The cause of turbidity in lyophilised plasmas and its effects on coagulation tests

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Abstract

Aims: To investigate the cause of turbidity in reconstituted lyophilised plasmas and to determine its effect on coagulometers.

Methods: The turbidities of 20 normal plasmas and 16 reconstituted lyophilised plasmas were determined by comparing a 1 in 4 dilution in distilled water with a standard suspension in an Aminco Fluorocolorimeter (American Instrument Co) in nephelometric mode. The turbidities of five other plasmas were determined before and after lyophilisation. The turbid components of fresh and reconstituted lyophilised plasmas were studied using electron microscopy. The effects of turbidity on five types of coagulometer were determined by adding varying concentrations of a turbidity enhancing material.

Results: Reconstituted lyophilised plasmas were more turbid than normal plasmas, because of agglomerated liposomes. Serum depleted of chylomicrons and very low density lipoproteins was not rendered more turbid by lyophilisation. Three out of five types of automated coagulometer tested gave activated partial thromboplastin times which were appreciably affected by plasma turbidity. One of the instruments was unable to detect a clot in a moderately turbid plasma. A second instrument gave results which were significantly affected by turbidity. Turbidity of the substrate plasma did not affect specific factor VIII assays in two types of coagulometer.

Conclusions: Lyophilisation of plasma induces turbidity due to the agglomeration of lipids. Such turbidity can affect the results of coagulation tests. Suppliers of lyophilised plasmas should be aware of this problem.

Plasma is naturally turbid. Turbidity of serum is caused by the presence of lipid containing complexes, chylomicrons, and very low density lipoproteins (VLDL).¹ The turbidity of plasma is probably attributable to the same factors. Reconstituted lyophilised plasmas are invariably more turbid than the plasmas from which they are prepared. Such turbidity may be undesirable if the plasmas are to be used in coagulometers based on nephelometric or absorptiometric principles.

Methods

PLASMA TURBIDITY MEASUREMENTS

The technique was based on a method for the measurement of water turbidity, described by the American Water Works Association and the American Public Health Association.² Hydrazine sulphate ($[\text{NH}_2]_2\text{H}_2\text{SO}_4$) (Sigma) 1.0 g and hexamethylenetetramine (Sigma) 10.0 g were each dissolved in 100 ml of distilled water. Five ml of each solution were mixed, diluted to 100 ml with distilled water, and incubated at 26°C for 24 hours. This solution gradually develops a stable turbidity due to the formation of formazin polymer particles. A value of 400 nephelometric turbidity units (NTU) was ascribed to this suspension. From this a series of working standards from 0–200 NTU were prepared. The turbidities of 20 normal plasmas and 16 reconstituted lyophilised plasmas were determined by comparing a 1 in 4 dilution in distilled water with a standard suspension in an Aminco Fluorocolorimeter (American Instrument Company) in nephelometric mode. The 16 lyophilised plasmas comprised seven obtained commercially and nine lyophilised in our laboratory. The turbidities of five further plasmas were determined before and after lyophilisation.

ELECTRON MICROSCOPY

Electron microscopic studies were performed on fresh and reconstituted lyophilised plasmas. One millilitre of each plasma was added to 1.0 ml of distilled water and 3.0 ml of 30 g/l glutaraldehyde added. These preparations were centrifuged at $65\,000 \times g$ for one hour. Aliquots of the upper turbid layers were applied to pialoform (Agar Scientific) coated electron microscopy grids and dried in air. They were then examined in a Philips EM420 transmission electron microscope (magnification between $\times 10\,000$ and $\times 105\,000$).

TURBIDITY OF SERA DEPLETED OF LIPOPROTEINS

Four serum samples of varying lipid content were obtained from the Department of Biochemistry at Withington Hospital, Manchester. These comprised lipid rich, chylomicron and VLDL depleted, chylomicron VLDL and low density lipoprotein (LDL) depleted, and lipid free. A fifth serum, devoid of chylomicrons alone, was prepared by centrifugation of lipid rich serum at $3000 \times g$ and removal of the upper layer. The turbidity of these sera was determined before and after lyophilisation.

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Electron micrographs of the turbid fraction of natural (A) and reconstituted lyophilised (B) plasmas. Scale bar = 600 nm.

EFFECT OF PLASMA TURBIDITY ON COAGULOMETERS
The turbidity of several aliquots of one plasma was varied by adding standard volumes of the standard formazin suspension. Activated partial thromboplastin times (APTT) by the Manchester APTT method³ were performed on five types of coagulometer: Amelung KC10 (Baxter), IL 300R (Instrumentation Laboratory), IL 810 (Instrumentation Laboratory), MLA Electra 700 (Lab Medics) and Coagamate $\times 2$ (Organon Teknika). Factor VIII assays were also carried out on the plasma series using the two different models of coagulometer from Instrumentation Laboratory (IL 300R and IL 810). Formazin suspension was added to a relatively clear factor VIII deficient plasma to test the effect of substrate plasma turbidity in an APTT based on stage factor assay.

Results

The mean (SD) turbidities of fresh and reconstituted lyophilised plasmas were 344 (126) and 1179 (56) NTU, respectively. The mean (SD) of the commercially obtained and locally prepared reconstituted lyophilised plasmas were 1533 (637) and 904 (351), respectively. The turbidities of fresh and reconstituted plasmas were compared by unpaired *t* test and found to be significantly different ($p < 0.05$). The mean (SD) turbidity of the five plasmas tested before and after lyophilisation were 416 (181) and 715 (280), respectively. These were compared by paired *t* test and found to be significantly different ($p < 0.05$).

Electron microscopic examination showed that fresh and reconstituted lyophilised plasmas were significantly different. Fresh plasma contained lipid rich particles of between 150 and 300 nm in diameter. Reconstituted lyophilised plasma contained large lipid rich agglomerates of up to 4 μm diameter, which showed structural liposome rearrangement. The commercially obtained plasmas contained the largest agglomerates. Electron micrographs of fresh and reconstituted commercially obtained lyophilised plasmas are shown in the figure.

The turbidity values of the sera series depleted of liposomes are shown in table 1. Chylomicrons and VLDL were responsible for the increased turbidity of lyophilised plasmas.

The APTT values of aliquots of a single plasma with turbidity values artificially adjusted to between 264 and 3484 NTU, as measured on five coagulometers, are shown in table 2. The Amelung KC10, IL 300R, and MLA Electra 700 seemed to show no turbidity induced effects. The IL 810 was unable to detect the clot in a sample with a turbidity of more than 1216 NTU. The Coagamate $\times 2$ gave an APTT value which increased with turbidity. These relations were compared by regression analysis and the significance of the observed correlations was tested by the F test. A significant correlation ($p < 0.05$) between turbidity and APTT was found in the Coagamate $\times 2$ and the IL 300R. The tight precision

Table 1 Turbidity values before and after lyophilisation of a series of sera of differing lipoprotein content

Serum type	Pre-lyophilisation turbidity (NTU)	Post-lyophilisation turbidity (NTU)
Serum	500	720
Serum depleted of chylomicrons	440	720
Serum depleted of chylomicrons and VLDL	500	760
Serum depleted of chylomicrons VLDL and LDL	200	280
Lipid free serum	200	220

Table 2 APTT values (seconds) of a single plasma "spiked" with varying levels of turbidity tested on five coagulometers

Turbidity (NTU)	Amelung KC10	IL 810	IL 300R	Organon Coagamate × 2	MLA Electra 700
264	35.0	37.8	29.8	26.3	33.0
411	34.3	39.8	29.8	25.8	31.6
571	34.5	39.4	30.0	27.5	32.0
896	35.7	38.6	29.8	28.5	33.0
969	35.5	41.0	29.7	29.3	32.5
1067	34.4	41.3	30.0	29.2	32.6
1216	35.0	CE	30.2	30.0	32.4
1560	34.9	CE	30.0	29.4	32.4
1738	35.2	CE	30.5	29.6	32.1
2082	35.2	CE	30.4	29.6	32.6
2329	34.6	CE	31.1	30.3	32.2
2525	35.3	CE	30.5	30.3	33.0
2671	35.2	CE	30.7	30.4	32.6
3007	35.0	CE	30.9	30.1	32.4
3289	34.9	CE	30.6	31.0	32.5
3484	34.4	CE	30.2	31.2	32.3

CE = Coagulation error

(CV 1.4%) of the latter instrument allowed a very small turbidity induced effect to be noted. There was no significant correlation between turbidity and APTT in the other analysers ($p > 0.05$).

Factor VIII assays performed on both types of IL coagulometer provided no significant correlation between plasma turbidity and factor concentration (data not shown) (factor assays were performed on these instruments because they use different clot detection principles when performing factor assays). Increasing the turbidity of a factor VIII deficient plasma from 520 to 1520 and 3520 NTU did not significantly affect the results obtained when assaying factor VIII concentrations of 20 and 100 IU/dl on either model of IL coagulometer.

Discussion

Lyophilisation significantly increased plasma turbidity. Electron microscopy showed that the turbidity was due to structural rearrangement and agglomeration of the lipid vehicles in plasma. The different liposomes in plasma contain different quantities of cholesterol, triglyceride, and phospholipid. The latter, being present in the liposome surface with

apolipoprotein, give the liposomes increased water solubility. The observed effects were probably due to liposome membrane fusing as previously described.⁴⁻⁶ Lyophilised material has been found to be unreliable in the assay of cholesterol⁷ and apolipoproteins.⁸ This may be due to the changed characteristics of lyophilised liposomes.

The turbidity measurements of sera depleted in liposomes showed that lyophilisation induced turbidity is caused mainly by chylomicrons and VLDL.

The observed effects of plasma turbidity on coagulometers is important. The mean turbidity of lyophilised plasmas was only slightly less than the value which adversely affected the IL 810 (1179 v 1216 NTU). This instrument has been superseded but many remain in use. The Coagamate × 2 and the IL 300R gave APTT values which varied with turbidity. The high precision of the IL 300R allows a small upward drift to be detected but this is not of any real importance. The results with the Coagamate × 2 gave more cause for concern. A five second difference is evident between the least and most turbid plasmas. Users of this instrument would obtain a result on a reconstituted lyophilised plasma which could be significantly affected by its turbidity. Lipaemic samples could also give the same effect. Such plasmas have been shown to have turbidities of up to 6800 NTU.

In conclusion, turbidity induced by lyophilisation is caused by lipids and can affect the results of coagulation tests. Suppliers of lyophilised plasmas should be aware of this problem.

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