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Determination of atenolol in human urine by emission–excitation fluorescence matrices and unfolded partial least-squares with residual bilinearization

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ABSTRACT

A second-order multivariate calibration method based on a combination of unfolded partial least-squares (U-PLS) with residual bilinearization (RBL) has been applied to second-order data obtained from excitation–emission fluorescence matrices for determining atenolol in human urine, even in the presence of background interactions and fluorescence inner filter effects, which are both sample dependent. Atenolol is a cardioselective beta-blocker, which is considered a doping agent in shoot practice, so that its determination in urine can be required for monitoring the drug. Loss of trilinearity due to analyte–background interactions which may vary between samples, as well as inner filter effects, precludes the use of methods like parallel factor analysis (PARAFAC) that cannot handle trilinearity deviations, and justifies the employment of U-PLS. Successful analysis required to include the background in the calibration set. Unexpected components appear in new urine samples, different from those used in calibration set, requiring the second-order advantage which is obtained from a separate procedure known as residual bilinearization (RBL). Satisfactory results were obtained for artificially spiked urines, and also for real urine samples. They were statistically compared with those obtained applying a reference method based on high-performance liquid chromatography (HPLC).

1. Introduction

Atenolol (RS)-4-(2-hydroxy-3-isopropylaminopropoxy)phenylacetamide is a beta-adrenoreceptor antagonist, a beta-1 blocker, because it is cardioselective and mainly affects the heart, competing for receptor sites on the cardiac muscle. This slows down the strength of the heart contractions and reduces its oxygen requirements and the volume of blood it has to pump. It is indicated in hypertension (high blood pressure), because of its ability to increase the diameter of blood vessels, allowing blood to flow under less pressure. It is also used to treat myocardial infarction (heart attack) and arrhythmias (rhythm disorders), angina (chest pain), disorders arising from decreased circulation and vascular constriction, including migraine and also panic attack. It is considered as a doping agent in competition in different sports as chez, aeronautical sports, bridge, gymnastics, bowling, shoot, ski, swimming, and weights. In shoot it is also considered a doping agent out of competition, since it reduces the cardiac frequency and minimizes tremors. It has been included in the list of prohibited drugs (as beta-1 blockers) by the World Anti-Doping Code belonging to the World Antidoping Agency (2010) [1].

In humans, absorption of an oral dose, usually of 25, 50 or 100 mg once a day is rapid and consistent but incomplete. Approximately 50% of an oral dose is absorbed from the gastrointestinal tract, reaching the peak plasma level concentration between 2 and 4 h after ingestion. This absorbed portion is eliminated primarily by renal excretion; therefore the total amount of atenolol excreted in urine can be used as a measure of bioavailability [2–4]. Thus, the determination of atenolol in urine can be useful for monitoring the drug, performing a suitable dosage adjustment. Moreover, in doping control, urine analysis is preferred.

Several analytical methods have been reported for the determination of atenolol in biological fluids based on gas chromatography [5–7], high-performance liquid chromatography (HPLC) [8–12], capillary zone electrophoresis [13] and voltammetry [14–16].

These methods have some advantages such as sensitivity and selectivity, although they also present some disadvantages: they may require expensive equipment, toxic and expensive solvents (mainly HPLC methods) and usually tedious sample pretreatment when used for analyzing biological samples. Spectroscopic methodologies may be useful and suitable for this kind of laboratories. A few analytical methods based on spectrophotometric and spectrofluorometric determination of atenolol in tablets or artificial samples have been reported in the literature, one of them based on

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fluorescence atenolol detection, using a molecular imprinted polymer, introducing a new approach for atenolol fluorescent analysis [17–20].

Indeed, spectrofluorimetry can be applied for determining atenolol since it presents natural fluorescence. Moreover, fluorescence spectroscopy is intrinsically sensitivity and instruments are easily available [21,22]. However, when spectrofluorimetric methodologies are applied for the determination of analytes in biological fluids such as urine, they may suffer the effects of the presence of potential natural fluorescence interferences, and also from other unexpected fluorescence sample components, so that sample pretreatment steps must be required. This problem can also be overcome combining spectrofluorimetry and multivariate calibration techniques, so that interferences could be mathematically removed [23–26]. First-order methods can handle the presence of potential interferences if they are represented during the calibration stage. A sample containing unexpected components is marked as an outlier due to the poor fit of its spectrum to the calibration model (first-order advantage) [27–29] but analyte prediction are inaccurate. Moreover, two spectrophotometric methods assisted by chemometrics have been reported for the determination of atenolol but in samples as pharmaceutical formulations, based on processing UV spectral data with different first-order methods [19].

Second-order data are suitable for the quantitative determination of analytes in complex multi-component samples such as urine. Processed with appropriate second-order multivariate calibration algorithms, concentration of calibrated analytes can be obtained even in presence of uncalibrated components, exploiting the so-called second-order advantage [24–26,30–32].

In the present report, a rapid, sensitive and selective method suitable for routine laboratories, based on chemometrics-assisted spectrofluorimetry, is developed for determining atenolol in urine. Second-order trilinear data (excitation–emission fluorescence matrices) have been recorded and processed by second-order algorithms achieving the second-order advantage, allowing the determination of atenolol in urine even in the presence of interferences [32]. The selection of a suitable second-order algorithm is discussed, since this is a peculiar analytical situation. Interactions occur between the analyte atenolol and the urine background, as well as fluorescence inner filter effects, and vary from sample to sample, so that the background must be included in the calibration set. All these facts cause trilinearity losses. This situation cannot be handled by most second-order algorithms, being appropriate those that take into account trilinearity deviations, such as unfolded partial least squares (U-PLS). On the other hand, although the background is included in the calibration set, algorithms achieving the second-order advantage must be applied to predict atenolol concentrations in urine samples different than those used in calibration, since unexpected components could be present. Parallel factor analysis (PARAFAC) could be one of these methods, but it requires trilinearity, hence in principle it is not applicable to this case [33,34]. Residual bilinearization (RBL) could be applied for modeling unexpected signals.

In conclusion, in the present case, methods taking into account deviations of trilinearity, and exploiting the second-order advantage, such as U-PLS/RBL, are applied for the determination of atenolol in complex urine samples even in presence of background interactions, inner filter effects and unexpected components and without sample pretreatments [35,36]. Predictive ability, figures of merit and accuracy are discussed.

To the best of our knowledge, this is the first attempt to apply U-PLS/RBL for this purpose.

2. Experimental

2.1. Equipment

All fluorescence measurements were done on an Aminco Bowman Series 2 spectrofluorophotometer, equipped with a 150 W Xe lamp, and connected to a microcomputer running under OS/2 (through a GPIB IEEF-488 interface). In all cases, 1.00 cm quartz cells were used, excitation–emission matrices (EEMs) were registered in the range λ_{em} = 270–340 nm each 1 nm and λ_{exc} = 200–250 nm each 3 nm, making a total of 17 × 71 data points per sample matrix. Excitation and emission slit widths were both 4 nm and the scan rate was 10 nm min^{-1}. The matrix data were then transferred to an IBM-compatible microcomputer with an Intel core duo T7100, 1.80 GHz microprocessor and 2.00 Gb of RAM and processed by applying chemometric analysis based on second order algorithms, tipically unfolded partial least squares with residual bilinearization (U-PLS/RBL), written in MATLAB 7.0 (The MathWorks Inc., Natick, MA, USA) and available at www.chemometry.com, including a graphical user interface data input and parameter setting [37].

High-performance liquid chromatography (HPLC) was carried out with a Waters liquid chromatograph equipped with a 515 Waters high-pressure pump, a Rheodyne injector and UV–visible detector, using: (a) column Zorbax SB C_{18} 4.6 mm × 150 mm (5 μm particle size), (b) mobile phase methanol: NaH_2PO_4 0.34% (w/w) adjusted to pH = 3.00 with H_3PO_4 (20:80), (c) flow rate of 1.00 mL min^{-1}, (d) temperature maintained at 25 ± 1 °C, and (e) detection wavelength 223 nm (Section 2.7).

2.2. Solutions

A stock 1000 mg L^{-1} solution of analytical grade atenolol (Sigma) was prepared by dissolving the compound in doubly distilled water, sonicating for a few minutes and storing in the dark at 4 °C. Working solutions were prepared by suitable dilutions of the stock solution with double distilled water. Buffer solution was prepared from KH_2PO_4·Na_2HPO_4 (Merck) at pH = 7.4.

2.3. Calibration sample set

A linear relationship between fluorescence intensity and atenolol concentration was previously checked to have an upper limit of 0.60 mg L^{-1} (~2 × 10^{-6} mol L^{-1}). Thus, calibration was performed using a pool of healthy human urine samples as matrix, spiked with different amounts of atenolol stock solution, considering the reference concentration values of atenolol in urine in accordance to usual oral doses.

Oral doses of atenolol are approximately 56% absorbed and subsequently eliminated by renal excretion. Most of an orally absorbed dose (85–100%) is eliminated in urine within 24 h. Thus, considering pharmacokinetic data such as renal clearance and distribution volume, atenolol urine concentrations may be from 10 mg L^{-1} to 40 or 60 mg L^{-1} [2–4]. Hence, spiked urine samples were diluted 1:100 in order to obtain equally spaced concentrations in the range 0–0.60 mg L^{-1}, according to the previously checked linear concentration range. The calibration set was prepared at pH = 7.4 adding KH_2PO_4/Na_2HPO_4 buffer solution as well as the surfactant sodium dodecyl sulfate (SDS) at a concentration 2.1 mmol L^{-1}, higher than the critical micelle concentration (cmc) of 1.4 mmol L^{-1}.

The analytical technique was performed as follows: in 10.0 mL volumetric flasks, suitable amounts of atenolol stock solution were added, in order to obtain concentrations in the range 0–60 mg L^{-1}, in accordance to reference concentration values of atenolol in urine, completing to the mark with urine pools. Then 100 μL of these pool urine samples, suitable spiked with atenolol, and 250 μL of SDS 0.087 mol L^{-1} were added in another 10.0 mL volumetric flasks

and completed to the mark with KH_2PO_4/Na_2HPO_4 0.067 mol L^{-1} buffer, obtaining concentration values included within the linear calibration range.

Excitation–emission fluorescence matrices (EEMs) were registered and processed as described above. All determinations were performed in random order by triplicate. Blank samples, i.e., urine pool samples free of atenolol were also included.

2.4. Validation sample set

In order to validate the method, the same urine pool samples used in the calibration set (i.e., the same background matrix) were spiked with stock solutions of atenolol in suitable amount considering the reference atenolol urine concentration, according to the corresponding oral dose and its pharmacokinetic and bioavailability [2–4]. Then samples were treated as described above, adding SDS to enhance the sensitivity and diluted with buffer in order to work in the linear calibration range. All validation samples were prepared by triplicate. EEMs recording was performed and processed as it was described in Section 2.1.

2.5. Spiked test samples

Different urine samples belonging to healthy individuals were spiked with suitable amounts of atenolol stock solution in order to obtain concentrations in the range 10 mg L^{-1} to 40 or 60 mg L^{-1} in accordance to the pharmacokinetic data and the correspondent dose, as discussed above. Therefore, considering the linear concentration calibration range, samples were diluted 1:100. Also, SDS was added for enhancing sensitivity.

Again, suitable amounts of atenolol stock solution were placed in 10.0 mL volumetric flasks and completed to the mark with each of the selected urine samples. A volume of 0.100 μL of these spiked urines were placed in another 10.0 mL volumetric flask, added with SDS and completed to the mark with the selected buffer, as described above. Samples were prepared by triplicate and EEMs were recorded as described in previous sections.

2.6. Real samples

Real urine samples were taken from patients medicated with a daily dose of 25, 50 and 100 mg respectively. Samples were collected 24 h after dosing, so that approximately 40–50% of the oral dose was excreted in urine [2–4]. A volume of 100 μL of each of these urine samples and 250 μL of SDS were placed in 10.0 mL volumetric flasks and completed to the mark with the selected buffer solution. EEMs were registered as described above and multivariate calibration algorithms were applied. Samples were also studied using an adapted HPLC technique, based on Refs. [38,39] and described below, for comparison with the chemometrics-assisted fluorescence methodology.

2.7. Chromatographic procedure

A standard solution of atenolol 900 mg L^{-1} was prepared by dissolving the compound in the mobile phase methanol:NaH_2PO_4 0.34% (w/w) adjusted to pH = 3 with H_3PO_4 (phosphoric acid) (20:80). The linear calibration range was from 5 to 95 mg L^{-1}. Urine samples were centrifuged, filtered through a filter of 0.5 μm, and suitably diluted in mobile phase. All samples were injected on a column Zorbax SB C_{18} 4.6 mm × 150 mm, at a flow rate of 1.00 mL min^{-1}, detecting the signal at 223 nm and working at 25 ± 1 °C [38,39].

3. Theory

3.1. Data orders

The various types of instrumental data have been classified on the basis on tensor algebra [40–42]. Within this scheme, when a given instrument produces a single instrumental response for a chemical sample, this datum is a scalar or zeroth-order tensor. Vector data for each sample belong to the first-order type: for example, absorption or emission spectra [UV–visible spectrophotometry, spectrofluorimetry, infrared, near-infrared (NIR), etc.], electrochemical scans (voltammograms, chrono-amperograms), nuclear magnetic resonance spectra, etc. When two first-order instruments are coupled in tandem (e.g., GC–MS, MS–MS, etc.), the order increases from first- to second-order. The latter can also be produced using a single instrument: examples are a spectrofluorometer registering excitation–emission matrices (EEMs) or a diode-array spectrophotometer where a chemical reaction takes place. The data order can be further increased to three if, for example, EEMs are registered as a function of time [40–42].

3.2. Algorithms

3.2.1. U-PLS

Unfolded partial least squares (U-PLS) operates in a similar way to partial least squares-1 (PLS-1), except that second-order data are first vectorized or unfolded along one of the data dimensions, and then a conventional partial least-squares (PLS) model is built using these unfolded data and the nominal analyte concentrations [43,44].

Cross-validation can be employed to estimate the number of calibration latent variables. The I calibration data matrices are first vectorized into $JK \times 1$ vectors, and then a usual PLS model is built using these data together with the vector of calibration concentrations $\mathbf{y}$ (size $I \times 1$). This provides a set of loadings $\mathbf{P}$ and weight loadings $\mathbf{W}$ (both of size $JK \times A$, where A is the number of latent factors), as well as regression coefficients $\mathbf{v}$ (size $A \times 1$). The parameter A can be selected leave-one-out cross-validation [45,46]. If no unexpected components are present in the test sample, $\mathbf{v}$ could be used to estimate the analyte concentration according to

$$\mathbf{y_u} = \mathbf{t_u^T v} \tag{1}$$

where $\mathbf{t_u}$ is the test sample score, obtained by projecting the vectorized data for the test sample vec($\mathbf{X_u}$) onto the space of the A latent factors:

$$\mathbf{t_u} = (\mathbf{W^T P})^{-1} \mathbf{W^T} \mathrm{vec}(\mathbf{X_u}) \tag{2}$$

where vec(·) implies the vectorization operator.

3.2.2. U-PLS/RBL

If unexpected constituents occur in a test sample, the U-PLS scores for the latter sample cannot be used for analyte prediction using the trained model. In this case, it is necessary to resort to a technique which is able to: (1) detect the new sample as an outlier, indicating that further actions are necessary before prediction, and (2) isolate the contribution of the unexpected component from that of the calibrated analytes, in order to recalculate appropriate scores for the test sample. U-PLS will consider a sample as an outlier if the residuals of the test data reconstruction are abnormally large in comparison with the typical instrumental noise. In such a case, residual bilinearization (RBL) can be employed to model the presence of unexpected sample components using principal component analysis (PCA), which allows one to estimate profiles for the unexpected components in the three data dimensions [37,43,44]. The RBL procedure consists in keeping constant the matrix of calibration loadings, and varying the test sample scores in order to

model the test data as a sum of contributions: (1) one modeled by the calibration loadings and (2) one due to the potential interferents. The number of unexpected components in the PCA phase can be assessed by comparing the final residuals of the RBL model with the instrumental noise level. Once the RBL step is finished, and the correct test sample scores have been found, they are employed to provide the analyte concentration as is regularly done in all PLS models.

3.2.3. Figures of merit

Figures of merit such as sensitivity (SEN) can be estimated for all PLS models, including those coupled to RBL [47]. The sensitivity is usually estimated as the length of the vector of regression coefficients provided by the U-PLS model, with a suitable correction due to the presence of interferents. The analytical sensitivity (γ) and its inverse are also useful, because they are independent on the type of registered signal [48,49]. Finally, the limit of detection (LOD) can be estimated as 3.3 times the standard deviation for a sample of low or zero analyte concentration. This takes into account the so-called type I and II errors, according to IUPAC recommendations [47].

As the second-order advantage was applied, these figures are sample specific, thus average values for a set of samples were calculated.

4. Results and discussion

4.1. Selection of a suitable second-order algorithm

Fluorescence excitation and emission spectra of atenolol in aqueous solution, in urine spiked with atenolol, and in urine free of atenolol, are shown in Fig. 1. The spectrum of atenolol in urine obtained after subtracting the urine background is also shown. The corresponding excitation–emission fluorescence matrices as well as contour plots of these samples are shown in Fig. 2. A strong overlapping can be noticed between urine free of atenolol, urine added with atenolol and atenolol in aqueous solution in the excitation dimension.

On the other hand, it seems that the emission spectra of atenolol are different in urine than in aqueous solution. Even when the urine background is subtracted, the resulting spectrum of atenolol is slightly different than the aqueous one. The contour plot of an aqueous solution of atenolol shows a maximum emission at 290–300 nm (Fig. 2a), and in the presence of urine a broad emission band shifts from 290 nm to 340 nm (Fig. 2b) similar to what is obtained for urine free of atenolol (Fig. 2c). Furthermore, when the background is subtracted, the atenolol emission spectrum does also show this broad emission band at 290–320 nm (Fig. 2d).

It should also be noticed that the urine fluorescence intensity increases in the presence of atenolol, but the intensity of atenolol decreases in the presence of urine. The fluorescence intensity, in both dimensions, of atenolol 50 mg L^{-1} in urine is notoriously lower than the intensity of an aqueous solution of the analyte at the same concentration (Figs. 1 and 2). The natural urine excitation spectrum presents a maximum near 230 nm, possibly due to amino acids, strongly overlapped with the analyte spectrum, and likely causing excitation inner filter effects. On the other hand, the fluorescence urine emission exhibits mainly two peaks: one exciting at 290 nm due to a series of indole type urinary metabolites, and the other one representing the fluorescence of endogenous metabolites present in urine of healthy subjects such as 4-pyridoxic acid excited at 317 nm, hydroxyanthranilic acid excited at 320 nm and xanthine excited at 315 nm [50]. Taking into account the presently selected wavelength emission range of 270–340 nm, and the excitation wavelengths of these urine components, an emission inner filter effect could be expected, which explains the decrease of fluorescence intensity of atenolol in urine. Although the emission wavelengths of all these compounds are near 400 nm, out of the selected wavelength emission range, another emission band at 290–340 nm is present in natural urine and is strongly overlapped with the analyte (Figs. 1 and 2).

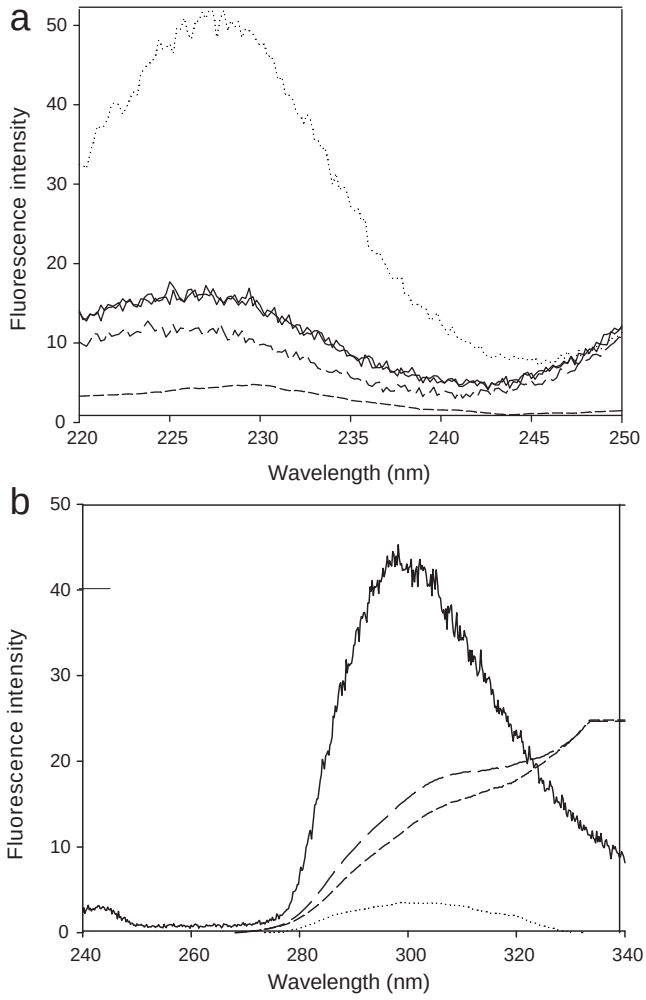

Fig. 1. (a) Fluorescence excitation spectra of: atenolol 50 mg L^{-1} diluted 1/100 in buffer phosphate pH = 7.4 (dotted line), urine spiked with atenolol 50 mg L^{-1} diluted 1/100 (solid line), urine free of atenolol diluted 1/100 (medium dashed line), atenolol 50 mg L^{-1} in urine diluted 1/100 and subtracting urine background (short dashed line). (b) Fluorescence emission spectra of: atenolol 50 mg L^{-1} diluted 1/100 in buffer phosphate pH = 7.4 (solid line), urine spiked with atenolol 50 mg L^{-1} diluted 1/100 (long dashed line), urine free of atenolol diluted 1/100 (short dashed line), atenolol 50 mg L^{-1} in urine diluted 1/100 and subtracting urine background (dotted line).

In order to enhance the analyte fluorescence intensity and the method sensitivity in the presence of urine, sodium dodecyl sulfate (SDS) was added at a concentration higher than the critical micelle concentration (cmc) of 1.4 mmol L^{-1}. The effect of SDS micelles on the spectrofluorimetric intensities of beta-blockers has been previously studied showing that fluorescence intensity is more pronounced at pH < pKa of atenolol (9.39 ± 0.05), at which the analyte remain as a cation. The fluorescence increase in a micellar medium is due to a stabilization and protection of the excited state singlet [51]. In the present work, the pH is fixed at 7.4.

Considering all these spectral phenomena, which seem to vary from sample to sample, it can be concluded that the determination of atenolol in urine cannot be carried out applying conventional methods, requiring, at least, sample pre-treatment to remove interferences. However, in order to avoid tedious sample pre-treatments, interferents can be mathematically removed

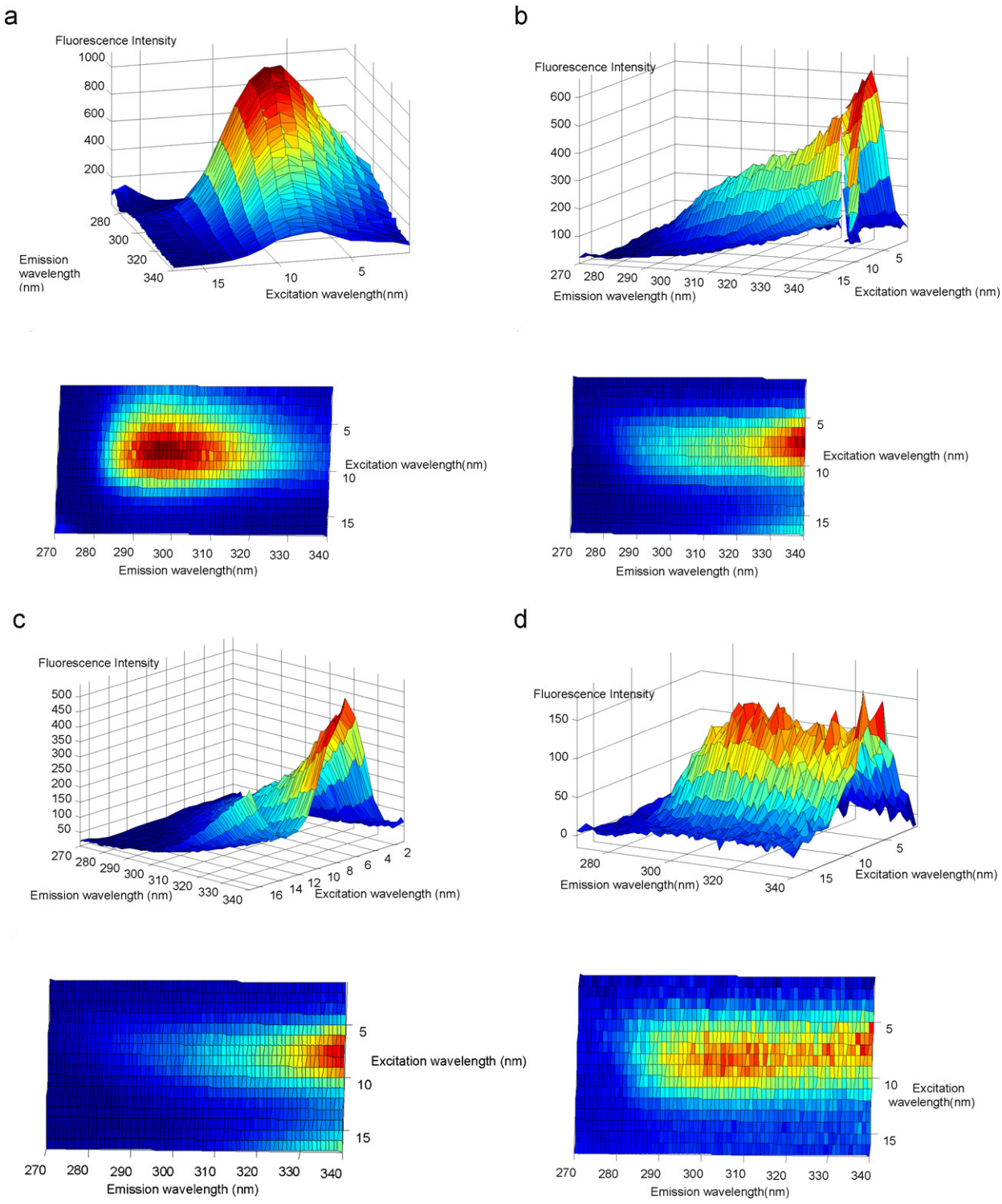

Fig. 2. Excitation–emission fluorescence matrices and contour plots of (a) atenolol 50 mg L^{-1}, (b) urine sample spiked with atenolol 50 mg L^{-1}, (c) urine sample free of atenolol, and (d) atenolol 50 mg L^{-1} in urine after subtracting the urine background.

applying chemometrics. For this purpose, second-order data such as excitation–emission fluorescence matrices were obtained and processed with second-order algorithms achieving the second-order advantage [36,37,40–44].

The applied algorithm should also take into account, as discussed above, interactions between the analyte and the urine background, which are sample dependent. These interactions originate a matrix effect, requiring to include the urine background in the calibration set and precluding the use of an external calibration curve, even when the second-order advantage is used. Inclusion of the background in the calibration can cause a loss of the trilinearity condition, necessary for multivariate algorithms like PARAFAC, due to sample to sample spectral variations. Furthermore, the fluorescence inner filter effect does also cause trilinearity deviations that cannot be resolved by most second-order algorithms. On the other hand, an incomplete calibration is used, meaning an incomplete knowledge of calibration constituents, since urine is included in the calibration set, and hence second-order algorithms like BLLS/RBL

Table 1
U-PLS Leave-one-out cross validation results for calibration samples.

Component	PRESS	SEP	*F*	*p*
1.0000E+000	2.0533E−001	1.8499E−001	1.6204E+001	9.9700E−001
2.0000E+000	**1.4383E−002**	**4.8960E−002**	**1.1350E+000**	**5.5900E−001**
3.0000E+000	1.2757E−002	4.6110E−002	1.0067E+000	5.0300E−001
4.0000E+000	1.2671E−002	4.5955E−002	1.0000E+000	4.9900E−001

PRESS, predicted error sum of squares; SEP, standard error of prediction; *F*, statistical parameter for determining the optimum number of latent variables; *p*, probability associated to *F*; and optimum value is highlighted in boldface.

Table 2
Validation set samples: prediction results for U-PLS/RBL and PARAFAC.

Atenolol ($mg\,L^{-1}$)	PREDICTED[a] atenolol ($mg\,L^{-1}$)	
	U-PLS/RBL[b]	PARAFAC[c]
0.05	0.05	0.05
0.10	0.10	0.10
0.21	0.21	0.22
0.31	0.31	0.30
0.42	0.40	0.38
0.53	0.56	0.57
0.06	0.06	0.07
0.17	0.17	0.17
0.34	0.34	0.28
0.68	0.69	0.72
0.51	0.50	0.50
RMSE ($mg\,L^{-1}$)	0.01	0.03
REP%	3.89	9.63

RMSE = root mean square error.
REP% = relative error of prediction.
[a] Concentration average of triplicate analysis reported in a volumetric flask for urine diluted 1:100.
[b] U-PLS $N = 2$, RBL $N_{unx} = 0$.
[c] $N = 2$, core consistency = 100.

could not be applied. As has recently been discussed in the literature, this situation can be resolved by U-PLS, achieving the second-order advantage by RBL [36,52]. The second-order algorithm U-PLS/RBL can be applied even when trilinearity deviations and incomplete calibration are present.

4.2. Validation samples

As described above, a set of 11 samples containing different amounts of atenolol in random order and the same urine matrix used in the calibration set was analyzed in order to test the predictive ability of the selected second-order multivariate methodology U-PLS/RBL.

Two latent variables are necessary in order to describe 99% of variability present in the calibration set ($A = 2$), estimated applying leave-one-out cross-validation, in accordance to the Haaland and Thomas criterion (Table 1) [45]. The number of unexpected components was 0 ($N_{unx} = 0$), calculated according to error analysis. This is expected considering that the validation samples composition is the same as that used in the calibration set, so that the second-order advantage is not necessary. The results are satisfactory and shown in Table 2.

Although the second-order advantage is not necessary for these samples, the advantage of using second-order data like excitation–emission fluorescence matrices (EEMs) instead of first-order data is the higher sensitivity as well as the lower number of calibration samples that would be required.

Validation samples show similar contour plots as calibration samples, as expected, so prediction applying PARAFAC was also satisfactory (Fig. 3, Table 2).

4.3. Spiked test samples

Urine samples, different from those used in the calibration and validation sets, were added with suitable random concentrations

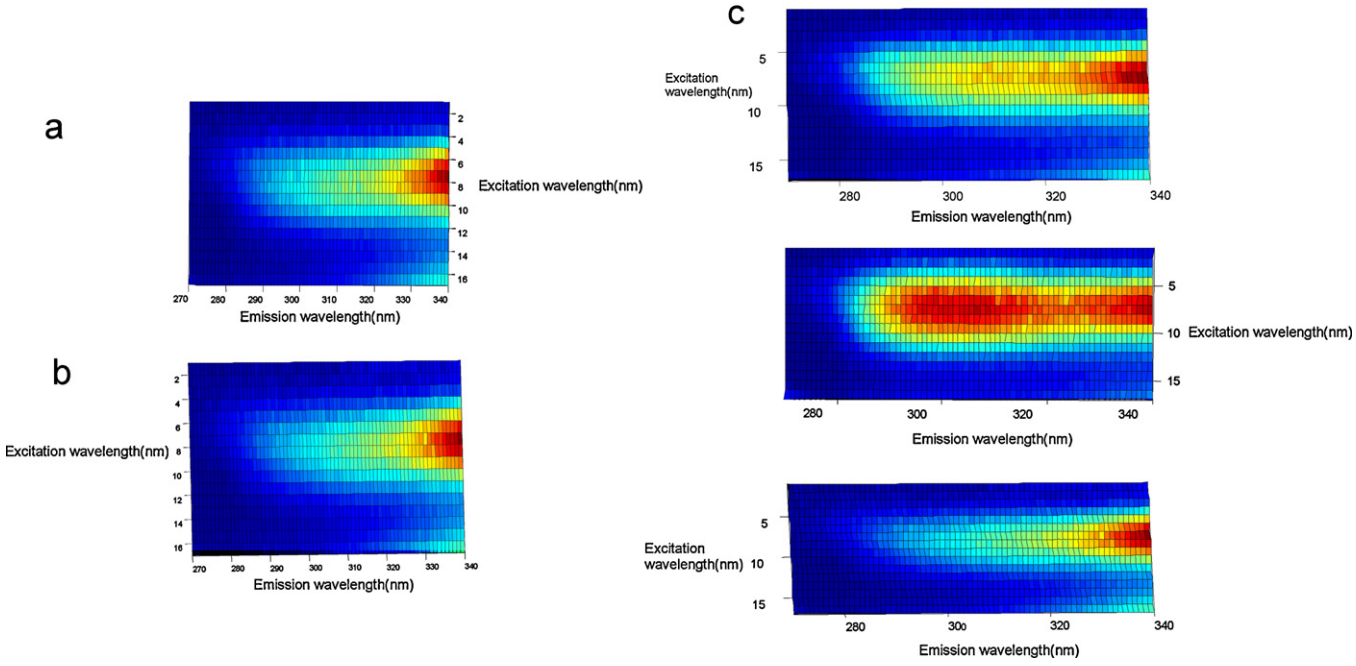

Fig. 3. Contour plots of: (a) calibration sample, (b) validation sample, and (c) three different test samples.

of atenolol, as described above (Section 2.5) for testing the predictive ability of the proposed second-order algorithm U-PLS/RBL, even when the background signal changes from sample to sample, considering that the analyte background interactions may also vary. As can be noticed in Fig. 3c, contour plots of test samples were not only different from each other, but also from those obtained for calibration and validation samples. Applying U-PLS/RBL, the number of calibration latent variables was two ($A = 2$) as was defined for both calibration and validation samples, modeling the analyte and the interaction with the urine background. As urine has been included in the calibration, it could be expected that the number of unknown or unexpected factors or components (N_{unx}) would also be zero and that the second-order advantage introduced by RBL would not be necessary. However, the number of unexpected components determined by analyzing the relationship between U-PLS prediction residuals before and after applying RBL suggests that these residuals are stabilized at $N_{unx} = 1$ or 2, according to each sample features (Fig. 4a). The fact that N_{unx} was different from zero indicates that although the urine background was included in the calibration, the second-order advantage is necessary considering that the algorithm uses an incomplete calibration. The prediction results are shown in Table 3, being satisfactory as indicated by the elliptical joint confidence region (EJCR) that shows a good correlation between predicted and nominal concentrations (Fig. 5a). Prediction results applying PARAFAC are also shown in this table, and are not satisfactory in accordance to the system features as discussed before. The low core consistency values for test samples, negative in some cases, indicate trilinearity deficiency or inconsistency [32].

Figures of merit (FOM) have also been estimated being: sensitivity (SEN) 540.1 AFU $(\text{mg L}^{-1})^{-1}$ expressed as the ratio between the arbitraty units of fluorescence and concentration, analytical sensitivity $\gamma = 73.0\ \text{L mg}^{-1}$, inverse of analytical sensitivity $0.014\ \text{mg L}^{-1}$ and LOD $0.03\ \text{mg L}^{-1}$. These figures are sample dependent so all these values are averages over the whole set of samples since the second-order advantage has been applied.

Another beta blockers like propanolol has already been determined in urine, showing two excitation maxima at 240 and 290 nm and a emission maximum at 375 nm thus considering the spectral ranges using in the present work, an inner filter effect could be expected that could be resolved by the selected algorithm [53].

However, potential interference effects from other beta blockers are not discussed in this report. When two or more beta blockers are simultaneously administered, in addition to a more marked decrease in the blood pressure, heart failure may develop (www.medicine.org.uk). Thus, simultaneous beta-receptor block-

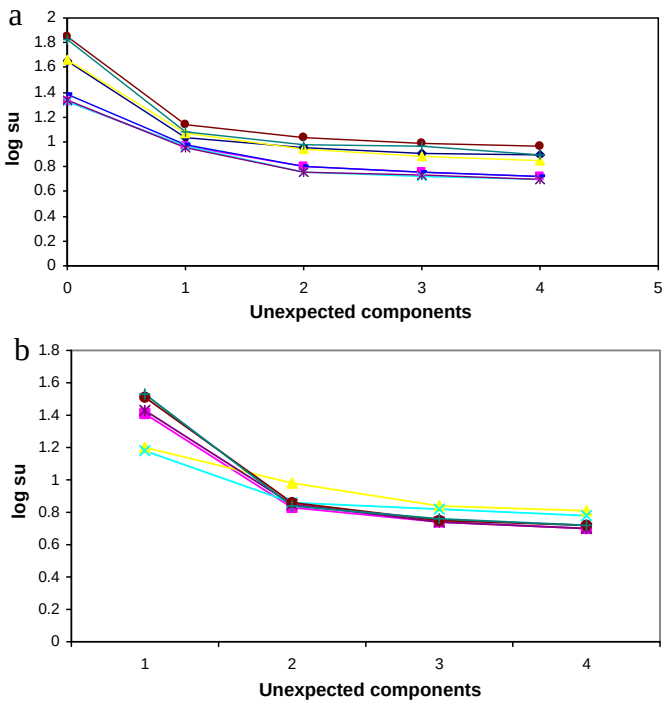

Fig. 4. Logarithms of U-PLS prediction residuals ($\log s_u$) as a function of the number of unexpected components for: (a) test samples and (b) real urine samples.

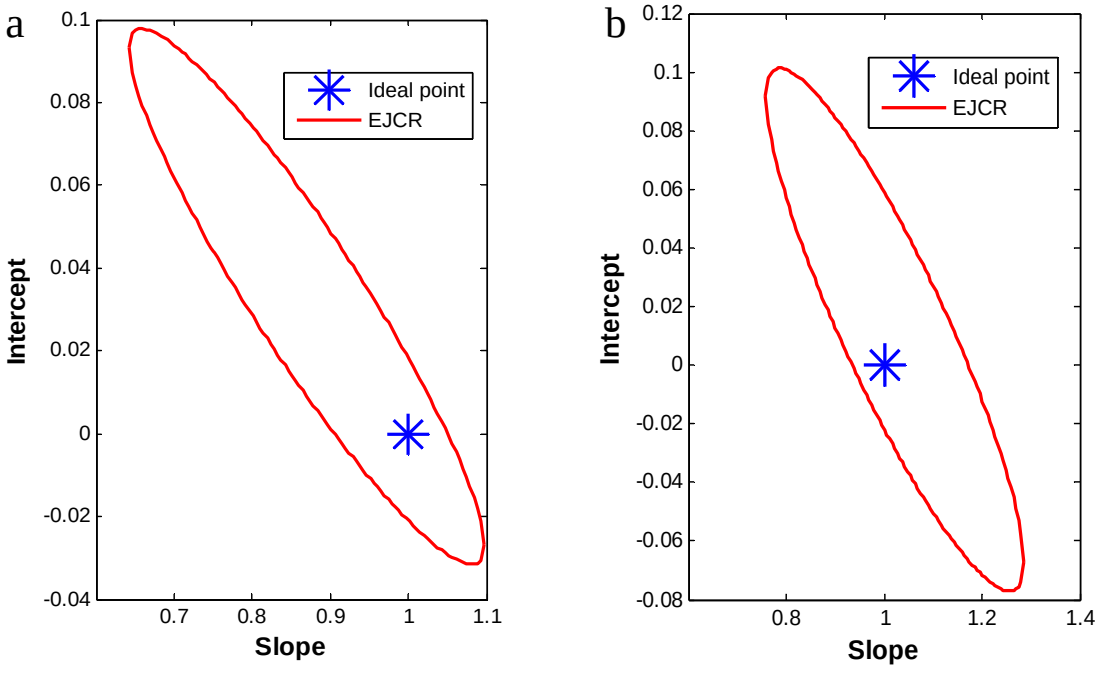

Fig. 5. Elliptical joint confidence region for the slope and intercept of the regression of predicted concentration vs. nominal values: (a) for the test set and (b) for real urine samples.

Table 3
Results for prediction test samples.

Atenolol (mg L^{-1})	Predicted atenolol (mg L^{-1})[a]		
	U-PLS/RBL[b]		PARAFAC
	N_{unx} = 0	Suitable selected N_{unx}[c]	N= 3
0.26	1.10	0.25	0.98
0.20	0.85	0.20	0.38
0.20	1.32	0.21	0.87
0.16	0.49	0.16	0.35
0.20	0.60	0.18	0.50
0.50	2.50	0.42	2.08
0.30	2.15	0.31	1.12
0.19	0.79	0.18	0.89
0.40	0.14	0.40	1.31
0.15	0.00	0.16	0.35
0.40	0.60	0.40	0.93
0.20	0.00	0.22	0.60
0.20	0.00	0.23	0.50
0.20	0.00	0.18	0.50
0.40	1.42	0.36	0.70
0.40	1.31	0.42	1.69
RMSE (mg L^{-1})	0.92	0.03	0.72
REP%	317	9.00	250

RMSE = root mean square error.
REP% = relative error of prediction.
[a] Predicted concentrations are averages of triplicate analyses and are reported in a volumetric flask for urine diluted 1:100.
[b] Latent variables for U-PLS A = 2.
[c] Unexpected components N_{unx} for stabilizing U-PLS residuals s_u N_{unx} = 1 or N_{unx} = 2 depending on urine samples.

Table 4
Results for real urine samples.

Sample[a]	U-PLS/RBL[b,c] (mg L^{-1})[d]	HPLC[b,c] (mg L^{-1})[d]
R1	13 (2)	13.1 (2)
R2	12 (2)	12.8 (2)
R3	29 (2)	26.4 (8)
R4	30 (2)	28.0 (8)
R5	51 (1)	51.0 (3)
R6	53 (1)	52.0 (3)

[a] R1 and R2 urine samples for oral doses of 25 mg; R3 and R4 oral doses of 50 mg; and R5 and R6 oral doses of 100 mg L^{-1}.
[b] Standard error in parenthesis obtained after three replicates analysis.
[c] Predicted values statistically compared to HPLC values, for paired t-test comparison, calculated t value 1.93, degree of freedom n = 5; critical t value 2.015 for n = 6, α = 0.05.
[d] Atenolol concentration in urine of 24 h.

ers must be avoided, and therefore it is unlikely that these drugs will be present in urine at the same time. Moreover, simultaneous determination of many beta blockers have been developed in different recent reports, mainly based on HPLC and capillary electrophoresis, using a mixture of betablockers for calibration in order to indentify their peaks and determine simultaneously in artificial spiked urine samples. However, in real urine samples only one of them was determined at a time, since these samples were obtained from real patients medicated with only one beta blocker [54,55].

4.4. Real samples

Real urine samples were analyzed applying the present fluorescence method and processing data with the algorithm U-PLS/RBL. The number of latent variables was also 2 (A = 2). Analyzing U-PLS prediction residuals, it can be concluded that N_{unx} should be different from zero, and stabilized at N_{unx} = 1 or 2 depending on the samples (Fig. 4b). These samples were also analyzed applying a reference method based on high performance liquid chromatography (HPLC). Results for both methods are shown in Table 4, being statistically comparable using paired t-statistics (calculated t values are lower than tabulated t value for the corresponding degrees of freedom in all cases). The elliptical joint confidence region (EJCR) of the regression of predictions by the fluorescence method vs. HPLC predictions is also studied. Fig. 5b shows that all confidence regions include the ideal point of unit slope and zero intercept, also indicating accuracy. Moreover, all concentration values that are obtained are in accordance with those expected, considering doses and pharmacokinetics of atenolol [2–4]. Figures of merit were also calculated as an average of all samples: sensitivity (SEN) 618.1 AFU (mg L^{-1})$^{-1}$, analytical sensitivity γ = 80.0 L mg^{-1}, inverse of analytical sensitivity 0.013 mg L^{-1} and LOD 0.02 mg L^{-1}.

5. Conclusions

A chemometrics-assisted spectrofluorimetric method was developed for the determination of atenolol in a complex biological urine matrices. The method is based on second-order data (excitation–emission fluorescence matrices, EEMs), which are prime examples of trilinear data, processed by the second-order algorithm unfolded partial least-squares (U-PLS) achieving the second-order advantage by applying residual bilinearization (RBL). Background signals are included in the calibration set due to the presence of analyte–background interactions, which are sample dependent. Fluorescence inner filter effects are also occurring. These facts cause deviations of trilinearity, that are well resolved applying U-PLS. Unexpected components appear in urines different that those used in calibration, requiring the second-order advantage provided by RBL. Satisfactory results are obtained for spiked urine samples. The results obtained for real samples are statistically comparable to those obtained applying the reference method based on high performance liquid chromatography (HPLC).

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