# We are IntechOpen,
# the world's leading publisher of
# Open Access books
# Built by scientists, for scientists

**6,900**
Open access books available

**185,000**
International authors and editors

**200M**
Downloads

**154**
Countries delivered to

Our authors are among the

**TOP 1%**
most cited scientists

**12.2%**
Contributors from top 500 universities

CLARIVATE ANALYTICS
**BOOK CITATION INDEX**
INDEXED

**WEB OF SCIENCE**™

Selection of our books indexed in the Book Citation Index
in Web of Science™ Core Collection (BKCI)

## Interested in publishing with us?
## Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com

# Mass Spectrometry for the Sensitive Analysis of Intracellular Nucleotides and Analogues

Kateřina Mičová, David Friedecký and Tomáš Adam

Additional information is available at the end of the chapter

## Abstract

Nowadays, mass spectrometry is very important and widely applied tool in nucleotide analysis. As a result of technological advances in sample preparation, separation and mass spectrometry detection, the developed methods allow sensitive and selective measurement of polar compounds occurring in low levels in various biological matrices. This enables more potential uses in clinical field. Direct methods require no special sample pre-treatment before analysis in contrast to indirect methods, where fractionation, dephosphorylation and purification are needed. The use of ion-pairing agent, ion exchange chromatography with pH gradient, porous graphitic carbon columns and HILIC in liquid chromatography represents the most common methods of nucleotide analysis. High separation efficiency is also achieved with the use of CE with MS detection. Analysis of nucleotides was also described by the means of MALDI-TOF, but poor reproducibility and lack of applications make a limitation for this approach. The chapter summarizes different techniques and approaches for determination of endogenous nucleotides and its analogues in various clinical applications.

**Keywords:** nucleotides, nucleotide analogues, mass spectrometry, capillary electrophoresis, liquid chromatography, MALDI-TOF, HILIC-MS/MS, triple quadrupole, selected reaction monitoring

## 1. Introduction

Nucleotides and nucleosides, together with their deoxidized forms, are involved in several important cellular processes. The structure of the nucleoside molecule consists of a heterocyclic base (adenine, guanine, cytosine, uracil and thymine) joined to a molecule of pentose (ribose

or deoxyribose) through a glycosidic bond. Nucleotides are formed by phosphorylation of nucleoside molecule. They represent a fundamental building block of the structure of nucleic acids formed by the polymerase-mediated synthesis of DNA and RNA from deoxynucleotides and nucleotides, respectively. All deoxynucleotides are synthesized from the corresponding nucleotides. Nucleotides play another important role in enzyme activation and metabolism. They are incorporated into important cofactors of enzymatic reactions such as transfer of various groups by coenzyme A (e.g. acetyl-CoA and succinyl-CoA) in many metabolic processes, NAD, NADP and FAD as coenzymes of oxidoreductases catalyses redox reactions in the cell. ATP and other nucleoside triphosphates are engaged as source energy providers. In the form of coenzymes, they transfer phosphate residues or nucleotide constituent and participate in the activation (e.g. uridine diphosphate glucose for pasting glucose units to polymeric saccharides or cytidine diphosphate-choline for phospholipid synthesis). Cyclic nucleotides act as secondary messengers in many signal transduction pathways. The most important nucleotide signal molecule is cyclic adenosine monophosphate (cAMP), which is an activator of protein kinases and participates in many metabolic pathways by transferring the effect of hormones into cells. Analogical molecule—cyclic guanosine monophosphate (cGMP)—also acts as a secondary messenger in the phototransduction process.

Nucleotide metabolism is one of the main therapeutic cellular targets. Synthetic analogues of these compounds have been widely applied in anti-cancer, anti-viral and immunosuppressive therapies [1–6]. Nucleoside analogues (e.g. cytarabine, gemcitabine and zidovudine) are modified at the base or sugar moiety. This 'prodrug' form requires intracellular activation by phosphorylation to mono, di or triphosphates. Related drugs are nucleobase analogues (6-mercaptopurine and thioguanine) without sugar-phosphate moiety and also fluoropyrimidines (fluorouracil, capecitabine and floxuridine). Its mechanism of action is based on the structural similarity to its natural endogenous precursor. Therefore, the analogues are called 'anti-metabolites' [4, 7].

Over 30 inherited disorders resulting from errors of purine and pyrimidine metabolism are known, and therefore the determination of the levels of nucleosides and nucleotides is used in their diagnosis and treatment monitoring. Disorders of purine and pyrimidine metabolism are clinically very diverse—manifestations include immunodeficiencies, urolithiases, mental defects and others. Adenosine deaminase deficiency, resulting in deoxyadenosine accumulation; purine nucleoside phosphorylase deficiency, characterized by elevated deoxyguanosine; hyperuricemia; hypoxanthine-guanine phosphoribosyl transferase deficiency, leading to high levels of uric acid, and phosphoribosylpyrophosphate synthetase overactivity, resulting in the overproduction of purine and uric acid. The most common pyrimidine metabolism disorder is orotic aciduria, where the defect of UMP-synthase causes the disease and leads to the excessive excretion of orotic acid in the urine [8].

Similarly, the determination of nucleotides and their intermediates is an important tool for understanding the metabolism of endogenous nucleotides and their synthetic analogues and also for the establishment of a diagnosis of disorders related to defects in the biosynthesis and degradation processes of purine/pyrimidine nucleotides [9]. The concentration of particular deoxy/nucleotides differs among various cell types and extracellular material

(plasma, urine—very low concentrations are physiologically present). Intracellular nucleotide analogue levels can also vary from patient to patient, which can result in under-treatment or toxicities [10]. In consequence of the often very low intracellular levels of nucleotide analogues in comparison to their natural variants, highly sensitive and selective analytical methods are necessary for the quantification [9–11].

Sample preparation is the major critical step in the determination of endogenous nucleotides and their analogues in biological material. The pre-analytical phase, including sampling, transport of material and sample preparation, plays an important role in the whole analytical process. The treatment of the sample is based on the material used for analysis, such as whole blood [12], erythrocytes [13–15], mononuclear cells [14, 16–24], cultured cells [25–35], plasma [12, 19, 20, 36, 37], urine [38], dry blood spots [15] and others [36, 39–47]. The enzymes involved in purine and pyrimidine metabolism can fundamentally change the nucleotide pool during the pre-analytical process and this should be stopped as soon as possible.

To date, many and various approaches using different analytical techniques have been developed for the determination of nucleotides and their analogues (in this chapter globally called nucleotides). Historically, the ATP concentration was measured by means of the luciferin-luciferase luminescence assay technique [48]. Radioimmunoassays were quite sensitive but had poor specificity as a result of cross-reactivity with structurally similar molecules [49, 50]. For separation of nucleotides, thin layer chromatography [51], capillary electrophoresis (CE) [52–56] and liquid chromatography [9, 12–15, 18, 19, 29, 30, 32, 36, 40, 42, 45, 46, 57–62] have been used. Because of the requirement of the simultaneous determination of several nucleotides and low-level deoxynucleotides occurring in complex matrices, separation techniques coupled predominantly to UV/DAD or mass spectrometry (MS) detection have become essential tool for analysis. Although UV detection has been widely applied for the detection of nucleotides in biological matrix [63–72], it suffers from very low sensitivity and specificity [9]. Sensitivity for adenosine nucleotides was significantly improved by fluorescence detection. However, analysis by means of this approach is time-consuming as a result of required derivatization step [73].

The first application of mass spectrometry to the analysis of amino acids and peptides was reported in 1958 [74]. With the advent of clinically useful mass spectrometers, this technology prevails for nucleotide detection in biological matrices. Because of the increasing requirements of clinical laboratories to analyse multiple analytes in a broad concentration range simultaneously, the high sensitivity and specificity of the technique and its growing price availability, it has become a prominent tool in most clinical research and routine practice.

Capillary electrophoresis (CE) can be used for the separation of mainly ionic and highly polar compounds with high resolution, which makes this technique suitable for nucleotide analysis. In combination with mass spectrometry, it has been used for nucleotide analysis in several applications [10, 52, 53, 55, 56, 75]. However, the most crucial problem in the coupling of CE to MS detection is the limited range of available and convenient interface techniques. Moreover, the electrophoresis run buffer has to be compatible with MS detection. Because of the practicality and wide possibilities of adjustment, presently liquid chromatography in

combination with mass spectrometry is the most frequently used separation technique for nucleotide determination in many biological materials.

Liquid chromatography has become an essential tool for the analysis of nucleotide in recent decades. The polarity of the nucleotides increases with the number of phosphate groups. Reversed-phase (RP) liquid chromatography is frequently used for the separation of nucleosides and also nucleoside monophosphates [14, 15, 22, 28, 30, 33, 36, 37, 58]. Because of their hydrophilicity, triphosphate nucleosides have very poor retention when separated on traditional reverse-phase high-performance liquid chromatography columns, and thus another chromatographic approach is required. The possibility is using ion-pairing [12, 16, 19–21, 23, 26, 31, 32, 41, 47] or anion exchange chromatography [13, 17, 18, 27, 42]. These approaches enhance the retention on RP columns, but they are, however, compromised by the high levels of additives in mobile phases (ammonium salts and alkylamines), which cause significant ion suppression, poor robustness and instability of the ion exchange columns, and also source pollution, which remains a major problem [10, 16, 60, 76]. The analysis of nucleotides on RP-HPLC is also possible by indirect methods based mainly on dephosphorylation using uric acid or alkaline phosphatase and subsequent analysis of particular nucleosides, the retention of which is significantly better on reverse-phase analytical columns compared to their triphosphate precursors [14, 15, 22, 33, 77, 78]. The indirect methods using fractionation and dephosphorylation simplify the final determination of significantly better ionizable nucleosides, but the overall complexity and time required for the use of the method rises and many errors could be introduced into the sample pre-treatment. Another possible way to measure nucleosides and their monophosphates (MP), diphosphates (DP) and triphosphates (TP) by liquid chromatography under MS-friendly conditions is using hydrophilic interaction liquid chromatography. Polar columns using mobile phase rich in organic solvent with increasing amount of water content during analytical run are suitable for coupling with MS [34, 39, 40, 43, 79].

In the last decade, many reviews on the analysis of endogenous nucleotides and low-level nucleotide analogues in biological matrices by various separation techniques but especially with liquid chromatography [9] and detection by mass spectrometry [10, 11] have been published. This chapter is focused on describing several approaches to the determination of endogenous nucleotides and low-level nucleotide analogues in biological matrices by means of various separation techniques in combination with mass spectrometry detection. In practical part, we are showing analysis of low-level of nucleotide analogues 5'-ethynyl-2'-deoxycytidine and 5'-ethynyl-2'-deoxyuridine, their mono-, di-, and triphosphates in the presence of the high levels of the physiological nucleotides by LC-MS/MS method.

## 2. Sample preparation

The sample preparation is probably the most critical step in separation methods. The mostly used mass spectrometers need a clean sample without salts and lipids causing ion suppression, signal response reduction and poor robustness of analytical methods. Gradual pollution

of the ion source and other parts of the instrument causes poor analytical quality and harms the long-term stability of the instrument. The pre-treatment of the sample and extraction of the nucleotides depend on the matrix sample types, such as peripheral blood mononuclear cells (PBMCs), cultured cells, tissue, plasma and so on, and also on the separation and detection mode. All matrices contain many active enzymes included in the biochemical pathways responsible for the conversions or transformations of nucleotides. Thus, cell lysis and inactivation of certain enzymes is the first required step. The extraction solution should therefore completely extract nucleotides and precipitate and inactivate proteins without compromising the stability of these analytes. The nucleotide structure contains phosphate groups and therefore the compounds have ionic character. It must be taken into account that during the preparation of the sample the organic agents used for each step of the sample preparation have enough polarity to prevent precipitation of the nucleotides. It is different for mono-, di-, and triphosphates, with triphosphates especially being sensitive to the choice of less polar solvents. In general, the sample preparation is initiated by the protein precipitation and extraction of the nucleotides, eventually purified from phospholipids by solid phase extraction (SPE) or liquid-liquid extraction (LLE), pre-concentrated by evaporating under the nitrogen flow or freeze drying and reconstituting in predominantly water solution.

Protein precipitation (PP) is usually performed with a strong acid such as perchloric acid (PCA) [17, 25, 36, 41, 62, 80] or trichloroacetic acid (TCA) [42, 75] and most often by organic solvents such as methanol (MetOH) [12, 18, 26–28, 39, 43, 55, 60, 81, 82] or ethanol (EtOH) [19] or acetonitrile (AcCN) [37]. Because of the instability of nucleotides at a low pH, a neutralization step after the PP with strong acid is required. Neutralization was performed using NaOH with a subsequent centrifugation step [80]. The PCA extracts were treated with KOH and the resulting $KClO_4$ precipitate was removed by centrifugation. The supernatant was directly stored at −20°C [25] or lyophilized [41] prior to the LC-MS analysis. The PP and extraction of the cyclic nucleotides by PCA was introduced in a study by Oeckl et al. and it was demonstrated on plasma and brain tissues. The materials were homogenized with 0.4 M PCA using sonication, and after centrifugation, the supernatant was filtered through cellulose membrane and stored at −80°C until analysis [36, 62].

PP with TCA is less frequently described because of its poor compatibility with MS detection; nevertheless Friedecký et al. used TCA for the deproteination of incubated cells followed by back-extraction into ether. This procedure provided an increased pH value and better stability of the nucleotides. Water phase extracts were used for the CE-MS analyses [75].

At present, precipitation by organic solvent is predominantly used. Derissen et al. described the usage of pure MetOH for the lysis of PBMC, and after mixing and centrifugation, the sample extract was stored at −70°C until analysis [18]. MetOH was used for the PP of the plasma samples designed for the determination of the nucleotides [12]. Soga et al. also used MetOH for cell lysis, but this procedure was followed by LLE based on the addition of chloroform and water to remove the phospholipids. After centrifugation, the resulting water-MetOH layer was lyophilized and dissolved in pure water before CE-ESI-MS analysis [55]. In a general metabolomic study covering 164 compounds including several nucleotides, 80% MetOH at the temperature of 75°C was used for three consecutive metabolite extractions [43]. Cell lysis,

PP and nucleotide extraction were also performed with ice-cold 70% MetOH [27, 39] followed by storage at −20°C [28, 82] or snap-frozen in liquid nitrogen [29]. A similar procedure can be performed with a 60% MetOH solution followed by incubation at −20°C for complete extraction and PP [26, 81] or frozen in liquid $N_2$ [60]. In a study of rat heart tissue, the extraction of the homogenate was performed with pre-cooled MetOH-water (1:1, v/v) and followed by centrifugation, and the supernatant was directly analysed [45]. Cyclic intracellular nucleotides were extracted by a mixture of AcCN/MetOH/water (2:2:1, v/v). The extract was heated to 95°C, cooled down, centrifuged, dried under flow of nitrogen and resuspended in water for analysis [30].

Several studies compared extraction procedures. Klawitter et al. compared four extraction agents [12% PCA, 70% AcCN, 70% MetOH and MetOH/chloroform (1:1, v/v)] applied to homogenized kidney tissue. The extracted homogenates were centrifuged, and the supernatant was collected, pellet was re-suspended in water and centrifuged and the supernatants were combined. In case of the PCA extraction, an additional neutralizing step (2 M KOH followed by 1 M $KHCO_3$) was performed. The resulting $KClO_4$ precipitate was removed and extracts were consequently lyophilized. The PCA extraction provided the best absolute recovery and best reproducibility among the four extraction procedures used in this study. The recovery varied from 75.6 to 89.1%, depending on the type of nucleotide [41]. Cordell et al. compared extraction solvents such as AcCN, ethanol (EtOH), MetOH, AcCN:water (8:2, v/v), EtOH:water (8:2, v/v), MetOH:water (8:2, v/v), 0.1 M formic acid, 0.5 M PCA and 0.1 M formic acid in MetOH using Chinese hamster ovary (CHO) cells. The authors also studied the effect of sonication on ice prior to the removal of the extraction fluid as well as the hexane addition in the case of methanol extraction. Methanol cooled to −20°C offered the most efficient extraction, with an average recovery of 53%. The addition of a hexane extraction step improves the sample clean-up and peak shapes and removes many problems attributed to the phospholipids [31]. Derissen et al. tested four extraction agents: MetOH, EtOH, AcCN and 1.35 M PCA at different volume ratios. Finally, 100 μL of methanol added to the 60 μL of the PBMC suspension, followed by extensive vortex mixing and direct centrifugation according to the extraction procedure 1 provided the most favourable results [18].

Sample pre-treatment by PP seems to be a suitable method for removing proteins and the extraction of nucleotides from biological samples. Since the concentration of some nucleotide analogues is often very low, and further clean-up procedures (ultrafiltration) and pre-concentration [solid phase extraction (SPE), LLE] are needed. Cordell et al. showed that phospholipids can be removed from the sample by LLE by hexane [31]. A similar procedure was reported by Soga et al. using chloroform and water being added to a methanol extract of *Escherichia coli* culture to remove phospholipids liberated from the cell membranes [55]. An additional sample clean-up was used in the study of Crauste et al., where cell extracts were purified by a weak anion exchange (WAX) SPE column after PP by MetOH. The mean extraction recoveries were between 60 and 81%, depending on the analyte. The effect of ion suppression on the peaks before and after SPE was also compared and the results indicated that SPE extraction allows the matrix effect to be reduced [81]. An additional sample clean-up with WAX-SPE after PP was introduced in several studies [39, 42, 83]. Comparison of extraction efficiency in protein-precipitated samples with and without consequent WAX SPE clean-up

procedure was described by Kamčeva et al. Extraction recovery for the procedure without SPE was achieved in the range 59.2–91.9% and with SPE in the range from 94.2 to 121.8%. The lower recovery for the first method can be explained by higher ion suppression; however, the usage of isotope-labelled triphosphates (ATP, GTP, UTP, dATP, dCTP, dGTP, dTTP) as internal standards simplified the nucleotide extraction step. The analytes and their isotopically labelled standards are affected equally by the cell matrix and the interference originating from the cell matrix influencing the recovery is reduced. Thus, additional steps for sample clean-up such as time-consuming and expensive SPE were unnecessary [60]. Cell lysis, protein precipitation and nucleotides extraction can be also carried out by MetOH in combination with Tris-HCl buffer [16, 20]. Another cell sample treatment was introduced by Teleki et al. Biomasses of *E. coli* were quenched by liquid nitrogen, diluted to the defined extraction concentration, immediately pre-incubated at 100°C with short-time vortexing in a water bath for enzymatic inactivation and then chilled on ice water. The metabolite extracts were separated from the cell debris by centrifugation and stored at −70°C [40]. The analysis of the nucleotide content in red blood cells (RBC) is described in a study by Hofmann et al. RBC mixed with EDTA, dithiothreitol and IS were heated at 95°C in a water bath for protein denaturation. The samples were subsequently extracted by MetOH followed with dichloromethane. After centrifugation, the supernatant was used for LC-MS/MS analysis [13].

Another procedure for sample clean-up was reported. In a study of Klawitter et al. comparing four extraction procedures (see above), additional online desalting was applied. The final extracts were loaded onto a C18 cartridge desalting column and washed with dibutylammonium formate buffer/methanol (95:5). Thereafter, the valve was switched back onto the analytical column. This switching procedure led to better reproducibility of the HPLC retention times and significantly improved the stability of the electrospray (ESI) as a result of the lower amounts of salt precipitated in the spray chamber [41]. In a study by Wu et al., supernatant resulted from centrifugation after PP was additionally cleaned by ultrafiltration with a molecular weight cut-off of 3000 Da [32]. According to Becher et al., Tris-HCl/MetOH cell lysates were treated by immunoaffinity extraction [21].

Further study used ice-cold MetOH and subsequent cell lysis by fast heating or using of sonic dismembrator for the islets of Langerhans and *E. coli* cells in MALDI-TOF analysis. The samples were mixed with matrix (9-aminoacridine) and deposited onto a MALDI target plate using the dried-droplet method [44]. In a study by van Kampen of AZT-triphosphate and nucleotide triphosphates in PBMCs by the means of MALDI-TOF technique, the pre-treatment of the sample was also based on ice-cold MetOH extraction followed by overnight incubation at 4°C, centrifugation, freeze drying and reconstitution. Before analysis, the sample was pipetted onto an Anchor Chip target plate, onto which the matrix had previously been applied and dried [84].

For indirect methods, where parental nucleoside resulting from the dephosphorylation of nucleotides is detected, the preparation of the sample is based on PP and the extraction of nucleotides from the matrix and subsequent fractionation of the sample to MP, DP and TP, usually by means of a salt gradient applied to an anion exchange SPE cartridge. Particular fractions are exposed to dephosphorylation on parent nucleosides, mostly by means of an

acid or alkaline phosphatase, eventually desalted and pre-concentrated by additional SPE. Cells or other matrices were precipitated by the means of 70% aqueous MetOH and the extract was separated into MP, DP and TP fractions using strong anion exchange cartridge with a potassium chloride gradient. The resulted fractions were treated by acid [14, 22, 33, 59] or alkaline [15] phosphatase for dephosphorylation to parental nucleoside. After hydrolysis, samples were desalted and purified by SPE and analysed. Preparation of the sample for indirect analysis is time and material consuming. Even if the final nucleoside analysis is quite simple, each sample fraction must be analysed separately. Each step in an indirect method could be the cause of a definite error resulting in inaccurate determination of the nucleotides.

In summary, sample preparation is the most important and critical step in the analysis of nucleotides. The proper extraction and clean-up procedures depend on various matrices and separation and detection techniques and should be chosen carefully.

## 3. Capillary electrophoresis with mass spectrometry detection in analysis of nucleotides

Capillary electrophoresis (CE) is an analytical technique enabling the separation of compounds in a fused silica capillary using electrolyte solution (separation buffer) under an electrical field application. Ionized molecules migrate through an electrolyte solution (separation buffer) under an electrical field application. In CE, cations migrate towards the cathode, whereas anions move in the opposite direction. Molecules migrate to the detector at different velocities that are based on their electrophoretic mobilities. This depends on their charge and size, the pH buffer, ionic strength, buffer composition and viscosity. The next factor that significantly affects the electrophoretic migration and separation of analytes is the electro-osmotic flow (EOF). The silica capillary surface is negatively charged under the alkaline conditions resulting from the ionization of silanol groups. The EOF can be modified, inversed or deleted by covalent or dynamic capillary wall modifications using surfactants or neutral or ionized polymers.

Numerous modes in CE enable the separation of a wide spectrum of compounds and thanks to the different separation mechanisms that can provide complementary information. The most widely used is capillary zone electrophoresis (CZE), where the mobility of an analyte is a vector sum of its electrophoretic mobility and the electro-osmotic mobility of the buffer. Micellar electrokinetic chromatography (MEKC) can be used for the separation of charged as well as neutral less polar compounds. The running buffer is enriched by surfactants forming micelles that can interact with neutral less polar compounds in a chromatographic manner through both hydrophobic and electrostatic interactions. CE can also be used with a capillary filled with different phases, resulting in different separation modes. A capillary filled with gel buffer enables the separation of molecules according to their size (capillary gel electrophoresis, CGE) and is used mainly for macromolecules such as protein and DNA analysis. CE with a chromatographic stationary phase capillary filling, mixture of ampholytes creating a pH gradient in capillary and several running buffers with different conductivities is called

capillary electrochromatography (CEC), capillary isoelectric focusing (CIEF) and capillary isotachophoresis (ITP), respectively.

In the last three decades, CE represented the main technique for the direct determination of nucleotides. However, with the development of new stationary phases compatible with highly polar analytes, liquid chromatography plays a more important role in the analysis of nucleotides. Despite this fact, CE represents a valuable alternative to chromatographic techniques for nucleotide analysis. Separation is fast, with very high efficiency. Low solvent and sample consumption and low running costs represent other significant advantages in the application of the technique, especially in combination with mass spectrometry detection.

Several methods using CE-MS have been developed for the determination of nucleotide analogues in clinical applications. Cai et al. introduced a method for the simultaneous analysis of the nucleoside reverse transcriptase inhibitor (NRTI) Ziagen (Abacavir) and its phosphorylated metabolites such as carbovir monophosphate, carbovir diphosphate and carbovir triphosphate that is used for the treatment of human immunodeficiency virus (HIV) type 1. This method enables the separation and detection of positively charged nucleoside analogues and negatively charged nucleotides in a single electrophoretic run thanks to the application of a time-segment program. The linearity of the method was established in the range 2–100 μM and the limits of detection for all the analytes were less than 2 μM. The capability of the method was demonstrated on human PBMC extracts spiked with Ziagen and its phosphorylated metabolites at 20 μM levels. Some endogenous nucleotides, such as adenosine 5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP) and adenosine 5′-monophosphate (AMP), were also detected in the cellular extracts [53].

Liu et al. used CE-ESI-MS for the determination of 14 compounds, including antiretroviral dideoxynucleosides (ddN), their nucleotides and a set of endogenous ribonucleosides and ribonucleotides. Separation by means of CE was performed on an untreated column using a volatile background electrolyte for ESI-MS. All the analytes were profiled within single 18 min CZE run. The method was applied to detect natural nucleotides, lamivudine and its mono-, di-, and triphosphates in the human hepatoma cell line Hep G2 [56].

The conversion of 5-amino-4-imidazolecarboxamide riboside (ZR) into mono-, di-, and triphosphates in human erythrocytes was demonstrated in a study by Friedecký et al. The erythrocytes were incubated with ZR in order to simulate the situation in the inherited metabolic disorder of the purine metabolism AICA-ribosiduria. Characteristic AICA-ribotides, together with the naturally occurring nucleotides AMP, ADP and ATP, were separated by means of capillary electrophoresis in an acetate buffer (20 mmol L⁻¹, pH 4.4) and identified online by mass spectrometry in negative mode [75].

Bezy et al. introduced the CE-ESI-MS/MS method for the simultaneous measurement of nucleoside 5′-triphosphate and 5′-monophosphate anabolites of the anti-HIV drugs, didanosine (ddATP, ddAMP) and stavudine (d4T-MP, d4T-TP), among a pool of 14 endogenous 5′-mono-, di- and triphosphate nucleosides in extracted PBMC. The running electrolyte consisted of an acetic acid/ammonia buffer with pH = 10 and ionic strength of 40 mM. Finally, the quantification of d4T-TP and ddATP was validated in this CE-MS/MS system [52].

The simultaneous analysis of naturally occurring nucleotides, nicotinamide-adenine dinucleotides and CoA compounds was introduced by Soga et al. using a pressure-assisted CE-MS. The method allowed simultaneous analysis of various compounds and offered sufficient sensitivity and selectivity, reproducibility, robustness and linearity. The required reproducibility of the system was achieved by exchanging the running electrolyte after every analysis using a buffer replenishment system. Under optimized conditions, 14 phosphorylated compounds were well determined in less than 20 min with detection limits between 0.5 and 1.7 µM. The usefulness of the method is demonstrated through the analysis of intracellular nucleotides and CoA compounds extracted from *E. coli* wild-type and pfkA and pfkB knockout mutants [55].

The combination of CE-enabling rapid analysis with efficient resolution and MS with excellent selectivity and sensitivity makes a very powerful technique for nucleotide analysis and a good alternative to the conventionally used LC-MS technique. Nevertheless, the development of the method for the separation of the nucleotides by means of CE coupled with MS detection still represents a big challenge. The hyphenation of CE with MS is technically demanding. Moreover, the commonly used electrophoresis running buffers are not compatible with MS detection as a result of the high concentration of electrolytes, which may create high background signals in ESI-MS analyses.

# 4. Liquid chromatography-mass spectrometry for the determination of nucleotides

Liquid chromatography (LC) is an analytical technique enabling very efficient and sensitive separation of compounds in complex sample matrices. The principle of the technique is based on the interactions and distribution of sample components between the stationary and mobile phase. The strength of the interaction determines the order of the elution of particular analytes. Because of the requirement for better efficiency of separation and shorter analysis time, high-performance liquid chromatography (HPLC) was investigated. The application of high pressure with a faster flow rate and separation columns with particles smaller than 2 µm offers highly efficient compound separation with a shorter analysis time in comparison to common column (low-pressure) chromatography. Currently, most applications for the analysis of nucleotides are based on liquid chromatography coupled with tandem mass spectrometry, and therefore descriptions of approaches involving these represent a major part of this chapter.

The majority of polar analytes are separated using reversed-phase high-performance liquid chromatography (RP-HPLC), where the stationary phase is usually non-polar [silica gel coated by octadecyl- (C18), octyl- (C8) or cyanogroup (CN)] and the mobile phase is composed of polar organic solvents (methanol or acetonitrile) in combination with a water buffer. The retention of compounds depends on hydrophobic interactions with the stationary phase. Using gradient elution, the polarity of the mobile phase is declined with a greater amount of organic solvent, resulting in the faster elution of non-polar analytes from the column. The

polarity of nucleotides increases with the number of phosphate groups. In contrast to nucleoside monophosphates (MP), nucleoside triphosphates (TP) and nucleoside diphosphates (DP) are therefore only minimally retained and are separated from other nucleotides on the commonly used reversed-phase HPLC columns under traditional conditions with a typical aqueous-organic mobile phase. In addition, many interferences from the matrix are poorly retained and the specificity and selectivity of the method is reduced even more. Introducing an ion-pairing (IP) reagent or anion exchange (AX) chromatography enabled the usage of RP-HPLC columns for the analysis of nucleotides. Although IP and AX chromatography are the most suitable methods for nucleotide analysis, high concentrations of a non-volatile ion-pairing reagent and/or a high salt concentration preclude the use of mass spectrometry for detection. Therefore, the type and concentration of the ion-pairing agent must be optimized. Moreover, the strong retention of ion-pairing agents inside the mass spectrometer makes it impossible to use instrumentation for other methods because of the interference of the IP agents with the compounds being analysed.

## 4.1. Reversed-phase liquid chromatography

As mentioned above, RP-HPLC-MS does not enable the analysis of nucleoside di- and triphosphates because of the low retention on the column. Therefore, RP-HPLC in a common setting is acceptable for the determination of nucleotides by indirect methods. The methods are usually based on the separation of the sample extract into MP, DP and TP fractions and subsequent dephosphorylation with alkaline or acid phosphatases on nucleoside forms. Consequently, nucleosides can be determined in a common RP-HPLC-MS setting.

Rodriguez et al. developed an indirect method for the quantification of the intracellular NRTIs zidovudine (ZDV) and lamivudine (3TC) triphosphates in patients with HIV infection. Extracted peripheral blood mononuclear cells (PBMCs) were separated into MP, DP and TP fractions using strong anion-exchange Sep-Pak plus (SAX-QMA) cartridges with a potassium chloride gradient. The cleavage of the phosphate groups was performed by acid phosphatase, the samples were desalted and the parental nucleosides ZDV, 3TC and AZdU (azidodeoxyuridine; internal standard added to the extract after enzyme digestion) were extracted by means of a solid phase extraction (SPE) XAD resin column. HPLC separation was performed on an RP column with a mobile phase consisting of methanol and acetonitrile mixture (3:1, v/v) with 0.25% acetic acid. The concentrations of the analytes were determined by using ZDV/AZdU and 3TC/AZdU peak area ratios, and calibration curves from ZDV-TP and 3TC-TP standard solution were prepared every time a series of samples was analysed [22].

The next indirect method was developed for the analysis of intracellular dideoxyadenosine triphosphate (ddATP). Cells were treated with 70% MetOH buffered to pH 7.4 and the samples were incubated on ice for 15 min and stored at −20°C. Before analysis the cell extract was evaporated to dryness and reconstituted in Tris (1 M, pH 7.4). The samples were loaded onto a QMA anion exchange SPE cartridge, MP and DP were eluted by 75 mM KCl, and finally ddATP was eluted with 500 mM KCl. The pH of the eluate was adjusted to 6.0 and it was incubated with acid phosphatase for 30 min at 37°C for dephosphorylation to ddA (dideoxyadenosine). The samples were desalted and purified using C18 SPE cartridges. The ddA

eluted by AcCN was evaporated to dryness and stored at −20°C until analysis. The overall recovery of QMA and C18 SPE was over 95%. The separation was performed on a Purospher-RP-18e column with a mobile phase consisting of MetOH/water (25/75, v/v) containing 1% formic acid. The overall analysis was achieved within 1 min. The LOQ of ddA in the CEM-T4 cells that were analysed was 0.02 ng mL$^{-1}$. This procedure could be used to perform simultaneous detection of five NRTIs, such as AZT, 3TC, ddA, ddC, and d4T [33].

An indirect method for the determination of the nucleoside analogues MP, DP and TP in an intracellular matrix of red blood cells (RBC) and PBMCs was described by Bushman et al. Cell extracts were loaded onto QMA SPE cartridges and MP, DP and TP fractions were eluted by means of a KCl concentration gradient. The isolated fractions were then treated with acid phosphatase for dephosphorylation to parental nucleoside and the samples were desalted and concentrated with a Strata X-SPE column, and the eluent was dried under nitrogen at 40°C and reconstituted in water. Separation was achieved on a Synergi Polar RP column. The phase for tenofovir (TFV) contained 2% AcCN and 0.1% formic acid in pure water with a run time of 8 min, and for zidovudine (ZDV) it contained 6% 2-propanol and 0.1% acetic acid in pure water with a run time of 12 min, both at an isocratic flow. The method was successfully applied to clinical research generating novel intracellular information for TFV, emtricitabine (FTC), ZDV and 3TC nucleotides [14].

Another indirect method for the determination of adenosine, guanosine and inosine nucleotides was introduced by Jimmerson et al. The analyses were performed on human PBMCs, red blood cells (RBCs) and dry blood spots (DBSs). Methanolic extracts of lysed cells and DBSs were fractionated to MP, DP and TP using an anion exchange cartridge and a potassium chloride salt gradient. The resulting separated fractions were dephosphorylated to the parental nucleosides adenosine, guanosine, and inosine by alkaline phosphatase. Subsequently, the samples were desalted and concentrated on phenylboronic (PBA) SPE cartridges. Analytical separation was performed on an RP column with an aqueous mobile phase containing 4% AcCN and 0.1% formic acid. The detection of nucleosides was performed in positive ESI MRM mode [15]. This group of authors previously described a similar method for the determination of intracellular ribavirin (RBV) MP, DP and TP, in which the particular fractions were isolated from a lysed intracellular matrix using strong anion exchange SPE, dephosphorylated to parent RBV, desalted and concentrated on PBA SPE cartridges and subsequently analysed. Chromatography was performed on a Reversed-Phase-Aqueous column (Develosil C30) with a mobile phase consisting of 2% AcCN and 0.1% formic acid in water at an isocratic flow and with a run time of 4 min. Each injection was followed by strong and weak needle washing. The method was used on human PBMCs and the RBC and DBS of patients taking RBV for the treatment of chronic hepatitis C virus infection [59].

Wang et al. described a method for the determination of creatine phosphate, creatine and 12 nucleotides in rat heart tissue. The extract was separated on a polar endcapped C18 column that was suitable for reversed-phase separations using highly aqueous mobile phases. The mobile phase consisted of 2 mM ammonium acetate in water adjusted with ammonia to pH 10.0 and isocratic elution was applied. The detection was performed by negative electrospray ionization in selection reaction monitoring mode. The study was, however, focused only on group of nucleotides of which the concentration in the tissue is relatively high [45].

The determination of 3′-deoxy-3′-fluorothymidine (FLT) and FLT-MP was used for the monitoring of serum thymidine kinase 1 (TK 1) activity. Separation was achieved on an Aquasil C18 column coupled with a security guard column (Gemini C18) using 0.1% formic acid in water (MF A) and 0.1% formic acid in AcCN (MF B). Elution was carried out under gradient conditions with a total analysis time of 7 min. The applicability of the method for measuring serum TK 1 activity was demonstrated in hepatocellular carcinoma patient serum samples and age-matched control sera [58].

Several methods for the determination of cyclic nucleotides with separation on RP-HPLC columns have been developed. Oeckl et al. described a method for the analysis of cAMP and cGMP in plasma, CSF and the brain tissue of mice. For separation a Varian MetaSil RP column with a mobile phase consisting of 0.1% formic acid in water (A) and acetonitrile (B) was used. The analysis time per sample was 3.5 min [36]. The method was subsequently applied in measuring cAMP and cGMP concentrations in CSF in patients with Creutzfeldt-Jakob disease, Parkinson's disease and amyotrophic lateral sclerosis. It demonstrated that the CSF determination of cAMP and cGMP may be included in the diagnosis of CJD and could be helpful in monitoring the progress of the disease as well as in therapy control [62]. The same separation conditions were used for the determination of cGMP in human plasma in a study by Zhang et al. [37]. In a method for the measurement of 2′,3′-cAMP, 2′,3′-cCMP, 2′,3′-cGMP and 2′,3′-cUMP, separation on a Zorbax eclipse XDB-C18 column connected to a security guard column with MetOH/water (3:97, v/v) as MF A and MetOH/water (97:3, v/v), each containing 5 mM ammonium acetate and 0.1% acetic acid, was achieved. The analysis was applied to the analysis of nucleotides in two different cell lines, Hek293T and HuT-78 [30].

Another use of reversed-phase analytical columns for the determination of nucleotides is possible with the addition of an ion-pairing reagent into the mobile phase [31, 41, 82]. This is described in detail in the chapter 'Ion-pairing chromatography'.

### 4.2. Ion exchange chromatography

Ion exchange chromatography (IXC) is a separation technique applied to the analysis of charged molecules, including, e.g. ions, polar nucleotides and amino acids, on the basis of their affinity to the ion exchanger. For positively charged analytes, cation exchange chromatography with negatively charged solid support is used and vice versa; negatively charged analytes are attracted to a positively charged solid support in anion exchange chromatography (AX). The elution of bounded negatively charged molecules is performed with an eluent containing anions in a higher concentration or by changing the pH of the column. The elution of strongly charged nucleotides requires a high concentration of competitive ions in the mobile phase and, thus, hyphenation with MS detection cannot be employed.

However, an alternative elution mechanism introduced by Shi et al. based on the application of a pH gradient enabled the direct quantification of intracellular nucleoside triphosphates (NTPs) without any need for high concentrations of non-volatile competing ions for elution. Extracts of human PBMCs were analysed by weak anion exchange (WAX) liquid chromatography coupled to mass spectrometry detection. An inverse ammonium acetate gradient (10–1 mM) enabled separation between MPs, DPs and TPs within 2 min. The method was

validated and used for the determination of the NRTI dexelvucitabine triphosphate metabolite in human PBMC samples from clinical studies [85].

Veltkamp et al. developed a sensitive and specific assay for the quantification of gemcitabine triphosphate (dFdCTP) in human PBMCs. It is based on a WAX LC-MS/MS approach, which makes the method more sensitive [17] than previously published work based on UV detection [70].

Jansen et al. developed and validated a method for the quantitative analysis of cladribine mono-, di- and triphosphates that was applied to a culture medium and MDCKII cell lysate. WAX LC-MS/MS in positive ion mode was applied via a fused-silica electrospray capillary instead of a stainless steel electrospray capillary to minimize the adsorption of analytes and, thus, reduce the variation in the analyte signals [27].

A simultaneous quantification method for 11 relevant nucleotide metabolites of thiopurine drugs (eleven mono-, di- and triphosphates of thioguanosine, methylthioinosine, methylthioguanosine and thioinosine) in RBCs was introduced by Hofmann et al. Ion exchange HPLC on a WAX column using a pH gradient from 6 to 10.5 and a decreasing ammonium acetate concentration from 10 to 1 mM was used for the separation of nucleoside mono-, di- and triphosphates. All eleven metabolites could be determined in RBCs from patients with inflammatory bowel diseases and long-term azathioprine therapy indicating high inter-individual variability of the metabolite levels. Using stable isotope-labelled analogues of the metabolites enabled the reproducible and accurate determination of all the analytes [13].

Anion exchange chromatography was also used for the determination of 2′-C-methylguanosine triphosphate concentration in mouse liver in a study by Rashidzadeh et al. The samples were eluted onto a Luna $NH_2$ chromatographic column with 1 mM ammonium acetate in water/AcCN (70:30, v/v), pH 8.0 (MP A) and 20 mM ammonium acetate in water/AcCN (70:30, v/v), pH 10. The total run time was 10 min per sample. The assay was linear over a 50–10,000 pmol mL$^{-1}$ concentration range in liver homogenate [42].

In a paper by Derissen et al., the development of an LC-MS/MS assay for the quantification of the widely used chemotherapeutic capecitabine with the active component 5-fluorouracile (FU) and its active metabolites FUTP, FdUTP and FdUMP was described. Because of the low concentrations of the analytes, the optimization of the sample preparation, including cell lysis and nucleotide extraction and subsequent chromatographic separation, was necessary. The samples were loaded onto a BioBasic AX WAX column coupled with a guard column; the mobile phase A and B composition was 10 mM ammonium acetate with pH 6.0 and 1 mM ammonium acetate with pH 10.5, respectively, both in AcCN/water (30:70, v/v). The pH gradient was applied to achieve effective separation of the MP, DP and TP of the nucleoside. The total analysis time was 7 min. The method was validated for the concentration ranges 0.488–19.9, 1.66–67.7 and 0.748–30.7 nM for FUTP, FdUTP and FdUMP, respectively. The assay was successfully applied to quantify 5-FU nucleotides in PBMC samples from patients treated with capecitabine and patients receiving 5-FU intravenously [18]. The method was also used for the quantification of the 5-FU nucleotides in PBMCs in a study exploring the intracellular pharmacokinetics of the 5-FU nucleotides during capecitabine treatment [57].

The WAX principle can also be used for sample preparation by SPE with Wax extraction cartridges followed by separation on RP or PGC columns [81, 83]. This was described previously in the chapter 'Sample preparation'.

### 4.3. Porous graphitic carbon columns

Another way to achieve the required separation of nucleosides is the use of porous graphitic carbon columns (PGC) because of their unique properties as a stationary phase. Their retention and selectivity for polar and structurally related compounds is different from conventional C18 columns. PGC is very suitable for the analysis of polar and ionic compounds, which are retained on the carbon surface without a need for an IP reagent, and the subsequent elution is not dependent on a high salt concentration. This enables the development of methods compatible with MS detection. PCG columns have the advantage of physical and chemical stability accompanied by toleration of a wide pH range (0–14). Retention increases with increasing analyte hydrophobicity [86]. However, PGC behaves as a strongly retentive alkyl-bonded silica gel for non-polar analytes. Thus, the disadvantage is the difficulty of desorption of non-polar compounds adsorbed on the surface and a general loss of retention on PGC within a run and over a period of time, which can limit their use [87].

A general LC-MS method for the analysis of nucleosides and their mono-, di- and triphosphates using a PGC column was developed by Xing et al. The method was optimized using different organic mobile phases and modifiers. The concentration of ammonium acetate was proved to be a critical step for retention during gradient elution with water/acetonitrile. Diethylamine (DEA) was found to improve the peak shapes of di- and triphosphates for mass spectrometric detection. Finally, the separation of 16 nucleosides and nucleotides was achieved during 15 min under the conditions of a gradient of ACN in water with 50 mM ammonium acetate and 0.1% DEA. In the study, comparison of several silica-based columns for separation of polar compounds was also performed. Hypercarb column provided the best results for separation and quantitation of the nucleotides and their phosphates [88].

Wang et al. described an LC-MS/MS method for the determination of AMP, ADP and ATP in the extract of HepG-2 cells. Chromatographic separation was performed on a PGC analytical column with a basic mobile phase. Negative-ion mode ESI-MS with basic mobile phase condition improved the sensitivity of the MS analysis. The method was successfully applied to determine ATP, ADP and AMP in HepG-2 cells treated with benzo[a]pyrene [25]. This method served as a model for a study focused on the quantification of gemcitabine (2′-2′-difluorodeoxycytidine, dFdC) and its metabolites 2′-2′-difluorodeoxyuridine (dFdU) and 2′-2′-difluorodeoxycytidine-5′ (dFdCTP) in pancreatic ductal adenocarcinoma tumour tissue and plasma from genetically engineered mouse models of pancreatic cancer. The tumour tissue was homogenized in ice-cold 50% AcCN containing tetrahydrouridine. An aliquot of homogenate was mixed with ice-cold 50% AcCN containing IS. After vortex mixing and centrifugation the supernatant was evaporated to dryness and reconstituted in water before analysis. With respect to plasma, it proceeded in the same way as the tumour homogenate by the addition of ice-cold 85% AcCN containing IS. The analytes were separated on a PGC Hypercarb column fitted with a guard column with 10 mM ammonium acetate, pH 10 (MF A) and acetonitrile

(MF B); the total run time was 15 min. In order to minimize carry-over between injections, the needle and injection path were flushed using the external wash procedure with water, 100% AcCN, 50% AcCN and water [46].

Jansen et al. also introduced a method for the separation of 2′-2′-difluorodeoxycytidine (gemcitabine, dFdC), 2′-2′-difluorodeoxyuridine (dFdU) and their mono-, di- and triphosphates using a porous graphitic carbon Hypercarb column. The separation of all the analytes was achieved using an ammonium bicarbonate gradient (0–25 mM) in acetonitrile/water. The rate of pH and redox state of the column had to be controlled in order to maintain the separation conditions within multiple runs [89]. Subsequently, this method was validated for quantification in PBMCs and successfully applied to clinical samples [90].

Another method utilizing PGC-HPLC column separation with MS detection was developed for the quantification of cytarabine triphosphate (araCTP), CTP and dCTP in a human follicular lymphoma cell line. The separation principle is based on an LC-MS/MS ion pair using a PGC analytical column. The mobile phase consisted of 5 mM hexylamine with 0.4% dimethylhexyl amine with a pH of 10 and an ACN/water mixture. The mass spectrometer operated in negative ESI and multiple reaction monitoring mode. The method was able to achieve a low limit of quantification (LLOQ) of 0.1 $\mu g\ mL^{-1}$ for araCTP and of 0.01 $\mu g\ mL^{-1}$ for both CTP and dCTP. The method was validated and used for the quantification of araCTP, CTP and dCTP formed after the incubation of araC and an araCMP prodrug in the human follicular lymphoma cell line RL [81].

The development and validation of an assay for the quantification of endogenous nucleoside MP and TP by LC-MS/MS in combination with online SPE extraction was introduced by Machon et al. An Oasis®WAX SPE column served for online extraction, whereas PGC Hypercarb® was used as an analytical separation column. The elution solvents were 0.25% $NH_4OH$ adjusted to pH 10 with acetic acid (A), water (B) and acetonitrile (C). The IP reagent was used for the reconstitution of the evaporated samples (5 mM hexylamine—0.5% DEA in water) to prevent peak tailing of nucleoside triphosphates. The total duration of a single run was 37 min. The method also allowed the separation and the detection of other nucleoside MP, DP and TP, deoxyribonucleotides and ribonucleotides. The advantage of the method was that it was based on online SPE, which was less time-consuming. After validation, the method was applied to the evaluation of the effects of gemcitabine and hydroxyurea on nucleotide pools in Messa cells [29].

Kamčeva et al. also introduced a combination of ion-pairing chromatography with PGC column separation. The development of a sensitive LC-MS/MS method for the separation and quantification of eight endogenous nucleotides (ATP, CTP, GTP, UTP, dATP, dCTP, dGTP and dTTP) and 2′, 2′-difluoro-2′-deoxycytidine triphosphate (dFdCTP) in PBMCs was described. The analytes were extracted by simple protein precipitation and chromatographic separation was performed on a Hypercarb column with delivery of a mobile phase containing 5 mM HA, 0.5% DEA, acetonitrile and water in gradient elution adjustment. The total analysis time was 68 min. The method was applied to monitor dFdCTP and changes in endogenous nucleotides in patients who were receiving gemcitabine infusions [60].

Nowadays, methods using PGC column separation represent a good alternative for nucleotide analysis compatible with MS detection in a robust and reproducible manner without the need for an ion-pairing agent or a high salt concentration. The optimization of mobile phase composition and an appropriate elution program facilitating consistent analyte retention remove the need for column regeneration, minimize run times and thus allow PGC columns to be used to their full potential [88].

### 4.4. Ion-pairing chromatography

Separation by ion-pairing (IP) chromatography (IPC) is based on the interaction between the negatively charged nucleotides and the positively charged IP reagent. The stationary phase is usually the commonly used non-polar C18 or C8 phase. The mobile phase contains cationic reagents such as alkylamines. The previously used tetrabutylammonium salts were effective for the separation of nucleotides by HPLC in combination with UV detection. However, non-volatile tetrabutylammonium salts were incompatible with electrospray-mass spectrometry detection. Thus, a more volatile salt is one of the possible ways to overcome this problem. Triethylamines, tributylamines, dibutylammonium salts, dimethyhexylamine or hexylamine are commonly used. The properties of the mobile phase are optimized for the efficient desorption of analytes using organic modifiers such as methanol or acetonitrile and also by the pH gradient. The concentration of the ion-pairing reagent plays a crucial role in LC separation coupled with MS detection. The amount has to be optimized for successful separation and for minimizing the mass spectrometry ion source contamination. Two of the solutions for reducing the electrospray ion source pollution are to minimize the flow rate of the mobile phase into the MS system or to use trialkylamine with a longer chain, promoting interactions between the IP reagent and a hydrophobic stationary phase, resulting in a reduction in the amount of the IP agent required. Another way is column miniaturization, which provides better chromatographic resolution and sensitivity of the LC-MS system [9].

The first study introducing IP capillary HPLC-MS described the separation of cyclic nucleotides on a C18 reversed-phase column using tetrabutylammonium (TBA) bromide as the IP agent. Different mobile phase compositions were evaluated in the concentration range of 50–500 µM of TBA salt with a combination of low flow rates (5–10 µL min$^{-1}$) in combination with negative electrospray ionization. Despite the relative non-volatility of TBA salt, the system was able to operate for several days without a reduced signal caused by source pollution being observed. Optimal conditions offered linear detection response in the femtomole to picomole range [91].

Claire et al. introduced the IP-HPLC-MS method for the determination of intracellular emtricitabine triphosphate (FTC-TP) levels in human PBMCs that was also adaptable to all purine- and pyrimidine-based nucleotides. All the nucleotides were extracted by means of aqueous methanol, isolated by IP-SPE and then directly analysed by LC-MS/MS. The mobile phase contained 10 mM ammonium phosphate, pH 6.4, with 2 mM tetrabutylammonium hydroxide and 15% acetonitrile. The method was linear in the range from 0.08 to 80 picomoles on-column. The low amount of TBA, thin column with a 1.0-mm internal diameter, slow flow

rate of 50 µL min⁻¹ and continuous flow of 20% methanol through the MS ion source enabled effective separation and detection stability [92]. Hawkins et al. applied the method for the simultaneous determination of the intracellular concentrations and pharmacokinetics of tenofovir diphosphate, carbovir triphosphate and lamivudine triphosphate in samples of patients on a triple-nucleoside regimen [93].

The IP-RP-LC-MS/MS method was developed for the quantitative detection of adefovir and its phosphorylated metabolites in cellular samples by Vela et al. Hep G2 cells were incubated with 10 µM adefovir for 24 h and then extracted using 70% methanol. Chromatographic separation was performed on microbore reversed-phase columns and a mobile phase containing TBA and ammonium phosphate was delivered to the MS system with a low flow of acetonitrile gradient. Further optimization of the method lowered the concentration of TBA and phosphate, reduced the pH and applied a linear gradient of acetonitrile. Thus, the method was found to have sufficient sensitivity, accuracy and precision to be broadly applicable. But despite all the improvements and optimization of the method, using TBA in the injection solvent caused significant ion suppression and phosphate clusters, and their ammoniated adducts also created a number of high-intensity interfering peaks [28]. Finally, TBA in combination with phosphates causes mass spectrometer ion source pollution, ion suppression and background interference that impose some limitations on the determination of nucleotides using IP-LC-MS/MS. Regardless of this, methods using TBA with a limited ion-pair concentration and maintaining low flow rates have been successfully used in many applications.

Trialkylamines are more suitable for MS detection because of their higher volatility than tetraalkylammonium salts. Apffel et al. developed a method for the analysis of oligonucleotides by RP-HPLC-ESI-MS. The mobile phase contained a novel additive, hexafluoropropanol (HFIP), adjusted to pH 7.0 with triethylamine (TEA). This combination resulted in good HPLC separation and efficient negative electrospray ionization. Application was demonstrated for synthetic homopolymers of thymidine, mapping plasmid sequence fragments and phosphorothioate ester antisense oligonucleotides with sensitivity below the 10 pmol level [94].

The IP LC-MS/MS method for the simultaneous determination of intracellular nucleoside triphosphates and other polar metabolites using a TEA-HFIP IP mobile phase was described by Wu et al. Compared to the less volatile ion-pair reagent triethylammonium acetate (100 mM, pH 7.0), the combination of 100 mM HFIP and 8.6 mM TEA increased the MS signal intensity about 50-fold, while retaining comparable chromatographic resolution. A gradient elution program with a total analysis time of 10 min was developed. The method was optimized and validated for the simultaneous and unambiguous determination of eight nucleoside triphosphates (including ATP, CTP, GTP, UTP, dATP, dCTP, dGTP and dTTP) and applied to samples of human lung cancer cell lines. The method can be used for the quantitative profiling of 74 polar metabolites with minor modifications and a prolonged mobile phase gradient [32].

Another widely utilized IP agent is N,N- or 1,5-dimethylhexylamine (DMHA). DMHA served as an ion-pairing agent for the identification and quantification of bisphosphonates, synthetic analogues of pyrophosphate that are used in the treatment of metabolic bone diseases. They can be metabolized intracellularly into non-hydrolyzable nucleotide analogues. The

separation was performed on a C18 reversed-phase LC column with MS detection in negative ESI mode [95, 96].

Tuytten et al. demonstrated the influence of different N,N-DMHA concentrations on the chromatographic and mass spectrometric performance. They developed the IP-LC-MS method using a short capillary column for the separation of 12 nucleotides eluted by a binary gradient of methanol/water/DMHA. Chromatographic performance and MS detection were improved by the addition of ammonium dihydrogen phosphate [97].

Another routine method for the simultaneous determination of the intracellular nucleotides of NRTI stavudine (d4T), as well as the natural corresponding triphosphate in human PBMCs, was developed and validated by Pruvost et al. Separation was performed on a reversed-phase microbore column with an IP reagent coupled to tandem mass spectrometry with negative electrospray ionization and multiple reaction monitoring detection mode. The LOQ for d4T-TP was 138 fmol per 7 mL blood. The method was applied to samples from patients treated with stavudine and could be used on more than 35 samples per day. However, the robustness was poor as a result of column instability because of the application of a high pH [23]. The method was subsequently improved; several HPLC columns were compared in order to enhance the stability of the peak shape over time. The SMT C18 column was replaced with a Supelcogel ODP-50, which is more stable under high-pH conditions. This method was successfully applied to clinical samples of HIV-positive patients receiving antiretroviral therapy containing d4T, ddI (didanosine) and/or 3TC (lamivudine) for the simultaneous determination of its triphosphate metabolites [98]. The problem with interference that occurred in the method was resolved by changing the polarity of the ion source during analysis and looking for another fragmentation pattern of the interfering molecules, resulting in the formation of different product ions without loss of sensitivity. Validation of the improved method for the simultaneous determination of carbovir triphosphate (CBV-TP), lamivudine triphosphate (3TC-TP) and tenofovir diphosphate (TFV-DP) was performed. The composition of the mobile phase was not changed (1,5-DMHA, pH = 10.5). The total run time was 12 min, as opposed to 26 min for NRTI analysis. The positive ESI offered better specificity and slightly better sensitivity than the negative ESI mode for these compounds and resulted in enhanced specificity and more robust assay methods. This assay was applied to PBMC samples from HIV-infected patients with NRTI therapy [16].

The separation of several nucleotides and related phosphate-containing metabolites using IP-LC with DMHA was introduced in the study of Cordell et al. A reversed-phase chromatographic column was loaded with a mobile phase consisting of water and MetOH in various ratio (95:5 for MF A and 20:80 for MF B), with the addition of DMHA in the concentration range 0.5–20 mM and the pH being adjusted by means of acetic acid. The optimized method enabled the simultaneous detection of 24 nucleotides and related phosphorylated compounds in negative ESI mode. The method that had been developed was then applied to profile endogenous levels of intracellular nucleotides in cultured CHO cells [31].

Chen et al. also introduced the IP-LC-MS/MS method for the analysis of intracellular nucleoside triphosphate levels using DMHA as the IP reagent in MF. The analysis was performed on a Supelcogel ODP-50 column coupled to an Xterra MS C18 guard column with a mobile phase

comprising 5 mM DMHA in water buffered to pH 7 by formic acid as MF A and 5 mM DMHA in AcCN (50:50, v/v) as MF B. The method was applied to five different human leukemia cell lines and bone marrow samples of leukemia patients. It enabled the determination of CTP, dCTP, UTP, GTP, dTTP, dGTP/ATP, dATP and 2-chloroadenosine (IS) within a run time of 40 min [26].

An analytical method for 2′-fluoro-5-methyl-beta-L-arabinofuranosyluracil (L-FMAU) tri-phosphate, a novel L-nucleoside analogue of thymidine known as an inhibitor of the hepatitis B virus, was introduced for the determination of its levels in PBMCs. Ion-pairing chromatography coupled with negative ion electrospray ionization tandem mass spectrometry showed accurate and repeatable detection, the intra- and inter-day precision was lower than 11.2%, and the accuracy was between 97.1 and 106.9%. The method was applied to HBV-infected patients undergoing L-FMAU treatment [99].

Becher et al. developed a method for the direct determination of intracellular levels of zidovudine (AZT) triphosphate in human PBMCs. Mobile phase A consisted of 10 mM DMHA and 3 mM ammonium formate, the pH being approximately 11.5, and mobile phase B contained 20 mM DMHA and 6 mM ammonium formate/acetonitrile (1:1). Separation proceeded on an SMT C18 column and the total analysis time was 26 min. The method was applied to PBMCs from HIV-infected AZT-treated or AZT-free patients [21]. Compain et al. extended the method to ATP monophosphate and the analytical run time was reduced to less than 10 min. The low limits of quantification were at 150 and 300 fmol per sample for AZT-TP and AZT-MP, respectively. The improved method offered the possibility of simultaneous determination of other nucleotide phosphates such as d4T-TP or dTTP [100]. Durand-Gasselin et al. [20] evaluated potential NRTI toxicity in newborns from a human immunodeficiency virus-infected mother with antiretroviral prophylaxis by the determination of zidovudine (AZT) and lamivudine (3TC) metabolites in PBMCs together with the level of the parent drug in plasma. Till then the pharmacological monitoring of these drugs in newborns had been limited to plasma and cord blood. The LC-MS/MS method for PBMC samples was adapted from previously reported assays [21, 100], with minor modifications concerning AZT-MP monitoring [20]. The method was improved by Kinai et al. The extraction of PBMC using a double tube filled with silicon oil completely removed residual plasma phosphates and sodium salts. Together with mobile phase alkalization, using of alkaline-stable HPLC column and tetrabutylammonium hydroxide as the IP reagent resulted in higher sensitivity in comparison with the previous method [19].

Hernandez-Santiago et al. developed a method with gradient elution of decreasing DMHA concentration during analysis. Their MS system operated in negative electrospray mode. The assay was applied predominantly for the determination of β-D-N4-Hydroxycytidine (NHC) triphosphate, a metabolite of a nucleoside analogue with selective anti-hepatitis C virus activity. In pre-clinical studies, the metabolism of the drug was investigated in various liver cells and primary human hepatocytes. Although a decreasing concentration of IP reagent was applied, the MS ion source and the whole LC column had to be regularly cleaned with a water-methanol mixture (50:50, v/v) at the end of each day [101].

Luo et al. focused on the identification and quantification of intracellular metabolites involved in central carbon metabolism, including glycolysis, the pentose phosphate pathway and the

tricarboxylic acid cycle. Separation was performed on an RP C18 column with a mobile phase consisting of 10 mM tributylamine aqueous solution with the pH adjusted to 4.95 with 15 mM acetic acid as eluent A and methanol as eluent B. The total analysis time was 80 min, and before each run, the column was equilibrated for 10 min. The method enabled the determination of 29 negatively charged compounds, including several nucleotides [47].

Hexylamine (HA) as an IP agent was used in the study of Fromentin et al., which was aimed at the determination of several clinically relevant nucleotide analogues and endogenous nucleotides. Separation was optimized for a Hypersil Gold C18 column, which offered the best results compared to other columns that were tested. The mobile phases A and B used in the method for partial validation consisted of 2 mM ammonium phosphate buffer containing 3 mM HA and acetonitrile, respectively. A gradient elution program was applied. The study was applied to human PBMC and macrophages, but it could also be applied for the quantification of other NRTIs with slight modifications [82].

A combination of IPC separation on PGC columns was also introduced in some studies. For a detailed description see the chapter 'Porous graphitic carbon columns' [60, 81].

Thanks to the reduced amount of IP reagent and salts entering the ion source, several robust methods for nucleotide determination with acceptable ion suppression have been developed and applied. However, pollution of the ion source remains the major problem.

## 4.5. Hydrophilic interaction liquid chromatography

Hydrophilic interaction liquid chromatography (HILIC) is an emerging separation mode of LC. In this variant of normal-phase LC, polar columns with a hydrophilic stationary phase are used in combination with a mobile phase consisting of reversed-phase-type eluents rich in organic solvents. Stationary phases used in HILIC configuration can contain a simple non-polar silica phase or can be modified by amino, anionic, amide, cationic or zwitterionic bonded phases. A typical mobile phase of HILIC mode consists especially of acetonitrile with a small amount of water or also alcohol in a higher concentration than the aprotic solvent to achieve the same retention behaviour. Polar analytes are eluted from the column by increasing the water content of the mobile phase. In HILIC, the analyte is distributed between a water-rich layer on the surface of the polar stationary phase and the organic mobile phase layer, creating a liquid-liquid extraction system. The mechanism of HILIC also includes hydrogen donor interactions between neutral polar species as well as weak electrostatic mechanisms under the high organic solvent conditions used for retention. Hence, this separation mode partly overlaps with ion chromatography and reversed-phase liquid chromatography. The retention behaviour of analytes, peak shape and chromatographic tailing are also controlled by the pH of the mobile phase and the ion strength formed by ionic additives, such as ammonium acetate and ammonium formate. The applied mobile phases are highly volatile, and thus the hyphenation with MS is friendly and favourable. HILIC mode showed very high efficiency for the retention of polar analytes that offered a different selectivity in comparison with the traditionally used RP-HPLC. High throughput of HILIC-MS/MS analysis at a high flow rate was allowed as a result of the very low column backpressure contributed by the high-organic mobile phase. Direct injection of the organic solvent extracts through LLE, SPE and PP onto the HILIC column is possible, in contrast to regular RP-HPLC. HILIC-MS/MS

has been demonstrated to be a very important supplement to the RP-HPLC-MS/MS for the analysis of polar compounds [102, 103].

The HILIC-MS method for the simultaneous determination of 141 endogenous metabolites, including several nucleotides, was introduced by Bajad et al. Separation was performed on an aminopropyl column with an alkaline mobile phase consisting of ammonium acetate and ammonium hydroxide as solvent A with a pH of 9.45 and acetonitrile as solvent B. A gradient elution program was used and the total analysis time was 40 min with five time segments in positive mode and 50 min with four time segments in negative mode. The method was applied to extracts of *E. coli* grown in [12C] vs. [13C] glucose and revealed appropriate 12C- and 13C-peaks of 79 different metabolites [43].

The complex metabolomics method using HILIC separation mode was developed by Karlíková et al. Separation was performed on the Luna NH$_2$ aminopropyl column with MP A consisted of 20 mM ammonium acetate at pH 9.75 and the mobile phase B consisted of AcCN. Gradient program was used for elution of targeted analytes. The total analysis time was 17 min. The method allowed analysis of 354 compounds as a result of continuous switching of positive and negative mode and detection of analytes using scheduled MRM mode. The method was applied on the metabolite profiling of the plasma and leukocytes of chronic myeloid leukemia patients [104].

Similar separation conditions were used for the determination of 2-methylcytidine triphosphate in a study by Pucci et al. Separation was performed on an aminopropyl column with ammonium acetate, pH 9.45 and acetonitrile. The chromatographic gradient was modified and reduced to 30 min, and the detection of the ions was performed in the negative ESI MRM mode. The calibration curve was linear over the 0.05–50 μM concentration range. The method has been successfully applied for pharmacokinetic studies of 2′-C-methyl-cytidine-triphosphate in rat liver tissue samples [39].

Teleki et al. also introduced alkaline conditions in HILIC mode for the quantitative profiling of more than 50 hydrophilic intracellular key metabolites. Separation was performed on a ZIC-pHILIC column. Optimization was focused on the pH of the mobile phase, buffer concentration, flow rate, column temperature and the gradient slope of the polar eluent. In optimized chromatographic conditions the mobile phase consisted of 10% aqueous buffer solution (10 mM ammonium acetate) and 90% acetonitrile for eluent A and 90% aqueous buffer with 10% acetonitrile for eluent B, both adjusted to a pH of 5.6 with acetic acid or a pH of 9.2 with ammonium hydroxide. The column was kept at 40°C, with a flow rate of 0.2 mL min$^{-1}$. Under alkaline conditions, 98% of the metabolites showed an absolute time shift of less than 0.04 min, in contrast to 80% under acidic conditions. Significant differences were also observed for the diphosphate and triphosphate metabolites and NADP, as well as for several amino acids with respect to the detection limits under alkaline conditions, which were approximately 20–50 times lower. Moreover, 70% of the metabolites presented more than 1.5-fold higher signal intensities under alkaline mobile phase conditions. The quality of the method was demonstrated by absolute quantification of selected metabolites in intracellular extracts of *E. coli* biomasses using standard-based external calibration, isotope dilution and standard addition as calibration strategies [40].

The HILIC-MS/MS method for the quantification of AMP, GMP, UMP, CMP and IMP in infant formula was developed by Inoue et al. The mobile phase used for the separation consisted of 30 mM ammonium formate in water with a pH of 2.5 and methanol. Data acquisition was achieved by positive ESI MRM; the LOD and LOQ were 5–10 and 10–30 $\mu$g mL$^{-1}$ for standard solution, respectively [79].

The HILIC-MS/MS method was developed for simultaneous quantitative analysis of cAMP and ATP for measuring the effect of (partial) agonists on cAMP accumulation *in vitro*. The separation of the analytes proceeded on a ZIC®-pHILIC column with mobile phase A consisting of 10 mM ammonium bicarbonate buffer adjusted to a pH of 9.4 with ammonium hydroxide in 20% AcCN in water and mobile phase B consisting of 100% AcCN. The total run time using pre-sampling was less than 6 min. The method was applied to Chinese hamster ovarian cells cloned and expressing the human dopamine D2L receptor [34].

Pesek et al. developed a method with the use of a silica hydride-based stationary phase for the aqueous normal-phase (ANP) retention of nucleotides. RP columns with a hydride surface underneath, as well as those with an unmodified or a minimally modified hydride material, were tested. The ANP retention of the hydrophilic nucleotides was dependent on mobile phases with a high organic content in combination with an additive to control ionic strength and pH. Both isocratic and gradient elution programs were used for separation optimization, and repeatability in both modes was excellent [105].

A combination of HILIC chromatography and an IP reagent was introduced by Zhang et al. This novel method enabled sensitive and short run-time analysis for adenine nucleotides. The novel aspect of this method is based on the application of DEA-HFIP in the mobile phase in combination with bare silica or $NH_2$ HILIC columns. This enabled successful separation of isobaric isomers (ATP vs. dGTP, etc.), among others. The highest sensitivity and chromatographic separation capacity was achieved using 100 mM HFIP and 0.5% DEA in MF at a pH of 8.9. The calibration curves showed excellent linear response over the concentration range 10–1000 ng mL$^{-1}$ for AMP and ADP and 2–200 ng mL$^{-1}$ for ATP; the low levels of 10 and 2 ng mL$^{-1}$ represent the LLOQ for individual analytes [12].

Mateos-Vivas et al. also described the HILIC-MS method for the separation of nucleosides and nucleotide mono-, di- and triphosphates in the presence of hydrophilic ion-pairing reagents. During optimization three analytical columns were tested with mobile phases containing a mixture of organic solvent (acetonitrile) and aqueous media at different pH values with different concentrations of salts. HILIC XBridge-Amide was found to be the most suitable column for the separation of 20 nucleosides and nucleotides. The optimized mobile phase consisted of ACN/UHQ water mixtures with 50 mM diethylamine and 100 mM hexafluoro-2-propanol. The total analysis time was 8 min [61].

HILIC mode represents a powerful approach offering highly selective and sensitive separation of a wide range of analytes in complex studies enabling the simultaneous analysis of a large number of compounds in a single run. Using volatile additives in mobile phases enables smooth hyphenation with mass spectrometry detection and this approach is becoming widely used for various analyses today.

# 5. Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF)

MALDI is a soft ionization technique used in mass spectrometry commonly applied for the analysis of large molecules (DNA, proteins, peptides, etc.). Analogously to ESI, it offers a soft ionisation to obtain ions in the gas phase. Briefly, the sample is applied to a metal plate together with a suitable matrix such as 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid), $\alpha$-cyano-4-hydroxycinnamic acid ($\alpha$-CHCA, alpha-cyano or alpha-matrix) or 2,5-dihydroxybenzoic acid (DHB). The sample is consequently irradiated by means of a pulsed laser, resulting in ablation and desorption of the sample and matrix material. Finally, the analytes are ionized by (de)protonation by laser ionization energy transferred through the matrix molecules and can then be accelerated into the mass spectrometer. The advantage of the method is its higher tolerance to salts and other contaminants from samples in comparison with other MS methods. MALDI-TOF is a sensitive technique that able to detect analytes in very low concentrations, which also result in a small sample amount being required for the analysis and the reduction of the sample preparation time.

Thanks to all of these facts van Kampen et al. developed a MALDI-TOF method for the determination of nucleotide analogue zidovudine (AZT) triphosphate, used as an NRTI for the treatment of HIV infection, and other (deoxy) nucleotide triphosphates. Four different matrices were compared in order to ascertain the sensitivity and reproducibility of the method. Anthranilic acid in combination with nicotinic acid was selected as the most suitable matrix. AZT-TP, ATP and GTP were analysed with LOD of 0.5 fmol per sample. The method was applied for the determination of intracellular levels of AZT-TP and (deoxy) nucleotides in PBMCs [84].

The determination of some endogenous nucleotides was included in a metabolomic study by Edwards and Kennedy. MALDI-TOF was used for the analysis of anionic metabolites, with 9-aminoacridin as the matrix. The detection limits for the standards were from the nM to $\mu$M concentration range. The method enabled detection of overall 105 different metabolites in analysis of Langerhans islets extract, direct analysis of single Langerhans islet and *E. coli* extract. It was able to detect different metabolic states depending on environmental conditions such as glucose concentration and the sample type [44].

A shotgun metabolomics approach using MALDI-TOF/TOF mass spectrometry was developed for the rapid analysis of negatively charged water-soluble cellular metabolites by Sun et al. Neutral organic extraction solvents were used to inactivate endogenous enzyme activities. As a result of conjunction with a matrix, that had minimal background noise, identification of 285 peaks corresponding to negatively charged metabolites was possible. The identification of the metabolite peaks was based on mass accuracy and confirmation was performed using tandem mass spectrometry for 90 of the identified metabolite peaks. Assignments of ions from isomeric metabolites and their relative abundance quantitation were achieved through fragmentation ions originating in tandem mass spectrometry (e.g. discrimination of ATP from dGTP). The method is highly sensitive and facilitates the detection of extremely low-abundance metabolites, including signalling molecules such as IP3, cAMP and cGMP. The method was applied to the metabolite profiling of mouse heart extracts [106].

MALDI-TOF is generally less popular for the identification of compounds with low molecular weight because of the presence of a variety of abundant matrix-related ions in the low-mass range of MS spectra and also the non-homogenous co-crystallization of analytes with traditional organic matrices such as 2,5-dihydroxybenzoic and sinapic acid. To overcome the problem, surface-assisted laser desorption/ionization (SALDI) was developed to eliminate matrix ion interferences and improve sample homogeneity. Recently, nanoparticles have been investigated as an effective SALDI matrix. Huang and Chang introduced an analysis for the determination of ATP and glutathione using aptamer-modified gold nanoparticles (Apt-AuNPs) as selective probes and gold nanoparticles (AuNPs) as the SALDI matrices with mass spectrometry detection. In comparison with conventional organic matrices, AuNPs as laser desorption/ionization (LDI) matrices provide a number of advantages, such as ease of preparation, selectivity, sensitivity and repeatability. In combination with Apt-AuNPs as a selective probe for ATP with less efficient LDI compared to AuNPs, the MS approach provided a very good sensitivity for ATP of 0.48 μM. The method was successfully applied to the analysis of ATP and GSH in human cell lysates. This approach demonstrates the practical monitoring of the bioactivity of cells through ATP and GSH levels [35].

Negative MALDI-TOF-MS appears to be a promising tool for nucleotide analysis providing complementary information to the armamentarium of metabolomic techniques. This technique can provide high levels of throughput because of the simple sample preparation, without the need for chromatographic separation and short analysis time, but its usability for low-mass compounds is still limited.

## 6. Summary

Nowadays, mass spectrometry is very important and widely applied tool in nucleotides analysis. As a result of technological advances in sample purification methods and mass spectrometry detection, the developed methods allow sensitive and selective measurement of polar compounds occurring in low levels in various biological matrices. This enables more potential uses in clinical field. Direct methods require no special sample pre-treatment before analysis in contrast to indirect methods, where fractionation, dephosphorylation and purification are needed. Previously time-consuming chromatographic separation based on the number of phosphate groups was improved as a result of wide spectrum of separation possibilities and settings. The use of IP agent in optimal amount made the methods relatively MS friendly. Alternative approach is the ion exchange chromatography with pH gradient used for elution. High selectivity in separation of closely related nucleotides can be achieved by using porous graphitic carbon columns. HILIC represents a promising approach for analysis of wide spectra of analytes including polar compounds such as nucleotides. High separation efficiency is also achieved with the use of CE with MS detection. Analysis of nucleotides is also described by the means of MALDI-TOF, but poor reproducibility and lack of applications make a limitation for this approach. Selected methods for determination of endogenous nucleotides and nucleotide analogues by mass spectrometry are summarized in **Tables 1** and **2**, respectively.

| Analysed nucleotides | Matrix/application | Technique | Nucleotides extraction | Separation | MS polarity | References |
|---|---|---|---|---|---|---|
| cGMP (cAMP, cIMP, AMP, GMP, IMP) | Human plasma | LC-MS/MS | PP (AcCN) | Metasil AQ C18, (RP) | MRM − | [37] |
| cAMP, cCMP, cGMP, cUMP | Hek293T cells | LC-MS/MS | PP (AcCN:MetOH:$H_2O$, 2:2:1, v/v) | Zorbax eclipse XDB-C18 (RP) | SRM + | [30] |
| UXP, CMP, AXP, GXP, TTP, dATP | Mice islet of Langerhans; *E. coli* | MALDI-TOF | PP (MetOH) | - | MS − | [44] |
| AXP | HepG-2 cells | LC-MS/MS | PP (PCA) | Hypercarb (PGC) | MS − | [25] |
| AXP, CXP, GXP, IXP, UXP, dAXP, dCXP, dGXP, dTXP, dUXP, cAMP | *E. coli* | LC-MS/MS | PP (80% MetOH) | Luna $NH_2$ (HILIC) | SRM ± | [43] |
| CTP, dCTP, UTP, GTP, dTTP, dGTP/ ATP, dATP | human leukemia cells lines (K562, NB4, ML-1, MV4-11, THP-1) | LC-MS/MS | PP (60% MetOH) | Supelcogel ODP-50 (IPC) | MRM − | [26] |
| TXP, cAMP, cGMP, AXP, GXP, CXP | *E. coli* | CE-MS (LC-MS/MS) | PP + LLE (MetOH + chloroform + $H_2O$) | Fused-silica 50 μm × 100 cm (Asahipak ODP-50 2D) | MS − (MRM −) | [55] |
| AXP, CDP, CTP, GDP, GTP, UDP, UTP | Rat kidney tissue | LC/LC-MS | PP (PCA) + online desalting | Zorbax C18 + Synergy Hydro C18, (IPC) | MS + | [41] |
| AXP, GXP, CXP, UXP, cAMP | *E. coli* | LC-MS/MS | PP (liquid $N_2$) | ZIC-pHILIC (HILIC) | MRM − | [40] |
| ATP | MDA-MB-231 cells | SALDI-MS | Sonication, filtration | - | MS − | [35] |
| CXP, UXP, AXP, GXP, cAMP, cGMP | Chinese hamster ovary cells (CHO) | LC-MS/MS | PP (MetOH) + LLE (hexane) | Symmetry (IPC) | MRM − | [31] |
| AMP/dGMP, ADP/dGDP, ATP/dGTP | Human blood and plasma | LC-MS/MS | PP (MetOH) | Luna $NH_2$ (IP-HILIC) | MRM − | [12] |
| AXP, cGMP | *E. coli* | LC-MS/MS | PP (60% MetOH) | Synergi Hydro-C18 (IPC) | MRM − | [47] |
| AXP, CTP, GXP, UTP, dATP, dCTP, dGXP, dTXP (CDP, dAMP, dCDP, dUMP, UDP) | H23 cells | LC-MS/MS | PP (60% MetOH) + ultrafiltration | Atlantis T3 (IPC) | MRM − | [32] |

| Analysed nucleotides | Matrix/application | Technique | Nucleotides extraction | Separation | MS polarity | References |
|---|---|---|---|---|---|---|
| AMP, ATP, dAMP, dATP, UMP, UTP, TMP, TTP, CMP, CTP, dCMP, dCTP, GMP, GTP, dGMP, dGTP | Messa cells | LC-MS/MS | PP (70% MetOH) + online SPE (WAX) | Hypercarb (PGC) | MRM + | [29] |
| GTP, ATP, UTP, CTP, GDP, ADP, UDP, CDP, GMP, AMP, UMP, CMP | Rat heart tissue | LC-MS/MS | PP (50% MetOH) | Hypersil Gold AQ C18 | MRM − | [45] |
| cAMP, ATP | CHO-K1 cells | LC-MS/MS | Lysis buffer (63% AcCN, 10 mM $NH_4HCO_3$) | ZIC-pHILIC (HILIC) | MRM − | [34] |
| cAMP, cGMP | Mice brain tissue, plasma, CSF | LC-MS/MS | PP (PCA) | Varian Metasil AQ C18 (RP) | MRM + | [36] |
| Adenosine, guanosine, inosine (AMP, ATP, GMP, GTP, IMP, ITP) | PBMC, RBC, DBS | LC-MS/MS Indirect | PP (70% MetOH) + SPE + dephosphorylation + SPE | Develosil C30, RP aqueous | MRM + | [15] |

**Table 1.** Selected methods for determination of endogenous nucleotides using mass spectrometry detection.

| Analysed nucleotides | Matrix/application | Technique | Nucleotides extraction | Separation | MS polarity | References |
|---|---|---|---|---|---|---|
| AZT, AZT-XP, 5-methyl-UTP, AZdU | PBMC/plasma | LC-MS/MS | PP (EtOH) | InnertSustain/ InnertSil ODS-30 (IPC) | MRM + | [19] |
| AZT, AZT-MP, AZT-TP, 3TC, 3TC-TP, dT-TP | PBMC/plasma | LC-MS/MS | PP (Tris-HCl/MetOH) | SMT C18 (IPC) | MRM ± | [20] |
| Clofarabine-TP | PBMC | LC-MS/MS | PP (AcCN) | CAPCELL PAK CN | MRM − | [24] |
| d4T, d4T-XP, dT-TP | PBMC | LC-MS/MS | PP (Tris-HCl/MetOH) | SMT C18 (IPC) | MRM − | [23] |
| AZT-TP | PBMC | LC-MS/MS | PP (TrisHCl/MetOH) + immunoaffinity extraction | Supelcogel ODP-50 (IPC) | MRM − | [21] |
| FLT, FLT-MP | Human serum | LC-MS/MS | PP (MetOH) | Aquasil C18 (RP) | MRM + | [58] |
| dFdC, dFdU, dFdC-TP | Mice pancreatic tumour tissue | LC-MS/MS | PP (aqueous AcCN) | Hypercarb (PGC) | MRM ± | [46] |
| MeGTP | Mouse liver tissue | LC-MS/MS | PP (TCA) + SPE-WAX | Luna $NH_2$ (IXC) | MRM + | [42] |

| Analysed nucleotides | Matrix/application | Technique | Nucleotides extraction | Separation | MS polarity | References |
|---|---|---|---|---|---|---|
| MeCTP | Rat liver tissue | LC-MS/MS | PP (70% MetOH)+SPE | Luna $NH_2$ (HILIC) | MRM − | [39] |
| 2CdA-XP | MDCKII cells | LC-MS/MS | PP (70% MetOH) | Biobasic AX (IXC) | MRM + | [27] |
| TFV-DP, 3TC-TP, CBV-TP | Human PBMC | LC-MS/MS | PP (Tris-HCl/MetOH) | Supelcogel ODP-50 (IPC) | MRM + | [16] |
| MeTG-XP, MeTI-XP, TG-XP, TI-MP, TI-TP | RBC | LC-MS/MS | PP (heating, MetOH, $CHCl_3$) | Biobasic AX (IXC) | MRM + | [13] |
| dFdC-TP | PBMC | LC-MS/MS | PP (PCA) | Biobasic AX (IXC) | MRM − | [17] |
| FUTP, FdUXP | PBMC | LC-MS/MS | PP (MetOH) | Biobasic AX (IXC) | MRM − | [18] |
| TFV(XP), ZDV(XP), 3TC(XP), FTC(XP) | RBC, PBMC | LC-MS/MS indirect | PP (70% MetOH), SPE, dephosphorylation, SPE | Synergi Polar RP (RP) | SRM + | [14] |
| RBV(XP) | RBC, PBMC, DBS | LC-MS/MS Indirect | PP (70% MetOH), SPE, dephosphorylation, SPE | Develosil C30- RP aqueous (RP) | SRM + | [59] |
| ZDV(TP), 3TC(TP) | PBMC | LC-MS/MS indirect | PP (70% MetOH), SPE, dephosphorylation, SPE | Hypersil C18 (RP) | SRM + | [22] |
| ddATP | CEM-T4cells | LC-MS/MS indirect | PP (70% MetOH), SPE, dephosphorylation, SPE | Purospher RP-18e (RP) | SRM + | [33] |
| Adefovir, Adefovir-MP, Adefovir-DP, Tenofovir-DP, dATP | HepG2 | LC-MS/MS | PP (70% MetOH) | C18: Xterra/YMC /Luna | MRM + | [28] |

**Table 2.** Selected methods for determination of nucleotide analogues using mass spectrometry detection.

# 7. Nucleotide profile of cell lines by HILIC chromatography

## 7.1. Introduction

We adopted and optimized the metabolomics method to cover intermediates in the majority of the metabolic pathways in the cellular metabolome, as well as common metabolites present in urine, plasma and other biofluids, originally published by Bajad in 2006 [43]. It is based on HILIC separation mode, where polar negatively charged metabolites are separated on an aminopropyl column under strongly alkaline conditions (pH > 9). The gradient of the mobile phase, pH and particle size selection (3 μm) in the column was optimized. The list of metabolites was significantly extended by measurement in scheduled MRM mode and applying polarity switching. Under the final conditions, the method allows the separation of 350 metabolites during 17 min of analysis. The method is routinely used in our laboratory [104, 107] and it was applied to the sensitive measurement of selected endogenous

nucleotides and nucleotide analogues in intracellular content [104, 107]. In this chapter, 5-ethynyl-2′-deoxyuridine (EdU) and its mono-, di- and triphosphate analogues resulting from the incubation of cancerous cell lines with EdU and 5-ethynyl-2′-deoxycytidine (EdC), together with endogenous purine and pyrimidine nucleotides, are presented.

## 7.2. Material and methods

### 7.2.1. Cell cultures

The cell line 143B PML BK TK [bone, osteosarcoma, contains a herpes simplex virus type 1 thymidine kinase (hsv-1 TK+) plasmid; 143B] was cultivated in DMEM supplemented with 3.7 gL$^{-1}$ of sodium bicarbonate and HAT (0.1 mM hypoxanthine, 400 nM aminopterin and 0.16 mM dT, Sigma Aldrich). The medium was also supplemented with 10% foetal bovine serum (Gibco) and 50 µg mL$^{-1}$ gentamicin. One week before the experiment, the culture medium was exchanged for a HAT-free medium. For more details see the study by Ligasová et al. [108].

### 7.2.2. Chemicals

Water, acetonitrile, methanol, acetic acid, ammonium hydroxide (all LC-MS purity grade), DMEM and dimethylsulphoxide (DMSO) were all purchased from Sigma Aldrich (St. Louis, MO, USA). 5-Ethynyl-2′-deoxyuridine (EdU) was purchased from Carbosynth (Compton, Berkshire, United Kingdom) and 5-ethynyl-2′-deoxycytidine (EdC) and EdU triphosphate (EdUTP) from Jena Biosciences (Jena, Germany). EdC monophosphate (EdCMP), EdC diphosphate (EdCDP) and EdC triphosphate (EdCTP) were synthesized by Dr. Liboska from the Institute of Organic Chemistry and Biochemistry, CAS, v.v.vi.

### 7.2.3. Instrumentation

Analyses of endogenous nucleosides, nucleotides, the nucleoside analogues and their phosphorylated metabolites were performed using the Ultimate 3000 RS high-performance liquid chromatographic system (Dionex, Sunnyvale, CA, USA) coupled with a Triple Quad 6500 tandem mass spectrometer (Sciex, Framingham, MA, USA) equipped with an electrospray IonDrive Turbo V Source. A Luna NH$_2$ aminopropyl column (2 × 100 mm, 3 µM, Phenomenex) protected by a 4 × 2-mm ID guard column made of the same material (Phenomenex, Torrance, USA) was used for separation.

### 7.2.4. Sample preparation

The sample preparation of the cells treated with EdU or EdC is described previously in the study by Ligasová et al. [108]. Briefly, cells cultivated in a medium with or without nucleoside analogue for a definite time were immediately vacuum-filtered. The membrane filter with the captured cells was transferred to a pre-cooled Petri dish, extracted with a cold extraction solution (AcCN:MetOH:0.5 M FA/30:10:10), and returned to a −20°C freezer for 30 min. The filter was rinsed with the extraction solution in the dish, the solution was transferred into centrifuge microtubes, and then the filter was re-rinsed with 1 mL of fresh cold extraction

solution, which was thereafter combined with the initial cell extract. After centrifugation, the supernatant was put into the freezer, pellet resuspended in fresh extraction solution, frozen and centrifuged, and the supernatant was combined with the previous one. The extract was neutralized with $NH_4HCO_3$ and centrifuged and the supernatant was lyophilized and resuspended in 100 µL of water mobile phase before the LC-MS/MS analysis. All the procedures were performed in an ice bath to prevent undesirable metabolic processes in the cells during the sample preparation.

### 7.2.5. Standard stock solutions

Standard solutions of EdCMP, EdCDP, EdCTP and EdUTP were dissolved in deionized water in concentrations of 1.96, 3.6, 4.7 and 5 µM, respectively. EdU and EdC were dissolved in DMSO to a concentration of a 10-mM stock solution. The working solutions of all standards for mass spectrometry optimization were obtained by serial dilutions to the required concentrations with water of LC-MS purity grade. All the solutions were stored at −80°C.

### 7.2.6. LC-MS/MS analysis conditions

The separation and mass spectrometry method was previously described by Ligasová et al. [108]. Briefly, mobile phase A consisted of 20 mM ammonium acetate buffer solution, pH = 9.75, and mobile phase B was acetonitrile. The gradient elution program used for the separation started with 95% B and during 7 min was reduced to 10% B and held for the next 7 min. Then, initial conditions were achieved in 1 min and equilibration took 3 min. The column was maintained at 35°C with a flow rate of 0.3 mL min$^{-1}$. The total analysis time was 17 min. The targeted metabolites were detected in multiple reaction monitoring (MRM) in both positive and negative electrospray ionization mode. The mass spectrometry parameters were optimized using a standard solution of the targeted analytes dissolved in a mixture of mobile phase A and B (50:50, v/v). The final MS conditions are summarized in **Tables 3** and **4** for positive and negative mode, respectively. Both quadrupoles (Q1 and Q3) were set to 'unit' resolution. The ion source parameters were optimized to the following settings: an ionization spray voltage of (−) 4500 V, a curtain gas of 30 psi, a collision gas of 8 psi, a heater gas and turbo ion spray gas of 40 psi, a source temperature of 400°C, and an entrance potential of (−) 10 V. High-purity nitrogen was used as the collision gas. The Analyst 1.6.2 and MultiQuant 3.0 software (Sciex, USA) were used for data acquisition and evaluation. Standards for EdUMP and EdUDP were not available and the mass spectrometry conditions were adopted from the optimization experiments of EdC/EdCMP/EdCDP/EdCTP. For more details, see the previously published study by Ligasová et al. [108].

### 7.3. Results and discussion

The first step in the mass spectrometry optimization was finding the optimal parameters for targeted metabolite analyses. Selected reaction monitoring (SRM) requires the accurate setting of molecular ion and the most sensitive and selective fragment, the optimal collision energy, declustering potential and entrance and exit potentials. A standard solution of each

| ID | Q1 | Q3 | DP | CE | ID | Q1 | Q3 | DP | CE | ID | Q1 | Q3 | DP | CE |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 135.9 | 118.9 | 171 | 31 | dHr | 252.9 | 136.9 | 51 | 19 | GMP | 364.0 | 152.0 | 36 | 21 |
| AMP | 348.0 | 135.9 | 116 | 27 | dUR | 228.9 | 113.0 | 91 | 23 | Gr | 284.0 | 151.9 | 51 | 27 |
| Ar | 268.2 | 136.1 | 41 | 29 | EdC | 252.0 | 136.1 | 46 | 15 | Hr | 268.9 | 136.9 | 81 | 21 |
| C | 111.9 | 94.8 | 96 | 27 | EdCDP | 412.0 | 216.1 | 51 | 13 | HX | 136.9 | 109.9 | 136 | 29 |
| CMP | 323.8 | 111.9 | 46 | 21 | EdCMP | 332.0 | 136.1 | 36 | 17 | IMP | 349.0 | 136.9 | 31 | 19 |
| Cr | 244.0 | 111.9 | 31 | 27 | EdCTP | 491.9 | 136.1 | 71 | 19 | T | 126.9 | 109.9 | 96 | 23 |
| dAMP | 331.9 | 135.9 | 41 | 23 | EdU | 253.0 | 137.1 | 116 | 17 | Tr | 242.9 | 126.8 | 61 | 17 |
| dAr | 251.9 | 118.9 | 36 | 59 | EdUMP | 333.0 | 81.1 | 36 | 53 | Xr | 284.9 | 152.9 | 96 | 15 |
| dCMP | 307.9 | 112.0 | 56 | 19 | EdUTP | 492.9 | 81.0 | 111 | 55 | | | | | |
| dGMP | 348.1 | 80.9 | 10 | 31 | G | 152.0 | 81.9 | 76 | 37 | | | | | |

**Table 3.** MS parameters for targeted analytes in positive mode.

| ID | Q1 | Q3 | DP | CE | ID | Q1 | Q3 | DP | CE | ID | Q1 | Q3 | DP | CE |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ADP | 425.8 | 133.9 | −95 | −32 | dTDP | 400.8 | 158.8 | −60 | −34 | EdUMP | 331.0 | 135.0 | −55 | −26 |
| ATP | 506.1 | 159.0 | −75 | −30 | dTMP | 320.8 | 194.9 | −35 | −24 | EdUTP | 490.9 | 158.8 | −70 | −34 |
| CDP | 401.8 | 158.9 | −60 | −32 | dTTP | 480.7 | 158.8 | −70 | −46 | GDP | 441.8 | 158.8 | −95 | −36 |
| CTP | 481.7 | 158.9 | −70 | −44 | dUMP | 306.8 | 195.0 | −50 | −22 | GTP | 522.0 | 424.0 | −90 | −25 |
| dADP | 409.8 | 158.8 | −30 | −32 | dUTP | 466.7 | 158.9 | −55 | −36 | IDP | 426.8 | 134.9 | −75 | −32 |
| dATP | 489.7 | 158.7 | −60 | −36 | EdC | 249.9 | 136.0 | −55 | −12 | ITP | 506.7 | 158.7 | −60 | −48 |
| dCDP | 385.8 | 158.9 | −30 | −30 | EdCDP | 409.9 | 274.7 | −45 | −26 | U | 111.0 | 42.1 | −35 | −22 |
| dCTP | 465.7 | 158.9 | −65 | −36 | EdCMP | 329.9 | 194.8 | −55 | −22 | UDP | 402.9 | 158.8 | −70 | −34 |
| dGDP | 425.8 | 158.9 | −70 | −32 | EdCTP | 489.8 | 158.8 | −5 | −34 | UMP | 322.8 | 210.9 | −65 | −22 |
| dGTP | 506.1 | 159.0 | −75 | −30 | EdU | 250.9 | 134.8 | −50 | −16 | UTP | 482.7 | 158.7 | −80 | −48 |
| dITP | 490.7 | 158.8 | −65 | −40 | EdUDP | 411.0 | 78.9 | −70 | −102 | X | 150.9 | 108.0 | −55 | −24 |

**Table 4.** MS parameters for targeted analytes in negative mode.

nucleotide was directly infused into the mass spectrometer and the parameters were tuned automatically. The most suitable ion for SRM was selected from the fragmentation spectra. The fragmentation spectra of EdU and EdUTP are shown in **Figure 1**. In positive mode, the cleavage of hydroxyl groups from deoxyribose (m/z of 235.0 and 217.2) and whole deoxyribose (137.0, 117.0 and 99.0) and cleavage of the pyrimidine ring (94.0) were typical fragmentation behaviour of EdU. In negative mode, the fragmentation is not so predictable and fragments of whole deoxyriboside (135.0) and the pyrimidine ring (207.8 and 92.0) and deoxyriboside (160.8) were obtained. EdUTP was fragmented in positive mode, similarly to the cleavage of the pyrimidine ring (137.0 and 119.0), whole EdU without hydroxyl groups (217.2) and one terminal phosphate group (81.0), respectively. In negative mode, the fragmentation pattern of EdUTP contained cleavage in six different positions of the triphosphate group (79.0, 97.0/393.0, 159.0, 176.8, 238.8 and 257.0) and the pyrimidine ring (135.0/355.0), respectively.

The fragmentation behaviour of deoxyriboside can be applied to other ribosides/deoxyriboside and their analogues. The cleavage of sugar in both positive and negative ionization modes offers the most intensive product ion of the pyrimidine or purine ring, which is commonly used as a characteristic transition for SRM. Similarly, deoxy/nucleoside di- and triphosphates and their analogues offer the most intensive fragmentation in negative mode, with cleavage of one, two or three phosphates. The situation is different in the case of deoxy/nucleoside
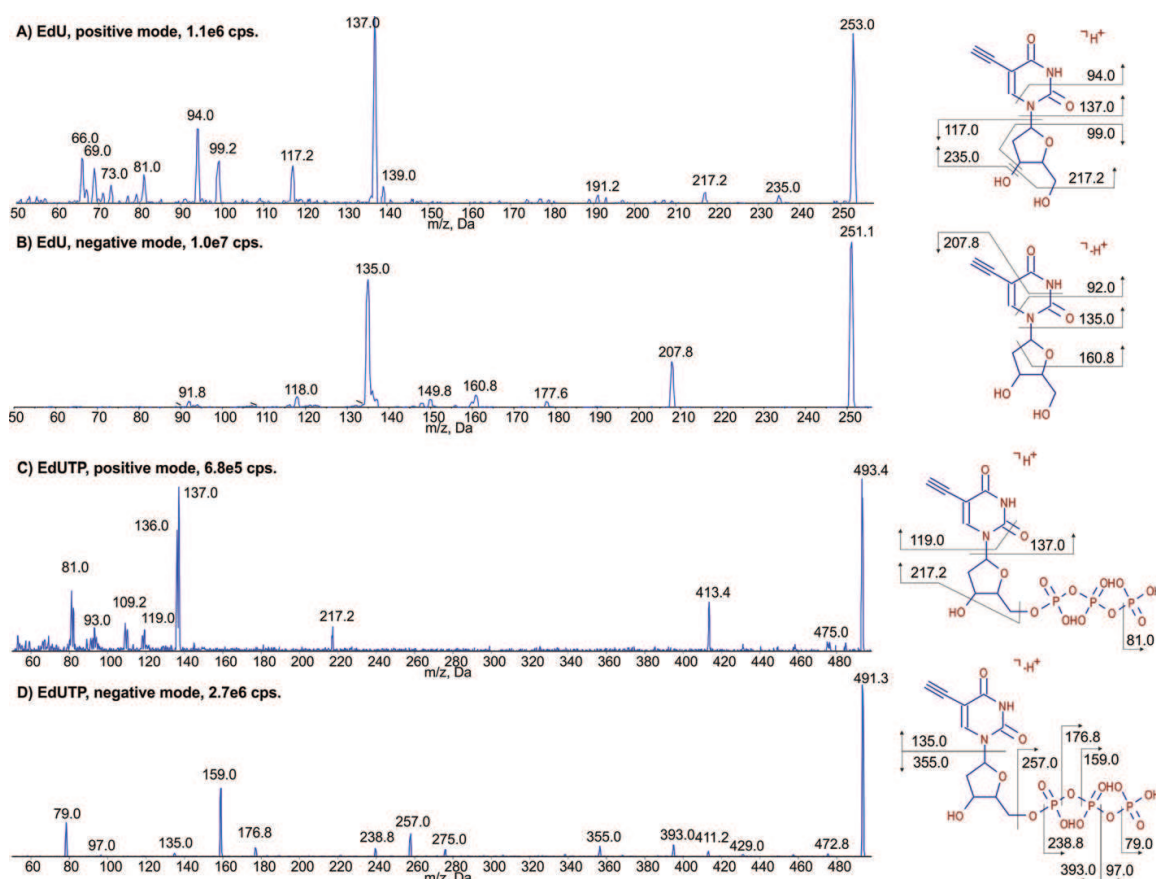


**Figure 1.** Fragmentation spectra of EdU and EdUTP in positive and negative ESI mode, respectively.

monophosphates, which provide a more intensive signal in positive mode with cleavage of the purine or pyrimidine ring. This approach can be successfully applied for many different nucleosides or nucleotides even when standards are not commercially available. The other crucial parameter is the collision energy, which has to be carefully optimized. Again, it can be adopted from available standards during the compound tuning and optimization procedure. This general phenomenon is documented in **Tables 3** and **4**, which show the SRM transitions and other MS parameters of all the nucleotides included in the method.

For the separation and accurate determination of nucleotides, it is necessary to choose optimal conditions for the stationary and mobile phase, gradient program and flow rate. Nucleotides are very polar analytes, with a hydrophilic-close-to-ionic character. Therefore, the analysis is not successful on common chromatography systems with reversed-phase columns. Using HILIC separation mode based on aminopropyl or amidic stationary phases has advantages in the separation of many polar analytes with negatively charged functional groups, which are commonly present in biofluids or cell extracts. Moreover, analytes with a strong ionic character are retained even more. Typically, purine or pyrimidine analytes from bases up to nucleotide triphosphates can be separated in one analysis (**Figures 2** and **3**). The most important parameter for the separation is a pH with a value above 9.5, where triphosphates are not so strongly retained and the analysis time can be reduced to less than 20 min. The first general issue of using an aminopropyl column is the limited lifetime of the column (<400 analyses), which can be overcome by using an amidic stationary phase (although the separation behaviour is different). A second issue is the presence of significant ion suppression and lower sensitivity for more retained compounds which are eluted by the alkaline buffer in water. This can be overcome by using a thinner column with an inner diameter of 1 mm and by subsequently reducing the flow rate to 50 $\mu$L min$^{-1}$ and by increasing the time taken for the analysis to 1 h.

The profiles of adenine, guanine, cytosine and uracil bases, ribosides and nucleotides in the cell line 143B incubated with EdU are shown in **Figures 2** and **3**. Purine bases, ribosides and monophosphates are most sensitive in positive ionization mode compared to di- and triphosphates, which are significantly more ionizable in negative mode. On the contrary, the analysis of uracil and EdU and its nucleotides is more sensitive in negative mode for all the compounds. Three peaks in the range 7–12 min, corresponding to the electrospray fragmentation of nucleotides on bases, can be seen in a chromatogram of adenine SRM transition. Similarly, this phenomenon is also present in other chromatograms (AMP, ADP, GDP …). The new generation of triple quadrupoles brings the dynamic range of intensities up to seven orders, which is very useful for the multicomponent analysis of compounds with a large span of detector responses, such as biofluids. For example, adenosine has an intensity that is three orders higher compared to the uridine in the same sample. The disadvantage of the method is its inability to analyse isobaric compounds with the same separation behaviour: AMP/dGMP, ADP/dGDP and ATP/dGTP.

The cell line 143B was incubated with EdU and the corresponding nucleotides were followed. Because of the toxicity of EdU, the concentration in the incubation mixture was relatively low (10 $\mu$mol L$^{-1}$) and therefore the production of nucleotides was limited to nmol-$\mu$mol L$^{-1}$
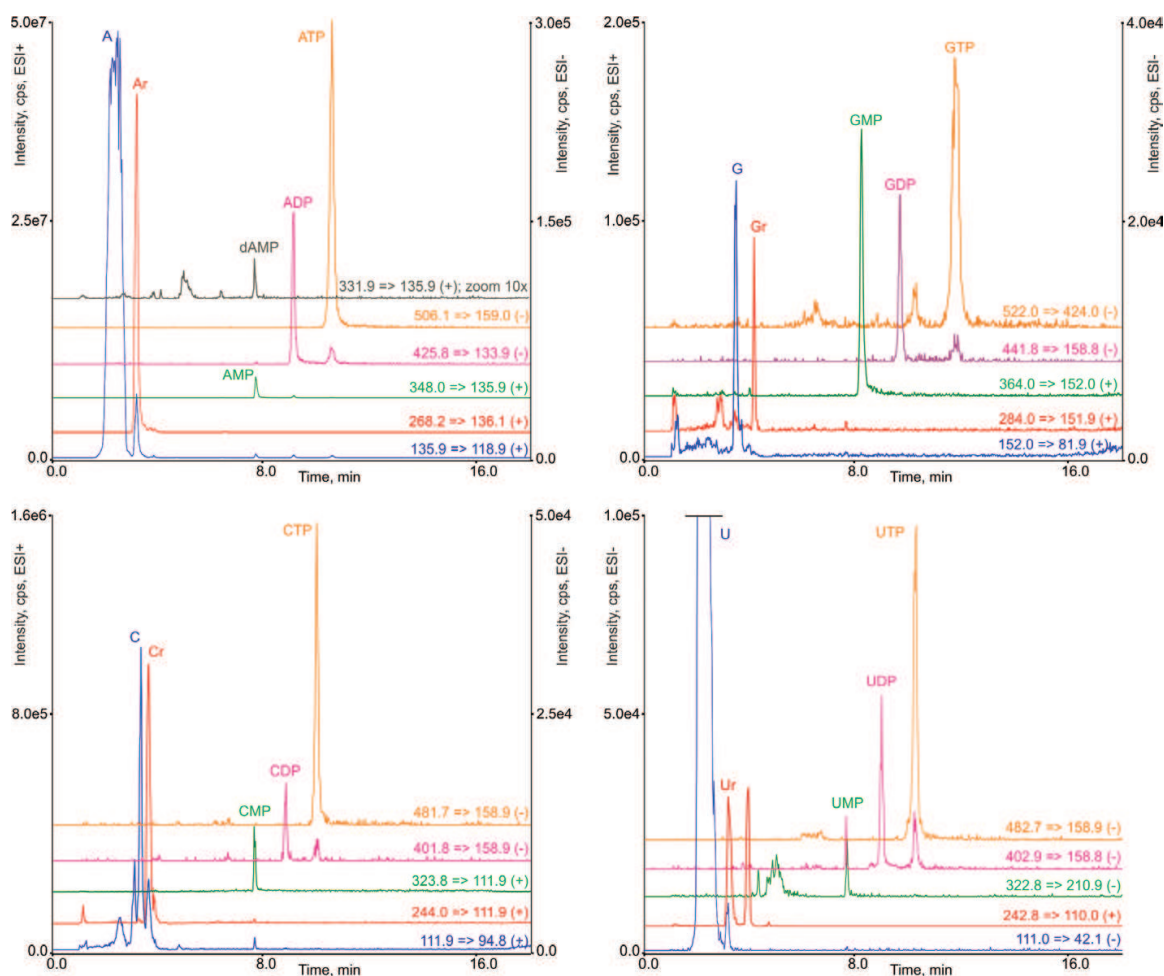
**Figure 2.** Extracted SRM chromatograms of selected endogenous bases, ribosides and nucleotides in intracellular content of 143B cell line.

concentration levels. **Figure 3** shows chromatograms of EdU and modified nucleotides in the intracellular extract of the incubated cell lines and controls without the addition of EdU. The optimization of the SRM transitions for commercially available EdU and EdUTP was performed. EdUMP and EdUDP were calculated theoretically and SRM transitions with fragments of the pyrimidine base and one phosphate group for EdUMP and EdUDMP offered the most sensitive signal. The approximate concentrations of these two commercially unavailable nucleotides were calculated from the ratio of the analogous SRM of the standards of adenine nucleotides.

### 7.4. Conclusion

Highly sensitive LC-MS/MS method for determination of intracellular levels of nucleotide analogues EdU and EdC and its phosphorylated metabolites and several endogenous nucleotides was described. The use of aminopropyl column in HILIC adjustment enables efficient separation of polar analytes during 17 min. Despite the limitations of the HILIC separation approach described above, it is very promising and probably the best way to analyse a wide range of
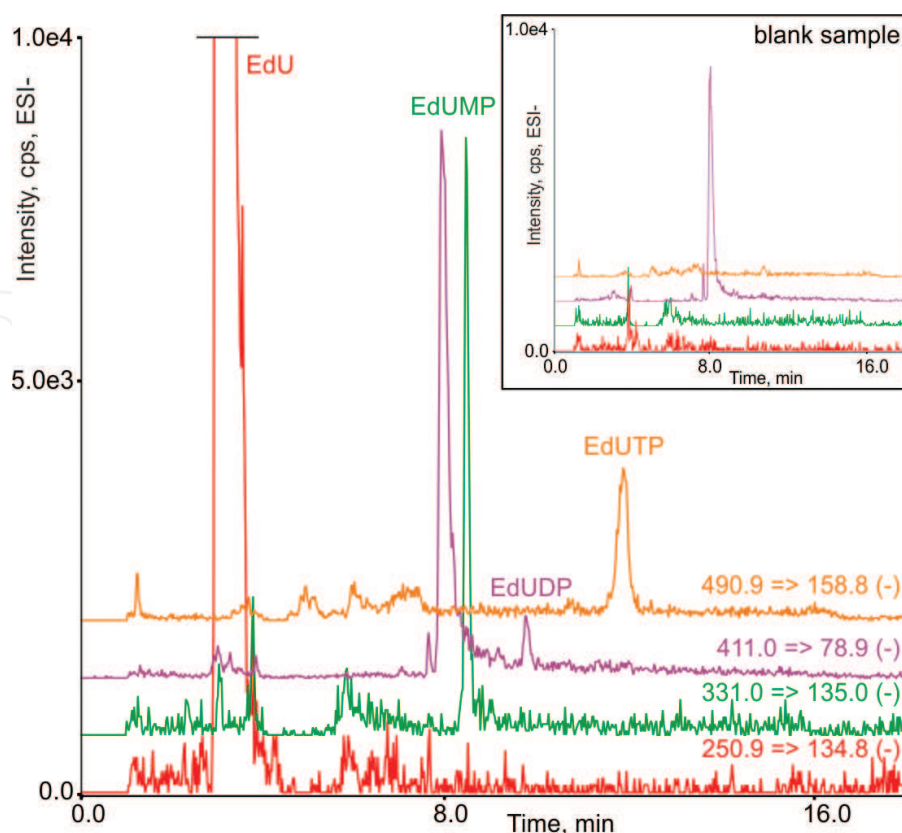
**Figure 3.** Extracted SRM chromatograms of EdU, EdUMP, EdUDP and EdUTP in intracellular content of 143B cell line treated by EdU.

purine and pyrimidine bases, ribosides and nucleotides within single analysis. Coupled with triple quadrupole mass spectrometry, it offers excellent selectivity and sensitivity, with a high linear response. Sample preparation based on cell lysis and protein precipitation by methanol without a need of SPE extraction allows application of the method on clinical studies. It can be applied on drug monitoring, pharmacokinetics studies and provides an insight into the influence of the nucleotide analogue on intracellular nucleotide pool *in vitro*.

# Acknowledgements

# Abbreviations

| | |
|---|---|
| 2cDA | Chlorodeoxyadenosine/cladribine |
| 3TC | Lamivudine |
| AcCN | Acetonitrile |
| ADP | Adenosine diphosphate |
| AICA | 5-Aminoimidazole-4-carboxamide |

| | |
|---|---|
| AMP | Adenosine monophosphate |
| araC | Cytarabine |
| araCMP | Cytarabine monophosphate |
| araCTP | Cytarabine triphosphate |
| ATP | Adenosine triphosphate |
| AX | Anion exchange |
| AZdU | Azidodeoxyuridine |
| AZT | Zidovudine |
| BrATP | Bromoadenosine triphosphate |
| cAMP | Cyclic adenosine monophosphate |
| CBV | Carbovir |
| cCMP | Cyclic cytidine monophosphate |
| CDP | Cytidine diphosphate |
| CE | Capillary electrophoresis |
| CEC | Capillary electrochromatography |
| CGE | Capillary gel electrophoresis |
| cGMP | Cyclic guanosine monophosphate |
| CIEF | Capillary isoelectric focusing |
| cIMP | Cyclic inosine monophosphate |
| CoA | Coenzyme A |
| CSF | Cerebrospinal fluid |
| CTP | Cytidine triphosphate |
| CU | Chlorouracil |
| cUMP | Cyclic uridine monophosphate |
| CZE | Capillary zone electrophoresis |
| d4T | Stavudine |
| dATP | Deoxyadenosine triphosphate |
| dCTP | Deoxycytidine triphosphate |
| ddA | Dideoxyadenosine |
| ddAMP | Dideoxyadenosine monophosphate |
| ddATP | Dideoxyadenosine triphosphate |
| ddI | Didanosine |
| ddN | Dideoxynucleotides |
| DEA | Diethylamine |
| dFdC | Difluorodeoxycytidine/gemcitabine |
| dFdU | Difluorodeoxyuridine |

| | |
|---|---|
| dGTP | Deoxyguanosine triphosphate |
| DMHA | Dimethylhexylamine |
| DNA | Deoxyribonucleic acid |
| DP | Diphosphate/declustering potential |
| dTTP | Deoxythymidine triphosphate |
| EdC | Ethynyldeoxycytidine |
| EDTA | Ethylenediaminetetraacetic acid |
| EOF | Electro-osmotic flow |
| ESI | Electrospray ionization |
| EtOH | Ethanol |
| FAD | Flavinadeninedinucleotide |
| FdUMP | Fluorodeoxyuridine monophosphate |
| FdUrd | Fluorodeoxyuridine |
| FdUTP | Fluorodeoxyuridine triphosphate |
| FLT | 3′-Deoxy-3′-fluorothymidine |
| FTC | Emtricitabine |
| FU | Fluorouracil |
| FUrd | Fluorouridine |
| FUTP | Fluorouridine triphosphate |
| GMP | Guanosine monophosphate |
| GDP | Guanosine diphosphate |
| GTP | Guanosine triphosphate |
| HA | Hexylamine |
| HFIP | Hexafluoropropanol |
| HILIC | Hydrophilic interaction liquid chromatography |
| HIV | Human immunodeficiency virus |
| HPLC | High-performance liquid chromatography |
| CHO | Chinese hamster ovarian |
| IDP | Inosine diphosphate |
| IMP | Inosine monophosphate |
| IP | Ion-pairing |
| IPC | Ion-pairing chromatography |
| ITP | Isotachophoresis |
| IX | Ion exchange |
| LC | Liquid chromatography |

| | |
|---|---|
| LLE | Liquid-liquid extraction |
| LLOQ | Lower limit of quantification |
| MALDI-TOF | Matrix-assisted laser desorption/ionization-time of flight |
| MeCTP | Methylcytidine triphosphate |
| MeGTP | Methylguanosine triphosphate |
| MEKC | Micellar electrokinetic chromatography |
| MeTI | Methylthioinosine |
| MetOH | Methanol |
| MeTG | Methylthioguanosine |
| MP | Monophosphate |
| MRM | Multiple reaction monitoring |
| MS | Mass spectrometry |
| MS/MS | Tandem mass spectrometry |
| NAD | Nicotinamide adenine dinucleotide |
| NADP | Nicotinamide adenine dinucleotide phosphate |
| NHC | N-hydroxycytidine |
| NRTI | Nucleotide reverse transcriptase inhibitor |
| NTPs | Nucleotide triphosphates |
| PBMC | Peripheral blood mononuclear cells |
| PBS | Phosphate buffer saline |
| PCA | Perchloric acid |
| PGC | Porous graphitic carbon |
| PP | Protein precipitation |
| RBC | Red blood cells |
| RBV | Ribavirin |
| RNA | Ribonucleotide acid |
| RP | Reversed phase |
| SPE | Solid phase extraction |
| TBA | Tetrabutylammonium |
| TCA | Trichloroacetic acid |
| TDP | Thymidine diphosphate |
| TEA | Triethylamine CTP |
| TI | Thioinosine |
| TFV | Tenofovir |
| TG | Thioguanosine |

| TK 1 | Thymidine kinase 1 |
| TMP | Thymidine monophosphate |
| TP | Triphosphate |
| TTP | Thymidine triphosphate |
| UDP | Uridine diphosphate |
| UMP | Uridine monophosphate |
| UTP | Uridine triphosphate |
| UV/DAD | Ultraviolet/diode array detection |
| WAX | Weak anion exchange |
| ZDV | Zidovudine |
| ZR | 5-Amino-4-imidazolecarboxamide riboside |

## Author details

Kateřina Mičová[1,2], David Friedecký[1,2]* and Tomáš Adam[1,2]

*Address all correspondence to: david.friedecky@gmail.com

1 Faculty of Medicine and Dentistry, Institute of Molecular and Translational Medicine, Palacky University, Olomouc, Czech Republic

2 Department of Clinical Chemistry, University Hospital Olomouc, Olomouc, Czech Republic

## References

[1] Korycka A, Lech-Marańda E, Robak T. Novel purine nucleoside analogues for hematological malignancies. Recent Patents on Anti Cancer Drug Discovery. January 2008;**3**(2):123–136

[2] Gesto DS, Cerqueira NMFSA, Fernandes PA, Ramos MJ. Gemcitabine: A critical nucleoside for cancer therapy. Current Medicinal Chemistry. 2012;**19**(7):1076–1087

[3] Robak T, Robak P. Purine nucleoside analogs in the treatment of rarer chronic lymphoid leukemias. Current Pharmaceutical Design. 2012;**18**(23):3373–3388

[4] Jordheim LP, Durantel D, Zoulim F, Dumontet C. Advances in the development of nucleoside and nucleotide analogues for cancer and viral diseases. Nature Reviews Drug Discovery. 31 May 2013;**12**(6):447–464. DOI: 10.1038/nrd4010

[5] Sarvaria A, Topp Z, Saven A. Current therapy and new directions in the treatment of hairy Cell leukemia. JAMA Oncology. 1 January 2016;**2**(1):123. DOI: 10.1001/jamaoncol.2015.4134

[6]   Hiramatsu N, Yamada R, Takehara T. The suppressive effect of nucleos(t)ide analogue treatment on the incidence of hepatocellular carcinoma in chronic hepatitis B patients. Journal of Gastroenterology and Hepatology. March 2016;**31**(3):546–552. DOI: 10.1111/ jgh.13229.

[7]   Galmarini CM, Mackey JR, Dumontet C. Nucleoside analogues and nucleobases in cancer treatment. Lancet Oncology. 2002;**3**(7):415–424. DOI: 10.1016/S1470-2045(02)00788-X

[8]   Nyhan WL. Disorders of purine and pyrimidine metabolism. Molecular Genetics and Metabolism. September 2005;**86**(1-2):25–33. DOI: 10.1016/j.ymgme.2005.07.027

[9]   Cohen S, Jordheim LP, Megherbi M, Dumontet C, Guitton J. Liquid chromatographic methods for the determination of endogenous nucleotides and nucleotide analogs used in cancer therapy: A review. The Journal of Chromatography B. 2010;**878**(22):1912–1928. DOI: 10.1016/j.jchromb.2010.05.016

[10]  Jansen RS, Rosing H, Schellens JHM, Beijnen JH. Mass spectrometry in the quantitative analysis of therapeutic intracellular nucleotide analogs. Mass Spectrometry Reviews. March 2011;**30**(2):321–343. DOI: 10.1002/mas.20280

[11]  Dudley E, Bond L. Mass spectrometry analysis of nucleosides and nucleotides. Mass Spectrometry Reviews. 2014;**33**(4):302–331. DOI: 10.1002/mas.21388

[12]  Zhang G, Walker AD, Lin Z, Han X, Blatnik M, Steenwyk RC, et al. Strategies for quantitation of endogenous adenine nucleotides in human plasma using novel ion-pair hydrophilic interaction chromatography coupled with tandem mass spectrometry. The Journal of Chromatography A. 2014;**1325**:129–136. DOI: 10.1016/j.chroma.2013.12.017

[13]  Hofmann U, Heinkele G, Angelberger S, Schaeffeler E, Lichtenberger C, Jaeger S, et al. Simultaneous quantification of eleven thiopurine nucleotides by liquid chromatography-tandem mass spectrometry. Analytical Chemistry. 2012;**84**(3):1294–1301. DOI: 10.1021/ac2031699

[14]  Bushman LR, Kiser JJ, Rower JE, Klein B, Zheng JH, Ray ML, et al. Determination of nucleoside analog mono-, di-, and tri-phosphates in cellular matrix by solid phase extraction and ultra-sensitive LC-MS/MS detection. Journal of Pharmaceutical and Biomedical Analysis. 2011;**56**(2):390–401. DOI: 10.1016/j.jpba.2011.05.039

[15]  Jimmerson LC, Bushman LR, Ray ML, Anderson PL, Kiser JJ. A LC-MS/MS method for quantifying adenosine, guanosine and inosine nucleotides in human cells. Pharmaceutical Research. 2017;**34**(1):73–83. DOI: 10.1007/s11095-016-2040-z

[16]  Pruvost A, Théodoro F, Agrofoglio L, Negredo E, Bénech H. Specificity enhancement with LC-positive ESI-MS/MS for the measurement of nucleotides: Application to the quantitative determination of carbovir triphosphate, lamivudine triphosphate and tenofovir diphosphate in human peripheral blood mononuclear cells. The Journal of Mass Spectrometry. February 2008;**43**(2):224–233. DOI: 10.1002/jms.1294

[17]  Veltkamp SA, Hillebrand MJX, Rosing H, Jansen RS, Wickremsinhe ER, Perkins EJ, et al. Quantitative analysis of gemcitabine triphosphate in human peripheral blood

mononuclear cells using weak anion-exchange liquid chromatography coupled with tandem mass spectrometry. The Journal of Mass Spectrometry. December 2006;**41**(12):1633–1642. DOI: 10.1002/jms.1133

[18] Derissen EJB, Hillebrand MJX, Rosing H, Schellens JHM, Beijnen JH. Development of an LC-MS/MS assay for the quantitative determination of the intracellular 5-fluorouracil nucleotides responsible for the anticancer effect of 5-fluorouracil. Journal of Pharmaceutical and Biomedical Analysis. 2015;**110**:58–66. DOI: 10.1016/j.jpba.2015.02.051

[19] Kinai E, Gatanaga H, Kikuchi Y, Oka S, Kato S. Ultrasensitive method to quantify intracellular zidovudine mono-, di-and triphosphate concentrations in peripheral blood mononuclear cells by liquid chromatography–tandem mass spectrometry. The Journal of Mass Spectrometry. 2015;**50**(6):783–791. DOI: 10.1002/jms.3577

[20] Durand-Gasselin L, Pruvost A, Dehée A, Vaudre G, Tabone M-D, Grassi J, et al. High levels of zidovudine (AZT) and its intracellular phosphate metabolites in AZT- and AZT-lamivudine-treated newborns of human immunodeficiency virus-infected mothers. Antimicrobial Agents and Chemotherapy. July 2008;**52**(7):2555–2563. DOI: 10.1128/AAC.01130-07

[21] Becher F, Schlemmer D, Pruvost A, Nevers M-C, Goujard C, Jorajuria S, et al. Development of a direct assay for measuring intracellular AZT triphosphate in humans peripheral blood mononuclear cells. Analytical Chemistry. 15 August 2002;**74**(16):4220–4227

[22] Rodriguez JF, Rodriguez JL, Santana J, Garcia H, Rosario O. Simultaneous quantitation of intracellular zidovudine and lamivudine triphosphates in human immunodeficiency virus-infected individuals. Antimicrobial Agents and Chemotherapy. 2000;**44**(11):3097–3100. DOI: 10.1128/AAC.44.11.3097-3100.2000

[23] Pruvost A, Becher F, Bardouille P, Guerrero C, Creminon C, Delfraissy JF, et al. Direct determination of phosphorylated intracellular anabolites of stavudine (d4T) by liquid chromatography/tandem mass spectrometry. Rapid Communications in Mass Spectrometry. 2001;**15**(16):1401–1408. DOI: 10.1002/rcm.384

[24] Tu X, Lu Y, Zhong D, Zhang Y, Chen X. A sensitive LC-MS/MS method for quantifying clofarabine triphosphate concentrations in human peripheral blood mononuclear cells. The Journal of Chromatography B. 2014;**964**:202–207. DOI: 10.1016/j.jchromb.2014.01.021

[25] Wang J, Lin T, Lai J, Cai Z, Yang MS. Analysis of adenosine phosphates in HepG-2 cell by a HPLC-ESI-MS system with porous graphitic carbon as stationary phase. Journal of Chromatography B Analytical Technologies in the Biomedical and Life Sciences. 2009;**877**(22):2019–2024. DOI: 10.1016/j.jchromb.2009.05.027

[26] Chen P, Liu Z, Liu S, Xie Z, Aimiuwu J, Pang J, et al. A LC-MS/MS method for the analysis of intracellular nucleoside triphosphate levels. Pharmaceutical Research. June 2009;**26**(6):1504–1515. DOI: 10.1007/s11095-009-9863-9

[27] Jansen RS, Rosing H, De Wolf CJF, Beijnen JH. Development and validation of an assay for the quantitative determination of cladribine nucleotides in MDCKII cells and

culture medium using weak anion-exchange liquid chromatography coupled with tandem mass spectrometry. Rapid Communications in Mass Spectrometry. 30 December 2007;**21**(24):4049–4059. DOI: 10.1002/rcm.3318

[28] Vela JE, Olson LY, Huang A, Fridland A, Ray AS. Simultaneous quantitation of the nucleotide analog adefovir, its phosphorylated anabolites and 2′-deoxyadenosine triphosphate by ion-pairing LC/MS/MS. Journal of Chromatography B Analytical Technologies in the Biomedical and Life Sciences. 2007;**848**(2):335–343. DOI: 10.1016/j.jchromb.2006.10.063

[29] Machon C, Jordheim LP, Puy JY, Lefebvre I, Dumontet C, Guitton J. Fully validated assay for the quantification of endogenous nucleoside mono- and triphosphates using online extraction coupled with liquid chromatography-tandem mass spectrometry. Analytical and Bioanalytical Chemistry. 2014;**406**(12):2925–2941. DOI: 10.1007/s00216-014-7711-1

[30] Bähre H, Kaever V. Measurement of 2′,3′-cyclic nucleotides by liquid chromatography-tandem mass spectrometry in cells. Journal of Chromatography B Analytical Technologies in the Biomedical and Life Sciences. 2014;**964**:208–211. DOI: 10.1016/j.jchromb.2014.02.046

[31] Cordell RL, Hill SJ, Ortori CA, Barrett DA. Quantitative profiling of nucleotides and related phosphate-containing metabolites in cultured mammalian cells by liquid chromatography tandem electrospray mass spectrometry. Journal of Chromatography B Analytical Technologies in the Biomedical and Life Sciences. 2008;**871**(1):115–124. DOI: 10.1016/j.jchromb.2008.07.005

[32] Wu J, Zhang Y, Wiegand R, Wang J, Bepler G, Li J. Quantitative analysis of intracellular nucleoside triphosphates and other polar metabolites using ion pair reversed-phase liquid chromatography coupled with tandem mass spectrometry. Journal of Chromatography B Analytical Technologies in the Biomedical and Life Sciences. 2015;**1006**:167–178. DOI: 10.1016/j.jchromb.2015.10.030

[33] Cahours X, Tran TT, Mesplet N, Kieda C, Morin P, Agrofoglio LA. Analysis of intracellular didanosine triphosphate at sub-ppb level using LC-MS/MS. Journal of Pharmaceutical and Biomedical Analysis. 2001;**26**(5-6):819–827. DOI: 10.1016/S0731-7085(01)00457-5

[34] Goutier W, Spaans PA, van der Neut MAW, McCreary AC, Reinders JH. Development and application of an LC-MS/MS method for measuring the effect of (partial) agonists on cAMP accumulation in vitro. The Journal of Neuroscience Methods. 2010;**188**(1):24–31. DOI: 10.1016/j.jneumeth.2010.01.027

[35] Huang YF, Chang HT. Analysis of adenosine triphosphate and glutathione through gold nanoparticles assisted laser desorption/ionization mass spectrometry. Analytical Chemistry. 2007;**79**(13):4852–4859. DOI: 10.1021/ac070023x

[36] Oeckl P, Ferger B. Simultaneous LC-MS/MS analysis of the biomarkers cAMP and cGMP in plasma, CSF and brain tissue. The Journal of Neuroscience Methods. 2012;**203**(2):338–343. DOI: 10.1016/j.jneumeth.2011.09.032

[37] Zhang Y, Dufield D, Klover J, Li W, Szekely-Klepser G, Lepsy C, et al. Development and validation of an LC-MS/MS method for quantification of cyclic guanosine 3′,5′-monophosphate (cGMP) in clinical applications: A comparison with a EIA method. Journal of Chromatography B Analytical Technologies in the Biomedical and Life Sciences. 2009;**877**(5-6):513–520. DOI: 10.1016/j.jchromb.2008.12.063

[38] Dudley E, El-shakawi S, Games DE, Newton RP. Development of a purification procedure for the isolation of nucleosides from urine prior to mass spectrometric analysis. Nucleosides, Nucleotides and Nucleic Acids. March 2000;**19**(3):545–558. DOI: 10.1080/15257770008035006

[39] Pucci V, Giuliano C, Zhang R, Koeplinger KA, Leone JF, Monteagudo E, et al. HILIC LC-MS for the determination of 2′-C-methyl-cytidine-triphosphate in rat liver. Journal of Separation Science. May 2009;**32**(9):1275–1283. DOI: 10.1002/jssc.200800722

[40] Teleki A, Sánchez-Kopper A, Takors R. Alkaline conditions in hydrophilic interaction liquid chromatography for intracellular metabolite quantification using tandem mass spectrometry. Analytical Biochemistry. 15 April 2015;**475**:4–13. DOI: 10.1016/j.ab.2015.01.002

[41] Klawitter J, Schmitz V, Klawitter J, Leibfritz D, Christians U. Development and validation of an assay for the quantification of 11 nucleotides using LC/LC-electrospray ionization-MS. Analytical Biochemistry. 15 June 2007;**365**(2):230–239. DOI: 10.1016/j.ab.2007.03.018

[42] Rashidzadeh H, Bhadresa S, Good SS, Cohen ML, Gupta KS, Rush WR. Overcoming stability challenges in the quantification of tissue nucleotides: Determination of 2′-C-methylguanosine triphosphate concentration in mouse liver. Biological and Pharmaceutical Bulletin. 2015;**38**(3):380–388. DOI: 10.1248/bpb.b14-00565

[43] Bajad SU, Lu W, Kimball EH, Yuan J, Peterson C, Rabinowitz JD. Separation and quantitation of water soluble cellular metabolites by hydrophilic interaction chromatography-tandem mass spectrometry. The Journal of Chromatography A. 2006;**1125**(1):76–88. DOI: 10.1016/j.chroma.2006.05.019

[44] Edwards JL, Kennedy RT. Metabolomic analysis of eukaryotic tissue and prokaryotes using negative mode MALDI time-of-flight mass spectrometry. Analytical Chemistry. 2005;**77**(7):2201–2209. DOI: 10.1021/ac048323r

[45] Wang JM, Chu Y, Li W, Wang XY, Guo JH, Yan LL, et al. Simultaneous determination of creatine phosphate, creatine and 12 nucleotides in rat heart by LC-MS/MS. Journal of Chromatography B Analytical Technologies in the Biomedical and Life Sciences. 2014;**958**:96–101. DOI: 10.1016/j.jchromb.2014.03.008

[46] Bapiro TE, Richards FM, Goldgraben MA, Olive KP, Madhu B, Frese KK, et al. A novel method for quantification of gemcitabine and its metabolites 2′,2′-difluorodeoxyuridine and gemcitabine triphosphate in tumour tissue by LC-MS/MS: Comparison with 19F NMR spectroscopy. Cancer Chemotherapy Pharmacology. 2011;**68**(5):1243–1253. DOI: 10.1007/s00280-011-1613-0

[47] Luo B, Groenke K, Takors R, Wandrey C, Oldiges M. Simultaneous determination of multiple intracellular metabolites in glycolysis, pentose phosphate pathway and

tricarboxylic acid cycle by liquid chromatography-mass spectrometry. The Journal of Chromatography A. 2007;**1147**(2):153–164. DOI: 10.1016/j.chroma.2007.02.034

[48] Neufeld HA, Towner RD, Pace J. A rapid method for determining ATP by the firefly luciferin-luciferase system. Experientia. 1975;**31**(3):391–392

[49] Aherne GW, Hardcastle A, Raynaud F, Jackman AL. Immunoreactive dUMP and TTP pools as an index of thymidylate synthase inhibition; effect of tomudex (ZD1694) and a nonpolyglutamated quinazoline antifolate (CB30900) in L1210 mouse leukaemia cells. Biochemical Pharmacology. May 1996;**51**(10):1293–1301. DOI: 10.1016/0006-2952(96)00035-4

[50] Piall EM, Aherne GW, Marks V. The quantitative determination of 2′-deoxycytidine-5′-triphosphate in cell extracts by radioimmunoassay. Analytical Biochemistry. April 1986;**154**(1):276–281. DOI: 10.1016/0003-2697(86)90526-9

[51] Ohyama T, Matsubara C, Takamura K. Highly sensitive densitometry for sugarphosphoesters and nucleotides using enzymatic hydrolysis on a TLC plate. Chemical and Pharmaceutical bulletin. 1996;**44**(6):1252–1254

[52] Bezy V, Chaimbault P, Morin P, Unger SE, Bernard M-C, Agrofoglio LA. Analysis and validation of the phosphorylated metabolites of two anti-human immunodeficiency virus nucleotides (stavudine and didanosine) by pressure-assisted CE-ESI-MS/MS in cell extracts: Sensitivity enhancement by the use of perfluorinated acids and al. Electrophoresis. June 2006;**27**(12):2464–2476. DOI: 10.1002/elps.200500850

[53] Cai Z, Fung EN, Sinhababu AK. Capillary electrophoresis-ion trap mass spectrometry analysis of Ziagen and its phosphorylated metabolites. Electrophoresis. September 2003;**24**(18):3160–3164. DOI: 10.1002/elps.200305425

[54] Friedecký D, Tomková J, Maier VV, Janost'áková A, Procházka M, Adam TT, et al. Capillary electrophoretic method for nucleotide analysis in cells: Application on inherited metabolic disorders. Electrophoresis. February 2007;**28**(3):373–380. DOI: 10.1002/elps.200600262

[55] Soga T, Ishikawa T, Igarashi S, Sugawara K, Kakazu Y, Tomita M. Analysis of nucleotides by pressure-assisted capillary electrophoresis-mass spectrometry using silanol mask technique. The Journal of Chromatography A. 2007;**1159**(1-2):125–133. DOI: 10.1016/j.chroma.2007.05.054

[56] Liu CC, Huang JS, Tyrrell DLJ, Dovichi NJ. Capillary electrophoresis-electrospray-mass spectrometry of nucleosides and nucleotides: application to phosphorylation studies of anti-human immunodeficiency virus nucleosides in a human hepatoma cell line. Electrophoresis. April 2005;**26**(7-8):1424–1431. DOI: 10.1002/elps.200410423

[57] Derissen EJB, Jacobs BAW, Huitema ADR, Rosing H, Schellens JHM, Beijnen JH. Exploring the intracellular pharmacokinetics of the 5-fluorouracil nucleotides during capecitabine treatment. British Journal of Clinical Pharmacology. 2016;**81**(5):949–957. DOI: 10.1111/bcp.12877

[58]  Faria M, Halquist MS, Kindt E, Li W, Karnes HT, O'Brien PJ. Liquid chromatography–tandem mass spectrometry method for quantification of thymidine kinase activity in human serum by monitoring the conversion of 3'-deoxy-3'-fluorothymidine to 3'-deoxy-3'-fluorothymidine monophosphate. The Journal of Chromatography B. 2012;**907**:13–20. DOI: 10.1016/j.jchromb.2012.08.024

[59]  Jimmerson LC, Ray ML, Bushman LR, Anderson PL, Klein B, Rower JE, et al. Measurement of intracellular ribavirin mono-, di- and triphosphate using solid phase extraction and LC-MS/MS quantification. Journal of Chromatography B Analytical Technologies in the Biomedical and Life Sciences. 2015;**978-979**(C):163–172. DOI: 10.1016/j.jchromb.2014.11.032

[60]  Kamčeva T, Bjånes T, Svardal A, Riedel B, Schjøtt J, Eide T. Liquid chromatography/tandem mass spectrometry method for simultaneous quantification of eight endogenous nucleotides and the intracellular gemcitabine metabolite dFdCTP in human peripheral blood mononuclear cells. The Journal of Chromatography B. September 2015;**1001**:212–220. DOI: 10.1016/j.jchromb.2015.07.041

[61]  Mateos-Vivas M, Rodríguez-Gonzalo E, García-Gómez D, Carabias-Martínez R. Hydrophilic interaction chromatography coupled to tandem mass spectrometry in the presence of hydrophilic ion-pairing reagents for the separation of nucleosides and nucleotide mono-, di- and triphosphates. The Journal of Chromatography A. 2015;**1414**:129–137. DOI: 10.1016/j.chroma.2015.08.040

[62]  Oeckl P, Steinacker P, Lehnert S, Jesse S, Kretzschmar HA, Ludolph AC, et al. Csf concentrations of camp and cgmp are lower in patients with creutzfeldt-jakob disease but not Parkinson's disease and amyotrophic lateral sclerosis. PLoS One. 2012;**7**(3):e32664. DOI: 10.1371/journal.pone.0032664

[63]  Coolen EJCM, Arts ICW, Swennen ELR, Bast A, Stuart MAC, Dagnelie PC. Simultaneous determination of adenosine triphosphate and its metabolites in human whole blood by RP-HPLC and UV-detection. The Journal of Chromatography B. 15 March 2008;**864**(1-2):43–51. DOI: 10.1016/j.jchromb.2008.01.033

[64]  Caruso R, Campolo J, Dellanoce C, Mariele R, Parodi O, Accinni R. Critical study of preanalytical and analytical phases of adenine and pyridine nucleotide assay in human whole blood. Analytical Biochemistry. 1 July 2004;**330**(1):43–51. DOI: 10.1016/j.ab.2004.03.063

[65]  Yeung P, Ding L, Casley WL. HPLC assay with UV detection for determination of RBC purine nucleotide concentrations and application for biomarker study in vivo. Journal of Pharmaceutical and Biomedical Analysis. 9 June 2008;**47**(2):377–382. DOI: 10.1016/j.jpba.2008.01.020

[66]  Czarnecka J, Cieślak M, Michał K. Application of solid phase extraction and high-performance liquid chromatography to qualitative and quantitative analysis of nucleotides and nucleosides in human cerebrospinal fluid. Journal of Chromatography B Analytical Technologies in the Biomedical and Life Sciences. 5 August 2005;**822**(1-2):85–90. DOI: 10.1016/j.jchromb.2005.05.026

[67]  Huang D, Zhang Y, Chen X. Analysis of intracellular nucleoside triphosphate levels in normal and tumor cell lines by high-performance liquid chromatography. Journal of Chromatography B Analytical Technologies in the Biomedical and Life Sciences. 25 January 2003;**784**(1):101–109

[68]  Losa R, Sierra M, Gion M, Esteban E, Buesa J. Simultaneous determination of gemcitabine di- and triphosphate in human blood mononuclear and cancer cells by RP-HPLC and UV detection. The Journal of Chromatography B. 7 August 2006;**840**(1):44–49. DOI: 10.1016/j.jchromb.2006.04.036

[69]  Nishi R, Yamauchi T, Ueda T. A new, simple method for quantifying gemcitabine triphosphate in cancer cells using isocratic high-performance liquid chromatography. Cancer Science. November 2006;**97**(11):1274–1278. DOI: 10.1111/j.1349-7006.2006.00323.x

[70]  Sparidans RW, Crul M, Schellens JH, Beijnen JH. Isocratic ion-exchange chromatographic assay for the nucleotide gemcitabine triphosphate in human white blood cells. The Journal of Chromatography B. 2002;**780**(2):423–430. DOI: 10.1016/S1570-0232(02)00638-4

[71]  Yamauchi T, Ueda T. Simple and sensitive method for quantification of fludarabine triphosphate intracellular concentration in leukemic cells using isocratic liquid chromatography. Journal of Chromatography B Analytical Technologies in the Biomedical and Life Sciences. 5 January 2004;**799**(1):81–86

[72]  Rodriguez CO, Plunkett W, Paff MT, Du M, Nowak B, Ramakrishna P, et al. High-performance liquid chromatography method for the determination and quantitation of arabinosylguanine triphosphate and fludarabine triphosphate in human cells. Journal of Chromatography B Biomedical Science and Applications. 18 August 2000;**745**(2):421–430. DOI: 10.1128/AAC.44.11.3097-3100.2000

[73]  Haink G, Deussen A. Liquid chromatography method for the analysis of adenosine compounds. The Journal of Chromatography B. January 2003;**784**(1):189–193. DOI: 10.1016/S1570-0232(02)00752-3

[74]  Andersson C-O, Sørensen P, Stenhagen E, Hartiala K, Veige S, Diczfalusy E. Mass spectrometric studies on amino acid and peptide derivatives. Acta Chemica Scandinavica. 1958;**12**:1353. DOI: 10.3891/acta.chem.scand.12-1353a

[75]  Friedecký D, Bednar P, Prochazka M, Adam T. Analysis of intracellular nucleotides by capillary electrophoresis. Mass spectrometry. Nucleosides, Nucleotides and Nucleic Acids. 2006;**25**(9-11):1233–1236. DOI: 10.1080/15257770600894667

[76]  Seifar RM, Ras C, van Dam JC, van Gulik WM, Heijnen JJ, van Winden WA. Simultaneous quantification of free nucleotides in complex biological samples using ion pair reversed phase liquid chromatography isotope dilution tandem mass spectrometry. Analytical Biochemistry. May 2009;**388**(2):213–219. DOI: 10.1016/j.ab.2009.02.025

[77]  Williams L, Vontungeln L, Beland F, Doerge D. Liquid chromatographic–mass spectrometric determination of the metabolism and disposition of the anti-retroviral nucleoside analogs zidovudine and lamivudine in C57BL/6N and B6C3F1 mice. The Journal of Chromatography B. 5 December 2003;**798**(1):55–62. DOI: 10.1016/j.jchromb.2003.08.051

[78] King T, Bushman L, Anderson PL, Delahunty T, Ray M, Fletcher CV. Quantitation of zidovudine triphosphate concentrations from human peripheral blood mononuclear cells by anion exchange solid phase extraction and liquid chromatography-tandem mass spectroscopy; An indirect quantitation methodology. Journal of Chromatography B Analytical Technologies in the Biomedical and Life Sciences. 2006;**831**(1-2):248–257. DOI: 10.1016/j.jchromb.2005.12.033

[79] Inoue K, Obara R, Hino T, Oka H. Development and application of an HILIC-MS/MS method for the quantitation of nucleotides in infant formula. Journal of Agricultural and Food Chemistry. 22 September 2010;**58**(18):9918–9924. DOI: 10.1021/jf102023p

[80] Gouy M-H, Fabre H, Blanchin MD, Peyrottes S, Périgaud C, Lefebvre I. Quantification of 5′-monophosphate cytosine arabinoside (Ara-CMP) in cell extracts using liquid chromatography–electrospray mass spectrometry. Analytica Chimica Acta. 2006;**566**:178–184. DOI: 10.1016/j.aca.2006.02.058

[81] Crauste C, Lefebvre I, Hovaneissian M, Puy JY, Roy B, Peyrottes S, et al. Development of a sensitive and selective LC/MS/MS method for the simultaneous determination of intracellular 1-beta-d-arabinofuranosylcytosine triphosphate (araCTP), cytidine triphosphate (CTP) and deoxycytidine triphosphate (dCTP) in a human follicular l. Journal of Chromatography B Analytical Technologies in the Biomedical and Life Sciences. 2009;**877**(14-15):1417–1425. DOI: 10.1016/j.jchromb.2009.02.071

[82] Fromentin E, Gavegnano C, Obikhod A, Schinazi RF. Simultaneous quantification of intracellular natural and antiretroviral nucleosides and nucleotides by liquid chromatography-tandem mass spectrometry. Analytical Chemistry. 1 March 2010;**82**(5):1982–1989. DOI: 10.1021/ac902737j

[83] Carli D, Honorat M, Cohen S, Megherbi M, Vignal B, Dumontet C, et al. Simultaneous quantification of 5-FU, 5-FUrd, 5-FdUrd, 5-FdUMP, dUMP and TMP in cultured cell models by LC-MS/MS. Journal of Chromatography B Analytical Technologies in the Biomedical and Life Sciences. 2009;**877**(27):2937–2944. DOI: 10.1016/j.jchromb.2009.07.004

[84] Van Kampen JJA, Fraaij PLA, Hira V, Van Rossum AMC, Hartwig NG, De Groot R, et al. A new method for analysis of AZT-triphosphate and nucleotide-triphosphates. Biochemical and Biophysical Research Communications. 2004;**315**(1):151–159. DOI: 10.1016/j.bbrc.2004.01.032

[85] Shi G, Wu J, Li Y, Geleziunas R, Gallagher K, Emm T, et al. Novel direct detection method for quantitative determination of intracellular nucleoside triphosphates using weak anion exchange liquid chromatography/tandem mass spectrometry. Rapid Communications in Mass Spectrometry. 15 June 2002;**16**(11):1092–1099. DOI: 10.1002/rcm.684

[86] Hanai T. Separation of polar compounds using carbon columns. The Journal of Chromatography A. 14 March 2003;**989**(2):183–196

[87] Bapiro TE, Richards FM, Jodrell DI. Understanding the complexity of porous graphitic carbon (PGC) chromatography: Modulation of mobile-stationary phase interactions overcomes loss of retention and reduces variability. Analytical Chemistry. 21 June 2016;**88**(12):6190–6194. DOI: 10.1021/acs.analchem.6b01167

[88] Xing J, Apedo A, Tymiak A, Zhao N. Liquid chromatographic analysis of nucleosides and their mono-, di- and triphosphates using porous graphitic carbon stationary phase coupled with electrospray mass spectrometry. Rapid Communications in Mass Spectrometry. 30 July 2004;**18**(14):1599–1606. DOI: 10.1002/rcm.1524

[89] Jansen RS, Rosing H, Schellens JHM, Beijnen JH. Retention studies of 2′-2′-difluorodeoxycytidine and 2′-2′-difluorodeoxyuridine nucleosides and nucleotides on porous graphitic carbon: Development of a liquid chromatography-tandem mass spectrometry method. The Journal of Chromatography A. 2009;**1216**(15):3168–3174. DOI: 10.1016/j.chroma.2009.02.002

[90] Jansen RS, Rosing H, Schellens JHM, Beijnen JH. Simultaneous quantification of 2′,2′-difluorodeoxycytidine and 2′,2′-difluorodeoxyuridine nucleosides and nucleotides in white blood cells using porous graphitic carbon chromatography coupled with tandem mass spectrometry. Rapid Communications in Mass Spectrometry. 15 October 2009;**23**(19):3040–3050. DOI: 10.1002/rcm.4212

[91] Witters E, Van Dongen W, Esmans EL, Van Onckelen HA. Ion-pair liquid chromatography-electrospray mass spectrometry for the analysis of cyclic nucleotides. Journal of Chromatography B Biomedical Science and Applications. 20 June 1997;**694**(1):55–63

[92] Claire RL. Positive ion electrospray ionization tandem mass spectrometry coupled to ion-pairing high-performance liquid chromatography with a phosphate buffer for the quantitative analysis of intracellular nucleotides. Rapid Communications in Mass Spectrometry. 15 September 2000;**14**(17):1625–1634. DOI: 10.1002/1097-0231(20000915)14:17<1625:AID-RCM72>3.0.CO;2-0

[93] Hawkins T, Veikley W, St Claire RL, Guyer B, Clark N, Kearney BP. Intracellular pharmacokinetics of tenofovir diphosphate, carbovir triphosphate, and lamivudine triphosphate in patients receiving triple-nucleoside regimens. Journal of Acquired Immune Deficiency Syndromes. 2005;**39**(4):406–411. DOI: 10.1097/01.qai.0000167155.44980.e8

[94] Apffel A, Chakel JA, Fischer S, Lichtenwalter K, Hancock WS. New procedure for the use of high-performance liquid chromatography–electrospray ionization mass spectrometry for the analysis of nucleotides and oligonucleotides. The Journal of Chromatography A. 1997;**777**(7):3–21. DOI: 10.1021/ac960916h

[95] Auriola S, Frith J, Rogers MJ, Koivuniemi A, Mönkkönen J. Identification of adenine nucleotide-containing metabolites of bisphosphonate drugs using ion-pair liquid chromatography-electrospray mass spectrometry. Journal of Chromatography B Biomedical Applications. 1997;**704**(1-2):187–195. DOI: 10.1016/S0378-4347(97)00490-8

[96] Mönkkönen H, Moilanen P, Mönkkönen J, Frith JC, Rogers MJ, Auriola S. Analysis of an adenine nucleotide-containing metabolite of clodronate using ion pair high-performance liquid chromatography-electrospray ionisation mass spectrometry. Journal of Chromatography B Biomedical Science and Applications. 2000;**738**(2):395–403. DOI: 10.1016/S0378-4347(99)00559-9

[97] Tuytten R, Lemière F, Dongen WV, Esmans EL, Slegers H. Short capillary ion-pair high-performance liquid chromatography coupled to electrospray (tandem) mass spectrometry for the simultaneous analysis of nucleoside mono-, di- and triphosphates. Newton RP, editor. Rapid Communications in Mass Spectrometry. 30 June 2002;**16**(12):1205–1215. DOI: 10.1002/rcm.704

[98] Becher F, Pruvost A, Goujard C, Guerreiro C, Delfraissy J-F, Grassi J, et al. Improved method for the simultaneous determination of d4T, 3TC and ddl intracellular phosphorylated anabolites in human peripheral-blood mononuclear cells using high-performance liquid chromatography/tandem mass spectrometry. Rapid Communications in Mass Spectrometry. 30 March 2002;**16**(6):555–565. DOI: 10.1002/rcm.605

[99] Lee J, Yoo B-C, Lee H-S, Yoo H-W, Yoo H-H, Kang MJ, et al. Rapid quantitative determination of L-FMAU-TP from human peripheral-blood mononuclear cells of hepatitis B virus-infected patients treated with L-FMAU by ion-pairing, reverse-phase, liquid chromatography/electrospray tandem mass spectrometry. Therapeutic Drug Monitoring. February 2006;**28**(1):131–137

[100] Compain S, Durand-Gasselin L, Grassi J, Benech H. Improved method to quantify intracellular zidovudine mono- and triphosphate in peripheral blood mononuclear cells by liquid chromatography-tandem mass spectrometry. Journal of Mass Spectrometry. March 2007;**42**(3):389-404. DOI: 10.1002/jms.1176

[101] Hernandez-Santiago BI, Beltran T, Stuyver L, Chu CK, Schinazi RF. Metabolism of the anti-hepatitis C virus nucleoside β-D-N4-hydroxycytidine in different liver cells. Antimicrobial Agents and Chemotherapy. 2004;**48**(12):4636-4642. DOI: 10.1128/AAC.48.12.4636

[102] Tang D-Q, Zou L, Yin X-X, Ong CN. HILIC-MS for metabolomics: An attractive and complementary approach to RPLC-MS. Mass Spectrometry Reviews. September 2016;**35**(5):574-600. DOI: 10.1002/mas.21445

[103] Hsieh Y. Potential of HILIC-MS in quantitative bioanalysis of drugs and drug metabolites. Journal of Separation Science. 2008;**31**(9):1481-1491. DOI: 10.1002/jssc.200700451

[104] Karlíková R, Široká J, Friedecký D, Faber E, Hrdá M, Mičová K, et al. Metabolite profiling of the plasma and leukocytes of chronic myeloid leukemia patients. Journal of Proteome Research. 2 September 2016;**15**(9):3158-3166. DOI: 10.1021/acs.jproteome.6b00356

[105] Pesek JJ, Matyska MT, Hearn MTW, Boysen RI. Aqueous normal-phase retention of nucleotides on silica hydride columns. The Journal of Chromatography A. 2009;**1216**(7):1140-1146. DOI: 10.1016/j.chroma.2008.12.046

[106] Sun G, Yang K, Zhao Z, Guan S, Han X, Gross RW. Shotgun metabolomics approach for the analysis of negatively charged water-soluble cellular metabolites from mouse heart tissue shotgun metabolomics approach for the analysis of negatively charged water-soluble cellular metabolites from mouse heart tissue. Analytical Chemistry. 2007;**79**(17):6629-6640. DOI: 10.1021/ac070843

[107] Karlíková R, Široká J, Jahn P, Friedecký D, Gardlo A, Janečková H, et al. Equine atypical myopathy: A metabolic study. Veterinary Journal. 2016;**216**:125-132. DOI: 10.1016/j.tvjl.2016.07.015

[108] Ligasová A, Liboska R, Friedecký D, Mičová K, Adam T, Oždian T, et al. Dr Jekyll and Mr Hyde: A strange case of 5-ethynyl-2'-deoxyuridine and 5-ethynyl-2'-deoxycytidine. Open Biology. January 2016;**6**(1):150172. DOI: 10.1098/rsob.150172