A rapid and simple method for measurement of total protein in very low density lipoproteins by the Lowry assay

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Summary When total protein in very low density lipoprotein samples is measured by the method of Lowry et al. (1951. *J. Biol. Chem.* **193:** 265–275), turbidity remains in the final color reaction. Addition of 0.1 ml of 2.5% (v/v) solution of Triton X-100 removes this turbidity immediately and effectively. This simple modification is faster, less cumbersome, and more economical than removing turbidity with ethyl ether or chloroform. Addition of 0.1 ml of 2.5% (w/v) sodium dodecyl sulfate to the final reaction mixture is also effective in removing turbidity and can be used as an alternative method.—**Kashyap, M. L., B. A. Hynd, and K. Robinson.** A rapid and simple method for measurement of total protein in very low density lipoproteins by the Lowry assay. *J. Lipid Res.* 1980. **21:** 491–495.

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The method of Lowry et al. (1) is used extensively for protein measurement. When this method is used for determining protein content in very low density lipoproteins (VLDL), turbidity is noted in the final solution prior to spectrophotometry. Ethyl ether (2, 3) and chloroform (4, 5) have been added to the final reaction mixture to remove this turbidity. Sodium dodecyl sulfate (SDS) has also been used for measuring proteins in insoluble proteolipids (6–8) and to a very limited extent for lipoproteins (9). Markwell et al. (9) added SDS to the alkali reagent and measured the protein concentration in lipoprotein samples. This method was compared with the measurement of lipoprotein protein after lipid extraction with diethyl ether. Data on the absorbance of the final color development of a single sample of low density lipoproteins (which do not give rise to a turbidity problem) was reported. Similar values for both methods were found. No comparison of this method was made with chloro-

Abbreviations: TG, triglycerides; VLDL, very low density lipoproteins; SDS, sodium dodecyl sulfate; EDTA-saline, 0.15 M sodium chloride in 1 mM diosodium ethylenediaminetetraacetic acid; BSA, crystalline bovine serum albumin.

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form extraction. Thus, an exact description or validation of the SDS method as applied to *turbid* VLDL samples is not available. Kruski and Narayan (10) described a method in which turbid samples were applied to filter paper discs, dried, and the lipid extracted with ether or chloroform. The dried residue was then quantitated for protein by the method of Lowry et al. Removal of turbidity with these organic solvent extraction methods is time consuming and cumbersome. More importantly, if not performed with adequate care to remove turbidity, the procedure may yield spuriously elevated values. Different procedures using ethyl ether have been described and often the exact method employed is not stated. Gustavson (2) described the removal of turbidity with ethyl ether, and other workers (3) indicated that more than one wash with ether was required to remove turbidity completely.

Preliminary work in our laboratory indicated that upon addition of polyethylene glycol p-isooctylphenyl ether (specifically Triton X-100, a registered trademark of Rohm and Haas Co.), the turbidity was removed immediately. Since this is a simple procedure, we compared this modification to the two methods commonly used for removing turbidity, viz. ethyl ether and chloroform. In addition, the observation of Markwell et al. (9) regarding the use of SDS for VLDL protein measurement has been modified and compared with the Triton X-100, ethyl ether, and chloroform methods.

MATERIALS AND METHODS

Venous blood was drawn from normal subjects and patients being managed for hypertriglyceridemia at the Lipid Clinic. Blood was drawn in tubes containing the disodium salt of ethylenediaminetetraacetic acid (EDTA) to yield a final concentration of 1.5 mg/ml blood. Following centrifugation at 4°C to obtain plasma, VLDL was isolated using a 40.3 Beckman rotor (11). The supernate from the first ultracentrifugation was layered with EDTA-saline and spun to obtain 'washed' VLDL. Triglycerides (TG) were measured according to the Lipid Research Clinic's methodology (12). Triton X-100 was purchased from Packard Instrument Company, Inc., Downers Grove, IL. Ethyl ether was purchased from Burdick and Jackson Laboratories, Inc., Muskegon, MI. Chloroform was obtained from Matheson, Coleman, and Bell, Norwood, OH. Sodium dodecyl sulfate was purchased from Canalco, Inc., Rockville, MD. Crystalline bovine serum albumin (BSA) was obtained from Sigma Chemical Co., St. Louis, MO.

The procedure for measuring protein was followed according to Lowry et al. (1) using BSA as standard. The absorbance was read at 750 nM using a Gilford 250 spectrophotometer. All sample measurements were done at least in triplicate. Appropriate dilution of the VLDL sample was made to ensure that its absorbance was in that part of the standard curve where absorbance was proportional to the BSA standard. The turbidity in the final sample was removed according to the following methods.

Triton X-100

Triton X-100 (0.1 ml of a 2.5% (v/v) stock solution) was added (to a final concentration of 0.1%) and the mixture was gently stirred using a vortex mixer. The effects of different concentrations of Triton X-100 on the standard curve with BSA and on serial dilutions of VLDL samples were also assessed.

Chloroform: one-wash method

Two ml of chloroform was added to the final reaction mixture and mixed vigorously over a vortex shaker. The tube was spun in a refrigerated centrifuge at 2000 rpm for 5 min. The supernate was transferred to a cuvette for spectrophotometry.

Chloroform: two-wash method

Two ml of chloroform was added to the supernate of the single wash procedure and shaken. Centrifugation was repeated as above and the supernate from this second chloroform was used for spectrophotometry.

Ethyl ether

Two ml of ethyl ether was added to the final reaction mixture, shaken and centrifuged at 2000 rpm for 5 min in a refrigerated centrifuge. The ether supernate was pipetted off and another 2.0 ml of ethyl ether was added and the procedure was repeated. The infranate was transferred to a cuvette for spectrophotometry.

Sodium dodecyl sulfate (SDS)

One-tenth ml of 2.5% (w/v) solution was added to the final reaction mixture, mixed, and the contents were transferred to a cuvette and its absorbance was determined.

RESULTS

At the suggested concentration of 0.1% Triton X-100 in the final mixture, no displacement of the standard curve with BSA was noted. Above a concentration of 0.5% a gradual shift of the curve to the right was noted. The standard BSA curve was unaffected

by treatment with chloroform, ethyl ether, or addition of 0.1 ml 2.5% SDS solution to the final reaction mixture. **Table 1** shows the results of protein determination in six pairs of VLDL samples by each of the four methods. The data indicate that over a wide range of VLDL TG concentrations studied (53 mg/dl to 1528 mg/dl), no appreciable difference was noted using Triton X-100 and any of the other methods except for the chloroform (single wash) method. The single wash chloroform method gave a mean concentration that was 19.8% higher than the results obtained by the Triton X-100 method. The concentrations obtained by the single chloroform wash method were consistently higher than results obtained by the Triton X-100 method. The correlation coefficient of the Triton X-100 method and the chloroform (single wash) chloroform (two washes), ethyl ether and SDS were 0.994, 0.998, 0.997, and 0.999 respectively. The standard deviations of the means of the set of a pair were within 4% of each other. An exception was the chloroform (single wash) method where a greater degree of scatter was observed for the same set of samples measured by the Triton X-100 method. The following additional observations were made using these methods.

TABLE 1. Comparison of protein concentrations of VLDL using Triton X-100, chloroform ethyl ether, and sodium dodecyl sulfate

A. Triton X-100 vs Chloroform (One Wash)[a] Method

Sample Number	Triglyceride Concentration	VLDL protein concentration	
		Triton X-100	Chloroform
	mg/dl	*mg/ml*	
1	1528	4.10	5.20
2	1325	2.65	2.90
3	433	0.84	1.04
4	240	0.59	0.66
5	892	2.26	2.60
6	607	1.71	2.14
Mean ± S.D.		2.02 ± 1.29	2.42 ± 1.62

B. Triton X-100 vs Chloroform (Two Washes) Method

Sample Number	Triglyceride Concentration	VLDL protein concentration	
		Triton X-100	Chloroform
	mg/dl	*mg/ml*	
1	1528	4.04	3.96
2	1325	2.60	2.50
3	433	0.82	0.80
4	240	0.59	0.65
5	892	2.16	2.30
6	607	1.67	1.72
Mean ± S.D.		1.98 ± 1.27	1.99 ± 1.23

C. Triton X-100 vs Ethyl Ether Method

Sample Number	Triglyceride Concentration	VLDL protein concentration	
		Triton X-100	Ethyl Ether
	mg/dl	*mg/ml*	
1	117	0.37	0.37
2	1395	2.73	2.75
3	1030	2.50	2.65
4	365	1.36	1.32
5	672	2.02	1.96
6	53	0.19	0.18
Mean ± S.D.		1.53 ± 1.07	1.54 ± 1.11

D. Triton X-100 vs Sodium Dodecyl Sulfate Method

Sample Number	Triglyceride Concentration	VLDL protein concentration	
		Triton X-100	SDS
	mg/dl	*mg/ml*	
1	1528	4.14	4.12
2	446	1.16	1.20
3	607	1.74	1.64
4	78	0.13	0.13
5	1300	3.30	3.40
6	720	1.80	1.80
Mean ± S.D.		2.04 ± 1.45	2.05 ± 1.47

[a] See text for details.

Triton X-100

Addition of this agent to the final reaction mixture resulted in an almost immediate clearing of turbidity and a clear blue solution. The absorbance measurements at 750 nm were stable and the digital readout readings did not fluctuate as much as when ethyl ether was used (see below). When Triton X-100 was added to the lipoprotein solution prior to addition of the reagents used in the analysis, a white precipitate formed. The coefficient of variation of the same sample measured ten times was 3.6%.

Ethyl ether

Preliminary results indicated that a single wash with 2.0 ml of diethyl ether did not in all cases even visibly remove turbidity. This has been previously noted (3). In more lipemic samples, two or more washes were necessary to remove turbidity. Compared to the results obtained by the Triton X-100 method, results with ethyl ether were higher unless the sample was washed several (two or more) times with this reagent. The absorbance was not stable, i.e. the readings were erratic and failed to stabilize for as long as 5 min with some samples. When properly performed, the coefficient of variation by this method was 3.8% (n = 10).

Chloroform

Two washes of chloroform were necessary to remove turbidity due to VLDL samples. One wash gave higher values (Table 1) over the range of VLDL TG concentrations studied. Results using the two-wash method were similar to those obtained with Triton X-

100 method. The coefficient of variation by this method was 3.6% (n = 10).

Sodium dodecyl sulfate

This method gave results that were comparable with those obtained using Triton X-100. However, with some lipemic samples the absorbance reading fluctuated for as long as 1 min before stabilizing. The coefficient of variation by this method was 3.5% (n = 10).

DISCUSSION

The two common methods for removal of turbidity in the final color reaction used in protein measurement of VLDL samples by the method of Lowry et al. are ethyl ether and, less commonly, chloroform. A limited description on the use of SDS to remove the turbidity was reported (9), but the method was not validated against the methods commonly used for VLDL protein measurement. Several publications deal with a modification of Lowry's method when Triton X-100 (which interferes with the assay) has been used for solubilizing proteins (13–18). The concentration of Triton X-100 used in this assay was much lower than that used for dissolving insoluble proteins. Our results indicate that no shift in the standard curve occurred with the low concentration used in our assay system.

In several publications dealing with measurement of VLDL protein, no statement is made regarding the removal of turbidity in the samples. Thus it is possible that the concentrations expressed may have been higher because of incomplete removal of turbidity. These methods, including the method described by Kruski and Narayan (10), are cumbersome, time consuming, more expensive, and potentially less accurate compared to the simple methods using either Triton X-100 or sodium dodecyl sulfate. Triton X-100 is perhaps the method of choice since it gives more stable absorbance readings in our experience. A white precipitate was seen if Triton X-100 was added prior to the addition of assay reagents. This confirms previous observations (13, 14) and emphasizes that this reagent should be added to the final reaction mixture prior to absorbance determinations. One wash of chloroform for turbidity removal gave higher values than the two-wash method. The two-wash method gave results comparable to the Triton X-100 method. The ethyl ether method was more time consuming and gave erratic absorbance readings. At least two washes were necessary. Both organic solvent extraction methods required two centrifugations. If the washing with organic solvents is not done carefully, the values obtained can be questionable regarding their accuracy. Ethyl ether has an added fire hazard if the treated sample is centrifuged in a nonprotected centrifuge. Potential discrepancies between different laboratories in the absolute values of protein concentrations of VLDL samples may partially be explained by differences in the exact method employed. Adoption of the Triton X-100 method for the protein measurement of VLDL samples may be useful for comparison of results between different laboratories.

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