

# Application of GC-MS/MS technique for the determination of benzodiazepines in blood samples

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## Abstract

Benzodiazepines (BZD) are widely used as pharmaceuticals in medicine. However, due to psychostimulatory effect of BZD, in the last decade, they are more and more often used as drugs of abuse. These compounds produce similar effects to classical illicit drugs and are sold as "legal" alternatives to them as new psychoactive substances (NPS). Currently new BZD, named designer benzodiazepines (DBZD), are synthesized each year by a simple modification of registered drug structure or their metabolites. The misuse and abuse of benzodiazepines has become an increasing problem in many countries. Due to this phenomenon there is a strong need to develop new analytical methods for the determination of a whole range of substances in biological specimens for forensic toxicology.

A rapid, sensitive and robust GC-MS/MS-based method with a simple liquid-liquid extraction for the determination of 10 benzodiazepines in whole blood samples was developed. The assay achieved satisfactory validation parameters, such as: inter-day accuracy (91.8–118.6 %), and precision (2.8–14.9%). The limit of detection (LOD) and limit of quantification (LOQ) were in the range of 0.02-0.53ng/mL and 1-2 ng/mL, respectively. The developed procedure can be widely applicable for rapid screening of new drugs of abuse in forensic or clinical cases.

**Key words:** GC-MS/MS, benzodiazepines, BZD, new psychoactive substances, NPS, blood samples

## 1. Introduction

Benzodiazepines (BZD), due to their anxiolytic, hypnotic, anticonvulsive and muscle-relaxant properties, are widely used in medicine for treatment of various mental illnesses. These compounds are typically prescribed by medical professionals for insomnia, anxiety, agitation, muscle spasms, seizures, alcohol withdrawal or as a premedication for medical procedure because of a wide spectrum of therapeutic result (1,2). However, due to psychostimulatory effect of BZD, a phenomenon involving misuse or abuse these type of compounds (as drugs of abuse) is observed. Moreover, in recent years, many new compounds belonging to BZD group were synthesized as modification of existing substances and were introduced into illicit trade. New BZD are typically sold as “designer benzodiazepines” (DBZD) giving recreation feeling similar to the other new psychoactive substances (NPS) (3,4). The easy availability both classical and designer benzodiazepines consists a serious societal problem and leads to health risks.

Chemically benzodiazepines are the fusion of benzene and diazepine ring with various substituent of side chains, what gives these compounds unique physic-chemical properties. The extensive research associated with these structures resulted in an introduction a variety of psychoactive substances that never got a marketing authorization which leads to illegal distribution these substances in drug market and appearance of more new derivatives.

Problems associated with the detection of benzodiazepines in complex matrices, such as blood and urine, consist an interesting area in various scopes to develop rapid and sensitive analytical procedures. Currently, many methods for BZD determination have been used. They mainly utilizing immunoassays techniques and hyphenated techniques (LC-MS/MS and GC-MS), allowing to obtain a valuable source data set useful in clinical research, quality control and legal studies (5). However, in toxicology analysis, new analytical procedures are still strongly required in order to simplify sample preparation step and to reduce volume of sample required for the extraction. The determination of BZD in biological specimen is still challenging owing mainly due to a large number of these compounds available. Such results can be useful for toxicological

analysis in order to establish magnitude of the abuse problem of these compounds or in the cause of death (6).

Despite the large number of published data associated with the determination of benzodiazepines, to the best of our knowledge, there is a lack of information concerning the application of GC-MS/MS for the analysis of these compounds in human biological fluids. Therefore, the aim of this study was to verify potentiality of application of a GC-MS/MS technique for the detection and quantification of benzodiazepines in whole blood samples for occupational and forensic purposes.

## **2. Experimental**

### *2.1 Standards, reagents and chemicals*

The certified standards of benzodiazepines used in this study were purchased from LGC Standards (London, UK), Cayman Chemical Company (Ann Arbor, MI, USA) and Cerilliant (Round Rock, Texas, USA) as separate solutions in methanol at a concentration of 1 mg/mL. The standards have a minimum purity of 98%. Diazepam-D<sub>5</sub> was used as the internal standard (IS). Diisopropyl ether, ethyl acetate and methanol were supplied by Sigma-Aldrich (St. Louis, MO, USA). All solvents used were of HPLC grade. Analytical-grade sodium carbonate (Na<sub>2</sub>CO<sub>3</sub>) and sodium bicarbonate (NaHCO<sub>3</sub>) powder were obtained from POCH S.A. (Gliwice, Poland). Both salts were used for the preparation of carbonate buffer solution at pH 9.2 by dissolving appropriate mass of powder in ultrapure water. Water was purified by a Millipore Milli-Q Gradient A10 water system (Merck, Warszawa, Poland).

Stock solutions of analytes (as a mixture) and the IS stock solution were prepared by dilution of the certified standards with methanol and were stored at -20°C until use.

### *2.2 Biological specimens*

Blank (drug-free) blood samples were collected from volunteers non-consumers of any drugs and were obtained from a regional blood donation bank (Gdańsk, Poland). Blank blood samples were used for the development and validation of the method and were also stored in -20°C prior to analysis.



## 2.2 Instrumentation

The analysis were performed using GC-2010 PLUS system with TQ8050 triple quadrupole mass spectrometer (Shimadzu, Kyoto, Japan). Chromatographic separation of the analytes was achieved on Zebron ZB-5MSi capillary column (30 m × 0.25 mm id, 0.25 μm; Torrance, Calif., U.S.A.). Helium (grade 5.0) was used as carrier gas with an initial flow rate of 1 mL/min. Then, a constant gas linear velocity was maintained at 38 cm/s. The splitless injection mode for 1 min under High Pressure Injection mode (300 kPa; 0.5 min) was used followed by split mode (20:1). The oven temperature gradient program was as follows: from 150°C (held 1 min) to 200°C at 20°C/min (held 1 min), then ramp to 285°C at 10°C/min (held 5 min) and finally ramp to 310°C at 10°C/min (held 4 min). The temperature of the injection port, MS transfer line and ion source were set at 260, 285 and 250°C, respectively. MS was operated in positive electronic ionization (EI) mode. The ion source temperature was set at 250°C. Argon (grade 5.0) was used as the collision-induced dissociation (CID) gas. Quantification was performed using multiple reaction monitoring (MRM) transitions. The specific transitions with optimum collision energies (CEs) for all compounds are shown in Table 1. Data acquisition and processing were performed using the GCMS Solution and Insight GCMS software (version 4.45, Shimadzu Corporation). Injection volume was 2 μL.

<Insert Table 1>

## 2.3 Extraction, calibration and method validation

The analytes were extracted from 0.5 mL of blood sample using diisopropyl ether after addition of the IS stock solution and carbonate buffer to obtain alkaline condition (pH 9.2). After evaporation of solvent, dry residue was dissolved in 100 μl of ethyl acetate. The calibration solutions were prepared in triplicate ( $n=3$ ) in the range of 1-200 ng/mL by spiking blank blood samples with the appropriate volume of the stock solution of the analytes and the IS stock solution. Then, extraction procedure was performed. Quality control (QC) samples were prepared similar to the calibration solutions at three concentration levels (at various steps depending on



calibration range) listed in Table 3. QC samples were used for the evaluation of precision and accuracy.

The developed GC-MS/MS-based method for the quantification of benzodiazepines in blood samples was validated according to the international guidelines (7,8). Several parameters relevant to a quantitative method such as: linearity, precision, accuracy, matrix effect (ME), limit of detection (LOD) and limit of quantification (LOQ) were evaluated.

### 3. Results and discussion

A LLE-based method utilizing GC-MS/MS technique for the determination of 10 benzodiazepines in whole blood samples was developed. Specific MRM transitions and CEs were optimized using Shimadzu MRM Optimization Tool and Shimadzu Smart Database software. Chromatography separation time was short and peaks for the analytes were totally resolved, excepting peaks for Diazepam and Diazepam-d<sub>5</sub> (Fig. 1). However, due to use of specific MRM transitions, chromatographic separation is not required in such cases. Up to now, in most GC-based procedures published in the literature (9), derivatization has been performed to obtain low LOD and LOQ. In our developed method, due to the use of MS/MS detection, the derivatization step could be omitted and low LOD and LOQ were obtained at similar levels as for LC-MS/MS-based methods (10).

<Insert Fig.1>

The validation data are shown in Tables 2 and 3. Calibration curves were linear in various range depending on MS responses with correlation coefficient ( $r$ ) above 0.99. Weighted least squares regression was applied to the calibration curves to improve the accuracy, especially at in low concentration level range. The limit of detection, based on a signal-noise ratio ( $S/N$ ) equal to 3, was between 0.02-0.53 ng/mL and the limit of quantification was assumed to be the lowest point of the calibration curve (1 or 2 ng/mL) due to the linearity.

<Insert Table 2>

GC-MS/MS matrix effect (ME) was calculated by comparing the MS response for extract of blank blood spiked with analytes at known amounts with MS response recorded for sample prepared in methanol at the same concentration level. The negative and positive values for ME were obtained what indicates signal enhancement and suppression. Therefore, matrix-match IS calibration, instead of external calibration, was used in this study. The intra-day accuracy varied from 83.7 to 117.6 %, while the inter-day accuracy was 91.8 to 118.6 %, respectively. Precision (as coefficient of variation; CV) varied from 0.1 to 12.7 % for intra-day and from 1.9 to 14.9 % for inter-day assay.

<Insert Table 3>

#### **4. Conclusions**

A LLE procedure followed by GC-MS/MS analysis was developed for the quantification of 10 benzodiazepines in whole blood samples using only 500  $\mu$ L of sample. The whole procedure was rapid and simple. Blood is one of the most commonly used matrix in forensic toxicology analysis for drug or other toxic substances screening. Importantly, the developed procedure can be easily expanded for more substances. Based on the obtained validation parameters, our procedure can be used as an alternative tool to LC-MS/MS-based methods for the determination of benzodiazepines for clinical laboratories and forensic toxicology.

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## Figures

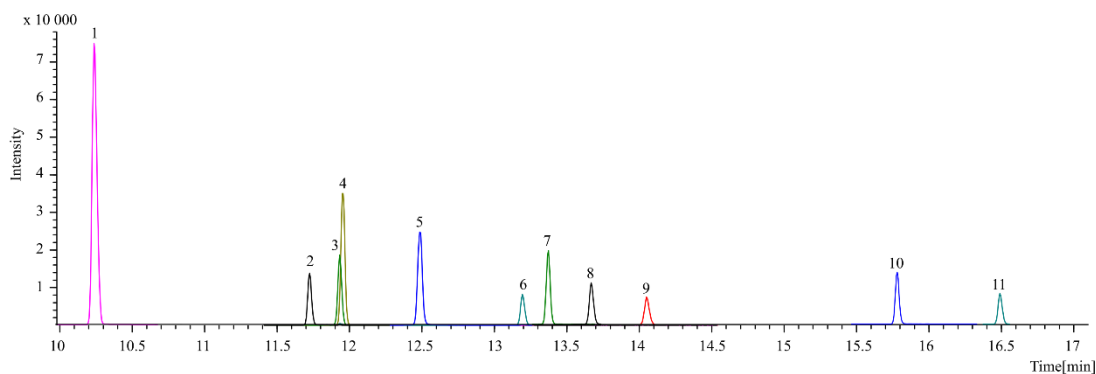


Fig 1. GC-MS/MS chromatogram (in MRM mode) of standard benzodiazepine mixture in blood in blood samples

## Tables

**Table 1.** List of compounds covered by developed method with their chemical names, retention times and parameters for MRM mode (CE – collision energy, RT – retention time, RA – relative abundance of quantifier/qualifier transitions)

| No. | Compound                | RT<br>[min] | Quantifier    |        | Qualifier     |        | RA     |
|-----|-------------------------|-------------|---------------|--------|---------------|--------|--------|
|     |                         |             | Transition    | CE [V] | Transition    | CE [V] |        |
| 1   | Medazepam               | 10.24       | 242.1 → 207.2 | 15     | 207.1 → 165.1 | 24     | 100/38 |
| 2   | Lorazepam               | 11.72       | 274.0 → 239.1 | 15     | 239.0 → 177.1 | 24     | 100/79 |
| 3   | Diazepam-D <sub>5</sub> | 11.93       | 261.0 → 226.2 | 12     | 261.0 → 170.2 | 30     | 100/31 |
| 4   | Diazepam                | 11.95       | 256.1 → 221.1 | 12     | 256.1 → 165.1 | 36     | 100/50 |
| 5   | Nordiazepam             | 12.48       | 242.0 → 207.1 | 12     | 242.0 → 152.1 | 33     | 100/47 |
| 6   | Midazolam               | 13.19       | 310.1 → 95.1  | 9      | 310.1 → 75.1  | 42     | 100/38 |
| 7   | Flunitrazepam           | 13.37       | 312.1 → 266.1 | 36     | 286.1 → 240.1 | 42     | 100/59 |
| 8   | Prazepam                | 13.67       | 269.1 → 240.1 | 15     | 269.1 → 116.1 | 33     | 100/86 |
| 9   | Lormetazepam            | 14.05       | 305.0 → 111.1 | 15     | 305.0 → 193.0 | 15     | 100/59 |
| 10  | Clonazepam              | 15.78       | 280.1 → 234.1 | 15     | 314.0 → 268.0 | 15     | 100/76 |
| 11  | Estazolam               | 16.49       | 259.1 → 205.1 | 12     | 205.1 → 151.1 | 15     | 100/47 |

**Table 2.** Quantification and calibration data (LOD-limit of detection, LOQ-limit of quantification, r- correlation coefficient)

| Compound      | Calibration curve range<br>[ng/ml] | Weighting factor | Calibration curves    | r      | LOD<br>[ng/ml] | LOQ<br>[ng/ml] |
|---------------|------------------------------------|------------------|-----------------------|--------|----------------|----------------|
| Medazepam     | 1 – 100                            | 1/x              | y = 0.07220x + 0.0137 | 0.9994 | 0.21           | 1              |
| Lorazepam     | 2 – 100                            | -                | y = 0.00701x - 0.0040 | 0.9992 | 0.30           | 2              |
| Diazepam      | 1 – 100                            | 1/x              | y = 0.03164x + 0.0020 | 0.9994 | 0.09           | 1              |
| Nordiazepam   | 1 – 100                            | 1/x              | y = 0.02498x - 0.0024 | 0.9995 | 0.09           | 1              |
| Midazolam     | 1 – 200                            | 1/x              | y = 0.00691x - 0.0006 | 0.9998 | 0.08           | 1              |
| Flunitrazepam | 2 – 200                            | 1/x <sup>2</sup> | y = 0.01329x - 0.0006 | 0.9953 | 0.11           | 2              |
| Prazepam      | 1 – 100                            | 1/x              | y = 0.01005x - 0.0003 | 0.9996 | 0.12           | 1              |
| Lormetazepam  | 2 – 100                            | 1/x <sup>2</sup> | y = 0.00137x - 0.0010 | 0.9951 | 0.44           | 2              |
| Clonazepam    | 2 – 100                            | 1/x              | y = 0.09301x - 0.0080 | 0.9966 | 0.02           | 2              |
| Estazolam     | 2 – 200                            | -                | y = 0.01179x - 0.0111 | 0.9998 | 0.53           | 2              |



**Table 3.** Summary of the validation test performed in this study: accuracy, precision (CV) and matrix effect (ME) [%]

| Compound      | C<br>[ng/ml] | Intra-day assay |        | Inter-day assay |        | ME [%] |
|---------------|--------------|-----------------|--------|-----------------|--------|--------|
|               |              | Accuracy<br>[%] | CV [%] | Accuracy [%]    | CV [%] |        |
| Medazepam     | 5            | 101.1           | 4.6    | 91.9            | 10.4   | -6     |
|               | 20           | 98.8            | 7.0    | 97.6            | 9.7    | 11     |
|               | 80           | 104.5           | 4.5    | 118.6           | 14.9   | 0      |
| Lorazepam     | 5            | 105.6           | 0.8    | 106.4           | 10.8   | 36     |
|               | 20           | 111.8           | 2.7    | 113.3           | 9.9    | 2      |
|               | 80           | 117.6           | 12.7   | 117.9           | 9.8    | 37     |
| Diazepam      | 5            | 96.0            | 1.3    | 93.5            | 9.8    | -10    |
|               | 20           | 103.7           | 0.5    | 95.4            | 9.0    | 7      |
|               | 80           | 103.8           | 1.9    | 113.0           | 13.8   | 18     |
| Nordiazepam   | 5            | 97.1            | 1.8    | 96.8            | 9.3    | 0      |
|               | 20           | 103.6           | 0.9    | 96.8            | 7.1    | 12     |
|               | 80           | 107.6           | 0.4    | 115.2           | 10.6   | 37     |
| Midazolam     | 5            | 98.4            | 1.0    | 95.1            | 6.9    | 1      |
|               | 50           | 101.9           | 3.8    | 109.5           | 9.8    | 2      |
|               | 150          | 107.9           | 0.5    | 114.2           | 7.7    | 12     |
| Flunitrazepam | 10           | 95.7            | 1.3    | 91.8            | 3.8    | 5      |
|               | 50           | 104.6           | 4.6    | 112.1           | 3.7    | 1      |
|               | 150          | 104.7           | 1.3    | 112.9           | 7.5    | 10     |
| Prazepam      | 5            | 99.3            | 0.1    | 97.1            | 9.0    | -10    |
|               | 20           | 103.2           | 1.0    | 95.9            | 8.6    | 8      |
|               | 80           | 103.6           | 1.7    | 112.1           | 1.9    | 22     |
| Lormetazepam  | 5            | 102.5           | 0.2    | 104.4           | 4.6    | - *    |
|               | 20           | 106.5           | 3.1    | 102.4           | 6.7    | -2     |
|               | 80           | 110.1           | 3.5    | 105.8           | 3.5    | 52     |
| Clonazepam    | 5            | 109.1           | 4.8    | 109.9           | 2.8    | 49     |
|               | 20           | 115.4           | 1.4    | 106.9           | 7.6    | 4      |
|               | 80           | 94.6            | 2.2    | 111.5           | 8.6    | 44     |
| Estazolam     | 10           | 83.7            | 0.9    | 99.2            | 6.4    | 3      |
|               | 50           | 99.5            | 3.5    | 118.4           | 5.7    | -3     |
|               | 150          | 112.2           | 5.1    | 115.1           | 9.5    | 6      |

\*Due to high signal enhancement in matrix, intensity of peak (S/N) in the solvent was below 3.