Department of Analytical Chemistry[1], Faculty of Pharmacy, Ankara University, Tandoğan-Ankara, Department of Pharmacology and Toxicology[2], Department of Internal Medicine[3], Faculty of Veterinary Medicine, Ankara University Dışkapı, Ankara-Turkey

Simultaneous determination of trimethoprim and sulphamethoxazole in veterinary formulations by chromatographic multivariate methods

E. Dinç[1], A. Bilgili[2], B. Hanedan[3]

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Assoc. Prof. Dr. Erdal Dinç, Department of Analytical Chemistry, Faculty of Pharmacy, Ankara University, 06100 Tandogan-Ankara, Turkey
dinc@pharmacy.ankara.edu.tr

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A comparative chromatographic study was developed for the simultaneous quantitative resolution of trimethoprim (TMP) and sulphamethoxazole (SMX) in veterinary formulations. Multi-wavelength chromatograms were recorded by using diode array detector (DAD) system at the five-wavelength set consisting of 220, 230, 240, 250 and 260 nm. In the first step, five different calibration equations at the above wavelengths for each drug were obtained by using the relationship between concentration and peak area. These calibration graphs were used for the quantitative evaluation of TMP and SMX in samples. These single-wavelength applications were called traditional LC method. In the second step, principal component regression (PCR) and partial least-squares (PLS) calibrations were applied to the above mentioned multi-wavelength chromatograms. The amount of two investigated drugs in samples was determined by the constructed PCR and PLS calibrations. The experimental results obtained from each single-wavelength calibration graph were compared with those obtained by the chemometric approaches and chromatographic multivariate approaches give successful results more than traditional LC method.

1. Introduction

Trimethoprim (TMP) is a very well- known antibacterial agent used in combination with sulfamethoxazole (SMX) in both veterinary and medicinal practice. SMX, which is a member of sulfonamides, is rapidly absorbed and its excessive use in veterinary practice may bear the risk of food product contamination. Thus, quality control and routine analysis of the binary mixture of TMP and SMX in veterinary and medicine commercial products is required.

Several reports on the simultaneous quantitative determination of TMP and SMX have been published. These include various spectrophotometric methods (Markopoulou et al. 2004; Granero et al. 2002; López-Martínez 2002; Ribone et al. 2000), capillary zone electrophoresis (Berzas Nevado et al. 2001a), and liquid chromatography (Akay and Ozkan 2002; Berzas Nevado et al. 2001b).

Recently, multivariate LC calibration has been used for the quantitative evaluation of analytes in samples (Dinc and Üstündağ 2005a; Dinc et al. 2005b, 2005c, 2006a, 2006b). This approach is based on the application of chemometric calibrations to multi-wavelength chromatograms. The advantages of the multivariate chromatographic calibrations are: to increase the precision and accuracy of results, without using the selection of a specific wavelength, to eliminate or reduce the errors coming from injections and compensation of fluctuation of peaks arisen from column and other chromatographic conditions.

In this study, a traditional LC method based on the calibration function obtained from a single wavelength was developed for the simultaneous determination of TMP and SMX in two veterinary formulations. Chromatographic separation of TMP and SMX was performed in presence of chlorzoxazone (IS) which was used as internal standard. As alternative approaches, PCR and PLS calibrations were subjected to the multi-chromatograms at the five-wavelength set using DAD. The use of a LC method in combination with chemometric techniques was denoted LC-PCR and LC-PLS. Traditional and chemometric LC approaches were validated by analyzing various mixtures of TMP and SMX in the working concentration range. The analytical approaches developed in this paper were statistically compared with each other. We observed that LC-PCR and LC-PLS give better results than traditional LC method.

2. Investigations, results and discussion

2.1. Multivariate LC methods

In this LC-chemometric study, the multivariate LC data were obtained by plotting the multi-chromatograms at five different wavelengths and detector responses were measured as peak area. PCR and PLS calibrations were applied to the multivariate LC data set consisting of the ratio of chromatographic peak area of drug and IS. Che-

mometric calibrations and their methodologies to the LC multivariate data were explained in the following sub-sections.

2.1.1. LC-PCR method

The ratio of the peak area of individual drug and the drug concentration set were reprocessed by mean-centering as R_o and C_o, respectively and the covariance dispersion matrix of the centered matrix R_o was calculated. By using the square covariance matrix, the normalized eigenvalues and eigenvectors were computed. Identifying the highest values of the eigenvalues we are able to select the number of the optimal principal components (eigenvectors (P)).
Therefore the remaining eigenvalues and their corresponding eigenvectors are omitted. To follow this objective the coefficient b defined as $b = P \times q$ was calculated, where P is the matrix of eigenvectors and q is the C-loadings given by $q = D \times T^T \times R_o$. T^T represents the transpose of the score matrix T and D is a diagonal matrix having the components the inverse of the selected eigenvalues. The drug content in samples was obtained using the equation: $C_{prediction} = b \times R_{sample}$. PLS toolbox 3.0 in Matlab 7.0 software was used for the data treatment.

2.1.2. LC-PLS method

The PLS calibration using the orthogonalized PLS algorithm is based on the simultaneous use of both independent and dependent variables on the data compression and decomposition operations.
In the proposed data analysis, the LC-PLS calibration is obtained by decomposition of both concentration and the ratio of peak area matrix into latent variables, $R = T \times P^T + E$ and $C = U \times Q^T + F$. After that, the linear regression, $C_{prediction} = b \times R_{sample}$, is used for the estimation of the drugs in the samples. The vector, b has the expression $b = W \times (P^T \times W)^{-1} \times Q$, where W denotes a weight matrix.
In application of this algorithm, PLS toolbox 3.0 in Matlab 7.0 software was used for the mathematical calculations.

2.2. Multivariate chromatographic method development

The fundamental aim of this study can be summarized follow as: The first step was to develop a traditional LC method. The second step was to apply two multivariate calibration techniques (PCR and PLS) to the multi-chromatograms under the same chromatographic conditions with traditional LC method. Finally, the experimental results obtained from both traditional LC and multivariate LC approaches were compared with each other.
The simultaneous registrations of the multiple chromatograms at selected multiple wavelengths were achieved by the PDA detector used in the LC instrument. This PDA detector system provides the recording of a multiwavelength chromatographic data set of chromatograms possessing different peak areas.
To bypass the disadvantages such as repeated injections which require a long period of time, peak fluctuations in column systems, selection of specific wavelength, noises coming from chromatographic instrumentations and other errors coming from environmental conditions in application of traditional LC method based on single wavelength calibration, multivariate LC calibration techniques are good analytical approaches for the quantitative analysis of TMP and SMX drugs in samples. In the case of the multivariate LC approaches, multiwavelength LC data analysis was obtained by computing the peak-area ratio of each drug to the IS.

2.3. Chromatographic data processing and method application

A concentration set of seven mixtures consisting of SMX and TMP in the linear concentration range of 1.0–30.0 μg/mL with a constant concentration of IS at 10.0 μg/mL was prepared in the solvent system containing acetonitrile and 0.1 M $(NH_4)_2CO_3$ (50 : 50). The chromatographic peak areas of the concentration set were plotted at 5 wavelengths (220, 230, 240, 250 and 260 nm) and the retention times were 2.61 min for SMX, 3.45 min for TMP and 4.59 min for IS. The 3D-multivariate chromatograms of the concentration set for both drugs with IS were indicated in the figure. In the first step, 5 linear regression equations for each drug at a single wavelength were obtained using the relationship between concentration and peak area-ratio (SMX/IS or TMP/IS). In the second step, PCR and PLS were applied to the concentration set and its corresponding chromatographic peak area-ratio at the 5 wavelengths.

2.4. LC-PCR method

The PCR algorithm briefly described in the section of multivariate LC methods was used to obtain the LC-PCR calibration for the simultaneous prediction of SMX and TMP in samples. The application of the PCR method to the multivariate chromatographic data is based on the relationship between the concentration set and peak-area ratio. The data sets shown in Table 1, which corresponds to the figure, was used for the LC-PCR calibration method. In Table 1, the concentration set of 7 mixtures of SMX and TMP in the presence of the constant concentration of IS corresponds to the y-block while the peak-area ratios of drug to IS corresponds to the x-block in PCR calibration. The LC-PCR calibration was used for the simultaneous quantitative prediction of the 2 drugs in the synthetic mixtures and two commercial veterinary formulations. The LC-PCR calibration and data analysis were performed by using PLS toolbox 3.0 in Matlab 7.0 and Microsoft Excel, respectively.

2.5. LC-PLS method

The PLS algorithm as in PCR calibration method was applied to the LC data summarized in Table 1 and corresponding to the figure. In this LC-PLS calibration method both the peak-area data and the concentration set were simultaneously decomposed. LC-PLS calibration is based on the use of the relationship between the decomposed peak-area data (x-block) and concentration set (y-block). The concentrations of SMX and TMP in the samples were simultaneously predicted by the constracted LC-PLS calibration. The mathematical treatments were performed by means of the same PLS toolbox 3.0 in Matlab 7.0 software and Microsoft Excel.

2.6. Traditional LC method

The chromatograms for SMX and TMP in the concentration range of 1.0–30 μg/mL in the presence of IS at 10 μg/mL were plotted from PDA detection data obtained

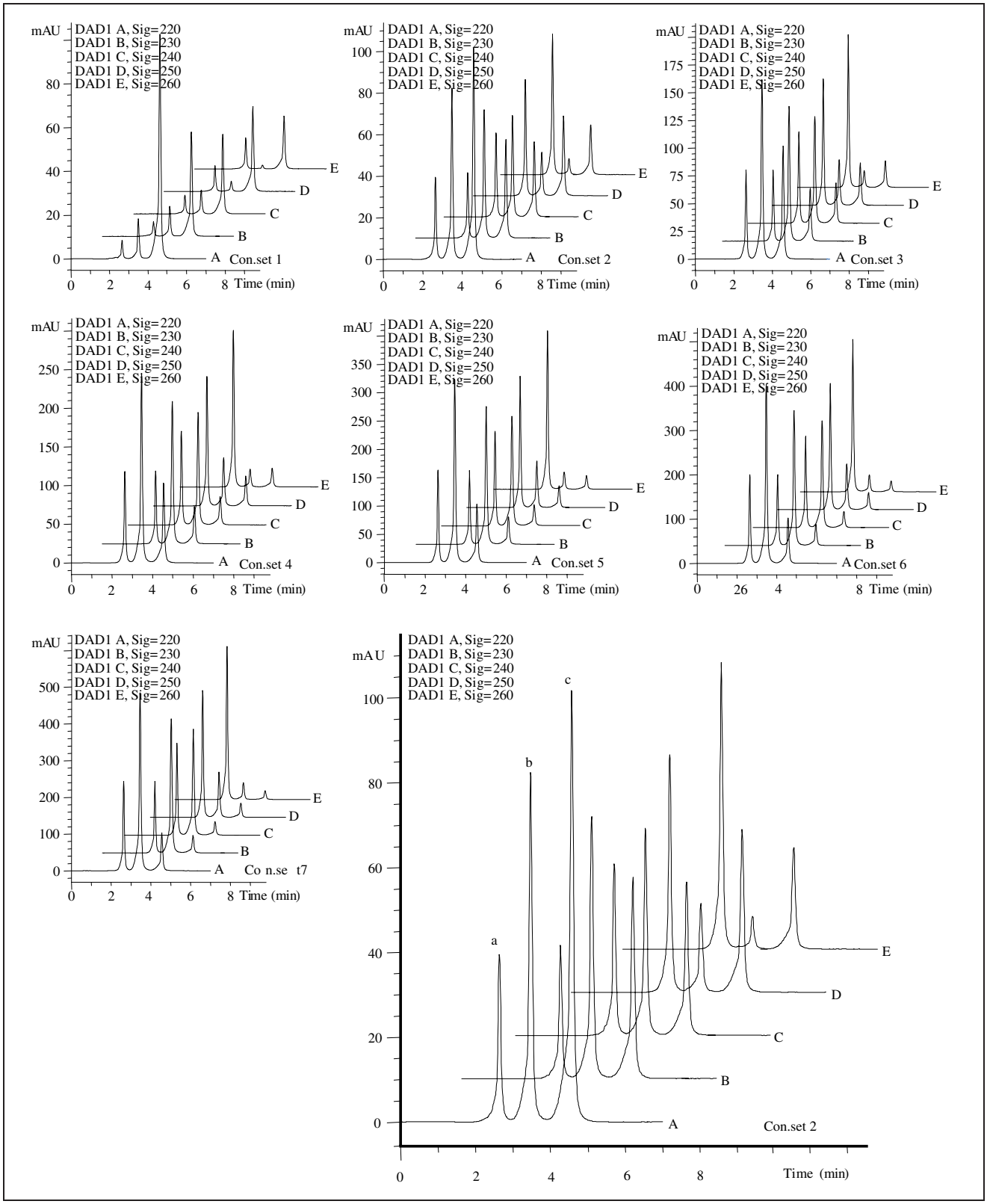

Fig.: 3D-Chromatograms of 5 μg/ml SMX (a), 5 μg/ml TMP (b) and 10 μg/ml IS (c), the small chromatograms correspond to the concentration set (1–7).

at the 5 different wavelengths (see figure). The detector responses were measured in terms of peak-area. With a mobile phase consisting of acetonitrile, water and $(NH_4)_2CO_3$ (0.1 M) (50 : 10 : 40), a good chromatographic separation was obtained at ambient temperature on a Waters Symmetry C18 column, 5 μm, 4.6 × 250 mm. The flow rate was set at 0.8 mL/min with a 20 μL injection volume. Chlorzoxazone as the IS was found to be suitable for optimum chromatographic separation. Before chromatographic conditions were optimized, different mobile phases and other chromatographic conditions were tested, and the above-mentioned chromatographic conditions were found to be suitable for satisfactory separation in the presence of the IS. These same chromatographic conditions were used for both the multivariate LC and traditional LC approaches. At a flow rate of 0.8 mL/min, reten-

Table 1: Concentration set and its corresponding peak-area-ratio

Concentration set μg/mL			Ratio of peak area (SMX/IS)					Ratio of peak area (TMP/IS)				
SMX	TMP	IS	220	230	240	250	260	220	230	240	250	260
1.0	1.0	10.0	0.0610	0.0938	0.1734	0.2250	0.4470	0.1442	0.2348	0.2417	0.0758	0.0443
5.0	5.0	10.0	0.2999	0.5053	0.8911	1.1554	2.2829	0.6982	1.1329	1.1932	0.4765	0.2536
10.0	10.0	10.0	0.5851	1.0101	1.7499	2.2631	4.3848	1.4012	2.2827	2.4109	0.9539	0.5066
15.0	15.0	10.0	0.8601	1.4713	2.5904	3.3755	6.5988	2.0909	3.3661	3.6042	1.4365	0.8388
20.0	20.0	10.0	1.1471	1.9738	3.5016	4.5253	8.8667	2.7869	4.5154	4.8815	1.9323	1.1425
25.0	25.0	10.0	1.4395	2.4673	4.2120	5.7002	11.1471	3.5007	5.6651	5.9572	2.4415	1.4456
30.0	30.0	10.0	1.7205	2.9536	5.0359	6.8672	13.4195	4.1850	6.7827	7.1198	2.9549	1.7533

Table 2: Linear regression analysis and its statistical results

λ (nm)	SMX					TMP				
	220	230	240	250	260	220	230	240	250	260
Slope (m)	0.0571	0.0983	0.1677	0.2283	0.4462	0.1395	0.2259	0.2382	0.0989	0.0595
Intercept(n)	0.0091	0.0082	0.0544	−0.0129	−0.0218	0.0026	0.0056	0.0232	−0.0300	−0.0462
r	0.9999	0.9999	0.9996	0.9996	0.9999	1.0000	1.0000	0.9998	0.9999	0.9994
SE(r)	0.0052	0.0118	0.0557	0.0281	0.0613	0.0062	0.0163	0.0504	0.0147	0.0246
SE(m)	0.0002	0.0006	0.0022	0.0011	0.0024	0.0002	0.0006	0.0019	0.0006	0.0010
SE(n)	0.0036	0.0081	0.0388	0.0195	0.0427	0.0043	0.0114	0.0351	0.0102	0.0171
LOD (μg/ml)	0.12	0.20	0.21	0.23	0.22	0.25	0.21	0.11	0.17	0.12
LOQ (μg/ml)	0.40	0.66	0.69	0.75	0.74	0.83	0.70	0.36	0.56	0.40

r = Correlation coefficient of linear regration equation
SE (r) = Standard error of correlation coefficient
SE (m) = Standard error of slope
SE (n) = Standard error of intercept
LOD = Limit of detection
LOQ = Limit of quantitation

Table 3: Recovery data obtained by applying the proposed analytical approaches to the synthetic mixtures

Mixture		SMX							TMP						
SMX	TMP	Traditional LC					LC-chemometry		Traditional LC					LC-chemometry	
(μg/mL)	(μg/mL)	220	230	240	250	260	PCR	PLS	220	230	240	250	260	PCR	PLS
1.0	5.0	111.2	109.1	106.0	134.0	119.1	104.7	104.5	106.9	106.3	104.1	104.9	101.6	103.6	102.4
10.0	5.0	101.6	103.4	101.0	100.3	99.9	101.3	101.3	99.4	99.9	97.8	101.0	88.8	97.4	97.4
20.0	5.0	100.7	102.6	101.2	98.1	98.1	100.1	100.1	103.3	101.6	99.7	103.0	86.6	98.5	98.5
30.0	5.0	101.6	103.7	102.7	99.2	98.6	101.2	101.2	104.8	101.9	100.1	103.5	85.8	98.6	98.7
25.0	1.0	102.9	104.6	103.5	100.4	99.7	102.2	101.4	101.5	101.5	91.7	107.0	119.6	104.3	104.3
25.0	10.0	100.9	103.5	102.8	99.2	99.5	101.2	100.4	100.6	101.7	100.7	100.4	102.2	101.1	101.1
25.0	20.0	101.5	102.6	104.9	100.7	101.2	102.2	101.4	96.4	100.0	101.2	98.6	99.8	99.8	99.8
25.0	30.0	100.8	103.9	102.6	98.8	100.0	101.2	100.0	99.9	100.0	99.3	100.6	100.8	100.1	100.1
	Mean	102.6	104.2	103.1	103.8	102.0	101.8	101.3	101.6	101.6	99.3	102.4	98.2	100.4	100.3
	SD	3.52	2.10	1.70	12.21	6.97	1.37	1.43	3.34	2.07	3.57	2.73	11.15	2.46	2.27
	RSD	3.43	2.02	1.65	11.76	6.84	1.35	1.42	3.28	2.04	3.59	2.67	11.36	2.45	2.26

SD = Standard deviation
RSD = Relative standard deviation

tion times were 2.61 min for SMX, 3.45 min for TMP, and 4.59 min for IS as shown in the figure.

The ratios of the peak areas (SMX/IS and TMP/IS) were calculated using the values obtained from the recorded chromatograms at the 5-wavelength set [220 nm (A), 230 nm (B), 240 nm (C), 250 nm (D), and 260 nm (E); Table 1]. In the present study, 5 linear regression equations at each wavelength mentioned above for each drug were separately calculated using the relation between concentration and peak-area ratio. The linear regression equations and their statistical parameters were given in Table 2.

These single linear regression equations were tested by analyzing mixtures containing the analyzed drugs, SMX and TMP. Mean recovery results and relative standard deviations obtained by applying the traditional LC method were between 102.0–104.2% and 1.65–11.76% for SMX, and 98.2–102.4% and 2.04–11.36% for TMP, respectively. As it can be seen from Table 3, the traditional LC approach may not give better results at every wavelength. One of the main problems of the traditional LC method is the selection of the optimal wavelength for obtaining a single linear regression to evaluate the content of drugs in samples. This drawback can be eliminated using a multivariate LC approach, which will be explained below.

2.7. Statistical evaluation of multivariate LC method

The standard error of calibration (SEC) values for SMX and TMP drugs were calculated using the data obtained from the difference between the added and predicted concentrations in the calibration steps. The results of the linear regression analysis including the SEC values are given

Table 4: Standard addition method and its recovery results by the proposed analytical methods

Actual Conc. (μg/mL)		SMX							TMP						
SMX	TMP	220	230	240	250	260	PCR	PLS	220	230	240	250	260	PCR	PLS
10.0	2.5	95.9	108.9	118.9	93.9	94.9	98.9	99.9	107.5	111.1	110.3	109.5	108.7	104.3	105.1
15.0	5.0	97.6	105.6	112.3	92.3	92.3	99.0	99.6	113.2	115.0	114.6	114.2	113.8	110.2	109.8
20.0	10.0	104.7	106.2	111.2	96.2	104.7	101.2	101.2	108.2	109.1	108.9	108.7	108.5	107.4	107.0
25.0	15.0	96.1	103.7	107.7	91.7	95.7	99.7	101.3	104.5	105.1	105.0	104.9	104.7	104.2	104.0
	Mean	122.9	130.9	138.0	124.8	129.5	99.7	100.5	130.7	134.1	135.8	137.5	139.2	106.5	106.5
	SD	4.15	2.13	4.66	2.00	5.40	1.07	0.87	3.60	4.12	3.97	3.84	3.73	2.86	2.54
	RSD	3.38	1.63	3.38	1.60	4.17	1.08	0.86	2.76	3.07	2.92	2.79	2.68	2.69	2.39

Table 5: Experimental result obtained by application of the analytical methods to the commercial veterinary formulations

Drug	Method		Mean		SD		RSD		SE		CL	
			(I)	(II)	(I)	(II)	(I)	(II)	(I)	(II)	(I)	(II)
SMX	Traditional LC	220	1115.8	199.6	17.85	7.92	1.6	3.97	7.29	3.23	14.29	6.34
		230	1085.0	199.2	40.78	6.93	3.76	3.48	16.65	2.83	32.63	5.55
		240	1110.1	196.2	50.00	6.06	4.5	3.09	20.41	2.47	40.00	4.85
		250	1085.4	194.9	21.85	2.28	2.01	1.17	8.92	0.93	17.49	1.83
		260	1073.6	197.6	39.73	2.43	3.70	1.23	16.22	0.99	31.79	1.95
	Chemometry	LC-PCR	1075.9	199.7	15.95	1.19	1.48	0.60	6.51	0.49	12.76	0.96
		LC-PLS	1051.7	199.7	14.75	1.13	1.4	0.57	6.02	0.46	11.81	0.90
TMP	Traditional LC	220	208.3	37.2	3.93	0.37	1.89	0.99	1.60	0.15	3.14	0.30
		230	206.6	36.4	3.50	0.61	1.69	1.68	1.43	0.25	2.80	0.49
		240	184.3	33.9	6.61	0.28	3.58	0.84	2.70	0.12	5.29	0.23
		250	198.1	36.8	4.91	1.42	2.48	3.85	2.01	0.58	3.93	1.13
		260	205.7	41.7	7.19	0.59	3.5	1.41	2.94	0.24	5.76	0.47
	Chemometry	PCR	220.8	39.7	3.07	1.50	1.39	3.76	1.25	0.61	2.46	1.20
		PLS	203.5	39.6	3.37	1.52	1.66	3.85	1.38	0.62	2.70	1.22

(I) = Otrizol® oblet which contains 1000 mg SMX, and 200 mg TMP,
(II) = Co-Trimoxazole® 24% Injectable which contains 200 mg SMX, and 40 mg TMP
LC = Confidence limit ($p = 0.05$, $n = 6$)

in Table 4. According to the cross-validation procedure, the first one factor for LC-PCR and LC-PLS gives a good prediction for both drugs.

The standard error of prediction (SEP) values and the related statistical values were calculated using a procedure similar to that used to calculate the SEC values in the calibration step. The values obtained for SEP, correlation coefficient (r), slope (m), and intercept (n) are presented in Table 4. The statistical data indicate that the minimum values of SEC and SEP give acceptable results under optimized conditions in the calibration and prediction steps.

2.8. Validation of the methods

The linearity of the LC detector response for the determination of SMX and TMP at the 5 different wavelengths was established by analyzing a series of different concentrations of each drug. In accordance with the guidelines of the International Conference on Harmonization (ICH; 12), at least 6 concentrations must be used. In our study, 7 concentrations were planned, ranging from 1.0–30.0 μg/mL for SMX and TMP. Each concentration was tested 3 times to provide information on the variation in the peak areas samples having the same analyte concentrations. The linearity of the individual calibration functions of the drugs at the 5 different wavelengths was confirmed by the high value of the correlation coefficient (Tables 2 and 4). For the traditional LC method, linear regression functions are shown in Table 2.

The precision of the LC-CLS, LC-PCR, LC-PLS, and traditional LC methods was tested by 8 replicate determinations at different concentration levels. The relative standard deviations were between 2.26 and 5.29% as shown in Table 5.

The accuracy of the applied methods was tested by analyzing mixtures containing the analytes at different concentration levels. The average recoveries ranged from 96.9 to 103.2% (Table 5). A good agreement between the results was obtained for the LC-PCR, LC-PLS, and some traditional LC methods. During the analytical procedure, interference and systematical error were not observed.

According to the ICH guidelines (12), the limit of detection (LOD) and limit of quantitation (LOQ) are calculated by using the standard deviation of the response and the slope of the calibration equation (Table 2). The results are presented in Table 2.

The calibration range, based on the concentrations of each drug in market sample products, was designated in the practical range, to give an accurate, precise, and linear response.

The matrix effect of the excipients in the commercial samples was tested by the elaborated methods. The results and the corresponding standard deviations were calculated (Table 3). The recoveries are averages obtained from the analysis of 5 replicates for each colorant. According to the results of the standard editions assay, we observed that the matrix effect was not a source of any error for the determinations.

2.9. Analysis of commercial veterinary formulations

The elaborated LC-chemometric and traditional LC methods were applied to the simultaneous determination of

SMX and TMP in samples. The assay results of commercial veterinary preparations were shown in Table 6. Statistical parameters, standard deviation, and percent relative standard deviations are also summarized in Table 6. These experimental results indicated that disadvantages of the traditional LC method could be eliminated by the LC-chemometric approaches. The mathematical properties of the chemometric calibration techniques were to find the best linear correlation between concentration set and its corresponding multiwavelength chromatographic data set.

In this study, all of the traditional LC application corresponding to single wavelength calibration did not give better results than the LC-chemometric methods.

We think that the effect of selection of working wavelength, peak fluctuation in column system, noise coming from LC instrumentation and experimental medium on the experimental results get to this unwanted situation in the application of traditional LC calibration. LC-chemometric method give better results in the analysis of commercial samples.

3. Experimental

3.1. Chromatographic conditions

The chromatographic system consisted of Agilent 1100 series LC system (Agilent Technologies, Inc., Palo, CA) equipped with a quaternary pomp, a thermostat autosampler, a thermostatted column compartment, and a multiwavelength photo DAD. The control of the LC instrument was carried out by using HP ChemStation software (Agilent Technologies, Inc.). The single- and multi-chromatograms were recorded and processed by means of the above mentioned system and software. The analytical column used was a Waters symmetry® C18, 5 μm, 4.6 × 250 mm. The mobile phase consisting of acetonitrile-water-$(NH_4)_2CO_3$ (0.1 M) (50 : 10 : 40) was used for the chromatographic separation at a constant flow rate of 0.8 ml/min. The mobile phase was prepared daily and filtered through a 0.45 μm membrane filter. The liquid chromatograph was operated at ambient temperature and the injection volume was 20 μL.

3.2. Reagents

Solutions were prepared with de-ionized water (Mill-Q quality). Acetonitrile was of LC grade. Acetonitrile and $(NH_4)_2CO_3$ were from Merck (interlab, Ankara, Turkey).

TMP and SMX were kindly donated from Sanovel Pharm. Ind. and Ege Vet Hayvancılık San. Tic. Ltd. Şti., Turkey.

3.3. Stock solutions

Stock solutions were prepared at a concentration 12.5 mg/50 ml by dissolving TMP, SMX and IS in the solvent system consisting of acetonitrile and 0.1 M $(NH_4)_2CO_3$ (50 : 50). A series of standard solutions of TMP and SMX over the concentration range of 1–30 μg/mL was prepared from the stock solution. A validation set of 8 solutions containing both drugs at the above concentration range was prepared. A constant concentration of IS at 10 μg/mL was used for the preparation of the samples through chromatographic analysis. In case of the standard addition method, 5 solutions including the stock solutions and the solutions of the commercial preparations were used. All the solutions were prepared daily and all the solutions were filtered through a 0.45 μm membrane filter. After that, chromatograms of all the prepared samples were plotted under chromatographic conditions given in this study.

3.4. Analysis of commercial veterinary formulation

Two commercial veterinary formulations, Otrizol® Oblet (produced by Sanovel Pharm. Ind., Turkey) containing 1000 mg SMX, and 200 mg TMP, and Co-Trimoxazole® 24% Injectable (produced by Ege Vet Hayvancılık San. Tic. Ltd. Şti) containing 200 mg SMX, and 40 mg TMP were analyzed using the proposed single and multivariate approaches.

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