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Analysis of the interactions of multicomponents in *Cornus officinalis* Sieb. et Zucc. with human serum albumin using on-line dialysis coupled with HPLC

Xiao Liu [a], Zhi-Ping Han [a,b], Yu-Ling Wang [a], Yue Gao [a], Zhi-Qi Zhang [a,*]

[a] *Key Laboratory of the Ministry of Education for Medicinal Plant Resources and Natural Pharmaceutical Chemistry, Shaanxi Normal University, Xi'an 710062, China*
[b] *Department of Chemistry and Chemical Engineering, Yulin College, Yulin 719000, China*

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ABSTRACT

Interactions of three iridoid glycosides extracted from *Cornus officinalis* Sieb. et Zucc. (CIG) with protein were simultaneously explored by on-line dialysis sampling coupled with high-performance liquid chromatography (DS–HPLC). Three main compounds in CIG were unequivocally identified as loganin, sweroside and cornuside by comparing their t_R, MS data and UV spectra with those of reference compounds. Dialysis recoveries and quantitative characteristics of DS–HPLC for three iridoid glycosides were determined. Recoveries of dialysis sampling ranged from 73.9 to 91.7% with the RSD below 3.0%. Based on the determination of concentrations before and after interaction with human serum albumin (HSA), the binding parameters of loganin, sweroside and cornuside with HSA were obtained and the binding mechanisms were investigated.

1. Introduction

Cornus officinalis Sieb. et Zucc. is considered a precious herb and food material in China, and is widely used in many traditional Chinese medicine prescriptions. It belongs to the Cornaceae family. In the Pharmacopoeia of the People's Republic of China it is recorded that *C. officinalis* is an astringent and has a nourishing effect on the liver and kidney. As a classic Chinese herb, *C. officinalis* has been studied for several decades. Modern pharmaceutical studies have indicated that *C. officinalis* not only exhibits anti-inflammatory and anti-bacterial effects, but also has some therapeutic effects on diabetes, cancer, and shock. The extract of *C. officinalis* is composed of organic acids, polysaccharides, saponins, and iridoids [1], such as oleanolic acid, ursolic acid, loganin, sweroside, morroniside and cornuside [2]. Some of its essential components, like loganin and sweroside are regarded as biologically active components [3,4]. In principle, the pharmacological activity of many synthetic drugs is significantly affected by reversible interactions with plasma proteins, such as α1-acid glycoprotein (AGP) and human serum albumin (HSA). These interactions play an important role in drug distribution, efficacy and toxicity. Thus, the pharmacological activity of *C. officinalis* in the human body can be the result of the combined interactions of some active compounds and proteins [5].

Spectrophotometry [6,7] and fluorometry [8,9] are extensively used for the direct study of drug–protein binding and are based on monitoring the change of physicochemical properties of the drug–protein system. Ion-selective electrode [10], modified electrode [11] and quartz crystal resonant sensor techniques [12] have been developed for the direct study of drug–protein interactions. Several articles have been published in recent years regarding the application of high-performance affinity [13,14] chromatography in drug–protein interactions.

Dialysis sampling (DS) has emerged as an effective tool for both *in vivo* and *in vitro* experiments [15,16], and is a dynamic sampling technique based on analyte diffusion across a semi-permeable membrane driven by a concentration gradient [17,18]. This method has many advantages such as simple operation, time saving, requires a lower quantity of drug, no shifting of the equilibrium between bound and unbound drug and easy connection to analytical instruments [19]. This sampling technique with HPLC [20] has been applied in pharmacological studies [21,22]. A convenient method for saponin isolation in tumor therapy has been used by combining dialysis and HPLC [23]. Zou et al. studied the interactions of multi-components in herbal medicine with protein [24], DNA [25], and MCF-7 cells [26] using dialysis coupled with HPLC–MS for screening and analyzing the bioactive compounds in herbal medicines. However, off-line operation may suffer from sample loss and time consumption during handling, while on-line dialysis sampling coupled with HPLC could overcome these disadvantages and has been used for drug monitoring [27–29]. There are no reports on the evaluation of interactions between multi-components and biological systems using DS–HPLC.

In this study, the binding characteristics of iridoid glycosides extracted from *C. officinalis* (CIG) with HSA were studied using

* Corresponding author. Tel.: +86 29 85303939; fax: +86 29 85307774.
E-mail address: zqzhang@snnu.edu.cn (Z.-Q. Zhang).

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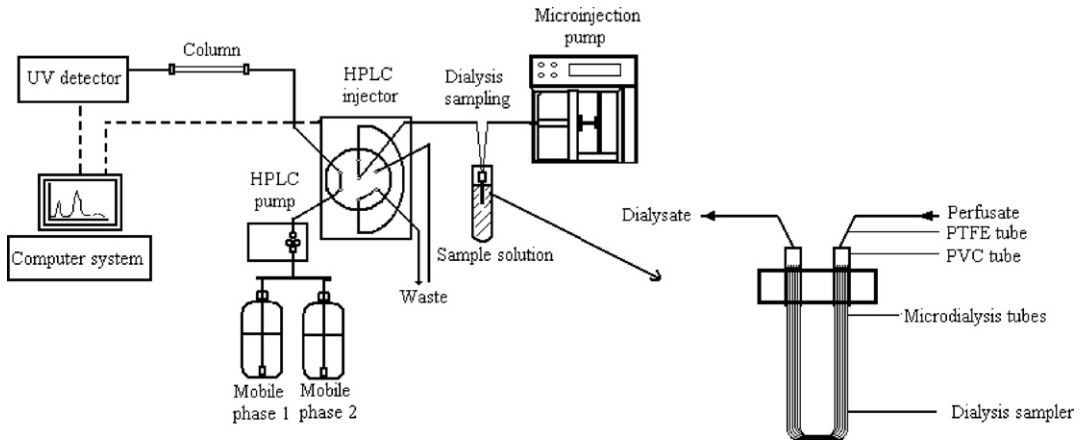

Fig. 1. Schematic diagram of the DS–HPLC system.

DS–HPLC. The binding parameters and thermodynamic parameters for three iridoid glycosides were obtained.

2. Experimental

2.1. Reagents and materials

The sample of *C. officinalis*, produced at Shannan, Shaanxi, was purchased from the Drug Market in Xi'an, China. Human serum albumin (HSA) was purchased from Sigma (St. Louis, MO, USA). The standard for loganin was purchased from the Institute of Chinese Pharmaceutical and Biological Product Inspection (Beijing, China); sweroside and cornuside were purchased from the Shang Rui Chemical Co., Ltd. (Shanghai, China). Acetonitrile, HPLC purity, was bought from Fisher Science (NJ, USA). Ultra-pure water (18.2 MΩ cm) produced with a Millipore purification system (USA) was used to prepare mobile phases and other related solutions in the experiments. Other chemical reagents were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2. DS–HPLC system and sampling

A schematic diagram of the DS–HPLC system is shown in Fig. 1. Dialysis sampling was performed using a home-made dialysis sampler coupled with a LSP02-1B microinjection pump (Baoding Longer Precision Pump Co. Ltd., Baoding, China). The dialysis syringe (20 mL volume) containing perfusate was connected to the inlet of the sampler with PTFE tubing. Each dialysate perfused through a dialysis probe was collected in a sample loop on a HPLC injector for chromatographic detection. The HPLC used was a Waters Breeze liquid chromatography system (Waters Corporation, Milford, MA, USA), which comprised a 1525 binary high-pressure pump, 7725i injection valves with a 20 μL loop, thermostatted column compartment, 2996 diode-array detector (DAD) and an Empower workstation.

The home-made dialysis sampler was made of several microdialysis tubes (0.12 mm i.d., 0.32 mm o.d., 8 cm length, 3000 Da cut-off molecular weight, Instech Laboratories, Plymouth Meeting, PA, USA) and two PTFE tubes (5 mm o.d., 3 cm length).

The perfusate (15 μL/min) was used to remove bubbles from inside the sampler for at least 30 min prior to use. The sampler was then placed into the sample solution. In order to obtain a stable dialytic recovery the dialysate was discarded for original 30 min, and then the dialysis sampling system was connected to the HPLC injector and the dialysate was collected for 35 min at 2 μL/min.

2.3. HPLC conditions

The sample was separated on a TC-C18 column (5 μm, 4.6 mm × 250 mm, Agilent, USA). All mobile phases for the chromatographic analysis were degassed for at least 15 min and filtered through a 0.45 μm filter prior to use. The mobile phase consisted of acetonitrile (A) and water containing 0.1% (v/v) formic acid (B). A gradient elution was programmed as follows: 0–45 min, 10–40% (v/v) A; 45–48 min, 40–10% A; 48–50 min, maintaining 10% A at a flow rate of 0.6 mL/min. The analytes were detected at 240 nm and the column temperature was set at 23 °C. Injection volume was 20 μL.

The mass spectra were acquired using an Esquire 6000 ion trap mass spectrometer (Bruker Daltonic, Bremen, Germany) connected to an Agilent 1100 Series HPLC. The ionization source was electrospray (ESI) and was operated in negative mode. MS parameters were as follows: nebulizer gas (N_2) 35.0 psi; dry gas (N_2) 8.0 L/min; dry gas temperature 350 °C; capillary 4500 V; scan range 100–1000 (m/z).

2.4. Preparation of the sample

Five grams of *C. officinalis* were crushed with a grinder, immersed in 50 mL of dehydrated alcohol for 12 h and then ultrasonicated for 30 min at 40 °C. The extraction procedure was repeated twice and the mixtures obtained were filtered under reduced pressure in a vacuum filtration device. The extracts were combined and concentrated under reduced pressure with a RE-52A rotary evaporator. The solvent was removed by rotary evaporation under vacuum at 40 °C and the residue was dissolved in phosphate buffer (0.1 mol/L Na_2HPO_4–NaH_2PO_4 and 0.1 mol/L NaCl). This was then filtered through a 0.45 μm membrane, and the filtrate was stored at 4 °C in the absence of light for further experiments.

The HSA sample of 1.0000 g was placed into a 10 mL volumetric flask, phosphate buffer was added to scale, and the sample was stored at 4 °C in the absence of light for further experiments.

The CIG solution of 2.5 mL was transferred into a 5 mL centrifuge tube, and HSA solutions of 0 mL, 1.00 mL and 2.00 mL, respectively, were added to obtain a series of sample solutions. The solutions were incubated for 12 h at 25 °C before sampling to ensure fully balanced combinations between the components in CIG and HSA for further perfusion and HPLC analysis.

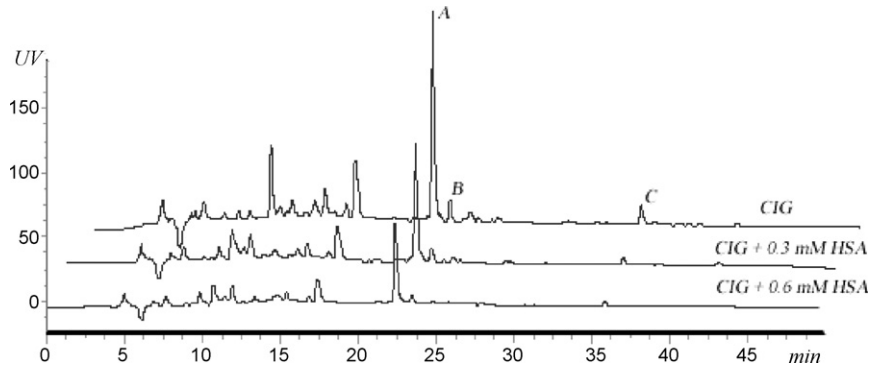

Fig. 2. Chromatograms of CIG and combined prescriptions of CIG with HSA.

3. Results and discussion

3.1. Identification of constituents in the dialysate of CIG

The chromatogram of CIG dialysate monitored at 240 nm is shown in Fig. 2. It is evident that there were more than 10 components present in the sample. Some of these components were identified using LC–MS and DAD. The molecular ion of peak A was 389 and its maximum UV absorption wavelength was 238 nm. When compared with the standard and reference, peak A was identified as loganin. The molecular ion of peak B was 357 and its maximum UV absorption wavelength was 246 nm. Compared with the standard and reference, peak B was identified as sweroside. The molecular ion of peak C was 541 and its maximum UV absorption wavelengths were 219 and 272 nm. Compared with the standard and reference, peak C was identified as cornuside [30].

3.2. Recovery of dialysis

Dialytic recovery is the most important factor in determining the free drug concentration in drug–protein solution. At perfusion rates typically used for dialysis, equilibrium is not established across the dialysis membrane. Therefore, the concentration of analyte collected in the dialysate is a fraction of the actual concentration of the solution. The dialytic recovery (R) is defined as the ratio between the concentrations of a particular substance dialyzed into perfusate solution and the actual concentration in solution.

$$R\ (\%) = \frac{C_{in}}{C_{out}} \times 100 \quad (1)$$

where C_{in} is the substance concentration in the dialysis solution, and C_{out} is the same substance concentration outside the dialysis tubes.

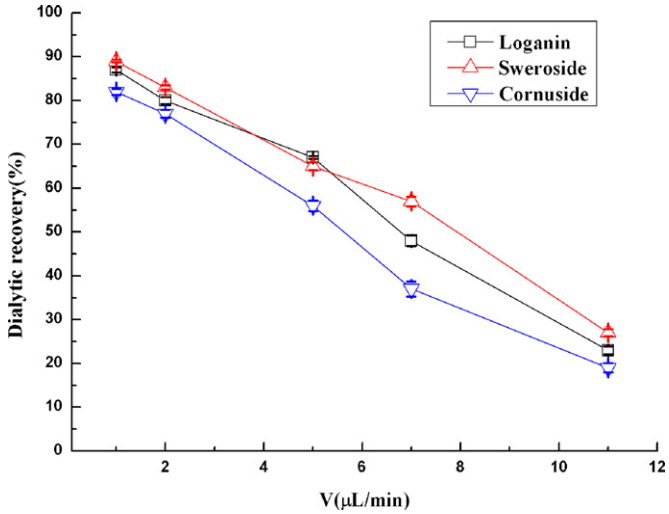

Fig. 3. Effect of dialysis flow rate on dialytic recovery.

Table 1
Relative dialysis recovery of CIG compounds.

Peaks	Retention time (min)	Relative recovery (%)	RSD (%) (n = 6)
Loganin	22.41	80.00	2.31
Sweroside	23.80	91.72	0.43
Cornuside	35.81	73.94	0.71

To obtain an acceptable R in a reasonable operating time, the effect of perfusion rate used in this study on the precision of recovery was studied in the range of 1–11 μL/min. As shown in Fig. 3, the recoveries gradually decreased when the dialysis flow rate increased. Although a low perfusion flow rate increases R, it takes time to collect sufficient dialysate to conduct an analysis. Given the relationship between sampling time and detection sensitivity, a perfusion rate of 2 μL/min was selected as the optimum dialysis flow rate for subsequent experiments.

There was 50 μL of dead volume in the whole HPLC injector and it would take about 25 min to fill the injector with dialysate. The optimum sampling time was chosen as 35 min. This sampling time could obtain a high concentration of analyte for HPLC analysis and ensure the accuracy of measurement.

Under the optimized conditions, the dialytic R was evaluated for three water-soluble components in CIG, and the results are listed in Table 1. In general, the smaller and more hydrophilic the component, the higher the dialytic recovery is [31]. Sweroside with a smaller molecular weight and multiple hydrophilic groups had the highest dialytic recovery, and cornuside with the biggest molecular weight (542) had the lowest dialytic recovery.

The RSD of the dialytic recoveries was below 2.5%. It can be concluded that DS–HPLC provided accurate determinations of the concentrations of the compounds in the CIG extract.

3.3. Quantitative characteristics of DS–HPLC for three iridoid glycosides

As the effect of temperature in the study was very small and the experimental time was short, the dialytic recovery of the sampler could be regarded as a constant. Therefore, the calibration curves prepared with standard solutions in the DS–HPLC system could be used directly to determine the total concentration of unbound drugs without calculating the dialytic efficiency, which makes this method much simpler and more reliable than other methods reported [32].

Table 2
The analytical characteristics for the quantification of three compounds in CIG.

Compound	Linear equation	Regression coefficient	Linear range (mol/L)	LOD (mol/L)
Loganin	$y=(1.00\pm0.12)\times10^{-10}x-(2.08\pm0.11)\times10^{-5}$	0.9998	3.0×10^{-4}–3.7×10^{-2}	1.0×10^{-4}
Sweroside	$y=(4.12\pm0.30)\times10^{-10}x-(3.41\pm0.25)\times10^{-5}$	0.9997	4.8×10^{-4}–4.9×10^{-3}	1.5×10^{-4}
Cornuside	$y=(2.26\pm0.10)\times10^{-10}x+(2.09\pm0.04)\times10^{-6}$	0.9999	5.5×10^{-4}–2.9×10^{-3}	1.7×10^{-4}

Three iridoid glycosides were quantitatively analyzed with the calibration curves obtained with standard solutions in DS–HPLC system, in which it is not required to calculate and use the dialytic recoveries. The linear equation, linear range, and the limit of detection (LOD, S/N ratio = 3) of these glycosides are listed in Table 2. The calibration curve for each compound was constructed with at least five appropriate concentrations.

3.4. Interaction of three iridoid glycosides with HSA

Human serum albumin (HSA) is the most abundant protein in human plasma with a concentration of 0.6 mM [5]. This globular protein consists of a single polypeptide chain of 585 amino acids, and it has many important physiological functions. HSA contributes to osmotic blood pressure, the transportation and distribution of many molecules and metabolites, such as fatty acids, amino acids, hormones, cations, anions and many diverse drugs. Binding to protein alters the free drug concentration. Thus, it strongly affects the distribution, metabolism, elimination and pharmacological effects of drugs. Therefore, the study of protein binding with drugs and the determination of related parameters have great practical and theoretical importance in the study of pharmacokinetics and pharmacodynamic properties.

3.4.1. Binding at pH 7.4

The binding results of the three iridoid glycosides with HSA at pH 7.4 were analyzed using DS–HPLC system and the results are shown in Fig. 2.

It can be seen from Fig. 2 that the integration of the peak areas in the chromatograms decreased following the interaction with different concentrations of HSA. This showed that the binding strength was influenced by the concentration of HSA, i.e. the higher the concentration, the higher the binding strength.

The degree of binding of the compounds in CIG with HSA was defined by Eq. (2):

$$\text{Binding degree}\,(\%) = \frac{C_o - C_s}{C_o} \times 100 \tag{2}$$

where C_o is the total concentration in the sample, and C_s is the actual unbound free concentration in solution.

It is clearly shown in Fig. 4 that cornuside had higher degrees of binding at 77.6% (0.3 mM HSA) and 80.8% (0.6 mM HSA), while loganin had lower degrees of binding at 49.6% (0.3 mM HSA) and 69.3% (0.6 mM HSA). These results may be explained by the polarity of the compounds and the configuration of HSA. The hydrophobic pocket of HSA easily binds with compounds of weak polarity. On the other hand, the degree of binding may be associated with the molecular configuration.

3.4.2. Influence of pH on binding degree

Solution pH is a critical factor affecting the molecular state of both biopolymers and drugs. When the solution pH is higher than the isoelectric point of HSA (pH 4.0), HSA has a negative charge. In addition, the molecular state of the compounds in CIG is changed with variation in pH. The influence of pH on binding degree is shown in Fig. 5.

The binding degree of loganin was 69.3% at pH 7.4, 74.5% at pH 6.0 and 47.6% at pH 8.9, while binding of sweroside and cornuside to HSA was observed to decrease at pH 6.0 and 8.9, respectively, when the concentration of HSA was 0.6 mM. We can conclude that pH influences the interaction between the compounds in CIG and HSA as a result of changes in the binding site structures of HSA and the molecular state of active compounds.

3.5. Estimation of binding parameters for the three iridoid glycosides

The binding parameters were estimated by the following equations for Scatchard analysis, which is widely applied in the study of interactions between drugs and proteins [33,34].

$$\frac{\nu}{[\mathrm{p}]} = nK - \nu K \tag{3}$$

where [p] is the concentration of the free drug, ν is the ratio of bound drug to the protein in molar concentration, n is the number

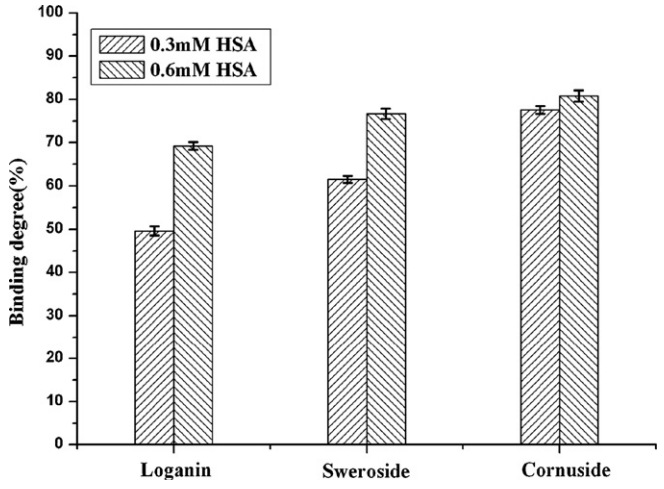

Fig. 4. Binding degrees of CIG reacted with HSA.

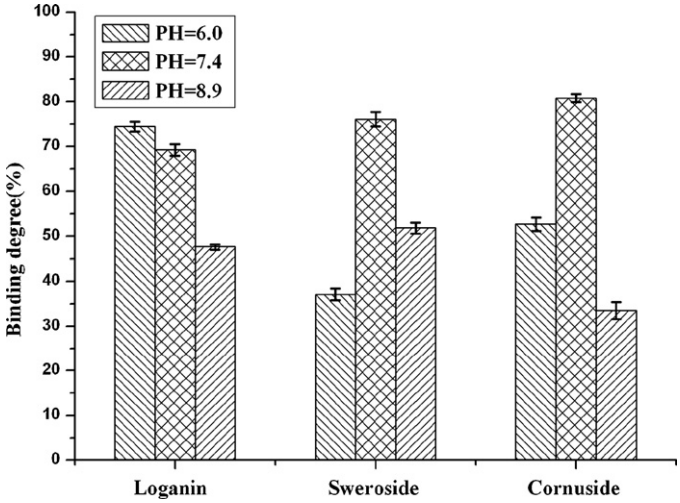

Fig. 5. Binding degrees of components in CIG with HSA under different pH values. The concentration of HSA was 0.6 mM/L.

Table 3
The drug–HSA binding parameters at different pH.

Com pounds	K (L/mol) (296 K, pH 6.0)	n	K (L/mol) (296 K, pH 7.4)	n	K (L/mol) (296 K, pH 8.9)	n	K (L/mol) (310 K, pH 7.4)	n
Loganin	$(2.13 \pm 0.32) \times 10^3$	0.94	$(1.66 \pm 0.21) \times 10^3$	0.83	$(1.17 \pm 0.15) \times 10^3$	0.71	$(2.34 \pm 0.31) \times 10^3$	0.88
Sweroside	$(1.40 \pm 0.17) \times 10^4$	0.64	$(2.00 \pm 0.14) \times 10^4$	0.67	$(1.08 \pm 0.97) \times 10^4$	0.53	$(2.46 \pm 0.10) \times 10^4$	0.61
Cornuside	$(4.63 \pm 0.11) \times 10^4$	1.27	$(5.08 \pm 0.08) \times 10^4$	1.02	$(3.58 \pm 0.76) \times 10^4$	1.08	$(3.32 \pm 0.48) \times 10^4$	1.18

of binding sites on one protein molecule and K is the association constant. Once ν/[p] and ν were determined, n and K could be estimated with the linear equation between ν/[p] and ν (where the slope is $-K$ and the intercept is nK).

The free drug concentrations for the three iridoid glycosides were determined with the DS–HPLC system, and the Scatchard plots are shown in Fig. 6. Their association constants K and number of binding sites n were obtained according to Fig. 6, which are listed in Table 3. The binding constant for cornuside decreased with increasing temperature when the pH was 7.4, while the binding constants for loganin and sweroside increased with increasing of temperature. As this is the first report on the binding parameters of loganin, sweroside and cornuside, there are no reference data in the literature to compare these parameters.

3.6. Determination of the acting force

Small organic molecules such as drugs and biological protein macromolecules often form supramolecular complexes by hydrophobic force, hydrogen bonding, van der Waals force and electrostatic forces, the reaction types of this interaction with different drugs and proteins are different. The thermodynamic characteristics of combinations of biological macromolecules can help to elucidate the interaction mechanism and function of the protein configuration changes, which can provide data on the pharmacology in the body and other important information.

This DS–HPLC system was further extended to determine the acting force. The selected temperatures were 23 °C and 37 °C at which HSA does not undergo any structural degradation. Because the temperature effect was very small and the range was not too wide during the study, the interaction enthalpy change could be regarded as a constant. Therefore, the thermodynamic parameters can be determined from the binding constants at different temperatures using the following equations:

$$ln\left(\frac{K_2}{K_1}\right) = \frac{\Delta H((1/T_1) - (1/T_2))}{R} \quad (4)$$

$$\Delta G = -RT \ \ln K \quad (5)$$

$$\Delta G = \Delta H - T\Delta S \quad (6)$$

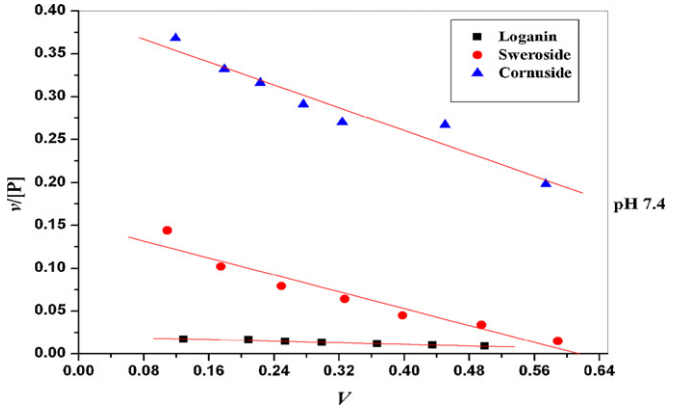

Fig. 6. The Scatchard plot for CIG–HSA binding at 310 °C.

Table 4
Thermodynamic parameters for the interaction of drugs with HSA.

Compounds	ΔH (kJ mol^{-1})	ΔG (kJ mol^{-1})	ΔS (J mol^{-1})
Loganin	18.68 ± 0.33	−18.24 ± 0.32	124.74 ± 1.20
Sweroside	11.34 ± 0.28	−24.36 ± 0.18	120.61 ± 0.85
Cornuside	−23.18 ± 0.21	−26.67 ± 0.04	11.80 ± 0.60

where K is the binding constant at the corresponding temperature, R is the gas constant; ΔG is the free energy change, ΔH is the enthalpy change, and ΔS is the entropy change. According to the findings of Némethy and Scheraga [35], the acting force is confirmed by the following relationship: (1) hydrophobic interaction played a major role in binding which was characterized by a positive value for entropy change and a positive value for enthalpy change, (2) electrostatic interaction played a major role in binding which was characterized by a positive value for entropy change and a negative value for enthalpy change (almost zero), (3) H-bonds, and van der Waals interaction played a major role in binding which was characterized by a negative value for entropy change and a negative value for enthalpy change.

As indicated in Table 4, the negative values for free energy change and positive values for entropy change for the formation of CIG–HSA coordination compounds revealed that the binding process was a spontaneous and exothermic process. The enthalpy change values for loganin and sweroside in the binding reactions are positive, and it could be deduced that the acting forces are mainly hydrophobic interactions. According to Némethy's study, the negative enthalpy change value suggests that cornuside and HSA combine with electrostatic force. It should be noted that due to the complexity of the protein structure, with its combination of small molecules, drugs tend to have a variety of forces.

4. Conclusion

In this study, on-line dialysis coupled with HPLC-PDA detection was successfully applied to the simultaneous investigation of the interactions between multi-components of the iridoid glycoside extract from *C. officinalis* and HSA. This study reported firstly the binding parameters and the acting forces for three iridoid glycosides, loganin, sweroside and cornuside. The results demonstrated that the DS–HPLC method can be used to screen the bioactive components in Traditional Chinese Medicine and to rapidly assess the binding properties of biological systems. The proposed approach would be beneficial in revealing the holistic combined action of herbal medicine in biological systems and to facilitate the discovery of drug candidates.

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