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Sensitive synchronous spectrofluorimetric methods for the determination of naproxen and diflunisal in serum

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Abstract The interaction of diflunisal and naproxen with several surfactants was studied. Spectrofluorimetric methods were developed for the determination of both drugs in sodium dodecylsulfate micellar medium. The mixture of these drugs was resolved by synchronous fluorescence spectrometry using two scans. At $\Delta\lambda$ = 20 nm, only naproxen yields a detectable signal that is unaffected by the presence of diflunisal. At $\Delta\lambda$ = 110 nm the signal of diflunisal is not influenced by the presence of an up to 3-fold excess of naproxen. Mixtures containing naproxen/diflunisal in ratios from 50:1 to 1:50 were analyzed with good results. The linear calibration ranges of both drugs were ca 0.02–2.0 μg mL^{-1}. The method has satisfactorily been applied to a mixture of both drugs in serum.

Introduction

Naproxen [NAP: (+)-2-(6-methoxy-2-naphthyl) propionic acid] and diflunixal [DIF: 2-hydroxy-5-(2, 4-difluorophenyl) benzoic acid] are non-steroidal anti-inflammatory drugs, which have primarily been used as analgesics.

Binary drug mixtures are increasingly used in the therapy of a variety of diseases [1]. The simultaneous control of these pharmaceuticals in biological fluids may present some difficulties due mainly to their similar analytical properties and the interference from the matrix. Gas-liquid chromatography and high-performance liquid chromatography are the most widely recommended techniques, although most of these procedures are time-consuming and requiring sample pretreatment prior to measurement [2, 3].

Several fluorimetric methods have been applied to the individual determination of NAP and DIF based on their intrinsic fluorescence [4, 7] or on derivative reactions [8]. Synchronous fluorescence spectrometry was used to analyze mixtures of salicylic acid and NAP or DIF. The resolution of these binary mixtures was accomplished using partial least squares multivariate calibration [9] and second-derivative synchronous fluorimetry [10, 11]. However, no reports have been found concerning the simultaneous determination of both compounds,. Therefore, the development of a method allowing the accurate determination of NAP and DIF in biological fluids could be considered of clinical value.

In the present paper, a non-chromatographic method for the determination of NAP and DIF in serum is reported. It is based on the intrinsic fluorescence of both compounds in sodium dodecylsulfate micellar medium. Synchronous spectrofluorimetry permitted the most accurate determination of NAP, whereas first-derivative synchronous fluorescence spectrometry was selected for the determination of DIF.

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Experimental

Reagents

The chemicals used for the preparation of the solutions were of the highest grade available. Demineralized water from a Milli-Q system (Millipore-Iberica, Madrid, Spain) was used for the preparation of the solutions.

Aqueous stock solutions of 250 μg mL^{-1} NAP and DIF (Sigma, Madrid, Spain) were prepared at pH 12. Working standard solutions were prepared by suitable dilution with demineralized water.

Stock solutions of 25 μg mL^{-1} NAP and DIF were also prepared in 0.1 mol L^{-1} sodium dodecylsulfate (SDS) (Merck, Darmstadt, Germany). Working standard solutions were prepared by appropriate dilution with 0.1 mol L^{-1} SDS.

Stock solutions of 0.5 mol L^{-1} sodium dodecylsulfate (SDS), 0.5 mol L^{-1} cetyltrimethylammonium bromide (CTAB), 0.01 mol L^{-1} cetylpyridinium bromide (CPB), 1% (w/v) Brij-35, 0.2% (w/v) Triton X-100 (Tx100), 0.2% (w/v) polyvinyl alcohol (PVA), 0.05 mol L^{-1} α-cyclodextrin (α-cydex) and 0.05 mol L^{-1} β-cyclodextrin (β-cydex) were prepared by dissolving the required amount in water.

Apparatus

An SLM-Aminco Bowman (Urbana, IL., USA) Series 2 spectrofluorimeter fitted with a 150 W continuous xenon lamp and inter-

faced to a PC 386 microcomputer was used for the collection of all fluorescence spectra and intensity measurements. Data acquisition and data analysis were performed with AB2 software version 2.0 running under OS/2 2.0. The software provides mathematical manipulation of the spectra and calculates first and second derivatives by the simplified least-squares procedure of Savitzky and Golay [12]. A spectral band-pass of 4 nm was set for excitation and emission monochromators. For synchronous fluorescence measurements, both excitation and emission monochromators were locked together and scanned simultaneously at a rate of 2 nm s^{-1}. All measurements were performed in a 10-mm quartz cell at 25° C, using a thermostatic cell holder and a Colora (Frankenthal, Germany) thermostatic bath.

Procedures for the determination of NAP-DIF mixtures

Method A. A sample volume containing NAP (0.2–20 μg) and DIF (0.2–23 μg) was diluted in a 10 mL calibrated flask with 0.1 mol L^{-1} SDS. For each sample two synchronous spectra were obtained by scanning both monochromators simultaneously at constant wavelength differences of $\Delta\lambda$ = 20 nm (λ_{ex} = 290–360 nm, λ_{em} = 310–380 nm) and $\Delta\lambda$ = 110 nm (λ_{ex} = 260–370 nm, λ_{em} = 370–480 nm) for NAP and DIF, respectively. Hereafter all wavelengths referring to synchronous spectra are taken as equal to those of the corresponding emission wavelength. The fluorescence intensity measurements were made at the synchronous maxima of each compound: for NAP $\lambda^0_{s,em}$ = 351 nm ($\Delta\lambda$ = 20 nm) and for DIF $\lambda^0_{s,em}$ = 413 nm ($\Delta\lambda$ = 110 nm). This method is only useful for molar ratios NAP/DIF less than 3:1.

Method B. A sample volume containing NAP (0.2–20 μg) and DIF (0.2–23 μg) was diluted in a 10 mL calibrated flask with 0.1 mol L^{-1} SDS. NAP was determined following the procedure described in method A. The first-derivative synchronous spectra were recorded by scanning both monochromators simultaneously with a constant 110 nm difference between them. DIF was determined through the derivative signal obtained by measuring the vertical distance to the base line at 435 nm.

Determination of NAP and DIF in serum. Serum samples were spiked with appropriate amounts of NAP and DIF to obtain a final concentration of these compounds within their therapeutic range (20–250 μg mL^{-1}). A volume of 400 μL of each sample was placed in a test-tube and 1 mL of 0.1 mol L^{-1} trichloroacetic acid and 1 ml of 0.2 mol L^{-1} SDS were added. The mixture was vortexed for 2 min and centrifuged for 3 min at 4000 g. A volume of 500 μL of the supernatant was analyzed following the above described procedure for determining NAP and DIF in method B.

Results and discussion

Aqueous alkaline solutions of DIF show an intense intrinsic fluorescence with excitation maxima at 272 and 310 nm and an emission maximum at 420 nm [13]. The fluorescence properties of NAP in alkaline aqueous solution have also been reported, and the excitation maxima are centered at 275 and 330 nm and the emission maximum at 358 nm [14].

To ascertain whether the determination of a DIF-NAP mixture using their native fluorescence was possible, the spectral characteristics of DIF and NAP in different micellar media were studied. The results obtained (Table 1) showed that both compounds yield the best luminescence in SDS micellar solution. In all cases, relatively high surfactant concentrations (at least three times the c.m.c.) are required to achieve maximum fluorescence intensity. However, none of the micellar media is suitable for the determination of the DIF-NAP mixture by conventional fluorimetry.

The influence of pH on the fluorescence intensities of DIF and NAP in 0.1 ml L^{-1} SDS micellar medium was studied using universal buffer solutions over the pH-range 2–12. The intensities remained constant over this pH-range.

The extent of the spectral overlap of these compounds was examined by obtaining their total spectrofluorimetric information available in the excitation-emission matrix. The two-dimensional (contour plots) of the fluorescence spectrum of DIF and NAP in SDS micelles are shown in Fig. 1. The parallel diagonal lines superimposed on the

Table 1 Fluorescence data for DIF and NAP in various micellar media[a]

Drug	Surfactant	λ_{ex}/nm	λ_{em}/nm	I_F
DIF	SDS (0.1 mol L^{-1})	286; *314*	421	1.9
	CTAB (0.05 mol L^{-1})	288; *314*	417	1.1
	CPB (0.01 mol/L^{-1})	285; *313*	421	1.4
	Triton X-100 (0.2% w/v)	286; *314*	421	0.8
	PVA (0.2% w/v)	286; *314*	420	1.0
	Brij-35 (0.01 mol L^{-1})	286; *314*	421	0.7
	α-cyclodextrin (0.02 mol L^{-1})	314	421	1.0
	β-cyclodextrin (0.02 mol L^{-1})	314	421	0.9
	Ethanol-water (1 : 1 v/v)	316	418	1.5
NAP	SDS (0.1 mol L^{-1})	236; 279; *331*	354	3.6
	CTAB (0.05 mol L^{-1})	236; 275; *335*	355	2.2
	CPB (0.01 mol L^{-1})	236; 279; *331*	355	1.9
	Triton X-100 (0.2% w/v)	236; 279; *331*	354	1.5
	PVA (0.2% w/v)	236; 279; *331*	354	2.1
	Brij-35 (0.01 mol L^{-1})	236; 278; *331*	354	1.1
	α-cyclodextrin (0.02 mol L^{-1})	331	356	2.0
	β-cyclodextrin (0.02 mol L^{-1})	331	356	1.8
	Ethanol-water (1 : 1 w/v)	332	355	3.1

[a] Values in italics are maximum excitation wavelengths

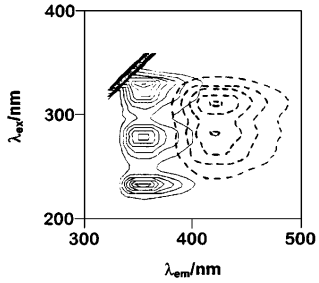

Fig. 1 Contour plot of the excitation-emission matrix in SDS micellar medium of DIF (*dashed line*) and NAP (*solid line*)

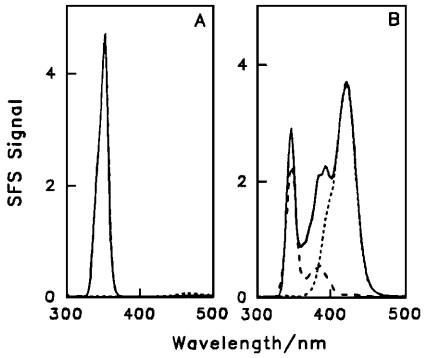

Fig. 2 A, B Synchronous fluorescence spectra of DIF (*dotted line*), NAP (*dashed line*) and their mixture (*solid line*) in 0.1 mol L^{-1} sodium dodecylsulfate. $C_{DIF} = C_{NAP} = 6 \cdot 10^{-6}$ mol L^{-1}. (A) $\Delta\lambda$ = 20 nm and (B) $\Delta\lambda$ = 110 nm

contour plots represents the scan paths through the excitation-emission matrix that would be obtained with synchronous scans at the wavelength interval shown.

It is evident that the maximum signal for NAP with the minimum interference from DIF is obtained at $\Delta\lambda$ = 20 nm, which corresponds to the difference between the emission and excitation maxima of NAP. At $\Delta\lambda$ = 110 nm, DIF yields the maximum signal and is not influenced by the presence of NAP. The $\Delta\lambda$ values between 20 and 110 nm are not suitable for measuring NAP-DIF mixtures because of the lower fluorescence intensities for both drugs and their strong spectral overlap. Figure 2 shows the synchronous fluorescence spectra of the NAP-DIF mixture at $\Delta\lambda$ values of 20 and 110 nm. The analytical signals of NAP (at $\Delta\lambda$ = 20 nm) and DIF (at $\Delta\lambda$ = 110 nm) are not dependent on the presence of the other drug. Therefore, $\Delta\lambda$ values of 20 and 110 nm were selected for the determination of NAP and DIF, respectively.

Analytical features

The determination of DIF and NAP in binary mixtures is carried out by synchronous fluorescence spectrometry (SFS) using two scans. The method involves the construction of independent calibration graphs for each component at $\Delta\lambda$ values corresponding to the difference between the main excitation and emission maxima of both compounds. When both monochromators are scanned together with a 110 nm constant difference between them, the concentration of DIF and the fluorescence intensity measured at 423 nm are linearly related over a sample concentration range of 0.02–2.3 μg mL^{-1}. At $\Delta\lambda$ = 20 nm, NAP concentration and the fluorescence measured at 351 nm are linearly related over the range 0.02–2.0 μg mL^{-1}. The correlation coefficients for the standard calibration graph were 0.9992 and 0.9993 (n = 11) for DIF and NAP, respectively. The limits of detection (LOD) and quantification (LOQ) were estimated according to IUPAC recommendations [15]. The LOD were 0.006 and 0.004 μg mL^{-1} and the LOQ were 0.013 and 0.01 μg mL^{-1} for DIF and NAP, respectively.

The proposed method was applied to the determination of DIF and NAP in synthetic mixtures containing different ratios of both drugs. Good recoveries were achieved for the two drugs in NAP/DIF ratios between 3 and 0.02.

Determination of DIF and NAP in binary mixtures

In order to apply the SFS technique to resolve mixtures of DIF and NAP, a detailed study on the influence of the excess of each compound on the analytical signal of the other was performed. The analytical signal of NAP obtained by SFS ($\Delta\lambda$ = 20 nm) is unaffected by the presence of up to a 50-fold excess of DIF (maximum ratio tested). The analytical signal of DIF ($\Delta\lambda$ = 110 nm) is not influenced by the presence of up to a 3-fold excess of NAP, while at larger excesses the signal of DIF increased considerably with increasing NAP concentration. This spectral overlapping can be resolved, however, by differentiation of the synchronous spectra.

The use of first derivative synchronous fluorescence spectrometry (FDSFS) was tried using the most appropriate parameters. A scan speed of 120 nm/min was selected after verifying that this parameter hardly affect the derivative signal.

Figure 3 shows first derivative synchronous fluorescence ($\Delta\lambda$ = 110 nm) spectra of NAP and DIF and a mixture of both drugs. By applying the zero-crossing technique to the first derivative synchronous spectrum of the

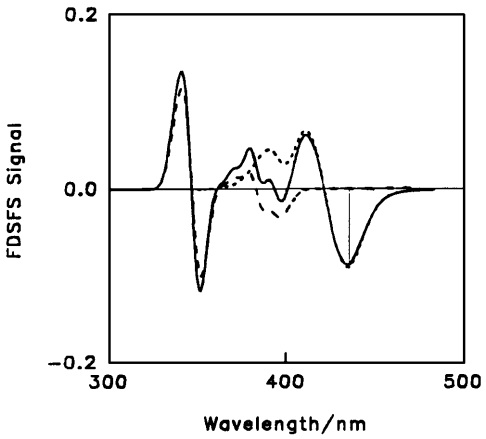

Fig. 3 First derivative synchronous fluorescence spectra of DIF (*dotted line*), NAP (*dashed line*) and their mixture (*solid line*). $C_{DIF} = 1 \cdot 10^{-6}$ mol L^{-1}, CNAP = $5 \cdot 10^{-6}$ mol L^{-1}. $\Delta\lambda$ = 110

mixture, DIF can be determined by measuring the vertical distance to the zero line at 435 nm. The use of FDSFS allows a substantial increase in the DIF:NAP ratio up to 1:50.

Interferences

In order to assess the possible analytical applications of the SFS and FDSFS procedures described above, the influence of foreign compounds that commonly accompany DIF and NAP in real samples was studied by analyzing synthetic sample solutions containing 0.2 μg mL^{-1} of each analyte and various excess amounts of the foreign compound up to 60 μg mL^{-1}. The tolerance ratio of each foreign substance was taken as the largest amount yielding an error less than 5% of the analytical signal of DIF or NAP. Table 2 summarizes the results obtained.

Table 2 Tolerance for different substances in the determination of DIF and NAP

Substance added	Tolerance ratio Interferent: drug[a]	
	NAP	DIF
Saccharine	300[b]	300
Caffeine	300	300
Lysine	300	300
Ethanol	300	300
Polyvinylpyrrolidone	300	300
Polyethyleneglycol	300	300
Lidocaine hydrochloride	100	100
Salicylic acid	5	1

[a] Drug concentration: 0.2 μg mL^{-1}
[b] Maximum ratio tested

Table 3 Determination of DIF and NAP in serum samples

Concentration/μg mL^{-1}				Recovery (%) ± SD	
Added		Found[a]			
NAP	DIF	NAP	DIF	NAP	DIF
18.0	160.0	17.4	155.0	97 ± 3	97 ± 1
54.0	120.0	52.6	121.0	97 ± 2	101 ± 2
90.0	100.0	88.2	98.4	98 ± 1	98 ± 3
150.0	20.0	154.4	18.8	103 ± 1	96 ± 2

[a] Average of three determinations

Serum samples

For the application of the method, pooled serum samples were spiked with different quantities of DIF and NAP, in such a form that their concentration were in the range 20–250 μg mL^{-1}.

The proposed procedure B was then applied as described above. The amounts of each drug present in the binary mixtures were calculated either by interpolation of the working lines or by the standard additions method. The percentage recoveries ranged from 96 to 101 for DIF and from 97 to 106 for NAP.

Conclusions

The study of DIF and NAP in the presence of different micelles revealed that SDS produces the greatest enhancement of the fluorescence of these compound in aqueous solutions.

Synchronous spectrofluorimetry combined with first-derivative fluorescence spectrometry is a useful approach for resolving a DIF-NAP mixture. The proposed method, for example, permits the determination of DIF and NAP in serum. Moreover, the results obtained demonstrate that this procedure can be used for determining a mixture of these drugs wherever simplicity, rapidity and cost-effectiveness are sought as alternatives to chromatography.

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