Comprehensive Glycomic Analysis Reveals That Human Serum Albumin Glycation Specifically Affects the Pharmacokinetics and Efficacy of Different Anticoagulant Drugs in Diabetes

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Long-term hyperglycemia in patients with diabetes leads to human serum albumin (HSA) glycation, which may impair HSA function as a transport protein and affect the therapeutic efficacy of anticoagulants in patients with diabetes. In this study, a novel mass spectrometry approach was developed to reveal the differences in the profiles of HSA glycation sites between patients with diabetes and healthy subjects. K199 was the glycation site most significantly changed in patients with diabetes, contributing to different interactions of glycated HSA and normal HSA with two types of anticoagulant drugs, heparin and warfarin. An in vitro experiment showed that the binding affinity to warfarin became stronger when HSA was glycated, while HSA binding to heparin was not significantly influenced by glycation. A pharmacokinetic study showed a decreased level of free warfarin in the plasma of diabetic rats. A preliminary retrospective clinical study also revealed that there was a statistically significant difference in the anticoagulant efficacy between patients with diabetes and patients without diabetes who had been treated with warfarin. Our work suggests that larger studies are needed to provide additional specific guidance for patients with diabetes when they are administered anticoagulant drugs or drugs for treating other chronic diseases.

Diabetes is one of the most common chronic diseases, with characteristic hyperglycemia, and is also a leading cause of death and disability worldwide (1,2). People suffering from diabetes are more likely to be at serious risk of cerebrocardiovascular diseases, including strokes and coronary heart disease, and they have a higher mortality rate than people without diabetes (3,4).

Long-term hyperglycemia in patients with diabetes leads to various pathological changes, including the excessive production of advanced glycation end products (AGEs). AGEs are the collective name given to proteins, lipids, and nucleic acids that undergo irreversible, covalent modification by reducing sugars or sugar-derived products without the participation of glycosyltransferases. This process, known as nonenzymatic glycation, significantly affects the structure and function of the modified molecules (5). Glycation involves several steps, including the reaction of carbonyl groups of reducing sugars with amino groups of

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proteins to form Schiff bases, rearrangement of Schiff bases to form more stable Amadori products, and the spontaneous reactions of Amadori products to produce AGEs, resulting in different products of posttranslational modifications, such as aldimine, ketoamine, and N_{ε}-(carboxymethyl)lysine and N_{ε}-(carboxyethyl)lysine adducts (6). Our study focuses on the Amadori products because they are relatively stable and present in larger quantities than other glycation products in plasma (7). Human serum albumin (HSA) is the most abundant protein in plasma, and it has a greater opportunity than other circulating proteins to undergo nonenzymatic glycation in diabetes (8). The level of glycated HSA (gHSA) is remarkably higher in patients with diabetes than in healthy individuals (6,9,10). HSA has many important physiological and pharmacological functions, such as maintaining colloid osmotic pressure and transporting fatty acids, hormones, drugs, and metabolites. As a dominant drug transport protein in blood, HSA plays a critical role in the pharmacokinetics and pharmacodynamics of many classes of drugs (11,12). The modification of specific amino acid residues on HSA might affect the binding between HSA and drug molecules, which may consequently influence drug efficacy and safety for patients with diabetes. The mapping of HSA glycation sites is fundamental for understanding functional changes in this carrier protein in patients with diabetes. Many analytical approaches have been developed to identify the HSA glycation sites, which primarily occur on arginine and lysine residues (7,8,13–16). However, a more definitive method that can precisely reveal additional glycation sites and accurately quantify these sites is still needed.

Because the incidence of cardiovascular disease is significantly higher in patients with diabetes than in patients without diabetes (17,18), patients with diabetes are more likely to be administered anticoagulant drugs than patients without diabetes. Warfarin and heparin are two major types of anticoagulant drugs used in the clinic and have different structures and mechanisms of action (Supplementary Fig. 1). Warfarin is a small-molecule drug that decreases the clotting ability of clotting factors by inhibiting vitamin K epoxide reductase (19), while heparin is a polycomponent drug consisting of polysaccharide chains that inactivate clotting factors through the binding and activation of antithrombin (20). Both warfarin and heparin are mainly carried by HSA, but they are likely to have different binding sites on HSA due to their distinctively different properties and molecular sizes. In patients with diabetes, the glycation of HSA may differentially influence the binding and free drug levels of warfarin and heparin. Both anticoagulant drugs must be carefully monitored due to their bleeding side effects. Moreover, warfarin has a narrow therapeutic index due to the toxicity of free warfarin, making understanding changes in the pharmacokinetics of this drug critical for medication safety (21). Therefore, it is important to delineate the specific changes in HSA glycation taking place in diabetes by using state-of-the-art analytical techniques. This improved understanding should help to elucidate variations in the pharmacokinetics of these anticoagulant drugs in people with diabetes.

Herein, we developed a novel analytical approach using nano–liquid chromatography–multistage mass spectrometry ($nLC\text{-}MS^n$) assay combined with the use of isobaric tags for relative and absolute quantification (iTRAQ) to qualitatively and quantitatively reveal differences in HSA glycation between healthy individuals and patients with diabetes. In vitro and in vivo experiments have been carried out to evaluate the impact of glycation on the binding of HSA to two anticoagulant drugs, warfarin and heparin. A preliminary retrospective clinical study was also conducted to better understand the risks of treating patients with diabetes with specific anticoagulant drugs.

RESEARCH DESIGN AND METHODS

Ethics Statement and Sample Collection

We recruited 32 patients with diabetes and 33 healthy individuals from Provincial Hospital Affiliated to Shandong University. All patients with diabetes were diagnosed according to the guidelines for diabetes diagnosis (22). This study has been approved by the Ethics Committee of Shandong Provincial Hospital and conformed to the Declaration of Helsinki. All subjects were informed of the nature of the study and were provided informed consent. All procedures were performed in compliance with relevant laws and institutional guidelines. The animal studies were approved by the Institutional Animal Care and Use Committees of the Scientific Investigation Board of Shandong University.

Identification of Glycation Sites on HSA Using $nLC\text{-}MS^2$

The gHSA was extracted from plasma using a procedure previously reported by our laboratory (14). The workflow is summarized in Supplementary Fig. 2*A*. Briefly, immunoglobulins were removed by polyethylene glycol precipitation and the gHSA was separated using affinity chromatography with a TSKgel Boronate-5PW column.

Trypsin-digested gHSA was analyzed using a Thermo Scientific LTQ Orbitrap Velos Pro mass spectrometer. A ReproSil-Pur C18-AQ analytical column (3 μm, 75 μm × 250 mm) was used to separate tryptic peptides. The mass spectrometer was operated in the positive-ion mode, and the data were acquired in the data-dependent mode for collision-induced dissociation (CID)-MS^2. An additional $nLC\text{-}MS^2$ with the dissociation mode of electron transfer dissociation (ETD) was also performed to confirm the exact location of glycation.

Quantitation of Altered Glycation in Patients With Diabetes Using iTRAQ Labeling and $nLC\text{-}MS^3$

The workflow for the quantitation of glycation sites was summarized in Supplementary Fig. 2*B*. Human plasma samples were precipitated with polyethylene glycol to remove immunoglobulins and digested with trypsin. The digests were labeled with iTRAQ 8plex reagents according to the manufacturer's protocol. After labeling, the eight samples with different mass tags were evenly mixed. Glycated peptides with iTRAQ labels were extracted from the pooled samples using boronate affinity chromatography.

Quantitative analysis was performed on a Thermo Scientific Easy-nLC 1000 system and a Thermo Scientific LTQ Orbitrap Fusion mass spectrometer. The separation conditions were the same as the qualitative method. The mass spectrometer was operated in the data-dependent mode for CID-MS^2 and high-energy collision dissociation (HCD)-MS^3. The difference in the glycation degree, at each HSA site, between patients with diabetes and healthy subjects was calculated from the ratio of corresponding reporter fragments in MS^3.

Validation of the Glycation Site K199 Using Liquid Chromatography–Tandem Mass Spectrometry–Multiple Reaction Monitoring Analysis

Human plasma with equal amounts of total proteins was directly digested with trypsin. A Thermo Scientific Easy-nLC 1000 system coupled with a TSQ Quantum Ultra mass spectrometer was used. The multiple reaction monitoring (MRM) analysis was performed at four transitions: 555.30 → 528.30 (from $[MH_2]^{2+}$ to $[MH_2\text{-}3H_2O]^{2+}$), 555.30 → 513.30 (from $[MH_2]^{2+}$ to $[MH_2\text{-}3H_2O\text{-}HCHO]^{2+}$), 555.30 → 537.30 (from $[MH_2]^{2+}$ to $[MH_2\text{-}2H_2O]^{2+}$), and 555.30 → 546.30 (from $[MH_2]^{2+}$ to $[y_5^+]$). The quantity of glycated peptides containing the glycation site K199 was calculated by integrating the signals of the four transitions.

Docking Simulation

AutoDock 4.2.6 software with the AutoDockTools 1.5.6 was used for docking studies. Site-specific gHSA and normal HSA were docked with warfarin or a heparin octasaccharide. The following parameters were used: grid maps, 126 × 126 × 126 points; grid-point spacing, 0.375 Å; number of genetic algorithms, 10; population size, 150; maximum number of energy evaluations, 2.5×10^6; and maximum number of generations, 27,000. PyMOL software was used to generate high-resolution figures to present the crystal structures.

Mutation of HSA K199 to M199 and Comparison of Warfarin Binding of Nonglycated and Glycated Mutant of HSA

The HSA K199 was mutated to M199 (23). The sequence-containing mutant site was cloned between Sgf I and Mlu I sites of multiple cloning sites in pCMV6 vector with a FLAG tag. The mutated HSA (K199M) was expressed in 293T cells and purified by the FLAG tag fusion protein purification kits. Purity of HSA (K199M) was analyzed by Western blot. A portion of 10 μmol/L HSA (K199M) was glycated in 30 mmol/L glucose until its glycation level became equivalent to that of HSA in patients with diabetes (determined by liquid chromatography–mass spectrometry [LC-MS]) and then warfarin was added to the solutions to a final concentration of 5 μmol/L warfarin. After 30 min, free warfarin was isolated and detected by LC–tandem MS–MRM (LC-MS/MS-MRM).

In Vitro Measurement of Free Anticoagulant Drugs in Plasma

The anticoagulant drugs warfarin and heparin (enoxaparin) were added to human plasma freshly collected from subjects with diabetes and healthy subjects. The administered concentrations of anticoagulant drugs were based on their standard clinical doses (6.25 μg warfarin or 10.31 μg enoxaparin in 0.2 mL plasma). After incubation at 37°C for 30 min, the free drugs were recovered using centrifugal filter devices and analyzed with two different LC-MS/MS-MRM methods. For warfarin, MRM was performed on an AB SCIEX Triple Quad 4500 mass spectrometer. The transition of mass/charge ratio (m/z) 309.2 → 163.2 in the positive ion mode was used. Enoxaparin was digested to its disaccharide building blocks using heparinases and then labeled with 2-aminoacridone according to our previously described method (24). Analysis was done with a Thermo Scientific TSQ Quantum Ultra mass spectrometer in the negative mode by monitoring eight basic heparin disaccharides.

Pharmacokinetic Analysis of Anticoagulant Drugs in Diabetic and Healthy Rats

A diabetes rat model was established by feeding male specific-pathogen-free Sprague-Dawley rats (weighing ~250 g; SPF (Beijing) Biotechnology Co., Ltd) with a high-fat diet for >2 weeks followed by the intraperitoneal injection of streptozocin (35 mg/kg body wt). After 5 days, rats with glucose levels >16.7 mmol/L were considered hyperglycemic (25). Diabetic rats were fed for ~21 additional days before being used in pharmacokinetic experiments. For warfarin, a single dose of 2 mg/kg drug was gavaged to rats. For enoxaparin, a single dose of 3.3 mg/kg drug was intravenously injected. Portions of 0.5 mL blood were collected at specific time points to determine levels of free warfarin and enoxaparin.

Preliminary Retrospective Clinical Study

The medical data records of 53 individuals with treatment by warfarin (13 patients with diabetes and 14 patients without diabetes) and the heparin group (13 patients with diabetes and 13 patients without diabetes) were collected from the electronic medical database at the hospital. When collecting patient medical records for each group, we ensured that these factors (age, sex, blood pressure, liver and kidney function, antibiotics, and herbal products) did not differ significantly between groups. The characteristics of these patients were shown in Supplementary Table 1, and the outcomes were international normalized ratio (INR) 24 h after medicine and INR difference (between INR 24 h after medicine and INR before medicine). A multivariable linear regression model was used for analysis of the data, and the Tukey method was used for post hoc pairwise comparison. The statistical differences were analyzed using an unpaired t test with a 95% CI.

Statistical Analysis

Data were presented as mean ± SD. The unpaired Student two-tailed t tests were used to detect significant changes. Statistical analysis was performed using GraphPad Prism v8, and $P < 0.05$ was considered statistically significant.

Data and Resource Availability

All the raw data generated during this current study are available from the corresponding author upon reasonable request. No applicable resources were generated or analyzed during the current study.

RESULTS

Identification of Glycation Sites on HSA Using nLC-MS^2

The glycation sites in gHSA in patients with diabetes and healthy subjects were unambiguously assigned by high-resolution MS and tandem MS. A representative MS spectrum of peptide E_{542}QLKAVMDDFAAFVEK$_{557}$ with and without glycation is shown in Fig. 1*A*. Further dissociation of the glycated peptide provided enough fragment ions to recover its full sequence and designate the K545 residue as a glycation site, which was confirmed by ETD-MS/MS (Supplementary Fig. 3). A total of 49 glycation sites on gHSA were successfully identified using this approach. By comparison with previously known glycation sites (summarized in Supplementary Table 2), seven glycation sites, R81, R117, R186, R257, K313, R410, and K541, were discovered for the first time in the current study. Among the glycation sites, residues K4, R81, R117, K439, K519, K538, K541, K557, and K573 were specifically present in patients with diabetes, while two sites, R410 and K436, were found only in healthy subjects (Fig. 1*B*). Most glycation sites occurred on lysine residues, while only six arginine residues, R81, R98, R117, R186, R257, and R410, were subject to glycation modification. No glycation was found either on cysteine residues or at the N-terminus of HSA. Most glycation sites were distributed on the surface of the HSA spatial structure (Supplementary Fig. 4), with the exception of site K199, which was not completely exposed.

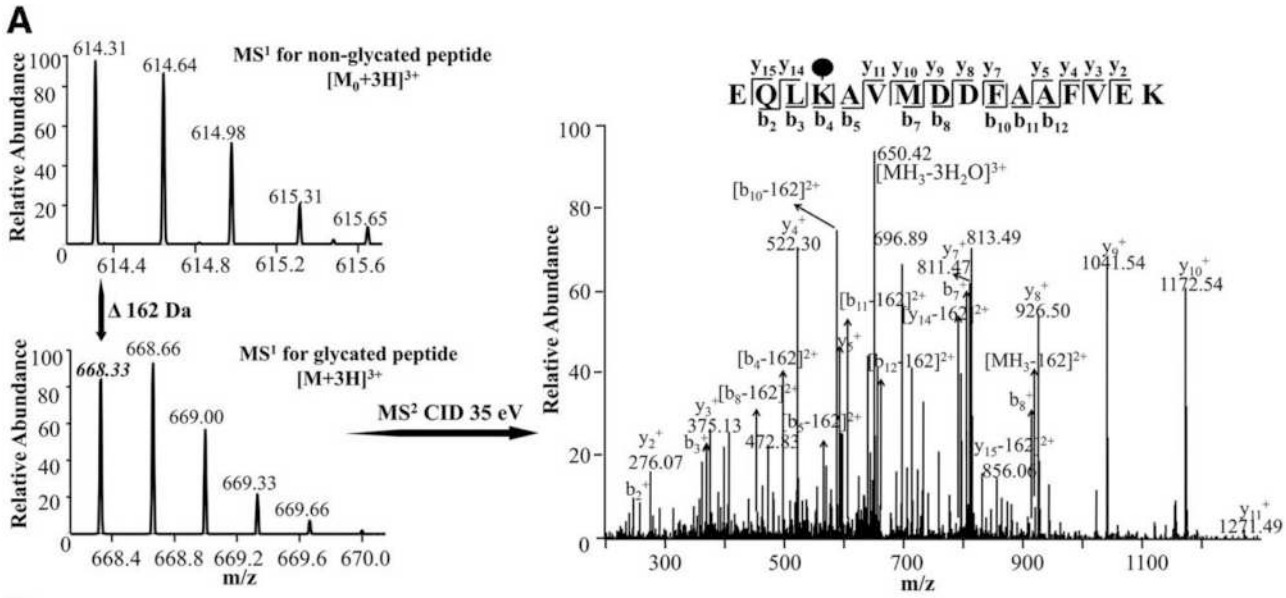
Quantification of Changes in Glycation on HSA in Patients With Diabetes

Sites on HSA are specifically glycated in diabetes because their various locations in the spatial structure lead to different accessibilities to glucose in blood. In the previous qualitative analysis, 38 glycation sites were shared by patients with diabetes and healthy subjects, while 11 sites were specifically glycated either for diabetes or healthy people. For further investigation of the quantitative change of these 38 sites (Fig. 1*B*), iTRAQ labeling and nLC-MS^3 experiments were performed. An example of the quantification of the glycated peptide K_{525Glc}QTALVELVK$_{534}$ is shown in Fig. 2*A*. A total of 21 glycation sites were successfully quantified (Fig. 2*B*), and 19 of them, including K51, K64, K93, K162, K199, K233, K262, K313, K323, K378, K402, K414, K466, K475, K525, K545, K557, K564, and K574, have statistically enhanced glycation during diabetes. Only sites K12 and K573 were not significantly different. Among the sites with significantly different levels of glycation, K162, K199, K233, K262, K402, K414, K466, and K475 were located in subdomains IIA and IIIA of HSA. Since these two subdomains are the major binding regions on HSA for many drugs (26), the modification of these sites is likely to affect the binding of HSA to drug molecules. The glycation level on site K199 had the most significant changes ($P < 0.01$); therefore, it was selected for further validation using the LC-MS/MS-MRM (Fig. 2*C*). The dissociating parent ion of the site K199 containing peptide $L_{198}K_{Glc}$CASLQK$_{205}$ (m/z 555.30, +2 charge) generated four abundant fragment ions, which were included in the MRM transitions for quantifying the amount of glycated K199. The result showed that the difference in the K199 glycation of patients with diabetes increased to 1.77-fold compared with that of healthy control subjects (Fig. 2*D*), which exhibited the same trend as in the iTRAQ labeling and nLC-MS^3 analysis.

K199 Glycation Affects the Binding of HSA to Anticoagulant Drugs

Molecular docking was used to simulate the binding between HSA and two widely used anticoagulant drugs, warfarin and enoxaparin heparin, when site K199 was glycated and not glycated. Since enoxaparin is a mixture of oligosaccharides with different lengths and sulfation levels, a heparin octasaccharide with the uniform sequence of ΔGlcA2SGlcNS6SIdoA2SGlcNS6SIdoA2SGlcNS6SIdoA2SGlcNS6S (identification 1FQ9) was used for docking. As shown in Fig. 3*A*, the binding location of warfarin (2BXD) was located in domain II and adjacent to the K199 residue in native HSA (1AO6). The binding domain generated by simulation is almost identical to the crystal structure previously reported by Petitpas et al. (27). When K199 was modified by glucose, the original binding site of warfarin was occupied, and warfarin moved to the gap among domain I, II, and III (Fig. 3*B*). The relocation of the binding site of warfarin led to a stronger binding affinity, calculated from the modeling simulation. The dissociation constant (*Kd*) between warfarin and K199-gHSA ($Kd = 1.72 \times 10^{-6}$ mol/L) was nearly two times lower than between warfarin and native HSA ($Kd = 3.49 \times 10^{-6}$ mol/L). Meanwhile, the binding location of heparin octasaccharide was relatively far from the K199 residue in native HSA, so glycation on K199 showed a negligible impact on binding between HSA and heparin (Fig. 3*C* and *D*).

Besides K199, other glycation sites located in or adjacent to the drug-binding subdomains were also evaluated for their impacts on change of binding affinity between HSA and warfarin before and after glycation. LC-MS/MS-MRM analysis of plasma from 14 patients with diabetes and 15 healthy subjects revealed that glycation on K233, K414, K262, K475, K402, K162, K436, and K439 was significantly enhanced during diabetes, while K205, R257, K274, and K466 were not significantly changed (Supplementary Fig. 5). Their contribution to warfarin-binding changes of HSA after glycation is presented in Supplementary Table 3 and was calculated by multiplying the fold of affinity change after glycation by fold of glycation change during diabetes. Site K199 contributed the most to the increased binding affinity to warfarin during diabetes,



B

1- DAHKSEVAHR	11- FKDLGEENFK	21- ALVLIAFAQY	31- LQQCPFEDHV	41- KLVNEVTEFA
51- KTCVADESAE	61- NCDKSLHTLF	71- GDKLCTVATL	81- RETYGEMADC	91- CAKQEPERNE
101- CFLQHKDDNP	111- NLPRLVRPEV	121- DVMCTAFHDN	131- EETFLKKYLY	141- EIARRHPYFY
151- APELLFFAKR	161- YKAAFTECCQ	171- AADKAACLLP	181- KLDELRDEGK	191- ASSAKQRLKC
201- ASLQKFGERA	211- FKAWAVARLS	221- QRFPKAEFAE	231- VSKLVTDLTK	241- VHTECCHGDL
251- LECADDRADL	261- AKYICENQDS	271- ISSKLKECCE	281- KPLLEKSHCI	291- AEVENDEMPA
301- DLPSLAADFV	311- ESKDVCKNYA	321- EAKDVFLGMF	331- LYEYARRHPD	341- YSVVLLLRLA
351- KTYETTLEKC	361- CAAADPHECY	371- AKVFDEFKPL	381- VEEPQNLIKQ	391- NCELFEQLGE
401- YKFQNALLVR	411- YTKKVPQVST	421- PTLVEVSRNL	431- GKVGSKCCKH	441- PEAKRMPCAE
451- DYLSVVLNQL	461- CVLHEKTPVS	471- DRVTKCCTES	481- LVNRRPCFSA	491- LEVDETYVPK
501- EFNAETFTFH	511- ADICTLSEKE	521- RQIKKQTALV	531- ELVKHKPKAT	541- KEQLKAVMDD
551- FAAFVEKCCK	561- ADDKETCFAE	571- EGKKLVAASQ	581- AALGL	

○ -Glycated sites only identified in healthy subjects

● -Glycated sites only identified in patients with diabetes

◑ -Glycated sites identified in both subjects with diabetes and healthy subjects

= -New sites identified in this experiment

Figure 1— Identification of glycation sites on HSA. *A*: An example mass spectrum of the peptide E_{542}QLKAVMDDFAAFVEK$_{557}$ is displayed to demonstrate the determination of a glycation site. Glycation on this peptide resulted in a 162 Da mass increase (from m/z 614.31 to 668.33, +3 charge). The ion 668.33 (in italics) was chosen for further fragmentation with MS^2 analysis. Sufficient fragment ions were generated, allowing for the complete sequencing of this glycated peptide, which located K545 as the glycation site. *B*: All HSA glycation sites identified in patients with diabetes ($n = 3$) and healthy subjects ($n = 3$) using this approach are summarized. Thirty-eight glycation sites were shared by patients with diabetes and healthy subjects, nine sites were detected only in patients with diabetes, and two sites were found only in healthy subjects. Seven glycation sites were disclosed in vivo for the first time.

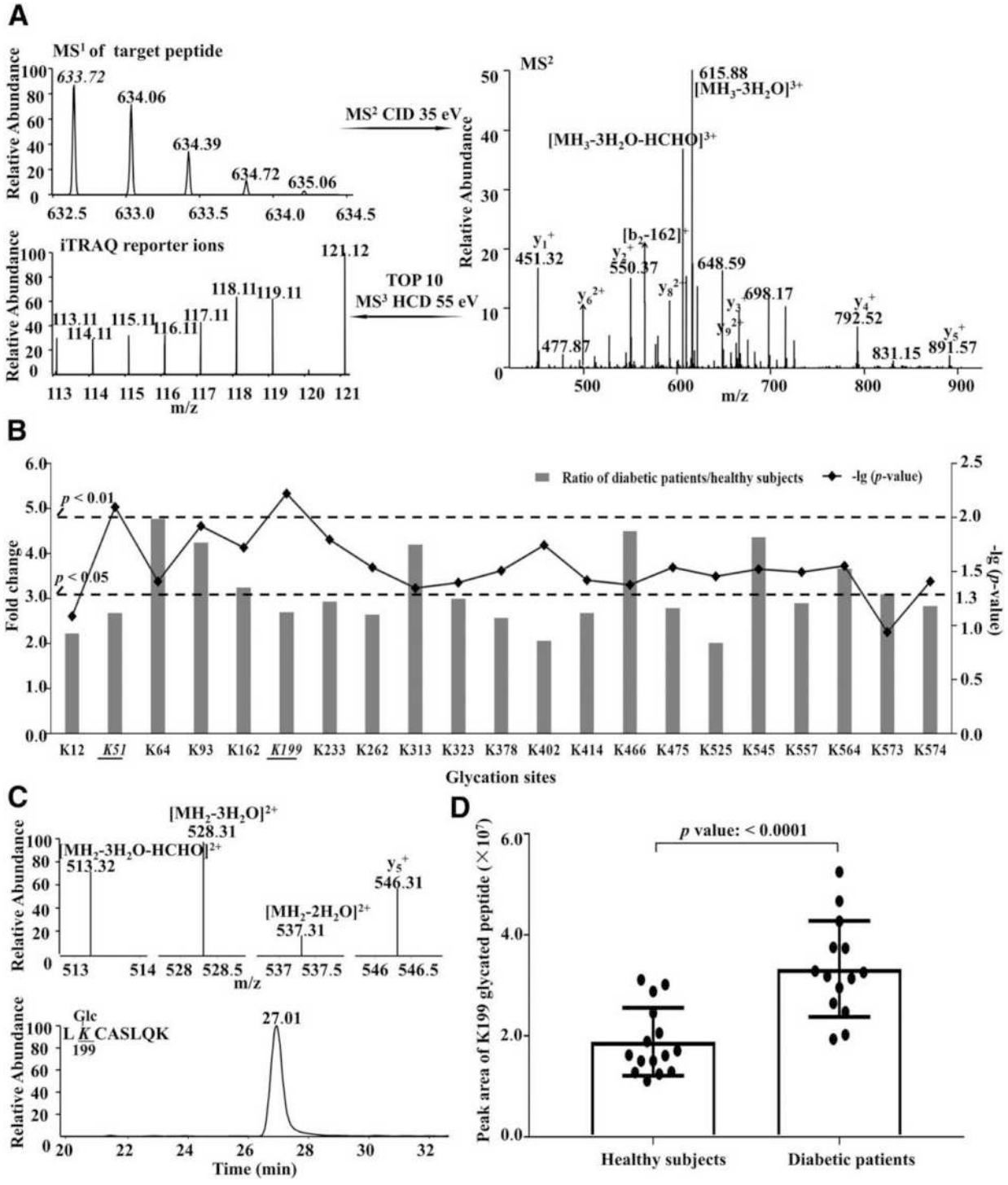


Figure 2— Comparative quantitation of the glycation levels of HSA glycation sites in patients with diabetes and healthy subjects. *A*: The example mass spectrum used for quantitation. The parent ion (m/z 633.72, +3 charge) of glycated peptide K_{525Glc}QTALVELVK$_{534}$ in MS^1 was fragmented to generate the MS^2 spectrum, and the top 10 most intense ions in the MS^2 were further fragmented to generate the MS^3 spectrum. In the MS^3 spectrum, the reporter ions 113, 114, 115, and 116 represent the abundances of glycated peptide K_{525Glc}QTALVELVK$_{534}$ from healthy subjects, while ions 117, 118, 119, and 121 represent those from patients with diabetes. *B*: The fold change in the glycation level at each glycation site on HSA between patients with diabetes ($n = 4$) and healthy subjects ($n = 4$). The significant differences were determined using an unpaired *t* test. Two dotted lines indicating $P < 0.05$ and $P < 0.01$, respectively. Glycation levels at sites K199 and K51 (underlined) were most significantly increased ($P < 0.01$) in patients with diabetes. *C*: The elevated glycation at K199 in patients with diabetes was validated by LC-MS/MS-MRM analysis of the glycated peptide $L_{198}K_{Glc}$CASLQK$_{205}$ (m/z 555.30, +2 charge). The following four transitions were selected to detect this glycated peptide at a retention time of 27.0 min: $[MH_2\text{-}3H_2O]^{2+}$, 528.3; $[MH_2\text{-}3H_2O\text{-}HCHO]^{2+}$, 513.3; $[MH_2\text{-}2H_2O]^{2+}$, 537.3; and y_5^+, 546.3. *D*: The amount of glycation at K199 was analyzed for healthy subjects ($n = 15$) and patients with diabetes ($n = 14$) and plotted as an unpaired *t* test of peak areas. It was confirmed that glycation at K199 was significantly increased in patients with diabetes ($P < 0.0001$).

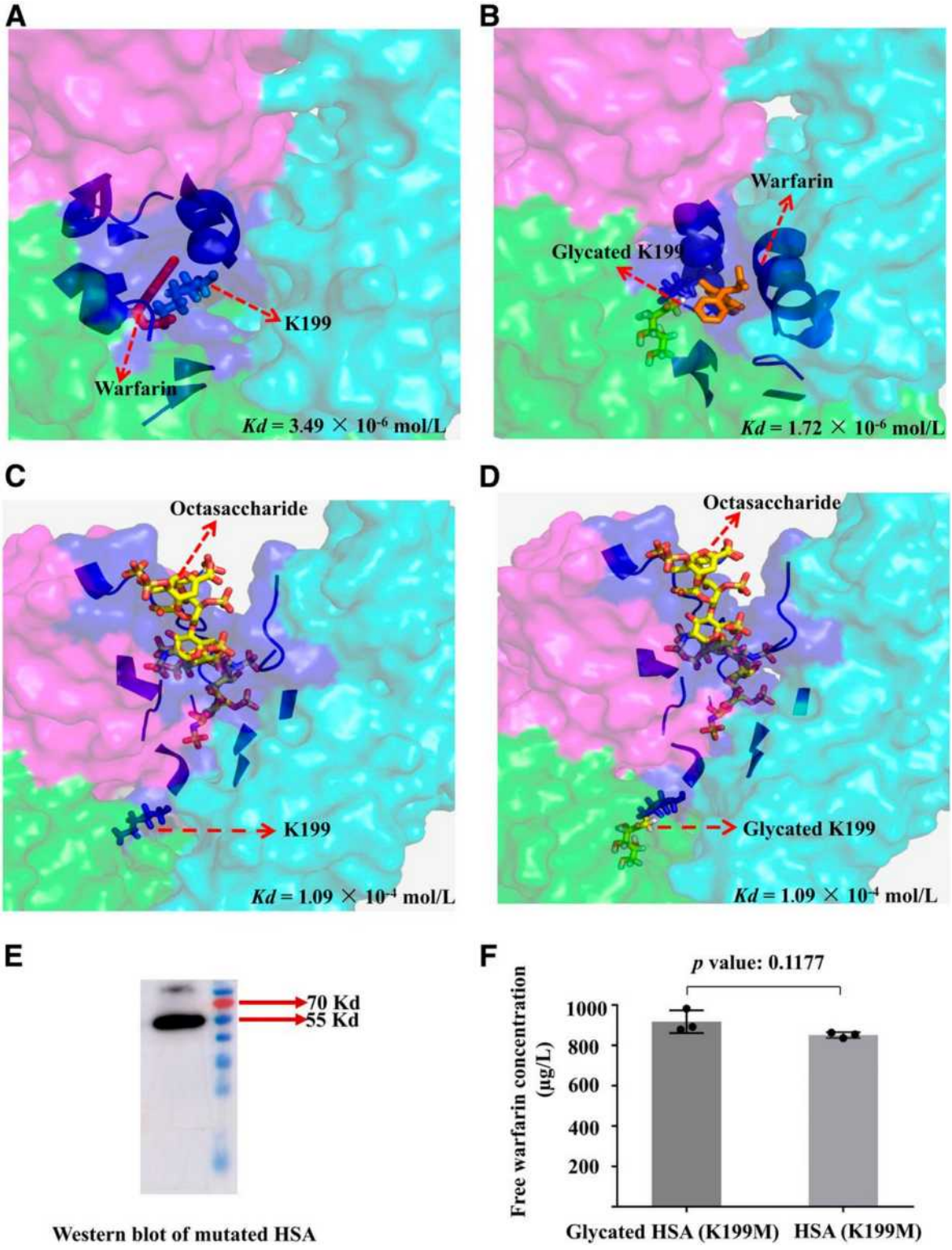


Figure 3—Docking simulation of native and K199-gHSA binding to anticoagulant drugs. Native HSA binds to warfarin (*A*), K199-gHSA binds to warfarin (*B*), native HSA binds to a heparin octasaccharide (*C*), and K199-gHSA binds to a heparin octasaccharide (*D*). Domains I, II, and III of HSA are in magenta, green, and cyan, respectively. The K199 residue is represented as a blue stick structure, warfarin is represented as orange sticks, and heparin octasaccharide is represented as a yellow stick structure. The amino acids involved in the interactions are shown in a blue ribbon structure. *E*: Western blot for purified HSA (K199M). *F*: Free warfarin concentration between nonglycated HSA (K199M) ($n = 3$) and gHSA (K199M) ($n = 3$).

while others sites increased or decreased the HSA binding affinity to warfarin to a less extent. To confirm that glycation on K199 was mainly responsible for the change of warfarin binding, we mutated K199 of HSA to M199 (Fig. 3*E*). Binding affinities of nonglycated K199M HSA and glycated K199M HSA to warfarin were compared by measuring free warfarin after incubating them with warfarin. No significant change in warfarin binding was observed (Fig. 3*F*).

The Different Concentrations of Free Anticoagulant Drugs Incubated in Plasma From Subjects With Diabetes and Healthy Subjects

For confirmation of the docking simulation result that the glycation of HSA had distinct effects on the binding to different anticoagulant drugs, warfarin and enoxaparin heparin were separately incubated with freshly collected plasma from subjects with diabetes and healthy subjects. Saturating binding study suggested that the binding between warfarin and HSA had reached equilibrium since 30 min after addition of warfarin to plasma (Supplementary Fig. 6). Concentrations of free drugs were determined by LC-MS/MS-MRM. As shown in Fig. 4*A*, free warfarin in diabetic plasma was averaged at 138 μg/L, which was significantly lower ($P < 0.005$) than the concentration of free warfarin in the plasma of healthy individuals (average concentration 222 μg/L). This finding demonstrated that the binding affinity of warfarin to HSA significantly increased in patients with diabetes compared with healthy subjects. It was a net result from multiple sites with decreased binding affinity, no change in affinity, or increased binding affinity after glycation during diabetes (Supplementary Table 3). Meanwhile, no significant change ($P = 0.17$) in the free drug concentration of enoxaparin was observed between subjects with diabetes (average concentration 6,174 μg/L) and healthy subjects (average concentration 6,498 μg/L), suggesting that the binding affinity of enoxaparin to HSA was not affected by glycation (Fig. 4*B*).

The Different Pharmacokinetic Profiles of Anticoagulant Drugs in Diabetic and Healthy Rats

The rat albumin (identification P02770 in the UniProt database) is structurally similar to HSA with regard to aspects of sequence (73%), disulfide bonds (identical), and shape (heart like). They have similar major drug-binding regions, and the crucial K199 residues are located in the subdomain IIA for both rat and human albumins. Therefore, the pharmacokinetic profiles of warfarin and enoxaparin heparin in diabetic and healthy rats were compared by measurement of free drug concentrations to further evaluate the above results. Before administration of anticoagulant drugs to rats, the albumin of diabetic rats was first confirmed to be glycated at a level similar to that in patients with diabetes (Supplementary Fig. 7). Moreover, the profiles of warfarin pharmacokinetics between diabetic and healthy rats were shown to be noticeably different (Fig. 4*C*). The concentrations of free warfarin in the plasma of diabetic rats were 6.06 μg/L at 8 h and 2.67 μg/L at 24 h. In the plasma of the control group, the values were 20.34 μg/L at 8 h and 7.59 μg/L at 24 h. A more than threefold decrease in free warfarin concentration was observed in diabetic rats compared with healthy rats.

Total warfarin in plasma from diabetic and healthy rats was also measured (Supplementary Fig. 8). By integration of the areas under the total warfarin and free warfarin curves, it turned out that total warfarin from diabetic rats was ~81% of that from normal rats, while free warfarin content from diabetic rats was ~51% of that from normal rats. These results suggested that both enhanced warfarin binding of gHSA and metabolism were reasons for decreased free warfarin in plasma from diabetic rats, but the impact of HSA glycation was more significant. In contrast, no significant differences were observed in the pharmacokinetics of enoxaparin between diabetic and healthy rats (Fig. 4*D*).

Preliminary Retrospective Clinical Study on the Efficacy of Anticoagulant Drugs in Patients With Diabetes

A preliminary retrospective clinical study that included 53 subjects was carried out to assess the clinical significance of the impact of HSA glycation on the efficacy of anticoagulant drugs in patients with diabetes. The sex, age, ethnicity, and other influencing factors were not significantly different between the patients with diabetes and subjects without diabetes. The INR 24 h after medicine of patients with diabetes taking warfarin was significantly lower than that of patients without diabetes ($P < 0.05$). For subjects taking heparin, INR was not significantly different between patients with diabetes and patients without diabetes (Fig. 4*E*). A lower INR in patients with diabetes means the blood coagulates more easily. Moreover, in subjects taking warfarin, the INR difference before and after medicine in patients without diabetes was significantly higher than that of patients with diabetes ($P < 0.05$). For subjects taking heparin, the INR difference showed no significant difference between patients with diabetes and patients without diabetes (Fig. 4*F*).

DISCUSSION

In this study, we have qualified and quantified the nonenzymatic glycation sites on HSA by developing a novel iTRAQ labeling and nLC-MS^n approach. The glycation level of K199 was found to have the most significant increase in subjects with diabetes, indicating that K199 was more sensitive to changes in plasma glucose concentration than any other amino acid residue. Moreover, the glycation of HSA remarkably enhanced the binding affinity of HSA to warfarin, which resulted in decreased plasma concentration of free warfarin and its altered pharmacokinetic profile. In addition, the in vitro and in vivo effects of HSA glycation on heparin were negligible. A retrospective clinical study was conducted to evaluate the clinical significance of HSA glycation in patients with diabetes and patients without diabetes administered anticoagulants and

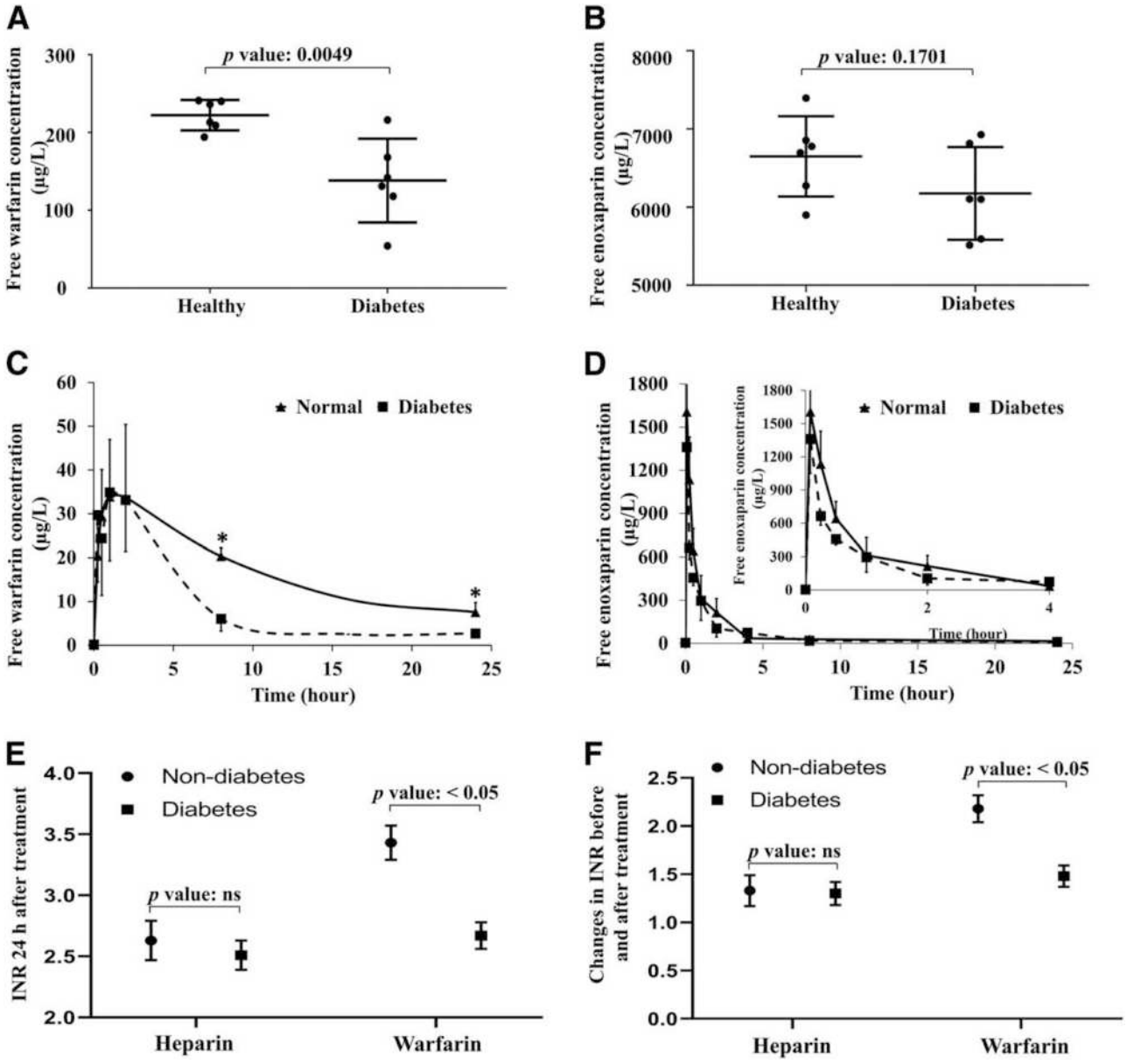


Figure 4—HSA glycation significantly affected the binding of HSA to warfarin but not to heparin. Free drug levels of warfarin (*A*) and heparin (*B*) after incubation for 30 min with freshly collected plasma from patients with diabetes ($n = 6$) and healthy subjects ($n = 6$). Statistical differences were compared by unpaired *t* test. Pharmacokinetics of warfarin (*C*) ($n = 6$) and heparin (*D*) ($n = 6$) were obtained by measuring the free drug concentrations in rat plasma at different time points. Change over time curves in the first 4 h for heparin are magnified. Solid lines represent the changes in the free drug concentrations over time in normal rats, while the dotted lines represent the changes in free drug concentrations over time in diabetic rats. *Time points at which the free drug concentrations were significantly different between normal and diabetic rats. The results of preliminary retrospective clinical study are shown as follows. *E*: Comparison of INR 24 h after medicine between subjects with diabetes ($n = 13$ for warfarin and $n = 13$ for heparin) and subjects without diabetes ($n = 14$ for warfarin and $n = 13$ for heparin). *F*: Comparison of the INR changes before and after treatment in subjects with diabetes ($n = 13$ for warfarin and $n = 13$ for heparin) and subjects without diabetes ($n = 14$ for warfarin and $n = 13$ for heparin).

showed that the efficacy of warfarin was decreased in patients with diabetes.

The mapping analysis of gHSA resulted in the successful identification of 49 glycation sites, which to our knowledge is the largest number of sites identified in one single study. Seven HSA glycation sites (R81, R117, R186, R257, K313, R410, and K541) were disclosed in vivo for the first time. At present, the measurement of HSA glycation available in the clinic is limited to the overall level of change, which does not provide adequate information for evaluating the specific condition of individual patients with diabetes. Herein, we have developed a novel and reliable HSA glycation quantitation method. Traditional iTRAQ labeling and MS/MS methods have accuracy problems of systematically

underestimating ratios because contaminate peptide precursor ions are unavoidably coselected and cofragmented with target precursor ions and then generate false reporter ions (28). Glycated peptides more easily generate neutral loss fragment ions, such as ions from loss of glucose molecules or water molecules during MS^2. They are suitable to be selected for further MS^3 fragmentation to generate sufficient backbone breakage ions for identification as well as specific reporter ions for quantitation. The new iTRAQ and MS^3 approach improved the specificity and accuracy of glycation quantitation. Differences in 21 glycation sites were determined, and two of these sites (K313 and K564) had never been quantified. The degree of glycation at most sites increased significantly under high blood glucose concentrations, as 19 sites showed significant differences between patients with diabetes and healthy subjects ($P < 0.05$). The most significantly changed site was K199 ($P < 0.01$). Patients with diabetes had an ~1.77-fold increase in glycated K199 compared with healthy subjects ($P < 0.0001$), as validated by analyzing more samples using LC-MS/MS-MRM. The underlying mechanism of why K199 is highly sensitive to changes in blood glucose concentration might be because the ε-amino group of the K199 residue has a low pK_a prone to glycation reaction and also because K199 is located in a basic amino acid residue–rich sequence (Lys_{195}-Gln-Arg-Leu-Lys_{199}). Therefore, glycation on K199 may possess two significant functions. First, it may represent a potential marker with high sensitivity and specificity for early diagnosis and for monitoring diabetes. Second, K199 is located in the subdomain IIA and is adjacent to the subdomain IIIA of HSA in the three-dimensional structure. Since these two subdomains play major roles in the binding of HSA to drug molecules, the glycation of K199 is speculated to have a significant impact on drug transport and efficacy in patients with diabetes.

Anticoagulant drugs are widely used in the clinic to treat or prevent blood clots in the veins and arteries. Due to the high incidences of cardiovascular disease in patients with diabetes, these patients are highly likely to be administered anticoagulant drugs. Warfarin and heparin are two major types of anticoagulants with distinctly different molecular weights, structures, and mechanisms of action, and both are transported by HSA in blood. Since only the unbound drug molecules have pharmacological relevance, the binding affinity of HSA to drug molecules will determine the free drug level and profoundly impact therapeutic efficacy. However, the impact of glycation in HSA on the efficacy of these two anticoagulants has never been evaluated, and this modification presents a potential risk for determining the choice and dosage of medications of patients with diabetes. Docking simulation suggested that K199 is located in the interaction domain between warfarin and HSA. Because the warfarin molecule is hydrophobic, K199 actually attenuates its binding affinity through its cationic amino group on the side chain. In the case of glycation, the binding affinity of gHSA to warfarin also became stronger when K199 was modified by glucose.

Both in vitro and in vivo experimental results have verified that HSA glycation noticeably increased the binding of HSA to warfarin but had little impact on heparin. For warfarin, the results showed that the free drug concentration of warfarin significantly decreased following incubation in diabetic plasma compared with healthy plasma in the in vitro experiment. Furthermore, the plasma concentration of free warfarin decreased much more quickly after the peak concentration in diabetic rats than it did in healthy rats, resulting in distinct pharmacokinetic profiles. It is expected that the anticoagulant effect of warfarin will be weaker in diabetic rats as a result of the stronger binding affinity of gHSA. In the case of heparin, neither the free drug concentrations, detected from in vitro incubation, nor the pharmacokinetic profiles exhibited significant difference. It is also noteworthy that measuring heparin concentration in vivo is extremely difficult due to its large molecular weight and heterogeneity. The LC-MS/MS-MRM method established herein offers a valuable approach for analyzing such polysaccharide drugs in disease model animals or in clinical trials.

These experimental observations were supported by a preliminary retrospective clinical study on the efficacy of anticoagulant drugs in patients with diabetes. INR is a basic index for assessing the efficacy of anticoagulant drugs. The INR value at 24 h after medication as well as the difference between INR at 24 h after medication and before medication was retrieved and analyzed. For patients taking warfarin, both INR at 24 h and the INR difference were lower in patients with diabetes than in patients without diabetes. Since INR is a consequence of the free anticoagulants present in plasma, it can be speculated that HSA binds to more warfarin molecules in patients with diabetes due to the high level of glycation. Because the increased activation of clotting factors and platelets makes the blood of patients with diabetes more hypercoagulable (29), the risk of developing a blood clot is higher for patients with diabetes with lower INR. Heparin binding was not affected by HSA glycation, and both INR 24 h and the INR difference were found to be equivalent in patients with diabetes and patients without diabetes. Moreover, heparin was almost completely metabolized after 8 h, while warfarin remained in plasma after 24 h. The long circulating and effective period of warfarin in plasma requires more careful monitoring of the INR of patients to prevent bleeding. In addition, warfarin is known to have a narrow therapeutic window. Combined with the significantly different pharmacokinetic behavior and efficacy of warfarin in patients with diabetes and patients without diabetes, additional caution must be taken when administrating warfarin as an anticoagulant to patients with diabetes. Heparin seems to be a safer and more effective anticoagulant in terms of the aspect of drug equivalence between patients with diabetes and patients without diabetes.

The glycation of circulating protein, especially HSA, the most abundant protein in plasma, is common in patients with diabetes. As a major transport protein, HSA is responsible

for delivering a variety of drug molecules. Glycation may affect the binding affinity of HSA to these drugs and consequently change their concentrations as free molecules in blood. In this case, therapeutic efficacy and drug safety are different for patients with diabetes and patients without diabetes. Because diabetes occurs frequently in elderly people, the coincidence of diabetes with other chronic diseases, such as cardiovascular disease and tumors, is considerable. In our study, two types of anticoagulant drugs administered to patients with diabetes were assessed and exhibited distinct trends. The drugs for treating other chronic diseases should be evaluated to provide additional specific guidance for patients with diabetes.

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Author Contributions. H.Q. completed the majority of experiments and wrote the manuscript. Z.W., L.C., and Q.Z. together designed the study. L.J. performed the docking. J.C. was involved in data analysis. M.S. and F.S. were involved in the LC-MS analysis. M.W. performed the retrospective clinical study. D.L. and X.X. developed the iTRAQ and LC-MS^3 approach. X.S. and X.Z. were involved in the in vitro and in vivo experiments. X.Y. and W.L. were involved in the mutation experiment. R.J.L. advised this study and revised the manuscript. L.C. and Q.Z. are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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