RESEARCH PAPER

Liquid–Liquid Extraction versus Protein Precipitation: A Comparative Assessment for Antiretroviral Drug Estimation in Human Plasma

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Abstract

Accurate quantification of antiretroviral (ARV) drugs in plasma is essential for pharmacokinetic studies and therapeutic drug monitoring. Sample preparation significantly influences the selectivity, sensitivity and reproducibility of bioanalytical methods. Among the commonly used techniques, protein precipitation (PPT), liquid–liquid extraction (LLE) and solid phase extraction (SPE) are widely applied prior to liquid chromatography tendem mass spectroscopy (LC–MS/MS) analysis. In this study, we have comparatively evaluated PPT and LLE for the quantification of lamivudine (3TC), abacavir (ABC), lopinavir (LPV), ritonavir (RTV) and Tenofovir (TFV) as Internal Standard (IS) in human plasma using LC–MS/MS technique. Comparison between PPT and LLE is based in terms of extraction recovery, selectivity, sensitivity, cost and time.PPT offers simplicity, rapid processing and cost-effectiveness. In contrast, LLE provides cleaner extracts and improved sensitivity, though it is more labour-intensive and solvent-intensive but sometimes suffers higher matrix interference. The findings suggest that PPT is suitable for high-throughput routine analysis of ARV drugs.

Keywords: ARV Drugs, LC-MS/MS, LLE, PPT, Human Immunodeficiency.

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1.Introduction

Antiretroviral (ARV) drugs play a crucial role in the treatment of Human Immunodeficiency Virus (HIV) infection by inhibiting viral replication and reducing viral load. Accurate quantification of ARVs in plasma is essential for pharmacokinetic studies, bioavailability assessment and therapeutic drug monitoring [(1,2)]. Highly Active Antiretroviral Therapy (HAART) involves a potent combination of at least two or three different antiretroviral (ARV) drugs from two different classes, reducing morbidity and mortality rates [(3-5)]. In June 2022, Cipla introduced Quadrimune, a new "4-in-1" fixed-dose combination drug approved by the South African Health Products Regulatory Authority (SAHPRA) for combating drug resistance [(6)]. A key challenge in developing a single assay for these drugs is their significantly different physicochemical characteristics. For example, protease inhibitors (PIs) like lopinavir and ritonavir are hydrophobic, while nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs/NtRTIs) such as lamivudine, abacavir, and tenofovir are hydrophilic. This disparity makes it difficult to extract all drugs effectively and simultaneously from plasma and to identify a suitable chromatographic column for their separation [(7)]. We developed a highly efficient, selective, and sensitive LC–MS/MS assay for the simultaneous quantification of lamivudine, abacavir, lopinavir, and ritonavir concentrations in plasma.

LC–MS/MS methods are crucial for bioequivalence, pharmacokinetic, toxicokinetic and HIV adherence studies. The performance of these methods relies on efficient sample preparation that accounts for both the diverse physicochemical properties of antiretroviral drugs and the complexity of biological matrices [(8)]. Proteins, lipids, and other endogenous substrates present in biological samples can interfere with target analyte detection. Consequently, accurate sample preparation is a pivotal step in bioanalysis because it eliminates matrix interference, enhances analyte concentration and ultimately improves the analytical system's performance and reliability [(9,10)]. Therefore, sample preparation is a critical step in bioanalytical workflows to enhance selectivity and sensitivity. Among various sample preparation techniques, protein precipitation (PPT) and liquid–liquid extraction (LLE) is the most widely used due to their practicality and effectiveness [(11)]. Each technique has distinct

advantages and limitations depending on the nature of the analyte and analytical requirements.

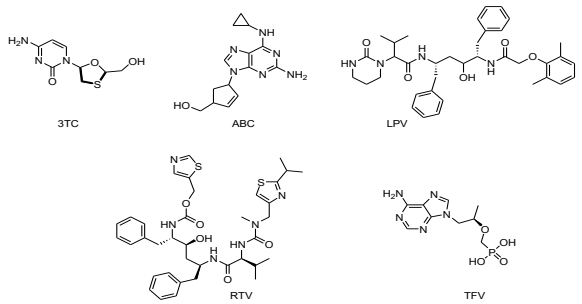

Figure.1: Chemical structures of Lamivudine (3TC), Abacavir (ABC), Lopinavir (LPV), Ritonavir (RTV), Tenofovir (TFV)

2. Materials and Methods

This study presents a comparative evaluation of PPT and LLE based on reported literature and typical bioanalytical workflows used for ARV drug estimation in plasma.

2.1 Chemicals and Reagents:

All chemicals used were of analytical grade. Cipla developed Quadrimune, a capsule containing 15 mg lamivudine, 30 mg abacavir, 40 mg lopinavir, and 10 mg ritonavir. Med Chem Express (MCE) provided analytical grade active pharmaceutical ingredients (APIs) of these drugs, as well as tenofovir, which was used as an internal standard (IS). Methanol, tert-butyl methyl ether (TBME), formic acid and acetonitrile were purchased from Loba Chemie Pvt Ltd. High-purity water was prepared in-house using a Milli-Q water-purification system and plasma was collected in-house from healthy, disease-free human subjects via vein puncture.

2.2 Chromatographic Conditions:

The analysis was performed using a SCIEX API 4000 LC/MS/MS triple quadrupole system equipped with an electrospray ionization (ESI) source, operating in multiple reaction monitoring (MRM) mode. Chromatographic separation was achieved on a Thermo Hypersil C18 column (4.6 X 100 mm, 5.0 µm particle size) maintained at 30°C. The autosampler temperature was kept at 10 °C. The mobile phase consisted of 0.1% formic acid and acetonitrile (10:90, v/v), delivered at a flow rate of 0.5 mL/min using a gradient elution. The injection volume was 20 µL, and the total run time was 4 minutes.

Preparation of Stock Solution and Spiking Solution:

The master stock solutions of 3TC, ABC, LPV and RTV having concentration 5 mg/ml are prepared in 50% methanol (diluent). Master stock solutions of each analyte were prepared at a concentration of 500 µg/mL. From these, secondary stock solutions of 50 µg/mL were prepared using the appropriate diluent. Subsequently, working stock solutions of 1 µg/mL were prepared by further dilution of the 50 µg/mL solutions. A 950 µL aliquot of blank plasma was spiked with 50 µL of stock solution (50 µg/mL of each analyte), vortexed for 30 second and used as the ULOQ plasma sample. A 950 µL aliquot of blank plasma was spiked with 50 µL of stock solution (1 µg/mL of each analyte), vortexed for 30 second and used as the LLOQ plasma sample. From 10 µg/ml solution of Tenofovir; 1 µg/mL Tenofovir solution was prepared and used as the internal standard (IS) for sample analysis.

2.1 Protein Precipitation (PPT)

PPT involves the addition of organic solvents such as acetonitrile or methanol to plasma samples, leading to protein denaturation and precipitation. The mixture is centrifuged and the clear supernatant containing analytes is subjected to analysis [12]. 250 µL of spiked plasma with the internal standard were taken in a Ria vial. Add 1 mL of acetonitrile to precipitate and denature proteins. Vortex the vial for 30 seconds on vertexer, then centrifuge at 12,000 RPM for 10 minutes at 10°C. Transfer 500 µL of the supernatant to a labelled autosampler vial for analysis [13]. Acetonitrile shows good extraction efficiency for all the four analytes along with internal standard Tenofovir [14]

2.2 Liquid–Liquid Extraction (LLE)

LLE is based on the partitioning of analytes between an aqueous phase and an immiscible organic solvent. The analytes are selectively extracted into the organic phase, which is then evaporated and reconstituted prior to analysis [15]**.** Sterilized human plasma, stored at –10°C, was thawed to room temperature and gently mixed prior to sample preparation. A 250 µL aliquot of the spiked plasma was then transferred to an RIA vial, mixed with tenofovir, and combined with 5 mL of TBME extraction solvent. This mixture was vortexed for 2 minutes and centrifuged at 12,000 rpm for 10 minutes at 10°C. Following centrifugation, the organic layer was collected and evaporated under nitrogen evaporator at 50°C for 15 minutes. The residue was reconstituted in 500 µL of mobile phase, vortexed for 30 seconds, and transferred to an autosampler vial for analysis. Before injection into the LC-MS/MS system, the extract was filtered through a 0.45 µm Acrodisc syringe filter. TBME was selected as the extraction solvent because its low volatility and good analyte compatibility facilitated efficient extraction of all four analytes [16-18].

3. Results and Comparative Evaluation

To confirm with ICH guidelines, we systematically evaluated common extraction techniques for their effectiveness in simultaneously extracting ARV drugs. Our evaluation focused on the specificity, selectivity and recovery of liquid–liquid extraction and protein precipitation to ensure accurate analysis, free from interferences. Selecting an appropriate extraction method is crucial for reliable LC–MS/MS analysis of antiretroviral drugs because of their varying polarities [19,20]. Table 1. summarizes the comparative performance of protein precipitation (PPT) and liquid-liquid extraction (LLE), which was assessed using blank samples and samples at the lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ). PPT yielded cleaner extracts and enhanced sensitivity for both polar and lipophilic compounds. However, LLE provided acceptable performance specifically for the lipophilic protease inhibitors [21-24].

Table 1: Comparison of protein precipitation (PPT) and liquid–liquid extraction (LLE) methods for antiretroviral drugs based on analyte polarity and extraction performance.

ARV Drugs	Class	Polarity	Liquid–Liquid Extraction with TBME	Protein Precipitation with acetonitrile
Lamivudine (3TC)	NRTI	Hydrophilic (Polar)	Low recovery and selectivity issues	Clean extract with good sensitivity
Abacavir (ABC)	NRTI	Hydrophilic (Polar)	Possible matrix interference	Good recovery with minimal interference
Lopinavir (LPV)	PI	Lipophilic (non-polar)	Acceptable extraction performance	Good extraction performance
Ritonavir (RTV)	PI	Lipophilic (non-polar)	Possible matrix interference	Good extraction performance

Selectivity and Specificity

The analytical method should be evaluated for its ability to detect endogenous matrix components (e.g., metabolites, impurities, or degradation products). Interfering components should not produce a response greater than 20% of the analyte response at the lower limit of quantification (LLOQ), or greater than 5% of the internal standard response in the LLOQ sample [25,26].

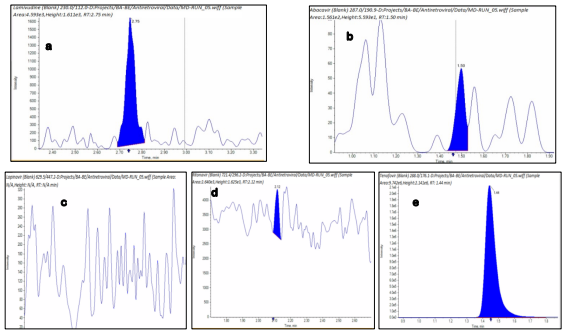

Figure 2: LC-MS/MS Chromatogram for 3TC (a), ABC (b), LPV (c), RTV (d), TFV (e) Blank sample extract from LLE with TBME

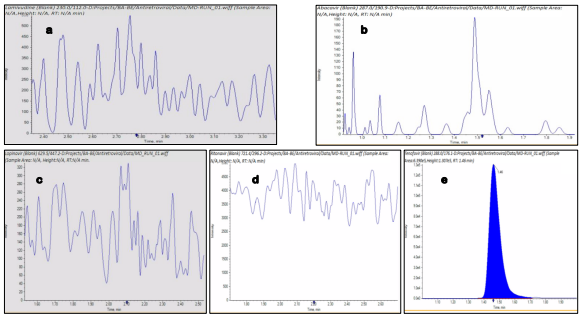

Figure 3: LC-MS/MS Chromatogram for 3TC (a), ABC (b), LPV (c), RTV (d), TFV (e) blank sample extract from PPT with acetonitrile.

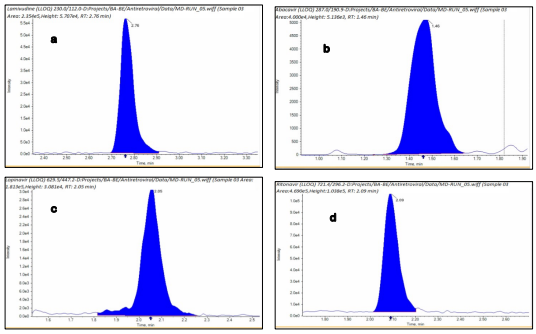

Figure 4: LC-MS/MS Chromatogram for plasma spiked with 3TC (a), ABC (b), LPV (c), RTV (d) at the lower limit of quantification (LLOQ) Concentration 50 ng/ml by LLE

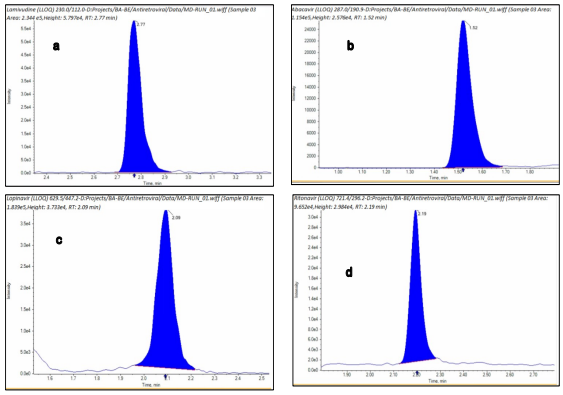

Figure 5: LC-MS/MS Chromatogram for plasma spiked with 3TC (a), ABC (b), LPV (c), RTV (d) at the lower limit of quantification (LLOQ) Concentration 50 ng/ml by PPT

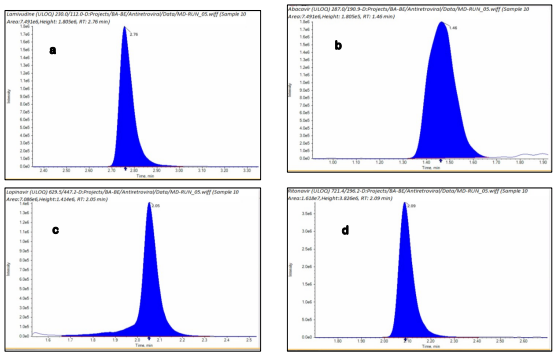

Figure 6: LC-MS/MS Chromatogram for 3TC (a), ABC (b), LPV (c), RTV (d) Upper limit of quantification (ULOQ) Concentration 2500 ng/ml by LLE

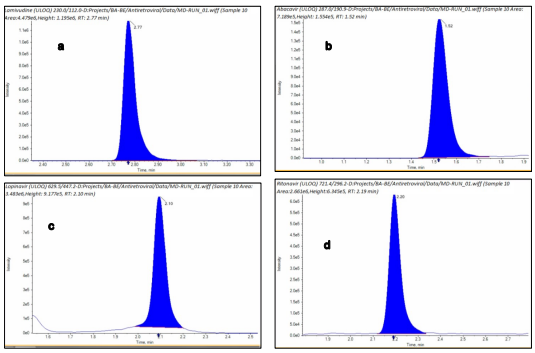

Figure 7: LC-MS/MS Chromatogram for 3TC (a), ABC (b), LPV (c), RTV (d) Upper limit of quantification (ULOQ) Concentration 2500 ng/ml by PPT

3.1 Extraction Recovery

Liquid–liquid extraction (LLE) can result in low or inconsistent recovery due to incomplete analyte partitioning during sample preparation. In contrast, protein precipitation (PPT) may exhibit higher apparent recovery, particularly for hydrophilic analytes. However, analyte binding to precipitated proteins can compromise accuracy and reproducibility [27,28].

3.2 Selectivity and Specificity

The PPT extraction technique demonstrated good selectivity and specificity, as the analyte peaks were eluted without interference from endogenous components, as illustrated in Figure 3, thereby ensuring accurate detection and quantification. In contrast, LLE showed noticeable interference from co-extracted endogenous compounds, which may affect analytical performance under the given experimental conditions as illustrated in Figure 2.

3.3 Throughput and Time Efficiency

PPT is rapid and suitable for high-throughput analysis, requiring minimal sample handling. LLE is comparatively time-consuming due to multiple steps such as phase separation and solvent evaporation.

3.4 Cost and Solvent Consumption

PPT is cost-effective and uses minimal solvents, whereas LLE requires larger volumes of organic solvents, increasing cost and environmental concerns.

3.5 Chromatographic Observation

PPT provided a clean baseline, acceptable signal response and efficient elution with well-defined peak shapes at the LLOQ. Retention times were 2.77 min for 3TC, 1.52 min for ABC, 2.09 min for LPV, and 2.19 min for RTV (Fig. 5). At the ULOQ, both extraction techniques produced well-defined peaks (Figs. 6 and 7); however, PPT resulted in superior peak clarity and baseline stability. While analyte polarity influenced the performance of both extraction techniques, protein precipitation yielded cleaner extracts and improved sensitivity

4. Discussion

Both PPT and LLE are widely used for the extraction of ARV drugs such as lamivudine, abacavir, lopinavir and ritonavir from plasma matrices. The choice of technique depends largely on the physicochemical properties of the analytes and the analytical objectives. PPT is advantageous for routine pharmacokinetic studies where high throughput is required. However, its limitations in selectivity and matrix interference may compromise analytical performance, especially at lower concentration levels. LLE, on the other hand, offers improved selectivity and cleaner extracts, making it suitable for highly sensitive LC–MS/MS methods. Despite being labor-intensive, it is preferred when matrix effects must be minimized and assay sensitivity is critical. A combined or hybrid approach, such as PPT followed by further cleanup, may offer a balance between efficiency and analytical performance.

Interfering peaks of endogenous compounds (fig.2.a, b, d) was observed at the retention times of the analytes 2.75 min,1.50 min,2.12 min, respectively for 3TC, ABC, RTV, as compared to 1.44 min TFV as IS in blank human plasma containing K2-EDTA as the anti-coagulant. Whereas LPV is a highly lipophilic HIV protease inhibitor (log P ≈ 5.6) with low aqueous solubility [29,30], which promotes efficient partitioning into organic solvents during LLE, resulting in a clean chromatographic profile with minimal or no interference (fig.2 c). No significant interference was observed in blank extract using PPT (fig.3); While LLE showed increased baseline noise, particularly for the more polar drugs 3TC, ABC (fig. 4).

At the LLOQ level, PPT provided a clean baseline with acceptable signal response, provided efficient elution with well-defined peak shapes, facilitating retention times of 2.77min,1.52 min,2.09 min, 2.19 min, for 3TC, ABC, LPV and RTV (fig.5), Both extraction techniques produced well-defined peaks at the ULOQ level (fig.6,7); however, PPT demonstrated superior peak clarity and baseline stability. The performance of both extraction techniques was found to be strongly influenced by analyte polarity. Protein precipitation provided cleaner extracts and better sensitivity for polar NRTI/NtRTI drugs such as 3TC, ABC and lipophilic PI including LPV and RTV [31]. The PPT extraction technique exhibited selectivity, as the analyte peaks were eluted without interference from other components present, as shown in Figure 3, ensuring accurate detection and quantification.

5. Conclusion

Protein precipitation and liquid–liquid extraction are both effective techniques for plasma sample preparation in ARV drug analysis. PPT is ideal for rapid, cost-effective, high-throughput applications, whereas LLE is better suited for sensitive and selective analyses requiring minimal matrix interference. The selection of the appropriate technique should be based on analytical requirements, drug properties and regulatory expectations.

PPT and LLE techniques were investigated for sample preparation to reduce interference of endogenous compound such as metabolites or impurities, improve extraction recovery and peak sharpness and prolong analytical column life. LLE offers a simple sample preparation approach but is time-consuming and suited for lipophilic analytes; however, differences in polarity can result in low extraction recovery. PPT is a well-established and effective method for processing plasma samples. Due to its simplicity, speed, reduced solvent consumption, and high extraction recovery, PPT is commonly used in ARV bioanalysis.

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