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Micellar liquid chromatographic separation of sulfonamides in physiological samples using direct on-column injection

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Abstract

A mixture of twelve sulfonamides was separated by micellar liquid chromatography (MLC) using sodium dodecyl sulfate (SDS) micelles and a hydrophilic endcapped C_{18} column. Retention behavior and selectivity pattern of sulfonamides in MLC were examined with the change of SDS concentration and volume fraction of an organic modifier (1-propanol). The suitable condition was found to be 0.070 *M* SDS and 6.0% 1-propanol for the separation of these twelve sulfonamides. Under this condition, the isocratic separation of the sulfonamides was achieved within 15 min with a relatively high column efficiency for MLC (ca. 7000 plates/25 cm column). Retention times of these twelve sulfonamides were found to be very repeatable, which is due to the highly reproducible retention behavior in MLC. The same twelve sulfonamides were successfully separated in the spiked physiological fluids (human urine and cow milk) through direct on-column injection by MLC.

1. Introduction

Micellar liquid chromatography (MLC) has been regarded as a powerful alternative to both conventional reversed-phase LC (RPLC) with hydro–organic eluents and ion-pair chromatography (IPC) for the analysis of biosamples [1–3]. Due to the existence of micelles, MLC has several unique advantages such as capability of simultaneous separation of ionic and non-ionic compounds, possibility of simultaneous enhancement of solvent strength and separation selectivity, reproducible and predictable retention behavior, possibility of direct on-column injection of physiological samples, enhanced luminescence detectability, safety and cost [4–7]. The possibility of direct on-column injection of physiological samples is one of the important merits of MLC for bioanalysis as compared to conventional RPLC and IPC. This is due to the fact that the protein matrix of a biological sample can be solubilized by micelles [e.g., sodium dodecyl sulfate (SDS) micelles] and eluted with the solvent front. This obviates the need for the time consuming and tedious sample preparation step, thus making direct, on-column injections of physiological fluids possible. This method has been used for the analysis of therapeutic drugs [8], nucleosides and bases [9], illegal drugs [10], cephalosporins [11], anticancer 6-thiopurine compounds [12] and steroids [13] in biological fluids.

Sulfonamides are a group of antibacterial compounds commonly used for the prevention and the treatment of diseases in livestock products. The sulfonamides residues in treated animals may pose a health threat to consumers through allergic or toxic reactions, or through

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induction of antibiotic resistance in pathogenic organisms [14,15]. Separation of these drugs by RPLC, GC, and capillary electrophoresis (CE) has already been reported [14–19]. Protein precipitation and extraction processes are often used to remove the protein matrix from physiological fluid samples in RPLC [14,16]. These processes are tedious, time consuming and may result in the loss of the analytes.

In this work, the retention behavior of sulfonamides in MLC is examined. A mixture of twelve sulfonamides in physiological fluids was separated under isocratic conditions in MLC and by direct on-column injection.

2. Experimental

2.1. Chromatographic system

An HPLC pump (Model 400; Applied Biosystems, Foster City, CA, USA) and a variable-wavelength absorbance detector (Model 783A, Applied Biosystems) set at 254 nm and a VIGI injector (Valco, Houston, TX, USA) were utilized in this work. The HPLC system was controlled by the Chemresearch chromatographic data management system controller software (ISCO, Lincoln, NE, USA) running on a PC-88 Turbo personal computer (IDS, Paramount, CA, USA). A 5-μm particle size, 250 × 4.6 mm YMC-Pack ODS-AQ column (YMC, Wilmington, NC, USA) was used as the analytical column. The column was thermostated at 40°C by a water circulator bath (Lauda Model MT-6; Brinkmann Instruments, Westbury, NY, USA). A silica precolumn was used to saturate the mobile phase with silicates and protect the analytical column.

2.2. Reagents

All the sulfonamides were obtained from Sigma (St. Louis, MO, USA) and are identified in Table 1. The structures of these sulfonamides are shown in Fig. 1. The sample solutions were prepared by diluting the stock solutions (5 mg/ml in methanol) with the mobile phase. The stock solution of SDS (Sigma) was prepared by dissolving the required amount of surfactant in doubly distilled, deionized water and was filtered through a 0.45-μm nylon-66 membrane filter (Rainin Instruments, Woburn, MA, USA). The ionic strength of the mobile phase was adjusted

Table 1
Retention and efficiency of sulfonamides in MLC

Compound	t_R (min)	k'	N (plates/25 cm column)[a]	h[b]
(1) Sulfacetamide	3.43	0.87	7988	6.26
(2) Sulfadiazine	3.77	1.06	9027	5.54
(3) Sulfamerazine	4.56	1.49	7297	6.85
(4) Sulfathiazole	5.17	1.82	8450	5.92
(5) Sulfamethazine	5.56	2.03	8223	6.08
(6) Sulfamethoxypyridazine	6.46	2.52	7814	6.40
(7) Sulfachloropyridazine	6.81	2.71	8696	5.75
(8) Sulfamonomethoxine	7.24	2.94	6894	7.25
(9) Sulfabenzamide	8.56	3.67	6886	7.26
(10) Sulfadimethoxine	10.78	4.88	6153	8.13
(11) Sulfaquinoxaline	13.09	6.14	6335	7.89
(12) Sulfisomidine	15.00	7.18	6710	7.45
Average			7012	7.13

Mobile phase: 0.070 M SDS, 6.0% 1-propanol, 0.020 M NaH_2PO_4, pH 3.0, YMC-Pack ODS-AQ column.
[a] Calculated by using Foley-Dorsey equation [23].
[b] Reduced plate height.

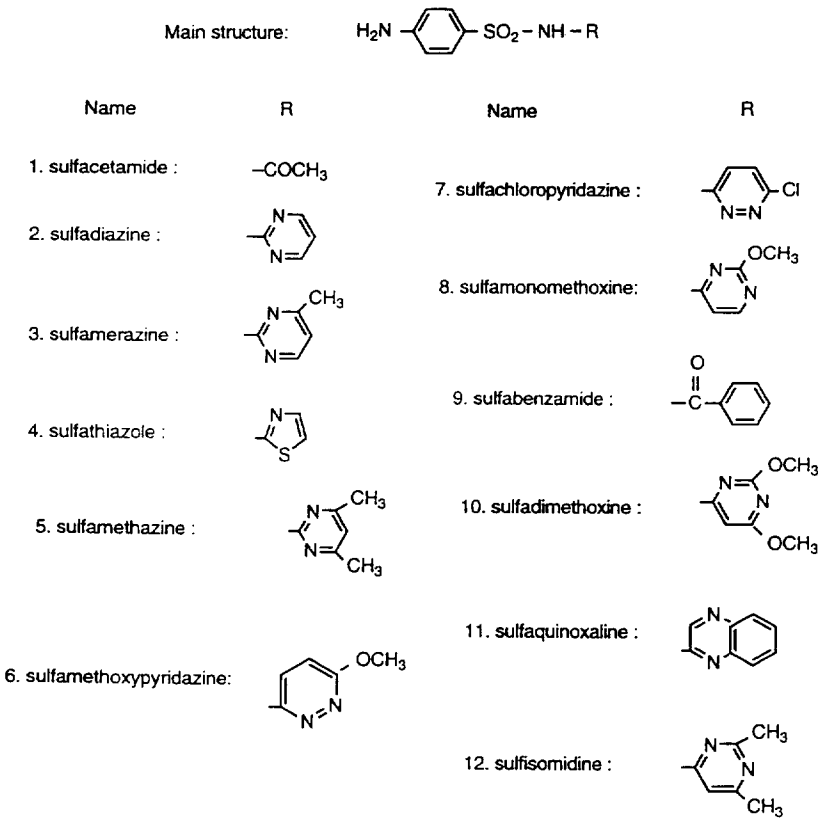

Fig. 1. The structures of sulfonamides.

by adding phosphate buffer so that the total buffer concentration of the final solution was 0.020 *M*. After adding the required amount of 1-propanol the pH was adjusted to 3.0. The urine matrix was from a healthy volunteer and filtered through a 10-mm glass microfiber filter (Rainin Instruments) and a 0.45-μm nylon-66 filter (Rainin Instruments) prior to on-column injection. The milk matrix was obtained from a local grocery store and was also filtered through a 10-mm glass microfiber filter (Rainin Instruments) before use. Due to the high viscosity of the milk sample, its filtration through a 0.45-μm filter paper under vacuum was not successful.

3. Results and discussion

MLC separations can be influenced by the types/concentrations of both surfactant and organic modifier, pH, ionic strength and temperature [3,4,6]. In this work, the effects of SDS concentration and volume fraction of 1-propanol on the retention behavior and separation of sulfonamides were investigated.

3.1. Effect of mobile phase composition on the separation of sulfonamides

The dependence of retention factor on volume fraction of organic modifier and micelle concentration in the MLC system can be described by Eqs. 1 and 2 [5,20].

$$\ln k' = -S\varphi_{org} + \ln k'_0 \quad (1)$$

$$1/k' = (K_{mw}[M] + 1)/(P_{sw}\phi) \quad (2)$$

where k' is the retention factor of a solute, φ_{org} is the volume fraction of organic modifier (1-propanol), k'_0 is the retention factor in a purely

aqueous micellar mobile phase, S is the solvent strength parameter, [M] is the micelle (SDS) concentration, ϕ is the phase ratio, K_{mw} is the binding constant of solute to micelles and P_{sw} is the partition coefficient of a compound from mobile phase into stationary phase. Fig. 2 shows the retention behavior of sulfonamides as a function of volume fraction of 1-propanol and concentration of SDS.

The dependence of selectivity ($\alpha = k'_2/k'_1$) in MLC systems on the volume fraction of organic solvent and micelle concentration can be described by Eqs. 3 and 4 [5,20].

$$\ln \alpha = -(S_2 - S_1)\varphi_{org} + (\ln k'_{0,2} - \ln k'_{0,1}) \quad (3)$$

$$\alpha = \frac{(\alpha_{sw})([M] + 1/K_{mw,1})}{(\alpha_{mw})([M] + 1/K_{mw,2})} \quad (4)$$

where α_{sw} is the stationary phase partitioning selectivity ($P_{sw,2}/P_{sw,1}$) and α_{mw} is the selectivity of binding to (or partitioning into) micelles ($K_{mw,2}/K_{mw,1}$). This is shown in Fig. 3 for sulfonamides with the changes of volume fraction of 1-propanol and concentration of SDS.

Micelle concentration is equal to the difference of the concentration of surfactant and its

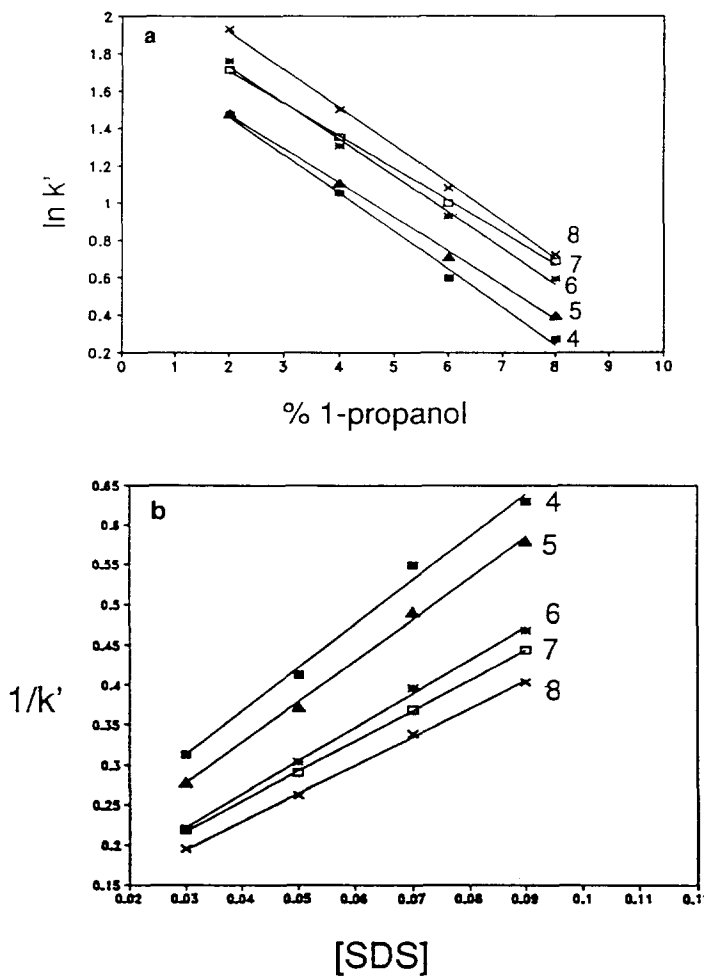

Fig. 2. The effect of (a) the volume fraction of 1-propanol (0.070 M SDS) and (b) the SDS micelle concentration (6.0% 1-propanol) on the retention behavior of sulfonamides.

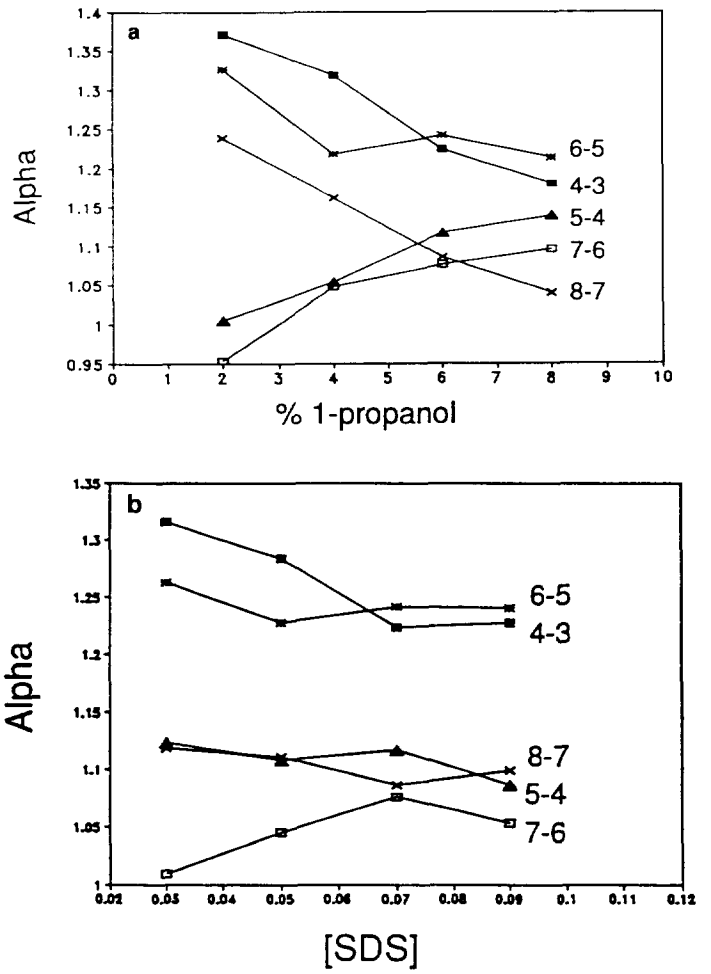

Fig. 3. The effect of (a) the volume fraction of 1-propanol (0.070 *M* SDS) and (b) the SDS micelle concentration (6.0% 1-propanol) on the selectivity of sulfonamides.

critical micelle concentration (CMC) (i.e., [M] = C_{sf} − CMC). The CMC value of a surfactant depends on many solution conditions. For example, the CMC value for SDS was found to be ca. 8 m*M* in aqueous solution at 25°C [4]. However, the CMC values for SDS were not available at our experimental conditions (i.e., 40°C, 2–8% 1-propanol, 0.020 *M* phosphate buffer and pH 3.0). Eq. 2 describes the relationship between retention factor and micelle concentration. At a constant volume fraction of organic modifier (e.g., 6% 1-propanol), $1/k'$ is linearly dependent on the concentration of SDS as shown in Fig. 2b assuming that CMC is a constant ($R^2 > 0.99$ for all of the sulfonamides). Therefore, we assume that the concentration of SDS, instead of SDS micelles concentration, can be used to find a suitable mobile phase condition without changing the results significantly.

Based on the experimental results in Figs. 2 and 3, the suitable mobile phase condition was found to be 0.070 *M* SDS, 6.0% 1-propanol for the separation of compounds 4–8. The mobile phase conditions did not have as much effect on the separation of the early-eluting peaks (1–3) and late-eluting peaks (9–12). In other words, compounds 4–8 were the "critical" pairs, and their separation was crucial. Therefore, the suit-

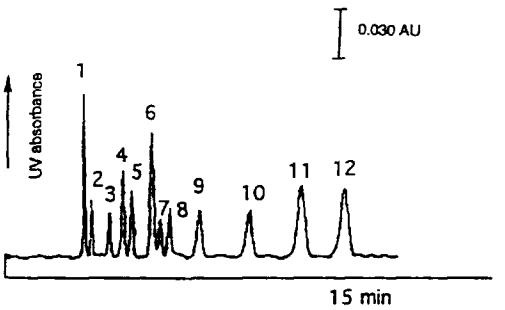

Fig. 4. The isocratic separation of 12 sulfonamides. The suitable mobile phase conditions: 0.070 *M* SDS, 6.0% 1-propanol, 0.020 *M* NaH_2PO_4, pH 3.0.

able mobile phase condition for the separation of compounds 4–8 was also suitable for the whole mixture containing twelve sulfonamides. Fig. 4 shows the good separation of these 12 sulfonamides in 15 min under this mobile phase conditions. High column efficiencies ($N \approx 7000$ plates/25 cm column) were achieved using the hydrophilic endcapped YMC ODS-AQ column for the sulfonamides as shown in Table 1. The achieved column efficiency using the ODS-AQ column is much higher than that typically obtained in MLC using the conventional C_{18} columns [20,21] and is slightly higher than that using a Flurooctyl (FO) column [21]. This results further confirm the belief that the stationary phase effect is significant in MLC and deserves more attention [21,22]. Column efficiencies were calculated by using Foley and Dorsey's equation [23].

$$N = \frac{41.7 \cdot \left(\frac{t_R}{W_{0.1}}\right)^2}{\frac{B}{A} + 1.25} \quad (5)$$

where N is the column efficiency, t_R is the retention time of a solute, $W_{0.1}$ is the width at the 10% peak height, and B/A is the asymmetric factor of the peak.

Retention behavior of sulfonamides was repeatable with high precision using micellar eluent as shown in Table 2, which is due to the highly reproducible retention behavior in MLC separations [5,7,20,21].

3.2. Direct on-column separation of sulfonamides in physiological fluids

Physiological fluid samples initially used in this work were human urine and cow milk. There were no pretreatments for these samples except filtration and dilution. The chromatograms of urine and milk blanks are shown in Figs. 5 and 6, respectively. As shown in Fig. 5, there are some unidentified components in the urine matrix, but

Table 2
Repeatability of sulfonamides' retention in MLC

Compound	t_R (min)	Average	S.D.
1	3.40, 3.43, 3.43, 3.43, 3.43, 3.43, 3.43, 3.43	3.43	0.01061
2	3.76, 3.77, 3.77, 3.77, 3.77, 3.77	3.77	0.00408
3	4.56, 4.56, 4.57, 4.56, 4.57, 4.56	4.56	0.00516
4	5.16, 5.17, 5.17, 5.17, 5.17, 5.17	5.17	0.00408
5	5.56, 5.56, 5.55, 5.56, 5.55	5.56	0.00548
6	6.46, 6.46, 6.46, 6.46	6.46	0
7	6.82, 6.82, 6.81, 6.82, 6.82, 6.82, 6.81	6.82	0.00488
8	7.24, 7.23, 7.24, 7.24, 7.23, 7.23	7.24	0.00548
9	8.58, 8.57, 8.57, 8.57, 8.57, 8.56	8.57	0.00632
10	10.81, 10.81, 10.76, 10.76, 10.76	10.78	0.02739
11	13.13, 13.06, 13.09	13.09	0.03512
12	15.00, 15.00, 15.01, 15.01, 15.00	15.00	0.00548

Retention times of sulfonamides were randomly collected in one day.

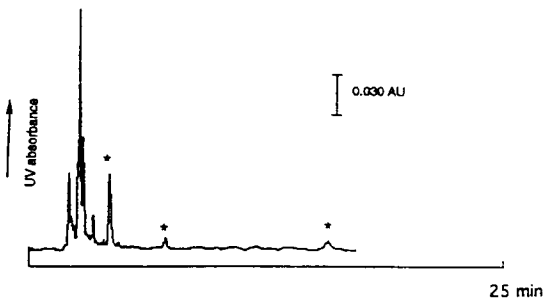

Fig. 5. Urine blank. Mobile phase conditions as in Fig. 4. Full scale of detection is 0.250. * = Unidentified components in urine matrix.

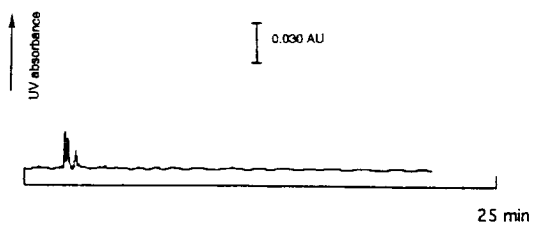

Fig. 6. Milk blank (1% milk–water–mobile phase, 1:1:1) under the mobile phase conditions given in Fig. 4. Full scale of detection is 0.250.

they did not interfere with the separation of these twelve sulfonamides. The separation of sulfonamides in spiked urine and milk are demonstrated in Figs. 7 and 8, respectively. Good separations of these twelve sulfonamides were

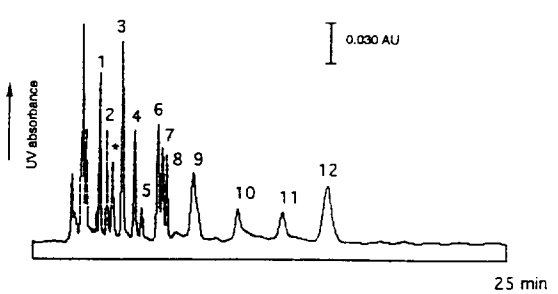

Fig. 7. Separation of sulfonamides in urine sample under the mobile phase conditions given in Fig. 4. Urine sample was spiked by 2.5 μg of compounds 8 and 10, 1 μg of compound 12 and about 0.5 μg of any other sulfonamides in 1 ml urine matrix. Full scale of detection is 0.250. * = Unidentified component in urine matrix.

Fig. 8. Separation of sulfonamides in milk sample. Mobile phase conditions as in Fig. 4. Milk sample was prepared by spiking 2.5 μg of compounds 8 and 10, 1 μg of compound 12 and about 0.5 μg of any other sulfonamides in 1 ml milk blank. Full scale of detection is 0.250.

achieved in these two physiological fluid samples, which suggests that MLC is a suitable technique for the rapid analysis of sulfonamides in physiological fluids. The retention time of sulfisomidine (compound 12) in the milk sample (Fig. 8) was relatively lower than those in the urine sample (Fig. 7) or without sample matrix (Fig. 4). This might be due to sample matrix effects. As mentioned in the experimental section, filtration of the milk sample through a 0.45-μm filter paper under vacuum was not successful due to its high viscosity. The infiltrated milk matrix may have caused some damages to the packing materials of the column that lead to deterioration of peak shapes and reduction of retention times. Further studies will concentrate on the separation of different pharmaceutical drugs in various physiological fluid samples.

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