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# 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 $C_{18}$ (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<br>B = 95:5 acetonitrile: 20mM ammonium acetate |

| Time (min) | A (%) | B (%) | Curve number |
|---|---|---|---|
| 0 | 100 | 0 | 1 |
| 0.5 | 75 | 25 | 1 |
| 8 | 40 | 60 | 7 (concave) |
| 11 | 40 | 60 | 6 (linear) |
| 12 | 100 | 0 | 1 |
| 15 | 100 | 0 | 1 |

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

MS conditions

| | |
|---|---|
| Mass spectrometer: | Micromass Quattro *Ultima* (**Figure 1**) |
| Ionisation Mode: | ES positive ion |
| Capillary voltage : | 3kV |
| MS/MS: | Collision gas Argon at 2.5 x $10^{-3}$ mbar |

MICROMASS MS TECHNOLOGIES

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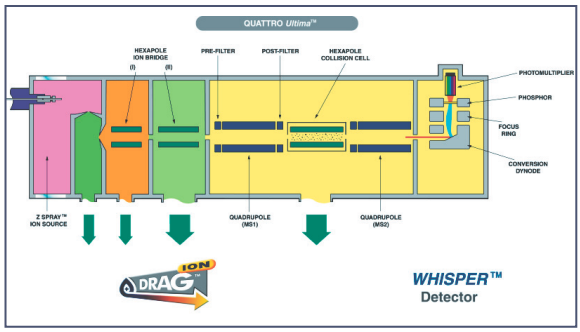


*Figure 1. The Quattro Ultima triple quadrupole mass spectrometer incorporating 'Z' Spray source*

## 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.

| Compound | Precursor ion (m/z) | Product ion (m/z) | Cone voltage (V) | Collision energy (eV) |
|---|---|---|---|---|
| Alprazolam | 308.8 | 280.9 | 70 | 25 |
| Alprazolam-d5 | 313.8 | 285.8 | 100 | 25 |
| Clonazepam | 315.8 | 269.8 | 80 | 25 |
| Clonazepam-d4 | 319.9 | 273.8 | 100 | 25 |
| Diazepam | 284.9 | 154.0 | 60 | 25 |
| Diazepam-d5 | 289.8 | 153.7 | 80 | 25 |
| Flunitrazepam | 313.9 | 267.9 | 80 | 25 |
| Flunitrazepam-d7 | 320.8 | 274.8 | 80 | 25 |
| Lorazepam | 320.8 | 274.7 | 60 | 23 |
| Lorazepam-d4† | 326.8 | 280.8 | 80 | 23 |
| Nordiazepam | 270.9 | 139.8 | 80 | 25 |
| Nordiazepam-d5 | 275.9 | 139.8 | 60 | 25 |
| Oxazepam | 287.0 | 240.8 | 60 | 26 |
| Oxazepam-d5 | 291.7 | 245.8 | 80 | 26 |
| Prazepam | 324.9 | 270.9 | 80 | 25 |
| Prazepam-d5 | 330.0 | 276.0 | 80 | 25 |
| Temazepam | 300.9 | 255.0 | 60 | 25 |
| Temazepam-d5 | 305.8 | 259.8 | 60 | 25 |
| Triazolam | 342.9 | 307.7 | 60 | 25 |
| Triazolam-d4† | 349.0 | 313.9 | 60 | 25 |

*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.*

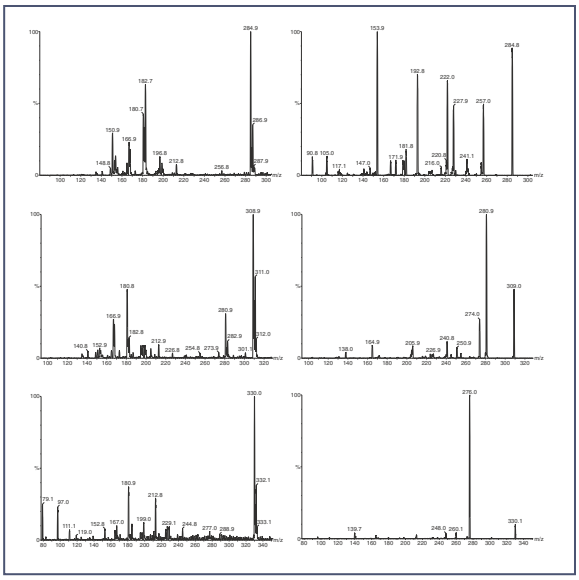

*Figure 2. MS and MS/MS spectra of (top to bottom) diazepam, alprazolam and the deuerated internal standard for prazepam (d5).*

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).

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**Table 2** compares the results for 20 plasma samples which were analysed using HPLC-DAD, LC-MS/MS and ELISA.

| Plasma sample | HPLC-DAD | LC-MS/MS | ELISA* |
|---|---|---|---|
| 1 | Lora (24), Mida (25) | Lora (23) | negative (35) |
| 2 | D (59), ND (< 20), Lora (24) | D (53), ND (7), Lora (29) | positive (566) |
| 3 | Alp (45) | Alp (48) | positive (506) |
| 4 | D (150), ND (34), Clon (<10) | D (129), ND (28), Clon (12), Ox (3), Tem (4) | positive (708) |
| 5 | Lora (30) | Lora (35) | negative (12) |
| 6 | D (1785), ND (1350) | D (1682), ND (1262), Ox (73), Tem (62) | positive (746) |
| 7 | ND (575) + Praz intox | ND (567), Ox (57), Pra (13) | positive (692) |
| 8 | D (850), ND (470) | D (763), ND (398), Ox (10), Tem (41) | positive (742) |
| 9 | Alp (15) | Alp (13) | positive (396) |
| 10 | D (280), ND (<20) | D (250), ND (5), Tem (3) | positive (681) |
| 11 | Brom (390), Lora (24) | Lora (30), D (10) | positive (353) |
| 12 | Alp (420) | Alp (364) | positive (767) |
| 13 | Lora (103) | Lora (117), D (17), ND (11) | positive (612) |
| 14 | Brom (200) | D (72), ND (20) | positive (589) |
| 15 | Ox (1300) | Clon (8), Flu (11), Ox (>800) | positive (792) |
| 16 | Alp (140) | Alp (147) | positive (648) |
| 17 | Brom (176), D (1290), ND (710) | D (1196), ND (655), Ox (47), Tem (46) | positive (806) |
| 18 | Alp (20), Lora (32) | Alp (19), Lora (34) | positive (294) |
| 19 | Flu (34) | Flu (26) | negative (6) |
| 20 | Brom (8300), D (3380), ND (2250) | D ( 3117), ND (2086), Ox (187), Tem (305) | positive (805) |

*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.*

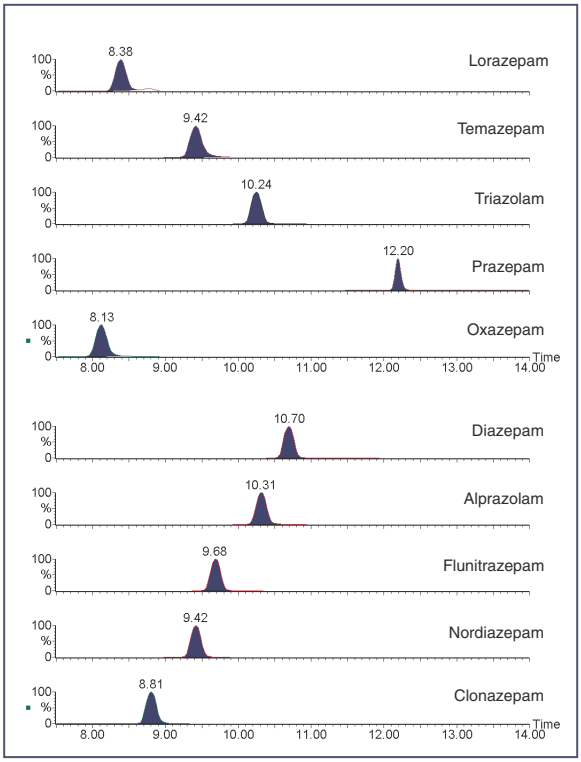


*Figure 3. MRM chromatograms for (top to bottom): lorazepam, temazepam, triazolam, prazepam, oxazepam, diazepam, alprazolam, flunitrazepam, nordiazepam and clonazepam. Responses were obtained with a 10µL injection of the 10µg/L plasma calibrator*

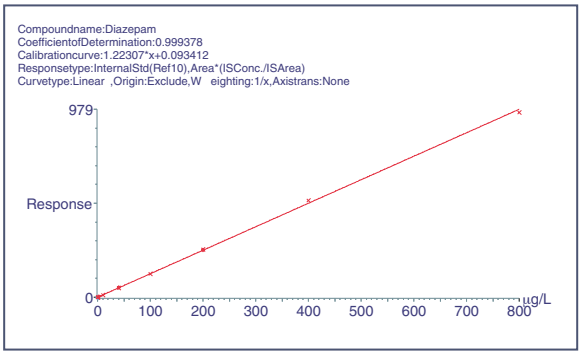


*Figure 4. Typical response for plasma containing diazepam. Diazepam spiked plasma was firstly extracted using acetonitrile prior to analysis using HPLC/MRM. Benzodiazepines were quantified by reference to their deuterated internal standards*

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## 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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