International Journal of Life science & Pharma Research

SPECTROFLUORIMETRIC DETERMINATION OF LEVOFLOXACIN IN PHARMACEUTICALS AND IN HUMAN URINE

ARNALDO PEIXOTO DA SILVA[1], ADERVAL SEVERINO LUNA[1*], THAIS MALCHER DA SILVA COSTA[1], RICARDO QUEIROZ AUCELIO[2], JEZ WILLIAN BATISTA BRAGA[3], RICARD BOQUÉ[4] AND JOAN FERRÉ[4]

[1]Department of Analytical Chemistry, Rio de Janeiro State University, CEP 20550-013, Rio de Janeiro – RJ
[2]Department of Chemistry, Pontifícia Universidade Católica do Rio de Janeiro, CEP 22453-900, Rio de Janeiro – RJ, Brazil
[3]Department of Chemistry, Brasilia University, CEP 70910-900, Brasília – DF, Brazil
[4]Department of Analytical Chemistry and Organic Chemistry, University Rovirai Virgili, Tarragona, Spain

ABSTRACT

Two spectrofluorimetric methods are proposed to determine levofloxacin in pharmaceutical tablets and spiked human urine. The first method allowed the determination of levofloxacin in aqueous solution using univariate (zero order) calibration. The analytical curve was linear to a concentration of levofloxacin of, at least, 300 ng mL^{-1} and the coefficient of correlation was 0.9988. The accuracy was evaluated using three different concentrations and the mean recovery was 97.6 ± 6.5% and the mean precision was lower than 2.0%, except for the concentration of 180 ng mL^{-1} for the analyst 2. The limit of detection (LOD) was 0.46 ng mL^{-1}, which can be considered adequate for this purpose. The method showed a good concordance when it was applied to Brazilian pharmaceutical formulation with a relative standard deviation of 3.5%. The second method used parallel factor analysis with standard additions for the determination of levofloxacin in urine. The scores, related to levofloxacin, were used to quantify levofloxacin in human urine, using linear regression and the standard additions method. The LOD was 1.4 ng mL^{-1} for urine sample diluted 1000 times with a mean precision of 3.0 ng. mL^{-1} and a root mean square error of calibration of 8.0 ng mL^{-1}. An additional application of this method was carried out to monitoring the levofloxacin in urine sample from a healthy man until of a complete excretion of this antibiotic with success.

Key words : levofloxacin, human urine, second order standard addition method, PARAFAC.

1. INTRODUCTION

Levofloxacin (LEVO), the levorotatory isomer of ofloxacin, exhibits activity against a broad spectrum of Gram-positive and Gram-negative bacteria. It is used to treat various infectious diseases such as community acquired and nosocomial pneumonia, skin structure infection, urinary tract infections or sepsis.[1]

Several analytical techniques have been used for the determination of levofloxacin in different matrices, including adsorptive square-wave anodic stripping voltammetry,[2] flow injection analysis with absorption photometric, potentiometry and conductometry detection.[3] However, these methods do not present selective signals to discriminate a single analyte in mixtures so separation procedures

or multivariate calibration algorithms are needed. A literature survey reveals various separation methods for the determination of levofloxacin such as reversed-phase high performance liquid chromatography (HPLC) with UV absorption spectrophotometric detection,[4–12] HPLC with fluorescence detection[1, 13–16] and capillary electrophoresis with chemiluminescence detection.[17] These methods are quite complex, mostly because of the sample preparation, which involves solid-liquid extraction,[18] liquid-liquid extraction[19] and protein precipitation combined with centrifugation steps.[15] Therefore, these methods are relatively expensive and time consuming for routine use in clinical pharmacology, and chemical and pharmaceutical laboratories. Recently, room-temperature phosphorimetry was used to selectively detect LEVO in samples containing ciprofloxacin and norfloxacin, but interferences were found when applied to urine samples.[20]

The use of spectrofluorimetric methods for determining drugs in biological fluids is difficult due to the presence of natural fluorescent interferences. In the last years, different strategies to circumvent this problem have been proposed, by combining spectrofluorimetric data and three-way chemometric tools, mainly parallel factor analysis (PARAFAC).[21-24] Therefore, in some cases, tedious preliminary steps can be avoided, replacing the physical separation of interferences by a mathematical separation of their signals. This combination has allowed simplifying the experimental procedure.

The objective of this article was the development of a method for the direct determination of LEVO in pharmaceutical tablet form and in human urine using spectrofluorimetry. The approaches aimed at a minimal sample manipulation. For the determination of LEVO in pharmaceutical tablets, a traditional univariate (zero order) calibration was used. For the determination of LEVO in human urine, the methodology developed exploited the second-order advantage of the three-way spectrofluorimetric data, through the use of PARAFAC (second order calibration) and second-order standard addition method (SOSAM). For the described methods, figures of merit, such as sensitivity, accuracy and limit of detection are reported.

1.1 Univariate calibration

The linear regression model uses the relationship shown below, where the observed signal or response *y* is given by

$$y = F(x) + e_y \quad (1)$$

with

$$F(x) = B + S = B + Ax \quad (2)$$

where S denotes the net signal; B the blank (or background or baseline, as appropriate); x the analyte amount or concentration; and A the sensitivity. The error e_y is taken to be random and normal, with zero mean (no bias) and dispersion parameter σ (standard deviation).[25]

1.1.1 Figures of merit

For the validation of the univariate method the mean precision, accuracy and limit of detection (LOD) were determined. The mean precision of the analytical results was evaluated by performing 10 determinations on three different concentration solutions by two analysts.

The LOD was estimated in accordance with the $3\sigma/b$, where σ is the standard deviation of the residuals and b the sensitivity of the method (slope of the analytical curve).[26]

The accuracy was evaluated by performing 10 determinations on three different concentration levels by two analysts.

1.2 Second-order multivariate calibration

Parallel factor analysis (PARAFAC) is a commonly used method for modeling fluorescence excitation-emission data (EEM). The mathematical model behind PARAFAC agrees with the physicochemical model that generates spectrofluorimetric data.[21] PARAFAC decomposes the fluorescence signals *X* into *F* tri-linear components according to the number of fluorophores present in the samples:[27]

$$x_{ijk} = \sum_{f=1}^{F} a_{if} b_{jf} c_{kf} + e_{ijk} \quad (3)$$

where x_{ijk} is the intensity of the measured light for sample *i* at excitation wavelength *j* and emission wavelength *k* and e_{ijk} is the error term. The *i*th score for the *f*th component is denoted by a_{if} and is related to the concentration of fluorophore *f* in sample *i*; b_{jf} and c_{kf} are the *j*th and *k*th element of

the *f*th excitation and emission loading respectively.[27]

PARAFAC is a second order calibration method that provides the second-order advantage, that is, the analyte can be quantified even in the presence of unmodelled interferences.

After the decomposition is completed, the identification of the chemical component is done by comparing the spectral profiles obtained by the PARAFAC model with those for a standard solution of the compound of interest.

Absolute concentrations for the analyte in the sample can be obtained by the regression of the scores a_i for the analyte versus the reference concentrations. When the PARAFAC model are combined with the standard additions method, the concentration of the samples is estimated by extrapolation of the linear univariate equation obtained by the regression of the scores versus the added concentrations in the sample. In this case, the whole procedure is called Second Order Standard Addition Method (SOSAM).[28]

1.2.1 Figures of Merit

The estimation of figures of merit in multivariate calibration is an active area of research in chemometrics, which is based on the concept of net analyte signal (NAS), first developed by Lorber,[29] or in analogy to univariate regression, as suggested by Rodríguez-Cuesta et alli.[30]

Following the approach proposed by Olivieri and Faber, the estimation of sensitivity for PARAFAC models can be performed by a general expression,[31] expressed as:

$$SEN_n = z_n\{[(\mathbf{B}_{exp}^T\mathbf{P}_{b,unx}\mathbf{B}_{exp})(\mathbf{C}_{exp}^T\mathbf{P}_{c,unx}\mathbf{C}_{exp})]^{-1}\}^{-1/2} \quad (4)$$

where $\mathbf{B}_{exp}$ and $\mathbf{C}_{exp}$ are the excitation and emission spectral profiles, respectively, for the calibrated analytes provided by the PARAFAC; $\mathbf{P}_{b,unx}$ and $\mathbf{P}_{c,unx}$ are projection matrices, that project onto the space that is orthogonal to the space spanned by the interferences in each mode:[31]

$$\mathbf{P}_{b,unx} = \mathbf{I} - \mathbf{B}_{unx}\mathbf{B}_{unx}^{+} \quad (5)$$

$$\mathbf{P}_{c,unx} = \mathbf{I} - \mathbf{C}_{unx}\mathbf{C}_{unx}^{+} \quad (6)$$

and z_n is an appropriate scaling factor. In PARAFAC, z_n is the parameter converting loadings to concentration.[32] Since the SEN values depend on the presence of interferences, which may be specific of a sample, the SEN cannot be defined for the whole multivariate method. In such cases, an average value for a set of samples is usually estimated and reported.

The limit of detection (LOD) is an important figure of merit that has recently been discussed for several first and second-order multivariate techniques.[33-35] An approximation to the LOD can be obtained by the expression:[32,35]

$$LOD_n = 3\frac{s_r}{SEN_n} \quad (7)$$

where s_r its an estimate of the instrumental noise. Since the SEN is given as an average value, LOD is also reported as an average figure.

The average prediction error of a method is a useful and simple parameter for method comparison and evaluation of the fit of the model. It is generally estimated as the mean prediction error for a set of test samples. However, for applications employing SOSAM just the root mean square error of calibration (RMSEC) is obtained, since the analytical curve is dependent and specific for each sample. The RMSEC can be estimated as:

$$RMSEC = \sqrt{\sum_{n=1}^{I}\frac{(y_{ref,i} - y_{u,i})^2}{I-2}} \quad (8)$$

where $y_{ref,i}$ and $y_{u,i}$ are the reference and the estimated concentrations values for each of the I calibration samples. Alternatively, recovery values for the concentration of the analyte in the sample can be estimated from spiked samples or when a reference method is available.

2. MATERIALS AND METHODS

2.1 Reagents

Levofloxacin (≥ 98% in purity) was purchased from Fluka Analytical (Sigma-Aldrich, Switzerland). A 100 mg L^{-1} stock solution of LEVO was prepared with high purity water (Gehaka, Brazil) and from this solution a set of 9 aqueous samples were prepared and used for calibration with the univariate and PARAFAC

models (C1 – C9 with concentrations from 50 to 300 ng mL^{-1}).

2.2 Sample preparation

2.2.1 Pharmaceutical tablets

The first proposed method for the determination of LEVO was applied to a Brazilian commercialized pharmaceutical formulation (Levofloxacino, EMS, Brazil). Each tablet of levofloxacin contains 500 mg levofloxacin as active substance. The tablets also contain the following excipients: crospovidone, hypromellose, microcrystalline cellulose and sodium stearyl fumarate. The film coating contains hypromellose + macrogol, titanium dioxide (E 171), yellow ferric oxide (E 172) and red ferric oxide (E 172) (levofloxacin information from EMS, Brazil). The total content of ten tablets was weighed and grounded to a fine powder using a pestle and a mortar. Powder was dissolved in ethanol-water (3:97), filtered through a membrane filter 0.45 µm and diluted to the mark in a 100 mL calibrated flask. Convenient aliquots from this solution were taken for the determination of LEVO by spectrofluorimertry (50 – 300 ng mL^{-1}). The calibration standards used to build the analytical curve consisted of 9 aqueous solutions (C1 – C9). The set of 17 pharmaceutical tablet samples (P1 – P17) were prepared with analyte concentrations different from those employed for calibration but within the calibration range. All the solutions were prepared in triplicate.

2.2.2 Urine samples

Urine samples were obtained from healthy men (from 20 to 50 years old) in the morning. It was assumed that the LEVO concentration of all these urine samples was zero. Urine samples were diluted 100, 500 and 1000 times with high purity water. After this, urine samples were spiked with convenient amounts of the LEVO stock solution. The final LEVO concentrations ranged from 0 – 300 ng mL^{-1}. All the solutions were prepared in triplicate.

Emission spectra and EEMs were measured in random order. Real samples were measured on different days from those of calibration.

2.3 Monitoring the LEVO in urine sample

A urine sample was obtained from a healthy man (50 years old) that took one tablet of LEVO (500 mg) in the morning. The urine samples were sampled before the ingestion of the medicine and until the complete excretion from the body (72 hours later). At the beginning, the first urine sample was collected and diluted 1000 times with high purity water. This sample was sub-sampled and spiked with LEVO from 0 up to 600 ng/mL. It was assumed that the LEVO concentration in this urine sample was zero. After two hours, the new urine sample was collected and diluted 1000 times with purity water, and splitted in two sub-samples. One of them was spiked with LEVO 50 ng/mL. This procedure was repeated until the complete excretion of the medicine from the body.

2.4 Apparatus and software

The spectra were obtained in a Panorama Spectrofluorimeter (Lumex, Russia) equipped with a Xenon lamp, with the PanoramaPro software, version 2.1 and using a 10.00 mm quartz cuvette. All spectral excitation-emission matrices (EEM) were obtained in the excitation range from 240 to 370 nm (step 2 nm) and in the emission range from 380 to 550 nm (step 1 nm). The excitation and emission spectral bandwidths were both set to 8.0 nm.

Calculations were done in MATLAB version 7.7 (The MathWorks, Natick, USA). The PLS Toolbox for MatLab, version 5.5 (Eigenvector, USA) was used for PARAFAC calculations.

2.5 Calibration Procedure

2.5.1 Zero-order calibration for pharmaceutical tablets

The univariate method was developed based on classical least squares regression (briefly described in section 1.1*)* and measurements of fluorescence of the 9 standard solutions (C1 – C9) at 489 nm using an excitation wavelength of 290 nm against a blank solution. The validation was performed by determining the concentrations of LEVO in the pharmaceutical tablet solutions (P1 – P17) and by estimation of the figures of merit. Besides, a recovery study was carried out by adding a known quantity of LEVO of 50, 100 and 150 ng mL^{-1} to the 100 ng mL^{-1} solution of the pharmaceutical dosage samples.

2.5.2 Second-order calibration for urine samples

The urine samples were diluted 100, 500 and 1000 times in order to reduce the background fluorescence intensity. For each individual diluted urine sample, a standard addition curve was obtained. The first one was carried out with the original diluted urine sample and three spiked samples from 0 to 150 ng mL^{-1} of LEVO. The second one was also carried out with the original diluted urine spiked with 50 ng mL^{-1} of LEVO and three spiked urine samples from 50 to 200 ng mL^{-1} of LEVO. The third one was carried out with diluted urine spiked with 100 ng mL^{-1} of LEVO and three spiked urine sample from 100 to 250 ng mL^{-1} of LEVO and the last one was carried out with diluted urine spiked 150 ng mL^{-1} of LEVO and three spiked urine samples from 150 to 300 ng mL^{-1} of LEVO (Table 3). The diluted urine samples were spiked at different concentrations to prove that the method worked can be applied in the range of concentrations at from 0 to 150 ng mL^{-1}. The urine samples were diluted 100, 500 and 1000 times, meaning that 48 (3 different dilutions x 4 levels of LEVO concentration x 4 patients) standard addition curves were analyzed. All the determinations were carried out in triplicate for the whole procedure.

In order to remove the scattering of radiation, only a subset of each excitation-emission matrix (EEM) was used in the excitation range from 274 nm to 318 nm and in the emission range from 459 nm to 529 nm.

2.5.3 Monitoring the LEVO in the urine sample

The monitoring of the LEVO concentrations in urine samples was developed with 1000 times dilutions in order to reduce the background fluorescence intensity. The determinations were performed by a standard addition analytical curve developed for the first collected and diluted urine sample. Different additions of a standard solution were performed to obtain nine different concentration levels (0 (blank sample), 50, 100, 150, 200, 300, 400, 500 and 600 ng/mL) . After two hours, a new urine sample was collected and diluted 1000 times, with purity water, and splitted in two sub-samples, which one of them was spiked with LEVO 50 ng/mL. This procedure was repeated at the following time intervals 4, 6, 8, 10, 12, 24, 26, 30, 34, 48, 60 and 72 hours after the ingestion of the medicine. All the solutions measured in triplicate were arranged in a cube and decomposed with the PARAFAC model. The concentrations of LEVO were estimated in all time intervals from the analytical curve developed for the urine sample collected at time 0 hour.

3. RESULTS AND DISCUSSIONS

3.1 Pharmaceutical tablets

The analytical curve obtained for the determination of LEVO in pharmaceutical tablets within 0 and 300 ng mL^{-1} was:

$y = 0.0539 (\pm 4.7 \times 10^{-5}) C + 0.0296 (\pm 0.0082)$ ($r = 0.9988$; $n = 35$)

where C = LEVO concentration expressed as ng mL^{-1}; y = fluorescence intensity, in arbitrary units. The analysis of variance (ANOVA) showed that the linear regression model was highly statistically significant ($p < 0.05$).

The analytical figures of merit for the determination of LEVO in pharmaceutical tablets are reported in Table 1. The analytical curve was linear to a concentration of LEVO of, at least, 300 ng mL^{-1}, the residuals presented a homoscedastic behavior and the coefficient of correlation was 0.9998. Note that even with the many excipients in the tablet that could potentially interfere on this determination no deviation from the linearity was observed. Accuracy was evaluated using three different concentrations (50, 100 and 150 ng mL^{-1}). The mean recovery for the univariate method was 97.6 ± 6.5% (Table 2), which can be considered satisfactory. This method was applied for the quantification of levofloxacin in the Brazilian commercial pharmaceutical dosage. A good concordance was found between the nominal and experimental values (500 and 468 mg, respectively) with a relative standard deviation (RSD) of 3.5%, indication that the method is able to determine the LEVO concentrations in pharmaceutical formulations.

Table 1 – ***Figures of merit from univariate calibration method***

Parameter	Value	Standard Error
Intercept	0.0296	0.0082
Slope	0.0539	4.7×10^{-5}
Adjusted R^2	0.99753	
LOD, ng mL^{-1}	0.46	
Precision,%	Repeatability (CV ≤ 2.0%)	
Concentration	Analyst 1: mean ± rsd (CV, %)	Analyst 2: mean ± rsd (CV, %)
120 ng mL^{-1}	125.2 ± 1.5 (1.2%)	124.7 ± 1.2 (0.9%)
150 ng mL^{-1}	143.8 ± 2.0 (1.4%)	147.7 ± 2.3 (1.6%)
180 ng mL^{-1}	187.2 ± 2.1 (1.1%)	182.1 ± 3.9 (2.2%)
Accuracy,% (98-102%)	Analyst 1: recovery mean ± rsd	Analyst 2: recovery mean ± rsd
120 ng mL^{-1}	104.4 ± 1.2	103.9 ± 1.0
150 ng mL^{-1}	95.9 ± 1.3	98.5 ± 1.5
180 ng mL^{-1}	104.0 ± 1.2	101.2 ± 2.2
Average	101.4 ± 1.2	101.2 ± 1.6

Table 2 – ***Recovery study of the levofloxacin in pharmaceutical tablets applying univariate calibration.***

Addition of, ng mL^{-1}	Estimated value, ng mL^{-1}	Recovery (%)
50	50.0	100.0
100	90.2	90.2
150	153.8	102.5
Recovery mean, %	97.6	
Standard deviation, %	6.5	

The mean precision results showed that the coefficient of variation (CV) was lower than < 2.0% for all solutions, except for the 180 ng mL^{-1} solution analyzed by the analyst 2. After applying the Student's *t*-test, no difference between the results obtained by the two analysts was found at a 95% confidence level. The precision of the results over different days could not be calculated, since all the solutions degrade even when they are stored at 4^0C and protected from the light.

The LOD was 0.46 ng mL^{-1}, which can be considered adequate for this purpose. The mean recoveries were 101.4 and 101.2 %, respectively, by the two analysts Once more, after applying the Student´s *t*-test, no difference between the recoveries obtained by the two analysts was found at a 95% of confidence level.

3.2 Urine samples

Figure 1 shows the three-dimensional EEM of a training sample (150 ng mL^{-1} standard aqueous solution). A better insight is obtained by considering the corresponding contour plot (Figure 2). A subset of the EEMs was used in order to avoid the presence of the Raman and Rayleigh scattering and the second harmonic from the diffraction grating, which are uncorrelated with the concentrations of the analytes. The excitation and emission ranges where only the analyte contributes to the overall fluorescence intensity were: emission from 459 to 529 nm at 1 nm intervals (J = 71 data points) and excitation from 274 to 318 nm at 2 nm intervals (K = 23 data points).

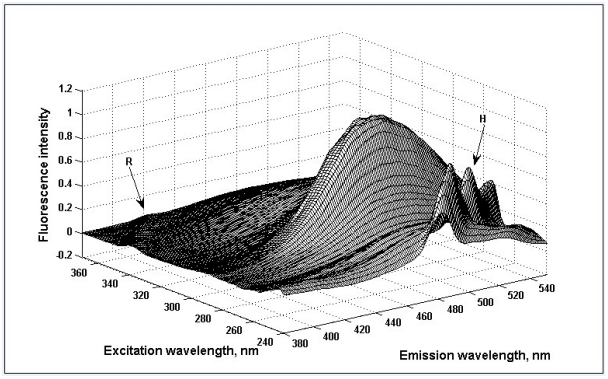

Figure 1. Three-dimensional plot of the excitation-emission fluorescence matrix (EEM) for standard solution of LEVO 150 ng ml^{-1}, showing the presence of a diffraction grating harmonics (H) and Raman (R) scattering as indicated.

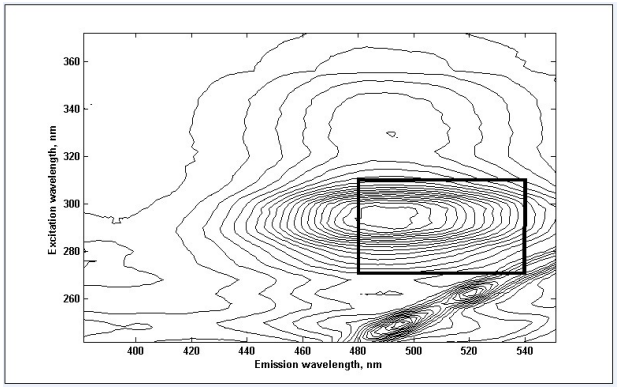

Figure 2. Contour plot for the same EEM. The rectangle illustrates the spectral excitation and emission ranges selected for calibration with PARAFAC.

The results (not shown) obtained with univariate regression for the determination of LEVO in spiked human urine clearly illustrates the necessity of the second order advantage. The models built with calibration samples C1 – C9 were not able to produce acceptable results on samples (U1 – U16). This is undoubtedly due to the presence of fluorescent urine compounds whose influence has not been taken into account in the calibration set. These compounds not only exhibit emission intensities that overlap with fluorescence signals from the analyte, but are also variable from patient to patient.

The EEMs of the calibration samples (C1 – C9), together with the original human urine plus spiked human urine samples (U1 - U16), were grouped in a cube of size 25 × 23 × 71 ($I \times J \times K$). PARAFAC was then applied to this cube. Figures 3 and 4 show the excitation and emission profiles B and C obtained. Comparison with the normalized experimental emission and excitation spectra, obtained for a pure standard solution and shown in the same figures, allowed us to ascribe the components of the PARAFAC model to LEVO and the matrix interference. In this particular case, the interference accounted for most of the data variability, indicating that it is the main source of fluorescence intensity across this particular data cube. Figure 5 clearly shows that the calibration set is completely different from the test set (urine sample plus spiked urine samples) when the PARAFAC model was used for all dilutions tested.

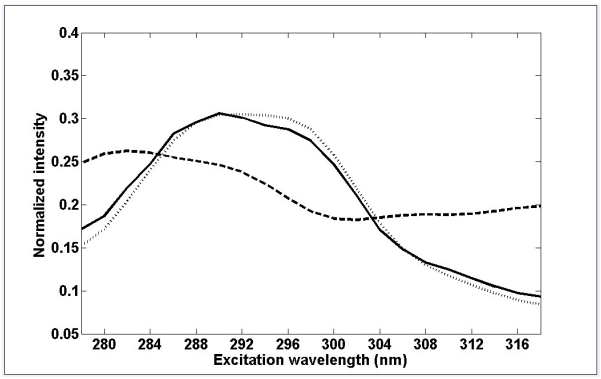

Figure 3. Deconvonluted excitation fluorescence spectra obtained from the loadings of the PARAFAC model (B matrix). Solid line: LEVO from aqueous solution; dotted line: PARAFAC component 1; dashed line: interference.

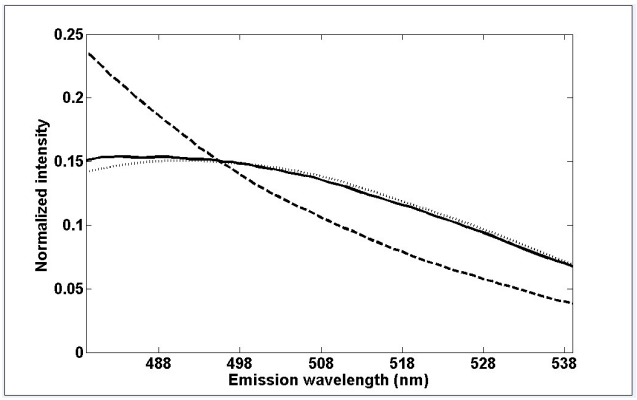

Figure 4. Deconvonluted emission fluorescence spectra obtained from the loadings of the PARAFAC model (C matrix). Solid line: LEVO from aqueous solution; dotted line: PARAFAC component 1; dashed line: interference.

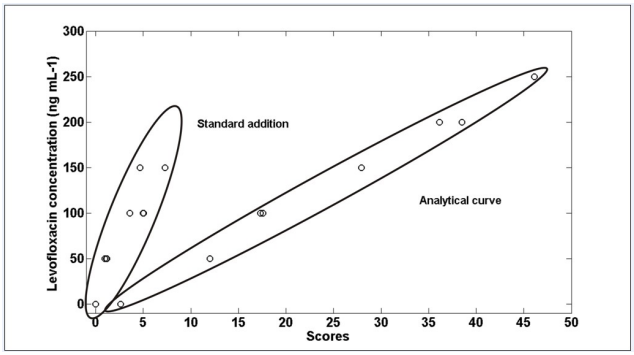

Figure 5. Predicted concentration of LEVO versus scores obtained from PARAFAC for calibration set from aqueous solution (analytical curve) and training set from spiked urine samples (standard addition method).

Figure 5 shows that it was mandatory to use an extension of the standard addition method for multi-way data, named second order standard addition method (SOSAM),[29] and coupled to PARAFAC to analyze the spiked human urine. In this case, the samples of each patient are decomposed separately and the loadings related to the sample mode (scores) were used for calibration through a pseudounivariate linear regression. The results for the determination of the LEVO in the

urine samples are shown in Table 3, together with the percentage of recovery. Prediction of the four test samples with three different dilutions using the SOSAM model led to reasonably good recoveries with slightly worse results for the 100 times dilution. For the 100 times dilution a positive systematic error is observed, while the other dilution levels have a non significant bias. The LEVO average concentration in the different urine samples were 3.9; 0.7 and 0.3 ng mL^{-1} for 100, 500 and 1000 times dilution. Note that at the first concentration level the urine sample do not contain LEVO. Therefore, the reference concentration is theoretically 0 ng mL^{-1}, indication that the 500 and 1000 times dilution present the better results.

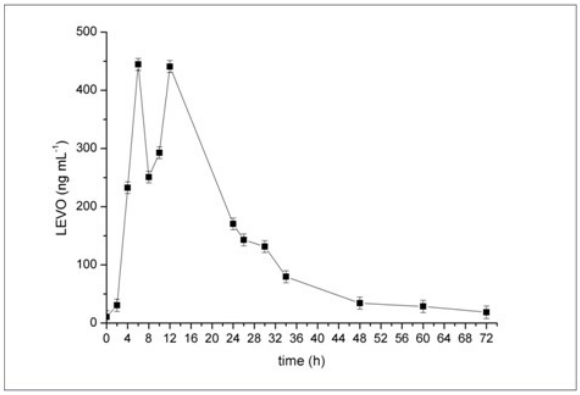

Figure 6. Concentration profile of the LEVO concentrations in urine over the time. Error bars indicate the standard deviations of triplicates.

Table 3 – ***Results obtained when applying second-order standard method to human urine spiked with LEVO.***

Concentration added, ng mL^{-1}	Patient	Urine sample diluted 100X		Urine sample diluted 500X		Urine sample diluted 1000X	
		Concentration predicted, ng mL^{-1}	Recovery, %	Concentration predicted, ng mL^{-1}	Recovery, %	Concentration predicted, ng mL^{-1}	Recovery, %
0	1	3.0		0.4		0.5	
0	2	5.9		0.9		0.3	
0	3	1.0		0.2		0.0	
0	4	5.7		1.3		0.5	
50	1	55.8	105.6	49.8	98.8	48.3	95.6
50	2	63.1	114.4	52.9	104.0	51.4	102.2
50	3	55.0	108.0	50.8	101.2	50.4	100.8
50	4	55.2	99.0	53.5	104.4	52.3	103.6
100	1	110.0	107.0	98.4	98.0	98.3	97.8
100	2	118.1	112.2	103.9	103.0	100.8	100.5
100	3	107.4	106.4	106.1	105.9	103.6	103.6
100	4	110.6	104.9	111.3	110.0	101.7	101.2
150	1	158.0	103.3	156.0	103.7	162.2	107.8
150	2	169.8	109.3	147.9	98.0	163.9	109.1
150	3	161.8	107.2	164.4	109.5	162.1	108.1
150	4	174.2	112.3	171.0	113.1	152.2	101.1
Recovery mean (rsd)			107.5 (4.2)		104.1 (4.9)		102.6 (4.1)

rsd: relative standard deviation

The figures of merit of the method are shown in Table 4. The lowest RMSEC, estimated from the data of the four patients, is obtained with the highest level of dilution and indicates a model

with an acceptable uncertainty. The 1000 times dilution also shows the best precision (lowest standard deviation). Concerning sensitivity and LOD, it was observed that the 500 and 1000 times dilutions show the best (and equivalent) results. Based on these results, the 1000 times dilution of the urine sample was considered the better condition for the method.

Table 4. ***Figures of merit of the determination of LEVO in urine samples by PARAFAC-SOSAM.***

Parameter	Dilution level		
	100X	500X	1000X
RMSEC, ng mL^{-1}	16	10	8.0
Mean Precision, ng mL^{-1}	4.9	5.8	3.0
Sensitivity, mL ng^{-1}	0.01	0.02	0.02
LOD, ng mL^{-1}	2.5	1.3	1.4

3.3 Monitoring the LEVO in the urine sample

The results from the monitoring of levofloxacin in urine are presented in Table 5 and Figure 6. Table 5 show the estimated concentrations for the additions of 50 ng L^{-1} carried out in each aliquot for all time intervals. A good agreement for most of the samples can be observed. Considering all data points of the monitoring an RMSEP of 10.1 ng mL^{-1} was obtained. However, a large error was observed for the aliquot at 12 h, which presented only a 48 % recovery. When this sample was not considered for the estimation of the RMSEP, the average recovery and standard deviation were 7.2 ng mL^{-1}, 100 % and 15 %, respectively. These results indicate that the model provide results for the LEVO concentration with an acceptable uncertainty and able the monitoring of its concentration in the urine.

Table 5. ***Results for the estimated concentrations for the addition of 50 ng L^{-1} LEVO carried out in each time interval.***

Time, h	Estimated concentration, ng mL^{-1}	Recovery (%)
2	52.1	104.3
4	47.7	95.5
6	35.1	70.2
8	54.9	109.9
10	55.0	109.9
12	24.1	48.2
24	62.8	125.6
26	43.6	87.1
30	38.8	77.5
34	47.2	94.3
48	54.2	108.5
60	51.2	102.4
72	55.6	111.1
Recovery mean (standard deviation)		96 (± 21)
RMSEP, ng mL^{-1}		10.1

The estimated concentrations of LEVO, obtained from the urine samples without additions are shown in Figure 6. It is interesting to note that the concentration of LEVO increases from 0 to 6 h, but at 8 and 10 h there is a decrease in the concentration of LEVO. At 12 h a new increase of concentration was observed. This observation can be explained considering that the volunteer that took the medicine and provided the urine samples drank a great quantity of liquid after 6 hours of the beginning of this experiment. Therefore, the samples collected at 8 and 10 hours were diluted, and the estimated concentrations of levofloxacin

reduced when compared to the other samples. It can also be observed in Figure 6 that only after 72 hours from the beginning of the experiment the estimated concentration of LEVO in urine decreased to the initial concentration level.

4. CONCLUSIONS

The application of the univariate (zero order) calibration method gave good results for levofloxacin determination in pharmaceutical samples.

The combination of fluorescence excitation – emission measurements and the PARAFAC were highly useful for the analysis of human urine spiked with levofloxacin, by exploiting the so-called second-order advantage. In this case, it was possible to determine levofloxacin in urine sample from healthy men with a simple dilution from 500 to 1000 times using fluorescence combined with second order standard addition method. The best results were obtained for a dilution of 1000 times of the urine sample.

The SOSAM model combined with the spectrofluorimetry was applied to monitoring the levofloxacin in urine samples from a healthy man, who took a medicine. Good recovery values were obtained, what shows the accuracy and effectiveness of the procedure, and indicates that it can be adopted for monitoring the concentration of LEVO in urine samples diluted 1000 times and employing a PARAFAC-SOSAM method.

5. ACKNOWLEDGEMENTS

The authors thank the volunteers who provided the urine samples. T. M. S. C. thanks FAPERJ for a fellowship and A. S. L. thanks CNPq and FAPERJ for a financial support and Programa Prociência from UERJ. A. S. L. and R. Q. A. thank CNPq for research scholarship.

6. REFERENCES

1. Siewert, S.; *J. Pharm. Biomed. Anal*. **2006**, 41, 1360.
2. Radi,A.; El-Sherif, Z.; *Talanta* **2002**, 58, 319.
3. Altiokka, G.; Atkosar, Z.; Can, N. O.; *J. Pharm. Biomed. Anal*. **2002**, 30, 881.
4. Kothekar, K. M.; Jayakar, B.; Khandhar, A. P.; Mishra, R.K.; *Eurasian J. Anal. Chem*. **2007**, 2, 21.
5. Arayne, M. S.; Sultana, N.; Siddiqui, F. A.; *Pak. J. Pharm. Sci*. **2007**, 20,100.
6. Yan, H.; Row,K. H.; *Anal. Chim. Acta* **2007**, 584,160.
7. Patel, S. A.; Prajapath, A. M.; Patel, P. U.; Patel, N. J.; Vaghmasi, J. B.; *J. AOAC Intern.* **2008**, 91, 756.
8. González, J. A.O.; Móchon, M. C.; Rosa, F. J. B.; *Microchim. Acta* **2005**, 151, 39.
9. Djabarouti, S.; Boselli, E.; Allaouchiche, B.; Ba, B.; Nguyen, A. T.; Gordien, J. B.; Bernadou, J. M.; Saux, M. C.; Breilh, D.; *J. Chromatog. B* **2004**, 799, 165.
10. El-Gindy, A.; Emara, S.; Mostafa, A.; *J. AOAC Intern*. **2007**, 90,1258.
11. Nemutlu, E.; Kir, S.; Özyuncu, Ö.; Beksaç, M. S.; *Chromatographia* **2007**, 66, S15.
12. Santoro, M. I. R. M.; Kassab, N. M.; Singh, A. K.; Kedor-Hackmam, E. R. M.; *J. Pharm. Biomed. Anal*. **2006**, 40, 179.
13. Neckel, U.; Joukhadar, C.; Frossard, M.; Jäger, W.; Muller, M.; Mayer, B. X;. *Anal. Chim. Acta* **2002**, 463, 199.
14. Böttcher, S.; Baum, H. V.; Hoppe-Tichy, T.; Benz, C.; Sonntag, H. –G.; *J. Pharm. Biomed. Anal*. **2001**, 25, 197.
15. Nguyen, H. A.; Grellet, J.; Ba, B. B.; Quentin, C.; Saux, M. C.; *J. Chromatogr. B* **2004**, 810, 77.
16. Esponda, S. M.; Padrón, M. E. T.; Ferrera, Z. S.; Rodríguez, J.J. S.; *Anal. Bioanal. Chem.* **2009**, 394, 927.
17. Liu, Y. –M.; Cao, J. –T.; Tian, W.; Zheng, Y. –L.; *Electrophoresis* **2008**, 29, 3207.
18. Epinosa-Mansilla, A.; de La Pena, A. M.; Gomez, D. G.; Salinas, F.; *J. Chromatogr. B* **2005**, 822, 185.
19. Liang, H.; Kays, M. B.; Sowinski, K. M.; *J. Chromatogr. B* **2002**, 772, 53.
20. Nava-Junior, I.; Aucelio, R. Q.; *Spectrochim. Acta Part A* **2009**, 72, 429.

21. Silva, L. C.; Trevisan, M.G.; Poppi, R. J.; Sena, M. M.; *Anal. Chim. Acta* **2007**, 595, 282.
22. de la Peña, A. M.; Mansilla, A. E.; Gómez, D. G.; Olivieri, A. C.; Goicoechea, H. C.; *Anal. Chem*. **2003**, 75, 2640.
23. Giménez, D.; Sarabia, L.; Ortiz, M. C.; *Anal. Chim. Acta* **2005**, 544, 327.
24. Ortiz, M. C.; Sarabia, L. A.; Sánchez, M. S.; Giménez, D.; *Anal. Chim. Acta* **2009**, 642, 193.
25. Currie, L. A.; *Anal. Chim. Acta* **1999**, 391, 105.
26. Ribeiro, F. A. L.; Ferreira, M. M. C.; Morano, S. C.; Silva, L. R.; Schneider, R. P.; *Quím. Nova* **2008,** 31,164.
27. Andersen, C.M.; Bro, R.; *J. Chemometr*. **2003**, 17, 200.
28. Booksh, K.; Henshaw, J. M.; Burguess, L. W.; Kowalski, B. R.; *J. Chemometr*. **1995**, 9, 263.
29. Lorber, A.; *Anal. Chem*. **1986**, 58, 1167.
30. Rodríguez-Cuesta, M. J.; Boqué, R.; Rius, F. X.; Zamora, D. P.; Galera, M. M.; Frenich, A. G.; *Anal. Chim. Acta* **2003**, 491, 47.
31. Olivieri, A. C.; Faber, N. M.; *J. Chemometr.* 2005, 19, 583.
32. Braga, J. W. B.; Bottoli, C. B. G. ; Jardim, I. C. S. F.; Goicoechea, H. C. ; Olivieri, A. C.; Poppi, R. J.; *J. Chromatogr. A* **2007**, 1148, 200.
33. R. Boqué, R.; Larrechi, M. S.; Rius, F. X.; *Chemom. Intell. Lab. Syst*. **1999**, 45, 397.
34. Boqué, R.; Ferré, J.; Faber, N. M.; Rius, F. X.; *Anal. Chim. Acta* **2002**, 451, 313.
35. Olivieri, A. C.; Faber, N. M.; *Chemom. Intell. Lab. Syst*. **2004**, 70, 75.