

# LC/MS/MS Method for the Determination of Kevetrin in Dog Plasma

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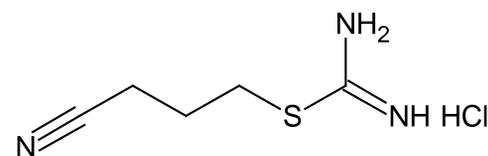
## ABSTRACT

Kevetrin™, a novel small molecule, was effective in reducing tumor growth in human lung, breast, and colon xenograft tumor models, including multi-drug resistant phenotypes. Kevetrin not only activates p53, but also activates both transcription-dependent and transcription-independent pathways to promote apoptosis (programmed cell death). Kevetrin also alters E3 processivity of MDM2. Monoubiquitination of p53 by Kevetrin stabilizes both wild type and mutant p53 and induces apoptosis in mutant p53 tumors. Thus, Kevetrin showed potent efficacy in both mutant and wild type tumor xenograft models. Analysis of Kevetrin is challenging due to the extreme hydrophilicity and small size of the molecule.

A novel HPLC-MS/MS method has been developed and validated for the determination of Kevetrin in dog plasma containing K<sub>3</sub>EDTA. Samples were prepared by protein precipitation with acetonitrile and have been used to support on-going Toxicology studies. Analysis was performed with an Applied Biosystems Sciex 4000 triple quadrupole mass spectrometer using a turboionspray source. Positive ions were measured using the multiple reaction monitoring (MRM) mode. The mass transitions measured were m/z 144.1 → 102.2 (Kevetrin) and m/z 147.2 → 104.2 (<sup>13</sup>C<sub>2</sub>, <sup>15</sup>N Kevetrin). The total run time was 10 minutes. The assay was validated over the range of 0.5 to 50 µg/mL with a 25 µL sample aliquot. Samples were stored at -70°C prior to analysis.

A validation was performed to evaluate precision, accuracy, specificity recovery and stability. The validation data presented demonstrate that the assay method meets the performance requirements needed to support pre-clinical toxicology studies.

Figure 1 Chemical Structure



Kevetrin Hydrochloride C<sub>5</sub>H<sub>10</sub>N<sub>3</sub>SCI

## Validation Samples

Calibration standards containing Kevetrin were prepared in K3EDTA dog plasma at eight concentrations spanning the concentration range. Quality control (QC) pools containing the analyte were prepared at the lower limit of quantitation and three additional concentrations spanning the quantitation range for the evaluation of assay performance, stability and recovery.

## METHODS

### Sample Preparation:

1. Samples are allowed to thaw on ice
2. Aliquot 25 µL of dog plasma sample
3. Add 30 µL of 2.5 µg/mL Internal Standard solution in 10 mM Ammonium Formate
4. Add 200 µL of acetonitrile within one hour of sample thawing, vortex and centrifuge
5. Transfer 100 µL of the supernatant from each sample into a borosilicate test tube and dry completely
6. Reconstitute each sample with 200 µL of 10 mM Ammonium Formate

### HPLC Conditions

Instrumentation: Shimadzu LC10ADVP pumps  
Leap HTC PAL autosampler  
Column: Waters Atlantis T3 C<sub>18</sub>, 4.6 × 250 mm, 5 µm  
Column Temp: 25 °C  
Flow Rate: 1 mL/minute  
Injection Volume: 10 µL  
Autosampler Temp: 4 °C  
Run Time: 10 minutes  
Retention Time: 7.5 minutes  
Mobile Phase: 10 mM Ammonium Formate in Water pH 3.0  
Divert Valve: HPLC flow diverted to waste except for peak elution window  
Flow Split: 1:5

### Mass Spectrometry Conditions

Instrument: SCIEX API 4000  
Detection: Turbo-Ion Spray, positive mode  
Resolution: Q1 = unit, Q3 = unit  
Peak Detection: Multiple Reaction Monitoring (MRM)

Compound	Parent Ion	Daughter Ion	Retention Time (min)
Kevetrin	144.1	102.0	7.5
<sup>13</sup> C <sub>2</sub> , <sup>15</sup> N Kevetrin	147.2	104.2	7.5

Figure 2 Linear Regression

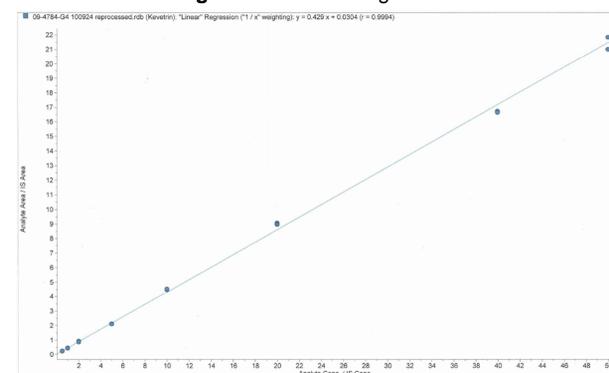
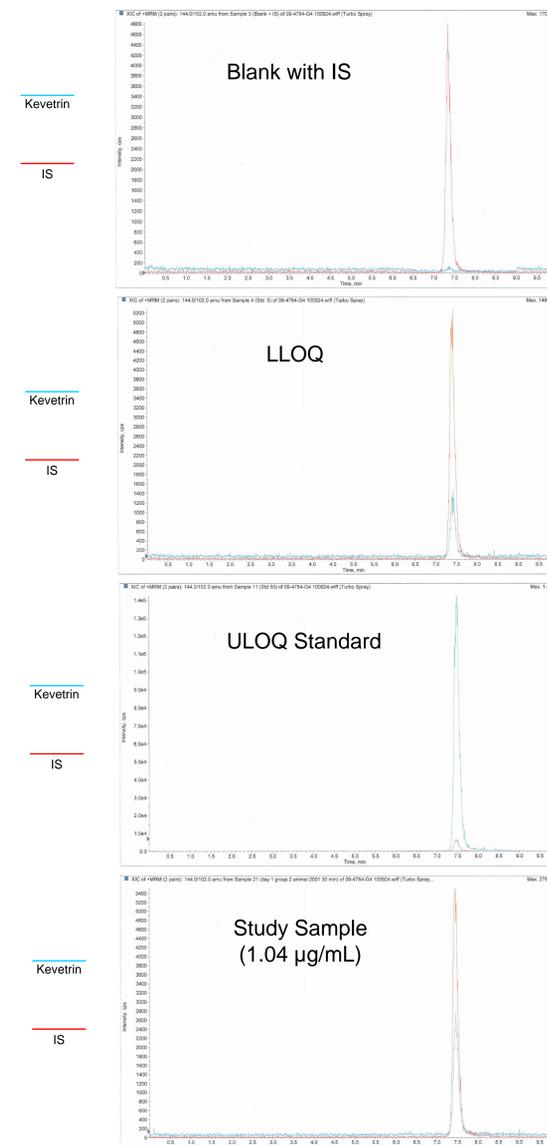


Figure 3 Chromatograms



Chromatography was reproducible provided all of the organic solvent was removed during sample processing

Figure 4 Day 28 Data from a Multiple Dose Dog Toxicology Study

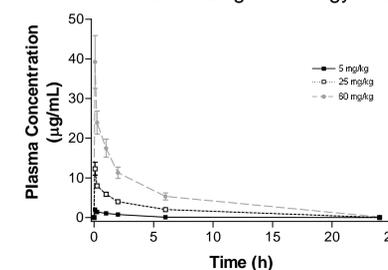


TABLE 1 Precision & Accuracy

	Theoretical Concentration (µg/mL)		
	Low QC	Mid QC	High QC
Mean Concentration	1.40	12.4	38.9
% CV	4.40	4.83	7.93
% Recovery	93.0	99.0	104.0

Interday			
	Theoretical Concentration (µg/mL)		
	Low QC	Mid QC	High QC
Mean Concentration	1.37	12.3	38.2
% CV	13.8	6.10	7.61
% Recovery	91.6	98.0	102

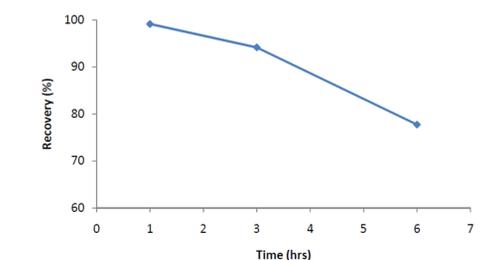
TABLE 2 Impact of Matrix Effect

	Low QC	Mid QC	High QC
Recovery (%)	115	84.9	89.6
Matrix Effect (%)	93.1	98.8	100

TABLE 3 Stability

	Low QC	High QC	Solution
Freeze-Thaw 3 Cycles	93.3%	113%	
8 Hr Room Temperature	75.4%	101%	
41 Hour Extract	95.4%	104%	
70 Hour Extract	119%	104%	
Reinjection Reproducibility	93.7%	108%	
118 Hour Stock Solution			98.8%
25 Day Refrigerated			97.9%
55 Day Refrigerated			97.3%

Figure 5 Room Temperature Stability of Kevetrin in Plasma



A time profile of the room temperature stability of Kevetrin in dog plasma was performed after a 24 hour room temperature stability of Kevetrin in plasma failed for all QC levels and an 8 hour room temperature stability failed for the low QC. To account for the instability, samples can be processed on ice or be precipitated within one hour of thawing.

## CONCLUSIONS

An HPLC-MS/MS assay for the determination of Kevetrin in Dog plasma containing K<sub>3</sub>EDTA has been successfully developed and validated. The assay is suitable for the assay of pre-clinical toxicology samples as demonstrated by its precision, accuracy, specificity recovery and stability.

## Acknowledgements

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