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Optimization of Separation of Several Sulfonamides in Acetonitrile-Water Binary Mixtures by Using Symmetry Shield RP-8 Column

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Abstract

In this work, the proportion of organic modifier of the hydro-organic mobile phase was optimized in order to separate twelve compounds namely sulfaguanidine, sulfanilamide, sulfadiazine, sulfathiazole, sulfamethazine, sulfamerazine, sulfamethoxypyridazine, sulfamonomethoxyne, sulfachloropyridazine, sulfadoxine, sulfafurazole and dapson using Symmetry Shield RP-8 Column. Mobile phase was optimized by establishing relationships between retention parameters and Reichardt's E^T_N scale of solvent polarity. In addition, selectivity (α) and resolution (R_S) values were used in order to establish a general model relating elution behavior of substances with the composition of mobile phase. Logarithm of retention factor corresponding to pure water, log k_w, which is the most commonly employed chromatographic hydrophobicity parameter determined by using chromatographic data.

INTRODUCTION

Sulfonamides were the first antimicrobial drugs, and paved the way for the antibiotic revolution in medicine. Sulfonamides (SAs, substituted amides of sulfanilic acid) are anti-bacterial and anti-infective compounds used for conditions such as acne and urinary tract infections, and are receiving renewed interest for the treatment of infections caused by bacteria resistant to other antibiotics. They also used preferably in farm animals for the treatment of a variety of bacterial infections. In food-producing animals SAs are used not only for the treatment of several diseases but also for prophylactic purposes and/or for promotion of growth. A major concern with the use of these compounds is that residues may be present in animal food products and may pose a health threat to consumers [1,2]. Residues of SAs can appear in animal food products, such as milk, honey, eggs, and meat. Within the European Union (EU) the maximum residue limit is 100 μg.kg^{-1} in milk, muscle, kidney and liver in any animal, which is used for food production [3]. Nowadays, pharmaceutical and veterinary products frequently contain sulfonamides in conjunction with other compounds in order to increase their activities. Thus, interest in the development of new, fast, easy, reliable and sensitive analytical methods to separate and to analyze these compounds is needed.

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Spectrophotometric methods on the basis of the Bratton-Marshall reaction are commonly used for determining the total sulfonamide content, but when the aim is the identification and quantification of individual compounds in mixtures containing sulfonamides, potenciators, and/or vitamins, the separation techniques provide analytical methods for resolving these combinations properly [4]. Over the years, several separation techniques with several detectors have been developed and used for the determination of SAs in various samples. Lots of them are based on LC/MS determinations [5-7] and more recently on LC/MS/MS [8], others in HPLC with diode-array [9-11] or fluorescence detection [12,13], miceller liquid chromatography [14], capillary electrophoresis [15], etc. However in routine analysis, in order to establish separation methodologies and determine SAs in biological materials and foodstuffs, liquid chromatography (LC) is widely used analytical technique and the method of choice because of its versatility, low-cost instrumentation, precision and sensitivity, and results are obtained in a reasonable time.

Optimization of the chromatographic resolution of ionogenic solutes in liquid chromatographic techniques is a task that has been actively researched [16-18]. Because of the specific ionization characteristics of these types of solutes, the two most useful optimization parameters are polarity and the pH of the mobile phase. The approach for optimizing the organic modifier concentration in the mobile phase during chromatographic separation of SAs has been made by establishing a relationship between the retention parameters and Reichardt's scale of solvent polarity [19]. Moreover, the pH of the mobile phase is a major factor influencing the chromatographic retention of ionizable compounds. Retention in such systems is influenced by the ionization state of functional groups present on the analytes.

In this work the components were detected by using their natural absorbances in the UV region. The relationships between the retention parameters (k) of the compounds and Reichard's scale of solvent polarity were used to optimize the proportion of organic modifier in the mobile phase.

EXPERIMENTAL

Chemicals and Reagents

The standard SAs studied are shown in Table 1. They were purchased from Sigma and used without further purification. Water, with conductivity lower than 0.05 mScm^{-1} was obtained with a Milli Q water purification system (Milli Pore Corp.). Acetonitrile (MeCN) was of HPLC grade and used as the organic component of the mobile phase. Stock standard solutions of SAs were freshly prepared in water at concentrations of approximately 200 mg L^{-1} and stored in amber bottles in refrigerator (4°C). Working solutions were diluted with corresponding mobile phase to 10 mg L^{-1}. These solutions were passed through a 0.45 mm nylon filter membrane (MSI) before injections.

Apparatus

A chromatographic system consisted of Shimadzu Model LC 10 ADVP pump with an auto injector (SIL 10 AD VP) and diode array detector system (SPDM 10 A DAD) was used for studies. This equipment has column oven (CTO 10 AVP) and degasser system (DGU 14 A). A Symmetry Shield RP-8 (5 μm x 3.9 mm, 150 mm Waters) end-capped column was used at ambient temperature. Flow rate was maintained at 1.0 mL min^{-1}.

The e.m.f. measurements used to evaluate the pH of the mobile phase were performed using Metleer-Toledo MA 235 pH/ion analyser with a Hanna HI 1332 combination pH electrode. The calibration solutions were thermostated externally at 25 ± 0.1^{o}C

Table 1. Chemical structures of studied compounds.

Compounds/Chemical Structure	
Sulfaguanidine (4-Amino-N-guanylbenzenesulfonamide)	Sulfanilamide
Sulfadiazine, (4-Amino-N-(2-pyrimidin-2-yl) benzenesulfonamide)	Sulfathiazole, (4-Amino-N-(1,3-thiazol-2-yl) benzenesulfonamide)
Sulfamerazine, (4-Amino-N-(4-methylpyrimidin-2-yl) benzenesulfonamide)	Sulfamethazine, (Sulfadimidine), (4-Amino-N-(4,6-dimethylpyrimidin-2-yl) benzenesulfonamide)
Sulfamethoxypyridazine, (4-Amino-N-(6-methoxypyridazin-3-yl) benzenesulfonamide)	Sulfamonomethoxyne, (4-Amino-N-(6-methoxy-4-pirimidinil) benzenesulfonamide)
Sulfachloropyridazine, (4-Amino-N-(6-chloro-3-pyridazinyl) benzenesulfonamide)	Sulfafurazole (5-amino-3,4-dimethylisoxazole)
Sulfadoxine, (4-Amino-N-(5,6-dimethoxy-4-pyrimidinyl) benzenesulfonamide)	Dapson 4-(4-Aminophenyl)sulfonylaniline

with a cooler system water bath (HETO CBN 8-30 and temperature control unit HETO HMT 200) when adjusting the pH. The electrode was stabilized in the appropriate MeCN–water mixture prior to e.m.f. measurements. pH measurements of the mobile phases were performed in triplicate to ensure stability and reproducibility of the potentiometric system. The use of organic solvent – water mixtures requires the correct measurements of pH in these media. Measurements are performed in a similar way to those performed in water using IUPAC standardization rules [20]. The pH values of the mobile phases were measured against a 0.05 mol/kg potassium hydrogen phthalate solution as primary reference standard solution, dissolved in the appropriate MeCN–water medium. This standard (potassium hydrogen phthalate dried at 110°C before use, Fluka) has been selected because of their pH values at different MeCN percentages up to 70% (v/v)) are already known [21]. In this work, the pH of the mobile phase is measured after mixing the aqueous buffer and the organic modifier.

Chromatographic procedure

Throughout this study, the compounds were separated using isocratic system and the mobile phases assayed were MeCN–water (15:85, 17.5:82.5 and 20:80, v/v) with 65 mM o-phosphoric acid. The pH of the mobile phase was adjusted to 4.50 with 1 M sodium hydroxide. The mobile phase was prepared daily, filtered, sonicated before use and delivered at a flow rate of 1 mL min^{-1} and the effluent was monitored at 270 nm. The mobile phase mixtures were filtered through a 0.45 µm pore nylon membrane filters (Millipore, Bedford, MA). A total of 20 µL of each solution was injected and chromatograms were recorded.

In general, for each MeCN content, the chromatographic retention was studied in acidic region at pH 4.50 ± 0.01. The column was pre-conditioned during at least 1 h at low flow-rate (0.5 mLmin^{-1}) with mobile phase before the first injection. Retention factors were calculated as;

$$k = (t_r - t_o) / t_o \qquad (1)$$

here t_o indicates the retention time of potassium bromide (hold-up time), which was measured for every mobile phase composition by injection of 0.01% (w/v) potassium bromide (Merck) solution in water for each mobile phase composition and pH studied.

RESULTS AND DISCUSSIONS

SAs contain ionogenic functions such as aromatic amine ($H_3N^{(+)}$-C_6H_4-SO_2-NHR) and sulfonic (H_2N-C_6H_4-SO_2-$N^{(-)}$-R) groups, respectively. SAs are ordinary ampholyte compounds so that their ionization can be described as a two-step protolysis (Figure 1). In ordinary ampholytes, when the difference (ΔpK_a) between acidic pK_a and basic pK_a is greater than 3, only one kind of group (acidic or basic) can be ionized to any extent at a time. However, when ΔpK_a is less than 3, the ionization of the other group will no longer be negligible, causing the existence of a small proportion of the zwitterionic species. When pH is about equal to average pK_a, the neutral form is the dominant species in ampholytes. Their retention on column depends on the percentage of ionized and non-ionized species of each compound. The hydrophobic nature of the neutral species is naturally greater than those of the associated ions [22,23]. Thus, the pH of the mobile phase was adjusted to 4.50 in order to keep the compounds in neutral form. Then the effect of concentration of organic modifier was investigated on peak symmetry and retention times for twelve compounds taking into the pH values as constant.

Figure 1. Protolytic equilibria of sulfonamides.

Symmetry Shield HPLC columns that used in separation have set a new standard of performance for peak shape of basic compounds. This reversed phase column is based on embedded polar group technology that literally “shields” the silica’s residual surface silanols from highly basic analytes. The embedded polar group, which is in close proximity to the silica surface, further reduces the activity of the surface silanols in column packing materials. As a result, these types of columns deliver improved peak shape and resolution over a much broader pH range and exhibit less retention of basic compounds than conventional C-18 and C-8 colunms.

The concentration of organic solvent in the mobile phase is one of the most important factor in RPLC. It has not only a large impact on both the retention and selectivity, but is also a very convenient property, owing to the flexibility and accuracy in the implementation of changes. In order to optimize the proportion of organic modifier, the normalized scale of solvent polarity proposed by Reichardt [19] was used:

$$\log k = C + eE_T^N \quad (2)$$

Retention factor values for sulfonamides studied were obtained experimentally in MeCN-water binary mixtures which values are in the range of 0.903-0.924 (15-20% (v/v) MeCN). The correlation between the experimental log *k* values of the solutes studied over the whole experimental range of MeCN contents and the values of the mobile phases are shown in Table 2.

According to Eq. (2), logarithm of the retention factors correlate linearly ($r \geq 0.99$) with the polarity of the mobile phase for the compounds studied. This linearity allows prediction of the elution behavior of SAs hence optimization of the composition for the best separation from only two experimental

Table 2. Relationships between logk for studied compounds and E^N_T parameters, with correlation coefficients (R^2).

Compounds	Equation	R^2
Sulfaguanidine	$\log k = 17.506 - 16.381E^N_T$	0.978
Sulfanilamide	$\log k = 4.684 - 4.351E^N_T$	0.989
Sulfadiazine	$\log k = 8.016 - 6.941E^N_T$	0.993
Sulfathiazole	$\log k = 10.403 - 9.070E^N_T$	0.989
Sulfamerazine	$\log k = 7.039 - 5.909E^N_T$	0.995
Sulfamethazine	$\log k = 24.782 - 22.144E^N_T$	0.994
Sulfamethoxy pyridazine	$\log k = 10.631 - 9.014E^N_T$	0.999
Sulfamono methoxyne	$\log k = 11.331 - 9.453E^N_T$	0.992
Sulfachloro pyridazine	$\log k = 15.999 - 13.593E^N_T$	0.989
Sulfadoxine	$\log k = 14.292 - 12.056E^N_T$	0.978
Sulfafurazole	$\log k = 16.105 - 13.553E^N_T$	0.997
Dapson	$\log k = 18.078 - 15.359E^N_T$	0.952

measurements of the capacity factors for each analyte.

The retention behavior can be modeled accurately by establishing a quadratic relationship between the logarithm of the retention factor, k, and the volume fraction of organic solvent in the aqueous-organic mobile phase, φ:

$$\log k = c_0 + c_1\varphi + c_2\varphi^2 \quad (3)$$

where c_i are regression coefficients with characteristic values for a given solute and column / solvent system. This model can be simplified for narrow ranges of organic solvent to a linear relationship:

$$\log k = \log k_w - S\varphi \quad (4)$$

The intercept of the fitted straight-line refers to the extrapolated log k for water as the mobile phase, and the slope indicates the sensitivity of retention to changes in elution strength. The log k_w and S (slope) values of the studied twelve compounds were calculated by Eq. (4) and are presented in Table 3.

The correlation between the percentage of organic modifier and the logarithm of the retention factor, log k, in HPLC are the reason for using the method of RP-HPLC for determination of log $P_{o/w}$. The chromatographic retention is governed by hydrophobic forces, and therefore RPLC retention data have been suggested for calculating the log $P_{o/w}$ value of compounds. In the literature, correlation between organic content of the mobile phase and log$k$ is described [24-26].

As known, achieving a good resolution between all of the working compounds is the main goal of chromatographic separation. In terms of fundamental chromatographic parameters the resolution, R_s, is affected by three independent variables:

Table 3. The relationships between log k and volume fraction of organic solvent (MeCN).

Compounds	$\log k = \log k_w - S\varphi$	R^2
Sulfaguanidine	$\log k = 0.902 - 0.074\varphi$	0.983
Sulfanilamide	$\log k = 0.269 - 0.020\varphi$	0.984
Sulfadiazine	$\log k = 0.968 - 0.037\varphi$	0.991
Sulfathiazole	$\log k = 1.193 - 0.044\varphi$	0.985
Sulfamerazine	$\log k = 1.036 - 0.030\varphi$	0.993
Sulfamethazine	$\log k = 2.318 - 0.104\varphi$	0.997
Sulfamethoxypyridazine	$\log k = 1.476 - 0.045\varphi$	0.998
Sulfamonomethoxyne	$\log k = 1.728 - 0.047\varphi$	0.989
Sulfachloropyridazine	$\log k = 2.191 - 0.067\varphi$	0.985
Sulfadoxine	$\log k = 2.041 - 0.060\varphi$	0.971
Sulfafurazole	$\log k = 2.338 - 0.068\varphi$	0.991
Dapson	$\log k = 2.470 - 0.075\varphi$	0.960

$$R_s = \underbrace{1/4N^{1/2}}_{\text{Efficiency}} \; \underbrace{[(\alpha-1)/\alpha]}_{\text{Selectivity}} \; \underbrace{[k/(1+k)]}_{\text{Retention}} \quad (5)$$

where N is the column plate number, k is the capacity factor. The selectivity factors for adjacent solute pairs were calculated in the usual way Although the selectivity term is generally regarded as the most important in RPLC, full attention must be given to all of the terms in resolution in Eq. (5). To obtain the best resolution of two peaks in the shortest analysis time, all three of these factors should be optimized. Also efficiency, selectivity and retention parameters of studied SAs are given in Table 4.

We chose as the optimal composition 15% (v/v) MeCN in the mobile phase due to the appropriate selectivity, resolution and retention time.

The best chromatogram (Figure 2), with complete separation of all analytes as symmetrical sharp

Table 4. Chromatographic capacity factors, selectivity and resolutions of studied compounds at 15% (v/v) MeCN.

Compounds	k	α	(α-1)/ α	$k_2/1+k_2$	(α-1)/ α x $k_2/1+k_2$	N	¼ √N	R_s
Sulfaguanidine	0.592	-	-	-	-	-	-	-
Sulfanilamide	0.956	1.614	0.380	0.489	0.186	4142	16.090	2.991
Sulfadiazine	2.958	3.096	0.677	0.747	0.506	6291	19.829	10.032
Sulfathiazole	3.561	1.204	0.169	0.781	0.132	7253	21.291	2.813
Sulfamerazine	3.974	1.116	0.104	0.799	0.083	7332	21.407	1.780
Sulfamethazine	5.483	1.380	0.275	0.846	0.233	7550	21.723	5.056
Sulfamethoxypyridazine	6.468	1.180	0.152	0.866	0.132	8202	22.641	2.985
Sulfamonomethoxyne	10.645	1.646	0.392	0.914	0.359	3399	14.575	5.228
Sulfachloropyridazine	11.929	1.121	0.108	0.923	0.099	8281	22.750	2.260
Sulfadoxine	14.716	1.234	0.189	0.936	0.177	8433	22.957	4.072
Sulfafurazole	16.520	1.123	0.109	0.943	0.103	9163	23.931	2.464
Dapson	18.337	1.110	0.099	0.948	0.094	8880	23.558	2.213

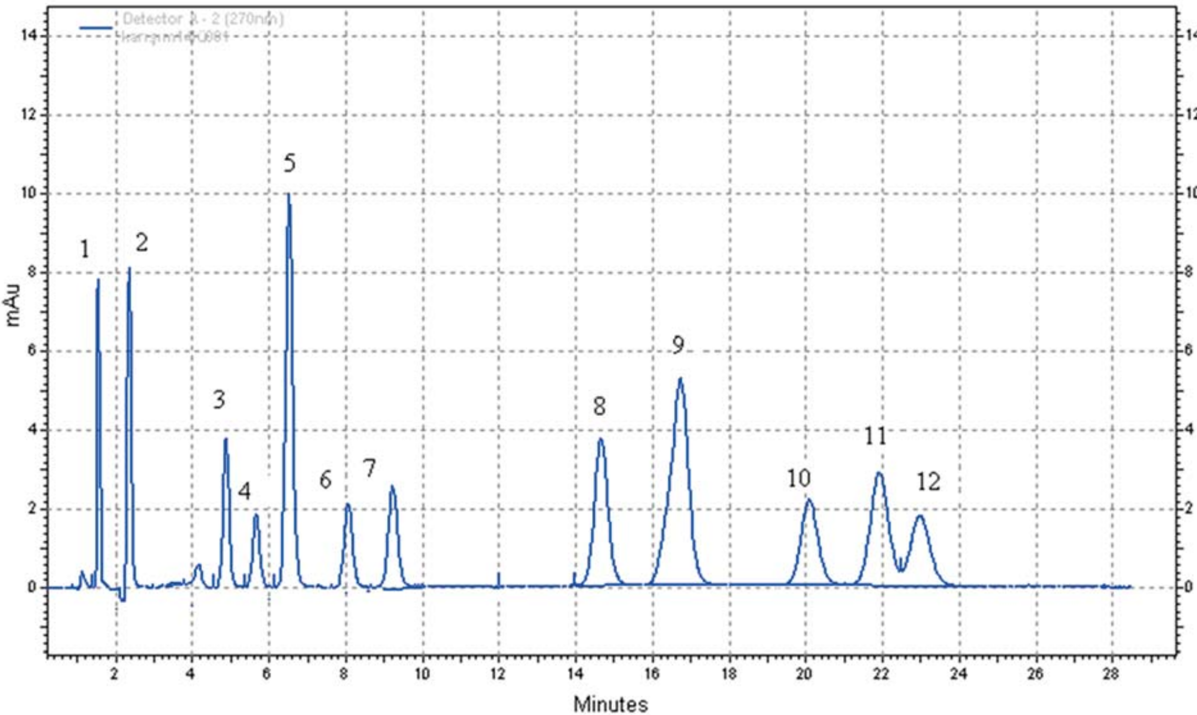

Fig. 2. Chromatogram of a standard mixture of SAs, with a mobile phase of MeCN–water (15:85, v/v) containing 65 mM phosphoric acid, with the pH adjusted to 4.50 with NaOH. Sulfaguanidine (1), sulfanilamide (2), sulfadiazine (3), sulfathiazole (4), sulfamerazine (5), sulfamethazine (6), sulfamethoxypyridazine (7), sulfamonomethoxine (8), sulfachloropyridazine (9), sulfadoxine (10), sulfafurazole (11) and dapson (12) respectively.

peaks in a short analysis time, was obtained by use of 65 mM o-phosphoric acid solution (pH 4.5) in 15% (v/v) MeCN-water mobile phase at a flow rate of 1.0 $mLmin^{-1}$ at ambient temperature. The UV detector was operated at 270 nm, which was compromise among the wavelengths of maximum absorption of twelve SAs. The total time required for analysis of one sample contained all of SAs was less than 24 minutes.

CONCLUSION

From this study, it can be concluded that at pH 4.50 baseline separations was achieved for twelve analytes. The retention time was stable and the peak shape was quite good. They present a maximum hydrophobicity around this pH. The greatest retention was obtained for sulfonamides bearing additional methyl or methoxy groups on the

R side chain. This suggests that the R side chain plays an important role in the hydrophobic interaction of the compounds with the reversed phase column.

The advantage of optimizing the sulfonamide separation is that, as we know the behavior of each compound over a wide range of conditions (organic phase and pH), we can predict the retention time for each percentage of organic phase and pH. As a consequence, when an interfering compound appears it is possible to change the chromatographic conditions in order to avoid co-elution.

In literature, most of the HPLC separations of SAs are achieved in gradient mode. Isocratic elution presents some advantages such as greater simplicity, lower cost, simple instrumentation, and no need of column re-equilibration between consecutive injections. For the series of sulfonamide compounds studied, the optimized mobile phase is composed of MeCN–water (15:85 v/v) with isocratic elution. This method can be applied for the separation of the SAs possibly present in meat, dairy products and drugs.

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