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Simple, Fast and Selective, Bioanalytical Sample Extraction for the Therapeutic Drug, Lenalidomide From Plasma Using Oasis™ MCX SPE

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Abstract

The following work demonstrates a simple, generic, broadly applicable, fully automated bioanalytical sample preparation strategy, requiring no method development, for the small-molecule therapeutic drug lenalidomide for several common bioanalytical extraction techniques including protein precipitation (PPT), liquid-liquid extraction (LLE), and solid phase extraction (SPE). LC-MS bioanalytical quantitation performance using mixed-mode SPE with Oasis WCX from plasma is highlighted using the Andrew+™ Pipetting Robot in combination with the Extraction+ Connected Device for fully automated liquid handling and solid phase extraction (SPE) sample preparation.

Benefits

- Simplified bioanalytical sample preparation strategy, requiring no method development for successful analyte extraction from biomatrices
- Generic extraction method protocols, yielding high analyte recovery, and achieving reproducible results, from

extracted plasma with no optimization required

- Fast bioanalytical extraction (<30 minutes) using the 96-well µElution extraction plate format.
- Automated sample extraction, for "walk-away" method execution, mitigating risk of manual error, improving analytical performance, and freeing up scientist time
- Fast, 3-minute UPLC™ analysis using the ACQUITY™ Premier HSS T3 Column
- Excellent quantitative performance from 0.5–100 ng/mL, QC accuracies between 96–112% with RSDs ≤5%

Introduction

Lenalidomide (Revlimid®) is a small molecule therapeutic used in the treatment of multiple myeloma and as a maintenance drug for stem cell transplantation a treatment for obesity.[1,2] With continued research and development of this drug and next generation therapies, there is continued need for their robust, sensitive, and selective bioanalytical sample preparation and liquid chromatography mass spectrometry (LC-MS) analysis. Bioanalytical method development focuses on three major aspects: 1- detection, 2- separation, and 3- extraction of analytes from the various biomatrix components to ensure sensitive, selective, and reproducible quantification of the analyte target.

In this work, several common bioanalytical techniques including protein precipitation (PPT), PPT with phospholipid (PL) removal, liquid-liquid extraction (LLE), reversed-phase (RP) SPE, RP-SPE with PL removal, and mixed-mode SPE were evaluated for the extraction of the therapeutic drug, lenalidomide from plasma. All extraction techniques were evaluated using generic, manufacturers' recommended protocols, with recovery and sample cleanliness evaluated. Final sample preparation and extraction was then automated on the Andrew+ Pipetting Robot to simplify and streamline the workflow, maximizing productivity, and ensuring overall analytical method performance.

Experimental

Materials

Lenalidomide was purchased from Sigma-Aldrich D5-lenalidomide, used as internal standard (IS) was obtained from Cayman Chemicals (www.caymanchem.com <https://www.caymanchem.com/>) LC-MS grade formic acid, phosphoric acid, and ammonium hydroxide, and ethyl acetate were purchased from Sigma Aldrich (www.sigmaaldrich.com <http://www.sigmaaldrich.com/>). Methanol and Acetonitrile were purchased from Honeywell (lab.honeywell.com <http://lab.honeywell.com>).

LC/MS Analysis

LC-MS/MS analysis was performed using a Xevo™ TQ-XS Tandem Quadrupole MS (ESI-) and chromatographic separation using an ACQUITY I-Class PLUS UPLC System and ACQUITY UPLC HSS T3 Column (p/n: 186003538 <https://www.waters.com/nextgen/us/en/shop/columns/186003538-acquity-uplc-hss-t3-column-100a-18--m-21-mm-x-50-mm-1-pk.html>). A flow rate of 0.7 mL/min using 0.1% formic acid in water (MP A) and 0.1% formic acid in acetonitrile (MP B). Starting LC conditions were held for 0.5 minutes, followed by an increase to 30% MP at one minute. MP B was then increased to 90% to flush the column and returned to starting gradient conditions at two minutes. Total analysis time was three minutes. The injection volumes of the extracted samples were 5 µL.

Sample Preparation

Stock solutions of lenalidomide and d5-lenalidomide (1 mg/mL) were prepared in methanol (MeOH). Working stock solutions (10 µg/mL) were also prepared in methanol and were used to prepare calibrators and QC samples in plasma. Lenalidomide was added to commercially available Sprague-Dawley rat plasma. Calibration curve samples were prepared between 0.250–500 ng/mL, while quality control (QC) samples were prepared at 0.75, 7.5, and 75 ng/mL. Standard curve and QC plasma samples were prepared in triplicate.

Sample Extraction

Extraction protocols for each bioanalytical extraction technique are listed in Table 1 with graphical representation listed in Figure 1. In each case, the manufacturers' guidance was used for the protocols.

PPT: Waters Sirocco Plate (p/n186002448)

1 PRECIPITATION	▪ Add 300 µL ACN to well of Sirocco Plate ▪ Add 100 µL plasma to ACN
2 VORTEX	▪ Vortex for 30 seconds **(Off deck)**
3 ELUTE	▪ Elute under with 5–15 psi

PPT Phospholipid Removal: Waters Ostro Plate (p/n 186005518)

1 SAMPLE	▪ Add 100 µL plasma to well of Ostro Plate
2 PRECIPITATION	▪ Add 300 µL ACN with 1% FA to plasma ▪ Aspirate 6x to mix
3 ELUTION	▪ Elute under vacuum 5 –15 psi

Liquid-Liquid extraction

1 LOAD	▪ Load 250 µL plasma ▪ Add 500 µL of ethyl acetate ▪ Vortex until no visible liquid layers remain
2 SEPARATE	▪ Centrifuge for 10 minutes at 5,000 RPM ▪ Transfer 250 µL of supernatant into a collection plate
3 EVAPORATE	▪ Evaporate to dryness under nitrogen stream
4 RECONSTITUTE	▪ Reconstitute with 125 µL water

Reversed-Phase SPE: Waters Oasis HLB Plate (p/n 186001828BA)

1 SAMPLE DILUTION	▪ Dilute 100 µL plasma 1:1 with 4% H_3PO_4
2 LOAD	▪ Load 100 µL diluted sample on Oasis HLB plate
3 WASH	▪ 200 µL of 95:5 Water:MeOH
4 ELUTE	▪ 2 x 50 µL MeOH
5 DILUTION	▪ Dilute with 100 µL water

Reversed-Phase SPE with Phospholipid Removal: Waters Oasis PRiME HLB Plate (p/n 186008052)

1 SAMPLE DILUTION	▪ Dilute 100 µL plasma 1:1 with 4% H_3PO_4
2 LOAD	▪ Load 100 µL diluted sample into well of Oasis PRiME HLB plate
3 WASH	▪ 200 µL of 95:5 Water:MeOH
4 ELUTE	▪ 2 x 50 µL 90:10 ACN:MeOH
5 DILUTION	▪ Dilute with 100 µL water

Mixed-Mode SPE: Waters Oasis MCX Plate (p/n 186001830BA)

1 SAMPLE DILUTION	▪ Dilute 100 µL plasma 1:1 with 4% H_3PO_4
2 LOAD	▪ Load 100 µL diluted sample onto well of Oasis MCX plate
3 WASH	▪ 200 µL of 2% formic acid in water ▪ 200 µL of MeOH
4 ELUTE	▪ 2 x 50 µL 70% MeOH with 1% NH_4OH
5 DILUTION	▪ Dilute with 100 µL water

Figure 1. Graphical representation of all sample preparation extraction

protocols used for various extraction methods evaluated. All methods were based on manufacturers' guidance for volumes and solvents contained in their respective care and use manual.

Sample preparation extraction technique	Sample preparation/extraction device	Part number	Care and use manual/protocol reference
Protein precipitation (PPT)	Sirocco Protein Precipitation Plates	186002448	https://www.waters.com/webassest/cms/support/docs/720003377en.pdf
Pass through: PPT with phospholipid removal	Ostro Protein Precipitation and Phospholipid Removal Plates	186005518	https://www.waters.com/webassest/cms/support/docs/720003672en.pdf
Reversed-phase SPE	Oasis HLB 96-well µElution Plate	186001828BA	https://www.waters.com/webassest/cms/support/docs/715000109en.pdf
Reversed-phase SPE with phospholipid removal	Oasis PRiME HLB 96-Well µElution Plate	186008052	https://www.waters.com/webassest/cms/support/docs/720001692en.pdf
Mixed-mode SPE	Oasis MCX 96-well µElution Plate	186001830BA	https://www.waters.com/waters/library.htm?cid=511436&lid=10009764&lcid=10009763

Table 1. Table of sample extraction methods, references for care and use instructions, and starting protocols used for lenalidomide extraction method evaluation.

Recovery and Matrix Effects Calculations

Analyte recovery was calculated according to the following equation:

$$\%Recovery = \left(\frac{Area\ A}{Area\ B} \right) x100\%$$

Where Area A = the peak area of an extracted sample and Area B = the peak area of an extracted matrix sample in which the compounds were added post-extraction.

Matrix effects were calculated according to the following equation:

$$Matrix\ Effects = \left(\left(\frac{Peak\ area\ in\ the\ presence\ of\ matrix}{Peak\ area\ in\ the\ absence\ of\ matrix} \right) - 1 \right) x\ 100\%$$

The peak area in the presence of matrix refers to the peak area of an extracted blank matrix sample in which the compounds were added post-extraction. The peak area in the absence of matrix refers to analytes in a neat solvent solution.

Phospholipid Monitoring

Residual phospholipids (PPLs), used as a measure of assessing sample cleanliness, were analyzed using the same UPLC gradient as lenalidomide. The MS system was operated in ESI+ using a parent scan ion of 184.1, scanning a mass range 400–1000 *m/z* and scan time of 1 second. Cone voltage was set to 30 V and a collision energy of 30 eV was applied conditions.

Automation Platform

The Andrew+ Pipetting Robot, equipped with the Extraction+ connected device and controlled with the cloud based OneLab™ Software, was used to design and execute the final sample preparation and bioanalytical extraction protocol used for quantification assessment of lenalidomide from plasma.

Results and Discussion

LC-MS/MS quantification of lenalidomide was performed using an ACQUITY UPLC I-Class coupled to the Xevo TQ-XS MS System operated in ESI+ mode. MS/MS multiple reaction monitoring (MRM) transition used for detection and quantitation where this analysis was 260.2>148.85 (CE 25 eV) and 265.0>149.9 (CE 20 eV), for lenalidomide and d5-lenalidomide ISTD. Lenalidomide is extremely polar, with a cLogP of -0.685. For this reason, the ACQUITY UPLC HSS T3 100 Å, 1.8 µm, 2.1 mm x 50 mm Column was used for this analysis to ensure best chromatographic retention. This chromatographic performance for lenalidomide and d5-lenalidomide, used as ISTD is illustrated in Figure 2. The LC gradient separation was employed using mobile phases consisting of water (MP A) and acetonitrile (MP B), both containing 0.1% formic acid. MP A was held at 100% for 0.5 minutes followed by a shallow gradient to 70% over 0.5 min followed by high organic flush. Column temperature was 45

˚C while the flow rate was 0.7 mL/min. Total analysis time was three minutes.

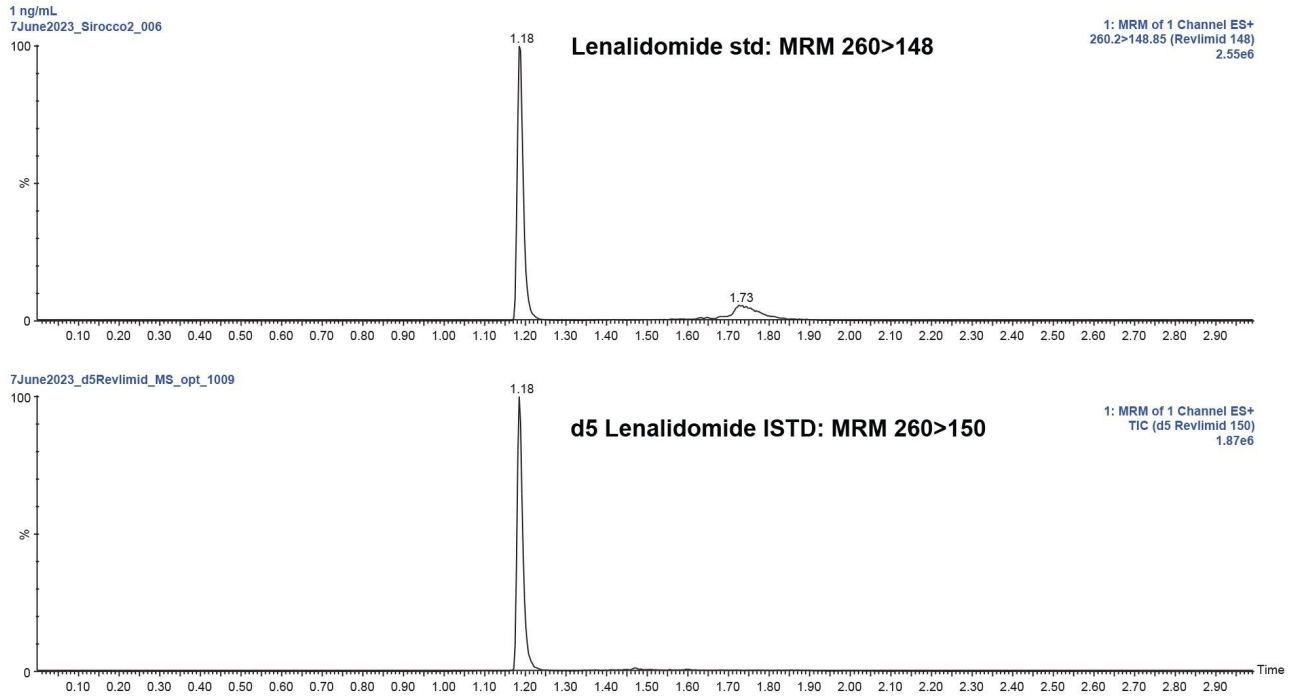

Figure 2. LC chromatographic separation of lenalidomide and lenalidomide d5 ISTD (1 ng/mL) using the ACQUITY UPLC HSS T3 100 Å, 1.8 µm, 2.1 mm x 50 mm Column.

Recovery and Matrix Effects

A key step in any bioanalytical procedure is the evaluation of extraction efficiency and cleanliness. This is done by calculating recovery and matrix effects for the target analytes across the various extraction techniques. The equations are provided in the Materials and Methods Section. Figure 3 shows the recovery and matrix effects (ME) results from the various generic sample extraction procedures recommended by the vendor. The sample preparation techniques are ordered by increasing selectivity, starting with the more universal methods such as protein precipitation, and progressing to the more selective and specific mixed-mode SPE procedures. A general trend of improved recovery and decreased matrix effects was seen with the more specific SPE methods. The Sirocco PPT, Ostro PPT with PL removal, and Oasis MCX provided best recoveries (>80%), while the SSLE and reversed phase HLB SPE prepared samples had the least recovery of all techniques. It should be noted that minimal optimization was performed for this or any technique, as one of our goals was to follow the

recommended vendor protocols for all products. With reversed-phase SPE, low recoveries were not surprising due to lenalidomide's extreme polarity (cLogP of -0.685), resulting in the loss of retention during the load step. Conversely, great recovery (88%) was seen when using the MCX mixed-mode sorbent. In this case the basic nature of lenalidomide, with pKa of 10.75 was leveraged in combination with negatively charged MCX sorbent, for ultimate retention on SPE loading. Matrix effects were greatly reduced from standard Sirocco PPT (-26.7%) using Ostro PPT with PL removal (-8.4%), while matrix effects were 9.3% using Oasis MCX SPE, indicating extraction selectivity.

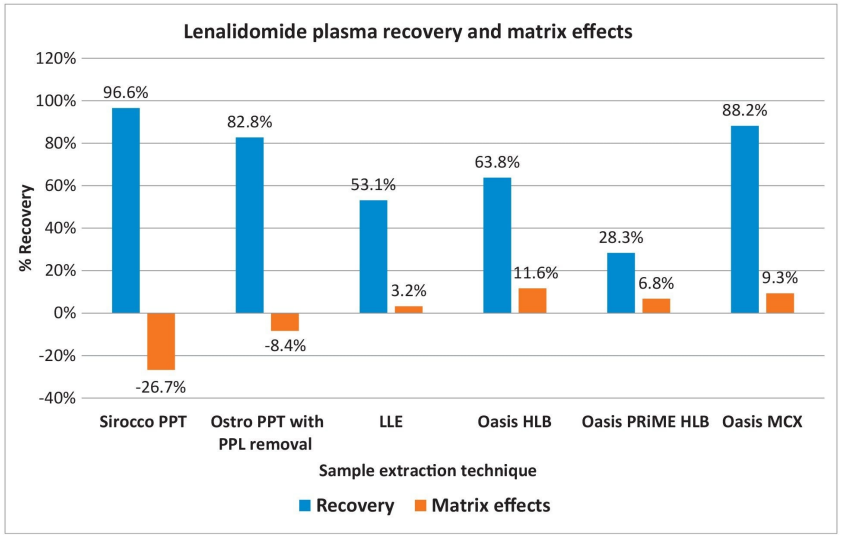

Figure 3. Representative recovery and matrix effects resulting from the extraction of lenalidomide from plasma using the techniques listed in Figure 1/Table 1. Best balance of recovery and matrix effects seen with Ostro PPT with phospholipid removal (Rec 82.8 and MEs -8.4%) and Oasis MCX SPE (Rec 88.2 and MEs 9.3%).

Residual Phospholipids

Another measure of sample cleanliness often evaluated is residual phospholipids (PLs) in the extracted samples. Major components of plasma are phospholipids, with major subcomponents being phosphatidylcholines. We can leverage MS detection and monitor precursors of 184 *m/z*, which corresponds to the polar head group of phosphatidylcholine species. Monitoring this transition allows detection of the various phosphatidylcholines

remaining in the extracted sample. This procedure was described in the Materials and Methods section. The MS intensities of the residual PL traces, corrected for sample concentration and or dilution across the various extraction protocols and normalized to standard organic PPT extraction, is illustrated in Figure 4. Unsurprisingly, simple PPT resulted in the highest presence of PLs. Ostro PPT with PL removal and Oasis MCX SPE significantly reduced the PL content in the extracted sample, removing over 98% as compared to the standard PPT with the Sirocco PPT plate.

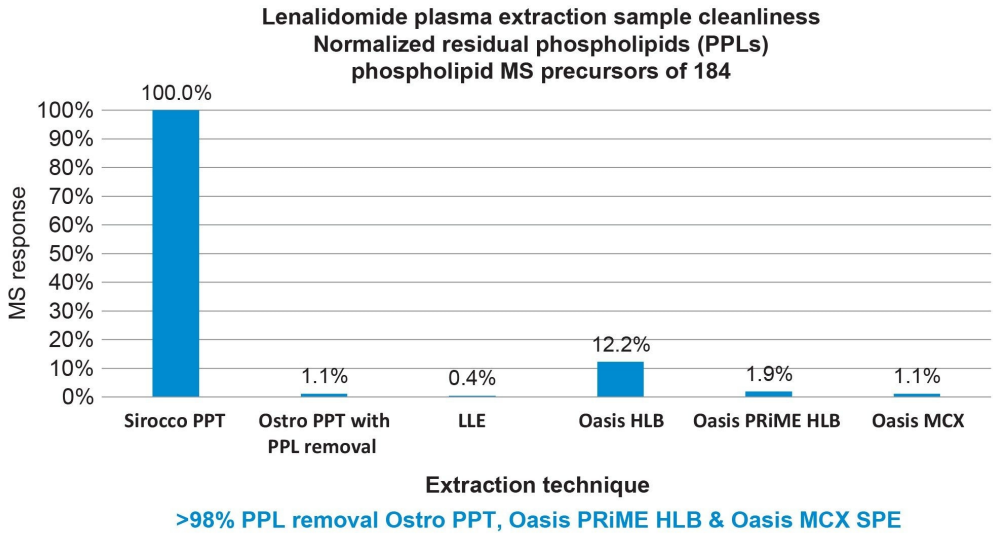

Figure 4. Graphical illustration of sample extraction technique cleanliness, highlighting best phospholipid removal with Ostro PPT and Oasis PRiME HLB with phospholipid removal and Oasis mixed mode MCX extraction (normalized to Sirocco protein precipitation). For this evaluation, MS intensity (MS Precursors of 184 Scan) of residual phospholipids detected with each sample preparation technique was corrected for starting sample volume and any differences in resulting sample eluate analyzed.

Bioanalytical Quantitation

The Oasis MCX SPE 96-well plate was ultimately chosen for extraction of lenalidomide from plasma, as it provided the best balance of recovery, matrix effects and residual phospholipids. Table 2 lists a summary of the quantitative results resulting from this extraction of the standards (A) and QC (B) in plasma, fully prepared and extracted using the Andrew+ Pipetting Robot configured with the Extraction+ Connected device. Linear dynamic

range was determined to be 0.5–100 ng/mL (>0.996 linear fit), calibration accuracy range of 92.7–115.9 and % RSD range from 1.38–16.6%. QC performance was similar with accuracies between 96.5–111.8% across the QC levels and RSDs ranging from 2.84–4.81%. This quantitative performance easily meets recommended bioanalytical method validation guidance criteria.[3] Representative chromatograms for the QCs samples, as compared to the blank is illustrated in Figure 4.

A	Lenalidomide calibration curve statistics			
Dynamic range (ng/mL)	**Linear fit (R^2)**	**Weighting**	**% Accuracy range**	**%RSD range**
0.5–100	0.996	1/x	92.7–115.9	1.38–16.6

B	Lenalidomide quality control statistics			
QC level	**Expected concentration (ng/mL)**	**Observed concentration (ng/mL)**	**% Accuracy**	**%RSD**
LQC	0.75	0.661	111.8	2.84
MQC	7.5	7.77	96.5	3.44
HQC	75	74.1	101.2	4.81

Table 2. Lenalidomide plasma quantification performance, prepared and extracted using the Andrew+ Pipetting Robot and Oasis MCX 96-well plate extraction. Calibration curve (A) and QC (B) statistics of semaglutide with accuracy and RSDs ≤15%.

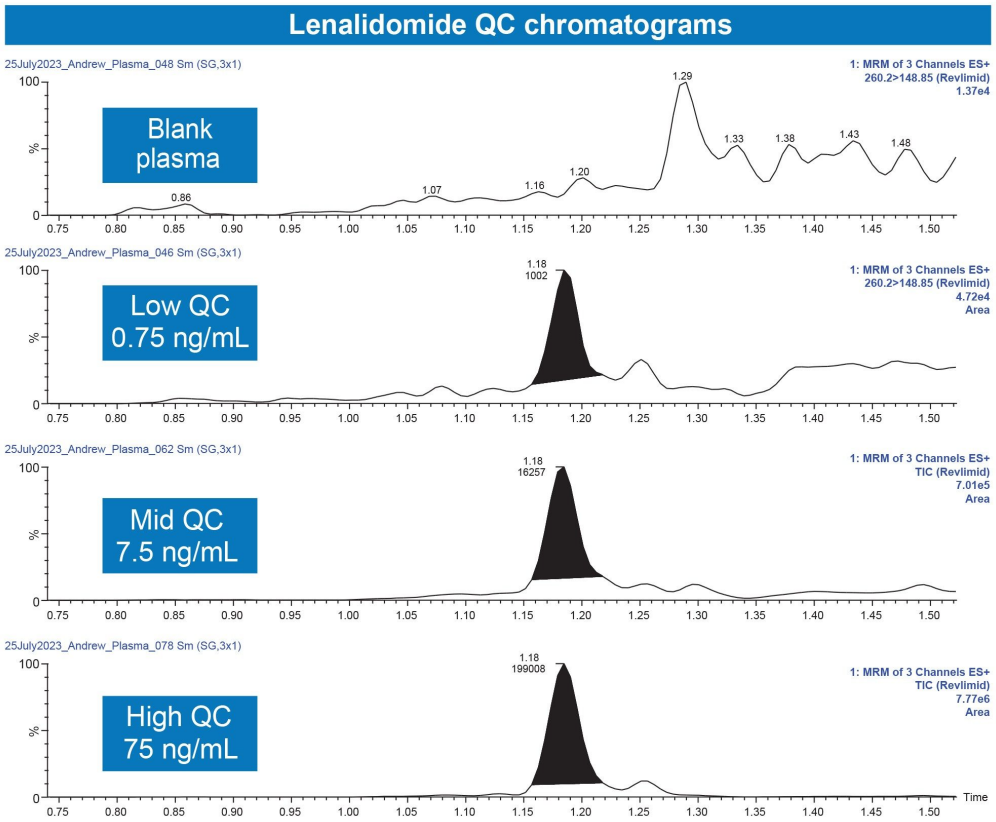

Figure 5. Representative chromatograms of lenalidomide QC samples extracted from plasma using Oasis MCX SPE.

Conclusion

This application highlights the successful SPE extraction and LC-MS/MS quantification of lenalidomide from plasma, requiring no method development. SPE extraction using Oasis MCX, achieved high analyte recovery (88%), low matrix effects (<10%), with lowest residual phospholipids in the extracted sample. The combination of generic protocols and automated sample preparation with the Andrew+ Pipetting Robot configured with the Extraction+ connected device greatly simplified and streamlined sample extraction, and maximized lab productivity, reduced errors, and ensured overall analytical method performance.

References

1. https://go.drugbank.com/drugs/DB00480 <https://go.drugbank.com/drugs/DB00480> .
2. https://www.chemexper.com/cheminfo/servlet/org.dbcreator.MainServlet?from=1&format=google2008&action=PowerSear 72-6&target=entry <https://www.chemexper.com/cheminfo/servlet/org.dbcreator.MainServlet?from=1&format=google2008&action=PowerSearch&sort=%3E%7Etopranking.value&realQuery=rn.value%3D191732-72-6&target=entry> .
3. Bansal, S.; DeStefano, A. Key Elements of Bioanalytical Method Validation for Small Molecules. *The AAPS Journal* 2007, *9* (1), E109–E114.

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