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High-performance liquid chromatographic determination of pyrazoloacridine, a nitro-9-methoxyacridine anticancer agent, in human plasma

Anura L. Jayewardene, Jennifer E. Santoro, John G. Gambertoglio*

Drug Research Unit, Department of Clinical Pharmacy, School of Pharmacy, University of California, San Francisco, CA 94143-0622, USA

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Abstract

Pyrazoloacridine (PZA) is a 9-methoxy substituted acridine with a reducible nitro group. PZA has shown selective solid tumor cytotoxicity with activity against hypoxic cells, non-cycling cells and cells expressing the multidrug resistant phenotype. A high-performance liquid chromatographic (HPLC) assay was developed and validated for the determination of PZA in human plasma to support phase II clinical trials. PZA and ethyl orange, the internal standard, were isolated from human plasma by precipitating plasma proteins with methanol, and centrifuging to pellet the proteins. The resulting supernatant was injected onto a cyanopropyl HPLC column eluted isocratically with a mobile phase consisting of 125 m*M* ammonium acetate buffer pH 4.75–acetonitrile (76:24, v/v). A single wavelength at 460 nm was used for detection. Relative standard deviations for the assay ranged from 5.0% to 12.2% for four different drug concentrations and the limit of quantitation was 100 ng/ml. During the validation short term stability of the drug in plasma and stability of PZA on repeated freezing and thawing of plasma was evaluated. Overall recovery of PZA was 88%. This simple assay was found suitable for studying the clinical pharmacokinetics of PZA. © 1997 Elsevier Science B.V.

Keywords: Pyrazoloacridine; Nitro-9-methoxyacridine

1. Introduction

Pyrazoloacridine, 9-methoxy-*N*,*N*-dimethyl-5-nitropyrazolo [3,4,5-*kl*]-acridine-2(6*H*)-propanamine as mono methanesulfonate salt (PZA), is a member of a class of antitumor acridines characterized by the presence of a reducible 5-nitro substituent. PZA was developed as one of a series of acridines which constitute a class of anticancer agents with selectivity against solid tumor cells [1]. PZA was synthesized by Capps et al. [2] and is one of 98 acridine compounds evaluated by Warner-Lambert/Parke-Davis [3]. It displays a unique set of properties uncharacteristic of most cytotoxic agents; it exhibits preferential activity against plateau phase cells, hypoxic cells and solid tumors [4]. In addition it shows activity in cells expressing the multidrug resistant phenotype. It has shown broad antitumor

*Corresponding author.

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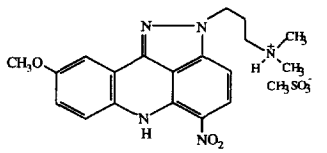
activity within in vitro and in vivo tumor systems [5]. Due to these properties, it is hoped that this compound will act against several different types of carcinomas. Although its precise mechanism of action is unknown, PZA appears to act as an intercalator, inhibiting DNA and RNA synthesis, with preferential inhibition of RNA synthesis [6].

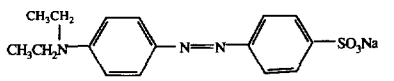
Two methods have been reported for determination of PZA in murine and monkey plasma samples. One assay developed by Berg et al., used for PZA determination in plasma of rhesus monkeys, involves an extraction by organic solvent of the PZA from plasma for which extraction efficiency must be determined [6]. After extraction is complete, samples are reconstituted in mobile phase for injection. Though an internal standard is used, it absorbs light at 340 nm while PZA absorbs at a wavelength of 460 nm; therefore, both wavelengths must be monitored in this assay.

The second and most recent assay was reported by Foster et al. to determine PZA in murine plasma [7]. This assay involves a solid-phase extraction of the drug from murine plasma samples following precipitation of plasma proteins with methanol. The extraction column is washed and the drug is finally eluted with a HCl–methanol (1:19, v/v) mixture. The eluate is evaporated and reconstituted in mobile phase and then transferred to vials for injection. No internal standard is used. Two solvent pumps are required for this HPLC assay. A simpler method of determining PZA concentration in plasma using an isocratic elution and less complicated sample preparation was needed.

The following assay was developed to determine PZA in human plasma. A cyanopropyl column was used for analysis and a one-step extraction procedure was employed to simplify the sample preparation. An internal standard, ethyl orange, that absorbs at the same wavelength of 460 nm as PZA was used so that the two peaks could be monitored at the same wavelength. Studies of the extraction efficiency and stability of PZA after extraction and during repeated freeze–thaw cycles were also undertaken. The structures of PZA parent compound and of ethyl orange are shown in Fig. 1. This assay was successfully applied to and is currently in use for the Phase II clinical study of PZA.



Pyrazoloacridine [PZA]
[$C_{20}H_{25}N_5O_6$ M.W. 463.5]



Ethyl Orange
[$C_{16}H_{14}N_3O_3SNa$ M.W. 355.4]

Fig. 1. Structural formulas of pyrazoloacridine [9-methoxy-*N*,*N*-dimethyl-5-nitropyrazolo [3,4,5-*kl*]-acridine-2(6*H*)-propanamine mono methanesulfonate salt (PZA) and ethyl orange [4-[*p*-(diethylamino) phenylazo] benzenesulfonic acid].

2. Experimental

2.1. Chemicals

All chemicals were HPLC or reagent-grade unless otherwise noted. Acetonitrile, methanol, ammonium acetate, and HCl were obtained from Fischer Scientific (Fair Lawn, NJ, USA). Ethyl orange, 90% dye content, was obtained from Aldrich (St. Louis, MO, USA). PZA was provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD, USA). Drug free human plasma was obtained from the Long Hospital Blood Bank and the Irwin Memorial Blood Center (UC San Francisco, San Francisco, CA, USA).

2.2. Chromatographic equipment and method

The HPLC system consisted of a Waters M-510 HPLC pump, an M-717 Waters autosampler, and a M-490 programmable UV detector from Waters Associates (Milford, MA, USA). An HP 3396 integrator was used to collect data (Hewlett-Packard, Santa Clara, CA, USA). A Beckman ultrasphere CN column, 150 mm×4.6 mm I.D., packed with 5 μm particles and a CN guard column were used for the assay (Fullerton, CA, USA). The M490 multi-

wavelength UV–visible detector was used to monitor drug and internal standard at a wavelength of 460 nm, with a sensitivity of 0.08 a.u.f.s.. The mobile phase was delivered isocratically at a flow-rate of 0.9 ml/min. Sample injection volume was 20–40 μl.

2.3. Mobile phase

The mobile phase consisted of 125 m*M* ammonium acetate buffer pH 4.75–acetonitrile (76:24, v/v). The buffer was prepared by dissolving 38.7 g of ammonium acetate in 4.0 l of distilled water. The pH was adjusted to 4.75 with 25% HCl. A mixture of 3040 ml of this buffer and 960 ml of acetonitrile was prepared and passed through a 0.22 μm filter and degassed by sonication under a vacuum prior to use.

2.4. Preparation of standards and controls

Calibration standards were prepared by spiking 5 ml aliquots of drug-free plasma with different concentrations of PZA stock solutions to give a range of concentrations from 100–10 000 ng/ml of PZA. Quality assurance/quality control (QA/QC) samples were prepared at four different concentrations of PZA using separate stock solutions of PZA. Aliquots of 250 μl of calibration standards and QA/QC samples were pipetted into culture tubes and frozen at −40°C until required for analysis.

2.5. Processing of plasma

Frozen 250 μl aliquots of plasma from study subjects, QA/QC samples and calibration standards were thawed as needed. The same procedure was followed for all samples. Aliquots of 40 μl of the internal standard, ethyl orange, at a concentration of 4 μg/ml were added to each tube. Tubes were vortexed for 10 s and 600 μl absolute methanol was added to each to precipitate the plasma proteins. The tubes were vortexed for 20 s, placed on ice for 15 min, and centrifuged at 1000 *g* for 10 min. The supernatant was then filled into autosampler vials for the HPLC assay.

2.6. Data analysis

Calibration standards containing 100, 250, 500, 1000, 5000 and 10 000 ng/ml of PZA were used to establish calibration curves for assay validation and for clinical assays. PZA concentrations versus peak height ratio values were plotted for the calibration curve samples and linear regression was performed to obtain the equation for the best fit line. The mean slope was $7.24 \cdot 10^{-4}$, with a standard deviation (S.D.) of $1.17 \cdot 10^{-4}$ and a relative standard deviation (R.S.D.) of 16.3%. The *y*-intercept mean was $-3.41 \cdot 10^{-2}$ and the mean coefficient of determination was 0.999 with a standard deviation of $4.79 \cdot 10^{-4}$ and an R.S.D. of 0.05% ($n=7$). The equation of the line was used to calculate the drug concentration in pooled frozen control and clinical samples using the peak height ratios calculated from the corresponding chromatograms.

2.7. Selectivity

Selectivity was assessed by using ethyl orange, a compound with an absorbance similar to PZA (460 nm) as the internal standard. Ethyl orange eluted before PZA, avoiding interference with PZA and resulting in two distinct peaks on the chromatograms. Five different blank plasma samples were assayed to establish that no endogenous plasma peaks interfered with the drug and internal standard peaks. None of the predose plasma samples from patients showed any interference. In addition, using detection at a wavelength of 460 nm allowed for the avoidance of interference.

A chromatogram of an extract of blank plasma is shown in Fig. 2a.

2.8. Inter-assay and intra-assay precision

Inter-assay and intra-assay precision were evaluated using previously frozen controls at four concentrations, extra-low, low, medium and high. For inter-assay precision, six samples of each concentration were assayed on six different days using six standard curves. R.S.D.s for the calculated drug concentrations were determined. For intra-assay pre-

cision six sets of six control samples from four different drug concentrations were assayed with a single calibration curve. R.S.D.s for the calculated drug concentrations were determined.

2.9. Lower limit of quantitation

The lower limit of quantitation was determined by spiking six aliquots of blank plasma with PZA at the concentration of the lowest plasma calibrator and assaying with a set of calibration curve samples. This determination was repeated on four different days. The mean R.S.D. was calculated and the accuracy of calculated concentration of drug compared to target concentration was determined.

2.10. Recovery

For assay recovery, aqueous samples were spiked with the same drug concentrations as plasma calibration standards. The internal standard was added to three replicates of each concentration for both aqueous samples and plasma calibration standards (after extraction). The samples for recovery were assayed on the same day. Assay recovery was determined by comparing peak height ratios from aqueous samples with peak height ratios from the corresponding plasma calibration standards to calculate a percent mean overall recovery.

2.11. Stability at room temperature

To assess the stability of PZA at room temperature, a set of six each of plasma QA/QC frozen controls were thawed and stored at room temperature for 24 h. Another set of six each of frozen controls were thawed and stored at room temperature for 48 h. At the end of the respective time periods, all quality controls were assayed along with a set of six each of freshly thawed controls. Stability at room temperature was evaluated by comparing the peak height ratios of PZA to ethyl orange to determine the mean decrease in PZA concentration over 24 and 48 h.

2.12. Freeze–thaw stability

To determine freeze–thaw stability of PZA, three sets of controls were thawed. One set was assayed while the remaining two sets were refrozen. These two sets were thawed on a subsequent day and one set was assayed while the other set was refrozen. The last set of quality controls was thawed on a third day and assayed. The peak height ratios from the assays of the three sets of quality controls were compared to evaluate the stability of PZA samples subjected to repeated thawing and freezing.

2.13. Stability of PZA in plasma after extraction

To establish the stability of PZA after plasma proteins were extracted, three sets of thawed quality controls were extracted and stored at 4°C for 30 h. After 30 h, the three sets of QA/QC samples were assayed. Three sets of freshly thawed control samples were assayed at the same time. The peak height ratios of the samples stored after extraction were compared to the peak height ratios of the freshly extracted control samples.

2.14. Applicability of the method

The applicability of the assay for completing a pharmacokinetic analysis of PZA was assessed by analyzing plasma samples from patients with advanced hormone refractory prostate cancer who had received a 3 h i.v. infusion of PZA at a 750 mg/m^2 dose level as part of a phase II trial. Heparinized blood samples were obtained prior to and serially during the 3 h infusion up to 72 h after the infusion. Patient plasma samples were processed and stored at −40°C until analysis. Pharmacokinetic data was obtained and analyzed using a noncompartmental analysis.

3. Results

3.1. Data collection and calculation

Peak height ratios of calibration standards were proportional to the concentrations of PZA in plasma

over the range tested. The calibration curves appeared linear and were described by linear regression lines with coefficients of determination greater than 0.999. The mean concentrations of the calibration standards ($n=7$) were within 95.6% of the spiked concentrations.

3.2. Selectivity

The assay was selective for PZA in human plasma. The retention times of PZA and ethyl orange were 13.7±0.5 min and 9.5±0.3 min, respectively. Chromatograms of drug free human plasma were free of peaks at the retention times of PZA and ethyl orange. A chromatogram of an extract of blank human plasma is shown in Fig. 2a. A chromatogram of extracts of human plasma with internal standard is shown in Fig. 2b, while Fig. 2c shows a plasma sample containing 903 ng/ml PZA from a patient after a dose of 1313 mg of PZA.

3.3. Inter-assay and intra-assay precision

The inter-assay precision for calculated drug concentrations had R.S.D. values ranging from 1.35% to 4.22% for QA/QC controls (Table 1). For intra-assay precision, R.S.D. values for the calculated drug concentrations ranged from 4.96% to 8.31% and the mean accuracy was 98.6% (Table 2).

3.4. Lower limit of quantitation

The lower limit of quantitation at 100 ng/ml had a mean accuracy of 105% of the target concentration and an R.S.D. of 12.5% for PZA in human plasma.

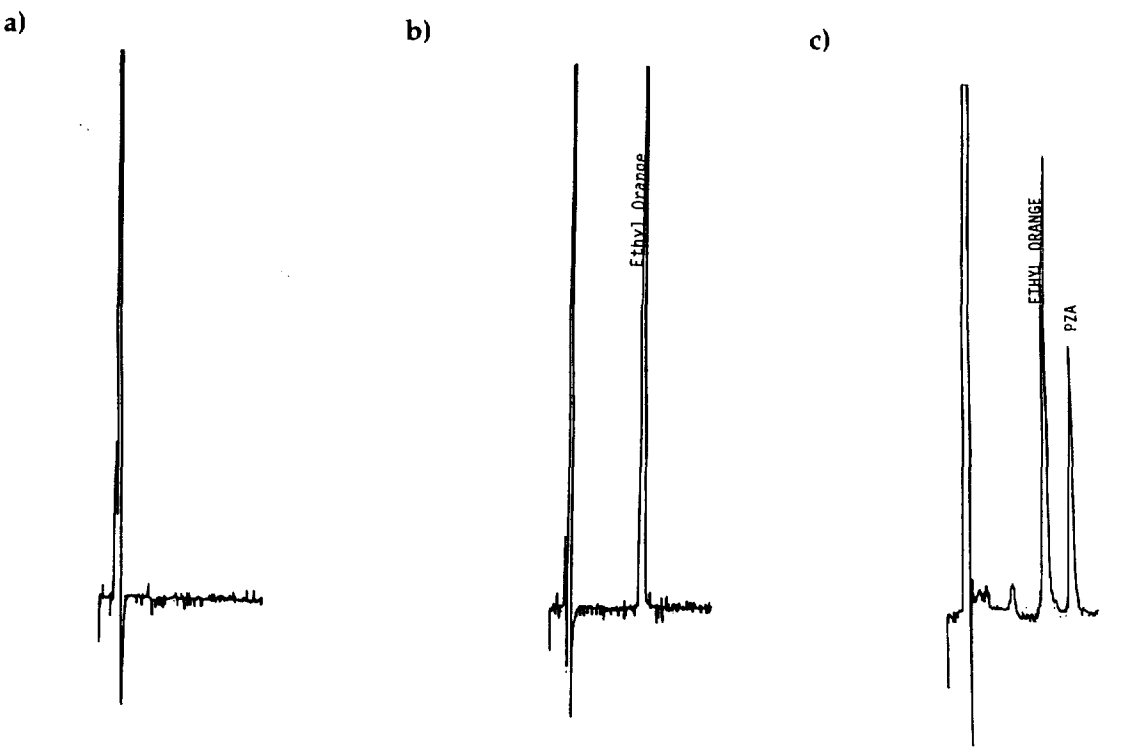


Fig. 2. Chromatograms of (a) blank plasma extract, (b) blank plasma extract with internal standard and (c) plasma extract from a patient after a 1313 mg dose of PZA with internal standard and a measured concentration of 903 ng/ml PZA. For chomatographic conditions refer to Section 2.2. Blank and spiked plasma samples were extracted as described in Section 2.5.

Table 1
PZA in plasma: inter-assay precision and accuracy

Day	PZA concentration[a] (ng/ml)			
	Extra low (275)	Low (935)	Medium (2320)	High (9270)
1	312	1029	2493	8901
2	303	1003	2396	8984
3	332	1036	2440	8970
4	311	1014	2467	9293
5	322	1034	2451	9115
6	295	994	2431	8980
Mean	313	1018	2446	9041
S.D.	13	17	33	142
R.S.D. (%)	4.22	1.71	1.35	1.57
Accuracy (%)	113.8	108.8	104.1	97.6

[a] Each value represents the mean of six samples.

Table 2
PZA intra-assay precision

Day	PZA concentration[a] (ng/ml)			
	Extra low (275)	Low (935)	Medium (2320)	High (9270)
1	256	982	2413	9159
2	258	883	2229	7849
3	312	929	2135	8876
4	297	1005	2163	7971
5	269	901	2450	9508
6	293	944	2329	8940
Mean	281	941	2287	8717
S.D.	23	47	131	664
R.S.D. (%)	8.31	4.96	5.73	7.62

[a] Each value represents mean of six samples

Table 3
Recovery of PZA from plasma

PZA concentration (ng/ml)	Peak height ratio				Recovery (%)
	Plasma		Water		
	Mean[a]	S.D.	Mean[a]	S.D.	Recovery (%)
100.00	0.0665	0.01	0.0752	0.01	88.38
250.00	0.2074	0.01	0.2224	0.01	93.25
500.00	0.4066	0.01	0.4539	0.01	89.25
1000.00	0.9450	0.07	1.1540	0.05	81.87
2500.00	2.0491	0.14	2.5885	0.03	79.14
5000.00	4.0571	0.07	4.7608	0.42	85.60
10000.00	8.8751	0.19	9.6466	0.34	93.11
Overall recovery of PZA					87.3±5.4

[a] $n=3$.

3.5. Recovery

Mean overall recovery of PZA from human plasma determined from three replicates of each calibration standard was 87.3% (R.S.D. 3.7%) (Table 3).

3.6. Stability of PZA in plasma

Concentrations of the drug in QA/QC samples that were thawed and kept at room temperature for 24 h decreased by 3.4%, and in those kept at room temperature for 48 h the percent decrease was 17%. The mean reduction in PZA concentration observed over three freeze–thaw cycles was 11.2% (Table 4). It was found that PZA and ethyl orange were stable when stored for up to 30 h after extraction from plasma.

3.7. Applicability of method

Fig. 3 depicts a plasma concentration–time curve of PZA obtained after a prostate cancer patient was

Table 4
Stability of PZA in plasma at room temperature and after repeat freeze–thaw

Stability	PZA control sample concentration (ng/ml)			
	Extra low (275)	Low (935)	Medium (2320)	High (9270)
At room temperature				
Stored for 24 h				
Mean PHR[a]	0.1152	0.4851	1.2402	4.1927
S.D.	0.0090	0.0131	0.0318	0.3441
R.S.D. (%)	7.86	2.69	2.57	8.33
Stored for 48 h				
Mean PHR[a]	0.0901	0.4041	1.0815	4.1046
S.D.	0.0081	0.0206	0.0364	0.2196
R.S.D. (%)	8.99	5.10	3.37	5.35
Fresh samples				
Mean PHR[a]	0.1183	0.4913	1.2721	4.4498
S.D.	0.0098	0.0221	0.0114	0.0838
R.S.D. (%)	8.29	4.50	0.90	1.88
PZA (%) after 24 h	97.4	98.7	97.5	94.2
PZA (%) after 48 h	76.2	82.3	85.0	92.2
Overall mean PZA concentration after 24 h storage				93.0%
Overall mean PZA concentration after 48 h storage				83.9%
Freeze–thaw				
First thawing				
Mean PHR[a]	0.1295	0.5041	1.2173	3.8725
S.D.	0.0048	0.0133	0.0198	0.1948
R.S.D. (%)	3.74	2.64	1.62	5.03
Second thawing				
Mean PHR[a]	0.1279	0.4866	1.1898	3.7110
S.D.	0.0049	0.0118	0.0225	0.1965
R.S.D. (%)	3.87	2.43	1.89	5.30
Third thawing				
Mean PHR[a]	0.1148	0.4129	1.1808	3.3627
S.D.	0.0149	0.0275	0.0837	0.0996
R.S.D. (%)	12.97	6.67	7.09	2.96
Change in PZA concentration after third thawing (%)	−11.4	−18.1	−3.0	−13.2

[a] Mean peak height ratio of six replicates.

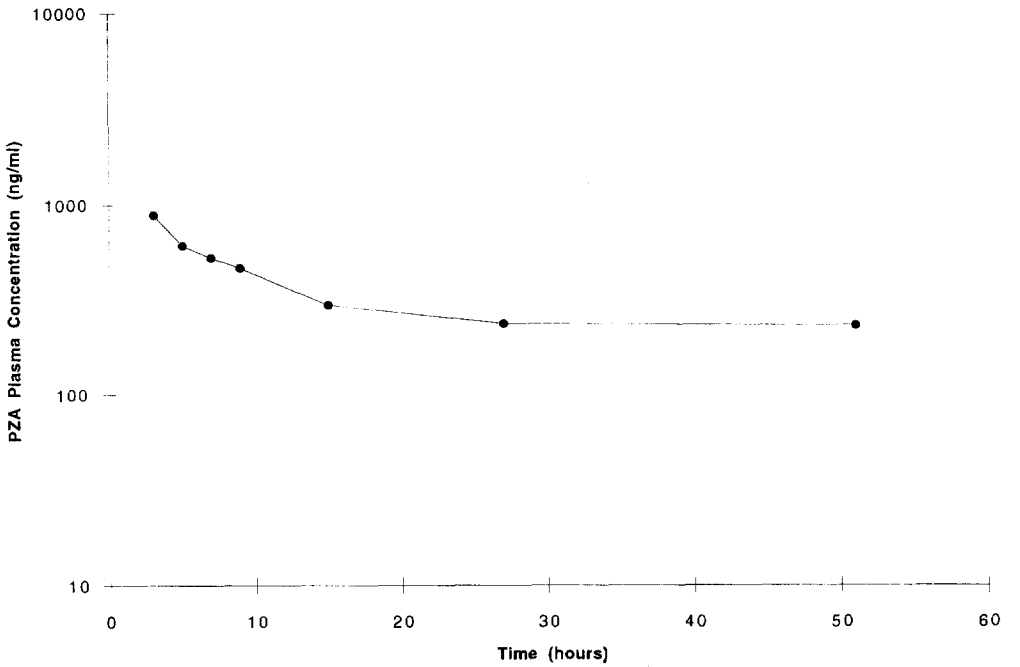


Fig. 3. Plasma concentration versus time curve for the IV administration of PZA. Experimental details are given in Section 2.

administered a 750 mg/m^2 dose of PZA by a 3 h infusion. Plasma samples for pharmacokinetic analysis were taken at 1 min, 2 h, 4 h, 6 h, 12 h, 24 h, 48 h and 72 h after the end of the infusion. The values for clearance, half-life and volume of distribution were 338.4 ml/min, 19.3 h and 523 l, respectively.

4. Conclusions

An isocratic HPLC assay for PZA in human plasma has been developed and validated. The assay is selective, precise and linear over the ranges studied. It involves simple sample preparation. This method was successfully used for pharmacokinetic analysis of PZA and is currently in use for the Phase II clinical study of PZA.

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