MILLIPORE

Application Note

Date: **April 2004**

Lit. No.: **AN2004EN00**

Title: **High throughput sample preparation for the quantitation of drug compounds in serum samples**

ABSTRACT

A rapid and automatable high throughput sample preparation method for total drug analysis of 50 to 300 µL serum or plasma samples based on solvent precipitation of protein has been developed using a solvent resistant 96-well filter plate (MultiScreen® Deep Well Solvinert filter plates). The method has been used with LC-MS/MS analysis to assess correlation and reproducibility for three different compounds (propranolol, testosterone and warfarin) in adult bovine serum. This filtration-based method provides comparable results to centrifugal isolation with the advantage of ease, speed, automation, better reproducibility, greater recovery of sample and better removal of precipitated protein.

Results demonstrate that the MultiScreen Deep Well Solvinert filter plate is a robust and reliable platform that generates reproducible results when coupled with an appropriate protocol. Samples isolated from serum by protein precipitation and filtration through the Deep Well Solvinert filter plate are essentially protein free and show high compound recovery and no interference from extractables.

BACKGROUND

Determining the concentration of drug in plasma or serum at various time points after administration is necessary to calculate the pharmacokinetics (PK) of a drug. PK, in turn, is an important component in the absorption, distribution, metabolism, and excretion (ADME) profile for a drug. Precise knowledge of ADME properties enables accurate determination of the proper drug dosage to maintain therapeutic drug levels without risking toxicity.

With the high demand on the pharmaceutical industry to introduce new drugs to market quickly and in a cost-effective manner, the availability of fast and effective sample preparation techniques and bioanalytical methods is critical. In total drug analysis applications, the compound must be quantitatively separated from the plasma proteins and solvated in a form that makes the compound amenable to subsequent analysis. Quantitative analysis from plasma using LC-MS/MS requires sample preparation such as solid phase extraction, liquid/liquid extraction, or protein precipitation. Protein precipitation is preferred in a high throughput setting, since it is fast and requires little method development.

A popular sample preparation method for total drug analysis in a high throughput workflow is protein precipitation with a water miscible organic solvent such as acetonitrile (ACN), followed by separation of the precipitated proteins and salts from the

aqueous organic solution with subsequent analysis using a method such as LC-MS/MS.[1,2] Centrifugation is one method to separate precipitated proteins; however, filter plates have also proven to be effective.[3] Besides easier automation and handling, filtration with a high retentive membrane can improve data quality because even the slightest particulate contamination during transfer can have a detrimental impact on the LC-MS/MS analysis.[4] It is important that the filtration method have no interference with quantitation either through extractables or non-specific binding.

INTRODUCTION

The MultiScreen Deep Well Solvinert filter plate has been optimized for removal of precipitated proteins and particulates prior to analysis. The plate has been validated for complete precipitated protein retention, with fast, discreet and complete filtrate transfer to provide an integrated, high-throughput, automation-compatible platform for sample preparation prior to total drug analysis.

Since high quality polyolefin materials and a polytetrafluoroethylene (PTFE) membrane are used, the plate exhibits very low NSB, and has little or no UV or Mass Spectrometry (MS) detectable or interfering extractable components. The MultiScreen Deep Well Solvinert filter plate is compatible with both vacuum and centrifugal modes of filtration and is designed to fit with standard 96- deep well receiver blocks for use in filtrate collection.

This Application Note details the use of the MultiScreen Deep Well Solvinert filter plate and supporting methodology for determining the total amount of drug in plasma or serum samples. Filtration with the device provides a protein-free filtrate that is compatible with HPLC-MS or HPLC-UV analysis. A comparison of filtration and centrifugation for removal of precipitated proteins is presented. Reproducibility and reliability were also evaluated. Furthermore, nonspecific binding and extractables data were measured to support the claim that the device does not interfere with sample recovery either by retaining compound (NSB) or by interfering with analysis as a consequence of releasing impurities. Results are presented for three different compounds (Testosterone, Propranolol, and Warfarin) at five different initial concentrations in the adult bovine serum (0.1 µM, 0.5 µM, 1 µM, 5 µM, and 10 µM).

MATERIALS & METHODS

- Testosterone (T-1500), Propranolol (P-8688) and Warfarin (A-2250) were purchased from Sigma (St. Louis, MO)
- Radiolabeled (^{3}H) compounds were purchased from different sources:
    - Testosterone (NET-553), Propranolol (NET-515) and Digoxin (NET-222) were purchased from Perkin Elmer Life Sciences, Inc. (Boston, MA).
    - Methotrexate (MT701), Taxol (MT552) and Warfarin (MT1620) were purchased from Moravek Biochemicals (Brea, CA)
    - Ibuprofen (ART 392) and Verapamil (ART 667) were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO)
- Bovine Adult Serum #B-2771 (Sigma—St. Louis, MO)
- Acetonitrile #A998^{SK}-1 (Fisher Scientific—Atlanta, GA)
- Milli-Q water (Millipore, Billerica, MA)

PROTOCOL

For specific details on the use of the MultiScreen Deep Well Solvinert plate with a phobic PTFE membrane and pre-filter for total drug analysis, including sample volume and important mixing procedures, see Millipore Protocol note PC1052EN00. For a more generic protocol with manual sample handling, see Millipore Protocol note PC1041EN00.

General procedure for protein precipitation with automation or programmable pipettes:

To a MultiScreen Deep Well Solvinert filter plate (catalog # MDRP NP4 05), 800 µl of acetonitrile was added. Using a programmable Biohit Proline™ pipette (Biohit—Helsinki, Finland), 200 µl of serum was pulled into the pipette tip(s) followed by 200 µl of acetonitrile from the 800 µl contained in the appropriate well(s) of the MultiScreen Solvinert Deep Well filter plate to initiate precipitation. The 1:1 ACN:serum solution was added to the 600 µL of acetonitrile remaining in the well(s) to afford a 4:1 ACN:serum mixture with a total volume of 1 mL. The plate was shaken for 2 minutes, incubated at 4 ^{o}C for 1 hr,[5] then filtered at 20" Hg or higher.

Three drugs, testosterone, propranolol, and warfarin, were tested at initial concentrations in the serum of 0.1 µM, 0.5 µM, 1 µM, 5 µM, and 10 µM. Samples were analyzed after being filtered. All samples were diluted with an equal volume of water prior to LC-MS/MS analysis to improve retention on the stationary phase of the LC (6 replicate wells with 3 LC-MS/MS injections per well).

Centrifugation All centrifugation steps were performed in a Greiner polypropylene 96 well Masterblock (catalog # 780285) in a Jouan CR 312 Centrifuge at 2000 x g for 5 minutes. Supernatants were removed for further analysis following centrifugation.

LC-MS/MS analysis: Unless otherwise indicated, LC-MS/MS analyses were performed using a Sciex API-2000 mass spectrometer coupled with an Agilent 1100 HPLC and well plate autosampler. A Phenomenex Synergi Hydro-RP (4 µm, 50x2 mm) C-18 column was used with a guard cartridge. For ESI-MS, Solvent A was 0.1% formic acid in water, solvent B was 100 % methanol. For APCI, solvent A was water. **Warfarin:** Injection volume: 15 µL/sample, flow rate = 300 µL/min, HPLC solvent of 80 % A to 10 % A in 4 min, then to 80 % A in 1 min. A TurboIonSpray (ESI) source was used in the positive mode monitored at m/z = 309/163. **Propranolol:** Injection volume: 5 µL/sample, flow rate = 300 µL/min, HPLC solvent of 100 % A for 2 min, gradient to 40 % A in 3 min, then returns to 100 % A in 1 min. A TurboIonSpray (ESI) source was used in the positive mode monitored at m/z = 260/116. **Testosterone:** Injection volume: 30 µL, flow rate = 500 µL/min, 50 % A to 0 % over 2.5 min, 0 % A to 50 % over 1.5 min, then remained at 50 % A for 2 min. A Heated Nebulizer (APCI) source was used in the positive mode, MS/MS monitored at 289/97.

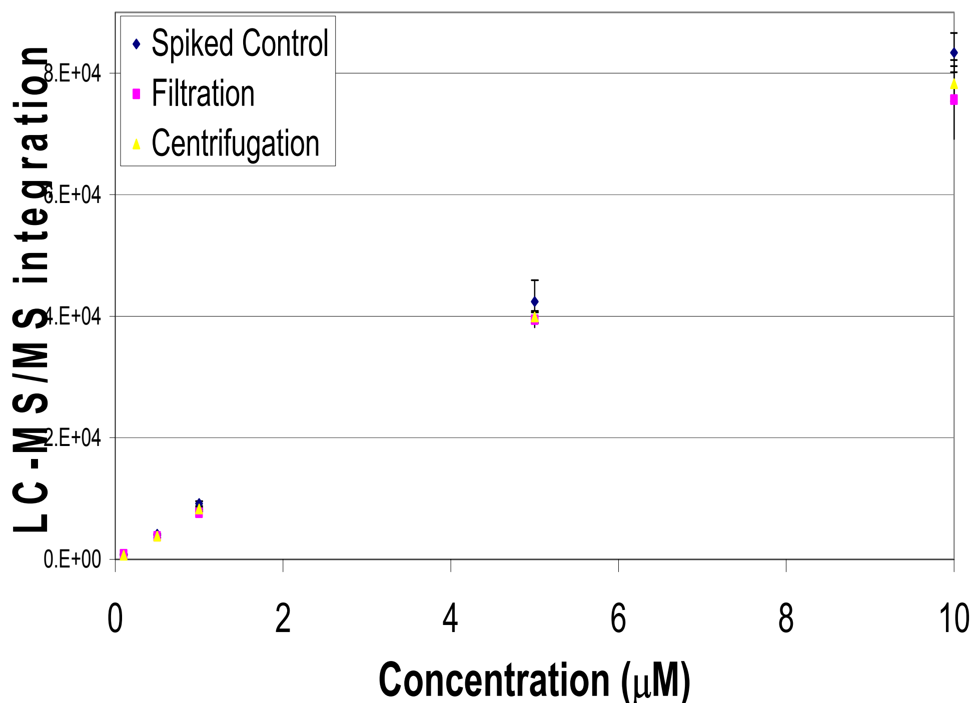
RESULTS AND DISCUSSION

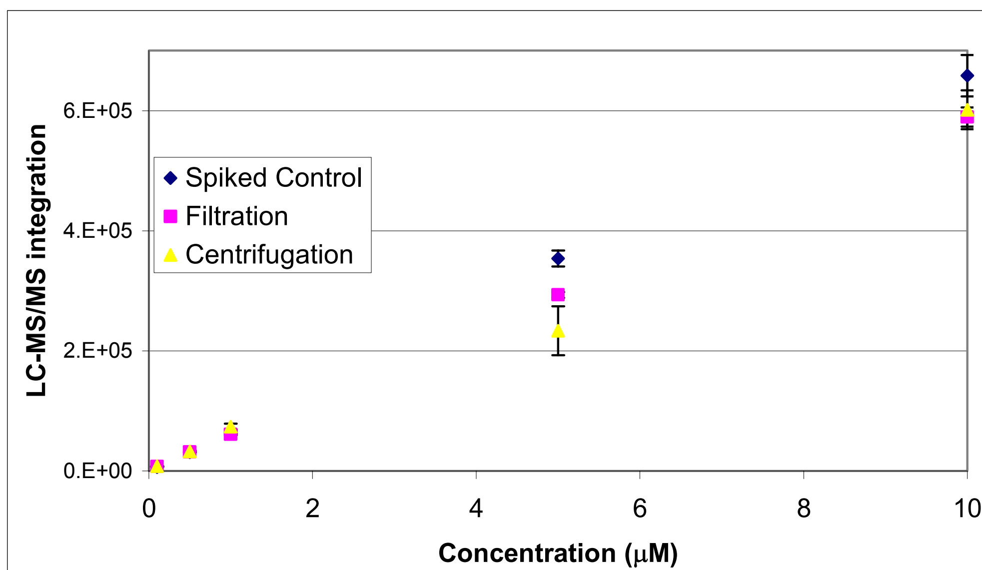
Correlation and Reproducibility of Filtration Method

Quantititive results obtained using the filtration method and the centrifugation method were compared to protein-free, ACN-treated serum ("spiked control"). Three

different compounds (Testosterone, Propranolol, and Warfarin) at five different initial concentrations in the serum (0.1 μM, 0.5 μM, 1 μM, 5 μM, and 10 μM) were analyzed to represent neutral, positively charged and negatively charged compounds. All compounds are highly bound (85-99.5%) by plasma proteins (Propranolol, 89%; Testosterone, 95.5%; Warfarin, 99.3%).[6]

Results obtained for testosterone and propranolol presented in **Figures 1A-B** indicate good agreement of the results obtained by the filtration method with those obtained by the centrifugation method and the spiked control. Method correlation was also observed for warfarin (Table 1). The filtration method showed good recovery (~90% or better) of the compound from the protein, even for these highly bound drugs which is as good or better than results published with a similar method.[3] The coefficient of variance (%CV) averaged from 18 replicates was also determined for each compound. The reproducibility of the filtration method was consistently very good (%CV less than 10% for all tested compounds). The centrifuge method had sporadic issues with reproducibility, presumably due to inconsistent protein removal. The reproducibility in the centrifuge method was unusually elevated in some cases (up to 24%) as displayed in Table 1.





Figures 1A and 1B: LC-MS/MS results for testosterone (figure 1A) and propranolol (figure 1B) incubated with serum at 5 different concentrations followed by protein precipitation using acetonitrile and subsequent removal by either centrifugation or filtration.

Table 1. Warfarin (n=18)

	Spiked Control		Filtration Method		Centrifuge Method	
Actual Concentration	Measured	%CV	Measured	%CV	Measured	%CV
0.1 µM	0.082	4%	0.083	5%	0.093	12%
0.5 µM	0.50	4%	0.42	4%	0.46	6%
1 µM	0.98	4%	0.85	7%	0.99	24%
5 µM	5.1	4%	4.4	4%	4.4	8%
10 µM	10.0	7%	8.8	3%	10.0	16%

The possibility of compound loss due to non-specific binding was evaluated using three approaches:

- Testosterone, Warfarin, and Propranolol were spiked (at various concentrations ranging from 0.1 to 10 µM) into 80% ACN in water and then filtered through the MultiScreen Deep Well Solvinert plate. The amount of drug present in each of the filtered samples was compared to levels present in unfiltered samples.

Testosterone, Warfarin and Propranolol in the range of 0.1 – 10µM were all > 95% recovered.

- 1 mL of 1, 3, and 10 nM solutions of Warfarin (80% ACN in water) were filtered through the MultiScreen Deep Well Solvinert plate. The amount of Warfarin present in the filtrate was compared to levels present in unfiltered samples Warfarin at the lower concentration range (1 to 10 nM) was consistently > 88 % recovered (data not shown),
- 1mL of different radiolabelled compounds (see Figure 2) solutions, each at a concentration of 1 uM in the filtrate obtained from a 4:1 mixture of ACN:serum, were filtered through the MultiScreen Deep Well Solvinert plate. The amount of radioactivity in the filtrate was compared to the level of radioactivity present in the unfiltered solutions and the results for the panel of 10 compounds shows recovery >94% (**Figure 2**).

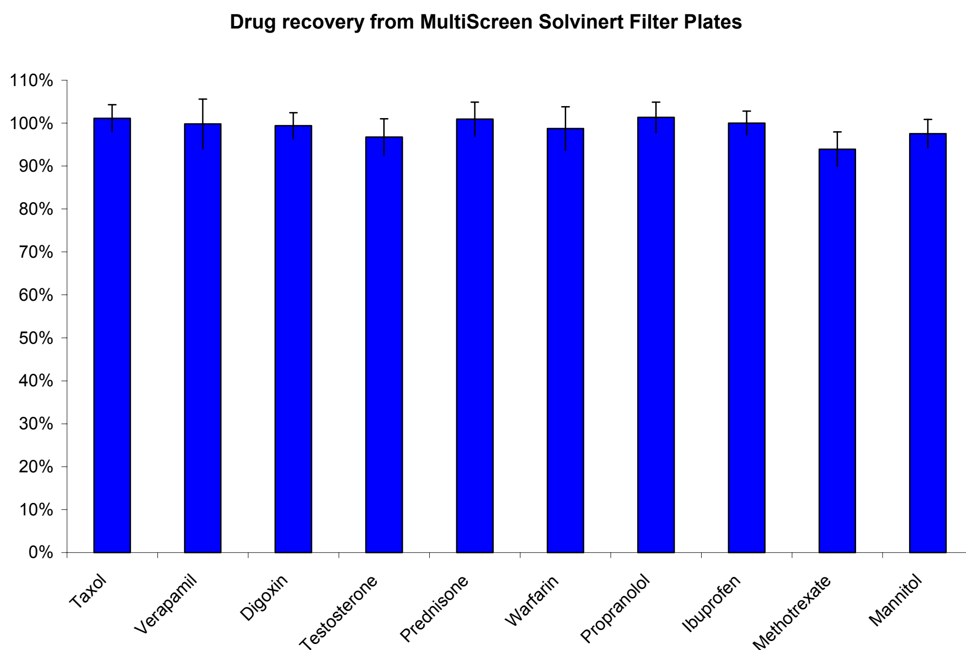


Figure 2: Drug Recovery at 1.0 µM: Drugs were added to a protein free filtrate obtained from previously ACN precipitated filtered serum, incubated and filtered. Recovery was determined as compared to non-filtered standards.

Protein-free filtrates

Complete protein retention was measured as a function of filtrate clarity as determined using light scattering at 800 nm by recording percent transmittance (%T). It was determined that a %T value of 96% for these measurements corresponds to the presence of approximately 20 to 25 µg/mL of protein in aqueous acetonitrile which is equivalent to greater than 99.95% removal of the precipitated protein. A particulate-free sample will have a % T of approximately 100% while precipitates in the filtrate will result in a proportional decrease of transmitted light. Filtrate analysis from total drug analysis conducted in and filtered through a MultiScreen Deep Well Solvinert filter plate has an average %T of 99.5 ± 0.3% (n = 480; 5 96-well plates) which is essentially a protein free solution.

Both centrifugation and filtration methods are able to remove > 99.5 % of proteins from ACN precipitated serum as determined by two separate analytical techniques: radiometric (presence of radiolabelled BSA in the prepared sample) and a Coomassie™ protein assay of the prepared sample.[7] While filtration produces a homogeneous sample and allows for nearly quantitative volume recovery, the amount of protein in the centrifugal supernatant is inversely proportional to its proximity to the protein pellet as illustrated in **Figure 3**. Consequently, the amount of sample recovered following centrifugation may vary and will depend on specific analytical needs related to protein interference.

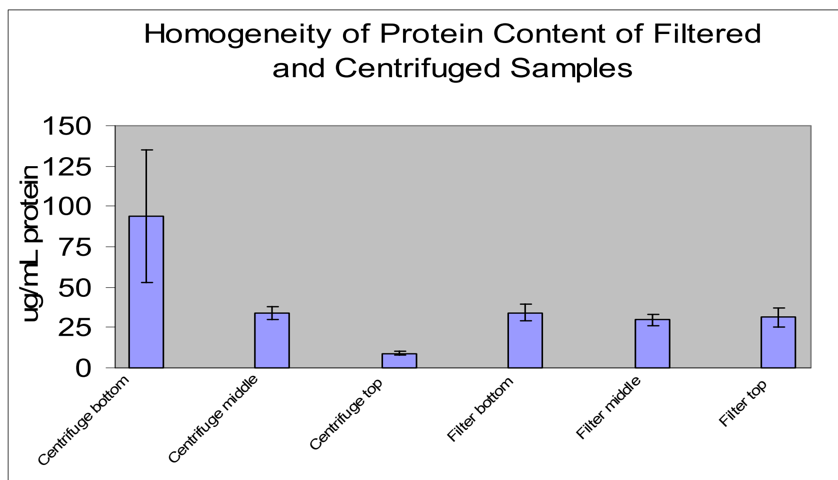


Figure 3: Protein concentration of the supernatant based on the approximate distance of the extraction point from the bottom of the well.

Robust and Reliable

To establish the robustness and reliability of the filtration performance required for automation, twenty plates were evaluated under application conditions (200µl of serum mixed with 800 µl of ACN) with the following results:

- High yield of filtrate with uniform filtration (940 ± 25 µl, %CV = 2.6 %).

- Fast filtration with no plugging: all wells on all plates emptied in less than 30 seconds.
- No drip outs after 10 min shaking at room temperature.
- In a separate experiment, discrete transfer of the filtrate was demonstrated with a checkerboard pattern of a sodium fluorescein and every other well was checked for cross-contamination (i.e., cross-talk). No cross-talk events were detected in 10 plates (960 wells) after filtration of 1 mL an 80% ACN solution.

Determination of Extractables and Ion Suppression

Extractable compounds can potentially interfere with total drug analysis in two different ways: Extractable compounds may contribute species that interfere with the detection, and/or; extractable compounds may contribute to ion suppression and elevate background. Filtrates from the MultiScreen Deep Well Solvinert plate were analyzed by HPLC-UV for the presence of extractable compounds. Sample plates were incubated in ACN for 1 hour and then the filtrate was collected and analyzed by HPLC –UV. The results of the analysis at 214 nm are presented in Figure 4 along with HPLC-UV of warfarin at 5.0 μM . Results obtained by monitoring at 254 nm were similar (data not shown).

While extractable compounds would not be expected to falsely elevate the amount of a specific analyte detected by LC-MS/MS, extractable species have the potential to contribute to ion suppression. **Figures 5A** and **B** show the LC-MS/MS analysis for warfarin added to 4:1 ACN:serum filtrate at 1.0 nM (**Figure 5A**), then filtered through the device (**Figure 5B**). Drug recovery in the filtered samples was determined to be consistent compared to an unfiltered sample in the same matrix showing no ion suppression from the device.

Formatted: Font: Bold

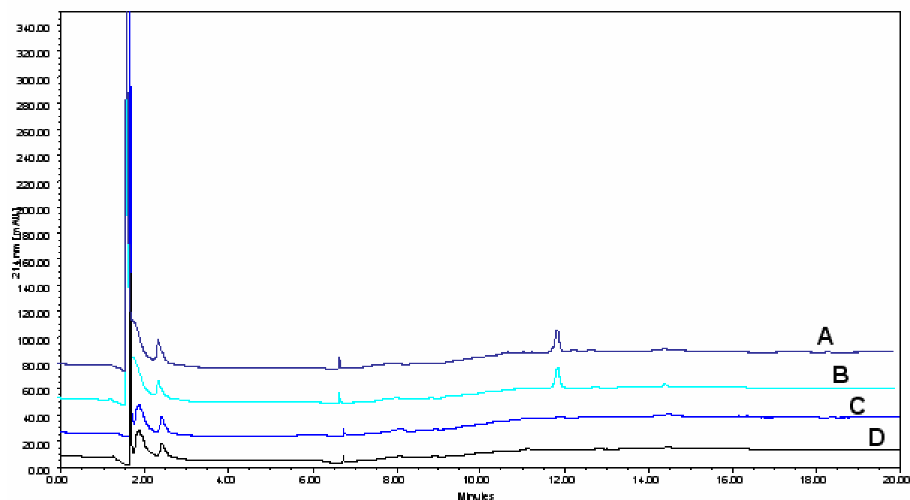


Figure 4: HPLC-UV analysis at 214 nm for extractable species. Chromatogram A: control sample for warfarin at 5 µM in 80 % aqueous acetonitrile; B: Warfarin at 5 µM in 80 % ACN after 1 hr incubation and filtration through the device; C: 80 % aqueous ACN after 1 hr incubation and filtration through the device (extractables); and D: solvent control.

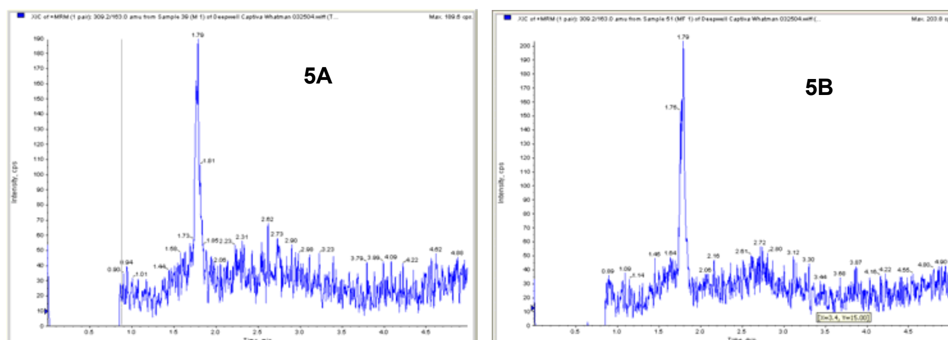


Figure 5: LC-MS/MS traces for (5A) 1.0 nM warfarin spiked into serum supernatant, then 1.0 nM warfarin filtered through a MultiScreen Solvinert Deep Well plate (5B).

CONCLUSION

The MultiScreen Deep Well Solvinert filter plate provides an automation compatible, high throughput platform for total drug analysis using in-plate precipitation of proteins from plasma or serum. This sample preparation method is a fast, simple and convenient and was used to process serum 96 samples for analysis in less than 10 minutes. Isolation of compound solutions from precipitated proteins by vacuum filtration is complete and

eliminates risk of cross-contamination. Furthermore, the filtrates are particulate free since due to use of a 0.4 µm membrane, thus providing a better sample for HPLC analysis. The plate and methodology provide for high drug recovery, low non-specific binding, and no interference from extractable compounds. The described methodology is robust and reliable, and generated highly reproducible data in conjunction with LC-MS/MS.

---

[1] Hsieh, Y., et al., Direct cocktail analysis of drug discovery compounds in pooled serum samples using liquid chromatography—tandem mass spectrometry. *J. Chrom.B,* **2002.** *767*, 353-362.

[2] Žunic, G., et al., Optimization of a free separation of 30 free amino acids and peptides by capillary zone electrophoresis with indirect absorbance detection: a potential for quantification in physiological fluids. *J. Chrom. B*, **2002**. *772***,** 19-33.

[3] Biddlecomb et al., Automated protein precipitation by filtration in the 96-well format. *Journal of Chromatography B*, 734 (1999) 257–265

[4] Dams, Riet; Huestis, Marilyn A.; Lambert, Willy E.; Murphy, Constance M., Matrix effect in bio-analysis of illicit drugs with LC-MS/MS: influence of ionization type, sample preparation, and biofluid. *J. Am. Soc. Mass. Spec.* **2003.** *14***,** 1290-1294.

[5] Polson, C.; Sarkar, P.; Incledon, B.; Raguvaran, V.; Grant, R. Optimization of protein precipitation based upon effectiveness of protein removal and ionization effect in liquid chromatography–tandem mass spectrometry, *J. Chrom. B*, **2003**. *785*, 263-275.

[6] MultiScreen Filter Assembly with Ultracel®-PPB Membrane Application Note: Drug plasma protein binding (PPB) assays: reproducibility and correlation (AN1732EN00)

[7] Bradford, M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye-binding. *Anal. Biochem*. **1976**, *72*, 248-254.

Millipore, MultiScreen and Ultracel are registered trademarks of Millipore Corporation.

Proline is a trademark of BioHit Oy.

Coomassie is a trademark of BASF Aktiengesellschaft.