Journal of Chromatography B, 868 (2008) 1–12

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Review

Nucleoside reverse transcriptase inhibitors and their phosphorylated metabolites in human immunodeficiency virus-infected human matrices

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ARTICLE INFO

Article history:
Received 31 October 2007
Accepted 13 April 2008
Available online 9 May 2008

Keywords:
Nucleoside reverse transcriptase inhibitors
Phosphorylated metabolites
High performance liquid chromatography
Tandem mass spectrometry
Electrophoresis/electrochromatography

ABSTRACT

Highly active antiretroviral therapy (HAART) is the common treatment strategy for human immunodeficiency virus (HIV)-infected patients at present. Generally, HAART regimens apply multitherapy drugs that contain nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), non-nucleoside/nucleotide reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PIs). Unlike NNRTIs and PIs, the active form of NRTIs is not the drug itself but its triphosphorylated (TP) metabolites in intracellular medium. Analysis of both the prodrugs or NRTIs and their intracellular metabolites is needed to provide overall information in pharmacokinetic and therapeutic effects to HIV-infected patients. Numerous publications have reported the assays for NRTIs and their phosphorylated metabolites in various biological matrices. The methods involved liquid chromatography (LC) with UV detection (LC-UV), LC with tandem mass spectrometry (LC–MS/MS), capillary electrophoresis/electrochromatography (CE/CEC) with UV detection (CE/CEC-UV) or/and MS/MS detection (CE-MS/MS). Due to the extremely low concentration of NRTIs and the phosphorylated metabolites as well as the complex biological matrices, sample pretreatment methods such as protein precipitation (PP), liquid–liquid extraction (LLE) and solid-phase extraction (SPE) have played important role in the successful analytical method development.

Contents

Abbreviations: 3TC, lamivudine; ABC, abacavir; ACN, acetonitrile; AIDS, acquired immunodeficiency syndrome; APCI, atmospheric pressure chemical ionization; AZDU, 3′-azido-2′,3′-dideoxyuridine; AZT, zidovudine; BGE, background electrolyte; CE, capillary electrophoresis; CID, collision-induced dissociation; CPS, clinical pharmacokinetic study; CZE, capillary zone electrophoresis; d4T, stavudine; ddC, zalcitabine; ddI, didanosine; DP, diphosphate; ESI, electrospray ionization; HAART, highly active antiretroviral therapy; HIV-1, human immunodeficiency virus type-1; HPLC, high-performance liquid chromatography; IPR, ion-pairing reagent; I.S., internal standard; LC, liquid chromatography; LLE, liquid–liquid extraction; LOD, limit of detection; MEKC, micellar electrokinetic chromatography; MeOH, methanol; MP, monophosphate; MPA, mycophenolic acid; MRM, multiple reaction monitoring; MS, mass spectrometry; MS/MS, tandem mass spectrometry (tandem quadropole mass spectrometry); NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; NRTI-TP, triphosphorylated nucleoside reverse transcriptase inhibitors; PBMCs, peripheral blood mononuclear cells; PGC, porous graphitic carbon; PIs, protease inhibitors; PP, protein precipitation; PRBCs, peripheral red blood cells; QC, quality control; RIA, radioimmunoassay; SRM, selected reaction monitoring; SPE, solid-phase extraction; TCA, trichloroacetic acid; TDM, therapeutic drug monitoring; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TMACl, tetramethylammonium chloride; TMAP, tetramethylammonium perchlorate; TP, triphosphate; UV, ultraviolet (also UV absorption); ZDV, zidovudine.

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doi:10.1016/j.jchromb.2008.04.012

1. Introduction

The current common treatment strategy for human immunodeficiency virus (HIV)-infected patients by highly active antiretroviral therapy (HAART) involves multitherapy drugs that contain two nucleoside reverse transcriptase inhibitors (NRTIs) combined with one non-nucleoside reverse transcriptase inhibitors (NNRTIs) or one protease inhibitors (PIs) or both. To date, six NRTIs, abacavir (ABC), didanosine (ddI), lamivudine (3TC), stavudine (d4T), zalcitabine (ddC) and zidovudine (AZT), have been approved by the U.S. Food and Drug Administration (FDA). In spite of the development of new antiretroviral drugs, the combination regimens remain as the backbone of the multitherapies against HIV. Unlike NNRTIs and PIs, the active form of NRTIs is not the drug itself but its triphosphorylated (TP) metabolites in intracellular medium in the infected lymphocytes. NRTIs require three intracellular phosphorylation steps to yield their corresponding biologically active nucleoside triphosphates [1–4]. The produced NRTI triphosphates competitively inhibit HIV reverse transcriptase with incorporation into the proviral DNA, resulting in DNA chain termination and prevention of viral replication [5]. Plasma concentration measurement of the phosphorylated metabolites is important to check adherence to NRTIs, to guide the dosing in patients with renal failure and to evaluate drug–drug interactions [6,7]. Analysis of both the parent drugs and the intracellular metabolites will give overall information about the variability in individual drug pharmacokinetics and provide information on the complete drug behavior from the digest tract to the site of action, and at the intracellular level [7–15]. Furthermore, the underlying and possible relationship between NRTI plasma concentrations and virological response [16] or intracellular concentrations of the corresponding triphosphate derivate should be intensively studied [14,15,17–21]. Thus, sensitive, selective and reproducible methods are needed for the analysis NRTIs and their metabolites in various biological matrices.

The separation methods for NRTIs were reviewed in 2001 by Pereira and Tidwell [22]. Armagan Onal published a review on HPLC analysis of antiretroviral drug including NNRTIs, NRTIs and PIs in biological matrices for therapeutic drug monitoring (TDM) in 2006 [23]. The review covered publications on HPLC method with various detection techniques for the analysis of the parent drugs. CE and CEC as well as their hyphenated methods, however, were not included. Furthermore, the review did not cover the analysis of NRTIs' metabolites which are actual active form of the drugs and are thus more important therapeutically. This paper provides comprehensive review on analytical methods for NRTIs and their phosphorylated metabolites in human immunodeficiency virus-infected human matrices, including the most recent publications. The samples include various biological matrices such as human plasma, serum, urine, saliva, cerebrospinal fluid (CSF) and seminal fluid. The review covers analytical methods using radioimmunoassays, liquid chromatography coupled with UV detection (LC-UV), reversed-phase LC coupled with MS/MS (RPLC–MS/MS), ion-pairing LC coupled with tandem mass spectrometry (IPLC–MS/MS), capillary electrophoresis (CE) coupled with UV detection (CE-UV) and CE-MS/MS. In this review, publications were searched from the Web of Science with the keywords of the six individual NRTIs as well as HPLC–UV, LC–MS(/MS) and CE-MS(/MS). Analytical methods cited from the obtained articles were also checked. The review also includes the pretreatment and enrichment methods due to the extremely low concentration of NRTIs and phosphate metabolites in biological samples.

2. Sample pretreatment

Although the samples used for the analysis of NRTIs and phosphorylated metabolites included plasma, serum, urine, saliva, CSF, seminal fluid, etc., only the pretreatment procedures of plasma and serum samples were reviewed in this section because of the specific challenge of the plasma and serum analysis. Plasma and serum are the most frequently analyzed samples for NRTIs and phosphorylated metabolites, although they are more complicated than other biological fluids. Moreover, the concentrations of NRTIs and metabolites in plasma and serum are often lower than those in other sample. To extract the analytes from the biological samples, pretreatment methods such as protein precipitation (PP), liquid–liquid extraction (LLE) and solid-phase extraction (SPE) were often used.

2.1. Protein precipitation

PP is the simplest procedure to remove proteins in plasma and serum. Briefly, the inorganic acid, organic acid or organic solvent such as perchloric acid (PCA), trichloroacetic acid (TCA) and acetonitrile (ACN) were used to precipitate proteins in plasma or serum sample. The mixture then was centrifuged to remove the denatured proteins. Veldkamp et al. used 75 μL of 20% (w/w) solution of PCA to treat 300 μL human plasma for 30 s. After the centrifugation for 15 min at 13,000 × *g*, 250 mL of the clear supernatant was collected and directly injected into HPLC for analysis of ABC without further treatment [24]. Bartlett research group applied 20 mL of 2 mol L^{-1} of PCA to extract 100 μL of plasma or amniotic fluid. The mixture was then centrifuged at 9000 rpm for 10 min. The supernatants were transferred to injection vials for HPLC analysis of 3′-azido-2′,3′-dideoxyuridine (AZDU), which is a potential candidate for the treatment of pregnant mothers to prevent prenatal transmission of HIV/AIDS to their unborn children [25]. The same group also applied the similar procedure to extract rat plasma and amniotic fluid for the analysis of acyclovir (9-[(2-hydroxyethoxy)-methyl]guanosine, ACV) and AZT [26] as well as 3TC in plasma and amniotic fluid [27]. Briefly, 15 μL of 2 mol L^{-1} PCA was used to precipitate proteins in 100 μL of plasma or amniotic fluid. Samples were then vortexed and centrifuged at 13,000 × *g* for 10 min. The supernatant was aspirated and neutralized with 2 mol L^{-1} of NH_4OH. The sample extracts were filtered through 0.2 μm nylon filters for the further analysis.

Zhou et al. used TCA to extract human serum for the measurement of 3TC. In this procedure, 20 μL of 20% TCA solution was used to precipitate proteins in 100 μL human serum. The mixture was centrifuged at 16,000 × *g* for 5 min. Approximate 100 μL of the supernatant was transferred into a 300-μL auto-sample vial and injected for the HPLC analysis [28]. Sparidans et al. isolated the tenofovir (TFV) from plasma sample by using TCA as the PP reagent. Briefly, 50 μL of 20% (w/v) TCA in water was used to precipitate 200 μL of plasma sample. After the centrifugation at 15,000 × *g* for 5 min, the supernatant was directly used for the analysis of TFV [29]. Alnouti et al. used ACN as the PP reagent. In their procedure, 400 μL of cold ACN was added to 100 μL sample. After being centrifuged at 13,000 rpm for 10 min the supernatant was dried under vacuum. The samples were then reconstituted in 100 μL of distilled water for the further analysis [27]. In another paper, the ice-cold ACN was used to precipitate proteins in plasma and amniotic fluid for the analysis of 2′,3′-dideoxycytidine that was used for the treatment of pregnant women to prevent prenatal transmission of HIV/AIDS to the unborn children [30]. Kano et al. also developed a HPLC method for the quantification of 3TC after the human plasma sample was extracted by using ACN [31].

PP has been showed to efficiently remove proteins in plasma and other biological matrices. However, the concentrations of NRTIs in plasma or serum are often extremely low. To achieve enough sensitivity for the sample analysis, the samples are often needed to be further concentrated by using procedures such as LLE and SPE.

2.2. Liquid–liquid extraction

Donnerer et al. described an analytical procedure for monitoring the plasma concentrations of seven drugs: ABC, AZT, efavirenz, nevirapine, indinavir, lopinavir, and nelfinavir [32]. The ABC, AZT and nevirapine in plasma samples were extracted by using LLE with the mixture of chloroform and isopropyl alcohol and subsequently subjected to HPLC analysis. Briefly, 0.5 mL of plasma was extracted with 6 mL of the mixture of chloroform and isopropyl alcohol (95:5, v/v) via vortex for 15 s. After the phase separation, 5 mL of the organic phase was transferred to a 15-mL sterile tube and evaporated under a stream of nitrogen. The residue was reconstituted in 400 μL of mobile phase for HPLC analysis. Ferrer et al. developed a rapid and simple HPLC method for the determination of ABC in human plasma. It included a single LLE procedure with a mixture of ethyl acetate-diethyl ether prior to the RP-HPLC analysis on a C_{18} column [33]. Bahrami et al. described a LLE method using dichloromethane–isopropyl alcohol (1:1, v/v) for the extraction of 3TC and internal standard (I.S., famotidine) [34]. The authors suggested that the LLE method provided advantages over the previously published PP and SPE extraction methods for removing endogenous interferences and salts from the biological samples. Ramachandran et al. reported a simple and fast method combining LLE with ethyl acetate and isocratic RP-HPLC for the simultaneous determination of AZT and nevirapine in plasma [35]. Prior to the LLE, 25 μL of 3-isobutyl 1-methyl xanthine (I.S.) was added to 250 μL of each of calibration standards and test samples at a concentration of 100 μg mL^{-1}. After 1 ml of ethyl acetate was added, the samples were vortexed vigorously, centrifuged at 1000 × *g*. 500 μL of the organic phase was collected and evaporated to dryness. The dried residue was reconstituted in 50 μL of mobile phase for HPLC analysis. The method was specific and sensitive enough to allow quantification of AZT and nevirapine at the concentrations observed clinically. The average recoveries of AZT and nevirapine from plasma were 95 and 94%, respectively. The method was applied to a pharmacokinetic study in HIV-infected patients who were receiving antiretroviral treatment with AZT and nevirapine containing regimens.

2.3. SPE

LLE is a simple and efficient method for the separation and concentration of relatively hydrophobic compounds. However, for more polar compounds such as the phosphorylated metabolites of NRTI, LLE may not be an effective extraction procedure. Most of the NRTI metabolites in plasma and intracellular medium present in mono-, di- or triphosphate forms. Thus, it is difficult to extract completely those extremely polar metabolites with organic solvent from human fluid. SPE is a more effective extraction procedure for NRTIs and their metabolites. The most frequently used SPE materials are C_{18} and polymer cartridges.

2.3.1. SPE with C_{18} column

Roth and Kelley applied a C_{18} SPE column to isolate and concentrate 2′-beta-fluoro-2′,3′-dideoxyadenosine, a synthetic ddI analogue that has been designed to overcome the acid stability problems of the anti-AIDS drug ddI [36]. Nadal et al. used a Bond Elut C_{18} extraction column to separate AZT and G-AZT in plasma [37]. The recoveries of AZT and G-AZT were 80.8–89.3% and 80.6–91.6%, respectively. Tan and Boudinot also applied Bond Elut C_{18} cartridges to extract AZT and AZT-MP in mouse plasma and peripheral red blood cells (PRBCs) [38]. The average extraction recoveries of AZT-MP and AZT in plasma were approximately 85 and 97%, respectively. The recoveries of AZT-MP and AZT from PRBCs averaged 56 and 69%, respectively. Caufield and Stewart also used the Bond Elut C_{18} SPE cartridge to extract AZT and levofloxacin in human plasma and obtained the mean recoveries of 94.1 and 91.2% for AZT and levofloxacin, respectively [39]. Clark et al. utilized a C_{18} SPE cartridge to extract AZT and AZDU from rat maternal plasma, amniotic fluid, placental and fetal tissue samples with recoveries ranged from 81 to 96% for AZDU and from 82 to 96% for AZT in the different matrices [25]. In another study, the same group applied a Sep-Pak-Vac C_{18} SPE cartridge to extract ddI and d4T in maternal rat plasma, amniotic fluid, placental and fetal tissue [40]. The obtained recoveries were above 70% for both compounds in all four matrices. Wiesner et al. used the same SPE cartridge to extract d4T in plasma with the mean recovery of 94% [41]. Quevedo et al. also applied the similar extraction cartridges containing 500 mg of RP silica to extract 3′-azido-3′-deoxy-5′-*O*-isonicotinoylthymidine (AZT-Iso) that is a novel derivative of the antiretroviral AZT in human, rat and rabbit plasma samples with the recoveries of between 93.1 and 99.4% [42].

2.3.2. SPE with polymer column

Sarasa et al. applied an Oasis® SPE cartridge to extract d4T in plasma and obtained the satisfactory recovery of 81.0–85.4% [43]. Fan and Stewart used Waters Oasis™ HLB cartridge for the extraction of AZT and 3TC in human plasma [44] and AZT/ddI/nevirapine (mixture A) or AZT/ddI/ritonavir (mixture B) in human serum [45]. Ding et al. also utilized the Waters Oasis® HLB SPE cartridge to extract 2′,3′-dideoxycytidine (DDC) and 3TC from rat placental and fetal homogenates. The relative recovery for DDC in each of the matrices ranged from 87.8 to 103.0% [30]. Pereira et al. also successfully used the similar cartridge to extract different PIs, NRTIs (d4T, AZT, ddI) and NNRTIs in serum. The extraction recoveries were higher than 90% with exception of efavirenz that was 77.4% [46]. Based on the performance characteristics, the proposed method was found suitable for the determination of AZT, ddI and efavirenz in serum samples. Bezy et al. also applied the Waters Oasis® HLB cartridges for the extraction of ddC, 3TC, ddI, d4T, ABC, AZT, TNF and its administrated form (TFV diisoproxyl fumarate, TDF) in rat plasma [47]. Verweij-Van Wissenab et al. applied Oasis MAX cartridges to extract 3TC, ddI, d4T, AZT and ABC in plasma with the extraction recovery of higher than 97% [48]. Notari et al. described

an automated SPE method with the Oasis HLB cartridge to extract seven NRTIs (ABC, ddI, emtricitabine, 3TC, d4T, ddC, and AZT); seven HIV PIs and two NNRTIs from human plasma samples with the absolute recovery between 88 and 120% [49]. Liu et al. also utilized Waters Oasis® HLB extraction cartridges to extract 3TC, oxymatrine and its active metabolite matrine in dog plasma. The average recoveries for 3TC, oxymatrin and matrine were 78.9, 69.9 and 74.5%, respectively [50]. Compain et al. applied the cartridge packed with 30 mg of a polymeric sorbent (ATH) to extract AZT, d4T, ddC, ddI, 3TC and ABC in plasma and intracellular medium [51]. Chianella et al. synthesized a high selective molecularly imprinted polymer (MIP) and used as the SPE material for the extraction of ABC. The MIP showed a surprisingly high binding capacity, up to 157 mg of drug/g of adsorbent. The high binding capacity could make this polymer suitable for the industrial applications to purify and/or concentrate the drugs [52].

2.3.3. SPE with other columns

Burger et al. applied a silica gel SPE column to extract the d4T in human plasma [53]. The recoveries of d4T in spiked samples at levels of 50, 500 and 5000 ng mL^{-1} were 92.5–101.8%. One year later, the same group also developed a HPLC method coupled with a cation-exchange column for the determination of 3′-amino-3′-deoxythymidine (AMT), a cytotoxic metabolite of AZT, in human plasma [54]. It was reported that among the silica, CN, and cation-exchange columns, only the cation-exchange column provided acceptable recovery for AMT. The optimal pH during the SPE procedure was 5. At pH > 6, the recovery of AMT almost decreased to 0. Schrive and Plasse suggested an on-line SPE method to extract AZT and G-AZT from plasma and urine with a Perisorb RP8 extraction column [55]. The on-line method provided rapid sample pretreatment because it circumvented the evaporation under nitrogen flow. Solas et al. developed an analytical methodology to quantify the intracellular nucleotides including mono-, di-, and triphosphates and the diphosphocholine derivative of 3TC in PBMCs [10]. The procedure included the extraction of 3TC nucleotides by SPE on an anion-exchange cartridge, with subsequent enzyme digestion of the resulting phosphates to the parent drug that is ultimately quantified by HPLC-UV. Rodriguez et al. applied a strong anion-exchange Sep-Pak plus cartridges to extract the ZDV-TP and 3TC-TP in PBMCs. For all concentrations studied, recoveries were >93% and the CV was <10% [56]. Zheng et al. used a cartridge packed with cyclohexane-silica for the extraction of 3TC and 3-isobutyl-methylxanthine (I.S.) in human plasma with the extraction recovery of higher than 95% for both 3TC and the I.S. [57]. Marchei et al. also used the same cartridge to extract AZT and nevirapine in human plasma. Extraction recoveries were higher than 90%, for both AZT and nevirapine [58]. Estrela et al. applied an on-line SPE procedure to extract ddI in human serum with a HySphere GP 10–12 μm cartridge. The absolute recoveries were 99.8% (10 ng mL^{-1}), 98.4% (30 ng mL^{-1}), 91.5% (700 ng mL^{-1}) and 94.7% (1200 ng mL^{-1}) [59]. One year later, the same group reported the application of the similar extraction procedure to separate 3TC and AZT in human serum [60]. The sample pretreatment methods for the analysis of NRTIs and their metabolites are summarized in Table 1.

3. Analytical methods

3.1. CE and CEC

3.1.1. CE and CEC with UV detection

In order to establish a relationship between efficacy and toxicity, an effective separation and sensitive detection method for the analysis of NRTIs and their metabolites at intracellular levels are needed (Table 2). Mesplet et al. investigated a capillary electrochromatography (CEC) method for the simultaneous quantitation of AZT, 3TC, ddI, d4T and their metabolites. In this method, a beta-cyclodextrin-bonded silica packed column was applied to separate the NRTIs, taking advantage of the internal hydrophobicity of the polysaccharide. The influence of several parameters such as buffer pH, ionic strength, ACN content, temperature and voltage has been investigated using the short-end injection technique to achieve baseline separation in a short-time analysis [61,62].

Mallampati et al. [63] presented a selective MEKC method for the analysis of ddI in bulk samples. Successful separation of ddI from 13 of its potential impurities that were derived from the various synthetic preparation procedures was achieved. The use of MEKC allowed the separation in a significantly shorter time than conventional HPLC. An anionic long-chain surfactant, namely lithium dodecyl sulfate (LiDS), was used as the pseudostationary phase and the sodium tetraborate buffer as the aqueous phase. The optimized electrophoretic conditions included the use of an uncoated fused-silica capillary with a total length of 40 cm and an ID of 50 μm, a background electrolyte (BGE) containing 40 mM sodium tetraborate and 110 mM LiDS at pH 8.0, an applied voltage of 18.0 kV, and the capillary temperature maintained at 15 °C [63]. Fan and Stewart developed a MEKC method to separate and quantify the anti-HIV drug mixtures A and B containing d4T/ddI/saquinavir and d4T/ddI/efavirenz, respectively, in human serum [64]. The effects of various factors such as buffer type, concentration of buffer and surfactant, and pH on the separation of the analytes were investigated. The optimized resolution of both mixtures was achieved with a run buffer containing 18 mM sodium dodecyl-sulfate (SDS) in 15 mM phosphate and borate buffer (pH 9.0). An uncoated 52 cm (effective length 30 cm) × 50 μm BD fused-silica capillary, operated at 30 °C, was used in the analysis with UV detection at 210 nm. All analytes were separated within 15 min [64]. Meanwhile, Sekar et al. also investigated a MEKC method for the simultaneous separation and determination of 3TC and AZT in pharmaceutical formulation [65]. In their study, the factors that affect the separation, such as buffer pH, surfactant concentration (SDS), organic solvents and applied voltage were optimized. Buffer consisting of 12.5 mM sodium tetraborate decahydrate and 15 mM boric acid adjusted at pH 10.8, containing 90 mM SDS and 5% (v/v) ACN, was found to be suitable for the separation of the drugs.

3.1.2. CE and CEC with MS/MS detection

Agrofolio et al. employed a method of CE coupled with ESI-MS/MS for the simultaneous determination of AZT, d4T, 3TC and ddI. The influences of several parameters (pH and ionic strength of the buffer of formic acid/ammonia) as well as the magnesium cation upon electroosmotic flow, electrophoretic mobility and peak efficiency were investigated [66]. Cahours and co-workers also described the analysis of AZT and d4T by using capillary zone electrophoresis (CZE)-ESI-MS/MS in positive ion mode. Several volatile formic acid–ammonia buffers with the same ionic strength (50 mm) but different pH values varying from pH 9 to 11 were prepared and tested to determine the best electrophoretic migration conditions [67]. Cai et al. developed a CE-ion trap MS method to simultaneously analyze ABC and its phosphorylated metabolites such as ABC-MP, ABC-DP and ABC-TP [68]. By using the developed time-segment program, the positively charged nucleoside analog and negatively charged nucleotides were separated and detected in a single electrophoretic run (Fig. 1). The capability of the method was demonstrated by analyzing ABC and its phosphorylated metabolites that were spiked in cellular extracts of human PBMCs at 20 μM levels. Liu et al. applied CE-ESI-MS for

Table 1
The sample pretreatment methods for analysis of NRTIs and metabolites

Sample types	Pretreatment methods	Analytes	References
Plasma	PP	ABC	[24,90]
		3TC	[27,31,95]
		AZT	[26,95]
	LLE	ABC	[33,35]
		AZT	[35]
	SPE	AZT	[26,38–41,43,44,47–49,55,58,78,96,108,109]
		d4T	[41,43,46–49,51,53,110]
		3TC	[44,47,48,50,51,57,83,94,96,109]
		ABC	[48,49,51,52,83]
		ddI	[40,47–49,51,83]
		ddC	[47,49,51,108]
Serum	PP	3TC	[28]
	LLE	AZT	[35]
		3TC	[34]
	SPE	AZT	[45,46,60,94,100,111]
		d4T	[46,64,94]
		3TC	[44,60,94,100,112]
PBMCs	SPE	AZT	[19,21,56]
		d4T	[10]
		3TC	[56]
CSF			[80,113–118]
Saliva			[80,119]
Semen			[93,120,121]
Urine			[43,55,122–124]

Note: PBMCs, peripheral blood mononuclear cells; PP, protein precipitation; LLE, liquid–liquid extraction; SPE, solid-phase extraction.

the determination of 14 compounds of nucleosides and nucleotides [69]. A CE system for the separation of the targeted compounds was developed based on a basic buffer with a volatile electrolyte suitable for ESI-MS detection in an untreated capillary column. The 14 nucleosides and nucleotides were profiled in a single CZE separation within 18 min. Bezy et al. developed and validated a CE-ESI/MS/MS method for the simultaneous measurement of nucleoside 5′-triphosphate and 5′-monophosphate anabolites of the ddI (ddA-MP, ddA-TP) and d4T (d4T-MP, d4T-TP). An acetic acid/ammonia buffer (pH 10, ionic strength of 40 mM) was selected as running electrolyte [70]. CE-MS/MS is an excellent technique for the separation and quantitation of various anti-HIV nucleosides but the interface technique between CE and MS still needs to be improved. Furthermore, the high concentration of electrolytes used in CE may produce high background signals in the ESI-MS analysis.

3.2. HPLC with fluorescence detection

Knupp et al. evaluated the steady-state pharmacokinetics of ddI and ketoconazole (KET) in plasma and urine by using HPLC-UV and -fluorescence methods [71]. A specific and sensitive HPLC assay for the determination of 3′-amino-3′-deoxythymidine (AMT) with pre-column derivatization and fluorescence detection was developed

Table 2
Summary of the analytical method for NRTIs and metabolites

NRTIs and metabolites	Analytical methods	References
ABC	CE(C)-MS(/MS)	[68]
	LC-UV	[24,32,33,35,48,49,75,76,83,84]
	LC–MS(/MS)	[51,90]
ddI	CE(C)-UV	[45,63,64,112]
	CE(C)-MS(/MS)	[66,70,126]
	LC-UV	[48,51,53,71,82–84,127–129]
	LC–MS(/MS)	[11,15,40,51,59,70,130]
3TC	CE(C)-UV	[65]
	CE(C)-MS(/MS)	[65,66,69]
	LC-UV	[27,28,31,34,80,83–86,88,131]
	LC–MS(MS)	[51,60,93–96,100,102,104,111,130,132]
d4T	CE(C)-UV	[46,61,62,64,133]
	CE(C)-MS(/MS)	[66,67,70,126]
	LC-UV	[53,83–86,88,131]
	LC–MS(/MS)	[41,51,94,104,130,134]
AZT	CE(C)-UV	[45,46,61,62,65,135,136]
	CE(C)-MS(/MS)	[66,67,70]
	LC-UV	[32,37,54,83–85,137]
	LC–MS(/MS)	[21,51,60,93,95,100,102,104,111,130,138–141]
ddC	LC-UV	[84]
	LC–MS(/MS)	[51]

Note: CE(C), capillary electrophoresis (electrochromatography); LC, liquid chromatography; MS(/MS), (tandem) mass spectrometry; UV, UV detection.

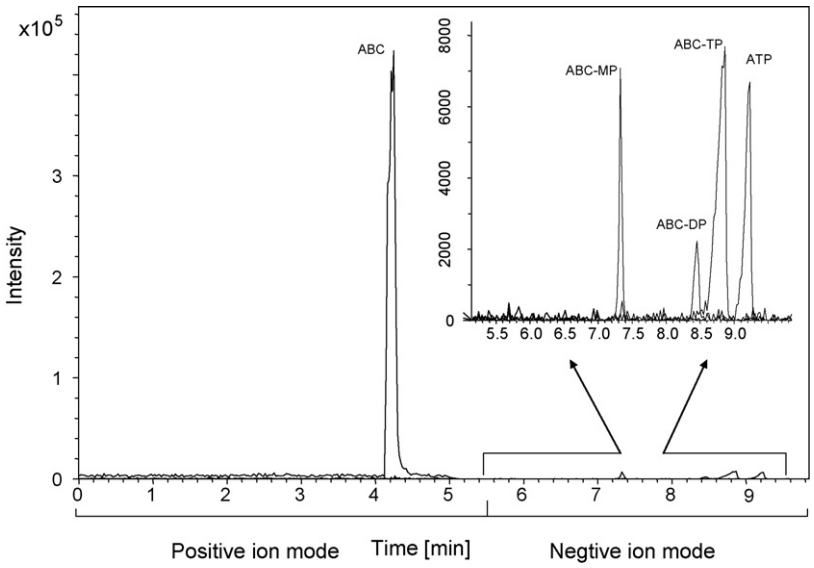

Fig. 1. CE-ESIMS analysis of a mixture of ABC and its phosphorylated metabolites spiked in cellular extracts of human PBMCs at 20 μM levels by using a time-segment program of polarity switching from positive to negative ion mode at the retention time of 5 min.

by Zhou and Sommadossi [72]. After the extraction with *tert*-butyl methyl ether-1-butanol (6:4, v/v) and back-extraction into the basified aqueous phase, the solution was neutralized with phosphate buffer and the analyte was derivatized with fluorescamine. The fluorescamine-labeled AMT was separated on a RP C_{18} column using a mixture of phosphate buffer–methanol–ACN (47:48:5, v/v/v) as mobile phase and fluorescence detection at excitation wavelength of 265 nm and emission wavelength of 475 nm. The limit of quantification was 3 ng mL^{-1} [72]. Yeh et al. used HPLC with UV, fluorescence or radioimmunoassay detection to study the pharmacokinetic interaction between 2′,3′-dideoxyinosine (ddI) and pentamidine [73]. Dai et al. developed a sensitive pre-column derivatization method to measure 5′-triphosphate of 2′-beta-fluoro-2′,3′-dideoxyadenosine (F-ddA, lodenosine) that was a new anti-HIV drug in human lymphocytes by HPLC using fluorescence detection [74]. Reaction of chloroacetaldehyde with F-ddA triphosphate in extracts from human lymphocytes produced a highly fluorescent etheno adduct. The method was applied to measure F-ddATP in PBMCs collected from HIV-infected patients. Sparidans et al. reported a combined bioanalytical assay for ABC and mycophenolic acid (MPA), based on RP-HPLC with both UV and fluorescence detections. MPA was detected by using fluorescence detection at 345 and 430 nm for excitation and emission wavelength, respectively [75]. Two years later, the same group developed and validated another sensitive and selective RP-HPLC assay for the determination of TFV in human plasma with fluorescence detection. The fluorescent 1,*N*-6-etheno derivative is formed at 98 °C in the buffered extract with chloroacetaldehyde. The derivative was analyzed by using fluorescence detection at 254 nm for excitation and 425 nm for emission [29]. Fluorescence detection was found more sensitive than UV detection. However, the application of fluorescence detection is limited because most of NRTIs or/and NRTIs metabolites do not produce fluorescence and thus need to be derivated prior to analysis. The derivation process is often complicated and time consuming.

3.3. HPLC–UV detection

3.3.1. Analysis of ABC

Veldkamp et al. described a simple and rapid HPLC method for the quantification of ABC in human plasma, which appeared to be suitable for supporting pharmacokinetic researches. The drug was separated from endogenous compounds by using an isocratic RP-HPLC method with UV detection [24]. To achieve the optimal drug concentrations for viral suppression and avoidance of drug toxicity, monitoring on the drug levels in biological matrices has been considered essential. Ferrer et al. developed a rapid and simple HPLC method for the determination of ABC in human plasma with the UV detection [33]. Ozkan et al. described a fully validated, simple, rapid, selective, and sensitive HPLC method with UV detection for the direct determination of ABC in pharmaceutical dosage forms, raw materials, spiked human serum and samples from drug dissolution studies without the need of complicated sample pretreatment [76]. Seshachalam et al. developed a chiral LC method for the separation of the enantiomers of ABC sulfate [77]. The enantiomers were resolved in a Chiralcel OD column with *n*-hexane–ethanol–TFA as mobile phase. The resolution between the enantiomers was not less than 3.5. The TFA had a very important effect on the separation of enantiomers. The method was extensively validated to prove its suitability and robustness. Solution stability and mobile-phase stability were good during the study period of 48 h. The method was found to be suitable for the analysis of the commercial drug substances in quality-control laboratories [77].

3.3.2. Analysis of zidovudine (AZT, ZDV) and metabolites

Borvak et al. determined AZT concentrations in spiked human plasma by RP-HPLC. Samples were first cleaned-up using the Silipore C_{18} SPE column [78]. A simple, sensitive, and reproducible HPLC method for the determination of AZT and theophylline was developed by Radwan [79]. To investigate the bioconversion and pharmacokinetic profiles of AZT and AZT-MP, Tan et al. developed a HPLC-UV detection method to determine AZT and AZT-TP in mouse plasma and PRBCs [38]. Chromatographic separation was performed by using a Hypersil ODS column. Caufield et al. developed a HPLC assay for the simultaneous determination of AZT and levofloxacin in human plasma [39]. Brown et al. developed and validated a sensitive and reproducible HPLC assay for the separation and analysis of AZT and acyclovir from plasma, amniotic fluid, placental homogenate and fetal homogenate [26]. The mobile phase consisted of 30 mM acetate/citrate buffer (pH 3) and methanol. The plasma and amniotic fluid samples were prepared by using a combination of PP and filtration, while the

more complex tissues were prepared by using a SPE. The assay was applied to a pharmacokinetic study involving the co-administration of AZT and acyclovir in pregnant rats [26]. Quevedo et al. presented a HPLC method for the quantification of 3′-azido-3′-deoxy-5′-*O*-isonicotinoylthymidine (AZT-Iso) in rat plasma with UV detection. The method was proved to be selective, sensitive and accurate [42]. Marchei et al. suggested a simple RP-HPLC method with UV detection at 265 nm for the simultaneous determination of AZT and nevirapine in human plasma [58]. Recently, Ramachandran et al. also described a simple and fast isocratic RP-HPLC method for the simultaneous determination of AZT and nevirapine in plasma with UV detection. The system required a C_{18} column and a mobile phase composed of potassium dihydrogen phosphate and ACN in the ratio of 80:20 (v/v) [35]. Due to the poor retention of NRTIs and phosphates on the RP column, ion-pairing reagent (IPR) was added to the aqueous mobile phase to prolong the retention time. Peter et al. reported a validated separation assay for the measurement of ZDV and its three phosphorylated anabolites, namely ZDV mono-, di- and triphosphates, in PBMCs by using the ion-pairing mobile phase on a RP-HPLC column. The mobile phase consisted of a sodium dihydrogenphosphate–disodium–hydrogenphosphate buffer (140 mM), tetrabutyl–ammonium dihydrogen–phosphate (1 M) (pH 7.5), and ACN (957.3:7.7:35, v/v/v) [19].

3.3.3. Analysis of lamivudine (3TC)

Zhou et al. developed and validated a rapid, sensitive and specific HPLC assay for the measurement of 3TC in human serum. The supernatant from the sample pretreatment by PP was directly injected onto HPLC and 3TC was separated on a RP C_{18} column using an isocratic program with a mixture of phosphate buffer and methanol and monitored with UV detection [28]. Zheng et al. also developed a HPLC-UV method for the quantification of 3TC in human plasma. The ion-pair chromatographic separation was performed using a YMC phenyl column with the UV detection at 270 nm [57]. Hoetelmans et al. developed a RP-HPLC method for the quantitative determination of 3TC in human plasma, saliva and CSF samples. 3TC was extracted from the samples by using silica extraction columns prior to the RP-HPLC with UV detection at 270 nm [80]. Alnouti et al. reported an HPLC method for the quantification of 3TC in rat plasma, amniotic fluid, placental and fetal tissues for the study of the placental transport of the drug in pregnant rats [27]. RP-HPLC was performed using a phenyl column. Recently, Liu et al. developed and validated a simple and highly selective method of IP-HPLC with UV detection for the determination of 3TC, oxymatrine and its active metabolite matrine in dog plasma [50].

3.3.4. Analysis of other single NRTIs

Sarasa et al. developed a sensitive HPLC assays for the quantification of d4T in human plasma and urine [43]. The analytical column, mobile phases, instrumentation and chromatographic conditions were investigated. The methods have been validated, and stability tests under various conditions have been performed. Uslu et al. developed and validated a HPLC method for the determination of ddC in bulk form, pharmaceutical dosage forms and human serum. The proposed method was conducted using a RP technique with the UV monitoring at 265 nm [81]. de Oliveira et al. developed a simple, rapid, sensitive and specific RP-HPLC method with UV detection for the analysis of ddI in drug substance and formulated products, tablets [82]. Chromatographic separation was carried out on a pre-packed, Lichrospher 100 RP-8 column by using 0.01 M sodium acetate solution–methanol (85:15, v/v) adjusted to pH 6.5 with acetic acid as mobile phase. Hypoxantine was confirmed as the main degradation product. The assay was linear over the concentration range of 50–150 $\mu g\,mL^{-1}$. The method was validated with acceptable accuracy and precision [82].

3.3.5. Simultaneous analysis of multi-NRTIs and metabolites

As discussion in Section 1, HAART regimens typically are composed a backbone of two NRTIs combined with either a PI or an NNRTI or both. In recent years, many publications have focused on the method development for the simultaneous analysis of multi-NRTIs and metabolites. Rodriguez et al. described a method for the simultaneous quantitation of 3TC-TP and AZT-TP in PBMCs collected from HIV-infected patients. The method was successfully applied for the determination of *in vivo* pharmacokinetic profiles of the ZDV-TP and 3TC-TP from HIV-infected patients receiving HAART [56]. Aymard et al. presented a RP-HPLC assay for the simultaneous determination of PIs, NRTIs and NNRTIs in 1-mL plasma samples [83]. A SPE procedure was coupled with two separated RP-HPLC systems; one for the determination of six PIs with the chromatographic run time of 32 min and one for the determination of ABC, ddI, 3TC, d4T, nevirapine and AZT with the run time of 40 min. The second system requires three mobile phases (potassium phosphate buffer plus ion-pairing agent and ACN) for different elution through a C_{18} Symmetry Shield column. Donnerer et al. set up an analytical procedure for simultaneously monitoring the plasma concentrations of ABC, AZT, efavirenz, nevirapine, indinavir, lopinavir, and nelfinavir [32]. The ABC, AZT, and nevirapine were monitored by UV detection at 266 nm. Two different HPLC eluents on a C_8 RP-HPLC column were used to monitor all of the seven compounds. Rezk et al. described an accurate, sensitive and specific RP-HPLC assay for the simultaneous determination of ddC, 3TC, ddI, d4T, AZT, ABC and nevirapine in human blood plasma [84]. The Polarity dC_{18} silica column used in this method was found to provide better resolution and peak shape than all other columns tested. Also, four different UV wavelengths were used for the accurate and specific quantitation of the analytes. Djurdjevic et al. developed and validated a method for the separation of ABC, 3TC, and AZT by HPLC on a C_{18} column with UV detection at 270 nm [85]. The effects of pH and proportion of methanol in the mobile phase were studied to optimize the separation. Bezy et al. developed a specific RP-HPLC method for the analysis of 3TC, ddC, ddI, d4T, carbovir, AZT and TNF in rat plasma. HPLC separation was optimized on an Atlantis™ C_{18} column with acetic acid–hydroxylamine buffer–ACN elution gradient and the analytes were detected at 260 nm [47]. Verweij-Van Wissenab et al. developed a RP-HPLC method for the simultaneous determination of d4T, 3TC, ddI, AZT and ABC in plasma. The method involved SPE with the Oasis MAX cartridge, followed by HPLC with a SymmetryShield RP 18 column and UV detection at 260 nm [48]. Anbazhagan et al. presented a RP-HPLC method for the simultaneous quantification of d4T, 3TC and nevirapine in tablets by UV spectroscopic detection [86]. In the UV multi-component spectral method, d4T, 3TC and nevirapine were quantified at 266, 271 and 315 nm, respectively. In the RP-HPLC method, the drugs were resolved on a C_{18}-ODS-Hypersil column in isocratic mode using a mobile phase of 20 mM sodium phosphate buffer–ACN (4:1, v/v). Notari et al. developed a HPLC-UV method to simultaneously quantify seven HIV PIs, seven NRTIs (ABC, ddI, erntricitabine, 3TC, d4T, ddC, and AZT) and two NNRTIs in human plasma [49]. The analytes were eluted on an analytical C_{18} Symmetry™ column. The mobile phase (0.01 M KH_2PO_4 and ACN) was delivered at 1.0 mL min^{-1} with linear gradient elution. Kapoor et al. reported an accurate, sensitive and specific RP-HPLC method for the simultaneous determination of 3TC, AZT and nevirapine in pharmaceutical fixed dose combinations [87]. Chromatographic separation was carried on a C_{18} column by gradient elution with two mobile-phase components. Sarkar et al. developed and validated a HPLC method with UV detection for the quantitative analysis of three antiretro-

viral drugs viz. 3TC, d4T and nevirapine that constituted one of the first line regimens in antiretroviral therapy [88]. Chromatography was carried out by isocratic technique on a RP C_{18} Symmetry column with mobile phases optimized depending on the polarity of the molecules. The UV detections were performed at 270, 265 and 313 nm for 3TC, d4T and nevirapine, respectively. Two methods for the simultaneous determination of 3TC and d4T in combined pharmaceutical tablets were described by Kapoor et al. [89]. The first method depended on the derivative UV-spectrophotometry with zero-crossing measurement technique. The derivative absorbance at 280 and 300 nm was selected for the determination of d4T and 3TC, respectively. The second method is based on the separation of both drugs by HPLC using methanol–water as the mobile phase at 0.6 mL min^{-1} on a RP column with the UV detection at 270 nm. Both methods showed good linearity, reproducibility and precision. No spectral or chromatographic interferences from the tablet recipients were found. The proposed methods were suitably applied to the assay of commercial formulations. The procedures were rapid, simple and suitable for routine application of quality control. The analysis of NRTIs using HPLC with UV detection was summarized in Table 3.

3.4. HPLC with mass spectrometric detection

3.4.1. Analysis of prodrugs by LC–MS

3.4.1.1. Analysis of individual prodrug by LC–MS. Wiesner et al. developed a sensitive method for the determination of d4T in plasma by using HPLC separation with MS/MS detection [41]. Chromatographic separation was performed on a Supelco Discovery(R) C_{18} column. Detection was performed with multiple reaction monitoring (MRM) mode on a triple quadrupole mass spectrometer (Applied Biosystems API 2000). Atmospheric pressure chemical ionization (APCI) was used for the analysis. The method with the increased sensitivity and selectivity of tandem mass spectrometric (MS/MS) detection allowed for a rapid and selective determination of d4T in human plasma. Clark et al. developed and validated a rapid and efficient HPLC–MS/MS method for the determination of the carbocyclic nucleoside antiviral ABC in maternal rat plasma, amniotic fluid, placental and fetal tissue samples [90]. Separation of the analyte and I.S. from the matrices was achieved on a C_8 analytical column.

3.4.1.2. Analysis of multiple prodrugs by LC–MS/MS. Font et al. developed a negative ion electrospray ionization (ESI) and collision-

Table 3
HPLC-UV analysis of NRTIs and phosphated metabolites

Drug and metabolites	Sample	Stationary phase	Mobile phase	Wavelength (nm)	References
ABC	Human plasma	Symmetry C_{18}	ACN–phosphate (pH 7.0, 15:85, v/v)	285	[24]
ABC	Human plasma	Luna C_{18}	Water–ACN (83:17, v/v)	285	[33]
ABC	Spiked human serum	Waters Spherisorb ODS	MeOH–ACN–KH_2PO_4 (36:2.6:61.4, v/v/v)		[76]
ABC sulfate	Standards	Chiralcel OD	*n*-hexane–ethanol–TFA (92:8:0.1, v/v/v)	210	[77]
AZT	Spiked human serum	Separon SGX C18	MeOH–KH_2PO_4 (pH 8.5, 50 mM)	267	[78]
AZT, theophylline	Rat serum	Novapak C_{18}	ACN–HAc (0.2%) (7.5:92.5, v/v)	270	[79]
AZT, AZT-TP	Mouse plasma, PRBCs	Hypersil ODS	ACN–phosphate (pH 7.5) (2.9:97.1, v/v)	267	[38]
AZT, levofloxacin	Human plasma	ODS	NaH_2PO_4–TFA (pH 2.4)–ACN (86:14, v/v)	266	[39]
AZT, acyclovir	Plasma, amniotic fluid, placental homogente, fetal homogente	Eclipse XDB C_8	Acetate/citrate buffer (pH 3)–MeOH	254	[26]
AZT-Iso	Human plasma	Alltech Allsphere ODS-1	Water–MeOH (40:60, v/v)	267	[42]
AZT, nevirapine	Human plasma	Zorbax SB-C18	ACN–phosphate buffer	265	[58]
AZT, nevirapine	Plasma	C_{18}	KH_2PO_4 (15 mM, pH 7.5)–ACN (80:20, v/v)	260	[35]
AZT, AZT mono-, di-, triphosphate	PBMCs	Novapak C_{18}	NaH_2PO_4/Na_2HPO_4–BADHP–ACN (957.3:3.7:35, v/v/v)		[19]
3TC	Human serum	C_{18}	Phosphate–MeOH (88.3:11.7, v/v)	280	[28]
3TC	Human plasma	YMC phenyl	ACN–0.085% Phosphoric acid (12:88, v/v)	270	[57]
3TC	Human plasma, saliva, CSF	μBondapack phenyl	Water (pH 6.8)–MeOH (92:8, v/v)	270	[80]
3TC	Rat plasma, amniotic fluid, placental and fetal tissues		Phosphate buffer (pH 6)–MeOH (95:5, v/v)		[27]
3TC, oxymatrine	Dog plasma	C_{18}	ACN–water (13:87, v/v, 5 mM sodium heptanesulfonate, at pH 3.2).	–	[50]
d4T	Human plasma, urine	Nova Pak C_{18}	ACN–phosphate buffer	266	[43]
ddC	Bulk form, dosage form, human serum	C_{18}	MeOH–0.01 M NaH_2PO_4 (85:15, v/v)	265	[81]
ddI	Drug, formulated products, tablets	Lichrospher 100 RP-8	NaAc (0.01 M)–MeOH	248	[82]
ABC, ddI, 3TC, d4T, AZT, nevirapine	Human plasma	C_{18} Symmetry Shield	Phosphate–ACN	260	[83]
ABC, AZT, nevirapine	Human plasma	C_8 RP	450 mL/LACN and 50 ml/L MeOH in 15 mM phosphate buffer	266	[32]
ddC, 3TC, ddI, d4T, AZT, ABC, nevirapine	Human plasma	Polarity dC_{18} silica	NH_4Ac–ACN–MeOH	–	[84]
ABC, 3TC, AZT	Tablets	Eorbax Extend C-18	Water (0.2% TEA, pH 3.2)–MeOH (60:40, v/v)	270	[85]
3TC, ddC, ddI, d4T, AZT, TNF, carbovir	Rat plasma	Atlantis C_{18}	HAc–NH_4Ac (pH7.0)–ACN	260	[47]
d4T, 3TC, ddI, AZT, ABC	Plasma	Symmetry Shield RP C_{18}	Acetate buffer–ACN	260	[48]
d4T, 3TC, nevirapine	Tablets	ODS Hypersil C_{18}	Phosphate (20 mM)–ACN (4:1, v/v)	266, 271, 315	[86]
ABC, ddI, 3TC, d4T, ddC, AZT, emtricitabine	Human plasma	Symmetry Shield RP C_{18}	KH_2PO_4 (0.01 M)–ACN ( )	240, 260	[49]
3Tc, AZT, nevirapine	Fixed dose combination	C_{18}	A: acetate buffer–MeOH (20%); B: ACN–IPA (1:1, v/v)	270	[87]
3Tc, d4T, nevirapine	Tablets	Symmetry Shield RP C_{18}	MeOH–water	270, 265, 313	[88]
3Tc, d4T	Combined tablet	RP column	MeOH–water (20:80, v/v)	270	[89]

Table 4
The concentrations of ion-pairing reagent for different size of column

Column type	Column size	Conc. of IPRs	References
Merck Purospher C_{18}e	125 mm × 3 mm i.d.	20 mM of DMHA	[97]
SMT-C_{18}	150 mm × 2.1 mm i.d.	20 mM of DMHA	[101]
SMT-C_{18}, PLRP-S, X-Terra MS, Supelcogel ODP-50	150 mm × 2.1 mm i.d.	20 mM of DMHA	[104]
Microbore Column	100 mm × 1 mm i.d.	10 mM of DMHA	[125]
Waters, Xterra C_8	100 mm × 1 mm i.d.	8 mM of DMHA	[105]
Zorbax XDB C_{18}	150 mm × 0.5 mm i.d.	5 mM of DMHA	[125]

induced dissociation mass spectrometry (CID-MS/MS) method for the analysis of AZT, ddI, ddC and 3TC [91]. Fragmentations of the compounds were obtained at different cone voltages and collision energies. It was reported that fragmentation of purines and pyrimidines occurred with different pathways. For purines (ddI), the fragmentation was similar to those found in endogenous nucleosides. The pseudo molecular ion was mainly present and a cleavage through the glycosidic bond was observed. For pyrimidines (AZT, ddC, 3TC), the fragmentation pathways were different from the endogenous nucleosides. For AZT, the fragmentation occurred primarily through the elimination of the azido group at the 3′-position, whereas ddC and 3TC presented more complex fragmentation patterns. For ddC, fragmentation appeared to be dominated by a retro Diels–Alder mechanism. For 3TC, the sulfur atom in the sugar moiety provided greater stability to the charge, producing fragments where the charge initially resided in the dideoxyribose [91].

A HPLC–MS/MS method was developed and validated to measure 3TC and AZT simultaneously in small volumes of human seminal plasma by Pereira et al. [92]. Sample preparation was simple and rapid, requiring only 25 μL of sample that was ultrafiltrated through a molecular mass cut-off membrane. The method used isotopically labeled 3TC and AZT as I.S. Fan et al. developed and validated a LC–MS/MS method with ionization polarity switch for the analysis of human serum samples collected from a 3TC/d4T/efavirenz combination HIV therapy [93]. The precursor and major product ions of the drugs were monitored on a triple quadrupole mass spectrometer in MRM mode. A method based on SPE-HPLC coupled with positive ESI-MS/MS for the simultaneous determination of 3TC and AZT in human serum was developed by Estrela et al. [60]. The acquisition was performed in MRM mode by monitoring the transitions m/z 230.0 → 111.8 for 3TC, m/z 268.1 → 126.8 for AZT and m/z 237.2 → 136.8 for ddI. Alnouti et al. developed and validated a simple LC–MS/MS method for the simultaneous determination of AZT and 3TC in rat plasma, amniotic fluid, placental, and fetal tissues. Chromatographic separation was performed using a C_{18} column with the mobile phase consisting of 30% methanol and 7.5 mM ammonium acetate (pH 6.5) [94]. Compain et al. developed an LC–MS/MS method to separate, detect and quantify AZT, d4T, ddC, ddI, 3TC and ABC in plasma and intracellular medium [51]. The LC separation was carried out on a Zorbax Stable Bond C_{18} column followed by ESI-MS/MS analysis in either negative or positive mode. The positive ionization mode was applied at the beginning of the run to detect ddC and 3TC, then the ionization mode was changed to negative for the detection of ddI, d4T, AZT and I.S. Recently, Notari et al. reported a MALDI-TOF/TOF method for the determination of 3TC, lopinavir, and ritonavir concentration in the plasma of HIV-infected patients [95]. Concentrations of 3TC, lopinavir and ritonavir determined by MALDI-TOF/TOF were in excellent agreement with those obtained by HPLC-UV and HPLC–MS/MS. MALDI-TOF/TOF experiments also allowed the detection of the ritonavir metabolite R5. However, AZT was undetectable by MALDI-TOF/TOF analysis because the minimal laser intensity might induce the anti-HIV drug photolysis. The MALDI-TOF/TOF technique was showed useful to determine the anti-HIV drugs at very low concentrations (0.0025–0.010 pmol μL^{-1}).

Table 5
HPLC–MS/(MS) analysis of NRTIs and phosphated metabolites

Drug and metabolites	Sample	Stationary phase	Mobile phase	Detector	References
d4T	Human plasma	Supelco Discovery C_{18}	NH_4Ac–ACN–MeOH (800:100:100, v/v/v)	APCI	[41]
ABC	Maternal rat plasma, amniotic fluid, placental and fetal tissues	C_8	NH_4Ac (10 mM)–ACN		[90]
AZT, ddI, ddC, 3TC	–	–	–	ESI, CID	[91]
3TC, AZT	Human seminal plasma	Aquasil	ACN–water	ESI	[92]
3TC, d4T, efavirenz	Human serum	Hexylsilane	NH_4Ac (20 mM)–ACN	ESI	[93]
3TC, AZT	Human serum	LiChrospher 100 RP-18	20 mM NH_4OAc–MeOH (60:40)		[60]
3TC, AZT	Rat plasma, amniotic fluid, placental and fetal tissues	C_{18}	NH_4Ac (7.5 mM, pH 6.5)–MeOH (7:3, v/v)		[94]
AZT, d4T, ddC, 3TC, ABC	Plasma, intracellular medium	Zorbax Stable Bond C_{18}	A: 5% MeOH; B: 40% ACN	ESI	[51]
3TC, lopinavir, ritonavir	Plasma	Vydac	Water–ACN	MALDI-TOF/TOF	[95]
AZT, AZT-TP, 3TC, 3TC-TP, d4T, d4T-TP	PBMCs	Colunbus C_{18}	10 mM NH_4Ac–ACN (86:14)	ESI	[101]
ddATP	Intracellular medium	Purospher RP-18e	MeOH–H_2O (1% FA) (25:75, v/v)	ESI	[11]
ZDV, ZDV-MP, ZDV-MP, ZDV-DP	Intracellular medium	XTerra™ RP18	0.1%AA–10%ACN	ESI	[21]
Nucleoside TP	PBMCs	Keystone BioBasic	A: 10 mM NH_4Ac (ACN–water = 30:70); B: 1 mM NH_4Ac (ACN–water = 30:70)	ESI	[102]
d4T metabolites	PBMCs	SMT-C_{18}	A: 10 mM DMH, 3 mM $HCOONH_4$; B: 20 mM DMH, 6 mM $HCOONH_4$/ACN	ESI	[97]
ABC-MP, ABC-DP, ABC-TP	Human liver cells	Luna C_8	A: 20 mM DMHA; B: MeOH–water (80:20, v/v)	ESI	[103]
d4T-TP, ddA-TP, 3TC-TP	PBMCs	Microbore column	A: 10 mM DMH, 3 mM $HCOONH_4$; B: 20 mM DMH, 6 mM $HCOONH_4$/ACN	ESI	[104]
ATP, dGTP, AZT-TP	Cells	–	–	MALDI-TOF MS	[107]

3.4.2. Analysis of phosphorylated metabolites of NRTIs by LC–MS/MS

As mentioned in Section 1, unlike NNRTIs and PIs, the active form of NRTIs is not the drug itself but its TP metabolites. NRTIs require three intracellular phosphorylation steps to yield their corresponding biological active components, namely the nucleoside triphosphate. To clearly determine the correlation between NRTI plasma concentrations and virological response or intracellular concentrations of the corresponding triphosphate derivates, measurement of the metabolites of NRTIs is more important. However, the analysis of NRTIs phosphates is a big challenge because the trace levels of the metabolites exist together with strong interference background of endogenous phosphates in biological samples. Moreover, the phosphorylated metabolites are extremely polar due to the presence of multiple phosphate groups and thus have poor retention under conventional reversed-phase chromatographic conditions. LC–MS has become an attractive tool for providing high sensitivity and specificity to overcome the analytical challenge of the phosphorylated metabolites of NRTIs. However, poor retention has often caused analytical problems for the trace analysis of complex samples. Specific chromatographic conditions such as ion pairing are often not amendable with ESI-MS analysis. Significant effort has been made to achieve longer chromatographic retention of the phosphorylated metabolites by using modified RP columns such as Symmetry Shield RP C_{18} [48,49], Polarity dC_{18} silica [84] and Microbore column [104]. Clark et al. developed and validated a rapid and efficient HPLC–MS/MS method for the determination of ddI concentrations in maternal rat plasma, amniotic fluid, placental and fetal tissue samples [40]. HPLC coupled with a triple quadrupole mass spectrometer (Micromass Quattro II) was used for the sample analyses. Chromatographic resolution was achieved on a Nova-Pak phenyl analytical column using 60% methanol in 10 mM ammonium acetate buffer mobile phase.

Several analytical approaches have been developed to tackle the problem of poor retention of the phosphorylated metabolites of NRTIs. Many of early studies have employed indirect analytical methods, in which the NRTIs metabolites in cellular extracts were dephosphorylated into the corresponding nucleosides by using enzymatic assays and the produced nucleosides were separated with an anion-exchange SPE [10,99,100] and analyzed by LC with UV detection [10,100], radio immunoassay [10,99,100] and LC–MS/MS [11,40,96,101]. Moore et al. developed a method of HPLC coupled with triple quadrupole MS for the simultaneous measurement of the intracellular nucleoside 5′-triphosphate anabolites of AZT (ZDV-TP), 3TC (3TC-TP), and d4T (d4T-TP) [101]. The compounds were extracted from patient PBMCs collected from the sites of HIV replication and drug action. Ion-exchange SPE followed by enzymatic digestion with alkaline phosphatase was utilized to yield the measurable nucleoside forms of the nucleotides. RP C_{18} SPE with the addition of a nucleoside I.S., 3′-azido-2′,3′-dideoxyuridine (AzdU) allowed for the indirect measurement of the original 5′-triphosphate concentration by HPLC–MS/MS. Quantitation was performed from calibration curves generated from authentic 5′-TP standards spiked in PBMCs from healthy volunteers. This method can be utilized to measure the intracellular 5′-TP levels in HIV-infected patients receiving antiretroviral therapy containing the NRTIs 3TC, d4T, or ZDV [101]. Cahours et al. developed a LC–MS/MS method for the analysis of intracellular didanosine triphosphate (ddATP) at sub-ppb level [11]. ddATP was extracted from CEM-T4 cells, isolated using an anion exchange SPE procedure, enzymatically dephosphorylated and then analyzed by LC–MS/MS. The influence of several parameters (ESI interface, acidic modifiers of the mobile phase) was investigated. Using LC–MS/MS detection in selected reaction monitoring mode. Furthermore, it was reported that this procedure could be used to simultaneously detect five NRTIs, such as AZT, 3TC, ddA, ddC and d4T and might allow LC–MS/MS being the method of choice for TDM in a clinical environment. King et al. reported a LC–MS/MS method coupled with dephosphorylation procedure to facilitate the assessment of drug safety and determination of phamacokinetics [21]. Human PBMCs were harvested from whole blood, lysed, and a suspension of intracellular ZDV-TP was produced. ZDV-TP was isolated from ZDV, ZDV-MP and ZDV-DP that existed in the cell lysate by performing a salt gradient anion exchange SPE. The isolated ZDV-TP was dephosphorylated with acid phosphatase to its parent drug form, i.e., ZDV. ZDV was then desalted and concentrated for MS/MS detection.

The indirect analytical procedure, however, is complicated, time consuming and often not specific. In recent years, ion-exchange, ion-suppression and ion-pairing HPLC have been employed to improve the retention and separation of the nucleotides and nucleotide analogs on RP-HPLC columns [98,102–106]. A novel analytical method for the direct quantification of intracellular nucleoside TP was developed by Shi et al. [102]. Lysates of PBMCs were extracted by PP, and the filtered extracts were analyzed by weak anion exchange LC coupled with MS detection. Compared with IPLC–MS/MS, the new method completely avoids the usage of IPR and has a shorter analytical time of only 2 min. The MS-compatible direct detection method was validated and was used to determine the amount of the triphosphate metabolite of D-D4FC (DPC817), an investigational HIV NRTI, in human PBMC samples. Pruvost et al. developed and validated a routine LC–MS/MS assay for active intracellular anabolites of d4T in human PBMC, which was applicable to pharmacokinetic studies and treatment monitoring [97]. After the cellular lysis in a Tris/methanol buffer, the extract is directly injected into the LC–MS/MS system. Phosphorylated metabolites of d4T as well as deoxythymidine-TP, the competitor on the reverse transcriptase, were separated from d4T on a RP-HPLC microbore column with IPR. The detection is performed in MRM mode with negative ion ESI ionization. Fung et al. developed an ion-paring HPLC–MS/MS method with positive ion ESI ionization to simultaneously quantify phosphorylated metabolites of ABC as well as ABC-MP, ABC-DP and ABC-TP [103]. DMHA was used as the IPR. The presence of this IPR allowed the retention and separation of the four compounds on a RP-HPLC column as well as the detection of the nucleotides with positive ion mode ESI. The method has been successfully applied to the analysis of these compounds in human liver cells treated with ABC. Becher et al. improved their previously described direct LC–MS/MS assay for d4T-TP [104]. Substitution of the narrowbore column by a microbore column (150 mm × 0.32 mm) was presented and discussed. The improved method was successfully applied to the simultaneous determination of d4T-TP, ddA-TP and 3TC-TP in PBMCs. The method was subsequently applied to clinical samples from HIV-positive patients receiving antiretroviral therapy containing d4T, ddI and/or 3TC. It was demonstrated that the developed method could be simply and routinely used for the analysis of approximately 200 samples per week, using commercially available instruments combined with a simple cell lyses as sample treatment [104].

Although the ion-paring and ion-exchange methods worked well with the UV detection, they were often “not-friendly” to LC–MS analysis. The ion-interaction reagents applied in the mobile phase may generate a lot interfering noise, cause ion suppression for the ESI-MS analysis, and results in the detection sensitivity and specificity. Efforts have been made to mitigate the ion suppression and to improve the method sensitivity [103,104]. One of efficient solutions was to apply the small-diameter columns so that the levels of IPR could be reduced while still being able to meet the chromatographic and mass spectrometric requirements for analyzing nucleotides [102]. The concentration of IPR in mobile phase was critical to the retention of the nucleotides and to the

ESI-MS sensitivity. Smaller HPLC columns could provide stronger chromatographic resolving power and better detection sensitivity because ESI-MS is concentration dependent technique. Lower delivery of mobile-phase solutions containing the IPR when using smaller columns may also significantly reduce the source contamination. For example, the use of lower concentration of the IPR dimethylhexylamine (DMHA) in the capillary HPLC method resulted in a sensitivity increase of 5- to 10-folds for the positive ion ESI-MS analysis of the nucleotides. The common concentrations of DMHA used for the different size of column were summarized in Table 4. Although the use of smaller column could more or less reduce the needed amount of IPR and increasing the sensitivity, the high interference background produced by the IRP could not completely eliminated. Therefore, further investigation of LC–MS methods without the use of IPR is still of great interest.

In addition to the LC–MS methods, other mass spectrometry technique has been applied for the analysis of NRTIs and their metabolites. Van Kampen et al. developed a new method based on matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOFMS) for the analysis of AZT-TP and (deoxy)nucleotide-TP and applied for monitoring the NRTI treatment in HIV-1 infected children and adults [107]. Four different matrices were compared for sensitivity and reproducibility of AZT-TP detection and anthranilic acid mixed with nicotinic acid was found the most suitable. Solutions of AZT-TP, ATP, and dGTP were detected up to 0.5 fmol per sample. Furthermore, intracellular AZT-TP, ATP and dGTP were detected in PBMCs. AZT-TP, ATP and dGTP yielded identical mass spectra. It was reported that MALDI-TOF post-source decay analysis could be used for discrimination between these compounds [107]. The analysis of NRTIs using HPLC with MS/(MS) detection was summarized in Table 5.

4. Conclusions

The methods using LC-UV, LC–MS/MS, CE/CEC-UV and CE/CEC-MS/MS for analysis of NRTIs and their phosphorylated metabolites were reviewed. Among these methodologies, LC-UV is the most commonly used for the analysis of NRTIs but is often not sensitive enough to detect the low concentration of the phosphorylated metabolites in biological samples. CE/CEC-UV exhibits high analytical resolution but has the similar problem as that of LC-UV. CE-MS/MS is an efficient technique for the separation and quantitation of various NRTIs and metabolites, but the interface technique between CE and MS still needs to be improved. Furthermore, the high salt background produced from the electrolytes used in CE often results in the decrease of ESI-MS detection sensitivity. Currently, the more powerful and popular methods for the analysis of NRTIs and their phosphorylated metabolites is RP-LC coupled with MS/MS detection. However, the poor retention of highly polar NRTIs phosphorylated metabolites often occurs on the typical RP C_{18} column. Although the use of IPR could increase the retention time of NRTIs metabolites on C_{18} column, the problems of high ion signal background produced from the IPR and the significant ion suppression on ESI-MS analysis have resulted in dramatic decrease in sensitivity. Thus, continuous efforts are needed to develop more selective and sensitive methods to overcome the challenge in analytical chemistry for the trace analysis of the phosphorylated metabolites of NRTIs in biological samples.

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