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HPLC BEHAVIOR OF SULFONAMIDES ON MOLECULARLY IMPRINTED POLYMERIC STATIONARY PHASES

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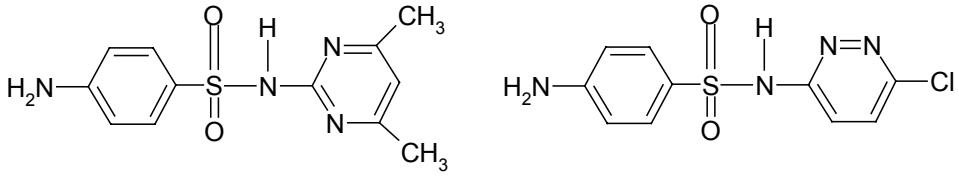
SUMMARY

The purpose of this study was to develop an HPLC procedure for determination of sulfamethazine (SMZ) and sulfachloropyridazine (SCP) on a molecularly imprinted polymeric (MIP) stationary phase. The MIPs were prepared by noncovalent polymerization. Sulfamethazine (SMZ), methacrylic acid (MAA), and ethylene glycoldimethacrylate (EGDMA) were used as template, functional monomer, and cross-linker, respectively, in the presence of chloroform as solvent. The MIPs obtained were packed into a stainless steel column (150 mm × 4.6 mm i.d.) and used as the stationary phase for HPLC. The mobile phase was 23 mM NaH_2PO_4 buffer solution–acetonitrile, 2:1 (*v*/*v*), at a flow-rate of 1 mL min^{-1}. The sample volume injected was 20 μL and detection was by UV absorbance at 272 nm. The retention times of SMZ and SCP under these conditions were approximately 7.015 ± 0.182 min and 11.282 ± 0.925 min. The total run time for each sample was 12 min. The selectivity of the MIPs was evaluated by analysis of different substances with molecular structures similar to that of SMZ. Separation factors (α) were used to compare chromatographic data from the stationary phases. The values of α obtained, in the range 1.57–1.72, showed that use of the MIPs enabled recognition of subtle structural differences from the template molecule. The limits of detection (LOD) of SMZ and SCP were 0.035 and 0.041 μg L^{-1}, respectively, and the respective limits of quantitation (LOQ) were 0.117 and 0.137 μg L^{-1}. The results showed that the SMZ-selective polymer was suitable for separating both SMZ and SCP.

INTRODUCTION

Sulfonamides are compounds of both biological and chemical importance which are widely used in veterinary medicine to treat livestock diseases such as urinary tract infections, Chlamydia, rheumatic fever, toxoplasmosis, and malaria. Sulfamethazine (SMZ) and sulfachloropyridazine (SCP) (Fig. 1) are commonly used sulfonamides. The adverse effects of the sulfonamides are numerous and may affect nearly all organs. To ensure safety the Department of Health, Executive Yuan (Taiwan), has adopted a maximum sulfonamide residue level (MRL) of 0.1 ppm in foods, including muscle, liver, kidney, eggs, and milk [1]. Analysis of sulfonamide residues has been performed by several methods, e.g. spectrophotometry [2–5], polarography [6,7], and gas chromatography–mass spectrometry [8–10], which not only requires expensive equipment but also extensive sample preparation and derivation [11,12]. In this study we have explored the use of molecularly imprinted polymers (MIP) as stationary phases for HPLC. The potential of MIP has previously been successfully demonstrated in a variety of applications [13–16]. Use of MIP as stationary phases for HPLC is by far the best studied application of imprinted polymers, largely because it is a convenient method for quantitative assessment of the quality of imprints produced by a particular procedure or strategy [17,18].



Sulfamethazine (SMZ) Sulfachloropyridazine (SCP)

Fig. 1

The structural formulae of SMZ and SCP

Most column chromatography packings made by molecular imprinting use bulk polymers that are ground and sieved before packing into the column. During imprinting the template creates a specifically imprinted cavity in the polymer with a permanent memory for recognition of the template. The selectivity of an MIP is evaluated by measuring its ability to resolve structural analogs in the adsorption process. Selectivity experiments revealed that SMZ MIPs were able to recognize structural differences be-

tween the template and compounds with similar structures. Evaluation of the affinity and selectivity of the imprinted polymer indicated it was a potential separation material with selectivity for both SMZ and SCP.

EXPERIMENTAL

Chemicals, Reagents, and Standards

All chemicals were of HPLC or analytical grade. Deionized water was passed through a Millipore (Bedford, MA, USA) system. Sulfamethazine (SMZ) and sulfachloropyridazine (SCP) were obtained from Sigma (St Louis, MO, USA). Methacrylic acid (MAA, 99%) and ethylene glycol dimethacrylate (EGDMA, 98%) were obtained from Merck (Darmstadt, Germany) and were purified by distillation before use. 2,2′-Azo-bis-isobutyronitrile (AIBN), methanol, ethanol, acetone, sodium dihydrogen phosphate, and acetonitrile of HPLC grade were obtained from Tedia (Fairfield, OH, USA). Chloroform and acetic acid (GC grade) were from J.T. Baker (Phillipsburg, NJ, USA).

For quantitative work, stock solutions of SMZ and SCP were prepared at a concentration of 1.0 mg mL^{-1} in acetonitrile. Working solutions of SMZ and SCP were prepared in acetonitrile by dilution of the stock solutions in the range 0.2–0.8 mg mL^{-1}.

Preparation of Polymers

The method used for preparation of the molecularly imprinted polymeric stationary phase is shown in Fig. 2. Sulfamethazine (template; 0.0400 g), MAA (functional monomer; 0.0742 g), and EGDMA (cross-linking agent; 2.6492 g) were dissolved in 10 mL chloroform and polymerization was initiated by addition of AIBN and irradiation with UV

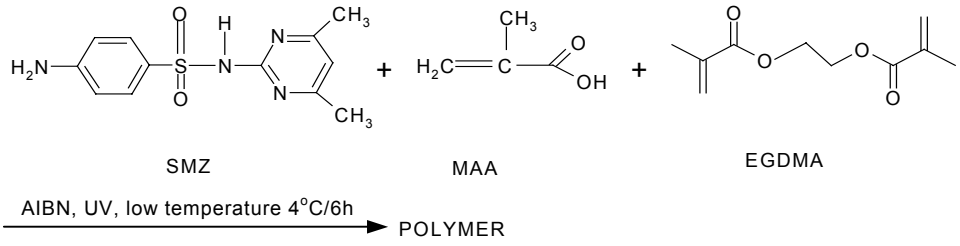


Fig. 2

Schematic illustration of the preparation of SMZ-imprinted polymers

light (100 W, 365 nm) for 6 h at 4°C. The rigid polymer obtained was ground and sieved through a 25–44-μm filter. The polymer (3.3200 g) was subsequently suspended in 30 mL methanol and the suspension was sonicated for 3 min, placed in a slurry reservoir, and then packed in a stainless steel column (150 mm × 4.6 mm i.d.), by use of an air-driven fluid pump, with 300 mL acetone as packing solvent. The template molecule, SMZ, was extracted by on-line washing with methanol–acetic acid, 9:1 (*v*/*v*). This washing step was repeated until a stable baseline was achieved. Blank polymer was prepared in the same manner but without addition of SMZ.

Chromatographic System and Conditions

HPLC was performed with a Jasco PU-2080 system (Japan) incorporating a Jasco UV-2075 variable-wavelength detector and a Rheodyne 7725 sample-loading injector (20 μL). The mobile phase was a mixture of pure acetonitrile and 23 mM sodium phosphate solution in the desired proportion. Mobile phase pH was measured by use of a calibrated pH meter. For data analysis, peak integration was performed with a Peak ABC Chromatography Workstation Ver. 2.10 integrator. The apparatus provided assurance that all the UV-absorbing components were detected if present in sufficient quantity. A solution of SMZ or SCP, or a mixture of both, was injected and elution was performed isocratically at a flow-rate of 1.0 mL min^{-1} with detection at 272 nm. The void volume of the column was determined by injecting toluene. The capacity factors (k) and separation factors (α) were calculated by use of the equations:

$$k_{SCP} = (t_{SCP} - t_0)/t_0 \text{ and } k_{SMZ} = (t_{SMZ} - t_0)/t_0$$

and

$$\alpha = k_{SCP}/k_{SMZ}$$

where k_{SCP} and k_{SMZ} are the capacity factors of SCP and SMZ, respectively, t_{SCP} and t_{SMZ} are the retention times of the SCP and SMZ, respectively, and t_0 is the elution time of the void marker, toluene.

Sample Preparation

SMZ and SCP tablets were purchased from a local pharmacy. The amount of SMZ and SCP present in the tablets was 25 mg. The SMZ-containing tablet also contained the excipients starch, stearic acid, magnesium stearate, and microcrystalline cellulose; the SCP-containing tablet also contained corn starch, hydroxypropylcellulose, stearic acid, magne-

sium stearate, and cellulose. Solutions of both products were prepared by grinding ten tablets in a mortar, transferring the powder to a 500-mL volumetric flask, and dissolving in sodium phosphate solution (0.05 M, 150 mL). The solutions were stirred magnetically for 1 h then diluted to 400 mL with acetonitrile. After mechanical shaking for 45 min a portion of the suspension was centrifuged for 3 min at 1000*g* to furnish clear supernatant solution which was then filtered into a 500-mL calibrated flask through Whatman no. 42 filter paper. The sample solution was injected without further treatment.

RESULTS AND DISCUSSION

The results presented in Tables I and II and in Fig. 3 reveal that rapid and selective separation of SMZ and SCP can be achieved by HPLC with a molecularly imprinted polymer as the stationary phase. Table I shows the retention times, Δt_R values, capacity factors, and separation factors (α) obtained after separation by HPLC. SMZ and SCP are readily and rapidly separated by use of 23 mM NaH_2PO_4 buffer solution–acetonitrile, 2:1 (*v*/*v*) as mobile phase. Table II shows the effect on the separation factor of the acetonitrile content of the mobile phase. Figure 3 shows a typical chromatogram recorded at 272 nm, showing the elution profile of SMZ and SCP.

Table I

Retention times and selectivity (α) for HPLC of SMZ and SCP with an MIP as the stationary phase

Solution no.	Concentration in sample (g L^{-1})[a]		Retention time (min)		Δt_R (min)	Capacity factor, *k*		α
	SMZ	SCP	SMZ	SCP		SMZ	SCP	
1	1.0	0	6.817	–	–	8.98	–	–
2	0.2	0.8	6.912	11.363	4.451	9.12	15.64	1.71
3	0.3	0.7	6.979	11.444	4.465	9.21	15.76	1.71
4	0.4	0.6	7.008	11.586	4.578	9.26	15.96	1.72
5	0.5	0.5	6.899	10.775	3.876	9.10	14.78	1.62
6	0.6	0.4	6.834	10.357	3.523	9.00	14.16	1.57
7	0	1.0	–	11.668	–	–	16.08	–

[a] Total concentration of binary solution 1 g L^{-1}

The void volume of the column was approx. 27.41% (determined by injection of toluene; the retention time of toluene was 0.683 min)

The retention times of SMZ and SCP were 3.502 min and 4.019 min when a non-MIP stationary phase was used for test solution no. 5

Table II

The mobile phases investigated in this study

Ratio of 23 mM buffer to acetonitrile (*v*/*v*)	Retention time		Separation ($R_{SCP} - R_{SMZ}$)	Separation factor (α)
	SMZ	SCP		
1:2 (pH 4.01 ± 0.01)	No resolution		–	–
2:2 (pH 3.99 ± 0.01)	No resolution		–	–
3:2 (pH 3.78 ± 0.01)	6.912	10.117	3.205	1.51
4:2 (pH 3.70 ± 0.01)	7.008	11.586	4.578	1.72
5:2 (pH 3.69 ± 0.01)	7.143	10.357	3.214	1.49

The concentration of the solution (g L^{-1}) was 0.4:0.6 SMZ:SCP

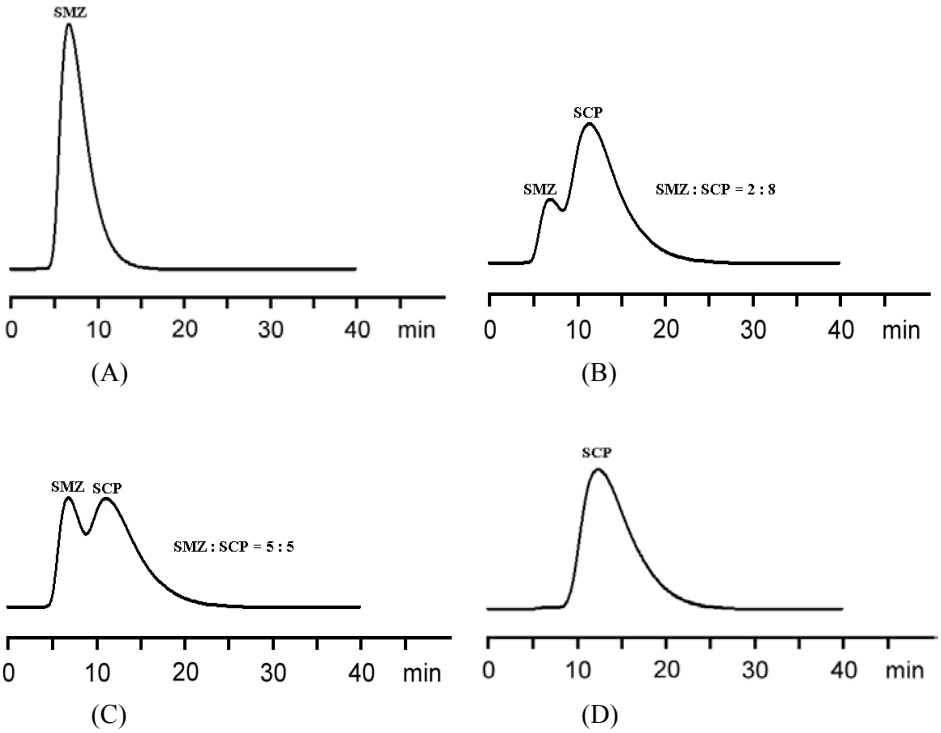


Fig. 3

Chromatograms obtained from SMZ and SCP, at 272 nm, by use of HPLC with a molecularly imprinted polymer as the stationary phase. (A) 1.0 g L^{-1} SMZ. (B) Mixture of 0.2 g L^{-1} SMZ and 0.8 g L^{-1} SCP. (C) Mixture of 0.5 g L^{-1} SMZ and 0.5 g L^{-1} SCP. (D) 1.0 g L^{-1} SCP. The mobile phase was buffer–acetonitrile, 2:1 (*v*/*v*)

From Table I the retention times of SMZ and SCP are 6.817–7.008 min and 10.357–11.668 min, respectively. The difference, Δt_R, between the retention times of SMZ and SCP was 3.523–4.578 min. The chromatographic run was complete in less than 12 min. We attribute the results for these two compounds to their capacity factors (k) and the selectivity (α)=k_{SCP}/k_{SMZ}. The separation factor for SMZ from SCP for different combinations of concentrations with a total concentration of 1 g L^{-1} ranged from 1.57 to 1.72, depending on the amount of SMZ loaded on the column. The retention times for SMZ and SCP were 3.502 min and 4.019 min when a blank (non-MIP) polymer was used as the stationary phase, so Δt_R was 0.517 min.

In this study MAA and SMZ were used as functional monomer and template for forming the MAA–SMZ complex before polymerization. After removal of the template molecules from the polymers a clear cavity of SMZ is left in the particles. Binding sites were formed from the many free carboxyl groups remaining in the copolymer matrix. The stability of MAA–SMZ complexes present in the solution before polymerization plays a dominant role in determining the recognition performance of the polymers. The morphology of the cavity is similar to that of SMZ, so SMZ and SCP were retained by the cavity and the free carboxyl groups of the stationary phase. SMZ has two methyl groups on C4 and C6 whereas SCP has a chlorine atom on C5, so the molecular structure of SMZ is larger than that of SCP. The primary amine and sulfonamide groups of SMZ interact with the MAA to produce the shape-selective MIP. Because of steric hindrance, however, the stationary phase seemed to interact more strongly with the chlorine atom of SCP causing it to be retained for longer than SMZ. This phenomenon has the effect that the SMZ template is suitable for recognition of SMZ only.

This study shows that HPLC with MIPs as the stationary phase enables analysis of sulfonamides without derivation or purification and with minimum sample preparation, and results in high sensitivity and selectivity. Reversed-phase HPLC methods used for analysis of sulfonamides usually require elaborate sample pretreatment, including extraction of the analytes and removal of interferences, frequently leading to low and variable recovery.

The purpose of the work reported here was to develop MIP for use as stationary phases in HPLC for determination of sulfonamides in different drug formulations. Most imprinted polymers have been synthesized using EGDMA as cross-linker and MAA as functional monomer for low-

temperature UV-light-initiated or high-temperature thermally initiated polymerization [19,20]. The MIPs studied here were synthesized in chloroform at low temperature. Chloroform is a poor hydrogen-bonding solvent; this promotes high selectivity and strong binding that relies on hydrogen-bonding and dipole–dipole interactions between the functional monomer and SMZ. To remove the imprint molecule from the polymer particle after polymerization it is necessary to break the non-covalent bond between the SMZ molecule and the carboxyl group of the MIP. Thus MIPs have high selectivity for SMZ and SCP when used as stationary phases for HPLC.

Because the template SMZ used for MIP preparation in this work has a simple structure, the mobile phase is very important in the resolution of the template and SCP. As shown in Table II, five different mobile phases were used. It is apparent from the results that the optimum proportion of buffer solution to acetonitrile was 2:1 (*v*/*v*). In this study the mobile phase was a mixture of buffer solution and acetonitrile. A mobile phase containing polar substances was used to weaken the interaction between the target molecules and the stationary phase and release them from the imprinted cavity. It is believed acetonitrile molecules were likely to bind to the stationary phase and displace the target molecules; as a result, the template molecules are eluted from the column.

Calibration and Limits of Detection and Quantification

The linearity of the calibration plot for SMZ and SCP in acetonitrile was determined. Calibration plots were established for SMZ or SCP by use of standard solutions of seven different concentrations (0.2, 0.3, 0.4, 0.5, 0.6, 0.7, and 0.8 g L^{-1}). Straight lines passing through the origin with correlation coefficients (r^2) of 0.9985 and 0.9973, respectively, were obtained; this indicates the linearity of the calibration plots was adequate. Limits of detection (LOD) and quantitation (LOQ) were calculated from

Table III

Calibration data from use of MIPs as the stationary phase for HPLC

Analyte	Calibration equation	r^2	Calibration range (g L^{-1})	LOD (g L^{-1})	LOQ (g L^{-1})
SMZ	$y = -0.031 + 7.283x$	0.9985	0.2–0.8	0.035	0.117
SCP	$y = 0.0026 + 6.258x$	0.9973	0.2–0.8	0.041	0.137

The calibration plot relates peak area (y) to the concentration of the analyte (x) in g L^{-1}. The number of calibration points, n, = 7 and r^2 is the correlation coefficient

chromatogram peak areas as the concentrations for which the signal-to-noise ratios were 3 and 10, respectively. The regression data, LOD, and LOQ are given in Table III.

The SMZ and SCP content of tablets from a local pharmacy were quantified by the HPLC method described above; the results are shown in Table IV. Use of a molecularly imprinted polymer as stationary phase with a mobile-phase flow rate of 1.0 mL min^{-1} resulted in a total analysis time of less than 12 min. Accuracy was 0.36 to 0.72% for the SMZ tablets and 0.28 to 0.68% for the SCP tablets. This is an advantage over the current methods of analysis, which require separate quantitation of the sulfonamides.

Table IV

Results from quantitative analysis of SMZ and SCP in tablet formulations

Sample[a]	Amount found (g per tablet)	*RSD*[b] (%)	Accuracy[c] (%)
SMZ tablet (25 mg per tablet)			
SMZ tablet 1	25.12	98.26 ± 0.17	0.48
SMZ tablet 2	25.09	101.43 ± 0.26	0.36
SMZ tablet 3	24.82	96.56 ± 0.05	0.72
SCP tablet (25 mg per tablet)			
SCP tablet 1	24.92	96.80 ± 0.63	0.32
SCP tablet 2	25.07	96.78 ± 0.92	0.28
SCP tablet 3	24.83	96.65 ± 0.32	0.68

[a] SMZ, sulfamethazine; SCP, sulfachloropyridazine
[b] Relative standard deviation. *RSD* values were estimated from repeatability
[c] Accuracy (%) = [(Amount found − amount added)/amount added] × 100

CONCLUSIONS

Molecularly imprinted polymers were synthesized and their molecular recognition properties were studied. Validation of the use of a molecularly imprinted polymer as the stationary phase for HPLC determination of SMZ and SCP confirmed the satisfactory behavior of the procedures used. Results obtained from drug analysis indicate the method is suitable for routine tests. Our experience shows that this method could be used for direct determination of sulfonamides in tablets or in food residues with low cost and high speed.

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