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Photoinduced color changes in two different qualities of riboflavin in the solid state and in various tablet formulations Photoreactivity of biologically active compounds. XX.

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There has been a gradual change in the relative amounts of synthetic and biosynthetic bulk riboflavin (RF) supplied to the overall market over the past years. The two sources of drug substance seem to have different photochemical properties that cannot be readily predicted. Alternating between the two qualities of RF therefore seems to influence the photochemical properties of the final product in a rather unpredictable way. A change in production method introduces the possibility of a change in polymorphic form which may alter the photoreactivity of the substance. The drug substance and tablets become green upon light exposure. The biosynthetic bulk material appears to be less photostable than the synthetic bulk material after inadvertent exposure to radiation or at elevated humidity. The observed color change cannot be explained by the formation of degradation products but is strongly dependent on the humidity level within the drug substance or preparation. The change in color was dramatically increased (by a factor up to 7) when the drug substance was formulated as tablets. Interactions were observed between RF and individual tablet components by mixing and compression at low pressure prior to exposure.

1. Introduction

Riboflavin (RF) is nutritionally important as vitamin B_2 and is a common ingredient in multi-vitamin tablets. It is, however, well known that RF is photolabile and can, depending on the environment, participate in a number of photoinduced reactions (Heelis 1991). The majority of the data reported concerns RF in solution while the influence of polymorphic form and excipients on the photostability of RF in the solid state is less well studied. Photoinduced changes in the solid state are a surface phenomenon because the radiation will penetrate only fractions of a millimeter into a solid sample. The molecules on a tablet surface have a very restricted mobility. Depending on the type of photoreaction (e.g. oxidation, radical formation) the resulting products may or may not be the same as in solution. Solid state photoreactions can have two outcomes; physical changes that may or may not give rise to changes in external appearance like the color, and induction of the chemical degradation of the active substance or other components in the formulation. Change in appearance does not always have a direct relation to observed chemical degradation, possibly because some degradants have a very strong hue and exert a large effect on appearance at levels which are not detectable using standard chromatographic techniques and detection. Thus a change in appearance may not affect the therapeutic efficacy of the tablet. A marked difference between the photostability of various physical forms (e.g. polymorphic forms, salt form changes, presence of amorphous) of a solid sample can be observed (Tønnesen 2004). This can be ascribed to differences in inter- and intramolecular binding, differences in diffusability (crystalline vs amorphous structure), and differences in water content (crystal water, adsorbed water). Water can dissolve in amorphous compounds because of the disordered state of the solid and locally dissolve the substance. The observed photochemistry will then be a mixture of solid state processes and reactions typical for a solution. The observed reactions can be further influenced by a change in humidity or the presence of excipients in the formulation.

There has been a gradual change in the relative amounts of synthetic and biosynthetic bulk riboflavin (RF) supplied to the overall market over the past years. A change in production method introduces the possibility of a change in physical characteristics, including crystallinity, polymorphic form, particle size, surface area/morphology, presence of amorphous material etc., which may alter the photoreactivity of the substance. In the case of RF, it is observed that certain batches of vitamin tablets undergo severe discoloration after inadvertent exposure to light although the tablets are quantitatively sound (personal communication from the manufacturer). The two sources of drug substance seem to have different photochemical properties that cannot be readily predicted. Alternating between the two qualities of RF therefore seems to influence

the photochemical properties of the final product in a rather unpredictable way. In the present work, we have focused on the photoinduced color change of RF in the solid state combined with calorimetric analysis, qualitative HPLC and TLC, and UV-VIS spectrophotometry. The investigations have been made on two commercially obtained qualities of the vitamin. The two qualities are produced by chemical synthesis and microbial fermentation respectively, and they seem to represent two different polymorphic forms of the compound. The vitamin was studied in the form of native drug substance, in a compressed form and in various tablet formulations.

2. Investigations and results

2.1. Bulk powder and compressed bulk powder samples

Bulk powder samples of the two RF qualities were exposed using a xenon lamp according to Option 1 in the ICH Guideline on photostability testing of drugs (ICH 1997). A powder sample holder was constructed in order to measure the color of the sample prior to and after exposure. The major color change appeared almost instantly; i.e. at a radiation dose ≤ 450 kJ/m^2 (Fig. 1). Initially, the largest change in color was observed in the biosynthetic sample. After this initial change, the color hardly changed upon further exposure. The synthetic sample showed a lower initial color change ($\Delta E^*ab \sim 4$ vs 5.5 in biosynthetic RF at 450 kJ/m^2) but unlike the biosynthetic sample the color continued to change gradually at continuing exposure. By the end of the experiment (dose 22045 kJ/m^2), the observed color change was therefore largest in the synthetic sample ($\Delta E^*ab \sim 7.5$ vs 6 in biosynthetic RF). In practice this means that the biosynthetic bulk material would appear to be less photostable than the synthetic bulk material when occasionally exposed to radiation, but upon continuous exposure, the synthetic bulk sample would demonstrate the largest discoloration. The RF samples showed changes towards both green and blue as demonstrated by an increase in the negative a^* and b^* values in the $L^*a^*b^*$ color space (CIELAB). The observed change in the color coordinators was identical in synthetic and biosynthetic RF ($-\Delta a^* = 4$, $-\Delta b^* = 6$). The color change (ΔE^*ab) did increase if the drug substance was compressed by use of an IR press operated at high pressure (10 tons for 4–6 min) prior to exposure. The biosynthetic RF then showed the largest increase in color change by the end of exposure, although both samples experienced a change in apparent melting point after compression (Table 1). An alteration of melting point might indicate a change in crystal form as a function of compression. However, DSC analysis also indicates overlap of melting with decomposition. In such cases a change in apparent melting point does not necessarily reflect a change in crystal form but can occur as a result of changes in impurity level or particle size.

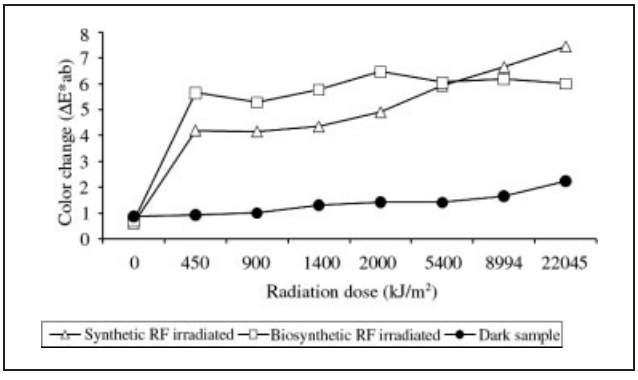

Fig. 1: Observed total color change (ΔE^*_{ab}) in riboflavin (RF) drug substance (synthetic and biosynthetic) as a function of radiation dose (kJ/m^2)

2.2. Compressed mixtures of RF and individual tablet components

RF (both qualities) was mixed (1 : 1) with selected, individual components present in the standard tablet formulation (TAB1) and manually compressed into tablets by a single punch tabletting machine. The resulting compressed samples were exposed to radiation followed by characterization with color measurements, DSC and qualitative HPLC. The results are presented in Table 2. The two RF qualities seem to react somewhat differently with respect to the observed color change. In the case of synthetic RF, the largest changes are observed in the presence of icing sugar, lactose and wheat starch while in the case of biosynthetic RF, the largest changes are observed in the presence of nicotinamid, lactose, talc and sodium starch glycolate. The changes in a^* and b^* values were within the same range, although the changes were largest in samples containing synthetic RF (synthetic RF: $-\Delta a^*$ 4–7, $-\Delta b^*$ 1–6; biosynthetic RF: $-\Delta a^*$ 1–3, $-\Delta b$ 1–3). All the excipients with the exception of talc (data not shown) did induce a change in RF melting point upon mixture and compression prior to radiation (examples are shown in Table 4, see below). Traces of lumichrome were detected in four of the samples of which three contained synthetic RF. The presence of lactose seemed to induce the formation of lumichrome independent of RF quality (Table 2). Some minor, unidentified peaks occurred in some of the HPLC chromatograms. TLC analysis of the bulk substance did not show any degradation products.

2.3. Influence of humidity

Riboflavin drug substance and compressed mixtures (1 : 1) of RF and selected excipients were exposed to elevated humidity prior to radiation. DSC analysis of the drug substance indicates that the biosynthetic RF sample has adsorbed water after 24 h incubation at elevated humidity (Figs. 2 and 3) while the synthetic sample apparently does

Table 1: Observed color change (ΔE^*_{ab}) and melting point (m.p; given as the peak value, °C) as a function of compression and exposure of the drug substance

	ΔE^*_{ab} Bulk Exposed	ΔE^*_{ab} Compressed Exposed	m.p (°C) Bulk Non exposed	m.p (°C) Compressed Non exposed	m.p (°C) Compressed Exposed
RF synthetic	5.0 (SD = 1.1)	4.2 (SD = 1.2)	305.9	299.7	299.7
RF biosynthetic	4.3 (SD = 2.1)	10.0 (SD = 0.1)	287.9	273.6	273.7

Exposed: radiation dose 22045 kJ/m^2 at intensity 765 W/m^2, RF = riboflavin, Bulk = drug substance (powder), Compressed = drug substance manually compressed into tablets by use of a single punch tabletting machine, SD = standard deviation (n = 3 or 6)

Table 2: Observed changes in color and melting point, and formation of degradation products in compressed mixtures (1 : 1) of RF and selected excipients present in the standard tablet formulation (TAB1) after light exposure

Excipients	RF synthetic			RF biosynthetic		
	ΔE^*_{ab}	DSC changes after exposure	Degradation products	ΔE^*_{ab}	DSC changes after exposure	Degradation products
None	8.15 (SD = 1.25)	−	Lc/?	0.67 (SD = 1.24)	−	−
Mg-stearate	9.40 (SD = 0.63)	−		0.59 (SD = 1.67)	−	
Thiamine	6.64 (SD = 1.35)	−	−	−0.42 (SD = 1.61)	−	−
Nicotinamide	7.61 (SD = 1.45)	−	Lc/?	2.10 (SD = 1.76)	+	−
Icing sugar	4.41 (SD = 1.18)	−		0.69 (SD = 0.29)	−	
Lactose	4.76 (SD = 1.18)	−	Lc/?	1.95 (SD = 1.24)	−	Lc/?
Talc	7.15 (SD = 2.09)	−		2.27 (SD = 1.54)	−	
Wheat starch	4.95 (SD = 1.57)	−		0.57 (SD = 0.83)	−	
Sodium starch glycolate	7.16 (SD = 0.97)	−	−	−2.41 (SD = 1.33)	−	−

The (1 : 1) mixture of RF and selected excipient was manually compressed into a tablet by use of a single punch tabletting machine
ΔE^*_{ab}: total color change at radiation dose 22045 kJ/m^2 (radiation intensity 765 W/m^2)
DSC changes after exposure: (+) denotes a change in melting point of the mixture, (−) indicates no change
Degradation product: Lc indicates lumichrome, ? indicates one or several unknown products
SD = standard deviation (n = 6)

not adsorb water even after 5 days incubation (data not shown). The water adsorption is demonstrated by the endothermic peak at ~75 °C which may be ascribed to water evaporation from the sample. This peak is absent in biosynthetic drug substance stored under ambient conditions and in the synthetic sample. Further, the compressed powder samples were exposed to elevated humidity for 24 h followed by immediate exposure to radiation. For this experiment RF was mixed with thiamine, nicotinamide, lactose and sodium starch glycolate, respectively. The first two substances are regular components in most vitamin B tablets while the latter two are commonly used tablet excipients which seem to interact with RF (see above). The change in color was measured by the end of the exposure time and DSC analyses were performed. The resulting color changes are presented in Table 3 and show that all the components influence the color of both qualities of RF at elevated humidity. The effects are slightly different from the observations made on the compressed samples at ambient humidity (Table 2). The changes are largest in synthetic RF. The DSC results indicate that only thiamine interacts differently with RF after irradiation at elevated humidity compared to a sample irradiated at ambient humidity as the RF melting point (peak value) is shifted towards a lower value (Table 4). The melting point remains almost unchanged in the other samples at elevated humidity after irradiation. The water peak at ~75 °C is present in all the thermograms of samples containing biosynthetic RF exposed to elevated humidity. The melting point of the plain RF drug substance is virtually unaffected by compression at low pressure (as compared to normal tablets and compression by an IR press), irradiation and elevated humidity (Table 4).

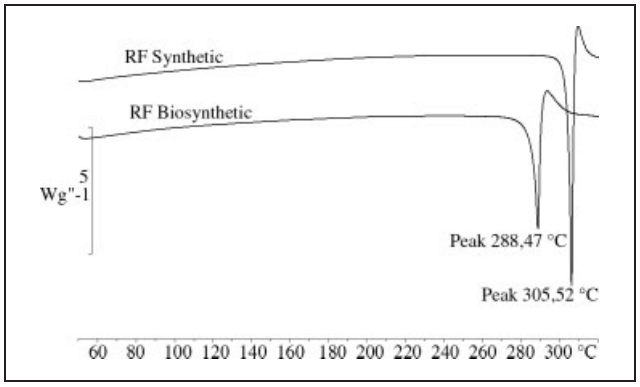

Fig. 2: DSC thermogram of riboflavin (RF) drug substance (synthetic and biosynthetic) stored at ambient humidity

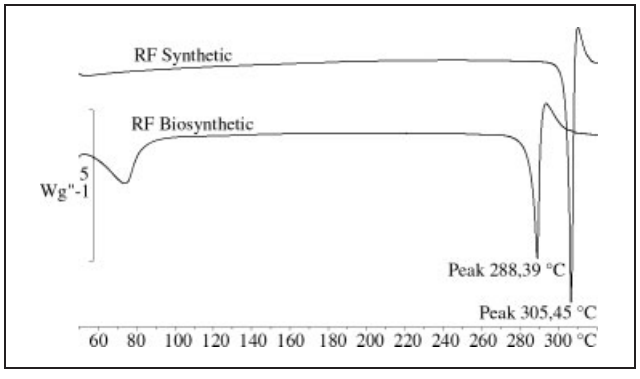

Fig. 3: DSC thermogram of riboflavin (RF) drug substance (synthetic and biosynthetic) stored at elevated humidity 24 h prior to analysis

2.4. Tablet formulations

The ICH Guideline does not demand a certain irradiance or irradiation time but requires a total energy in the UV and visible range of the spectrum (ICH 1997). The irradiance can be set to different levels and the testing time would be adjusted accordingly, e.g. varying between 7.1 h at 765 W/m^2 and 21.8 h at 250 W/m^2. It has previously been observed that the light-induced color change in tablets can be dependent on the conditions within the radiation chamber (e.g. exposure time and irradiance level) (Tønnesen et al. 2007). In the present study the standard tablet formulation (TAB1) containing either the synthetic or biosynthetic RF was therefore tested at different irradiances (250 W/m^2, 550 W/m^2 and 765 W/m^2) until certain illumination energies were reached. The color changes (ΔE^*ab) are presented in Figs. 4 and 5. The overall change was apparently independent of the irradiance by the end of the test, but initially the change seems to be larger at low irradiance (250 W/m^2). This is demonstrated for both qualities of RF. Most of the color change took place early in the experiment (dose < 900 kJ/m^2) independent of RF quality. This is consistent with the observation

Table 3: Observed changes in color of compressed mixtures (1 : 1) of riboflavin (RF) and selected excipients present in the standard tablet formulation (TAB1) exposed to elevated humidity for 24 h prior to light exposure

Excipients	RF synthetic			RF biosynthetic		
	ΔE^*_{ab}	a^* (average value)	b^* (average value)	ΔE^*_{ab}	a^* (average value)	b^* (average value)
None	7.65 (SD = 0.59)	7	4.5	0.35 (SD = 0.39)	1	1
Thiamine	1.23 (SD = 1.88)	−5	xx)	8.05 (SD = 3.63)	−1	−2
Nicotinamide	13.77 (SD = 1.42)	−3	−10	6.51 (SD = 0.42)	1	−7
Lactose	−0.07 (SD = 0.98)	−2	xx)	1.66 (SD = 0.48)	1	2
Sodium starch glycolate	7.92x)	−6	−4	−1.21 (SD = 1.42)	1	1

The (1 : 1) mixture of RF and selected excipient was manually compressed into a tablet by use of a single punch tabletting machine
ΔE^*_{ab}: total color change at radiation dose 22045 kJ/m² (radiation intensity 765 W/m²)
a^*: CIELAB color coordinate that correlates with red (+a^*) and green (−a^*)
b^*: CIELAB color coordinate that correlates with yellow (+b^*) and blue (−b^*)
SD = standard deviation (n = 6)
x): standard deviation not measured due to low number of measurements (n = 1)
xx): both negative and positive values observed

Table 4: Melting point (°C, peak value) of riboflavin (RF) drug substance (powder) and compressed mixtures (1 : 1) of RF and selected excipients present in the standard tablet formulation (TAB1) exposed to elevated humidity for 24 h prior to light exposure

Excipient	Synthetic RF					Biosynthetic RF				
	Drug substance dark	Compr dark	Compr light	Compr dark humid	Compr light humid	Drug substance dark	Compr dark	Compr light	Compr dark humid	Compr light humid
None	305	305	305	305	302	288	289	287	288*	288*
Thiamine		250	250		217		241	242		227*
Nicotinamide		129	129		129		129	129		126*
Sodium starch glycol.		271	274		270		265	264		263*
Lactose		283	282		285		269	269		269*

The (1 : 1) mixture of RF and selected excipient was manually compressed into a tablet by use of a single punch tabletting machine
Compr: compressed samples
Dark: non exposed
Light: irradiated to 22045 kJ/m² at 765 W/m²
Humid: stored at elevated humidity for 24 h prior to irradiation
Glycol.: glycolate
* The thermogram contains an endotherm peak at ~75 °C assumed to represent evaporation of water

made on the bulk material. In both preparations and under all conditions, the major color change was towards blue as demonstrated by an increase in $-b^*$ value in the $L^*a^*b^*$ color space (CIELAB) (Table 5). The darkening of the tablets was confirmed by an increase in $-L^*$ value. If the change in color could be ascribed only to photobleaching of RF (i.e. a reduction of $+b^*$) this would have been associated with an increase in the $+L^*$ value. The change in $-L^*$ and $-b^*$ values was virtually independent of irradiance, while the change in $-a^*$ value seemed to be slightly higher at higher irradiance. Visually, the tablets become green after light exposure as the original tablets are yellow and the major change is towards blue. Theoretically, the visual appearance of green may also be due to loss of yellowness from the RF revealing a green color from another component in the tablet. However, neither of the excipients present has a green color nor becomes green after light exposure. The color change in these wet granulated tablets is most apparent in the samples containing biosynthetic RF. However, this quality showed the least color

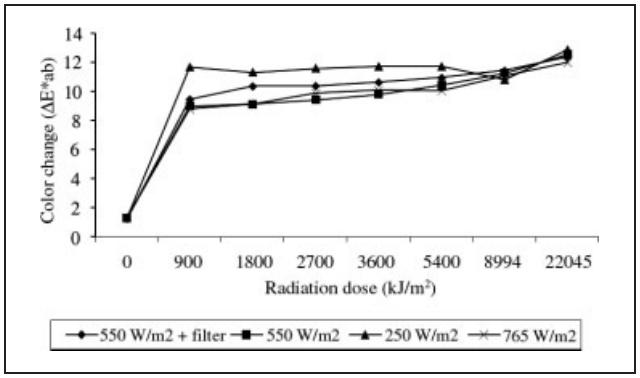

Fig. 4: Total color change (ΔE^*_{ab}) of TAB1 containing synthetic riboflavin (RF) as a function of radiation dose (kJ/m²) and irradiance (W/m²). Filter: the samples were covered with a UV filter (cut-off 406 nm; 50% T at 418 nm)

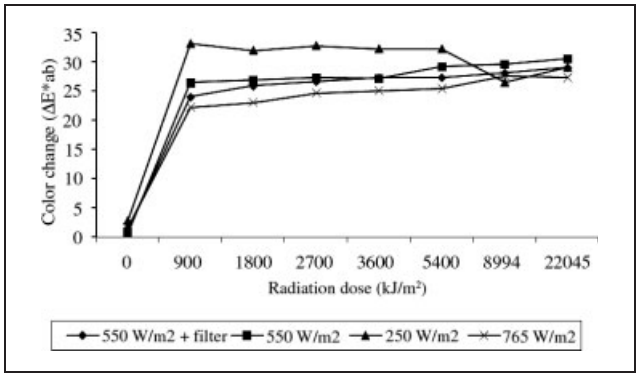

Fig. 5: Total color change (ΔE^*_{ab}) of TAB1 containing biosynthetic riboflavin (RF) as a function of radiation dose (kJ/m²) and irradiance (W/m²). Filter: the samples were covered with a UV filter (cut-off 406 nm; 50% T at 418 nm)

Table 5: Change in L^*, a^* and b^* values in riboflavin (RF) tablets as a function of radiation dose (kJ/m^2) and irradiance (W/m^2)

Irradiance (W/m^2)	Radiation Dose (kJ/m^2)	Tablets Synthetic RF			Tablets Biosynthetic RF		
		ΔL^*	Δa^*	Δb^*	ΔL^*	Δa^*	Δb^*
250 W/m^2	900	−4.18 (SD = 0.17)	−2.02 (SD = 0.36)	−10.43 (SD = 0.98)	−14.44 (SD = 0.46)	−4.14 (SD = 0.31)	−29.30 (SD = 1.25)
250 W/m^2	22045	−4.39 (SD = 0.20)	−3.68 (SD = 0.14)	−11.37 (SD = 0.79)	−11.85 (SD = 0.28)	−3.37 (SD = 0.25)	−26.16 (SD = 0.87)
550 W/m^2	900	−3.88 (SD = 0.26)	−1.51 (SD = 0.15)	−8.02 (SD = 0.58)	−11.23 (SD = 0.69)	−2.83 (SD = 0.25)	−23.19 (SD = 1.39)
550 W/m^2	22045	−5.47 (SD = 0.22)	−3.64 (SD = 0.17)	−10.51 (SD = 0.48)	−12.73 (SD = 0.48)	−3.38 (SD = 0.37)	−27.38 (SD = 0.58)
765 W/m^2	900	−3.72 (SD = 0.38)	−1.31 (SD = 0.41)	−7.57 (SD = 1.32)	−9.42 (SD = 0.50)	−1.79 (SD = 0.19)	−19.79 (SD = 1.05)
765 W/m^2	22045	−5.48 (SD = 0.58)	−3.25 (SD = 0.19)	−10.55 (SD = 0.99)	−11.31 (SD = 0.26)	−2.66 (SD = 0.15)	−24.74 (SD = 0.45)
550 W/m^2 +UV filter*	900	−3.22 (±0.01)	−1.41 (±0.08)	−8.91 (±0.01)	−9.01 (±0.11)	−1.98 (±0.05)	−21.64 (±0.16)
550 W/m^2 +UV filter*	22045	−4.66 (±0.02)	−3.27 (±0.05)	−10.98 (±0.04)	−11.78 (±0.13)	−3.68 (±0.01)	−27.40 (±0.07)

UV filter: cut-off 406 nm; 50% T at 418 nm
SD = standard deviation (n = 6)
* n = 2

change when exposed as a bulk sample and as a compressed bulk sample at ambient and elevated humidity (see above). HPLC analysis of the colored tablet layer showed trace amounts of lumichrom for both RF qualities. No other degradation products could be detected under the given conditions. TLC analysis of the colored tablet layer did not show any degradation products. The tablets were then covered by a UV-filter (cut-off 406 nm; 50% T at 418 nm) and exposed to 22045 kJ/m^2 at 550 W/m^2. The total color change (Figs. 4 and 5) and change in $-a^*$, $-b^*$ and $-L^*$ values were almost identical to the uncovered tablets (Table 5). This indicates that the color change is induced by visible light (> 400 nm) rather than the UV part of the spectrum. RF in water has one absorption maximum at 446 nm, i.e. in the visible range of the spectrum (Al-Shammary et al. 1990). A series of tablets were produced by modification of the standard tablet (TAB1). Either the production method or the excipients were changed (TAB2A-D). The total color change after exposure is presented in Table 6. The tablets prepared by wet granulation containing biosynthetic RF undergo the most extensive color change (TAB1b, TAB2Bb, TAB2Cb, TAB2Db). This is different from the observations made on the RF bulk material and compressed bulk material at the same radiation dose, but consistent with the observations made on drug substance compressed at high pressure. This emphasizes that both the total composition of the tablets (i.e. microenvironment) and the applied compression force may influence the photoreactivity of RF in the vitamin tablets. The color change in the tablets is nearly independent of the presence and concentration of hydrochloric acid in the granulation fluid (TAB1, TAB2B, TAB2C). The change in $-b^*$ color coordinate is dominant for both qualities of RF. This is manifested by a greenish appearance of the tablets. The tablets prepared by direct compression (TAB2A) undergo a smaller color change which is independent of the RF quality. A change in production method from wet granulation to direct compression therefore seems to have a color stabilizing effect on the tablets, particularly those containing biosynthetic RF. A substitution of wheat starch by microcrystalline cellulose (TAB2D) has little effect on the colorfastness of the tablets independent of RF quality.

Table 6: Observed color change in modified riboflavin (RF) tablets after exposure to radiation

Sample	ΔE^*_{ab} (22045 kJ/m^2)	a^* (average value)	b^* (average value)
TAB1s	8.83 (SD = 0.60)	−3	−7
TAB1b	23.33 (SD = 0.77)	1	−22
TAB2As	5.91 (SD = 0.65)	−3	−4
TAB2Ab	5.80 (SD = 0.37)	−3	−4
TAB2Bs	7.42 (SD = 0.20)	−3	−6
TAB2Bb	20.50 (SD = 0.54)	0.5	−19
TAB2Cs	8.61 (SD = 1.06)	−3	−7
TAB2Cb	16.37 (SD = 0.32)	−1	−16
TAB2Ds	10.91 (SD = 0.66)	−3	−10
TAB2Db	17.91 (SD = 0.67)	2	−17

(radiation dose 22045 kJ/m^2 at 765 W/m^2)
TAB1: standard formulation. TAB2A: made by direct compression, TAB2B : 20% HCl, TAB2C: water, TAB2D: Avicel PH101 (see experimental for details).
s = synthetic riboflavin, b = biosynthetic riboflavin
ΔE^*_{ab} : total color change at radiation dose 22045 kJ/m^2 (radiation intensity 765 W/m^2)
a^*: CIELAB color coordinate that correlates with red ($+a^*$) and green ($-a^*$)
b^*: CIELAB color coordinate that correlates with yellow ($+b^*$) and blue ($-b^*$)
SD = standard deviation (n = 6)

2.5. UV-VIS absorption measurements

Visual inspection indicated that the green color disappeared when the irradiated samples were dissolved in methanol or aqueous solvents. This observation was confirmed by UV-VIS absorption measurements in the wavelength range 290–700 nm. The UV-VIS absorption spectrum of the dissolved samples remained unchanged after exposure to light. This observation was valid both for the drug substance and the colored layer that was scraped off the tablets. The spectra of the exposed samples were normalized to the same maximum absorption value as the non exposed samples. The spectra were then perfectly superposed (data not shown).

3. Discussion

Change in production method of RF obviously has an influence on the light sensitivity of the substance. This is

manifested as a change in the visual surface color towards green (i.e. the blue and green color coordinates increase) in RF tablets and drug substance. The observed color changes cannot be explained by the formation of degradation products. Small amounts of lumichrome are detected in certain samples, but lumichrome is a yellow compound. Also trace amounts of some unknown degradation products are present in the HPLC chromatograms of certain samples, but a variation in the detection wavelength indicates that neither of these products are blue or green. TLC analysis did not reveal the formation of any new products. The UV-VIS absorption spectrum of the dissolved samples remained unchanged independent of light exposure prior to dissolution. This observation further indicates that the color change might be a result of physical changes on the tablet or powder surface rather than chemical degradation. The dissolution with an appropriate solvent will eliminate a color change that is not a result of bond-breaking changes in the RF structure. These findings are also consistent with our previous observations indicating that the color change is reversible and dependent on adsorbed water (Sue-Chu et al. 2008). The influence of trace amounts of water on the spectral properties of the samples was investigated by addition of 0.1% water to samples in methanol. Such a low amount will not lead to spectral changes due to a change in solvent refractive index or dielectric constant. Trace amounts of water did however, not influence the absorption spectrum of the samples in solution. The reactions taking place at the tablet or powder surface will be further evaluated by use of alternative techniques (e.g. TOF-SIMS) in an upcoming study.

From the DSC analysis it is apparent that the biosynthetic drug substance has the ability to adsorb water at elevated humidity. This could be ascribed to the presence of an amorphous phase within the biosynthetic material. The DSC data indicate an exotherm immediately after the endotherm ascribed to the melting process which suggests that the transition involves decomposition in addition to melting. The differences in the DSC traces between synthetic and biosynthetic RF suggest physical differences between the samples involving one or more of the following; different polymorphic forms, different levels of amorphous material present in the crystalline form or different particle size. Inspection of the samples confirms a difference in bulk powder characteristics; e.g. synthetic sample has lower bulk density and better powder flow and compressibility than the biosynthetic quality. A further study evaluating the physical characteristics of the two sources of RF by use of other techniques will be carried out and reported elsewhere.

Water molecules initially adsorbed on the surface may form a monomolecular layer and increase the van der Waals forces, thereby increasing the interactions between the molecules and smooth out surface micro-irregularities that might influence the wavelength distribution of the reflected light, leading to an apparent change in color (Nokhodchi and Javadzadeh 2007). The presence of water molecules can also change the polarity of the microenvironment which may lead to solvatochromism (Gordon and Gregory 1987). Adsorbed water can further influence the reactant conformation, intra- and intermolecular hydrogen bonding properties or facilitate hydrogen atom abstraction. All these parameters are important with respect to color changes in the solid state (Nokhodchi and Javadzadeh 2007). As discussed in a previous paper, the color change in solid state RF show elements of photochromism (i.e. reversible color change upon exposure to light) (Sue-Chu et al. 2008). This further emphasizes the importance of adsorbed water on the change in surface color.

According to the manufacturer, all the tablets that were withdrawn from the market due to discoloration were quantitatively sound (personal communication). This is consistent with observations made previously; i.e. that a change of appearance does not always have a direct correlation to the chemical degradation and may therefore not affects the efficacy of the preparation (Tønnesen et al. 2007; Chen et al. 2005). The patients might however; feel uneasy to use medications that are discoloured and the compliance is therefore reduced. As mentioned above, trace amounts of lumichrome could be detected in irradiated solid samples. Lumichrome is however, a major photodegradation product of riboflavin in solution (Ahmad et al. 2004). Further, lumiflavin is claimed to be the major photodecomposition product of RF in the solid state but was not identified under the present conditions (Roth et al. 1985). This illustrates the complexity of solid state reactions; i.e. that the observed photoinduced changes can be a mixture of solid state processes (e.g. physical changes) and reactions typical for the sample in solution. Also, a change in reaction rate with respect to color change during the process is very clearly demonstrated in the case of RF where most of the color change took place almost immediately followed by a minor if any change throughout the remaining experiment. This can be ascribed to alterations of the quantity of light reflected and absorbed at the surface, and possible absorption of certain amounts of energy by the decomposition products or other compounds formed (Lachman et al. 1962). When the surface darkens as illustrated by an increase in $-L^*$ value, it can provide protection for underlying layers. The extent of RF loss at the surface might be severe, but still the tablet can be quantitatively sound when assaying the whole tablet due to the dilution effect of the unexposed (and hence intact) material. The use of a general mathematical model to describe the rate of a solid state photoreaction is of little value because the radiation is concentrated on the surface and not evenly distributed throughout the sample (Sande 1996).

The observed color change of RF was dramatically increased (by a factor up to 7) when the drug substance was formulated as tablets. It was therefore suspected that one or more of the excipients might exhibit a catalyzing effect on the process(es) leading to the observed change in color. Interactions between RF and individual tablet components (with the exception of talc) were indicated by DSC after mixing and compression at low pressure prior to exposure. The lack of effect of talc could be attributed to the fact that this excipient is poorly compressible and hence may protect riboflavin from the effect of compression as discussed above. Excipients like lactose, sugar and starches are liable to participate in photochemical processes. They are susceptible to free radical attack in that they have abstractable hydrogens (Moore 2004). Talc is regarded to be photo inert but may contain trace amounts of iron that can influence RF photoreactivity (Tzeng and Lee 1989). The free radical and iron induced photodegradation reactions seemed however, to play a minor role in the case of the observed color change in the solid state RF. Riboflavin and its degradation products lumichrome and lumiflavin are further known to form ground state complexes with various electron donating substances like indoles, phenols and purines as well as other compounds not having notable electron donor properties (Foster 1969). Such complex formation can induce color changes and enhance absorp-

tion at long wavelength (in the ground state) as well as influencing the photoreactivity of RF and its degradation products. In some cases, even a photostabilizing effect is observed (Foster 1969; Slifkin 1971). Formation of a weak ground state complex would have the potential to induce or enhance a color change that might disappear upon dissolution. Based on the results reported above, it seems possible that RF undergoes some kind of complex formation or physical interaction with the excipients in the tablet. RF in a hydrogen bonded state will be a better electron acceptor than in the unbonded state as H-bonding leaves the isooalloxazine ring more electropositive (Slifkin 1971). Adsorbed water will favour a hydrogen bonded state of the RF molecules, thereby enhancing the electron acceptor properties and facilitate ground state complex formation with excipients. Hydrogen bonding is reported to have less effects on interactions with hydrogen donors (Slifkin 1971). The fact that direct compression made the tablets, particularly those containing biosynthetic RF, less susceptible to a light induced color change further supports the hypothesis that hydrogen bonding of RF influences its color-fastness as the water content would be low in directly compressed tablets. The effect is dependent on the physical form of riboflavin.

The formation of lumiflavine and lumichrome is generally favoured in the absence of an excess of readily oxidable substrates (Kostenbauer et al. 1965). The fact that these degradation products are absent or only occur in minor amounts in the samples indicates that RF might be protected from photodegradation by a tentative ground state complex formation or alternative excited state processes. Charge-transfer transitions in RF have been observed in neutral as well as strongly acidic environment (Foster 1969). The addition of hydrochloric acid to the granulation fluid had however, no effect on the observed tablet color change in the present work.

The above results indicate that the biosynthetic drug substance would appear to be more susceptible to undergo a color change than the synthetic drug substance after inadvertent exposure to radiation. This visual observation is not necessarily reflected in the photostability test performed according to the ICH Guideline. The recommended protocol is an end-point test, and for the RF drug substance, the synthetic sample would according to the test, demonstrate the largest discoloration. This emphasizes the importance of performing additional measurements trying to mimic "in use" conditions. The color tolerance limit must be set individually for each product and should take into account the results from visual examination of the preparations (Tønnesen et al. 2007). The above results also illustrate how difficult it can be to predict photostability of a final preparation from the observations made on the pure drug substance; i.e. the biosynthetic RF is the most stable in bulk (at end-point criteria) but the least stable in tablets in the presence of adsorbed water or at high humidity levels.

The water content, either in the form of granulation fluid or adsorbed from the environment therefore seems to be the key issue with respect to the color-fastness of the RF tablets. The biosynthetically produced RF is apparently more hygroscopic than the synthetic quality. As a conclusion direct compression should be preferred and the container should be air tight and non-transmitant to light. The tablets could alternatively be packed in unit dose containers or at least the tablet box should contain a drying material (e.g. dried silica) to prevent adsorption of humidity from the environment, thereby reducing light sensitivity under "in use" conditions.

4. Experimental

4.1. Materials

Synthetic riboflavin (m.p. 305 °C), biosynthetic riboflavin (m.p 288 °C), icing sugar, sodium starch glycolate, magnesium stearate and talc were generously provided as a gift by Weifa A/S (Oslo, Norway). Thiamine hydrochloride, lactose and wheat starch were purchased from Norsk Medisinaldepot (Oslo, Norway), nicotinamide, lumichrome and lumiflavin were purchased from Sigma Chemicals Co. (St.Louis, MO, USA) and microcrystalline cellulose (Avicel PH 101) was purchased from FMC Biopolymers (Drammen, Norway).

4.2. Methods

4.2.1. Irradiation of the samples

Irradiation was performed in a SUNTEST CPS+ (Atlas, Gelnhausen, Germany). The light source was a xenon lamp (1.5 kW) equipped with a 6 mm special glass filter, transmitting light corresponding to exposure behind window-glass (cut-off approximately 310 nm). The cabinet was equipped with a SunCool™ device (Atlas) which maintains a chamber temperature < 30 °C. The intensity was measured by using a XenoCal Sensor (Atlas) with a spectral range 300–800 nm. Dark controls were added to evaluate temperature effects. The samples were exposed according to the ICH Guideline (ICH 1997).

4.2.1.1. Drug substance

A powder sample holder was constructed in order to measure the color of the sample prior to and after exposure. The device was made from Plexiglas. The actual sample compartment had a diameter of 10 mm and a depth of 2 mm. The sample was covered by a quartz lid (95% transmission at 195 nm). The sample holder was placed in the Suntest CPS$^+$ test chamber and transferred to the color measuring device at selected intervals.

4.2.1.2. Compressed drug substance and tablets

The compressed powder samples and ordinary tablets were presented in glass dishes. In the Suntest CPS$^+$ the dishes were covered with a thin plastic wrap (Gladpack). The plastic wrap transmits only 85% of the incident radiation above 310 nm and this is taken into account in the calculation of energy levels. The temperature inside the dishes was detected by use of a temperature recording strip (sensitivity range 37–65 °C). In most cases the temperature was below the lower detection limit of the strip.

4.2.2. Color measurements

The color of the samples was determined by use of a Minolta CM-3500 spectrophotometer. The sample color was measured by use of the 1976 CIE $L^*a^*b^*$ (CIELAB) color space. The model is based on the assumption that three pairs of opposing color sensations can produce all colors; red and green; yellow and blue; and black and white (Marcus 1998). The CIELAB color coordinate a^* correlates with red ($+a^*$) and green ($-a^*$) while the CIELAB coordinate b^* correlates with yellow ($+b^*$) and blue ($-b^*$). L^* correlates with perceived lightness in the CIELAB color space. A perfect white would have an L^* of 100, and a perfect black would have an L^* of zero. Color difference ΔE^*_{ab} in the $L^*a^*b^*$ color space, which indicates the degree of color difference but not the direction, is defined by the following equation (Eq.1):

$$\Delta E^*_{ab} = \sqrt{((\Delta L^*)^2 + (\Delta a^*)^2 + (b^*)^2)} \quad (1)$$

where ΔL^*, Δa^*, and Δb^* are differences in L^*, a^* and b^* values respectively, between the specimen color and the target (reference) color (Editorial 1998).

The color was determined at 3 different points of the sample surface and each point was measured 3 times. Six samples were used in each study.

4.2.3. Differential scanning calorimetry (DSC)

The analyses were performed by use of a Mettler-Toledo DSC822^e differential scanning calorimeter. The sample (1–2 mg) was filled in a 40 µl aluminium pan equipped with a perforated lid. The heating rate was 10 °C/min in the temperature interval 50 °C–320 °C.

4.2.4. Qualitative HPLC analysis

The HPLC system consisted of a Shimadzu LC-9A Liquid Chromatograph pump, a Shimadzu SPD-10A UV Spectrophotometric detector, a Shimadzu SIL-9A auto sampler and a Shimadzu C-R5A recorder. The stationary phase was a Water Nova-Pak® C_{18} 3.9 · 150mm (i.d) column. The mobile phase consisted of water/methanol (13 : 7). Flow 0.5 ml/min, injection volume 20 µl, detection wavelength 374 nm. Retention times: riboflavin ~5 min, lumiflavin ~7 min, lumichrome ~20 min. The samples were prepared by dissolving irradiated and non-irradiated samples in distilled water

at a theoretical RF concentration in the range 1.3×10^{-5} M-6.6×10^{-6} M. The color layer of the tablets after exposure to 22045 kJ/m^2 at 765 W/m^2 (TAB1, prepared from both synthetic and biosynthetic RF) was enriched by scraping off the surface (approximately 5 mg per tablet to be dissolved in 10 ml distilled water; total tablet weight 75 mg). The samples were filtered (Spartan 13/0.45 RC, Whatman) prior to analysis. The samples were spiked with lumichrome or lumiflavin for identification of degradation products.

4.2.5. Qualitative TLC analysis

The color layer of the tablets after exposure to 2700 kJ/m^2 at 765 W/m^2 (TAB1, containing 16% RF, prepared from biosynthetic RF) was enriched by scraping off the surface. Approximately 1 mg was scraped off per tablet and the colored layer from 5 tablets (i.e. 5 mg) was dissolved in 5ml methanol. A reference sample was identically prepared from non exposed tablets. Further, exposed and non exposed drug substance (1 mg) and lumichrome were dissolved in methanol (5 ml). The samples were analysed by means of TLC. Stationary phase: Silica gel 60 F_{254} (Merck); mobile phase: glacial acetic acid/acetone/methanol/benzene (5 : 5:20 : 70). R_f riboflavin $\sim$ 0.20; R_f lumichrome $\sim$ 0.65.

4.2.6. UV-VIS spectrometry

Exposed and non exposed tablets (see Qualitative TLC analysis) were dissolved in methanol, methanol +0.1% water, and water respectively. Exposed and non exposed drug substance and lumichrome (see Qualitative TLC analysis) were dissolved in methanol. All samples were diluted until an absorbance <1 was obtained. The UV-VIS spectra of the samples were recorded in the wavelength range 290–700 nm by use of a UV-2401PC UV-VIS Recording Spectrophotometer (Shimadzu). The aqueous samples were filtered (Spartan 13/0.45 RC, Whatman) prior to analysis.

4.2.7. Tablet formulations

The standard tablet formulation (TAB1) consisted of riboflavin, thiamine hydrochloride, nicotinamide, wheat starch, lactose, icing sugar, sodium starch glycolate, magnesium stearate and talc. The tablets were prepared by wet granulation. The granulation fluid was made from 10% hydrochloric acid, water, ethanol, methanol, gelatine and liquid paraffin which are used in the commercial manufacture of the product. This tablet formulation was then modified as follows: TAB2A is identical to TAB1 but is made by direct compression, in TAB2B the concentration of hydrochloric acid in the granulation fluid is increased to 20%, in TAB2C hydrochloric acid is replaced by water in the granulation fluid, and in TAB2D wheat starch is replaced by microcrystalline cellulose (Avicel PH 101).

4.2.8. Compression of powder mixtures and production of tablets

RF was mixed with the individual tablet components (1 : 1) and manually compressed into tablets by use of a single punch tabletting machine (Kongsberg). The pressure applied was not recorded for practical reasons. The thickness was 1–2 mm and the diameter was 8 mm. A single punch Diaf Vibe Mølle tabletting machine was used for the production of ordinary tablets of approximately the same thickness. The resulting compressed samples and ordinary tablets were exposed in the Suntest CPS$^+$ and analysed by color measurements, DSC and HPLC. Dark controls were treated similarly.

4.2.9. Exposure to elevated humidity

Riboflavin drug substance and compressed samples of RF mixed with selected excipients were exposed to elevated humidity prior to radiation. The samples were placed in a Petri dish together with a small container filled with water. The dish was sealed with Parafilm and placed in an incubator at 40 °C for 24 h or 5 days. The samples were then immediately transferred to the appropriate sample holder (see above) and irradiated.

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