Technical methods

Influence of sample preparation on estimates of blood fibronectin concentration

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In view of the growing interest in blood concentrations of fibronectin (FN) it is appropriate to sound a note of caution regarding the preparation and storage of specimens for FN measurement. We used blood from 12 volunteers to investigate the effect of using different anticoagulants in plasma preparation, different clotting times in serum preparation, and storage of these samples, on FN concentrations.

Material and methods

Fibronectin concentrations were determined by single radial immunodiffusion¹ on 1% agarose supplemented with 3% polyethylene glycol, using a specific antiserum to FN and standards supplied by Behringwerke, Marburg, Lahn, West Germany. The plates were incubated at room temperature for four days to reach completion, and stained with Coomassie blue.

Blood was obtained from 12 volunteers, six men and six women. Fibronectin was measured in the plasma or serum obtained after spinning down a portion of whole blood. Portions of whole blood were treated as shown in Table 1.

The sera produced by methods 2–4 inclusive were each divided into two portions; one was stored at

–20°C, one at –70°C. The FN concentrations were determined immediately after preparation of the plasma or serum, and subsequently after freezing and thawing the samples five times, FN being measured after each thawing. The time interval between each thawing was one week.

Results

The results are presented in Figs. 1–3 and Table 2. Leaving blood to clot for 5 to 6 h resulted in the same FN concentration, which was lower by 33–69% than that in blood clotted for 1 h. The temperature of storage, whether –20°C or –70°C, made no appreciable difference to FN concentrations (Fig. 1). The effect of storage on serum prepared by clotting blood at 4°C and 37°C (method 1, 5) was similar to that on serum prepared at 21°C (method 2). It was observed that the higher the clotting temperature, the greater the amount of FN retained in serum (Table 2).

Although EDTA plasma had the highest FN concentrations when assayed immediately after storage, these became unpredictable in that on sequential freezing and thawing the amount of FN fluctuated extensively and concentrations ranged

Table 2 *Effects of sample preparation on the initial value of FN*

Method			Initial fibronectin mean $\pm$ SD (mg/l)
1	Clotted at 4°C	in glass tubes	232 $\pm$ 31
2	Clotted at 21°C		266 $\pm$ 47
5	Clotted at 37°C		288 $\pm$ 52
6	Clotted at 21°C	in plastic tube	283 $\pm$ 51
7	EDTA plasma		352 $\pm$ 53
8	Heparin plasma		238 $\pm$ 49
9	Citrate plasma	tubes containing the anticoagulant	322 $\pm$ 41

Table 1 *Treatment of samples for FN measurement*

Method	Tube	Clotting		Anticoagulant added	Comment
		Temperature (°C)	Time (h)		
1	Glass	4	1	—	Stirred to effect haemolysis
2	Glass	21	1	—	
3	Glass	21	5	—	
4	Glass	21	6	—	
5	Glass	37	1	—	
6	Plastic	21	1	—	EDTA Citrate Heparin
7	Plastic	21	1	—	
8	Plastic	21	1	—	
9	Plastic	21	1	—

Fig. 1 Change in FN concentrations after storage of serum prepared by methods 2 and 3. (Storage temperatures and duration shown for each method.)

Fig. 3 Effect of clotting blood in a plastic tube (method 6) compared to a glass tube (method 2) under the same conditions.

Fig. 2 FN concentrations after storage of plasma prepared by methods 8 and 9.

from 20 to >500 mg/l in any one sample. The effect of using other anticoagulants is shown in Fig. 2. The loss of FN after storage when the blood was clotted in a plastic tube is seen in Fig. 3.

Discussion

Serum is a poor indicator of circulating FN concentrations as FN binds to the fibrin clot and the measured concentration is influenced by rate of clot formation, ambient temperature and delay in separation of serum from the clot.

Fresh EDTA plasma shows the highest FN concentration of the plasma samples, but in this anticoagulant, as with heparin, concentrations are extremely inconsistent after storage. Citrate plasma was the most stable medium for the measurement of FN after storage. It is not certain what causes massive fluctuations in FN in some samples. Fibronectin is particularly sensitive to degradation by plasma proteases² so the formation of fragments with the antigenic properties of FN may result in an apparently high value on radial immunodiffusion (RID). Alternatively, aggregation of FN molecules may produce particles too large to diffuse in agarose gel so would result in an apparently low value on RID. Heparin would be expected to affect FN concentrations as it is well known to form a cold precipitable complex with FN.³ EDTA prevents the activation of complement and damages platelets,⁴ with which FN is known to interact.⁵

When methods such as RID are being used, especially in clinical research, it is important to be able to use stored samples so that the assays can be performed in economical batches. We have demonstrated that citrated plasma is the only suitable medium to use for the measurement of FN concentrations in stored samples. We suggest that, for routine collection of samples for fibronectin measurement, blood is taken into a plastic tube containing 1 vol of trisodium citrate dihydrate (3·13%) per 9 vol blood, mixed thoroughly and separated within 3 h by spinning at 3000 rpm for 15 min. The separated plasma should be stored at -20°C in a plastic tube until assayed.

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References

- ¹ Mancini G, Carbonara AO, Heremans JF. Immunochemical quantitation of antigens by single radialimmunodiffusion. *Immunochemistry* 1965;2:235-54.
- ² Vaheri A, Vartio T, Stenman S, Saksela O, Hedman K, Alitalo K. Fibronectin and proteinases in tumor invasion. In: Strauli P, Barrett AJ, Baici A, eds. *Proteinases and tumor invasion*. New York: Raven Press, 1980:49-57.
- ³ Mosesson MW, Amrani DL. Review: the structure and biologic activities of plasma fibronectin. *Blood* 1980;56:145-58.
- ⁴ Mollison PL. *Blood transfusion in clinical medicine*. 6th ed. Oxford: Blackwell Scientific Publications, 1979.
- ⁵ Pearlstein E, Gold LJ, Garcia-Pardo A. Fibronectin: a review of its structure and biological activity. *Mol Cell Biochem* 1980;29:103-28.

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Measurement of conjugated and unconjugated serum bile acid concentrations using 3 α -hydroxysteroid dehydrogenase

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Many reports have highlighted the importance of distinguishing between conjugated and unconjugated serum bile acids¹⁻⁵ and thus have revived interest in earlier studies.^{6,7} Serum bile acids may be separated into their unconjugated and conjugated fractions by the method of Alme *et al.*⁸ Serum extracts are first subjected to cation exchange (with Amberlyst A-15) and the bile acids are then separated using the lipophilic anion exchanger diethylaminohydroxypropyl Sephadex LH-20 (Lipidex-DEAP). Once separated, the bile acids can be measured using gas-liquid chromatography,⁵ or

radioimmunoassay.⁹ However, when we applied the hydroxysteroid dehydrogenase-fluorimetric assay for bile acids¹⁰ we obtained absurdly high recoveries from serum samples. This interference appeared to be associated with the use of Amberlyst A-15. When SP-Sephadex was substituted for Amberlyst A-15,¹¹ no interference with subsequent enzyme analysis was observed.

Material and methods

Serum (usually 2 ml aliquots) was diluted with 0.1 M NaOH (10 ml) and methanolic extracts were prepared using either XAD-2 and the batch procedure¹² or, recently, 0.01 M NaOH and Sep-Pak-C₁₈ cartridges.¹³ An aliquot of the methanolic extract was kept for direct analysis of total 3 α -hydroxy bile acids and the remainder evaporated to dryness. The residue was dissolved in 72% ethanol (vol/vol 2.5 ml) and applied to a Pasteur pipette column of SP-Sephadex (Sigma Chemical Co; 50 mm $\times$ 5 mm [internal diameter], taken to the H⁺ form with 0.05 M HCl in 72% ethanol). The eluate from the SP-Sephadex column was allowed to flow directly on to the Lipidex-DEAP column (Packard Instrument Ltd; 18 mm $\times$ 12 mm [internal diameter]; prepared as described⁸). A further 2.5 ml 72% ethanol was added to the SP-Sephadex and the eluate again allowed to flow directly on to the Lipidex-DEAP column. The SP-Sephadex column was then discarded. The Lipidex-DEAP column was then eluted with the following solutions, prepared as described by Alme *et al.*,⁸ each eluate being collected separately before the next solution was applied:

(i) 72% ethanol, 5 ml; (ii) 0.05 M acetate pH 3.8, 3 ml; (iii) 0.1 M acetate pH 3.8, 1 ml; (iv) 0.1 M acetate pH 3.8, 2 ml; (v) 0.3 M acetate pH 5.0, 3 ml; (vi) 0.3 M acetate pH 9.6, 3 ml. Eluates 2 and 3 were pooled, as were 4, 5, and 6. Each pooled fraction was evaporated to dryness and the residue dissolved in methanol. The methanolic extracts were then analysed using the enzymatic fluorimetric technique. The pooled fraction of eluates 2 and 3 contained the unconjugated bile acids, that of eluates 4, 5, and 6, the conjugated bile acids.

Results and discussion

During development of the method, separation was evaluated using standard solutions and labelled bile acids (¹⁴C-cholate, ¹⁴C-glycocholate, ¹⁴C-taurocholate and ³H-glycochenodeoxycholate). The recoveries of labelled and non-labelled bile acids from both aqueous solutions and serum samples that were taken through the entire procedure were high with negligible quantities appearing in other fractions (Tables 1 and 2). The results in 18 healthy subjects (laboratory personnel, nine women) were: fasting total 3 α -hydroxy bile acids, 9.1 μ mol/l $\pm$ 3.5 (mean $\pm$ SD; range 3.9-16.8 μ mol/l), the corresponding unconjugated bile acid fraction was 32.4% $\pm$ 18.1