**Optimal Conditions for Extraction and Simultaneous Determination of Sulfamethoxazole and Trimethoprim in Pharmaceuticals by Micellar Electrokinetic Capillary Chromatography**

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**ABSTRACT**

A micellar electrokinetic capillary chromatography was performed at 25°C and 30 kV (under pressure of 15 mbar), with 30 mM borate buffer (pH 9.0), 60 mM sodium dodecysulfate, and 10% (v/v) ethanol as background electrolyte for the determination of sulfamethoxazole and trimethoprim. UV detection was at 205 nm. Recoveries were optimal and acceptable after extraction with ethanol / deionized water (1:1, v/v) for both investigated compounds from laboratory mixtures of standards. The method was shown to be specific, accurate (recoveries were 99.9 ± 0.4% for sulfamethoxazole and 99.8 ± 0.3% for trimethoprim), linear over the tested ranges (correlation coefficients ≥ 0.9990) and precise (RSD below 0.6%). The method was applied to determine sulfamethoxazole and trimethoprim in tablets, powder for cutaneous use and solution for infusion.

Key words: sulfamethoxazole,trimethoprim, LLE, micellar electrokinetic capillary chromatography (MEKC), pharmaceuticals

**INTRODUCTION**

Co-trimoxazole is a fixed anti-microbial combination of two chemotherapeutics, sulfonamide sulfamethoxazole (SUL) and trimethoprim (TRI), in a 5:1 (w/w) ratio. Sulfamethoxazole is an antibiotic of broad spectrum that competitively inhibits the bacterial enzyme dihydropteroate synthetase. Trimethoprim is a dihydrofolate-reductase inhibitor that increases activity of SUL. SUL and TRI are active ingredients in several oral suspensions, solution for infusion, and solid dosage forms (1).

Pharmaceuticals containing SUL and TRI are usually analyzed by three principal methods: TLC, spectrophotometry and HPLC. Agbaba et al. (2) developed simultaneous TLC determination of co-trimoxazole as well as sulfanilamide and sulfanilic acid impurities in pharmaceuticals. SUL and TRI were determined in tablets by ratio spectra derivative spectrophotometry (3), second derivative spectrophotometry in the presence of hydroxypropyl-β-cyclodextrin (4), first derivative ratio spectrometry (5), and a flow injection sensor using Sephadex SP-C-25 for continuous on-line separation and solid phase UV transduction (6). Flow-injection spectrophotometric determination was used for sulfadiazine and sulfamethoxazole in pharmaceuticals and urine (7). Lopez-Martinez et al. (8), performed simultaneous determination of binary mixtures of TRI and SUL or sulphamethoxypyridazine by the bivariate calibration spectrophotometric method. Different HPLC systems were used for determination of SUL and/or TRI in suspensions with methyl- and propyl-paraben (9), serum in human immunodeficiency virus-infected patients (10), bovine milk using an on-line clean-up column (11), sera in donkeys, mules and horses (12), and in tablets after preparing SUL-imprinted polymer in acetonitrile (13). Yang et al. (14) used LC-MS-MS to determine sulfonamides and tetracyclines in water. LC with a fluorescence detector was also successfully used for determination of sulfanamide residues (including SUL) in honey (15), as well as nuclear magnetic resonance spectroscopy for quantitative analysis of miconazole, metronidazole and sulfamethoxazole in pharmaceutical and urine samples (16). Molecular imprinting-chemiluminescence determination of TRI using TRI-imprinted polymer as recognition material was performed for tablets and human urine (17).

Capillary electrophoresis (CE) has become an important liquid separation technique. Capillary zone electrophoresis was used for determination of SUL, sulfadiazine and associated compounds in pharmaceutical preparations (18), as well as for determination SUL and...
TRI in human plasma \( ^{19} \). Continuous on-line concentration based on dynamic pH junction for TRI and SUL by microfluidic capillary electrophoresis combined with flow injection analysis system was demonstrated through the separation and determination of SUL and TRI in pharmaceutical preparations \( ^{20} \).

One of the commonly used CE modes is micellar electrokinetic capillary chromatography (MEKC). It is efficient for the separation of both ionic and neutral analytes. Success of separation is based mainly on appropriate selection of the surfactant. Nevado \( et \ al. \) \( ^{21} \) developed a method for the determination of SUL and TRI and their main metabolites in human serum by MEKC. They used 20 mM borate buffer (pH 9.3), 25 mM sodium dodecylsulphate (SDS) and 5% (v/v) acetonitrile as electrolyte.

According to previous studies no publications for determination of SUL and TRI in pharmaceuticals by MEKC are available.

Experiments in this study have been related to the effects of different parameters such as influence of pH, buffer composition, running voltage, running pressure, concentration of surfactant and organic modifier on the determination of SUL and TRI by MEKC and the method validation, as well as influence of different solvent for extraction on recoveries. The method was tested with extracts from co-trimoxazole tablets (from Serbia and Bangladesh), commercial powder mixture Co-trimox \( ^{\circ} \) for cutaneous use (Serbia) and solution for infusion (from Serbia).

**MATERIALS AND METHODS**

I. Instruments

An Agilent 3D-CE capillary electrophoresis system (Waldbronn, Germany) with a diode-array detector and controlled by HP ChemStation software was used to carry out MEKC. Compounds were determined on a 56 cm (50 cm to the detector) × 50 μm i.d. fused silica capillary (with bubble cell, 150 μm) (Agilent, Waldbronn, Germany).

A Metrohm 691 pH meter by Herisau (Switzerland) was used for pH measurements.

II. Reagents and Solutions

All solvents and reagents were of analytical grade unless indicated otherwise. Solutions were prepared with deionized water (Millie-Q-quality). Sulfamethoxazole and trimethoprim were obtained from Sigma (Deisenhofen, Germany) and Fluka (Buchs, Switzerland), respectively (both USP quality).

Buffer solutions were prepared by dissolving appropriate amount of Na\(_2\)B\(_4\)O\(_7\) in deionized water. The pH was adjusted to 9.0 with HCl. Sodium dodecylsulphate (SDS) was from Riedel-de Haën AG (Seelze, Germany). Na\(_2\)B\(_4\)O\(_7\)·10H\(_2\)O was p.a. from Kemika (Zagreb, Croatia).

Commercial buffer with 50 mM borate and 100 mM SDS, pH 9.3 (Agilent, Waldbronn, Germany), was diluted (the pH was adjusted to 9.0 with HCl) and used for MEKC method after the determination of optimal conditions.

The background electrolyte (BGE) was 30 mM borate buffer, pH 9.0, containing 60 mM SDS and 10% (v/v) ethanol.

III. Preparation of Standard Stock Solutions

Standard stock solutions of SUL and TRI were prepared by weighing 25 mg of the drugs and dissolving in 50 mL of ethanol / deionized water (1:1, v/v). Solutions were stable under refrigeration until use. Solutions were prepared by standard stock solutions preparation, was selected since the same solvent composition has been confirmed as the best for extraction of pharmaceutical products.

IV. Samples

The pharmaceutical formulations Co-trimoxazol \( ^{\circ} \) (tablets) containing 400 mg SUL and 80 mg TRI, Cotrim \( ^{\circ} \) (tablets) containing 800 mg SUL and 160 mg TRI, commercial powder mixture Co-trimox \( ^{\circ} \) for cutaneous use containing SUL:TRI in ratio 5:1 (w/w), and Bactrim \( ^{\circ} \) (ampoules) containing 400 mg SUL and 80 mg TRI per 5 mL ampoule dissolved in ethanol / water for injections (1:9, v/v) were obtained from Jugoremedija (Zrenjanin, Serbia), Square Pharmaceuticals LTD Bangladesh (Dhaka, Bangladesh), SB Trade (Belgrade, Serbia) and Galenika a.d. (Belgrade - Zemun, Serbia), respectively.

V. Sample Preparation and Extraction

SUL and TRI were extracted from the tablets using the following procedure. First, 20 tablets from Jugoremedija (average weight 599.62 ± 9.88 mg) or 20 tablets from Square Pharmaceuticals (average weight 1098.65 ± 8.15 mg) were accurately weighed, finely ground to powder and thoroughly mixed. Amounts corresponding to 24 mg of declared active principle (calculated as 20 mg of SUL and 4 mg of TRI), as well as commercial mixture powder were weighed and transferred into a plastic volumetric flask. Samples were four-fold extracted (4 × 5 mL) with ethanol / deionized water (1:1, v/v) by shaking and storage in an ultrasonic bath for 15 min. The extracts were combined, filtered (0.22 μm nylon filter), transferred to 25-mL volumetric flask, and filled up with ethanol / deionized water (1:1, v/v). Different known aliquots (10 – 375 μL) were placed in 1.5 mL calibrated vials and filled up to volume by automatic pipette with running buffer.

Ampoule of 5 mL with SUL and TRI first was diluted with 5 mL of absolute ethanol, without extraction, and different known volumes were diluted with running buffer.
VI. Operating Conditions

The capillary was conditioned prior to its first use by flushing with 0.1 M NaOH for 20 min and then with water for 10 min. The capillary was conditioned using the optimized method at the beginning of each day with methanol under high pressure for 3 min, water for 0.5 min, and then rinsed for 2 min with 0.1 M NaOH and 3 min with background electrolyte. Conditioned procedure was followed by hydrodynamic sample injection at 600 mbars. Assays were carried out at 30 kV and 25°C (under a running pressure of 15 mbar) in 10 min, and the current was 63-65 µA. The analytes were monitored and quantified at each maximum absorption wavelength in order to obtain the maximum signal-to-noise ratio. Accordingly, the selected wavelengths were 205 and 274 for SUL and 207, 230 and 280 nm for TRI. The selected maximum absorption for both investigated compounds was at 205 nm.

RESULTS AND DISCUSSION

I. Optimization of Extraction Conditions

According to the 4th European Pharmacopoeia, SUL is practically insoluble in water, freely soluble in acetone, sparingly in alcohol and slightly in ether, where as TRI is very slightly soluble in water and slightly in alcohol (22).

SUL and TRI were usually extracted from pharmaceuticals with ethanol / deionized water mixtures in different ratios (3, 4, 6, 18, 20) or with ethanol / ammonium buffer solution 2:5 (v/v) (8). Methanol and acetonitrile were also used as solvents for extraction of SUL and TRI from pharmaceuticals (9, 13).

Influences of organic solvent on recoveries were developed. Methanol, ethanol and acetonitrile were tested in concentrations from 0 to 100%. Extraction solutions were prepared by mixing organic solvent with deionized water. The influence of organic solvent on the recoveries is shown in Figure 1. The results demonstrate that type and percent of solvent have influence on recoveries of SUL and TRI. A ratio of 1:1 (v/v) ethanol / deionized water was found to give the best recoveries for both drugs.

A. Influence of pH and Buffer on the Separation

The influence of pH was examined over the range of 6.0 to 10.0 using phosphate buffer and borate buffer, respectively, as electrolyte in deionised water and adjusting with HCl or NaOH to the required pH. The results show that determination was best at pH 9.0 with borate buffer. The borate and phosphate buffers were varied from 5 to 50 mM using the experimental conditions mentioned above. A 30 mM concentration of borate buffer was considered suitable peak shape without shoulders, as well as the best resolution of peaks.

B. Influence of the Organic Modifier and SDS Concentration

At low concentration of surfactant, the main problem was the same or very similar migration times of investigated compounds. The peaks in preliminary studies overlapped and showed shoulders with higher SDS concentration. Also, the symmetry of the peaks was not good. Addition of organic modifier can be essential for purity of peak and quality of separation. Ethanol (Figure 2A), methanol (Figure 2B) and acetonitrile (Figure 2C) were tested in concentrations from 0 to 15%. The presence of a 10% (v/v) of ethanol in the BGE resulted in better resolution and removal of shoulders (Figure 3). SDS concentration was tested from 10 to 100 mM, and a concentration of 60 mM was found to give the best shape and resolution (Figure 4).

C. Influence of Running Pressure, Voltage and Temperature

Running pressure was tested in the range 0-30 mbar using the above experimental conditions. Migration times were slightly decreased, with increasing pressure. Optimal
Figure 2. Effect of: (A) ethanol, (B) methanol, (C) acetonitrile, as organic modifier on migration time and composition of separation SUL and TRI. The BGE was 30 mM borate buffer, pH 9.0, containing 60 mM SDS, the temperature and voltage were 25°C and 30 kV, respectively, with running pressure of 15 mbar.

Figure 3. Effect of ethanol concentration (5, 10 and 15%, v/v) in 30 mM borate buffer, pH 9.0, containing 60 mM SDS. Temperature and voltage were 25°C and 30 kV, respectively, with running pressure of 15 mbar. UV detection was set at 205 nm. Electropherograms were obtained for 0.2 mg/mL of SUL and 0.04 mg/mL of TRI from pharmaceuticals.
A BGE of 30 mM borate (pH 9.0), containing 60 mM sodium dodecysulfate and 10% (v/v) ethanol, without running pressure at 25°C, was used for the determination of running voltages effects in the range 5-30 kV. An acceptable level of baseline noise was achieved by performing experiments at 25°C and 30 kV.

A micellar electrokinetic capillary chromatography was performed at 25°C and 30 kV (under pressure of 15 mbar), using 30 mM borate buffer (pH 9.0) containing 60 mM sodium dodecylsulfate (SDS) and 10% (v/v) ethanol, as background electrolyte for separation of SUL and TRI with resolution of 2.4 and 1.6 for standards (Figure 5A) and tested samples (Figure 5B), respectively. UV detection was carried out at 205 nm. From the data obtained at 205 nm, the electrophoretic mobilities of SUL and TRI in standard solutions were $1.63 \times 10^{-4}$ cm$^2$/Vs and $1.54 \times 10^{-4}$ cm$^2$/Vs, as well as in test samples were $1.62 \times 10^{-4}$ cm$^2$/Vs and $1.51 \times 10^{-4}$ cm$^2$/Vs, respectively.

### III. Validation of the Test Method

Validation procedures were those described in USP 24[23], the International Conference of Harmonization (ICH) Guidelines[24,25], and other literature[26-28].

**I) Selectivity**

Selectivity of the method was investigated by observing interfering peaks from matrix present in the pharmaceuticals. Four different matrices were tested. There was no interference in MEKC results by the matrices ingredients in any of the tested sample, indicating that the method is selective (Figure 5).

**II) Linearity**

Linearity of the assay was determined by analysis of at least five different concentrations of standards[24,25]. Linearity was checked in range 0.5 to 200 mg/L for each

![Figure 4](C:\HPCHEM\1\DATA\BAKTRIM\STAND02.D)

![Figure 5](C:\HPCHEM\1\DATA\BAKTRIM\STAND61.D)

**Figure 4.** Effect of SDS concentration (20 – 100 mM) in 30 mM borate buffer, pH 9.0, containing 10% (v/v) ethanol. Temperature and voltage were 25°C and 30 kV, respectively, with running pressure of 15 mbar. UV detection was set at 205 nm. Electropherograms were obtained for 0.2 mg/mL of SUL (higher peak) and 0.04 mg/mL of TRI from pharmaceuticals.

**Figure 5.** Electropherograms obtained for (A) 0.05 mg/mL of both standards (B) 0.2 mg/mL of SUL and 0.04 mg/mL of TRI from pharmaceuticals; under the optimized conditions, at 205 nm. The BGE was 30 mM borate buffer, pH 9.0, containing 60 mM SDS and 10% (v/v) ethanol, the temperature and voltage were 25°C and 30 kV, respectively with running pressure of 15 mbar.
investigated compound (0.5, 5.0, 10.0, 25.0, 50.0, 75.0, 100.0, 125.0, 150.0 and 200.0 mg/L). Linearity of calibration the curves (peak area vs. concentration) for SUL and TRI was established across the concentration ranges 6.5 – 100.0 mg/L and 4.0 – 102.5 mg/L with correlation coefficients of 0.9990 and 0.9992, respectively (Table 1).

(III) Limit of Detection (LOD) and Limit of Quantification (LOQ)

LOD and LOQ were estimated by the baseline noise method. Baseline noise was evaluated by recording the detector response over a period of ten times the peak width. LOD and LOQ, were defined as the analyte concentrations resulting in peaks of height three and ten times the baseline noise level, respectively(29). LOD and LOQ are 1.3 mg/L for TRI and 1.8 mg/L for SUL and 3.4 mg/L for TRI and 5.9 mg/L for SUL, respectively (Table 1).

(IV) Accuracy and Precision

Accuracy of the method was determined by analyzing solutions of known concentrations (working standard solutions) and comparing the measured and known values. The mean recoveries for SUL and TRI were 99.9 ± 0.4% and 99.8 ± 0.3% (n = 5 for each of presented concentration), proving a good accuracy of the method (Table 2).

Although precision can be measured as repeatability, reproducibility, and intermediate precision, this study investigated only repeatability and intermediate precision.

(V) Repeatability

A repeatability test was performed to determine intra-day variation in peak’s areas and migration times. Standard solutions of concentrations 25, 50 and 100 mg/L (n = 6) were analyzed (Table 3). The RSD values for migration times (0.15% for SUL and 0.25% for TRI) and for peak areas (0.19-0.57% SUL, 0.31-0.36% TRI) indicate that repeatability of the method is acceptable.

(VI) Intermediate Precision

Intermediate precision was evaluated over three days (inter-day repeatability) using working solutions (concentrations 10-100 mg/L). These solutions were injected daily under the same conditions and the results were used for the repeatability study. The solutions were stored at room temperature (25 ± 2°C) in sunlight, decreasing recovery values from 99.9 to 98.3% for SUL and 100.1 to 97.5% for TRI in ethanol / deionized water (1:1, v/v). When stored in refrigerator in the dark, the recovery ranged from 100.1 to 99.5% over three days for both drugs. The RSD values (0.11-0.24% for SUL and 0.13-0.29% for TRI) indicate that the intermediate precision is acceptable.

(VII) Robustness

The optimum MEKC conditions set for this method have been slightly modified in order to evaluate the robustness. The effects of different concentrations of SDS (30 ± 1 mM), organic modifier (10 ± 0.5% ethanol) in the BGE, buffer pH (9.0 ± 0.06), capillary temperature (25 ± 5°C), running pressure (15 ± 1 mbar), running voltage (30 ± 1 kV), and detection wavelength (± 3 nm) were determined. The fractional factorial design(29) was applied. No significant variations in accuracy, specificity and precision were found over the tested ranges, which indicated that the method conditions are robust.

| Table 1. Statistical parameters of the calibration curve for SUL and TRI (linear regression), with LODs and LOQs |
|-----------|--------------|--------------|-----------|
|          | SUL          | TRI          |           |
| Intercept| 17.268 ± 3.2 | -21.335 ± 4.5|           |
| Slope    | 6570.7 ± 17.3| 11835.0 ± 23.1|           |
| Correlation coefficient | 0.9990 | 0.9992 |
| Linear range (mg/L) | 6.5 – 100.0 | 4.0 – 102.5 |
| LOD (mg/L) | 1.8 | 1.3 |
| LOQ (mg/L) | 5.9 | 3.4 |

| Table 2. Determination of accuracy in samples of known concentration of SUL and TRI |
|-----------|------|----------|------|------|
| Theoretical concentration (mg/L) | SUL | TRI | SUL | TRI |
| 10 | 9.98 | 9.95 | 99.80 | 99.50 |
| 20 | 19.94 | 19.96 | 99.70 | 99.80 |
| 30 | 30.11 | 29.87 | 100.37 | 99.57 |
| 40 | 39.79 | 40.07 | 99.48 | 100.17 |
| 50 | 50.13 | 50.01 | 100.26 | 100.02 |

Mean ± SD 99.92 ± 0.38 99.81 ± 0.29

| Table 3. Determination of repeatability |
|-----------|------------|-----------|
| Theoretical concentration (mg/L) | Migration timea (min) | Peak areaa (mAU) |
| SUL | | |
| 25 | 8.546 ± 0.009 | 181.29 ± 0.34 |
| 50 | 8.542 ± 0.013 | 342.45 ± 4.73 |
| TRI | | |
| 25 | 9.217 ± 0.012 | 275.45 ± 0.86 |
| 50 | 9.196 ± 0.027 | 569.41 ± 2.09 |

aMean ± SD (n = 6).
III. Drugs Stability

Stability of SUL and TRI in ethanol / deionized water (1:1, v/v) solutions was checked at room temperature (25 ± 2°C) for 72 hr and the recoveries were 101.2 ± 0.3% and 100.8 ± 0.4%, respectively. Stability in the same above mentioned solvent was also checked at 72 hr at 4°C (refrigerator). Recovery was 100.1 ± 0.2% for SUL and 99.9 ± 0.2% for TRI, indicating good stability. For exact results, samples have to be prepared in ethanol / deionized water (1:1, v/v) and refrigerated until usage.

IV. Application

The proposed assay method of investigated compounds was applied for quality control of different pharmaceutical products. The Serbian pharmaceutical industry currently has three different commercial formulations containing SUL and TRI, tablets, commercial powder mixture for cutaneous use and solution for infusion. Also, tablets from Bangladesh have been analyzed for the comparison with European products.

In the analysis of the commercial products, the found amounts and recoveries were determined by calibration curves of standards solution. The results show agreement between the declared and found values (Table 4).

The advantage of this method over the HPLC and TLC methods described in literature\(^2,9,13\) for analysis SUL and TRI in pharmaceuticals is its lower running costs and higher environmental friendliness. An HPLC analysis with flow-rate of 1.0\(^{13}\) or 1.8\(^9\) mL/min and analysis time of 15 min each, requires 15 or 27 mL of acetonitrile/water or acetonitrile/water/triethanolamine as the mobile phase, respectively. In the developed and proposed method, 20-30 analyses with MEKC require 3 mL of borate buffer containing SDS and 10% (v/v) ethanol, while 20 analyses by HPLC require 300 or 540 mL of mobile phase. Another advantage of proposed method over SPF methods\(^3,4,8\) for analysis SUL and TRI in pharmaceuticals is better accuracy of MEKC. On the other hand, a disadvantage of developed MEKC method is lower sensitivity in contrast to SPF methods.

Table 4. Application results

<table>
<thead>
<tr>
<th>Tested sample</th>
<th>Amount expected (mg)</th>
<th>Amount found (mg)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SUL</td>
<td>TRI</td>
<td>SUL</td>
</tr>
<tr>
<td>Co-trimoxazol®</td>
<td>400</td>
<td>80</td>
<td>406.5 ± 0.8</td>
</tr>
<tr>
<td>Cotrim®</td>
<td>800</td>
<td>160</td>
<td>804.1 ± 0.6</td>
</tr>
<tr>
<td>Co-trimox®</td>
<td>5</td>
<td>1</td>
<td>4.9 ± 0.1</td>
</tr>
<tr>
<td>Bactrim®</td>
<td>400</td>
<td>80</td>
<td>399.8 ± 0.2</td>
</tr>
</tbody>
</table>

CONCLUSIONS

The new experimental condition for MEKC is presented as a useful technique for rapid determination of SUL and TRI using SDS as surfactant (60 mM) and ethanol 10% (v/v) as organic modifier and borate buffer (30 mM, pH 9.0). A running pressure of 15 mbar was applied and it gives the best shape of peaks. This system was also applied successfully to their SUL and TRI identification and determination in pharmaceuticals as tablets, powder and solution for infusion. Ethanol / deionized water (1:1, v/v) was essential for efficient extraction and recoveries over 99.8% for both analysed drugs.

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