DEVELOPMENT OF A RAPID AND SENSITIVE METHOD FOR THE QUANTIFICATION OF BENZODIAZEPINES IN HUMAN PLASMA BY LC-MS/MS

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OBJECTIVE
To develop and validate a rapid and sensitive LC-MS/MS method for the simultaneous quantitation of benzodiazepines in plasma. The method should require minimal sample preparation.

INTRODUCTION
- Benzodiazepines are the most widely prescribed psychoactive drugs in the world for the symptomatic treatment of anxiety and sleep disorders.

- Here we describe the development of a rapid and sensitive LC-MS/MS method for the quantification of 10 benzodiazepines. Limits of detection 0.4µg/L or better were achieved when just 50µL plasma was used.

- Results were compared with those obtained using a commercial ELISA kit and a validated reference HPLC-DAD method.

EXPERIMENTAL
Validation Samples
Plasma samples were obtained from the Hospital Emergency Department.

HPLC-DAD
Plasma samples were initially screened for a broad range of medicinal drugs by HPLC-DAD. Briefly, after a liquid extraction of 1mL of plasma at pH 9.2 with 1-chlorobutane, the organic phase was evaporated and redissolved in 150µL of mobile phase (phosphate buffer pH 3.8- acetonitrile 67/33 (v/v) ). Fifty microlitres was injected on a Waters Symmetry C18 (3.9 x 150 mm, 5µm) column at 33°C.

ELISA
Plasma samples were also screened using a commercial ELISA kit (Cozart, UK) according to the manufacturer’s instructions. Negative and positive calibrators (oxazepam in the range 0-500 µg/L) were pipetted (in duplicate) onto the same microtitre plates as the unknown samples.

LC-MS/MS conditions
HPLC
HPLC System: Waters Alliance 2690
Column: Zorbax SB-phenyl column (2.1 x 150mm, 5µm)
Mobile phase : A = 10:10:80 acetonitrile:methanol: 20mM ammonium acetate
B = 95:5 acetonitrile:20mM ammonium acetate

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>A (%)</th>
<th>B (%)</th>
<th>Curve number</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>0.5</td>
<td>75</td>
<td>25</td>
<td>1</td>
</tr>
<tr>
<td>8</td>
<td>40</td>
<td>60</td>
<td>7 (concave)</td>
</tr>
<tr>
<td>11</td>
<td>40</td>
<td>60</td>
<td>6 (linear)</td>
</tr>
<tr>
<td>12</td>
<td>100</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>15</td>
<td>100</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

Flow rate: 0.25 mL/min
Injection volume: 10 µL

MS conditions
Mass spectrometer: Micromass Quattro Ultima
(Figure 1)
Ionisation Mode: ESI positive ion
Capillary voltage : 3kV
MS/MS: Collision gas Argon at 2.5 x 10⁻³ mbar
RESULTS AND DISCUSSION

Figure 2 shows the MS and MS/MS spectra for a selection of the benzodiazepines. The MRM transitions and conditions used for the measurement of the benzodiazepines and their respective deuterated analogues are summarised in Table 1. The latter were used as internal standards for quantification purposes.

Table 1. MRM transitions and conditions for the measurement of 10 benzodiazepines. †For these 2 compounds the isobaric nature between the analogue and their respective non-deuterated compounds meant that an alternative precursor ion was necessary.

A series of calibrators (1, 10, 40, 100, 200, 400 and 800 µg/L) were prepared by adding the benzodiazepines to drug-free plasma. Plasma samples were isolated from the matrix using a simple acetonitrile clean up procedure which also incorporates the addition of the internal standards.

Figure 3 shows the MRM chromatograms of the benzodiazepines obtained with a 10µL injection of the 10µg/L plasma calibrator. Quantification was performed by integration of the area under the specific MRM chromatograms. Figure 4 shows a typical standard curve for diazepam in plasma. Responses were linear, in all cases, over the range investigated (Coefficient of Determination > 0.99).
Table 2 compares the results for 20 plasma samples which were analysed using HPLC-DAD, LC-MS/MS and ELISA.

Table 2. Benzodiazepine quantification in 20 plasma samples using HPLC-DAD, LC-MS/MS and ELISA.

<table>
<thead>
<tr>
<th>Plasma sample</th>
<th>HPLC-DAD</th>
<th>LC-MS/MS</th>
<th>ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Lor (20), Mida (25)</td>
<td>Lor (25)</td>
<td>negative (55)</td>
</tr>
<tr>
<td>2</td>
<td>D (59), ND (20), Lor (24)</td>
<td>D (57), ND (15), Lor (29)</td>
<td>positive (386)</td>
</tr>
<tr>
<td>3</td>
<td>Al (44)</td>
<td>Al (44)</td>
<td>positive (359)</td>
</tr>
<tr>
<td>4</td>
<td>D (10), ND (14), Lor (13)</td>
<td>D (12), ND (26), Tem (12), Ox (1), Tor (4)</td>
<td>positive (198)</td>
</tr>
<tr>
<td>5</td>
<td>Lor (30)</td>
<td>Lor (39)</td>
<td>negative (12)</td>
</tr>
<tr>
<td>6</td>
<td>D (150), ND (159)</td>
<td>D (102), ND (132), O (57), Tor (82)</td>
<td>positive (304)</td>
</tr>
<tr>
<td>7</td>
<td>ND (&lt;150), Ox (100)</td>
<td>Ox (100), Ox (100), Tor (80)</td>
<td>positive (492)</td>
</tr>
<tr>
<td>8</td>
<td>D (150), ND (150)</td>
<td>D (150), ND (150), Ox (100), Tor (80)</td>
<td>positive (342)</td>
</tr>
<tr>
<td>9</td>
<td>Al (150)</td>
<td>Al (150)</td>
<td>positive (356)</td>
</tr>
<tr>
<td>10</td>
<td>D (20), ND (20)</td>
<td>D (20), ND (20), Tem (10)</td>
<td>positive (411)</td>
</tr>
<tr>
<td>11</td>
<td>Lor (50), Lor (24)</td>
<td>Lor (35), D (15)</td>
<td>positive (353)</td>
</tr>
<tr>
<td>12</td>
<td>Al (450)</td>
<td>Al (450)</td>
<td>positive (357)</td>
</tr>
<tr>
<td>13</td>
<td>Lor (100)</td>
<td>Lor (110), D (27), ND (31)</td>
<td>positive (312)</td>
</tr>
<tr>
<td>14</td>
<td>D (150)</td>
<td>D (150)</td>
<td>positive (389)</td>
</tr>
<tr>
<td>15</td>
<td>Ox (150)</td>
<td>Ox (150), Flu (150), Ox (200)</td>
<td>positive (392)</td>
</tr>
<tr>
<td>16</td>
<td>Al (145)</td>
<td>Al (145)</td>
<td>positive (348)</td>
</tr>
<tr>
<td>17</td>
<td>Lor (150), D (120), ND (150)</td>
<td>D (170), ND (200), Ox (150), Tor (300)</td>
<td>positive (386)</td>
</tr>
<tr>
<td>18</td>
<td>Al (20), Lor (15)</td>
<td>Al (19), Lor (19)</td>
<td>positive (294)</td>
</tr>
<tr>
<td>19</td>
<td>Ox (16)</td>
<td>Ox (16)</td>
<td>negative (10)</td>
</tr>
<tr>
<td>20</td>
<td>Lor (500), D (300), ND (220)</td>
<td>D (710), ND (200), Ox (140), Tor (120)</td>
<td>positive (304)</td>
</tr>
</tbody>
</table>

Table 2. Benzodiazepine quantification in 20 plasma samples using HPLC-DAD, LC-MS/MS and ELISA.

Key: D=Diazepam, ND=nordiazepam, Tem=temazepam, Ox=oxazepam, Lora=lorazepam, Alp=alprazolam, Pra=prazepam, Mida=midazolam, Clon=clonazepam, Brom=bromazepam. Bracketed values are drug concentration in µg/L.

* A cut-off value of 100µg/L was applied to the ELISA results.

Figure 3. MRM chromatograms for (top to bottom): lorazepam, temazepam, triazolam, prazepam, oxazepam, diazepam, alprazolam, flunitrazepam, nordiazepam and clonazepam. Responses were obtained with a 10µL injection of the 10µg/L plasma calibrator.

Figure 4. Typical response for plasma containing diazepam. Diazepam spiked plasma was firstly extracted using acetonitrile prior to analysis using HPLC/MRM. Benzodiazepines were quantified by reference to their deuterated internal standards.
CONCLUSION

LC-MS/MS
We have developed a simple, rapid method that allows the simultaneous quantification of 10 benzodiazepines in a single chromatographic run. Only 50µL sample is required. The procedure involves a simple protein precipitation step with acetonitrile followed by HPLC/MRM analysis and is less time-consuming and labour-intensive than the existing GC/MS and LC-DAD methods. The developed method has been successfully applied to the analysis of plasma samples collected from current benzodiazepine users and compared with the results of a validated reference HPLC-DAD screening method.

ELISA
A cut-off value of 100 µg/L was applied to the data which resulted in an accuracy of 89 %, a sensitivity of 87 % and a specificity of 100 % for a total of 62 samples. Lowering the cut-off to 50 µg/L does not change the outcome significantly. When a cut-off of 10 µg/L is applied, the accuracy is 90 %, sensitivity 93 % and specificity only 71 %. A lower cut-off value leads to a number of false positives, also due to the difference in the nature of the matrix of the samples. False negatives were only obtained for low concentrations of lorazepam and flunitrazepam.

FUTURE AIMS

- To validate the method for alternative specimens.
- To apply the method to insects recovered from decomposing human remains (forensic entomology) and post-mortem samples (hair, body fluids, tissue).
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