Correlation between Diazepam in Plasma and Dose in Patients in Long-Term Treatment

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SUMMARY. Diazepam, a benzodiazepine drug, has many therapeutic uses but little is known about the diazepam dose/plasma level ratio when the drug is administered for a long period of time. In the present study we determined plasma diazepam concentration in 26 patients receiving 5 and 10 mg/day of the drug. A gas chromatography/electron-capture detector method was validated for diazepam quantification in plasma using an OV 17-3% in Chromosorb W 80/100 mesh column. The use of 70:30 n-hexane: dichloromethane (v/v) as extraction solvent yielded good results. The following data were obtained: linearity from 10 to 1000 ng mL⁻¹, detection and quantification limits of 5 and 10 ng.mL⁻¹, respectively; intra and interassay average precision of 4.6 and 7.9%, respectively, mean recovery of 80.6 %. The drug remained stable in the plasma sample for at least 30 days when stored at -20 °C. The relation between dose and plasma concentration did not increase in linearity when the dose was increased.

INTRODUCTION

Diazepam, considered to be a prototype of the benzodiazepine class, has been extensively studied in terms of neuropsychopharmacological profile, showing anxiolytic, anticonvulsant, sedative-hypnotic, muscle relaxant and amnesic properties. It is also routinely prescribed as the standard first-line treatment for acute convulsions and prolonged status epilepticus 1.

Some studies have demonstrated that the therapeutic effect of benzodiazepines is better correlated with plasma levels than with administered dose 2,3. Thus, pharmacotherapy may induce adverse reactions due to high plasma concentrations, such as the absence of an adequate effect due to sub therapeutic blood concentrations caused by the use of a non-ideal dose. To increase the efficacy of a drug and to reduce its adverse effects, different dosage regimens must be prescribed to different individuals, who present variations in absorption, distribution, biotransformation, excretion and response when treated with the same medication 4,5.

Fraser 6 called attention for the importance of monitoring closely for the clinical use of benzodiazepines because of the great awareness of their adverse effects after long-term use and as well as because of the potential for misuse and abuse. For that reason identification of diazepam in human biological fluids is very important for forensic and clinical toxicology as well as pharmacokinetics studies.

Several analytical techniques for quantifying diazepam in biological fluids have been reported, including gas chromatography (GC) with nitrogen-sensitive 7,8 or electron-capture detection (ECD) 9 and high performance liquid chromatographic (HPLC) methods 10-15. Most of the methods are limited by laborious extraction procedures 13 and long retention times 13,15. In plasma samples treatment, liquid-liquid extraction is still the most used technique, followed by solid-phase extraction.

In view of the importance of the dose x plasma concentration and plasma concentration x therapeutic effect relationships for the therapeutic monitoring of patients submitted to prolonged treatment with diazepam, the objective of monitoring closely for the clinical use of benzodiazepines because of the great awareness of their adverse effects after long-term use and as well as because of the potential for misuse and abuse. For that reason identification of diazepam in human biological fluids is very important for forensic and clinical toxicology as well as pharmacokinetics studies.
of the present study was to investigate the correlation between dose x plasma concentration of diazepam in volunteers submitted to prolonged treatment with the drug, after validating a GC/ECD method for diazepam analysis in plasma samples.

MATERIALS AND METHODS

Chemicals and reagents

Diazepam and medazepam were obtained from Sigma®. Standard stock solutions were prepared in ethanol, at 1 mg ml⁻¹ for diazepam and at 2 mg.ml⁻¹ for medazepam (internal standard). Working solutions were prepared by appropriate serial dilution of the stock solutions with ethanol. Acetone, ethanol, dichloromethane and sodium hydrogenophosphate, from Merck®, were of analytical grade, and n-hexane nanograde from Mallinckrodt®.

Chromatographic conditions

Chromatography was performed in a GC-Ciola e Gregori® apparatus equipped with an electron capture detector, ⁶³Ni source (GC/ECD). A 1.2 m x 4.5 mm i.d. glass column OV 17-3% on 80-100 mesh Chromosorb W/HP was used under isotherm condition. After optimization, the chromatographic conditions established were: column temperature, 260 °C; injector temperature, 275 °C; detector temperature, 310 °C. Nitrogen was used as the mobile phase at a flow rate of 50 ml.min⁻¹, and 2 µl of the sample was injected.

Extraction procedure

Plasma sample (1 mL) was added with 2 ml sodium hydrogenophosphate solution (pH 9.0) and 200 µl of the internal standard and extracted with 5 ml n-hexane: dichloromethane, 70:30 v/v in 15-ml PTFE-lined screw capped glass centrifuge tubes. The mixture was vortex-mixed for 3 min and then centrifuged at 1500 g for 5 min. The organic phase was separated and evaporated to dryness in a water bath at 60 °C under N₂ flow. The residue was reconstituted with 200 µl n-hexane: acetone, 80:20 v/v, and 2 µl was injected into the GC apparatus.

Validation

For validation of the analytical method, samples (n = 5 for each level) spiked at concentration of 1, 10, 50, 100, 200, 400, 600, 800 and 1000 ng.ml⁻¹ to blank plasma were prepared. The linearity of the analytical method was evaluated with plasma samples spiked with diazepam (n = 5 for each concentration) over the range of 10- 1000 ng.ml⁻¹ (8 points). Calibration curves were fitted by linear regression analysis using the ratios area (y) of diazepam to the internal standard versus the concentrations (x) of the nominal standards and the relationships were determined by linear least-squares regression analysis. The limit of detection (LOD) for diazepam was estimated as a signal-to-noise ratio of 3:1, whereas the limit of quantification (LOQ) using a signal-to-noise ratio of 10:1. Precision was determined using plasma samples spiked with diazepam at three concentrations (50, 400 and 800 ng.ml⁻¹). The precision was calculated as the coefficients of variation (CVs) within a single run, intra-day precision (five replicates per concentration) and inter-days precision (six consecutive days, duplicate per day). Relative recovery was determined submitting the samples spiked with 100, 400 and 700 ng.ml⁻¹ to liquid-liquid extraction, and comparing the peak areas obtained with those resulting from the direct addition of the standards to the blank plasma extract.

Samples from human volunteers

The study was performed in 26 patients treated with diazepam for at least 30 days. These volunteers were divided into 2 groups according to the daily dose of diazepam ingested, i.e., 5 mg (n = 12) and 10 mg (n = 14). Both groups included adult patients, men and women, smokers and non-smokers, aged 23 to 70 years. Children, adolescents, pregnant women and individuals ingesting alcoholic drinks were excluded. The study was approved by the Research Ethics Committee of the Institution and the subjects signed an informed consent form to participate. Samples from volunteers using no drug (plasma blank) and spiked with the analyte and internal standard were used to optimize and validation of the method.

Plasma samples were collected using a heparinized vacuum collection system, and stored at -20 °C for a maximum of 15 days before analysis. Samples were collected immediately before the next drug dose (minimum concentration). Biochemical (blood glycosis, total cholesterol, transaminases – SGPT and SGOT - urea, triglycerides, total proteins, creatinine in serum and urine and type I urine) and hematological (hemogram) tests were carried out, showing that all patients were in good health, as informed by them when asked about.
RESULTS AND DISCUSSION

Gas chromatography with an electron capture detector permitted the detection of low diazepam levels (ng ml⁻¹) due to the presence of an electronegative group in position 7 and one carboxyl group in position 2 in the diazepam molecule. Figure 1 shows that diazepam is well separated from the internal standard and from the biological background under the described optimized chromatographic conditions. The relative retention time diazepam/medazepam was 1.4 min. The peaks were of good shape and completely resolved from one another. No interference with constituents from plasma was observed.

![Figure 1. Plasma samples chromatograms. A = blank plasma; B = blank plasma spiked with 2 ng. mL⁻¹ diazepam; C = plasma from patient treated with diazepam (5 mg.day⁻¹); (1) medazepam (internal standard); (2) diazepam.](image)

The method showed linearity in the range of 10 to 1000 ng.ml⁻¹. The mean regression linear obtained was y = 0.00213x + 0.1278 (r = 0.9981) and standard errors of the slope and of the intercept were 0.00628 and 0.01509, respectively. Analysis of variance of the correlation coefficients indicated non-significant differences (p > 0.05) confirming the linearity of the standard curves in the range studied.

This range permits the analysis of diazepam in plasma aiming clinical or pharmacokinetics application. The detection and quantification limits obtained were, respectively, 5 and 10 ng ml⁻¹, similar to those cited by other authors. A HPLC method used by Abu-Qare & Abou-Donia presented detection and quantification limits of 50 and 100 ng mL⁻¹, although Rouini et al. reported quantification limits of 2 ng.ml⁻¹ for several benzodiazepines in plasma, also analyzed by HPLC/UV, after solid-phase microextraction.

The intra and inter-assay coefficients of variation (%) for samples containing 50, 400 and 800 ng diazepam ml⁻¹ plasma were 7.2, 3.7 and 2.9 (intra) and 9.2, 8.0 and 6.1 (inter), respectively. Mean recovery for concentrations of 100, 500 and 700 ng diazepam.ml⁻¹ plasma was 80.6 ± 0.45. The analyte proved to be stable in samples stored frozen at -20 °C for the 30 day study period (CV < 5%).

The assay method was successfully used to quantitatively measure the concentrations of diazepam in plasma obtained from patients who had been taking the drug for a prolonged period of time as an anxiolytic. The criteria of blood collection for therapeutic monitoring are based on the rates of absorption, distribution and elimination of the drug, the time of drug administration, and differentiation between minimum and maximum concentrations.

Samples were collected immediately before administration of the drug, i.e., 24 h after the last dose (minimum dose), a procedure that seems to be more appropriate for monitoring. In the present study, only patients taking the drug for at least one month were investigated. According to Kaplan et al. the time needed for plasma diazepam to reach a steady-state level is 2 to 3 weeks. No reports were found in the literature about the most indicated time for blood sample collection for diazepam analysis for monitoring purposes. According to Sznitowska et al., the mean $T_{max}$ is 20 min, in animals that received 2 mg/kg of a diazepam solution.

Tables 1 and 2 respectively present the plasma concentrations of diazepam for the 12 pa-
tients treated with a daily dose of 5 mg and for the 14 patients treated with 10 mg day–1.

A mean ± SD plasma diazepam concentration of 173.5 ± 52.0 ng ml–1 (CV = 29.9%) was determined in patients ingesting 5 mg day–1 and 249.5 ng ml–1 ± 44.8 (CV = 17.9) in those taking 10 mg/day, a value close to that reported by Kaplan et al. 27, 226.8 ng ml–1, in samples collected 24 hours after drug administration. No studies were found in the literature correlating the diazepam dose administered and the plasma concentration obtained in patients receiving the drug for long-term.

The correlation between dose ingested and plasma levels, taking in account all volunteers of the study, give a Pearson coefficient (r) of 0.62. To verify the distribution of plasma diazepam, Shapiro-Wilk test was used and show that plasma concentration does not increase linearly with increased dose. Greenblatt et al. 29 demonstrate the non-Gaussian distribution of plasma diazepam when the minimum plasma concentration is considered due mainly to the interindividual variation of the pharmacokinetic parameters.

Some authors reported that age and gender may influence the diazepam plasma levels. In this study, patients receiving 5 mg day–1 diazepam show significant positive correlation between plasma levels and age (r = 0.8036, p ≤ 0.002) but that was not true for those receiving 10 mg day–1. Maybe differences in number, age interval and gender in the volunteers of the two groups can explain the data.

<table>
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<tr>
<th>Patient</th>
<th>Age (years old)</th>
<th>Gender</th>
<th>Lenght of treatment (month)</th>
<th>Plasma diazepam. (ng ml–1)</th>
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Mean ± SD 173.83 ± 52.0 % CV 29.9

<table>
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<tr>
<th>Patient</th>
<th>Age (years old)</th>
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<th>Lenght of treatment (months)</th>
<th>Plasma diazepam. (ng. mL –1 )</th>
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Mean ± SD 249.54 ± 44.8 % CV 17.9

Table 1. Plasma levels, lenght of drug treatment, age and gender in patients treated daily with 5 mg diazepam.

Table 2. Plasma levels, lenght of treatment, age, gender and drug associated in patients treated daily with 10 mg diazepam.
In this study, no significant correlation was detected between gender and plasma diazepam levels. Giles et al. reported no significant difference in the clearance of diazepam between males and females. McLeod et al. reported gender differences in the extent of binding of diazepam to plasma protein, assessed in a series of patients with renal insufficiency, and concluded that the intensity and duration of diazepam’s clinical action in patients with renal insufficiency might differ between males and females.

Large interindividual variability in plasma diazepam levels was found in this study, higher in the group taking 5 mg day⁻¹. Greenblatt et al. reported coefficients of variation as higher as 21.8% in diazepam levels. These differences may be explained by the differences in pharmacokinetics of the drug among human beings.

The pharmacokinetic profiles in human in a clinical setting, patients of various age groups may possess a variety of pathological, genetic, and environmental characteristics that may influence the metabolism of drugs in general and then, their plasma levels. Age, disease state and drug interaction may also affect the physiological disposition profile of a drug – such changes may be reflected in specific changes in the pharmacokinetic parameters.

CONCLUSION

The analytical method described for diazepam analysis in plasma is fast, simple and showed conditions that allow its use in routine clinical analysis of the drug. In patients taking the drug for long-term, when the daily dose of diazepam was doubled, the same did not occur for the plasma concentration, showing the nonlinear correlation between dose versus plasma concentration.

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REFERENCES