The influence of storage time on ponazuril concentrations in feline plasma

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Background. The pharmacokinetics of ponazuril have been determined in several species however, there is very little information on the stability of the drug after storage for long periods of time. This study was undertaken to determine the stability of ponazuril in plasma samples stored at -80°C, which is the temperature most commonly used in the author's laboratory.

Method. Spiked plasma samples (0.3, 7.5, and 15 μg/mL) were stored at -80°C for three months. Analysis occurred on the first day and then once a week for the following twelve weeks. The drug was extracted using a chloroform extraction and separated by high performance liquid chromatography using ultraviolet detection.

Results. There was no loss of drug for any concentration for the first four weeks of storage. There was an average loss of less than 5% from day 35 through day 70 and an average loss of 6% on day 77 and 84. The data suggest that ponazuril is stable for 4 weeks when stored at -80°C and undergoes minimal loss in the remaining 8 weeks.

2   The Influence of Storage Time on Ponazuril Concentrations in Feline Plasma

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17 **Abstract**

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20 species however, there is very little information on the stability of the drug after storage

21 for long periods of time. This study was undertaken to determine the stability of

22 ponazuril in plasma samples stored at -80ºC, which is the temperature most commonly

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27 high performance liquid chromatography using ultraviolet detection.

28 **Results.** There was no loss of drug for any concentration for the first four weeks of

29 storage. There was an average loss of less than 5% from day 35 through day 70 and an

30 average loss of 6% on day 77 and 84. The data suggest that ponazuril is stable for 4

31 weeks when stored at -80ºC and undergoes minimal loss in the remaining 8 weeks.

Introduction

34   Ponazuril (also known as toltrazuril sulfone) is from the triazine class of

35 antiprotozoal drugs and is a metabolite of the drug toltrazuril (Love, et al., 2015).

36 Triazine compounds have been used in veterinary medicine to treat the harmful effects

37 of protozoa such as *Toxoplasma gondii*, *Coccidia* spp., and *Isopora*, (Stock, et al.,

38 2018).

39 - -   The use of drugs in different species requires knowledge of their

40 pharmacokinetics. An understanding of the pharmacokinetics is necessary for

41 determining the most appropriate dose, route of administration and dosing interval. The

42 determination of accurate drug concentrations is extremely important in the estimation

43 of pharmacokinetic parameters and may impact design of the drug regimen. To

44 determine these properties, it is essential to calculate the concentration of the

45 compound of interest after administration. Because of the importance placed on the

46 validation of bioanalytical methods that are used in the determination of data for

47 pharmacokinetic studies the FDA has established guidelines (FDA, Guidance for

48 industry: Bioanalytical Method Validation). The information in the guidelines applies to

49 bioanalytical procedures such as chromatographic methods. A necessity for the

50 generation of the data is a validated analytical procedure. However, if proper conditions

51 are not maintained for storage the results may not accurately indicate the sample

52 concentration at the time it was determined. For most studies, samples are stored until

53 there is an adequate quantity for analysis, which requires them to be stored, usually

54   frozen for a period of time. To guarantee accurate results it is essential to determine

55   drug degradation from samples using the storage conditions anticipated for the study.

56   Pharmacokinetic studies of ponazuril have been determined in several species

57   (Laczay, et al., 1995; Epe, et al., 2005; Ghanem, et al., 2008; Love, et al., 2015; Prado,

58   et al., 2011; Dirikolu, et al., 2011; Zou, et al., 2014; Stock, et al., 2018; Furr and

59   Kennedy, 2020). To the author's knowledge there are no other stability articles involving

60   drugs used in felines or triazine drugs. Thus, the intention of this research was to

61   determine if concentrations of ponazuril in feline plasma decreased after storage for 12

62   weeks at -80ºC using a validated method (Cox, et al., 2021).

63   **Materials & Methods**

64
65   **Equipment**

66   The equipment used for ponazuril assessment consisted of a 2695 separation

67   module and a 2487 ultraviolet detector (Waters Milford, MA, USA). Separation was

68   achieved on a Waters Symmetry Shield RP_{18} (4.6 mm x 150 mm, 5 µm) column. The

69   mobile phase consisted of 0.1% formic acid in water and acetonitrile (50:50 v/v). The

70   ultraviolet detector was set at 254 nm and the flow rate was 1.1 mL/min.

71   **Reagent and solutions**

72   Ponazuril and diclazuril were purchased from Cayman Chemical (Ann Arbor, MI,

73   USA). Both compounds had a purity greater than 99%. All other solvents and chemicals

74   were acquired from Fisher Scientific (Pittsburg, PA, USA). Stock solutions of ponazuril

75   (100 µg/mL) and diclazuril (100 µg/mL), which is the internal standard, were prepared

76   by dissolving appropriate amounts of each compound in methanol. Cat plasma was

77 fortified with ponazuril to construct daily standard curves (0.1 – 25 µg/mL). These were

78 accepted if the coefficient of determination (r^2) was greater than 0.99.

Sample Collection

80   Six adult cats from UTCVM faculty and staff were determined to be healthy

81 based on results of physical examination and history were used. Healthy animals are

82 routinely used in pharmacokinetic studies in order to prevent distortion of results due to

83 abnormal metabolism, disease processes or interference from drugs the animal is

84 taking. Venous blood was collected from the jugular vein of each cat and placed in

85 lithium heparin tubes. The volume of blood collected from each cat did not exceed 1%

86 of body weight in kilograms. Enough collected blood was centrifuged to provide plasma

87 for the study. Plasma was pooled into one container and then divided into 3 separate

88 tubes, and then spiked with one of the ponazuril calibration standards (0.3, 7.5, and 15

89 µg/mL) that were used in the study. Aliquots from each of the 3 individual standards

90 were then placed in separate vials labeled Day 1, 7, 14, 21, 28, 35, 42, 49, 56, 63, 70,

91 77, and 84 for analysis on those dates. These concentrations were chosen as low

92 medium and high standards within the linear curve used. Samples were then placed in

93 the -80ºC. Day 1 samples were analyzed on that day. Because of animal size and

94 availability enough plasma was obtained to analyze only one sample for each ponazuril

95 concentration evaluated.

Extraction method

97   Ponazuril was extracted from plasma samples that were thawed one time using a

98 liquid-liquid extraction method (Cox et al., 2021). Frozen plasma samples were thawed

99 at room temperature (22ºC) for ~30 minutes, vortex-mixed, and 100 µl of plasma was

100   transferred to a screw top tube followed by 10 µl of diclazuril (100 µg/mL, internal

101   standard) and 2 mL chloroform. The tubes were placed on a rocker for 15 minutes and

102   then underwent centrifugation for 20 min at 1000 x g. The organic layer was transferred

103   to a glass tube and evaporated to dryness with nitrogen gas. Samples were

104   reconstituted in 250 µL of mobile phase and 100 µL was analyzed.

105   **Statistical analysis**

106   Comparative changes in the actual values of different measured ponazuril

107   concentrations in plasma samples were used to summarize the effect of storage length

108   on stability of the drug.

109   **Results**

110

111   Samples were analyzed on days 1, 7, 14, 21, 28, 35, 42, 49, 56, 63, 70, 77, and

112   84 (Figure 1A-D and Table 1). The actual values of the different measured ponazuril

113   concentrations and the percent variation of the concentration compared to the starting

114   concentration are reported in Table 1. Ponazuril concentrations were determined in all

115   samples. There was no loss of drug for any concentration (low, medium or high) for the

116   first four weeks (days 1 through 28) of storage at -80ºC. Please refer to the Figure and

117   Table for the remainder of the results.

118   The ponazuril method of analysis that was used for the stability study was

119   previously validated (Cox et al., 2021). The method produced a linear curve over the

120   concentration range used (0.1 – 25 µg/ml) with a lower limit of quantification of 0.1

121   µg/mL. The intraday and interday variability ranged from 3.7 to 10% and 2.1 to 5.5%

122   while the average ponazuril recovery was 99% and the average recovery of diclazuril

123   was 94%. Calibration curves were analyzed each day of plasma analysis.

Discussion

124 **Discussion**

125

126   In order to determine the optimal storage time for ponazuril samples at -80ºC,

127 plasma was collected from healthy felines and pooled in order to reduce the effects of

128 inter-individual variability. A validated method of analysis was applied to the samples to

129 determine the effect of storage duration. Plasma samples spiked with various amounts

130 of ponazuril that fell within a validated linear concentration range and stored in a -80ºC

131 freezer for 84 days were used. One limitation to the study was a lack of duplicate

132 samples for the analysis. The analysis of only one sample did not allow for any

133 statistical analysis.

134   Ponazuril was detected in all concentrations for the entire 84 days. For the first

135 28 days, there was no change in any of the concentrations. Indicating that the drug is

136 stable after storage for that length of time. After 28 days, there was a slight loss of drug

137 for all three concentrations. The average loss was 4% or less for days 35 through 63,

138 which would still not have an impact on higher concentrations and have a minimal

139 impact on lower concentrations. The samples should still be viable for this period of

140 time. On day 70, there was an average loss of 5% and on days 77 and 84 there was an

141 average loss of 6%. The loss on day 84 was greater for the 0.3 and 7.5 than the 15

142 µg/mL standard. Overall, the average loss from day 7 though day 84 was 2%, 4% and

143 2% for 0.3, 7.5 and 15 µg/mL, respectively.

144   To the author's knowledge there are no other stability articles involving drugs

145 used in felines or triazine drugs. There is a paucity of information in the literature about

146 the storage stability of drugs in biological samples in general. Determination of the

147 concentrations in biological samples is fundamental to describing pharmacokinetic

148 parameters which in turn leads to appropriate dosing regimens. Therefore, it is

149 imperative to determine the effect that storage can have on drug stability.

150

Conclusions

152

153   The intention of this study was to determine the effect of storage duration at -

154 80ºC on the stability of ponazuril concentrations in plasma. In summary, plasma

155 samples, containing ponazuril can be stored for 28 days with no loss of drug. By

156 applying the FDA Bioanalytical Guidelines (FDA, Guidance for industry: Bioanalytical

157 Method Validation) for method development that states a value should be within 15%

158 deviation from the standard all of the samples were stable for 84 days when stored at -

159 80ºC. This study provides much needed information on the stability of ponazuril plasma

160 samples stored at -80ºC.

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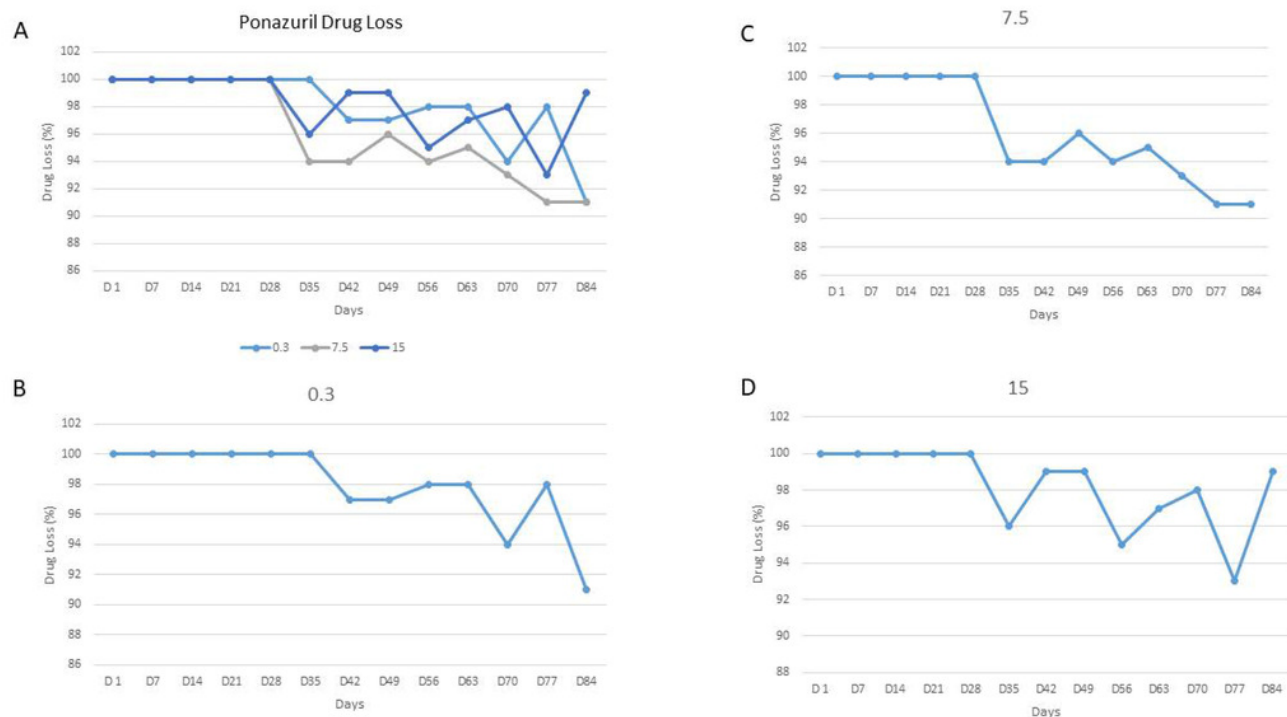
Figure 1

Ponazuril Drug Loss

Percent ponazuril loss in plasma over 84 days (A) all 3 concentrations (B) 0.3 µg/mL, (C) 7.5 µg/ml, (D) 15 µg/ml.

Ponazuril stability information for 84 days after storage at -80°C.

Concentration results reported in µg/mL; n = 1; * Day 1 samples were not frozen but analyzed immediately after spiking.

1   Table 1. Ponazuril stability information for 84 days after storage at -80°C.

Starting conc.	Concentration at different days of storage												
	Day 1*	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49	Day 56	Day 63	Day 70	Day 77	Day 84
0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.291	0.291	0.294	0.294	0.282	0.294	0.273
7.5	7.5	7.5	7.5	7.5	7.5	7.05	7.05	7.2	7.05	7.1	7.0	6.8	6.8
15	15.0	15.0	15.0	15.0	15.0	14.4	14.85	14.85	14.25	14.55	14.7	13.95	14.85
	Percentage change in concentration from starting concentration												
0.3	0	0	0	0	0	0	-3	-3	-2	-.2	-6	-2	-9
7.5	0	0	0	0	0	-6	-6	-4	-6	-5	-6	-9	-9
15	0	0	0	0	0	-4	-1	-1	-5	-3	-2	-7	-1

2   Concentration results reported in µg/mL; n = 1; * Day 1 samples were not frozen but analyzed
3   immediately after spiking.