Application Note

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

ABSTRACT

A new, solvent resistant 96-well filter plate (MultiScreen® Solvinert filter plate) was evaluated to determine its performance in total drug analysis applications. Non-specific drug binding (NSB) and drug recovery were measured following typical protein precipitation protocols (e.g., 3:1 acetonitrile addition followed by 1 hour of room temperature incubation). The drug recovery of eight different compounds (digoxin, propranolol, taxol, testosterone, verapamil, warfarin, ibuprofen, and methotrexate) was measured in multiple wells of different MultiScreen Solvinert filter plates. Reproducibility from well-to-well, plate-to-plate and day-to-day was evaluated with in-plate and out-of-plate protein precipitation methods. NSB was measured for each of the compounds, which tested with greater than 90% drug recovery. Additionally, multiple solvent systems were examined using the methodology detailed below, illustrating the robustness and flexibility of the assay and the Multiscreen Solvinert filter plate.

BACKGROUND

In order to calculate the pharmacokinetics (PK) of a drug, its concentration in plasma at various points in time after administration must be determined. PK, in turn, is an important component of a drug's absorption, distribution, metabolism, and excretion (ADME) profile. Precise knowledge of a drug's ADME properties enables accurate determination of the proper drug dosage necessary to maintain therapeutic drug levels in the blood without risking toxicity.

The pharmaceutical industry faces a high demand for new drugs to be introduced to the market, so the availability of fast and effective sample preparation techniques and bioanalytical methods is essential. UV-Vis and Mass Spectrometry (MS) after separation with high performance liquid chromatography (HPLC) are the methods most commonly used in the pharmaceutical industry to measure the concentration of a drug in plasma. Prior to analysis, it is typically necessary to extract the solute (drug) by precipitating proteins using acetonitrile, trifluoroacetic acid in acetonitrile, methanol, or some other water-miscible solvent combination. Failure to remove protein and eliminate interfering ions (e.g., salts) may shorten the lifespan of the HPLC column and produce spurious results due to ion suppression.

In many protein precipitation procedures, three or four volumes of organic solvent are added to the plasma sample and the mixture is mixed and incubated for up to an hour at room temperature. At this point, some means to separate the denatured protein from the sample (containing the compound and non-protein plasma constituents) is employed. The MultiScreen Solvinert filter plate was evaluated for its ability to be used to perform this separation step in an integrated, automation-compatible workflow.

INTRODUCTION

The MultiScreen Solvinert filter plate is a single-use, automation-compatible 96-well device for use in processing a wide range of aqueous and organic solutions. The device is intended for the high throughput processing of these solutions in the 0.1– 0.5 mL volume range. The device can be used in conjunction with vacuum filtration or filtration by centrifugation and is designed to fit with standard 96-well receiver plates for use in filtrate collection. The MultiScreen Solvinert filter plate has been developed and optimized for use in a wide range of applications including total drug analysis, solid phase combinatorial chemistry, peptide synthesis, and sample preparation prior to HPLC. The chemically-resistant, 0.45 µm PTFE

membrane used in the MultiScreen Solvinert filter plate has excellent flow properties, exhibits low NSB and low protein binding, and has little or no UV or MS detectible extractable components. The device meets Society for Biomolecular Screening (SBS) dimensional guidelines and tolerances for automation compatibility.

The assay workflow illustrated in **Figure 1** produces 96 samples for the determination of (total) drug in serum following protein precipitation. The precipitation of proteins from the serum sample can be done either within the individual wells of the MultiScreen Solvinert filter plate or outside of the plate prior to filtration. Any number of different solvents or solvent combinations can be used.

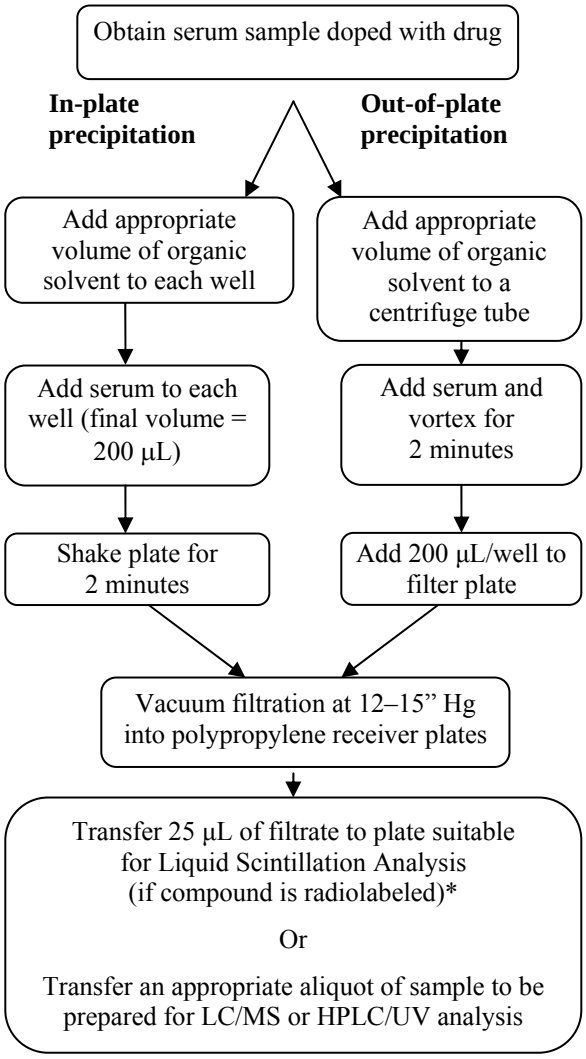


Figure 1. MultiScreen Solvinert Filter Plate Drug Analysis Flow Chart

Starting with equilibrated drug–serum samples[1], serum proteins are precipitated prior to filtration by one of two methods:

- In-plate precipitation: The appropriate amount of organic solvent (*i.e.,* acetonitrile, acetone, aqueous 20% TCA or methanol) is added to each well of the MultiScreen Solvinert filter plate, followed by an aliquot of the incubated drug solution to achieve the desired dilution factor.[2,3,4,5] The plate is then shaken for 2 minutes.[6]

- Out-of-plate precipitation: The incubated drug solution is added to an organic solvent in a centrifuge tube to achieve the desired dilution factor, then mixed by vortex for 2 minutes. The solution is then added to the MultiScreen Solvinert filter plate at 200 µL/well.

The MultiScreen Solvinert filter plate is filtered by vacuum mode at a pressure of 12 to 15" Hg into a polypropylene receiver plate. Following removal of the receiver plate from the vacuum manifold, 25 µL of each filtrate sample is removed and analyzed for drug concentration in order to determine the Percent Recovery from the initial serum/drug solution.

Additionally, NSB is analyzed in a similar manner. A 3:1 acetonitrile:serum solution is mixed for 2 minutes and filtered through a MultiScreen Solvinert filter plate. The drug is added to the filtrate to achieve a final concentration of 5 µM, and then filtered through another MultiScreen Solvinert filter plate into a polypropylene receiver plate. Then, 25 µL of each filtrate sample is removed and analyzed for drug concentration to determine the Percent Recovery or Percent Bound to the membrane/filter plate.

**Although this application note highlights the ease of high throughput sample preparation for analysis by HPLC-UV or LC/MS/MS, the results presented herein were tabulated employing radiometric detection with tritiated, commercially available drugs. This alternative detection method was selected for its reliability, ease of use and high reproducibility.*

MATERIALS & METHODS

Reagents

- Radiolabeled (^{3}H) compounds were purchased from different sources:
  - Testosterone (NET-553), Propranolol (NET-515) and Digoxin (NET-222) were purchased from PerkinElmer 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).
- Non-radiolabeled compounds were purchased from Sigma (St. Louis, MO): Testosterone (T-1500), Verapamil (V-4629), Taxol (T-1912), Digoxin (D-6003), Ibuprofen (I-4883), Methotrexate (M-8407), Propranolol (P-8688) and Warfarin (A-2250)
- PBS, pH 7.4 pouches #P-3813 (Sigma—St. Louis, MO)
- Bovine Adult Serum #B-2771 (Sigma—St. Louis, MO)
- Acetonitrile #A998^{SK}-1 (Fisher Scientific—Atlanta, GA)
- Scintillation cocktail #1200-439 (PerkinElmer Life Sciences, Inc.—Boston, MA)

Materials

- MultiScreen Solvinert filter plates #MSRL N04 10 and #MSRP N04 10 (Millipore—Bedford, MA)
- Biohit™ Proline single and multi-channel pipetters (Biohit—Helsinki, Finland)
- Wheaton Redi-Pak® 1oz. Wide-mouth amber glass bottles #02-911-7 (Fisher Scientific—Atlanta, GA)
- 15 mL graduated centrifuge tubes #05-527-90 (Fisher Scientific—Atlanta, GA)
- 50 mL graduated centrifuge tubes #14-432-23 (Fisher Scientific—Atlanta, GA)
- Non-sterile troughs #13-681-100 (Fisher Scientific—Atlanta, GA)
- Greiner Polypropylene 96 well V-bottom plates #M-8185 (Sigma—St. Louis, MO)
- Microbeta® sample plates #1450-401 (PerkinElmer—Boston, MA)

Equipment

- Thermolyne 37600 Mixer
- Lab-Line® Incubator
- Multiscreen Vacuum Manifold #MAVM 096 0R (Millipore—Bedford, MA)
- Lab-Line titer plate shaker #4625 (Lab Line—Dubuque, IA)
- MicroBeta® TriLux Liquid Scintillation Counter (PerkinElmer—Boston, MA)

Protocol—Protein Precipitation Assay

1) **Preparation of drug solutions in serum**: Drug and radiolabeled drug were added to 5 mL of bovine adult serum so that the final drug concentration was approximately 5 µm and the specific activity was in the range of 0.2 mCi/µMole. The drug serum mixture was incubated for at least 1 hour at 37 °C so that protein binding equilibrium was reached.

2) **Precipitation of protein from drug solutions (2 options)**

   a) Out-of-plate precipitation

      i) Add a 2 mL aliquot of each incubated drug solution to a separate centrifuge tube.

      ii) Add 6 mL of acetonitrile to the tube (3:1 acetonitrile:serum dilution) and mix by vortex for two minutes. The serum proteins will precipitate and coagulate on the bottom of the tube.

      iii) From the supernatant (acetonitrile, non-protein serum constituents and drug samples) add 200 µL/well to one row of each MultiScreen Solvinert filter plate being tested.

   b) In-plate precipitation

      i) Add 150 µL of acetonitrile to each well of each MultiScreen Solvinert filter plate being tested.

      ii) Add each incubated drug solution to one row of each plate at a volume of 50 µL/well and mix for two minutes by shaking the plates moderately on a plate shaker. Do not shake too vigorously to avoid sample spillover.

3) **Preparation of standards**

   a) A separate set of drug solutions is made up identical to those made in Step 1 except that PBS is substituted for bovine adult serum.

   b) Add 150 µL of acetonitrile to each well of the "standards" MultiScreen Solvinert filter plate.

   c) Add each incubated PBS drug solution to one row of the "standards" plate at a volume of 50 µL/well. Mix for two minutes by shaking moderately on a plate shaker, taking care to avoid spillover of the samples.

4) **Filtration and sample preparation**

   a) Position a Greiner polypropylene V-bottom plate onto the base of the Multiscreen vacuum manifold to be used as a receiver plate for the filtrate samples.

   b) Assemble each MultiScreen Solvinert filter plate (including the standard) individually onto the vacuum manifold and apply vacuum at a pressure of 12 to 15″ Hg for approximately 30 seconds.

   c) Add 25 µL of each filtrate sample to 100 µL of scintillation cocktail in a MicroBeta sample plate and mix thoroughly by pipetting five times.

   d) Read each MicroBeta sample plate for tritium CPMs in the TriLux Liquid Scintillation Counter.

DATA ANALYSIS

Percent Recovery Calculation. For each drug in the "standards" plate, the average CPM value is calculated (n=8). The calculation is repeated for each drug in each filtrate receiver plate. The Percent Recovery is determined as a ratio of these two values calculated as follows:

$$\%\ \text{Recovery} = \left(\frac{(\text{Average CPM}_{filtrate})}{(\text{Average CPM}_{\text{standard}})} \right) \times 100\%$$

RESULTS

Reproducibility

The reproducibility of the protein precipitation assay in the MultiScreen Solvinert filter plate was determined using a panel of six drugs with a variety of low, medium and high binding affinities for serum proteins. Accurate measurement of the "standards" was critical for achieving good reproducibility.

Well to well

The protein precipitation assay required two plates—the first involved in-plate precipitation and the second followed the procedure for out-of-plate precipitation. The panel of drugs was added as a solution, one drug to a single row of each plate (n = 12 wells per drug for each precipitation condition). The Percent Recovery results are summarized in **Table 1:**

Table 1

	In-plate precipitation		Out-of-plate precipitation	
Drug	**Average**	**Standard Deviation**	**Average**	**Standard Deviation**
Digoxin	96%	2%	98%	3%
Propranolol	98%	4%	95%	3%
Taxol	92%	1%	91%	2%
Testosterone	92%	2%	91%	2%
Verapamil	97%	1%	92%	2%
Warfarin	93%	2%	90%	2%

Plate to plate

The protein precipitation assay was run on four plates within the same day—two with in-plate precipitation and two with out-of-plate precipitation. The panel of drugs were assayed with a one drug to a single row of each plate (n = 12 wells per drug per plate) tested. Plate to plate reproducibility is shown in the Percent Recovery results in **Table 2**:

Table 2

	In-plate precipitation		Out-of-plate precipitation	
Drug	**Plate #1**	**Plate #2**	**Plate #3**	**Plate #4**
Digoxin	93±3%	96±2%	98±3%	94±1%
Propranolol	97±2%	98±4%	95±3%	96±2%
Taxol	93±3%	92±1%	91±2%	88±2%
Testosterone	94±3%	92±2%	91±2%	90±1%
Verapamil	95±3%	97±1%	92±2%	98±1%
Warfarin	91±3%	93±2%	90±2%	90±3%

Day to day

The protein precipitation assay was completed over three separate days using a total of 12 plates—six with in-plate precipitation and six with out-of-plate precipitation. Two plates were examined with each precipitation method on each day. The panel of drugs were assayed following the exact same assignments as the plate to plate reproducibility studies, with each drug in one row of each plate (n = 24 wells per drug per day). The day to day reproducibility is shown in the Percent Recovery results in **Table 3**:

Table 3

	In-plate precipitation			Out-of-plate precipitation		
Drug	**Day 1**	**Day 2**	**Day 3**	**Day 1**	**Day 2**	**Day 3**
Digoxin	94±2%	95±3%	103±16%	98±1%	96±2%	81±7%
Propranolol	96±2%	98±3%	88±11%	97±3%	96±3%	81±6%
Taxol	92±3%	92±2%	96±10%	91±2%	90±2%	99±9%
Testosterone	106±3%	93±2%	104±11	102±2%	90±2%	88±8%
Verapamil	90±3%	96±2%	99±14%	91±2%	95±2%	102±11%
Warfarin	92±3%	92±3%	91±8%	90±2%	90±2%	83±8%

Method Correlation

The Percent Recovery of six drugs in bovine adult serum was calculated following protein precipitation and vacuum filtration using the MultiScreen Solvinert filter plate. One of two methods was used to prepare the samples prior to filtration: a) protein precipitation from the serum solutions with acetonitrile in the wells of the filter plate or b) outside of the plate in a centrifuge tube. The averages and standard deviations are presented in **Table 4** for the plates tested. The Percent Recovery of each drug does not differ significantly and is greater than 95% for all methods employed.

Table 4

	In-plate precipitation with 3:1 Acetonitrile : Serum		Out-of-plate precipitation with 3:1 Acetonitrile : Serum	
Drug	**Average**	**Standard Deviation**	**Average**	**Standard Deviation**
Digoxin	97%	7%	91%	3%
Propranolol	94%	5%	91%	4%
Taxol	93%	5%	93%	4%
Testosterone	101%	5%	93%	4%
Verapamil	95%	6%	96%	5%
Warfarin	92%	4%	87%	4%

There are many alternative solvent systems employed for Total Drug Analysis in which a solvent is added to precipitate any protein and solubilize any bound drug. The amount of drug recovered from a serum sample can have a strong dependence on the solvent system used for protein precipitation. In an effort to illustrate the compatibility of the MultiScreen Solvinert filter plate under a wide variety of conditions, selected samples are displayed below. **Table 5** lists the Percent Recovery of radio-labeled drugs in a variety of solvent systems following the in-plate protein precipitation method. The averages and standard deviations are presented for the solvent system utilized. The Percent Recovery of each drug does not differ significantly and is greater than 80% for all methods.

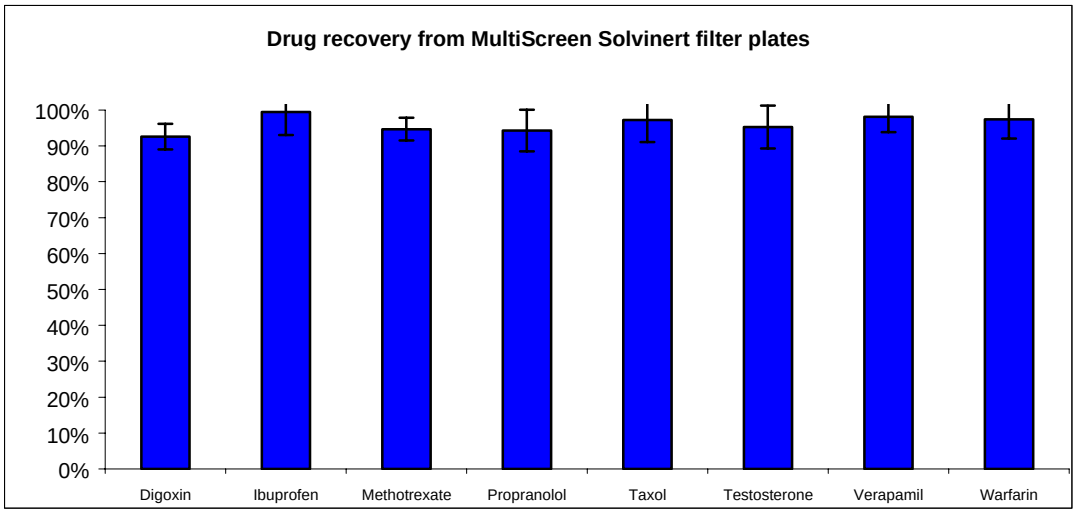
Table 5

Drug	Solvent System	Average	Standard Deviation
Ibuprofen	5:1 Acetonitrile:Serum	100%	7%
Methotrexate	3:1 Methanol:Serum	90%	10%
Propranolol	3:1 Methanol:Serum	80%	9%

Non-specific binding (NSB)

The eight drugs examined for total drug analysis were also radiometrically analyzed for their drug recovery in a Multiscreen Solvinert filter plate using a 100 nM solution of each drug in a 3:1 acetonitrile:serum solution previously filtered. The results are displayed in **Figure 2.** Drug solutions were added to two plates (one row per drug at 200 µL/well, n = 24 wells /drug) and filtered by vacuum at 12 to 15" Hg. The drug concentration in the filtrate (CPM/mL) is compared to the starting concentration. Although the drug concentration used here is only 100 nM, the drug recovery is greater than 90% for each drug tested.

Figure 2



CONCLUSION

The MultiScreen Solvinert filter plate provides an automation compatible, high throughput means for total drug analysis. Sample preparation using a MultiScreen Solvinert filter plate is a robust method that can process up to 96 samples for analysis in less than one hour. The Percent Drug Recovery values obtained using this product with numerous methods has been shown to be reproducible and consistent. For all compounds tested, the Percent Drug Recovery was consistently greater than 90%. The Percent Recovery results from well-to-well, plate-to-plate and day-to day were consistent across both sample preparation methods. The chemical resistance of the MultiScreen Solvinert filter plate allows for a wide range of operating conditions and provides reliable and precise total drug analysis that is easily integrated into existing chemical profiling and early ADME workflows.

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