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COMPETITIVE BINDING OF DRUGS TO THE MULTIPLE BINDING SITES ON HUMAN SERUM ALBUMIN
A calorimetric study

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Abstract

A multiple-site competitive model has been developed to evaluate quantitatively the equilibrium competition of drugs that bind to multiple classes of binding sites on human serum albumin (HSA). The equations, which are based on the multiple-class binding site model, assume that competition exists at individual sites, that the binding parameters for drug or drug competitor pertain to individual sites, and also that the binding parameters for drug or competitor at any given site are independent of drug or competitor bound at other sites. For the drug-competitor pairs, ethacrynic acid (EA) -caproic acid (C6), -lauric acid (C12), and -palmitic acid (C16), the reaction heat of EA binding to HSA was measured in the absence and the presence of fatty acids at the molar ratio of 3:1 with HSA at pH 7.4 and 37°C by isothermal titration microcalorimetry. The calorimetric titration data induced by the presence of fatty acids were directly compaired to the computer simulation curves by the corresponding multiple-site competititve models, which were precedently calculated from binding parameters of EA and fatty acids. In the case of EA-C12 or -C16 competitive binding, EA binding at the first and the second classes of binding sites on HSA were instantaneously inhibited by C12 or C16, resulting that the binding constant of the first class of binding sites of EA were decreased and that the second class of binding sites on HSA entirely disappeared. In the competition between EA and C6, the first class of binding sites of EA was diminished by C6, resulting in the decrease of the binding constants and the number of binding sites in the first class of EA, whereas, the second class of binding sites was unaffected. The multiple-site competitive model assuming site-site competition could be directly comparable to the calorimetric data and be suitable to account for the competitive processes for drugs bound to the multiple-class of binding sites on HSA.

Keywords: calorimetry, competitive drugs binding, ethacrynic acid, fatty acids, HSA

Introduction

Most of ionic drugs are bound to multiple classes of binding sites on human serum albumin (HSA) by relatively weak forces such as hydrophobic, van der Waals, or hydrogen bonding interaction. Consequently, binding constants generally are small and binding is readily reversible. Acidic drugs commonly bind to HSA, competing substances such as endogeneous fatty acids and other drugs may displace one another from their binding sites [1–3].

In a series of thermodynamic studies on the interaction of ionic drugs with human blood components, human plasma protein, HSA, α_1-acid glycoprotein, and

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erythrocytes, we have demonstrated the good-to-excellent linear relationships in the enthalpy and entropy changes for ionic drug binding to the common binding sites of blood components by isothermal titration calorimetry [4–6], and also reported that the binding of consecutive mono- and di-aliphatic carboxylic acids to HSA resulted in correlationships between thermodynamic functions and alkyl-chain length/hydrophobicity. HSA binding sites for ionic drugs and fatty acids were then classified into three groups of S1, S2 and S3 by the molecular recognition of HSA [7–9]. Group S1 included high-affinity binding sites for site II-bound drugs [10, 11], such as ibuprofen, flufenamic acid, and ethacrynic acid, and short- or medium-length alkyl-chain fatty acids; group S2 included low-affinity binding sites of site II-bound drugs and long-length alkyl-chain fatty acids; and group S3 contained the high-affinity bindings sites for site I-bound drugs [10, 11], such as phenylbutazone, oxyphenylbutazone, and warfarin, and long-length alkyl-chain fatty acids.

It is therefore of great importance to elucidate the processes of competitive binding of drugs to HSA by fatty acid and/or other drugs. Graphical analyses such as Scatchard plots and Lineweaver-Burk plots indicate that the displacement from the high-affinity sites is apparently competitive in the complete displacement of each ligand by the other occurs. However, the competitive binding does not always occur at the same binding sites or same region on HSA molecule, and the displacement from the numerous low-affinity sites cannot be disregarded especially for drug-competitor paires bind to multiple classes of binding sites on HSA. We have proposed a new competitive binding model, which formulates the mathematics in terms of binding constants and the number of binding sites of individual classes on HSA for drug and competitor pairs, assuming that all classes of binding sites preexist and every class of a competitor competes independently for available classes of a drug. The computer simulations of the multiple-site competitive models were directly compaired to the calorimetric titration data for the reaction heat of drug binding of drug with HSA. We have chosen in this report the competitions between ethacrynic acid and caproic acid, lauric acid, and palmitic acid on the base of the classification of HSA binding sites, to verify the competitive binding of acidic drug with fatty acids to HSA.

Experimental

Materials

Defatted human serum albumin (Fraction V, less than 0.005% fatty acids, HSA) and fraction V human serum albumin (HSAV) obtained from Sigma Chemical (St. Louis, MO, USA) was used without further purification. HSA was dissolved in a 1/30 M phosphate buffer, physiological pH 7.4 to give the concentration initially of 0.2% w/v. Caproic acid (C6), lauric acid (C12) and palmitic acid (C16) were supplied by Applied Science Laboratories Inc. (State College, PA, USA), and added to HSA buffer solution at the molar ratio of 3:1 with HSA. Ethacrynic acid (EA) was purchased from Sigma Chemical. A relatively concentrated solution of EA dissolved in 0.01 M NaOH was prepared and diluted with 1/30 M phosphate buffer, pH 7.4 to provide the desired initial concentration (10^{-6}~10^{-3} M).

Microcalorimetry

Calorimetric titrations were carried out at 37±0.001°C using isothermal microcalorimeters, a TAM 2277-204 with flow-mix system (ThermoMetric, Sweden) [12] and a differential flow microcalorimeter with twin-cell flow system [13]. The instruments were calibrated both electrically and chemically with a sensitivity on the 3 ~10 μW scale. The reaction solutions were introduced at equal flow rates (0.1 mL min^{-1}) into the calorimeter through Tygon tubing with a peristaltic pump (Gilson minipuls 2, Villers-Le-Bel, France). Detailed experimental procedures were as reported [9].

The concentration of HSA in the initial and final calorimetric solutions were determined by Lowry method [14] and/or the UV absorption at 278 nm using an extinction coefficient E (1% 1 cm) of 5.30 and the molecular mass of 69 000. The total concentrations of C6, C16 and C12 were determined by the radio activities of ^{14}C-labeled fatty acids, using a Packard Tri-Carb 2660 liquid scintillation counter (Downers Grove, IL, USA) [7].

Computer analysis and simulation

The direct analysis of calorimetric titration curves for drug ligand binding to protein allowed the determination of the binding enthalpy change (ΔH_{app}) of drug ligand and the apparent binding constant (K_i) with the number of binding sites (n_i) in the *i*th class of binding sites, as follows:

$$Q = \Delta H_{app} F_r [P_t] \sum_{i=1}^{m} \frac{n_i K_i [A_f]}{1 + K_i [A_f]} \quad (1)$$

where Q is reaction heat (μW) at the constant flow rate (F_r), and $[P_t]$ and $[A_f]$ are the total concentration of protein and the unbound concentration of drug ligand, respectively. The binding and thermodynamic parameters, K_i, n_i and ΔH_{app}, for single-class (m=1) and two-class (m=2) binding model were computed from the actual calorimetric data with an iterative non-linear least-squares regression program for minimizing the value of $\Sigma(Q_{exp}-Q_{calc})^2$. The initial value of ΔH_{app} was obtained experimentally form the slope of the initial linear part of a calorimetric titration curve. The molar enthalpy change of protein for complete binding of all sites (ΔH_{max}) which is estimated from a plateau value of a calorimetric titration curve represents a composite quantity resulting from the algebric summation of the intrinsic enthalpy change of the *i*th class of binding site (ΔH_i).

$$\Delta H_{max} = \sum_{i=1}^{m} n_i \Delta H_i \quad (2)$$

In the competitive binding between drug A and competitor B to protein P, both A and B bind to the competitive classes (i=1~l) and the non-competitive classes (j=1~m) of binding sites on P molecule to form two kinds of complexes, PA and PB, respectively. The concentrations of PA and PB, ([PA] and [PB], respectively) are expressed by following equations.

$$\frac{[PA]}{[P_t]} = \sum_{i=1}^{l} \frac{K_{Ai}[A_f]}{1 + K_{Ai}[A_f] + K_{Bi}[B_f]} + \sum_{j=1}^{m} \frac{K_{Aj}[A_f]}{1 + K_{Aj}[A_f]} \quad (3)$$

$$\frac{[PB]}{[P_t]} = \sum_{i=1}^{l} \frac{K_{Bi}[B_f]}{1 + K_{Bi}[B_f] + K_{Ai}[A_f]} + \sum_{j=1}^{m} \frac{K_{Bj}[B_f]}{1 + K_{Bj}[B_f]} \quad (4)$$

where $[A_f]$ and $[B_f]$ are unbound concentrations of A and B, respectively. The total concentrations of A, B and P, ($[A_t]$, $[B_t]$ and $[P_t]$, respectively) are given by Eqs (5)–(7). $K_{A_{i,j}}$ and $K_{B_{i,j}}$ represent the binding constants of *i*th or *j*th individual binding site of drug and competitor, respectively. The assumption consistent with the multiple-site binding model is that the binding constants for drug or competitor at any given site are independent of drug or competitor bind at other sites on P, HSA.

$$[A_t]=[A_f]+[PA] \quad (5)$$

$$[B_t]=[B_f]+[PB] \quad (6)$$

$$[P_t]=[P_f]+[PA]+[PB] \quad (7)$$

Thus, the value of Q is proportional to $[PA]$ and $[PB]$ at a fixed $[P_t]$ and flow rate F_r by use of Eqs (3) and (4).

$$Q = (\Delta H_{A,\,app}[PA] + \Delta H_{B,\,app}[PB])F_r \quad (8)$$

where, $\Delta H_{A,app}$ and $\Delta H_{B,app}$ are the binding enthalpy changes of *PA* and *PB*, respectively. Theoretical curve of heat quantities as a function of total drug concentrations ($[A_t]$) was generated by computer according to the multiple-site competitive models and compared to the experimental calorimetric data.

Results and discussion

Effect of fatty acids on the binding of EA to HSA

Figure 1 shows the reaction heat of EA binding to HSA in the absence and the presence of constant concentrations of C6, C12 or C16 at the molar ratio of 3:1 with HSA. The calorimetric data are represented best as a plot of the heat of binding per mole of HSA (kJ mol^{-1}) *vs.* the total EA concentration in the final calorimetric solution. In our previous paper [9], it was reported that EA was bound to HSA at least two classes of independent binding sites containing one high-affinity site (n_1=1.1±0.2) with the binding constant of K_1=1.65±0.14×10^5 M^{-1} (the first class of binding sites) and some low-affinity binding sites (n_2=3.2±0.6) with the value of K_2=3.89±0.76×10^3 M^{-1} (the second class of binding sites) and that the value of ΔH_{app} for EA binding to HSA was –71.0±3.4 kJ mol^{-1}. Thus, the calorimetric data for EA binding to HSAV and to HSA mixing with C6, C12 and C16 was examined by computer-fitting to both single- and two-class binding models using a fixed value of ΔH_{app}=–71.0 kJ mol^{-1}, and the best-fit values of binding parameters were listed in Table 1, together with MSE (a mean of squared prediction errors).

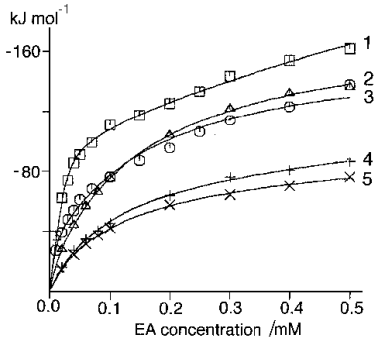

Fig. 1 Effect of fatty acids on the reaction heat of ethacrynic acid (EA) binding to defatted human serum albumin (HSA) at 37°C and pH 7.4 in 1/30 M phosphate buffer. The initial concentration of albumin were ca. 0.2% (w/v) and the final concentrations in the calorimetric solutions were determined to be 1.41 to 1.50×10^{-5} M. Points show the experimental data and solid lines represent computer-generated best-fit curves assuming single- or two-class binding model. Curves of 1, 2, 3, 4 and 5 are the reaction heat of EA binding to HSA, fraction-V human serum albumin (HSAV), HSA in the presence of caproic acid (C6), lauric acid (C12), and palmitic acid (C16) at the molar ratio of 3:1 with HSA, respectively

In the presence of C12 or C16, the heat of EA binding to HSA markedly reduced, resulting in n_1=1.1 with the apparent binding constants of $1.0\sim7.0\times10^4$ M^{-1}, which was 1/10~1/2 less than those in the absence of C12 and C16 (Table 1). The apparent binding parameters of the second class of binding sites were unable to be calculated from two-class binding model. A better fit to one-class binding model was obtained rather than to the two-class binding model. The binding of EA to HSA was clearly inhibited by C12 and C16, where the first class of binding sites was reduced and the second class of binding sites was entirely disappeared. On the other hand, EA binding to HSA was also reduced by the presence of C6. The values of the binding parameters in the first class of binding sites for EA were decreased to be K_1= 7.89×10^4 M^{-1} and n_1=0.3, although the apparent binding parameters in the second class were almost equal to those in the absence of C6.

Simulations of EA-C16 and -C12 competitive binding to HSA

The binding of C12 and C16 as the competitors to HSA were also characterized by one high-affinity site and some low-affinity sites for which K_1=4.98± 0.05×10^5 M^{-1} (n_1=0.9) and K_2=9.01±0.07×10^3 M^{-1} (n_2=5.6) of C12 binding to HSA with the binding enthalpy change (ΔH_{app}=–42.7 kJ mol^{-1}) and K_1=2.17± 0.03×10^6 M^{-1} (n_1=1.2) and K_2=2.27±0.11×10^4 M^{-1} (n_2=6.7) or C16 binding to HSA with ΔH_{app} (=–38.9 kJ mol^{-1}) were determined by using microcalorimetry [7]. The competitions between drug and competitor are not usually account for by only one site binding but rather by a concomitant binding at a high-affinity site to a varying number of sites with lower-affinity. For the case of the competitive binding between EA and C16 to HSA, both of drug A and competitor B bound to two classes of binding sites on HSA molecule to form the complexes of PA_1 and PA_2 for EA binding to the first and the second class of binding sites of HSA, respectively, and those of PB_1

Table 1 Binding and thermodynamic parameters of ethacrynic acid binding to HSA

HSA	K_1/ 10^5 M^{-1}	n_1	K_2/ 10^3 M^{-1}	n_2	$-\Delta H_{max}$/ kJ mol^{-1}	MSE
HSA	1.650*	1.1*	3.89*	3.2*	308*	–
HSA V	0.858	0.9	4.45	2.4	248	0.052
HSA in the presence of C6	0.789	0.3	5.95	2.1	173	0.071
HSA in the presence of C12	0.735	1.1	–	–	79.9	0.067
HSA in the presence of C16	0.102	1.1	–	–	71.0	0.079

* mean values of the best-fit parameters computed from three calorimetric titration curves [9]

and PB_2 for C16 binding to the first and the second class of binding sites of HSA, respectively. The one-site competitive model was initially simulated, assuming that the competitive binding between EA and C16 was dependent on only one class of binding sites and that other classes of binding sites were independent of the presence of the competitor C16. The one-site competitive binding model was classified into following four types expressed by the reversible interactions: $PA_1 \leftrightarrow PB_1$, $PA_1 \leftrightarrow PB_2$, $PA_2 \leftrightarrow PB_1$, and $PA_2 \leftrightarrow PB_2$. Thus, $PA_1 \leftrightarrow PB_1$ and $PA_1 \leftrightarrow PB_2$ are represented by that the complexation for EA binding to the first class of binding sites of HSA (PA_1) is inhibited by C16 binding complex at the first class (PB_1) and the second class (PB_2) and also $PA_2 \leftrightarrow PB_1$ and $PA_2 \leftrightarrow PB_2$ by that the complexation of EA binding to the second class of binding sites of HSA (PA_2) is inhibited by C16 binding complex at the first class (PB_1) and the second class (PB_2), respectively. The simulations were made using the two-site competitive model classified into three types by $PA_i \leftrightarrow PB_1$, $PA_i \leftrightarrow PB_2$ and $PA_i \leftrightarrow PB_i$. Thus, $PA_i \leftrightarrow PB_1$ and $PA_i \leftrightarrow PB_2$ are represented by that the complexation for EA binding to two classes of binding sites of HSA (PA_1 and PA_2) is simultaneously inhibited by C16 binding complex at the first class of binding sites (PB_1) and by C16 binding complex at the second class (PB_2), and also $PA_i \leftrightarrow PB_i$ by that the PA_1 and PA_2 of EA are inhibited by PB_1 and PB_2 of C16, respectively.

Figures 2 and 3 show the computer simulations of the one-site and two-site competitive models, respectively, for the EA-C16 competitive binding to HSA. Points show the experimental data in the presence of C16. The solid line represents the computer-generated curve for the reaction heat of EA binding to HSA in the absence of C16 and each broken line the simulation curve computed from the multiple-site competitive model. Although any models did not fit to the actual calorimetric data in Fig. 2, a reasonably good fit to all the data was obtained from the two-site competitive model of $PA_1 \leftrightarrow PB_i$ as shown in Fig. 3. The result indicated that EA binding of the first class and the second class of binding sites to HSA was concomitantly inhibited by C16 bound at two classes of binding sites on HSA.

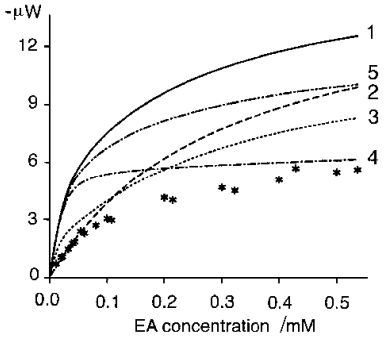

Fig. 2 Computer simulations of one-site competitive model for the EA-C16 competitive binding to HSA. Points show the calorimetric titration data measured as a function of final concentration of EA when the final concentration of HSA and C16 were 1.98×10^{-5} M and 6.83×10^{-5} M, respectively. Solid line of 1 represents a computer-generated curve for the reaction heat of EA binding to HSA in the absence of C16. Broken lines of 2, 3, 4 and 5 are the computer simulation curves of the one-site competitive models of $PA_1 \leftrightarrow PB_1$, $PA_2 \leftrightarrow PB_1$, $PA_1 \leftrightarrow PB_2$, and $PA_2 \leftrightarrow PB_2$, respectively

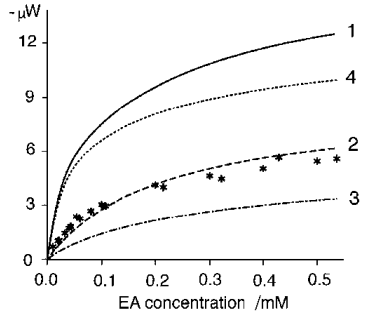

Fig. 3 Computer simulations of two-site competitive model for the EA-C16 competitive binding to HSA. Points and solid line are same as shown in Fig. 2. Broken lines of 2, 3, and 4 are the computer simulation curves of the two-site competitive models of $PA_i \leftrightarrow PB_i$, $PA_i \leftrightarrow PB_1$, and $PA_i \leftrightarrow PB_2$, respectively

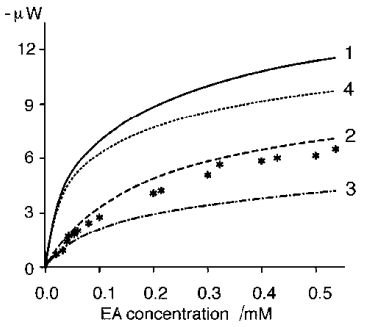

Fig. 4 Computer simulations of two-site competitive model for the EA-C12 competitive binding to HSA. Points show the calorimetric titration data as a function of final concentration of EA measured when the total concentrations of HSA and C12 were 1.81×10^{-5} M and 5.44×10^{-5} M, respectively. Solid line of 1 represents a computer-generated curve for the reaction heat of EA binding to HSA in the absence of C12. Broken lines of 2, 3, and 4 are the computer simulation curves of the two-site competitive models of $PA_i \leftrightarrow PB_i$, $PA_i \leftrightarrow PB_1$, and $PA_i \leftrightarrow PB_2$, respectively

In the case of the competition between EA and C12, the two-site competitive model of $PA_i \leftrightarrow PB_i$ also fit best to the reduced calorimetric data by the presence of C12 (Fig. 4).

Simulations of EA-C6 competitive binding to HSA

The binding of C6 to HSA was characterized by one-class binding model with $K_1=1.65\pm0.1\times10^4$ M^{-1}, $n_1=2.3$, and $\Delta H_{app}=-36.2$ kJ mol^{-1} [7]. In the competitive binding between EA and C6, the drug A bind to two classes of binding sites to form PA_1 and PA_2, whereas the competitor B forms only one kind of complex PB_1. The computer simulations based on three types of the competitive model, $PA_1 \leftrightarrow PB_1$, $PA_2 \leftrightarrow PB_1$, and $PA_i \leftrightarrow PB_1$, were performed as shown in Fig. 5. The actual data fit

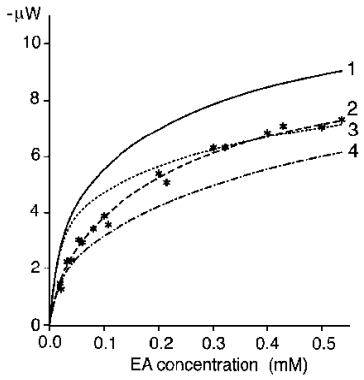

Fig. 5 Computer simulations of the competitive model for the EA-C6 competitive binding to HSA. Points show the calorimetric titration data as a function of final concentration of EA when the total concentrations of HSA and C6 were 1.45×10^{-5} M and 5.45×10^{-5} M, respectively. Solid line of 1 represents a computer-generated curve for the reaction heat of EA binding to HSA in the absence of C6. Broken lines of 2, 3, and 4 are the computer simulation curves of the competitive models of $PA_1\leftrightarrow PB_1$, $PA_2\leftrightarrow PB_1$, and $PA_i\leftrightarrow PB_1$, respectively

well to the model of $PA_1\leftrightarrow PB_1$, indicating that EA binding at the first class is inhibited by the presence of C6.

Competitive processes in the EA binding to HSA by fatty acids

The competitive model of $PA_1\leftrightarrow PB_1$ excellently fit to the data of EA-C6 competition to HSA (Fig. 5) and the $PA_i\leftrightarrow PB_i$ model was best fit to the EA competitive binding by C12 and C16 (Figs 3 and 4), indicating that some different processes in the competition between EA and fatty acids is relevant to the alkyl-chain length of fatty acids. At the first class of EA and C6 binding sites of HSA included in group S1 by the classification of the binding sites, the values of the binding parameters of EA, $K_1=0.789\times10^5$ M^{-1} and $n_1=0.3$, were decreased by the presence of C6 (Table 1). The second class of binding sites of EA contained to group S2 was, however, not influenced by the presence of C6. Thus, it is suggested that the competition between EA and C6 occurred at the common binding sites in group S1, where the first class of EA binding are occupied first by C6, leaving only the second class of binding sites for binding at higher EA concentrations.

The first classes of binding sites of C12 and C16 included to group S3 is clearly different from the first class of EA binding sites. A competitive binding could not occur by the binding to the same sites. As the longer-chain fatty acids have a greater freedom of rotation around its single bonds to be more flexible, the increased freedom of movement allows the hydrocarbon side chain to be present on the surface of the HSA molecule [15] and/or to be easy to interfere with EA binding to HSA in decreasing the binding affinity at the first class of binding sites. On the other hand, the second classes of EA, C12 and C16 were included to group S2, where they should exist predominantly in an anionic form bound to HSA at physiological pH 7.4 due to the pKa values of EA (pKa=3.59) and fatty acids (pKa=4.3~4.9). EA binding would

be then completely inhibited by C12 and C16 with higher binding constants at the second class of binding sites.

Conclusions

In the present work, the computer simulations of the multiple-site competitive model were performed for the reaction heat of EA competitive binding to HSA in the presence of fatty acids. In these cases, it is important to emphasize the necessity of including both competitive-site and noncompetitive-site binding of drug and competitor in the formulation of the competitive model. A general treatment of individual site binding has been presented with a partition function in which the noncompetitive site binding was ignored. It is not so easy to separate experimentally the competitive-site binding of drug from the noncompetitive-site binding that only the unbound concentration of either drug or competitor has been monitored. The calorimetric titration method is easily able to measure the reaction heat of drug binding which is proportional to the bound concentrations of drug and competitor to all the binding sites, and to estimate stoichiometries with the intrinsic or the apparent competitive binding constants.

In conclusion, the present multiple-site competitive models are completely accounted for and fitted by the calorimetric titration data for the competitive binding EA and fatty acids to HSA. The microcalorimetric method would be useful to evaluate quantitatively the competitive binding of drugs at the multiple classes of binding sites in blood components.

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