INTRODUCTION

The formation of reactive oxygen species within the cells causes damage of biomolecules and it plays an important role in carcinogenesis and aging. An important type of oxidative DNA damage is hydroxylation of DNA bases, leading to formation of 8-hydroxy-2-deoxyguanosine (8-OhdG) and 5-hydroxymethyluracil (5-HMU). Measurements of these biomarkers in urine samples are challenging due to the low levels of the analytes and the complex matrix.

In this work a semi-automatic MEPS combined with UHPLC-PDA was developed in order to quantify urinary biomarkers of oxidative DNA damage, 8-OhdG and 5-HMU.

A previous optimization of the MEPS experimental influence parameters was carried out.

EXPERIMENTAL

Sampling

Urine sample diluted 1:10 and pH = 6

MEPS procedure with the automated analytical syringe eVol®

1. Solubilizing
   250 µL MeOH + 250 µL H2O (0.01% formic acid)

2. Sampling
   5 x 50 µL urine sample

3. Washing
   50 µL H2O (0.01% formic acid)

4. Elution
   3 x 30 µL H2O (0.01% formic acid) MeOH (80:20, v/v)

Analysis

UHPLC-PDA conditions

- Column: ACQUITY UPLC HSS T3
- (100 mm, 2.1 mm, 1.8 µm particle size)
- Column temperature: 30°C
- Flow rate: 250 ml min⁻¹
- UV detector wavelengths: 215 nm; 246 nm; 285 nm
- Injection volume: 2 µL
- Mobile phase: 0.01% formic acid with 20% methanol

RESULTS

Optimization of MEPS influencing parameters

Figure 1. Effect of the main MEPS influencing extraction parameters: (a) comparison of the performance of five different MEPS sorbents; (b) influence of pH values. Error bars represent standard error of the mean (n=3 for each data point).

Figure 2. Effect of the main MEPS - influencing extraction parameters: (a) influence of number of extraction cycles and volume of spiked urine sample; (b) influence of elution volume in UPLC-PDA response. Error bars represent standard error of the mean (n=3 for each data point).

Figure 3. Representative chromatogram of standard mixture and human urine, of oxidative stress biomarkers: (a) standard mixture; (b) Urine Sample; (c) HMU; (d) IS - Internal Standard (8β-OhdG acquired (Ag)) at different wavelengths.

Analytical MEPS/UHPLC-PDA-based methodology performance

Table 1. Validation process data showing the concentration range inside which the linearity was tested, retention times (RT), and results of regression for total area versus concentration and analytical performance for the bioactive metabolites (biomarkers of oxidative DNA damage) on synthetic urine determined by the newly developed methodology (eVol® MEPS®/UHPLC-PDA).

<table>
<thead>
<tr>
<th>Peak no.</th>
<th>RT (min)</th>
<th>Bimarker</th>
<th>m/z (Da)</th>
<th>Conc. Range (µg mL⁻¹)</th>
<th>Regression equation</th>
<th>r²</th>
<th>LOD* (µg mL⁻¹)</th>
<th>LOQ* (µg mL⁻¹)</th>
<th>% Matrix effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.05</td>
<td>SHMU</td>
<td>215</td>
<td>0.0005 - 0.11</td>
<td>127.5* a + 0.8318</td>
<td>0.9906</td>
<td>0.00005</td>
<td>0.00003</td>
<td>80.11</td>
</tr>
<tr>
<td>2</td>
<td>1.60</td>
<td>IS</td>
<td>218</td>
<td>5</td>
<td>-</td>
<td>0.9946</td>
<td>0.04</td>
<td>0.13</td>
<td>82.11</td>
</tr>
<tr>
<td>3</td>
<td>1.80</td>
<td>OHdG</td>
<td>205</td>
<td>0.1 - 5</td>
<td>0.0724x + 0.0052</td>
<td>0.9994</td>
<td>0.04</td>
<td>0.13</td>
<td>82.11</td>
</tr>
</tbody>
</table>

*Linearity of determination (r²): calibration curve obtained in PDA system detection.
*LOD and LOQ: limits of detection and quantification.
*Matrix effect:

CONCLUSIONS

A ultra fast (running time <3min), sensitive, reusable, time and resource saving analytical method is reported for the successfully measurement of oxidative DNA biomarkers.

The linearity of the method is demonstrated all along the range of concentration investigated, with correlation coefficient (R²) ranging from 0.9906 to 0.9946 depending on analytes.

The LODs and LOQs were in the range of 0.00005 - 0.04 µg mL⁻¹ and 0.00023 - 0.13 µg mL⁻¹, respectively.

The comparison between the biomarkers were found to be statistically different (p<0.05).

The method provides a potential non-invasive screening for cancer diagnosis.

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