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Development and validation of a sensitive solid-phase extraction and high-performance liquid chromatographic assay for the novel bio-reductive anti-tumor agent RH1 in human and mouse plasma

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Abstract

A HPLC assay and solid-phase extraction technique from human plasma has been developed and validated for the experimental anticancer agent, RH1 (2,5-diaziridinyl-3-hydroxymethyl-6-methyl-1,4-benzoquinone) which is currently being evaluated by the CRC phase I/II committee. A 500 mg amino propyl solid-phase extraction cartridge was used to isolate RH1 from human plasma. Analysis was performed on a reversed-phase chromatography system using a 15 cm cyanopropyl column and isocratic elution with a 10% methanol–90% water (double distilled) solution. The lower limit of quantitation for RH1 was found to be 0.00375 μg/ml (3.75 ng/ml±8.3%) in water and following extraction from plasma. Recovery of >80%(±11.9%) was achieved over a five-day validation study. This method was used to carry out pre-clinical studies in BDF mice (standard strain of hybrid mice) at three dose levels (2, 5 and 10 mg/kg of RH1 in 0.9% (w/v) saline via an intraperotoneal injection). Standard Version of PC Winnonlin pharmacokinetic modelling software was used to model the data. A none-compartmental model was used to describe the disposition of RH1 in mice plasma. RH1 was rapidly eliminated from plasma with a mean plasma clearance of 23.4 ml/min, mean volume of distribution of 321.6 ml and mean $t_{1/2}$ α and β decays of 4.8 and 9.6 min, respectively. RH1 in human and mouse whole blood and plasma was found to be stable up to 2 h.

Keywords: RH1

1. Introduction

RH1 (Fig. 1) 2,5-diaziridinyl-3-hydroxymethyl-6-methyl-1,4-benzoquinone is a water soluble (>0.5 mg/ml, 25°C) synthetic aziridinyl benzoquinone which is structurally related to the experimental agent MeDZQ (2,5-diaziridinyl-3,6-dimethyl-1,4-benzoquinone) [1,2], a promising anti-tumor agent whose progress to the clinic has been hampered by difficulties in formulation (aq. solubility <0.05 mg/ml at 25°C).

The aim of cancer chemotherapy is to use cytotox-

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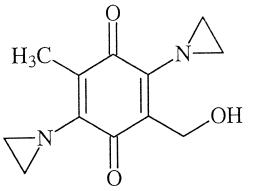

Fig. 1. The structure of RH1 2,5-diaziridinyl-3-hydroxymethyl-6-methyl-1,4-benzoquinone (OH–MeDZQ).

ic agents that are selectively toxic to cancer cells with minimal toxicity to normal tissues. One approach to achieving selective toxicity is to target enzymes that are over expressed in cancer cells relative to normal cells. One such potential target enzyme is DTD (DT-Diaphorase) (NAD(P)H-quinone oxidoreductase [3–5].

DTD is an obligate two electron reductase which is mainly found in the liver but is also expressed in brain, heart, lungs and kidney. The levels of DTD are generally higher in cancerous tissues compared to normal. This is particularly so in non small cell lung cancers (NSCLC) [6]. A recent study has demonstrated that DTD expression can be increased up to 80-fold in non-small cell lung cancers relative to normal lung, and 20–35-fold in NSCLC relative to small cell lung cancers (SCLC) [7].

It is hoped to exploit these higher concentrations of DTD by designing bio-reductively activated prodrugs which would target tumors over expressing DTD such as has been found in lung tissue.

RH1 is activated via a two electron reduction of the quinone to the hydroquinone derivative by DTD [8]. This is capable of selective alkylation and cross linking of DNA at 5′-GNC-3′ (G=guanine, N=any other DNA base, C=cytosine) sequences (Fig. 2). This results in inhibition of the DNA replication process thereby preventing tumor growth [2,9]. RH1 is a good DNA cross linker and shows large cytotoxicity differentials between cell lines which have low and high levels of DTD [3].

The aim of RH1 therapy will be to target patients whose tumors over express DTD e.g NSCLC, and to try and improve on current therapies such as the use of mitomycin C which results in a relatively poor prognosis for the patients involved [3,4].

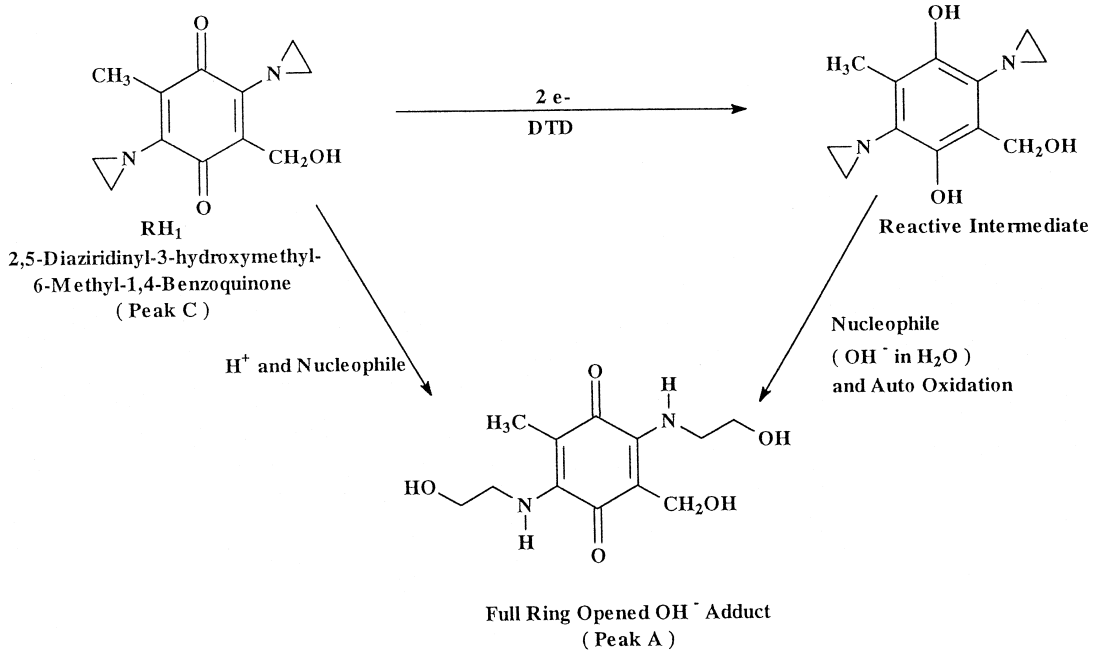

Fig. 2. Mechanism of activation of RH1 via a reactive intermediate

The study described here discusses the development of a solid-phase extraction and high-performance liquid chromatography assay which allows RH1 and its metabolites to be isolated from plasma and quantified over a range of concentrations. These methods have been used in mice and have allowed us to model the data to generate pharmacokinetic parameters. The ultimate aim is to have RH1 introduced into the clinic and the analytical methods developed here will be applicable in the analysis of patient samples.

2. Experimental

2.1. Chemicals and reagents

RH1 was synthesised in house and was >99% pure as assayed by TLC and high resolution mass spectrometry [10]. A solution of RH1 (15.4 μg/ml) in double distilled water was used to develop the HPLC assay. HPLC grade acetonitrile and methanol were obtained from Rathburns, (Scotland, UK). Human plasma was obtained from the blood bank at Christie Hospital. Double distilled water was used throughout the study and was obtained in-house from all glass distillation apparatus, from alkaline permanganate.

Aminopropyl solid-phase extraction cartridges (500 mg) were obtained from Anachem (Luton, UK).

2.2. Chromatographic system

HPLC analysis of RH1 was carried out using a Gilson 306 solvent delivery system, a Gilson 117UV–Vis detector and an ASPEC XL autoinjector. The system was controlled using Gilson 715 controller software. This included data capture and processing. A wavelength of 340nm was used for the duration of this work.

A 5 m spherisorb cyanopropyl column (4.6×150 mm I.D) (Anachem, Luton, UK) was used at ambient temperature and a pH of 6.0, under isocratic conditions of 10% methanol and 90% water (double distilled) to allow separation between the RH1 any impurities and remaining plasma components.

2.3. Sample preparation

A solid-phase extraction technique for RH1 from human plasma was developed. Several solid-phase extraction cartridges were evaluated including phenyl, cyanopropyl C18, C8, C2 and aminopropyl. Reproducible extraction was best obtained using a 500 mg aminopropyl cartridge under the following conditions at room temperature:-condition the cartridge with (1) 1 ml of acetonitrile; (2) condition the cartridge with 1 ml of water; (3) load 0.5 ml of plasma containing RH1; (4) allow plasma proteins to elute to waste (set push volume on ASPEC to 1 ml/min); (5) elute the retained drug with 1 ml of water and (6) centrifuge samples in a microcentrifuge (13000 *g*) for 10 min.

The samples were injected into a 200 μl sample injection loop and chromatographed at a flow of 1 ml/min, and monitored at 340 nm as described above.

2.4. Method validation

2.4.1. Standard curve

A stock solution 15.4 μg/ml of RH1 in double distilled water was made up and stored at 4°C protected from light. A standard calibration curve of this stock was generated by serial dilution (1–2) from 15.4 μg/ml–0.00375 μg/ml. Each sample was chromatographed in triplicate and the mean used for the calibration curve. A linear regression analysis was applied to the concentration versus area data.

2.4.2. Plasma standard curves

Fresh stock's of RH1 (15.4 μg/ml) in water were used to prepare calibration samples by adding 0.5 ml of, 15.4, 7.7, 3.85, 1.9, 0.96, 0.48, 0.24, 0.12 0.06, 0.03, 0.015 μg/ml of RH1 to 0.5 ml aliquots of pooled human plasma. Half a millilitre of each sample was extracted as described with elution into 1 ml of water.

Each extracted sample was centrifuged for 10 min and the supernatant was chromatographed using the developed assay described in Section 2.2. Three replicate analyses were made at each concentration level. To construct the calibration curves mean areas were plotted against each concentration and a simple

linear regression analysis was carried out on the data. This procedure was repeated over 5 days.

2.4.3. Within day precision and accuracy

The within day precision and accuracy of this method was determined by the extraction and analysis of RH1 samples from pooled human plasma at four concentrations 3.8, 0.48, 0.03, and 0.00375 μg/ml on the same day. Three replicate analyses were made at each concentration.

2.4.4. Between day precision and accuracy

The between day precision and accuracy of this method was determined by the extraction and analysis of RH1 samples from pooled human plasma at four concentrations 3.8, 0.48, 0.03 and 0.00375 μg/ml on five different days. Three replicate analyses were made at each concentration.

2.4.5. Determination of percentage recovery

Following preparation of samples (page 7, 2.4 Method Validation, Plasma Standard Curves) each sample was analysed in triplicate. The mean area obtained at each concentration was expressed as a ratio of the mean area obtained from the non extracted standard (calibration curve.)

2.4.6. Application of developed methodology to an in vivo study in mice

The validated method described above was used to extract RH1 from mouse plasma at three dose levels and to carry out pharmacokinetic analyses of the data obtained.

2.5. Drug administration and blood collection

Three dose levels of RH1 were made up in 0.9% isotonic saline to give final concentrations of 1, 0.5, and 0.2 mg/ml (10, 5 and 2 mg/kg), 200 μl were injected into the peritoneum of each mouse (average weight 20 g).

Fourteen mice were used for each experiment (2 mice used per time point) with samples taken at time 0 (control, no drug given), 5, 10, 15, 25, 30 and 60 min following injection. Mouse blood was collected via a cardiac puncture using a 1 ml syringe containing 50 μl of heparin. Blood was centrifuged immediately at 13 000 *g* for 10 min and the supernatant was removed for analysis. A sample volume of 300 μl was used for extraction throughout these experiments.

2.6. Stability study in human whole blood/human plasma and mouse whole blood

The stability of RH1 was studied in both human plasma/whole blood and mouse whole blood.

RH1 was spiked into whole blood and plasma (plasma was isolated from the whole blood by centrifugation at 13000 g for 20 min) obtained from three healthy volunteers and into pooled whole blood obtained from BDF mice. Sampling took place at 0, 2, 5, 10, 15, 30, 60 and 120 min. Each sample was covered in foil and kept on ice prior to extraction and analysis following the addition of RH1 to each sample.

In a separate study the stability of RH1 (3.75 μg/ml final conc.) in human plasma was monitored under various storage conditions including light (room temp), dark (room temperature), also at −20°C, −80°C and 4°C. All samples were monitored over a period of 7 days, each sample was extracted using the solid-phase extraction method.

3. Results

A solid-phase extraction and high-performance liquid chromatography assay has been developed and validated for the novel anti-tumor agent RH1 in both human and animal plasma.

From Fig. 3C, it can be seen that RH1 elutes at a retention time of 8.20 min (peak c) and is well separated from its ring opened species both are more polar than the RH1 and elute at 6.2 (peak a) and 6.8 min (peak b). Fig. 3A illustrates extracted drug free plasma, indicating no drug between retention times 6 and 8 min, and Fig. 3B which illustrates RH1 at its LLQ of 3.75 μg/ml.

RH1, in water was used to construct standard curves which were linear over the concentration range 0.00375–3.9 μg/ml (±0.2–8.3%). The lower limit of quantification of RH1 was calculated as 0.00375 μg/ml (slope=15.871×10^{6}, Intercept=0.0373 and correlation coefficient=0.9998) this was the same following extraction of RH1 from human

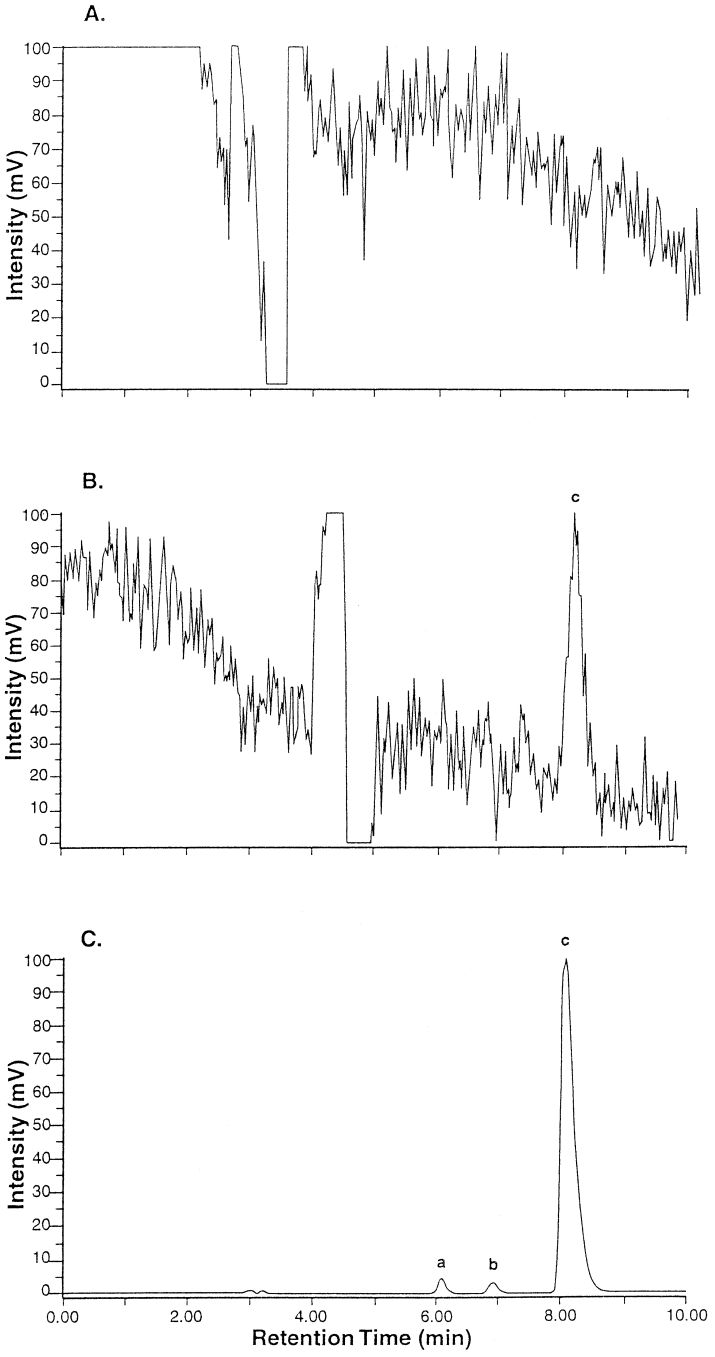

Fig. 3. (A) Chromatogram of drug free extracted plasma; (B) chromatogram of RH1 at its LLQ of 3.75 μg/ml; (C) chromatogram of RH1 (peak c), half ring opened aziridinyl species (peak b) and fully ring opened aziridinyl species (peak a).

Table 1
Calibration data for RH1 extracted from human plasma over a 5 day validation study

Validation day	Slope	Intercept	Correlation coeff.
1	10.455×10^6	0.0229	0.9997
2	11.999×10^6	0.015	0.9962
3	9.6536×10^6	0.337	0.9983
4	15.809×10^6	0.1267	0.9994
5	12.145×10^6	0.0424	0.9969

plasma. Simple linear regression analysis was performed on the peak areas versus concentration data from each day of the validation study and is shown in Table 1. Percentage extraction recoveries obtained over the concentration range 3.9–0.00375 μg/ml over the 5 day validation study can be seen in Table 2. Inter- and intra-day precision and accuracy data for the four selected concentrations analysed using the developed method can be seen in Table 3.

Fig. 4 illustrates the pharmacokinetic profile obtained when BDF1 mice were dosed with 10, 5 and 2 mg/kg of RH1 in saline. The pharmacokinetic data obtained from this study can be seen in Table 4.

Fig. 5 illustrates the stability profile of RH1 in both human whole blood and plasma and mouse whole blood monitored over 2 h. Stability of RH1 in human plasma over 7 days can be seen in Fig. 6.

4. Discussion

The method described above allowed the isolation and quantitation of RH1 and its two main metabolites from human plasma and whole blood and allowed the pharmacokinetic behaviour of RH1 in mice to be studied.

Table 3

Actual Conc. μg/ml	Measured μg/ml	Conc. % RSD	% Accuracy
Within day precision and accuracy of the method			
3.8	2.48±0.18	7.23	65.26
0.48	0.39±0.004	1.05	81.25
0.03	0.019±0.02	10.55	63.33
0.00375	0.004±0.000017	0.40	106.66
Between day precision and accuracy of the method			
3.8	2.57±0.37	14.29	67.63
0.48	0.34±0.057	16.72	70.83
0.03	0.025±0.004	16.90	83.33
0.00375	0.0035±0.00046	12.97	93.33

As can be seen from Fig. 2, RH1 elutes at a retention time of 8.2 min and is well separated from its aziridine ring opened species (full ring opened –OH adduct) A and (half ring opened –OH adduct) B, at acidic pH the aziridine ring becomes protonated and this may facilitate aziridine ring opening.

It is proposed that the mechanism of activation of RH1 is by a two electron reduction of the quinone to its hydroquinone form facilitated by the enzyme DT-Diaphorase (Fig. 2), generating a reactive intermediate which is capable of cross linking DNA. The differential levels of DTD present in different types of cells in both tumorous and non tumorous tissues can be utilised to target specific types of tumors expressing high levels of DTD such as NSCLC.

Three dose levels of RH1 (2, 5 and 10 mg/kg in 0.9% saline) were used to carry out pharmacokinetic analysis in mice. The area under the plasma con-

Table 2
Percentage recovery data for RH1 extracted from human plasma over a 5 day validation study

Day	RH1 Concentration μg/ml										
	3.8	1.9	0.95	0.48	0.24	0.12	0.06	0.03	0.015	0.0075	0.0375
1	61.88	68.32	78.09	71.00	67.24	61.61	50.79	60.38	83.96	81.32	114.54
2	75.71	87.89	67.42	73.88	72.24	101.92	78.16	68.64	99.46	73.54	84.32
3	58.43	64.9	69.78	68.3	73.21	68.56	84.39	77.19	82.93	61.44	88.59
4	82.73	89.71	78.09	80.92	76.98	97.06	91.07	87.14	82.6	67.87	91.35
5	77.63	73.72	65.76	82.54	98.73	93.39	70.46	90.05	75.07	69.28	85.98
Mean	71.28	76.91	71.83	75.33	77.68	84.51	74.97	76.68	84.80	70.69	92.96
SD	9.43	10.13	5.27	5.54	10.97	16.23	13.88	11.12	7.98	6.58	11.05
RSD%	13.23	13.17	7.34	7.35	14.12	19.2	18.5	14.5	9.41	9.31	11.89
n	5	5	5	5	5	5	5	5	5	5	5

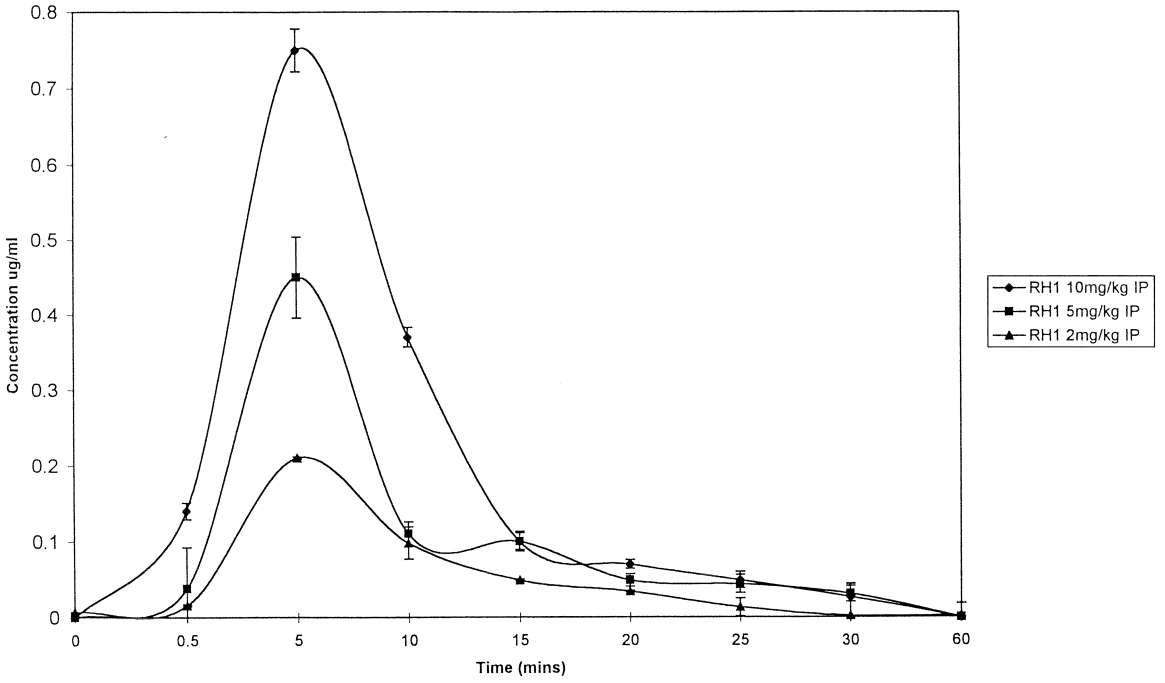

Fig. 4. Pharmacokinetic profiles of RH1 given to BDF1 mice in saline at three dose levels 10, 5 and 2 mg/kg.

centration–time curve (AUC) for RH1 was calculated using the linear-logarithmic trapezoidal method extrapolated to infinity using the rate constant of the terminal phase of the plasma concentration–time curve. It was found that the AUC was linearly related to dose (Table 4) and ranged from 2.09–7.32 μg/ml/min. The elimination half-life was calculated from the relationship $t_{1/2}$=ln 2/k (min) where k is the elimination rate constant.

Rapid elimination of RH1 was observed with a terminal $t_{1/2}\beta$ of 8.44–10.71 min. Total plasma clearance (calculated by dose/AUC (ml/min)) varied between 18.96 and 27.41 ml/min. The volume of distribution (calculated by Cl/k) varied between 289.83 and 384 ml (±16.82%).

Table 4
Pharmacokinetic parameters

	10 mg/kg	5 mg/kg	2 mg/kg
T_{max}	5 min	5 min	5 min
C_{max}	0.75 μg/ml	0.45 μg/ml	0.21 μg/ml
$t_{1/2}\alpha$	4.9 min	4.61 min	4.75 min
$t_{1/2}\beta$	9.72 min	8.44 min	10.71 min
CL	27.41 ml/min	23.79 ml/min	18.96 ml/min
Vd	384 ml	289.83 ml	290.89 ml
AUC	7.32 μg/ml/min	4.29 μg/ml/min	2.09 μg/ml/min

Although a $t_{1/2}\alpha$ of only 5 min has been seen for this drug it can be argued that the concentrations of RH1 necessary for in vitro cytotoxicity of RH1 as seen in several cell lines [11] are very low and may be achieved for a sufficient duration in vivo to allow anti-tumor activity.

(K562 chronic myelogenous leukaemia=0.15 n*M* of RH1; BE human colon carcinoma=2.5 n*M*. H460 non small cell carcinoma=0.05 n*M*; A549 non small cell carcinoma=0.04 n*M*).

A shorter exposure may be enough to achieve sufficient tumor kill especially for the lung tumors which contain the very large quantities of the enzyme DTD and where very small concentrations of RH1 are required to achieve the IC 50.

The lower limit of quantitation achieved in this work was 3.75 ng/ml (0.016 n*M*), which is lower than the IC50 levels seen in cultured cell lines e.g (A549=0.04 n*M*). We have therefore a sensitive and selective chromatography assay which will enable quantitation of RH1 at levels necessary for biological activity.

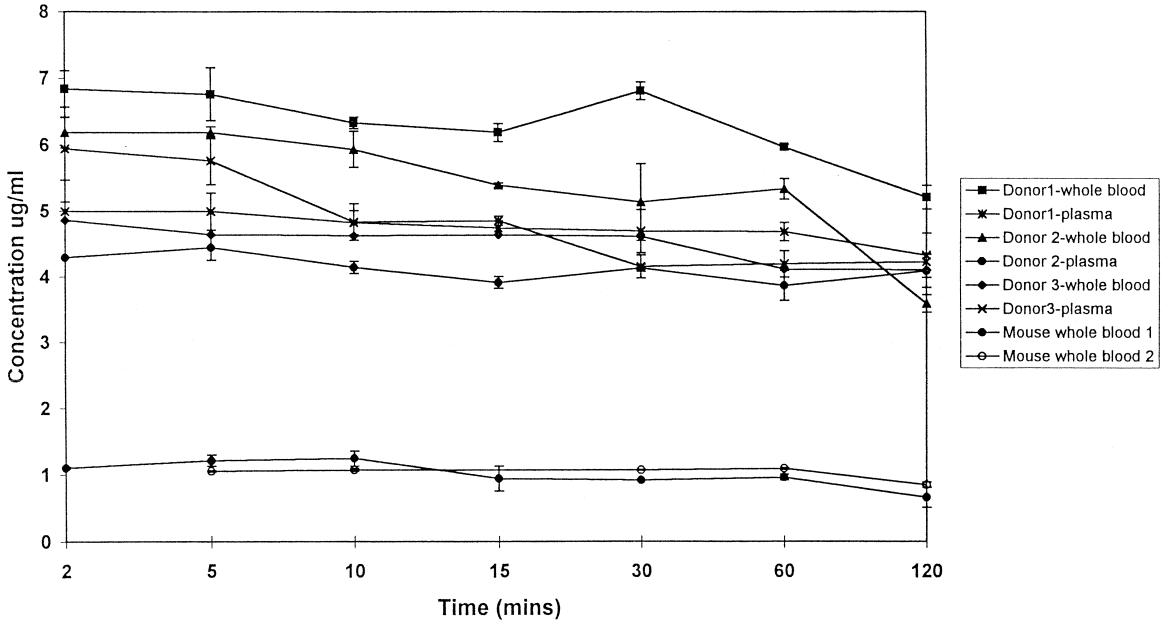

Fig. 5. Graph to show the stability of 3.75 μg/ml of RH1 in both human whole blood and plasma and in mouse whole blood.

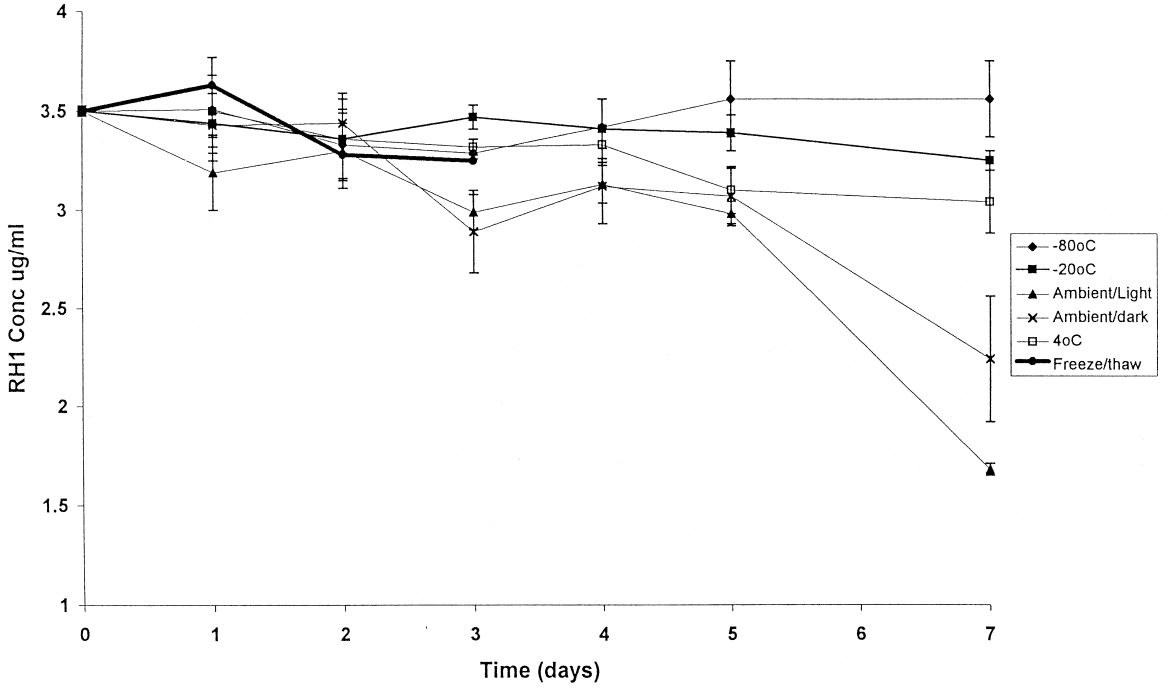

Fig. 6. Graph to show the stability of RH1 in human plasma, monitored over 7 days under different environmental conditions.

In a parallel study as part of the assay method validation, the stability of RH1 was investigated in both human whole blood and plasma together with mouse whole blood. It was found that in all samples analysed RH1 degradation was less than 40% over a period of 2 h. This reduction is considerably slower than that observed in the in vivo mouse experiments at three dose levels indicating a rapid decline of RH1 where the $t_{1/2}$ α was found to be 4.75 min (±1.98%).

RH1 was stable up to a period of 4 days under conditions of ambient/light, ambient/dark, 4°C, 20°C 80°C. There was a rapid loss of RH1 between days 4 and 7, particularly of samples stored in the light of greater than 50%. Samples stored at −80°C indicated little loss of RH1.

Several studies have now demonstrated that systemic exposure to certain anti- cancer drugs is correlated with toxicity and efficacy. These studies support the rationale behind the use of pharmacokinetics as a method to increase the effectiveness and/or to decrease the toxicity of a patients drug therapy.

The methods described will enable the maximum tolerated dose (the highest dose that could be given to a patient to produce manageable and reversible toxicity) to be defined via a series of dose escalation steps and provide other related information for planning of subsequent trials based on pharmacokinetic analysis.

5. Conclusion

A sensitive, specific, accurate and reproducible solid-phase extraction and HPLC assay has been developed for the experimental water soluble anti-tumor agent RH1 which allows the isolation and quantitation of RH1 from human plasma down to concentrations of 3.7 ng/ml (0.7 ng on column). This methodology has been used in a preliminary study in mice and can be used for the analysis of clinical samples from the phase one investigation of this agent currently under discussion.

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