Chem. Eng. Comm., 194:828–834, 2007

ISSN: 0098-6445 print/1563-5201 online
DOI: 10.1080/00986440701193845

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Partition Coefficients of Some Antibiotics, Peptides and Amino Acids in Liquid-Liquid Partitioning of the Acetonitrile-Water System at Subzero Temperatures

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Liquid-liquid equilibrium (LLE) occurs at a subzero temperature for the acetonitrile-water binary system. When the equilibrium temperature is below its LLE critical temperature of $-1.32^{\circ}C$, the top phase is acetonitrile-rich and the bottom phase water-rich. It was observed that a human growth hormone analog with 191 amino acids partitioned exclusively into the bottom phase and its bioactivity was retained. This suggests that the liquid-liquid partition system can be used for bioseparations. The new system gives a different selectivity and the low temperature environment offers better stabilities for biomolecules compared to conventional solvent extraction systems. This work presented the partition coefficients of some biomolecules including trypsin, thyroglobulin, transferrin, ribonuclease A; L-phenylalanine, L-tryptophan; val-ala-ala-phe, phe-gly-gly-phe; erythromycin, chloramphenicol, vancomycin, spiramycin, gramicidin D, cycloheximide, fusidic acid, antimycin and tetracycline. Temperature and pH effects, as well as partition mechanisms were probed.

Keywords Acetonitrile; Amino acids; Antibiotics; Phase partition; Proteins

Introduction

In the increasingly competitive biotechnology industry, it is important to have efficient separation, recovery and purification of biological substances as the scale of production increases. Liquid-liquid partition and the subsequent separation of bioproducts can easily be operated continuously at steady state, which is beneficial both for large-scale production and for quality control (Ahuja, 2000). It can be a part of a multistage downstream process for product purification. Apart from the traditional solvent extraction systems, newer liquid-liquid partition systems such as aqueous two-phase systems offer better retention of bioactivities for larger biomolecules. To look for a different selectivity, Soto et al. (2005) used a room temperature ionic fluid with water to form two liquid phases for the separation of biomolecules. In addition to selecting different chemical compounds to form two phases, selectivity for separation can also be modulated by adjusting temperature and pH, or by adding ion pairs (Belter et al., 1988; Gu, 2000).

Acetonitrile (ACN)-water binary mixtures split into two liquid phases at equilibrium when the temperature is below $-1.32^{\circ}C$. The top phase is ACN-rich and

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the bottom phase is water-rich. An accidental experimental observation of an acetonitrile-water mobile phase fraction collected from a reversed phase chromatography run showed that the hGHG120R protein, a human growth hormone analog with 191 amino acids, partitioned into the water-rich bottom phase exclusively at $-17^{\circ}C$ without losing its bioactivity (Gu et al., 1994). This means that the liquid-liquid partition system is suitable for bioseparations.

Pence and Gu (1996) reported the liquid-liquid equilibrium data for the binary system and a thermodynamic model for the data. Compared with the traditional two-phase organic solvent-water systems (Johansson, 1985), two-phase aqueous polymer systems (Albertsson, 1971), two-phase aqueous surfactant systems (Liu et al., 1995) and two-phase extraction with reversed micelle systems (Pires, 1996), the ACN-water two-phase partitioning system at low temperatures offers a number of desirable features, such as increased stabilities for biomolecules at low temperatures, the presence of water in both phases and different partition behavior. So far, there has been no published literature showing partition coefficients for common groups of biomolecules in this system.

Materials and Experimental Methods

Deionized water came from an in-house reverse osmosis cartridge system. Acetonitrile (from Fisher Scientific, Pittsburgh, PA) was HPLC grade with a minimum purity of 99.9%. All buffers, acids and bases used to adjust pH values were purchased from Fisher Scientific. They were as follows: 0.1 M hydrochloric acid (Certified Grade), 0.1 M sodium hydroxide (Certified Grade), standard buffer solutions (pH 2.00, 4.00, 7.00, 10.00, all Certified Grade). The biomolecules for partition experiments were all from Sigma Chemicals (St. Louis, MO). They included trypsin, thyroglobulin, transferrin, ribonuclease A; L-phenylalanine, L-tryptophan; val-ala-ala-phe, phe-gly-gly-phe; erythromycin, chloramphenicol, vancomycin, spiramycin, gramicidin D, cycloheximide, fusidic acid, antimycin and tetracycline.

A Neslab (Portsmouth, NH) Model RTE-100LP refrigerated temperature bath ($\pm 0.1^{\circ}C$ accuracy) filled with 60 wt% ethylene glycol and 40 wt% water was used. Temperature was measured with an ASTM-62C ($0.1^{\circ}C$ subdivisions) thermometer from Fisher Scientific. All pH values were measured using a Corning 320 pH meter with a Model 476530 general-purpose pH electrode (both from Fisher Scientific).

Standard solutions with various concentrations for calibration in the UV detection of biomolecule concentrations were prepared. Concentrations varied from 0.1–4.0 mg/ml for different biomolecules that had different UV absorbance. For hydrophilic substances water solutions were prepared. They were then buffered to the desired pH with an appropriate buffer. 0.1 M NaOH and 0.1 M HCl were used to carry out minor adjustments of the pH to the desired point if the pH to be tested was different from the buffer pH. All pH measurements were performed prior to the addition of ACN due to the difficulty of pH measurements in the presence of an organic solvent. It was assumed that the pH did not vary significantly due to the presence of the buffer. The water solution was mixed with ACN at a volumetric water:ACN ratio of 1:1.5. For hydrophobic substances, solutions were prepared in ACN and then mixed with buffered water with the water:ACN ratio of 1:1.5. Test tubes containing liquid mixtures were put into the temperature bath and allowed to equilibrate overnight to form a two-phase system. This time period was found to be sufficient to achieve equilibrium because longer times didn't change the phase

compositions. After equilibrium, disposable glass pipettes were used to sample the top and bottom phases for composition analyses. The partition coefficient K was defined as the ratio of the biomolecule's molar concentration in the ACN-rich top phase (C_t) to that in the water-rich bottom phase (C_b), i.e., $K = C_t/C_b$. For the measurement of K, the concentration of a biomolecule in each coexisting liquid phase was determined by measuring UV absorbance at a wavelength that provided a peak absorbance value.

Results and Discussion

Table I shows the experimentally determined K values in the ACN-water system without adding a buffer. Most compounds in the list have $K < 1$, indicating that they prefer the water-rich bottom phase. Large molecules tend to stay exclusively in the bottom phase. Smaller molecules, such as amino acids and short-chain peptides tend to partition into both phases. It is also clear that some hydrophobic antibiotics prefer to stay mostly in the ACN-rich top phase.

The order for proteins favoring the bottom water-rich phase is thyroglobulin > transferrin > trypsin > ribonuclease A. This observed trend is consistent with the notion that interfacial energy between a biomolecule and the two-phase system plays the dominant role in determining the observed partitioning behavior. This is supported by the molecular weight order of thyroglobulin (669 k), transferrin (77 k), trypsin (15 k) and ribonuclease A (13.5 k). It is likely that the larger the molecule, the more hydrophilic sites it has, resulting in an increased preference for the water-rich bottom phase. In this work, bioactivity retention was not verified after phase partitioning.

Effect of pH

Figures 1 to 3 and Table II show the experimental K values at different pH values. For proteins, peptides and amino acids, the partitioning coefficients increase when pH values decrease. This might be due to the selective solvation properties of ACN-water mixture. It was reported that ACN prefers positively charged silver ion to negatively charged nitrate ion in a solution of silver nitrate solution (Coetzee

Table I. Partition coefficients of some biomolecules in the ACN-water two-phase system at $-10.0^{\circ}C$

Compound	K	Compound	K
Ribonuclease A	0.067	Vancomycin	0.039
Trypsin	0.046	Spiramycin	0.249
Transferrin	0.015	Tetracycline	0.248
Thyroglobulin	0.008	Erythromycin	2.217
Val-Ala-Ala-Phe	0.063	Cycloheximide	5.52
Phe-Gly-Gly-Phe	0.26	Chloramphenicol	6.248
Tryptophan	0.183	Fusidic Acid	7.146
Phenylalnine	0.131	Gramicidin D	25.098
		Antimycin	60.418

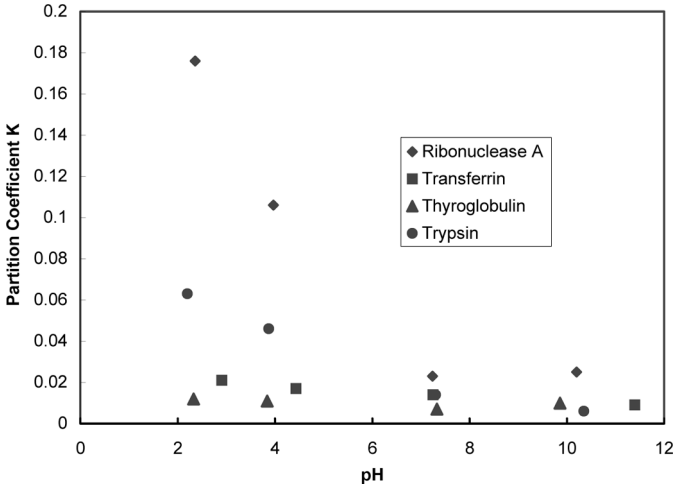

Figure 1. Partition coefficients of four proteins in the ACN-water system at −10.0°C.

and Ritchie, 1969). At lower pH values, the biomolecules tested are positively charged. This may explain the tendency for their favoring the ACN-rich top phase.

The partition behavior of antibiotics seems to follow the rule of extractive separation of acids and bases (Robinson and Cha, 1985). Weak bases favor the ACN-rich top phase at high pH values, while weak acids favor the top phase at low pH values.

Shapes of Equilibrium Curves and Their Implications

The equilibrium isotherm of erythromycin is presented in Figure 4. Concentrations are expressed in UV absorbance at 288 nm. Within the tested range, its absorbance

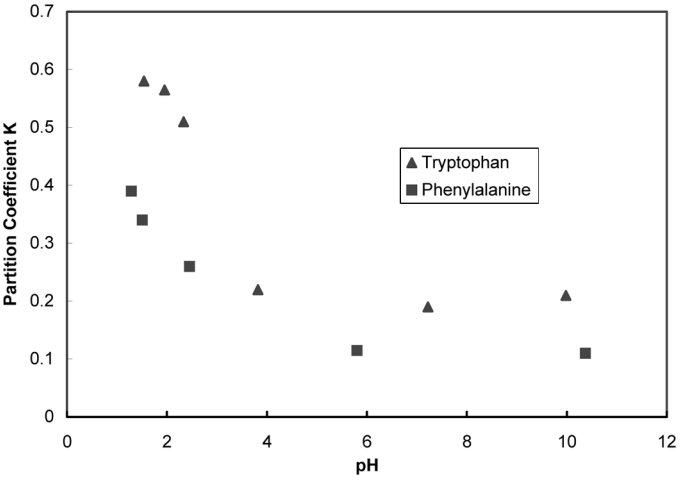

Figure 2. Partition coefficients of two amino acids in the ACN-water system at −10.0°C.

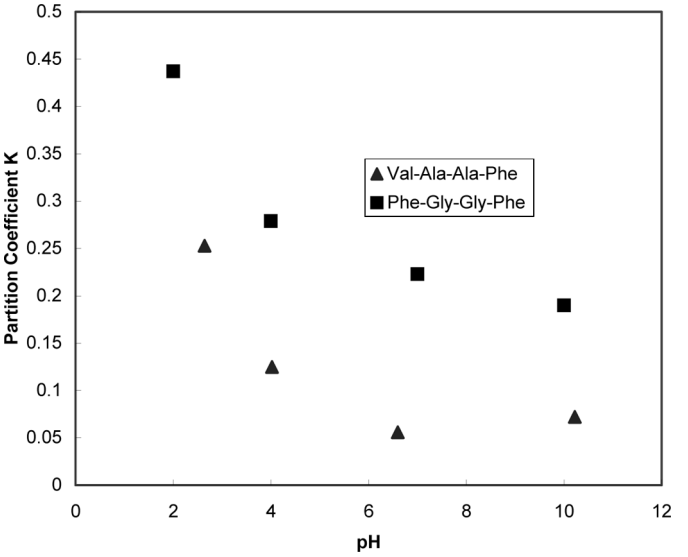

Figure 3. Partition coefficients of two peptides in the ACN-water system at -10.0°C.

vs. concentration is linear. According to Blumberg (1988), the shape of an equilibrium curve gives a practical presentation of the distribution behavior of the solute as a function of its concentration. Different shapes of the isotherms indicate different partition mechanisms. The linear relationship in Figure 4 indicates that the mode of transfer is simply a function of relative solubility of the solute in the competing

Table II. Partition coefficients of antibiotics in the ACN-Water system at -10.0°C

Compound	pH	K	Compound	pH	K
Spiramycin	4	0.193	Chloramphenicol	4	5.886
	no buffer	0.249		no buffer	6.248
	10	1.854		10	4.485
Vancomycin	4	0.012	Antimycin	4	70.09
	no buffer	0.039		no buffer	60.42
	10	0.024		10	15.05
Cycloheximide	4	5.511	Gramicidin D	4	49.62
	no buffer	5.52		no buffer	25.1
	10	2.668		10	23.07
Erythromycin	4	0.279	Fusidic Acid	4	12.29
	no buffer	2.217		no buffer	7.15
	10	2.888		10	0.72
Tetracycline	4	0.316			
	no buffer	0.214			
	10	0.127			

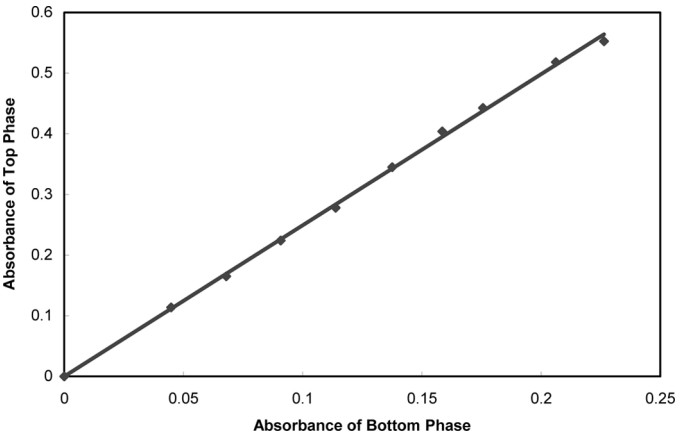

Figure 4. Equilibrium isotherm of erythromycin in the ACN-water system at $-10.0^{\circ}C$.

solvent ACN, without strong interactions for erythromycin. Similar results were obtained for phenylalanine.

Effect of Temperature

Figure 5 shows the experimental data of partition coefficient K values for tryptophan and phenylalanine as a function of temperature between $-2.8^{\circ}C$ to $-14.5^{\circ}C$. As the temperature decreases further below the critical temperature $T_c = -1.32^{\circ}C$, K decreases and deviates further from unity for both species. This is because the difference in ACN concentrations for the two coexisting phases increases as the temperature moves away from the critical temperature at which the two liquid phases have the same ACN concentration in water (Pence and Gu, 1996).

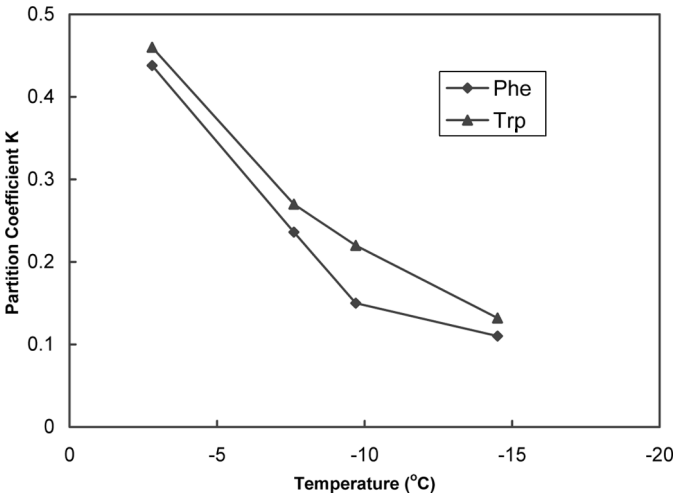

Figure 5. Partition coefficients of phenylalanine and tryptophan at different temperatures.

Conclusions

Partition coefficients of the tested biomolecules indicate that the ACN-water system can be used for bioseparations due to K values that deviate from unity. pH affects the partition behavior of proteins, peptides and amino acids as a protonization force. Antibiotics tested in this work behave as common weak bases or weak acids with the change of pH in the ACN-water two-phase system. For large molecules such as proteins, interfacial energy seems to be the dominant factor that determines the partition behavior. For small molecules, such as short-chain peptides, amino acids and antibiotics, the mode of transfer is a function of relative solubility of the solute in ACN.

References

Ahuja, S. ed. (2000). *Handbook of Bioseparations*, Academic Press, New York.

Albertsson, P. A. (1971). *Partition of Cell Particles and Macromolecules*, 2nd ed., 58–73, Wiley-Interscience, New York.

Belter, P. A., Cussler, E. L., Hu, W. S. (1988). *Bioseparations: Downstream Processing for Biotechnology*, 103–109, Wiley-Interscience, New York.

Blumberg, R. (1988). *Liquid-Liquid Extraction*, 2–6, Academic Press, New York.

Coetzee, J. F. and Ritchie, C. D. (1969). *Solute-Solvent Interactions*, 301–302, Marcel Dekker, New York.

Gu, T. (2000). Liquid-liquid partitioning methods for bioseparations, in: *Handbook of Bioseparations*, ed. S. Ahuja, Vol. 2, 329–364, Academic Press, New York.

Gu, T., Gu, Y., Zheng, Y., Wiehl, P. E., and Kopchick, J. J. (1994). Phase separation of acetonitrile-water mixture in protein purification, *Sep. Technol.*, **4**, 258–260.

Johansson, G. (1985). Partitioning of proteins, in: *Partitioning in Aqueous Two-Phase Systems – Theory, Methods, Uses, and Applications to Biotechnology*, eds. H. Walter, D. E. Brooks, and D. Fisher, 161–227, Academic Press, New York.

Liu, C. L., Nikas, Y. J., and Blankschtein, D. (1995). Partitioning of proteins using two-phase aqueous surfactant systems, *AIChE J.*, **41**, 991–995.

Pence, D. N. and Gu, T. (1996). Liquid-liquid equilibrium in the acetonitrile-water system, *Sep. Technol.*, **6**, 261–264.

Pires, M. J., Aires-Barros, M. R., and Cabral, J. M. S. (1996). Liquid-liquid extraction of proteins with reversed micelles, *Biotechnol. Prog.*, **12**, 290–301.

Robinson, R. G. and Cha, D. Y. (1985). Controlled pH extraction in the separation of weak acids and bases. Increasing extraction selectivity in multistage extractions, *Biotechnol. Prog.*, **1**, 18–25.

Soto, A., Arce, A., and Khoshkbarchi, M. K. (2005). Partitioning of antibiotics in a two-liquid phase system formed by water and a room temperature ionic liquid, *Sep. Purif. Technol.*, **44**, 242–246.