Use of a rapid and highly sensitive fluorescamine-based procedure for the assay of plasma lipoproteins

G. Michael Funk,[1,*] Charles E. Hunt,[*] Dennis E. Epps,[**] and Patricia K. Brown[*]

Pathology and Toxicology Research,[*] and Physical and Analytical Chemistry Research,[**] The Upjohn Company, 301 Henrietta Street, Kalamazoo, MI 49001

Summary A rapid and sensitive method for determining protein concentrations using fluorescamine has been characterized for use in the analysis of intact lipoproteins. It was shown that there is no interference with the assay due to the presence of lipid-associated turbidity or primary amine content. The assay was shown to be sensitive to as little as 0.3 μg of lipoprotein and to yield similar results when compared to the Lowry method. — **Funk, G. M., C. E. Hunt, D. E. Epps, and P. K. Brown.** Use of a rapid and highly sensitive fluorescamine-based procedure for the assay of plasma lipoproteins. *J. Lipid Res.* 1986. **27:** 792–795.

Supplementary key words lipoproteins • fluorescamine • protein assay • Lowry method

The accurate quantification of proteins and peptides in solution is of paramount importance to many areas of biological and biochemical research. Numerous quantitative methods for the determination of proteins and peptides have been published; however, the classic biuret (1) and Lowry (2) procedures are most often employed, with the method of Lowry et al. (2) being the method of choice for the assay of lipoproteins. Neither method is adequate when the quantification of very low protein concentrations is desired.

Fluorescamine, 4-phenylspiro[furan-2(3H), 1'-phthalan]-3,3'-dione, was originally synthesized as a reagent for the quantitative fluorometric determination of amino acids, peptides, and proteins in the picomole range (3). The use of this reagent was extended to the quantification of apoproteins, peptides derived from proteins, and the monitoring of column fractions to detect eluted peptides or proteins. Fluorescamine is highly reactive (almost instantaneous) with primary amines and only the reaction products yield fluorescence. However, some peptides must be subjected to alkaline hydrolysis in order to be detected with fluorescamine.

Lipoproteins and lipoprotein fractions have traditionally been assayed by the procedure of Lowry et al. (2) in the presence of sodium lauryl sulfate. However, when analyzing lipoproteins from such sources as small animals, interstitial fluids, or isopyknic gradients, the concentration of protein is frequently insufficient for accurate quantification by the Lowry procedure. In the course of analyzing the lipid composition of small animal lipoproteins subjected to isopyknic gradient ultracentrifugation, we found that many fractions contained too little protein but sufficient lipid (as analyzed by gas–liquid chromatography (4)) for quantification. We were thus unable to compare lipid/protein ratios between fractions with any degree of confidence. We therefore undertook the characterization of this fluorescamine assay for the determination of lipoprotein concentrations. In this work, we show the applicability of fluorescamine for the quantification of intact plasma lipoproteins. Protein aliquots as low as 0.3 μg can be quantified accurately. It is linear over a wide range and is not adversely affected by the lipids associated with lipoproteins. This assay as presented is an attractive alternative to the procedure of Lowry et al. (2) when higher sensitivity is desired.

MATERIALS AND METHODS

Bovine serum albumin, fraction V, fatty acid-free and fluorescamine were obtained from Sigma Chemical Company. Acetone was from Burdick and Jackson Laboratories, Inc. Iodine-125 was purchased from New England Nuclear. All other reagents were analytical grade, obtained through standard suppliers.

Human lipoproteins were obtained from normal male subjects who fasted overnight. Blood was collected via venipuncture into Na_2EDTA, 1.5 mg/ml. The plasma (80 ml) was separated by centrifugation at 1000 *g* for 30 min at 4°C, and the lipoproteins were isolated by preparative ultracentrifugation at the following densities (g/ml) by the addition of solid KBr: VLDL < 1.006, LDL 1.019–1.063, and HDL 1.063–1.21. All ultracentrifuge procedures were carried out in a Beckman L8-80 ultracentrifuge (Beckman Instruments) at 5°C in Beckman 60Ti or 70.1Ti rotors at 45,000 to 55,000 rpm for 20–23 hr. All preparations were washed one time and then exhaustively dialyzed against 0.9% NaCl, 0.01% Na_2EDTA, 10^{-3} M NaN_3, pH 7.4 (lipoprotein buffer).

Lipoproteins were iodinated using the iodine monochloride method (5). Unbound ^{125}I was removed by passing the preparations through prepacked desalting columns (Pharmacia) after which they were dialyzed against lipoprotein buffer to remove the remaining unbound iodine. The percentage of ^{125}I bound to lipid was determined by counting an aliquot of the lower phase obtained from extracted (6) iodinated preparations. The lipid label values were 11.0% for VLDL, 4.5% for LDL, and 1.8% for HDL. Soluble ^{125}I in the preparations was <1% as

Abbreviations: VLDL, LDL, and HDL, very low, low, and high density lipoproteins, respectively.

[1]To whom reprint requests should be addressed.

measured by precipitation in 20% trichloroacetic acid. All ^{125}I quantification was performed in a Packard Autogamma 5780 gamma scintillation counter (Packard Instrument Co.) operating at a counting efficiency of 78%.

Iodinated lipoprotein aliquots containing 3000–5000 cpm $^{125}I/\mu g$ of protein were delipidated with 2 ml of chloroform–methanol ($CHCl_3$–MeOH) 1:1 at 4°C overnight. The tubes were then centrifuged at 1000 *g* for 25 min at 4°C, after which 1 ml of the $CHCl_3$–MeOH supernatant was removed and 1 ml of cold $CHCl_3$–MeOH 2:1 was added and mixed. This wash procedure was performed seven times which resulted in removal by serial dilution of greater than 99% of the lipid as confirmed by gas–liquid chromatographic total cholesterol analysis of nonextracted lipoprotein aliquots versus the $CHCl_3$–MeOH supernatants obtained from the delipidations. After the final wash, the remaining milliliter of $CHCl_3$–MeOH was evaporated under nitrogen. Each tube was counted prior to and after delipidation and protein recovery was calculated using these counts, the TCA-soluble counts in the iodinated preparations, and the lipid label data. Protein recoveries ranged from 87 to 97%. These tubes were assayed and compared to nondelipidated assay tubes to ascertain whether or not the presence of lipids and/or turbidity affected the determination of protein concentration.

The standard protein assay was by a modification of the method of Lowry et al. (2) using sodium lauryl sulfate as described by Markwell et al. (7).

The fluorescamine assay was performed as described previously (3) virtually unmodified. Briefly, aliquots of either BSA standard or sample were pipetted into 13 × 100 mm borosilicate tubes and dried in a forced air oven at 110°C. After cooling, 0.5 ml of 0.5 N NaOH was added to each tube and alkaline hydrolysis was performed at 122°C in an Amsco laboratory sterilizer (American Sterilizer Company) in the automatic liquids cycle with the sterilization time set at 20 min. Upon cooling, 0.4 ml of 0.5 N HCl was added and mixed followed by addition of 2 ml of 0.5 M sodium borate buffer, pH 8.5. The fluorescamine (0.23 ml of a 30 mg/dl solution in acetone made fresh biweekly) was added to the tubes while stirring on a vortex mixer. Mixing was continued for several seconds. The contents of each tube was transferred into a quartz standard ultraviolet transmitting cell (Spectrocell) for measurement of relative fluorescence. Fluorescence measurements were made on an SLM 4800S (SLM Instruments) in the steady-state mode. Rhodamine was used in the reference channel as a quantum counter to correct for variations in excitation light intensity. Fluorescence emission was measured in the ratio mode as the ratio of the light intensity (voltage) at the emission photomultiplier tube to that of the reference photomultiplier tube. The excitation and emission slits were 4 and 8, respectively. Fluorescamine–protein fluorescence was determined with excitation at 390 nm and emission at 475 nm. Placement of a 470 nm cutoff filter (Schott Optics) in the emission path did not significantly alter the background or improve the linearity or intensity of the fluorescence. Standards and unknowns were measured in quadruplicate.

Coefficient of variation (CV) for assays was calculated by dividing the standard deviation of the data set by the mean.

RESULTS

When lipoproteins are assayed using the fluorescamine system, significant turbidity results with VLDL exhibiting more than LDL and HDL. Also, since fluorescamine reacts with primary amines, we wanted to determine whether or not the presence of lipid-associated primary amines, e.g., phosphatidylethanolamine and phosphatidylserine, caused significant contribution to the lipoprotein fluorescence. In the first set of experiments, we compared lipoprotein samples in which the lipids had been extracted using $CHCl_3$–MeOH alongside the same samples that were not extracted. Those that were not extracted exhibited the turbidity noted above while the extracted samples were clear. Assays comparing the two were run simultaneously on the same mixtures of ^{125}I-labeled lipoproteins and uniodinated lipoproteins. The assays of the extracted samples were corrected for ^{125}I-labeled protein recovery from the extractions, which took into account TCA-soluble ^{125}I and counts due to lipid label. Results are shown in **Table 1.** We obtained similar concentrations for the lipoprotein fractions whether or not the lipids were removed.

In the second group of experiments, we compared the Lowry and fluorescamine methods on the same lipoprotein samples on the same day. The fluorescamine assay compared well with the Lowry assay on each lipoprotein fraction. Results are shown in **Table 2.**

TABLE 1. Protein concentrations for nonextracted and extracted lipoprotein samples

Lipoprotein	Protein Concentration	
	Nonextracted	Extracted
	μg/ul	
VLDL (n = 10)	0.66 ± 0.03	0.66 ± 0.05
LDL (n = 10)	3.28 ± 0.35	3.40 ± 0.04
HDL (n = 10)	7.31 ± 0.26	7.47 ± 0.28

Values are mean ± SD for the indicated numbers of determinations.

Coefficients of variation were measured on seven assays performed over a period of 4 weeks using aliquots of BSA standard and lipoprotein sample that were pipetted in quadruplicate, stored at −80°C, and thawed as needed. Variance of the intra-assay fluorescence of the standards ranged from 0.024 to 0.035, while that of the lipoproteins ranged from 0.025 to 0.035. The interassay CVs of the lipoprotein concentrations were 1.6% for VLDL, 2.3% for LDL, and 4.0% for HDL.

We then addressed the issue of the sensitivity of the fluorescamine system. Sensitivity is ultimately limited by the fact that there is light scattering by the borate buffer alone. This background scattering was not attenuated by instrument adjustment or by inclusion of a 470 nm cutoff filter. However, we were still able to achieve sensitivity of 0.3 μg for each of the lipoprotein preparations and BSA. A representative 0.3–5.0 μg curve is shown in **Fig. 1.** We observed linearity to as high as 50 μg (data not shown). Since the sensitivity of the instrument had to be increased with decreasing protein concentration, it may be necessary to limit the range of the standard curve in some cases to preserve a good signal-to-noise ratio.

Fluorescence intensity was measured for each of the lipoproteins and BSA over a period of 2 hr after addition of fluorescamine to the samples (data not shown). The fluorescence was stable during the first hour, varying less than 1.5% for all preparations. However, fluorescence decreased as much as 5.9% by the end of 2 hr. We performed all measurements between 15 min and 1 hr after the addition of fluorescamine to the samples.

DISCUSSION

The fluorescamine procedure described for assaying plasma lipoproteins is very useful when increased sensitivity and reproducibility are essential, e.g., in interstitial fluids, isolates from isopyknic gradients, or samples from small animals. We have determined that lipoproteins can be measured accurately over a wide range of concentrations using this method, including aliquots as small as 0.3 μg.

Fluorescamine is highly reactive toward primary amines and the possibility that some phospholipids (e.g., phosphatidylethanolamine) might contribute significantly to fluorescence had to be ruled out. Accordingly, lipoproteins were quantified in the presence and absence of bound lipid (Table 1). There was no interference with the assay; results were similar whether or not associated lipids were extracted from the proteins. We also found that there is no interference with the assay due to turbidity caused by protein-associated lipids. The assay is linear (Fig. 1) with an excellent correlation coefficient and, in the case of human lipoproteins, yields similar results when compared with the Lowry method (Table 2). Something to be considered, when comparing protein assay by fluorescamine and by the Lowry method, is that when using the Lowry assay one apparently is measuring the relative dye-binding characteristics of the reference and sample proteins that are due to their tyrosine content (8). It has been shown that, in membrane protein preparations and presumably in soluble lipoprotein preparations, the Lowry method can yield significantly higher values when compared to quantitative amino acid analysis (9). When measuring protein by the fluorescamine reaction, one is assuming that the quantum yield of fluorescence from the amino acid content of the reference protein is similar to that of the sample protein (8). It would be of interest to know which assay assumption is more valid. While there is little intuitive reason why the Lowry and fluorescamine assays should compare favorably, it is none the less beneficial that they do, as the Lowry method is widely used in the assay of lipoproteins.

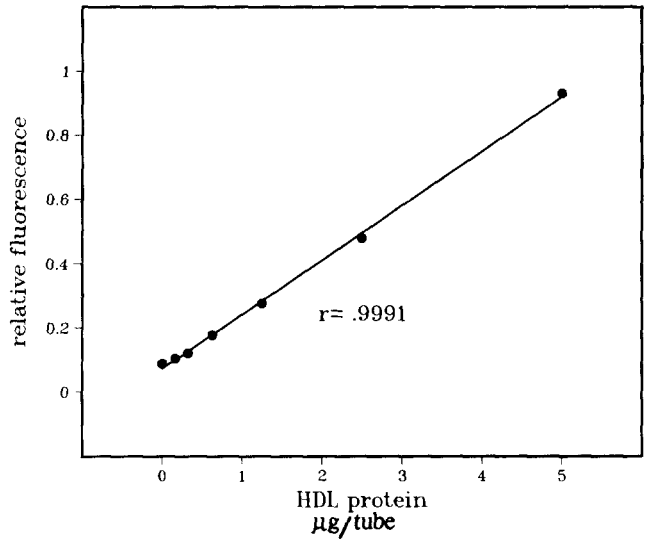

Fig. 1. A representative 0.3–5.0 μg high density lipoprotein curve using the fluorescamine-based assay as presented. Relative fluorescence is plotted on the ordinate against mass (μg/tube) on the abscissa. Each point represents the average of four replicate determinations.

TABLE 2. Protein concentrations for lipoprotein samples assayed by the Lowry and fluorescamine methods

Lipoprotein	Protein Concentration	
	Lowry	Fluorescamine
	μg/μl	
VLDL (n = 10)	0.73 ± 0.01	0.73 ± 0.01
LDL (n = 10)	3.31 ± 0.04	3.41 ± 0.12
HDL (n = 10)	7.90 ± 0.17	8.14 ± 0.29

Values are mean ± SD for the indicated numbers of determinations.

The authors wish to thank Mr. David Powell for preparing the manuscript.

Manuscript received 5 August 1985.

REFERENCES

1. Gornall, A. G., C. J. Bardawill, and M. M. David. 1949. Determination of serum proteins by means of the biuret reaction. *J. Biol. Chem.* **177:** 751-766.
2. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265-275.
3. Nakai, N., C. Y. Lai, and B. L. Horecker. 1974. Use of fluorescamine in the chromatographic analysis of peptides from proteins. *Anal. Biochem.* **58:** 563-570.
4. Kaluzny, M. A., L. A. Duncan, M. V. Merritt, and D. E. Epps. 1985. Rapid separation of lipid classes in high yield and purity using bonded phase columns. *J. Lipid Res.* **26:** 135-140.
5. Bilheimer, D. W., J. L. Goldstein, S. M. Grundy, and M. S. Brown. 1975. Reduction in cholesterol and low density lipoprotein synthesis after portacaval shunt surgery in a patient with homozygous familial hypercholesterolemia. *J. Clin. Invest.* **56:** 1420-1430.
6. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226:** 497-509.
7. Markwell, M. A. K., S. M. Haas, N. E. Tolbert, and L. L. Bieber. 1981. Protein determination in membrane and lipoprotein samples: manual and automated procedures. *Methods Enzymol.* **72:** 296-303.
8. Bohlen, P., S. Stein, W. Dairman, and S. Udenfriend. 1973. Fluorometric assay of proteins in the nanogram range. *Arch. Biochem. Biophys.* **155:** 213-220.
9. Peters, W. H. M., A. M. M. Fleuren-Jakobs, K. M. P. Kamps, J. J. H. H. M. dePont, and S. L. Bonting. 1982. Lowry protein determination on membrane preparations: need for standardization by amino acid analysis. *Anal. Biochem.* **124:** 349-352.