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Direct and Indirect Quantification of Phosphate Metabolites of Nucleoside Analogs in Biological Samples

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Abstract

Nucleoside reverse transcriptase inhibitors (NRTIs) are prodrugs that require intracellular phosphorylation to active triphosphate nucleotide metabolites (NMs) for their pharmacological activity. However, monitoring these pharmacologically active NMs is challenging due to their instability, high hydrophilicity, and their low concentrations in blood and tissues. Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) is the gold standard technique for the quantification of NRTIs and their phosphorylated NMs. In this review, an overview of the publications describing the quantitative analysis of intracellular and total tissue concentration of NMs is presented. The focus of this review is the comparison of the different approaches and challenges associated with sample collection, tissue homogenization, cell lysis, cell counting, analyte extraction, sample storage conditions, and LC-MS analysis. Quantification methods of NMs via LC-MS can be categorized into direct and indirect methods. In the direct LC-MS methods, chromatographic retention of the NMs is accomplished by ion-exchange (IEX), ion-pairing (IP), hydrophilic interaction (HILIC), porous graphitic carbon (PGC) chromatography, or capillary electrophoresis (CE). In indirect methods, parent nucleosides are 1st generated from the dephosphorylation of NMs during sample preparation and are then quantified by reverse phase LC-MS as surrogates for their corresponding NMs. Both approaches have advantages and disadvantages associated with them, which are discussed in this review.

Keywords

Nucleotide metabolites; Nucleoside Analogs; LC-MS/MS; Direct quantification; Indirect quantification

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1. Introduction

Nucleosides are the natural building blocks of DNA and RNA, and their structures composed of a sugar and a nitrogen base. The nitrogen base consists of a pyrimidine base (cytosine (C), thymine (T), uracil (U) or a purine base (adenine (A), guanine (G), whereas the sugar moiety consists of a ribose or a deoxyribose pentose. Nucleoside analogs (NAs) are synthetic, chemically modified nucleosides, which mimic physiological nucleosides in terms of uptake and metabolism and are incorporated into newly synthesized DNA, resulting in synthesis inhibition and chain termination. Some NAs also inhibit key enzymes involved in the generation of the purine and pyrimidine nucleotides and RNA synthesis [1-3].

Therapeutic NAs are used in many diseases including cancer, AIDS, hepatitis, and as immunosuppressive therapies. In particular, nucleoside reverse transcriptase inhibitors (NRTIs) are widely used as antiretroviral drugs. NRTIs are prodrugs that require intracellular phosphorylation to active triphosphate (TP) nucleotide metabolites [4, 5]. NRTIs are converted intracellularly to nucleotide metabolites (NMs) including mono-, di-, and then the active tri-phosphates (TPs) by various nucleoside phosphate kinases [6, 7]. Phosphorylation of nucleoside analogs is a key factor for their efficacy as anti-HIV agents [6, 7].

Monitoring the intracellular TP metabolites of NRTI, which exist at the femtomole levels, is important to understand their pharmacological effects [8, 9]. Therefore, very sensitive and selective methods are required for their quantification. Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) is the gold standard technique for the quantification of NRTIs and their phosphorylated nucleotide metabolites (NMs) [8]. The same approaches followed for the bioanalysis of therapeutic NAs and their metabolites are used for the analysis of endogenous nucleosides and nucleotides [9-14]. However, additional challenges are associated with the bioanalysis of endogenous compounds due to the lack of blank matrices to build calibration curves, which we have discussed previously [15]. Quantification of endogenous nucleotides is of interest for investigation of numerous cellular biochemical processes, such as energy metabolism and signal transduction [12].

NMs contain 1-3 phosphate groups, deoxyribose or ribose pentose sugar, and a purine or a pyrimidine nitrogen base [16] (Figure 1). Due to their hydrophilicity, chromatographic separation of NMs is challenging using traditional reverse phase chromatography. Therefore, alternative approaches are used for the chromatographic separation of NMs, which can be grouped into two categories, direct and indirect approaches [8, 17, 18]. Direct methods rely on the direct quantification of NMs under non-reverse phase LC conditions. In these methods, chromatographic retention of the nucleotides is accomplished by ion-exchange (IEX), ion-pairing (IP), hydrophilic interaction (HILIC), or porous graphitic carbon (PGC) chromatography. Whereas, indirect methods rely on the quantification of the parent nucleosides resulting from the dephosphorylation of NMs during sample preparation, under reverse phase LC conditions. Both approaches have their advantages and disadvantages, as we will discuss in details.

In this review, we present an overview of the publications describing the quantitative analysis of therapeutic NMs using direct and indirect analyses. Tables 1 and 2 lists publications on the direct and indirect LC-MS methods for the quantification of NMs, respectively. We also discussed the challenges associated with the various steps of sample preparation and analysis of NMs. Different approaches are compared and good practices are presented.

2. Sample preparation

The pre-analytical phase, including sample collection, transport, storage, and preparation for analysis, plays an important role in NM analyses. Sample preparation depends on the matrix to be analyzed, but all sample preparation approaches for NM analyses will follow these steps:

2.1. Sample preparation before extraction

2.1.1. Intracellular NMs

2.1.1.1. Cell isolation: NMs are typically measured in peripheral blood mononuclear cells (PBMCs). PBMCs can be isolated from whole blood by density gradient centrifugation. Tubes prefilled with density gradient medium are commercially available for this purpose, such as BD Vacutainer® CPT™ (BD Biosciences, Franklin Lakes, NJ) and SepMate™ (Stem Cells, Cambridge, MA). During PBMC isolation, whole blood is layered over a density gradient made of Ficoll-paque. Based on their different densities, in the order from low to high; plasma, platelets, buffy coat/lymphocyte and monocyte band, density gradient fluid, gel barrier, and erythrocytes (red blood cells) and neutrophils layers are formed from top to bottom in the tube after centrifugation [18]. The buffy coat layer contains mostly PBMCs along with minor portions of granulocytes and erythrocytes. PBMC fractions with a pink or red color, an indication of erythrocyte contamination, shows a stronger and more variable matrix effect caused by endogenous nucleotides from erythrocytes compared to clear fractions [19]. Importantly, erythrocytes and granulocytes are capable of phosphorylating some NMs ex vivo after sample collection. To prevent erythrocyte contamination, an extra erythrocyte lysis step can be performed [18].

Another variable to control during PBMCs isolation, is the formation of NMs ex vivo by PBMCs themselves, i.e. after sample collection. This is achieved by performing the cell isolation step as quickly as possible, and preferably on ice [20]. In addition, extra measures should be in place to minimize the possibility of intracellular NMs leaking outside the cells during cell isolation, washing, and counting. These measures also include cell isolation as quickly as possible under low temperature, using less number of buffer washes, or using oil wash during cell isolation steps [21].

2.1.1.2. Cell counting: To express NMs concentration as amount per cell, the number of cells isolated should be quantified. Cell counting, however, can be a considerable source of variability, which may negatively affect the reliability of the quantitative analysis [18, 22]. Conventional approaches for cell counting include hemocytometer, microscope, or flow

cytometry, but other indirect approaches using protein [23] and DNA [24] determination have also proven to be useful [18].

Manual cell counting using hemocytometry is often considered the gold standard of cell counting methods in many laboratories [18, 25], [26]. However, this method has several limitations including: (1) Operator-dependent variability in distinguishing cells from cell clusters, cell debris or other particles; (2) errors in sample loading volume, dilution, and pipetting; (3) Trypan-blue toxicity to viable cells leading to cell lysis, which underestimates the actual number of viable cells in the sample; and (4) accuracy and precision associated with manual counting of cells. Moreover, manual counting is very tedious and time-consuming. In recent years automated cell counting has become an attractive alternative to manual hemocytometer-based cell counting, which utilizes the same principles of manual hemocytometry, but offers more reliable results in a fraction of the time needed for manual counting [18, 27]. However, automated cell counters only quantify cells between 2–70 μm in size, leaving the possibility that some types of cells and aggregated cells may not be counted [28].

Flow cytometry is also often used for cell counting [29], which simultaneously measures multiple physical characteristics of single cells as they flow in a fluid stream through a light beam. With a flow cytometer, viable cells can be distinguished from nonviable cells based on the differential permeability of two DNA-binding dyes. Unfortunately, this method is unable to quantify larger cell aggregates and cannot be applied for high throughput applications due to elaborate sample processing steps [28].

Cells can also be counted indirectly by determining their DNA content [18, 30, 31]. First, total DNA is extracted and hydrolysed from cells, incubated with fluorogenic DNA intercalants such as SYBR green, and quantified by fluorocytometry [22]. DNA-based cell counting is considered a high throughput method compared to manual cell counting and flow cytometry, which can be used to analyze a large number of samples simultaneously. Another advantage of this approach, and in contrast to the other approaches, is that it counts both dead and alive cells as long as they have intact cell membrane. Therefore, intracellularly NMs concentration expressed per number of cells is more accurate using this method [18]. One disadvantage of this method is that SYBR Green dye fades relatively rapidly; therefore, signal measurement must be done promptly, and readings from each sample must be completed relatively quickly after the addition of SYBR Green [32]

2.1.1.3. Cell lysis: After their isolation, cells are lysed to release intracellular NMs before further sample analysis. Cells could be lysed using organic solvents such as methanol, acetonitrile, alcohols, ether, or chloroform; strong acids like perchloric acid; surfactants, like sodium dodecyl sulfate (SDS); or chaotropic agents such as urea or guanidine. The choice of cell lysis solvents and reagents depends on the chromatographic method to be used for sample analysis after their extraction, as well as the stability profiles of analytes in the cell lysate.

2.1.2. Total NMs using whole tissue homogenization—Total intra- and extra-cellular NMs concentration can also be expressed per mg of tissue proteins without the need

for intracellular quantification [33, 34]. Single-step breakdown of the tissue followed by extraction of total analytes as opposed to cell isolation followed by extraction of intracellular analytes is more rugged and reproducible [35]. One disadvantage of this approach vs. intracellular quantification is that most of the data in NMs literature is reported as intracellular concentration per million cells rather than total NA concentration per mg/tissue. Conversion of concentrations expressed per mg protein into per number of cells concentrations can be challenging [28]. Another disadvantage of whole tissue homogenization is the relatively high matrix effect in LC-MS/MS analysis of tissue compared to cell. Also, NA levels may differ between cell subtypes in the same tissue [8]; which cannot be distinguished after whole tissue homogenization. Specially, in anti-HIV therapy, determination of the intracellular concentrations of NRTIs in specific subtypes of immune cells is important for pharmacodynamic correlations. On the other hand, it is not always easy to isolate cells and differentiate the ones of interest from tissues, which makes whole tissue homogenization a useful approach, given its ruggedness and high throughput.

For total tissue concentration determination, tissues are first homogenized via mechanical homogenization, sonication, cryogrinding, or bead beating [36]. Generally, two to five volumes of diluent is sufficient for all homogenization methods. Hypotonic diluents such as water are preferred because they also help in cell lysis by creating osmotic pressure inside the cells, which lead to plasma membrane burst [37]. Regardless of which homogenization method is used, sample temperature has to be controlled to minimize analyte degradation during homogenization, which is accomplished by using homogenizers equipped with refrigeration units. In mechanical homogenization, a stainless-steel probe-style blender with a generator and a set of blades causes vigorous mixing and turbulence as well as physically shearing with tissue and diluent. In sonication, tissues are cut into small pieces and mixed with diluent, then sonicated at specific frequency using a specially designed probe placed directly into the tissue-diluent mixture. Sonication can also be combined with cryogrinding, where a tissue is snap frozen and ground to a fine powder using a mortar and pestle in a liquid nitrogen bath. In bead beating, small stainless steel beads are added to the vial containing tissue and diluent. The beads are accelerated and continuously bombard the tissue by mechanical shaking of the vial at a high frequency or by an agitating probe placed inside the vial [38, 39].

Among these methods, bead beating, has many advantages because it is considered a one-step high throughput method, but bead size has to be optimized for sample size and the tissue type. In addition, in bead beating, either aqueous or organic diluents can be used for homogenization. When organic diluents are used, this also can serve as an extraction step before LC-MS analysis. However, in other homogenization methods, use of organic diluents may lead to tissue precipitation or clogging of instrument parts.

2.2. Sample extraction

The choice of sample extraction technique depends on the chromatographic method to be used for sample analysis after their extraction. Therefore, we divided sample extraction techniques into two categories:

2.2.1. Sample extraction for direct analytical methods—Typically, protein precipitation with organic solvents is used for sample extraction associated with direct quantification of NMs. In these methods, the cell lysis or tissue homogenization steps will also serve as extraction steps; therefore, solvents that ensure high extraction efficiency and stability of NMs as well as efficient protein precipitation are used [8, 17]. Moreover, these organic solvents should be compatible with the subsequent chromatography separation and MS detection. Therefore, volatile organic solvents such as acetonitrile (ACN) and methanol (MeOH) are typically used for both protein precipitation as well as cell lysis or tissue homogenization. After cell lysis/tissue homogenization, samples are centrifuged and supernatants are directly analyzed by LC-MS, or first concentrated by evaporation and reconstitution in reduced volumes before LC-MS analysis [8]. In addition, samples can be further extracted after protein precipitation with ion-pairing solid phase extraction (SPE) before LC-MS injection [34, 40-42]. This SPE step can help improve selectivity by removing interfering salts present in cell lysate or homogenate.

To minimize dilution and improve sensitivity, minimal volumes of solvents are used for cell lysis and sample extraction without compromising the extraction efficiency. Otherwise, larger volumes are used to increase extraction efficiency, but sample extracts are then concentrated by evaporation and reconstitution in smaller volumes. However, sample evaporation may decrease analyte recovery due to analyte instability during evaporation and reconstitution [17].

Sample extraction for direct methods is generally a one-step simple protein precipitation, which makes it a high throughput process and minimizes instability issues associated with multistep sample processing. On the other hand, protein precipitation is not as rugged and reproducible sample extraction procedure with LC-MS quantification due to the strong and inconsistent matrix effect.

2.2.2. Sample extraction for indirect analytical methods—In indirect methods and unlike direct methods, cell lysis and tissue homogenization are separate steps from sample extraction. Therefore, there are more options available for cell lysis that are not necessarily compatible with LC-MS, but may be more efficient in cell lysis such as strong acids like perchloric acid; surfactants, like sodium dodecyl sulfate (SDS); or chaotropic agents such as urea and guanidine.

Sample extraction of cell lysates or whole tissue homogenates for indirect methods can be divided into three steps: (i) anion exchange solid-phase extraction (SPE), (ii) dephosphorylation, and (iii) reversed phase SPE (Figure 2).

(i) Anion exchange solid-phase extraction (SPE): In the indirect methods, parent nucleosides are reverse generated from their nucleotide metabolites during sample preparation using enzymatic dephosphorylation via acidic or alkaline phosphatases. Therefore, nucleotide metabolites have to be separated into MP, DP, and TP fractions before conversion into their corresponding parent nucleosides because they all produce the same parent nucleoside upon dephosphorylation. NMs fractionation is usually achieved using strong (SAX) or weak (WAX) ion-exchange SPE [43-48].

Sep-Pak Accell Plus QMA is a SAX SPE cartridge (Waters, Milford, MA) frequently used to separate MP, DP, and TP metabolites [43, 44, 46, 48-55]. The cartridges are preconditioned with concentrated salt (500-2000 mM KCl) followed by diluted salt (5 mM) or H₂O. The supernatant from cell lysate is loaded onto the cartridges and then washed with 5 mM KCl or H₂O for the removal of the parent nucleoside. Followed by three consecutive elution steps of MP, DP, and TP metabolites fractions using low (50-70 mM), intermediate (70-120 mM), and high-concentration KCL solutions (400-2000 mM), respectively. The concentrations of these KCl solutions are optimized to maximize separation and minimize co-elution of the various fractions.

Because of the unavailability of commercial standards for the MP and DP metabolites and to confirm the separation of MP, DP, and TP fractions by WAX/SAX SPE, MP, DP, and TP metabolites of their corresponding nitrogen bases are commonly used as surrogate standards followed by HPLC-UV or scintillation counter monitoring. For example, guanosine, cytidine, and thymidine, were used as surrogate standards for carbovir (CBV), lamivudine (3TC), and zidovudine (ZDV), respectively [17, 44]. In addition, TPs are usually of special importance compared to DPs and MPs, due to their pharmacological activity. Therefore, the fractionation procedure is commonly simplified using 2-step elution with intermediate and high KCL solutions to separate TPs from all other species combined, i.e. parent, MP, and DP.

(ii) Dephosphorylation: The three NM fractions eluted from anion-exchange SPE including MPs, DPs, and TPs, are individually subjected to enzymatic phosphorylation to liberate their corresponding parent nucleoside. Dephosphorylation can be performed using acidic or alkaline phosphatases. For acidic phosphatases, optimal pH ranges from 4.2 to 5.5 using ammonium or sodium acetate buffer, and the amount of enzyme used is 0.1 –1 unit per ml of sample, for an incubation time of 0.5 - 2 h at 37 °C [17, 44]. For alkaline phosphatases, optimal pH ranges from 7.5 to 9.5 using Tris-HCl and the remaining conditions are similar to the acidic phosphatases [11].

(iii) Desalting with reversed phase SPE: Parent nucleosides formed in the dephosphorylation step are then subjected to a second SPE step to remove excess buffers and salts used in the first SPE and enzymatic conversion steps. Samples are loaded onto reverse phase SPE cartridges, washed with water or buffer, and parent nucleosides are then eluted with an organic solvent compatible with LC-MS analysis such as MeOH and ACN.

In general, sample preparation for indirect methods is labor intensive and time consuming because it includes three steps of fractionation, dephosphorylation, and de-salting [8, 44]. Every one of the MP, DP, and TP fractions, undergo dephosphorylation, de-salting, and LC-MS analysis, separately, which makes it an even lower-throughput approach. Also, sample preparation for indirect methods suffers from relatively low and irreproducible recovery. Analytes are lost in the 1st and 2nd SPE steps during sample loading and washing, and may not completely elute from the cartridge. Analyte recovery may also be inconsistent from the dephosphorylation step due to incomplete dephosphorylation, variability in incubation time and conditions, and loss of enzymatic activity during storage. Therefore, to improve overall recovery, analyte losses of every step of sample extraction should be quantified and

addressed separately. For example, in our previous study for the quantification of 3TC-TP and CVB-TP, recoveries at individual steps involved in the extraction were 70-90% from the anion-exchange first SPE, 95-100% from dephosphorylation, and 90-100% from the reversed-phase second SPE step. Overall recovery was 60-90 % for both 3TC-TP and CVB-TP. Therefore, analytes losses were primarily associated with the first SPE step, whereas recoveries from second SPE, dephosphorylation, and matrix effect were near 100 % [17].

2.3. NMs stability during sample collection, preparation, storage, and analysis

NMs are highly susceptible to enzymatic and non-enzymatic degradation of their labile phosphate bonds. Therefore, stability evaluation is required throughout sample preparation, analysis, and storage. The time from sample collection until sample analysis can be divided into three stages: (i) time from tissues collection until cell isolation or tissue homogenization; (ii) time from cell isolation until cell lysis (for intracellular NMs quantification); (iii) time after cell lysis or tissue homogenization until sample analysis.

For the 1st stage, tissues are not usually processed immediately after collection and are stored for variable periods of times until further processing. A lower temperature around 4 °C is recommended, but storage of tissues at very low temperature or freezing is not recommended either, because it may interfere with cell isolation. Therefore, NMs stability have to be monitored during that period, which may need to be standardized with certain time limits. This is difficult to quantify because these tissues should already contain the analyte from animals treated with the drug of interest during an in vivo experiment rather than adding analyte standards to blank tissues. We have previously accomplished that by cutting tissues from animals pretreated with NAs into small pieces, and analyzing portions of these pieces over time. For example, we have found that 3TC-TP and CBV-TP were stable in spleen tissues from mice treated with 3TC and ABC and stored at 4 °C for at least 48 h before cell isolation [17]. In case of blood storage before PBMCs isolation, storage stability studies were not performed previously, but the maximum storage time recommend is typically 6 h according to the suppliers' protocols [56].

The 2nd stage associated with sample preparation for intracellular NMs determination, is the storage of isolated cells before cell lysis. Similar to total tissue analysis, cells already containing analytes should be stored under low temperature and for the shortest amount of time possible until the time they are processed and lysed. If cell storage is required, storage of cells as a pellet rather than in suspension at -20 °C or -80 °C is preferred to minimize degradation as well as leakage outside the cells. For example, we have previously reported that 3TC-TP or CBV-TP were stable in PBMCs previously loaded with 3TC and ABC and can be stored at least for 24 h in PBS at 4 °C before cell lysis [17]. In other studies, d4T-TP (stavudine-TP) and ddA-TP (2',3'-dideoxyadenosine triphosphate) were found to be stable in PBMCs pellets isolated from human blood as a part of a clinical trial for up to 3-months at -20 °C, and for up to 6 months at -80 °C [24, 57]. TFV-DP was also stable in PBMCs pellet isolated from human blood for up to 345 days at -70 °C [18]

The 3rd stage represents NMs stability in tissues after homogenization as well as cells after lysis until samples are analyzed. This is easier to accomplish at this stage because stability can be monitored as a part of standard method validation protocols by spiking tissue

homogenates or cell lysates with an analyte standard and monitor its stability over time under various conditions. Normally and as a part of bioanalytical method validation, freeze-thaw, bench-top, post-extraction storage, autosampler, and long-term storage stability are performed. Stability for NMs in cell lysates or tissue homogenates should be tested at temperatures relevant to their processing, storage, and analysis conditions; typically, 4 °C, –20 or –80 °C, and room temperature, respectively. These stability studies were almost always performed entirely or partially, as part of method development, for NMs such as AZT-TP, d4T-TP, 3TC-DP, TFV-DP (tenofovir diphosphate), FTC-TP (emtricitabine-TP), and CBV-TP [17, 43, 47, 48, 58].

Use of internal standard (IS) during sample storage and preparation improves NMs quantification accuracy. In general, stable isotopically labeled (SIL) internal standards yield better assay performance compared to structural analog ISs. However, purity and stability of the SIL-IS label should be monitored to prevent the introduction of unlabeled compound into samples.

3. Quantification methods

Nucleotide metabolites are quantified by direct or indirect approaches.

3.1. Direct methods

In the direct LC-MS methods, chromatographic retention of NMs is accomplished by IEX, IP, hydrophilic interaction (HILIC), porous graphitic carbon (PGC) chromatography, or capillary electrophoresis (CE) (Table 1).

3.1.1. Ion exchange (IEX) chromatography—In IEX chromatography, analytes are retained by electrostatic binding to the oppositely charged functional groups of the column. Analytes are then eluted via a pH- or a counter-ion mobile phase gradient, to displace the column-bound analytes [8, 9, 33, 59]. Both strong and weak IEX columns are used. Strong IEX stationary phases have strong acidic or basic functional groups, which are permanently charged regardless of the buffer pH, such as quaternary ammonium, sulfonate, and sulfopropyl groups. In contrast, weak IEX columns, have weak acidic or basic functional groups with pH-dependent ionization such as diethylaminoethyl (DEAE) or carboxymethyl (CM) groups [9, 60].

Because of the negatively charged phosphate groups, anion-exchange chromatography is used for NMs. For LC-MS methods of NMs in particular, WAX chromatography is typically used because it utilizes relatively more volatile and more electrospray (ESI)-compatible mobile phases compared to SAX chromatography [9, 60, 61]. In general, IEX chromatography is incompatible with ESI-MS because of ionization suppression by the mobile phase additives, unless volatile buffers and counter-ions are utilized. In addition, ion exchange-MS methods are generally not rugged nor reproducible due to salt build-up in the column, LC system, and MS source, overtime. IEX methods are also more sensitive to matrix components than reverse phase chromatography, which makes chromatography of the same analyte, matrix-dependent. Therefore, elaborate sample extraction to remove most of matrix components is required before LC-analysis [62]. In addition, relatively extreme pHs

are often used, which may damage the column and other components of the LC system, relatively fast. Finally, sensitivity of IEX methods is usually low because of ionization suppression by the mobile phase components.

3.1.2. Ion-pairing chromatography (IPC)—In IPC, charged analytes form “ion-pairs” via electrostatic binding with oppositely charged IP agents, which are used as additives to mobile phase. Therefore, formation of these ion-pairs mask the charge of the analyte, and facilitate its retention on reverse-phase columns. Similar to IEX, IPC in general, is also irreproducible, not rugged, insensitive, matrix-dependent, and incompatible with ESI-MS. In addition, IP mobile phases cause damages to the column, LC, and MS systems in a relatively short period of time due to the build-up of salts in the system over time. When used with ESI-MS, relatively volatile shorter-chain and positively charged/acidic IP agents, which usually form weaker ion-pairs, are used such as tripropylamine, hexylamine, triethylamine, tributylammonium acetate, tetrabutylammonium acetate, Tetrabutyl ammonium hydroxide (TBAH), trifluoroacetic acid, N,N- dimethylhexylamine (DMHA), and 1,5-DMHA [8, 9, 62-83]. To minimize the adverse effects of IP agents on the LC-MS system, IP agents can be added to the sample rather than to the mobile phase to decrease the LC-MS system exposure to these agents. However, this comes at the expense of analyte retention and separation efficiency [63].

Retention of NMs with acidic ion pairing agents depends on the number of charges of NMs; therefore, TPs are retained the most followed by DPs, then MPs. Concentration of the IP agent, the organic solvent, and pH of the mobile phase play important role in the separation of these analytes. Optimal concentration of IP agent depends on many factors but we found it to be about ten times the concentration of nucleotides in the sample [84]. Higher concentrations of IP agents, will increase the affinity of the NMs for the stationary phase causing extended retention times and peak broadening. Elution of analytes from the column is achieved using a gradient profile that gradually increase the concentration of the organic mobile phase, the concentration of the cationic counter ion of the aqueous mobile phase, or H^+ concentration [60, 84]. Nucleotide separation is usually carried out between pH 6.0 and 8.0. Lower pHs, may decrease NMs retention by decreasing ionization of the analytes, and higher pHs will also decrease retention by decreasing the ionization of the IP agents.

3.1.3. Porous graphitic carbon (PCG) columns—In PGC chromatography, the stationary phase is made of intertwined graphitic ribbons, which is a conducting crystalline material. The graphitic sheets are constituted of sp^2 hybridized carbon atoms, laid out in hexagonal arrangements. Both polar and nonpolar compounds are retained because polar or polarizable analytes such as nucleotides form a charge-induced dipole with the graphite surface of the column [85], while nonpolar analytes are retained via hydrophobic interactions with the planer hexagonal graphite carbons. Therefore, PGC has the advantage of not requiring high salt concentrations or IP agents, which are needed in IEX and IP chromatography, respectively [86, 87]. However, similar to IEX and IP, PGC is also irreproducible and is associated with loss of retention capacity over time due to contamination with matrix components and oxidation of the packing material over time, which can cause changes to column surface and charge over time [86, 88].

3.1.4. Hydrophilic interaction liquid chromatography (HILIC)—HILIC

chromatography uses normal-phase stationary phases in combination with reverse-phase mobile phases, for a better retention of polar analytes. When the aqueous mobile phase contains polar solvents such as water, the polar solvent preferentially adsorbs onto the polar stationary phase, creating a semi-stagnant water layer around the stationary phase, where analytes are retained [34, 89]. Analyte retention is dependent on the extent of partitioning between the mobile phase and the aqueous-encapsulated stationary phase [89]. In contrast to reverse-phase chromatography, retained analytes elute from the column by increasing the aqueous rather than the organic content of the mobile phase [8, 89]. Similar to IEX, IP, and PGC, HILIC is, overall, sensitive to matrix effects and shows variability in retention times. The polar endogenous components of biological matrices are strongly retained on the column, which gradually deteriorates its performance. Consequently, a typical problem with HILIC is column overloading with matrix components, which results in peak distortion, peak splitting, and shift in retention time; therefore, requires elaborate sample extraction before LC-analysis [89]. On the other hand, similar to PGC, HILIC uses ESI-MS compatible mobile phases and does not require the use of salts or IP agents in the mobile phase.

3.1.5. Capillary electrophoresis—Capillary electrophoresis (CE) is an analytical technique that separates analytes in a fused silica capillary using electrolyte solution (separation buffer) under an electrical field application [90, 91]. Electrophoretic mobility of analytes depends on their charge and size, buffer pH, ionic strength, buffer composition and viscosity, and electro-osmotic flow. Efficient separation, low solvent and sample consumption, and low running costs represent significant advantages of this method for NMs analysis. Despite the high salt concentrations used in the separation buffer, the small (micro) flow used in CE minimizes matrix effect in the ESI source and makes CE a good separation technique to combine with MS and a good alternative to LC-MS. However, CE-MS method development requires optimization of various parameters that makes it more challenging than LC-MS method development. Also, CE separations are not as rugged or reproducible as LC methods [90-92].

3.1.6. MS detection without chromatographic separation—Matrix-assisted laser desorption/ionization (MALDI) is a soft ionization technique used in mass spectrometry commonly applied for the analysis of large molecules (DNA, proteins, peptides, etc.). MALDI offers the advantage of more tolerance to the effect matrix components on analytes ionization [93]. In addition, raw samples without any extraction and without chromatographic separation are directly analyzed by MALDI-MS, which markedly shorten analysis time and increase its throughput. MALDI is often hyphenated with Time of flight (TOF) MS analyzer, which offers high sensitivity and resolution [94]. On the other hand, MALDI is primarily used for macromolecule analysis and its application for small molecules is limited [95]. Direct MALDI-TOF analysis without chromatographic separation was used for the quantification of AZT-TP from human PBMCs [93].

3.2. Indirect methods

Parent nucleosides generated from the dephosphorylation of NMs during sample preparation are separated on reversed-phase or modified reverse phase chromatography as part of their

LC-MS quantification (Table 2). Even though not as polar as NMs, nucleosides are still hydrophilic analytes, which are hard to retain on reverse phase columns. Therefore, modified reversed-phase columns with embedded polar groups (e.g., amide, phenyl, Pentafluorophenyl) are typically used instead of conventional reversed-phase columns [17, 43, 54, 96]. Retention on these columns is still minimal using minimal amounts of organic mobile phases, yet is acceptable due to the additional selectivity of MS detection. The organic composition of the mobile phase can be as low as 5% to increase nucleosides retention, which adversely affect analyte ionization in the ESI source and can lower analytical sensitivity [44, 97]. In addition, low organic content is, in general, associated with lower reproducibility of retention time. In general, very low amounts of organic content of the mobile phase leads to the collapse of reverse-phase stationary phases, which results in peak tailing, broadening, and shift in retention time [97]. Also, low organic content decreases the overall performance of the LC-MS system by promoting the build-up of endogenous components of samples and microbial growth over time. Alternatively, other modes of chromatography are used such as HILC and IPC [98].

The main advantage of indirect methods is the ruggedness and sensitivity associated with reversed-phase/modified reversed-phase chromatography as opposed to the other modes of chromatography (IEX, IPC, PGC, etc.) utilized in direct methods. The mobile phase modifiers used in these direct methods cause inconsistency in analytes retention over time and leads to ionization suppression in the MS source. Both problems are avoided with reversed phase chromatography methods. For example, LLOQ of 20 and 204, fmol/million were reported with two CBV-TP direct methods, whereas LLOQ of the same analyte were 0.8 and 2 fmol/million with two indirect methods [17, 44, 77]. Similarly, three direct methods reported LLOQ of 100-1000 fmol/million for 3TC-TP, while LLOQ as low as 2.1 fmol/million was reported with indirect methods for 3TC-TP [17, 44, 52, 76, 77]. Also LLOQ ranged from 15 to 150 fmol/million for direct ZDV-TP methods as compared to ~4 fmol/million for indirect methods [44, 47, 48, 74, 76, 99].

In addition, isotope-labeled NAs or their structural analogs could be used as internal standards for indirect methods [17]. These compounds are cheap and commercially available from multiple sources. In contrast, isotope-labeled mono, di, and tri-phosphates or their structural analogs should be used for direct methods, which can be very expensive and are not commercially available in most cases [44]. On the other hand, the sample preparation associated with direct methods is much faster and simpler compared to the complex sample preparation of indirect methods [8, 17].

4. Detection

4.1 Mass spectrometry (MS)

LC-MS/MS has become the technique of choice for quantitative analyses because of its sensitivity, selectivity, and speed. In LC-MS/MS, the effluent from the LC system enters the ionization source, where analytes are ionized before mass-separation by the MS analyzer [15]. Atmospheric pressure ionization (API), especially electrospray ionization (ESI) is the most commonly used MS source [100, 101]. Nucleotides carry permanently negative charges on their phosphate groups; therefore, are expected to ionize efficiently in the

negative ionization mode of the ESI source. However, NMs usually produce a stronger signal in the positive mode due to the positive charge on the nitrogen base [8, 42, 77, 102]. These positively charged nitrogen bases are also typically the most abundant fragments produced in the collision cell of the MS/MS system [18, 77]. One reason for the weaker signal of the negative mode is due to the use of ion-pairing agents in direct methods, which could mask the negative charges on the phosphate moiety [102]. In addition the negative mode produce ions with multiple charges from the three phosphate groups and this distribution of charge state could decrease sensitivity for particular ions [18]. Also, the negative mode may have lower selectivity because many endogenous nucleotides share the same fragmentation pattern, which can produce interfering signals in the negative mode, unless separated chromatographically [8, 18]. However, some methods have successfully used the negative ionization mode with comparable detection limits for the quantification of TPs [74, 80].

Another challenge with the MS detection of NMs is the overlap in signal between the mono, di, and tri-phosphates [8]. Even though, these metabolites have different masses, they still have to be chromatographically separated, because of the cross-talk between them due to the production of the same fragment ions in the source and/or the collision cell [18]. Otherwise, false signals can be produced from the formation of mono- or di-phosphates from the tri-phosphate metabolites in the MS system [8, 85].

4.2. Fluorescence, enzymatic, and UV methods

LC-MS is currently the method choice for NMs quantification due to its superior sensitivity, selectivity and resolution. Therefore, it replaced most of the older methods used for NMs quantification including HPLC-UV, fluorescence, and enzyme immunoassays [103, 104]. In HPLC-UV methods, there is more demand on chromatographic resolution compared to LC-MS because of the absence of the additional MS selectivity. Therefore, long separations are needed with HPLC-UV methods using modified reverse phase, IP, HILIC, and IEX chromatography. On the other hand, UV or fluorescence detection has the advantage of compatibility with non-volatile IP agents and salts in the mobile phase, which causes better retention and resolution with IP or IEX chromatography [103, 104].

HPLC methods with fluorescence detection were developed to improve sensitivity and selectivity compared to UV detection. In these methods, fluorophores such as 1,N⁶-ethenoadenosine are used to derivatize NMs into fluorescent analytes [103, 104]. Enzymatic immunoassays, commercially available as kits, were also used for the direct quantification of NMs. In these assays, NMs are bound to secondary antibodies, which interact with surfaces coated with primary antibodies, and are detected by fluorescence or radioactivity detectors [105].

5. Conclusions

We presented an overview of the publications describing the quantitative analysis of intracellular and total tissue NMs. The focus was on comparing the different approaches and challenges associated with sample collection, tissue homogenization, cell lysis, cell counting, analyte extraction, sample storage conditions, and LC-MS analysis.

During cell isolation, contamination from other cell types and ex vivo formation and/or losses of NMs should be monitored and minimized. We also described various cell counting methods and their advantages and disadvantages. Similarly, various approaches for tissue homogenization before total vs. intracellular NMs quantification were compared and good practice were presented. For sample extraction, generally one-step simple protein precipitation is used with direct LC-MS methods. In contrast, multi-step and complex processes are used to prepare samples for indirect LC-MS methods including NMs fractionation by SPE followed by dephosphorylation into parent nucleosides and further desalting via another SPE step. NMs are highly susceptible to enzymatic and non-enzymatic degradation of their labile phosphate bonds. Therefore, evaluation of stability is required throughout sample preparation, analysis, and storage.

Quantification methods of NMs via LC-MS can be categorized into direct and indirect methods. In the direct LC-MS methods, chromatographic retention of the NMs is accomplished by IEX, IP, hydrophilic interaction (HILIC), porous graphitic carbon (PGC) chromatography, or capillary electrophoresis (CE). In indirect methods, parent nucleosides are 1st generated from the dephosphorylation of NMs during sample preparation and then quantified by reverse phase LC-MS as surrogates for their corresponding NMs. Direct methods have the advantage of simple sample preparation, while indirect methods are more rugged and sensitive due to the compatible LC-MS conditions.

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REFERENCES

- [1]. Cherry CL, Wesselingh SL, Nucleoside analogues and HIV: the combined cost to mitochondria, *J Antimicrob Chemother* 51(5) (2003) 1091–3. [PubMed: 12668570]
- [2]. Galmarini CM, Mackey JR, Dumontet C, Nucleoside analogues: mechanisms of drug resistance and reversal strategies, *Leukemia* 15(6) (2001) 875–90. [PubMed: 11417472]
- [3]. el Kouni MH, Trends in the design of nucleoside analogues as anti-HIV drugs, *Curr Pharm Des* 8(8) (2002) 581–93. [PubMed: 11945160]
- [4]. Balzarini J, Aquaro S, Hassan-Abdallah A, Daluge SM, Perno CF, McGuigan C, Improved antiviral activity of the aryloxymethoxyalaninyl phosphoramidate (APA) prodrug of abacavir (ABC) is due to the formation of markedly increased carbovir 5'-triphosphate metabolite levels, *FEBS Lett* 573(1-3) (2004) 38–44. [PubMed: 15327972]
- [5]. Coulier L, Gerritsen H, van Kampen JJ, Reedijk ML, Luiders TM, Osterhaus AD, Gruters RA, Brull L, Comprehensive analysis of the intracellular metabolism of antiretroviral nucleosides and nucleotides using liquid chromatography-tandem mass spectrometry and method improvement by using ultra performance liquid chromatography, *J Chromatogr B Analyt Technol Biomed Life Sci* 879(26) (2011) 2772–82.
- [6]. Van Rompay AR, Johansson M, Karlsson A, Phosphorylation of nucleosides and nucleoside analogs by mammalian nucleoside monophosphate kinases, *Pharmacol Ther* 87(2-3) (2000) 189–98. [PubMed: 11008000]
- [7]. Jordheim LP, Durantel D, Zoulim F, Dumontet C, Advances in the development of nucleoside and nucleotide analogues for cancer and viral diseases, *Nat Rev Drug Discov* 12(6) (2013) 447–64. [PubMed: 23722347]

- [8]. Jansen RS, Rosing H, Schellens JH, Beijnen JH, Mass spectrometry in the quantitative analysis of therapeutic intracellular nucleotide analogs, *Mass Spectrom Rev* 30(2) (2011) 321–43. [PubMed: 20623700]
- [9]. Thomas D, Herold N, Keppler OT, Geisslinger G, Ferreiros N, Quantitation of endogenous nucleoside triphosphates and nucleosides in human cells by liquid chromatography tandem mass spectrometry, *Anal Bioanal Chem* 407(13) (2015) 3693–704. [PubMed: 25749797]
- [10]. Fromentin E, Gavegnano C, Obikhod A, Schinazi RF, Simultaneous quantification of intracellular natural and antiretroviral nucleosides and nucleotides by liquid chromatography-tandem mass spectrometry, *Anal Chem* 82(5) (2010) 1982–9. [PubMed: 20143781]
- [11]. 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, *Pharm Res* 34(1) (2017) 73–83. [PubMed: 27633886]
- [12]. Kamceva T, Bjanec T, Svandal A, Riedel B, Schjott 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, *J Chromatogr B Analyt Technol Biomed Life Sci* 1001 (2015) 212–20.
- [13]. Zhang G, Walker AD, Lin Z, Han X, Blatnik M, Steenwyk RC, Groeber EA, Strategies for quantitation of endogenous adenine nucleotides in human plasma using novel ion-pair hydrophilic interaction chromatography coupled with tandem mass spectrometry, *J Chromatogr A* 1325 (2014) 129–36. [PubMed: 24377733]
- [14]. Cohen S, Megherbi M, Jordheim LP, Lefebvre I, Perigaud C, Dumontet C, Guitton J, Simultaneous analysis of eight nucleoside triphosphates in cell lines by liquid chromatography coupled with tandem mass spectrometry, *J Chromatogr B Analyt Technol Biomed Life Sci* 877(30) (2009) 3831–40.
- [15]. Thakare R, Chhonker YS, Gautam N, Alamoudi JA, Alnouti Y, Quantitative analysis of endogenous compounds, *J Pharm Biomed Anal* 128 (2016) 426–437. [PubMed: 27344632]
- [16]. 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* 1006 (2015) 167–78. [PubMed: 26551209]
- [17]. Gautam N, Lin Z, Banoub MG, Smith NA, Maayah A, McMillan J, Gendelman HE, Alnouti Y, Simultaneous quantification of intracellular lamivudine and abacavir triphosphate metabolites by LC-MS/MS, *J Pharm Biomed Anal* 153 (2018) 248–259. [PubMed: 29518644]
- [18]. Xiao D, Ling KHJ, Custodio J, Majeed SR, Tarnowski T, Quantitation of intracellular triphosphate metabolites of antiretroviral agents in peripheral blood mononuclear cells (PBMCs) and corresponding cell count determinations: review of current methods and challenges, *Expert Opin Drug Metab Toxicol* 14(8) (2018) 781–802. [PubMed: 30010446]
- [19]. Furman PA, Fyfe JA, St Clair MH, Weinhold K, Rideout JL, Freeman GA, Lehrman SN, Bolognesi DP, Broder S, Mitsuya H, et al., Phosphorylation of 3'-azido-3'-deoxythymidine and selective interaction of the 5'-triphosphate with human immunodeficiency virus reverse transcriptase, *Proc Natl Acad Sci U S A* 83(21) (1986) 8333–7. [PubMed: 2430286]
- [20]. Lan X, Mingdao L, Huilin G, Wei G, Lvjiang H, Yan Z, Gang L, Simultaneous determination of 2',3'-dideoxyinosine and the active metabolite, 2',3'-dideoxyadenosine-5'-triphosphate in human peripheral-blood mononuclear cell by HPLC-MS/MS and the application to cell pharmacokinetics, *J Chromatogr B Analyt Technol Biomed Life Sci* 1002 (2015) 337–42.
- [21]. Cory TJ, Winchester LC, Robbins BL, Fletcher CV, A rapid spin through oil results in higher cell-associated concentrations of antiretrovirals compared with conventional cell washing, *Bioanalysis* 7(12) (2015) 1447–55. [PubMed: 26168252]
- [22]. Moore JD, Acosta EP, Johnson VA, Bassett R, Eron JJ, Fischl MA, Long MC, Kuritzkes DR, Sommadossi JP, Intracellular nucleoside triphosphate concentrations in HIV-infected patients on dual nucleoside reverse transcriptase inhibitor therapy, *Antivir Ther* 12(6) (2007) 981–6. [PubMed: 17926654]

- [23]. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal Biochem* 72 (1976) 248–54. [PubMed: 942051]
- [24]. Becher F, Landman R, Mboup S, Kane CN, Canestri A, Liegeois F, Vray M, Prevot MH, Leleu G, Benech H, Monitoring of didanosine and stavudine intracellular trisphosphorylated anabolite concentrations in HIV-infected patients, *AIDS* 18(2) (2004) 181–7. [PubMed: 15075534]
- [25]. Bazzoli C, Jullien V, Le Tiec C, Rey E, Mentre F, Taburet AM, Intracellular Pharmacokinetics of Antiretroviral Drugs in HIV-Infected Patients, and their Correlation with Drug Action, *Clin Pharmacokinet* 49(1) (2010) 17–45. [PubMed: 20000887]
- [26]. Yan Z, Sun J, Wang J, Xu Y, Chang Y, Meng P, Zhu M, Fu Q, Sun Y, He Z, Simultaneous determination of didanosine and its amino acid prodrug, valdidanosine by hydrophilic interaction chromatography coupled with electrospray ionization tandem mass spectrometry: application to a pharmacokinetic study in rats, *J Chromatogr B Analyt Technol Biomed Life Sci* 878(3–4) (2010) 466–70.
- [27]. Cadena-Herrera D, Esparza-De Lara JE, Ramirez-Ibanez ND, Lopez-Morales CA, Perez NO, Flores-Ortiz LF, Medina-Rivero E, Validation of three viable-cell counting methods: Manual, semi-automated, and automated, *Biotechnol Rep (Amst)* 7 (2015) 9–16. [PubMed: 28626709]
- [28]. Dyavar SR, Ye Z, Byrareddy SN, Scarsi KK, Winchester LC, Weinhold JA, Fletcher CV, Podany AT, Normalization of cell associated antiretroviral drug concentrations with a novel RPP30 droplet digital PCR assay, *Sci Rep* 8(1) (2018) 3626. [PubMed: 29483619]
- [29]. Shi G, Wu JT, Li Y, Geleziunas R, Gallagher K, Emm T, Olah T, Unger S, Novel direct detection method for quantitative determination of intracellular nucleoside triphosphates using weak anion exchange liquid chromatography/tandem mass spectrometry, *Rapid Commun Mass Spectrom* 16(11) (2002) 1092–9. [PubMed: 11992513]
- [30]. Jansen RS, Rosing H, Schellens JH, Beijnen JH, Protein versus DNA as a marker for peripheral blood mononuclear cell counting, *Anal Bioanal Chem* 395(3) (2009) 863–7. [PubMed: 19685233]
- [31]. Colombo S, Beguin A, Telenti A, Biollaz J, Buclin T, Rochat B, Decosterd LA, Intracellular measurements of anti-HIV drugs indinavir, amprenavir, saquinavir, ritonavir, nelfinavir, lopinavir, atazanavir, efavirenz and nevirapine in peripheral blood mononuclear cells by liquid chromatography coupled to tandem mass spectrometry, *J Chromatogr B Analyt Technol Biomed Life Sci* 819(2) (2005) 259–76.
- [32]. Suzuki T, Fujikura K, Higashiyama T, Takata K, DNA staining for fluorescence and laser confocal microscopy, *J Histochem Cytochem* 45(1) (1997) 49–53. [PubMed: 9010468]
- [33]. Liu A, Lute J, Gu H, Wang B, Trouba KJ, Arnold ME, Aubry AF, Wang J, Challenges and solutions in the bioanalysis of BMS-986094 and its metabolites including a highly polar, active nucleoside triphosphate in plasma and tissues using LC-MS/MS, *J Chromatogr B Analyt Technol Biomed Life Sci* 1000 (2015) 29–40.
- [34]. Pucci V, Giuliano C, Zhang R, Koeplinger KA, Leone JF, Monteagudo E, Bonelli F, HILIC LC-MS for the determination of 2'-C-methyl-cytidine-triphosphate in rat liver, *J Sep Sci* 32(9) (2009) 1275–83. [PubMed: 19347863]
- [35]. Boulieu R, Sample preparation for the determination of purine nucleotide analogues in tissues, *J Chromatogr A* 729(1–2) (1996) 197–200. [PubMed: 9004940]
- [36]. Goldberg S, Mechanical/physical methods of cell disruption and tissue homogenization, *Methods Mol Biol* 424 (2008) 3–22. [PubMed: 18369848]
- [37]. Goodhead LK, MacMillan FM, Measuring osmosis and hemolysis of red blood cells, *Adv Physiol Educ* 41(2) (2017) 298–305. [PubMed: 28526694]
- [38]. Goldberg S, Mechanical/physical methods of cell distribution and tissue homogenization, *Methods Mol Biol* 1295 (2015) 1–20. [PubMed: 25820709]
- [39]. Liang X, Ubhayakar S, Liederer BM, Dean B, Ran-Ran Qin A, Shahidi-Latham S, Deng Y, Evaluation of homogenization techniques for the preparation of mouse tissue samples to support drug discovery, *Bioanalysis* 3(17) (2011) 1923–33. [PubMed: 21899502]

- [40]. Wang LH, Begley J, St Claire RL 3rd, Harris J, Wakeford C, Rousseau FS, Pharmacokinetic and pharmacodynamic characteristics of emtricitabine support its once daily dosing for the treatment of HIV infection, *AIDS Res Hum Retroviruses* 20(11) (2004) 1173–82. [PubMed: 15588339]
- [41]. Hawkins T, Veikley W, St Claire RL 3rd, Guyer B, Clark N, Kearney BP, Intracellular pharmacokinetics of tenofovir diphosphate, carbovir triphosphate, and lamivudine triphosphate in patients receiving triple-nucleoside regimens, *J Acquir Immune Defic Syndr* 39(4) (2005) 406–11. [PubMed: 16010161]
- [42]. Claire RL 3rd, 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 Commun Mass Spectrom* 14(17) (2000) 1625–34. [PubMed: 10960917]
- [43]. Bushman LR, Kiser JJ, Rower JE, Klein B, Zheng JH, Ray ML, Anderson PL, 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* 56(2) (2011) 390–401. [PubMed: 21715120]
- [44]. Robbins BL, Poston PA, Neal EF, Slaughter C, Rodman JH, Simultaneous measurement of intracellular triphosphate metabolites of zidovudine, lamivudine and abacavir (carbovir) in human peripheral blood mononuclear cells by combined anion exchange solid phase extraction and LC-MS/MS, *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences* 850(1-2) (2007) 310–7. [PubMed: 17197254]
- [45]. Robbins BL, Waibel BH, Fridland A, Quantitation of intracellular zidovudine phosphates by use of combined cartridge-radioimmunoassay methodology, *Antimicrob Agents Chemother* 40(11) (1996) 2651–4. [PubMed: 8913483]
- [46]. Moyle G, Boffito M, Fletcher C, Higgs C, Hay PE, Song IH, Lou Y, Yuen GJ, Min SS, Guerini EM, Steady-state pharmacokinetics of abacavir in plasma and intracellular carbovir triphosphate following administration of abacavir at 600 milligrams once daily and 300 milligrams twice daily in human immunodeficiency virus-infected subjects, *Antimicrob Agents Chemother* 53(4) (2009) 1532–8. [PubMed: 19188387]
- [47]. Becher F, Schlemmer D, Pruvost A, Nevers MC, Goujard C, Jorajuria S, Guerreiro C, Brossette T, Lebeau L, Creminon C, Grassi J, Benech H, Development of a direct assay for measuring intracellular AZT triphosphate in humans peripheral blood mononuclear cells, *Anal Chem* 74(16) (2002) 4220–7. [PubMed: 12199596]
- [48]. 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, *J Chromatogr B Analyt Technol Biomed Life Sci* 831(1-2) (2006) 248–57.
- [49]. Moore JD, Valette G, Darque A, Zhou XJ, Sommadossi JP, Simultaneous quantitation of the 5'-triphosphate metabolites of zidovudine, lamivudine, and stavudine in peripheral mononuclear blood cells of HIV infected patients by high-performance liquid chromatography tandem mass spectrometry, *J Am Soc Mass Spectrom* 11(12) (2000) 1134–43. [PubMed: 11118121]
- [50]. Rodriguez JF, Rodriguez JL, Santana J, Garcia H, Rosario O, Simultaneous quantitation of intracellular zidovudine and lamivudine triphosphates in human immunodeficiency virus-infected individuals, *Antimicrob Agents Chemother* 44(11) (2000) 3097–100. [PubMed: 11036029]
- [51]. Yuen GJ, Lou Y, Bumgarner NF, Bishop JP, Smith GA, Otto VR, Hoelscher DD, Equivalent steady-state pharmacokinetics of lamivudine in plasma and lamivudine triphosphate within cells following administration of lamivudine at 300 milligrams once daily and 150 milligrams twice daily, *Antimicrob Agents Chemother* 48(1) (2004) 176–82. [PubMed: 14693537]
- [52]. Holdich T, Shiveley LA, Sawyer J, Effect of Lamivudine on the plasma and intracellular pharmacokinetics of apricitabine, a novel nucleoside reverse transcriptase inhibitor, in healthy volunteers, *Antimicrob Agents Chemother* 51(8) (2007) 2943–7. [PubMed: 17242147]
- [53]. Goicoechea M, Jain S, Bi L, Sun S, Smith G, Ha B, Richman D, Louie S, Haubrich R, California Collaborative Treatment G, Interlaboratory measurement differences in intracellular carbovir triphosphate concentrations in HIV-infected patients: sources of variability in processing, shipping, and quantitation, *J Clin Pharmacol* 50(8) (2010) 968–74. [PubMed: 19952374]

- [54]. Chen J, Flexner C, Liberman RG, Skipper PL, Louissaint NA, Tannenbaum SR, Hendrix CW, Fuchs EJ, Biphasic elimination of tenofovir diphosphate and nonlinear pharmacokinetics of zidovudine triphosphate in a microdosing study, *J Acquir Immune Defic Syndr* 61(5) (2012) 593–9. [PubMed: 23187888]
- [55]. Jimmerson LC, Ray ML, Bushman LR, Anderson PL, Klein B, Rower JE, Zheng JH, Kiser JJ, Measurement of intracellular ribavirin mono-, di- and triphosphate using solid phase extraction and LC-MS/MS quantification, *J Chromatogr B Analyt Technol Biomed Life Sci* 978-979 (2015) 163–72.
- [56]. Kim DW, Jang YY, Shin MG, Shin JH, Suh SP, Ryang DW, Yoon M, Lee JJ, Kim SK, Cho D, Overnight storage of blood in ACD tubes at 4{degrees}C increases NK cell fraction in peripheral blood mononuclear cells, *Ann Clin Lab Sci* 43(3) (2013) 267–73. [PubMed: 23884220]
- [57]. Pruvost A, Becher F, Bardouille P, Guerrero C, Creminon C, Delfraissy JF, Goujard C, Grassi J, Benech H, Direct determination of phosphorylated intracellular anabolites of stavudine (d4T) by liquid chromatography/tandem mass spectrometry, *Rapid Commun Mass Spectrom* 15(16) (2001) 1401–8. [PubMed: 11507751]
- [58]. Becher F, Pruvost A, Gale J, Couerbe P, Goujard C, Boutet V, Ezan E, Grassi J, Benech H, A strategy for liquid chromatography/tandem mass spectrometric assays of intracellular drugs: application to the validation of the triphosphorylated anabolite of antiretrovirals in peripheral blood mononuclear cells, *J Mass Spectrom* 38(8) (2003) 879–90. [PubMed: 12938109]
- [59]. Jansen RS, Rosing H, de Wolf CJ, 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 Commun Mass Spectrom* 21(24) (2007) 4049–59. [PubMed: 18008286]
- [60]. Lammerhofer M, Nogueira R, Lindner W, Multi-modal applicability of a reversed-phase/weak-anion exchange material in reversed-phase, anion-exchange, ion-exclusion, hydrophilic interaction and hydrophobic interaction chromatography modes, *Anal Bioanal Chem* 400(8) (2011) 2517–30. [PubMed: 21336792]
- [61]. Sun L, Chavez-Eng C, Fillgrove KL, Lu B, Xie I, Rudd DJ, Breidinger S, Anderson M, Yeh S, Zhang R, Woolf EJ, Toward highly sensitive and reproducible LC-MS/MS analysis of MK-8591 phosphorylated anabolites in human peripheral blood mononuclear cells, *Bioanalysis* (2019).
- [62]. Kostianen R, Kauppila TJ, Effect of eluent on the ionization process in liquid chromatography-mass spectrometry, *J Chromatogr A* 1216(4) (2009) 685–99. [PubMed: 18799161]
- [63]. Zhao Y, Liu G, Liu Y, Yuan L, Hawthorne D, Shen JX, Guha M, Aubry A, Improved ruggedness of an ion-pairing liquid chromatography/tandem mass spectrometry assay for the quantitative analysis of the triphosphate metabolite of a nucleoside reverse transcriptase inhibitor in peripheral blood mononuclear cells, *Rapid Commun Mass Spectrom* 27(3) (2013) 481–8. [PubMed: 23280981]
- [64]. Holcapek M, Volna K, Jandera P, Kolarova L, Lemr K, Exner M, Cirkva A, Effects of ion-pairing reagents on the electrospray signal suppression of sulphonated dyes and intermediates, *J Mass Spectrom* 39(1) (2004) 43–50. [PubMed: 14760612]
- [65]. Monkkonen H, Moilanen P, Monkkonen 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, *J Chromatogr B Biomed Sci Appl* 738(2) (2000) 395–403. [PubMed: 10718657]
- [66]. Lynch T, Eisenberg G, Kernan M, LC/MS determination of the intracellular concentration of two novel aryl phosphoramidate prodrugs of PMPA and their metabolites in dog PBMC, *Nucleosides Nucleotides Nucleic Acids* 20(4-7) (2001) 1415–9. [PubMed: 11563034]
- [67]. Hernandez-Santiago BI, Beltran T, Stuyver L, Chu CK, Schinazi RF, Metabolism of the anti-hepatitis C virus nucleoside beta-D-N4-hydroxycytidine in different liver cells, *Antimicrob Agents Chemother* 48(12) (2004) 4636–42. [PubMed: 15561837]
- [68]. Ray AS, Myrick F, Vela JE, Olson LY, Eisenberg EJ, Borroto-Esodo K, Miller MD, Fridland A, Lack of a metabolic and antiviral drug interaction between tenofovir, abacavir and lamivudine, *Antivir Ther* 10(3) (2005), 451–7. [PubMed: 15918336]

- [69]. Ray AS, Vela JE, Olson L, Fridland A, Effective metabolism and long intracellular half life of the anti-hepatitis B agent adefovir in hepatic cells, *Biochem Pharmacol* 68(9) (2004) 1825–31. [PubMed: 15450948]
- [70]. Pruvost A, Negredo E, Benech H, Theodoro F, Puig J, Grau E, Garcia E, Molto J, Grassi J, Clotet B, Measurement of intracellular didanosine and tenofovir phosphorylated metabolites and possible interaction of the two drugs in human immunodeficiency virus-infected patients, *Antimicrob Agents Chemother* 49(5) (2005) 1907–14. [PubMed: 15855513]
- [71]. Delaney W.E.t., Ray AS, Yang H, Qi X, Xiong S, Zhu Y, Miller MD, Intracellular metabolism and in vitro activity of tenofovir against hepatitis B virus, *Antimicrob Agents Chemother* 50(7) (2006) 2471–7. [PubMed: 16801428]
- [72]. Borroto-Esoda K, Vela JE, Myrick F, Ray AS, Miller MD, In vitro evaluation of the anti-HIV activity and metabolic interactions of tenofovir and emtricitabine, *Antivir Ther* 11(3) (2006) 377–84. [PubMed: 16759055]
- [73]. Lee J, Yoo BC, Lee HS, Yoo HW, Yoo HH, Kang MJ, Kim DH, 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, *Ther Drug Monit* 28(1) (2006) 131–7. [PubMed: 16418707]
- [74]. 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, *J Mass Spectrom* 42(3) (2007) 389–404. [PubMed: 17238240]
- [75]. 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, *J Chromatogr B Analyt Technol Biomed Life Sci* 848(2) (2007) 335–43.
- [76]. Durand-Gasselin L, Da Silva D, Benech H, Pruvost A, Grassi J, Evidence and possible consequences of the phosphorylation of nucleoside reverse transcriptase inhibitors in human red blood cells, *Antimicrob Agents Chemother* 51(6) (2007) 2105–11. [PubMed: 17438052]
- [77]. Pruvost A, Theodoro F, Agrofoglio L, Negredo E, Benech 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, *J Mass Spectrom* 43(2) (2008) 224–33. [PubMed: 17935070]
- [78]. Ray AS, Vela JE, Booramra CG, Zhang L, Hui H, Callebaut C, Stray K, Lin KY, Gao Y, Mackman RL, Cihlar T, Intracellular metabolism of the nucleotide prodrug GS-9131, a potent anti-human immunodeficiency virus agent, *Antimicrob Agents Chemother* 52(2) (2008) 648–54. [PubMed: 18056281]
- [79]. Reiser H, Wang J, Chong L, Watkins WJ, Ray AS, Shibata R, Birkus G, Cihlar T, Wu S, Li B, Liu X, Henne IN, Wolfgang GH, Desai M, Rhodes GR, Fridland A, Lee WA, Plunkett W, Vail D, Thamm DH, Jeraj R, Tumas DB, GS-9219--a novel acyclic nucleotide analogue with potent antineoplastic activity in dogs with spontaneous non-Hodgkin's lymphoma, *Clin Cancer Res* 14(9) (2008) 2824–32. [PubMed: 18451250]
- [80]. Coulier L, van Kampen JJ, de Groot R, Gerritsen HW, Bas RC, van Dongen WD, Brull LP, Luidert TM, Simultaneous determination of endogenous deoxynucleotides and phosphorylated nucleoside reverse transcriptase inhibitors in peripheral blood mononuclear cells using ion-pair liquid chromatography coupled to mass spectrometry, *Proteomics Clin Appl* 2(10-11) (2008) 1557–62. [PubMed: 21136803]
- [81]. Kuklenyik Z, Martin A, Pau CP, Holder A, Youngpairoj AS, Zheng Q, Cong ME, Garcia-Lerma JG, Heneine W, Pirkle JL, Barr JR, On-line coupling of anion exchange and ion-pair chromatography for measurement of intracellular triphosphate metabolites of reverse transcriptase inhibitors, *J Chromatogr B Analyt Technol Biomed Life Sci* 877(29) (2009) 3659–66.
- [82]. Else LJ, Jackson A, Puls R, Hill A, Fahey P, Lin E, Amara A, Siccardi M, Watson V, Tjia J, Emery S, Khoo S, Back DJ, Boffito M, Pharmacokinetics of lamivudine and lamivudine-triphosphate after administration of 300 milligrams and 150 milligrams once daily to healthy

volunteers: results of the ENCORE 2 study, *Antimicrob Agents Chemother* 56(3) (2012) 1427–33. [PubMed: 22183172]

- [83]. 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, *J Mass Spectrom* 50(6) (2015) 783–91. [PubMed: 26169132]
- [84]. Werner A, Reversed-phase and ion-pair separations of nucleotides, nucleosides and nucleobases: analysis of biological samples in health and disease, *J Chromatogr* 618(1-2) (1993) 3–14. [PubMed: 8227262]
- [85]. Jansen RS, Rosing H, Schellens JH, 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, *J Chromatogr A* 1216(15) (2009) 3168–74. [PubMed: 19237159]
- [86]. Xia YQ, Jemal M, Zheng N, Shen X, Utility of porous graphitic carbon stationary phase in quantitative liquid chromatography/tandem mass spectrometry bioanalysis: quantitation of diastereomers in plasma, *Rapid Commun Mass Spectrom* 20(12) (2006) 1831–7. [PubMed: 16705646]
- [87]. Huang L, Lizak P, Aweeka F, Long-Boyle J, Determination of intracellular fludarabine triphosphate in human peripheral blood mononuclear cells by LC-MS/MS, *J Pharm Biomed Anal* 86 (2013) 198–203. [PubMed: 24013121]
- [88]. 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, *Anal Chem* 88(12) (2016) 6190–4. [PubMed: 27228284]
- [89]. Jian W, Edom RW, Xu Y, Weng N, Recent advances in application of hydrophilic interaction chromatography for quantitative bioanalysis, *J Sep Sci* 33(6-7) (2010) 681–97. [PubMed: 20155745]
- [90]. Cai Z, Fung EN, Sinhababu AK, Capillary electrophoresis-ion trap mass spectrometry analysis of Ziagen and its phosphorylated metabolites, *Electrophoresis* 24(18) (2003) 3160–4. [PubMed: 14518039]
- [91]. Liu CC, Huang JS, Tyrrell DL, 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* 26(7-8) (2005) 1424–31. [PubMed: 15761917]
- [92]. Bezy V, Chaimbault P, Morin P, Unger SE, Bernard MC, 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 alcohols as coaxial sheath-liquid make-up constituents, *Electrophoresis* 27(12) (2006) 2464–76. [PubMed: 16786481]
- [93]. van Kampen JJ, Fraaij PL, Hira V, van Rossum AM, Hartwig NG, de Groot R, Luiders TM, A new method for analysis of AZT-triphosphate and nucleotide-triphosphates, *Biochem Biophys Res Commun* 315(1) (2004) 151–9. [PubMed: 15013439]
- [94]. 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, *Anal Chem* 79(17) (2007) 6629–40. [PubMed: 17665876]
- [95]. Zhang S, Liu J, Chen Y, Xiong S, Wang G, Chen J, Yang G, A novel strategy for MALDI-TOF MS analysis of small molecules, *J Am Soc Mass Spectrom* 21(1) (2010) 154–60. [PubMed: 19906541]
- [96]. Kim J, Chou TF, Griesgraber GW, Wagner CR, Direct measurement of nucleoside monophosphate delivery from a phosphoramidate pronucleotide by stable isotope labeling and LC-ESI(-)-MS/MS, *Mol Pharm* 1(2) (2004) 102–11. [PubMed: 15832506]
- [97]. Jandera P, Hajek T, Sromova Z, Mobile phase effects in reversed-phase and hydrophilic interaction liquid chromatography revisited, *J Chromatogr A* 1543 (2018) 48–57. [PubMed: 29486886]

- [98]. Chen Y, Bicker W, Wu J, Xie M, Lindner W, Simultaneous determination of 16 nucleosides and nucleobases by hydrophilic interaction chromatography and its application to the quality evaluation of Ganoderma, *J Agric Food Chem* 60(17) (2012) 4243–52. [PubMed: 22500559]
- [99]. Font E, Rosario O, Santana J, Garcia H, Sommadossi JP, Rodriguez JF, Determination of zidovudine triphosphate intracellular concentrations in peripheral blood mononuclear cells from human immunodeficiency virus-infected individuals by tandem mass spectrometry, *Antimicrob Agents Chemother* 43(12) (1999) 2964–8. [PubMed: 10582890]
- [100]. Niessen WM, Progress in liquid chromatography-mass spectrometry instrumentation and its impact on high-throughput screening, *J Chromatogr A* 1000(1-2) (2003) 413–36. [PubMed: 12877182]
- [101]. Korfmacher WA, Principles and applications of LC-MS in new drug discovery, *Drug Discov Today* 10(20) (2005) 1357–67. [PubMed: 16253874]
- [102]. Fung EN, Cai Z, Burnette TC, Sinhababu AK, Simultaneous determination of Ziagen and its phosphorylated metabolites by ion-pairing high-performance liquid chromatography-tandem mass spectrometry, *J Chromatogr B Biomed Sci Appl* 754(2) (2001) 285–95. [PubMed: 11339272]
- [103]. Bhatt DP, Chen X, Geiger JD, Rosenberger TA, A sensitive HPLC-based method to quantify adenine nucleotides in primary astrocyte cell cultures, *J Chromatogr B Analyt Technol Biomed Life Sci* 889-890 (2012) 110–5.
- [104]. Ramos-Salazar A, Baines AD, Fluorometric determination of adenine nucleotides and adenosine by ion-paired, reverse-phase, high-performance liquid chromatography, *Anal Biochem* 145(1) (1985) 9–13. [PubMed: 4003764]
- [105]. Horton JK, Martin RC, Kalinka S, Cushing A, Kitcher JP, O'Sullivan MJ, Baxendale PM, Enzyme immunoassays for the estimation of adenosine 3',5' cyclic monophosphate and guanosine 3',5' cyclic monophosphate in biological fluids, *J Immunol Methods* 155(1) (1992) 31–40. [PubMed: 1328396]

Highlights:

- Direct and indirect quantification of phosphate metabolites of nucleoside analogs.
- Quantification of intracellular and total tissue concentrations of nucleotide metabolites.
- Compare methods for sample collection, preparation, storage conditions, and LC-MS analysis.

Figure 1:
Representative chemical structure of nucleotide metabolite.

Figure 2:
Schematic representation of sample preparation steps involved in indirect methods for mono-, di-, and triphosphate nucleotide metabolites quantification.

Table 1:

Direct LC-MS/MS methods for the quantification of intracellular nucleotide metabolites

Analyte	Matrix	Sample extraction	Chromatography	Column	Reference
FTC-TP	Human PBMCs	PP with MeOH + ion pairing SPE	Ion-pairing with TBAH	Xterra RP18 (1.0 × 100 mm)	[42]
Clodronate-TP (AppCClp)	Human PBMCs	PP with ACN	Ion-pairing with DMHA	Genesis C18 (2 × 50 mm)	[65]
ABC-MP, CBV-MP, CBV-DP, CBV-TP	In vitro Human PBMCs, Hep G2	PP with MeOH	Ion-pairing with DMHA	Luna C 8 (2 × 50 mm)	[102]
d4T-MP, d4T-DP d4T-TP	Human PBMCs	PP with MeOH	Ion-pairing with DMHA	SMT C18 (2.1 × 150 mm)	[57]
PMPA-DP	Dog PBMCs	PP with MeOH	Ion-pairing with DMHA	Luna C8 (2.1 × 50 mm)	[66]
D-D4FC-TP	Human PBMCs	PP with MeOH	Anion-exchange with NH ₄ OH	BioBasic AX (1.0 × 20 mm)	[29]
AZT-TP	Human PBMCs	PP with MeOH	Ion-pairing with DMHA	Supelcogel ODP-50 (2.1 × 150 mm)	[47]
NHC-TP	In vitro HepG2 Huh-7	PP with MeOH	Ion-pairing with DMHA	Hypersyl BDS C8 (4.6 × 150 mm)	[67]
FTC-TP	Human PBMCs	PP with MeOH + ion pairing SPE	Ion-pairing with TBAH	Xterra MS C18 (1.0 × 100 mm)	[40]
AZT-MP	Human PBMCs	PP with MeOH	Reverse phase	Zorbax XDB-C18 (0.5 × 150 mm)	[96]
Adefovir-DP	In vitro HepG2	PP with MeOH	Ion-pairing with TBAH	Xterra MS C18 (1.0 × 100 mm)	[69]
3TC-TP, CBV-TP TFV-DP	Human PBMCs	PP with MeOH + ion pairing SPE	Ion-pairing with TBAH	Xterra MS C18 (1.0 × 100 mm)	[41]
CBV-TP, TFV-MP, TFV-DP	In vitro Human PBMCs, CCRF-CEM	PP with MeOH	Ion-pairing with TBAH	Xterra MS C18 (1.0 × 100 mm)	[68]
TFV-DP, ddA-TP	Human PBMCs	PP with MeOH	Ion-pairing with DMHA	Supelcogel ODP-50 (2.1 × 150 mm)	[70]
TFV-DP	In vitro HepG2	PP with MeOH	Ion-pairing with TBAH	Xterra MS C18 (1.0 × 100 mm)	[71]
L-FMAU-TP	Human PBMCs	PP with MeOH	Ion-pairing with DMHA	Hydrosphere C18 (2.0 × 50 mm)	[73]
TFV-DP, FTC-TP	In vitro Human PBMCs, CCRF-CEM	PP with MeOH	Ion-pairing with TBAH	Xterra MS C18 (1.0 × 100 mm)	[72]
Adefovir-MP, Adefovir-DP	In vitro Hep G2	PP with MeOH	Ion-pairing with TBAH, or TBAA	Xterra MS C18 (1.0 × 100 mm), YMC C18 (1.0 × 150 mm), Luna C18 (1.0 × 100 mm)	[75]
AZT-MP, AZT-TP, d4T-TP	Human PBMCs	PP with MeOH	Ion-pairing with DMHA	Supelcogel ODP-50 (2.1 × 50 mm)	[74]
TFV-DP, AZT-MP, AZT-TP, 3TC-TP	Human PBMCs, RBCs	PP with MeOH	Ion-pairing with DMHA	Supelcogel ODP-50 (2.1 × 50 mm)	[76]
2CdA-MP, 2CdA-DP, 2CdA-TP	In vitro MDCK-II	PP with MeOH	Anionic exchange with NH ₄ OH	BioBasic AX (2.1 × 50 mm)	[59]
Fd4AP-DP, TFV-MP, TFV-DP	Human PBMCs	PP with MeOH	Ion-pairing with TBAA	Luna C18 (1.0 × 100 mm)	[78]

Analyte	Matrix	Sample extraction	Chromatography	Column	Reference
PMEG-DP	Human PBMCs	PP with MeOH	Ion-pairing with TBAA	Luna C18 (1.0 × 100 mm)	[79]
TFV-DP, 3TC-TP, CBV-TP	Human PBMCs	PP with MeOH	Ion-pairing with DMHA	Supelcogel ODP-50 (2.1 × 50 mm)	[77]
AZT-MP, AZT-DP, AZT-TP, d4T-MP, d4T-DP, d4T-TP 3TC-MP, 3TC-DP, 3TC-TP	Human PBMCs	PP with MeOH	Ion-pairing with Hexylamine	Inertsil ODS-3 (3.0 × 100 mm)	[80]
TFV-DP, FTC-TP, 3TC-TP	Monkey PBMCs	PP with MeOH	Ion-pairing with DMHA	Biomax AX (2.1 × 10 mm) Persuit C18 (2.1 × 10 mm) Persuit C18 (2.1 × 50 mm)	[81]
dFdC-MP, dFdC-DP, dFdC-TP, dFdU-MP, dFdU-DP, dFdU-TP	Human PBMCs	PP with MeOH	PGC	Hyper-carb (2.1 × 100mm)	[85]
2'-Me-Cy-TP	Rat tissue Homogenate	PP with MeOH+ Anion-exchange SPE	HILIC	Aminopropyl (2.0 × 100 mm)	[34]
3TC-TP	Human PBMCs	PP with ACN	Ion-pairing with DMHA	Fortis C18 (2.1 × 150 mm)	[82]
BMS-986001-TP	Monkey PBMCs	PP with MeOH	Ion-pairing with DMHA	Luna C18 (1.0 × 50 mm)	[63]
F-ara-A-TP	Human PBMCs	PP with MeOH	PGC	Hyper-carb (2.1 × 100mm)	[87]
AZT-MP, AZT-DP, AZT-TP	Human PBMCs	PP with MeOH	Ion-pairing with TBAH	InnertSustain (1.5 × 50 mm)	[83]
INX-09114 (TP)	Monkey Tissue homogenate	PP with organic	Anion-exchange with NH ₄ OH	Scherzo SM C18 (2.0 × 50 mm)	[33]
3TC-TP, CBV-TP	Human PBMCs	PP with MeOH	Anion-exchange with NH ₄ OH	BioBasic AX (2.1 × 150 mm)	[17]
MK-8591-TP	Human PBMCs	PP with MeOH	Anion-exchange with NH ₄ OH	BioBasic AX (1.0 × 50 mm)	[61]

TBAA, Tetrabutyl ammonium acetate; TBAH, Tetrabutyl ammonium hydroxide; DMHA, N, N-Dimethylhexylamine; APh, Ammonium Phosphate; PMPA, 9-[2-R-(phosphonomethoxy) propyl] adenine; PMEG, 9-(2-phosphonylmethoxyethyl) guanine; Fd4AP, 5-(6-amino-purin-9-yl)-4-fluoro-2,5-dihydrofuran-2-ylloxymethyl]-phosphonic acid, d4T, stavudine; NHC, Beta-D-N4-hydroxycytidine; L-FMAU, 2'-fluoro-5-methyl-β-l-arabinofuranosyl uracil; 2CdA, 2-chloro, 2'-deoxyadenosine; dFdC, 2'-2'-difluorodeoxycytidine; dFdU, 2'-2'-difluorodeoxyuridine; F-ara-A, 9-beta-D-arabinofuranosyl-2-fluoroadenine; INX-09114, Triphosphate metabolite of prodrug BMS-986094; D-D4FC-TP, beta-D-2',3'-dideoxy-2',3'-dideoxy-5-fluorocytosine-5'-triphosphate, 2'-Me-Cy-TP, 2'-C-methyl-cytidine triphosphate; PP, Protein precipitation.

Table 2:

Indirect LC-MS/MS methods for the quantification of nucleotide metabolites

Compound	Matrix	Sample extraction	Chromatography	Column	References
3TC-TP, d4T-TP, ZDV-TP	Human PBMCs	(i) PP with MeOH, (ii) QMA SPE, (iii) ALP, (iv) SEP-PAK C18 SPE	Reverse phase	Columbus C18 (1.0 × 100 mm)	[49]
3TC-TP, ZDV-TP	Human PBMCs	(i) PP with MeOH, (ii) QMA SPE, (iii) ACP, (iv) XAD column SPE	Reverse phase	Hypersil C18 (2.1 × 100 mm)	[50]
3TC-TP	Human PBMCs	(i) PP with MeOH, (ii) QMA SPE, (iii) ALP, (iv) SEP-PAK C18 SPE	Reverse phase	Columbus C18 (1.0 × 100 mm)	[51]
3TC-TP, ATC-TP	Human PBMCs	(i) PP with MeOH, (ii) QMA SPE, (iii) ALP, (iv) Varian C18 SPE	Reverse phase	YMC ODS-AQ C18 (2.0 × 100 mm)	[52]
3TC-TP, CBV-TP, ZDV-TP	Human PBMCs	(i) PP with MeOH, (ii) QMA SPE, (iii) ACP, (iv) Oasis HLB C18 SPE	Reverse phase	AQUASIL C18 (2.1 × 50 mm)	[44]
CBV-TP	Human PBMCs	(i) PP with MeOH, (ii) QMA SPE, (iii) ALP, (iv) Varian C18 SPE	Reverse phase	YMC ODS-AQ (2.0 × 50 mm)	[46]
CBV-MP CBV-DP CBV-TP	Human PBMCs	(i) PP with MeOH, (ii) QMA SPE, (iii) ACP, (iv) Varian C18 SPE	Reverse phase	YMC ODS-AQ (2.0 × 50 mm)	[53]
3TC-TP, TFV-DP, ZDV-TP, FTC-TP	Human PBMCs	(i) PP with MeOH, (ii) QMA SPE, (iii) ACP, (iv) Strata-X SPE	Reverse phase	Synergi Polar RP (2.0 × 100 mm)	[43]
ZDV-TP, TFV-DP	Human PBMCs	(i) PP with MeOH, (ii) QMA SPE, (iii) na (iv) Oasis HLB C18 SPE	Reverse phase	Xterra MS C18 (2.1×50 mm)	[54]
RBV-MP, RBV-DP, RBV-TP	Human PBMCs, RBCs	(i) PP with MeOH, (ii) QMA SPE, (iii) ACP, (iv) Varian C18 SPE	Reverse phase	Develosil C30 (2.1 × 100 mm)	[55]
3TC-TP CBV-TP	Mouse PBMCs, other cells	(i) PP with MeOH, (ii) QMA SPE, (iii) ACP, (iv) Oasis HLB C18 SPE	Reverse phase	CSH C18 (2.1 × 100 mm)	[17]

RBV, Ribavirin; ATC, Apricitabine; QMA-SPE, Sep-Pak QMA-anion-exchange SPE cartridge; ACP, Acid phosphatases; ALP, Alkaline phosphatases; na, information not available