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Micellar-enhanced synchronous-derivative fluorescence determination of derivatized folic acid in pharmaceutical preparations

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Abstract

A sensitive and inexpensive micellar-enhanced synchronous-derivative spectrofluorimetric method is described for the determination of folic acid in pharmaceutical preparations. The method is based upon derivatization of the vitamin with fluorescamine in acid solution and enhancement of the fluorescence with a surfactant. Linear fluorimetric analytical curves were obtained for folic acid concentrations of 0.05–6 μg ml^{-1} (detection limit 0.012 μg ml^{-1}); the RSD for 1 μg ml^{-1} of folic acid was 2.82%. The method has been applied to the determination of folic acid synthetic mixtures and pharmaceutical preparations.

Keywords: Fluorescamine; Folic acid; Micellar media; Pharmaceutical analysis; Synchronous-derivative spectrofluorimetry

1. Introduction

Folic acid (vitamin B_9) (*N*(4-((2-amino-1,4-di-hydro-4-oxo-6-pteridinylmethyl)amino)-benzoyl)-L-glutamic acid), which occurs naturally in cereals, is essential in humans [1]. It is one of the most important components of the haemopoietic system, being the coenzyme that controls the generation of ferrohaeme.

The interest in the determination of this compound lies in recent research which indicates that numerous diseases (for example, macrocytic anaemia associated with leucopenia, psychiatric disorders), especially those concerned with malformations during pregnancy and carcinogenic processes, are related to its deficiency. It is, therefore, very important to establish highly sensitive methods for the quantitative determination of trace amounts of folic acid.

A number of methods have consequently been reported for the determination of folic acid in different samples. The most widely used are those based on gas chromatography or high-performance liquid chromatography (HPLC) with absorption [2,3], fluorescence [4,5] or phosphorescence [6] detection, together with bioassays [7] and voltamperometry [8].

The main difficultly associated with such determinations is the determination of trace amounts in complex samples matrices so that selectivity and sensitivity are important requirements.

Fluorescence spectroscopy is a widely used technique in biochemical [9,10] and clinical research [11] as well as in chemical analysis [12]. However, despite its advantages, the emission spectra of most fluorophores are broad, unstructured and usually overlap on the wavelength scale. A technique which offers improved selectivity for luminescence analysis

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without having to sacrifice simplicity is the synchronous technique [13]. Its main attributes are its band-narrowing effect and the simplification of spectral profiles.

Advantages can be taken of these attributes to enhance sensitivity in the determination of compounds characterized by broad spectra. This is a relatively frequent situation with organic compounds, especially those where the intrinsic fluorescence is obtained by the use of derivatizating agents, such as fluorescamine [14–16]. Under these circumstances, the band-narrowing effect obtained by synchronous scanning may be very valuable if derivatives of the signal output are taken [17,18].

The combination of these two techniques, first suggested by John Soutar [19], is of great value to increasing sensitivity, and presents a further strategy for improved resolution and rejection of matrix interference, as has been well demonstrated in many fields of analytical chemistry [20–27].

Since the findings of Singh and Hinze [28] on the micellar-enhanced fluorimetric determination of dansyl and *o*-phthalaldehyde (OPA) derivatives of amino-acids, providing fluorescence enhancement factors from 8 to 20, a few applications have been reported [29–32].

In the present paper, the use of different surfactant solutions in the fluorescence analysis of fluorescamine-derivatized folic acid have been investigated, with the aim of developing a simple and selective fluorescence method for the analysis of the vitamin in real samples.

2. Experimental

2.1. Reagents

All chemical were of analytical-reagent grade, and the solutions were prepared with double-distilled, demineralized water.

Folic acid (Sigma) standard solution (1 mg ml^{-1}) was prepared by dissolving 25 mg of folic acid in 0.1 M ammonium hydroxide and diluting the solution to 25 ml with phosphate buffer solution (pH 6.5; 0.1 M). The solution was stable for at least 2 months at 4 °C. Working solutions were prepared by appropriate dilution with the standard solution.

Analytical-reagent-grade fluorescamine (4--Phenylspiro(furan - 2(3*H*),1′ - phthalan) - 3,3′ - dione, Sigma) solution (1 mg ml^{-1}) was prepared by dissolving fluorescamine in acetone.

Phosphate buffer solution (pH 6.5; 0.1 M) and phthalate–HCl buffer solution (pH 3.0; 0.1 M) were prepared from solutions of their corresponding sodium salts (Merck) by mixing appropriate volumes with hydrochloric acid.

The surfactants isooctylphenoxypolyethoxyethanol (Triton X-100; c.m.c. = 0.24 mM), sodium lauryl sulphate (SDS; c.m.c. = 9.2×10^{-4} M), *N,N,N*-trimethyl-1-hexadecaminium bromide (BrCTA; 8.27 mM) and polyoxyethylene 23-lauryl ether (Brij-35; c.m.c. = 0.09 mM), were obtained from Sigma.

2.2 Equipment

Fluorescence was monitored with a Perkin-Elmer Model MPF-66 spectrofluorimeter, equipped with a 150 W xenon arc lamp and a R-928 photomultiplier. All measurements took place in a standard 1-cm quartz cell thermostatically controlled at 25.0 ± 0.5 °C with a water-bath circulator (S-382 Frigiterm). Excitation and emission monochromators were locked together and scanned simultaneously with a constant difference $\Delta\lambda = \lambda_{em} - \lambda_{ex} = 90$ nm. The scan speed and response time of the spectrometer were set at 480 nm min^{-1} and 0.5 s, respectively. The spectrometer was connected to a Perkin-Elmer Model 7300 Professional Computer provided with PETLS application software (C 646-0280). Fluorescence data are given without spectral correction.

A Crison Digit-501 pH-meter was used for all pH measurements.

2.3. General procedure

Suitable aliquots of folic acid standard solution to obtain final concentration of 0.05–6 μg ml^{-1} were transferred to 10 ml calibration flasks. Then, 3 ml of phthalate–HCl buffer solution (pH 3.0) was added, followed by 300 μl of fluorescamine solution, and the mixture was diluted to 10 ml with the corresponding surfactant solution to obtain a final surfactant concentration near its c.m.c. (critical micellar concentration). A blank solution containing all the reagents except the analyte was prepared and treated in the same way as described for the sample.

The fluorescence intensity was measured at 494 nm, with excitation at 399 nm. For synchronous derivative measurements, a wavelength scanning interval ($\Delta\lambda$) of 90 nm and 25 experimental smooth points with 25 $\Delta\lambda$ data

were used. Synchronous first-derivative measurements were made as the vertical distance from the corresponding spectrum to the baseline at 430 nm.

The resulting relative units were converted into folic acid concentration values by applying the corresponding regression equation.

2.4. Determination of the vitamin in pharmaceuticals

Two different commercial products were analyzed. *Acfol* (Torlan, S.A. Laboratories, Barcelona, Spain) had a nominal content of 5 mg of folic acid. A multivitamin complex, *Gestamater* (Lederle, Madrid, Spain), had a nominal content of 1 mg of folic acid with vitamin B_{12} 10 μg, thiamine mononitrate 2 mg, riboflavine 2 mg, nicotinamide 7 mg, ascorbic acid 35 mg, ferrous sulphate 6 mg (Fe), manganese sulphate 0.12 mg (Mn), calcium biphosphate 250 mg (Ca) and 190 mg (P), vitamin D 400 U and vitamin A 2000 U.

Both products were weighed and ground to powder, and a portion of the powder was weighed and dissolved in 0.1 M NaOH, shaken and heated at 70 °C for 20 min. The solutions were filtered and diluted with the phosphate buffer solution (pH 6.5) to obtain a final folic acid concentration of 100 μg ml^{-1}. Aliquots of this solution were treated as indicated in Section 2.3.

3. Results and discussion

Despite its native fluorescence, folic acid was first derivatized with fluorescamine in acid solution to produce a high intensity fluorophor. Several experiments had been performed previously to establish optimal fluorescence intensity conditions before micellar medium treatment of the derivatized compound [33].

3.1. Effect of micellar media and pH

In order to obtain better analytical characteristics for the determination of folic acid and taking into account the findings of Singh and Hinze [28], a study on the effect of different cationic, anionic and non-ionic surfactants on the fluorescence spectra of a fluorescamine–folic acid derivative was carried out. Fluorescamine-derivatized solutions of folic acid were treated with equal volumes of the different cationic, anionic and non-ionic surfactants to ensure concentrations higher than the c.m.c. of each medium. A blank water-treated fluorophore (the aqueous medium) was prepared in the same way to study the enhancing effect on the fluorescence emission of the derivative.

The different enhancement ratios, defined as the ratios between the fluorescence intensity of the aqueous fluorescamine-derivatized solution and those treated with the different enhancers (surfactants) are summarized in Table 1. The findings correspond with those of Baeyens and co-workers [31,32], where an increase in sensitivity of up to nearly three times the fluorescence signal was observed. This is especially seen when non-ionic surfactants, such as Triton X-100 and Brij-35, are employed.

For the different surfactants tested, an overall bathochromic shift of a few nanometers in both the excitation and emission maxima was noted on changing from the anionic (SDS) to the non-ionic surfactants (Triton X-100 and Brij-35); a markedly hypsochromic shift was observed with the cationic medium BrCTA.

Because all the previous experiments with the four surfactants tested were carried out at a fixed pH of 3.0, the effect of different pH values on the relative fluorescence intensity of the corresponding fluorescamine–folic acid fluorophore was studied. Fig. 1 shows the effect of a wide range of pH on the fluorescence emission of the different solutions. It is observed that both non-ionic surfactants exhibit the same behaviour with the maximum emission at pH values between 2.5 and 3.5; in contrast, the anionic and cationic media both give a lower relative fluorescence intensity, and no special changes are observed with different pH values.

Since the best enhancement factor was obtained with the solution of fluorescamine–folic

Table 1
Influence of the micellar medium on the excitation and emission maxima of folic acid

Enhancer	λ_{ex} (nm)	λ_{em} (nm)	Enhancement ratio
Aqueous solution	399	499	1.0
SDS	400	498	1.2
BrCTA	390	480	1.4
Triton X-100	410	496	2.2
Brij-35	402	494	2.8

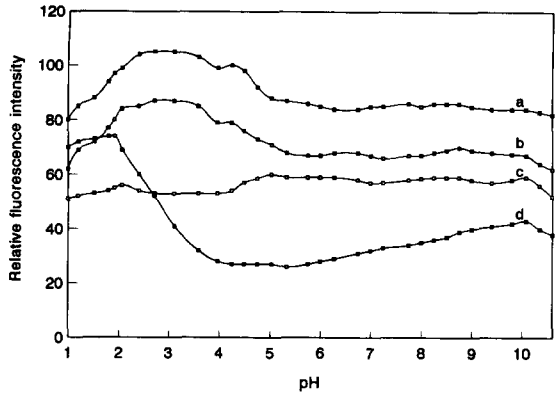

Fig. 1. Influence of pH on the fluorescence intensity of fluorescamine–folic acid fluorophore (1 μg ml^{-1}) in (a) Brij-35, (b) Triton X-100, (c) SDS and (d) BrCTA micellar media. Enhancer concentration = 50 c.m.c.; slits (ex) 4 nm, (em) 4 nm.

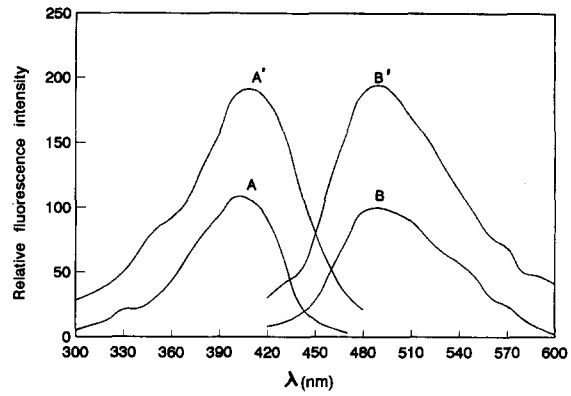

Fig. 3. Excitation and emission spectra of fluorescamine–folic acid fluorophore (1 μg ml^{-1}) in aqueous (A, B) and Brij-35 (A', B') medium. [Brij-35] = 50 c.m.c.; pH = 3.2; slits (ex) 5 nm, (em) 5 nm.

acid treated with Brij-35 at pH 3.2 using a phthalate–HCl buffer solution, this medium was selected for the rest of the experiment work.

3.2. *Effect of Brij-35 concentration and temperature*

Once the best micellar medium and pH had been selected to obtain the maximum fluorescence intensity of the fluorescamine–folic acid fluorophore, the effect of different Brij-35 concentrations on the enhancement ratio was carefully studied.

The results are illustrated in Fig. 2. Brij-35 causes a progressive increase in the fluorescence intensity of the fluorophore, reaching maximum when using a concentration which corresponds to 50 c.m.c. Higher concentrations could not be used owing to limited solubility.

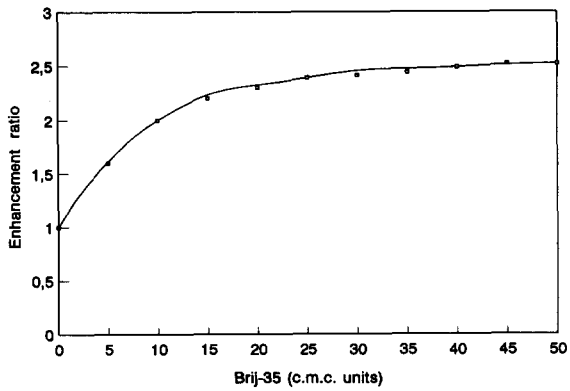

Fig. 2. Enhancement ratios of fluorescamine–folic acid fluorophore (1 μg ml^{-1}) treated with increased concentrations of Brij-35. pH = 3.2; slits (ex) 5 nm, (em) 5 nm.

To achieve complete solubilization of the mixture, an ultrasonic water-bath was used. A study of the influence of mixing time on the fluorescence intensity of the derivatized folic acid in Brij-35 medium demonstrated that 45 min mixing time was necessary to obtain the highest and most reproducible results.

A study of the influence of temperature on the fluorescence signals showed that 25 °C gave the best results. The temperature was kept constant using a water-bath circulator throughout the experimental work. The samples prepared under these conditions remained stable for at least 3 days after their preparation.

Fig. 3 shows that the use of a non-ionic surfactant, such as Brij-35, on folic acid derivatized with fluorescamine is an easy way to substantially increase the fluorescence intensity of the fluorophore, and would permit a more sensitive analysis of the vitamin in real samples.

3.3 *Selection of instrumental parameters for synchronous-derivative scanning*

In this technique, the difference between the two wavelength monochromators ($\Delta\lambda$) can considerably modify the spectrum shape. For the selection of the appropriate value, various synchronous spectra at different $\Delta\lambda$ have been recorded. As shown in Fig. 4, the band widths of the different synchronous spectra of fluorescamine–folic acid fluorophore in Brij-35 medium are greatly increased or decreased when $\Delta\lambda$ is above or below the Stokes shift (92 nm), respectively. From these spectra, it is evident that a $\Delta\lambda$ of 90 nm gave the narrowest

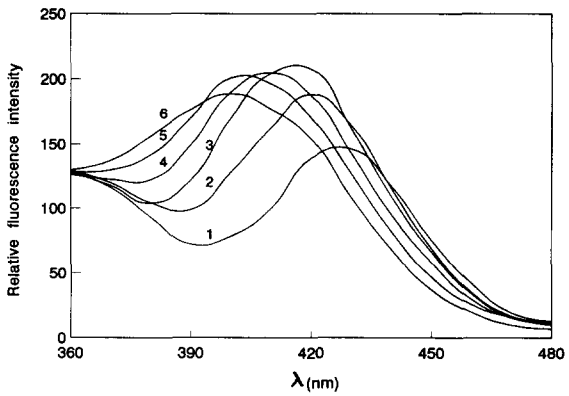

Fig. 4. Effect of the wavelength scanning interval ($\Delta\lambda$) on the synchronous spectra of fluorescamine–folic acid fluorophore (1 μg ml^{-1}). (1) 70, (2) 80, (3) 90, (4) 100, (5) 110 and (6) 120 nm. [Brij-35] = 50 c.m.c.; pH = 3.2; slits (ex) 5 nm, (em) 5 nm.

spectrum, the highest relative fluorescence intensity and the maximum simplicity. Choice of the appropriate scanning interval is especially dictated by the requirements of resolution and sensitivity. As seen in Fig. 5(a), using a scanning interval of 90 nm, the peak half-width is greatly reduced from 70 nm in the normal excitation spectrum to 53 nm in the synchronous spectrum. The band-narrowing effect obtained by the synchronous scanning and the direct relationship between analyte concentration and the slope of the spectra provided by the derivative technique permits the synchronous-derivative approach to enhance considerably the sensitivity of the present analytical method (see Fig. 5(b)).

The instrumental parameters affecting the shape of the derivative spectra are the scan speed and the response time. After verifying that these parameters did not appreciably affect the derivative signal obtained, a scan speed of 480 nm min^{-1} with a response time of 0.5 s were chosen for the rest of the experimental work.

Using this technique, the height h at a wavelength of 430 nm from the peak to the baseline, indicated in Fig. 5(b), was proportional to the fluorophore concentration and it was used in the preparation of the calibration graph.

3.4. Analytical characteristics

Under the optimum operating conditions previously selected, a calibration graph was

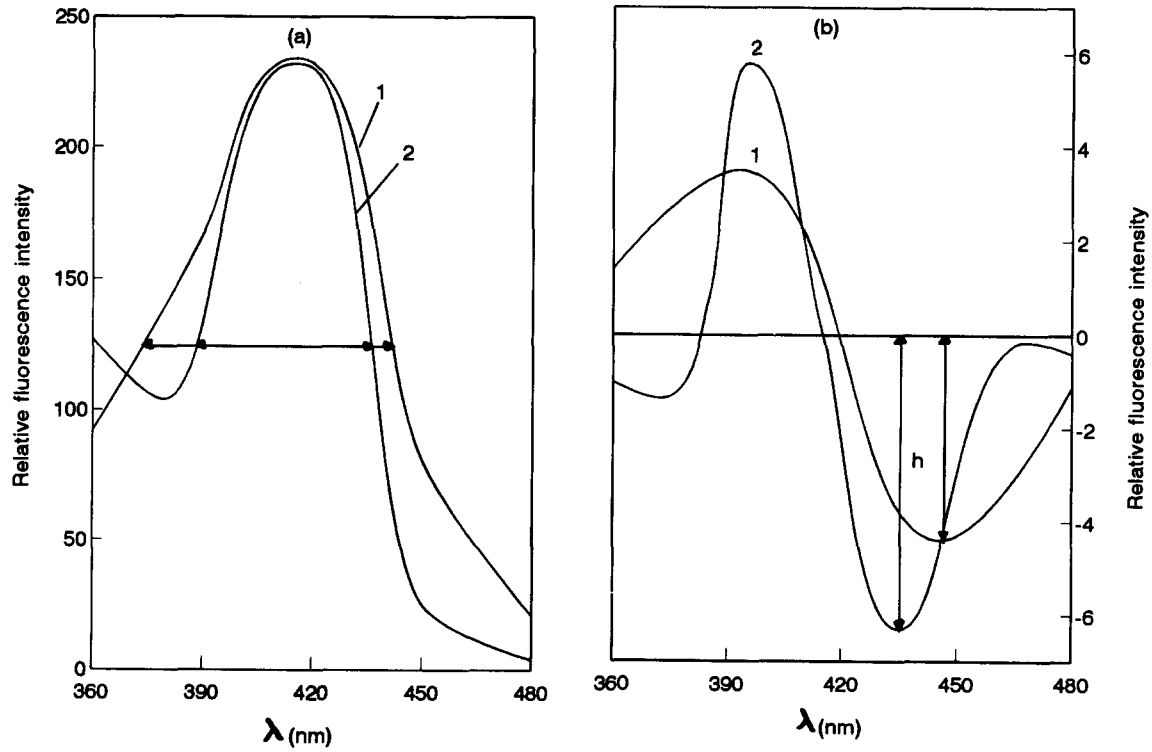

Fig. 5. (a) Excitation (1) and synchronous (2) spectra at $\Delta\lambda = 90$ nm of the fluorescamine–folic acid fluorophore (1 μg ml^{-1}). The double-headed arrows show the reduction in band-width. (b) First-derivative of fluorescence excitation spectrum (1) and of synchronous spectrum at $\Delta\lambda = 90$ nm (2) of the same solution. The double-headed arrows indicate the analytical measurements in each case. Conditions as in Fig. 4.

Table 2
Interference study on the determination of 1 μg ml^{-1} of folic acid

[Interferent]/[folic acid]	Interferent	Recovery (%)
3000[a]	Mg^{2+}	102
2500	Ca^{2+}	99
1200	P	102
600	Vitamin B_6	101
500	Panthothenic acid	102
	Vitamin B_3	99
	Vitamin B_1	98
100	Vitamin E	103
	I^-	101
	F^-	102
	Zn^{2+}	99
50	Cu^{2+}	101
	Fe^{3+}	97

[a] Maximum ratio tested.

obtained by plotting the peak height (h) of the first-derivative synchronous spectra versus folic acid concentration; the graph was linear in the range 0.05–6.00 μg ml^{-1}, with Pearson's correlation coefficient [34] of 0.9998, and the detection and determination limits [35] were 12 and 40 mg ml^{-1}, respectively. The relative standard deviation (RSD) for 1 μg ml^{-1} vitamin concentration (10 determinations) was 2.82%.

3.5. Interference

Since the aim of this work was determination of folic acid in pharmaceutical preparations, the effects of common vitamins and minerals which normally accompany the analyte were considered. Table 2 shows the results for the effects of eight minerals and five vitamins on folic acid determination. As can be seen, the proposed procedure is highly selective. The tolerance limit was taken as the concentration causing an error of not more than ± 3% in the folic acid recovery.

3.6. Applications

The proposed method was applied satisfactorily to the determination of folic acid in two different pharmaceutical preparations. Table 3 summarizes the results.

For the two kinds of commercial products tested, the statistical calculations for the assay results showed satisfactory precision of the first-derivative synchronous method proposed, with no significant differences between the declared and experimental results. It has been demonstrated that the use of the synchronous-derivative technique in the presence of micellar media to enhance the sensitivity and selectivity of a derivatization reaction enables a simple and time-saving assay to be developed for the analysis of small amounts of a substance in real samples.

Table 3
Application of the proposed method to pharmaceutical preparations

Sample	Content		Recovery[a] (%)
	Declared (mg)	Found[a] (mg)	
Acfol (Torlan)	5.00	4.87	97.3 ± 3.4
Gestamater (Lederle)	1.00	0.96	96.1 ± 3.6

[a] Mean values ± SD of three determinations.

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