Application Note 575

Quantitative Profiling of Nucleotides Using Capillary IC-MS/MS

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Key Words
Dionex ICS-5000 RFIC, TSQ Quantum Access MAX, Capillary IC, nucleotide, metabolomics

Goal
To develop a capillary IC-MS/MS method for simultaneous quantitation of mono-, di-, and triphosphate nucleotides and to evaluate its performance.

Introduction
Nucleoside and nucleotide analogues constitute a major class of antiviral and antitumor agents. These compounds are phosphorylated intracellularly to pharmacologically active nucleoside triphosphates that inhibit DNA synthesis and, subsequently, viral or cell proliferation. Elucidation of the intracellular pharmacology of therapeutic nucleosides and nucleotides is essential for determining drug efficacy and toxicity, and for guiding dosing regimens. Analytical methods capable of simultaneous quantitation of nucleotide metabolites at pharmacologically relevant concentrations in complex matrices will facilitate optimization of current drug therapies and development of new nucleoside/nucleotide analogues and prodrugs.[1,2]

Due to high hydrophilicity, nucleotides are commonly analyzed by techniques using ion-exchange chromatography or ion-pair reverse-phase high-performance liquid chromatography. UV-based detection methods are not selective or sensitive enough to detect therapeutic levels of nucleotides in complex biological samples. Radiolabels are commonly used for nucleotide quantitation but are difficult and expensive to synthesize. Mass spectrometry (MS) detection offers exceptional sensitivity and selectivity for direct quantitative analysis, but is incompatible with the high-ionic-strength mobile phases and the relatively nonvolatile reagents that are typically utilized in ion-exchange and ion-pair chromatographic methods.[1,2] Dephosphorylation of nucleotides to nucleosides prior to liquid chromatography/mass spectrometry (LC/MS) analysis is a strategy that has been used to circumvent MS compatibility issues. However, this indirect method of nucleotide detection requires laborious and time-consuming sample preparation.[2] Hydrophilic interaction chromatography (HILIC) utilizes MS-compatible buffers, but generally yields poor separations and unsatisfactory peak shapes for multiply phosphorylated analytes.[3] These analytical challenges underscore the need for an MS-compatible chromatographic approach for direct and simultaneous determination of nucleoside mono-, di-, and triphosphate metabolites.

Capillary ion chromatography (Cap IC) with electrolytic suppression technology using a Thermo Scientific™ Dionex™ ICS-5000 Reagent-Free™ IC (RFIC™) system enables seamless coupling of highly efficient IC separations with electrospray ionization MS (ESI-MS) detection.[3] Post-column suppressor removes potassium ions and converts potassium hydroxide mobile phases to deionized water to ensure compatibility with ESI-MS analysis. The Thermo Scientific Dionex IonSwift™ capillary column uses monolith technology to enhance IC separation speed, resolution, and peak shape, and is optimized for use with hydroxide gradients. In addition to improved sensitivity, IC on the capillary scale reduces eluent consumption, decreases waste, and allows continuous system operation ultimately leading to reduction in total cost of ownership. The Thermo Scientific TSQ Quantum Access MAX™ triple quadrupole MS facilitates highly sensitive quantitation in matrix-rich samples and offers selected-reaction monitoring (SRM) for greater selectivity. In this note, we demonstrate highly sensitive, accurate, and reproducible detection and quantitation of 19 native and 2 modified nucleotides on a capillary IC-MS/MS platform comprising a Dionex ICS-5000 RFIC system coupled to a TSQ Quantum Access MAX triple quadrupole MS.

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Experimental

Preparation of Nucleotide Standards

Nucleotide standards (Table 1) were purchased from Sigma Aldrich (St. Louis, MO, USA).

Table 1. Nucleotide standards

Nucleotide Standard	Catalog Number
Adenosine monophosphate (AMP)	A1752
Adenosine diphosphate (ADP)	A2754
Adenosine triphosphate (ATP)	A2383
Cytidine monophosphate (CMP)	C1006
Cytidine diphosphate (CDP)	C9755
Cytidine triphosphate (CTP)	C9274
Guanosine monophosphate (GMP)	G8377
Guanosine diphosphate (GDP)	G7127
Guanosine triphosphate (GTP)	G8877
Uridine monophosphate (UMP)	U6376
Uridine diphosphate (UDP)	U4125
Uridine triphosphate (UTP)	U6655
2'-Deoxyadenosine triphosphate (dATP)	D6920
2'-Deoxycytidine triphosphate (dCTP)	D7045
2'-Deoxyguanosine triphosphate (dGTP)	D7170
Thymidine triphosphate (TTP)	T7791
Inosine monophosphate (IMP)	I2879
Inosine diphosphate (IDP)	I4375
Inosine triphosphate (ITP)	I0879

Isotope labeled internal standards (Table 2) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Table 2. Internal standards

Internal Standards	Catalog Number
ATP-$^{13}C_{10}$	710695
CTP-$^{13}C_{9}$	711020
GTP-$^{13}C_{10}$	710687
UTP-$^{13}C_{9}$	711012

Stock solutions of each internal standard were prepared in deionized water at 100 µM. Standard stock solutions were prepared at 1, 10, and 100 µM in deionized water. Calibration standards were prepared in deionized water by serial dilution at 1, 5, 10, 20, 50, 100, 200, 500, and 1000 nM with each level containing each of the 4 internal standards at 100 nM. Modified nucleotides N^2-CEGTP and N^2-CEdGTP were prepared in house.

Preparation of HEK293T Cell Extracts

HEK293T cells were cultured in American Type Culture Collection (ATCC)-recommended medium at 37 °C and in 5% CO_2 atmosphere. All media were supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 100 µg/mL streptomycin. After growing to 70% confluence (at a density of 106 cells/mL), cells were washed with phosphate-buffered saline (PBS) twice and then cultured in PBS containing 0 and 250 µM methylglyoxal for 3 hours.

After methylglyoxal treatment, cell pellets were harvested from the drug- or mock-treated cells (~2 x 10^7 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 4 µL of 50 mM dithiothreitol (DTT) and 50 µL 100 mM D-penicillimine were added. 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 molecular weight cut-off Microcon® ultracentrifugation units. The treated and untreated cell extracts were diluted 100-fold in deionized water and 5 µL of diluted samples were injected for capillary IC-MS/MS analysis.

Instrumentation

Capillary IC-MS/MS analysis was performed on a Dionex ICS-5000 RFIC system coupled to a TSQ Quantum Access MAX triple quadrupole mass spectrometer. A schematic of the capillary IC-MS/MS system configuration is shown in Figure 1. The RFIC system consists of a DP pump with capillary flow capability, an EG eluent generator, a DC detector/chromatography compartment, and an AS-AP autosampler. Post-column conversion of potassium hydroxide mobile phase to deionized water was achieved with an anion capillary electrolytic suppressor. Precision-cut tubing was used throughout the system to reduce dead volumes caused by connections. A MicroTee (P-890, Upchurch Scientific) was used to combine the IC stream with acetonitrile desolvation solvent prior to the ESI interface.

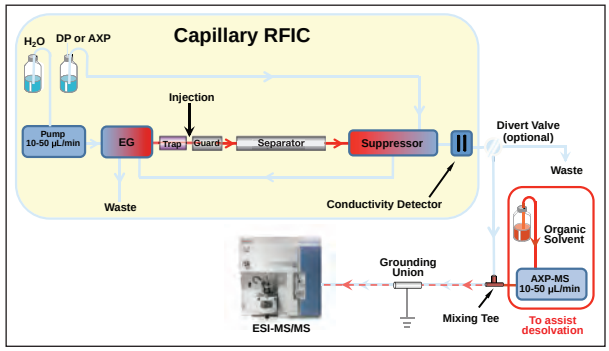


Figure 1. Capillary IC-MS/MS system

Chromatographic Conditions		
System	Dionex ICS-5000 capillary RFIC	
Column	Dionex IonSwift™ MAX-100 monolith capillary column (0.25 x 250 mm, P/N 074246) with guard column (0.25 x 50 mm, P/N 074247)	
Eluent source	Thermo Scientific Dionex EGC-KOH capillary cartridge (P/N 072076)	
Eluent	Potassium hydroxide gradient	
	Time (min)	Conc. (mM)
	-5.0	10
	0.1	10
	25.0	75
	29.9	75
	30.0	100
	39.9	100
	40.0	10
Flow rate	15 µL/min	
Injection	5 µL	
Temperature	40 °C	
Suppressor	Thermo Scientific Dionex ACES™ 300 anion capillary electrolytic suppressor (P/N 072052) in external water mode	
Suppressor current	20 mA	
Regenerant	DI water at 30 µL/min	
Desolvation solvent	Acetonitrile at 20 µL/min	

Mass Spectrometric Conditions	
Interface	Heated electrospray ionization with HESI II probe and low-flow needle insert (P/N OPTON-53011)
Spray voltage	3500 V
Vaporizer temperature	150 ºC
Sheath gas pressure	25 arbitrary units
Auxiliary gas pressure	15 arbitrary units
Capillary temperature	200 ºC

Results and Discussion

Capillary IC Separation and MS Detection of Targeted Nucleotides

A nucleotide comprises a nucleobase, a pentose sugar, and 1–3 phosphate groups. The chemical structures of the targeted nucleoside triphosphates are depicted in Figure 2. Nucleotides are highly polar compounds that are readily separated by IC and, with suppressor technology, are amenable to subsequent ESI-MS/MS detection.

Compared to conventional IC, capillary IC enhances MS mass sensitivity and reduces eluent consumption. Standard ESI interfaces are generally optimized based on analytical flow (100 µL to several mL/min) or nanoflow (<1 µL/min) ranges. Capillary IC flow rate, typically in the 10–50 µL/min range, requires modification and optimization of existing ESI interfaces. For this work, the ESI probe was fitted with a low-flow metal needle for optimum sensitivity at a flow rate of 15 µL/min. Acetonitrile was utilized as a desolvation solvent to further improve MS sensitivity. Other ESI interface parameters (e.g. ESI probe depth, ESI voltage, and proximity of the spray tip to the MS) were also optimized for nucleotide analytes.

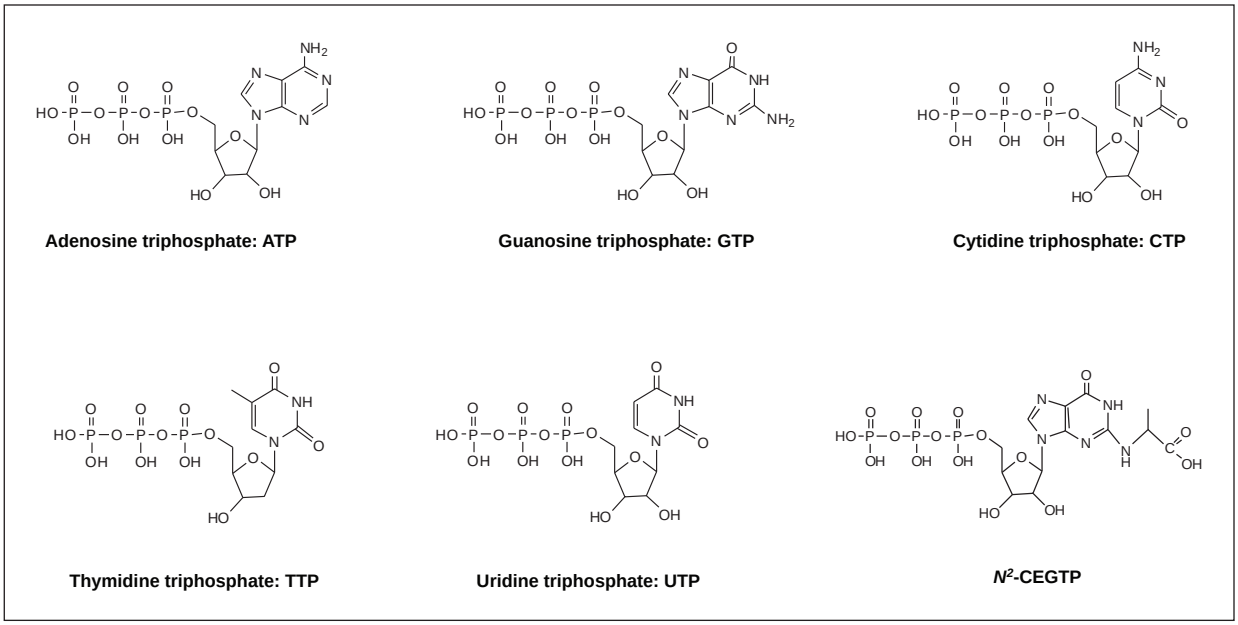


Figure 2. Chemical structures of native and modified nucleoside triphosphates

Separate SRM transitions were used for the quantitation (Q-SRM) and confirmation (C-SRM) of each target analyte with collision energy (CE) optimized for each SRM transition. Detailed SRM scan events are summarized in Table 3. Using a Dionex ICS-5000 RFIC system coupled to a TSQ Quantum Access MAX triple quadrupole MS, a Dionex IonSwift MAX-100 anion-exchange column and an electrolytically generated hydroxide gradient, 19 native and 2 modified nucleotides were separated and detected within 40 minutes (Figure 3). All analytes were well resolved and all chromatographic peaks were sharp and narrow, ensuring maximum detection sensitivity and robust quantitation. Chromatographic separation is particularly critical in preventing possible interference by in-source fragments between structurally related analytes such as ADP and ATP.

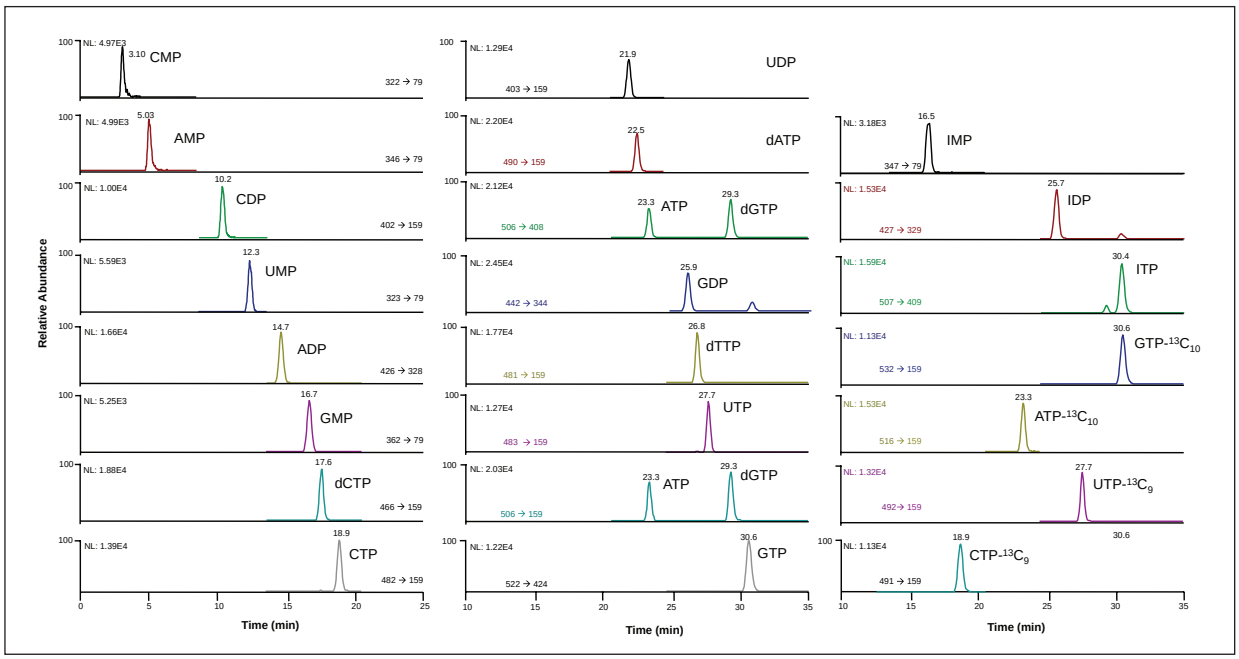


Figure 3. Chromatograms of nucleotide Q-SRM transitions

Table 3. SRM scan events for mono-, di- and triphosphate nucleotides

Retention Time (min)	Analyte	Precursor Ion (*m/z*)	Q-SRM Product Ion (*m/z* (collision energy))	C-SRM Product Ion (*m/z* (collision energy))
3.2	CMP	322.0	79 (40)	97 (24)
5.5	AMP	346.0	79 (37)	134 (36)
10.7	CDP	402.0	159 (25)	384 (20)
13.0	UMP	323.0	79 (37)	97 (25)
15.3	ADP	426.0	328 (19)	159 (27)
17.2	IMP	347.0	79 (36)	135 (32)
17.4	GMP	362.0	211 (21)	79 (37)
18.0	dCTP	466.0	159 (32)	368 (23)
19.4	CTP-$^{13}C_9$	491.0	159 (28)	393 (19)
19.5	CTP	482.0	159 (27)	384 (21)
22.8	UDP	403.0	159 (27)	111 (22)
23.4	dATP	490.0	159 (26)	392 (25)
24.4	ATP-$^{13}C_{10}$	516.0	159 (31)	418 (25)
24.4	ATP	506.0	408 (23)	159 (30)
27.6	IDP	427.0	329 (19)	135 (25)
27.8	GDP	442.0	344 (20)	150 (27)
29.2	dTTP	481.0	159 (27)	383 (20)
30.7	UTP-$^{13}C_9$	492.0	159 (31)	394 (17)
30.7	UTP	483.0	385 (22)	159 (36)
33.2	dGTP	506.0	159 (36)	408 (22)
33.7	ITP	507.0	409 (21)	159 (36)
33.8	GTP-$^{13}C_{10}$	532.0	159 (27)	434 (22)
33.8	GTP	522.0	424 (22)	159 (27)
36.1	N^2-CEdGTP	578.0	480 (22)	159 (40)
37.0	N^2-CEGTP	594.0	496 (22)	159 (40)

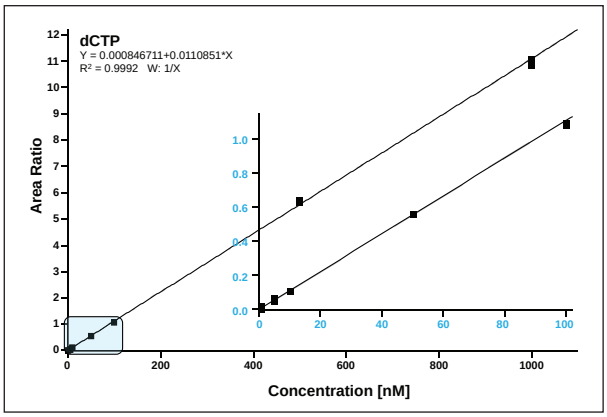
Capillary IC-MS Method Performance

Four isotope-labeled internal standards were used to ensure optimal quantitative reliability. Excellent linearity was observed over the range of 1–1000 nM, with coefficients of determination (r^2) greater than 0.99 for all nucleotides (Table 4). Representative calibration curves are shown in Figure 4. Limits of quantitation of 1 nM (5 fmol on column) were achieved for all analytes.

Method reproducibility was investigated by analyzing five replicate injections of standard solutions at 10 nM, 100 nM, and 500 nM (Table 4). Quantitative precision ranged from 1.65% RSD (IDP, 100 nM) to 17.4% RSD (UDP, 10 nM). Accuracy was determined to range from 86.4% (GMP, 10 nM) to 107% (GDP, 100 nM) (Table 4).

Table 4. Method quantitative performance

Analytes	IS	r^2	10 nM			100 nM			500 nM		
			Mean	%RSD	%Accuracy	Mean	%RSD	%Accuracy	Mean	%RSD	%Accuracy
ADP	ATP-IS	0.9951	10.2	8.37	102	96.7	4.02	96.7	504	2.29	101
AMP	ATP-IS	0.998	9.72	10	97.2	101	3.8	101	507	1.78	101
ATP	ATP-IS	0.998	10.1	16.3	101	96.4	2.57	96.4	500	1.81	100
CDP	CTP-IS	0.9938	9.45	6.25	94.5	94	3.15	94	478	2.09	95.6
CMP	CTP-IS	0.9978	9.91	8.56	99.1	98.5	4.15	98.5	476	3.57	95.2
CTP	CTP-IS	0.9988	10.4	10.8	104	97	3.11	97	487	1.47	97.4
dATP	ATP-IS	0.9987	10.5	8.86	105	96.8	1.77	96.8	510	1.72	102
dCTP	CTP-IS	0.9992	10.2	4.63	102	98.2	2.81	98.2	497	2.07	99.4
dGTP	GTP-IS	0.9966	10.5	9.89	105	98.8	3.01	98.8	516	2.27	103
dTTP	UTP-IS	0.9977	9.95	11.4	99.5	99.6	3.04	99.6	544	3.29	109
GDP	GTP-IS	0.9944	10.2	12.2	102	104	1.75	104	534	2.73	107
GMP	GTP-IS	0.9951	8.64	15.2	86.4	106	4.42	106	519	2.4	104
GTP	GTP-IS	0.996	10.5	12.4	105	97.5	4.14	97.5	502	3.95	100
IDP	ATP-IS	0.9971	9.48	11.8	94.8	99	1.65	99	522	3.49	104
IMP	CTP-IS	0.9946	8.77	15	87.7	92.7	3.52	92.7	472	4.55	94.4
ITP	GTP-IS	0.9966	9.96	9.38	99.6	98.1	5.8	98.1	498	3.78	99.6
UDP	UTP-IS	0.9974	10.1	17.4	101	99.2	4.61	99.2	533	3.81	107
UMP	UTP-IS	0.9968	9.7	9.92	97	97.9	2.85	97.9	526	3.72	105
UTP	UTP-IS	0.9976	9.62	15.7	96.2	94.8	2.25	94.8	519	2.64	104



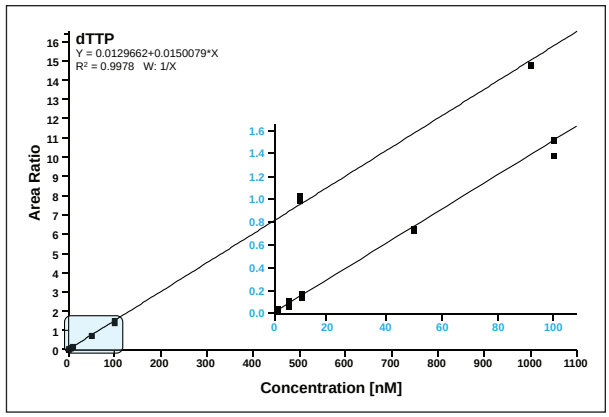


Figure 4. Calibration curves of dCTP and dTTP

Determination of Intracellular Nucleotide Pools by Capillary IC-MS/MS

Accurate quantitation of intracellular nucleotide pools is critical for elucidating the mechanism of action and for determining the efficacy of pharmacological agents that are designed to perturb nucleotide metabolism and disrupt DNA synthesis. Methylglyoxal is an endogenous dicarbonyl metabolite that reacts with the guanyl bases of nucleotides and nucleosides to produce methylglyoxal-derived moieties such as *R,S*-N^2-(1-carboxyethyl)-2'-deoxyguanosine (N^2-CEdGTP). Methylglyoxal-modified nucleotides inhibit DNA synthesis and could theoretically have therapeutic significance as, for example, targeted pharmacological agents for tumor cells with high glycolytic activity.[4] The capillary IC-MS/MS method was used to measure the nucleotide pool content of extracts from 293T cells that were treated with methylglyoxal and cells that were left untreated. Following methylglyoxal treatment, perturbations in the levels of CDP, ADP, IMP, CTP, UDP, ATP, GDP, GTP, dTTP and UTP were detected (Table 5). The targeted modified triphosphate nucleotides N^2-CEGTP and N^2-CEdGTP were not detected in either untreated or treated cell extracts, possibly due to dephosphorylation to their corresponding mono- and/or diphosphate forms.

Table 5. Nucleotide pool content of methylglyoxal-treated and untreated extracts from 293T cells

	Treated (µM)	Untreated (µM)
ADP	13.8	21.2
AMP	> 100	> 100
ATP	0.524	2.53
CDP	5	8.66
CMP	> 40	> 40
CTP	0.351	1.17
dATP	ND	ND
dCTP	ND	ND
dGTP	ND	ND
dTTP	0.085	0.100
GDP	9.05	10.1
GMP	> 100	> 100
GTP	0.374	1.45
IDP	ND	ND
IMP	37.2	23.8
ITP	ND	ND
UDP	7.64	11.6
UMP	> 100	> 100
UTP	0.227	1.52

ND = Not detected

Conclusion

A sensitive and robust capillary IC-MS/MS method for targeted quantitative profiling of nucleotides was developed. Excellent chromatographic separations were achieved for target analytes, ensuring robust quantitation. Sensitive and selective MS/MS assays enabled accurate and reproducible quantitation of nucleotides at concentrations as low as 1 nM. This assay was used to quantify intracellular nucleotide pools in cell extracts. Capillary IC-MS/MS offers unique chromatographic selectivity for polar metabolites and can be used as a complementary technique to reversed-phase LC-MS in metabolomic and clinical research applications.

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