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Short communication

Selection of an analytical method for evaluating bovine serum albumin concentrations in pharmaceutical polymeric formulations

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ABSTRACT

Bovine serum albumin (BSA) is a commonly used model protein in the development of pharmaceutical formulations. In order to assay its release from various dosage forms, either the bicinchoninic acid (BCA) assay or a more specific size-exclusion high performance liquid chromatography (SE-HPLC) method are commonly employed. However, these can give erroneous results in the presence of some commonly used pharmaceutical excipients. We therefore investigated the ability of these methods to accurately determine BSA concentrations in pharmaceutical formulations that also contained various polymers and compared them with a new reverse-phase (RP)-HPLC technique. We found that the RP-HPLC technique was the most suitable method. It gave a linear response in the range of 0.5–100 μg/ml with a correlation co-efficient of 0.9999, a limit of detection of 0.11 μg/ml and quantification of 0.33 μg/ml. The performed '*t*'-test for the estimated and theoretical concentrations indicated no significant difference between them providing the accuracy. Low % relative standard deviation values (0.8–1.39%) indicate the precision of the method. Furthermore, the method was used to quantify *in vitro* BSA release from polymeric freeze-dried formulations.

1. Introduction

With the development of complex drug delivery systems containing proteins in complex polymer matrices comes a need for methods of determining both the drug loading in these systems and of determining rate of drug release from them. Currently, column separation techniques are the most widely used. For example, the retention mechanism originates from electrostatic interactions in ion-exchange chromatography [1], mixed modes of mechanisms in biospecific affinity chromatography [2] and metal chelate interaction chromatography [3]. In pharmaceutical formulations comprised of protein and other non-active ingredients, the native structure of the protein can be disrupted and, subsequently, a loss or decrease in the biological activity may occur [4]. The concept of incorporating proteins into polymers for controlled release has been recognized before several years. Currently, there are limited published methods for protein determination from polymeric controlled release formulations [5].

In this study, bovine serum albumin (BSA) is used as a model protein because of its stability, its lack of effect in many biochemical reactions, and its low cost to develop a suitable method to analyze such proteins without interference of polymers or other excipients. Several analytical methods have been developed and published for the quantification of BSA including SE-HPLC with fluorescence detector and buffers as mobile phase [6], with pre- and post-derivatization [7], online immobilized-enzyme HPLC method [8], HPLC–MS method [9], fluorescence quenching technique [10], HPLC with ionization mass spectrometry [11], and the BCA protein assay kit. The BCA protein assay uses detergent-compatible reagents for the colorimetric detection and quantification of total protein. The BCA method is not a true end-point method; that is, the final colour continues to develop as well as various substances react with bicinchoninic acid and so may give an artificially high estimation of protein concentration [12]. A number of fluorescent- and luminescent-based methods for peptide quantification are also in use [13,14]. Various modified HPLC procedures exist that incorporate for example extraction procedures, use of pH adjusted buffers in the mobile phase [15] and use of organic modifiers viz. cyanine dye [16], or BCA, which reduces column life.

Protein assays based on colorimetric principle, including the Biuret-based Lowry [17] and BCA assays, are occasionally utilized to quantify proteins. RP-HPLC based methods have the advantage of being accessible to most analytical laboratories, since they do not require expensive dedicated instruments [7]. We therefore investigated the ability of two existing techniques: BCA assay and SE-HPLC technique with a newly developed RP-HPLC method that can determine BSA concentrations without using buffers. The ability of these techniques to determine BSA concentrations in the presence of the polymers polycarbophil, poly(methyl vinyl ether/maleic anhydride), hydroxy ethylcellulose

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(HEC), hydroxypropyl methylcellulose (HPMC), polyvinylpyrrolidone (PVP), carboxymethylcellulose sodium (Na-CMC) or chitosan were validated before selecting one method with which to perform drug release studies from freeze-dried polymer-based formulations.

2. Materials and methods

2.1. Materials

BSA (fraction V, approx. 99%, lyophilized powder), HPMC (1,00,000 cps 2%w/v in water), Na-CMC (2,50,000 M.Wt), PVP and chitosan (2,00,000 cps) were purchased from Sigma–Aldrich Inc., MO, USA. Poly(methyl vinyl ether/maleic anhydride) (Gantrez S97BF, 1,980,000 M.Wt, ISP Technologies, Inc., Koln, Germany), polycarbophil (Noveon AA1, Surfachem Group Ltd., Leeds, UK), HEC (4500–6500 cps) (Hercules GmBH, Düsseldorf, Germany) were purchased. Trifluoroacetic acid, sodium hydroxide and glacial acetic acid were of analytical grades. Acetonitrile and water were of HPLC grade. Nunc F96 Micro-Well™ plates were supplied by Thermo Fischer Scientific, Roskilde, Denmark. The BCA assay kit was purchased from Pierce (Thermo Fisher Scientific Inc., Rockford, IL, USA).

2.2. Methods

2.2.1. Manufacture of BSA loaded freeze-dried polymeric tablets

Accurately weighed 3 g of each polymer was added to water to give 100 g total mass and hydrated overnight. Gels containing Noveon and Gantrez were neutralized with sodium hydroxide solution, while that containing chitosan was cross-linked with diluted glacial acetic acid. Accurately weighed 1 g of BSA was mixed to form uniform gels in a SpeedMixer™ (Synergy devices Ltd., Hauschild, Germany) at 2000 rpm for 2 min. 0.65 g of prepared gel was immediately filled in PVC blisters with upper diameter of 15 mm, lower diameter of 8 mm and depth of 4 mm resulting in a BSA dose of 6.5 mg per tablet and freeze-dried (Freeze dryer, Advantage, VirTis, Gardiner, NY, USA). The resultant tablets were analyzed on the same day of manufacture.

2.2.2. Analytical methods

2.2.2.1. BCA assay kit. A stock solution of BSA (1 mg/ml) was prepared by dissolving 100 mg in 100 ml of water in 100 ml volumetric flask. Working-standard solutions were prepared in water from the stock solution range from 0.5 to 200 μg/ml ($n = 9$). The same procedure was used to prepare BSA standard solutions for quantification by HPLC methods. 150 μl of each standard BSA was transferred to a microplate (five replicates) followed by 150 μl of BCA standard working reagent (SWR) (prepared by mixing 25 parts of BCA reagent A and 24 parts of reagent B with 1 part of reagent C [12]. The microplate was covered with the supplied lids and incubated at 37 °C for 2 h. The plate was then cooled to room temperature and the absorbance at 562 nm measured (PowerWave Microplate Spectrophotometer XS2, BioTek, Mason Technology, Dublin, Ireland).

2.2.2.2. Size-exclusion HPLC method. BSA was quantified using SE-HPLC with ultraviolet detection. Chromatographic conditions were as follows: Perkin-Elmer HPLC system; BioSep-SEC-S 3000 (300 nm × 7.8 nm i.d., 5 μm particle size) column, with security guard cartridge of the same material (Phenomenex, UK); run temperature 25 °C; isocratic mode; 0.05 M ammonium acetate buffer pH 5.5 mobile phase; run time 20 min, UV detection at 280 nm; flow rate 1.0 ml/min; injection volume 100 μl; BSA retention time 8.86 min. A linear calibration plot for BSA was obtained over the range 0.5–200 μg/ml ($n = 9$) ($R^2 = 0.999$).

2.2.2.3. Reverse-phase HPLC method. Chromatographic conditions for quantification of BSA by RP-HPLC method were as follows: Perkin-Elmer HPLC system; Jupiter 5 μm C5 300A column 4.6 mm i.d. × 250 mm length; with security guard cartridge of the same material (Phenomenex, UK); run temperature 25 °C; gradient flow (0.1%TFA in water (A), 0.1%TFA in acetonitrile (B); A/B from 95:5 to 35:65 in 20 min) mobile phase, total run time 25 min, UV detection at 220 nm; flow rate 1.0 ml/min; injection volume 100 μl; BSA retention time 17.6 min. A linear calibration plot for BSA was obtained over the range 0.5–200 μg/ml ($n = 9$) ($R^2 = 0.999$).

2.2.3. BSA/polymeric solution interaction studies

5 ml of BSA solution (70 μg/ml for BCA assay kit and 100 μg/ml for HPLC method) was mixed with 5 ml of different polymeric solutions (300 μg/ml) and quantified by three different methods as described in Section 2.2.2. The results were compared by determining concentrations in case of BCA assay kit and by chromatographs in case of HPLC methods.

2.2.4. In vitro BSA release studies from freeze-dried polymeric tablets

Polymeric freeze-dried tablets (containing different amounts of BSA) were placed in sealed borosilicate glass bottles with 100 ml of release medium (water), placed in a shaking incubator (60 rpm, with 2.5 cm throw) and maintained at 37 ± 0.5 °C. 1.5 ml of aliquot was withdrawn and replaced by fresh medium at regular time intervals. The samples were analyzed for BSA content by RP-HPLC method as described in Section 2.2.2.3. The % of BSA released from different polymeric formulations was calculated and reported in Table 3.

2.2.5. Statistical analysis

Data is expressed as the mean ± standard deviation (SD) of obtained results. All statistical calculations were performed using GraphPad InStat (version 3.0a for Macintosh, GraphPad Software, San Diego, CA, USA). '*t*'-Calculated > '*t*'-tabulated was considered as statistically significant.

3. Results and discussion

3.1. Linearity and validation

Linearity was determined from a calibration curve with standard BSA solutions in the range 0.5–200 μg/ml in water and with five replicates. Under the experimental conditions described in Section 2.2.2, linear relationship was observed between the absorbance or peak area of BSA to the concentration of BSA in water with all three methods as reported in Table 1 with regression co-efficient 0.999. The limit of detection D_L and the limit of quantification Q_L (Table 1) were attained as defined by IUPAC [18] and ICH Topic Q2B [19] using the slope (b) of the calibration curve and the standard deviation, Sa, of the intercept (a) by the following:

$$D_L = 3.3 \times \frac{Sa}{b} \quad (1)$$

$$Q_L = 10 \times \frac{Sa}{b} \quad (2)$$

The values of D_L and Q_L show that the RP-HPLC method is more sensitive and selective than the other two methods. Accuracy values were determined (Eq. (3)) over 2 days and were always within acceptable limits (±5%) at all validation concentrations (Table 1) for values determined by the BCA kit and the RP-HPLC method, but not for the SE-HPLC method.

$$\text{Accuracy} = \frac{(\text{True value} - \text{measured value}) \times 100}{\text{True value}} \quad (3)$$

Table 1
Validation results of various analytical methods for BSA.

Conc. μg/ml	R^a	Calibration equation[b]	Mean (SD)		% RSD[c]		% Accuracy		D_L[d] μg/ml	Q_L[e] μg/ml
			Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day		
BCA kit										
10	0.999	$Y = 0.04076(\pm 0.00033) \times X + 0.00512(\pm 0.00502)$	9.92 (0.17)	10.09 (0.25)	1.80	2.51	99.28	100.88	0.40	1.23
20			19.73 (0.11)	20.18 (0.33)	0.56	1.65	98.66	100.9		
40			40.03 (0.36)	40.26 (0.49)	0.90	1.22	100.07	100.64		
SEC-HPLC method										
10	0.999	$Y = 2025.1(\pm 37.9) \times X + 502.6(\pm 12.0)$	9.52 (0.50)	10.10 (0.39)	5.31	3.90	95.22	100.94	0.21	0.63
20			18.99 (0.23)	19.78 (0.96)	1.23	4.88	94.98	98.89		
40			42.09 (1.50)	39.90 (2.23)	3.56	5.57	105.24	99.755		
RP-HPLC method										
10	0.999	$Y = 31349.2(\pm 149.1) \times X + 3948.0(\pm 105.0)$	10.03 (0.08)	10.06 (0.14)	0.80	1.34	101.48	100.62	0.11	0.33
20			19.98 (0.15)	20.10 (0.24)	0.78	1.20	99.92	100.49		
40			40.59 (0.56)	40.06 (0.34)	1.39	0.85	101.48	100.16		

[a] Regression co-efficient.
[b] The peak area of the BSA, *Y*, vs. concentration of BSA, *X*, in μg/ml; nine standards ($n = 5$).
[c] Relative standard deviation.
[d] Limit of detection.
[e] Limit of quantification.

Table 2
BSA, different polymers and BSA with polymers together analysis by BCA kit.

Types of polymers	Blank polymer concentration (μg/ml) Mean ± SD	BSA Concentration in presence of polymers (μg/ml) Mean ± SD		
		Theoretical[a]	Practical	*t*-Value
Gantrez	3.36 ± 1.16	38.36	16.63 ± 4.09	12.46*
Noveon AA	3.32 ± 0.60	38.32	19.71 ± 4.47	9.00*
HEC	0.98 ± 0.46	35.98	36.27 ± 0.61	−0.81
HPMC	1.74 ± 0.05	36.74	36.75 ± 0.19	−0.12
PVP	4.25 ± 0.65	39.25	22.56 ± 7.68	5.13*
Na-CMC	0.52 ± 0.09	35.52	34.97 ± 0.20	0.38
Chitosan	0.63 ± 0.09	35.63	35.91 ± 0.35	−2.11
BSA 35 μg/ml	34.99 ± 0.06	34.99	–	–

t-Tabulated value is 2.77 at degree of freedom 4.
[a] Theoretical = 35 μg/ml of BSA + respective blank concentration in μg/ml.
* Statistically different.

Precision was determined by the elaboration of three standard calibration curves, two from the same day (intra-day precision) and third one from a different day (inter-day precision). Intra-day and inter-day precision of methods were assessed by analyzing BSA in water at low (10 μg/ml), medium (20 μg/ml) and high (40 μg/ml) concentrations. Five replicates of each concentration were analyzed as shown in Table 1. To evaluate intra-day precision, the mean values and the % RSD values were calculated for each concentration. The precision was found to be acceptable, with the % RSD values ranging from 0.56 to 1.80% by BCA kit and 0.78 to 1.39% by RP-HPLC method, but it was more than 5% for the SE-HPLC method. The inter-day data for precision was also determined by analyzing three sample batches of BSA in water at low, medium and high concentration on 2 days. The inter-day % RSD values ranged from 1.22 to 2.65% and 0.85 to 1.34% for values determined by the BCA kit and RP-HPLC method, while they ranged from 3.90 to 5.57% for values determined by the SE-HPLC method (Table 1). Therefore the accuracy and precision data revealed that BCA kit and RP-HPLC method are more accurate and precise than the SE-HPLC method.

3.2. BSA/polymer interaction studies

The results from the BSA/polymer interaction studies using the BCA kit are reported in Table 2 and those determined by SE-HPLC and RP-HPLC are shown in Figs. 1 and 2 respectively.

3.2.1. BCA assay kit

There was a significant difference found between the theoretical and practical BSA concentrations as determined by the BCA kit in the presence of Noveon (*t*-value, 12.46), Gantrez (*t*-value, 9.00) and

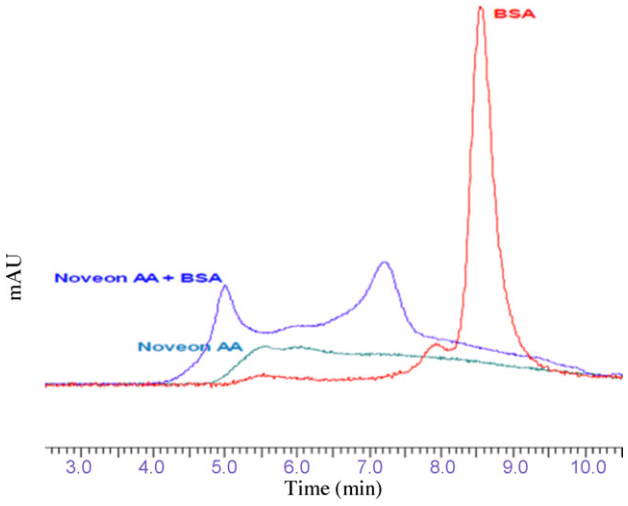

Fig. 1. Chromatographs of (a) Noveon, BSA and Noveon + BSA solution and (b) Gantrez, BSA and Gantrez + BSA solutions obtained by the SE-HPLC method.

Table 3
% BSA released from polymeric freeze-dried tablets.

Time in min	% BSA released from freeze-dried polymeric tablets (Mean ± SD)						
	Noveon AA	Gantrez	HEC	HPMC	PVP	Na-CMC	Chitosan
5	–	4.02 ± 1.84	–	–	8.27 ± 3.12	7.40 ± 1.07	66.58 ± 5.83
10	–	78.04 ± 9.43	–	–	94.82 ± 1.39	47.48 ± 7.88	85.98 ± 7.69
15	3.98 ± 1.82	91.03 ± 5.27	7.50 ± 2.83	7.42 ± 1.07	97.00 ± 1.22	56.91 ± 14.18	99.08 ± 0.48
30	10.83 ± 4.53	99.87 ± 1.01	25.74 ± 2.66	16.48 ± 1.63	99.49 ± 1.64	94.81 ± 1.56	
60	24.19 ± 6.18	–	52.23 ± 9.57	23.82 ± 4.74	–	–	–
120	52.21 ± 8.13	–	85.01 ± 5.55	37.80 ± 6.58	–	–	–
240	85.16 ± 5.96	–	96.40 ± 4.26	62.73 ± 5.83	–	–	–
360	99.39 ± 2.94	–	99.73 ± 1.83	77.32 ± 8.61	–	–	–
480	–	–	–	87.66 ± 3.67	–	–	–
1440	–	––	–	100.25 ± 3.65			

PVP (*t*-value, 5.13). These polymers showed an interaction with BCA working reagent and interfered with BSA analysis with the BCA kit method (Table 2). The BCA kit method uses BCA as a detection reagent for Cu^{+}, which is formed when Cu^{2+} (present as one of the reagents in BCA kit) is reduced by carboxylic acids and amine groups present on the protein surface in an alkaline environment [20]. A purple complex is formed by chelation of two molecules of BCA with one Cu^{+} ion and exhibits a strong absorbance at 562 nm. Noveon [21], Gantrez [22] and PVP (at high concentration) [23] compete with BSA to form a complex with the Cu^{2+} ions. None of the other polymers investigated interfered with the BCA assay.

3.2.2. SE-HPLC and RP-HPLC methods

In this study the SE-HPLC method gave reasonable results although they were not as accurate, precise or sensitive as the other methods (Table 1). During the BSA/polymer interaction study, it was found that Noveon and Gantrez interfered at the low pH (5.5) of the mobile phase 0.05 M ammonium acetate buffer (Fig. 1). This is caused by formation of stable water-soluble polycomplexes between BSA and the anionic polyacrylic acid in aqueous solutions at pH below the isoelectric point (pI) of BSA (pH 6–7) [24]. These eluted at a retention time of 5.2 min (Fig. 1) [25]. There was some free BSA left in the mixture that eluted at 7.5 min, which is earlier than the peak for pure BSA (8.6 min). This indicates the formation of aggregates of BSA in the presence of Noveon. In the literature, it is also reported that the fluorescence intensity of BSA sharply decreased when a different amount of polyacrylic acid was added [24]. It is hypothesized that the polymeric maleic acid derivatives present in Gantrez may play the same kind of role as the polyacrylic acid derivatives present in Noveon and form soluble polycomplexes

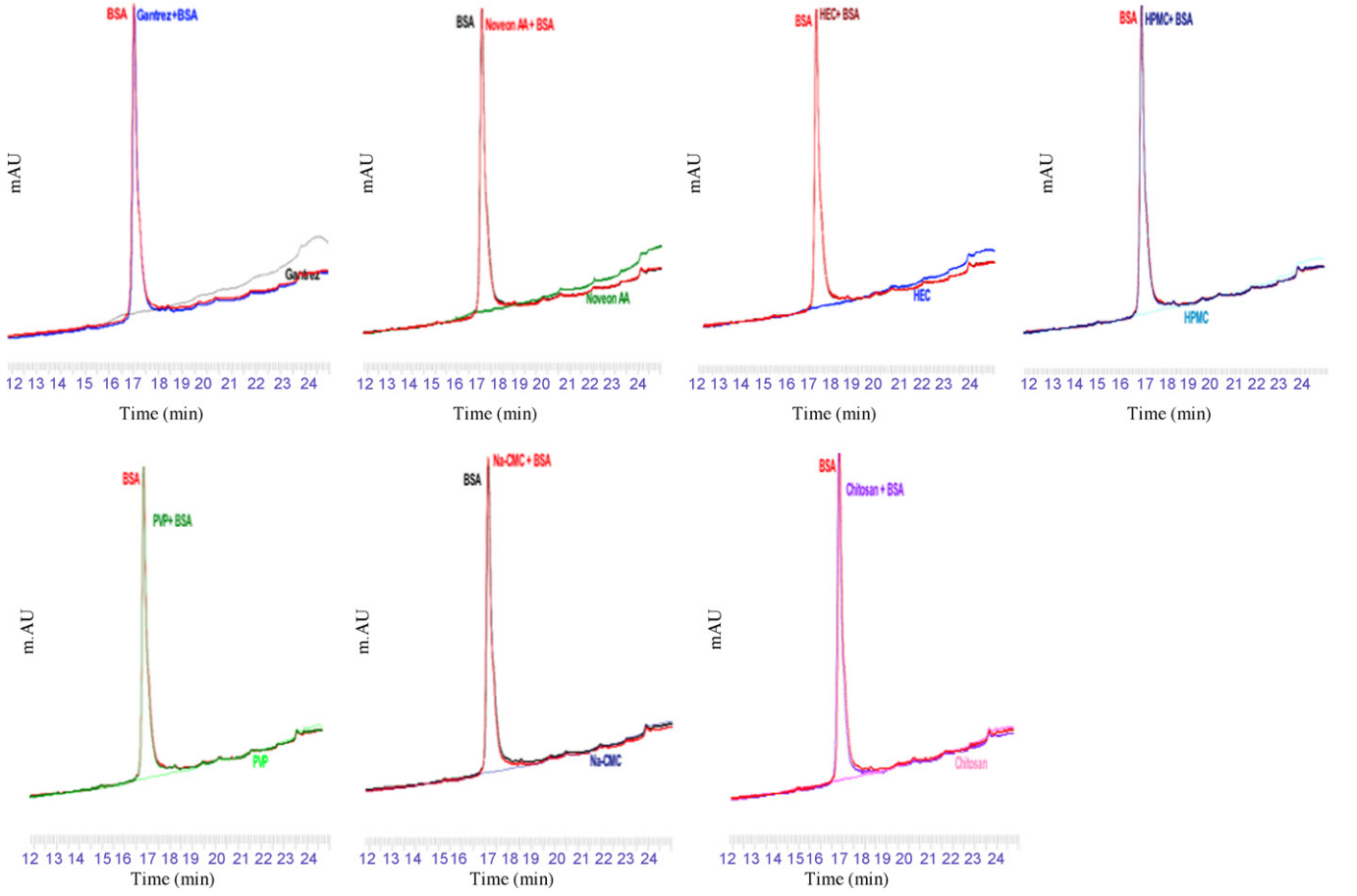

Fig. 2. Chromatographs showing responses from solutions of BSA, BSA in the presence of polymer and response of the polymer solution alone. Data obtained by the RP-HPLC method.

which eluted at 5.6 min retention time. A decrease in the peak area due to BSA was found in the presence of Gantrez and Noveon.

In this study, BSA/polymer interaction study was also evaluated by a newly developed RP-HPLC method. Ideally in RP-HPLC method, there is an adsorption of hydrophobic molecules onto a hydrophobic stationary phase in a polar mobile phase. BSA has a great affinity to adsorb on hydrophobic surfaces [26], which can be reduced by decreasing the mobile phase polarity by using organic solvents (acetonitrile) resulting in desorbtion and elution from the reverse-phase column. With the use of a gradient method, the amount of 0.1% TFA in acetonitrile was increased from 5 to 65% over 20 min, which helped the BSA to desorb. BSA was eluted at 17.6 min (Fig. 2) the peak shape and intensity were as for the pure BSA samples in the presence of all polymers. Due to the hydrophilic nature of the polymers, they were not adsorbed on stationary phase and did not interfere with the BSA determination.

3.3. Application of RP-HPLC method in in vitro BSA released study

The % BSA released from different polymeric freeze-dried tablets is shown in Table 3. These results indicated that RP-HPLC method is a suitable method to determine the release of BSA from these polymer matrices. 100% of BSA was released from chitosan in 15 min, from Gantrez, Na-CMC, PVP polymeric tablets in 30 min, while it took 6 h to be released from Noveon and HEC and 24 h to be released from HPMC. The order of % BSA released in time from different polymers was found as follows: Chitosan < Gantrez = Na-CMC = PVP < Noveon AA = HEC and < HPMC. Thus, Noveon, HEC and HPMC are ideal polymers to sustain the release of BSA from freeze-dried tablets for a longer period of time in a controlled manner.

4. Conclusion

The BCA kit, SE-HPLC and RP-HPLC techniques were compared for their ability to determine BSA concentrations in the presence of polymers. RP-HPLC was selected on the basis that the polymers did not interfere and that it gave good selectivity and sensitivity. The method was also shown to be highly reproducible.

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