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NONENZYMATIC GLUCOSYLATION OF HUMAN SERUM ALBUMIN AND ITS INFLUENCE ON BINDING CAPACITY OF SULFONYLUREAS

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Abstract—To estimate the functional change occurring in human serum albumin by nonenzymatic glucosylation, glucosylated human serum albumin was prepared by *in vitro* incubation with glucose. The rate of glucosylation proceeded as a first-order reaction. The binding of sulfonylureas to serum albumin was determined by equilibrium gel filtration. Through this method, it was possible to estimate the binding capacity of a low water solubility drug with a high affinity to protein. The amounts of the sulfonylureas bound to glucosylated HSA decreased by 44% with tolazamide and acetohexamide, 50% with glibenclamide, and 52% with tolbutamide, compared to human serum albumin (HSA). This suggests that a high concentration of glucosylated HSA in diabetic patients may possibly cause an increase in free drug concentration exceeding normal levels. This study shows that the decrease in the binding capacity of sulfonylureas with protein is due to the modification of albumin molecules by the covalent binding of glucose.

The nonenzymatic glucosylation of a variety of proteins, such as hemoglobin [1], albumin [2, 3], collagen [4], erythrocyte membrane proteins [5], lens crystallines [6] and certain enzymes [7, 8], has been the subject of considerable research activity. Diabetic patients have high blood glucose levels and significantly more glucosylated albumin in the plasma (16.1%) than normal (8.1%) [9]. Although a direct correlation between an increase in glucosylated proteins and the pathophysiology of diabetes has not been found, alterations in the biochemical properties of albumin subsequent to glucosylation have been described: glucosylated albumin is catabolized faster and accumulated more rapidly by the liver than native albumin [9] and also is deposited on the walls of the microvasculature [10].

It is well known that serum albumin is capable of reversible binding of drug molecules to its surface and of functioning as buffer and transporter in drug distribution and elimination processes. The binding of a drug to protein is altered when the drug is displaced by other drugs or physiological and pathological substances. Thus, the binding of a drug to serum albumin *in vivo* should be more complex than that occurring in a conventional dialyzing cell. The effects of glucose are particularly noteworthy, because this constituent is normally present in the blood. Little investigation has been carried out on the binding properties of glucosylated albumin except for a report indicating that glucosylated serum albumin decreases salicylate binding accompanied by a reduction in the number of classes of binding sites [11].

Sulfonylureas, the hypoglycemics, bind strongly to serum albumin [12, 13], and thus it should be worthwhile to determine the change in the binding capacity of sulfonylureas to glucosylated serum albumin. The results of such a study should provide some information about the biochemical and pathophysiological characteristics of glucosylated albumin in diabetic patients.

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MATERIALS AND METHODS

Human serum albumin (HSA) was obtained from Fujirebio Inc. (Tokyo). Glibenclamide, tolbutamide, acetohexamide and tolazamide were donated by the Yamanouchi Pharmaceutical Co., Ltd., the Ono Pharmaceutical Co., Ltd., the Shionogi Pharmaceutical Co., Ltd. and the Japan Upjohn Co., Ltd., respectively. Glucose and thiobarbituric acid were of guaranteed reagent grade, from Wako Pure Chemical Industry Ltd. 5-(Hydroxymethyl)-2-furaldehyde (HMF) was from the Tokyo Kasei Co., Ltd. All other chemicals were of analytical grade. Matrex gel (PBA-10), *m*-aminophenyl-boronic acid immobilized on agarose, was obtained from the Amicon Corp.

Preparation of glucosylated HSA. HSA was dissolved in 0.067 M phosphate buffer (pH 7.4) containing 200 mM glucose to a 4% concentration. This solution was filtered through a millipore filter (millex-GS, 0.22 μm) and poured into a dry-heat sterilized test tube followed by incubation at 37° for 240 hr. The mixture was then adequately dialyzed against distilled water to remove any unreactive glucose, followed by lyophilization. The same procedure was followed without glucose for the control. To investigate the effects of glucose concentration and incubation time on the formation of glucosylated HSA, 4% HSA containing various concentrations of glucose was incubated for an appropriate period of time.

Determination of glucosylated HSA. Glucosylated serum albumin was determined by the thiobarbituric acid method (TBA method) [14]. An aqueous solution of serum albumin was hydrolyzed with 1 M oxalic acid at 100° for 4.5 hr and, following the removal of the precipitated protein by the addition of 40% trichloroacetic acid, 0.05 M TBA was added before incubation at 40° for 40 min. The absorbance at 443 nm was measured. Standard HMF solutions ranging in concentration from 1.00×10^{-7} M to 1.00×10^{-5} M were colored quantitatively with TBA. The concentration of serum albumin was measured using $E^{\%}_{1cm} = 5.31$.

Separation of glucosylated HSA from HSA. The PBA-10 column (1.4 × 16 cm) was equilibrated with 25 mM phosphate buffer (pH 8.5), and 100 mg of the lyophilized reaction mixture, dissolved in the buffer (5 ml), was charged. The column was washed with the buffer (250 ml), and the glucosylated HSA was then eluted with the buffer containing 100 mM sorbitol.

Binding of sulfonylureas by equilibrium gel filtration. The Sephadex G-25 (fine) column was equilibrated with 0.067 M phosphate buffer (pH 7.4) containing 1×10^{-5} M sulfonylurea at 37° [15]. Two milligrams of HSA or glucosylated HSA was dissolved in the drug-containing buffer (2 ml) and charged on the column. Then serum albumin was eluted with the drug-containing buffer. Three milliliters of each effluent was quantitated by a spectrophotometer at 230 nm for glibenclamide and tolbutamide, and 250 and 225 nm for acetohexamide and tolazamide respectively. The amount of bound drug to serum albumin was obtained from equation 1.

Amount of bound sulfonylurea

$$= \frac{\Sigma_{n=i}^{j}(A - A_n)}{\varepsilon} \cdot \frac{1000}{3} \qquad (1)$$

where ε is the drug molar extinction coefficient, and A and A_n show the absorbance of the equilibrated drug level and that of the n fraction when a trough was formed by the ith to jth fractions respectively.

To estimate the quantitativeness of the binding studies by equilibrium gel filtration, 2 ml of a drug-free buffer was charged on the column equilibrated with a drug-containing buffer. The trough was used to calculate the decrease in the amount of the drug, i.e. the moles not detected, by equation 1. The recovery was calculated by equation 2.

$$\text{Recovery } (\%) = \frac{\text{moles not detected}}{2 \times 10^{-8} \text{ moles}} \times 100 \qquad (2)$$

A recovery of 99.46% to 101.02% was obtained for four sulfonylureas, indicating that the trough on the equilibrium gel filtration chromatogram accurately shows the decrease in drug amount.

RESULTS

The effects of glucose concentration on the preparation of glucosylated HSA and its time course were investigated. The results are listed in Table 1. The higher the glucose concentration and the longer the incubation time, the more was HSA glucosylated. However, HSA without glucose in the incubation mixture showed no increase in HMF content during incubation, though 0.76×10^{-2} mole of HMF per mole of HSA was observed at zero time. It is considered that the period from 0 to 24 hr gives the initial reaction rate of glucosylation. The initial reaction rate rose linearly with glucose concentration. From the slope of the straight line, an apparent reaction rate constant of 0.0146 hr^{-1} was obtained, indicating that glucose was added nonenzymatically to the HSA molecule as a first-order process.

To obtain glucosylated HSA from the reaction mixture, PBA-10 affinity chromatography was carried out. As shown in Fig. 1a, 90% of the total protein was eluted by the sorbitol-containing buffer. HSA without incubation with glucose was charged in the same manner as the control, and only 6.9% of the total protein was eluted by the sorbitol-containing buffer. Each unbound and bound fraction was dialyzed against distilled water for 24 hr. After lyophilization, 0.9 mg of each protein was assayed by the TBA method (Fig. 1b). Though the amount of bound fraction of HSA incubated with glucose was about thirteen times larger than the control HSA (Fig. 1a), the HMF level per mole of albumin in the bound fraction was about 1 mole in both incubated and non-incubated HSA (Fig. 1b). This indicates that incubation with a high concentration of glucose

Table 1. Glucosylation of human serum albumin*

Concentration of glucose (mM)	HMF/HSA (× 10^{-2} mole/mole)							
	Incubation time (hr)							
	0	24	48	72	96	144	192	240
0	0.76							0.80
5	0.56	2.10	2.50	3.10	3.60	4.0	3.50	4.30
10	0.60	2.20			4.00			
25	0.79	3.20	3.80	4.40	5.80	6.10	7.90	8.80
50	0.75	3.80			9.60			15.40
75	0.74	4.50			12.10			24.90
100	0.69	6.50			17.20			31.40
150	0.79	7.00	13.30	20.00	25.40	30.40	39.70	44.20
200	0.76							86.50

* 4% Human serum albumin and various concentrations of glucose were incubated for 0–240 hr. Glucosylated human serum albumin was assayed by the thiobarbituric acid method.

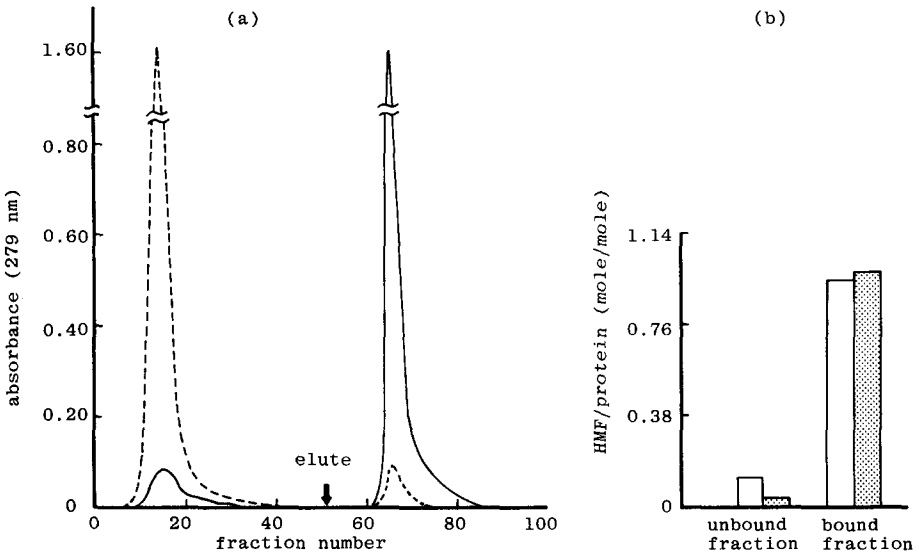

Fig. 1. (a) Phenylboronic acid affinity chromatography of human serum albumin (HSA). The wash buffer was 25 mM phosphate buffer (pH 8.5), the elution buffer was 100 mM sorbitol in wash buffer, the flow rate was 1 ml/min, and the fraction size was 5 ml. (b) Assay by thiobarbituric acid method for bound and unbound fraction of phenylboronic acid affinity chromatography. A sample of 0.9 mg was used for assay. Key: (——, ☐) HSA incubated with 200 mM glucose for 240 hr; and (-----, ▨) HSA without incubation.

accelerated the formation of the same glucose adduct as that formed under physiological conditions. But the unbound fraction (Fig. 1b) had a lower HMF level than the bound fraction. In this experiment, the bound fraction was used as glucosylated HSA for the binding study of sulfonylureas. The unbound fraction is referred to as non-glucosylated HSA in the subsequent text. The TBA method used in this experiment consisted of the hydrolysis of the ketoamine adduct of serum albumin and the reaction of the released HMF with TBA. To estimate the reproducibility of this method, 0.9 mg of the glucosylated HSA was dissolved in 0.067 M phosphate buffer (pH 7.4) and assayed. The measurement (N = 10) indicated good reproducibility (CV = 2.9%) of hydrolysis and colorimetry. When glucosylated HSA was added to non-glucosylated HSA, in various ratios, followed by the TBA method, the concentration of glucosylated HSA correlated well with the formation of HMF ($r = 0.9999$).

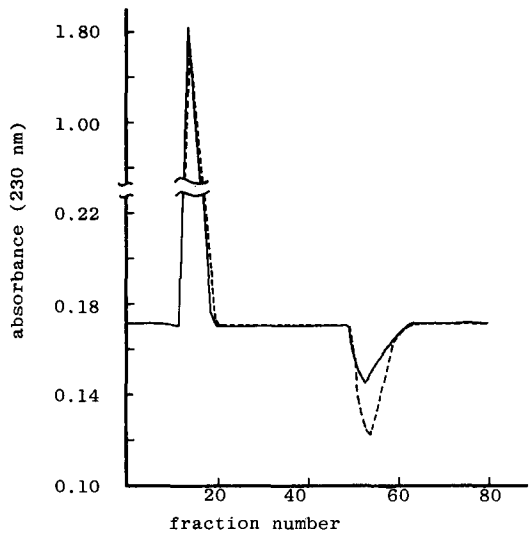

Fig. 2. Elution profile of equilibrium gel filtration for glibenclamide. The elution buffer was 0.067 M phosphate buffer (pH 7.4) containing 1×10^{-5} M glibenclamide, the flow rate was 20 ml/hr, and the fraction size was 3 ml. Key: (-----) human serum albumin, and (——) glucosylated human serum albumin.

The binding of sulfonylureas to HSA and glucosylated HSA was studied by equilibrium gel filtration. A typical elution profile is illustrated in Fig. 2. It is obvious that the area of the trough obtained from glucosylated HSA is smaller than that from HSA. This indicates that a smaller amount of glibenclamide bound to glucosylated HSA than to HSA. The other sulfonylureas, tolbutamide, acetohexamide and tolazamide, were similar to glibenclamide in their elution profiles. Table 2 summarizes the binding of four sulfonylureas and the binding ratios of glucosylated HSA to HSA. The amounts of sulfonylureas bound to glucosylated HSA decreased in comparison with those bound to HSA. The ratios were 44% with tolazamide and acetohexamide, 50% with glibenclamide, and 52% with tolbutamide. HSA following incubation for 240 hr without glucose showed the same binding capacity for sulfonylureas as HSA without incubation.

DISCUSSION

Bunn *et al.* [1] have reported that nonenzymatically glucosylated hemoglobins change in their oxygen-binding equilibria. Similarly, the glucosylation

Table 2. Binding of sulfonylureas to human serum albumin (HSA) and glucosylated HSA

Drug	Protein	Amount of bound drug* ($\times 10^{-8}$ mole)	Amount of bound drug/protein (mole/mole)	Ratio (glucosylated HSA/HSA)
Glibenclamide	HSA	5.85	2.02	0.50
	Glucosylated HSA	2.92	1.01	
Tolbutamide	HSA	1.89	0.65	0.52
	Glucosylated HSA	1.00	0.34	
Acetohexamide	HSA	2.87	0.99	0.44
	Glucosylated HSA	1.28	0.44	
Tolazamide	HSA	0.79	0.27	0.44
	Glucosylated HSA	0.35	0.12	

* Calculated by equation 1 in the text (average of two runs).

of HSA may possibly affect its affinity toward a variety of ligands. In the *in vitro* glucosylation of rat serum albumin studied by Day *et al.* [3], the reaction was a first-order process with respect to glucose and albumin concentrations. In the present experiment, the glucosylation of HSA was a first-order process with respect to glucose concentration, with a higher rate constant than that of rat serum albumin. The reaction of glucose and albumin is theoretically a second-order process. However, this reaction showed an apparent first order since the Schiff-base formation between glucose and protein was fast and reversible, but the subsequent Amadori rearrangement was slow. In the formation of hemoglobin A_{1c}, the rate of the latter process was one-hundredth that of the former [16].

The formation rate of nonenzymatically glucosylated HSA in the presence of 25 mM glucose, corresponding to hyperglycemia (400 mg/100 ml), approximately doubled compared to that of glucosylated HSA in the presence of 5 mM glucose, corresponding to the blood glucose level of normal subjects (90 mg/100 ml).

The TBA assay for nonenzymatically glucosylated protein was first applied to provide structural evidence of hexose bound to hemoglobin A [17]. The free glucose in serum interferes with an accurate measurement of glucosylated serum protein [18]. Thus, in this experiment, the free glucose was removed by an overnight dialysis of the reaction mixture against distilled water. During the dialysis, the labile Schiff-base was dissociated and removed from the tubing [19]. Though the experimental conditions were milder than those of Ney *et al.* [20] for the hydrolysis of the stable ketoamine adduct of the glucosylated albumin in the TBA method, an excellent correlation of quantitativeness was obtained.

In phenylboronate affinity chromatography, immobilized boronic acid specifically binds to the cis-diol groups. Hemoglobin A_{1c} has been shown to be separated from hemoglobin by phenylboronate affinity chromatography with good agreement to the results by the TBA method and ion-exchange liquid chromatography [21]. Mortensen and Christophersen [22], in their mechanism study of hemoglobin A_{1c}, proposed that the product of the Amadori rearrangement is best represented as a β-D-pyranose configuration having a 4,5-cis-diol group. It seems reasonable to assign glucosylated HSA separated by PBA-10 chromatography to the pyranoid ring structure. In this experiment, it was found that native HSA contained 6.9% glucosylated HSA (Fig. 1), as was also demonstrated by the TBA method (Table 1.)

Many studies on protein binding have been carried out by equilibrium dialyses and ultrafiltration, followed by estimation of free drug concentration. On the other hand, in equilibrium gel filtration, the stronger the binding of the drug to the protein, the larger becomes the area of the trough of the elution profile, indicating improvement in the accuracy of estimating the binding capacity of a drug with high affinity to the protein. The order of the amounts of sulfonylureas bound to HSA was glibenclamide> acetohexamide > tolbutamide > tolazamide (Table 2). It is considered that these amounts reflect the binding capacity of these drugs to the primary class of binding sites, since the drug concentration (1×10^{-5} M) was lower than that of the serum albumin (1.4×10^{-5} M). The order of the binding degree of these drugs agree with that of the product of the binding constant and the number of the binding sites of the primary class, as obtained from equilibrium dialysis using ^{14}C-labeled drugs [12, 13]. This indicates the reliability of equilibrium gel filtration in determining the capacity of sulfonylurea binding to serum albumin. The order of binding of sulfonylureas to glucosylated HSA was essentially the same as that for HSA. The binding ratio of glucosylated HSA to HSA ranged from 44 to 52%. The denaturation of HSA during incubation could be ignored. A decrease by about half of the binding of these four sulfonylureas to glucosylated HSA resulted in an increase of the free drug concentration. Thus, a high concentration of glucosylated HSA in diabetic patients may possibly result in an increase in free drug concentration beyond that expected in normal persons.

The alteration in drug–protein binding is generally considered to arise from displacement through competition of one drug with another or with endogenous and pathological substances. However, in this study, it was found that the decrease in binding of sulfonylureas to protein was due to modification of the albumin molecules by covalently bound glucose.

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