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Gradient high-performance liquid chromatographic assay for the determination of the novel indoloquinone antitumour agent E09 in biological specimens

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ABSTRACT

A high-performance liquid chromatographic method for the determination of the novel indoloquinone antitumour agent E09, 3-hydroxymethyl-5-aziridinyl-1-methyl-2-(1*H*-indole-4,7-dione)prop-β-en-α-ol, in mouse plasma and urine is described Following protein precipitation by means of methanol (2 volumes), separation and quantification of parent drug, metabolites and internal standard E012 (5-morpholine substituted analogue) were achieved on a 5-μm Resolve C_{18} Rad-Pak with a 15-min linear gradient of 10–30% acetonitrile in a 0 02 *M* pH 7 4 sodium phosphate buffer with UV detection at 280 and 310 nm. The utility of the assay is also demonstrated for the aziridine ring-opened analogue E05A, 3-hydroxymethyl-5-β-hydroxyethylamino-2-(1*H*-indole-4,7-dione)prop-β-en-α-ol. Plots of area ratios of analytes *versus* internal standard were linear in the range 50–15 000 ng/ml The detection limit for indoloquinones in plasma was *ca.* 30 ng/ml The within-assay and day-to-day variation were consistently lower than 12.5%. The assay was applied in preliminary pharmacokinetic investigations One minor metabolite of E09 could be identified, further metabolites were characterized by ultraviolet–visible spectra.

INTRODUCTION

Hypoxic cells in solid tumours are considered to limit their curability by radiotherapy and chemotherapy [1]. In 1972, it was hypothesized that these cells might have a greater capacity for reductive processes compared to normal well oxygenated cells [2]. This proposal gave rise to the development of so-called bioreductive drugs which exhibit selective cytotoxicity towards hypoxic cells. Three classes are presently known: (a) nitroimidazoles, such as misonidazole, pimonidazole (Ro 03-8799) and etanidazole (SR 2508) [3]; (b) benzotriazine di-N-oxides, *e.g.* SR 4233 [4]; and (c) quinone-type agents, such as the naturally occurring mitomycin C and daunorubicin [5].

Recently, a family of novel indoloquinones designed to form alkylating intermediates under bioreductive conditions has been synthesized [6]. On the basis of succesful *in vitro* and *in vivo* antitumour activity E09, 3-hydroxymethyl-5-aziridinyl-1-methyl-2-(1*H*-indole-4,7-dione)prop-β-en-α-ol, was selected by the EORTC New Drug Development Committee for clinical studies [7,8].

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In order to obtain an insight into the pharmacokinetics of this drug and to optimize the schedule of administration during the planned Phase I studies, a sensitive, rapid and readily applicable assay for the detection of drug and metabolites in body fluids was required. We therefore developed a gradient high-performance liquid chromatographic (HPLC) technique with ultraviolet (UV) detection for the quantification of E09 in biological specimens employing the indoloquinone E012, 3-hydroxymethyl-5-morpholino-1-methyl-2-(1*H*-indole-4,7-dione)prop-β-en-α-ol, as internal standard (structures are given in Fig. 1). The proposed metabolite E05A, 3-hydroxymethyl-5-β-hydroxyethylamino-2- (1*H*-indole-4,7-dione)prop-β-en-α-ol (Fig. 1), resulting from the hydrolysis of the aziridine ring of E09 was also included in our investigations. A further intention of this work was to provide information on the structure of observed bioconversion products by means of UV–visible spectra.

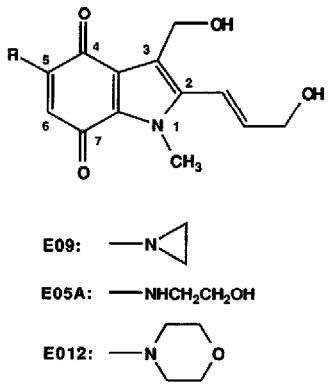

Fig 1. Structures of E09 [3-hydroxymethyl-5-aziridinyl-1-methyl-2-(1*H*-indole-4,7-dione)prop-β-en-α-ol], E05A [3-hydroxymethyl-5-β-hydroxyethylamino-2-(1*H*-indole-4,7-dione)prop-β-en-α-ol] and E012 [3-hydroxymethyl-5-morpholino-1-methyl-2-(1*H*-indole-4,7-dione)prop-β-en-α-ol]

EXPERIMENTAL

Chemicals and reagents

E05A, E09 and E012 were obtained from Dr. E. A. Oostveen (Laboratory of Organic Chemistry, University of Amsterdam, Amsterdam, The Netherlands) and through the EORTC New Drug Development Office (Free University Hospital, Amsterdam, The Netherlands). The chemical purity of all drugs was confirmed by reversed-phase HPLC to be >99%.

Methanol and acetonitrile (HPLC grade) were purchased from Rathburn (Walkerburn, U.K.). All chemicals were analytical grade or better and obtained from commercial sources. Water was distilled once and deionized using a four-cartridge Milli-Q water purification system (Millipore, Molsheim, France).

High-performance liquid chromatography

The modular HPLC system (Waters Assoc., Milford, MA, U.S.A.) comprised a Model 710B WISP automated sample injector, two Model 6000A pumps, a Model 660 solvent programmer and a Model 990 photodiode array detector together with a Model APC IV Power Mate 2 personal computer (NEC, Boxborough, MA, U.S.A.), a Model CP6 pinwriter (NEC) and a Model 990 plotter (Waters) with the chart speed set at 10 mm/min. Separation was achieved with a Resolve C_{18} Rad-Pak column (10 cm × 8 mm I.D., 5 μm particle size, Waters) under compression from a Z-module (Waters) and protected by a Resolve C_{18} Guard-Pak precolumn.

The mobile phase A consisted of 5% (v/v) acetonitrile in 0 02 *M* phosphate buffer, pH 7.4, and mobile phase B of 50% (v/v) acetonitrile in the same buffer. Prior to use the mobile phase was filtered through 0.45-μm Millipore filters and thoroughly degassed under vacuum. A linear gradient was run over 15 min starting at 90% A–10% B and finishing at 45% A–55% B. The flow-rate was 3.0 ml/min at ambient temperature.

Solutions and calibration standards

Stock standard solutions containing 1 mg/ml E05A, E09 and E012 in dimethyl sulphoxide (DMSO) were kept at −20°C in the dark for up to four weeks. Aliquots of these solutions were freshly diluted with methanol to yield concentrations of 0.05–15.0 μg/ml E05A and E09 and 1.0 and 12.0 μg/ml E012, respectively.

Animals and drug administration

Adult inbred male C3H/He mice were obtained from our own breeding colony. They were allowed laboratory chow and water *ad libitum* and were used at 25–30 g body weight.

E05A and E09 were dissolved in DMSO and diluted 1:20 with phosphate-buffered saline (PBS, pH 7.4) immediately before intravenous (i.v.) injection in a volume of 0.01 ml/g body mass at doses of 6 and 12 mg/kg, respectively. In the case of urinary recovery experiments, an additional 1 ml of PBS was injected by the intraperitoneal (i.p.) route. Blood samples (approximately 1 ml) were taken under diethyl ether anaesthesia by cardiac puncture into heparinised syringes. Five mice per group were bled at 1, 2, 4, 6, 10 and 30 min after injection. Samples were centrifuged at 3000 *g* for 15 min to obtain plasma. For urinary recovery studies groups of five mice were contained in a Urimax metabolism cage, and urine was collected in 4-h intervals for 24 h on dry ice. Urine samples and separated plasma fractions were stored at −20°C and analyzed within 24 h.

Sample preparation

Plasma samples. Samples (400 μl) were deproteinised by the addition of 2 vol. of methanol containing the internal standard E012. After thorough vortex-mix-

ing and centrifugation (3000 *g*, 15 min), 800 μl of the supernatant were removed and dried under vacuum using a Speed Vac sample concentrator coupled to a Model RT100-A refrigerated condensation trap (Savant, Farmingdale, NY, U.S.A.). Residues were resuspended in 300 μl of mobile phase A, and 50-μl aliquots were injected for triplicate analysis.

Urine samples. Samples (300 μl) were deproteinised by the addition of 2 vol. of methanol containing the internal standard E012. Samples were thoroughly vortex-mixed and centrifuged (3000 *g*, 5 min). Aliquots of 50 μl were used for triplicate analysis.

Calibration, recovery and reproducibility

To establish calibration curves, biological specimens were spiked with standard solutions. For urine, concentrations of 0.1, 0.5, 1.0, 3.0, 6.0, 9.0, 12.0 and 15.0 μg/ml for E05A and E09 were used and the internal standard E012 was 12.0 μg/ml. For plasma, concentrations of 0.05, 0.1, 0.25, 0.5, 0.75, 1.0, 2.0 and 4.0 μg/ml for E05A and E09 were used and the internal standard was 1 μg/ml Samples were processed as described above. The calculated ratio of the peak area of the compounds divided by the peak area of the internal standard was plotted against the spike concentrations, and least-squares linear regression analyses were performed.

Recoveries of analytes from urine and plasma samples were calculated by comparison of peak areas of drugs in biological specimens *versus* peak area of standard solutions obtained by direct injection of standard solutions onto the column.

The within-day precision and day-to-day reproducibility were studied by calculating the relative standard deviations at different concentrations of E05A and E09 in biological material.

RESULTS AND DISCUSSION

Chromatography of E09 and a variety of derived indoloquinones was first attempted on several different packings: cyanonitrile (CN), octylsilane (C_8) and octadecylsilane (C_{18}). Best resolution and peak shapes were obtained with Resolve and Novapak C_{18} Rad-Pak columns. The influence of the acetonitrile concentration (c_{CH_3CN}) of the mobile phase on the capacity factor k' of E05A, E09 and E012 using a 5-μm Resolve C_{18} column was also investigated. The equations used to fit the regression in the range 16–26% acetonitrile are as follows:

$$k'_{E05A} = 121.7\ (10\ \exp\ -0.09599 \cdot c_{CH_3CN})$$
$$k'_{E09} = 128.4\ (10\ \exp\ -0.07995 \cdot c_{CH_3CN})$$
$$k'_{E012} = 464.2\ (10\ \exp\ -0.09498 \cdot c_{CH_3CN})$$

The goodness of fit was calculated to be 99.8, 99.7 and 99.6%, respectively.

A gradient technique was found to be necessary for an adequate retention and resolution of drugs and metabolites due to their wide range of polarities. We found a linear gradient of 10–30% acetonitrile in 15 min gave best results. To prevent hydrolysis of the aziridine group of E09 during analysis, the mobile phase was buffered at pH 7.4 with 0.02 *M* sodium phosphate. Chromatograms of the biological specimens after precipitation of protein revealed no significant interference peaks at the retention times of E09, E05A or internal standard. E012 was chosen as standard because of its relative stability and achieved resolution.

Fig. 2a shows a chromatogram of resuspended dried methanolic extract from drug-free C3H/He mouse plasma and Fig. 2b shows a chromatogram of plasma from a mouse receiving 12 mg/kg E09 i.v. 1 min previously. Fig. 3a depicts a chromatogram of urine from untreated mice and Fig. 3b depicts a chromatogram of pooled urine collected during the first 4 h after an i.v. dose of 6 mg/kg. In order to further demonstrate the utility of the devised assay, the same gradient technique was also employed for the determination of the injected aziridine ring-opened drug E05A and its metabolites in pooled mouse urine (details are given in the legend of Fig. 4). Identification of compounds is based on experiments showing superimposition of retention time with authentic material, as well as comparison of the recorded UV–visible spectra in the range 220–600 nm.

Drugs were eluted with capacity factors of 6.5, 8.5 and 9.9 for E05A, E09 and E012, respectively, resulting in separation factors (α) of 1.3 and 1.2, respectively. The efficiency (N) of the separation for E05A, E09 and E012 using a 5-μm Resolve C_{18} Rad-Pak column protected with a Resolve C_{18} Guard-Pak precolumn was 11 000, 14 000 and 24 000 plates, respectively.

A simple precipitation of protein by addition of 2 vol. of methanol to biological specimens prior to analysis proved to be sufficient to prevent fast contamination of the precolumn/column. The absolute recovery for analytes from plasma for this simple clean-up step was typically 90%. The relative recovery (peak area analyte/peak area internal standard) of E05A from plasma over a concentration range of 50–4000 ng/ml ranged from 89.2% (at 50 ng/ml) to 109.3% (at 1000 ng/ml), averaging (± S.D.) 102.0 ± 7.0% ($n=8$). The relative recovery of E09 was between 98.6% (at 500 ng/ml) and 113.1% (at 100 ng/ml), averaging 103.2 ± 5.9% ($n=8$). The absolute recovery for analytes from urine was quantitative. The relative mean recovery of E05A was 98.9 ± 5.7% and 101.9 ± 4 0% for E09 ($n=7$). We found that solid-phase extraction from plasma did significantly decrease recovery of E09 whereas a liquid–liquid extraction of urine samples with ethyl acetate or chloroform was unsuitable for metabolite extraction.

Plots of peak-area ratios of analytes *versus* area of internal standard in plasma and urine were linear in the μg/ml range. Linearity data are provided in Table I. The within-day and day-to-day coefficients of variation as well as precision (S.D / mean) and accuracy (observed concentration/expected concentration) of the devised method for E05A and E09 are summarized in Table II. Respective detection limits in plasma were approximately 30 ng/ml and 50 ng/ml in urine at a signal-to-noise ratio of 3, representing an on-column injection of *ca.* 2 ng.

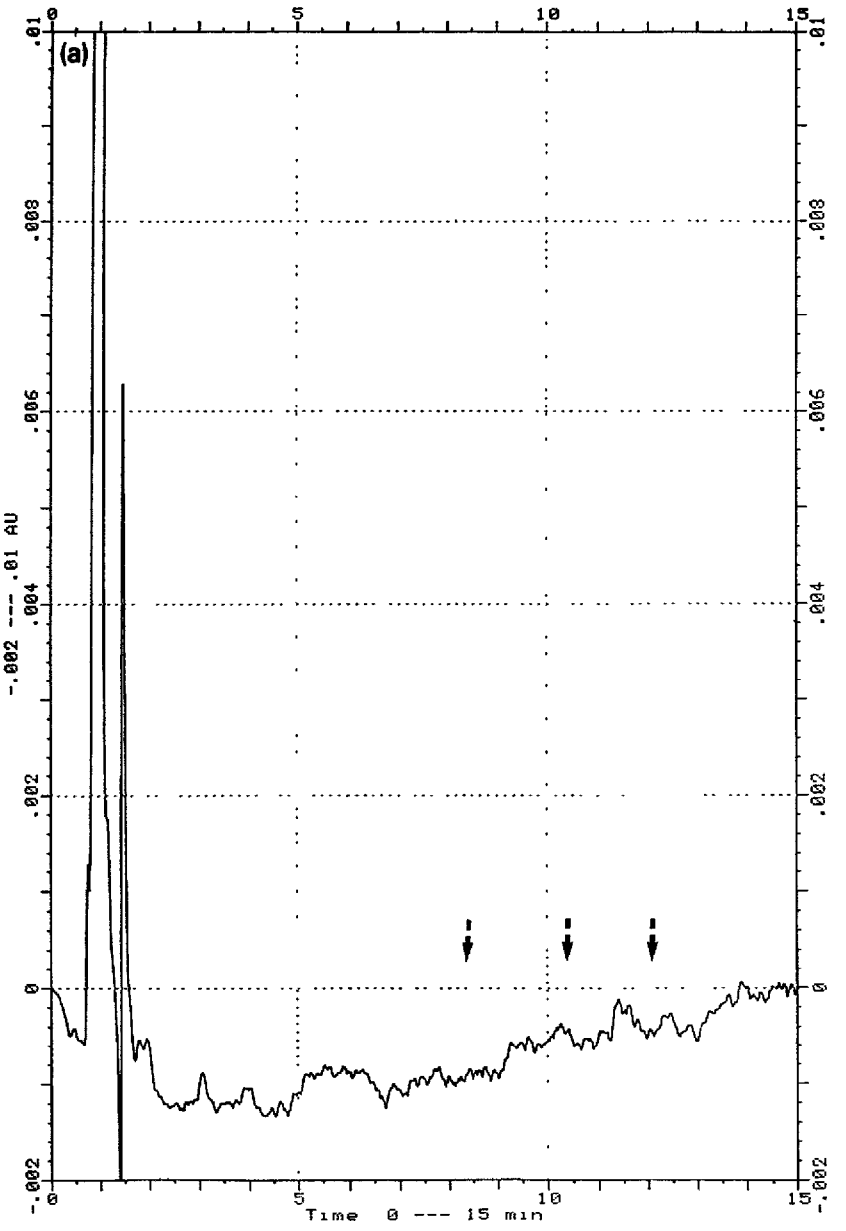

Fig. 2.

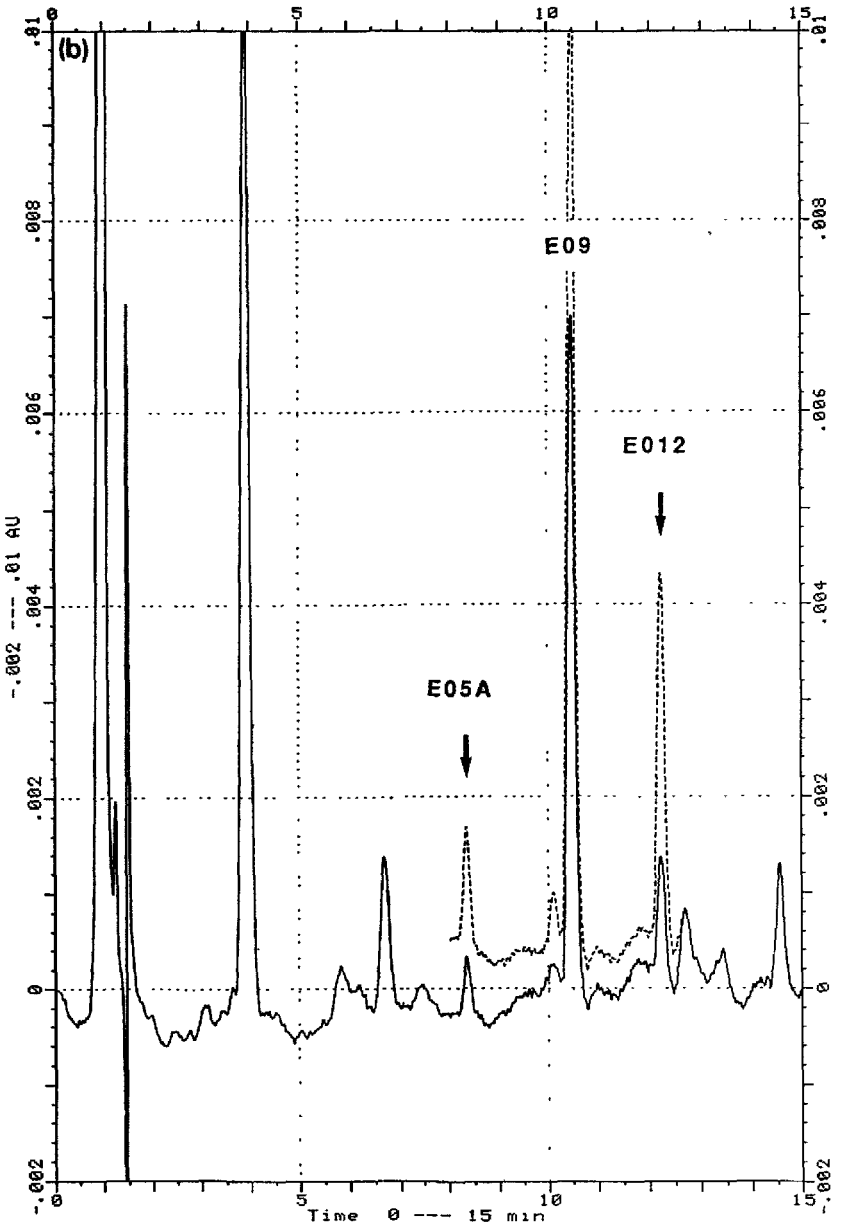

Fig. 2. Chromatograms of resuspended dried methanolic extracts of blood plasma from male C3H/He mice. (a) Blank plasma, dotted arrows denote retention times of E05A, E09 and internal standard (b) Sample taken 1 min after i.v. injection of 12 mg/kg E09 containing 1965 ng/ml drug, 190 ng/ml metabolite E05A and 1000 ng/ml internal standard E012 Chromatographic conditions are described in the Experimental section; UV detection at 280 nm (- - - -) and 310 nm (———)

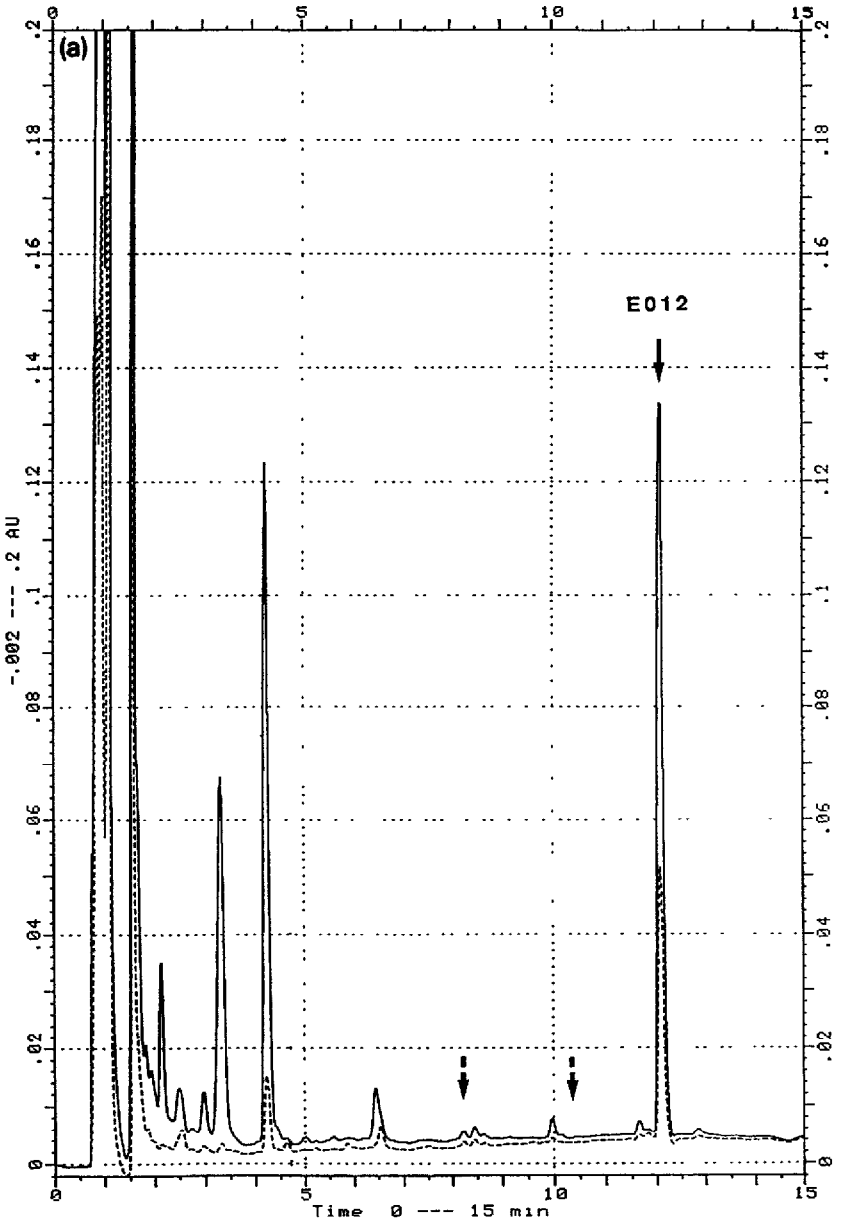

Fig 3

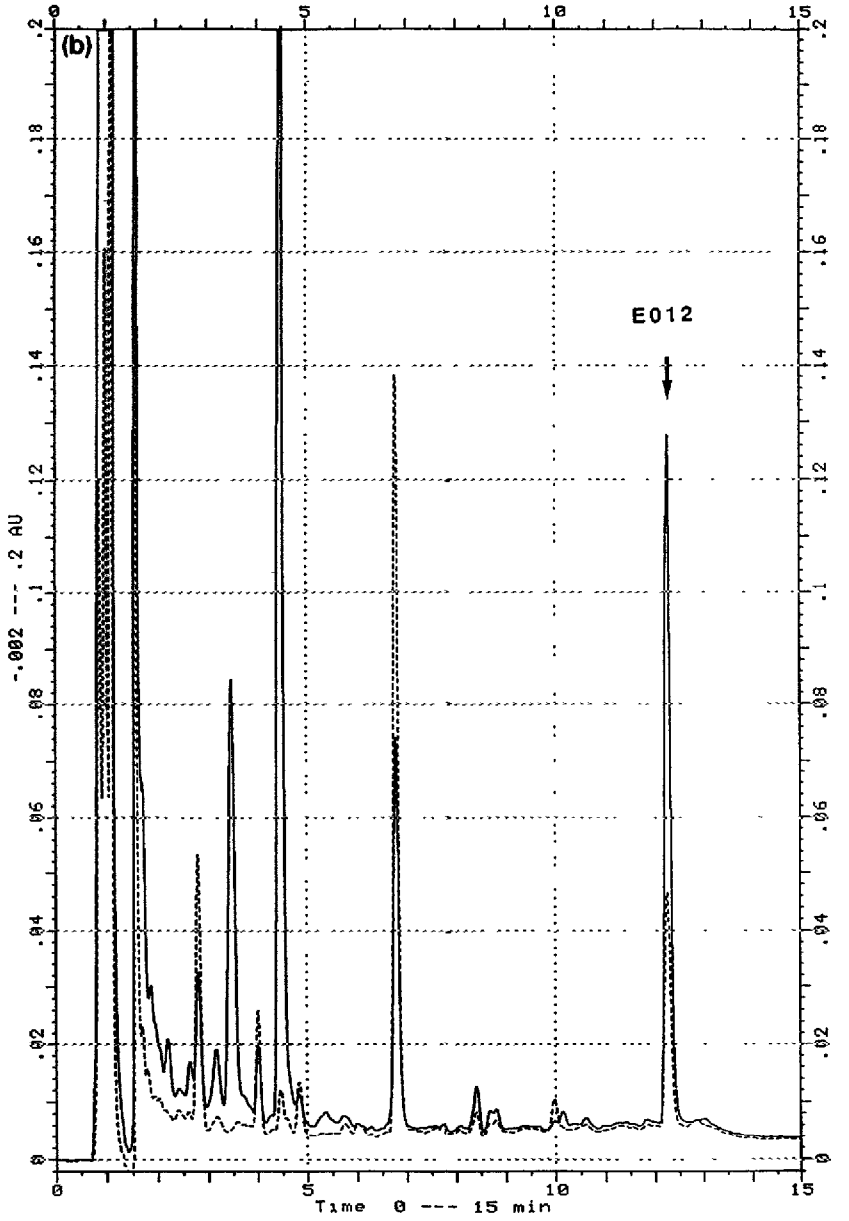

Fig. 3. Chromatograms of an extract of 1:3 diluted urine from male C3H/He mice ($n = 5$) (a) Blank urine; dotted arrows denote retention times of E05A and E09 (b) Urine sample collected for 4 h after I.V. injection of 6 mg/kg E09 containing 12 μg/ml internal standard E012. No parent drug was detected. Chromatographic conditions were as for Fig 2, UV detection at 280 nm (———) and 310 nm (- - - -)

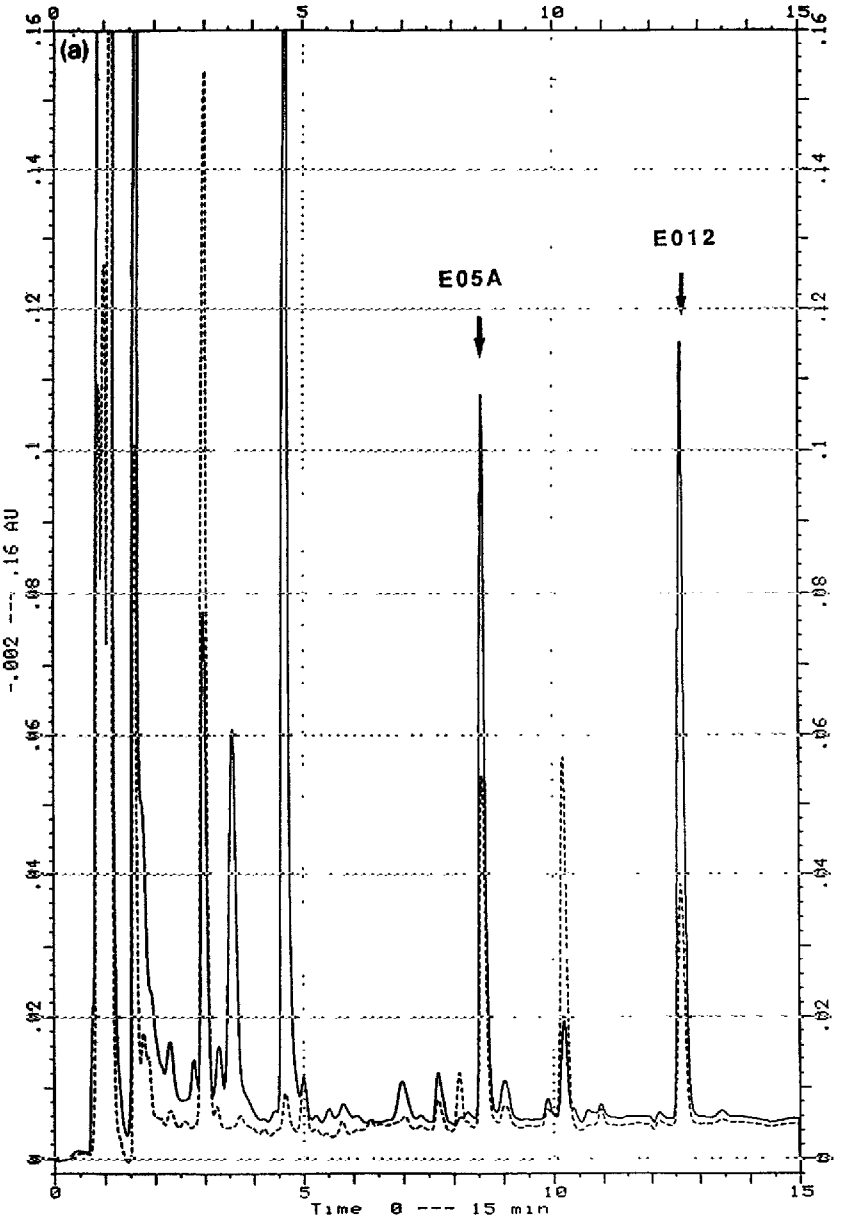

Fig. 4.

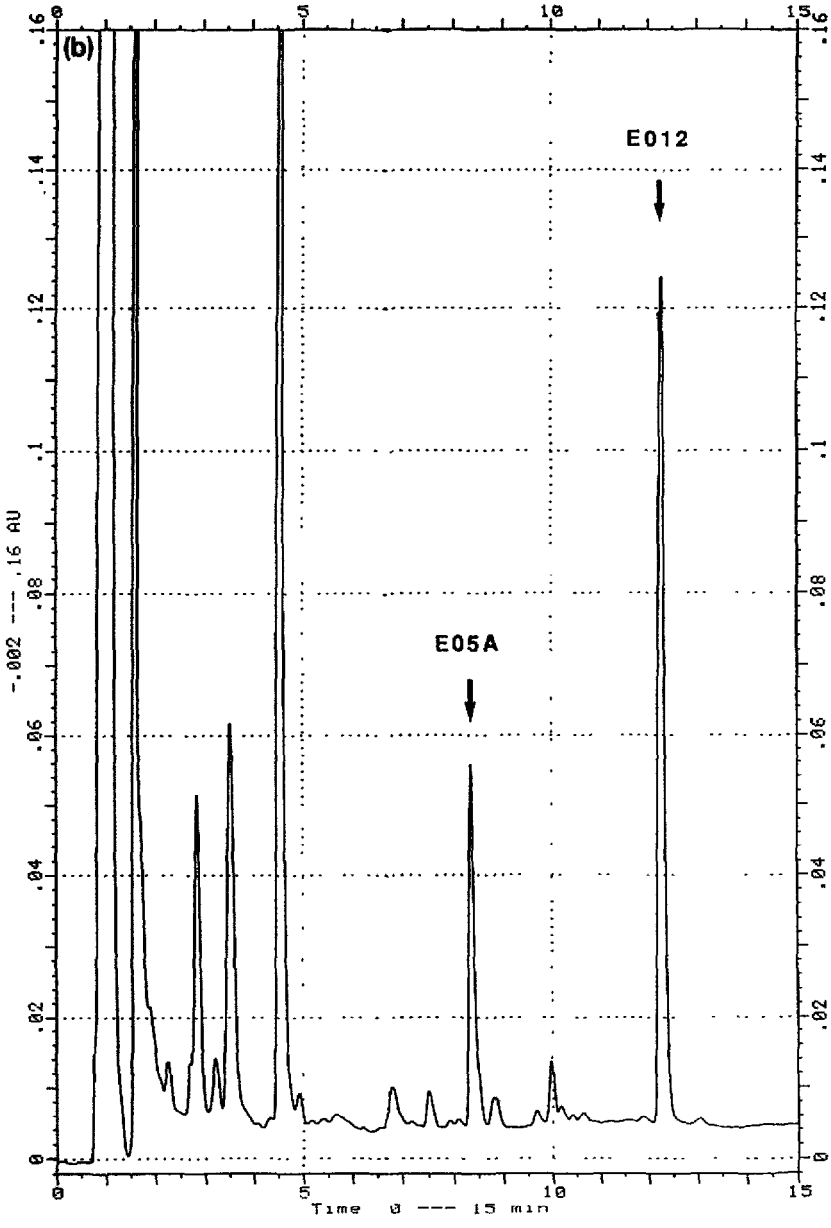

Fig. 4. Chromatograms of an extract of 1:3 diluted urine from male C3H/He mice ($n = 5$). (a) Urine sample collected for 4 h after i.v. injection of 6 mg/kg E05A containing 12 5 μg/ml drug and 12 μg/ml internal standard E012. (b) Urine sample taken 4–8 h after drug administration containing 6.7 μg/ml drug. Chromatographic conditions were as for Fig. 2; UV detection at 280 nm (———) and 310 nm (- - - -)

TABLE I

LINEARITY

Drug	Matrix	Concentration (μg/ml)	Intercept	Slope	Correlation coefficient
E05A	Urine[a]	0.10–15 00	0.00565	0 08486	0 9996
	Plasma[b]	0.05–4 00	0.03277	1 29630	0.9999
E09	Urine[a]	0.10–15.00	0.00642	0.07721	0.9988
	Plasma[b]	0.05–4.00	0 03111	1 38890	0 9999

[a] Spiked with 12 μg/ml E012.
[b] Spiked with 1 μg/ml E012

Following the optimization of chromatographic conditions and validation of the assay, the method was applied in preliminary pharmacokinetic investigations of E09 in mice. As shown in Table III, after i.v. administration of 12 mg/kg E09 the drug level could be measured in the plasma of individual mice. The method was used to demonstrate the extremely rapid elimination of the drug, with a

TABLE II

WITHIN-ASSAY AND DAY-TO-DAY PRECISION AND ACCURACY

Drug	Matrix	Expected concentration (μg/ml)	Observed concentration[a] (μg/ml)	Precision (%)	Accuracy (%)
Within-assay					
E05A	Urine	9.00	8.99 ± 0 13	1 5	99 9
		1 00	1.10 ± 0 04	3 5	110.0
	Plasma	0 75	0.74 ± 0.01	1 4	98 6
		0.50	0.50 ± 0.01	2.8	100.8
E09	Urine	9 00	8.92 ± 0.05	0.6	99.1
		1.00	0.94 ± 0.01	1 1	94.0
	Plasma	0.75	0 76 ± 0 04	4 9	100.8
		0.50	0 51 ± 0 01	2 2	101 1
Day-to-day					
E05A	Urine	6 00	6.25 ± 0.17	2 7	104 2
		0.50	0.52 ± 0.05	9.1	104.1
	Plasma	4.00	4.03 ± 0.04	1.0	100.8
		0.25	0.24 ± 0 03	12 5	96.0
E09	Urine	6.00	5.89 ± 0.27	4 6	98.2
		0.50	0.58 ± 0 02	3.4	116 0
	Plasma	4 00	4.08 ± 0 18	4 4	102.0
		0 25	0.23 ± 0 01	4 3	92 2

[a] Concentrations were calculated as the mean of triplicate HPLC determinations ± S D.

TABLE III

PLASMA CONCENTRATIONS OF E09 IN MICE AFTER INTRAVENOUS INJECTION OF 12 mg/kg

Time (min)	Concentration of E09 (ng/ml)				
	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5
1	2073	891	1964	1236	1552
4	284	313	371	286	410
10	64	54	24	44	71

plasma half-life of 1.9 min. The high sensitivity and excellent reproducibility of the method permitted the quantification of drug for at least five half-lives. Chromatograms showed two major plasma metabolites which eluted after 3.9 and 6.8 min, together with a number of other smaller peaks not present in blank plasma (Fig. 2). The minor metabolite at 8.2 min was assigned as 5-β-hydroxyethylami-

TABLE IV

UV–VISIBLE SPECTROSCOPIC PROPERTIES OF E09, E05A AND THEIR METABOLITES

++ denotes main metabolite; sh denotes a shoulder.

Compound	Retention time[a] (min)	Urine	Plasma	λ_{max} (nm)
E09				
Metabolite	2.8[b]	+	+	307, 375 (sh), 550
Metabolite	3.1	−	+	240, 310, 375 (sh), >500[c]
Metabolite	3.9	+	++	305, 375 (sh), 510
Metabolite	6.2	−	+	280, 325 (sh), >500[c]
Metabolite	6.8	++	+	308, 375 (sh), 550
Metabolite E05A	8.2	−	+	277, 325 (sh), 375 (sh), 550
Drug E09	10.4	−	+	280, 325 (sh), 375 (sh), 510
Metabolite	12.8	+	+	325, 380 (sh), 500
Metabolite	14.5	−	+	275, 325 (sh), 375 (sh), >500[c]
E05A				
Metabolite	2.8[b]	++	++	307, 375 (sh), 550
Metabolite	7.2	+	−	288, 375 (sh), 550
Metabolite	7.7	+	+	243, 321, 375 (sh), 550
Drug E05A	8.2	+	+	277, 325 (sh), 375 (sh), 550
Metabolite	9.8	+	+	320, 350 (sh), 535

[a] Chromatographic conditions are described in Experimental section

[b] Identical metabolite detected after i.v injection of E09 and E05A

[c] Visible absorption not accurate detectable.

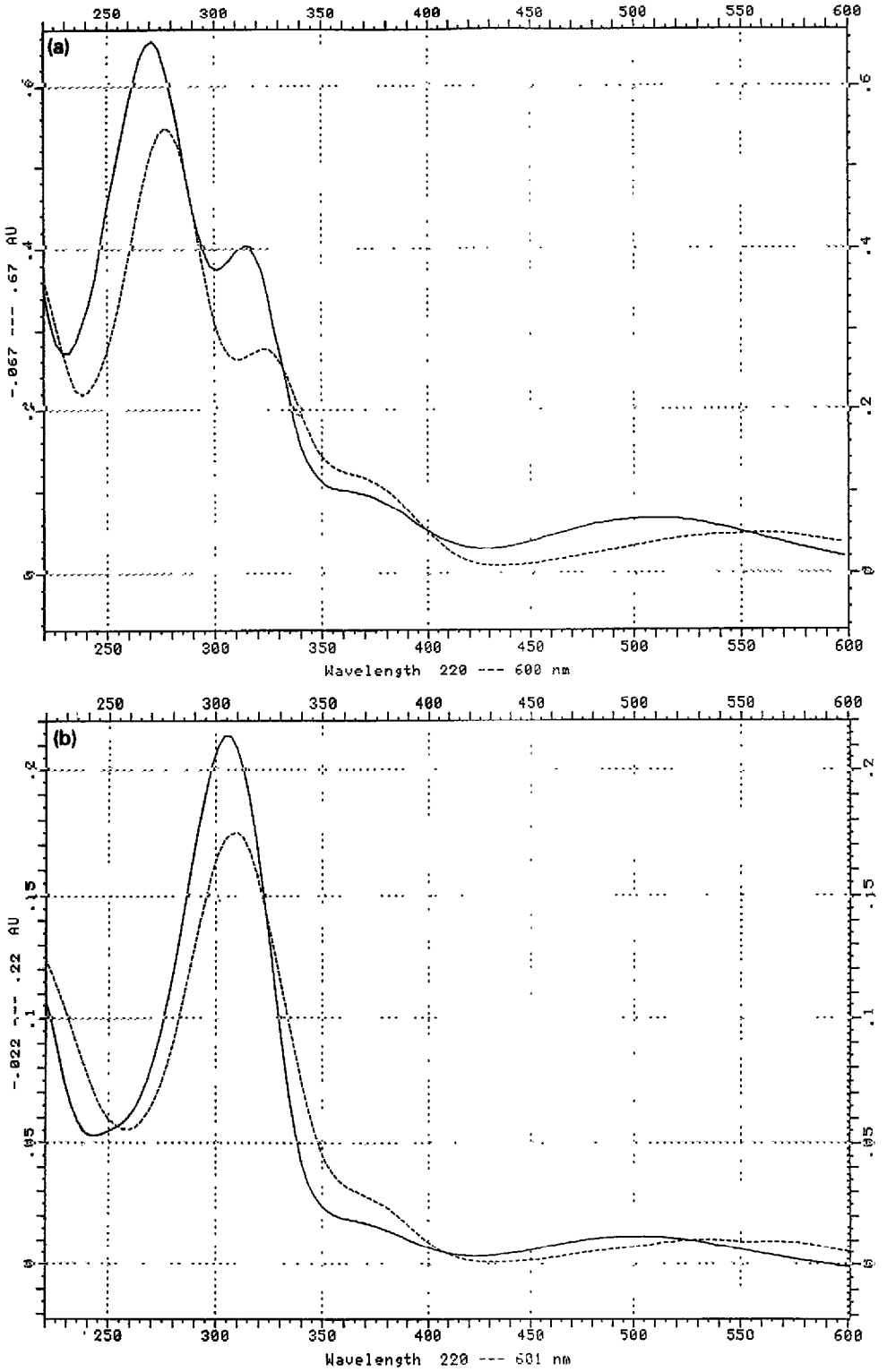

Fig. 5. (a) UV–visible spectra of E09 (———) and E05A (- - - -) in mobile phase. (b) UV–visible spectra o E09 metabolites eluted after 3.9 min (———) and 6 8 min (- - - -). Details of the chromatographic condi tions are described in the Experimental section.

noindoloquinone E05A. It is interesting to note that all relatively hydrophilic metabolites show a shift of the short wavelength absorbance from *ca.* 280 to 310 nm. Consequently, the use of dual-wavelength detection at both absorbance maxima for highest sensitivity was imperative.

In the case of urine, neither E09 nor metabolite E05A could be detected after injection of E09 (Fig. 3b). Non-metabolised E05A was, however, detected for up to 20 h after i.v. administration of 6 mg/kg E05A itself (Fig. 4). In addition, the urine chromatograms showed the presence of numerous metabolites. UV–visible data for these metabolites as well as metabolites detected in plasma are summarized in Table IV.

By analogy with ^{1}H NMR, ^{13}C NMR, IR and UV–visible data for coloured aminobenzoquinones [6,9] it appears that the visible absorption of E09 is also due to an intramolecular amino donor–quinone acceptor interaction. Thus, depending on the inductive/mesomeric effect of the substituent in position 5, the long wavelength maximum is shifted; *e.g.* E09 absorbs at 510 nm whereas E05A absorbs at *ca.* 560 nm (see Fig. 5a) presumably because of hydrogen bonding between the secondary amine substituent with the neighbouring carbonyl group resulting in an enforced conjugation of the donor lone pair electrons with the quinone moiety [10]. Similar effects were observed in the UV–visible spectra of analogues. It is therefore likely that the aziridine group of the metabolite eluted after 3.9 min and absorbing at 510 nm is still intact but is hydrolyzed in case of the metabolite eluted after 6.8 min with a shifted visible maximum (see Fig. 5b). However, due to the limited information obtained by UV–visible spectra the structure of these metabolites cannot be assigned at this stage. Further experiments are aimed at determining the structure of metabolites by means of liquid chromatography–mass spectrometry.

The HPLC method here described is currently being used for further pharmacokinetic studies of E09 and its analogues in rodents. We anticipate that this method will also be used in Phase I clinical studies which will commence shortly.

CONCLUSIONS

A linear gradient HPLC technique following a single clean-up step has been developed for the determination of the promising new antitumour agent E09 in biological specimens. The devised method is sufficiently sensitive and readily applicable to rodent pharmacokinetic studies and may also be extended to pharmacokinetic studies of the drug in humans.

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