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Study on PEG-$(NH_4)_2SO_4$ Aqueous Two-Phase System and Distribution Behavior of Drugs

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The distribution behavior of chlorpromazine hydrochloride (CPZ), procaine hydrochloride (PCN) and procaine amide hydrochloride (PCNA) in polyethylene glycol (PEG800 or PEG1500)-$(NH_4)_2SO_4$ aqueous two-phase systems has been investigated. The result shows that the PEG-$(NH_4)_2SO_4$ aqueous two-phase system has potential extraction capability in small molecular drug separation. In PEG800-$(NH_4)_2SO_4$ system, the extraction efficiencies (E) of CPZ, PCN and PCNA amount to 92.8%, 74.5% and 74.4%, respectively, with the distribution coefficients (K_D) being 25.7, 5.9 and 5.8, correspondingly. In PEG1500-$(NH_4)_2SO_4$ system, the extraction efficiencies (E) of CPZ, PCN and PCNA are 93.7%, 71.3% and 63.2%, respectively, with distribution coefficients (K_D) of 39.6, 6.6 and 5.0, correspondingly. Based on the study on ultraviolet and fluorescence spectra and also distribution behavior of the drugs in PEG-$(NH_4)_2SO_4$ aqueous two-phase system, extraction mechanism was further proposed that both hydrogen bond and hydrophobic interaction are involved in extraction.

Keywords aqueous two-phase system, polyethylene glycol, extraction mechanism of drug

Introduction

Progress in biochemistry and cell biology depends to a great extent on the development of efficient separation methods.[1(a)] Methods such as high-speed centrifugation, capillary electrophoresis, liquid chromatography, extraction, *etc*., are pressingly demanded in the fields of biological research. The polymer aqueous two-phase system is a mild and efficient extraction technique which does not use any organic solvent, thus is friendly to the environment. Compared with the conventional organic-solvent extraction, it is considered to be a "green" extraction technique.[2] Furthermore, this aqueous two-phase system extraction technique has many other advantages[2,3] such as an effective cost, relatively easy mass production, an offer of gently non-toxic environment for bioentities and a high enriching factor. It is also easy to couple this technique with analytical instruments such as FIA-Biosensor,[4] AES[5] and CE.[6] Since 1950's, aqueous two-phase system technique has mainly been applied to separation and purification of bio-active substances such as proteins, nucleic acids and cell particles.[7-12] In recent years, it has been applied to effective separation of drugs, such as ecdysone, 20-hydroxyecdysone, baicalin and geniposide, *etc*., from natural plants.[13-16] However, application of drug extraction and separation in aqueous two-phase system is still limited, and the mechanism of the drug extraction has not been well elucidated. Our previous study has suggested guidelines on salt selection and primary extraction mechanism of drugs in non-ionic surfactant-salt aqueous two-phase system.[17]

In this paper, polyethylene glycol (PEG800 or PEG1500)-$(NH_4)_2SO_4$ system was used to form an aqueous two-phase system. The effects of the PEG concentration, salt species and the equilibrium time were investigated. In order to study the extraction mechanism of drugs more effectively, we selected chlorpromazine hydrochloride (CPZ), procaine hydrochloride (PCN) and procaine amide hydrochloride (PCNA), which have the similar molecular structure, as model drugs. Extraction mechanism was discussed based on ultraviolet and molecular fluorescence spectra and the extraction behavior. In the meanwhile, the effect of PEG molecular weight on the extraction behavior of drugs was also explained.

Experimental

Materials and instruments

A Shimadzu UV-265 spectrophotometer and a Shimadzu UV-120-02 spectrophotometer were used for the determination of ultraviolet absorbance. A Shimadzu RF-540 fluorescence photometer was used for molecular fluorescence measurement. A Model-821 pH meter (Zhongshan University, China) was used for the pH measurement.

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Polyethylene glycol (PEG800 or PEG1500) (Beijing Xudong Chemical Factory, China, A.R.) was prepared in a 50% (*w*/*V*) water solution. Chlorpromazine hydrochloride (CPZ) (Sigma, 99%), procaine hydrochloride (PCN) (Acros, 99%), and procaine amide hydrochloride (PCNA) (Acros, 99%) were prepared as water solutions of 1.00 mg/mL. A series of Britton-Robinson (B.R.) buffer solutions containing phosphoric acid, acetic acid, and boric acid, each with a concentration of 0.25 mol/L at pH 2.0—7.0,[18] $(NH_4)_2SO_4$, $(NH_4)_3PO_4$ and Na_2SO_4 (Beijing Chemical Factory, China, A.R.) were employed.

Method for phase separation

Into a 10 mL color comparison tube, 3.0 mL of 50% PEG800 or PEG1500 solution and 3.0 mL of B.R. buffer solution of pH 6.0 were added. The contents were diluted to the 10 mL mark with distilled water and mixed thoroughly. After that, 2.5 g of $(NH_4)_2SO_4$ were added, then the tube was shaken till all the salt was dissolved. A stable aqueous two-phase system was formed after 10 min.

Determination of the drugs

An aqueous two-phase system was prepared according to the similar method for phase separation except that a certain amount of the drug was added. The upper phase containing PEG and the drug mainly was transferred completely into another tube. Then 3.0 mL of B.R. buffer solution of pH 6.0 was added followed by the addition of water to the 10 mL mark. The absorbance of solution was measured by UV-Vis spectrophotometry at the absorption maximum wavelength (λ_{max}) of the drugs (256, 283 and 293 nm for CPZ, PCNA and PCN, respectively) with a reference of blank PEG solution without drug prepared in the same way.

Results and discussion

Phase diagram

At room temperature, the amount of salt needed for the PEG solution to form aqueous two-phase system depends on the following factors: concentration of PEG, the property of salt and acidity of the solution. $(NH_4)_2SO_4$, $(NH_4)_3PO_4$ and Na_2SO_4 were chosen for the test. As shown in Figure 1, at the same concentration of PEG solution, the amount of salt needed to form phase separation varied with salt species. For the same salt, minimum amount of salt for the formation of phase separation decreased with the increase of PEG molecular weight. It could be due to that with the increase of PEG molecular weight the number of -OH group in the PEG with the same mass decreases, causing the sites where PEG and water molecules can form hydrogen bonds to be reduced. $(NH_4)_2SO_4$ was chosen as the phase separation salt based on the following separation effect: (1) $(NH_4)_2SO_4$ shows no influence on the determination of drug concentration. (2) $(NH_4)_2SO_4$ can be dissolved readily in water. Its solubility (g/100 g H_2O) is 75.4 at 20 ℃,[19(a)] while those of Na_2SO_4 and $(NH_4)_3PO_4 \cdot 3H_2O$ are 19.5 at 20 ℃[19(b)] and 26.1 at 25 ℃,[19(c)] respectively. Moreover, $(NH_4)_3PO_4$ solution is not stable. (3) By using $(NH_4)_2SO_4$, phase separation can be rapidly obtained and the interface between the two phases is clear. (4) $(NH_4)_2SO_4$ is a cost-effective salt to be applied to large scale production. In this paper, according to the phase diagram (see Figure 1), 15% PEG water solution and 2.5 g of $(NH_4)_2SO_4$ were chosen as the phase separation conditions.

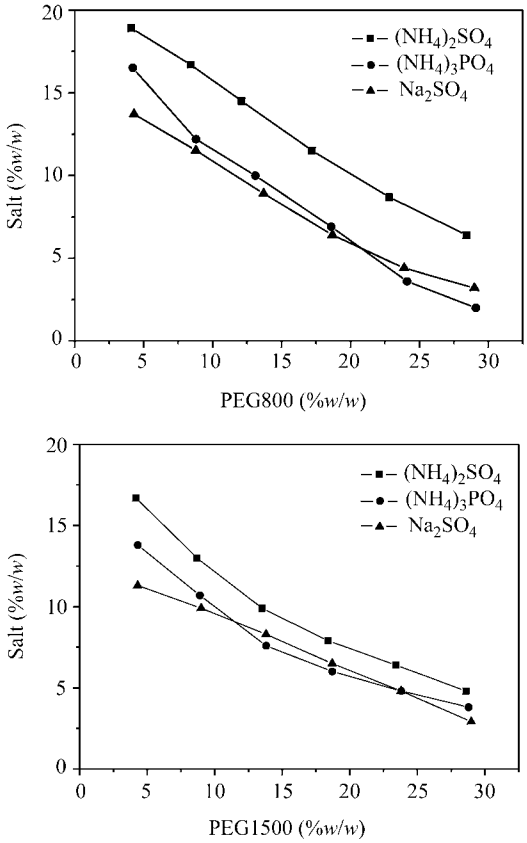

Figure 1 Phase diagrams of PEG-salt aqueous two-phase systems.

Effect of pH on phase separation

Within pH 2.0—7.0, the volume ratios of phases do not change obviously: $V_{\text{upper phase}}/V_{\text{lower phase}}$ is 0.71 in PEG1500 system, while in PEG800 system it is 0.50 (see Figure 2), indicating that pH has little effect on phase separation. This is because that within pH 2.0—7.0 the forms of PEG, NH_4^+ (pK_a=9.4) and SO_4^{2-} (pK_a of HSO_4^- is 1.8) have little change. In this paper the B.R. buffer solution was used to control the acidity at pH 6.0.

Effect of equilibrium time on phase separation

According to the method for phase separation, a stable aqueous two-phase system with a clear interface would form 10 min later, with the upper phase composed mainly of PEG aqueous solution, and the lower phase primarily salt solution. The volume ratio of the two phases had no further variation after 10 min.

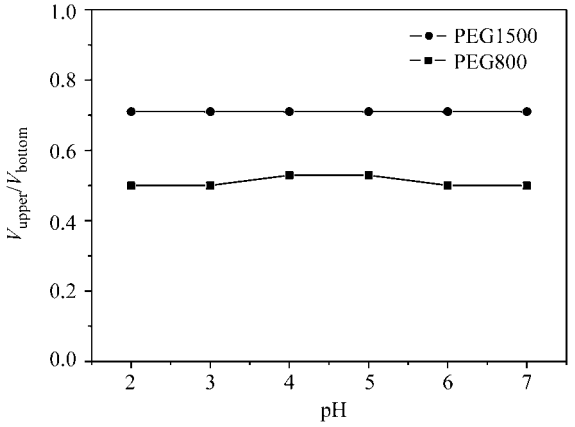

Figure 2 Effect of pH on phase separation. V_{upper} and V_{bottom} are the volumes of upper and bottom phases, respectively.

Effect of pH on drug extraction efficiencies

The effect of pH on extraction efficiencies (E) of the drugs is shown in Figure 3. Due to the effect of basic solution on $(NH_4)_2SO_4$ concentration, the range of pH 2.0—7.0 was selected. Within this range little change in the extraction efficiency of CPZ was observed. The extraction efficiencies of PCN and PCNA increased with increasing pH values at first and were steady within pH 4.5—7.0. Thus pH 6.0 was chosen for phase separation and the drug extraction. This can be interpreted by the structures of the three drugs (Figure 4). The -$CH_2N(Et)_2$ and Ph-NH_2 forms of PCN and PCNA vary with pH change. At pH 2.0—7.0, -$CH_2N(Et)_2H^+$ (pK_a=10.7[20]) always exists as -$CH_2N(Et)_2H^+$, while Ph-NH_3^+ (pK_a =4.5[20]) takes the main form of Ph-NH_3^+ at 2.0—4.5 and Ph-NH_2 at 4.5—7.0. Ph-NH_3^+ is more hydrophilic than Ph-NH_2 in water, therefore, the extraction efficiencies of PCN and PCNA are smaller at the lower pH, while greater at higher pH and vary little at 4.5—7.0. For CPZ, the -$CH_2N(Me)_2H^+$ (pK_a=9.8[20]) part always exists as -$CH_2N(Me)_2H^+$ form within pH 2.0—7.0, so the extraction efficiency of CPZ nearly keeps constant in this pH range.

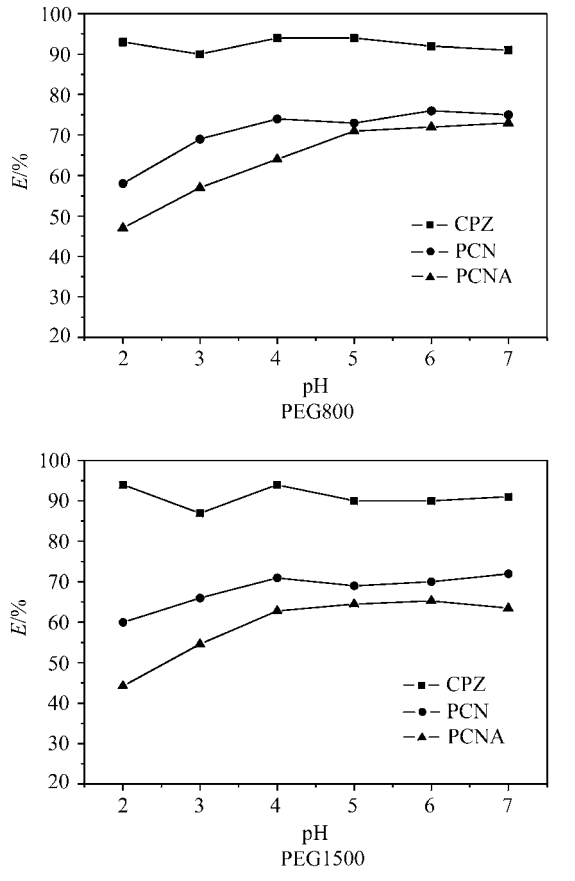

Figure 3 Effect of pH on extraction efficiencies of drugs. The concerntration of each drug was 10 μg•mL^{-1}.

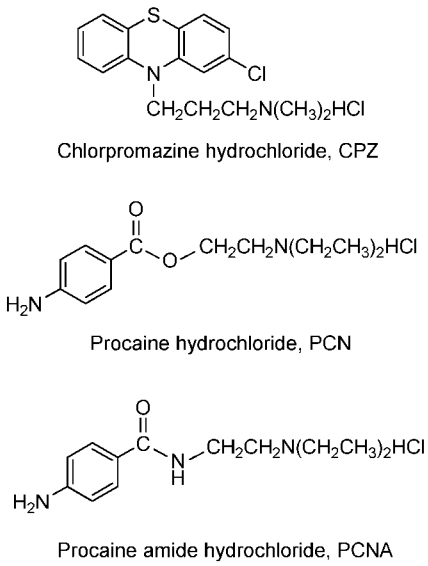

Figure 4 Structures of the tested drugs.

Drug distribution coefficients and extraction efficiencies

According to the method for the determination, the drugs in the two phases were measured, and their distribution coefficients (K_D) and extraction efficiencies (E) were calculated and summarized in Table 1 and Table 2, respectively. The following conclusion can be drawn from the results.

(1) Within tested concentration, the extraction efficiencies and distribution coefficients of the three drugs show little variation with the increase of drug concentration. It means that when the conditions of the PEG-$(NH_4)_2SO_4$ system are set, the distribution of drugs in the system depends only on the properties of the drugs. This is consistent with Brönsted's equation:[1(b)] $K_D = c_E/c_W = \exp(\lambda M/kT)$, where M is the molecular weight, λ a factor which depends on properties other than molecular weight, T the absolute temperature and k Boltzmann constant.

(2) In the same aqueous two-phase system, the average values of the distribution coefficients of the three drugs are sorted as $K_D^{CPZ} > K_D^{PCN} > K_D^{PCNA}$ and consistent with Brönsted's equation. K_D^{CPZ} is much greater

than K_D^{PCN} and K_D^{PCNA}, while K_D^{PCN} and K_D^{PCNA} are not significantly different. When the conditions of the PEG-$(NH_4)_2SO_4$ aqueous two-phase system are set, λ will be determined by the chemical structure and molecular weights of the drug molecules. The molecular weights of CPZ, PCN and PCNA are in the order of M_{CPZ} (355.3)$>M_{PCN}$ (272.8)$>M_{PCNA}$ (271.8). Due to the similarities in structure and molecular weight, distribution coefficients and extraction efficiencies of PCN and PCNA are similar. CPZ has a very different structure form and a much larger molecular weight than PCN and PCNA, so its distribution coefficients and extraction efficiencies are very different from those of PCN and PCNA.

(3) In PEG800-$(NH_4)_2SO_4$-H_2O system, $\Delta E_1 = E_{CPZ} - E_{PCN} = 18.3\%$, $\Delta E_2 = E_{PCN} - E_{PCNA} = 0.1\%$, $\Delta K_D^1 = K_D^{CPZ} - K_D^{PCN} = 19.8$, $\Delta K_D^2 = K_D^{PCN} - K_D^{PCNA} = 0.1$, while in PEG1500-$(NH_4)_2SO_4$-$H_2O$ system, $\Delta E_1 = 22.4\%$, $\Delta E_2 = 8.1\%$, $\Delta K_D^1 = 33.0$, $\Delta K_D^2 = 1.6$. All the differences between the drugs in the PEG1500 system are greater than those in the PEG800 system. These results indicate that with the increase of PEG molecular weight the hydrophobicity of PEG increases, the differences of the extraction and distribution behavior between different drugs, even with similar ior between different drugs, even with similar structures such as PCN and PCNA, are enlarged.

Drug extraction mechanism

Extraction mechanism of the drugs in PEG-$(NH_4)_2SO_4$ aqueous two-phase system has been studied based on ultraviolet and fluorescence spectra of the three drugs. As shown in Figure 4, CPZ has two unconjugated aromatic rings, while PCN has conjugated ester group on its aromatic ring and PCNA has conjugated amide group on its aromatic ring. The ultraviolet spectra of CPZ, PCN and PCNA (see Figure 5) all show red shift and increase of ultraviolet intensities in 15% PEG1500 phase (similar in PEG800 phase). The shifts of ultraviolet spectra of the drugs are: $\Delta\lambda_{CPZ} = 2$ nm, $\Delta\lambda_{PCN} = 3$ nm and $\Delta\lambda_{PCNA} = 5$ nm. It might be due to that hydrogen bonds between drug and PEG lead to the redistribution of the electron cloud within PCN molecule and the decrease of π-π*, n-π* electronic transition energy which cause the red shift of the absorption. As PCNA shows the more intense the tendency of forming intermolecular hydrogen bond, the more significant the shift.

Table 1 Distribution coefficients (K_D) of drugs in PEG-$(NH_4)_2SO_4$ aqueous two-phase system[a]

	Distribution coefficient	Drug concentration/(μg•mL^{-1})								$\bar{K}_D$ [b]	RSD[c]/%
		4.0	6.0	8.0	10.0	12.0	14.0	16.0	20.0		
PEG800	K_D^{CPZ}	25.9	25.1	25.3	25.4	26.4	25.6	25.9	25.9	25.7	1.6
	K_D^{PCN}	6.1	6.2	6.1	5.9	5.7	5.7	5.8	5.9	5.9	3.2
	K_D^{PCNA}	6.2	5.9	6.0	5.6	5.8	5.7	5.4	5.7	5.8	4.2
PEG1500	K_D^{CPZ}	40.9	39.7	38.2	39.6	38.0	40.2	39.9	40.6	39.6	2.6
	K_D^{PCN}	6.1	7.0	6.7	6.9	6.6	6.4	6.5	6.5	6.6	4.3
	K_D^{PCNA}	4.5	5.4	5.8	4.7	4.8	5.0	4.9	5.2	5.0	8.3

[a] $K_D = c_E/c_W$, c_E and c_W are the concentration of the drug in the PEG phase and in the water phase, respectively. [b] The average value of the distribution coefficients. [c] The relative standard deviation.

Table 2 Effect of drug concentration on the extraction efficiencies (E)[a]

	Extraction efficiency/%	Drug concentration/(μg•mL^{-1})								$\bar{E}$ [b]/%	RSD[c]/%
		4.0	6.0	8.0	10.0	12.0	14.0	16.0	20.0		
PEG800	E_{CPZ}	92.8	92.6	92.7	92.7	93.0	92.7	92.8	92.8	92.8	0.1
	E_{PCN}	75.2	75.7	75.2	74.6	74.1	74.0	73.4	73.6	74.5	1.1
	E_{PCNA}	75.1	74.6	74.9	73.6	74.2	74.0	74.9	74.0	74.4	0.7
PEG1500	E_{CPZ}	93.7	93.5	93.3	93.5	93.2	93.6	94.5	94.1	93.7	0.5
	E_{PCN}	71.0	71.8	73.2	72.1	70.5	71.5	70.4	70.1	71.3	1.5
	E_{PCNA}	62.0	62.3	64.1	63.2	63.7	64.4	62.7	63.5	63.2	1.3

[a] $E = n_E/n_T$, n_E represents the drug amount in the PEG phase and n_T represents the total drug amount in the system. [b] The average value of the extraction efficiencies. [c] The relative standard deviation.

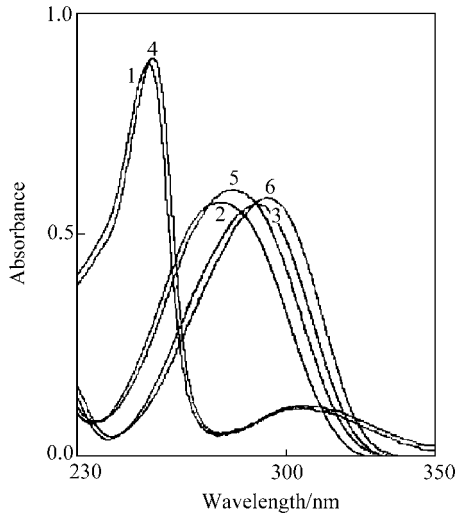

Figure 5 Absorption spectra of the drugs. c=10 μg•mL^{-1}, pH =6.0; 1, 2, 3—CPZ, PCNA, PCN in water phase (water as blank); 4, 5, 6—CPZ, PCNA, PCN in 15% PEG1500 phase (15% PEG1500 as blank); λ_{max1}=254 nm, λ_{max2}=278 nm, λ_{max3}=290 nm, λ_{max4}=256 nm, λ_{max5}=283 nm, λ_{max6}=293 nm.

Seen from the fluorescence spectra in Figure 6, fluorescence intensities of the drugs in 15% PEG1500 phase (similar in PEG800 phase) are constantly greater than those in water phase. The first is as a result of that these three drugs can form hydrogen bonds with PEG1500, thus molecular rigidity of the drugs is increased. The second, PEG as a non-ionic surfactant can alter many characteristics of the solvent such as the polarity, viscosity and dielectric constant, which result in "solvent cages" that protects those fluorescent molecules in the singlet state and shields them from quencher such as dissolved oxygen. In the same experimental conditions, the latter effect on the three drug molecules

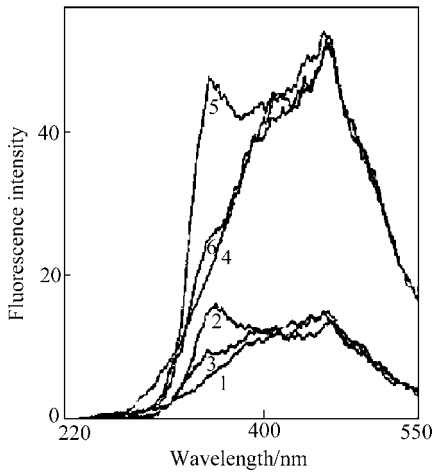

Figure 6 Fluorescence spectra of the drugs. λ_{EX}=210 nm, c= 10 μg•mL^{-1}; pH=6.0. 1, 2, 3—CPZ, PCNA, PCN in water phase (water as blank); 4, 5, 6—CPZ, PCNA, PCN in 15% PEG1500 phase (15% PEG1500 as blank).

should be similar. However, the increases of fluorescence intensity at 350 nm of PCN and PCNA in PEG1500 are greater than that of CPZ, which obviously indicates that PCN and PCNA are more likely to form hydrogen bonds, thus they tend to be preserved in water phase and exhibit lower extraction efficiencies than CPZ in PEG phase. In addition, the fluorescence intensity of PCNA is greater than PCN, especially at 350 nm, PCNA shows a clear raising effect. This is because the amide moiety "-CONH-" of PCNA forms hydrogen bonds with PEG1500 more easily than the ester moiety "-COO-" of PCN. It is consistent with the conclusion drawn from the ultraviolet spectra.

Based on the above spectral results, the extraction mechanism of drugs was further proposed. If hydrogen bond between drug and water dominates the extraction, the drug will exhibit smaller distribution coefficient and extraction efficiency, and if hydrophobic interaction between drug and PEG dominates the process, the drug will show greater distribution coefficient and extraction efficiency. CPZ is more hydrophobic and has weaker hydrogen bonding ability than PCN and PCNA, so it has larger distribution coefficient and extraction efficiency in PEG-$(NH_4)_2SO_4$ aqueous two-phase system. Moreover, the mechanism deduced in this paper can also explain the difference of distribution behavior of the drugs between the two different aqueous two-phase systems. Having less hydroxyl groups than PEG800, PEG1500 shows much stronger hydrophobicity than PEG800. As a result, CPZ with stronger hydrophobicity than hydrogen bond ability toward PEG, enters the PEG phase in the PEG1500-$(NH_4)_2SO_4$ system more easily than that in PEG800-$(NH_4)_2SO_4$ system, thus has a larger K_D in the former system than in the latter system. On the other hand, PCNA, which has hydrogen bond ability stronger than hydrophobicity with PEG, more easily enters the water phase, and consequently has a smaller K_D in the PEG800 system than in the PEG1500 system.

In addition, according to the results described above, another conclusion can be drawn that only in the system in which PEG is more hydrophobic, can the difference of distribution behavior between the similar drugs with stronger hydrogen bond ability than hydrophobicity be enlarged, and consequently can the drugs be separated. Likewise, only in the system in which PEG is less hydrophobic, can the similarly hydrophobic drugs be separated.

Conclusions

The phase separation conditions of PEG (PEG800 or PEG1500)-$(NH_4)_2SO_4$ aqueous two-phase system have been investigated. The distribution coefficients of CPZ, PCN and PCNA are determined to be 25.7, 5.9 and 5.8, respectively in PEG800-$(NH_4)_2SO_4$ aqueous two-phase system, while in PEG1500-$(NH_4)_2SO_4$-H_2O aqueous two-phase system the parameters are 39.6, 6.6 and 5.0, respectively. The differences of extraction behavior among the three drugs were enlarged in the latter system

because PEG1500 had a stronger hydrophobicity than PEG800. CPZ, the extraction efficiencies of which in PEG800 and PEG 1500 are 92.8% and 93.7%, respectively, can be almost completely extracted into the PEG phase. Through the study of ultraviolet and fluorescence spectra of the three drugs in PEG-$(NH_4)_2SO_4$ aqueous two-phase system and their extraction behaviors, extraction mechanism of the drugs was further proposed that extraction and separation were the result of the hydrogen bonding and hydrophobic interactions. This proposed mechanism can well explain the differences of distribution coefficients and extraction efficiencies of the three drugs, and will possibly provide guidelines for selection of the aqueous two-phase systems to successfully separate and preconcentrate drugs. The whole procedure mentioned in this study involved no organic solvent. The method was simple and showed good reproducibility.

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