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Combined liquid and solid-surface room temperature fluorimetric determination of naproxen and salicylate in serum

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Abstract

A rapid and sensitive method for the determination of naproxen and salicylate in serum is presented. The employed strategy combines solid-phase extraction on a reverse-phase membrane with spectrofluorimetry. Solid-phase extraction under optimum pH conditions makes NX to be retained over the solid surface (where it is directly determined by a fluorimetric technique). Salicylate passes through the disk and is also fluorimetrically determined, but in solution. The linear calibration ranges for NX in the membrane and salicylate in solution were 0.014–0.250 and 0.010–0.250 $\mu g\,ml^{-1}$, respectively. The lowest value, in each case, is the corresponding limit of quantitation. The performance of the method is demonstrated with the successful determination of both drugs in spiked and real human serum samples.

Keywords: Room temperature fluorimetry; Solid-phase extraction; Naproxen; Salicylate

1. Introduction

Naproxen (NX) [(+)-2-(6-methoxy-2-naphtyl)propionic acid] and salicylate (SA) [2-hydroxybenzoate] (Fig. 1), are two non-steroidal anti-inflammatory drugs with analgesic, anti-inflammatory and antipyretic properties [1].

Serum SA mainly comes from the hydrolysis of the widely used acetylsalicylic acid (aspirin), whose in vivo anti-inflammatory activity has been demonstrated to be essentially due to SA [1]. Comparative studies showed that there are no significant differences as regards the therapeutic efficacy of the studied compounds, but NX is better tolerated than aspirin [2]. The concomitant administration of NX with aspirin was shown to be more effective than of aspirin alone [3]. When anti-inflammatory treatments become chronic, as in the case rheumatoid arthritis, the patients are exposed to the drugs for prolonged time periods. Therefore, the monitoring of serum concentrations is of great value in preventing toxicity.

Several high-performance liquid chromatographic (HPLC) methods have been proposed for the evaluation of NX [4–6], SA [7–10] or both [11,12] in serum or plasma samples. Because of the luminescence properties of both active principles, various analytical methodologies using this type of signals have also been reported [13–19]. The simultaneous control of these drugs in serum by fluorescence techniques presents difficulties, due to their mutual interference and that arising from the matrix. To solve this problem, different approaches have been used, such as second-derivative synchronous spectrofluorimetry [20], or first-order [21,22] and second-order [23] multivariate calibration of fluorescence data. As a new alternative to both chromatographic and the rather complex chemometric methods, we have evaluated a simple and effective approach for the determination of NX and SA in human serum in a single experiment. Specifically, the neutral form of NX is extracted from the sample by using an octadecyl extraction membrane and is fluorimetrically determined on the solid surface. The un-retained SA is then spectrofluoriemtrically determined in the sample extract.

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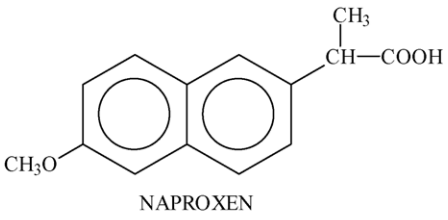

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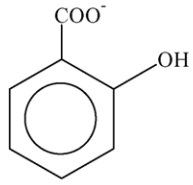

SALICYLATE

Fig. 1. Structures of the studied compounds.

Solid-phase extraction (SPE) typically refers to a procedure that concentrates and purifies analytes from solution by retention onto a solid phase, followed by elution of the analyte with an adequate solvent for instrumental analysis (generally, liquid and gas chromatographies). However, in the present case, the solid-surface serves as a retention material for NX and as support for its fluorescence determination. Obviously, the elution step mentioned above is not necessary. Recently, the phosphorimetric determination of NX on a substrate filter paper was carried out [24]. However, to the extent of our literature search, NX has not been fluorimetrically determined in a solid-phase. In general, methods involving solid-phase spectrofluorimetry compare very favourably with other analytical techniques in terms of sensitivity and selectivity [25–28], and we have successfully applied this methodology for the determination of piroxicam in pharmaceutical formulations and serum [29,30]. According to our experience, the procedure is of low-cost and very simple manipulation, and represents a promising methodology for trace analyses.

2. Experimental

2.1. Reagents and solutions

Analytical-reagent grade chemicals were used in all experiments. Stock solutions (approximately 4000 $\mu g\,ml^{-1}$) of naproxen sodium salt (Sigma, St. Louis, MO, USA) and sodium salicylate (Merck, Darmstadt, Germany) were prepared by dissolving the adequate weight of each compound in doubly distilled water. From these concentrated solutions, 20.0 $\mu g\,ml^{-1}$ working solutions were prepared. A buffer solution of acetate/acetic acid of pH 4.0 was prepared in a 250 ml volumetric flask by adding HCl 1.0 M to 6.6 g of sodium acetate (Cicarelli, San Lorenzo, Argentina) and diluting to the mark with water. Methanol was purchased from Merck (Darmstadt, Germany).

2.2. SPE-solid-surface fluorescence

The solid-phase extraction was performed with SPEC® (solid-phase extraction concentrator) octadecyl (C18 AR) disks, which are indicated for retaining non-polar compounds. These membranes were obtained from Ansys Diagnostics (Lake Forest, CA, USA). Membranes (47 mm) were dissected into 13 mm disks. The smaller disks were loaded into a 13 mm stainless steel filter syringe kit (Alltech, Deerfield, IL, USA) and placed into a 5 ml syringe (Hamilton, Reno, NV, USA). Prior to sample application, the membrane was conditioned with 1 ml of methanol. Positive pressure was used to force all liquid solutions (5 ml) through the membrane in approximately 30 s per sample. This flow rate is in the optimum range for maximum breakthrough volume (10–30 ml min^{-1}) [31]. Following the NX extraction, the sample was dried by forcing two 25 ml volumes of air through the disk using a 25 ml syringe (Hamilton, Reno, NV, USA). The disk was then placed in a laboratory-made SPE membrane holder [32] and the fluorescence spectra were collected at 90°, irradiating at 272 nm and obtaining the emission at 350 nm. The SA content was spectrofluorimetrically investigated in the un-extracted solution, using 1.00 cm quartz cells, slit widths of 4 nm, excitation at 297 nm and fluorescence emission obtained at 405 nm. The temperature was maintained at 20 °C. Spectrofluorimetric measurements were performed using an Aminco Bowman (Rochester, NY, USA) Series 2 luminescence spectrometer equipped with a 150 W xenon lamp, a 486 personal computer, a GPIB (IEEE-488) interface card for computer instrument communication and a thermostated cell holder. Data acquisition and data analysis were performed by use of the AB2 Series 2 software, running under OS/2 version 4.0.

2.3. Calibration curves

Different dilutions of the 20.0 $\mu g\,ml^{-1}$ NX standard solution with acetate buffer (pH 4.0) were made in order to obtain concentrations in the range 0–0.250 $\mu g\,ml^{-1}$. Then, SPE and fluorescence measurements were performed by the procedure described above. Independent calibration solutions of SA were prepared by appropriate dilutions of the stock solution with acetate buffer (pH 4) in order to achieve concentrations in the range 0–0.250 $\mu g\,ml^{-1}$. The fluorescence intensities of the latter solutions were directly measured. Each calibration point was performed by triplicate in all cases. Exploratory determinations were performed over artificial aqueous samples prepared with different concentrations of both NX and SA, in the corresponding ranges mentioned above, following a random design.

2.4. Serum samples

It was verified that a maximum plasma concentration of SA following a single oral administration of 500 mg of aspirin is reached between 2 and 3 h, with values around 30 $\mu g\,ml^{-1}$

[9]. However, toxicity often occurs at SA concentrations about 300 μg ml^{-1} [33]. On the other hand, mean values of maximum plasma NX concentration under therapeutic doses are between 40 and 65 μg ml^{-1}, depending on the treatment days [34]. Overdoses of NX produce plasma concentrations larger than 200 μg ml^{-1} [1].

For serum analysis, a set of eleven samples was prepared by spiking normal human sera with both NX and SA aqueous solutions in order to obtain maximum concentrations which are near to those known to cause overdose or toxicity. Nine of these samples corresponded to the concentrations provided by a full factorial design at three levels. This design is described as a 3^k-design, where the base 3 stands for the number of factor levels (the analysed concentrations) and k expresses the number of compounds. The three tested NX concentrations were: 97, 181 and 300 μg ml^{-1}, and those corresponding to SA were: 101, 181 and 309 μg ml^{-1}. The remaining two samples contained only one of the studied analytes at an intermediate concentration (C_{NX} = 213 μg ml^{-1} and C_{SA} = 181 μg ml^{-1}). The extraction of the studied analytes from serum into methanol was tested and a quantitative recovery was corroborated. Thus, a volume of 40.0 μl of a given spiked serum sample was placed in a capped flask and 1.00 ml of methanol was added. The tube was shaken and centrifuged (10 min at 2500 × g). An aliquot of 100.0 μl of the supernatant was transferred to a flask, evaporated by use of dry nitrogen, and 5 ml of acetate buffer were added to the flask in order to obtain final concentrations for NX and SA which were within the linear ranges for the analytes. It is important to point out that these series of operations took only a few minutes. Each serum sample was prepared in triplicate. The corresponding fluorescence spectra were read in either surface or solution for the NX and SA determination, respectively.

The concentrations of both NX and SA in unknown samples were corroborated by high-pressure liquid chromatography (HPLC) following the chromatographic conditions suggested in the literature [11]. HPLC was carried out on a liquid chromatograph equipped with a Waters (Milford, MA, USA) 515 HPLC pump and a UV detector. A 20.0 μl loop was employed to spread the sample on to a Zorbax SB C18 column (5 μm average particle size, 150 mm × 4.6 mm i.d.). Detections were at 254 and 297 nm for NX and SA, respectively.

For these measurements, samples were prepared as follows: 200.0 μl of serum sample were treated with 1.00 ml of methanol and 50.0 μl of $HClO_4$ (18%) and, after 2 min vortexed, it was centrifuged (10 min at 2500 × g). The supernatant was separated, filtered and 20.0 μl were injected into the HPLC column under the reference conditions.

3. Results and discussion

In a previous paper, the overlapping between the fluorescence emission for a normal human serum, NX and SA at neutral pH was demonstrated [23]. This fact, in principle, hinders the direct fluorimetric determination of the investigated compounds in this type of matrix. As was previously verified, this problem could be overcome by applying strategies involving chemometric tools [23]. However, the physical separation is an interesting alternative for workers not familiarised with chemometric analysis. Thus, the performance of solid-phase extraction prior to fluorescence detection was investigated.

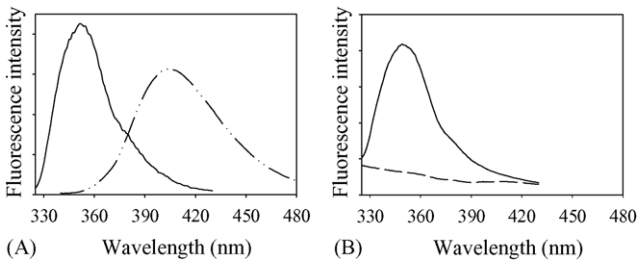

Fig. 2. Fluorescence spectra for (A) aqueous solutions of (—) NX and (– · · –) SA (C_{NX} = C_{SA} = 0.150 μg ml^{-1}, pH 4.0, λ_{ex} (NX) = 272 nm, λ_{ex} (SA) = 297 nm, PMT sensitivity = 650 V); and (B) for (—) NX contained in a diluted serum sample and extracted on a C18AR membrane and (– –) the background membrane signal (C_{NX} = 0.050 μg ml^{-1}, PMT sensitivity = 300 V), λ_{ex} = 272 nm.

3.1. Naproxen solid-phase extraction and salicylate in the sample extract

The ionization state of the analyte in the working solution is essential for either the adsorption or elution from the solid surface used as support. Because of the nature of the selected membrane (reversed-phase) for the adequate retention of NX, this compound must be in its neutral form. According to the acidity constant value of NX (pK_a = 5.09 [19]), below pH 5 the carboxylic group of NX is mostly protonated, leading to an uncharged molecule able for retention on the mentioned membrane.

Fig. 2 shows the fluorescence spectra of NX both in aqueous solution and adsorbed on the extraction surface. When compared to the analyte signal on the membrane, the background signal of the disk is low, allowing NX determination at low concentration levels. The comparison of this spectrum with that in aqueous solution shows that the SPE membrane does not promote significant changes in the fluorescence emission of NX.

On the other hand, the other studied compound (salicylic acid, pK_{a1} = 2.80, pK_{a2} = 13.4 [35]) must be in its charged form for a complete passage through the disk without retention. The study of the fluorescence intensity dependence of salicylic acid with the pH was previously investigated [36]. The results indicated that a high and pH-independent fluorescence emission is attained above pH 4, where salicylic acid is present in solution in its deprotonated (negatively charged) form. The C18AR membrane does not retain the anionic form and therefore SA can be spectrofluorimetrically determined in the un-extracted solution at the same pH used

Table 1
Fluorescence analytical figures of merit of NX in C18AR membrane and SA in solution (pH 4.0, $t = 20\,^{\circ}C$)[a]

	NX	SA
Linear range ($\mu g\,ml^{-1}$)	0.014–0.250	0.010–0.250
Correlation coefficient (r)	0.999	0.999
h[b]	5.8 (0.4)	1.1 (0.2)
m[c]	503 (3)	373 (2)
γ[d] (μg^{-1} ml)	473	648
LOD[e] ($\mu g\,ml^{-1}$)	0.005	0.003
LOQ[f] ($\mu g\,ml^{-1}$)	0.014	0.010
R.S.D.[g] (%)	0.7	0.6

[a] The number of data for each calibration curve corresponds to nine different concentration levels, with three replicates for each level ($n = 27$).
[b] Intercept (standard deviation within parenthesis).
[c] Slope (standard deviation within parenthesis).
[d] Analytical sensitivity: $\gamma = m/Ss$, where Ss is the standard deviation of the regression residuals [42].
[e] Limit of detection: calculated according to Clayton et al. [43] using 0.05 as assurance probabilities.
[f] Limit of quantitation.
[g] Relative standard deviation: $C_{NX} = C_{SA} = 0.150\,\mu g\,ml^{-1}$ (five replicates were measured in each case, in different days).

for NX separation (Fig. 2). Thus, pH 4.0 was selected as the working pH, which is easily obtained by using an appropriate acetate/acetic buffer solution.

Both the complete retention of NX on the disk and the total elution of SA in the non-extracted solution were confirmed by comparing the fluorescence intensities of the solutions before and after the extraction procedure. For this experiment, solutions containing either NX or SA at concentrations within the linear range of the calibration curve were prepared and analysed. It is important to indicate that the sample with the highest concentration of NX ($0.250\,\mu g\,ml^{-1}$) contains 1.25 μg of this compound, and therefore it does not exceed the extraction capacity of the C18 membrane (about 1–10%, w/w) [37]. The recoveries of NX and SA from solutions containing four different concentration levels (0.016, 0.050, 0.150 and $0.250\,\mu g\,ml^{-1}$) of each analyte ranged from 99.4 to 100.0% for NX (with standard deviations in the range 0.1 to 0.3%, calculated on triplicate samples), and from 96 to 99% for SA (with standard deviations in the range 1 to 2%, calculated on triplicate samples). Using the latter information, a statistical analysis showed no significant differences, based on a t-test for comparing (at 95% confidence level) the ideal 100% recovery with each of those experimentally obtained.

3.2. Analytical figures of merit

Table 1 displays the analytical figures of merit calculated for NX on the extraction membrane and SA in liquid solution. The linearity for each curve was tested by an ANOVA method [38]. As can be observed, the analytical parameters are of high quality, with special attention to γ (analytical sensitivity) values and limits of detection and quantitation. Table 2 summarises limits of detection previously described for NX by using different methodologies. The method presently proposed compares very favorably with previous ones, taking into account that in our case the rigorous Clayton definition was applied for limits of detection calculation. In Table 3, results are shown as regards a preliminary quantitative study carried out with different binary synthetic samples. A rapid inspection of the values obtained seems to indicate that the used strategy yields good results. In order to gain further insight into the accuracy of the method, linear regression analysis of nominal versus found concentration values of both compounds was applied. The estimated intercept and slope ($\widehat{a}$ and $\widehat{b}$, respectively) were compared with their ideal values of 0 and 1 using the elliptical joint confidence region (EJCR) test [39] (Fig. 3).

As can be seen, the EJCR contains the theoretical ($a = 0$, $b = 1$) point, concluding that the proposed approach yields statistically accurate results.

Table 2
Limits of detection (LOD) for NX using different methods of determination

Technique[a]	LOD ($\mu g\,ml^{-1}$)	Reference
SF	0.03[b]	[21]
MS–RTF	0.004[c]	[14]
MS–RTP	0.014[b]	[18]
CS–RTP	0.03[d]	[19]
HPLC–UV	0.05[e]	[4]
HPLC–DA	0.05[e]	[4]
HPLC–F	0.02[e]	[4]
GC–MS	0.02[e]	[4]
SS–RTP	0.0027[b]	[24]
SS–RTF	0.005[d]	This work

[a] SF: spectrofluorimetry; MS–RTF: micelle-stabilised room-temperature fluorescence; micelle-stabilise room-temperature phosphorescence; CS–RTP: cyclodextrins-stabilised room-temperature phosphorescence; HPLC–UV: high-performance liquid chromatography–ultraviolet detection; HPLC–DA: high-performance liquid chromatography–diode-array detection; HPLC–F: high-performance liquid chromatography–fluorescence detection; GC–MS: gas chromatography–mass spectrometry, RTP: solid-surface room-temperature phosphorescence; SS–RTF: solid-surface room-temperature fluorescence.
[b] Calculated as a signal:noise ratio = 3.
[c] Calculated according to Long and Winefordner [44].
[d] Calculated according to Clayton et al. [43].
[e] Method for calculation not indicated.

Table 3
Determination of NX and SA in binary artificial mixtures[a]

	Actual concentration ($\mu g\,ml^{-1}$)	Found concentration ($\mu g\,ml^{-1}$)	Recover (%)
NX	0.032	0.030 (0.001)	94
SA	0.206	0.203 (0.001)	99
NX	0.100	0.105 (0.009)	105
SA	0.150	0.149 (0.004)	99
NX	0.150	0.153 (0.003)	102
SA	0.100	0.101 (0.001)	101
NX	0.250	0.250 (0.002)	100
SA	0.032	0.030 (0.001)	94
NX	0.250	0.251 (0.001)	100
SA	0.206	0.202 (0.004)	98

[a] Standard deviation (average of three replicates) in parenthesis.

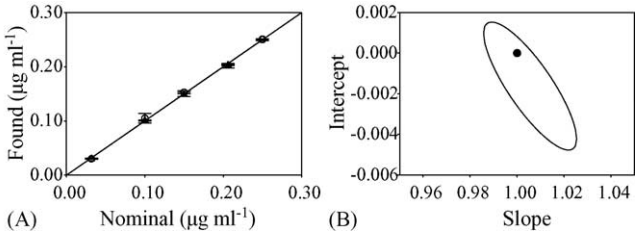

Fig. 3. (A) Plot of the NX (◯) and SA (Δ) concentrations predicted by the proposed method in binary artificial samples, as a function of the nominal values. Error bars indicate the standard deviations of the triplicate sample analysis (see Table 3). The solid line is the perfect fit. (B) Elliptical joint region (at 95% confidence level) for the slope and intercept of the weighted least-squares regression of the data shown in plot (A). The circle marks the theoretical ($a = 0$, $b = 1$) point.

3.3. Effect of foreign species

To evaluate the potential effect of other compounds, a systematic study of the effect produced by these species on the determination of serum samples containing both NX and SA was carried out. The investigated potential interferences were other anti-inflammatory drugs, analgesics and vitamins which could be found in the serum of patients under anti-inflammatory therapy.

The detection of NX might be susceptible to the interference of analytes concomitantly retained by the membrane at the working pH. These interferences can either increase the resulting signal (if they emit near the NX signal), or produce a less intense signal by inner filter effect. On the other hand, SA might be sensitive to the interference of compounds not-retained by the disk, which can also fluoresce itself or decrease the SA signal by inner filter mechanism. Compounds partially retained on the membrane should be investigated both in the solid surface and in solution. Therefore, the ionization state at the working pH and the absorption and emission wavelengths of each compound was taken into account.

This study was performed by adding known amounts of each tested species to a solution containing 0.150 μg ml^{-1} of both NX and SA. The amounts were such that the relationship (μg ml^{-1} foreign species/μg ml^{-1} NX or SA) ranged up to a value of 50. If interference occurred, the concentration of the foreign species was progressively reduced until the interference ceased. Tolerance was defined as the relative amount of interfering species that produced an error not exceeding ±2.1 and ±1.8% in the determination of NX and SA, respectively. These latter values represent three times the relative standard deviation (R.S.D.) corresponding to each analyte (see Table 1).

Table 4 collects the results obtained. An inspection of this Table indicates that the lower tolerance values for NX correspond to mefenamic acid, piroxicam, caffeine and cyanocobalamin, whose serum concentrations at toxic levels are 25, 24, 15 and >0.0014 μg ml^{-1}, respectively [40]. Therefore, if the dilution produced by the serum treatment indicated in the experimental section is taken into account, one can conclude that these compounds do not constitute a real interference. A similar conclusion is obtained when the interferences on the SA determination are analyzed. The serum concentration value of pyridoxine at a toxic level (0.0018 μg ml^{-1} [40]) is significantly lower than the accepted tolerance.

3.4. Validation and application of the method

3.4.1. Spiked serum samples

Table 5 shows the nominal and predicted NX and SA concentrations in spiked serum samples. The absence of interference from the matrix was confirmed by the very good recoveries. The EJCR test was also successfully applied (Fig. 4). As was mentioned in the experimental section, the monitoring of drugs such as anti-inflammatories becomes of special interest under situations where overdose is a health risk. Therefore, the suggested protocol was designed to ensure that samples with the highest concentrations could be appropriately diluted, in order to be measured within the linear ranges of the corresponding calibration curves. This was obtained by working with a few microlitters of serum, and

Table 4
Effect of foreign species on the determination of 0.150 μg ml^{-1} of NX and SA

Foreign species	Therapeutic action	pK_a's	λ_{abs} or λ_{ex} (nm)	λ_{em} (nm)	Interference/NX[a] (μg ml^{-1}/μg ml^{-1})	Interference/SA (μg ml^{-1}/μg ml^{-1})
Ibuprofen	Anti-inflammatory-analgesic	4.12	262	288	10	50[b]
Mefenamic acid	Anti-inflammatory-analgesic	4.2	279, 350	413	0.1	50[b]
Piroxicam	Anti-inflammatory-analgesic	1.81, 5.12	320	340	0.1	[c]
Paracetamol	Analgesic	9.91	240		50[b]	[c]
Dextropropoxyphene	Analgesic	6.3	257		[d]	50[b]
Phenylephrine	Sympathomimetic agent	9.02, 10.1	277	300	[d]	50[b]
Caffeine	Central nervous stimulant	3.60	270		0.9	50[b]
Pyridoxine	Vitamin	4.94, 8.89	285	392	[d]	0.2
Cyanocobalamin	Vitamin		351, 276		0.5	50[b]
Ascorbic acid	Vitamin	4.03, 10.95	243		50[b]	50[b]

[a] The concentration of NX corresponds to the solution before the extraction step.
[b] Maximum tested tolerance level.
[c] Compound completely retained on the membrane.
[d] Compound not retained on the membrane.

Table 5
Determination of NX and SA in spiked and real serum samples[a]

Spiked samples	Nominal concentration ($\mu g\,ml^{-1}$)	Found by the proposed method ($\mu g\,ml^{-1}$)
NX	213	220 (1) [103]
SA	0	0 (0.5) [100]
NX	181	173 (1) [96]
SA	181	175 (1) [97]
NX	300	312 (4) [104]
SA	309	302 (1) [98]
NX	97	92 (2) [95]
SA	101	98 (3) [97]
NX	0	0 (0.5) [100]
SA	181	176 (1) [97]
NX	181	181 (3) [100]
SA	309	303 (8) [98]
NX	181	177 (4) [98]
SA	101	95 (1) [94]
NX	300	292 (1) [97]
SA	181	178 (1) [98]
NX	97	97 (4) [100]
SA	309	298 (4) [96]
NX	300	300 (2) [100]
SA	101	94 (1) [93]
NX	97	99 (0.5) [102]
SA	181	186 (0.5) [103]
Real samples	Found by HPLC ($\mu g\,ml^{-1}$)	
NX	42 (2)	42 (1)
SA	30 (4)	31 (1)
NX	39 (1)	38 (1)
SA	32 (2)	33 (1)

[a] Standard deviation (average of three replicates) in parenthesis. Recoveries (in %), are given within squares brackets.

making a significant dilution of the sample. In other words, the concentration ranges in which the analytes can be determined in serum following the indicated treatment are: 18–325 and 13–325 $\mu g\,ml^{-1}$ for NX and SA, respectively. These figures follow from conversion of the quantitation limits and upper linear values, respectively, taken into account the dilution factor. Although these ranges also cover values found under normal therapeutic doses [21], screening serum samples containing these and lower levels would be better done by reducing the degree of dilution discussed above. In these latter cases, very low concentrations (in the order of 0.02 $\mu g\,ml^{-1}$) could in principle be measured in serum. If this statement is taken into account, it can be claimed that the proposed method satisfactorily compares with the sensitivity of other described techniques. Limits of detection ranging between 0.01–0.1 $\mu g\,ml^{-1}$ [4,7,8], and quantitation of 0.1–4.3 $\mu g\,ml^{-1}$ [6,9,10] have been reported for the studied compounds using HPLC. On the other hand, limits of detection of 0.03 and 0.05 $\mu g\,ml^{-1}$ for NX and SA, respectively, have been described using techniques involving multivariate calibration [21].

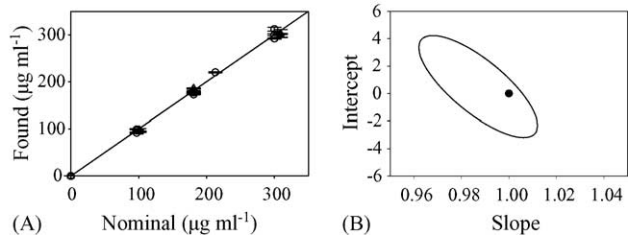

Fig. 4. (A) Plot of the NX (◯) and SA (Δ) concentrations predicted by the proposed method in serum samples, as a function of the nominal values. Error bars indicate the standard deviations of the triplicate sample analysis (see Table 5). The solid line is the perfect fit. (B) Elliptical joint region (at 95% confidence level) for the slope and intercept of the weighted least-squares regression of the data shown in plot (A). The circle marks the theoretical ($a = 0$, $b = 1$) point.

3.4.2. Real serum samples

With the purpose of evaluating the proposed method in real samples, two different sera of patients administered with single oral doses of both 250 mg of NX and 500 mg of aspirin were investigated. In this case, an HPLC method was used as a reference method [11]. The values obtained, summarised in Table 5, indicate that both methods (spectrofluorimetric and chromatographic) yield results in mutual agreement when tested using suitable statistical procedures [41].

4. Conclusions

The fluorimetric determination of NX and SA in serum appears to be viable using a combination of solid-phase and solution fluorescence. Only a single experiment of simple manipulation is required to quantify these compounds in a wide range of concentrations and with good sensitivity. The selectivity of the reverse-phase membrane is coupled to that present by fluorimetric methods. In addition, both the short analysis time and low solvent consumption considerably reduce the analysis cost for numerous samples.

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