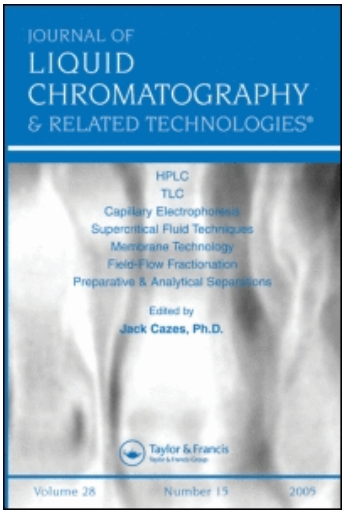

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Analysis of 9-Hydroxy Ellipticine by High-Performance Liquid Chromatography

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ANALYSIS OF 9-HYDROXY ELLIPTICINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

9-hydroxyl derivative of ellipticine, compound of current interest known for its antitumor activity, shows a degradation in a reverse-phase column during High-Performance Liquid Chromatography analyses. In this paper, a chemical explanation of this phenomenon is proposed with the help of a degradation mechanism previously plubished, to understand the appearance of additionnal peaks corresponding to other products.

INTRODUCTION

Ellipticine, a natural indolic alkaloid first extracted from plant Ochrosia elliptica Labill. (1) is known for its antitumor activity (2-5).

Numerous derivatives of this alkaloïd have been synthetized such as 9-hydroxy, 9-methoxy derivatives and N-quaternized compounds. They all display significant anticancerous activity (6,7). Clinical trials (8-10) have shown the efficiency of this type of compounds in the treatment of breast, uterus and lung cancers, sarcomas and cerebral tumours.

The synthesis of such compounds, and particularly 9-hydroxy and 9-methoxy ellipticine, can be realized either by chemical routes (11 and mentionned references), or by biological way (12,13). Whatever the choice of the route, it is necessary to determine the quantity of synthetized compounds. High Performance Liquid Chromatography was classically employed to quantify ellipticine and its derivatives (12,14-17). But this method, the conditions of which are described in

literature, needs the stabilization of the ellipticine derivatives molecules, with for example sodium phosphate buffer, to prevent their instability in solution (15,16).

Despite these operating conditions, we show in this paper that instability of 9-hydroxylated ellipticine is a problem in HPLC. This compound undergoes a degradation mechanism, which occurs within the reverse-phase column, and which explains the appearance of additionnal peaks when it is injected alone.

MATERIALS

Apparatus

The chromatographic system consisted of a Waters® system (Milford, MA, USA) with two high-pressure pumps (Waters, Model 501), a mixing chamber and an injection device (type U6K). Ultraviolet detector (Waters, Model 481) and an integrator (Waters, type 745) complete the system. A Waters μBondapak C_{18} reversed-phase column (300 x 3.9 mm I.D; particle size 10 μm) was eluted isocratically at room temperature. Chromatograms were traced on the integrator at a speed of 1 $cm.mn^{-1}$.

Ultraviolet and visible absorption spectra were recorded on a Kontron (Uvikon 860) spectrophotometer.

Chemicals and reagents

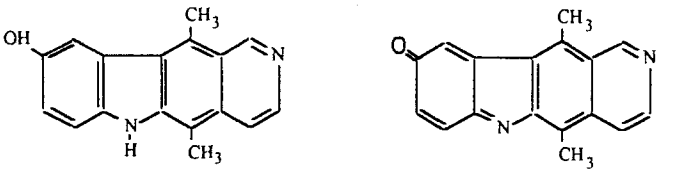
Analytical grade chemicals were employed. 9-hydroxy ellipticine **1** (262 $g.mol^{-1}$) (Figure 1) was obtained as gift from Sanofi (Paris, France). Water, methanol, ammonium acetate and acetic acid were chromatographic grade and were provided by Carlo Erba® (Italy). All mobile phase was filtered through a 0.22 μm Millipore-filter, and renewed every day.

Synthesis of 9-oxo ellipticine **2** (260 $g.mol^{-1}$) (Figure 1) was carried out as following : 320 mg of manganese dioxide was added to a suspension of 200 mg of **1** in 500 ml of chloroform. After 30 minutes of stirring at room temperature, the mixture was filtered. The filtrate was dried and concentrated, the solid obtained was purified by column chromatography (silicagel, benzene/chloroform/ethanol 3/2/1,v/v/v). After removing the solvent under vacuum, **2** was obtained (18). The 91% yield and the NMR spectrum are in agreement with the results existing in the literature (19,20).

METHODS

Procedure

The operating conditions for analyses were experimentally determined. This study was performed using the methanol-water (75/25, v/v) mobile phase containing 5.10^{-2} M ammonium



1 : 9-hydroxy ellipticine **2** : 9-oxo ellipticine

FIGURE 1 : Structure of 9-hydroxy ellipticine **1** and 9-oxo ellipticine **2**

acetate and adjusted to pH 6.0 with glacial acetic acid, at a 1.8 $ml.min^{-1}$ flow-rate. The detection was carried out by reading absorbance at 293 nm, which corresponds to the maximum of absorbance of the 9-hydroxy ellipticine and at 493 nm. Other wavelenghts (λ = 254, 308 nm) mentionned in literature (15,16,21) were tried to compare absorbance. 10 µl of the sample, prepared as described below, were injected within the column for each analysis.

Preparation of samples

Samples were solubilized in methanol, previously degazed under vacuum and different solutions were prepared to obtain 9-hydroxy ellipticine final concentrations ranging from 1 to 10 $\mu g.l^{-1}$.

A guard-column was not used for the "preparation" of samples. It is used usually to avoid too fast a degradation of the column (after few measurements, a lost of concentration of the introduced substance of about 25% was established using a guard-column). It was sufficient to clean regularly the filter of the column head.

RESULTS

High-Performance Liquid Chromatography analysis of the 9-hydroxy ellipticine for quantitative determination can give rise to uncertainties based on the appearance of many peaks during the analysis of a solution containing that molecule. Indeed, although operating conditions described in the literature were used (12,15,17) concerning especially the column type, the temperature and the composition of the mobile phase (presence of ammonium acetate buffer in order to stabilize the 9-hydroxy ellipticine), appearance of additional peaks was observed related to a modification and/or a degradation of the 9-hydroxy ellipticine injected.

The detection of 9-hydroxy ellipticine has been carried out at λ = 293 nm (Table 1) which corresponds to its maximum of absorbance in the ultraviolet range. Tables 2 and 3 present all the retention times and areas as a function of the detection wavelenght for these analyses of 9-hydroxy

TABLE 1 : Retention time and area from peaks detected at λ = 293 nm.
(Results in bold type indicate the major peaks)

Compound	Concentration ($\mu g.ml^{-1}$)	Retention time (min)	Peak Area (%)	Total (%)
9-hydroxy ellipticine	2.5	2.01 **3.13** 4.01 4.96	5.2 **91.3** 1.5 1.8	99.8
	5	1.99 2.35 **3.13** 4.0 4.96 7.56	3.4 1.9 **91** 1.5 1.1 1	99.9
9-oxo ellipticine	2.5	1.94 **3.16** 6.07 10.82	7 **72.5** 17.2 3.2	99.9
	5	1.97 **3.15** 6.01 10.72	5.4 **81** 6.5 7	99.9
	7.5	1.95 **3.14** 3.96 6.04 10.48	2.3 **80.3** 6 7.2 3.6	99.4
	10	1.91 2.29 **3.09** 3.72 3.96 6.0 10.46	2.5 5.6 **63.9** 4.1 10.2 9.1 3.7	99.1

ellipticine. In order to make sure that 9-hydroxy ellipticine was determinable by HPLC at 293 nm for all concentrations, the linearity of the detector responses was verified as a function of the concentrations of this compound injected from 1 to 10 $\mu g.ml^{-1}$. Area/concentration ratio, if we only consider the major peak (retention time of 3.1 min.), is correctly respected up to 7.5.$\mu g.ml^{-1}$ but the variation is not linear beyond this value (Figure 2). The literature mentions however other wavelenghts (λ = 254 ou 308 nm) of detection (12,15).Calibration curves were also plotted on Figure 2.

It was noted that when the detection had been carried out in the Ultraviolet range, a few collected fractions showed an absorbance in the visible range. It is known that 9-hydroxy ellipticine oxidizes rapidly and easily into 9-oxo ellipticine in liquid phase (21,22) and that 9-oxo ellipticine is a red compound which absorbs in the visible range at 493 nm (ε=10500) (22).

TABLE 2 : Retention time and area from peaks detected at $\lambda = 254$ nm.
(Results in bold type indicate the major peaks)

Compound	Concentration ($\mu g.ml^{-1}$)	Retention time (min)	Peak Area (%)	Total (%)
9-hydroxy ellipticine	2.5	1.99 2.4 **3.22** 4.13	9.9 7.4 **65.3** 17.3	99.9
	5	2.01 2.44 2.82 **3.25** 4.15 8.59	3.7 4.3 1.6 **65** 14.8 10.5	99.9
	10	2.02 2.44 **3.21** 4.11 8.33 11.53	1.6 1.3 **60.3** 23.5 8.3 4.9	99.9
9-oxo ellipticine	2.5	2.01 **3.28** 4.12 10.93	7 **64.5** 4 24.5	100
	5	1.93 **3.25** 4.09 6.42 8.31 10.93	2.6 **42** 2.4 1.7 37.6 13.6	99.9
	10	1.9 **3.24** 4.07 5.05 10.34 10.85	1.1 **77.9** 6.1 2.4 7.4 4.9	99.8

Therefore we wanted to know if it was possible to detect peaks issued from the 9-hydroxy ellipticine at this wavelenght (Figure 2 showed also the calibration curve of the 9-hydroxy ellipticine at $\lambda = 493$ nm). Figure 3A and 3B present the peaks obtained at $\lambda = 493$ nm for the 9-hydroxy ellipticine and the 9-oxo ellipticine at a concentration of 5 $\mu g.ml^{-1}$.

At last, it was interesting to plot a calibration curve of the 9-oxo ellipticine **2** at all the wavelenght previously mentionned, 254, 293, 308 and 493 nm (Figure 4). Tables 1 to 4 present retention times and areas obtained for 9-hydroxy ellipticine and for 9-oxo ellipticine as a function of the wavelenght of detection in ultraviolet and visible range.

TABLE 3 : Retention time and area from peaks detected at $\lambda = 308$ nm.
(Results in bold type indicate the major peaks)

Compound	Concentration ($\mu g.ml^{-1}$)	Retention time (min)	Peak Area (%)	Total (%)
9-hydroxy ellipticine	2.5	1.91 2.27 **3.01** 3.83 7.13	9.7 6.3 **58.5** 1.8 23.5	99.8
	5	1.97 **3.16** 4.98	5.3 **92.5** 2.1	99.9
	10	1.91 2.23 **3.1** 3.91 4.94	2.5 5 **74** 11.8 6.3	99.6
9-oxo ellipticine	2.5	1.97 **3.14** 3.93	9.3 **87.3** 3.3	99.9
	5	1.94 **3.17** 4.02 10.76	22.4 **52** 1.5 24	99.9
	10	1.98 **3.24** 5.06 10.85 13.15	3.4 **86.8** 3 3.6 3.2	100

DISCUSSION

The examination of the results of 9-hydroxy ellipticine chromatographic analysis at 293, 254 or 308 nm shows the appearance of many peaks. If we only consider the major peak (retention time of 3.1 min., area of 90%) this compound presents a correct linearity for a concentration range from 1 to 7.5 $\mu g.ml^{-1}$ (Figure 2). At $\lambda = 254$ or 308 nm, the linearity area/concentration of the major peak was respected for concentrations of 9-hydroxy ellipticine higher than those which were detected at $\lambda = 293$ nm, but the slopes were much weaker (Figure 2) and consequently, the sensitivity and the accuracy of the measurements carried out at these wavelenghts were much lower. The 9-hydroxy ellipticine is therefore, *a priori*, determinable by high-performance liquid chromatography.

To explain the appearance of additional peaks, we can remember that numerous authors have mentionned that the 9-hydroxy ellipticine, like all indolic compounds (19-23), can be oxidized in liquid phase (21) and that an equilibrium exists between 9-hydroxy ellipticine and 9-oxo ellipticine (19). As the 9-hydroxy ellipticine cannot react, in sufficient quantity, at the

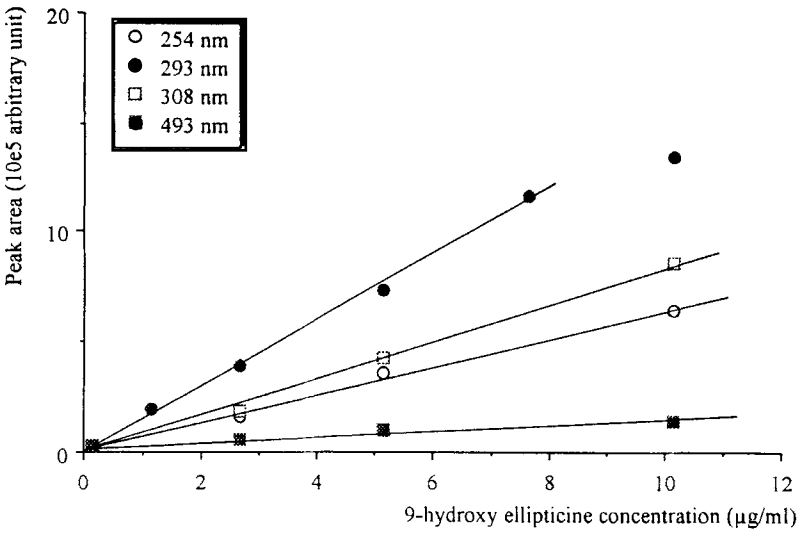


FIGURE 2 : Peaks areas expressed in arbitrary units versus 9-hydroxy ellipticine concentration ($\mu g.ml^{-1}$) at λ = 293 nm and compared absorbance of this 9-hydroxy ellipticine at three different wavelenght : λ = 254 nm, λ = 308 nm and λ = 493 nm.

analysis temperature (room temperature) with mobile phase compounds, the degradation products must be, therefore, generated either by reaction with the residual oxygen of the mobile phase or of the solution, or with the stationnary phase within the column since its oxidizing nature is known owing to the grafted silica constitutives. The first hypothesis is not very plausible since the solvent and the mobile phase were systematically degazed and saturated with nitrogen in order to avoid or to limit the possible oxidation by oxygen during the elution and the results have always shown the appearance of additionnal peaks in equal number and quantity. For these reasons, it is logical to think that the compounds which appear during the analysis must proceed of an oxidation of 9-hydroxy ellipticine within the column.

In order to verify this hypothesis, the iminoquinone **2** has been synthetized according to the operating conditions previously described and analyzed by HPLC using the operating conditions mentionned above. It is reasonable to think that the peak which is attributable to the 9-oxo ellipticine is the major peak, the retention time of which is close to 3.3. Indeed, the 9-hydroxy ellipticine is colourless in solution and cannot, consequently, be detected in the visible range. Tables 1 to 4 show that most of the peaks which have been detected in the ultraviolet range are equally detected in the visible range (equal or very close retention time) : the detected compounds must be therefore highly coloured. However, the areas of the corresponding peaks are different, which shows that these compounds do not absorb in an equivalent way in the two ranges of the

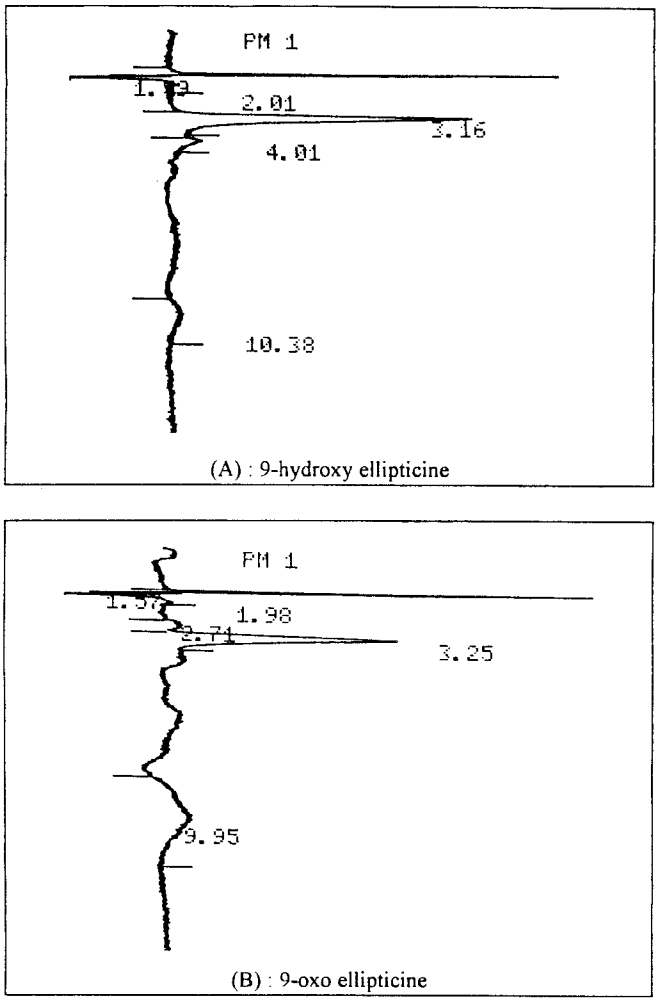


FIGURE 3 : Chromatograms of 9-hydroxy ellipticine (A) and 9-oxo ellipticine (B). Compounds are injected alone at a concentration of 5µg.ml^{-1} in a mobile phase methanol-water (75/25 v/v) containing 5.10^{-2} mol.l^{-1} ammonium acetate and are detected at λ = 493 nm.

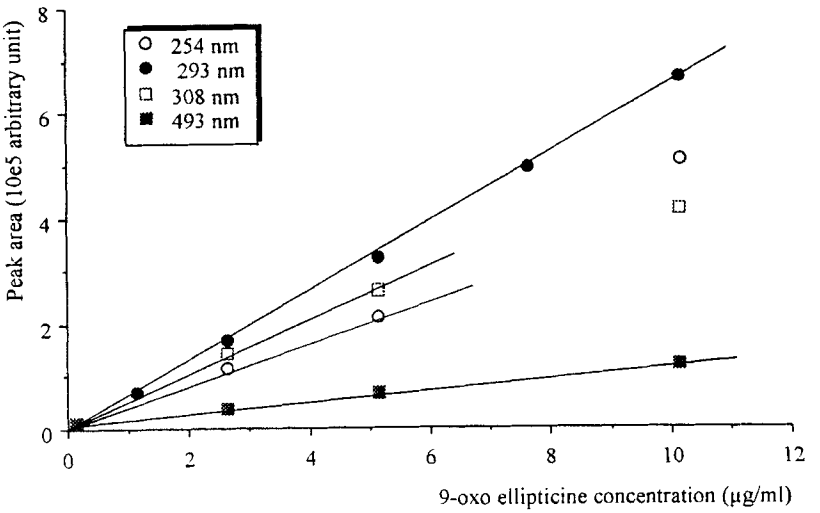


FIGURE 4 : Peaks areas expressed in arbitrary units versus 9-oxo ellipticine concentration ($\mu g.ml^{-1}$) at four different wavelenght (λ = 254, 293, 308 and 493 nm).

TABLE 4 : Retention time and area from peaks detected at λ = 493 nm.
(Results in bold type indicate the major peaks)

Compound	Concentration ($\mu g.ml^{-1}$)	Retention time (min)	Peak Area (%)	Total (%)
9-hydroxy ellipticine	2.5	2.02 **3.19**	36.2 **63.8**	100
	5	2.01 **3.16** 4.01 10.38	23.7 **64.3** 2.9 8.9	99.8
	10	1.99 **3.16** 4.04 12.1	12.3 **60.4** 6.9 20.3	99.9
9-oxo ellipticine	2.5	1.75 2.03 **3.3**	6.8 31.8 **61.3**	99.9
	5	1.98 2.71 **3.25** 9.95	12.4 3.1 **41** 43.4	99.9
	10	1.97 **3.3** 7.21	5.1 **61.1** 33.7	99.9

electronic absorption. In all cases (different concentrations of the injected solutions), the detected peaks in the visible range were found again in the ultraviolet range, especially that attributable to the 9-oxo ellipticine which absorbs in the ultraviolet range because of its aromaticity. However, a few very minor peaks one of which is attributable to the 9-hydroxy ellipticine, were not observed. The 9-hydroxy ellipticine therefore is transformed in totality during the elution using the operating conditions described above.

The presence of minor peaks indicates the formation of other compounds issued from the 9-oxo ellipticine or from the 9-hydroxy ellipticine. In a previous publication, the mechanism was precisely stated of the oxidation of the 9-hydroxy ellipticine in a methanol solution (22) :

- Oxidation of the 9-hydroxy ellipticine into 9-oxo ellipticine **2** (red) : the first step of this mechanism explains exactly the relative stabilizating power of an acidification of the mobile phase by adding ammonium acetate. Indeed, in an acidic medium, the phenolate intermediate ion is formed with much difficulty. But, the pH cannot be too acidic, because in this case, a destruction of the aromaticity, and, consequently, of the molecule to measure occurs (24). However, this addition of salts is not sufficient to avoid completely the appeareance of additionnal peaks and stop the oxidation of the 9-hydroxy ellipticine.
- Oxidation of **2** which yields the 9,10-dioxo ellipticine **3** (yellow).
- Formation of 9-hydroxy,10-methoxy ellipticine **4** (pink) by Michael addition of methanol on the cetoimine **2**.
- Formation of 9-oxo,10-methoxy ellipticine **5** (purple) by oxidation of **4**.
- Formation of 9-oxo,10,10-dimethoxy ellipticine **6** (orange) by Michael addition of methanol on **5**.

It is not surprising to find the additional peaks caused by the products issued of an oxidation of the 9-oxo ellipticine or of a Michael addition on the resulting products. The analyses were carried out with solutions of 9-hydroxy ellipticine or 9-oxo ellipticine with different concentrations and the variability of the results (number of peaks, areas) can be attributed to the modification of the oxidation power of the column and/or to the displacements of the redox equilibria and/or to the addition reactions, which are a function of the concentrations. At the three detection wavelenghts in the ultraviolet range that were used, the results of the analyses are quite different. This is not surprising since, for the compounds **2** to **6**, the aspect of the curves and their maxima of absorption in the ultraviolet range cannot be identical because of the different substitutes existing on the heterocyclic system of the ellipticine.

It is possible to determine quantitatively the 9-hydroxy ellipticine by HPLC with the condition to take into account the peak issued from the 9-oxo ellipticine, but also the peaks caused by the degradation of the 9-hydroxy ellipticine (determinable in the UV range) and the reaction products of the 9-oxo ellipticine (determinable in the UV and visible range). Taking into account all the peaks permits the obtention of the total mass of the products. However, for a comparative determination only one peak can be taken because the ε must be different according to the products. This result is corroborated by the analyses that were carried out on the same solutions of 9-hydroxy ellipticine or 9-oxo ellipticine by a method of colour measurement (22).

A 'column phenomenon' occurs during the determination of the 9-hydroxy ellipticine. The 9-hydroxy ellipticine oxidation during its chromatographic analysis is indeed impossible to avoid by the conventional means of stabilization : decreasing the pH, presence of nitrogen. The quantitative determination of the 9-hydroxy ellipticine is, in fact, a determination of 9-oxo ellipticine in solution.

This study can be concluded with the following points:

* The 9-hydroxy ellipticine is a compound of which the quantitative determination is impossible due to its aptitude to be oxidized within the chromatographic column.
* The stabilization with buffer salts which make the mobile phase acidic is not adapted to stabilize the 9-hydroxy ellipticine well enough.
* In return, if all the eluted peaks are taken into account, the quantitative determination can be realized.
* The major product eluted is the 9-oxo ellipticine.

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