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Determination of plasminogen/plasmin levels in horse, cattle, sheep and pig plasma by the use of synthetic chromogenic substrate

Oznaczanie stężenia plazminogenu/plazminy w osoczu krwi koni, bydła, owiec i świń przy użyciu syntetycznego substratu chromonogennego

The fibrinolytic system comprises a proenzyme, plasminogen, which can be converted to the active enzyme, plasmin, which degrades fibrin. Plasminogen activation is mediated by plasminogen activators, which are classified as either tissue-type plasminogen activators (t-PA) or urokinase-type plasminogen activators (u-PA). Inhibition of the fibrinolytic system may occur at the level of the activators or at the level of activated plasmin.

Plasmin has a low substrate specificity, and when circulating freely in the blood it degrades several proteins including fibrinogen, factor V, and factor VIII. Plasma does, however, contain a fast-acting plasmin inhibitor, α_2-antiplasmin, which inhibits free plasmin extremely rapidly but which reacts much slower with plasmin bound to fibrin (7).

Plasminogen activation is the main regulatory process in fibrinolytic system. Fibrinolysis may occur by three different ways: a) by t-PA-dependent way, b) by factor XII-dependent way and c) by factor XII-independent way (17).

t-PA is secreted by endothelial cells and transported to blood circulation. The concentration of this protein is 5 - 50 ng/ml (33) and biological half-life 2.5 min. (3). At this level plasminogen activator inhibitor (PAI) is active (37); liver or vascular endothelium may be the sources of PAI. Units of t-AP activity are based on urokinase units (15). The level of t-AP in blood circulation in man is 0.1 - 0.4 IU/ml and the level of PAI is 5 - 10 IU/ml (38). This, in normal conditions an excess of PAI exists. Diurnal changes in fibrinolytic activity are caused by the changes in PAI activity (21).The decreased t-AP activity in early postoperative period and after heart infarct is also caused by PAI activity increase (12). The rapid changes observed in these states are caused by short half-life of PAI (11). Factor XII-dependent plasminogen activation is one of the most intriguing haemostasis processes. Factor XII after activation to factor XIIa cause the change of precallicrein to callicrein and the last one is a strong plasminogen activator (10).

Factor XII-independent way is urokinase-dependent (3). Leukocyte-derived plasminogen activators enhanced excretion is the cause of coagulation disorders in Chediak-Higashi syndrome (13).

Plasmin is a key enzyme in fibrinolytic system. The enzyme shows a broad, trypsin-like specificity and its unspecific proteolytic activity is regulated in blood circulation by protease inhibitors, such as α_2-antiplasmin. Its inheritant deficiency or absence cause bleeding tendency

(19, 20). Both plasminogen and -antiplasmin are modulated through many factors. In normal conditions, only 60% of plasminogen is available for activation; the remaining portion of this protein is connected with circulating histidine-rich glycoprotein (HRG) (28). Another plasminogen-binding protein, tetranectin, also exists (6).

Thrombolytic activity of t-AP was examined on animal experimental models of lung infarct (4, 30), venous thrombosis (8, 9) and coronary artery thrombosis (14, 16).

The synthesis of synthetic substrate, sensitive to plasmin activity, was essential in the development of the methods of precise plasminogen/plasmin level estimation in human blood plasma (5, 18).

In previous publications (24, 25, 26, 27) we have stated the usefulness of chromogenic substrates for the estimation of platelet factor 3, endogenous heparin and factor VII and VIII levels in blood plasma of domestic animals.

The aim of this study was to evaluate the suitability of the synthetic tripeptide substrate, H-D-Valyl-Leucyl-Lysyl-p-nitroanilide (S-2251) in the estimation of plasminogen/plasmin concentration in blood plasma of domestic animals.

MATERIAL AND METHODS

Principle of the procedure

Plasminogen + Streptokinase (excess) ----------> Plasminogen-streptokinase complex

H-D-Val-Leu-Lys-p-NA $\xrightarrow{\text{Plasminogen-streptokinase complex}}$ H-D-Val-Leu-Lys + p-NA

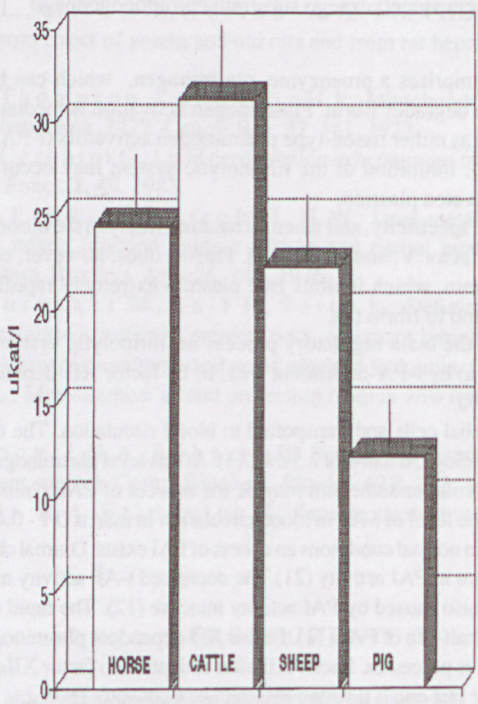

Fig. 1. Plasminogen/plasmin levels in horse (n = 21), cattle (n = 20), sheep (n = 19) and pig (n = 24) plasma. Mean ± S.D.

Plasminogen is converted into an active plasminogen-streptokinase complex when an excess of streptokinase is added. The complex catalyzes splitting of p-nitroaniline (p-NA) from the substrate. The rate at which p-NA is released is measured photometrically at 405 nm.

The blood of 21 horses, 20 cows, 19 sheep and 24 pigs (9 vol) was mixed with 1 vol. of 0.1 M sodium citrate solution and centrifuged at 2000 x g for 20 min.

To standard curve construction, a human plasmin according to 1st British Standard for Plasmin (National Institute for Biological Standards and Controls, London, England) was used for no availability of animal plasmin specimen or standards. The 2 mM substrate (S-2251) solution and streptokinase (4 000 IU/ml) were purchased from Kabi - Vitrum (Molndal, Sweden).

Before estimations, 50 µl plasma was mixed with 2 ml of Tris-HCl buffer (0.05 M, pH = 7.4).

To thermostated (37° C) spectrophotometer cuvette (Specord UV-VIS, Karl Zeiss, Jena, Germany) 200 µl of diluted plasma was poured, and after 4-5 min 200 µl of streptokinase solution was added. Mixture was incubated for 10 min. After incubation period 200 of S-2251 was added and E_{405}/min was measured.

One katal (kat) is defined as the amount of enzyme activity that will release one mole of p-nitroaniline per second.

RESULTS

Figure 1 shows the plasmin activity in blood plasma of examined animal species. The highest activity of plasmin was stated in cattle, the lowest one in pig.

Figure 2 shows the velocity of p-nitroaniline release from S-2251 substrate as dependent on the time of reaction and plasmin concentration. By the incubations

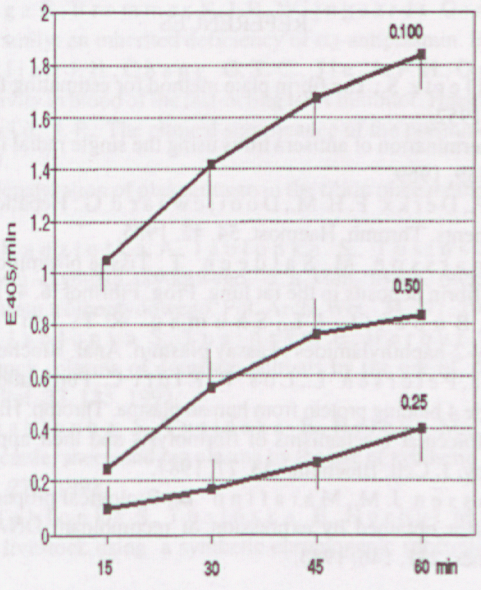

Fig. 2. The rate of p-nitroaniline release from chromogenic substrate as dependent on incubation time (horse plasma, n = 21). Mean ± S.D.

time above 10 min. a dependence was observed. To keep the proportionality between hydrolysis rate and enzyme concentration, the time of incubation of 10 min was chosen.

DISCUSSION

The elevated plasminogen levels were stated in acute bacterial infections, inflammatory states, thrombophlebitis, after major surgery, in myocardial infarction, in pregnancy, the lowered one in disseminated intravascular coagulation (DIC) and in liver cirrhosis (22). The plasminogen level is routinely measured by the use of caseinolytic (18, 34) and fibrinolytic (1, 23) methods, immunological methods are also available (2, 29). However, the long incubation time in radial immunodiffusion methods (a dozen or so hours) keep away the rapid plasminogen level estimations. The caseinolytic method is both laborious and time-consuming.

The amidolytic methods with the use of chromogenic substrates based on the observation that streptokinase forms an equimolar complex with plasminogen (32, 35). Introduction of synthetic substrates (5, 31, 36) allows to elaborate sensitive and rapid methods, useable in laboratory practice.

The results presented above suggest that the S-2251 substrate is entirely useful in plasminogen activity determination in blood plasma of large domestic animals.

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STRESZCZNIE

Oznaczano stężenia plazminogenu/plazminy w osoczu krwi koni, bydła, owiec i świń przy użyciu chromogennego substratu trójpeptydowego p-nitroanilidu H-D-valilo-leucylo-lizyny (S-2251). Najwyższy poziom tego czynnika (31,2 mkat/l) stwierdzono w osoczu krwi bydła, najniższy zaś (12,3 mkat/l) u świń. W osoczu krwi koni i owiec poziomy te wynosiły odpowiednio 24,5 mkat/l i 22,3 mkat/l. Szybkość uwalniania p-nitroaniliny z substratu w zależności od czasu inkubacji w osoczu krwi koni wzrastała z 1,05 do 1,84 (ΔE_{405}/min) przy 0,1 jednostek plazminy w objętości próbki, z 0,25 do 0,83 przy 0,5 jednostki plazminy w objętości próbki, z 0,1 do 0,4 przy 0,25 jednostki plazminy w objętości próbki

Stwierdzono przydatność substratu S-2251 do oznaczania stężenia plazminogenu/plazminy w osoczu krwi zwierząt gospodarskich.