



# The Determination of a Tetra-Phosphate Compound in Rat Plasma by LC/MS/MS

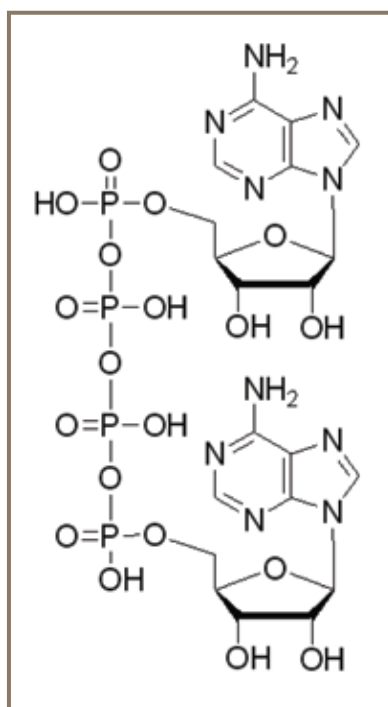
## Authors

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## Purpose

A tetra-phosphate compound of proprietary structure and similar to the internal standard used in this assay was developed as a potential drug candidate. The method applies weak ion-exchange SPE and ion-pairing technique to achieve a 10.0 ng/mL LLOQ using only 50.0  $\mu$ L of rat plasma.

FIGURE 1. P<sup>1</sup>,P<sup>4</sup>-Di(adenosine-5') tetraphosphate (ATPD as Internal Standard)





## Method

During method development, four types of ion-exchange SPE plates were tested to determine the best recovery. The SPE profiles are presented in Figure 1. The results show that WAX provides the best retention for both, the analyte and internal standard. In addition to the high recovery, the profile indicates that a wash step with 100% methanol ensures maximum elimination of the matrix interference, while the compound and internal standard are safely retained.

P1, P4-Di(adenosine-5') tetraphosphate (ATPD) was used as an internal standard. A solution of 100 mM of ammonium acetate buffer at pH 4.5 was added to the spiked rat plasma and the mixture was then transferred to the conditioned Waters Oasis WAX SPE plate. The plate was washed with 2% formic acid, water, and methanol respectively to eliminate the matrix interference. The analyte was then eluted with 5% ammonium hydroxide in methanol:water (90:10, v:v). The eluate was evaporated to dryness and reconstituted with an aqueous solution of 1% of N, N-Dimethylhexylamine (DMHA). A Phenomenex Gemini C6-phenyl, 2.1 x 50 mm, 4  $\mu$ m column was used for chromatographic separation, coupled with a Sciex API-4000 mass spectrometer.

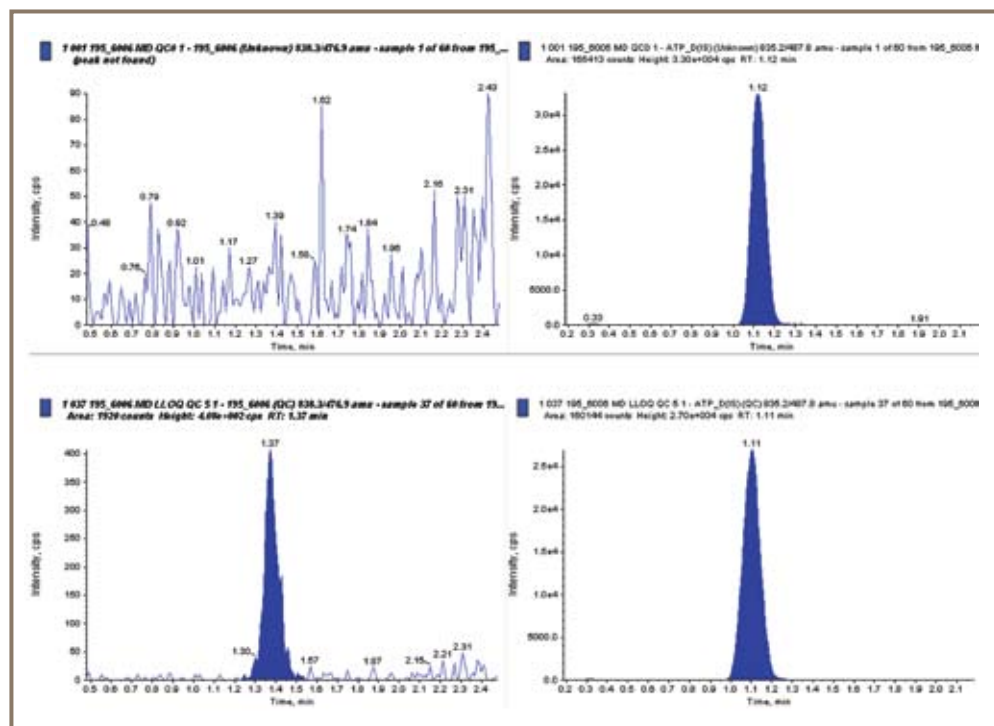
A gradient elution was used, with a mobile phase A consisting of a 5 mM ammonium bicarbonate solution in water and mobile phase B consisting of a solution of 5 mM ammonium bicarbonate in acetonitrile. The slightly basic mobile phase facilitated ionization in the negative ion mode. The total flow was 0.3 mL/min and the total run time, including column wash and re-equilibrium, was 4 minutes.

MRM transitions of 838.1  $\rightarrow$  477.1 and 835.1  $\rightarrow$  488.2 were selected to monitor the compound and IS in negative ion mode. The retention times were 1.3 and 1.2 minute for analyte and IS, respectively.



## Method continued

FIGURE 2. Extracted blank sample with IS and extracted LLOQ (10 ng/mL in Rat Plasma)



## Results

DMHA was used as an ion-pairing agent to ensure adequate retention time for analyte and IS. Using an ion-pairing agent in the reconstitution solution, instead of adding it to the mobile phase, prevented mass spectrometer signal loss and column damage. The calibration range was linear from 10.0 to 5000 ng/mL in rat plasma. A linear regression,  $1/x^2$  was used to process the data. Accuracy was within 91.8 and 99.3% of the nominal concentration and precision was better than 9.4%. Method selectivity was tested in 10 individual lots of rat plasma. Bench top, freeze-thaw, long term stability and recovery were evaluated.



## Results continued

**TABLE 1:**  
**Precision and Accuracy, Selectivity**

Lot of blank plasma	LLOQ QC (ng/mL)	LLOQ SPEC1 (ng/mL)	LLOQ SPEC2 (ng/mL)	LLOQ SPEC3 (ng/mL)	QC1 (ng/mL)	QC2 (ng/mL)	QC3 (ng/mL)	QC4 (ng/mL)
Theor. Conc.	10.0	10.0	10.0	10.0	30.0	200	2000	4000
Found Conc.								
#1	8.77	10.6	9.18	10.4	27.4	186	1870	3810
#2	9.55	11.1	9.25	11.2	28.8	200	1740	4140
#3	8.60	9.76	9.27	9.72	30.2	196	1920	3920
#4	10.1	9.90	10.0	9.35	27.2	209	2130	3940
#5	8.70	9.56	9.82	9.15	27.6	205	1890	~ 3120
#6	8.93	9.23	10.1	10.8	32.7	~ 168	1940	4000
#7		9.77	9.47	11.3				
#8		9.35	11.5	9.18				
#9		9.83	~ 12.3	8.78				
#10		9.70	8.42	9.32				
Mean	9.11	9.88	9.93	9.92	29.0	194	1920	3820
S.D.	0.592	0.565	1.16	0.926	2.14	15.0	127	360
%CV	6.5	5.7	11.7	9.3	7.4	7.7	6.6	9.4
%Theoretical	91.1	98.8	99.3	99.2	96.7	97.0	96	95.5
n	6	10	10	10	6	6	6	6

~ %Deviation from nominal concentration greater than  $\pm 15\%$



**Results** continued

TABLE 2: Bench top and Freeze-thaw Stability						
	QC1 3cy FT (ng/mL)	QC4 3cy FT (ng/mL)	QC1 6hr BT (ng/mL)	QC4 6hr BT (ng/mL)	QC1 96hr BT (ng/mL)	QC4 96hr BT (ng/mL)
Theor. Conc.	30.0	4000	30.0	4000	30.0	4000
Found Conc.						
#1	30.3	3850	28.6	3770	25.9	3950
#2	31.0	3750	31.3	3710	~ 25.2	3980
#3	30.0	3620	30.5	3790	~ 25.1	3770
#4	28.1	3640	30.3	3590	~ 24.8	3590
#5	28.9	3830	31.4	3860	~ 23.6	3570
#6	29.4	* NA	* NA	3860	~ 24.7	3420
Mean	29.6	3740	30.4	3760	24.9	3710
S.D.	1.04	106	1.13	102	0.757	225
%CV	3.5	2.8	3.7	2.7	3.0	6.1

~ %Deviation from nominal concentration greater than  $\pm 15\%$

\* no peak detected



## Conclusion

A fast and sensitive weak ion exchange SPE method was developed for the assay of a nucleotide in rat plasma. The method combines SPE technique, unique ion-pairing usage, and negative MRM detection mode on a Sciex API-4000 mass spectrometer.

FIGURE 1 Ion exchange SPE profile

