

Development and Validation of HPLC-UV Method for Determination of Bovine Serum Albumin and Myoglobin Proteins

Fuad Al-Rimawi^{1*}

¹*Faculty of Science and Technology, Al-Quds University, P.O. Box 20002, East Jerusalem,
Palestine.*

Author's contribution

This whole work was carried out by the author FAR.

Original Research Article

Received 7th February 2014
Accepted 23rd March 2014
Published 22nd May 2014

ABSTRACT

A simple HPLC with UV detection method is developed and validated for determination of Bovine Serum Albumin and Myoglobin proteins in a standard solution of the two protein as well as in a plasma spiked with these two proteins. Separation was achieved on a reversed-phase C18, 5.0 μm , 150mm \times 4.6 mm inner diameter column using a mobile phase consisting of solution A (900 mL of water, 100 mL Acetonitrile, 10 mL Trifluoroacetic acid) and solution B (900 mL of acetonitrile, 100 mL water, 10 mL Trifluoroacetic acid) with gradient elution of 5 to 50% of solution B in 15 minutes. This new method is validated in accordance with requirements for new methods, which include accuracy, precision, selectivity, linearity and range, robustness, limit of detection and limit of quantitation. The method demonstrates good linearity over the range of 1-1000 ppm for the two proteins with r^2 greater than 0.998. The percentage recovery of the method is within 97.9 to 102.0% for the two proteins. Precision of this method reflected by relative standard deviation of the area of six replicate injections of each protein at five concentration levels (1.0, 100.0, 300.0, 500, and 1000.0 ppm) was found to be less than 2%. Limit of quantitation of the two proteins is low which enables the determination of these proteins at low concentration.

*Corresponding author: Email: falrimawi@science.alquds.edu;

Keywords: Bovine serum albumin; myoglobin; protein; HPLC-UV; validation.

1. INTRODUCTION

Bovine serum albumin (BSA) is a serum albumin protein derived from cows with 607 amino acids. BSA has numerous biochemical applications including ELISAs (Enzyme-Linked Immunosorbent Assay), immunoblots, and immunohistochemistry. It is also used as a nutrient in cell and microbial culture [1]. Myoglobin is an iron- and oxygen-binding protein found in the muscle tissue of vertebrates in general and in almost all mammals. It is related to hemoglobin, which is the iron- and oxygen-binding protein in blood, specifically in the red blood cells. Myoglobin is only found in the bloodstream after muscle injury. It is an abnormal finding, and can be diagnostically relevant when found in blood [2]. Myoglobin is a single-chain globular protein of 153 or 154 amino acids, containing a heme (iron-containing porphyrin) prosthetic group in the center around which the remaining apoprotein folds. It has eight alpha helices and a hydrophobic core. It has a molecular weight of 17,699 daltons (with heme). Unlike the blood-borne hemoglobin, to which it is structurally related, this protein does not exhibit cooperative binding of oxygen, since positive cooperativity is a property of multimeric/oligomeric proteins only [3].

Many methods has been developed for analysis of bovine serum albumin [4-5] and myoglobin [6-7] by HPLC, however a simple reversed phase HPLC method for simultaneous determination of the two proteins was not reported so far. In this respect, a method for simultaneous determination of these two proteins is needed and presented in this work. The analytical method employed for the analysis of these proteins in the current work is simple where HPLC technique is employed. Furthermore, UV detector was employed for the detection of these proteins where it is available in most of analytical labs in contrary to other detectors like fluorescence and mass spectrometry which are not available in many analysis labs. Additionally this method was validated in accordance of new methods which include linearity and range, precision, accuracy, selectivity, limit of detection, limit of quantitation, and ruggedness.

2. EXPERIMENTALS

2.1 Chemicals

Acetonitrile (HPLC grade), Trifluoroacetic acid, and standards of the two proteins are from Merck (Darmstadt, Germany).

2.2 Apparatus

HPLC system (Merck Hitachi Lachrom Elite HPLC system, Japan) with a pump, an autosampler, column oven, and a UV-detector was employed. The chromatographic column C18 (15cm length, 4.6 mm inner diameter, and 4.6 μ m particles) is from Waters Corporation (Milford, Massachusetts, USA).

2.3 Standard Solutions Preparation

2.3.1 Standard solutions for linearity and range

Stock standard solution of the two proteins (1000 ppm) was prepared in phosphate buffer pH

7.2. Different concentrations of each protein were then prepared from the stock solution by dilution using phosphate buffer pH 7.2 as diluent. The following diluted concentrations were prepared: 1.0, 20.0, 100.0, 300, 500.0, 700.0, and 1000.0 ppm. Each of these solutions was injected into the HPLC and peak areas were recorded and plotted versus the concentration of the protein.

2.3.2 Percentage recovery (Accuracy) of the method

Five recovery solutions were prepared by spiking the proteins in phosphate buffer pH 7.2 at five concentration levels (1.0, 100.0, 300, 500.0, and 1000.0 ppm).

2.3.3 Limit of detection (LOD) and limit of quantitation (LOQ)

LOD of the proteins was determined by preparing a set of solutions with low concentrations of the two proteins (0.1, 0.3, 0.7, and 1.0 ppm) that is expected to produce a response that is 3-20 times baseline noise. The solutions are injected and the signal to noise ratio (S/N) are recorded. LOD is selected as the concentration of protein that gives a S/N ratio of 3-10. LOQ is determined in the same manner and selected as the concentration of protein that gives an S/N ratio of 10-20.

3. RESULTS AND DISCUSSION

3.1 Method Development

Preliminary studies involved trying C8 and C18 reversed-phase columns and testing several mobile phase compositions and using different chromatographic parameters for the separation of the two proteins. The chromatographic separation was started with C8 column (12.5 and 25.0 cm length) but it did not show good separation (poor resolution, less than 1.5). Therefore C18 column was the next choice for separation of the two proteins. C18 column with 15 and 25cm length was tested for separation of the two proteins, and it was found that C18 column with 25 cm length gave higher retention times with a low theoretical plates, while C18 column (15 cm) gave a good separation with a reasonable retention time, and good theoretical plates. Therefore C18 column with 15 cm length (4.6 mm inner diameter, and 5.0 μ m particles) was used for separation of the two proteins. Regarding the mobile phase, different mixtures of acetonitrile and water as well as mixtures of methanol and water was tested in both isocratic and gradient modes, but the separation was poor (low resolution) and the shape of the peaks is not good (low theoretical plates). However when trifluoroacetic acid was added to a mixture of water and acetonitrile, the shape of the peaks of the proteins become good. The volume fraction of trifluoroacetic acid in the mobile phase was optimized and found to be 1%. Isocratic elution gave poor separation of the two proteins; therefore gradient elution was used for separation of these two proteins. Gradient elution was optimized for solution A (900 mL of water, 100 mL Acetonitrile, 10 mL Trifluoroacetic acid) and solution B (900 mL of acetonitrile, 100 mL water, 10 mL Trifluoroacetic acid), and found that 5 to 50% of solution B in 15 minutes at a flow rate of 0.8 ml/min is the optimum condition for separation of the two proteins. The apparent pH of solution A and B is 2. Concerning the wavelength of the detector, it was chosen to be 220 nm as it gave higher peak heights and areas and consequently higher sensitivity for both proteins. Wavelength lower than 220 nm gave higher peak areas (higher sensitivity) but the selectivity decreases since mobile phase absorb at this wavelength. On the other hand, higher wavelength gave low sensitivity, therefore 220 nm was the best wavelength for determination of these proteins. Injection

volume was chosen to be 20 μ L as it gave good peak areas even at low concentration of the two proteins. Higher and lower injection volumes can also be used, but low injection volumes give low sensitivity, while higher injection volumes give broad peaks and nonlinear peak areas. Therefore an injection volume of 20 μ L was used for all standards and samples in this study. Fig. 1 shows a chromatogram of bovine serum albumin and myoglobin standards, while Fig. 2 shows a chromatogram of a serum sample spiked with bovine serum albumin and myoglobin at 50 ppm concentration of each protein.

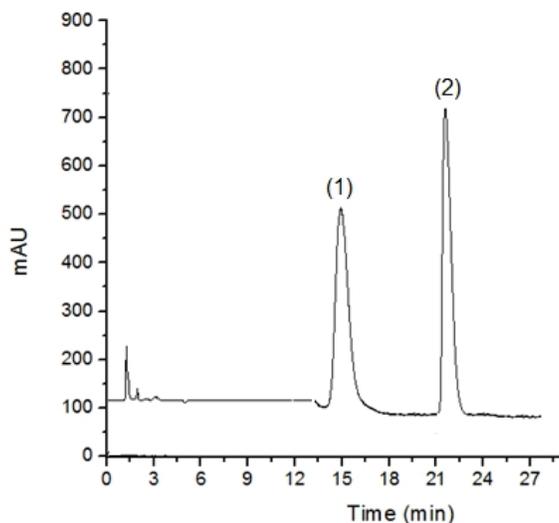


Fig. 1. Chromatogram of bovine serum albumin BSA (1) and Myoglobin (2). Column: C18, 5 μ m, 15 cm length, 4.6 mm inner diameter, UV detection: 220 nm. Mobile phase: gradient of solution A (900 mL of water, 100 mL Acetonitrile, 10 mL Trifluoroacetic acid) and solution B (900 mL of acetonitrile, 100 mL water, 10 mL Trifluoroacetic acid), 5 to 50% of B in 15 minutes, flow rate: 0.8 ml/min, injection volume: 20 μ L

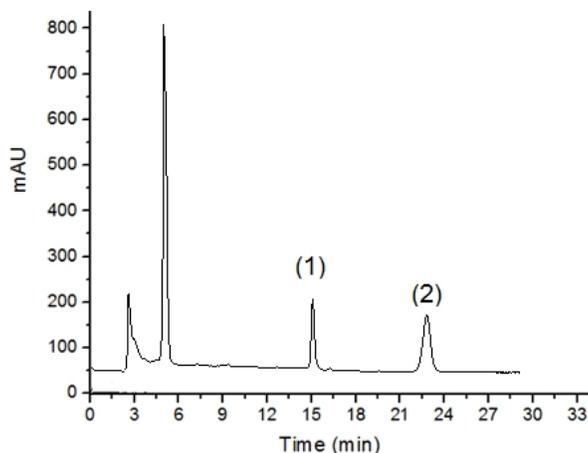


Fig. 2. Chromatogram of Bovine Serum Albumin BSA (1) and Myoglobin (2) spiked at a concentration of 50ppm in a serum sample. For other chromatographic conditions, see the legend of Fig. 1.

3.2 Method Validation

3.2.1 Linearity and range

Linearity is the ability of a method to elicit test results that are directly proportional to analyte concentration within a given range. Range is the interval between the upper and lower levels of analyte that have been demonstrated to be determined with precision, accuracy, and linearity using the method as written. A minimum of five concentration levels, along with certain minimum specified ranges are required. Acceptance criteria for linearity are that the correlation coefficient (r^2) is not less than 0.990 for the least squares method of analysis of the line [8].

To evaluate linearity of the current method for determination of the two proteins, different calibration standards of the proteins were analyzed by HPLC-UV and the responses are recorded. A plot of the peak areas of the proteins versus concentration (in ppm) was found to be linear in the range of 1-1000 ppm for the two proteins with r^2 greater than 0.998, see Fig. 3. This result indicates that these proteins can be determined at wide concentration range. The concentration range of linearity (1-1000 ppm) was selected in order to test the ability of the method for determination/quantitation of the proteins at wide concentration range.

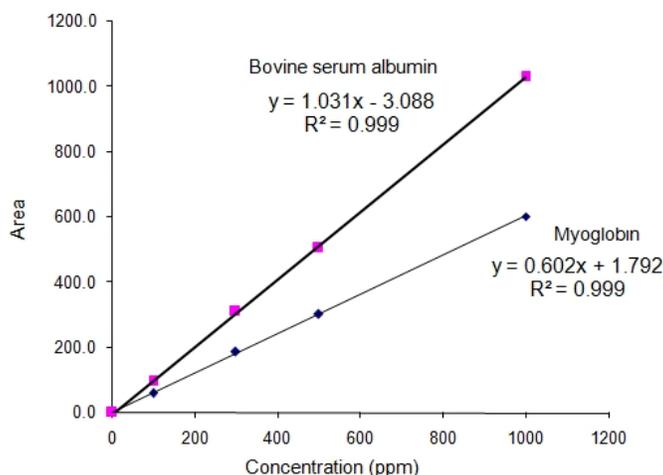


Fig. 3. Plot of peak areas vs. concentration of myoglobin and Bovine serum Albumin (linearity test)

3.2.2 Percentage recovery (accuracy)

The accuracy of an analytical method measures the closeness between the value, which is accepted either as a conventional true value or an accepted reference value, and value found (i.e., accuracy is a measure of exactness of an analytical method). Accuracy is measured as the percent of analyte recovered after spiking samples in a blank. To document accuracy, a minimum of nine determinations over a minimum of three concentration levels covering the specified range (for example, three concentrations, three replicates for each) were collected. It is performed at three concentrations covering the range of the method. At each level studied, replicate samples are evaluated. The RSD of the replicates provides the analysis variation and gives an indication of the precision of the test method. Moreover, the

mean of the replicates, expressed as % of label claim, indicates the accuracy of the test method. The mean recovery of the assay should be within $100 \pm 5.0\%$ at each concentration over the studied range [9].

For determination of the percentage recovery of the investigated proteins, these proteins are spiked in phosphate buffer pH 7.2 followed by analysis by HPLC-UV. The average recovery for each level has been calculated by proportion of the area of the peak of proteins resulted from the spiked solution to the area of the peak of that protein resulted from a standard solution prepared from standard of that protein. The average recovery and the RSD for each level have been calculated. Results have showed that the current method has good recovery (from 97.9 to 102.0%) for the two proteins at the five concentration levels studied (1.0, 100.0, 300.0, 500.0, and 1000.0 ppm), and with a relative standard deviation lower than 2.0%, see Table 1.

Table 1. % recovery of bovine serum albumin and myoglobin at five concentration levels (1, 100, 300.0, 500.0, and 1000 ppm)

Protein	% Recovery				
	Concentration (ppm)				
	1.0	100.0	300.0	500.0	1000.0
Bovine serum albumin	102.0, 101.3, 100.7	98.4, 97.9, 101.0	98.3, 98.6, 99.2	101.6, 101.0, 102.0	102.0, 101.1, 100.7
	Mean: 101.3	Mean: 99.1	Mean: 98.7	Mean: 101.5	Mean: 101.3
	SD: 0.65	SD: 1.66	SD: 0.46	SD: 0.50	SD: 0.67
	RSD: 0.64	RSD: 1.68%	RSD: 0.46%	RSD: 0.50%	RSD: 0.66
Myoglobin	97.9, 98.1, 99.1	99.6, 100.7, 101.2	98.2, 99.3, 99.1	100.6, 101.3, 101.9	99.8, 99.1, 100.3
	Mean: 98.4	Mean: 100.5	Mean: 98.9	Mean: 101.3	Mean: 99.7
	SD: 0.64	SD: 0.82	SD: 0.59	SD: 0.65	SD: 0.60
	RSD: 0.65%	RSD: 0.81%	RSD: 0.59%	RSD: 0.64%	RSD: 0.60%

*SD: Standard Deviation.

**RSD: Relative Standard Deviation

3.2.3 Precision

Precision is the measure of the degree of repeatability of an analytical method under normal operation and is normally expressed as the RSD for a statistically significant number of samples. There are two types of precision: repeatability and intermediate precision (ruggedness).

3.2.3.1 Repeatability

Repeatability is the closeness of agreement between mutually independent test results obtained with the same method on identical test material in the same laboratory by the same operator using the same equipment within short intervals of time. It is determined from a minimum of nine determinations covering the specified range of the procedure (for example, three levels, three repetitions each). RSD for replicate injections should not be greater than 1.5% [10].

Repeatability of the current method for determination of the two proteins was evaluated by calculating the RSD of the peak areas of six replicate injections of standard solutions with

five concentrations (1.0, 100.0, 300.0, 500.0, and 1000.0 ppm), which was found to be less than 1.0% (Table 2). These results show that the current method for determination of the proteins is repeatable.

Table 2. Repeatability of the method for determination of bovine serum albumin and myoglobin proteins

Concentration (ppm)	Bovine Serum Albumin protein		Myoglobin protein	
	SD of the peak areas of six replicate injections	RSD of the peak areas of six replicate injections	SD of the peak areas of six replicate injections	RSD of the peak areas of six replicate injections
1	0.27	0.30%	0.43	0.47%
100	0.77	0.81%	0.72	0.70%
300	0.76	0.79%	0.85	0.88%
500	0.61	0.63%	0.60	0.61%
1000	0.90	0.88%	0.53	0.50%

*SD: Standard Deviation.

**RSD: Relative Standard Deviation

3.2.3.2 Intermediate precision (ruggedness)

Intermediate precision (also called ruggedness) of a method measures the repeatability of the result obtained with the same method, on the same sample, in the same laboratory, but by different operators and in different day. Intermediate precision of the current method was evaluated by calculating the % recovery of the proteins at five concentration levels (1.0, 100.0, 300.0, 500.0, and 1000.0 ppm) by another analyst in different day. Results of this study showed that the % recovery obtained by the second analyst is comparable to that obtained by the main analyst and ranges from 98.1 to 101.8% (data not shown), indicating that this method is rugged.

3.2.4 Selectivity

Selectivity of a method is the ability to assess unequivocally the analyte in the presence of other analytes and other components that may be expected to be present in the matrix or sample [11]. It is a measure of the degree of interferences from such components, ensuring that a response is due to a single component only. Selectivity of the current method was demonstrated by good separation of the two proteins from each other with good resolution (resolution is 4.1, see Fig. 1). Additionally this method is specific for determination of these two proteins in serum sample, where the two proteins are good separated (resolution is higher than 3), and the bovine serum albumin (peak 1) is separated from the adjacent peak (which is from the serum sample) with a resolution higher than 3, see Fig. 2.

3.2.5 Robustness

Robustness measures how a method stands up to slight variations in the operating parameters of the method like flow rate, wavelength, percentage of mobile phase composition. Robustness of the current method was investigated by measuring the % recovery of the proteins at 100ppm concentration using the same developed method in this study but changing deliberately one chromatographic condition each time. The chromatographic conditions which were changed are (a) flow rate (0.6 and 1.0 mL/min vs.

the original flow rate of 0.8 mL/min), and (b) wavelength (218 and 222 nm vs. the original wavelength of 220 nm). Results have shown that separation is not affected by changing slightly the chromatographic conditions; resolution between adjacent proteins remained good. Additionally, the % recovery of the proteins was not affected significantly by changing the chromatographic conditions (flow rate, and wavelength), see Table 3.

Table 3. Robustness testing for determination of bovine serum albumin and myoglobin

Parameter		% Recovery	
		Bovine serum albumin	Myoglobin
Flow rate	0.6	100.7%	101.3%
	0.8	99.1%	100.5%
	1.0	99.8%	100.7%
Wavelength	218	100.7%	98.5%
	220	99.1%	100.5%
	222	99.8%	100.7%

3.2.6 Limit of detection (LOD) and limit of quantitation (LOQ)

LOD is the lowest concentration of analyte in a sample that can be detected but not necessarily quantitated under the stated experimental conditions. It can be determined by preparing a solution that is expected to produce a response that is about 3 to 10 times base line noise. The solution is injected three times, and the signal to noise ratio for each injection are recorded. The concentration of the solution is considered as a LOD if the S/N ratio is between 3 and 10. LOQ can be determined in the same manner but with S/N ratio of 10-20.

LOD and LOQ of the proteins investigated in this study using the current method were found to be low (see Table 4) which enables the detection and quantitation of these proteins at low concentration levels.

Table 4. LOD and LOQ of bovine serum albumin and myoglobin using the method employed in this study

Protein	LOD (in ppm)	LOQ (in ppm)
Bovine serum albumin	0.3	1.0
Myoglobin	0.4	1.0

4. CONCLUSION

A simple, accurate, precise, and selective HPLC method has been developed and validated for the simultaneous determination of bovine serum albumin and myoglobin. The method is linear for determination of these proteins with a wide dynamic range (1-1000 ppm). This method is also accurate where the % recovery of the proteins is within 97.9 to 102.0%. Precision of the method is confirmed by low RSD of replicate samples of the proteins. Low LOD and LOQ of the proteins analyzed in this study enable the detection and quantitation of them at low concentrations. The method of analysis is simple, the analysis time is short, elution is isocratic, and the detector is UV detector which is available in most of separation labs.

COMPETING INTERESTS

Author has declared that no competing interests exist.

REFERENCES

1. Ge S, Kojio K, Takahara A, Kajiyama T. Bovine serum albumin adsorption onto immobilized organotrichlorosilane surface: Influence of the phase separation on protein adsorption patterns. *J Biomater Sci Polym Ed.* 1998;9(2):131–150.
2. Nelson DL, Cox MM. *Lehninger Principles of Biochemistry* (3rd ed.). New York: Worth Publishers. 2000;206. ISBN 0-7167-6203-X.
3. Hendgen-Cotta UB, Kelm M, Rassaf T. A highlight of myoglobin diversity: The nitrite reductase activity during myocardial ischemia-reperfusion. *Nitric Oxide.* 2010;22(2):75–82.
4. Hamidi M, Zarei N. A reversed-phase high-performance liquid chromatography method for bovine serum albumin assay in pharmaceutical dosage forms and protein/antigen delivery systems. *Drug Test Anal.* 2009;1(5):214-8.
5. De Frutos M, Cifuentes A, Díez-Masa JC, Camafeita E, Méndez E. Multiple Peaks in HPLC of Proteins: Bovine Serum Albumin Eluted in a Reversed-Phase System. *J High Resolut Chromatogr.* 1998;21(1):18-24.
6. Mayr BM, Kohlbacher O, Reinert K, Sturm M, Gro C, Lange E, Klein C, Huber CG. Absolute Myoglobin Quantitation in Serum by Combining Two-Dimensional Liquid Chromatography-Electrospray Ionization Mass Spectrometry and Novel Data Analysis Algorithms. *J Proteome Res.* 2006;5:414-421.
7. Powell SC, Friedlander ER, Shihabi ZK. Myoglobin determination by high-performance liquid chromatography. *J Chromatogr.* 1984;28(317):87-92.
8. International Conference on Harmonization (ICH). "Validation of Analytical Procedures-PA/PH/OMCL (05) 47 DEF". Elaborated by OMCL Network/EDQM of the Council of Europe; 2005.
9. Green M. A Practical Guide to Analytical Method Validation. *Analytical Chemistry News and Features* P. 1996;309.
10. Huber L. Validation of Analytical Methods. In "Validation and Qualification in the Analytical Laboratories". Interpharm Press, Buffalo Grove, IL. 1998;107.
11. A WHO Guide to Good Manufacturing Practice (GMP) Requirements, Part 2: Validation, World Health Organization, Geneva; 1997.

© 2014 Al-Rimawi; This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/3.0>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Peer-review history:

The peer review history for this paper can be accessed here:

<http://www.sciencedomain.org/review-history.php?iid=536&id=7&aid=4651>