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Simultaneous determination of naproxen and its desmethyl metabolite in human serum by second-derivative synchronous fluorescence spectrometry

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Abstract

A simple, rapid, sensitive and selective method for the simultaneous determination of the non-steroidal anti-inflammatory drug naproxen (NAP) and its main metabolite the 6-demethylated derivative (DNAP) in human serum is described. The method is based on the intrinsic fluorescence of both acids in alkaline aqueous solution. The broad-band overlapping conventional spectra of both compounds are resolved by means of second-derivative synchronous fluorescence spectrometry, thus obviating the need for pre-analysis sample separation techniques. NAP and DNAP are determined in serum supernatant solution after removal of proteins with acetonitrile at pH 11.7. Recoveries from sera spiked with NAP (5–70 μg ml^{-1}) and DNAP (0.25–5 μg ml^{-1}) ranged from 95 to 106% (mean 99.6%) and from 95 to 104% (mean 100.1%), respectively.

Keywords: Fluorimetry; Second-derivative synchronous fluorescence spectrometry; Naproxen; 6-*O*-Desmethylnaproxen; Serum

1. INTRODUCTION

Naproxen [(+)-6-methoxy-α-methyl-2-naphthaleneacetic acid] (NAP), a nonsteroidal anti-inflammatory drug with analgesic and antipyretic properties, has been widely used in the treatment of rheumatoid arthritis and osteoarthritis. Naproxen is completely absorbed in humans [1]. Its major metabolite in man has been identified as 6-*O*-desmethylnaproxen (DNAP). In plasma, naproxen is the predominant species and only traces of DNAP have been measured [2]. Glucuronide conjugates of NAP and DNAP have been detected in urine only [3]. Analytical methods allowing the simultaneous determination of naproxen and trace amounts of its desmethyl metabolite would be of interest in pharmacokinetic studies of naproxen in serum or plasma.

Among the techniques used for determining NAP in biological fluids only a few allow the simultaneous determination of NAP and its 6-demethylated metabolite. These methods include liquid chromatography (LC) [4], gas–liquid chromatography [5] and thin-layer chromatography with UV detection [6].

In this article a non-chromatographic method for the simultaneous determination of NAP and DNAP added to a single serum sample by second-derivative synchronous (scanning) fluorescence spectrometry (SDSFS) is reported. The method is based on the intrinsic fluorescence of both compounds in alkaline aqueous solutions. By the method described here, NAP and DNAP were determined in serum supernatant after simple deproteination of the sample with acetoni-

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trile. The results obtained show that complete analysis of NAP–DNAP mixtures can readily be achieved by SDSFS, and small amounts of DNAP can be determined reliably in the presence of large amounts of NAP. Studies on samples containing various concomitantly administered drugs were performed in order to demonstrate the specificity of the proposed method.

2. EXPERIMENTAL

2.1. Apparatus

A Model 512 fluorescence spectrometer (Perkin-Elmer, Norwalk, CT), equipped with a 150-W xenon arc lamp and a magnetic stirrer under the cell holder, interfaced to an Amstrad CPC-6128 microcomputer, was used [7,8]. Home-made software [7] was used for spectral acquisition, calculations of the spectrum derivatives and automatic evaluation and presentation of the analytical signals. Smoothed and derivative spectra were defined using the Savitzky-Golay method [9].

2.2. Reagents

All solutions were prepared in deionized, distilled water from reagent-grade materials, unless otherwise stated. Naproxen was obtained from Sigma and 6-desmethylnaproxen was synthesized according to the method described in [5]. Aqueous stock solutions of NAP and DNAP containing 1000 mg l^{-1} and 100 mg l^{-1}, respectively, were prepared at pH 11.6 and working standard solutions were prepared by suitable dilutions. Phosphate buffer (0.05 mol l^{-1}) of pH 11.6 was prepared. Acetonitrile was obtained from Ferak. For recovery experiments normal serum (Ciba-Corning, Quality Control Serum) was used.

2.3. Procedures

Sample treatment

Place 0.2 ml of serum containing 1.0–14 μg of NAP and 0.05–1.0 μg of DNAP into a test-tube. Add 0.4 ml of acetonitrile, vortex the mixture for 1 min and centrifuge for 3 min at 1500 g.

Spectrofluorimetry

Transfer 0.3 ml of the supernatant liquid into the cuvette, add 2.0 ml of phosphate buffer and start the stirrer. Obtain the synchronous fluorescence spectra by scanning both monochromators simultaneously at a constant wavelength difference $\Delta\lambda = 20$ nm ($\lambda_{ex} = 250$–400 nm) and $\Delta\lambda = 180$ nm ($\lambda_{ex} = 200$–350 nm) for NAP and DNAP, respectively. (Hereafter all wavelengths referring to synchronous spectra are taken to be equal to those of the corresponding excitation wavelengths.) Evaluate the derivative signal of NAP, ΔI_{NAP}, within the spectral range 310–334 nm and the signal of DNAP, ΔI_{DNAP}, within the spectral range 228–252 nm. Calculate the concentrations of NAP and DNAP in serum from a calibration graph obtained with control serum standards spiked with NAP (concentration range 5.0–70.0 μg ml^{-1}) and DNAP (0.25–5.0 μg ml^{-1}) and treated similarly. Standard curves based on aqueous NAP or DNAP standards were prepared by mixing one volume of the NAP or DNAP standard solutions with two volumes of acetonitrile and the above procedure for NAP and DNAP was followed.

All instrumental parameters are summarized in Table 1.

3. RESULTS AND DISCUSSION

3.1. Comparison of spectra

Aqueous NAP solutions show a strong intrinsic fluorescence which is not dependent over the pH

Table 1
Instrumental parameters for the determination of NAP and DNAP

Parameter	Compound	
	NAP	DNAP
Slit width/ex., em. (nm)	10, 10	20, 20
$\Delta\lambda$ (nm)	20	180
Savitzky–Golay filter size/points	11	11
Synchronous spectrum scanning range/λ_{ex} (nm)	250–400	200–350
ΔI evaluation range/λ_{ex} (nm)	310–334	228–252

1–14 range [10]. Aqueous DNAP solutions also show an intrinsic fluorescence which strongly increases with increasing pH. The maximum fluorescence intensity occurs at pH > 11. This increase can be attributed to the ionization of the phenolic group. The conventional excitation and emission spectra of NAP and DNAP at various pH are shown in Fig. 1. The ionization constant of the phenolic hydroxyl of DNAP was determined using a potentiometric/ fluorimetric method. The average pK_a value estimated graphically from the spectrofluorimetric data by the method of Bridges et al. [11] was found to be 9.5 ± 0.1. As can be seen from Fig. 1, the emission bands of NAP ($\lambda_{em} = 355$ nm) and DNAP ($\lambda_{em} = 420$ nm) are quite satisfactorily resolved, so as to be useful for the direct and simultaneous determination of both compounds by conventional spectrofluorimetry. However, spectral overlaps may occur in their binary mixtures when one of the components is present in a large excess. Moreover, the strong overlap of the excitation and emission spectra of DNAP and NAP makes it quite difficult to determine simultaneously binary mixtures of these compounds by using conventional spectrofluorimetry. For separating binary mixtures of NAP and DNAP the synchronous scanning approach [12] was used in combination with the second derivatives of the synchronous spectra [13].

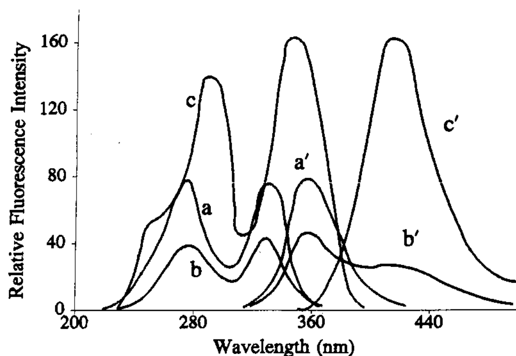

Fig. 1. Fluorescence excitation (a–c) and emission (a′–c′) spectra of aqueous solutions of NAP (pH 7.2 and 11.6), $C = 0.9$ μg ml^{-1}, $\lambda_{ex} = 276$ nm, $\lambda_{em} = 356$ nm (a,a′); DNAP (pH 7.2), $C = 0.9$ μg ml^{-1}, $\lambda_{ex} = 290$ nm, $\lambda_{em} = 420$ nm (b,b′) and DNAP (pH 11.6) (c,c′).

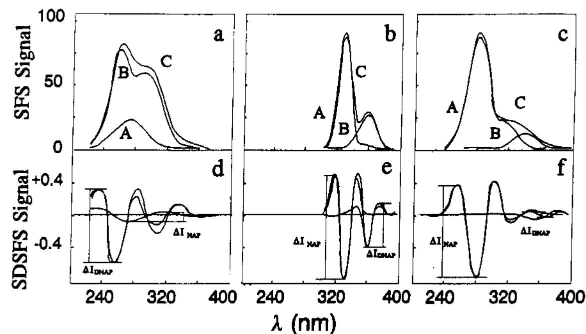

Fig. 2. Synchronous fluorescence spectra (a,b,c) of (A) NAP; (B) DNAP; (C) NAP–DNAP mixture and their second-derivative spectra (d,e,f) in alkaline aqueous solutions (pH 11.6). (a) $C_{DNAP}/C_{NAP} = 1:50$, $\Delta\lambda = 180$ nm, (b) $C_{DNAP}/C_{NAP} = 1:1$, $\Delta\lambda = 20$ nm and (c) $C_{DNAP}/C_{NAP} = 1:20$, $\Delta\lambda = 70$ nm.

Fig. 2 shows the synchronous spectra and their corresponding second-derivatives obtained for NAP, DNAP and NAP–DNAP binary mixtures at different constant wavelength differences ($\Delta\lambda$) between excitation and emission monochromators. These $\Delta\lambda$ values correspond to the differences between the main excitation and emission maxima of both compounds. As can be seen from Fig. 2 the combination of synchronous and derivative fluorescence techniques results in an adequate resolution of the mixtures. It is also shown that better resolution of DNAP from an excess of NAP is obtained at $\Delta\lambda = 180$ nm (Fig. 2a), while NAP could be determined equally well at low DNAP concentrations at $\Delta\lambda = 20$ and 70 nm (Fig. 2b and c).

3.2. Selection of $\Delta\lambda$

In synchronous scanning derivative spectrofluorimetry the choice of the appropriate scanning interval ($\Delta\lambda$) is dictated by the requirements of resolution, sensitivity and features. For this reason a wide range of $\Delta\lambda$ values (20–200 nm) was examined. The position of the minima of the second-derivative spectrum and the signals for NAP and DNAP as functions of $\Delta\lambda$ are shown in Fig. 3A and B, respectively. As optimum wavelength intervals for NAP and DNAP, $\Delta\lambda$ values of 20 and 180 nm, respectively, were selected, so as to minimize the spectral interference caused

by each compound in the mixture and to minimize the loss of sensitivity. The election of the optimum $\Delta\lambda$ for these compounds has also been dictated by the interference caused by the serum matrix. Fig. 4 shows the position of the minimum of the second-derivative spectrum and the signal for the background fluorescence. As can be seen from Fig. 4 the maximum signal from the serum matrix occurs at $\Delta\lambda = 70$ nm, while at $\Delta\lambda = 20$ and 180 nm this signal is minimal.

3.3. General analytical characteristics

The determination of NAP and DNAP in binary mixtures is carried out in two scans. The method involved the construction of independent calibration curves for each component. NAP and DNAP final concentrations and synchronous scanning second-derivative signals were linearly related over the range 0.02 to 15 μg ml^{-1}. Pearson's correlation coefficients (r) for the calibration graphs were 0.9998 (NAP) and 0.9990 (DNAP) ($n = 10$). The detection limits obtained by SDSFS were 1.6 and 1.9 ng ml^{-1} for NAP and DNAP respectively. In order to test the precision of the method, three series of samples covering the ranges of interest for NAP (0.098, 0.99 and 10.0 μg ml^{-1}) and for DNAP (0.050, 0.99, 10.0 μg ml^{-1}) were analyzed and the corresponding relative standard deviations (R.S.D.) ($n = 10$) were found to be 3.4, 2.1 and 0.8% for NAP and 3.6, 1.8 and 0.7% for DNAP.

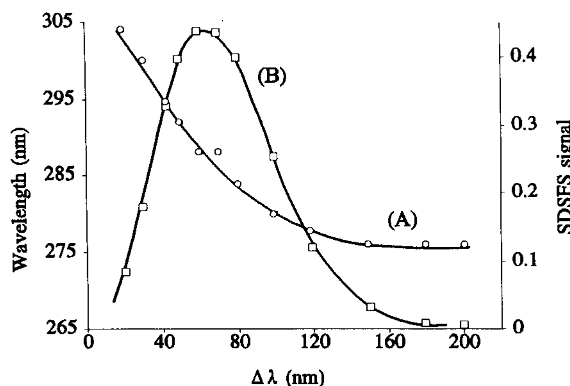

Fig. 4. Effect of $\Delta\lambda$ on the second-derivative synchronous spectra of drug-free serum supernate. (A) Effect on the wavelength corresponding to the minimum of second-derivative peak; (B) effect on the analytical signal.

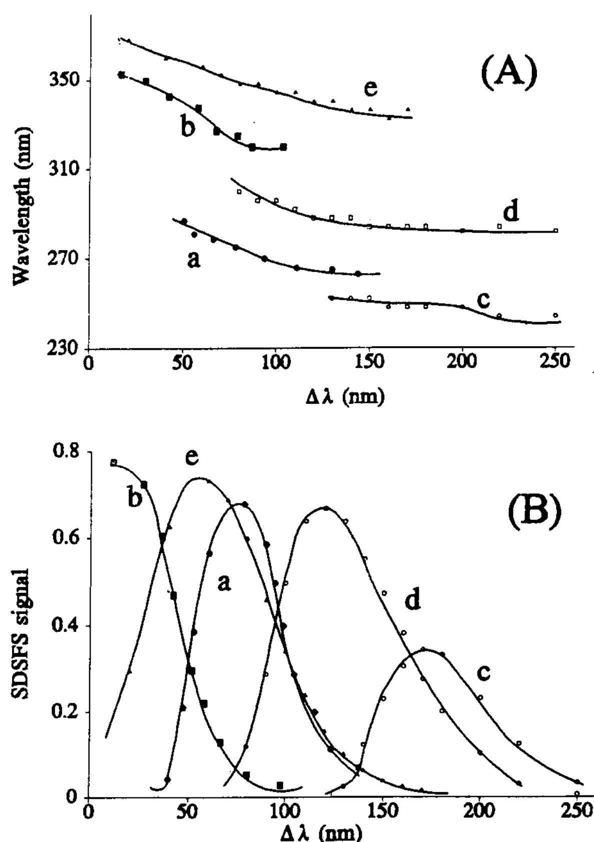

Fig. 3. Effects of $\Delta\lambda$ on the second-derivative synchronous spectra of NAP (lines a and b) and DNAP (lines c, d and e). (A) Effect on the wavelength corresponding to the minimum of second-derivative peaks; (B) effect on the analytical signals.

3.4. Determination of NAP and DNAP in binary mixtures

In order to apply the SDSFS technique to the simultaneous determination of NAP and DNAP in their mixture, a detailed study on the influence of the excess of each acid on the analytical signal of the other was performed. As can be seen from Fig. 5A, the analytical signal of NAP obtained by SDSFS is practically not influenced by the presence of up to a 10-fold excess of DNAP, while at larger excess the signal of NAP decreased considerably with increasing DNAP concentration be-

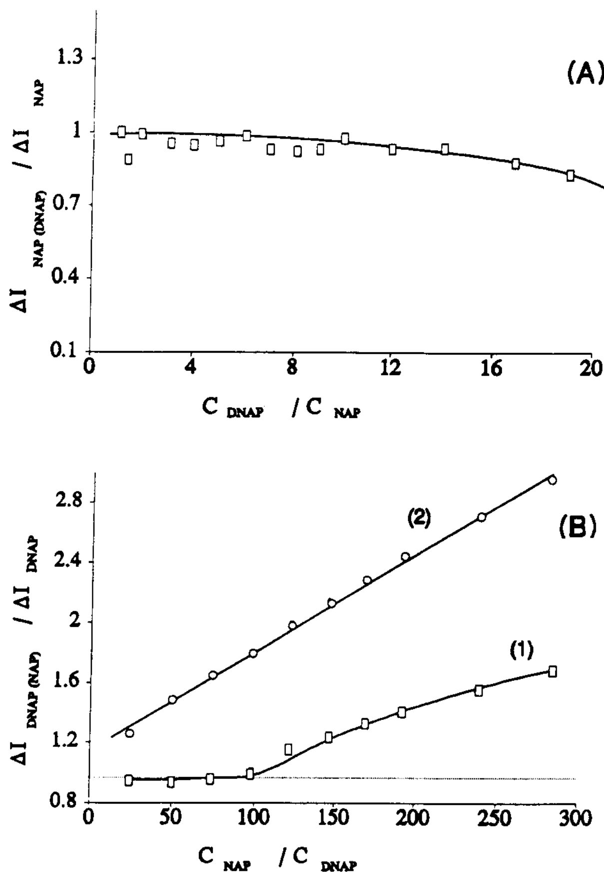

Fig. 5. Effect of the analytical signal (A) of DNAP on the signal of NAP (expressed as signal of NAP in the presence of DNAP, $\Delta I_{NAP(DNAP)}$, to the signal of NAP, ΔI_{NAP}) and (B) of NAP on the second-derivative signal of DNAP (curve 1) and on the signal of DNAP obtained by synchronous fluorimetry (curve 2).

cause of the strong overlap between the excitation spectrum of DNAP and the emission spectrum of NAP. The analytical signal of DNAP obtained by SDSFS (Fig. 5B, curve 1) remains practically constant in the presence of up to a 100-fold excess of NAP. At higher excess, the apparent analytical signal of DNAP increased considerably with increasing NAP concentration. On the contrary, the analytical signal of DNAP obtained by synchronous scanning fluorimetry (Fig. 5B, curve 2) increased even at low concentrations of NAP. The above ratios cover satisfactorily the expected ratios of NAP to DNAP in serum samples.

3.5. Serum samples

The recommended procedure to determine NAP and DNAP in serum samples after removal of proteins with acetonitrile was used. Because of the presence of acetonitrile in the final solution (9%), its influence on the second-derivative spectra of NAP ($\Delta\lambda = 20$ nm) and DNAP ($\Delta\lambda = 180$ nm) was studied. The position of the second-derivative peaks for both compounds was not influenced by the presence of up to 15% of acetonitrile, while the analytical signals were slightly increased with increasing acetonitrile concentration. To avoid any influence on the analytical signals caused by the presence of acetonitrile, aqueous calibration standards in the same manner as with serum standards. Serum samples containing NAP and DNAP gave signals smaller than those obtained with aqueous standard solutions. This is due to binding with precipitated proteins. Recovery experiments on serum samples containing NAP and DNAP at several component concentrations gave values of 68.5–3.0% ($n = 7$) and 81.0–2.7% ($n = 7$) for NAP and DNAP respectively. NAP and DNAP in serum can be quantified by using (i) working curves obtained from control serum spiked with NAP or DNAP and (ii) the standard calibration graphs and applying the appropriate correction factor. In this study the first approach was used. Recovery data for NAP–DNAP synthetic mixtures, added to serum are summarized in Table 2. Most of the selected concentrations for binary mixtures are typical for NAP and DNAP levels in serum during the first 24 h from a typical subject following an oral dose of 500 mg of naproxen [14]. The absence of detectable blanks in naproxen-free serum indicates that the constituents of normal serum do not interfere with fluorescence measurements.

3.6. Interference studies

Twelve commonly used drugs (acetylsalicylic acid, salicylic acid, diflunisal, phenacetin, carbamazepine, amiloride, caffeine, imipramine, in-

Table 2
Recovery data for NAP and DNAP in synthetic serum mixtures

Concentration added (μg ml^{-1})		Concentration found [a] (μg ml^{-1})		Recovery ± S.D. (%)	
NAP	DNAP	NAP	DNAP	NAP	DNAP
9.0	0.25	9.1	0.26	101 ± 1	104 ± 5
25.0	0.50	24.1	0.50	96 ± 2	100 ± 3
70.0	1.0	69.3	0.99	99 ± 2	99 ± 1
50.0	1.5	50.0	1.43	100 ± 1	95 ± 3
30.0	3.0	31.7	3.13	106 ± 2	104 ± 2
25.0	5.0	23.7	4.99	95 ± 1	100 ± 4
5.0	5.0	5.0	4.96	100 ± 5	99 ± 3
			Mean,	99.6	100.1

[a] Average of three measurements.

domethacin, amoxyline, levedopa, theophylline, amitriptyline) were tested for their potential interference in the method by supplementing pooled serum containing NAP (25 μg ml^{-1}) and DNAP (2.0 μg ml^{-1}) with amounts of the drug under investigation. No interference was noted by using SDSFS with any of the above drugs except for diflunisal and salicylate which interfere with the determination of DNAP at mass ratios (drug to DNAP), > 2:1 and > 15:1, respectively.

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