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Short communication

Rapid and simultaneous determination of sulfamethoxazole and trimethoprim in human plasma by high-performance liquid chromatography

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Abstract

A simple and reproducible high-performance liquid chromatographic method was developed for simultaneous determination of sulfamethoxazole (SMX) and trimethoprim (TMP) in human plasma. The method entailed injection of the samples after deproteination with perchloric acid and subsequent neutralizing. Primidone was used as internal standard. Chromatography was performed on a C_{18} column (250 mm × 4.6 mm, 5 μm) under isocratic elution with 50 mM aqueous sodium dihydrogen phosphate–acetonitrile–triethylamine (100:25:0.5, v/v), pH 5.9. Detection was made at 240 nm and analyses were run at a flow-rate of 1.5 ml/min at a temperature of 35 °C. The recovery was 83.4, 88.5 and 98.2% for TMP, SMX and internal standard, respectively. The precision of the method was 2.6–9.8% over the concentration range of 0.125–2 μg/ml for TMP and 0.39–50 μg/ml for SMX. The limit of quantification (LOQ) in plasma was 0.125 and 0.39 μg/ml for TMP and SMX, respectively. The method was used for a bioequivalence study.

Keywords: Sulfamethoxazole; Trimethoprim

1. Introduction

Co-trimoxazole, a broad-spectrum antimicrobial combination of sulfamethoxazole (SMX) and trimethoprim (TMP) (Fig. 1) is used in the treatment of many common infections such as urinary and respiratory tract infections. SMX inhibits bacterial synthesis of dihydrofolic acid by competing with para-aminobenzoic acid. TMP blocks the production of tetrahydrofolic acid from dihydrofolic acid by binding to and reversibly inhibiting the required enzyme, dihydrofolate reductase [1].

Simultaneous determination of SMX and TMP in biological fluids by high-performance liquid chromatography has been the subject of several investigations [2–10]. In spite of structure dissimilarity between SMX and TMP, organic solvent [2,3] or solid-phase [4,5] extraction have been developed but they are time-consuming and may show variable recoveries at different concentrations [3,5]. A column-switching method [6] has the problem of the availability of the required instruments. Plasma protein precipitation [7–10] provides a simple and fast sample processing, but subsequent chromatographic separation with sufficient sensitivity is not easily achieved. Different authors have reported methods requiring either separation of SMX and TMP using two elution solvents and two successive chromatographic operations [8], or insufficient limit of quantification of 0.5 μg/ml for TMP [7,8,11].

In this paper, we investigated various factors affecting separation and sensitivity. The results lead to developing an optimized separation of SMX and TMP as well as the internal standard and the metabolite in plasma after protein precipitation and obtaining sufficient sensitivity and specificity. The method was used for a bioequivalence study of co-trimoxazole tablets in healthy human volunteers.

2. Experimental

2.1. Reagents

SMX (Virchow Laboratories, Hyderabad, India), TMP (Behdashtkar Co., Rasht, Iran) and primidone (Sigma, ST Louis, MO 63178, USA) was kindly provided by Sobhan Pharmaceuti-

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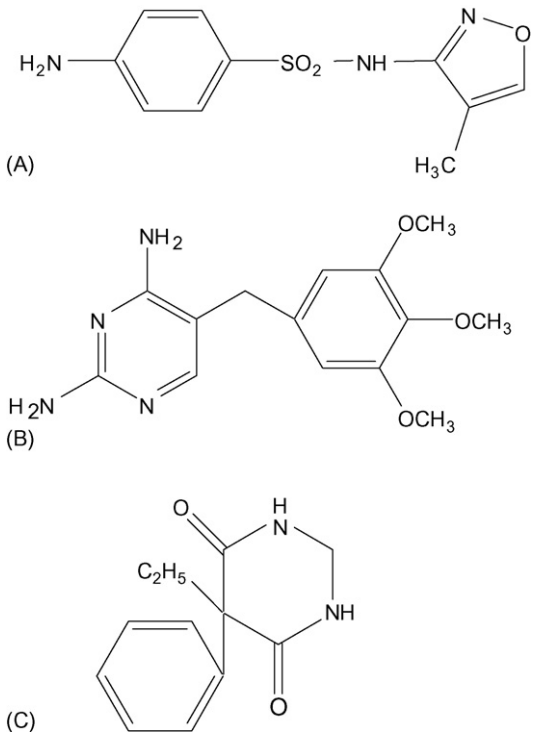

Fig. 1. Chemical structure of sulfamethoxazole (A), trimethoprim (B) and the internal standard primidone (C).

cal Co. (Rasht, Iran). Analytical grade phosphoric acid, sodium dihydrogen phosphate, dipotassium hydrogen phosphate, triethylamine and HPLC grade methanol were purchased from E. Merck (Darmstadt, Germany). HPLC grade acetonitrile and analytical grade 65% perchloric acid was obtained from Carlo Erba Reagenti (Rodano, Italy). All other reagents were of analytical grade.

2.2. Instrumentation

The analyses were performed on a Shimadzu chromatographic system (Kyoto, Japan) equipped with an LC-6A solvent delivery pump, SPD-10AVP ultraviolet detector (operated at 240 nm), CTO-6A column heater, a Rheodyne 7725 injector and C-R8A integrator. A Shimadzu C_{18} vp column (250 mm × 4.6 mm i.d.; 5 μm particle size) was used for the chromatographic separation. The mobile phase comprised of 50 mM aqueous sodium dihydrogen phosphate–acetonitrile–triethylamine (100:25:0.5, v/v), pH 5.9. Analyses were run at flow rate of 1.5 ml/min at 35 °C.

2.3. Standard solutions

The internal standard (primidone), SMX and TMP were dissolved in methanol to make concentrations of 0.8, 5 and 0.1 mg/ml, respectively, and stored at −20 °C.

2.4. Calibration curves and quantitation

Calibration curves were constructed by plotting peak height ratio (y) of SMX or TMP to the internal standard versus SMX or TMP concentrations (x). A linear regression was used for quantitation. Calibration standards were prepared in human plasma by spiking a pool of plasma to a known concentration and then serially diluting it with blank plasma to attain the desired concentration range 0.125–2 μg/ml for TMP and 0.39–50 μg/ml for SMX. The first concentration of TMP (2 μg/ml) and SMX (50 μg/ml) have been combined in one plasma sample and then by serially diluting it, individual standards of 1, 0.5, 0.25 and 0.125 μg/ml for TMP, and 25, 12.5, 6.25, 3.125, 1.56, 0.78 and 0.39 μg/ml for SMX was obtained. The prepared calibration standards (250 μl) were pipetted into 1.5 ml microcentrifuge tubes and stored at −20 °C pending analysis.

2.5. Extraction procedure

A 250 μl volume of plasma was transferred to a 1.5 ml microcentrifuge tube and 20 μl of the internal standard (equal to 16 μg primidone) was added and vortex-mixed for 10 s. Then, 20 μl of 65% perchloric acid were added, vortex-mixed for 30 s and the tube was centrifuged for 5 min at 11,300 × g. The supernatant (100 μl) was transferred to another tube, 50 μl of 2 M dipotassium hydrogen phosphate was added, mixed for 5 s and centrifuged for 1 min at 11,300 × g. A 20 μl aliquot of supernatant was injected onto the HPLC system for analysis.

2.6. Assay validation

Blank human plasma, obtained from 12 healthy volunteers, was assessed by the procedure as described above and compared with spiked plasma samples with SMX and TMP and also plasma samples from volunteers after dosing with co-trimoxazole to evaluate selectivity of the method. The precision and accuracy of the method were examined by adding known amounts of SMX and TMP to pool plasma (quality control samples). Quality control samples were made from a stock solution separate from that used to prepare standards and were not used for constructing calibration curves. For intra-day precision and accuracy five replicate quality control samples at each concentration were assayed on the same day. The inter-day precision and accuracy were evaluated on 5 different days within 2 weeks along analyzing plasma samples of volunteers. The absolute recoveries ($n = 5$) was calculated by comparing peak heights obtained from prepared sample extracts with those found by direct injection of drug solution made in 0.1 M potassium dihydrogen phosphate at the same concentration. The limit of quantification (LOQ) was estimated by analyzing SMX and TMP at low concentrations of the calibration curves. The LOQ was defined as a concentration level where accuracy and precision were still better than 10%. To determine the limit of detection (LOD), lower plasma concentrations than the lower end of the calibration curves were used. The LOD was then defined as the concentration which caused a signal three times the noise (S/N = 3/1).

2.7. Application

The assay was used for a comparative bioavailibility study of two co-trimoxazole tablets containing 400 mg SMX and 80 mg

TMP. The reference product was Bactrim (Roche, Neuilly-sur-Seine, Cedex, France) and the test product was from Sobhan pharmaceutical Co. Twelve healthy volunteers participated in the study. The study was conducted using a two-way crossover design, as a single dose (800/160 mg, two tablets), randomized trial. The two formulations were administrated on two treatment days, separated by a washout period of 7 days, to fasted subjects who received one of the study medications. Food and drinks were not allowed until 3 h after ingestion of the tablet. Multiple blood samples (3 ml) were collected before and 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 10 and 30 h post dosing. The plasma was immediately separated by centrifugation and frozen at −20 °C until analysis.

3. Results and discussion

3.1. Chromatographic conditions

3.1.1. Chromatographic column and mobile phase

The retention of SMX and TMP on Shimpack C_{18}, C_8 and CN columns (250 mm × 4.6 mm; 5 μm) was evaluated with acetonitrile-phosphate buffer as mobile phase. SMX and TMP had similar retention on C_{18} and C_8 columns and shorter retention on CN column. No preference was found between tested columns regarding the separation of interested peaks from plasma interferences. Addition of triethylamine to the mobile phase [12] produced a sharper peak for TMP, although in a constant pH, it slightly decreased and increased the retention of TMP and SMX, respectively. The beneficial effect of triethylamine was likely due to blocking free silanol groups in silica-based column which could improve chromatography of basic drug like TMP. The mobile phase pH had different influences on the retention of TMP and SMX (Fig. 2).

3.1.2. Temperature

To our knowledge, none of the previous investigators have been reported the effects of temperature on the retention of SMX and TMP, and most of the separations have been done on ambient

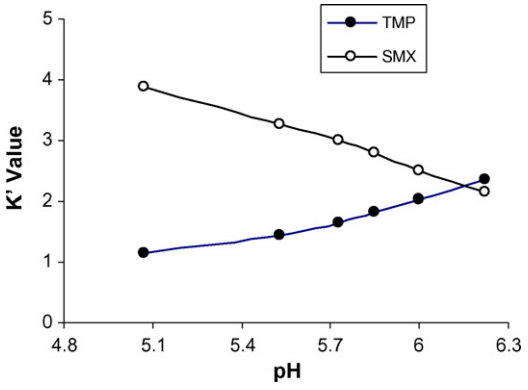

Fig. 2. The effects of the mobile phase pH on the retention of SMX and TMP. The chromatographic conditions were as follows: column, C_{18} (250 mm × 4.6 mm, 5 μm); mobile phase, 50 mM NaH_2PO_4–acetonitrile–triethylamine (100:25:0.5, v/v); *t*, 50° C; flow rate, 1.5 ml/min; detection, 240 nm.

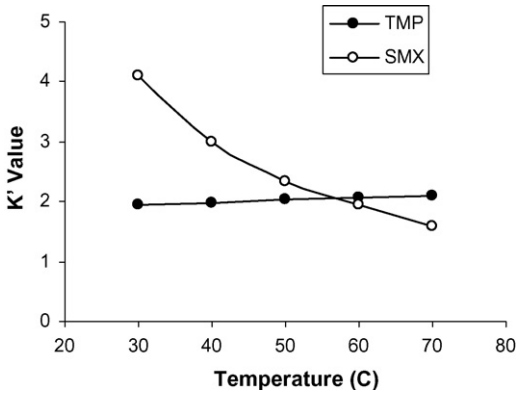

Fig. 3. The effects of temperature on the retention of SMX and TMP. The chromatographic conditions were as follows: column, C_{18} (250 mm × 4.6 mm, 5 μm); mobile phase, 50 mM NaH_2PO_4–acetonitrile–triethylamine (100:25:0.5, v/v) pH 6; flow rate, 1.5 ml/min; detection, 240 nm.

temperature [2–12]. Interestingly, we found that temperature had a dual effect on the retention of TMP and SMX (Fig. 3). While the retention of SMX is rapidly decreased by elevating temperature, the retention of TMP is slightly increased in the same condition. It has been reported that retention times of some bases shows anomalous increases with column temperature [13,14]. The most possible explanation is that the pK_a of bases generally decreases with increasing temperature [15]. At constant pH, as the strength of the base decreases (pK_a becomes smaller), bases will become less protonated (increasingly neutral) which will result in increased retention by hydrophobic processes. Alternatively, ionic retention caused by interactions between solute and silanols should be reduced [13]. This finding is especially useful for TMP, which is usually eluted earlier and has a higher risk to be interfered with plasma endogenous peaks. In the present method, chromatography at temperature of 35 °C produced the best separation.

3.1.3. Optimum UV wavelength

The optimum UV wavelength of the present study (240 nm) was selected on the basis of higher sensitivity for TMP (which had much lower plasma concentrations than SMX) and less plasma interferences. Higher UV wavelengths such as 280 nm [3,11] was associated with less plasma interferences, however, the sensitivity for TMP was not satisfactory. TMP had a better UV absorbance at 225 nm [7,12], but interferences from plasma are relatively excessive at this wavelength. SMX had a relatively good UV absorbance in all tested wavelengths.

3.1.4. Sample preparation

Since direct injection of supernatant of plasma after precipitation of proteins with perchloric acid could damage chromatographic column, a simple and efficient neutralizing method for the acidic supernatant was used. The salt of dipotassium hydrogen phosphate was very soluble in water, but potassium perchlorate had a limited solubility in water and mostly was removed by centrifugation. Using this method and without using guard column, the column pressure was just increased slightly after injection of more than 300 samples.

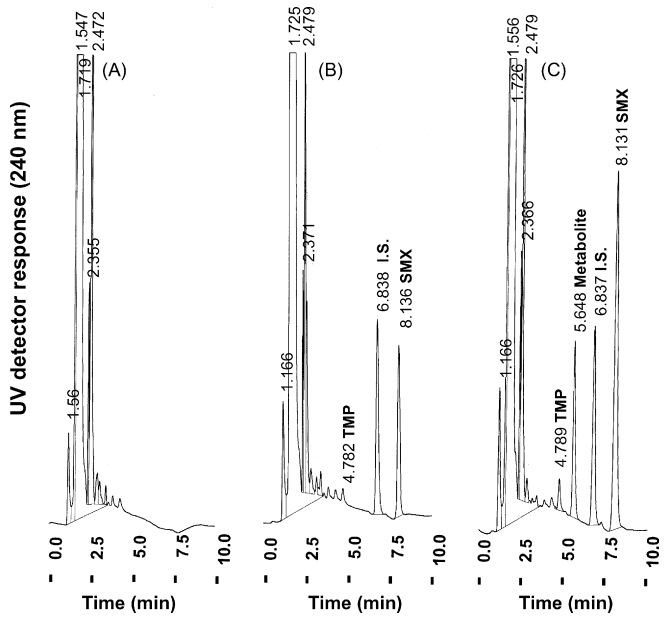

Fig. 4. Representative chromatograms of (A) a blank plasma, (B) a plasma spiked with 0.125 μg/ml TMP and 3.125 μg/ml SMX and (C) a plasma sample from a volunteer, 30 h after dosing with co-trimoxazole (800 mg SMX/160 mg TMP). The concentrations of TMP and SMX in the sample were 0.324 and 6.43 μg/ml, respectively. The retention times of TMP, metabolite, I.S. and SMX were 4.8, 5.6, 6.8 and 8.1 min, respectively. Attenuation, 2; chart speed, 4 mm/min.

3.1.5. Selection of internal standard

The internal standard primidone was selected due its suitable retention time, recovery and lack of interference with endogenous peaks. It had a relatively neutral nature, was not affected by the mobile phase pH and was affected with lower rate by temperature in comparison with SMX. These phenomena helped its good separation from other peaks.

3.2. Method validation

Representative chromatograms of drug-free plasma, plasma spiked with SMX and TMP, and a volunteer sample 30 h after administration of a single dose of co-trimoxazole are shown in Fig. 4. The retention times for TMP, internal standard and SMX were 4.8, 6.8 and 8.1 min, respectively. The analysis run time was less than 9 min. No interfering peaks from the endogenous plasma components were observed in the retention time of SMX, TMP or internal standard in blank plasma samples from 12 volunteers. All analyzed volunteer samples showed a metabolite peak at 5.6 min.

The calibration curves in plasma were linear over the concentration range of 0.125–2 μg/ml for TMP and 0.39–50 μg/ml for SMX. The linearity of this method was statistically confirmed. For each calibration curve, the intercept was not statistically different from zero. The correlation coefficients (r) for calibration curves were better than 0.999. The slops of plasma standard curves in the five different preparations were practically the same (the CVs were less than 1.8 and 2.5% for the slopes of plasma standard curves of TMP and SMX, respectively).

The LOQ was 0.125 and 0.39 μg/ml, and the LOD was about 0.1 and 0.03 μg/ml for TMP and SMX assay, respectively. The values obtained for intra-day and inter-day precision and accuracy during the 5-day validation for plasma are shown in Table 1. The mean absolute recoveries for TMP, SMX and internal standard using the present extraction procedure were 83.4, 88.5 and 98.2%, respectively.

3.3. Pharmacokinetic results

The method was successfully applied for a comparative bioavailability study and mean plasma concentration-time profiles for TMP, SMX and metabolite following administration of

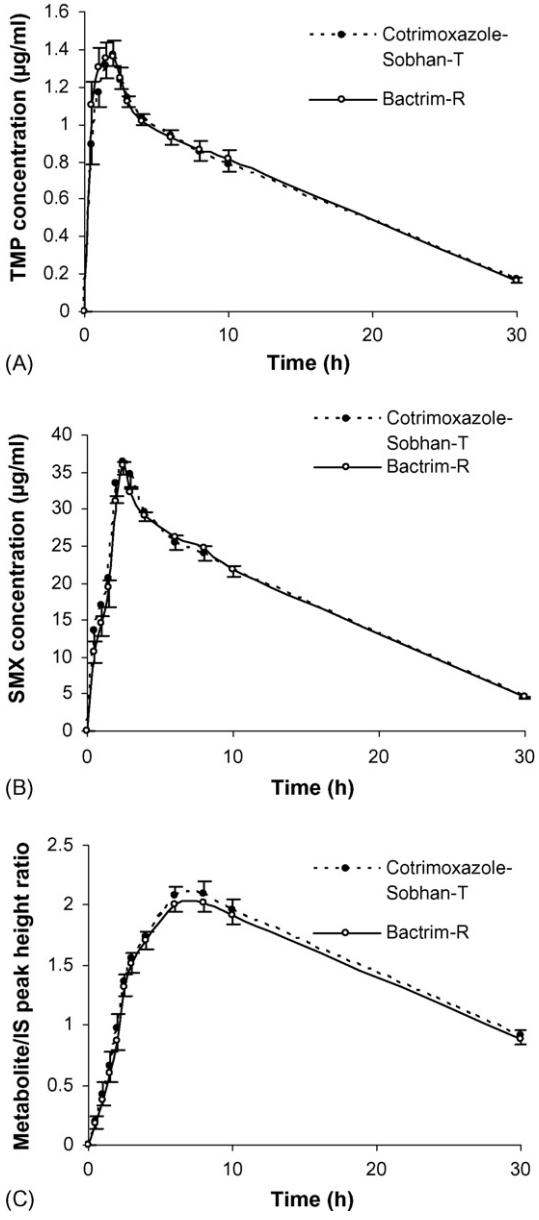

Fig. 5. Mean (±S.E.M.) plasma concentration-time profiles of TMP, SMX and metabolite in 12 healthy volunteers following a 800/160 mg oral dose of two different formulations of co-trimoxazole (Bactrim vs. test formulation from Sobhan Pharmaceutical Co.) in a two-way crossover study.

Table 1
The intra- and inter-day precision and accuracy, and recovery data for the measurement of TMP (A) and SMX (B) in human plasma ($n = 5$)

	Recovery (%)	Intra-day			Inter-day		
		Mean ± S.D.	Precision (%)	Accuracy (%)	Mean ± S.D.	Precision (%)	Accuracy (%)
(A) Nominal TMP concentration (μg/ml)							
0.125	81.3 ± 6.1	0.129 ± 0.010	7.7	3.2	0.123 ± 0.012	9.8	−1.6
0.5	85.2 ± 5.2	0.483 ± 0.02	4.1	−1	0.492 ± 0.03	6.1	−1.6
2	83.7 ± 4.2	2.04 ± 0.09	4.4	2	1.94 ± 0.10	5.2	−3
(B) Nominal SMX concentration (μg/ml)							
0.39	85.3 ± 5.4	0.384 ± 0.03	7.8	−1.5	0.379 ± 0.025	6.6	−2.8
6.25	89.4 ± 2.8	6.29 ± 0.17	2.7	0.6	6.31 ± 0.22	3.5	1
50	90.7 ± 3.4	50.34 ± 1.29	2.6	0.7	49.4 ± 1.51	3.1	−1.2

Table 2
Mean (±S.E.M.) pharmacokinetic parameters of TMP and SMX in 12 volunteers after administration of a single 800/160 mg oral dose of test or reference product

Parameter/prep.	TMP		SMX	
	Test	Reference	Test	Reference
C_{max} (μg/ml)	1.488 (0.06)	1.522 (0.08)	41.30 (1.34)	39.57 (1.04)
t_{max} (h)	1.5 (0.2)	1.5 (0.2)	2.2 (0.2)	2.6 (0.2)
$AUC_{0–30}$ (μg h/ml)	19.36 (0.86)	19.80 (0.87)	516.0 (16.2)	512.7 (18.2)
$AUC_{0–\infty}$ (μg h/ml)	21.85 (1.03)	22.23 (1.08)	581.5 (19.4)	577.7 (21.1)
$t_{1/2}$ (h)	9.7 (0.5)	9.6 (0.5)	9.7 (0.3)	9.6 (0.3)

two formulations is presented in Fig. 5. Table 2 shows the values of pharmacokinetic parameters for two formulations.

4. Conclusion

The present method provides a new HPLC method for the simultaneous determination of SMX and TMP in human plasma based protein precipitation of plasma. Effects of several factors on chromatographic behavior of TMP and SMX are discussed which helps other investigators to reproduce the method and optimize the separation. The method is simple, rapid and sensitive, and is suitable for pharmacokinetic, bioavailability or bioequivalence studies.

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