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Research paper

Effect of ethanol or/and captopril on the secondary structure of human serum albumin before and after protein binding

Shan-Yang Lin[a,*], Yen-Shan Wei[a], Mei-Jane Li[a], Shun-Li Wang[b]

[a]*Biopharmaceutics Laboratory, Department of Medical Research and Education, Veterans General Hospital-Taipei, Taipei, Taiwan, ROC*
[b]*Department of Applied Chemistry, National ChiaYi University, ChiaYi, Taiwan, ROC*

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Abstract

The attenuated total reflection/Fourier transform infrared technique has been utilized to characterize secondary structural changes in human serum albumin (HSA) before and after protein binding via incubation of HSA in different concentrations of ethanol, captopril or ethanol/captopril mixture. The results indicate that ethanol induced a transition from β-sheet to an α-helical structure and promoted conversion of intramolecular hydrogen-bonded β-sheet to intermolecular hydrogen-bonded β-sheet. In contrast, captopril or captopril/ethanol mixture induced conversion of intramolecular hydrogen-bonded β-sheet to intermolecular hydrogen-bonded β-sheet and resulted in exposure of the aromatic side-chain groups in the unfolding conformation of HSA. Thus, protein binding between HSA and captopril or captopril/ethanol seems to play an important role in protein secondary structure.

Keywords: Human serum albumin; Attenuated total reflection/Fourier transform infrared; Secondary structure; Ethanol; Captopril; Protein binding

1. Introduction

Peptides or proteins in aqueous solution have conformations matchable to their physiological activities. Recently, a challenge in biochemistry is how to exactly determine the protein structure and how to correlate this structure with its activity. To aware the structure of a protein, the primary amino acid sequence along with its folding patterns at the secondary and tertiary structure levels should be investigated. Several useful methods for the determination of secondary structure of protein including nuclear magnetic resonance spectroscopy (NMR), circular dichroism (CD), Fourier transform infrared spectroscopy (FTIR), and X-ray crystallography have been utilized [1].

The FTIR spectroscopy is a powerful tool with non-destructive and molecular 'fingerprinting' capabilities for studying the structural characterization of proteins, particularly hydrogen bonding. By connecting with computerized software, FTIR spectroscopy with an attenuated total reflection (ATR) is one of the sensitive techniques for an extensive study on the secondary conformation of proteins in aqueous solution via subtracting the water contribution from the protein aqueous solution [1–3]. This ATR–FTIR spectroscopy can easily gain information on the structure of biological molecules from amide I–III bands. The amide I region is widely used, as it is more sensitive to the change of protein secondary structure than other amide bands [4,5]. The conformational sensitivity of the amide bands includes hydrogen bonding and protein secondary structure, which is sensitive for FTIR spectroscopic determinations.

Human serum albumin (HSA) is the most abundant protein constituent of blood plasma and has many important physiological functions [6]. HSA is a globular protein consisting of a single polypeptide chain of 585 amino acid residues and a molecular weight of 66,500 Da [7]. It cannot only serves as a transporter or/and depot carrier for many endogenous and exogenous compounds, but also maintain the osmotic pressure and play a role in coagulation and thrombosis. It has also been reported that the most important contribution to drug binding in the plasma is made by HSA, which comprises about one half of the total plasma proteins. Binding to HSA may affect the distribution and elimination of drug, as well as both pharmacokinetic

* Corresponding author. Biopharmaceutics Laboratory, Department of Medical Research and Education, Veterans General Hospital-Taipei, Shih-Pai, Taipei, Taiwan, ROC. Tel.: +886-22-875-7397; fax: +886-22-873-7200.
E-mail address: sylin@vghtpe.gov.tw (S.-Y. Lin).

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and pharmacodynamic effects of drug. Co-administration of multiple drugs that bind to HSA produces complex alterations in pharmacokinetic and pharmacodynamic properties of drugs and may cause unexpected drug responses or side effects [8,9]. It is interesting to note whether the drug–protein interaction may alter the protein secondary structure of HSA, thus to modify biophysical and biochemical properties of HSA.

Ethanol produces three distinct effects on protein structure: (a) destruction of rigid native structure, (b) induction of α-helical structure, and (c) dissolution of peptide or its aggregates [10]. Ethanol-induced changes in native protein structure may lead to direct binding of ethanol to the specific hydrophobic binding sites and/or displacement of endogenous ligands from those sites or via replacement of hydrogen-bonded water by ethanol [11,12]. Furthermore, alcohol-induced denaturation of protein might result in stabilization of extended helical rods in which the hydrophobic side-chains are exposed but the polar amide groups are shielded from the solvent [13]. This is supported by other reports, in which ethanol altered warfarin–HSA binding and beer consumption produced changes in native protein functions, due to alterations in compactability, stability, and structural deviations of HSA [14,15].

Captopril is a novel angiotensin converting enzyme (ACE) inhibitor that contains one unique free sulfhydryl (SH) group and binds proteins via sulfhydryl (SH)/disulfide (SS) interaction. It binds to HSA via non-covalent and covalent binding, in which the non-covalent binding decreased rapidly with time but the covalent binding increased gradually [16]. Captopril forms disulfide bonds with albumin or other proteins, but the reactivity in serum is about 10-fold higher than that obtained for HSA solutions. Parameters such as pH, drugs, oxygen and metal ions may be responsible for these differences [17].

Although several studies have been reported to investigate the interaction of single drug with HSA by FTIR spectroscopy [18,19], there are few report to study the co-effect of two drugs on the protein structure of HSA. The purpose of this study was to investigate the effect of ethanol or/and captopril on the secondary structural conformation of HSA before and after protein binding process using ATR/FTIR spectroscopy.

2. Materials and methods

2.1. Materials

HSA (A-1887) was purchased from Sigma Chem. Co. (St Louis, MO) and used without further purification. Ethyl alcohol (ethanol >99.5%) was of analytical reagent grade and obtained from Nakalai Tesque, Kyoto, Japan. Pharmaceutical grade captopril was purchased from Bulk Med. and Pharm., Hamburg, Germany.

2.2. Preparation of different HSA–ethanol stock solutions

Different HSA–ethanol stock solutions containing HSA (5%, w/v) with or without addition of ethanol (10 or 25%, v/v) were, respectively, prepared by dissolving it in pH 7.4 Mcllvaine buffer solution. The pH of solution was maintained to 7.4 by using 1 N NaOH or 1 N HCl aqueous solution, and then incubated at 25 °C. At the pre-described intervals, the incubated solution was sampled for FTIR determination.

2.3. Preparation of different HSA–captopril or HSA–captopril–ethanol stock solutions

Different HSA–captopril or HSA–captopril–ethanol stock solutions containing HSA (5%, w/v) with addition of captopril (2.5%, w/v) alone or captopril (2.5 or 5.0%, w/v) and ethanol (10%, v/v) were prepared by dissolving in pH 7.4 Mcllvaine buffer solution. The pH of solution was maintained at 7.4 by using 1 N NaOH or 1 N HCl aqueous solution and subsequent incubation at 25 °C. At the pre-described intervals, the incubated solution was sampled for FTIR determination.

2.4. ATR/FTIR spectroscopic studies

Each HSA sample solution was analyzed using an ATR/FTIR spectrometer (FT/IR-620, Jasco, Tokyo, Japan) equipped with a deuterated L-alanine triglycine sulfate (DLATGS) detector [20,21]. The sample solution was placed in a horizontal ATR accessory (Pike Tech., WI, USA) with zinc selenide prism. All spectra were carried out at 100 scans and a resolution of 4 cm^{-1}. Solvent spectra were also examined in the same accessory and instrument conditions as sample spectra. Each different spectrum was obtained by digitally subtracting solvent spectrum from the corresponding sample spectrum. A scaling factor was applied to the solvent spectrum with varying in the iterative procedure until the baseline was flat. The first-derivative minimization method was utilized to determine the flattest baseline [22]. The final scaling factor was determined by halving the minimum summation between 1750 and 1950 cm^{-1}. Each sample solution was divided into three batches and analyzed separately. The individual spectrum of three determinations to each sample was obtained and averaged to produce a single spectrum for subsequent data processing.

2.5. Data acquisition and handling

Spectral manager software (Jasco Co., Tokyo, Japan) was used for data acquisition and handling. Second-derivative spectral analysis was applied to locate the positions of the overlapping components of the amide I band and assign them to different secondary structures [20–22]. The protein secondary structure and the composition of each component

in amide I band of these IR spectra were estimated quantitatively by a least-square fitting program iterating the curve-fitting process according to the Gaussian function. Adjustment was performed until the synthetic curve matched the experimental one with a minimum standard error. The proportion of each component was computed to be the fractional area of the corresponding peak divided by the sum of the areas of all the peaks.

3. Results

Fig. 1 shows the effect of ethanol concentration and incubation time on the IR spectra of HSA in the pH 7.4 buffer solution. Obviously, there was no alteration to protein structure of HSA in the absence of ethanol. Two major absorption peaks in the spectral region were observed: the amide I band at 1650 cm^{-1} and the amide II band at 1547 cm^{-1}. Both peaks are indicative of a predominant structural contribution from α-helices [5,23]. Five main peaks at 1682, 1652, 1631, 1547 and 1515 cm^{-1} were observed in the second-derivative IR spectra. The peaks at 1682 and 1632 cm^{-1} represented β-turn and β-sheet structures, respectively, and the peaks at 1652 and 1547 cm^{-1} represented an α-helix conformation. The peak at 1515 cm^{-1} was most likely due to tyrosine absorption. After incubation of ethanol, the IR spectra of HAS produced a shift in the 1632 cm^{-1} peak to 1629 cm^{-1} (10% ethanol) or 1626 cm^{-1} (25% ethanol), indicating a structural transformation from an intramolecular hydrogen-bonded β-sheet to an intermolecular hydrogen-bonded β-sheet [24].

The curve-fitted amide I band of HSA and its components, assignments and compositions with the increase of ethanol concentration and incubation time are illustrated in Fig. 2. The structural composition of native HSA in the absence of ethanol consisted of 52% α-helix, 37% β-sheet and 11% β-turn, which was consistent with other reports [18,25]. The α-helix composition of HSA increased in proportion to ethanol concentration and incubation time, whereas total β-sheet composition decreased, and β-turn composition remained unchanged.

Time-dependent changes in IR spectra resulting from captopril and HSA–captopril mixture in pH 7.4 buffer solution are shown in Fig. 3. Captopril or HSA–captopril mixture produced no change in original and second-derivative IR spectra. However, the second-derivative IR spectra of HSA after subtracting captopril from HSA–captopril mixture demonstrated subtle changes in protein structure with increased incubation time; the peak at 1628 cm^{-1} assigned to the intramolecular hydrogen-bonded β-sheet gradually shifted to 1622 cm^{-1} due to the intermolecular hydrogen-bonded β-sheet.

Fig. 4 demonstrates the original and second-derivative IR spectra of HSA after subtracting 10% ethanol and 2.5%

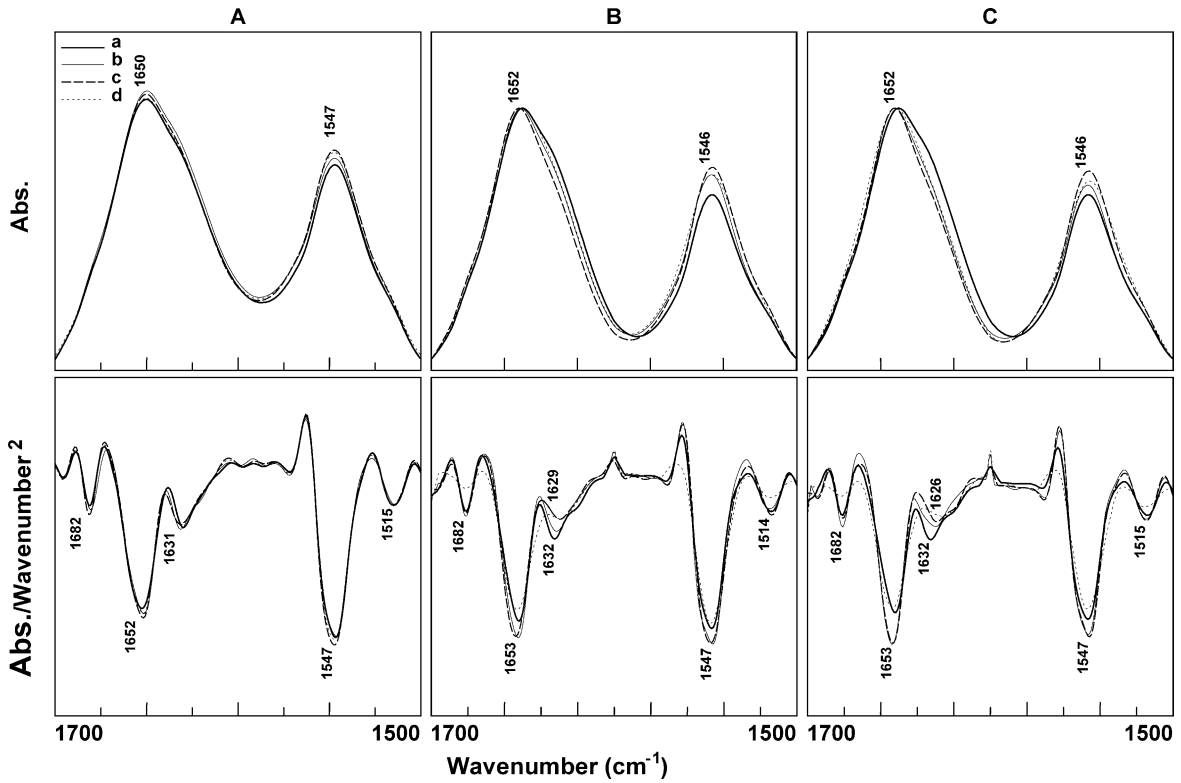
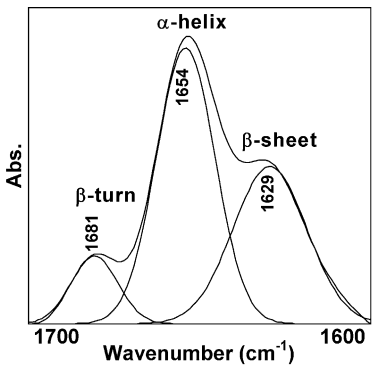


Fig. 1. Effect of different ethanol concentration and incubation time on the IR spectra of HSA in pH 7.4 buffer solution. Key: (A) HSA before treatment with ethanol. (B) HSA after treatment with 10% ethanol. (C) HSA after treatment with 25% ethanol. (a) Native HSA; incubation time: (b) 0.1; (c) 5.0; (d) 24.0 h.



Sructural components	Compositions (%)								
	No treatment	10% Ethanol				25% Ethanol			
		0.1hr	5hr	10hr	24hr	0.1hr	5hr	10hr	24hr
α -helix	52	56	58	60	62	57	60	63	65
β-sheet	37	34	33	31	29	32	28	27	26
β-turn	11	10	9	9	9	11	12	10	9

Fig. 2. The effect of ethanol on the components, assignments and compositions of the curve-fitted amide I band of HSA before and after treatment with different ethanol concentrations.

captopril or HSA after subtracting 10% ethanol and 5.0% captopril from the HSA–captopril–ethanol mixture. HSA precipitated with longer incubation times, producing a gradual reduction in IR absorption of HSA in pH 7.4 buffer solution. In addition, the peak at 1630 cm^{-1} gradually shifted to 1623 or 1620 cm^{-1}, and additional peaks were observed around 1600–1605 cm^{-1}, likely reflecting aromatic side-chain groups [5,23].

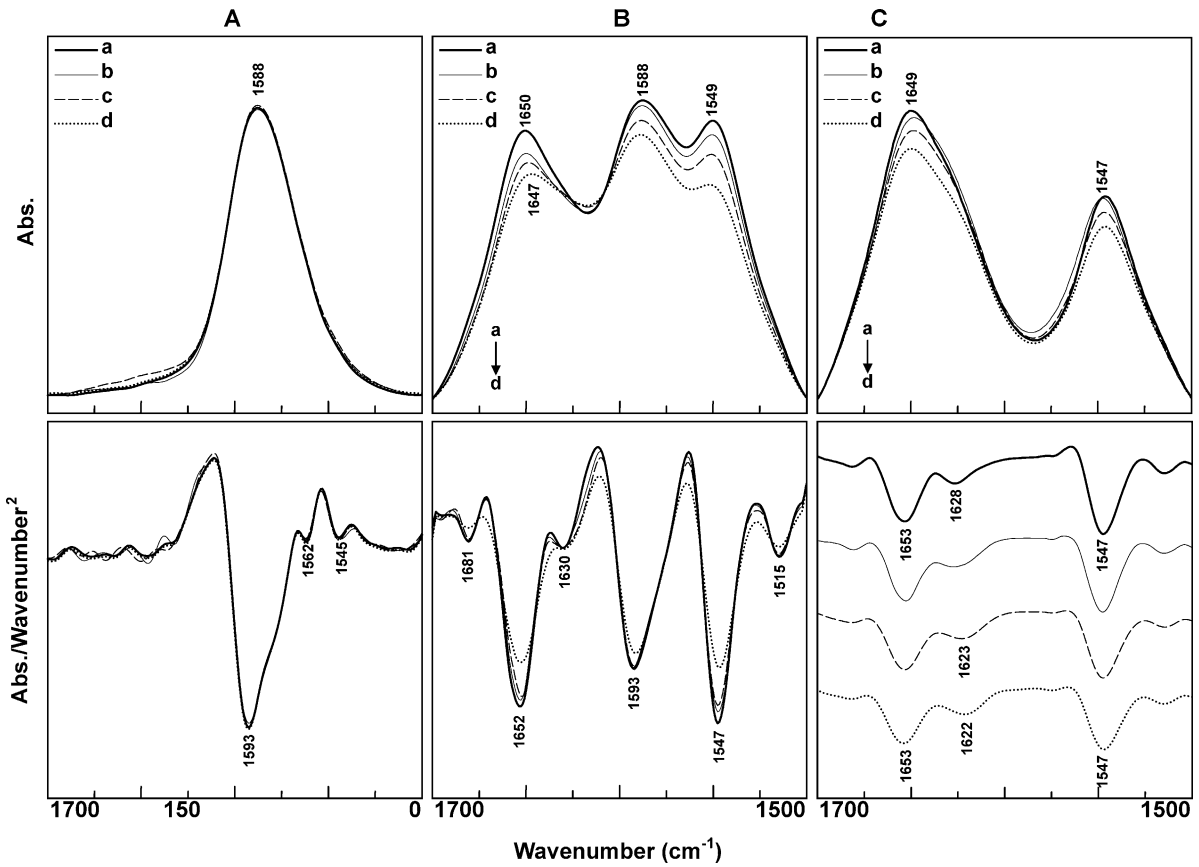


Fig. 3. The original and second-derivative IR spectra of captopril (A), captopril–HSA mixture (B) and HSA (C) subtracted captopril from HSA–captopril mixture in pH 7.4 buffer solution. Key: incubation time: (a) 0.1; (b) 1.0; (c) 3.0; (d) 5.0 h.

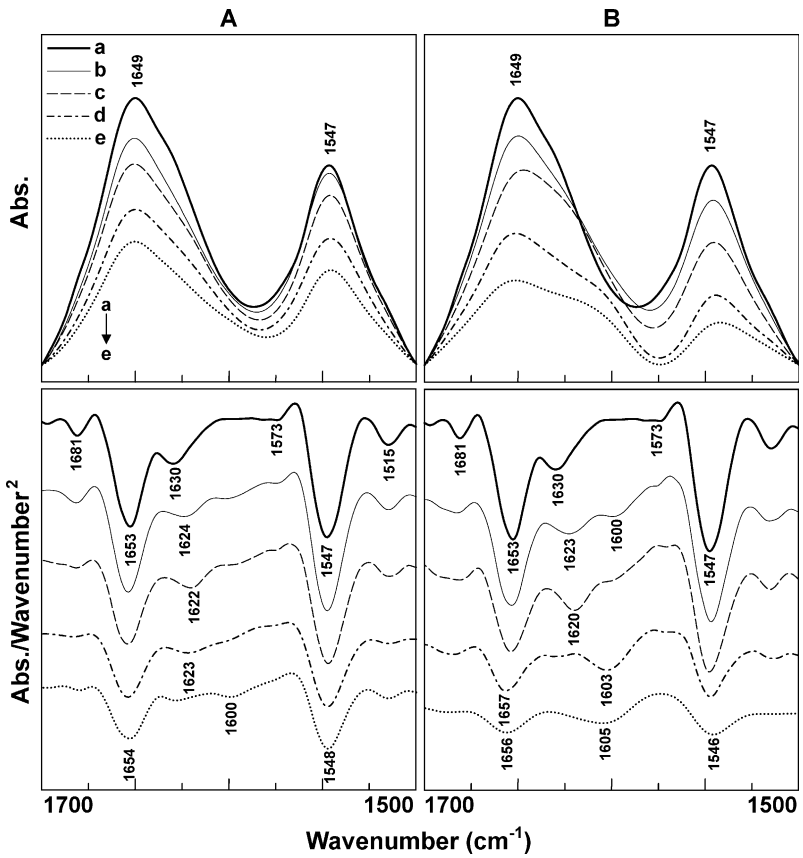


Fig. 4. The original and second-derivative IR spectra of HSA (A) after subtracting 10% ethanol and 2.5% captopril or HSA (B) after subtracting 10% ethanol and 5.0% captopril from the HSA–captopril–ethanol mixture. Key: (a) native HSA; incubation time: (b) 0.1; (c) 1.0; (d) 3.0; (e) 5.0 h.

4. Discussion

HSA is a 585 residue polypeptide that comprises a significant portion of human plasma and provides binding sites for many endogenous and exogenous bioactive substances [6,26]. It is composed of three homologous domains (I–III); with each domain consisting of two subdomains that are predominantly helical and extensively cross-linked by disulfide bonds. Previous studies have demonstrated that HSA has two primary drug-binding sites located in subdomains IIA and IIIA, and an additional third site is located elsewhere in the albumin molecule [13,26]. HSA–ligand interactions are particularly important as unbound ligand, which might significantly influence the pharmacokinetics and pharmacological effect of drugs [27]. Thus, competitive binding to HSA or within ligands may alter drug properties or may produce electrostatic effects, resulting in conformational modifications of HSA molecule [26].

The secondary structure of a protein can be characterized via IR spectrum and the use of amide I and II frequencies as indicators. Using the amide I band as a sensitive indicator of conformation and structure [5,23], the present study demonstrated that native HSA in pH 7.4 buffer solution consisted of 52% α-helix, 37% β-sheet and 11% β-turn (Fig. 2), which was similar to other reports [18,25].

It has been reported that various alcohols might denature the native state of proteins and induce α-helical structure, but which was depending on the alcohols species [28]. The direct interaction of the hydrophobic groups of alcohols with the hydrophobic groups of proteins might be responsible for the mechanism of alcohol effects. In this study, the increase of the ethanol concentration and incubation time might enhance α-helical content from 56 (initial) to 62% (24 h) for HSA after treatment with 10% ethanol or from 57 (initial) to 65% (24 h) for HSA after treatment with 25% ethanol, respectively. Moreover, β-sheet content was reduced from 37 (34%) to 29 or 26%, suggesting a transition from β-sheet to α-helical structure after treatment with ethanol. Our results are also consistent with other study, in which methanol and ethylene glycol increased the helix secondary structure of HSA [29]. In fact, many investigations have also reported that alcohol-induced denaturation of globular proteins was typically accompanied by a characteristic increase in α-helix content [30,31]. The mechanism by which alcohols induced α-helical conformations may be proposed by several physical mechanisms or by a helical-HP (hydrophobic/polar) lattice model [32–34].

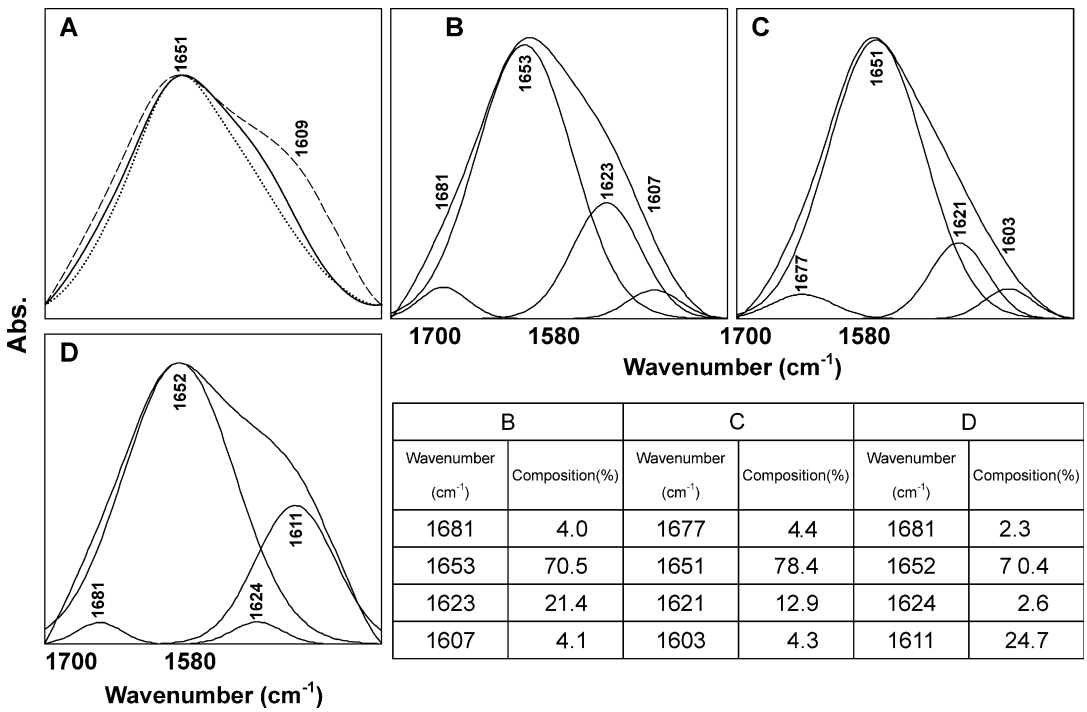
It is interesting to note that the structural conformation of HSA from blood plasma of patients with coronary atherosclerosis may be transited from α-helix to β-sheet

structure after beer consumption [15]. This was consistent with other reports that denaturing reagents or heat treatment might produce unfolding of α-helices, aggregation and precipitation, leading to an increase in the formation of intermolecular β-sheets [35,36]. However, it was against some other studies in which alcohols might induce α-helical formation [10,28–31]. Although the change in compactability of HSA as a result of ethanol consumption has been proposed [15], this controversial result is worth studying.

Captopril is reported to have only 22.8% protein binding in vitro but 30.0% protein binding in vivo [37], implying that captopril may bind to other plasma proteins. The IR spectra shown in Fig. 3C seems to confirm weak HSA binding, as there was no change for the predominant peaks near 1653 and 1547 cm^{-1} assigned to the α-helix structure. However, the peak at 1628 cm^{-1} assigned to the intramolecular hydrogen-bonded β-sheet gradually shifted to 1622 cm^{-1} assigned to the intermolecular hydrogen-bonded β-sheet following incubation with captopril for 5 h. This shifting phenomenon may result from non-covalent binding and/or covalent binding between captopril and HSA, as NMR studies have demonstrated rapid formation of non-covalent HSA–captopril complexes and captopril–HSA by thiol/disulfide interchange reactions after long-term incubation [16].

The fraction of unbound drug is a major determinant of pharmacological effects and toxicity. Thus, co-administration of drugs or ingestion of ethanol may alter pharmacokinetics, pharmacodynamics and metabolism of drugs [38,39]. The original and second-derivative IR spectra of HSA after subtraction of 10% ethanol and 2.5 or 5.0% captopril from the HSA–captopril–ethanol mixture is shown in Fig. 4. A shift from 1630 to 1623 or 1620 cm^{-1} was observed, likely secondary to captopril-induced formation of HSA intermolecular β-sheets. Another IR peak appeared near 1600–1605 cm^{-1} and may represent an increment of HSA unfolding in hydro-ethanolic cosolvent, in which the aromatic residues such as tryptophan became more accessible to the solvent [33,40].

Fig. 5 shows the comparison of original, curve-fitted IR spectra and the compositions of HSA after incubation with 2.5% captopril alone, 2.5% captopril/10% ethanol or 5.0% captopril/10% ethanol for 5 h. The secondary structural composition of HSA after 5-h incubation with 2.5% captopril consisted of 70.5% α-helix (1653 cm^{-1}), 21.4% β-sheet (1623 cm^{-1}), 4.0% β-turn (1681 cm^{-1}) and 4.1% amino acid side-chain (1607 cm^{-1}). Once 10% ethanol and 2.5% captopril were co-incubated with HSA for 5 h, the compositions of HSA changed to 78.4% α-helix (1651 cm^{-1}), 12.9% β-sheet (1621 cm^{-1}), 4.4% β-turn (1677 cm^{-1}) and 4.3% amino acid side-chain (1607 cm^{-1}). When the concentration of captopril increased from 2.5 to 5.0%, however, the secondary compositions of HSA was comprised of 70.4% α-helix (1652 cm^{-1}), 2.6% β-sheet (1624 cm^{-1}), 2.3% β-turn (1681 cm^{-1}) and 24.7% amino acid side-chain (1611 cm^{-1}). These data indicate that



B		C		D	
Wavenumber (cm^{-1})	Composition(%)	Wavenumber (cm^{-1})	Composition(%)	Wavenumber (cm^{-1})	Composition(%)
1681	4.0	1677	4.4	1681	2.3
1653	70.5	1651	78.4	1652	7 0.4
1623	21.4	1621	12.9	1624	2.6
1607	4.1	1603	4.3	1611	24.7

Fig. 5. The original, curve-fitted IR spectra and the compositions of HSA after incubation with 2.5% captopril, 2.5% captopril/10% ethanol or 5.0% captopril/10% ethanol for 5 h. Key: (A) solid line, IR spectra of HSA incubated with 2.5% captopril; dotted line, IR spectra of HSA incubated with 2.5% captopril/10% ethanol; dashed line, IR spectra of HSA incubated with 5.0% captopril/10% ethanol; (B) curve-fitted IR spectra and composition of HSA incubated with 2.5% captopril; (C) curve-fitted IR spectra and composition of HSA incubated with 2.5% captopril/10% ethanol; (D) curve-fitted IR spectra and composition of HSA incubated with 5.0% captopril/10% ethanol.

interactions between captopril, HSA and ethanol can affect HSA protein secondary structure.

In conclusion, the present results indicate that ethanol induced a transition in HSA conformation from β-sheet to α-helical structure and promoted conversion of intramolecular hydrogen-bonded β-sheet to intermolecular hydrogen-bonded β-sheet. Further, captopril or captopril/ethanol mixture mainly promoted conversion of intramolecular hydrogen-bonded β-sheet to intermolecular hydrogen-bonded β-sheet. It also resulted in exposure of aromatic side-chain groups in the unfolding conformation of HSA.

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