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Short Communication

The use of fluorescamine (Fluram) in fluorimetric trace analysis of primary amines of pharmaceutical and biological interest*

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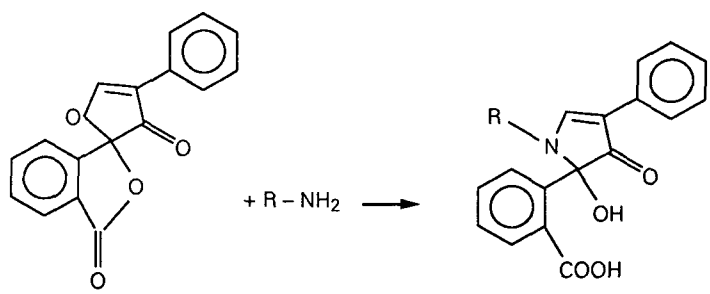
Introduction

Flurescamine (also known as Fluram) is 4-phenylspiro(furan-2(3H),1-phthalan)-3,3′-dione; it is a reagent which reacts instantaneously with a primary amino-group in solution at pH 8–9 to give the highly fluorescent compound, pyrrolinone [1, 2].

Reaction with secondary amines gives the non-fluorescent compound, aminoenone [2–4]. Fluorescamine can be used for the general [5] and selective [6–8] fluorimetric determination of compounds that possess a primary amino-group; examples are amino acids [9, 10] peptides and proteins [11–13]. This compound has also been used as a derivatizing reagent in HPLC [14], as a fluorimetric detection reagent in capillary electrophoresis [15] and in HPTLC [16]. The fluorescence quantum yield of many compounds is very sensitive to the environment of the excited state [17, 18]; for example, it has been shown that water molecules are capable of interacting with the excited state of the fluorophores [19] to form an excited state complex (exciplex). This reaction is competitive with fluorescence and therefore it can reduce the quantum yield of fluorescence. The presence of inorganic ions in aqueous media also decrease the intensity of fluorescence. Fluorescamine itself is rapidly hydrolysed to form a non-fluorescent product under certain reaction conditions [9]. The factors mentioned previously affect the sensitivity and precision of the fluorimetric method. A logical approach to the prevention of exciplex formation and the elimination of inorganic ions is to carry out the assay with organic media [18].

The aim of this work was to develop a sensitive and precise fluorimetric method for the analysis of traces of primary amines based on the extraction of pyrrolinone after treatment of the analyte with fluorescamine in aqueous buffered solution.



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Experimental

Chemicals and reagents

Fluorescamine (Fluram) was purchased from Fluka (Buchs, Switzerland), noradrenaline acid tartrate was from Aldrich-Chemie (UK); daunorubicin was from Laboratorie Roger Bellon (Italy); amino acids, organic solvents and all other reagents were from E. Merck (Germany). The solutions were prepared in triple-distilled water.

Apparatus

Fluorimetric measurements were made on a Shimadzu spectrofluorimeter model RF-540 U-4.0 with a xenon lamp and 1-cm quartz cells.

Solutions

Stock solutions of amino acids were prepared by dissolving 0.1–10 μg of each compound in 10 ml of water. Noradrenaline acid tartrate (NE), dopamine (DA) and daunorubicin stock solutions were prepared by dissolving 1 mg of each compound in 10 ml of water.

Solutions of 1 M sodium bicarbonate, 0.66 M boric acid and 0.075 M borax were prepared. A 1% v/v solution of ascorbic acid was prepared. A 5 g mass of ethylenediamine tetra-acetic acid disodium salt (EDTA) was dissolved in water and the solution was diluted to 100 ml with water. Borate buffer (0.2 M, pH 9), phosphate buffer (0.2 M, pH 8.5) and ammonia buffer (2 M, pH 9) were prepared. 0.05% (w/v) fluorescamine stock solution was prepared in acetone. A 100 × 10 mm i.d. column containing 0.3–0.9 mm carboxylic resin (Na form) was used.

Calibration graphs

Aqueous standard solutions of amino acids

Stage 1. 50-ml portions of aqueous solutions containing 0.5–50 μg of each amino acid (0.01–1 μg ml^{-1}) were transferred to 250-ml separation funnels; the pH of each solution was adjusted to 9.0 by addition of 1 ml of borate buffer.

Stage 2. To each solution 1 ml of fluorescamine solution was added, the mixture was shaken for 10 s and 5 ml of organic solvent and 1 ml of 6 M sulphuric acid were added rapidly. The mixture was further shaken for 10 s; after 1 min the intensity of fluorescence of the organic phase was measured.

Aqueous standard solutions of catecholamines. A 5 ml volume of each aqueous solution containing 0.01–1 μg ml^{-1} of each catecholamine was transferred to 150 × 14 mm i.d. glass test tubes; 0.5 ml of EDTA solution and 0.5 ml of ascorbic acid solution were added and the pH was adjusted to 6.2 with $NaHCO_3$ solution. The mixture was poured through the ion-exchange column at 2.5 cm min^{-1} and the column was washed with 6 ml of water. The catecholamine was eluted with 4 ml of boric acid solution and the eluate was transferred to the 250-ml separation funnels; the pH was adjusted to 9.0 with phosphate buffer. The assay was continued as described for the second stage of the procedure for amino acids.

Aqueous standard solutions of danourubicin. A 5 ml volume of aqueous solutions containing 0.1–1 μg ml^{-1} of drug were transferred to 100-ml separation funnels. The pH was adjusted to 7–10 with 2 ml of ammonia buffer; the drug was extracted with 5 ml of chloroform, then it was back-extracted into 5 ml of 0.1 M HCl. The pH of the aqueous solution was adjusted to 9.0 with 5 ml of ammonia buffer and the assay was continued as described for the second stage of the procedure for amino acids.

General procedure for total catecholamines (NE + DA) in urine

Two sample solutions: (1) containing urine; and (2) containing the same urine plus 0.25 ml of 5 μg ml^{-1} standard solution of noradrenaline were prepared. The assay was carried out as in the procedure for aqueous standard solutions of catecholamines.

General procedure for daunorubicin in human plasma

A 5 ml volume of clean human plasma was spiked with 0.5 μg ml^{-1} of drug and the analysis procedure was carried out as described for aqueous standard solutions of daunorubicin. The blank value obtained for the same volume of plasma was subtracted from the fluorescence intensity of the spiked plasma.

Results and Discussion

Determination of excitation and emission spectra

The spectra obtained are shown in Fig. 1. The emission spectrum shows a maximum at

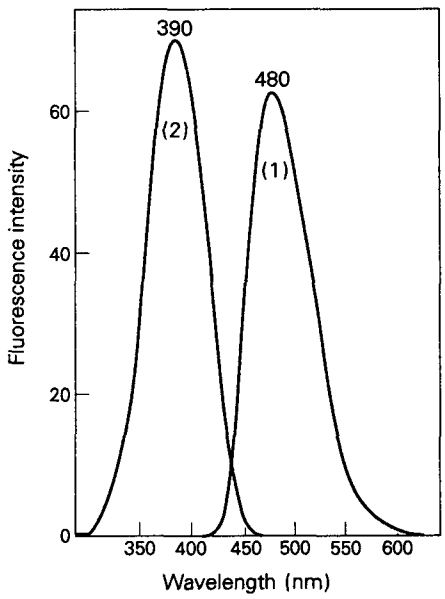

Figure 1
Fluorescence spectrum of pyrrolinone. (1) Excitation spectrum with emission at 480 nm; (2) emission spectrum with excitation at 350 nm.

wavelength 480 nm with excitation at 350 nm. The excitation spectrum shows a maximum at 390 nm with emission at 480 nm. The spectral characteristics of pyrrolinones obtained from different compounds are similar. The fluorescence intensity of the examined compounds except for NE and DA varies slightly with the nature of the analyte.

Choice of organic solvent for the extraction procedure

Different solvents such as dichloromethane, chloroform and carbon tetrachloride were examined for the extraction of pyrrolinone. Maximum extraction efficiency and fluorescence intensity were observed with dichloromethane.

Effect of pH on pyrrolinone extraction

Since it was known that compounds possessing a primary amino-group react with fluorescamine optimally in the presence of phosphate (pH 7–8) or borate (pH 9–10) buffer [1], attempts were made to improve pH control in the extraction step of pyrrolinone. Figure 2 illustrates the fluorescence intensity of the organic phase versus pH of the aqueous phase. When the pH of aqueous solutions was adjusted to ≤ 1 with 6 M sulphuric acid, maximum fluorescence intensity was achieved; the fluorescence intensity of the organic phase

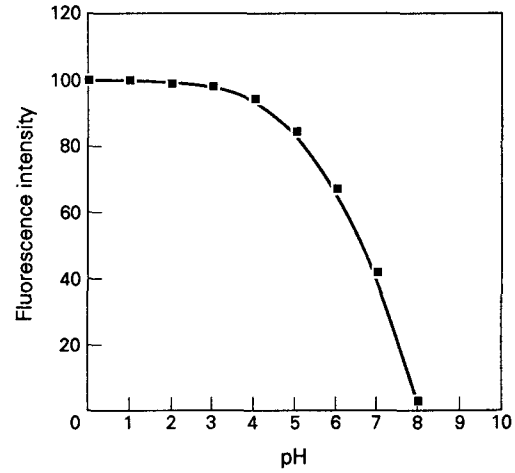

Figure 2
Effect of pH on the extraction of pyrrolinone in dichloromethane.

was equal to that of the blank solution for the second extraction. Therefore it can be concluded that the extraction efficiency of pyrrolinone is over 99% for a single-step extraction.

Enhancement of fluorescence intensity in an organic solvent

Results for the fluorescence intensity of the pyrrolinone formed between fluorescamine and glycine are presented in Fig. 3 in water media (a) and in an organic medium (b). At least a 4-fold increase in fluorescence intensity was observed in dichloromethane. This enhancement is probably due to the elimination

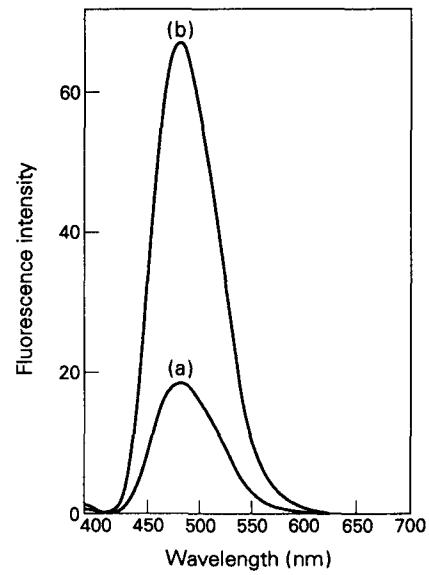

Figure 3
Fluorescence intensity of 1 ppm pyrrolinone (a) in water and (b) in dichloromethane.

of water molecules, inorganic ions and other compounds with quenching effects. By virtue of this enhancement of fluorescence, a relative improvement in sensitivity was confirmed.

Stability of pyrrolinone and fluorescence intensity

The emission spectrum of pyrrolinone formed between fluorescamine and glycine were measured at 5-min intervals in water (Fig. 4) and in the organic phase (Fig. 5). From the results obtained a 12 and 6% decrease was observed in fluorescence intensity in the aqueous and organic phases, respectively, after 5 min. The relative increase in fluorescence stability in the organic phase was confirmed.

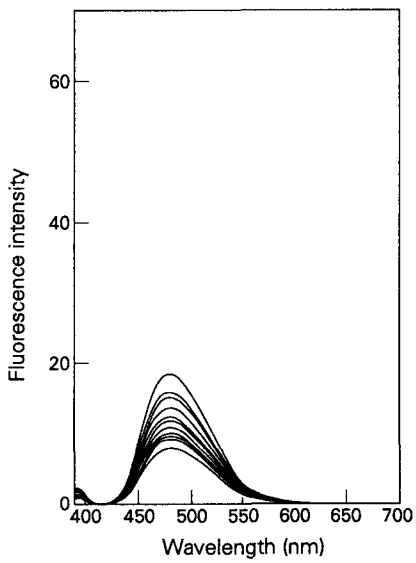

Figure 4
Fluorescence intensity of pyrrolinone in water measured at 5-min intervals.

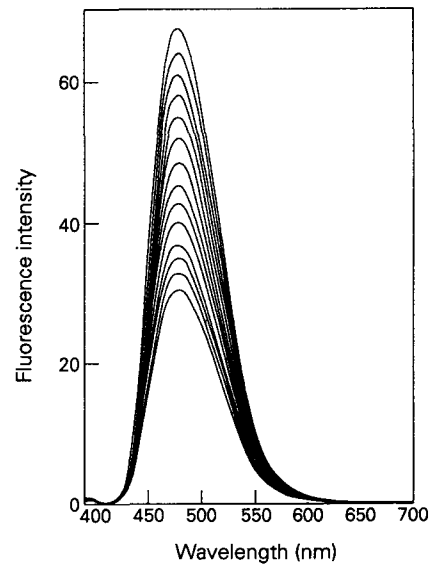

Figure 5
Fluorescence intensity of pyrrolinone in the organic phase measured at 5-min intervals.

Correlation between fluorescence intensity and the concentrations of the substance to be analysed

From the results obtained (Table 1), the fluorescence intensity was proportional to the concentration of glycine and phenylalanine over the range 10–100 ng ml^{-1} and over the range 0.1–1 μg ml^{-1} for tryptophan, noradrenaline, dopamine and daunorubicin.

Determination of total catecholamines (CA = NE + DA) in urine

As described in the general procedure, the internal standard addition method has been used for this purpose. Therefore the total concentration of CA can be calculated using the following relationship:

Table 1
The regression equation* and lower limits of detection of analytes

Analyte	Conc. (ppm)	r	A	B	LOD (ppm)
Glycine	0.01–0.1	0.999	894.71	31.18	0.007
Glycine	0.1–1	0.999	78.12	2.25	0.008
Phenylalanine	0.01–0.1	0.997	717.75	26.33	0.005
Phenylalanine	0.1–1	0.999	63.61	1.46	0.008
Tryptophan	0.01–0.1	0.974	1071	12.33	0.004
Tryptophan	0.1–1	0.999	108.27	2.35	0.005
Noradrenaline	0.1–1	0.997	97.66	0.015	0.003
Dopamine	0.1–1	0.998	97.66	0.015	0.003
Daunorubicin	0.1–1	0.999	77.42	7.67	0.004

*$y = Ax + B$.
LOD = Limit of detection.

$$C_0 = C \times E/(E - E_0),$$

where: C_0 = concentration of CA in urine sample, C = added standard concentration in organic phase, E_0 = fluorescence intensity of urine sample, and E = fluorescence intensity of urine sample plus added NE.

The precision of the proposed method was studied by the determination of CA five times for the urine sample (375 ng ml^{-1}). The mean value, standard deviation, true values and relative error (95% confidence limits) were 376.8 ng ml^{-1}; 3.27 ng ml^{-1}; 376.8 ± 4.06 ng ml^{-1} and 1.08%, respectively.

Determination of daunorubicine in human plasma

The concentration of the drug was calculated, using the results obtained by the general procedure. The precision of the method was studied by determination of the drug five times in spiked human plasma (500 ng ml^{-1}). The mean value, standard deviation, true value and relative error (95% confidence limits) were 496.8 ng ml^{-1}; 12.79 ng ml^{-1}; 496.8 ± 15.90 ng ml^{-1} and 3.2%, respectively.

Conclusions

From the results obtained, fluorescamine is a useful reagent for the extraction and fluorimetric determination of compounds with a primary amine group. The efficiency of the extraction procedure is excellent and the sensitivity of the method is good. The precision with dichloromethane as solvent is better than that with aqueous media. The method is also applicable to the determination of drugs and compounds of biological interest with a primary amine group.

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