# Challenges with nucleoside triphosphate method development and analysis during the life cycle of a HCV program

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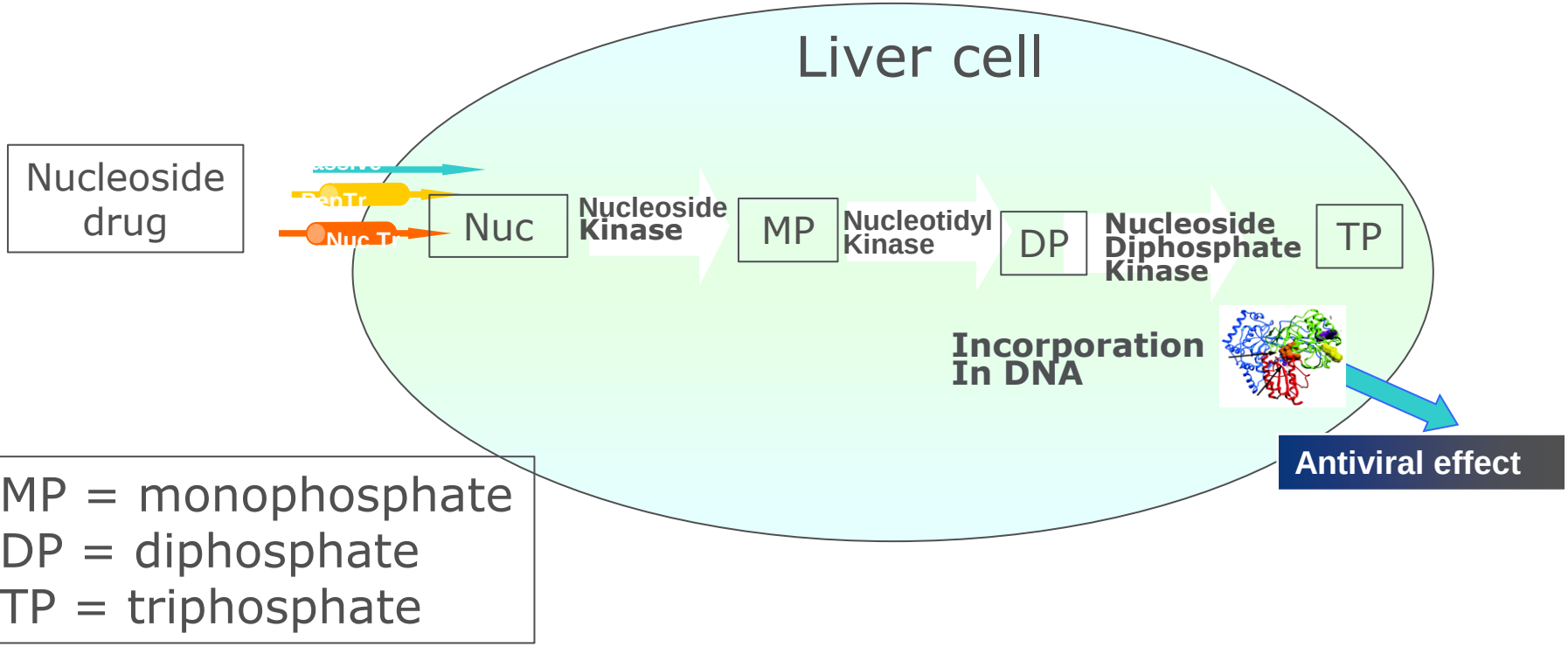
# Outline of the presentation

- Background – situation of project
- Method development for nucleoside triphosphate quantitation
  - Challenges
  - Extraction recovery
  - Stability
  - LCMSMS
- Application of method
  - *In vivo*
  - *In vitro* support
- Method development for endogenous triphosphates
- Conclusion

# Background
# Hepatitis C Virus (HCV)

- Up to 3% of population infected (170 million worldwide)
- 80% will remain chronically infected for decades
- Nucleoside drugs under development for treatment of HCV




MP = monophosphate
DP = diphosphate
TP = triphosphate

# Method development for TP quantitation

- Request for triphosphate (TP) analysis in pre-NME toxicity study in liver

- Literature: LC methods
  - Ion-pairing: non-volatile solvent
  - Ion-exchange: pH gradient, conc buffer gradient

  MS methods
  - Negative mode: phosphate groups, more sensitivity
  - Positive mode: base, better selectivity

Lit ref: J.Sep.Sci. 2009, 32, 1275-1283

# Method development for TP quantitation Challenges

- Challenges:
  - Extraction
  - Sample handling
  - Stability
  - Robust chromatography

# Method development for TP quantitation Challenges

- Standard protocol for tissue homogenisation and extraction
  - 1/10 w/w homogenisation in aqueous buffer
  - Extraction with 3 volumes of organic (methanol)
- Stability issues anticipated:
  - homogenates in MeOH/EDTA-EGTA
  - inactivate phosphatase activity – solubility of TP

# Method development for TP quantitation Extraction recovery

- 100 µl homogenate (70/30 Methanol/20mM EDTA-EGTA (4° C))
- 100 µl TP in water
- Add 200 µl methanol and vortex
- centrifugate (9000g) - directly inject supernatant OR
- evaporate to dryness, redissolve in 200 µl 25mM $NH_4Ac$/MeOH (70/30)

| MATRIX | % recovery vs reference | |
|---|---|---|
| | with evaporation | without evaporation |
| methanol/water 70/30 (EDTA/EGTA 20 mM) | 86 | 109 |
| liver homogenate | 37 | 42 |

# Method development for TP quantitation
# Extraction recovery

- 100 µl homogenate (70/30 Methanol/20mM EDTA-EGTA (4° C))
- 100 µl TP solution in water
- Vortex
- **NO ADDITION OF extra METHANOL**
- centrifugate (9000g) - directly inject supernatant (SN)

| | % recovery | | |
|---|---|---|---|
| SN homogenate | **83** | **87** | **95** |
| **Resuspension solvent** | **water** | **methanol** | **methanol/water 70/30** |
| first resuspension in 200 µl solvent | **21** | **0.5** | **8** |
| 2nd resuspension in 200 µl solvent | **3** | **0** | **3** |
| total | **107** | **87** | **102** |

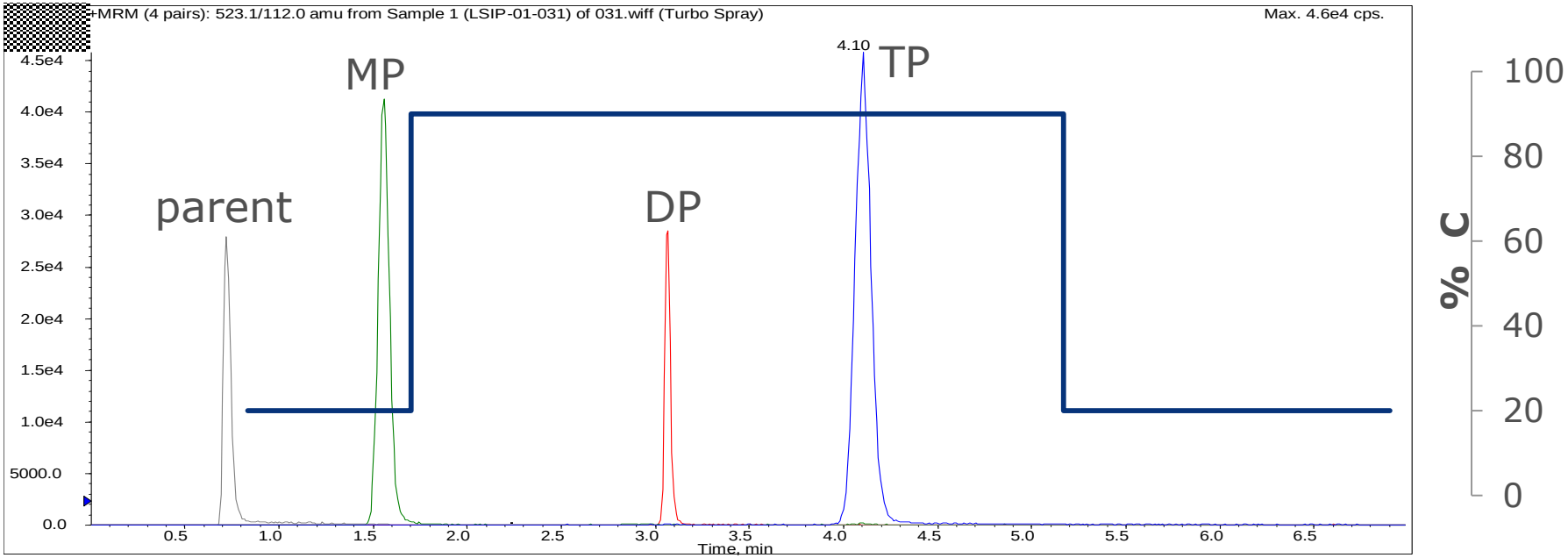
# Method development for TP quantitation
# Benchtop stability in liver homogenate and SN

| | | Incubation Condition | % of reference (t=0 min) 30 min | 60 min | 120 min |
|---|---|---|---|---|---|
| Liver homogenate | TP | 4°C | 100 | 99 | 98 |
| | | RT | 99 | 97 | 89 |
| | DP | 4°C | 100 | 103 | 112 |
| | | RT | 107 | 118 | 154 |
| Supernatant | TP | 4°C | 108 | 109 | 108 |
| | | RT | 99 | 97 | 89 |
| | DP | 4°C | 102 | 103 | 105 |
| | | RT | 104 | 103 | 115 |

# Method development for TP quantitation LC-MS/MS

Column: Biobasic AX (Thermo) 50x4.6 mm, 5µm
flow rate 0.8 ml/min

A: 0.025M ammonium acetate pH 6.0 (adjusted with HCOOH)
B: acetonitrile (constant @ 10%)
C: 0.025M ammonium acetate pH 10.0 (adjusted with $NH_4OH$ 25%)



# Method development for TP quantitation LC-MS/MS

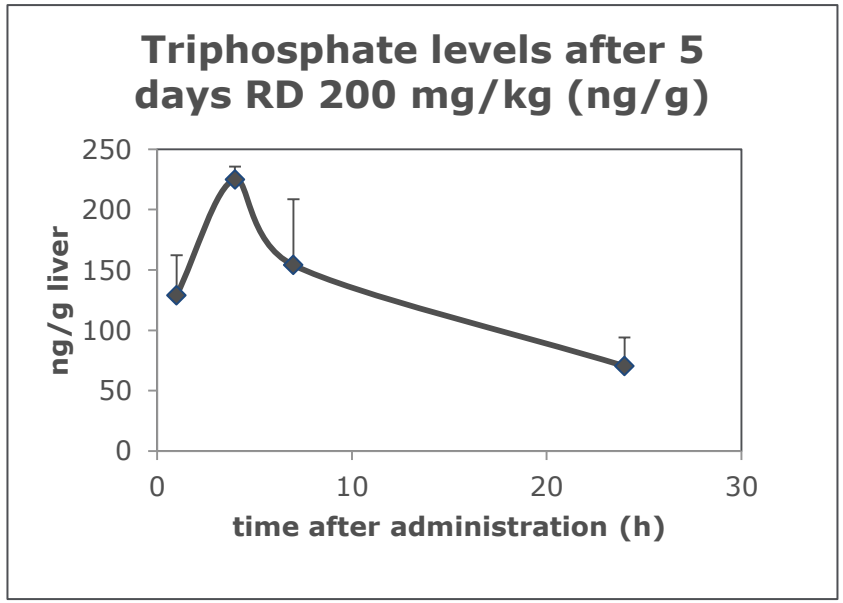
- Ion exchange column
  - variability in column batches – need for optimisation
  - difference in retention time (Rt)
  - adapt buffer concentration for similar retention times
  - fresh preparation of 0.025M $NH_4Ac$ pH10 to avoid shift in Rt
- Other HPLC columns were evaluated ($C_{18}$, $C_8$, Hilic, Amide)
  - no sensitive alternative method was found
- For some TP analogues a post-column make up flow with MeOH is needed to obtain good sensitivity

# Application of method In vivo rat study

- Quantitate NTP in liver in rat pre-NME tox study
  - Liver sampled and stored @ -80 ° C
  - Homogenisation @ time of analysis in ice cold MeOH/EDTA-EGTA
  - Batch acceptance criteria OK

No TPs were detected in study samples

- Repeat study
  - Liver sampled and immediate homogenisation
  - TPs detected



# Application of method
# Next steps

Questions:

- Are TPs stable in solid tissues post sampling?

- Impact of homogenisation procedure on results
  - whole liver versus biopts
  - snap freezing (in liquid $N_2$)

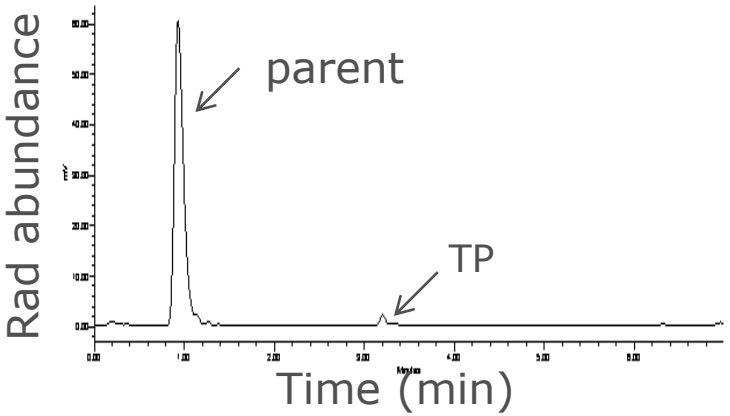
| | conc ng/g | % of reference |
|---|---|---|
| immediately homogenise liver in ice cooled MeOH/EGTA-EDTA | 2405 | 100 |
| snap-freeze liver in liquid $N_2$, homogenise after 2h | 613 | 26 |
| cut liver into pieces, liquid $N_2$, homogenise after 2h | 246 | 10 |

# Application of method
# Dog liver biopts

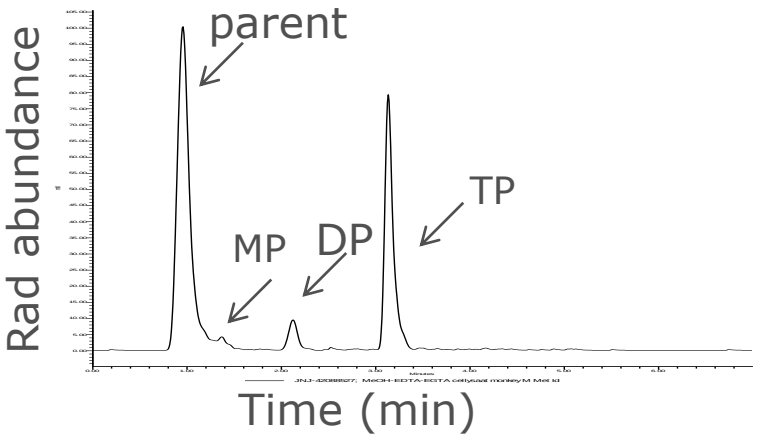
- 20 mg biopts sampled under laparoscopy
  - Snap frozen in liquid $N_2$ at the time of sampling
  - addition of 70% MeOH/20mM EDTA-EGTA
  - Immediate homogenisation with ultrasonic probe
- Sample analysis within 24 h
- **Results:**
  - Liver triphosphate levels (ng/g) after 7 d RD in dog
    - 1150 ng/g @ 1h – 899 ng/g @ 6h following last dose

# Application of method *In vitro* radioactive studies

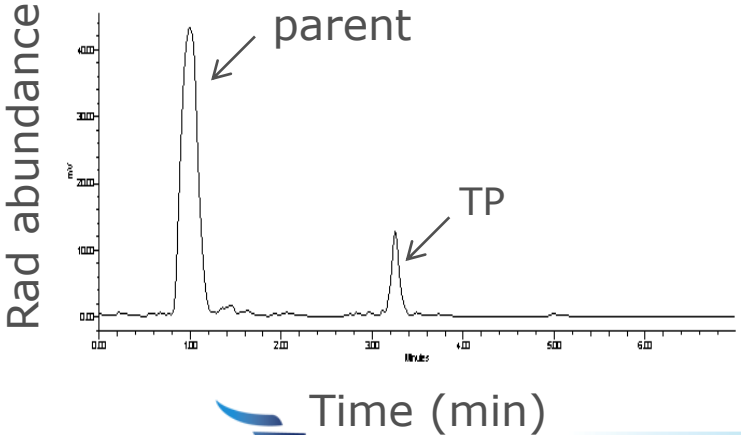
Rat hepatocytes – cpd A



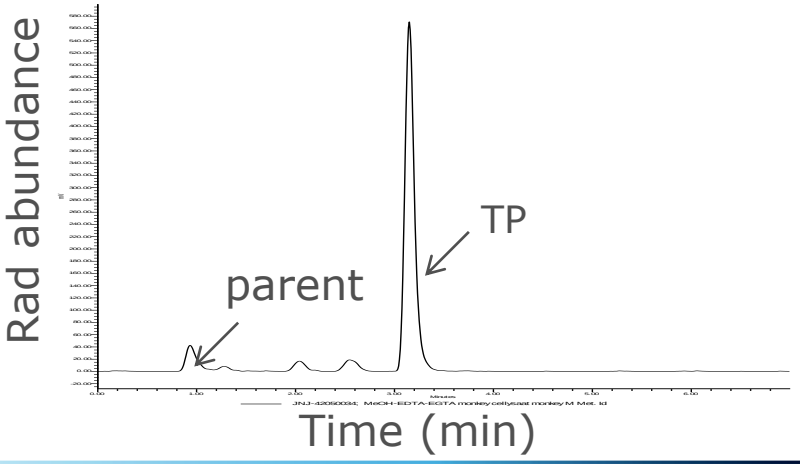

Monkey hepatocytes – cpd A




Rat hepatocytes – cpd B




Monkey hepatocytes – cpd B



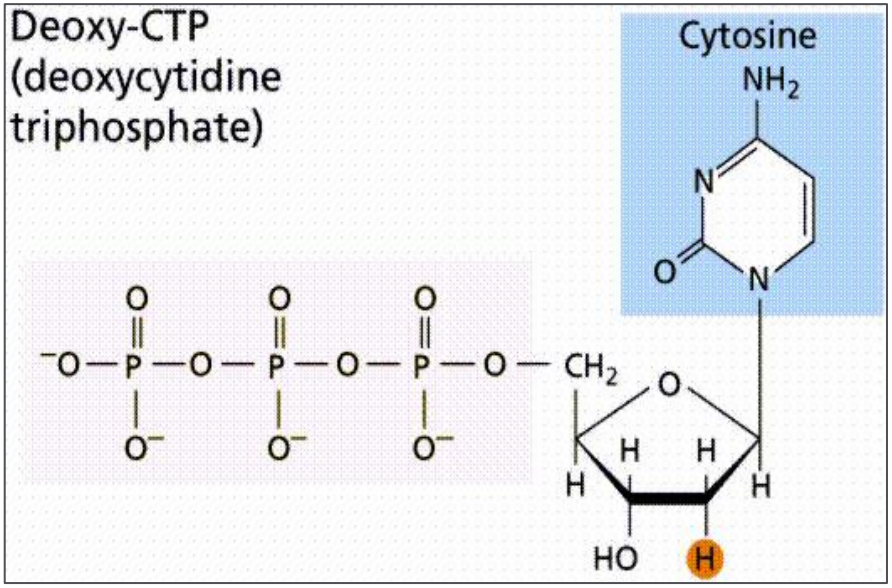
# Conclusion part 1

- Successful TP analysis depends on:
  - Ion exchange LC
  - MS/MS in positive ion mode
  - After sampling, immediate homogenisation in 70/30 MeOH/ 20mM EDTA-EGTA

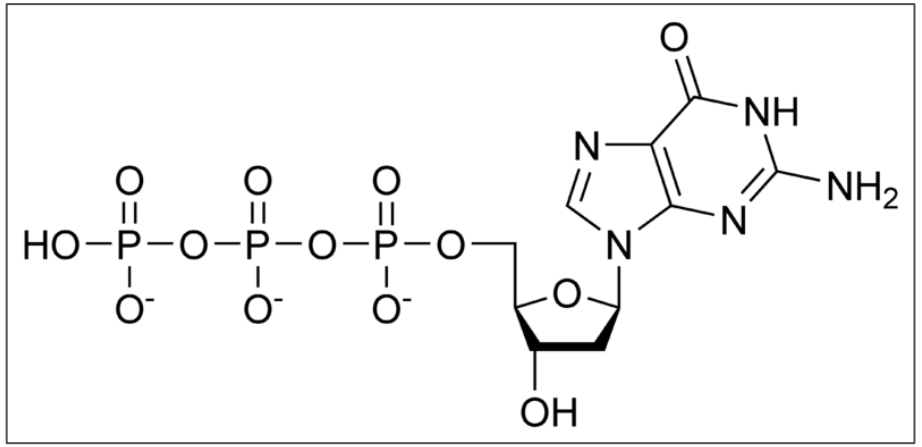
# Method development for endogenous TP quantitation

- Lead project: need for analysis of endogenous TP levels in tissues (liver and bone marrow) and in *in vitro* cell lines
- Analytes of interest: d-CTP, CTP and d-GTP

Deoxy-CTP
(deoxycytidine
triphosphate)




Deoxy- GTP
(deoxyguanosine triphosphate)



# Method development for endogenous TP quantitation

- Liver and cell samples were processed as optimized for exogenous TP analysis
- Bone marrow was extracted from femur under air pressure and immediately sonicated in ice cold MeOH/20mM EDTA-EGTA solution
- Stable isotope labelled IS (STIL) added to compensate for differences in response in different matrices
- LC adapted for separation between different NTP

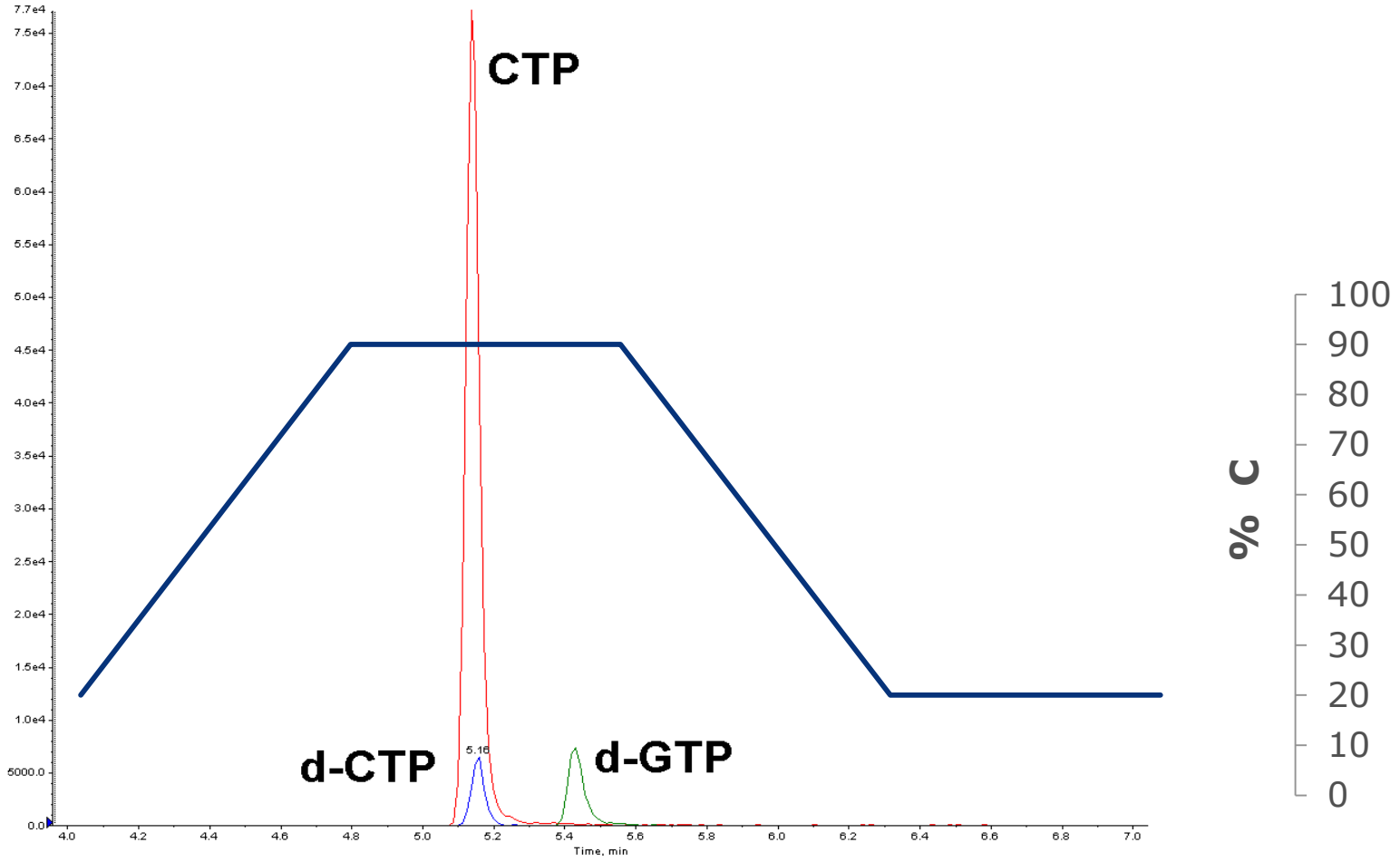
# Method development for endogenous TP quantitation

column: Biobasic AX (Thermo) 50x4.6 mm, 5µm
Flow 0.500 ml/min

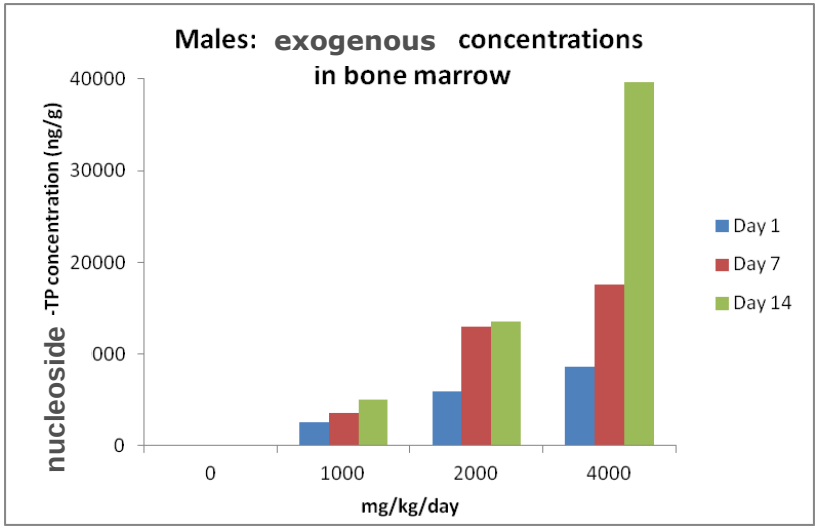
A: 0.01M ammonium acetate pH 5.4 (adjusted with $CH_3COOH$)
B: acetonitrile
C: 0.01M ammonium acetate pH 10.7 (adjusted with $NH_4OH$ 25%)

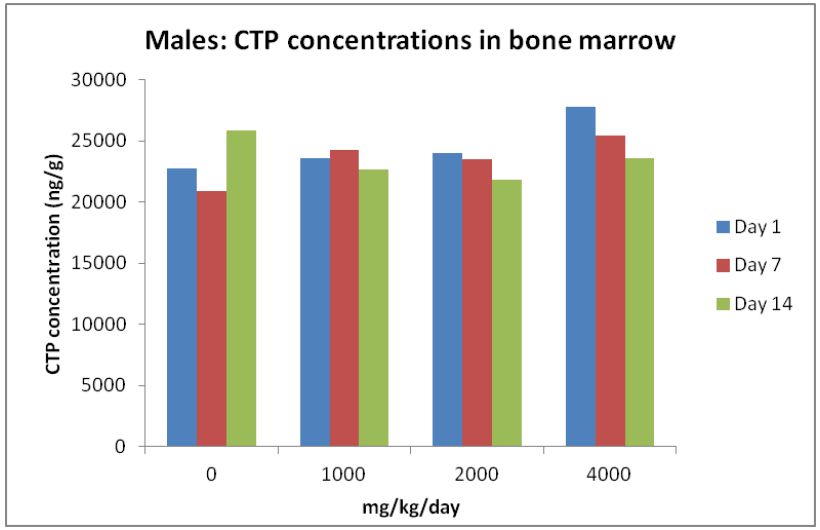
| MS parameters | | |
|---|---|---|
| | Q1 | Q3 |
| d-CTP | 468 | 112 |
| STIL d-CTP | 480 | 119 |
| CTP | 484 | 112 |
| STIL CTP | 496 | 119 |
| d-GTP | 508 | 152 |
| STIL d-GTP | 523 | 162 |

# Application: Endogenous nucleoside triphosphate quantitation



# Application: Endogenous/exogenous nucleoside triphosphate quantitation



# Conclusion

- Endogenous TP analysis:
  - LC method with minor modifications for separation of endogenous triphosphates
  - MS/MS in positive ion mode – selective base ion in Q3
  - Sampling method optimised per sample type (different tissues, *in vitro* samples)

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