In Vitro Assessment of Plasma Protein Binding and Metabolic Stability of PTK 0796 (BAY 73-6944)

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ABSTRACT

Background PTK 0796 (7-dimethylamino, 9-(2,2-dimethyl-propyl)-aminomethylcycline) is a novel antibiotic of the tetracycline family with potent and enhanced activity against susceptible and resistant strains of both gram positive and gram negative bacteria.

Method Protein binding of PTK 0796 in mouse, rat, and monkey plasma was estimated using an equilibrium dialysis based method. The metabolic stability of PTK 0796 to the cytochrome P450 enzymes was assessed by incubation for up to 60 minutes with liver microsomes from mice, rats, and monkeys. The sample analysis in both assays was carried out on a High Turbulence Liquid Chromatography based on-line extraction system coupled with a triple quadrupole mass spectrometer. The metabolic stability was assessed by monitoring the disappearance of the parent molecule.

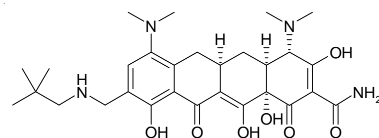
Results PTK 0796 was found to be substantially unbound in plasma at concentrations ranging from 0.5 µg/ml to 10 µg/ml. At these concentrations, the plasma protein binding for reference tetracyclines (doxycycline and minocycline) ranged from 79-87 %. The data were quite similar across the species. In the presence of liver homogenates from all the species tested in this study, PTK 0796 appears to be metabolically stable with no detectable loss of compound over 60 minutes. These results are consistent with data for tetracyclines such as doxycycline and minocycline which also do not yield any major metabolites and are known to be metabolically stable.

Conclusions PTK 0796 exhibits little protein binding and metabolism in mouse, rat, and monkey, both of which are considered to be favorable pharmacologic characteristics consistent with potential effectiveness in treating bacterial infections. These characteristics likely contribute to the excellent efficacy in animal models of infection reported elsewhere.

INTRODUCTION

- PTK 0796 (BAY 73-6944) is a novel aminomethylcycline of the tetracycline family with potent antimicrobial activity against both gram positive and gram negative bacteria.
- PTK 0796 (BAY 73-6944) has demonstrated excellent efficacy in animal models of infection.
- When administered intravenously, PTK 0796 (BAY 73-6944) is rapidly distributed in the tissue.

STRUCTURE OF PTK0796 (BAY 73-6944)



METHODS

Plasma Protein Binding We employed equilibrium dialysis in the Equlibrium Dialyzer-96Tm from Harvard Bioscience, Holliston, MA, USA, using turbulent flow chromatography based on-line extraction system in conjunction with a triple quadrupole mass spectrometer to compute the free plasma drug concentration of PTK 0796 in mouse plasma.

Sample preparation Serial dilution was performed using thawed plasma to prepare 10, 5, 1, 0.5 and 0.1µg /mL of PTK 0796, Doxycycline and Minocycline plasma samples. An identical series of samples was prepared using 0.1M potassium phosphate buffer (pH 7.4). The dialyzer unit consisted of a top chamber and a bottom chamber separated by a semipermeable membrane with a molecular weight cut-off of 10-kDa. Duplicate aliquots of 150µL each of plasma samples were added to the top wells of the microtiter plate. 150µL of potassium phosphate buffer were added to the corresponding bottom wells. This set of samples will be referred to as the plasma/buffer set. To another set of duplicate rows, were added 150µL of buffer samples to the top wells and 150µL of blank buffer in the corresponding bottom wells. This set of samples will be referred to as the buffer/buffer set. The remainder of the plasma and buffer samples were saved to generate a standard curve for computing the free drug concentration. The samples were allowed to dialyze at 37^{0}c for 3 hr on a plate rotator, 100µL aliquots were transferred to an HPLC vial. 10µL of the norgestrel internal standard solution were added to each sample. Samples for the generation of standard curve were prepared in an identical manner from the remainder of the plasma and buffer samples. The vials were vortexed briefly and set up for LC-MS analysis.

LC-MS analysis

- The LC-MS analysis was accomplished on the CohesiveAria/Sciex 2000 LC-MS system.
- Loading column- Cohesive HTLC –silica-C-8; 1x 50mm; 50 micron
- Eluting column- Agilent Eclipse XDB C-8 catridge
- Mobile phase A- Water + 0.1% TFA
- Mobile phase B- methanol : acetonitrile (50:50) + 0.1 % TFA
- The loading step was accomplished using Buffer A and the samples were eluted using a 10/90 isocratic gradient of Buffer A and Buffer B.
- The MS-MS analysis was carried out in the MRM mode. Each sample was run in duplicate on the LC-MS system.

Calculations The LC-MS data were calculated using the quantitation software supplied by Applied Biosystems as part of Analyst 1.2 software with the API 3000 Mass Spectrometer. Statistical analysis was employed to generate the means, standard deviations, coefficient of variation and regression parameters. The peak area ratio of the test item : internal standard was calculated for each sample. A calibration line was generated using the test item: internal standard area ratio plotted against the actual test item concentration to generate a standard calibration line. The free drug concentrations were calculated by using the following formula:

$$\text{\% of Free Drug} = \frac{\text{drug concentration in bottom well}}{\text{drug concentration in top well}} \times 100$$

Metabolic Stability Liver microsomes from rat, mouse and monkey were used to assess the metabolic stability of PTK 0796. Doxycycline and minocycline were included as comparators. The experiments were performed as per the manufacturer's (In Vitro Technolgies, Inc., Baltimore, MD, USA) recommendations. 7-ethoxy coumarin was examined as the control in all experiments.

Preparation of samples for LC-MS analysis 225 µL aliquots were taken out from each sample, and 25µL of norgestrel (50µg/mL) were added to each sample. The samples were then set up for analysis on the cohesive turbulent flow on-line extraction system coupled with an API 2000 triple quadrupole mass spectrometer. The LC-MS analysis was carried out as described under Plasma Protein Binding.

Calculations The data were calculated using the quantitation software supplied by Applied Biosystems as part of Analyst 1.2 software with the API 2000 Mass Spectrometer. Statistical analysis was employed to generate the means, standard deviations, coefficient of variation and regression parameters. The ratios of peak areas obtained at different time points to that at zero time point were multiplied by 100 to get the percent parent drug left intact.

RESULTS

Plasma Protein Binding An equal distribution of PTK 0796 between the top and bottom wells of the buffer/buffer set demonstrated the drug reached an equilibrium state (Table 1). PTK 0796 was almost evenly distributed between the plasma containing top well and the buffer containing bottom well at concentrations of 0.5 µg/ml and above suggesting that it is only minimally bound to plasma proteins at concentrations of 0.5 µg/ml and above. However, at a concentration of 0.1µg/ml, PTK 0796 was about 14-20% bound revealing the presence of some low capacity binding sites at lower concentrations (Table 2). At elevated concentrations, the binding is insignificant suggesting a saturation of the binding sites present on the plasma proteins. In sharp contrast, Doxycycline (Table 3) and Minocycline (Table 4), under identical experimental conditions, show significantly higher binding to the plasma proteins of all the three species included in this study. The percentage free drug obtained for Doxycycline and Minocycline are in general agreement with those reported for human plasma . Although Doxycycline at higher concentrations appears to be slightly less bound to mouse plasma proteins than Minocycline, the latter demonstrates somewhat lower protein binding in rat and monkey plasma than the former.

Table 1. Equilibrium dialysis of Phosphate Buffer Samples Spiked With PTK 0796

Concentration Tested (µg/ml)	Concentration Top Well (µg/ml)	Concentration Bottom (µg/ml)	% Free Drug Concentration
0.1	0.06	0.06	100
0.5	0.27	0.27	100
1	0.50	0.54	100
5	2.5	2.6	100
10	5.4	5.6	100

Table 2. Equilibrium dialysis of Mouse, Rat and Monkey Plasma Samples Spiked With PTK 0796

Concentration Tested (µg/ml)	% Free drug concentration		
	Mouse Plasma	Rat Plasma	Monkey plasma
0.1	80	82	86
0.5	97	100	100
1	98	100	100
5	100	100	100
10	100	100	100

Table 3. Equilibrium dialysis of Mouse, Rat and Monkey Plasma Samples Spiked With Doxycycline

Concentration Tested (µg/ml)	% Free drug concentration		
	Mouse Plasma	Rat Plasma	Monkey plasma
0.1	21.8	18.0	28.0
0.5	23.0	17.6	18.3
1	24.0	13.9	17.8
5	26.7	18.6	17.9
10	32.0	19.7	15.9

Table 4. Equilibrium dialysis of Mouse, Rat and Monkey Plasma Samples Spiked With Minocycline

Concentration Tested (µg/ml)	% Free drug concentration		
	Mouse Plasma	Rat Plasma	Monkey plasma
0.1	26.5	21.7	25.0
0.5	19.7	16.5	28.0
1	17.9	15.9	19.5
5	22.6	22.8	17.7
10	22.9	26.0	25.5

Figure 1. Metabolic Stability of PTK 0796 upon incubation with mouse liver microsomes

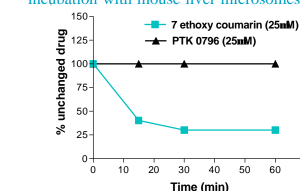


Figure 2. Metabolic Stability of PTK 0796 upon incubation with rat liver microsomes

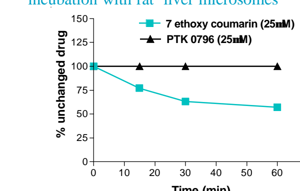
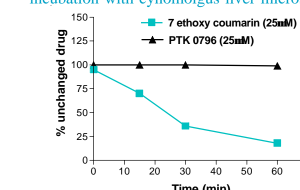


Figure 3. Metabolic stability of PTK 0796 upon incubation with cynomolgus liver microsomes



Metabolic Stability

The results for PTK 0796 upon incubation with mouse, rat and cynomolgus plasma are shown in Figures 1, 2 and 3. The control substrate, 7 ethoxy coumarin, was metabolized between 43-82% with microsomes from the 3 different species. These results established that the liver microsomes used in these experiments preserved most of the cytochrome P450 enzyme activities. The enzyme blank sample showed no loss of the parent compound indicating that the compound stayed stable in the absence of the microsomal enzymes. PTK 0796 appeared to be extremely stable upon incubation with liver microsomes from mouse, rat or Cynomolgus monkey. There was no detectable loss of the drug after 60 min of incubation with the mouse, rat or Cynomolgus liver microsomes.

The results for PTK 0796 and the comparators, Minocycline and Doxycycline, along with the control substrate are summarized in Table 5. Like PTK 0796, Minocycline was found to be stable upon incubation with mouse, rat and Cynomolgus liver microsomes. The other comparator, Doxycycline was also found to be metabolically stable with no detectable disappearance of the parent drug upon incubation for up to 60 min with mouse liver microsomes. However, about 7-9% of Doxycycline was observed to be metabolized upon incubation with rat and Cynomolgus liver microsomes. Minocycline has been shown to be metabolized in humans only. Doxycycline, on the other hand, has been shown to yield some metabolites in several species including human.

Table 5. Metabolic Stability of PTK 0796, Minocycline and Doxycycline

Compound	% Unchanged Drug at 60 min		
	Mouse liver microsomes	Rat liver microsomes	Cynomolgus liver microsomes
7 ethoxy coumarin (control substrate)	30	57	18
PTK 0796	**100**	**100**	**99**
Minocycline	100	100	100
Doxycycline	100	92.5	91

CONCLUSIONS

- PTK 0796 (BAY 73-6944) exhibited minimal protein binding to mouse, rat and monkey plasma at concentrations above 0.1µg/ml. However, at a concentration of 0.1µg/ml, it shows low (14-20%) but appreciable binding to plasma proteins. Doxycycline and Minocycline are substantially more bound (68-86%) for the concentrations tested in this study.
- The low plasma protein binding of PTK 0796 (BAY 73-6944) would result in a considerably higher fraction unbound (fu) facilitating better target tissue distribution of this drug.
- PTK 0796 (BAY 73-6944) is metabolically stable. The results are generally consistent with those reported for Doxycycline and Minocycline, which are known to be only modestly metabolized.
- This low protein binding and metabolic stability could have a strong positive influence on the pharmacokinetics, pharmacodynamics and toxicity of this drug leading to better potency and efficacy in animal models of infection.