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Spectrophotometric and Spectrofluorimetric Determination of Fluorouracil in the Presence of its Degradation Products

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Abstract

Three reliable spectrophotometric and spectrofluorimetric procedures are described for the determination of fluorouracil in bulk powder and ampoules in the presence of its degradation products.

One spectrophotometric procedure, based on measurement at 555 nm of the violet-coloured complex formed by fluorouracil with cobalt(II), has a detection limit of 0·03 mg mL^{-1}. Two sensitive spectrofluorimetric procedures are also proposed. One is based on measurement of the intrinsic fluorescence of the liberated fluorouracil at 375 nm, after precipitation as its cobalt(II) complex, decomposition of the precipitate with sulphuric acid and excitation at 295 nm. The second depends on excitation of the fluorouracil–cobalt(II) complex at 395 nm and measuring its fluorescence at 483 nm. The limits of detection of the two spectrofluorimetric procedures are 0·5 and 2 $\mu g\,mL^{-1}$, respectively.

The three procedures have been used successfully for the determination of fluorouracil ampoules. The validity of the methods has been assessed by applying the standard addition technique.

Fluorouracil, or fluorinated pyrimidine, was developed in 1966 as a potent antineoplastic agent. Its usefulness is confined to solid tumors (Bagly et al 1974). Most pharmacopoeias recommended non-aqueous procedures for its assay. Other analytical procedures reported in the literature include nuclear magnetic resonance (Bertucci et al 1995), polarography (Liang 1991) and analysis via its fluoride content (Hintsche et al 1990). Thin-layer chromatography, gas chromatography and high-performance liquid chromatography have been also reported (Buyuktimkin 1983; William et al 1985; Compagnon et al 1996).

The literature also contains reports of three spectrophotometric procedures exploiting complexation of the drug with copper or cobalt after extraction (Banerjee & Sumathi 1993; Banerjee & Rao 1994; Sumathi & Rao 1994), two indirect spectrophotometric procedures (Cavrini et al 1991; Salem et al 1994) via the second and fourth derivatives, and two spectrofluorimetric procedures (Ohkura 1988; Kemena et al 1991) using reactions with chloroacetaldehyde and coumarin derivatives. Most of the above procedures require sophisticated instrumentation not yet available in many control laboratories, especially in developing countries. It seems, therefore, necessary to develop simple and sensitive stability-indicating procedures for the assay. Fluorouracil has three known degradation products, sodium fluoroacetate, urea and formaldehyde. It degrades under alkaline conditions at pH > 9 and its degradation is enhanced by increasing pH and temperature (Rudy & Seknowsky 1973). In the current communication fluorouracil is determined either spectrophotometrically or spectrofluorimetrically, in presence of its degradation products, by making use of its cobalt(II) complex.

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Materials and Methods

Reagents

All chemicals were of analytical grade. Cobaltous chloride hexahydrate was 10^{-2} M in dimethylsulphoxide or in ethanol.

Samples
Pure samples of fluorouracil from Aldrich were assayed as 99·6 ± 0·56% according to the United States Pharmacopeia (USP) (1995). Fluorouracil ampoules, claimed to contain 250 mg, were purchased from Hoffmann-La Roche and Biosyn; the batch numbers were 038591 and 86445, respectively. Pure samples of sodium fluoroacetate, urea and 37% formalin were purchased from Aldrich, Morgan and Sigma, respectively.

Standard stock solutions
Standard solutions of fluorouracil, sodium fluoroacetate and urea (3, 1 and 0·1 mg mL^{-1} of each) were prepared by dissolving 0·3 g of each in dimethylsulphoxide, 0·1 g of each in ethanol and 0·01 g of each in dimethylsulphoxide, in separate 100-mL volumetric flasks, and diluting to volume with the same solvent each time.

Standard solutions containing 3, 1 and 0·1 mg mL^{-1} formaldehyde in 100-mL volumetric flasks were prepared in dimethylsulphoxide, ethanol or dimethylsulphoxide, respectively.

Stock solution of fluorouracil degradation products (3 mg mL^{-1}) was prepared by dissolving fluorouracil (0·3 g) in sodium hydroxide solution (4 M; 20 mL) and hydrolysing by heating the alkaline fluorouracil sample on a boiling water bath for 3 h, neutralizing with sulphuric acid (10 M) and diluting to 100 mL with either dimethylsulphoxide or ethanol. The hydrolysis was monitored for completeness by TLC on plastic-backed plates, with intact fluorouracil as reference; ethyl acetate–acetone–water, 70 + 40 + 10 was used as mobile phase. Spots were visualized by UV illumination.

Spectrophotometric procedure (Method A)
Bulk powder. A sample of well-mixed powder equivalent to 0·3–1 mg fluorouracil was accurately weighed into a 10-mL volumetric flask, dissolved in dimethylsulphoxide (1 mL) and cobaltous reagent in dimethylsulphoxide (2 mL) then pyrrolidine (0·1 mL) were added. The mixture was agitated and diluted to volume with dimethylsulphoxide. The absorbance of the resulting violet colour was measured at 555 nm (Beckman DU-7 UV-Vis spectrophotometer) and the concentration of fluorouracil calculated either by use of a previously determined calibration curve or by use of the appropriate regression equation (Table 1).

Ampoules. The contents of 10 ampoules were mixed and the volume measured. A solution (1 mg mL^{-1}) was prepared in dimethylsulphoxide and a sample of this solution containing 0·3–1 mg fluorouracil was treated with cobaltous reagent in dimethylsulphoxide (2 mL) then pyrrolidine (0·1 mL) and the analysis continued as for bulk powder.

Spectrofluorimetric procedure exploiting the intrinsic fluorescence of fluorouracil (Method B)
Bulk powder. Well mixed powder equivalent to 5–30 mg fluorouracil was accurately weighed and dissolved in ethanol (8 mL) in a 20-mL test tube and cobaltous reagent (2 mL) in ethanol the pyrrolidine (0·1 mL) were added. The mixture was agitated and filtered and the resulting precipitate was washed with ethanol (5 mL) and transferred to a 100-mL volumetric flask. Sulphuric acid (10 M, one drop) was added to dissolve the precipitate and to liberate fluorouracil from its cobalt(II) complex and the solution was diluted to volume with de-ionized water. This solution was diluted 1 : 100 with de-ionized water and the fluorescence intensity measured at 375 nm (Shimadzu RF-450 recording spectrofluorophotometer) using 295 nm as the excitation wavelength; the ordinate scale was 4 (×8) and the sensitivity high (×100). The concentration of fluorouracil was calculated either by use of a previously determined calibration curve or by use of the regression equation.

Ampoules. The contents of 10 ampoules were mixed and the volume measured. A solution (3 mg mL^{-1}) was prepared in ethanol and a sample of this solution containing 5–30 mg fluorouracil was analysed as for the bulk powder.

Spectrofluorimetric procedure with the cobalt(II) complex (Method C)
Bulk powder. Well mixed powder equivalent to 0·02–0·08 mg fluorouracil was accurately weighed and dissolved in dimethylsulphoxide (1 mL) in a 10-mL volumetric flask. Cobalt(II) reagent in dimethylsulphoxide (0·2 mL) and pyrrolidine (0·1 mL) were added and the mixture was agitated and diluted to volume with dimethylsulphoxide. The fluorescence intensity was measured at 483 nm using 395 nm as the excitation wavelength; the ordinate scale was 2 (×4) and the sensitivity high (×100). The concentration of fluorouracil was calculated either by use of a previously determined calibration curve or by use of the regression equation.

Ampoules. The contents of 10 ampoules were mixed and the volume measured. A solution (0·1 mg mL^{-1}) was prepared in ethanol and a sample of this solution containing 0·02–0·08 mg fluorouracil was analysed as for the bulk powder.

Validity tests

Recovery of fluorouracil in presence of its degradation products. Standard fluorouracil solutions (3 mg mL^{-1}, 1 mg mL^{-1} and 0·1 mg mL^{-1}) were mixed separately with different proportions of the equivalent standard solutions of the degradation products (sodium fluoroacetate, urea and formaldehyde). These solutions were analysed as described above and the percentage recoveries of fluorouracil calculated.

Adaptation of the procedures to samples from the hydrolysis of fluorouracil. Standard solutions of fluorouracil, 3 mg mL^{-1}, 1 mg mL^{-1} and 0·1 mg mL^{-1}, were mixed with different proportions of the laboratory-degraded sample of fluorouracil stock solution. These synthetically prepared mixtures were analysed as described above for standard solutions of the degradation products.

Results and Discussion

The reaction of cobalt(II) ions with a ring imide structure, in the presence of organic amine (the Koppani-Zwilker reaction) has been recommended for the analysis of these imides (Stevens 1986). Having two imide groups in its ring, fluorouracil undergoes this reaction, forming the fluorouracil–cobalt(II) complex which can be measured spectrophotometrically and spectrofluorimetrically. The degradation products of fluorouracil, fluoroacetate, urea and formaldehyde, are devoid of a ring imide structure and so the reaction can be used as the basis for a convenient indicating assay for fluorouracilul—cobalt(II) reacts only with intact fluorouracil and not with its degradation products.

Spectrophotometric investigation

Although 266 nm has been recommended for measurement of the absorbance of aqueous solutions of fluorouracil (USP 1995), the products of alkaline-induced degradation of fluorouracil, which absorb at 249, 260 and 264 nm (formaldehyde, urea and sodium fluoroacetate, respectively) interfere with the analysis of the intact drug. The spectrophotometric absorbance of the fluorouracil–cobalt(II) complex at 555 nm was, however, found to be free from interference from the degradation products (Figure 1).

Spectrofluorimetric investigation

Although the fluorescence intensity of methanolic solutions of fluorouracil has been measured at 391 nm after excitation at 315 nm (USP 1995), the fluorescence of the compounds' degradation products (405 and 415 nm for urea and sodium fluoroacetate, respectively) was found to interfere after excitation at this wavelength.

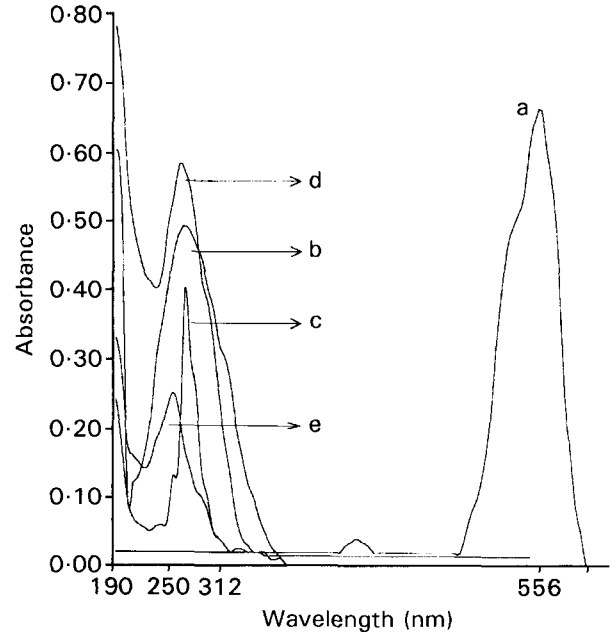

Figure 1. Absorption spectra of 0·07 mg mL^{-1} Co(II)-fluorouracil complex (a) and 0·01 mg mL^{-1} fluorouracil (b), sodium fluoroacetate (c), urea (d) and formaldehyde (e).

In method B, precipitation of fluorouracil as its cobalt(II) complex was used to separate the intact drug from its degradation products. Liberation of fluorouracil from its metal complex in acid medium enabled measurement of its fluorescence at 375 nm, after excitation at 295 nm, without any interference (Figure 2).

In method C, formation of a complex between fluorouracil and cobalt(II), resulted in physicochemical modification of the intrinsic fluorescence of fluorouracil, the emission wavelength becoming higher and the emission intensity lower. These results accord with a literature report (Robert 1988) of intramolecular transitions between ligand and metal ion leading to a shift in the photoluminescence of the ligand. Thus, the fluorouracil–cobalt(II) complex can be measured at 483 nm after excitation at 395 nm without any interference from its degradation products (Figure 3).

The three suggested methods need no extraction step.

During optimization of the assays it was found that the absorbance of the complex increased as the concentration of cobalt(II) was increased to 47·6% (m/m); for cobalt(II) concentrations above this the absorbance remained constant.

When the reaction was conducted in ethanol or dimethylsulphoxide, the blue cobalt(II) which resulted had four ligands. Use of aqueous media results in pink cobalt(II) which is reported to have

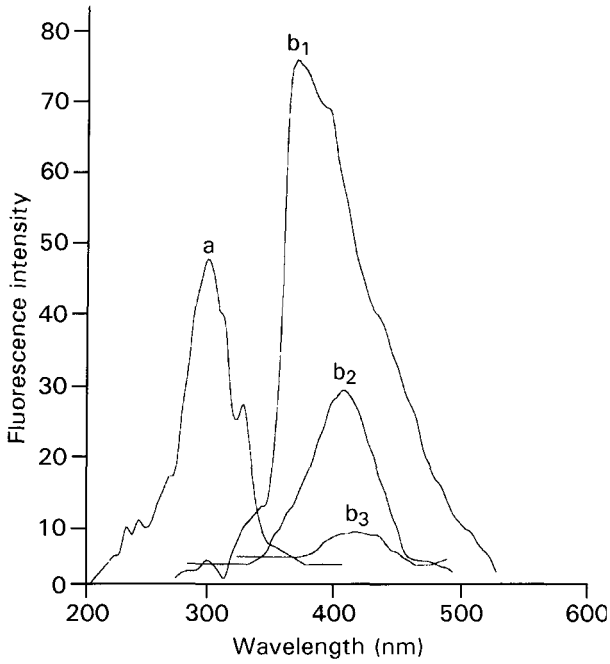

Figure 2. Excitation (a) and emission spectra of 2·5 μg mL^{-1} aqueous fluorouracil solution (b_1), urea (b_2) and sodium fluoroacetate (b_3).

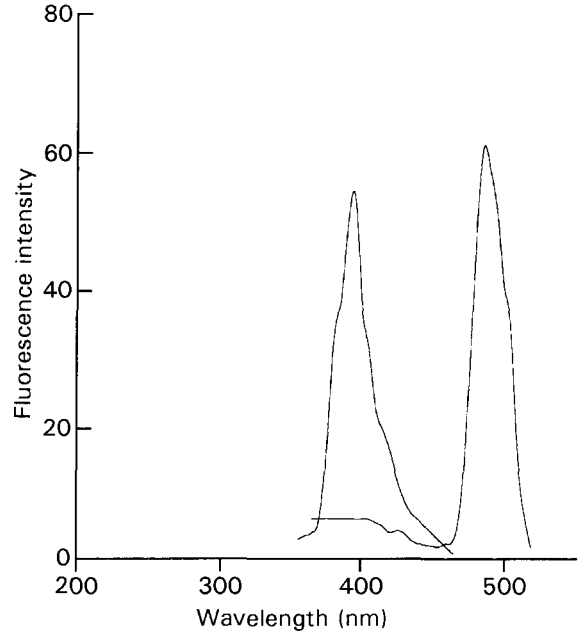

Figure 3. Excitation (a) and emission (b) spectra of cobalt-(II)–fluorouracil complex obtained from 5 μg mL^{-1} fluorouracil.

six ligands. The pink cobalt ions retard complex formation, as is apparent from the equation (Moeller & Bailar 1984):

$$[CoCl_4]^{2-} + 6H_2O \rightleftharpoons [Co(H_2O)_6]^{2+} + 4Cl^- \quad (1)$$

On the other hand, use of the ethanolic medium and available organic solvents, e.g. benzene, toluene, butyl acetate, acetone and dichloromethane, resulted in precipitation of the complex; use of dimethylsulphoxide achieves maximum stability and solubility of the fluorouracil–Co(II) complex.

A basic reagent, preferably an organic base such as piperazine, piperidine and pyrrolidine, is essential for colour development. In the proposed procedures 0·1 mL pyrrolidine is optimum; its final concentration is 1% (v/v). Full colour development is instantaneous and both absorbance and fluorescence intensities remain unchanged for 10 h.

The stoichiometric ratio of fluorouracil to cobalt(II) in the complex was determined by continuous variation of the method; the results showed the formation of a 1 : 1 complex, a result which was confirmed by elemental analysis of the cobalt(II)–fluorouracil complex.

Calibration graphs were constructed for the three proposed methods using the optimum conditions described above. A summary of the regression analysis data is presented in Table 1. The relative standard deviations were found to be less than 2%, indicative of the high reproducibility of the procedures.

Fluorouracil ampoules were analysed by the proposed procedures and the results obtained compared with those obtained from the official USP 1995 method (Table 2). Control experiments were performed to evaluate the accuracy of the procedures when applied to ampoules; known concentrations of fluorouracil were added to samples of the analysed ampoules and the recovery of the added drug was computed as a criterion of the accuracy of the proposed procedures (Table 3).

Statistical analysis of the results revealed no significant difference between those from the proposed methods and from the official USP 1995 method, indicating that the proposed methods are as precise and accurate as the official method (Table 4).

The results presented in Tables 5 and 6 show that the proposed procedures are satisfactory for synthetic mixtures containing different ratios of the drug and its degradation products.

In conclusion, these studies prove that the described spectrophotometric and spectrofluorimetric procedures are satisfactory, reliable and selective and that they enable rapid and accurate determination of fluorouracil. The methods have simple work-up procedures, require no sophisticated instrumentation or costly reagents and determine only the therapeutically active, undegraded drug.

Table 1. Analytical parameters of the methods proposed for the determination of fluorouracil.

Parameter	Method A	Method B	Method C
Maximum wavelength (nm)	555	λ_{ex} 295 λ_{em} 375	λ_{ex} 395 λ_{ex} 483
Concentration range ($\mu g\,mL^{-1}$)	30–100	0·5–3	2–8
Linear regression:			
Intercept	0·002	−0·747	3·489
Slope	9·402	30·046	11·211
Correlation coefficient	0·999	0·999	0·996
Mean recovery	100·2	99·7	100·4
Relative standard deviation (%)*	0·41	0·86	0·73

*Five replicate determinations.

Table 2. Determination of fluorouracil in ampoules by the proposed methods and by the US Pharmacopeia method.

Pharmaceutical preparation	Recovery ± relative standard deviation (%)†			
	Method A	Method B	Method C	US Pharmacopeia method‡
Fluorouracil Roche	101·3 ± 0·46	100·1 ± 0·92	101·1 ± 1·22	99·9 ± 0·23
Fluorouracil Biosyn	100·1 ± 0·58	98·8 ± 0·93	100·8 ± 0·73	100·8 ± 0·18

*Claimed to contain 250 mg per ampoule. †Mean of six replicate determinations. ‡United States Pharmacopeia (1995).

Table 3. Determination of fluorouracil in ampoules by application of the standard addition technique with the proposed methods.

Exp.	Method A		Method B		Method C	
	Amount of standard added ($\mu g\,mL^{-1}$)	Recovery (%)	Amount of standard added ($\mu g\,mL^{-1}$)	Recovery (%)	Amount of standard added ($\mu g\,mL^{-1}$)	Recovery (%)
1	20	100·2	0·5	99·2	2	100·5
2	30	99·8	1·0	98·1	3	100·4
3	40	100·7	1·5	100·4	4	100·9
Mean ± relative standard deviation (%)		100·2 ± 0·45		99·2 ± 1·15		100·6 ± 0·26

Table 4. Statistical comparison of the determination of fluorouracil by the proposed methods and by the US Pharmacopeia method.

Values	Method A	Method B	Method C	US Pharmacopeia method†
Mean	100·2	99·7	100·4	99·6
Standard deviation	0·41	0·86	0·73	0·56
Variance	0·1681	0·7396	0·5329	0·3136
n	8	6	7	4
F	1·87 (4·35)*	2·36 (9·01)*	1·70 (8·94)*	
Student's t-test	0·56 (2·36)*	0·21 (2·57)*	1·89 (2·45)*	

*Tabulated values at 95% confidence limit. †United States Pharmacopeia (1995).

Table 5. Relative recoveries of fluorouracil from synthetic mixtures with its degradation products, by use of the proposed methods.

Sample no.	Degradation products (%)			Found (%) of pure added fluorouracil*		
	Urea*	Formaldehyde*	Fluoroacetate*	Method A	Method B	Method C
1	0	0	0	100·32	99·87	99·99
2	20	20	20	98·92	98·90	100·43
3	40	40	40	99·16	98·83	99·76
4	60	60	60	100·11	100·84	99·50
5	80	80	80	99·45	98·93	99·12
6	100	100	100	0	0	0
Mean ± relative standard deviation (%)				99·59 ± 0·605	99·47 ± 0·885	99·76 ± 0·496

*Solutions of the same concentrations were used in different ratios for each method.

Table 6. Relative recoveries of fluorouracil in synthetic mixtures with its hydrolytic sample, by use of the proposed methods.

Sample no.	Hydrolytic sample (%)	Amount of pure added fluorouracil found (%)		
		Method A	Method B	Method C
1	0	100·33	100·48	99·97
2	20	100·86	98·21	100·50
3	40	100·12	99·73	98·93
4	60	99·27	100·15	100·03
5	80	100·65	99·56	100·22
6	100	0	0	0
Mean ± standard deviation		100·25 ± 0·616	99·62 ± 0·874	99·93 ± 0·596

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