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Investigating noncovalent interactions of rutin – serum albumin by capillary electrophoresis – frontal analysis

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ABSTRACT

The application of capillary electrophoresis-frontal analysis (CE-FA) to study noncovalent interaction between rutin and serum albumin (bovine serum albumin, BSA and human serum albumin, HSA) in phosphate buffer solution (67 mM, pH 7.4) at 37 °C is presented. Using fixed HSA or BSA concentration and increasing rutin concentration, the number of primary binding sites per HSA or BSA molecules, and the affinity constants were obtained. Both affinity constants are in a comparable range suggesting the similarity of affinity properties of HSA and BSA towards rutin. The proposed CE-FA method is simple, rapid and cost-effective which may be useful in further high-throughput protein binding studies of multi-components in traditional herbal medicines for pharmacological effect evaluations.

1. Introduction

Serum albumin (bovine serum albumin, BSA and human serum albumin, HSA), the most abundant protein in blood plasma, reversibly binds a wide range of endogenous and exogenous substances. Because of its clinical and pharmaceutical importance, a great deal of attention has been paid to the interaction of serum albumin with a number of active natural or synthetic ligands, including the study of binding constants and the number of binding sites.

Capillary electrophoresis-frontal analysis (CE-FA) has been proved to be an efficient method of studying noncovalent interactions [1–3]. Advantages of this method include low sample consumption, relatively short analysis time and ease of automation. These properties make CE-FA an ideal method to study small molecules binding to protein. In CE-FA, relatively large sample plug, consisting of a pre-equilibrated mixture of analyte and protein, is injected into the fused-silica capillary, then giving rise to a square-shaped peak. The presence of plateau leads to a robust measurement because plateau height is seldom affected by the changes of analyte's migration time, EOF, capillary length and voltage. The key requirement of CE-FA is that the complex and free analyte must have different electrophoretic mobilities. Experimental conditions should be optimized so that the equilibrium is kept, while a plateau of free analyte arises out of the complex (protein + analyte) zone.

Rutin (Fig. 1, 3-rhamnosyl-glucosyl-quercetin) is a flavonol glycoside found in many herbs, which exerts a wide range of therapeutic activities in traditional medicines practices, including antimicrobial, antiplatelet, blood vessel protecting, and antioxidant effects [4–7]. However, there were only very limited reports mainly using fluorescence techniques [8–13] on the evaluating rutin–serum albumin interactions.

This study employed the method of CE-FA to determine affinity parameters for two kinds of proteins (BSA and HSA) with rutin in the phosphate buffer solution (67 mM, pH 7.4). To our knowledge, this is the first report using CE-FA method to determine rutin–serum albumin affinities at simulated physiological condition. Using a fixed HSA or BSA concentration and increasing rutin concentration, the number of primary binding sites per HSA or BSA molecules, and the affinity constants were obtained.

2. Experimental

2.1. Instrumentation

An Agilent CE (Waldbronn, Germany) equipped with a diode-array detector and the Agilent 3D-CE ChemStation software (Version A.09.03) was used throughout. A 50 μm inner diameter (ID) and 365 μm outer diameter (OD) fused-silica capillary was used (Yongnian Optical Fibre Corporation, Hebei, China). The capil-

Abbreviations: CE-FA, capillary electrophoresis frontal analysis; BSA, bovin serum albumin; HSA, human serum albumin.

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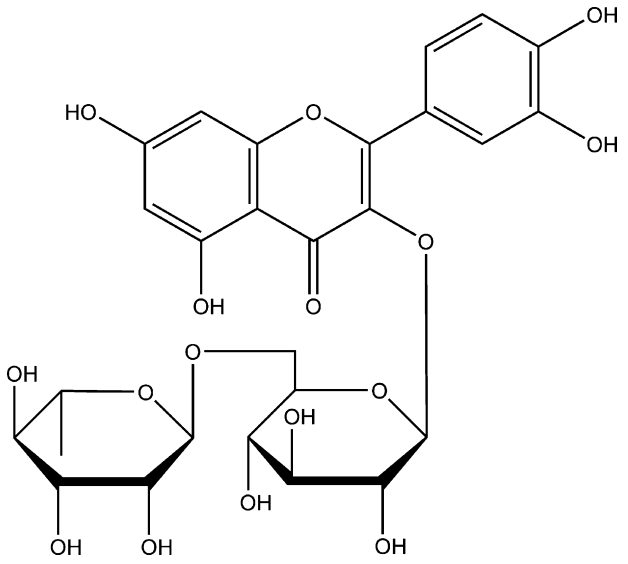

Fig. 1. Structure of rutin.

lary cassette temperature was controlled at 37.0 °C and UV detector was set at 270 nm. In rutin–BSA study, a capillary was employed with total and effective length 70 and 61.5 cm, respectively, and a running voltage 14 kV was used. In rutin–HSA study, the total and effective length of capillary was 35 and 26.5 cm, respectively, and a running voltage of 8 kV was used. A Sartorius PB-10 pH meter (Sartorius, Germany) was employed to adjust the pH of the following buffer. Water was purified by Sartorius 631UV-3 deionization system (Goettingen, Germany).

2.2. Chemicals and samples

All chemicals were of analytical grade and used as obtained. The running buffer in CE experiments was 67 mM phosphate solution pH 7.4 (PBS) and was prepared by dissolving the appropriate amount of sodium dihydrogen phosphate dehydrate in water and adjusting the pH at 7.4 with 1 M NaOH. 200 mg/ml HSA stock solution (Lot. No. 200602010) was purchased from Shanghai Institute of Biological Products (Shanghai, China). HSA working solutions were daily prepared by diluting the corresponding stock solution with PBS to 40 μM. BSA lyophilized powder was purchased from Roche (Lot. 10735108001, Germany). BSA working solutions were daily prepared by diluting the corresponding amount of BSA powder with PBS to 40 μM. Rutin (Lot. 0080-9705) was obtained from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Rutin stock solution (1 mM) was prepared in PBS, containing 3% v/v methanol. Rutin working solutions were prepared by dilution with PBS from the stock solution to final concentration ranged between 40 and 500 μM. Acetone (0.5% v/v) was used as EOF marker in CE running. All solutions were filtered through a 0.45 μm filter (Micron Separation, Westborough, MA, USA) prior to use.

2.3. Procedures

New capillaries were conditioned successively with 1 M NaOH, water and PBS, each for 30 min at 940 mbar. In order to obtain good peak shape and reproducible migration time, the capillaries were daily consecutively flushed with 1 M NaOH, water and PBS, each for 10 min at 940 mbar before starting experiments. Between measurements, the capillaries were flushed with 0.1 M NaOH, water and PBS.

Series of mixtures with increasing rutin concentration (from 40 to 500 μM) and a fixed concentration of BSA (40 μM) were prepared in duplicate by dilution of the stock solutions of rutin and BSA with PBS. All these mixtures were allowed to reach equilibrium for at least 30 min before injected into the capillary system. Samples were introduced through pressure (50 mbar for 80 s) and running voltage of 14 kV was applied. The free rutin concentration was determined from the height of the plateau peaks using calibration curves obtained from working solution of rutin. All measurements were in triplicate.

Procedure for the study of rutin–HSA interaction was the same as rutin–BSA except for injection (15 s at 50 mbar) and running voltage 8 kV. All measurements were in triplicate.

3. Results and discussion

The binding density, "*r*", is a measure of the average degree of binding expressed as the number of the moles of rutin bound per serum albumin protein [3]:

$$r = \frac{[D]_b}{[P]_t} = \sum_{i=1}^{m} n_i \frac{K_i[D]_f}{1 + K_i[D]_f} \quad (1)$$

where $[D]_b$ and $[D]_f$ are the bound and free rutin concentrations, respectively; $[P]_t$ is the total protein concentration; m is the number of independent types of binding sites; n is the number of binding sites of type i; K_i is the association constant of the site of type i.

By developing a method capable of measuring either the free or bound rutin concentration without disturbing their equilibrium, it is possible to construct a binding curve or plot of r versus $[D]_f$,

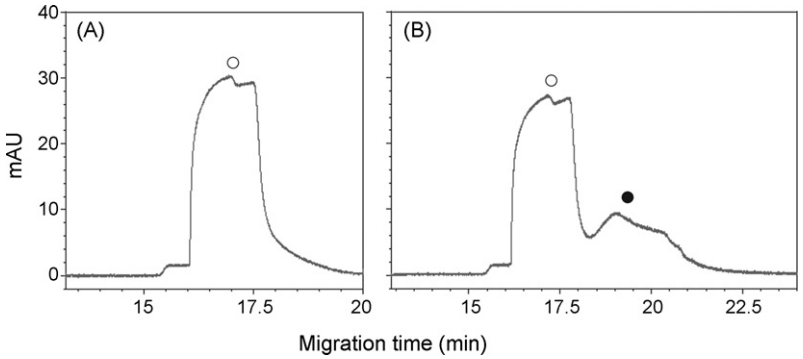

Fig. 2. CE-FA electropherograms of (A) 300 μM rutin; (B) 300 μM rutin and 40 μM BSA in 67 mM phosphate buffer (pH 7.4) at 37 °C. (○) Free rutin; (●) BSA–rutin complex + free BSA. Experimental conditions: total capillary length, 70 cm (61.5 cm effective length); 14 kV; λ = 270 nm; hydrodynamic injection for 80 s at 50 mbar.

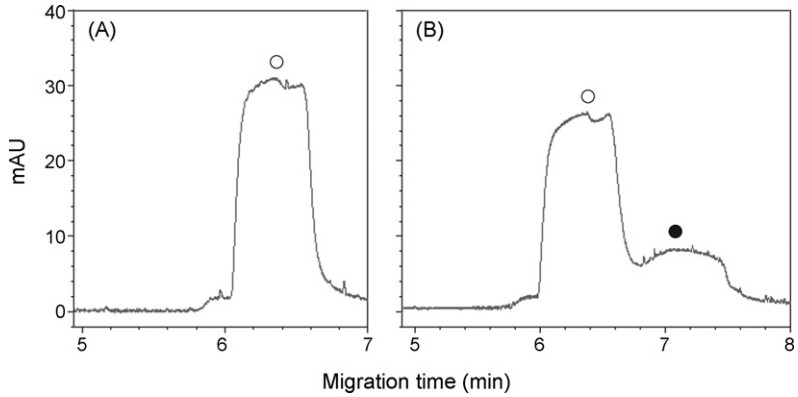

Fig. 3. CE-FA electropherograms of (A) 300 μM rutin; (B) 300 μM rutin and 40 μM HSA in 67 mM phosphate buffer (pH 7.4) at 37 °C. (○) Free rutin; (●) HSA–rutin complex + free HSA. Experimental conditions: total capillary length, 35 cm (26.5 cm effective length); 8 kV; λ = 270 nm; hydrodynamic injection for 15 s at 50 mbar.

which can thus be used to extract values by a nonlinear regression for m, n, and K_i.

The mobility of each HSA–rutin and BSA–rutin complex was determined similarly as that of the corresponding protein while have enough differences from that of rutin (data not shown here), which fulfilled the above requirements. Electropherograms with two plateaus will thus result. These plateaus are attributed to free rutin and the rutin–protein complex, co-eluting with free protein.

By comparison of the free rutin plateau height (designated as h_f) obtained during electrophoresis of a rutin–protein complex sample to that of known concentration ($[D]_s$) rutin-only calibration standard (designated as h_s), it was possible to determine the free rutin concentration $[D]_f$ [14], Eq. (2).

$$[D]_f = \frac{[D]_s}{h_s} h_f \quad (2)$$

From the free rutin concentration, it is possible to find the concentration of rutin bound to protein, Eq. (3), and $[D]_b$ can be used to calculate the fraction r the rutin molecules bound per protein molecule, as shown in Eq. (1).

$$[D]_b = [D]_t - [D]_f \quad (3)$$

Representative electropherograms of rutin-only and rutin–BSA sample and rutin–HSA sample are shown in Figs. 2 and 3. Here, the free rutin is seen as a plateau ("○") following the plateau attributed to the protein–rutin complex (which co-elutes with free protein) ("●"). The plateau heights and from comparable electropherograms (not shown) recorded for 10 samples containing 40 μM BSA or 40 μM HSA premixed with various concentrations of rutin, ranging from 40 to 500 μM were used in conjunction with Eqs. (2) and

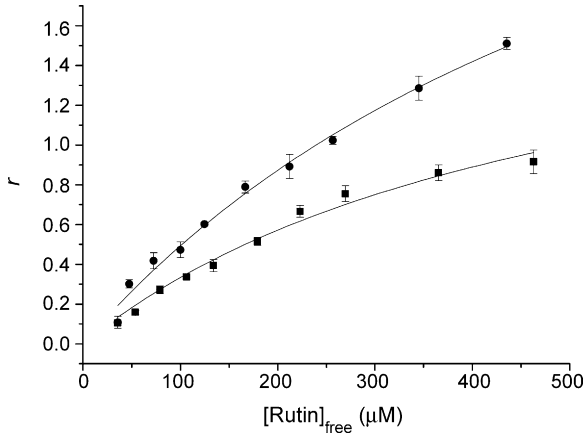

Fig. 4. Binding curves obtained by CE-FA for (●) rutin binding to HSA; (■) rutin binding to BSA with the CE-FA method. Separation buffer, 67 mM phosphate buffer (pH 7.4); samples, 40 μM serum albumin + varying concentrations of rutin (40, 60, 90, 120, 150, 200, 250, 300, 400 and 500 μM); all other conditions were the same as in Figs. 2 and 3.

Table 1
Binding parameters of rutin to BSA or HSA

Binding system	Method	[Albumin] (μM)	[Rutin] (μM)	Conditions	$n \pm$ error	$K \pm$ error (10^3 M^{-1})	Ref.
Rutin–BSA	CE-FA	40	40–500	0.067 M phosphate buffer (pH 7.4), 37 °C	2.00 ± 0.25	2.00 ± 0.40	This study
	Fluorescence enhancement	75		0.05 M phosphate buffer -0.1 M NaCl (pH 7.4), 25 °C	1.44 ± 0.56	10.7 ± 6.6	[8]
	Fluorescence enhancement	75		0.02 M phosphate buffer (pH 7.4), 25 °C	2.66 ± 0.21	8.6 ± 1.1	[9]
	Fluorescence quenching		0–80	0.1 M phosphate buffer -0.1 M NaCl (pH 7.4), 35 °C		1250	[10]
Rutin–HSA	CE-FA	40	40–500	0.067 M phosphate buffer (pH 7.4), 37 °C	3.68 ± 0.30	1.57 ± 0.19	This study
	Fluorescence quenching		0–80	0.1 M phosphate buffer -0.1 M NaCl (pH 7.4), 35 °C.		1670	[10]
	Fluorescence quenching	1	0–28	0.05 M Tris–HCl-0.1 M NaCl (pH 7.4), 26 °C.	1.06~1.60	54.8–128	[11]
	Fluorescence quenching	1	0–28	0.05 M Tris–HCl-0.1 M NaCl (pH 7.4), 37 °C.		41.5–105	[11]
	Fluorescence polarization	40	40	20 °C		30.8	[12]
	Fluorescence quenching	4	2–80	0.01 M Tris–HCl (pH 8.0), 25 °C.	1	68.7 ± 2.2	[13]

(3) in order to obtain the concentration of bound rutin $[D]_b$ in each sample.

The rutin concentration ranges in these studies balanced the need to achieve sufficient sensitivity while maintaining sufficient solubility without inclusion of a high percentage of organic solvent in rutin–protein sample mixtures. The excellent linear response observed between plateau height and injected rutin concentration demonstrates the feasibility of determining $[D]_b$ in this fashion. Each calculated value of $[D]_b$, in turn, was used to determine the fraction "r" of rutin molecules bound per protein molecule, according to Eq. (1). The best-fit values for binding parameters (Fig. 4) were achieved by using Origin Software (Version 7.022, Northampton, MA, USA). It is possible to extract the relevant noncovalent binding parameters for rutin–BSA at pH 7.4, which are: $m = 1$; $n = 2.00 \pm 0.25$ and $K_a = (2.00 \pm 0.40) \times 10^3\ M^{-1}$. The noncovalent binding parameters for rutin–HSA at pH 7.4 are calculated as: $m = 1$; $n = 3.68 \pm 0.30$ and $K_a = (1.57 \pm 0.19) \times 10^3\ M^{-1}$. Both affinity constants are in the comparable range suggesting the similarity of affinity properties of HSA and BSA towards rutin. The data are also in the similar range compared with most data obtained in the publications by conventional fluorescence methods (Table 1).

4. Conclusions

It is believed that not just a few active compounds but all the components in traditional herbal medicines are responsible for the integral pharmaceutical effects. It is essential to study active components' binding to plasma proteins step by step to understand their pharmacokinetic and pharmacodynamic properties. As an example, in the present paper, the CE-FA method was applied to determine the noncovalent affinity parameters for rutin with serum albumin (BSA and HSA) at simulated physiological conditions (pH 7.4, 37 °C). The proposed CE-FA method is simple, rapid and cost-effective.

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