Supporting Information

Sample collection and pretreatment. The initial clinical samples were collected according to standard protocols by venipuncture for the analysis of hemoglobin A1C (HbA1C) using a 5 mL EDTA plasma collection tube. The anticoagulated whole blood was processed to give plasma by using centrifugation at 2000 × g for 10 min to separate the plasma from the cells. After centrifugation, the plasma was removed from the tube. A 1-2 mL portion of de-identified and remnant EDTA plasma was set aside and frozen at –20ºC until shipment. The frozen specimens were placed on dry ice during shipping and were stored at -80ºC upon receipt and until further use.

To isolate HSA and *in vivo* glycated HSA from plasma or control serum, 20 μL of a plasma or serum sample was added to a Vivaspin 6 spin-filter column that contained a fresh 400 μL portion of a 50% packed resin slurry with polyclonal anti-HSA antibody fragments immobilized onto cross-linked agarose beads. The initial buffer in this slurry was given by the manufacturer as being a proprietary pH 7.0-7.5 Tris buffer with a low salt concentration. The antibody-containing beads had a reported minimum binding capacity of 2 mg HSA/mL resin. The sample/resin mixture was placed on a rotary shaker for 15 min at room temperature. After this incubation step, the spin-filter column was centrifuged at 400 × g for 2 min and washed with 200 μL of pH 7.4, 0.1 M Tris buffer for 1 min at 400 × g. This washing procedure was repeated two times to reduce non-specific binding of sample components to the resin. To elute the retained HSA and glycated HSA, the spin-filter column was washed six times with 200 μL of pH 2.8, 0.1 M glycine/HCl buffer for 2 min at 400 × g, with the collected HSA/glycated HSA fractions being pooled. Immediately after each elution step, the collected fractions were adjusted

to approximately pH 7.0 by adding pH 8.0, 1 M Tris buffer. If additional HSA/glycated HSA was needed for other experiments (e.g., protein assays or mass spectrometry, as used in ongoing studies), this process was repeated for several cycles, each with a fresh spin-filter column, and the isolated proteins were pooled prior to use or further analysis.

The collected HSA/glycated HSA for a given sample was placed into a sterile Slide-A-Lyzer dialysis cassette (10 kDa MW cutoff; 0.1–0.5 mL sample volume from Thermo Scientific, Rockford, IL) and dialyzed twice against two portions of 2.5 L water with gentle stirring for 2 h at room temperature. The sample was dialyzed against water a third time without stirring at 4°C for 14–18 h, with the protein then being lyophilized and stored at -80°C for later use in immobilization or analysis (Note: An alternative procedure that can be used is to dialyze the sample against an appropriate buffer and then use the soluble protein directly for immobilization). To determine if these isolation steps affected the activity of HSA, binding studies based on ultrafiltration, as described later in this section, were performed before and after representative samples of the control HSA and glycated HSA were treated with the low pH buffer, dialyzed and lyophilized. It was found that this process gave no significant changes, at the 95% confidence level, in the binding of HSA with the sulfonylurea drugs that were examined in this study.

Ultrafiltration studies. Ultrafiltration was performed using Centrifree micropartition devices (30 kDa MW cutoff, 0.15–1.0 mL sample capacity) from Amicon (Danvers, MA, USA). These devices were used along with a 5702RH temperature-controlled centrifuge from Eppendorf (New York, NY) and a fixed-angle centrifuge rotor from VWR (West Chester, PA). The flow-injection analysis experiments that were used to measure the drug content in each filtrate were carried out by using a Jasco 2000 HPLC

system (Easton, MD, USA) that contained a DG-2080-53 three solvent degasser, two PU-2080 isocratic pumps, an AS-2057 autosampler equipped with a 100 μL sample loop (operated in the partial loop injection mode), and a UV-2075 absorbance detector.

For the ultrafiltration-based binding studies, each mixture of tolbutamide and HSA or glycated HSA was incubated at 37ºC for 45 min; the use of longer incubation times (i.e., up to 90 min) did not produce any significant change in the final results. Prior to ultrafiltration, the membrane in each Centrifree ultrafiltration device was rinsed three times with water and pH 7.4, 0.067 M potassium phosphate buffer to reduce interferences by any contaminants or preservatives that may have been present in the device. The ultrafiltration device was spun for at least 15 min at 1,500 × g after rinsing to avoid dilution errors due to the presence of any remaining rinse solution in the device; the volume of rinse solution that remained after this step was less than ~10 μL. The ultrafiltration experiments were carried out by placing 1 mL of each drug/protein mixture into the ultrafiltration device and spinning this mixture at 1,500 × g for 25 min at 37 ºC. The filtrate was collected and analyzed for its drug content by making triplicate 5 μL injections onto a flow-injection analysis system at 0.5 mL/min and in the presence of pH 7.4, 0.067 M potassium phosphate buffer. The elution of tolbutamide was monitored at 227 nm. A correction for any contaminants or preservatives remaining in the filtrate was made by carrying out similar measurements on filtrates for 0.5 mL samples that contained only glycated HSA in pH 7.4, 0.067 M potassium phosphate buffer.

Notes on other experimental methods. In the bicinchoninic acid (BCA) protein assay that was performed to determine the protein content of each HSA/glycated HSA support, there was no measureable difference in the response for standards based on

normal HSA versus glycated HSA in this assay. Although sulfonylurea drugs are weak acids with pK_a values of 5.2-6.2, a change in pH of less than 0.05 units was seen in the pH 7.4 buffer when these drugs were present even at the highest concentrations that were utilized in the frontal analysis and zonal elution experiments.

In the frontal analysis studies shown in Figure 2, the relative standard deviations for the measured binding capacities ranged from only ± 0.01% to ± 5.5% (average, ± 1.1%) for triplicate measurements at the sixteen tested drug concentrations. Similar precisions were obtained for the other columns and drugs that were examined in this study. The zonal elution results in Figure 3(a-b) gave retention factors with relative standard deviations that ranged from ± 0.08% to ± 0.93% (average, ± 0.24%) and ± 0.60% to ± 5.0% (average, ± 1.9%) for triplicate injections of *R*-warfarin or L-tryptophan as the injected probe, respectively, and over seven concentrations of tolbutamide or acetohexamide in the mobile phase for the *in vivo* glycated HSA columns. Similar precisions were again obtained with the other drugs that were examined.

Analysis of glycation products and glycation patterns. Both qualitative and quantitative studies based on MALDI–TOF-MS were used to examine the types and levels of modification that occurred for the *in vivo* glycated HSA, as based on methods described in Refs. 1s-4s. The results are summarized in Tables 1s and 2s for clinical sample HSA-CS1. These data include the regions of the *in vivo* glycated HSA that were found to be glycated, as well as the suspected residues at which these regions were modified and the types of modifications which may have been present.

The data in Tables 1s and 2s show that there were several modifications in this sample of *in vivo* glycated HSA that occurred at or near Sudlow sites I and II.[1s-5s]

Regions that were found to be modified and that occurred around these sites included residues 160-181, 189-208, 196-212, 275-286 and 286-297, all of which were located within Sudlow Site I. In addition, residues 359-376, 415-436 and 476-500 are located at or near Sudlow site II and were also found to be modified during *in vivo* glycation. These results agree with those seen in this report for the binding studies using the same preparation of *in vivo* glycated HSA, in which shifts in affinity were noted for several of the sulfonylurea drugs at Sudlow sites I and II when comparing this protein preparation to normal HSA.

Table 1s summarizes the results of quantitative studies that made of use of MALDI-TOF-MS and $^{16}O/^{18}O$-labeling with the *in vivo* glycated HSA. In this case, peptides from residues 5-20, 7-17, 94-106, 275-286 and 286-297, among others, were found to be modified and have elevated $^{16}O/^{18}O$ ratios versus control samples that were prepared using normal HSA. These regions exhibited considerable overlap with the regions of modification that were previously seen for *in vitro* glycated HSA samples that were prepared under similar conditions and used in the binding studies described in Refs. 25-27 (as cited in the main body of the text).

The glycation products formed on the *in vivo* glycated HSA were identified by determining the peptide ions with mass values that were unique to the *in vivo* glycated HSA samples.[3s,4s] The corresponding modifications were then determined by comparing these masses with a theoretical list of modified peptides that might result from a glycated HSA digest. When this approach was used, several early and late-stage glycation adducts were assigned to the *in vivo* glycated HSA sample, as listed in Table 2s. For this sample, 18 peptides were found to be modified to form a mixture of early glycation products and

AGEs. Seven of those modified peptides were also found to be modified in prior work that used preparations of *in vitro* glycated HSA, with another two regions showing significant areas of overlap. For example, modifications were found in regions 189-208, 275-292, 312-321 and 542-585 for both the *in vivo* glycated HSA and previously-examined samples of *in vitro* glycated HSA.[1s-4s]

Two different peptides (K190/K199) were found to be modified on the *in vivo* glycated HSA to produce CML and LL (see list of abbreviations and full names for these products that are provided in Tables 1s and 2s); previous studies using *in vitro* glycated HSA with various stages of glycation showed modifications assigned to K199 due to FL at early stages of glycation and due to Pyr during later stages of glycation.[2s,4s] These results corroborate that K199 is prone to both *in vivo* and *in vitro* glycation, in agreement with various reports that have found K199 to be a major glycation site on HSA.[1s-4s,6s] In addition, K276 and K281 within residues 275-286 were found to be modified to form two Pyr or C2-CML+AL in the *in vivo* glycated HSA. In previous studies using *in vitro* samples, K281 was modified to form FL-related modifications such as FL-1H_2O/FL-2H_2O, as well as CEL and/or Pyr.[4s] This information is consistent with other reports that have denoted K281 as another major modification site in glycated HSA.[3s,6s] Within the region 5-20, two peptides (R10/K12) were found to be modified during *in vivo* glycation to produce a FL/G-H1 precursor or CML+3,4-dihydroxy-2-imidazoline. This same region was found to be modified to form FL on K12 when using an *in vitro* glycated HSA sample in Ref. 4s.

References

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(3s) Wa, C.; Cerny, R.L.; Clarke, W.A.; Hage, D.S. *Clin. Chim. Acta* 2007, 385, 48-60.

(4s) Barnaby, O.S.; Cerny, R.L.; Clarke, W.; Hage, D.S. *Clin. Chim. Acta* 2011, 412, 277-285.

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Table 1s. Glycation versus control (G/C) indices obtained through $^{16}O/^{18}O$- labeling and MALDI-TOF-MS and indicating the presence of an increase in glycation-related modifications for *in vivo* glycated HSA versus normal HSA as a control[1s,2s]

Residues (Digest)	**Modification site**	**Potential adducts[a]**	**G/C Index[b]**
275-286 (Trypsin)	K_{276}/K_{281}	CML & Pyr/Pyr	1.9 (± 0.2)
557-565 (Glu-C) 561-574 (Trypsin)	K_{564}	FL/AFGP & FL/CML	1.7 (± 0.2)[c]
286-297 (Glu-C) 274-286 (Trypsin)	K_{286}	AFGP	1.9 (± 0.2)[c]
566-585 (Glu-C) 561-574 (Trypsin)	K_{573}/K_{574}	FL/CML & FL/FL	1.7 (± 0.2)[c]
94-106 (Lys-C)	R_{98}	G-H1	2.0 (± 0.1)
5-20 (Lys-C) 7-17 (Glu-C)	R_{10}/K_{12}	FL/G-H1 precursor; CML/3,4-dihydroxy-2-imidazoline	1.3 (± 0.1)[d]
476-500 (Lys-C) 480-492 (Glu-C)	R_{484}/R_{485}	ArgP/MG-H1	1.8 (± 0.1)[d]

[a]Abbreviations: AFGP, 1-alkyl-2-formyl-3,4-glycol-pyrole; ArgP, argpyrimidine; CML, N_ε-carboxymethyl-lysine; FL, fructosyl-lysine; G-H1, hydroimidazolone derived from glyoxal, or N_ε-(5-hydro-4-imidazolon-2-yl)ornithine; MG-H1, hydroimidazolone derived

from methylglyoxal, or N_{ε}-(5-hydro-5-methyl-4-imidazolon-2-yl)ornithine; Pyr, pyrraline.

[b]The values in parentheses represent ± 1 standard error of the mean.

[c]This result was obtained for the tryptic digest. The result for K286 is the value obtained for residues 275-286.

[d]This result was obtained for the Glu-C digest.

Table 2s. Suspected modification sites found in various digests of *in vivo* glycated HSA

Residues (digest)	**Suspected sites (modification)[a]**
275-286 (Trypsin)	K276 & K281 (Pyr+Pyr) K276 & K281 (C2-CML+AL)
415-436 (Trypsin)	K432 (FL-H_2O) & R428 (oxMG-H1)
542-560 (Trypsin)	K545 & K557 (Pyr+AL)
189-208 (Glu-C)	K190 & K199 (CML+LL)
278-292 (Glu-C)	K281 & K286 (AL+AL)
312-321 (Glu-C)	K313 or K317 (CML); K313 & K317 (FL-2H_2O+AFGP)
359-376 (Glu-C)	K372 (FL-1H_2O)
566-585 (Glu-C)	K573/K574 (FL-1H_2O+C2-CML or CML+FL-2H_2O; FL+FL)
557-565 (Glu-C)	K557, K560 or K564 (FL-1H_2O+AFGP)
557-571 (Glu-C)	K560 & K564 (FL+CML)
5-20 (Lys)	R10 & K12 (FL+C2-G-H1 or CML+3,4-dihydroxy-2-imidazoline)

94-106 (Lys)	R98 (G-H1)
160-181 (Lys)	K162 & K174 or R160 & K162/K174 (Pyr+Pyr; CEL+FL-1H_2O; FL+MG-H1; CEL+3-DG-H1; CEL+THP)
160-174 (Lys)	R160 & K162 (AFGP+MG-H1 or FL+3,4-dihydroxy-2-imidazoline)
196-212 (Lys)	K199/K205 & R197/R209 (C2-CML+oxMG-H1 or G-H1+oxMG-H1)
476-500 (Lys)	R484 & R485 (ArgP+oxMG-H1)

[a]Abbreviations: AFGP, 1-alkyl-2-formyl-3,4-glycol-pyrole; AL, allysine; ArgP, argpyrimidine; C2-CML, N_ε-carboxymethyl-lysine precursor (C2-imine); C2-G-H1, precursor for hydroimidazolone derived from glyoxal (C2-imine); CEL, N_ε-carboxyethyl-lysine; 3-DG-H1, hydroimidazolone derived from 3-deoxyglucosone; CML, N_ε-carboxymethyl-lysine; FL, fructosyl-lysine; FL-xH_2O, dehydrated fructosyl-lysine; G-H1, hydroimidazolone derived from glyoxal, or N_ε-(5-hydro-4-imidazolon-2-yl)ornithine; LL, 3-(*N*'-lysino)-lactic acid; MG-H1, ; oxMG-H1, oxidized N_ε-(5-hydro-5-methyl-4-imidazolon-2-yl)ornithine; Pyr, pyrraline; THP, tetrahydropyrimidine.

Figure Legends

Figure 1s. Typical chromatograms obtained at 0.5 mL/min by carrying out frontal analysis on an affinity microcolumn containing glycated HSA-clinical sample 2 (glycated HSA-CS2) and using solutions that contained 1.0, 2.5 or 5.0 μM acetohexamide (bottom-to-top). Other conditions are given in the text.

Figure 2s. Fit of frontal analysis data obtained for acetohexamide on the glycated HSA-CS1 column when using a one-site model, as described by eq (1s).[7s] Each data point represents the average of three measurements for m_{Lapp}, the moles of analyte required to reach the mean point of the breakthrough curve at a given molar concentration of the applied analyte. The error bars represent ± 1 S.D. for this value and ranged in size from ± 0.01-5.5% (average, ± 1.1%). Terms: molar concentration of applied analyte A, [A]; K_{a1} and K_{a2}, association equilibrium constants for the analyte at binding sites 1 and 2; m_{L1} and m_{L2}, total moles of binding sites 1 and 2 in the column.

Figure 3s. Zonal elution results were generated at 0.5 mL/min on the glycated HSA clinical sample 1 column (glycated HSA-CS1) by using 5 μM *R*-warfarin as the injected probe and mobile phases that contained 0, 1, 5, 10, 15 or 20 μM acetohexamide (bottom-to-top). Other conditions are given in the text.

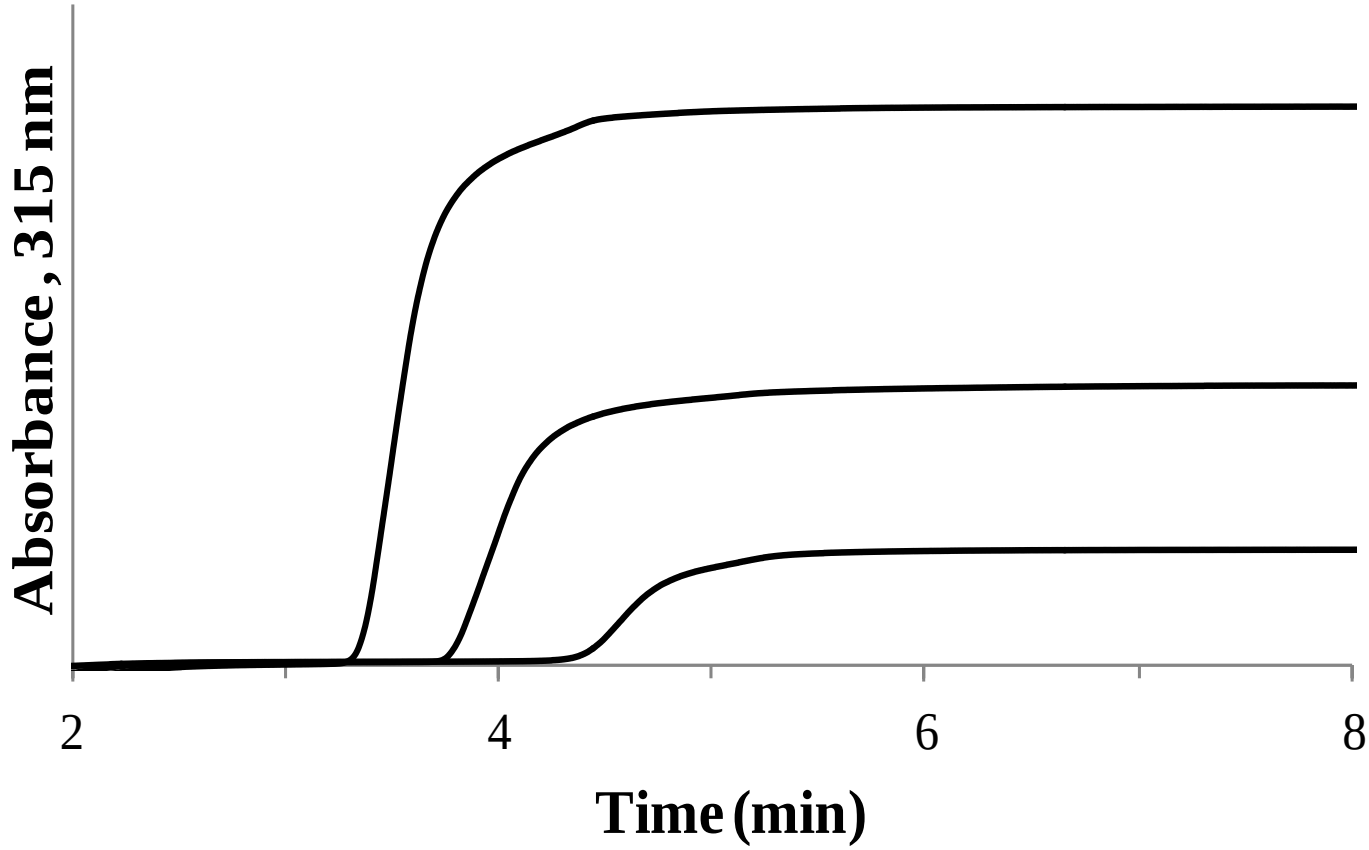

Figure 1s

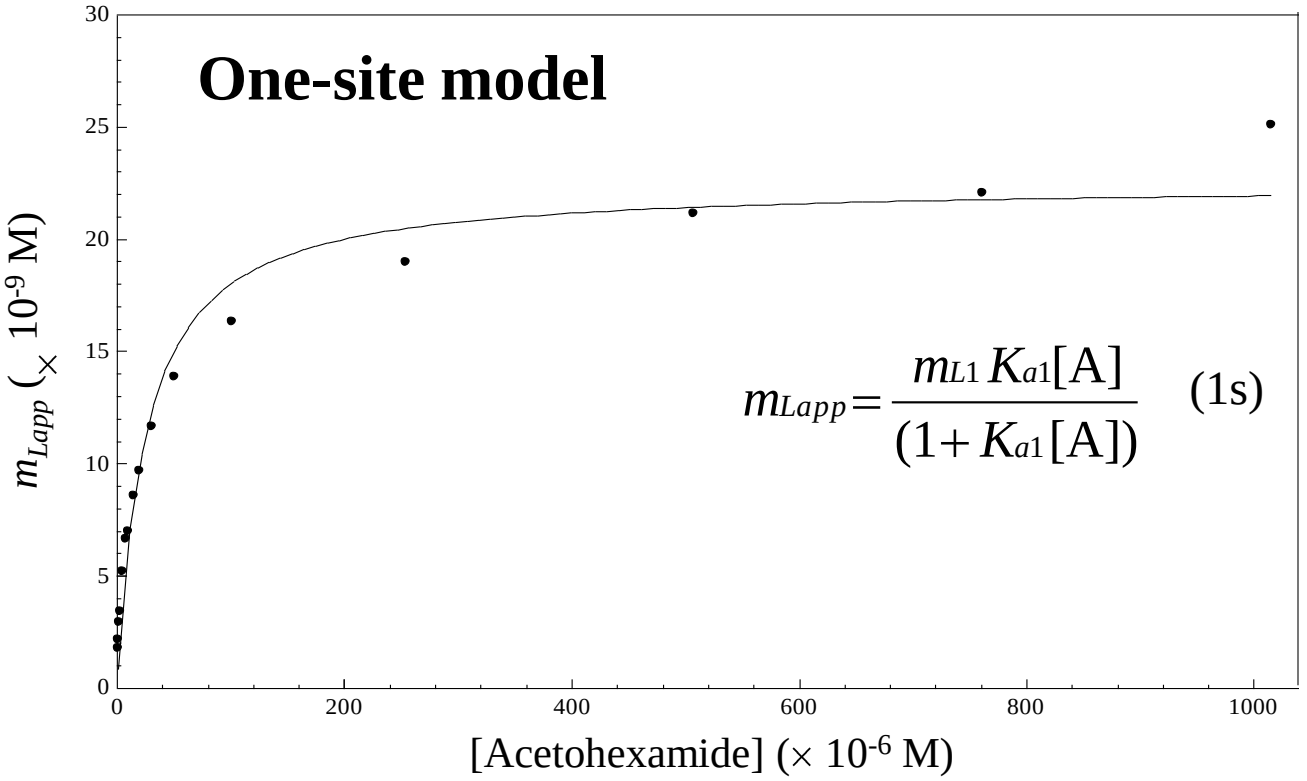
One-site model
$m_{Lapp} = \frac{m_{L1} K_{a1}[A]}{(1 + K_{a1}[A])}$ (1s)
m_{Lapp} (× 10^{-9} M)
[Acetohexamide] (× 10^{-6} M)
0
5
10
15
20
25
30
0
200
400
600
800
1000

Figure 2s

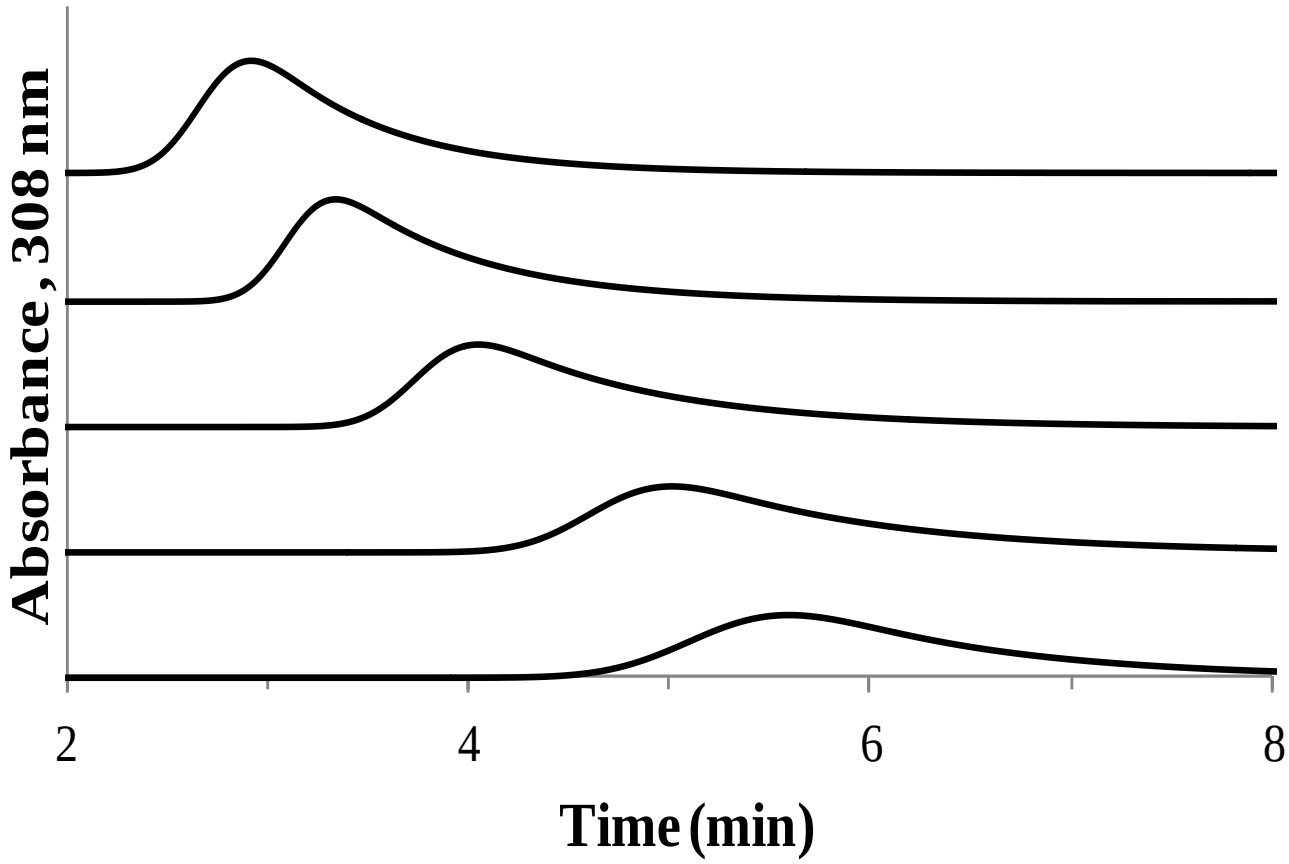

Figure 3s