# Profiling Analysis of Mono-, Di- and Tri-phosphate Nucleotides Using Capillary Ion Chromatography Mass Spectrometry

**Terri Christison[1], Yinsheng Wang[2], Ken Cook[3], Linda Lopez[1], Yingying Huang[4]**
**Thermo Fisher Scientific, [1]Sunnyvale, CA, USA; [2]University of California, Riverside, CA, USA; [3]Hemel Hempstead, U.K.; [4]San Jose, CA, USA.**

## Overview

**Purpose:** To develop and evaluate a quantitative method for targeted metabolic profiling analysis of mono-, di-, and tri-phosphate nucleotides using capillary ion chromatography tandem mass spectrometry (Cap IC-MS-MS).

**Methods:** A 5 µL sample was separated on a Thermo Scientific™ Dionex™ IonSwift™ MAX-100 monolith anion-exchange capillary column using an hydroxide gradient elution. Target analytes were detected using a Thermo Scientific™ Quantum TSQ Access MAX™ triple quadrupole mass spectrometer in selected reaction monitoring (SRM) mode. Potassium hydroxide mobile phase was converted to water, post-column by an Thermo Scientific™ Dionex™ ACES™ 300 anion capillary electrolytic suppressor. Acetonitrile was added to IC stream to assist in desolvation and to improve sensitivity.

**Results:** Target nucleotides were chromatographically resolved within 40 min; two SRM transitions were used for quantitation and confirmation for each analyte. The chromatographic separation was essential to eliminate the SRM interferences from structurally related analytes, e.g. ADP and ATP. The Limit of Quantitation was 1 nM for each analyte (5 fmol on column). Calibration range was 1–1000 nM with a coefficient of determination ($r^2$) > 0.99. Good quantitation accuracy and precision at 10 nM, 100 nM and 500 nM, were also achieved, 86.4% to 107% and %RSD less than 6% (< 17.4% for 10 nM), respectively.

## Introduction

Nucleotides are essential compounds active in many cell functions such as energy storage and release, signal transduction and synthesis of DNA and RNA. In recent years, there have been extensive studies of using nucleoside analogs as prodrugs in anti-cancer, anti-viral and immunosuppressive therapy[1,2], and monitoring of their activated nucleotides metabolites is of paramount importance to understand the pharmacology. Reported methods for nucleotides quantification include liquid chromatography (LC) with ion paring reagent[3], hydrophilic interaction liquid chromatography[4] (HILIC), ion chromatography[5,6] (IC) and capillary electrophoresis[7~9] (CE) with different detections such as conductivity, UV and mass spectrometry (MS). These methods usually do not have sufficient retention or complete chromatographic resolution to establish nucleotides profile, or required sensitivity to quantify nucleotides at trace levels.

This study describes a capillary IC tandem MS method for profiling analysis of 19 native and two modified nucleotides. Chromatographic separation was achieved on a monolith column with baseline resolutions for most of the target analytes. MS was operated in selected reaction monitoring (SRM) mode to achieve sensitive and selective quantitation. Ion exchange column chemistry with the monolith format provides the required chromatographic selectivity and the speed for the analyses. The reduced flow rate used in the method further improves the MS detection sensitivity, thus ensuring the accurate quantitation at low nM level. Three isotope labeled internal standards were used for better quantitation accuracy. Potassium hydroxide mobile phase was converted to water by a suppressor ensuring compatibility with MS detection.

## Methods

### Sample Preparation

HEK 293T Cells were cultured in ATCC-recommended medium at 37 °C and in 5% $CO_2$ atmosphere. All media were supplemented with 10% fetal bovine serum, 100 I.U./mL penicillin, and 100 µg/mL streptomycin. After growing to 70% confluence (at a density of 106 cells/mL), cells were washed with PBS twice then cultured in PBS containing 0 µM and 250 µM methylglyoxal for 3 hours.

After methylglyoxal treatment, cell pellets were harvested from the drug- or mock-treated cells (~ 2×107 cells), washed with PBS buffer and resuspended in 10 mM sodium citrate (pH 4.5) at a final volume of 400 µL.

The mixture was sonicated for 1 min, to which solution was added 4 µL of 50 mM DTT and 50 µL 100 mM D-P. The resulting solution was incubated at 37 °C for 30 min. After centrifugation at 13,000 rpm for 5 min, the supernatant was transferred out and filtrated using 3000-MW cut-off Microcon® ultra centrifugation units. The ultrafiltrates were then subjected to IC-MS-MS analysis

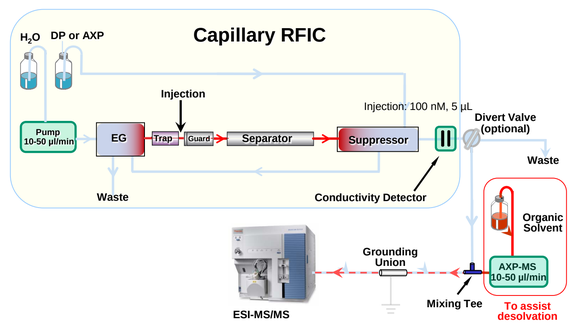
### Ion Chromatography

- Thermo Scientific™ Dionex™ ICS-5000 RFIC™ capillary IC system (capillary DP Dual Pump, EG Eluent Generator, DC Detector Column modules)
- Thermo Scientific™ Dionex™ IC Cube™ with 6-port injection valve
- Thermo Scientific ™ Dionex ™ AS-AP Autosampler

Columns: Dionex Ion Swift MAX 100 with guard, 0.25 × 250 mm
Eluent Source: Thermo Scientific Dionex EGC-KOH capillary cartridge
KOH Gradient: 10 mM (-5–0 min), 10–75 mM (0.1–25 min), 75 mM (25–29.9 min), 75–100 mM (29.9–30 min), 100 mM KOH (30–39.9 min), 100–10 mM (39.9–40 min)
Flow Rate: 0.015 mL/min
Inj. Volume: 5 µL
Column Temp.:40 ◦C

All tubing were precision cut to avoid extra dead volumes. A MicroTee® (P-890, Upchurch Scientific) was used to blend the IC stream with acetonitrile desolvation solvent before entering ESI ionization interface. The preferred Cap IC-MS-MS flow schematic is shown in Figure 1.

**FIGURE 1. Preferred Cap IC-MS-MS schematics**




### Mass Spectrometry

A Quantum TSQ Access MAX™ triple quadrupole mass spectrometer was used in this study and coupled to the Cap RFIC system with a Ion Max source and heated ESI probe (HESI II) with low-flow metal capillary kit. The source parameters were: spray voltage (3500 V), vaporizer temperature (150 ºC), sheath gas pressure (25 arbitrary unit), aux gas pressure (15 arbitrary unit), capillary temperature (200 ºC). Two SRM transitions were used for the quantitation (Q-SRM) and confirmation (C-SRM) of each target analyte with collision energy (CID) optimized for each SRM transition. Detailed SRM scan events are listed in Table 1.

### Data Analysis

Thermo Scientific Xcalibur 2.2 SP1 with Foundation 2.0 SP1 and TSQ 2.3 SP3.

Thermo Scientific DCMS$^{Link}$ 2.11.

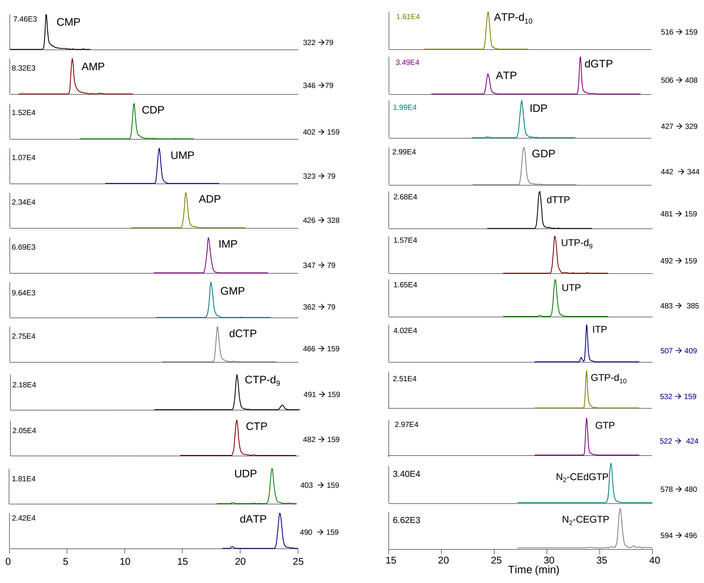
## Results

### Method Performance

As seen in Figure 2 of the SRM chromatograms, all target analytes were separated with necessary chromatographic resolution, and closely eluted peaks were successfully differentiated by SRM.

As summarized in Table 2, the calibration for each analyte from 1 nM to 1000 nM were very good, $r^2$ > 0.99. The accuracy and precision were evaluated at 10, 100 and 500 nM levels. Accuracy was observed in the range from 86.4% (GMP, 10 nM) to 107% (GDP, 100 nM). Precision was addressed as %RSD and observed in the range from 1.65% (IDP, 100 nM) to 17.4% (UDP, 10 nM).

**FIGURE 2. Profiling 19 native and two modified nucleotides using Cap IC-MS-MS**




100 nM; 5 µL injection

**TABLE 1. SRM scan events and quantified nucleotides in cell extracts**

| R.T. (min) | Analytes | Precursor | Q-SRM | C-SRM | Treated* | Untreated* |
|---|---|---|---|---|---|---|
| 3.2 | CMP | 322.0 | 79 (40) | 97 (24) | > 40 | > 40 |
| 5.5 | AMP | 346.0 | 79 (37) | 134 (36) | > 100 | > 100 |
| 10.7 | CDP | 402.0 | 159 (25) | 384 (20) | 5 | 8.66 |
| 13.0 | UMP | 323.0 | 79 (37) | 97 (25) | > 100 | > 100 |
| 15.3 | ADP | 426.0 | 328 (19) | 159 (27) | 13.8 | 21.2 |
| 17.2 | IMP | 347.0 | 79 (36) | 135 (32) | 37.2 | 23.8 |
| 17.4 | GMP | 362.0 | 211 (21) | 79 (37) | > 100 | > 100 |
| 18.0 | dCTP | 466.0 | 159 (32) | 368 (23) | ND | ND |
| 19.4 | CTP-$d_9$ | 491.0 | 159 (28) | 393 (19) | | |
| 19.5 | CTP | 482.0 | 159 (27) | 384 (21) | 0.351 | 1.17 |
| 22.8 | UDP | 403.0 | 159 (27) | 111 (22) | 7.64 | 11.6 |
| 23.4 | dATP | 490.0 | 159 (26) | 392 (25) | ND | ND |
| 24.4 | ATP-$d_{10}$ | 516.0 | 159 (31) | 418 (25) | | |
| 24.4 | ATP | 506.0 | 408 (23) | 159 (30) | 0.524 | 2.53 |
| 27.6 | IDP | 427.0 | 329 (19) | 135 (25) | ND | ND |
| 27.8 | GDP | 442.0 | 344 (20) | 150 (27) | 9.05 | 10.1 |
| 29.2 | dTTP | 481.0 | 159 (27) | 383 (20) | 0.085 | 0.100 |
| 30.7 | UTP-$d_9$ | 492.0 | 159 (31) | 394 (17) | | |
| 30.7 | UTP | 483.0 | 385 (22) | 159 (36) | 0.227 | 1.52 |
| 33.2 | dGTP | 506.0 | 159 (36) | 408 (22) | ND | ND |
| 33.7 | ITP | 507.0 | 409 (21) | 159 (36) | ND | ND |
| 33.8 | GTP-$d_{10}$ | 532.0 | 159 (27) | 434 (22) | | |
| 33.8 | GTP | 522.0 | 424 (22) | 159 (27) | 0.374 | 1.45 |
| 36.1 | $N_2$-CEdGTP | 578.0 | 480 (22) | 159 (40) | ND | ND |
| 37.0 | $N_2$-CEGTP | 594.0 | 496 (22) | 159 (40) | ND | ND |

**TABLE 2. Method Performance**

| Analytes | $r^2$ | 10 nM | | | 100 nM | | | 500 nM | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Mean | %RSD | %Accuracy | Mean | %RSD | %Accuracy | Mean | %RSD | %Accuracy |
| CMP | 0.998 | 9.91 | 8.6 | 99.1 | 98.5 | 4.2 | 98.5 | 476 | 3.6 | 95.2 |
| AMP | 0.998 | 9.72 | 10 | 97.2 | 101 | 3.8 | 101 | 507 | 1.8 | 101 |
| CDP | 0.994 | 9.45 | 6.3 | 94.5 | 94.0 | 3.2 | 94.0 | 478 | 2.1 | 95.6 |
| UMP | 0.997 | 9.70 | 9.9 | 97.0 | 97.9 | 2.9 | 97.9 | 526 | 3.7 | 105 |
| ADP | 0.995 | 10.2 | 8.4 | 102 | 96.7 | 4.0 | 96.7 | 504 | 2.3 | 101 |
| GMP | 0.995 | 8.64 | 15 | 86.4 | 106 | 4.4 | 106 | 519 | 2.4 | 104 |
| dCTP | 0.999 | 10.2 | 4.6 | 102 | 98.2 | 2.8 | 98.2 | 497 | 2.1 | 99.4 |
| IMP | 0.995 | 8.77 | 15 | 87.7 | 92.7 | 3.5 | 92.7 | 472 | 4.6 | 94.4 |
| CTP | 0.999 | 10.4 | 11 | 104 | 97.0 | 3.1 | 97.0 | 487 | 1.5 | 97.4 |
| UDP | 0.997 | 10.1 | 17 | 101 | 99.2 | 4.6 | 99.2 | 533 | 3.8 | 107 |
| dATP | 0.999 | 10.5 | 8.9 | 105 | 96.8 | 1.8 | 96.8 | 510 | 1.7 | 102 |
| ATP | 0.998 | 10.1 | 16 | 101 | 96.4 | 2.6 | 96.4 | 500 | 1.8 | 100 |
| GDP | 0.994 | 10.2 | 12 | 102 | 104 | 1.8 | 104 | 534 | 2.7 | 107 |
| dTTP | 0.998 | 9.95 | 11 | 99.5 | 99.6 | 3.0 | 99.6 | 544 | 3.3 | 109 |
| IDP | 0.997 | 9.48 | 12 | 94.8 | 99.0 | 1.7 | 99.0 | 522 | 3.5 | 104 |
| UTP | 0.998 | 9.62 | 16 | 96.2 | 94.8 | 2.3 | 94.8 | 519 | 2.6 | 104 |
| dGTP | 0.997 | 10.5 | 9.9 | 105 | 98.8 | 3.0 | 98.8 | 516 | 2.3 | 103 |
| GTP | 0.996 | 10.5 | 12 | 105 | 97.5 | 4.14 | 97.5 | 502 | 4.0 | 100 |
| ITP | 0.997 | 9.96 | 9.4 | 99.6 | 98.1 | 5.8 | 98.1 | 498 | 3.8 | 99.6 |

The treated and untreated cell extracts were diluted 100-fold with deionized water and 5 µL of diluted samples were injected for Cap IC-MS-MS analysis. Most native nucleotides were detected (Table 1). The mono-phosphate nucleotides were the most abundant ones. However, modified triphosphate nucleotides were not detected in either sample probably due to degradation to di- or mono-phosphates.

## Conclusion

A Cap IC-MS-MS method was developed for the targeted profiling quantitation of nucleotides in biological samples. This method was evaluated and demonstrated:

- Excellent chromatographic separation for target analytes;
- Selective SRM MS-MS detection capable of differentiating closely eluted analytes;
- Ultra-sensitive detection of target analytes down to 1 nM with only few µL sample consumption;
- Successful application for biological sample analysis.

## References

1. Cohen, S.; Jordheim, L. P.; Megherbi, M.; etal. *J. Chromatogr. B* **2010**, 878 (22), 1912-1928.
2. Jansen, R. S.; Rosing, H.; Schellens, J. H. M.; Beijnen, J. H. *Mass Spectrom. Rev.* **2011**, 30 (2), 321-343.
3. Seifar, R. M.; Ras, C.; van Dam, J. C.; etal. *Anal. Biochem.* **2009**, 388 (2), 213-219.
4. Johnsen, E.; Wilson, S. R.; Odsbu, I.; Krapp, A.; etal. *J. Chromatogr. A* **2011**, 1218 (35), 5981-5986
5. C. Caldwell, I. *J. Chromatogr. A* **1969**, 44 (0), 331-341.
6. Inoue, K.; Obara, R.; Akiba, T.; Hino, T.; etal. *J. Agric. Food. Chem.* **2008**, 56 (16), 6863-6867
7. Nguyen, A. L.; Luong, J. H. T.; Masson, C. *Anal. Chem.* **1990**, 62 (22), 2490-2493.
8. Soga, T.; Ohashi, Y.; Ueno, Y.; Naraoka, H.; etal. *Journal of Proteome Research* **2003**, 2 (5), 488-494



Thermo SCIENTIFIC