Effectiveness of Solid Phase Extraction in Urinary Cortisone and Hydrocortisone Analysis

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Solid Phase Extraction (SPE) has long been viewed as a simple interference/impurities/matrix removal and/or pre-concentration technique. The plethora of different SPE products available presents both opportunities and challenges in selecting the right SPE material and conditions. In this experiment, the cleanliness and recovery of cortisone and hydrocortisone extracts from urine by different SPE phases, are examined. The tested phases included are silica-based and surface-modified polymeric resin beads. Each phase represents one or more modes of retention mechanism such as; hydrophobic, cationic or anionic, or any combination thereof.
We evaluated urine pool extracts produced from different SPE sorbents based on their respective general recommended extraction procedures. The SPE media was divided into two main groups, silica particles and polymeric resin beads. The silica-based SPE consists of end-capped C18 ligands which present a strong hydrophobic retention mechanism. The polymeric resin comprised of a polystyrene backbone with a quaternary amine moiety (strong anion-exchange), a sulfite moiety (strong cation-exchange), or a pyrrolidone moiety for neutral compounds (see below). The styrene backbone provides both hydrophobic and π-π interaction and/or retention mechanism.

Cortisone and hydrocortisone (cortisol) are two of the prominent corticosteroids. Urinary levels of these hormones are commonly analyzed to determine metabolic disorder. While both compounds are chemically neutral, they possess multiple polar sites with varying degrees of reactivity.

The evaluation criteria included: the cleanliness of the extract (ion suppression and/or enhancement throughout the chromatogram), a total ion chromatogram of wide mass range covering most small molecules (100-1000 Da), and general recovery. As a reference, a urine sample subjected to simple protein precipitation by acetonitrile was included in the ion suppression study. The experiments were carried out on an AB SCIEX API 4000™ LC/MS/MS system under positive polarity with an ESI source. The recovery and ion-suppression studies are conducted under an MRM scan function while a Q1 scan was used for the total ion chromatogram. An Agilent® 1200 SL with a binary pump and high pressure autosampler provided the chromatographic separation on a Kinetex® 2.6µm core-shell C18, 50 x 2.1mm column. Ammonium acetate and methanol combination provided optimal sensitivity and separation of the two compounds.
### MS/MS Instrument

**AB SCIEX API 4000**
**Pos ESI, MRM function**

<table>
<thead>
<tr>
<th>Compound ID</th>
<th>Q1 Mass (Da)</th>
<th>Q3 Mass (Da)</th>
<th>CE (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortisone 1</td>
<td>361.1</td>
<td>163.2</td>
<td>30</td>
</tr>
<tr>
<td>Cortisone 2</td>
<td>361.1</td>
<td>121.2</td>
<td>30</td>
</tr>
<tr>
<td>Cortisol 1</td>
<td>363.1</td>
<td>120.9</td>
<td>30</td>
</tr>
<tr>
<td>Cortisol 2</td>
<td>363.1</td>
<td>309.1</td>
<td>30</td>
</tr>
<tr>
<td>Cortisol-D4 (I.S.)</td>
<td>367.1</td>
<td>120.9</td>
<td>28</td>
</tr>
<tr>
<td>Cortisol-D4 (I.S.)</td>
<td>367.1</td>
<td>309.2</td>
<td>28</td>
</tr>
</tbody>
</table>

**Q1 Scan**
- 100-1000 amu, 1 sec time
- Profile Scan @ 0.1 amu step

**Ion Source Conditions:**
Same conditions were used for both MRM and Q1 scan (except for CAD gas and CXP)

| CUR: | 25  |
| GS1: | 50  |
| GS2: | 50  |
| IS:  | 5000|
| TEM: | 300 |
HPLC Conditions

- **Column:** Kinetex 2.6 µm C18
- **Dimensions:** 50 x 2.1 mm
- **Part No.:** 00B-4462-AN
- **Mobile Phase:**
  - A: 10 mM Ammonium Acetate in Water
  - B: 10 mM Ammonium Acetate in Methanol
- **Gradient:**
  - Time (min) % B
  - 0.01 40
  - 0.5 40
  - 2.0 90
  - 3.0 90
  - 3.01 40
  - 5.0 40
- **Flow Rate:** 0.4 mL/min
- **Column Temp:** 40 °C
- **Injection Volume:** 10 µL
- **LC system:** Agilent 1200SL with binary pumps

Sample Prep Method

**SPE Media**
- Strata® C18-E (end-capped) 50 mg/1 mL
- Strata™-X (polymer) 60 mg/3 mL
- Strata-X-A (anion-exchange polymer) 60 mg/3 mL
- Strata-X-C (cation-exchange polymer) 60 mg/3 mL

<table>
<thead>
<tr>
<th>Media</th>
<th>End-Capped C18, Silica</th>
<th>Polymeric Resin</th>
<th>Anion-Exchange Polymeric Resin</th>
<th>Cation-Exchange Polymeric Resin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conditioning</td>
<td>1 mL MeOH</td>
<td>1 mL MeOH</td>
<td>1 mL MeOH</td>
<td>1 mL MeOH</td>
</tr>
<tr>
<td>Equilibration</td>
<td>1 mL DI H₂O</td>
<td>1 mL DI H₂O</td>
<td>1 mL DI H₂O</td>
<td>1 mL DI H₂O</td>
</tr>
<tr>
<td>Loading¹</td>
<td>1:2 Diluted Urine</td>
<td>1:2 Diluted Urine</td>
<td>1:2 Diluted Urine</td>
<td>1:2 Diluted Urine</td>
</tr>
<tr>
<td>Weak Wash</td>
<td>1 mL DI H₂O</td>
<td>1 mL DI H₂O</td>
<td>1 mL DI H₂O</td>
<td>1 mL DI H₂O</td>
</tr>
<tr>
<td>Strong Wash</td>
<td>1 mL 50 % MeOH in DI H₂O</td>
<td>1 mL 50 % MeOH in DI H₂O</td>
<td>1 mL 50 % MeOH in DI H₂O</td>
<td>1 mL 50 % MeOH in DI H₂O</td>
</tr>
<tr>
<td>Final Elution²</td>
<td>2x 0.8 mL EtOAc/IPA (85:15)</td>
<td>2x 0.8 mL EtOAc/IPA (85:15)</td>
<td>2x 0.8 mL EtOAc/IPA (85:15)</td>
<td>2x 0.8 mL EtOAc/IPA (85:15)</td>
</tr>
</tbody>
</table>

¹ Urine samples diluted with DI water or 10 mM Ammonium acetate
² The elution solution for end-capped C18 and polymeric resin was fortified to contain 2 % formic acid

Abbreviation Key: MeOH = Methanol; DI H₂O = Deionized Water; EtOAc = Ethyl acetate; IPA = Isopropanol
Cortisone and hydrocortisone peaks are well-resolved using the Kinetex core-shell C18 column (Figure 1) and are adequately separated away from the solvent front (~0.4 min). A 10µg/mL solution of cortisone and hydrocortisone was infused into the mobile phase stream post-column and before the mobile phase entry point into the ionization source. The resulting region of ion suppression at t₀ is most extensive for the sample subjected to protein precipitation. In contrast, the same region show very little suppression for the samples processed by SPE methods (Figures 2 and 3). Overall, the enhancement and the suppression regions are comparable for all extracts.

The SPE extracts, both blank and urine samples, were further analyzed by a qualitative Q1 scan ranging from mass 100 to 1000 Da (m/z). Comparison of the extract’s blank subtracted spectra revealed two clusters of peaks. One group from 300 to 320 Da and the second around mass 453 Da (Figure 4). The cation-exchange extract, however, displayed the least intense signal for the ions in the 453 region and virtually no peak at mass 310 Da. The identity of these peaks are not known. However, given the retention mechanism of the SPE ligands, it is plausible to consider these ions to contain one or more amine functional group.

In addition, the cation-exchange resin produced the lowest recovery of cortisone and hydrocortisone of about 55%. By comparison, quantitative recoveries were obtained by simple polymeric and cation-exchange resin. The data suggests that cortisone and hydrocortisone may have a stronger attraction to the sulfite group of the cation-exchange resin.
Figure 1. Representative chromatogram of cortisone and hydrocortisone extracted from urine.
Figure 2.
Overlaid chromatograms of ion suppression tests for Cortisone primary MRM channel. Cortisone peak is also overlaid for comparison.
Figure 3.
Overlaid chromatograms of ion suppression tests for Hydrocortisone primary MRM channel. Hydrocortisone peak is also overlaid for comparison.
Figure 4. Blank subtracted spectra of various SPE extracts. The spectra collected from 100-1000 Da.
All SPE sorbents produced reasonably clean extracts from a urine pool when tested for cortisone and hydrocortisone. While both cortisone and hydrocortisone are considered neutral, they exhibited substantial attraction to the strong cation-exchange resin. The strong cation-exchange resin produced the strongest retention of some matrix components.
We would like to thank Jeff Layne, Sean Orlowicz, and Shahana Huq for their technical assistance.

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