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Analysis of flucytosine dosage forms by derivative UV spectroscopy and liquid chromatography*

VANNI CAVRINI,† DANIELA BONAZZI and ANNA MARIA DI PIETRA

Department of Pharmaceutical Sciences, University of Bologna, Bologna, via Belmeloro 6, 40126, Italy

Abstract: A simple second-order derivative spectrophotometric method was developed for the selective determination of flucytosine (an antimycotic drug) in the presence of 5-fluorouracil (a cytotoxic agent), its synthetic precursor and degradation product. Traces of 5-fluorouracil in flucytosine were also determined by derivative UV spectroscopy; flucytosine was removed by a selective solid-phase extraction (SPE) procedure using a strong cation-exchange sorbent. The spectrophotometric methods were applied successfully to the quality control of commercial dosage forms of flucytosine and the results were compared with those obtained by a HPLC procedure (cyano column) developed as a reference method.

Keywords: *Flucytosine; 5-fluorouracil; derivative UV spectrophotometry; high-performance liquid chromatograpy.*

Introduction

Flucytosine, 4-amino-5-fluoro-2(1H)-pyrimidinone, is an antimycotic drug used in the treatment of severe *Candida* and *Cryptococcus* infections. The drug and its formulations may contain traces of 5-fluorouracil (a cytotoxic agent) its synthetic precursor and also its major degradation product [1, 2]. Pharmacopoeial methods include a semi-quantitative evaluation of 5-fluorouracil in flucytosine by thin-layer chromatography [3–5] with a limit of 0.1% (w/w) relative to flucytosine [4]; meanwhile a non-specific conventional spectrophotometric method has been proposed for the assay of flucytosine in its dosage forms [3, 4]. For the selective determination of 5-fluorouracil in flucytosine formulations, methods based on high-performance liquid chromatography (HPLC) [6–8], high-performance thin-layer chromatography (HPTLC) and derivative UV spectroscopy [9] have been reported. HPLC was also applied to stability studies of flucytosine in aqueous solutions [10]. Column overloading (HPLC methods) and measurements at low wavelengths (derivative UV method) can represent drawbacks for some of the cited methods. As a result, it was considered of interest to develop practical, alternative spectrophotometric methods suitable for the reliable quality control of flucytosine dosage forms, whose use appears to be restricted by the potential presence of 5-fluorouracil.

In the present paper a rapid, stability-indicating, second-order derivative spectrophotometric method is proposed for the selective determination of flucytosine in the presence of 5-fluorouracil. The derivative UV technique, in combination with a preliminary solid-phase extraction (SPE) procedure, also offers the opportunity of determining traces of 5-fluorouracil in flucytosine formulations. The SPE step uses a strong cation-exchange sorbent and allows flucytosine to be completely removed from the acidic sample solution. The proposed derivative UV methods were applied to the analysis of commercial dosage forms of flucytosine and the results were compared with those obtained by a new HPLC procedure developed as a reference method.

Experimental

Materials

5-Fluorocytosine (flucytosine), 5-fluorouracil and thymine (5-methyluracil) were obtained from Fluka (Buchs, Switzerland) and sodium heptanesulphonate from Eastman Kodak (Rochester, NY, USA). Cartridges of SCX Tech-Elut each containing 500 mg of strong cation-exchange sorbent were from HPLC Technology (Macclesfield, Cheshire,

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† Author to whom correspondence should be addressed.

UK). All other chemicals were RPE grade from C. Erba-Farmitalia (Milan, Italy). Methanol of HPLC grade (C. Erba-Farmitalia) and de-ionized, double-distilled water were used for the chromatographic procedure.

A phosphate buffer solution (pH 3.0, 0.07 M) was prepared by adding sufficient hydrochloric acid to 0.07 M potassium dihydrogen phosphate to adjust the pH to 3.0.

Stock solutions of flucytosine and 5-fluorouracil were prepared in water (100 μg ml^{-1}) for spectrophotometric analyses or in buffer solution (pH 3.0) (60 and 50 μg ml^{-1}) for chromatographic analyses. A stock solution of thymine (the internal standard) was prepared in buffer solution (pH 3.0) (27 μg ml^{-1}).

Apparatus

Spectrophotometric analyses were performed with a Jasco Uvidec-610 double-beam spectrophotometer using 10-mm quartz cells. Suitable settings were: slit width 1.0 nm; scan speed 100 nm min^{-1}; and λ scale 20 nm cm^{-1}. For the derivative mode, $\Delta\lambda = 6$ nm was selected and an absorbance scale expansion of ×10 (flucytosine determination) or ×15 (5-fluorouracil determination) was used. HPLC separations were performed on a Varian 5020 liquid chromatograph equipped with Rheodyne model 7125 injector with a 20-μl sample loop. Measurements were made at ambient temperature using a variable wavelength detector (Varian UV-50) connected to a Varian 4270 integrator. The detector wavelength was set at 266 nm (Attenuation 64). Routine analyses were carried out isocratically on a 5-μm Spherisorb-CN column (250 × 4.6 mm i.d.) using 0.009 M sodium heptanesulphonate (adjusted to pH 2.8 with phosphoric acid) as the mobile phase, at a flow rate of 0.8 ml min^{-1}. SPEs were carried out using the Baker-10 SPE system connected to a water aspirator.

Calibration graphs

Spectrophotometric method.

Flucytosine. Standard solutions of the drug were prepared in an acidic medium (pH 3.0) in concentrations of 2.5–20.0 μg ml^{-1}. The second-order derivative spectrum of each standard solution was recorded against the solvent blank and the amplitudes $^2D_{310.8}$ were plotted against the corresponding concentration to obtain the calibration graph.

5-Fluorouracil. Mixed standard solutions containing a fixed concentration of flucytosine (1 mg ml^{-1}) and different concentrations of 5-fluorouracil [3.0–30.0 μg ml^{-1} corresponding to 0.3–3.0% (w/w) of 5-fluorouracil in flucytosine] were prepared in the buffer solution (pH 3.0). A 2-ml aliquot of each standard solution was applied to a SCX extraction column, previously conditioned by rinsing with 6 ml of methanol and five 2-ml portions of buffer solution (pH 3.0). After application of the sample, the column was washed with 1.0 ml of buffer solution (pH 3.0) and the combined filtrates (≈3 ml) containing 5-fluorouracil were subjected to derivative UV spectrophotometric analysis. The second-order UV spectrum was recorded using buffer solution (pH 3.0) as the blank; the peak-to-peak amplitude $^2D_{292.4,267.2}$ was measured and plotted against the corresponding concentration to obtain the calibration graph.

Chromatographic method.

Flucytosine and 5-fluorouracil. Mixed standard solutions containing 3.0–15.0 μg ml^{-1} of flucytosine, 1.0–12.5 μg ml^{-1} of 5-fluorouracil and 4.0 μg ml^{-1} of thymine (the internal standard) were prepared in buffer solution (pH 3.0). A 20-μl aliquot of each solution was injected into the chromatograph in triplicate. The area ratios of the analyte (flucytosine and 5-fluorouracil) to the internal standard (thymine) were plotted against the corresponding concentrations to obtain the respective calibration graph.

5-Fluorouracil. The SPE filtrates (spectrophotometric method) were used to obtain the calibration graph for the chromatographic method. A 2.0-ml aliquot of the SPE filtrate was mixed with 1.0 ml of the internal standard solution (6.7 μg ml^{-1}) and then subjected to chromatographic analysis as described above.

Determination of flucytosine in pharmaceutical formulations

Sample preparation. A portion of powdered tablets, equivalent to approximately 5 mg of flucytosine, was extracted with 50 ml of water with magnetic stirring for 15 min. The extract was filtered to obtain a clear sample solution. Flucytosine solution for intravenous infusion was diluted with water to yield a sample solution containing about 100 μg ml^{-1} of the drug.

Spectrophotometric determination. A 4.0-ml aliquot of the sample solution was diluted to 20 ml with buffer solution (pH 3.0) and the second-derivative spectrum of the resulting solution was recorded. The amplitude $^2D_{310.8}$ was measured and the drug content in each sample was calculated by direct comparison with flucytosine standard solution (20 μg ml^{-1}).

HPLC determination. A 1.0-ml aliquot of sample solution was transferred into a 10-ml calibrated flask containing 1.5 ml of the internal standard (thymine) solution and the volume was adjusted to 10 ml with buffer solution (pH 3.0). The sample solutions were chromatographed concurrently with the appropriate standard solution (10 μg ml^{-1}).

Determination of 5-fluorouracil in flucytosine formulations

Bulk material, tablets and solutions were treated as described above to provide sample solutions (pH 3.0) containing 1 mg ml^{-1} of flucytosine. A 2.0-ml aliquot of each solution was subjected to the SPE procedure and the filtrate was analysed by both the derivative spectrophotometric and HPLC methods, as described under Calibration graphs.

Results and Discussion

Derivative UV spectroscopy and HPLC were used to provide methods suitable for the reliable quality control of flucytosine dosage forms: the selective assay of the drug; and the sensitive determination of the potential impurity 5-fluorouracil.

Chromatography

All HPLC methods reported [6–8, 10] for the simultaneous analysis of flucytosine and 5-fluorouracil employ reversed-phase column packings in combination with ion-pair type elution. In these chromatographic systems, 5-fluorouracil appears to be poorly retained and to be eluted close to the solvent peak; with the aim of improving retention of the compound a nitrile column was chosen for the present work. Using a Spherisorb-CN column, a simple aqueous mobile phase comprising 0.009 M sodium heptanesulphonate (pH 2.8) was found to be suitable for the rapid and fully resolved separation of 5-fluorouracil (retention time = 4.00 min), thymine (the internal standard)

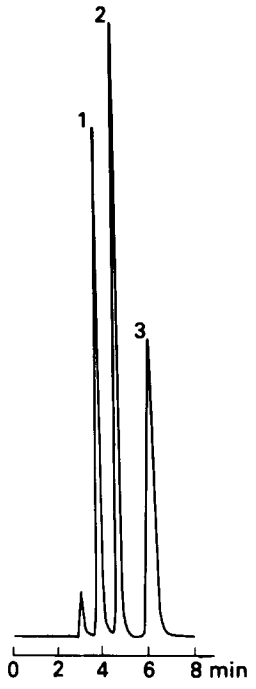

Figure 1
HPLC separation of 1,5-fluorouracil; 2, thymine; and 3, flucytosine. Column: Spherisorb-CN (5 μm); mobile phase: 0.009 M sodium heptanesulphonate (pH 2.8) at a flow rate of 0.8 ml min^{-1}; detection UV at 266 nm.

(retention time = 4.85 min) and flucytosine (retention time = 6.30 min) (Fig. 1). When heptanesulphonate was omitted and a simple phosphate buffer solution (pH 2.8) was used as the mobile phase, 5-flucytosine was better retained (retention time = 9.7 min), with longer analysis times. Therefore, the chromatographic conditions of Fig. 1 were chosen to carry out rapid routine analyses; linear relationships between area ratios of analyte (flucytosine and 5-fluorouracil) to the internal standard (thymine) were obtained (Table 1).

The precision of the method was good as indicated by the relative standard deviation (RSD = 0.60–0.84%) of the area ratio (analyte to internal standard) obtained from replicate (n = 8) injections of a single standard solution.

Determination of flucytosine

The determination of flucytosine by a conventional spectrophotometric method can be susceptible to interference from 5-fluorouracil, its potential impurity [Fig. 2(a)]. In contrast, for the second-order derivative mode [Fig. 2(b)], the satellite peak at 310.8 nm in the flucytosine spectrum offers an opportunity for

Table 1
Calibration graphs for the determination of flucytosine and 5-fluorouracil

Method	Compound	Slope	Intercept	Correlation coefficient	Concentration range (μg ml^{-1})
$^2D_{310.8}$	Flucytosine	0.01000	0.00170	0.9997	2.5–20.0
$^2D_{310.8}$	Flucytosine*	0.01010	0.00141	0.9998	2.5–20.0
$^2D_{292.4,267.2}$	5-Fluorouracil	0.00923	−0.00120	0.9998	3.0–30.0
$^2D_{292.4,267.2}$	5-Fluorouracil†	0.00936	−0.00150	0.9997	3.0–30.0
HPLC	Flucytosine	0.19400	−0.02000	0.9996	3.0–15.0
HPLC	5-Fluorouracil	0.22440	0.01147	0.9998	1.0–12.5

*From flucytosine standard solutions containing 5-fluorouracil [10% (w/w) relative to flucytosine].
†From mixed standard solutions of flucytosine and 5-fluorouracil, subjected to the SPE procedure.

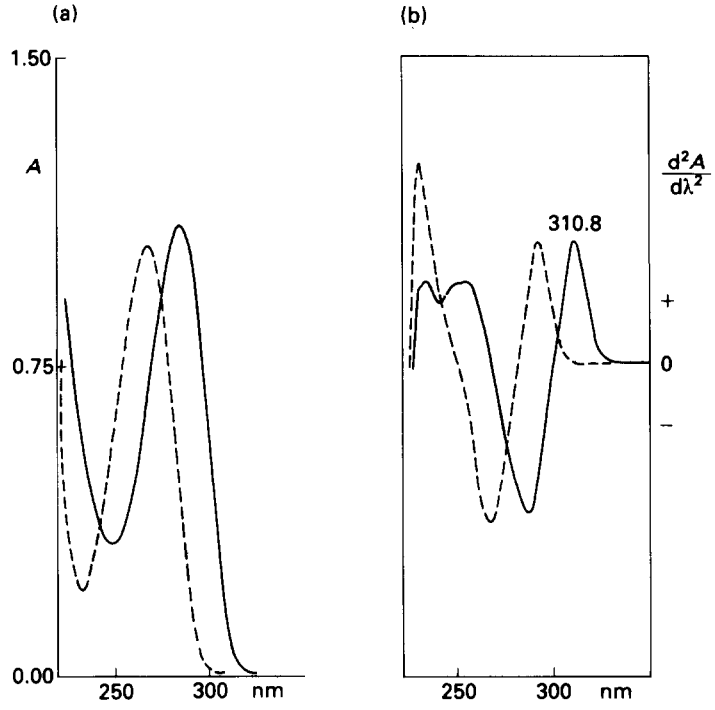

Figure 2
Ultraviolet spectra of flucytosine (solid line) and 5-fluorouracil (broken line) in buffer solution (pH 3.0; 20 μg ml^{-1}): (a) absorption zero-order spectra; (b) second-order derivative spectra.

the selective assay of the drug. Essentially identical, linear relationships were found between the amplitude $^2D_{310.8}$ and the flucytosine concentration in the absence and in the presence (10%, w/w) of 5-fluorouracil (Table 1). The UV spectra were recorded in an acidic medium (pH 3.0) because it provided the best resolution of the absorption maxima. The precision of the method was evaluated by replicate (n = 8) analyses of the same flucytosine standard solution (10 μg ml^{-1}); the RSD was about 0.53%, indicating good precision.

The derivative spectrophotometric method was then applied to the analysis of commercial flucytosine dosage forms (tablets and solutions) and the results are summarized in Table 2. The results were in good agreement with the claimed contents and with those obtained by the HPLC method. The accuracy of the proposed methods was verified by analysing synthetic preparations which reproduced the composition of the commercial tablets; quantitative recoveries were obtained using both the spectrophotometric and the HPLC methods.

Analysis of traces of 5-fluorouracil

The selective determination of 5-fluorouracil in flucytosine formulations was first attempted with a derivative UV method based on the

Table 2
Results for the determination of flucytosine and 5-fluorouracil in commercial flucytosine dosage forms. Results are mean values of five determinations and are expressed as a percentage of the claimed content (flucytosine) and as a percentage of flucytosine (5-fluorouracil)

Formulation*	Compound	$^2D_{310.8}$ Found	$^2D_{310.8}$ RSD (%)	$^2D_{292.4,267.2}$ Found	$^2D_{292.4,267.2}$ RSD (%)	HPLC Found	HPLC RSD (%)
Tablets	Flucytosine	99.4	1.20			99.16	0.42
	5-Fluorouracil			—		—	
Solutions†							
A	Flucytosine	100.31	1.00			100.10	1.23
	5-Fluorouracil			0.43	3.2	0.45	2.50
B	Flucytosine	100.02	0.95			99.90	1.32
	5-Fluorouracil			—		—	
C	Flucytosine	99.28	1.10			101.10	1.44
	5-Fluorouracil			—		—	
D	Flucytosine	100.02	0.80			100.21	1.35
	5-Fluorouracil			0.31	3.5	0.32	2.10

*Other ingredients. Tablets: lactose, starch, magnesium stearate, microcrystalline cellulose and colloidal silica. Solutions: trometamol (2-amino-2-hydroxymethyl-1,3-propanediol) buffer and sodium chloride.
†Obtained from different sources. Solutions B and D were from the same batch and solution C was the most recently manufactured preparation.
—, Significant quantities were not observed.

measurement of the negative peak amplitude at 267.6 nm, where the second-derivative of flucytosine is zero (zero-crossing method) [Fig. 2(b)]. That procedure, however, proved to be inaccurate for the determination of 5-fluorouracil levels of ≤3% (w/w) relative to flucytosine.

The different acid–base properties of flucytosine [2, 8] and 5-fluorouracil [8, 11] led to the development of a preliminary SPE procedure to remove completely the flucytosine. When an acidic flucytosine solution (pH 3.0) was applied to an appropriately conditioned strong cation-exchange SPE column, the protonated flucytosine was found to be quantitatively retained on the column, whereas the potential impurity 5-fluorouracil in the neutral form was quantitatively recovered in the filtrate. The filtrate was then subjected to derivative spectrophotometric and HPLC analyses for the determination of 5-fluorouracil. The performance of the SPE procedure was checked by both the chromatographic and spectroscopic techniques. Figure 3 shows chromatograms obtained from a standard solution of flucytosine (1 mg ml^{-1}) containing 5-fluorouracil at the 1% (w/w) level relative to flucytosine before [Fig. 3(a)] and after [Fig. 3(b)] the SPE step. Flucytosine was completely eliminated and 5-fluorouracil was quantitatively recovered. Thus essentially identical HPLC calibration graphs were obtained using 5-fluorouracil standard solutions and the corresponding SPE filtrates. The conventional

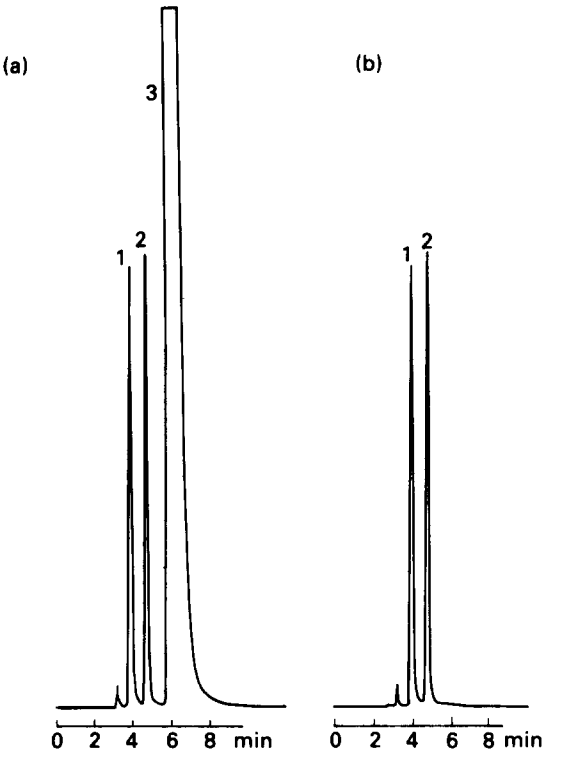

Figure 3
HPLC chromatograms obtained from: (a) sample solution containing flucytosine (1 mg ml^{-1}) and 5-fluorouracil (10 μg ml^{-1}); (b) the same sample solution after the SPE step. Chromatographic conditions as in Fig. 1. 1,5-fluorouracil; 2, thymine; and 3, flucytosine.

zero-order and second-order derivative UV spectra of a SPE filtrate were recorded and compared (Fig. 4) with those of a flucytosine standard solution. It was shown that with the

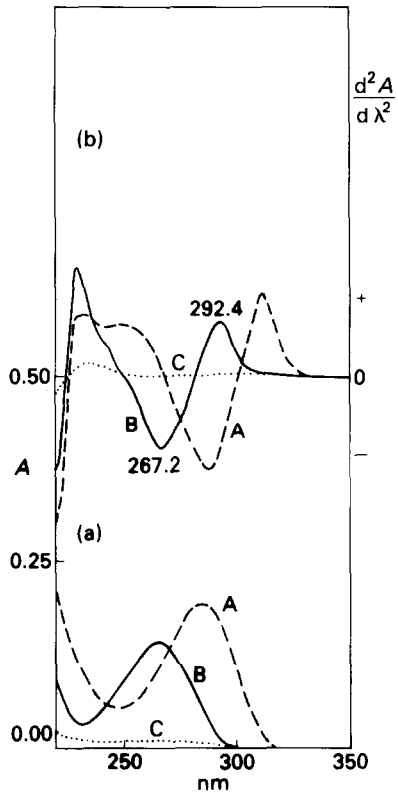

Figure 4
Ultraviolet spectra of A, flucytosone [10 μg ml^{-1} in buffer solution (pH 3.0)]; B, SPE filtrate (pH 3.0) from 5-fluorouracil solution (10 μg ml^{-1}) and C, buffer solution (pH 3.0) filtered through the SPE column. (a) Absorption zero-order spectra; (b) second-order derivative UV spectra.

derivative mode, 5-fluorouracil traces could be unequivocally identified and distinguished from flucytosine; it was confirmed that flucytosine was eliminated by the SPE procedure (HPLC monitoring). The weak absorption contribution of the filtered buffer solution observed in the conventional UV spectrum, but suppressed using the derivative mode (curve C, Fig. 4), was found to be due to the SPE column.

For quantitative applications, the peak-to-peak amplitude $^2D_{292.4,267.2}$ obtained from the second-order derivative spectrum was found to be linearly correlated with the 5-fluorouracil concentration; furthermore, essentially identical calibration graphs were obtained from 5-fluorouracil standard solutions and mixed standard solutions containing flucytosine, that had been subjected to the SPE procedure (Table 1). The RSD of the selected amplitude $^2D_{292.4,267.2}$, derived from replicate (n = 8) analyses of a single mixed standard solution [1% (w/w) of 5-fluorouracil relative to flucytosine], was 0.7%, indicating satisfactory precision. The lower detection limit for 5-fluorouracil obtained by a statistical treatment of the calibration data [12] was about 0.60 μg ml^{-1}, equivalent to 0.06% (w/w) relative to flucytosine. This value is lower than the pharmacopoeial limit (0.1%, w/w) [4].

Commercial tablets and solutions for intravenous infusion were analysed for the potential presence of 5-fluorouracil and the results are reported in Table 2. The presence of appreciable quantities of 5-fluorouracil was observed only in certain solutions and was probably dependent on the age and stability of the preparation. The solvolytic deamination of flucytosine to 5-fluorouracil is known to depend on pH and temperature [10]; pH 6.4 was optimum for drug stability and a temperature range of 18–25°C was recommended for the storage of commercial flucytosine solutions. At the pH used in the proposed assay methods the degradation of flucytosine to 5-fluorouracil was not observed during the analytical procedure; in this acidic region 5-fluorouracil does not decompose [10]. A limited, exploratory stability test on a commercial flucytosine intravenous solution was carried out. The commercial 5-fluorouracil-free solution of the drug was maintained at 40°C and monitored for the formation of 5-fluorouracil by both the HPLC and derivative UV methods. Essentially concordant results showed 5-fluorouracil to be absent up to the fifth day but to attain levels (relative to that of flucytosine) of 0.16, 0.4 and 1.0% (w/w) after 10, 20 and 30 days, respectively.

Conclusions

Reliable quality control of flucytosine formulations requires the availability of selective analytical procedures. The selective determination of flucytosine in the presence of 5-fluorouracil can be easily accomplished by HPLC and second-order derivative spectrophotometric methods. Traces of 5-fluorouracil in flucytosine formulations can be detected and determined by a second-order derivative UV assay, after preliminary removal of protonated flucytosine using a strong cation-exchange column. The combination of this SPE procedure with a HPLC method enables a highly sensitive assay of traces of 5-fluorouracil to be performed without column overloading. For

the determination of traces of 5-fluorouracil HPLC is the method of choice; derivative spectroscopy can constitute a useful and convenient alternative when the HPLC instrumentation is not available.

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