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Identification and Quantification of Antiretroviral Drugs in Simulated Bodily Fluids using HPLC–MS

Experiment carried out on 20th March 2025

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1. Abstract

High-performance liquid chromatography with mass spectrometry (HPLC–MS) was utilised to identify and quantify antiretroviral (ARV) drugs in a simulated bodily fluid. Calibration curves were constructed, within a range of concentrations from 1–100 μg mL^{-1} for the ARV drugs Efavirenz, Emtricitabine, Lopinavir, Ritonavir, and Tenofovir disoproxil fumarate (TDF). Analysis of an unknown sample revealed the presence of Lopinavir and Ritonavir, identified by comparison of retention times (RT). Concentration values of these drugs were determined using the experimental calibration data. The results demonstrated the effectiveness of HPLC–MS for the detection and quantification of ARV drugs in simulated bodily fluid.

2. Introduction

Antiretroviral (ARV) drugs are a significant class of compounds developed to prevent the replication of RNA viruses, most prominently the Human Immunodeficiency Virus (HIV). Although structurally diverse, these drugs are functionally grouped into four main functional classes-nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), integrase strand transfer inhibitors (INSTIs), and protease inhibitors (PIs). NRTIs incorporate into the viral DNA, terminating chain elongation; NNRTIs bind allosterically to the reverse transcriptase enzyme; INSTIs prevent integration of viral DNA into the host genome; and PIs inhibit the maturation of infectious viral particles.[1,2]

Current HIV treatment regimens are based on combination antiretroviral therapy (cART), which involves the simultaneous use of multiple drug classes. This approach effectively reduces the chance of viral mutations that could lead to drug resistance.[3] For instance, the World Health Organization previously reported recommendations of Dolutegravir as the preferred first line and second-line treatment for all populations.[4] This combination includes Emtricitabine and Tenofovir Disoproxil Fumarate (TDF), both classified as nucleoside reverse transcriptase inhibitors, and Dolutegravir, an integrase inhibitor.

HPLC–MS is widely used for therapeutic drug monitoring and pharmacokinetic profiling of ARVs due to its high sensitivity, selectivity, and ability to analyse complex biological matrices.[5] Accurate quantification of ARV levels is essential in therapeutic drug monitoring to ensure efficacy while avoiding resistance or toxicity. In this experiment, HPLC–MS was employed to identify and quantify ARVs in a simulated bodily fluid. Calibration curves were generated for five standard ARV drugs-Efavirenz, Emtricitabine, Lopinavir, Ritonavir, and TDF over a concentration range of 1–100 μg mL^{-1}. An unknown sample (MK–U) containing two unidentified ARVs was analysed to evaluate the method's suitability for quantitative ARV analysis.

3. Experimental

3.1 Chemicals and Reagents

All analytical standards were provided as part of the laboratory materials and included the following ARV drugs: Efavirenz, Emtricitabine, Lopinavir, Ritonavir, and Tenofovir Disoproxil Fumarate. Stock solutions of each ARV were freshly prepared in methanol and water (1:1) at concentrations of 1 mg mL^{-1} and were further diluted to prepare calibration standards ranging from 1–100 μg mL^{-1} as shown in Table 1. All solvents used were of HPLC grade. Deionised water was used throughout for aqueous preparations.

Table 1 Serial dilution scheme for preparation of calibration concentrations

Concentration (μg mL^{-1})	Solution Used	Volume of Solution (mL)	Volume of Solvent (mL)
100	cART SS	1.00	9.00
50	100 μg mL^{-1}	5.00	5.00
25	50 μg mL^{-1}	5.00	5.00
10	100 μg mL^{-1}	1.00	9.00
1	10 μg mL^{-1}	1.00	9.00

3.2 Instrumentation and Analytical Conditions

Chromatographic separation was performed on an ACE 5 C18 column (15.00 cm × 2.10 mm, 5.00 μm particle size, Advanced Chromatography Technologies, UK). The column temperature was maintained at ambient laboratory conditions. The autosampler was set to an injection volume of 5.00 μL.

The mobile phase consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). Gradient elution was carried out in line with the standard method, which includes re-equilibration and column wash steps intended to minimise carryover and promote reproducibility.

The HPLC system used was an Agilent 1100 Series alongside a Thermo Scientific LTQ Orbitrap XL mass spectrometer, operating in positive electrospray ionisation mode (ESI). Data was collected across the full mass range relevant to each analyte's molecular ion and processed using standard instrument software protocols.

3.3 Calibration Curve Preparation

Calibration curves at five concentrations (1–100 μg mL^{-1}) were prepared from the serial dilutions, as outlined in Table

1. Each concentration was analysed via HPLC–MS, all standard injections were performed using the Agilent 1100 Series HPLC system and LTQ Orbitrap XL mass spectrometer as described. For each ARV compound, peak areas were assigned based on their respective retention times.

The simulated bodily fluid used for unknown sample preparation consisted of a 1:1 methanol-water mixture. This solvent system was selected for its compatibility with HPLC–MS analysis and allowed for consistent preparation of all solutions without the complications associated with biological matrices.

A single blank sample was run prior to each set of five standard injections to establish a background, and the resulting peak area values were subtracted from all calibration peaks to obtain adjusted peak areas. These adjusted areas (AA) were then plotted against concentration to generate calibration curves. The slope, intercept, and R^2 values were recorded for quantitative analysis of unknown samples.

3.4 Unknown Sample Analysis

The unknown sample labelled MK–U was analysed using the established HPLC–MS method at the same time as the calibration concentrations. The chromatographic signals were screened for all five ARV drugs by comparing the RT against the standard compounds.

Two analytes were successfully identified based on strong matches in retention time, Lopinavir and Ritonavir. The AA for each detected drug was adjusted by subtracting the corresponding blank AA obtained at the start of the run. These adjusted areas were substituted into the calibration curve equations to estimate the concentrations of each drug present in the unknown sample as shown by *Equation (1).*

$$y = mx + c \longrightarrow x = \frac{y - c}{m} \qquad (1)$$

y = Adjusted peak area of the unknown sample
m = Slope of the calibration curves line of best fit
c = Intercept of the calibration curve
x = Concentration of analyte (μg mL^{-1})

The unknown concentration calculations were compared against the validated calibration range (1–100 μg mL^{-1}), and quantification was reliability assessed using residual plots and the linearity of the calibration curves.

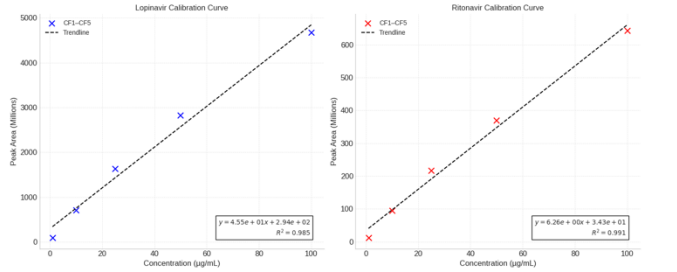
4. Results and Discussion

4.1 Calibration Curves

Experimental HPLC–MS data obtained, as shown in Table A1, were used to generate standard calibration curves for the five ARV drugs: Efavirenz, Emtricitabine, Lopinavir, Ritonavir and TDF. Each drug was analysed at five concentrations between 1 and 100 μg mL^{-1} (CF1-CF5), with AA plotted against concentration to form linear models. During processing, CF3 and CF4 were identified as mislabelled; this was corrected to maintain consistency.

All five calibration curves demonstrated strong linearity across the validated concentration range. Coefficients of determination (R^2) ranged from 0.954 for Emtricitabine to 0.996 for Ritonavir, whilst values above 0.99 for most analytes indicate excellent correlation between AA and concentration. The lower R^2 for Emtricitabine suggests slightly greater variability in the data and potentially reduced predictive reliability. Slope and intercept values for each regression model were recorded. Representative calibration plots are shown in Fig. 1 full-scale data are provided in Fig. B1 in Appendix B. These models were later used to calculate concentrations in the unknown MK–U sample.

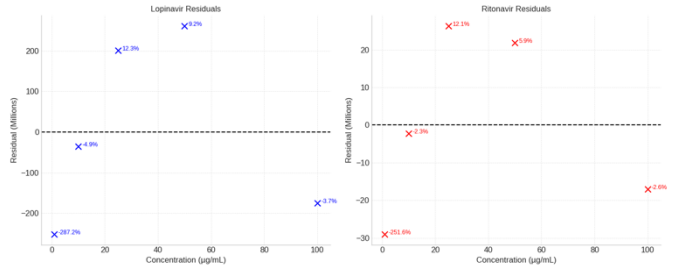
Fig. 1 Calibration curves for Lopinavir and Ritonavir (1–100 μg mL^{-1}), with linear regression applied to adjusted peak areas (AA).



4.2 Residuals and Error Analysis

To assess how well the linear model fit the data, residuals, which are the differences between the observed and predicted peak areas were plotted. Ideally, these residuals should scatter randomly around zero. Any clear pattern might suggest a flaw in the setup or model. [6]

Fig. 2 Residual plots for Lopinavir and Ritonavir calibration curves, showing non-random residual trends across concentration points, with percentage error values displayed for each.



The residual plots for both Ritonavir and Lopinavir displayed a clear, non-random pattern across the calibration range, as shown in Fig. 2. For both analytes, negative residuals were observed at the lowest (1 μg mL^{-1}), highest (100 μg mL^{-1}), and one intermediate concentration (10 μg mL^{-1}), with the residual at 10 μg mL^{-1} being closest to zero. In contrast, the mid-range concentrations (25 and 50 μg mL^{-1}) exhibited positive

residuals, forming a consistent curved trend across both compounds.

This shared residual profile suggests the presence of systematic error, potentially arising from pipetting inaccuracies during dilution preparation, slight detector non-linearity, or injection sequence effects. [6] Which may slightly affect model predictive accuracy, particularly at concentration extremes. The similarity in behaviour across both compounds reinforces the likelihood of a procedural or instrumental factor rather than compound variability.

To support interpretation, percentage error values were included for each calibration point. Most values, particularly in the mid to high concentration range (10-100 μg mL^{-1}), were within ±15%, which falls within accepted criteria for nominal concentrations. [7] The lowest concentration (1 μg mL^{-1}) displayed a markedly higher relative error, influenced by reduced signal-to-noise ratio and increased background variability. At this level, the error exceeded ±100%, significantly surpassing the accepted ± 20%, for the lower limit of quantification (LLOQ).[7] Despite this, the calibration models were consistent and reliable across the remaining validated range, though results near the LLOQ require cautious interpretation.

4.4 LOD and LOQ Analysis

The MK–U sample was analysed using calibration curves generated from experimental data to estimate analyte concentrations. Five replicate injections were performed, and the resulting chromatograms were examined for signals matching the expected RT of the target antiretroviral drugs.

Initial analysis indicated the presence of four ARVs: Lopinavir (RT ≈ 18.74 min), Ritonavir (RT ≈ 18.14 min) Emtricitabine (RT ≈ 3.29 min), and TDF (RT ≈ 13.39 min). These were identified with high confidence based on retention time; however, the presence of signal alone was not deemed sufficient for confident quantification.

$$\mathrm{LOD} = \frac{3.3 \times \sigma}{S} \quad and \quad \mathrm{LOQ} = \frac{10 \times \sigma}{S} \qquad (2)$$

To assess whether the observed signals could be reliably quantified, the limits of detection (LOD) and limits of quantification (LOQ) were calculated for each analyte using *Equation (2).* [8] These values were estimated using the standard deviation of baseline noise (σ) obtained from blank injections, along with the slope of the corresponding calibration curve.

The adjusted peak areas from the MK–U sample, shown in Table A2, were compared against the calculated LOQ and LOD values shown in Table 2 to determine which analytes could be reliably quantified.

Table 2 Limit of detection (LOD) and limit of quantification (LOQ) values, calculated using blank noise (σ) and calibration slope (m)

Drug	Slope (m)	σ (Noise Estimate)	LOD (μg mL^{-1})	LOQ (μg mL^{-1})
Lopinavir	4550000	43300	0.031	0.095
Ritonavir	6260000	43700	0.023	0.070
TDF	29100000	71600	0.008	0.025
Efavirenz	916000	4930	0.018	0.054
Emtricitabine	2420000	12600	0.017	0.052

Although signals were observed for TDF and Emtricitabine, their adjusted peak areas were significantly lower than those of Lopinavir and Ritonavir. This may reflect either trace-level presence, background interference, systematic error or contamination in sample preparation making reliable quantification uncertain. Consequently, these two analytes were qualitatively identified but not quantified. No measurable signal was detected for Efavirenz.

In contrast, the adjusted peak areas for Lopinavir and Ritonavir significantly exceeded their corresponding LOQ and LOD thresholds, supporting reliable quantification. As a result, these two drugs were identified as the confirmed ARV drugs of the unknown MK–U sample.

4.5 Quantification of Unknown Sample (MK–U)

Quantification was carried out using the calibration curves in Fig. 1. Adjusted peak areas from the MK–U injections were compared to the calibration curves and concentrations were calculated using *Equation (1)*.

Ritonavir:

$$x = \frac{1.98 \times 10^9 - 2.94 \times 10^4}{6.26 \times 10^6} = \mathbf{310.58\ \mu g\ mL^{-1}} \qquad (3)$$

Lopinavir:

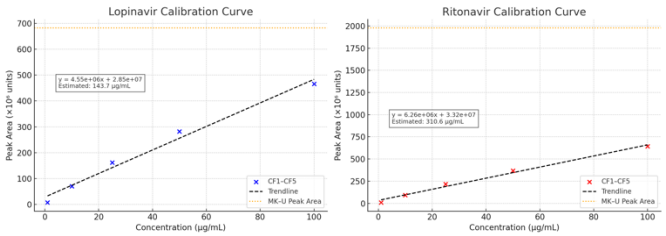
$$x = \frac{6.82 \times 10^8 - 3.43 \times 10^4}{4.55 \times 10^6} = \mathbf{143.66\ \mu g\ mL^{-1}} \qquad (4)$$

Both Ritonavir and Lopinavir were quantified at concentrations exceeding the validated calibration range of 1–100 μg mL^{-1}, with estimated values of 310.6 μg mL^{-1} and 143.7 μg mL^{-1}, respectively shown by *Equations (3) and (4*).

Given that the MK–U sample was prepared using diluted ARV standards intended to remain within this range, the observed values likely reflect a systematic error during preparation such

as pipetting error, incorrect dilution or use of undiluted stock solution.

Fig. 3 Lopinavir and Ritonavir calibration curves (1–100 μg mL^{-1}) with MK–U sample points overlaid to determine estimated concentrations.



While these concentrations fall outside the validated quantification range and should be interpreted with caution, the method itself demonstrated high sensitivity and consistent linear response across the tested range. The findings support the method's suitability for ARV identification and quantification in simulated biological matrices, with the caveat that results beyond calibration boundaries require further validation through dilution and reanalysis.

5. Conclusion

High-performance liquid chromatography with mass spectrometry (HPLC–MS) was used to identify and quantify antiretroviral drugs in a simulated bodily fluid. Calibration curves were generated for five ARVs and showed strong linearity across the tested range, with all R^2 values above 0.95. Residual and error analysis suggested good fit overall, though some deviations were noted at intermediate concentrations and high error at the lower limit of quantification (LLOQ).

When applied to the unknown sample (MK–U), Ritonavir and Lopinavir were successfully identified based on consistent retention times and high adjusted peak areas. TDF and Emtricitabine were also detected, but their responses fell below quantifiable thresholds. Efavirenz was not observed in the sample. Lopinavir was quantified at an estimated concentration of 143.7 μg mL^{-1}, and Ritonavir at 310.6 μg mL^{-1}, both significantly exceeding the validated calibration range of 1–100 μg mL^{-1}.

Given that the unknown was prepared using diluted standards intended to remain within the validated range, these values are likely the result of procedural or systematic error. Potential causes include inaccurate dilution from intermediate stocks as described in Table 1, unintended over-spiking, or failure to adjust the total sample volume following the addition of multiple drug standards. Additionally, no replicate runs were performed in this experiment, limiting assessment of precision and repeatability.

Despite these discrepancies, the method demonstrated strong sensitivity and performance for ARV identification in simulated matrices. In future iterations of this experiment, the solution preparation protocol would be revised to favour direct dilutions from original stock solutions, thereby reducing compounding error. Further improvements should include replicate injections, verification of dilution accuracy, and the use of internal standards to support reliable quantification and enhance method accuracy.

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Appendix A - Raw Data Tables

Table A1 Raw calibration data (CF1-CF5) for five antiretroviral drugs, including peak area (AA), noise level (NL), retention time (RT), and adjusted values

Drug	Conc. (μg mL^{-1})	Sample AA	Sample NL	RT (min)	Blank AA	Blank NL	Adjusted AA
Efavirenz	1.00	480,485.87	53,179.00	19.25	27,563.80	2,399.81	452922.06
	10.00	3,908,004.80	486,447.56	19.24	27,563.80	2,399.81	3,880,441.00
	25.00	16,440,300.86	2,079,307.15	19.24	27,563.80	2,399.81	16,412,737.06
	50.00	9,640,076.60	1,246,068.84	19.24	27,563.80	2,399.81	9,612,512.80
	100.00	26,060,832.85	3,311,988.85	19.24	27,563.80	2,399.81	26,033,269.05
Tenofovir	1.00	8,826,100.54	1,174,734.78	13.37	1,696,868.53	141,915.60	7,129,232.01
	10.00	142,638,709.31	24,531,759.03	13.38	1,696,868.53	141,915.60	140,941,840.78
	25.00	555,957,566.48	100,007,960.99	13.36	1,696,868.53	141,915.60	554,260,697.95
	50.00	318,936,973.78	58,109,857.86	13.39	1,696,868.53	141,915.60	317,240,105.25
	100.00	1,707,870,631.12	291,754,657.02	13.36	1,696,868.53	141,915.60	1,706,173,762.59
Ritonavir	1.00	11,525,627.42	1,356,326.15	18.13	1,122,060.93	87,907.85	10,403,566.49
	10.00	94,675,797.59	11,785,602.77	18.10	1,122,060.93	87,907.85	93,553,736.66
	25.00	369,214,518.90	45,639,947.43	18.12	1,122,060.93	87,907.85	368,092,457.97
	50.00	217,109,230.04	27,098,584.59	18.13	1,122,060.93	87,907.85	215,987,169.11
	100.00	643,295,664.40	81,294,709.99	18.12	1,122,060.93	87,907.85	642,173,603.48
Lopinavir	1.00	8,764,950.02	1,033,542.88	18.73	892,628.42	66,458.13	7,872,321.60
	10.00	71,366,257.64	8,820,264.58	18.71	892,628.42	66,458.13	70,473,629.22
	25.00	283,044,063.50	34,669,073.05	18.72	892,628.42	66,458.13	282,151,435.08
	50.00	163,203,690.33	20,342,148.79	18.73	892,628.42	66,458.13	162,311,061.90
	100.00	466,990,594.44	56,624,091.28	18.72	892,628.42	66,458.13	466,097,966.02
Emtricitabine	1.00	17,944,554.19	1,592,633.53	3.29	74,925.16	7,242.75	17,869,629.03
	10.00	64,126,675.92	4,116,823.47	3.40	74,925.16	7,242.75	64,051,750.76
	25.00	146,223,963.80	10,260,066.77	3.28	74,925.16	7,242.75	146,149,038.64
	50.00	101,796,879.37	6,693,517.72	3.33	74,925.16	7,242.75	101,721,954.21
	100.00	217,717,681.56	14,372,391.04	3.27	74,925.16	7,242.75	217,642,756.40

Table A2 LOD and LOQ analysis data for MK–U sample, including retention times, blank response, and adjusted peak areas

Drug	RT (min)	Blank AA	MK–U AA	Adjusted AA
Tenofovir	13.39	192600.99	2747696.93	2555095.93
Ritonavir	18.14	169521.81	1977737053	1977567531.19
Lopinavir	18.74	641903.39	682886810	682244906.61
Emtricitabine	3.29	54165.78	5369268.86	5315103.08
Efavirenz	NF	67416.32	NF	NF

Appendix B - Full Calibration Curve Data

Fig. B1 Full calibration curves for antiretroviral drugs (Efavirenz, Emtricitabine, Lopinavir, Ritonavir, and Tenofovir), showing linear regression fits across the range 1–100 μg mL^{-1}.