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Journal of Chromatography A, 1061 (2004) 141–148
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Human insulin and desamido human insulin isotherms in ethanol–water reversed phase systems

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Received 22 June 2004; received in revised form 13 October 2004; accepted 26 October 2004

Abstract

The multi-component isotherms for human insulin (HI) and desamido human insulin (dHI) over reversed phase packing (C_{18}) and with 29.8% (w/w) ethanol–water as mobile phase have been determined experimentally. The isotherms of HI in ethanol–water differ from those obtained with the more commonly applied methanol–water and acetonitrile–water mobile phase, as described in this paper. The isotherm exhibits anti Langmuirian behavior and can be very well modeled by an anti Langmuir isotherm presented in this paper. The HI and dHI anti Langmuir isotherm are determined as:

$$q_{HI} = \frac{8.4C_{HI} + 3C_{HI}C_{dHI}}{1 - 0.05C_{HI} - 0.14C_{dHI} + 0.04C_{HI}C_{dHI}} \quad \text{and} \quad q_{dHI} = \frac{11.4C_{dHI} + 2C_{HI}C_{dHI}}{1 - 0.05C_{HI} - 0.14C_{dHI} + 0.04C_{HI}C_{dHI}}$$

Keywords: Human insulin; Desamido human insulin; Adsorption isotherms; Anti-Langmuir isotherm; Reversed phase chromatography; Multimerization

1. Introduction

The production of recombinant human insulin (HI) is a large-scale process since the current world consumption is in the order of several tons per year, as opposed to kilograms for other recombinant protein therapeutics. Typical downstream processes consist of several reversed phase HPLC (RP-HPLC) steps to remove various impurities [1,2]. Nowadays, there is a tendency to optimize the preparative chromatography based on modeling instead of using a purely empirical approach. Sufficient knowledge of the isotherms of HI and its derivatives is essential for the thorough understanding of the properties of nonlinear chromatography and for modeling the process [3,4].

HI consists of chain A and chain B containing 51 amino acid residues with molecular weight of 5807.6 Da. HI exists as monomer only at concentration below 0.6 mg/L. It dimerizes at higher concentrations in acid and neutral solutions and in the pH range 4–8, in the presence of zinc ions, three dimers assemble at concentrations above 0.6 g/L further into a hexamer. At concentrations higher than 11 g/L, the hexamer is formed at neutral pH without assistance of zinc ions [5,6]. The hydrodynamic size is about 26 Å for monomer and 40 Å for dimer [7]. Abundance of hydrophobic amino acid residues makes insulin very hydrophobic but these hydrophobic interfaces are often buried in the internal of dimer or hexamer.

One of the derivatives of HI that needs to be removed in the downstream processing is desamido HI (dHI). Two asparagine residues located at A^{21} and B^{3} are exposed and susceptible to deamidation. The resulting products are des-A^{21} at low pH and des-B^{3} at pH7 or slightly above [6]. Deamidation does not have a significant change of the biological potency of HI but it needs to be removed in order to meet

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doi:10.1016/j.chroma.2004.10.089

Nomenclature

List of symbols

AD	adsorption desorption method for isotherm determination
C_i	liquid phase concentration (g/L)
k_A	adsorption rate constant (–)
k_B	desorption rate constant (–)
K	partition value (–)
b_i	Langmuir constants (–)
m_i	total amount of compound in liquid phase and solid phase (g)
q_i	solid phase concentration (g/L)
q_{max}	column saturation capacity (g/L)
q_s	column saturation capacity (g/L)
t_0	retention time for non-retained compound (s)
t_R	retention time of sample (s)
V_c	column volume (m^3)

Greek letter

ε_t	total porosity of column (–)

the strict criteria of drug manufacturing. The deamidation changes the conformation of the insulin and makes it even more hydrophobic. As a result, it is more retained by the reversed phase. RP-HPLC is used for both detection and removal of desamido during analysis and purification [2,7].

In our laboratory, simulated moving bed (SMB) chromatography has been used to improve the purification of therapeutics including antibiotics and proteins including HI [8–10]. SMB systems are complicated chromatographic processes that cannot be designed and optimized simply by empirical procedures only, but require model aided design including accurate isotherm models [11]. The retention mechanism for HI and its variants has been described by isotherm models proposed by several groups [4,12,13]. The result illustrates that the isotherm is very dependent on type and composition of mobile phase and type of reversed-phase adsorbent. A typical Langmuir-type isotherm was observed at certain high concentration. Until now, most of the work on HI isotherms is done using acetonitrile–water or methanol–water as the mobile phase. One of the disadvantages of using acetonitrile and methanol is their toxicity, which is a concern for medicine for human use. For safety reasons, ethanol as a food-grade solvent, is preferably chosen in industrial RP-HPLC processes [1].

The lack of data and understanding in these sorts of industrially relevant systems justifies the investigation into the isotherms of HI and dHI in ethanol–water mobile phase. In this paper, we present the isotherm data for HI and dHI in ethanol–water mobile phase and report their unique behavior different from those found in acetonitrile–water mobile phase.

An anti-Langmuir isotherm model is built based on the concentrations of the two components and interactions between them. An alternative model approach may take the multimerization of HI and dHI into account via dissociation equilibria and application of a multi-component competitive Langmuir type isotherm model. In this paper, we only elaborate on the first approach, and the properties of such and multimerization model will be briefly introduced and its properties commented upon. The models above might provide us a tool for interpolation between protein concentrations and solvent concentrations, and thereby optimization of a process at given condition of fixed resin, proteins and solvent.

2. Theory and principles

2.1. Anti-Langmuir isotherm model

There are many models to describe multi-component isotherms [14–22]. The one most often used is the classical competitive Langmuir isotherm, which is described by Eq. (1):

$$q_i = \frac{q_s b_i C_i}{1 + b_i C_i + b_j C_j} \tag{1}$$

where q_i and C_i are the concentrations of compound i in the stationary and mobile phases at equilibrium between the two phases, q_s the column saturation capacity and $b = k_A/k_D$ which is the ratio between the adsorption rate constant and desorption rate constant. When the concentrations of the components are low, as often is the case at analytical scale, the isotherm becomes linear. The $q_s b$ value actually equals the K value in linear isotherm, which can be calculated from chromatographic retention data using Eq. (2)

$$K = \frac{(t_R - t_0)\varepsilon_t}{t_0(1 - \varepsilon_t)} \tag{2}$$

with ε_t total column porosity, t_R retention time of sample, t_0 retention time for non-retained compound.

The classical competitive Langmuir isotherm is thermodynamically consistent only if the saturation capacities of all the components are equal, which is certainly not always true. A modified Langmuir model proposed by Guiochon is based on statistical thermodynamics and also applicable for situations with unequal saturation capacities [14]. The corresponding kinetic equations for the two compounds are as follows [23]:

$$\frac{\partial q_1}{\partial t} = K_{a1}(q_s - \alpha_{11}q_1 - \alpha_{12}q_2)C_1 - K_{d1} \times (C_s - \beta_{11}C_1 - \beta_{12}C_2)q_1 \tag{3a}$$

$$\frac{\partial q_2}{\partial t} = K_{a2}(q_s - \alpha_{21}q_1 - \alpha_{22}q_2)C_2 - K_{d2}(C_s - \beta_{21}C_1 - \beta_{22}C_2)q_2 \tag{3b}$$

where K_a and K_d are adsorption and desorption rates, respectively, q_i and C_i the concentrations of the two components of the mixture in the stationary phase and mobile phase, while q_s and C_s are the saturation concentrations in the stationary and mobile phase, respectively. The α_{ij} and β_{ij} factor are dependent on the size of the molecules and interactions between them. Assuming pseudo steady state Eq. (3a) and (3b) can be solved for q. After mathematical rearrangement and grouping of coefficients we get the equations for the modified Langmuir isotherms:

$$q_1 = \frac{A_1C_1 + A_{12}C_1C_2 + A_{11}C_1^2}{1 + B_1C_1 + B_2C_2 + B_{12}C_1C_2 + B_{11}C_1^2 + B_{22}C_2^2} \quad (4a)$$

$$q_2 = \frac{A_2C_2 + A_{21}C_1C_2 + A_{22}C_2^2}{1 + B_1C_1 + B_2C_2 + B_{12}C_1C_2 + B_{11}C_1^2 + B_{22}C_2^2} \quad (4b)$$

Since A_{11}, A_{22}, B_{11} and B_{22} only depend on the original coefficients β_{12} and β_{21}, which account for the influence of the concentration of one component on the rate of desorption of the other, they become zero when the above influence is neglected as and first approximation. In doing so, the higher order terms C_1^2 and C_2^2 vanish, and the equations are simplified into:

$$q_1 = \frac{A_1C_1 + A_{12}C_1C_2}{1 + B_1C_1 + B_2C_2 + B_{12}C_1C_2} \quad (5a)$$

$$q_2 = \frac{A_2C_2 + A_{21}C_1C_2}{1 + B_1C_1 + B_2C_2 + B_{12}C_1C_2} \quad (5b)$$

Coefficients A_1, B_1, B_2 are the same as in the single component isotherm. A_{12}, A_{21}, B_{12} account for interaction between the two components and have to be determined for the binary mixture. The reason for this neglecting is because the preceding coefficients (A_{11}, A_{22}, B_{11}, B_{22}) in the isotherm for quadratic terms become zero. The parameters before C_1^2 and C_2^2 account for the influence of the concentration of one component of the binary mixture on the rate of desorption of the other. This influence is relatively smaller than that on the rate of adsorption and thus can be neglected as a first approximation. This is approximation has been applied before, and the model has been applied in several cases and proved to generate data which fitted the experimental data well [14,20,25]. We, however, checked the approximation by modeling the system with and without the quadratic terms. Including the quadratic terms yields a good fit with the experimental data only if the preceding parameters (A_{11}, A_{22}, B_{11}, B_{22}) have a very low value, i.e. three orders of magnitude lower than the other parameters. We therefore can conclude that the approximation seems valid, and the quadratic term may be skipped.

In general, sometimes the fitting is poor and empirical correction coefficients are introduced into isotherm equations. The equations then cannot be completely interpreted physicochemically any more. However, they are still justified because of its capability as a tool to describe the protein behavior at given conditions [22]. In this work, however, we use Eq. (5a) and (5b) based on sound statistical thermodynamical theory for the isotherm model construction [14,26].

3. Materials and methods

3.1. Stationary phases and columns

Reversed-phase adsorbent ODS-AM C18 from YMC (Schermbeck/Weselerwald, Germany) with a particle diameter of 5 μm and an average pore size of 120 Å is pre-packed by the manufacturer into a 4.6 mm × 150 mm i.d. column. Total porosity of this column $\varepsilon_t = 0.611$. This HPLC column is used for pulse and breakthrough experiments because of its high plate number. Another column (10 mm × 150 mm) is self-packed with adsorbent with 50 μm particle diameter. The total porosity of this column is $\varepsilon_t = 0.615$. This column is used for the adsorption–desorption (AD) experiment to determine the isotherm and it is the same type of column used for rHI SMB separation (not reported in this paper).

3.2. Mobile phase and chemicals

The mobile phase consist of ethanol (HPLC grade, J.T. Baker, Belgium) and water from a Millipore water generator, at ethanol–water composition of 29.8%:70.2% (w/w). The mobile phase is buffered with 50 mM ammonium sulfate at pH 3.5 and is filtered (0.22 μm) before use. HI was kindly provided by NovoNordisk (Gentofte, Denmark). dHI was prepared by conversion of HI in a hydrochloride acid solution with pH 2.5 at 50 °C for 72 h.

3.3. Chromatography equipment

The pulse experiment and breakthrough experiment were done on a Waters Alliance HPLC (Milford, MA, USA), consisting of a 2690 separation module and a 996-photodiode-array detector. The equipment was fully controlled and the data was stored and processed with Waters Millennium 32 software interfaced with an IBM-compatible personal computer.

3.4. Methods

3.4.1. HPLC analysis and breakthrough experiment

The purity of the sample was checked with the analytical column eluted with the mobile phase mentioned above in isocratic mode, with a flow rate of 1 mL/min and at a temperature of 25 °C. For breakthrough experiments, the same column was loaded with about 45 mL HI and dHI (98:2) dissolved in the mobile phase, and eluted with a flow rate of 0.5 mL/min and at a temperature of 25 °C.

3.4.2. Pulse experiment for K value determination

HI and dHI was loaded separately on the preparative column and eluted with the same condition as HPLC analysis experiment. From the retention time, the K values of HI and dHI were derived at this condition according to Eq. (2).

3.4.3. Adsorption–desorption experiments

The adsorption–desorption method is used for isotherm determination due to its accuracy and simplicity although it is tedious [27]. HI and dHI are dissolved in the mobile phase (Section 3.2) at total concentration of 0.25, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0 g/L, with different ratios of HI/dHI at 54%:46%, 71%:29%, 98%:2% (w/w), respectively. The column packed with 50 μm particle (Section 3.1) is chosen for the AD experiment because the same type of packing material and columns are used for later SMB experiments. The possibility of the influence of different particles sizes on isotherm is then excluded. The columns are first equilibrated with the mobile phase and then saturated with the above sample. Afterwards they are eluted with the mobile phase. A flow rate of 12 CV/min and temperature of 25 °C is used all the time. The eluent is analyzed for the composition of HI and dHI by HPLC analysis, from which the concentration of each component was derived. Control experiments are done to ensure that all compounds are eluted off the column. Using the equation below, q_i is calculated according to Eq. (6) based on a mass balance [28].

$$q_i = \frac{m_i - \varepsilon_t V_c C_i}{(1-\varepsilon_t)V_c} \tag{6}$$

3.4.4. Data processing

For the anti-Langmuir isotherm model, the coefficients in Eq. (5a) are obtained by the following strategy: A_1 equals the K value for the human insulin, measured at analytical scale; A_2 equals the K value for the desamido human insulin, measured at analytical scale; B_1 is derived from the adsorption–desorption experiment at the very low concentration of HI, when the items with C_{desHI} in Eq. (5a) approximate into zero; B_2 could be derived similarly at very low concentration of HI. The remaining A_{21}, A_{12} and B_{12} are obtained by curve fitting in Microsoft Excel.

4. Results and discussion

4.1. HPLC analysis and breakthrough experiment

On the analytical column, baseline separation was achieved for HI and dHI (Fig. 1), which enabled us to check the purity and composition of our samples accurately. The curves in the breakthrough experiment have dispersive front parts and sharp rear parts (Fig. 2) that are opposite to the typical breakthrough curve from components exhibiting a Langmuir adsorption isotherm. The higher the concentration is, the more obvious the effect is. On the front part, HI elutes first, forming the first sub-plateau, followed by a second sub-plateau formed by HI and dHI.

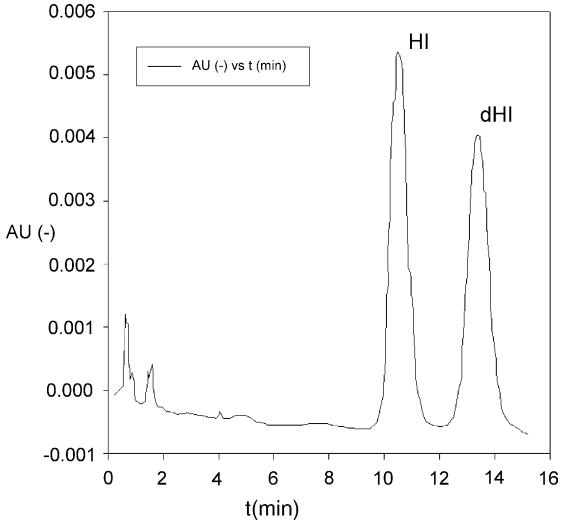

Fig. 1. Separation of HI and dHI in a pulse experiment. 4.6 mm × 150 mm RP-HPLC column, packed with C_{18} particle with 5 μm particle size and 120 A pore size, $\varepsilon_t = 0.611$. Mobile phase: 29.8% (w/w) ethanol in aqueous phase, 50 mM $(NH_4)_2SO_4$, pH 3.5, flow rate: 24 CV/h, temperature 25 °C. Two peaks correspond to HI and dHI with retention time of 10.23 and 13.01 min, respectively. Non-retained compounds present in the sample elute before 2 min.

4.2. Pulse experiments

The determined K values for HI and dHI are 8.4 and 12.4, respectively, in mobile phase of ethanol–water

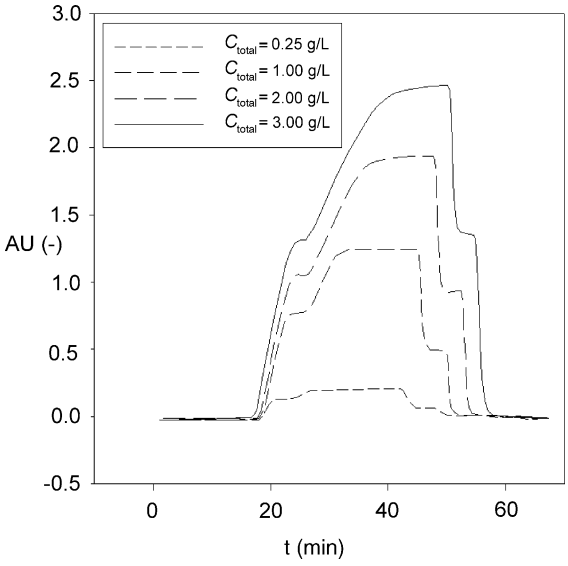

Fig. 2. Breakthrough curve of HI and des-HI done on 4.6 mm × l50 mm RP-HPLC column, packed with C_{18} particle with 5 μm particle size and 120 A pore size, $\varepsilon_t = 0.611$. Mobile phase: 29.8% (w/w) ethanol in aqueous phase, 50 mM $(NH_4)_2SO_4$, pH 3.5, flow rate: 12 CV/h, temperature 25 °C. The four curves from bottom to top correspond to feed sample with different total concentration of 0.25, 1.0, 2.0, 3.0 g/L, respectively, all at the ratio of 98%:2% (HI:dHI).

(29.8%:70.2%, w/w), 50 mM ammonium sulfate, pH 3.5, at temperature of 25 °C, measured on YMC ODS-AM C18 adsorbent with 50 μm particle size.

4.3. Influence adsorbent particle size

The 5-μm diameter stationary phase particles are used in the analytical column, for concentration determination of HI and dHI, and to demonstrate the unique elution curve during the breakthrough experiment. The larger 50-μm particles are used to determine the *K* values and partition behavior. The model is based on retention data on the larger particles. Size may matter regarding adsorption behavior. There is a slight difference of the *K* values for HI and dHI, measured between 5-μm (8, 11.25) and 50-μm particles (8.4, 11.4).

4.4. Adsorption–desorption experiments

The breakthrough curves obtained from AD experiment performed on the 5 μm adsorbent column have dispersive front parts and sharp rear parts that are opposite to the typical breakthrough curve from Langmuir isotherm (see Fig. 2). The higher the concentration is, the clearer the effect is. On the front part, HI elutes first, forming the first sub-plateau, followed by a second sub-plateau, which formed by HI and dHI. The efficiency of the column with 50 μm adsorbents particles is too low to allow us see the first sub-plateau, but the dispersive front part and sharp rear part are observed during experiments.

The calculated isotherms for HI and dHI from adsorption–desorption experiments are listed in Table 1. They are plotted in Figs. 3 and 4, together with the curves predicted

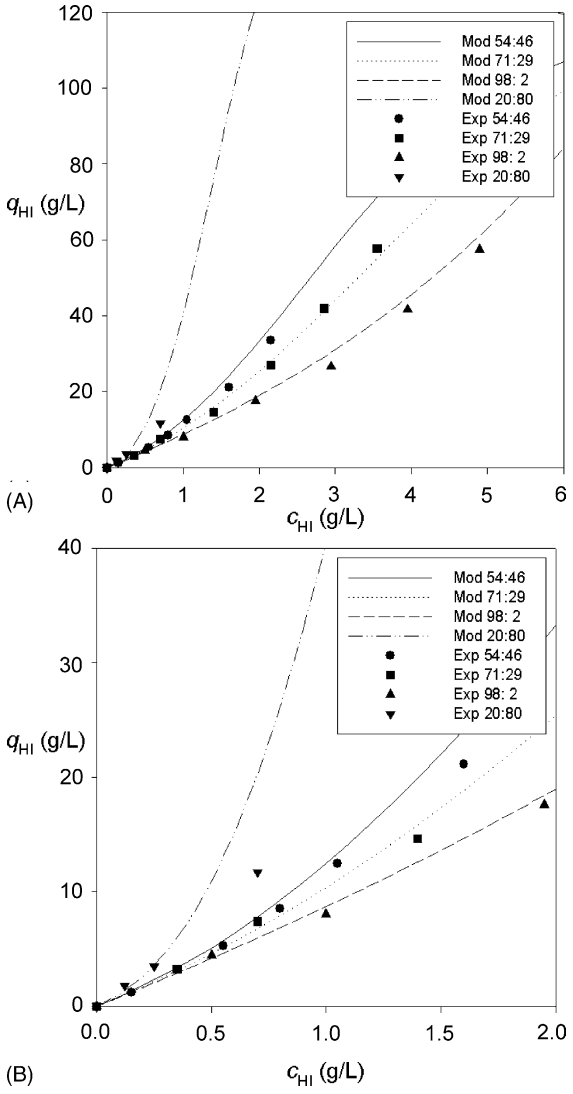

Fig. 3. (A) Measured and modeled HI isotherm using Eqs. (7) and (8). Different lines correspond to different ratios C_{HI}/C_{dHI}: 54%:46% (Q1), 71%:29% (Q2), 98%:2% (Q3) and 20%:80% (Q4). Model curves and experimental points are for HI. Experiments are done at $T = 25\,^{\circ}C$, pH 3.5, 50 mM $(NH_4)_2SO_4$, 29.8% (w/w) $EtOH/H_2O$; (B) As (A) but magnified.

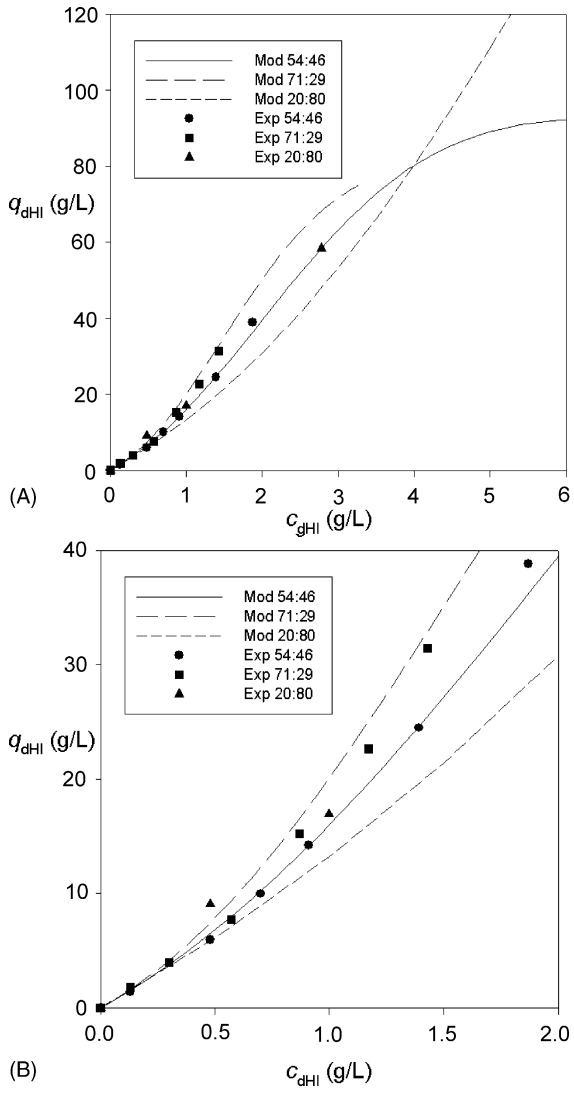

Fig. 4. (A) Measured and modeled dHI isotherm using Eqs. (7) and (8). Different lines correspond to different ratios C_{HI}/C_{dHI}: 54%:46% (Q1), 71%:29% (Q2), 98%:2% (Q3) and 20%:80% (Q4). Model curves and experimental points are for dHI. Experiments are done at $T = 25\,^{\circ}C$, pH 3.5, 50 mM $(NH_4)_2SO_4$, 29.8% (w/w) $EtOH/H_2O$; (B) As (A) but magnified.

Table 1
Isotherm data of HI and dHI measured from adsorption–desorption experiment in ethanol–water 29.8% (w/w), $T = 25\,^{\circ}C$ (for additional conditions, see Fig. 3) at different C_{HI}/C_{dHI} ratios

C_{total} (g/L)	HI			dHI		
	C_{HI} (g/L)	q_{HI} (g/L)	q_{HI}/C_{HI}	C_{dHI} (g/L)	q_{dHI} (g/L)	q_{dHI}/C_{dHI}
Ratio 1 (54%:46%)						
0.25	0.14	1.16	8.60	0.12	1.42	12.35
1.00	0.54	5.28	9.87	0.47	5.95	12.80
1.50	0.80	8.51	10.60	0.70	9.98	14.31
2.00	1.07	12.45	11.63	0.93	14.19	15.25
3.00	1.61	21.12	13.16	1.40	24.49	17.56
4.00	2.14	33.46	15.64	1.86	38.84	20.88
Ratio 2 (71%:29%)						
0.50	0.36	3.21	9.10	0.15	1.81	12.51
1.00	0.71	7.40	10.64	0.29	3.96	13.13
2.00	1.42	14.62	10.30	0.58	7.71	13.29
3.00	2.13	27.04	12.69	0.87	15.24	17.52
4.00	2.84	41.94	14.77	1.16	22.66	19.54
5.00	3.55	57.67	16.25	1.45	31.43	21.68
Ratio 3 (98%:2%)						
0.50	0.49	4.45	9.08	0.01	NA[a]	NA
1.00	0.98	8.03	8.19	0.02	NA	NA
2.00	1.95	17.57	9.01	0.05	NA	NA
3.00	2.95	26.63	9.03	0.05	NA	NA
4.00	3.95	41.66	10.55	0.05	NA	NA
5.00	4.90	57.45	11.72	0.10	NA	NA
Ratio 4 (20%:80%)						
0.5	0.12	1.80	15.00	0.48	9.08	18.92
1.25	0.25	3.48	13.92	1.00	16.95	16.95
3.50	0.70	11.70	16.71	2.80	58.39	20.85

[a] Not available.

by the model. In the case of anti-Langmuir isotherm model, the following coefficients for Eq. (5a) and (5b) are derived:

$$q_{HI} = \frac{8.4C_{HI} + 3C_{HI}C_{dHI}}{1 - 0.05C_{HI} - 0.14C_{dHI} + 0.04C_{HI}C_{dHI}} \quad (7)$$

$$q_{dHI} = \frac{11.4C_{dHI} + 2C_{HI}C_{dHI}}{1 - 0.05C_{HI} - 0.14C_{dHI} + 0.04C_{HI}C_{dHI}} \quad (8)$$

The isotherm model curve fits the experimental data reasonably well, as can be seen in Figs. 3 and 4. All the curves are S-shaped. Within the concentration range we worked, the slope of the curve keeps on increasing, indicating solid phase concentration increases faster than the increasing of liquid phase. This is typical for an anti-Langmuir isotherm. The negative sign of B_1 and B_2 correspond to the attractive interactions between HI and dHI during adsorption. The model also predicts that at higher concentration, the slope of the curves will decrease and finally a plateau is reached indicating the saturation of solid phase. Interactions between HI and dHI are cooperative instead of competitive. With experimental data, when we fix the liquid concentration of one component and increase the liquid concentration of the second component, the solid concentration increases for the first component, indicating cooperative interaction. The S-shaped isotherm curve is an indication of cooperativity. We observed this type of curve at different ratio of HI and dHI (98:2, 54:48 and 20:80). So, the cooperative adsorption seems to occur in HI–HI and HI–dHI systems. As for dHI–dHI, since no experiments were done for pure dHI, we cannot draw such conclusion for the system dHI–dHI.

Interestingly, Geng and Regnier studied the dependence of the elution curve and adsorption isotherms of insulin on composition of mobile phase of frontal analysis in reversed phase liquid chromatography [29]. They used 32% ethanol in water (v/v) as mobile phase, with trifluoroacetic acid (TFA) as ion-pairing agent and found Langmuir behavior for insulin at low concentration of 0.1 – 0.5 g/L. It would be worthwhile to investigate at high concentration of insulin if there is anti-Langmuir behavior with the presence of TFA. From our extensive literature search, we did not find any article dealing with the cooperative isotherm for human insulin that we report in this paper (see Table 2). A recent article of Mihlbachler also reported the cooperative isotherms, but then for the two enantiomers of Troger's base [25].

4.5. Influence ethanol concentration

The main objective of the study presented in this paper is to provide isotherm data for simulated moving bed design for purification of HI in isocratic mode. Therefore, we only need to know the adsorption behavior of HI and dHI at a specific ethanol concentration. We did check a few other ethanol

Table 2
Literature data on single component insulin (of various origin) isotherms compared to this work

Reference	Insuline	Mobile phase	Stationary phase	Isotherm model
Geng and Regnier [12]	Bovine	EtOH, water, TFA (32:68:0.1, v/v)	SynChrompakC18, 5.6 μm, 300 Å	Langmuir and SDM-A[a] single component $\log c_s = \beta + nr/Z \log c_m$
Liu et al. [4]	Human bovine porcine	ACN, water, TFA (31:69:0.1, v/v)	YMC ODS-A C18, 5 μm, 120 Å	Toth model single component $q = \frac{q_s bC}{[1+(bC)^n]^{1/n}}$
Sabharwal and Chase [24]	Bovine porcine	ACN, water, TFA (30–36%:70–64%:0.1, v/v)	Whatman BioPrep C4, 20 μm, 150 Å	Langmuir model single component. Competition between bovine and porcine insulin in the adsorption
This work	Human	EtOH, water (30:70, w/w), pH 3.5, 50 mM $(NH_4)_2SO_4$	YMC ODS-AM C18, 50 μm, 120 Å	Anti-Langmuir for binary system $q_1 = \frac{A_1C_1 + A_{12}C_1C_2}{1 + B_1C_1 + B_2C_2 + B_{12}C_1C_2}$ $q_2 = \frac{A_2C_2 + A_{12}C_1C_2}{1 + B_1C_1 + B_2C_2 + B_{12}C_1C_2}$

[a] SDM-A: Stoichiometric displacement model for adsorption. *Z* in the model is the sum of *nr* and *q*, corresponding to the contribution from adsorption and partition.

concentrations as well, but do not present them in this paper. For optimal SMB design, the SMB might be operated in gradient mode, and then the influence of the ethanol concentration needs to be known very accurately. That work will be discussed in a next paper.

4.6. Model performance

The evaluation of the models capability to model single component pure HI and dHI is limited by the fact that getting pure HI and dHI is expensive and difficult, especially when large amounts of the sample are needed for adsorption–desorption experiments. The highest purity of our HI sample is 98%, which we used in pulse experiment for *K* value determination. Therefore, we can only compare our model to this maximum HI purity data. From the results we can see that the model can predict the single component "pure" HI data very well.

4.7. Multimerization

The highly hydrophobic nature, the tendency for multimerization and moderate molecular size of HI and dHI might be responsible for their special isotherms. As discussed earlier in this paper, a first layer is formed by the adsorption of solute to the stationary phase. The bound molecules promote second-layer adsorption of solute to the first layer due to the association between these molecules. The molecular weight of HI dimer is 11,600 Da, which is supposed to be one of the main multimerization forms of HI in the liquid phase, with the hydrodynamic size in the range of 26 – 55 Å, which is not as large as other proteins. Enough adsorption surfaces are available before the saturation is reached. The scenario which happens in the Langmuir isotherm situation might not be so apparently present in our case. Another explanation is that the influence of multimizeration changes the pattern of a normal Langmuir isotherm.

It is well known that HI has a tendency to aggregate and form multimers in solution. Therefore also a model was composed including liquid phase multimerization and regular Langmuir adsorption behavior to see whether the modeled adsorption using the apparent liquid phase HI concentration was able to describe the observed anti-langmuirian behavior. With this model it could be shown that the inflection point could be described well, but *not* the influence of the ratio of HI/dHI. Taking liquid phase multimerization of HI into account might be a possible alternative route to describing apparent anti langmuirian behavior.

4.8. Influence mobile phase

Exploration of the reasons for different behavior of HI in aqueous phase contains methanol, ethanol and acetonitrile would be intriguing. Guo and Purcell have investigated the thermondynamic properties of HI on RP-HPLC, using methanol–water and acetonitrile–water as mobile phase respectively [30,31]. They found that the stability of protein conformation is associated with thermondynamic properties. For the above two different mobile phases, the so-called entropy–temperature curve is different, indicating insulin seems more stable in methanol–water than acetonitrile–water. This was correlated with the fact that methaonl is a H-bond donor while acetonitrile is a H-bond acceptor. As ethanol has a similar H-bonding ability as methanol, we would expect that HI is also stable in ethanol. But how this correlates with the different isotherm type is still a matter for future research. Does the more stable conformation of HI accelerate the adsorption of the protein on the solid phase, or the multimerization of HI?

The interaction of proteins to interfaces and their related conformation change is a very complicated process, which is not very well understood. A deep and intensive research at molecular level is necessary to help us gain further and better understanding of the mechanisms involved. One approach to give more insight and understanding could be the study of protein conformation change by computer simulation via methods like molecular dynamics and Monte Carlo study [32,33], but this lies beyond the scope of the present paper.

5. Conclusions

HI and dHI have different isotherms in ethanol–water reversed phase systems with respect to the more commonly used methanol–water and acetonitrile–water solvents. An anti-Langmuir model has been successfully used for the description of the adsorption behavior of the two compounds.

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