Comparison of the Impact of Sample Preparation Techniques on Matrix Effects in Electrospray LC-MS/MS

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Introduction

Sample preparation is essential when analyzing drugs in biological fluids, even when a selective detector such as MS/MS is used. The degree of sample clean-up varies significantly depending on the sample preparation technique used. For example, extracts obtained by protein precipitation contain relatively high levels of co-extracted material; whereas extracts obtained using a selective technique such as mixed-mode solid phase extraction (SPE) contain significantly less.

This presentation aims to compare the effect of different sample preparation techniques on the clean-up of human plasma samples, in particular the impact on ion suppression in LC-MS/MS.

The following techniques will be investigated:

- Protein precipitation
- Supported liquid extraction (SLE)
- Non-polar SPE (polymer-based sorbent)
- Non-polar SPE (silica-based sorbent)
- Mixed-mode SPE (silica-based sorbent)

Experimental Procedure

Sample Preparation

100 µL of blank, pooled human plasma was extracted using standard methodology for each technique (details below). Following sample preparation, the extracts were evaporated to dryness, and reconstituted in appropriate mobile phase for subsequent analysis. Duplicate samples were extracted for the HPLC-UV and FIA LC-MS/MS experiments. All sample preparation procedures were carried out using 96-well format sample preparation plates.

a) Protein Precipitation

ISOLUTE® Array PPT+ (p/n 120-2040-RP)

1. Add 300 µL acetonitrile to each well
2. Add 100 µL plasma to each well
3. Allow to stand for 2 minutes
4. Apply vacuum at -20 "Hg and collect filtrate
5. Evaporate and reconstitute as appropriate

b) Supported Liquid Extraction (SLE)

ISOLUTE Array HM-N 200 mg/1 mL (800-0200-RP)

1. Apply 100 µL plasma to each well
2. Apply a short pulse of vacuum to initiate flow
3. Allow to stand for 5 minutes, and dry with a short pulse of vacuum
4. Apply 4 x 200 µL hexane and elute under gravity
5. Evaporate and reconstitute as appropriate

IST Sample Preparation

Biotage

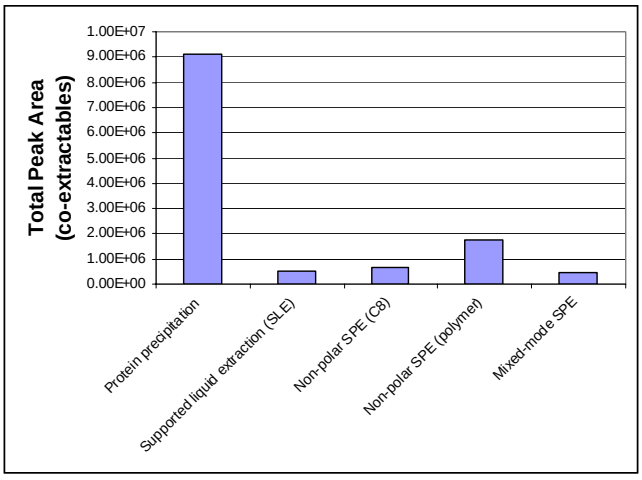
c) Non-polar SPE (silica based sorbent)
ISOLUTE Array C8 25 mg/1 mL (290-0025-RP)
1. Condition each well with methanol (1 mL)
2. Equilibrate with 1% formic acid (1 mL)
3. Load plasma sample (100 µL diluted 1:1 (v/v) with 1% formic acid)
4. Wash with 1% formic acid:methanol (95:5, v/v, 1 mL)
5. Elute with methanol (1 mL)
6. Evaporate and reconstitute as appropriate

d) Non-polar SPE (polymer-based sorbent)
EVOLUTE™ Array 25 mg/1 mL (p/n 600-0025-RP)
1. Condition each well with methanol (1 mL)
2. Equilibrate with 0.1 % formic acid (1 mL)
3. Load plasma sample (100 µL diluted 1:3 (v/v) with 1% formic acid)
4. Wash with water:methanol (95:5, v/v, 1 mL)
5. Elute with methanol (1 mL)
6. Evaporate and reconstitute as appropriate

e) Mixed-mode SPE (silica-based sorbent)
ISOLUTE Array HCX 25 mg/1 mL (p/n 902-0025-RP)
1. Condition each well with methanol (1 mL)
2. Equilibrate with ammonium acetate buffer (0.05 M, pH 6, 1 mL)
3. Load diluted plasma sample (100 µL)
4. Wash with: (i) ammonium acetate buffer (0.05 M, pH 6, 1 mL), (ii) acetic acid (1 M, 1 mL), (iii) methanol (1 mL)
5. Elute with methanol:NH4OH (95:5, v/v)
6. Evaporate and reconstitute as appropriate

HPLC-UV Conditions
Following extraction, extracts were evaporated to dryness and reconstituted in mobile phase (500 µL).
Column: Genesis® C18, 150 x 4.6 mm, 4 µm
Mobile Phase: 60:40 (v/v) 0.2% phosphoric acid pH 2.5: methanol containing 200 µL/L diethylamine
Flow Rate: 1.4 mL/minute Wavelength: 220 nm
Column Temp: 40 °C **Injection Volume:** 40 µL

Results



HPLC-UV Analysis
In order to compare the amount of co-extracted material detectable by HPLC-UV, chromatograms were integrated by drawing a baseline under the whole chromatogram, to include the area of all peaks present. Total peak areas are shown graphically in **Figure 1**.

Figure 1. Comparison of the amount of co-extracted matrix components in plasma extracts generated using different sample preparation techniques (HPLC-UV)

IST Sample Preparation

Biotage

Chromatograms for protein precipitation, EVOLUTE ABN and ISOLUTE HCX extracts are shown for illustrative purposes (see **Figure 2**).

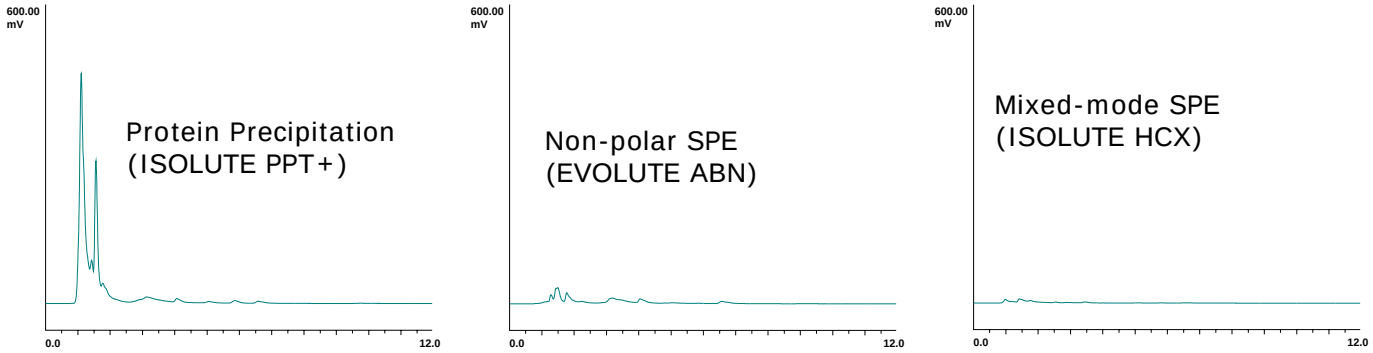


Figure 2. HPLC-UV (220 nm) chromatograms illustrating the relative cleanliness of extracts produced using: protein precipitation, non-polar SPE (polymer), mixed-mode SPE

FIA LC-MS/MS Conditions

Following extraction, plasma extracts were evaporated to dryness and reconstituted in mobile phase spiked with caffeine at a concentration of 1 µg/mL. The MS-MS signal intensity observed from the spiked plasma sample extracts was then compared with that observed for pure mobile phase spiked at the same concentration (1 µg/mL) with caffeine.

Column: None
Mobile Phase: Water:acetonitrile:methanol (50:45:5, v/v) acidified with 0.1% (v/v) formic acid
Flow Rate: 0.25 mL/minute
Injection Volume: 5 µL
Instrument: Varian 1200L triple quadrupole
Ionization: Electrospray, +ve
Drying Gas Temperature: 260 °C
SRM Transition for Caffeine: m/z 195>138

FIA LC-MS/MS Results

The degree of ion suppression caused by co-extracted matrix components from each sample preparation technique was measured relative to the signal produced by pure spiked mobile phase (no plasma sample present). This is shown graphically in **Figure 3**.

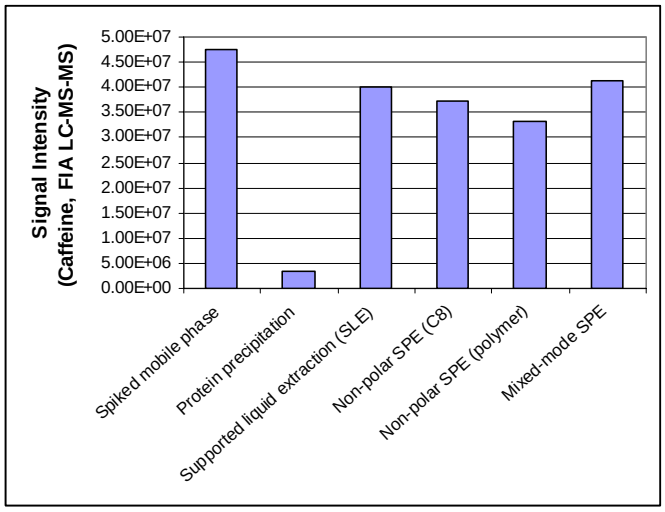


Figure 3. Comparison of the degree of ion suppression caused by co-extracted matrix components in extracts generated using different sample preparation techniques relative to spiked mobile phase (FIA LC-MS/MS)

Comments

Flow injection analysis (FIA) LC-MS/MS is a useful tool for assessing the total ion suppression / enhancement effects due to co-extracted matrix components resulting from a particular sample preparation technique. However, it does not give an indication of ion suppression or enhancement due to specific individual matrix components. Whether or not matrix effects are observed for a particular analyte will depend on the separation conditions used. Continued…

However, with increasing throughput requirements leading to wider use of short LC runtimes, the cleaner the extract the less likely matrix effects such as ion suppression / enhancement are to affect the analysis.

Correlation Between HPLC-UV and FIA LC-MS/MS Results

The total HPLC-UV co-extractables peak areas were plotted against the % ion suppression observed for each sample preparation technique (see **Figure 4**).

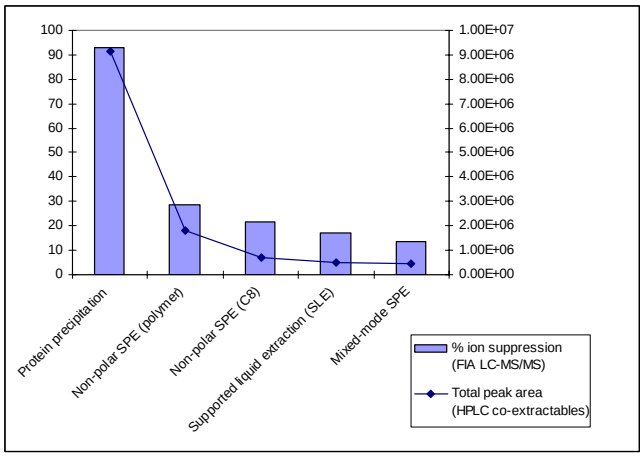


Figure 4. Correlation between HPLC-UV and FIA-LC-MS/MS data

Comments

Of the sample preparation techniques investigated, the techniques giving the cleanest extracts (lowest levels of co-extracted material in HPLC-UV analysis) also gave the lowest levels of ion suppression in LC-MS/MS.

However, when choosing the most appropriate sample preparation technique for a particular assay, other factors such as applicability to a wide range of compounds, speed and simplicity of use and automation compatibility are also important.

Discussion

Protein Precipitation

- Extracts contain significantly more co-extracted material than the other techniques investigated, with LC-MS/MS signal suppressed by approximately 93%.
- Relatively non-selective technique, applicable to a wide range of acidic, basic and neutral compounds.
- Fast and simple sample preparation technique (96 samples can be processed in approximately 7 minutes).
- Using this 'solvent first' methodology with ISOLUTE PPT+ plates, the technique is very easy to automate fully (no manual intervention such as vortex mixing required).

Supported Liquid Extraction (SLE)

- Using this method (hexane as extraction solvent) very low levels of co-extracted material are observed, with LC-MS/MS signal suppressed by only 17 %.
- If other, more polar extraction solvents are used, we predict higher levels of matrix components would be extracted. This will be investigated more fully in future work.
- Can be used to extract acidic, basic or neutral compounds (method optimization required). As with traditional liquid-liquid extraction, applicability is limited by solubility of analytes of interest in water immiscible solvents.
- No manual steps involved, so technique is fully automation compatible.
- Sample volume limited (max 200 µl per well).

Non-polar SPE (Silica-based Sorbent)

- Relatively low levels of co-extracted matrix components observed, with LC-MS/MS signal suppressed by 21%.
    - Slightly cleaner than non-polar resin based SPE, due to the less hydrophobic character of base material.

- Not as clean as mixed-mode SPE, as single retention mechanism does not allow 100% solvent wash step.
- Applicable to extraction of acidic, basic and neutral compounds – some method optimization may be required. May not be as suitable for very polar dugs and metabolites as non-polar polymer based SPE.
- No manual steps involved, so technique is fully automation compatible.

Non-polar SPE (Polymer-based Sorbent)

- Relatively low levels of co-extracted matrix components observed, with LC-MS/MS signal suppressed by 29%.
  - Despite increased hydrophobic character (compared with C8) the optimized pore structure minimizes the level of co-extracted matrix components (compared with other polymer based SPE sorbents).
- Applicable to a very wide range of acidic, basic and neutral compounds, including very polar drugs and metabolites, using a single method.
- No manual steps involved, so technique is fully automation compatible.

Mixed-mode SPE (Silica-based Sorbent)

- Cleanest extracts of all sample preparation techniques investigated, with LC-MS/MS signal suppressed by 14%.
  - Dual retention mechanism allows rigorous interference elution regime.
  - Increased scope for removal of specific ion suppressing components.
- Only suitable for extraction of basic compounds.
- Mixed-mode approach is also suitable for acidic compounds using alternative sorbents.
- No manual steps involved, so technique is fully automation compatible.

Overall Conclusions

In this study, sample preparation techniques providing the visually cleanest extracts (HPLC-UV analysis) also gave the lowest ion suppression effects (FIA LC-MS-MS). Future work will investigate the effect of different sample preparation techniques on removal of specific matrix components known to cause ion suppression / enhancement.

In terms of extract cleanliness, the sample preparation techniques studied can be ranked as follows (cleanest first):

1. Mixed-mode SPE (silica-based sorbent)
2. Supported liquid extraction
3. Non-polar SPE (silica-based sorbent)
4. Non-polar SPE (polymer-based sorbent)
5. Protein precipitation

Selection of a particular sample preparation technique for a particular assay should not be based on extract cleanliness alone. A number of other factors (as discussed above) should also be considered.

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