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Development and validation of an assay for the simultaneous determination of zidovudine, abacavir, emtricitabine, lamivudine, tenofovir and ribavirin in human plasma using liquid chromatography–tandem mass spectrometry

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ABSTRACT

This paper describes the development and validation of an assay for the simultaneous quantification of the antiviral and antiretroviral drugs zidovudine, abacavir, emtricitabine, lamivudine, tenofovir and ribavirin in human plasma using liquid chromatography coupled to tandem mass spectrometry. Sample pretreatment consisted of protein precipitation with 0.1% (v/v) formic acid in methanol, evaporation and reconstitution. Chromatographic separation was performed on a Synergy Polar reversed phase C18 column (150 mm × 2.0 mm ID, particle size 4 μm) using a stepwise gradient with 0.1% (v/v) formic acid in water and 0.1% (v/v) formic acid in methanol at a flow rate of 300 μL/min. A triple quadrupole mass spectrometer operating in the positive ionization mode was used for drug detection and quantification. Isotopically labeled zidovudine, lamivudine, tenofovir and ribavirin were used as internal standards. The method was validated over a clinical range of 20–2500 ng/mL for zidovudine, lamivudine and tenofovir, 4–500 ng/mL for abacavir and emtricitabine and 160–20,000 ng/mL for ribavirin. The inter and intra-assay accuracies and precisions were between −8.47% and 14.2% for zidovudine, emtricitabine and ribavirin. For abacavir, lamivudine and tenofovir, the inter and intra-assay accuracies and precisions at the lower limit of quantification were between −11.0% and 18.3%, whereas at all other levels these accuracies and precisions were between −11.7% and 12.0%. The described method is suitable for the determination of zidovudine, abacavir, emtricitabine, lamivudine, tenofovir and ribavirin in human plasma in clinical practice to monitor plasma concentrations in selected cases to optimize therapy.

1. Introduction

Nucleoside (NRTI) and nucleotide (NtRTI) reverse transcriptase inhibitors are synthetic nucleoside and nucleotide analogs used for the treatment of viral infections, like the human immunodeficiency virus (HIV) type 1 and 2, hepatitis C virus (HCV) or hepatitis B virus (HBV). HIV-infected patients are often co-infected with HBV due to a similar transmission route. Additionally, many HIV-infected patients are HCV co-infected.

In HIV-infected patients, dual therapy of the N(t)RTIs tenofovir, emtricitabine, lamivudine, zidovudine and/or abacavir (see Table 1) shows superior efficacy compared to single therapy. Therefore, a dual combination is currently the standard of care backbone of the combined antiretroviral therapy [1,2]. Ribavirin (see Table 1) is used in the treatment of HCV, together with pegylated interferon and, in case of HCV genotype 1, a protease inhibitor (e.g. boceprevir or telaprevir) [3]. In HBV/HIV co-infection current guidelines recommend tenofovir and lamivudine or emtricitabine as a first-line treatment [1]. Additionally, a dual combination of tenofovir and emtricitabine showed efficacy as pre-exposure prophylaxis for HIV infection in at-risk individuals [4].

After intracellular uptake, N(t)RTIs are phosphorylated by endogenous kinases to their active 5′-triphosphate moieties. These active anabolites, except those from ribavirin, are incorporated into the viral DNA or RNA after which translation is terminated. Ribavirin anabolites interfere with RNA or mRNA synthesis. Although the N(t)RTIs are inactive prodrugs, therapeutic drug monitoring may be helpful to prevent treatment failure or toxicity in anti-HIV, anti-HCV and anti-HBV treatment. For instance, high ribavirin plasma concentrations have been related to increased sustained virologic responses in HCV and HCV/HIV co-infection [5,6]. Also, increased CD4 cell counts and decreased HIV RNA levels have been correlated

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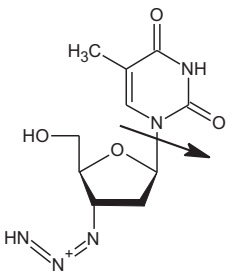
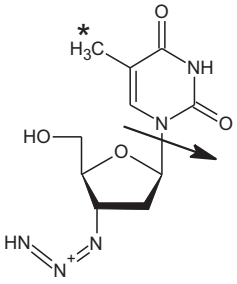
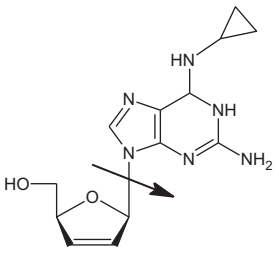
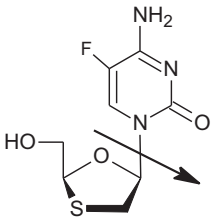
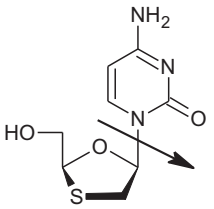
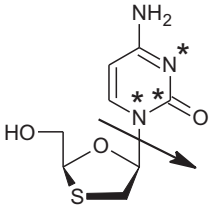
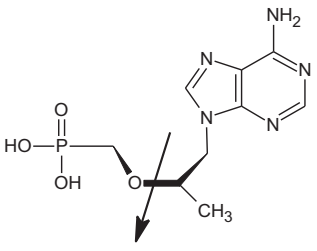
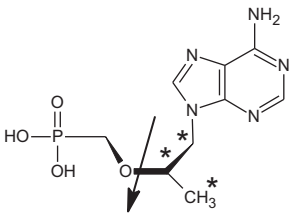
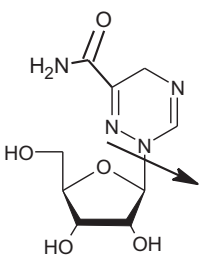
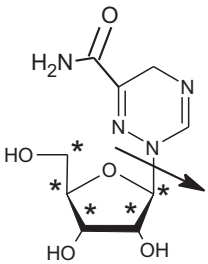
Table 1
Selected transitions and fragmentation pathways of all analytes and internal standards.

Analyte	Mass transition (*m/z*)	Proposed fragmentation pathway	
		Analyte	Internal standard
Zidovudine	268 → 127		
$^{13}C,^{2}H_3$-zidovudine	272 → 131		
Abacavir	287 → 191		–
Emtricitabine	248 → 130		–
Lamivudine	230 → 112		
$^{15}N_2,^{13}C$-lamivudine	233 → 115		
Tenofovir	288 → 176		
$^{2}H_6$-tenofovir	294 → 182		
Ribavirine	245 → 113		
$^{13}C_5$-ribavirin	250 → 113		

* Representation of the stable isotope of the corresponding element (e.g. ^{13}C, ^{2}H or ^{15}N).

to increased abacavir maximum plasma concentrations [7]. Additionally, tenofovir associated renal dysfunction and zidovudine induced anemia have been related to high plasma concentrations [8–10]. Thus, monitoring N(t)RTI plasma concentrations can be a useful tool in selected cases and ideally these analytes will be analyzed simultaneously, because most patients receive more than one N(t)RTI.

Previously, several assays have been published describing the determination of two or more N(t)RTIs in plasma using liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) [11–27]. Seven of these assays described the simultaneous determination of the NtRTI tenofovir in combination with other NRTIs, which can be a challenging task due to the different physical–chemical properties of tenofovir in comparison with the other NRTIs [13,16,17,21–23,25]. None of the described methods quantified the HIV N(t)RTIs together with the HCV NRTI ribavirin, although quantification of ribavirin has been described before [28–30].

Therefore, this paper describes the development and validation of a bioanalytical assay to determine zidovudine, abacavir, emtricitabine, lamivudine, tenofvir and ribavirin plasma concentrations using LC–MS/MS.

2. Experimental

2.1. Chemicals and reagents

Zidovudine, emtricitabine and tenofovir originated from Sequoia Research Products (Pangbourne, United Kingdom), lamivudine from VWR (Amsterdam, The Netherlands) and ribavirin from Merck Sharp & Dohme (Haarlem, The Netherlands). Abacavir was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. The internal standards $^{13}C,^{2}H_3$-zidovudine, $^{13}C_5$-ribavirin and $^{2}H_6$-tenofovir were purchased from Alsachim (Illkirch Graffenstaden, France), while $^{15}N_2,^{13}C$-lamivudine originated from Toronto Research Chemical (North York, ON, Canada) (see Table 1). Methanol was obtained from Biosolve Ltd. (Valkenswaard, The Netherlands). Distilled water originated from B. Braun (Melsungen, Germany). Formic acid was from Merck (Amsterdam, The Netherlands). Drug free plasma was obtained from healthy volunteers (Slotervaart Hospital, Amsterdam, The Netherlands).

2.2. Chromatographic conditions

Chromatographic separation of all analytes was carried out using an HPLC system (Agilent Technologies, Palo Alto, CA, USA) consisting of an HP1100 binary pump, a cooled autosampler (4 °C) and a column oven (40 °C). Compounds were eluted using a stepwise gradient at a flow rate of 300 μL/min. Mobile phase A consisted of 0.1% (v/v) formic acid in water and mobile phase B consisted of 0.1% (v/v) formic acid in methanol. At time zero the flow consisted of 98% mobile phase A and 2% mobile phase B. After 3 min 30% of mobile phase A was mixed with 70% of mobile phase B for 4 min, which was followed by 5% of mobile phase A with 95% of mobile phase B for 2 min. The last 2 min the column was reconditioned with 98% of mobile phase A and 2% of mobile phase B, before the next injection. A Synergi Polar reversed phase C18 column (150 mm × 2.0 mm ID, 4 μm particle size; Phenomenex, Rotterdam, The Netherlands) protected with an inline filter was used for separation. The column outlet was connected to the electrospray sample inlet through a divert valve. The divert valve was directed to waste during the first 1.3 min and last 2 min of the run to prevent the introduction of endogenous compounds into the mass spectrometer. Total run time was 11 min and sample injection volumes were 10 μL.

2.3. Mass spectrometric conditions

An API 3000 triple quadrupole mass spectrometer (AB Sciex, Framingham, MA, USA) with an electrospay ionization source operating in the positive ion mode was used. The nebulizer gas (air), curtain gas and collision gas (both nitrogen) were set at 8.0, 10 and 8.0 psi, respectively. The ion spray voltage was 5.5 kV and the ion source temperature was 550 °C. For quantification, multiple reaction monitoring (MRM) chromatograms were acquired. Table 2 shows the analyte specific mass spectrometric parameters, whereas Table 1 shows the mass transitions and potential fragmentation patterns of all analytes and internal standard. Data was acquired and processed using Analyst software (AB Sciex, Framingham, MA, USA).

2.4. Preparation of calibration standards and quality control samples

Stock solutions of all analytes were prepared from independent weightings; one for calibration standards (CAL) and one for validation samples (VS). For each analyte, approximately 1 mg was accurately weighted (compound weighting was corrected for purity) and dissolved in 1 mL of water (tenofovir) or methanol (zidovudine, abacavir, emtricitabine and lamivudine) to obtain 1 mg/mL stock solutions. A capsule containing 200 mg ribavirin was dissolved in 100 mL of water in a volumetric flask to give a 2 mg/mL stock solution. The stock solutions of the internal standards were also prepared in water ($^{13}C_5$-ribavirin and $^{2}H_6$-tenofovir) or methanol ($^{13}C,^{2}H_3$-zidovudine and $^{15}N_2,^{13}C$-lamivudine) with an approximate concentration of 1 mg/mL.

For the preparation of the calibration standards, working solutions in the range from 400 to 50,000 ng/mL (zidovudine, lamivudine and tenofovir), 80 to 10,000 ng/mL (abacavir and emtricitabine) and 3200 to 400,000 ng/mL (ribavirin) were used. The working solutions were prepared by diluting the stock solutions of all analytes in water. To obtain calibration standards, 50 μL of the working solutions was added to 950 μL of control human plasma. In this way, calibration standards in the range from 20 to 2500 ng/ml for zidovudine, lamivudine and tenofovir, 4 to 500 ng/mL for abacavir and emtricitabine and 160 to 20,000 ng/mL for ribavirin were prepared.

For the preparation of the validation samples working solutions in the range of 400–40,000 ng/mL (zidovudine, lamivudine and tenofovir), 80–8000 ng/mL (abacavir and emtricitabine) and 3200–320,000 ng/mL (ribavirin) were prepared by dilution of independently prepared stock solutions. To obtain validation samples of 20, 60, 200 and 2000 ng/mL for zidovudine, lamivudine and tenofovir, 4, 12, 40 and 400 ng/mL for abacavir and emtricitabine and 160, 480, 1600 and 16,000 ng/mL for ribavirin 50 μL of the working solution was added to 950 μL of plasma. The stock and working solutions were stored at nominally −20 °C until use.

2.5. Sample pre-treatment

To precipitate plasma proteins 100 μL 0.1% (v/v) formic acid in methanol was added to 50 μL plasma. Before precipitation, 10 μL internal standard working solution with 3000 ng/mL $^{13}C,^{2}H_3$-zidovudine, 5000 ng/mL $^{15}N_2,^{13}C$-lamivudine, 4000 ng/mL $^{2}H_6$-tenofovir and 24,000 ng/mL $^{13}C_5$-ribavirin in water was added. After vortex mixing for 10 s, samples were centrifuged at 15,000 × *g* for 5 min and 100 μL of the supernatant was transferred to an Eppendorf and evaporated to dryness. The residue was reconstituted in 100 μL reconstitution solution containing 95% mobile

Table 2
Analyte specific mass spectrometric parameters for the analysis of all analytes.

Analyte specific parameter	Zidovudine	Abacavir	Emtricitabine	Lamivudine	Tenofovir	Ribavirin
Parent mass (*m/z*)	268	287	248	230	288	245
Product mass (*m/z*)	127	191	130	112	176	113
Declustering potential (V)	10	10	10	8	20	20
Focussing potential (V)	50	50	50	50	50	50
Entrance potential (V)	5	15	4	10	11	10
Collision energy (V)	15	50	15	19	35	15
Collision exit potential (V)	12	14	12	6	16	10
Dwell time (ms)	100	100	100	100	100	100
Typical retention time (min)	7.0	6.6	6.4	3.7	2.8	1.9

phase A and 5% mobile phase B. This solution was transferred to an autosampler vial and 10 μL was injected onto the column. The internal standard working solution was stored at nominally −20 °C until use, whereas the reconstitution solution was stored at room temperature.

2.6. Validation

For the validation of the assay the linearity, accuracy, precision, specificity, selectivity, recovery, matrix effect, carry over and stability under several conditions were determined according to the FDA and EMA guidelines for validation of bioanalytical assays [31,32].

3. Results

3.1. Assay development

To find the optimal balance in chromatography, sample pretreatment and mass spectrometric parameters for the studied compounds, several points of consideration were identified. First, an analytical column with stability under highly aqueous mobile phase conditions was required to obtain adequate retention of the highly polar analytes ribavirin and tenofovir, thereby minimizing ionization effects. Furthermore, an extraction procedure for polar analytes from plasma was required. Finally, an acidic mobile phase was preferred to increase the MS/MS signal of all N(t)RTIs, since most analytes are not ionized at basic pH.

Two different columns (Synergy Polar reversed phase C18 and Synergy Fusion reversed phase C18), with polar retention capacities and aqueous stability, were tested with different acidic mobile phases of whom the Synergy Polar reverse phase C18 showed best peak shape and retention with 0.1% (v/v) formic acid in water and 0.1% (v/v) formic acid in methanol in a stepwise gradient, allowing analytes with different polarities to elute within 11 min (see Fig. 1).

Previously, adequate extraction recoveries for tenofovir have been found using solid phase extraction [17,18,23,25]. However, solid phase extraction is a time-consuming procedure and therefore a simple protein precipitation was preferred. Evaluation of several protein precipitation solvents showed the best extraction recoveries for tenofovir (90–100%) in 0.1% formic acid in methanol, although recoveries for ribavirin (~50%) were relatively low with this extraction solvent. However, this extraction solvent was chosen, since high tenofovir extraction recovery was required to increase sensitivity, whereas for ribavirin this was not critical due to relatively high plasma concentrations (2500 ± 900 ng/mL) obtained in clinical samples [33]. After precipitation, reconstitution in an acidic aqueous solution was required to prevent peak tailing of tenofovir.

The fragmentation pattern of the NRTIs typically showed the loss of the purine (abacavir) or pyrimidine (emtricitabine, lamivudine, zidovudine and ribavirine) moiety from the sugar group, whereas the NtRTI tenofovir loses the phosphomethoxy group (see Table 2).

During assay development an interfering endogenous peak was observed at the transition windows of ribavirin in all plasma batches, which has been observed before [28–30]. Most likely, the peak in the transition window of ribavirin originated from uridine, which has a similar parent and product ion as ribavirin [34]. Since the uridine peak co-eluted with ribavirin attempts were made to identify a specific product ion for ribavirin. However, due to a high similarity in chemical structure of ribavirin and uridine this was unsuccessful. Acceptable separation of the two peaks was achieved by increasing the aqueous mobile phase content from 90% to 98% at the start of the gradient. This gradient was also able to separate the endogenous peak at the transition windows of emtricitabin.

3.2. Validation

3.2.1. Linearity

Eight non-zero calibration samples were prepared and analyzed in duplicate in three separate runs. The best model that described the linear regression of the ratio of the areas of the analyte and the internal standard peaks versus the concentration was fitted using linear regression with a weighting factor of $1/x^2$. The assay was linear over the validated range from 20 to 2500 ng/mL for zidovudine, lamivudine and tenofovir, 4–500 ng/mL for abacavir and emtricitabine and 160–20,000 ng/mL for ribavirine. In accordance with the FDA and EMA guidelines, calibration curves were accepted if two-thirds of the non-zero calibration standards, including a lower limit of quantification (LLOQ) and an upper limit of quantification (ULOQ), have a deviation within ±15% of nominal [31,32]. All calibration curves met these acceptance criteria and a correlation coefficient (r^2) of at least 0.993 was obtained.

3.2.2. Accuracy and precision

Accuracy and precision were assessed by quantification of validation samples with analyte concentrations at the LLOQ and in the low, mid and high ranges of the calibration curves. Each validation sample was analyzed in 5 replicates in 3 separate analytical runs. Table 3 summarizes the inter-assay performance for the tested concentration levels. The intra-assay accuracies and precisions were between −8.23% and 14.2% for zidovudine, emtricitabine and ribavirin. For abacavir, lamivudine and tenofovir, the intra-assay accuracies and precisions at the LLOQ level were between −11.0% and 18.3%, whereas at the all other levels these accuracies and precisions were between −11.7% and 12.0%. Therefore, the accuracy and precision were within the acceptance criteria (within ±20% at the LLOQ and ±15% for the low, mid and high concentrations) of the FDA and EMA guidelines [31,32].

3.2.3. Selectivity

Six different batches of control drug-free plasma were prepared as double blanks and spiked at the LLOQ level to determine whether endogenous compounds from plasma interfered with the detection of the analytes or internal standards. Peaks appearing in

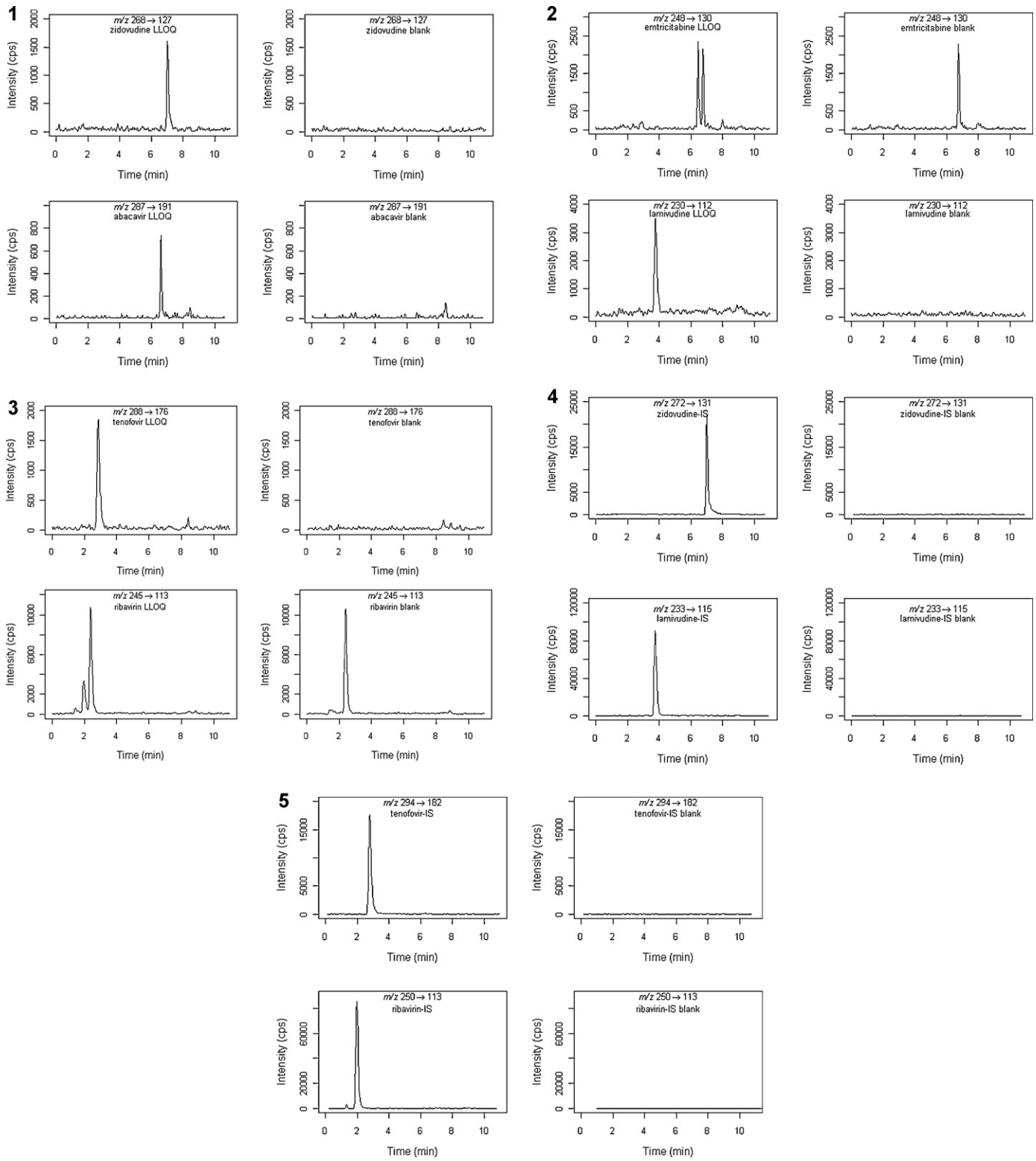


Fig. 1. Multiple reaction monitoring chromatograms of the LLOQ (left) and a blank (right) of respectively zidovudine, abacavir, emtricitabine, lamivudine, tenofovir, ribavirin, $^{13}C,^{2}H_3$-zidovudine (zidovudine-IS), $^{15}N_2,^{13}C$-lamivudine (lamivudine-IS), $^{2}H_6$-tenofovir (tenofovir-IS) and $^{13}C_5$-ribavirin (ribavirin-IS).

double-blank samples of these batches and co-eluting with an analyte or internal standard were maximally 9.2%, 5.3% and 6.5% of the LLOQ peak area of respectively lamivudine, emtricitabine and zidovudine and 0.2% and 0.1% of the peak area of $^{15}N_2,^{13}C$-lamivudine and $^{13}C,^{2}H_3$-zidovudine, respectively. For abacavir, tenofovir, ribavirin, $^{2}H_6$-tenofovir, $^{13}C_5$-ribavirin no interferences were found. Additionally, at least two-thirds of the LLOQ levels were within ±20%. Therefore, selectivity was found to be acceptable [31,32].

Cross-analyte interference was tested by spiking plasma with one of the analytes (at the ULOQ level) or at the level of the internal standards used in the method. The maximum cross-analyte

Table 3
Assay performance data for zidovudine, abacavir, emtricitabine, lamivudine, tenofovir and ribavirin.

Analyte	Nominal concentration (ng/mL)	Inter-assay accuracy (% DEV)	Within-run precision (% CV)	Between-run precision (%CV)	No. of replicates
Zidovudine	20.0	7.35	9.81	6.13	15
	60.0	0.73	7.81	[a]	15
	200	−1.28	4.66	[a]	15
	1999	−0.05	7.52	4.12	15
Abacavir	3.76	4.63	16.4	9.23	15
	11.3	−1.64	11.9	[a]	15
	37.6	−3.05	6.13	2.88	15
	376	−8.47	6.72	3.38	15
Emtricitabine	4.06	0.10	10.6	6.16	15
	12.2	1.78	8.04	4.88	15
	40.6	1.23	9.46	[a]	15
	406	−0.48	6.83	[a]	15
Lamivudine	20.3	5.46	18.3	3.98	15
	60.8	2.41	6.91	2.81	15
	203	5.36	4.66	[a]	15
	2026	−7.04	5.68	[a]	15
Tenofovir	19.8	−1.20	16.2	7.61	15
	59.3	−4.88	5.86	[a]	15
	198	−1.20	4.03	3.07	15
	1975	5.59	6.14	3.78	15
Ribavirin	160	−3.21	10.4	6.14	15
	480	−1.21	5.59	[a]	15
	1600	−2.67	4.64	1.63	15
	16,000	−0.38	6.60	[a]	15

[a] The between-run precision could not be calculated (mean square between groups is less than mean square within groups) DEV, deviation; CV, coefficient of variation; no., number.

interference that was observed originated from emtricitabine, generating a peak of 13.5% of the LLOQ of zidovudine at the transition window of zidovudine. The internal standards did not generate a peak at the transition window of the corresponding analyte. Therefore, the cross-analyte and internal standard interferences were considered acceptable since the interferences were less than 20% of the peak area of the analyte at the LLOQ level and less than 5% for the internal standards [31,32].

3.2.4. Recovery and matrix factor

Recovery of all analytes were determined at three concentrations in triplicate by comparing the analyte-internal standard area ratio of the processed validation samples (supernatant obtained after protein precipitation) with those of processed blank samples spiked with analyte. Table 4 shows that recovery was high (80.6–111%) and reproducible (RSD ≤ 11.3%) at all concentration levels for all analytes, except for ribavirin. The mean recovery of ribavirin was 49% with a good reproducibility (RSD ≤ 8.0%), which was considered acceptable, as mentioned before.

The matrix factor (e.g. ion suppression or enhancement) was examined by comparing the analyte area of processed samples with samples processed in water at three concentrations in triplicate in one batch for zidovudine, lamivudine, tenofovir and ribavirin. These four analytes are quantified using an isotopically labeled internal standard. Therefore, assessment of the matrix factor in one batch was considered acceptable [35]. The mean matrix factor was 1.10, 1.06, 0.90 and 1.46, respectively, whereas the internal standard-normalized matrix factor was close to 1 (1.10, 0.90, 1.0 and 1.01, respectively). This means the internal standards corrected for possible matrix effects. For abacavir and emtricitabine, the matrix factor was examined in 6 different batches. The mean internal standard-normalized matrix factor was 1.45 and 1.24, respectively, with a CV ≤ 12.1%, which is within the acceptance criteria [35].

3.2.5. Carry-over

Carry-over was tested by injecting two processed blank matrix samples sequentially after injecting an ULOQ sample. For zidovudine, lamivudine, tenofovir and ribavirine no carry-over was observed, whereas for emtricitabine and abacavir the carry-over detected in the first blanc matrix was 6.4% and 17.2% of the response detected in a LLOQ sample. There was no carry-over for all internal standards in the first blank matrix. The carry-over of the analytes and internal standards was found to be acceptable [31,32].

3.2.6. Stability

The stability of all analytes was investigated during various steps of the analysis. The stock solution stability was investigated after storage at −20 °C. The stock solutions were considered stable when 90–110% of the nominal concentration was found compared to the freshly prepared stock solution. All stocks fulfilled these criteria after the indicated storage times (see Table 5).

The stability of all analytes in plasma was investigated in triplicate at low and high concentration levels and the analytes were considered stable if the difference was within ±15% when calculated against the initial concentration. After three freeze–thaw cycles at −20 °C and after 72 h at room temperature all analytes were stable (see Table 4). Long term stability in plasma at −20 °C is ongoing, although previous studies showed stability of zidovudine, abacavir, emtricitabine, lamivudine and tenofovir up to 6 months at −20 °C [22]. Ribavirin was stable for at least 5 months at −20 °C [29].

The final extract stability was determined after storage at 2–8 °C at three concentrations in triplicate and considered stable after 3 days (−9.1 to 5.3% of the initial concentration was recovered when compared with freshly prepared calibration standards).

3.2.7. Reinjection reproducibility

Reinjection of the validation samples low, mid and high in triplicate was reproducible after 22 days of storage at 4 °C since

Table 4
Extraction recoveries for zidovudine, abacavir, emtricitabine, lamivudine, tenofovir and ribavirine in human plasma.

Analyte	Nominal conc. (ng/mL)	Mean recovery (%)	RSD (%)	No. of replicates
Zidovudine	60.0	103	5.12	3
	200	98.0	10.6	3
	1999	107	9.89	3
Abacavir	11.3	104	11.3	3
	37.6	99.6	11.0	3
	376	111	2.02	3
Emtricitabine	12.2	82.4	8.38	3
	40.6	80.9	8.43	3
	406	96.5	4.28	3
Lamivudine	60.8	111	3.66	3
	203	99.7	8.36	3
	2026	107	6.36	3
Tenofovir	59.3	108	6.12	3
	198	108	6.36	3
	1975	106	4.60	3
Ribavirin	480	47.8	2.56	3
	1600	47.1	4.22	3
	16,000	52.4	7.99	3

Conc., concentration; RSD, relative standard deviation; no., number.

Table 5
Stability of zidovudine, abacavir, emtricitabine, lamivudine, tenfovir and ribavirin in working solution and biomatrix.

Matrix	Condition	Analyte	Initial conc. (ng/mL)	Meas conc. (ng/mL)	DEV (%)	CV (%)	No. of replicates
Methanol (stock)	12 months, −20 °C	Zidovudine	1,000,000	1,037,200	3.72	10.1	3
	26 months, −20 °C	Abacavir	1,000,000	949,200	−5.08	1.41	3
	33 months, −20 °C	Emtricitabine	1,000,000	1,096,068	9.61	0.90	3
		Lamivudine	1,000,000	1,033,300	3.33	4.42	3
Water (stock)	12 months, −20 °C	Tenofovir	1,000,000	982,700	−1.73	2.74	3
	3 months, −20 °C	Ribavirine	2,000,000	2,025,800	1.29	3.94	3
Plasma	3 freeze (−20 °C)/thaw cycles	Zidovudine	60.0	64.1	6.87	5.32	3
		Zidovudine	1999	1890	−5.45	9.73	3
		Abacavir	11.3	12.0	6.18	5.56	3
		Abacavir	376	340	−9.61	12.7	3
		Emtricitabine	12.2	13.6	11.8	12.2	3
		Emtricitabine	406	392	−3.67	11.7	3
		Lamivudine	60.8	66.0	8.53	4.36	3
		Lamivudine	2026	1960	−3.26	7.94	3
		Tenofovir	59.3	59.5	0.40	1.66	3
		Tenofovir	1975	2135	8.10	7.62	3
		Ribavirine	480	477	−0.63	4.80	3
		Ribavirine	16,000	16,950	5.94	2.09	3
	72 h RT	Zidovudine	60.0	61.4	2.42	11.9	3
		Zidovudine	1999	2040	2.05	6.24	3
		Abacavir	11.3	11.5	1.75	11.0	3
		Abacavir	376	352	−6.28	5.22	3
		Emtricitabine	12.2	13.7	12.1	0.84	3
		Emtricitabine	406	421	3.47	6.56	3
		Lamivudine	60.8	68.9	13.3	1.34	2
		Lamivudine	2026	2025	−0.05	6.64	3
		Tenofovir	59.3	61.1	3.05	9.42	3
		Tenofovir	1975	2090	5.82	2.71	3
		Ribavirine	480	467	−2.78	4.86	3
		Ribavirine	16,000	16,100	0.63	4.39	3
Final extract	3 days, 2–8 °C	Zidovudine	60.0	54.5	−9.08	7.15	3
		Zidovudine	1999	1873	−6.29	6.41	3
		Abacavir	11.3	11.2	−0.62	4.72	3
		Abacavir	376	334	−11.2	5.16	3
		Emtricitabine	12.2	12.8	5.28	2.95	3
		Emtricitabine	406	412	1.30	11.1	3
		Lamivudine	60.8	60.2	−1.01	9.02	3
		Lamivudine	2026	1947	−3.92	12.5	3
		Tenofovir	59.3	58.1	−1.96	4.56	3
		Tenofovir	1975	1977	0.08	3.09	3
		Ribavirine	480	479	−0.21	5.68	3
		Ribavirine	16,000	15,500	−3.13	1.94	3

Conc., concentration; Meas., measured; DEV, deviation; CV, coefficient of variation; NA, not applicable; no., number; RT, room temperature.

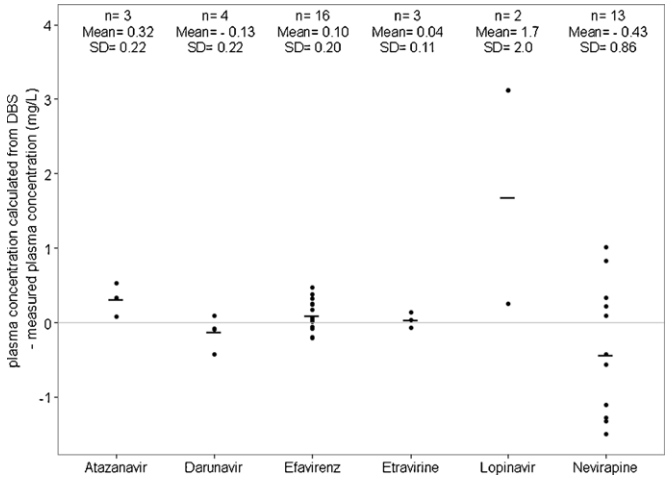


Fig. 2. Observed concentrations in 36 plasma samples (● represents an individual concentration and – represents the median).

88.2–108% of all analytes at the nominal concentration was recovered.

3.3. Clinical application

This method has successfully been used in clinical practice for therapeutic drug monitoring in specific situations (e.g. suspicion of non-adherence and renal failure) and in a clinical trial. Fig. 2 shows the concentrations observed in 36 plasma samples collected on different time points from 19 patients. In total, 10 patients were HBV infected and on tenofovir monotherapy, 2 patients were HIV/HVC co-infected and received ribavirin together with tenofovir and emtricitabine, whereas the other 7 patients were HIV infected and received either a combination of tenofovir and emtricitabine ($n = 3$), abacavir and lamivudine ($n = 2$) or lamivudine and zidovudine ($n = 2$).

4. Discussion

The described bioanalytical method provides an efficient tool for therapeutic drug monitoring of zidovudine, abacavir, emtricitabine, lamivudine, tenofovir and ribavirin and is the first method to quantify these seven analytes simultaneously.

Previously, seven assays have been published describing the simultaneous determination of NRTIs and tenofovir, with its different physical–chemical properties, in plasma [13,16,17,21–23,25]. Three of these assays, analyzing only two analytes, showed a very short run time, but required a solid phase extraction procedure which can be time consuming and expensive since trained personnel and specialized equipment is required [17,23,25]. Most likely, this extensive sample pretreatment was required to obtain a clean extract since no separation of endogenous compounds on the column was obtained with these short run times. However, for therapeutic drug monitoring purposes a simple sample pretreatment is preferable. Assays using a simple protein precipitation have been described [13,16,21,22]. However, two of these assays required large sample volume (≥200 μL) for their analysis, whereas Kuklenyik et al. used a system which required three isocratic pumps which can be problematic in a regular hospital setting [13,16,21]. Thus, only the assay from Le Saux et al. appeared to be practically applicable for therapeutic drug monitoring purposes. However, the currently described assay has a shorter run time, requires less sample volume and allows the simultaneous quantification of ribavirin, making this assay ideal for clinical application [22].

5. Conclusion

A bioanalytical method for the determination of zidovudine, abacavir, emtricitabine, lamivudine, tenofovir and ribavirine has been developed. This is the first method describing the simultaneous quantification of the anti-HCV drug ribavirin together with the anti-HIV drugs zidovudine, abacavir, emtricitabine, lamivudine and tenofovir. The method is simple, sensitive, specific and reproducible and can be used in clinical practice to monitor N(t)RTI plasma concentrations in selected cases to optimize therapy.

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