RAPID AUTOMATED PROTEIN CRASH METHOD USING NOVEL TIP-ON-TIP FILTRATION

TECHNICAL NOTE

BLOOD TIP-ON-TIP

The bottleneck in blood analysis is most often sample preparation. We propose a novel patent-pending automated method which uses wide bore tip mixing followed by "in-tip" filtration as an alternative to protein precipitation and centrifugation. A wide bore tip thoroughly mixes the sample solution with organic solvent to provide efficient protein precipitation. The wide bore tip containing precipitated sample solution is then transferred and pressure fitted into a filter tip. The sample is dispensed through the filter tip into a clean well plate for analysis or further sample preparation. To enhance sample clean-up during the filtration process, a variety of solid phase extraction sorbents can be added.

We present three filtration options: filter only, C18 sorbent, or phospholipid removal sorbent. The filter only option is ideal for metabolomics and drug discovery, or for specimens that need additional sample preparation post-filtration. Matrix components that may bind irreversibly to the C18 sorbent are not introduced into the LC-MS/MS system, as the sorbent acts as a disposable guard column. Addition of a phospholipid removal sorbent is ideal to remove ion suppression effects (1–3). This process can perform blood protein precipitation, filtration, and simultaneous clean-up of up to 96 samples in less than 5 minutes, significantly increasing throughput and simplifying of blood analysis. The method is readily automated and elminates the need for a centrifuge or positive pressure manifold.

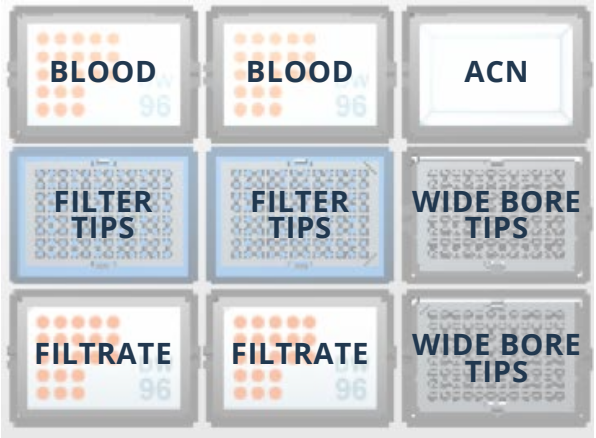

Figure 1. Proposed automated platform deck layout showing DPX Tip-On-Tip sample preparation.

INSTRUMENTATION AND METHODS

All drug standards were purchased from Cerilliant Corporation (Round Rock, TX). Analyses were performed using a Thermo TSQ Vantage™ triple quadrupole mass spectrometer (Milwaukee, WI) coupled to an Agilent 1260 Series HPLC (Agilent Technologies, Santa Clara, CA)

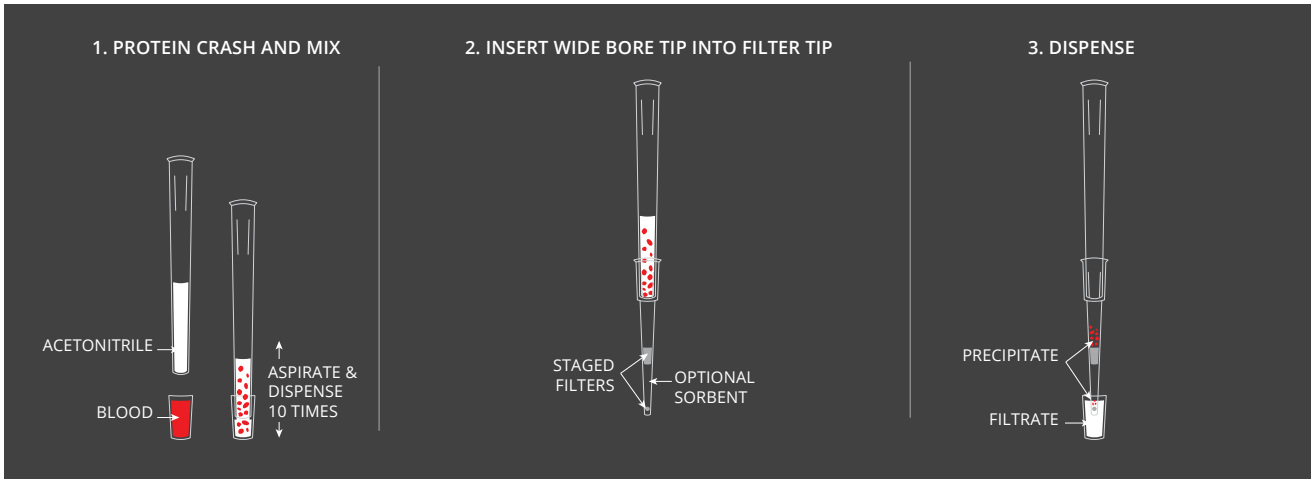

Figure 2. Schematic of the DPX Tip-On-Tip method for automated protein crash of blood.

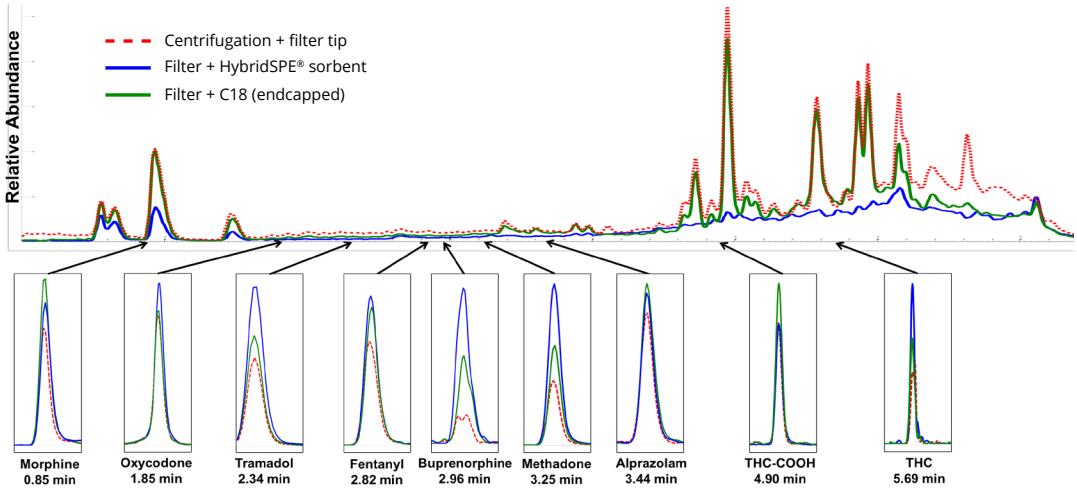

Figure 3. Top: Total ion chromatogram of a LC/MS scan (m/z = 200–1500 u). Bottom: Extracted ion chromatograms of LC-MS/MS data for select compounds from a mix of 40 drugs.

equipped with an Agilent Poroshell EC-C18 column (3.0 × 50 mm, 2.7 µm) with column temperature held at 50 °C. Sample injections of 10 µL were made using a 6 port (0.25 mm) Cheminert C2V injection valve (Houston, TX) incorporated on a dual rail GERSTEL MPS autosampler (Linthicum, MD). Column flow rate was 0.65 mL/min. The mobile phase was composed of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The gradient was 5% B for 0.2 min, ramped to 95% B at 5.1 min, held for one minute, then re-equilibrated to 5% B for a total run time of 7.2 minutes. Mass spectrometer parameters were: electrospray, 4000 V; auxiliary gas, 8 psi; sheath gas, 30 psi; and capillary temperature, 300 °C.

An automated platform was loaded with a 96 well plate of blank pooled blood in 100 µL aliquots, a reservoir of acetonitrile (5% acetic acid for HybridSPE® sorbent), a blank well plate, DPX filter tips, and 1 mL wide bore tips. Figure 1 shows the preparation of two consecutive well plates on the automated platform. Figure 2 illustrates the DPX Tip-On-Tip method. The filtrate was solvent evaporated and reconstituted in 100 µL of 10% methanol in water for injection. The filter tip contained 25 mg of HybridSPE® sorbent and 10 mg of C18.

RESULTS AND DISCUSSION

Previous studies performed in our laboratory have shown that repeated wide bore tip aspiration and dispensing of acetonitrile and blood provides consistent drug recoveries when compared to 30 seconds of rigorous vortex mixing. This suggests that efficient protein precipitation can be performed using an automated liquid handler with simple pipetting commands. To remove the precipitate, we also discovered that the wide bore tip can be readily fitted into a smaller filter tip, to provide a filtration step without the need for a centrifuge or positive pressure manifold.

In this study, we combined wide bore mixing of acetonitrile with whole blood spiked with 40 drugs of abuse at 50 ng/mL. Figure 3 shows the total ion chromatogram of a LC/MS scan of the acetonitrile crashed whole blood. The chromatograms show the matrix peaks associated with protein crashed blood. The red dashed plot is the centrifuged sample which appears identical to the filter tip without any sorbent. The 40 drugs analyzed by LC-MS/MS were all readily detected, even with the high background associated with the matrix. As noted in the extracted ion chromatograms, peak intensities were good and provided reasonable S/N ratios even with no cleanup. Not surprisingly, use of C18 (green plot) reduces the background and matrix peaks. While use of

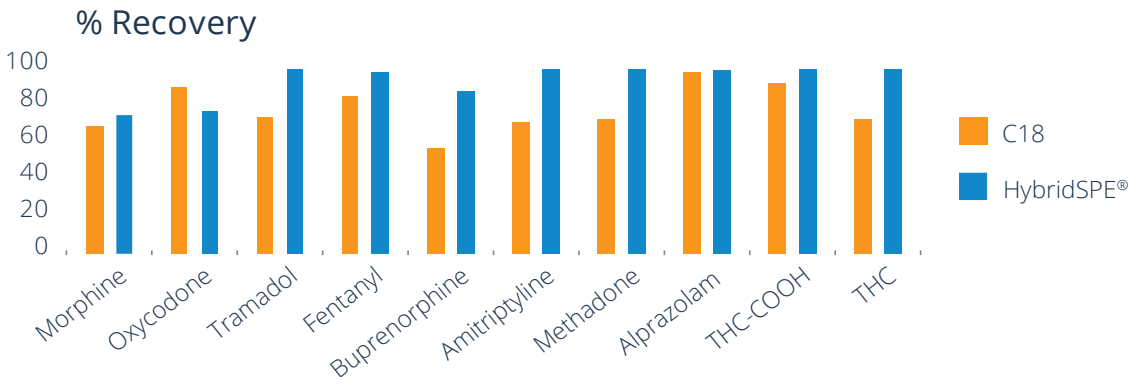

Figure 4. Recoveries of representative compounds out of the 40 drugs analyzed with C18 and HybridSPE® sorbent incorporated in the filter tip (calculated post protein precipitation using matrix-matched standards).

C18 negatively affects certain drug recoveries, our Tip-On-Tip method may protect the LC/MS system by preventing introduction and accumulation of matrix components in the column and ionization source (Figure 4). Drug recoveries could be enhanced by increasing the amount of solvent in the tip or by reducing the amount of C18. Active sites from silica may have reduced recoveries with some of the commercial C18 products tested. Additional improvement may be found by simply adding acid to improve the solubility of basic drugs in solution. More work will be conducted to determine optimal parameters. Nevertheless, this method provided acceptable recoveries (>70%) for most of the analytes studied.

We also incorporated HybridSPE® sorbent in the filter (references below). Recoveries of basic analytes were poor when using 100% acetonitrile; however, addition of 5% acetic acid greatly improved drug recoveries. The baseline of the total ion chromatogram (LC/MS scan) was greatly reduced as shown in Figure 3 (blue plot). Background reduction results in reduced ion suppression, leading to higher peak intensities as shown in the extracted ion chromatograms (Figure 3).

CONCLUSION

We are currently evaluating our Tip-On-Tip method for analysis of vitamin D metabolites in serum, testosterone and other steroids in serum and plasma, immunosuppressants in whole blood, and comprehensive screening of whole blood for forensic toxicological analyses. Other applications include metabolomics and analyses of solid organ specimens.

Our Tip-On-Tip filtration technique is amenable to a variety of heterogeneous mixtures. Customized sorbents may be incorporated to facilitate sample cleanup and enhance sensitivity.

References

1. Aurand, Craig “Isolation and LC-MS Characterization of Illicit Bath Salts in Urine” Reporter US Volume 30.3
2. Lu, Xiaoning; Ye, Michael “Enrichment of Phospholipids in Biological Samples Using HybridSPE-PL” Reporter US Volume 28.3
3. Aurand, Craig “Minimizing Phospholipid Matrix Effects in the HILIC LC-MS” Reporter US Volume 28