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Simultaneous spectrophotometric determination of chlordiazepoxide and clidinium using multivariate calibration techniques

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Three multivariate modelling approaches including partial least squares regression (PLS), genetic algorithm-partial least squares regression (GA-PLS), and principal components-artificial neural network (PC-ANN) analysis were investigated for their application to the simultaneous determination of chlordiazepoxide and clidinium levels in pharmaceuticals. A set of synthetic mixtures of drugs in ethanol and 0.1 M HCL was made, and the prediction abilities of the aforementioned methods were examined using RSE% (relative standard error of the prediction). The PLS and PC-ANN methods were found to be comparable, and GA-PLS produced slightly better results. The predictive models that we built were successfully applied to simultaneously determine the levels of chlordiazepoxide and clidinium in coated tablets. **Copyright © 2010 John Wiley & Sons, Ltd.**

Keywords: chemometrics; chlordiazepoxide; clidinium; simultaneous determination; spectrophotometry

Introduction

Combining different active pharmaceuticals into a single form is an appealing therapeutic approach because it reduces cost, improves patient compliance, and simplifies disease management.[1] The combination of chlordiazepoxide (a benzodiazepine) and clidinium (an antimuscarinic agent) is used to treat gastrointestinal problems, especially peptic ulcers and irritable bowel syndrome.[2]

Accurate time-effective determination of active pharmaceuticals when in their final form is a major concern for quality control and quality assurance in the pharmaceutical industry. Conventionally, different analytical techniques including official methods,[3–5] high performance liquid chromatography (HPLC),[6–8] polarography,[9] fluorometry,[10] and spectrofluorometry[11] have been used for simultaneous determination of chlordiazepoxide and clidinium. However, the complex, expensive, and time-consuming nature of these techniques makes them less favourable for routine analyses.

Spectrophotometric techniques with acceptable precision and accuracy are suitable alternatives to the above-mentioned traditional approaches. The most critical limitation when using traditional ultraviolet-visible (UV-Vis) spectrophotometry for the simultaneous determination of multicomponent systems is the overlapping of drug absorption spectra. To obtain quantitative information from these types of systems without performing any chemical separations prior to examination, chemometric methods have been developed. Only two reports have performed simultaneous determinations of chlordiazepoxide and clidinium by spectrophotometry. In the first report, a derivative technique was employed for the simultaneous determination of these two components using a preceding acetonitrile extraction.[12] In the second report, a few well-known full-spectrum chemometrics techniques including principal component regression (PCR), partial least squares regression (PLS), and artificial neural network (ANN) analysis were employed to overcome the challenges posed by the overlapping spectra of chlordiazepoxide and clidinium in methanol.[13] The United States Pharmacopoeia endorses a reduction in the amount of reagents and materials (including acetonitrile and methanol) used in pharmaceutical assays that may cause harm to human health or the environment.[3]

In the case of UV-VIS spectrophotometry, selecting the optimal number of wavelengths from the full range of the electromagnetic spectrum that results in maximum accuracy is still a challenging task, especially when spectra display strong overlapping features. Spectral wavelength selection is a process that optimizes the prediction capacity through choosing those wavelengths at which the analyte of interest absorbs but does not overlap with absorbances of other interfering species. Ultimately, this process leads to the rejection of wavelengths that are not related to the analyte of interest.

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Among the different strategies for variable selection, genetic algorithms (GAs)[14–17] are widely used alternatives. The mechanisms of natural selection used in GA methods progress by random search techniques and make these techniques robust and suitable for parallel processing implementations. A complete discussion of genetic algorithms can be found in the literature.[18–21]

The use of artificial neural networks in chemometrics has increased in recent years.[22–26] Their ability to handle linearities as well as non-linearities makes them a valuable tool, especially when classical multivariate calibration methods fail. In addition, by combining principal component analysis and artificial neural network (PCA-ANN) analysis, an improved ANN model can be obtained. In such a model, the principal component analysis of the spectral data of the calibrated mixtures is calculated first, and the scores from this computation are used as the network inputs instead of original data. Therefore, the number of inputs can be reduced to a much smaller PCS. Consequently, training time, repetition, and redundancy in the system decrease to yield a more accurate network.

This paper reports the simultaneous spectrophotometric determination of chlordiazepoxide and clidinium in synthetic samples or pharmaceuticals. The most important aspect of this work is comparison of the predictive ability of three chemometric methods including PLS, principal components-artificial neural network (PC-ANN) analysis and genetic algorithm-partial least squares regression (GA-PLS) as wavelength selection techniques.

Experimental

Apparatus

A dual-beam GBC Cintra 101 spectrophotometer with 1-cm quartz cells, a scan rate of 1000 nm min^{-1} and a slit width of 2 nm was used for collecting the absorbance spectra over the wavelength of 200–400 nm with one data point per nanometer. All spectral measurements were performed using a blank solution as reference.

Reagents and solutions

All reagents used were of analytical grade (Merck and Fluka) and were used directly without further purification. Chlordiazepoxide and clidinium bromide were kindly donated by SOBHAN pharmaceutical company (Tehran, Iran). Triple-distilled water was used to prepare buffer and reagent solutions. Stock solution containing 100 μg ml^{-1} of chlordiazepoxide and clidinium bromide were prepared by dissolving appropriate weights of these drugs in 100 ml of ethanol and 0.1 M HCL, respectively. All solutions were prepared fresh daily.

Pharmaceutical preparation

Three commercial pharmaceutical preparations (Clidinium-C® tablets) manufactured by SOBHAN (Tehran, Iran), HAKIM (Tehran, Iran) and AMIN (Isfahan, Iran) Pharmaceutical laboratories were purchased from local drug stores and assayed. Their declared contents were as follows: 2.5 mg clidinium and 5 mg chlordiazepoxide in each coated tablet.

Procedures

Individual calibration

In two 100-ml volumetric flasks, 100-μg ml^{-1} solutions of chlordiazepoxide and clidinium were prepared from their stock solutions by dissolving 100 mg of each compound in ethanol and 0.1 M HCl in a 3 : 1 ratio. The absorbance values of the chlordiazepoxide and clidinium solutions were measured between 200 and 400 nm against a solvent blank.

Multivariate calibration

Each calibration, prediction, and synthetic mixture was prepared by adding an appropriate amount of each ingredient (from 100-μg ml^{-1} stock solutions) to a 10-ml volumetric flask that was then filled with ethanol and 0.1 M HCL (3 : 1). Two sets of standard solutions were prepared. The calibration set contained 25 standard solutions, and the prediction set contained 16 standard solutions. The concentration range in these mixtures was 1–10 μg ml^{-1} for chlordiazepoxide and 1–15 μg ml^{-1} for clidinium. The concentration ranges were chosen so that absorbance values obtained for all standard samples were less than 1.5 absorbance units. This criterion guaranteed the accuracy and precision of the measurements and yielded reliable results. The composition of the samples was determined by a five-level factorial design to ensure the elucidation of a non-correlated concentration profile. The calibration matrix is shown in Table 1. PLS, a genetic algorithm coupled with PLS and PC-ANN were used for chemometric analysis of the data. For all calculations, Matlab for Windows (version 7.0)[27] was used. The GA-PLS and ANN methods were carried out with the PLS-Toolbox[28] and the Neural Network Toolbox[29] within the Matlab software program, respectively.

Sample preparation

Ten tablets were ground and mixed in a mortar. An amount of powder equivalent to one tablet was accurately weighed and transferred into a volumetric flask using ethanol and 0.1 M HCl at a 3 : 1 ratio and dissolved by mechanical shake for 30 min. The solution was filtered into a 100-ml volumetric flask through Whatman No. 42 filter paper and diluted with the same solvent. This stock solution provided suitable working sample solutions for UV measurements.

Results and Discussion

Individual calibrations

Figure 1 shows the absorption spectra for the active ingredient solutions. Individual univariate calibration (linear regression) curves were constructed as absorbance versus concentration in the range 0.5–10 μg ml^{-1} for chlordiazepoxide and 0.5–15 μg ml^{-1} for clidinium. The intercepts on the ordinates are negligible in the calibration lines. Limits of detection, calculated as $3s_0$ per slope (where s_0 is the standard deviation of the intercept on the ordinate), were chlordiazepoxide (0.31 μg ml^{-1}) and clidinium (0.37 μg ml^{-1}), respectively. These results are shown in Table 2.

Multivariate calibration

The first step in the simultaneous determination of the composition of the mixture of active ingredients by multivariate calibration methods involves constructing the calibration matrix for binary mixtures of chlordiazepoxide and clidinium. A total of 25 mixtures were selected by five-level factorial design as the calibration set. The calibration model in each chemometric method was validated with 16 synthetic mixtures containing concentration proportions

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Table 1. Composition of the calibration samples for chlordiazepoxide and clidinium

Number	Chlordiazepoxide ($\mu g\ ml^{-1}$)	Clidinium ($\mu g\ ml^{-1}$)	Number	Chlordiazepoxide ($\mu g\ ml^{-1}$)	Clidinium ($\mu g\ ml^{-1}$)
1	3	7	14	9	9
2	9	12	15	7	12
3	7	5	16	9	5
4	7	9	17	3	1
5	5	1	18	9	7
6	5	5	19	1	7
7	7	1	20	3	9
8	1	1	21	5	9
9	1	5	22	3	12
10	7	7	23	5	7
11	1	12	24	3	5
12	9	1	25	5	12
13	1	9			

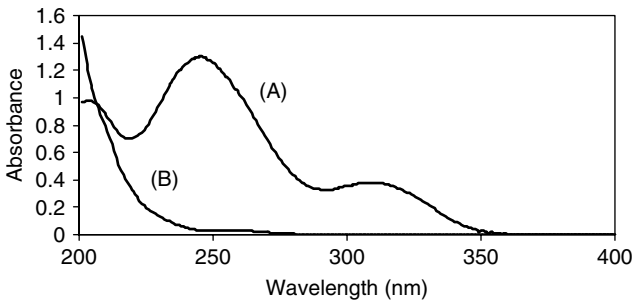

Figure 1. Absorbance spectra of: (A) 10 $\mu g\ ml^{-1}$ chlordiazepoxide and (B) 10 $\mu g\ ml^{-1}$ clidinium in ethanol and 0.1 M HCL (3 : 1 ratio).

Table 2. Parameters for the linear regression equations for each drug compound

Parameter	Chlordiazepoxide	Clidinium
Sample number	24	16
Linear range ($\mu g\ ml^{-1}$)	0.5–10	0.5–15
Intercept of calibration curve	1.1×10^{-2}	-7×10^{-3}
Standard error of intercept	6.2×10^{-3}	6.1×10^{-3}
Confidence limit of intercept	-1.18×10^{-3} to 2.45×10^{-2}	-1.99×10^{-2} to 6.49×10^{-3}
Slope of calibration curve	1.7×10^{-1}	1.0×10^{-1}
Standard error of slope	1.1×10^{-3}	6.9×10^{-4}
Confidence limit of slope	1.69×10^{-1} to 1.74×10^{-1}	1.02×10^{-1} to 1.05×10^{-1}
Correlation coefficient	0.9995	0.9997
Limit of detection ($\mu g\ ml^{-1}$)	0.31	0.37

created by a four-level factorial design. The predictive abilities of PLS, GA-PLS, and ANN were examined for simultaneous determination of chlordiazepoxide and clidinium in sample mixtures. A requirement that was common for all of the methods is that the unknown samples and standards must be of the same nature. Therefore, all contributions from drugs, interferences and matrix effects present in the samples must be modeled implicitly.[30]

In the PLS algorithm, the information regarding responses and concentrations are considered simultaneously. Once the model is built, it can predict the concentrations of unknown samples. The selection of the optimum number of factors was estimated by cross-validation.[31] The prediction error was calculated for each drug based on a prediction set containing no samples that were used for the construction of the model. The error was expressed as the prediction residual error sum of squares (PRESS). PRESS was calculated for the first latent variable (factor), which was extracted from the PLS calibration step. Then the next factor was added and PRESS was calculated again. These calculations were repeated for up to the 25 factors that were used in PLS modelling. This procedure was repeated for each drug. A plot of PRESS against the number of factors for each individual component displays a minimum value that encompasses the optimal number of factors. To find the smallest model with the fewest factors, **F** statistics were used. The optimal number of factors for chlordiazepoxide and clidinium were found to be three. The results obtained from simultaneous determination of chlordiazepoxide and clidinium by applying the model constructed from the PLS1 algorithm are given in Tables 3 and 4, respectively. In these tables, the prediction error of each drug in the mixture was estimated by calculating the relative standard error of the predicted concentration (RSE%).

Variable selection (wavelength) in the PLS model improved the prediction ability of the model.[32–34] In this study, the GA was run for 201 variables (in the range of 200–400) using a validated PLS regression method. After the most appropriate wavelengths were selected, the PLS was run again. To obtain the best set of wavelengths for determining the content of each drug, the GA procedure was repeated 10 times. If the percent selection for a variable exceeded the critical value with thresholds of 90% and 75% for chlordiazepoxide and clidinium, respectively, the wavelength was selected. The criteria for this selection were adjusted to reach the minimum error of prediction for each drug. The selected wavelengths were 236, 249, 252, 258, 260, 309, and 321 nm for chlordiazepoxide and 208, 210, 212, 219, 221, and 229 for clidinium. The prediction ability of each method was determined using 16 binary drug mixtures (Tables 3 and 4). As can be seen, the RSE% of the GA-PLS is slightly smaller than the results

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Table 3. Composition of the prediction samples and predicted values for chlordiazepoxide

Number	C_{real} (μg ml^{-1})	$C^a_{calc.PLS}$ (μg ml^{-1})	$C^a_{calc.GAPLS}$ (μg ml^{-1})	$C^a_{calc.PCA-ANN}$ (μg ml^{-1})
1	8.00	8.41	8.01	8.20
2	4.00	4.16	4.05	4.11
3	1.00	1.08	1.08	1.18
4	4.00	4.04	4.04	4.50
5	6.00	5.99	5.99	5.88
6	8.00	8.33	8.02	8.18
7	4.00	4.04	4.04	4.12
8	8.00	8.19	8.09	8.05
9	6.00	6.18	6.18	6.08
10	8.00	8.38	8.02	8.12
11	1.00	0.91	0.91	0.99
12	6.00	5.80	5.98	5.92
13	1.00	1.01	1.01	1.03
14	4.00	4.08	4.08	4.12
15	1.00	0.91	0.99	0.95
16	6.00	6.04	6.04	6.16
RSE (%)		3.57	1.23	3.16
Mean % recovery [b]		101.0	100.6	102.7
S.D. % recovery		4.66	3.26	5.47

[a] Calculated concentration.
[b] Comparison between the mean % recoveries showed no significant differences ([F(2,30)=2.645, p>0.05].

Table 4. Composition of prediction samples and predicted values for clidinium

Number	C_{real} (μg ml^{-1})	$C^a_{calc.PLS}$ (μg ml^{-1})	$C^a_{calc.GAPLS}$ (μg ml^{-1})	$C^a_{calc.PCA-ANN}$ (μg ml^{-1})
1	5.00	4.83	4.99	4.96
2	1.00	1.09	1.05	1.11
3	12.00	12.01	12.05	12.08
4	5.00	5.24	5.03	5.36
5	10.00	9.90	9.98	9.95
6	10.00	10.02	10.30	10.50
7	10.00	10.01	9.99	10.99
8	12.00	12.04	12.01	12.05
9	1.00	1.03	1.05	1.09
10	1.00	0.93	0.99	1.10
11	5.00	4.92	4.98	4.98
12	5.00	5.09	5.02	5.02
13	1.00	1.04	1.02	1.06
14	12.00	11.99	11.99	12.02
15	10.00	10.50	9.96	10.08
16	12.00	12.90	11.80	12.02
RSE (%)		3.31	1.15	3.62
Mean % recovery[b]		101.4	100.8	103.7
S.D. % recovery		4.0	2.0	4.5

[a] Calculated concentration.
[b] Comparison between mean % recoveries showed no significant differences ([F(2,30)=3.865, p>0.05].

for the PLS model, which shows that the GA-PLS might be more suitable for simultaneous determination of these drugs.

In this study, the number of factors obtained by PLS cross-validation for each drug was 3, which means that non-linearity may exist in the mixture due to interactions. So, the artificial neural network calibration (ANN) was used for evaluating the prediction ability of the non-linear model. The theory of ANN can be found elsewhere.[22–26]

By using the score matrix obtained by principal component analysis (PCA) performed on the original absorbance data, the training time and robustness of the ANN model increased. Consequently, a more accurate and precise network could make better predictions. To construct the network, two sets of data were used: the calibration set (Table 1) and the prediction set (Tables 3 and 4). The optimized PCA-ANN networks were constructed with these two sets to reach the minimum error of prediction. To avoid overfitting, the weights of the network were calculated from the calibration set and the prediction set simultaneously. The concentration of the prediction set was determined. After construction of the network, the third set (the validation set), which belonged neither to the calibration nor to the prediction set, was used to externally validate the network (data not shown). This procedure guaranteed the prediction performance of the network. Hence, this PCA-ANN network was used to predict the chlordiazepoxide and clidinium concentrations simultaneously. The construction of the optimized ANN model is summarized in Table 5. The predictions resulting from the application of this model are shown in Tables 3 and 4.

In spite of the better RSE% obtained by GA-PLS compared to PLS and PC-ANN, the statistical analysis of the mean % recovery of the chlordiazepoxide and clidinium by one-factor repeated-measured analysis of variance (ANOVA) showed no significant differences between the proposed methods (Tables 3 and 4).

Table 5. Optimized parameters values for ANN

Drug	Number of PCs	Number of epoch	Number of neuron	Momentum constant	Learning rate
Chlordiazepoxide	2	450	4	0.800	0.15
Clidinium	5	600	5	0.825	0.15

Analysis of real samples

The proposed methods were applied to the determination of chlordiazepoxide and clidinium in several drug samples. Five replicate measurements were made to evaluate the agreement between the results and the claimed values. As can be seen in Table 6, satisfactory results were obtained in all cases by the proposed procedure for simultaneous determination of chlordiazepoxide and clidinium in real samples. The lower standard deviation of the GA-PLS method shows that the precision of this method seems to be better for the determination of these two drugs. However, statistical analysis of the data shown in Table 6 by ANOVA shows similar results because the experimental F values in all comparisons were less than the critical F ($\alpha = 0.05$) found to be 3.88. Consequently, all three of the abovementioned methods yield comparable results regarding the contents of chlordiazepoxide and clidinium in our studied tablets. To check the validity of the

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Table 6. Assayed results of the simultaneous determination of chlrodiazepoxide and clidinium in commercial tablets by the proposed methods.

Commercial formulation	Chlordiazepoxide			Clidinium		
	PLS	GA-PLS	ANN	PLS	GA-PLS	ANN
Clidinium-C (Sobhan)						
Amount on the label (mg)	5.0	5.0	5.0	2.5	2.5	2.5
Mean % recovery [a]	101.3	100.3	101.8	99.5	100.2	101.4
S.D. % recovery[a]	0.76	0.34	0.79	0.78	0.36	0.89
Clidinium-C (Amin)						
Amount on the label (mg)	5.0	5.0	5.0	2.5	2.5	2.5
Mean % recovery [a]	102.1	99.9	100.9	100.4	100.5	103.1
SD % recovery [a]	0.55	0.31	0.87	0.79	0.21	0.65
Clidinium-C (Hakim)						
Amount on the label (mg)	5.0	5.0	5.0	2.5	2.5	2.5
Mean % recovery[a]	99.4	100.1	101.0	101.2	99.2	101.4
SD % recovery[a]	0.61	0.45	0.68	0.75	0.22	0.89

[a] Mean and SD for five determinations. Mean and SD. % recovery compared to the claimed amount.

Table 7. Recovery studies of chlordiazepoxide and clidinium in commercial tablets (Clidinium-C) using the proposed methods

Clidinium-C	Chlordiazepoxide			Clidinium		
	PLS	GA-PLG	PC-ANN	PLS	GA-PLG	PC-ANN
Mean % recovery[a]	100.2	99.9	98.5	99.7	100.1	101.1
SD % recovery	0.66	0.32	0.51	0.44	0.23	0.45

[a] For standard addition of 50% of the declared content.

proposed methods, the standard addition method was used. After adding the known amounts of chlordiazepoxide and clidinium to the commercial formulations, the recovery of each drug was determined by dividing the concentrations obtained from the spiked samples by those of the pure drug. As is shown in Table 7, the high recovery values obtained suggest a high accuracy of the proposed methods that does not suffer from interferences from excipients that are present in commercial formulations.

Conclusion

Spectrophotometry as a rapid and low-cost method is a good choice for quantitative determination of drugs in routine quality control programmes. However, overlapping absorbance spectra are often a problem when trying to simultaneously determine the content of drugs. Combination of spectrophotometry and chemometrics can overcome such limitations and enhance the quality and efficacy of the analysis. Our study proves the feasibility of simultaneous spectrophotometric determination of chlordiazepoxide and clidinium in synthetic and pharmaceutical preparations without the need to apply any extraction step because of our employment of chemometrics methods. PLS, GA-PLS, and PC-ANN were chosen as our analysis methods, and their abilities to simultaneously determine the presence of chlordiazepoxide and clidinium were compared. All methods showed high accuracy and precision with slightly better results resulting from the GA-PLS method. The superiority of GA-PLS over the other applied multivariate methods is due to its wavelength selection using PLS calibration and the genetic algorithm, which resulted in no loss of prediction capacity. The proposed methods have been successfully applied to synthetic mixtures and real samples, and the high percentage of recovery shows the abilities of the methods to determine chlordiazepoxide and clidinium simultaneously without interference from excipients in commercial formulations.

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