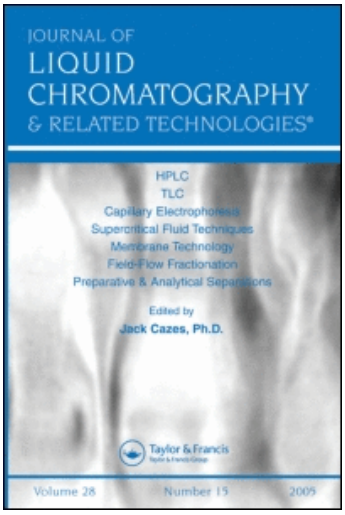

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A QUANTITATIVE HPLC DETECTION METHOD FOR WHI-P154 [4-(3'-BROMO-4'-HYDROXYLPHENYL)-AMINO-6,7-DIMETHOXYQUINAZOLINE]

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ABSTRACT

WHI-P154 [4-(3'-bromo-4'hydroxylphenyl)-amino-6,7 di-methoxyquinazoline] is a novel anti-tumor agent with unique cytotoxic activity against human glioblastoma cells (Clin. Cancer. Res. 4:1405-1414, 1998). Further development of WHI-P154 will require detailed pharmacodynamic studies in preclinical animal models. Therefore, we established a sensitive and accurate high performance liquid chromatography (HPLC)-based quantitative detection method for WHI-P154. This method allows the measurement of WHI-P154 levels in plasma, as well as in target human glioblastoma cells. Plasma and cell lysates were extracted with chloroform, dried with nitrogen gas and reconstituted in methanol : water (9:1, v/v).

An aliquot was injected into a Hewlett Packard HPLC system employing a 250 x 4mm Lichrospher 100, RP-18 (5 µm) analytical column in conjunction with a 4 x 4 mm Lichrospher 100, RP-18 (5 µm) guard column. The eluted compounds were detected by a diode array detector set at a wavelength of 335 nm. Acetonitrile/water containing 0.1% trifluoroacetic acid and 0.1% triethylamine (28:72, v/v) was used as a mobile phase. The average extraction recovery of WHI-P154 was 78.3% for plasma and 96.0% for U373 human glioblastoma cells.

The assay was linear (r>0.999) within the concentration range of 0.1 - 20 µM in 100 µL plasma and within the quantity range of 0.025 - 5 nmol per 2.5 million U373 glioblastoma cells. The intra- and inter-assay variabilities were less than 6% and the lowest detection limit of WHI-P154 was 0.05 µM in plasma and 0.01 nmol in U373 cells, respectively. The practical utility of this new HPLC method was confirmed in pilot pharmacokinetic studies using rats as well as cellular uptake studies using U373 human glioblastoma cells.

INTRODUCTION

Glioblastoma multiforme (GM) is a highly invasive primary tumor of the human central nervous system.[1] Total surgical resection of GM is rarely possible because of a diffuse infiltration of the surrounding normal brain parenchyma by glioblastoma cells and the lack of a distinct tumor-free margin.[2-4] The ability of glioblastoma cells to migrate within the context of the extracellular matrix (ECM) is thought to play a major role in local recurrence and progression of GM after multimodality treatment programs employing surgery in combination with radiochemotherapy.[5-11] Therefore, new agents that can inhibit the infiltration of normal brain parenchyma by glioblastoma cells may provide the basis for more effective multimodality treatment programs for GM.

In a recent study, we discovered that the novel quinazoline derivative, 4-(3'-bromo-4'hydroxylphenyl)-amino-6,7-dimethoxyquinazoline (WHI-P154) exhibits significant cytotoxicity against human glioblastoma cell lines causing apoptotic cell death at micromolar concentrations.[12] In a subsequent study, we found that WHI-P154 is a potent inhibitor of glioblastoma cell adhesion and migration in the context of ECM. At non-cytotoxic concentrations, WHI-P154 inhibited (a) integrin-mediated glioblastoma cell adhesion to the ECM proteins laminin, type IV collagen, and fibronectin, (b) integrin-independent EGF-induced adhesion of glioblastoma cells to poly-L-lysine coated tissue culture plates, (c) fetal bovine serum-induced polymerization of actin and actin stress

fiber formation as well EGF-stimulated formation of focal adhesion plaques in serum-starved glioblastoma cells, and (d) glioblastoma cell migration in in vitro assays of invasiveness using tumor cell spheroids and/or Matrigel-coated Boyden chambers.[13]

Further development of WHI-P154 will require detailed pharmacodynamic studies in preclinical animal models. Currently, there are no analytical methods available for detecting WHI-P154 in biological fluids and in human glioblastoma cells. Therefore, we set out to establish a sensitive and accurate detection method for WHI-P154. Here, we first describe a high performance liquid chromatography (HPLC)-based quantitative detection method which allows the measurement of WHI-P154 levels in plasma, as well as in target human glioblastoma cells in pharmacokinetic studies.

EXPERIMENTAL

Chemicals

Deionized distilled water was obtained from U.S. Filter (United States Filter Corporation, Cowell, MA). Methanol, acetonitrile, chloroform, triethylamine (TEA), and trifluoroacetic acid (TFA) were obtained from Fisher Chemicals (Fair Lawn, NJ). Cell culture medium (Minimal Essential Medium with Earle salts and L-glutamine), nonessential amino acid (NEAA), and sodium pyruvate and were purchased from GIBCO BRL (Gaithersburg, MD). Fetal bovine serum was from Summit (Fort Collins, CO) and was heated at 56°C for 30 minutes before use.

Cell Lines and Cultures

Human glioblastoma cell line U373 from American Type Culture Collection (ATCC) (Rockville, MD)[12,13] was propagated in MEM with Earle salts and L-glutamine, supplemented with heat-inactivated FBS (10% vol/vol), 0.1 mM NEAA, and 1mM sodium pyruvate. The cell cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere.

Drugs

WHI-P154 and its internal standard WHI-P131 were synthesized as previously described.[12,13] Their structures (Figure 1A) and physicochemical properties were previously reported.[12,13] Stock solutions of WHI-P154 and

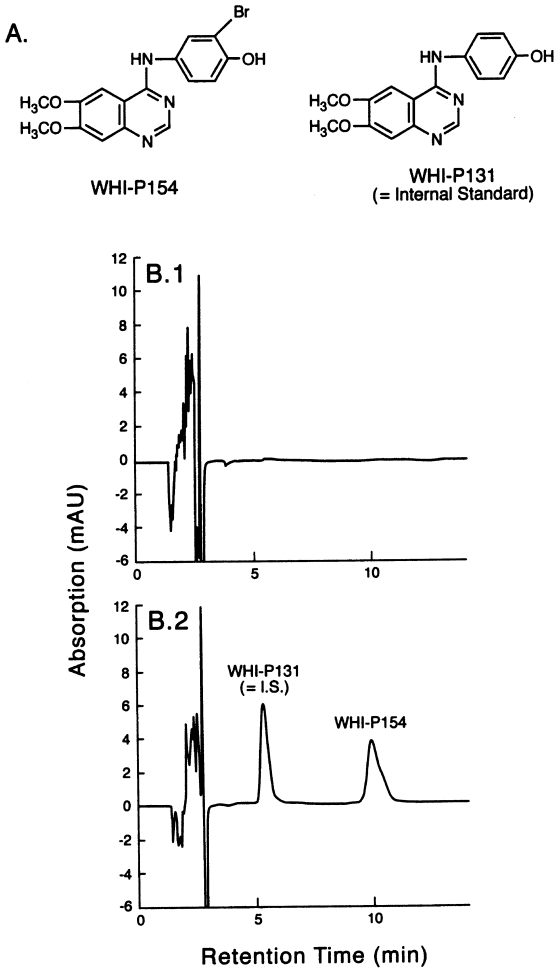

Figure 1. (A) Chemical structures of WHI-P154 and its internal standard WHI-P131. Molecular weight for WHI-P154 was 376 and for WHI-P131 was 297. Representative chromatograms from (B.1) blank plasma, (B.2) plasma sample 10 min after i.p. injection of 10 μmol/kg WHI-P154. I.S. = internal standard.

WHI-P131 were prepared in methanol/water (50:50) at a concentration of 200 μM and 1 mM, respectively, and stored at -20°C. The stock solutions were diluted further in 50% methanol to yield appropriate working solutions for the preparation of standards to calibrate.

Apparatus and Chromatographic Conditions

The HPLC system consisted of a Hewlett Packard (HP) series 1100 equipped with an automated electronic degasser, a quaternary pump, an autosampler, an automatic thermostatic column compartment, diode array detector and a computer with a Chemstation software program for data analysis. A 250 x 4 mm Lichrospher 100, RP-18 (5 µm) analytical column and a 4 x 4 mm Lichrospher 100, RP-18 (5 µm) guard column were obtained from Hewlett Packard Inc. (Palo Alto, CA). Acetonitrile/water containing 0.1% of trifluoroacetic acid (TFA) and 0.1% triethylamine (TEA) (28:72, v/v) was used as the mobile phase. The mobile phase was degassed automatically by the electronic degasser system.

The analytical column was equilibrated and eluted under isocratic conditions utilizing a flow rate of 1.0 mL/min at ambient temperature. The wavelength of detection was set at 335 nm. Peak width, response time, and slit were set at >0.03 min, 0.5 s and 8 nm, respectively.

Extraction Procedures

For determination of WHI-P154 levels in plasma, 10 µL of the internal standard WHI-P131 (50 µM) was added to a 100 µL plasma sample. For extraction, 7 mL chloroform was then added to the plasma sample, and the mixture was vortexed thoroughly for 3 min.

Following centrifugation (1500 rpm, 5 min), the aqueous layer was frozen using acetone/dry ice and the organic phase was transferred into a clean test tube. The chloroform extracts were dried under a slow steady stream of nitrogen. The residue was reconstituted in 100 µL of methanol: water (9:1) and a 50 µL aliquot of this solution was used for HPLC analysis.

For extraction of WHI-P154 from 2.5 million cells, 10 µL of the internal standard WHI-P131 (50 µM) (=0.5 nmol) was added to cell lysate in 100 µL water and then the mixture was extracted with chloroform as described above. Replicate (N=5) plasma samples (100 µL/sample) were spiked with known amounts of WHI-P154 to yield a final concentration of 0.5 µM and 10 µM of WHI-P154, and replicate lysates samples (N=6) were spiked with 0.5 nmol WHI-P154. The samples were extracted following the above described extraction procedures. The extraction recovery (ER) was calculated using the formula: ER ={Peak Area $[\text{WHI-P154}]_{\text{extracted}}$/Peak Area $[\text{WHI-P154}]_{\text{unextracted}}$}x100.

Calibration Curves

A calibration curve was generated to confirm the linear relationship between the peak area ratio (peak area of WHI-P154 over the peak area of internal standard WHI-P131), and the concentration of WHI-P154 in the test samples. WHI-P154 was added to plasma to yield final concentrations of 0.1, 0.25, 0.5, 1, 5, 10, and 20 μM, and to untreated U373 cell lysates ($2.5x10^6$ cells) to yield the quantities of 0.025, 0.05, 0.1, 0.5, 1, and 5 nmol. Subsequently, 10 μL of the internal standard WHI-P131 (50 μM) (=0.5 nmol) was added to each sample. The plasma and cell lysate samples with known amounts of WHI-P154 and its internal standard were extracted as previously described, and the standard curves were generated by plotting the peak area ratios (WHI-P154/WHI-P131) against the drug concentrations tested. Linear regression analysis of the standard curve was performed by using the CA-Cricket Graph III computer program, Version 1.1 (Computer Association, Inc., Islandia, NY).

The standard samples of WHI-P154 (0.7 μM and 7.5 μM in plasma, and 0.5 nmol in cell lysate) were used as calibrators. The WHI-P154 contents of these standards were calculated by interpolating the peak area ratio with the calibration curve.

Intra-Assay and Inter-Assay Accuracy and Precision

To evaluate the intra-assay accuracy and precision, WHI-P154 and its internal standard WHI-P131were added to drug-free plasma at concentrations of 0.75 and 7.5 μM. These standard samples were prepared and analyzed within a single day following addition of the internal standards. The concentrations were calculated using a standard curve. The ratio of the calculated concentration over the known concentration of WHI-P154 was used as the accuracy of the analytical method, and the coefficient of variance was used as an index of precision. The inter-assay accuracy and precision were determined using 6 independent experiments. To evaluate the intra-assay and inter-assay accuracy and precision of the assay for measurement of WHI-P154 content in cells, 0.5 nmol WHI-P154 was added to lysates from untreated $2.5x10^6$ U373 cells, and these lysate samples were analyzed as described above.

Drug Administration to Rats and Blood Sampling

Lewis male rats (260-310 g) purchased from Harlan Sprague Dawley (Indianapolis, IN) were housed in a controlled environment (12-h light/l2-h dark photo-period, 22±1°C, 60±10% relative humidity), which is fully accredited by the USDA (United States Department of Agriculture). The rats were allowed

free access to pelleted food and tap water throughout the experiments. Animal studies were approved by Hughes Institute Animal Care and Use Committee and all animal care procedures conformed to the guidelines found in Hughes Institute Animal Care and Use Manual.

Drug Administration and Sampling

WHI-P154 was dissolved in DMSO and diluted with alkaline PBS (pH ~8.5). Three rats were injected intraperitoneally with a 10 µmol/kg (i.e. 1 mg /per rat) bolus dose of WHI-P154. Blood samples (~0.3 mL) were collected from the tail vein prior to drug administration as well as at 5, 10, 15, 30, 45 min and 1, 2, 4, and 7 hr after drug administration. These blood samples were heparinized and centrifuged at 7,000 g for 10 min in a microcentrifuge to obtain plasma.

The plasma samples were stored at -20°C until analysis. 100 µL aliquots of plasma were used for extraction and HPLC assays.

Cellular Pharmacokinetics in U373 Cells

U373 cells were grown in tissue culture flasks until they were nearly confluent. They were then trypsinized, spun down, and resuspended in fresh medium at a cell density of $1x10^6$/mL. The appropriate volume of cell suspension was then added to duplicate polystyrene tissue culture dishes to achieve 2.5 million cells/dish. Cells were allowed to adhere over-night prior to the experiment. Cells were treated with 25 µM of WHI-P154 at 37°C in a humidified 5% CO_2 atmosphere.

At specific time-points (15, 30 min., and 1, 4, 24 h), cells were removed from the dishes using a cell scraper, and transferred into clean tubes. After 3 min centrifugation at 300 g, cell pellets were lysed in water by freezing and thawing, extracted and analyzed by HPLC as described above. The cell volume was determined from the average cell diameter using the formula: Volume = 4/3 * Ξ*r^3. Cellular concentrations of WHI-P154 were calculated using the formula: Concentration = Quantity/Volume, where the volume is 4.2×10^{-12} L, corresponding to an average diameter of 20 µm for U373 glioblastoma cells.

Pharmacokinetic Analysis

Data fitting and pharmacokinetic parameter calculations were carried out using the pharmacokinetic software, WinNonlin program, version 1.1.

(Scientific Consulting Inc., Cary, NC).[14] An appropriate pharmacokinetic model was chosen on the basis of lowest weighted squared residuals, lowest Schwartz criterion (SC), lowest Akaike's information criterion (AIC) value, lowest standard errors of the fitted parameters, and dispersion of the residuals.

RESULTS AND DISCUSSION

Sensitivity and Accuracy of the Detection Method for Measurement of WHI-P154 Levels in Plasma and Human Glioblastoma Cell Lysates

Several combinations of acetonitrile, methanol and water (with 0.1% TFA and 0.1% TEA) were evaluated as possible mobile phases. Without TFA, WHI-P154 bond tightly to column and could not be eluted by combination of acetonitrile and water or with 100% acetonitrile. TEA has been demonstrated to be an important modifier for most of nitrogen-containing compounds.[15-19] Addition of TEA in the described mobile phase shortens the retention times and sharpens the peak for the compound WHI-P154. Therefore, TEA is also an important modifier for this system.

Under the described chromatographic separation conditions, the retention times for WHI-P154 and its internal standard WHI-P131 were 9.5 minutes and 5.0 minutes, respectively. At the retention time, the WHI-P154 and WHI-P131 were eluted without any interference peaks from the blank plasma (Figure 1B.1 and 1B.2) and also from cell lysates. With the described extraction conditions, the extraction recovery of WHI-P154 was 78.3% from plasma and 96.0% from cells (Table 1). The extraction recovery of WHI-P131 was 88.4% from plasma and 91.9% from cells.

The standard curves obtained from extraction of plasma and cell lysate samples containing known amounts of WHI-P154 were linear over the concentration-dose ranges tested. The calibration curve was linear and could be described by the regression equations: $Y = 3.728*X-0.01399$ ($r = 0.9999$) for the plasma, and $Y=0.523*X-0.086$ for the cell lysate, in which Y is the agent recovered (in µM in plasma and nmol in cell lysate), and X is the peak area ratio (WHI-P154/WHI-P131). The lowest limits of detection of WHI-P154 were 0.05 µM in plasma and 0.01 nmol in cells, respectively.

The obtained results indicate that the intra-assay and inter-assay coefficients of variance (C.V.) in plasma and in cells were less than 6%. The accuracy of this HPLC detection method was 99.7% in both plasma and in cells (Table 2).

Table 1

Extraction Recovery of WHI-P154 from Plasma and from Cells

Added (μM)	Peak Area (mAU*s) Unextracted	Peak Area (mAU*s) Extracted	Extraction Recovery (%)	Average (%)
In Plasma[a]				
0.5μM	25.9±0.3	20.0±1.0	77.2±3.9	
10μM	542.9±4.2	430.8±21.7	79.4±4.0	78.3
In Cells[b]				
0.5 nmol	162.1±0.5	155.6±5.8	96.0±3.6	96.0

* All data are presented as mean ± SD.
[a] N = 5; b_N = 6.

Table 2

Intra-assay and Inter-assay Accuracy and Precision of the Determination of WHI-P154 in Plasma and in U373 Human Glioblastoma Cells

	Added	Found	Accuracy (%)	C.V.* (%)
Intra-assay (n=6)				
In Plasma	0.75μM	0.75±0.03	100.2±4.7	4.7
	7.5 μM	7.31±0.34	97.5±4.5	4.6
In Cells	0.5 nmol	0.50±0.03	100.3±5.2	5.2
Inter-assay (n=6)				
In Plasma	0.75μM	0.74±0.02	98.4±3.0	3.1
	7.5 μM	7.71 ±0.31	102.6±4.2	4.0
In Cells	0.5 nmol	0.49±0.28	99.0±5.5	5.6

* C.V. = coefficient of variance. The data are presented as mean ± SD.

Table 3

Pharmacokinetic Parameters (Mean±SD) of WHI-P154 in Rats and U373 Cells

Parameters	C_{max}	t_{max} (h)	$t_{1/2ka}$ (h)	AUC
In Rats[a]	2.81±0.97 (μM)	0.37±0.29	0.22±0.22	2.45±0.51* (μM·h)
In Cells[b]	0.64 (nmol/10^6 cells)	13.79	0.63	13.84** [(nmol/10^6 cells)·h]

[a] Pharmacokinetic analysis on each rat, [b] pharmacokinetic analysis on pooled data. Abbreviation: t_{max} is the time for reaching the maximum plasma concentration or cellular drug content (C_{max}). $t_{1/2ka}$ is the half-life for drug absorption in rats or uptake in cells, AUC is the area under the concentration-time curve. *AUC from 0 -to ∝ h, ** AUC from 0 to 24 h.

Detection of WHI-P154 in Plasma After Systemic Administration

The plasma WHI-P154 concentrations ranged from 0.1 to 5.0 μM in rats during the first 2 h following i.p. administration of a 10 μmol/kg bolus dose (Figure 2A). However, the plasma WHI-P154 concentrations were below the detection limit at later time points. A single compartment pharmacokinetic model was used to describe the pharmacokinetics of WHI-P154. Some of the key pharmacokinetic parameters are presented in Table 3. WHI-P154 was absorbed with an absorption half-life of 0.22 h and a t_{max} of 0.37 h. WHI-P154 was also rapidly eliminated with elimination half-life of 0.36 h.

Detection of WHI-P154 in Lysates of U373 Human Glioblastoma Cells

The cellular uptake profile of WHI-P154 by human U373 glioblastoma cells following treatment with 25 μM WHI-P154 is shown in Figure 2B. The calculated cellular pharmacokinetic parameters are shown in Table 3. WHI-P154 showed a rapid uptake with half-life of 0.63 h, and t_{max} of 13.79 h. The predicted maximum cellular exposure dose (i.e., drug content) of WHI-P154 was 0.64 nmols per $1x10^6$ U373 cells, which corresponds to a concentration of 0.152 mM (= 0.64 nmols/$4.2x10^{-12}$ L). Thus, WHI-P154 accumulated in human glioblastoma cells to reach peak concentrations ~6-fold higher than the concentration in the treatment medium (i.e, 25 μM).

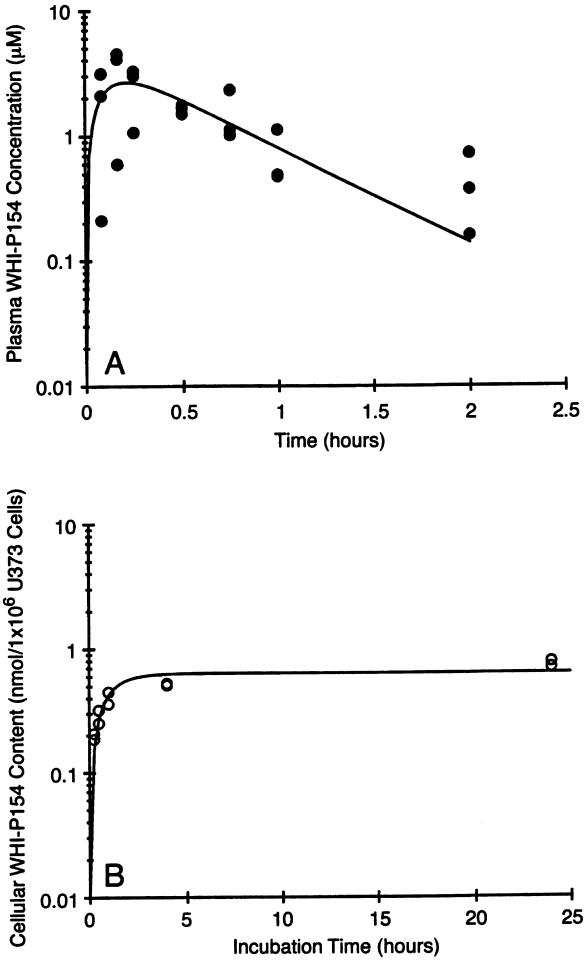

Figure 2. (A) Plasma WHI-P154 concentration-time profiles in rats following intraperitoneal administration of WHI-P154 (10 μmol/kg; n = 3). (B) Cellular WHI-P54 concentration-time profiles in U373 cells following incubation with 25 μM of WHI-P154 (•, ○ observed; — predicted).

While the molecular mechanism for this favorable cellular accumulation of WHI-P154 in human glioblastoma cells is unknown, it may in part explain the potent anti-glioblastoma cell activity of this novel quinazoline derivative. We hypothesize that WHI-P154 may be taken up via a pump-mediated active transportation route in addition to passive diffusion.

In summary, we have developed a highly sensitive and accurate analytical HPLC method for quantitative detection of the novel anti-glioblastoma agent WHI-P154 in plasma and cells. The availability of this assay will now permit detailed pharmacodynamic studies of WHI-P154 and facilitate our translational research efforts aimed at developing novel and effective treatment strategies for glioblastoma.

REFERENCES

1. D. C. Russel, L. Rubinstein, "Tumors of Central Neuroepithelial Origin," in, **Pathology of Tumors of the Nervous System**, Ed. 5., E. Arnold, ed., Hodder & Stoughton, London, 1989, pp. 83-289.

2. B. C. Devaux, J. R. O'Fallon, P. J. Kelly, J. Neurosurg., **78**, 767-775 (1993).

3. F. W. Kreth, P. C. Warnke, R. Scherement, C. B. Ostertag, J. Neurosurg., **78**, 762-766 (1993).

4. N. R. Quigley, J. C. Maroon, Neurosurgery, **29**, 385-389 (1991).

5. M. E. Berens, J. T. Rutka, M. L. Rosenblum, Neurosurg Clinics N. Am., **1**, 1-18 (1990).

6. S. K. Chintala, Z. L. Hokaslan, Y. Go, R. Sawaya, G. L. Nicolson, J. S. Rao, Clin. Exp. Metastasis, **14**, 358-366 (1996).

7. J. L. Finlay, "Chemotherapeutic Strategies for High Grade Astrocytoma of Childhood," in **Pediatric Neuro-Oncology**, R. J. Packer, W. A. Bleyer, C. Pochedly, eds., Harwood Academics, Philadelphia, 1992, pp. 278-297.

8. S. A. Grossman, L. K. Norris, Sem. Oncol., **22**, 530-539 (1995).

9. M. D. Pardos, C. Russo, Sem. Surgical Oncol., **14**, 88-95 (1998).

10. A. A. Brandes, M. V. Fiorentino, Cancer Invest., **14**, 551-559 (1996).

11. M. D. Pardos, C. B. Wilson, "Neoplasms of the Central Nervous System," in **Cancer Medicine**, Vol. I., J. F. Holland, R. C. Bast, Jr., D. L. Morton, E. Frei III, D.W. Kufe, R. R. Weichselbaum, eds., Williams and Wilkins, 1997, pp. 1471-1514.

12. R. K. Narla, X. Liu, D. E. Myers, F. M. Uckun, Clin. Cancer Res., **4**, 1405-1414 (1998).

13. R. K. Narla, X -P, Liu, D. Klis, F. M. Uckun, Clin. Cancer Res., **4**, 2463-2471 (1998).

14. **User's Guide**, WinNonlin, Scientific Consulting, Inc., Cary, NC, 1995.

15. C. L. Chen, B. A. Lesseley, C. R. Clarke, J. D. Roder, S. Sangiah, K. D. Berlin, G. L. Garrison, B. J. Scherlag, R. Lazzara, E. Patterson, J. Chromatogr. Biomed. Appl., **583**, 274-279 (1992).

16. C. L. Chen, S. Sangiah, J. D. Roder, H. Chen, K. D. Berlin, G. L. Garrison, B. J. Scherlag, R. Lazzara, Analytical Letters, **26**, 1125-1135 (1993).

17. C. L. Chen, S. Sangiah, J. D. Roder, H. Chen, K. D. Berlin, G. L. Garrison, B. J. Scherlag, R. Lazzara, Analytical Sciences, **9**, 429-431, (1993).

18. C. L. Chen, S. Sangiah, J. D. Roder, H. Chen, K. D. Berlin, G. L. Garrison, B. J. Scherlag, R. Lazzara, J. Liquid Chromatogr., **17**, 913-927 (1994).

19. C. L. Chen, S. Sangiah, H. Chen, K. D. Berlin, G. L. Garrison, B. J. Scherlag, R. Lazzara, J. Liquid Chromatogr., **17**, 3681-3694 (1994).

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