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Review

Liquid chromatographic methods for the determination of endogenous nucleotides and nucleotide analogs used in cancer therapy: A review

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ABSTRACT

Endogenous ribonucleotides and deoxyribonucleotides play a crucial role in cell function. The determination of their levels is of fundamental interest in numerous applications such as energy metabolism, biochemical processes, or in understanding the mechanism of nucleoside analog compounds. Nucleoside analogs are widely used in anticancer therapy. Their mechanisms of action are related to their structural similarity with natural nucleotides. Numerous assays have been described for the determination of endogenous nucleotides or anticancer nucleotide analogs in different matrices such as cellular cultures, tissue or peripheral blood mononuclear cells. The determination of these compounds is challenging due to the large difference of concentrations between ribonucleotides and deoxyribonucleotides, the presence of numerous endogenous interferences in complex matrices and the high polarity of the molecules due to the phosphate moiety. The extraction was generally performed at low temperature and was based on protein precipitation using acid or solvent mixture. This first phase could be coupled with extraction or cleaning step of the supernatant. Liquid chromatography coupled with UV detection and based on ion-exchange chromatography using non-volatile high salt concentrations was largely described for the quantification of nucleotides. However, the development of LC–MS and LC–MS/MS during the last ten years has constituted a sensitive and specific tool. In this case, analytical column was mostly constituted by graphite or C18 stationary phase. Mobile phase was usually based on a mixture of ammonium buffer and acetonitrile and in several assays included a volatile ion-pairing agent. Mass spectrometry detection was performed either with positive or negative electrospray mode according to compounds and mobile phase components. The purpose of the current review is to provide an overview of the most recent chromatographic assays (over the past ten years) developed for the determination of endogenous nucleotides and nucleotide analogs used in cancer therapy. We focused on sample preparation, chromatographic separation and quantitative considerations.

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1. Introduction

Nucleotides are essential for life processes as activated precursors of nucleic acids, essential components of signal-transduction pathways, or source energy providers such as adenosine triphosphate (ATP) and guanosine triphosphate (GTP). The typical cell contains 5–10 times as much RNA as DNA. Consequently, the majority of nucleotide biosynthesis leads to the production of ribonucleoside triphosphates (NTP). However, deoxyribonucleoside triphosphates (dNTP) production also occurs in cells for the replication of their genomes, and all deoxyribonucleotides are synthesized from the corresponding nucleotides. Nucleoside analogs are used extensively in cellular and molecular biology research but are above all available for the treatment of various diseases and used as antiviral products or anticancer drugs. These analogs are metabolised into pharmacologically active nucleotides in cells and compete with natural compounds. Due to this relationship, the determination of natural as well as the analog nucleotides levels is of fundamental interest in numerous applications [1–5]. Over the last decades, liquid chromatography with UV detector (LC-UV) based on ion-exchange (IE) chromatography using non-volatile high salt concentrations was mostly described for the quantification of nucleotides. The development of liquid chromatography with mass spectrometry (LC–MS) and tandem mass spectrometry (LC–MS/MS) during the last ten years has constituted a sensitive and specific tool. However, LC–MS analysis required a new approach concerning the composition of mobile phase compared to IE chromatography. Indeed, typical mobile phase would use a combination of water and methanol or acetonitrile and modifiers such as buffer or counter-ion which would be volatile compounds.

The purpose of the current review was to provide a survey of the latest chromatographic assays (since ten years) for the determination of endogenous nucleotides and nucleotide analogs used in cancer therapy. We focused on the sample pre-treatment from biological samples, the chromatographic separation and the detection mode. Tables summarize the main data concerning the validated methods. In order to perform an easier comparison, the limit of detection (LOD) and/or the limit of quantification (LOQ) were expressed as quantity of compound injected when the data provided in the study allowed the calculation.

This review highlights the difficulties to quantify the natural nucleotides and their analogs in biological matrices due to their chemical similarities, chemical and biological instabilities, high polarity, anionic nature, highly variable concentrations, and presence of numerous endogenous interferences.

2. Brief overview on metabolism and mechanisms of action of nucleoside analogs

The nucleotide metabolism is one of the cellular targets in cancer treatment. Nucleoside analogs (cytarabine, gemcitabine, fludarabine, cladribine and clofarabine) and nucleobases (6-mercaptopurine and thioguanine) as well as fluoropyrimidines (fluorouracil, capecitabine and floxuridine) are all structurally related to endogenous compounds involved in the nucleotide metabolic pathway [6,7]. Their metabolism and mechanism of action are based on interaction with membrane transporters, kinases and intracellular enzymes usually transporting, phosphorylating and transforming endogenous/physiological nucleosides and nucleotides (Fig. 1). The competition between the natural molecules and the drugs at the level of each protein indicates that the pools of endogenous nucleotides and the relative concentration of each derivative could influence on the cytotoxic activity of the drugs. In addition, the exogenous purine and pyrimidine derivatives often induce modifications in endogenous pools. Here

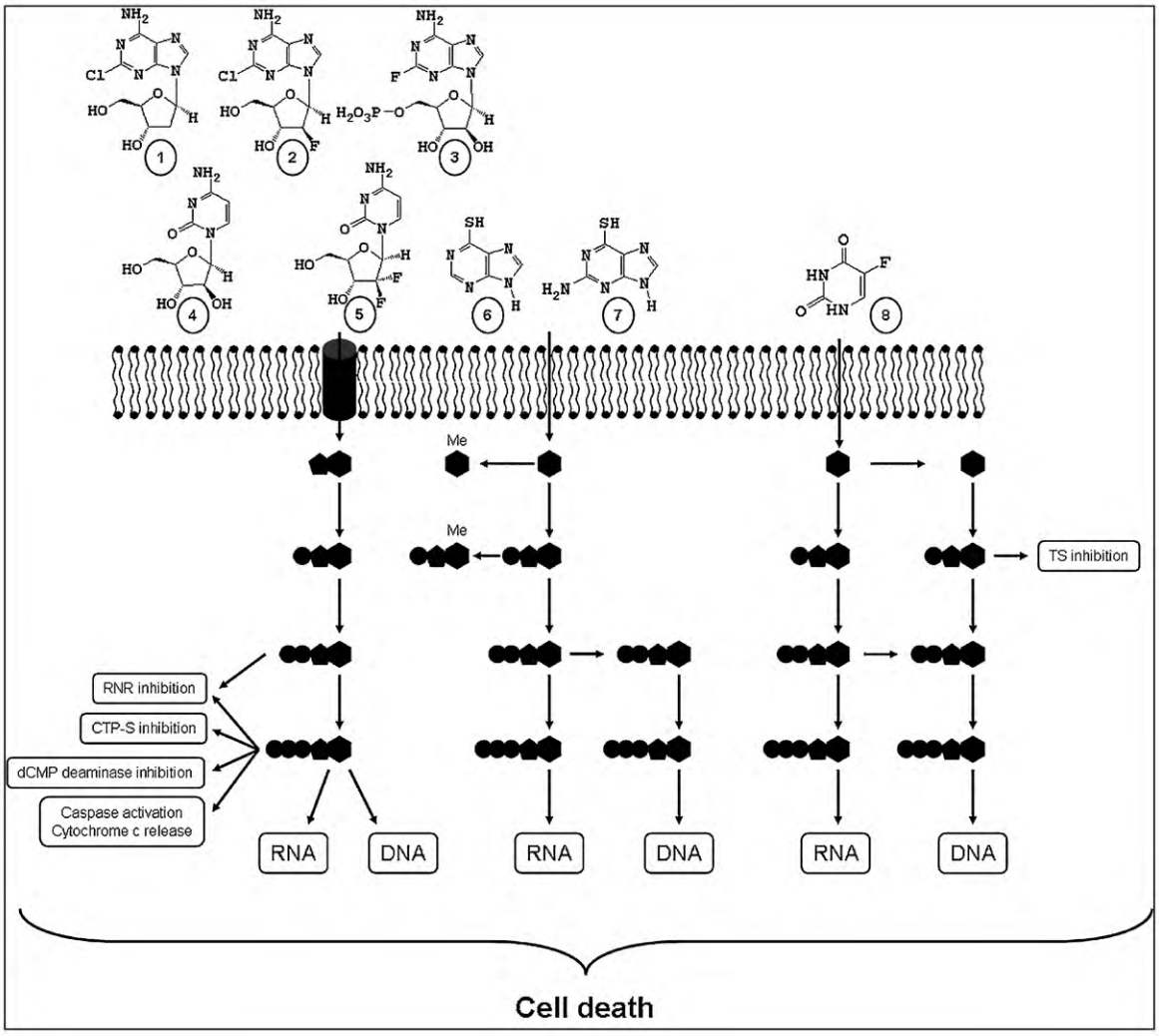

Fig. 1. Intracellular metabolism of nucleoside analogs (left), nucleobases (middle) and fluoropyrimidines (right). 1: Cladribine; 2: Clofarabine; 3: Fludarabine; 4: Cytarabine; 5: Gemcitabine; 6: 6-mercaptopurine; 7: 6-thioguanine; 8: 5-fluorouracil; Me: methyl group; RNR: ribonucleotide reductase; CTP-S: CTP-synthase; TS: thymidylate synthase; FUDR: fluorodeoxyuridine; ⬢: nucleobase; ⬟: (deoxy)ribose; ●: phosphate.

we will shortly describe the mechanism of action of these drugs and emphasize the interaction with the metabolism of endogenous nucleotides.

2.1. Nucleoside analogs

Nucleoside analogs enter the cells by the means of specific nucleoside transporters and are activated by phosphorylation by intracellular kinases. The main cytotoxic effect is obtained after formation of tri-phosphorylated derivatives and their incorporation into DNA leading to the production of non-natural nucleic acids, chain termination, cell cycle arrest and subsequently to cell death.

2.1.1. Deoxycytidine analogs

The triphosphorylated form of deoxycytidine analogs are in direct competition with the natural molecules. Therefore, decreased concentration of deoxycytidine triphosphate (dCTP) should theoretically sensitize the cancer cells to gemcitabine or cytarabine [8,9]. The gemcitabine diphosphate (dFdCDP) inhibits the ribonucleotide reductase (RNR) [10]. dFdCDP acts as a substrate and binds to the active site of the large subunit of RNR [11]. The formation of triphosphosphorylated cytarabine in quiescent B-chronic lymphocytic leukaemia (B-CLL) cells is correlated to inhibition of RNA synthesis [12].

2.1.2. Deoxyadenosine analogs

The triphophorylated forms of deoxyadenosine analogs are potent inhibitors of RNR [13–16]. Cladribine triphosphate, clofarabine triphosphate and fludarabine triphosphate are supposed to bind to the regulatory site for overall activity usually interacting with ATP (activator) or deoxyadenosine triphosphate (dATP) (inhibitor) [11]. These molecules are active on non-proliferating cells, indicating a mechanism of action independent of DNA synthesis. These molecules can induce caspase activation in cell extracts and release of cytochrome c from mitochondria [17–20]. This might be a part of its mechanism of action, but here, dATP would have the same impact on the cell. Fludarabine inhibits RNA polymerases and is incorporated into the polyadenylation part of mRNA, modifying its properties and the capacity of protein synthesis [21,22]. It seems that this action on RNA mediates the major part of the cytotoxicity of fludarabine on CLL cells [23]. In a murine L1210 cell line resistant to fludarabine (L1210/F), it was shown that the incorporation of fludarabine in DNA was decreased as a result of increased dATP concentration in the cells [1]. Co-incubation of these cells with hydroxyurea, a RNR inhibitor, increased the DNA-incorporated fludarabine while dATP was decreased. This clearly indicates that modifications in intracellular deoxyribonucleotide pools can influence on the activity of nucleoside analogs. It has recently been shown that polyadenylation is inhibited by triphos-

phorylated adenosine analogs [24]. This is done by competition with intracellular ATP.

Another, less studied nucleoside analog, 8-chloroadenosine (8-Cl-Ado) was recently shown to exert its activity through inhibition of ATP synthase [25]. This results in decreased ATP concentrations in 8-Cl-Ado treated cells. Other enzymes inhibited by phosphorylated nucleoside analogs are cytidine triphosphate (CTP) synthase and deoxycytidine monophosphate (dCMP) deaminase [26,27].

2.2. Nucleobases

6-Mercaptopurine and thioguanine are purine bases analogs to hypoxathine used for the treatment of leukemias and inflammatory diseases [28,29]. These molecules are transformed into deoxythioguanine triphosphate (dthioGTP) that is subsequently incorporated into DNA and RNA [30]. The induction of cell death seems to implicate DNA mismatch repair [31]. These molecules and other metabolites of 6-mercaptopurine and thioguanine are potent inhibitors of enzymes involved in the *de novo* synthesis of nucleotides, thus inducing purine starvation and apoptosis in treated cells. One major enzyme involved in the metabolism of these nucleobases is thiopurine methyltransferase (TPMT) catalyzing the methylation of the bases and the corresponding monophosphorylated nucleosides [32]. The methylated metabolites also possess cytotoxic properties.

2.3. Fluoropyrimidines

5-Fluorouracil (5-FU) is a fluorinated pyrimidine base analog largely used in the treatment of breast and colon cancer. Floxuridine is its ribonucleoside derivative that is used for treatment of colorectal cancer. The metabolism of floxuridine leads to the formation of 5-FU, and thereafter the two compounds sheare the same metabolic pathway and mechanims of action. Induction of cell death is due to RNA and DNA incorporation of triphosphorylated 5-fluorouridine (5-FUTP) and 5-fluorodeoxyuridine (5-FdUrd) respectively, and to inhibition of the enzyme thymidylate synthase (TS) by fluorodeoxyuridine monophosphate (5-FdUMP) [33]. In addition to the formation of damaged DNA and RNA, TS inhibition leads to depletion in thymidine triphosphate (TTP) and increased deoxyuridine monophosphate (dUMP), thus enhancing the toxic effects of the molecule. This underscores the interest in the dosage also of monophosphorylated nucleosides such as 5-FdUMP, dUMP and thymidine monophosphate (TMP).

2.4. In conclusion

Several approaches were developed in order to increase activity of nucleoside analogs in transporter or kinase deficient cancer cells. Some of these were based on the development of pronucleotides, where monophosphorylated nucleoside analogs are released inside the cells and subsequently phosphorylated into active metabolites. This approach has been particularly studied with antiviral nucleoside analogs, but has been adapted more recently to cytotoxic compounds [34–37]. In this setting, it is of great interest to know the decomposition of the prodrug and the production of monophosphorylated nucleoside analogs in biological media (cell extract, whole cell, sera, . . .). The rapid overview of metabolism and mechanisms of action of these drugs clearly highlights the importance of knowing how to determine concentrations of endogenous ribonucleotides and deoxyribonucleotides in biological samples such as tumor cells. Such techniques are of great interest in the search for the mechanism of action of new drug candidates interacting with purine and pyrimidine metabolism. In addition, such dosages before or during chemotherapy could predictive information as to the outcome of a treatment based on nucleoside analogs, nucleobases or fluoropyrimidines.

3. Analysis of endogenous nucleotides

3.1. Nucleoside monophosphates

3.1.1. Sample pre-treatment

The analysis of nucleotides have been performed in various matrix such as whole blood [38,39], erythrocytes [40], peripheral blood mononucleous cells (PBMC) [41–43], cultured cells [4,42,44–49], cerebrospinal fluid (CSF) [50] or tissue [51–53]. These matrixes contain enzymes involved in the purines and pyrimidines metabolism. Thus, the first step to sample pre-treatment is to lyse the cells or organelles first and block the metabolism of nucleotides immediately by inactivation of the enzymes. In this aim, the extraction was generally performed at low temperature [44,50,54,55]. The sample preparation is probably the major critical step for the determination of endogenous nucleotides and nucleotide analogs in biological samples. This is demonstrated by the number and the variety of methods described since the last decade. There is no consensus and the method preparation depended in part to the detection mode and sample matrix. A precipitation step constituted the single phase common to all methods described. In some cases, at this step a solvent or a solid phase treatments were associated to extract nucleotides or to clean the supernatant obtained after protein precipitation. Finally, in few studies, the solvent treatment was also followed by an on-line approach.

For protein precipitation, perchloric acid (PCA) was mainly employed when analysis were performed either with LC-UV or LC–MS devices [38,39,42,45,47,52,53,55,56] (Tables 1 and 2). This step was followed by adding a basic solution such as KOH [38,44,45,47], K_2HPO_4 [42], K_2CO_3 [38] or borate buffer [39] in order to precipitate perchlorates and to neutralize the supernatant. The use of trichloroacetic acid (TCA) was less frequently described due to its poor compatibility with detection by MS [40,57]. However, for analysis of gemcitabine phosphate derivatives and for some endogen nucleotides from PBMC, TCA was preferred to PCA due to its negative effect on the retention behaviour of nucleotides in ion-pair chromatography [58]. The retention time of ATP, adenosine diphosphate (ADP) and adenosine monophosphate (AMP) decreased according to the PCA concentration added in the sample and the pH of the mobile phase. This may be linked to the ion-pairing capacity of perchlorate. Moreover, after several injections, the ATP peak split in two components [59]. In order to remove excess of TCA from the lysate, a cleaning step with diethyl ether [57] or methyl *tert*-butyl ether [40] was then performed.

Protein precipitation was also described using pure methanol [49,60] or a methanol/water mixture with different proportions of both compounds: v/v: 80/20 [4], 70/30 [43], 60/40 [51]. To enhance efficiency of protein precipitation, several authors proposed a supplementary step consisting to a freezing–thawing cycle [4,40,42,56,61] or to a sonication in an ice bath [4,44]. Extraction recoveries using protein precipitation were mostly greater than 60% [38–40,47,49,55]. However, as suggested by several studies, protein precipitation efficiency could be matrix-dependent and results were conflicting between studies. For analysis of eleven nucleotides from tissue samples by LC–MS, authors compared four mixtures: PCA 12%, methanol/water (70/30, v/v), acetonitrile/water (70/30, v/v) and methanol/chloroform (1/1, v/v) [55]. PCA extraction yielded the highest recovery and the best reproducibility (CV < 10%). For the quantification of ATP in mouse brain, Khan [62] concluded that association of PCA with ultraturax homogenizer allowed a clear sensitivity enhancement. From cell cultures samples, Cordell et al. [49] also evaluated several extraction sol-

Table 1
Analytical conditions used in validated assays based on UV detection mode.

Compound	Sample	Extraction	Stationary phase	Mobile phase	Run	Comments	Reference
ATP, ADP, AMP, adenosine, adenine, inosine, hypoxanthine, uric acid	Whole blood	Protein precipitation, $HClO_4$ + neutralization (K_2CO_3 + KOH)	Hypersil ODS C18, 150 mm × 4.6 mm, 3 μm (Thermofisher)	Binary gradient program A: 50 mM phosphate buffer pH 6 B: MeOH	19 min		[38]
ADP, ATP, CTP, GTP, UTP, dATP, dCTP, dGTP, TTP	Cultured cells	Protein precipitation, TCA and neutralization (5 M K_2CO_3)	Symmetry C18, 150 mm × 4.6 mm, 3.5 μm (Waters)	Binary gradient program A: 10 mM TBAH, 10 mM KH_2PO_4, 0.25% MeOH, pH 6.9 B: 5.6 mM TBAH, 50 mM KH_2PO_4, 30% MeOH, pH 7	60 min		[54]
Adenosine, cytidine, guanosine, uridine, AMP, CMP, GMP, UMP, ADP, CDP, GDP, UDP, ATP, CTP, GTP, UTP	Human cerebrospinal fluid	SPE (Strata X Phenomenex) + lyophilization + lipid removal with n-heptane	Sulpelcosil LC-18-DB, 250 mm × 4.0 mm (Sigma–Aldrich)	Isocratic mode 150 mM phosphate buffer pH 6, 100 mM KCl, 10 mM TBAH			[50]
ATP, ADP, AMP, GTP, GDP, GMP	Rat erythrocytes	Protein precipitation, 10% TCA + clean step with methyl *tert*-butyl ether + non-retentive SPE (Chromosep CN bonded cartridge Chromatographic SpecialtiesInc.)	Supelcosil LC-18-T, 250 mm × 3.0 mm, 5 μm (Supelco)	Isocratic mode 0.5 mM TBAH sulphate in 0.1 M KH_2PO_4/ACN/MeOH (v/v/v: 9.6/0.3/0.1) pH 6.3	20 min	GMP was not measurable in the RBC lysate due to interference from the biologic samples	[40]
ATP, ADP, AMP, NAD(P), NAD(P)H	Whole blood	Protein precipitation, $HClO_4$ + neutralization (1 M borate buffer)	Discovery C18, 250 mm × 4.6 mm, 5 μm (Supelco)	Binary gradient program A: 0.1 M KH_2PO_4 pH 6.0 B: 0.1 M KH_2PO_4 pH 6.0–MeOH (v/v: 90/10)	35 min		[39]
dFdCTP	PBMCs	Protein precipitation $HClO_4$ + neutralization (KOH)	Partisil 10 SAX, 250 mm × 4.6 mm, 10 μm (Whatman)	Isocratic mode 412.5 mM ammonium phosphate buffer pH 2.6/ACN (v/v: 75/25)	30 min		[97]
dFdCTP, dFdCDP, ATP, ADP	PBMCs and cultured A2780 and SKOV-3 ovarian cancer cells	Protein precipitation TCA + neutralization and clean-step with trioctylamine-freon mixture (v/v, 1/4)	Tracer Excel ODSA C18, 100 mm × 4.6 mm, 3 μm (Technocroma S.Coop)	Isocratic mode: solvent A-solvent B (v/v: 64/36) A: KH_2PO_4 10 mM/TBACl 10 mM pH 7 with 0.25% MeOH B: KH_2PO_4 250 mM/TBACl 10 mM pH 7/MeOH (v/v: 85/15)	26 min	A clean step after each injection is necessary to remove all compounds from the chromatographic column 9 NTD in pure solution (CTP, dCTP, UTP, GTP, dFdCTP, ADP, TTP, dFdCTP, ATP) were separated with these conditions	[58]
dFdCTP	Cultured human leukemia HL 60 cells and PBMCs	Protein precipitation $HClO_4$ + neutralization (KOH)	TSK gel DEAE-2SW, 250 mm × 4.6 mm, 5 μm (anion exchange column) (Tosoh Corp.)	Isocratic mode 0.06 M Na_2HPO_4 pH 6.9/ACN (v/v: 80/20)	120 min		[96]
F-ara-ATP	Cultured human leukemia HL 60 cells	Protein precipitation $HClO_4$ + neutralization (KOH)	TSK gel DEAE-2 SW, 250 mm × 4.6 mm, 5 μm (anion exchange column) (Tosoh corp.)	Isocratic mode 0.06 M Na_2HPO_4 pH 6.9/ACN (v/v: 80/20)	120 min		[101]
F-ara-ATP, Ara-GTP, CTP, UTP, ATP, GTP	PBMCs	Protein precipitation $HClO_4$ + neutralization (KOH)	10SAX Partisil, 250 mm × 4.6 mm (Whatman)	Binary gradient program A: 0.005M $NH_4H_2PO_4$ pH 2.8 B: 0.75M $NH_4H_2PO_4$ pH 3.6	50 min		[102]

ACN: Acetonitrile; dFdCMP: 2′,2′-difluorodeoxycytidine monophosphate (gemcitabine monophosphate); dFdCDP: 2′,2′-difluorodeoxycytidine diphosphate (gemcitabine diphosphate); dFdCTP: 2′,2′-difluorodeoxycytidine 5′triphosphate (gemcitabine triphosphate); F-ara-ATP: 9-β-D-arabinofuranosyl-2-fluoroadenine triphosphate (fludarabine triphosphate); MeOH: methanol; PBMCs: peripheral blood mononuclear cells; TBAH: tetrabutylammonium hydroxide; TBACl: tetrabutylammonium hydrochloride; TCA: trichloroacetic acid.

Table 2
Analytical conditions used in validated assays based on mass spectrometry detection mode.

Compound	Sample	Extraction	Stationary phase	Mobile phase	Run	Detection method	Comments	Reference
ATP, ADP, AMP	Cultured human hepatoma HepG-2 cells	Protein precipitaion $HClO_4$ + Na-EDTA 1 mM + neutralization (KOH)	PGC Hypercarb 150 mm × 2.0 mm, 5 μm (Thermo Fhisher)	Binary gradient program A: 10 mM NH_4Ac buffer pH 10 B: ACN	19 min	Ion trap MS ESI negative mode		[47]
dATP, TTP, dCTP, dGTP	PBMCs	Protein precipitation (tris–HCl–MeOH (v/v 30:70))	Supelcogel ODP-50, 150 mm × 2.1 mm, 5 μm (Sigma–Aldrich)	Binary gradient program A: 6 mM DMHA in 20 mM ammonium formiate (sol. 1)/H_2O(v/v: 50/50) B: sol. 1/ACN (v/v: 50/50) pH ≈ 11	26 min for dATP, TTP, dCTP 39 min for dGTP	MS/MS triple quadrupole ESI negative mode	Two binary gradient programs were applied: one for analysis of dATP, TTP, dCTP and another one for dGTP analysis dCTP and dGTP are analysed after periodate oxidation	[88]
Adenine, adenosine, cAMP, AMP, ADP, ATP, cGMP, GMP, GDP, GTP, UMP, UDP, UTP, CMP, CDP, CTP, NAD, NADH, NADP, NADPH, NAADP, FAD, FMN, cyclic ADP ribose	Cultured Chinese hamster ovary cells	Protein precipitation MeOH + hexane extraction for lipid removal	Symmetry C18, 150 mm × 2 mm, 3 μm (Waters)	Binary gradient program A: MeOH–H_2O (v/v: 5/95), DMHA 5 mM pH 7 B: MeOH–H_2O (v/v: 80/20), DMHA 5 mM	35 min	MS/MS triple quadrupole ESI negative mode		[49]
AMP, ADP, ATP, CDP, CTP, FAD, GDP, GTP, UDP, UTP, NAD^+	Rat tissues	Protein precipitation $HClO_4$ + online desalting column (Agilent, Zorbax C18, 50 mm × 4.6 mm, 5 μm)	Synergy hydro C18, 250 mm × 4.6 mm, 3 μm (Phenomenex)	Binary gradient program A: 2 mM dibutyl ammonium formate buffer B: MeOH	31 min	MS single quadrupole ESI negative mode	Use an online desalting column before analytical column	[55]
TTP	PBMCs	Protein precipitation MeOH 70%	Sentry C8, 20 mm × 3.9 mm, 5 μm (Waters)	Isocratic mode ACN–2 mM ammonium buffer pH 7.5 (v/v: 55/45)	4 min	MS/MS triple quadrupole ESI negative mode	To prepare blank matrix, endogenous TTP was decomposed by heating the BPMC extract at 90 °C in hot water for 12–14 h until TTP was undetectable by LC–MS/MS assay	[61]
dATP, dGTP, dCTP, TTP, ATP, GTP, CTP, UTP	Cultured human leukemia cells (K562, NB4, ML-1, MV4-11, THP-1) and BM of a leukemia patient	Protein precipitation MeOH 60%	Supelcogel ODP-50, 150 mm × 2.1 mm, 5 μm (Supelco) with a Waters XTerra MS C18 10 mm × 2.1 mm, 3.5 μm	Binary gradient program A: 5 mM DMHA in H_2O pH 7 (HCOOH) B: 5 mM DMHA in ACN 50%	40 min	MS/MS ion trap ESI negative mode	Blank cell matrix was prepared by dephosphorylation of NTP and dNTP with acid phosphatase GTP and dGTP were coeluted and were not mass-resolved using MRM mode since they are isobaric in mass	[75]
CdAMP, CdADP, CdATP	Cultured Martin-Darby canine kydney II (MDCKII) cells and culture medium	Cells: Protein precipitation MeOH 70% Culture medium: stabilization with MeOH 30%	Biobasic AX, 50 mm × 2.1 mm, 5 μm (ThermoFisher)	Binary gradient program A: 10 mM NH_4Ac in ACN/H_2O (v/v 30:70) pH 6 B: 1 mM NH_4Ac in ACN/H_2O (v/v: 30/70) pH 10.5	10 min	MS/MS triple quadrupople ESI positive mode		[48]

dFdCTP, dFdCDP, dFdCMP	PBMCs	Protein precipitation $HClO_4$ + neutralization (NH_4Ac 1 M NH_3 10%)	Biobasic AX 50 mm × 2.1 mm, 5 μm (ThermoFisher)	Binary gradient program A: 10 mM NH_4Ac in ACN/H_2O (v/v 30:70) pH 6 B: 1 mM NH_4Ac in ACN/H_2O (v/v: 30/70) pH 10.5	10 min	MS/MS triple quadrupole ESI negative mode		[98]
dFdC, dFdU, dFdCMP, dFdCDP, dFdCTP, dFdUMP, dFdUDP, dFdUTP	PBMCs	Protein precipitation MeOH	PGC Hypercarb, 50 mm × 2.1 mm, 5 μm (Thermo Fhisher)	Binary gradient program. A: 1 mM ammonium acetate in ACN/H2O (v/v: 15/85) adjusted with acetic acid at pH 5 B: 25 mM ammonium bicarbonate in ACN/H2O (v/v: 15:85)	15 min	MS/MS triple quadrupole ESI positive mode except for dFdU	Before each run the column was treated with H_2O_2. Between each sample 100 μL of diluted formic acid was injected on the column in a flow of 100% A After each analytical batch the column was back flushed with tetrahydrofuran (ca. 20 column volumes) before storage	[100]
F-ara-ATP	Cultured Jurkat cells	Protein precipitation $HClO_4$	Prodigy C8, 100 mm × 2.0 mm, 5 μm (Phenomenex)	A: 0.0025% (v/v) DMHA, 20 mM formic acid pH 7.5 B: MeOH	16.5 min	MS single quadrupole ESI negative mode		[103]
F-ara-ATP	T-lymphocytes	Protein precipitation $HClO_4$ + neutralization ($KHCO_3$)	YMC-Pack Hydrosphere C18, 150 mm × 2.0 mm, 3 μm (Waters)	Binary gradient program A: 20 mM ammonium formate pH 8 B: MeOH	8 min	MS		[104]
Ara-CMP	Cell extract	Protein precipitation $HClO_4$ + NaOH 1.25 M	PGC Hypercarb, 50 mm × 2.1 mm, 5 μm (Thermo Fhisher)	Binary gradient program A: H_2O–formic acid 0.1% (v/v) B: ACN/H_2O (v/v: 80/20), formic acid 0.1% (v/v)	43 min	MS single quadrupole ESI positive mode		[94]
Ara-CTP, CTP, dCTP	Cultured RL7 and RL-G cells	Protein precipitation MeOH 60% + IE-SPE-Oasis WAX cartridges (Waters)	PGC Hypercarb, 50 mm × 2.1 mm, 5 μm (Thermo Fhisher)	Binary gradient program A: hexylamine 5 mM, DEA 0.4% (v/v) pH 10 B: ACN–H_2O (v/v: 1/1)	51 min	MS/MS triple quadrupole ESI negative mode		[87]
5-FU, 5-FUrd, 5-FdUrd, 5-FdUMP, dUMP, TMP	Cultured human breast MCF7 and human kidney HEK 293T cells Culture medium	For cellular quantification: protein precipitation MeOH For quantification in cell culture medium: *5-FU*: direct injection of the cell culture medium *5-FdUMP*: SPE Oasis WAX cartridge (Waters)	Atlantis dC18, 100 mm × 2.1 mm, 3.5 μm (Waters)	Isocratic mode 5 mM ammonium formate buffer pH 4–MeOH–H_2O (v/v: 5/5/90)	6.2 min	MS/MS triple quadrupole ESI negative mode		[60]

ACN: Acetonitrile; AraCMP: 9-β-D-arabinosylcytosine 5′monophosphate; AraCTP: 9-β-D-arabinosylcytosine 5′triphosphate; AraGTP: arabynosylguanosine triphosphate; CdA: 2-clorodeoxyadenosine (cladribine), CdAMP: cladribine-5′monophosphate, CdADP: cladribine-5′diphosphate, CdATP: cladribine-5′triphosphate; DEA: diethylamine; dFdC: 2′,2′-difluorodeoxycytidine = gemcitabine; dFdCMP: 2′,2′-difluorodeoxycytidine monophosphate (gemcitabine monophosphate); dFdCDP: 2′,2′-difluorodeoxycytidine diphosphate (gemcitabine diphosphate); dFdCTP: 2′,2′-difluorodeoxycytidine 5′triphosphate (gemcitabine triphosphate); DMHA: dimethylhexylamine; 5-FU: 5-fluorouracil; 5-FdUMP: 5-fluoro-2′-deoxyuridine-5′-monophosphate; 5-FdUrd: 5-fluoro-2′-deoxyuridine; F-ara-ATP: 9-β-D-arabinofuranosyl-2-fluoroadenine triphosphate = fludarabine triphosphate; MeOH: methanol; NAADP: nicotinic acid-adenine dinucleotide phosphate; NH_4Ac: ammonium acetate; PBMCs: peripheral blood mononuclear cells; BM: bone marrow.

vents: acetonitrile, ethanol, methanol/acetonitrile (80/20, v/v), methanol/water (80/20, v/v), 0.1 M formic acid, 0.5 M PCA and 0.1 M formic acid in methanol. Poor extraction recoveries were observed with ethanol, acetonitrile and with mixture containing 20% water or formic acid. In this last both cases, authors suggested a solvent's inability to stop immediately cellular processes and/or the co-interfering compounds extraction. Although the use of PCA was previously reported, authors concluded the incompatibility of this acid with LC–MS detection. Taken altogether, the best extraction procedure proposed was the addition of methanol cooled at −20 °C with sonication and followed by hexane extraction for lipid removal [49]. Thus, clean up phase procedure to eliminate lipids was also added after the protein precipitation based on liquid/liquid approach [49]. In this way, Losa et al. [58] performed a clean up procedure with freon/trioctylamine (4/1) in order to clean and neutralize the supernatant obtained after TCA precipitation. Czarnecka et al. [50] tested different solvent such as: PCA (1 M), $P_2O_3WO_3$ (0.1 M) and CH_3COOUO_2 (0.2 M) for nucleotides extraction from CSF by protein precipitation. In all cases, low and variable extraction efficiencies were observed due to the low nucleotide concentrations in CSF and the lability of the pyrophosphate bond.

The use of solid phase extraction (SPE) was less often described. For the determination of purine nucleotides in erythrocytes, a non-retentive SPE with a CN bonded cartridges (Chromosep®, Chromatographic specialities Inc.) was performed after precipitation with TCA 10% and methyl *tert*-butyl ether treatment [40]. The SPE step was to remove excess TCA and others drugs potentially present. Zhang et al. [63] reported a method for purification of nucleotides using anion-exchanging SPE column (LC-NH_2 from Supelco, Sigma) with a gradient of salt concentration and pH value. The monophosphate derivatives were eluted from the column with 2 ml of 100 mM ammonium dihydrogenophosphate at pH 3.0. In these conditions, the recovery was evaluated about 95% for uridine monophosphate (UMP). Due to the low extraction efficiency obtained by precipitation with different acids, as described above, Czarnecka et al. [50] described a method for quantification of nucleotides in CSF based on SPE using reversed phase polymeric column (Strata X, Phenomenex). This column has hydrophilic, hydrophobic and π–π retention mechanisms and authors established the conditions of an ion-pair (IP) chromatography using an ion pairing agent (25 mM ethanolamine, pH 8) for conditioning this support. Compounds were eluted with 50% and then with 100% methanol. Triphosphate derivatives elution required a higher percentage of methanol than diphosphate derivatives. It was also observed that CTP exhibited stronger interactions with the support than ATP. Finally, this extraction procedure allowed obtaining a satisfactory yield above 90% for all compounds tested. Aussenac et al. [64] proposed a method for the isolation of monophosphate nucleotides from Champagne wine by ultra filtration allowing a 25-fold concentration of the analytes, followed by a semi-preparative HPLC step using a strong anion-exchange column. Then, fraction was passed through a reverse phase (RP) column in order to remove salt previously LC–MS analysis. However this method was difficult to implement with biological samples because a large volume of sample was needed (250 ml).

The sample obtained after a protein precipitation step could be directly injected into an analytical device [42,47,57,65]. However, in some cases, the sample underwent concentration by evaporation [46,49,60] or a freeze-drying [4,56]. Some assays also included during the pre-treatment procedure an on-line phase. For example, the extract was desalted using an on-line C18 cardridge column washed with 4 mM dibutylammonium formate buffer methanol (v/v: 95/5) before being back-flushed from the desalting column onto the analytical column [55]. This on-line column switching procedure led to better reproducibility of HPLC retention times and improves significantly electrospray ionisation (ESI) due to a lesser salt precipitated in the spray chamber [55]. Moreover, analytes were concentrated at the top of the desalting column and eluted immediately as a sharp band when back flushed, resulting that column switching improved chromatographic separation due to narrower peaks.

A pilot study explored an alternative way to enrich and pre-purify biological samples containing nucleoside mono-, di- and triphosphates [57]. The on-line sample clean-up and preconcentration of nucleotides were performed by immobilised metal affinity chromatography (IMAC) prior to their determination by LC–MS. Nucleotides were trapped on a Poros 20 MC IMAC column, which was conditioned with Fe^{3+} [57]. After a wash step, the elution of compounds was obtained by a 20 μL injection of NH_4OH (pH 10) and nucleotides were switched to the analytical column. With the system of column switching proposed, the IMAC precolumn was never put on-line with the analytical column that minimized contamination of MS by possible metal leaks. However it was noted that some of the nucleoside monophosphates were eluted during the wash step.

3.1.2. High performance liquid chromatography

Nucleotides are chemical compounds with two different regions: a negatively charged phosphate group (pK_a around 1 [66]), and the ribose basic group, less polar [67]. An estimate of the pK_a of the 2′-OH group of a nucleotide is based on a value of 12.5. However, the presence of the phosphate group could modify the pK_a value. Thus the value is established in the range of 12.17–13.59 [68,69]. Their polarity increases with the number of phosphate group. Unlike nucleoside triphosphates highly polar, monophosphate nucleosides can be retained on RP-HPLC column [38,39,60,65,70]. Lu et al. [71] described a RP-HPLC separation for 90 nitrogen-containing cellular metabolites including nine nucleoside monophosphates (AMP, cytidine monophosphate (CMP), UMP, guanosine monophosphate (GMP), TMP, deoxyadenosine monophosphate (dAMP), dCMP, dUMP, deoxyguanosine monophosphate (dGMP)) using a Synergi Fusion RP column (Phenomenex) and a mixture water/formic acid/methanol as mobile phase delivered by a gradient program. These stationary phase used a polar embedded and a hydrophobic ligand to achieve improved selectivity. The polar embedded group provided enhancement of polar retention compared to a conventional RP C18 column. However, like most methods described the simultaneous determination of nucleoside mono-, di- and triphosphates, assays based on reverse phase chromatography were scarce and inappropriate.

Uehara et al. [4] reported an assay focused on endogenous phosphorus metabolites based on a normal phase (NP) liquid chromatography using an amino propyl silica gel column (Luna amino column, Phenomenex) with basic ammonium carbonate buffer (pH 10). These chromatographic conditions allowed a good retention and separation of phosphorus compounds because of both ionic and hydrophilic interaction between the compounds and the solid phase.

IP [40,42,43,45,46,49–52,55–57,64,66,67,72–75] and anion exchange (AE) chromatography [44,76] are in principle the most suitable methods for the determination of nucleotides. Tomiya et al. [44] compared an IP chromatography and an AE chromatography for the determination of nucleotides and sugar nucleotides. Their study showed that the separation for the nucleotides was satisfactory with both methods and the analysis times were similar [44]. However AE chromatography suffered from a poor compatibility with mass spectrometry [51]. The elution of charged compounds required high concentrations of competing ions in the mobile phase which caused ion suppression and contamination in the ionisation source [51].

IP chromatography is considered as the most appropriate chromatographic method in term of compatibility with the MS detection for analysis of charged compounds [51]. The separation

is based on the formation of ion pairs between the negatively charged nucleotide and the positively charged ion-pairing reagent. The stationary phase is a conventional apolar phase C18 [40,42,46,49–52,55,67,73,77] or C8 [45,74]. The literature reported the use of different cationic reagents (alkylamines) as ion-pairing reagents in the mobile phase. The tetrabutylammonium salts was successfully used for HPLC analysis of mono-, di- and triphosphates nucleoside coupled with UV detection mode [40,50,52,67]. However, tetrabutylammonium salts were inherently non-volatile and therefore incompatible with electrospray mass spectrometer [66]. One solution was to use more volatile amines such as triethylamine (TEA) [42,64], tributylamine [66], dibutylammonium salt [51,55], dimethylhexylamine (DMHA) [45,49,74,77], or hexylamine (HA) [73]. Desorption of the analytes was generally achieved using pH gradient [73] and/or gradients of organic modifiers like methanol [49,57,66,67,73,74,77] or acetonitrile [45–47,51,52,78]. In some cases, ammonium phosphate was added to the mobile phase to improve chromatographic performance by introducing competitive anion [57]. However ammonium phosphate is not volatile and thus not compatible with MS detection [51]. A selective desorption could be carried out by introducing magnesium complexation as additional secondary equilibrium [42]. By adding Mg^{2+} as competing counter ion in the mobile phase, Mg-nucleotide complexes were formed, which were much less retained than the corresponding TEA-nucleotide species [42]. Aussenac et al. [64] showed that the use of 1,1,1,3,3,3-hexafluoro2-propanol (HFIP) as a mobile phase additive with TEA gave better results in terms of separation and electrospray performance that TEA alone. These conditions were previously described by Apffel et al. [79]. HFIP used instead of acetate as counter ion of TEA reduced the phenomenon of ion suppression observed during the coupling LC–MS thanks to the highly volatile of HFIP. However, disadvantages of the HFIP-TEA were the background signal generated by the reagents and the corrosiveness of HFIP [64,79].

The concentration of ion pairing agent in the mobile phase and the pH were critical and had to be optimized to obtain good retention and peak shapes and minimized contamination when spectrometric mass detection was used. The concentration of trialkylamine used as ion pairing agent could be reduced by increasing the length of the alkyl chain and thus promoting interactions between the hydrophobic stationary phase and the ion pairing agent. One solution for reducing contamination of the ESI source and mass spectrometer caused by sample matrix and ion pairing reagent present in the mobile phase was the decrease in flow of the mobile phase by using a splitter before the entrance of the mass spectrometer [51,56,64,73]. Another way was to use column miniaturization [42,45]. Used a small column reduced the amount of ion pairing agent required and consequently increased ESI-MS sensitivity. In addition the use a small diameter HPLC column provided better chromatographic resolution and sensitivity for LC–MS since the sensitivity of the ESI-MS was dependant on the concentration of analytes. Using a short capillary column, Tuytten et al. [77] showed that the addition in the mobile phase of $NH_4H_2PO_4$ 5 mM improved the chromatographic performance without impairing the MS detection. Uehara et al. [4] reported a nano-LC-ESI-MS/MS method for quantification of phosphorus metabolites in cells. Good separation of compounds was achieved with forty to hundred-fold increase of sensitivity compared with a semi-micro-LC–MS. The decreasing pH of the mobile phase led to observe a shorter retention times and degradation in peak shape with peaks broadening. The increase in retention time due to the increase in pH could be explained by changes in ionisation of the phosphate moiety increasing ion-pair formation. At pH 3 compounds were barely retained on the column and all eluted at very similar times [49]. A mobile phase pH between 6 and 7 was most often used in several nucleotide separation methods involving the use of alkylamine as ion pairing agent [40,42,45,49,51,57,64,73,74,78]. One publication described LC methods for cyclic AMP (cAMP) analysis employing a mobile phase at pH 3 with either tetrabutylammonium sulphate (TBAS) or DMHA as ion-pairing agent and they achieved satisfactory retention and peak shape for cAMP [63].

Another way to achieve the separation of nucleotides was the use of carbon column [47,72]. The physical and chemical stability of graphitic carbon columns performed applications in chromatography especially for polar compounds those were difficult in silica-based packing materials [80]. The retention mechanisms on porous graphitic carbon (PGC) are complex [80]. The PGC is involved in π–π interactions and dispersive interactions with aromatic solutes. Retention increases as the hydrophobicity of the molecule increases. Furthermore, the graphitic carbon surface acts as a Lewis base toward polar solutes [80]. When an electrostatic charge of a polar analyte approaches the graphite surface, charge-induced dipole at the graphite surface is generated, increasing the attraction between the analyte and support. The retention mechanism corresponds both to hydrophobic interactions and electrostatic interactions. The strength of analyte interactions with the support is dependent on the molecular area in contact with the graphite surface, at the points of contact. Furthermore, PGC has the advantage to tolerate a large range of pH (0–14) [80]. Wang et al. [47] showed that adenosine phosphates were separated using a PGC column under the RP conditions. Xing et al. [72] developed on a PGC column a general LC–MS method for the analysis of nucleosides and their mono-, di- and triphosphates. Different organic mobile phases and modifiers suitable were evaluated with MS detection. It was showed that the concentration of ammonium acetate was critical for elution of nucleotides and for maximizing MS detector sensitivity. The presence of diethylamine reduced retention of monophosphates and enhanced their MS signal intensity [72]. One disadvantage of PGC column was difficult desorption of non-polar compounds adsorbed on the surface.

3.1.3. Detection mode

Although UV detection mode was used to analyze ribonucleoside monophosphates in biological matrix [38–42,44,46,50,67,76], this detection mode lacked of sensitivity [42] and specificity [40,67]. The wavelength mostly used for the determination was set to 254 nm [39,40,42]. The limits of detection for published methods were in the order of tens of pmol (Table 3).

The fluorescence detection mode greatly improved the sensitivity for adenosine phosphates analysis [52,63,81]. However, this detection mode required derivatization of the analytes with 2-chloroacetaldehyde. This procedure was time-consuming to implement and required 20–40 min at 80 °C according to the authors [52,63,81]. The limits of detection varied between 1 and 50 fmol [52,81].

LC coupled with MS detection has been, since the last decade, the most widely used analytical tool for nucleotides analysis. Detection was based on either triple quadrupole mass spectrometer or ion trap mass spectrometer. Since cellular endogenous deoxyribonucleotides concentration are low, their detection could not be performed with an ESI ion trap mass spectrometer in full scan mode [43]. Due to its higher sensitivity a triple quadrupole mass spectrometer in multiple ion monitoring mode was required for this purposes [43]. For exemple, LC–MS/MS device lowered the limit of detection by a factor of 100 compared to the UV detection mode and improved significantly the specificity [4,39,41,43,45,49,51,66,67] (Table 4). LC–MS/MS analysis were then performed either by negative [49,82,83] and or positive ESI mode [70,71]. The negative mode seemed to be the logical choice for the analysis of negatively charged compounds such as nucleotides [49]. However an other assay using positive ESI mode for the quantification of AMP, GMP, cAMP and cyclic GMP (cGMP) in human platelet

Table 3
Performance and validation parameters for the quantification of endogenous nucleotides and nucleotide analogs using UV detection mode.

Compound	Sample	Calibration	Internal standard	Linear range (quantity injected)	LOQ (quantity injected)	Recovery	Comments	Reference
ATP, ADP, AMP, adenosine, adenine, inosine, hypoxanthine, uric acid	Whole blood	Method: addition	None	24–1180 pmol	AMP: 0.41 pmol ADP: 0.56 pmol ATP: 0.38 pmol	85.4–108.2%	LOQ = 10 × noise SD	[38]
ADP, ATP, CTP, GTP, UTP, dATP, dCTP, dGTP, TTP	Cultured cells	Medium: aqueous standard	None	ADP: 1–16 nmol NTP: 0.8–20 nmol dNTP: 240–4300 pmol		93.8–120.5% ($n = 3$)		[54]
Adenosine, cytidine, guanosine, uridine, AMP, CMP, GMP, UMP, ADP, CDP, GDP, UDP, ATP, CTP, GTP, UTP	Human cerebrospinal fluid		CTP	0–500 pmol		86.7–93%	Quantification was validated for AMP, ADP, ATP, GDP, GTP	[50]
ATP, ADP, AMP, GTP, GDP, GMP	Rats erythrocytes	Method: addition	3.7-dimethyl uric acid	ATP, ADP: 0.1–2 mg/ml AMP: GTP, GDP: 0.02–0.4 mg/ml		>70%	GDP was not quantified due to low concentration in samples	[40]
ATP, ADP, AMP, NAD(P), NAD(P)H	Whole blood	Medium: aqueous standard	None	0.05–20 nmol		93.4–100.8%		[39]
dFdCTP	PBMCs	Medium: human red blood cells	None	2.4–1.2 nmol	0.024 nmol	96–99%		[97]
dFdCTP, dFdCDP, ATP, ADP	PBMCs and cultured A2780 and SKOV-3 ovarian cancer cells	Medium: aqueous standard	None	dFdCTP: 0.09–1.2 nmol dFdCDP: 0.085–1.4 nmol ATP: 0.79–16.5 nmol ADP: 0.14–7 nmol	dFdCTP: 0.09 nmol dFdCDP: 0.06 nmol ATP: 0.72 nmol ADP: 0.14 nmol	96–108%		[58]
dFdCTP	Cultured human leukemia HL 60 cells and PBMCs	Medium: aqueous standard	None	20–2000 pmol	20 pmol			[96]
F-ara-ATP	Cultured human leukemia HL 60 cells	Medium: aqueous standard	None	0.05–20 nmol	0.05 nmol			[101]
F-ara-ATP, Ara-GTP, CTP, UTP, ATP, GTP	PBMCs	Medium: aqueous standard	None	0.025–10 nmol	0.025 nmol			[102]

dFdCMP: 2′,2′-Difluorodeoxycytidine monophosphate (gemcitabine monophosphate); dFdCDP: 2′,2′-difluorodeoxycytidine diphosphate (gemcitabine diphosphate); dFdCTP: 2′,2′-difluorodeoxycytidine 5′triphosphate (gemcitabine triphosphate); F-ara-ATP: 9-β-D-arabinofuranosyl-2-fluoroadenine triphosphate (fludarabine triphosphate); PBMCs: peripheral blood mononuclear cells; SD: standard deviation.

Table 4
Performance and validation parameters for the quantification of endogenous nucleotides and nucleotide analogs using mass spectrometry detection mode.

Compound	Sample	Calibration	Internal standard	Linear range (quantity injected)	LOQ (quantity injected)	Recovery %	Reference
ATP, ADP, AMP	Cultured human hepatoma HepG-2 cell	Medium: aqueous standard	None	AMP: 0.22–57.80 μM ADP: 0.59–117.37 μM ATP: 0.49–98.81 μM	AMP: 0.22 μM ADP: 0.59 μM ATP: 0.49 μM	78.5–87.1%	[47]
dATP, TTP, dCTP, dGTP	PBMCs	Method: addition	Cl-ATP	0.3–20 pmol	TTP, dCTP: 0.3 pmol dATP: 0.4 pmol dGTP: ND		[88]
Adenine, adenosine, cAMP, AMP, ADP, ATP, cGMP, GMP, GDP, GTP, UMP, UDP, UTP, CMP, CDP, CTP, NAD, NADH, NADP, NADPH, NAADP, FAD, FMN, cADP ribose	Cultured Chinese hamster ovary cells	Method: addition	BrAMPc, Br-ATP, nicotinamide, 1,N^6-etheoadenine dinucleotide	5–1000 pmol		>53%	[49]
AMP, ADP, ATP, CDP, CTP, FAD, GDP, GTP, UDP, UTP, NAD^+	Rat tissues	Medium: 6% bovine serum albumin in isotonic saline solution	N^6-(6-aminohexyl)adenosine 3′,5′-diphosphate	AMP: 2–500 μM ADP, CTP, GDP, GTP, UTP: 2–50 μM ATP, CDP, UDP: 1–50 μM NAD: 3–50 μM FAD: 0.25–25 μM	0.25–3 μM	75.6–9.1%	[55]
TTP	PBMCs	Medium: blank cell extract	Methyl indinavir sulphate	0.02–20 pmol	0.02 pmol	103.4%	[61]
dATP, dGTP, dCTP, TTP, ATP, GTP, CTP, UTP	Cultured human leukemia cells (K562, NB4, ML-1, MV4-11, THP-1) and BM of a leukemia patient	Medium: blank cell matrix	Cl-ATP	2.5–50 pmol and 50–500 pmol	2.5 pmol	53–71%	[25]
CdAMP, CdADP, CdATP	Cultured Martin-Darby canine kydney II (MDCKII) cells and culture medium	Medium: blank stabilized medium	CMP ($^{13}C/^{15}N$) CDP ($^{13}C/^{15}N$) CTP ($^{13}C/^{15}N$)	CdAMP: 25–630 fmol CdADP: 12.5–1250 fmol CdATP: 30–1190 pmol	CdAMP: 25 fmol CdADP: 12.5 fmol CdATP: 30 fmol	85–106%	[48]
dFdCTP, dFdCDP, dFdCMP	PBMCs	Medium: PBMCs	CTP ($^{13}C/^{15}N$)	dFdCTP: 33–830 fmol	dFdCTP: 33 fmol		[98]
dFdC, dFdU, dFdCMP, dFdCDP, dFdCTP, dFdUMP, dFdUDP, dFdUTP	PBMCs	Medium: standard in blank PBMC	Mixture of ^{13}C, ^{15}N labelled isotopes of dFdC and dFdU nucleotides	dFdC: 0.01–1 pmol dFdCMP, dFdCDP: 0.07–7 pmol dFdCTP, 0.09–9 pmol dFdU, dFdUDP: 0.1–10 pmol dFdUMP: 0.06–6 pmol dFdUTP: 0.13–13 pmol	dFdC: 0.01 pmol dFdCMP, dFdCDP: 0.07 pmol dFdCTP, 0.09 pmol dFdU, dFdUDP: 0.1 pmol dFdUMP: 0.06 pmol dFdUTP: 0.13 pmol		[99]
F-ara-ATP	Cultured Jurkat cells	Medium: aqueous standard	Cl-ATP	0.62–10 pmol	50 fmol		[103]
F-ara-ATP	T-lymphocytes		Cl-ATP		50 fmol		[104]
Ara-CMP	Cell extract		ddCP	0.053–2.6 nmol	0.053 nmol	68.3–73.1%	[94]
Ara-CTP, CTP, dCTP	Cultured RL7 and RL-G cells	dCTP (^{13}C, ^{15}N) and CTP (^{13}C, ^{15}N) stable isotope labelled	Br-ATP	araCTP: 0.2–16 nmol/ml CTP (^{13}C, ^{15}N): 0.02–60 nmol/ml dCTP (^{13}C, ^{15}N): 0.02–16 nmol/ml	araCTP: 0.2 nmol/ml CTP (^{13}C, ^{15}N): 0.02 nmol/ml dCTP (^{13}C, ^{15}N): 0.02 nmol/ml	60–85%	[87]
5-FU, 5-FUrd, 5-FdUrd, 5-FdUMP, dUMP, TMP	Human breast MCF7 and kidney HEK 293T cells Culture medium	Medium: methanol solution	5-CU (dUMP for 5FdUMP in culture medium)	5-FdUMP: 0.15–3.0 pmol dUMP: 0.16–3.2 pmol TTP: 0.078–1.56 pmol	5-FdUMP: 150 fmol dUMP: 160 fmol TTP: 78 fmol	≥90%	[60]

ACN: Acetonitrile; AraCMP: 9-β-D-arabinosylcytosine 5′monophosphate; ddCP: [1′-(5′,6′-dideoxy-β-D-arabino-5′-hexenofuranosyl)cytosyl]-6′-phosphonic acid AraCTP: 9-β-D-arabinosylcytosine 5′triphosphate; AraGTP: arabynosylguanosine triphosphate; $BrAMP_C$: 8-bromoadenosine 3′,5′-cyclic monophosphate; CdA: 2-clorodeoxyadenosine (cladribine), CdAMP: cladribine-5′monophosphate, CdADP: cladribine-5′diphosphate, CdATP: cladribine-5′triphosphate; Cl-ATP: 2-chloroadenosine 5′-triphosphate; DEA: diethylamine; dFdC: 2′,2′-difluorodeoxycytidine = gemcitabine; dFdCMP: 2′,2′-difluorodeoxycytidine monophosphate = gemcitabine monophosphate; dFdCDP: 2′,2′-difluorodeoxycytidine diphosphate = gemcitabine diphosphate; dFdCTP: 2′,2′-difluorodeoxycytidine 5′triphosphate = gemcitabine triphosphate; DMHA: dimethylhexylamine; 5-FU: 5-fluorouracil; 5-FdUMP: 5-fluoro-2′-deoxyuridine-5′-monophosphate; 5-FdUrd: 5-fluoro-2′-deoxyuridine; F-ara-ATP: 9-β-D-arabinofuranosyl-2-fluoroadenine triphosphate = fludarabine triphosphate; MeOH: methanol; NAADP: nicotinic acid-adenine dinucleotide phosphate; NH_4Ac: ammonium acetate; PBMCs: peripheral blood mononuclear cells; BM: bone marrow.

extracts have shown a better sensitivity than the negative ion mode [65].

In several studies, DMHA was included in mobile phase [45,70,74]. Thus the nucleotide protonation was enhanced and provided much better sensitivity with positive rather than negative ion mode [74]. In this condition, the most abundant relevant ion was the adduct ion $[M+DMHA+H]^+$ and not the $[M+H]^+$ ion. The influence of DMHA on the MS detection was evaluated in ESI positive and negative modes and it was suggested that effective ion-pair formation is a *prerequisite* to enhance sensitivity in ESI-MS detection [77]. It was shown in negative mode that a significant excess of DMHA is needed to generate positive effect on the signal intensity of the deprotonated nucleotides $[M-H]^-$ [77]. This effect was assigned to a reduction in surface tension, which facilitated ion evaporation in the electrospray process. Similar measurements in ESI positive mode indicated that ion pair formation effectively occurred and that a signal of $[M+DMHA+H]^+$ could be detected together with the protonated molecule $[M+H]^+$ [77]. However, more complex full scan spectra were observed with ESI positive mode due to highest background contribution of the protonated DMHA ion and the presence multiple molecular ion species [49,77]. This phenomenon led to reduce sensitivity and nucleotides such as cGMP and GMP failed to produce a signal [49]. The same observation was described using hexylamine as the ion-pair agent [73].

Using negative or positive ESI modes, the most significant fragmentation results from the cleavage of the glycosidic bond, the most abundant fragment ion corresponding to the nucleic base (purine or pyrimidine) [49,51,56,70,84]. Besides its better the sensitivity, the use of MS detection has other advantages over the detection by UV or fluorescence. Although some of compounds co-elute, it was possible to distinctly quantify them due to the separation via the m/z ratio [56]. However, some nucleotides such as AMP and dGMP had the same elemental composition and thus molecular mass. Furthermore these nucleotides had very close retention times and were therefore difficult to distinguish. AMP and dGMP could be separated using MS–MS mode after loss of the purine unit, i.e. adenine (m/z: 135) and guanine (m/z: 151). On the other hand, CMP and UMP with only one unit of m/z difference should be chromatographically separated. When CMP and UMP were co-eluted the peak area contribution from [M+1] isotopes of CMP on the observed transition for UMP should be corrected by considering the abundances of these isotopes [51].

3.2. Nucleoside diphosphates

3.2.1. Sample pre-treatment

Nucleoside diphosphates were generally analysed under conditions close to those used for monophosphate nucleosides. Thus the extraction procedures described were similar for both family compounds [4,38–41,43–45,47,49,51–57,84]. The protein precipitation was the most widely sample preparation performed and the SPE was less often employed [50,63]. Zhang et al. [63] using an anion exchange SPE showed that nucleoside diphosphate were eluted with a salt concentration ($NH_4H_2PO_4$ 300 mM, pH 3.3) greater than that used for monophosphate compounds. It should be noted that at pH 3 the nucleoside diphosphates were unstable and were likely to lose one phosphate group [49].

3.2.2. High performance liquid chromatography

The chromatographic conditions were similar to those described for monophosphate nucleosides. Diphosphate nucleosides due to their stronger polarity were less retained using RP-HPLC than monophosphate nucleosides [38,39,56]. Conversely, when the ion pairing agent was used [40,41,45,49,50,52,53,55–57,84] or using the normal phase LC assay described by Uehara et al. [4] nucleoside diphosphates were better retained than their monophosphate analogs. Several authors achieved a satisfactory separation and retention with a PGC column [47,72]. The method described by Xing et al. [72] showed that addition of diethylamine (DEA) in mobile phase improved peak shapes for diphosphate nucleotides without modifying their retention times and decreased their signal intensity.

3.2.3. Detection mode

Diphosphate nucleosides were mostly detected at 254 nm or at very closed wavelengths such as 249 or 260 nm.

As previously described for monophosphate nucleosides, ESI source was used with both positive [45,55,74,76] or negative [4,47,49,51,56,57,66,73,77,85] ionisation mode. However, in positive ionisation mode, uridine diphosphate (UDP) fel to produce a signal [49]. This may be related to the basicity of the nucleobase. Indeed, Fung et al. [83] suggested that nucleotides containing a weaker base had more efficient ionisation in the negative mode than in the positive one. According to the authors, the monitored ion fragments were either the pyrophosphate ion (m/z: 159) or the ion corresponding to the removal of a phosphate group from the precursor ion [75] or nucleic bases ion [51,73,74]. Deoxyguanosine diphosphate (dGDP) and ADP which have the same molecular weight could be distinguished each other using the MS/MS mode when bases fragments were monitored [73]. As described for monophosphates, cytidine diphosphate (CDP) and UDP shift of 1 amu which required either a complete chromatographic separation or a higher mass resolution [51]. Seifar et al. [51] suggested to correct the peak area contributions from $[M+1]^+$ isotopes of CDP on the observed transition for UDP by considering abundances of these isotopes.

3.3. Nucleoside triphosphates

3.3.1. Sample pre-treatment

Like others nucleotides, the most common procedure to extract nucleoside triphosphates was a simple protein precipitation [76,86]. Several authors proposed a sample preparation based on SPE [50,63,82,87] using anion exchange column and NH_4OH to elute triphosphate nucleotides [63,82,87]. It should be noted that, such as diphosphate nucleosides, triphosphate are unstable at pH 3 and likely to lose one or two phosphate groups. This effect has been also notified by Cai et al. [86] thus a particular attention must be paid to this possibility.

3.3.2. High performance liquid chromatography

Although nucleoside triphosphates were very polar compounds, several assays using RP-HPLC were performed [38,39,56,61]. For example, Chi et al. [61] described a RP-HPLC method for TTP analysis on a short C8 column. The mobile phase consisted of acetonitril–2 mM ammonium buffer (pH 7.5) (55:45) delivered with the isocratic mode. In these conditions TTP was eluted very early (retention time; 0.77 min). Coolen et al. [38] used a C18 RP column with a phosphate buffer and methanol as mobile phase delivered by gradient program for determination of adenosine phosphates. In this condition, ATP has a short retention time at 3.5 min. At our knowledge, no publication reported the determination of all nucleoside and deoxynucleoside triphosphates using an RP-HPLC method.

As above described for monophosphate nucleosides, the retention achieved for nucleoside triphosphates was satisfactory using either IP chromatography [39,40,45,49–52,54,55,57,58,66,67,72–75,77,78,84,86,88–90] or PGC column [47,82,87]. The complete resolution between the eight intracellular nucleoside triphosphates (ATP, CTP, guanosine triphosphate (GTP), uridine triphosphate (UTP), dATP, dCTP, deoxyguanosine triphosphate (dGTP), TTP) was achieved using an IP-HPLC with high salts concentrations

in the mobile phase (10 mM tetrabutylammonium hydroxide, 10–50 mM K_2HPO_4) [54]. However such high concentrations for non-volatile salts were not compatible with the mass spectrometric detection.

Claire [90] described an IP-LC–MS method with phosphate buffer and tetrabutylammonium hydroxide for the quantitative analysis of intracellular nucleotides. Due to the presence of non-volatile compounds in the mobile phase coupled with MS detection, a microbore HPLC column was used in order to reduce the flow-rate injected into the source of mass spectrometer.

Since chemical structure and properties of several nucleotides are close, coelution was frequent as observed with ATP and dGTP. Hennere et al. [88] used a sample pre-treatment based on a periodate oxidation. This procedure led to the oxidation of ribonucleoside triphosphates by sodium periodate and to its degradation products by methylamine. This assay was time consuming and two separate injections to analyze nucleotides and deoxynucleotides were performed. Cai et al. [86] using a capillary IP-HPLC achieved a separation of ATP and dGTP. The two compounds were injected from pure solution at the same concentration. However the specific conditions in which they tested the chromatographic separation were not consistent with the cellular physiological conditions since the endogenous ATP concentration in cells is about 1000 times greater than dGTP [91].

3.3.3. Detection mode

As previously described for mono and diphosphate derivatives, triphosphate compounds were analysed with UV detection at 254 nm or closed to wavelength.

However, the more sensitive and specific detection mode for the determination and quantification of endogenous nucleoside triphosphates was the mass spectrometry. The most common ionisation mode used was the negative ion ESI mode [4,43,47,49,51,55–57,61,66,73,77,87,92]. Several authors showed that the sensitivity was greater using the positive ion ESI mode [45,74,86]. However, nucleotides with a weaker base CTP, UTP and TTP had more efficient ionisation in the negative ion mode than in the positive ion mode [49,82]. The choice between the two modes of ionisation was intensively discussed in the literature without clear consensus.

With the negative ion mode, according to the methods the most abundant fragment was either the pyrophosphate ion (m/z: 159) [61,82,87,88] or the base fragment ion [51] or the fragment ion corresponding to the removal of a phosphate moiety [75]. Using the positive ion mode, the monitored ion fragment was the nucleic base ion [74,82].

MS detection allowed distinguishing co-eluted molecules of ATP and dGTP. The first possibility was to monitor the ion base fragment after DEA-adduct formation [82]. The second one was the analysis of the ion fragment corresponding to the loss of the purine unit; i.e. adenine (m/z: 135) and guanine (m/z: 151) thanks to MS/MS/MS detection [73]. In both cases, the fragmentation pathway corresponded to the cleavage of the glycosidic bond. Furthermore it was necessary to obtain a good chromatographic separation between CTP and UTP since these two compounds had a molecular weight which differed by only one amu and they had the same fragmentation pathway [51,82]. This was also observed for TTP and CTP [82].

3.4. Endogenous nucleotides and quantification

The quantification of endogenous compound was challenging since nucleotides were naturally present in the matrix. Various quantification methods were described. Calibration curves and quality control (QC) samples were performed from an aqueous matrix [39,41,45,47,54,67,73,74,78,86]. This method was easier to implement however it did not took into account a possible matrix effect leading to a modification of the response with MS detection.

The addition calibration method was widely used [38,40,49,52,56,66,88]. However, due to the large variability in nucleotides levels according to cell extracts, this method was difficult to implement. For the quantification of TTP in cells, Chi et al. [61] used a blank matrix achieved by heating the PBMC extract at 90 °C in hot water for 12–14 h until TTP was undetectable by LC–MS/MS assay. This method was not representative of an analysis performed in a cellular environment that has not undergone thermal degradation.

Recently, few methods reported the use of stable isotope labelled analogs (^{13}C, ^{15}N) for the quantification of endogenous nucleotides [82,87,93]. The stable isotopes labelled compounds have a molecular weight different from that of endogenous compounds and are not naturally present in the matrix. The calibration curve was then considered to be equivalent to that of an exogenous compound.

Few fully validated assays were published and we reported most of them in Table 2. Several validated assays used an external calibration [38,42,45,47,53,54,57,73,86]. Among these, almost were based on UV detection. However, internal standard calibration methods were mostly described. Numerous internal standard such as stable isotopes [43,51] chloro or bromo halogen derivatives [49,75,82,88] or miscellaneous compounds [40,43,55] were used depending the matrix and the compounds analysed.

4. Nucleoside analogs

4.1. Cytarabine

Cytarabine (1-beta-D-arabinofuranosylcytosine, araC) is a synthetic nucleoside which differs from the normal nucleosides cytidine and deoxycytidine in that the sugar moiety is arabinose rather than ribose or deoxyribose. This drug was indicated for remission induction in acute non-lymphocytic leukemia of adults and children. It was also found useful in the treatment of acute lymphocytic leukemia and the blast phase of chronic myelocytic leukemia [87].

4.1.1. Sample pre-treatment

Cytarabine monophosphate (araCMP) was extracted from biological matrix using a simple precipitation with PCA, and adjusting the pH of the supernatant at 2 with NaOH [94]. It should be noted that araCMP was rapidly hydrolyzed to araC at room temperature. The extraction should be performed in an ice bath to prevent this dephosphorylation. AraCMP was stable in the extract after adjustment of the pH during 24 h [94].

The extraction of araCTP (cytarabine triphosphate) was based on a protein precipitation with 60% methanol followed by an SPE using a weak anion exchange cartridge [87]. Target compounds were eluted with a mixture of methanol–water–NH_4OH (24/5/1, v/v/v) resulting in recovery of about 75% for araCTP. Authors showed that araCTP was stable in cells extract before the SPE a least 24 h at −20 °C and during the extraction procedure under acidic and basic conditions. Authors noted that SPE reduced significantly the matrix effect when this procedure was added to the protein precipitation [87].

4.1.2. High performance liquid chromatography and detection

AraC phosphate derivatives (araCMP, araCDP (cytarabine diphosphate), araCTP) and corresponding endogenous phosphate cytidine derivatives (CMP, CDP, and CTP) are stereoisomers. Thus, they had the same MS/MS transition. The intracellular determination of araCMP was performed using a RP-LC–MS method employing a PGC column separation and a gradient elution with

a water/acetonitrile mobile phase containing formic acid [94]. In acidic medium araCMP was slightly negatively charged and its retention was due to specific electronic interactions between the analyte and delocalized electrons on PGC [95]. With these chromatographic conditions authors achieved a satisfactory resolution between araCMP and CMP. Analysis was performed using the positive ion mode [94].

Crauste et al. [87] evaluated the previous chromatographic conditions to analyze araCTP, CTP and dCTP. It was showed that formic acid even at high concentration fel to elute nucleoside triphosphates, presumably due to insufficient competition with electronic interaction between triphosphates and the PGC surface. The elution of nucleotide triphosphates needed the addition in the mobile phase of an ion-pairing agent such as alkylamines. Indeed, the addition in the mobile phase of HA acting as counter ion facilitated the elution of triphosphates compounds. The addition of DEA improved the peak shape by reducing interactions between nucleotides phosphate group and PGC. With these chromatographic conditions the resolution between CMP, araCMP, CDP, araCDP, CTP and araCTP was complete. For araCTP, CTP, dCTP and bromo-adenosine triphosphate (Br-ATP) the fragment ion monitored using the negative ion mode was the pyrophosphate ion (m/z: 159) [87].

Nishi et al. [96] proposed an HPLC method for simultaneous determination of araCTP and gemcitabine triphosphate (dFdCTP) in cancer cells using an anion exchange column coupled with UV detection at 254 nm. Samples were eluted with isocratic mode using a mixture of phosphate buffer (60 mM, pH 6.9) and acetonitrile (80/20, v/v). They achieved a satisfactory separation between araCTP, CTP, UTP, ATP, GTP and dFdCTP, however the run time of the analysis was very long (~2 h). They showed that deoxyribonucleotide triphosphates were eluted at retention times almost identical to those of the corresponding ribonucleotide triphosphates [96].

4.1.3. Quantification

The quantification of the phosphorylated cytarabine metabolites were based on an internal standard calibration with either Br-ATP commercially available [87] or [1′-(5′,6′-dideoxy-beta-D-arabino-5′-hexenofuranosyl)cytosyl]-6′-phosphonic acid [94] requiring home made synthesis. In both studies, calibrations curves were performed in biological matrix (cultured cell lines) [87,94] (Table 1).

4.2. Gemcitabine

Gemcitabine (2,2-difluoro-2-deoxycytidine, dFdC) is a pyrimidine antimetabolite used in therapeutic in several cases such as breast, ovarian, haematological, lung, pancreatic, bladder or urothelial cancer. Gemcitabine is metabolised by deoxycytidine kinase to a mono-, di- and triphosphate analogs [58].

4.2.1. Sample pre treatement

dFdCTP was extracted from biological matrix using a protein precipitation with PCA and neutralization with KOH [96,97]. The protein precipitation could be performed using TCA and then the supernatant was processed with a mixture trioctylamine–freon to allow the cleaning and neutralization of the sample (step repeated three times) [58]. The stability of dFdCTP in intact mononuclear blood cells on ice was strongly limited (~100 min) and after freezing half-life of the analyte in the cellular lysate is ~30 min [97]. No degradation was observed for dFdCTP for 24 h in PCA extracts on ice or in neutralized extracts at room temperature. dFdCTP was stable in PCA extract stored at −80 °C during at least 7 month [97]. The extraction could be also achieved using an IP-SPE with tetrabutylamine as ion-pairing agent after protein precipitation with methanol [2]. The elution of nucleosides and nucleotides was then performed with a mixture of methanol/water (60/40, v/v).

4.2.2. High performance liquid chromatography and detection

Several chromatographic modes were employed for the determination of the nucleoside gemcitabine monophosphate (dFdCMP), diphosphate (dFdCDP) and triphosphate: either the IE-HPLC [96–98], or IP-HPLC [2,58] or RP-HPLC on a PGC column [99]. Veltkamp et al. [98] developed an assay based on a weak anion exchange liquid chromatography coupled to a detection with MS/MS detection. The separation of dFdCMP, dFdCDP and dFdCTP was achieved using a pH gradient from 6 to 10.5 associated with a decrease of the ammonium acetate concentration in the mobile phase. The mass spectrometer operated in the negative ionisation mode and the monitored fragment was the pyrophosphate ion $m/z = 159$. The method proposed by Jansen et al. [99] for the simultaneous determination of gemcitabine and its metabolites (dFdU, dFdCMP, dFdUMP, dFdCDP, dFdUDP, dFdCTP and dFdUTP) used a PGC column without ion pairing agent and MS/MS detection. Since uridine and cytidine only differed to 1 amu and exhibited similar fragmentation patterns, all the correspondent phosphorylated derivatives (dFdU/dFdC, dFdUMP/dFdCMP, ...) required a complete chromatographic resolution in order to avoid miss quantification due to the isotopic abundance. Authors achieved a baseline separation between compounds with gradient mode associated to a complex mobile phase [99,100]. In these conditions, the PGC column behaves like an ion-exchange column with bicarbonate ions acting as eluting ion. However direct analysis of a second sample was not possible because of a loss in retention of the nucleotides. Thus, an injection of acidic solution was requiring between each samples in order to restore the separation capability of the PGC column. After 60 sample injections, the column was rinsed with a mixture containing 0.05% H_2O_2 in ammonium acetate buffer (1 mM) to maintain the retention times and preventing the slow reduction of the graphite surface [99]. Finally, before the storage, analytical column was back-flushed with 20 volumes of tetrahydrofuran and then stored in ammonium acetate buffer (1 mM)/acetonitrile (85/15, v/v) [100]. Three studies used UV detection for the quantification of di- and triphosphate derivatives of gemcitabine [58,96,97]. According to study, the wavelengths were setting to 254, 271 and 275 nm.

4.2.3. Quantification

Two modes of quantification were described: an external quantification [96,97] or an internal standard quantification with the stable isotope CTP as internal standard ($^{13}C_9$, $^{15}N_3$-cytidine triphosphate) [98]. Calibration curves were performed using either aqueous standards [58,96] or on matrix spiked standards [97,98].

4.3. Fludarabine

Fludarabine (2-Fluoro-9-beta-D-arabinofuranosyladenine, F-ara) is mainly used in the treatment of B-CLL. Fludarabine is converted into cells by successive phosphorylation into the active triphosphate metabolite [101].

4.3.1. Sample pre-treatment

The triphosphate metabolite of fludarabine, F-ara-ATP, was extracted from the biological matrix (cells, PBMC, lymphocytes) by protein precipitation with PCA and neutralization with KOH [101,102] or $KHCO_3$ [103,104]. It was shown that these compounds were stable during at least 12 h in the neutralized supernatant [103].

4.3.2. High performance liquid chromatography and detection

Like other nucleosidic analog triphosphates or endogenous nucleoside triphosphates, the assays for the determination of F-ara-phosphates were based on IP-HPLC [103] or IE-HPLC [101,102] or RP-HPLC [104]. The literature showed that UV detection lacked of sensitivity and specificity and required a delicate and reproducible chromatographic separation corresponding to long run times [101,102] since the synthetic nucleosides and their phosphorylated metabolites had similar UV properties to those of endogenous nucleosides and nucleotides. According to these studies, the wavelength used for fludarabine triphosphate was 261 nm [101] or 246 nm [102] and for fludarabine monophosphate 272 nm [105]. Mass spectrometry, operating in the negative ionisation mode, appeared to be the detection mode of choice [103,104].

4.3.3. Quantification

Quantification of F-ara-ATP in biological matrix was performed using either an external calibration [101,102] or an internal standard calibration with chloro-adenosine triphosphate (Cl-ATP) [103,104] or aqueous solutions of F-ara-ATP as standards [101–103]. In this case, to validate the method in biological samples, quality control were performed in cell extracts [101,102].

4.4. Cladribine

Cladribine (2-Chloro-2′-deoxy-beta-adenosine, 2CdA) is used in anticancer therapy against hairy cell and chronic lymphocytic leukemias. Into cells cladribine is phosphorylated to 5′-monophosphate by deoxycytidine and deoxyguanosine kinases, then 5′-diphosphate and 5′-triphosphate, the active nucleotide, are yield [48,106]. At this date, only two articles reported the determination of cladribine nucleotides. Bierau et al. [106] reported an RP-HPLC method for the determination of 2CdA and his monophosphate metabolite (2CdAMP) with UV detection at 265 nm. The assay published by Jansen et al. [48] for cladribine triphosphate (2CdATP) involved an IE-HPLC. This method was based on weak anion-exchange liquid chromatography using a pH gradient coupled with tandem MS operating in the positive ion mode. A satisfactory validation was obtained for the quantification of 2CdAMP, the diphosphorylated metabolite (2CdADP) and 2CdATP in cells and in cellular medium.

4.5. Clofarabine

Clofarabine (2-Chloro-9-(2-deoxy-2-fluoro-beta-D-arabinofuranosyl)adenine) is indicated for the treatment of patients with relapsed or refractory acute lymphoblastic leukaemia. Clofarabine is phosphorylated intracellularly to the cytotoxic 5′-triphosphate metabolite [107]. To our knowledge, to date, no assay described the analysis of the clofarabine mono, di or triphosphate derivatives.

4.6. 6-Mercaptopurine and 6-thioguanine

6-Mercaptopurine is a thiopurine-derivative antimetabolite with antineoplastic and immunosuppressive activities. Produced through the metabolism of mercaptopurine by hypoxanthine-guanine phosphoribosyltransferase, mercaptopurine metabolites 6-thioguanosine-5′-phosphate and 6-thioinosine inhibit nucleotide interconversions and *de novo* purine synthesis. This drug is also incorporated into DNA in the form of deoxythioguanosine. 6-Mercaptopurine is used for the treatment of several malignancy syndromes such as acute lymphoblastic leukaemia, acute promyelocytic leukaemia, chronic myelogenous leukaemia, or lymphoblastic lymphoma. 6-Thioguanine is a synthetic guanosine analog antimetabolite structurally close to 6-mercaptopurine. This drug is phosphorylated by hypoxanthine-guanine phosphoribosyltransferase into cells. 6-Thioguanine is used in therapeutic against acute lymphoblastic leukaemia, acute myeloid leukaemia, or lymphoblastic non-Hodgkins lymphoma [30].

Over the past ten years, to our knowledge, only one assay for the determination of phosphate derivatives has been proposed [46]. The analytical method was poorly described and a validation procedure was lacking. This assay was based on IP liquid chromatography with tetrabutylammonium as ion pairing agent coupled to UV detection. The aim of the work was the study of the metabolism and transport of the thiopurine metabolites from cell lines. Other chromatographic methods which analyzed phosphorylated metabolites of thiopurine without hydrolysis pre-treatment were earlier than 2000s. These methods were based on an anion exchange liquid chromatography with UV [108] or fluorimetric detection after oxidation of the thioguanine nucleotides [109–111].

Methods proposed over the past decade derived from work published prior to 2000 by Lennard [112,113] or Dervieux and Boulieu [114]. In both approaches, a dephosphorylation was performed to convert nucleotides into nucleosides. Thus, these methods do not allow distinguishing the part of mono, di and triphosphate derivatives. These methods were based on acid hydrolysis of the thiopurine nucleotides moieties to thiopurine bases. The hydrolysis was obtained with perchloric acid [114,115] or sulphuric acid [112,116] in the presence of dithiothreitol (DTT) to protect thiol groups from oxidation. Thiopurine nucleotides were measured either in washed erythrocytes or in whole blood as a surrogate to intracellular concentrations of these metabolites in the target tissues. The chemical stability study of phosphorylated metabolites after sampling showed that the concentrations of 6-thioguanine nucleotides in erythrocytes drop drastically after 7 days at room temperature. 6-Thioguanine nucleotides (6-TGN) and S-methyl-mercaptopurine nucleotide (6-MMPN) were stables when samples were stored 7 days at +4 °C [117,118], one month at −20 °C [115] and 6 month at −80 °C [116].

4.7. 5-Fluorouracil

5-Fluorouracil is widely used for over fifty years against several human solid tumors including colorectal, breast, stomach or pancreatic cancer. 5-FU undergoes a complex metabolic pathway leading to its conversion in cells to the active metabolites 5-fluoroxyuridine monophosphate and 5-5-fluoro-2′-deoxyuridine-5′-O-monophosphate. Then, 5-FUTP and 5-fluorodeoxyuridine triphosphate (5-FdUTP) cytotoxic metabolites are produced and incorporated into RNA and DNA, respectively [33].

Several assays were described the quantification of 5-FU and 5-fluorouridine (5-FUrd) and 5-FdUrd. However assays for the determination of the 5-FU phosphorylated metabolites (5-fluorouridine monophosphate (5-FUMP) and 5-FdUMP) were sparse. During the last decade the literature reported three types of assays by liquid chromatography. The first one used tritium radiolabeled 5-FU ([^{3}H-5FU]) and a chromatographic separation with a counter ion. The quantification of 5-FU and its phosphate metabolites was successfully developed in cell lines [119,120]. The second one for the simultaneous quantification of 5-FdUMP, dUMP and TMP was performed using RP-LC–MS/MS and a fully validation procedure was described [60]. For the determination of 5-FdUMP in extra cellular medium, sample preparation was based on ion-exchange SPE.

5. Applications and conclusion

Numerous and varied applications were described in the framework of endogenous deoxyribonucleotides and ribonucleotides

determination. First, the quantification of nucleotides such as adenosine and guanosine derivatives assessed the energy charge i.e. the energy status of cells as an overall quality measure for the sample workup and analytical procedure [73]. Their quantification in microorganisms contributed to the understanding of microbial metabolic networks [56]. The determination of cellular concentrations could provided valuable information for understanding the mechanisms of cell damage and death under different culture conditions such as the effects of different chemical toxicants on cell metabolism in different cell lines [45]. On the other hand, endogenous nucleotides are a target of numerous pharmacological nucleoside analogs such as immunosuppressive, antiviral or anticancer agents. The quantification of intracellular nucleotides provided a valuable tool to better understand the *in vivo* pharmacology of analogs. Wilson et al. [121] compared the effects upon nucleotide metabolism in two different cell lines of three nucleoside analogs (cladribine, fludarabine and pentostatin) to assess possible mechanism of cytotoxicity. Van Moorsel et al. [91] investigated whether changes in ribonucleotide and deoxyribonucleotide levels induced by different drugs may explain the different sensitivity of these drugs in cell lines. The activity and toxicity of nucleoside analogs depend not only on their intracellular phosphorylated active concentrations but also on the intracellular levels of natural nucleotides and mostly on the concentration ratio between the active form of the drug and the physiologic nucleotide counterpart. Finally, the quantification of phosphorylated analogs is also important to study the metabolism and flux of these drugs [122,123].

Ribonucleotides and deoxyribonucleotides are essential cell constituents participating in synthesis of DNA and RNA, in cellular signalling, in metabolic regulation or as source of energy. This review summarized the methods based on the liquid chromatography published during the past 10 years. The list of our references is not exhaustive since numerous assays have been published. Among analytical assays described for the nucleotides determination, LC has been the method of separation predominantly developed with ion exchange or ion pairing agent associated to a gradient. LC separation allowed generally an adequate resolution for UV detection and numerous techniques before 2000 described LC-UV approach. However during the last decade MS has became the detector mostly coupled with LC. The sensitivity and the specificity of MS, and all the more so MS/MS, constituted a major asset for difficult analysis such as the quantification of nucleotides and their analogs in complex biological samples. With their negative charge, this group of compounds may be easily resolved by capillary electrophoresis (CE) with a reduced analysis time. Recent studies highlight the promising potential of this alternative technique, and particularly when the mass detector was coupled [124–127]. Finally, nanoLC–MS could also constitute a future improvement of the nucleotide analysis offering a major advance in term of sensitivity and selectivity.

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