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Validation of liquid chromatography assay for the quantitation of (*Z*)-3-[2,4-dimethyl-5-(2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-1H-pyrrol-3-yl]propionic acid (SU006668) in human plasma and its application to a phase I clinical trial

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Abstract

The validation of an analytical method to quantify the antiangiogenic, (*Z*)-3-[2,4-dimethyl-5-(2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-1H-pyrrol-3-yl]propionic acid (SU006668) for pharmacokinetic determination in a phase I clinical trial, is described. HPLC, with a gradient mobile phase and UV detection at 440 nm, was used. SU006668 was extracted from plasma by precipitation of proteins with acetonitrile. The assay was linear from 25 to 2000 ng/ml (r^2=0.997); sensitive (limit of quantification 25 ng/ml), accurate (RE 2.6–11.9%) and reproducible (inter-batch precision C.V. 3.2%). Pharmacokinetic data for six patients are presented. They show linear pharmacokinetics with a low volume of distribution and induction at doses of 50, 100 and 200 mg/m^2.

Keywords: SU006668

1. Introduction

Recent in vitro studies have shown SU006668, a novel anticancer agent, to be a potent inhibitor of signalling through the receptor tyrosine kinases Flk-1, platelet derived growth factor receptor (PDGFR) and fibroblast growth factor receptor (FGFR) [1]. Binding of their respective growth factors, vascular endothelial growth factor, PDGF and FGF leads to autophosphorylation of the split kinase domain of these receptors initiating the signalling cascade that leads to angiogenesis [2]. Angiogenesis is the process of formation of new blood vessels and is vital for small tumours if they are to grow beyond 1–2 mm in size and metastasise [3]. It has been shown that the quantity of new vessel formation in primary tumours relates directly to poor prognosis in many carcinomas including breast [4], bladder [5] and colon [6]. Preclinical studies in athymic mice showed that SU006668 achieved better than 75% growth inhibition against a wide range of human tumour xenografts [7]. In addition, inhibition of angiogenesis was confirmed by an observed reduc-

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tion in microvessel formation compared with controls [7]. SU006668 also induces regression in established tumour xenografts [7]. These factors suggested SU006668 was a suitable clinical candidate for patients with solid tumours and a clinical trial of the drug was initiated. A phase I single centre dose escalation study of the intravenous formulation of SU006668 was undertaken at The Royal Marsden Hospital under the auspices of the Cancer Research Campaign Phase I/II Committee. The primary objectives of the trial were to establish the maximum tolerated dose of SU006668; the toxicity profile of the study compound and the dose limiting toxicity; and thus to propose a safe dose for phase II evaluation. A method to quantify the compound in human plasma has been developed and validated according to established guidelines [8]. This method was used to support pharmacokinetically guided dose escalation in the phase I trial. This study describes the chromatographic conditions as well as sample extraction and complete validation of the method. It also presents the pharmacokinetic (PK) analysis of the first six patients dosed with intravenous SU006668.

2. Experimental

2.1. Apparatus

HPLC analyses were performed on a P4000 quaternary pump equipped with an AS3000 autosampler and UV6000 detector all supplied by Thermo-Finnigan (Hemel Hempstead, UK). All peak area integration and quantification were carried out using ChromQuest™ software associated with the HPLC system. Calibration curves were generated from plotting the peak area ratios of SU006668 to the internal standard (IS) SU009905 against concentration of SU006668 spiked into plasma and weighted as $1/y$, the dependent variable.

2.2. Chromatography

Initial chromatography was performed on a Luna C_{18} 50×4.6 mm I.D. column (Phenomenex, Macclesfield, UK) using a gradient mobile phase starting at 20:80 Acetonitrile:20 m*M* ammonium acetate. The resulting calibration line was not linear over the required range. We decided to use an ion-exchange 150×4.6 mm I.D. 5 μm ABZ+ column with a 20×2 mm guard column (Supelco Dorset, UK) with a linear gradient mobile phase from 100% water to 100% acetonitrile over 30 min. Neither SU006668 or the I.S. eluted under these conditions. Optimal chromatography and linearity were finally achieved using reversed-phase ion-exchange chromatography on this ABZ+ column with a mobile phase of 100% 30 m*M* ammonium acetate, decreasing linearly to 50% ammonium acetate and 50% acetonitrile over a 10-min gradient then decreasing linearly to 0% ammonium acetate 100% acetonitrile. The flow-rate was a constant 0.8 ml/min (see Table 1).

The maximum absorbance (λ_{max}) of SU006668 is 440 nm hence this was chosen as the UV detection wavelength.

2.3. Reagents

Both methanol and acetonitrile were Laserpure HPLC grade obtained from Laserchrom Chemicals Ltd. (Rochester, Kent, UK) All other reagents were supplied as analytical grade.

2.4. Preparation of calibration and quality control (QC) samples

Calibration samples were prepared at concentrations of 25, 50, 100, 200, 500, 1000 and 2000 ng/ml, using 200 μl of human plasma and 50 μl of the appropriate spiking solution in methanol. Quality Control (QC) samples were prepared at the lower limit of quantification, (LLOQ), 25 ng/ml and at low, medium and high concentrations (60, 250 and 1500 ng/ml, respectively). 100 ng of I.S. was added

Table 1
Elution gradient used for separation of SU006668 and I.S.

Time (min)	Acetonitrile (%)	Ammonium acetate 100 m*M* (%)	Water (%)
0	0	30	70
10	50	30	20
15	100	0	0
16	100	0	0
17	0	30	70
20	0	30	70

to 200 μl aliquots of each QC and calibration plasma sample prior to extraction and analysis.

2.5. Extraction of SU006668 and internal standard from plasma

Plasma samples were acidified with 10 μl of 10% v/v, hydrochloric acid in water and vortex mixed for approximately 5 s. Proteins were precipitated using 1.5 ml of acetonitrile and the supernatant extract removed after centrifugation at 7000 *g* for 10 min. The extracts were evaporated to dryness, on heat setting 1, in a speed vac sample concentrator (Joane, France) and redissolved in 200 μl of methanol. Ten μl was injected onto the HPLC column.

2.6. Method validation

Validation of the method consisted of three precision batches. Each batch comprised duplicate human plasma standard curves, control human plasma, human plasma spiked only with I.S. and replicate ($n=6$) high, medium, low and LLOQ QC samples. The following parameters were evaluated.

2.7. Specificity

Six independent sources of control human plasma were checked for chromatographic peaks of endogenous origin that could affect assay sensitivity and accuracy. Methanol washes were injected after samples of high concentration to assess injection carryover.

2.8. Linearity of the human plasma standard curves

The reproducibility of calibration was assessed from the three duplicate standard curves. The effect of weighting on slope, intercept and coefficient of regression was evaluated.

2.9. Precision

Intra-batch and inter-batch coefficients of variation at the LLOQ and at low, medium and high concentrations were calculated from replicate ($n=6$) analysis of QC samples in assay batches 1–3.

2.10. Accuracy

The observed concentrations of QC samples were compared with the prepared concentrations. Results are expressed as relative errors (RE), calculated using the following equation:

$$\text{RE}\,(\%) = \frac{(\text{mean observed concentration} - \text{prepared concentration})}{\text{prepared concentration}} \times 100$$

2.11. Sensitivity

The precision and accuracy at the lower limit of quantification was assessed from replicate assay of the LLOQ QC sample (25 ng/ml) in assay batches 1–3.

2.12. Stability

The stability of standard stock solutions and working solutions was assessed by periodic HPLC–UV comparisons of stored solutions and freshly prepared stock solutions.

2.13. Freeze–thaw stability

Six aliquots (200 μl) at low and high concentration were designated freeze–thaw stability samples. These were thawed at room temperature for 2 h, refrozen three times for at least 24 h and analysed immediately after the third freeze–thaw cycle. The relative errors of observed concentrations of the stability samples were calculated.

2.14. Effect of diluting human plasma with plasma

It is likely that clinical samples may have SU006668 concentrations above the upper limit of quantification. The effect of diluting over-range samples to within the calibration range was therefore investigated. The accuracy (RE) and precision (C.V.) of dilution control samples were assessed.

2.15. Dosing schedule

The starting dose was 50 mg/m^2 with treatment delivered as an intravenous infusion using a concentration of 4 mg/ml and administered at 150 ml/h. The treatment plan involved two phases. Firstly, a 2-week induction phase to provide single and repeat dose PKs with treatment on day 1 and day 8–12 inclusive. Secondly, a weekly maintenance phase, with therapy once weekly in cycles of 6 weeks. All patients were required to have solid tumours refractory to standard therapy.

2.16. Pharmacokinetics in humans

Concentrations of SU006668 were measured in human plasma samples after i.v. administration of 50, 100 or 200 mg/m^2 on days 1, 8 and 12. Blood was collected via cannulation, in heparinised tubes, at the following time points: pre and post infusion, then at 5, 10, 20, 30, 45, 60, 90, 120 and 240 min for days 1 and 8, and the same time points in addition to 6, 12, 24 and 48 h post infusion for day 12. Samples were centrifuged and the plasma extracted and stored at −70 °C until required for analysis.

2.17. Pharmacokinetic analysis

Pharmacokinetic parameters were calculated with non-compartmental analysis (WinNonlin Pro version 3.0, Pharsight, Mountain View, CA). Model 202, a constant infusion for plasma model, was used. Days 1, 8 and 12 were modelled as separate days.

Pharmacokinetic parameters estimated in the model include: C_{max}: maximum concentration; T_{max}: Time of maximum observed concentration (end of infusion); AUC_{last}: Area under the curve from the time of dosing to the last measurable concentration. $t_{1/2}$lambda-z: terminal half-life ln2/λz (λz: first order rate constant associated with the terminal log-linear portion of the curve); MRT_{last}: Mean residence time from the time of dosing to the last measurable concentration = ($AUMC_{last}$/AUC_{last})-TI/2 (TI time of infusion); Vss observed: = MRTxCl; Vz, Vz_F: Volume of distribution based on the terminal phase = dose/λz* AUC_{inf}; Cl: total body clearance = Dose/AUC_{inf}; $AUC_{0-4\ h}$: Area under the curve from the time of dosing to 4 h post administration.

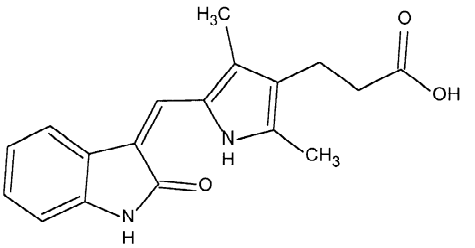

Fig. 1. Structure of SU006668.

3. Results

3.1. HPLC

The HPLC–UV method using the 15 cm ABZ+ reversed-phase column, with mobile phase at a flow-rate of 0.8 ml/min, gave a retention time for SU006668 (Fig. 1) and the I.S. (Fig. 2) of approximately 12.4 and 14.2 min, respectively. Representative chromatograms are shown in Figs. 3–6. They show chromatograms for control human plasma containing 0 ng/ml, 25 ng/ml (LLOQ) and 2000 ng/ml of SU006668.

3.2. Specificity

No peaks of significance were observed that would compromise the method following the analysis of control human plasma. No peaks co-eluted with either SU006668 or the I.S. Neither compound was detected in the wash samples injected after the 2000 ng/ml standard verifying that autoinjector carry over was not observed (Fig. 7).

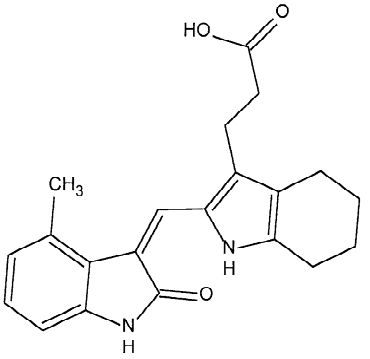

Fig. 2. Structure of SU009905 (Internal Standard).

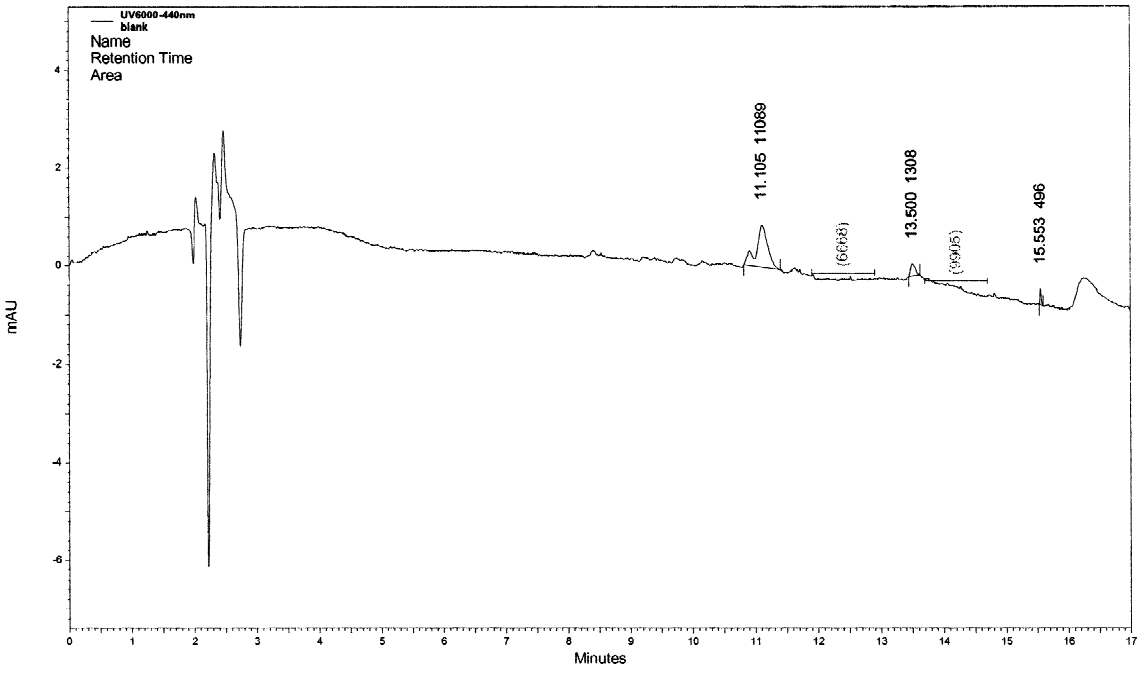

Fig. 3. Representative chromatogram of extracted blank human plasma.

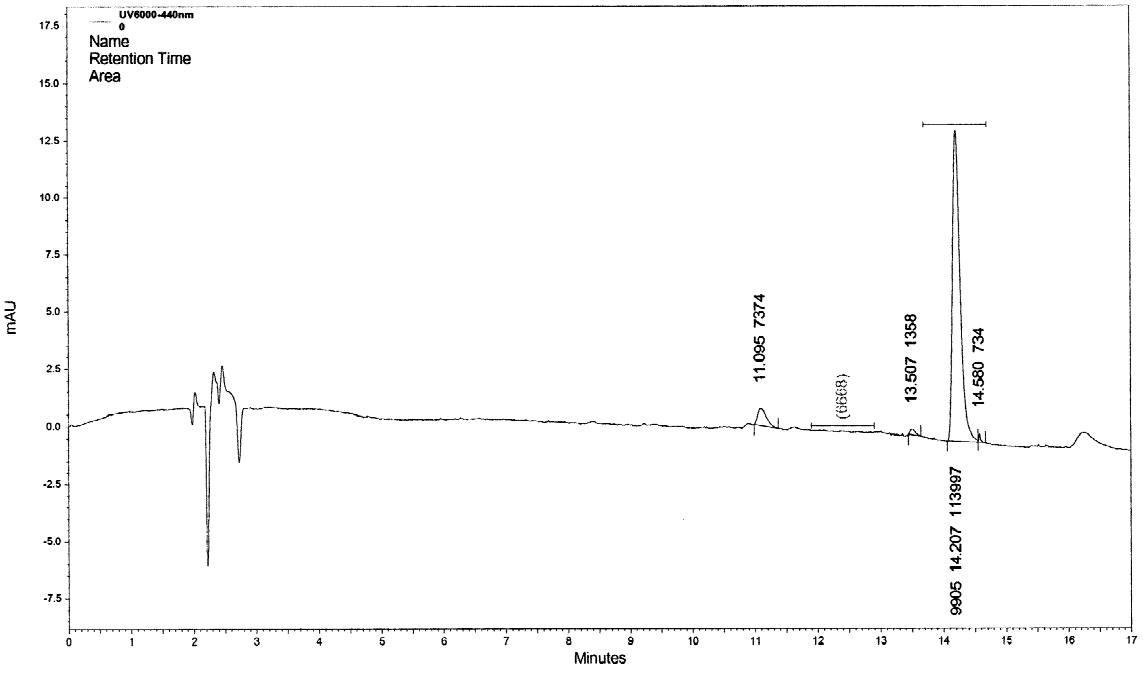

Fig. 4. Representative chromatogram of extracted zero (0 ng/ml) calibration standard.

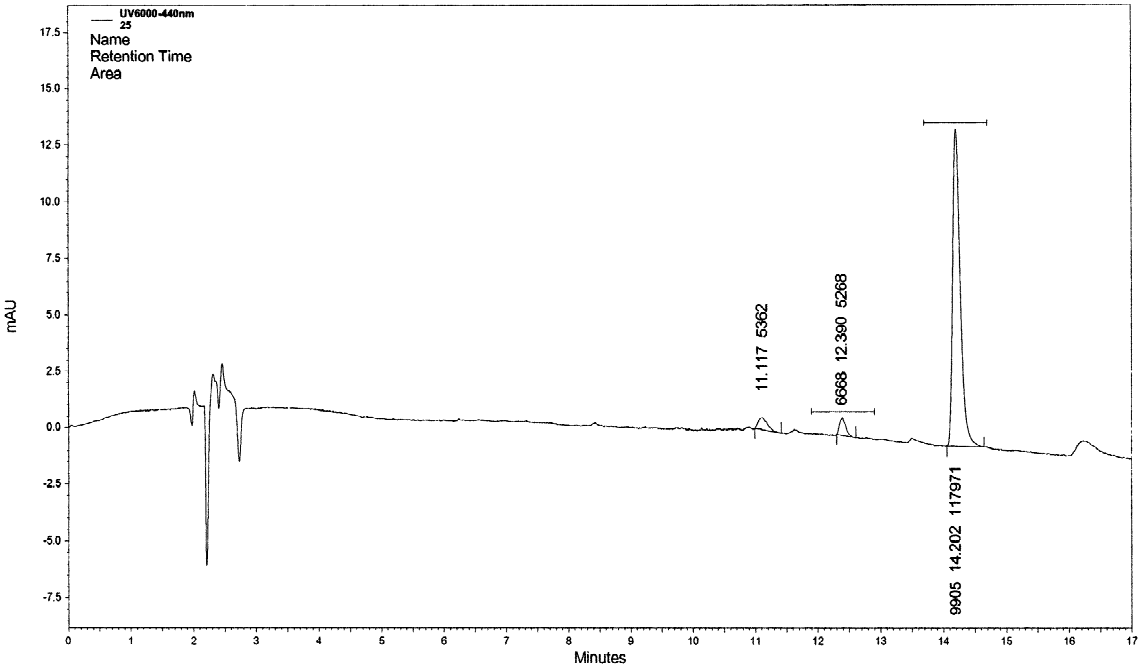

Fig. 5. Representative chromatogram of extracted LLOQ (25 ng/ml) calibration standard.

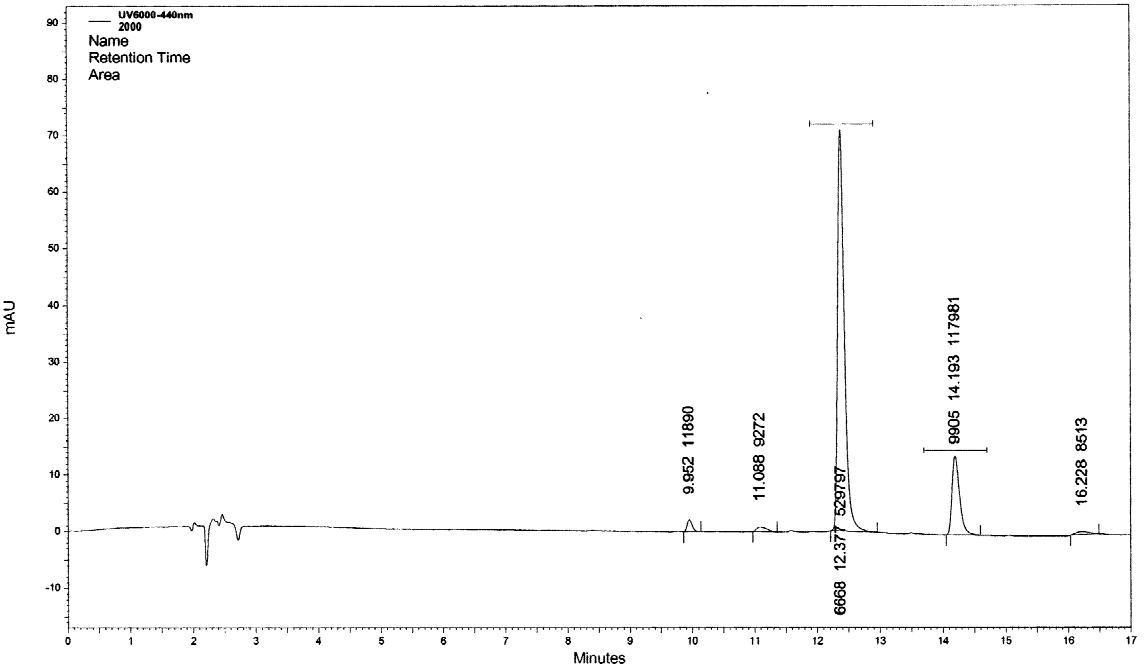

Fig. 6. Representative chromatogram of extracted 2000 ng/ml calibration standard.

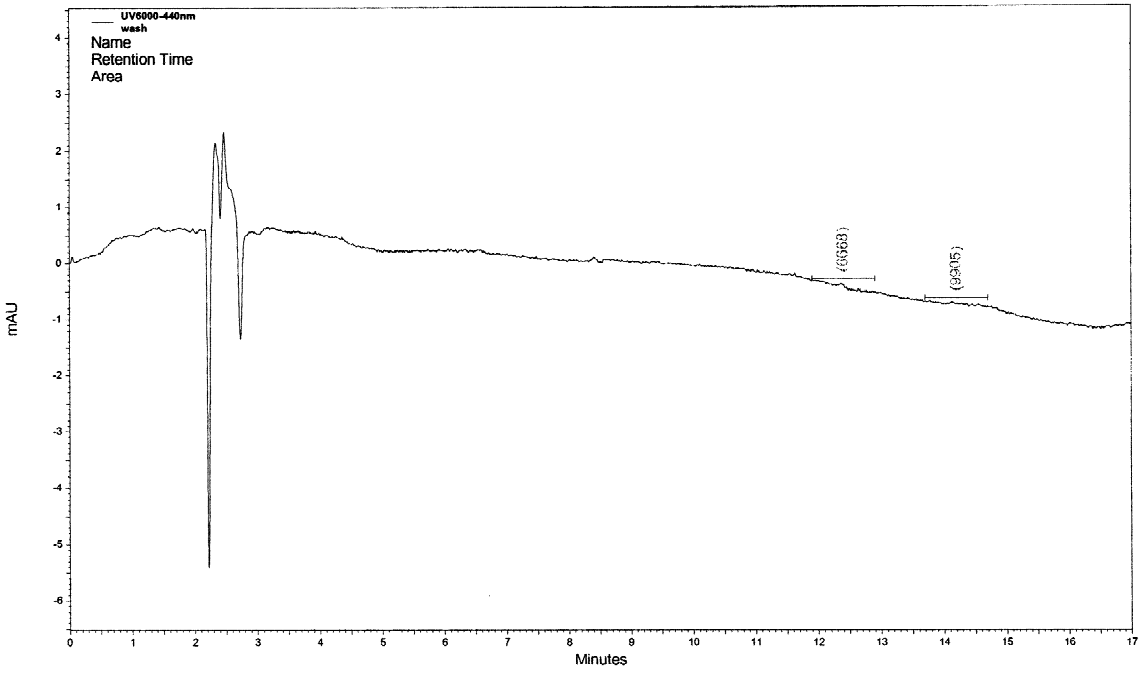

Fig. 7. Chromatogram of a methanol wash following 2000 ng/ml standard.

3.3. Intra-batch precision

The intra-batch precision for replicate ($n=6$) analysis of QC samples in assay batches 1–3 are expressed as C.V.'s of observed concentrations at each level. C.V.'s ranged from 2.0 to 2.2% at 25 ng/ml (LLOQ), 1.0 to 3.8% at 60 ng/ml, 0.4 to 0.8% at 250 ng/ml and 0.4 to 1.2% at 1500 ng/ml (Table 2a–c).

3.4. Inter-batch precision

The inter-batch precision of QC samples are expressed as C.V.'s ($n=18$) at each level for assay batches 1–3. C.V.'s were 3.2% at 25 ng/ml (LLOQ), 2.8% at 60 ng/ml, 2.3% at 250 ng/ml, and 2.6% at 1500 ng/ml (Table 3).

3.5. Accuracy

The REs of observed concentrations at each QC level were 6.6% at 25 ng/ml (LLOQ), 2.6% at 60 ng/ml, 4.4% at 250 ng/ml, and 11.9% at 1500 ng/ml (Table 3).

3.6. Sensitivity

At the LLOQ (25 ng/ml) the intra-batch precision, expressed as C.V.'s, ranged from 2.0 to 2.2%. The C.V. for inter-batch precision was 3.2%, with a RE of 6.6% ($n=18$) (Tables 2 and 3).

3.7. Stability

3.7.1. Stability of stock solutions

SU006668 was stable in standard stock solutions and working solutions for at least 1 month at -20 °C. I.S. was stable in solution for at least 1 month at -20 °C.

3.7.2. Stability in human plasma

SU006668 was stable in control human plasma at *ca* -70 °C following three freeze–thaw cycles (Table 4a and b).

3.8. Dilution of human plasma

The observed concentrations for dilution controls were in good agreement with the prepared con-

Table 2
Quality control samples

QC	LLOQ 25 ng/ml	QC Low 60 ng/ml	QC Medium 250 ng/ml	QC High 1500 ng/ml
(a) Results for batch 1 ($n=6$)				
1	24.966	62.906	266.080	1727.441
2	26.515	60.984	270.219	1751.530
3	25.726	62.751	271.883	1748.834
4	26.224	62.327	269.712	1714.500
5	26.013	61.627	267.087	1730.470
6	26.279	64.370	267.279	1709.830
Mean	25.954	62.494	268.710	1730.434
SD	0.552	1.167	2.234	17.152
C.V. (%)	2.1	1.9	0.8	1.0
(b) Results for batch 2 ($n=6$)				
1	26.094	59.866	256.990	1635.793
2	26.779	61.635	258.473	1683.164
3	25.857	61.469	261.585	1682.266
4	27.221	61.370	261.342	1679.686
5	25.970	61.172	258.315	1678.573
6	26.684	55.733	257.793	–
Mean	26.434	60.208	259.083	1671.896
SD	0.541	2.282	1.917	20.268
C.V. (%)	2.0	3.8	0.7	1.2
(c) Results for batch 3 ($n=6$)				
1	28.128	61.345	253.300	1634.380
2	26.888	61.537	254.801	1629.742
3	26.914	62.786	256.093	1619.354
4	27.431	61.841	254.47	1641.173
5	28.094	62.587	254.716	1634.951
6	28.118	61.624	255.831	1633.310
Mean	27.596	61.953	254.869	1632.152
SD	0.600	0.593	1.007	7.282
C.V. (%)	2.2	1.0	0.4	0.4

– = No data.

Table 3
Precision and accuracy summary of inter-batch quality controls ($n=18$)

	LLOQ (25 ng/ml)	QC Low (60 ng/ml)	QC Medium (250 ng/ml)	QC High (1500 ng/ml)
Mean	26.661	61.552	260.887	1678.529
SD	0.861	1.696	6.021	43.837
C.V. (%)	3.2	2.8	2.3	2.6
RE (%)	6.6	2.6	4.4	11.9
n	18	18	18	17

Table 4
Freeze–thaw stability results for SU006668 in human plasma

QC	1 freeze–thaw	3 freeze–thaws
Low (60 ng/ml)		
1	61.066	58.616
2	60.035	61.709
3		59.939
4		60.71
5		61.173
6		60.939
Mean	60.551	60.514
SD		1.097
C.V. (%)		1.8
Stability (%)		99.9
High (1500 ng/ml)		
1	1645.518	1660.014
2	1622.741	1712.649
3		1654.489
4		1684.656
5		1689.228
6		1680.005
Mean	1634.13	1680.174
SD		21.085
C.V. (%)		1.3
Stability (%)		102.8

Table 7
Summary of calibration parameters from batches 1–3

Batch number	Slope	Intercept	Correlation coefficient
1	0.00216	−0.00055	0.99797
2	0.00224	−0.00438	0.99749
3	0.00212	−0.01196	0.99702
Mean	0.00217	−0.00563	0.99749
C.V. (%)	2.71	–	0.05

centrations. The REs were −7.8, −0.4 and +5.1% after 100-, 1000- and 4000-fold dilutions, respectively. The corresponding C.V.'s were 1.4, 3.1 and 3.6% (Table 5).

3.9. Human plasma standard curve

The assay was linear over the range 25–2000 ng/ml ($r = 0.9975$, $n = 3$). Weighted least squares regression (1/y) was used. Values for peak area ratios, slope and correlation coefficient were repeatable, and a negligible intercept was seen (Tables 6 and 7).

Table 5
Precision and accuracy summary for the dilution quality controls

Dilution QC Conc. (μg/ml)	Dilution factor	Mean assayed Conc. (μg/ml)	RE (%)	C.V. (%)	*n*
800	4000	840.71	5.1	3.6	6
800	1000	796.716	−0.4	3.1	6
8	100	7.38	−7.8	1.4	6

Table 6
Peak area ratios of calibration points for all three validation batches

	Concentration (ng/ml)						
	25	50	100	200	500	1000	2000
Batch 1	0.051	0.117	0.212	0.403	1.194	2.043	4.371
Batch 1	0.045	0.100	0.199	0.402	1.165	2.136	4.491
Batch 2	0.054	0.113	0.214	0.403	1.217	2.095	4.569
Batch 2	0.050	0.100	0.191	0.393	1.112	2.063	4.655
Batch 3	0.046	0.094	0.186	0.394	1.128	1.960	4.353
Batch 3	0.044	0.088	0.190	0.377	1.024	1.950	4.084
Mean	0.048	0.102	0.198	0.395	1.140	2.041	4.420
SD	0.004	0.011	0.012	0.010	0.069	0.074	0.201
C.V.%	8.5	10.9	6.0	2.5	6.1	3.6	4.5

Table 8
Patient pharmacokinetic parameters derived from WinNonlin

Patient	Course (day)	C_{max} (μg/l)	T_{max} (h)	AUC_{last} (h* μg/l)	$t_{1/2}$_Lambda_z (h)
1	1	24624	0.14	18723	1.21
	8	24584	0.14	21808	1.26
	12	23375	0.13	14093	4.01
2	1	41524	0.38	46378	1.03
	8	37482	0.28	37773	1.07
	12	39584	0.28	32388	4.22
	57	34358	0.28	30668	0.91
3	1	37167	0.68	71854	1.34
	8	46272	0.75	85875	2.32
	12	33184	0.7	78896	7.61
4	1	30455	0.53	27146	1.13
	8	25969	0.53	21879	1.31
	12	28920	0.58	21764	23.99
5	1	37451	0.6	31807	0.68
	8	44847	0.58	46843	0.88
	12	23135	0.72	27516	8.56
6	1	35100	0.53	48630	1.06

3.10. Clinical trial

Six patients were treated at three dose levels, 50 mg/m^2 (1 patient); 100 mg/m^2 (1 patient); 200 mg/m^2 (4 patients). Possible drug related grade 3 diarrhoea at 200 mg/m^2 in one patient lead to dose expansion of this cohort. SU006668 was in general well tolerated at the doses tested. Other drug related toxicity seen included mild fatigue (2 patients), mild nausea (2 patients), mild reversible elevation in hepatic transaminases (2 patients) and pain felt in the tumour following the treatment infusion (2 patients). The trial was halted due to unforeseen instability problems with the intravenous formulation of SU006668.

3.11. Pharmacokinetic analysis and results

The PKs of SU006668 show interpatient variability with 2.6-fold variation in patients' day 1 AUC at the dose of 200 mg/m^2. The 4 h AUC appears to increase linearly with dose but not significantly ($r = 0.61$). The AUC versus dose graph, for all patients, is shown in Fig. 8.

Accurate evaluation of the terminal half-life ($t_{1/2}$lambda-*z*), clearance (Cl) and volume of distribution (Vz, Vz_F) on days 1 and 8 was not possible due to the short sampling times. In all patients, the $AUC_{0-4\ h}$ on day 12 was lower than the $AUC_{0-4\ h}$ on day 1. The mean volume of distribution, determined on day 12, was 13.1±7.8 l. PK parameters are tabulated in Tables 8 and 9. Concentration of SU006668 vs. time graphs for days 1, 8 and 12 are shown in Figs. 9–11, respectively.

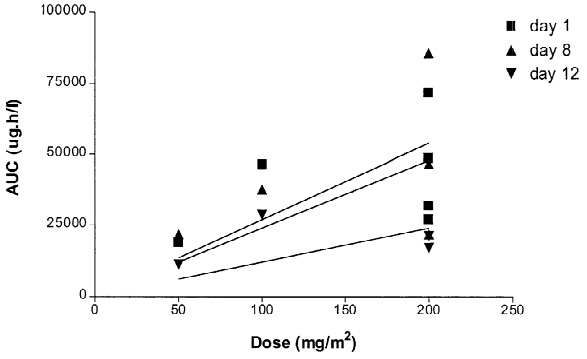

Fig. 8. AUC vs. Dose for all patients.

Table 9
Patient pharmacokinetic parameters derived from WinNonlin

Patient	Course (day)	AUC_{1-4} (h* μg/l)	MRT_{last} (h)	Vss (observed) (l)	Cl (observed) (l/h)
	1	18723	1.02	5.66	4.08
1	8	21808	1.03	4.89	3.49
	12	11169	1.64	10.7	5.81
	1	46378	1.18	2.49	1.65
2	8	37773	1.03	2.66	2.06
	12	28755	1.77	5.43	2.51
	57	30668	0.91	2.91	2.53
	1	71854	1.62	2.16	1.04
3	8	85875	1.83	2.43	0.7
	12	48736*	8.12	10.1	1.01
	1	27146	1.23	4.91	2.63
4	8	21879	1.04	4.9	3.53
	12	17081	3.41	26.2	3.6
	1	31807	0.98	2.83	2.5
5	8	46843	1.16	2.25	1.69
	12	21258	3.72	13.1	2.96
6	1	48630	1.31	2.54	1.59

*Four-hour time point missing.

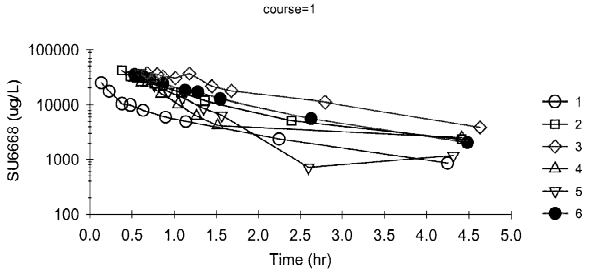

Fig. 9. Concentration of SU006668 vs. Time graph day 1.

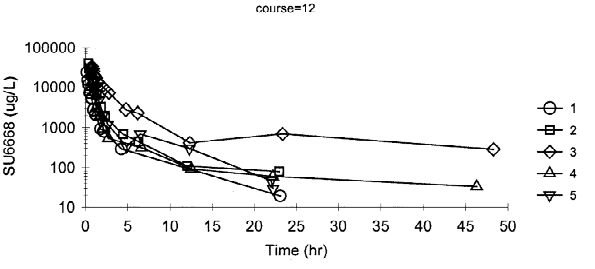

Fig. 11. Concentration of SU006668 vs. Time graph day 12.

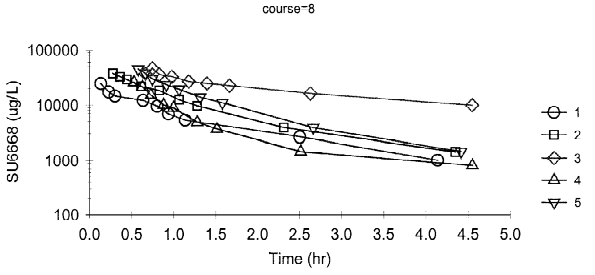

Fig. 10. Concentration of SU006668 vs. Time graph day 8.

4. Discussion

This paper describes an assay, based on HPLC with UV detection, to quantify the novel antiangiogenic agent SU006668 in human plasma. SU006668 inhibits Flk-1 and FGF receptor tyrosine kinases with IC_{50} s of 0.41 and 9.3 μ*M*, respectively [1]. Therefore, the calibration range of the assay should and does encompass these values (25–2000 ng/ml which is equivalent to 0.08–6.45 μ*M*).

SU5416 is another antiangiogenic agent that displays structural similarity with SU006668. Both compounds have an oxy-indole core and a substituted pyrrole ring; however SU006668 differs from SU5416 due to an extra propionic acid side chain on the 3 position of the pyrrole ring. An assay has previously been developed for the quantification of SU5416 in human plasma [9]. As with SU006668, the drug was extracted from plasma by protein precipitation with acetonitrile. The sensitivity of this assay was comparable with that for SU006668 (calibration range of 10–2000 ng/ml)

In the clinical trial, the intravenous formulation of SU006668 was well tolerated at the dose levels tested. Dose limiting toxicity was not identified and a maximum tolerated dose was not defined due to the premature closure of the study. It is interesting to note that two patients developed tumour pain after treatment infusion. These episodes were recurrent with every infusion and in one case involved bone pain in a site which at the time was not yet proven to be a known metastatic deposit but was subsequently identified on bone scan. Such pain may be the result of a treatment related change in vascular permeability.

The current method has been used to evaluate the pharmacokinetics of six patients following intravenous administration of 50, 100 or 200 mg/m^2 SU006668 on days 1, 8 and 12. The $AUC_{0-4\ h}$ on day 12 was lower than the $AUC_{0-4\ h}$ on day 1 suggesting that induction of drug metabolism had occurred. Since patients did not have similar concomitant treatments, it is possible that the drug induces its own metabolism. The mean volume of distribution determined on day 12 was low (13.1 ± 7.8 l), indicating that the drug does not distribute very well outside the vascular compartment. Despite the limited number of patients in this study, our results indicate clear induction, following multiple administration, with a low volume of distribution of SU006668. Conversely, SU5416 displays a high volume of distribution (39–215 l) and clears very rapidly from the circulation (46–215 l/h) [9].

In conclusion the developed assay is robust, sensitive, accurate and reproducible and was successfully used to support the clinical study.

5. Nomenclature

PK	pharmacokinetics
QC	Quality Control
LLOQ	Lower Limit of Quantification
RE	Relative Error
C.V.	Coefficient of Variation
HPLC	High Performance Liquid Chromatography
UV	Ultra violet
h	hour
i.v.	intravenous
IS	Internal Standard
nm	nanometer

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