# Fast and Simple Method for Simultaneous Detection and Quantification of Diazepam and Desmethyldiazepam in Plasma Samples in Psychiatric Patients by GC-MS-FID

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*The aim of this study was to develop a method for identification and quantification of diazepam and its major metabolite, desmethyldiazepam in plasma samples in psychiatric patients. Liquid - liquid extraction (LLE) procedure was applied in order to simplify the pre-analytical phase, followed by gas chromatography – mass spectrometry (GC-MS) detection and gas chromatography – flame ionization (GC-FID) quantitation.*

*Keywords: liquid-liquid extraction, diazepam, GC-MS-FID*

Because benzodiazepines are frequently encountered in emergency rooms and toxicology laboratory, various reliable and specific techniques made the purpose of identification and confirmation their presence in human body [1-4].

Benzodiazepines are widely used for the treatment of anxiety and sleep disorders, being prescribed as psychoactive drugs. Diazepam is a long-acting 1,4-benzodiazepine, relatively safe, frequently prescribed for the treatment of stress, panic disorders, sleep disorders, muscle spasms, alcohol withdrawal, and seizures [5]. Nordiazepam is the primary active metabolite resulted after diazepam demethylation following administration [6]. In order to predict the concentration profile of these drugs in biological fluids, the modern toxicological analysis mostly uses hyphenated chromatographic techniques, e.g. HPLC-DAD, HPLC-MS or GC-MS [7, 8]. This newfound interest in using the modern analytical techniques led to the necessity for the development of sophisticated methods for the simultaneous determination of the most commonly used benzodiazepines in various biological specimens [9-11].

A direct consequence of the advances made in therapeutic drug management within both the clinical and diagnostic communities has encouraged analysts to develop strategies in monitoring the response to different therapies in which analytical analysis of drug plasma concentration plays an important role [12].

Considering the important linkage between drug effect and concentration of drug in the body, we followed a chromatographic technique that can be used routinely for the identification and quantification of free fraction of diazepam and its major metabolite, nordiazepam in biological fluids. Our purpose was to develop an efficient and simple extraction step without derivatization for bio-sample preparation that can be successfully applied for the analysis of the concentration profile after single dose administration of diazepam in psychiatric patients.

## Experimental part

### *Materials and methods*

*Chemicals and reagents.* Diazepam and desmethyl-diazepam were purchased from LGC Standards. Methanol, diethyl ether, ethyl acetate, 1-chlorobutane, all GC grade; potassium carbonate, chloroform, isopropanol and amitriptyline were supplied by Sigma-Aldrich. Midazolam (internal standard used for identification) was supplied by Roche Diagnostics. All chemicals used were of reagent grade or better.

### *Human specimens*

Blank plasma samples were sponsored by Regional Center of Blood Transfusion Timisoara.

*Specimen collection.* Biological specimen was collected at specific intervals from a total number of ten patients presenting with different psychiatric disorders at the Psychiatry Clinic. For plasma analysis, whole blood samples were collected in $K_3$EDTA tubes and centrifuged at 3000rpm, 4°C for 5 min, after which the plasma was removed for analysis. Plasma samples were stored at -20°C prior to analysis.

### *Stock solutions*

Diazepam, nordiazepam and amitriptyline, internal standard (IS used for quantification) were prepared by weighing the appropriate amounts in methanol. Working solutions were prepared by appropriate dilution of the stock solutions in methanol. All solutions were stored at 4°C. Quality control (QC) samples were prepared independently of the preparation of the calibration solutions.

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*Sample preparation*

Independent of bio-sample, a suitable preparation was necessary before GC-MS and GC-FID analysis. Compounds of interest were isolated from heterogeneous matrice by liquid-liquid extraction (LLE) procedure. Plasma samples (800µL) were mixed with 0.1 mL IS, and were extracted with diethyl ether (2 mL), followed by vortex-mixing (1min) and centrifugation (3500rpm for 5 min). After phase separation, the organic extract was transferred into a labeled flask and evaporated to dryness. The aqueous residue was extracted a second time with 2 mL of the extraction solvent. This organic extract was transferred into the same flask and evaporated at 60°C under nitrogen stream. The combined residues were dissolved in 100 µL 1-chlorobutane. *Mass spectro-metry.* Mass spectrometry was performed using a 240 MS Ion Trap (Varian). The ionization parameters were set as follows: ion trap temperature at 170 °C, the transfer line temperature at 230 °C using helium as mobile phase. Ionization was performed in EI mode at 70eV. Nominal mass was used for recording full spectra and single ion storage technique was applied for the identification of drugs from endogenous compounds of the matrices. Each standard was injected separately in order to check if the fragmentation data matched the database information. Fragmentation patterns were extracted from the peaks and referred to spectral database (NIST, Wiley, PMW) for the confirmation of the identity of the separated compounds.

*Chromatographic conditions*

We performed the chromatography on 450 GC Varian, using hydrogen as carrier gas at a constant flow rate of 1.5 mL/min, and a linear velocity of 50.1 cm/s. For qualitative analysis we used a low polarity column Factor Four VF-5ms (30m x 0.25mmID, 0.25µm film), (Varian). The column used for quantitative analysis was an BR-5ms (30m x 0.25mmID, 0.25µm film), (Brucker Daltonics), maintained at a temperature of 170 °C, 1 min, raised to 280 °C with 10 °C/min, held for 3 min. The temperature of the injection pool was set at 280 °C. Samples were injected at a volume of 1 µL using split ratio for 5min, followed by splitless mode 10min. Quantitation was obtained by maintaining the flame ionization detector temperature at 300 °C.

## Results and discussions

*Calibration curve.* In order to evaluate the linearity we investigated the relative response of the target analytes to the amount of internal standard. The calibration curve was linear on six level concentrations and three measurements of each level were performed. For blank plasma calibration, linearity was obtained in the range 0.05-2.5 µg/mL for both diazepam and nordiazepam. To check the precision three levels of quality control (high, medium and low) were prepared by successive dilutions of working solutions for both analytes in blank specimens. For standards prepared in pure biological fluids, a weighted ($1/x^2$) linear regression fit was achieved with $R^2 > 0.998$ and deviations from the fitted curve < 10%. At the lowest concentration yielding imprecision was < 20%.

*Extraction recoveries and carryover.*

We evaluated the extraction recoveries in 10 lots of biological fluids, each from a different donor. Calculation was based on peak area ratio before and after extraction procedure [13]. In the development phase of our method, we tested the precision and accuracy in five different concentrations (table 1). For both benzodiazepines the extraction recovery was > 85% in plasma samples. IS recovery was > 30% in plasma samples (table 2). During the analysis of blank samples, no clear interferences were detected. Carryover was < 1% for both analytes and IS.

For daily checking of the apparatus performance, a methanolic solution of diazepam, nordiazepam and IS was injected into the GC-MS and we compared the acquired response to the database fragmentation pattern. Identification of plasma extracts was realized using the analytical parameters presented in table 3. The obtained data was analyzed based on internal standard procedure using MS Workstation software.

| Nominal concn, µg/mL | Diazepam, RSD% | Nordiazepam, RSD% |
|---|---|---|
| | Plasma | |
| 0.05 | 7.45 | 4.46 |
| 0.1 | 2.17 | 1.55 |
| 0.5 | 0.65 | 0.55 |
| 1 | 0.82 | 1.20 |
| 1.5 | 1.86 | 8.90 |

**Table 1**
CONFIDENCE PARAMETERS (MEAN) OF VALIDATED METHOD FOR THE DETERMINATION OF DIAZEPAM AND NORDIAZEPAM IN PLASMA

**Table 2**
RECOVERIES OF DIAZEPAM AND NORDIAZEPAM IN PLASMA SAMPLES (n=5)

| Extraction solvent | Diazepam, recovery (%) | Nordiazepam, recovery (%) |
|---|---|---|
| | plasma | |
| Diethylether | 90.05 | 85,61 |
| Chloroform : isopropanol (9:1) | 42.02 | 41.56 |
| Ethyl acetate | 55.14 | 51.03 |

| Analyte | Retention time [min] | Main ions [m/z] in MS spectra* |
|---|---|---|
| Amitriptyline | 11,139 | 58.0, 276.1 |
| Diazepam | 12.832 | 221.1, 256.1, 283.1, 285.0 |
| Nordiazepam | 13.480 | 235.1, 242.1, 270.1, 271.1 |
| Midazolam | 13.623 | 310.2, 312.1, 325.1 |

**Table 3**
ANALYTICAL PARAMETERS OF COMPOUNDS IDENTIFIED IN ANALYZED SPECIMENS

**, ion with the highest intensity on MS spectrum is underlined.*

| Patient | Sex | Age, years | Dose, mg | $C_{1\,h}$ µg/L[a] | | $C_{3\,h}$ µg/L[b] | | $C_{5\,h}$ µg/L[c] | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | DZ[d] | ND[e] | DZ | ND | DZ | ND |
| 1 | F | 55 | 20 | 360 | NC[f] | 180 | 562 | 70 | 331 |
| 2 | F | 40 | | 440 | NC | 266 | 430 | 85 | 366 |
| 3 | F | 52 | | 320 | NC | 196 | 621 | 650 | 477 |
| 4 | M | 66 | | 600 | NC | 440 | 301 | 120 | 172 |
| 5 | M | 58 | | 440 | NC | 311 | 702 | 103 | 141 |
| 6 | M | 23 | | 1200 | NC | 650 | 661 | 150 | 299 |
| 7 | M | 58 | | 840 | NC | 445 | 344 | 160 | 165 |
| 8 | M | 24 | | 955 | NC | 330 | 860 | 165 | 440 |
| 9 | M | 29 | | 1400 | NC | 545 | 805 | 206 | 550 |
| 10 | M | 53 | | 850 | NC | 421 | 350 | 100 | 276 |

**Table 4**
PRELIMINARY RESULTS IN PLASMA SAMPLES FOLLOWING A SINGLE ORAL DOSE OF DIAZEPAM.

*[a] Concentration at t = 1 h; [b] Concentration at t = 3 h; [c] Concentration at t = 5 h; [d] DZ, diazepam; [e] ND, desmethyldiazepam; [f] NC, not calculable (too few points to determine)*

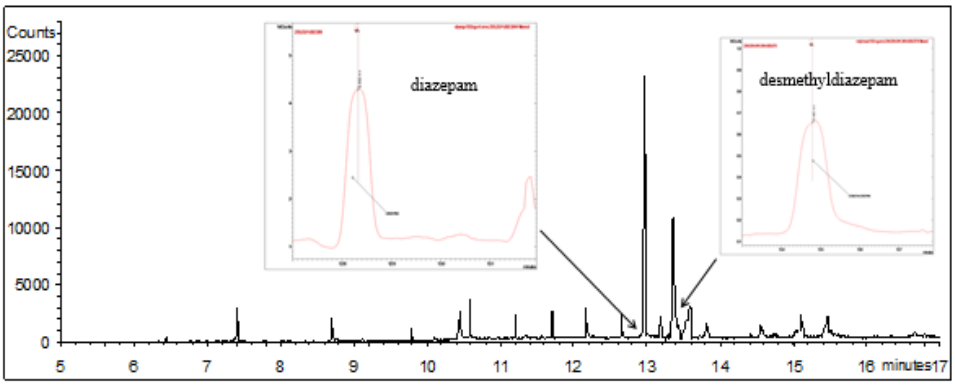


Fig. 1. Chromatogram of total ion current (TIC) of extract of patient plasma. Single ion storage. Chromatogram of diazepam and desmethyldiazepam.

*Samples*

Blood samples were collected without difficulty at specific intervals prior to analytical phase. 10 patients (3 women and 7 men), oral administered 20 mg single dose diazepam were tested. Results are presented in table 4.

Chromatogram is presented in figure 1.

## Conclusions

The method we proposed in this report is specific, reproducible and accurate for the identification and quantification of diazepam and its major metabolite, nordiazepam in human plasma samples. In order to eliminate impurities and increase sensitivity we included a double liquid - liquid extraction step in bio-sample preparation step.

The method described herein was applied to simultaneously identify and quantify plasma concentration of diazepam and nordiazepam in psychiatric patients after oral administration of a therapeutic dose (20 mg). The simplicity of bio-sample preparation offers a guaranteed recovery and is no time consuming, giving a plus due to the lack of time in emergency rooms and clinics.

The usefulness of monitoring diazepam and desmethyldiazepam concentration in plasma samples, allows the optimization of drug management from psychologically perspective, in addition to method simplicity, which makes it suitable for clinics and hospital applicability.

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