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Multicomponent spectrophotometric assay of riboflavine and photoproducts

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Abstract: A multicomponent spectrophotometric method has been developed for the simultaneous determination of riboflavine, formylmethylflavine and degradation products in photolysed solutions. It is based on partial separation of the photoproducts by chloroform extraction at pH 2.0 in a potassium chloride–hydrochloric acid solution and subsequent determination, in the aqueous phase, of riboflavine and formylmethylflavine at 445 and 385 nm. The chloroform extract containing lumichrome and lumiflavine is evaporated to dryness, the residue dissolved in acetate buffer (pH 4.5) and the products determined at 356 and 445 nm. The reproducibility of the method, based on the analysis of synthetic mixtures, is within ±5%. Absorption corrections for minor products and interfering substances have been proposed. Chromatographic, spectrophotometric and distribution coefficient data for riboflavine and photoproducts are reported. The method is specific, rapid and convenient for photodegradation studies of riboflavine and formylmethylflavine.

Keywords: *Riboflavine; formylmethylflavine; lumichrome; lumiflavine; spectrophotometric assay; photoproducts.*

Introduction

Spectrophotometric methods are widely used for the assay of riboflavine in pharmaceutical preparations [1] and photolysed solutions [2–20]. The multicomponent spectrophotometric assay of riboflavine in vitamin preparations [21] and photobleached solutions [22, 23], and of its major photoproducts, formylmethylflavine [24–26] and lumichrome [27] in degraded solutions, has been described. In most of the single component assays no attempt has been made to correct for the absorbance of compounds, other than riboflavine or an analogue, at 445 nm, produced during photolysis or hydrolysis. Thus the rate constants determined from such data [28–34] may be misleading.

The structural similarity of flavines makes it difficult to assay these compounds simultaneously and hence an accurate analysis of riboflavine and photoproducts is not possible unless a more specific and relatively sensitive method is available. The fluorescence maxima [9] and redox potentials [29] of these compounds are too close for any simultaneous determination by fluorimetry or polarography. The use of quantitative thin-layer chromatography (TLC) [22] in kinetic studies is time consuming, inconvenient and has poor precision.

The present investigation is based on the development of a multicomponent spectrophotometric method for the assay of riboflavine, formylmethylflavine and their major photoproducts, lumichrome and lumiflavine in degraded solutions. In addition to these products, the photolysed solutions also contain minor components [13] which may cause interference in the assay. It is sometimes necessary to apply a correction for irrelevant absorption depending upon the spectral characteristics of the interfering substances.

A multipoint correction for irrelevant absorption varying linearly with wavelength

The variation of absorptivity, a, with wavelength is determined from the spectrum of the pure component. The value of a at a particular wavelength is taken for reference and multiplying this value by the appropriate factor, the values for a at different wavelengths are obtained. Thus

$$a_i = k_i\, a \qquad (1)$$

where a_i and k_i are the values for absorptivity

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and the factor appropriate to the wavelength λ_i, respectively.

The irrelevant absorption ($_1a$) is assumed to obey the following relation:

$$_1a_i = m\,\lambda_i + c \qquad (2)$$

where m and c are constants for any one solution containing the component of interest and the interfering substances.

Thus the total absorbance A_i at λ_i is

$$A_i = {}_1Ck_i\,a + m\,\lambda_i + c \qquad (3)$$

From this one can find the most probable concentration, $_1C$, of the component from a series of n absorbance measurements, A_i, at the wavelengths $i = 1$ to n. The unknowns are $_1C$, m and c. The knowns are

λ_i, the wavelength of ith measurement;
A_i, from absorbance measurement;
k_i, and a from the pure substance, a is usually taken as the value at the maximum of a characteristic absorption band.

Irrelevant absorption in a two-component assay can be corrected for by expressing the data in the form of the matrix equation,

$$\begin{bmatrix} A_1 \\ A_2 \\ A_3 \\ A_4 \end{bmatrix} \begin{bmatrix} {}_1K_1 & {}_2K_1 & \lambda_1 & 1 \\ {}_1K_2 & {}_2K_2 & \lambda_2 & 1 \\ {}_1K_3 & {}_2K_3 & \lambda_3 & 1 \\ {}_1K_4 & {}_2K_4 & \lambda_4 & 1 \end{bmatrix} \begin{bmatrix} {}_1C \\ {}_2C \\ m \\ c \end{bmatrix} \qquad (4)$$

where A_1, A_2, A_3, A_4 are the absorbancies at four appropriately chosen wavelengths λ_1, λ_2, λ_3, λ_4; K is the absorptivity-cell path product; $_1C$ and $_2C$ are concentrations of the two components and m and c are constants. To obtain $_1C$ and $_2C$, the solution of only two equations is required.

A multipoint correction for irrelevant absorption varying non-linearly with wavelength

The variation of the irrelevant absorption can sometimes be expressed as a polynomial in λ_i. If in this case equation (2) can be replaced by a quadratic equation, then

$$_1a_i = m\,\lambda_i^2 + n\,\lambda_i + c \qquad (5)$$

and the matrix equation contains an extra element in each row and column. The solution of four or more matrix equations can be obtained with the help of a suitably programmed computer.

Experimental

Materials

Riboflavine (RF) was obtained from Roche Products Ltd and was recrystallized from 2 M acetic acid. Formylmethylflavine (FMF) and carboxymethylflavine (CMF) were synthesized by the methods of Fall and Petering [35] and Fukumachi and Sakurai [36], respectively. Lumichrome (LC) and lumiflavine (LF) were prepared by the method of McNutt [37] and purified by cellulose column chromatography (Whatman CC 31). All solvents and reagents were analytical grade from BDH. The following buffers were used throughout: KCl + HCl, pH 2.0; HCOOH + HCOONa, pH 3.5; CH_3COOH + CH_3COONa, pH 4.0–5.0; KH_2PO_4 + Na_2HPO_4, pH 6.0–8.0; $NaHCO_3$ + NaOH, pH 9.0; the ionic strength was 0.1 M in each case.

Photolysis

10^{-4} M RF or FMF at the appropriate pH was placed in 1-l. pyrex vessel and de-oxygenated by bubbling with nitrogen gas for 1 h. The solution was then irradiated with a Mazda M2 4.5 W low pressure mercury discharge lamp (emission at 365, 405 and 435 nm) fitted at the bottom of the vessel, while continuously bubbling the solution with nitrogen. The temperature of the solution was maintained at 25 ± 1°C by circulation of water from a constant temperature bath and on occasions in conjunction with a Grant laboratory refrigeration unit. Samples were withdrawn at appropriate intervals for TLC [26] and assay. The pH of unbuffered solutions was controlled by an autotitrator that added 0.01–0.10 M HCl or NaOH solution.

Assay method

Ten millilitres of the photolysed solution of RF or FMF was placed in a 25-ml beaker and the pH was adjusted to 2.0 with 0.1 M HCl solution. The solution was quantitatively transferred to a 20-ml volumetric flask, diluted to volume with KCl–HCl solution (pH 2.0) and extracted with 3 × 20-ml portions of chloroform. The aqueous phase was centrifuged, if necessary, to break any emulsion. The chloroform extract was washed with water, dried over anhydrous Na_2SO_4 and evaporated to dryness

at room temperature. The residue was dissolved in 1–2 ml of 0.5–1.0 M acetic acid and diluted to 20 ml with acetate buffer (pH 4.5). Absorption spectra were recorded on both solutions using a Unicam SP 700 UV and visible spectrophotometer and the concentrations were determined by single- or two-component assay [38, 39] (see Analytical Scheme).

The assay procedure was carried out in a dark room provided with Kodak yellow light.

Determination of distribution coefficient

Distribution coefficients of RF, FMF, LF and LC were determined between water and chloroform at 25 ± 1°C. 10 ml of suitable concentrations of each flavine (0.625–2.50 × 10^{-5} M) at pH 2.0 and 4.5, was transferred to a 50-ml separating funnel and 10 ml of chloroform, saturated with water at the appropriate pH, was added. The solution was thoroughly shaken to allow the distribution to attain equilibrium. Concentrations of flavines in each phase were determined spectrophotometrically and the apparent values of the distribution coefficients were obtained from the ratio of the two concentrations. The distribution coefficient, K_D, was found to be independent of flavine concentration over the range studied.

Analytical Scheme

For the assay of FMF, RF and photoproducts

Photolysed solutions (pH 2–9), containing non-degraded FMF or RF and several products, were pre-adjusted to pH 2.0 and extracted with chloroform. TLC was used to monitor qualitatively, variations in the composition of photoproducts (Fig. 2).

FMF and photoproducts	
Aqueous phase	Chloroform extract
FMF, minor components*	LC
Single component FMF assay at 385 nm, with sometimes a linear (equation 2) or non-linear (equation 5) correction for irrelevant absorption depending upon the spectral characteristics of interfering substances	Single component assay at 356 nm LF, LC Two-component assay at 445 and 356 nm
RF and photoproducts	
Aqueous phase	Chloroform extract
RF, FMF, minor components*	LC
Two-component assay (RF, FMF) at 445 and 385 nm, with sometimes a linear (equation 2) or non-linear (equation 5) correction for irrelevant absorption depending upon the spectral characteristics of interfering substances	Single component assay at 356 nm LF, LC Two-component assay at 445 and 356 nm

*Assumed not to interfere with the assay.

Results and Discussion

Choice of wavelengths

The choice of appropriate analytical wavelengths is most important for the success of multicomponent spectrophotometric analysis. Wavelengths showing high sensitivity of measured absorbance and minimum interference from instrumental characteristics are ideal for analytical work. A visual examination of the absorption spectra of RF and FMF (pH 2.0) and LF and LC (pH 4.5) (Fig. 1) provides adequate information for the choice of certain wavelengths which also correspond to the absorption maxima of these compounds (Table 1) and, therefore, contribute to the maximum specificity and sensitivity of the method (see Analytical Scheme). Several factors influence the choice of analytical wavelengths [40] and

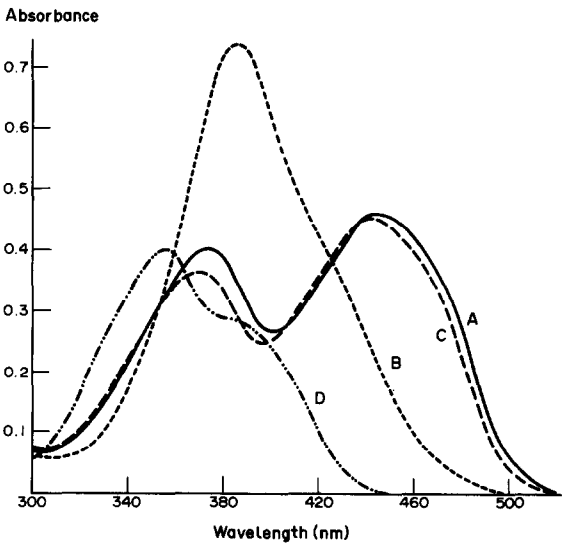

Figure 1
Absorption spectra of RF and photoproducts: (A) RF (3.6 × 10^{-5} M) and (B) FMF (4.5 × 10^{-5} M) at pH 2.0 (KCl–HCl solution); (C) LF (4.3 × 10^{-5} M) and (D) LC (3.7 × 10^{-5} M) at pH 4.5 (acetate buffer).

Table 1
Molar absorptivities (M^{-1} cm^{-1} × 10^{-3}) of riboflavine and photoproducts*

Compound	pH	356 nm	385 nm	445 nm
Riboflavine	2.0		9.7	12.5
Formylmethylflavine	2.0		16.4	4.7
Lumichrome	4.5	10.8		0.13
Lumiflavine	4.5	7.4		10.4

*Each value is a mean of three to five determinations.

more precise methods have been proposed to optimize wavelength selection [41–43]. Ultimately the true choice of the most appropriate absorption correction procedure is more important in the analysis of complex mixtures.

The wavelengths chosen for the correction of irrelevant absorption depend upon the spectral characteristics of interfering substances. The assumption of a particular type of irrelevant absorption curve throughout the reaction may be invalid due to variations with time in the relative composition of substances responsible for such absorption. Since various unknown products were detected in the photolysed solutions of RF and FMF by TLC (Fig. 2), several sets of analytical wavelengths were used for absorption corrections, and that giving better self-consistent data was selected for the degradation studies.

Validity of Beer's law

The validity of Beer's law for RF and photoproducts, in the concentration range $0.5–10 \times 10^{-5}$ M, alone or in mixtures and at the appropriate pH, at the various analytical wavelengths, was established prior to the assay. The molar absorptivities (Table 1), used for calculations, represent the means of determinations at at least five different concentrations (correlation coefficient 0.995–0.999) over the range likely to be found in the reaction mixtures. These are in agreement with the values reported by Fife and Moore [44], Duren *et al.* [27], McBride and Metzler [24], Koziol [9], Holmstrom [5] and Fall and Petering [35]. The effects of environment on the position of absorption maxima and the molar absorptivities of the flavine chromophore have been discussed by Penzer and Radda [45] and Visser and Muller [46].

When the presence of certain photoproducts in degraded solutions had been recognized by TLC and their identity was established by spectral and other physicochemical characteristics, the assay procedure involved preliminary extraction with chloroform to achieve partial separation of the reaction mixture. A somewhat similar procedure was used by Holmstrom [5] for the assay of RF, LC and an unknown substance in aerobically photolysed solutions at pH 6.7. Two series of synthetic mixtures were required to check the validity of Beer's law for RF, FMF and their photoproducts. Assays were, therefore, performed at pH 2.0 (aqueous phase) and pH 4.5 (chloroform residue dissolved in acetate buffer) and the results are given in Table 2. From the calculated values of percentage recovery, the reproducibility of the method appears to be within ±5%. The adherence of flavines to Beer's law suggests that there is no or little significant interaction among these compounds, since the percentage deviation appears to be randomly distributed.

The relatively high fluorescence intensity of flavines would suggest the possibility of some kind of energy transfer among the various species [47] and thus cause deviations from Beer's law. However, since RF, FMF and LF exhibit same fluorescence maxima (525 nm) [48] which do not correspond to any absorption band of these compounds, this possibility, with the exception of LC, would be expected to be slight. The fluorescence maximum of LC at 485 nm [9] partly corresponds to the absorption bands of other flavines. Some interference must, therefore, be anticipated. However, the chloroform extraction procedure gives a mixture of LC and LF only in neutral and alkaline reactions (pH 7–9) in which the latter is a minor product [25].

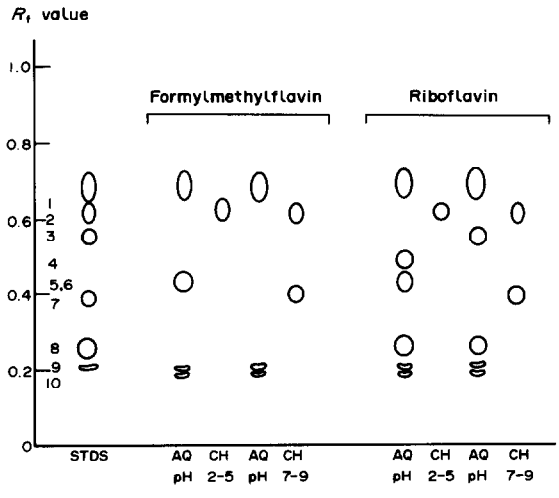

Figure 2
Thin-layer chromatograms (cellulose, Whatman CC 41) of anaerobically photolysed solutions of RF and FMF after extraction with chloroform at pH 2.0 (solvent system: 1-butanol–1-propanol–acetic acid–water (50:30:2:18) [26]). STDS, standards; AQ and CH, aqueous and chloroform extracts. 1, FMF; 2, LC; 3, AH_1; 7, LF; 8, RF; 9, CMF; 4–6 and 10, unknown products. All spots show yellow-green fluorescence under UV light (350 nm) except LC (light-blue) and AH_1 (blue-violet); 5 and 6 have same R_f value and are produced in the photolysis of RF and FMF, respectively. AH_1 has been tentatively identified as 6,7-dimethyl-4-D-ribityl-2,3-dioxo-tetrahydroquinoxaline, a ring cleavage product of RF.

Table 2
Analyses of synthetic mixtures of riboflavine and photoproducts*

Three component analyses of formylmethylflavine, lumichrome and lumiflavine

Formylmethylflavine				Lumichrome				Lumiflavine			
Added ($M \times 10^5$)	Found ($M \times 10^5$)	Recovery (%)	RSD (%)	Added ($M \times 10^5$)	Found ($M \times 10^5$)	Recovery (%)	RSD (%)	Added ($M \times 10^5$)	Found ($M \times 10^5$)	Recovery (%)	RSD (%)
1.800	1.818	101.0	1.6	0.681	0.658	96.6	1.7	0.309	0.296	95.8	3.7
1.283	1.292	100.7	1.8	1.135	1.165	102.6	2.9	0.463	0.463	100.0	2.5
0.770	0.776	100.8	1.8	1.600	1.594	99.6	2.6	0.694	0.672	96.8	3.9
0.257	0.255	99.2	1.4	2.044	2.135	104.5	2.4	0.926	0.952	102.8	2.8
0.257	0.253	98.4	1.4	2.280	2.287	100.3	2.5	1.251	1.258	101.1	2.7

Four component analyses of riboflavine, formylmethylflavine, lumichrome and lumiflavine

Riboflavine				Formylmethylflavine				Lumichrome				Lumiflavine			
Added ($M \times 10^5$)	Found ($M \times 10^5$)	Recovery (%)	RSD (%)	Added ($M \times 10^5$)	Found ($M \times 10^5$)	Recovery (%)	RSD (%)	Added ($M \times 10^5$)	Found ($M \times 10^5$)	Recovery (%)	RSD (%)	Added ($M \times 10^5$)	Found ($M \times 10^5$)	Recovery (%)	RSD (%)
2.255	2.280	101.1	1.2	0.251	0.261	104.0	3.1	0.363	0.373	102.7	1.9	0.154	0.155	100.6	2.3
1.754	1.789	102.0	1.7	0.754	0.760	100.8	1.4	0.681	0.682	100.1	0.5	0.154	0.151	98.1	2.2
1.253	1.273	101.6	1.3	1.257	1.257	100.0	0.4	0.545	0.552	101.3	2.0	0.309	0.307	99.4	4.1
1.002	1.007	100.5	1.3	1.509	1.524	101.0	0.7	1.362	1.348	99.0	1.3	0.770	0.770	100.0	3.8
0.501	0.496	99.0	1.4	1.800	1.790	99.4	0.5	1.600	1.606	100.4	1.5	0.926	0.933	100.7	3.1

*Values expressed as a mean of three determinations.

Choice of assay pH

RF and FMF are unstable in neutral and alkaline solutions. It was, therefore, considered necessary to record all the spectra in sufficiently acidic solutions. The distribution coefficients of FMF, LC and LF for the chloroform/water system (Table 3) suggest that pH 2.0 is most suitable for extraction since RF and FMF (pK_a 3.5) [49] would be retained in the aqueous phase. Three chloroform extractions were found to be sufficient to remove LC and LF from the photolysed solutions. This was also confirmed by TLC analysis. The absorption spectrum of FMF in the protonated state (pH 2.0) is quite distinct from that of RF thus making a two-component assay relatively simple. Similarly, LC and LF can be conveniently analysed at pH 4.5 (Fig. 1). An approximate quantitative analysis of RF, LC and a cyclic intermediate [23] and FMF and LC [24] has been reported at pH 7.0 in anaerobically photolysed solutions.

Table 3
Distribution coefficients for riboflavine and photoproducts between water and chloroform*

Compound	pH	K_D†
Riboflavine	2.0	0.00
Formylmethylflavine	2.0	0.005
Formylmethylflavine	4.5	0.11
Lumichrome	2.0	1.96
Lumichrome	4.5	2.04
Lumiflavine	2.0	2.62
Lumiflavine	4.5	3.59

*Each value is a mean of three determinations.
†Ionic strength of the aqueous phase was 0.10 M.

The absorption spectra of flavines are sensitive to changes in pH and solvent polarity [50, 51] and hence Beer's law may not be obeyed at different pH values at all the analytical wavelengths. The absorbance measurements were, therefore, carried out at a constant pH, i.e. 2.0 and 4.5, so as to minimize the analytical errors and to achieve uniformity of the assay results.

The degradation of RF and FMF, if any, in the dark, during pH adjustment and extraction must be negligible at pH 2.0 since these compounds are stable at this pH. Similarly, LC and LF do not appear to degrade at pH 4.5 in the dark. However, the instability of minor unknown products during assay, which may be pH sensitive, could contribute to the overall analytical error.

Reproducibility and applications of the assay

The validity of Beer's law, and the reproducibility of the results of synthetic mixtures within ±5%, suggest that the proposed assay procedure, with the correct assumption of the compounds analysed for, is adequately reliable for kinetic studies. The method has been used to study the photodegradation and alkaline hydrolysis of FMF by Heelis *et al.* [25] and Ahmad *et al.* [26], respectively.

A set of typical results for the analyses of three to four products in the photolysed solutions of FMF and RF at pH 9.0 is reported in Table 4. The assay method, when applied to the photolysis of FMF, gave uniformly increasing values of LC and LF as expected and an almost constant molar balance, with time. The values of the molar balance are in good agreement with the initial concentration of FMF. Similar assay data were obtained for RF and photoproducts although the molar balance indicated a gradual increase of approximately 7%. This may be due to the presence of unknown degradation products [13] and chromatographically detected minor components such as CMF and AH_1 in the aqueous phase (Fig. 2), which could not be accounted for in the assay. In view of the existence of several minor photoproducts of RF, it is not an unsatisfactory situation considering the nature

Table 4
Analyses of the products of FMF/RF photolysis at pH 9.0

Concentrations of FMF and photoproducts ($M \times 10^5$)

Time (h)	FMF	LC	LF	Total
0	9.50	0.00	0.00	9.50
0.25	8.67	0.61	0.22	9.50
0.50	8.08	1.05	0.27	9.40
1.00	7.68	1.56	0.30	9.54
1.50	6.97	2.15	0.38	9.50
2.00	6.63	2.56	0.45	9.64
2.50	6.24	2.99	0.36	9.59
3.00	5.78	3.31	0.44	9.53
3.50	5.37	3.65	0.51	9.53
4.00	4.91	4.02	0.57	9.50

Concentrations of RF and photoproducts ($M \times 10^5$)

Time (h)	RF	FMF	LC	LF	Total
0	10.00	0.00	0.00	0.00	10.00
2	9.12	0.40	0.27	0.24	10.03
4	8.40	0.72	0.46	0.51	10.09
6	7.82	1.07	0.99	0.43	10.31
8	7.50	1.10	1.42	0.51	10.53
10	6.91	1.37	1.43	0.86	10.57
12	6.31	1.44	2.10	0.87	10.72

and limitations of the multicomponent assay method.

In the study of complex systems such as RF photolysis, even if a multicomponent assay is made, it is sometimes necessary to correct for the impurities and interfering substances in order to satisfactorily interpret the kinetic data according to certain established criteria. Such an interpretation must also be consistent with the qualitative information on the nature and composition of the reaction mixture if meaningful conclusions are to be drawn. A careful assessment of the analytical data over appropriate lengths of time may provide additional information on various factors contributing to the extent of molar imbalance in such reactions and hence the overall analytical error. The method, however, is specific and rapid and may be used for photodegradation studies.

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