J Fluoresc (2012) 22:1483–1492
DOI 10.1007/s10895-012-1085-6

ORIGINAL PAPER

Simultaneous Determination of Mefenamic and Tolfenamic Acids in Real Samples by Terbium-Sensitized Luminescence

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Received: 21 March 2012 / Accepted: 20 June 2012 / Published online: 1 July 2012

Abstract A simple luminescent methodology for the simultaneous determination of mefenamic and tolfenamic acids in pharmaceutical preparations and human urine is proposed. Since the native fluorescence of both analytes is not intense, the method takes advantage of the lanthanide-sensitized luminescence, which provides a higher sensitivity. Due to the strong overlapping between the luminescence spectra of both terbium complexes, the use of luminescence decay curves to resolve mixtures of the analytes is proposed, since these curves are more selective. A factorial design with three levels per factor coupled to a central composite design was selected to obtain a calibration matrix of thirteen standards plus eight blank samples that was processed using a partial least-squares (PLS) analysis. In order to assess the goodness of the proposed method, a prediction set of synthetic samples was analyzed, obtaining recovery percentages between 90 and 104 %. Limits of detection, calculated by means of a new criterion, were 14.85 μg L^{-1} and 15.89 μg L^{-1} for tolfenamic and mefenamic acids, respectively. The method was tested in a pharmaceutical preparation containing mefenamic acid, obtaining recovery percentages close to 100 %. Finally, the simultaneous determination of both fenamates in human urine samples was successfully carried out by means of a correction of the above-explained model. No extraction method neither prior separation of the analytes were needed.

Keywords Mefenamic acid · Tolfenamic acid · Lanthanide-sensitized luminescence

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Abbreviations

TF	Tolfenamic acid
MF	Mefenamic acid
NSAID	Non-steroidal anti-inflammatory drug
6-MNA	6-methoxy-2-naphtylacetic acid
TOPO	Trioctylphosphine oxide
PLS	Partial least-squares
DW	Durbin-Watson parameter
PRESS	Predict error sum of squares
SEP	Standard error of prediction
REP	Relative error of prediction
s_d	Standard deviation
RSD	Relative standard deviation

Introduction

Tolfenamic (TF) and Mefenamic (MF) acids are two fenamates, a group of medicines that are members of the non-steroidal anti-inflammatory drug (NSAID) family. They possess anti-inflammatory, antipyretic and analgesic effects due to the inhibition of the ciclooxygenase enzymes, responsible for the synthesis of prostaglandins. These drugs are frequently orally administrated and their secretion takes place mainly with urine.

Most methods in literature make use of liquid or gas chromatographic techniques for the simultaneous determination of TF and MF in several matrices, such as urine [1–8], blood serum [2, 6–11], pharmaceutical preparations [1, 2] or bovine milk [12]. A method that makes use of isotachophoresis and conductimetric detection to determinate the analytes in medicines has also been reported [13]. All these methods, however, require complex and expensive instrumentation, and frequently a previous step of derivatization or extraction of the analytes.

Since the native fuorescence of TF and MF in water is not especially intense, lanthanide-sensitized luminescence has been studied as a very attractive approach for the development of spectrofluorimetric methods to determine these drugs [14, 15]. After luminescent excitation of the analytes, an intersystem crossing to their triplet state takes place and, afterwards, the energy is transferred to the 4f level of the lanthanide ion (Tb(III), in this case). The emission is observed as characteristic line-type bands, the intensity of which depends only on the concentration of the analytes in solution if the concentration of the lanthanide ion is kept constant. Arnaud and Georges [15] reported that the TF-Tb and MF-Tb complexes yielded a very intense luminescence, in particular in the presence of trioctylphosphine oxide (TOPO) as a synergistic ligand and Triton X-100 as a surfactant. The synergistic ligand removes water molecules from the coordination sphere of the lanthanide ion, since its presence is deleterious to luminescence emission. In addition, the micellar environment protects the chelates against non-radiative processes [16–18].

Since the emission always takes place from the lanthanide energy levels, luminescence spectra of different analyte-lanthanide complexes overlap completely. However, the development of analytical methods that achieve their simultaneous determination has been possible thanks to differences in the lifetimes of the complexes, and multivariate calibration. For instance, Ibáñez [19] reported the simultaneous determination of tetracycline and oxytetracycline in bovine serum using europium as a sensitizer.

The purpose of this work is to develop a new method to determine simultaneously TF and MF combining the advantages of lanthanide-sensitized luminescence, time-resolved measurements and multivariate calibration techniques. Synthetic samples were used to validate the results, and the method was successfully applied to biological matrices, namely urine. Unlike all the previous works found in literature, the proposed method requires no prior derivatization or extraction steps and, therefore, it is simpler, faster and less expensive.

Experimental

Chemicals

All solution were prepared using ultrapure distilled water with a total organic carbon (TOC) level <5 μg L^{-1}, which was obtained from a Milli-Q 185 plus system.

Stock solutions (50.0 mg L^{-1}) of both analytes (MF and TF) were prepared dissolving the required amounts (Sigma Aldrich, St. Louis, USA) in NaOH (0.01 mol L^{-1}), and were stored at 4 °C. These solutions were found to be stable for, at least, a fortnight.

Terbium (III) chloride hexahydrate, Triton X-100 and TOPO were purchased from Sigma Aldrich too. Stock solutions of Terbium ($2 \cdot 10^{-2}$ mol L^{-1}), Triton X-100 (1 % v/v) and TOPO (10^{-2} mol L^{-1}) were prepared in distilled water and used in the determination of the analytes. A 10 % v/v of ethanol was also needed to dissolve TOPO completely.

A buffer solution (1 mol L^{-1} with a pH of 5.4) was prepared using the appropriate amount of Sodium Acetate Anhydrous (Panreac, Barcelona, Spain). Solutions of NaOH and HCl were used for pH adjustments and were also obtained from Panreac.

Finally, stock solutions of the tested interferences were prepared from the compounds obtained from Sigma Aldrich, except 6-methoxy-2-naphtylacetic acid (6-MNA), which was purchased from ICN Biomedical Inc (Aurora, Ohio, USA).

The medicine Coslan (Parke-Davis S.L., Grupo Pfizer, Alcobendas, Madrid, Spain) is sold in capsules containing 250 mg of MF, and was bought in a drugstore. Human urine was obtained from fasting and healthy people at first time in the morning, and kept at 4 °C until it was used.

Instrumentation

Measurements of pH were performed using a Crison 2001 pH-meter with a glass-saturated calomel combination electrode. A Selecta ultrasons bath was used for the preparation of the solutions from pharmaceutical preparations.

The luminescence decay curves of the analytes were obtained using a Photon Technology International (PTI) TimeMaster fluorometer equipped with a Xenon integral pulsed lamp, two Czerny-Turner monochromators (on the excitation and emission ports, respectively) and a photomultiplier detector. The system is connected to a PC via Ethernet and is governed by the Felix32 software.

This equipment also possesses a Peltier based cuvette holder for Quartz cuvettes with a pathlength of 1.0 cm × 1.0 cm, the temperature of which can be controlled between −20 and 100 °C.

In order to carry out the PLS regression, the decay curves obtained in the Felix32 were exported as ASCII files and transferred to a PC fitted with MatLab 7.0.1 and the routine MVC1, designed by Olivieri et al. [20], which is freely available on the Internet.

Study of Luminescence Lifetimes of the Analytes

In order to study the luminescence lifetimes of the analytes, decay curves of both compounds were obtained and analyzed using an algorithm based on the Marquardt studies [21] that is included in the Felix32 software.

The goodness of the fit was evaluated using chi-squared (χ^2) and Durbin-Watson parameters, residuals distribution and autocorrelation function. χ^2 parameter acceptable values should be included between 0.8 and 1.3, since higher or lower values mean a significant bias respect to the theoretical fit. In the case of the Durbin-Watson (DW) parameter, a

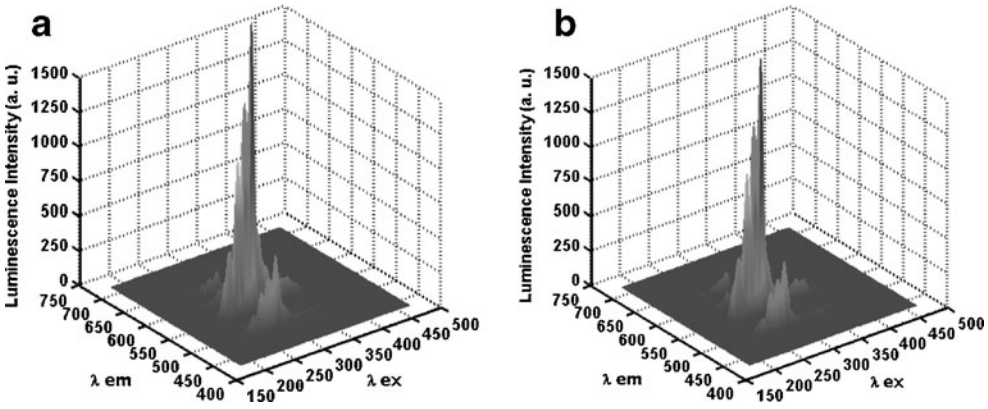

Fig. 1 Three dimensional luminescence spectra of (**a**) TF-Tb and (**b**) MF-Tb complexes. Conditions: 10^{-3} mol L^{-1} of Tb, 0.04 % of Triton X-100, 10^{-4} mol L^{-1} of TOPO, 5 % v/v of EtOH

good fit should return a value higher than 1.7. As for residuals distribution and autocorrelation function, desired results should fulfill the homocedasticity criteria, showing a random distribution centred around 0.

Variables Affecting Luminescence Intensity

So as to obtain the maximum sensitivity, all the variables affecting the luminescence intensity were studied by measuring solutions containing 500.0 μg L^{-1} of MF or TF at the different conditions. Possible variations in lifetimes or pH values during optimization were checked.

Selection of the Calibration and Validation Matrices

Analyte concentrations in the calibration matrix ranged from 0 to 400.0 μg $L^{-1}$for MF and from 0 to 300.0 μg L^{-1} for TF. In order to represent accurately the whole concentration intervals selected, a factorial design with three levels per factor coupled to a central composite design was used to obtain a matrix of thirteen standards. Since we decided to add eight analyte blanks, the calibration matrix finally consisted of twenty-one standards. A study of the prediction ability of the model was carried out with the use of a validation matrix. This validation matrix consisted of ten samples containing both analytes in different concentrations that covered the intervals written above, and were obtained using a home-made random number generator software [22].

Procedure for Calibration and Validation Samples

Calibration and validation samples were prepared adding the appropriate amount of each analyte from stock solutions: 0.50 mL of Tb $2{\cdot}10^{-2}$ mol L^{-1}, 0.40 mL of Triton X-100 1 %, 0.1 mL of TOPO 10^{-2} mol L^{-1} and 1.0 mL of the buffer stock solution into a 10 mL volumetric flask that was brought to volume with distilled water and ethanol (95/5 % v/v). Measurements were performed, using the instrumental optimized conditions showed below, by collecting decay curves and exporting them as ASCII files, which were afterwards introduced into the MVC1 routine [20], controlled by Matlab, and analyzed using the PLS-1 algorithm.

Procedure for Pharmaceutical Preparation Samples

Each Coslan capsule content was dissolved in sodium hydroxide (0.01 mol L^{-1}), then filtered using a filter paper for quantitative analysis DP 145 125 (Albet). The filtered solution was placed in a 0.5 l volumetric flask in order to obtain a solution of 100 mg L^{-1} of MF.

Three replicates of Coslan were prepared, and then diluted, so that concentrations were within calibration range. The resulting solutions were measured and predicted as unknown samples using the optimized chemical and instrumental conditions.

Procedure for Urine Samples

Urine from three healthy people was collected freshly in the morning and centrifuged immediately (20 °C, 15 min,

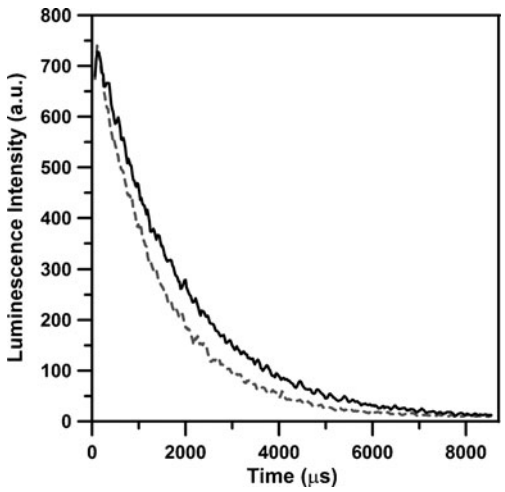

Fig. 2 Experimental luminescence decay curves for TF-Tb (*solid line*) and MF-Tb (*dashed line*). Conditions: 10^{-3} mol L^{-1} of Tb, 0.04 % of Triton X-100, 10^{-4} mol L^{-1} of TOPO, 5 % v/v of EtOH, λ_{exc}=287 nm and λ_{em}=546 nm

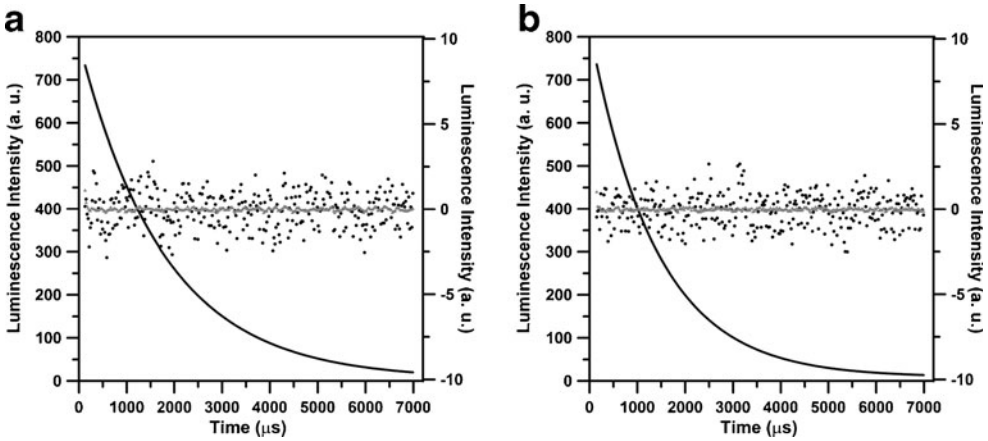

Fig. 3 Fitted curves (*solid lines*), residual distributions (*black dots*) and autocorrelation functions (*gray dots*) for (**a**) TF-Tb and (**b**) MF-Tb complexes. Data obtained by applying the algorithm of Marquardt [21], contained in the Felix32 software, to the decay curves showed in Fig. 2. Conditions: 10^{-3} mol L^{-1} of Tb, 0.04 % of Triton X-100, 10^{-4} mol L^{-1} of TOPO, 5 % v/v of EtOH, λ_{exc}= 287 nm and λ_{em}=546 nm

350 rpm). Dilutions were prepared and stored properly. In order to test the developed calibration model, aliquots of urine were spiked with convenient amounts of TF and MF into a 10 mL volumetric flask containing 0.50 mL of Tb $2{\cdot}10^{-2}$ mol L^{-1}, 0.40 mL of Triton X-100 1 %, 0.1 mL of TOPO 10^{-2} mol L^{-1} and 1.0 mL of the buffer stock solution. The 10 mL volumetric flask was subsequently brought to volume with distilled water and ethanol (95/5 % v/v). In this case, the study was also performed by collecting decay curves and exporting them as ASCII files, which were afterwards introduced into the MVC1 routine, controlled by Matlab, and analyzed using the PLS-1 algorithm.

Results and Discussion

Spectral Characteristics of the Analytes, Their Tb-Complexes and Human Urine

Unless pH is very acid, native luminescence emission of both analytes in water is not very intense [14, 15]. However, if terbium is present, the luminescent signal rises dramatically. Three-dimensional spectra of both lanthanide complexes are shown in Fig. 1. For both analytes, excitation and emission maxima are 287 and 546 nm, respectively. The latter corresponds to an emission peak of terbium, which confirms that the transference of energy from analyte to lanthanide actually takes place. Due to this fact, both spectra are almost identical and overlap completely. Consequently, they are not suitable to analyze MF and TF simultaneously by means of conventional fluorometry. Human urine shows no intrinsic luminescence at the selected excitation and emission wavelengths [23].

Luminescence Lifetimes of the Analytes Complexes

The measured decay curves (λ_{exc}=287; λ_{em}=546) for both terbium-fenamate complexes are shown in Fig. 2. These curves were fitted (Fig. 3) by using the algorithm of Marquardt [21], contained in the Felix32 software, obtaining the results shown in Table 1. The averages of the measured lifetimes for MF-Tb and TF-Tb complexes are around 1,380 and 1,790 μs, respectively. This small difference avoids the time-resolved simultaneous determination of both compounds. However, the decay curves are different enough to allow the simultaneous determination of the analytes using multivariate calibration methods.

Variables Affecting Luminescence Intensity

Chemical Variables

First of all, the influence of the pH on intensity and lifetimes was studied adding different amounts of HCl and NaOH to each sample. As can be seen in Fig. 4, the maximum signal appeared at around pH=6 for both analytes.

Dihydrogen carbonate/hydrogen carbonate, citrate, dihydrogen phosphate/hydrogen phosphate and acetate buffers were tested. Only the latter was found to buffer the samples efficiently, keeping an intense luminescence emission at the same time. Therefore, acetate buffer was chosen, although it was necessary to select a slightly lower pH (5.4) in order to assure a good buffer capacity of acetate (its pKa is 4.7). An optimum concentration of 0.1 mol L^{-1} was chosen, since signal dropped at higher buffer concentrations.

Both analytes showed identical tendencies when other chemical parameters were changed. To obtain the maximum possible intensities, the following concentrations were selected for working solutions: 10^{-3} mol L^{-1} of Tb, 0.04 % of Triton

Table 1 Lifetimes and quality statistical parameters for MF-Tb and TF- Tb complexes

Parameter	MF-Tb	TF-Tb
χ^2	0.91	1.02
DW	2.28	2.03
Pre-exponential	724±33	784.5±3.7
Lifetime (μs)	1381.1±7.2	1787±11

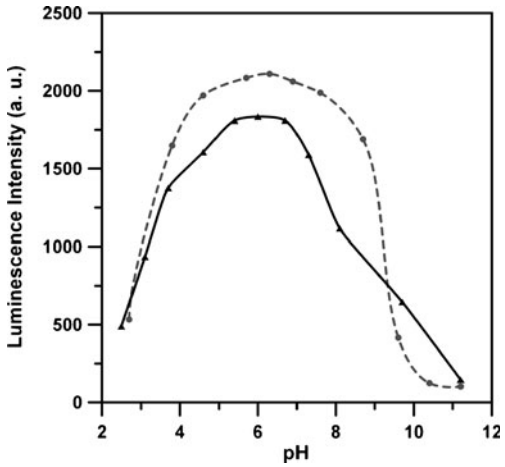

Fig. 4 Effect of pH on the luminescence intensity of TF-Tb (*solid line*) and MF-Tb (*dashed line*) complexes. Conditions: 10^{-3} mol L^{-1} of Tb, 0.04 % of Triton X-100, 10^{-4} mol L^{-1} of TOPO, 5 % v/v of EtOH, λ_{exc}=287 nm and λ_{em}=546 nm

X-100, 10^{-4} mol L^{-1} of TOPO and 5 % v/v of ethanol. No significant changes were observed in lifetime values with these optimized chemical conditions.

Instrumental Variables

The influence of the instrumental variables on the luminescence intensities and lifetimes was also studied in order to obtain the better signal-to-noise ratio and acceptable acquisition times. The following values were found to provide the best signal-to-noise ratios for both analytes: integration time of 400 μs, 11 lamp shots for each measurement, 5 averages for each decay, 400 channels (points per decay) and a pulse frequency of 100 Hz. No significant changes were observed in lifetime values with these optimized instrumental conditions.

Linear Response of Terbium Complexes

A study of the linearity between luminescence intensity and concentration of the analytes was carried out. Different solutions containing increasing analyte concentration values were prepared and measured under the optimum conditions explained above. The results showed that luminescence intensity and concentration have a linear dependence up to 500.0 μg L^{-1} for TF and up to 750.0 μg L^{-1} for MF.

Application of the PLS-1 Model

Studies involving the use of multivariate calibration techniques for multi-component resolution of UV/visible or fluorescence data revealed no significant differences between the principal component regression (PCR) and PLS predictions. However, considering its theoretical advantages and optimal performance over a wide range of conditions, PLS is the method of choice [24]. We adopted the PLS-1 algorithm, which performs PLS analysis one component at a time, to carry out the determination. PLS algorithms consist of two steps: calibration and prediction.

Optimising Data for Calibration

The simplest calibration design consists of two levels per component (four calibration samples). However, this model showed to be too simple, since the results were poor, owing to the inadequate number of standards employed and the fact that the design failed to consider samples containing only one of the components in the mixture. As an alternative, a central star design (five calibrations samples) includes samples with only one analyte, although not a simultaneous combination of high or low concentrations. In addition, the number of samples was too small. That is why a combination of both designs was finally considered. Four more intermediate samples as well as eight analyte blanks were also added to this model, in order to statistically maximize the information contained in the decay curve, so finally a calibration matrix of twenty-one standards was used: a combination of a factorial design with three levels per factor, a central star design and eight blank samples. Concentrations were varied over the range from 0 to 400.0 μg $L^{-1}$for MF and from 0 to 300.0 μg L^{-1} for TF.

Decay curves of samples, prepared under the previously optimised experimental conditions described above, were recorded from 0 to 8,600 μs with 400 experimental points (channels) per curve. Since full-spectrum methods such as PLS can use many experimental points, range selection is seemingly unnecessary, so all available points are often used. However, measurements considering points of a decay curve that are non-informative in a model often degrade performance. That is why the range between 5 and 395 points was selected both for MF and TF, since doing so the best results were obtained.

Selection of the Optimum Number of Factors

An appropriate choice in the number of principal components, or factors, is necessary for PLS to perform properly.

Table 2 Statistical parameters after cross-validation

Analyte	Number of factors	SEP[a]	% REP[b]	R^2
MF	2	13	10	0.990
TF	2	6.3	6.8	0.996

[a] Standard Error of Prediction

[b] Relative Error of Prediction

Table 3 Results obtained on the prediction of synthetic samples containing MF and TF

Mefenamic acid					
Sample	Actual [MF] (μg L^{-1})	Predicted [MF] (μg L^{-1})	s_d[a]	RSD[b] (%)	Recovery (%)
1	72.0	74.0	7.6	3.0	102.8
2	54.0	48.9	6.9	2.9	90.6
3	172.0	177.5	5.0	2.8	103.2
4	80.0	81.9	4.3	3.2	102.3
5	110.0	107.3	5.4	2.6	97.5
6	320.0	317.1	6.6	2.6	99.1
7	236.0	243.8	5.7	2.6	103.3
8	260.0	256.1	6.0	2.2	98.5
9	144.0	135.0	5.4	2.9	93.7
10	353.0	355.9	6.9	2.5	100.8
Tolfenamic acid					
Sample	Actual [TF] (μg L^{-1})	Predicted [TF] (μg L^{-1})	s_d[a]	RSD[b] (%)	Recovery (%)
1	248.0	251.8	3.8	3.0	101.5
2	230.0	228.1	3.5	2.9	99.2
3	140.0	145.5	2.5	2.8	103.9
4	108.0	104.8	2.2	3.2	97.0
5	194.0	194.4	2.7	2.6	100.2
6	188.0	183.0	3.3	2.6	97.3
7	195.0	190.0	2.9	2.6	97.5
8	220.0	215.9	3.0	2.2	98.1
9	160.0	160.2	2.7	3.0	100.1
10	184.0	184.2	3.5	2.5	100.1

[a]Standard Deviation
[b]Relative Standard Deviation

The number of factors should account as much as possible (since the more factors, the lower noise level of the data is), but without resulting in overfitting.

After performing cross-validation and leave-one-out method with the PLS-1 algorithm, the predicted concentrations were compared with the known concentrations of the compounds in each calibration sample in order to calculate the predict error sum of squares (PRESS) [25], which was recalculated upon addition of each new factor to the PLS-1 model.

Table 2 shows the statistical results obtained after cross-validation. Following the Haaland and Thomas criterion [25], two was selected as the optimum number of factors for both MF and TF. This is usually expected for a binary mixture.

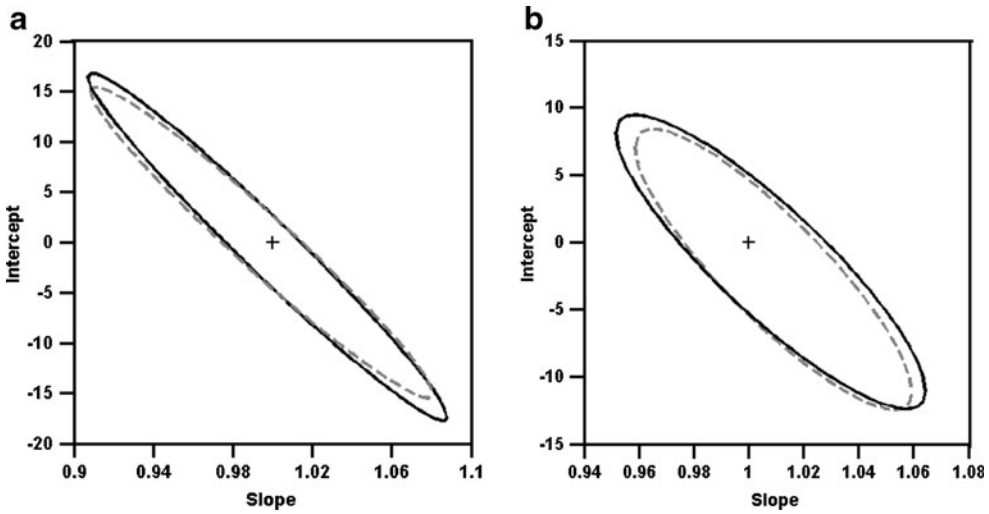

Fig. 5 Elliptical joint confidence region for the intercept and the slope obtained from the calibration matrix for (**a**) TF-Tb and (**b**) MF-Tb, considering both an Ordinary Least-Squared Regression (*solid line*) and a Weighted Least-Squared Regression (*dashed line*)

Table 4 Study of NSAID interferences on MF (150.0 μg mL^{-1}) and TF (150.0 μg mL^{-1}) determination

NSAID added	Proportion MF/NSAID	Recovery (%)
Flufenamic Acid	1/0.50	96.2
Meclofenamic Acid	1/0.50	95.3
Diclofenac	1/0.50	102.5
Salicylic Acid	1/0.25	102.3
Diflunisal	1/0.5	99.7
Nabumetone	1/50	100.6
6-MNA	1/0.5	96.7
NSAID added	Proportion TF/NSAID	Recovery (%)
Flufenamic Acid	1/0.1	105.9
Meclofenamic Acid	1/0.50	105.7
Diclofenac	1/0.5	98.1
Salicylic Acid	1/1.0	95.4
Diflunisal	1/0.1	94.7
Nabumetone	1/50	96.7
6-MNA	1/0.5	100.9

Values of SEP (standard error of prediction) and REP (relative error of prediction) give an idea of the prediction ability of the PLS model. R^2 shows whether a good linear correlation exists between the values of predicted and actual analyte concentrations.

Prediction of Synthetic Samples (Validation of the Method)

The prediction ability of the proposed method was assessed by applying the developed PLS-1 method to a series of ten unknown mixtures. Concentrations of MF and TF were chosen from a distribution of random numbers [22] falling within the ranges of the calibration matrix.

Actual and predicted concentrations for each sample and analyte are shown in Table 3, as well as standard deviation (s_d) and relative standard deviation (RSD). The recoveries of the analytes in the synthetic samples (between 90 and 104 %) prove that the model is capable of predicting synthetic or unknown samples properly.

Calibration graphs of predicted versus actual concentration values showed the following linear equations: $y = (-2.0 \pm 3.5) + (1.009 \pm 0.017) \times (R^2 = 0.998)$ for MF and $y = (-0.4 \pm 5.8) + (0.997 \pm 0.030) \times (R^2 = 0.993)$ for TF. Figure 5 shows the elliptical joint confidence region between the intercept and the slope, considering both an Ordinary Least-Squared Regression and a Weighted Least-Squared Regression. Since the point (0,1) is located inside of the ellipse, it is possible to assume that there are no statistically significant differences between the experimental results and the theoretical ones at a confidence level of 95 % [26].

Limits of Detection

Our research group recently developed a new criterion to calculate the limit of detection of a multivariate calibration method [27, 28]. This criterion is based on the IUPAC definition [29], which only considers the standard deviation of the blank and the slope of the calibration graph to calculate the limit of detection. Analogously, our criterion considers the standard deviation of ten replicates of a blank solution and the slope of the PLS-predicted concentration versus actual concentration graph to calculate the limit of detection.

Following the above-explained criterion, 14.85 μg L^{-1} was obtained for TF and 15.89 μg L^{-1} for MF, values that are expected from terbium-sensitized methods for the determination of fenamates [15].

Precision Study

To carry on this study, ten replicates of a sample containing 160.0 μg L^{-1} of TF and 290.0 μg L^{-1} of MF were analyzed using the proposed method. The average concentrations obtained were 159.9 μg L^{-1} (standard deviation: 2.6; relative error: 0.07 %) for TF and 289.4 μg L^{-1} (standard deviation: 3.0; relative error: 0.2 %) for MF, which shows the high precision and accuracy of the proposed method.

Selectivity Study

In order to assess the selectivity, TF and MF were determined in the presence of different non-steroidal anti-inflammatory drugs (namely salicylic acid, meclofenamic acid, tolfenamic acid, diclofenac, diflunisal, nabumetone, and 6-MNA). Table 4 shows the maximum proportion in

Table 5 Results of the analysis of samples of the pharmaceutical preparation Coslan, containing MF and spiked quantities of TF

Coslan Sample	[Analyte] in the medicine (μg L^{-1})		Spiked [Analyte] (μg L^{-1})		Predicted [Analyte] (μg L^{-1})		Recovery (%)	
	MF	TF	MF	TF	MF	TF	MF	TF
1	300	–	–	0	293.7	–	97.8	–
2	200	–	–	60	203.3	61.5	101.6	102.4
3	200	–	–	130	207.3	128.0	103.6	98.4

Table 6 Statistical results of the analysis of variance. F′ indicates whether a statistically significant difference exist among the different experimental sets, while F″ evaluates whether their slopes are comparable

	F′	F″
Critical Value (95 % confidence)	3.63	4.26
MF	$19.8 \cdot 10^{-2}$	$8.67 \cdot 10^{-2}$
TF	$7.88 \cdot 10^{-2}$	$1.06 \cdot 10^{-2}$

which the presence of these drugs does not affect significatively the determination of TF and MF, as well as the recovery percentages in each case. Other members of the fenamate group were found to be the main interferences, while the presence of nabumetone hardly affected the determination of TF or MF, even in the highest proportions. Salicylic acid and diflunisal cause interference to the determination of MF and TF, respectively. This fact is due to the proximity of the luminescence lifetimes of the analytes and interferences in these conditions (around 1,200 and 1,890 μs, for salicylic acid and diflunisal, respectively).

Applications

Application to Pharmaceutical Preparations

Before dealing with more complex matrices, the proposed method was applied to the analysis of Coslan, a pharmaceutical preparation that contains MF. However, some test samples were conveniently spiked with different amounts of TF, so as to assess completely the prediction capacity of the PLS-1 model in medicines.

The results are expressed as the average of the determination of three replicates in Table 5. In all cases, the recovery percentages for Coslan agree well enough with the nominal content, and the precision is quite satisfactory. Therefore, the proposed method can be used in routine analyzes of pharmaceutical preparations, since the excipients commonly found in them show no interference.

Application to Human Urine Samples

In order to test the accuracy and applicability of the method to biological samples, the optimised matrix obtained by the PLS-1 model was applied to human urine samples from volunteers, fortified with the two fenamates of interest. The dilution factor applied to the urine samples was fixed according to the excreted amounts of these drugs in urine under habitual therapy.

Although urine does not show fluorescence at the wavelengths used in this work [23], its presence was found to mean an interference in the determination of MF and TF, due to a strong matrix effect, since unacceptable errors were observed in the prediction of spiked samples. However, the graphs of predicted versus actual concentration values showed a linear dependence.

The experiments were carried out using urines from three different people (dilution 1/250) and accuracy of data was evaluated using analysis of variance. The validity of this analysis assumes that the residual error variance does not change from one sample to another or from one calibration graph to another. To carry out an analysis of variance, the variance ratio (experimental F) must be calculated and compared to a critical value of F, for adequate degrees of freedom, at 95 % confidence level.

Table 6 shows that in all cases the experimental values of F are smaller than the critical ones. Therefore, the variation between the three sets of data is not statistically significant and global calibration lines could be calculated and used to determinate the analytes in urine (Fig. 6). The equations of these lines are $y = (6.4 \pm 1.7) + (0.3789 \pm 0.0081) \times (R^2 = 0.993)$ for TF and $y = (38.4 \pm 1.9) + (0.514 \pm 0.010) \times (R^2 = 0.994)$ for MF. These results show that, with a slight modification, the method is valid for biologic samples. It is important to highlight

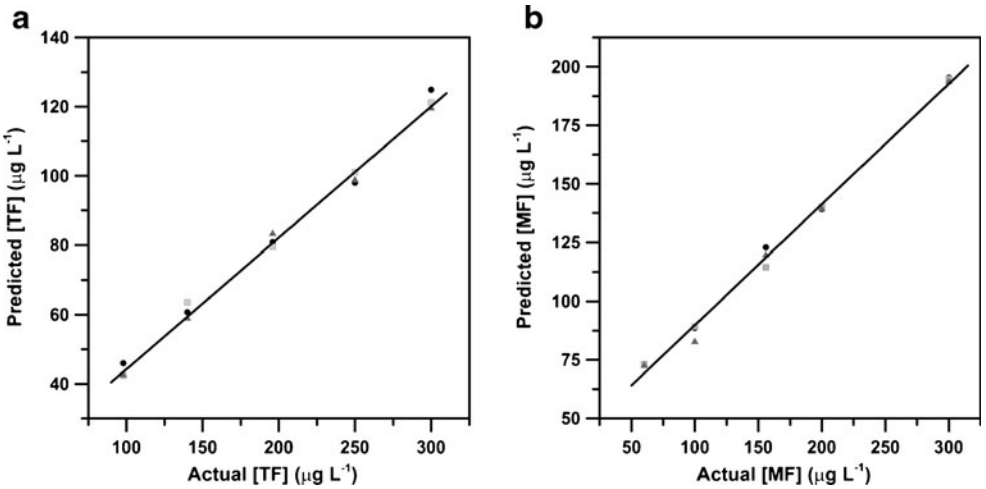

Fig. 6 Global calibration lines for TF-Tb (**a**) and MF-Tb (**b**) in urine, obtained by an Ordinary Partial Lest-Squared regression. The equations of these lines are $y = (6.4 \pm 1.7) + (0.3789 \pm 0.0081) \times (R^2 = 0.993)$ for TF and $y = (38.4 \pm 1.9) + (0.514 \pm 0.010) \times (R^2 = 0.994)$ for MF

that extraction steps prior the analysis were not needed. This is an important advantage because time and reagents are saved.

Conclusions

Lanthanide-sensitized luminescence is an excellent tool for the fluorometric determination of molecules that show negligible native luminescence. The use of the luminescence decays as analytical signals in multivariate calibration has proved to be successful to resolve complex mixtures of compounds with extremely overlapped luminescence spectra.

In this case, the simultaneous determination of a mixture of mefenamic acid and tolfenamic acid using PLS-1 and a calibration matrix with a factorial design with three levels per factor coupled to a central composite design, provide reliable and precise results, and could be applied both to routine analyses of pharmaceutical preparations and to real samples, such as urine, with limits of detection of a few μg L^{-1}. The proposed method shows a great improvement compared to previously published ones, since the latter require complex sample pre-treatments and expensive instrumentation that is costly to operate, while the first is simple and takes much less time. Finally, this is the first method in literature that performs the simultaneous determination of mefenamic and tolfenamic acids without prior separation of the analytes.

Acknowledgments The authors gratefully acknowledge financial support from the "Consejería de Educación y Cultura, Junta de Comunidades de Castilla-La Mancha" (Project Nº PCI-08-0120).

Fernando Martínez Ferreras thanks the Spanish Ministerio de Educación for a FPU (Formación del Profesorado Universitario) fellowship.

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