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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

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ABSTRACT

Nucleoside reverse transcriptase inhibitors (NRTIs) are a key class of drugs for the treatment of HIV infection. NRTIs are intracellularly phosphorylated to their active triphosphate metabolites and compete with endogenous deoxynucleotides (dNTP) for substrate binding. It is therefore important to analyze the intracellular concentrations of these compounds to understand drug efficacy and toxicity. To that purpose an analytical platform was developed that is capable of analyzing 8 NRTIs, 12 phosphorylated NRTIs and 4 dNTPs in small numbers of peripheral blood mononuclear cells, i.e. 1×10^6 cells. The platform consists of two liquid chromatography–tandem mass spectrometry (LC–MS/MS) methods: a reversed-phase method for NRTIs using positive electrospray ionization (ESI) and an ion-pair LC–MS/MS method for the phosphorylated compounds using negative ESI. The methods use the same LC–MS system and column and changing from one method to the other only includes changing the mobile phase. The methods were partially validated, focussing on sensitivity, accuracy and precision. Successful transfer of the methods to ultra performance liquid chromatography (UPLC) led to a significant improvement of speed for the analysis of NRTIs and sensitivity for both NRTIs and phosphorylated NRTIs. The latter was demonstrated by the improved separation by UHPLC of dGTP vs. AZT-TP and ATP which made direct analysis of dGTP possible using the optimal MS/MS transition thereby significantly improving the detection limit of dGTP. Typically LLOQs observed for both the NRTIs and phosphorylated NRTIs were 1 nM, while the mean accuracy varied between 82 and 120% and inter- and intra-assay precision was generally <20%.

1. Introduction

Nucleoside reverse transcriptase inhibitors (NRTIs) remain the backbone of highly active antiretroviral therapy (HAART) for patients infected with HIV. NRTIs require intracellular phosphorylation to achieve their antiretroviral effects. As a result it is essential to monitor not only the NRTIs but also the active metabolites, i.e. NRTI-triphosphates (NRTI-TPs), intracellularly. Moreover the resulting active NRTI-TPs compete with endogeneous deoxynucleotide triphosphates (dNTPs) for substrate binding to reverse transcriptase. Thus to fully understand the drug efficacy and toxicity it is also necessary to follow the NRTI-TPs and dNTPs in peripheral blood mononuclear cells (PBMCs).

Several papers on the analysis of NRTIs, phosphorylated NRTI (NRTI-Ps) and dNTPs in PBMCs have been published [1–10] and more recently summarize [11,12]. The analysis of phosphorylated compounds with LC–MS is not straightforward due to their strong acidity and polarity. Analysis by reversed-phase methods is not possible and therefore other types of chromatography like anion exchange [7,10] and ion-pair [2–5,9] chromatography have been applied for these types of compounds in the past. However, most of these studies only focus on a few NRTIs or their phosphorylated metabolites and/or did not include dNTPs.

The objective of this work was to develop a comprehensive analytical platform in which all (commercially available) NRTIs, NRTI-Ps and dNTP could be analyzed. It was foreseen that this could not be achieved using a single LC–MS/MS method due to the strong deviating chemical properties of the NRTIs vs. phosphorylated compounds. The goal was therefore to develop methods on the same LC–MS system with the same column but only a

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different mobile phase composition and gradient so that the analysis of both classes of compounds could be achieved by 'simple' switching of the mobile phase. Furthermore the aim was to make the analytical platform capable of analyzing the relevant targets in small numbers of PBMCs, i.e. 1×10^6 cells. The reason for this is to make it possible to analyze samples of children with HIV from which only a limited volume of blood can be withdrawn.

In this paper we describe the development and of two LC–MS/MS methods capable of analyzing various NRTIs, NRTI-Ps and dNTP in PBMCs at clinically relevant concentrations. For NRTIs a straightforward reversed-phase LC–MS is used and for the phosphorylated compounds an ion-pair LC–MS/MS method, using hexylamine as ion-pair agent. The ion-pair LC–MS/MS method was derived from an ion-pair LC–MS method developed for microbial metabolomics applications [13]. The first step in developing an ion-pair LC–MS/MS method for phosphorylated NRTIs was published before [8]. Furthermore, we show for the first time the transfer of methods for NRTIs and NRTI-Ps to ultra performance liquid chromatography (UPLC).

2. Experimental

2.1. Chemicals and reagents

Reference standards of NRTIs (lamivudine (3TC), zidovudine (AZT), stavudine (d4T), abacavir (ABC), dideoxycytidine (ddC), didanosine (ddI), emtricitabine (FTC), tenofovir (TDF)) and phosphorylated NRTIs (NRTI-Ps); monophosphates (MP): AZT, TDF, 3TC, d4T, diphosphates (DP): AZT, d4T, TDF, triphosphates (TP): d4T, 3TC, AZT, CBV (carbovir), ddA (dideoxyadenosine), were obtained from Jena Bioscience (Jena, Germany) and Moravek (Brea, CA, USA). Reference standards of the endogenous deoxynucleotides (dNTPs; dATP, dTTP, dCTP, dGTP) and 2-chloroadenosine (Cl-A) were obtained from Sigma–Aldrich (Zwijndrecht, the Netherlands). $^{13}C^{15}N$-labelled internal standards of dGMP, dGTP, dTTP, dCTP were obtained from Cambridge Isotope Laboratories (Andover, MA, USA).

Hexylamine, acetic acid, ammonium acetate and ammonia were obtained from Sigma–Aldrich (Zwijndrecht, the Netherlands). Acetonitrile (HPLC-S grade) was purchased from Biosolve (Valkenswaard, the Netherlands). Milli-Q water (Milli-Q Advantage unit, Millipore, Amsterdam, the Netherlands) was used in all analyses. Heparin was obtained from Leo Pharma BV (Weesp, the Netherlands), and Lymphoprep from Axis-Shield PoC AS (Oslo, Norway).

2.2. LC–MS/MS system

For HPLC separation a LC-10AD LC pump (Shimadzu, Manchester, UK), a SIL-HTC autosampler (Shimadzu) and a CTO-10AS column oven (Shimadzu) was used. Liquid chromatography was performed on an XBridge C18 column (column dimensions 3.0 mm × 100 mm, particle size 3.5 μm; Waters, Etten-Leur, the Netherlands). For UPLC separation an ACQUITY UPLC™ system was used with an autosampler (Waters). The instrument was operated under Analyst v 1.4 software (Applied Biosystems) and ICOP 1.4 driversoftware (Waters). Liquid chromatography was performed on an Acquity BEH C18 column (column dimensions 2.1 mm × 50 mm, particle size 1.7 μm).

Both LC systems were coupled to an API4000 triple quadrupole mass spectrometer equipped with a Turbo Ionspray interface (Sciex, Applied Biosystems, Toronto, Canada). For data acquisition and data treatment Analist software v.1.4 (Applied Biosystems) was used.

2.3. LC–MS/MS conditions

For HPLC, the autosampler temperature was set at 15 °C and the column was maintained at 30 °C. For separation of NRTIs the following mobile phase gradient was applied with a constant flow of 0.4 mL/min: 99.5% A (0.1% formic acid in water) and 0.5% B (0.1% formic acid in acetonitrile) for 2 min followed by an increase towards 50% B in 4 min, followed by an increase towards 100% B in 1 min and finally 100% B for 2 min (total runtime 9 min). The injection volume was 25 μL. For separation of NRTI-Ps and dNTPs the following mobile phase gradient was applied with a constant flow of 0.4 mL/min: 100% A (5 mM hexylamine in water adjusted to pH 6.3 with acetic acid) to 50% B (5 mM hexylamine and 10 mM ammoniumacetate in 90% acetonitrile/10% water adjusted to pH 8.5 with ammonia) in 12 min followed by an increase towards 100% B in 1 min and finally 5 min at 100% B (total runtime 18 min). The injection volume was 50 μL.

For UPLC, the autosampler temperature was set at 10 °C. For separation of NRTIs the following mobile phase gradient was applied with a constant flow of 0.6 mL/min and a column temperature of 60 °C: 100% A (0.1% formic acid in 90% water/10% methanol) to 40% B (0.1% formic acid in methanol) in 2 min followed by an increase towards 100% B in 0.1 min and finally 0.4 min at 100% B (total runtime 2.5 min). The injection volume was 10 μL. For separation of NRTI-Ps and dNTPs the following mobile phase gradient was applied with a constant flow of 0.7 mL/min and a column temperature of 40 °C: 95% A (5 mM hexylamine in water adjusted to pH 6.3 with acetic acid) to 18.2% B (5 mM hexylamine and 10 mM ammoniumacetate in 90% acetonitrile/10% water adjusted to pH 8.5 with ammonia) in 14 min followed by an increase to 100% B in 0.1 min and finally 100% B for 1.4 min (total runtime 15.5 min). The injection volume was 15 μL.

For the analysis of NRTIs the triple quadrupole mass spectrometer was operated in the positive ionization mode with a Turbo Ion spray temperature of 500 °C and a nebuliser potential of 4500 V. Nebulizer, turbo, and curtain gas used nitrogen delivered at 40, 40 and 30 psi, respectively. NRTIs were detected in the multiple reaction monitoring (MRM) mode (see Table 1).

NRTI-Ps and dNTPs were analyzed in the negative ionization mode with a Turbo Ion spray temperature of 450 °C (HPLC) or 550 °C (UPLC) and a nebulizer potential of −4500 V (HPLC) or −4200 V (UPLC). Nebulizer, turbo, and curtain gas used nitrogen delivered at 40, 40 and 30 psi for RPLC and 40, 40 and 20 psi for UPLC, respectively. NRTI-Ps and dNTPs were detected in the multiple reaction monitoring (MRM) mode (see Table 2).

A schematic overview of the various LC methods applied is shown in Fig. 1.

2.4. Preparation of (internal) standard solutions

Stock solutions containing 2 mM of the individual NRTIs, NRTI-Ps, dNTPs and internal standards (3.5 mM for Cl-A) were made in methanol/water (1:1, v/v) either by dissolving of solid material or dilution of commercially obtained solutions. Stock solutions were stored at −80 °C. Calibration standards covering the range of 0.1–100 nM for NRTIs and NRTI-Ps, 10–200 nM for dTTP, dCTP, dATP and 25–200 nM for dGTP were prepared by dilution in methanol/water (1:1, v/v) to the appropriate concentrations.

2.5. PBMC extraction

PBMCs were isolated from buffy coat (Sanquin, Rotterdam, the Netherlands) as described earlier [14]. PBMC pellets (10×10^6 cells) were suspended in 100 μL PBS and stored at −80 °C. Extraction of PBMCs was carried out by adding 100 μL methanol/water (1:1, v/v) to 1×10^6 cells followed by extraction overnight at 5 °C. Prior

Table 1
Optimized mass spectrometric parameters for the quantification of NRTIs and internal standard (IS) in PBMCs in positive electrospray ionization (ESI) using multiple reaction monitoring (MRM) mode.

Analyte	Parent ion (*m*/*z*)	Fragment ion (*m*/*z*)	Declustering potential (DP) (V)	Collision energy (CE) (V)	Collision cell energy (CXP) (V)
AZT	268.0	127.0	36	17	8
3TC	230.0	112.0	36	19	8
d4T	225.1	127.0	26	15	10
ABC	287.1	191.2	61	29	16
ddI	237.0	137.0	31	11	12
FTC	248.0	130.0	36	17	12
ddC	212.0	112.0	26	13	8
Cl-A (IS)	286.0	170.1	61	27	30

Table 2
Optimized mass spectrometric parameters for the quantification of NRTI-Ps, dNTPs and internal standards (IS) in PBMCs in negative electrospray ionization (ESI) using MRM mode.

Analyte	Parent ion (*m*/*z*)	Fragment ion (*m*/*z*)	Declustering potential (DP) (V)	Collision energy (CE) (V)	Collision cell energy (CXP) (V)
TDF	286.1	134.2	−80	−34	−7
TDF-MP	366.0	79.0	−50	−50	−1
TDF-DP	446.0	158.8	−60	−36	−9
AZT-MP	346.0	177.1	−70	−30	−5
AZT-DP	426.2	176.7	−55	−28	−1
AZT-TP	506.2	159.1	−85	−36	−1
d4T-MP	303.1	176.9	−60	−18	−3
d4T-DP	383.0	256.8	−55	−24	−7
d4T-TP	463.0	158.9	−90	−30	−9
3TC-MP	308.0	78.9	−50	−52	−15
3TC-TP	468.0	158.8	−80	−28	−5
ddA-TP	474.1	159.0	−70	−30	−1
CBV-TP	485.9	158.9	−75	−32	−3
$^{13}C^{15}N$-dGMP (IS)	361.0	78.9	−75	−30	−11
$^{13}C^{15}N$-dGTP (IS)	521.0	158.7	−75	−30	−11
$^{13}C^{15}N$-dCTP (IS)	478.0	158.5	−55	−46	−31
$^{13}C^{15}N$-dTTP (IS)	493.0	158.7	−80	−38	−3
dTTP	481.0	159.0	−90	−36	−3
dATP	490.0	158.7	−95	−48	−1
dCTP	466.0	158.9	−90	−40	−11
dGTP	506.0	256.8	−80	−40	−19

to analyses the samples were diluted 1:1 with water (containing the internal standards and analytes). The final concentration of the internal standards was 20 nM (17.5 nM for Cl-A). Isolation and inactivation of samples from HIV infected patients was carried out as described by van Kampen et al. [14].

2.6. Analytical performance

The HPLC–MS/MS methods, both for NRTIs and NRTI-Ps/dNTPs, were partially validated for all commercially available analytes, including the endogenous deoxynucleotides, focussing on sensitivity, precision and accuracy. The validation procedure consisted of analyzing three validation batches, including calibration curves and quality control (QC) samples, on three consecutive days. Each batch included blanks, two six-point calibration curves and zero samples prepared in PBMC and quality controls at three different levels in five-fold. Peak identification and quantification was performed using Analyst software v.1.4 (Applied Biosystems). Data processing was performed using in-house programmed calculation Excel sheets for regression analysis, calculation of accuracy, and inter- and intra-batch variation. Validation of the UPLC–MS/MS methods was more limited, i.e. each batch included quality controls at three different levels in duplicate and for only a selection of analytes. For each batch of validation experiments a different batch of blank PBMCs was used.

2.7. Linearity/lower limit of quantification (LLOQ)

For each analyte, a calibration curve was constructed using a zero sample and calibration standards at six concentrations in the

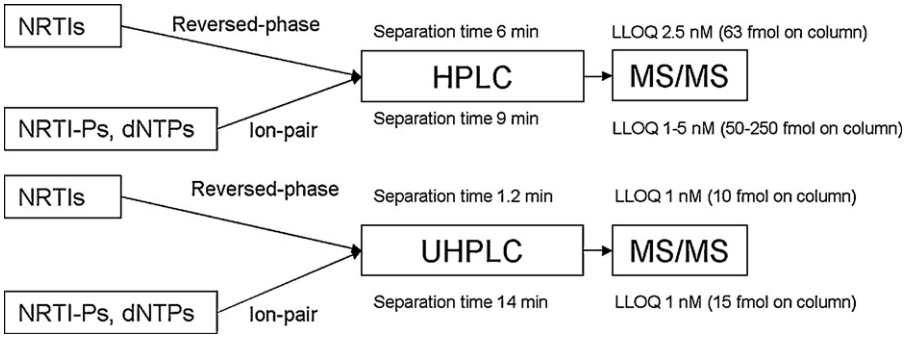


Fig. 1. Schematic overview and typical characteristics of the various LC–MS/MS methods for NRTIs, NRTI-Ps and dNTPs in PBMCs developed and validated.

range 0.1–100 nM for NRTIs and NRTI-Ps, 10–200 nM for dTTP, dCTP, dATP and 25–200 nM for dGTP. Quantification was carried out using linear regression on the response ratios (peak area analyte/peak area internal standard) from the calibration curve as a function of the corresponding concentration. For each analyte, the best weighing factor was determined according to the evolution of variance with respect to the concentration. Deviation from the actual concentration within 15% for all calibration standards was found acceptable, except 20% for the LLOQ. The LLOQ was taken as the lowest calibration standard to accurately and precisely quantify within this 20% and with a response at least 5 times higher compared to a blank response.

2.8. Accuracy and precision

Accuracy and precision of the methods were determined for NRTIs and NRTI-Ps using QC samples spiked at three concentrations in the range 1–40 nM, depending on the LLOQ. For dTTP, dCTP and dATP the QC levels were 17, 40 and 75 nM and for dGTP 200 and 470 nM. QC samples were analyzed in five-fold in three batches. For accuracy, the mean value for the concentration should be within 15% of the actual value except at LLOQ (<20%). Inter- and intra-batch precision was expressed as the variation coefficient (CV) within and between the batches, respectively. Acceptance criteria were 15% for precision, except for the LLOQs (<20%).

3. Results and discussion

3.1. HPLC–MS/MS characteristics

3.1.1. NRTIs

A reversed-phase LC method using a C18 column and a mobile phase gradient from 0.1% formic acid in water to 0.1% formic acid to acetonitrile was sufficient to separate the different NRTIs within ~6 min. An example of an LC–MS/MS chromatogram of NRTIs spiked into PBMCs at LLOQ level is shown in Fig. 2. The only exception was tenofovir (TDF), this compound is a so-called nucleotide reverse transcriptase inhibitor (NtRTI), i.e. the parent drug contains already a phosphate group. It was therefore decided to incorporate TDF into the method for phosphorylated compounds.

Detection of NRTIs was carried out using electrospray ionization (ESI) in the positive ionization mode. The sensitivity in the positive ionization mode was for all NRTIs better or equal to that in the negative ionization mode in contrary to Compain et al. [5] who found better sensitivity for some NRTIs in the negative ionization mode. However these authors did not use any additives in their mobile phases while in our case the aqueous mobile phase contained 0.1% formic acid (pH 2–3) which is likely to improve the ionization of NRTIs in positive ionization mode thereby increasing the sensitivity of these compounds in the positive ionization mode as observed in this study.

3.1.2. NRTI-Ps and dNTPs

Ion-pair chromatography was used to separate the different phosphorylated compounds. Other possibilities to deal with these highly polar compounds are anion exchange chromatography [10], capillary electrophoresis [15,16], hydrophilic interaction chromatography (HILIC) [17] or dephosphorylation prior to analysis [1,7]. Despite possible disadvantages of using ion-pair agents in combination with MS with respect to for instance increased ion-suppression we selected ion-pair chromatography to analyze NRTI-Ps and dNTPs based on earlier experience [8,13]. An advantage of ion-pair chromatography is that standard C18 columns can be used in contrary to anion exchange chromatography m HILIC, thereby profiting from the extreme robustness and reproducibility of current generation of C18 columns. Different types of ion-pair agents can be used although alkylamines are most commonly used in combination with LC–MS [2–4,6,8,9]. Hexylamine (HA) was used as ion-pair agents in this study based on good experiences in the past [8,13]. However other alkylamines, like dimethylhexylamine, would probably give very similar results. Finally, an ion-pair LC method was set-up in which the various NRTI-Ps and dNTPs could be separated within 12 min using a mobile phase gradient from 5 mM HA in water (pH 6.3) to 5 mM HA in 90% acetonitrile/10% water (pH 8.5). An example of an ion-pair LC–MS/MS chromatogram for a selection of NRTI-Ps at LLOQ level and dNTPs in PBMCs is shown in Fig. 3. Despite the high pH of the mobile phase, i.e. pH 8.5, the column used did not show any significant deterioration during method development and validation.

Detection was carried out with electrospray in the negative ionization mode giving better or equal sensitivity compared to the positive mode. In addition, negative ionization mode gave mainly $[M-H]^-$-ions and no significant adduct formation was observed. Various MS/MS transitions were assessed for each compound. While the objective was to detect the compounds at concentrations as low as possible, only the most sensitive MS/MS transition was used (see Table 2). All compounds could be separated based on retention time and/or MS/MS transition. This includes also AZT-TP which has the same mass (m/z 507) and main MS/MS transition (m/z 506 → 159) as ATP and dGTP but in this method AZT-TP had a significant different retention time (see Fig. 3). Only dGTP gave problems, as expected, while it has the same mass, elemental composition, main MS/MS transitions and retention time as ATP (see Table 2, Figs. 3 and 4). Furthermore, the concentration of ATP in PBMCs is generally orders of magnitude higher than that of dGTP. Most papers seem to have ignored this problem by focusing on NRTI/dNTP combinations that did not include dGTP. An exception is the work by Hennere et al. [4] who describe a periodate oxidation procedure to degrade NTPs, like ATP, and leaving the dNTPs intact. Our aim was to keep the methods as simple as possible without including additional sample preparation. Hence a MS/MS transition was chosen that was unique for dGTP and would thus not be disturbed by ATP, i.e. m/z 506 → 257 (see Fig. 4). A drawback is the fact that this is a minor MS/MS transition and thus will probably not show the best sensitivity for dGTP.

3.2. Analytical performance

Partial validation of the ion-pair LC–MS/MS method was only carried out for the compounds listed in Table 3. More compounds could be detected, especially other mono- and di-phosphates of NRTIs, but these compounds were either not commercially available or were not considered pure enough to be used for validation.

Validation of endogenous dNTPs was also carried out but due to the basal levels of these compounds in PBMCs the LLOQ cannot be accurately determined and QC concentrations can only be used when significantly above the basal level.

Stability was not assessed in this study due to the large number of analytes included in the methods and the fact that the main interest was on method development with sensitivity as the main validation criteria.

3.2.1. NRTIs measured in a cellular matrix

The data on the LLOQ (expressed in nM, fmol and pmol/1×10^6 cells) and linearity for NRTIs are shown in Table 3. Calibration curves (0.1–100 nM) were constructed using linear regression of the NRTI/Cl-A ratio. It can be seen that the RPLC–MS/MS method shows good linearity with a correlation coefficient (r^2) better than 0.990 for all analytes. The LLOQ was 2.5 nM, i.e. 0.5 pmol/10^6 cells, for all NRTIs. This LLOQ is higher than reported by Compain et al.

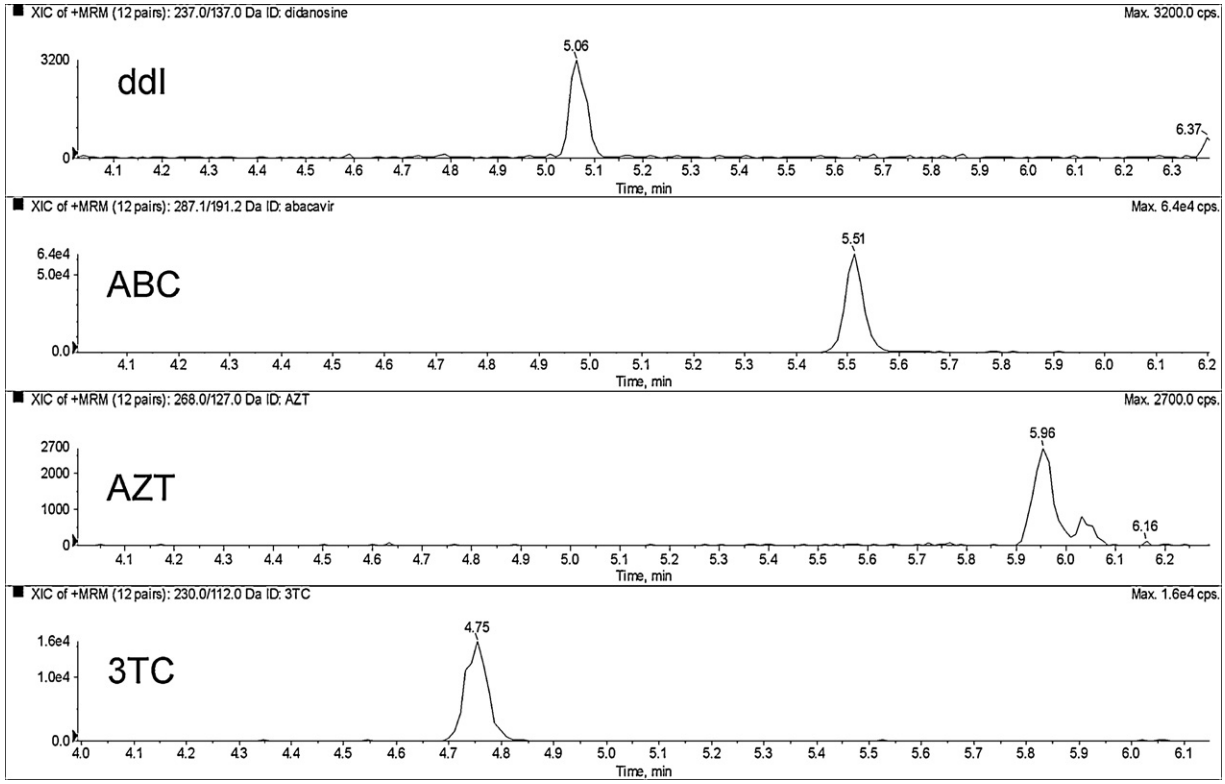


Fig. 2. RPLC–MS/MS chromatogram of the NRTIs 3TC, AZT, ABC and ddI spiked to a PBMC extract at LLOQ level (2.5 nM).

[5] who reported a LLOQ of 25 pg/cell pellet of $(7–10) \times 10^6$ cells which corresponds roughly to ~10 fmol/10^6 cells. However, these authors used 7–10 times higher PBMCs concentrations. Taking into account their sample work-up the LLOQs observed in this study are similar.

Table 4 summarizes the results obtained for the accuracy and inter- and intra-assay precision of NRTIs in PBMCs showing that the mean accuracy was between 94 and 116% for all NRTIs at all three QC concentrations. For all NRTIs both the intra- and inter-assay precision was lower than <20% at LLOQ level and ≤15% for the other QC concentration levels, except for 3TC at 10 nM which showed a CV of 16%.

Matrix effects were not extensively studied. For each batch of validation experiments for determining the inter-batch variation a different batch of blank PBMC matrix was used. No interferences were observed from blank PBMC sample extracts at the retention

Table 3
Linearity and LLOQ for NRTIs, NRTI-Ps and dNTPs.

	Linearity (R^2)	LLOQ		
		nM	fmol (on column)	pmol/10^6 cells
ddI	0.993	2.5	63	0.5
FTC	0.995	2.5	63	0.5
AZT	0.996	2.5	63	0.5
ddC	0.992	2.5	63	0.5
ABC	0.996	2.5	63	0.5
3TC	0.996	2.5	63	0.5
d4T	0.992	2.5	63	0.5
TDF	0.997	5	250	1.0
ddA-TP	0.995	1	50	0.2
d4T-MP	0.996	5	250	1.0
d4T-DP	0.993	5	250	1.0
d4T-TP	0.995	1	50	0.2
AZT-MP	0.998	5	250	1.0
AZT-DP	0.997	5	250	1.0
AZT-TP	0.998	1	50	0.2
CBV-TP	0.999	1	50	0.2
TDF-MP	0.995	5	250	1.0
TDF-DP	0.995	1	50	0.2
3TC-MP	0.997	5	250	1.0
3TC-TP	0.994	1	50	0.2
dTTP	0.997	17	850	3.4
dGTP	0.992	200	10,000	40
dATP	0.993	17	850	3.4
dCTP	0.993	17	850	3.4

Table 4
Accuracy and inter- and intra-assay precision for NRTIs in PBMCs.

Compound	QC (nM)	Accuracy (%, $n = 15$)	Intra-batch variation (%CV, $n = 15$)	Inter-batch variation (%CV, $n = 3$)
ddI	2.5	99	19	19
	10	100	8	13
	40	103	5	12
FTC	2.5	106	9	10
	10	110	6	6
	40	95	5	13
AZT	2.5	101	12	12
	10	109	6	10
	40	99	6	7
ddC	2.5	114	13	13
	10	108	11	14
	40	94	7	15
ABC	2.5	102	11	14
	10	113	6	13
	40	102	5	6
3TC	2.5	104	13	17
	10	103	7	16
	40	98	6	14
d4T	2.5	109	16	19
	10	116	10	12
	40	108	15	15
TDF	5	102	9	9
	12.5	102	7	7
	40	104	4	4

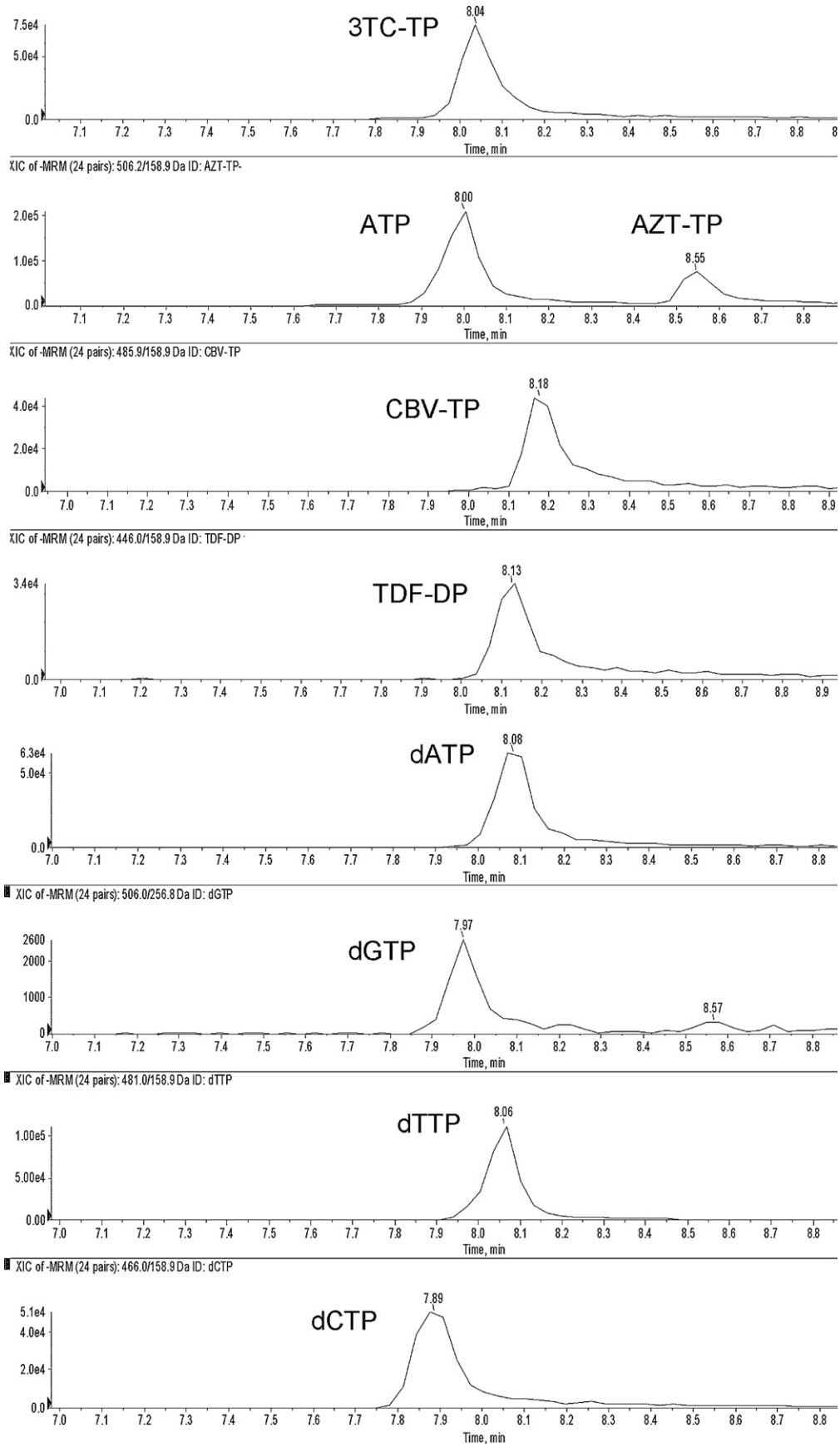
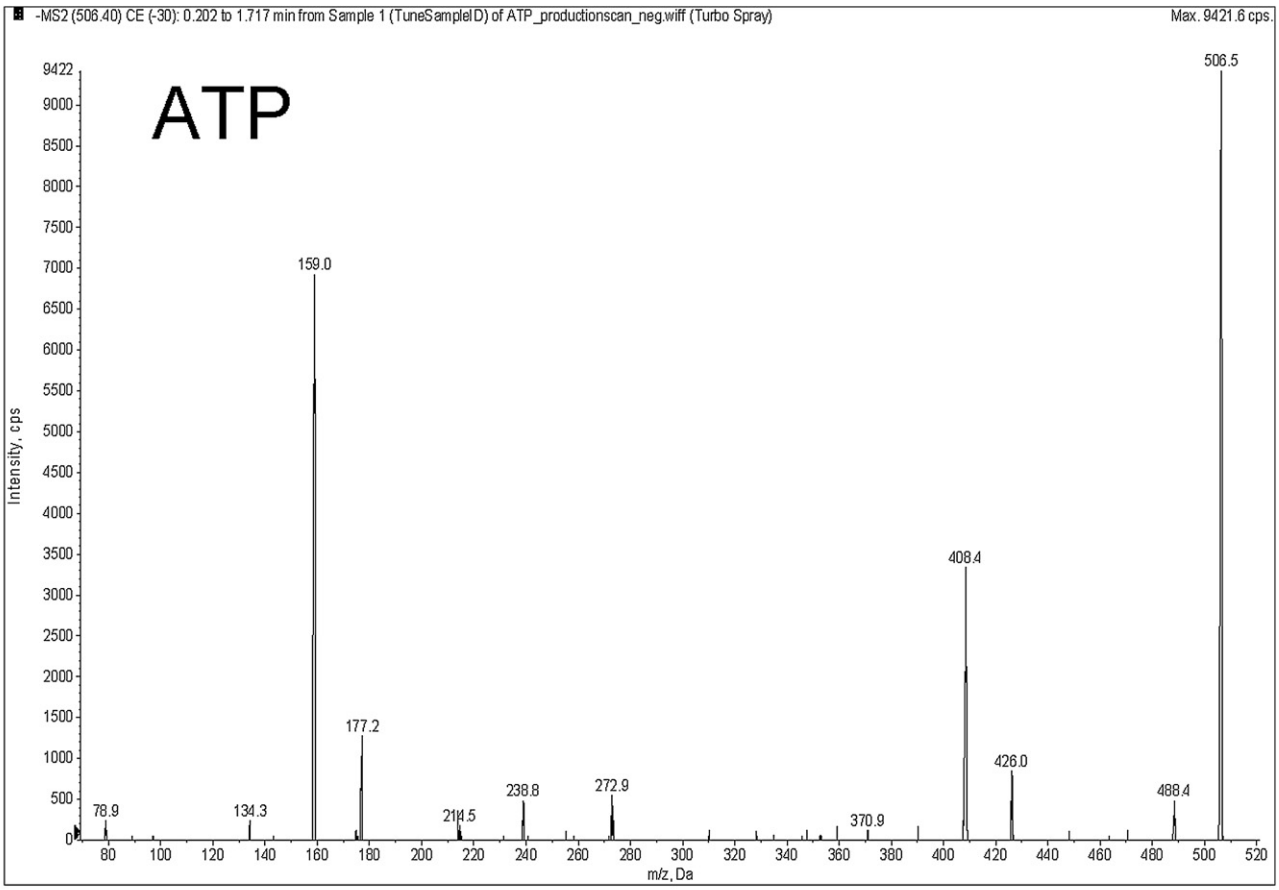


Fig. 3. Ion-pair HPLC–MS/MS chromatograms of the NRTI-TPs 3TC-TP, AZT-TP, CBV-TP, TDF-DP at LLOQ level (1 nM for 3TC-TP and TDF-DP and 5 nM for AZT-TP and CBV-TP) and the dNTPs dATP, dTTP, dGTP and dCTP spiked at LLOQ level to a PBMC extract (17 nM for dATP, dTTP and dCTP and 200 nM for dGTP).



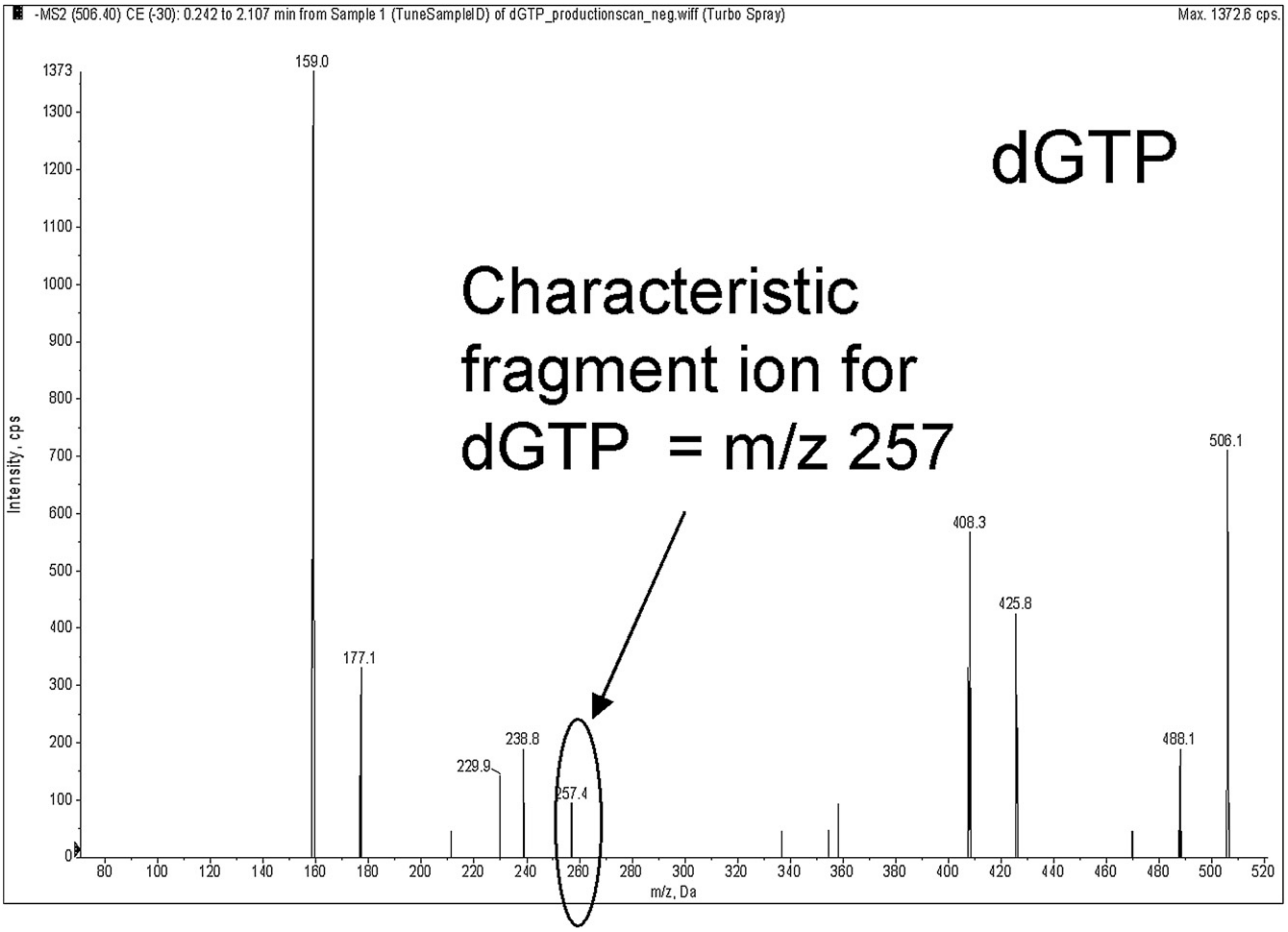


Fig. 4. Product ion scans of (A) ATP and (B) dGTP showing the great similarity but also the specific product ion for dGTP.

times of the NRTIs or internal standard. Analysis of extracts containing different concentrations of PBMCs ((10–50) × 10^6 cells/mL) but identical concentrations of NRTIs did not reveal significant matrix effects.

It was concluded that the RPLC–MS/MS method for NRTIs in PBMCs showed overall good analytical performance.

3.2.2. NRTI-Ps and dNTPs measured in a cellular matrix

The ion-pair LC–MS/MS method shows good linearity in the calibration range of 1–100 nm with correlation coefficients higher than 0.990 (Table 3). The LLOQ for NRTI-Ps varies between 1 and 5 nM, i.e. 0.2–1 pmol/10^6 cells, with the triphosphates showing generally better sensitivity than the mono- and di-phosphates. The LLOQs expressed in nM or fmol are equal or in some cases better than reported in literature [2,6,9,10]. Most studies use significantly higher concentrations of cells, i.e. (7–15) × 10^6 PBMCs in ~150 μl [2–4,6], than in this study, i.e. 1 × 10^6 PBMCs in 200 μL, resulting in seemingly lower LLOQs when expressed as fmol/1 × 10^6 cells.

The mean accuracy varies between 82 and 120% for all NRTI-Ps and dNTPs at all QC levels (Table 5). The intra-assay precision was <20% at LLOQ level for all compounds tested. The same holds true for the inter-assay precision at LLOQ level although for two compounds a slightly higher CV was observed (d4T-TP (CV 21%) and TDF-DP (CV 22%)). The intra- and inter-assay precision at the other QC concentrations was ≤15% for all compounds.

Interferences, i.e. endogenous (deoxy)nucleotides, were observed in blank PBMC sample extracts at the retention times of some of the NRTI-Ps. Especially ATP co-eluted with some of the dNTPs and NRTI-Ps but this only resulted in a high detection limit of dGTP. The other compounds did not seem to be influenced. In combination with the differences in retention time and MS/MS transition between the different target compounds, it was concluded that the specificity and selectivity of the method was satisfactory. The same holds true for the matrix effect. For each batch of validation experiments a different batch of blank PBMC matrix was used. No interferences were observed for the different target compounds. Different amounts of PBMCs ((10–50) × 10^6 cells/mL) resulted in different amounts of dNTPs and endogenous nucleotides like ATP, but had no significant influence on the analytical performance of the NRTI-Ps.

It was concluded that the ion-pair LC–MS/MS method for NRTI-Ps and dNTPs in PBMCs showed overall satisfactory analytical performance.

Table 5
Accuracy and inter- and intra-assay precision for NRTI-Ps and dNTPs in PBMCs.

Compound	QC (nM)	Accuracy (%, n = 15)	Intra-batch variation (%CV, n = 15)	Inter-batch variation (%CV, n = 3)
ddATP-TP	1	116	9	17
	5	111	10	11
	12.5	104	7	10
D4T-MP	5	99	10	10
	12.5	102	6	6
	40	103	5	6
D4T-DP	5	90	9	15
	12.5	93	8	11
	40	96	8	12
D4T-TP	1	95	16	21
	5	95	7	13
	12.5	99	8	9
AZT-MP	5	97	18	18
	12.5	112	9	11
	40	111	9	11
AZT-DP	5	116	17	19
	12.5	106	13	13
	40	114	7	13
AZT-TP	5	109	12	12
	12.5	107	7	8
	40	109	9	10
CBV-TP	5	87	5	6
	12.5	107	9	11
	40	106	6	7
TDF-MP	5	107	14	19
	12.5	111	9	9
	40	112	6	10
TDF-DP	1	83	18	22
	5	95	10	12
	12.5	100	8	9
3TC-MP	5	96	13	17
	12.5	91	11	12
	40	91	6	7
3TC-TP	1	82	17	17
	5	101	13	15
	12.5	100	7	14
dTTP	17	106	14	14
	40	106	10	13
	75	109	7	10
dGTP	200	97	5	5
	370	101	8	10
dATP	17	108	12	12
	40	107	6	6
	75	106	7	7
dCTP	17	120	10	10
	40	119	6	11
	75	111	8	8

3.3. Method transfer from HPLC to UPLC

Columns packed with sub-2-μm particles in ultra performance conditions (UPLC) are replacing HPLC methods more and more, especially in pharmaceutical applications like bioanalysis, drug metabolism and metabolomics [18]. The big advantage of UPLC is the ability to transfer existing HPLC methods directly and UPLC systems can be coupled easily to different types of MS systems. Usually UPLC methods should give higher throughput and better sensitivity and separation efficiency. It was therefore tried to transfer both the methods for NRTIs and phosphorylated compounds to UPLC coupled to the same mass spectrometer.

3.3.1. NRTIs

The main differences between the RPLC and UPLC methods were a different column with small particles size, a higher flow rate and a higher column temperature. This resulted in separation of NRTIs within only two minutes (see Fig. 5). The UPLC–MS/MS method for NRTIs was partially validated for a selection of NRTIs by analyzing QCs at two concentrations in triplicate on 2 days (Table 6). The LLOQ was 1 nM for all NRTIs which was lower than the LLOQ of 2.5 nM observed for the RPLC–MS/MS. Taking into account the lower injection volume used for UPLC–MS/MS, i.e. 10 μL instead of 25 μL, the difference in LLOQ expressed in fmol on column is even more significant, i.e. 10 fmol vs. 63 fmol. The accuracy ranged between 85 and 118% for both QC concentrations, while the inter-batch precision was <15% at both QC concentration. From these results it can be concluded that transfer of the method for NRTIs in PBMCs to UPLC significantly increases the speed and sensitivity as summarized in Fig. 1.

3.3.2. NRTI-Ps and dNTPs

The transfer of the ion-pair LC–MS/MS method for NRTI-Ps and dNTPs to UPLC was less straightforward. Generally, a column with small particles in combination with a high flow rate and high column temperature should result in the optimal chromatographic conditions for UPLC with respect to speed and separation. However, for the ion-pair LC–MS/MS method the pH of the mobile phase is very critical to obtain good separation and peak shapes [13]. The high pressure in UPLC due to the small particles in combination with a high flow rate did not have a significant influence on pH and a

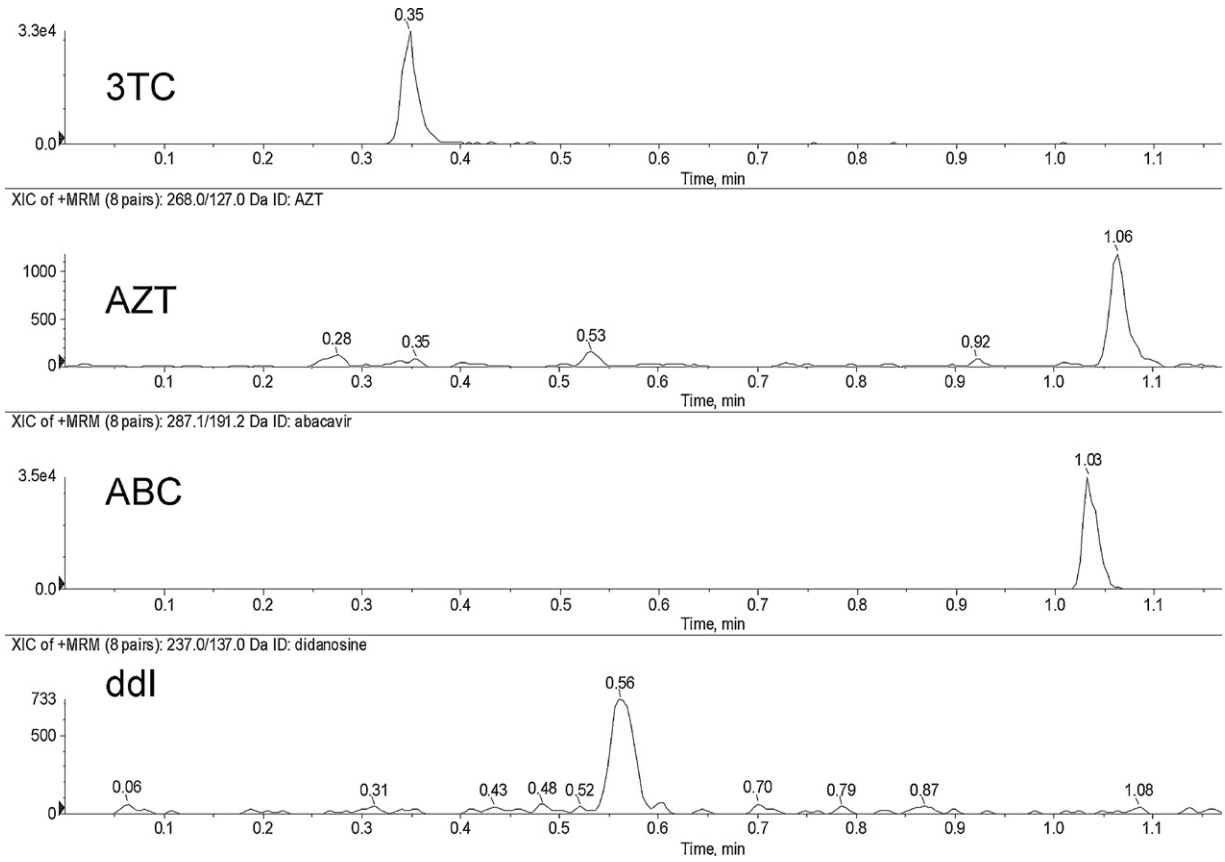


Fig. 5. UPLC–MS/MS chromatogram of the NRTIs 3TC, AZT, ABC and ddI spiked to a PBMC extract at LLOQ level (1 nM).

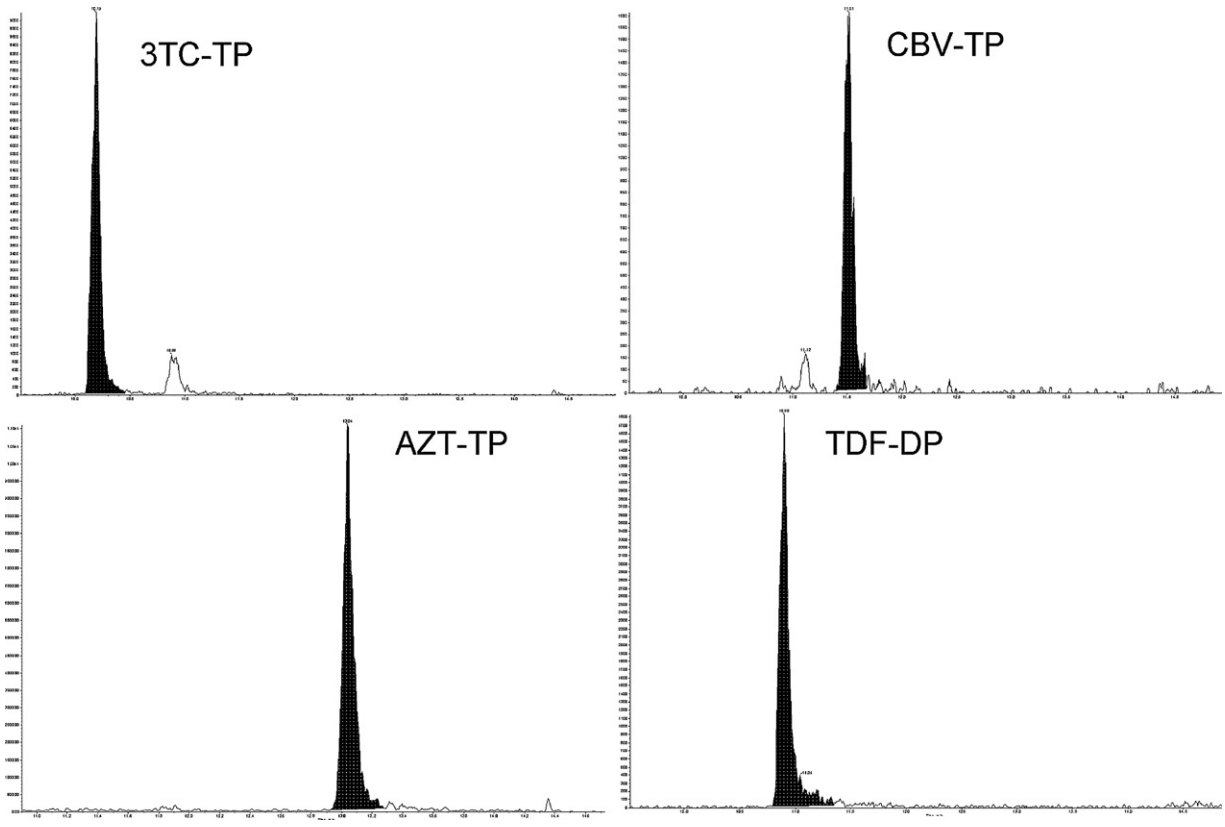


Fig. 6. Ion-pair UPLC–MS/MS chromatogram of NRTI-Ps 3TC-TP, TDF-DP, CBV-TP and AZT-TP spiked to a PBMC extract at LLOQ level (1 nM).

negative effect on the separation and peak shape. However, changing the column temperature to 60 °C, had an enormous influence on the pH due to the volatility of the ion-pair agent and resulted in bad separation and peak shapes. It was therefore decided to maintain the column temperature at 40 °C and investigate whether these conditions lead to improvement of the separation of NRTI-Ps and dNTPs. Optimization of the mobile phase containing the ion-pair agent with respect to high column temperatures and optimal pH is still recommended for future experiments.

It can be clearly seen from Figs. 3 and 6 that UPLC does not result in a significant increase of the speed of analysis, i.e. 15.5 runtime for UPLC vs. 18 min for HPLC, but a significant improvement of the separation is clearly visible. NRTI-TPs like 3TC-TP, CBV-TP and TDF-DP showed significant overlap in Fig. 3 but are baseline separated from each other with UPLC as can be seen in Fig. 6. This improved separation is further demonstrated in Fig. 7 which shows the m/z 506 → 159 MS/MS transition for the ion-pair UPLC–MS/MS method obtained for two QC at different concentrations. With the ion-pair LC–MS/MS method AZT-TP could already be separated from the other endogenous nucleotides but dGTP and ATP showed complete overlap resulting in a high detection limit of dGTP (Table 3). With UPLC AZT-TP is further separated from dGTP and ATP, i.e. 1 min separation instead of 0.5 min, but more important dGTP is largely separated from ATP. As a result the m/z 506 → 159 MS/MS transition can now be used for quantifying dGTP which leads to lowering of the LLOQ from 200 nM to 20 nM. It can also be seen in Fig. 7 that the UPLC gives generally sharper peaks and results in lower LLOQs for many of the NRTI-Ps, i.e. 1 nM instead of 5 nM (Table 6). Again, taking into account the significant lower injection volume used for UPLC, i.e. 15 μL instead of 50 μL, the LLOQ expressed as

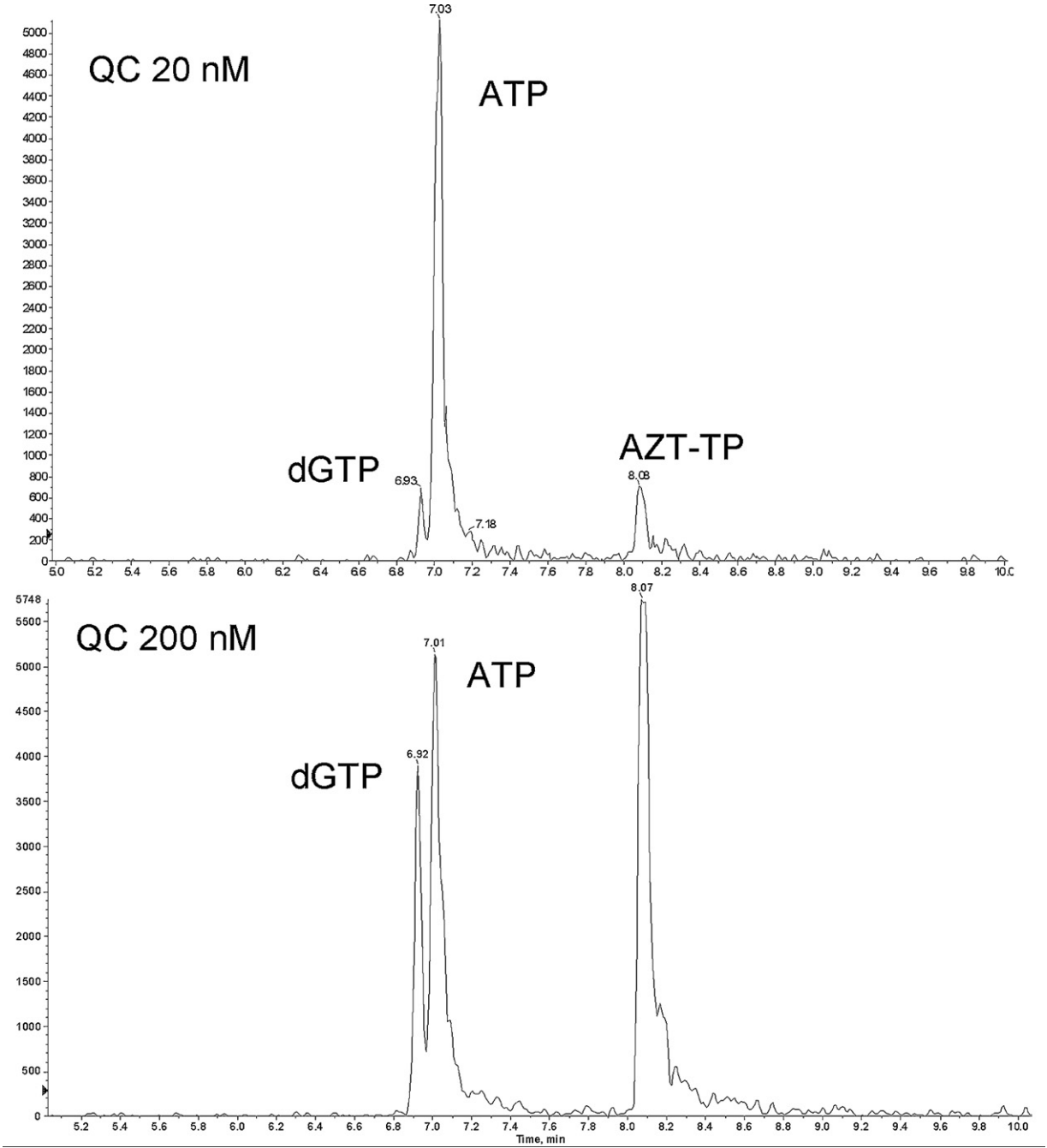


Fig. 7. Ion-pair UPLC–MS/MS chromatogram (m/z 506 → 159) of two QC samples showing the separation of dGTP and ATP.

Table 6
Validation results for NRTIs and NRTI-Ps in PBMCs analyzed by (ion-pair) UPLC–MS/MS.

Compound	QC (nM)	Accuracy (%, $n=6$)	Inter-batch variation (%CV, $n=3$)
AZT	1	98	11
	5	99	14
ABC	1	104	4
	5	97	9
3TC	1	85	8
	5	103	4
TDF	1	118	14
	5	110	6
3TC-MP	1	87	12
	5	97	13
3TC-TP	1	94	15
	5	91	11
AZT-MP	1	102	10
	5	98	11
AZT-TP	1	97	16
	5	96	8
TDF-MP	1	89	18
	5	95	9
TDF-DP	1	97	12
	5	95	9

fmol on column is significantly lower for all compounds using UPLC, i.e. 15 fmol, than HPLC, i.e. ≥50 fmol. Partial validation of the ion-pair UPLC–MS/MS method showed good analytical performance, i.e. accuracy between 87 and 102% and inter-batch precision ≤18% at LLOQ level and ≤13% at a QC concentration of 5 nM (see Table 6). It can be concluded that the ion pair LC–MS method for NRTI-Ps and dNTPs using UPLC does not increase the speed of the analysis but does lead to an improvement of the separation and increase of sensitivity. Further improvement might be possible when the column temperature is increased without disturbing the fragile balance of pH of the mobile phases which is critical for good separation and peak shapes [12].

3.4. Human PBMC samples

Finally, a set of human PBMC samples from patients infected with HIV were analyzed by the methods described here earlier to test whether NRTIs and NRTI-Ps can be detected in clinical samples. The set of samples were obtained from different patients who all obtained 3TC. The samples all contained 1×10^6 cells and where either processed directly after the blood sample was taken in 200 μL water/MeOH (1:1, v/v) or after leaving the blood for 4 h at the bench top. All samples were analyzed by RPLC–MS/MS and ion-pair LC–MS/MS and in all samples 3TC, 3TC-MP and 3TC-TP could be quantified (see Table 7). 3TC-DP was not included while quantification was not possible because no appropriate reference material was available. It can be seen from the results in Table 7 that storage of blood for 4 h leads to significant decrease in 3TC and increase of 3TC-TP concentrations in PBMC samples for all four patients showing that intracellular phosphorylation clearly proceeds during storage at room temperature and immediate isolation and subsequent storage of PBMCs is essential to obtain reliable results. Future work will focus on analyzing larger sample sets of clinical samples containing various NRTIs.

Table 7
Concentrations of NRTIs and NRTI-Ps in PBMCs that were processed directly after blood sampling or processed after 4 h for four HIV-infected subjects receiving 3TC.

pmol/10^6 cells	3TC	3TC-MP	3TC-TP
Pat. 1 $t=0$	1.74	3.28	3.04
Pat. 1 $t=4$	0.96	3.60	6.90
Pat. 2 $t=0$	2.41	3.04	2.10
Pat. 2 $t=4$	0.72	2.22	4.66
Pat. 3 $t=0$	1.98	1.34	1.74
Pat. 3 $t=4$	0.55	0.78	4.26
Pat. 4 $t=0$	1.84	1.04	3.78
Pat. 4 $t=4$	0.88	2.34	7.28

4. Conclusions

A RPLC–MS/MS method was developed for detecting multiple NRTIs in PBMCs and an ion-pair LC–MS/MS method for detecting various phosphorylated NRTIs and dNTPs in PBMCs. These methods were developed as such that it was easy switching between methods by basically only changing the mobile phases. Starting from these methods, for the first time UPLC–MS/MS methods were developed that clearly resulted in improvement of speed, sensitivity and separation. Furthermore, the methods were specifically set-up for samples containing only 1×10^6 cells per 200 μL solvent, thereby making the methods especially interesting for analysis of samples from HIV-infected children. Taking into account these PBMC concentrations, the typical LLOQ observed for both the NRTIs and phosphorylated NRTIs are 1 nM with the improved UPLC–MS/MS methods.

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