Drug Development and Industrial Pharmacy, 2010; 36(3): 307–314

informa
healthcare

ORIGINAL ARTICLE

Drug–protein-binding determination of stilbene glucoside using cloud-point extraction and comparison with ultrafiltration and equilibrium dialysis

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Abstract

Aim: We did a prospective study to investigate the binding of stilbene glucoside (2,3,5,4′-tetrahydroxystilbene-2-O-β-D-glucoside, TSG) to plasma, albumin, and α1-AGP (2.0, 10.0, or 50 μg/mL) by three different methods: ultrafiltration, equilibrium dialysis and cloud-point extraction (CPE). *Method*: Drug stability in plasma was assessed at different temperatures (4, 25, and 37°C) and on the condition of thawing and freezing. A previously validated high-performance liquid chromatography (HPLC) method was used to analyze the total concentration of drug and free fraction in the samples. *Results*: The binding of TSG to plasma increased with adding drug concentration. The binding to albumin was constant (about 60%) within concentration range studied while the binding to α1-AGP decreased with increasing drug concentration indicating that albumin is more important in clinical settings. *Conclusions*: α1-AGP might be important when plasma proteins change with disease. The results to plasma obtained by CPE were in good agreement to those observed by ultrafiltration and equilibrium dialysis, indicating that CPE was a highly sensitive and selective method for the measurement to plasma protein binding of TSG. The results obtained in our studies are important before clinical trials and to perform pharmacokinetic studies.

Key words: *Cloud-point extraction; drug–protein binding; equilibrium dialysis; ultrafiltration; stilbene glucoside*

Introduction

Stilbene glucoside (2,3,5,4′-tetrahydroxystilbene-2-*O*-β-D-glucoside, TSG) is one of the water-soluble bioactive components in *Radix polygoni multiflori* and *Caulis polygoni multiflori*. The amount of TSG in *Radix polygoni multiflori* could reach to 3.3%[1]. Because of the high content, TSG could be separated from *Radix polygoni multiflori* more readily[2]. Many studies have documented the beneficial properties of TSG, including its strong antioxidant and free-radical scavenging[3–5]. Pharmacokinetics and tissue distribution of TSG in animals had been studied in our previous papers[6,7], but the plasma–protein binding of TSG has not been determined.

There are several proteins in plasma, including serum albumin, α1-acid glycoprotein (α1-AGP), and lipoproteins, which contribute to plasma protein binding of a drug. The degree of plasma–protein binding has a significant effect on pharmacokinetic and pharmacodynamic outcomes in vivo. Many critical pharmacokinetic parameters such as hepatic metabolism rate, renal excretion rate, biomembrane partition rate, and steady-state distribution volume are a function of unbound drug fraction (unbound/bound concentration ratio). Therefore, quantitative determination of drug–protein binding is important in clinical drug development.

Many methodologies have been investigated for quantitative determination of drug–protein binding. Among those, equilibrium dialysis and ultrafiltration followed by high-performance liquid chromatography (HPLC) analysis have been conventionally and most commonly used[8,9]. These conventional methods suffer

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(Received 5 Jan 2009; accepted 29 Jun 2009)

ISSN 0363-9045 print/ISSN 1520-5762 online © Informa UK, Ltd.
DOI: 10.3109/03639040903154192

from relatively long analysis time (hours for equilibrium dialysis), nonspecific binding of drugs onto the membrane, and leakage of bound drug through the membrane, which make them not very applicable to highly protein-bound drugs[10].

As an alternative to solvent extraction methods, cloud-point extraction (CPE) is being used by analytical chemists because of its efficiency, cost effectiveness, and environmental friendliness. Micellar aqueous solutions prepared with many nonionic surfactants undergo phase separation above a certain temperature also known as the cloud-point temperature. During the cloud-point phase-separation process, these micellar vesicles, which attract nonpolar analytes because of hydrophobic interactions, aggregate into a surfactant-rich phase. The CPE process is attractive to analytical chemists because of features such as its capacity to concentrate a variety of analytes with high concentration factors; its safety and cost benefits (it is an excellent alternative to conventional sample concentration methods that require the use of large amounts of toxic and flammable organic solvents); the ease with which surfactant used in the process can be incinerated in the presence of waste acetone or ethanol; its capacity to enhance detection by reconcentrating the analyte; the compatibility of the surfactant-rich phase with micellar liquid chromatographic techniques; the preclusion of analyte losses during the evaporation of solvents used in traditional liquid–liquid extraction techniques; and the inhibition of adsorption of nonpolar analytes to glass surfaces[11].

CPE is now widely used for the selective extraction of various compounds from biological and environmental media[12–14]. We developed a simple protocol for the determination of the free fraction of TSG in plasma and solutions of serum albumin and α1-AGP with this technique. CPE transfers the plasma proteins and the protein-bound drugs into a surfactant-rich phase. The free fraction of the drug remains in the water phase and its concentration can be determined by HPLC[15,16]. A comparison of the method with equilibrium dialysis and ultrafiltration has also been made.

Experimental

Chemicals and reagents

The nonionic surfactant Triton X-114 and α1-AGP were obtained from Sigma-Aldrich (St. Louis, MO, USA). Ultrafiltration units (Microcon YM-10; MWCO 10 K) and filtrate collection tubes were obtained from Millipore (Bedford, MA, USA). The dialysis membranes (molecular weight cutoff 10,000) were purchased from Spectrum Laboratories Inc. (Los Angeles, CA, USA). Bovine serum albumin (Roche) was packed by Sino-American Biotec (Beijing, China). A pool of control human plasma was obtained from the blood center (Shijiazhuang, China) and was stored at −20°C until use. All mobile phase solvents were HPLC grade, and all reagents were analytical reagent grade. Deionized distilled water was used for the preparation of all solutions.

HPLC method

A previously validated HPLC method[6] was used to analyze the drug in the samples. Briefly, sample preparation consisted of adding 200 μL of methanol to 100 μL sample aliquot to precipitate proteins. The samples were vortex-mixed for 25 seconds and centrifuged at $6800 \times g$ for 10 minutes. The supernatant was transferred into a microvial for injection into the Agilent Technologies 1200 series HPLC system (Agilent Technologies, Foster City, CA, USA) equipped with quadruple pump, vacuum degasser, autosampler, and photodiode array detector. Agilent chemstation software was used for data acquisition and mathematical calculations. Samples were analyzed using an Diamonsil® C_{18} HPLC column (4.6×250 mm, 5 μm particle size) preceded by a guard column (3.9×20 mm, 4 μm particle size), operating at 30°C, and were eluted at 1.0 mL/min using a mobile phase consisting of acetonitrile, methanol, and 0.1% formic acid (v/v) in water (15:18:67, v/v). The detection was carried out at 320 nm.

Sample preparation and stability of TSG in sample

The stock solutions (0.05, 0.25, and 1.25 mg/mL) of TSG were prepared by dissolving appropriate amount of reference substance in methanol. Forty microliters of the above solutions was added to glass test tubes and evaporated to dryness under a stream of nitrogen in the thermostatically controlled water bath maintained at 55°C for about 20 minutes. Thereafter, 1 mL of the drug-free plasma, albumin (40 g/L in plasma water), or α1-AGP (0.9 g/L in plasma water) was then added to it and vortexed for 45 seconds to give final concentrations of 2.0, 10.0, or 50 μg/mL. The mixtures were incubated at 4°C, 25°C, and 37°C, and at appropriate times (2, 4, 6, 8, 12, 24, and 48 hours) samples were withdrawn and stored at −20°C until analysis. In parallel, the stability of TSG after repeated thawing and freezing of samples at 8, 12, 24, 36, and 48 hours was assessed. All samples were analyzed in duplicates and stored at −20°C.

Equilibrium dialysis

Equilibrium dialysis was performed as described elsewhere in cells with a compartment volume of 1 mL.

Before use, dialysis membranes were soaked overnight in distilled water.

Five hundred microliters of the sample was introduced in one side of the cell, 500 μL phosphate buffer (pH 7.4, for the phosphate buffer, 34.5 g $NaH_2PO_4 \cdot H_2O$ and 22.2 g $Na_2HPO_4 \cdot 2H_2O$ were each dissolved in 250 mL of water, giving concentrations of 1.00 and 0.50 M, respectively. Fifteen milliliters of the solution containing Na_2HPO_4 was diluted to 0.015 M and the pH was adjusted to 7.4 with the solution containing NaH_2PO_4) were added on the other side of the membrane. Equilibrium dialysis was carried out for 5 hours at 37°C. After equilibrium, samples were withdrawn from both chambers and stored at −20°C. The concentrations of TSG in plasma (C_{tot}) and dialysis buffer (C_f) were measured by HPLC. The percentage fraction of bound drug [F_b (%)] was calculated according to the equation:

$$F_b(\%) = \frac{C_{tot} - C_f}{C_{tot}} \times 100.$$

All experiments of protein binding in plasma, albumin, and α1-AGP ran in five different determinations and the results were expressed as mean values ± SD. Preliminary experiments were carried out in phosphate buffer (pH 7.4) in order to determine adsorption of the drug to the dialysis membrane; however, no considerable differences in the concentration of TSG in both sides after equilibrium were observed. The stability of TSG during the incubation time was determined in phosphate buffer by comparing the concentration of the drug at zero time with the concentration after 5 hours incubation at 37°C.

Ultrafiltration

Ultrafiltration units consisted of a low-binding regenerated cellulose ultrafilter and a microcentrifuge tube for filtrate collection. This device is designed for rapid separation of the unbound fraction of the drugs in small volumes of plasma, albumin, or α1-AGP solutions. A 100 μL sample aliquot was removed for the analysis of total concentration (C_{tot}) in each sample using the method described previously. An aliquot of 0.5 mL sample was introduced in the sample reservoir and allowed to equilibrate at 37°C for 10 minutes. The ultrafiltrate was obtained by sample centrifugation at 5000 × *g* for 30 minutes at 25°C in a fixed angle rotor and yielded less than 15% of the total sample volume. A 20 μL aliquot of filtrate was directly analyzed for free drug concentration (C_f) using HPLC. The bound fraction was calculated as described above for equilibrium dialysis. TSG (2.0, 10.0, and 50 μg/mL) in plasma water ran together with the samples to assess the bound of the drug to the membrane. It was determined that the binding of the compound to the ultrafiltration membrane was 25%, independent to the compound concentration. This factor was taken into consideration for the protein binding determination.

Cloud-point extraction

In a test tube 1.0 mL of a 5% (w/v) aqueous solution of Triton X-114 was added to 0.2 mL of sample. The tube was placed in a temperature-controlled water bath with a positive temperature gradient of approximately 1°C/5 min. The phase separation was observed at 35°C. The extraction mixture was kept at this temperature for 10 minutes. After centrifugation at 3000 × *g* for 5 minutes the upper dilute aqueous phase with the free fraction of TSG was removed from the surfactant-rich lower phase and transferred into a tube for the measurement of volume (V_f), and then the concentration of TSG (C_f') was determined as described above. The percentage fraction of bound drug [F_b (%)] was calculated according to the equation:

$$F_b(\%) = \frac{C_{tot} - C_f' \times \dfrac{V_f}{0.2}}{C_{tot}} \times 100.$$

All experiments ran in triplicate and the results were expressed as mean values ± SD.

Results and discussion

Analytical methodology

The analytes were well separated using the present chromatographic conditions. Figure 1a–f showed the chromatograms of blank plasma, blank albumin (40 g/L in plasma water), blank α1-AGP (0.9 g/L in plasma water), blank 5% Triton X-114, TSG methanol solution, and blank plasma spiked with TSG (2 μg/mL). The calibration curves were linear over the concentration range of 0.25–70 μg/mL with determination coefficients >0.999. The limit of quantification was 0.25 μg/mL. The accuracy of the method was >90%. The inter-day relative SDs were 8.54%, 4.42%, and 3.25% at 0.25, 5, and 70 μg/mL, respectively.

Stability of TSG in sample

The stability of TSG (2.0, 10.0, or 50 μg/mL) was studied in plasma at different time points. As shown in Figure 2a, TSG was not so stable at 4°C, 25°C, and 37°C after 12 hours. After repeatedly freezing and thawing cycles, the drug

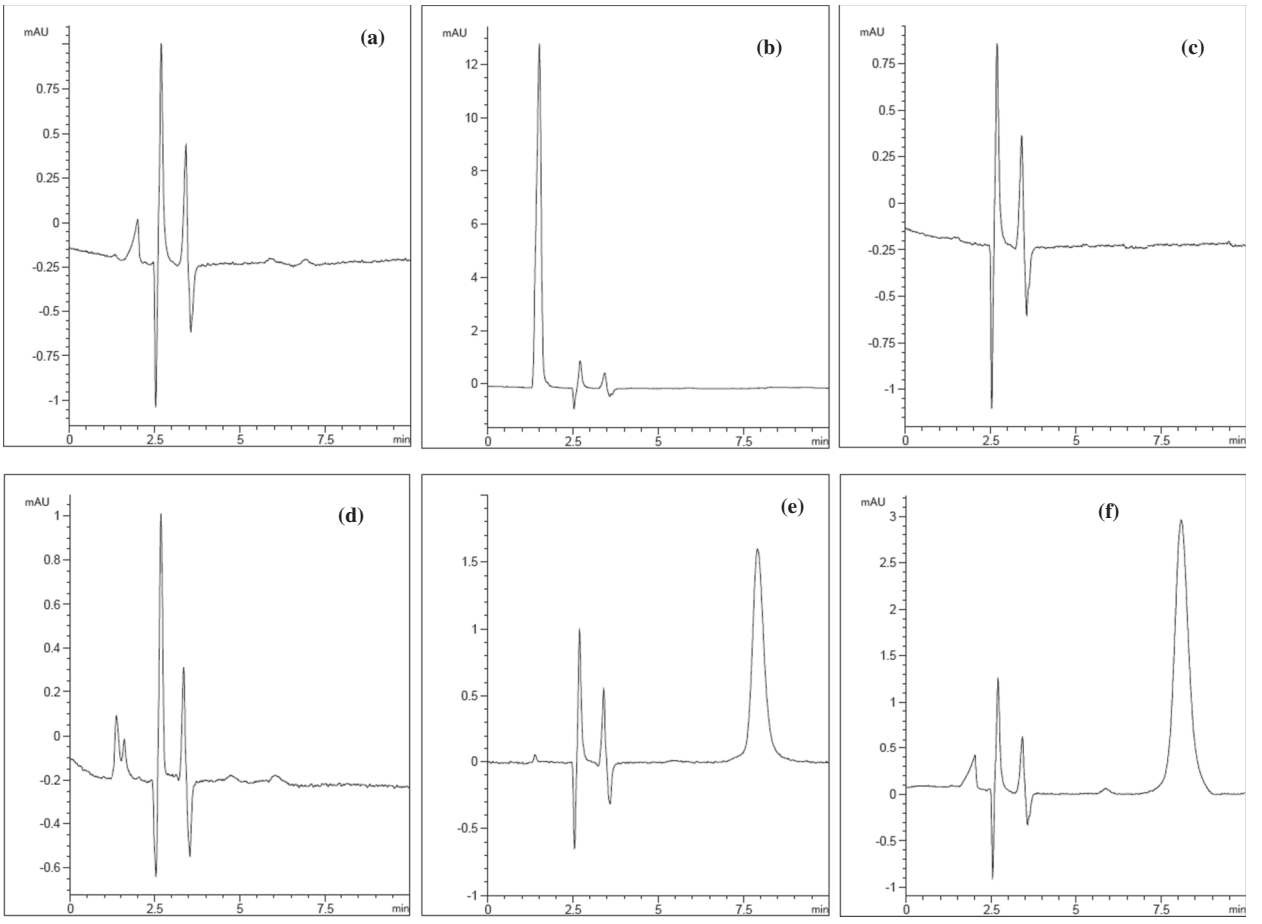

Figure 1. Chromatograms obtained from (a) blank plasma, (b) blank albumin (40 g/L in plasma water), (c) blank α1-AGP (0.9 g/L in plasma water), (d) blank 5% Triton X-114, (e) TSG methanol solution, and (f) blank plasma water spiked with TSG (2 μg/mL).

remained stable (Figure 2b). Therefore, all experiments of protein binding in plasma, albumin, and α1-AGP were run within 12 hours.

Protein binding of TSG

Ultrafiltration and equilibrium dialysis are two widely used methods for the determination of protein binding. Both methods have advantages and disadvantages; the ultrafiltration system is favorable because of its simplicity and relative fast generation of the ultrafiltrate. The equilibrium dialysis is a simple technique and can be easily operated at different temperatures, the disadvantage is the time required for the drug to reach the equilibrium, which can be associated with a volume shift from the dialysate to the plasma side as was described by Lima et al. and recently by Banker et al.[17] In our study, no adsorption of the drug to the dialysis membrane was observed, but the binding of the drug to the ultrafiltration membrane was 25%, independent of the compound concentration.

The binding of drug to plasma protein was lower at higher temperatures that cause bond breakage and hence lower binding capacity[18]. So we selected the same temperature (35°C) for all investigations.

The binding of TSG to plasma, albumin, and α1-AGP was investigated by three different methods. As can be seen from Table 1, the binding of TSG to plasma obtained by CPE were in good agreement with those obtained by ultrafiltration and equilibrium dialysis. The results of albumin (Table 2) and α1-AGP (Table 3) obtained by equilibrium dialysis were similar to those obtained by ultrafiltration, whereas the results obtained by CPE were very different from those measured by both ultrafiltration and equilibrium dialysis. Although the reason for this difference was not yet known, there were some influencing factors that circumscribed the application of CPE, such as lipophilicity of drug, hydrophilicity of protein, and the influence on the binding of drug during the phase separation.

As shown in Figure 3 (the results of ultrafiltration are similar to those obtained by equilibrium dialysis), the binding of TSG to plasma increased with increasing drug concentration, and binding albumin was constant (about 60%) within concentration range studied

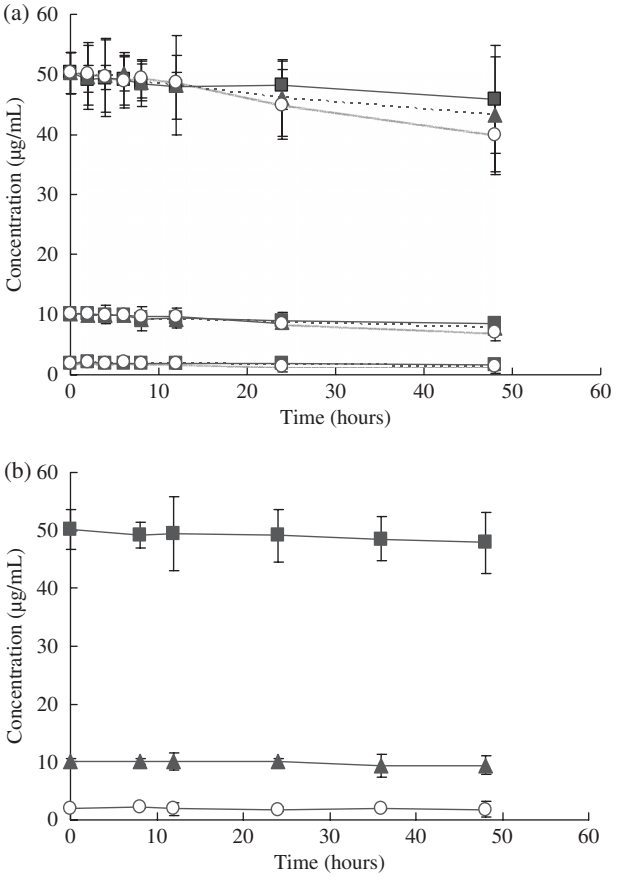

Figure 2. Stability of TSG in plasma: (a) the stability of TSG at 4°C (■), 25°C (▲), and 37°C (○) was assessed during 48 hours; (b) the effect of repeated thawing and freezing (–20°C) of the samples was studied during a period of 48 hours for TSG at the concentrations 2.0 μg/mL (▲), 10 μg/mL (■), and 50 μg/mL (○).

Table 1. Percentage of the bound fraction of TSG to plasma proteins determined by ultrafiltration, equilibrium dialysis, and CPE methods.

Concentration (μg/mL)	Ultrafiltration	Equilibrium dialysis	CPE
2	55.8 ± 4.1	53.4 ± 4.4	51.9 ± 5.0
10	79.6 ± 3.3	74.2 ± 3.3*	72.5 ± 2.6*
50	89.8 ± 1.8	85.0 ± 3.6	87.4 ± 1.3

The results are expressed as mean values from five different determinations ± SD.
*$P < 0.05$.

whereas the binding to α1-AGP decreased with increasing drug concentration.

These results indicate that both albumin and α1-AGP are important for TSG protein binding. As it is observed that the binding to albumin did not change with increasing concentration while the binding to α1-AGP decreased with increasing concentration, indicating that albumin is more important in clinical settings, whereas α1-AGP might be important when plasma proteins change with disease. The binding to α1-AGP decreased by increasing concentration of the drug, which may indicate that TSG is bound to specific sites on that TSG becomes saturated with higher concentrations. It seems to be that the binding of TSG to albumin is nonspecific and did not reach saturation level within the concentration studied.

Table 2. Percentage of the bound fraction of TSG to albumin determined by ultrafiltration, equilibrium dialysis, and CPE methods.

Concentration (μg/mL)	Ultrafiltration	Equilibrium dialysis	CPE
2	60.1 ± 3.2	59.7 ± 3.5	75.8 ± 4.3*
10	63.0 ± 1.7	64.4 ± 3.0	81.1 ± 4.4*
50	59.9 ± 2.1	63.5 ± 4.7	87.2 ± 3.9*

The results are expressed as mean values from five different determinations ± SD.
*$P < 0.01$.

Table 3. Percentage of the bound fraction of TSG to α1-AGP determined by ultrafiltration, equilibrium dialysis, and CPE methods.

Concentration (μg/mL)	Ultrafiltration	Equilibrium dialysis	CPE
2	57.3 ± 2.4	68.2 ± 4.2**	79.2 ± 3.4**
10	39.9 ± 4.6	49.5 ± 4.0*	86.3 ± 2.5**
50	15.4 ± 4.0	20.5 ± 3.9**	91.9 ± 2.6

The results are expressed as mean values from five different determinations ± SD.
*$P < 0.05$, **$P < 0.01$.

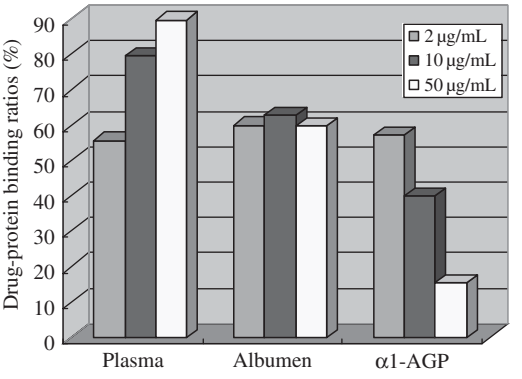

Figure 3. The binding of TSG to plasma, albumin, and α1-AGP under the concentrations of 2, 10, and 50 μg/mL, respectively.

Tables 1 and 3 showed that the binding of TSG to albumin obtained by equilibrium dialysis was lower than this obtained by ultrafiltration, the binding to α1-AGP measured by equilibrium dialysis was higher than that obtained by ultrafiltration, indicating that the volume shifts that may occur for the long time required for equilibrium had some effect on TSG binding to plasma proteins.

As an effective separation method, CPE has recently been successfully used for the selective extraction or

pre-concentration of various compounds from biological and environmental media, including estrogens[19], vitamin A, vitamin E[11], terazosin hydrochloride[20], arbidol[21], amino acid[22], metal[23–29], trace element[30], as well as some other chemical compounds[13,14,31–33].

The technique is based on forming surfactant micelles that have been applied to analysis of bioactive compounds in various matrices. In most of these analyses, nonionic surfactants (such as Triton X-100[31], Triton X-114[19,21–24,26,27,29,30,32], PONPE 7.5[20,25,28], Genapol X-114[11,14], SDSA (sodium dodecane sulfonic acid)[34], PEG4000[33], Genapol X-080[13], and so on) were found to be the optimal micelle-forming agents.

Aqueous solutions of nonionic surfactants may separate in two phases in a narrow temperature range called the cloud point. The micellar solution separates in a surfactant-rich phase of a small volume and in a dilute aqueous phase, in which the surfactant concentration is close to the critical micellar concentration. In optimal conditions of pH, surfactant type, and concentration, any hydrophobic species (such as metal chelates, plasma proteins, protein-bound drugs[35], and so on) were transferred into the surfactant-rich phase, being extracted or preconcentrated.

Among the several surfactants, Triton X-100 cannot be used because their cloud-point temperature is too high. The other surfactants gave convenient temperatures. Triton X-114 showed up in the chromatogram only as a small peak. Therefore Triton X-114 was selected as the optimal micelle-forming agents on this study.

Table 4 showed the influence of surfactant concentration and pH on the found free fraction of TSG. A stable phase separation took place by adding 1 mL of 5% Triton X-114 solution to 0.2 mL of plasma (10 μg/mL) and the free fraction of TSG remains constant. Therefore the studies were performed with 0.2 mL of solution and 1 mL of 5% Triton X-114. Neutral pH gave the lowest cloud-point temperature; therefore it was not necessary to adjust the pH of the extraction mixture (with hydrochloric acid or sodium hydroxide).

Because TSG is the water-soluble bioactive component, so after CPE, the plasma proteins and the protein-bound drugs transfer into a surfactant-rich phase and the free fraction of the drug remains in the water phase.

Table 4. Influence of cloud-point extraction conditions on the found free fraction and on the cloud-point temperature.

Conditions (Triton X-114)	TSG-free fraction (%)	Cloud-point temperature
10%, pH 6	14.2	45
2.5%, pH 6	15.5	27
5%, pH 6	15.1	35
5%, pH 3	15.8	37
5%, pH 10	15.4	40

The coefficients of variation of the CPE procedure were determined with 10 identical plasma samples. We found the RSD was lower than 9.5%. This precision is adequate for pharmacokinetic studies.

In our study, the binding of TSG to plasma obtained by CPE were in good agreement to these observed by ultrafiltration and equilibrium dialysis, indicating that CPE was a highly sensitive and selective method for the measurement to plasma protein binding of TSG. CPE has several advantages: The required volume of plasma is small, namely 200 μL, the procedure is cheap, and it is simple to carry out (it is simpler than, e.g., equilibrium dialysis). The main advantage of CPE is its capability to concentrate a variety of analytes with high concentration factors. If a liquid–liquid extraction step is added, further concentration of the analytes is achieved, which allows the quantification of trace concentration of drugs. However, if the concentration of the analyte is high enough a liquid–liquid extraction is not necessary and the aqueous phase can directly be injected into a reversed-phase HPLC separation system. It is also possible to use CPE for the direct determination of the protein-bound fraction of a drug. This fraction transfers into the surfactant-rich phase. CPE can be used for routine assays that investigate the influence of various factors on protein–drug binding or the interactions of drugs in vivo, and for studies of the complex relationships between the total and free fractions of a drug and their influence on pharmakokinetics.

Conclusions

In this study, TSG was found to be bound to both albumin and α1-AGP. However, albumin concentration in plasma is 600 μM compared with 20 μM of α1-AGP, which may indicate that albumin is more relevant for TSG protein binding in clinical settings. The binding to albumin was constant (about 60%) within concentration range. The binding to α1-AGP exhibits a concentration-dependent (saturable) protein binding within their therapeutic range so that higher concentrations are associated with lower drug–protein binding ratios. So α1-AGP might be important when plasma proteins change with disease. The binding of TSG to plasma increased with increasing drug concentration, and the results obtained by CPE were in good agreement to these observed by ultrafiltration and equilibrium dialysis, indicating that CPE was a highly sensitive and selective method for the measurement to plasma protein binding of TSG. TSG was stable during 12 hours at different temperatures in plasma. This is an important observation to be considered when handling the samples.

Acknowledgments

This work was supported by Program for Natural Science Foundation of Hebei Province (C2006000791).

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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