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SHORT COMMUNICATION

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Development and validation of a sensitive solid-phase-extraction and high-performance liquid chromatography assay for the bioreductive agent tirapazamine and its major metabolites in mouse and human plasma for pharmacokinetically guided dose escalation

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Abstract A sensitive solid-phase-extraction and high-performance liquid chromatography (HPLC) method has been developed to investigate the pharmacokinetics and metabolism of the hypoxic-cell cytotoxic agent tirapazamine (1,2,4-benzotriazine-3-amine 1,4-di-N-oxide; WIN 59075, SR 4233), currently in phase I/II studies in the United Kingdom and United States. A sample extraction and concentration process was devised using strong cation-exchange Bond Elut cartridges. Tirapazamine, the mono and zero-*N*-oxide metabolites (WIN 64012, WIN 60109) were isocratically resolved using a μBondapak phenyl HPLC column and measured using photodiode-array detection. The minimal quantifiable level (MQL) of tirapazamine was 40 ng/ml in mouse plasma and 20 ng/ml in human plasma. Recovery was consistently greater than 80% for all compounds over the concentration range of 20 ng/ml to 20 μg/ml. No significant decomposition was observed following up to three freeze/thaw cycles and storage at −70 °C for 52 days. The assay was accurate and reproducible, with measured values lying within the limits of defined acceptance criteria. Additional studies to investigate the degree of plasma protein binding showed that tirapazamine did not bind extensively to plasma proteins (binding, 9.7% ± 0.1% and 18.7% ± 1.3% in mouse and human plasma, respectively). These small species differences in protein binding are unlikely to have any major impact on the extrapolation of pharmacokinetic data from mice to humans. The assay has now been successfully applied to investigate the pharmacokinetics and metabolism of tirapazamine in mice and patients as part of a pharmacokinetically guided dose-escalation strategy for phase I clinical trials.

Key words Tirapazamine · Bioreductive agents · Assay · Pharmacokinetically guided dose escalation

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Introduction

The hypoxic-cell cytotoxic agent 1,2,4-benzotriazine-3-amine 1,4-di-*N*-oxide (tirapazamine; WIN 59075, SR 4233) is the lead compound in a new series of highly selective bioreductive agents currently in phase I/II trials in the United Kingdom and United States. The impressive differential toxicity noted between oxic and hypoxic tumour cells [2, 15] has been attributed to metabolic one-electron (1e-) reduction of tirapazamine to a highly reactive (oxidizing) nitroxide radical by cytochrome P450 and P450 reductase [9, 12–14]. Nitroxide radical formation is thought to result in DNA strand cleavage by hydrogen atom abstraction, which ultimately results in cell death. Further 1e- reduction by P450/P450 reductase or direct 2e- and 4e- reduction by DT-diaphorase leads to the formation of the inactive reduction products WIN 64012 and WIN 60109 (Fig. 1) [9, 12–14].

As part of ongoing investigations to examine the utility of pharmacokinetically guided dose-escalation strategies in phase I trial design [3, 6–8], a highly sensitive assay was required to investigate the pharmacokinetics and metabolism of tirapazamine in mice at one-tenth the dose lethal to 10% of mice (LD_{10}) and in patients at the phase I starting dose. The present study describes the validation of a sensitive and robust solid-phase extraction method for tirapazamine and its reduction products in mouse and human plasma. The study also investigates any species differences in the extent of plasma protein binding as a prelude to clinical studies.

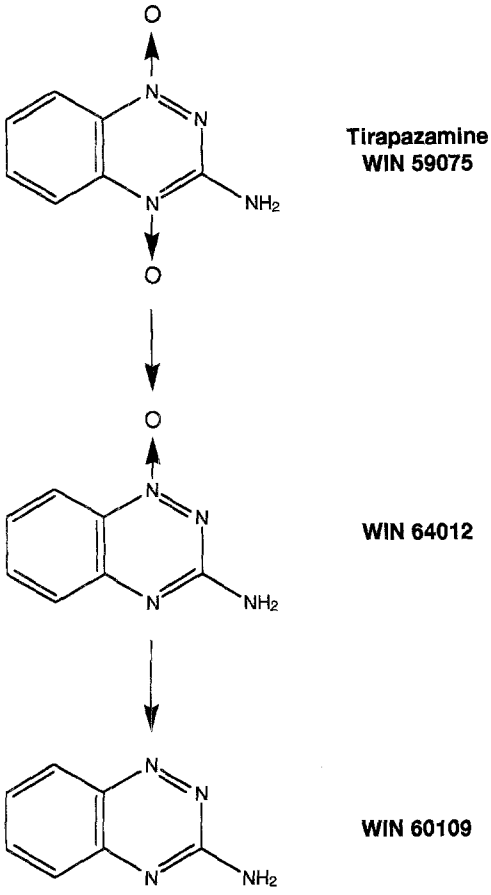

Fig. 1 The bioreductive metabolism of WIN 59075 to the 2e- (*WIN 64012*) and 4e- (*WIN 60109*) reduced metabolites

Materials and methods

Materials

Tirapazamine was obtained from Sterling Winthrop Pharmaceuticals Research Division (Rensselaer, N. Y., USA). 1,2,4-Benzotriazine-3-amine 1-mono-*N*-oxide (WIN 64012; SR 4317), 1,2,4-Benzotriazine-3-amine (WIN 60109, SR 4330) and the internal standard 3-Ethylamino-1,2,4-benzotriazine 1,4,-di-N-oxide (WIN 59852) were obtained from SRI International (Menlo Park, Calif., USA). All compounds were >99% pure as determined by high-performance liquid chromatography (HPLC) and were used without further purification. All reagents were of HPLC or analytical reagent grade. Pooled human plasma (lithium heparin anticoagulant) was supplied by the Blood Transfusion Service (Glasgow, Scotland, UK). Female BALB/c mouse plasma was provided by Harlan Olac Ltd. (Oxon, UK). Strong-cation-exchange (SCX) benzenesulphonic acid Bond Elut cartridges were supplied by Crawford Scientific (Strathaven, Scotland, UK).

Apparatus

The HPLC equipment comprised a Waters model 991 photodiode-array detector, a NEC powermate SC/16 computer running Waters 991 photodiode-array software, a model 660 gradient controller with a quaternary pump and a WISP model 712 autosampler (Millipore Ltd/Waters Chromatography Division, Harrow, Middlesex, UK).

Chromatographic conditions

Tirapazamine, WIN 64012, WIN 60109 and the internal standard were resolved using a μBondapak (10 μm) phenyl analytical column in an RCM 8 × 10 radial compression module. μBondapak phenyl pre-column inserts were used and replaced when an excessive increase in back pressure (>1500 psi) was observed. The mobile phase consisted of 20% methanol in water, run at a flow rate of 2 ml/min. All solvents were filtered through a 0.45 μm membrane (PTFE/polypropylene) and de-gassed with helium prior to use. Photodiode-array detection was performed at 462 nm (tirapazamine), 400 nm (metabolites) and 500 nm (internal standard). Quantification was based on peak area against the appropriate reference standards. Peak identification was confirmed by retention time in comparison with authentic standards and by diode-array analysis of the spectra.

Standard stock solutions

Tirapazamine, WIN 64012 and WIN 60109 were weighed (5 mg of each compound) into a single glass vial and dissolved in 25 ml dimethyl sulphoxide (DMSO). Nine concentrations ranging from 0.02 to 200 μg/ml were prepared in DMSO and frozen as 100 μl aliquots at −70 °C until use. The internal standard solution was prepared by dissolving 5 mg of WIN 59852 in 1000 ml of 0.01 *M* TRIS/HCl buffer (pH 6.0) to give a final concentration of 5 μg/ml.

Calibration curves

Aliquots of standard stock solution (50 μl) were added to 450 μl aliquots of mouse plasma or 950 μl human plasma to give concentration ranges of 40 ng/ml to 20 μg/ml and 20 ng/ml to 10 μg/ml for mouse and human plasma, respectively. Each concentration level was prepared in singlet except for the lowest (20 ng/ml, 40 ng/ml), intermediate (0.5 μg/ml, 1 μg/ml) and highest (10 μg/ml, 20 μg/ml) calibration levels, which were prepared in triplicate. Equal volumes of the internal standard solution were added to plasma samples immediately before the extraction procedure. The percentage of recovery was determined at the low, intermediate and high concentration levels in triplicate by adding 50 μl of the respective standard stock solutions to 250 μl HPLC mobile phase and directly injecting 250 μl aliquots without extraction.

Assay validation

Validation samples were prepared at five concentration levels in mouse and human plasma as follows: (a) mouse plasma 40 ng/ml (minimal quantifiable level, MQL), 44 ng/ml (low level), 1 μg/ml (medium level), 18 μg/ml (high level), 20 μg/ml (top level) and blank plasma; and (b) human plasma 20 ng/ml (MQL), 22 ng/ml (low level), 0.5 μg/ml (medium level), 9 μg/ml (high level), 10 μg/ml (top level) and blank plasma. Each level ($n = 6$ samples per level) were quantitated as unknown samples using a freshly prepared calibration curve on day 1 of analysis (within-day validation). Between-day validation was performed by assaying the five validation levels in singlet on 5 separate analytical days following frozen storage at −70 °C.

Stability studies

The long-term stability of tirapazamine, WIN 64012 and WIN 60109 in mouse and human plasma was assessed at low and intermediate concentration levels. Plasma samples were analysed in triplicate on days 1 and 52 following storage at −70 °C. An additional pool of samples was prepared at the intermediate concentration level. Aliquots in triplicate (0.5 and 1.0 ml for mouse and human plasma, respectively) were assayed fresh and following three consecutive cycles of freezing and thawing.

Solid-phase-extraction procedure

Bond Elut SCX cartridges were conditioned with 2 ml methanol and 5 ml buffer (0.01 M TRIS/HCL, pH 6.0). Plasma samples were mixed with an equal volume of pH 6.0 buffer containing the internal standard and passed through the solvated columns. Plasma components were washed to waste with 1 ml methanol and the drug was then eluted with 4.5 ml methanol/aqueous ammonia (19:1, v/v). The extracts were then dried under vacuum at 40 °C, reconstituted in 300 µl HPLC mobile phase and clarified by centrifugation (10 min, 1000 *g*) and 250 µl aliquots were analysed by HPLC.

Statistical analysis

Assay precision was defined as the coefficient of variation expressed as percent (%CV) for each validation level. The %CV was defined as the standard deviation divided by the mean × 100. The accuracy was defined as the mean percentage difference from the nominal value at each validation level. The MQL was defined as the lowest concentration of analyte that could be reliably estimated (signal-to-noise ratio, >3:1). Recovery was calculated by dividing the peak area of the extracted samples by the peak area of the corresponding unextracted standards expressed as a percentage.

The following acceptance criteria based on Food and Drug Administration (FDA) guidelines (10) were used to evaluate assay performance: (1) 75% of all samples within 15% of the nominal value (20% low level), (2) 4 of 6 samples at each validation level within the nominal value and (3) mean percentage of difference and %CV for each level within 15% (20% at low level).

Plasma protein binding

The plasma protein binding of tirapazamine was studied in freshly collected mouse and human plasma. [^{14}C]-WIN 59075 (specific activity, 100 µCi/mg) was dissolved at 1 mg/ml in methanol. Aliquots (50 µl) of the stock solution were diluted (in triplicate) in 950 µl of 0.01 *M* TRIS/HCl buffer (pH 7.4), BALB/c mouse plasma or human plasma for centrifugal ultrafiltration (final concentration, 50 µg/ml). A further set of standards (in triplicate) were prepared in buffer and were not filtered. These standards were used to determine the degree of non-specific binding to the filter.

The samples were placed in sealed test tubes and incubated at 37 °C for 15 min. The samples (1 ml) were then transferred to Amicon Centrifree micropartition filters (Amicon Corp., Upper Mill, Stonehouse, Glous., UK) and centrifuged at 3000 *g* for 15 min using a fixed-angle rotor. Aliquots (10 µl) of the ultrafiltrate or unfiltered standards were then mixed with 10 ml Ecoscint-A scintillation fluid (National Diagnostics, Kennedy Blvd., Manville, N. J.) and ^{14}C levels were determined by liquid scintillation counting using a Tricarb 1600 TR liquid scintillation analyser (Canberra Packard, Station Road, Pangbourne, Berks). The results are expressed as mean values ± SD ($n = 3$ determinations).

Results

Chromatographic performance

Complete chromatographic resolution was achieved for tirapazamine, the reduced metabolites and the internal standard using a µBondapak phenyl column. No interfering peaks were observed at the selected wavelengths (Fig. 2). Approximate retention times were 7.0 min (tirapazamine), 15.5 min (WIN 60109), 18 min (WIN 64012) and 20.5 min (internal standard, WIN 59852; Fig. 2). Peak identity was confirmed by co-elution with authentic standards and by diode-array analysis of the spectra.

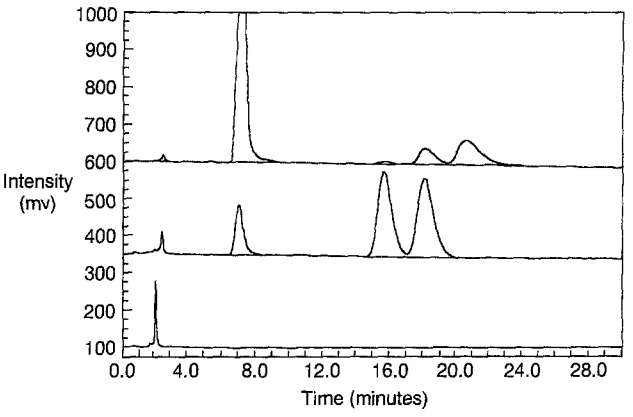

Fig. 2 Human plasma extract containing tirapazamine (7.0 min), WIN 60109 (15.5 min) and WIN 64012 (18 min) at 9 µg/ml. Internal standard: WIN 59852 at 5 µg/ml (20.5 min). Detection was carried out at 462 nm (*upper trace*) and 400 nm (*middle trace*). The *lower trace* shows control human plasma at 462 nm

Minimum quantifiable level

The MQL for tirapazamine, WIN 64012 and WIN 60109 was 20 ng/ml and 40 ng/ml in human and mouse plasma, respectively (approximately 15 ng on the column). This

Table 1 Within-day and between-day precision and accuracy of the assay for tirapazamine, WIN 64012 and WIN 60109 in mouse plasma (*A* Tirapazamine, *B* WIN 64012, *C* WIN 60109). *conc.* concentration, *obs.* observed, *%Dev* percentage of deviation

Within-day:				Between-day:			
Nominal conc. (µg/ml)	Mean obs. conc. (µg/ml)	Mean %Dev	%CV	Nominal conc. (µg/ml)	Mean obs. conc. (µg/ml)	Mean %Dev	%CV
0.040:				0.040:			
A	0.040	0	6.0	A	0.041	5.0	3.0
B	0.042	5.0	3.0	B	0.042	7.0	5.0
C	0.045	11.0	3.0	C	0.044	11.0	2.0
0.044:				0.044:			
A	0.047	7.0	4.0	A	0.046	3.0	7.5
B	0.047	6.0	4.0	B	0.046	3.0	3.8
C	0.049	12.0	1.0	C	0.048	10.0	4.7
1.0:				1.0:			
A	0.900	−11.0	1.0	A	0.906	−9.0	2.0
B	0.880	−12.0	2.0	B	0.950	−5.0	7.0
C	0.850	−15.0	3.0	C	0.947	−5.0	7.0
18:				18:			
A	17.70	−2.0	1.0	A	19.05	5.0	8.0
B	19.40	8.0	1.0	B	17.39	−3.0	9.0
C	16.20	−10.0	2.0	C	16.73	−7.0	8.0
20:				20:			
A	20.20	1.0	1.0	A	19.54	−2.0	3.0
B	20.90	6.0	3.0	B	19.46	−2.0	4.0
C	19.70	−2.0	2.0	C	19.34	−3.0	6.0

Table 2 Within-day and between-day precision and accuracy of the assay for tirapazamine, WIN 64012 and WIN 60109 in human plasma (*A* Tirapazamine, *B* WIN 64012, *C* WIN 60109). *conc.* concentration, *obs.* observed, *%Dev* percentage of deviation

Within-day:				Between-day:			
Nominal conc. (μg/ml	Mean obs. conc. (μg/ml)	Mean %Dev	%CV	Nominal conc. (μg/ml)	Mean obs. conc. (μg/ml)	Mean %Dev	%CV
0.020:				0.020:			
A	0.019	−5.0	3.7	A	0.019	−4.1	8.3
B	0.020	3.5	2.6	B	0.022	11.7	3.8
C	0.019	−1.6	4.1	C	0.019	−3.1	9.8
0.022:				0.022:			
A	0.022	−2.5	9.3	A	0.021	−2.5	7.5
B	0.026	16.0	3.6	B	0.025	12.0	3.8
C	0.023	−6.5	6.0	C	0.023	5.8	4.7
0.5:				0.5:			
A	0.459	−8.2	2.0	A	0.451	−9.9	4.0
B	0.478	−4.5	3.6	B	0.472	5.7	14.2
C	0.515	3.0	3.4	C	0.451	−1.9	5.5
9:				9:			
A	8.48	−5.8	2.7	A	8.65	−3.9	3.7
B	9.05	0.5	4.1	B	8.50	5.6	3.6
C	8.12	−9.8	4.5	C	8.48	−4.4	6.3
10:				10:			
A	9.41	−5.8	3.8	A	9.22	−7.8	3.8
B	9.08	−9.2	2.5	B	9.39	6.1	1.7
C	8.82	−11.8	6.4	C	9.01	−9.9	2.4

level of sensitivity was suitable for pharmacokinetic determinations at one-tenth the LD_{10} in mice and at the phase I starting dose in humans.

Precision and accuracy

The within-day and between-day assay precision and accuracy were assessed at the five validation levels. Coefficients of determination were $<15\%$ for tirapazamine, WIN 64012 and WIN 60109 in both mouse and human plasma (Tables 1, 2).

Recovery

Calibration curves were linear in mouse and human plasma over the concentration ranges tested (data not shown). The overall recovery was 90%, 91% and 86% for tirapazamine, WIN 64012 and WIN 60109, respectively, in mouse plasma and 83%, 96% and 78%, respectively, in human plasma.

Frozen stability in plasma

The frozen stability of the drug metabolites was evaluated in mouse and human plasma at the low and intermediate concentrations. No systematic deviation from nominal values was observed for up to 52 days at −70 °C (Table 3). Three freeze/thaw cycles were performed at the intermediate level. No significant deviation from freshly prepared and analysed samples was observed (Table 4).

Table 3 Long-term frozen stability of tirapazamine, WIN 64012 and WIN 60109 in mouse and human plasma (*A* Tirapazamine, *B* WIN 64012, *C* WIN 60109). *conc.* concentration, *obs.* observed, *%Dev* percentage of deviation

Mouse plasma:				Human plasma:			
Nominal conc. (μg/ml)	Mean obs. conc. (μg/ml)	Mean %Dev	%CV	Nominal conc. (μg/ml)	Mean obs. conc. (μg/ml)	Mean %Dev	%CV
0.044:				0.022:			
A	0.047	7.0	1.0	A	0.022	2.1	5.1
B	0.048	10.0	5.0	B	0.025	17.0	0
C	0.050	13.0	2.0	C	0.026	16.4	5.6
1.0:				0.5:			
A	0.903	−10.0	5.0	A	0.464	−7.2	1.3
B	0.924	−8.0	2.0	B	0.498	−0.5	3.0
C	0.943	−6.0	4.0	C	0.527	5.2	0

Plasma protein binding of WIN 59075

The extent of plasma protein binding was $9.7\% \pm 0.06\%$ and $18.7\% \pm 1.3\%$ in mouse and human plasma, respectively. Although WIN 59075 was not extensively bound to plasma protein in either mouse or human plasma, a small species difference in the extent of binding was observed.

Table 4 Freeze/thaw stability of tirapazamine, WIN 64012 and WIN 60109 in mouse and human plasma (*A* Tirapazamine, *B* WIN 64012, *C* WIN 60109). *conc.* concentration, *obs.* observed, *%Dev* percentage of deviation

Mouse plasma:				Human plasma:			
Nominal conc. (1.0 μg/ml)	Mean obs. conc. (μg/ml)	Mean %Dev	%CV	Nominal conc. (0.5 μg/ml)	Mean obs. conc. (μg/ml)	Mean %Dev	%CV
Fresh:				Fresh:			
A	0.915	−9.0	3.0	A	0.475	−5.0	5.0
B	0.927	−7.0	3.0	B	0.506	1.3	2.9
C	0.945	−6.0	2.0	C	0.538	12.0	3.3
Cycle 1:				Cycle 1:			
A	0.898	−10.0	2.0	A	0.465	−7.1	1.5
B	0.932	−7.0	2.0	B	0.506	1.3	2.9
C	0.954	−5.0	2.0	C	0.548	9.6	3.3
Cycle 2:				Cycle 2:			
A	0.912	−9.0	1.0	A	0.454	−9.2	1.7
B	0.917	−8.0	4.0	B	0.481	−3.9	3.1
C	0.956	−4.0	4.0	C	0.511	2.1	3.1
Cycle 3:				Cycle 3:			
A	0.894	−11.0	2.0	A	0.454	−9.2	1.3
B	0.898	−10.0	3.0	B	0.463	−7.3	0
C	0.939	−6.0	3.0	C	0.517	3.3	3.5

Discussion

The contribution of pharmacokinetics studies during early stages of anti-cancer drug development has become increasingly important in recent years [6]. Interspecies variability in pharmacokinetics has been identified as a contributing factor for the disparity between the LD_{10} in mice and the maximum tolerated dose (MTD) in patients. However, pharmacokinetically guided phase I studies that correct for species differences in drug clearance have already achieved beneficial results [1, 4–7]. However, several problems, including species differences in plasma protein binding and metabolism, non-linear pharmacokinetics and inadequate assay sensitivity at the phase I starting dose, have complicated this approach [6, 7].

A sensitive, reliable and validated analytical method for tirapazamine and its metabolites was required to investigate drug disposition and metabolism at low doses in mice and humans. Several approaches to develop an assay were evaluated, including protein precipitation, liquid-liquid extraction and solid-phase extraction. The best recovery and selectivity was achieved using a strong cation-exchange (SCX) bonded phase sorbent. The interaction of tirapazamine with the SCX sorbent phase at pH 6.0 probably comprises a mixture of electrostatic (cationic) interactions with the negatively charged benzenesulphonic acid and secondary H-bond interactions with residual silanol groups. This strong interaction between the drug and the sorbent bed permits rigorous washing with methanol prior to elution with methanol/ammonia, which results in negligible interference for endogenous plasma components. A high degree of selectivity is also achieved by utilizing wavelengths for the various analytes in the visible end of the spectra (400–500 nm). Using this selective isolation procedure and sample concentration step, the lower limit of detection for tirapazamine and its metabolites was found to be 20 ng/ml in human plasma and 40 ng/ml in mouse plasma. This is a significant improvement over previously published analytical methods for tirapazamine with limits of detection in the range of 300–400 ng/ml [11].

Using this assay, we investigated the pharmacokinetics of tirapazamine and its principal bioreductive metabolites in mice at doses as low as one-tenth the LD_{10} in mice and at the phase I starting dose (36 mg/m^2) as part of a pharmacokinetically guided dose-escalation (PGDE) strategy [8]. Although small inter-species differences in plasma protein binding were observed, it is unlikely that this will contribute to any species difference in tirapazamine pharmacokinetics. Indeed, initial pharmacokinetics investigations indicate that the pharmacokinetics of tirapazamine are similar between the species with respect to drug clearance and the formation of bioreductive metabolites [8]. Studies are now under way to establish the MTD of tirapazamine in patients, to investigate if therapeutically relevant plasma level can be achieved at tolerable doses and to determine the utility of the PGDE approach for this new class of bioreductive agent.

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