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Research Paper

A Validated and Rapid HPLC Method for Quantification of Human Serum Albumin in Interferon beta-1a Biopharmaceutical Formulation

Somayyeh Heidari [1], Mohsen Imani[2], Seyed Mojtaba Mostafavi[3]*

[1] Department of Cell and Molecular Biology, Universiti Putra Malaysia, Serdang, Malaysia

[2] Cinnagen Pharmaceutical Company, Karaj, Iran

[3] Department of Chemistry, Faculty of Science, K. N. Toosi University of Technology, Tehran, Iran

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Abstract

Multiple sclerosis, or MS, is a long-lasting disease that can affect your brain, spinal cord, and the optic nerves in your eyes. It can cause problems with vision, balance, muscle control, and other basic body functions. IFN-beta treatment reduces the relapse rate in multiple sclerosis (MS), but the exact mechanism of action of the drug has remained elusive. CD73 (ecto-5'-nucleotidase) is an ectoenzyme, which produces adenosine from adenosine monophosphate (AMP) precursor by enzymatic dephosphorylation. In this study, a validated and rapid HPLC method for quantification of human serum albumin in interferon beta-1a biopharmaceutical formulation was developed and linearity, robustness, specificity, accuracy and precision of samples were determined.

Keywords: Multiple Sclerosis (MS); Human Serum Albumin; Interferon beta-1a; robustness; specificity;

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1. Introduction

Human serum albumin (HSA), biosynthesized by liver, is the most abundant plasma protein and plays a crucial role in several physiological processes, including maintenance of the blood osmotic pressure, hormone transport, fatty acid binding, ion transport, blood pH buffering and other applications [1,2]. For medical purposes, this protein is used to restore and maintain circulating blood volumes in case of hypovolemic shock, kidney disorders, burns, hepatic failure, hypoproteinemia and other applications [3-5]. Moreover, because of its nutritional, anti-oxidant and cryoprotective properties, HSA is an important ingredient of culture, freezing and thawing media, which are used for assisted reproductive techniques (ART), including in vitro fertilization (IVF), intracytoplasmic sperm injection (ICSI) procedures, and cryopreservation of gametes and embryos [5-9].

Human serum albumin (HSA) is a single chain non-glycosylated polypeptide [10]. However, studies have demonstrated that it is composed of a complex mixture of variants such as mercapto and non-mercapto [11], glycated [12] and polymerized [13,14] forms. Conditions of storage and manufacture can further increase heterogeneity by introducing deamidation, oxidation or polymerization [14]. Chromatographic separations of HSA components based on size-exclusion [11, 15, 16], ion-exchange [17-18] and reversed-phase [19] HPLC techniques have been reported.

Methodologies using high performance chromatographic and electrophoretic separation techniques have been increasingly used over the last two decades for the purification, characterization and quality assessment of biomolecules [6,7]. These methods are particularly useful for biopharmaceuticals, especially for biologically important proteins and glycoproteins produced through recombinant DNA technology.

Reversed-phase HPLC (RP-HPLC) is one of the most versatile chromatographic modes for studying proteins and their impurities, owing to advances made in microparticulate stationary phases and to the development of a wide range of columns of differing selectivities [8,9].

Due to the abundant concentration of HSA as the stabilizer in comparison with the active pharmaceutical molecule content in the formulations of biopharmaceuticals, quantitation of such excipient will be challenging. In this study, we introduce a precise, accurate, robust and also time-saving reversed phase high-performance liquid chromatography (HPLC) method for quantification of HSA concentration as the stabilizer in presence of IFN-β-1a in drug product formulation.

Table 1. Chromatographic condition

Column	**VYDAC 214TP C4 (250mm × 4.6 mm I.D. ; Grace)**
Mobile phase	A: 0.1% TFA (v/v) aqueous solution B: 0.1% TFA (v/v) in 99.9% Acetonitrile
Gradient elution	Gradient program (time, %B): 0 min, 20% B; 0-5 min, 40% B; 5-8 min, 45% B; 8-10 min 60% B; 10-11 min 100% B; 11-13 min, 100% B; 13-14min, 20% B; 14-20 min 20% B
Flow rate	1 mL/min
Injection volume	20 µL
Column temperature	40 °C
Sample temperature	2-8 °C
Detection wavelength	280 nm
Run time	20 min

2. Materials and methods

2.1. Chemicals and Reagents

Human serum albumin 20% solution (200 g/l) was purchased from CSL Behring AG (Switzerland). All of the standard solution of HSA were prepared from this concentrated solution. CinnoVex (CinnaGen, Iran) was used as a representative IFN-β1a reference material. The specification of the medicine indicated that there were 15 mg of HSA and 30 µg protein in each vial along with Sodium Chloride (5.8 mg), Sodium di-hydrogen phosphate (5.7 mg) and di-Sodium hydrogen phosphate (1.2 mg). N-acetyl-L-tryptophan were purchased from Sigma. Milli-Q water (Millipore, Germany) was used throughout the study and the gradient grade Acetonitrile and Trifluoroacetic acid (pure for Analysis) purchased from Merck.

2.2. HSA standard sample solutions

All of the standard solutions of HSA were prepared from the HSA 20%standard solution using MilliQ water as the diluent. The working standard solution were prepared using the HSA 20% standard and marketed IFN-β1a (CinnoVex) that spiked into DP-Buffer to obtain desire concentration, respectively. 4 HSA standard solutions consist of 7.5, 12, 15 and 18 mg/ml were prepared by dilution of HSA 20% standard solution via MilliQ water.

2.3 Chromatographic system and condition

An Agilent 1260 HPLC system equipped with a quaternary pump, a multi-sampler with thermostat, a column oven and a photodiode array detector was used to performing all chromatographic analyses. The HPLC system was controlled by OpenLab software. Chromatographic method is summarized in table 1.

2.4. Analytical method validation

Method validation was performed according to the International Conference on Harmonization ICH Q2 (R1) guidelines relating to the validation of analytical procedure [20] and therefore the linearity and range, precision (repeatability and intermediate precision), accuracy, robustness and specificity have been evaluated after peak identification.

Based on guideline, a linear relationship should be evaluated across the range of the analytical procedure [20]. For the establishment of linearity, calibration curve was used consisting of 4 HSA standard solutions of 7.5, 12, 15 and 18 mg/ml, prepared in triplicate and injected to HPLC. According to linear regression, R2 of regression line should be $\geqslant 0.99$ and 95% C.I of slope and Y-intercept for regression line should contain 1 and 0 respectively [21].

The standard deviation (SD) of the analytical response and the slope of the linear regression curve (S) were used in estimation of the limit of detection (LOD) and the limit of quantification (LOQ) that may be calculated by (3.3× SD/S) and (10×SD/S) formulas, respectively.

Repeatability was assessed by quantification of 6 spiked HSA solutions in the target concentration (15 mg/ml). Intermediate precision was determined by analyzing 6 spiked HSA samples of 15 mg/ml on different days. To determine the accuracy of the measurement, the recovery percentage of spiked HSA solutions in linearity assessment should not show any significant difference with ideal recovery percentage (100%).

3. Results and discussion

3.1 Method development

The HPLC method, which has been developed previously for the determination of human serum

albumin (HSA) [22], were applied for the assay of HSA in presence of IFN-β1a. In brief, we used a VYDAC 214TP C4 Column (250 mm × 4.6 mm i.d.; Grace) 5 µm with pore size of 300 Å. The large pores of the 300 Å TP silica allows polypeptide molecules to have complete access to the interior area of the silica pores [23]. A gradient elution was applied over a period of 20 min, with 0.1% TFA (v/v) aqueous solution mobile phase A and 0.1% TFA (v/v) in 100% acetonitrile (ACN) mobile phase B [22].

3.2 Peak identification

Due to the presence of N-Acetyl-Tryptophanate in HSA 20% standard solution, the corresponding peak for N-Acetyl-Tryptophanate should be characterized. To identify all peaks, samples of HSA containing N-Ac-Trp and IFN-β1a solution were analyzed respectively. Separation on the chromatographic condition shows three peaks, as demonstrated in figure 1. The gradient of mobile phases caused the elution of N-Acetyl-Trp, HSA and IFN-β1a respectively. To identify all peaks, samples of N-Acetyl-Trp, HSA and IFN-β1a were analyzed separately. The chromatograms reveal that the first peak (Rt =4.87 min, n=3, RSD=0.21%) corresponds to N-Acetyl-Trp standard solution peak (Rt=4.83, n=3 ,RSD=0.20%). Moreover, the identification of HSA peak (Rt =8.24 , n=3 ,RSD=0.09%) is confirmed by the analysis of standard HSA (Rt = 8.23 ,n=3 ,RSD=0.12%). The third peak, (Rt=12.5 n=3, RSD=0.07%), matched to IFN-β1a that was confirmed by the analysis of IFN-β1a standard solution.

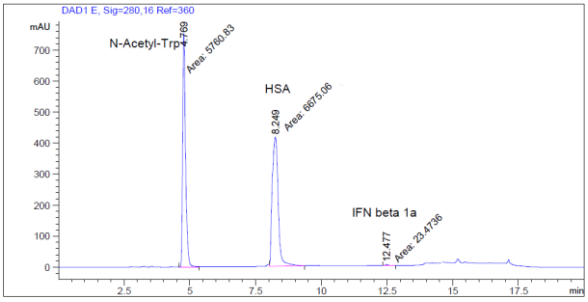

Figure 1. Chromatographic analysis of an HSA in presence of IFN-β1a.

3.3 Analytical method validation

The Human Serum Albumin 20% was used throughout the study, as reference standard material. The working standard solutions were prepared daily immediately before analysis by dilution with Milli-Q water.

3.3.1 Linearity and Range

To determine the linearity, a linear relation was evaluated across the range 50 to 120 percent of the target concentration [25]. Table 2 shows the calibration function of the 5 independent runs. The relation between the area under the peak (AUP) and HSA concentrations was linear over the range 7.5-18 mg/ml. The 95% C.I. of intercept (a) and slope (b) for regression line should contain 0 and 1, respectively. The LOD and LOQ were estimated as indicate in experimental section (figure 2).

Table 2. Linearity and range of the method.

Parameters	Value
Intercept (a)	0.072
Slope (b)	0.992
S (a)[a]	0.182
P-value (%)[b]	0.000
R^2 [c]	0.998
Limit of detection (LOD) (mg/ml)[d]	3.375
Limit of quantification (LOQ) (mg/ml)[d]	10.22
Practical linear range (mg/ml)	7.5-18.00

[a] Standard deviation of the intercept.
[b] Probability of intercept significant.
[C] Determination coefficient.
[d] Estimated from the SD of the intercept (a).

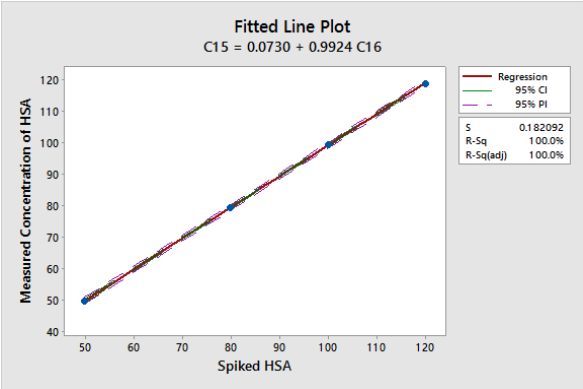

Figure 2. Standard curve of HSA, with a concentration range between 7.5-18 mg/ml.

3.3.2. Accuracy

Accuracy should be established across the specified range of the analytical procedure as stated in the ICH Q2(R1) guidelines [3]. As shown in table 3, the recovery percent of measured contents of spiked HSA samples in linearity assessment, didn't have any significant difference with ideal recovery percent (100%). The P-value of comparison were more than 5%, indicating that the recovery values in all cases were near to 100%.

3.3.3. Precision

To calculate the repeatability of the method, six replicate injection from sample preparation at 100% of the target concentration were analyzed by one analyst on one day. The effects of random events on the precision of the analytical procedure (Intermediate precision) established by typical

variation include days. Table 4, showing RSD of less than 2% that is a satisfactory result for both intra-day and inter-day precision (figure 3).

Table 3. Accuracy of the method.

Concentration tested (mg/ml)	AUP	Calc. concentration (mg/ml)	Recovery [a] (%)
7.500	3351.746	7.462	99.494
12.000	5344.393	11.895	99.122
15.000	6705.492	14.924	99.494
18.000	8026.540	17.864	99.245
		Average recovery (%)	99.338
		Standard deviation	0.186
		RSD(%)	**0.187**

[a]Recovery value obtain from three samples prepared from standard.

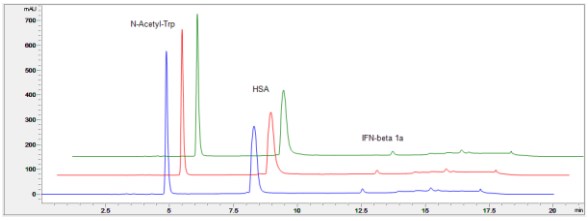

Figure 3. Chromatograms of three intra-day analysis replicates in HSA target concentration (15 mg/ml)

3.3.4. Specificity

Forced degradation studies were performed using five samples that were kept in 45°C for three days and the HSA concentrations measured by the method were compared with samples in suitable condition. Due to degradation of samples, AUPs of the degraded samples were statistically different from AUPs of samples kept in 2-8°C by P-value of 0.000 (Table 5).

Table 4. Intra-day and inter-day precision of the method.

Precision (RSD, %)[b]	
Intra-day	**Inter-day (3 days)**
0.24	0.36

[b] Relative standard deviation from six standard samples.

Table 5. Specificity of the method to differentiate the stressed samples from normal ones.

AUP of HSA Peak in 15 mg/ml concentration	
Samples stored in 2-8°C condition	**Samples stored in stressed condition**
6675.956	6100.279
6689.092	6111.491
6655.742	6128.092
6687.813	6116.103
6656.771	6094.824

3.3.5. Robustness

Robustness of the method was evaluated by deliberate but small changes in UV wavelength, column temperature and composition of the mobile phase (Content of TFA). A Full Factional Design DOE for robustness study of the method is used which the results are as in table 6.

For the AUP of HSA peak, the method is not robust for the considered variabilities of mentioned factors according to the estimated P-values. According to the above statistical assessments and graphs, the effect of wavelength variability on AUP values are significantly greater than two other factors between experiments at its lower and upper levels. It is determined that the method results are significantly affected by the variation of mentioned factors, so they should be controlled appropriately during method execution.

Table 6. Full Factional Design DOE for robustness study

Standard Order	Run Order	Center Point	Blocks	Wavelength (nm)	Column Temperature (ºC)	TFA (%)	HSA Peak AUP	HSA Peak Retention time (min)
3	1	1	1	278	42	0.090	6877.9	8.261
1	2	1	1	278	38	0.090	6882.0	8.276
2	3	1	1	282	38	0.090	6347.5	8.276
5	4	1	1	278	38	0.011	6874.4	8.474
8	5	1	1	282	42	100.1	6223.9	8.474
7	6	1	1	282	42	10.01	6816.2	8.445
11	7	0	1	280	40	0.010	6678.9	8.416
9	8	0	1	280	40	0.010	6693.1	8.417
4	9	1	1	282	42	0.090	6295.7	8.303
10	10	0	1	280	40	0.010	6693.3	8.415
6	11	1	1	282	38	10.01	6268.8	8.477

For the Retention time, the method is not robust for TFA variabilities according to the estimated P-value (0.000). According to the above statistical assessments and graphs, the effect of variability of TFA content in the mobile phase on retention time of HSA is 0.1885 min between experiments at its lower and upper levels. It is determined that the method results are significantly affected by the variation of TFA, so it should be controlled appropriately during method execution.

References

[1] M.A.Rothschild,M. Oratz and S.S. Schrieber, Hepatology,1988, 8, 385.
[2] G. J. Quinlan, G.S.Martin and T.w.Evans, Hepatology, 2005, 41, 1211.
[3] C. A. Janeway, S. T. Gibson, L. M. Woodruff, J. T. Heyl, O. T. Bailey and L. R.Newhouse, J. Clin. Invest. 1944, 23,491.
[4] A. Cournand, R. P. Noble, E. S. Breed, H.D. Lauson, E. De F. Baldwin, G. B. Pinchot and D.W. Richards Jr, J. Clin. Invest, 1944, 23,491.
[5] J. L.Tullis, J. Am. Med. Assoc., 1977, 237, 460.
[6] Y. Menezo, J. Testart and D.Perone, Fertil. Steril, 1984, 42, 750.
[7] I.Khan, C. Staessen, P. Devroey and A. C. Van Steirteghem, Fertil. Steril, 1991, 56, 98.
[8] N.Hoist, K. Bertheussen, E.Forsdahl, M.B. Hakonsen, L.J. Hansen and H.I. Nielsen, J. Assist. Reprod. Genrt, 2005, 7, 47.
[9] M. Roche, P. Rondeau, N.R. Singh, E. Tarnus and E. Bourdon, FEBS Lett., 2008, 582, 1783.
[10] D. C. Cartex, X. He, S. H. Munson, P. D. Twigg, K. L. Gernert, M. B. Broom, T. Y. J. Miller, Science, 244, 1195 (1989).
[11] M. Sogami, S.Nagoka, S. Era, M. Honda, K. Noguchi, Int. J. Pept. Protein Res. 24, 96 (1984).
[12] I. Syrovy, J.Biomed. Biophys. Met. 28, 115 (1994).
[13] G. Jordan, S. Yoshioka, T.Terao, J.Pharm. Pharmacol. 46,182 (1994)
[14] T. Moreira, L.Cabrera, A. Gutierrez, A.Cadiz, M.E. Castel-Lano, Acta Pharm. Nord. 4, 59 (1992).
[15] M. Sogami, S. Era, S. Nagaoka, K. Kuwata, K. Kida, J.Shigemi, J. Chromatogr. 332, 19 (1985).
[16] B. M. Van Liedekerke, H. J. Nelis, J.A. Kint, F.W. Vannest, A.P. De Leenheer, J. Pharm. Sci. 80 (1), 11 (1991).
[17] A. Sugii, K. Harada, K. Nishimura, R. Hanaoka, S.Masuda,J. Chromatogr. 472,357 (1989).
[18] K. Harada, k. Kitahara, M. Nakayama, T. Eoth, A. Sugii, Y. Ujila, H. Okabe, Analytical Science 10, 461 (1994).
[19] P. Vidol, E.Nielsen, B. S. Welinder, J. Chromatogr. 573, 201 (1992).
[20] International Conference on Harmonization (ICH) guidelines ICH Q2 (R1), Validation of analytical procedures; text and methodology, 2005.
[21] Parenteral drug association (PDA), Analytical method validation and transfer for biotechnology products, Technical report No.57.
[22] Frank Eertmans, Veerle Bogaert, Barbara Puype, Anal. Methods, 2011, 3, 1296.
[23] Microscale Structure Analysis of a High Molecular Weight Hydrophobic Membrane Glycoprotein Fraction with Platelet Derived Growth Factor-Dependent Kinase Activity, P. Tempst, D. Woo, D. Teplow, R. Aebersold, L. Hood, and S. Kent, J. Chrom. 359, 403-412 (1986).