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Note

Detection of fibrinogen heterogeneity by capillary isotachophoresis

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The heterogeneity of human fibrinogen has been demonstrated by the differences in the solubilities of various clottable fractions, as well as by differences in their electrophoretic mobilities and in their molecular masses [1–4]. Sodium dodecyl sulphate (SDS) electrophoresis studies [5,6] of fibrinogen heterogeneity have established that fibrinogen consists of two major and one minor clottable fractions differing in molecular mass. The two lower-molecular-mass fractions are believed to be proteolytic derivatives of high-molecular-mass fibrinogen. The mechanism by which this limited proteolysis takes place is so far unknown. It has been postulated that different proteolytic mechanisms are involved in the elaboration of clottable and unclottable derivatives (fibrinogen degradation products, FDPs) [7]. An endothelial cell effect has been proposed, but not proved, and thrombin and plasmin have been previously excluded [8–11]. The relative amounts of the fractions have been reported to vary in some diseases such as cancer, diabetes, and occlusive vascular diseases [12].

In this work we used analytical capillary isotachophoresis (ITP) to study human fibrinogen. This technique has been used as a method for the assay of several proteins. The most interesting feature of capillary ITP lies in the fact that it provides qualitative and quantitative information about proteins without requiring their denaturation or staining [13]. Moreover this technique is a highly potent tool for the separation of compounds exhibiting very small differences in their net mobility.

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EXPERIMENTAL

Reagents

Chemicals were obtained from the following manufacturers: hydroxypropylmethylcellulose (HPMC) from Dow Chemicals (Midland, MI, U.S.A.); 2-N-(morpholino)ethanesulphonic acid (MES) from Merck (Darmstadt, F.R.G.); β-analine and valine from Merck; 2-amino-2-methyl-1,3-propanediol (Ammediol) and 6-aminohexanoic acid from Merck-Schuchardt (Munich, F.R.G.); glycine from Canalco (Bethesda, MD, U.S.A.); poly(ethylene glycol) (PEG) 6000 and barium hydroxide from BDH (Poole, U.K.); hirudin from Sigma (St. Louis, MO, U.S.A.); aprotinine (Trasylol) from Bayer (Milan, Italy); thrombin from Boehringer (Mannheim, F.R.G.).

Plasma preparation

Plasma was obtained from citrated venous blood collected with aprotinine (12 000 kI.U./ml) after centrifugation for 10 min at 1500 *g* at 4°C. Plasma samples were collected from healthy donors after informed consent and from children affected by leukaemia, during asparaginase treatment, or by diabetes. All plasma samples were immediately utilized for the purification of fibrinogen.

Fibrinogen purification

Fibrinogen was purified by the method of Vila et al. [14]. This method permits the isolation of fibrinogen using PEG 6000 and salting-out procedures. Isolated fibrinogen was dissolved in phosphate-buffered saline (pH 7.4) and immediately analysed by ITP.

Isotachophoresis

The apparatus used was the LKB 2127 Tachophor (LKB, Bromma, Sweden). Separation was carried out in a 23-cm PTFE capillary tube, kept at a constant temperature of 20°C. The apparatus was equipped with a UV detector set at 280 nm.

The leading electrolyte was 5 m*M* Mes–10 m*M* Ammediol–0.5% HPMC (pH 9.1). The terminating electrolyte was 10 m*M* 6-aminohexanoic acid–10 m*M* Ammediol, adjusted to pH 10.8 with barium hydroxide. The initial current was 200 μA, maintained until a voltage of 15 kV had been reached. The current was then reduced to 50 μA. During detection, under a constant current of 50 μA, the voltage rose from 5 to 16 kV. The separation time was less than 30 min. The samples were 3 μl of purified fibrinogen samples; ranging from 4 to 140 mg/dl. The calibration curves were obtained by measuring peak areas (cm^2). In some experiments 1 μl of spacer solution (1.6 mg/ml glycine and 1.6 mg/ml valine) were used [15]. In other experiments, bovine thrombin (1 U/ml) was added to purified fibrinogen solutions. After clot formation, samples were centrifuged. The clot was discharged and 3 μl of the supernatants were injected.

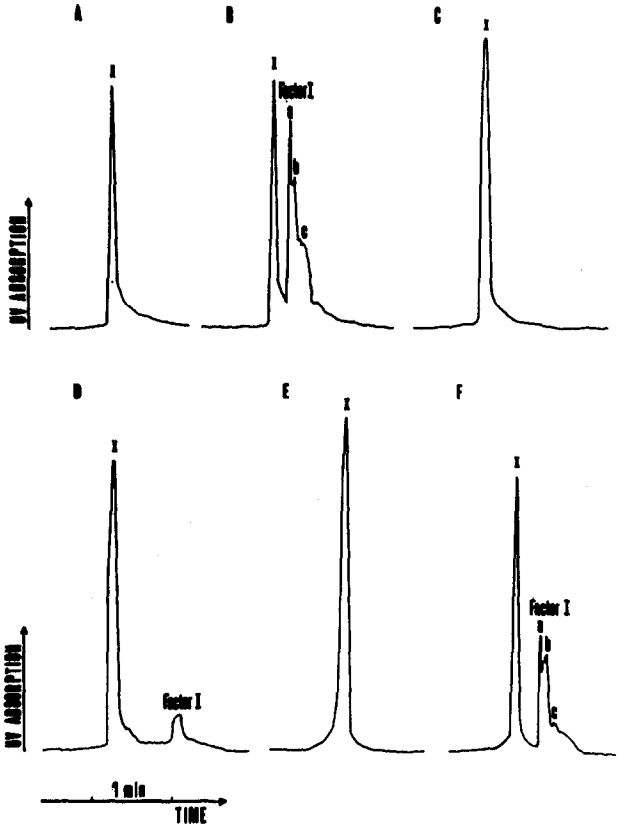


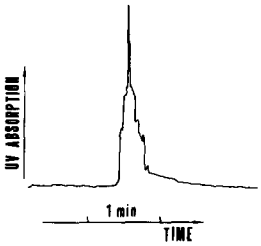
Fig. 1. Representative UV trace of human fibrinogen (panel B, Factor 1). The peak X indicates the leading impurity (panel A). The UV trace of thrombin-treated fibrinogen solutions is shown in panel D, while panel F represents the isotachopherogram obtained by adding fibrinogen to these preparations again, after inhibition of thrombin by hirudin. The UV traces of thrombin alone or thrombin plus hirudin are shown in panels C and E.

Standard fibrinogen assay

Fibrinogen was assayed in plasma or purified fibrinogen solutions by the method of Clauss [16] or by an immunological method [17]. FDPs were assayed as previously reported [18].

RESULTS AND DISCUSSION

Fig. 1B shows a characteristic profile of fibrinogen prepared from healthy donors. This profile may be divided in three parts (Fig. 1B, a,b,c). When the UV-undetectable amino acids (glycine and valine) were injected no separation of these consecutive zones took place. This may suggest that the above-mentioned zones represent fibrinogen fractions. In fact these amino acids, which act as discrete spacers, allow the separation of fibrinogen in the ITP analysis of human plasma (data not shown). Unclottable proteins are virtually absent in our fibrinogen preparations. Indeed, in the isotachopherograms of the supernatants,



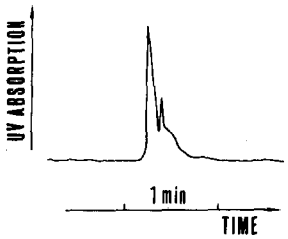


Fig. 2. ITP profile of purified fibrinogen taken from a patient affected by microangiopathia.

Fig. 3. ITP profile of fibrinogen obtained from a leukaemic patient during asparaginase treatment.

obtained from thrombin-treated fibrinogen preparations after clot removal, these peaks do not occur (Fig. 1D). They reappear when purified fibrinogen is added to these supernatants after inhibition of thrombin by hirudin (2 U/ml). Injection of thrombin-treated samples increases the peak corresponding to leading impurity (Fig. 1A). We consider that this increase is caused by the addition of thrombin in the samples to trigger the transformation of the fibrinogen in the water-insoluble protein, i.e. fibrin. Hence it appears that thrombin forms a mixed zone with leading impurities (Fig. 1C). On the other hand, it is unlikely that the increase in the first peak is caused by split products of the fibrinogen, because the fibrin monomers migrate very slow [19] and the fibrinopeptides are undetectable by the electrolyte system used in these experiments.

It has already been demonstrated that the isolation of fibrinogen from human plasma by precipitation with PEG yields a final product that is not contaminated with other plasma proteins, is not denatured and maintains its biological functions [14]. In particular, plasmin or thrombin contamination has not been found [14]. We were unable to detect FDPs in our preparations. We can conclude that ITP may be useful in the study of fibrinogen heterogeneity.

An interesting finding is that ITP profiles changed under some pathological conditions. Fig. 2 shows a representative isotachopherogram of fibrinogen taken from a diabetic patient affected by microangiopathia. Fig. 3 shows the profile of fibrinogen from a leukaemic patient during asparaginase therapy, which is known to cause a- or dys-fibrinogenaemia. The changes in the ITP profiles indicate a different pattern of fibrinogen fractions. This could correspond to a variation of the ratio between the high-molecular-mass and the low-molecular-mass fractions. The pathogenic implications of these variations have been already reported [12].

Reproducibility of ITP was checked by carrying out five consecutive determinations on the same sample of fibrinogen. A coefficient of variation of less than 3% was found. The relationship between injected fibrinogen and peak area was linear over the range 4–14 mg/dl. In fact, linear regression analysis of peak area (y) on fibrinogen concentration (x) gave the equation $y=1.17+0.21x$, with a correlation coefficient (r) of 0.998 ($n=6; p<0.001$). The lower limit of detection of fibrinogen by ITP was 4 mg/dl.

This technique may improve the analysis of the protein under various patho-

logical conditions. It must be recalled that the fibrinogen samples have to be prepurified before the ITP study. In fact, the plasma samples themselves cannot be used, because under these experimental conditions the fibrinogen may form mixed zones with immunoglobulin A [1]. However, the overall yield of isolated fibrinogen we have managed to obtain from the original plasma is ca. 60%, as also previously reported [14]. These data demonstrate that ITP can also be used to investigate the properties of fibrinogen in hypo- or dys-fibrinogenaemic patients. The advantage of the method are its sensitivity and reproducibility, and the possibility of analysing fibrinogen in a short time without denaturation of the protein.

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REFERENCES

1 B. Blomback and M. Blomback, Ark. Kemi., 10 (1956) 415.
2 P.J. Gaffney, Nature (New Biol.), 230 (1971) 54.
3 G.A. Jamieson and P.J. Gaffney, Biochim. Biophys. Acta, 154 (1968) 96.
4 M.W. Mosesson, J.S. Finlayson and R.A. Umfleet, J. Biol. Chem., 247 (1972) 5210.
5 I. Lipinska, B. Lipinski and V. Gurewich, J. Lab. Clin. Med., 84 (1974) 509.
6 B. Holm and G.H. Godal, Thromb. Res., 35 (1984) 279.
7 V. Surevich, I. Lipinska, E.E. Gordon and M. Palmi, Thromb. Res., 22 (1981) 535.
8 I. Lipinska, B. Lipinksi, V. Gurewich and W.D. Hoffman, Am. J. Clin. Pathol., 66 (1976) 258.
9 H. Hasegawa, H. Nagata, M. Yamauchi, I. Murakoshi, Y. Oguma and H. Takei, Acta Haematol. Jpn., 44 (1981) 149.
10 D. Mills and S. Karpatkin, Biochim. Biophys. Acta, 251 (1971) 121.
11 B. Holm, D.W.T. Nilsen and H.C. Godal, Thromb. Res., 41 (1986) 879.
12 I. Lipinska, B. Lipinski, W. Gurewich and K.D. Hoffman, Am. J. Clin. Pathol., 66 (1976) 958.
13 P. Delmotte, in A. Adam and C. Schots (Editors), Biochemical and Biological Application of Isotachophoresis, Elsevier, Amsterdam, 1980, p. 259.
14 V. Vila, E. Reganon, F. Llopis and J. Aznar, Thromb. Res., 39 (1985) 651.
15 D. Del Principe, C. Colistra, A. Menichelli, G. Biancini, C. D'Arcangelo, G. Multari, B. Werner, M. Lemmo and G. Digilio, J. Chromatogr., 342 (1985) 285.
16 J. Clauss, Acta Haematol., 17 (1957) 237.
17 G. Mancini, J.P. Vaerman, A.O. Carbonara and J.F. Heremans, in H. Peeters (Editor), Peptides of the Biological Fluids, Elsevier, Amsterdam, 1964, p. 370.
18 D.J. Castelan, J. Hirsh and M. Martin, J. Clin. Pathol., 21 (1968) 638.
19 D. Del Principe, G. Biancini, C. D'Arcangelo, G. Mancuso, M. Persiani and A. Menichelli, Pediatr. Res., 15 (1981) 1185.