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A reversed-phase high-performance liquid chromatography method for bovine serum albumin assay in pharmaceutical dosage forms and protein/antigen delivery systems

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Bovine serum albumin (BSA) is among the most widely used proteins in protein formulations as well as in the development of novel delivery systems as a typical model for therapeutic/diagnostic proteins and the new versions of vaccines. The development of reliable and easily available assay methods for quantitation of this protein would therefore play a crucial role in these types of studies. A simple gradient reversed-phase high-performance liquid chromatography with ultra-violet detection (HPLC-UV) method has been developed for quantitation of BSA in dosage forms and protein delivery systems. The method produced linear responses throughout the wide BSA concentration range of 1 to 100 μg/mL. The average within-run and between-run variations of the method within the linear concentration range of BSA were 2.46% and 2.20%, respectively, with accuracies of 104.49% and 104.58% for within-run and between-run samples, respectively. The limits of detection (LOD) and quantitation (LOQ) of the method were 0.5 and 1 μg/mL, respectively. The method showed acceptable system suitability indices, which enabled us to use it successfully during our particulate vaccine delivery research project.

Keywords: bovine serum albumin; high-performance liquid chromatography; protein analysis

Introduction

Albumin, the most abundant blood protein in mammals, contributes mainly to blood's buffer property as well as to blood colloid osmotic pressure and plays an important role in the transport and deposition of a variety of endogenous and exogenous substances in the circulatory system.[1] Bovine serum albumin (BSA), one of the main naturally occurring forms of albumin, is very similar to human serum albumin (HSA) in terms of structure and physicochemical properties. Having a molecular weight in the range of 66 to 69 KD, the BSA molecule consists of a single long chain of about 582 amino acid residues, which is characterized by a low content of tryptophan and methionine and a high content of cysteine, aspartic acid and glutamic acid. Bovine serum albumin was clinically used for the first time as a diluent for anti-RH serum in 1945.[2] Since then, it has been used extensively as stabilizer, cryoprotectant and diluent in parenteral peptide/protein formulations, as blocking and coating agent in immunoassays, as a main component of biological buffers, as a cell-culture supplement and as a basic component of several biological diagnostic reagents, mainly used for immunohematological tests.[2]

Besides the these extensive applications, BSA has found an important role in recent decades as a typical model protein in novel drug delivery research[3–11] as well as a model antigen[12–15] in studies on particulate and/or conjugated delivery systems. Various methods of analysis have been attempted to determine the BSA concentrations in formulations and protein/antigen delivery systems, mainly based on general colorimetric protein analysis methods such as lowry,[3] bio-rad,[4] bromocresol green,[16] coomassie blue,[5] bradford,[6] bichinchoninic acid (BCA)[7] as well as enzyme-based techniques,[12,17] electrophoresis,[18,19] ligand-specific analysis,[20,21] and reversed-phase,[8,22,23] affinity[24] and size-exclusion chromatography.[9–11] Most of the non-chromatographic methods presented for this purpose have limitations regarding their popularity[12,17–19] as well as specificity[3–7,16] limitations. The HPLC methods developed so far for BSA assay also have some inherent limitations, including the high cost of analysis,[9–11,25–27] long sample preparation and/or analysis times and, sometimes, low sensitivity.[11,24] The development of a simple, easily available and validated method for the assay of this protein in the matrices most often encountered in delivery systems researches would therefore be of central importance in the field.

In this article, a simple, easily available and reliable reversed-phase HPLC method with UV-detection has been developed and validated for the determination of BSA concentrations in formulations and novel delivery systems. This simple method can be used popularly, considering its relatively short analysis time, lack of interferences, relatively low cost for the high number of samples, acceptable sensitivity, and robustness.

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Experimental

Chemicals

Bovine serum albumin (Sigma, St Louis, United States; Art. No. A2153) was purchased locally. Other chemicals and solvents were of chemical laboratory or HPLC purity grades, as needed, and purchased locally.

Instrument and HPLC method

The reversed-phase HPLC method consisted of a gradient system of 0.1% trifluoroacetic acid (TFA) in water (A) and 0.08% TFA in acetonitrile (B) with initial A/B ratio of 80/20, which changed linearly to the final ratio of 35/65 (A/B) within 15 min. The reversal to the initial condition then occurred within 2 min and, finally, the system was re-equilibrated over 8 min (total run time of 25 min). The flow rate was 1 mL/min over all the gradient steps. The analyte separation was carried out using a Symmetry 300® C_4 protein analysis column (50 × 4.6 mm; particle size 5 µm; pore size 300 Å; Waters, MA, USA) operated at 40 °C and equipped by a guard column with the same packing (Waters, MA, USA). The solvent delivery system was a double-reciprocating pump (model 600, Waters, MA, USA). A UV-detector (model 746, Waters, MA, USA) was used in a wavelength of 220 nm for analyte detection. Finally, the outputs were processed and recorded by a compatible integrator (model 486, Waters, MA, USA). Sample injection was made by a loop injector (Rheodyne®, Cotati, CA, USA) equipped by a 50 µl loop.

Sample preparation

A stock solution of BSA with the concentration of 100 µg/mL was prepared in phosphate buffer (KH_2PO_4 0.08 M; pH = 7.4) and the concentrations of 1, 2, 4, 8, 10, 25 and 50 µg/mL were prepared by serially diluting this solution with the proper amount of the same buffer.

System suitability tests

The following parameters were calculated as system suitability indices of the developed method, using a sample with the BSA concentration of 10 µg/mL:

$$\text{number of theoretical plates (N)} = 16\,(t_R/W)^2$$

$$\text{peak symmetry} = W/2f$$

$$\text{retentability } (k') = (t_R/t_a)_{-1}$$

where, t_R is the retention time of analyte peak, W is the peak width at 0.05 peak height, f, is the front half-width of the peak at 0.05 peak height and t_a is the retention time of non-retained peak (solvent front).

Analysis validation tests

Standard curve (linear range)

Serial samples, prepared as described, were injected directly to the chromatograph and then the linear regression analysis was carried out on known added (nominal) concentrations of BSA against the corresponding peak heights. The regression coefficient (r), slope, and intercept of the resulting calibration curves were determined.

Within-run variations

In one run, three samples with BSA concentrations of 4, 10 and 100 µg/mL (from high-, middle- and low-concentration regions of the standard curve) were prepared in triplicate, analysed using the HPLC method that was developed. The coefficient of variation (CV%) of the corresponding determined concentrations were calculated in each case.

Between-run variations

In three different runs, samples from upper, middle and lower concentration regions used for the construction of the standard curve (the same concentrations used in within-run variations test) were prepared and were analysed using the HPLC method. The corresponding CV% values were then calculated in each case.

Absolute recovery (accuracy)

For each sample tested for within- and between-run variations, the absolute recovery of the method was determined as the ratio of the measured (based on the standard curve) concentration to the corresponding added (nominal) concentration. The recovery was determined without consideration of any matrix effects.

Limits of detection and quantitation

Limit of detection (LOD) of the method was determined as the lowest BSA concentration producing a signal-to-noise (S/N) ratio of about 3. The limit of quantitation (LOQ) was determined as the lowest BSA concentration capable of being quantitated with acceptable accuracy and precision.

Specificity

A series of pharmaceutical matrices from different categories, including, mainly among others, hydrogel-based matrices, PLGA-based vehicles, cellulose-based formulations, lipid-based matrices and the most complex cellular carriers were tested during our extensive vaccine delivery studies using BSA as model antigen, to assay this protein in different delivery systems. In each case, any evidence of the signals in the corresponding time of the chromatogram were monitored as a sign of potential interferences.

Sample stability

To exploit the stability of analyte in samples during analysis as well as sample storage times, samples of BSA with known concentrations of 2, 25 and 100 µg/mL were analysed immediately after preparation as well as after 12, 24 and 72 h in room temperature. Then, the percentage ratios of concentrations determined in each case to known added concentrations were calculated.

Method applicability

The applicability of the developed analysis method was evaluated by applying the method on BSA-containing samples obtained during an ongoing study on a particulate antigen delivery system by our research group.

Results and Discussion

Method development

In response to lack of an easily available, reliable and easy-to-use method for determination of BSA concentrations in pharmaceutical matrices as well as delivery systems containing this popular model protein/antigen, a gradient reversed-phase HPLC method was developed for quantitation of BSA. We examined several HPLC method variables with respect to their corresponding effects on the results of the analysis. For this purpose, a series of gradient systems consisting of two mobile phase components typically used in protein analysis (solutions of TFA in water and acetonitril) with different concentrations, initial and final ratios and total gradient and run times were tested. The other parameters in this factorial design were gradient mode, temperature, flow rate, detection wavelength (UV detection was used in all cases owing to its popularity), and volume of injection. Considering the whole body of the data obtained from this extensive study, the set of conditions indicated earlier in this article was selected for further validation. A typical chromatogram of the HPLC method developed is shown in Figure 1.

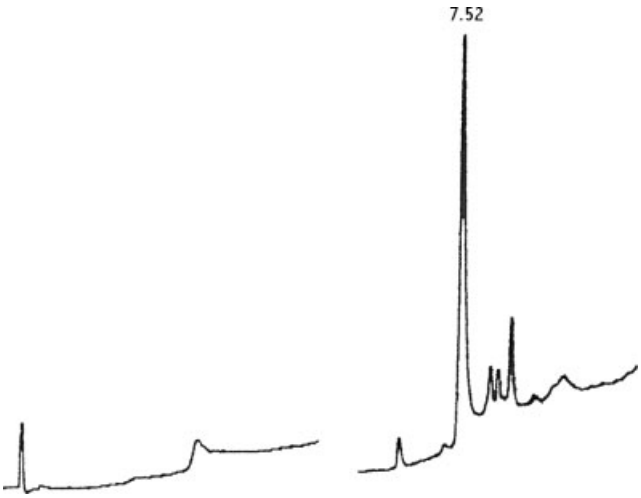

Figure 1. Typical chromatograms of blank phosphate buffer (left) and phosphate buffer containing 20 µg/mL of BSA (right) analysed by developed HPLC method.

System suitability tests

The number of theoretical plates (N), peak symmetry, and retentability (K′) of the method were 1413, 1.18 and 7.35, respectively. These data showed that the method developed is of appropriate separation efficiency and peak shape, both of which are important factors in evaluation of the chromatographic method outputs.

Method validation tests

Linearity

The method produced linear responses throughout the BSA concentration range of 1–100 µg/mL. A typical linear regression equation of the method was $y = 2282.2, x = -4485.9$ with x and y representing BSA concentration (in µg/mL) and peak height (in

Table 1. Within-run variations of the HPLC method for quantitation of BSA (n = 3)

Nominal added concentration (µg/mL)	Run number	Measured concentration (µg/mL)	Mean ± SD (µg/mL)	CV%	Accuracy	Mean ± SD
4.00	1	4.19	4.39 ± 0.19	4.35	104.75	109.83 ± 4.78
	2	4.57			114.25	
	3	4.42			110.50	
10.00	1	10.17	10.14 ± 0.24	2.42	101.70	101.46 ± 2.45
	2	10.38			103.80	
	3	9.89			98.90	
100.00	1	101.53	102.19 ± 0.62	0.61	101.53	102.19 ± 0.62
	2	102.28			102.28	
	3	102.77			102.77	

Table 2. Between-run variations of the HPLC method for quantitation of BSA (n = 3)

Nominal Added Concentration (µg/mL)	Run Number	Measured Concentration (µg/mL)	Mean ± SD (µg/mL)	CV%	Accuracy	Mean±SD
4.00	1	4.39	4.40 ± 0.14	3.30	109.75	110.00 ± 3.63
	2	4.55			113.75	
	3	4.26			106.50	
10.00	1	9.89	10.18 ± 0.26	2.60	98.90	101.80 ± 2.65
	2	10.24			102.40	
	3	10.41			104.1	
100.00	1	102.21	101.96 ± 0.72	0.71	102.21	101.96 ± 0.72
	2	101.15			101.15	
	3	102.54			102.54	

Table 3. Sample stability of BSA in room temperature (n = 3)

Concentration (μg/mL)	Percent recovered			
	Fresh sample	*12 h sample*	*24 h sample*	*72 h sample*
2.00	98.64 (2.35)[a]	98.13 (5.56)	95.73 (3.89)	96.76 (5.22)
50.00	101.21 (4.97)	98.77 (4.12)	96.41 (4.69)	95.71 (2.31)
100.00	102.24 (5.31)	99.43 (5.1)	97.12 (3.24)	96.22 (4.51)

[a] Men (SD).

arbitrary units), respectively, with the regression coefficient (r) of 0.9996.

Accuracy

The mean absolute recovery values of the method throughout the linear range are shown in Tables 1 and 2. From these data, it is obvious that the method is remarkably accurate, which ensures that this method produces reliable results.

Precision

The within- and between-run variations of the developed HPLC method are shown in Tables 1 and 2. These data indicate a considerable degree of precision and reproducibility for the method both during one analytical run and between different runs.

Limit tests

The limits of detection (LOD) and quantitation (LOQ) of the method were 0.5 μg/mL and 1 μg/mL, respectively with the CV% values of the peak heights of six successive injections in each case being 6.8% and 5.4%, respectively. These values reflect the high sensitivity of the method, which is of great importance in most studies on novel delivery systems of this protein. Considering the high molecular weight of BSA, this protein is routinely used in milligram amounts in drug delivery studies in order to fulfill the intended roles. The sensitivity reached in this method is therefore high enough to let it to be used in such a study. Some of the methods already used for BSA analysis offer better sensitivity than the current method (enzyme-based methods). However, those sensitivities are not required in delivery/formulation research and those methods have their own limitations as described earlier in this article.

Specificity

No evidence of interfering peaks was recorded in any of the pharmaceutical matrices tested. Therefore, this approach can be used reliably for BSA assay in a variety of delivery studies.

Sample stability

The stability of samples with different concentrations of BSA is shown in Table 3. These data showed that BSA has remarkable stability under test condition. Considering the fact that the stability of analysis samples is a serious concern in all protein/peptide studies, the results of this test indicate that the method can be used reliably in these studies.

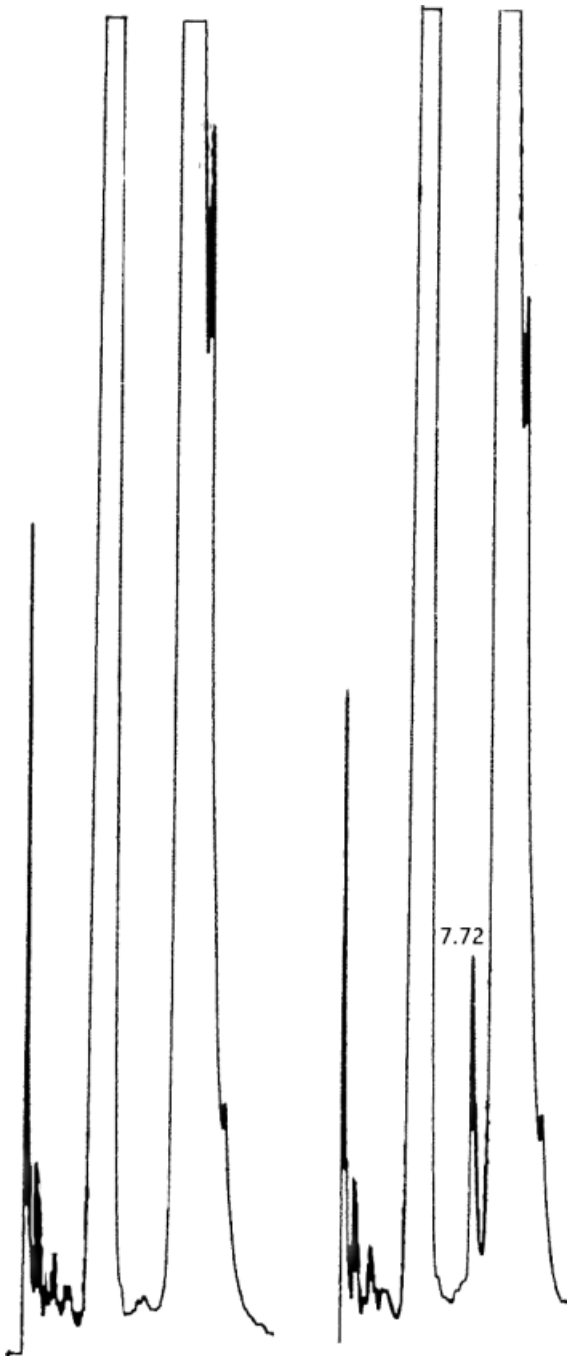

Figure 2. Chromatograms of a typical blank (BSA-free; left) and BSA-containing carrier erythrocyte samples (20 μg/mL; right). The peak corresponding to BSA has the retention time of 7.72 min.

Applicability tests

The wide linear range with considerable accuracy, precision and sensitivity allows the method to be used for BSA assay during a variety of in vitro experiments including the development of new formulations as well as novel systems for the delivery of this protein. For example, the method is being used successfully by authors during experiments on encapsulation of this protein in erythrocyte carriers. The typical chromatograms of the blank matrices as well as BSA-containing samples have been shown in Figure 2.

Conclusion

A simple and easily available HPLC method was developed in this study for the quantitation of BSA in pharmaceutical matrices. The main advantages of this method over the previously reported HPLC methods for BSA analysis[11,24] are its considerably shorter run times, its availability and its popularity. All of these properties are very important in practice, particularly when a large number of samples is to be analysed. The results of validation tests were, collectively, indicative for a method with a relatively wide linear range, acceptable precision and accuracy both within and between analytical runs, and practically reliable sensitivity. Our preliminary experiments with a wide range of matrices showed no interferences between the BSA peak and background peaks in chromatograms. This method is currently being used successfully as a part of a vaccine delivery project in our laboratory, the results of which have already been published separately.[28,29]

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