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ORGANIC-HIGH IONIC STRENGTH AQUEOUS SOLVENT SYSTEMS FOR SPIRAL COUNTER-CURRENT CHROMATOGRAPHY: GRAPHIC OPTIMIZATION OF PARTITION COEFFICIENT

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Abstract

A new series of organic-high ionic strength aqueous two-phase solvents systems was designed for separation of highly polar compounds by spiral high-speed counter-current chromatography. A total of 21 solvent systems composed of 1-butanol-ethanol-saturated ammonium sulfate-water at various volume ratios are arranged according to an increasing order of polarity. Selection of the two-phase solvent system for a single compound or a multiple sample mixture can be achieved by two steps of partition coefficient measurements using a graphic method. The capability of the method is demonstrated by optimization of partition coefficient for seven highly polar samples including tartrazine (K=0.77), tryptophan (K=1.00), methyl green (K= 0.93), tyrosine (0.81), metanephrine (K=0.89), tyramine (K=0.98), and normetanephrine (K=0.96). Three sulfonic acid components in D&C Green No. 8 were successfully separated by HSCCC using the graphic selection of the two-phase solvent system.

Keywords

Spiral high-speed counter-current chromatography; Organic-high ionic strength aqueous solvent system series; Graphic optimization of partition coefficient; Sulfonic acids; Catecholamines; Zwitter ions

INTRODUCTION

High-speed counter-current chromatography (HSCCC) [1–6] requires tedious work for selecting the suitable two-phase solvent systems by measuring the partition coefficients (K) of target compounds. It often consumes 90% of researchers' work force only for optimizing

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the K values [7]. In order to solve this problem, methods of systematical search for the suitable solvent systems have been devised for separation of moderately polar compounds. These solvent series include hexane-ethyl acetate--methanol or ethanol-water system [8, 9], and methyl *tert.*-butyl ether-acetonitrile-1-butanol-water system each at various volume ratios, the latter of which is conveniently used for pH-zone-refining counter-current chromatography [10]. However, there are many biologically active polar compounds which are still not easily separated by the conventional HSCCC. Recently, we have introduced a series of polar organic-aqueous two-phase solvent systems composed of i-butanol-ethanol-saturated ammonium sulfate-water at various volume ratios which can be applied for separation of highly polar compounds using spiral column counter-current chromatography [11]. This new solvent system series consisted of 10 steps with a gradual change of polarity so that the search can be performed by following the series up or down until the suitable K values for the target compounds are obtained. However, when the log K values of 4 polar samples were plotted against the solvent system numbers, it was found that each curve formed a near straight line. This trend suggested that the K values of each component in all solvent systems could be determined simply by plotting the K values of two solvent systems on a graph paper.

The present paper introduces a new series of polar solvent systems consisting of 21 steps from the same set of solvents. In this paper we devised a simple graphic method for selecting a two-phase solvent system which gives suitable K values for a single target compound or a mixture of multiple compounds. Using this graphic method an ideal two-phase solvent system can be found by two steps of K measurements. The utility of the system is demonstrated by several test samples with high polarity including amino acids, catecholamines, sulfonic acids, etc.

EXPERIMENTAL

Apparatus

The HSCCC instrument used in the present studies is a type-J coil planet centrifuge (Ito Multi-Layer Coil Separator-Extractor, P.C. Inc, Potomac, MD, USA). It holds a separation column and a counter-weight symmetrically at a distance of 10 cm from the central axis of the apparatus. The spiral tube support for holding the multi-layer spiral column was purchased from CCBiotech, Rockville, MD, USA. The spiral column was prepared in our laboratory inserting a single piece of PTFE (polytetrafluoro-ethylene) tubing (Zeus industrial Products, Orangeburg, SC, USA) after extruding it through a narrow slit followed by twisting along its axis (flat-twisted spiral column) [12]. The column consisted about 50 m of 1.6 mm ID PTFE tubing with a total capacity of 70 ml. It has 10 layers of spiral tubing each containing 4 interwoven spiral tubing connected in series. The revolution speed of the apparatus can be regulated up to 1200 rpm with a speed controller, but the present experiments were performed at 800 rpm. The solvent was pumped with a HPLC pump (Model: LC-10ADvp, Shimadzu Corp., Kyoto, Japan) and the effluent was monitored with a UV detector (Model: Uvicord SII, LKB Instruments, Bromma, Sweden). The chromatogram was recorded with a strip-chart recorder (Model: Pharmacia LKB-Rec 102, Sweden). The partition coefficient values of test samples were determined with a spectrophotometer (Model: Genesys 10 UV, Thermo Spectronic, Rochester, NY, USA).

Reagents

Absolute ethanol of analytical grade was purchased from Warner Graham Co., Cockeysville, MD, USA, 1-butanol of HPLC grade from Fisher Scientific Co., Fair Lawn, NJ, USA, and ammonium sulfate of analytical grade from Mallinckrodt Chemicals, Phillipsburg, NJ, USA. Test samples including methyl green (Matheson Coleman and Bell, Cincinnati, OH, USA),

L-tyrosine (United States Biochemical Corp., Cleveland, OH, USA), DL-tryptophan (Janssen Chimica, Geel, Belgium), tyramine hydrochloride (Aldrich, Milwaukee, WI, USA), DL-normetanephrine (Sigma, St. Louis, MO, USA), DL-metanephrine (Sigma) were purchased from the companies given in the parentheses. The sulfonated dyes, D&C Yellow No. 5 (tartrazine) and D&C Green No. 8 (pyranine), were research samples provided by Dr. A. Weisz of U.S. Food and Drug Administration, College Park, MD. The samples of 1,3,6-pyrenetrisulfonate (P3S) and 8-hydroxy-1,3,6-pyrenetrisulfonate (HP3S) were prepared previously [13], and the sample of 1,3,6,8-pyrenetetrasulfonate (~98%, monohydrate, P4S) was obtained from Sigma-Aldrich, St. Louis, MO, USA.

Preparation of Two-Phase Solvent Systems

First, ammonium sulfate saturated solution was prepared as follows: To a large glass bottle containing water placed on a magnetic mixer, ammonium sulfate was added until the solution became cloudy. The mixing was continued for several hours until the solution returned to room temperature. The bottle was kept for overnight to yield clear supernatant which was used for the preparation of two-phase solvent systems as described below. This solution contains ca. 4 M of ammonium sulfate with density at 1.260 g/cm^3.

In the present study a set of 21 two-phase solvent systems from systems 0 to 20 were prepared as listed in Table 1. Each solvent system was made by mixing 1-butanol, ethanol, saturated ammonium sulfate solution and water at the indicated volume ratios each in a 50 ml capacity container. The contents were well mixed to equilibrate the two phases. After preparation, the physical properties of the solvent system such as a volume ratio of the two phases (upper/lower), density difference between the two phases and the settling time were determined. The settling time of the two phases was measured according to the standard technique [14] as follows: 2 ml of each phase (total 4 ml) of the equilibrated solvent system was delivered into a test tube (13 × 100 mm). After capping the test tube, the contents were vigorously shaken for 5 times and left vertically on the test tube rack to measure the time required to form clear two layers. The test was repeated 3 times to get the average value.

Measurement of Partition Coefficient (K) of Test Samples

Partition coefficient values of the test samples in the selected two-phase solvent systems were determined using a spectrophotometer. In each measurement a small amount of the sample was added to 4 ml of two-phase solvent system (2 ml each phase) in a test tube. After capping the test tube, the contents were vigorously stirred with a vortex mixer until the sample was completely dissolved and partitioned into the two phases. After two phases were separated, an aliquot (usually 100 μl) of each phase was separately mixed with 2 ml of solvent (water for the lower phase and 50% ethanol for the upper phase) to measure the absorbance at a suitable wavelength using a spectrophotometer. The K values are expressed by the absorbance value of the upper phase divided by that of the lower phase.

HSCCC Separation of Test Samples

Separation of sulfonic dyes was performed with an optimized two-phase solvent system composed of 1-butanol-ethanol-saturated ammonium sulfate-water at a volume ratio of 1.2:0.8:1:1 (System 8). The experiment was initiated by filling the column entirely with the upper organic phase followed by sample injection. Then the column was rotated at 800 rpm while the lower aqueous mobile phase was pumped into the internal head terminal of the spiral column at a flow rate of 0.5 ml/min. The effluent from the outlet of the column was continuously monitored with a UV detector at 280 nm and fractionated into test tubes at 4 minute intervals. After all peaks were eluted, the column contents were pushed out by pressured air into a graduated cylinder to measure the volume of the stationary phase retained in the column.

RESULTS AND DISCUSSION

Organic-High Ionic Strength Aqueous Two-Phase Solvent System Series

Table 1 illustrates compositions of a set of 21 two-phase solvent systems which are arranged from the top to the bottom in an order of increasing polarity. Important physical properties of the solvent system such as a volume ratio between the two phases, settling time, and density difference between the two phases are also presented. All systems show acceptable volume ratios between the two phases. The settling times of all these systems are longer that 20 seconds suggesting that the conventional multilayer coil may not be efficiently used for separation. However, the spiral tube configuration used in the present studies can retain a satisfactory volume of the stationary phase at over 50% by utilizing the centrifugal force gradients acting along the spiral channel. A large difference in density between the two phases due to a high concentration of ammonium sulfate in the lower phase also enhances the retention of the stationary phase in the spiral column. A set of these polar solvent systems can be used to determine the suitable two-phase solvent systems for a single compound or a multiple sample mixture by only two steps of K measurements using a simple graphic analysis as described in the following sections. Although lower phase fraction contains a large amount of ammonium sulfate, it is largely removed by adding methanol followed by centrifugation.

Graphic Selection of Two-Phase Solvent System for a Single Compound

A suitable K value for separation of a single target compound is near unity. In order to achieve this goal, one may often spend an enormous amount of time to optimize the solvent system. However, the ideal two-phase solvent system can be easily found from the solvent series listed in Table 1 by two-step K measurements followed by a simple graphic analysis as follows: For each sample prepare three two-phase solvent systems, Systems 0, 10 and 20 in Table 1. Then, measure the partition coefficient of the sample in System 10 to get K_{10}. If the sample is a pure standard as in the present study, K value may be determined with a spectrophotometer at a suitable wavelength. If the sample is a crude mixture, the standard HPLC method should be used to get the K value of the target compound. Then if K_{10} is greater than 1, the second K measurement is performed in System 0 to get $K_{0.}$ If K_{10} is above 1, the second measurement is performed in System 20 to get K_{20}. Then, these two Log K values are plotted in the graph as shown in Figure 1. Then, these two points were connected with a straight line to get the crossing point to the Log K = 0 line which indicates the ideal solvent system for the separation of this compound. Several compounds including tartrazine, methyl green, tryptophan, and catecholamines have been tested, the results of which are shown in Table 2. It should be noted that all these compounds are not efficiently separated by the standard HSCCC method with existing two-phase solvent systems due to their low K values.

Graphic Selection of Two-Phase Solvent System for Multiple Target Compounds

The present method can also be applied to the selection of the two-phase solvent system for separation of multiple compounds as follows: The sample mixture is first distributed in System 10. If the averaged K values are greater than 1, the second measurement is performed in System 0. But if the averaged K value is smaller than 1, the second measurement is performed in System 20. In either case, a pair of log K values for each target compound is plotted on the graph and connected with a straight line. In Fig. 2, a set of three sulfonic acids, P3S, HP3S and P4S were each plotted according to this procedure. From this graph the separation of these three compounds is easily visualized by distribution of three lines at each solvent system. In this case System 8 or 9 will provide satisfactory peak resolution between three compounds. Fig. 3 shows the separation of D&C Green No. 8 which contains three compounds at a flow rate of 1 ml/min at 800 rpm.

CONCLUSIONS

In order to expand the utility of HSCCC, a novel series of organic-high ionic strength aqueous two-solvent systems have been designed. It consists of 1-butanol-ethanol-saturated ammonium sulfate-water at various volume ratios which are arranged according to the order of increasing polarity. Using a graphic analysis, the suitable two-phase solvent system for a single component or multiple sample mixture is determined by 2-step partition coefficient measurement. The capability of the method is demonstrated by separation of sulfonic dyes.

Acknowledgments

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References

1. Ito, Y. Countercurrent Chromatography, Theory and Practice. Mandava, NB.; Ito, Y., editors. Marcel Dekker; New York: 1988. p. 79-442.
2. Conway, WD. Countercurrent Chromatography, Apparatus and Applications. VCH; New York: 1990.
3. Ito, Y.; Conway, WD., editors. High-Speed Countercurrent Chromatography. Wiley-Interscience; New York: 1996. p. 1-44.
4. Menet, JM.; Thiébaut, D., editors. Countercurrent Chromatography. Vol. 3. Marcel Dekker; New York: 1999. p. 87-119.
5. Berthod, A. Countercurrent Chromatography: The support-free liquid stationary phase. Elsevier Science; B.V. Amsterdam, The Netherlands: 2002.
6. Ito Y. High-Speed Countercurrent Chromatography. CRC Crit Rev Anal Chem. 1986; 17:65.
7. Ito Y. Golden Rules and Pitfalls in Selecting Optimum Conditions for High-Speed Counter-Current Chromatography. J Chromatogr A. 2005; 1065:145–168. [PubMed: 15782961]
8. Oka F, Oka H, Ito Y. J Chromatogr A. 1991; 538:99–108.
9. Friesen B, Pauli GGESS. A Generally Useful Estimate of Solvent Systems for CCC. J Liq Chrom & Rel Technol. 2005; 28:2777–2806.
10. Ito Y, Ma Y. pH-Zone-Refining Countercurrent Chromatography. J Chromatogr A. 1997; 753:1–36. [PubMed: 8962503]
11. Zeng Y, Liu G, Ma Y, Chen X-Y, Ito Y. Organic-High Ionic Strength Aqueous Two-Phase Solvent System Series for Separation of Ultra-Polar Compounds by Spiral Counter-Current Chromatography. J Chromatogr A. in press.
12. Yang Y, Aisa HA, Ito Y. Flat-Twisted Tubing: Novel Column Design for Spiral High-Speed Counter-Current Chromatography. J Chromatogr A. 2009; 1216:5265–5271. [PubMed: 19486987]
13. Weisz A, Mazzola EP, Ito Y. Preparative Separation of 1,3,6-Pyrenetrisulfonic Acid Trisodium Salt from the Color Additive D&C Green No. 8 by Affinity-Ligand pH-Zone- Refining Counter-Current Chromatography. J Chromatogr A. 2011; 1218:8249–8254. [PubMed: 21982993]
14. Ito Y, Conway WD. Experimental Observations of the Hydrodynamic Behavior of Solvent Systems in High-Speed Counter-Current Chromatography III. Effects of Physical Properties of the Solvent Systems and Operating temperature on the Distribution of Two-Phase solvent Systems. J Chromatogr. 1984; 301:405–414. [PubMed: 6501497]

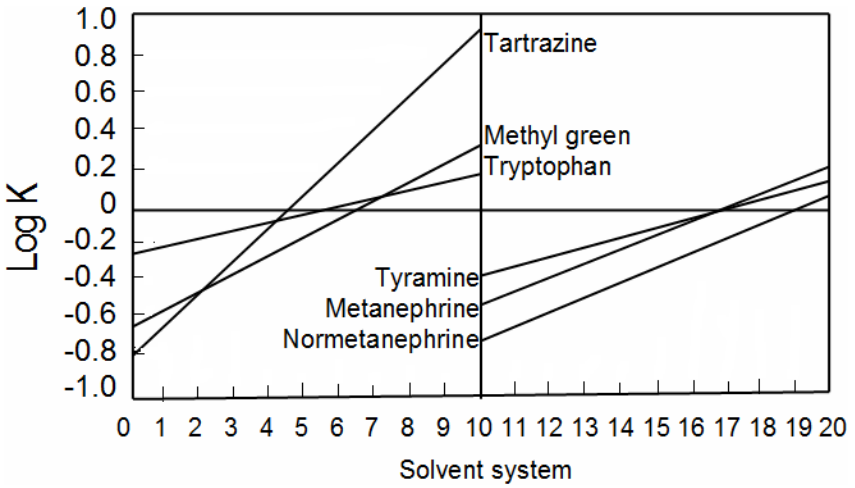

Figure 1.
Graphic selection of two-phase solvent system for single polar compounds by two-step partition coefficient measurements. Optimum K values and solvent systems are listed in Table 1.

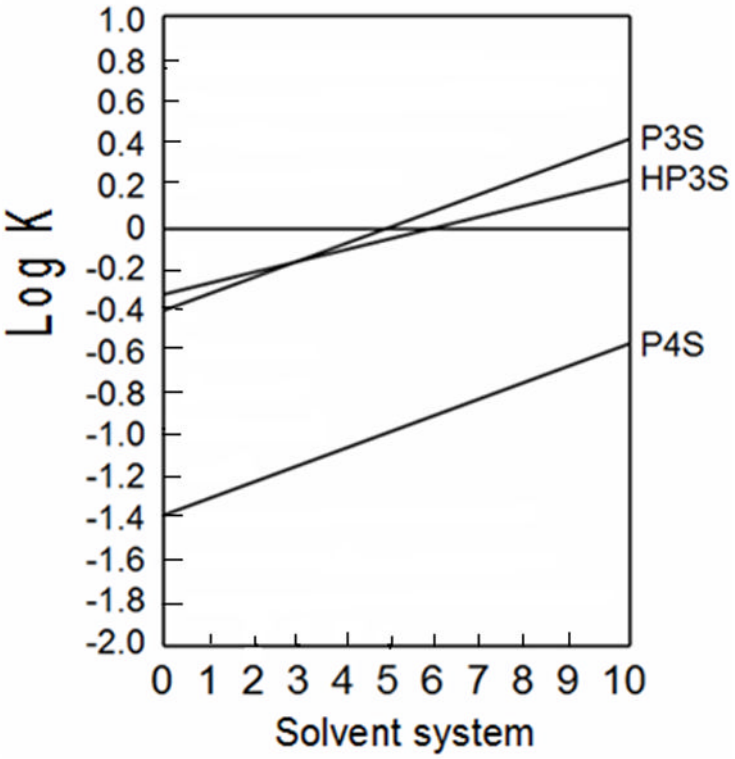

Figure 2.
Graphic selection of two-phase solvent system for a set of sulfonic acids by two-step partition coefficient measurements. Sample: 1,3,6-pyrenetrisulfonic acid (P3S), 8-hydroxy-1,3,6-pyrenetrisulfonic acid (HP3S) and 1,3,6,8-pyrenetetrasulfonic acid (P4S)

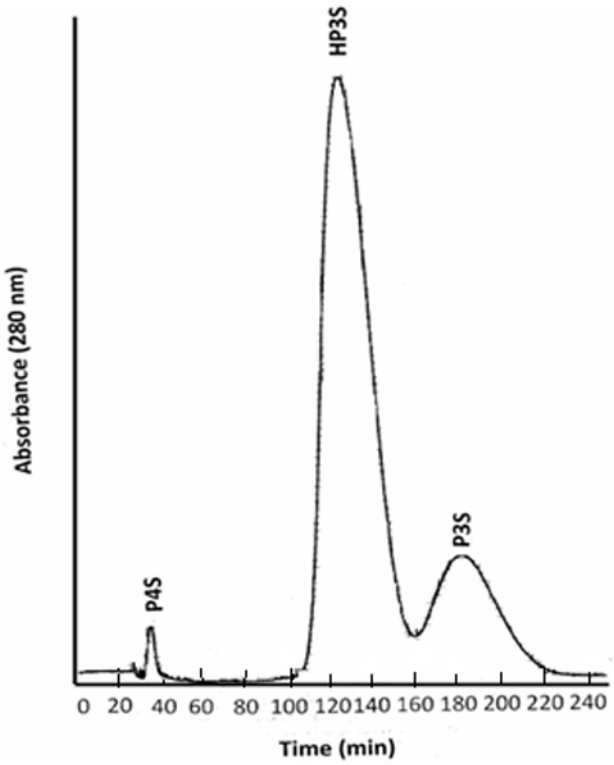

Figure 3.

Separation of sulfonic dyes by spiral HSCCC with Solvent system 9. Experimental conditions: apparatus: type-J HSCCC coil planet centrifuge with 10 cm revolution radius; separation column: 1.6 mm ID, flat-twisted PTFE spiral column with 70 ml capacity; solvent system: System 9 composed of 1-butanol-ethanol-saturated ammonium sulfate-water (1.2:0.8:1:1, v/v); elution mode: lower phase eluted through the internal head toward outer tail terminal of the spiral column; sample: three sulfonic acids in D&C Green No. 8, 5 mg [1,3,6-pyrenetrisulfonic acid (P3S), 8-hydroxy-1,3,6-pyrenetrisulfonic acid (HP3S) and 1,3,6,8-pyrenetetrasulfonic acid (P4S)]; flow rate: 1 ml/min; revolution speed: 800 rpm; detection: 280 nm; retention of the stationary phase: 71%; maximum column pressure: 120 psi. -

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Table 1

Organic-high ionic strength aqueous two-phase solvent systems

System No.	1-BuOH	EtOH	AS*	H_2O	UP/LP Volume	Set.Time (s)	Densisty difference (g/cm3)
0	2	0	1	1	1.1	23	0.16
1	1.9	0.1	1	1	1.1	25	0.33
2	1.8	0.2	1	1	1.2	25	0.33
3	1.7	0.3	1	1	1.2	27	0.33
4	1.6	0.4	1	1	1.2	27	0.33
5	1.5	0.5	1	1	1.2	26	0.36
6	1.4	0.6	1	1	1.3	26	0.35
7	1.3	0.7	1	1	1.3	30	0.34
8	1.2	0.8	1	1	1.3	40	0.33
9	1.1	0.9	1	1	1.3	26	0.33
10	1	1	1	1	1.3	26	0.36
11	0.9	1	1	1	1.3	26	0.34
12	0.8	1	1	1	1.3	26	0.31
13	0.7	1	1	1	1.1	29	0.31
14	0.6	1	1	1	1.1	31	0.34
15	0.5	1	1	1	1.1	31	0.3
16	0.4	1	1	1	1.1	35	0.3
17	0.3	1	1	1	1.1	39	0.29
18	0.2	1	1	1	1.1	41	0.26
19	0.1	1	1	1	1.1	41	0.23
20	0	1	1	1	1.2	65	0.15

*Saturated ammonium sulfate stock solution.

Table 2

Optimized Log K at the crossing point by a graphic method

	Log K at system				
Compound	**0**	**10**	**20**	**Optimized Log K at crossing point**	**K**
tartrazine	−0.793	0.96		−0.113 at system 5	0.77
tryptophan	−0.25	0.145		−0.003 at system 6	1
methyl green		−0.726	0.29	−0.030 at system 7	0.93
tyrosine		−0.09		−0.090 at system 10	0.81
metanephrine		−0.509	0.223	−0.049 at system 17	0.89
tyramine		−0.37	0.179	−0.008 at system 17	0.98
normetanephrine		−0.699	0.093	−0.019 at system 19	0.96

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