Spectrofluorimetric determination of anthranilic acid derivatives based on terbium sensitized fluorescence

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Terbium sensitized fluorescence was used to develop a sensitive and simple spectrofluorimetric method for the determination of the anthranilic acid derivatives furosemide and mefenamic and tolfenamic acids. The method makes use of radiative energy transfer from anthranilates to terbium ions in alkaline methanolic solutions. Optimum conditions for the formation of the anthranilate–Tb^{3+} complexes were investigated. Under optimized conditions, the detection limits are 6×10^{-9}, 1.4×10^{-8} and 9.0×10^{-9} mol l^{-1} for furosemide, mefenamic acids and tolfenamic acid, respectively. The range of application is 2.5×10^{-8}–5.0×10^{-5} mol l^{-1} for all three drugs. The method was successfully applied to the determination of furosemide and mefenamic and tolfenamic acids in serum after extraction of the samples with ethyl acetate, evaporation of the organic layer under a stream of nitrogen at 40 °C and reconstitution of the residue with alkaline methanolic terbium solution prior to instrumental measurement. The mean recoveries from serum samples spiked with furosemide (5.0×10^{-7}, 2.0×10^{-6} and 8.0×10^{-6} mol l^{-1}), mefenamic acid (3.0×10^{-6}, 9.0×10^{-6} and 3.0×10^{-5} mol l^{-1}) and tolfenamic acid (3.1×10^{-6}, 12.5×10^{-6} and 2.5×10^{-5} mol l^{-1}) were 96 ± 8, 101 ± 5 and 98 ± 7%, respectively. The within-run precision (RSD) for the method for two serum samples of each drug varied from 2 to 8% and the day-to-day precision for two concentration levels varied from 2 to 13%.

Introduction

The sensitization of the fluorescence of lanthanide ions by organic ligands, especially that of europium and terbium, has been extensively used during the past 20 years in various applications, including the investigation of biological systems,[1,2] immunoassays and DNA hybridization assays,[3–6] quantification of organic compounds[7–9] and chromatographic detection.[7,10] The specific strong fluorescence of lanthanide ions, which usually occurs as a result of intramolecular energy transfer through the excited triplet state of the ligand (donor) to the emitting level of the lanthanide ion (acceptor), is characterized by large Stokes shifts, narrow emission bands and long fluorescence lifetimes.[11] Less effective, diffusion controlled energy transfer can also occur through the excited state of a non-chelated organic compound to the lanthanide ion.[12]

Terbium ions can form fluorescent complexes with several classes of organic compounds.[8,9,13,14] Depending on the type of organic ligand, the formation of terbium complexes may occur either in slightly acidic (pH 5–6) or in strongly alkaline solutions (pH > 12). For example, salicylic acid and its derivatives (sulfosalicylic acid, diflunizal, tiron, *etc.*) form fluorescent ternary complexes with terbium and EDTA in alkaline solutions (pH ≈ 12.5),[14] whereas fluorescent terbium complexes with benzoic acid derivatives, quinolones and pyridinecarboxylic acid derivatives are formed in slightly acidic solutions (pH ≈ 6) in the presence of trioctylphosphine oxide (TOPO), which serves as a synergistic agent.[8,9]

Furosemide, a potent short-acting loop diuretic, and mefenamic and tolfenamic acids, two non-steroidal anti-inflammatory drugs, are derivatives of anthranilic acid (Fig. 1). Furosemide (4-chloro-2-furfurylamino-5-sulfamoylbenzoic acid) (FR) is one of the most widely used loop diuretics for the treatment of oedema and hypertension.[15] Along with other diuretics, FR has been misused and abused in sports to reduce the body mass rapidly or to decrease the urine concentration of doping agents so as to avoid their detection.[16] Hence rapid and sensitive methods are required for the determination of diuretics in therapeutic drug monitoring and in the control of doping. Methods for the determination of furosemide in biological fluids mainly include HPLC with spectrophotometric or fluorimetric detection.[17–20]

Mefenamic acid [*N*-(2,3-xylyl)anthranilic acid] (MF) and tolfenamic acid [*N*-(2-methyl-3-chlorophenyl)anthranilic acid] (TF) are both potent prostaglandin synthetase inhibitors widely used clinically as non-steroidal anti-inflammatory and analgesic–antipyretic drugs.[21,22] For the determination of mefenamic and tolfenamic acids in biological fluids, a number of HPLC methods with spectrophotometric detection have been reported.[21–24] Although fluorimetry is widely used as a sensitive detection system, there is only one report on the fluorimetric

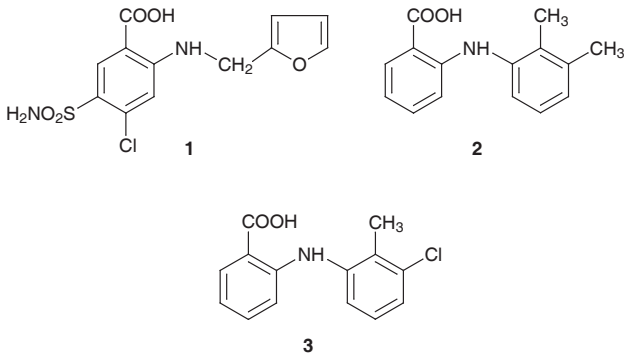

Fig. 1 Structures of furosemide (**1**), mefenamic acid (**2**) and tolfenamic acid (**3**).

determination of mefenamic acid in pharmaceuticals based on the formation of a fluorescent compound with Al(III).[25]

In our previous work on terbium sensitized fluorimetry in aqueous solutions, it was mentioned that furosemide interferes with the determination of fluoroquinolone antibiotics.[8] In preliminary experiments, we found that anthranilic acid and its derivatives can form fluorescent complexes with terbium ions in weakly alkaline (pH ≈ 8) aqueous solution. Moreover, we observed a substantial enhancement of terbium sensitized fluorescence by anthranilates when the reaction was performed in organic solvents such as dimethylformamide (DMF), dimethyl sulfoxide (DMSO) and alcohols.

In this paper, we report for the first time the results of a study on the intrinsic fluorescent properties of the three anthranilic acid derivatives and on their ability to sensitize terbium ion fluorescence in both aqueous and non-aqueous solutions. We also report a sensitive and simple method for the determination of furosemide and mefenamic and tolfenamic acids in serum based on terbium sensitized fluorescence in methanolic solutions. The method is compared with the spectrofluorimetric determination of these compounds based on their native fluorescence.

Experimental

Apparatus

A Model 512-A double beam fluorescence spectrophotometer (Perkin-Elmer, Norwalk, CT, USA) equipped with a 150 W xenon arc lamp and a magnetic stirrer under the cell holder was used. The instrument was interfaced with an IBM-PC 386DX microcomputer for data acquisition and calculations.[8] All measurements took place in a standard 10 mm pathlength quartz cell, thermostated at 25.0 °C. The energy mode and excitation and emission monochromator bandwidths of 20 nm were used.

Reagents and solutions

Furosemide and mefenamic and tolfenamic acids were kindly donated by pharmaceutical companies. All other chemicals were obtained from Merck (Darmstadt, Germany) and Sigma (St. Louis, MO, USA), unless stated otherwise. Water from a Millipore (Molsheim, France) Milli-Q RG ultra-pure water system was used to prepare all aqueous solutions.

A stock standard solution of terbium containing 0.1 mol l^{-1} was prepared by dissolving the required amount of terbium(III) chloride hexahydrate ($TbCl_3 \cdot 6H_2O$, 99.9%) (Aldrich, Milwaukee, WI, USA) in methanol. Stock standard solutions of FR, MF and TF containing 0.01 mol l^{-1} were prepared in methanol. Working standard solutions were prepared daily by dilution with methanol. The stock standard solutions of FR, MF and TF were stable for several months under refrigeration. Aqueous stock solutions of FR, MF and TF containing 0.01 mol l^{-1} were also prepared. These solutions were used for recovery experiments in serum. A working solution of terbium (0.5×10^{-3} mol l^{-1})–triethylamine (TEA) (2.0×10^{-3} mol l^{-1}) in methanol was used as a single reagent.

Sample preparation and measurement

Serum samples and standards were prepared in human serum pool from healthy subjects spiked with the appropriate drug in the concentration range 5.0×10^{-7}–1.6×10^{-5} mol l^{-1} for FR and 1.5×10^{-6}–5.0×10^{-5} mol l^{-1} for MF and TF.

To 0.250 ml of serum sample or standard of furosemide (0.200 ml for MF or TF samples), add 0.060 ml (0.050 ml for MF or TF) of 8.5 mol l^{-1} acetic acid solution, vortex mix briefly and add 2.00 ml (1.60 ml for MF or TF samples) of ethyl acetate. Then, sonicate the mixture for 20 min and centrifuge for 15 min at 1500*g*. Transfer 1.70 ml (1.40 ml for MF or TF samples) of the organic layer into a glass tube and evaporate the organic layer under a stream of nitrogen at 40 °C. Reconstitute the residue with 2.00 ml of working terbium solution and measure the fluorescence intensity at $\lambda_{ex}/\lambda_{em}$ = 350/550 nm for FR and 360/550 nm for MF and TF *versus* a blank in which drug-free serum is substituted for the standard or sample. Calculate the unknown concentration from the calibration graph.

Results and discussion

Luminescence spectra

Acidic (pH ≈ 3) aqueous solutions of FR, MF and TF show strong intrinsic fluorescence with $\lambda_{ex}/\lambda_{em}$ maxima at 350/400, 385/435 and 380/435 nm for FR, MF and TF, respectively. Excitation and emission spectra of the intrinsic fluorescence for all three compounds are shown in Fig. 2. The fluorescence intensity is pH dependent with the maximum fluorescence observed over the pH range 2.0–3.5 for the three anthranilates. At pH > 4.0 the intrinsic fluorescence is negligible and a hypsochromic shift in the excitation and emission maxima is observed for all anthranilates due to ionization of carboxylic group (pK_a = 3.9 and 4.2 for FR and MF, respectively). As an example, the fluorescence spectra of FR *versus* pH are shown in Fig. 3. The native fluorescence of FR is increased in the presence of up to 40% v/v of methanol, whereas the fluorescence of MF and TF is strongly decreased upon addition of methanol (Fig. 4). This difference in fluorescence emission between FR and the other two anthranilates could be explained by the hypsochromic shift of about 60 nm in both the excitation and emission maxima for TF and MF observed upon addition of methanol. In contrast, there is no substantial shift in the excitation or emission maxima for FR. Hence the optimum conditions for measuring the intrinsic fluorescence of anthranilates are as follows: methanol–aqueous solution, 40% v/v, with apparent pH 2.5 for FR and aqueous solution of pH 3.0 for MF and TF.

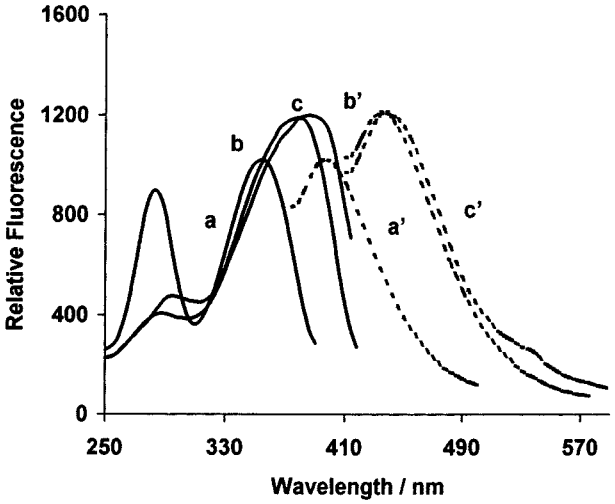

Fig. 2 Fluorescence excitation (a–c) and emission (a′–c′) spectra (uncorrected) of aqueous solutions (pH 3.0) of FR, $C = 1.0 \times 10^{-6}$ mol l^{-1}, λ_{ex} = 350 nm, λ_{em} = 400 nm (a, a′), MF, $C = 1.0 \times 10^{-6}$ mol l^{-1}, λ_{ex} = 385 nm, λ_{em} = 435 nm (b, b′) and TF, $C = 1.0 \times 10^{-6}$ mol l^{-1}, λ_{ex} = 380 nm, λ_{em} = 435 nm (c, c′).

Complex formation of anthranilates with Tb^{3+}

The addition of terbium ions to weakly alkaline (pH 8.0) aqueous solutions of FR, MF and TF results in the appearance of new emission bands in the range 450–610 nm (λ_{max} = 550 nm), characteristic of terbium ion fluorescence, and is due to the complex formation of anthranilates with terbium (Fig. 5).

The effect of pH on the fluorescence intensity of terbium complexes with FR, MF and TF in aqueous solutions was studied. To avoid precipitation of terbium hydroxide in alkaline solutions, a 1% v/v solution of triethylamine was used for the adjustment of pH.[26] It was found that the optimum pH for the complex formation of terbium with FR, MF and TF was in the range 8.0–10.0.

It is well known that water molecules strongly quench terbium sensitized luminescence.[27] For this reason, synergistic agents such as TOPO (for complex formation in slightly acidic solutions) or EDTA (for complex formation in alkaline solutions) are usually employed to exclude water molecules from the coordination sphere of terbium ions. The use of organic solvents can also increase the efficiency of the energy transfer from the organic ligand to terbium ions.[27] Indeed, we observed a considerable increase (about 40-fold) in the fluorescence intensity of the terbium complexes with FR, MF and TF in mixed aqueous–methanolic solutions with the maximum fluorescence observed in pure methanolic solutions. We studied the effect of various organic solvents, such as DMF, DMSO, acetonitrile, acetone, butanol, ethanol and methanol, on the fluorescence of terbium complexes with furosemide and the results (expressed as the ratio of the relative fluorescence of the complexes in particular solvent to that in aqueous solutions) are shown in Fig. 6. As can be seen, methanolic solutions of the complexes show the most intense fluorescence. The effect of acidity on the fluorescence intensity of terbium complexes with FR, MF and TF in methanolic solutions was studied. The maximum fluorescence signal for all three compounds was obtained in alkaline TEA solutions at TEA concentrations in the range (1.5–4.0) $\times$ 10^{-3} mol l^{-1}. A TEA concentration of 2.0 $\times$ 10^{-3} mol l^{-1} was selected for subsequent measurements.

We also studied the effect of terbium concentration on the fluorescence intensity for all three complexes in the range (0.25–2.5) $\times$ 10^{-3} mol l^{-1} at a final concentration of the drug of 1.0 $\times$ 10^{-6} mol l^{-1}. The maximum fluorescence signal was observed at terbium concentration in the range (0.3–1.5) $\times$ 10^{-3} mol l^{-1} and a concentration of 0.5 $\times$ 10^{-3} mol l^{-1} was selected for subsequent measurements.

General analytical characteristics

Under optimized conditions for intrinsic fluorescence, the analyte final concentration and relative fluorescence intensity were linearly related over the range 1.0 $\times$ 10^{-7}–5.0 $\times$ 10^{-5}

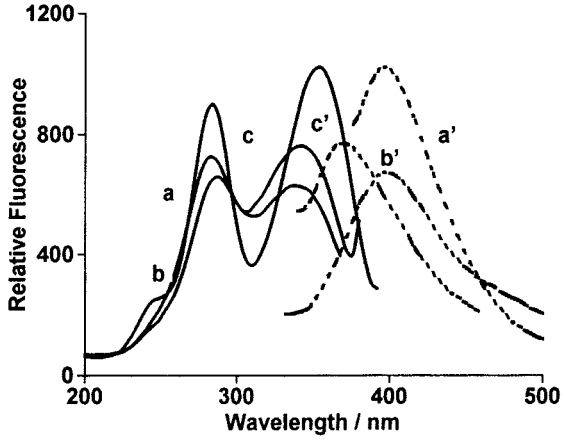

Fig. 3 Fluorescence excitation (a-c) and emission (a′-c′) spectra of aqueous solutions of FR at pH 2.6, C = 1.0 $\times$ 10^{-6} mol l^{-1} (a, a′), pH 6.0, C = 5.0 $\times$ 10^{-5} mol l^{-1} (b, b′) and pH 11.6, C = 5.0 $\times$ 10^{-5} mol l^{-1} (c, c′); λ_{ex} = 350 nm, λ_{em} = 400 nm.

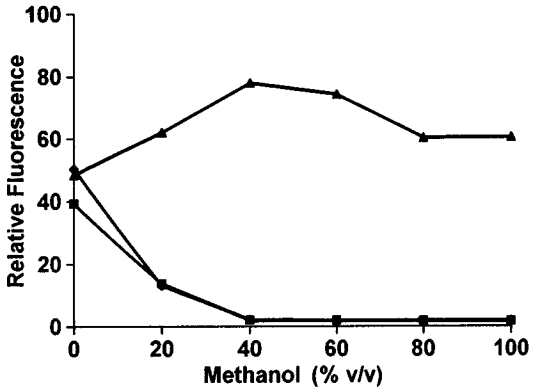

Fig. 4 Effect of methanol concentration on the intrinsic fluorescence (apparent pH = 3.0) of (▲) FR, C = 1.0 $\times$ 10^{-6} mol l^{-1}, λ_{ex} = 350 nm, λ_{em} = 400 nm, (◆) MF, C = 1.0 $\times$ 10^{-6} mol l^{-1}, λ_{ex} = 385 nm, λ_{em} = 435 nm and (■) TF, C = 1.0 $\times$ 10^{-6} mol l^{-1}, λ_{ex} = 380 nm, λ_{em} = 435 nm.

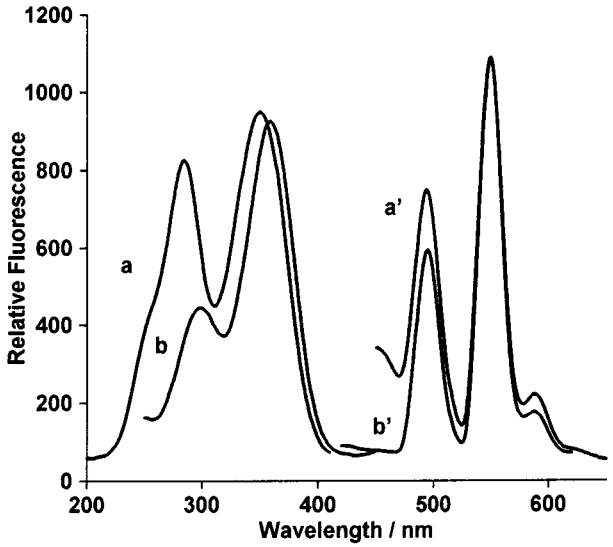

Fig. 5 Fluorescence excitation (a, b) and emission (a′, b′) spectra (uncorrected) of methanolic solutions of FR–Tb^{3+} complex, C_{FR} = 1.0 $\times$ 10^{-6} mol l^{-1}, C_{Tb} = 1.0 $\times$ 10^{-3} mol l^{-1}, λ_{ex} = 350 nm, λ_{em} = 550 nm (a, a′) and TF–Tb^{3+} complex, C_{TF} = 1.0 $\times$ 10^{-6} mol l^{-1}, C_{Tb} = 1.0 $\times$ 10^{-3} mol l^{-1}, λ_{ex} = 360 nm, λ_{em} = 550 nm (b, b′).

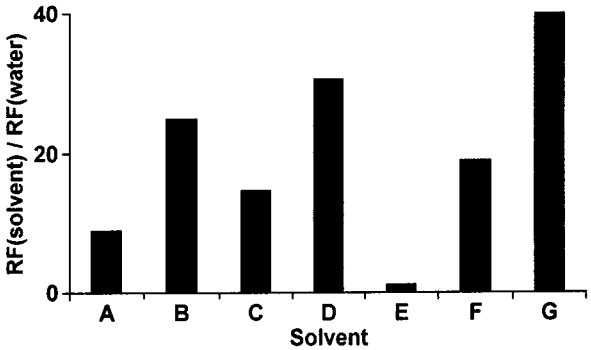

Fig. 6 Effect of organic solvents on the fluorescence of the FR–Tb^{3+} complex: (A) acetone; (B) acetonitrile; (C) DMF; (D) DMSO; (E) butanol; (F) ethanol; and (G) methanol. C_{FR} = 1.0 $\times$ 10^{-6} mol l^{-1}, C_{Tb} = 5.0 $\times$ 10^{-4} mol l^{-1}, λ_{ex} = 350 nm, λ_{em} = 550 nm. RF = relative fluorescence intensity.

mol l^{-1} for FR and 5.0×10^{-7}–5.0×10^{-5} mol l^{-1} for MF and TF. For terbium sensitized fluorescence, the linear range was 2.5×10^{-8}–5.0×10^{-5} mol l^{-1} for all three compounds. Pearson's correlation coefficients (r) for the calibration graphs were 0.999, 0.9994 and 0.998 (intrinsic fluorescence) and 0.9993, 0.996 and 0.998 (terbium sensitized fluorescence) for FR, MF and TF, respectively. The detection limits, defined as the concentration corresponding to a signal equal to three times the standard deviation of the lowest concentration, were 2.0×10^{-8}, 9.0×10^{-8} and 1.7×10^{-7} mol l^{-1} (intrinsic fluorescence) and 6.0×10^{-9}, 1.4×10^{-8} and 9.0×10^{-9} mol l^{-1} (terbium sensitized fluorescence) for FR, MF and TF, respectively. The relative standard deviations (RSDs), covering the range of interest for FR (5.0×10^{-8}, 1.0×10^{-6} and 1.0×10^{-5} mol l^{-1}) and MF and TF (5.0×10^{-7}, 5.0×10^{-6} and 5.0×10^{-7} mol l^{-1}) varied from 1.0 to 4.0%.

Serum samples

To apply the method to the determination of anthranilates in serum, we performed a detailed study of the deproteinization of serum by methanol and acetonitrile. After precipitation of proteins with methanol, high background signals were observed, probably owing to insufficient deproteinization of the sample. Acetonitrile was found to be the most efficient deproteinization agent. However, about a 20-fold decrease in analytical signals in final mixed aqueous–acetonitrile–methanolic solutions was observed. To obtain the maximum analytical signals (by performing measurements in methanolic solutions), we followed the reported extraction procedure for furosemide[18] with some modifications. Acidified with acetic acid, serum samples were extracted with ethyl acetate, the organic layer was evaporated to dryness under a stream of nitrogen at 40 °C and the residue was reconstituted in alkaline methanolic terbium solution. Extraction of furosemide was performed under sonication. For the optimization of the extraction procedure, we studied the effects of sonication time and the acidity of the samples prior to the extraction on the recovery of the drug. The maximum recovery of furosemide was achieved from serum samples acidified with acetic acid at a final concentration of 2.0 mol l^{-1} and using a sonication time of 20 min.

Under the optimum conditions for the extraction procedure, we compared the calibration graphs obtained for furosemide with aqueous and serum standards. The slope of the calibration graph (fluorescence intensity *versus* concentration of FR, mol l^{-1}) with aqueous furosemide standards was found to be $1.1 \times 10^7 \pm 2.1 \times 10^5$ and was higher ($t_{exp.} = 19.57 \gg t_{theor.} = 2.45$, $n = 8$, $P = 0.05$) than the slope obtained with serum standards, which was found to be $6.7 \times 10^6 \pm 4.5 \times 10^4$. This is probably due to some kind of binding of furosemide with serum proteins. On the other hand, the background signals obtained from drug-free serum were found to be very low and did not vary from sample to sample. Therefore, for the determination of furosemide and the other anthranilates in serum, calibration graphs obtained using serum standards were used.

The method was successfully applied to the determination of FR, MF and TF in synthetic serum samples. The range of concentrations of FR, MF and TF in serum corresponds to the pharmacokinetic profile of the drugs after oral administration of 40 mg of FR[17] and of 2 mg kg^{-1} body mass for MF[21] and TF[23] and ranged from 5.0×10^{-7} to 8.0×10^{-6} mol l^{-1} for FR and from 3.0×10^{-6} to 3.0×10^{-5} mol l^{-1} for MF and TF.

We also investigated the possibility of measuring anthranilates in serum by using their native fluorescence. For this purpose, we followed the whole procedure for serum samples of furosemide and redissolved the residue (after removing the ethyl acetate) with aqueous–methanolic solution of pH 2.5. The ratio of the slopes of the calibration graphs obtained by measuring terbium sensitized luminescence and intrinsic fluorescence for furosemide standards was the same as obtained for aqueous standards. However, the background signals obtained from drug-free serum were more than 20 times higher than those obtained by measuring terbium sensitized luminescence, thus making intrinsic fluorescence unsuitable for the fluorimetric measurement of these compounds in serum.

Recovery and precision

The analytical recovery was assessed by analysing serum samples spiked with FR, MF and TF at three different concentrations and the results are summarized in Table 1. The results obtained are satisfactory and the recovery ranged from 90 to 106%. To determine the within-run precision of the method, two serum pools containing different concentrations of each drug were measured eight times each. To assess the day-to-day precision, repeated analyses of two serum samples over 2 weeks were performed and the results are given in Table 2.

Effect of foreign substances

The influence of other drugs which can also form fluorescent complexes with terbium, such as derivatives of salicylic acid (diflunizal, salol, salicylates) and fluoroquinolone antibiotics, on the determination of anthranilic acid derivatives in serum was investigated. Amounts of the substances under investigation were added to give a final concentration of 5.0×10^{-6} mol l^{-1} in serum samples spiked with FR and the samples where subjected to the whole procedure. Fluoroquinolone antibiotics do not interfere in the determination of anthranilates by this method, whereas salicylates, diflunizal and salol caused positive errors.

In humans FR, MF and TF are mainly metabolized into acyl glucuronides.[28,29] The pharmacokinetics of furosemide are the most extensively studied. In serum, furosemide is the predominant species[30] and only traces of FR glucuronide have been detected.[28] Taking into account the involvement of the free

Table 1 Analytical recoveries of FR, MF and TF in serum samples

Analyte	Added/ mol $l^{-1} \times 10^6$	Found[a] ± s/ mol $l^{-1} \times 10^6$	Recovery (%)
FR	0.5	0.47 ± 0.05	94
	2.0	2.1 ± 0.1	105
	8.0	7.2 ± 0.1	90
MF	3.0	2.9 ± 0.2	97
	9.0	8.9 ± 0.1	99
	30.0	31.9 ± 0.1	106
TF	3.1	3.2 ± 0.2	103
	12.5	12.7 ± 0.5	102
	25.0	22.7 ± 0.3	91

[a] Average of three measurements.

Table 2 Precision data

Analyte	Mean concentration/ mol $l^{-1} \times 10^6$	Within-run precision[a] (RSD) (%)	Mean concentration/ mol $l^{-1} \times 10^6$	Day-to-day precision[b] (RSD) (%)
FR	0.86	8.4	0.58	7.9
	2.1	5.6	1.1	5.6
MF	2.8	4.2	2.2	12.8
	22.0	2.0	30.0	2.4
TF	3.7	5.0	3.2	5.6
	22.0	2.5	24.0	5.6

[a] Average of eight measurements. [b] Average of five measurements.

carboxyl group of anthranilates in the formation of the complexes with terbium, along with the very low concentration of glucuronides in serum,[28] no interference from these metabolites is expected in the determination of anthranilic acid derivatives by the proposed method.

Conclusions

The sensitization of terbium ion luminescence in methanolic solutions by furosemide and mefenamic and tolfenamic acid was investigated for the first time and was used for measuring these anthranilates in serum. The spectrofluorimetric method developed is relatively simple and more sensitive and selective than the method which makes use of the native fluorescence of these drugs. The proposed method could be easily applied in pharmakokinetic studies and also for the rapid screening of these drugs in serum. The chemical system developed could also be used as a very sensitive and selective detection system for furosemide and mefenamic and tolfenamic acid after the separation of these drugs by HPLC.

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