Acta vet. scand. 1987, *28*, 271–277.

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Quantitative Determination of Bovine Plasma Fibrinogen by Rocket Immunoelectrophoresis and by Refractometry after Heat-Precipitation

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Liberg, P.: Quantitative determination of bovine plasma fibrinogen by rocket immunoelectrophoresis and by refractometry after heat-precipitation. Acta vet. scand. 1987, 28, 271–277. – A standard syneresis method for measuring plasma fibrinogen concentrations in cattle was compared with a rocket immunoelectrophoresis method and with a simple refractometer method; the correlations between the results obtained by the standard method and by the other 2 methods were high ($r = 0.94$ and 0.89 respectively, $p < 0.001$). The immunoelectrophoretic method is efficient and simple to perform. In contrast with the standard syneresis method, the other methods allow for the fibrinogen degradation products produced by fibrinolysis in vitro; they can therefore tolerate longer sample storage before analysis. The refractometer method is exceptionally simple and rapid and can be recommended for use under field conditions.

cow; refractometer method; inflammation.

Introduction

The measurement of plasma fibrinogen is useful for the clinical evaluation of hemorrhagic and inflammatory disorders. The standard syneresis method of *Morrison* (1947) which utilizes thrombin for the indirect measurement of clottable protein has generally been accepted as one of the most accurate methods for measuring plasma fibrinogen. The original method or one of its modifications (*Jacobsson* 1955, *Blombäck & Blombäck* 1956, *Nilsson & Olow* 1962) is often used as a reference standard for the evaluation of other methods. The standard syneresis method is highly specific but it is laborious and time-consuming. Immunological methods for the quantitative determination of fibrinogen in human plasma by rocket immunoelectrophoresis have been reported by *Nielsen & Weeke* (1971) and *Ferrari & Fabucci* (1976).

The objective of the present investigation was to evaluate the determination of fibrinogen in bovine plasma by rocket immunoelectrophoresis and by a rapid test based on heat precipitation with a standard syneresis method as reference.

Materials and methods

Animals and samples

In routine diagnostic work during a period of approx. 3 years blood samples were collected by venopuncture from 220 dairy cows with a variety of clinical disorders; the samples were prevented from clotting by the

addition of Na_2-EDTA (2 mg/ml). Plasma was separated by centrifugation. Fibrinogen determinations were performed by thrombin clotting (see below) on fresh plasma. The rest of the plasma was stored at –20°C until required.

Antiserum

A monospecific antiserum was produced in rabbits. A commercial bovine fibrinogen (Sigma, clottable protein 97 %) was used as antigen. The antigen was dissolved in physiological saline to a concentration of 1.25 mg/ml. On days 0, 14, and 28 two rabbits were given subcutaneous injections of 50 µl antigen solution + 50 µl Freund's incomplete adjuvant. On day 50 blood was withdrawn from an ear vein. The antisera produced were tested for specificity and titre by immunodiffusion and immunoelectrophoresis, and then pooled.

Determination of fibrinogen

Rocket immunoelectrophoresis. The electrophoresis was performed at 4–5 V/cm for 16 h at about 10°C. The composition of the gel for the plates (11 cm × 20.5 cm) was: agarose (Miles Seravac) 250 mg, rabbit anti-bovine fibrinogen serum 0.3 ml, barbital buffer (pH 8.6, ionic strength 0.06) 35 ml. The electrode vessels contained the same buffer as the supporting gel. Plasma from 15 clinically healthy cows was pooled and used as a reference sample. The protein concentration (5.15 g/l) was determined, after fibrin clotting, by N-analysis by the Kjeldahl method. The reference plasma sample was divided into portions corresponding to daily requirements and stored at –20°C. To obtain satisfactory immunoprecipitates for quantitation, the plasma was carbamylated as for the determination of human fibrinogen (*Nielsen & Weeke* 1971). Equal volumes of plasma and freshly prepared potassium cyanide (2 mol/l) were allowed to react at 37°C for 30 min and were then placed in cold water. For the establishment of a reference curve the carbamylated plasma was diluted 241-, 121-, 81-, and 61-fold. The carbamylated samples were diluted 201-fold.

Fibrinogen determination by refractometer. The determinations were performed by the method of *Schalm et al.* (1970). After centrifugation in a microhematocrit centrifuge for 5 min the total protein of the supernatant was estimated with a Goldberg hand refractometer (TS-meter, American Optimal Company) before and after heating at 58°C for 3 min and re-centrifugation. The difference between the two values represents the fibrinogen level in plasma.

Standard syneresis method with thrombin clotting. The method used was a modification of that devised by *Jacobsson* (1955). The plasma was clotted with thrombin (Topostasin, Hoffman LaRoch, Switzerland) 30 National Institute of Health units to 1 ml plasma, and the clot was dissolved in boiling 1 N NaOH. The optical density of the solution was determined spectrophotometrically at 280 nm and its nitrogen content was then determined by the Kjeldahl method. The factor for the conversion of optical density to g/l fibrin(ogen) was calculated to be 4.7 (SD 0.1).

The fibrinogen concentration of fresh plasma was determined by the standard syneresis method. According to the original plans the fibrinogen concentrations in stored plasma samples, determined by all 3 methods, should be compared. As shown in Fig. 1 the values of the thrombin clotting were on an average considerably lower in stored than in fresh plasma. Therefore comparisons were made between fibrinogen concentrations determined in fresh plasma by the standard

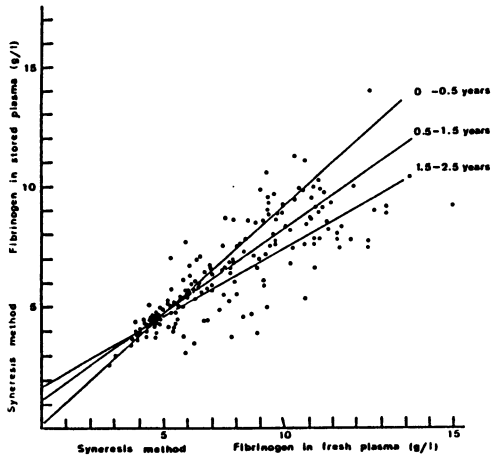

Figure 1. The correlation between the concentrations of fibrinogen determined by the standard syneresis method in plasma stored at 4°C for up to 1 week and in plasma at –20°C for 3 different storage time intervals; regressions: on top (0–6 months) y = 0.88x + 0.15, r = 0.91, middle 1/2–1 1/2 years) y = 0.69x + 1.17, r = 0.85, at the bottom (1 1/2–2 1/2 years) y = 0.54x + 1.74, r = 0.87.

syneresis method, and those determined in stored plasma by rocket immunoelectrophoresis and refractometer.

Statistics

The analytical variation (S_a) was calculated according to the formula $S_a = \sqrt{\frac{\sum d^2}{2n}}$, where d is the difference between two single determinations on the same sample and n is the number of duplicate determinations. When using rocket immunoelectrophoresis the variation was calculated as intraplate variation between double determinations of 4 standards and of the clinical material (n = 220), determined in 23 different plates, and as interplate variation of the standards, in the total material (n = 23).

All measurements of variation were expressed relative to the appropriate mean:

$$\left(\frac{S_a \times 100}{\bar{x}}\ \%\right).$$

Results

Anodic precipitates of bovine fibrinogen suitable for quantitative measurements were obtained by the rocket immunoelectrophoresis method (Fig. 2).

The analytical variation observed when using the rocket immunoelectrophoresis method is shown in Table 1. The intraplate variation was between 1.5 and 2.9 % and the interplate variation was between 4.8 and 6.6 % . The relative standard deviations of duplicate determinations by the standard syneresis method and by the refractometer method were 3.1 and 9.7 % respectively.

There was a strong linear correlation (r = 0.94; $p < 0.001$) between the results of the standard syneresis method in fresh plasma and the results of the rocket immunoelectrophoresis method in stored plasma (Fig. 3). On average the rocket immunoelectro-

Table 1. The analytical variation of plasma fifrinogen concentration by rocket immunoelectrophoresis.

The intraplate variations between double determinations for 4 standards repeated in 23 plates, and of the duplicates of the clinical material are given. The interplate variations for the standards between the 23 different plates are also presented.

Standard (mg/l)	Rocket height ($\bar{x}$, mm)	Analytical variation (%)	
		intraplate	interplate
11	15.6	2.8	6.6
21	24.1	2.9	4.8
31	31.1	1.5	4.9
42	35.5	2.3	5.5
Clinical material		2.9	

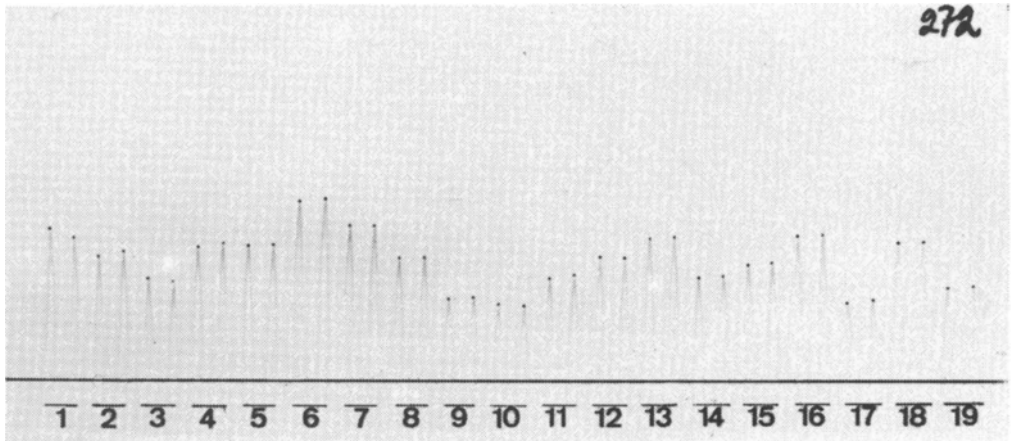

Figure 2. Bovine plasma fibrinogen measured by rocket immunoelectrophoresis in anti-fibrinogen containing agarose gel. 4 µl of carbamylated reference plasma was applied in duplicate in the wells with positions 6–9 and unknown plasma samples were applied in duplicate in the wells with positions 1–5 and 10–19. Dilutions were 1/60, 1/80, 1/120 and 1/240 for the pooled reference plasma and 1/200 for the unknown samples.

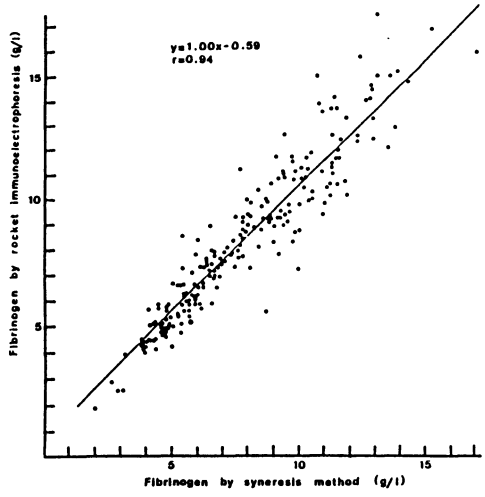

Figure 3. The correlation between the concentrations of bovine fibrinogen determined by the standard syneresis method in plasma stored at 4°C for up to 1 week and by the rocket immunoelectrophoresis method in plasma stored at –20°C for up to 3 years.

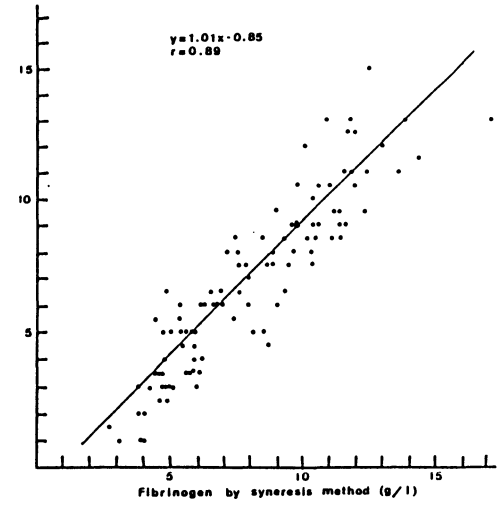

Figure 4. The correlation between the concentrations of bovine fibrinogen determined by the standard syneresis method in plasma stored at 4°C for up to 1 week and by refractometry after heat precipitation in plasma stored at –20°C for up to 3 years.

phoresis determination gave slightly but significantly higher values than the standard syneresis method ($\bar{x}_d$ = + 0.6 g/l; $p < 0.001$).

There was a similarly strong correlation ($r = 0.89$; $p < 0.001$) between the results of the standard syneresis method in fresh plasma and the results of the refractometer method in stored plasma (Fig. 4). On the average the refractometer method gave slightly lower values ($\bar{x}_d$ = − 0.8 g/l; $p < 0.001$) than the standard syneresis method.

The coefficient of correlation between the results of the rocket immunoelectrophoresis and the results of the refractometer method was 0.85 and the linear regression equation was: $y = 0.92x - 0.47$, where y = refractometer fibrinogen, and x = rocket fibrinogen.

Discussion

Plasma fibrinogen concentration has usually been measured by techniques based on the addition of thrombin to plasma. The technical variants of this method usually give reliable results, but the methods are slow and laborious. Polymerisation time methods are readily performed and are possible to automate but have to be closely standardised against other techniques.

Immunological methods are particularly powerful for the determination of plasma protein concentrations and, if specific antisera are available, most plasma proteins can be estimated by the same technique. By using 2 antisera, 2 different proteins can be determined simultaneously. Rocket immunoelectrophoresis is a simple, quick and reproducible method for the determination of many plasma proteins (*Weeke* 1973). The strong correlation ($r = 0.94$) between bovine plasma fibrinogen determined by the standard syneresis method and by rocket immunoelectrophoresis is in agreement with results obtained with human plasma (*Nielsen & Weeke* 1971, *Ferrari & Fabucci* 1976). Rocket immunoelectrophoresis can thus be used routinely to determine plasma fibrinogen in cattle over a wide range of concentrations.

Storage at −20°C reduced the plasma concentration of fibrinogen measured by the standard syneresis method (Fig. 1). Since the reproducibility of this method was good it is probable that the losses of fibrinogen during storage were caused primarily by fibrinolysis in vitro. No synthetic competitive plasminogen inhibitor was added to the samples to depress fibrinolytic activity.

However, fibrinolysis during storage will probably not influence the measurement of fibrinogen concentrations by either the rocket immunoelectrophoresis or the refractometer method. It has been clearly demonstrated that the quantitatively dominant fibrinogen degradation products produced by fibrinolysis have the same antigenic determinants as the intact fibrinogen molecules, whereas only a refraction of the degradation products retain their clottability (*Marder et al.* 1969). As a result fibrinolysis in vitro should not interfere with an immunological determination of fibrinogen. Furthermore, although the clottability characeristics of the intact fibrinogen molecule are lost as a result of fibrinolysis, the heat precipitating characteristics of most of the degradation products are preserved (*Marder et al.* 1969). For these reasons the results of the rocket immunoelectrophoresis and the refractometer methods obtained in stored plasma have been compared with the results of the standard syneresis method obtained in fresh plasma. The mean fibrinogen concentration determined by immunoelectrophoresis in stored plasma was slightly higher than the mean concentration determined by the standard syneresis method in fresh plasma. This dif-

ference suggests that there may already have been fibrinogen degradation products in the fresh plasma which were not clotted by thrombin in the standard method but which were measured by the rocket immunoelectrophoresis and refractometer methods. The results obtained by the standard syneresis method and by the refractometer method also showed a strong correlation ($r = 0.89$, $p < 0.001$) in agreement with the observations of *Sutton* (1977). With the refractometer the fibrinogen concentration can be read only to the nearest g/l, although the repeatability of the method (relative standard deviation of duplicate 9.7 %) is acceptable. The values obtained by the refractometer method were slightly lower ($\bar{x}_d = -0.8$ g/l) than those obtained by the standard syneresis method and there was no explanation for this difference. In considering whether the errors of the refractometer method limit its clinical usefulness the samples can be classified as either normal or increased by the 2 methods. Using the author's established upper normal limit of fibrinogen concentration in the cow ($\bar{x} + 2$ SD = 5.4 g/l) 85 % of samples could be classified as either normal or increased by both methods.

In conclusion, rocket immunoelectrophoresis in an efficient, simple, and reproducible method for plasma fibrinogen determinations in cattle. The refractometer method is particularly rapid and simple and is also considered to be useful, particularly under field conditions.

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Sammendrag

Kvantitativ bestämning av bovint plasma fibrinogen med raketimmunoelektrofores och med refraktometri efter värmeprecipitation.

En standard syneresmetod för bestämning av plasmafibrinogen hos nötkreatur jämfördes med raketimmunoelektroforesbestämning och med en enkel refraktometermetod; korrelationen mellan standardmetoden och de andra metoderna var god ($r = 0{,}94$ och 0,89 respektive, $p < 0.001$). Den immunoelektroforetiska metoden visades vara en effektiv och enkel laboratoriemetod for fibrinogenbestämning. Refraktometermetoden är utom-

ordentligt enkel och snabb och får anses specielt lämplig för bestämningar under fältmässiga förhållanden.

I motsats till standard syneresmetoden tar de andra metoderna hänsyn till fibrinogennedbrytningsprodukter uppkomna genom fibrinolys in vitro. Användandet av dessa metoder kan således tillåta längre lagringstider av proverna med viss in vitro nedbrytning av fibrinogenet och ändå ge en korrekt spegling av ursprungskoncentrationen av intakt fibrinogen i provet.

(Received March 9, 1987).

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