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Research Article

Correction for Irrelevant Absorption in Multicomponent Spectrophotometric Assay of Riboflavin, Formylmethylflavin, and Degradation Products: Kinetic Applications

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Abstract. In the spectrophotometric assay of multicomponent systems involved in drug degradation studies, some minor or unknown degradation products may be present. These products may interfere in the assay and thus invalidate the results due to their absorption in the range of analytical wavelengths. This interference may be eliminated by the application of an appropriate correction procedure to obtain reliable data for kinetic treatment. The present study is based on the application of linear and non-linear irrelevant absorption corrections in the multicomponent spectrophotometric assay of riboflavin and formylmethylflavin during the photolysis and hydrolysis studies. The correction procedures take into account the interference caused by minor or unknown products and have shown considerable improvement in the assay data in terms of the molar balance. The treatment of the corrected data has led to more accurate kinetic results in degradation studies.

KEY WORDS: degradation kinetics; formylmethylflavin; irrelevant absorption; riboflavin; spectrophotometric assay.

INTRODUCTION

Several studies have been conducted to propose correction procedures for irrelevant absorption in the spectrophotometric assay of drug substances. The early applications included the correction for irrelevant absorption in the spectrophotometric determination of vitamin A in liver oils by Morton and Stubbs (1–3). It was based on the assumption that the irrelevant absorption curve is linear over the range 310–340 nm, with the absorption maximum of vitamin A around 328 nm (1–3). A similar correction procedure was suggested considering the absorption maximum of vitamin A at 326 nm (4). Later, a geometric correction of absorption curves was proposed to determine the true concentration of vitamin A in cod liver oil (5). Morton and Stubbs correction for linear irrelevant absorption was also applied to the assay of ergosterol extracted from yeast (6). An advancement in the correction procedures for irrelevant absorption in the spectrophotometric assay of griseofulvin was made (7,8). It was based on the consideration of impurities to exhibit quadratic absorption spectra resulting in non-linear absorption. The application of zero-order UV spectrophotometric and graphical background correction methods for the assay of active components in a changeable matrix has been suggested (9). The orthogonal function method has been applied to the simultaneous determination of nifuroxime and furazolidone in pharmaceutical formulations. In this method, the correction of interference by either component is carried out using a calculated absorbance ratio at the absorption maxima (10).

In the spectrophotometric assay of multicomponent systems such as that involved in drug degradation studies, some minor or unknown degradation products may be present. The absorption characteristics of these products may not be known and hence their interference may invalidate the assay results, particularly, in kinetic studies. This may be overcome by the application of an appropriate correction procedure in the analysis of complex mixtures using suitable wavelengths. The wavelengths selected for the correction of irrelevant absorption due to interfering compounds would depend on the absorption characteristics of these compounds. It has been suggested that the assumption of a particular type of irrelevant absorption curve during the degradation reaction may not produce the desirable results (11). This is due to the fact that the irrelevant absorption curve may vary, with time, with respect to the relative concentrations of the compounds

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present. It is, therefore, advisable to use several sets of wavelengths for a particular irrelevant absorption correction and to select the best set that gives more self-consistent data for the assay of the major components of a drug in a degradation study.

The present study is based on the application of linear and non-linear irrelevant absorption correction procedures in the multicomponent assay of riboflavin and photoproducts and formylmethylflavin and hydrolytic products during the degradation reactions. The correction procedures have shown improvement in the analytical data for achieving more accurate and reliable kinetic results in these studies. The commonly used correction procedures for irrelevant absorption (11–13) may be described as follows.

Correction for Irrelevant Absorption Varying Linearly with Wavelength

The irrelevant absorption ($_1e$) in a particular assay is considered to obey the following relation.

$$_1e_i = m\lambda_i + c \tag{1}$$

where m and c are constants for any one mixture containing the components of interest and the undesirable compounds.

The total absorbance A_i at λ_i is:

$$A_i = {}_1Ck_ie + m\lambda_i + c \tag{2}$$

Using this relation, it is possible to determine the most probable concentration, $_1C$, of the component from a series of n absorbance measurements, A_i, at the wavelengths, λ_i=1 to n.

The unknowns in these determinations are:

$_1C$, m, and c.

The knowns are:

λ_i the wavelength of the ith measurement
A_i the absorbance measurement at λ_i

The e and k_i are the values of absorptivity of the pure compound and the appropriate factor to obtain the value of e at the wavelength, λ_i, respectively. The e is usually the value of absorptivity at the absorption maximum.

The following matrix equation can be used for the five appropriately chosen wavelengths which are required for a linear irrelevant absorption correction in a three-component spectrophotometric assay:

$$\begin{bmatrix} A_1 \\ A_2 \\ A_3 \\ A_4 \\ A_5 \end{bmatrix} = \begin{bmatrix} {}_1K_1 & {}_2K_1 & {}_3K_1 & \lambda_1 & 1 \\ {}_1K_2 & {}_2K_2 & {}_3K_2 & \lambda_2 & 1 \\ {}_1K_3 & {}_2K_3 & {}_3K_3 & \lambda_3 & 1 \\ {}_1K_4 & {}_2K_4 & {}_3K_4 & \lambda_4 & 1 \\ {}_1K_5 & {}_2K_5 & {}_3K_5 & \lambda_5 & 1 \end{bmatrix} \begin{bmatrix} {}_1C \\ {}_2C \\ {}_3C \\ m \\ c \end{bmatrix} \tag{3}$$

Where A_1, A_2, A_3, A_4, and A_5 are the absorbancies at the wavelengths λ_1, λ_2, λ_3, λ_4, and λ_5; K is the absorptivity-cell path product; $_1C$, $_2C$, and $_3C$ are concentrations of the three components. The determination of $_1C$, $_2C$, and $_3C$ requires the solution of three equations. The constants m and c are not normally calculated. A similar matrix equation can be used for linear irrelevant absorption correction in one- or two-component spectrophotometric assays.

Correction for Irrelevant Absorption Varying Non-Linearly with Wavelength

The variation of non-linear irrelevant absorption in a mixture may be expressed as a polynomial in λ_i. In this case Eq. (1) can be replaced by a quadratic equation:

$$_1e_i = m\lambda_i^2 + n\lambda_i + c \tag{4}$$

Therefore, the matrix equation contains an extra element in each row and column and for a three-component assay the equation is:

$$\begin{bmatrix} A_1 \\ A_2 \\ A_3 \\ A_4 \\ A_5 \\ A_6 \end{bmatrix} = \begin{bmatrix} {}_1K_1 & {}_2K_1 & {}_3K_1 & \lambda_1^2 & \lambda_1 & 1 \\ {}_1K_2 & {}_2K_2 & {}_3K_2 & \lambda_2^2 & \lambda_2 & 1 \\ {}_1K_3 & {}_2K_3 & {}_3K_3 & \lambda_3^2 & \lambda_3 & 1 \\ {}_1K_4 & {}_2K_4 & {}_3K_4 & \lambda_4^2 & \lambda_4 & 1 \\ {}_1K_5 & {}_2K_5 & {}_3K_5 & \lambda_5^2 & \lambda_5 & 1 \\ {}_1K_6 & {}_2K_6 & {}_3K_6 & \lambda_6^2 & \lambda_6 & 1 \end{bmatrix} \begin{bmatrix} {}_1C \\ {}_2C \\ {}_3C \\ m \\ n \\ c \end{bmatrix} \tag{5}$$

Where A_1, A_2, A_3, A_4, A_5, and A_6 are absorbencies at the appropriately selected wavelengths, λ_1, λ_2, λ_3, λ_4, λ_5, and λ_6. K is absorptivity-cell product, $_1C$, $_2C$, and $_3C$ are the concentrations of the three components and m, n, and c are constants.

In the present work, the solution of these matrix equations has been obtained with the help of a suitably programmed computer.

MATERIALS AND METHODS

Riboflavin (RF), lumiflavin (LF), and lumichrome (LC) were obtained from Sigma Chemicals Co. St. Louis, MD. Formylmethylflavin (FMF), carboxymethylflavin (CMF), and cyclodehydroriboflavin (CDRF) were prepared according to the methods of Fall and Petering (14), Fukumachi and Sakurai (15) and Schuman Jorns *et al.* (16), respectively. All reagents and solvents were of analytical grade or of the purest form available from Merck & Co., Whitehouse Station, NJ.

Photolysis of RF

A 10^{-4} M aqueous solution of RF containing 2.00 M Na_2HPO_4 was adjusted to pH 7.0 using 5.0 M HCl solution. A 200-ml volume of the solution was placed in a 250-ml Pyrex flask and immersed in a water bath maintained at 25±1°C. The solution was exposed to a Philips HPLN 125-W high-pressure mercury vapor fluorescent lamp (Lieschout, Netherlands) emitting at 405 and 436 nm (the 436-nm band is close to the 445-nm absorption maximum of RF). The lamp was fixed at a distance of 30 cm from the center of the flask. The solution was continuously bubbled with air during the irradiation. Samples were withdrawn at appropriate intervals for thin-layer chromatography and spectrophotometric assay.

Photolysis of FMF

A 10^{-4} M aqueous solution of FMF was adjusted to pH 2.0 with 0.1 M HCl solution and placed in a 1-L reaction vessel. The solution was deoxygenated for about 1 h by bubbling oxygen free nitrogen and irradiated with a Mazda M2 4.5-W low-pressure mercury discharge lamp (Associated Electronic Industries, London, UK), emitting at 350 and 440 nm (the 440-nm band corresponds to the 445-nm absorption maximum of FMF). The lamp was fixed in a cavity at the bottom of the vessel. The pH of the solution was maintained by the addition of 0.1 M HCl solution using an autotitrator. The solution was continuously bubbled with nitrogen during the irradiation. The reaction was carried out at 25±1°C by circulating water in the vessel from a thermostat bath. The rate of the reaction was followed by thin-layer chromatography and spectrophotometry.

Hydrolysis of FMF

A 10^{-4} M aqueous solution of FMF (1 L) was prepared and the pH adjusted to 11.0 with 0.1 M NaOH solution. The solution was placed in a water bath (25±1°C) to carry out the hydrolysis. The pH of the solution was maintained by the addition of 0.1 M NaOH solution using an autotitrator. The reaction was continued till the complete hydrolysis of FMF has taken place as observed by thin-layer chromatography (TLC). Samples were withdrawn at appropriate intervals for chromatographic examinations and spectrophotometric assay.

Thin-Layer Chromatography (TLC)

TLC of the degraded solutions of RF and FMF was performed on 250-μm cellulose plates (Whatman CC41) employing the solvent system: (a) 1-butanol/1-propanol/acetic acid/water (50:30:2:18, *v*/*v*) (17) and 250-μm silica gel G plates (Merck) employing the solvent system: (b) chloroform/methanol (90:20 *v*/*v*) (16). The compounds were detected by their characteristic fluorescence under 365-nm excitation or by visual observation.

Spectral Determination

The spectral determinations on pure and photolyzed/hydrolyzed solutions of the compounds were performed on a Shimadzu UV-240 recording spectrophotometer using quartz cells of 10-mm path length.

Light Intensity Measurement

The measurement of the intensity of the irradiation source was carried out using potassium ferrioxalate actinometry (18). A value of the emission of $2.19\pm0.12\times10^{18}$ quanta s^{-1} was obtained for the Philips 125-W lamp and $3.71\pm0.16\times10^{17}$ quanta s^{-1} for the Mazda 4.5-W lamp.

Spectrophotometric Assay

RF and photoproducts (FMF, LC, LF, and CDRF) in photolyzed solutions were determined by a specific multicomponent spectrophotometric assay reported by Ahmad *et al.* (11,19). It involves the adjustment of the photolyzed solutions to pH 2.0 (HCl–KCl buffer), removal of LF and LC by extraction with chloroform, dissolution of the chloroform residue in acetate buffer (pH 4.5) and their determination by a two-component assay at 356 and 445 nm. In the aqueous phase (pH 2.0), absorbance measurements are made at 385, 410, and 445 nm and the determination of FMF, CDRF, and RF is carried out by a three-component assay using a suitable software. The wavelengths chosen for the assay of all the compounds correspond to their absorption maxima (RF 445 nm, CDRF 410 nm, FMF 385 nm, LF 445 nm, and LC 356 nm) (11,19).

The assay of FMF and its major photoproduct (LC) in acid solution and hydrolytic products (LC and LF) in alkaline solution has been carried out by the multicomponent spectrophotometric method of Ahmad *et al.* (11,17). The degraded solutions of FMF were pre-adjusted to pH 2.0 (HCl–KCl buffer), LC and LF were extracted with chloroform and their assay was carried out as described above in the case of RF. The assay of FMF present in the aqueous phase was performed at 385 nm.

Fluorescence Measurement

The measurement of fluorescence of RF solutions was performed at room temperature (~25°C) with a Spectromax 5 fluorimeter (Molecular Devices, Sunnyvale, CA, USA) in the end point mode using 374 nm as the excitation wavelength and 525 nm as the fluorescence wavelength (20). The fluorescence was measured in relative fluorescence units using a pure 0.05 mM RF solution as standard.

RESULTS AND DISCUSSION

Composition of Degraded Solutions

The degradation products of RF (photolysis) and FMF (hydrolysis) in aqueous solution are known (11,12,16,17,19–34). In order to ascertain the presence of these products in degraded solutions TLC has been applied using well-established solvent systems developed by Ahmad *et al.* (11,17) and Schuman Jorns *et al.* (16) for the detection of these compounds. The confirmation of the degradation products of RF and FMF formed under the present reaction conditions is necessary to carry out the specific spectrophotometric assay for the determination of these compounds in degraded solutions.

RF Photolysis

The TLC study of the composition of aerobically photodegraded solutions of RF in the presence of phosphate buffer (pH 7.0) showed the presence of the following compounds exhibiting characteristic fluorescence or color, using solvent system (a): R_f values and fluorescence, major (undegraded RF, 0.27, yellow green; FMF, 0.70, yellow green; LF, 0.43, yellow green; LC, 0.63, sky blue) and minor products (CMF, 0.20, yellow green); and solvent system (b): major (undegraded RF, 0.37, yellow green; CDRF, 0.46, red color) and unknown minor products (0.07, pale color; 0.63, blue fluorescence; 0.71, red color; 0.76, blue fluorescence). FMF,

CDRF, LF, LC, and CMF have previously been identified as the photodegradation products of RF in the presence and absence of phosphate buffer (pH 7.0) (19,21–25). The presence of 2.0 M phosphate reduces the fluorescence intensity of RF to the extent of ~36%, indicating a complexation between RF and HPO_4^{2-} ions and a change in reaction pathway to form CDRF (21). In addition to these compounds, the above mentioned unknown minor degradation products were also detected by TLC. The presence of a large number of known and unknown products in the photodegraded solutions of RF indicated the complexity of the mixture and hence a consideration of the interference caused by the unknown minor products in the assay of RF and major degradation products.

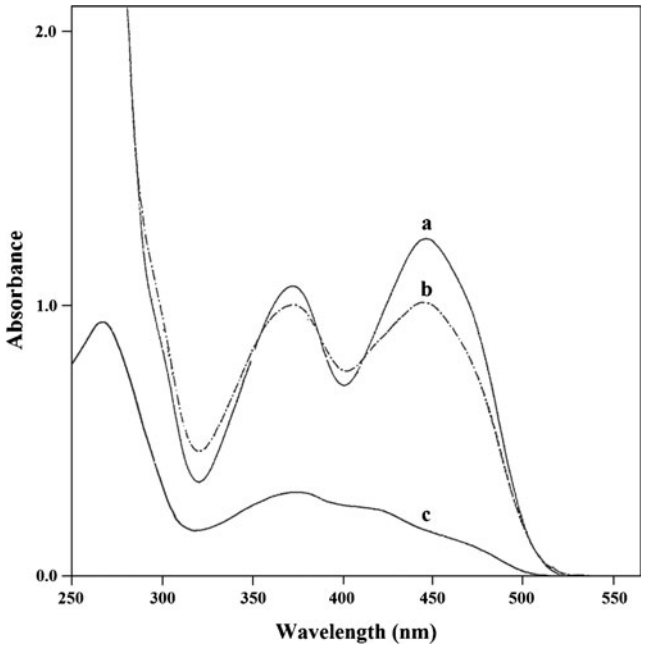

Fig. 1. Absorption spectra (pH 2.0) of *a* 10^{-4} M RF; *b* aqueous phase of photodegraded solution of RF (~20%) and *c* same (~80%)

FMF Photolysis

A TLC study of the anaerobic photolysis of FMF at pH 2.0 showed the presence of the following compounds using solvent system (a): major (undegraded FMF, LC), minor (CMF), unknown product (R_f 0.44, yellow green fluorescence), LC and CMF are the known photodegradation products of FMF in aqueous solution (26–28). The presence of an unknown product of FMF has also been observed at other pH values. CMF and the unknown product may interfere in the assay of FMF in degraded solutions.

FMF Hydrolysis at pH 11.0

FMF is known to undergo hydrolysis in the dark to LC and LF and to a minor oxidation product, CMF, in the neutral and alkaline media (11,17,27,28). The R_f values and fluorescence of these compounds is reported under RF photolysis. A completely hydrolyzed solution of FMF still contains products that are formed by the alkaline cleavage of the isoalloxazine ring, the nucleus present in FMF. These products absorb in the 300–500-nm region (29–31) and would interfere in the assay of FMF in hydrolyzed solutions.

Nature of Interfering Compounds

The present study involves multicomponent spectrophotometric assay of RF and its major photolysis and photoaddition products (FMF, CDRF, LC, and LF) (19) and FMF and its major photodegradation (LC) (11,27) and hydrolysis products (LC and LF) (17,32) in aqueous solution by the methods developed by Ahmad and Rapson (11) and Ahmad *et al.* (19). However, the composition of the photodegraded solutions of RF containing several known and unknown products may vary with the light intensity and wavelengths of the irradiation source (22) and thus affect the accuracy of the assay with the correct assumption of the compounds analyzed for.

In the present study, the unknown products might absorb in the region of the analytical wavelengths (300–500 nm) and thus cause interference in the assay of RF and its major photodegradation products (FMF, CDRF) or FMF present in the aqueous phase (pH 2.0) after extraction with chloroform to remove LC and LF (11,17). TLC examination has shown that the chloroform layer does not contain any other

Table I. Photolysis of 10^{-4} M RF at pH 7.0 (2.0 M Na_2HPO_4). Concentrations of RF and Photoproducts before Irrelevant Absorption Correction

Time (min)	RF ($M\times10^5$)	CDRF ($M\times10^5$)	FMF ($M\times10^5$)	LC ($M\times10^5$)	LF ($M\times10^5$)	Total ($M\times10^5$)
0	10.00	–	–	–	–	10.00
60	8.70	0.72	0.18	0.88	0.16	10.64
120	7.90	1.38	0.25	1.67	0.28	11.48
180	6.92	1.74	0.42	2.20	0.37	11.65
240	6.15	2.10	0.58	2.59	0.46	11.88
300	5.50	2.32	0.68	2.73	0.51	11.74
360	4.78	2.45	0.88	2.96	0.59	11.66
420	4.24	2.67	0.95	3.12	0.62	11.60
480	3.80	2.81	1.05	3.21	0.65	11.52

Radiation source: Philips HPLN 125-W high-pressure mercury vapor fluorescent lamp
RF riboflavin, *CDRF* cyclodehydroriboflavin, *FMF* formylmethylflavin, *LC* lumichrome, *LF* lumiflavin

Table II. Photolysis of 10^{-4} M RF at pH 7.0 (2.0 M Na_2HPO_4). Concentrations of RF and Photoproducts after Linear Irrelevant Absorption Correction

Time (min)	RF ($M\times10^5$)	CDRF ($M\times10^5$)	FMF ($M\times10^5$)	LC ($M\times10^5$)	LF ($M\times10^5$)	Total ($M\times10^5$)
0	10.00	–	–	–	–	10.00
60	8.44	0.69	0.15	0.88	0.16	10.32
120	7.25	1.07	0.20	1.67	0.28	10.47
180	6.17	1.41	0.34	2.20	0.37	10.49
240	5.25	1.70	0.45	2.59	0.46	10.45
300	4.70	1.85	0.52	2.73	0.51	10.31
360	4.02	1.95	0.65	2.96	0.59	10.18
420	3.45	2.16	0.75	3.12	0.62	10.10
480	2.97	2.28	0.81	3.21	0.65	9.92

Radiation source: Philips HPLN 125-W high-pressure mercury vapor fluorescent lamp
RF riboflavin, *CDRF* cyclodehydroriboflavin, *FMF* formylmethylflavin, *LC* lumichrome, *LF* lumiflavin

component and, therefore, can be assayed for LC alone or for LC and LF simultaneously without interference. CMF is a minor product of FMF oxidation (27) and has not been accounted for in the assay of the major products of RF and FMF. It amounts to ~0.5% of a 10^{-4} M FMF solution photolyzed up to 50% at pH 7.0 (28). In view of the composition of the photodegraded solutions of RF and FMF, interference in the assay of RF, FMF, CDRF, or FMF alone (aqueous phase) could be expected from CMF and the unknown minor products. The presence of the isoalloxazine ring cleavage products (29–31) in the hydrolyzed solutions of FMF would also be a source of interference in the assay of FMF. The nature and magnitude of this interference would depend on the compositional variations during the photolysis or hydrolysis reactions and hence the resultant spectral characteristics of the minor compounds.

Correction for Irrelevant Absorption

RF Photolysis

In view of the presence of interfering compounds in the assay of RF and its major photodegradation products (RF, FMF, and CDRF in aqueous phase) formed in the presence of 2.0 M phosphate buffer (pH 7.0), by the method of Ahmad *et al.* (19), an attempt was made to apply a correction for irrelevant absorption to improve the analytical data in terms of the molar balance. The spectrum of the aqueous phase of a 10^{-4} M RF solution at ~80% degradation exhibited a region almost linear with wavelength over the range 375–465 nm (Fig. 1), covered by the analytical wavelengths. Assuming the absorption of the mixture of minor products approximated by an amount inversely proportional to wavelength, the aqueous phase (pH 2.0) was assayed for RF and photodegradation products (FMF and CDRF) by applying a linear irrelevant absorption correction to allow for the presence of minor interfering products. The various wavelengths chosen for such corrections included three sets of wavelengths, *i.e.*, 375 and 460 nm, 375 and 432 nm, and 400 and 460 nm, over the range, in addition to the wavelengths of 385, 410, and 445 nm used for the three-component assay (19). The analytical data obtained using 375- and 460-nm corrections by the solution of the matrix Eq. (3) were found to be satisfactory. The values of uncorrected and corrected concentrations of RF and photodegradation products determined during the reaction are reported in Tables I and II, respectively.

The application of the correction procedure has reduced the molar balance (10.0×10^{-5} M) from 11.9 to 10.5×10^{-5}

Table III. Photolysis of 10^{-4} M FMF at pH 2.0. Concentrations of FMF and Degradation Products

	Before irrelevant absorption correction			After non-linear irrelevant absorption correction		
Time (min)	FMF ($M\times10^5$)	LC ($M\times10^5$)	Total ($M\times10^5$)	FMF ($M\times10^5$)	LC ($M\times10^5$)	Total ($M\times10^5$)
0	10.00	–	10.00	10.00	–	10.00
24	9.14	1.36	10.50	8.66	1.36	10.02
48	8.56	2.28	10.84	7.69	2.28	9.97
72	8.29	2.66	10.95	7.28	2.66	9.94
96	7.91	3.18	11.09	6.78	3.18	9.96
120	7.68	3.46	11.14	6.46	3.46	9.92
144	7.13	3.58	10.71	6.37	3.58	9.95
168	6.86	3.76	10.62	6.22	3.76	9.98
192	6.64	3.92	10.56	5.96	3.92	9.88
216	6.32	4.20	10.52	5.65	4.20	9.85
240	6.04	4.45	10.49	5.35	4.45	9.80

pH adjusted with 0.1 M HCl solution
FMF formylmethylflavin, *LC* lumichrome

Table IV. Hydrolysis of 10^{-4} M Formylmethylflavin (FMF) at pH 11.0. Concentrations of FMF and Degraded Products

	Before irrelevant absorption correction				After linear irrelevant absorption correction			
Time (min)	FMF ($M\times10^5$)	LC ($M\times10^5$)	LF ($M\times10^5$)	Total ($M\times10^5$)	FMF ($M\times10^5$)	LC ($M\times10^5$)	LF ($M\times10^5$)	Total ($M\times10^5$)
0	10.00	–	–	10.00	10.00	–	–	10.00
15	7.55	1.76	0.74	10.05	7.50	1.76	0.74	10.00
30	5.87	3.15	1.21	10.23	5.66	3.15	1.21	10.02
45	5.04	3.77	1.60	10.41	4.69	3.77	1.60	10.06
60	4.50	4.25	1.92	10.67	3.88	4.25	1.92	10.05
90	3.83	4.64	2.37	10.84	3.07	4.64	2.37	10.08
120	2.55	5.59	2.64	10.78	1.88	5.59	2.64	10.11
150	2.07	5.79	2.94	10.80	1.32	5.79	2.94	10.05
180	1.90	5.82	3.14	10.86	1.15	5.82	3.14	10.11
210	1.63	5.95	3.36	10.94	0.77	5.95	3.35	10.07
240	1.47	6.10	3.42	10.99	0.58	6.10	3.42	10.10

pH adjusted with 0.1 M NaOH solution
FMF formylmethylflavin, *LC* lumichrome, *LF* lumiflavin

M, *i.e.*, ~12%, indicating the magnitude of absorption contribution and interference from the minor products. The greater increase in molar balance in the middle of the data (120–240 min) may be due to variations, with time, in the relative composition and photosensitivity of the products contributing to irrelevant absorption during the reaction. The pseudo first-order plot for the photodegradation of RF has been found to be linear indicating the accuracy of the analytical data after correction.

FMF Photolysis

FMF is highly sensitive to light in aqueous solution (20,27,33). Its anaerobic photolysis has been carried out using a low-intensity radiation source at pH 2.0, at which FMF exists in the protonated form (pK_a 3.5) (35), and is resistant to photodegradation (28). It was irradiated for 240 h causing ~40% degradation. The analytical results showed a total molar balance (FMF and LC) exceeding ~11% of the initial concentration of FMF due to interference from CMF and the unknown products (Table III). A multipoint non-linear irrelevant absorption correction, assuming partial contribution from CMF and minor degradation products present in aqueous phase, was applied. Out of the two concentrations calculated using two sets of wavelengths, *i.e.*, 345, 375, and 410 nm and 360, 395, and 425 nm, the data from the former set gave better results (Table III). These data also fitted better the second-order plots for the bimolecular reaction undergone by FMF on photodegradation. This indicates the importance of the application of a suitable correction procedure to the assay to obtain reliable analytical data for kinetic studies.

FMF Hydrolysis

FMF undergoes hydrolysis to LC and LF in alkaline solution along with the formation of CMF as a minor oxidation product (27,28). Initially, it was expected that FMF would hydrolyze almost completely to LC and LF which are chloroform soluble and, therefore, the aqueous phase (pH 2.0) would contain FMF only. However, the spectra of the aqueous phase after complete hydrolysis of FMF (TLC evidence) showed a distinct increase in absorbance at 360 nm and a decrease in the 370–440-nm region. This was probably due to the formation of the isoalloxazine ring cleavage products (29–31) absorbing in this region. Similar products are formed on the alkaline hydrolysis of RF (34). The increase in molar balance, with time, indicated greater interference in the assay due to the ring cleavage products (Table IV). Since the absorption spectra of the aqueous phase at the end of the reaction exhibited a region linear with wavelength over the range 350–500 nm, it was analyzed for FMF using a linear irrelevant absorption correction to allow for the presence of the ring cleavage products. The corrected analytical data for FMF during the reaction are given in Table IV. The data show improved molar balance up to ~11% and are close to the initial concentration of FMF. The corrected concentrations of FMF could provide more accurate kinetic results for the hydrolysis of FMF in alkaline solution.

CONCLUSION

The spectrophotometric assay of pharmaceutical compounds may be affected by the presence of minor impurities and/or related compounds. This is particularly relevant to the assay of a compound in the presence of its degradation products. In this situation, an error may be introduced in the assay values, depending on the spectral characteristics of the interfering compounds. This may adversely affect the kinetic results in a degradation study. In order to overcome this problem, the application of linear and non-linear irrelevant absorption corrections in multicomponent spectrophotometric assays of riboflavin and formylmethylflavin during the photolysis and hydrolysis studies has been demonstrated. These correction procedures lead to improvement in the assay data for kinetic treatment in degradation studies. The multicomponent spectrophotometric method used for the quantitative determination of riboflavin, formylmethylflavin, and their degradation products is rapid, convenient, and reliable for kinetic studies. It has the advantage of specificity and reproducibility for the compounds analyzed as compared to other methods such as LC-MS, which is time-consuming and expensive, with a possibility of further degradation of riboflavin and

formylmethylflavin on the column and is suitable for the identification of degradation products.

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