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Fluorometric Determination of Mixtures of Quinolones by Means of Partial Least Squares and Neural Networks

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Multivariate calibration methods (partial least squares calibration, back propagation multilayer perceptrons networks, radial basis functions and generalized regression neural networks) were applied to the simultaneous fluorometric quantification of levofloxacin, garenoxacin and grepafloxacin, without previous separation steps. A data matrix was obtained by registering the emission spectra of mixtures of the three quinolones in urine (with concentrations ranging over 0.00 – 0.40 μg mL^{-1} for each quinolone) with a 283 nm excitation at pH 4.0. The generalized regression neural network model proved to be the most adequate model for simultaneous quantification of the three quinolones in urine samples.

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During the last two decades, quinolones have become an increasingly important and expanding family of antibacterial agents. The appearance in the market of new quinolones with greatly enhanced effectiveness has led to a wider spectrum of applications and better bioavailabilities to combat infections caused by microorganisms resistant to other bacteria.[1]

The quinolones levofloxacin, garenoxacin and grepafloxacin (Fig. 1) have activity against both Gram(+) and Gram(–) bacteria (aerobic and anaerobic) through inhibition of the bacterial enzyme (DNA gyrase) that maintains the super-twisted helical structure of DNA.[2] These quinolones are administered to patients with urinary, respiratory or cutaneous infections in 500, 600 and 400 mg day^{-1} doses, respectively.

Due to the overlap of their absorption and emission spectra and matrix effects found when working with biological samples (emission or absorption of the matrix components, attenuation of analyte signals, *etc*.), the determination of a mixture of these (or other) quinolones, by means of spectrophotometric or spectrofluorometric methods, traditionally requires a prior separation stage. This step is usually performed by HPLC techniques.[3,4] Nevertheless, the application of diverse multivariate techniques can avoid these difficulties without the need of a previous separation stage.

Partial least squares (PLS) regression can be used to quantify mixtures of pharmaceuticals and excipients using absorption or fluorescence emission spectra when linear responses are expected. This technique has been applied to the multicomponent quantification of diverse pharmaceutical mixtures,[5–7] including fluoroquinolones.[8,9] Partial least squares is a factor analysis that constructs a model to specify the linear relationship between dependent variables or responses (Y_i) and a set of predictor variables (X_i). For many data-analysis problems, an estimation of linear relationships between variables is adequate to describe the observed data or to make reasonable predictions for new observations.

Some authors have studied the spectrophotometric resolution of mixtures of pharmaceuticals by means of neural networks (NN).[10-12] NN are very sophisticated modelling techniques that simulate a biological nervous system and perform discriminant models and regression. They are especially useful when other statistical techniques are not able to predict complicated phenomena.[13,14] In order to carry out regression studies, different types of NN, such as multilayer perceptron (MLP), radial basis functions network (RBFN) and generalized regression neural networks (GRNN) can be applied.

Multilayer perceptrons are feedforward multilayer networks consisting in neurones arranged in layers (an input layer, various hidden layers and an output layer), being the connections (weights) unidirectional from input to output.

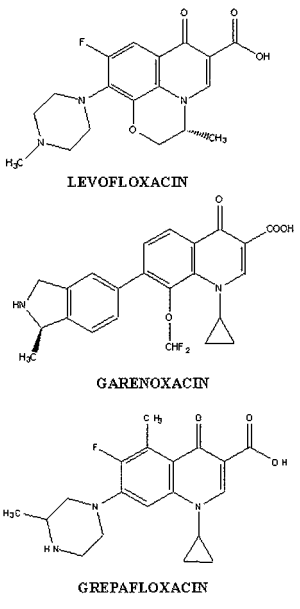

Fig. 1 Structures of levofloxacin, garenoxacin and grepafloxacin.

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Since NN learn from the data set, it is necessary to divide the cases in different subsets, a training set that allows learning the relationships between inputs and outputs and a test set that shows the NN prediction ability. When the networks learn not only the gross structure of a data set, but also the fine one, it leads to bad performance of the constructed model. In order to avoid these overfitting problems, a third subset of cases, verification set, may be included. MLP-NN are usually trained by back-propagation (BP), minimizing the prediction error made by the network.[13,15]

Radial basis function networks are three-layered networks containing input, hidden and output layers. The input layer serves to introduce the values of the input variables. Each node in the hidden layer represents a radial function that models a Gaussian response surface characterized by a central point (μ), which reflects a natural clustering of the data, and a deviation (σ) or smoothing factor. The hidden layer is connected to the output layer and the response of each output node is a linear function of its inputs.[16] RBFN training is performed by setting the two mentioned parameters: μ and σ.

Lastly, generalized regression neural networks are a generalization of probabilistic neural networks, but perform regression rather than a classification task. GRNN present a four-layer architecture: input, output and 2 hidden layers. The first hidden layer (pattern layer) contains radial units copied directly from the training data. There is a node in the pattern layer for each case of the training set. Each neurone models a Gaussian function centered at the training case, using kernel-based approximation to form an estimate of the probability density functions. The second hidden layer (summation layer) contains units that help to estimate the weighted average.[17-20] The only control factor in GRNN is a smoothing factor (the radial deviation of the Gaussian functions). The appropriate figure can be established experimentally as the number that produces the lowest verification error.

The scope of this work is to study the efficiency of PLS, BP-MLP, RBFN and GRNN to carry out multivariate calibrations for quantifying mixtures of levofloxacin, garenoxacin and grepafloxacin in urine without the need of a previous separation step. A comparison of these multivariate calibration models is discussed.

Materials and Methods

Apparatus and software

Fluorescence spectra were registered on a Cary Eclipse (Varian, Australia) luminescence spectrometer in a standard 10 mm pathlength quartz cell, thermostated at 25.0 ± 0.5˚C, with 5 nm bandwidths for emission and excitation monochromators. The pH was measured on a Crison (Barcelona, Spain) micropH 2002 pH-meter. Deionized water was obtained from a Milli-Q system (Millipore, Bedford, MA).

Principal component analysis and partial least squares were performed using the software package Statistica 6.0, and ANN studies were carried out using Statistica Neural Network, both purchased from Statsoft Inc. (Tulsa, USA).

Reagents and samples

Levofloxacin, garenoxacin and grepafloxacin were kindly provided by Hoechst Marion Roussel (France), Bristol Myers Squibb (USA) and GlaxoSmithKline (UK), respectively. Standard solutions of each quinolone (100 μg ml^{-1}) were prepared. These solutions, stored in the dark at 4˚C, were stable for more than 1 month. A 0.1 M sodium acetate–acetic acid (pH 4.0) buffer solution was daily prepared from analytical-quality reagents.

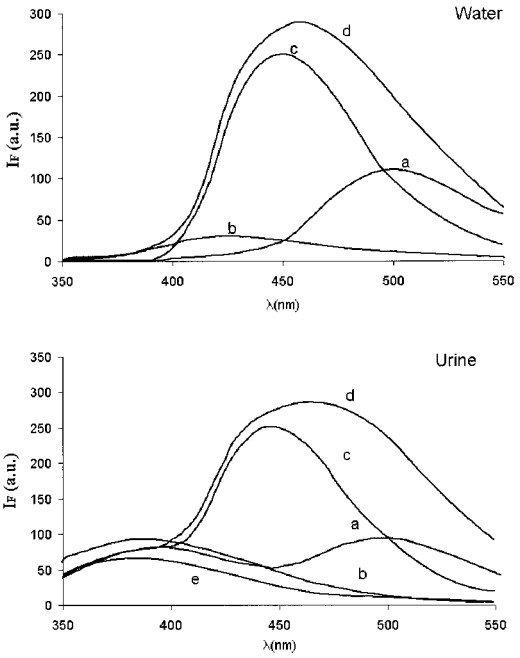

Fig. 2 Fluorescence emission spectra obtained for 0.175 μg mL^{-1} of (a) levofloxacin, (b) garenoxacin, (c) grepafloxacin, (d) a mixture of the three quinolones in water and urine samples and (e) an urine blank (λ_{ex} = 283 nm, pH 4.0).

Fluorescence emission spectra measurement

Urine samples were collected from 7 different healthy volunteers. Portions of 50 μl of urine were spiked with different amounts of the 3 quionolones for concentrations over the range 0.0 – 200.0 μg mL^{-1}. After the addition of 5 mL of a 0.1 M sodium acetate–acetic acid (pH 4.0) buffer solution, the samples were diluted to obtain final concentrations in the range of 0.00 – 0.40 μg mL^{-1}. Urine blanks and solutions containing 0.4 μg mL^{-1} of each quinolone were prepared as the lowest and highest points of the set of samples, respectively. Between these points, mixtures of 1, 2 or 3 quinolones were prepared covering this range of concentrations, while varying the urine used to prepare the solutions and the proportion of each compound. The obtained solutions were thermostatted at 25 ± 0.1˚C and the fluorescence emission spectra from 350 to 550 nm were registered, using an excitation wavelength of 283 nm. In this way, 83 different spectra (including the corresponding 8 urine blanks) were obtained.

Results and Discussions

Preliminary studies

Preliminary studies showed that levofloxacin, garenoxacin and grepafloxacin exhibit native fluorescence in acid medium, with maximum signals having excitation and emission wavelengths of 292 and 502 nm for levofloxacin, 281 and 422 nm for garenoxacin and 277 and 449 nm for grepafloxacin, respectively. Thus, a 283 nm excitation wavelength was selected for studying mixtures of these quinolones.

Figure 2 shows the emission spectra for each quinolone and for their mixture under these conditions in water and urine.

Table 1 Mean error, error standard deviation and coefficient of correlation obtained for levofloxacin, garenoxacin and grepafloxacin when applying the different calibration models to mixtures of this quinolones

Calibration model		Levofloxacin			Garenoxacin			Grepafloxacin		
		Tr	V	T	Tr	V	T	Tr	V	T
PLS	Mean error	1.1×10^{-4}	-6.6×10^{-4}	-1.1×10^{-3}	-5.9×10^{-3}	-1.3×10^{-2}	2.0×10^{-3}	-5.1×10^{-3}	-6.2×10^{-3}	4.8×10^{-3}
	Error SD	8.2×10^{-3}	2.4×10^{-2}	2.5×10^{-2}	3.5×10^{-2}	5.7×10^{-2}	5.4×10^{-2}	3.2×10^{-2}	3.4×10^{-2}	1.6×10^{-2}
	r	0.998	0.982	0.981	0.966	0.908	0.939	0.968	0.964	0.994
4:41:4:3 GRNN	Mean error	-9.3×10^{-7}	-2.4×10^{-6}	-1.5×10^{-6}	1.9×10^{-5}	-2.2×10^{-5}	-1.7×10^{-6}	1.8×10^{-5}	-1.9×10^{-5}	1.3×10^{-7}
	Error SD	1.4×10^{-5}	1.7×10^{-5}	1.8×10^{-5}	2.7×10^{-4}	9.6×10^{-5}	8.2×10^{-6}	2.6×10^{-4}	9.0×10^{-5}	5.1×10^{-7}
	r	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
4:38:3 RBFN	Mean error	-1.0×10^{-14}	-9.7×10^{-4}	7.7×10^{-4}	-2.0×10^{-14}	1.3×10^{-3}	2.5×10^{-3}	-3.5×10^{-14}	6.7×10^{-4}	6.9×10^{-4}
	Error SD	2.1×10^{-4}	4.1×10^{-3}	5.5×10^{-3}	1.7×10^{-4}	8.9×10^{-3}	2.8×10^{-2}	2.1×10^{-4}	6.3×10^{-3}	1.5×10^{-2}
	r	1.000	1.000	0.999	1.000	0.998	0.981	1.000	0.999	0.995
4:8:3 BP-MLP	Mean error	-8.6×10^{-3}	-5.9×10^{-3}	-9.2×10^{-3}	1.3×10^{-2}	5.7×10^{-4}	9.8×10^{-3}	4.5×10^{-3}	-3.0×10^{-4}	5.9×10^{-3}
	Error SD	1.6×10^{-2}	2.2×10^{-2}	2.3×10^{-2}	3.4×10^{-2}	3.2×10^{-2}	3.4×10^{-2}	2.5×10^{-2}	1.6×10^{-2}	1.4×10^{-2}
	r	0.991	0.984	0.984	0.965	0.969	0.963	0.981	0.992	0.996

Tr, Training set; V, verification set; T, test set; SD, standard deviation; *r*, coefficient of correlation.

Compared with the spectra for aqueous samples, emission increase can be observed in the 350 – 410 nm range. Thus, this emission can be assigned to the native fluorescence of urine components, with maximum emission at about 390 nm.

Figure 3 shows the influence of the pH in the emission intensity for the three quinolones using a 283 nm excitation wavelength. The three quinolones showed a constant emission in similar pH ranges (3.0 – 5.0 for levofloxacin, 2.5 – 4.5 for garenoxacin and 3.2 – 5.0 for grepafloxacin), corresponding to the maximum emission for levofloxacin and grepafloxacin. Garenoxacin showed its maximum emission over a pH range of 1.3 – 2.1, but the emission of levofloxacin and grepafloxacin at these pH values showed a notable decrease with respect to their maximum values. Taking into account these results, pH 4.0 was selected as a compromise value to study mixtures of these quinolones.

The linear fluorescence–concentration range was checked for each analyte. Linear calibration curves for aqueous solutions could be established in the range 0.015 – 1.125, 0.06 – 0.60 and 0.01 – 0.50 μg mL^{-1} for levofloxacin, garenoxacin and grepafloxacin, respectively.

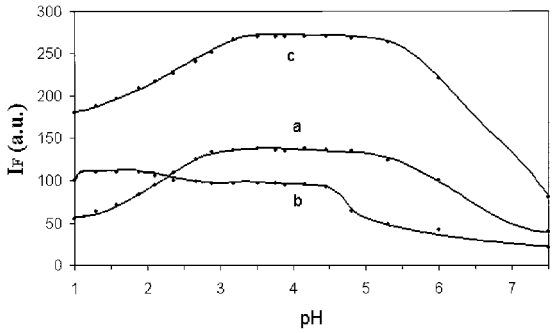

Fig. 3 Influence of the pH in the fluorescence emission of (a) levofloxacin, (b) garenoxacin and (c) grepafloxacin.

Chemometric studies

The efficiency of the regression for 4 different statistical techniques, such as partial least squares, radial basis function networks, generalized regression neural networks and back propagation multilayer perceptrons neural networks, have been studied. A data matrix containing 83 different mixtures of quinolones in urine (including 8 urine blanks) as cases and 200 variables, corresponding to their fluorescence emission intensities at each emission wavelength, were constructed in order to perform PLS studies. The data set was divided in 3 subsets: a training set (50% of the cases) to construct the PLS model, a verification set (25%) to cross-validate this model and a test set (25%) to study its performance efficiency. A preliminary principal component analysis (PCA) was performed in order to reduce the number of variables to use NN analysis.[21] The Kaiser criterion was used to determine the number of components to be retained. Taking into account that the data are autoscaled, each observed variable contributes one unit of variance to the total variance in the data set. The Kaiser criterion retains components with eigenvalues greater than 1, because these components account for a greater amount of variance than one observed variable.[22] In this case, the first four principal components (PC) were extracted. The percentage of variance explained by these PC was 99.97%. Then, to carry out NN studies, the data matrix was constructed using different mixtures of quinolones as cases and the factor scores obtained as variables. Like in PLS studies, the data set was divided in three subsets: a training set (50% of the cases) that allows learning the relationships between inputs and outputs and construct the prediction model, a verification set (25%) and a test set (25%). The performance efficiency of the constructed models was evaluated by looking at the mean error (the mean of the differences between the target and predicted outputs), the error standard deviation (SD) and the standard Pearson correlation coefficient (*r*) between the original and predicted outputs.

PLS models were constructed by extracting the appropriate number of factors that minimize the sums of squares of the prediction residuals obtained for the observations included in the verification set. The number of components extracted was 6.

After a number of NN structures were tested, NN models leading to the minimum verification mean-square error were trained and used for performing the calibration of the three mentioned quinolones. In this way, a BP-MLP model consisting in a 4:8:3 architecture, *i.e.* 4 neurones in the input layer connected to 8 hidden neurones and three output nodes that predict levofloxacin, garenoxacin and grepafloxacin concentrations was constructed. The learning rate and momentum were set at 0.15 and 0.52, respectively, and the

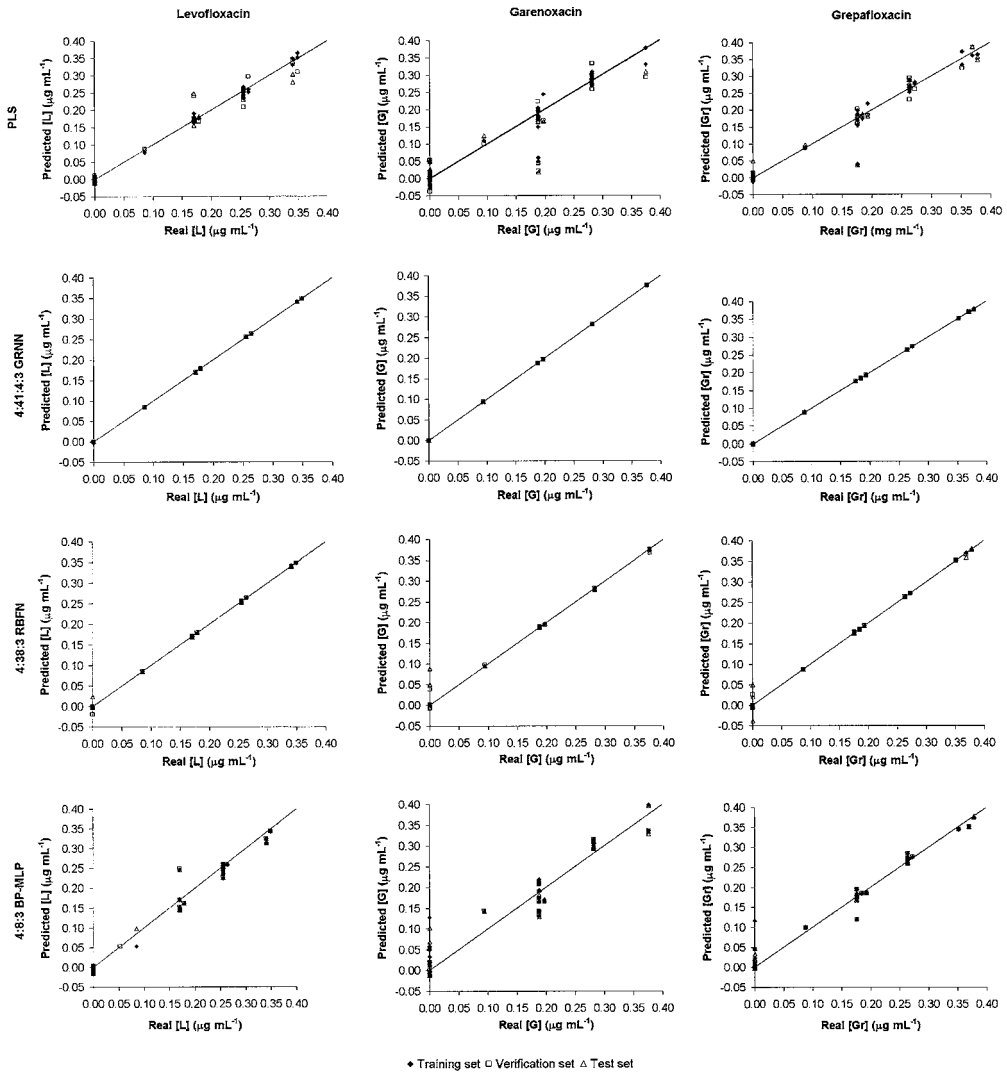

Fig. 4 Plots of predicted concentration obtained with different constructed calibration models *versus* real concentrations of levofloxacin (L), garenoxacin (G) and grepafloxacin (Gr) in urine samples.

training was performed during 1000 epochs. Secondly, a 4:38:3 RBFN model was constructed. The central point and deviation of the Gaussian functions were set using the K-means and K-nearest neighbors algorithms, respectively.[19] Finally, a 4:41:4:3 GRNN for carrying out the calibration in urine, being the smoothing factor 0.0202. In all cases, the input units correspond to the factor scores calculated in PCA and the outputs to the analyte concentrations.

The mean error and error standard deviation obtained when applying the different calibration models to the cases belonging to the different sets, as well as the coefficient of the correlation obtained when comparing the original concentrations with the predicted ones are given in Table 1. In addition, plots of the predicted concentrations *versus* the real ones are depicted in Fig. 4. When performing the calibration of the 3 quinolones in urine, GRNN and RBFN models presented similar efficiency in the prediction of levofloxacin in the training, verification and test sets. These techniques showed mean errors close to zero and lowest error SD compared to PLS and MLP. Both generalized regression and radial basis function models lead to a high correlation between the predicted and the actual levofloxacin concentrations. As can be observed in Fig. 4, PLS presented the worst result for verification and test sets. In the case of garenoxacin, the GRNN model presented the best results in the training, verification and test sets, with the mean error being close to zero, the lowest error SD and coefficients of correlation of 1. The worst results were obtained with the PLS and MLP models. RBFN showed good results at the different garenoxacin concentration levels, but the model was not able to estimate the urine blanks, as can be seen in Fig. 4. The same can be observed in the case of grepafloxacin, but the global results of RBF, MLP and PLS were better than that obtained for garenoxacin. The GRNN model fitted well the training and verification data sets, and presented good prediction ability, with γ-values close to 1 and the lowest error SD.

The highest error SD, and consequently the lowest γ-values, were obtained for garenoxacin quantification. This fact can be explained because garenoxacin presents the weakest native fluorescence and a high spectral overlap with grepafloxacin. The reason can also be the matrix effect due to the fluorescence emission of urine, taking into account (Fig. 2) that garenoxacin maximum emission wavelength (422 nm) is the nearest to the urine fluorescence emission range (350 – 410 nm). In addition, at the same concentration of the three quinolones, garenoxacin

presents the lowest fluorescence intensity.

Looking at these results, ANN models performed better than PLS. This fact can be explained because ANN can be used for linear and nonlinear systems, though PLS is especially recommended for linear ones.

The best performance efficiency was achieved using the GRNN model, with a correlation coefficient of 1, mean errors close to zero and the lowest error SD for the 3 studied quinolones. This can be explained because the inputs for NN were PC scores (combination of the original variables) which, according to the central limit theorem, can be assumed to be nearly normally distributed. Because RBFN and GRNN present hidden layers that contain radial units modelling a Gaussian response surface, they fit better to the data than MLP. In addition, the BP algorithm requires an optimal number of epochs to minimize the prediction error, but sometimes the training process sticks at a local minimum, leading to results with low accuracy. Finally, as mentioned in the introduction, each node in the hidden layer of RBFN represents a radial function centered in a natural clustering of data, while GRNN does it in each training case. Thus, if the training set is optimally selected, the GRNN can fit better to new cases because similar objects are already included in the model.

Conclusions

The application of multivariate calibration models, such as partial least squares, back propagation multilayer perceptrons, radial basis functions networks and generalized regression neural networks, allows the simultaneous quantification of levofloxacin, garenoxacin and grepafloxacin, without previous separation steps. From comparisons of these multivariate calibration models, the best performance efficiency was achieved using the GRNN. GRNN is a suitable regression model for carrying out the simultaneous quantification of levofloxacin, garenoxacin and grepafloxacin mixtures in urine samples.

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