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Simultaneous determination of sulfamethoxazole and trimethoprim in human plasma by RP-HPLC

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ABSTRACT

A simple and precise reversed-phase high performance liquid chromatography (HPLC) method for simultaneous determination of sulfamethoxazole (SUZ), and trimethoprim (TMP) in human plasma was developed and validated. Using cefmetazole sodium as an internal standard (IS), separation was achieved on Symmetry Shield C18 (4.6×150 mm, 4 μm) column. The mobile phase, 30 mM sodium phosphate (pH 5.8), acetonitrile, and 0.05% triethylamine (83:17:0.05,) v/v, was delivered at a flow rate of 1.2 ml/min. The eluent was monitored using spectrophotometric detection at 235 nm. Plasma samples were precipitated using acetonitrile, and extracts were evaporated and reconstituted in sodium phosphate buffer. No interference in blank plasma or of commonly used drugs was observed. The relationships between the concentrations of TMP and SUZ with their corresponding peak height ratios to the IS were linear over the range of 0.10-6.0 and 1.0-70 μg/ml, respectively. The intra-day and inter-day coefficients of variation were ≤5.2% and 8.7% and ≤7.0 and 11.3% for TMP and SUZ, respectively. The extraction recovery of TMP, SUZ, and the IS from plasma samples were 95, 85, and 80%, respectively. The method was applied to assess the stability of TMP and SUZ under various conditions generally encountered in the clinical laboratory. TMP and SUZ in plasma were stable for at least 24 hr at RT, 8 weeks at -20ºC; and after three freeze-thaw cycles. TMP and SUZ in processed samples were stable at least 24 hr at RT, and 48 hr. at -20ºC. Stock solutions of TMP and SUZ in mobile phase were stable at 24 hr at RT, 8 weeks at -20ºC.

KEYWORDS

Sulfamethoxazole;
Trimethoprim;
Cefmetazole;
RP-HPLC.

INTRODUCTION

Sulfonamides are one of the oldest recognized groups of antibacterial agent. During recent years, these agents have been used in combination with other drugs; such as sulfaquinixaline/pyrimethamine, sulfadiazine/trimethoprim, and sulfamethaoxazole (SUZ)/trimethoprim (TMP) to potentiate their antibacterial effect. The most widely used combination is SUZ and TMP, is effective in treating infections due to various types of bactria[1,2]. Due to the common use of drug combination in formulations and the formation of metabolites there has been need for creating reliable methods that can be used for simultaneously quantification both the drugs.

Several analytical methods have been reported in literature for the determination of SUZ individually or combination with others agents. A spectrophotometeric method based on the Bratton-Marshall procedure has

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been widely used for the determination of total content of sulfonamides[3]. The binary mixture of SUZ and TMP has been studied by spectrophotometric or spectro fluorometeric methods for the simultaneous determination in authentic mixtures and pharmaceutical preparations[4-10]. While most of these methods are simple, they however, still require a lot of data manipulation, making their clinical application difficult. Further, they could not be applied directly for the determination of SUZ and TMP in biological fluids, where endogenous metabolic products and commonly used pharmacological agents may interfere. For routine analysis, where the analyte appears together with other compounds, separation techniques are often required, and HPLC is more commonly used[11-26]. Most of the reported HPLC methods have comparable detection limits, however, require large sample volume. In regard to stability of SUZ and TMP only limited data is available[27-30]. We describe the validation of a simple and reliable HPLC method for the simultaneous quantitative determination of therapeutic levels of SUZ and TMP in 250 µl of human plasma. The method was applied to determine the stability SUZ and TMP under various conditions encountered in the clinical laboratory.

EXPERIMENTAL

Apparatus

Chromatography was performed on Waters HPLC System (Waters Associates Inc, Milford, MA, USA) consisting of an autosampler (717 plus), 515 HPLC pump, and 2487 UV dual 2690 λ absorbance detector. A reversed-phase Symmetry Shield C18 (4.6×150 mm, 4-µm) column in conjunction with a guard Pak pre-column module with Bondapak C18, 4-µm insert were used for separation. The data were collected with a pentium IV computer using empower chromatography manager software.

Chemicals and reagents

All reagents were of analytical-reagent grade unless stated otherwise. Acetonitrile (HPLC grade), triethylamine, Potassium phosphate, and phosphoric acid were purchased from Fisher Scientific, Fairlawn, NJ, USA. Di-potassium hydrogen ortho phosphate was purchased from BDH Chemicals Ltd, Poole, England. Sodium acetate was purchased from Chemia, Switzerland. Water for the HPLC analysis was generated by "reverse-osmosis" using Milli-Q-Water (Millipore Co., Bedford, MA, USA). TMP, SUZ, efmetazole sodium, and dibasic anhydrous sodium phosphate were obtained from sigma-aldrich chemie, Steinheim, Germany.

Chromatographic conditions

The mobile phase composed of 30 mM sodium phosphate (pH 5.8 $\pm$ 0.05, adjusted with phosphoric acid), acetonitrile, and triethylamine (83:17:0.05) (v/v). Before delivering into the system, the mobile phase was filtered through 0.45 µm polyestersulfone membrane and sonicated under vacuum for 5 minutes. The analysis was carried out under isocratic conditions using a flow rate of 1.2 ml/min at room temperature (23^0C) and a run time of 10 minutes. Chromatograms were recorded at 235 nm using a UV detector.

Preparation of stock and working solutions

The stock solutions (1000 µg/ml) of trimethoprim, sulfamethoxazole, and the cefmetazole sodium (internal standard, IS), were prepared separately by dissolving 25 mg each in 25 ml mobile phase. Working solutions of TMP and SUZ were prepared by diluting 500 and 1000 µl of the stock solutions of TMP and SUZ up to 10 ml in blank plasma, to produce working solutions of 50 and 100µg/ml respectively. 1000 µl of stock solution of IS was added to 9 ml of 30 mM sodium phosphate buffer (pH 5.8) to produce a working solution of 100 µg/ml. The working solutions were used within one week of their preparation.

Calibration standards/Quality control samples

Calibration standards were prepared by mixing nine different volumes of TMP and SUZ working solutions in blank human plasma to produce final concentrations in the range of 0.10-6.0 µg/ml and 1-70 µg/ml for TMP and SUZ, respectively. Quality control samples were prepared by mixing four different volumes of TMP and SUZ working solutions in blank human plasma to produce Quality control (QC) samples with final concentrations of 0.1, 0.3, 3.0, and 5.4 µg/ml (TMP) and 1.0, 3.0, 35 and 63 µg/ml (SUZ). Samples were vortexed for 20 seconds then 250 µl aliquots were transferred into 1.5 ml eppendrof microcentrifuge tubes and stored at-20^0C until used.

Sample preparation

To 250 µl of human plasma, calibration standards, or quality control samples placed in a 1.5 ml eppendrof microcentrifuge tubes, 50 µl of the IS working solution (5.0 µg of IS) was added. The solutions were vortexed for 20 seconds and then 375 µl acetonitrile and 100 µl saturated potassium phosphate were added. The solutions were vortexed again for 20 seconds and then centrifuged at 10,000 rpm for 10 min at room temperature (23°C). The clear supernatant solution was colleted and dried under a gentle steam of nitrogen. The dried samples were reconstituted in 250 µl of 30 mM sodium phosphate buffer and 100 µl was auto injected into the HPLC system.

Stability studies

A total of 40 aliquots of the following QC samples were freshly prepared: TMP 0.3 and 5.4 µg/ml and SUZ 3.0 and 63 µg/ml. Five aliquots of each QC sample were extracted and immediately analyzed (baseline), five aliquots were allowed to stand on the bench-top for 24 hours at room temperature before being processed and analyzed (counter stability, 24 hours at room temperature), five aliquots were stored at -20^{0}C for eight weeks before being processed and analyzed (long term freezer storage stability), and five aliquots were processed, reconstituted, and stored at room temperature for 24 hours or 48 hours at -20^{0}C before analysis (autosampler stability). Finally, fifteen aliquots of each QC sample were stored at -20^{0}C for 24 hours. They were then left to completely thaw unassisted at room temperature. Five aliquots of each sample were extracted and analyzed and the rest returned to -20^{0}C for another 24 hours. The cycle was repeated three times (freeze-thaw stability).

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

Figure 1 depicts the chemical structures of the compounds used in the present study. In order to optimize the absorbance wavelength for simultaneous detection of SUZ and TMP, we performed the analysis at 235 nm. Wavelength was selected based on photodiode array (PDA) extracted spectra (Figure 2). A mobile

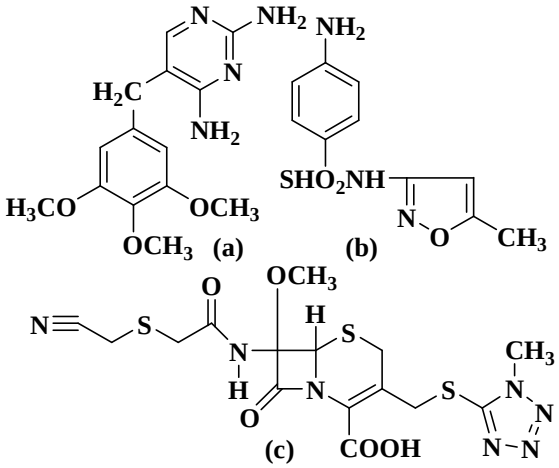

Figure 1: Chemical structures of (a) Trimethoprim, (b) Sulfamethoxazole and (c) Cefmetazole (internal standard, IS)

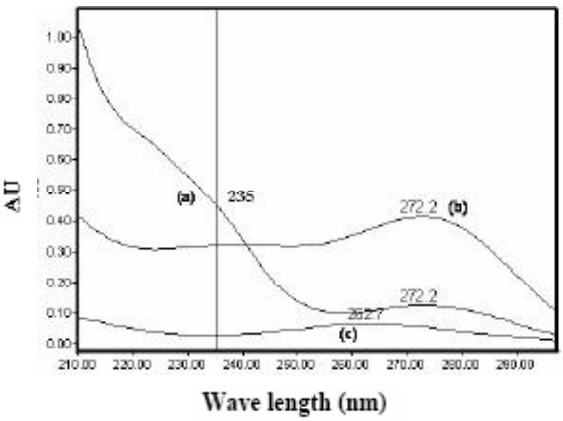

Figure 2: PDA extracted ultraviolet spectra of (a) Trimetho prim (b) Sulfamethoxazole and (c) Cefmetazole (internal standard, IS)

phase composed of acetonitrile and sodium phosphate buffer was initially employed to achieve base-line separation of these compounds and minimize background absorbance. A satisfactory resolution of the peaks of interest was obtained. However, the addition of a small amount of triethylamine improved peak symmetry, resolution, and signal intensity. Under the described conditions, the IS, TMP, and SUZ were well resolved within a run time of 10 minutes, and their retention factors (k′) were 1.42, 2.34, and 4.84, respectively. An Overlay of calibration curve chromatograms of SUZ and TMP is shown in figure 3.

Validation of method

The procedures used for validation are as described in US food and drug administration (FDA) bioanalytical

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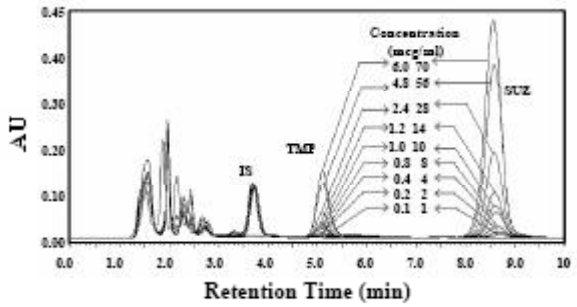

Figure.3: Overlay of Calibration curve chromatograms for simultaneous determination of sulfamethoxazole (SUZ) and trimethoprim (TMP) with IS (Internal standard)

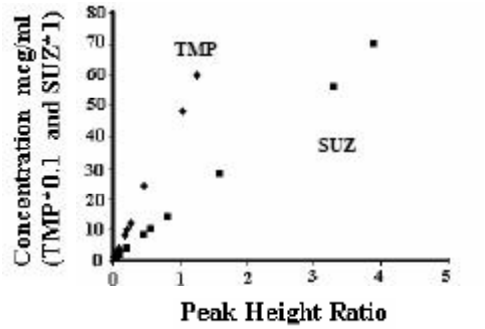

Figure 4: Representative calibration curves of combined trimethoprim (TMP) and sulfamethoxazole (SUZ) assay

method validation guidance[31].

Specificity

To evaluate the assay specificity, we screened for potential interference six batches of human plasma and eight frequently used medications for potential interference, namely: acetaminophen, diclofenac sodium, lansoprazole, ranitidine, nicotinic acid, ascorbic acid, caffeine, and omeprazole. None was found to co-elute with TMP, SUZ, or the IS. Caffeine, ranitidine, nicotinic acid, acetaminophen, ascorbic acid, and omperazole eluted during the run time of the assay. Their retention factors (k′) were 0.74, 0.33, 0.01, 0.77, 0.01, and 6.03, respectively.

Linearity

Linearity was evaluated by analysis of a series of standards at nine different concentrations over the range of 0.10-5.4 μg/ml for TMP and 1.0-70 μg/ml for SUZ. The peak height ratio and concentration of each drug was subjected to regressive analysis. The mean regressive equations obtained were $Y=4.6961\times-0.0152$, $r^2=0.9966$ (n=8) for TMP and $Y=18.0400\times-0.0947$, $r^2=0.9983$ (n=8) for SUZ. Figure 4 depicts representative calibration curves of TMP and SUZ in the combined assay.

Limit of quantification

The limit of quantification (LOQ) was established at an average signal to noise ratio of 10. The LOQ of SUZ and TMP were found to be 1.0 (n=5) μg/ml and 0.10 (n=5) μg/ml, respectively.

Accuracy and precision

Accuracy and precision were determine by applying the method to mixtures of drugs in blank human plasma to which known quantities of each drug substance corresponding to LOQ, low (3 ×LOQ), middle 0.5×upper limit of quantification, (ULQ), and high (0.9×ULQ) concentrations. The intra-and inter-day

TABLE 1: Precision and accuracy of combined trimethoprim and sulfamethoxazole assay

Trimethoprim sulfamethoxazole							
Nominal concentration (μg/ml)	Found concentration (μg/ml)	*Precision (CV, %)	**Accuracy (%)	Normal concentration (μg/ml)	Found concentration (μg/ml)	*Precision (CV,%)	*Accuracy (%)
Intra-day (n=10)							
0.1	0.107±0.006	3.9	107	1.0	0.877±0.104	7.0	88
0.3	0.289±0.011	2.7	96	3.0	2.922±0.136	2.5	97
3.0	2.965±0.091	2.1	99	35	33.901±0.884	1.6	97
5.4	5.163±0.321	5.2	96	63	61.413±3.446	3.4	97
Inter-day (n=20)							
0.1	0.100±0.013	8.7	100	1.0	0.958 ±0.192	11.3	96
0.3	0.288±0.026	4.8	96	3.0	2.801 ±0.105	3.9	94
3.0	3.032±0.184	3.2	101	35	34.063 ±1.887	3.0	97
5.4	5.215±0.265	3.8	97	63	60.496 ±1.898	3.6	96

***Precision as coefficient of variation (CV, %) = Standard Deviation divided by mean measured concentration×100, **Accuracy=Mean measured concentration/Nominal concentration**

TABLE 2: Stability of trimethoprim and sulfamethoxazole under various conditions

Nominal oncentration (μg/ml)	Stability (%)								
	Plasma samples							*Stock solution	
	Unprocessed		Processed		Freeze-Thaw Cycle				
	24 hrs RT	8 wks-20°C	24 hrs RT	48 hrs -20°C	1	2	3	24 hrs RT	8 wks -20°C
Trimethoprim									
0.3	98	100	108	98	102	100	93		
5.4	93	106	102	101	93	97	92		
10	-	-	-	-	-	-	-	99	90
Sulfamethoxazole									
3.0	93	96	106	103	108	103	95		
63.0	95	109	103	96	97	95	94		
10	-	-	-	-	-	-	-	101	98

***Trimethoprim and sulfamethoxazole stock solution, 1 mg/ml in mobile phase.**

precision and accuracy of the assay, determined over three different days. The intra-day precisions measured over ten replicates were ≤5.2% and 7.0% for TMP and SUZ, respectively. The inter-day precisions were ≤8.7 % and 11.3%, for TMP and SUZ, respectively. The intra-day and inter-day accuracy were in the range of 88%-107% for both compounds, results are represented in TABLE 1.

Recovery

The recoveries of SUZ and TMP were evaluated at four different concentrations of LOQ, low, middle, and high from peak height ratios of plasma to mobile phase samples. The mean recovery of SUZ, and TMP was 85% and 95%, respectively. The mean recovery of the IS at concentration of 5 μg /ml was 80%.

Robustness

The robustness of a method is a measure of its capacity to remain unaffected by small variations in method conditions. It provides an indication of the reliability of the method during normal applications. The robustness of the proposed method was evaluated by slightly altering the strength of sodium phosphate buffer, pH, and amount of acetonitrile in mobile phase. No significant effects were observed. Further, the chromatographic resolution and peak responses were stable over about 600 injections of processed plasma samples.

Stability

The stability of the SUZ and TMP in plasma and processed samples, during analysis and usual storage conditions was investigated. No decrease in the measured concentration or change in chromatographic behavior of the SUZ, TMP, or IS were observed. The stock/working solutions, plasma samples, or processed samples were stable after being maintained at room temperature for period of up to 24 hours. Plasma samples (TMP: 0.30 and 5.4 μg/ml, SUZ: 3.0 and 63.0 μg/ml) stored at -20°C were found to be stable for at least 8 weeks and at least after three freeze-thaw cycles. TABLE 2 summarizes the stability studies of SUZ, TMP, and IS.

CONCLUSION

In summary, the HPLC method for the simultaneous determination of TMP and SUZ described here is rapid, sensitive, reliable, and reproducible. It has been applied for studying TMP and SUZ stability under various clinical laboratory conditions. Potentially, it could be used for the simultaneous determination of therapeutic levels of TMP and SUZ in small volume of human plasma.

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Full Paper

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