Direct determination of closely overlapping drug mixtures of diflunisal and salicylic acid in serum by means of derivative matrix isopotential synchronous fluorescence spectrometry

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Abstract

A direct method for the simultaneous fluorometric determination of two anti-inflammatory drugs in serum is proposed. The combination of matrix isopotential synchronous fluorescence (MISF) and first derivative technique provides good analytical results and permits the simultaneous determination of diflunisal and salicylic acid in human serum. MISF spectra are obtained by calculating the isopotential trajectory in the three-dimensional fluorescence spectrum for a serum solution. In the spectral contour, the trajectory is taken to be the portion of the line that passes by the fluorescence maxima of both compounds ensuring a sensitivity level similar to that of a direct determination in absence of background fluorescence. Analysis was carried out in water using a pH of 7.2 provided by 0.1 M sodium dihydrogen phosphate buffer. Serum samples are diluted 100 times and provide linear calibration plots at diflunisal and salicylic acid concentrations up to 800 ng mL\(^{-1}\). The goodness of the analytical signal was checked by using variance analysis. Signals recorded throughout the calibration range were subjected to three calibrations per each analyte, both in the absence and in the presence of variable amounts of the other analyte. Differences between individual calibrations and slopes were compared with those of individual calibrations. Based on the results, diflunisal and salicylic acid can be accurately quantified in the presence of each other. The limit of detection calculated according to Clayton who uses error propagation throughout the calibration curve and a non-centralized security factor was 36.8 and 37.3 ng mL\(^{-1}\) for diflunisal and salicylic acid, respectively.

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1. Introduction

The non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used in the treatment of many arthritis types. NSAIDs help combat arthritis pain by interfering with the inflammatory process, the NSAIDs work by blocking the activity of the enzyme cyclooxygenase (known as COX). Research has revealed that there are two forms of the COX enzyme, known as COX-1 and COX-2. COX-1 is involved in maintaining healthy tissue and COX-2 is involved in the inflammatory pathway.

Diflunisal is a difluorophenyl derivative of salicylic acid, used for the treatment of inflammation and pain that results from many forms of arthritis, including rheumatoid arthritis and osteoarthritis, as well as soft tissue injuries, such as tendinitis and bursitis.

Diflunisal is immediately released into the blood and is also used for rapid relief of mild to moderate pain and menstrual cramps.

Acetylsalicylic acid breaks down into salicylic acid 20 min after entering the bloodstream. It is the salicylic acid that is responsible for the beneficial effects of aspirin. Salicylic acid itself is too caustic to be taken orally.

Salicylic acid is responsible for the anti-inflammatory action of aspirin, and may cause the reduced risk of colorectal cancer observed in those who take aspirin. Yet salicylic acid and other salicylates occur naturally in fruits and plants, while diets rich in these are believed to reduce the risk of colorectal cancer.

The similar structures of salicylic acid and diflunisal result in mutual interferences with the determination of each other. Thus, diflunisal interferes with immunoassay and UV–vis spectrophotometric methods [1–3] for salicylate. This interference could cause serious problems in salicylate assay for therapeutic drug monitoring and drug abuse screening purposes. Although chromatographic methods [4,5] avoid this problem, they are
time-consuming, requiring sample pretreatment prior to the measurement, different internal standards, when applied to serum or urine, and the use of organic solvents which is an inherent disadvantage of chromatographic techniques.

A method for the simultaneous determination of these compounds in serum, based on their intrinsic fluorescence in chloroform-acetic acid solutions and the use of second derivative synchronous scanning with prior protein precipitation with trichloroacetic acid, was reported [6]. The same sample pretreatment was used by Muñoz de la Peña et al. [7], who proposed a method for the simultaneous determination of these analgesics in serum samples by combination of synchronous fluorometry and partial least squares (PLS) multivariate calibration. Later, a method based on the formation of ternary Tb-EDTA-difunisal and Tb-EDTA-salicylate complexes was reported [8]. The mixtures of difunisal and salicylate in the form of these ternary complexes were resolved by using second derivative synchronous fluorescence spectrometry technique and adequate mathematical treatment of the analytical signals. Panadero et al. proposed a method for the simultaneous determination of these compounds in serum combining the lanthanide-sensitized luminescence with kinetic and equilibrium measurements of both systems [9].

Also, a method for the determination of difunisal and salicylic acid by capillary electrophoresis with post-column addition of terbium and sensitized lanthanide-ion luminescence detection has been proposed [10].

Fluorescence spectroscopy is widely used in quantitative analysis because of its great sensitivity and selectivity as well as its relative low cost. However, satisfactory analysis of a fluorescent multicomponent system without any separation or derivatization step is not possible when there are interferences in the spectra due to an overlap of the fluorescence bands. For this reason, development of techniques which improve the selectivity of fluorimetric methods is desirable. Among these techniques, synchronous [11] and derivative [12] fluorescence spectrometry are the most commonly used.

A fluorimetric technique called matrix isopotential synchronous fluorescence (MISF) has been applied for determinations of porphyrins [13], analgesics [14], anthracenes [15], antibiotics [16] and anti-inflammatory drugs [17,18], in the presence of unknown fluorescence backgrounds or fluorescence matrix, such as urine, faeces or serum. This technique combines high sensitivity with improved selectivity compared with other synchronous fluorimetric techniques.

The MISF technique consists on performing synchronous scans through a trajectory joining points of equal intensity on a fluorescence matrix three-dimensional spectrum. Provided the matrix to be used exhibits an almost invariable composition, a constant background signal can be maintained even if the fluorescence intensity changes. This is possible if a portion of the total fluorescence spectrum along a trajectory connecting points of identical intensity (isopotential trajectory) is selected from an initial point to final excitation and emission wavelengths. Such as trajectory can be obtained by using a software program developed by us in BASIC [19]. In order to ensure a sensitivity level similar to that of a direct determination in absence of background fluorescence, trajectories must pass by the fluorescence intensity peaks of both analytes.

The performance of this technique can be improved by using derivative methods.

Human serum contains a variety of organic substances. Most are present at low concentrations or possess low fluorescence efficiency. In fact, only a few of these substances are fluorescent and exhibit different excitation and emission maxima [20,21] that contributes significantly to the overall fluorescence spectrum of serum. This ultimately results in a high background fluorescence that interferes with the direct determination of difunisal and salicylic acid.

MISF spectrometry has so far been applied to the individual determination of a variety drugs in biological fluids with excellent result. In this work, a method for the simultaneous determination of difunisal and salicylic acid in serum using a single isopotential trajectory without the need for a prior separation is proposed.

2. Experimental

2.1. Apparatus

All fluorometric measurements were performed on an Aminco Bowman series 2 luminescence spectrometer connected with a personal computer fitted with the AB2 software. The instrument is equipped with a continuous 150 W xenon lamp for fluorescence measurements and also with a 7 W xenon integral pulsed lamp for phosphorescence measurements.

All measurements were made on Quartz glass cuvettes with a pathlength of 1.0 cm × 1.0 cm, thermostatically controlled by a Selecta frigierm thermostatic bath.

A Crison Model 2001 pH-meter with a glass-saturated calomel combination electrode and a Selecta ultrasonic bath were also used.

2.2. Software

The AB2 program allows the instrument control, operation and acquisition of excitation, emission and total fluorescence spectra. This program also allows to export the fluorescence data into ASCII file format.

Flotal [19] software is a home made program which provides the best characterization of the fluorescence of any fluorescent compound by performing a total luminescence spectral analysis. To create a file the data are taken from the ASCII file obtained with the AB2 program, when the file has been generated, the three-dimensional spectra can be obtained and presented as isometric projection or as contour maps. This program allows to autoscale and to remove light scattering, which allows taking full advantage of the fluorescent characteristics of the compounds. Moreover, the three-dimensional spectra can be processed by means of mathematical operations, such as smoothing and taking the derivative.

However, the main advantage is that this program allows to generate any bi-dimensional spectrum (excitation, emission, conventional synchronous, constant energy synchronous, matrix
isopotential synchronous, linear variable angle synchronous and
non-linear variable synchronous spectra) from any trajectory
in the total luminescence spectrum. These spectra can also
be subjected to arithmetic operations, such as derivation or
smoothing. For the MISF spectrum Fitobal obtains the intensity
values by applying Lagrange's interpolation method to the
excitation wavelengths calculated by means of the particular
function that join points of equal intensity on the matrix total
fluorescence spectrum.

A program which enables us to obtain the values of $\lambda_{\text{exc}}$
and $\lambda_{\text{em}}$ for any constant value of fluorescence intensity from
a three-dimensional spectrum was also used. Once the trajectory
has been defined, the spectrum is obtained by means of the
Fitobal program.

The statistical analysis is totally covered by means of a pro-
gram that has a menu which includes procedures, such as least
median of squares regression (detection of outlier and lever-
age points), least squares regression with and without replicates,
weighted least squares regression, tests of regression and correla-
tion, detection and determination limits (IUPAC [22,23], error
propagation theory [24,25] and Clayton et al. [26]), ellipse graph
for the 95% confidence region for the true slope and intercept
on the $y$-axis estimated from the regression method, dispersion
and confidence bands for the calibration graph and ANOVA
test for linearity and for comparison of several regression
lines.

2.3. Reagents

All experiments were performed with analytical reagent
grade chemicals, pure solvent and Mill-Q water. Sodium dihy-
drogen phosphate for buffer solutions was obtained from Pan-
reac Química S.A. (Barcelona, Spain).

Salicylic acid and difunisal were obtained from Sigma
Chemical Co. (St. Louis, USA). The stock solutions of these
standards (5 mg dissolved in 100 mL of water) were stored at
less 5 °C and kept away from light, under these conditions the
solutions were stable for at least 2 months. Standard working
solutions of salicylic acid and difunisal were prepared between
a range of 1 and 8 $\mu$g of salicylic acid and difunisal into a
10 mL volumetric flask, and were stable for at least 2 h at room
temperature.

The NSAID's standards for the interferency study were
obtained from Sigma Chemical Co., except 6-methoxy-2-
naphthylacetic acid (6-MNA) which was obtained from ICN
Biomedicals Inc. (Aurora, OH, USA).

A 0.5 M buffer solution with pH 7.2 was prepared by mixing
appropriate amounts of sodium dihydrogen phosphate and
sodium hydroxide.

Human serum was obtained from fasting and healthy people,
a dilution of 4 mL of serum into 100 mL of water was prepared
and stored below 5 °C.

2.4. General procedure

To prepare the calibration graph place an appropriate aliquot
of the stock standard solutions containing 1–8 $\mu$g of salicylic
acid and difunisal into a 10 mL volumetric flask, add 2 mL of
buffer solution (pH 7.2), 2.5 mL of serum dilution and dilute with
water to a final volume of 10 mL. For the assay, a 1:100 dilution
was adopted that ensures high enough sensitivity to determine
appropriate concentration of these anti-inflammatory drugs in
therapeutic doses.

Acquire the three-dimensional spectrum of each sample (61
excitation spectra of 192 nm width in steps of 0.4 nm, varying the
excitation wavelength in 3.2 nm steps), export it into ASCII files
and get the total luminescence spectrum by means of the Fitobal
program. Then the adequate trajectory and matrix isopotential
synchronous spectra were obtained for each sample. Calculate
the first derivative, according to the Savitzky and Golay
algorithm [27,28], and finally determine difunisal and salicylic
acid by measuring the derivative signal at $\lambda_{\text{exc}}$/ $\lambda_{\text{em}}$ 249.6/399.4
and 264/420.8 nm, respectively. Finally, the concentration of
both drugs was determined by an appropriate calibration

3. Results and discussion

3.1. Spectral characteristics

The fluorescence of the analytes is best characterized from
a three-dimensional spectrum. Such spectrum can be repre-
sented as the isometric projection (a plot of the emission
spectra at stepped increments of the excitation wavelength).
A reversed projection of the data occasionally indicates the
presence of bands hidden by the foreground. In this projection,
the emission spectra are plotted at decreased excitation
wavelengths. Alternatively, three-dimensional spectra can be
effectively transformed into two-dimensional plots (with the
excitation and emission wavelength as coordinates) by connect-
ing points of the same intensity to form contours. As a rule,
contours are more useful than isometric projection to detect the
presence of hidden bands and select the best possible trajectory
in order to obtain optimum results with the synchronous scan
technique.

Fig. 1 shows the total fluorescence spectra for (Fig. 1(A))
a 1:100 dilution of serum, (Fig. 1(B)) a standard solution
500 ng mL$^{-1}$ of salicylic acid and (Fig. 1(C)) a standard solu-
tion of 600 ng mL$^{-1}$ of difunisal. Salicylic acid exhibits two
excitation bands at 233.2 and 296 nm, respectively, at the same
emission maximum (408 nm).

Difunisal also exhibits an excitation peak at 258 nm and
two shoulders at 232.4 and 292 nm at the same emission
maximum (422 nm). Finally, the serum spectrum for 1:100
exhibits only a band at excitation/emission wavelengths of
281.6/336.4 nm.

Previous experiments [17,14] showed the qualitative com-
position of fluorescent metabolites in serum from healthy indi-
viduals of both sexes but variable age and on different diets to
be virtually the same (a necessary condition for application of
this new synchronous technique). The different serum samples
exhibited the same type of fluorescence, with barely any differ-
ences spectral shape or fluorescence maxima. Some differences
in intensity were observed, however.
3.2. Factors affecting fluorescence intensity

Chemical variables were studied and optimized in order to ensure the best possible measurement conditions, maximum fluorescence intensity and adequate selectivity.

First of all the solubility of the analytes was studied. Salicylic acid is soluble in water, and difunisial is lightly soluble in water; but its solubility is increased in basic medium, so no organic solvent was required.

The influence of the pH was studied by adding different amounts of HCl and NaOH. Salicylic acid and difunisial shows an increment on fluorescence intensity up to a value of 4 units, over this the intensity of the salicylic acid remains constant, and difunisial intensity increases up to a value of 7 units and finally shows a decrease on the higher values. The influence of pH on the fluorescence intensity of serum was examined in previous work [29] and found to be nearly constant over the range 2-12. Thus, a pH value of 7.2 was selected as optimal. To adjust the pH value on the working solutions a buffer solution of sodium dihydrogen phosphate was added. The influence of the buffer solution (sodium dihydrogen phosphate) concentration into the fluorescence intensity was studied by adding different amounts of the buffer solution (between 0.02 and 0.3 M) into the measurements solutions.

This study conclusion was that the fluorescence intensity of the salicylic acid is not affected by the buffer concentration on the studied range, and the fluorescence intensity of difunisial increases up to a concentration value of 0.1 M, at higher concentrations the intensity remains constant with a lightly decay. The concentration selected was 0.1 M due to a sufficient buffering capacity.

The fluorescence intensity of difunisial, salicylic acid, and serum showed a decrease when the temperature increased from 5 to 65 °C. A temperature of 20 °C, close to room temperature, was selected for the determination.

The influence of analytes concentration on the fluorescence intensity was studied under these conditions. Salicylic acid shows a linearity response up to a concentration of 5 μg mL⁻¹ and difunisial up to 2.5 μg mL⁻¹.

Finally, a diluted solution was recorded every 15 min and remained stable for at least 2 h.

3.3. Simultaneous determination

As can be seen in Fig. 2, the fluorescence maxima of difunisial and salicylic acid were located in the UV region, where serum exhibits a broad peak that precludes determining the analytes without its prior separation.

In addressing a fluorimetric determination in serum, it is preferable to quantify the analytes at high wavelengths. Based on selectivity, sensitivity and reproducibility criteria, the bands of characteristic wavelengths λₜₐₓ = 258 nm, λₑₘ = 422 nm and

Fig. 1. Isometric representation of the total fluorescence spectra of: serum (dilution 1/100) (A), salicylic acid (500 ng mL⁻¹) (B) and difunisial (600 ng mL⁻¹) (C).

Fig. 2. Total fluorescence spectrum of: serum (1/100) (black), difunisial (600 ng mL⁻¹) (grey; broken line), salicylic acid (600 ng mL⁻¹) (grey; continuous line) and the trajectory selected as optimum (dark black).
\[ \lambda_{\text{exc}} = 296 \text{ nm}, \quad \lambda_{\text{em}} = 408 \text{ nm} \]

were selected to determine diflunisal and salicylic acid, respectively, for the following reasons:

- they resulted in an increased fluorescence intensity for salicylic acid;
- they decreased the fluorescence background of serum, thereby diminishing the internal filter of the matrix;
- the contour lines for serum were apart, so differences in MISF among samples were smaller;
- some interferences were lessened.

From careful inspection of the overlapped contour maps (Fig. 2), a simple isopotential trajectory was selected for the simultaneous determination of diflunisal and salicylic acid. The total fluorescence spectra for diflunisal, salicylic acid and serum are shown as contour lines in Fig. 2. Note the overlap between diflunisal and salicylic acid and the fluorescence background exhibited by the serum at the fluorescence maxima of the two analytes. The isopotential trajectory, also shown in Fig. 2, was obtained from the average of five total fluorescence spectra for as many serum samples. With this synchronous fluorimetric technique, the analytical signal is obtained as difference between the maximum intensity and the constant intensity value to which the trajectory tends asymptotically at both ends of the MISF spectrum. Fig. 3 shows the MISF spectra for a mixture of 600 ng mL\(^{-1}\) salicylic acid, 600 ng mL\(^{-1}\) diflunisal and serum diluted 100 times, as well as those for the individual analytes at the same concentration levels in serum. The fact that the fluorescence reaches a constant value on the right end allows one to determine these drugs by measuring the maximum fluorescence intensity with respect to the initial end of the selected trajectory. However, spectral overlap between diflunisal and salicylic acid precludes their determination using the MISF spectrum as such, so its first derivative must be calculated (Fig. 4). Although the first derivative does not resolve the bands, satisfactory results can be obtained by applying the zero-crossing technique. The determination of salicylic acid and diflunisal was made by measuring the derivative signal at the excitation wavelengths of 264 and 249.6 nm, respectively. Obviously, the signal of serum is cancelled as the trajectory is obtained isopotentially.

MISF spectra were derived by using the Savitzky and Golay [27,28] algorithm. Five filter factors with 5, 9, 15, 21 and 25 points of smoothing were employed to this end using two concentration levels for each analyte. Low levels require a filter factor with 21 smoothing points, which provided derivative spectra with adequate signal-to-noise ratios.

3.4. Calibration and statistical analysis of the proposed method

3.4.1. Individual calibrations

Six individual calibrations (three per analyte) were done. Samples for the first individual calibration for each analyte were prepared in the absence of the other, the remaining ones containing it at two different concentration levels. The same procedure was used with the second analyte (Table 1). Each individual calibration was evaluated by statistical analysis of experimental data that were fitted to an \( y = a + bx \) mathematical model using least median of squares regression (LMS) and least-squares regression (LS). LMS [30] allows to determine the presence of outliers in the true line; these outliers cause errors when experimental data are fitted according to the LS regression. LS should be applied once outliers have been discarded by using a robust regression method (LMS). Tables 1 and 2 show the most salient results of the statistical analysis. As can be seen, the slopes of the LMS and LS were all similar to one another. This indicates the absence of outliers, and, also, because of the similarity among LS slopes, one can consider the LS lines for the individual calibrations to be nor different from a global LS line. The intercepts on the \( y \)-axis were negligible throughout the concentration range studied; in fact, Student's \( t \) values smaller than the theoretical ones at the 95% confidence level and also an adequate number of degrees of freedom (Table 2). As can also be seen from tables, determination coefficients were very close unity and standard deviations of the estimates were very small.

3.4.2. ANOVA test for global calibrations

A global LS procedure was used to obtain the most representative calibration graph. The regression line was obtained by
including every data pair and applying LMS and LS procedures. In order to check whether the wavelengths selected for zero-crossing led to the analytical signal for the individual analytes in a mixture of both in serum, variance analysis was used to compare the three calibration lines for each analyte [31]. Two different F statistics were calculated.

\[ F_1(\alpha, 2k - 2, N - 2k) = \frac{\sum_{j=1}^{k}\sum_{i=1}^{n}(Y_{i,j} - \bar{Y})^2 - b^2 \left( \sum_{j=1}^{k}\sum_{i=1}^{n}(X_{i,j} - \bar{X})(Y_{i,j} - \bar{Y}) \right)}{\sum_{j=1}^{k}\sum_{i=1}^{n}(Y_{i,j} - \bar{Y})^2} \]

\[ F_2(\alpha, k - 1, N - 2k) = \frac{\sum_{j=1}^{k}\sum_{i=1}^{n}(Y_{i,j} - \bar{Y})^2 - b^2 \left( \sum_{j=1}^{k}\sum_{i=1}^{n}(X_{i,j} - \bar{X})(Y_{i,j} - \bar{Y}) \right)}{\sum_{j=1}^{k}\sum_{i=1}^{n}(Y_{i,j} - \bar{Y})^2} \]

Thus, \( F_1 \) compared the total deviations from the overall linear region with respect to the deviations within each calibration in order to test the suitability of the single overall regression line. If the experimental \( F_1 \) value was less than its theoretical value, then departure of the individual sets from the overall regression line was not significant. \( F_2 \) compared differences among slopes in relation to the deviations within each calibration and thus tested for the differences between slopes. If the experimental \( F_2 \) value was less than the theoretical one, then there are no significant differences between the individual slopes and the overall regression slope may be taken as the representative one. In all cases the validity of the overall regression lines is proven by the values of experimental \( F_1 \) and \( F_2 \) values (Table 2) which were smaller than the theoretical ones at 95% confidence level. (Theoretical \( F_1(4, 12, 95\%) = 3.26 \); theoretical \( F_2(2, 12, 95\%) = 3.89 \).)

The global regression lines used to determine difunisal and salicylic acid exhibited a uniform variance of residuals. Measurements errors were thus assumed to be independent of the difunisal and salicylic acid concentration, and the calibration graphs homoscedastic [31,32].

Finally, an analysis of variance was used to test for linearity in the calibration graphs was conducted [31,32] by comparing the lack of fit with respect to the pure calibration error. The lack of fit term (tables) was negligible so the model adopted provides an accurate description of the true relationship between the analytical signal and the analyte concentration (i.e., the hypothesis of linearity of calibration can be accepted since the experimental value of the \( F \) statistic was less than the theoretical value at 95% confidence level).

3.4.3. Precision of estimate and limits of detection

In order to estimate the error in a subsequent determination, a series of 10 solutions containing 300 ng mL\(^{-1}\) of salicylic
Table 3

<table>
<thead>
<tr>
<th>NSAID added</th>
<th>Proportion compound added/salicylic acid</th>
<th>Recovery (%)</th>
</tr>
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<tr>
<td>Fenoprofen</td>
<td>1/2</td>
<td>92</td>
</tr>
<tr>
<td>6-MNA</td>
<td>5/1</td>
<td>94</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>1/2</td>
<td>98</td>
</tr>
<tr>
<td>Sulindac</td>
<td>3/1</td>
<td>101</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>4/1</td>
<td>97</td>
</tr>
<tr>
<td>Flxicam</td>
<td>1/2</td>
<td>92</td>
</tr>
<tr>
<td>Nimesalide</td>
<td>5/1</td>
<td>105</td>
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</tbody>
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<table>
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<tr>
<th>Proportion compound added/difunisal</th>
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<tr>
<td>Fenoprofen</td>
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<td>6-MNA</td>
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<td>Flxicam</td>
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<td>Nimesalide</td>
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acid and 300 ng mL\(^{-1}\) of difunisal in 1:100 serum was prepared under the same conditions as the calibration standards. The proposed method was applied and the absolute values of the first derivative MISF scan were measured at \(\lambda_{exci}/\lambda_{em} \) 249.6/399.4 and 264/420.8 nm to quantify difunisal and salicylic acid, respectively. On the assumption of error propagation, the resulting standard errors at the 95\% confidence levels were 37 and 11 ng mL\(^{-1}\) for salicylic acid and difunisal, respectively. The respective relative standard deviations for the replicates were 9.1 and 7.7 ng mL\(^{-1}\) for salicylic acid and difunisal, respectively.

The detection limits of the two analytes were studied by applying the different definitions, according to the IUPAC definition [22,23], based only on three times the standard deviation of the blank a detection limit of 7.5 and 9.7 ng mL\(^{-1}\) was obtained for salicylic acid and difunisal, respectively. The errors propagation definition will give a detection limit consistent with the reliability of the blank measurements (IUPAC) and besides the signal measurements of the standards [24,25]. In this case a detection limit of 19.2 and 22.5 ng mL\(^{-1}\), respectively, was obtained. And according to Clayton definitions [26], considers the probability of positive false and negative false, the detection limit being 36.8 and 37.3 ng mL\(^{-1}\), respectively. Because of the limit of detection according to Clayton considers all possible errors described above, it is suitable to assume this type of detection limit.

3.4.4. Study of interferences

Finally, a study of interferences was also performed by adding other NSAID’s into a dilution of the analytes at the same conditions than the calibration dilutions. The concentration used of salicylic acid and difunisal was 250 ng mL\(^{-1}\). In Table 3, the recovery percentage values of the analytes at the maximum concentration of NSAID’s that did not show interferences for the determination of both compounds is shown.

4. Conclusion

A new method for the simultaneous direct fluorimetric determination of difunisal and salicylic acid in serum by matrix isopotential synchronous fluorescence that requires no separation is proposed. The simultaneous determination is accomplished by using a single isopotential trajectory that passes by the maxima in the fluorescence bands of difunisal and salicylic acid in order to ensure a sensitivity level similar to that of a direct determination in absence of background fluorescence. Although the MISF technique suppresses the fluorescence background of serum, the overlap between the difunisal and salicylic acid MISF spectra precludes the simultaneous determination of the two compounds. Satisfactory results, however, can be obtained by measuring the analytical signals at the zero-crossing points in the first derivative of matrix isopotential synchronous scan, namely, \(\lambda_{exci}/\lambda_{em} \) 249.6/399.4 nm for difunisal and 264/420.8 nm for salicylic acid.

The proposed method is a fast, simple and reliable alternative to the simultaneous quantification of these anti-inflammatory drugs in serum at therapeutic concentration levels.

The main advantages of this method compared with the previous reported are as follows:

- aqueous are used throughout the assay, a significant improvement over other methods where the use of organic solvents is required [33,36];
- it can be directly applied to untreated serum samples [6,34,35];
- limits of detection are similar or lower than obtained in the other described methods [8,10].

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