Compositional Analysis of Aldehyde-Modified HSA

In the next step, the aldehyde-modified HSA was incubated for 7 days (methylglyoxal-HSA, glyoxal-HSA, glyceraldehyde-HSA, glycolaldehyde-HSA) and 4 weeks (glucose-HSA, glucosone-HSA, 3DG-HSA) were evaluated amino acid analysis. As shown in Figure 3, glycolaldehyde modified Lys residues more selectively than Arg residues, whereas methylglyoxal and glyoxal reacted more favorably with Arg residues than lysine residues. However, in the present study, no significant modification was detected in glucose-HSA. These results indicate that the amino residues susceptible to each aldehyde are different, and that modification ratio of Lys and Arg residues by methylglyoxal, glyoxal, glyceraldehyde, glycolaldehyde, and 3DG are higher than that of glucose. The modification ratios of lysine and arginine residues estimated by spectroscopic methods were highly correlated with the data obtained by an amino acid analysis (Fig. 3C and D).

Immunoreactivity of Aldehyde-Modified HSA With Monoclonal Antibodies Against Several AGE Structures

To determine the effect of reactive aldehydes on AGE formation, HSA was incubated with reactive aldehydes, followed by the determination of several AGE structures by noncompetitive ELISA. As shown in Figure 4A, all aldehydes-modified HSA reacted with anti-AGE antibody (6D12), indicating that AGE was generated in all aldehyde-modified samples. Consistent with previous reports, CML formation was observed in glyoxal-, glyceraldehyde-, and glycolaldehyde-HSA, and CEL was generated by incubation with methylglyoxal (Fig. 4C). Although GA-pyridine was specifically detected in glycolaldehyde-HSA, imidazolone formation was observed in several aldehydes-modified HSA (Fig. 4D and E). In contrast, in the present study, anti-pyrraline antibody (H-12) did not show any reactivity with aldehydes-modified HSA (Fig. 4F). These results

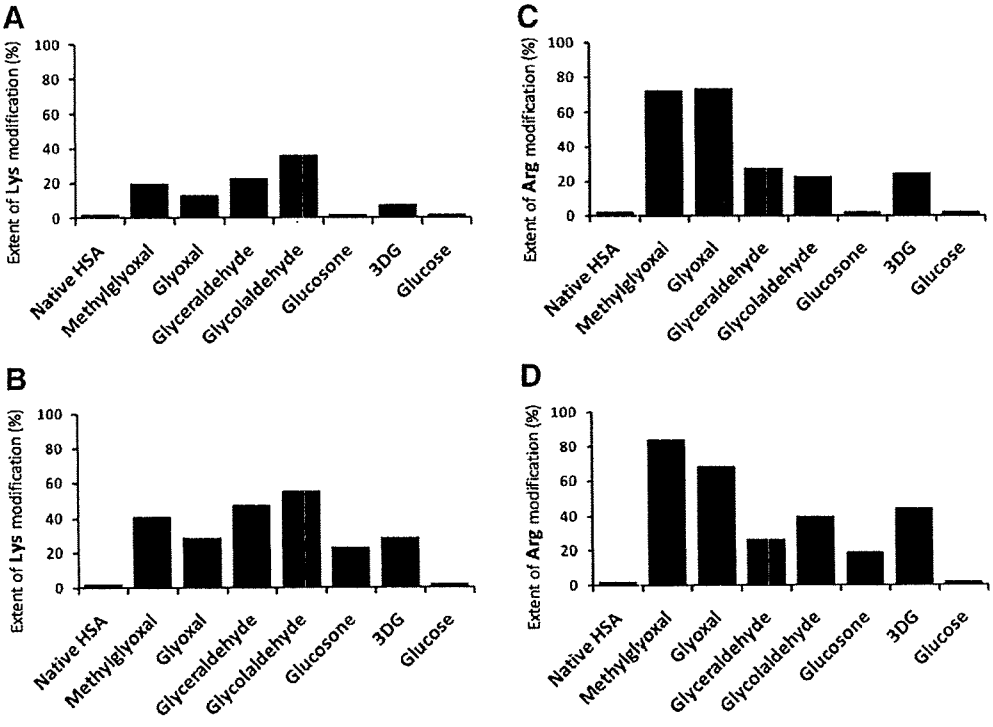

Figure 3. Compositional analysis of aldehyde-modified HSA. Methylglyoxal-, glyoxal-, glyceraldehyde-, and glycolaldehyde-HSA were prepared by incubation for 7 days, and glucose-, glucosone-, and 3DG-HSA were also prepared by incubation for 4 weeks. The extent of lysine (A) and arginine (C) modification was determined by an amino acid analysis after acid hydrolysis. The extent of lysine (B) and arginine (D) modification was also estimated by 2,4,6-trinitrobenzenesulfonic acid (TNBS) and 9,10-phenanthrenequinone, respectively.

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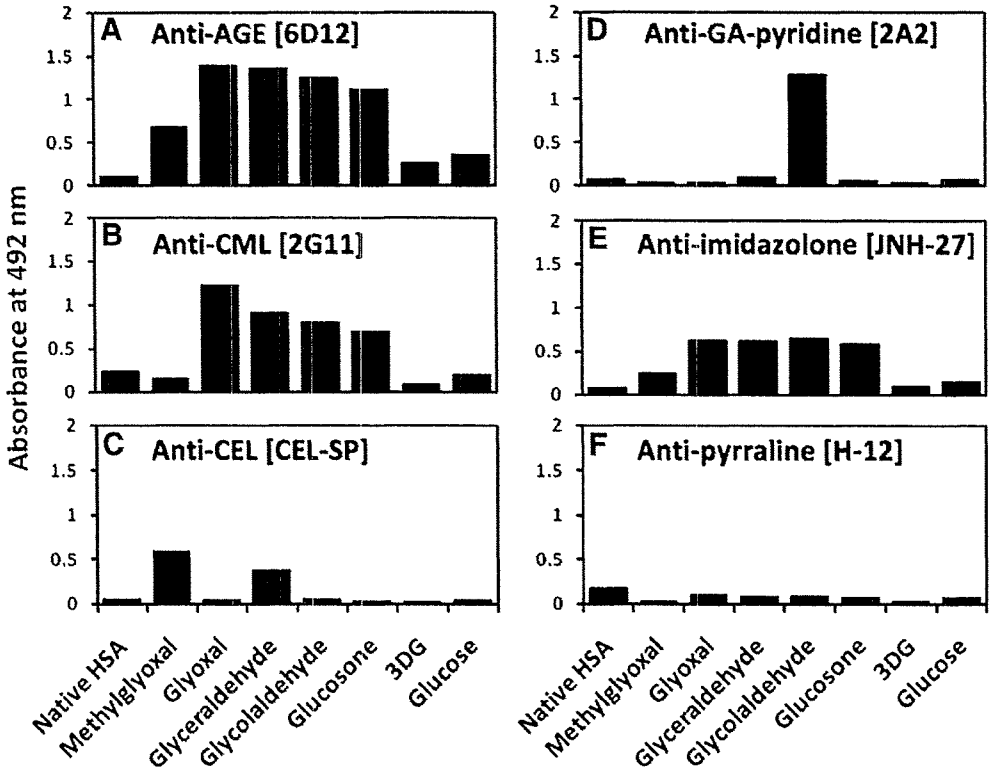

Figure 4. Immunoreactivity of aldehyde-modified HSA with monoclonal antibodies against several AGE structures. Methylglyoxal-, glyoxal-, glyceraldehyde-, and glycolaldehyde-HSA were prepared by incubation for 7 days, and glucose-, glucosone-, and 3DG-HSA were prepared by incubation for 4 weeks. The reactivity of aldehyde-modified HSA with monoclonal anti-AGE antibody (6D12; A), monoclonal anti-CML antibody (2G11; B), monoclonal anti-CEL antibody (CEL-SP; C), monoclonal anti-GA-pyridine antibody (2A2; D), monoclonal anti-imidazolone antibody (JNH-27; E), and monoclonal anti-pyrraline antibody (H-12; F) was determined by noncompetitive ELISA.

indicated that each aldehyde generated different AGE structures.

Drug Binding Activity of Aldehyde-Modified HSA

The binding activity of HSA to warfarin and ketoprofen was decreased by preincubation with 10 mM reactive aldehydes in a time-dependent manner (Fig. 5), whereas the effect of glucose on the drug binding activity of HSA was negligible (Fig. 5). However, a significant decrease of the drug binding activity was observed following preincubation with 10 mM glucose for 40 weeks (data not shown). The dose-dependent effect of reactive aldehydes (0.01–1 mM) on the binding activity was then measured next. The binding activity of HSA to warfarin decreased when HSA was incubated with aldehydes such as methylglyoxal and glyceraldehyde (Fig. 6), and that to ketoprofen was decreased after incubation with methylglyoxal, glyoxal, glyceraldehyde, or glycolaldehyde (Fig. 7). These results indicated that each aldehyde showed a different effect on the binding activity of HSA. A strong negative correlation was observed between the drug binding activity of HSA and increased molecular mass and negative charge (Tab. 1). Furthermore, the drug binding activity of HSA was more closely correlated with the extent of Arg modification than Lys modification determined by amino acid analysis (Tab. 1, Fig. 8). Similar patterns were also obtained when the lysine and arginine modification ratio estimated by spectroscopic methods (Supplementary Fig. 1). These results indicated that an increase in the negative charge and formed AGEs are dependent on each aldehyde and that modification of arginine residues may

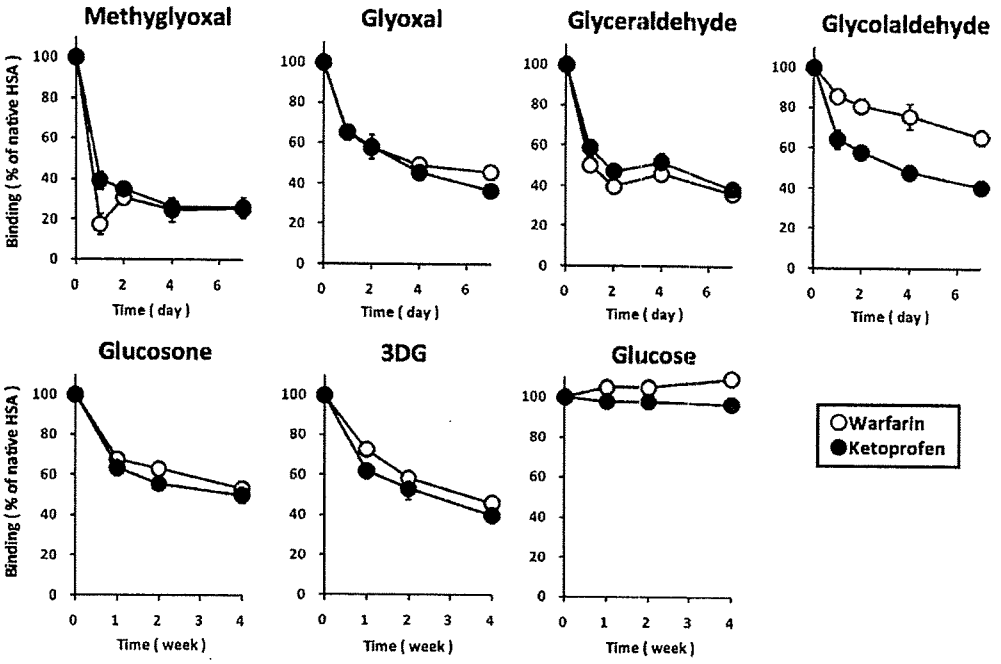

Figure 5. Binding of warfarin and ketoprofen to aldehyde-modified HSA. The binding of warfarin (5 μM) and ketoprofen (5 μM) to the aldehyde-modified HSA (10 μM) in PBS (pH 7.4) was examined by ultrafiltration as described in the Materials and Methods Section. Data are presented as the mean ± SD ($n = 3$).

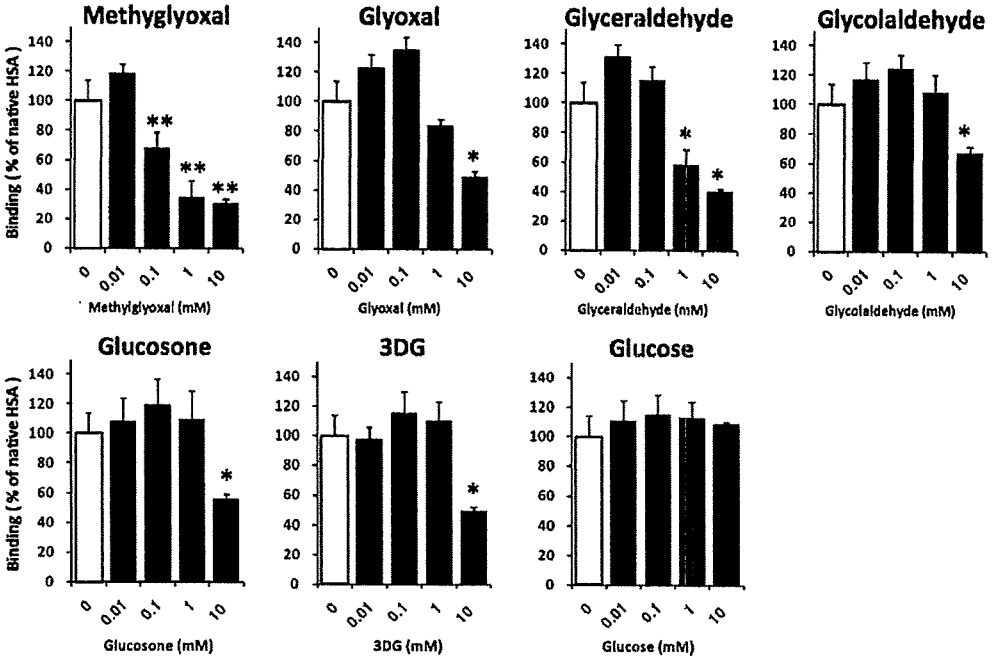

Figure 6. Dose-dependent effect of aldehydes on the warfarin binding to aldehyde-modified HSA. The samples were prepared by the incubation of HSA with the indicated concentrations of aldehydes. Methylglyoxal-, glyoxal-, glyceraldehyde-, and glycolaldehyde-HSA were prepared by incubation for 7 days, and glucose-, glucosone-, and 3DG-HSA were prepared by incubation for 4 weeks. The binding of warfarin (5 μM) to the aldehyde-modified HSA (10 μM) in PBS (pH 7.4) was examined by ultrafiltration as described in the Materials and Methods Section. Data are presented as the mean ± SD ($n = 3$). $^{*}p < 0.05$ and $^{**}p < 0.01$ compare to 0 mM (native-HSA).

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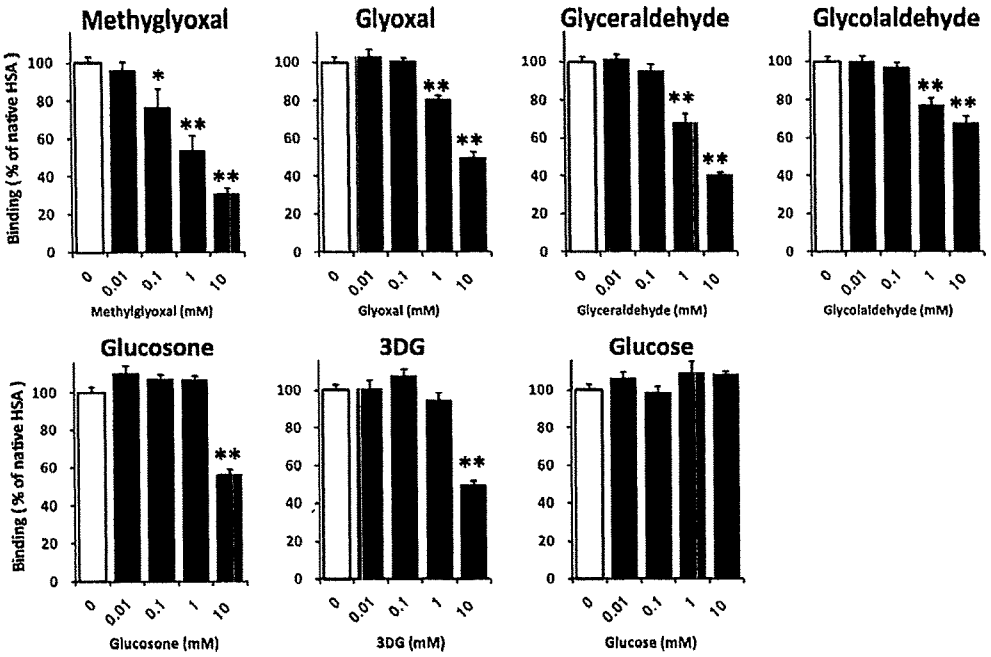

Figure 7. Dose-dependent effect of aldehydes on the ketoprofen binding to aldehyde-modified HSA. Samples were prepared by incubation of HSA with the indicated concentrations of aldehyde compounds. Methylglyoxal-, glyoxal-, glyceraldehyde-, and glycolaldehyde-HSA were prepared by incubation for 7 days, and glucose-, glucosone-, and 3DG-HSA were prepared by incubation for 4 weeks. The binding of ketoprofen (5 μM) to the aldehyde-modified HSA (10 μM) in PBS (pH 7.4) was examined by ultrafiltration as described in the Materials and Methods Section. Data are expressed as the mean ± SD ($n = 3$). $^{*}p < 0.05$ and $^{**}p < 0.01$ compare to 0 mM (native-HSA).

be an important factor for the drug binding activity of HSA.

DISCUSSION

Recent studies have demonstrated that AGEs are generated not only from glucose but also from reactive aldehydes which are generated *in vivo* through several pathways such as glycolysis and inflammation. In streptozotocin-induced diabetic rats, the administration of the trapping reagents for reactive aldehydes such as aminoguanidine, pyridoxaimine, thiamine, and benfotiamine, reduced AGEs accumulation *in vivo* and inhibited the development of diabetic nephropathy[9,33,34] and retinopathy,[10,35] whereas these reagents did not affect the blood glucose level. The findings of

Table 1. Correlation Between Conformational Change and Drug Binding Activity

	R (p-Value)				
	HSA-Molecular Mass	HSA-REM	Lys Modification	Arg Modification	Warfarin Binding
HSA-REM	0.656 ($p < 0.001$)				
Lys modification	0.674 ($p < 0.001$)	0.648 ($p < 0.001$)			
Arg modification	0.527 ($p = 0.006$)	0.367 ($p = 0.066$)	0.369 ($p = 0.064$)		
Warfarin binding	0.756 ($p < 0.001$)	0.680 ($p < 0.001$)	0.412 ($p = 0.036$)	0.752 ($p < 0.001$)	
Ketoprofen binding	0.841 ($p < 0.001$)	0.809 ($p < 0.001$)	0.568 ($p = 0.003$)	0.657 ($p < 0.001$)	0.905 ($p < 0.001$)

HSA-REM, relative electrophoretic mobility.
Pearson's correlation coefficients and significance (p).

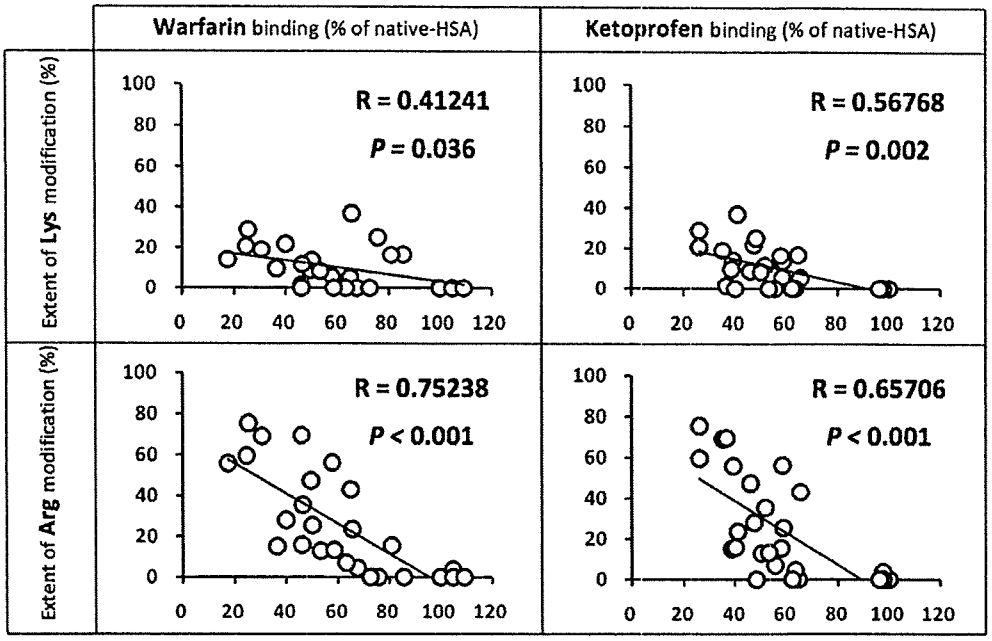

Figure 8. Correlation between amino acid analysis and drug binding activity. The correlation between the drug binding activity of HSA and Arg or Lys modification determined by amino acid analysis.

these reports indicate that the modification of proteins with aldehydes *in vivo* may contribute to the pathogenesis of diabetic complications by enhancing the posttranslational modification of various tissues. The present study compared the physicochemical properties and AGE structures formed by incubation of HSA with reactive aldehydes. Modification of HSA with aldehydes such as methylglyoxal and glyoxal is dramatically faster than that with glucose even at lower concentrations and shorter incubation periods, indicating that those reactive aldehydes may play an important role in the denaturalization and AGEs formation of proteins.

Although the molecular weight of glyceraldehyde- and glycolaldehyde-HSA reached a plateau level at day 2, it increased up to day 4 in methylglyoxal-HSA (Fig. 1). In the present study, we measured the time- and aldehydes-dependent protein modification by measuring the p*I* of albumin since the measurement of p*I* is one of the reliable methods to estimate protein modification. The increase in the net negative charge of methylglyoxal-HSA was observed from 1 day (Fig. 2), by correlating these findings with those obtained in previous reports.[25,36] Interestingly, the net negative charge of glycolaldehyde-modified HSA steeply increased in time-dependent manner (Fig. 2). It is probably because glycolaldehyde-alkylimine (Schiff base adduct of glycolaldehyde with amine) is rearranged to form an aldoamine,[37,38] which further reacts with another amino group of proteins to generate AGEs.[39] Since methylglyoxal dramatically modified arginine residues and inhibited the binding of HSA to warfarin and ketoprofen, arginine residues may contribute to the binding of those drugs. Although 470.7 nM methylglyoxal is observed in the plasma in patients with diabetes,[8] the level of continuously released methylglyoxal from the Embden–Meyerhof pathway to the blood stream may be higher than the detected methylglyoxal concentration because it rapidly reacts with lysine and arginine residues to form AGEs, whereas dicarbonyl detection reagents such as 2,3-diaminonaphthalene detects only the free form of methylglyoxal. Therefore, Ahmed et al.[36] prepared minimally modified methylglyoxal-protein by the incubating proteins with 0.5 mM methylglyoxal. In the present study, the binding of warfarin and ketoprofen to HSA was significantly inhibited even with the modification with 0.1 mM methylglyoxal for 7 days (Figs. 6 and 7). Thornalley et al. reported that only a few percent of HSA was modified by methylglyoxal even in diabetic patients[40] and the molecular mass of HSA increases by only <500 Da in uremic patients.[41] Further study will be required to clarify

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the physiological effect of *in vivo* glycation on the drug binding capacity of HSA.

Although Nakajou et al.[1] demonstrated that the site II of HSA is more susceptive to glycation than site I, the modification of HSA with methylglyoxal significantly inhibited the binding of warfarin which is known to bind in site I of HSA, thus, indicating that methylglyoxal modifies the amino residues at site I which are important for the binding of such drugs as warfarin. In addition, Arg 410 located in the site II of HSA is important not only for the selective modification by methylglyoxal but also the binding of ketoprofen.[36] Taken together, the modification of HSA by methylglyoxal may be an important factor in reducing the function of HSA. HSA has at least two distinct sites for high-affinity binding of several physiologically important compounds and a large number of drugs, known as sites I and II. Site I binds a great diversity of both endogenous and exogenous substances, such as warfarin, phenytoin, and bilirubin and site II mainly interacts with ligands possessing a carboxylate at one end of extended hydrophobic molecules, such as ketoprofen and medium chain fatty acids.[42] The present study demonstrated that reactive aldehydes, especially methylglyoxal, decreased the binding activity of HSA to warfarin and ketoprofen. This result suggests that reactive aldehydes may affect the binding activity of HSA to physiologically important compounds such as bilirubin and medium chain fatty acids. Further studies will be required to confirm the biological significance of HSA modification by methylglyoxal since the modification in the current study was induced at comparatively higher concentrations of methylglyoxal than that detected in pathophysiological conditions. However, the present study was conducted in order to clarify which aldehydes have the potential to decrease the function of HSA. Furthermore, although instrumental analyses are superior to immunological detection in the quantification of AGEs, detection of AGEs by anti-AGE antibodies is useful for detecting them in a short period using conventional assays. The current study also provides evidence that formed AGEs, detected by antibodies against CML, CEL, pyrraline, and GA-pyridine, on HSA are highly dependent on the kind of aldehydes.

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Editorial

Albumins with New Functions and Clinical Applications

Full text of this paper is available at http://www.jstage.jst.go.jp/browse/dmpk

The focus of DMPK Theme Issues is state of the art and recent developments in research areas that have significant relevance for the academic world as well as for the pharmaceutical industry. The three issues that have appeared to date deal with drug-metabolizing enzymes, membrane transporters and pharmacokinetics and pharmacodynamics. This issue is dedicated to a water-soluble protein transporter, namely human serum albumin (HSA), and presents strategies for utilizing its unique ligand binding capability, and the latest news concerning its metabolism. This information can be useful in terms of the development of new compounds – drugs, albumins or albumin-ligand complexes – that can be used diagnostically or therapeutically.

A considerable proportion of compounds with other biological activity fails to progress to later stages of drug development due to problems associated with absorption, distribution, metabolism and elimination (ADME). Because ADME is a complex issue, the cost of development of new drugs is increased considerable. This factor constitutes one of the main driving forces in the search for techniques to improve ADME characteristics in the early stages of drug development. The ligand binding properties of HSA and its abundance in the body couples the protein closely to the ADME problem. Therefore, detailed information regarding binding sites and ligand-induced conformational changes of HSA are critical to solving this problem. Recent X-ray crystallography and site-directed mutagenesis studies have contributed significant, new information in this area. Such information can be used to determine whether a new drug *in spe* should be constructed in such a way that it makes use of, or avoids, albumin transport and depot function. Molecular information is also very useful in related contexts, because it provids basic information on how to modify a protein or ligand for producing complexes with new properties. For example, modified albumin-heme complexes have the ability to reversibly bind to molecular oxygen; the aim of this research has been to construct an artificial hemoprotein. Furthermore, a complex of HSA and carboxy-C_{60}-fullerene may become a useful photosensitizer for photodynamic therapy of cancer.

In addition to the reversible binding of ligands, HSA binds to xenobiotics and endogenous compounds in a covalent manner and thereby effects the metabolic fate and clearance of certain drugs. However, complex formation may produce a protein with new functions and possible clinical applications. Thus, HSA can be *S*-nitrosylated by nitric oxide, and the resulting compound may have antibacterial and cytoprotective properties. The production of fusion proteins involves a completely different type of covalent binding. Because of its relatively high *in vivo* half-life of approximately 19 days, HSA is an attractive fusion partner for extending the half-life of peptides and small proteins.

Little information is available concerning interplay between reversibly bound ligands and covalent binding of other compounds. However, this interplay may be valuable when designing or improving new albumin functions. For example, the reversible binding of endogenous fatty acids appears to improve the cytoprotective effect of *S*-nitrosylated HSA by facilitating *S*-transnitrosation from HSA to cellular target proteins.

The binding of a drug to albumin has significant effect on the pharmacokinetics and pharmacodynamics of many drugs, but such binding can also be clinically very relevant outside the body. Extracorporeal albumin dialysis serves to illustrate this point. By this technique, it is possible to remove albumin binding toxins and drugs from the body. The method will probably be improved in the future by constructing HSA mutants with increased affinity for the compound that is intended to be removed from a patient's body.

The *in vivo* binding properties of HSA can be modified by many factors. If an unambiguous link exists between the provoking factor and the type of modified binding, this can be used diagnostically. Thus, in myocardial ischemia, the N-terminal part of HSA is modified in such a way that its ability to bind certain metal ions is lost. In practice, binding is monitored by using cobalt ions as a representative ligand.

Clinically, HSA is used for the restoration of blood volume, emergency treatment of shock, acute management of burns and other situations associated with hypoproteinemia. In such situations it would be beneficial to have access to albumins with prolonged life-time in the circulation. Therefore, much research is currently being conducted on albumin-isoforms, -fragments and -dimers, in attempt to find such a protein. If the protein carries a useful drug or other therapeutical agent, it would improve the clinical situation even further. Modifyied HSA is used for other *in vivo* purposes such as targeting individual types of cells or organs. The main purpose of such targeting is to obtain specific delivery of

an albumin-bound drug. For example, lactosylation, mannosylation and succinylation target HSA to hepatocytes, Kuppfer cells and endothelial cells of the liver, respectively.

A recent landmark in our understanding of the normal metabolism of HSA is the discovery of its pH-dependent interaction with the intracellular neonatal Fc receptor (FcRn). This receptor prevents a large fraction of albumin and IgG from degradation in lysozomes and sends it back for reuse in the circulation and other extracellular spaces. Interference with the interaction between the protein and receptor would likely modify the half-life of albumin, albumin-bound drugs and albumin fusion proteins in a therapeutically useful way.

Thus, modern albumin research has progressed from the mere determination of binding site number and corresponding association constants to the development and characterization of albumin isoforms and albumin-ligand complexes with new therapeutical or diagnostic functions. Several examples of such possibilities are described and discussed in greater detail, including methodological aspects, in the seven expert reviews in this Theme Issue. The reports were first presented at an international symposium entitled "Development of albumins with new functions and clinical applications" held on October 30, 2008 in Kumamoto, Japan. We hope that these reviews will stimulate more work and the development of new ideas in this promising and growing field of research. Finally, we wish to sincerely thank all the contributors and readers of this fourth Theme Issue of DMPK.

Masaki OTAGIRI
Graduate School of Pharmaceutical Sciences,
Kumamoto University

Ulrich KRAGH-HANSEN
Department of Medical Biochemistry,
University of Aarhus

Teruko IMAI
Graduate School of Pharmaceutical Sciences,
Kumamoto University

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Altered chain-length and glycosylation modify the pharmacokinetics of human serum albumin

Yasunori Iwao [a], Mikako Hiraike [a], Ulrich Kragh-Hansen [b], Keiichi Kawai [c], Ayaka Suenaga [a], Toru Maruyama [a], Masaki Otagiri [a,*]

[a] Department of Biopharmaceutics, Graduate School of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Kumamoto 862-0973, Japan
[b] Department of Medical Biochemistry, University of Aarhus, DK-8000 Aarhus C, Denmark
[c] School of Health Sciences, Faculty of Medicine, Kanazawa University, Ishikawa 920-0942, Japan

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ABSTRACT

Human serum albumin with modified plasma half-life will be useful for clinical purposes. Therefore, the pharmacokinetics of three of each of the following types of genetic variants, and of their corresponding normal albumin, were examined in mice: N-terminally elongated, C-terminally truncated and glycosylated albumins. Isoforms differing from the normal protein by three or more amino acids, especially two of the truncated forms, had shorter half-lives. The effect of glycosylation depended on the position of attachment: in domain II it increased half-life, whereas in domain I and III it had no significant effect. Liver, kidney and spleen uptake clearances were also modified. The pronounced changes in half-life of the two truncated variants and the glycosylated isoform could be explained, at least partly, by large changes in organ uptakes; in the remaining six cases, different effects were registered. Such information should be useful when designing therapeutical albumin products for, e.g., drug delivery systems. In addition to various types of cell endocytosis, leading to intracellular destruction or recycling of the proteins, the metabolism of the alloalbumins could be affected by plasma enzymes. No correlation was found between mutation-induced changes in the pharmacokinetic parameters and changes in α-helical content or changes in heat stability as represented by ΔH_v.

1. Introduction

Human serum albumin (HSA) is produced in the parenchymal cells of the liver, and it is the most abundant plasma protein. It is an important circulating depot protein and transport protein for 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 it has a molecular mass of approximately 66.5 kDa [2]. According to X-ray crystallographic analyses of HSA and of its recombinant version (rHSA), 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]. A combined phosphorescence depolarization-hydrodynamic modeling study has proposed that the overall conformation of HSA in neutral solution is very similar to that observed in the crystal form [5].

Clinically, HSA is used for urgent restoration of blood volume, emergency treatment of chock, acute management of burns and other situations associated with hypoproteinemia [2]. 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 can be in limited supply. Because of these problems, rHSA, which is highly expressed by *Pichia pastoris*, most probably will be commercially available in the near future [6]. Another benefit of this approach is that protein engineering will enable the creation of rHSAs with modified properties such as extended half-life in the circulation. In this connection HSA dimers seem to be useful candidates. Matsushita et al. [7] found that rHSA dimers had a high retention rate in the circulatory blood and a lower vascular permeability than native rHSA in normal rats and in mice with paw edema. Similar observations have been made by Komatsu et al. [8], who examined the pharmacokinetics of chemically crosslinked rHSA dimers in the rat. On the other hand, recombinant albumin domain(s) are cleared very fast. Sheffield et al. [9] found that recombinant domain I, I+II and III of rabbit serum albumin all had very short mean terminal catabolic half-lives in rabbits due to a fast elimination in the urine.

Abbreviations: HSA, human serum albumin; rHSA, recombinant HSA; Alb, albumin; Alb A, normal (wild-type) albumin; CD, circular dichroism; ΔH_v, van't Hoff enthalpy; RAGE, receptor for advanced glycation end products

* Corresponding author. Tel.: +81 96 371 4150; fax: +81 96 362 7690.
E-mail address: otagirim@gpo.kumamoto-u.ac.jp (M. Otagiri).

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Because of a relatively high in vivo half-life of ca. 19 days [2], HSA is an attractive fusion partner to extend the half-life, and potentially the therapeutic utility, of recombinant peptides and proteins. Among recent examples are rHSA genetically fused to type 1 interferons [10], glucagon-like peptide-1 [11] and interleukin-2 [12]. However, although an extension of the half-life of therapeutic peptides and proteins often is desirable, an extension to that of albumin could be excessive.

Although HSA-preparations with a modified half-life thus could be very useful, not much has been done to design or find such preparations. In our search for useful candidates, we have paid our attention to HSA genetic variants. Until now, 65 inherited variants of HSA, including proalbumin variants, have been identified and structurally characterized [13]. Usually, these genetic variants are expressed in heterozygous form and without any known association to disease [13]. Therefore, unlike lethal mutations, such 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.

Recently, we have studied the pharmacokinetic properties in mice of 17 alloalbumins with single-residue mutations [14]. The study showed that, for example, only a few of the variants had a significantly modified half-life in the blood. In an attempt to find genetic variants with a more pronounced impact on pharmacokinetics, we now have expanded that study by determining the plasma half-lives and organ uptakes of three HSAs with a slightly longer chain-length (proalbumin variants), three with a slightly shorter chain-length (C-terminal variants) and three alloalbumins *N*-glycosylated in domain I, II and III, respectively. For being able to make a more detailed comparison between molecular characteristics and pharmacokinetic properties, we have estimated the effect of the molecular modifications on the α-helical content of the alloalbumins by using circular dichroism (CD). Previously, the effect of genetic variation on the thermal stability of HSA has been quantified in terms of, for example, changes in the van't Hoff enthalpy (ΔH_v) [15]. In the present work, the pharmacokinetic results have also been related to changes in ΔH_v.

2. Materials and methods

2.1. Protein samples

The genetic variants of HSA and their normal (wild-type) counterpart (endogenous Alb A) were isolated from serum from heterozygous carriers by ion-exchange chromatography. The locations of the structural changes of the nine variants are indicated in Fig. 1. After isolation, the 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; and Dr. D. Donaldson, East Surrey Hospital, Redhill, UK. Before use, the albumins were delipidated by treatment with hydroxyalkoxypropyldextran at pH 3.0, as described in a previous paper [16]. 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 [17], deionized, freeze-dried and then stored at −20 °C until used.

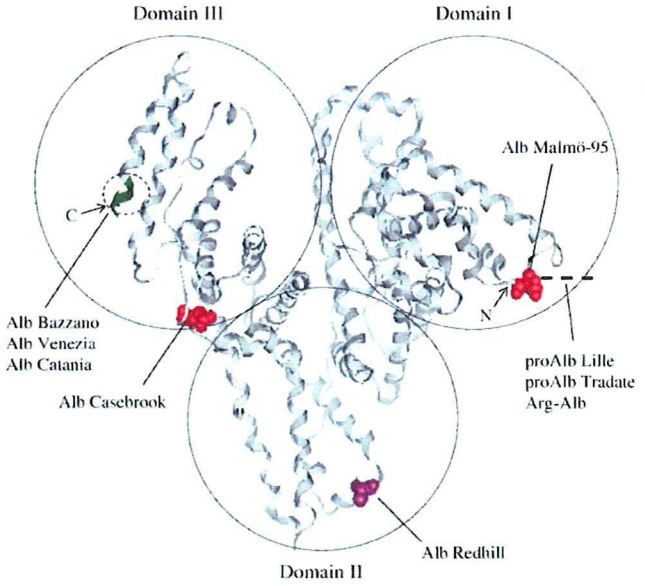

Fig. 1. The crystal structure of HSA indicating the locations of the mutations of the three C-terminal variants and the three proAlb variants used in this study. The locations of the glycosylated 63 Asn (Alb Malmö-95), 318 Asn (Alb Redhill) and 494 Asn (Alb Casebrook) are also shown. The subdivision of HSA into domains is marked; N and C stand for the N-terminal and the C-terminal ends, respectively. The broken, black line added to the N-terminal end indicates the prosegment of HSA.

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.

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. [18], as described elsewhere [19]. 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-labeled 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 [20]. 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 [21]. The two-compartment model was fitted according to the Akaike information criterion by Eq. (1).

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

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.

2.4. Far–UV CD spectra

The protein concentration was 1.5 μM, as determined by the method of Bradford [22], and the buffer was 67 mM sodium phosphate, pH 7.4, 25 °C. Far–UV intrinsic spectra were recorded from 200 to 250 nm using a Jasco J-720 spectropolarimeter (Tokyo, Japan). For calculation of the mean residue ellipticity, [θ], the molecular masses were assumed to be 65.8 kDa for Alb Venezia, 67.1 kDa for proAlb Lille and Tradate and 66.5 kDa for the remaining variants and Alb A. The α-helical content of the proteins was estimated from the ellipticity values at 222 nm as described by Chen et al. [23].

2.5. Analysis of experimental data

The effects of the molecular changes were evaluated by using the following relationship:

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

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

2.6. 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. The genetic variants

The alloalbumins used in this study have been named after the place from where the first detected carrier originates, and their molecular changes are summarized in Table 1.

Proalbumin (proAlb) is an albumin molecule to which the propeptide, Arg-Gly-Val-Phe-Arg-Arg-, is still bound at the N-terminus (Fig. 1). The positions of the propeptide are numbered from −6 to −1 (the juxtaposition to albumin). Normally, proAlb does not occur in detectable amounts in the circulation, because the propeptide is cleaved off by propeptidase within the liver cells. However, substitution of −1 Arg or −2 Arg (as in proAlb Lille [24] and proAlb Tradate [25]) inhibits the proteolytic cleavage of the propeptide but not the secretion of the protein, and such proalbumin variants, in contrast to wild-type proalbumin, can be isolated from the serum. In vivo, the prepro-form of proAlb Tradate (−2 Arg→Cys) is often cleaved after the mutated residue giving rise to HSA retaining −1 Arg (Arg-Alb) [25].

Among the C-terminal variants most are truncated albumins (Table 1). Thus, Alb Catania is three amino acids shorter than Alb A, and the three last residues in the new C-terminal end are changed from Gln-Ala-Ala to Lys-Leu-Pro [26]. Alb Venezia has been shortened by seven amino acids, and the new C-terminal end is changed from Gly-Lys-Lys-Leu-Val-Ala-Ala to Pro-Thr-Met-Arg-Ile-Arg-Glu [26]. Alb Bazzano has been shortened by three amino acids, and 14 of the last 16 amino acids in the new C-terminal end have been substituted: from Cys-Phe-Ala-Glu-Glu-Gly-Lys-Lys-Leu-Val-Ala-Ala-Ser-Gln-Ala-Ala to Ala-Leu-Pro-Arg-Arg-Val-Lys-Asn-Leu-Leu-Leu-Gln-Val-Lys-Leu-Pro [27]. Here the 567 Cys→Ala substitution has caused the loss of the C-terminal disulfide bridge.

It is uncommon for an amino acid substitution to result in the formation of an oligosaccharide attachment sequence. However, that has happened to Alb Malmö-95 [28], Alb Redhill [29,30] and Alb Casebrook [31,32], which are glycosylated in domain I, II and III, respectively (Fig. 1). In all three cases, the glycan is a disialylated (mainly or totally) biantennary complex type oligosaccharide N-linked to an asparagine residue [30]. Alb Redhill is unique, because it is

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

Variant name (mutation)	Domain		Half-life[a] (min)	Clearance (μL/hr)[a]		
				Liver	Kidney	Spleen
proAlb Lille (−2Arg->His)	I	Variant	251.1±4.31*	56.13±8.07	60.06±6.76	82.47±15.96
		Alb A	264.3±4.58	42.66±7.52	79.69±8.04	90.11±7.97
proAlb Tradate (−2Arg->Cys)	I	Variant	249.4±5.89	111.99±14.99	89.14±11.11**	74.83±13.57
		Alb A	252.4±7.62	109.99± 14.01	46.01±6.89	87.86±6.36
Arg-Alb (Alb A having −1 Arg)	I	Variant	262.1±6.53	21.16±3.72**	99.98±23.18	105.77±14.52
		Alb A	253.5±4.98	94.38±5.17	119.38±10.29	91.38±6.51
Alb Bazzano (567–582 substituted, 583–585 deleted)	III	Variant	231.1±5.31*	189.77±26.11**	253.69±36.22**	84.66±7.00**
		Alb A	245.2±6.93	41.35±4.68	119.95±20.05	57.94±5.11
Alb Venezia (572–578 substituted, 579–585 deleted)	III	Variant	225.1±5.49**	134.32±11.41**	136.11±13.55**	88.48±10.15
		Alb A	247.2±6.83	41.24±4.81	62.87±3.28	67.89±15.03
Alb Catania (580–582 substituted, 583–585 deleted)	III	Variant	248.6±5.46	12.29±1.18*	140.09±15.01**	45.97±5.15
		Alb A	251.3±3.99	48.99±18.18	81.53±12.02	52.69±7.64
Alb Malmö-95 (63 Asp->Asn, glycosylated at 63 Asn)	I	Variant	261.3±7.62	145.82±13.84**	113.96±15.72	53.21±4.21**
		Alb A	264.2±6.14	79.72±11.74	111.64±18.18	82.68±4.09
Alb Redhill (−1 Arg retained, 320 Ala->Thr, glycosylated at 318 Asn)	II	Variant	260.3±7.43*	25.69±3.51**	62.62±7.05**	45.68±6.91**
		Alb A	245.2±6.34	93.49±8.95	125.84±15.21	84.39±7.26
Alb Casebrook (494 Asp->Asn, glycosylated at 494 Asn)	III	Variant	251.2±4.88	134.79±13.97**	141.01±8.14	56.94±6.10
		Alb A	249.1±4.54	81.45±9.19	148.71±21.18	57.73±4.25

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

the only example so far of an albumin with two mutations. One is the 320 Ala→Thr, which leads to glycosylation of 318 Asn; the other is −2 Arg→Cys, which results in abnormal hydrolysis of the prepro-form within the liver cells and to the formation of albumin still possessing −1 Arg [29].

According to the literature cited [24–32], none of the mutations seem to affect the oligomeric state of albumin.

3.2. 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. As seen, in all 9 cases the mutation affected, to different degrees, the elimination of HSA. Table 1 gives the plasma half-lives, calculated by β-phase using the nonlinear least-square program MULTI and Eq.(1), and liver, kidney and spleen 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-lives for Alb A and commercial HSA are 252.5 min and 268.2 ± 7.2 min (n=6), respectively. The liver, kidney and spleen uptake clearances for Alb A are on an average 70.36 μL/h, 99.51 μL/h and 74.74 μL/h, respectively, whereas

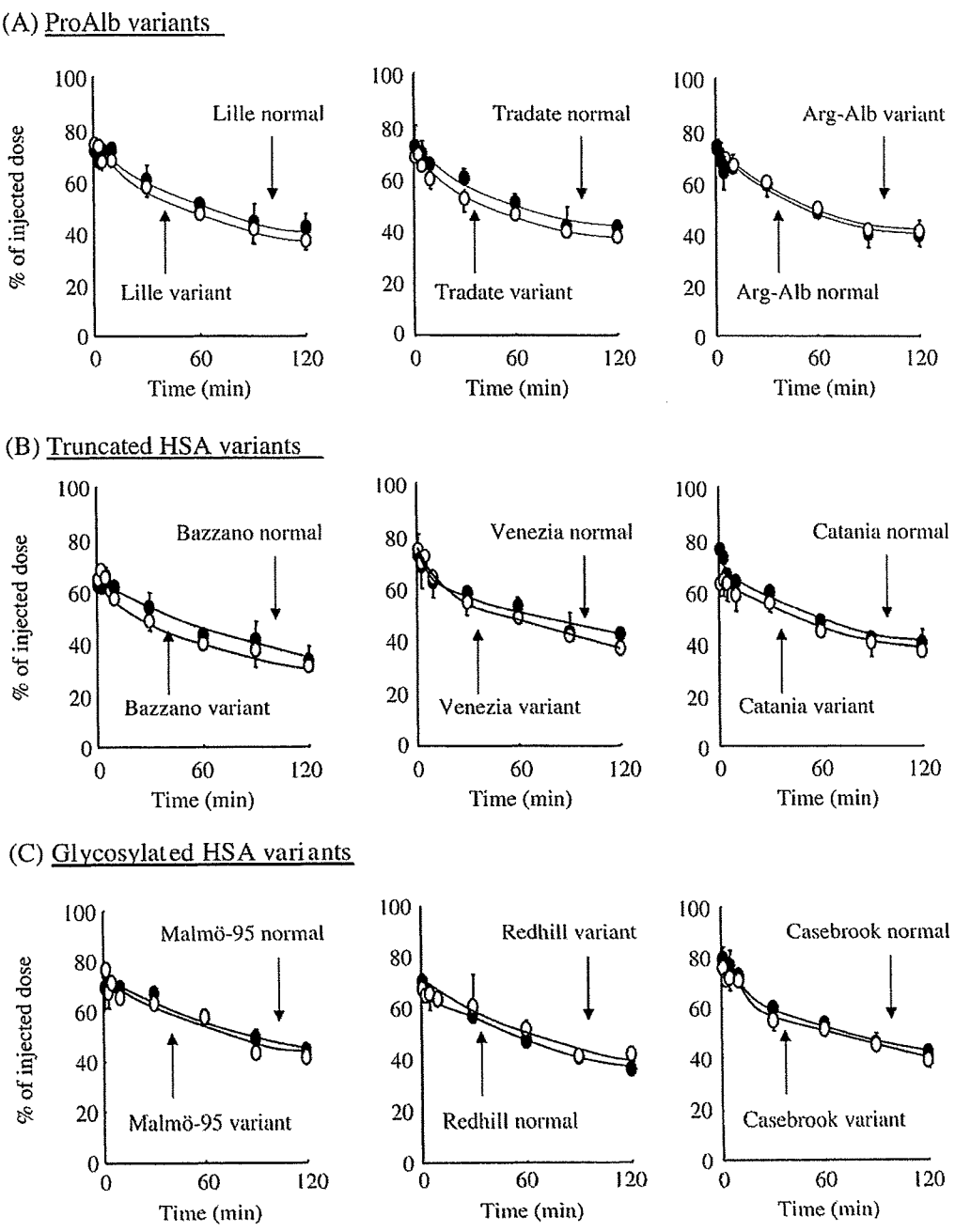

Fig. 2. Relative plasma amounts of ^{111}In-labeled proAlb variants (A), truncated HSA variants (B) and glycosylated HSA variants (C) and their corresponding Alb A after intravenous administration in mice. ^{111}In-albumin was injected as a bolus dose into the tail vein. Relative amounts are plotted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

those for commercial HSA are 54.31±8.23 μL/h, 83.12±5.43 μL/h and 63.65±3.56 μL/h, respectively. The slightly lower half-life and the slightly higher organ uptake clearances found for Alb A are most probably due to differences in isolation and/or defatting procedures.

The effects of the mutations on plasma half-lives and organ uptake clearances, calculated according to Eq.(3), are given in Table 2. As seen, all the proAlb variants and the truncated variants have diminished plasma half-lives, i.e., negative percent changes; this is most evident for Alb Venezia. By contrast, the effect of glycosylation varied: Almost no effect in the case of Alb Malmö-95 and Casebrook but a significant prolongation of the half-life for Alb Redhill. The increase of the half-life for Alb Redhill can partly be explained by the presence of −1 Arg (Table 2). In all 9 cases, the molecular changes resulted in a modified liver, kidney and spleen uptake clearance (Table 2). The most pronounced effects were found for the truncated variants Alb Bazzano and Venezia; the uptakes by the three organs were increased very much. At the other extreme, the uptakes of Alb Redhill were in all cases significantly decreased. For all three variants, the changes in organ uptakes can explain, at least partly, the changes in plasma half-lives. For the remaining six alloalbumins, the effects on organ uptakes are more complex, i.e., both increases and decreases in uptake were found for the same variant. The most pronounced changes, i.e., more than 100%, were observed for liver and kidney uptakes of Alb Bazzano and Venezia.

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

The effects of genetic variation on the albumin structure were studied by far-UV intrinsic CD. From Fig. 3 it is apparent that the variants have spectra which differ to varying extents from that of their corresponding Alb A. In most cases, the structural modification results in a less negative spectrum. However, in two cases (Arg-Alb and Alb Venezia) the variant has a more negative spectrum. The most pronounced spectral changes were found for two variants having a modified chain length (proAlb Tradate and Alb Bazzano). We used the ellipticities at 222 nm to calculate the α-helical content of all the albumins. The impact of genetic variation on that parameter is given in a quantitative way in Table 2. Because the alloalbumins had both modified pharmacokinetic properties (section 3.2.) and modified α-helical contents we investigated, whether there is a direct correlation between these parameters. That was found not to be the case. When plotting the changes in half-lives as a function of changes in α-helical content a poor correlation was found; a straight line had a *P*-value of 0.48 (not shown). Likewise, no correlations were found between the changes in organ uptake clearances and changes in α-helical content; the *P*-values were in the range 0.16–0.52 (not shown).

Previously, we have studied the effects of genetic variation on the thermal stability of HSA [15]. The stability was quantified by determining van't Hoff enthalpies (ΔH_v-values), and the results of that study are included in Table 2. We also examined whether a correlation exists between changes in the ΔH_v-values and the changes in pharmacokinetic properties. However, no such correlation was found. When plotting the changes in half-lives versus the changes in ΔH_v-values, the *P*-value was only 0.68 (not shown). In addition, no correlations were found between the changes in organ uptakes and changes in ΔH_v-values; the *P*-values were in the range 0.38–0.83 (not shown).

4. Discussion

Clinically, it would be useful, if protein engineering could result in the production of rHSA preparations 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 and as a carrier in drug delivery systems. However, also in the two latter types of examples it would be advantageous to be able to modify the plasma half-life of the protein product. For finding such isoforms, in the present study, we investigated the half-life and organ uptakes of a series of genetic HSA variants with relatively large molecular changes, i.e., modified chain-length or glycosylated. The most clear results were obtained for two truncated alloalbumins (Alb Venezia and Bazzano) and a glycosylated variant (Alb Redhill). For the other six variants less clear and individual results were obtained.

All the alloalbumins with chain-lengths deviating by three or more residues from Alb A have diminished half-lives in the circulation (Table 2). This is most evident for Alb Bazzano and Venezia the half-lives of which were reduced by 6–9%. These results must be due to mutation induced changes in protein charge and/or conformation. In this connection it is relevant to note that Alb Bazzano has lost its C-terminal disulfide bridge because of the 567 Cys→Ala substitution (see Section 3.1.). Our previous study [14], making use of single-residue mutations, revealed that the half-life of Alb Hawkes Bay is shortened by ca. 30%. This finding is due to the mutation 177 Cys→Phe [33], which results in the loss of the disulfide bond between 168 Cys and 177 Cys. Thus, the lifespan of HSA is dependent on the existence of its 17 stabilizing disulfide bridges [1–4].

The effect of glycosylation depends on the position of attachment. Glycosylation of 63 Asn in domain I (Alb Malmö-95) or 494 Asn in domain III (Alb Casebrook) has no significant effect on variant half-life. By contrast, glycosylation of 318 Asn in domain II (Alb Redhill) results in a significant increase in half-life; however, part of the increment is caused by the presence of −1 Arg. Sheffield et al. [9] also observed position-dependent effects of *N*-glycosylation when studying the half-life of mutated rabbit serum albumin in rabbits. These authors reported no effect of glycosylation of 12 Asn in the 14 Val→Thr variant. By contrast, a similar modification of 494 Asn in the 494 Asp→Asn isoform resulted in a reduction of the mean terminal catabolic half-life from 4.32 days to 2.87 days. The different results obtained in our and their study for the latter variant is probably due to species differences between protein and/or test animal.

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 (%)	Spleen clearance (%)	α-helical content (%)	ΔH_v[a] (%)
proAlb Lille (−2Arg->His)	−4.99	31.58	−24.63	−8.48	−9.60	−6.08
proAlb Tradate (−2Arg->Cys)	−1.19	1.82	93.74	−14.84	−24.15	N.D.[b]
Arg-Alb (Alb A having −1 Arg)	3.39	−77.58	−16.25	15.75	5.64	3.80
Alb Bazzano (567–582 substituted, 583–585 deleted)	−5.73	358.94	111.49	46.10	−20.09	−4.49
Alb Venezia (572–578 substituted, 579–585 deleted)	−8.94	225.74	116.50	30.32	5.41	34.50
Alb Catania (580–582 substituted, 583–585 deleted)	−1.07	−74.92	71.83	−12.75	−1.09	−19.71
Alb Malmö-95 (63 Asp->Asn, glycosylated at 63 Asn)	−1.10	82.91	2.07	−35.65	−0.31	−30.75
Alb Redhill (−1 Arg retained, 320 Ala->Thr, glycosylated at 318 Asn)	6.16	−72.52	−50.24	−45.63	−3.26	−4.48
Alb Casebrook (494 Asp->Asn, glycosylated at 494 Asn)	0.84	65.49	−5.17	−1.36	−7.06	17.54

[a] The values for ΔH_v are taken from Kragh-Hansen et al. [15].
[b] ND, Not determined.

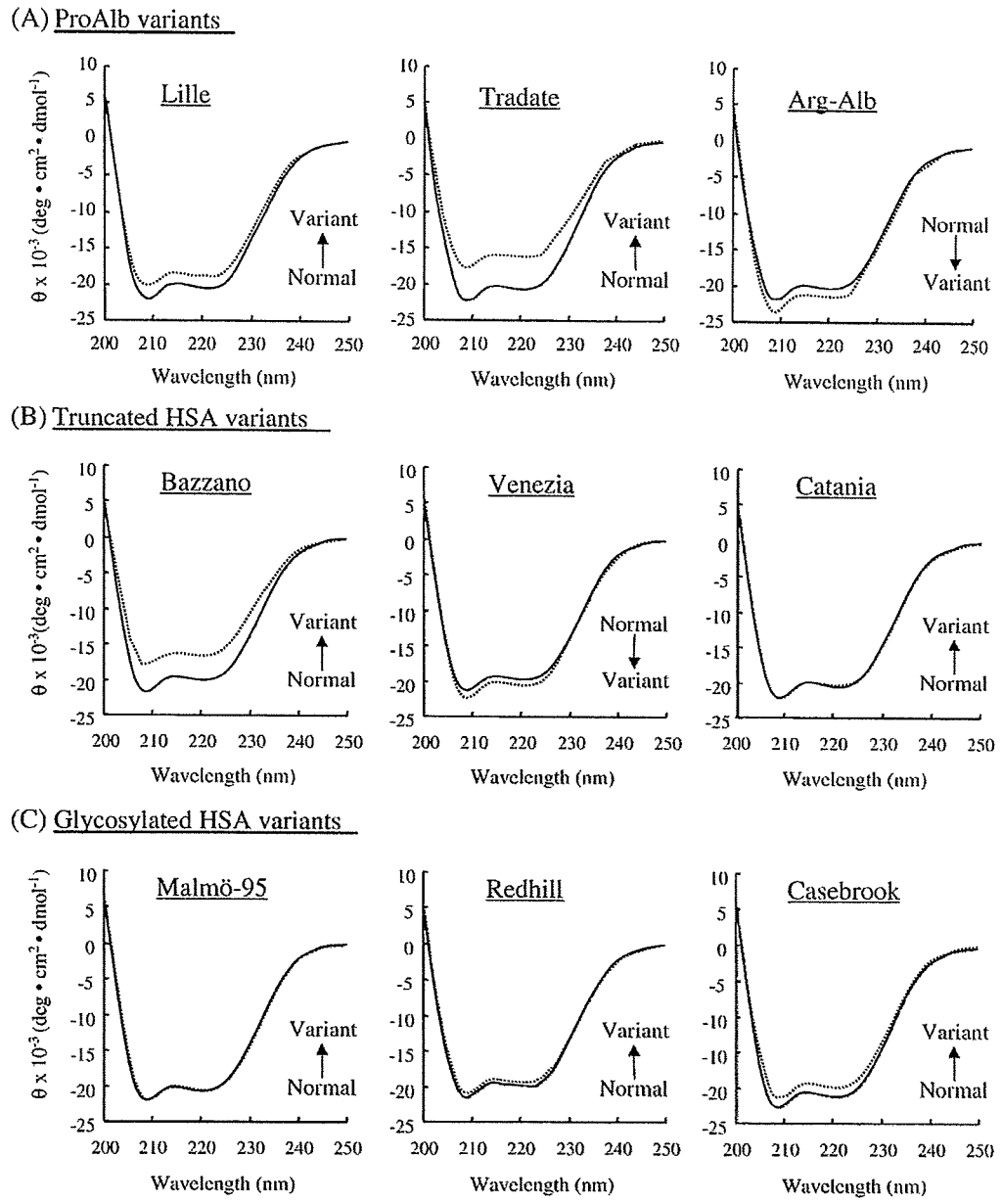

Fig. 3. Far-UV CD spectra of proAlb variants (A), truncated HSA variants (B) and glycosylated HSA variants (C) and their corresponding Alb A. The dotted and full curves represent variant and normal albumin, respectively. The curves are averages for three experiments.

In addition to change in chain-length and glycosylation we investigated whether the modified plasma half-lives could be correlated to other molecular parameters. Therefore, we estimated the effect of genetic variation on the α-helical content of albumin and related any changes to the changes in plasma half-life and organ uptakes. However, no clear correlation could be found between the changes in α-helical content and the different pharmacokinetic parameters (not shown). The changes in half-life and organ uptakes were also compared to mutation-induced changes in heat stability; quantified by using ΔH_v [15]. However, also in this case no correlations were found (not shown).

For being able to explain, at least in part, the modified plasma half-lives of the genetic variants we investigated their liver, kidney and spleen uptake clearances (Table 1). The diminished plasma half-lives of Alb Bazzano and Venezia are in full accordance with pronounced increases in liver, kidney and spleen uptake clearances. Likewise, the increased half-life of Alb Redhill can fully, or partly, be caused by diminished uptake by the three organs. Although most of the remaining variants have an increased liver clearance and a reduced spleen uptake individual results were obtained for these six alloalbumins. However, for all variants also other metabolic factors could be modified, see below.

Uptake of HSA by liver, kidney and spleen is mainly due to the presence of cell membrane receptors which recognize the protein and then internalize it by endocytosis. Some of these receptors interact with native protein, whereas other receptors interact with modified protein, and both types of interactions could be affected by genetic variation of albumin. Thus, binding of HSA to the membrane-bound receptor gp60 initiating a transcellular pathway for albumin across the endothelial cell wall to the underlying interstitium [34] could be affected. Sometimes, a glycosylated albumin variant looses one or both of the sialic acid residues on the antennae. Since the remaining of

the glycans has galactose and mannose units, these forms could interact with increased affinity with the galactosyl receptor-mediated (asialoglycoprotein receptor-mediated) endocytotic pathway of the hepatocytes and the mannose receptor-mediated endocytotic pathway of non-parenchymal cells in the liver. The liver also possesses receptors for rapid uptake of oxidized albumin and albumin with advanced glycation end products. Whether the present molecular changes can initiate endocytosis by scavenger receptors such as gp18, gp30, stabilin-1 or stabilin-2 is at present only speculative. However, liver uptake by adsorptive endocytosis could be influenced by the molecular changes, 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, part of the protein reabsorbed by the proximal tubule cells, most probably via the endocytic receptors megalin and cubulin, is degradated in the lysosomes. Whether genetic modification of HSA affects glomerular filtration and/or reabsorbtion of the protein remains to be clarified. However, since the size and charge of albumin influence glomerular filtration, especially the glycosylated variants could have an altered handling by the glomeruli. It should be noted that no radioactivity was detected in the urine during the present experimental period. An alternative, or supplementary, explanation for the modified uptake of the albumin isoforms by the kidney could be the presence of tubular RAGE which is known to cause the internalization of proteins with advanced glycation end products. Irrespective of the detailed mechanism, the net effect of deleting three or more C-terminal amino acids is an increased kidney uptake clearance (Tables 1 and 2). The effect of glycosylation depends on the position of attachment, because glycosylation of domain II (Alb Redhill) decreases uptake, whereas glycosylation of domain I (Alb Malmö-95) and domain III (Alb Casebrook) has no significant effect on kidney uptake.

Glycosylation diminishes uptake by the spleen (Tables 1 and 2). This is most evident for Alb Redhill, especially when taking into account that the presence of −1 Arg increases uptake. The presence of a modified propeptide also decreases clearance uptake. By contrast, the truncated variants with the most pronounced molecular changes (Alb Bazzano and Venezia) have significantly increased uptakes. Apparently, less is known about endocytosis-associated membrane receptors in the spleen than in the liver and kidneys. However, the organ most probably also has endothelial cells with the receptor gp60 [34]. In addition, it harbors scavenger receptors such as stabilin-1 and stabilin-2. To what extent albumin-receptor interactions are affected by the mutations remains to be elucidated.

More recently, another type of endocytosis of HSA has been identified in virtually all nucleated cells which results in reuse of the protein [35–37]. 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. Anderson et al. [35] have proposed that the intracellular binding of HSA to FcRn is due to a hydrophobic interaction, whereas Chaudhury et al. [36] suggested interaction(s) between histidine residue(s) in the receptor and histidine residues in domain III of albumin. By contrast, Andersen et al. [37] suggested that FcRn interacts with negatively charged and surface exposed residues on domain III of HSA. However, the authors cited agree that domain III of albumin seems to contain all of the FcRn binding activity, why especially genetic variants with domain III changes could have modified plasma half-lives due to a modified HSA–FcRn recycling process.

The metabolism of HSA can be affected by enzymes such as aminopeptidase(s) and carboxypeptidase(s) in the circulation. That has been observed in, for example, a patient with a severe traumatic injury, who had an increased activity of carboxypeptidase A resulting in hydrolysis of the C-terminal leucine and a fast elimination of albumin from the blood; the half-life was changed from ca. 19 days to less than 80 h [38]. In addition, modified albumin can be a substrate for endopeptidase(s) in the blood. Thus, oxidized bovine serum albumin, but not the native protein, can be cleaved by oxidized protein hydrolase [39]. Because the hydrolase is found in the blood, also this enzyme could hydrolyse some of the genetic variants in the mouse circulation and thereby render them more exposed to organ uptake.

In conclusion, the pharmacokinetics of HSA can be modified by changes in chain-length and by glycosylation. Three of the alloalbumins with a modified chain-length had a significantly shorter half-life in the circulation, whereas a glycosylated protein had an increased half-life (Table 1). These findings are useful when trying to construct an isoform with a modified stability in plasma. If an isoform with a shorter half-life is wanted, it is probably preferable to choose one with a C-terminally shortened chain-length rather than one with a N-terminally elongated chain-length, because the presence of a propeptide is known to block the high-affinity binding site for metal ions such as Cu^{++} and Ni^{++} [40]. It is a disadvantage to block this binding site, because albumin binding of Cu^{++} is an element in our anti-oxidative defense. Liver, kidney and spleen clearances were determined, and eight of the nine genetic variants had one or more modified organ uptakes. The uptakes measured are net effects of organ uptake leading to destruction in the lysosomes or to recycling via the FcRn receptor. The uptakes themselves and the two intracellular pathways could be affected differently by the protein modifications. The results revealed that if an increased uptake in the three organs is wanted for, for example, drug delivery systems, then truncated variants like Alb Bazzano and Venezia are good candidates. If the opposite is wanted, namely decreased uptakes in the organs, then the glycosylated Alb Redhill could be useful. Finally, we investigated whether blood half-lives or organ uptakes are directly related to mutation-induced changes in the proteins α-helical content or to changes in ΔH_v, representing thermal stability. However, that was found not to be the case.

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Review

Updates on Contemporary Protein Binding Techniques

Victor Tuan Giam CHUANG[1], Toru MARUYAMA[2,3] and Masaki OTAGIRI[2,4,*]

[1]*School of Pharmacy, Curtin University of Technology, Perth, Australia*
[2]*Department of Biopharmaceutics, Graduate School of Pharmaceutical Sciences, Kumamoto University*
[3]*Center for Pharmaceutical Sciences,Graduate School of Pharmaceutical Sciences, Kumamoto University, Kumamoto, Japan*
[4]*Department of Biopharmaceutics, School of Pharmacy, Sojo University, Kumamoto, Japan*

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Summary: Automated high-throughput techniques have become key to improving existing as well as new techniques associated with protein binding analysis. A wide variety of methods and experimental conditions are used for estimating protein binding as well as binding affinity, such as ultrafiltration and affinity chromatography. These methods rely either on the separation of a bound and free drug for subsequent conventional analysis or change in intrinsic parameters such as conformational properties of the protein. More recently developed techniques include surface plasmon resonance and solid-phase microextraction. Photoaffinity labeling, site-directed mutagenesis and x-ray crystallography are valuable techniques to identify the locations of binding sites on a protein. A new high-throughput assay based on the distribution of a drug among plasma water, plasma proteins, and solid-supported lipid membranes (Transil) has been reported to produce valid results, even for drugs that are strongly bound to plasma proteins. This new method may be suited for examining highly lipophilic drugs that adsorb onto surfaces due to their low solubility in aqueous media. Such a method may promote drug discovery and development for high-throughput determination of protein binding.

Keywords: protein bindings; albumin; binding affinity; techniques for binding studies; binding site

Introduction

Human serum albumin (HSA) is the most abundant protein in the circulatory system, and one principal function is the transport of endogenous substances and drugs. Binding to HSA not only controls the free, active concentration of a drug, but also affects the progress of some diseases. Co-administered drugs, food, especially lipids and pathological conditions, can modify the binding properties of HSA to a significant extent. This is especially crucial in cases where free concentrations of drugs that have a relatively narrow therapeutic index are affected.[1)]

Quantification of the binding of new chemical entities to a protein is an important early screening step during drug discovery and is of fundamental interest for estimating safety margins during drug development. Investigation of binding parameters has received significant attention since its importance was recognized at the beginning of the 20th century. New techniques emerge and old ones are being refined further to incorporate automated high-throughput screening, to make procedures more efficient with improved time and cost effectiveness. Interestingly, many recently developed drugs have been reported to bind strongly to serum protein (**Table 1**).

Determination of Binding Affinity

A wide variety of methods and experimental conditions may be used for estimating the degree of protein binding as well as binding affinity, such as equilibrium dialysis, ultrafiltration, ultracentrifugation, spectroscopic methods, affinity chromatography and electrophoretic methods. These methods rely on separation of bound and free drugs for subsequent conventional analysis or

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**To whom correspondence should be addressed:* Masaki OTAGIRI, Ph.D., *Department of Biopharmaceutics, Graduate School of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Kumamoto 862-0973, Japan. Tel.: +81-96-371-4150, Fax. +81-96-362-7690, E-mail: otagirim@gpo.kumamoto-u.ac.jp*
Abbreviations: HSA; human serum albumin, ED; equilibrium dialysis, RED; rapid equilibrium dialysis, HPFA; high-performance frontal analysis, SPME; solid-phase microextraction, SPR; surface plasma resonance, ITC; isothermal titration calorimetry

Table 1. Protein binding data of drugs approved in Japan recently

Drug	Percentage bound (%)	Drug	Protein binding rate (%)
Blonanserin	>99.7	Lamotrigine	53.1–56.2
Naratriptan	29	Pirfenidone	54–62
Sorafenib	99.5	Etravirine	approx. 99
Sitafloxacin	46–55	Maraviroc	approx. 67
Irbesartan	approx. 97	Nalfurafine	73.3–76.3
Deferasirox	approx. 99	Minodronic Acid	61.2–61.9
Diazoxide	90–93	Dasatinib	approx. 96
Sunitinib	95	Nilotinib	approx. 98
raltegravir	approx. 83	Rifabutin	93

change in intrinsic parameters such as conformational properties of the protein or spectroscopic properties of a drug-protein complex.

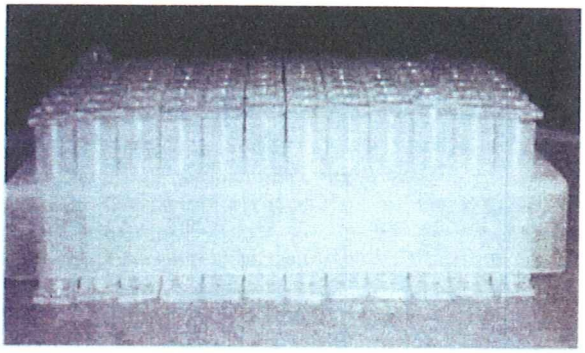
Quantification of free fraction

Equilibrium dialysis (ED) is the 'gold standard' for measuring plasma protein binding. Although it is the preferred method, other methods that involve different principles such as circular dichroism and fluorescence are also in use in studies on binding mechanisms.[2–4] ED is labour intensive, time consuming, cost prohibitive and difficult to automate. Another inherent shortcoming of this method is that volume shifts from the dialysate to the plasma side due to differences in osmotic pressure. Kragh-Hansen reported that the rate-of-dialysis technique is much faster and the primary binding constants determined are similar to those obtained by equilibrium dialysis. The results are less scattered and the technique is well suited for binding studies that involve high protein concentrations, because it is not affected by osmosis or Donnan effects.[5]

The standard ED system is not amenable to high throughput screening due to incompatibility with automated liquid handling systems currently in use. The development of a high throughput ED device in a 96-well ED assay format has been reported by Kariv *et al.* (**Fig. 1**). The apparent free fraction of three drugs, propranolol, paroxetine, and losartan, obtained using this 96-well format equilibrium dialysis plate has been found to correlate with published values determined by traditional equilibrium dialysis techniques.[6]

Banker *et al.* designed and constructed a 96-well equilibrium dialysis block that is reusable and can be shaken to facilitate dissolution of test compounds. This may lessen the time required to reach equilibrium. This dialysis apparatus aligns the dialysis membrane vertically in the well, to maximize the surface-to-volume ratio and permit the investigator to dispense and aspirate from either or both the sample and dialysate sides from the top of the apparatus via use of a robotic system. As a result,

A. 96-well dispo-equilibrium biodialyzer plate



B. Schematic representation of the compound sampling

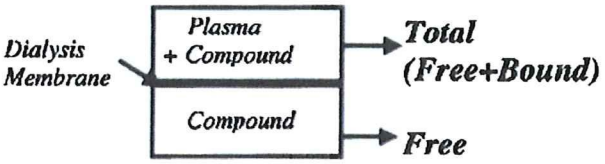


Fig. 1. 96-well equilibrium device (from reference 6)

the assay can be readily automated. Plasma protein binding values obtained for 10 diverse compounds using standard dialysis equipment and the 96-well dialysis block validate this method.[7]

The rapid equilibrium dialysis (RED) device with a shorter preparation and dialysis time, capable of being automated as a high-throughput assay for the determination of protein binding has recently been introduced by Pierce Biotechnology. Owing to the high membrane surface-to-volume ratio which allows for rapid dialysis, equilibrium can be reached in 4 hours with high levels of reproducibility and accuracy. Incubation time as little as 100 minutes is possible with proper agitation.[8]

Ultrafiltration is a relatively fast and simple method for obtaining protein binding data.[9] Despite the intrinsic problem of non-specific binding, ultrafiltration has been shown a technique capable of producing protein binding data similar to that obtained using ED.[10–12]

Continuous ultrafiltration has been developed for the rapid determination of the extent of binding of a drug to a protein. The results obtained with continuous ultrafiltration show good correlation with binding data reported in other reports.[13,14] However, this method is not able to provide binding constants. Xie *et al.* describe a new ultrafiltration method using drop coating deposition raman difference spectroscopy suitable for the detection of raman active compounds with dissociation constants in the order of 10 microM.[15]

Ultracentrifugation techniques involve simply spinning the sample to separate the protein-free phase from the protein-containing phase and, thus does not use a