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Simple, rapid and sensitive spectrofluorimetric determination of diflunisal in serum and urine based on its ternary complex with terbium and EDTA

Pinelopi C. Ioannou *, Evriklia S. Lianidou, Dimitrios G. Konstantianos

University of Athens, Laboratory of Analytical Chemistry, Panepistimiopolis, Zografou, 157 71 Athens, Greece

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Abstract

A very simple, rapid and highly sensitive fluorimetric method for the determination of diflunisal in serum and urine is described. The method is based on the formation of a ternary complex between diflunisal, Tb^{3+} and EDTA in alkaline aqueous solutions. This complex exhibits very intense terbium ion luminescence with a main emission maximum at 546 nm when excited at 284 nm. Optimum conditions for the complex formation have been investigated. The detection limit for diflunisal is 2.4 μg l^{-1}, while the range of application is 0.01–6.00 mg l^{-1}. The method has been successfully applied for the determination of diflunisal in untreated human serum and urine samples. Analytical recoveries from serum and urine samples spiked with diflunisal were in the ranges of 96.8–101.2% and 98.0–102.0%, respectively.

Keywords: Fluorimetry; Diflunisal; Serum; Terbium; Urine

1. Introduction

Diflunisal (DIF, 2,4-difluoro-4-hydroxy-3-biphenylcarboxylic acid) is a derivative of salicylic acid with similar analgesic and anti-inflammatory properties to aspirin. Different methods for the determination of DIF have been reported. The proposed methods for monitoring diflunisal in biological fluids are liquid chromatography (LC) [1], gas–liquid chromatography [2] and fluorimetry [3]. These methods are time-consuming and require removal of proteins prior to the measurement. Fluorimetric methods for the determination of DIF are based on its intrinsic fluorescence in various solvents. A method for the simultaneous determination of diflunisal and salicylic acid in serum based on their intrinsic fluorescence in chloroform–acetic acid solutions has also been reported [4].

Fluorescent europium and terbium chelates are characterized by unique spectroscopic properties such as line emission spectra, large Stoke's shift and long-lived fluorescence [5]. When excited at wavelengths absorbed by an organic ligand, these complexes emit characteristic lanthanide ion line emission spectra. This is due to an intramolecular energy transfer through the excited triplet state of the ligand to the emitting level of the lanthanide ion. Several very important applications of europium and terbium complexes have already been reported [6] with the most attractive ones being their use as fluorescent labels for highly sensitive time-resolved fluorescence immunoassays and nucleic acid hybridization assays [7,8]. Another area with interesting applications is the fluorimetric determination of

* Corresponding author.

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organic molecules capable of transferring their triplet energy to lanthanide ions either via complexation or by intermolecular energy transfer regulated by diffusion processes. These methods are known as lanthanide-sensitized luminescence and are applicable to many aromatic aldehydes and ketones and a variety of biologically important compounds [6].

Terbium ions form strongly fluorescent ternary complexes with aminopolycarboxylic acids such as EDTA and phenol derivatives such as salicylic and sulfosalicylic acids [9,10]. A method for the determination of salicylate in plasma based on its ternary complex with terbium and EDTA has been reported [11]. Diflunisal also forms a fluorescent ternary complex with terbium and EDTA. This complex has already been applied for the development of a highly sensitive enzyme-amplified lanthanide immunoassay (EALL) [12].

Here, we report a very simple, rapid and sensitive fluorimetric method for the determination of diflunisal based on its ternary complex with terbium and EDTA. Optimum conditions for the complex formation have been investigated. The method has successfully been applied for the determination of diflunisal in human serum and urine samples without any pretreatment. Because of its simplicity and high sensitivity this method is advantageous for therapeutic drug monitoring and drug abuse screening of diflunisal in biological fluids.

2. Experimental

2.1. Apparatus

A Model 512 fluorescence spectrophotometer (Perkin-Elmer, Norwalk, CT), equipped with a 150-W xenon lamp and a magnetic stirrer under the cell holder was used. Instrument settings were as follows: energy mode; excitation wavelength 284 nm, with a bandwidth of 20 nm; emission wavelength 546 nm, with a bandwidth of 20 nm. A constant temperature of 25.0°C in the 1.000 cm (pathlength) sample cell was maintained with a thermostated water bath.

2.2. Reagents

All solutions were prepared in deionised distilled water from reagent grade materials, unless otherwise stated.

Aqueous stock solution of DIF (Sigma) containing 500 mg l^{-1} was prepared at pH 12.0. This solution was stable for at least 1 month at room temperature. Working standard solutions of diflunisal were prepared by appropriate dilution. A terbium solution, 0.01 mol l^{-1}, was prepared by dissolving the appropriate amount of terbium oxide (Merck) in concentrated nitric acid, evaporating to dryness and dissolving the residue in 0.01 mol l^{-1} hydrochloric acid. An EDTA solution of 0.01 mol l^{-1} and a CAPS (3-[cyclohexylamino]-1-propanesulfonic acid) (Sigma) buffer solution, 0.1 mol l^{-1} of pH 12.7 were prepared. A working solution containing terbium and EDTA at a final concentration of 1.0×10^{-3} mol l^{-1} was prepared by mixing the appropriate volumes of terbium and EDTA solutions and diluting with CAPS buffer (final concentration of CAPS, 0.08 mol l^{-1}). This working solution was used as a single reagent for the measurements.

2.3. Procedure

Transfer 10 μl of the sample (serum or urine) or aqueous standard into the cuvette, add 2.0 ml of the mixed Tb–EDTA reagent and start the stirrer. Measure the fluorescence intensity versus a reagent blank in which 10 μl of water is substituted for standard or sample. Calculate the unknown concentration, C_{DIF}, from the calibration graph. Calculate the concentration of DIF in serum, $C_{DIF,s}$, and urine, $C_{DIF,u}$, from Eqs. 1 and 2, respectively:

$$C_{DIF,s} = 1.80 C_{DIF} \quad (1)$$

$$C_{DIF,u} = 1.32 C_{DIF} \quad (2)$$

where 1.80 and 1.32 are correction factors and their values depend on the actual dilution factors (200-fold according to the above procedure) of serum and urine samples.

3. Results and discussion

Aqueous alkaline solution of diflunisal show an intense intrinsic fluorescence with excitation maxima at 272 and 310 nm and an emission maximum at 420 nm [4]. In the presence of the Tb–EDTA mixture a strongly fluorescent ternary complex is formed in alkaline aqueous solution with excitation maxima shifted

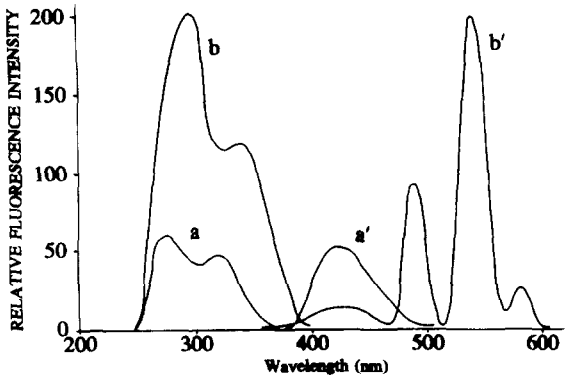

Fig. 1. Fluorescence excitation (a,b) and emission spectra (a',b') of: aqueous solutions (pH 12.6) of diflunisal, $C = 4.0 \times 10^{-6}$ mol l^{-1}, $\lambda_{ex} = 272$ nm, $\lambda_{em} = 420$ nm (a,a') and of the DIF–Tb–EDTA ternary complex, $C_{Tb\text{-}EDTA} = 1.0 \times 10^{-3}$ mol l^{-1}, $\lambda_{ex} = 284$ nm, $\lambda_{em} = 546$ nm (b,b').

towards longer wavelengths with respect to those for diflunisal ($\lambda_{ex} = 284$ and 336 nm) and a narrow line terbium ion luminescence spectrum with maxima at 488, 547 and 596 nm (Fig. 1). The main emission band at 547 nm was selected for the measurements with excitation at 284 nm.

3.1. Optimum conditions for DIF–Tb–EDTA ternary complex formation

Effect of pH

The fluorescence intensity of diflunisal solutions in the presence of excess Tb–EDTA mixture was measured over a pH range from 9.0 to 13.5, by using Tris buffer and adjusting the pH with hydrochloric acid and sodium hydroxide solutions. The maximum fluorescence intensity was observed at a pH between 12.5 and 13.0 (Fig. 2). Among several buffer systems tested (Tris, CAPS, 2-amino-2-methyl-1-propanol (AMP), glycine, borate), a CAPS buffer solution at a concentration of 0.08 mol l^{-1} was found to be the most suitable for this pH range.

Reagent excess and stability of the complex

The effect of Tb–EDTA mixture excess on the fluorescence intensity of the ternary complex was investigated. The maximum fluorescence signal of the complex is observed at about 500-fold molar excess of Tb–EDTA (1:1) mixture (Fig. 3, curve a), while the intrinsic fluorescence of diflunisal practically disappeared (Fig. 3, curve b). It was found that a concentration of 1.0×10^{-3} mol l^{-1} of Tb–EDTA mixture is sufficient for the analytical range of application for diflunisal and was selected for the analytical procedure. The ternary complex is formed immediately when diflunisal is added to the alkaline Tb–EDTA solution. The fluorescence signal remains constant for at least 24 h if there are no changes in pH.

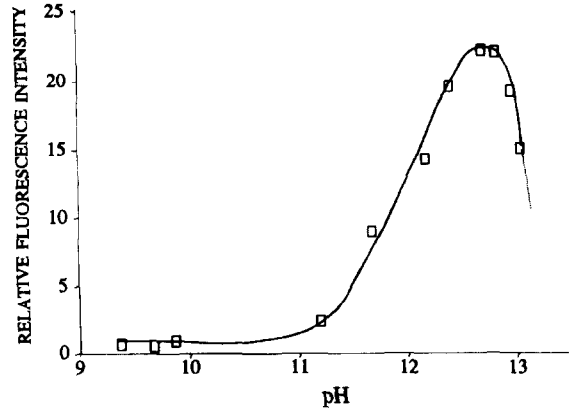

Fig. 2. Effect of pH on the fluorescence signal of the DIF–Tb–EDTA ternary complex ($\lambda_{ex} = 284$ nm, $\lambda_{em} = 546$ nm, $C_{DIF} = 4.0 \times 10^{-7}$ mol l^{-1}, $C_{Tb\text{-}EDTA} = 1.0 \times 10^{-3}$ mol l^{-1}).

3.2. General analytical characteristics

The fluorescence signal was linearly related to the concentration of diflunisal in the range of 0.01–6.00 μg ml^{-1}. Pearson's correlation coefficient (r) for the standard calibration graph was 0.9991. The detection limit was found to be 2.4 ng ml^{-1}. To test the precision,

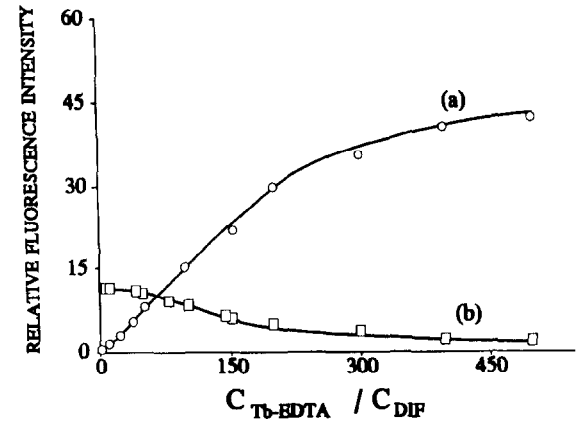

Fig. 3. Effect of the molar excess of Tb–EDTA mixture on: (a) the analytical signal of the DIF–Tb–EDTA ternary complex ($\lambda_{ex} = 284$ nm, $\lambda_{em} = 546$ nm) and (b), on the intrinsic fluorescence of diflunisal ($\lambda_{ex} = 272$ nm, $\lambda_{em} = 420$ nm).

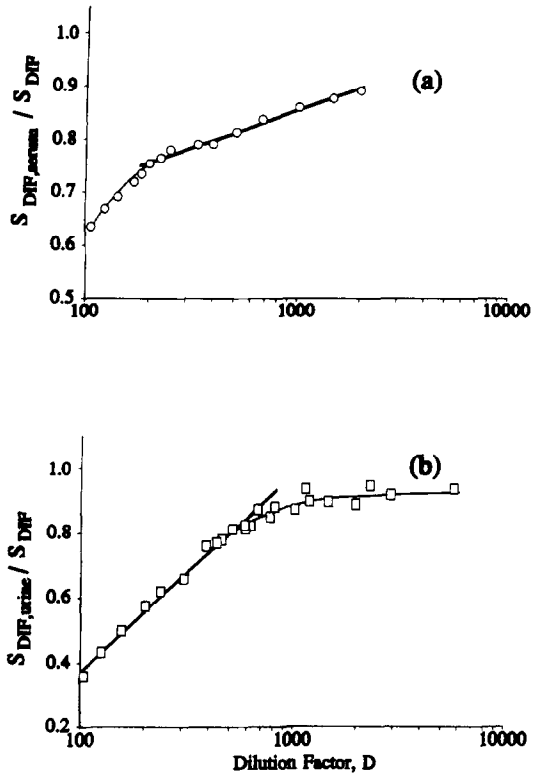

Fig. 4. Effect of the dilution factor, D, of serum (a) and urine (b) samples on the slope of the calibration graph for diflunisal.

three series of samples covering the range of interest for DIF (0.05, 0.50 and 5.00 $\mu g\ ml^{-1}$) were analysed and the corresponding R.S.D. (%) values were found to be 8.5, 3.0 and 2.0, respectively ($n=9$).

3.3. Determination of diflunisal added to serum and urine samples

For measuring diflunisal in serum and urine, untreated samples were used. The fluorescence signals

Table 1
Analytical recovery of DIF added to serum samples

Concentration added ($\mu g\ ml^{-1}$)	Concentration found [a] ($\mu g\ ml^{-1}$)	Recovery ± S.D. (%)
5.0	4.95	99 ± 2
10.0	9.68	97 ± 2
20.0	19.60	98 ± 3
50.0	48.80	98 ± 1
100.0	101.30	101 ± 3
Mean		98.6

[a] Mean of four measurements at different dilutions for each sample (1000-, 340-, 200- and 150-fold dilutions).

Table 2
Analytical recovery of DIF added to urine samples

Concentration added ($\mu g\ ml^{-1}$)	Concentration found [a] ($\mu g\ ml^{-1}$)	Recovery ± S.D. (%)
3.0	3.10	103 ± 4
6.0	5.96	99 ± 1
10.0	9.80	98 ± 4
20.0	19.70	98 ± 2
40.0	40.60	101 ± 2
100.0	99.40	99 ± 5
Mean		99.7

[a] Mean of three measurements (200-fold dilution).

obtained for serum and urine samples spiked with diflunisal were lower than those obtained with the corresponding DIF aqueous standards, depending on the dilution factor of the sample. To choose the better procedure for measuring DIF in serum and urine (direct calibration or standard addition), a detailed study of the effect of the dilution factor of the sample (D) on the slope of DIF calibration graphs was carried out (Fig. 4). As can be seen from Fig. 4a, the slope of the calibration graph linearly increased with the logarithm of the dilution factor between 200 and 1100 for serum according to Eq. 3. For higher dilutions the slope of the calibration graph in serum practically reaches that of aqueous standard solutions. For urine samples (Fig. 4b) the slope of the calibration graph increased linearly with the logarithm of the dilution factor between 150 and 700 according to Eq. 4, reaching that of aqueous standards at higher dilutions (>700).

$$\text{Slope}_{DIF,s}/\text{Slope}_{DIF} = 0.152(\pm 0.01)\log D + 0.407(\pm 0.027) \quad (r=0.9950) \tag{3}$$

$$\text{Slope}_{DIF,u}/\text{Slope}_{DIF} = 0.592(\pm 0.023)\log D - 0.806(\pm 0.056) \quad (r=0.9930) \tag{4}$$

Thus, diflunisal added to serum and urine can be quantified by using: (i) the standard additions technique or (ii) a direct calibration graph and applying the correction as dictated by Eqs. 1 and 2. In this study, the second approach was used. Analytical recovery experiments on serum and urine samples spiked with DIF (using different dilution factors for each sample)

are shown in Tables 1 and 2, respectively. The concentrations selected are typical for DIF concentration levels in human serum and urine for 50 h after a single 750 mg oral dose [13,14].

3.4. Interference studies

The absence of detectable blanks in diflunisal-free serum and urine indicates that the constituents of the biological samples do not interfere with the fluorescence measurements. Interference from other drugs was tested by analysing serum and urine samples spiked with DIF where amounts of the drug under investigation were also added. None of the drugs tested (naproxen, indomethacin, amitriptyline, phenacetin, caffeine, antipyrine, theophylline) interfered with the determination of DIF even at concentrations higher than those achieved therapeutically. The only interference occurs from salicylic acid which also forms a ternary complex with Tb–EDTA at mass ratios (salicylic acid/diflunisal) > 0.3. This interference could be avoided by using synchronous scanning second-derivative fluorescence spectrometry (SDSFS) for measurements. Preliminary experiments have shown that, by using SDSFS, it is possible to determine simultaneously diflunisal and salicylic acid without any preliminary separation step. This work is in progress.

4. Conclusions

The present method for the determination of diflunisal is highly sensitive with detection limits 5 and 70 times lower than those obtained by the previously reported fluorimetric [4] and LC [1] methods, respectively. The method is very simple and rapid; it allows the determination of diflunisal in biological fluids without any preliminary steps, and requires only a small sample volume (10 μl). Moreover, the method can be easily adopted to LC or flow-injection techniques.

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