain statistically sound results when testing a pharmaceutical product, it is compulsory to establish normal ranges and determine whether these results compare to that of the rabbits in the treatment groups, leading us into this chapter.

5.1 INTRODUCTION

Coagulation and haemostasis are important processes in the human body and the haemostatic system involves primary haemostasis (platelet aggregation), secondary haemostasis (coagulation) and tertiary haemostasis platelet (fibrinolysis) (Stassen *et al*., 2004). During the coagulation process, activation cascades of various factors and proteins in the intrinsic and extrinsic pathway play and important role (Troy, 1988; Kalafatis *et al*., 1997) and different proteins are involved, including von Willebrand's factor, fibrinogen, fibronectin, and specific glycoprotein receptors on platelet surfaces (Troy, 1988; Hawiger, 1987). Fibrinogen and calcium are also required for successful aggregation, while thrombin is important for formation of fibrin and for exposure of platelet receptors for adhesive molecules and for "activation of factors V and VIII" (Ofosu *et al*., 1996; Torbet, 1995). The contact of factor XII with a negatively charged surface, e.g. collagen, activates the intrinsic system. The common pathway is propagated through a series of reactions with prekallikrein, high-molecular weight kininogen (HMWK), and factors XI, IX, VIII. The common pathway and factor IX in the intrinsic pathway are stimulated by the extrinsic, or tissue factor system (Troy, 1988; Lammle and Griffin, 1985). The fact that the intrinsic pathway is stimulated by factor VII in the extrinsic pathway has inspired reassessment of the biological importance of the extrinsic system. The common pathway includes factor X and V and causes thrombin to convert fibrinogen to fibrin. Calcium and

platelet phospholipids are substances that have vital roles in steps in the coagulation scheme. Once fibrin is formed, factor XIII interacts with the substance, providing a stabilizing effect (Troy 1988; Mcvey, 1999)(Diagram 5.1).

Diagram 5.1: Coagulation pathway in the human



HMMK – High molecular mass kininogen; ⟶ - conversion of inactive protein to active enzyme; - - -> Active enzyme as cofactor for activation of inactive protein; ·····> - active factor VIII working as cofactor on active factor IX to activate factor X; P/TPh – platelet or tissue phospholipids; $Fibrin_o$ – unstable fibrin; $Fibrin_s$ – stable fibrin. Diagram adapted from Meyer and Meij, 1996.

Because the various proteins and factors involved in the coagulation process in humans is such a complex process and sometimes difficult to study, we investigate here the suitability to use an animal model. Traditionally, mice are used as animal model. However, a few studies have used rabbits, and from these results it seems as if results from rabbits compare well with those of humans. However, studies of rabbit values compared to that of humans are limited. Clarke and co-workers studied factor VII antigen and factor VIIa in the plasma of 36 normal rabbits Clarke *et al*. in 2003. The concentrations for the factor VII antigen ranged between 38 -198% in

the 36 normal rabbits, with a mean value of 766ng/ml plasma and these values correlate with those found in humans (Howards *et al.* 1994). The quantitation of activated FVII has not been described previously in rabbit plasma. On average, 1.1% of rabbit plasma FVII exists as FVIIa and its plasma concentration varied over 15-fold range in normal rabbits (Clarke *et al.* 2003). These values also correspond with data published for FVIIa concentration in human plasma (Morrissey *et al.,* 1993).

Noguchi *et al*. in 1989 performed a comparative study for measuring condition of activated partial thromboplastin time and contact factors in experimental animals. For establishing the optimal incubation time (OIT) for measurement of activated partial thromboplastin time (aPTT), they determined the shortest clotting time of the plasma from dogs, rabbits, guinea pigs, rats and mice. These findings were compared with that of human plasma. The OIT for aPTT determination was 15 – 30 seconds in guinea pigs, rats and mice and 5 – 10 minutes in dogs and rabbits. The mouse aPTT (about 30 seconds) with the OIT thus determined was similar to human aPTT, and relatively longer than aPTT in other animal species (10-20 seconds). The plasma of each animal species was tested for clarification of the mechanism for species differences in OIT, Noguchi et al. examined for the activity of the contact factors XII, XI, high molecular weight kininogen (HMWK) and prekallikrein (PK) and their effect on the coagulation of contact factor-deficient plasma. The total activity of contact factors was higher in dogs and guinea pigs and lower in rabbits and mice than that in humans. Species difference was noted in clotting time but not in OIT for factor XII, XI and HMWK. Due to the difference in activity of the plasma contact factors and the mode of coagulation for each contact factor there is a probable difference in OIT for aPTT in the different species.

Thus, the aim of this chapter was therefore to obtain a possible answer for the research question leading this part of the study: *Is there a difference between the blood clotting time, coagulation profile and different coagulation factors of the control and aspartame treated groups and how does these values compare to that of humans?*

Thus, this chapter focused on a comparison between the control and aspartame treated groups and to compare concentrations of different coagulation factors between the human and rabbit.

Specific attention was given to factors associated with the common pathway, namely factors II, V, X and fibrinogen; factors in the intrinsic pathway, namely Factors VIII, IX as well as factor VII, found in the extrinsic pathway. The *prothrombin time* (PT; measures how long blood takes to form a clot) and activated *partial thromboplastin time* (aPTT; measures recalcification time of plasma) was also measured.

5.2 MATERIALS AND METHODS

5.2.1 Test animals and collection of samples

Eight adult New Zealand white rabbits (2 male and 7 female, ±4 kg), obtained from the University of Witwatersrand, Johannesburg, were used for testing of aspartame. The rabbits were single housed by means of cage housing, without bedding at the University of Limpopo, Medunsa campus. Rabbits were weighed on a weekly basis to ensure that they were not losing any weight. Normal blood clotting factor ranges for rabbits was established by testing blood from the seven rabbits as there is no reference to these values in literature. It is also necessary to calibrate the instrument being used for testing of the coagulation factors found in rabbit blood. In this way, the normal ranges for the different coagulation factors can be determined.

1.5ml blood was drawn once weekly for four weeks, where after the rabbits were allowed to recuperate for two weeks (time needed to replace drawn blood). This procedure was repeated once more (blood drawn once weekly for four weeks, followed by a two week recuperation period). The data obtained from the samples from the above mentioned eight weeks was utilised to establish the normal ranges before treatment with the aspartame commenced.

5.2.2 Preparation of animals for drawing of blood samples:

Rabbits were placed in the modified restraint (as shown in chapter 3) before any blood was drawn. To increase visibility of the marginal ear vein of the rabbit, the area surrounding the vein was wiped with an alcohol swab. The ears were also flicked with the middle finger to improve the visibility of the vein.

5.2.3 Handling of the rabbits:

The person handling the rabbits used his/her thumb and little finger of one hand/ left hand/ non-dominant hand to grip the skin of the back of the neck and the head of the rabbit to lift the rabbit applying minimal pressure. The two hind paws were grabbed with the right hand/ dominant hand/ other hand and the rabbit turned so that its legs faced away from the handler. A firm grip on the paws were maintained and none on the back of the rabbit as it is fragile and could easily be injured.

5.2.4 Obtaining the blood samples:

1.5ml blood was drawn from the marginal ear vein of the rabbits (without sedation) at the Biology section of the Production unit (University of Limpopo, Medunsa campus). After the normal ranges were established, the rabbits were divided into 2 groups: 3 rabbits (1 male and 2 female) in the control group and 5 rabbits (1 male and 4 females) in the aspartame-treated group. The minimum amount of rabbits was chosen to decrease the number of animals that was treated with aspartame. The control group was utilized to ensure that normal ranges were maintained. Rabbits in the aspartame-treated group were fed aspartame by means of force-feeding with a syringe into the back of the mouth of the rabbit, as described in chapter 3. Aspartame was utilised in its pure form. The concentrations of aspartame that were studied was calculated according to the projected daily ingestion by humans (34mg/kg body weight) and also at 2 doses considered to be abuse concentrations (100 and 150mg/kg body weight) (Stegink *et al*. 1981). Rabbits in the control group only received double distilled water, by means of force-feeding with a syringe into the side of the mouth of the rabbit. Both the control and test groups received the same volume of fluid. The aspartame-treated rabbits received 25 exposures of aspartame over a period of 37 days. No aspartame was fed to the rabbits over weekends.

After exposure to the aspartame/double distilled water, 1.5ml blood was drawn from the marginal ear vein of ears of the rabbits (without sedation) in both the control and aspartame treated groups. Blood was drawn with a vacutainer needle and transferred into a 5ml vacutainer tube with a blue top, containing anti-coagulant (0.109M or 3.2% trisodium citrate) for testing. Two weeks of recovery time was allowed between consecutive concentrations. Blood was drawn on three set intervals - after 3 exposures, 13 exposures and 25 exposures to aspartame.

5.2.5 Preparation of sample for Start 4 instrument

The 1.5ml blood was centrifuged at 2500rpm for 10 minutes. The plasma was pippeted into small Eppendorf tubes and frozen at -80^{o}C till needed for use on the Start 4. The plasma could only be stored for 1 month.

All coagulation experiments were performed on the Start 4 Diagnostica Stago (Paris, France). This machine is the smaller, manual version of the larger machines routinely used in all haematology laboratories to determine the concentration of specific coagulation factors and profiles. The programming of the parameters into the Start4 was completed before any of the assays were attempted, and these parameters remained in the memory of the Start4. These parameters could only change when altered by the researcher. Examples of these parameters are maximum incubation time, precision (5%) and whether the experiment is run singularly or in duplicate. These parameters were dictated by the assays that were being performed.

5.2.5.1 Assays kits that were utilized:

- Neoplastine® Cl Plus (determination of Prothrombin time [PT])
- STA-Deficient II (substrate plasma for Factor II assay; Prothrombin)
- STA-Deficient V (substrate plasma for Factor V assay)
- STA-Deficient VII (Immuno-depleted plasma for Factor VII assay)
- STA-Deficient X (Immuno-depleted plasma for Factor X assay)
- STA-PTT Automate with silica activator (Sensitivities: Factors +++; Heparin: +++; Lupus ++; determination of Activated Partial Thromboplastin Time [aPTT])
- STA-Deficient VIII (Immuno-depleted plasma for Factor VIII assay)
- STA-Deficient IX (Immuno-depleted plasma for Factor IX assay)
- STA-Fibri-prest Automate (Clauss method; Quantitative determination of Fibrinogen)

All procedures were done according to the standardized operating procedures for determining human values.

The PT value may vary according to the origin of the thromboplastin reagent and to the instrument used to measure it. A solution for standardization adopted by the World Health Organization is a 'system of international reference standards for thromboplastins permitting the definition of an international scale for the intensity of anticoagulant therapy'. In this system the PT ratio is converted into the International Normalized Ratio (INR). The INR value corresponds to the value of the ratio of the patient's PT to that of the standard PT raised to the ISI (International Sensitivity Index) power of the thromboplastin used:

$$INR = \left(\frac{\text{Patient's PT}}{\text{Mean normal PT}} \right)^{ISI}$$

The ISI value of a given thromboplastin is determined by testing normal plasmas and Coumadin-treated patient plasmas with that thromboplastin and with the International Reference Preparation for thromboplastin. The PT values obtained with the two thromboplastins are plotted on log-log graph paper, and the orthogonal regression line is drawn. The slope of this line multiplied by the ISI value of the reference thromboplastin represents the ISI value of the thromboplastin of interest. The use of INR is recommended for the assessment of the PT in patients under oral anticoagulant therapy (Package insert - Neoplastine® CI Plus 2; Ref 00376).

5.2.5.2 Preparation of standards, patients' samples and controls

The unicalibrator (standard) was used for establishing the calibration curve for all of the assays performed. The dilutions that were to be used for each assay were determined after the normal ranges were set. The package insert of the unicalibrator supplies the researcher with information regarding the amount of active ingredient present for each specific assay. This value may vary from package to package, thus care was taken to use the correct package insert at any given time. From here, the concentration of active ingredient was calculated, which in turn was used for setting of the calibration curve.

For example:

Unicalibrator package insert values for factors II and V
1 : 10 = 100%
Dilutions utilized: 1:2, 1:3, 1:5, 1:10 and 1:20
1 : 20 = 50%
1 : 10 = 100%
1 : 5 = 200%
1 : 3 = 333.33%
1 : 2 = 500%

The plasma samples obtained were defrosted and the necessary dilutions were made for the different assays. For determining the concentration of factors II, V, VII, X, VIII and IX present in rabbit blood, 50µl rabbit plasma was added to 500µl Owren-Köller solution (1:10 dilution). For determining the concentration of fibrinogen present in the plasma, a 1:20 dilution was made with 25µl rabbit plasma and 475µl Owren-Köller solution. For determining the PT and aPTT, the plasma samples were used undiluted. A normal and pathological control was tested consecutively with all the assays performed, and their dilutions were the same as that of the plasma being tested for that assay.

Standards (utilizing unicalibrator) were diluted as follows:

a.) PT – 1:2, 1:3 and 1:4

b.) aPTT – no calibration necessary

c.) Factors II, V, VII, X, VIII, IX – 1:2, 1:3, 1:5, 1:10 and 1:20

d.) Fibrinogen – 1:7, 1:10; 1:20 and 1:40

The values obtained from the Start 4, together with the percentages calculated (as described above) were then used to draw the standard curve.

5.2.5.3 Assays

The reagents from the different assay kits were prepared according to their package inserts. The indicated amount of distilled water was added to each reagent, left for 5 minutes to homogenize and then swirled to improve optimal mixture of reagent. Reagents were then left to stand for 30 minutes to stabilize. Care was taken with the timing of preparation of factors VIII and IX, as they were only stable for four hours. Thus, they were prepared just before they were needed.

Table 5.1: Assays performed with different solutions and activators for each assay

Assay	**Dispensed into cuvette**	**Incubation period**	**Activator (pre-warmed at 37 ^{o} C)**
PT	50µl plasma	60 seconds	100µl Neoplastine CI plus
Deficient II	50µl plasma + 50µl Deficient II	60 seconds	100µl Neoplastine CI plus
Deficient V	50µl plasma + 50µl Deficient V	60 seconds	100µl Neoplastine CI plus
Deficient VII	50µl plasma + 50µl Deficient VII	60 seconds	100µl Neoplastine CI plus
Deficient X	50µl plasma + 50µl Deficient X	60 seconds	100µl Neoplastine CI plus
aPTT	50µl plasma + 50µl PTT automate	180 seconds	50µl $CaCl_2$
Deficient VIII	50µl plasma + 50µl Deficient VIII	180 seconds	50µl $CaCl_2$
Deficient IX	50µl plasma + 50µl Deficient IX	180 seconds	50µl $CaCl_2$
Fibrinogen	100µl plasma	60 seconds	50µl FibriPrest

5.3 RESULTS

The prothrombin time (PT; measures how long blood takes to form a clot) and activated partial thromboplastin time (aPTT) was determined. aPTT (measures recalcification time of plasma) is used to screen for deficiencies in coagulation factors involved in the intrinsic pathway (factor VII and factor VIII excluded). The aPTT is also utilized to evaluate the effect of therapy and to monitor and regulate heparin therapy (Proctor and Rapaport, 1961). PT measures the presence and activity of factors I, II, V, VII and X. A Normal PT time, for a healthy human is 10 – 13 seconds with the International Normalized Ratio (INR) being 1.0 – 1.4 (WebMD Medical Tests, 2006). Partial thromboplastin time in a normal healthy human is 30 – 45 seconds, while the activated partial thromboplastin time is 25 – 39 seconds (WebMD Medical Tests, 2006).

Literature does not describe normal values for the different coagulation factors in rabbits, thus the normal ranges for rabbits for the different factors were established.

Analysis indicated that rabbits have higher concentration levels for the factors tested. Therefore, the standard curve was extrapolated to determine the values for rabbits. The instrument automatically extrapolates the curve to 200%, but the values for rabbits were more than 200%. Statistically, extrapolation was done from the standard curve obtained, to determine the true percentage from the actual seconds (raw data) measured.

5.3.1 Determining normal ranges

The normal ranges for the different coagulation factors within the group of rabbits were obtained and are represented in Table 5.2.

Table 5.2: Normal ranges for the different coagulation factors for the group of rabbits

Assay performed	***Lower spectrum***	***Higher spectrum***
PT (Prothrombin time) %	93.15	107.13
INR	1.19	1.39
Deficient factor II	170.72	264.35
Deficient factor V	609.11	670.06
Deficient factor VII	319.89	422.06
Deficient factor X	240.37	351.76
APTT (activated partial prothrombin time)	45.98	69.89
Deficient factor II	488.49	713.3
Deficient factor II	76.44	355.33
Fibrinogen	1.81	3.58

These values was used during the study do make certain that the controls remained within the normal range limits, demonstrating that the results obtained for the rabbits in the treatment group was indeed due to the aspartame and not due to continues drawing of blood. The values were also used to determine whether the aspartame had any adverse effects on the concentration of the factors discussed and the time to form a blood clot (prothrombin time and activated partial thromboplastin time).

The values obtained for the rabbits were placed in a table (Table 5.3) and compared to the normal values of human beings.

Table 5.3: Comparison between normal ranges determined for rabbit plasma and human plasma

Assay Performed	**Values obtained for determining normal ranges for rabbits**	**Expected values for normal human adults**
PT **%** (n = 41) **INR** (n= 42)	93.15 – 107.13 % 1.19 – 1.39 INR 7.16 ± 0.55 sec	Greater than 70% 1.0 – 1.4 INR 10 – 13 sec
Factor II (n = 33)	170.72 – 264.35 %	70 – 120 % (Bezeaud *et al.* 1979)
Factor V (n = 39)	496.69 – 556.55 %	70 – 120 %
Factor VII (n = 37)	319.89 – 422.06 %	55 – 170 %
Factor X (n = 40)	240.37 – 351.76 %	70 – 120 % (Marian *et al.* 1999)
aPTT (n = 35)	45.98– 69.89 sec	25 – 39 sec (Levin and Lusher, 1982)
Factor VIII (n = 41)	488.49 – 713.3 %	60 – 150 %
Factor IX (n = 42)	76.44 - 355.33 %	60 – 150 %
Fibrinogen (n = 37)	1.81 – 3.58 g/l	2 – 4 g/l (Bezeaud *et al*. 1979)

Values for humans obtained from package inserts of the assays performed; Values for rabbits were obtained statistically by utilizing mean ± 2 standard deviations. n indicates the number of values used when obtaining the means.

When the results obtained for the rabbits were compared with those of humans, it was clear that the PT (in seconds) is shorter than that of the human although the percentage and INR values

correspond with that of the human. The percentage of factor II found in rabbits was found to be twice as much as that of a human. The amount of factor V and VIII is four to six times greater for rabbits. The rabbit contains two to three times more factor VII and X. The lower end of the range for factor IX in rabbits fall inside the lower spectrum set for humans and the higher end of the spectrum is two times more than that of a human. The rabbit also has a much longer activated partial thromboplastin time when compared to a human. And finally, and certainly most importantly, the amount of fibrinogen is comparable between humans and rabbits.

5.3.2 Blood clotting time and coagulation profile after treatment with aspartame

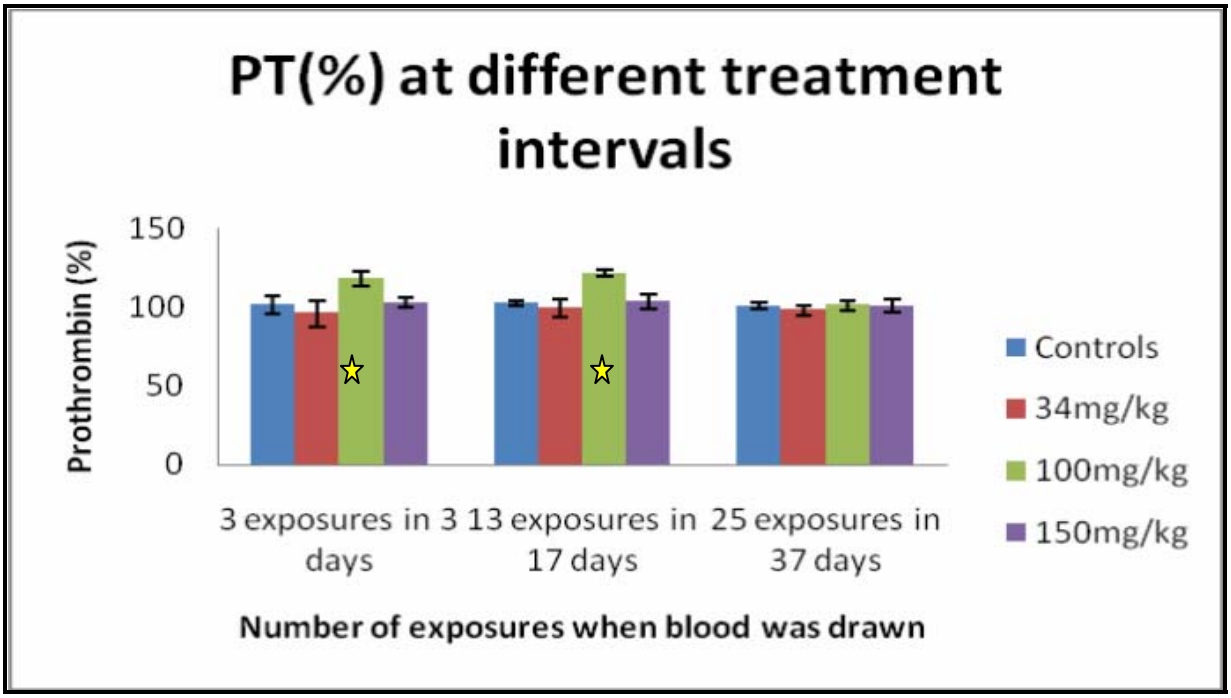
Table 5.4 and graph 5.1 indicate the values for PT (%) after treatment with the three different concentrations of aspartame at the set intervals for drawing blood.

Table 5.4: Effects of aspartame on the PT (%) for the three different concentrations at the set intervals for drawing blood

PT (%)	3 exposures in 3 days	13 exposures in 17 days	25 exposures in 37 days	Normal range
Controls	101.06	102.49	100.41	93.15 - 107.13
34mg/kg	95.78	99.35	97.85	
100mg/kg	117.94	121.29	100.99	
150mg/kg	102.55	103.81	100.59	

Values highlighted in yellow fall outside the normal set ranges

Graph 5.1: Effects of three different concentrations of aspartame on the PT (%) of the rabbit



Yellow star indicates the values that fall outside the normal set ranges

The results indicate that the prothrombin time (PT) never exceeded the values set for normal ranges during the 34mg/kg aspartame treatment. However, the PT was prolonged after the 100mg/kg aspartame treatment. The two-way ANOVA was performed on the data highlighted in yellow, and a statistically significant (P=0.04) difference was found between the rows of data, but not the columns. This indicates that the different intervals for drawing blood after a certain number of exposures were not significant, but the effects of the different concentrations were significant. These results were confirmed by performing the t-Test: Paired Two Sample for Means. There was a significant difference (P=0.034 two-tailed) between the control and the first two values obtained for the 100mg/kg aspartame treatment group. It would seem that the rabbits were mostly affected during the earlier stages of treatment (3 and 13 exposures) with the 100mg/kg aspartame. The values obtained for the 150mg/kg aspartame treatment never exceeded the values set as normal ranges.

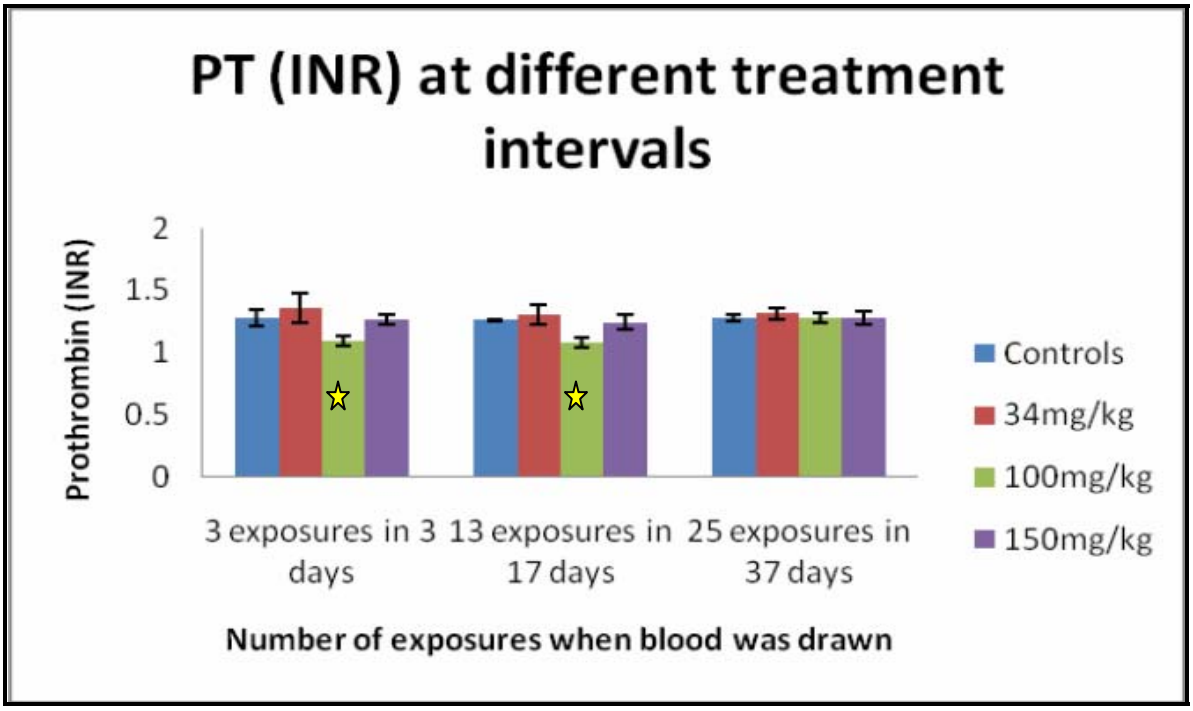
Table 5.5 and graph 5.2 indicate the values for PT (INR) after treatment with the three different concentrations of aspartame at the set intervals for drawing blood.

Table 5.5: Effects of aspartame on the PT (INR) for the three different concentrations at the set intervals for drawing blood

PT (INR)	3 exposures in 3 days	13 exposures in 17 days	25 exposures in 37 days	Normal range
Controls	1.28	1.26	1.28	1.19 - 1.39
34mg/kg	1.36	1.3	1.31	
100mg/kg	1.09	1.08	1.28	
150mg/kg	1.26	1.24	1.28	

Values highlighted in yellow fall outside the normal range

Graph 5.2: Effects of aspartame on the PT (INR) of the rabbit after treatment with the three concentrations of aspartame



Yellow stars indicate values that fall outside the normal range

The results for PT (INR) were the same as for PT (%), seeing that only the measurement units were different. A significant difference (*P*=0.017 two-tailed) were seen between the control and 100mg/kg aspartame.

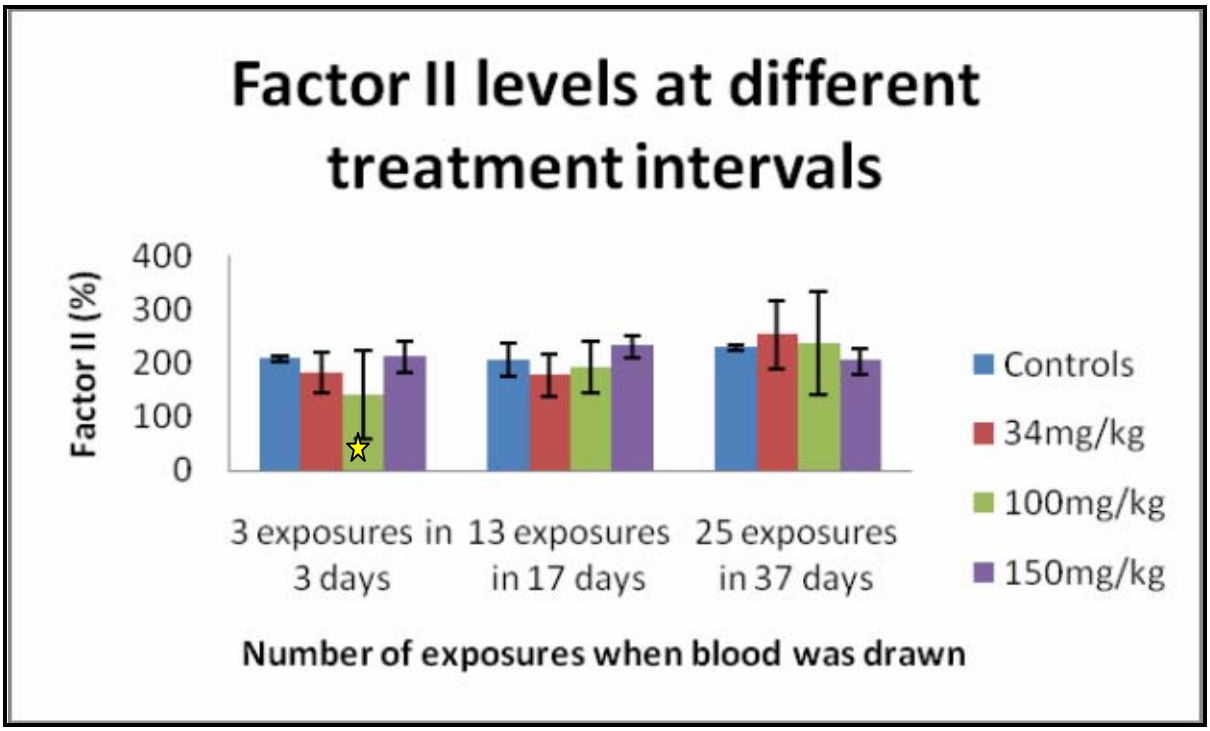
Table 5.6 and graph 5.3 indicate the values for Factor II after treatment with the three different concentrations of aspartame at the set intervals for drawing blood.

Table 5.6: Effects of aspartame on the concentration of F II (%) after treatment with three concentrations aspartame at the three intervals set for drawing blood

Factor II	3 exposures in 3 days	13 exposures in 17 days	25 exposures in 37 days	Normal range
Controls	208.35	206.38	229.3	170.72 - 264.35
34mg/kg	181.92	178.55	252.5	
100mg/kg	140.78	192.47	236.34	
150mg/kg	210.4	231.24	203.94	

Values highlighted in yellow fall outside the normal range

Graph 5.3: Effects of aspartame on the concentration of F II (%) after treatment with the three concentrations of aspartame



Yellow stars indicate values that fall outside normal range

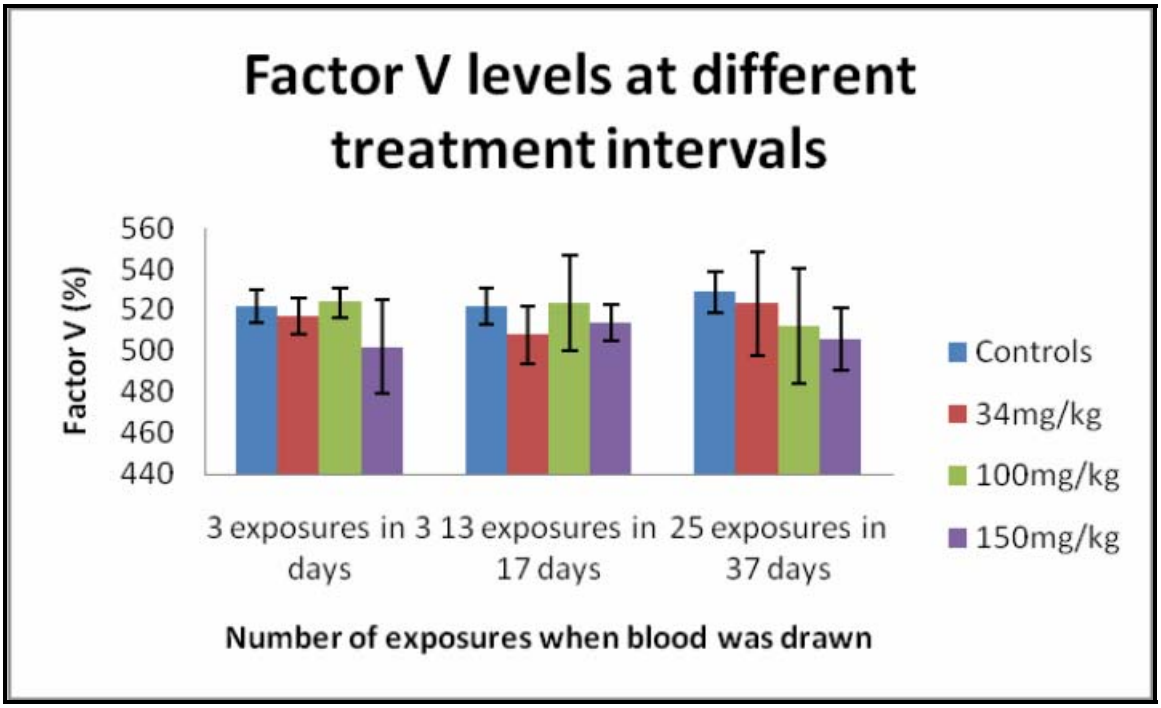
The results indicate that the concentration of factor II were not adversely affected by any of the concentrations of aspartame tested. The value highlighted in yellow does not differ significantly from the control, as proven by the two-way ANOVA and t-Test: Paired Two Sample for Means.

Table 5.7 and graph 5.4 indicate the values for Factor V after treatment with the three different concentrations of aspartame at the set intervals for drawing blood.

Table 5.7: Effects of aspartame on the concentration of factor V (%) for the three different concentrations at set intervals for drawing blood

Factor V	3 exposures in 3 days	13 exposures in 17 days	25 exposures in 37 days	Normal range
Controls	521.67	521.94	528.67	496.69 - 556.55
34mg/kg	517.04	507.91	523.05	
100mg/kg	523.64	523.23	512.28	
150mg/kg	501.96	513.77	505.69	

Graph 5.4: Effects of aspartame on the concentration of factor V (%) at different concentrations



All the values obtained for factor V fell within the normal set ranges, thus it would appear as if factor V was not adversely affected by the aspartame.

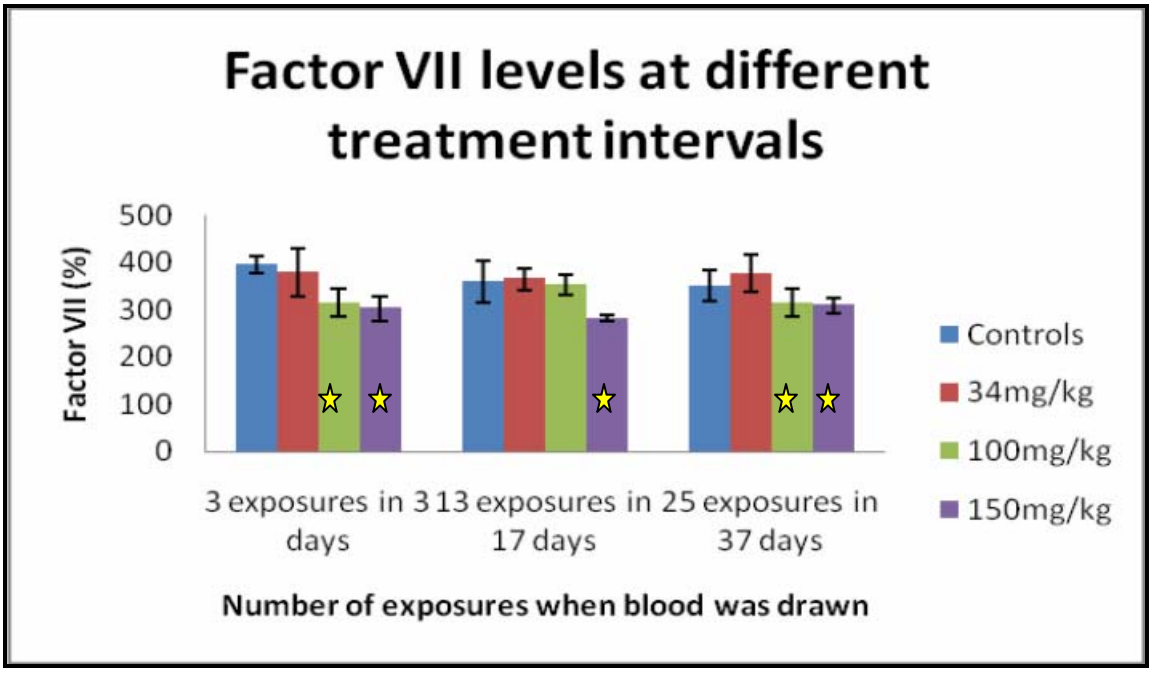
Table 5.8 and graph 5.5 indicate the values for Factor VII after treatment with the three different concentrations of aspartame at the set intervals for drawing blood.

Table 5.8: Effects of aspartame on the concentration of factor VII (%) after treatment with the three concentrations at set intervals for drawing blood

Factor VII	3 exposures in 3 days	13 exposures in 17 days	25 exposures in 37 days	Normal range
Controls	395.42	358.78	350.46	319.89 - 422.06
34mg/kg	379.26	365.26	376.76	
100mg/kg	315.16	353.39	314.56	
150mg/kg	302.86	282.05	309.05	

Values highlighted in yellow fall outside the normal range

Graph 5.5: Effects of aspartame on the concentration of factor VII (%) after treatment with different concentrations



Yellow stars indicate values that fall outside the normal range

The obtained results indicate that the concentration of factor VII decreased at 34mg/kg aspartame, but the values still remained within the normal range of values. The values obtained for 100mg/kg aspartame were even lower, with two of the values falling outside the normal range. A two-way ANOVA was performed on this data and it was found that there was a statistically significant (*P*=0.010) difference between rows, but not between columns. Thus, the aspartame treated groups differed significantly from the controls, but there was no significant difference between the values for the different exposure time intervals for drawing blood. The effect of the aspartame remained the same throughout the exposure period. Furthermore, a t-Test was performed on the values obtained for the 100mg/kg aspartame, and no significant difference were found between the controls and affected values. When comparing the values obtained for the 150mg/kg aspartame-treated group with the controls (t-Test), there was a statistically significant (*P*=0.043 two-tailed) decrease in the concentration of factor VII. Factor VII, which forms part of the extrinsic pathway, is necessary for activation of factor IX *in vivo*. Factor VII in turn is activated by tissue factor in the presence of Ca^{2+}. Tissue factor is an integral protein of cell membranes (Meyer and Meij, 1996). Thus, it could be stated that a decreased concentration of factor VII could induce a decreased concentration of factor IX, leading to bleeding disorders. If there is deficient amounts of tissue factor or Ca^{2+} in the test animals, factor VII formation will also be decreased.

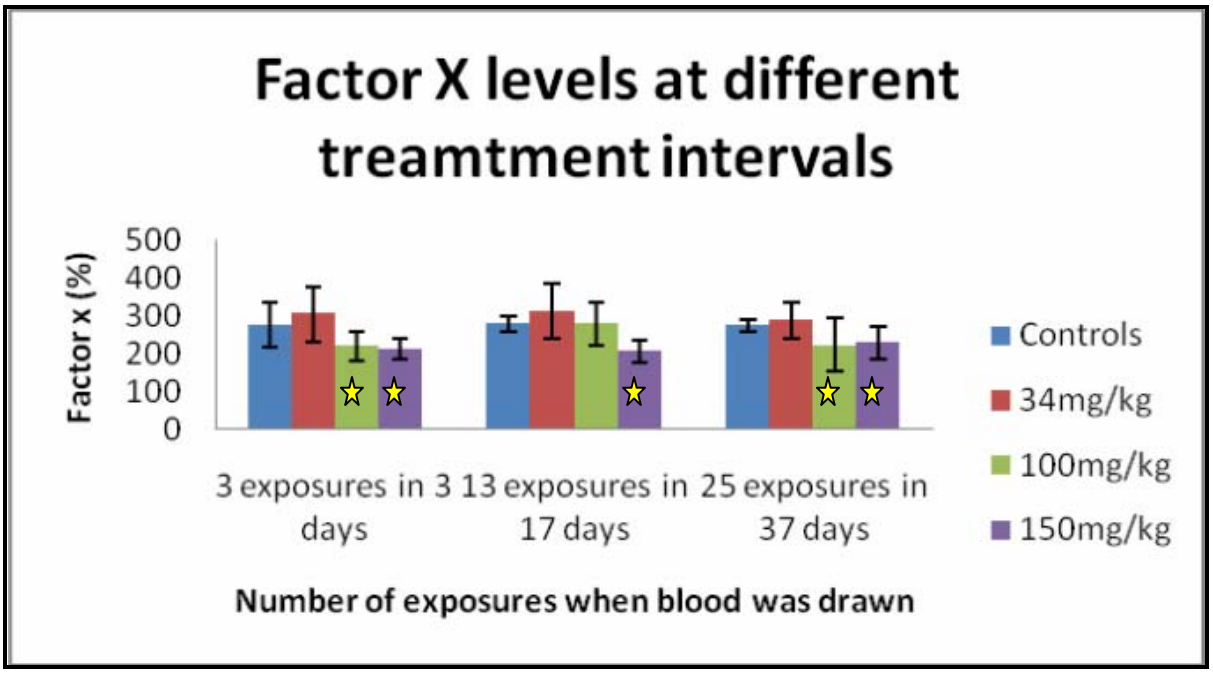
Table 5.9 and graph 5.6 indicate the values for Factor X after treatment with the three different concentrations of aspartame at the set intervals for drawing blood.

Table 5.9: Effects of aspartame on the concentration of factor X (%) after treatment with the three different concentrations at set intervals for drawing blood

Factor X	3 exposures in 3 days	13 exposures in 17 days	25 exposures in 37 days	Normal range
Controls	275.92	278.82	274.54	240.37 - 351.76
34mg/kg	304.34	312.11	288.46	
100mg/kg	219.91	278.33	222.94	
150mg/kg	213.03	205.54	228	

Values highlighted in yellow fall outside the normal ranges

Graph 5.6: Effects of aspartame on the concentration of factor X (%) after treatment with the different concentrations



Yellow stars indicate values that fall outside the normal range

From the results obtained it is clear that the concentration of factor X was not adversely affected by the 34mg/kg aspartame treatment. However, values from the 100mg/kg and 150mg/kg aspartame treatment groups fall outside the normal rage. A two-way ANOVA test was performed on the data, and the values in the rows were found to differ significantly (P=0.005) from the controls. There was no significant difference between the values in the columns, thus the aspartame had no different effect on the concentration of factor X at the different treatment intervals. The values that fall outside the normal range for the 100mg/kg and 150mg/kg aspartame treatments were subjected to further testing (t-Test). No significant difference were found between the control and 100mg/kg aspartame, but a statistically significant (P=0.016 two-tailed) decrease were found between the control and 150mg/kg aspartame. Thus, the decrease of the concentration of factor X was significant. Factor IX (in its active form) works in on factor X and split this protein to form an active enzyme, F Xa. The active form of factor X can be produced by either the intrinsic or extrinsic pathways. The active form of factor X, in combination with factor V, platelet or tissue phospholipids and calcium ions, convert prothrombin to thrombin (Meyer and Meij, 1996). Thus, a decreased amount of factor X could lead to lower concentrations of thrombin, needed for conversion of fibrinogen to fibrin.

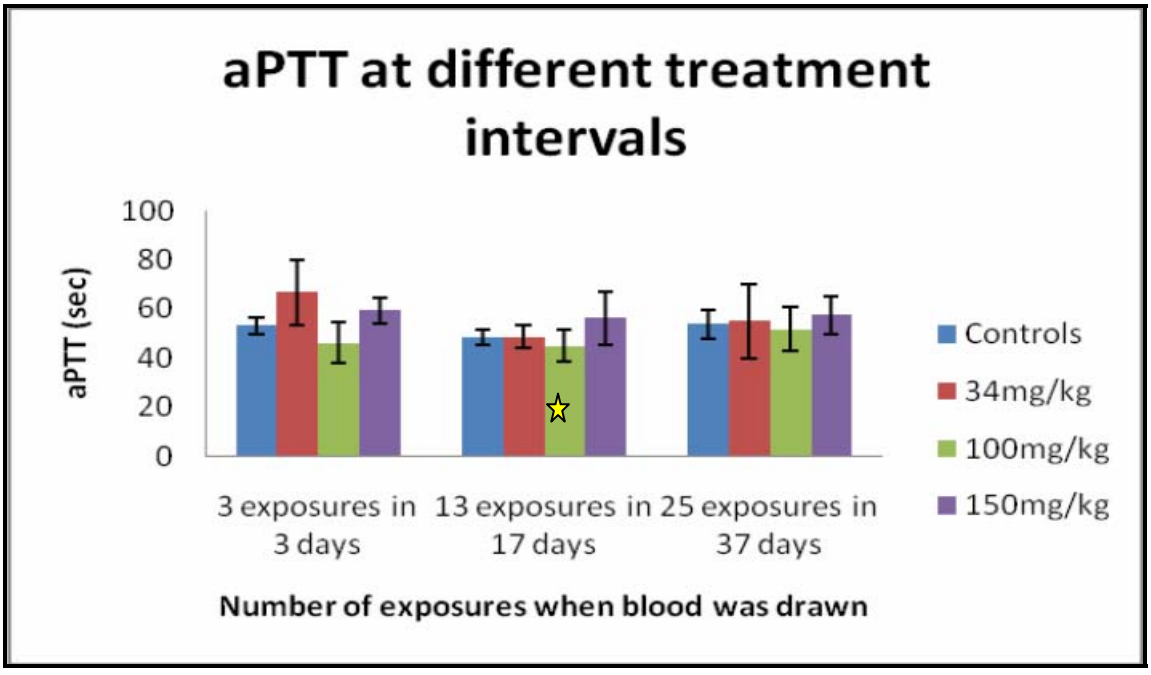
Table 5.10 and graph 5.7 indicate the values for aPTT after treatment with the three different concentrations of aspartame at the set intervals for drawing blood.

Table 5.10: Effects of aspartame on the aPTT (sec) after treatment with the three different concentrations at the set intervals for drawing blood

aPTT	3 exposures in 3 days	13 exposures in 17 days	25 exposures in 37 days	Normal range
Controls	53.16	48.28	53.73	45.98 - 69.89
34mg/kg	66.84	48.72	54.95	
100mg/kg	46.18	44.87	51.64	
150mg/kg	59.48	56.14	57.44	

Values highlighted in yellow fall outside the normal range

Graph 5.7: Effects of aspartame on the aPTT (sec) after treatment with the different concentrations



Yellow stars indicate values that fall outside normal range

From the results shown in table 10, it is clear that the time for recalcification of the plasma (aPTT) was not affected by the treatment with aspartame as the values all fell within the normal range. The one value for the 100mg/kg aspartame after 13 exposures that did fall outside the

normal range was subjected to a two-way ANOVA test and it was found that the value did not differ significantly from the control values.

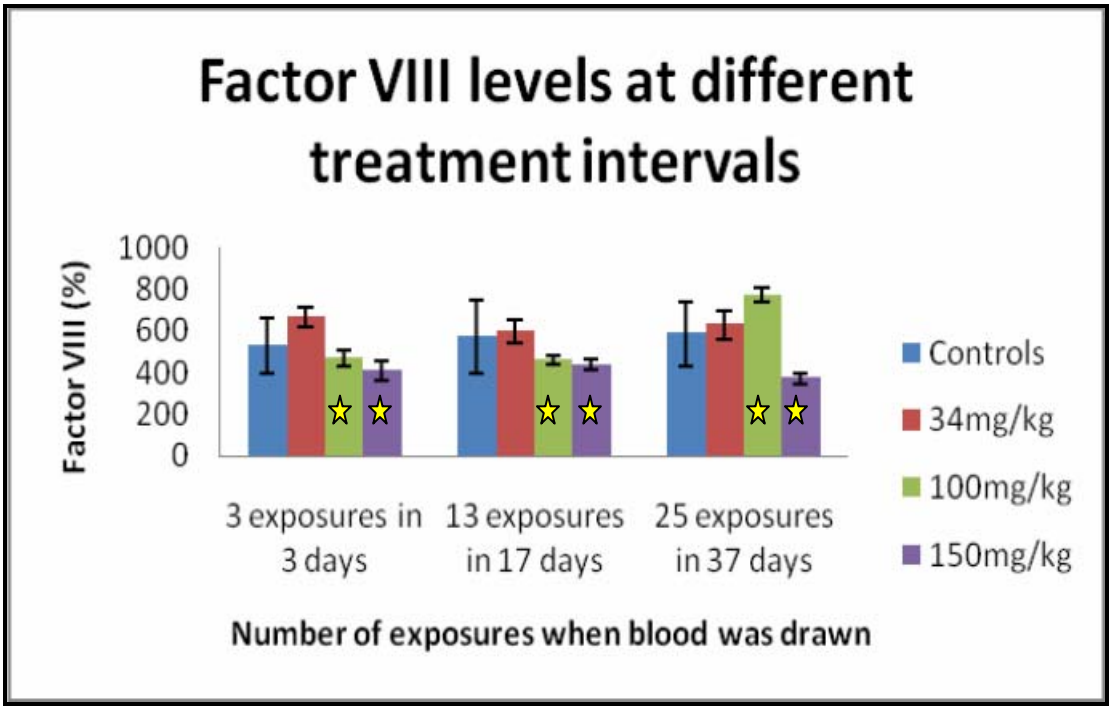
Table 5.11 and graph 5.8 indicate the values for Factor VIII after treatment with the three different concentrations of aspartame at the set intervals for drawing blood.

Table 5.11: Effects of aspartame on the concentration of factor VIII (%) after treatment with the three different concentrations at the set intervals for drawing blood

Factor VIII	3 exposures in 3 days	13 exposures in 17 days	25 exposures in 37 days	Normal range
Controls	528.72	569.75	585.54	488.49 - 713.3
34mg/kg	664.72	599.25	630.36	
100mg/kg	471.07	461.16	770.8	
150mg/kg	407.36	437.82	373.95	

Values highlighted in yellow fall outside the normal range

Graph 5.8: Effects of aspartame on the concentration of factor VIII (%) after treatment with the different concentrations



Yellow stars indicate values that fall outside the normal range

From the obtained results it appears as though 34mg/kg aspartame increased the concentration of factor VIII present in the tested rabbit plasma, but the values remained within the set normal ranges. It was noted that the values for both the 100mg/kg and 150mg/kg aspartame treatments fell outside the normal range. The data was analyzed by means of the two-way ANOVA, but no statistical significance was found between the rows and columns of data. The data was further subjected to analysis by means of the t-Test, to ensure that data was not significant. It was found that the values obtained for the 100mg/kg aspartame did indeed not differ significantly from that of the control, but it was not the same scenario for the 150mg/kg aspartame treatment. There was a statistically significant (P=0.032 two-tailed) decrease between the controls and 150mg/kg aspartame treatment groups. Thus, the concentration of factor VIII decreased significantly. Factor VIII is known as an antihemofillic globulin and is activated by thrombin, thus a decrease in the concentration of any of these factors can lead to bleeding disorders (Meyer and Meij,1996).

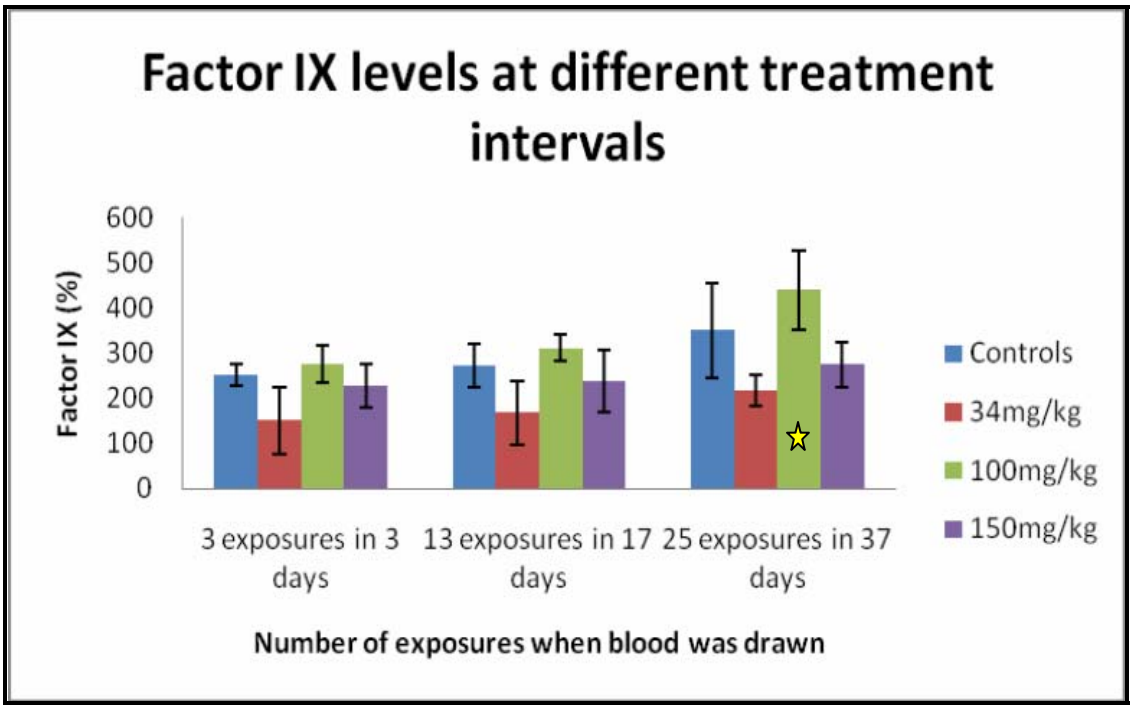
Table 5.12 and graph 5.9 indicate the values for Factor IX after treatment with the three different concentrations of aspartame at the set intervals for drawing blood.

Table 5.12: Effects of aspartame on the concentration of factor IX (%) after treatment with the three different concentrations at the set intervals for drawing blood

Factor IX	3 exposures in 3 days	13 exposures in 17 days	25 exposures in 37 days	Normal range
Controls	251.41	271	349.32	76.44 - 355.33
34mg/kg	150.42	167.5	216.97	
100mg/kg	274.22	310.13	438.3	
150mg/kg	226.5	237.3	274.06	

Values highlighted in yellow fall outside normal range

Graph 5.9: Effects of aspartame on the concentration of factor IX (%) after treatment with the different concentrations



Yellow star indicate values that fall outside the normal range

After analyzing the results obtained for factor IX, it was clear that aspartame did not adversely affect the concentration of factor IX in the plasma of the rabbit. The amount of factor IX decreased after treatment with 34mg/kg aspartame, but not to a degree that the values fall outside the normal range. The amount of factor IX increased readily from exposure interval to exposure interval for the 100mg/kg aspartame treatment, but the values did not fall outside the normal ranges. The value obtained for 100mg/kg aspartame after 25 exposures that did fall outside the normal range were tested with the t-Test, and the results indicated that there was no significant difference between the tested value and the values of the control.

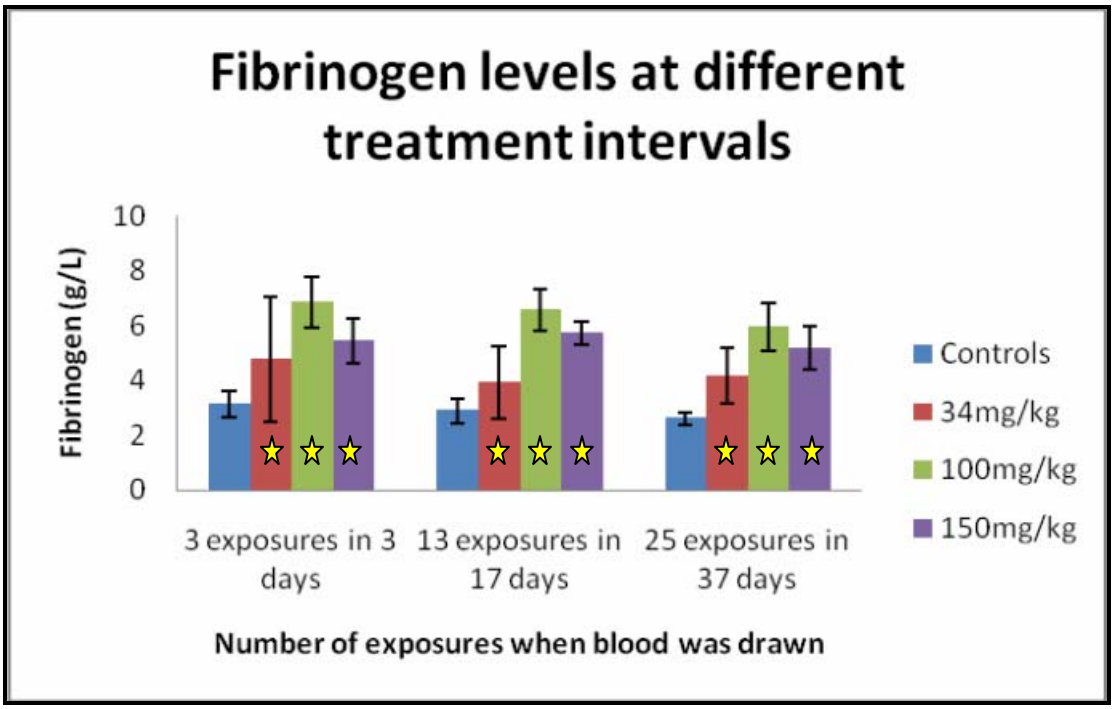
Table 5.13 and graph 5.10 indicate the values for Fibrinogen after treatment with the three different concentrations of aspartame at the set intervals for drawing blood.

Table 5.13: Effects of aspartame on the concentration of fibrinogen (g/L) after the three different concentrations at set intervals for drawing blood

Fibrinogen	3 exposures in 3 days	13 exposures in 17 days	25 exposures in 37 days	Normal range
Controls	3.15	2.9	2.62	1.81 - 3.58
34mg/kg	4.75	3.91	4.17	
100mg/kg	6.85	6.56	5.96	
150mg/kg	5.45	5.74	5.17	

Values highlighted in yellow fall outside the normal range

Graph 5.10: Effects of aspartame on the concentration of fibrinogen (g/L) after treatment with the different concentrations



Yellow stars indicate values that fall outside the normal range.

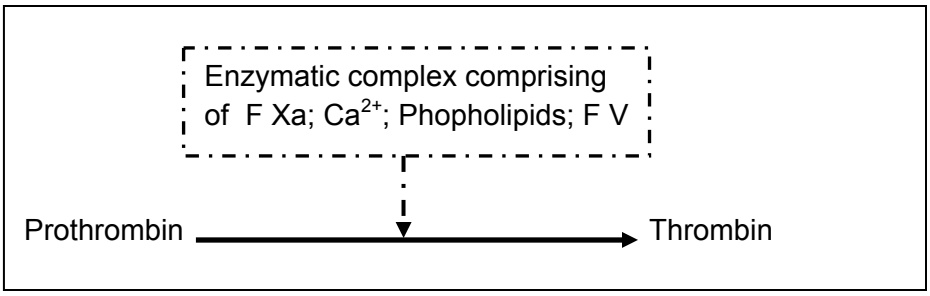
When looking at the effects of aspartame on the concentration of fibrinogen, it was clear that the amount of fibrinogen increased from the first treatment with aspartame at the lowest concentration (34mg/kg). A two-way ANOVA was performed on the results, indicating that there was a statistical significant (P=1.75x10^5) difference between the different concentrations and the controls. However there was no difference between the different numbers of exposures. The data was further subjected to analysis (t-Test) to determine whether there was any difference

between the different concentrations. The t-Test indicated that there was a statistically significant (*P*=0.018 two-tailed) increase between the concentration of fibrinogen of the control and 34mg/kg aspartame treated group. The t-Test was performed on the values of the control and 100mg/kg aspartame treated groups, and a statistically significant (*P*=0.001 two-tailed) increase were found between the control and aspartame treated groups. A statistically significant (*P*=0.0036 two-tailed) increase was also found between the control and 150mg/kg aspartame. The values of the 34mg/kg aspartame treatment group was compared to the values of the 100mg/kg aspartame treatment group (t-Test), and a significant increase (*P*=0.013 two-tailed) were found between the two concentrations. When comparing (t-Test) the 34mg/kg aspartame results with the 150mg/kg aspartame results, a one-tailed significance (*P*=0.037) were found between the results. There was a significant increase in the concentration of fibrinogen between the above mentioned two concentrations. The last comparison (t-Test) was made between the 100mg/kg values and the 150mg/kg values. A two-tailed significant (*P*=0.037) decrease were found between the values of the 150mg/kg aspartame and the 100mg/kg aspartame. However, the concentration of fibrinogen for the 150mg/kg aspartame was still higher after the decrease than the values for the 34mg/kg aspartame. Thus, an overall increase of the concentration of fibrinogen was noted after treatment with the aspartame.

5.4 SUMMARY AND EXPLANATION

The ***prothrombin time*** (PT) is a basic coagulation screening test, useful in the assessment of congenital and/or acquired deficiencies of the extrinsic coagulation pathway (factors II, V, VII and X). A prolonged PT has been observed in the following clinical states like treatment with vitamin K antagonists, hemorrhagic disease of the newborn, intestinal reabsorption disorders, liver failure (cirrhosis; hepatitis; jaundice), fibrinolysis and distributed intravascular coagulation (DIC) (Package insert – Neoplastine® CI Plus 2; Ref 00376). The PT was prolonged after treatment with aspartame with deficiencies in factors VII and X.

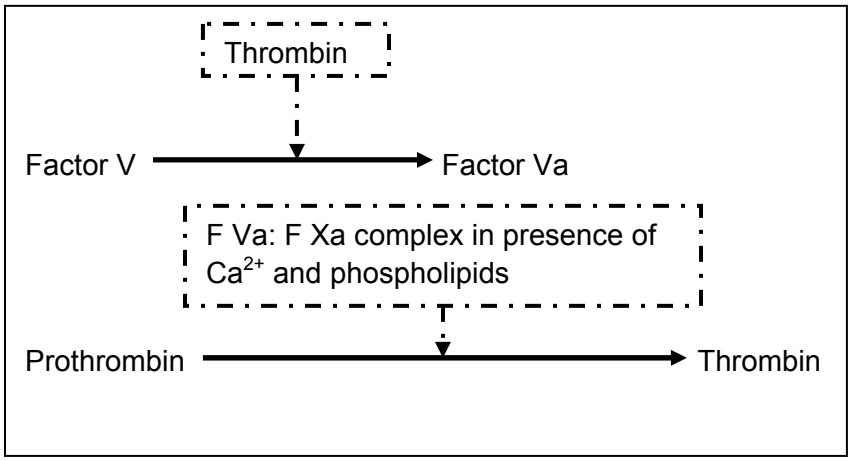
Factor II, also known as prothrombin, is synthesized in the liver. The complete synthesis of the prothrombin molecule requires the presence of vitamin K.



Solid arrow indicates activation of the inactive protein (F II; Prothrombin) to its active form (F IIa; Thrombin); dashed arrow and box indicate the enzymatic complex needed for the activation of prothrombin to thrombin.

Factor V must undergo a molecular modification under the influence of traces of thrombin to aid in the activation of thrombin. Acquired F II deficiencies can be found in several clinical states; during oral anticoagulation therapy (which depresses the vitamin-K dependant factors II, VII, IX and X), intake or absorption deficiencies of vitamin K, liver damage (cirrhosis; hepatitis), during fibrinolysis, disturbed intravascular coagulation (DIC) and the presence of factor II inhibitors (Package insert – STA Deficient II; Ref 00745). Aspartame did not affect the concentration of circulating factor II.

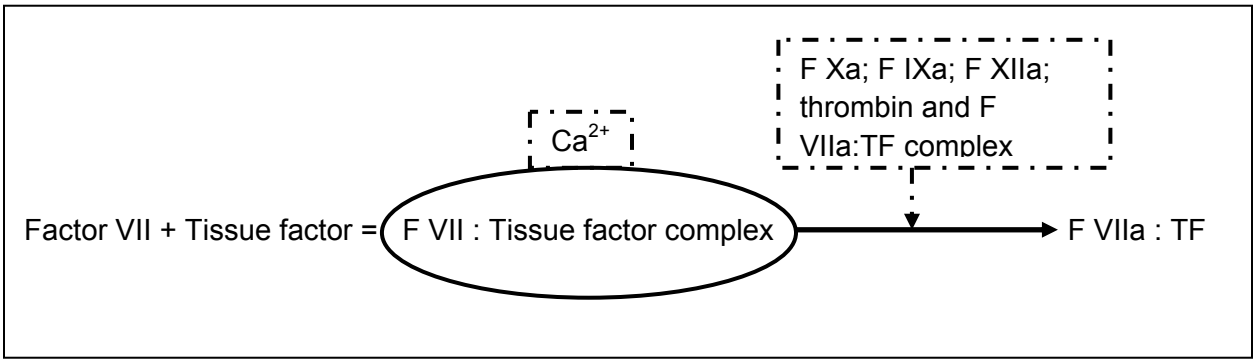
Factor V is synthesized by the reticuloendothelial system. The structure of the platelet factor V is closely related if not identical with that of the plasma factor V.



Solid arrow indicates activation of the inactive protein (F V) to its active form (F Va) with the dashed arrow and box indicating that thrombin is needed for activation; dashed arrow and box indicate the enzymatic complex needed for the activation of prothrombin to thrombin. Note that F Va is needed for activation.

Factor Va is inactivated by factor Xa, activated protein C and plasmin. Liver cells synthesize various coagulation factors, some of which require the presence of vitamin K for their elaboration (factors II, VII, IX and X). Any hepatic disorder can lead to a more or less important decrease in the level of these factors. Liver damage, therefore, can result in hemorrhagic disorders. Specific testing for the factors is of major interest for reasons of prognosis and diagnosis with respect to liver damage. If both factors VII and X are decreased prolonged hepatitis can be diagnosed (Package insert – STA Deficient V; Ref 00744). Factor X concentrations were decreased after treatment with aspartame. The enzyme complex between F Va and F Xa were thus compromised and so prothrombin could not be converted to thrombin.

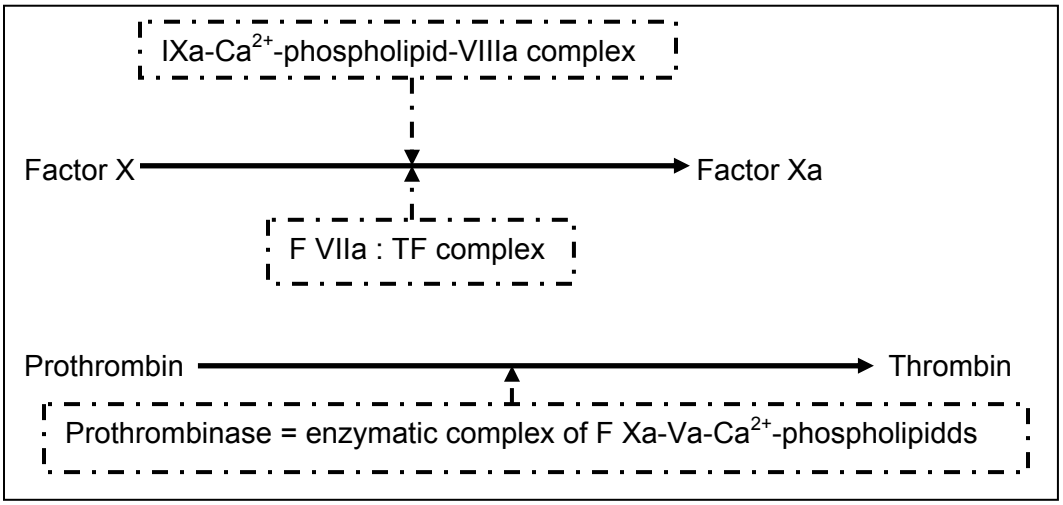
Factor VII is a vitamin K dependant glycoprotein synthesized in the liver.



F VII can only be activated if in complex with Tissue Factor (TF); Oval indicate F VII:TF complex; Solid arrow indicate activation of inactive protein (F VII) to is active counterpart (F VIIa); dashed arrow and box indicate factors/enzymatic complexes/co-factors needed for activation of protein.

F VIIa : TF complex can activate either factor X or factor IX and is inhibited by tissue factor pathway inhibitor (TFPI). TFPI forms a complex with factor Xa then with F VIIa : TF complex. This results in the formation of an inactive tetramolecular complex. F VIIa : TF complex could be inhibited by antithrombin in the presence of heparin. Acquired deficiency of factor VII associated with deficiencies of other coagulation factors: intake or absorption deficiency of vitamin K (hemorrhagic disease of newborn, obstructive icterus, antibiotic therapy); oral anticoagulant therapy; hepatic disorders; fibrinolysis; disturbed intravascular coagulation (DIC) (Package insert – STA Deficient VII; Ref 00743). Factor VII concentrations were decreased after exposure to aspartame. Decreased concentrations of factor VII, leads to decreased amounts of active enzyme of this factor, which in turn can lead to a decrease in conversion of factors X and IX to their active forms. F Xa is necessary for conversion of prothrombin to thrombin, which in turn converts fibrinogen to fibrin. Thus a deficiency of F Xa could indirectly lead to bleeding disorders as fibrin cannot be formed (lack of thrombin).

Factor X is a vitamin K dependant glycoprotein which is synthesized in the liver.

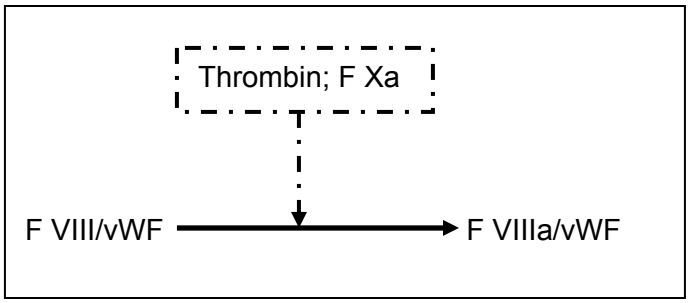


Solid arrows indicate activation of inactive protein (F X) to its active counterpart (F Xa); F X can be activated via 2 pathways, with 2 different enzymatic complexes indicated by dashed arrows and boxes; activated F Xa is needed for the conversion of prothrombin to thrombin as indicated by the dashed arrow and box.

Factor Xa can activate factor VII to F VIIa. Factor Xa is inhibited by antithrombin III, associated or not with heparin. Acquired deficiencies of factor X associated with deficiencies of the factors II, VII and IX: oral anticoagulant therapy; intake or absorption deficiency (hemorrhagic disease of the newborn; obstructive icterus; antibiotic therapy). Acquired deficiencies of factor X associated with deficiencies of the factors II, V and VII: hepatic disorders (cirrhosis; hepatitis); fibrinolysis; disrupted intravascular coagulation (Package insert – STA Deficient X; Ref 00738). Factor X was decreased after treatment with aspartame. Factor X can be activated either via IXa-Ca^{2+}-phospholipid-VIIIa complex or the F VIIa : TF complex. The amount of circulating F VII decreased after exposure to aspartame, thus activation via this route could be hindered due to inability of complex formation between F VII and Tissue factor occurring, which further prevents activation of F VII. The other pathway of activation involves F IXa, F VIIIa, Ca^{2+} and phospholipids. As was indicated, the amount of circulating F VIII was decreased after treatment with aspartame. Although the amount of F IX was not affected, there will be complications in the formation of the enzymatic complex for activation of F Xa. Factor X is needed in its activated form for conversion of prothrombin to thrombin, thus a deficiency of F X indirectly leads to a deficiency of thrombin.

Activated partial thromboplastin time (aPTT) is a general coagulation screening test of the intrinsic coagulation pathway (factors XII, XI, VIII, X, V, II and I). Acquired deficiencies and abnormal conditions are caused by liver diseases, consumptive coagulopathy, circulation anticoagulants (antiprothrombinase or circulating anticoagulant against a factor), during heparin or oral anticoagulant therapy, treatment with thrombin inhibitors (Package insert - PTT Automate 5; Ref 00482). The aPTT was not affected by the treatment with aspartame.

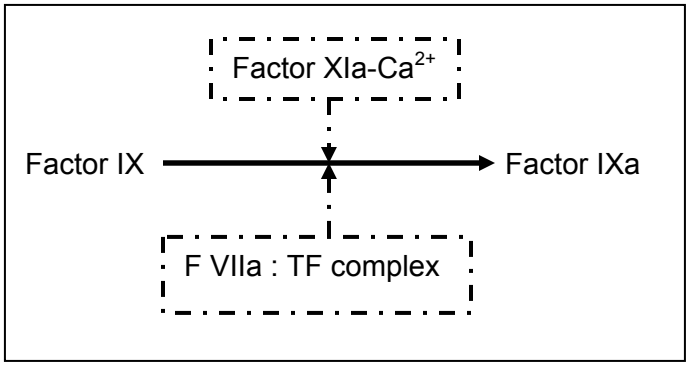
Factor VIII is a glycoprotein which is synthesized and released into the blood stream by the vascular endothelium (Rubin and Leopold, 1998). In the plasma, factor VIII circulates in a complexed form with the von Willebrand factor as F. VIII/vWF by means of a covalent linkage.



Solid arrow indicates the activation of the inactive protein (F VIII) to its enzymatic counterpart (F VIIIa). Note that F VIII can only be activated if in complex wit von Willebrand factor; dashed arrow and box indicate factors needed for activation of protein.

F VIIIa increases the activation of factor X by the F IXa in the presence of phospholipids and calcium (Package insert – STA Deficient VIII; Ref 00725). As seen from description for F X, the amount of factor VIII was decreased by treatment with the aspartame, thus activation of F X by the pathway including both factors VIIIa and IXa are indirectly inhibited. It was also noted that the amount of thrombin would be decreased due to the inability of F X to convert prothrombin to thrombin. It could therefore be stated that due to the decreased concentrations of both thrombin (indirectly caused by aspartame) and F X (caused directly by aspartame), a diminished capacity would exist for activation of factor VIII.

Factor IX is a glycoprotein synthesized by the liver and its synthesis is vitamin K dependant. This vitamin is necessary for the carboxylation of glutamic acid residues which are essential for the fixation of factor IX on platelet or tissue phospholipids in the presence of calcium ions.



Solid arrow indicates activation of the inactive protein (F IX) to its enzymatic counterpart (F IXa); Note that F IX can be activated via two pathways, either F XIa-Ca^{2+} complex or F VIIa:TF complex as indicated by the dashed arrows and boxes.

Factor IX can be activated via two pathways, either via a F XIa-Ca^{2+} complex or the F VIIa : TF complex. Factor IXa forms an enzymatic complex with phospholipids, Ca^{2+} and factor VIIIa which then activates factor X (Package insert – STA Deficient IX; Ref 00724). It was noted earlier in the summary that F VII concentrations were decreased by aspartame, thus it is hypothesized that factor IX was activated via the other pathway. Aspartame did not affect the concentration of circulating factor IX.

Fibrinogen is a glycoprotein that is synthesized in both the liver and by megakaryocytes. Fibrinogen is composed of six chains: two Aα, two Bβ and two γ. Thrombin (F IIa) breaks up the fibrinogen molecule to split out two fibrinopeptide A (FPA) fragments from the Aα chains and two fibrinopeptide B (FPB) fragments from the Bβ chains. The fibrin monomers that are produced from these reactions then aggregate to form fibrin, which is subsequently stabilized by factor XIIIa. The first step of stabilization consists of the binding of the two γ chains of the two fibrin monomers. This binding is the origin of the D-dimer, the degeneration product that is specific of fibrin. Fibrinogen can be degraded by plasmin. An increase in fibrinogen levels is usually found in cases of diabetes, inflammatory syndromes and obesity. A decrease of the fibrinogen level is observed in DIC and fibrinogenolysis. Furthermore, fibrinogen seems to be involved in the pathogenicity of thrombotic cardiovascular events (Package insert – Fibri-Prest

Automate 2; Ref 00854). Aspartame increased the concentration of circulating fibrinogen. This could be due to the fact that F VII levels and F X levels were decreased, and they are two key factors necessary for the conversion of prothrombin to thrombin. Decreased levels of thrombin leads to higher concentrations of circulation fibrinogen, which was the case after treatment with aspartame.

5.5 CONCLUSION

Blood coagulation involves a biological reinforcement system where a number of circulating proteins form part of a cascade of reactions to for a fibrin clot. The coagulation pathway is activated and coagulation occurs through two pathways known as the intrinsic and extrinsic pathways, made up of twelve different coagulation factors as shown in Diagram 5.1 in the introduction.

For the first part of the study which compares the concentration of the different factors tested between humans and rabbits, it was concluded that there were a number of differences in the quantities of the factors between humans and rabbits, when taking the results obtained in this study into account (Table 5.2). Literature indicates that the rabbit is a good candidate for testing pharmaceutical products. This study established normal ranges for rabbits and therefore rabbits can be used successfully as a model in testing the effect of pharmaceutical products on the different coagulation factors.

The second part of the study focused on the effects of aspartame on the concentration of different coagulation factors. Different factors in the different pathways were studied, and the following can be summarized:

- F II, F V, F IX and the recalcification time of the plasma (aPTT) was not adversely affected by treatment with aspartame.
- The first concentration of aspartame, 34mg/kg, did not adversely affect the tested factors, except for fibrinogen, where a significant increase (P=0.018 two-tailed) was noted. Thus it could be stated that if aspartame were to be taken in small doses, like

the above mentioned concentration, it would not adversely affect blood coagulation except for higher concentrations of circulation fibrinogen being present.

- The 100mg/kg aspartame treatment affected a number of the tested factors. The PT was prolonged, which could be an indication that a deficiency of/or the presence of inhibitors of FI (fibrinogen), factor II (FII), FV, FVII or FX were present. It was found during this study that the concentration of F VII and F X indeed decreased, but not significantly for this concentration. It also did not significantly adversely affect the concentration of F VIII and F IX. However, the concentration of fibrinogen was significantly higher (*P*=0.001 two-tailed) than the values obtained for the first and last concentration of aspartame.

- The values obtained for the PT for the last concentration of aspartame, 150mg/kg, never exceeded the normal set ranges. There was a significant decrease ($p<0.05$) of circulating amounts of F VII, FX and F VIII after treatment with this concentration. F IX was not adversely affected by the last concentration of aspartame. There was a significant increase (*P*=0.0036 two-tailed) in the amount of circulating fibrinogen after treatment with this concentration. A significantly higher concentration of fibrinogen was present than after treatment with 34mg/kg aspartame, but the amount of fibrinogen was significantly less than after treatment with 100mg/kg aspartame. However, an overall increase of fibrinogen was noted.

Factor VII is needed for activation of F IX. Thus lower concentrations of F VII could indicate an inability to activate F IX. Although F VII was decreased, F IX was not adversely affected by the treatment with aspartame. F VII is activated by tissue factor and circulating Ca^{2+} ions, thus a deficiency of any of these 2 cofactors and treatment with the aspartame could have lead to the decrease in circulating F VII. Activated F IX is needed for activation of F X, together with activated F VII, activated F VIII and Ca^{2+} ions. Lowered concentrations of activated F X leads to the inability of prothrombin to be converted to thrombin, which in turn is needed for activation of F VIII. Thus, lower concentrations of activated cofactors F VII and FVIII will indirectly lead to decreased concentrations of F X, which in turn will lead to decreased amounts of thrombin. These findings were supported by the results obtained on the START 4, where decreased concentrations of F VII, F X and F VIII were noted. Thus, it could be said that lower amounts of

prothrombin is converted to thrombin after treatment with aspartame, due to the decrease in concentrations of F VII, F X and F VIII. A decrease in the amount of thrombin could lead to the inability to convert fibrinogen to fibrin and activation of FXIII (needed to convert unstable fibrin to stable fibrin), which explains the high amounts of circulating fibrinogen found in the results.

The results obtained on the START 4 were also corroborated with results obtained in other chapters of this thesis. When the plasma, obtained from the aspartame treated rabbits, was utilized for creating fibrin clots (Chapter 5), the high amount of circulating fibrinogen was converted to fibrin by addition of human thrombin to the plasma. The clots obtained were studied with SEM and it was evident that the change in the amount of fibrin fibres present when compared to the controls and the change in the morphology of the major fibres and mat-like minor fibres could be contributed to the high concentrations of circulating fibrinogen.

Thus, it can be concluded that overuse of aspartame (100mg/kg aspartame and higher per day) may cause bleeding disorders as a number of the important factors (F VII, F X and F VIII) needed for production of thrombin were significantly decreased. Deficiency of thrombin leads to the inability of conversion of fibrinogen to fibrin, and activating of F XIII to stabilize fibrin fibres were also compromised. Thus, seeing that the rabbit and humans have similar fibrin network and platelet aggregate morphology (Chapter 4) and similar amounts of circulating fibrinogen (Table 5.3) it could be postulated that humans with bleeding tendencies should minimize their consumption of aspartame.