RESEARCH PAPER

Quantitation of endogenous nucleoside triphosphates and nucleosides in human cells by liquid chromatography tandem mass spectrometry

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Abstract Nucleosides and nucleoside triphosphates are the building blocks of nucleic acids and important bioactive metabolites, existing in all living cells. In the present study, two liquid chromatography tandem mass spectrometry methods were developed to quantify both groups of compounds from the same sample with a shared extraction procedure. After a simple protein precipitation with methanol, the nucleosides were separated with reversed phase chromatography on an Atlantis T3 column while for the separation of the nucleoside triphosphates, an anion exchange column (BioBasic AX) was used. No addition of ion pair reagent was required. A 5500 QTrap was used as analyzer, operating as triple quadrupole. The analytical method for the nucleoside triphosphates has been validated according to the guidelines of the US Food and Drug Administration. The lower limit of quantification values were determined as 10 pg on column (0.5 ng/mL in the injection solution) for deoxyadenosine triphosphate and deoxyguanosine triphosphate, 20 pg (1 ng/mL) for deoxycytidine triphosphate and thymidine triphosphate, 100 pg (5 ng/mL) for cytidine triphosphate and guanosine triphosphate, and 500 pg (25 ng/mL) for adenosine triphosphate und uridine triphosphate respectively. This methodology has been applied to the quantitation of nucleosides and nucleoside triphosphates in primary human CD4 T

lymphocytes and macrophages. As expected, the concentrations for ribonucleosides and ribonucleoside triphophates were considerably higher than those obtained for the deoxy derivatives. Upon T cell receptor activation, the levels of all analytes, with the notable exceptions of deoxyadenosine triphosphate and deoxyguanosine triphosphate, were found to be elevated in CD4 T cells.

Introduction

Nucleosides are biomolecules composed of a nucleobase and a five-carbon sugar. This sugar is ribose in ribonucleosides and 2-deoxyribose in deoxynucleosides (see Electronic Supplementary Material (ESM), Fig. S1). Nucleosides can be phosphorylated at the 5′ hydroxyl group to nucleoside mono-, di-, or triphosphates. Ribonucleoside triphosphates (NTP) are important energy carriers, signal molecules, and monomers of RNA, while DNA is built of 2-deoxyribonucleoside triphosphates (dNTP) [1].

The necessity of nucleosides (ribonucleosides and deoxyribonucleosides) and (d)NTP (NTP and dNTP) for virus replication is exploited for the treatment of several virus infections by using nucleoside analogues. Examples are the human immunodeficiency virus (HIV) (e.g., zidovudine, a thymidine analogue), herpes simplex virus (HSV; e.g., aciclovir, a guanosine analogue), or hepatitis C virus (HCV; e.g., ribavirin, a guanosine analogue). These substances are phosphorylated in the virus-infected cell to their corresponding triphosphates by cellular and viral kinases and act as competitors of the endogenous substrates or inhibit viral enzymes [2–4]. In case of ribavirin, the metabolite ribavirin monophosphate

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(RMP) also inhibits the cellular enzyme inosine monophosphate dehydrogenase (IMPDH). To monitor the pharmacological effects of antiviral drugs, the determination of the concentrations of endogenous (d)NTP and the corresponding nucleosides are of particular interest. Furthermore, quantitation methods are interesting for investigations of energy metabolism [5] or the activation state of immune cells. After activation, immune cells, e.g., T cells, rapidly proliferate and have increased requirements of nucleosides and (d)NTP as compared to their resting counterparts [6, 7]. In general, knowing the endogenous nucleoside and nucleotide concentrations in cells is interesting because this provides information about the cell cycle state [8].

Analytical methods for the quantitation of nucleosides and nucleotides have been widely described in the bibliography and include enzymatic assays [9, 10] and high-performance liquid chromatography (HPLC) coupled to UV detection [11, 12] or to a diode array detector (DAD) [13]. In the last decade, multiple mass spectrometric methods have been published as well, of which in particular tandem mass spectrometry offers improved selectivity and sensitivity. The use of mass spectrometry in the analysis of nucleosides and nucleotides has been reviewed in several papers [5, 14, 15].

For the quantitation of the highly polar (d)NTP, in most of the published LC-MS/MS methods, the addition of ion-pairing reagents such as trifluoracetic acid (TFA) or N, N-dimethylhexylamin (DMHA) [2, 16–23] to the mobile phases has been described. Unfortunately, these additives reduce sensitivity [24] and cause ion source contamination [25], leading to background noise and undesired signals in the following measurements. To overcome these impairments, approaches have been made without using ion pair reagents. Zhao et al. and Machon et al. reduced the use of ion pair reagents to a minimum by only adding it to the injection solution [25, 26]. Shi et al. reported the first weak anion exchange LC-MS/MS method for the determination of a single nucleotide, completely avoiding to work with ion pair reagents [27]. We developed a similar approach for the quantitative determination of the endogenous NTP adenosine triphosphate (ATP), guanosine triphosphate (GTP), cytidine triphosphate (CTP), and uridine triphosphate (UTP) as well as for the dNTP deoxyadenosine triphosphate (dATP), deoxyguanosine triphosphate (dGTP), deoxycytidine triphosphate (dCTP), and thymidine triphosphate (TTP) using a chromatographic separation based on a pH-dependent anion exchange.

Several methods have been reported for the quantitation of nucleosides as well, though most published analytical methods do not include the deoxy derivatives. UV detection [28, 29] is used as well as mass spectrometry [30–36]. Besides, multiple analytical methods are published for the quantitation of modified nucleosides [37–40].

Due to strong differences in polarity, a simultaneous quantitation of both families, nucleosides and (d)NTP, is very challenging. Only few methods using UV [41] or DAD [13] detection reported the simultaneous determination of analytes from both groups. However, these investigators used phosphate buffers which are incompatible with mass spectrometry. Cordell et al. developed an assay for the analysis of several nucleotides and the nucleoside adenosine by LC-MS/MS using DMHA [19]. Yamaoka and co-workers determined 23 endogenous nucleosides and nucleoside mono-, di-, and triphosphates by ion pair LC-MS but did not include any deoxy derivatives [36]. The same limitations were present in case of the LC-MS method developed by Xing et al. who quantified 16 endogenous nucleosides and their corresponding phosphate derivatives [42]. To our knowledge, to date, there is no analytical method describing the simultaneous quantitation of the endogenous nucleosides and (d)NTP present in RNA and DNA using mass spectrometry.

The aim of the present study was to develop and validate an analytical procedure for the quantitation of all endogenous (d)NTP, functioning as building blocks of RNA and DNA, in human cell samples. Furthermore, to maximize the gain of knowledge, the corresponding nucleosides should be quantifiable in the same sample. Due to the different polarity of the analytes, the simultaneous quantitation in one run is hardly possible and has not been described yet. For this reason, two LC-MS/MS methods for the determination of the studied analytes were developed. However, the sample preparation allows the quantitation of both substance groups from the same extracted sample. A second, time-consuming extraction procedure is avoided by dividing the sample into equal parts during the extraction. Thus, the newly developed methods provide the possibility of the quantitation of these two substance groups from the very same sample.

Materials and methods

Materials

The following nucleoside standards, nucleotide standards, and internal standards were obtained from Sigma-Aldrich (Steinheim, Germany): adenosine (A), adenosine-5′-triphosphate (ATP), adenosine-$^{13}C_{10}$,$^{15}N_5$-5′-triphosphate ($^{13}C_{10}$,$^{15}N_5$-ATP), 2′-deoxyadenosine (dA), 2′-deoxyadenosine-5′-triphosphate (dATP), 2′-deoxyadenosine-$^{13}C_{10}$,$^{15}N_5$-5′-triphosphate ($^{13}C_{10}$,$^{15}N_5$-dATP), guanosine (G), guanosine-5′-triphosphate (GTP), guanosine-$^{13}C_{10}$,$^{15}N_5$-5′-triphosphate ($^{13}C_{10}$,$^{15}N_5$-GTP), 2′-deoxyguanosine (dG), 2′-deoxyguanosine-5′-triphosphate (dGTP), 2′-deoxyguanosine-$^{13}C_{10}$,$^{15}N_5$-5′-triphosphate ($^{13}C_{10}$,$^{15}N_5$-dGTP), cytidine (C), cytidine-5′-triphosphate (CTP), cytidine-$^{13}C_9$,$^{15}N_3$-5′-triphosphate ($^{13}C_9$,$^{15}N_3$-CTP), 2′-deoxycytidine (dC), 2′-deoxycytidine-5′-triphosphate (dCTP), 2′-deoxycytidine-$^{13}C_9$,$^{15}N_3$-5′-triphosphate sodium

salt solution ($^{13}C_9$,$^{15}N_3$-dCTP), uridine (U), uridine-5′-triphosphate (UTP), uridine-$^{13}C_9$$^{15}N_2$-5′-triphosphate ($^{13}C_9$,$^{15}N_2$-UTP), 2′-deoxythymidine (T), and 2′-deoxythymidine-5′-triphosphate (dTTP). Thymidine-$^{13}C_{10}$,$^{15}N_2$-5′-triphosphate ($^{13}C_{10}$,$^{15}N_2$-dTTP) was obtained from Silantes (Munich, Germany). $^{13}C_5$-Adenosine ($^{13}C_5$-A), $^{13}C_5$-guanosine ($^{13}C_5$-G), ^{13}C,$^{15}N_3$-2′-deoxycytidine (^{13}C,$^{15}N_3$-dC), and $^{13}C_5$-uridine ($^{13}C_5$-U) were purchased from Alsachim (Illkirch, France). Water, acetonitrile (ACN), and methanol (all LC-MS grade) were purchased from Roth (Karlsruhe, Germany). Ammonia solution, ammonium acetate powder, and formic acid (pro analysis) were obtained from VWR (Darmstadt, Germany). Dulbecco's phosphate buffered saline (PBS) without calcium and magnesium was obtained from PAA Laboratories (Pasching, Austria).

Sample preparation

Primary human CD4 T cells were directly purified from buffy coats of healthy blood donors using the negative-selection RosetteSep Human CD4 T Cell Enrichment Cocktail (Stemcell Technologies, Vancouver, Canada) on a Ficoll gradient (Ficoll-Paque, Sigma-Aldrich, St. Louis, USA) according to the manufacturer's protocol. Cells were kept at a density of 2×10^6 cells per mL in RPMI1640 (Gibco, Life Technologies, Carlsbad, USA) supplemented with 10 % fetal calf serum (Biochrom, Berlin, Germany), 50 U/mL penicillin, and 50 μg/mL streptomycin (Gibco, Life Technologies, Carlsbad, USA). Activation was carried out by addition of 100 IU/mL interleukin-2 (Biomol, Hamburg, Germany) and 2 μg/mL of phytohaemagglutinin (Sigma-Aldrich) for 3 days. Before the cells were subjected to nucleoside/nucleotide measurements, cells were counted, washed once in PBS, pelleted, and stored at −80 °C.

Human M1 and M2 macrophages were kindly supplied by Dr. Andreas Weigert from the Institute of Biochemistry I of the Goethe University in Frankfurt. The macrophages were isolated as described before [43]. Three samples were provided each for differentially differentiated macrophages: One sample had been stimulated with interleukin-4 (IL-4) while a second sample had been stimulated with lipopolysaccharide (LPS) and interferon-γ (IFN-γ). A third sample had not been stimulated and was used as a reference control.

Instrumentation

Samples were analyzed by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS). The HPLC system consisted of an Agilent 1260 Series binary pump, degasser, and column oven (Agilent Technologies, Waldbronn, Germany) connected to a CTC PAL autosampler (CTC Analytics AG, Zwingen,

Switzerland). For detection, a hybrid triple quadrupole-ion trap mass spectrometer 5500 QTRAP (AB Sciex, Darmstadt, Germany) equipped with a Turbo Ion Spray source operated in positive ion mode was used.

(d)NTP

For the chromatographic separation of the NTP and dNTP, a BioBasic AX column (150 mm × 2.1 mm I.D., 5 μm particle size and 300 Å pore size; Thermo Fisher, Waltham, MA, USA) was used. The column oven was tempered at 25 °C, and the injection volume was 20 μL. A gradient program was used for the separation of the analytes with a flow rate of 450 μL/min within a total run time of 15 min. Buffer A consisted of 40 mL acetonitrile (ACN), 0.06 mL glacial acetic acid, and 1 mL ammonium formate solution (1 M) per 100 mL water (pH=5.6) while buffer B consisted of 30 mL ACN, 0.3 mL ammonia solution (25 %), and 0.1 mL ammonium formate solution (1 M) per 100 mL water (pH=10.6). The gradient started with 70 % buffer A, was linearly decreased to 30 % buffer A within 4 min and then, further to 10 % buffer A within 7 min. These conditions were held for 1 min. Finally, a change back to the initial conditions was made within half a minute and the column was re-equilibrated for 2.5 min.

The mass spectrometer was operated in positive multiple reaction monitoring mode (MRM) with a dwell time of 50 ms for all precursor-to-product ion transitions. The ionspray voltage was set at 4500 V with an ionization source temperature of 450 °C. Ion source gases 1 and 2 were both set at 50 psi while curtain gas was 35 psi and collision gas 12 psi. All quadrupoles were running at unit resolution. Entrance potential (EP) was 10 V for all analytes while declustering potential (DP), collision energy (CE), and collision cell exit potential (CXP) were optimized manually for every substance. The optimized parameters together with the precursor-to-product ion transitions used for quantitation and qualification are listed in Table 1.

Nucleosides

The nucleosides were separated using an Atlantis T3 column (100 mm × 2.1 mm I.D., 3 μm particle size and 100 Å pore size; Waters, Milford, MA, USA). The temperature of the column oven was 40 °C, and the flow rate was 300 μL/min. The injection volume was 20 μL. The separation of the analytes was achieved within a runtime of 15 min under gradient conditions with eluent A, 0.1 % acetic acid, and eluent B, methanol with 10 mM ammonium acetate. The gradient program started with 100 % A for 2.5 min; within 2.5 min, the fraction of A was linearly decreased to 10 % and remained so for 4.5 min. For 1.5 min, solvent A was linearly increased again to 100 % and the column was re-equilibrated for 4 min.

Table 1 Precursor-to-product ion transitions of the analytes and their internal standards used as quantifiers and qualifiers (italic) for the analytical method

Analyte/IS	Precursor ion [m/z]	Product ion [m/z]	DP [V]	CE [V]	CXP [V]
ATP	508.0	410.0	120	25	20
		136.1	120	54	12
$^{13}C_{10},^{15}N_5$-ATP	523.0	146.0	120	54	13
		425.0	120	26	19
dATP	492.0	136.1	130	40	13
		81.0	130	76	12
$^{13}C_{10},^{15}N_5$-dATP	507.0	146.1	140	44	16
		86.0	140	76	12
GTP	524.0	152.0	165	45	16
		135.0	165	98	17
$^{13}C_{10},^{15}N_5$-GTP	539.0	162.1	150	47	20
		282.0	150	40	15
dGTP	508.0	152.0	165	44	16
		232.2	165	22	20
$^{13}C_{10},^{15}N_5$-dGTP	523.0	162.1	140	43	15
		86.0	140	65	12
CTP	484.0	112.1	130	49	13
		208.1	130	23	15
$^{13}C_9,^{15}N_3$-CTP	496.0	119.0	90	53	10
		220.0	90	23	15
dCTP	468.1	112.1	110	43	17
		192.1	110	17	26
$^{13}C_9,^{15}N_3$-dCTP	480.1	119.0	140	47	17
		204.2	140	18	17
UTP	485.0	227.0	70	32	18
		97.0	70	50	18
$^{13}C_9,^{15}N_2$-UTP	496.0	102.0	170	45	14
		238.2	170	27	15
dTTP	483.0	81.0	170	55	17
		207.0	170	15	20
$^{13}C_{10},^{15}N_2$-dTTP	495.0	85.9	170	53	11
		219.1	170	15	18
A	268.1	136.2	60	33	20
		119.0	60	67	20
dA	252.1	136.2	60	30	13
		119.0	60	61	17
$^{13}C_5$-A	273.1	136.1	80	33	15
		119.0	80	65	15
G	284.0	152.0	70	28	20
		135.1	70	55	10
dG	268.2	152.0	50	25	17
		135.1	50	52	15
T	243.1	127.0	40	20	13
		110.0	40	42	11
$^{13}C_5$-G	289.1	152.0	50	27	15
		135.1	50	54	10

Table 1 (continued)

Analyte/IS	Precursor ion [m/z]	Product ion [m/z]	DP [V]	CE [V]	CXP [V]
C	244.2	112.1	50	28	13
		94.9	20	59	13
dC	228.1	112.0	40	27	13
		95.0	40	53	11
$^{13}C,^{15}N_3$-dC	232.1	116.0	40	18	17
		98.0	40	50	11
U	245.0	113.0	60	26	25
		96.0	60	49	20
$^{13}C_5$-U	250.2	113.0	50	21	15
		96.0	50	50	10

DP declustering potential, *CE* collision energy, *CXP* collision cell exit potential

For improving the spray stability, isopropanol was infused into the ion source at a flow rate of 100 μL/min after the column. The mass spectrometer settings were like those for the (d)NTP except for the ionspray voltage which was 5000 V and the collision gas which was 9 psi. An optimization of DP, CE, and CXP was done as well; the results are listed in Table 1. For quantitation, four different internal standards were used: $^{13}C_5$-A as IS for A and dA, $^{13}C_5$-G as IS for G, dG and T, $^{13}C,^{15}N_3$-dC as IS for C, and dC and $^{13}C_5$-U as IS for U.

Standard preparation

For the (d)NTP calibration curve, a standard mixture containing all (d)NTP in methanol was diluted with methanol to differently concentrated working solutions. Twenty microliters of every working solution was spiked into 10 μL PBS as blank matrix, mixed with 20 μL of the corresponding internal standard mixture (500 ng/mL in methanol), and diluted with 360 μL methanol. The tubes were vortexed and centrifuged at 20,238×g for 3.5 min (Eppendorf Microcentrifuge 5424, Wessling-Berzdorf, Germany). The supernatant was collected, and the solvent was evaporated under nitrogen at 45 °C. (d)NTP standard samples were resuspended in 50 μL of a mixture of 70 % buffer A and 30 % buffer B (see "Instrumentation"). The quality control samples were prepared the same way.

The nucleoside calibration curve was prepared in the same manner, except for working solutions made from a standard mixture containing all nucleosides in methanol. The concentration of the internal standard mixture was 100 ng/mL in methanol. After evaporation, the calibration standards were resuspended in 50 μL of 10 mM acetic acid/ sodium acetate buffer. For the concentrations of all (d)NTP and nucleoside calibration standards, see ESM, Table S1.

Sample extraction

The sample extraction was done on ice. The cells were mixed with 360 μL ice-cold methanol, 20 μL of the IS mixture for the nucleosides (100 ng/mL in methanol), and 20 μL of the IS mixture for the (d)NTP (500 ng/mL in methanol). The mixture was vortexed for 1 min and centrifuged at 20,238×g for 3 min. Two new tubes were filled with 190 μL of the supernatant each, and the organic solvent was evaporated at 45 °C under a slight stream of nitrogen. Samples were reconstituted in the appropriate solvent for (d)NTP or nucleoside analysis respectively.

(d)NTP method validation

The developed method for the quantitation of (d)NTP has been validated according to FDA guidelines [44] in terms of sensitivity (lower limit of quantification, LLOQ), linearity, precision, accuracy, recovery, and stability.

The LLOQ values for the dNTP were calculated on the basis of precision and accuracy values and the signal-to-noise ratio (S/N≥10). Due to the high amounts of NTP present in biological samples, the calibration ranges for these substances were chosen on the basis of the concentrations expected in the samples, although a more sensitive quantitation would have been possible.

The linearity was assayed with six calibration curves which were prepared as described in "Sample extraction". Each curve contained a blank sample (PBS + methanol), a zero sample (PBS + methanol + IS), and ten differently concentrated non-zero samples (for concentrations, see ESM, Table S1). The calibration curves were constructed by plotting the ratio of the analyte peak area/internal standard peak area against the nominal concentration followed by linear regression with the weighting factor of 1/x.

For assaying intraday and interday precision and accuracy, five samples corresponding to the LLOQ, medium and high concentrations, were prepared in sets of three replicates and analyzed at three consecutive days. For everyday and concentration, the intraday precision was calculated as the relative standard deviation (RSD) of the five measurements. The interday precision was determined according to Krouwer as total imprecision [45] and, for better comparability to RSD, divided by the mean value (MV) of all 15 results to calculate the "relative total imprecision" s_{Trel}. The relative error term (RE) was used to express accuracy. It was calculated according to the formula [(calculated concentration−nominal concentration)/nominal concentration]. The interday accuracy was calculated as the mean value of the three intraday values. Precision and accuracy were expressed in percentage and had to be less than 15 %, except for LLOQ values (≤20 %).

The same three different concentrations were used to assay recovery with five replicates each concentration. For calculating recovery, the amount of the analyte extracted from spiked PBS samples was divided by the concentration found in blank PBS samples spiked with the same standard concentration after the extraction. In both cases, the internal standard was spiked after the extraction.

The stability of the samples was checked under various conditions. Autosampler stability was investigated by determining the concentrations of the analytes before and after storing the extracted and reconstituted samples at 7 °C in the autosampler for 72 h. These conditions were tested in samples extracted from real matrix as well (see "Matrix effects and standard addition approach"). For freeze/thaw stability, samples were frozen at −40 °C immediately after preparation. Then, they were thawed and frozen again three times and the extraction was done. Short-term stability was investigated by spiking standard solutions into three PBS samples each and leaving them at room temperature for 4 h. For long-term stability, the samples were frozen immediately after preparation and stored at −40 °C for 120 days. After all described storage conditions, the samples were extracted and the (d)NTP concentrations were measured against a freshly prepared calibration curve. In all cases, stability was defined as the RE of the calculated value after the specific storing condition and the concentration at point zero to be less than 15 %.

Matrix effects and standard addition approach

Nucleosides and nucleotides are present in all kind of biological matrices, where very high concentrations of NTP can be found. For this reason, a calibration with standard samples extracted from spiked matrix is not possible and PBS was used as an analyte-free surrogate matrix. In some other publications, the validation is carried through with stable isotopes of the analytes [26].

For proofing suitability of PBS as a surrogate matrix, we used a standard addition approach for the T cell matrix and the macrophages. Furthermore, the stability of spiked matrix samples in the autosampler was assessed. For the human CD4 T cells, 2×10^7 cells were pooled and suspended in 140 μL PBS and 14 aliquots were prepared, each containing about 1.4×10^6 cells. Besides, 14 aliquots with 100,000 cells each were prepared from a pool of 1.4×10^6 macrophages. For both matrices, 11 aliquots were used to prepare a calibration curve for the standard addition approach, containing a blank sample, a zero sample spiked with internal standard, and nine non-zero samples spiked with internal standard and differently concentrated calibration standards. In both cases, the zero samples were included for constructing calibration curves, setting the nominal analyte concentration to zero. The slopes of the curves were compared to those of a calibration curve extracted from PBS to assess potential matrix effects on the analyte response.

The remaining three aliquots were used as quality control samples. They were spiked with internal standard and a low

concentrated calibration standard solution. These calibration standards increased the concentration extracted from the cell samples by 50 ng/mL in the injection solution for ATP and UTP, 10 ng/mL for GTP and CTP, 2 ng/mL for dCTP and dTTP, and 1 ng/mL for dATP and dGTP. These samples were used to assay precision and the autosampler stability for 72 h for samples extracted from real matrix.

Application to biological samples

The method was proven to be suitable for several cell classes by determining the concentrations of (d)NTP and nucleosides in human CD4 T cells and macrophages. The T cell samples contained two million cells each. For the macrophages, one million cells were used for the quantitation of the nucleosides and the dNTP, while only 25,000 cells were used for NTP quantitation. The analyzed concentrations were compared for activated versus resting T cells and for activated versus resting M1 and M2 macrophages.

Results

Method optimization

Several approaches were made to include all analytes in one LC-MS/MS method, but none was successful. An indispensable target in our experiments was the complete absence of ion pair additives in the mobile phases due to disadvantages already mentioned in the introduction. Unfortunately, the (d)NTP were not retained on the Atlantis column which was found to give the best results for the nucleosides. Different solvents and gradient conditions were tried out, as well as other columns suitable for polar analytes, but retention was not achieved. Conversely, the anion exchange column did not work for the separation of the nucleosides as they are not ionic in a weakly acidic solution. A combined method would have been desirable, but applying the dual approach presented in this work, both classes can be readily quantified from one sample just by dividing the supernatant after the protein precipitation.

Under the described conditions, the (d)NTP elute in three groups. A total baseline separation was not achieved, although different gradient programs and pH values of the mobile phases were tried out. However, due to different precursor-to-product ion transitions, a clear differentiation for a correct quantitation is easily possible. Furthermore, a stable isotope as internal standard is available for every analyte and can compensate the potential interferences.

For the nucleosides, we found that the postcolumn addition of isopropanol after the chromatographic separation greatly improved both precision and repeatability. Without isopropanol, the ionspray was unstable due to high water content resulting in big variability of the results when the same sample was injected several times. Furthermore, we found that acetic acid in the mobile phase led to higher sensitivity than formic acid.

(d)NTP method validation

Lower limit of quantitation

We determined LLOQ values of 10 pg on column (o.c.) (0.5 ng/mL in the injection solution) for dATP and dGTP, 20 pg o.c. (1 ng/mL) for dCTP and dTTP, 100 pg o.c. (5 ng/mL) for CTP and GTP, and 500 pg o.c. (25 ng/mL) for ATP und UTP respectively. For the NTP, these values could be set lower if necessary. The validated values gave an S/N ratio higher than 70 for CTP and UTP, 100 for GTP, and 700 for ATP. In this case, the range of the calibration curve was chosen on the basis of the expected concentrations. In contrast, the dNTP concentrations were set as low as possible. Figure 1 shows chromatograms of the LLOQ concentrations (A, B). The peak of U is broader than those of the other nucleosides, and the peaks of T, dA, and dATP show small shoulders. These peak shapes could not further be optimized without affecting the peak shapes of the other analytes or impairing the separation. Nevertheless, all LLOQ values were confirmed by determining precision and accuracy (see "Precision and accuracy").

Linearity and calibration range

The calibration ranges were chosen on the basis of the expected concentrations in various cell types which were estimated according to test samples. All analytes showed good linearity with regression coefficients better than 0.999 (see ESM, Table S2). In case of ATP and UTP, higher ULOQ was desirable, but not possible, due to column overload and signal saturation.

Recovery

All recovery values were close to 100 %, demonstrating the suitability of the sample extraction procedure. Table 2 gives an overview over the collected data.

Precision and accuracy

All analytes showed very good precision and accuracy values. The results for the interday precision (relative total imprecision) were always below 7.5 %, while all accuracy values were better than 9 %. An overview of these results is given in Table 2. The same was found for the intraday data (data not shown).
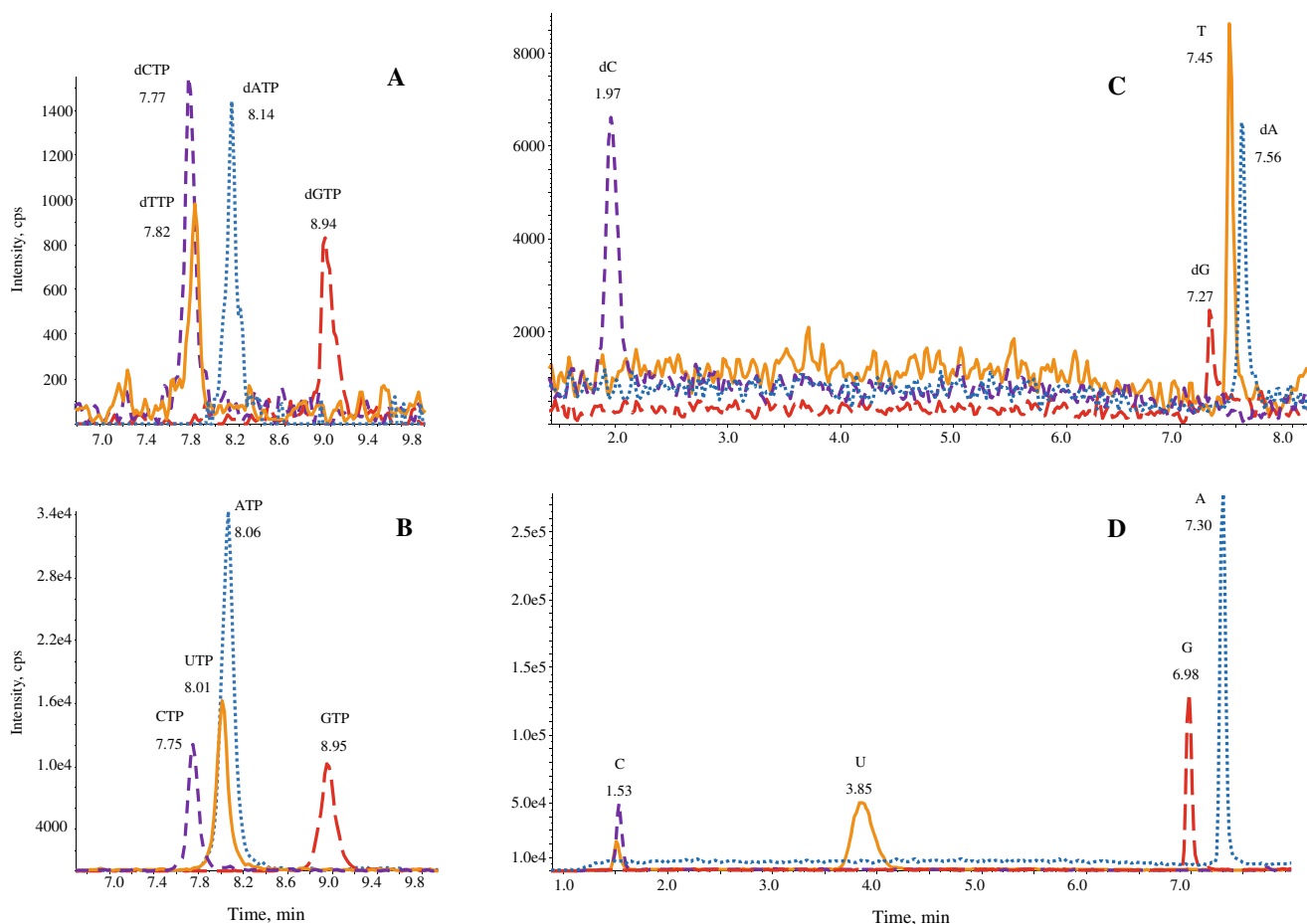
Fig. 1 Chromatograms of the lowest concentrated calibration standard of the ribonucleosides (**A**, **B**) and the deoxynucleosides (**C**, **D**) extracted from spiked PBS samples. The concentrations of the (d)NTP are 0.5 ng/mL in the injection solution for dATP and dGTP, 1 ng/mL for dCTP and dTTP, 5 ng/mL for CTP and GTP, and 25 ng/mL for ATP und UTP respectively. The concentrations of the nucleosides were 0.1 ng/mL in the injection solution for dA, dG, and dC; 0.5 ng/mL for C, G, and T; and 5 ng/mL for A and G

Stability

The samples were stable under all conditions tested. After 4 h at room temperature, the RE was always lower than 9 %. The stability for extracted samples in the injection solution at 7 °C in the autosampler was demonstrated for 72 h with RE not higher than 8 % except for dATP (RE=7.46±5.42 %). This was confirmed for the analytes extracted from CD4 T cells and macrophages (see "Matrix effects"). Furthermore, the samples are possible to be frozen and thawed for at least three times. The results for the long-term stability showed that samples can be stored at −40 °C for 120 days. Detailed results are given in Table 3.

Matrix effects

The calibration curves extracted from PBS, macrophages, and CD4 T cells showed similar slopes (see ESM, Table S3). As a consequence, we can conclude that the slope of the calibration curve is not influenced by substances present in the matrix and that PBS can be used as a surrogate matrix.

The calculated concentrations of the quality control samples showed good precision, and all analytes extracted from the matrices were stable at 7 °C in the autosampler for 72 h. For the NTP extracted from the macrophages, the found concentrations were above the calibration curve. Nevertheless, the calculated results could be replicated after 72 h, and we were able to confirm that the stability behavior of the analytes is independent of their concentration. Additionally, all analytes extracted from CD4 T cells were in the range of the calibration curve and stability was proven. Table 4 gives an overview of the collected data.

Nucleosides

For the deoxynucleosides, the concentrations with an S/N higher than ten were chosen as lowest concentrations of the calibration curve. Analogously to the NTP, the ribonucleosides are present in much higher concentrations in biological

Table 2 Interday accuracy and precision values of the analytes for the three studied concentrations measured at three different days ($n=5$) and relative recovery values obtained for the analytes in PBS samples at three different concentrations ($n=5$)

(d)NTP	Concentration [ng/mL]	Interday accuracy	Interday precision		Relative recovery
		RE (MV±SD) [%]	MV [ng/mL]	s_T/MV (RSD total) [%]	(MV±SD) [%]
ATP	25	4.5±2.1	24.3	3.4	98.74±5.22
	125	3.4±2.0	128.1	3.5	101.89±4.47
	800	2.4±0.7	814.1	2.4	98.86±2.93
dATP	0.5	4.1±1.6	0.5	3.7	97.41±8.94
	2.5	4.6±1.0	2.6	5.7	102.73±2.23
	16	2.8±1.2	16.4	2.6	98.32±1.82
GTP	5	6.2±2.5	5.2	3.7	99.65±3.73
	25	2.9±1.4	25.3	4.0	102.48±2.22
	160	2.0±0.3	161.7	2.0	99.63±3.69
dGTP	0.5	6.6±1.4	0.5	7.5	97.76±9.43
	2.5	3.6±1.6	2.5	5.0	102.33±4.70
	16	3.9±1.2	16.4	4.1	101.63±2.31
CTP	5	6.2±1.3	5.1	4.4	100.67±7.82
	25	2.7±1.2	25.3	4.1	100.40±3.30
	160	2.3±0.7	163.6	2.0	97.57±2.00
dCTP	1	4.0±1.7	1.0	4.4	96.01±5.13
	5	5.2±1.1	5.1	6.3	101.86±2.43
	32	3.1±1.8	32.8	3.0	98.43±4.10
UTP	25	6.0±2.1	25.3	5.1	97.56±4.51
	125	4.8±2.2	126.7	6.0	102.23±6.11
	800	3.1±2.0	818.9	3.5	98.18±2.54
dTTP	1	4.5±2.3	1.0	4.7	95.25±11.86
	5	3.4±0.4	5.0	4.4	103.16±6.19
	32	3.4±2.6	33.2	4.5	97.81±3.12

MV mean value, *RE* relative error, *RSD* relative standard deviation, s_T total imprecision

samples. Consequently, the range of the calibration curve was adjusted to the expected concentrations although a more sensitive quantitation would in principle have been possible. Figure 1 shows chromatograms of the lowest concentrated standard of the calibration curve (C, D).

Application to biological samples

Activated and resting CD4 T lymphocytes

All analytes could be detected and quantified in CD4 T cells. Some exemplary chromatograms of the analytes extracted from one sample of activated cells can be found in the ESM, Fig. S2.

In Fig. 2, a box plot illustrates the concentration ranges of the analytes found in resting and in activated CD4 T cells. It is clearly visible that the concentration of (d)NTP is increased in activated CD4 T cells in line with previous studies using different methodology [6, 7]. Notably, the levels of dC, T, and ribonucleosides were also increased, while no changes could be observed for dA and dG.

Macrophages

In all samples, the analytes could be quantified. For the determination of the NTP, the extraction had to be repeated with less cells because, in the first approach, the detected concentrations were all clearly above the ULOQ. As expected, the concentrations of the NTP and the ribonucleosides were much higher than those of the deoxy derivatives, ranging from about factor 10 for the ratio G/dG to about factor 1000 for A/dA. Consistent differences for either the differently treated groups of macrophages or MΦ1 and MΦ2 were not observed. The analyte concentrations are listed in the ESM, Table S4.

Discussion

The developed analytical procedure provides the possibility to determine both nucleosides and (d)NTP from the same sample using a simple, fast, and inexpensive extraction procedure and two LC-MS/MS methods. Although the simultaneous

Table 3 Stability of spiked PBS samples under specific storing conditions for given time intervals ($n=3$)

(d)NTP	Concentration [ng/mL]	Short-term stability (20 °C, 4 h) RE (mean ± SD) [%]	Long-term stability (-40 °C, 120 days) RE (mean±SD) [%]	3 freeze/thaw cycles (-40 °C/20 °C) RE (mean±SD) [%]	Autosampler stability (4 °C, 72 h) RE (mean±SD) [%]
ATP	25	–	–	2.4±0.6	2.2±0.3
	50	2.9±1.7	3.6±2.4	–	–
	125	–	–	5.3±2.0	2.7±2.1
	150	3.3±1.9	7.2±2.3	–	–
	800	–	–	2.3±1.5	1.4±0.3
dATP	0.5	–	–	2.5±1.1	7.5±5.4
	1	5.4±3.0	3.3±2.5	–	–
	2.5	–	–	2.9±0.8	3.0±1.2
	3	3.1±0.5	4.6±3.1	–	–
	16	–	–	0.2±0.0	2.5±0.7
GTP	5	–	–	9.7±3.4	2.0±2.0
	10	4.4±3.6	5.5±6.1	–	–
	25	–	–	3.2±1.3	1.1±1.0
	30	2.6±1.2	4.7±3.0	–	–
	160	–	–	2.5±2.2	1.3±0.2
dGTP	0.5	–	–	7.8±2.2	4.2±3.0
	1	5.8±3.0	4.3±2.9	–	–
	2.5	–	–	2.2±1.0	1.5±1.1
	3	0.7±1.0	3.0±2.4	–	–
	16	–	–	3.6±0.6	4.8±2.9
CTP	5	–	–	1.3±0.2	3.4±3.8
	10	3.8±3.6	4.2±3.4	–	–
	25	–	–	0.5±0.2	1.1±1.1
	30	1.4±0.2	3.3±2.9	–	–
	160	–	–	0.8±0.3	2.00±2.1
dCTP	1	–	–	7.3±4.5	8.2±4.4
	2	3.3±1.8	4.2±3.5	–	–
	5	–	–	1.8±1.2	2.4±2.1
	6	5.1±0.6	2.4±2.2	–	–
	32	–	–	2.9±1.2	4.3±2.4
UTP	25	–	–	5.8±7.0	6.8±3.3
	50	4.9±3.3	2.7±2.5	–	–
	125	–	–	3.8±0.5	3.3±2.1
	150	5.4±1.7	1.4±1.9	–	–
	800	–	–	2.3±0.7	2.4±2.5
dTTP	1	–	–	6.0±5.8	5.1±6.4
	2	7.1±3.2	6.1±6.1	–	–
	5	–	–	4.5±1.9	3.0±1.5
	6	0.3±0.2	14.1±0.8	–	–
	32	–	–	1.4±1.3	1.5±0.5

quantitation of both compound families would be desirable, it seems to be not possible due to the high diverging in polarity of both groups and, thus, their chromatographic behavior if no ion pair reagents are used (tests concerning this issue were carried out but did not lead to satisfying results). These substances accelerate column deterioration and contaminate the ion source. Several approaches have been made to reduce the use of ion pair reagents to a minimum: adding them only to the reconstitution solution [25, 26] or including a column washing step to improve the robustness of the analytical method and

Table 4 Stability of spiked macrophage and T cell samples ($n=3$); results were calculated using a calibration curve extracted from PBS

(d)NTP	Matrix	Spiked concentration [ng/mL]	Mean value [ng/mL]	Precision RSD [%]	Autosampler stability (4 °C, 72 h) RE (mean±SD) [%]
ATP	CD4 T cells	50.0	639.7	1.2	1.8±1.5
	Macrophages	50.0	2590>ULOQ	6.0	3.6±2.5
dATP	CD4 T cells	1.0	1.6	7.0	4.2±3.5
	Macrophages	1.0	1.8	1.3	4.7±4.4
GTP	CD4 T cells	10.0	32.4	2.5	2.4±2.4
	Macrophages	10.0	259.3>ULOQ	2.9	1.4±1.4
dGTP	CD4 T cells	1.0	1.2	11.0	4.9±2.4
	Macrophages	1.0	1.5	10.6	8.1±4.5
CTP	CD4 T cells	10.0	30.2	4.9	2.7±1.9
	Macrophages	10.0	439.7>ULOQ	7.2	2.9±2.4
dCTP	CD4 T cells	2.0	2.6	10.1	6.5±3.0
	Macrophages	2.0	4.1	3.7	10.7±3.0
UTP	CD4 T cells	50.0	118.3	9.0	6.5±4.1
	Macrophages	50.0	1863>ULOQ	5.1	7.0±6.0
dTTP	CD4 T cells	2.0	2.6	6.2	6.4±5.3
	Macrophages	2.0	3.3	10.0	10.8±4.1

column lifetime [25]. However, even when a minimum of ion pair reagent is used, it is unavoidable that traces contaminate the mass spectrometer and interfere with the analyses of other substances that are carried out on the same instrument. Therefore, we avoided completely the use of ion pair reagents during the determination of nucleosides and (d)NTP.

The developed method for the quantitation of the endogenous (d)NTP provides highest sensitivity and has been validated for several primary human cell types. The simultaneous quantitation of NTP and dNTP is challenging because of their highly different concentrations in the samples [5]. Especially for the dNTP quantitation, high sensitivity is required due to
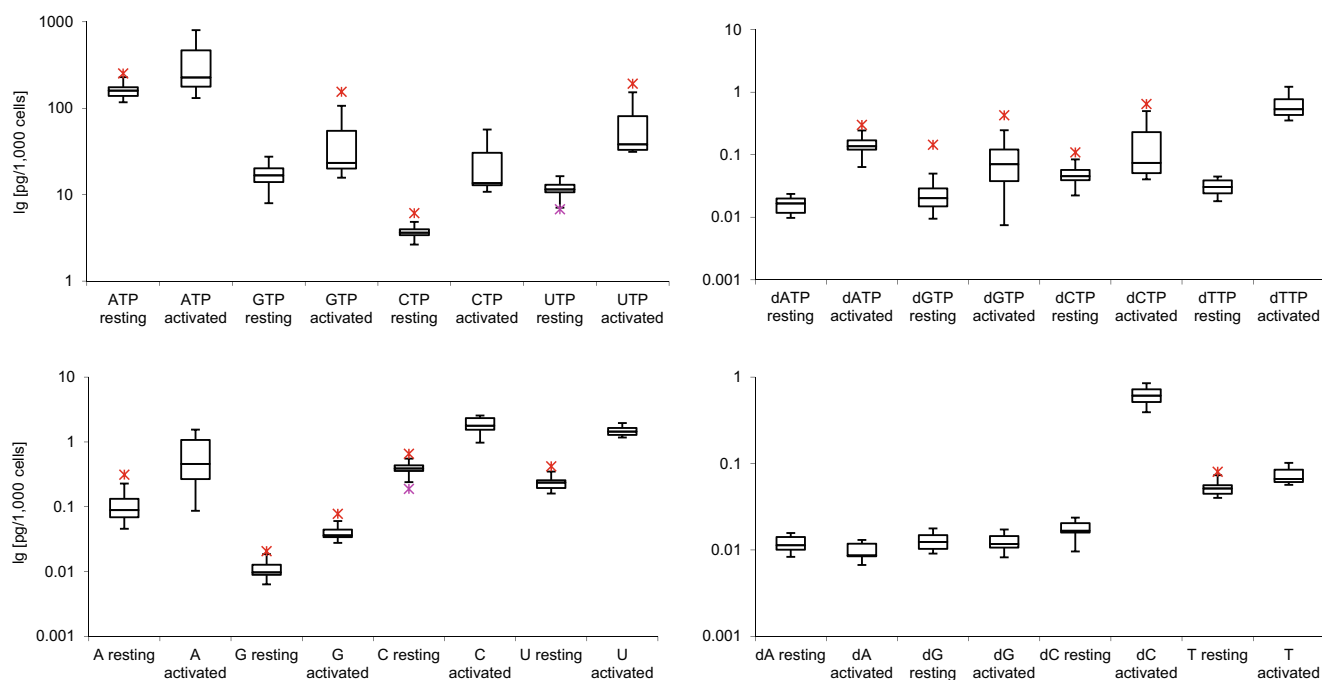

Fig. 2 Box plots of (d)NTP and nucleoside concentrations determined in 12 samples of resting CD4 T cells compared to 9 samples from activated CD4 T cells. The *top* and the *bottom* of the box represent the first and the third quartile while the median is shown as the band inside the box. The *ends of the whiskers* are set at 1.5*interquartile range (IQR) above the third quartile and 1.5*IQR below the first quartile maximum but end at the last data point included in this range. Minimum or maximum values outside this range are shown as outliers. Logarithmic scale on the *y-axis*

low endogenous concentrations. Therefore, the sample size must be adequate for being able to detect and quantify dNTP but must not be too large as then the concentrations of the NTP, especially ATP and UTP could be out of the calibration range. Many published methods for the quantitation of endogenous nucleotides have reported only the quantitation of NTP, but not of dNTP [19, 22, 23, 36]. The developed method achieves LLOQ values for the dNTP of 10 pg o.c. (0.5 ng/mL in the injection solution) for dATP and dGTP and 20 pg o.c. (1 ng/mL) for dCTP and dTTP respectively. To our knowledge, this is the most sensitive method for the simultaneous determination of (d)NTP described to date. Previously published studies reported LLOQ values for these four analytes in the range of 120–600 pg o.c. [17], 150–200 pg o.c. [18], 150–600 pg o.c. [26], 400–5000 pg o.c. [2], and 1250 pg o.c. [16] respectively. This method has been fully validated according to the FDA guidelines and successfully applied to biological samples, demonstrating changes in nucleotide pools of different primary human cell types.

As it can be seen for the determination of the analytes in T cells, there is a difference between activated and resting cells for all the measured compounds, except for deoxyadenosine and deoxyguanosine. The latter finding is not surprising as the purine deoxynucleotides in CD4 T cells are mainly synthesized by ribonucleotide reduction. As a consequence, the concentrations of deoxyadenosine and deoxyguanosine are expected to be very low and, importantly, independent from the cell cycle state [46].

The nucleoside method is the first LC-MS/MS method reported including all endogenous nucleosides occurring in DNA and RNA. It provides information about the cell cycle state in addition to the (d)NTP concentrations.

Collectively, the presented methods allow fast, easy, and reliable quantitation of the endogenous nucleosides and (d)NTP and, thus, the building blocks of tumor cell and pathogen replication. This, combined with the possibility to add nucleoside and nucleotide analogues to the analysis, is the basis for future studies of cytostatic and antiviral drugs.

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