

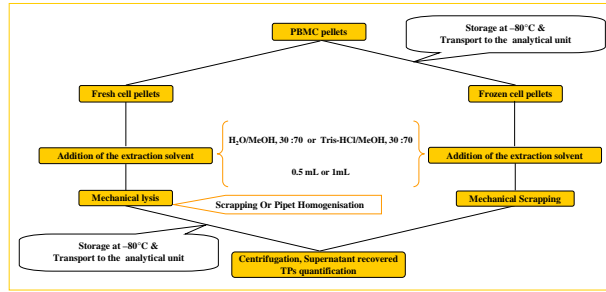
INTRODUCTION

- Nucleoside and nucleotide reverse transcriptase inhibitors (NRTIs and NtRTIs) are intracellularly anabolised in cells to their corresponding active triphosphate (TP) or diphosphate (DP) metabolites. Intracellular concentration of these metabolites should then play a critical role controlling both the drug efficacy and toxicity.
- Direct assays aimed to quantify intracellular NRTI-TP and NtRTI-TP have already been described.¹⁻⁵ These methods always include first an extraction step, involving cell lysis, in order to recover phosphate anabolites in solution. In a second step, NRTI or NtRTI phosphates are analysed usually using tandem mass spectrometry.
- Differences between different extraction procedures were recently outlined and the question was raised: "either to perform extraction on fresh cells in the clinical unit or on frozen cell in the analytical unit". So far, comparison between the two methods has not been published.

OBJECTIVES

Compare extraction methodologies (fresh versus frozen cells, volume and extraction medium) and their effect on natural deoxynucleotide triphosphates (dNTP), NRTI-TP and NtRTI-DP recoveries.

STUDY DESIGN :

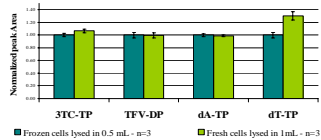


METHODS

- Clinical samples were obtained from blood of HIV infected patients, treated with various combinations of AZT, 3TC and TFV, within the framework of normal monitoring (MD. C. Goujard). Peripheral blood mononuclear cells (PBMCs) were pooled, washed twice in aqueous cold NaCl, evenly distributed and spin to 10^7 cells before extraction.
- PBMCs were also prepared from blood of healthy subjects (EFS, Rungis, France), either as blanks or as 'patient-mimic' after a 24 hour incubation of cultured PBMCs with d4T, TFV and 3TC (respectively 5, 2.5 and 4 μ M, 37°C). All Cells were washed twice in aqueous cold NaCl, evenly distributed and spin to 10^7 cells before extraction.
- 3TC-TP, d4T-TP, TFV-DP, dA-TP and dT-TP levels in PBMCs were simultaneously determined using validated LC-MS/MS assays involving an ion-pairing liquid chromatography coupled to a tandem mass spectrometry (negative ESI, Multiple Reaction Monitoring mode, TSQ Quantum Ultra, Thermo-Electron).^{1,2}
- Extraction was performed either on frozen cells by scrapping or on fresh cells using several methods (scrapping or homogenization using a pipet). Lysis were carried out with different volumes (0.5mL or 1mL) of different solvents (H₂O/MeOH: 3/7 or Tris-HCl(0.05M, pH7.4)/MeOH: 3:7).

A. 'IN VIVO' RESULTS : COMPARISON ON CLINICAL SAMPLES

Figure 1. Clinical samples (3TC/TFV) lysed either frozen in 0.5 ml or fresh in 1 ml Tris/MeOH (0.05M, PH=7.4; 3:7)



Both extraction methodologies lead to similar results for 3TC-TP, TFV-DP and dA-TP. dT-TP recovery is better using a 1mL lysis on fresh cells rather than a 0.5mL one on frozen cells.

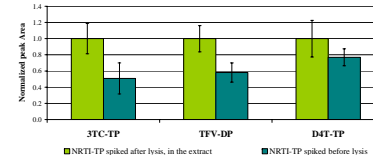
Detection of d4T-TP in AZT treated patient samples

Analysis of a pool of clinical samples from AZT treated patients were performed using both methodologies (Fresh cell extraction in 1mL and frozen cell extraction in 0.5mL):

d4T-TP was detected in all the extracts

B. EXTRACTION YIELD ON FRESH CELLS SPIKED WITH NRTI-TP AND NtRTI-TP

Figure 2. Extraction yield on PBMCs lysed fresh (3TC-TP : 400pmol/ 10^7 cells, TFV-DP : 2120fmol/ 10^7 cells, d4T-TP : 2500fmol/ 10^7 cells)



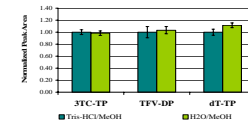
Even when performed on fresh cells, the extraction procedure does not allow 100% recovery.

*NRTI-TPs and NtRTI-TPs extraction yields are expressed as the ratio between blank samples (fresh pellets lysed before storage at -80°C) spiked with NRTI-TPs and NtRTI-TPs either before or after the lysis, in the extract.

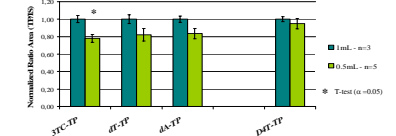
C. 'IN VITRO' RESULTS : INFLUENCE OF OTHER FACTORS ON THE EXTRACTION YIELD

Figure 3. TP levels from 'patient-mimic' fresh PBMCs using:

a. 1mL of different extraction solvents (n=6)

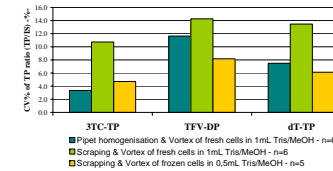


b. 0.5 or 1 mL of Tris/MeOH (0.05M, PH=7.4; 3:7)



The nature of the extraction medium (H₂O/MeOH or Tris 0.05M/MeOH) influences neither NRTI-TP nor dN-TP recoveries. However, they are improved when using 1mL extraction solvent instead of 500mL.

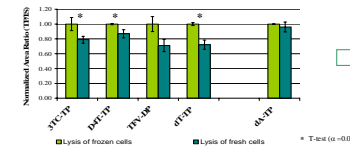
Figure 4. Variability on triphosphate extraction using different lysis method on 'patient-mimic' cells



Scrapping frozen cells and pipet-homogenizing of fresh cells provide the best results in terms of repeatability.

D. 'IN VITRO' RESULTS : EXTRACTION YIELD COMPARISON BETWEEN FRESH AND FROZEN CELL LYSIS

Figure 5. TP level in PBMCs lysed with 0.5mL of Tris/MeOH (0.05M, PH=7.4, 3:7) either before or after storage at -80°C (n=5)



All things being equal on other respects, cells lysed frozen gave higher recoveries for TFV-DP, 3TC-TP, d4T-TP and dT-TP than cells lysed fresh.

CONCLUSION

- Taking together the overall set of data, it appears that both extraction methods show very similar results for the recovery of NRTI-TP and NtRTI-TP on clinical samples.
- In our sense, freezing cells in the clinical unit is advantageous in that it simplifies the work of clinician and allows a better control of the extraction process which is entirely achieved in the analytical unit.

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