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Research paper

Impaired drug-binding capacities of *in vitro* and *in vivo* glycated albumin

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ABSTRACT

Albumin, the major circulating protein in blood, can undergo increased glycation in diabetes. One of the main properties of this plasma protein is its strong affinity to bind many therapeutic drugs, including warfarin and ketoprofen. In this study, we investigated whether or not there were any significant changes related to *in vitro* or *in vivo* glycation in the structural properties and the binding of human albumin to both therapeutic drugs. Structural parameters, including redox state and ketoamine contents of *in vitro* and *in vivo* glycated purified albumins, were investigated in parallel with their affinity for warfarin and ketoprofen. High-performance liquid chromatography was used to determine the free drug concentrations and dissociation constants according to the Scatchard method. An alternative method based on fluorescence spectroscopy was also used to assess drug-binding properties. Oxidation and glycation levels were found to be enhanced in albumin purified from diabetic patients or glycated with glucose or methylglyoxal, after determination of their ketoamine, free thiol, amino group and carbonyl contents. In parallel, significant impairments in the binding affinity of *in vitro* and *in vivo* glycated albumin, as indicated by the higher dissociation constant values and confirmed by higher free drug fractions, were observed. To a lesser extent, this alteration also significantly affected diabetic albumin affinity, indicated by a lower static quenching in fluorescence spectroscopy. This work provides useful information supporting *in vivo* diabetic albumin could be the best model of glycation for monitoring diabetic physiopathology and should be valuable to know if glycation of albumin could contribute to variability in drugs response during diabetes.

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1. Introduction

Diabetes has reached pandemic proportions and affects around 6% of the world's adult population in 2006. This disease is considered to be one of the world's most important causes of expenditure, mortality, disability, and lost economic growth. Diabetes mellitus is characterized by an elevated level of glucose in the blood, which can affect the main circulating proteins through a non-enzymatic process known as glycation or glycoxidation. This condensation reaction involves circulating proteins and glucose and leads to the formation of Amadori products, which can give rise to irreversible conjugates called advanced glycation end products (AGE) in the case of unchecked hyperglycemia [1]. Elevated levels of these adducts induce irreversible damage associated with the metabolic disorders observed in diabetes mellitus, such as retinopathy, nephropathy, neuropathy, and coronary artery disease [2,3].

Among the blood proteins, hemoglobin and albumin are the most common proteins that are likely to be glycated. The glycated hemoglobin (HbA_{1C}) level is considered as one of the main clinical parameters used for monitoring chronic glycemic control *via* blood glucose measurements (Self-Monitoring of Blood Glucose, SMBG) [4]. With a shorter half-life than hemoglobin in blood, glycated human serum albumin (HSA) appears to be an alternative marker for glycemic control as it can indicate blood glucose status over a short period (2–4 weeks) [5,6]. *In vivo*, the proportion of glycated albumin in healthy people is in the range of between 1% and 10% [7,8], and in the case of diabetes mellitus, this proportion can increase two- to three-fold [9].

Human serum albumin (HSA), as the most prominent protein in plasma, is the major carrier of various endogenous and exogenous metabolites in blood throughout the body. This plasma protein could serve as a circulating depot for many drugs [10]. In particular, it could play a major role in affecting therapeutic drug absorption, distribution, metabolism, and excretion [11]. Indeed, binding to human

Abbreviations: AGE, advanced glycation end-products; HSA, human serum albumin; HSA-ND and HSA-D, purified human serum albumin from non-diabetic and diabetic patients, respectively; HbA_{1C}, glycated hemoglobin level; PBS, phosphate-buffered saline; SMBG, self-monitoring of blood glucose; TNBS, 2,4,6-trinitrobenzenesulfonic acid.

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albumin increases the lifetime of a drug before it is metabolized, and it also allows some hydrophobic drugs to become more soluble in blood [12,13]. After delivery by albumin, unbound drugs could easily reach target organs through the plasma. In contrast, bound drugs can hardly reach the action site through the blood without losing their pharmacological activities and toxicity. Among the therapeutic drugs, some have a narrow therapeutic index, such as warfarin, and are easily toxic in the unbound state. Indeed, it was established that Minimal Effective Concentration (MEC) of warfarin is comprised between 1 and 7 μg/mL, while its Minimal Toxic Concentration (MTC) is above 10 μg/mL [14]. Some pathological states, which impair liver or renal functions, triggering hypoalbuminemia, could increase the effects of drugs highly bound to albumin [15].

Albumin has two major binding sites for drugs called Sudlow site I and Sudlow site II [16]. Because of its large size and adaptability, site I tends to bind bulky heterocyclic compounds including sulfonamides, salicylate and coumarin compounds such as warfarin [17,18]. In contrast, site II, which is smaller and less flexible, induces the more stereospecific binding of aromatic carboxylic acids and profens such as ibuprofen or ketoprofen [19,20]. The interaction of drugs with albumin can be affected by modifications of the protein such as glycation or oxidation [21,22]. Several reports have found that the main glycation reactions occur near both Sudlow sites [23,24]. But conflicting results have been reported on the impact of the glycation of albumin on its affinity to several drugs including warfarin [11,25–27]. These contradictory findings have resulted from several differences in: the mode of albumin purification, the way that *in vitro* glycation is performed, and the methods used for the measurement of ligand binding. These previous studies highlight the importance of having accurate knowledge of the preparation of *in vitro* glycated albumin models and the necessity of using an *in vitro* model of albumin with biochemical characteristics typical of those expected of serum albumin in diabetes.

Few studies have reported the binding affinities of albumin *in vivo*, in case of chronic hyperglycemia, for many therapeutic drugs. Regarding these previous findings, the drug-bindings properties of *in vitro* glycated albumin models should be investigated and compared with glycated albumin from diabetic patients. This drug-binding study should be performed on three different *in vitro* glycation models. Two of them, which are common glycated albumin models, are considered controversial and use suprapathological concentrations of glucose (100 mM) or methylglyoxal (10 mM). The third model, which uses a pathological concentration of glucose (25 mM), is more typical of diabetic albumin. The binding parameters of these *in vitro* models should be compared with native and diabetic HSA. Two therapeutic drugs were selected for this study. Warfarin, an anti-coagulant drug, and ketoprofen (Fig. 1), a non-steroidal anti-inflammatory drug with analgesic and antipyretic effects, were chosen as site-selective probes for Sudlow sites I and II, respectively.

The aim of this study is to identify differences between *in vitro* and *in vivo* modified albumin models through the determination of their binding properties for two probes. The warfarin and ketoprofen binding characteristics obtained for our different albumin preparations, by using high-performance liquid chromatography and fluorescence spectroscopy, as well, could provide a better knowledge of albumin structural modifications during its glycation process.

2. Material and methods

2.1. Chemicals and reagents

Aqueous methylglyoxal solutions (40%), D-glucose, anti-dinitrophenyl hydrazine (DNPH) antibody, the 3,3′,5,5′-tetramethyl-benzidine liquid substrate (TMB) system for ELISA, warfarin and ketoprofen were all purchased from Sigma.

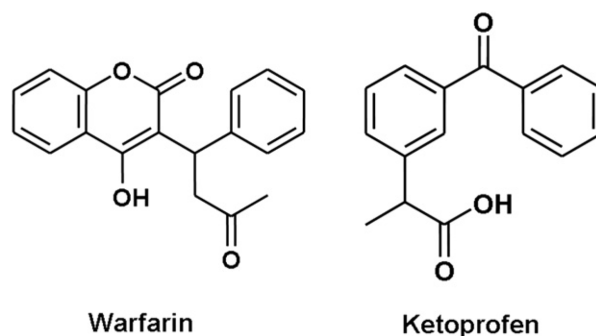

Fig. 1. Structures of warfarin and ketoprofen.

2.2. Human albumin purification

Blood was obtained from a pool of diabetic patients and non-diabetic subjects and anticoagulated in EDTA tubes (BD Vacutainer) at the Biochemistry laboratory of the Centre Hospitalier Universitaire (CHU, Saint-Denis, La Réunion). The procedures and the collection of human materials were approved by the local governmental French Ethical Committee. The albumin was purified using pooled serum from 39 diabetic (% $HbA_{1C} = 11.0 \pm 1.9$) and 14 non-diabetic (% $HbA_{1C} = 5.5 \pm 0.4$) patients. Pool of diabetics includes patients with both types of diabetes. Patients with a hemoglobin abnormality and/or hyperleukocytosis were excluded from the pool of plasma. All biological data related to both pools are given in Table 1.

The purification of serum albumin from fresh human plasma was based on extensive dialysis against Tris/HCl (pH 7.4; 50 mM) followed by affinity chromatography using Cibacron Blue 3G linked to agarose (Amersham cat#17-0948-01) as a ligand for the albumin. A NaCl (pH 7.4; 1.5 M) buffer was used for the desorption of bound albumin from Cibacron Blue-agarose following the elution of other plasma proteins with a Tris/HCl (pH 7.4; 50 mM) buffer. Each fraction of eluate was examined by gel electrophoresis and the most purified and concentrated extracts were pooled before the remaining immunoglobulins were removed with sepharose-protein A, followed by extensive dialysis against PBS and then storage at 80 °C.

2.3. Preparation of in vitro glycated HSA

Advanced glycation end products (AGE) were prepared as previously described [28] by incubating 0.37 mM purified HSA from a non-diabetic pool (HSA-ND) without and with glucose (25 mM or 100 mM) in PBS, pH 7.4, under sterile conditions, at 37 °C for 3 weeks, or with methylglyoxal (10 mM) for 2 days. The proteins

Table 1
Clinical and biochemical characteristics of diabetic and non-diabetic patients and plasma samples.

Compounds	Non diabetic (ND)	Diabetic (D)
N (females)	14 (7)	39 (16)
Age	33 ± 9	51.5 ± 20.1**
HbA1c (%)	5.5 ± 0.4	11.0 ± 1.9***
Fructosamines (ng/mL)	218.3 ± 10.9	390.0 ± 131.5***
Creatinin (μmol/L)	89.8 ± 12.3	88.0 ± 16.2
Albumin (g/L)	46.4 ± 2.6	38.8 ± 5.5***
Total protein (g/L)	73.8 ± 4.0	67.7 ± 7.7**
CRP (mg/L)	2.6 ± 2.5	7.0 ± 8.0*
Cholesterol (mmol/L)	4.6 ± 0.7	4.5 ± 1.3
Triglycerides (g/L)	0.9 ± 0.4	1.6 ± 1.0***

Data are expressed as means ± SD and were compared using the Student's *t* test for unpaired samples; ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

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were dialyzed against PBS, sterile-filtered through a 0.2 μm Millipore filter and stored at −80 °C.

2.4. Structural analysis of modified albumins

Fructosamine and ketoamine derivatives, useful index of diabetic control, were determined using the method developed by Johnson et al. [29] with the nitroblue tetrazolium (NBT) reagent. The thiol groups in native, modified albumin were measured by Ellman's assay using 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) [30], which was well described in a previous study [31].

The 2,4,6-trinitrobenzenesulfonic acid (TNBS) assay is a sensitive method for determining the primary free amino groups in proteins [32]. This method was described in detail in a previous study by our group [33]. The degree of carbonylation of glycoxidized albumin was determined by the carbonyl ELISA assay based on the recognition of protein-bound DNPH in carbonylated proteins with an anti-DNP antibody. This method was described in detail in previous studies by our group [33,34].

2.5. Affinity study: experimental design for the HPLC method

The experimental design for this study drew its inspiration from a work conducted by Olsen about the drug-binding capacity of pharmaceutical grade albumins [35], and is described below.

Stock solutions of warfarin (100 mM) and ketoprofen (100 mM) were prepared in NaOH (0.1 M). A standard range of concentrations from 0.01 to 2 mM was prepared from these stock solutions in PBS 1X, for both drugs.

The samples were prepared in Eppendorf tubes, where 150 nmol of proteins of different albumin samples were incubated at 37 °C, for 30 min with 60, 80, 100, 120, 130, 140, 150, 160, 1500 and 3000 nmol of warfarin or ketoprofen. After incubation, the fractions of free drugs and drugs bound to albumin were separated by ultrafiltration using the Amicon© Ultra system (Millipore) and centrifugation for 20 min at 13,000 rpm. The percentage of free drug fractions recovered using this method was about 93% (±2.1).

Then, the unbound (free) drug concentrations were determined by high performance liquid chromatography (HPLC), coupled to a UV spectrophotometer. A solution of acetonitrile–Tris–HCl (pH 7.4; 50 mM) (35:65, v/v) was used as the mobile phase, delivered at a flow rate of 1 mL/min in a LiChrospher 100 RP-18 column (250 × 4.6 mm i.d., s-5, 5 μm) (Merck, Darmstadt, Germany) at 30 °C. The pump was maintained at 147 bars and the analysis was performed on 50 μL of each sample. The measurements took place at 254 nm.

2.6. Affinity study: experimental design of the fluorescence spectroscopy method

This method is based on the quenching of albumin fluorescence induced by its interaction with drugs [36]. The intrinsic fluorescence of human albumin is mainly attributed to the tryptophan residue (Trp-214). Different series of assay solutions were prepared by mixing 10 μM of native or diabetic HSA with warfarin or ketoprofen at variable concentrations ranging from 5 to 70 μM. Each solution was heated for 5 min at 37 °C and transferred into a quartz cell. The fluorescence spectra were recorded in the range of 250–500 nm under excitation at 283 nm. The binding parameters (*i.e.* the binding constant K_A and the binding site number n) for both drugs were obtained from the equation given below [37]:

$$\log\frac{(F_0 - F_C)}{F_C} = \log K_A + n\log[C]$$

where F_0 and F_C are the tryptophan fluorescence intensities in the absence and presence of a drug at concentration $[C]$, respectively, and K_A is the formation constant of the complex formed between the drug and albumin, expressed as L/mol.

2.7. Statistical analysis

The data are expressed as the means ± standard deviation (SD) from a minimum of three experiments. Statistical significances were determined using one-way ANOVA (followed by the Student's *t* test) for multiple comparisons; a p value of less than 0.05 was required for significance.

3. Results

3.1. Biochemical characterization of the glycated HSA samples

Three *in vitro* models of glycated albumin were prepared and compared with native and *in vivo* diabetic albumin. The HSA-ND_{G25} and HSA-ND_{G100} models were prepared with 25 mM and 100 mM of glucose, while the HSA-ND_{MGO} model corresponded to HSA incubated with 10 mM of methylglyoxal, a side-product of different metabolic pathways, such as the degradation process of glucose. The group of Thornalley has shown that physiological glycation processes also involve the modification of proteins by reactive α-oxoaldehydes such as methylglyoxal. These derivatives could also be formed during glucose-induced protein glycation [38]. At the first step of glycation process, glucose modification of albumin gives rise to the formation of fructosamine while methylglyoxal induces the generation of another ketoamine derivative named as early glycation product.

As shown in Table 2, the glycated albumin obtained under typical hyperglycemia conditions (HSA-ND_{G25}) had a lower level of ketoamine, of 5.07 (±0.35) mol/mol HSA, than the other *in vitro* glycated albumin models. These albumin models were prepared with suprapathological concentrations of glucose (HSA-ND_{G100}) or methylglyoxal (HSA-ND_{MGO}) and contained 12.43 (±1.88) and 9.25 (±1.46) mol of ketoamine/mol HSA, respectively. If its ketoamine level is significantly higher (2.13 ± 0.58 mol) than that of native albumin (HSA-ND_{G0}) (1.11 ± 0.12 mol), diabetic HSA (HSA-D) has

Table 2
Oxidation parameters in various albumin preparations.

Methods	Ketoamine/HSA (mol/mol)	Thiols/HSA (mol/mol)	Free amino groups/HSA (mol/mol)	Carbonyl rate increase (%/control)
Purified HSA-ND				
HSA-ND_{G0}	1.11 ± 0.12	0.609 ± 0.013	13.36 ± 0.98	100.00 ± 7.35
HSA-ND_{G25}	5.07 ± 0.35***	0.333 ± 0.067**	12.44 ± 0.83	111.42 ± 10.89
HSA-ND_{G100}	12.43 ± 1.88***	0.286 ± 0.011***	10.42 ± 0.20**	224.84 ± 13.93***
HSA-ND_{MGO}	9.25 ± 1.46***	0.250 ± 0.026***	5.10 ± 0.54***	455.67 ± 69.68***
Purified HSA-D				
HSA-D_{G0}	2.13 ± 0.58*	0.307 ± 0.012***	10.38 ± 0.80*	95.66 ± 3.90**

1) Ketoamine levels obtained with the NBT assay; 2) free thiol group content as assessed by Ellman's method; 3) primary amino group contents in proteins by the TNBS assay; 4) percentage carbonyl rate as assessed by the ELISA carbonyl assay. All data are expressed as means ± SD from three independent experiments.
Effect of glycation or diabetes on purified HSA (*vs.* HSA-ND_{G0}); ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

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a very low level of glycation in comparison to HSA-ND$_{G25}$. The relatively lower ketoamine level of HSA$_{MGO}$, in comparison to HSA-ND$_{G100}$ could be explained by a higher reactivity of methylglyoxal, which gives rise to a more rapid formation of AGE.

Several oxidative parameters in these albumin preparations are also reported in Table 2. As expected, the levels of free thiol and amino groups significantly decreased in albumin with *in vitro* glycation by glucose or methylglyoxal and *in vivo* glycation, whereas the carbonyl level increased, except in diabetic albumin. The unexpected higher carbonylation level for HSA-NDG0, in comparison with diabetic albumin, could be partially explained by the 3 weeks incubation of native HSA at 37 °C (diabetic albumin did not undergo this incubation step). During this incubation time, oxidation process could occur.

This oxidative state was more marked in the case of methylglyoxal-modified HSA. Similarly, the rate of increase of carbonyl, attributed to the enhanced oxidation of albumin with *in vitro* modification, was more marked with methylglyoxal than with glucose. As expected, the oxidative parameters observed for diabetic HSA corresponded to those obtained for albumin modified with 25 mM of glucose. Both types of albumin displayed quite similar global net charges, as attested by the electrophoretic migration profile in native page electrophoresis (data not shown).

3.2. Binding of both drugs to glycated albumins

As shown in Fig. 2a and b, warfarin and ketoprofen were highly bound to native human albumin with unbound drug fractions of 3.9 and 2.7%, respectively. As noticed for both drugs, the free fractions were higher for both *in vitro* and *in vivo* glycated albumins. This increase, which was more marked for HSA-ND$_{MGO}$, was by approximately eight and seven times for warfarin and ketoprofen, whereas the diabetic albumin exhibited a free drug fraction of around twice higher as well for warfarin (7.3%) as for ketoprofen (5.4%) in comparison with native albumin.

Human albumin, glycated or not, has a lower affinity for ketoprofen than for warfarin. Deeper analyses were performed to obtain more information on the binding parameters for different glycated HSA samples with both drugs. Fig. 3 shows the results of the binding studies, in a Scatchard plot form, at various concentrations of warfarin (Fig. 3a) and ketoprofen (Fig. 3b) with HSA-ND$_{G0}$, HSA-ND$_{MGO}$, and HSA-D. The Scatchard plot was performed over a range of drug concentrations, giving an r value (mol number of bound drug per mol of albumin) of between 0.4 and 1.1, corresponding to the specific binding study. As seen in Fig. 3, the drug-binding yielded straight lines with correlation coefficients (r) above 0.985, which could indicate the number of specific binding sites (n) and the dissociation constant (K_d). These binding parameters for warfarin and ketoprofen are summarized in Table 3. The smaller is the dissociation constant, the stronger is the binding between the ligand (drug) and the receptor (albumin). As observed for previous unbound drug results, albumin exerted a higher affinity for ketoprofen (K_d = 6.98 μmol/L) than for warfarin (K_d = 9.52 μmol/L). These results also showed significant variations between native HSA and *in vitro* or *in vivo* glycated HSA. For both drugs, an increase in K_d values was observed with the *in vitro* glycation of albumin, which was more marked for HSA-ND$_{MGO}$, indicating a loss of affinity. The binding affinity for warfarin was reduced, according to an increase in K_d values and the number of binding sites (between 1.55 and 2.80). In contrast, the binding affinity for ketoprofen was affected, with glycation, but without significant changes in the number of binding sites (between 1.43 and 1.67). Similar to *in vitro* glycated albumin, but to a lesser extent, *in vivo* glycated albumin from diabetic patients had a lower affinity for both drugs with elevated dissociation constants for ketoprofen (K_d = 9.98 μmol/L) and warfarin (K_d = 11.74 μmol/L). This partial loss of affinity was not accompanied by an increase in the number of binding sites (around 1.3 for both drugs).

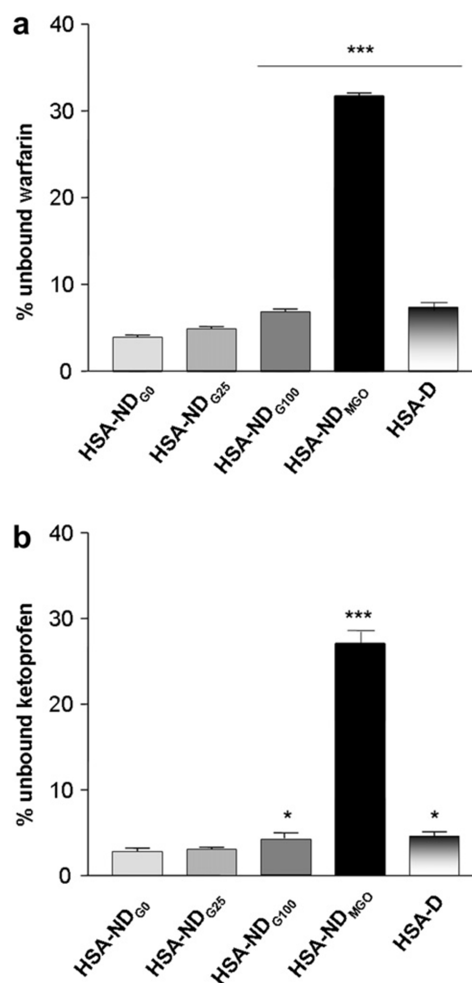

Fig. 2. Percentage of unbound warfarin and ketoprofen concentrations in various albumin preparations. All data are expressed as means ± SD from three independent experiments. *Effect of glycation or diabetes on purified HSA (vs. HSA-ND$_{G0}$); ***$p < 0.001$, *$p < 0.05$.

3.3. Binding of both drugs to in vivo glycated albumin

In order to strengthen the previous findings, the interactions between warfarin and ketoprofen and the native and diabetic HSA were also investigated using an alternative method based on fluorescence quenching. Fluorescence quenching corresponds to a process that reduces the intensity of fluorescence. As shown in the fluorescence emission spectrum of Fig. 4a and b, the maximum fluorescence intensity of native albumin at 337 nm, attributed to tryptophan, decreased with increasing concentrations of warfarin and ketoprofen. Both drugs acted as quenchers *via* interactions with the protein. However, the maximum tryptophan emission wavelength of around 337 nm for native HSA without drugs (thick lines) was not affected in the presence of warfarin or ketoprofen. The log plots ($(F_0 - F_C)/F_C$) of HSA-ND$_{G0}$ and HSA-D *versus* drug concentration (log [C]) are shown in Fig. 5a for warfarin and Fig. 5b for ketoprofen. These plots exhibit a good linearity with the correlation coefficients (r) above 0.986 (Table 4). The numbers of binding site n and the binding constants K_A, listed in Table 4 for warfarin and ketoprofen, were calculated using these plots. The

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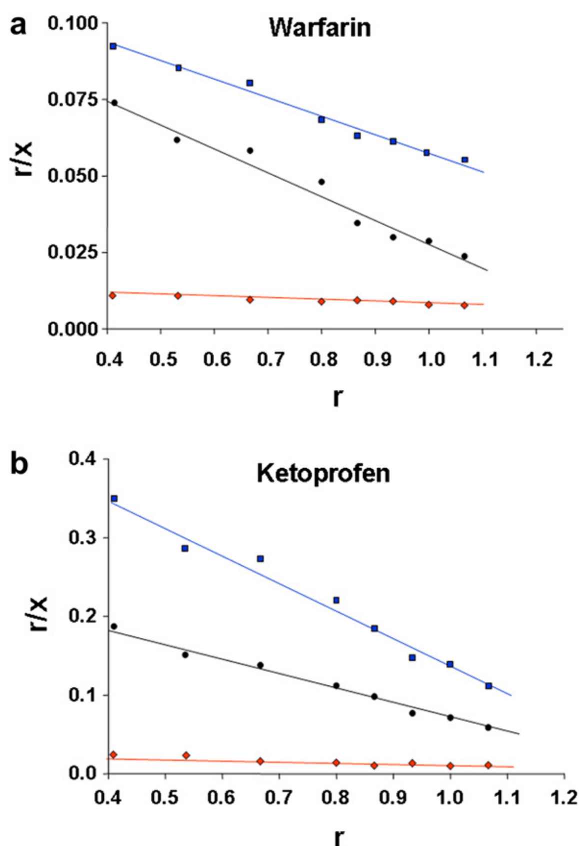

Fig. 3. Scatchard plots of warfarin and ketoprofen in various albumin preparations. Scatchard plot of: (a) warfarin and (b) ketoprofen binding in native human albumin HSA-ND_{G0} (■), *in vitro* glycated albumin HSA-ND_{MGO} (◆) and diabetic albumin HSA-D (●).

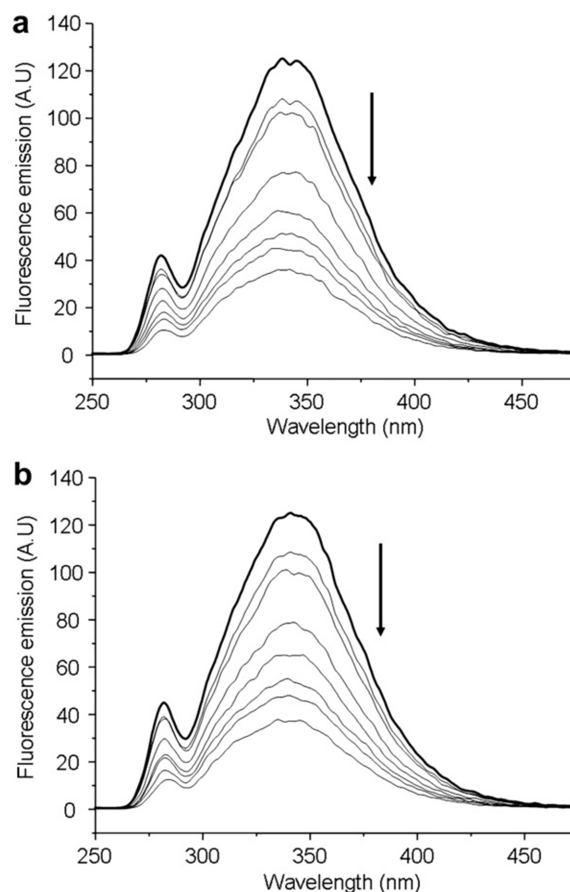

Fig. 4. Effects of warfarin and ketoprofen on the fluorescence emission spectrum of native HSA (HSA-ND_{G0}). Native albumin at 10 μM was incubated without (thick curve) and with different concentrations of (a) warfarin and (b) ketoprofen of between 5 and 70 μM at 37 °C for 5 min.

binding site n corresponds to the slope and log K_A to the intercept. The binding constant relative to native HSA with ketoprofen ($K_A = 6.15 \times 10^6$ L/mol) is higher than the one calculated for warfarin ($K_A = 2.23 \times 10^6$ L/mol), as was found with the HPLC method. In comparison to the native HSA, a decrease in the binding constant of diabetic albumin can be observed, which dropped considerably with both warfarin (120 times lower) and ketoprofen (200 times lower). These results suggest a partial loss in the affinity of diabetic albumin for both drugs, which was confirmed by the reduced number of binding sites to around $n = 1.03$ ($n = 1.08$) with the *in vivo* glycation of warfarin (ketoprofen) in comparison to the native albumin, which had around $n = 1.44$ ($n = 1.55$) binding sites.

Table 3
Binding parameters of warfarin and ketoprofen in various albumin preparations.

	Dissociation constant K_d (μmol/L)		Specific binding sites (n)	
	Warfarin	Ketoprofen	Warfarin	Ketoprofen
Purified HSA-ND				
HSA-ND_{G0}	9.52 ± 0.57	6.98 ± 1.49	1.55 ± 0.14	1.43 ± 0.1
HSA-ND_{G25}	13.3 ± 1.64**	6.65 ± 2.19	1.60 ± 0.02	1.49 ± 0.02
HSA-ND_{G100}	24.0 ± 4.14***	14.3 ± 5.07*	1.99 ± 0.06	2.10 ± 0.07
HSA-ND_{MGO}	120 ± 24.1***	58.5 ± 4.57***	2.80 ± 0.04	1.67 ± 0.13
Purified HSA-D				
HSA-D_{G0}	11.74 ± 1.46*	9.98 ± 1.86*	1.31 ± 0.1	1.35 ± 0.1

The binding studies with warfarin and ketoprofen were performed with 0.5 mM albumin samples. The binding parameters: specific binding site (n) and dissociation constant K_d were calculated according to Scatchard. All data are expressed as means ± SD from four or five independent experiments.
Effect of glycation or diabetes on purified HSA (*vs.* HSA-ND_{G0}): $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$.

These binding site numbers are listed in Table 4 and are quite similar to those obtained by the HPLC method.

4. Discussion

Uncontrolled hyperglycemia in severely diabetic patients is the main cause of the advanced glycation process of plasma proteins such as hemoglobin and albumin. These glycation-induced structural and functional changes in albumin are of particular interest because numerous *in vivo* studies have reported the strong involvement of glycated albumin in the development and progression of chronic diabetic complications [2,3]. Furthermore, this enhanced glycation process could potentially contribute to an alteration of the binding properties of albumin for many drugs, with a consequent change in the pharmacokinetic and pharmacodynamic properties of these drugs. There are several causes for the decreased binding of albumin, such as high concentrations of bilirubin or free fatty acids, which can displace drugs from the binding sites of albumin, or also hypoalbuminemia [39]. This way, glycation is one of several mechanisms that could induce an increase in unbound circulating fractions of many albumin-bound drugs. The unbound drug, which is considered to be pharmacologically active, appears to be responsible for dose-related therapeutic effects and also the adverse drug reactions. Glycation, in the diabetic context, could induce an alteration in the pharmacological properties of albumin for many drugs associated with adverse consequences. This effect of glycation on albumin affinity could be more relevant

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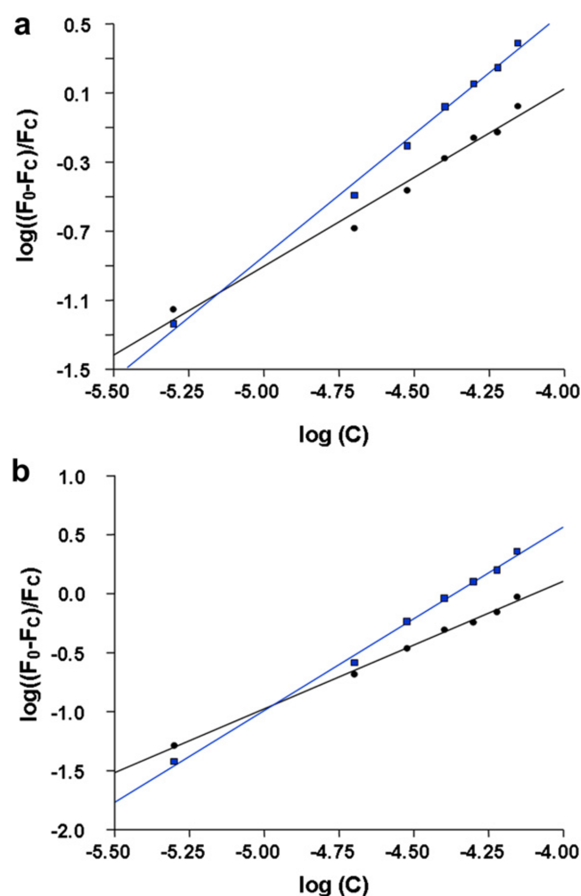

Fig. 5. The log plots $((F_0 - F_C)/F_C)$ *vs.* log $[C]$ for warfarin and ketoprofen binding with native and diabetic albumin. F_0 and F_C are the tryptophan fluorescence intensities of native albumin, HSA-ND$_{G0}$ (■) and diabetic albumin, HSA-D (●) in the absence and presence of (a) warfarin or (b) ketoprofen at different concentrations $[C]$.

for drugs that are highly bound (>95%) to albumin and have a low therapeutic index, such as warfarin. Warfarin is an anti-coagulant with a very low therapeutic index, which could trigger severe hemorrhaging if the correct degree of its pharmacological effect is not maintained. In contrast to warfarin, ketoprofen, a common medication for the treatment of inflammation or pain, has a wide therapeutic index. Major studies focused on the impact of glycation on the binding properties of albumin for therapeutic drugs were exclusively based on *in vitro* models of glycation, which do not necessarily meet the biological conditions expected in the context of diabetes. Moreover, among these numerous studies, some of them highlighted conflicting findings about the effect of glycation on the interaction between albumin and warfarin. For instance, in one study, the affinity of warfarin for the binding site I was shown to be unaffected in the case of human albumin glycated with glucose (2 mol glucose/mol albumin) [22], whereas a decrease in binding with early stage glycated albumin, which was attributed to conformational changes or to steric hindrance of the protein, was reported in other studies [40,41]. In contrast, an excessive glycation (60 days) of albumin with a large amount of glucose (9 mol glucose/mol albumin) was shown to enhance warfarin binding to recombinant albumin [26]. Only a few studies have focused on the drug-binding properties of *in vivo* glycated HSA.

Table 4
Binding parameters of warfarin and ketoprofen in native and diabetic albumin preparations obtained by spectroscopy.

	Binding constant K_A (L/mol)	Binding sites (n)	Correlation coefficient (r)
Warfarin			
HSA-ND$_{G0}$	2.23×10^6	1.44 ± 0.10	0.988
HSA-D$_{G0}$	1.77×10^4	1.03 ± 0.07	0.986
Ketoprofen			
HSA-ND$_{G0}$	6.15×10^6	1.55 ± 0.05	0.995
HSA-D$_{G0}$	2.97×10^4	1.08 ± 0.03	0.989

The binding studies of warfarin and ketoprofen were investigated with 10 μM albumin samples. The binding parameters: binding site number (n) and binding constant K_A were calculated as described in the Materials and methods section.

In this study, we first characterized our different glycated albumin models (*in vitro* and *in vivo*) by checking their redox status *via* their thiol and carbonyl contents and by assessing the glycation level through ketoamine and TNBS assays. Then, we investigated the interaction between two therapeutic drugs and modified albumin models by using two completely different techniques.

Three *in vitro* glycation models were characterized and compared with unmodified albumin (native) and *in vivo* glycated albumin purified from a pool of plasma from diabetic patients with very high glycated hemoglobin levels (HbA$_{1C}$ = 11.0% ± 1.9). The HSA-ND$_{G25}$ model, prepared using pathological concentrations of glucose, closely reflects the conditions found in diabetes, whereas HSA-ND$_{G100}$ and HSA-ND$_{MGO}$ are widely used models that greatly differ from this pathological condition. As expected, both suprapathological glycation models displayed a considerable level of glycation associated with an increased oxidative state. Furthermore, compared to the data obtained for *in vivo* glycated HSA (HSA-D), *in vitro* glycated HSA with 25 mM of glucose (HSA-ND$_{G25}$) appeared to be the most relevant *in vitro* model for mimicking hyperglycemia or the diabetes model. However, our *in vivo* glycated albumin remained the most suitable model for drug-binding studies.

The affinity of albumin for both therapeutic drugs was also evaluated with the help of binding parameters (dissociation constant and the number of binding sites) and the determination of free drug concentrations. The unbound fractions of warfarin and ketoprofen were measured by HPLC after ultrafiltration. An alternative would have been to perform an equilibrium dialysis instead of ultrafiltration in order to recover the unbound drug fraction. Lagrange et al. reported that both experimental modes generated quite similar data in the binding capacity of ketoprofen for HSA [20].

Although the experimental conditions used in our study were quite similar to those used by Olsen et al. [35], there were some differences in the results obtained for the interaction between native HSA and warfarin. Indeed, the free fraction of warfarin was five times higher (3.9%) than found by Olsen et al. in purified human albumin (0.7%). This difference could be attributed to the mode of purification of albumin in our study. After the chromatographic purification and immunoglobulin removal steps, our albumin samples were not defatted. Free fatty acids can reduce the binding affinity of albumin when they are bound to albumin. Besides this, the present study demonstrated that the increase in free drug fractions found for warfarin and ketoprofen with the glycation process was in agreement with the binding parameter values obtained by the Scatchard method. In parallel with an eightfold increase in the free warfarin fraction for the HSA-ND$_{MGO}$ model (*vs.* native HSA), there was an eleven-fold decrease in the strength of this binding, as indicated by the dissociation constant values. The significant decrease in binding for the most *in vitro* glycated albumins could be explained by the major structural changes in HSA after glycation. In particular, the tertiary structure conformation, as detected by tryptophan fluorescence, was significantly affected by glycation (data not shown). This structural modification has been shown to be accompanied by a partial opening of hydrophobic

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pockets, as detected by 1-anilinonaphthalene-8-sulfonate (ANS) fluorescence [42], among which are hydrophobic cavities in the subdomains IIA and IIIA of albumin, where Sudlow sites I and II are located. As expected, the glycation of albumin under conditions that most closely resemble those that occur in the blood during hyperglycemia led to fewer changes in the binding of both drugs compared to the suprapathological models. The reduced affinities for warfarin and ketoprofen observed in these latter glycation models featured conformational changes in the Sudlow binding sites I and II, respectively. These impairments, which occurred during the glycation process, resulted in an increased binding site number. Results in the literature have reported that warfarin and ketoprofen only bind to one highly specific binding site (Sudlow sites I and II, respectively) [16]. In this study, specific binding site values of between 1.43 and 1.55 were calculated, indicating the existence of a secondary binding site with a lower affinity. Several authors have suggested the existence of a secondary binding site for warfarin [43,44] and ketoprofen [20]. The increase in the number of binding sites for highly glycated albumin could indicate a displacement of drugs from their changed primary binding sites to binding sites with a lower specificity. In a recent study, we showed that albumin tertiary structure of albumin was more affected by methylglyoxal-induced glycation than by glucose [45]. We observed a drastic alteration in albumin tertiary structure associated with change in the protein hydrophobicity. This alteration could impair affinity of the Sudlow site I for warfarin in albumin and could lead to formation of a new unspecific binding site having low affinity.

Moreover, regarding diabetic albumin, our study highlights interesting findings. Indeed, the specific binding of warfarin was significantly reduced in diabetic albumin and was quite similar to the glycation model most typical of the conditions in diabetes ($HSA\text{-}ND_{G25}$). This partial loss of affinity was confirmed by a higher free warfarin fraction, which was more marked for diabetic albumin. This downward trend was also found for ketoprofen, except for $HSA\text{-}ND_{G25}$, which did not show a significant difference to native albumin with respect to the binding properties. Thus, these results are in agreement with a marked increase in the free fractions of both drugs, which were nearly doubled between the diabetic and native albumin. It could be hypothesized that, in the case of diabetes, this minor loss in affinity could significantly impact the biological responses of many drugs highly bound to albumin such as warfarin or ketoprofen. Up to now, no study has established any clinical relevance for this hypothesis in human pharmacology context. Warfarin constitutes a drug exhibiting narrow therapeutic window and important inter-individual and inter-ethnic differences in the dose required for its anticoagulation effect [46]. The main factors (40%) contributing to this inter-individual variation remain unknown [47]. Therefore, it is at utmost importance to determine the contribution of albumin modifications in these factors.

The effect of glycation on the interaction between both drugs and albumin was determined using the fluorescence quenching technique. The interaction of warfarin or ketoprofen with albumin molecules leads to a quenching of tryptophan fluorescence. There are many reasons to account for such quenching, including molecular rearrangement or a change in the microenvironment around tryptophan as a result of an unfolding process. For instance, the glycation process of albumin leads to a quenching of tryptophan fluorescence, as reported in numerous studies [9,33,45]. It can be noted that the sole tryptophan residue of human albumin is located in subdomain IIA near Sudlow site I [48]. In these interactions between the quencher (warfarin or ketoprofen) and the fluorophore (albumin), fluorescence quenching can occur *via* two different mechanisms: static and dynamic, respectively [49]. In the static quenching mode there is a ground state formation of a non-fluorescence complex between the fluorophore and the quencher. In the dynamic quenching mode, following the collision of a quencher during the lifetime of the exited state, the fluorophore returns to ground state, without the emission of photons. Previous studies reported that the quenching mechanism of HSA by ketoprofen or warfarin was due to complex formation in the static mode rather than by dynamic collision [36,50].

In the presence of ketoprofen or warfarin, a quenching of tryptophan fluorescence occurred for both native and diabetic albumin without any shift in the maximum emission wavelength. This result suggests complex formation between the drugs and albumin, involving changes in the microenvironment around tryptophan. Furthermore, we found that the binding constant relative to native HSA with ketoprofen ($K_A = 6.15 \times 10^6$ L/mol) was markedly higher than the one calculated ($K_A = 1.17 \times 10^4$ L/mol) in a recent study by Bi et al. [36]. Though the experimental conditions were quite similar to those in our study, this large difference could be explained by the use of commercial HSA by this group instead of fresh, purified albumin from plasma. In previous studies we reported that commercial albumin is often more oxidized than fresh, purified albumin [28,45]. Oxidation is one of several deleterious effects that could impact on the binding properties of plasma proteins [21].

Using this methodology, we focused on the differences in the interaction between drugs and native albumin and diabetic albumin by assessing the number of binding sites and the affinity constant. In accordance with the binding results calculated in the Scatchard method, the *in vivo* glycated albumin affinities appeared to be significantly and considerably impaired. Indeed, a reduction in the binding constant associated with a lower number of binding sites was found for diabetic albumin. Finally, the higher affinity of albumin for ketoprofen compared to warfarin, as determined by fluorescence spectroscopy, was in agreement with the results obtained by the HPLC method (Table 2).

The present study demonstrated the reduced binding capacity of glycated human albumin for warfarin and ketoprofen, which were used as probes for the main drug-specific sites of this protein. These results could provide supplementary information about the impact of glycation on changes in the structure of albumin, particularly around both Sudlow sites. Moreover, the relevant findings obtained for glycated albumin and, in particular, for the *in vivo* glycated albumin, emphasize the importance to assess the biological response of many drugs in diabetic pathology. This could be of utmost importance for therapeutic drugs highly bound to albumin and with narrow therapeutic index, such as warfarin whose dosing adjustment remains challenging in pathological context.

Author contributions

J.B.V. researched data, contributed discussion, reviewed manuscript. A.G.D. researched data, contributed discussion, reviewed manuscript. E.B. contributed discussion, reviewed manuscript. P.R. researched data, wrote, reviewed and edited manuscript.

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