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Changes of net charge and α-helical content affect the pharmacokinetic properties of human serum albumin

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Abstract

The pharmacokinetics of 17 genetic variants of human serum albumin with single-residue mutations and their corresponding normal albumin were studied in mice. In all cases, the plasma half-life was affected, but only variants with +2 changes in charge prolonged it, whereas changes in hydrophobicity decreased it. Good positive and negative correlations were found between changes in α-helical content taking place in domains I+III and domain II, respectively, and changes in half-lives. No correlation was found to type of mutation or to changes in heat stability as represented by ΔH_v. Liver and kidney uptake clearances were also modified: α-helical changes of domains I+III showed good negative correlations to both types of clearances, whereas changes in domain II only had a good positive correlation to kidney uptake clearance. No correlation between the other molecular changes and organ uptakes was observed. The relatively few correlations between changes in molecular characteristics and the organ uptakes of the variants are most probably due to different handling by plasma enzyme(s) and the various types of cell endocytosis. Of the latter, most lead to destruction of albumin, but at least one results in recycling of the protein. The present information should be useful when designing recombinant, therapeutical albumins or albumin products with a modified plasma half-life.

Keywords: Human serum albumin; Genetic variant; Pharmacokinetics; Half-life; Hepatic uptake; Renal disposition

1. Introduction

Human serum albumin (HSA) is produced in the parenchymal cells of the liver and after removal of its pre–pro sequences, it is secreted into the circulation, where it is the most abundant plasma protein. It is an important circulating carrier of endogenous and exogenous ligands in the blood, and contributes to the maintenance of osmotic pressure, plasma pH and to the Donnan-effect in the capillaries [1,2]. The protein is formed by a single polypeptide chain of 585 amino acids and has a molecular mass of approximately 67,000 [2]. According to X-ray crystallographic analyses of HSA and of its recombinant version, albumin has about 67% α-helix but no β-sheet. The analyses also showed that the polypeptide chain forms a heart-shaped protein with three homologous domains (I–III), each comprised of two subdomains (A and B) with distinct helical folding patterns that are connected by flexible loops [3,4]. HSA has 35 cysteine residues, and all of these but one, Cys 34, are involved in the formation of stabilizing disulfide bonds.

Clinically, HSA is used to treat severe hypoalbuminemia or traumatic shock [2], and the usual dosages of HSA are in excess of 10 g/dose. To date, albumin has been produced by fractionation of whole blood. However, there is the potential risk of HSA contamination with blood-derived pathogens. In addition, human plasma is in limited supply in countries like Japan. Therefore, the development of an alternative method of industrial production of HSA is desired as this would greatly assist in the general movement of, for example, Japan toward self-sufficiency in blood and blood products. Because of these

Abbreviations: HSA, human serum albumin; Alb, albumin; Alb A, normal (wild-type) albumin; ΔH_v, van't Hoff enthalpy

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problems, recombinant HSA, that is highly expressed by *Pichia pastoris*, most probably will be commercially available in the near future. Protein engineering will also enable the creation of recombinant HSAs with modified properties such as extended half-life in the circulation. A modified half-life will also be beneficial, when HSA is used as a fusion protein to extend the half-life of small proteins like soluble CD4 and hirudin [5,6]. Despite these possibilities, not much has been done to clarify the effects of mutations on the pharmacokinetics of HSA.

Previous studies with chemically modified albumin have demonstrated that changes in hydrophobicity and net charge on the protein surface affect its in vivo clearance [7,8]. In a previous study in which we produced six recombinant HSAs, our findings suggested that even single-residue mutations of surface or more internally placed amino acids can affect both the structural properties and the in vivo elimination of HSA [9]. Even though these studies indicated that the elimination of HSA is dependent on its structural properties more detailed studies of the relationships between structural and pharmacokinetic properties are needed to design useful recombinant HSAs with modified half-lives in the blood.

In our search for such candidates, we paid our attention to HSA genetic variants. Until now, more than 60 inherited variants of HSA have been identified and structurally characterized [10]. Usually, these genetic variants are expressed in heterozygous form and without any known association to disease [2]. Therefore, unlike lethal mutations, such as may occur for hemoglobin and coagulation factors, studying the pharmacokinetic properties of HSA variants is a good way of gaining information which can be used when designing recombinant HSAs, because we can consider the effects of molecular variation without worrying about complications such as antigenic effects.

Previously, we have shown that inherited single-residue substitutions affected the structure (α-helical content) and thermal stability (van't Hoff enthalpy (ΔH_v)) of HSA [11]. In addition, good linear correlations between mutation-induced changes of α-helical content and ΔH_v were found. In the present study, we examined the pharmacokinetics of 17 structurally different genetic variants with single-residue mutations isolated from heterozygote carriers, namely Alb Malmö-95 (D63N), Alb Tregasio (V122E), Alb Hawkes Bay (C177F), Alb Herborn (K240E), Alb Niigata (D269G), Alb Caserta (K276N), Alb Canterbury (K313N), Alb Brest (D314V), Alb Roma (E321K), Alb Sondrio (E333K), Alb Parklands (D365H), Alb Milano Slow (D375H), Alb Kashmir (E501K), Alb Maku (K541E), Alb Church Bay (K560E), Alb Verona (E570K) and Alb Milano Fast (K573E). We labeled the variants and their corresponding, endogenous normal albumin with ^{111}In, and examined the pharmacokinetic properties of these albumins in mice. Then, we determined the effects of the natural mutations on plasma half-life and organ uptakes. Finally, we related these results to the previously reported changes in α-helical content and ΔH_v.

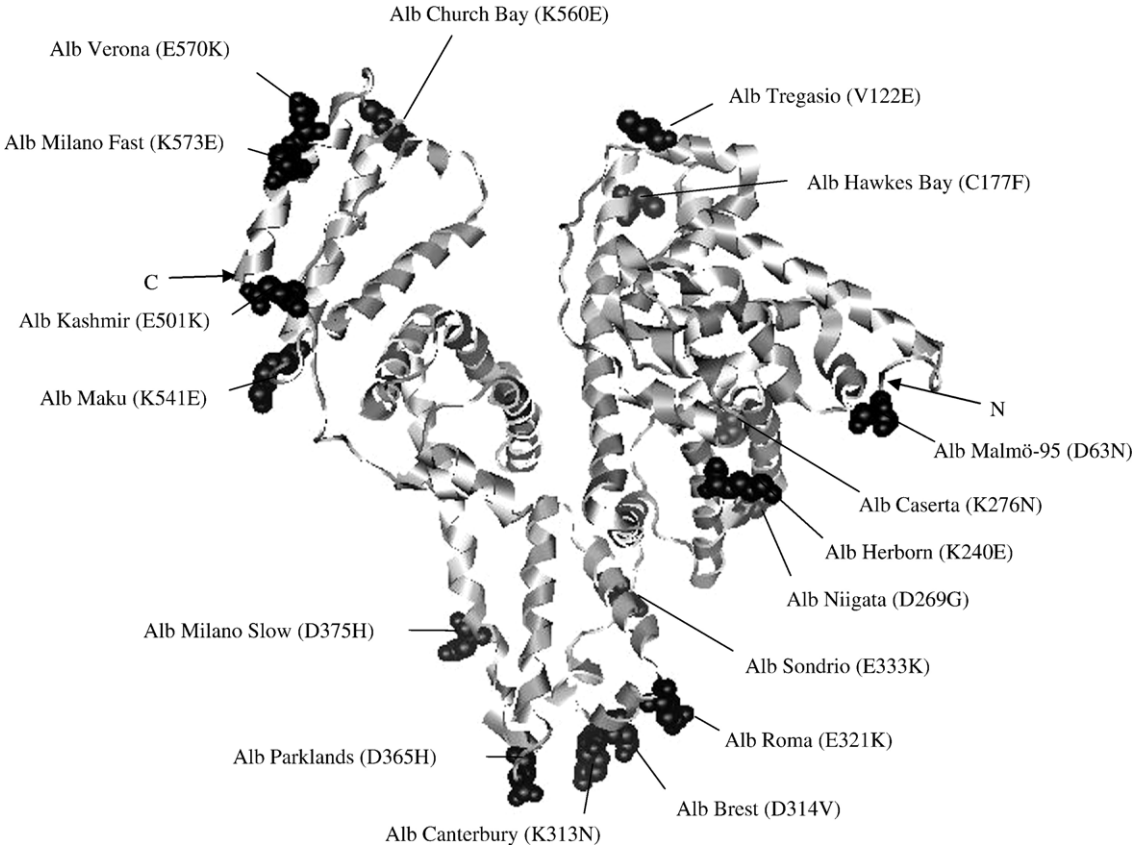

Fig. 1. The crystal structure of HSA showing the locations of the single-residue mutations of the 17 genetic variants used in this study. N and C stand for the N-terminal and the C-terminal ends, respectively.

2. Materials and methods

2.1. Protein samples

The 17 HSA genetic variants used in this study have been named after the place from where the first detected carrier originates. The variants have single-residue substitutions, and the location of the mutations is shown in Fig. 1. All variants, except one, have net charges which differ from that of normal (wild-type) HSA (endogenous Alb A) at physiological pH: Alb Roma (E321K) [12], Sondrio (E333K) [13], Kashmir (E501K) [14] and Verona (E570K) [15] are all +2 variants (i.e., they have two positive charges more than Alb A); Alb Malmö-95 (D63N) [16], Niigata (D269G) [17], Brest (D314V) [18], Parklands (D365H) [19] as well as Milano Slow (D375H) [20] are +1 variants; Alb Tregasio (V122E) [21], Caserta (K276N) [22] and Canterbury (K313N) [23] are −1 variants; and, finally, Alb Herborn (K240E) [24], Maku (K541E) [25], Church Bay (K560E) [26] and Milano Fast (K573E) [27] are −2 variants. The only variant with no change in net charge is Alb Hawkes Bay (C177F) [28]. Most of the substitutions are placed in domain II (residues 200–391), namely 9. Domains I (residues 1–199) and III (residues 392–585) are represented by three and five examples, respectively.

After isolation from heterozygous sera by ion-exchange chromatography, the individual albumins were checked for homogeneity by native electrophoresis, and no denaturation or significant (no more than 5%) cross-contamination between variant and Alb A was detected. The proteins were donated to us by Drs. M. Galliano and L. Minchiotti, University of Pavia, Pavia, Italy; Dr. S.O. Brennan, Canterbury Health Laboratories, Christchurch, New Zealand; Dr. A.L. Tárnoky, University of Reading, Reading, UK; and Dr. O. Sugita, Niigata University School of Medicine, Niigata, Japan. Before use, the albumins were delipidated by treatment with hydroxyalkoxypropyldextran at pH 3.0, as described elsewhere [29]. After defatting, the albumins were dialysed extensively against deionized water, lyophilized and stored at −20 °C until used. Thus, the albumins from a donor have been exposed to exactly the same conditions from the time the blood samples were taken until the present experiments were performed.

Fraction V HSA (96% pure), assumed to be Alb A, was donated by the Chemo-Sera-Therapeutic Research Institute (Kumamoto, Japan) and defatted using the charcoal procedure described by Chen [30], deionized, freeze-dried and then stored at −20 °C until used.

2.2. Chemicals and animals

$^{111}InCl_3$ (74 Mbq/mL in 0.02 N HCl) was donated by Nihon Medi-Physics (Takarazuka, Japan). All chemicals were of the highest grade commercially available, and all solutions were prepared using deionized, distilled water.

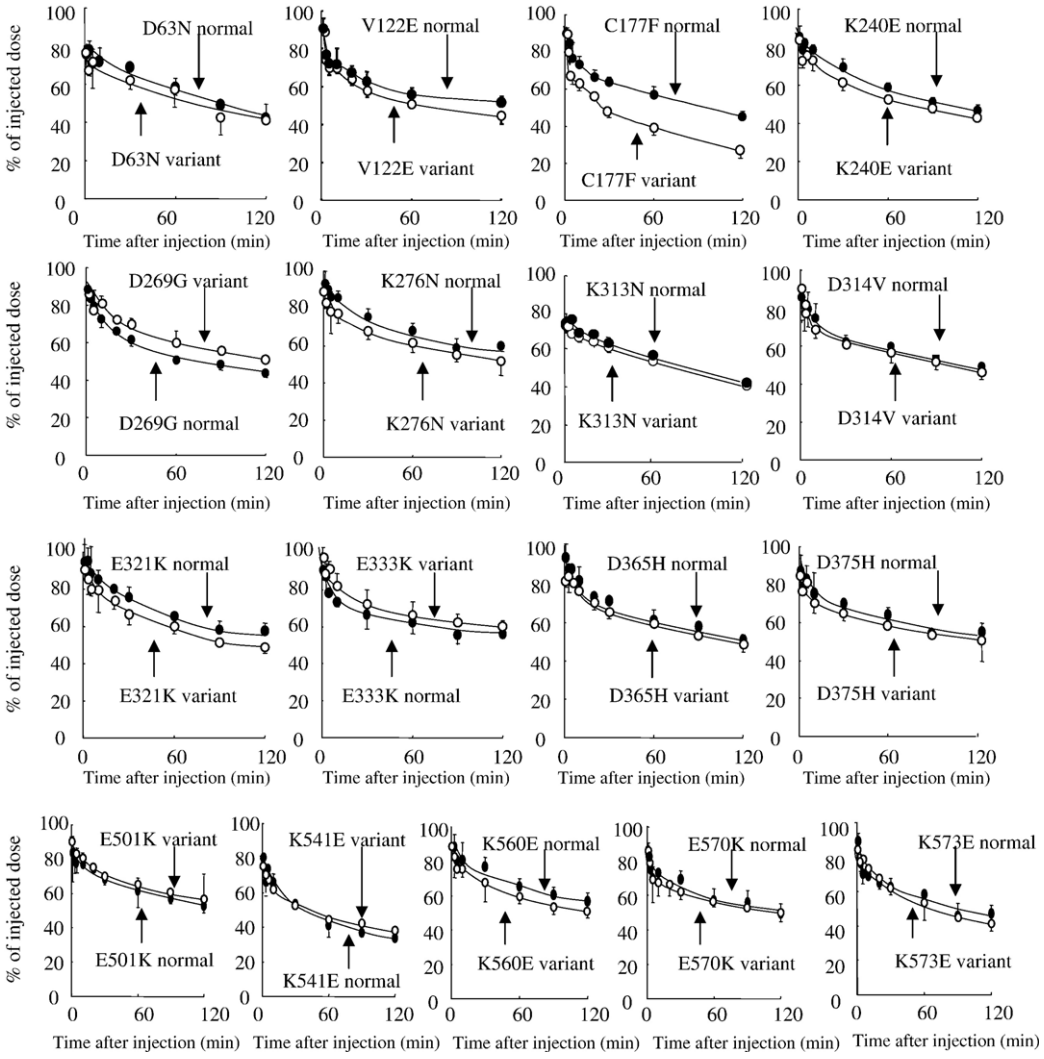

Fig. 2. Relative plasma amounts of ^{111}In-labeled HSA variants and their corresponding Alb A after intravenous administration in mice. ^{111}In-albumin was injected as a bolus dose into the tail vein. Relative amounts are plotted against time after injection. Each point represents an average value obtained for 3–6 mice (±S.D.). The open and closed circles represent variant and normal albumin, respectively.

Male ddY mice (26–32 g) were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan), and were maintained under conventional housing conditions. All animal experiments were conducted in accordance with the principles and procedures outlined in the National Institute of Health Guide for the Care and Use of Laboratory Animals.

2.3. In vivo experiments

All proteins were radiolabeled with ^{111}In using the bifunctional chelating reagent DTPA anhydride according to the method of Hnatowich et al. [31], which has been described in detail in a previous paper [32]. In previous works, we found no significant differences in pharmacokinetic properties among these albumins, when ^{111}In-labeled mouse, rat, bovine or human serum albumin was administered to mice (unpublished), suggesting that immunogenic behavior does not occur in mice. Therefore, we chose the mouse as a reasonable model for the study of the pharmacokinetics of the HSAs. Mice received tail vein injections of ^{111}In-labelled proteins in saline, at a dose of 0.1 mg/kg and were housed in metabolic cages to allow the collection of urine samples. Urine samples were collected throughout the 120 min of the experimental period. In the early period after injection, the efflux of ^{111}In radioactivity from organs is assumed to be negligible, because the degradation products of ^{111}In-labeled proteins using DTPA anhydride cannot easily pass through biological membranes [33]. This assumption was supported by the fact that no ^{111}In was detectable in the urine after 120 min. At 1, 3, 5, 10, 30, 60, 90 or 120 min after injection, blood was collected from the vena cava under ether anesthesia and plasma was obtained by centrifugation. After blood collection, the animals were sacrificed, organs were excised, rinsed with saline and weighed. The radioactivity of each blood and tissue sample was measured in a well-type NaI scintillation counter (ARC-500, Aloka, Tokyo).

Pharmacokinetic analyses were performed as follows. The plasma ^{111}In radioactivity concentrations (C_p) were normalized with respect to the percentage of injected dose and analyzed using the nonlinear least-square program MULTI [34]. The two-compartment model was fitted according to the Akaike information criterion by Eq. (1).

$$C_p = Ae^{-\alpha t} + Be^{-\beta t} \quad (1)$$

The half-lives of the HSAs were determined as β-phase elimination within a 120-min period. The tissue distribution patterns were evaluated using tissue uptake clearances (CL_{uptake}) according to the integration plot analysis. CL_{uptake} was calculated using Eq. (2).

$$CL_{uptake} = \frac{X_t/C_t}{AUC_{0-t}/C_t} \quad (2)$$

where X_t is the tissue accumulation at time t, AUC_{0-t} is the area under the plasma concentration time-curve from time 0 to t, and C_t is the plasma concentration at time t. CL_{uptake} was obtained from the slope of the plot of X_t/C_t versus AUC_{0-t}/C_t. We estimated the organ uptake clearances within a 30-min period.

Table 1
Half-lives and organ uptake clearances of ^{111}In-labeled variants and corresponding Alb A in mice

Variant name (mutation)		Half-life[a] (min)	Clearance (μL/h)[a]	
			Liver	Kidney
Alb Malmö-95 (63 Asp→Asn)	Variant	259.4±7.26	53.14±12.09*	65.09±18.83*
	Alb A	264.2±6.14	79.72±11.74	111.64±18.18
Alb Tregasio (122 Val→Glu)	Variant	250.3±6.65**	73.22±18.70*	71.42±12.74
	Alb A	270.4±1.23	129.13±11.87	104.87±24.94
Alb Hawkes Bay (177 Cys→Phe)	Variant	176.3±8.68**	494.40±38.82**	374.08±43.19**
	Alb A	250.8±5.58	23.99±2.68	69.11±11.67
Alb Herborn (240 Lys→Glu)	Variant	265.8±6.97	132.45±22.19**	137.86±25.61
	Alb A	269.3±4.51	41.10±5.37	110.69±12.94
Alb Niigata (269 Asp→Gly)	Variant	269.7±1.68	14.12±2.39	83.69±19.97
	Alb A	275.2±4.68	18.00±3.27	97.37±23.26
Alb Caserta (276 Lys→Asn)	Variant	254.8±4.89*	45.86±6.29**	203.48±35.37**
	Alb A	269.3±7.64	21.77±5.03	81.00±13.51
Alb Canterbury (313 Lys→Asn)	Variant	221.6±4.65	119.37±12.12	331.98±60.07**
	Alb A	231.6±4.64	128.80±29.97	64.36±20.01
Alb Brest (314 Asp→Val)	Variant	260.8±7.64*	35.48±11.53	102.87±13.27
	Alb A	276.4±5.46	25.31±6.10	99.75±16.32
Alb Roma (321 Glu→Lys)	Variant	269.4±4.51	56.18±5.89**	117.48±11.06*
	Alb A	266.1±3.65	13.34±2.91	83.49±14.23
Alb Sondrio (333 Glu→Lys)	Variant	251.1±4.98	44.72±10.58	172.70±40.37
	Alb A	247.6±7.89	28.92±8.71	169.51±16.82
Alb Parklands (365 Asp→His)	Variant	267.3±6.51	30.99±4.11**	119.35±14.93*
	Alb A	273.1±4.49	63.58±6.54	75.63±20.15
Alb Milano Slow (375 Asp→His)	Variant	268.7±7.61	43.88±10.33*	47.34±9.81
	Alb A	272.9±1.43	17.57±8.33	59.65±22.73
Alb Kashmir (501 Glu→Lys)	Variant	273.2±4.62	44.21±6.34*	76.48±16.91*
	Alb A	270.6±5.48	64.36±9.46	138.53±18.94
Alb Maku (541 Lys→Glu)	Variant	245.9±7.32	144.86±15.01**	285.50±44.74**
	Alb A	251.3±6.51	276.01±47.95	90.25±20.42
Alb Church Bay (560 Lys→Glu)	Variant	270.3±3.56*	13.37±4.39	77.06±16.01*
	Alb A	276.3±1.03	17.61±8.08	136.67±30.62
Alb Verona (570 Glu→Lys)	Variant	274.1±3.98*	99.12±18.83	128.12±20.68
	Alb A	262.4±4.14	63.85±11.72	121.06±16.44
Alb Milano Fast (573 Lys→Glu)	Variant	263.9±2.46	119.41±18.90	218.05±33.41**
	Alb A	263.5±6.45	85.88±11.35	89.64±10.02

[a] The data are average values of 3–6 experiments (±S.D.). *$P<0.05$, **$P<0.01$ as compared with endogenous Alb A.

2.4. Analysis of experimental data

The effects of the single-residue mutations were evaluated by using the following relationship:

$$\text{Percent change} = \frac{(\text{Result for variant}) - (\text{Result for Alb A})}{(\text{Result for Alb A})} \times 100\% \qquad (3)$$

In Eq. (3), the result can be a value determined for plasma half-life, organ uptake clearance, α-helical content or for ΔH_v.

2.5. Statistical analysis

Statistical analyses were performed by using the Student *t*-test. A probability value of $P<0.05$ was considered to indicate statistical significance.

3. Results

3.1. Pharmacokinetic properties of HSA variants

Fig. 2 shows the time courses for radioactivity in mouse plasma after intravenous administration of ^{111}In-labeled preparations of the variants and their corresponding Alb A. For all 17 variants, the mutation affected the elimination of HSA. In particular, Alb Hawkes Bay (C177F) showed rapid elimination as compared with Alb A. However, in this example the variant has undergone a major conformational change, see below, because it has lost a disulfide bond [28]. Table 1 gives the plasma half-lives, calculated by β-phase using the nonlinear least-square program MULTI and Eq. (1), and liver and kidney uptake clearances, determined by an integration plot analysis (Eq. (2)). As a control we have compared the pharmacokinetic results obtained for endogenous Alb A (Table 1) with those obtained with commercial HSA (not illustrated), because both types of preparations are assumed to represent the normal protein. The average half-life for Alb A is 264.2 min. This value is comparable to that determined for commercial HSA, i.e., 268.2±7.2 min ($n=6$). The liver and kidney uptake clearances for Alb A are on an average 64.64 μL/h and 100.2 μL/h, respectively, whereas those for commercial HSA are 54.31±8.23 μL/h and 83.12±5.43 μL/h, respectively. The slightly lower values found for commercial HSA are most probably due to differences in isolation and/or defatting procedures.

Table 2 shows the effects of the mutations on plasma half-lives and organ uptake clearances as determined by Eq. (3). As seen, 12 of the mutations diminished plasma half-life, i.e., negative percent changes, whereas one mutation had almost no effect (Alb Milano Fast). By contrast, the remaining variants, all of which are +2 variants, had a prolonged half-life. These findings are in accordance with those of Iwao et al. [9], who, by using oxidized and recombinant mutants, observed that increasing the net negative charge of HSA increased its elimination from the circulation. The results of Table 2 also propose that a change in hydrophobicity diminish plasma half-life. Thus, Alb Malmö-95, Niigata, Caserta, Canterbury, Brest, Parklands and Milano Slow all are more hydrophobic than Alb A, and all of them have negative values for the effect of mutation. Principally the same was found to be the case for Alb Tregasio which is less hydrophobic than its normal protein.

All genetic variants also have a modified liver and kidney uptake clearance (Table 2). About half of the mutants have diminished liver uptake clearance, and about half of them have a reduced kidney uptake. In general, the increases in organ uptake clearance are more pronounced than the decreases. Thus, all percent changes of organ uptake clearances of 100% or more are associated with increases in uptake. For liver uptake it is Alb Hawkes Bay (1961%), Roma (321%), Herborn (222%), Milano Slow (150%) and Caserta (111%), and for kidney uptake it is Alb Hawkes Bay (441%), Canterbury (416%), Maku (216%), Caserta (151%) and Milano Fast (143%). However, no clear correlation could be found between changes of protein net charge and hydrophobicity, respectively, and liver and kidney uptake clearance, respectively.

3.2. Relationships between structure, stability and pharmacokinetic properties of HSA variants

Previously, we have studied the effects of the single-residue substitutions on the α-helical content and thermal stability of

Table 2
Percent changes of half-life and organ uptake clearances of HSA variants in mice

Variant name (mutation)	Half-life (%)	Liver clearance (%)	Kidney clearance (%)	α-helical content[a] (%)	ΔH_v[a] (%)
Alb Malmö-95 (63 Asp→Asn)	−1.82	−33.34	−41.69	−9.68	−46.71
Alb Tregasio (122 Val→Glu)	−7.43	−43.30	−31.89	2.02	9.76
Alb Hawkes Bay (177 Cys→Phe)	−29.70	1960.58	441.31	−10.00	−8.53
Alb Herborn (240 Lys→Glu)	−1.30	222.26	24.54	−4.12	−21.59
Alb Niigata (269 Asp→Gly)	−2.00	−21.56	−14.05	−3.92	−18.83
Alb Caserta (276 Lys→Asn)	−5.38	110.68	151.21	0.68	5.74
Alb Canterbury (313 Lys→Asn)	−4.32	−7.32	415.82	1.47	3.28
Alb Brest (314 Asp→Val)	−5.64	40.17	3.13	1.57	10.53
Alb Roma (321 Glu→Lys)	1.24	321.26	40.70	−1.77	11.31
Alb Sondrio (333 Glu→Lys)	1.41	54.66	1.88	−1.57	−12.14
Alb Parklands (365 Asp→His)	−2.12	−51.26	57.81	−3.13	29.19
Alb Milano Slow (375 Asp→His)	−1.54	149.77	−20.63	−5.30	−32.23
Alb Kashmir (501 Glu→Lys)	0.96	−31.30	−44.79	0.37	−0.60
Alb Maku (541 Lys→Glu)	−2.15	−47.52	216.36	0.40	−20.76
Alb Church Bay (560 Lys→Glu)	−2.17	−24.12	−43.62	1.18	5.28
Alb Verona (570 Glu→Lys)	4.46	55.24	5.83	2.55	36.24
Alb Milano Fast (573 Lys→Glu)	0.17	39.03	143.24	−2.95	−16.64

[a] The values for α-helical content and ΔH_v are taken from Kragh-Hansen et al. [11].

albumin [11], and the results of that study are included in Table 2. The α-helical contents were estimated from far-UV intrinsic circular dichroism spectra by using the ellipticity values at 222 nm. The effect of the mutations on the reversible thermal denaturation (25–85 °C) was also monitored by circular dichroism at 222 nm. These additional data allow for a more detailed comparison between molecular characteristics and pharmacokinetic properties. Fig. 3 gives the correlations between mutation-induced changes in α-helical content and pharmacokinetic parameters. For the mutations in domain I and III, i.e., positions 63–177 and 501–573, respectively (Fig. 1), the same tendencies between the data were observed; therefore they have been gathered in Fig. 3A–C. For these mutations, an increase in α-helical content implies increased plasma half-lives, but decreased liver and kidney uptake clearances. Surprisingly, mutations in domain II, i.e., positions 240–375 (Fig. 1), have the opposite effects. In these cases, an increase in α-helical content results in decreased plasma half-lives (Fig. 3D) but in an increment of kidney uptake clearances (Fig. 3F). By contrast, there is no correlation between changes in α-helical content and liver uptake clearances (Fig. 3E).

The effect of the mutations on albumin stability was examined in terms of heat stability, which was quantified by determining van't Hoff enthalpies (ΔH_v-values) (Table 2). Fig. 4 shows that no correlation was found between the percent changes in ΔH_v and any of the pharmacokinetic parameters. Principally the same observations were made when pooling all the results for domains I–III; in that case the *P*-values were in the range 0.79–0.97 (not shown).

4. Discussion

From a clinical point of view it would be beneficial, if protein engineering could result in the production of HSA with a prolonged half-life in the circulation. In addition, because of its half-life of 19 days in humans, its ease of synthesis and its known structure albumin is an attractive candidate for use in recombinant fusion proteins. These proteins combine a therapeutic protein (that would otherwise be rapidly cleared) and a plasma protein with a slow clearance in a single polypeptide chain. Furthermore, HSA has been proposed as a carrier in drug delivery systems. Also in the two latter types of examples it

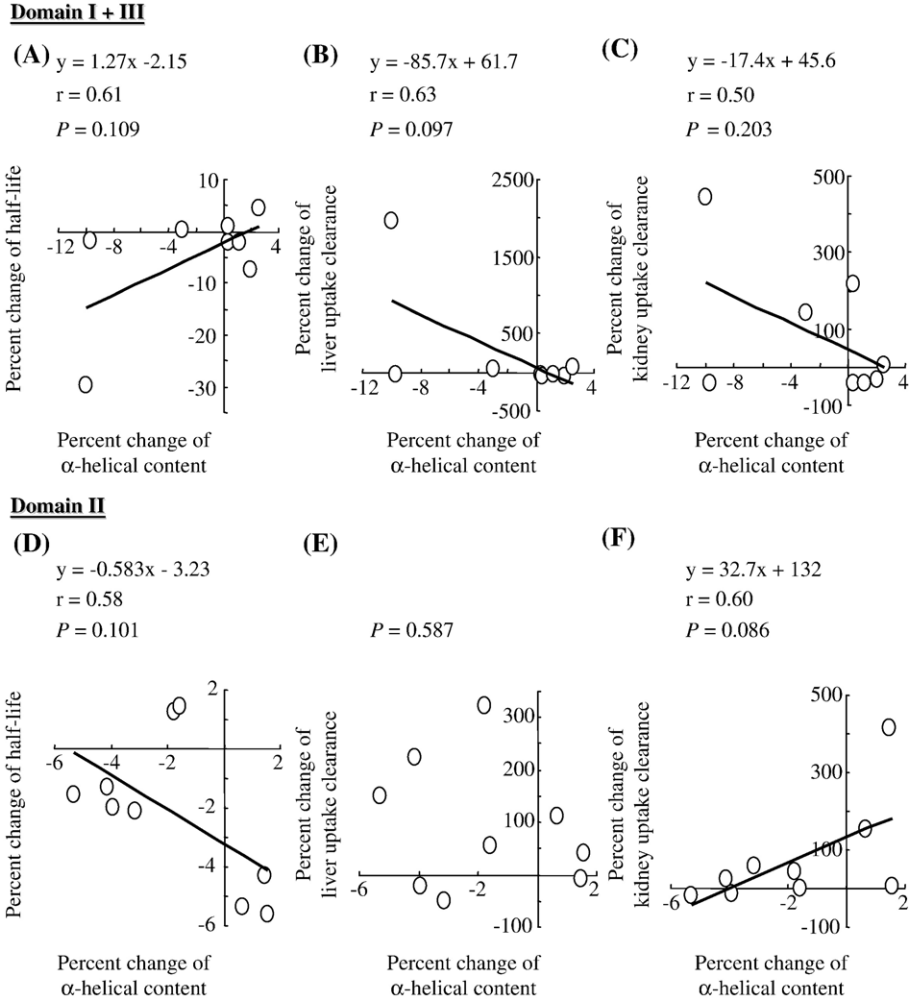

Fig. 3. Relationships between percent change of mutation-induced alterations of α-helical content and percent changes of half-life (A and D), liver uptake clearance (B and E) and kidney uptake clearance (C and F).

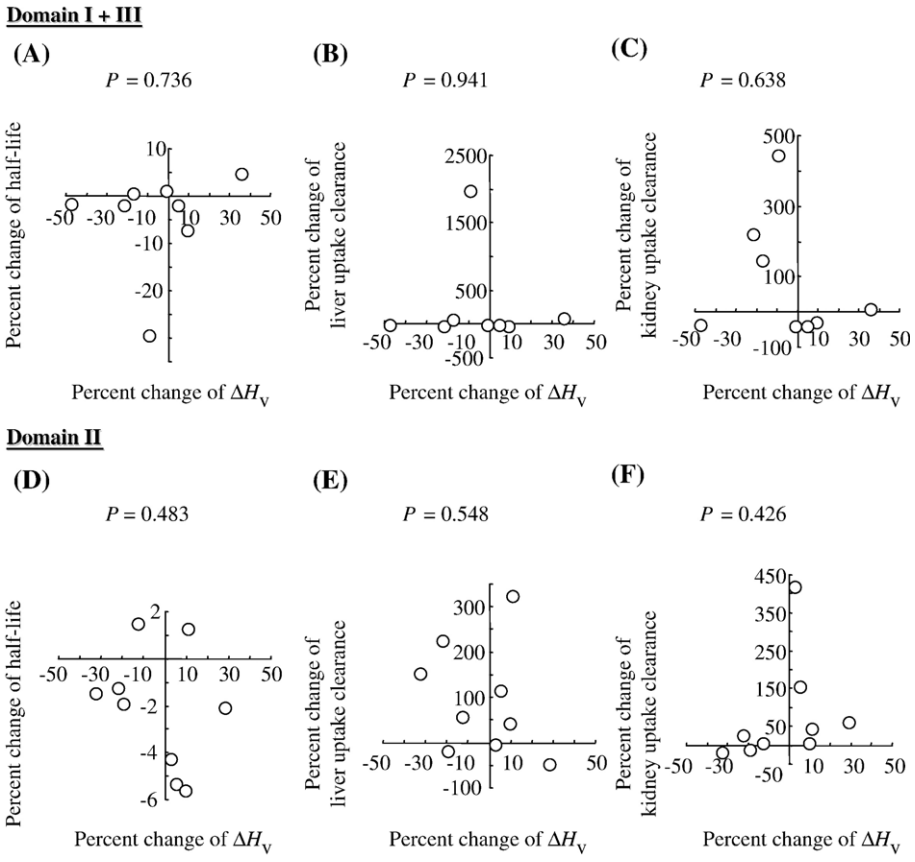

Fig. 4. Relationships between percent change of mutation-induced alterations of ΔH_v and percent changes of half-life (A and D), liver uptake clearance (B and E) and kidney uptake clearance (C and F).

would be advantageous to be able to modify the plasma half-life of the protein product. Therefore, in the present study, we investigated the half-life and organ uptakes of a series of genetic HSA variants. Since the isoforms have known single-residue mutations, we tried to identify molecular characteristics responsible for any modified pharmacokinetics.

The present variants have been detected by electrophoresis performed under native conditions. Therefore, their changes in charge are located on the surface of the protein, where they are exposed to the solvent. The only exception is Alb Hawkes Bay which rather is detected due to major conformational changes caused by the loss of the disulfide bond between Cys 168 and Cys 177 [28]. Thus, the amino acid substitutions affect the hydrophilic/hydrophobic characteristics of accessible regions of the protein. It should be mentioned that we did not quantify these effects experimentally. Instead, we used the knowledge of the amino acid substitutions, and when doing that we found that pronounced decreases in net charge (i.e., +2 variants) resulted in increased plasma half-lives. By contrast, increases in hydrophobicity implied shorter half-lives (Tables 1 and 2). These observations are in full accordance with those of Iwao et al. [9], who studied the effects of oxidation and recombinant mutations. Furthermore, Sheffield et al. [35], who used rabbit serum albumin, found that the recombinant mutant D494N, but not the charge-neutral mutant V14T, had a shorter half-life than the wild-type protein.

In addition to change in net charge and surface hydrophobicity we considered whether the modified plasma half-lives could be correlated to other molecular parameters. Therefore, in Fig. 3A and D the half-lives have been related to changes in α-helical content, which in this case were caused by single-residue substitutions; the results showed a good correlation between the two parameters ($P=0.1$). However, surprisingly, the effect depended on the location of the molecular change, because changes in α-helical content taking place in domains I and III showed a positive correlation to changes in half-life, whereas changes situated in domain II had the opposite effect. It can also be seen that most changes in α-helical content resulted in faster elimination from the circulation. The changes in half-life were also compared to mutation-induced changes in heat stability; quantified by using ΔH_v. However, in this case there was no correlation between the two parameters (Fig. 4A and D).

Iwao et al. [9] found that the diminished half-lives of their HSA preparations were accompanied by increased liver uptake clearances. Nakajou et al. [36] reported that a recombinant mutant of HSA in which the lysine residues in positions 199, 439 and 525 all had been substituted for alanine disappeared faster than the wild-type protein from the circulation. The authors suggested a faster than normal loss of the mutant to extravascular spaces. However, the triple-residue mutant also exhibited increased liver and kidney clearances. For being able to explain, at least in part, the modified plasma half-lives of the present

genetic variants we also investigated their liver and kidney uptake clearances (Table 1). No clear correlation could be found between organ uptakes and changes in protein charge or hydrophobicity, respectively. With respect to the effect of changes in α-helical content the domains again behaved differently. For changes in domains I and III good correlations were found (P=0.1–0.2) between increases in percent change of α-helical content and decreases in the change of liver (Fig. 3B) and kidney (Fig. 3C) uptakes. For domain II changes, a good correlation (P=0.1) was observed for kidney uptake (Fig. 3F), whereas no correlation was registered between the molecular alterations and changes in liver uptake clearance (Fig. 3E). As with the plasma half-lives, no correlations were found between changes in organ uptakes and changes in ΔH_v (Fig. 4B, C, E and F).

Uptake of HSA by liver and kidneys is mainly due to the presence of cell membrane receptors which recognize the protein and then internalize it by endocytosis. Hepatocytes and the nonparenchymal cells of the liver are involved in galactosyl receptor-mediated and mannose receptor-mediated endocytosis, respectively. Due to the specificities of these receptors, it is not likely that the effects of the present single-residue mutations on liver uptake clearance can be explained by one of these mechanisms. The liver also possesses receptors for rapid uptake of oxidized albumin and albumin with advanced glycation end products. Whether small molecular changes such as single-residue mutations can initiate endocytosis by scavenger receptors such as gp18 or gp30 is at present only speculative. On the other hand, liver uptake by adsorptive endocytosis could be influenced by the amino acid substitutions, because this type of uptake is dependent on the net charge of the protein.

Normally, glomerular filtration of HSA in the kidneys is followed by its return into the venous circulation without degradation (the albumin retrieval pathway). However, a small fraction is degraded in proximal tubular cells most probably after uptake by the endocytic receptors megalin and cubulin. Whether genetic modification of HSA results in increased glomerular filtration and increased uptake by this receptor-complex remains to be clarified. It should be noted that no radioactivity was detected in the urine during the present experimental time. An alternative explanation for the increased uptake of the albumin isoforms by the kidney could be uptake by tubular receptors for advanced glycation end products (RAGE-receptors). As with the scavenger receptors of the liver, it is not known whether single-residue mutations of HSA can initiate uptake by these receptors.

Fujino et al. [37] have found that oxidized bovine serum albumin, in contrast to the native protein, can be cleaved by oxidized protein hydrolase. Because this endopeptidase is found in the blood, it could partly hydrolyse some of the genetic variants in the mouse circulation and thereby render them more exposed to organ uptake. The enzyme selectively recognizes hydrophobic regions in its substrate. Therefore, this mechanism can be especially relevant for the genetic variants with increased hydrophobicity, which all have shorter plasma half-lives (Tables 1 and 2).

More recently, another type of endocytosis of HSA has been identified in virtually all nucleated cells which results in reuse of the protein [38]. After pinocytosis, albumin binds intracellularly and in a pH-dependent manner to the receptor FcRn. Thereby the protein is diverted from the lysosomal degradation pathway and exocytosed back to the circulation in an intact form extending its plasma half-life. Chaudhury et al. [39] have proposed that the intracellular binding of HSA to FcRn is caused by interaction(s) between histidine residue(s) in the receptor and histidine residues in domain III of albumin. By contrast, Andersen et al. [40] suggested that FcRn interacts with negatively charged and surface exposed residues on domain III of HSA. Thus, especially genetic variants with domain III substitutions could have modified plasma half-lives due to a modified HSA-FcRn recycling process.

In conclusion, the plasma half-life of HSA can be modified by single-residue mutations on its surface. No clear relation exists with respect to type of mutation, but +2 variants increase the half-life, whereas increased hydrophobicity decreases it. Changes in the proteins α-helical content have a positive effect on the half-life, if they take place in domain I or III, else they have a negative effect (domain II). By contrast, no correlation was found between the half-lives and changes in ΔH_v representing thermal stability. All mutations modified liver and kidney uptake clearances, and good correlations were found when relating liver (partly) and kidney uptakes to changes in α-helical content. No correlations were found to type of mutation, changes in charge or hydrophobicity or to changes in ΔH_v. Organ uptakes are brought about by different types of endocytosis with different characteristics. Some of these lead to protein destruction in the lysosomes, whereas interaction with FcRn results in recycling of HSA. The relatively few correlations between molecular albumin parameters and organ uptakes could be due to different effects of the mutations on the various forms of endocytosis. However, substitutions of domain III of HSA could modify binding to FcRn and thereby alter its recycling. Although several details with respect to organ uptakes still have to be shed light on, the present information should be useful when designing recombinant HSA mutants with a modified plasma half-life.

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References

[1] U. Kragh-Hansen, Molecular aspects of ligand binding to serum albumin, Pharmacol. Rev. 33 (1981) 17–53.
[2] T. Peters Jr., All About Albumin: Biochemistry, Genetics, and Medical Applications, Academic Press, San Diego, CA, 1996.
[3] D.C. Carter, J.X. Ho, Structure of serum albumin, Adv. Protein Chem. 45 (1994) 153–203.
[4] S. Sugio, A. Kashima, S. Mochizuki, M. Noda, K. Kobayashi, Crystal structure of human serum albumin at 2.5 A resolution, Protein Eng. 12 (1999) 439–446.
[5] P. Yeh, D. Landais, M. Lemaitre, I. Maury, J.Y. Crenne, J. Becquart, A. Murry-Brelier, F. Boucher, G. Montay, R. Fleer, P.-H. Hirel, J.-F. Mayaux, D. Klatzmann, Design of yeast-secreted albumin derivatives for human therapy: biological and antiviral properties of a serum albumin-CD4 genetic conjugate, Proc. Natl. Acad. Sci. U. S. A. 89 (1992) 1904–1908.

[6] S. Syed, P.D. Schuyler, M. Kulczycky, W.P. Sheffield, Potent antithrombin activity and delayed clearance from the circulation characterize recombinant hirudin genetically fused to albumin, Blood 89 (1997) 3243–3252.
[7] Y. Yamasaki, K. Sumimoto, M. Nishikawa, F. Yamashita, K. Yamaoka, M. Hashida, Y. Takakura, Pharmacokinetic analysis of in vivo disposition of succinylated proteins targeted to liver nonparenchymal cells via scavenger receptors: importance of molecular size and negative charge density for in vivo recognition by receptors, J. Pharmacol. Exp. Ther. 301 (2002) 467–477.
[8] S.F. Ma, M. Nishikawa, H. Katsumi, F. Yamashita, M. Hashida, Cationic charge-dependent hepatic delivery of amidated serum albumin, J. Control Release 102 (2005) 583–594.
[9] Y. Iwao, M. Anraku, K. Yamasaki, U. Kragh-Hansen, K. Kawai, T. Maruyama, M. Otagiri, Oxidation of Arg-410 promotes the elimination of human serum albumin, Biochim. Biophys. Acta 1764 (2006) 743–749.
[10] L. Minchiotti, M. Campagnoli, A. Rossi, M.E. Cosulich, M. Monti, P. Pucci, U. Kragh-Hansen, B. Granel, P. Disdier, P.J. Weiller, M. Galliano, A nucleotide insertion and frameshift cause albumin Kenitra, an extended and O-glycosylated mutant of human serum albumin with two additional disulfide bridges, Eur. J. Biochem. 268 (2001) 344–352.
[11] U. Kragh-Hansen, S. Saito, K. Nishi, M. Anraku, M. Otagiri, Effect of genetic variation on the thermal stability of human serum albumin, Biochim. Biophys. Acta 1747 (2005) 81–88.
[12] M. Galliano, L. Minchiotti, P. Iadarola, G. Ferri, M.C. Zapponi, A.A. Castellani, The amino acid substitution in albumin Roma: 321 Glu → Lys, FEBS Lett. 233 (1988) 100–104.
[13] L. Minchiotti, M. Galliano, M. Stoppini, G. Ferri, H. Crespeau, D. Rochu, F. Porta, Two alloalbumins with identical electrophoretic mobility are produced by differently charged amino acid substitutions, Biochim. Biophys. Acta 1119 (1992) 232–238.
[14] D. Savva, A.L. Tarnoky, M.F. Vickers, Genetic characterization of an alloalbumin, albumin Kashmir, using gene amplification and allele-specific oligonucleotides, Biochem. J. 266 (1990) 615–617.
[15] L. Minchiotti, M. Galliano, P. Iadarola, M. Stoppini, G. Ferri, A.A. Castellani, Structural characterization of two genetic variants of human serum albumin, Biochim. Biophys. Acta 916 (1987) 411–418.
[16] Y. Sakamoto, K. Kitamura, J. Madison, S. Watkins, C.B. Laurell, M. Nomura, T. Higashiyama, F.W. Putnam, Structural study of the glycosylated and unglycosylated forms of a genetic variant of human serum albumin (63 Asp → Asn), Biochim. Biophys. Acta 1252 (1995) 209–216.
[17] O. Sugita, N. Endo, T. Yamada, M. Yakata, S. Odani, The molecular abnormality of albumin Niigata: 269 Asp → Gly, Clin. Chim. Acta 164 (1987) 251–259.
[18] L. Minchiotti, U. Kragh-Hansen, H. Nielsen, E. Hardy, A.Y. Mercier, M. Galliano, Structural characterization, stability and fatty acid-binding properties of two French genetic variants of human serum albumin, Biochim. Biophys. Acta 1431 (1999) 223–231.
[19] S.O. Brennan, The molecular abnormality of albumin Parklands: 365 Asp → His, Biochim. Biophys. Acta 830 (1985) 320–324.
[20] M. Galliano, S. Watkins, J. Madison, F.W. Putnam, U. Kragh-Hansen, R. Cesati, L. Minchiotti, Structural characterization of three genetic variants of human serum albumin modified in subdomains IIB and IIIA, Eur. J. Biochem. 251 (1998) 329–334.
[21] L. Minchiotti, S. Watkins, J. Madison, F.W. Putnam, U. Kragh-Hansen, A. Amoresano, P. Pucci, R. Cesati, M. Galliano, Structural characterization of four genetic variants of human serum albumin associated with alloalbuminemia in Italy, Eur. J. Biochem. 247 (1997) 476–482.
[22] J. Madison, M. Galliano, S. Watkins, L. Minchiotti, F. Porta, A. Rossi, F.W. Putnam, Genetic variants of human serum albumin in Italy: point mutants and a carboxyl-terminal variant, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 6476–6480.
[23] S.O. Brennan, P. Herbert, Albumin Canterbury (313 Lys → Asn). A point mutation in the second domain of serum albumin, Biochim. Biophys. Acta 912 (1987) 191–197.
[24] L. Minchiotti, M. Galliano, M.C. Zapponi, R. Tenni, The structural characterization and bilirubin-binding properties of albumin Herborn, a [Lys240 → Glu] albumin mutant, Eur. J. Biochem. 214 (1993) 437–444.
[25] K. Arai, K. Huss, J. Madison, F.W. Putnam, F.M. Salzano, M.H. Franco, S.E. Santos, M.J. Freitas, Amino acid substitutions in albumin variants found in Brazil, Proc. Natl. Acad. Sci. U. S. A. 86 (1989) 1821–1825.
[26] E.K. Chua, S.O. Brennan, P.M. George, Albumin Church Bay: 560 Lys → Glu a new mutation detected by electrospray ionisation mass spectrometry, Biochim. Biophys. Acta 1382 (1998) 305–310.
[27] P. Iadarola, L. Minchiotti, M. Galliano, Localization of the amino acid substitution site in a fast migrating variant of human serum albumin, FEBS Lett. 180 (1985) 85–88.
[28] S.O. Brennan, A.P. Fellowes, Albumin Hawkes Bay; a low level variant caused by loss of a sulphydryl group at position 177, Biochim. Biophys. Acta 1182 (1993) 46–50.
[29] U. Kragh-Hansen, A micromethod for delipidation of aqueous proteins, Anal. Biochem. 210 (1993) 318–327.
[30] R.F. Chen, Removal of fatty acids from serum albumin by charcoal treatment, J. Biol. Chem. 242 (1967) 173–181.
[31] D.J. Hnatowich, W.W. Layne, R.L. Childs, The preparation and labeling of DTPA-coupled albumin, Int. J. Appl. Radiat. Isot. 33 (1982) 327–332.
[32] F. Staud, M. Nishikawa, K. Morimoto, Y. Takakura, M. Hashida, Disposition of radioactivity after injection of liver-targeted proteins labeled with 111In or 125I. Effect of labeling on distribution and excretion of radioactivity in rats, J. Pharm. Sci. 88 (1999) 577–585.
[33] J.R. Duncan, M.J. Welch, Intracellular metabolism of indium-111-DTPA-labeled receptor targeted proteins, J. Nucl. Med. 34 (1993) 1728–1738.
[34] K. Yamaoka, Y. Tanigawara, T. Nakagawa, T. Uno, A pharmacokinetic analysis program (multi) for microcomputer, J. Pharmacobio-Dyn. 4 (1981) 879–885.
[35] W.P. Sheffield, J.A. Marques, V. Bhakta, I.J. Smith, Modulation of clearance of recombinant serum albumin by either glycosylation or truncation, Thromb. Res. 99 (2000) 613–621.
[36] K. Nakajou, H. Watanabe, U. Kragh-Hansen, T. Maruyama, M. Otagiri, The effect of glycation on the structure, function and biological fate of human serum albumin as revealed by recombinant mutants, Biochim. Biophys. Acta 1623 (2003) 88–97.
[37] T. Fujino, M. Kojima, M. Beppu, K. Kikugawa, H. Yasuda, K. Takahashi, Identification of the cleavage sites of oxidized protein that are susceptible to oxidized protein hydrolase (OPH) in the primary and tertiary structures of the protein, J. Biochem. (Tokyo) 127 (2000) 1087–1093.
[38] C. Chaudhury, S. Mehnaz, J.M. Robinson, W.L. Hayton, D.K. Pearl, D.C. Roopenian, C.L. Anderson, The major histocompatibility complex-related Fc receptor for IgG (FcRn) binds albumin and prolongs its lifespan, J. Exp. Med. 197 (2003) 315–322.
[39] C. Chaudhury, C.L. Brooks, D.C. Carter, J.M. Robinson, C.L. Anderson, Albumin binding to FcRn: distinct from the FcRn–IgG interaction, Biochemistry 45 (2006) 4983–4990.
[40] J.T. Andersen, J. Dee Qian, I. Sandlie, The conserved histidine 166 residue of the human neonatal Fc receptor heavy chain is critical for the pH-dependent binding to albumin, Eur. J. Immunol. 36 (2006) 3044–3051.