

Spectral Classical Least Square Calibration Approach for the Simultaneous Determination and Stability Test of Sulphadiazine and Trimethoprim in Bolus

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Abstract: A simple spectral classical least square calibration approach were proposed for the simultaneous quantitative evaluation and stability testing of sulphadiazine and trimethoprim in an animal commercial preparation, bolus without requiring a priori separation step. Stability tests of sulphadiazine and trimethoprim in bolus were performed by using the following conditions: Room temperature, refrigerator and etuve (37°C) for each 3 month during 12 months. A spectral classical least square calibration based on the use of absorptivities at three-wavelength set (241, 257 and 288 nm) was computed by using the linear concentration range of 4.0-22.0 µg mL⁻¹ for sulphadiazine and 3.0-18.0 µg mL⁻¹ for trimethoprim. The proposed spectral least square calibration was validated by using various synthetic binary mixtures containing the above veterinary drugs. Simultaneous analysis and stability test of sulphadiazine and trimethoprim in commercial veterinary bolus was established by the spectral classical calibration method.

Key words: Spectral classical least square, simultaneous determination, stability test, sulphadiazine, trimethoprim, chemometry

INTRODUCTION

Sulphadiazine is N¹-(pyrimidin-2-yl) sulphanilamid. Its closed formula C₁₀H₁₀N₄O₂S and molecular weight 250.3 gmoL⁻¹ (Fig. 1). It is a crystal or powder in colored of white, yellowish white or pinkish. It does not dissolve in water, chloroform and ether. Its solubility in ethanol very low, it dissolves in acetone 1:200. However, it dissolves in mineral acids, alkaline hydroxide and carbonate solutions. It is a drug which using in *Nocardia* infections of dogs and combined with aminopenicillines and tetracyclines. It is combined with trimethoprim in the treatment of diseases caused by *Staphylococcus*, *Streptococcus*, *Clostridium*, *Corynebacterium*, *Proteus*, *Pasteurella*, *Salmonella*, *Klebsiella* and *Escherichia coli* in particular (Barragry, 1994; Bishop, 1996; Budavari *et al.*, 1996).

Trimethoprim is 5-(3,4,5-trimethoxybenzyl)pyrimidin-2, 4-diylidamine. Its closed formula C₁₄H₁₈N₄O₃ and molecular weight 290.3 gmoL⁻¹ (Fig. 2). White and yellowish white colored crystal or crystallized powder. It dissolves in water 1:2500, in ethanol 1:300, in chloroform

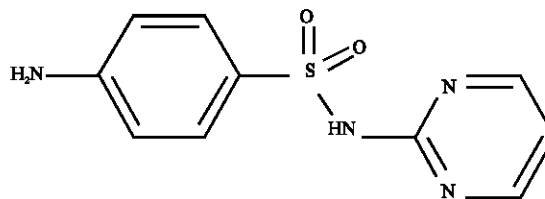


Fig. 1: The chemical structure of sulphadiazine (European Pharmacopoeia, 1992)

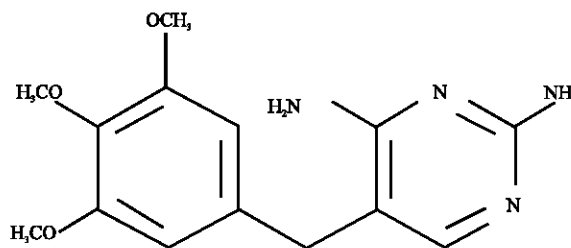


Fig. 2: The chemical structure of trimethoprim (European Pharmacopoeia, 1992)

1:55 and in methanol 1:80 and it does not dissolve in ether. Trimethoprim structurally resembles in pyteridine of dihydrofolic acid and is strong inhibitor of *dihydrofolat reductase* which converted dihydrofolat into tetrahydrofolat. This situation blocks purins and finally DNA, RNA and protein synthesis. Therefore, sulphanamid and trimethoprim combination is real example of synergism and lead to the sequential inhibition of folic acid synthesis. Bacterial *dihydrofolat reductase* is susceptible to trimethoprim 20-60.000 times than mammalian enzyme. Trimethoprim has bacteriostatic effect with broad-range of Gram positive and Gram negative bacteria and generally is ineffective to anaerobes (Barragry, 1994; Bishop, 1998).

In previous studies, the determination of sulphadiazine and trimethoprim in samples was performed by spectrophotometric methods (Dinc *et al.*, 2010; Markopoulou *et al.*, 2004; Granero *et al.*, 2002; Ribone *et al.*, 2000) and capillary zone electrophoresis (Nevado *et al.*, 2001).

In this research, a simple spectrophotometric classical least square was proposed and successfully applied to simultaneous analysis and stability test of sulphadiazine and trimethoprim in a commercial veterinary formulation. The recovery studies for the method validation were carried out by using the various synthetic mixtures. The proposed simple spectral calibration was successfully applied to the determination of the related veterinary drugs in bolus and a good agreement was observed.

MATERIALS AND METHODS

Experimental

Apparatus: The registration of the absorption spectra was obtained by using a Shimadzu UV-160 double beam UV-Vis spectrophotometer possessing a fixed slit width (2 nm) connected to a computer loaded with Shimadzu UVPC software. The absorption data of the active compounds and their samples were transformed into ASCII files and transferred to Microsoft EXCEL. Data treatments, regressions and statistical analysis were performed by using the EXCEL and Matlab 7.0 software.

Commercial veterinary formulation: A commercial veterinary product (BIOTRIN bolus, Sanofi Dif Ilac A.S., Istanbul, Turkey, containing 1000 mg sulphadiazine and 200 mg trimethoprim per bolus. Sulphadiazine and trimethoprim were obtained as a donation from Sanofi Dif Ilac A.S., Istanbul, Turkey.

Standard solutions: Stock solutions of sulphadiazine and 200 mg trimethoprim were prepared by dissolving of 25 mg sulphadiazine and trimethoprim in 50 mL methanol-water (50:50, v/v). A standard series in the concentration range of 4.0-22.0 $\mu\text{g mL}^{-1}$ sulphadiazine and 3.0-18.0 $\mu\text{g mL}^{-1}$ trimethoprim in the same solvent were obtained from the above stock solutions. For the method validation, the synthetic mixtures solutions in the above working concentration ranges were prepared.

Sample solutions preparation: For the analysis of the commercial samples, 1/10 bolus containing sulphadiazine and trimethoprim were carefully weighed and powdered. The quantity corresponding to one bolus was transferred into 100 mL calibrated flask and the volume was completed with methanol. The content of the flask was mechanically shaken for 30 min and then, filtrated by Whatman 42 (stock solution). In the following step, by adding 5 mL of the buffer solution (ammonium chloride/ammonium hydroxide, pH = 10) to 25 mL flask and 0.5 mL of the above filtrated sample solution was dissolved in 25 mL flask in methanol-water (50:50, v/v). The absorption spectra of the final solution was recorded and stored in computer. This procedure was repeated ten times.

For the stability test procedure of samples, the commercial bolus with the same batch numbers were opened up (twice from each formulation) and their analysis were done, the control data were obtained. Following that the others formulations in their original containers were stored in different conditions (room temperature, refrigerator, etuve) for next experimental periods. Their analysis was done after every 3 months during 12 months. The temperature and humidity of storage conditions were 3.9-4.1°C and 30-32% for refrigerator; +37°C and 17-19% for etuve and 20-29°C and 30-33% for room temperature depends on season.

RESULTS AND DISCUSSION

The absorption spectra of sulphadiazine and trimethoprim in the concentration range of 4.0-22.0 and 3.0-18.0 $\mu\text{g mL}^{-1}$ in methanol-water (50:50, v/v) were recorded between 210 and 330 nm, respectively. The absorption spectra of both compounds were presented in Fig. 3. The absorption spectra of the samples containing sulphadiazine and trimethoprim were obtained under experimental condition as well as calibration step.

As shown in Fig. 3, the simultaneous determination and stability test of two compounds in the same mixture

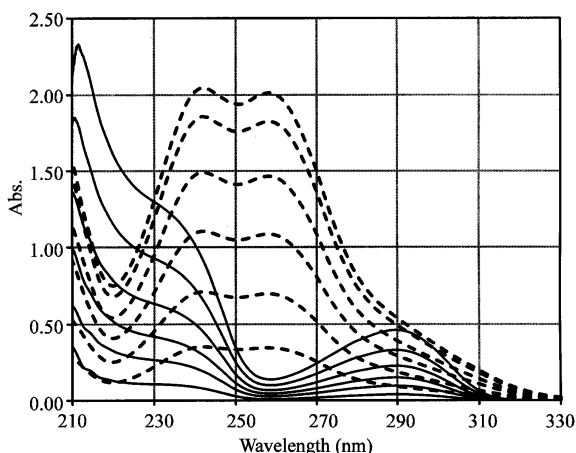


Fig. 3: Absorption spectra of the calibration solutions of sulphadiazine (4.0-22.0 $\mu\text{g mL}^{-1}$) (---) and trimethoprim (—) (3.0-18.0 $\mu\text{g mL}^{-1}$)

is not possible by using single calibration approach in the presence of the overlapping spectra of sulphadiazine and trimethoprim.

In order to overcome this problem, a simple spectrophotometric classical least square method based on the three wavelength set was proposed for simultaneous analysis and test of the subjected matter compounds.

Spectral classical least square method: In the application of spectral classical least square calibration, the standard series of each compound in the concentration range 4.0-22.0 $\mu\text{g mL}^{-1}$ for sulphadiazine and 3.0-18.0 $\mu\text{g mL}^{-1}$ for trimethoprim in methanol-water (50:50, v/v) was prepared. Their absorption spectra were recorded between 210-330 nm. Similar spectral registration procedure was used for the sample solutions.

The absorbances of the calibration solutions corresponding to standard series were measured at the three-wavelength set (241.0, 257.0 and 288.0 nm). The three wavelengths correspond to the critical points to build classical least square calibration. At the above three-wavelength set, the absorptivities for each compound were calculated by using the formula:

$$a \text{ (absorptivity)} = \frac{A \text{ (Absorbance)}}{C \text{ (Concentration, } \mu\text{g mL}^{-1}\text{)}}$$

The mean values of the calculated absorptivity values for each concentration level in the calibration series were used for the spectral classical least squares calibration. The mean absorptivity values at three-wavelength set for both were shown in Table 1.

Table 1: The absorptivities at the three-wavelength set for sulphadiazine and trimethoprim

Absorptivities λ (nm)	241.0	257.0	288.0
Sulphadiazine	9.08×10^{-2}	8.95×10^{-2}	2.55×10^{-2}
Trimethoprim	5.24×10^{-2}	6.95×10^{-3}	2.45×10^{-2}

According to the calculated absorptivity values at the three-wavelength set, the following equation calibration for the spectral classical least squares calibration was obtained as:

$$\begin{aligned} \lambda_{241.0} A_{\text{mix1}} &= 9.08 \times 10^{-2} C_{\text{SDZ}} + 5.24 \times 10^{-2} C_{\text{TMP}} \\ \lambda_{257.0} A_{\text{mix2}} &= 8.94 \times 10^{-2} C_{\text{SDZ}} + 6.95 \times 10^{-3} C_{\text{TMP}} \\ \lambda_{288.0} A_{\text{mix3}} &= 2.55 \times 10^{-2} C_{\text{SDZ}} + 2.45 \times 10^{-2} C_{\text{TMP}} \end{aligned} \quad (1)$$

Where:

A_{mix1} , A_{mix2} and A_{mix3} = The absorbances of the binary mixture samples at the wavelength, $\lambda_{241.0}$, $\lambda_{257.0}$ and $\lambda_{288.0}$, respectively

C_{SDZ} and C_{TMP} = The concentration ($\mu\text{g mL}^{-1}$) of sulphadiazine and trimethoprim in sample

Equation 1 can be formulated in matrix notation as:

$$\begin{bmatrix} A_{\text{mix1}} \\ A_{\text{mix2}} \\ A_{\text{mix3}} \end{bmatrix} = \begin{bmatrix} 9.08 \times 10^{-2} & 5.24 \times 10^{-2} \\ 8.94 \times 10^{-2} & 6.995 \times 10^{-2} \\ 2.55 \times 10^{-2} & 2.45 \times 10^{-2} \end{bmatrix} \times \begin{bmatrix} C_{\text{SDZ}} \\ C_{\text{TMP}} \end{bmatrix} \quad (2)$$

Equation 2 was used for the calculation of the amount of the sulphadiazine and trimethoprim in samples. This approach was denoted as the spectral classical least square calibration.

Validity of spectral classical least square method: For the method validation, a set of 12 synthetic mixtures containing sulphadiazine and trimethoprim in the working concentration range (in methanol-water 50:50, v/v) were prepared by using sulphadiazine and trimethoprim stock solutions (Table 2). The absorption spectra of these synthetic mixtures were recorded in the spectral region 210-330 nm. Sulphadiazine and trimethoprim in mixtures were determined by using spectral classical least square calibration. The mean recovery and relative standard deviation were given in Table 1. Good precision and accuracy was observed for the application of the spectral classical least square approach to the analysis of synthetic mixtures.

Analysis and stability test of the commercial bolus: In this study, the spectral classical least square calibration was proposed for the simultaneous quantitative

Table 2: The validation results of the synthetic mixtures of sulphadiazine and trimethoprim by spectral classical least square calibration

No.	Binary mixture ($\mu\text{g mL}^{-1}$)		Found ($\mu\text{g mL}^{-1}$)		Recovery (%)	
	Sulphadiazine	Trimethoprim	Sulphadiazine	Trimethoprim	Sulphadiazine	Trimethoprim
1	20.0	3.0	18.94	2.890	94.70	96.30
2	20.0	4.0	18.90	3.930	94.50	98.30
3	20.0	6.0	19.00	5.620	95.00	93.70
4	20.0	9.0	19.76	8.980	98.80	99.80
5	20.0	13.0	19.88	12.640	99.40	97.20
6	20.0	18.0	19.80	17.640	99.00	98.00
7	4.0	4.0	4.01	3.860	100.30	96.50
8	8.0	4.0	7.83	3.750	97.90	93.80
9	12.0	4.0	11.83	3.860	98.60	96.50
10	16.0	4.0	15.04	4.136	94.00	103.40
11	20.0	4.0	18.99	4.200	95.00	105.00
12	22.0	4.0	20.80	4.120	94.50	103.00
Mean					96.80	98.50
SD					2.36	3.68
RSD					2.44	3.73

SD = Standard Deviation, RSD = Relative Standard Deviation

Table 3: Assay results of sulphadiazine and trimethoprim by the spectral classical least square calibration

No.	Sulphadiazine (mg tablet^{-1})	Trimethoprim (mg tablet^{-1})
1	985.6	201.3
2	962.0	200.8
3	998.3	209.0
4	999.4	197.5
5	999.9	201.3
6	992.8	201.9
7	992.8	193.1
8	993.3	203.0
9	1002.7	202.4
10	990.6	196.4
Mean	991.7	200.6
SD	11.63	4.31
RSD	1.17	2.15
SE	3.68	1.36
CL	7.21	2.67

SD = Standard Deviation, CL = Confidence Level ($p = 0.05$)

evaluation and stability test of sulphadiazine and trimethoprim in animal bolus without a chemical separation step. In the first step, the proposed classical least square method based on the use of absorptivities at three-wavelength set was applied to sulphadiazine-trimethoprim bolus as explained in the above mentioned sample solutions preparation section. The assay results obtained by application of the simple chemometric approach were shown in Table 3. Mean values, standard deviation, relative standard deviation, standard error and confidence limit were calculated and shown in the Table 2.

In the second step, the analysis of bolus was performed for each 3 months during 12 months. These veterinary formulations kept up in their original containers until analyzing procedure and stored in different conditions (room temperature, refrigerator, etuve). Sample solution preparations as explained in sample solutions preparation were performed. Their absorption spectra were recorded between 210-330 nm. The absorption data of samples was measured at the three-wavelength set (241, 257 and 288 nm). Sulphadiazine and trimethoprim in

samples were analyzed by using Eq. 2. The shelf life of veterinary formulation is declared as 60 months. At the end of study, the degradation in the active compounds of veterinary formulation during 12 months was in the limits of its shelf life.

CONCLUSION

In this study, the spectral classical least square approach was applied to the simultaneous quantitative evaluation and stability test of sulphadiazine and trimethoprim in bolus without any pre-treatment and graphical procedure in the presence of very closely overlapped spectra. The application of classical least square method using special mathematical can be considered a suitable chemometric calibration for a precise, accurate, rapid and less expensive determination of subject two compounds in samples. This can be considered as an advantage of new spectral classical least square approach over other spectrophotometric methods for the quantitative resolution of bolus containing sulphadiazine and trimethoprim.

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