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Effective Parameters on the Partition Coefficient of Guanidine Hydrochloride in the Poly (Ethylene Glycol) +Phosphate +Water System at 298.15 K

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Abstract

The partition coefficient of guanidine hydrochloride for the PEG4000+ Phosphate + Water system is presented at 298.15 K. The partition coefficient of guanidine hydrochloride was near unity. It was shown that partition coefficient of guanidine hydrochloride was dependent on the concentration of guanidine hydrochloride, pH and PEG/phase forming salt (%w/w) ratio. In most systems the partitioning coefficient of Guanidine is more than one, which means guanidine preferred polymer-rich phase.

Keywords: *Aqueous Two Phase System, ATPS, Partition, Guanidine Hydrochloride*

1. Introduction

Mixing two or more incompatible polymers or a polymer and a structuring salt in aqueous conditions generally forms an aqueous two-phase system (ATPS), in which the percentage of water in both phases is 75-90% (v/v) [1]. Nowadays there is growing interest in the use of ATPS as a powerful but mild separation technique for mixtures of biomolecules [2]. So that these systems present a gentle, scalable and efficient procedure for separation of various biological materials such as recombinant proteins and enzymes [3-6], inclusion body refolding processes are poised to play a major role in the production of recombinant proteins. One step of the general strategy used to recover active protein from inclusion bodies is solubilization of the aggregated protein with denaturant such as guanidine hydrochloride and urea [7]. Aqueous two-phase systems, based on polyethylene glycol and sodium sulfate, have earlier been successfully used in the presence of urea for the recovery of active insulin like growth factor (IGF1) from inclusion bodies [8]. Also, ATPS based on PEG and phosphate has been used for the recovery of active recombinant xylanase from denatured enzyme solution using guanidine hydro-

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chloride as a chaotropic salt [6]. Recovery of active protein in phase systems containing PEG and a chaotropic salt such as guanidine hydrochloride has also been shown as a possibility [9]. Novel bioseparation research based on aqueous two-phase systems needs to focus on determining phase diagrams, partition coefficients and other thermodynamic data for the design of an industrial-scale process [1]. Some articles have been written about applying guanidine hydrochloride in aqueous two-phase system for the initial recovery step, but little has been published about the complex problem of how the guanidine hydrochloride affects the phase diagram behaviour, and determining the partition coefficient of guanidine hydrochloride in these systems.

A comprehensive review of the early experimental liquid–liquid equilibrium (LLE) of the aqueous two-phase systems containing two different kinds of polymers or a polymer and a salt have been reported by Albertsson (1986) [1] and Walter et al. (1985) [4], but the knowledge of phase systems containing chaotropic compounds is very limited [9-10], determining phase diagrams of poly(ethylene glycol)(PEG)/ sodium sulfate/ urea/ water and PEG/ dextran T-500(DEX)/ phosphate buffer/water at different concentrations of urea and different PEG molecular weights. Guanidine hydrochloride is preferred since urea solutions may contain, spontaneously producing cyanate [11], which can carbamylate the amino groups of the protein. In addition, inclusion body solubilization by urea is pH dependent and optimum pH conditions must be determined for each protein [12]. In the previous work the effect of guanidine hydrochloride on phase behavior of PEG4000/ phosphate/ guanidine hydrochloride/ water at different guanidine hydrochloride concentrations and pH were investigated [13]. In the present study the partition coefficient of guanidine hydrochloride and the parameters that affect it, e.g., pH and PEG/Salt weight percent ratio, were studied. These data would be useful to increase the knowledge of the separation processes based on the aqueous two-phase system and improving the yield of protein refolding.

2. Experimental Section

2.1. Materials

Polyethylene glycol, with a mass average 4000, dipotassium hydrogen phosphate and sodium di-hydrogen phosphate were of analytical grade (Merck) and were used without further purification. Guanidine hydrochloride was purchased from Sigma-Aldrich. Distilled water was used in all experiments. All other materials were analytical grade.

2.2. Apparatus and Procedure

Biphasic systems were prepared by a mixture of PEG 4000 and phosphate salt solution at required pH. The pH of the salt solution was adjusted by mixing an appropriate ratio of sodium di hydrogen phosphate and di potassium hydrogen phosphate. In this work, for preparation of the basic biphasic system, the experimental data reported by Haghtalab et al. (2004) [14] were used. For each of the mentioned systems four samples including 2.5 %, 5%, 7.5% and 10 % (w/w) of guanidine hydrochloride were arranged. All components were added into a graduated 15

ml tube as a dry powder or as stock solution at constant pH and temperature (298.15k), resulting in a 10 g system. The pH values of the solutions were measured precisely with a pH meter (JENWAY 3345). The resulted solution was mixed by rigorously vortexing the solution for 2 min. The tubes were placed at room temperature for 2 h; and then centrifuged at 2400 rpm for 10 min. In the results, two clear phases with a visible interface were obtained and the solution reaches equilibrium. The samples of the top and bottom phase were carefully withdrawn, with care being taken to leave a layer of solution at least 0.2 cm thick above the interface. The concentration of GuHCl in the phases was measured using the colorimetric assay described by Sullivan [15] using 1,2-naphthoquinone-4-sodium sulfonate, which leads to the development of a rose-red color due to the formation of 4- guanidonaphtho-quinone-1,2 in an acidic medium. To overcome the possible interference by the phase-forming components in the analyses, identical phase systems without the analyte (GuHCl) were used as blanks.

The concentration of phase forming salt was determined by conduct meter at 298.15K using a JENWAY 4510 model. Since the conductivity of phase samples depends on both guanidine hydrochloride and salt concentration, but is independent on PEG concentration, calibration plots of con-ductivity versus guanidine hydrochloride concentration were prepared for different concentrations of salt. The concentration of PEG was determined by refractive index measurements at 298.15K using an ATAGO-DTM1 model (Fig. 1). Since the refractive index of the phase samples depends on PEG, guanidine hydrochloride, and salt concentration, calibration plots of refractive index versus polymer concentration were prepared for different concentrations of salt and guanidine hydrochloride. Each experiment was repeated 3 times and the results were the average of these 3 replications.

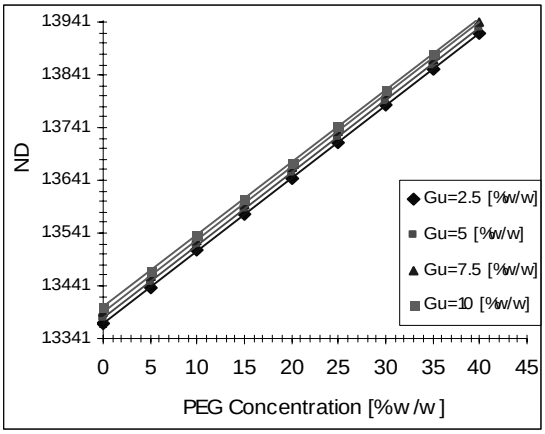

Figure 1. Refractive index calibration curves for PEG (4000) - K_2HPO_4-water at 298.15K. (Salt=1.5g)

3. Results and Discussion

In these systems opposing components were found. Considering the lyotropic series, $H_2PO_4^-$ and K^+ are so-called structure-making salts, while guanidine hydrochloride is described as the structure breaking agent [16]. The combination of these two competing components on phase separation is very interesting and cannot be predicted without experiments.

For measuring the concentration of components in the top and bottom phases of aqueous two-phase systems, standard calibration curves should be built. The PEG concentration has no effect on the calibration curve of the guanidine hydrochloride concentration in PEG/ guanidine

hydrochloride/ phosphate/ water system. In the results, the calibration curves were related to the concentrations of salt and guanidine hydrochloride. A change of PEG concentration in the range of 10- 50% (w/w) results in 0.05 ms (milli- Siemens) in the conductivity of the solution which is negligible, relative to the 20 ms changes related to the changes in GuHCl concentration in the same range [13].

A typical calibration curve for guanidine hydrochloride/ K_2HPO_4/ water system was shown in Fig. 2.

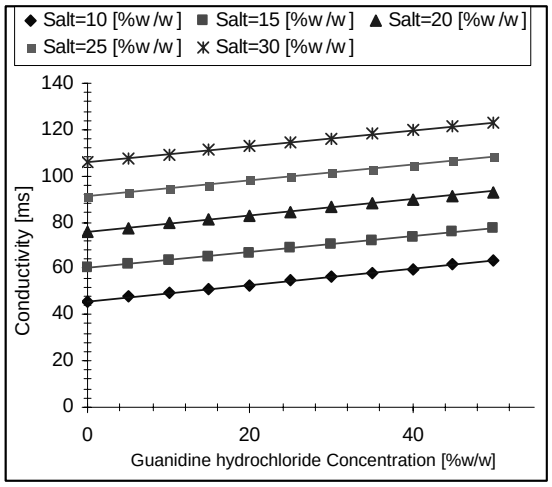

Figure 2. Conductivity calibration curves for PEG (4000)-K_2HPO_4-water at 298.15K in different guanidine and salt concentrations.

The guanidine concentrations varied between 0 and 50 (% w/w) and the concentration of phosphate varied between 10 and 30 (%w/w). To increase the knowledge of aqueous two-phase systems containing denaturants, the partitioning coefficient of guanidine hydrochloride for a broad range of systems based on PEG4000/ phosphate/ water at 298.15K and different pH (7.2, 9.1, and 10.8) were determined.

Addition of guanidine hydrochloride leads to a shift of the binodial away from the origin with increasing guanidine concentration. The more guanidine hydrochloride added to the phase systems, the smaller the liquid-liquid two-phase region. It could be related to the structural breaking properties of guanidine. At guanidine hydrochloride concentration more than 10% it precipitates due to the solubility limit of guanidine in PEG/ phosphate aqueous two-phase systems. The guanidine effect on the binodal diagram has been explained based on the fact that the PEG solubility is increased by the ions which are structuring breaking of the water restructures [17]. Guanidine hydrochloride also increases the solubility of the hydrophobic solutes in water; this effect is due to the capacity of guanidine hydrochloride to form hydrogen bound with the water molecules, replacing part of the water molecules ordered around the hydrophobic boundaries [18]. Guanidine hydrochloride, a non-charge solute, forms a stable complex with water molecules by hydrogen bound with the previous destruction of the water–water interaction; therefore, guanidine hydrochloride acts as a structure breaking solute.

As solute is added to ATPS, partitions between the phases, and its partition coefficient, K, is defined as the solute concentration in the upper phase divided by its concentration in the lower phase, therefore, the partition coefficient guanidine hydrochloride in the two-phase system is defined as:

$$K= [G]_{top}/ [G]_{bottom} \quad (1)$$

where, $[G]_{top}$ and $[G]_{bottom}$ are equilibrium concentrations of guanidine hydrochloride in the top and bottom phases, respectively. The PEG/phase forming salt (%w/w) ratio and partition coefficient of guanidine hydrochloride for pH=7.2 are given in Table 1.

Table1. Experimental Data for PEG4000/ Phosphate/ Water/ Guanidine Hydrochloride at pH=7.2

[PEG/Phase forming salt]	K	GuHCl
(%w/w)		Feed(%w/w)
1.07	0.95	2.5
1.10	1.00	2.5
0.66	0.91	2.5
1.18	1.02	2.5
1.10	0.96	2.5
1.05	0.98	2.5
0.80	0.98	5.0
0.92	1.05	5.0
0.90	0.93	5.0
1.16	1.05	5.0
1.11	0.99	5.0
1.38	1.01	5.0
0.80	1.01	7.5
0.92	1.08	7.5
0.92	0.95	7.5
1.16	1.06	7.5
1.13	1.03	7.5
1.41	1.04	7.5
0.81	1.06	10.0
0.94	1.05	10.0
0.93	1.04	10.0
1.16	1.10	10.0
1.14	1.05	10.0
1.50	1.06	10.0

Partitioning of guanidine hydrochloride as a function of PEG/ phase forming salt ratio and initial guanidine concentration for pH of 9.1 and 10.8 was shown in Tables 2 and 3, respectively.

Table 2. Experimental Data for PEG4000/ Phosphate/ Water/ Guanidine Hydrochloride at pH=9.1

[PEG/Phase forming salt]	K	GuHCl
(%w/w)		Feed(%w/w)
1.34	1.00	2.5
1.13	0.92	2.5
1.27	1.01	2.5
1.12	1.00	2.5
1.20	0.92	2.5
1.38	1.01	2.5
1.23	0.94	2.5
1.45	0.98	2.5
1.56	1.02	5.0
1.16	0.96	5.0
1.54	1.01	5.0
1.31	1.03	5.0
1.28	0.94	5.0
1.31	1.00	5.0
1.20	0.97	5.0
1.22	1.02	5.0
1.45	1.04	7.5
1.08	0.99	7.5
1.37	1.04	7.5
1.37	0.99	7.5
1.16	1.02	7.5
1.26	1.03	7.5
1.25	1.01	7.5
1.28	1.02	7.5
1.28	1.06	10.0
1.11	1.00	10.0
1.30	1.01	10.0
1.44	1.04	10.0
1.25	1.03	10.0
1.50	1.04	10.0
1.42	1.03	10.0
1.26	1.04	10.0

Table 3. Experimental Data for PEG4000/ Phosphate/ Water/ Guanidine Hydrochloride at pH=10.8

[PEG/phase forming salt]	K	GuHCl
(%w/w)		Feed(%w/w)
1.34	0.94	2.5
1.59	1.00	2.5
1.41	0.97	2.5
1.03	0.90	2.5
1.34	0.95	2.5
1.46	0.99	2.5
1.23	0.96	5.0
1.52	0.97	5.0
1.36	0.98	5.0
0.97	0.89	5.0
1.25	0.96	5.0
1.83	1.02	5.0
1.35	0.98	7.5
1.74	1.01	7.5
1.44	0.99	7.5
1.00	0.93	7.5
1.16	0.99	7.5
1.73	1.03	7.5
1.15	1.02	10.0
1.51	1.01	10.0
1.82	1.04	10.0
0.96	0.97	10.0
1.55	1.04	10.0
1.69	1.04	10.0

The partitioning factor of guanidine hydrochloride varies between 0.9 to 1.1, which is nearly similar to the partition of urea in PEG-salt systems [9-10].

A diagram of partitioning coefficient of guanidine hydrochloride vs. PEG/ phase forming salt (%w/w) ratio for different concentrations of guanidine has been shown in Fig. 3-5.

It can be seen that for a constant PEG/ phase forming salt (%w/w) ratio, partition coefficient of guanidine hydrochloride increased with increasing guanidine concentration. Also, the partitioning coefficient increased with increasing PEG / phase forming salt (%w/w) ratio at constant guanidine hydrochloride concentration. It seems that the chaotropic effect of guanidine is more pronounced and caused this result, pushing the guanidine to a PEG-rich phase.

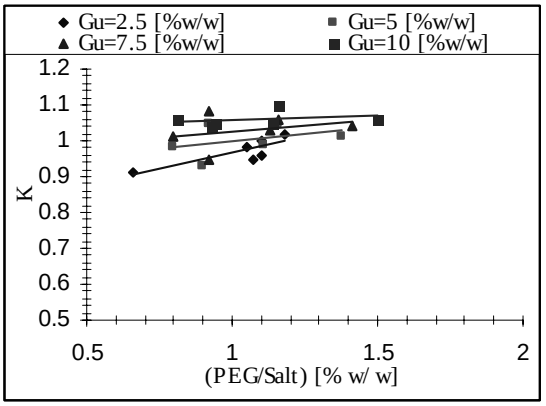

Figure 3. Effect of PEG/Salt (%w/w) ratio and guanidine hydrochloride concentration on partition coefficient of guanidine hydrochloride at pH = 7.2

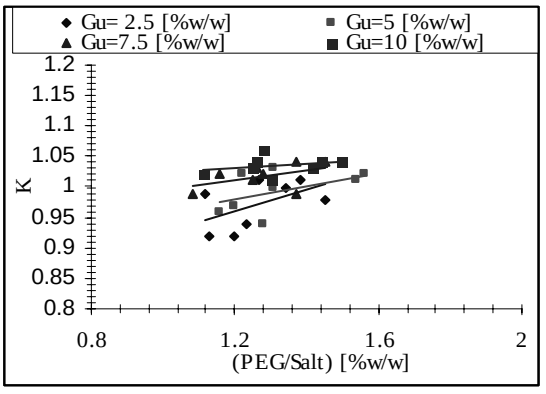

Figure 4. Effect of PEG/Salt (%w/w) ratio and guanidine hydrochloride concentration on partition coefficient of guanidine hydrochloride at pH = 9.1

The slope of partitioning coefficient of guanidine vs. PEG/ phase forming salt (%w/w) ratio decreased with increasing GuHCl concentration. It could be related to the volume excluding effect of the PEG network in which the compact PEG chains do not allow guanidine to diffuse to the polymer network.

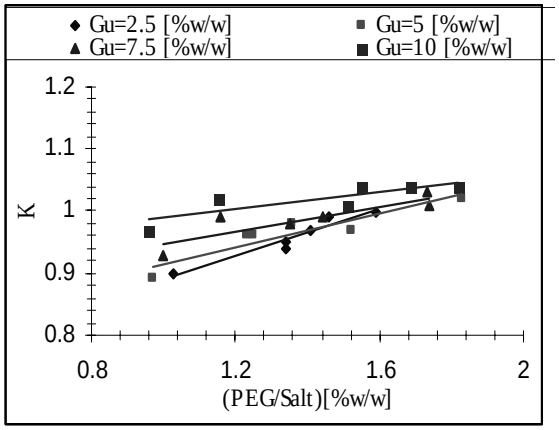

Figure 5. Effect of PEG/Salt (%w/w) ratio and guanidine hydrochloride concentration on partition coefficient of guanidine hydrochloride at pH = 10.8

The increase in guanidine hydrochloride concentration induces a decrease in the specific partial volume in agreement with a minor water hydration of PEG molecule due to the guanidine hydrochloride–water formation by the hydrogen bound [19].

Guanidine hydrochloride decreased the PEG specific partial volume in agreement with the known capacity to form a hydrogen bond with the water molecules around the ethylene chains of PEG. PEG has been demonstrated to be a hydrophilic molecule in which each oxi ethylene group interacts with 16 water molecules: two, by hydrogen binding formation with the oxygen ether and 14 with ethylene, forming an ordered water structure around it [20-21]. This ordered water is very sensitive to the temperature changes and to the presence of the solute which modifies the water structure. Salts have been classified as structure breaking and structure making to describe their effects on the water structure. The addition of salts to the aqueous PEG solution leads to an arrangement of ordered water molecules around the polymer molecule. This induces a decrease in the specific partial volume of the PEG molecule due to the loss of ordered water by the formation of a water layer around the cation. It also induces a more compact structure with a minor volume of the PEG molecule. As shown in Fig. 6-9, for constant concentration of guanidine hydrochloride, it is partitioned nearly equally to both phases at all pH and with increasing PEG/salt ratio, K_{GuHCl} decreased. The slope of the partitioning coefficient of guanidine vs. the PEG/phase forming salt (%w/w) ratio increased with increasing pH.

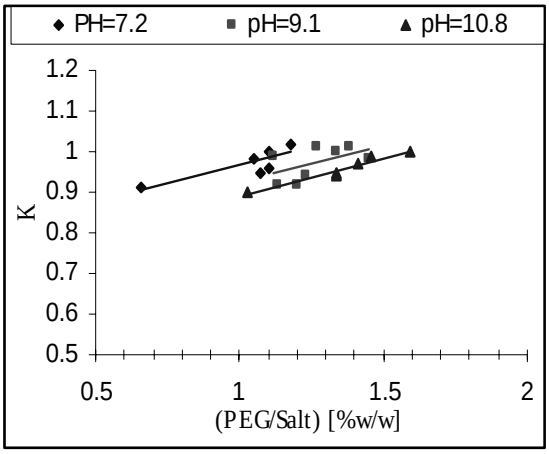

Figure 6. Effect of PEG/Salt (%w/w) ratio and pH on partition coefficient of guanidine hydrochloride at GuHCl =2.5 (%w/w)

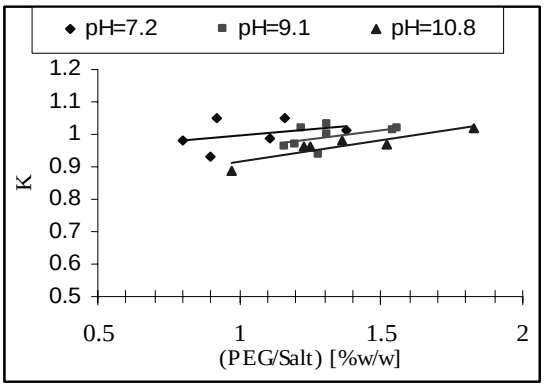

Figure 7. Effect of PEG/Salt (%w/w) ratio and pH on partition coefficient of guanidine hydrochloride at GuHCl =5 (%w/w)

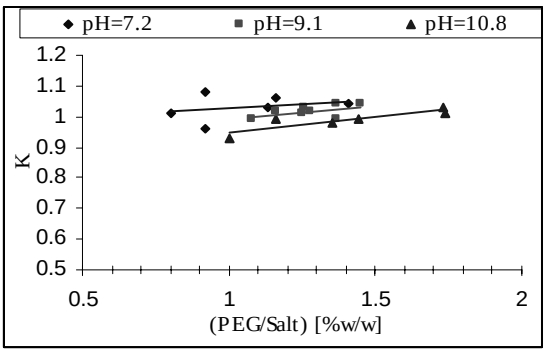

Figure 8. Effect of PEG/Salt (%w/w) ratio and pH on partition coefficient of guanidine hydrochloride at GuHCl =7 (%w/w)

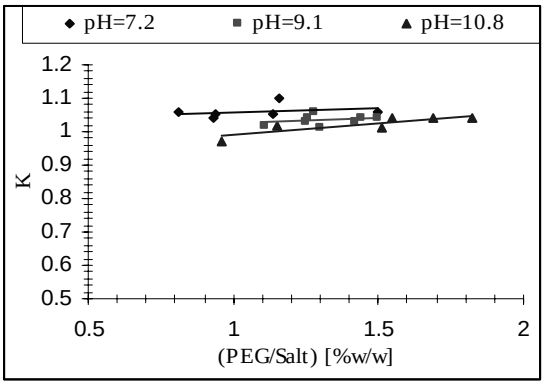

Figure 9. Effect of PEG/Salt (%w/w) ratio and pH on partition coefficient of guanidine hydrochloride at GuHCl =10 (%w/w)

4. Conclusions

To increase the knowledge based on an aqueous two-phase system containing co-solute which alters the water structure, we determined the partitioning coefficient of guanidine in different systems based on PEG–potassium phosphate in the presence of different guanidine hydrochloride concentrations. Guanidine hydrochloride has been described as a structure making solute because of its capacity to form a hydrogen bound with the water molecules. Our finding suggests a strong relation between the structure breaking co-solute capacity and their concentration in solution.

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