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Investigation of the Mechanism of Enhanced Effect of EGCG on Huperzine A's Inhibition of Acetylcholinesterase Activity in Rats by a Multispectroscopic Method

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The mechanism of enhanced effect of (−)-epigallocatechin-3-gallate (EGCG) on huperzine A's (HUP) inhibition of acetylcholinesterase (AChE) activity in rats was investigated. The inhibitory effects of HUP at 10 and 5 μg/kg on AChE activity were quite weak in the whole phase. In contrast, upon addition of EGCG (100 mg/kg) to the HUP 10 and 5 μg/kg groups, remarkably enhanced inhibitory effects with maximum inhibitory percentages of 90.94 and 88.13% were observed under the same conditions. EGCG also can greatly prolong the inhibitory time. The mechanism of the enhanced effects of EGCG on HUP's inhibition of AChE activity was investigated by steady fluorescence spectroscopy, infrared spectroscopy, and ultraviolet spectroscopy. HUP hardly interacted with the main transport protein, whereas there was a very strong binding interaction between EGCG and bovine serum albumin. The enhanced transport of HUP is a possible cause of the enhanced effect of EGCG on HUP bioactivity.

KEYWORDS: Huperzine A; (−)-epigallocatechin-3-gallate; acetylcholine esterase; BSA; fluorescence quenching

INTRODUCTION

(−)-Epigallocatechin-3-gallate (EGCG, **Figure 1**), which constitutes 50% of the catechins in tea, is a major mediator of the antioxidative effect of green tea and has attracted great interest among researchers (*1–4*). EGCG is the most widely studied polyphenol for disease prevention. Many of the putative health benefits of tea are presumed to be caused by its antioxidant effects (*5*).

Huperzine A (HUP, **Figure 1**) is a plant-based alkaloid separated from qian ceng ta (*Huperzia serrata*), which has been used for centuries to treat fever, inflammation, blood disorders, and schizophrenia (*6, 7*). HUP has been found to reverse or attenuate cognitive deficits in several animal models (*8–10*). HUP acts as a potent and reversible inhibitor of acetylcholinesterase (AChE). Its potency of AChE inhibition is similar or superior to those of physostigmine, rivastigmine, galanthamine, donepezil, and tacrine (*11, 12*). The latter three drugs are acethylcholineesterase inhibitors (AChEIs) approved for Alzheimer's disease (AD) in the United States and some European countries.

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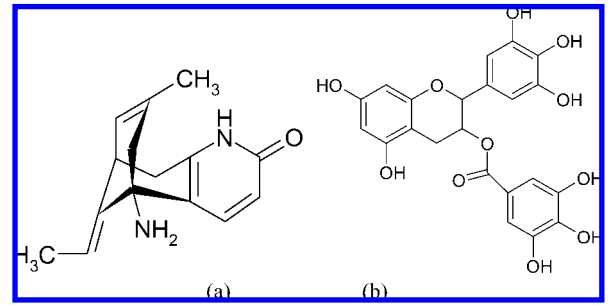

Figure 1. Chemical structures of HUP (**a**) and EGCG (**b**).

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Antioxidant therapy is another treatment pathway for AD (*13*). Injury to cell structures by highly reactive oxygen agents generated in normal cell metabolism is believed to play an important role in many age-related diseases (*14*). Clinical trials using antioxidants such as vitamin E have been proposed, supported by epidemiological evidence of these agents in reducing the risk of the development of AD (*15*).

To our knowledge, there are no papers examining the bioactivity of HUP combined with EGCG. Herein, we compare inhibitory effects of HUP on AChE activity with and without EGCG. The mechanism of enhanced effect of EGCG on HUP inhibiting AChE activity was investigated by means of a multispectroscopic method.

MATERIALS AND METHODS

Chemicals and Reagents. HUP (with a purity of >98%) was provided by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). EGCG (with a purity of >99%) was purchased from the Institute of Tea, Chinese Academy of Agriculture. Bovine serum albumin (BSA; fraction V) was purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents and solvents were of analytical reagent grade and used without further purification unless otherwise noted. All aqueous solutions were prepared using newly double-distilled water.

Experimental Animals. All experimental protocols involving animals were reviewed and approved by the institutional animal experimentation committee of the Central South University (Changsha, China). Adult male Sprague–Dawley rats (195–205 g) were supplied by the laboratory animal center of Xiangya Hospital, Central South University. They were housed in groups of three or four in a room maintained at 25 °C with a 12 h light/dark cycle and were allowed free access to water and food. The rats were treated with different doses of HUP (5, 10, 20, 40, 80, and 120 μg/kg), EGCG (10, 50, 100, 150, 200, and 300 mg/kg), and HUP + EGCG (HUP 5 μg/kg + EGCG 100 mg/kg; HUP 10 μg/kg + EGCG 100 mg/kg) by oral administration. Rats were sacrificed at intervals of 0.5, 1.0, 2.0, 4.0, 8.0, 12.0, or 24.0 h by decapitation. The group of rats sacrificed at 0 h was used as the control group. The brain was rapidly dissected on ice and then weighed and homogenized in 6 volumes of cold 75 mM sodium phosphate buffer, pH 7.4. Homogenates were centrifuged at 13000*g* for 30 min at 4 °C. The supernatant used as acetylcholinesterase source was divided into aliquots and stored at −20 °C.

Measurement of AChE Activity. AChE activity was measured according to the Ellman method (*16*). Briefly, 125 μL of 3 mM 5,5′-dithiobis(2-nitrobenzoic acid), 25 μL of 15 mM acetylthiocholine iodide for AChE activity, and 25 μL of sample dissolved in buffer containing not more than 10% methanol were added to microplate wells, followed by 25 μL of 0.28 unit/mL AChE. The microplate was then read at 405 nm every 5 s for 2 min by a CERESUV900C microplate reader (Bio-Tek Instrument,Winooski, VT). The velocities of the reactions were measured. AChE activity rates were calculated as a percentage of the velocities compared to that of the control group. AChE inhibitory rates were calculated by subtracting the percentage of AChE activity rates from 100%. Every experiment was done in triplicate. Stock solutions of samples in Trisma hydrochloride (Tris-HCl) buffer containing not more than 10% methanol were diluted serially with Tris-HCl buffer to obtain eight or nine different concentrations.

BSA Fluorescence Quenching Study. Fluorescence spectra were recorded on a JASCO *FP-6500* spectrofluorometer. Tris-HCl buffer (0.20 mol/L, pH 7.4) containing 0.10 mol/L NaCl was selected to keep the pH value and maintain the ionic strength of the solution. Appropriate quantities of EGCG and HUP (1.0×10^{-4} mol/L) solution were transferred to a 10 mL flask, treated with 1.0×10^{-5} mol/L BSA (1.0 mL), and made up with Tris-HCl buffer to a final volume of 10 mL and then incubated at 37 °C for 1 h. The solution was scanned on the fluorophotometer with the range of 290–400 nm. The fluorescence intensity at 340 nm was determined under the excitation at wavelength of 280 nm.

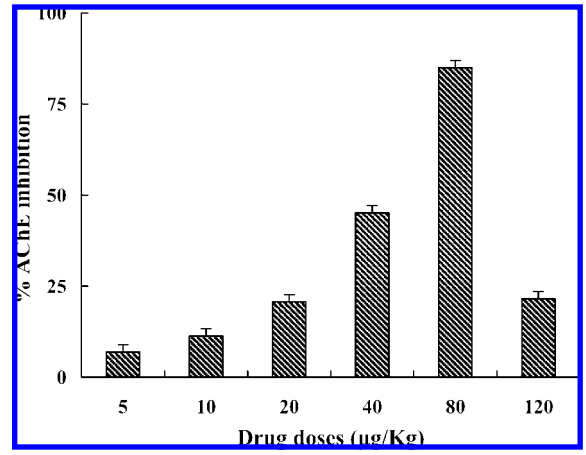

Figure 2. Dose−response curve of percent AChE inhibition of HUP (1 h after oral administration). Values are the mean of one typical experiment performed in triplicate.

For static quenching, the relationship between fluorescence quenching intensity and the concentration of quenchers can be described by the equation (*17, 18*)

$$\lg\frac{F_0 - F}{F} = \lg K_a + n \lg[Q]$$

where K_a is the binding constant and n is the number of binding sites per BSA.

Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR). ATR-FTIR measurements were carried out on a Nicolet Nexus 670 FT-IR spectrometer. All spectra were taken via the ATR method with a resolution of 4 cm^{-1} and using 60 scans. The spectra-processing procedure involved collecting spectra of the buffer solution under the same conditions. Next, the absorbency of the buffer solution was subtracted from the spectra of the sample solution to obtain the FT-IR spectra of BSA.

Statistical Analysis. Data from AChE activity were expressed as mean ± SE. Statistical analyses were carried out using parametric analysis (ANOVA) and Fisher's LSD test for post hoc testing to calculate the significance for both TDE-treated and control rats. Significance was set at $P < 0.05$.

RESULTS AND DISCUSSION

Inhibitory Effect of HUP on AChE Activity. As shown in **Figure 2**, HUP showed a dose-dependent inhibitory effect on AChE activity. The inhibitory percentages of AChE activity compared to that of the control group were 6.90, 11.29, and 84.95% for the 5, 10, and 80 μg/kg HUP groups, respectively (1 h after oral administration). The AChE levels of the HUP groups were significantly different from that of the control group ($P < 0.05$; **Figure 2**). When the dose of HUP was over 80 μg/kg, the AChE inhibitory rate began to decrease, but EGCG had no inhibitory effect on AChE activity within the range of 10–300 mg/kg (data not shown). No difference was found among these EGCG-treated groups ($P > 0.05$).

Effect of EGCG on HUP Inhibitory AChE Activity. The inhibitory percentage of the 80 μg/kg HUP group on AChE activity arrived at its maximum (84.68%) within 1 h and decreased rapidly 2h after administration. The 80 μg/kg HUP group had no inhibition effect on AChE activity at 24 h after oral administration (**Figure 3**, curve b). The inhibitory percentage of 80 μg/kg HUP group on AChE activity was higher than that of the HUP 10 μg/kg + EGCG 100 mg/kg group within 1 h of administration (**Figure 3**, curves a and b). However, 1 h after oral administration, the inhibitory percentage of the HUP 10 μg/kg + EGCG 100 mg/kg group on AChE activity was

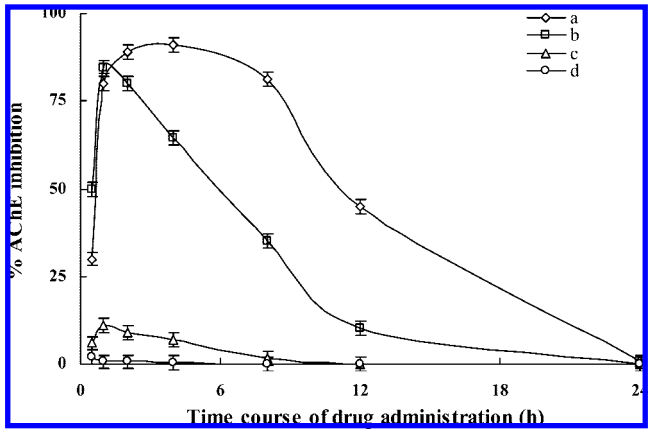

Figure 3. Time course of inhibition of AChE activity following oral administration: a, HUP 10 μg/kg + EGCG 100 mg/kg; b, HUP 80 μg/kg; c, HUP 10 μg/kg; d, EGCG 100 mg/kg.

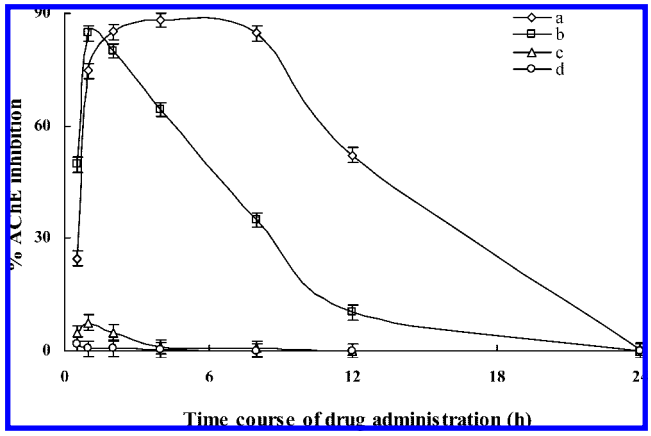

Figure 4. Time course of inhibition of AChE activity following oral administration: a, HUP 5 μg/kg + EGCG 100 mg/kg; b, HUP 80 μg/kg; c, HUP 5 μg/kg; d, EGCG 100 mg/kg.

higher than that of the 80 μg/kg HUP group (**Figure 3**), and there was a stable phase with high inhibition effect (>80%) on AChE activity from 1 to 8 h. Eight hours after oral administration, the inhibitory percentage of the HUP 10 μg/kg + EGCG 100 mg/kg group on AChE activity decreased rapidly, and there was no inhibition effect on AChE activity at 24 h after oral administration. The same results were also found in the HUP 5 μg/kg + EGCG 100 mg/kg group (**Figure 4**).

It can be seen that the inhibitory percentage of the HUP 5 and 10 μg/kg groups on AChE activity were quite weak in the whole phase (**Figure 3**, curve c, and **Figure 4**, curve c). In contrast, upon addition of EGCG (100 mg/kg) to the HUP 10 and 5 μg/kg groups, the remarkably enhanced inhibitory effects with maximum inhibitory percentages of 90.94 and 88.13% were observed under the same conditions (**Figure 3**, curve a, and **Figure 4**, curve a). EGCG also can greatly prolong the inhibitory time. These results indicated that addition of EGCG to HUP can reduce the dose of HUP to $^1/_8$–$^1/_{16}$ with higher activity than that of the high dose of HUP.

Fluorescence Quenching of BSA by EGCG. The fluorescence quenching of BSA induced by EGCG is shown in **Figure 5**. With the increasing concentration of EGCG (from 2.00 to 20.00 $\times$ 10^{-7} mol/L), the fluorescence intensity of BSA decreased remarkably. However, the high concentration of HUP (5.00 $\times$ 10^{-5} mol/L) hardly quenches BSA fluorescence (data not shown). It can be concluded that HUP hardly interacted with the main transport protein.

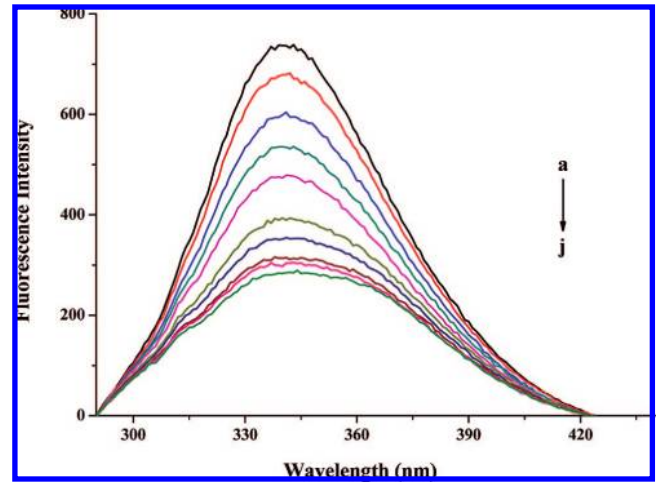

Figure 5. Quenching effect of EGCG on BSA fluorescence intensity: λ_{ex} = 280 nm; (a–j) BSA, 1.00 $\times$ 10^{-6} mol/L, 0.00, 2.00, 4.00, 6.00, 8.00, 12.00, 14.00, 16.00, 18.00, 20.00 ($\times$ 10^{-7} mol/L) of EGCG (pH 7.4, T = 37 °C).

According to eq 1, the apparent binding constant (K_a) between EGCG and BSA was 2.96 $\times$ 10^7 L/mol (R = 0.9991) and the binding sites value (n) was 1.27. Normally the binding constant between small molecules and BSA was 10^3–10^5 L mol^{-1} (*17–21*). The result illustrated that a very strong binding force formed between EGCG and BSA.

The most outstanding function of serum albumin is that it serves as a depot protein and a transport protein for many exogenous compounds (*19–24*). The drug–protein interaction may result in the formation of a stable protein–drug complex, which has important effects on the distribution, free concentration, and metabolism of drug in the blood stream. Thus, the drug–albumin complex may be considered as a model for gaining fundamental insights into drug–protein interactions. How did EGCG enhance the inhibitory effect of HUP on AChE activity? We hypothesized that HUP interacted with the EGCG–BSA complex, which enhanced the transport capacity of HUP in the blood. This process increased the pharmacodynamic effect of HUP in inhibiting AChE activity, so after the addition of EGCG to the low-dose groups of HUP, the remarkably enhanced inhibitory effects took place.

Analysis of BSA Conformation after EGCG and HUP Binding. We had ascertained that the binding of HUP to the EGCG–BSA complex causes the enhanced effects of EGCG in the inhibition of AChE activity of HUP, but it is still a puzzle about whether the binding affects the conformation and/or microenvironment of BSA. To further verify the binding of HUP to the EGCG–BSA complex and investigate BSA structure after HUP addition, synchronous fluorescence and infrared spectroscopies were utilized.

The synchronous fluorescence spectra of the EGCG–BSA and EGCG–BSA–HUP systems are presented in the **Figures 6** and **7**, respectively. As shown in **Figure 6A**, the fluorescence intensity of BSA weakened regularly along with the addition of EGCG, implying that EGCG bonded to BSA and located in close proximity to the Tyr residues (*25, 26*). A blue shift of the λ_{max} (~1.0 nm) is shown in Figure 6**B** with a gradual quenching, indicating that EGCG was close to the Trp residue and had an effect on the microenvironment around Trp residue.

As shown in **Figure 7A**, upon addition of HUP to the EGCG–BSA system, the fluorescence intensity decreased strongly with a blue shift of the λ_{max} (~1.5 nm), implying that HUP interacted wth EGCG–BSA.

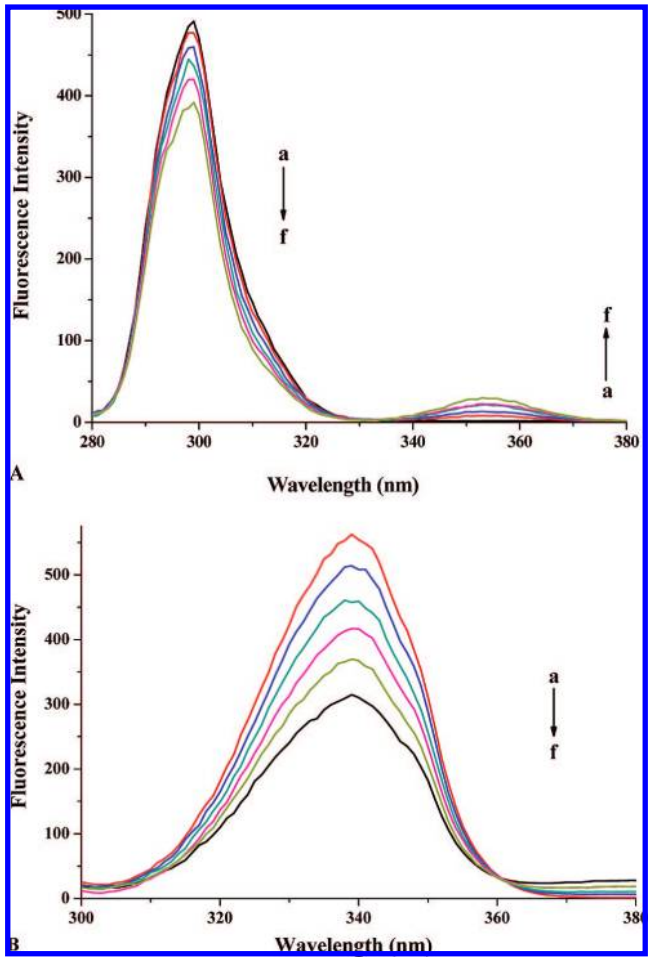

Figure 6. Synchronous fluorescence spectra of interaction between BSA and EGCG (**A**) at $\Delta\lambda = 15$ nm and (**B**) at $\Delta\lambda = 60$ nm. The concentration of BSA was 1.00×10^{-6} mol/L, whereas concentrations of EGCG were 0.00, 2.00, 4.00, 6.00, 8.00, 10.00×10^{-7} mol/L from a to f (pH 7.4, $T = 37$ °C).

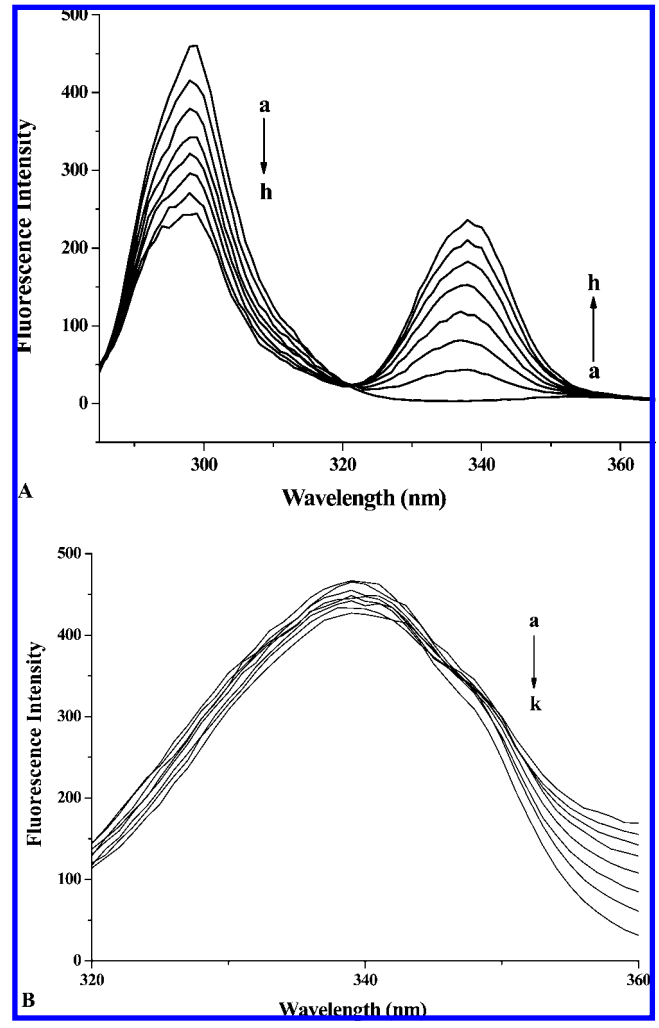

Figure 7. Synchronous fluorescence spectra of interaction between BSA−EGCG−HUP (**A**) at $\Delta\lambda = 15$ nm and (**B**) at $\Delta\lambda = 60$ nm. Concentrations of BSA and EGCG were 1.00×10^{-6} and 4.00×10^{-7} mol L^{-1}, whereas concentrations of HUP were 0.0, 0.50, 1.00, 1.50, 2.00, 2.50, 3.00, and 3.50×10^{-6} mol L^{-1} from a to h (pH 7.4, $T = 37$ °C).

When the NH_2 of the HUP molecule is close to the phenyl moiety of EGCG, the hydrogen bond interaction between HUP and EGCG may form. HUP weakens the interaction between EGCG and the Trp residue, which results in no quenching taking place when $\Delta\lambda$ is 60 nm (**Figure 7B**).

The FT-IR spectra of the EGCG-free and EGCG-bound form of BSA with its difference absorption spectrum are shown as panels **a** and **b** of **Figure 8**. The spectrum in **Figure 8a** was obtained by subtracting the absorption of the Tris-HCl buffer from the spectrum of the protein solution. The spectrum in **Figure 8b** was obtained by subtracting the spectrum of EGCG from that of the BSA−EGCG complex. The spectrum in **Figure 8c** was obtained by subtracting the spectrum of HUP from that of the BSA−HUP complex. The protein amide I in the region 1600–1700 cm^{-1} (mainly C═O stretch) and amide II band ≈1540 cm^{-1} (C−N stretch coupled with N−H bending mode) both have a relationship with the secondary structure of protein, and the amide I band is more sensitive to the change of protein secondary structure than amide II (*27*). As shown in **Figure 8a**,**b**, the peak position of the amide I band moved from 1653.21 to 1652.57 cm^{-1} and the amide II band moved from 1541.60 to 1541.04 cm^{-1}, which indicate that the protein secondary structure was changed after EGCG was added. As shown in **Figure 8c**, the peak positions of the amide I band and the amide II band were almost the same as that of BSA (**Figure 8a**), which confirmed that HUP hardly interacted with the main transport protein.

Lin et al. studied the pharmacokinetics of EGCG in rats (*2*). They found that the elimination half-lives of EGCG were 62 ± 11 and 48 ± 13 min for intravenous (10 mg/kg) and oral (100 mg/kg) administration, respectively. They found that EGCG had a high protein binding ratio. The protein binding in rat plasma was 92.4 ± 3.1% by means of ultrafiltration method.

The displacement of EGCG protein binding may affect the clotting time and lead to hemorrhage via drug−drug interaction. The compound galloyl groups such as EGCG and ECG potentially bind to proteins or membrane surfaces by hydrogen bonding via phenolic terminals, particularly to the proteins (*28*), which may be similar to our findings.

Several Alzheimer drugs approved by the U.S. Food and Drug Administration, such as tacrine and E2020, work by means of inhibiting AChE activity. HUP, which differs from these drugs in chemical structure but also inhibits AChE activity, is currently investigated in China and elsewhere as a possible Alzheimer's drug. Zeng et al. studied the synthesis and AChE inhibitory activity of a HUP−E2020 combined compound. The results

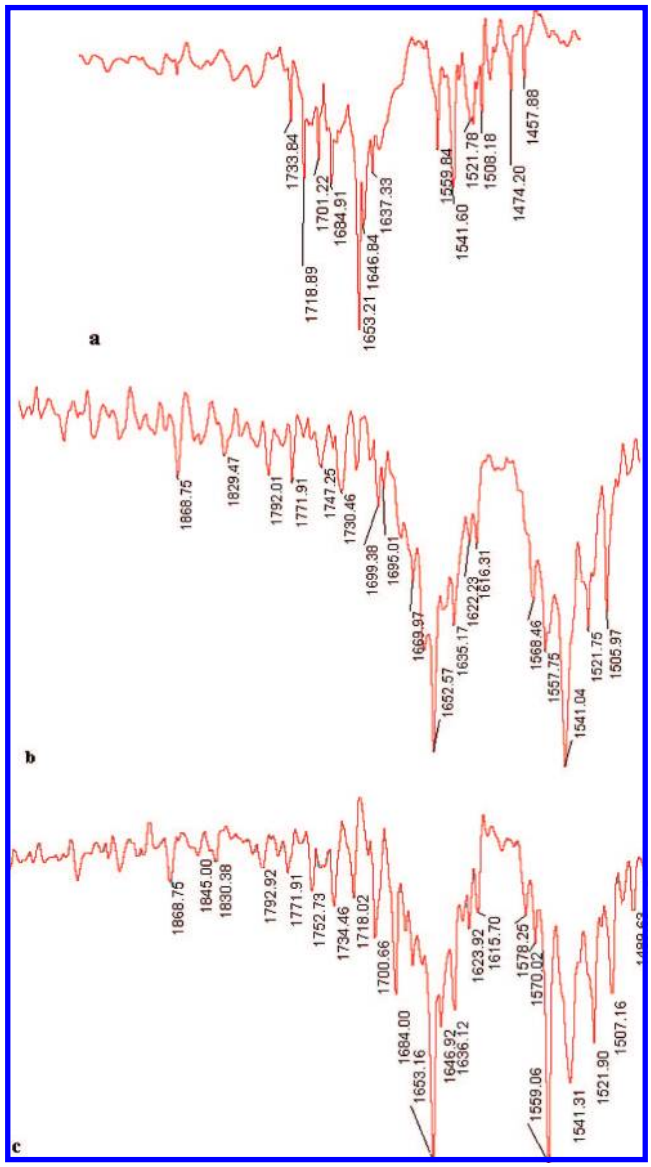

Figure 8. FT-IR spectra of (**a**) BSA, (**b**) BSA after interacting with EGCG at physiological pH (BSA, 1.0×10^{-5} mol/L; EGCG, 1.00×10^{-4} mol/L), and (**c**) BSA after interacting with HUP at physiological pH (BSA, 1.0×10^{-5} mol/L; HUP, 1.00×10^{-4} mol/L).

indicated that the HUP−E2020 combined compound was 850-fold less active than E2020 (*29*).

Isolation and purification of HUP from*H. serrata* is tedious work, because the content of HUP in the *H. serrata* is very low (<0.01%, w/w) (*30, 31*). The natural resources of HUP source plants are not abundant and found only in very specialized habitats. Because of the great health benefits that these plants provide, *H. serrata* has suffered from extensive collection in recent years, which may soon lead to its extinction (*32*). Thus, it is imperative not only to protect the natural resources of HUP but also to support efforts to develop synthetic derivative or alternative forms of HUP as a new drug for AD treatment. However, the synthesis of HUP and its derivatives appears to suffer from complexity and high cost with low yield (*33*). It is necessary to find complementary drugs that can enhance the pharmacological effect of HUP. EGCG is one of these complementary drugs. According to the new finding of this paper, it is worthwhile to test other catechins, such as (−)-epicatechin, (−)-epigallocatechin, and (−)-epicatechin-3-gallate.

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