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High-performance liquid chromatographic assay for the measurement of the novel microtubule inhibitor 1069C85 in biological tissues and fluids

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ABSTRACT

1069C85 is a novel tubulin binder developed to circumvent the resistance associated with the *Vinca* alkaloids. Cytotoxic activity has been demonstrated *in vitro* against a variety of tumour cell lines, including a variant of the P388 leukaemia with acquired resistance to vincristine. A phase I clinical trial is planned and an assay suitable for preclinical and clinical pharmacokinetics has been developed. A high-performance liquid chromatographic (HPLC) assay is described which allows measurement of 1069C85 in plasma, urine, and tissue samples. The method uses reversed-phase chromatography with isocratic elution and detection by fluorescence at 406 nm following excitation at 340 nm. The assay is specific, sensitive (limit of sensitivity 0.25 ng/ml) and reproducible (coefficient of variation $<5\%$). The method has been used to study the pharmacokinetics of 1069C85 in Balb C mice following a single oral dose of 1 mg/kg. The maximum plasma concentration was reached 15 min after administration and subsequent elimination was slow with a half life of 6.5 ± 2.2 h. The drug remained detectable in plasma, at 1 ± 0.5 ng/ml, 24 h after this dose. This assay will be used to determine the pharmacokinetic profile of 1069C85 in mice and in a forthcoming phase I clinical trial.

INTRODUCTION

Tubulin binding agents remain a major component of cancer chemotherapy, the *Vinca* alkaloids [1–3] being particularly important in the treatment of leukaemia and lymphoma. Interest in tubulin as a target for cancer chemotherapy has recently been renewed owing to the clinical activity against platinum complex refractory cancer of the taxanes, taxol and taxotere, agents which cause abnormal aggregation of microtubules [4]. The synthetic compound 1069C85 [N-6-(3,4,5-trimethoxybenzyloxy) imidazo 1,2-b-1-pyridazin-2-yl carbamate], see Fig. 1, was developed in an attempt to circumvent resistance to *Vinca* alkaloids. It retains the trimethoxybenzene ring common to podophyllotoxin and colchicin. 1069C85 shows potent cytotoxic activity against a variety of tumour cell lines *in vitro* with IC50 concentrations in the range 10^{-8}–10^{-9} *M*. 1069C85 also shows a lack of cross-resistance with a number of agents known to be susceptible to P-glycoprotein when tested against variants of the P388 leukaemia with acquired resistance to a variety of drugs [5].

The drug is scheduled for phase I clinical trial in 1993 under the auspices of the Cancer Research Campaign. Given the potency of 1069C85, a sensitive and reproducible method for the measurement of 1069C85 in biological samples was required. A novel HPLC method is described which is adapted from a previous method from J. Salmon (unpublished data). The

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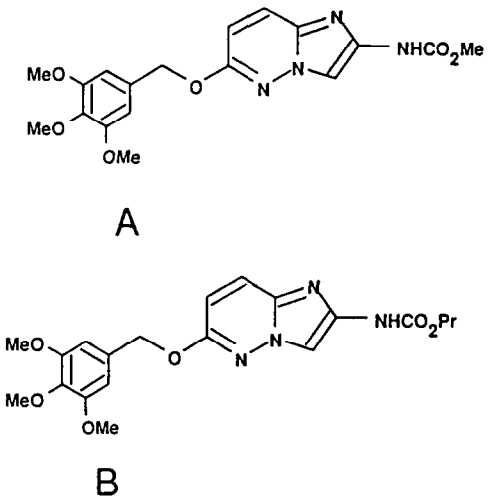

Fig. 1. Structure of (a) 1069C85 and (b) 27C87.

pharmacokinetics of 1069C85 will be studied in mice in order to investigate the relationship between toxicity and plasma concentration and to evaluate the feasibility of performing a pharmacokinetically guided dose escalation in man. An example of preclinical pharmacokinetic data obtained with this assay is included in this report.

EXPERIMENTAL

Chemicals

1069C85 and the internal standard 27C87 (Fig. 1) were provided by Wellcome (Beckenham, UK); HPLC-grade methanol and analar chloroform were purchased from BDH (Poole, Dorset). All other reagents were analytical grade.

Animals

Female Balb C mice were purchased from OLAC (Bicester, UK).

High-performance liquid chromatography

Separations were carried out on a C_{18} reversed-phase column (25 cm × 4.6 mm I.D.) packed with 5 μm particles of Hypersphere from Shandon. This was preceded by a guard column (5 cm × 2.1 mm I.D.) packed with 56 μm pellicular ODS for guard columns from Whatman (Maidstone, UK). The mobile phase consisted of methanol–deionized water (75:25, v/v), filtered prior to use (22-μm PTFE Whatman). Elution was performed isocratically at a flow-rate of 1.3 ml/min.

Samples were delivered using a Kontron 465 autosampler (Watford, Herts, UK) equipped with a 100-μl syringe. Detection was performed using a Perkin-Elmer LS4 fluorimeter set at 340 nm excitation and 406 nm emission with a slit width of 10 nm.

Data acquisition and analysis were performed using the Kontron MT2 data system. Peak areas were determined by electronic integration. Calibration curves were obtained by plotting the peak-area ratio of 1069C85 to 27C87 as a function of the spiked 1069C85 and equations were derived from the unweighted linear least-squares fit of the curves defined by these calibration standards. Drug identification was achieved by coelution with a reference standard and quantitation was done by using the peak-area ratios on the previously defined equation.

Plasma

Human plasma samples containing 1069C85 were used for method development. An organic extraction procedure was developed and a sonification step was found to be required following addition of the internal standard to ensure reproducibility.

Assays were performed using 0.5-ml aliquots of plasma, to which 50 ng of the internal standard 27C87, an analogue of 1069C85 (Fig. 1), and in the case of standard curve samples varying amounts of 1069C85, were added in 50 μl methanol. The resulting mixtures were sonicated for 2 min. Samples were then treated with 3 ml of chloroform, thoroughly mixed on a multitube vortex-mixer (Baxter, speed 4) for 40 s and centrifuged at 1500 *g* for 10 min. The aqueous phase was discarded and the organic phase evaporated to dryness under vacuum in a Speed Vac sample concentrator for 1 h without heating. Residues were reconstituted in 100 μl of methanol, sonicated for 1 min in a water bath and the supernatant transferred to capped plastic vials. Samples (50 μl) were injected into the HPLC system for analysis. Twenty calibrations standards consisted of two blank plasmas, and 9

standards in duplicate (0.625, 1.25, 2.5, 5, 10, 20, 40, 80, 160 ng/ml) made up in human plasma.

Quality control samples were included at 1.4, 10, 110 ng/ml. The reproducibility of the assay was established by running 10 quality controls of each type on 3 consecutive days. Routine assays included only one duplicate of each quality control sample. The lower limit of quantification was determined by diluting the lower plasma sample of the standard curve until intra-assay variation was greater than 20%.

In order to validate the assay for mouse plasma prior to pharmacokinetic studies, mouse plasma was diluted 1/5 with human plasma and then spiked with 0, 10, 50, 150 ng/ml 1069C85 and measured against a human plasma standard curve. A standard curve was also analysed in human urine using the same concentrations as for plasma.

Tissues

Samples of brain, liver, kidney and intestines were obtained from Balb C mice and homogenized with a Potter homogeniser for 25 s in 5 ml methanol/g tissue. The homogenate was then spiked with 50 μl of 1069C85 solution, at a concentration of 10, 20, 50, or 100 ng/ml. A fixed amount of 27C87, the internal standard, was then added to the samples and the resulting homogenates analysed by HPLC against a standard curve in methanol.

Solubility and stability

Solubility was determined in several different solvents including dimethylsulphoxide, chloroform, water, methanol, perchloric acid and sodium chloride. A saturated solution of 1069C85 was made in each solvent and an aliquot diluted in methanol and analysed by HPLC against a standard curve in methanol.

Stability was determined by comparing plasma samples spiked with a range of concentrations of 1069C85 which had been freshly prepared with others left at room temperature for 24 h.

Pharmacokinetics in mice

Female Balb C mice were caged on sawdust and adapted to laboratory conditions for 2 weeks prior to the experiment. Animals were allowed food and water *ad libitum*. Concentrations of 1069C85 were measured in mouse plasma after a single administration of 1 mg/kg, given orally in a suspension of 0.1% Tween 80–0.9% saline. The animals were sacrificed by carbon dioxide asphyxiation (3 animals per time point) and blood was obtained by cardiac puncture at 10, 20, 40 min and 1, 2, 4 and 8 h after drug administration, and placed in heparinized tubes. The blood samples were centrifuged, the plasma removed and stored at − 20°C until analysis.

Statistical analysis

Coefficients of variations were calculated using the Statgraphics ANOVA test [6]. The pharmacokinetic parameters were evaluated using the MK Model curve fitting programme [7].

RESULTS AND DISCUSSION

1069C85 is a potent tubulin binder which is shortly to enter phase I clinical trial. 1069C85 has similar cytotoxic potency and mode of action to that of the *Vinca* alkaloids. The understanding of the pharmacokinetics of these latter drugs in humans was delayed by the lack of a sufficiently sensitive analytical technique [9,10]. This paper describes a HPLC assay with fluorimetric detection for the measurement of 1069C85 in plasma, urine, brain, liver, kidney and intestine which has been validated according to regulatory guidelines [8]. The HPLC assay described achieves separation of 1069C85 and 27C87, no interfering peaks being found in any of the matrices studied (Figs. 2 and 3) (data not shown for brain, intestine and urine). The method is rapid, elution being complete by 10 min. Owing to the limited solubility of 1069C85 and the internal standard (Table I) it was necessary to sonicate the samples prior to extraction in order to ensure reproducibility. The plasma standard curve as calculated by the Kontron MT2 data system was always linear ($r > 0.99$) and the variation on the curve was below 5% (data not shown). The inter- and intra-assay coefficients of variation were below 5% (Table II). 1069C85 was stable in plasma for up to 24 h. The limit of quantification of the assay in human plasma was

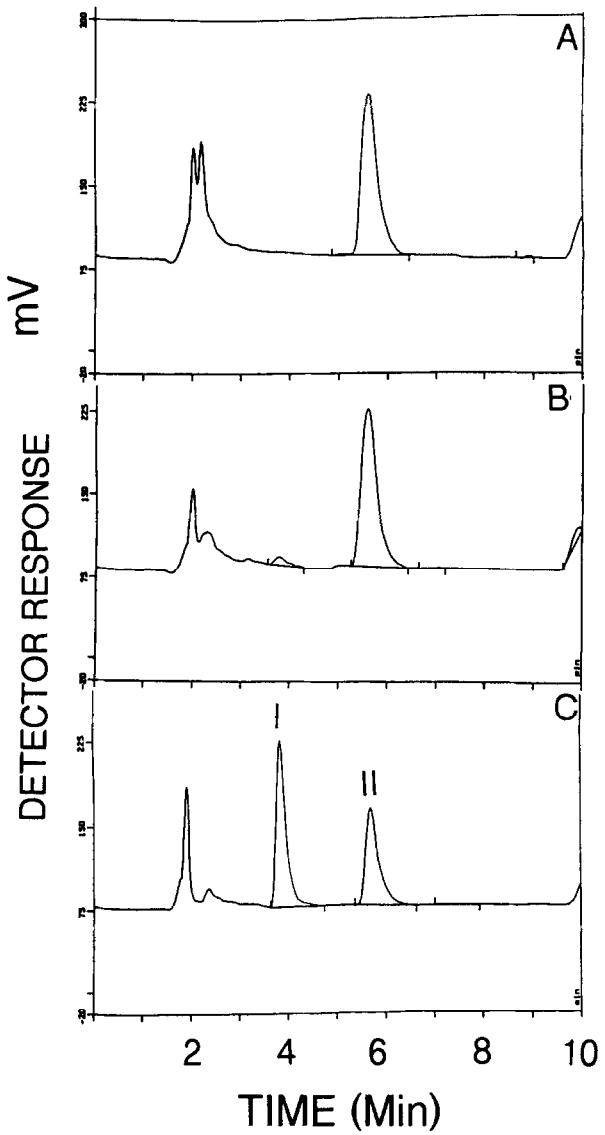

Fig. 2. A representative chromatogram of (a) blank plasma, (b) plasma spiked with 0.625 ng/ml 1069C85, and (c) plasma spiked with 160 ng/ml 1069C85. Peaks: I = 1069C85, II = 27C87. Chromatographic conditions; reversed-phase column (25 cm × 4.6 mm I.D.) 5 μm hypersphere, mobile phase (methanol–water, 75:25, v/v) run isocratically at 1.3 ml/min. Fluorimetric detection at 340 nm excitation, 406 nm detection. Samples were extracted with 6 volumes chloroform after addition of the internal standard.

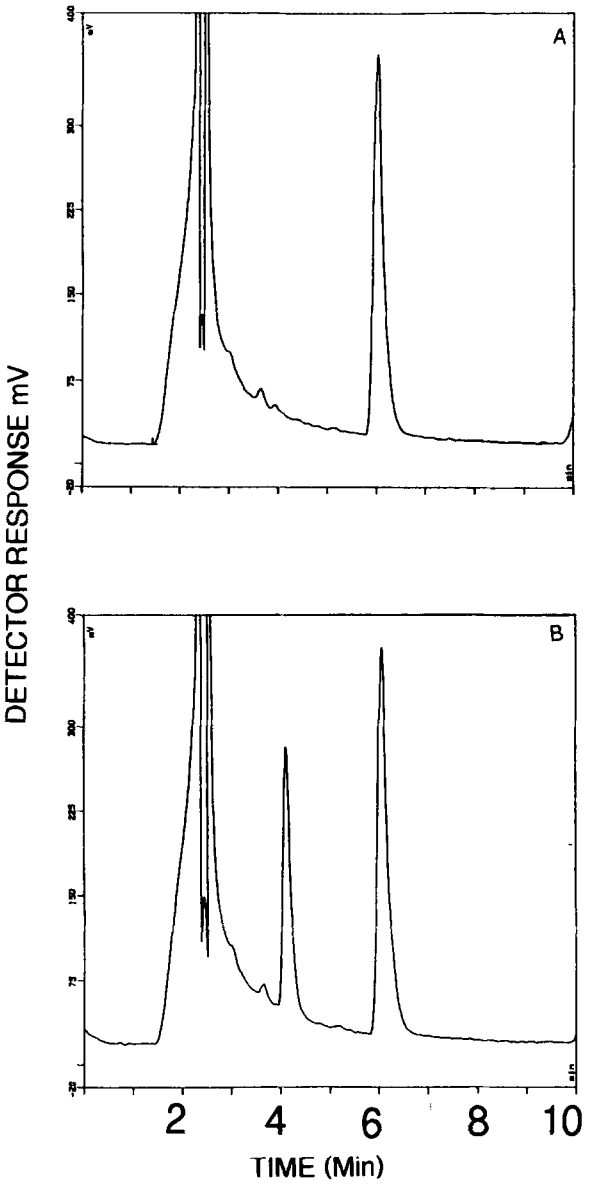

Fig. 3. Chromatogram of a blank mouse liver (20% homogenate in methanol) spiked with 27C87 (50 ng) and a mouse liver spiked with 100 ng/ml 1069C85. Chromatographic conditions as in Fig. 2.

0.25 ng/ml (0.6 n*M*) which corresponds to 80 pg on the column and is far below the cytotoxic levels of the drug [5].

Following oral administration of 1069C85 to mice, the drug was found to be rapidly absorbed and a maximum concentration of 34 ± 12 ng/ml was reached 15 min after administration (Fig. 4). This drug level is above cytotoxic concentrations. 1069C85 was eliminated slowly with a $t_{1/2}$ of 6.5

TABLE I

SOLUBILITY OF 1069C85 IN DIFFERENT SOLVENTS

Solvent	Solubility (μg/ml)
DMSO	3000
Chloroform	80
Water	0.8
Methanol	1.6
Perchloric acid	0.6
Sodium chloride	0.08

h and the drug was still detectable 24 h later at a concentration of 1 ± 0.5 ng/ml. Although the assay is very reproducible, large variations were observed in the mean concentrations of 1069C85 in the plasma. This could be the result of inter individual variation possibly caused by differential absorption.

The limit of quantification of this assay in plasma is below the *in vitro* cytotoxic concentration range (10^{-8}–10^{-9} *M*) and the assay will therefore be helpful in predicting toxic concentrations in man. Further studies in mice will be carried out to determine the linearity of the area under the concentration–time curve (AUC) with increasing doses of the drug and to investigate tissue and tumour distribution. It is known that 1 mg/kg is the maximum tolerated dose by the intravenous route and a comparison between the pharmacokinetics after intravenous and oral administration will be performed to estimate the oral bioavailability. These results should indicate the feasibility of performing a pharmacokinetically guided dose escalation in the forthcoming clinical trial.

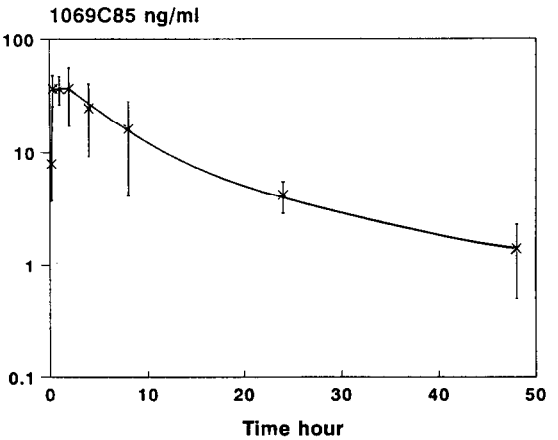

Fig. 4. Time course of 1069C85 concentrations in mice plasma after a single oral administration of 1 mg/kg to Balb C mice in a suspension in Tween 80–saline.

ACKNOWLEDGEMENT

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TABLE II

COEFFICIENTS OF VARIATION

Analysis of 10 replicates of low (1.4 mg/ml), medium (10.5 ng/ml) and high (110 ng/ml) concentrations of 1069C85 on three consecutive days. Results have been analysed with Statgraphics

Nominal concentration (ng/ml)	Concentration found (mean ± S.D.) (ng/ml)			Coefficient of variation (%)	
	Day 1	Day 2	Day 3	Inter-assay	Intra-assay
1.4	1.368 ± 0.0411	1.413 ± 0.0411	1.536 ± 0.0739	2.5	4.4
10.5	10.46 ± 0.163	10.45 ± 0.308	10.18 ± 0.249	1.3	2.4
110	104.1 ± 10.79	110.2 ± 2.76	111.3 ± 3.67	3.6	4.2

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