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Synchronous scanning second derivative spectrofluorimetry for the simultaneous determination of diflunisal and salicylic acid added to serum and urine as ternary complexes with terbium and EDTA

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Abstract

By using second derivative synchronous scanning fluorescence spectrometry the simple resolution of two nonsteroidal, antiinflammatory drugs diflunisal and salicylic acid, as ternary complexes with terbium and EDTA, is accomplished. The method developed is simple, sensitive and rapid, and has been successfully applied for the determination of both compounds in 10 μl of untreated human serum and urine samples. The detection limits for diflunisal were 0.9 and 1.8 mg l^{-1}, and for salicylic acid 1.2 and 1.7 mg l^{-1}, in serum and urine, respectively. The mean analytical recoveries from serum and urine samples spiked with diflunisal (25–100 mg l^{-1}) and salicylic acid (28–138 mg l^{-1}) were 99 ± 8% (serum) and 102 ± 8% (urine) for diflunisal and 102 ± 9% (serum) and 95 ± 8% (urine) for salicylic acid. The precision of all determinations varied from 2.5 to 10%.

Keywords: Fluorimetry; Chemiluminescence; Lanthanide sensitized luminescence; Diflunisal; Serum; Urine; Salicylic acid

1. Introduction

Diflunisal (DIF, 2′,4′-difluoro-4-hydroxy-3-biphenylcarboxylic acid), a difluorophenyl derivative of salicylic acid (SA) is a non-steroidal anti-inflammatory drug with antipyretic properties similar to aspirin. Due to the structural similarities, a high cross reactivity of diflunisal is observed in the fluorescence polarisation immunoassay and UV–visible spectrophotometric methods used for the determination of salicylates [1,2]. This interference could cause serious problems in salicylate assay for therapeutic drug monitoring (TDM) and drug abuse screening purposes. For salicylate screening in serum from patients treated with diflunisal, liquid chromatographic methods are recommended [3–6]. Most of these 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. In addition,

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it must be noted that no chromatographic method has been proposed for the simultaneous determination of both compounds. Therefore, the development of a method allowing the accurate determination of SA in the presence of DIF and their simultaneous determination in biological fluids could be considered of clinical value.

Recently, we have reported a simple spectrofluorimetric method for the simultaneous determination of DIF and SA in serum, based on the intrinsic fluorescence of both analytes in chloroform–1% acetic acid solution [7]. Mixtures of DIF and SA in serum were resolved after removal of proteins by using second derivative synchronous fluorescence spectrometry (SDSFS). Although simple and rapid, this method required an extraction with chloroform and could not be applied to urine samples because of matrix interference.

In the present paper, we report an improved spectrofluorimetric method for the simultaneous determination of DIF and SA in serum and urine samples not requiring the use of organic solvents. The method is based on the formation of ternary Tb–EDTA–DIF and Tb–EDTA–SA complexes. Both complexes emit a very strong terbium ion fluorescence with the main maximum at 546 nm, when excited at wavelengths characteristic for each ligand. This phenomenon, generally known as lanthanide-sensitised luminescence, is due to energy transfer through the excited triplet state of the ligand to the emitting level of the terbium ion [8]. Spectrofluorimetric methods for the determination of SA in serum [9] and, recently, for DIF in serum and urine [10] based on the above mentioned ternary complexes have been reported. Despite their simplicity and high sensitivity, these methods could not be used for the simultaneous determination of DIF and SA because of the overlap of the broad band excitation spectra of the ternary complexes. However, mixtures of DIF and SA in the form of these ternary complexes were readily resolved by using SDSFS technique, as is described in this paper. SDSFS is well known as an excellent improvement in terms of sensitivity and selectivity to conventional fluorimetry, particularly suitable for severely overlapping spectral bands [11]. The proposed method allows the simultaneous determination of DIF and SA added to serum or urine samples, in a single scan, and without removal of proteins.

2. Materials and methods

2.1. Instrumentation

All the spectrofluorimetric measurements were performed with a Model 512 fluorescence spectrometer (Perkin-Elmer, Norwalk, CT) interfaced to an IBM-PC 386DX microcomputer. Two 5-phase, 1000-steps per revolution stepper-motors, type 564/50 LH (Berger-Lahr, Germany) were used to bring under direct computer control the excitation and emission monochromators of the spectrometer. A stepping motor control card, type PCL-838, and a Multilab Analog and Digital I/O card, type PCL-812PG (American Advantech, Sunnyvale, CA) were used for stepper motor control and for fluorescence signal data acquisition (via a 12-bit analog-to-digital converter), respectively.

A homemade control program (FLUOR-MOD) written in TURBO-PASCAL 6.0 (Borland, Scotts Valley, CA) was used for overall system control, data collection and processing. The program provides a graphic display of emission, excitation or synchronous fluorescence spectra and/or of their respective 1st and 2nd derivatives. The Savitzky-Golay method is used for smoothing and calculation of the derivatives of the obtained spectra [12,13]. In derivative fluorescence spectroscopy the analytical signal $\Delta I_X^{(n)}$ of the analyte X is defined as:

$$\Delta I_X^{(n)} = \left(I_{max}^{(n)} - I_{min}^{(n)}\right)_{\lambda_1,\lambda_2} \quad (1)$$

where I is the relative fluorescence intensity, and $I_{max}^{(n)}$ and $I_{min}^{(n)}$ are the maximum and minimum values of the nth order derivative of I ($I^{(n)} = d^nI/d\lambda^n$), within a relatively narrow spectral range bounded by the wavelengths λ_1 and λ_2. The program allows the user to specify up to three spectral ranges, so that three different analytical signals, each with its own analytical significance, can be calculated and directly displayed after a single scan.

The spectra can be stored on disk files and/or in memory buffers so that they can be subjected to a variety of mathematical manipulations (addition, subtraction and normalizations). Spectra can appear directly as background-corrected after loading a spectrum file defined as "background". The system can be pre-programmed to perform a series of successive scannings under changing monochromators settings

(initial λ_{ex}, λ_{em} and scanning range). Such successive scannings are mainly used to create a series of spectral files which can subsequently be used for drawing of three-dimensional "total" fluorescence spectra, i.e., of the type $I = F(\lambda_{ex}, \lambda_{em})$. Total spectra can also conveniently be displayed as two-dimensional plots of equifluorescence lines (contour plots).

2.2. Materials

Diflunisal, salicylic acid, and CAPS (3-(cyclohexylamino)-1-propanesulfonic acid) (Sigma, St. Louis, MO), terbium oxide and EDTA (Merck, Darmstadt, Germany) were used.

2.3. Solutions

Diflunisal stock solution, 10.0 mmol l^{-1}, was prepared by dissolving 25.0 mg of DIF in doubly-distilled water, made to pH ~ 12 with NaOH solution and diluting to 10.0 ml. This solution was stable for at least 1 month at room temperature when kept in the dark.

Salicylic acid stock solution, 10.0 mmol l^{-1}, was prepared by dissolving 13.8 mg of SA as above and diluting to 10.0 ml. This solution was stable for at least 1 month at room temperature.

DIF-SA mixed standard solutions. For the construction of the calibration graph for DIF and the calculation of the equation for measuring SA concentration, four different standard mixtures containing DIF and SA were prepared fresh when needed by mixing the appropriate volumes of the DIF and SA stock solutions and diluting with water, control serum (normal) or drug-free urine. In these standard mixtures the final concentrations of DIF were 10, 20, 50, and 100 $\mu g\ l^{-1}$, while that of SA was 55.2 $\mu g\ ml^{-1}$.

Terbium stock solution, 10.0 mmol l^{-1}, was prepared by dissolving 365.8 mg of terbium oxide (Tb_2O_3) in spectrally pure concentrated nitric acid, evaporating to dryness and dissolving the residue in 200 ml of 10 mmol l^{-1} hydrochloric acid.

EDTA solution, 10 mmol l^{-1}.

Tb–EDTA–CAPS working solution, 1 mmol l^{-1} Tb–EDTA–100 mmol l^{-1} CAPS, pH 12.6. This solution was prepared by mixing terbium and EDTA stock solutions and 125 mmol l^{-1} CAPS buffer of pH 12.6 at a volume ratio of 1 + 1 + 8. This working solution was used as a single reagent for the measurements. It was stable for at least 1 month at 4°C.

2.4. Methods

2.4.1. Blank measurement

Place 2.00 ml of the Tb–EDTA–CAPS working solution and 10 μl of control serum (normal) or drug-free urine into the cuvette. Record the synchronous fluorescence spectrum by scanning both monochromators at a constant wavelength difference, $\Delta\lambda = 220$ nm ($\lambda_{ex} = 240$–360 nm, $\lambda_{em} = 460$–580 nm). Hereafter all wavelengths referring to synchronous spectra are taken as equal to those of the corresponding excitation wavelengths. The resulting synchronous spectrum is stored in memory as a "background spectrum" and it will be subtracted from all subsequently obtained spectra.

2.4.2. Sample measurement

Place 2.00 ml of the working solution and 10 μl of the sample (serum or urine) or standard mixture into the cuvette. Record the synchronous fluorescence (background corrected) spectrum in the same manner. Obtain the analytical signals $\Delta I^{(2)}_{DIF}$ (spectral range 248–278 nm), and $\Delta I^{(2)}_{DIF+SA}$ (or $\Delta I^{(2)}_{SA}$ in the absence of DIF) (spectral range of 324–348 nm), from the second derivative of the synchronous spectrum.

Calculate the concentration of DIF, C_{DIF}, in serum or urine from the calibration graph obtained by plotting the $\Delta I^{(2)}_{DIF}$ vs. DIF concentration in control serum or in drug-free urine spiked with DIF (concentration range 10–100 mg l^{-1}) and with SA (55.2 mg l^{-1}).

Calculate the unknown concentration of SA, C_{SA}, in serum or urine samples from the equation of general form:

$$\left(\Delta I^{(2)}_{DIF+SA}/\Delta I^{(2)}_{DIF}\right) = a + b \times \left(C_{SA}/C_{DIF}\right) \qquad (2)$$

where regression parameters a and b have previously been calculated from the linear plot of the ratios $\Delta I^{(2)}_{DIF+SA}/\Delta I^{(2)}_{DIF}$ vs. C_{SA}/C_{DIF} obtained using the mixed standards (DIF + SA) prepared in control serum (normal) or drug-free urine, respectively.

In diflunisal-free serum or urine samples, calculate the concentration of SA, C_{SA}, from the calibration graph obtained by plotting $\Delta I_{SA}^{(2)}$ vs. SA concentration in control normal serum or in drug-free urine spiked with SA (concentration range 5–100 mg l^{-1}).

3. Results and discussion

Alkaline aqueous Tb–EDTA solutions show very weak fluorescence which strongly increases in the presence of diflunisal and/or salicylic acid. This is due to the formation of the Tb–EDTA–DIF and/or Tb–EDTA–SA ternary complexes. The corresponding emission and excitation spectra are shown in Fig. 1.

As can be seen from Fig. 1, the excitation spectrum of the Tb–EDTA–DIF complex consists of two bands with $\lambda_{ex} = 284$ and 336 nm. The second band of the Tb–EDTA–DIF strongly overlaps with the single band of the Tb–EDTA–SA complex ($\lambda_{ex} =$ 326 nm). As a result, the simultaneous determination of these compounds in a single scan by conventional spectrofluorimetry is clearly unfeasible, requiring another approach for the resolution of mixtures of these two compounds such as synchronous fluorescence spectrometry.

The optimal chemical conditions (pH, reagent excess, etc.) for the formation of the ternary complexes of Tb with EDTA and DIF or SA have been reported previously [9,10].

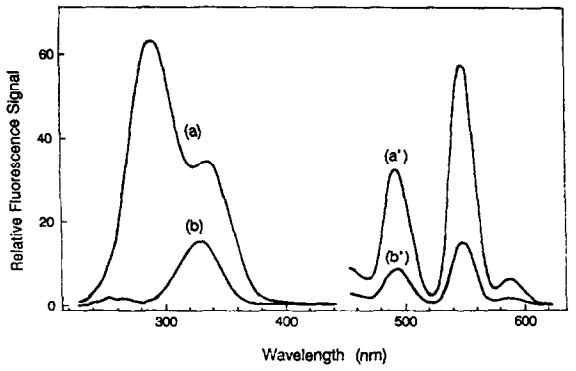

Fig. 1. Fluorescence excitation (a,b) and emission (a',b') spectra of Tb–EDTA–DIF ternary complex, $C_{DIF} = 1$ μmol l^{-1}, $C_{Tb-EDTA} = 1$ mmol l^{-1}, $\lambda_{ex} = 336$ nm, $\lambda_{em} = 546$ nm (a,a') and Tb–EDTA–SA ternary complex, $C_{SA} = 1$ μmol l^{-1}, $C_{Tb-EDTA} = 1$ mmol l^{-1}, $\lambda_{ex} = 326$ nm, $\lambda_{em} = 546$ nm (b,b').

3.1. Optimisation of variables

In order to determine the optimum $\Delta\lambda$ value for the resolution of the mixture by synchronous scanning fluorescence spectrometry we obtained the total fluorescence spectra of the Tb–EDTA–DIF and Tb–EDTA–SA complexes. The three-dimensional and two-dimensional (contour plots) representations of these spectra are shown in Fig. 2.

At the lower part of these spectra for both analytes a region of weak fluorescence can be seen, corresponding to the intrinsic fluorescence of free DIF and SA. The intense signal at the lower right side is due to scattering of the excitation beam. At the middle and particularly at the upper part of the plots there are regions of intense fluorescence corresponding to the fluorescence of the ternary complexes Tb–EDTA–DIF and Tb–EDTA–SA.

The contour-plots offer an easy way for finding the best trajectory (always at 45° for synchronous spectra) which must be followed to minimize spectral overlap. The trajectory used (dashed line corresponding to $\lambda_{em} = \lambda_{ex} + \Delta\lambda$, where $\Delta\lambda = 220$ nm), crosses two distinct regions A and B. In region A the ternary complex Tb–EDTA–DIF is the main contributor whereas the contribution of the ternary complex Tb–EDTA–SA is negligible. Thus, this region is used for the evaluation of the analytical signal of DIF, $\Delta I_{DIF}^{(2)}$. In region B both ternary complexes contribute, hence this region is used for the evaluation of the analytical signal of the sum DIF + SA, $\Delta I_{DIF+SA}^{(2)}$, or (in the absence of DIF) of the analytical signal of SA, ΔI_{SA}^{2}.

The synchronous fluorescence spectra of Tb–EDTA–DIF and Tb–EDTA–SA ternary complexes and that of their mixture taken at a constant wavelength difference $\Delta\lambda = 220$ nm are shown in Fig. 3a. Taking into account the strong overlapping of the synchronous spectra of both compounds we have employed the second derivatives of these spectra to make use of the additional resolution capability offered by derivative fluorescence spectrometry [14]. The second derivative synchronous spectra obtained for these compounds as well as the analytical signals for DIF and for DIF + SA are shown in Fig. 3b.

Hereafter, for the sake of simplicity, all signals corresponding to Tb–EDTA–DIF and Tb–EDTA–SA are referred to as DIF and SA signals.

3.2. Assay characteristics

The concentration of diflunisal and the signals $\Delta I^{(2)}_{DIF}$ are linearly related over a sample concentration range 3–1000 mg l^{-1}. Salicylic acid concentration and the second derivative signals $\Delta I^{(2)}_{SA}$ (in the absence of DIF) are linearly related over the range 4–800 mg l^{-1}. Pearson's correlation coefficients for the standard calibration graphs were 0.9996 and 0.999 ($n = 15$) for DIF and SA, respectively. The detection limits obtained for DIF and SA by SDSFS taken as the concentration corresponding to three times the standard deviation of the signal for the lowest concentration studied were 0.9 and 1.2 mg l^{-1} for serum and 1.8 and 1.7 mg l^{-1} for urine, respectively.

3.3. Resolution of binary DIF–SA mixtures

The application of the SDSFS for resolving mixtures with overlapping synchronous spectra requires a thorough investigation of the effect of each com-

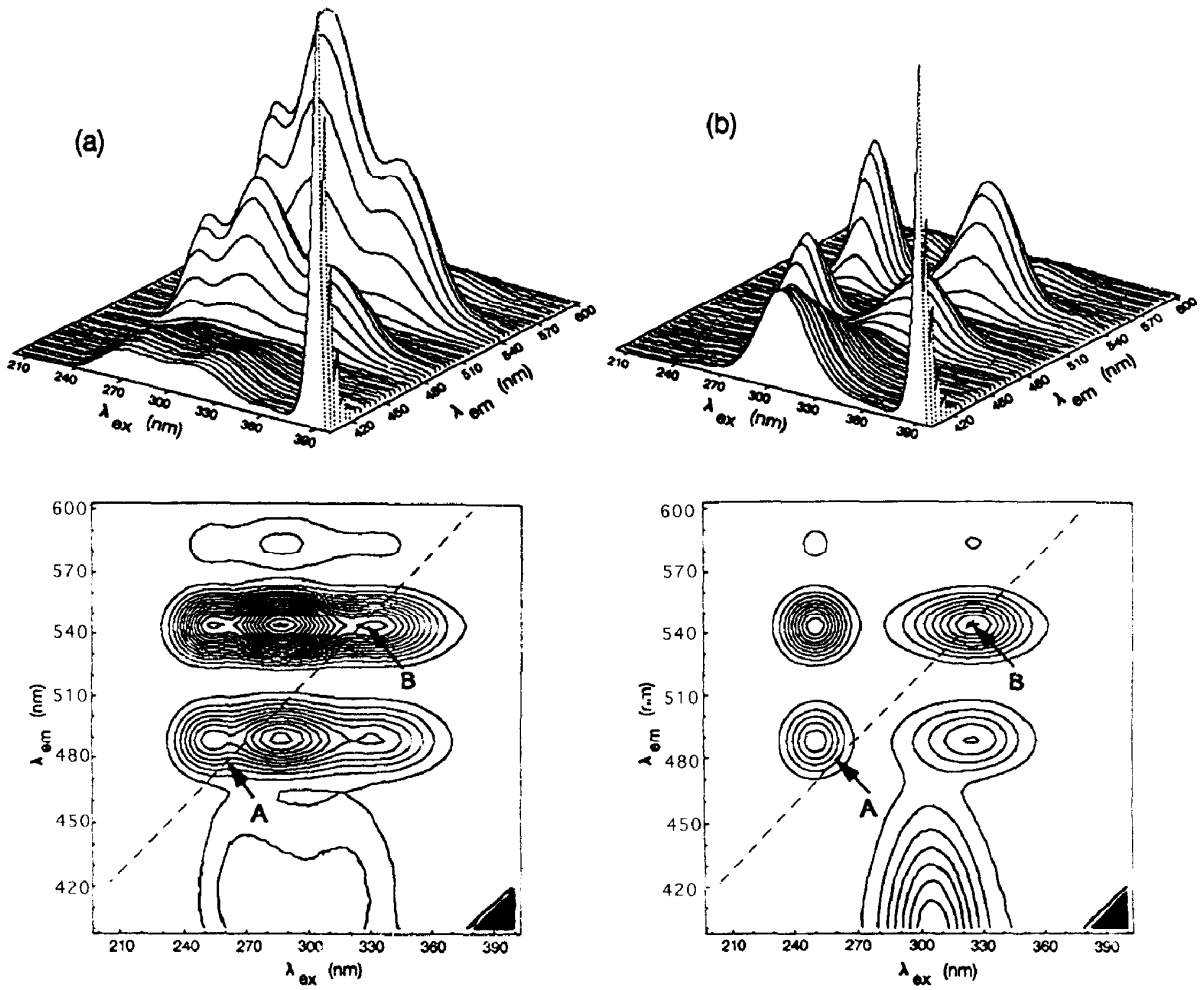

Fig. 2. Three-dimensional and two-dimensional (contour plots) "total" fluorescence spectra of Tb–EDTA–DIF (a) and Tb–EDTA–SA (b) (background uncorrected). The dashed line on the contour plots is the trajectory followed during synchronous scans.

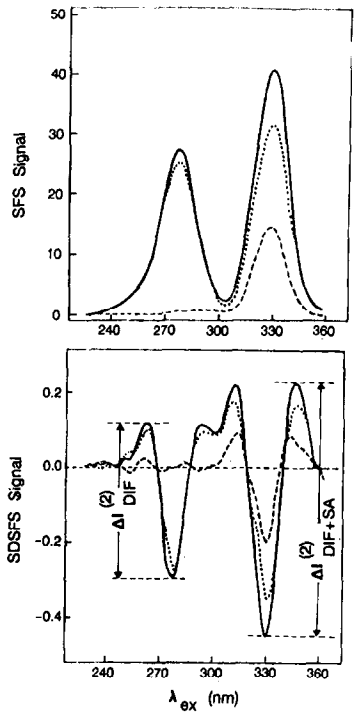

Fig. 3. Synchronous fluorescence spectra (SFS) and second derivative synchronous fluorescence spectra (SDSFS) of Tb–EDTA–DIF (dotted line), Tb–EDTA–SA (dashed line) and their mixture (solid line). $\Delta\lambda = 220$ nm, $C_{DIF} = 1$ μmol l^{-1}, $C_{SA} = 1$ μmol l^{-1}, $C_{Tb-EDTA} = 1$ mmol l^{-1}.

pound on the signal of the other. The effect of the concentration of SA on the analytical signal of DIF (curve a) and that on the analytical signal of the sum DIF + SA (curve b) in aqueous mixtures is shown in Fig. 4. The analytical signal of DIF is not affected by the presence of up to a 20-fold mass excess of SA (about 40-fold molar excess), while the ratio $\Delta I^{(2)}_{DIF+SA}/\Delta I^{(2)}_{DIF}$ is linearly related to the ratio C_{SA}/C_{DIF} up to a mass concentration ratio of 10 (molar ratio ca. 20) closely following Eq. 2.

The dimensionless regression parameters a and b are affected by the sample matrix; their experimental values are given in Table 1.

3.4. Serum and urine samples

The analytical signals of DIF in serum are smaller than those obtained with aqueous solutions, while the signals of DIF + SA are not affected by the serum matrix. This is probably due to the stronger binding

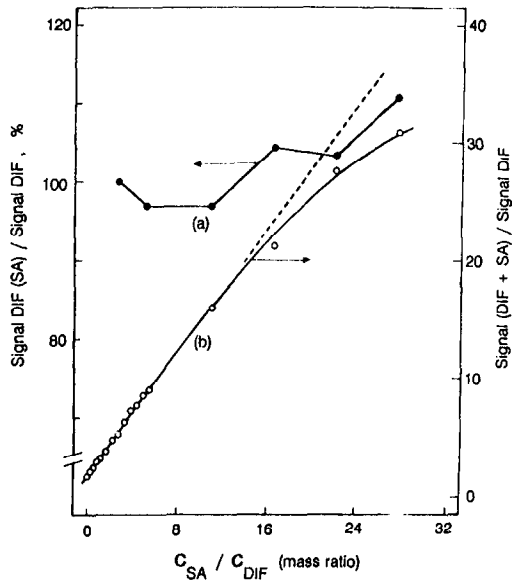

Fig. 4. Effect of the concentration of SA on the analytical signal of DIF, $\Delta I^{(2)}_{DIF}$, (curve a) and on the ratio $\Delta I^{(2)}_{DIF+SA}/\Delta I^{(2)}_{DIF}$ (curve b).

to serum proteins of the less polar 2,4-difluorophenyl group of DIF, which is responsible for the absorption of radiation at 272 nm.

Urine samples containing DIF and SA gave signals smaller than those obtained with aqueous solutions for both DIF and DIF + SA. Due to the similarity of the effect of the urine matrix on both signals this decrease may be attributed to the inner filter effect from the urine.

3.5. Analytical recovery

Serum and urine samples spiked with DIF and SA at different concentrations and at different mass ra-

Table 1
Experimental values of a and b linear regression parameters of Eq. 1 in various matrices [a]

Matrix	$a \pm$S.D.	$b \pm$S.D.	r
Water	1.79±0.16	1.59±0.06	0.999
Serum	2.10±0.23	1.97±0.15	0.998
Urine	1.95±0.15	1.26±0.07	0.996

[a] Calculated by measuring 16 samples spiked with DIF and SA, covering a concentration range from 10 to 100 mg l^{-1} (0.05 to 0.5 mg l^{-1} in the final solution) for DIF and mass concentration ratios C_{SA}/C_{DIF} from 0.28 to 5.5.

Table 2
Analytical recoveries of diflunisal and salicylic acid in normal serum synthetic mixtures

Concentration added (mg l^{-1})		Concentration found [a] (mg l^{-1})		Recovery (%) ± S.D. [a]	
DIF	SA	DIF	SA	DIF	SA
100.0	27.6	105.0	26.1	105 ± 6	106 ± 6
100.0	55.2	107.0	58.8	107 ± 2	106 + 6
50.0	27.6	52.2	26.9	104 ± 10	97 ± 8
50.0	138.0	48.1	158.0	96 ± 6	114 ± 5
25.0	69.0	22.1	60.5	88 ± 8	88 ± 10
25.0	138.0	23.0	135.0	92 ± 4	98 ± 6
			Mean	99 ± 6	102 ± 6

[a] Average of three measurements.

tios were prepared by adding known amounts of DIF and SA to control serum (normal) and drug-free urine samples. The above concentrations for DIF and SA were selected from pharmacokinetic data of both compounds from a typical subject following an oral dose of 650 mg of aspirin [5] and 500 mg of DIF [15]. The mean analytical recoveries in serum mixtures were 99 ± 8% and 102 ± 9% for DIF and SA, respectively, while in urine mixtures the corresponding mean recoveries were 102 ± 8% and 95 ± 8% as shown in Tables 2 and 3. Analytical recovery experiments for DIF in lipemic, icteric and hemolyzed serum samples as well as in three different pools of drug-free serum samples have been also performed in order to study the influence of endogenous substances in serum. The recovery data for these samples are summarized in Table 4 and show that there is no influence from the serum matrix on the determination of DIF and consequently on the determination of SA.

Table 3
Analytical recoveries of diflunisal and salicylic acid in drug-free urine synthetic mixtures

Concentration added (mg l^{-1})		Concentration found [a] (mg l^{-1})		Recovery (%) ± S.D. [a]	
DIF	SA	DIF	SA	DIF	SA
100.0	27.6	93.2	29.9	93 ± 6	108 ± 7
100.0	55.2	106.0	49.0	106 ± 5	89 ± 8
50.0	27.6	55.9	24.5	112 ± 5	89 ± 5
25.0	69.0	23.8	62.9	95 ± 5	91 ± 3
25.0	138.0	26.0	133.0	104 ± 5	96 ± 2
			Mean	102 + 8	95 + 8

[a] Average of three measurements.

Table 4
Analytical recovery of diflunisal added to different serum samples

Serum sample	DIF (mg l^{-1})		
	Added	Found [a]	Recovery (%) ± S.D. [a]
Icteric	10.0	9.1	91 ± 7
Lipemic	10.0	11.3	113 ± 22
Hemolyzed	10.0	9.7	97 ± 7
Pool 1	10.0	9.4	94 ± 11
Pool 2	10.0	10.0	100 ± 10
Pool 3	10.0	10.6	106 ± 5
Icteric	50.0	53.1	106 ± 4
Lipemic	50.0	53.0	106 ± 2
Hemolyzed	50.0	52.7	105 ± 3
Pool 1	50.0	51.0	102 ± 7
Pool 2	50.0	49.8	100 ± 8
Pool 3	50.0	52.1	104 ± 6
Icteric	100.0	99.0	99 ± 4
Lipemic	100.0	94.4	94 ± 5
Hemolyzed	100.0	96.9	97 ± 6
Pool 1	100.0	101.0	101 ± 6
Pool 2	100.0	107.0	107 ± 0
Pool 3	100.0	96.0	96 ± 5

[a] Average of three measurements.

3.6. Precision

Within-run precision of the proposed assay was determined by analyzing serum and urine samples

Table 5
Precision of the DIF–SA assay

Sample	Diflunisal (mg l^{-1})		Salicylic acid (mg l^{-1})	
	Mean	C.V. (%)	Mean	C.V. (%)
Serum, within-run ($n = 6$)				
1	108	2.5	49.8	8.9
2	45.6	3.6	145	3.5
3	24.5	9.6	143	6.4
Serum, day-to-day ($n = 5$)				
1	106	5.7	53.8	10.1
2	46.3	9.7	150	3.3
3	24.8	8.5	139	2.9
Urine, within-run ($n = 6$)				
1	105	2.5	50.7	9.1
2	59.0	5.6	199	3.2
3	24.0	9.2	162	5.3

spiked with SA and DIF at three different concentrations, 6 times each. Day-to-day precision was assessed by analyzing the same three serum samples 5 times for 5 days. All relevant data are given in Table 5.

4. Conclusions

The present method for the simultaneous determination of DIF and SA by SDSFS technique, based on the formation of ternary complexes with terbium and EDTA, represents a substantial improvement of our previous method, which was based on the intrinsic fluorescence of both compounds in chloroform–1% acetic acid solutions [7]. The main advantages of this method compared with the previous one are as follows:

(1) lower detection limits for diflunisal and salicylic acid (about 5 times) due to the formation of strongly fluorescent complexes which permit the use of as little as 10 μl of the biological sample;

(2) the unusually large Stoke's shift of terbium ion fluorescence, which eliminates the interferences from endogenous substances, thus making measurements in untreated samples feasible;

(3) considerable elimination of the interference from other substances usually found in biological fluids, because only few compounds form fluorescent complexes with terbium and EDTA;

(4) simplified procedure because of the use of the same mixed standards for obtaining the calibration graph for DIF and the equation for the determination of SA;

(5) aqueous solutions are used throughout the assay, a significant improvement over other methods where the use of organic solvents is required.

Acknowledgements

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