Journal of Pharmaceutical & Biomedical Analysis
Vol. 5, No. 4, pp. 341–351, 1987
Printed in Great Britain

0731–7085/87 $3.00 + 0.00

Oxidative biotransformation of the antitumour agent elliptinium acetate: structural characterization of its human and rat urinary metabolites

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Abstract: The electrophilic properties of the antitumour drug N^2-methyl-9-hydroxy-ellipticinium acetate (Celiptium®) are revealed by the detection of thiol-conjugate metabolites in man and rat urine. Besides the unchanged drug and its glucuronide, the cysteinyl- (in man) and the *N*-acetylcysteinyl- (in man and rat) conjugates have been unambiguously characterized using NMR, UV and mass spectral data. The urinary excretion profile exhibits total excreted products of 21% (in man) and 9% (in rat) with respect to the administered dose. The unchanged drug is found to be the major excreted compound from urine in both species (17% in man, 6.3% in rat); whereas the glucuronide (2.6% in man, 1.5% in rat), cysteinyl- (1.3% in man) and *N*-acetylcysteinyl- (0.2% in man, 1.2% in rat) conjugates represent the minor excreted compounds. The presence of the latter thio-conjugates provides an indirect proof of the *in vivo* generation of an oxidized intermediate form of the administered drug.†

Keywords: *Elliptinium acetate; antitumour drug; identification of urinary human and rat metabolites; oxidative biotransformation.*

Introduction

Elliptinium acetate (Celiptium®, NSC 264137, N^2-methyl-9-hydroxyellipticinium acetate, NMHE, Fig. 1) is a new antitumour agent in the ellipticine series presently undergoing clinical trials [1, 2]. NMHE has exhibited a favorable clinical activity mainly against osseous metastases of breast cancer [2].

As for many other antitumour agents, the mechanism of action of this drug is far from being completely understood. Like other ellipticine derivatives, elliptinium acetate

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†The abbreviations used in this paper are given in the Appendix.

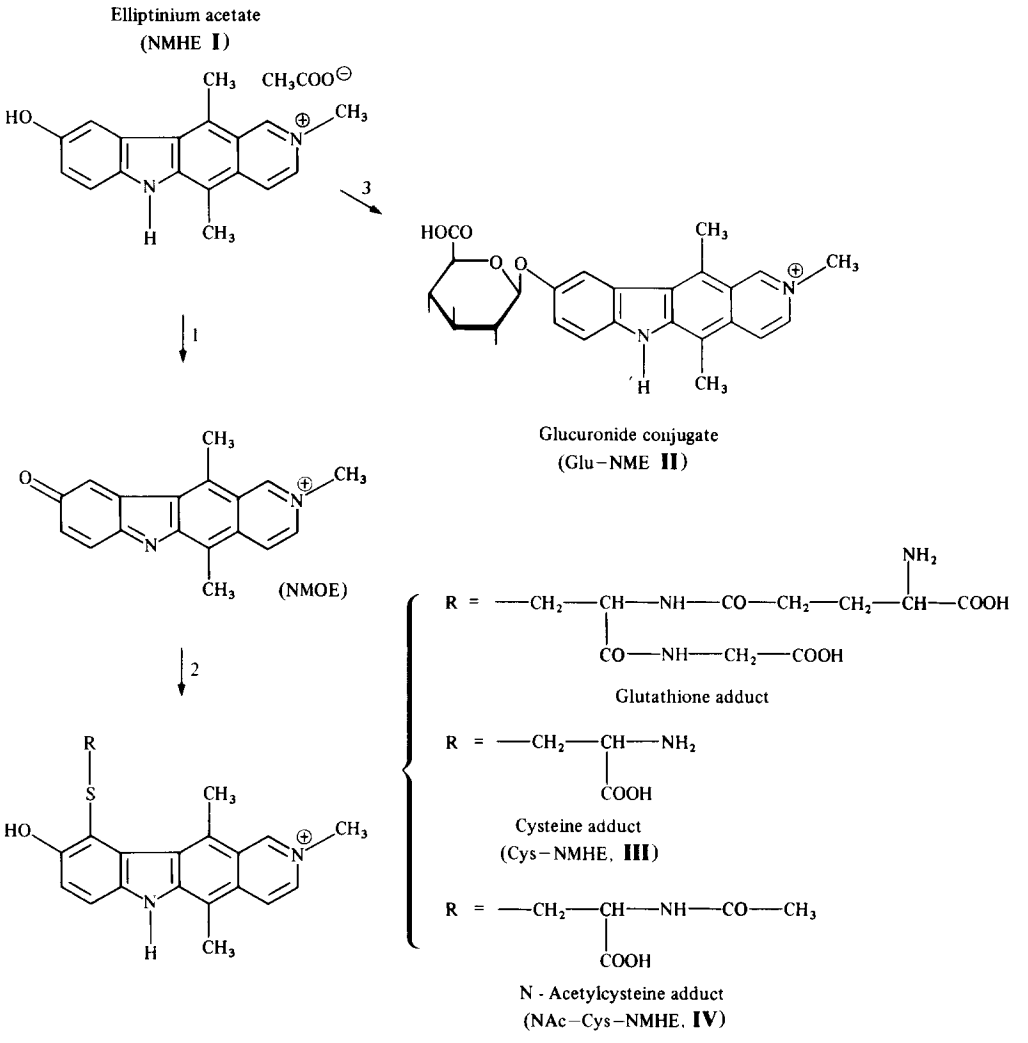


Figure 1
Synthesis of reference elliptinium derivatives. (1) HRP/H_2O_2; (2) RSH; (3) for the chemical synthesis, see ref. 10.

exhibits a strong affinity for DNA [3]. In addition, the presence of an hydroxyl group in the *para* position to the indolic N–H means that this molecule is easily oxidized by a peroxidase in the presence of hydrogen peroxide [4, 5]. The quinone–imine N^2-methyl-9-oxoellipticinium (NMOE) formed behaves as a strong electrophile capable of reaction with various nucleophiles to give adducts after fixation of hetero-atoms, N, S or O at position 10 (Fig. 1) [6–8].

Thus the activity of NMHE might be related to a possible alkylation *in vivo* of biological nucleophiles by this electrophilic intermediate NMOE, assuming that a preliminary biochemical oxidation product of the elliptinium acts as a proalkylant.

Among nucleophiles containing a S–H bond, three different adducts have been prepared via this biosynthetic route, which might be formed *in vivo* as detoxification

conjugates of the quinone–imine NMOE, namely the glutathionyl-, cysteinyl- and *N*-acetylcysteinyl–elliptinium adducts [see Fig. 1 and refs 6, 9 and 10 for preparations].

From preliminary studies on the biliary metabolism of elliptinium acetate in rat it is known that the O-glucuronide elliptinium conjugate is the main metabolite, whereas the glutathionyl–elliptinium appears as a detoxification conjugate [11]. Following this first indication of a "biooxidative alkylation" pathway for elliptinium acetate, at least as a detoxification process of the *in vivo* generated quinone–imine NMOE, it has been possible to observe cysteinyl–elliptinium conjugates in human and rat urines [12]. In that primary study, the human and rat urinary metabolites were only identified by their chromatographic behaviour. In the present work, their isolation and spectroscopic identification are described. The quantitative urinary elimination of the parent drug and its metabolites in man and rat also are reported.

Materials and Methods

Chemicals

NMHE or its formulation (Celiptium®) were provided by SANOFI Company (Paris). Other chemicals used were purchased from Prolabo (Paris). Reference compounds Cys–NMHE, NAc–Cys–NMHE, Glu–NME and GS–NMHE were prepared as previously described [6, 9, 10].

Animal studies

NMHE was administered as an i.v. bolus mode (4 min) into the caudal vein of four rats (male Sprague–Dawley, 200–250 g, Charles River, France) at a dose of 10 mg/kg in sodium acetate buffer 0.1 M (pH 5.0) for quantitation of urinary excretion, and to twenty rats for the isolation and characterization of the metabolites. Urine was collected quantitatively for various periods of time up to 96 h after drug administration and was kept frozen (−20°C) until analysis.

Administration and urine collection in patients

The study was carried out on two male (SB, CE) and one female (BS) patient (ranging in age from 57 to 73 years) admitted to the Claudius Regaud Centre (Toulouse) with clinical evidence of histologically confirmed malignant disease. They were treated for a clear cell renal adenocarcinoma (SB), a prostate adenocarcinoma (CE) and a parotid cylindroma (BS); the first two patients revealed bone metastasis and the last one liver metastasis. The patients after giving their informed consent received Celiptium as first-line treatment for their disease. The Celiptium (100 mg/m^2 = 200 mg SB , 170 mg CE and 145 mg BS) was dissolved in 250 ml of sterile isotonic 5% m/v glucose solution and infused i.v. at a constant rate over 1 h. During the first course, for each patient, whole urine was collected for various periods of time up to 42 h after drug treatment and was kept frozen (−20°C) until analysis.

High-performance liquid chromatography (HPLC)

HPLC analyses were performed as described in the preliminary study [12]. Urine samples (1–10 μl) were injected directly onto analytical columns without any additional pretreatment to avoid the loss of hydrophilic elliptinium metabolites associated with solvent extraction [10].

Semi-preparative HPLC was carried out on a μ-Bondapak C_{18} (30 cm × 7.8 mm i.d.)

column protected by a μ-Bondapak guard column. Isocratic elution was performed with methanol–5 mM ammonium acetate–acetic acid buffer (40:59.8:0.2, v/v/v) at a flow rate of 1.2 ml/min.

The excreted compounds were quantified by means of a calibration curve (HPLC peak areas versus concentration) obtained by analysing spiked control urine (human or rat) with various concentrations of NMHE or reference compounds Cys–NMHE, NAc-Cys–NMHE and Glu–NME. The calibration plots were linear in the range 0.1–10 μg/ml. The lowest detectable amounts of NMHE, Cys–NMHE, NAc–Cys–NMHE or Glu–NME, defined as five times the noise level, were in the range 1–5 ng. These amounts correspond, for the 10 μl injected into the loop column, to a concentration of 0.1–0.5 μg/ml. Urine samples spiked with 5 μg/ml NMHE, Cys–NMHE, NAc–Cys–NMHE or Glu–NME were assayed to check the status of the chromatographic system. Injections of 10 μl were made three times a day, over a period of ten days. Measured deviations were always below 5% of the indicated values.

Isolation of sulphur-containing metabolites III *and* IV *from human urine and* IV *from rat urine*

In a preliminary step, urine samples were purified on Sep–Pak C_{18} cartridges, prewashed with methanol and conditioned with water prior to use. Human urine (collected for the 15–20 h period after the drug treatment of patient SB, total volume 700 ml) was introduced as 3 ml aliquots onto the Sep–Pak cartridges. Each cartridge was washed with 5 ml of water and then eluted with a step gradient of methanol–water in order to separate the individual retained metabolites. The gradient consisted of successive 5 ml volumes containing 20, 25, 30, 35, 40, 50 and 100% of methanol. Only 20 cartridges were used for this purification procedure, each one being recycled 10–15 times after careful washing with methanol and then water. Detection of metabolites during Sep–Pak treatment was monitored by analytical HPLC. A similar procedure was used for rat urine (6–24 h pooled fractions from 20 rats, total volume 150 ml). The metabolite II was eluted with the 30–35% aqueous methanol, III and IV with the 40–45% aqueous methanol and the unchanged drug I with 50% aqueous methanol. All the fractions from the same gradient were pooled. After removal of the methanol under vacuum and water by lyophilization, each crude metabolite was purified by semi-preparative HPLC (*vide supra*). At the end, each metabolite was purified on a LH20 gel (Pharmacia) chromatography column (10 cm × 15 mm i.d.) with water as the eluent followed by lyophilization.

Proton magnetic resonance spectrometry

^{1}H-NMR spectra were obtained at 250 MHz with a Bruker WM 250 instrument. The chemical shifts are expressed in ppm downfield from sodium 4,4-dimethyl-4-silapentane sulphonate (DSS) used as external standard. Gated decoupling conditions were used to suppress the HDO signal.

^{1}H-NMR spectrum (10,000 scans) in D_2O at 296 K of human metabolite III (Cys–NMHE): 9.22 (*s*, 1*p*, H_1); 7.90 (*d*, 1*p*, H_3 or H_4); 7.84 (*d*, 1*p*, H_4 or H_3); 7.23 (*d*, 1*p*, H_7 or H_8); 7.12 (*d*, 1*p*, H_8 or H_7); 4.33 (*s*, 3 *p*, $\dot{N}$–Me); 3.73 (*m*, 1*p*, H_αCys); 3.20 (*m*, 1*p*, H_βCys); 3.07 (*s*, 3*p*, Me_{11}); 2.38 (*s*, 3*p*, Me_5); 2.03 (*s*, 3*p*, OAc^-).

^{1}H-NMR spectrum (13,000 scans) in D_2O at 296 K of human metabolite IV (NAc–Cys–NMHE): 9.51 (*s*, 1*p*, H_1); 8.25 (*d*, 1*p*, H_3 or H_4); 8.18 (*d*, 1*p*, H_4 or H_3); 7.31 (*d*, 1*p*, H_7 or H_8); 7.18 (*d*, 1*p*, H_7 or H_8); 4.49 (*s*, 3*p*, N^+–Me); 3.42 (*m*, 1*p*, H_αCys);

3.25 (*s*, 3*p*, Me_{11}); 2.83 (*m*, 1*p*, $H_\beta Cys$); 2.61 (*s*, 3*p*, Me_5); 2.02 (*s*, 3*p*, OAc^-); 0.90 (*s*, 3*p*, CO–Me).

^{1}H-NMR spectrum (10,600 scans) in D_2O at 323 K of rat metabolite IV (NAc–Cys–NMHE): 9.46 (*s*, 1*p*, H_1); 8.25 (*d*, 1*p*, H_3 or H_4); 8.15 (*d*, 1*p*, H_4 or H_3); 7.30 (*d*, 1*p*, H_7 or H_8); 7.17 (*d*, 1*p*, H_7 or H_8); 4.50 (*s*, 3*p*, N^+–Me); 3.52 (*m*, 1*p*, $H_\alpha Cys$); 3.25 (*s*, 3*p*, Me_{11}); 2.84 (*m*, 1*p*, $H_\beta Cys$); 2.65 (*s*, 3*p*, Me_5); 1.93 (*s*, 3*p*, OAc^-); 0.99 (*s*, 3*p*, COMe).

Mass spectrometry

A Ribermag 10–10 spectrometer was used for DCI^+ mass spectra (NH_3 as reactant gas).

Ultraviolet–visible spectroscopy

Spectra of elliptinium-conjugates were recorded on a Beckman Acta III instrument with water as solvent. For reference thio-compounds, ϵ in $cm^{-1}mol^{-1}$ were, respectively 3000, 5500, 32,200, 18,000 and 17,100 at 460, 386, 324, 284 and 250 nm, respectively (Cys–NMHE), 3000, 5450, 32,200, 18,200 and 17,300 at 460, 386, 324, 284 and 250 nm, respectively (NAc–Cys–NMHE).

Results

Identification of human urinary metabolites

For each patient, the urine (pooled for the 0–42 h period) was analysed by HPLC without prior extraction by organic solvents. In each case, four ellipticine derivatives were detected. Besides the unchanged drug (I) and the glucuronide conjugate (II), two other products (III and IV) were identified as thiol-conjugates by comparison of the retentions with those of authentic samples. These two compounds are the Cys–NMHE and NAc–Cys–NMHE, respectively. Because the cysteinyl and glutathionyl elliptinium adducts are co-eluted on the 10μ Waters column, the distinction between these two products was effected by means of HPLC on a 5μ Altex column. However, to avoid any ambiguity about the chemical structures, these metabolites were isolated from one patient's (SB) urine sample, purified then characterized by spectroscopic methods: UV–visible, mass and ^{1}H-NMR.

The glucuronide conjugate II effectively gave the parent elliptinium drug I after treatment by β-glucuronidase and was further identified spectroscopically using a reference sample of Glu–NME (the chemical synthesis and the β-glucuronidase treatment are described in ref. 10).

The two thiol metabolites III and IV also were identified by UV–visible, mass and ^{1}H-NMR spectra comparison with reference compounds prepared by biochemical synthesis (preparations described in ref. 6). The UV–visible spectra are compared with those of synthetic Cys–NMHE and NAc–Cys–NMHE in Fig. 2. Likewise the mass spectral data (Fig. 3) show that fragmentations for each metabolite are in good agreement with those of the reference compounds: in particular the fragmentation observed at *m/z* 309 and 323 confirms that cysteine residues are linked by the sulphur atom to the ellipticine skeleton in both cases. The ^{1}H-NMR spectra of metabolites III and IV (Figs 4A and 4B) unambiguously identified these conjugates as Cys–NMHE and NAc–Cys–NMHE, respectively. The absence of H_{10} resonance in both spectra confirms that the cysteinyl moiety is attached to the elliptinium molecule at carbon 10.

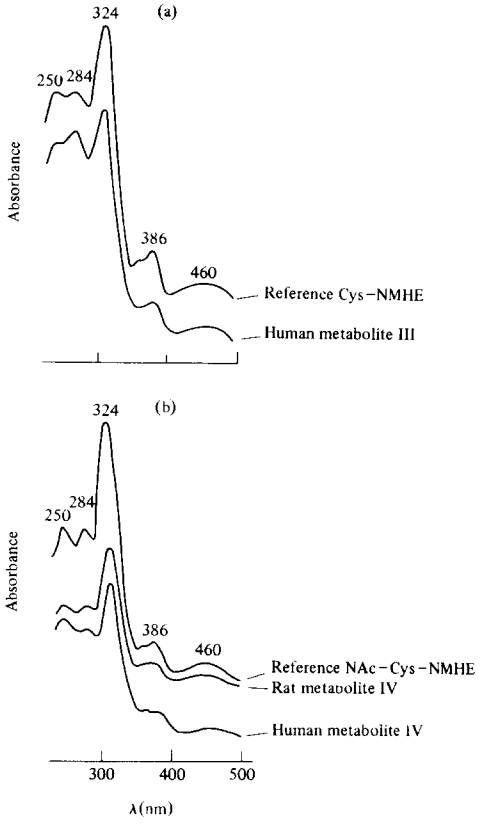


Figure 2
UV–visible spectra of metabolites III (human) (A) and IV (human and rat) (B) compared with reference compounds Cys–NMHE and NAc–Cys–NMHE.

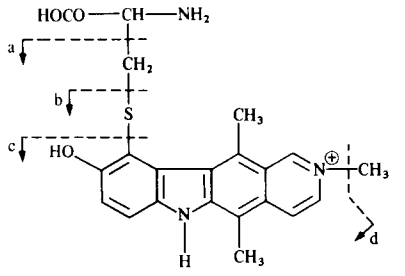
Identification of rat urinary thiol-conjugate

HPLC analyses of urine from the four rats treated with elliptinium acetate indicated the presence of three elliptinium derivatives of which compounds I and II were identified as the parent drug NMHE and the glucuronide Glu–NME, respectively. To confirm the molecular structure of metabolite IV, it was isolated and purified as described earlier. The UV–visible spectrum of this metabolite shows the same absorption maxima at 250, 284 and 324 nm as the reference compound NAc–Cys–NMHE (see Fig. 2B). The mass spectrum exhibits the same fragmentation pattern as NAc–Cys–NMHE (see Fig. 3B). Peaks at 309 and 323 indicate that the cysteinyl residue is attached by the sulphur atom to the elliptinium moiety. ^{1}H-NMR spectrum (see Fig. 4C) confirms that the sulphur–carbon linkage occurs at carbon 10.

Quantification of urinary elimination

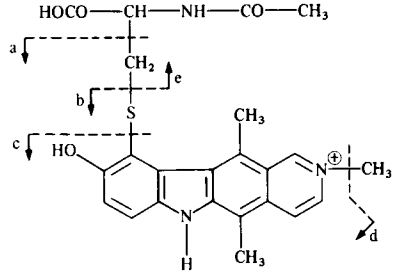
Human urinary excretion. The four excreted compounds, namely, the unchanged drug, the glucuronyl, the cysteinyl- and the *N*-acetylcysteinyl–elliptinium conjugates were determined by HPLC. Cumulative excretion data recovered in the 0–42 h period from one patient (BS) represents 21% of the administered dose (Fig. 5A). The unchanged drug is the major compound excreted in urine (17%), whereas the glucuronide-conjugate, the cysteinyl- and the *N*-acetylcysteinyl-conjugates represent, respectively 2.6%, 1.3% and 0.2% of the initial dose. The unchanged drug excretion for the two other patients was 23% (SB) and 13% (CE), respectively.

(a)



Ion	Reference	Human
m/z	Cys–NMHE*	metabolite III*
M+	—	—
a	323 (12.5%)	323 (16%)
b	309 (25%)	309 (16%)
c	277 (95%)	277 (100%)
d	263 (100%)	263 (65%)

(b)



Ion	Reference	Human	Rat
m/z	NAc–Cys–NMHE*	metabolite IV*	metabolite IV*
M+	—	—	—
a	323 (25%)	323 (15%)	323 (3%)
b	309 (30%)	309 (30%)	309 (5%)
c	277 (80%)	277 (57%)	277 (20%)
d	263 (100%)	263 (100%)	263 (45%)
e	130 (30%)	130 (40%)	130 (100%)

Figure 3
(A) Mass spectral data for human metabolite III and reference Cys–NMHE; (B) mass spectral data for human and rat metabolites IV and reference NAc–Cys–NMHE. * numbers represent ion mass and ion intensities in parentheses.

Rat urinary excretion. Three excreted compounds were quantified in rat urine (based on four animals) during a collection period of 96 h, namely, the unchanged drug, its glucuronide and the *N*-acetylcysteine conjugate. Total excretion in the 0–96 h period reached 9.0 ± 1.2% of the administered dose (Fig. 5B). Among these urinary metabolites, the unchanged drug is the major excreted compound: 6.3 ± 0.8% of the dose. The glucuronide and the *N*-acetylcysteine conjugate are only minor metabolites; 1.5 ± 0.3% and 1.2 ± 0.2% of the administered drug, respectively.

Discussion

Recent studies of the biochemical oxidation of elliptinium acetate directed attention to the possible drug transformation *in vivo* to an electrophilic quinone–imine intermediate [4–6]. This hypothesis was supported by the identification of a glutathione–elliptinium adduct in rat bile [11] (for a recent review on glutathione detoxification of anticancer

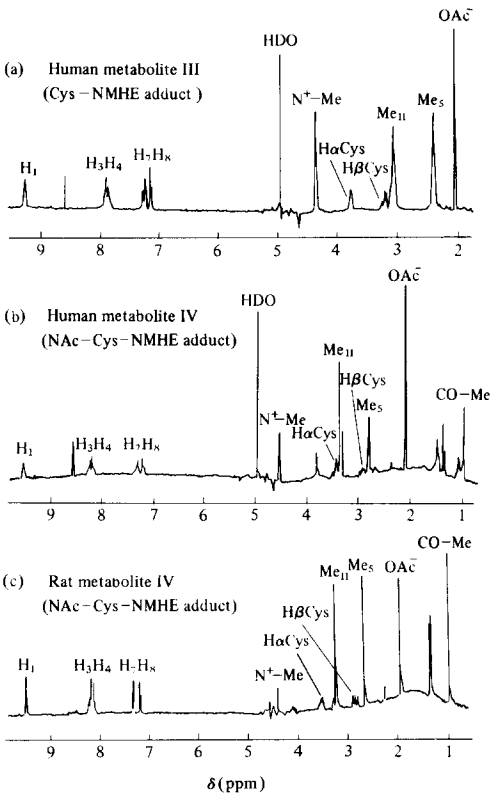


Figure 4
^{1}H-NMR spectra of: (A) human metabolite III; (B) human metabolite IV; (C) rat metabolite IV.

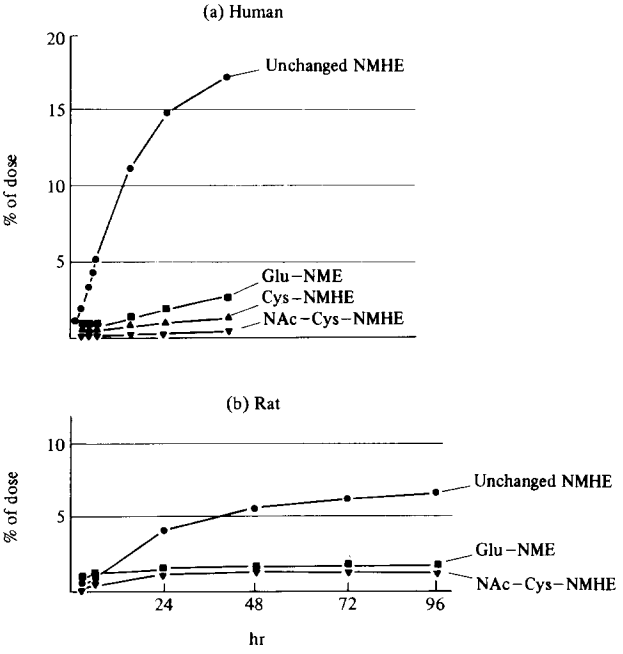


Figure 5
Cumulative excretion of ellipticinium acetate and its metabolites in human (A) and rat (B) urines.

drugs, see ref. 13). Consequently urinary metabolism studies on patients and animals treated with elliptinium acetate were undertaken in order to detect S-conjugates in urine samples and to provide further evidence for the biooxidative activation of the administered drug. Because of the present availability of the possible S-conjugates, such a study was more feasible than previous work on the pharmacokinetics and metabolism of the drug [14, 15].

The isolation and spectral evidence confirm completely that, besides the unchanged drug, three metabolites are present in human urine, namely, Glu–NME(II), Cys–NMHE(III) and NAc–Cys–NMHE(IV). Since glutathione conjugates are easily degraded into cysteine conjugates by peptidases [16], the cysteinyl–elliptinium conjugates are expected to be found in urine (for a recent commentary on renal processing of glutathione conjugates, see ref. 17). In human urine, the two possible cysteine derivatives are found viz., Cys–NMHE and its *N*-acetylated form. The formation of mercapturic acid derivatives in the kidney is known to be catalyzed by the specific enzyme cysteine S-conjugate *N*-acetyltransferase [18].

In rat urine, three elliptinium compounds are present viz., the parent drug I, its glucuronide II and the S-conjugate NAc–Cys–NMHE. The absence of the non-acetylated cysteine conjugate can easily be understood since rats are rapid acetylators [19]. The *N*-acetylation function is known to depend on the species and within the same species age-related changes have also been observed [20].

Concerning the ratio of different elliptinium derivatives excreted in human urine, the unchanged drug represents the main product: 81% of the total of four identified compounds excreted in urine corresponding to a 17% recovery of the administered dose (patient BS); the unchanged drug excretion for the three patients was 18 ± 5%; this result agrees with those already published [15]. The amount of glucuronide-conjugate is in the same order of magnitude as the other urine metabolites (12% of excreted elliptinium derivatives). More remarkable is the presence of two S-conjugated metabolites viz., the cysteinyl- (6%) and *N*-acetylcysteinyl (1%) elliptinium adducts. These two metabolites can be regarded as proof of the biooxidation of elliptinium acetate to an electrophilic derivative, the quinone–imine NMOE, which can bind to macromolecules *in vitro* [21, 22] or be detoxified by addition of a S–H containing peptide like glutathione. In fact, this primary detoxification metabolite has been previously identified in rat bile [11]. Here, two S-conjugates of NMHE were identified in human urine; they result from activities of enzymes that catalyse mercapturic acid formation from glutathione-adduct, namely, γ-glutamyltranspeptidase, aminopeptidases and *N*-acetyl transferase.

In the case of rat urine, we notice that the unchanged drug also is the main excreted elliptinium derivative (70% of excreted elliptinium compounds) unlike the excretion in rat bile [10, 11]. The glucuronide and the *N*-acetylcysteine conjugate are in the same range (17% and 13% of excreted products, respectively). Since the glutathione–elliptinium adduct has been identified as a rat biliary metabolite, it is not surprising to find one of its metabolites in urine (for the discussion on the absence of the cysteine–elliptinium adduct in rat bile, *vide supra*).

In conclusion, two further points can be mentioned:

(i) the unchanged drug is the main excreted compound in human and rat urine and its glucuronide conjugate is also detected;

(ii) two S-conjugates, the cysteinyl- and *N*-acetylcysteinyl–elliptinium, are present in human urine, whereas the latter one is only observed in rat urine.

So, this identification of S-conjugates of elliptinium provides a further indirect proof of the *in vivo* generation of the oxidized form of the antitumour agent elliptinium acetate, usually known for its DNA intercalating properties [3]. Consequently, elliptinium acetate probably presents a plurimodal mechanism of action in which, besides intercalating properties or radical formation [23], a "biooxidative alkylation" process might be involved in the explanation of its antitumour activity.

In order to establish if such a concept can be developed or not, the covalent binding of the oxidized form of the drug to RNA and DNA *in vitro* [22, 24] and also to RNA and DNA extracted from L1210 cells incubated with 0.1 μM of elliptinium acetate [25] has been obtained (this concentration corresponds to the $i.d._{50}$ value of the drug, see ref. 26 for preclinical data on the cytotoxicity and the antitumour activity of elliptinium acetate).

The authors are currently working on the role of the covalent binding of elliptinium acetate to nucleic acids and the possible relationship between the alkylation of intracellular macromolecules and the cytotoxicity.

Acknowledgements: We thank B. Dugue for the preparation of Glu–NME and R. Rao for ^1H nmr spectroscopic measurements. The authors are also indebted to the Sanofi Company for financial support.

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[First received for review 31 January 1986; revised manuscript received 21 April 1986]

Appendix

Abbreviations used are: NMHE, N^2-methyl-9-hydroxyellipticinium acetate or elliptinium acetate; NMOE, N^2-methyl-9-oxo-ellipticinium; GSH, glutathione; Glu–NME, N^2-methyl-9-(O)β-D-glucuronylellipticinium; GS–NMHE, N^2-methyl-9-hydroxy-10-(S)-glutathionylellipticinium; Cys–NMHE, N^2-methyl-9-hydroxy-10-(S)-cysteinylellipticinium; NAc–Cys–NMHE, N^2-methyl-9-hydroxy-10-(S)-*N*-acetylcysteinylellipticinium; HPLC, High-performance liquid chromatography; DCI^+, positive desorption chemical ionization; HRP, horseradish peroxidase.