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Simultaneous quantification of 9-(β-D-1,3-dioxolan-4-yl)guanine, Amdoxovir and Zidovudine in human plasma by liquid chromatography–tandem mass spectrometric assay

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ABSTRACT

A sensitive method was developed and validated for simultaneous measurement of an investigational antiviral nucleoside, Amdoxovir (DAPD), its deaminated metabolite 9-(β-D-1,3-dioxolan-4-yl)guanine (DXG), and Zidovudine (ZDV) in human plasma. This method employed high-performance liquid chromatography–tandem mass spectrometry with electrospray ionization. DXG and DAPD separation with sufficient resolution was necessary since they differ in only one mass to charge ratio, which increases the risk of overlapping MS/MS signals. However, the new method was observed to have functional sensitivity and specificity without interference. Samples were purified by ultrafiltration after protein precipitation with methanol. The total run time was 29 min. A linear calibration range from 2 to 3000 ng mL^{-1} and 2 to 5000 ng mL^{-1} was achieved for DAPD and DXG, and ZDV, respectively. Precisions and accuracies were both ±15% (±20% for the lower limit of quantification) and recoveries were higher than 90%. Matrix effects/ion suppressions were also investigated. The analytes were chemically stable under all relevant conditions and the method was successfully applied for the analysis of plasma samples from HIV-infected persons treated with combinations of DAPD and ZDV.

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1. Introduction

Nucleoside reverse transcriptase inhibitors (NRTI) currently constitute the backbone of combinatorial regimens for the treatment of HIV infections and are usually combined with protease (PI), non-nucleosides reverse transcriptase (NNRTI), integrase, or entry/fusion inhibitors. The dosing of antiretroviral agent can be complex due to a significant potential for drug interactions, adverse effects and adherence challenges [1]. Resistance is still a major concern for NRTI, as is true for all classes of HIV drugs. Therefore, new compounds with improved safety, effectiveness and with a high genetic barrier to resistance are warranted. A clinical study was conducted evaluating the combination of Amdoxovir, [(−)-β-D-2,6-diaminopurine dioxolane, DAPD, AMDX], an investigational NRTI at 500 mg twice daily (bid) with standard and reduced doses of 3′-azido-3′-deoxythymidine (Zidovudine, ZDV, AZT) at 200 or 300 mg bid, respectively [2].

DAPD, a prodrug of 9-(β-D-1,3-dioxolan-4-yl)guanine (DXG), is currently in phase 2b clinical testing for the treatment of HIV-1 infection, and has been safely administered to nearly 200 patients ([3]; www.rfspharma.com, last consulted on June 15, 2009). DXG has potent anti-HIV activity with a high genetic barrier to resistance, but is limited by its aqueous solubility. Therefore, DAPD was developed as a prodrug, which is rapidly deaminated by the ubiquitous enzyme adenosine deaminase (ADA) to DXG, followed by intracellular phosphorylation of DXG to its active metabolite DXG-5′-triphosphate which is a potent inhibitor of HIV-1 reverse transcriptase (HIV-1 RT) [4]. Therefore, it is important to accurately measure the concentrations of both compounds in human plasma, to assess the bioconversion efficiency of DAPD to DXG *in vivo* [4].

DAPD is currently the only guanosine nucleoside analog in clinical development, and has activity *in vitro* against wild type and drug-resistant forms of HIV-1, including viruses that are resistant to ZDV (mutations M41L, D67N, K70R, L210W, T215Y/F and K219Q/E) and 3TC (mutation M184V/I) [5–8]. ZDV, a thymidine nucleoside analog, was the first antiretroviral drug approved by the FDA, initially as a monotherapy regimen and subsequently as a component of HAART regimens [9,10]. The current approved oral dose of ZDV is 300 mg bid. ZDV undergoes intracellular phosphorylation, similar to other NRTI, to form the active ZDV-5′-triphosphate, which inhibits wild-type HIV-1 RT [11]. However, ZDV treatment is lim-

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ited by adverse effects, which may include nausea and malaise, as well as serious bone marrow cytotoxicity, including anemia and neutropenia [12–14]. A recent *in silico* study using population enzyme kinetic and pharmacokinetic data, suggested that decreasing the dose of ZDV from 300 to 200 mg, bid may decrease the amount of cellular ZDV-MP associated with hematological toxicity, without significantly reducing the cellular amount of ZDV-TP associated with antiviral efficacy [2]. Furthermore, a cellular pharmacology study demonstrated no drug–drug interaction at the phosphorylation level between ZDV and DXG [15]. Additionally, the development of HIV-1 resistant to DXG emerges slowly *in vitro* and viruses resistant to DXG had one of two mutations (K65R *or* L74V) within the viral polymerase gene [8,16–18]. ZDV has anti-K65R activity and therefore, could be potentially incorporated as a 'resistance repellent' for the K65R mutation that may result from prolonged treatment with DAPD and other K65R selecting drugs such as tenofovir disoproxil fumarate [2,16,18]. Furthermore, DXG demonstrated antiviral synergy in combination with ZDV in human peripheral blood mononuclear cells, and the combination of DAPD and ZDV completely prevented the development of DAPD or ZDV-associated resistance mutations through Week 28 [7,30].

A proof-of-concept randomized, placebo-controlled, single site study was conducted in HIV-infected persons to evaluate the safety, efficacy and pharmacokinetics of DAPD 500 mg po bid, in combination with ZDV 200 or 300 mg po bid. The study was conducted in Argentina and was approved by the site institutional review board/ethics review committee. The overall mean $CD4^+$ cell count was 417 cells/mm^3 (range 201–1071), HIV-1 RNA was 4.5 $\log_{10}$ copies/mL (range 3.6–6.0) at baseline, and the median age was 33 (range 21–52) with an equal gender distribution (50% male, 50% female). Both combinations were safe and well tolerated [31], and produced a similar 2-$\log_{10}$ decrease in mean plasma HIV-1 RNA from baseline at Day 10, supporting the earlier *in silico* ZDV study [2]. It was essential to develop a robust and validated LC–MS/MS method to measure the drug concentrations of DAPD, DXG and ZDV in plasma obtained from the pharmacokinetic component of the clinical study described above [32].

LC–MS/MS methodologies, such as reverse-phase chromatography tandem mass spectrometry and electrospray ionization, have been used for the last decade, have demonstrated improved specificity and sensitivity, and are capable of measuring very low concentrations of nucleosides in plasma and other tissues. Recently, levels of quantification as low as 0.5 ng mL^{-1} of ZDV in plasma were reported [19]. The extraction of the nucleosides from the plasma for these assays typically involve, either solid phase extraction [19–23], or simple sample clean-up using a robotic system and disposable Centricon 30 ultra-filtration units [24].

Simultaneous measurement of ZDV, DAPD and its major metabolite, DXG was accomplished, despite polarity differences between ZDV and the two guanosine analogs, and the similarity of DAPD and DXG differing by only one functional group (Fig. 1). A previously unpublished method for DAPD and DXG quantification [32] developed by Triangle Pharmaceuticals Inc. (acquired by Gilead Sciences in 2003) was modified to allow simultaneous quantification of ZDV in plasma. Herein, we present an optimized and improved method for extracting and simultaneously quantifying DAPD, DXG and ZDV in human plasma, as well as evidence of the reproducibility, accuracy and precision of this method.

2. Experimental

2.1. Material and reagent

2.1.1. Chemicals

Reference standards for DAPD and DXG were obtained from RFS Pharma, LLC (Tucker, GA). ZDV was obtained from Samchully Pharmaceuticals Co. Ltd. (Seoul, Korea). 2,6-Diaminopurine-2′-deoxyriboside (DPD) and 2′-deoxyadenosine (2′-dA) were purchased from Sigma (St Louis, MO, USA) and 2′-deoxycoformycin (DCF), a potent adenosine deaminase inhibitor, from Waterstone Technology LLC (Carmel, IN).

2.1.2. Liquid chromatography

Human blood from healthy subjects was obtained from the American Red Cross (Atlanta, USA) and used as control human plasma. Eppendorf centrifuge model 5417C (Eppendorf North America, NY, USA) was used for plasma preparation. HPLC-grade methanol was obtained from Thermo Fisher Scientific Inc. (Waltham, MA), ultrapure water from an ELGA Ultrapure equipped with US filters, formic acid from Fluka (St Louis, MO, USA) and ammonium formate (purity 99%) from Acros Organics (NJ, USA). High-pressure nitrogen and ultra high purity and high-pressure argon were purchased from Nexair (Suwanee, GA). Eppendorf 1.5 mL safe lock cones were used to preserve the samples.

The HPLC system was a Dionex Packing Ultimate 3000 modular LC system comprising of a quaternary pump, vacuum degasser, thermostated autosampler, and thermostated column compartment (Dionex, CA). A TSQ Quantum Ultra triple quadrupole mass spectrometer (Thermo Scientific, Waltham, MA, USA) was used for detection. Thermo Xcalibur software version 1.3 was used to operate HPLC, the mass spectrometer and to perform data analyses.

2.1.3. Stock standard solutions

Standard stock solutions were freshly prepared in ultrapure MeOH:H_2O (1:1) to achieve the following concentrations: 0.2 mg mL^{-1} for DAPD, 0.1 mg mL^{-1} for DXG, 0.5 mg mL^{-1} for ZDV and 1 mg mL^{-1} for DCF (for conversion to μM, refer to Tables 2 and 3). Standards were serially diluted to 100, 10 and 1 μg mL^{-1}. Calibration standards covering the range from 2 to 5000 ng mL^{-1} were prepared by adding appropriate volumes of serially diluted stock solutions to human plasma containing 10 μL of DCF at 1 mg mL^{-1} (final volume 5 mL). Eight calibration concen-

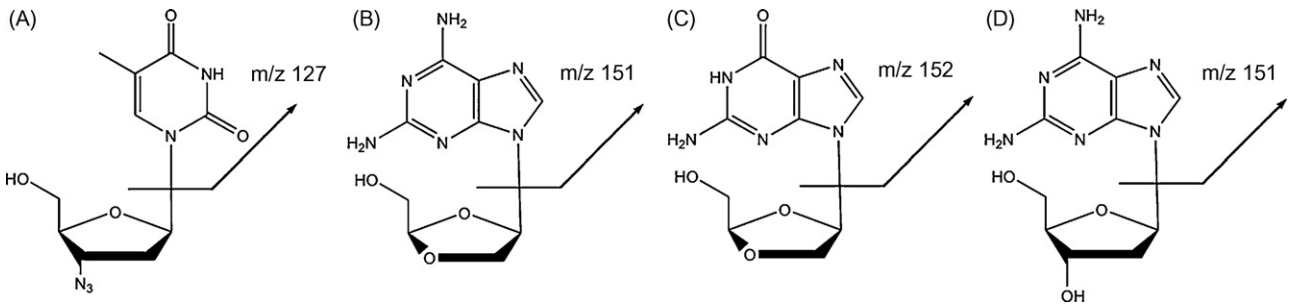

Fig. 1. Chemical structure of (A) Zidovudine, ZDV, (B) Amdoxovir, DAPD, (C) 9-(β-D-1,3-dioxolan-4-yl)guanine, DXG, (D) 2,6-diaminopurine-2′-deoxyriboside, DPD and the representation of the fragment used in the Selected Reaction Monitoring (SRM).

trations (2, 5, 10, 50, 100, 500, 1000 and 3000 ng mL^{-1}) were used to define the calibration curve for all three analytes and an additional concentration, 5000 ng mL^{-1} was used to ZDV calibration. Five quality control (QC) standards were also used (2, 5, 10, 500 and 3000 ng mL^{-1}) for assay validation. Aliquots of about 1 mL of calibration standards and QC samples were transferred to 1.5 mL polypropylene snap-cap tubes and stored frozen at −20 °C until analysis. All stock standard solutions were stored at −20 °C.

A stock solution of the internal standard, DPD was prepared at 1 mg mL^{-1} in methanol, and was diluted to a 250 ng mL^{-1} solution in methanol for use during sample preparation. A stock solution of 100 mM ammonium formate was prepared and adjusted once to pH 4.8 using formic acid followed by filtration under vacuum with nylon discs 0.2 μm (Whatman, New Jersey, USA) and was stored at 4 °C. A stock solution of 4 mM 2′-dA was prepared and filtered under vacuum using nylon discs 0.2 μm (Whatman, New Jersey, USA) and was stored at 4 °C.

2.2. Preparation of plasma samples and extraction procedure

DAPD and DXG were extracted from human plasma using a methanol-based protein precipitation procedure, followed by LC-MS/MS analysis. Prior to analysis, calibration standards, quality controls (QC) and clinical samples (collected at Aclires-Argentina SRL, Buenos Aires, Argentina), were thawed and allowed to equilibrate at room temperature. One hundred μL of plasma (calibration, QC and subject samples) were transferred to a 1.5 mL polypropylene snap-cap tubes and spiked with 400 μL of methanol-based solution containing internal standard (DPD, 250 ng mL^{-1}). The microcentrifuge vials were capped and vortex mixed for 1–2 s. The samples were allowed to sit for 15 min before being mixed under vortex at high speed for 30 s to inactivate any HIV present in the samples. The vials were centrifuged at 14,000 × *g* for 5 min followed by the removal of 200 μL of the supernatant to two microcentrifuge tubes, which was evaporated to dryness under a stream of air. The residue was reconstituted in 125 μL of 2 mM ammonium formate, pH 4.8 and 0.04 mM 2′-dA and briefly centrifuged at high speed. The supernatant was transferred to a Costar Spin-X microcentrifuge tube filter and centrifuged at 14,000 × *g* for 5 min. Fifty μL of the filtrate were transferred to 1.5 mL vials containing an insert of 200 μL, and 5 μL were injected directly into the chromatographic system.

2.3. LC–MS/MS conditions

2.3.1. Reverse-phase chromatography

Chromatographic separation was performed using a Betabasic-C18 column (100 × 1 mm, 3 μm particle size; Thermo Scientific, Waltham, MA, USA). This column was protected from remaining particles by a pre-column filter with 0.2 μm particle size (Thermo Scientific, Waltham, MA, USA). The mobile phase A consisted of 2 mM ammonium formate buffer, pH 4.8 containing 0.04 mM 2′-dA prepared daily from stock solutions. The mobile phase B consisted of methanol. The initial conditions were 94% A and 6% B at 50 μL min^{-1}. DXG, DAPD and DPD were eluted by this isocratic method during the first 7 min of analysis. From 7.5 min to 8.5 min, the flow rate was increased to 100 μL min^{-1}, from 8.5 min to 13 min, B was increased from 6% to 90% and immediately decreased to 6% at 14.3 min allowing ZDV elution. The flow rate was maintained at 100 μL min^{-1} until 25 min to accelerate the column re-equilibration and was decreased to 50 μL min^{-1} in 1 min. The total run time was 29 min, including time for column regeneration, which was optimized in order to maintain an efficient separation of DAPD and DXG on the following run. However, a shorter re-equilibration time led to a co-elution of DAPD and DXG and no benefit was achieved using longer re-equilibration times. The column temperature was kept constant at 30 °C. The column effluent was directed to waste via the divert valve of the mass spectrometer at 0–3 min, 8–13 min and 15–29 min. During these intervals, a cleaning solution containing 80% methanol and 0.4% formic acid in water was used at 50 μL min^{-1}. This cleaning of the ion source improved the sensitivity of detection. A solution consisting of 80% methanol and 0.1% formic acid in water was used for autosampler loop and syringe cleaning following injection.

2.3.2. MS/MS conditions

Analytes were protonated by electrospray ionization (ESI) in positive mode. Selected Reaction Monitoring (SRM) mode was used for the acquisition. The intensity of selected product ion in the MS/MS spectrum of each compound was optimized using direct infusion of the analytes in the corresponding mobile phase at 25 μg mL^{-1} and individually into the instrument using a syringe pump at 5 μL min^{-1}. The sheath and auxiliary gas (nitrogen) were set at 45 and 0.5 arbitrary units (au), respectively without ion sweep gas. The collision gas (argon) pressure was set at 1.3 mTorr. The spray voltage was 4000 V. The capillary was heated at 280 °C and 0.1 s scan time was used. The collision-induced dissociation (CID) was at −6 V. Scan parameters were as follows: precursor ion *m*/*z*, product ion *m*/*z*, collision energy, tube lens offset and the full width half mass (FWHM) resolution (unit resolution) for both quadrupole (Q1 and Q3) and are listed in Table 1. A representation of the hypothesized fragmentation for all nucleosides is shown in Fig. 1.

2.4. Validation

2.4.1. Limit of quantification

The limit of quantification (LOQ) was defined as the smallest quantity of analyte likely to be quantified accurately with a precision within ±20%. For each of the three analytes, QC and calibration standards were prepared at the lower limit of quantification (2 ng mL^{-1}).

2.4.2. Linearity

Ten calibration standards (2, 5, 10, 50, 100, 500, 1000, 3000 and 5000 ng mL^{-1}) and four QCs (2, 10, 500 and 3000 ng mL^{-1}) were prepared in control blank plasma pretreated with DCF, prior to processing the clinical samples. Standards were processed simultaneously with the patient samples and were assayed prior to patient samples. QCs were run along with the clinical samples to ensure confidence in the sample stability during the sequence and in the accuracy of the quantification. Calibration curves were calculated by linear regression using a weighting factor of 1/*x*. Linearity was evaluated by means of back-calculated concentrations of the calibration standards; these values should be within 15% of the nominal concentration and 20% of the nominal concentration at LLOQ to

Table 1
Scan parameters of the Thermo TSQ Quantum Ultra triple quadrupole mass spectrometer.

Analyte	Precursor ion (*m*/*z*)	Product ion (*m*/*z*)	Collision energy (V)	Tube lens offset (V)	FWHM resolution for Q1	FWHM resolution for Q3
DAPD	253	151	27	59	1.00	0.70
DXG	254	152	20	80	0.70	0.50
ZDV	268	127	24	51	0.70	0.50
DPD	267	151	25	68	0.70	0.70

be accepted. Based on the criteria, less than 25% of the calibration standards were rejected from the calibration curve.

2.4.3. Specificity and selectivity

Two sets of human blank plasma were prepared and analyzed in the same manner as the calibration standards and QCs, but without the internal standard. The objective was to determine whether any endogenous compounds interfere at the mass transition chosen for DAPD, DXG, DPD and ZDV. Interference can occur when co-eluting endogenous compounds, produce ions with the same m/z values that are used to monitor the analytes and internal standard. The peak area of any endogenous compounds co-eluting with the analyte should not exceed 20% of the analyte peak area at LLOQ or 5% of the internal standard area.

2.4.4. Recovery and matrix effect

The amount of analyte lost during sample preparation was calculated from the recovery values. Recoveries of DAPD, DXG and ZDV from plasma following sample preparation were assessed in triplicate by comparing the response of each analyte extracted from plasma with the response of the same analyte at the same concentration spiked in post-extracted blank plasma. It was also important to ensure the absence of a significant matrix effect. Significant ion suppression could occur when non-appropriate solvents are used or when endogenous compounds are simultaneously eluted with the analyte of interest resulting in interference with its ionization. The suitable dilution had to be determined to avoid decreasing the MS signal in the presence of increasing amounts of biological sample. The matrix effect was assessed by comparing the response of the post-extracted blank plasma spiked at known concentration and the response of the same analyte at the same concentration prepared in mobile phase. This value provided information about specific ion suppression in plasma. A low, medium and high-level concentration at 10, 100 and 1000 ng mL^{-1}, respectively was used to assess recovery and matrix effects.

2.4.5. Accuracy and precision

The intra- and inter-day precisions and accuracy were also evaluated at low, medium and high concentrations (10, 100 and 1000 ng mL^{-1}). For intra-assay precision, one control sample from each of the three concentrations was assayed on six runs in one sequence. For inter-day precision, one control sample from each of the three concentrations was assayed on four separate days (corresponding to four runs). The vial containing the control sample was maintained at −20 °C between injections. Inter-day extraction reproducibility was assessed by calculating the precision of five extracted spiked standards in plasma, analyzed on five different days. Intra- and inter-day variations were assessed by comparing means and standard deviations of drug concentrations at the three levels. The precision was evaluated as the relative standard deviation of the mean expressed as a percentage (coefficient variation – %CV) and had acceptance criteria of less than 20%. Accuracy was expressed as the mean absolute percentage deviation from the theoretically determined concentration with acceptance criteria of within 80-120%.

2.4.6. Stability

The stability of extracted standards from plasma in the autosampler was assayed by quantification of each analyte at 1000 ng mL^{-1} after storage for 40 h and 5 days at 4 °C. The stability of the standards during the extraction was assessed by quantification of each analyte at 1000 ng mL^{-1} after storage at room temperature for 24 h. The stability of extracted standards after three freeze/thaw cycles was also evaluated. The analyte was considered stable in biological matrix or extracts when 80–120% of the initial concentration was measured. The re-injection reproducibility was assessed to determine if an analytical run could be re-analyzed in the case of instrument failure.

2.4.7. Carryover

Carryover was evaluated by injecting two matrix blanks immediately following the upper limit of quantification (ULOQ) standard and the lower limit of quantification (LLOQ) standard. Carryover was acceptable as long as the mean carry over in the first blank was less than or equal to 30% of the peak area of the ULOQ and LLOQ and was less than or equal to 20% in the second blank.

3. Results

3.1. LC/MS/MS characteristics

Typical LC–MS/MS chromatograms for extracted DAPD, DXG, ZDV and DPD (internal standard) compared with standard extracted from plasma blank are shown in Fig. 2. The retention times of DAPD, DXG, DPD and ZDV were 5.49, 3.86, 6.10 and 13.65 min, respectively.

An additional peak was observed at 5.45 min on DXG reconstituted ion chromatogram (RIC) in both patient and standard, but not in blank plasma. This interference was observable at the same retention time as DAPD. This peak was approximately 10% of DAPD signal intensity and resulted from the isotope distribution of DAPD. The chromatographic separation was essential in order to accurately quantify DXG. A plasma sample from a patient was also analyzed without internal standard to confirm that no endogenous substances interfered with any of the analytes, including the internal standard.

The highest intensity for protonated ions was found in positive mode for all analytes and internal standard as they have an ability to accept protons. The introduction of a methanol–water–formic acid (80:20:0.4 v/v/v) solution to the electrospray ionization (ESI) source, while the effluent from the column was diverted to waste, increased the intensity and participated in rinsing the source. The optimization of capillary temperature and nitrogen flow was considered important as they both played an important role in minimizing ion suppression and increasing the sensitivity of the method.

3.2. Linearity and limit of quantification

The standard curve was obtained by fitting the ratio of peak height of DAPD, DXG and ZDV to that of the internal standard against the concentrations (2–3000 ng mL^{-1}) of DAPD and DXG and (2–5000 ng mL^{-1}) of ZDV using a $1/x$ weighted linear regression ($y = Ax + B$). The lower limit of quantification (LLOQ) was 2 ng mL^{-1}, which could be quantified accurately and precisely within ±20% for each analyte. This corresponds to an amount of 3.2 pg of analyte injected on column. The upper limit of quantification (ULOQ) was 3000 ng mL^{-1} for DAPD and DXG and 5000 ng mL^{-1} for ZDV.

3.3. Validation results

3.3.1. Recovery and matrix effect

Recovery was determined by measuring an extracted sample against a post-extracted spiked sample. Matrix effect was determined by measuring a post-extracted spiked sample and an un-extracted sample. Three concentrations (10, 100 and 1000 ng mL^{-1}) were used and assayed in duplicates (Table 2). Absolute values were determined by comparing areas and relative values determined by comparing ratios (area of the analyte over the area of the internal standard).

The relative recovery and matrix effect values reflect general analyte loss on both the analyte and the internal standard. The abso-

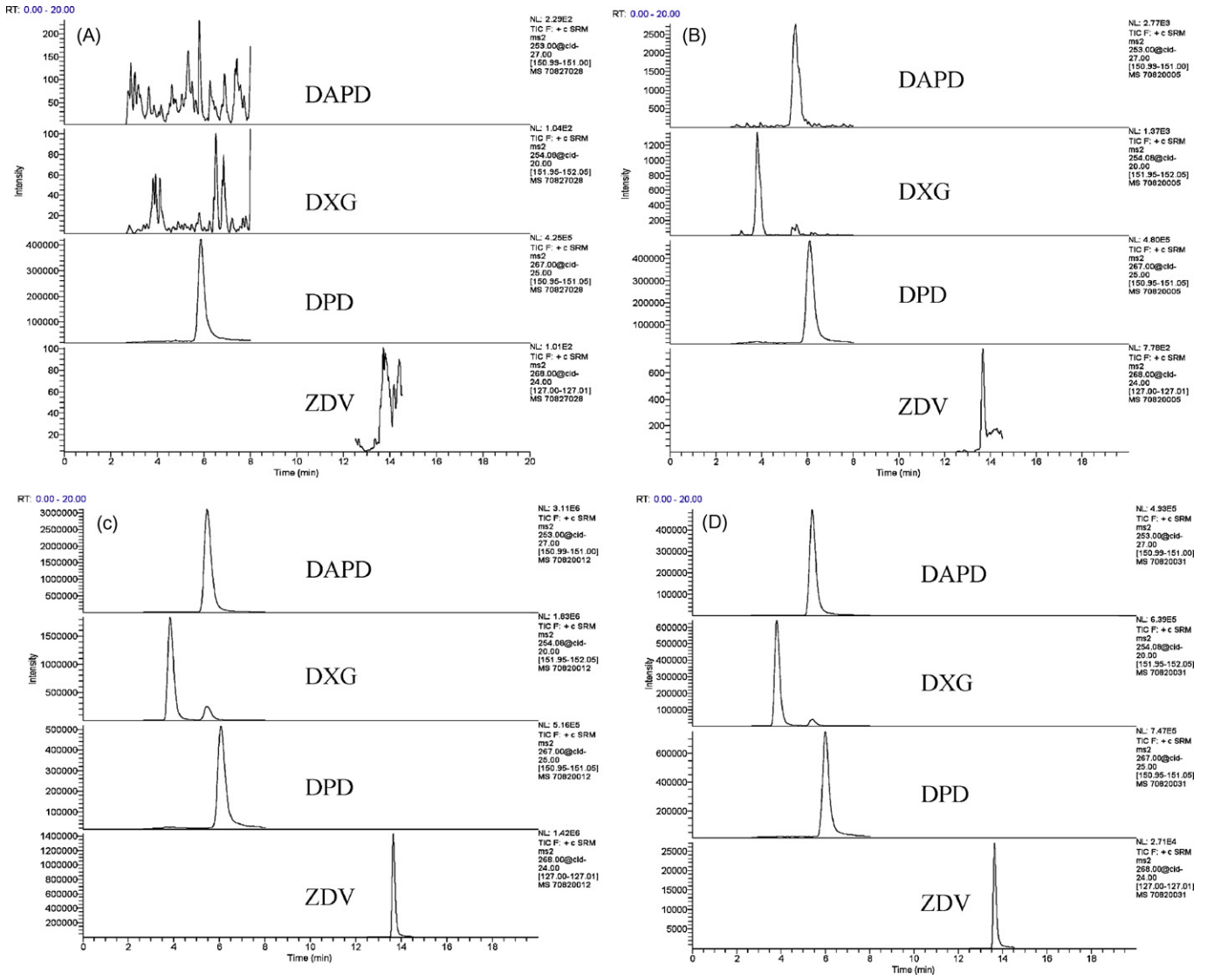

Fig. 2. Reconstituted ion chromatograms (RIC) for (A) patient plasma obtained on first day of treatment before first dose administration, (B) healthy subject plasma at 2 ng mL^{-1} of each standards, (C) healthy subject plasma at 3000 ng mL^{-1} of each standards and (D) patient plasma 4 h after the first dose administration; the calculated analyte concentrations were as follow: 328 ng mL^{-1}, 693 ng mL^{-1} and 43 ng mL^{-1} for DAPD, DXG and ZDV, respectively (all plasma samples from healthy subjects and patient were treated with methanol containing internal standard, DPD). The top trace is the RIC for DAPD, the second trace is the RIC for DXG, the third trace is the RIC for DPD and the bottom trace is the RIC for ZDV. The retention time appears on top of the peak.

lute values represent specific loss occurring during the extraction or specific ion suppression. For all analytes, the recovery values provided confidence in the extraction process. Greater matrix effect was observed for DXG compared with DAPD and ZDV, but the ion suppression did not significantly vary with increasing concentrations of DXG, allowing the linearity of the response.

3.3.2. *Accuracy and precision*

The results of intra-assay and inter-assay precision for three concentrations (low, medium and high) are summarized in Table 3. Instrument intra-assay imprecision was <5% and accuracy was >95% at all concentrations. Instrument inter-assay imprecision was <10% at all concentrations, accuracy was >80% at low concentration

Table 2
Recovery and matrix effect (10, 100 and 1000 ng mL^{-1}) in duplicate.

Analyte	Conc. (ng mL^{-1})[a]	Absolute recovery (%)	Relative recovery (%)	Absolute matrix effect (%)	Relative matrix effect (%)
DXG	*10*	81.8	91.8	33.1	23.0
DAPD		95.3	92.3	9.5	−3.0
ZDV		101.3	97.3	12.6	0.8
DXG	*100*	97.2	104.9	34.2	16.6
DAPD		89.3	94.5	21.0	−0.6
ZDV		91.4	95.6	22.5	2.5
DXG	*1000*	92.3	106.5	19.7	15.0
DAPD		86.9	93.2	3.3	−2.6
ZDV		67.4	80.4	5.1	−0.3

[a] To convert ng mL^{-1} to nM, the concentration should be divided by 253, 252, 267 for DXG, DAPD and ZDV, respectively.

Table 3
Intra- and inter-assay precision and inter-assay reproducibility for all three analytes.

Analyte	Conc.[a] (ng mL^{-1})	Intra-assay precision (n = 6 runs)		Inter-assay precision (n = 4 runs)		Inter-assay extraction reproducibility (n = 5 replicates)	
		Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)
DXG	*10*	104.6	3.4	90.8	2.7	99.1	9.2
DAPD		104.5	4.1	82.7	7.0	97.2	8.1
ZDV		97.2	1.4	85.0	5.1	101.7	10.9
DXG	*100*	108.2	3.2	107.4	8.5	103.6	10.5
DAPD		106.1	0.4	104.5	9.3	97.9	7.7
ZDV		103.7	3.1	102.4	3.5	103.1	13.5
DXG	*1000*	106.8	2.5	105.4	4.6	101.3	7.1
DAPD		106.1	1.4	102.9	4.8	94.1	10.3
ZDV		105.7	1.5	99.3	1.9	96.8	9.11

[a] To convert ng mL^{-1} to nM, the concentration should be divided by 253, 252, 267 for DXG, DAPD and ZDV, respectively.

and >99% at high and medium concentrations. Extraction reproducibility was in the range of 94.1–103.6% accuracy and 7.1–13.5% imprecision.

3.3.3. Stability

The analytes were found to be stable under all conditions tested and the variation in concentration was minimal with 84–110% recovery (Table 4).

3.3.4. Carryover

At LLOQ, for ZDV and DXG, the peaks in the blank following the injection at 2 ng mL^{-1} were in the range of the background noise of the reconstituted ion chromatogram and consequently were not integrated. For DAPD, at LLOQ, the carryover was 8% in the first blank and 4% in the second blank, which were acceptable. For all analytes, at ULOQ, the carryover was below 0.08% in the first blank and below 0.03% in the second.

4. Discussion

An improved method was developed and validated for high throughput simultaneous measurement of DAPD, DXG and ZDV levels in plasma, generating data necessary for clinical and pharmacokinetic analysis. LC–MS/MS methods have been used successfully for the last decade for quantification of antiretroviral agents, including NRTI in biological matrix. Limited work was available for DAPD and DXG. However, ZDV quantification by LC–MS/MS, using reverse phase chromatography, has been described previously [19,22–25]. Short runs were used for ZDV detection with mobile phase containing 0.1% of acetic acid [25] or at neutral pH [19,24]. However, at pH 4.8, which improved DAPD and DXG separation, ZDV retention was increased, which could be explained by the work of Bezy et al., who demonstrated that at a pH between 5 and 7, ZDV was neutral and its retention was increased, whereas at pH 9, ZDV became negatively charged with a low retention [23]. ZDV was detected either in positive [26,27] or negative mode [19,22–25], depending on the mobile phase used. In our case, the positive mode was found to have greater sensitivity for all analytes. The challenge was to obtain a discriminating and rapid separation of DAPD and DXG, which have similar molecular weights and fragmentation patterns, while still achieving simultaneous detection of ZDV, which is less polar than DAPD and DXG.

A binary method was developed, consisting of an isocratic elution of DAPD, DXG and DPD in the first 8 min of the run followed by a fast gradient for ZDV elution, because of its strong affinity for the stationary phase. Following the gradient, it became essential to optimize the equilibration time of the column with 6% methanol in order to maintain consistent retention times for DAPD and DXG for the subsequent injection, since these retention times were very sensitive to slight variations in methanol concentration. The peak shape of all analytes was improved by the addition of 0.04 mM of 2′-dA, creating a shift in baseline signal of the reconstituted ion chromatogram, and decreasing background noise. 2′-dA may act as a silanol-masking agent, which would prevent the non-specific binding of the analytes to the column. It may also produce an ion-pairing effect with other nucleosides, enhancing the shape of the peaks. The peak shape was also greatly improved by using a microbore column, with 3 μm particle size, which allowed the use of lower flow rates than wider columns with bigger particle size, while increasing the backpressure. Increasing the particle size to 5 μm and using a different column: Hypersil GOLD-C18 (100 × 1 mm, 5 μm particle size; Thermo Scientific, Waltham, MA, USA), caused a peak broadening. However, the use of shorter column to reduce the run time decreased the resolution between DXG and DAPD inducing quantification error due to signal overlap. Several gradients were tested, but the steepest slope was necessary to shorten the re-equilibration time. The use of this column improved the assay sensitivity by decreasing the need for sample dilution. Furthermore, a reduction in mobile phase volume introduced to the ion source provided the added benefit of decreasing the ion suppression over time, rendering high throughput analysis of the clinical trial samples possible. In addition, reducing the amount of mobile phase, and therefore the amount of organic solvent, was advantageous, since it reduced the cost of the analysis, and was also more ecological. For the clinical trial, 43 clinical samples were successfully assayed in sequences of 26 h, including standards and QCs.

In this method, the extraction was performed manually, which was time consuming and could be further adapted to allow for high throughput application. Kenney et al. used a robotic system, which decrease bias and imprecision, as well as increase the throughput [24]. The extraction imprecision and inaccuracy did not

Table 4
Stability of the analytes extracted from plasma spiked at 1000 ng mL^{-1} under temperature variation conditions.

Analyte	24 h at room temperature (% recovery)	40 h at 4 °C (% recovery)	5 days at 4 °C (% recovery)	4 freeze/thaw cycles at −20 °C (% recovery)
DXG	101	104	91	110
DAPD	92	100	95	99
ZDV	96	91	84	104

exceed 15% for 5 days, meeting the limits required to validate the method.

5. Conclusions

A sensitive and robust LC–MS/MS method was developed and validated for simultaneous measurement of DAPD, DXG and ZDV in plasma. The results obtained [32,34] using this optimized method were in accordance to previous reported studies [28–30,33] and the lower limit of quantification was sufficient to perform the pharmacokinetic analysis on clinical samples [34]. This new method should be useful to simultaneously measure DAPD, DXG and ZDV in clinical samples and will allow further assessment of potential drug–drug interactions.

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