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Note

Use of electrochemical detection in the high-performance liquid chromatographic determination of hydroxylated ellipticine derivatives

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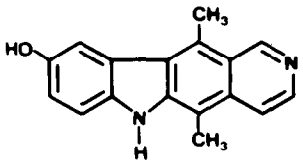
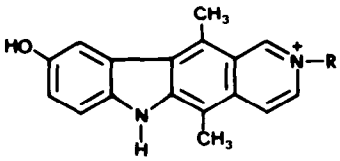
Ellipticine derivatives were first described as natural antitumour alkaloids [1, 2]. It was later shown that hydroxylation in position 9 (9-hydroxyellipticine, 9-OH-E) and quaternization of the pyridine nitrogen (N_2-methyl-9-hydroxyellipticinium acetate, 9-OH-NME) increase the antitumour activity (Fig. 1). This last compound (Celiptium®) is actually one of the most efficient anticancer drugs in this series and has been retained for clinical use in man [3, 4].



9-OH-E (1)



R = $-CH_3$: 9-OH-NME (2)

R = $-CH_2-CH_2-CH_3$: 9-OH-NPE (3)

Fig. 1. Chemical structure of ellipticines: 1 = 9-hydroxyellipticine (9-OH-E); 2 = N^2-methyl-9-hydroxyellipticinium acetate (9-OH-NME); 3 = N^2-propyl-9-hydroxyellipticinium acetate (9-OH-NPE).

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Up to now, a reversed-phase high-performance liquid chromatographic method using an internal standard (N^2-propyl-9-hydroxyellipticinium acetate, 9-OH-NPE) has been described [5]; detection was by ultraviolet (low sensitivity) or fluorescence (high sensitivity but requires extraction plus derivatization) measurement. It seemed of interest to develop a sensitive and specific detection method directly utilizable for biological samples without derivatization.

The 9-hydroxylated ellipticine derivatives are easily oxidized in a two electron process in *p*-quinone-imine structures [6, 7], for example by a peroxidase such as horseradish peroxidase in the presence of hydrogen peroxide. On the other hand, the one-electron electrochemical oxidation of 9-OH-E and 9-OH-NME has been described by Moiroux and Armbruster [8]. These two reactions are summarized in Fig. 2. We took advantage of this property to develop the electrochemical detection of the 9-hydroxylated ellipticines. Experimental conditions for the two compounds, 9-OH-E and 9-OH-NME, are discussed.

The proposed technique was used to carry out a pharmacokinetic investigation of Celiptium after intravenous infusion and compared with fluorescence detection.

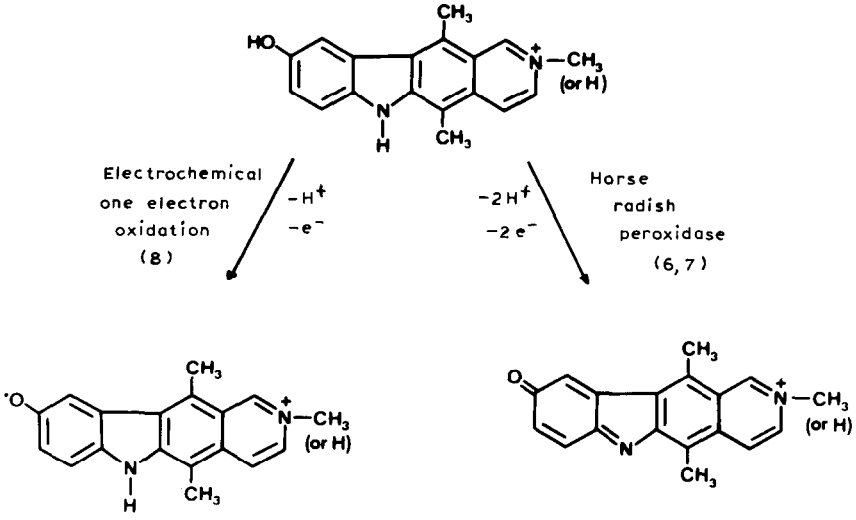

Fig. 2. Two reaction schemes for oxidation of 9-hydroxylated ellipticine.

MATERIALS AND METHODS

Chemicals and reagents

9-Hydroxyellipticine (9-OH-E), N^2-methyl-9-hydroxyellipticinium acetate (9-OH-NME) and N^2-propyl-9-hydroxyellipticinium acetate (9-OH-NPE) were provided by LABAZ Labs. (Sanofi Group, Sisteron, France). Aqueous solutions of the compounds were kept at 4°C for one month without any degradation.

9-OH-NPE was used as internal standard for the determination of 9-OH-NME.

Methanol was chromatographic grade (Merck, Darmstadt, F.R.G.); sodium tetraphenylborate, ammonium acetate and glacial acetic acid were obtained from Merck.

All mobile phases were filtered through a type FH 0.5-μm Millipore filter, and renewed every week.

Apparatus

The chromatographic system consisted of a high-pressure pump (solvent delivery system, Model 6000A, Waters Assoc., Milford, MA, U.S.A.), and an injection device (Model U6K, Waters Assoc.). The electrochemical detector comprised a Faraday cage enclosing a TL-5 glassy carbon electrode and an LC-4 controller, both from Bioanalytical Systems (West Lafayette, IN, U.S.A.). A Waters Assoc. μBondapak C_{18} reversed-phase column (30 X 3.9 mm I.D.; particle size 10 μm) was eluted isocratically at room temperature. Chromatograms were traced on a PE Servotrace recorder at a speed of 0.5 cm/min.

Procedure

The study of electrochemical detection raised two analytical problems: determination of the oxidative potential and optimization of the mobile phase.

The influence of the oxidative potential on the peak heights of 9-OH-NME, 9-OH-NPE and 9-OH-E was investigated by 0.05-V stepwise change of the electrode potential from 0.1 to 1.1 V. This study was performed using the mobile phase methanol—water (60:40) with 100 mmol/l ammonium acetate adjusted to pH 6 with glacial acetic acid, at a flow-rate of 1.5 ml/min.

In order to study the effects of pH, the mobile phase was adjusted to various pH values between 4.7 and 7.0 by decreasing the ratio of acetic acid. The influence of counter-ion concentration was investigated using increasing amounts of ammonium acetate from 10 to 250 mmol/l.

Preparation of biological samples

Blood samples were taken for a period of 7 h after administration of 160 mg of 9-OH-NME. They were collected in heparinized tubes and centrifuged at 1000 *g* for 5 min. Aliquots (200 μl) of plasma were spiked with 10 μl of 9-OH-NPE as internal standard (final concentration 100 ng/ml). Both drug and internal standard were extracted three times with ethyl acetate (3 X 1 ml) after addition of 5 μl of sodium tetraphenylborate (0.5%, w/v, final concentration) as counter-ion. For electrochemical determination, the combined upper organic phases were evaporated to dryness under a gentle stream of nitrogen and the residue was redissolved in 200 μl of mobile phase. For fluorimetric determination, we used the method described by Muzard and Le Pecq [5].

RESULTS AND DISCUSSION

Determination of oxidative potential

It is known from the literature [6] that 9-OH-NME is polarographically active. In Fig. 3, the relation is given between the potential applied to the glassy carbon electrode and the peak heights of 9-OH-NME, 9-OH-NPE and 9-OH-E. It can be seen that at a potential of about 600 mV, an adequate signal for the three compounds is obtained using the mobile phase methanol—water (60:40), with 100 mmol/l ammonium acetate, adjusted to pH 6 with acetic acid.

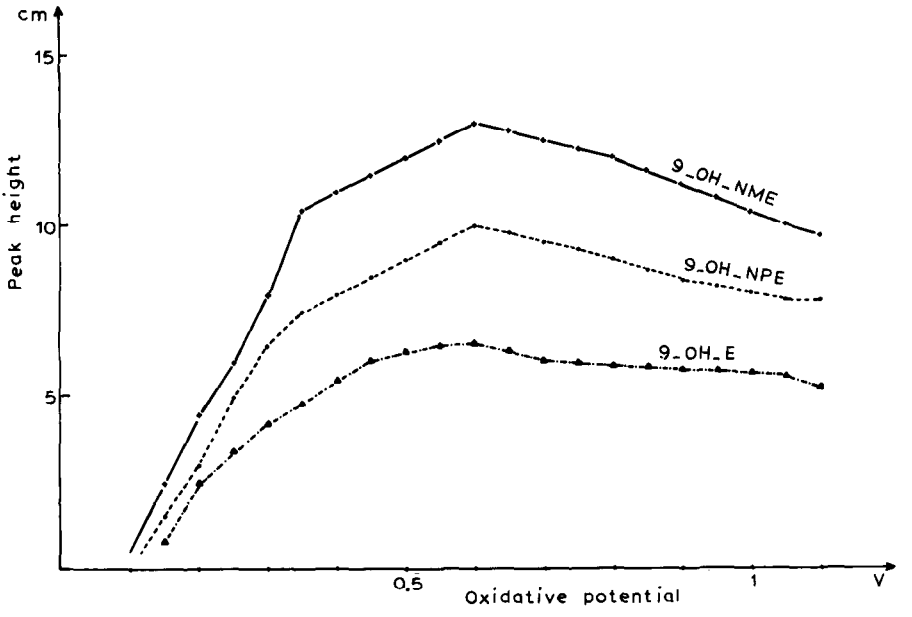

Fig. 3. Effect of oxidative potential on peak heights of different compounds.

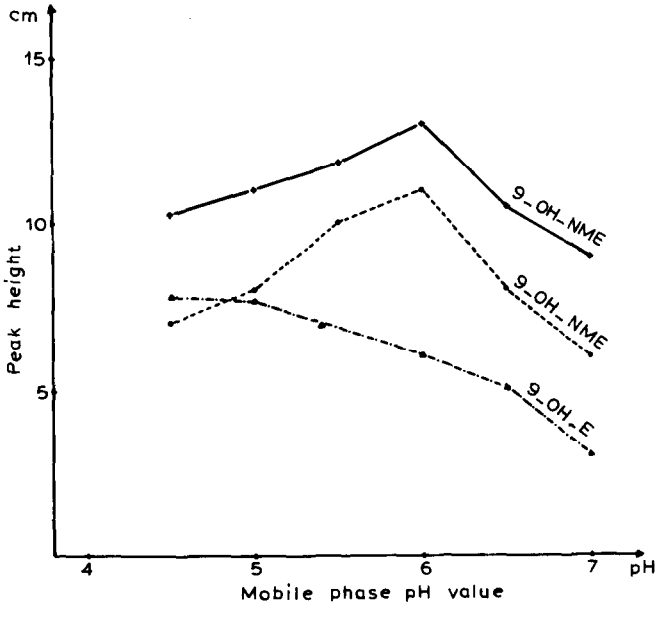

Fig. 4. Influence of pH of mobile phase on peak heights of different compounds.

Mobile phase composition

The peak heights of the three compounds were measured as a function of pH and ammonium acetate content of the mobile phase (Figs. 4 and 5). In the pH range 4.7—7.0, detection efficiencies for 9-OH-NME and 9-OH-NPE were maximum between 5.7 and 6.2, while that of 9-OH-E was between 4.5 and 5.

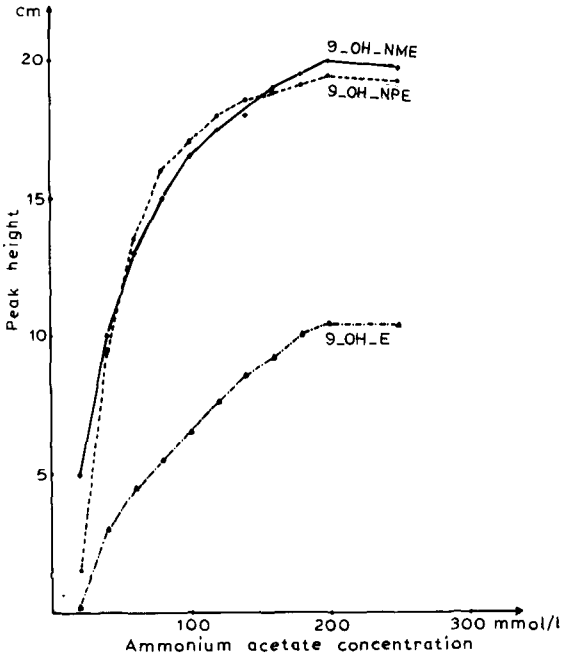

Fig. 5. Influence of counter-ion concentration on peak height of the three compounds.

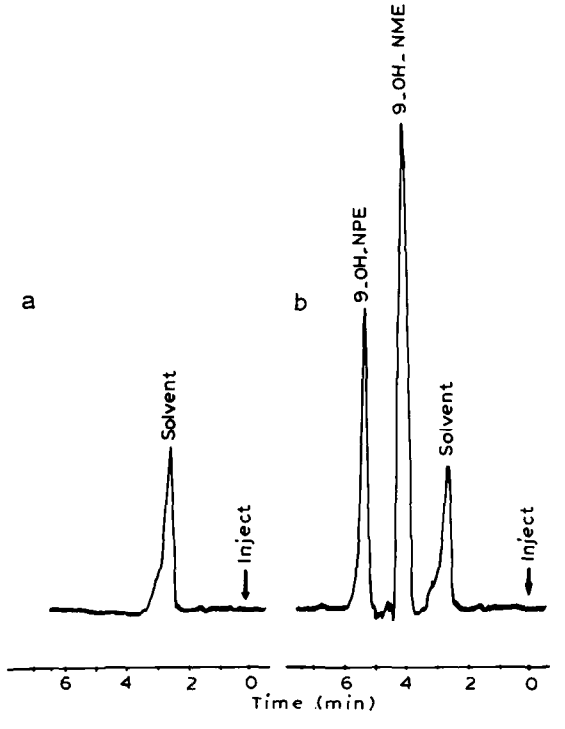

Fig. 6. Representative chromatograms of (a) a blank plasma extract, (b) an extract of plasma from a patient (adding 9-OH-NPE at 100 ng/ml of plasma) 1 h after administration of 160 mg of 9-OH-NME. Calculation from this chromatogram gave the original plasma concentration as 187 ng/ml.

At these pH values, addition of ammonium acetate has a beneficial effect on the efficiency of detection. It seems that a concentration of 200 mmol/l optimized the peak heights of the three compounds.

For determination of 9-OH-NME with use of 9-OH-NPE as internal standard, the optimal composition of the mobile phase was found to be methanol—water (60:40), 200 mmol/l ammonium acetate, adjusted to pH 6.0 with glacial acetic acid. In these conditions, the retention times of 9-OH-NME and 9-OH-NPE were 3.6 ± 0.2 min and 5.5 ± 0.2 min, (n = 50), respectively, Fig. 6a shows the chromatogram of a control plasma sample while Fig. 6b represents a chromatogram of a plasma sample containing 187 ng/ml 9-OH-NME. There was an absence of interfering peaks in control plasma for all subjects examined.

Calibration, linearity, sensitivity, reproducibility

Calibration curves, determined every day, were obtained by analysing spiked plasma from healthy volunteers with various concentrations of 9-OH-NME. They were linear over the range 30 ng/ml to 1 μg/ml. These calibration curves were used for calculating the 9-OH-NME concentration in samples from patients by the internal standard method.

The lowest amount of 9-OH-NME detectable, defined as five times the noise level, was 250 pg. This amount corresponds, for the 10 μl injected into the loop column, to a concentration of 25 ng/ml.

A serum spiked with 100 ng/ml 9-OH-NME was always assayed, along with plasma extracts, to check the status of the chromatographic system, after addition of 100 ng/ml 9-OH-NPE as internal standard. Injections of 10 μl were

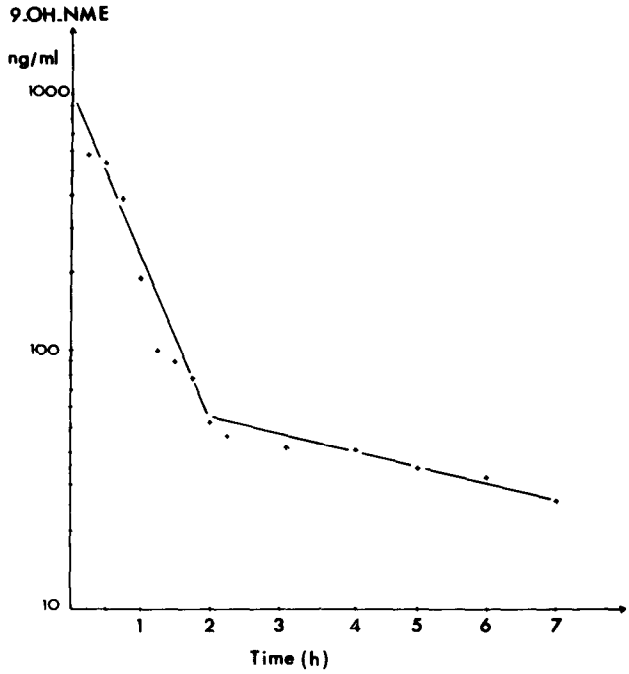

Fig. 7. Concentration of 9-OH-NME as a function of time obtained by analysis of plasma samples from a patient who had received 160 mg of 9-OH-NME.

made three times a day and over a period of ten days. The coefficients of variation were 1.5% and 4.8%, respectively.

Preliminary clinical application

The method described was tested and compared with fluorimetric detection on blood samples from a patient suffering from a metastatic breast carcinoma and receiving a 60-min infusion of 100 mg/m^2 9-OH-NME. Fig. 7 shows the plasma concentration—time course. The decline of blood level appears to be biphasic as described by Gouyette et al. [9]. The results obtained for fluorimetric and electrochemical detection were well correlated (r = 0.997).

The principle of the method could be potentially applicable to all ellipticine derivatives bearing a preserved phenolic function (i.e. glutathione or cysteine adducts recently observed in human and animal bile and urine [10, 11]).

ACKNOWLEDGEMENT

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