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Journal of Chromatography B, 754 (2001) 193–199

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

High-performance liquid chromatographic analysis of the tyrphostin AG1478, a specific inhibitor of the epidermal growth factor receptor tyrosine kinase, in mouse plasma

A.G. Ellis[a], E.C. Nice[b,1], J. Weinstock[b], A. Levitzki[c], A.W. Burgess[b], L.K. Webster[a,*]

[a]*Pharmacology and Developmental Therapeutics Unit, Trescowthick Research Laboratories, Peter MacCallum Cancer Institute, Locked Bag 1, A'Beckett Street, Melbourne, Victoria 8006, Australia*

[b]*Epithelial Biochemistry Laboratory, Ludwig Institute for Cancer Research, P.O. Box 2008 RMH, Melbourne, Victoria 3050, Australia*

[c]*Department of Biological Chemistry, Alexander Silverman Institute of Life Sciences, Hebrew University of Jerusalem, Jerusalem, Israel*

Received 8 September 2000; received in revised form 20 November 2000; accepted 20 November 2000

Abstract

The tyrphostin 4-(3-chloroanilino)-6,7-dimethoxyquinazoline (AG1478) is undergoing evaluation as a potential new anticancer agent. We have developed a specific and sensitive reversed-phase HPLC assay for AG1478 in mouse plasma. The method involves a rapid and simple extraction process followed by separation on a Symmetry C_8 stationary phase with a gradient of acetonitrile in ammonium acetate buffer. A linear response was achieved over the concentration range of 0.2–100 μM using multilevel calibration with internal standard method of calculation. Inter- and intra-assay accuracy and precision were better than $\pm 10\%$. The limit of quantitation was 0.2 μM. We have used this method to study the preclinical pharmacokinetics of this new agent in mice.

Keywords: Tyrphostins; AG1478

1. Introduction

A series of quinazoline analogues known as tyrphostins were recently developed as tyrosine kinase inhibitors by Levitzki and Gazit [1]. The tyrphostin 4-(3-chloroanilino)-6,7-dimethoxyquinazoline (AG1478, Fig. 1) is a highly potent and specific inhibitor of epidermal growth factor (EGF) receptor tyrosine kinase ([1] and Nice et al., in preparation). It is currently undergoing preclinical evaluation as a potential new anticancer agent. A specific and sensitive assay for AG1478 in mouse plasma is essential for preclinical pharmacokinetic studies, and can be adapted to suit human plasma. AG1478 is poorly soluble in water but exhibits an absorption peak maximum at 330 nm. Data regarding the physical or chemical characteristics are limited, but some information from in vitro cell culture experiments regarding its specific inhibition of the

*Corresponding author. Tel.: +61-3-9656-1276; fax: +61-3-9656-1411.

E-mail addresses: ed.nice@ludwig.edu.au (E.C. Nice), l.webster@pmci.unimelb.edu.au (L.K. Webster).

[1]Corresponding author. Tel.: +61-3-9341-3155; fax: +61-3-9341-3104.

0378-4347/01/$ – see front matter
PII: S0378-4347(00)00606-X

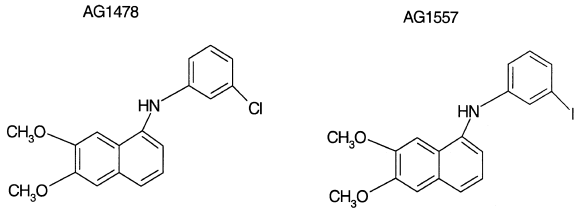

Fig. 1. The structures of the tyrphostins 4-(3-chloroanilino)-6,7-dimethoxyquinazoline (AG1478) and 4-(3-iodoanilino)-6,7-dimethoxyquinazoline (AG1557, internal standard).

EGF receptor has been published [2,3]. Reversed-phase high-performance liquid chromatography (HPLC) has been used previously to assess plasma levels of another tyrphostin, AG490, however the sensitivity of this assay was limited to only 1.0 μ*M* and metabolite separation was not addressed [3].

2. Experimental

2.1. Chemicals and reagents

AG1478 and the internal standard AG1557 [4-(3-iodoanilino)-6,7-dimethoxyquinazoline, Fig. 1] were kindly provided by A. Levitzki or were purchased from the Institute of Drug Technology (IDT) (Boronia, Australia). The water used was high-purity reverse osmosis, organic-filtered, 0.22-μm filtered (Milli-Q; Millipore, Bedford, MA, USA). All other reagents were chromatography grade with the exception of glacial acetic acid which was analytical grade. All solvents or buffers used in the HPLC system were vacuum-filtered through a 0.22-μm filter (GVWP; Millipore).

2.2. Standards and quality controls

Stock solutions of AG1478 and AG1557 were prepared by dissolving the compounds in dimethylsulfoxide (DMSO) at 50 m*M* and 10 m*M*, respectively. Stock solutions were stored at −70°C. Quality control (QC) samples were prepared in mouse plasma and stored at −70°C as individual 90-μl aliquots. QCs were made up prior to the commencement of a series of assays. Typically QCs are used at a range of relevant concentrations (low, medium and high positions within the standard curve range). However the limited availability of mouse plasma restricted the use of QCs in these studies to low (1.0 μ*M*) and medium–high (10.0 μ*M*). QC samples were deliberately prepared from a separate stock solution (separate weighing of drug) to that used to create standard curve samples. Aliquots of QCs were thawed and processed to monitor inter-assay variation.

2.3. Sample preparation

A solution of acetonitrile–water (50:50, v/v) (AW) was used to dilute stock solutions to a working concentration of 500 μ*M* AG1478, and serial dilutions into mouse plasma generated the following samples used to prepare the standard curve: 100, 50, 10, 2, 0.5 and 0.2 μ*M*. A sample of blank mouse plasma (0 μ*M* AG1478) was also prepared and extracted without the addition of internal standard. Samples were stored at −70°C and on the day of assay were thawed at room temperature, vortex-mixed and centrifuged (11,600 *g* for 5 min). To 90 μl of sample 180 μl of cold (0–4°C) extraction solution (acetonitrile containing 50 μ*M* internal standard) was added. Cold (0–4°C) acetonitrile, in place of extraction solution, was added to the blank plasma sample and any predose samples. Extracted samples were vortex-mixed (at least 30 s), centrifuged (10,500 *g* for 5 min) then supernatant transferred to a clean tube and evaporated to dryness at 42°C under a stream of air. The resulting residue was reconstituted in 45 μl acetonitrile followed by the addition of 45 μl high-purity water and centrifugation (10,500 *g* for 5 min) to pellet any undissolved debris. A single 50-μl injection was made per sample. Injections of non-extracted aqueous solutions of both AG1478 and internal standard were also made and used to calculate the recovery from extracted samples.

2.4. Chromatography

The system components were manufactured by Waters (Milford, MA, USA) unless specified otherwise and included a Model 2690 ''Alliance'' separations module, and a Model 996 photo diode array detector. System control, data acquisition and processing were performed via a Pentium personal

computer with Windows 95 (Microsoft, Redmond, WA, USA) and Millennium 32 version 3.05.01 chromatography software with the system suitability option installed. A stainless steel Symmetry C_8 column, 150×3.0 mm I.D., 5 μm particle size, was preceded in-line by a stainless steel Symmetry C_8 guard column, 20×3.9 mm I.D., 5 μm particle size. Separations were performed at ambient temperature. Detection was at 330 nm. The mobile phase was generated automatically via in-line mixing of solvents from individual solvent lines flowing through an in-line degasser. Two separate solvent lines were used: (A) 0.1 *M* ammonium acetate buffer, pH 6.0; and (B) acetonitrile. Gradient starting conditions of A–B (90:10) at a flow-rate of 0.2 ml/min were used before a linear gradient to A–B (5:95) at 0.2 ml/min over 38 min, followed by a linear return to starting conditions at 0.4 ml/min over 2 min and reequilibration at A–B (90:10) at 0.4 ml/min for 7 min. A delay of 1 min of A–B (90:10) at 0.2 ml/min was included prior to a subsequent injection. The total run time for each analysis was 47 min. A multilevel calibration used six calibration standards in mouse plasma and was derived from the plot of concentration (x) versus the analyte-to-internal standard ratio of peak area (response) observed (y). A linear regression curve weighted $1/x$ was fitted to the plotted points. Acceptability limits for standards and QCs were ±15% (or ±20% at the limit of quantitation, LOQ).

3. Results

3.1. Specificity

Analytical reversed-phase HPLC demonstrated that AG1478 and internal standard (AG1557) eluted essentially as single symmetrical peaks with retention times of 31.4 and 32.5 min, respectively (Fig. 2). There was no interference from endogenous compounds in drug-free mouse plasma. A number of compounds were tested as potential internal standards in the development of this assay: dimethylaminobenzaldehyde, a range of quinazoline analogues including dimethoxyquinazolinedione, 4-benzylamino-8-methoxyquinazoline, AG1557, progesterone and several other steroids. The quinazoline analogue AG1557 was chosen as internal standard, as it eluted after the analyte, had similar extraction characteristics and showed excellent stability under storage in the autosampler over the duration of an assay.

3.2. Standard curve

The standard curve showed linearity and reproducibility over the concentration range 0.2–100 μ*M* (r^2>0.999) (Table 1).

3.3. Accuracy and precision

3.3.1. Intra-assay

Intra-assay validation was performed using six QC samples at each of two concentrations (1.0 and 10.0 μ*M*) of AG1478 in mouse plasma that were assessed within one analytical run. The QC samples used were from two different batches that were prepared from separate weighings and in separate batches of blank plasma. Values are presented for the accuracy and precision of these QC samples (Table 2) and for intra-assay validation of aqueous injections (Table 3).

3.3.2. Inter-assay

Six replicates at concentrations of 1.0 or 10.0 μ*M* AG1478 in mouse plasma underwent individual extraction in six different assays. Acceptable values for inter-assay accuracy and precision were obtained for these samples and for aqueous samples containing 50 μ*M* of either AG1478 or AG1557 (Table 3).

3.4. Sensitivity

The limit of detection (LOD) was estimated by multiplying the magnitude (in peak height) of baseline noise (<0.0001 AU) by 3 to give an estimated LOD of 0.0003 AU. This was equivalent to one-eighth of the magnitude of the low standard (0.0024 AU, 0.2 μ*M*) or an LOD of 0.03 μ*M*. The LOQ was 0.2 μ*M* and this concentration of AG1478 was used as the low standard. Due to the limited availability of matrix (mouse plasma) attempts to establish a lower LOQ were not made. At 0.2 μ*M* the RSD from four separate assays was 7.3%.

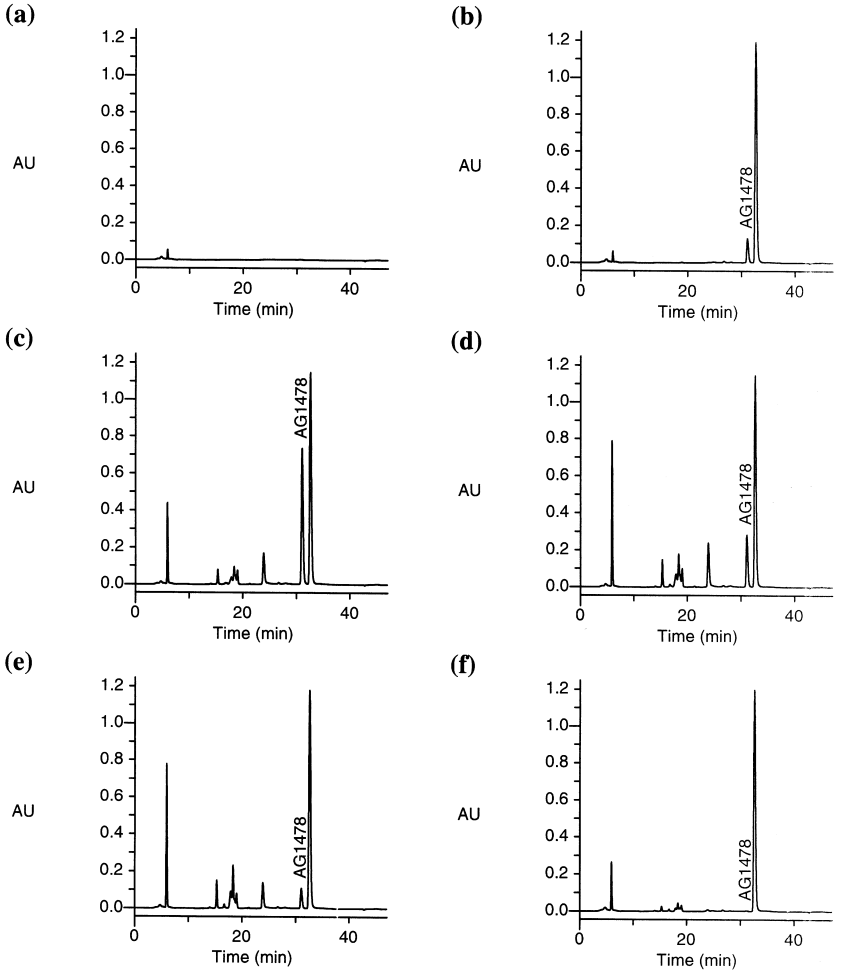

Fig. 2. Chromatograms of (a) blank mouse plasma sample containing no AG1478 or internal standard, (b) 10.0 μ*M* AG1478 QC sample with internal standard, or plasma from mice that had received a subcutaneous 88 mg/kg dose of AG1478 tartrate and had blood collected at (c) 30 min (d) 60 min (e) 120 min (f) 240 min post dose. Retention times for AG1478 and internal standard (AG1557) were 31.4 and 32.5 min, respectively.

3.5. Recovery

Recovery of AG1478 and internal standard following the extraction process was assessed by comparing the peak areas from samples of extracted mouse plasma with that achieved using injections of aqueous (non-extracted) samples containing 50 μ*M* analyte or internal standard in AW. Extracted samples included mouse plasma samples at each of the six calibration standard concentrations ranging from 0.2 to 100.0 μ*M*, each with an internal standard concentration of 50 μ*M*. The recoveries of AG1478 from five separate analytical runs for all of these samples were high (>91%) and consistent (RSD 9.7%, $n=29$). The recovery of internal standard (AG1557) for the same samples was also high (>86%) and consistent (RSD 4.3%, $n=30$).

3.6. Stability

3.6.1. Stability at −70°C of stock solutions and of plasma samples

Stock solutions of analyte in DMSO stored at −70°C were stable over at least 3 months. Following

Table 1
Inter-assay variation – accuracy, precision and linearity of standard curve samples from five separate assays[a]

Assay No.	Calculated AG1478 concentration (μ*M*)						Accuracy[b] (%)						r^2
	0.2	0.5	2.0	10.0	50.0	100.0	0.2	0.5	2.0	10.0	50.0	100.0	
1	0.22	0.46	1.95	9.94	50.27	99.86	10.09	−7.47	−2.39	−0.62	0.54	−0.14	0.999949
2	0.19	0.46	2.19	10.15	49.93	99.78	−3.28	−7.14	9.30	1.49	−0.14	−0.22	0.999855
3	0.19	0.49	2.12	10.18	50.51	99.21	−5.51	−2.63	6.10	1.82	1.02	−0.79	0.999854
4	–	0.50	1.98	10.00	51.76	98.26	–	−0.77	−1.04	0.02	3.52	−1.74	0.999398
5	0.19	0.59	1.83	9.32	50.94	99.83	−3.92	17.62	−8.66	−6.76	1.88	−0.17	0.999403
Mean	0.20	0.50	2.01	9.92	50.68	99.39							0.999692
SD[c]	0.01	0.05	0.14	0.35	0.71	0.68							0.000269
RSD[d] (%)	7.3	10.3	7.1	3.5	1.4	0.7							0.03

[a] A linear curve was fitted to the data for response versus theoretical concentration as described in Experimental. The calculated concentration was derived from reading the response for each standard sample against its corresponding curve.
[b] Accuracy=the deviation of the calculated concentration from the curve (theoretical concentration) expressed as percent.
[c] SD=Standard deviation.
[d] RSD (precision, relative standard deviation)=100×SD/mean.

dilution (1:1000 in AW) injection of the analyte stock solution resulted in peak areas for analyte with a RSD<4% (Table 3). Similarly following dilution (1:200 in AW) injection of the internal standard stock solution resulted in peak areas for internal standard with an RSD<9% (Table 3). The stability of analyte (at 1.0 and 10.0 μ*M*) in mouse plasma over at least 3 months of storage at −70°C was confirmed by the accuracy of QC samples in the inter-assay validation (Table 3). Where sample volume permitted, the repeat measurement of experimental samples after storage at −70°C also demonstrated acceptable stability over a period of 2 months.

3.6.2. Stability upon thawing and refreezing

Stability of analyte in mouse plasma samples was unaffected by three cycles of freezing and thawing. Duplicate aliquots of QC samples at both 1.0 and 10.0 μ*M* were frozen at −70°C then assayed after

Table 2
Intra-assay variation – accuracy and precision of replicate quality control (QC) samples within the same assay[a]

Replicate No.	QC 1.0 μ*M*		QC 10.0 μ*M*	
	Concentration (μ*M*)	Accuracy[b] (%)	Concentration (μ*M*)	Accuracy[b] (%)
1	0.95	5.2	9.94	0.6
2	1.14	14.3	10.74	7.4
3	1.05	5.2	10.98	9.8
4	1.01	1.2	10.85	8.5
5	1.05	5.4	10.82	8.2
6	1.05	4.6	10.76	7.6
Mean	1.04	6.0	10.68	7.0
SD[c]	0.06	4.4	0.38	3.2
RSD[d] (%)	6.1		3.5	

[a] Results from injection of individually extracted replicate samples in mouse plasma. The samples were from two different batches of QC samples (separate weighings of drug and separate batches of blank plasma).
[b] Accuracy=100×measured concentration/theoretical concentration.
[c] SD=Standard deviation.
[d] RSD (relative standard deviation)=100×SD/mean.

Table 3
Inter-assay variation – accuracy and precision data for mouse plasma samples and for aqueous samples[a,b]

Replicate No.	Mouse plasma samples				Aqueous samples[a]	
	1.0 μ*M* (AG1478)		10.0 μ*M* (AG1478)		50 μ*M* AG1478	50 μ*M* AG1557
	Concentration (μ*M*)	Accuracy[c] (%)	Concentration (μ*M*)	Accuracy[c] (%)	Peak area ($\cdot 10^6$)	Peak area ($\cdot 10^6$)
1	0.90	10.4	9.98	0.2	13.61	13.95
2	0.96	4.1	10.06	0.6	13.78	13.29
3	0.89	10.8	9.53	4.8	–	11.84
4	0.90	9.9	9.66	3.4	12.79	11.54
5	0.95	5.2	9.94	0.6	12.97	11.40
6	0.90	10.3	10.11	1.1	13.37	11.83
Mean	0.92	8.5	9.88	1.8	13.30	12.31
SD[d]	0.03		0.23		0.42	1.05
RSD[e] (%)	3.3		2.4		3.1	8.5

[a] Data from six separate assays. Mouse plasma samples were extracted as described in Experimental, and aqueous samples were injected without extraction. Assays were conducted over a period of 3 months.
[b] Aqueous samples were in acetonitrile–water (50:50, v/v).
[c] Accuracy=(100×measured concentration/theoretical concentration)−100.
[d] SD=Standard deviation.
[e] RSD (relative standard deviation)=100×SD/mean.

thawing at room temperature or after a further one or two freeze–thaw cycles. No significant difference was observed (Table 4).

3.6.3. Stability of samples within the autosampler

The stability of extracted mouse plasma samples in the autosampler was not assessed due to the limited availability of the matrix. The stability of analyte and internal standard in the autosampler was assessed using injections of 50 μ*M* aqueous solutions that were repeated over a period of 32 h (over 40 injections). Alternating injections of blank AW were also made to assess for any carryover effect. The peak areas achieved for both internal standard and analyte were consistent with no evidence of carryover. An earlier internal standard candidate for

Table 4
Stability of AG1478 in mouse plasma following up to three cycles of freezing and thawing[a]

No. freeze–thaw cycles	Replicate	Measured concentration (μ*M*)	
		1.0 μ*M* AG1478	10.0 μ*M* AG1478
1	a	0.90	10.11
	b	0.91	10.18
2	a	0.93	9.99
	b	0.90	9.93
3	a	0.92	9.90
	b	0.92	10.18

[a] Replicate aliquots at each concentration were frozen at −70°C and thawed at room temperature then either assayed or refrozen. No significant difference was found between cycles, one-way analysis of variance (ANOVA) ($P=0.533$).

this assay, dimethylaminobenzaldehyde, showed unacceptable stability under these conditions and was withdrawn from further development for this reason.

3.7. Pharmacokinetic studies

This method has been successfully applied to the analysis of mouse plasma obtained from preclinical pharmacokinetic studies (Fig. 2). Briefly, these studies involved administering the drug to mice using a variety of vehicles and administration routes. Plasma was generated for analysis from blood samples taken at a series of specific times post administration. In addition, a single batch of human plasma was tested and exhibited no interfering endogenous peaks. A single sample of this plasma containing 28 μ*M* AG1478 was measured against a standard curve from samples in mouse plasma and showed acceptable accuracy (−7.7%).

4. Discussion

The development of a novel method for the extraction, reversed-phase HPLC separation, and quantitation of the quinazoline derivative AG1478 has facilitated the investigation of the pharmacokinetics of this potential new anticancer pharmaceutical. Such preclinical information is necessary prior to progression to clinical trial. In the present study we describe a reversed-phase HPLC method that uses a rapid and simple extraction procedure and results in high and consistent recovery. The quantitation uses the internal standard method of calculation and a multilevel calibration curve with matrix-matched calibration standards. Sensitivity down to 0.2 μ*M* and good intra- and inter-assay accuracy and precision is achieved using only 90 μl of plasma. The gradient has been designed to facilitate the separation of potential metabolite peaks and parent compound – it would be possible to fractionate the eluate to further characterize these potential metabolites. The internal standard is of similar structure to the analyte and exhibits appropriate recovery, stability and chromatographic performance. In addition its elution after the analyte, rather than before, avoids any potential interference with peaks of more polar metabolites of the analyte. Importantly the analyte whether in matrix, in stock solution or in a reconstituted sample is stable under the storage conditions used. This method has been used to successfully quantitate samples from pharmacokinetic studies of AG1478 in mice (in preparation) and could easily be adapted to clinical samples or to samples of other biological fluids.

Acknowledgements

The authors would like to thank S. Rainone and N. Wark for assistance with pharmacokinetic studies, and A. Vitali for technical assistance.

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