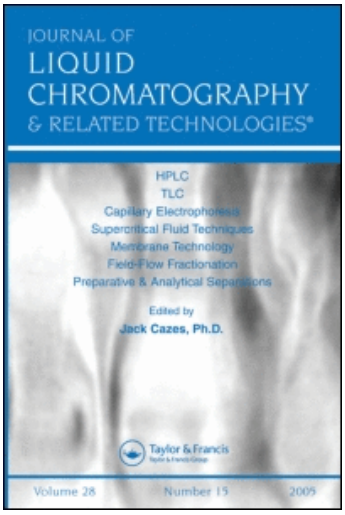

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

The Influence of Urea on the Retention of Protein in Hydrophobic Interaction Chromatography

W. Feng[ab]; X. Jiang[a]; X. Geng[a]
[a] Institute of Modern Separation Science Northwest University, Xi'an, The People's Republic of China [b] Institute of Analytical Chemistry, University of Vienna, Vienna, Austria

To cite this Article Feng, W. , Jiang, X. and Geng, X.(1995) 'The Influence of Urea on the Retention of Protein in Hydrophobic Interaction Chromatography', Journal of Liquid Chromatography & Related Technologies, 18: 2, 217 — 226
To link to this Article: DOI: 10.1080/10826079508009233
URL: http://dx.doi.org/10.1080/10826079508009233

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(2), 217-226 (1995)

THE INFLUENCE OF UREA ON THE RETENTION OF PROTEIN IN HYDROPHOBIC INTERACTION CHROMATOGRAPHY

WENKE FENG*, XINQI JIANG, AND XINDU GENG
Institute of Modern Separation Science
Northwest University
Xi'an 710069, The People's Republic of China

ABSTRACT

The retention behaviour of eight proteins in high performance hydrophobic interaction chromatography (HPHIC) in a mobile phase consisting of ammonium sulphate-potassium dihydrogen phosphate containing urea was studied. By using the stoichiometric displacemant model for retention (SDM-R) of solute in liquid chromatography, and by measuring surface tention of the mobile phase in the presence of urea, also and UV-spectra of proteins, it was found that in the presence of urea in the mobile phase of HIC, even though the urea somewhat strong solvent in terms of result, the change of the conformation of protein molecular in hydrophobic interaction systems due to the presence of urea is considered as the major contribution to the retention of proteins.

* Present address: Institute of Analytical Chemistry, University of Vienna, Waehringer Str. 38, A-1090, Vienna, Austria

INTRODUCTION

Urea and guanidine hydrochloride are solubilizing and denaturing agents for proteis, They are often used for the extraction of proteins from bacterium body [1] For instance,7.0mol/L guanidine hydrochloride or 8.0mol/L urea solution is used for the extraction of very strongly hydrophobic proteins but which causes denaturation. Therefore refolding and purification are needed in order to obtain bioactive and pure proteins. It has been demmostrated that this could be renatured by High performance hydrophobic interaction chromatography[2,3]. Because of the small injection volume, the urea or guanidine hydrochloride in the sample has a negligible influence on the retention of proteins. The addition of urea or guanidine hydrochloride to the eluent to increace the solubility of the proteins has a significant influence on the retention characteristic of proteins. In our previous works[4,5] we found that urea in a concentration of 3.75mol/L influenced the retention behaviour of proteins in HIC significantly. Because the concentration of urea is constant during the separation process, the molecular conformation of the protein didn't change, When the concentration of urea is not certain, but changeable during separation, for example, in the preparative chromatography large volume of sample is necessory, how does the denaturing agent influence the retention behaviour of protein? Many studies on the retention mechanism of protein in hydrophobic chromatography[6,7] did not include molecular conformation changes during separation into their theory. In a previous paper[8] we have investigated the influence of guanidine hydrochloride on retention. In this continuation paper reports the results of the influence of the concentration of urea in the mobile phase on the retention of proteins under the aspect of the change of the molecular conformation.

EXPERIMENTAL

Instrumentation

The chromatographic system consisted of three isocratic solvent pumps (Model LC-6A, Shimadzu, kyoto, Japan) with a system controller (Model SCL-6B,Shimadzu). Detection was performed by a UV-VIS detector (Model SPD-6AV, Shimadzu, Kyoto, Japan). Silica (7μm, 500Å) was modified to having ether end-group and packed into a 40mm ×2mm stainless-steel column by means of a column packing apparatus (Model 1224, Chemico, Osaka, Japan). The UV-spectra of protein solution were recorded by a Spectrophotometer (Perkin-Elmen, Norwalk, Connecticut, USA).

Chemicals

Cytochrome C (Cyt-C), myoglobin (MYO), ribonuclease (RNase), bovine serum albumin (BSA), concanavalin A(Con A),Ferritin (FER), a-chymotrypsinogen A (a-CTY-A) were obtained from Sigma (St. Louis, MO, USA), Lysozyme (LYS) from Dongfeng Biochemical Factory (Shanghai, China). Other chemicals were purchaesd from Xi'an Chemical Co. (Xi'an, China) and were analytical grade.

Eluent Composition

The mobile phases were prepared with three pumps using following solutions: solution A, 3mol/L ammonium sulphate-20mmol/L potassium dihydrogen phosphate, pH 7.0; solution B, 8mol/L urea-20mmol/L potassium dihydrogen phosphate, pH 7.0; solution C, 20mmol/L potassium dihydrogen phosphate, pH 7.0.

Operation

By mixing of the three solutions various mobile phases with different concentration of urea were obtained. The mobile phase was filtrated and sonicated (5min.) before using. The column was equilibrited with at least 5 ml new mobile phase before injecting a sample solution. The flow-rate was set up at 0.2ml/min and eluent was monitored at 280nm.

The dead volume of chromatographic system was deteremined with solution C. The concentration of water in mobile phase was measured according to the following equation[6]

$$[H_2O] = \frac{d_A\varphi_A + d_B\varphi_B + d_C\varphi_C - W_S}{0.018}(mol/L)$$

where d_A. d_B and d_C are the densities of solution of A, B, and C, respectively, φ_A, φ_B and φ_C are the volume fractions (V/V) of solutions A, B and C in the mobile phase, respectively, W_S is the amount of salt in the mobile phase. The surface tention of mobile phase was determined according to literature[9].

RESULTS AND DISCUSSION

Generally, in order to purify a certain protein which has a strong hydrophobicity from inclussion body or other origins, firstly make it denatured with 8.0mol/L urea, or 7.0mol/L guanidine hydrochloride, then make it refolded and purified. HIC has been considered to be not only a separation method but also a tool for protein refolding[2]. In the condition of the gradient elution model in HIC, the weak eluent is an aqueous solution of 2.5-3.0mol/L sodium sulphate or ammonium sulphate and so on. The addition of 8.0mol/L urea would cause the precipation of proteins and/or salts. According to the different proteins we used

urea of a high concentration as possible, but without causing any precipatation in chromatographic systems.

The retention values of BSA for various concentration of urea in HIC are presented in Fig.1. It shows that the retention of BSA drop with increacing concentrations of urea. The same results were obtained at other proteins (Table 1). The results suggest that the elution strength of urea in HIC is comparable the water.

Influence of the Surface Tention on the Retention of Proteins

It would be helpful that we measure the relationship between k′ value and surface tention of the mobile phase of ammonium sulphate-potassium dihydrogen phosphate containing urea of different concentrations. As shown in Fig.2, it was found that the surface tention increased with the concentration of urea.This is similar to the system of guanidine hydrochloride[8].At low concentrations of urea. the surface tention of eluent increased more quickly than at high concentrations, Horvath found that[10] the retention of proteins increases proportionally to the surface tention of mobile phase. Our results are in contradiction to the one of Horvath. He and co-workes , which used only non-denaturating salts in their eluent, found that the retention of proteins increases proportionally to the surface tention of the mobile phase. The addition of the denaturating urea to the eluent changes the retention behaviour of proteins. A nonlinear drop of the retention with increacing concentrations of urea was observed.

The results above show that the urea existed in the mobile phase of HIC don't cause only the change of surface tention of the system similar to water.

Relationship between [H_2 O] in Mobile Phase and k′

One of the authors[6,11] pointed out that the most rational retention mechanism of proteins in hydrophobic interaction chromatography and in reversed-phase

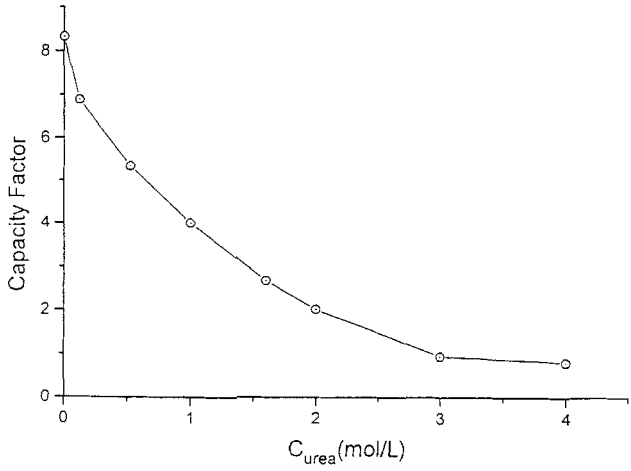

Fig. 1. Dependence of the retention of BSA on the concentration of urea. Condition: see experimental except eluent: 0.60mol/L $(NH_4)_2SO_4$ - 20mmol/L KH_2PO_4 -Urea.

TABLE 1 Capacity Factores of Eight Proteins in Different C_{urea}

C_{urea}	Cyt-t	MYO	RNase	LYS	BSA	Con.A	FER	α-CTY-A
0.00	10.67	14.00	11.33	28.93	8.33	11.67		
0.10				22.33				
0.12	10.33	13.33	11.33		6.87	8.00		
0.50				14.67				
0.52	8.33	9.33	11.33		5.33	5.33		35.67
1.0	6.00	6.80	9.67	8.33	4.00	3.33	6.00	17.33
1.5				4.67				
1.6	4.00	4.00	5.67		2.67	2.00	2.33	9.27
2.0	3.34	3.67	4.00	2.84	2.00	1.67	1.33	6.67
3.0	2.00	2.67	2.00	1.17	0.93	0.80	0.80	3.33
4.0	1.80	1.20	0.87	0.73	0.80	0.73	0.73	1.33
5.0	1.00	0.87	0.80					0.80
6.0	0.93	0.80						

Composition of the mobile phase: 20mol/L KH_2PO_4 -$(NH_4)_2SO_4$ 4-Urea. The used $C_{(NH_4)_2SO_4}$ for Cyt-C, Myo, RNase, Lys, BSA, Con A, Fer, α-CTY-A were separately 1.80, 0.75, 1.56, 1.05, 0.60, 1.12, 0.45, 0.75 mol/L

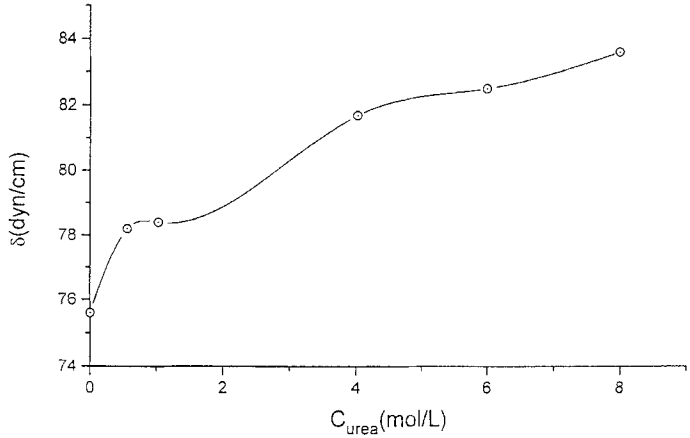

Fig. 2 The effect of urea on the surface tention.
Condition: aqueous solution of 1.40mol/L $(NH_4)_2 SO_4$ -20mmol/L $KH_2 PO_4$ -Urea. Others see experimental.

chromatography might be a stoichiometric displacement process between protein and solvent molecules, with water as displacing agent. Hence the plot of the logarithm of capacity factor of proteins *versus* the logarithm of concentration of water in the mobile phase should be linear. This was conformed for high concentration of water in the eluent. At low concentration a nonlinear relationship was found (Fig.3). This can be explainted by the fact that the concentration of urea is low when the concentration of water is high in the mobile phase[6]. The parameters of the plots of logk'-log[H_2 O] for the eight proteins are shown in Table 2.

It is important that retention increaces with the increasing concentration of water in the eluent. This result is contradictory to the system of ammonium sulphate-water. In other words, when the eluent contains urea, water couldn't be seen as the displacing agent in the system of HIC. But from the fact that the k' value of the proteins decrease with increacing concentrations of urea in high

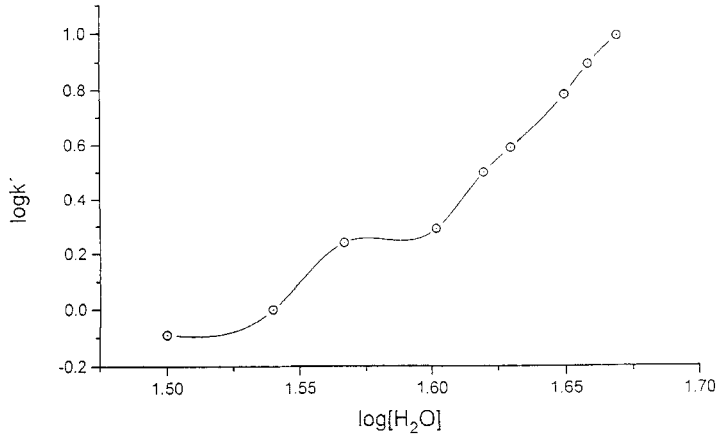

Fig. 3 Plot of logk′ of Cyt-C against log [H_2 O] in the mobile phase condition: see experimental except eluent: 0.40mol/L $(NH_4)_2 SO_4$ - 20mmol/L $KH_2 PO_4$ -Urea.

TABLE 2 The Parameters of the Plot of logk′-log[H_2 O]*

Protens	Cyt-c	MYO	RNase	LYS	BSA	Con. A	FER	α-CTY-A
C	0.9659	0.9685	0.9839	0.9847	0.9811	0.9644	0.9084	0.9803
Z	6.35	7.44	10.6	16.7	10.1	10.7	11.3	14.4
LogI	-9.71	-11.7	-16.8	-27.1	-16.3	-17.5	-18.5	-23.1

* The meanings of parameters C, Z, logI see ref.[6]

concentration, urea seems to be the displacing agent in HIC. However, when we ploted the logk′-logC_{urea} according to the stoichiometric displacement concept, the curve is not straight too. This means disagreement with the view of considering urea to be the displacing agent in HIC system.

The Change of Molecular Conformation of Proteins

It is well known that proteins change the three-dimesional structure in 8.0mol/L urea. But when the concentration of urea is relative low (0-6.0mol/L),

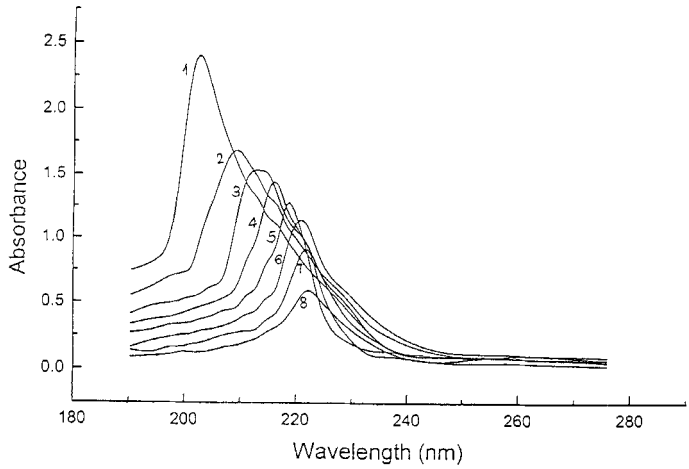

Fig. 4 UV-spectra of BSA in the solution with different C_{urea}.
1, 2, 3, 4, 5, 6, 7, 8 denote the different C_{urea}: 0.00, 0.52, 1.0, 2.0, 3.0, 4.0, 6.0, 8.0, respectively.

the question arises , if the molecular conformation of proteins really changes.

UV spectrophotometry is one of the most important tools for investigating the changes of the molecular conformation and refolding of proteins[1]. Aqueous protein solutions with and without urea were prepared, and the UV-spectra were recorded. The spectra were corrected by the blank solution without urea and proteins. They were compared with spectra of the undenatured proteins. Fig. 4 shows the UV-spectra of BSA in a solution consisting 0.62mol/L ammonium sulphate and different concentration of urea. It can be seen that the absorbance density of BSA is getting lower when the concentration of urea increaces. At the same time a shift of the maximum absorption towards longer wavelength is obeseved. From the results it can be concluded that besides solvophobic interaction between urea and protein others take place. Because of the change of molecular conformation of proteins in aqueous solution of urea, the stoichiometric

displacement model can not be used for the explanation of the retention behaviour of proteins in HIC.

REFERENCES

1. C. Ghelis and J. Yon, Protein Folding, Academic press, New York, 1982.
2. X. -D. Geng and X. Chang, J. Chromatogr., 599: 185-194(1992)
3. J. L. Fausnaugh, L. A. Kennedy and F. E. Regnier, J. Chromatogr., 317: 141-155 (1984)
4. J. Chang L. Guo, W. Feng, X.-D. Geng, Chromatographia, 34: 589-596 (1992)
5. L. Bian, W. Feng X. -D. Geng, Sepu, 9: 267-271 (1991)
6. X. -D. Geng, L Guo, J Chang, J. Chromatogr., 507: 1-23 (1990)
7. W. R.Melander, D. Corradini and Cs. Horvath, J. Chromatogr.,317:67-85 (1984)
8. W. Feng, X. Jiang, X. -D. Geng, J. Chem. of Chinese University, in press
9. Fudan University, Experiment of pysical Chemistry (A), High Education publisher, Beijing, 1986
10. Cs. Horvath, W. Melander, I. Melner, J. Chromatogr., 125: 129-146(1976)
11. X. -D. Geng and F. E. Regnier, J. Chromatogr., 296: 15-30 (1984)

Received: July 11, 1994
Accepted: July 26, 1994