

**VALIDATION OF AN LC/MS/MS  
ASSAY FOR TENOFOVIR IN HUMAN  
SERUM**

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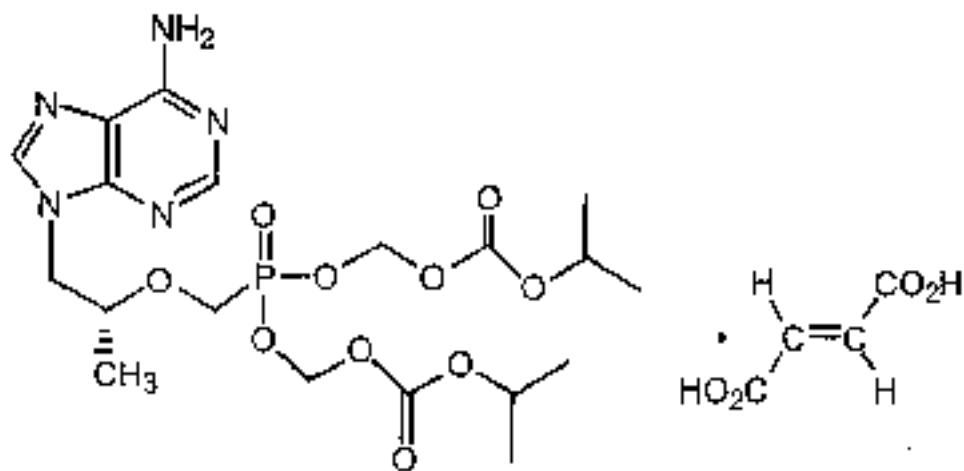
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*WEST LAFAYETTE, IN 47906*

# TENOFOVIR - HIV REVERSE TRANSCRIPTASE INHIBITOR

- Tenofovir disoproxil fumarate (prodrug)
- Prodrug requires initial diester hydrolysis for conversion to tenofovir and subsequent phosphorylation by cellular enzymes to form tenofovir diphosphate
- Tenofovir diphosphate competes with natural substrate deoxyadenosine 5'- triphosphate to inhibit activity of HIV reverse transcriptase and causes DNA chain termination

# STRUCTURES OF PRODRUG



**TENOFOVIR DISOPROXIL FUMARATE**

# METHOD DEVELOPMENT CONSIDERATIONS

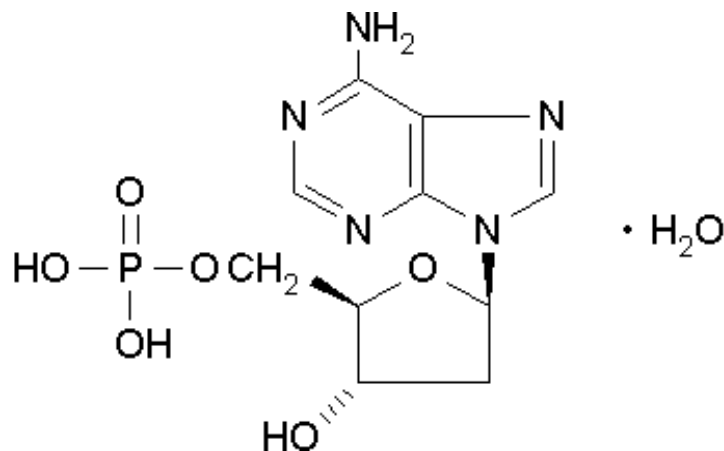
- Solubility and stability of analyte stock solution
- No stable label Internal Standard available. Therefore, important to choose an ISTD that had similar structural chemistry
- Identifying analytical column that gave good retention and ruggedness. In general most columns used gave poor analyte retention and reproducibility
- Identifying extraction chemistry that allowed for selective isolation of analyte of interest

# Stock Solution

- Common solvents like methanol, ACN tried unsuccessfully
- Finally, methanol with 1% ammonium hydroxide was used

# Selection of Internal Standard

2'-Deoxyadenosine-5'-monophosphoric acid, monohydrate was chosen because of structural similarities



# Identifying an Analytical Column

<b>Column Type</b>	<b>Mobile Phase</b>	<b>Analyte RT</b>
Betasil C 18	10% acetonitrile with 20mM ammonium formate	Not retained
Xterra C 18	10% acetonitrile with 20mM ammonium formate	2.72 min. Column lost peak shape within 50 injections
Ultra IBD	20% acetonitrile with 0.1% formic acid	3.72 min. Was able to run over 200-300 extracts on column without losing peak shape

# Optimizing Extraction Chemistry

## Variable

## Conclusion

Protein Precipitation

Ion suppression, therefore aborted.

SPE , anion exchange chemistry

Works well, no ion suppression.

Amount of Buffer and pH

200  $\mu$ L and 400 $\mu$ L of pH 10 and 12 evaluated. Optimized at 400 $\mu$ L of pH 12.

Amount of elution solution

200  $\mu$ L and 400 $\mu$ L of 2% formic acid evaluated. Optimized at 400 $\mu$ L.

# Final Optimized Conditions

- Column: Ultra IBD 100 x 4.6mm, 5 $\mu$ m
- SPE using Waters Oasis MAX(30mg)
- 50 mM Sodium Carbonate (pH12.0) Buffer
- Elution Solution: 2% formic acid in methanol
- Mobile Phase: 20% ACN/ 0.1% formic acid

## General Assay Procedure - Automation

- Load Samples onto 96-well plate
- Add ISTD in buffer
- Condition plate with methanol and water
- Load samples
- Rinse plate with water and methanol
- Elute with acidic solvent
- Evaporate to dryness
- Reconstitute
- Inject on LC/MS/MS

## Assay Specifics

Sample Volume: 200  $\mu$ L

Sample Preparation: SPE

Validated Range: 5.00 - 500 ng/mL

Column: Ultra IBD

Mobile Phase: 20% ACN/ 0.1% formic acid

Regression: Linear 1/x

Detection: LC/MS/MS

# Chromatogram of a Extracted Low Calibrator

Instrument: mseightball/sd

14 - Jun - 2002

18:48:56

STD 5.001

run 004\_012 Sm (Mn, 1x3)

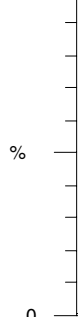
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M R M of 2 Channels ES+  
332.00 > 135.90  
3.26e5  
Area

run 004\_012 Sm (Mn, 1x3)

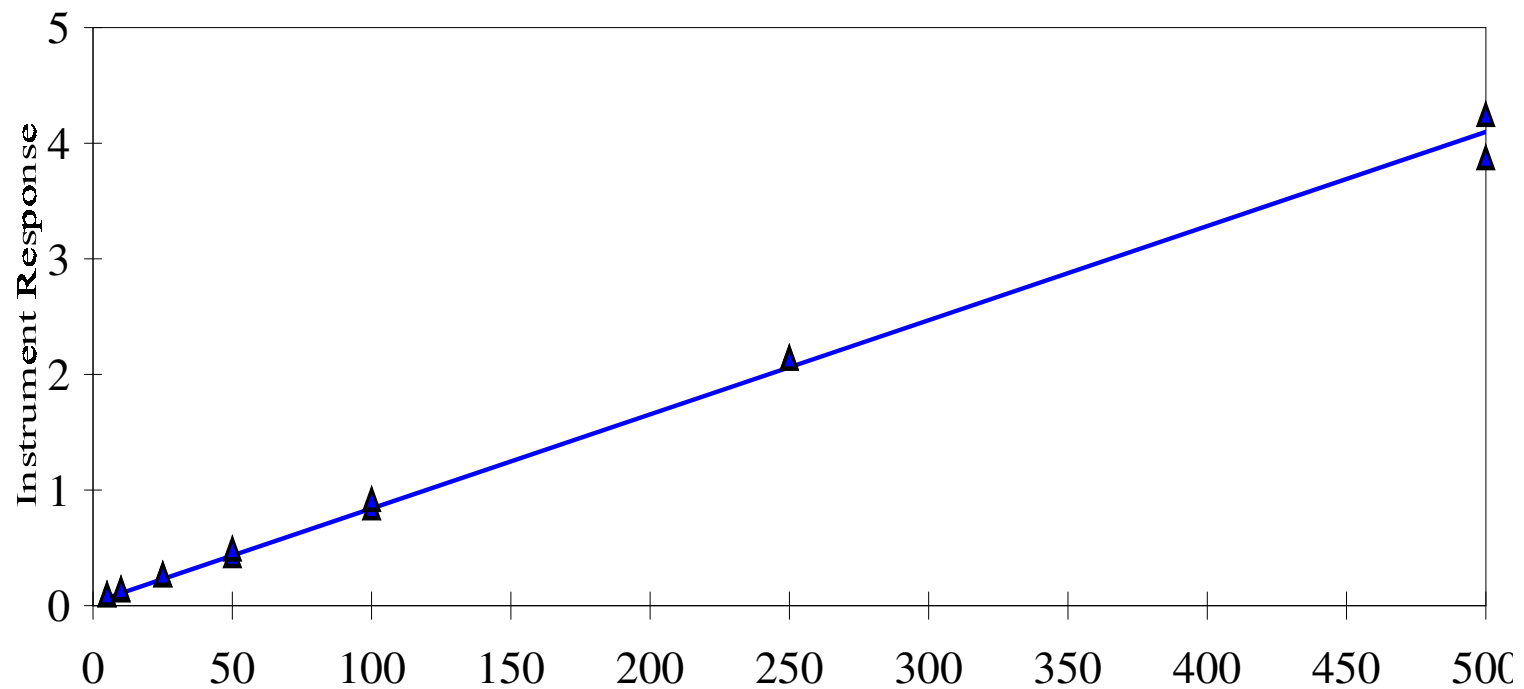
100



M R M of 2 Channels ES+  
288.00 > 175.90  
1.93e4  
Area

1.00 2.00 3.00 4.00 5.00 Time

# Typical Calibration Curve



# Calibration Standard Statistics (ng/mL)

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Nominal Concentration	5.00	10.0	25.0	50.0	100	250	500
Average Concentration	4.55	9.88	25.2	51.6	105	252	491
Standard Deviation	0.236	0.804	1.03	3.03	5.86	13.1	15.2
Precision (%)	5.2%	8.1%	4.1%	5.9%	5.6%	5.2%	3.1%
% Bias	-9.0%	-1.2%	0.8%	3.2%	4.8%	0.7%	-1.8%
N	6	8	8	8	8	8	7

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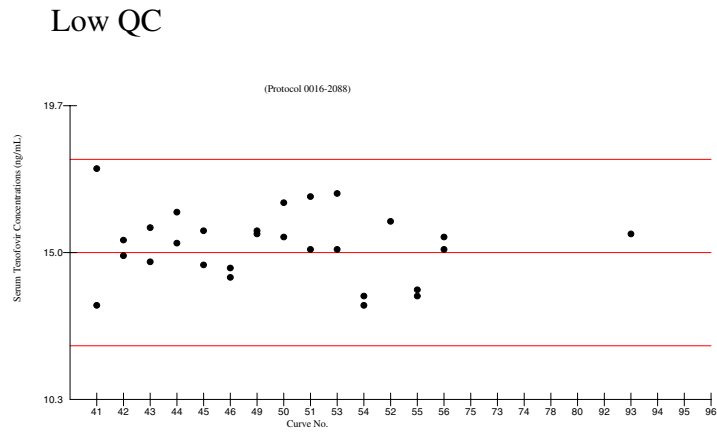
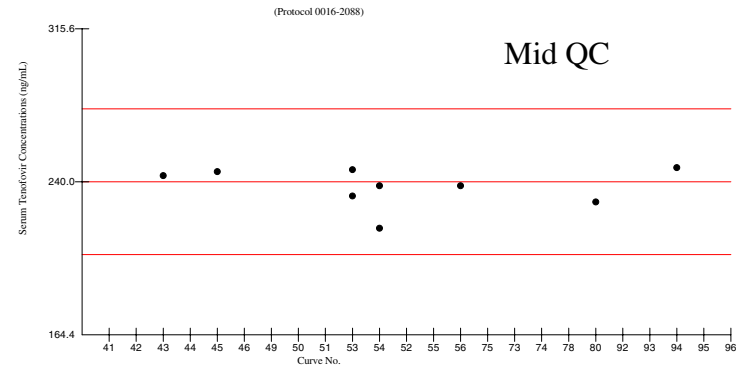
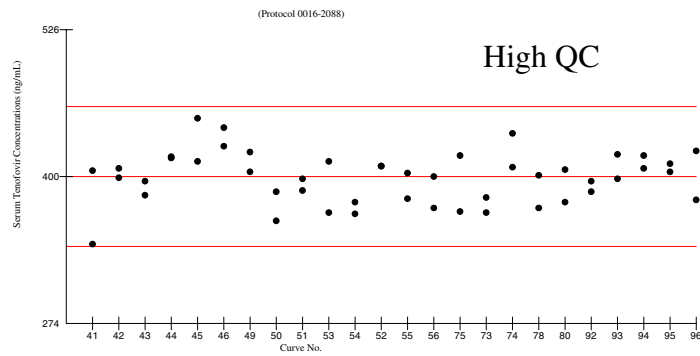
# Inter-Assay Quality Control Sample Statistics

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Nominal Concentration	400	240	15.0	5.00
Average Concentration	407	244	15.1	4.51
Standard Deviation	17.1	14.4	1.01	0.268
Precision (%)	4.2%	5.9%	6.7%	5.9%
% Bias	1.8%	1.5%	0.7%	-9.7%
N	20	20	20	17

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# Assay Performance over 50 batches



# Summary of Validation Results

- **Sample Matrix:** Human serum
- **Instrumental Technique:** LC/MS/MS
- **Sample Volume:** 200  $\mu\text{L}$
- **Calibration Standards:** 5 - 500 ng/mL
- **Regression:** Linear 1/x. Quantitation by peak area ratio.
- **Quality control samples:** 400 ng/mL, 240 ng/mL, 15.0 ng/mL
- **Freeze/thaw stability:** 4 cycles at  $\sim -20^{\circ}\text{C}$
- **Room temperature stability in matrix:** Demonstrated  $\sim 28$  hours
- **Processed extract stability:** Demonstrated  $\sim 47$  hours at room temperature
- **Heat Treatment Stability:** At least 40 minutes at  $\sim 56^{\circ}\text{C}$
- **Stock Solution Stability:** At least 51 days at  $\sim -20^{\circ}\text{C}$
- **Long term stability in frozen matrix:** At least 30 days at  $\sim -20^{\circ}\text{C}$

# Conclusions

- Tenofovir a nucleotide reverse transcriptase inhibitor is assayed by reverse phase HPLC using an isocratic mobile phase
- Assay is rugged, sensitive, and will aid in the pharmacokinetic measurements of Tenofovir