Application News

MALDI-8030 EasyCare

Quantification of HIV Antiretroviral Drugs in Human Plasma by Matrix Assisted Laser Desorption Ionisation Time-of-Flight Mass Spectrometry

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User Benefits

- Minimal sample preparation required prior to acquisition.
- Data can be rapidly acquired without the need for chromatographic separation.
- The quantification assay can be developed according to guidelines.

■ Introduction

Matrix assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) is widely used in many fields of application thanks to its robustness, simple operation and high sensitivity. The majority of applications are almost exclusively for qualitative analysis aimed at fast and accurate confirmation of the molecular mass. Quantitative analysis with MALDI-TOF MS has traditionally been viewed as impractical due to non-uniform distribution of the analyte(s) and matrix across the spot surface, giving rise to variations in the ion intensities. For this reason, LC–MS/MS is the mainstream technique for quantifying drugs in plasma. These unavoidable limitations can be overcome by adopting the 'internal standard (I.S.)' approach, where a fixed amount of an exogenous compound is added to the sample and the peak heights or areas of the endogenous analytes are corrected relative to it. The I.S. serves as a mimic of the analyte(s) and should exhibit similar chemical and physical properties, so that in all the stages of the assay (from sample preparation to analysis) it behaves in a manner close/identical to the sample analyte(s). A calibration curve is usually generated containing various amounts of a single analyte of interest and a fixed amount of the internal standard. This way, the 'corrected' ion intensities allow calculation of absolute amounts as the constant of proportionality can be established.

Here, we show a proof-of-principle example of determination and quantification of HIV protease inhibitors in human plasma using a Shimadzu benchtop MALDI-8030 EasyCare instrument (Figure 1). Lopinavir (LPV) was used as an example of HIV antiretroviral drug and quantified against Ritonavir (RTV) internal standard [1]. The quantitative assay was developed according to the FDA guidelines [2]. In this study, by employing MALDI-TOF MS, we achieved quantitative analysis that enables both simplified sample preparation and high-throughput measurements.

Figure 1. Shimadzu MALDI-8030 EasyCare MALDI-TOF mass spectrometer.

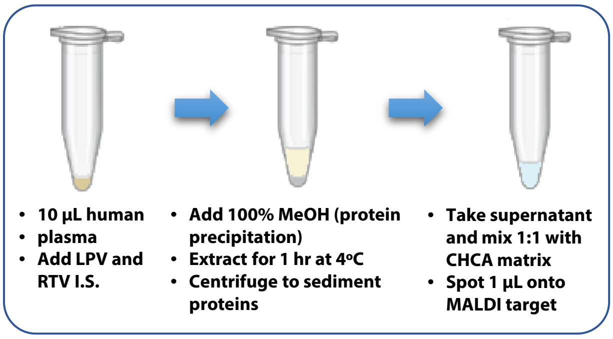

Figure 2. Sample preparation workflow.

■ Sample preparation

Human plasma from a healthy donor was purchased from Cambridge Bioscience (UK). Lopinavir and Ritonavir were purchased from Thermo Fisher Scientific (UK). Individual stock solutions of the drug standards were prepared in 50:50 methanol/water at various dilutions taking into account the final concentrations in the human plasma for the calibration curve. Sodium trifluoroacetate (NaTFA) was used as cationisation agent to promote the conversion of the drugs to sodiated species. α-Cyano-4-hydroxycinnamic acid (CHCA) was used as the MALDI matrix (5 mg/mL in 50:50 acetonitrile/water). NaTFA was added to the matrix to a final concentration of 50 mM.

The sample preparation workflow is illustrated in Figure 2: 10 µL of plasma were spiked with Lopinavir and Ritonavir I.S. (10 µL each). 70 µL of methanol were added to obtain a final volume of 100 µL resulting in a 1/10 dilution of the added drug stock solutions. The final concentration of RTV I.S. in all samples was 500 nM. The methanol also induced the precipitation of the proteins in the plasma. Samples were extracted for 1 hr at 4ºC, then centrifuged for 5 minutes at 15,000 rpm (21,130 rcf). An aliquot of the supernatant was transferred to a 0.5 µL microcentrifuge tube and an equal volume of MALDI matrix (containing 50 mM NaTFA) was added to achieve a 1:1 ratio.

Seven calibrator concentrations of Lopinavir were prepared to build the calibration curve: 1.5 µM, 1 µM, 750 nM, 500 nM, 250 nM, 100 nM and 75 nM (lower limit of quantification (LLOQ)). Five replicates were prepared for each calibrator. Quality controls were also prepared to check the validity of the calibration curve: low QC (≤3X LLOQ; 225 nM), mid QC (600 nM) and high QC (1.2 µM).

A 'blind' plasma sample was prepared containing LPV (400 nM) and RTV, which was used to test the accuracy of the generated LPV/RTV calibration curve.

For the MALDI analyses, 1 µL of the sample/matrix premix was spotted onto the MALDI target. Analyses were conducted in positive ion mode on a MALDI-8030 EasyCare instrument. The analysis conditions are summarised in Table 1.

Table 1. Analysis Conditions.

System	: MALDI-8030 EasyCare
Polarity	: Positive
Mass Range	: *m/z* 100-1000
Acquisition	: 100 shots per profile @ 200Hz
Blanking	: 400
Pulsed Extraction	: 650
Profiles	: 50

■ Results

Figure 3 shows the MALDI-MS spectra of Lopinavir and Ritonavir I.S. in human plasma at the various calibrator concentrations. Lopinavir and Ritonavir were detected as $[M + Na]^+$ species (LPV *m/z* 651.352 calculated; RTV *m/z* 743.302 calculated). To maximise mass accuracy, lock mass correction was applied to all data using the calculated *m/z* of Ritonavir. Blanks, i.e. human plasma not containing the drugs but processed in the same way, were analysed to ensure no endogenous species were potentially interfering with the Lopinavir and Ritonavir (data not shown).

Figure 4 shows the calibration curve which was constructed by plotting the LPV-to-RTV I.S. peak height ratios as the average of the five replicates against the concentration of LPV (nM) (blue dots). The low-, mid- and high-QC are also plotted (orange dots). As it can be seen, the curve exhibits excellent linearity with the coefficient of determination (R^2) equal to 0.9987.

For each concentration of calibrator and QCs, precision (CV%) and accuracy (% bias) were determined ensuring that they were within FDA criteria: *i)* precision (CV%) is ≤15% for all calibrators except the LLOQ for which is ≤20%; *ii)* accuracy (% bias) is ≤±15% for all calibrators except the LLOQ for which is ≤±20%. According to these criteria, the LLOQ was established as 75 nM. The simulated blind sample (400 nM) was also plotted on the curve (pink dot), and it gave a determined concentration of 372 nM. Table 2 shows the precision (CV%) and accuracy (% bias) values for all calibrators, QCs and blind sample. All samples meet the FDA criteria for precision and accuracy (values shown in parenthesis).

■ Conclusion

MALDI-TOF MS has historically been considered not fit for quantitative analysis due to variations in the ion intensities across the spot. However, these variations can be compensated for using an internal standard which normalises ion intensities and converts them to absolute amounts. Here, we showed a proof-of-principle application on the quantification of HIV antiretroviral drugs in human plasma by MALDI-TOF MS. The quantitative assay has been successfully developed according to guidelines.

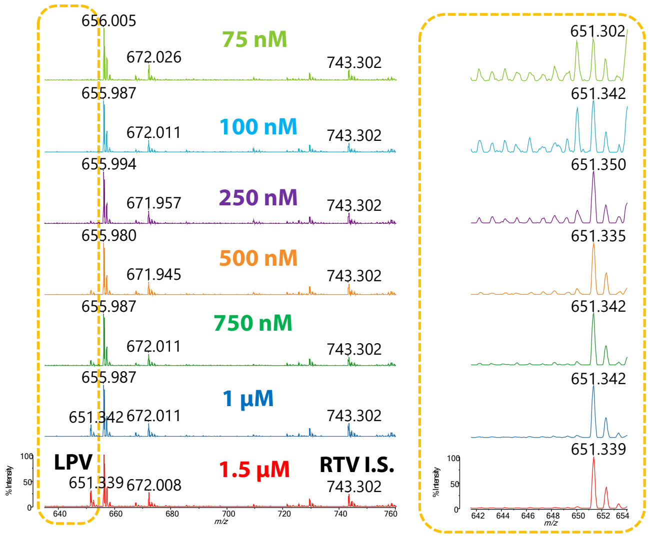

Figure 3. MALDI-MS spectra of LPV at the various calibrator concentrations and RTV I.S.

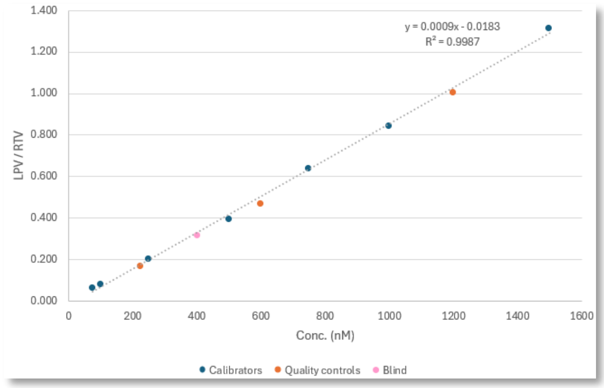

Figure 4. Calibration curve showing all calibrators of LPV (blue dots), QCs (orange dots) and simulated blind sample (pink dot). The data show good linearity over the calibration curve range.

Table 2. Precision (CV%) and accuracy (% bias) values for all calibrators of LPV, quality controls and simulated blind sample. Precision and accuracy values in parenthesis are the criteria set by the guidelines.

Sample type	Conc. (nM)	Precision (CV%)	Accuracy (% bias)
Calibrator	1500	2.5 (≤15)	1.4 (≤±15)
Calibrator	1000	2.8 (≤15)	4.4 (≤±15)
Calibrator	750	3.1 (≤15)	3.3 (≤±15)
Calibrator	500	3.8 (≤15)	8.8 (≤±15)
Calibrator	250	7.7 (≤15)	2.2 (≤±15)
Calibrator	100	11.2 (≤15)	-9.4 (≤±15)
Calibrator (LLOQ)	75	11.0 (≤20)	-18.0 (≤±20)
High QC	1200	7.6 (≤15)	5.5 (≤±15)
Mid QC	600	3.0 (≤15)	10.1 (≤±15)
Low QC	225	2.3 (≤15)	8.4 (≤±15)
Blind	400	6.6 (≤15)	6.9 (≤±15)

■ References

[1] van Kampen JJA, Reedijk ML, Burgers PC, Dekker LJM, Hartwig NG, et al. (2010) Ultra-Fast Analysis of Plasma and Intracellular Levels of HIV Protease Inhibitors in Children: A Clinical Application of MALDI Mass Spectrometry. PLoS ONE 5(7): e11409. doi:10.1371/journal.pone.0011409.

[2] Guidance for Industry: Bioanalytical Method Validation (2018). https://www.fda.gov/regulatory-information/search-fda-guidance-documents/bioanalytical-method-validation-guidance-industry

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