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Analytical methods for the determination of folic acid in a polymeric micellar carrier

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Abstract

Amphiphilic copolymers have been the object of growing scientific interest due to their ability to form polymeric micelles in aqueous environments entrapping lipophilic drugs in their inner core. In this study, polyvinylalcohol substituted with oleic acid was employed as an amphiphilic micellar carrier for folic acid (FA), a model drug similar for its chemical–physical characteristics to methotrexate. In order to investigate the stability of the polymeric micelles, the drug incorporation and the kinetic aspects of drug release from these systems, selective analytical methods are required. The development of three analytical methods suitable for selectively identifying and reliably determining FA contained in the micelles and in the delivery systems is reported. UV derivative (first and second order) spectrophotometry was first applied to the aqueous solution of the FA containing micelles obtained at pH 9.0 and provided a characteristic spectral profiling with sharp peaks, related to the analyte, whose amplitude was used for quantitative application. A second approach involved a solid phase extraction (strong anion exchanger), which provided an effective clean up of the FA micelles solution, allowing accurate analysis to be performed also by a conventional spectrophotometric method. A RP-HPLC method, selectively supplying the FA separation from the micelles' components, was then used as a reference method to determine the accuracy of the spectrophotometric methods. These methods were applied to various micelle composition and to the delivery system study.
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Keywords: Folic acid; Amphiphilic micelles; Derivative spectrophotometry; Solid phase extraction; High performance liquid chromatography

1. Introduction

In recent years, polymeric micelles have emerged as a potential carrier for low therapeutic index drugs and for their targeting to cancer cells avoiding side effects [1,2]. These systems may be employed for parental drug targeting, prolonging the permanence of short half life drugs in circulation or increasing the bioavailability of poorly soluble drugs, both for oral or transdermal route.

Amphiphilic copolymers are now a topic of growing scientific interest due to their ability to

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entrap lipophilic drugs in their inner core. They offer attractive characteristics such as a generally small size (100 nm) and a propensity to evade scavenging by the mononuclear phagocyte system. Polyvinylalcohol represents an interesting material for the preparation of amphiphilic polymers, due to its biocompatibility and the possibility for substitution through chemical linkage to its oxy-residues, able to modify its physico-chemical properties.

In this study, polyvinilalcohol (PVA) substituted with oleic acid was employed as a micellar carrier for folic acid (FA), a model compound for its high lipophylicity and similar for its chemical–physical characteristics to methotrexate, an antineoplastic drug. FA was chosen because it was used in a relatively large amount for optimization of this new formulation and it is easily available at a low cost and not toxic.

FA is a hematopoietic vitamin present free or combined with one or more additional molecules of L(+)-glutamic acid, in liver, kidney, mushrooms, spinach, yeast, green leaves, grasses [3]. Several HPLC methods were published to determined folates and their reduced forms in biological fluids [4–9], food [10] with solid phase extraction (SPE). However, the form of this new formulation and the planned release studies needed the development of accurate and specific extraction procedures and sensitive and selective assay methods.

In order to evaluate the performance of FA containing polymeric micelles, analytical methods able to ascertain the FA content entrapped in the micelles and to monitor the release of FA from the polymer were developed. These methods were based on conventional absorption spectrophotometry (after apreliminary SPE procedure), derivative (first and second order) spectrophotometry and liquid chromatography (HPLC).

2. Experimental

2.1. Materials

HPLC grade acetonitrile and methanol (Romil Ltd, Cambridge, UK) and water obtained from Milli-RX apparatus (Millipore, USA) were used to prepare solutions and mobile phases. All the other reagents were of analysis quality (Carlo Erba Reagenti, Milano, Italy). The buffer solutions were filtered through a 0.45 μm membrane filter and degassed before their use for HPLC. FA [L-glutamic acid, *N*-[4-[[(2-amino-1,4 dihydro-4-oxo-6-pteridinyl)methyl]amino]benzoyl] was purchased from Fluka.

Polyvinylalcohol substituted with oleic acid and polymeric micelles containing 0.45% FA were prepared according to previous paper [11].

Phosphate buffer solutions (0.01, 0.1 M, pH 9.0 and 0.1 M, pH 3.0) were prepared according to standard methods.

SPE were performed on Isolute SAX (strong anion exchanger (SAX)) cartridges (500 mg $\times$ 3 ml) from IST (UK), using a Baker-10 SPE vacuum manifold, connected to a water aspirator.

2.2. Apparatus

The spectrophotometric analyses were performed on a Jasco V-530 double beam spectrophotometer, using 1 cm quartz cell. Suitable settings were: width 2 nm, scan speed 400 nm min^{-1}, UV range 220–500 nm.

Derivative UV spectra were obtained using $\Delta\lambda = 10$ nm.

The solvent delivery system was a quaternary HP 1050 Ti series Pump, equipped with a Reodyne Model 7125 injector with a 20 μl sample loop. The eluents were monitored by a Multiwavelength HP 1050 Detector connected to a computer station (HP Chemstations, Vectra VT).

The chromatographic separations were carried out on a reversed-phase Phenomenex Luna C18, 5 μm (I.D. 150 $\times$ 2.0 mm) column, using a mobile phase consisting of methanol −0.01 M phosphate buffer (pH 5.0) containing 4 mM tetrabutylammonium hydrogensulfate (23:77) (v/v). The flow rate was 0.3 ml/min. and the detector wavelength was set at 270 nm.

2.3. Solid phase extraction

Before use, SAX sorbents were properly conditioned by rinsing with 6 ml of methanol and then with 6 ml 0.01 M Na phosphate buffer (pH 9.0). A

5.0 ml aliquot of FA standard solutions (2–50 μg/ml) in 0.01 M Na phosphate buffer (pH 9.0) was applied to the SPE SAX cartridge. The column was washed with 5 ml portion of distilled water and the FA was eluted either with 5 ml × 2 portions of acetonitrile-Na phosphate buffer 0.1 M (pH 3.0) containing 0.2 M potassium chlorate 50:50 (v/v), or with 5 ml portion of Na phosphate buffer 0.1 M pH 9.0 containing 0.3 M potassium chlorate.

Similarly, a 5.0 ml aliquot of FA polymeric micelle sample solution (FA = 10 μg/ml) was subjected to the same (SPE) treatment. The resulting eluate solutions after SPE procedure were analysed by a conventional UV method.

2.4. Spectrophotometry

2.4.1. Calibration graph

The zero order UV spectra of FA standard solutions (10–100 μg/ml) and FA free micelle (5.5 mg/ml) in Na phosphate buffer 0.01 M (pH 9.0) were recorded using the pH 9.0 buffer as blank; the first order and second order derivative spectra of each folic standard solution and micelles were then derived.

Using the first derivative mode, the amplitude of the negative peak at $\lambda = 387$ nm to zero line $^{1}D_{387}$, were measured for FA standard solutions. When the second order derivative was applied, the amplitude of the positive peak at $\lambda = 320$ to zero line $^{2}D_{320}$ was measured for FA.

The amplitude values, $^{1}D_{387}$ and $^{2}D_{320}$, were then plotted against the corresponding FA concentration to obtain the respective calibration graphs.

A calibration curve was also obtained by the conventional absorption method, in view of its application to the analysis of SPE eluate. Thus, the zero order UV spectra of FA (2–50 μg/ml) standard solutions in acetonitrile-Na phosphate buffer 0.1 M (pH 3.0) containing 0.2 M potassium chlorate 50:50 (v/v), were recorded using the same buffer as blank and the absorbance values at $\lambda_{max} = 359$ nm were plotted against the corresponding concentration to obtain the calibration graph.

2.4.2. Sample analysis

A 33 mg quantity of sample (FA containing micelles) was dissolved in 10 ml volumetric flask with Na phosphate buffer (0.01 M pH 9.0) under sonication. FA content was determined measuring the amplitude $^{1}D_{387}$ and $^{2}D_{320}$ from the derivative spectra, by comparison with a standard solution of FA (15 μg/ml).

2.5. Chromatographic (HPLC) analysis

2.5.1. Calibration graph

FA standard solutions (0.1–3.0 μg/ml) with a fixed concentration of benzoic acid (internal standard, 40 μg/ml) were prepared in the mobile phase. A 20 μl volume of each standard solution was injected in triplicate and the peak area ratios (analyte to internal standard) were plotted against the corresponding drug concentration to obtain the calibration graph (Table 1).

2.5.2. Sample analysis

A 60 mg quantity of micelle formulation was dissolved in a volumetric flask with 5 ml ammonium hydroxide (10%) and the volume adjusted with the mobile phase to 20 ml. An aliquot of 2 ml of this solution was transferred into a 20 ml volumetric flask, the amount of benzoic acid (internal standard) to obtain the concentration used for the calibration graph (40 μg/ml) was added and the volume was adjusted with the mobile phase.

3. Results and discussion

Analytical methods, based on UV spectroscopy and liquid chromatography (HPLC) were developed, suitable for determining FA in the micelle system and for monitoring the drug release. For the first application, the interference from the micelle matrix should be suppressed; this was achieved either by derivative UV spectrophotometric analysis of the whole sample or using a preliminary sample clean-up involving SPE. UV spectrophotometry was used to determine leucovorin, a reduced form of FA in combination with triamterene in biological fluids [12].

Table 1
Data for the calibration graphs ($n = 6$)

Method	Slope	Y intercept	Correlation coefficient (r^2)	Concentration range (µg/ml)
HPLC	2640.8 ± 29.6	−0.026 ± 0.042	0.9998	0.1–3.5
A_{359}	15.423 ± 0.135	0.025 ± 0.003	0.9998	2.0–50
$^1D_{387}$	0.528 ± 0.009	0.004 ± 0.001	0.9993	10–100
$^2D_{320}$	0.107 ± 0.006	0.0003 ± 0.003	0.9897	10–100

3.1. Spectrophotometry

3.1.1. First derivative method

Direct conventional (zero order) spectrophotometric analysis of FA entrapped in micelles was first approached. The basic solution (0.01M Na phosphate buffer pH 9.0) of a suitable amount of FA containing micelles (3.3 mg/ml) (Fig. 1) was submitted to the UV analysis and compared to FA standard solution. Over the UV range 230–380 nm a FA standard solution (pH 9.0) shows three absorption maxima, at 256, 283 and 360. However, direct UV determination of FA was not found feasible. In fact, at the characteristic FA absorption bands, a high absorption contribution by micelles, also in the visible region, interfered, giving inflated results in the direct determination (Fig. 1).

Therefore, the first derivative spectrum of standard and sample FA solutions (pH 9.0) was applied. When compared to the one obtained with the respective blank, the first derivative spectrum showed a specific negative peak at 387 nm, wavelength at which the blank is zero (Fig. 2). Therefore, the derivation procedure allows to suppress the large non-specific absorption by the polymeric matrix. In fact, the amplitude of this negative peak, $^1D_{387}$, was found to be linearly correlated to the concentration of FA in the micelles (Table 1) with negligible intercept.

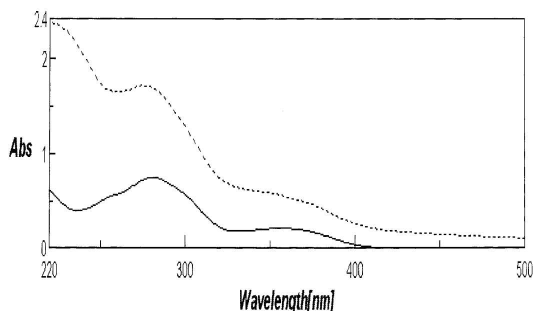

Fig. 1. UV spectra of FA standard (—) and FA in micelles (----) at 15 µg/ml in Na 0.01-M phosphate buffer pH 9.0.

Therefore, the direct application of the first derivative UV analyses to the (pH 9.0) sample solution was performed, allowing a selective determination of FA in the micelles (Table 2) to be achieved.

3.1.2. Second derivative method

Also the second derivative method, measuring the amplitude of the positive peak at 320 nm, where the blank spectrum is zero, proved to be useful for a selective and accurate determination of FA contained in the micelles preparation (Fig. 3). As expected, however, under these conditions, the method exhibits lower sensitivity.

A linear calibration graph was obtained by plotting $^2D_{320}$ against the FA standard corresponding concentration (Table 1). In Table 2 the assay results are reported. As can be seen, the derivative spectrophotometric assay results were in agreement with the HPLC method described, confirming the method accuracy.

3.2. Solid phase extraction

In order to eliminate the interference caused by the micelles in the UV direct spectrophotometric method and to provide a method useful to isolate FA, a SPE method with SAX cartridges was developed. This ion exchange methodology proved to be suitable for the clean up of micelle samples containing FA. FA, in the anion carboxylate form in the (pH 9.0) buffer solution, was retained by a SAX sorbent, while the substituted polyvinyl

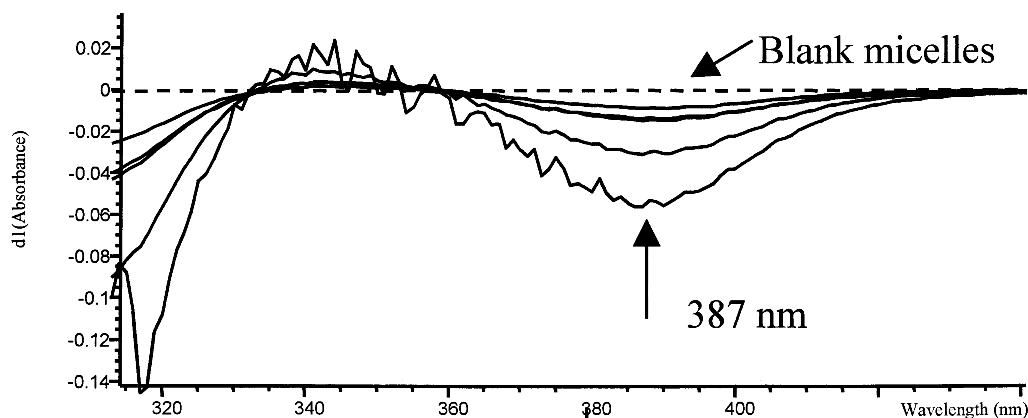

Fig. 2. First derivative UV spectra of FA in micelles 10–100 μg/ml and blank micelles (1.1 mg/ml).

Table 2
Assay results for the spectrophotometric and HPLC analyses of the micelles containing FA

Method	Spectrophotometry		HPLC	
	%Found	%R.S.D.	%Found	%R.S.D.
A_{359} after SPE	97.4[a]	2.6	99.46	1.9
$^{1}D_{387}$	98.1	2.1	–	–
$^{2}D_{320}$	100.8	2.3	–	–

The results are the average of five determinations and are expressed as a percentage of the claimed content.
[a] Calculated from the recovery 92%.

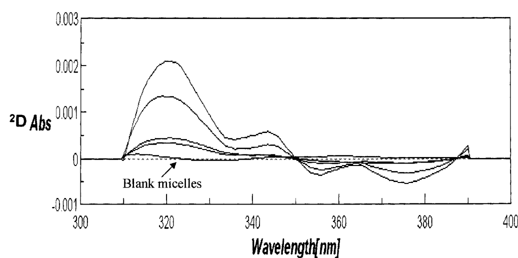

Fig. 3. Second derivative UV spectra of FA in micelles 2–20 μg/ml and blank micelles.

alcohol micelle matrix passed unretained through the sorbent (Fig. 4). An appropriate washing step with water cleaned the sorbent.

FA was recovered either by eluting with an acidic solvent system consisting of acetonitrile:Na phosphate buffer (0.1 M; pH 3.0) containing 0.2 M potassium chlorate 50:50 (v/v), as selective SAX competitor or with a basic solvent system consisting of Na phosphate buffer (0.1 M; pH 9.0), containing 0.3 M potassium chlorate. The resulting eluate was then subjected to subsequent conventional spectrophotometric analysis, which provided a FA UV spectrum with its correct profile, cleaned by the micelles interference (Fig. 5).

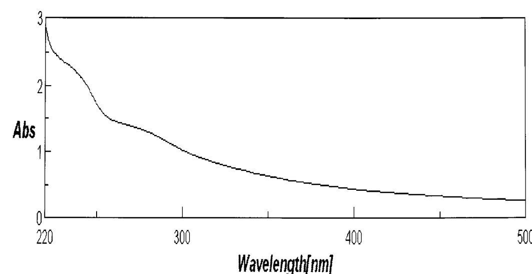

Fig. 4. UV spectra of SPE filtrate containing polymeric micelles (4.4 mg/ml) in 0.01 M phosphate buffer pH 9.0.

The difference between the two eluting system was that the FA recovery with the basic buffer was performed with 5 ml portion of eluent, whereas with the acidic eluent 2×5 ml portions were required. Therefore, a dilution effect resulted by using the acidic eluent.

Direct UV determination was then applicable at 358 and 283 nm. A linear calibration graph was obtained by plotting A_{358} against the FA standard corresponding concentrations (Table 1). In Table 2 the assay results are reported.

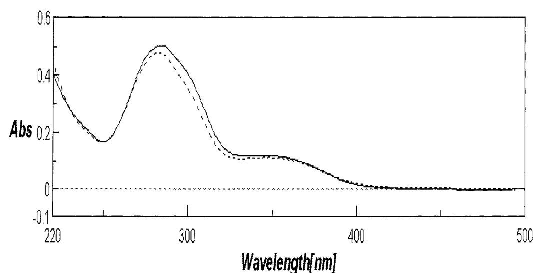

Fig. 5. UV spectra of SPE elution for FA standard (—) (10 μg/ml) and FA in micelles (----) Solvent mixture acetonitrile: Na phosphate buffer (0.1 M, pH 3.0) containing 0.2 M potassium chlorate 50:50 (v/v).

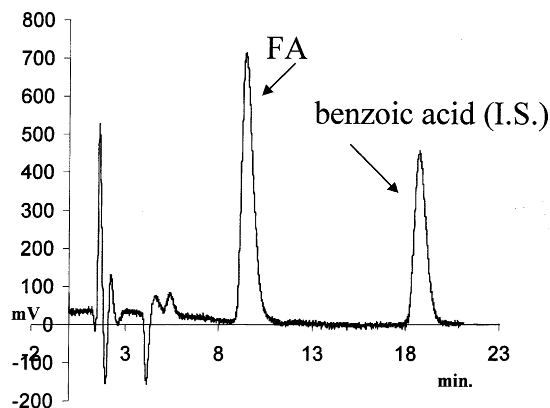

Fig. 6. HPLC separation of FA contained in micelles and benzoic acid (I.S.). Chromatographic conditions: stationary phase: Phenomenex Luna C18, 5 μm (I.D. 150 × 2.0 mm), mobile phase: methanol: 0.01 M phosphate buffer pH 5.0 containing 4 mM tetrabutylammonium sulfate (23:77) (v/v). Flow rate 0.3 ml/min. UV detector set at 270 nm.

The accuracy of the proposed method was assessed by analysing micelle blank fortified by known FA standard solutions. The recovery was 92 ± 3% as calculated by comparison to standard FA solutions.

The precision of the method was found satisfactory as indicated by the R.S.D. values reported in Table 2.

3.3. Chromatography

Several HPLC methods have been reported for the determination of FA and its reduced derivatives in food and biological systems [4–10]. The present study was directed to provide a reversed-phase HPLC method for determining FA in polymeric micellar systems, suitable to validate the proposed spectrophotometric methods and to monitor the drug release. Good chromatographic separations were achieved using a C18 Phenomenex Luna 5 μm (I.D. 150 × 2.0 mm) column, using a mobile phase consisting of methanol: 0.01-M phosphate buffer (pH 5.0) containing tetrabutylammonium acid sulfate 4 mM (23:77) (v/v). The flow rate was 0.3 ml/min. and the detector wavelength was set at 270 nm. This selected wavelength was suitable to detect benzoic acid (I.S.) and FA with satisfactory sensitivity.

Using this chromatographic system, no interference from micelles was observed (Fig. 6). Under the conditions of Fig. 6 linear relationships between the peak area ratios (analyte to internal standard) and FA concentration were obtained (Table 1).

The method was applied to the analysis of the FA containing micelles and the results were found in close agreement with the spectrophotometric data (Table 2). On applying the F-test and t-test at the 95% confidence level, no significant difference was found between the mean recoveries obtained with the three spectrophotometric methods.

The LOD and LOQ obtained with the HPLC method (Table 3) resulted lower than those obtained with the spectrophotometric approach. The higher sensititivity of the HPLC method was found particularly useful for release studies.

Table 3
Limit of detection and quantitation for the spectrophotometric and HPLC methods

Method	LOD (μg/ml)	LOQ (μg/ml)
HPLC	0.02	0.07
A_{359}	0.5	1.5
$^{1}D_{387}$	1.0	3.3
$^{2}D_{320}$	1.5	4.5

4. Conclusions

In order to assess the performance of new polymeric micelle systems, suitable analytical methods are necessary. In the present application, spectrophotometric methods (SPE-UV conventional and derivative) were found useful to perform the determination of FA in polymeric micellar carrier based on substituted PVA. The spectrophotometric derivative methods were found to be advantageous in terms of rapidity and feasibility, allowing the direct determination of FA content in the micelles.

SPE proved to be an effective tool for providing adequate sample clean up and isolating FA from the micelles, allowing direct spectrophotometric analyses to be performed. SPE-UV spectrophotometry can be considered to be a useful combination for the routine quality control of FA in polymeric micellar carrier. The proposed SPE procedure may also be adopted in chromatographic (HPLC) analyses to avoid overloading and deleterious effects upon the analytical column performance and lifetime.

HPLC chromatographic method resulted particularly adequate in release study to determine very low concentration of released drug. It was used as the reference method to validate the spectrophotometric and SPE methods. On the other hand, the spectrophotometric methods had the advantage of the analysis speed.

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