Simultaneous determination of ribonucleoside and deoxyribonucleoside triphosphates in biological samples by HILIC-MS/MS

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Introduction

Information about intracellular concentrations of ribonucleoside and deoxyribonucleoside triphosphates, (NTPs and dNTPs, respectively) is important for studies of the mechanisms of DNA replication and repair. The balance and the overall concentration of the four dNTPs are tightly regulated by multiple mechanisms. Defects in dNTP metabolism lead to increased mutation rates and are associated with various human disorders.

The overall concentration of dNTPs is very low in nondividing cells, where dNTPs are used primarily for DNA repair or mitochondrial DNA (mtDNA) synthesis, but increases in dividing cells in S phase, when dNTPs are used for the replication of nuclear DNA. However, even in actively dividing budding yeast cells, the concentration of dNTPs is between ~40- and ~200-fold lower than the concentration of the corresponding NTPs, depending on the individual dNTP/NTP pair. The low concentration of dNTPs and their chemical similarity to NTPs present a challenge for their measurement.

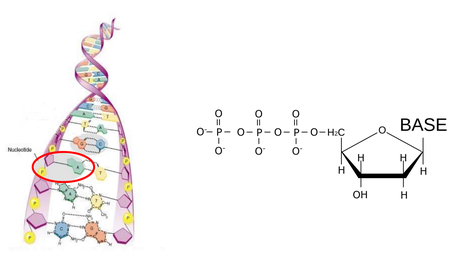

Figure 1. Illustration of dNTP, and dNTP pools

Several methods have been established over time, using different analytical techniques, but the determination and the quantification of cellular dNTP and NTP still remain a challenge, and surprisingly little is known about dNTP and NTP concentrations in various organisms and tissues.

We have developed and validated a sensitive, simple and efficient method for the determination of eight canonical dNTP and NTP using a bonded zwitterionic phosphoryl choline type stationary phase coupled with tandem mass spectrometry, and used this method to determine dNTPs and NTPs in actively dividing and in quiescent mouse fibroblasts*.

Methods

Intracellular concentrations of dNTP an NTP were analyzed on a LC-MS/MS system composed of an Agilent 1290 UHPLC (Agilent Technologies, Waldbron, Germany) coupled with Agilent 6490 triple quadrupole mass spectrometer (Agilent Technologies).

The analytes were separated on a 150×2.1 mm ZIC-cHILIC column with 3 µm particles (Merck Life Science, Darmstadt Germany). A stepwise gradient program was applied with mobile phase A (10 mM ammonium acetate, adjusted to pH = 7.7 with ammonia aqueous solution, in 90/10 water/acetonitrile) and mobile phase B (2.5 mM ammonium acetate adjusted to pH = 7.7 with ammonia aqueous solution in 90/10 acetonitrile/water) delivered at a flow rate of 200 µL/min (Table 1).

Time (min)	A (%)	B (%)	Flow (ml/min)
0.0	20	80	
7.0	20	80	
12.0	40	60	
17.0	40	60	200
19.0	20	80	
23.0	20	80	

Table 1. HPLC gradient for the separation of dNTPs and NTPs on the ZIC-cHILIC column

The HPLC eluate was introduced into the mass spectrometer through an electro-ionization spray interface, in which analytes and internal standards were ionized and carried a positive charge The injection volume was 5 µL and the separation was carried out at 35 °C.

The precursor ions, product ions and collision energies are listed in Table 2. The in-source parameters were set as follows: gas temperature 200°C; gas flow 14 L/min; nebulizer pressure 20 psi; sheath gas temperature 320°C; nebulizer gas flow 10 L/min; capillary voltage 4000 V; Nozzle voltage 400 V.

Each run was divided into three sections, in which the eluent from the column was diverted to waste during 0 to 7 min and 19 to 23 min to minimize the contamination of ion source.

	Precursor Ion [M+H]+	Product Ion (m/z)	Collision Energy (V)
dCTP	468	112	15
$dCTP_{13C,15N}$	480	119	15
dTTP	483	81	15
$dTTP_{13C,15N}$	495	86	15
dATP	492	136	15
$dATP_{13C,15N}$	507	146	15
dGTP	508	152	18
$dGTP_{13C,15N}$	523	162	18
CTP	484	112	25
CTP_{15N}	487	115	25
UTP	485	97	25
$UTP_{13,15N}$	496	102	25
ATP	508	136	30
$ATP_{13C,15N}$	523	146	30
GTP	524	152	25
GTP_{13C}	534	157	25

Table 2. Precursor/product ions and collision energies

Results

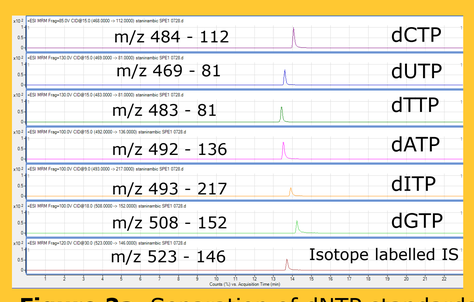

Figure 2a. Separation of dNTP standards

Mammalian cells

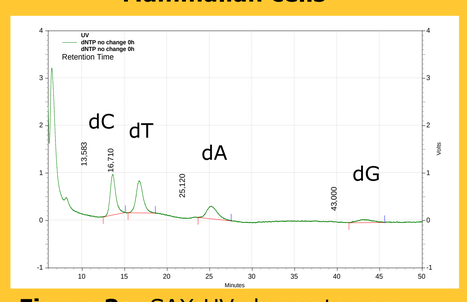

Figure 3a. SAX-UV chromatogram

Mouse Spleen

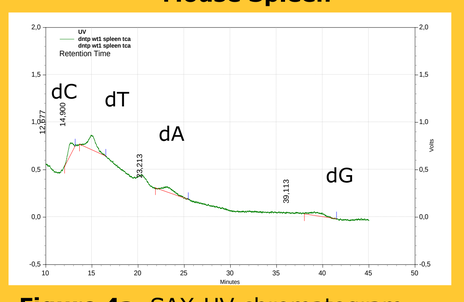

Figure 4a. SAX-UV chromatogram

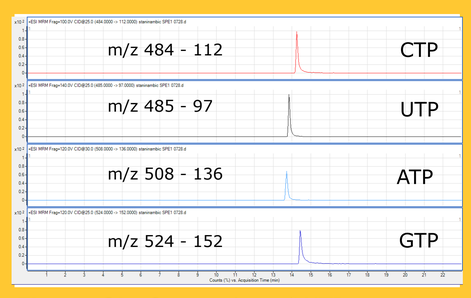

Figure 2b. Separation of NTP standards

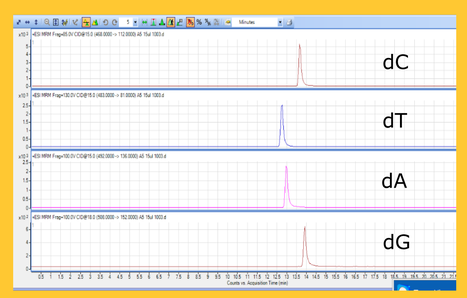

Figure 3b. HILIC-MS/MS data (same sample as in 3a)

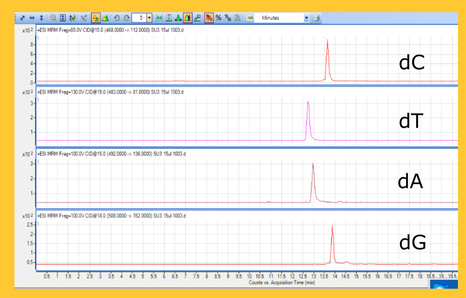

Figure 4b. HILIC-MS/MS data (same sample as in 3a)

Discussion

Separation and detection of dNTPs and NTPs:
A zwitterionic ZIC-cHILIC stationary phase was used in gradient mode. The mobile phase compositions were optimized with respect to both electrospray ionization (MS compatibility), and chromatographic separation efficiency (retentivity/resolution). All eight target analytes could be detected with good peak shapes, and the chromatographic resolution among all eight dNTP and NTP were sufficient. No isotopic interference was observed as can be seen in Figure 2a-b.

Method Validation:
Using data acquired from six concentration levels of standard solutions with dNTP and NTP, the method was shown to exhibit good linearity. The within day coefficients of variation (CVs, n=3) were less than 12.7% and 8.1% for dNTP and NTP, respectively, and the inter-day precision was less than 10.5% and 12.7% for dNTP and NTP, respectively. The stability of Balb/3T3 dNTP and NTP samples at -20 °C was tested for a period of 72 h and no significant difference was found among mean values of dNTPs and NTPs with Student t test.

Measurement of dNTP and NTP in biological samples:
The levels of dNTPs in eukaryotic cells are varying during the cell cycle, whereas the NTP levels are relatively constant. The dNTP levels are high in S phase when the DNA synthesis occurs, and low in G1 and in quiescent cells. To test the sensitivity of the developed LC-MS/MS method on biological samples, we measured dNTP and NTP levels in logarithmically growing cells cultures with a mixed population of cells in all different phases of the cell cycle and in serum-starved quiescent cells that are predominantly in G1/G0. The cell cycle profiles were verified using flow cytometry analysis. The cells were counted and the amount dNTP and NTP was determined

This new method can easily be adopted for the measurement of non-canonical minor dNTP species, such as dUTP, dITP, 8-oxo-dGTP and other physiologically relevant dNTPs.

* *"Simultaneous determination of ribonucleoside and deoxyribonucleoside triphosphates in biological samples by hydrophilic interaction liquid chromatography coupled with tandem mass spectrometry"*
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Summary

- The new method has an adequate linearity (R2 > 0.99) over a wide concentration-range, and could accurately quantify dNTPs and NTPs at low pmol levels.
- The intra-day and inter-day precision, were both better than 13%, and the relative recovery was ranging between 92% and 108%.
- The new method has short analysis time and require a simpler sample pretreatment, and do not require ion-pair reagents in the mobile phase.
- Using the new method, it is possible to determine dNTP and NTP concentrations in actively dividing and quiescent mouse fibroblasts.

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