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Sensitive isocratic high-performance liquid chromatographic determination of a novel indoloquinone cytotoxic drug (EO9) in human plasma and urine

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ABSTRACT

A reversed-phase isocratic high-performance liquid chromatographic method is described for the simultaneous determination of EO9, 3-hydroxymethyl-5-aziridinyl-1-methyl-2-(1*H*-indole-4,7-dione)prop-β-en-α-ol (I), and its ring-opened aziridine analogue EO5A (II), employing ultraviolet detection. Solid-phase sample extraction was used without addition of an internal standard. Plots of peak heights and areas of I and II were linear in the range 5–10 000 ng/ml. The lower limit of detection of both I and II in plasma was 2 ng/ml. The between-day variation of I was 13.9% at 5 ng/ml and lower than 6.2% for concentrations $\geq$ 10 ng/ml. The between-day variation of II at 5 ng/ml was 13.8% and lower than 4.5% for concentrations $\geq$ 10 ng/ml. The assay was developed to enable pharmacological guiding of a phase I study of I in solid tumour cancer patients.

INTRODUCTION

Compound I (Fig. 1) is the lead compound of a series of novel indoloquinone cytotoxic agents based on the prototype bioreductive agent mitomycin C [1]. It was selected for phase I clinical trials on the basis of good *in vitro* and *in vivo* antitumour activity against several human and murine tumour models [2]. Bioreductive activation is thought to occur via reduction of the aziridinyl-quinone ring. Subsequently the reactive metabolite may form DNA crosslinks [3]. The flavoenzyme DT-diaphorase (NAD[P]H:quinone acceptor oxidoreductase), obtained from rat Walker 256 mammary carcinoma and human HT 29 colon carcinoma, was shown to reduce I readily to its active metabolite(s) [4]. In mice and rats, I was cleared rapidly with an elimination half-life ($t_{1/2}$) of 1.9 min [5]. One of the principal metabolites was characterized as the ring-opened hydrolysis product II [5]. A gradient high-performance

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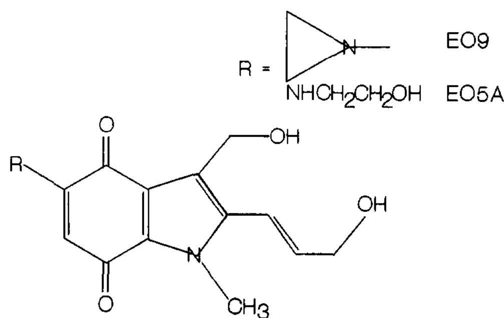

Fig. 1. Structures of EO9 (I), 3-hydroxymethyl-5-aziridinyl-1-methyl-2-(1*H*-indole-4,7-dione)prop-β-en-α-ol, and its ring-opened aziridine analogue EO5A (II), 3-hydroxymethyl-5-β-hydroxyethylamino-2-(1*H*-indole-4,7-dione)prop-β-en-α-ol.

liquid chromatographic (HPLC) assay for I was developed by Binger and Workman [6] with a lower limit of detection of 30 ng/ml and application of an internal standard. The methodology described in the present study was developed because the plasma concentrations in the clinical study were expected to be lower than 30 ng/ml and because no internal standard was available.

EXPERIMENTAL

Chemicals and reagents

Compound I was synthesized previously[a] and its chemical purity was confirmed by reversed-phase HPLC to be greater than 99%. Compound II was obtained by moderate acidic hydrolysis of I. To 5 ml of a 0.10 mg/ml solution of I in dimethyl sulphoxide (DMSO) 5 ml of perchloric acid (pH 2.0) were added. The mixture was left for 1 min at room temperature. Subsequently, 40 ml of a 0.02 *M* phosphate buffer solution (pH 8.0) were added. The efficiency of the hydrolysis was confirmed by the reversed-phase HPLC method and on-line assessment of UV spectra (see later).

[a] EO9 was synthesized by Dr. E. A. Oostveen (Laboratory of Organic Chemistry, University of Amsterdam, Amsterdam, Netherlands) and obtained through the EORTC New Drug Development Office (Free University Hospital, Amsterdam, Netherlands).

All chemicals (analytical grade or better) and organic solvents (HPLC grade) were obtained from Baker (Deventer, Netherlands). Water was purified using a four-cartridge Milli-Q purification and deionization system (Millipore, Etten-Leur, Netherlands).

Chromatographic system

The HPLC system consisted of a Model 710B WISP autosampler, a Model 510 pump, a Model 680 solvent programmer (Waters Assoc., Milford, MA, USA), a UV 2000 variable-wavelength UV spectrophotometer set at 270 nm (Spectra Physics, San Jose, CA, USA) and a CR3A integrator (Shimadzu, Kyoto, Japan) set at 5 mm/min. Separation was achieved with a Hewlett-Packard LiChrospher 60 RP-select B column (125 mm × 4 mm I.D., 5 μm particle size) and protected by a Hewlett-Packard LiChrospher 60 RP-select B precolumn (4 mm × 4 mm I.D., 5 μm particle size) obtained from LC-Service (Emmen, Netherlands). The column oven, from Spark Holland (Emmen, Netherlands), was set at 30°C.

The mobile phase consisted of a 0.02 *M* phosphate buffer (pH 7.0) with 10% acetonitrile and 1% tetrahydrofuran (v/v). Prior to use, it was filtered through 0.22-μm Millipore filters and degassed under helium. The flow-rate was 1.0 ml/min.

Sample preparation and calibration curves

To 1.0 ml of plasma in a glass centrifuge tube, 2.0 ml of 0.02 *M* phosphate buffer (pH 8.0) were added. After mixing, 2.5 ml were transferred to a column (C_{18}/14% 500 mg/3 ml, Spe-ed, Applied Separations, Lehigh Valley, PA, USA). The column was preconditioned by washing once with 0.02 *M* phosphate buffer (pH 8.0). The column was washed twice with 1 ml of Milli-Q water. After drying, the analyte was eluted with two 0.5-ml volumes of methanol. The pooled sample was evaporated to dryness under vacuum at 40°C in a Gyrovap (Howe, Banbury, UK). The residue was dissolved in 300 μl of 0.02 *M* phosphate buffer calibrated at pH 8.0 with 10% acetonitrile and 1% tetrahydrofuran (v/v), and transferred to

clean glass inserts. A volume of 100 μl was injected into the chromatograph.

In addition to solid-phase extraction, a liquid–liquid extraction method was also evaluated. To 1.0 ml of plasma, 5 ml of dichloromethane–diethyl ether–isobutyl alcohol (60:40:5 v/v/v) were added. After mixing on a whirl mixer and centrifugation for 5 min at 4000 *g*, 4 ml of the organic upper layer were accurately collected and transferred to a clean glass tube. Extraction was carried out twice. The pooled sample was evaporated to dryness under vacuum at 40°C in a Gyrovap. The residues were resuspended in the phosphate buffer as described above.

For each assay in plasma, a seven-point calibration curve was processed (six-point for II) identically and simultaneously. The stock solution of I was prepared in DMSO and that of II in 0.02 *M* phosphate buffer (pH 8.0). The concentration of I in its stock solution was 100 μg/ml, and that of II was 10 μg/ml. Five solutions were made from the stock solutions of I and II in 0.02 *M* phosphate buffer (pH 8.0). The final concentrations in these solutions were: for I, 10 000, 5000, 2000, 1000 and 100 ng/ml; and for II, 5000, 2000, 1000 and 100 ng/ml. These standards were used for the preparation of the calibration curves as outlined in Table I. The concentrations of I in the calibration curve were 0, 5, 25, 100, 200, 500 and 1000 ng/ml, and those of II were 0, 5, 25, 100, 200 and 500 ng/ml. Subsequently, the samples were carried through the analytical procedure.

After thawing and centrifugation of the urine, 1 ml was taken for analysis and 1 ml of 0.5 *M* phosphate buffer (pH 8.0) was added. The urine samples were analysed without further extraction. Calibration curves were prepared by adding I and II to final concentrations of 0, 50, 100, 500, 1000, 2000 and 4000 ng/ml. The injection volume was 100 μl.

Peak heights were used for quantitation. Least-squares linear regression analyses were performed. The between-day variation was studied by calculating the relative standard deviations at different concentrations of I and II of calibration curves which were freshly prepared on five consecutive days. The within-day variation was studied by reanalysing a calibration curve of I and II three times on one day.

The detection limit was defined as the concentration at which the signal-to-noise ratio was 2. The lower limit of quantitation was defined as the lowest measurable concentration with a between-day coefficient of variation (C.V.) of ≤15%. Assay data were accepted if more than 75% of all data were within 15% of the nominal values.

Stability of I and II

The stability of I and II was tested in plasma, whole blood and urine at different temperatures and pH values. The temperatures were room temperature (22°C), 37°C (water-bath), 0°C (melting ice-bath) and −80°C. The pH values were 7.0, 7.5 and 8.0. The pH was titrated by

TABLE I

PREPARATION OF THE CALIBRATION CURVE

A 0.02 *M* phosphate buffer (pH 8.0) was used. The concentrations 100–10 000 ng/ml are the concentrations of parent drug I and metabolite II in the standard solutions. The 10 000 ng/ml standard contains only I.

	0	5	25	100	200	500	1000 ng/ml
Plasma	900 μl	900 μl	900 μl	900 μl	900 μl	900 μl	900 μl
Buffer	100 μl	50 μl	75 μl				
100 ng/ml		50 μl					
1000 ng/ml			25 μl	100 μl			
2000 ng/ml					100 μl		
5000 ng/ml						100 μl	
10 000 ng/ml							100 μl

adding small volumes of 0.01 *M* hydrochloric acid or sodium hydroxide. The starting concentrations of I and II in the matrix were 1000 ng/ml. All experiments were carried out in triplicate. The stability experiments were carried out in separate series of test-tubes. The concentrations of I and II were determined at 0, 2, 4, 8 and 24 h after incubation for the three pH values and for the temperature values except −80°C. For the experiment at −80°C, samples were analysed after 0, 1, 2, 3, 4, 8 and 12 weeks.

Human experiments

The starting dose of the phase I study was 2.7 mg/m^2. One of the first patients was treated at the 5.4 mg/m^2 dose level. A dose of 9.5 mg was administered as a 5-min bolus injection into an antebrachial vein. Prior to intravenous administration, I was dissolved in sterile water to a concentration of 0.5 mg/ml. Fifteen blood samples and all voided urine were collected up to 24 h after administration. Immediately after sampling plasma was prepared by centrifugation (5 min at 4000 *g*). Aliquots were frozen at −80°C and assayed within 1 week.

RESULTS

Assay in plasma

Adequate retention and resolution could be obtained by the isocratic HPLC system. The retention times of I and II were 17 and 10 min, respectively (Fig. 2). No significant interfering peaks were found. The UV–visible spectrum of synthetised II co-segregated with the spectrum of the compound, after extraction of plasma of subjects given I intravenously, with a retention time of 10 min. Addition of synthetised II to a sample from a patient containing II proportionally increased the peak at 10 min.

The absolute recovery of I from plasma, applying solid-phase extraction, was 87 ± 5.8%, and that of II was 95 ± 4.2% (n = 15). The absolute recoveries using liquid–liquid extraction were 82 ± 5.0% (n = 5) for I and 52 ± 6.2% (n = 5) for II. Ethanol precipitation, as described by Binger

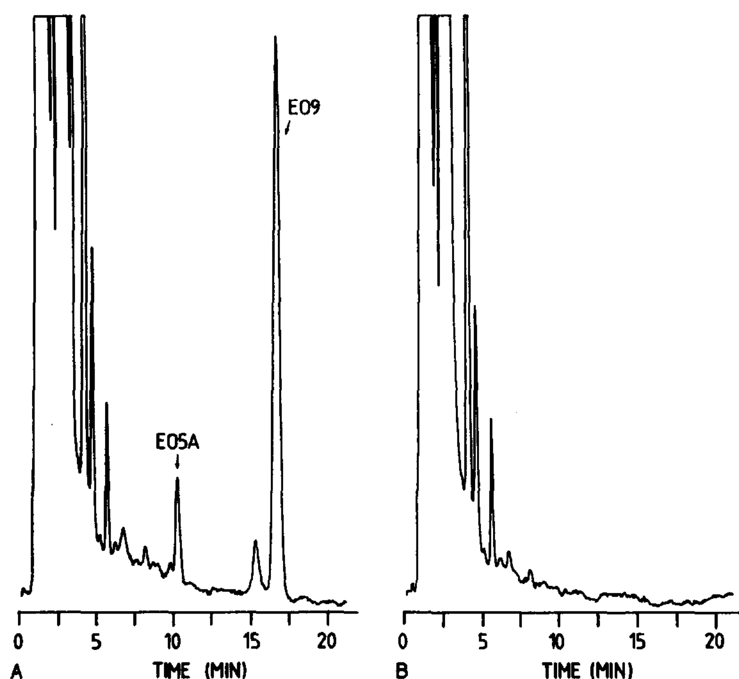

Fig. 2. Chromatograms of (A) I (113 ng/ml) and II (16 ng/ml) after extraction of a blood sample of a patient who was administered 9.5 mg I and (B) of the corresponding blank sample.

TABLE II

BETWEEN-DAY (n = 5) AND WITHIN-DAY (n = 3) VARIATION OF I AND II IN HUMAN PLASMA (5–1000 ng/ml)

	Peak height					
	5 ng/ml	25 ng/ml	100 ng/ml	200 ng/ml	500 ng/ml	1000 ng/ml
Between-day, I						
Mean	129	740	3009	6071	15 387	30 387
S.D.	17.9	45.5	176	303	772	1737
C.V. (%)	13.9	6.1	5.8	5.0	5.0	5.7
Within-day, I						
Mean	140	719	2997	6043	15 404	31 124
S.D.	9.5	18.0	62.9	121	277	591
C.V. (%)	6.8	2.5	2.1	2.0	1.8	1.9
Between-day, II						
Mean	218	1217	5115	10 332	26 114	
S.D.	30.0	51.9	130	461	593	
C.V. (%)	13.8	4.3	2.5	4.5	2.3	
Within-day, II						
Mean	246	1249	4937	9980	25 198	
S.D.	19.4	37.5	59.2	210	504	
C.V. (%)	7.9	3.0	1.2	2.1	2.0	

and Workman [6] for plasma samples of rodents, was unsuitable because of interfering peaks.

The calibration curves of I and II were linear in the studied concentration range with correlation coefficients of at least 0.9999. The detection limit of both I and II in plasma was 2 ng/ml. The between-day and within-day variations are given in Table II.

Stability of I and II

Compound I was found to be unstable in plasma and acidic solutions. After 24 h at pH 8.0 3% of the initial concentration of I was lost, at pH 7.5 10% and at pH 7.0 28%. Compound I was also unstable in urine at neutral or acidic pH. The stability of I in whole blood was temperature-dependent: at pH 7.35 and 37°C the $t_{1/2}$ was 100 min, at 22°C it was 4 h, and at 0°C (melting ice-bath) it was 10 h.

Compound II was stable under all described conditions, and was found to be stable for more than 3 months at −80°C. Compound I was unstable at −80°C: after 1 week none was lost, but after 3 months 30% had disappeared.

Assay in urine

In urine the recoveries of I and II were 106 ± 3 and 100 ± 2%, respectively. Calibration curves were linear in the studied concentration range with correlation coefficients of ≥0.9991 and ≥0.99991 for I and II, respectively. The between-day C.V. of II was 16% at 50 ng/ml, 6.7% at 100 ng/ml and ≤1.4% for all higher concentrations; the C.V. of I was ≤3.6% for concentrations higher than 100 ng/ml. Representative chromatograms of a urine sample of a patient and a blank urine sample are given in Fig. 3.

Human experiment

The plasma concentration–time curve after intravenous administration of 9.5 mg of I is given in Fig. 4. The areas under the plasma concentration–time curves of I and II were 990 ng min/ml and 197 ng min/ml, respectively. The $t_{1/2}$ of I and II were 2.9 and 4.6 min, respectively. Total plasma clearance of I was 9.8 l/min, and the volume of distribution was 40 l. The renal excretion of I and II in the 0–12 h urine was 105 μg and 64 μg, respectively. In the 12–24 h urine, no I or II could be detected.

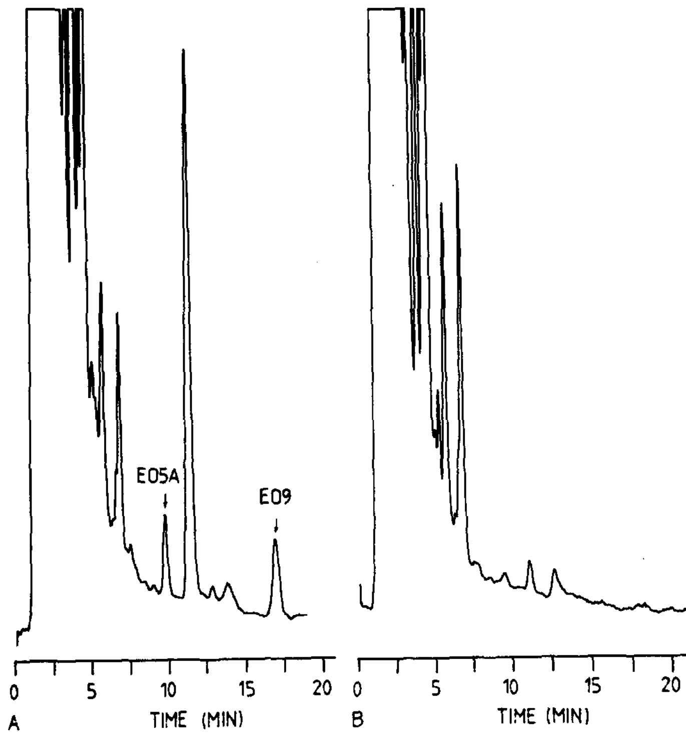

Fig. 3. Chromatograms of (A) I (70.7 ng/ml) and II (43.3 ng/ml) in a urine sample of a patient, and (B) the corresponding blank sample.

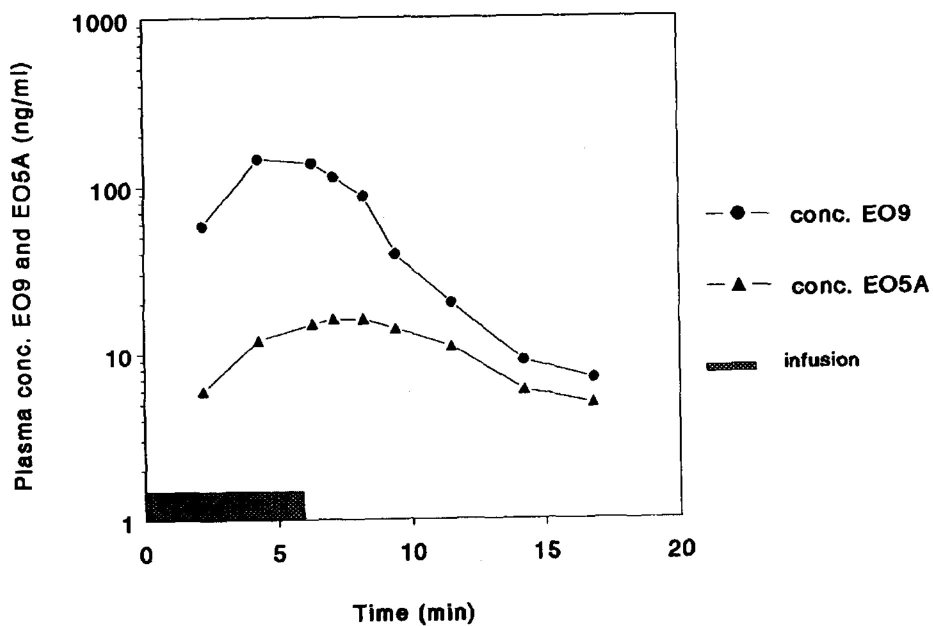

Fig. 4. Plasma concentration–time curves of (●) I and (▲) II, after intravenous administration of 9.5 mg of I to a patient.

DISCUSSION

The described methodology is appropriate for the measurement of I and its main metabolite II in plasma and urine. For the assay in plasma solid-phase extraction gave higher recoveries of both compounds and better reproducibility for II than liquid–liquid extraction. In urine no extraction procedure was necessary. Compound I was unstable at pH <7.5, and I was also found to be unstable at acidic pH in cell culture media [7]. The satisfactory lower limit of detection enabled the assessment of the maximum plasma concentration and the decline of the plasma concentration–time curve, even at the lowest administered dose of 2.7 mg/m^2. Thus, optimal pharmacokinetic monitoring of the present phase I study in cancer patients is feasible. The pharmacokinetics in one patient show a rapid decline of the plasma concentration–time curve with a terminal $t_{1/2}$ of I of 2.9 min. The total plasma clearance of 9.8 l/min indicates rapid elimination from the central compartment. Only very small amounts of I and metabolite II could be detected in the urine.

CONCLUSION

A sensitive and selective isocratic reversed-phase HPLC method is described for the simultaneous analysis of I, a novel indoloquinone cytotoxic compound, and its main metabolite II in plasma and urine. No internal standard was needed. Extraction from plasma was carried out by a solid-phase procedure. The methodology can be used for pharmacological guiding of drug administration to patients in a phase I clinical study, even at the lowest administered dose level.

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