Automation Compatible Protein Precipitation in High Throughput Filterplates

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INTRODUCTION

Protein precipitation is routinely used for removal of matrix components from biological fluids, typically plasma, prior to analysis. This approach has broad appeal, particularly in drug discovery support where a generic sample preparation approach, applicable to a broad range of compound types, is required. Recently, trapping of precipitated protein in microtitre filterplates and collection of the filtrate has become a popular approach due to its productivity advantages and ease of automation.

In order to achieve optimum precipitation, filterplate processing should begin with addition of precipitating solvent ('solvent first' approach). Many filterplates cannot be used with solvent first as they are unable to retain organic precipitating solvents. The applied solvent immediately passes through the filter under gravity minimizing contact with the plasma. This results in cloudy extracts containing increased levels of proteinaceous materials that can adversely affect quantitation by UV and tandem MS methods.

ISOLUTE PPT+ fixed well plate on VacMaster™-96 Sample Processing Manifold

We have developed a novel microplate (ISOLUTE® PPT+) with optimized filter material capable of retaining organic solvents. Recent work[1] on this 'solvent first' approach has shown that as well as maximizing protein precipitation efficiency, leading to reliable quantification of drugs, ISOLUTE PPT+ plates can be processed using a simplified procedure compared to other similar commercially available filterplates.

This poster describes the application of ISOLUTE PPT+ Protein Precipitation Plates in sample preparation of a broad range of analytes, and demonstrates the efficiency and automation compatibility of the processing procedure.

1. Efficiency of Protein Precipitation

Extraction of a wide range of drugs from plasma samples

ISOLUTE PPT+ plates were used to extract a range of drugs (see **Table 1**) of varying functionality (acidic, neutral and basic) and with a wide range of polarity.

EXPERIMENTAL SECTION

Experiment 1

The effect of the optimized filter material used in the ISOLUTE PPT+ plates on analyte recovery was investigated, to eliminate the possibility of low drug recoveries caused by non-specific binding of analytes to the filter material. This was accomplished by extracting the drugs from spiked deionized water samples (no plasma components present).

Experiment 2

The same drugs were then extracted from pooled human plasma samples, spiked at the same concentrations as **Experiment 1**. Analysis was by LC-MS/MS.

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EXPERIMENTAL CONDITIONS

Sample: Human plasma spiked with probe analytes, as listed in **Table 1**.

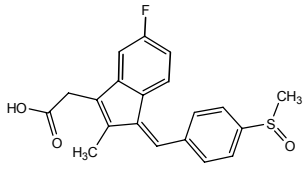
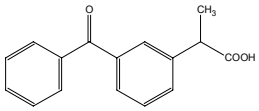
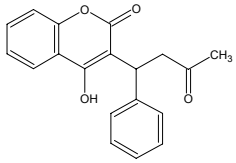
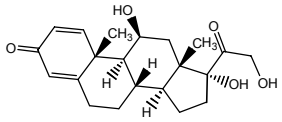
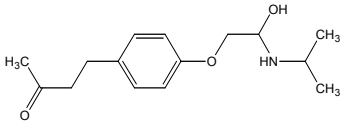
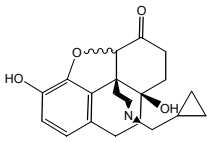
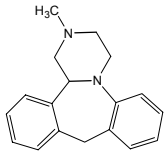
Classification	Compound	Structure	logP[1]	pK[1]	Plasma Sample Concentration (ng/µL)
Acidic, non-polar	Sulindac		3.59	3.6	0.1 ng
Acidic, polar	Ketoprofen		2.81	4.2	0.1 ng
Neutral, polar	Warfarin		3.42	4.5	0.1 ng
Neutral, polar	Prednisolone		1.49	N/A	1 ng
Basic, polar	Metoprolol		1.34	10.8	0.1 ng
Basic, polar	Naltrexone		1.80	9.2	0.1 ng
Basic, non-polar	Mianserin		3.67	8.3	1 ng

Table 1. Probe compounds

[1] pK and logP values were obtained from literature or calculated if not available.

EXPERIMENTAL CONDITIONS

Method

See Section 2 'Standard Procedure, No Vortex Mixing' for details on the procedure.

Experiment 1: Deionized water spiked at 0.1 or 1 ng/µL with 7 drugs (see **Table 1** for details).

Experiment 2: Pooled human plasma spiked at 0.1 or 1 ng/µL with 7 drugs (see **Table 1** for details).

LC-MS/MS Conditions

HPLC Conditions:
Column: Agilent Zorbax Eclipse XDB-C18 2.1 x 100 mm 3.5 µm with Agilent Eclipse XDB-C8 2.1 x 12.5 mm 5 µm guard column
Mobile Phase: A: Formic acid 0.1 % v/v
B: Acetonitrile
HPLC System: Varian ProStar binary pump
Flow rate: 250 µL/min

Gradient: Time (minutes)	%A	%B
0:00	80	20
5:00	64	36
7:00	20	80
8:00	20	80
8:06	80	20

MS/MS Conditions:
MS System: Varian 1200L Triple quadrupole
Ionization Mode: ESI+
MRM Transition:

Analyte	MRM Transitions	Collision Energy (eV)
Naltrexone	342.2>324	-18
Metoprolol	268.1>116	-16.5
Mianserin	265.2>208	-19
Prednisolone	361.3>147	-18.5
Sulindac	357.2>233	-28
Ketoprofen	255.1>209	-12.5
Warfarin	309.2>163	-13

RESULTS

Experiment 1: Analyte Recovery from Deionized Water

Analyte	% Recovery	% RSD
Naltrexone	104	8
Metoprolol	95	3
Mianserin	81	1
Prednisolone	98	6
Sulindac	95	9
Ketoprofen	97	3
Warfarin	102	8

Experiment 2: Analyte Recovery from Pooled Human Plasma Samples

Analyte	% Recovery	% RSD
Naltrexone	83	5
Metoprolol	81	6
Mianserin	74	2
Prednisolone	87	6
Sulindac	79	4
Ketoprofen	77	4
Warfarin	83	2

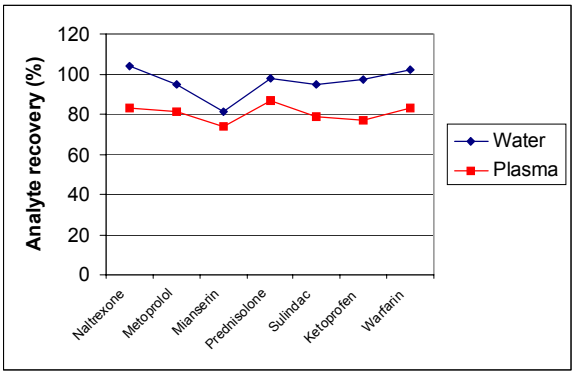


Figure 1. Recovery of analytes from spiked water and plasma samples. See Table 1 for analyte concentrations

CONCLUSIONS

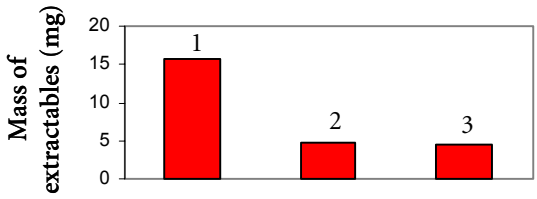
- **Experiment 1:** High analyte recoveries were obtained from spiked water samples, indicating no significant analyte loss caused by the optimized frit material used in ISOLUTE PPT+ plates.
- **Experiment 2:** High analyte recoveries were also obtained from spiked human plasma samples, demonstrating the applicability of ISOLUTE PPT+ Protein Precipitation Plates to the extraction of a wide range of drugs.
- Lower analyte recovery from plasma samples compared with spiked water can be attributed to a small percentage of analytes being trapped by the precipitating protein therefore not being detected in the filtrate.

Section 2. Automation Compatibility and Productivity Considerations

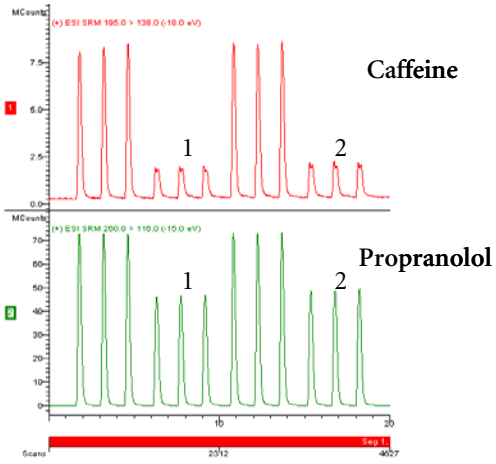
In previous work[1] we have demonstrated that the ISOLUTE PPT+ Protein Precipitation Plate efficiently removes proteinaceous material from plasma samples without the requirement for vortex mixing as part of the procedure.

Filtrates resulting from a protein precipitation procedure incorporating a vortex mixing step (as recommended for use with other commercially available protein precipitation plates) were found to have no significant difference in levels of proteinaceous material (determined gravimetrically) compared to filtrates from ISOLUTE PPT+ plates processed using the standard procedure (detailed in **Section 1**, no vortex mixing step required). In addition, these filtrates showed similar levels of ion suppression in LC-MS/MS analysis.

See **Figure 2** for gravimetric and LC-MS/MS results from this previous work.



There is no significant difference in the mass of plasma extractables between the ISOLUTE PPT+ plate processed with vortex mixing (2), and the ISOLUTE PPT+ plate processed without vortex mixing (3). The samples were then analysed by FIA LC-MS/MS, to investigate the effect of vortex mixing on the degree of ion suppression. Extractables from the 'plasma first' method are also shown (1).



1 = Vortexed, 2 = Not vortexed

Figure 2. Data from previous work[1] showing the effects of vortex mixing versus not vortexing

Elimination of a vortex mixing step has significant advantages in terms of ease of automation, negating the need for a time consuming step requiring manual intervention.

In this current work, we have investigated the effect of vortex mixing on analyte recovery.

EXPERIMENTAL CONDITIONS

Method

Sample: Pooled human plasma spiked at 0.1 or 1 ng/µL with 7 drugs (see **Table 1** for details).

Protein Precipitation (Standard Procedure, No Vortex Mixing)	Protein Precipitation (With Vortex Mixing)
ISOLUTE PPT+ fixed well protein precipitation plate (p/n 120-2040-P01)	ISOLUTE PPT+ fixed well protein precipitation plate (p/n 120-2040-P01)
• Pipette 300 µL acetonitrile into each well	• Pipette 300 µL acetonitrile into each well
• Pipette 100 µL sample into each well	• Pipette 100 µL sample into each well
• Allow to stand for 2 minutes	• Vortex for 2 minutes
• Apply vacuum at -15 "Hg for 3 minutes and collect the filtrate	• Apply vacuum at -15 "Hg for 3 minutes and collect the filtrate
• Evaporate and reconstitute in mobile phase (1 mL) prior to analysis	• Evaporate and reconstitute in mobile phase (1 mL) prior to analysis

Analytical Method

As described in **Section 1**.

RESULTS

No significant differences in analyte recovery were observed between the 'vortex mixing' and 'no vortex mixing' procedures carried out using the ISOLUTE PPT+ Protein Precipitation Plates (see **Figure 3**).

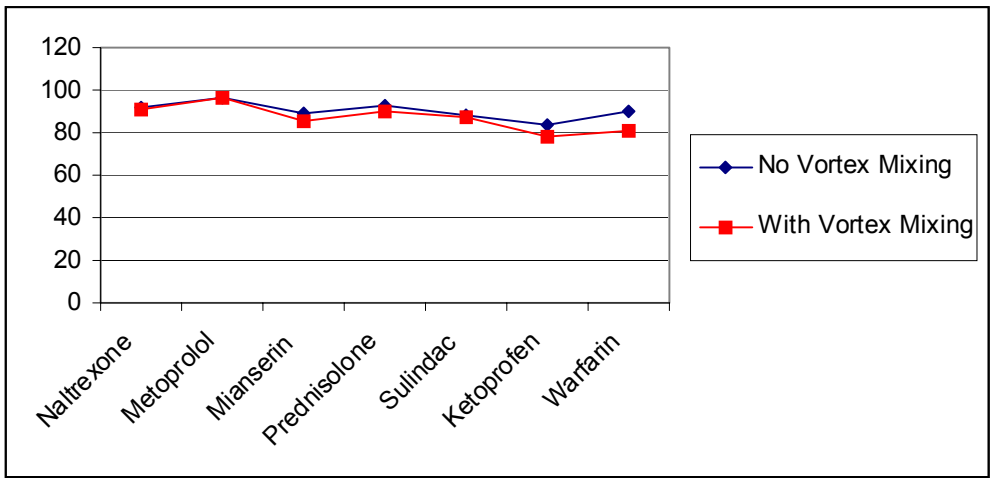


Figure 3. Comparison of analyte recovery using ISOLUTE PPT+ plates processed using the standard procedure and 'vortex mixing' procedure

CONCLUSIONS

Vortex mixing is not required during the ISOLUTE PPT+ protein precipitation procedure. No advantages are seen in terms of reduction of proteinaceous material present in the extract, reduction of ion suppression, or increase in analyte recovery when a vortex mixing step is incorporated.

OVERALL CONCLUSIONS

ISOLUTE PPT+ Protein Precipitation Plates can be used to efficiently extract a wide range of analytes from plasma samples with high analyte recoveries and low RSD.

ISOLUTE PPT+ plates are processed using a simple, efficient procedure, which does not require a time consuming, difficult to automate vortex mixing step.

References

1. Improved Protein Precipitation using a Novel Filtration Microplate by M. Cleeve et al. Presented at ASMS 2004.

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