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Note

Determination of the anthrapyrazole anticancer drug CI-941 in plasma and urine by solid-phase extraction and high-performance liquid chromatography

MARTIN A. GRAHAM*, DAVID R. NEWELL and A. HILARY CALVERT

Clinical Pharmacology Team, Drug Development Section, The Institute of Cancer Research, 15 Cotswold Road, Belmont, Sutton, Surrey SM2 5PX (U.K.)

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The anthrapyrazole CI-941 (I, Fig. 1) is one of a new series of DNA-complexing anticancer drugs currently undergoing clinical evaluation. I has been developed in an attempt to find a non-cardiotoxic alternative to doxorubicin with equivalent or superior antitumour activity. I is similar to doxorubicin in that it displays broad-spectrum high-level antitumour activity against a number of murine tumours [1,2]. However, the molecule does not undergo metabolic activation to drug-free radical species, induce lipid peroxidation or significant superoxide anion formation in rat liver microsomes [3]. Since these events have been implicated in doxorubicin cardiotoxicity [4,5], this anthrapyrazole is unlikely to induce heart damage in patients by a free radical-mediated mechanism.

In this study, a method has been developed to extract I from biological sam-

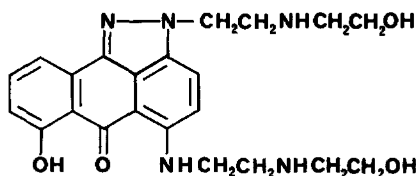

Fig. 1. Molecular structure of the anthrapyrazole I.

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ples and quantitate drug levels by high-performance liquid chromatography (HPLC). The method is based on solid-phase extraction prior to HPLC and is simple, reproducible and sensitive. The sample clean-up procedure yields an analyte with low-level background interference. The high sensitivity of the assay has facilitated the accurate determination of the pharmacokinetics of the drug in patients treated at the phase-I starting dose (5 mg/m^2) and should enable the dose to be escalated in a pharmacokinetically guided manner [6,7].

EXPERIMENTAL

Chemicals and reagents

The anthrapyrazole I was a generous gift from Warner-Lambert Pharmaceutical Research (Ann Arbor, MI, U.S.A.). All solvents and reagents were either analytical-reagent grade or HPLC grade. Methanol was purchased from May and Baker (Dagenham, U.K.) and acetonitrile from Romil Chemicals (Shepshed, U.K.). Ammonium formate, formic acid and concentrated hydrochloric acid were all purchased from Fisons (Loughborough, U.K.). Water was purified using a Milli-Q water purification system (resistance$>$12 MΩ) from Millipore (Harrow, U.K.). Plasma, used in validation for the method, was pooled human donor plasma stored at -40°C until use. Urine was collected from five healthy volunteers and used to determine drug recovery.

Apparatus

The following apparatus was used in the study. A Waters liquid chromatograph (Millipore) equipped with a Model 660 solvent programmer, Model 510 pumps, a WISP 710 B autosampler and a Model 490 programmable multiwavelength detector.

Pharmacokinetics in man

The drug was given as a single intravenous bolus injection, at the phase-I trial starting dose (5 mg/m^2). Blood was removed periodically via an indwelling intravenous cannula into heparinised tubes and plasma was prepared by centrifugation (1000 *g* for 5 min). Plasma and urine samples were stored at -20°C until analysis.

Extraction method and validation of the assay

The drug was isolated from plasma or urine by solid-phase extraction with 200 mg per 3 ml C_2 Bond Elut cartridges (Jones Chromatography, Llanbradach, U.K.). The cartridges were solvated prior to use with approximately 3 ml of methanol followed by 10 ml of deionised water. Anthrapyrazole I was extracted by passing either 1–2 ml of plasma or 1 ml urine through the cartridges. Plasma proteins/urinary contaminants were eluted in 10 ml deionised water prior to eluting the analyte in 2 ml concentrated hydrochloric acid (10.2

M) in methanol (1:19, v/v). The collected fractions were evaporated to dryness under a stream of nitrogen at 45°C. The plasma and urine samples were reconstituted in 200 and 500 μl of HPLC mobile phase, respectively. After vortexing, the reconstituted fractions were clarified by centrifugation at 1000 g for 5 min prior to analysis by HPLC (injection volumes: plasma, 150 μl; urine, 25 μl). Recovery was determined by comparison of the peak area of plasma and urine samples containing known amounts of I with the same amount of I dissolved in 200 μl HPLC mobile phase. Column performance, expressed as theoretical plate count (N), was calculated using the equation $N=16(R/W)^2$ (Sigma 4 method) where R=peak retention and W=peak width as measured by extrapolating tangents from the peak to the baseline.

High-performance liquid chromatography

The reconstituted fractions were assayed by HPLC using a 15 cm×0.46 cm Spherisorb C_6 analytical column (5 μm particle size) (Phase Separations, Queensferry, U.K.) fitted with a Co:Pell ODS (C_{18}) pre-column (Whatman, Clifton, NJ, U.S.A.). The analyte was eluted isocratically at a flow-rate of 1.5 ml/min at ambient temperature (back-pressure 120 bar). The pre-column was replaced when the back-pressure exceeded 240 bar. The mobile phase consisted of acetonitrile–methanol–0.25 M ammonium formate (adjusted to pH 3 with 98% formic acid) (1:1:8, v/v). Peaks were detected at the λ_{max} for I (492 and 385 nm), thereby introducing a high degree of selectivity.

Peak identification

The identity of I was confirmed by a multiple-wavelength ratio method. Plasma samples were taken, 5–15 min post administration, from six patients receiving an intravenous dose of I (30–50 mg/m^2). The drug was extracted from 0.5–1.0 ml aliquots of plasma and reconstituted in 200 μl of HPLC mobile phase as described above. Aliquots (25 μl) were analyzed by HPLC (flow-rate 1 ml/min) with detection at 254, 360, 385, 410, 465, 492 and 515 nm. Reference standards in triplicate, at 0.1 mg/ml I in HPLC mobile phase, were assayed directly by HPLC (10 μl injection volume) at the above wavelengths. The absorbance ratios, relative to the peak height at the λ_{max} for I (492 nm), were calculated for both the reference and patient samples.

Quantitation of clinical samples

Drug standards were prepared in plasma or urine at 50 and 500 ng/ml on each occasion samples were analysed. The standards and recovery standards (I diluted directly in 200 μl HPLC mobile phase) were each assayed in triplicate, before and after each batch of clinical samples ($n<15$). Chromatographic data were stored and analyzed, by integrating the area under the I peak, using a Trilab 3000 multichannel chromatography data system (Trivector, Sandy, U.K.). The levels of I in patient plasma (single determination) and urine sam-

ples (analysed in triplicate) were quantitated by external standardisation using the mean peak area of the 500 ng/ml standards prepared in plasma. The 50 ng/ml standards in plasma were analysed as unknown samples to confirm linearity.

Drug stability in human plasma stored at −20°C

The stability of I, stored over a period of ten weeks at −20°C, was determined by analyzing 2-ml aliquots of I at 500 ng/ml in control human plasma. The percentage recovery was calculated with reference to the appropriate drug standard in 200 μl of HPLC mobile phase. All patient samples were analysed within ten weeks of collection.

RESULTS

Figs. 2 and 3 show typical chromatograms of I after extraction from plasma and urine. Recovery from plasma was satisfactory (Table I) and linear over the concentration range 5–1000 ng/ml (correlation coefficient $r=0.999$, significance of regression $p<0.01$) and did not deteriorate on storage for ten weeks at −20°C (Table I). The coefficient of variation for the assay was <6%. Recovery of I from urine was also linear over the concentration range 100–1000 ng/ml (correlation coefficient $r=0.987$, significance of regression $p<0.01$) (Table II).

The drug was assayed by isocratic elution using a C_6, reversed-phase analytical column (Figs. 2 and 3). The use of a C_6 column in preference to a C_{18} or C_8 column resulted in an improved peak shape and higher plate count. The

TABLE I

RECOVERY OF I FROM PLASMA

Concentration added (ng/ml)	Recovery (mean ± S.D., $n=4$) (%)	Coefficient of variation (%)
1000	94 ± 3	3
400	82 ± 3	3
100	81 ± 3	4
50	93 ± 8	9
10	89 ± 9	10
5	101 ± 6	6
Mean	90 ± 8	8
Plasma samples stored at −20°C		
500 ng/ml week 1	91 ± 6	7
500 ng/ml week 10	94 ± 4	5

TABLE II

RECOVERY OF I FROM URINE CONTAINING 500 ng/ml I

Sample No.	Recovery (mean ± S.D., $n=3$) (%)	Coefficient of variation (%)
1	91 ± 10	10
2	84 ± 8	10
3	88 ± 7	8
4	82 ± 1	1
5	84 ± 1	1

mobile phase was optimised using the C_6 column and it was found that a mixture of acetonitrile and methanol was superior to methanol or acetonitrile alone. The ammonium formate component of the mobile phase was found to improve the peak shape by reducing peak tailing without having a significant effect on peak retention. Similarly, peak tailing was greatly reduced at pH 3 as opposed to pH 6. The optimal mobile phase, therefore, comprised acetonitrile–methanol–0.25 *M* ammonium formate, pH 3 (1:1:8, v/v) at a flow-rate of 1.5 ml/min. This system resulted in a satisfactory retention volume of 5.2 ml and theoretical plate count ($N = 10478\ m^{-1}$).

The identity of I in patient plasma samples was confirmed by a multiple-wavelength ratio method. The peaks detected in patient plasma samples co-eluted with standard I and gave identical wavelength ratio values at various

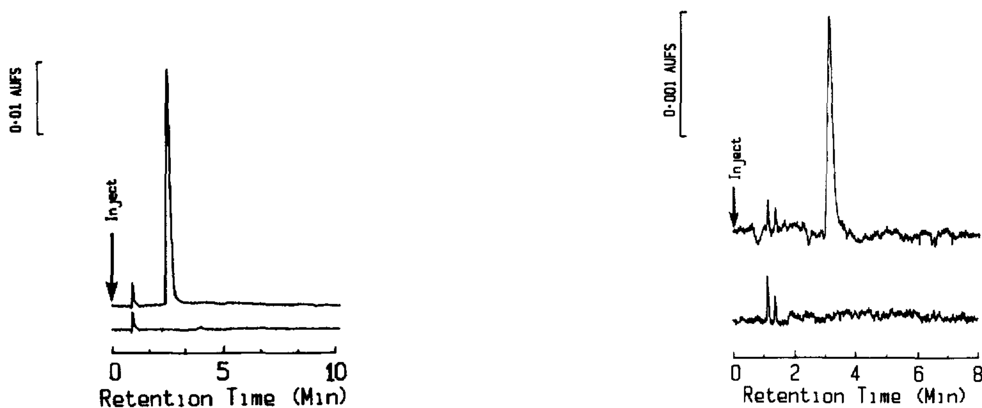

Fig. 2 (left). Chromatogram of I (500 ng/ml) after extraction from plasma using a C_2 Bond Elut cartridge (injection volume 150 µl). Lower trace shows control plasma. Detection at 492 nm.

Fig. 3 (right). Chromatogram of I (500 ng/ml) after extraction from urine using a C_2 Bond Elut cartridge (injection volume 25 µl). Lower trace shows control urine. Detection at 492 nm.

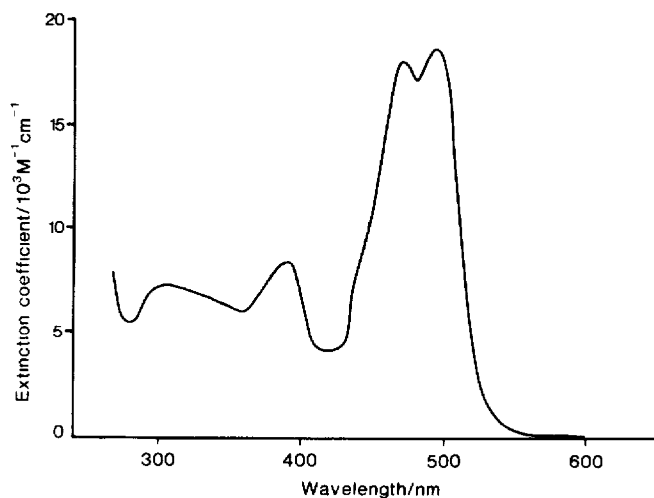

Fig. 4. Ultraviolet-visible spectrum of I.

TABLE III

IDENTIFICATION OF I IN PATIENT PLASMA SAMPLES

Wavelength (nm)	λ-Ratio[a]	
	Standard I ($n=3$)	Unknown peak ($n=6$)
254	0.633 ± 0.017	0.636 ± 0.019
360	0.239 ± 0.004	0.239 ± 0.005
385	0.350 ± 0.008	0.353 ± 0.004
410	0.128 ± 0.003	0.124 ± 0.002
465	0.762 ± 0.014	0.766 ± 0.011
492	1.0	1.0
515	0.152 ± 0.001	0.152 ± 0.005

[a]Ratio of the peak height to the peak height at 492 nm (λ_{max}) (mean ± S.D.).

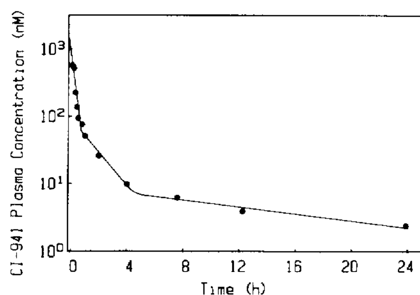

Fig. 5. Pharmacokinetics of I in a patient treated at the phase-I trial starting dose (5 mg/m²).

λ_{max} and λ_{min} across the I ultraviolet–visible spectrum (Fig. 4, Table III). The limit of detection for a 2-ml aliquot of plasma was 1 ng/ml, enabling the pharmacokinetics of I to be determined in patients treated at 5 mg/m^2 (Fig. 5).

DISCUSSION

A sensitive HPLC assay has been developed for the determination of I in human plasma and urine. The method involves the solid-phase extraction of the drug onto C_2 reversed-phase Bond Elut cartridges followed by a concentration step to enhance the sensitivity of the assay. In developing an assay for this drug, note was taken of methods published previously for the structurally related antitumour agent mitoxantrone.

Ehninger et al. [8] and Reynolds et al. [9] have described the use of XAD-2 columns for the extraction of mitoxantrone from plasma. These methods were not evaluated as the packing of XAD-2 columns is tedious and time-consuming and sensitivity can be poor, i.e. 75 ng/ml [9]. Other investigators have devised highly selective and sensitive assays based on organic phase extraction of mitoxantrone from alkalinised plasma. Van Belle et al. [10] have used hexanesulphonic acid as an ion-pair former and ametantrone as an internal standard. The drug was extracted into dichloromethane and the organic fraction evaporated to dryness and reconstituted in HPLC mobile phase prior to analysis. The authors report a limit of detection of 1 ng/ml which enabled the identification of the prolonged terminal γ-phase half-life in patients receiving a dose of 15 mg/m^2 [11]. A similar method was attempted for the extraction of I from alkalinised plasma (pH 12). Various organic solvents were evaluated with or without a sodium chloride-saturated aqueous phase. Typically extraction into ethyl acetate or diethyl ether was poor ($<40\%$) and variable (data not shown). Reconstitution of the evaporated fractions was also difficult due to the presence of contaminating plasma components, presumably lipids. Consequently, in order to achieve the desired sensitivity (<10 ng/ml), solid-phase extraction was evaluated as an alternative.

Peng et al. [12] have described a solid-phase extraction technique for the analysis of mitoxantrone which utilised C_{18} reversed-phase Bond Elut cartridges. The high recovery ($98\pm6\%$) and sensitivity (1 ng/ml) compares favourably with the best of other techniques. Therefore the extraction of I was evaluated using C_{18}, C_8 and C_2 Bond Elut cartridges. Unlike mitoxantrone, the C_{18} (and C_8) Bond Elut cartridges proved unsatisfactory due to poor (approximately 30%) and non-linear recovery at low drug concentrations (<100 ng/ml). However, results with the C_2 Bond Elut enabled levels of less than 5 ng/ml to be measured in plasma (mean recovery $90\pm7\%$, mean coefficient of variation 8%). Furthermore, recovery was linear over the concentration range 5–

1000 ng/ml and detection of the analyte at 492 nm introduced a high degree of selectivity into the assay.

The chromatographic properties of I were found to be markedly different from those of mitoxantrone. Numerous HPLC methods which employ C_{18} reversed-phase analytical columns have been published for mitoxantrone [8–10, 12–14]. These methods frequently require the inclusion of an ion-pair former, such as pentane- or hexanesulphonic acid, to improve peak shape. In the present study, by using a C_6 reversed-phase column, an excellent peak shape was obtained with a relatively simple solvent system which did not involve the use of an ion-pair reagent. The identity of I extracted from patients' plasma was confirmed by a multiple-wavelength ratio method. Confirmation by mass spectrometry was not possible, as I failed to form a detectable molecular ion using either electron-impact or chemical ionisation techniques.

The assay described herein is selective and precise. Coupled with its high sensitivity the assay has enabled the study of I pharmacokinetics in patients treated at the phase-I starting dose (5 mg/m^2). Drug was detectable at 24 h post injection enabling an accurate determination of the pharmacokinetics of I to be made. This information will in turn be used to expedite the clinical evaluation of this new anticancer drug.

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