Analytical Methods

Cite this: *Anal. Methods*, 2011, **3**, 1296

www.rsc.org/methods

PAPER

Development and validation of a high-performance liquid chromatography (HPLC) method for the determination of human serum albumin (HSA) in medical devices

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Received 16th March 2011, Accepted 14th April 2011
DOI: 10.1039/c1ay05148b

Because of its nutritional, anti-oxidative, and cryoprotective properties, human serum albumin (HSA) is an important ingredient of the culture and cryopreservation media for assisted reproductive techniques (ART) such as *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) procedures. Several tools are available for the determination of this serum protein in biological samples and pharmaceutical preparations, including colorimetric, electrophoretic, and immunological assays. However, because of inter-assay variability and accuracy problems of the above-mentioned assays, we have chosen to develop and validate a reverse phase (RP) high-performance liquid chromatography (HPLC) method to assess HSA content in ART-related media. Briefly, a gradient elution (a combination of acetonitrile/water, supplemented with 0.1% (v/v) trifluoroacetic acid) was used to separate samples on a C4 (*n*-butyl-coated silica) column. Two main peaks were observed at 4.970 and 8.715 min, representing the stabilizer *N*-acetyl-tryptophan (N-Ac-Trp) and HSA respectively. Validation of the method demonstrated that HSA can be determined in an accurate and precise manner, in a range between 0.4 and 25 mg ml^{-1}, without interference of matrix ingredients. The limit of detection (LOD) and lower limit of quantification (LLOQ) values were 0.128 and 0.386 mg ml^{-1}, respectively. In summary, this RP-HPLC method serves as a quality control for ART product release and stability studies. If required, the method can be easily adapted for assessment of HSA in biological samples and pharmaceutical preparations.

Introduction

Human serum albumin (HSA), produced in the liver, is the most abundant plasma protein and plays a crucial role in several physiological processes, including maintenance of the osmotic pressure, hormone transport, fatty acid binding, ion transport, blood pH buffering, …[1,2] For medical purposes, this protein is used to restore and maintain circulating blood volumes in case of (hypovolemic) shock, for treatment of kidney disorders, burns, hepatic failure, hypoproteinemia, …[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]

It is clear that HSA plays a key role in different fields of human medicine, whereby the applied concentration depends on its purpose. For this reason, accurate determination of HSA in ART media and other matrices such as pharmaceuticals and biological samples (*e.g.* blood and urine) is essential. Several tools are widely used, including the bromocresol green (BCG) and bromocresol purple (BCP) assays,[10] the biuret method,[11] electrophoretic techniques, and immunological tests.[12] However, besides inter-method variance in the estimation of HSA content, assay-specific problems may occur. For example, several publications report accuracy problems when using the colorimetric BCG and BCP tests, resulting in under- or overestimation of HSA.[13,14] Alternatively, very sensitive and accurate fluorometric tests are available, which have been developed for the determination of very low HSA concentrations in biological samples (*e.g.* in the case of microalbuminuria).[15] However, this type of assay is not suited for our purposes, as HSA content in ART products of interest varies between 0.4 and 2% (v/v).

In view of the reported inter-assay variability and accuracy problems, we have chosen to use high-performance liquid chromatography (HPLC) with gradient elution for the analysis of HSA in ART products. The advantages of a correctly developed HPLC assay include short analysis time, high resolution, high sensitivity, reproducibility, accuracy, and automation possibilities.[16] Several HPLC methods for the determination of HSA have been described, mostly based on size exclusion, ion exchange, and reverse phase (RP) separation.[17–21]

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This paper describes the development and validation of an RP-HPLC method, which allows quick (10 min) and reliable determination of HSA content in medical devices. The obtained data are used in-house for quality control (QC) purposes, including product release and stability studies. Although not tested, this method can be easily adapted for HSA determination in other matrices such as pharmaceuticals, and human biological samples, following appropriate sample preparation.

Materials and methods

Chemicals and reagents

Human serum albumin was purchased as a 25% isotonic solution from Talecris Biotherapeutics (Frankfurt am Main, Germany). Ultrapure water (level 1+) was made in-house by reverse osmosis on a Milli-RO 60 device (Millipore, Brussels, Belgium) and subsequent deionization and purification using an Elga Purelab Genetic device (Analis, Namen, Belgium). Recombinant HSA was obtained from Novozymes Belgium (Overijse, Belgium). *N*-Acetyl-tryptophan (N-Ac-Trp; ≥99%) was purchased from Sigma Aldrich (Bornem, Belgium). HPLC-grade acetonitrile (ACN; 99.9%) conforming to the European Pharmacopoeia was obtained from Acros Organics (Geel, Belgium) and Merck (VWR, Haasrode, Belgium). HPLC-grade trifluoroacetic acid (TFA; ≥99.5%) was purchased from Pierce (Thermo Fisher Scientific, Erembodegem, Belgium) and Acros Organics (Geel, Belgium). Isopropanol and methanol were obtained from Prolabo (VWR, Haasrode Belgium).

Equipment

An Agilent 1100 HPLC system was used, consisting of a quaternary pump (G1311-A), a vacuum membrane degasser (G1379-A), a thermostatted autosampler (G1330-B, G1367-A), a thermo-regulated column department (G1316-A), and a photodiode array detector (G1315-B). The HPLC system was controlled by Agilent's ChemStation software, version B.03.02 (341). Chromatographic separation was performed on a VYDAC® 214TP C4 column (250 mm × 4.6 mm i.d.; Grace Davison, Lokeren, Belgium), consisting of polymerically bonded, endcapped *n*-butyl-coated silica particles (5 μm) with a pore diameter of 300 Å. To protect the analytical column and to extend its life time, a guard column, containing identical C4 material as the former, was mounted in front.

Chromatographic conditions

A gradient elution was applied over a period of 20 min, with mobile phase A being a 0.1% TFA (v/v) aqueous solution and mobile phase B being 0.1% TFA (v/v) in 100% acetonitrile (ACN). Both liquids were filtered through a 0.22 μm filter before use. For more details concerning the gradient setup, we refer to Table 1. The autosampler was chilled to 4 °C. Eluent was pumped at a flow rate of 1 ml min^{-1}, the injection volume was 20 μl, the column department was heated to 40 °C and the detection wavelength was set to 280 ± 2 nm.

Table 1 Gradient elution scheme for chromatographic separation of HSA on a C4 RP column

Time (min)	% Mobile phase A	% Mobile phase B
0	80	20
5	60	40
8	55	45
10	40	60
11	0	100
13	0	100
14	80	20
20	80	20

Standard curve and quality control samples

To assess linearity and range, we used a calibration curve consisting of 7 HSA aqueous solutions of 0.4, 0.5, 1, 5, 10, 15, and 25 mg ml^{-1}, prepared by serial dilution of the 25% stock solution. Ultrapure water served as a blank. For each analytical run (n = 6), all standards were freshly prepared. To allow peak identification, pure (≥99%) *N*-acetyl-tryptophan (N-Ac-Trp) was dissolved in ultrapure water to a final concentration of 0.8 mM, which corresponds to the average N-Ac-Trp content in an HSA control sample of 10 mg ml^{-1}. To confirm the HSA peak, a solution of recombinant HSA (10 mg ml^{-1}) was prepared with ultrapure water. Both identification samples were run under identical conditions as the HSA-containing samples.

Data integration and statistical analysis

After each run, the ChemStation software automatically determined the baseline and integrated all peaks between 7.5 and 10 min (if present). Based on the area under the curve (AUC) values of the calibration (standard) samples, the software performed a linear regression analysis according to the least squares method, and calculated the coefficient of determination (R^2). The resulting formula ($y = a \times x + b$) was then applied for calculation of the HSA concentration of unknown samples, taking into account the dilution factor of the specific sample. The relative standard deviation (RSD) or coefficient of variation (CV), expressed as %, was calculated using the following formula: 100 × (standard deviation (SD)/average). General statistics were calculated using MedCalc statistical software (MedCalc, Mariakerke, Belgium).

Method validation

Method validation was based on the guidelines of the Center for Drug Evaluation and Research (CDER) and the International Conference on Harmonization (ICH).[22,23] Besides peak identification and determination of the linearity and range, the following parameters have been assessed: precision (repeatability and intermediate precision), accuracy, robustness, specificity, and sensitivity. Repeatability, representing intra-day variability, was determined using two different HSA solutions of 0.5 (low range) and 10 mg ml^{-1} (mid range), which were injected 10 and 6 times respectively on the same day. Inter-day precision was tested by analyzing an HSA control sample of 10 mg ml^{-1} on different days and executed by different operators (14 times). In

addition, different batches of media, with varying time from production, were analyzed. Accuracy was tested by measuring HSA three times at three different concentrations (1, 10, and 25 mg ml^{-1}). Robustness was assessed by changing the following parameters: flow rate (0.9, 1, and 1.1 ml min^{-1}), oven temperature (35, 40, and 45 °C), autosampler temperature (4, 10, and 20 °C), changes in TFA concentration of both mobile phases (0.05, 1 and 1.5% (v/v)), the use of different brands of HPLC-grade reagents (Pierce, Acros, Merck), and variable injection volumes (10, 15, and 20 µl). To evaluate the influence of matrix compounds (specificity), background was determined of all the ART products, prepared without HSA. Specificity was further confirmed by testing different batches of the same products (also see inter-day precision experiments). Sensitivity was assessed by calculating the limit of detection (LOD) and the lower limit of quantification (LLOQ), using the following formulas: LOD = (3.3 × SD)/S; LLOQ = (10 × SD)/S. Both SD and S values are derived from the linear calibration curve, whereby SD corresponds to the standard deviation of the analytical response (AUC), in other words, the SD of the y-intercept. The S value represents the slope of the linear regression curve. System suitability parameters, including plate number, resolution (R_s), capacity (k'), and tailing factor, were calculated using ChemStation.

Results

Peak identification

According to the manufacturer's certificate of analysis (COA), the 25% HSA solution contains not less than 96% HSA in monomeric form. Polymers of HSA and the globular fraction account for a maximum of 5%. To stabilize the product, sodium caprylate and N-Ac-Trp are added in a concentration varying between 16.6 and 23.6 mM.

Separation on the C4-RP column yielded two major peaks, as demonstrated in Fig. 1. When higher HSA concentrations (10 mg ml^{-1} or higher) were loaded on the column, a very small peak was visible at the left foot of the HSA-peak, with a retention time (t_R) of 8.392 min (n = 28; RSD = 0.544%). This peak was not taken into account during data integration (AUC calculation).

To reveal the identity of all peaks, samples of an N-Ac-Trp and recombinant HSA (rHSA) solution respectively were analyzed under identical conditions. Our data (not shown) confirmed that the first peak (t_R = 4.970 min; n = 69; RSD = 0.405%) was identical to the one obtained with the N-Ac-Trp standard solution (t_R = 4.945; n = 3; RSD = 0.100%). The second peak, showing an average t_R of 8.715 min (n = 69; RSD = 0.388%), corresponded to HSA. This was confirmed by the analysis of an rHSA solution, containing recombinant protein and sodium caprylate. Only one peak was observed at 8.767 min (n = 6; RSD = 0.276%).

Linearity and range. The relationship between the AUC and HSA content was linear over the range 0.4–25 mg ml^{-1}, with a coefficient of determination (R^2) of at least 0.999 (0.99998 in

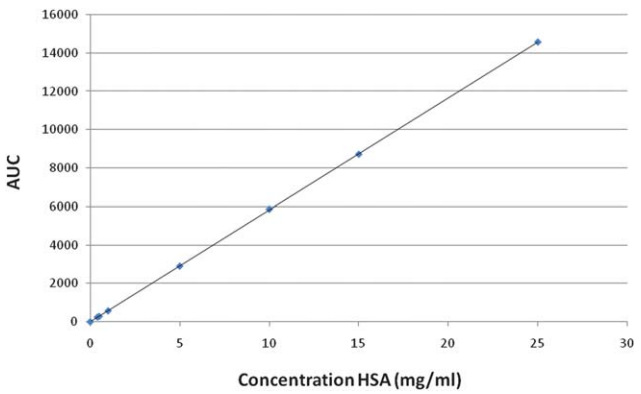

Fig. 2 Standard curve of HSA, with a concentration range varying between 0.4 and 25 mg ml^{-1}.

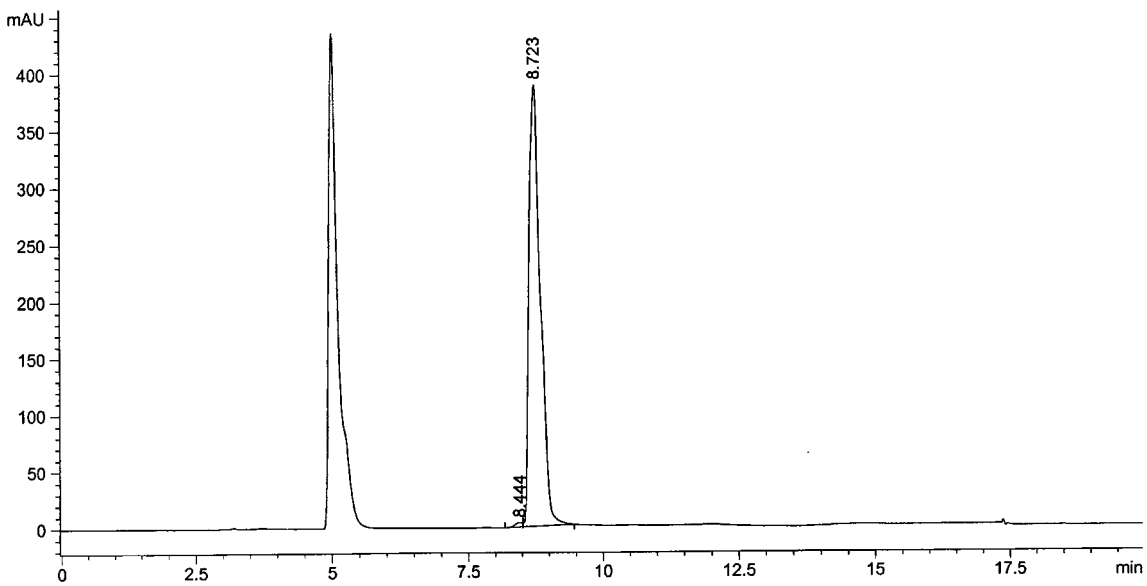

Fig. 1 Example of a chromatographic analysis of an HSA-sample (10 mg ml^{-1}).

this example). Fig. 2 shows the average standard curve of 6 independent runs (freshly prepared standards), yielding the following regression formula: $y = 582.91 \times x + 5.7519$.

The range of an analytical method is defined as the interval between the upper and lower levels (including these levels) that have been demonstrated to be determined with precision, accuracy and linearity using the test method. The ICH requires the minimum specified range to be 80 to 120 percent of the test concentration. The described HPLC method perfectly meets this demand, as the HSA content of the tested ART products varies between 4 and 20 mg ml^{-1}, thus lying within the linear range of the assay.

Precision. Intra-day variability (repeatability) was determined by analyzing two HSA concentrations in the low (0.5 mg ml^{-1}) and mid range (10 mg ml^{-1}), which were injected 10 and 6 times respectively. Data are presented in Table 2, showing an RSD of 0.643% (0.5 mg ml^{-1}) and 0.380% (10 mg ml^{-1}). These values meet the criterion that the RSD for intra-day variation must be lower than or equal to 1%. Intermediate precision (within-laboratory variation) was determined by running a control sample of 10 mg ml^{-1} (freshly prepared) 14 times on different days, with different batches of eluents, and two operators. An RSD of 0.660% was obtained (data not shown), which is lower than the acceptance criterion of 2%. The use of a different batch of C4 analytical and guard columns respectively did not alter retention times and corresponding AUCs. Good inter-assay variability is further reflected by low RSD values following analysis of different product batches (data not shown).

Recovery. We have used three different concentrations of HSA in the low (1 mg ml^{-1}), mid (10 mg ml^{-1}) and high (25 mg ml^{-1}) range to calculate the recovery percent of HSA. Details are described in Table 3. An average recovery of 99.73% (±1.89%) was achieved, indicating that our method is suitable for quantitative determination of HSA.

Selectivity/specificity. The presented HPLC method was able to detect HSA in a specific manner. No peaks were observed in samples containing ultrapure water, not even after running HSA at higher concentrations (25 mg ml^{-1}), indicating the absence of carry over. To assess matrix effects, samples of the same ART media but without HSA were run under identical test conditions. None of them yielded false positive results. Several of these products contain amino acids, or cryoprotectants (*e.g.* dimethyl sulfoxide (DMSO), ethylene glycol, …). These compounds elicited reproducible peaks in the respective chromatograms, without interfering in the HSA retention time region (data not shown). In summary, HSA concentrations can be determined in an accurate, precise, and specific manner, without interference of matrix ingredients.

Table 2 Intra-day variation (repeatability) assessed using two different HSA concentrations

Run/AUC	0.5 mg ml^{-1}	10 mg ml^{-1}
1	265.77	5738.53
2	262.51	5776.16
3	262.86	5784.46
4	262.98	5782.44
5	262.6	5793.86
6	263.75	5801.36
7	260.41	—
8	261.16	—
9	263.04	—
10	265.66	—
Average	263.07	5779.47
Stdev	1.69	21.94
RSD (%)	0.64	0.38

Table 3 Accuracy of the HPLC method for HSA determination

HSA concentration (mg ml^{-1})	AUC	RSD	Average	Calc. concentration	Recovery %
1	591.12 598.7 598.3	0.715	596.04	0.98	97.9
10	5859.64 5876.58 5865.06	0.147	5867.09	10.17	101.67
25	14 348.2 14 337.2 14 278.2	0.263	14 321.2	24.9	99.62
			Average recovery %		99.73
			Standard deviation		1.89
			RSD		1.9

Robustness. Robustness of the assay was evaluated by varying several parameters, which may (possibly) affect the chromatographic separation of HSA. Column temperature, flow rate, autosampler temperature, different suppliers of eluents, different eluent concentrations, and injection volume were assessed on freshly-prepared HSA-samples of 10 mg ml^{-1}. Only flow rate, TFA concentration, and injection volume affected the outcome. As shown in Fig. 3a and b, changing the TFA content resulted in decreased (average: 8.399 min with 0.05% TFA) or increased (average: 9.062 min with 1.5% TFA) retention times when compared to the routinely-used TFA concentration (average: 8.715 min with 1% (v/v) TFA).

Reducing the flow rate to 0.9 ml min^{-1} resulted in an increased AUC (+11.41%) and t_R (9.152 min). Opposite results were observed when the flow rate was raised to 1.1 ml min^{-1} (AUC—8.54%; $t_R = 8.311$ min). Examples are presented in Fig. 4a (0.9 ml min^{-1}) and Fig. 4b (1.1 ml min^{-1}).

Obviously, reduction of the injection volume resulted in decreased AUC values, but the retention time remained identical, while linearity was preserved. In other words, although lower volumes were injected, the HPLC method was still able to precisely determine HSA concentrations.

Sensitivity. The limit of detection (LOD) corresponds to the lowest concentration of the analyte in a sample that can be detected but not necessarily quantified. The limit of quantification (LOQ) can be divided into lower (LLOQ) and upper (ULOQ) values, representing the lowest and highest amount of the analyte respectively which produces quantitative measurements with acceptable precision and accuracy. In this case, only the LLOQ has been calculated, as the HSA concentration in the cell culture media (4–20 mg ml^{-1}) lies within the linear range of 0.4–25 mg ml^{-1}, which makes the determination of ULOQ irrelevant.

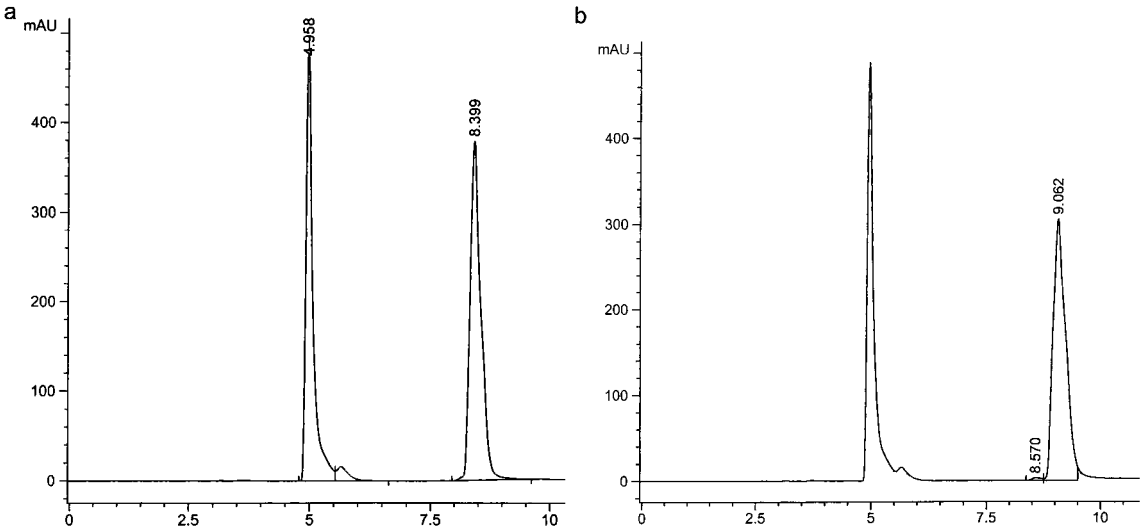

Fig. 3 Effect of TFA concentration on HSA retention time ((a): 0.05% TFA; (b): 1.5% TFA).

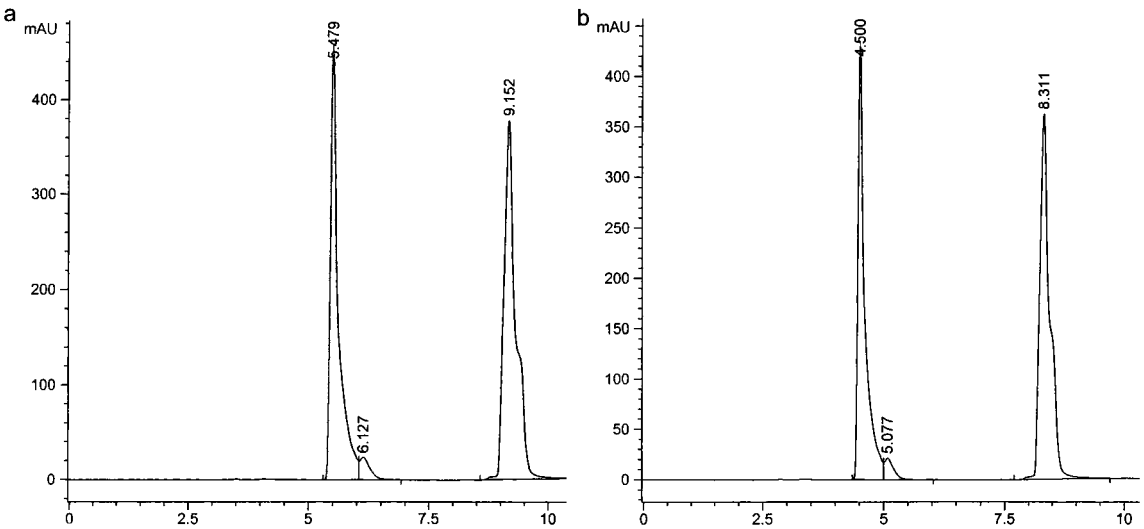

Fig. 4 Effect of flow rate on HSA retention time ((a): 0.9 ml min^{-1}; (b): 1.1 ml min^{-1}).

Several methods are available to determine LOD and LLOQ values, including calculation of the signal to noise ratio and the slope method[24] which was used in the present study. The SD of the analytical response (or *y*-intercept) and the slope of the linear regression curve were applied in the respective mathematical formulas, yielding LOD (3.3 × SD/*S*) and LLOQ (10 × SD/*S*) values of 0.128 and 0.386 mg ml^{-1} respectively.

System suitability. System suitability parameters, including the capacity factor (k'), resolution (R_s), the tailing factor, and theoretical plate number, have been calculated using the Chem-Station software. All parameters fulfilled the recommended criteria, as shown in Table 4.

Table 4 System suitability parameters

Parameter	Results	Recommended
Capacity factor (k')	2.495	>2
Tailing factor	1.390	≤2
Plate number	7556	>2000
Resolution (R_s)	13.795	>2

Discussion

Human serum albumin is not only widely-used for medical purposes,[3–5] but also plays an important role in the action of ART-related media. For example, in embryo culture media, HSA serves as a scavenger of oxidative and toxic agents, which have a negative impact on embryo quality. Furthermore, this plasma protein acts as a transporter molecule for nutrients, it prevents sticking of the embryo (or gamete) to plastic labware and enhances sperm motility. Freezing and thawing media for gametes and embryos also contain HSA, together with other ingredients such as DMSO or ethylene glycol, to prevent damaging during the freeze/thaw process.[5–9] Obviously, the

concentration of HSA depends on the ART application and must lie within product-specific limits. For QC purposes, including product release and stability studies, we have developed and validated an RP-HPLC method for the determination of the HSA content in ART-related products.

As HSA is a relatively large protein (66–67 kDa), we have chosen to use a C4 column with 300 Å pore size and 5 μm *n*-butyl-bound silica particles. Acetonitrile and TFA (0.1% v/v) served as the organic modifier and ion-pairing agent respectively. Under the given conditions (4 °C autosampler, 40 °C column, 20 μL injection volume and a flow rate of 1ml min^{-1}), the applied gradient elution resulted in reproducible chromatograms, with two main peaks at 4.970 min (N-Ac-Trp) and 8. 715 min (HSA). This was confirmed by running samples containing either N-Ac-Trp or recombinant HSA. Interestingly, in the latter chromatogram, only one peak at 8.767 min was observed. Based on the manufacturer's data, rHSA contains two ingredients, namely recombinant protein and sodium caprylate as the stabilizer. The absence of a peak for sodium caprylate may be explained by the fact that this compound is not retained by the column or is not detected at a wavelength of 280 nm. Indeed, this carboxylic acid (fatty acid) requires derivatization to allow decent LC separation and detection at higher UV wavelengths.[25]

Another difference between the chromatograms of commercial HSA and its recombinant variant, is the presence of a very small peak at 8.392 min when higher concentrations (≥10 mg ml^{-1}) of the former compound were tested. This may be explained by the presence of globulins in blood-derived HSA solutions, as a result of the fractionation process.[26] Obviously, this protein fraction cannot be present in recombinant HSA. Detection of this globular fraction has been described by Wilczynska and colleagues,[27] who investigated erythropoietin and HSA in medical products using a C8 column. Based on these data, one may conclude that the peak at 8.393 min corresponds to globulins.

The described RP-HPLC method is able to reliably quantify HSA concentration in the range of 0.4–25 mg ml^{-1}, with an LOD and LLOQ of 0.128 and 0.386 mg ml^{-1} respectively, as calculated using the slope method. For all analytical runs, ultrapure water served as a blank. Analysis of HSA-free control samples demonstrated the absence of interfering peaks at the t_R of HSA. Also, other ingredients (*e.g.* DMSO, amino acids, …) in the culture and freeze/thaw media did not interfere with the HSA determination, thereby confirming the assay specificity. For this reason, ultrapure water can be used as a blank without affecting the final outcome, which facilitates data integration. Additional validation experiments demonstrated good precision, accuracy, recovery, and system suitability.

Besides for product release purposes, we also use this HPLC method for stability studies, in order to determine HSA-concentration as a function of shelf-life. Batch analyses, performed over the complete shelf-life, yielded low RSD values, thereby indicating that HSA content remains stable during the shelf-life of the respective products.

In conclusion, this validated RP-HPLC method is able to detect HSA in the range of 0.4–25 mg ml^{-1}, without interference of other matrix ingredients. It allows the use of the assay for QC purposes, including product release and stability studies. In addition, this method can be easily adapted to measure HSA in pharmaceutical preparations and biological samples. In the latter case, good sample preparation will determine the analysis outcome.

Abbreviations

ACN	acetonitrile
ART	assisted reproductive techniques
AUC	area under the curve
BCG	bromocresol green
BCP	bromocresol purple
CDER	Center for Drug Evaluation and Research
CI	confidence interval
COA	certificate of analysis
CV	coefficient of variation
DMSO	dimethyl sulfoxide
HPLC	high-performance liquid chromatography
ICH	International Conference on Harmonization
ICSI	intracytoplasmic sperm injection
IVF	*in vitro* fertilization
HSA	human serum albumin
LOD	limit of detection
LOQ	limit of quantification
LLOQ	lower limit of quantification
N-Ac-Trp	*N*-acetyl-tryptophan
QC	quality control
rHSA	recombinant human serum albumin
RP	reverse phase
Rs	resolution
t_R	retention time
RSD	relative standard deviation
S	slope
SD	standard deviation
TFA	trifluoroacetic acid
ULOQ	upper limit of quantification

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