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A novel method for simultaneous determination of codeine and acetaminophen in plasma by combination of UV-Vis spectroscopy and artificial neural network

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A sensitive and selective method using combination of principal component analysis (PCA), artificial neural network (ANN) and UV-Visible spectroscopy has been developed for the simultaneous determination of acetaminophen (AMP) and codeine (COD) in plasma samples. The ANN trained by the back-propagation learning was employed to model the complex non-linear relationship between the PCs extracted from UV-visible spectra of medications and the absorbance values. Optimal ANN model were as follows: Number of input PCs: 6, number of neurons in hidden layer: 5. The linear calibration range was 10-70 μg ml^{-1} and 40-700 μg ml^{-1}, and the detection limit were 0.3 μg ml^{-1} and 1.3 μg ml^{-1}, for AMP and COD, respectively. The results have been compared with those obtained by the HPLC method.

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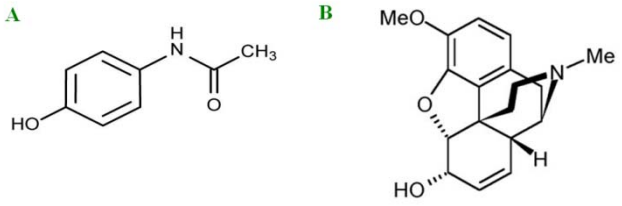
Introduction

Blood is very useful biological matrix in clinical and forensic toxicology due to its significance for determining short-term to drugs. Blood concentration of drugs and chemicals can be directly related to the experimental symptoms or behavior [1, 2]and its analysis is necessary to confirm, for example, if a subject is driving under the effect of pharmaceuticals.

Acetaminophen (AMP) (Scheme 1A) is extensively employed as an analgesic and as an antipyretic drug. Many analytical methods for determining of AMP have been reported including titrimetry[3], chromatography [4-6], fluorometry[7], colorimetry[8], UV spectrophotometry [9], and a variety of modes of electrochemistry techniques [10-17].

Codeine (COD) or 3-methylmorphine (a natural isomer of methylated morphine) (Scheme 1B) has long been employed as an efficient analgesic and antitussive agent in pharmaceutical preparations [18-20]. There are some reports for assaying of COD including gas chromatography, chemiluminescent, and high performance liquid chromatography, were reported for codeine determination [21-29].



Scheme 1. the structure of (A) acetaminophenand(B) codeine

In fact, in many cases, particularly fields outside the major stream of the analytical chemistry, or even biometrics and psychometrics, the linear regression methods undoubtedly are invaluable tool. The reason is that the underlying factors have little or no physical meaning so a linearly additive model in which each underlying factor can be interpreted chemically is not expected [30] .

Recently, as the researchers have get accustomed to the application of computers, as a result to the implementation of different complicated statistical techniques, they are trying to probe multivariate correlations between the input and output variables increasingly in detail. With the increasing accuracy and precision of analytical measuring techniques it become clear that all effects that are of interest cannot be explained completely by simple univariate and even not by the linear multivariate calibration techniques such as MLR and PLS. Therefore, the researchers are at all times eager to attempt all new techniques that are available to solve such problems. A set of approaches, that have newly found very intensive application among researchers are the artificial neural network (ANN) methods.

The ANNs are not easy to explain with a simple definition. A typical neural network can be considered as a black box having multiple inputs and multiple outputs and is able to predict an response when it recognizes a given input pattern [31]. ANN approaches operates employing a large number of mostly parallel connected simple processing units called neuron. The most important note that can be mentioned about all ANN techniques is that they work best if they are dealing with non-linear relationship between the inputs and outputs (Figure 1).

ANN methods can be used to explain or to find linear relationship as well, but the final result might often be worse than that if using other simpler linear statistical methods [31]. Because of the fact that at the beginning of establishment calibration between independent and dependent variables, researches often do not know whether the dependent variable is related to the independent variables in a linear on in a nonlinear way, a good advice is to attempt at all times a or more linear regression approach for interpreting the data parallel to the use of ANNs[31].

Codeine and Acetaminophen are often prescribed in admixture with each other or in combination with other drugs. Literature revealed that several techniques have been reported for the quantification of codeine and acetaminophen individually but no analytical method using UV-visible spectroscopy analysis for their simultaneous determination is reported.

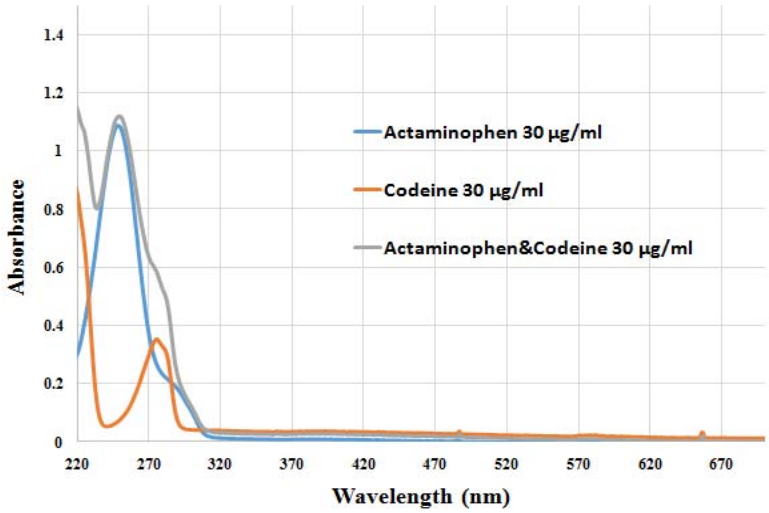


Fig. 1. UV absorbance spectra of methanolic solution of drugs studied in this study

Thus a precise and accurate method has been suggested in this study to quantify acetaminophen and codeine simultaneously by combination of ANN technique and UV-Visible spectroscopy. The determinations based on UV- visible spectroscopy are often preferred in quality control testing and ordinary laboratories due to its wide availability and appropriateness. The main goals of this research are to develop and validate a simple, rapid and specific UV spectrophotometric method for the simultaneous determination of codeine and acetaminophen in biological fluids. This method exhibited precise, accurate and inexpensive determination for these drugs in plasma.

Materials and methods

Chemicals, Instruments and software

Pure powders of codeine and acetaminophen were purchased from sigma. HPLC grade acetonitrile and methanol were obtained from Merck. Methanolic stock solutions (10^{-3}M) were used. All other chemicals used were of analytical grade quality and bidistilled water was used too. An hp spectrophotometer (Agilent 8453) with 10 mm quartz cells was used. A PCA and ANN required routines were written in Matlab package in our laboratory. The concentration of the mixture solutions were uniformly distributed over the range from 10 µg/ml to 70 µg/ml and from 40 µg/ml to 700 µg/mlfor AMP and COD, respectively. The UV–visible spectra of the mixtures were used over the wavelength range 220–650 nm in increments of 1nm.

Sample extraction

The plasma samples were stored at −20 ˚C and allowed to thaw at room temperature before processing. The analytes of interest (AMP and COD) were extracted from the plasma samples using a single-step liquid-liquid extraction procedure. 1 ml Clean human plasma samples (up to 1.0 ml) were pipetted into clean glass tubes and analytes with known concentration was added. The tube then was vortex-mixed for 2 min and allowed to stand at room temperature for some minute. Then, the samples was treated with 200 µlNaOH 1M and shake well. A total of 3 ml ethyl acetate was added. After vortexing for 3 min, and after 20 min centrifugation at 3500 rpm, the organic phase was transferred into a new glass tube and evaporated until completely dry under a nitrogen stream.

Univariate calibration

In order to find the linear dynamic concentration range of COD and AMP, A univariate calibration was carried out. Different volumes of a 1×10^{-3} solution of each medication were added into different 10 ml volumetric flasks and diluted to the mark with plasma. An extraction procedure described above was used. The absorbance spectra were recorded over the 210–650 nm spectral range versus a solvent blank. The linear dynamic range for each drug was determined by plotting the absorbance at its λ_{max} (274 nm for COD and 241 nm for AMP) versus sample concentration.

Standard mixture solutions

To build ANN model, a set of standard mixture solutions (i.e. training and test sets), plasma spiked with AMP and COD within the linear dynamic ranges were prepared.

As shown in Table 1, the calibration set contained 37 standard mixtures, and 9 mixtures were employed in the validation set. The respective concentrations of COD and AMP in the standard mixtures were in their linear range. For preparation of each solution, the required volumes of stock solution were added to a 10.0 ml volumetric flask, and the contents of the flask were diluted to volume with solvent. Then, the absorbance spectra of the mixture were recorded versus the solvent blank. The spectra were recorded in the wavelength range of 190–1100 nm but the range of 210–650 was used in the calibration step.

Table 1. Concentration data of the original dataset for mixture systems spiked in plasma

Sample No.	Codeine conc. μg/ml	Acetaminophen conc. μg/ml	Sample No.	Codeine conc. μg/ml	Acetaminophen conc. μg/ml
1	40	10	25	480	40
2	150	10	26	590	40
3	260	10	27*	40	50
4	370	10	28	150	50
5	480	10	29	260	50
6	590	10	30	370	50
7	700	10	31	480	50
8	40	20	32	700	50
9	150	20	33*	40	60
10	370	20	34*	150	60
11	480	20	35	260	60
12	590	20	36	370	60
13	700	20	37*	480	60
14	40	30	38	590	60
15	150	30	39	700	60
16	260	30	40	40	70
17	370	30	41	150	70
18	480	30	42*	260	70
19	590	30	43	370	70
20*	700	30	44	480	70
21	40	40	45*	590	70
22	150	40	46*	700	70
23*	260	40			
24	370	40			

*Solutions selected as test set

Calibration and validation sets splitting

In order to test the final model performances, about 20% of the samples (9 out of 46) were selected as external test set samples. The best situation of this step of model formation is dividing original matrix to guarantee that both training and test sets individually cover the total

space occupied by original dataset. Then ideal splitting of dataset as each of samples in test set is close to at least one of the samples in the training set. Various methods were used as tools for splitting the whole original dataset to the training and test sets. According to Tropsha, the best models would be built when Kennard and Stone algorithm was used [32, 33] .The Kennard–Stone [34] algorithm selects a set of samples in studied set of data, which are 'uniformly' distributed over the space defined by the original dataset.

This is a classic technique to extract a representative set of samples from a given dataset. In this technique the samples are selected consecutively. The first two samples are chosen by selecting the two farthest apart from each other. The third sample chosen is the one farthest from the first two samples, etc. Supposing that m samples have already been selected (m<n), the (m+1)th sample in the calibration set is chosen using the following criterion:

$$\max_{m<r\leq n}(\min(d_{1r}, d_{2r}, \ldots d_{mr})$$

where n stands for the number of samples in the training set, d_{jr}, j=1,...,m are the squared Euclidean distances from a candidate sample *r*, not yet included in the representative set, to the *m* samples already included in the representative set. One more benefit of the Kennard–Stone method is that it may be used to any matrix of predictors; there are no restrictions regarding the matrix multicollinearity. The other advantage is that the test samples all fall inside the measured region and the training set samples map the measured region of the input variable space completely with respect to the induced metric.

Principal Component Analysis (PCA)

Experimental spectrums were exported to the MATLAB routines for the purpose of PCA. The complete data set, defined by the absorbance of drugs in the columns (in this study, 440 column) and the samples in the rows, was autoscaled through mean centering by column. PCA models the maximum directions of variation in a data set by projecting the samples as a swarm of points in a space spanned by PC's. Each PC is a linear function of a number of original absorbance of drugs, resulting in a reduction of the original number of variables. PC's describe, in decreasing order, the most variation among the samples, and because they are calculated to be orthogonal to one another, each PC can be interpreted independently. This allows an overview of the data structure by revealing relationships between the samples as well as the detection of deviating samples. To find these sources of variation, the original data matrix of UV-Visible spectrums, defined by *X(n,m)*, is decomposed into the sample space, the descriptor space, and the error matrix. The latter represents the variation not explained by the extracted PC's and is dependent on the problem definition. The approach describing this decomposition is presented as:

$$X(n,m) = T(n,k)P(k,m)^T + E(n,m)$$

Where *X* is the independent absorbance matrix, *T* is the scores matrix, *P* is the loadings matrix, *E* is the error matrix, *n* is the number of samples, *m* is the number of columns in original data set, and *k* is the number of PC's used.

In PCR procedure, all calculated scores were collected in a single data matrix and the best subset of PCs was obtained by a stepwise regression.

Artificial neural network

One method to providing a more flexible form of linear regression is to use a feed-forward neural network with error back-propagation learning algorithm. This is a computational system whose design is based on the architecture of biological neural networks and which consists of artificial 'neurons' joined so that signals from one neuron can be passed to many others (Figure 1). Clarification of the theory of the artificial neural networks in details has been adequately described elsewhere [35] but little relevant remarks is presented. ANN are parallel computational tools consisting of computing units named neurons and connections between neurons named synapses that are arranged in a series of layers.

Back propagation artificial neural network includes three layers. The first layer namely input

layer has n_i neurons, and duty of this layer is reception of information (*i.e.* inputs) and transfers them to all neurons in the next layer called the hidden layer that number of them was indicated by n_h. The neurons in the hidden layer calculate a weighted sum of the inputs that is subsequently transformed by a linear or non-linear function. The last layer is the output layer and its neurons handle the output from the network and it is the calculated response vector. Duty of synapses is connection of input layer to hidden layer and hidden layer to output layer. The manner in which each node transforms its input depends on the "weights" and bias of the node, which are modifiable. On the other hand the output value of each node depends on both the weight, and biases values. In addition, depend on, the weighted sum of all network inputs, which are normally transformed by a nonlinear or linear transform function determine the outputs of the network.

The relation between response, Y_O, of the network and a vector input, X_i, can be written as following if number of neurons in the output layer is equal to 1 (same with our condition in here):

$$Y_O = \sum_{J=1}^{N_H} W_J \, f\left(\sum_{I=1}^{N_I} W_{JI} X_I + b_I \right) (1)$$

Where b_j is the bias term, W_{JI} is the weight of the connection between the *I*th neuron of the input layer and the *J*th neuron of the hidden layer, and *f* is the transformation function of the hidden layer. In the training process, the weights and bias of the network which are the adjustable parameters of the network are determined from a set of objects, which is known as training set.

Through the training of the network, the connection weights are regulated so that error of calculated responses and observed values was minimized. For this, a nonlinear transfer function makes a connection between the inputs and the outputs. Commonly neural network is adjusted, or trained, so that a particular input leads to a specific target output. There are numerous algorithms available for training ANN models. We used back propagation algorithm here for training of network. In this algorithm several steps for minimizing of networks were performed and the update of weight for the *(n + 1)*th pattern is given as:

$$W_{JI,n+1} = W_{JI,n} + \alpha \Delta W_{JI,n} (2)$$

With using following equation the descent down the error surface is calculated [36]:

$$\Delta W_{JI,n} = -\mu \frac{\partial E}{\partial W_{JI,n}} \quad (3)$$

Where α and μ are momentum and learning rate, respectively.

With respect to above demonstration, in the ANN some adjustable parameters exist including number of nodes in input and hidden layers, transfer function of hidden and transfer function output layers, momentum, number of iteration for training of network and learning rate that were evaluated by obtaining those which result in minimum in the error of prediction.

As mentioned above in order to avoid overfitting and underfitting, a validation set was used in the ANN modeling. Evaluation of ANN was performed on an external set (validation set) that consisted of samples belonging to neither the calibration set nor the test set.

High-performance liquid chromatography

The HPLC system consisted of a **Shimadzu** system equipped with two **LC-10AD-Vp** pumps, a model 1001 solvent delivery system and a model 2700 UV detector, equipped with Millennium chromatography manager for integration.

Separations were carried out on a Nucleosil-100 C8 column (250 mm × 4 mm i.d., 5 µm particle size) that protected by a Nucleosil-100 C8 Encapped guard (4 mm × 4 mm i.d., 5 µm particle size) obtained from Knauer (Germany).

The chromatographic run began with acetonitrile-trichloroacetic acid 0.025% (10: 90) solution. The mobile phase flow-rate was 0.7 ml min^{-1}. This HPLC procedure resulted in a total run time of 10 min for two drugs studied.

The injection volume was 20 µL for all the solutions and the detection was performed at the wavelength of 281 nm.

Results and Discussion

Fig. 1 shows the absorption spectra of AMP and COD extracted from plasma separately. Fig. 1 shows that the absorbance maximum of AMP and MEP are 241 nm and 275 nm, respectively. As can be seen, the difference in maximum of absorbance spectra is not so large to permit simultaneous determination of the analytes using conventional univariate calibration methods. Said another way, the simultaneous determination of AMP and COD in mixtures by conventional spectrophotometric methods is hindered by strong spectral overlap throughout the wavelength range. Such a determination could theoretically be facilitated by the application of multivariate calibration such as PCA.

Hence, PCR as a robust multivariate regression technique was used for simultaneous analysis of AMD and COD in pharmaceutical samples. It should be mentioned that the spectral regions employed in PCR were between 210 and 425 nm because methanol has strong absorbancies at wavelength regions lower than 210 nm.

Reproducibility and stability

The reproducibility of the proposed technique was assessed by measuring the uv-vis absorbance spectra for fresh solution of 1.0×10^{-5} M AMP and COD for some replicate measurements. The peak absorbance was almost identical after absorbance experiments and compared to the original spectrum, a small discrepancy was observed. This suggests that the proposed method possesses good reproducibility.

Conventional univariate calibration

In order to establish the optimal conditions for the joint determination of AMP and COD, the univariate calibration technique was applied to investigate the effect of experimental variables on the absorption spectra for the drugs studied.

In order to analyze the drugs, individual calibration curves were constructed with several points (Fig. 2 and 2), as absorbance vs. analytes concentration in their range of 10 μgml^{-1} to 70 μgml^{-1} and from 40 μgml^{-1} to 700 μgml^{-1} AMP and COD, respectively. The wavelengths employed to generate calibration curves were 241 and 275 nm for AMD and COD, respectively. Linear regression results, line equations, and R^2 are also shown in Fig. 2 and Fig.3.

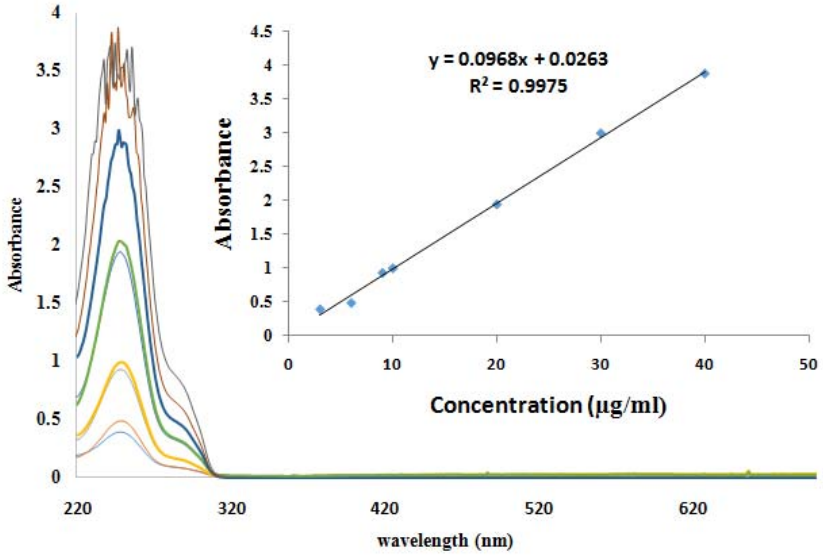


Fig. 2. Analytical curve for univariate determination of AMP.

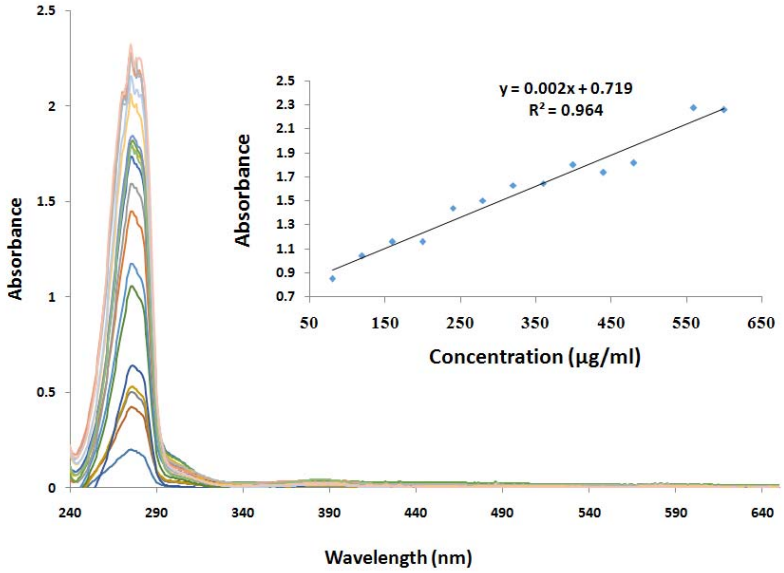


Fig. 3. Analytical curve for univariate determination of COD.

PC-ANN modeling

Because of the large number of columns of spectra, PCA was employed to solve the collinearity problem in and the principal components (PCs) were used as new variables for model building. After deleting zero variance columns of X block, PCA was carried out on the pool of all spectra. A first 20 significant PCs were reported in Table 2. In this Table, the eigenvalues, the percent of variances explained by each eigenvalue and the cumulative percent of variances are represented.

Table 2. Eigenvslues of calculated PCs, % of explained variances and cumulative variances obtained from PCA.

PC No.	Eigenvalue	% variance explained	cumulative variance
1	608.303	66.773	66.773
2	146.104	16.038	82.811
3	84.462	9.271	92.082
4	24.409	2.679	94.762
5	13.697	1.504	96.265
6	9.116	1.001	97.266
7	4.273	0.469	97.735
8	3.443	0.378	98.113
9	2.070	0.227	98.340
10	1.826	0.200	98.541
11	1.519	0.167	98.707
12	1.354	0.149	98.856
13	1.191	0.131	98.986
14	1.035	0.114	99.100
15	0.935	0.103	99.203
16	0.825	0.091	99.293
17	0.713	0.078	99.372
18	0.692	0.076	99.448
19	0.564	0.062	99.510
20	0.505	0.055	99.565

As can be seen in Table 2, PCA gives 6 significant PCs (% variance explained > 1), These PCs can explain more than 97.26 % of the variances in the original descriptors data matrix.

Two sets of standard solution of plasma samples including drugs were prepared.

The concentrations of each medication inthe resulting standard csolutions were in the range of linear dynamics determined for each drug.

A typical multivariate calibration technique needs a suitable experimental design of the standard composition of calibration set to give the best predictions. The best situation in this stage of model building is dividing data set to guarantee that both training set and test set individually cover the total space occupied by original data set. The possibility of overfitting of the developed model increases by the selection of more similar samples as calibration set. Hence, ideal splitting of data set can be performed in such a way that each of the samples in test set to be close to at least one of the samples in the calibration set. In order to chose the mixtures that provide more information using a few experimental trials from calibration set, Kennard and Stone method was used for determination of plasma samples in the calibration and prediction sets. In determining calibration and test set mixtures an important point should be considered; concentration ranges of the sets should be comprised in the linear ranges of their univariate calibration graphs. Application of splitting algorithm, led to preparation of training set of 28 samples with the compositions as shown in Table 1. The calibration set is used to train a neural net. A total of 9 synthetic mixtures containing the drugs spiked in plasma were also prepared for prediction (Table 1). The prediction set is used to determine the performance of a neural network on patterns that are trained during learning, and 9 mixtures (validation set) that were not included in the previous sets were employed as an independent test for finally checking the overall performance of a neural net. The validation set was also selected using Kennard and stone. After dividing the samples into two parts, calibration and test sets, based on Kennard and Stones algorithm, building of regression models using calibration set was performed.

The problem of the 'number of significant PCs' [37] is one of the most critical problems the researcher faces when using PCA in calibration model building.

The ability to identify relationships by generating linear combinations of absorbencies indicating common trends of variation can contribute substantially to the recognition of patterns in the matrix of spectra. However, the issue of determining whether or not a given PC is non-trivial remains obscure in many studies.

As discussed above, PCA performs a transformation on a set of *n* correlated/uncorrelated columns of matrix of spectra into linear combinations of a set of n pair-wise uncorrelated variables called PCs. PCs are built so that the first component explains the largest amount of total variance in the original matrix of spectra and each subsequent component is built so as to explain the largest amount of the remaining variance while remaining uncorrelated with (orthogonal to) previously built components. The set of *n* PCs is often reduced to a set of size p, where $1 \leq p < n$. This procedure is called dimension reduction.

The ultimate goal of dimension reduction is to make analysis and interpretation easier, while at the same time retaining most of the information (variation) contained in the original data. Clearly, the closer the value of *p* is to *n* the better the PCA model will fit the data since more information has been retained, while the closer p is to 1, the simpler the generated model.

In the ANN model, a network including a fully connected three layer, feed-forward neural network model trained with a back-propagation learning algorithm was used. The input of the network was the eigenvalue ranked PCs. PCs were applied as input of networks. The resulted model called PC-ANN. In order to evaluate the ANN, mean square error for test set (MSEtest) was used. The values resulting from hidden layer are transferred to the last layer, which contains a single neuron representing the predicted activity. For output layer a linear transfer function was chosen.

In order to find how many PCs (factors) are appropriate to describe the best developed model; MSEtest was evaluated when varying the number

of factors. As it was shown in fig.4 RMSECV minimized at 6 LVs and, after this number, RMSECV increase significantly. Thus the regression model was built using 6 LVs

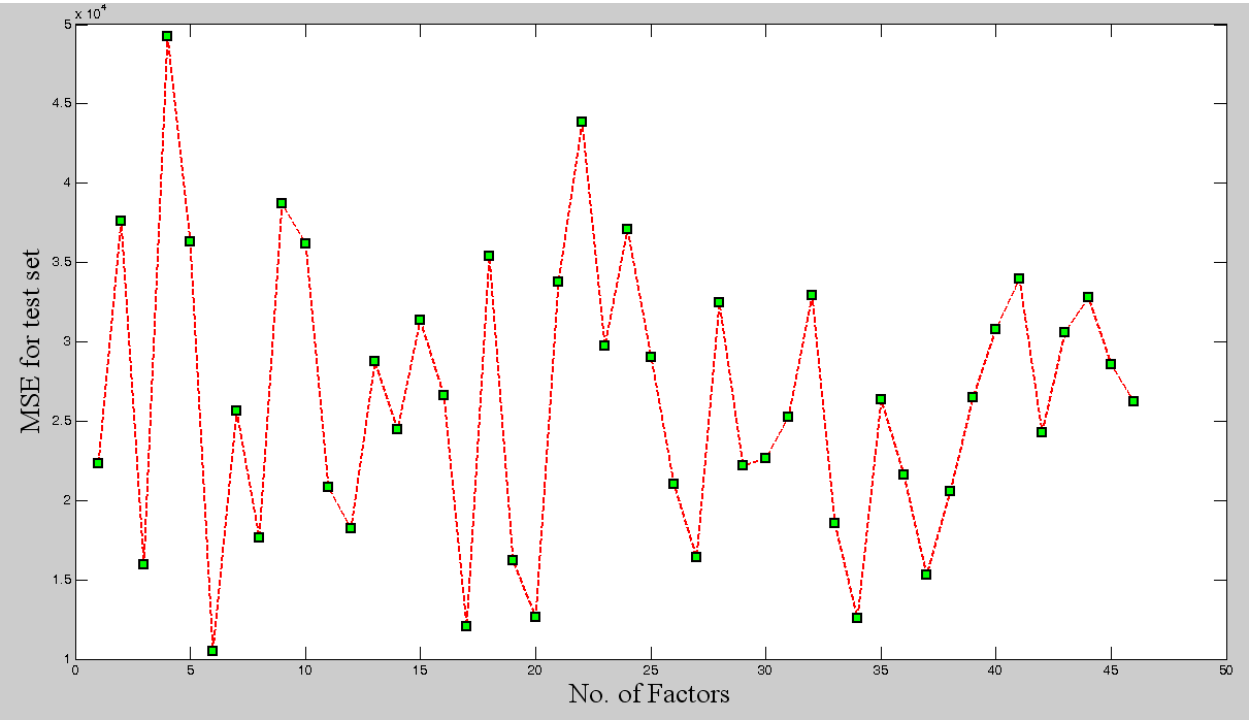


Fig. 4. Selection of optimum number of PCs for ANN model.

Various ANN architectures were run with the six selected PCs as input. In each run, the neuron architecture and parameters were optimized to reach the lowest MSE for test set as the performances of the resulted models. It must be considered that for inhibition of overfitting in the ANN model, the training of the network for the prediction of concentration must be stopped when the MSE of the test set commences to increase while MSE of training set continues to decrease. Therefore, training of the network was stopped when overtraining began.

As mentioned above, before training the network, the number of nodes in the hidden layer must be optimized. For this purpose, several training of network was performed with different numbers of hidden nodes from 1 to 12. The MSE for test set was obtained for different numbers of neurons at the hidden layer, and the minimum value of MSE was verified as the optimum value. A typical plot of MSE for test set versus the number of nodes in the hidden layer has been shown in Fig. 5. It is clear that 7 nodes in the hidden layer is the optimum value.

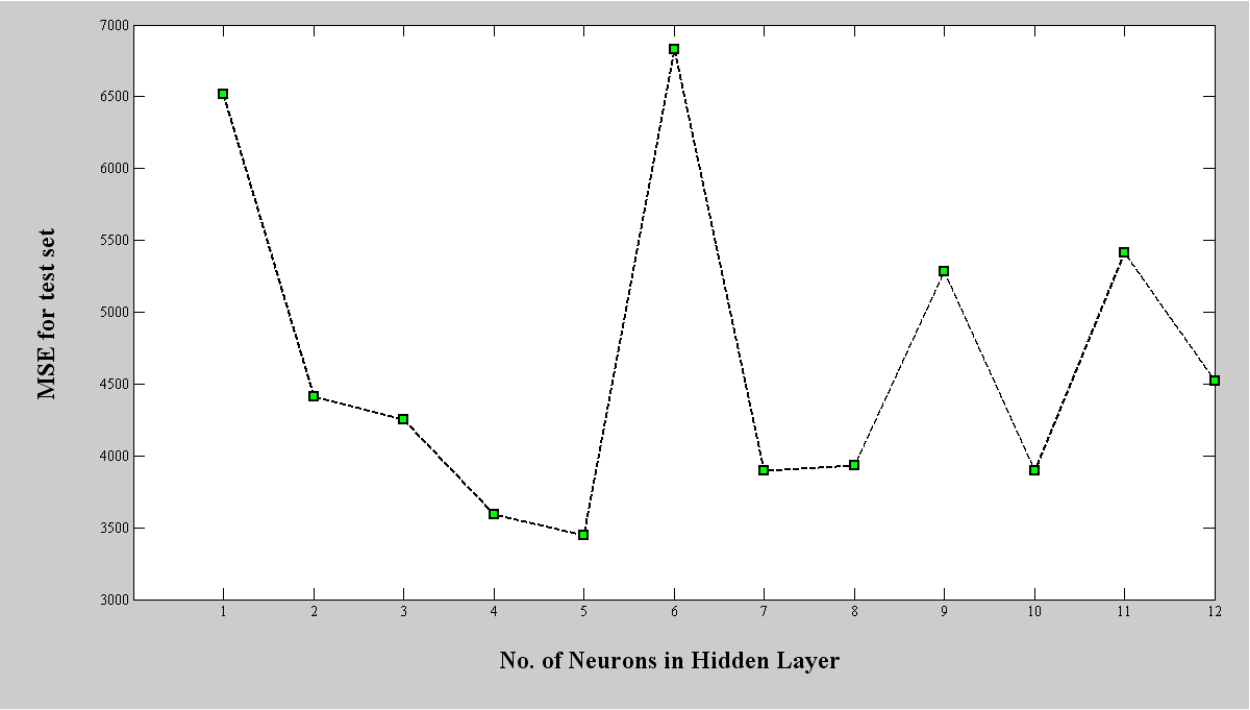


Fig. 5. Optimization of number of nodes in developed ANN model using MSE for test set.

A nonlinear transformation named the sigmoidal function was used between the input and output of a node in the hidden layer, and the pureline function was employed for the output layer nodes. For each permutation of network parameters (number of input nodes, number of hidden neurons, learning rate, and number of epochs), some separate networks were trained to reduce the effect of the random initial starting conditions.

In order to evaluate the performance of the model for simultaneous determination of AMP and COD, the calculated concentration againstexpected concentration for each drug using PC-ANN technique are listed in Table 3 and are plotted in Fig.6 and Fig.7. The plot of Fig.6 and Fig. 7 shows that the data are distributed around a straight line.

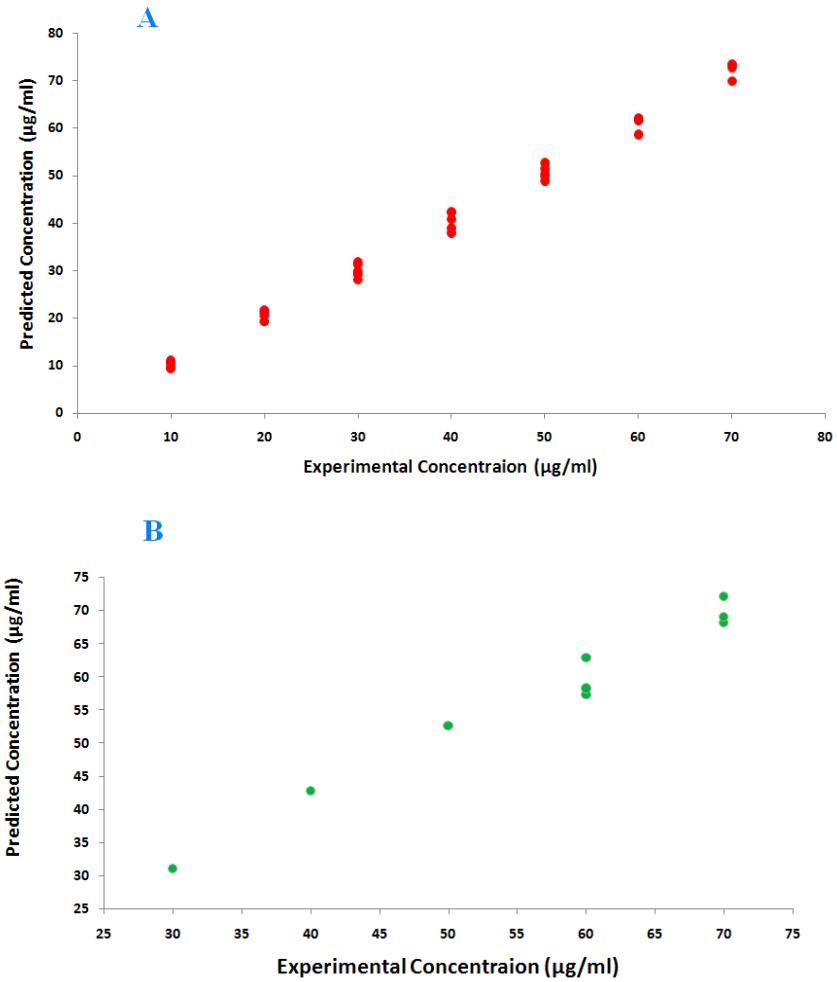


Fig. 6. Relationship between expected concentration of (A) AMP for calibration set(B) and test set and calculated concentration of drug that was calculated by PC-ANN.

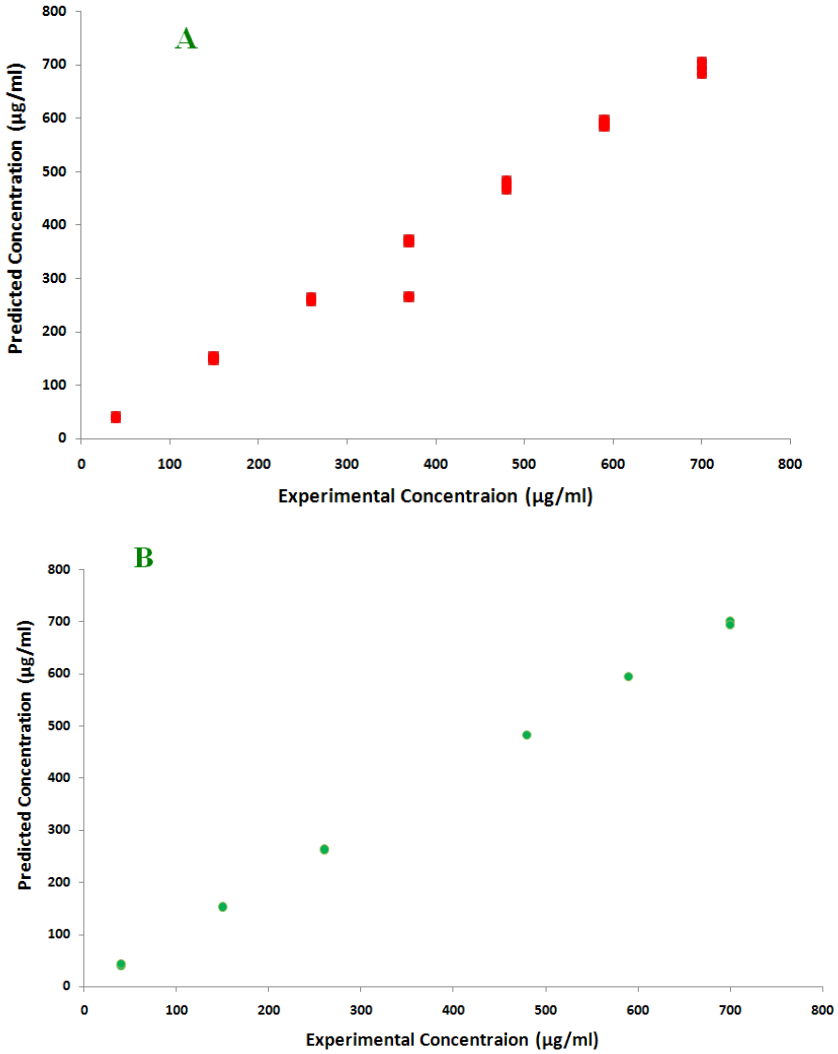


Fig. 7. Relationship between expected concentration of (A) COD for calibration set(B) and test set and calculated concentration of drug that was calculated by PC-ANN.

This model was validated by some statistics parameters such as R^2. Results are reported in the Table 4.

The good correlation coefficient (0.995 for AMP and 0.993 for COD) reveals the capability of the model. The linear dynamic range were 10–70.0 and 40–700.0 µg $ml^{-1}$for AMP and COD, respectively. In this study, the limit of detection (LOD) and has been calculated from the univariate definition as described by Garcia *et al.*[38] The spectra for five blank solutions (free-drug plasma) was obtained from 210 to 650 nm. From the developed PCA-ANN for each drug, the predicted concentrations were calculated. The standard deviation of predicted concentrations for each medication was estimated (S_b). Then, three times the S_b for each element was taken as the detection limit. Detection limits were 0.3 µg $ml^{-1}$for AMP and 0.80 µg ml^{-1} for COD.

Table 3. Predicted concentration for mixtures of drugs spiked in plasma using PC-ANN model

Sample No.	COD Con. (µg/ml)	% Rec	AMP Conc. (µg/ml)	%Rec
1	41.03	102.57	10.78	107.78
2	148.52	99.02	11.14	111.35
3	262.92	101.12	9.48	94.81
4	372.09	100.56	10.66	106.55
5	476.20	99.21	10.86	108.63
6	584.00	98.98	9.75	97.46
7	705.98	100.85	10.78	107.75
8	39.10	97.75	20.55	102.73
9	151.83	101.22	21.28	106.38
10	367.65	99.36	19.35	96.76
11	466.53	97.19	21.57	107.84
12	596.95	101.18	21.74	108.68
13	684.03	97.72	19.44	97.19
14	39.80	99.51	28.17	93.92
15	153.85	102.57	31.37	104.57
16	257.13	98.90	31.86	106.21
17	266.24	71.96	29.27	97.57
18	471.52	98.23	30.02	100.06
19	592.89	100.49	29.76	99.21
20	700.34	100.05	31.10	103.65
21	41.36	103.41	40.86	102.16
22	151.49	100.99	42.25	105.64
23	263.06	101.18	42.79	106.97
24	369.27	99.80	38.06	95.14
25	474.38	98.83	39.03	97.56
26	584.13	99.00	42.49	106.22
27	40.22	100.54	52.63	105.25
28	152.32	101.55	52.75	105.49
29	260.23	100.09	49.99	99.98
30	367.76	99.39	50.49	100.98
31	479.03	99.80	51.54	103.08
32	694.80	99.26	49.01	98.01
33	43.00	107.49	62.94	104.90
34	152.52	101.68	57.36	95.61
35	264.78	101.84	61.82	103.03
36	372.68	100.72	62.19	103.64
37	482.49	100.52	58.28	97.13
38	591.23	100.21	61.71	102.84
39	695.33	99.33	58.72	97.87
40	39.20	98.00	73.34	104.77
41	148.15	98.77	70.03	100.04
42	262.99	101.15	68.18	97.40
43	373.49	100.94	72.87	104.11
44	482.60	100.54	73.54	105.06
45	593.84	100.65	69.12	98.74
46	693.05	99.01	72.12	103.03

Comparison of the results of PC-ANN with HPLC

The aim of this part was to compare HPLC method for the simultaneous determination of AMP and COD in human plasma to developed PCA-ANN method. A satisfactory separation of each medication from biological endogenous components in human plasma was carried out.

The optimum wavelength for detection was 281 nm, at which much better detection responses were achieved at the optimized conditions, the retention times for AMP and COD were 7.52 and 4.72 min, respectively. No endogenous plasma component eluted at the retention time of COD and AMP.

One component analysis indicated that the chromatographic responses were linear in the concentration ranges, which were used in the PC-ANN discussed above.

Two sets of standard solutions resulted from synthetic mixtures of drugs spiked in the plasma were also used in HPLC experiment.

The calibration set was employed to construct the calibration curve for each drug separately, and the resulted calibration equation was employed to predict the concentration of the AMP and COD in the prediction set.

The approach was validated with regards to linearity, limit of detection and quantification, recovery, precision, accuracy and specificity.

Peak area (Y) of COD of calibration standards was proportional to the concentration (x) of drugs in plasma over the range tested. The peak area ratio (Y) of AMP calibration standards was proportional to the concentration (x) of AMP in plasma over the range tested. Blank human blood samples spiked with the corresponding compounds to give concentrations of 0.8, 2, 2.8, 4 and 6 μg/ml for COD, and 8, 30, 52, 74, 96, 118 and 140 μg/ml for AMP were analysed. For all analytes, excellent linearity was obtained in the specified concentration range.

As it can be seen in figure 8, the correlation coefficients for the calibration regression line were 0.999 and 0.988 for AMP for COD, respectively. The equations of the calibration lines were as follows: y=−2568 + 28589x for AMP and y=16276+ 634.03x for COD.

The LOQ and the LOD of COD and AMP were estimated to be 0.5, 0.25, 5 and 2.5 μg/ml, respectively.

The statistical parameters for the developed ANN models and the HPLC method are summarized in Table 4 too.

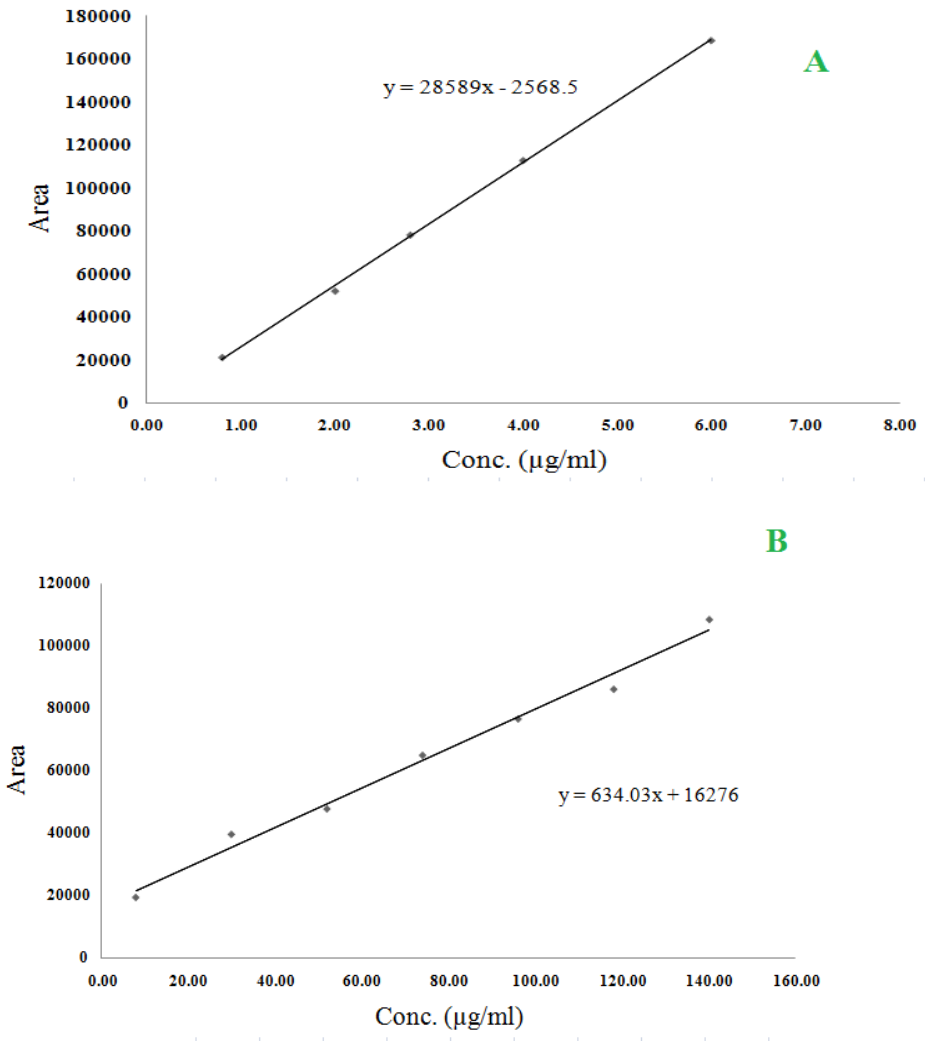


Fig. 8. Calibration curve for (A) AMP and (B) COD

The data supports that the analytical power and prediction ability of the HPLC method is relatively higher than that of the ANN model, but, the differences are not very significant.

Table 4. The results obtained using PCA-ANN model and HPLC

	PC-ANN		HPLC	
	AMP	**COD**	**AMP**	**COD**
R^2C	0.995	0.993	0.999	0.988
R^2P	0.976	0.999		
Linear Range (µg ml^{-1})	10–70.0	40–700	0.8–6	8-140
LOD	0.3	1.3	0.25	2.5
LOQ	0.99	4.29	0.5	5

Conclusion

A combination of PCA approach and artificial neural network was used to analyzethe binary mixtures of acetaminophen and codeine in thespiked plasma using their UV absorbance data. It was proposed combination of PCA, ANN and UV-Vis spectroscopy method is specific, accurate and precise for the simultaneous determination of codeine and acetaminophen.The explained approach is suitable for routine analysis of biological fluids containing these drugs either as such or in combination. On the whole, this work shows that even when a complicated system is present, a well-developed chemometrics method may be capable of giving a satisfactory performance for spectroscopic calibration in plasma samples.

In order to assess the results found by this procedure, a HPLC technique wasalso employed. The results revealed that the HPLC technique generated better results than PCA-ANN, however, the difference were not significant.

Conflict of Interests

Authors certify that no actual or potential conflict of interest in relation to this article exists.

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