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Molecular spectroscopic studies on the interaction between Ractopamine and bovine serum albumin

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ABSTRACT

To investigate the interaction between Ractopamine (RAC), an animal growth promoter, and bovine serum albumin (BSA), three spectroscopic approaches (fluorescence, UV–vis and FT-IR) and three different experiments (two mole-ratio and a Job's methods) were used to monitor the biological kinetic interaction procedure. The Stern–Volmer quenching constants K_{SV}, the binding constants K_a, and the number of binding sites n at 298, 301 and 304 K were evaluated by molecular spectroscopic approaches. The values of enthalpy ($-13.47\ kJ\ mol^{-1}$) and entropy ($78.39\ J\ mol^{-1}\ K^{-1}$) in the reaction indicated that RAC bound to BSA mainly by electrostatic and hydrophobic interaction. The site markers competitive experiments indicated that the binding of RAC to BSA primarily took place in site I. The spectra data matrix was further investigated with multivariate curve resolution-alternating least squares (MCR-ALS), and the concentration profiles and the pure spectra for three species (BSA, RAC and RAC-BSA) existed in the kinetic interaction procedure, as well as the apparent equilibrium constants, were obtained.

1. Introduction

Ractopamine (RAC), originally developed to treat respiratory diseases, was found to have an important side effect of causing a significant reduction in fat levels and a dramatic increase in the amount of muscling when administered to animals in high doses [1]. It is licensed for use as an animal growth promoter in more than 20 countries worldwide, including the United States and Canada, but is either not licensed or prohibited by over 150 others, including those within the European Union, because of their well-documented adverse effects on human health, such as cardiovascular and central nervous diseases, caused by food poisoning associated with the residues in livers. But RAC was the first β-agonist approved (2003) by the US Food and Drug Administration (FDA) for use in cattle finishing diets, and the FDA (2003) [2] reported that supplementing RAC (10 or 20 ppm) during the final stages of finishing did not affect palatability of beef from mixed-breed steers and heifers [3]. Therefore, the view is very contradictory from different countries, and generally consumers consider chemical growth promoter residues as unwholesome and unwelcome constituents in food. Though there are many researches on how to detect the quantity of RAC in animals [4,5], little work has been done to search the poisonous and pharmacology adverse effect [6]. Thus, because of the requirements of providing quality assurance for the consumer and satisfying legal testing obligations, the interaction between residues of the drug at low concentrations and proteins can not only provide useful information for appropriately understanding the toxicological action, but also illustrate its binding mechanism at a molecular level.

In this work, the investigation of bovine serum albumin (BSA) with RAC was carried out in aqueous solution at physiological conditions using fluorescence, UV–vis and FT-IR spectral approaches. Spectroscopic evidence regarding the drug binding mode, the association constant and the change of protein secondary structure are provided. Furthermore, the chemometrics method, multivariate curve resolution-alternating least squares (MCR-ALS) [7], was applied to resolve the two-way UV–vis and fluorescence spectral data so as to improve the understanding of complex kinetic processes and extract the equilibrium profiles of the reacting species.

2. Material and methods

2.1. Apparatus

Fluorescence spectra for samples were measured on a Perkin–Elmer LS-55 spectrofluorometer equipped with a thermo-

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static bath (Model ZC–10, Ningbo Tianheng Instruments Factory, China) and a 1.0 cm quartz cuvette. The excitation and emission slits were set at 10 nm, while the scanning rate was 1500 nm min^{-1}. The FL WinLab software (Perkin–Elmer) was used to correct the measured data. The UV–vis spectra were measured on an Agilent 8453 UV–vis spectrophotometer. FT-IR spectra were measured on a Thermo Nicolet 380 FT-IR spectrometer equipped with a deuterated triglycine sulfate (DTGS) detector in the rage of 4000–400 cm^{-1}. The web-based MCR-ALS programs [8] were used to process the collected spectral data if necessary. All the measurements were carried out at room temperature (25 ± 0.5 °C).

2.2. Materials

A stock solution of 1×10^{-3} mol L^{-1} Ractopamine hydrochloride (Sigma, the purity is not less than 95.1%) was prepared by dissolving its crystals (0.0169 g) in 50 mL distilled water. BSA (2×10^{-3} mol L^{-1}) was prepared by dissolving 1.36 g of the purified protein (M = 68,000 Da; The Bomei Biological Co. Ltd., Hefei) in 10 mL 50×10^{-3} mol L^{-1} sodium chloride solution and stored at 4 °C. Its purity was 99% based on a reference absorbance value of 0.667 at 278 nm for 1.0 g L^{-1} pure BSA [9]. Essentially fatty acid free HSA was obtained from Sigma Chemical Company (St. Louis, USA). The solution of HSA (1×10^{-3} mol L^{-1}) was prepared by dissolving 0.660 g in 10 mL 50×10^{-3} mol L^{-1} sodium chloride solution based on its molecular weight of 66,000. Warfarin (Medicine Co. Ltd., Shanghai) and ibuprofen (Baike Hengdi Pharmaceutical Co. Ltd., Hubei) stock solutions (2.5×10^{-3} mol L^{-1}) were prepared by dissolving the accurately weighed appropriate amounts of each compound and dissolving in water above. The solution was then diluted to the required volume with distilled water in practical use. All experimental solutions were adjusted with the Tris–HCl ((hydroxy methyl) amino methane–hydrogen chloride) buffer of pH 7.4. Other chemicals were of analytical grade reagents, and doubly distilled water was used throughout.

2.3. Procedures

2.3.1. Fluorescence and absorbance spectra

Fluorescence spectra of BSA (3.33×10^{-8} mol L^{-1}) and HSA (3.33×10^{-8} mol L^{-1}) in presence of RAC (0–1.33×10^{-7} mol L^{-1}, with an interval of 1.67×10^{-8} mol L^{-1}) were recorded at 298, 301 and 304 K. An excitation wavelength of 280 nm was chosen and the emission wavelength was recorded from 200 to 450.5 which was used to calculate the thermodynamic and binding parameters of RAC and BSA/HSA systems. Synchronous fluorescence spectra (SFS) of BSA (1.67×10^{-7} mol L^{-1}) in the presence of RAC were measured (λ_{ex} = 200–350 nm; $\Delta\lambda$ = 60 and 15 nm) at pH 7.4, the concentration of RAC was increased from 0 to 2.67×10^{-7} mol L^{-1} at an interval of 3.34×10^{-8} mol L^{-1} (total 9 samples).

2.3.2. FT-IR spectroscopy

FT-IR spectra are taken via the attenuated total refection (ATR) method with resolution of 4 cm^{-1} and 32 scans (range of 400 and 4000 cm^{-1}). The spectra of the buffer solution was firstly collected and then subtracted the spectra of buffer from the spectra of sample solution to get the FT-IR spectra of the protein.

2.3.3. The experiments to get the expanding data matrix

Three separate experiments were carried out in the Tris–HCl buffer of pH 7.4, and the experimental conditions and necessary variables are listed in Table 1.

Experiment 1 (mole-ratio method): the concentration of BSA was kept constant (3.33×10^{-8} mol L^{-1}), and different amounts (0–1.00×10^{-7} mol L^{-1}, at 5.00×10^{-9} mol L^{-1} interval) of RAC were added to the solution. Experiment 2 (mole-ratio method): the concentration of RAC was kept constant (6.67×10^{-8} mol L^{-1}) and different amounts of BSA were added with a range of 0–6.67×10^{-8} mol L^{-1} at an interval of 3.33×10^{-9} mol L^{-1}. Experiment 3 (Job's method, sometimes called continuous variation method): the concentration of RAC was kept change from 3.33×10^{-8} mol L^{-1} to 0 and different amounts of BSA were added in the range of 0–3.33×10^{-8} mol L^{-1}. The ratios of $r_{RAC:BSA}$ and $r_{BSA:RAC}$ were ranged from 1.0 to 0 and 0 to 1.0, respectively.

The solutions used in experiments 1–3 were prepared with 3.0 mL of pH 7.4 Tris–HCl buffer containing appropriate amounts of BSA and RAC. The total added volume (BSA and RAC) was less than 0.1 mL, so the volume variation was thus negligible. Titrations were performed manually using suitable micropipettes. The mixed solution was shaken thoroughly and equilibrated for 10 min and then the molecular fluorescence (200–450.5 nm) and UV–vis (200–450 nm) spectra were recorded at every 0.5 and 1 nm, respectively. Thus, six data matrices D_{UV}^{BSA}, D_{F}^{BSA} (experiment 1) D_{UV}^{RAC}, D_{F}^{RAC} (experiment 2) and D_{UV}^{Var}, D_{F}^{Var} (experiment 3) can be obtained, these column-wise and row-wise data matrices with two different measuring approaches for three different experiments were combined and expanding data matrix was obtained.

2.3.4. Fluorescence spectra in the presence of the site markers

In order to identify the binding site of RAC on BSA, warfarin and ibuprofen were used as the markers for sites I and II, respectively.

Method 1: the marker was added to the mixture of RAC and BSA, the ratio of $r_{RAC:BSA}$ was kept at 2, but the concentrations of the site marker I (warfarin) for one series, and II (ibuprofen) for the other series, were varied.

Method 2: site I marker of warfarin, with different concentration, was added to a series of test tubes containing BSA of 3.33×10^{-8} mol L^{-1}, total six solutions with different molar ratios of $r_{warfarin:BSA}$ (0–5) were prepared, and then RAC was added to these six tubes at different concentrations (from 0 to 1.33×10^{-7} mol L^{-1} at 1.67×10^{-8} mol L^{-1} interval, total 9 times, respectively. Fluorescence spectra were measured as previously described. The binding constants, K_a, of the RAC to BSA were then calculated according to the double-logarithm equation.

2.4. Theoretical background for chemometrics methods

Multivariate curve resolution-alternating least squares (MCR-ALS) has been used for the resolution of multiple component responses in unknown mixtures. UV–vis, fluorescence, near-infrared reflectance (NIR), HPLC and circular dichroism (CD) were used with MCR-ALS optimization step described elsewhere [10]. A short description of the MCR-ALS is given here.

The multivariate curve resolution model can be written as Eq. (1):

$$\boldsymbol{D} = \boldsymbol{C}\boldsymbol{S}^{T} + \boldsymbol{E} \quad (1)$$

The bilinear method to resolve an experimental data matrix $\boldsymbol{D}$ ($M \times N$) into the product of a column matrix $\boldsymbol{C}$ ($M \times F$) usually associated with concentration profiles, and a matrix of row profiles $\boldsymbol{S}^{T}$ ($F \times N$), usually associated with spectra. The superscript "T" means the transpose of matrix $\boldsymbol{S}$, where pure spectra are column profiles. $\boldsymbol{E}$ is the matrix of residuals and ideally should be close to the experimental error. M is the number of spectra recorded throughout the process and N is the number of the instrumental responses measured at each wavelength. The number of species, F, is directly related with the number of main components in matrix $\boldsymbol{D}$. This number is estimated by rank analysis, using singular value decomposition (SVD), principal component analysis (PCA), or some related techniques based on factor analysis, such as evolving factor analysis (EFA) [11] or pure-variable detection methods like SIMPLISMA