Sample Preparation Using ISOLUTE® PPT+ Protein Precipitation Plates

Protein precipitation is a routinely used, high throughput sample preparation technique for removal of protein from biological fluid (plasma, serum or blood) samples prior to analysis by LC-MS/MS.

Historically, protein precipitation has been carried out in vials or collection plates, followed by centrifugation; the supernatant is then transferred for analysis. Protein precipitation by filtration in the 96-well format has more recently been used as a high throughput, easy to automate alternative to the traditional centrifugation based technique. However, most filterplates require the plasma sample to be dispensed before the precipitating solvent is added (the 'plasma first' method), leading to leaking, cloudy filtrates and blocked wells.

ISOLUTE® PPT+ Protein Precipitation Plates have been designed to overcome these issues. The functionalized bottom frit holds up organic solvents, in particular acetonitrile, allowing the precipitating solvent to be dispensed into each well prior to sample addition. This 'solvent first' methodology is optimal for both high efficiency protein precipitation and automation, since the solvent first approach negates the need for vortex mixing.

The system has an optimized porosity distribution and acts as a depth filter, retaining the precipitated protein without well blockage.

'Solvent First' Methodology for Protein Precipitation

ISOLUTE PPT+ plates can be used to process plasma sample volumes of up to 400 µL, using the optimal acetonitrile/plasma ratio of 3:1 (v/v).

The method below describes the procedure for processing 100 µL plasma.

1. Place the ISOLUTE PPT+ plate onto a suitable 96-well sample processing manifold (e.g., a Biotage® VacMaster™-96 Sample Processing Manifold or Biotage® PRESSURE+ 96 Positive Pressure Manifold). Ensure that a 96-well collection plate is positioned inside the manifold to collect the filtrate.
2. Dispense 300 µL of acetonitrile into each well. No solvent 'dripping' through the filterplate will occur.
3. Add 100 µL of plasma sample with force to each well. Again, no 'dripping' through the filterplate will occur. Allow the plate to stand for approximately 5 minutes. Mix using repeated aspirate/dispense cycles if required.
4. Apply vacuum or pressure to filter the sample, and collect the purified filtrate.

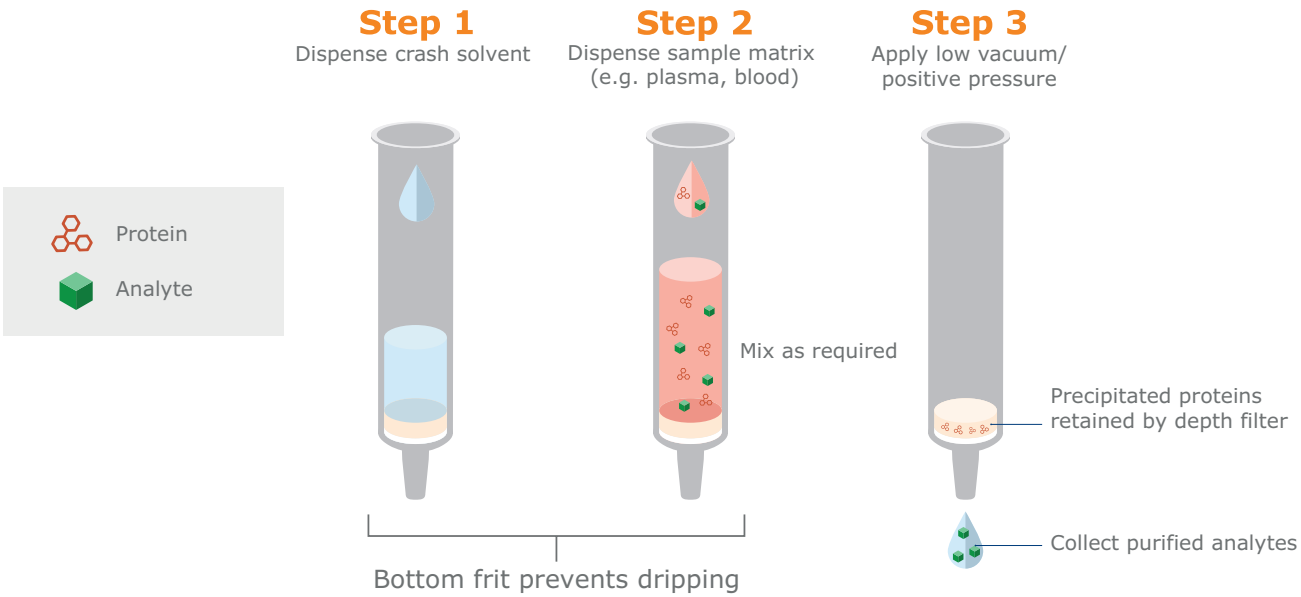

Figure 1. 'Solvent First' Procedure using ISOLUTE PPT+ Protein Precipitation Plates.

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Notes

1. The maximum practicable volume of plasma that can be processed is 400 µL, using 1200 µL of acetonitrile (total volume per well is 1600 µL) in the fixed well format.
2. Acetonitrile has been found to be the most effective solvent for precipitating protein. Alternatively, acid precipitation may be used e.g. , 1 M trichloroacetic acid (TCA) in a 3:1 (v/v) ratio (TCA/plasma). The same dispensing method should be used for these alternative precipitating solvents, and no dripping will occur until vacuum or pressure is applied.
3. We recommend that internal standard is added to the plasma sample and not the precipitating solvent, so that binding of the analyte to plasma components can be better mimicked. As the filtration system holds up organic solvents, the sample will not drip though the plate until vacuum or pressure is applied.
4. Serum or blood samples may also be processed, however, additional solvent may be required to fully precipitate the protein.
5. Recommended processing conditions:
   Vacuum: up to -15" Hg
   Positive pressure: 2–4 psi

Experimental Data

1. Analyte Recovery

Using the 'solvent first' methodology as described, ISOLUTE® PPT+ plates were used to isolate two probe compounds, propranolol and nadolol, from human plasma samples. Analyses were performed using HPLC-UV and LC-MS/MS respectively. Analysis conditions are shown below. An external standard was used for quantification. Following filtration, samples were evaporated at 40 °C, and reconstituted in 1 mL of mobile phase.

Results

Analyte	% Recovery	% RSD
Propranolol	93.6	7.6
Nadolol	75.4	6.1

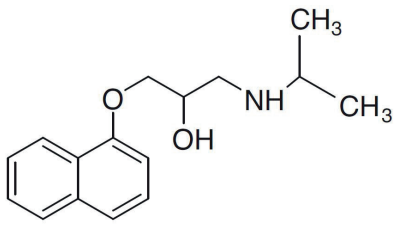
Conclusion

ISOLUTE® PPT+ plates can be use to achieve high, reproducible recovery of analytes from plasma samples.

Analytical Conditions

Propranolol

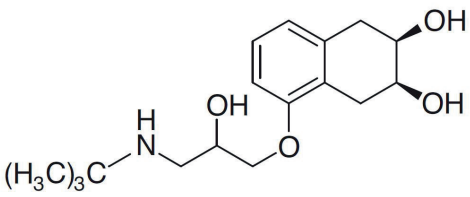
Concentration: 10 ng/100 µL plasma



HPLC-UV Conditions	
Column	Genesis 4 µm C18, 4.6 x 150 mm
Mobile Phase	0.2 % phosphoric acid pH 2.5 : MeOH (60:40, v/v) and 200 µL DEA
HPLC System	Agilent HP1100
Flow Rate	1.4 mL/min
Injection Volume	40 µL
Wavelength (λ)	220 nm

Nadolol

Concentration: 10 ng/100 µL plasma



HPLC and MS-MS Conditions	
Column	Polaris 3 µm C18-A,50 x 2.0 mm
Mobile Phase	0.1 % formic acid :MeCN : MeOH (70:25:5, v/v)
HPLC System	Varian ProStar binary pump
Injection Volume	40 µL
MS System	Varian 1200L Triple quadrupole
Ionization Mode:	ESI+
SRM Transition:	310 > 254 (-16 eV)

2. Protein Removal

Efficiency of protein removal using the 'solvent first' protein precipitation methodology was compared with the 'plasma first' method.

(i) Gravimetric Analysis of Residual Endogenous Material

Samples prepared using 'solvent first' and 'plasma first' methodology were compared to non-treated plasma.The amount of residual endogenous material remaining in the sample after filtration was determined gravimetrically.

For both methods, the filtrates from 5 wells were pooled together (total plasma volume 500 µL), and evaporated at 40 °C to constant weight. Non-treated plasma: 500 µL was dispensed into a pre-weighed vial and evaporated at 40 °C to constant weight.

Sample	Mass of Residuals in Filtrate	% Reduction
Non-treated plasma	44.0 mg	-
'Plasma first' method	15.7 mg	64%
'Solvent first' method	4.4 mg	90%

Results

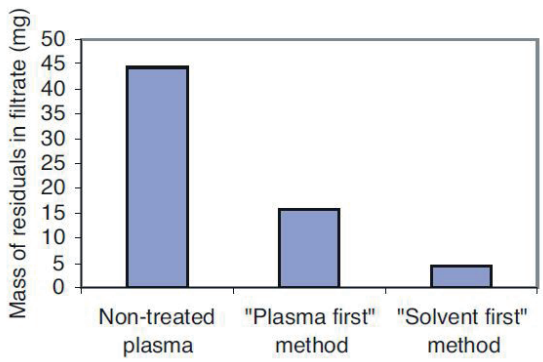


Figure 2.

Conclusion

The 'solvent first' method, using ISOLUTE PPT+ plates gives more efficient protein removal than the 'plasma first' method. Compared with non-treated plasma, ISOLUTE PPT+ plates reduce the amount of endogenous material (proteins, lipids, etc) in the prepared sample by up to 90%.

(ii) Effect on Ion Suppression

The effect of protein precipitation filtrates on analyte signal was investigated by flow injection analysis (FIA)[1] using electrospray LC-MS/MS.

100 µL blank human plasma was processed using either the 'plasma first' or 'solvent first' methods described previously. Blank plasma filtrates were evaporated and reconstituted with mobile phase containing caffeine at 1 µg/mL. 5 µL aliquots of the filtrates were injected directly into the ES interface without an HPLC column in place. Three replicate injections were analysed in SRM mode and compared mobile phase containing caffeine.

LC-MS/MS Conditions and Apparatus	
Mobile Phase	MeCN : Water (50:50, v/v) containing 0.1% formic acid
HPLC System	Varian ProStar binary pump/well plate sampler
MS System	Varian 1200L Triple Quadrupole
Ionizarion Mode	ESI+, SRM Transition: Caffeine 195 > 138

Results

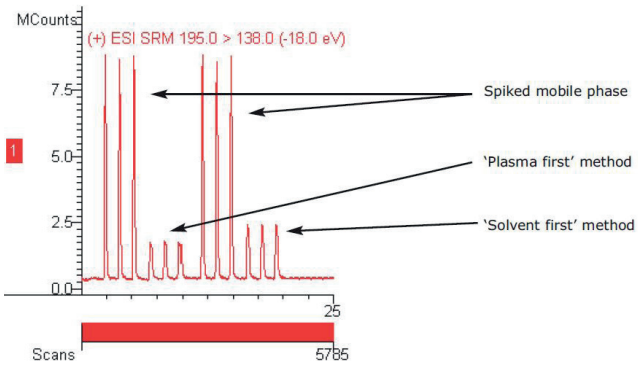


Figure 3. Mass chromatogram showing effect of residual endogenous material from 'plasma first' and 'solvent first' filtrates on signal response of caffeine, compared to spiked mobile phase.

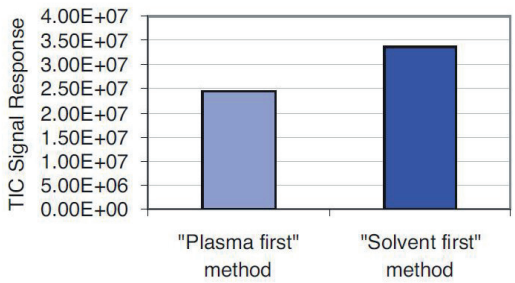


Figure 4.

Conclusion

The 'solvent first' method gives reduced ion suppression compared to the 'plasma first' method. Signal intensity is 37 % higher using the 'solvent first' method with ISOLUTE® PPT+ plates.

Ordering Information

Part Number	Description	Quantity
Fixed Well Plate		
120-2040-P01	ISOLUTE® PPT+ fixed well plate, 2 mL	1
Collection Plates		
121-5201	Collection plate, 350 µL	50
121-5202	Collection plate, 1mL	50
121-5203	Collection plate, 2mL	50
Vacuum Processing Manifold		
121-9600	Biotage® VacMaster™-96 Sample Processing Manifold	1
121-9601	Biotage® VacMaster VCU-1 Vacuum Control Unit	1
121-9602	Biotage® VacMaster VCU-2 Vacuum Control and Generation unit	1
Positive Pressure Processing Manifold		
PPM-96	Biotage® PRESSURE+ 96 Positive Pressure Manifold	1

References

1. R. Bonfiglio, R.C. King, T.V. Olah, K. Mwerkle; Rapid Commun. Mass Spectrom., (1999) 13 1175-1185.

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