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Rapid high-performance liquid chromatographic protein quantitation of purified recombinant Factor VIII containing interfering substances

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ABSTRACT

A simple and rapid high-performance liquid chromatography method for the quantitative estimation of protein content in highly purified recombinant Factor VIII (rFVIII) solutions containing substances which interfere in other methods has been developed. The method is an adaptation of a procedure [Stoffel *et al.*, *Hoppe-Seyler's Z. Physiol. Chem.*, 363 (1982) 1117] described for separating peptides from tryptic digests. Many detergents, stabilizing reagents, amino acids, or high salts may interfere in the more common protein determination assays, such as the Lowry, Bradford, bicinchoninic acid or A_{280} nm reading. This simple high-performance liquid chromatography method separates protein from interfering substances, such as amino acids, detergents, and high salts by size-distribution chromatography in the presence of 90% formic acid. The 90% formic acid mobile phase is an extremely effective solvent for proteins, including any aggregated protein which may be present. The formic acid dissociates non-covalent bonding and allows the proteins to move in a gel permeation system as a single peak which is well separated from UV interfering substances. Sample preparation is not necessary. Quantitation is based upon UV absorbance at 280 nm using a protein standard similar to the sample being quantitated. When analyzing highly purified proteins, the standard may be assigned a protein content based upon the amino acid composition of that protein. The method is accurate, fast, reproducible and relatively easy to perform.

INTRODUCTION

Protein assays such as the Lowry, Bradford, bicinchoninic acid (BCA) or A_{280} are affected by interfering substances[1]. Plasma-derived Factor VIII of recombinantly engineered Factor VIII (rFVIII) is an inherently unstable protein which requires the addition of stabilizers during its purification. The presence of these stabilizers pre-

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cludes the use of the classical protein quantitation methods. Sample manipulation such as dialysis or gel filtration to remove the interfering substances is unreliable, because of potential diminution of biological activity or absorptive protein losses. In order to assay unaltered rFVIII, it was necessary to develop an alternative method of quantitation which required no sample preparation.

This paper describes the simple and rapid quantification of highly purified rFVIII by high-performance gel chromatography in 90% formic acid. The method described is a high-performance liquid chromatography (HPLC) procedure which separates protein from interfering excipients and stabilizers with a molecular weight $\geqslant$ 12 500 dalton by size distribution. The mobile phase, 90% formic acid, dissociates non-covalent bonding and allows the protein to move in the gel permeation system as a single peak well separated from other UV-absorbing substances. Quantitation is determined by the peak area of the protein as measured at 280 nm and the results are expressed in μg/ml based on a standard curve generated with a rFVIII standard. Although this paper focuses on rFVIII, the procedure should be readily adaptable to other proteins such as human serum albumin and haptoglobin which were used to develop the assay.

MATERIALS AND METHODS

Apparatus and materials

The liquid chromatograph consisted of a Model 114 M constant-flow solvent pump, a Model 160 UV-absorbance detector equipped with a 280-nm filter and low-volume analytical flow cell, and a Model 340 system organizer fitted with a Model 7125 sample injector containing a 10-μl fixed loop (Beckman-Altex, San Ramon, CA, U.S.A.). The sample injector was a product of Rheodyne (Cotati, CA, U.S.A.). The LiChrosorb Si-100, 10 μm column (250 mm $\times$ 7 mm I.D.) was purchased from Alltech (Deerfield, IL, U.S.A.). An Uptight, short column (2 cm $\times$ 4 mm I.D., Upchurch, Oak Harbor, WA, U.S.A.) packed with pellicular silica (Alltech) served as the guard column. A Hewlett-Packard (Palo Alto, CA, U.S.A.) Model HP3393A digital electronic integrator plotted and integrated the signals received from the UV detector. The mobile phase, 90% formic acid, was prepared by diluting 900 ml of GR-grade formic acid (EM Science, Cherry Hill, NJ, U.S.A.) with 100 ml of HPLC-grade water (J. T. Baker, Phillipsburg, NJ, U.S.A.). The 90% formic acid solution was filtered through a 0.2-μm, 47 mm diameter polycarbonate membrane filter (Poretics, Livermore, CA, U.S.A.) using a 1-l ground-glass joint flask equipped with a 300-ml glass funnel and tubulated base (Millipore, Milford, MA, U.S.A.).

Chromatographic conditions

The mobile phase was 90% formic acid in HPLC-grade water. The flow-rate was 1.0 ml/min. The detector sensitivity was set at 0.020 a.u.f.s. (at 280 nm), the chart speed was 0.5 cm/min, and the temperature was ambient. A25-μl volume of sample was injected manually into the 10-μl sample loop using a Hamilton (Reno, NV, U.S.A.) blunt-tipped syringe. Prior to the next injection, the injector loop was rinsed with 250 μl of 90% formic acid to insure that there is no sample carryover.

Proteins

Human serum albumin (Sigma, St. Louis, MO, U.S.A.), 1 mg/ml was prepared by dissolving 10 mg into 10 ml of 50 m*M* sodium phosphate, 150 m*M* sodium chloride pH 7.2 buffer. Human haptoglobin (Sigma), 2 mg/ml was dissolved into 50 m*M* sodium phosphate, 150 m*M* sodium chloride, 1 m*M* EDTA, pH 7.1 buffer (PBS). Horse heart cytochrome *c* (Pierce, Rockford, IL, U.S.A.), 1 mg/ml was prepared by dissolving 1.12 mg into 1.12 ml of PBS. Horse spleen ferritin (Pierce), 10 mg/ml was diluted 1:10 with PBS to attain a 1.0 mg/ml solution. Insulin (Eli Lilly, Indianapolis, IN, U.S.A.), 5 mg/ml was diluted 1:5 with PBS for a final concentration of 1.0 mg/ml. The rFVIII samples were obtained during purification of this product for clinical evaluation[2].

Interfering substances

All of the reagents used in this study were known to interfere with at least one of the following protein assays: Coomassie blue (Bradford), Lowry, Biuret, BCA or A_{280} (refs. 1 and 3). The reagents were all ACS grade or equivalent. The stock solutions were prepared with water suitable for injection (WFI) or the appropriate buffer. The reagents investigated in this study included: Nonidet-P-40 (Sigma), sodium dodecylsulfate (SDS; Bio-Rad, Richmond, CA, U.S.A.), glycerol (Sigma), Thimerosal (Sigma), Tris (Sigma), Guanidine · HCl (Sigma), Tween-80 (Sigma, calcium chloride (J. T. Baker), imidazole (Kodak, Rochester, NY, U.S.A.), and L-tryptophan (Calbiochem, La Jolla, CA, U.S.A.).

Interfering substance study

Human serum albumin (HSA) at 1.0 mg/ml was diluted 1:10 with WFI to serve as the control for this study. HSA was also diluted 1:10 with the various interfering substances. Aliquots (25 μl) of the HSA samples were injected into the 10-μl fixed-loop injector and applied to the column. Injections of the various reagents without HSA were also made. All samples were run in duplicate or triplicate. The mean peak area of the sample containing interfering substances was divided by the mean peak area of the HSA control sample to determine the amount of interference (if any).

Protein retention time study

Aliquots (25 μl) of ferritin, cytochrome *c*, HSA, haptoglobin and insulin were individually injected into the 10μl loop and subsequently injected into the column. A pool of equal amounts of ferritin, cytochrome *c*, HSA and haptoglobin was made and a 25-μl injection of the pool was carried out. A pool of HSA and insulin was also injected into the column. The retention times for all samples in this study were recorded.

Quantitation of rFVIII

A pool of approximately twenty-five 1-ml aliquots of highly purified (>90%) rFVIII was made. A protein concentration value was assigned to the pool by performing amino acid analysis on an aliquot of the pool. The rFVIII standard pool was diluted with buffer to obtain a final concentration of 160 μg/ml. This stock solution is subsequently serially diluted with buffer to generate standards at 80, 40 and 20 μg/ml. A known amount of standard per 10 μl was injected. The peak area is directly related

to the amount of standard injected and is expressed as peak area × 10^4 per μg per 10 μl. Each standard curve point is made in duplicate unless where triplicate may be necessary. The mean ($\bar{x}$) peak area × 10^4 value is plotted *vs.* their respective μg load and calculated according to linear regression. The resulting y intercept and slope are used to calculate the $\bar{x}$ peak area × 10^4 values of the unknowns by the straight line formula $y = ax + b$. Controls are run with each assay to monitor intra-assay variation.

RESULTS AND DISCUSSION

Interfering substances

The combination of 90% formic acid as the mobile phase and LiChrosorb Si-100 as the analytical column allows for the separation of interfering substances from proteins. The formic acid dissociates non-covalent bonding associated with protein–protein interactions or protein–interfering substance interactions. This environment allows for the separation of proteins from low-molecular-weight substances by size-exclusion chromatography. Fig. 1a is a typical chromatogram demonstrating the elution position of the interfering substance, tryptophan. All the interfering substances listed in Table I had a retention time between 6 and 9 min. Fig. 1b shows the separation of tryptophan from human serum albumin. The presence of any of the interfering substances did not alter the chromatographic profile or retention time of HSA. Fig. 1c is a chromatogram of the integrated protein peak only, the plotter/integrator was stopped before the tryptophan eluted from the column. This system of evaluation (*i.e.*, HSA with and without additives) was carried out for each of the interfering substances described in this paper. The results (Table I) indicate that the substances known to interfere in other protein assays do not interfere in this assay. Since there are many substances known to interfere with one or more of the other protein assays, it was beyond the reasonable scope of this paper to evaluate each one. However, the results reported in this paper indicate that this method should be adaptable to many of the other known interfering substances of various protein assays.

Protein retention time

The proteins selected for this study were chosen because they represented a wide molecular weight range of 6 000–450 000 dalton. One of the objectives was to deter-

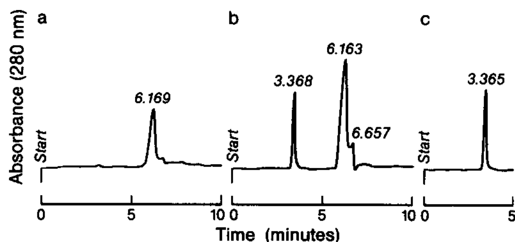

Fig. 1. HPLC chromatograms of (a) tryptophan, (b) human serum albumin and tryptophan and (c) human serum albumin as the only peak integrated and plotted. Mobile phase: 90% formic acid. Flow-rate: 1.0 ml/min. Chart speed: 0.5 cm/min. Detector: 0.02 (280 nm).

TABLE I

LABORATORY REAGENTS KNOWN TO SIGNIFICANTLY INTERFERE IN COMMON PROTEIN ASSAYS

Sample additive[a]	*Deviation from known concentration (%)*[b]
Nonidet-P-40 (0.9%)	− 3.2
SDS (0.9%)	0
Glycerol (13.5%)	+ 2.5
Thimerosal (0.9%)	+ 1.6
Tris (0.9 *M*)	− 5.1
Guanidine · HCl (3.6 *M*)	− 10.2
Tween-80 (0.09%)	+ 10.0
$CaCl_2$ (0.9 *M*)	− 9.9
Imidazole (180 m*M*)	− 8.1
L-Tryptophan (0.9 mg/ml)	− 5.0

[a] HSA sample contained these additives at the listed concentrations.
[b] Deviations $\leqslant 10\%$ are considered not significant. The percent deviation was determined by comparing the HSA additive samples with the HSA control which contained no additives.

mine the molecular weight at which separation of proteins occurs. Insulin (6000 dalton) had the only significantly different retention time (Table II). The other proteins shared nearly identical retention times as did a pool of 4 proteins. This information allowed us to conclude that the method is suitable for proteins with a molecular weight greater than 12 500 dalton. The advantage of this system is that the proteins elute as a single peak which allows for easy quantification of the peak area. Since insulin is baseline separated from the HSA (Fig. 2a–c), it is reasonable to assume that one could still quantitate the protein peak and/or the insulin peak. However, that type of assay would require a more complex standard.

Quantitation of rFVIII

The need to determine an accurate specific activity of rFVIII greatly influenced the development of this assay. The essential requirements for the assay included

TABLE II

RETENTION TIMES OF SIX PROTEINS

Protein	*Molecular weight (dalton)*	*Retention time (min)*
Insulin	6000	4.506
Cytochrome *c*	12 500	3.342
HSA	66 500	3.365
rFVIII	220 000	3.350
Haptoglobin	86 000–400 000	3.330
Ferritin	450 000	3.368
Pool of haptoglobin, HSA, ferritin, cytochrome *c*	12 500–450 000	3.348

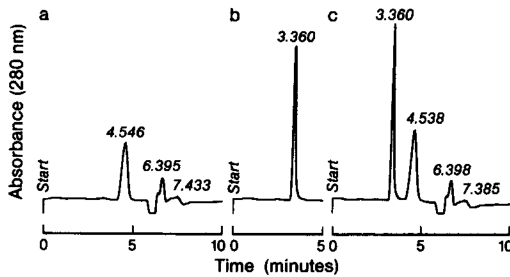

Fig. 2. HPLC chromatogram of (a) insulin, (b) human serum albumin and (c) human serum albumin plus insulin. Chromatographic conditions as in Fig. 1.

accuracy, sensitivity, small sample volume, reproducibility, and no sample preparation. Sample preparation, such as dialysis, can result in absorptive losses of protein and/or loss of biospecific activity of the sample. Such losses result in an inaccurate estimation of the sample's biospecific activity. Because highly purified rFVIII solutions contain substances that interfere with the more commonly used protein assays, it was imperative that the rFVIII sample could be assayed without any type of preparation. To insure that this assay was accurate, we prepared a rFVIII standard that was similar in composition to our rFVIII samples. This standard was subjected to amino acid composition analysis and assigned a protein concentration.

A typical linear regression curve generated by one of our rFVIII standards involving 32 separate experiments resulted in a coefficient of variation (C.V.) for each of the 4 standard curve points of 4.0–11.4%. The correlation coefficient was 0.9999. The C.V. for 26 separate intra-assay control samples was 6.25%, an indication of excellent reproducibility. The standard curve range reported in this paper covers 0.2–1.6 μg/10 μl injection. The range could be adjusted lower or higher to fit individual assay requirements. Typically, less than 100 μl sample is required to run in triplicate.

The HPLC protein assay using formic acid is easy to use, and it generates results quickly. Each run takes only 8 min when the flow-rate is 1 ml/min. The method can be automated. Very accurate sample protein content can be obtained, if a well defined standard similar to the sample of interest is used. Since sample preparation is not necessary, a direct relationship between protein content and protein biological activity can be made. This allows for a highly accurate estimation of biospecific activity.

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