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Determination of 9-hydroxyellipticine by redox colorimetry

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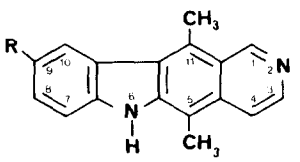
Abstract: The chemical reactions involved in the decomposition of 9-hydroxyellipticine (9-OH-E), an anticancer agent, in polar solvents is explained. The reactions, which involve the formation of 9-oxo-ellipticine and the addition of a nucleophilic acid on the C10 site of the heterocyclic system, have been used to measure 9-OH-E quantitatively by colorimetry in solution and by reflection on paper surfaces. A method for the stabilization of 9-OH-E in polar solvents is proposed.

Keywords: *9-Hydroxyellipticine; mechanism of instability; qualitative and quantitative analysis; thin-layer chromatography; colour measurement; stabilization.*

Introduction

Ellipticine **1** (Fig. 1), a natural indolic alkaloid, is extracted from the plant *Ochrosia elliptica* Labill (of the Apocynaceae family) [1, 2]. This compound has biological activity, particularly against malignant animal and human tumours [2–4]. An extremely interesting cytotoxic effect against certain human tumours and breast cancer has also been demonstrated [4, 5]. Derivatives of this compound have been synthesized such as the 9-hydroxyl derivative and 9-methoxy or N-quaternized compounds in position 2 of the indolic system (Fig. 1). They all possess efficient anticancer activity [5–7].

N-methyl-9-hydroxyellipticinium acetate (Celiptium®), one of the extensively studied derivatives obtained from 9-hydroxyellipticine **2** (9-OH-E, Fig. 1) by acetomethylation has the advantage of being more soluble in aqueous solution than its non-ionic homologue [7]. Clinical trials [6, 8–11] have shown the efficiency of this type of compound in the treatment of breast, uterus and lung cancers, sarcomas and cerebral tumours. 9-Hydroxyellipticine is obtained in industry from 5-methoxyindole [12] or from para-xylène [13] by chemical processes requiring a number of solvents or purification steps.



1 R = H
2 R = OH

Figure 1
Structure of ellipticine **1** and 9-hydroxyellipticine **2**.

9-Hydroxyellipticine is of current interest. In order to improve the conditions of its synthesis, use, and analysis, this study was carried out to confirm that the compound is unstable in polar solvents to describe the mechanism of its degradation to develop a method of analysis based on its instability, to propose a method for stabilizing this molecule when it exists in solution, and to propose a method for verifying that 9-OH-E is well stabilized.

Experimental

Apparatus

The infrared spectra were recorded on a Perkin–Elmer® 683 spectrophotometer and the mass spectra on a AE1-MS902 spectrometer using electron impact. The nuclear

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magnetic resonance spectra were determined on a Bruker® 300 MHz spectrometer using TMS as the internal standard. Ultraviolet and visible absorption spectra were recorded on a Kontron (Uvikon 860) spectrophotometer.

The colour apparatus used for this study was a Minolta® (type CR221) colorimeter which gave the colorimetric data in the CIELab colour chart (L^*,a^*,b^*,C^*,h^*). The measuring area of the sample carrier was chosen to be equal to 3 mm (small sample) and the illuminant standard used was the C (6774 K) or D_{65} (6504 K).

Reagents

Analytical grade chemicals were employed. Commercially available ellipticine **1** and 9-hydroxyellipticine **2** were obtained as gifts from Sanofi (14, rue Pierre et Marie Curie, F75005, Paris).

Stock solutions

Sodium hypochlorite. 9×10^{-3}, 3.21×10^{-2} and 6.42×10^{-2} M aqueous solutions were prepared.

Sodium methylate. A 0.174 M solution was prepared with sodium (200 mg) and anhydrous methanol (50 ml).

Synthesis of 9-oxoellipticine 3

To a suspension of 200 mg of **2** in 500 ml of chloroform, 320 mg of manganese dioxide was added. After 30 min of stirring at room temperature, the mixture was filtered. The filtrate was dried and concentrated and the solid obtained was purified by column chromatography (silica gel, benzene–chloroform–ethanol, 3:2:1, v/v/v). After removing the solvent under vacuum, **3** was obtained [14].

Yield: 91%; IR (KBr): 1635 ($\nu_{C=O}$),1610 ($\nu_{C=N}$), 1580 ($\nu_{C=C}$) cm^{-1}.

The NMR ($CDCl_3$, TMS, 300 MHz) spectrum conformed with the results existing in the literature [15]: 2.7802 (s, 3H, CH_3-5), 2.7969 (s, 3H, CH_3-11), 6.5110 (d, 1H, J_{H7-H8}=9, H-8), 6.8061 (s, 1H, H-10), 7.4422 (d, 1H, J_{H7-H8}=9, H-7), 7.7503 (d, 1H, J_{H3-H4}=6, H-4), 8.6457 (d, 1H, J_{H3-H4}=6, H-3), 9.3902 (s, 1H, H-1).

Colorimetry in solution

To a solution (10 ml) of **1** and/or its hydroxy derivative **2** (1.5×10^{-4} M to 1.5×10^{-2} M in methanol), a few microlitres of a dilute sodium hypochlorite solution (9×10^{-3} M) were added. The initial yellow tint transforms rapidly to brick-red. Within a period of 10 min, the brick-red colour fades and disappears in a few hours. In order to obtain good reproducibility, it is advisable to wait 5 min before recording the spectra.

The product produced by this method gave the same spectral data as the pure product **3** obtained by synthesis.

Colour measurement on paper

Solutions of 9-OH-E, varying in concentration (2.4×10^{-5} to 7.63×10^{-4} M), and sodium hypochlorite (3.21×10^{-2} or 6.42×10^{-2} N) in thoroughly degassed methanol were saturated with nitrogen. The adsorbent support was white paper of 1 mm thickness (Whatman 17 Chr). For each measurement, 20 µl of solution were deposited in such a manner that the stain was approximately 5 mm in diameter. To this was added 20 µl of an oxidizing solution. The colour measurement was made immediately as it is unstable with time.

Methoxylation of 9-oxoellipticine

To 150 mg of **3** dissolved in 50 ml of dichloromethane, a solution of sodium methylate (50 ml) was added drop-by-drop. The progress of the reaction was followed by thin-layer chromatography (silica gel Merck 60 F254; benzene–chloroform–absolute ethanol, 3:2:1, v/v/v) and the addition of methylate was stopped when the 9-oxo-ellipticine was completely transformed. After concentration under vacuum, the medium was analysed by thin layer preparative chromatography (four spots with R_f values of 0.21, 0.30, 0.46 and 0.68 were visible).

The two slowest migrating spots were each eluted from the silica by extraction with acetone and the two fastest migrating spots with chloroform. After evaporation of the solvents, the compounds were analysed by NMR and IR spectroscopy.

Spot at R_f = 0.21; 9-OH-E 2. The NMR (DMSO, TMS, 300 MHz) spectrum was in agreement with that already reported [16]: 2.7 (s, 3H, CH_3-5), 3.2 (s, 3H, CH_3-11), 6.9 (d, 1H, H-8), 7.2 (s, 1H, H-7), 7.6 (d, 1H, H-10), 7.7 (d, 1H, H-4), 8.2 (d, 1H, H-3), 9.7 (s, 1H, NH), 10 (s, 1H, H-1), 12 (s, 1H, OH). IR: 3400 ($\nu_{O=H}$), 3100 ($\nu_{N=H}$); m/z = 262.

Spot at R_f = *0.30; 9-hydroxy,10-methoxy-ellipticine* ***4***. NMR (CD_3COCD_3, TMS, 300 MHz): 2.75 (s, 3H, CH_3-11), 3.56 (s, 3H, CH_3-5), 3.93 (s, 3H, OCH_3), 7.17 (d, 1H, J_{H7-H8} = 10 Hz, H-8), 7.22 (d, 1H, J_{H7-H8} = 10 Hz, H-7), 7.90 (d, 1H, J_{H3-H4} = 6 Hz, H-4), 8.44 (d, 1H, J_{H3-H4} = 6 Hz, H-3), 9.70 (s, 1H, H-1), 10.2 (s, 1H, NH), 11.00 (s, 1H, OH). IR: 3400 ($\nu_{O=H}$), 3100 ($\nu_{N=H}$); *m/z* = *292.*

Spot at R_f = *0.46; 9-oxo,10,10 di-methoxy-ellipticine* ***6***. The NMR ($CDCl_3$, TMS, 300 MHz) spectrum was in agreement with that reported [17]: 2.74 (s, 3H, CH_3-11), 3.28 (s, 3H, OCH_3-10), 3.30 (s, 6H, OCH_3-10, CH_3-5), 6.24 (d, 1H, J_{H7-H8} = 10 Hz, H-8), 7.48 (d, 1H, J_{H7-H8} = 10 Hz, H-7), 7.75 (d, 1H, J_{H3-H4} = 6.3 Hz, H-4), 8.37 (d, 1H, J_{H3-H4} = 6.3 Hz, H-3), 9.06 (s, 1H, NH), 9.68 (s, 1H, H-1). IR: 3150 ($\nu_{N=H}$), 1680 ($\nu_{C=O}$).

Spot at R_f = *0.68; 9-oxo,10-methoxyellipti-cine* ***5***. The NMR (DMSO, TMS, 300 MHz) spectrum was in agreement with that reported [17]: 2.65 (s, 3H, CH_3-11), 2.95 (s, 3H, CH_3-5), 4.15 (s, 3H, OCH_3), 6.57 (d, 1H, J_{H7-H8} = 10 Hz, H-8), 7.54 (d, 1H, J_{H7-H8} = 10 Hz, H-7), 7.93 (d, 1H, J_{H3-H4} = 4 Hz, H-4), 8.63 (d, 1H, J_{H3-H4} = 4 Hz, H-3), 9.45 (s, 1H, H-1). IR: 1670 ($\nu_{C=O}$), 1640 ($\nu_{C=N}$).

Results

Instability of hydroxyellipticine in methanolic solution

Methanol has been widely used to dissolve compounds obtained from the synthesis of 9-hydroxy-ellipticine or its reaction products for the purpose of analysis.

The hydroxylated derivative **2** in solution with methanol (conc. from 1.5×10^{-4} to 1.5×10^{-2} M), turns red–orange very rapidly and becomes deeply coloured within a few hours. The compounds obtained from 9-OH-E have been qualitatively studied by thin-layer chromatography (silica gel 60F254 Merck; eluent: benzene–chloroform–ethanol, 3:2:1, v/v/v). The principal results (R_f, colour) are presented in Table 1. An examination of these results shows that even within the first few minutes after dissolution in methanol, 9-OH-E decomposes to form several compounds. Of these, the compound in highest yield is the orange product of R_f 0.21. A smaller quantity of a red product (R_f 0.65) and yellow and orange compounds of trace quantities (R_f values 0.50 and 0.43, respectively) were also formed. If the solution, thus prepared, is left a few days at room temperature, with or without light, the quantity of red and yellow compounds diminish significantly resulting in the formation of the orange product (R_f = 0.43). Similar results were observed when other polar solvents were used (acetonitrile, ethyl acetate, DMF, DMSO).

During the past few years, research has shown that 9-OH-E can be easily oxidized [18–20]. It has now been verified that the instability of this compound in polar solvents, particularly methanol, is due to an oxidation reaction. If the derivative **2** is dissolved under a nitrogen blanket in methanol which has been degassed under vacuum, the resulting solution remains orange in colour. Further, if the thin-layer chromatography is done under an inert atmosphere with solvents which have been thoroughly degassed and then saturated with nitrogen, the red spot (R_f = 0.65) appears only as a trace and only the orange spot (R_f = 0.21) persists, which can be attributed to 9-OH-E.

If 9-OH-E is oxidized in an alcoholic medium, either degassed or undegassed, by a classical oxidizing agent like hypochlorite, iodine or permanganate, the solution immediately becomes red and separation by chromatography results in two spots whose R_f values are 0.21 (9-OH-E) and 0.65. When this solution is left at room temperature, the red colour fades to become orange after approximately 30 min. A study of the oxidized solution by TLC shows the appearance of orange (R_f = 0.50) and yellow (R_f = 0.43) spots which were observed during the analysis of 9-OH-E in the non-degassed methanol solution. When an excess of the oxidizing agent is introduced into a solution of 9-OH-E, a transient red tint appears which rapidly disappears and the resulting yellow solution shows four spots by TLC. The main two spots have R_f values of 0.43 and 0.72 while the other two smaller spots have R_f values of 0.21 and 0.68.

From these experiments, it is concluded that the decomposition of 9-OH-E is due to an oxidation reaction by atmospheric oxygen dissolved in the solvents. The products thus formed are identical to those resulting from an oxidation reaction of **2** by an oxidizing agent such as iodine or hypochlorite ions.

Table 1
Qualitative study by thin-layer chromatography of R_f values 9-OH-E degradation compounds (bold are the major spots)

1	**2**	**2** decomposed	**2** /nitrogen	**2** oxidized	**2 oxidized** decomposed	**2 + excess of** oxidizing agent	**3**	**3** decomposed	**3** methoxylated	Compounds
						0.72 J		0.72 J		**7**
		0.68 V				0.68 V			**0.68 V**	**5**
	0.65 R		0.65 R	**0.65 R**			**0.65 R**	0.65 R		**3**
	0.50 J				0.50 J			0.50 J		**8**
0.43 J										**1**
	0.43 O	**0.43 O**			**0.43 O**	**0.43 O**		**0.43 O**	0.46 O	**6**
									0.30 r	**4**
	0.21 O	**0.21 O**	**0.21 O**	0.21 O	0.21 O	0.21 O		0.21 O	0.21 O	**2**

Quantification of 9-hydroxy-ellipticine based on its oxidation

Colorimetry in solution. Although the oxidation of 9-OH-E by atmospheric oxygen or oxidizing agents gives a red compound which reacts in a nucleophilic medium, it is stable for a few minutes. Quantitative colorimetric measurements in the visible region of the absorption spectrum of oxidized 9-OH-E, may therefore be carried out after appropriate calibration. This can be done with the aid of an oxidizing agent such as an aqueous solution of sodium hypochlorite, potassium permanganate or iodine. Only the oxidation reaction in the presence of hypochlorite ions is considered further.

When the substituted derivative **2** is mixed with sodium hypochorite, a red-brick tint appears; the spectra are shown in Fig. 2. The absorption at 490 nm (ϵ = 10500) by the oxidized derivative corresponded with the blue–green region of the visible range. When compound **2** was mixed with the ellipticine and an oxidizing agent (in the ratio 1:1, mol:mol), there was no reaction, since the absorbance of the solution was found to be unchanged. For quantitative analysis, a wavelength of 490 nm, at which maximum absorption occurs has been chosen.

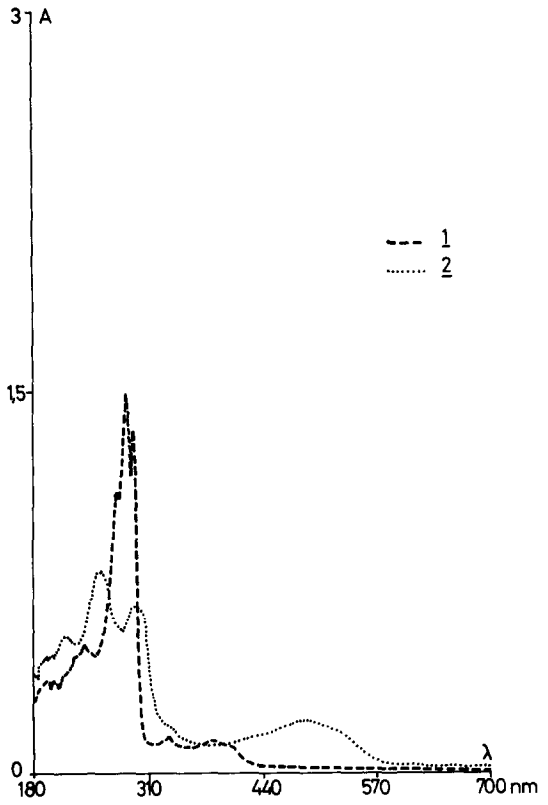

Figure 2
Absorption spectra of the oxidized solutions.

The red colour which occurred after the reaction between **2** and an oxidizing agent was not stable with time. The absorption requires 5 min, after the addition of sodium hypochlorite, to attain its maximum value, which is therefore the optimum time for quantitative analysis. All results have been obtained after this time interval.

Compliance with Beer's law at 490 nm was verified for concentrations of oxidized **2** ranging from 1 to 40 $\times$ 10^{-6} mol l^{-1} in methanol (calibration equation: absorbance = 7905.42C + 0.012, r = 0.997, where C is in mol l^{-1}).

In this range of concentrations and at the wavelength used, ellipticine does not absorb. It was, however, necessary to check if it interfered with the red colour formation, when present in significant amounts. Therefore, in case of 9-OH-E with ellipticine, a deviation of linearity beyond 4 $\times$ 10^{-5} mol l^{-1} was found and estimated error was about 30%.

The amount of sodium hypochlorite influenced the results of the analyses. In fact, an excess of oxidant favoured the decomposition of the reaction products. If the oxidizing agent was too concentrated, a transient red colour formation occurred in the presence of **2**, making further analysis impossible. For all quantitative analysis, 30 µl of a 9 $\times$ 10^{-3} mol l^{-1} solution of sodium hypochlorite, for every millilitre of organic solution was added.

By reflection from solid surfaces. For many years, numerical analysis of colour has been used to quantify the colour of various objects such as wood, plastic and food in order to improve their quality in industry [21]. This technique has been applied to the analysis of 9-OH-E in an oxidizing medium on an adsorbent support in order to provide a method of analysis which is simple, rapid and capable of computerization.

Colour is defined in terms of tint or 'hue', lightness or 'value', saturation or 'chroma'.

The CIELab system defines the following parameters:

L^* (value): lightness which varies from L^* = 0 for black to L^* = 100 for white;

a^* and b^* (hue) indicate two axes with a^* the red–green axis, and b^* the yellow–blue axis.

C^* (chroma) and h^* (hue angle) indicate the

polar coordinates of the colour of the object in the plane a^*–b^*.

$$C^* = (a^{*2} + b^{*2})^{1/2}$$

$$h^* = \arctan (b^*/a^*)$$

ΔH^* which is the hue difference between two samples:

$$\Delta H^* = (\Delta E^{*2} - \Delta L^{*2} - \Delta C^*_{ab}{}^2)^{1/2},$$

where ΔC^*_{ab} is the saturation difference between the two objects and ΔE^* the total colour difference

$$\Delta E^* = (\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2})^{1/2}.$$

The results of the colour measurements, obtained by variation of the concentration and the reaction time of 9-OH-E are shown in Figs 3 and 4. Each result was the average of four tests with three measurements done for each test.

From the results in Fig. 3 it can be inferred that when the concentration of 9-OH-E decreases, the value L^* increases and the coordinates a^* and b^* decrease. These variations are normal, because the hue difference ΔH^* increases as the red tint becomes increasingly weak. The colour angle h^* is practically unaffected for weak concentrations indicating that the standard CIE observer would not detect such chromatic differences.

L^*, a^* and b^* are time dependent. Figure 4 shows results obtained from the oxidation of a solution of 9-OH-E (3.82×10^{-4} M). The colour of the stain evolves becoming progressively weaker (L^* increases) and more yellow (h^* increases) which indicates the rapid decomposition of 9-oxo-ellipticine.

Sodium hypochlorite (6.42×10^{-2} N) has no influence of the colour formation provided that an excess of the oxidizing agent is not used, as discussed earlier. If the colour measurement is conducted in the presence of ellipticine, no influence on the measured colour by ellipticine is observed.

Stabilization of 9-hydroxyellipticine in a polar medium

9-OH-E is unstable in polar media, the decomposition being due to an oxidation reaction by atmospheric oxygen. To avoid or at least to minimize this type of reaction, several

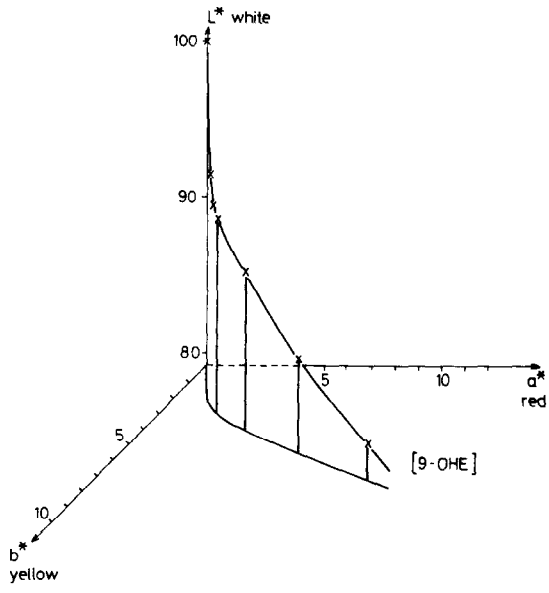

Figure 3
$L^*a^*b^*$ colour chart as a function of 9-OH-E concentration.

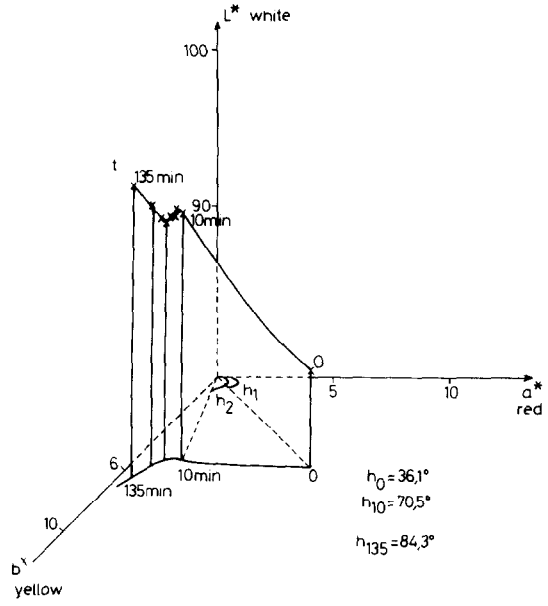

Figure 4
$L^*a^*b^*$ colour chart as a function of time (9-OH-E conc. = 1.91×10^{-4} M).

possibilities exist: (i) to keep the 9-OH-E in a solvent saturated with an inert gas and in the absence of air; this precaution may be a delicate operation during industrial synthesis or use of the substance; (ii) to work in acidic medium because the decomposition essentially takes place in a neutral or basic medium; (iii) to use a reducing agent which stabilizes the

indolic derivative. It is this last possibility that is discussed further.

After several tests, the most efficient and inert reducing agent *vis-a-vis* 9-OH-E is ascorbic acid, a natural substance widely used as an antioxidant [20, 22]. The oxidation of this antioxidant involves the same number of electrons as in a case of 9-OH-E, and hence, it is easier to adjust the concentrations of these two compounds. The addition of an equivalent quantity of vitamin C to an alcoholic solution of 9-OH-E completely oxidized by hypochlorite ions, destroys the red tint formed initially and regenerates 9-OH-E. Similarly, oxidation by other oxidizing agents in a 1:1 mixture of 9-OH-E and ascorbic acid does not lead to any colour formation even after a contact time of several hours. However, an excess of the oxidizing agent forms a red solution as observed earlier.

If a test with hypochlorite is conducted under similar conditions to those described above, it is possible to verify if the 9-OH-E is still intact. The formation of a red tint implies that the 9-OH-E is no longer stabilized because of a deficiency of ascorbic acid. Hence a known quantity of reducing agent may be added to effect the stabilization. This simple test allows the stability of the compound to be verified.

Discussion

Auclair [23] and Meunier [19] have suggested that the oxidation of **2** to a derivative of the type quinoneimine **3** takes place according to a reaction mechanism as shown in Fig. 5(b). Recently, it has been shown with indole derivatives that the first step of the oxidation takes place at the cyclic nitrogen atom [24]. In addition, the reduction of **3** to 9-OH-E is possible [20]. It therefore appears that the first decomposition of 9-OH-E is due to an oxidation reaction due to atmospheric oxygen and which leads to 9-oxo-ellipticine **3**. This compound has been synthesized through the action of manganese dioxide on 9-OH-E. The NMR

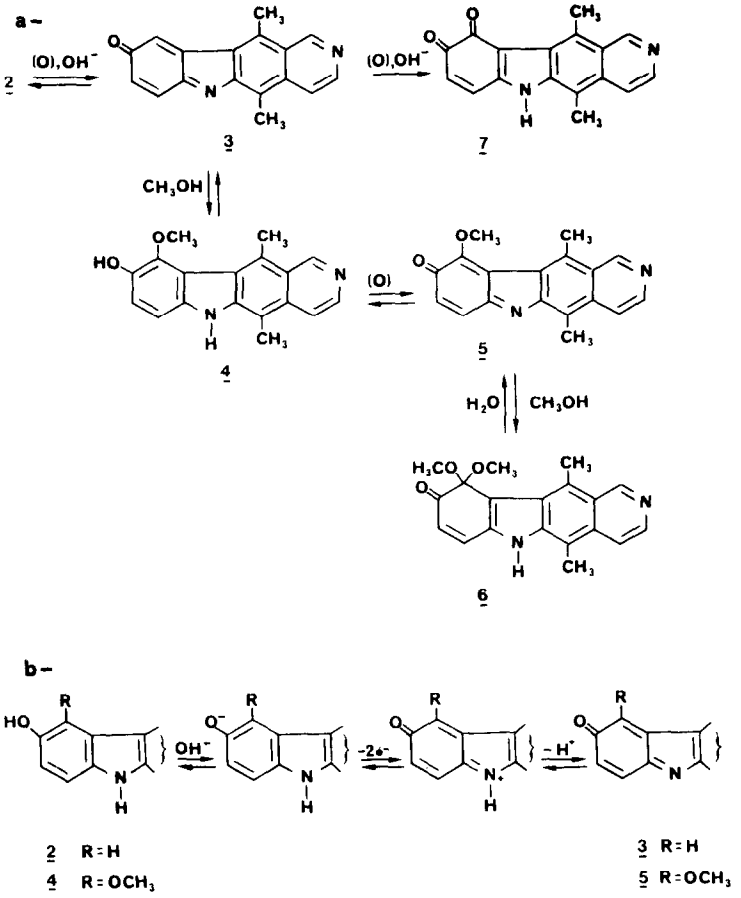

Figure 5
(a) Degradation mechanism; (b) oxidation mechanism.

spectrum of the product obtained conforms with that described in the literature. The IR spectrum of the coloured derivative shows a band at 1635 cm^{-1} which can be attributed to the vibration of a conjugated carbonyl and another at 1625 cm^{-1} which is characteristic of an imine bond. This imine, if dissolved in methanol solution and analysed by chromatography under the experimental conditions described earlier, leads to a red spot with a R_f value of 0.65. If the solution is left for a few hours under ambient conditions of temperature and pressure, the chromatographic analysis shows four spots with R_f values of 0.65, 0.50, 0.43 and 0.21 which are the same as those observed earlier during the analysis of 9-OH-E.

The quinoneimines are electrophilic and the reaction site at C10 is the most reactive. This peculiarity has been proved theoretically [19] as well as experimentally [17, 19, 25, 26]. The polar solvent that was used may react at this site to form adducts by Michael addition. The derivative **3** has been methoxylated by means of sodium methylate solution, and the TLC analysis of the resulting solution shows the formation of four compounds characterized by proton NMR and by IR as **3**: 9-OH-E (yellow, $R_f = 0.21$); **4**: 9-OH,10-MeO-E (pink, $R_f = 0.30$); **6**: 9-oxo,10,10-diMeO-E (orange, $R_f = 0.46$); **5**: 9-oxo,10-MeO-E (purple, $R_f = 0.68$). The transition of 9-oxo-E to the dimethoxylated compound **6** is known [15], but the intermediate **4** has not been described in the literature. It should be noted that of the oxidation of **4** by manganese dioxide and the hydrolysis of **6** gives rise to **5**. According to the results previously obtained, the reaction mechanism of the decomposition of 9-OH-E in methanol is proposed in Fig. 5(a): oxidation of **2** leading to the imine **3**, Michael addition to form the adduct **4** after aromatization, oxidation of **4** which forms the imine **5** and a new Michael addition leading to **6** after aromatization.

The same reactions take place during the analysis of 9-OH-E in an oxidizing medium and it is normal that the red colour due to the formation of the derivative **3** changes with time, since this compound reacts with the solvent to lead to adducts having different specific molar absorbance coefficients and wavelengths of maximum absorption. The decomposition of **2** in alcoholic media is extremely slow. In fact the oxidation reaction takes place only in basic or neutral media. A possible explanation of this phenomenon is based upon the reaction mechanism shown in Fig. 5(b). An acid medium does not favour the first step of formation of the phenolate ion and in addition destroys the aromaticity of the heterocyclic system [24]. Auclair [18] had recently elucidated the oxidation mechanism of 9-OH-E in basic or neutral media as in Fig. 5(b), by introducing a superoxide anion and a phenoxy radical. In this reaction sequence it has been shown that the manganese ions accelerated the reaction. If the oxidation of 9-OH-E is effected using potassium permanganate instead of sodium hypochlorite, the reaction is much more rapid since the red colour is obtained after 4 min.

Figure 3 shows that the colour changes in the CIELab mode, as a function of the concentration of 9-hydroxy-ellipticine are monotones. The colour angle was constant (Fig. 6), indicating the possibility of using a colorimeter to conduct the analysis at weak concentrations (the operator hardly observes the differences in colour in this mode). It is therefore possible to use this technique for the purposes of analysis, after calibration with standards.

In order to verify the validity of this mode of assay, an alcoholic solution of 9-OH-E (1.9×10^{-4} M) was analysed. By placing the obtained values for C^*, h^* and ΔE^* into the calibration curves in Fig. 6, an error of 3% was obtained in using the saturation curve, an important error (10–150%) in using h^* (because this parameter does not change very much), and finally an error of 0.5% in using the total colour difference ΔE^*. If this technique is used to carry out quantitative assay, it is helpful to use the colour difference which takes into account all the chromatic parameters. Moreover, this technique has the advantage of being fast and non-destructive. 9-Oxo-ellipticine was unstable with time. The curves in Fig. 7 show that the degradation reactions take place during the first 10 min; after this time, the colour is practically stable since the parameters C^*, h^* and ΔE^* do not change.

9-Oxo-ellipticine is transformed, in two steps into 9-10-dioxo-N_6-*H*-ellipticine and into 9-OH-E in aqueous medium [27], into 9,10-dioxo-N_2-*H*-ellipticine **7** by oxidation [27] and into 9-oxo-10,10-dimethyloxyellipticine **6** by oxidation and Michael addition.

During its synthesis or use, 9-hydroxy-

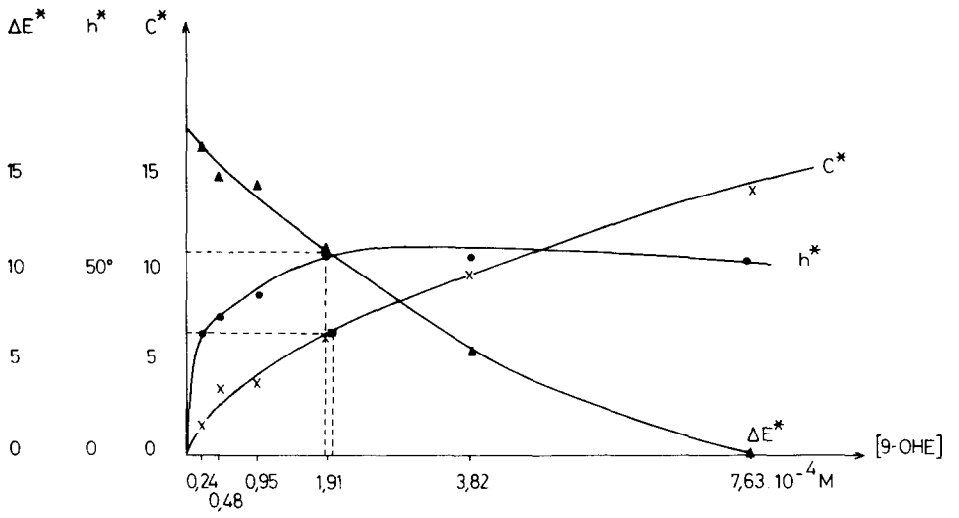

Figure 6
C^*, h^* and ΔE^* as a function of the 9-OH-E concentration.

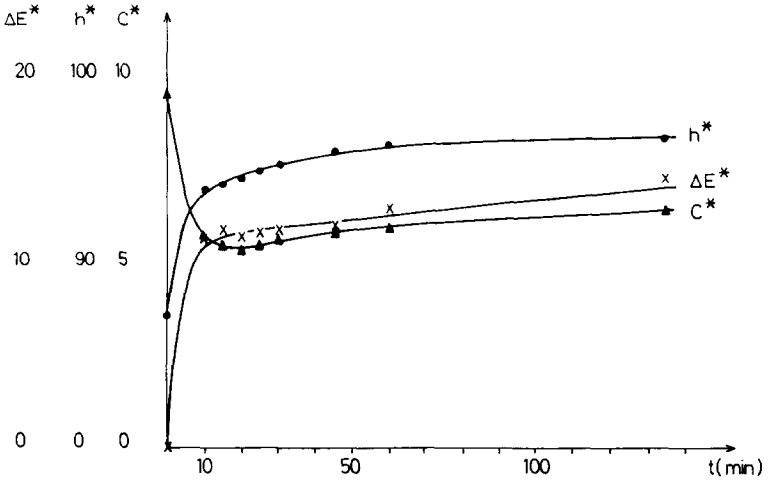

Figure 7
C^*, h^* and ΔE^* as a function of time (9-OH-E conc. = 1.91×10^{-4} M).

ellipticine is usually in a polar medium, which favours its decomposition and, as has been discussed these reactions take place after the oxidation of 9-oxo-E. It is impossible to avoid this phenomenon but the presence of an appropriate reducing agent stabilizes the molecule in polar solvents. The use of ascorbic acid has been preferred over all other reducing agents, since this derivative is of a natural origin and is widely used as an antioxidant in the food and pharmaceutical industries. The antitumour activities of 9-OH-E is, apparently, due to the possibility of producing a superoxide anion $(O_2^{\cdot})^-$ [18]. Thus, with a minimum amount of the reducing agent, it should be possible to stabilize 9-OH-E, in order to preserve its pharmacodynamic properties.

The colorimetric method that has been developed is simple, rapid and permits quantification of the 9-OH-ellipticine with reproducible results. The method does not require expensive apparatus and could be adapted to a solid support. The 9-OH-ellipticine is stable for several days in the presence of vitamin C (1:1, mol:mol). The method can be extended to any hydroxylated indolic system and allows the analysis of these compounds *in vitro*.

9-OH-E, *in vivo* a major anticancer agent, is known to result in alkylating agents which lead to serious internal lesions by formation of

radical derivatives. If it is possible to stabilize the 9-OH-E *in vivo* with ascorbic acid which is known to be a non-toxic protector avoiding formation of radical molecules [28], 9-OH-E may not oxidize to 9-oxo-E and other derivatives which have been described in the reaction mechanism in Fig. 5. But, for this to occur, vitamin C should not be degraded by other systems *in vivo*.

The degradation products do not participate in the redox reactions as they are formed from 9-oxo-E and produce a number of derivatives. If they were to participate in the reactions, the stabilization by means of a reducing agent would be impossible.

Conclusions

9-Hydroxy-ellipticine, an efficient antitumour agent, has been shown to be unstable in a nucleophilic medium. This compound can be oxidized by atmospheric oxygen to a quinoneimine, which in turn can react with polar solvents by a Michael addition. The reaction mechanism has been described and the intermediates isolated. Unlike other analytical methods in which the instability of the 9-OH-E is an inconvenience, the series of reactions can be used for the establishment of a quantitative colorimetric method in alcohol medium as well as by the measurement of transmission in the liquid phase or of reflection on a solid support by means of a colorimeter. The decomposition of hydroxylated ellipticine can be avoided by keeping the solution under an inert atmosphere or by adding a reducing agent such as ascorbic acid. This method is suitable as a stability-indicating assay.

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