‘Cut Out The Fat!’ A Unique Filtration Device for The Removal of Proteins and Lipids from Plasma Samples

Paul A Boguszewski[1], David Jones[2], James Stratta[1], Neil Walsh[1]

1) Varian UK Ltd, 10 Mead Road, Yarnton, Oxford OX5 1QU. Tel: +44 (0) 1865 291500 2) Varian, Inc. 25200 Commercentre, Lake Forest, CA 92630, USA.

Abstract

Bioanalytical method development is often a trade-off between achieving a needed sensitivity and the desire for minimal sample analysis time. When sensitivity is not paramount, methods based on dilution or protein precipitation are selected to reduce cost and analysis time. These methods, however, may not be as robust or clean as more involved techniques such as solid phase extraction. We investigate the possibility of creating robust methods across samples and /or species by selective removal of matrix interferences including proteins, phospholipids, and lysophospholipids. This filtration based device, known as Captiva ND*Lipids*, gives improved cleanliness over protein precipitation, yet avoids additional method development or sample processing time. The method also normalizes disparate samples and may lead to more robust methods for the entire bioanalytical process.

Lysophospholipids: A Major Cause of Ion Suppression

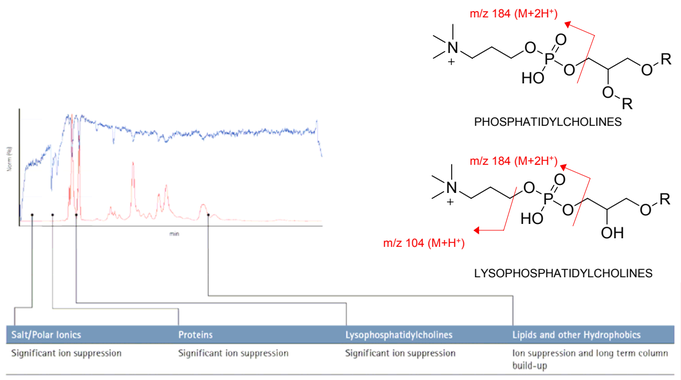
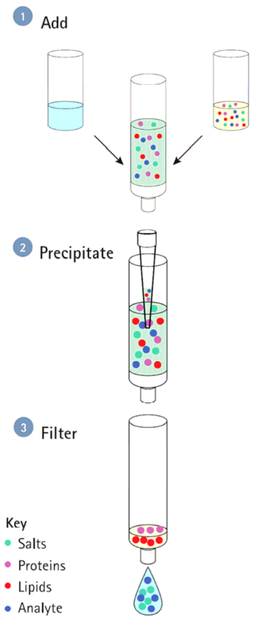


Figure 1 Different types of ion suppression (Blue: post column infusion experiment with albuterol, Red: MS lipid trace).

Methodology and Ease of Use

Captiva ND*Lipids* ease of use lies in the use of a non-drip, lipid depleting filter. The non-drip feature allows for protein precipitation and protein removal in a single well. The lipid depletion provides cleanliness nearing that of traditional solid phase extraction (SPE). The removal of matrix interferences in the filtration mode also allows analyte concentration similar to SPE.



Add Plasma:

50-200 µL Plasma

Add Crash Solvent:

- For optimal lipid and protein removal, 3:1 methanol to plasma ratios are recommended
- pH modification can be made to ionize the analyte of interest
- For basic compounds, we recommend 0.1% to 1.0% formic acid in methanol
- For acidic compounds, we recommend 5 mM to 10 mM ammonium formate buffers at pH 9
- Captiva ND*Lipids* can be used with various organic crash solvents, modifiers and ratios.

For thorough precipitation, pipette mixing is recommended

Mixing:

- 3 to 5 pipette strokes of 3/4 combined liquid volume is sufficient to thoroughly precipitate plasma proteins
- Orbital/vortex mixing of the 96 well plate is insufficient to thoroughly precipitate plasma proteins in methanol

Pass sample through filter and collect filtrate:

- Flow rate is highly dependent on plasma type, age, and mixing
- Samples should pass in under 5 min depending on volume
- Unmixed or difficult (rat) plasmas may take longer to pass through the 0.2 µm filter

Volume collected:

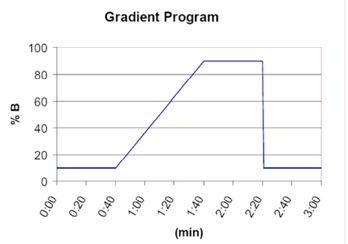
- Recovery volumes of 75-85% of the combined liquid volumes added should be expected
- The majority of volume reduction comes from the precipitation and removal of proteins. Filter cakes should be dried sufficiently to avoid additional losses

Results and Discussion

Sample cleanliness under a variety of precipitation conditions was explored using post-column infusion. Sample matrices from protein precipitated samples and the lipid filtered samples were injected on column while 10 ppm analytes were infused at 10 µL/min. At the same time, samples were monitored for phosphatidylcholines using the transitions described above. Figure 1 shows the typical ion suppression features in a post-column infusion experiment, as well as the correlation of ion-suppression to phosphatidylcholine elution.

Methods

Sample: Plasma with varying concentration of analytes
HPLC Column: Polaris™ C-18A 5 µm 50 x 2.0 mm (Varian, Inc.)
Solvents: A: 0.1% Formic Acid B: Acetonitrile
Gradient Flow: 500 µL/min
Post column infusion (where applicable): 10 ppm Analytes at 10 µL/min
Interference Detection (Varian 320 LC/MS/MS in +ESI):
184→184 (fatty acid non-selective, non-specific
phosphatidylcholine channel with in-source ionization)
104→104 (lysophoshatidylcholine specific,
fatty acid non-selective channel using in-source ionization)
Where R = fatty acid ester (e.g. 16:0, 18:0, 18:1, 20:4, etc.)



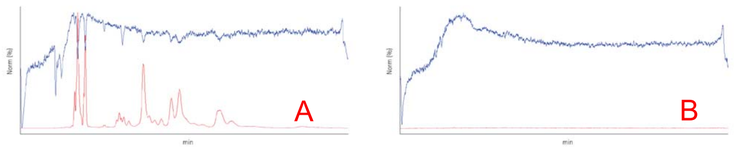
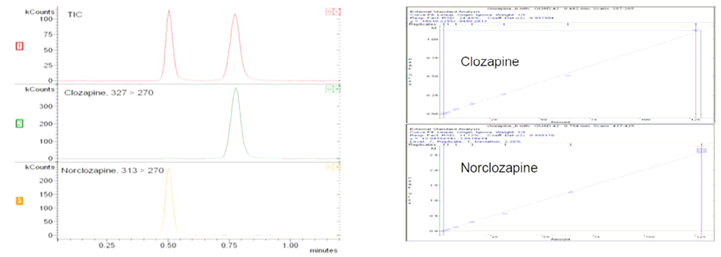


Figure 2 Post column infusion experiment (PCI) of albuterol (Blue:PCI, Red:MS lipid trace) with standard protein precipitation (A) and after using Captiva ND Lipids (B).

Determination of Clozapine and Norclozapine in Plasma

A mix of clozapine and norclozapine was spiked in plasma at 32 ng/mL, filtered through the Captiva ND*Lipids* filtration plate, and run on a Pursuit™ XRs Ultra 2.8 C18 HPLC column.

The Captiva ND*Lipids* method yielded higher average absolute recoveries compared to samples processed by protein precipitation. Also, the fluctuation in recoveries over the range tested was much wider for protein-precipitated samples (e.g. norclozapine had recoveries ranging from 12.1 to 101.7), while filtered samples had a narrower range (41.6 to 76.7). This could be attributed to a possible higher plasma protein-binding capacity to both these analytes (Figure 3). The process of filtration through Captiva ND*Lipids* could possibly shift the equilibrium of bound plasma protein-drug complex to the unbound state resulting in better recoveries and reproducibility.



Compound	Extraction Method	2 ppb	4 ppb	8 ppb	16 ppb	32 ppb	64 ppb	128 ppb	Average Recovery (%)
Clozapine	Captiva	98.9	67.2	70.9	78.0	57.7	68.0	84.4	75.0
	Protein Precipitation	79.9	39.1	50.2	83.9	67.8	71.9	64.6	65.3
Norclozapine	Captiva	74.8	41.8	69.4	73.5	54.4	66.6	76.7	65.3
	Protein Precipitation	36.4	12.1	38.3	101.7	74.3	74.9	60.7	56.9


CLOZAPINE


NORCLOZAPINE

Figure 3. Data bundle showing LC/MS response, linearity and recovery data for clozapine and norclozapine extraction from plasma using Captiva ND Lipids.

Universality of Method for Broad Ranging Analytes and Matrices

The unique membrane approach to lipid removal means that functional group tolerance to analytes within a plasma matrix is very good. Only when an analyte is of very high logP will performance reduce. Table 1 shows that the filter plate can be used for compounds with a logP up to ~ 6.5. Above this point, the relative recovery will fall in line with that observed with standard precipitation methods. Interestingly, although recoveries are lower, the exclusion of phospholipids, and hence the sensitivity increase, still remains. Figure 4 demonstrates that lipid removal efficacy is uniform across a large range of plasma sources, allowing excellent universality of the method in bioanalysis.

Analytes	Log P	Hydrophobicity	Relative Response vs. Precip alone
Tranylcypromine	1.40	-	251.4%
Nomifensine	2.94		162.6%
Zolpidem	3.32		109.6%
Amoxapine	3.40		171.4%
Warfarin	3.51		100.5%
Mianserin	3.52		167.4%
Sulindac	3.59		136.7%
Loratadine	3.65		103.4%
Nefazodone	4.70		144.9%
Maprotiline	5.10		159.0%
Vardenafil	6.01		106.5%
Loperamide	6.25	+	106.7%

Table 1. Relative response vs standard precipitation for analytes with a broad logP.

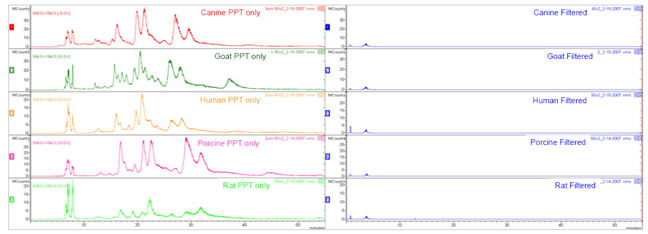


*Figure 4 Phopsholipid profiles of a range of plasma types with standard precipitation (left) and after Captiva ND*Lipids* (right).*

Extended Column Lifetimes for Fast LC Applications

When combining Captiva ND*Lipids* with sub 2 µm or sub 3 µm columns, such as Pursuit™ XRs Ultra 2.8 and Pursuit UPS 2.4, back pressure issues become a thing of the past. A longevity study analyzing beta blockers, spiked in rat plasma after filtration with Captiva ND*Lipids*, shows virtually no changes in back pressure, retention time and peak shape with UV and MS detection. Even after more than 6000 injections the column characteristics remain the same (Figure 5). Using Captiva ND*Lipids* helps prolong column lifetime and therefore reduces downtime due to column changing and/or conditioning. The integrated combination of sub 2 µm or 3 µm columns with such a unique filtration device increases productivity and reduces cost, two crucial elements for high throughput laboratories. Captiva ND*Lipids* can act as a protective shield for analytical columns and detectors. Ion sources require less maintenance, reducing disturbance of daily work flows.

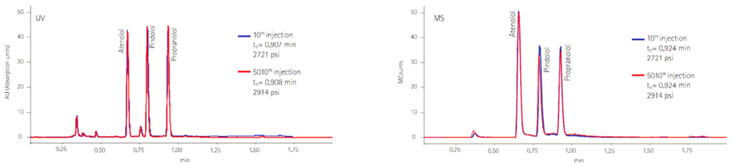


*Figure 5 No significant changes in back pressure, retention time, and peak shape with Captiva ND*Lipids* after 10 and 5010 injections for LC/MS or LC/MS/MS bioanalysis (left= UV detection; right = MS detection).*

Conclusions

- Captiva ND*Lipids* is a novel approach to a well adopted sample prep technique.
- The removal of phospholipids allows for reduced ion suppression and greater sensitivity.
- Relative recoveries are higher and more reproducible compared to standard precipitation methods.
- HPLC column lifetimes can be dramatically increased when adopting this technology.
- A broad range of analytes and plasma types can be used under very generic, facile conditions.