*Journal of Chromatography*, 577 (1992) 190–194
*Biomedical Applications*
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6306

# Short Communication

# Sensitive assay for triazolam in plasma following low oral doses

Timi Edeki, Deborah W. Robin, Chandra Prakash, Ian A. Blair and Alastair J. J. Wood*

*Division of Clinical Pharmacology, School of Medicine, Vanderbilt University, Nashville, TN 37232 (USA)*

(First received July 12th, 1991; revised manuscript received February 10th, 1992)

ABSTRACT

At low doses of triazolam currently recommended increased assay sensitivity is required for measurement of low plasma concentrations. A highly sensitive capillary gas chromatographic analytical method with a limit of detection of 0.02 ng/ml was developed and used to describe the pharmacokinetics of triazolam following the oral intake of 0.125, 0.250 and 0.375 mg. Six male subjects were studied with blood sampling at the following times: 0, 15, 30 and 45 min and 1, 1.5, 2.0, 2.5, 3, 4, 5, 6 and 8 h. The mean pharmacokinetic parameters for the three doses, respectively, were as follows: half-life, 2.7 ± 0.4, 3.2 ± 0.5 and 3.2 ± 0.6 h; apparent oral clearance, 302.3 ± 59.0, 260.2 ± 67.9 and 328.6 ± 77.8 ml/min; apparent volume of distribution, 64.3 ± 9.6, 62.0 ± 12.6 and 73.3 ± 7.7 l; time to maximum concentration, 0.7 ± 0.2, 0.6 ± 0.1 and 0.8 ± 0.3 h; maximum concentration, 2.2 ± 0.3, 4.3 ± 0.6 and 5.0 ± 0.5 ng/ml; and the area under the concentration–time curve (AUC) up to 8 h, 6.8 ± 1.2, 16.8 ± 2.9 and 19.6 ± 3.5 ng/ml h; and AUC extrapolated to infinity, 8.5 ± 1.7, 21.4 ± 4.4 and 26.3 ± 7.2 ng/ml h. There were no significant differences in the half-life, clearance, volume of distribution and time to maximum concentration among the three doses. The AUC was significantly different on the three occasions and was linearly correlated with dose: $r = 0.64$ ($p < 0.005$).

## INTRODUCTION

Triazolam is a triazolobenzodiazepine, one of the newer benzodiazepines used as a short-acting hypnotic in various conditions where a rapid onset and short duration of action is required [1,2]. Since its introduction it has been given over a relatively broad dosage range in several forms of therapy. The pharmacokinetics of triazolam have been determined with different doses in several groups of subjects [3–5]. Recent evidence suggests that toxicity occurs with higher doses of triazolam [6] and this has resulted in reduction of the recommended doses. In order to define the pharmacokinetics of triazolam at lower doses now recommended [6] we have developed a sensitive gas chromatographic (GC) assay which is an improvement over currently used methods [3,7–9].

## EXPERIMENTAL

### *Subjects*

Six healthy male volunteers, with a mean age of 23 (19–29) years and weight 76.3 (58–86) kg, after approval by the Vanderbilt Committee for the Protection of Human Subjects, gave written informed consent to participate in the study. No

0378-4347/92/$05.00 

subject had any clinically significant abnormalities on routine history, physical examination and biochemical testing. None had taken any medications for at least a week prior to the study and they did not take any medication or caffeine-containing beverages during the study.

*Collection of samples*

On four occasions separated by at least 48 h the subjects were given 0.125, 0.250 and 0.375 mg of triazolam or placebo, in a double-blind placebo-controlled randomized fashion. Each study day commenced between 8:00 and 9:00 a.m. A 10-ml control blood sample was taken, and the drug was administered orally with approximately 50–100 ml of water. Blood samples (10 ml) were collected in heparinized tubes before and at the following times after drug administration: 15, 30, 45 min and 1, 1.5, 2, 2.5, 3, 4, 5, 6 and 8 h. The plasma was separated and stored at −20°C until analysis.

*Materials*

Triazolam was a gift from Upjohn (Kalamazoo, MI, USA), flunitrazepam and borate phosphate were obtained from Sigma (St. Louis, MO, USA) and Fisher Scientific (Fair Lawn, NJ, USA), respectively. Dichloromethane, *n*-hexane and ethyl acetate were obtained form Baxter (McGraw Park, IL, USA) and were of high-purity grade. The carbonate buffer pH 10.3 was made from 1 *M* $Na_2CO_3$ and 1 *M* $NaHCO_3$. Stock solutions of triazolam and flunitrazepam were made up in ethyl acetate at concentrations of 1 mg/ml.

*Extraction procedure*

To 1 ml of plasma in round-bottom glass tubes were added 20 μl (4 ng) of flunitrazepam in ethyl acetate as internal standard and 200 μl of 1 *M* carbonate buffer pH 10.3. The mixture was vortex-mixed for a few seconds and 5 ml of hexane–dichloromethane (50:50) were added. This was then mixed on a shaker for 10 min and centrifuged for 10 min at 2000 *g*. A 4-ml portion of the organic phase was transferred to tapered glass tubes and evaporated to dryness under dry nitrogen at 37°C. The samples were reconstituted with 20 μl of ethyl acetate, vortex-mixed, and 1 μl was injected on column.

*Equipment*

A Shimadzu gas chromatograph (GC 14A; Columbia, MD, USA), with an electron-capture detector, was used for the analysis. GC was carried out on a fused-silica capillary column (SPB, Supelco, Bellefonte, PA, USA), 15 m × 0.25 mm I.D., 0.25 μm film thickness. The data were processed on a Shimadzu computer (CR 4A Chromatopac).

*Chromatographic conditions*

The samples were analyzed in the split mode of injection. Solenoid valves were programmed to close 1 min after injection of any sample. The carrier gas was helium with an auxiliary gas of 5% argon in methane at flow-rates of 0.6 and 30 ml/min, respectively. Both gases were of highest purity. The split and purge flow-rates were 40 and 11.5 ml/min, respectively. Thus the split ratio was approximately 1:68. The temperatures of the injector port and detector were 275 and 345°C, respectively. The approximate initial temperature of the column was 200°C, it was held isothermally for 1 min, then increased in a two-step programming procedure. The temperature was increased to 235°C at 35°C/min, then to 265°C at 5°C/min. It was held at 265°C for 1 min and then cooled to 200°C ready for the next injection.

*Standard curve*

Plasma standards were prepared by adding varying (known) amounts of triazolam in the range 0–10 ng/ml to plasma together with a fixed amount of flunitrazepam internal standard. The standards were processed through the extraction procedure and analyzed by GC–electron-capture detection. A standard curve was prepared by plotting the peak-height ratios of triazolam to flunitrazepam against the concentration of triazolam added to the plasma. Unknown samples were analyzed under identical conditions and plasma concentrations were calculated by interpolation from the standard curve.

*Pharmacokinetic analysis*

Pharmacokinetic parameters were calculated by initial curve striping using the computer program STRIPE [10], to obtain the initial estimates. Data were then analyzed by SIMP [11], a non-linear curve fitting program. The plasma profiles of the drug were fitted to a sum of exponentials with a first-order input function, using an iterative weighted curve fitting program which minimized the sum of squares. A reciprocal weighting factor was used throughout. The data were fitted to one- or two-compartment models and Akaike Index Criteria and sum of squares were used to determine the best fit, which in all instances was a one-compartment model.

The terminal elimination half-life ($t_{1/2}$) was obtained using eqn. 1 and the area under the concentration–time curve (AUC) calculated by linear trapezoidal method.

$$t_{1/2} = 0.693/K \quad (1)$$

The maximum observed plasma levels and the time taken were obtained directly from the analytical data. The apparent volume of distribution ($V_d$) and apparent oral clearance ($Cl$) were calculated from the formulae:

$$V_d\,(\mathrm{l}) = \frac{\mathrm{Dose}}{\mathrm{AUC} \cdot K} \quad (2)$$

$$Cl\,(\mathrm{ml/min}) = \frac{\mathrm{Dose}}{\mathrm{AUC}} \quad (3)$$

where $K$ is the elimination rate constant. The AUC extrapolated to infinity was obtained by adding the concentration of the last time point divided by $K$ to AUC. An absorption of 100% was assumed.

*Statistical analysis*

The differences in the pharmacokinetic parameters on the three occasions were compared using the repeated-measures analysis of variance. The minimal level of significance accepted was $P < 0.05$. The correlation between the dose and AUC was carried out by linear regression analysis.

RESULTS

The standard curve was linear in the range 0–10 ng/ml ($r = 0.999$). The retention times for flunitrazepam (internal standard) and triazolam were 4.6 and 8.7 min, respectively (Fig. 1). The between-day coefficient of variation for the concentrations 1 and 4 ng/ml were 8.2 and 6.5%, respectively. The accuracy of the assay was 2.2%.

Triazolam was rapidly absorbed with peak concentrations attained within the first hour (Table I; Fig. 2). Higher plasma concentrations were observed with increasing dose and some of the subjects had secondary peaks. The pharmacokinetic parameters of triazolam for the three doses

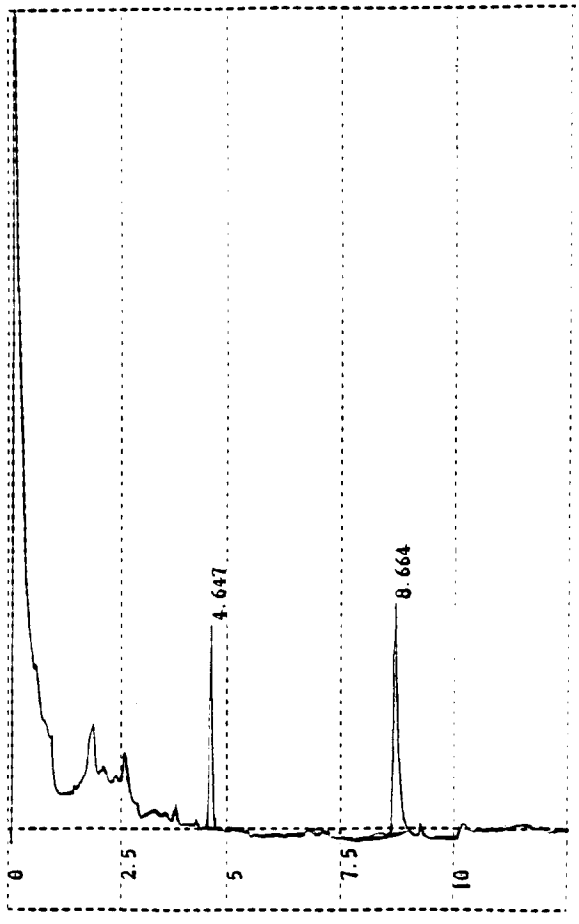


Fig. 1. Representative chromatogram in plasma of flunitrazepam (2 ng/ml) and triazolam (4 ng/ml) showing their respective retention times of 4.6 and 8.6 min.

TABLE I

MEAN (± STANDARD ERROR OF THE MEAN) PHARMACOKINETIC PARAMETERS OF SIX MALE SUBJECTS FOLLOWING THE ORAL INTAKE OF 0.125, 0.250 AND 0.375 mg OF TRIAZOLAM

Terminal elimination half-lives ($t_{1/2}$), clearance ($Cl$), apparent volume of distribution ($V_d$), maximal concentration ($C_{max}$), time to maximal concentration ($T_{max}$) and area under the concentration time curve to last time point ($AUC_{0-8h}$) and extrapolated to infinity ($AUC_{0-inf}$) were each compared by analysis of variance among the three doses.

| Parameter | 0.125 mg | 0.250 mg | 0.375 mg | Significance[a] |
|---|---|---|---|---|
| $t_{1/2}$ (h) | 2.71 ± 0.35 | 3.15 ± 0.5 | 3.15 ± 0.61 | N.S. |
| $Cl$ (ml/min) | 302.33 ± 58.98 | 260.15 ± 67.92 | 328.60 ± 77.78 | N.S. |
| $V_d$ (l) | 64.32 ± 9.59 | 61.98 ± 12.57 | 73.27 ± 7.66 | N.S. |
| $T_{max}$ (h) | 0.71 ± 0.16 | 0.63 ± 0.06 | 0.79 ± 0.25 | N.S. |
| $C_{max}$ (ng/ml) | 2.22 ± 0.27 | 4.31 ± 0.59 | 5.03 ± 0.45 | $P < 0.001$ |
| $AUC_{0-8h}$ (ng/ml h) | 6.84 ± 1.19 | 16.75 ± 2.85 | 19.59 ± 3.53 | $P < 0.001$ |
| $AUC_{0-inf}$ (ng/ml h) | 8.46 ± 1.68 | 21.37 ± 4.40 | 26.25 ± 7.23 | $P < 0.01$ |

[a] Significance level at $P$ less than or equal to 0.05, for each pharmacokinetic parameter with comparison among the three doses.

are presented in Table I. There were no significant differences in the $t_{1/2}$, $Cl$, $V_d$ and time to maximum concentration ($T_{max}$) for the three doses. The maximum concentration ($C_{max}$), $AUC_{0-8h}$ and $AUC_{inf}$ increased significantly with the dose administered (Table I and Fig. 2). The correlation of the $AUC_{0-8h}$ with the dose ($r = 0.64$; Fig. 3) was statistically significant ($P < 0.005$).

## DISCUSSION

The method described here is convenient for the analysis of a large number of samples, because of the relatively short run time of each analysis sample. The sensitivity and reduced plasma volume required for analysis is an improvement over the currently used GC methods of Jochemsen and Breimer [7], Coassolo *et al.* [8], Baktir *et al.* [3] and Baktir and Bircher [9]. The sensitivity allows for the adequate monitoring of plasma concentrations following low oral doses of triazolam or where prolonged sampling is involved. The volume of plasma (1 ml) used for the extraction is convenient and leaves enough for repeat analyses or other measurements. This extraction procedure is simple and does not involve a back-extraction step as used by Coassolo *et al.*

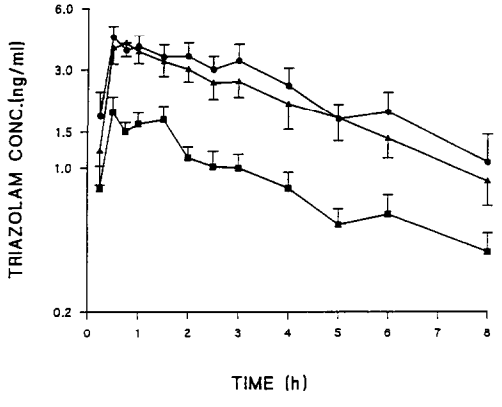


Fig. 2. Mean values (with standard errors) of triazolam concentration in six male subjects, following oral intake of 0.125 (■), 0.250 (▲) and 0.375 (●) mg.

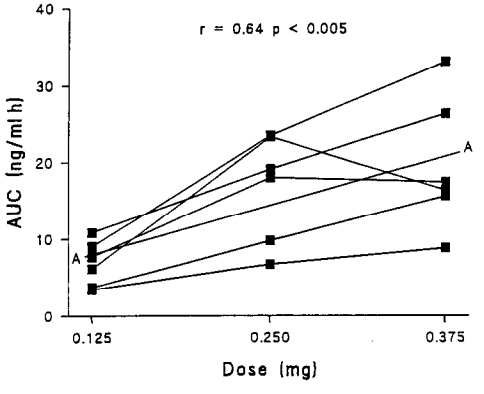


Fig. 3. Relationship of AUC to dose of triazolam in six male subjects with the mean regression line (A) superimposed.

[8]. The temperature programming adopted was a modification of that originally described by Grob and Grob [12]. Instead of the starting temperature of the column (oven) lower than that of the boiling point of the injecting solvent, the initial temperature was higher than that of the solvent's boiling point. This minimized the run time of each analysis but caused a slight loss in sensitivity. However, sensitivity is more than adequate for the concentrations seen following these doses.

A second peak of triazolam, observed in some of the subjects, is reflected in the plots of the mean concentration and time (Fig. 2), and was striking in some individuals. This was also observed by Bakti *et al.* [13], in which triazolam had occasional secondary peaks after an initial fall in plasma concentration. Although not commented on by the authors, an examination of the concentration–time plots in another study [14] also showed secondary peaks. The explanation for these secondary peaks has not been defined, but may be due to entero-hepatic circulation, as triazolam has been demonstrated in the bile of rats [15]. The secondary peak in plasma triazolam concentration may have implications in maintaining the hypnotic effect of the drug in some individuals.

ACKNOWLEDGEMENTS

This study was supported by NIH Grants RR00095, AG01395 and GM31304.

REFERENCES

1 G. E. Pakes, R. N. Brogden, R. C. Heel, T. M. Speight and G. S. Avery, *Drugs*, 22 (1981) 81.
2 P. D. Kroboth and R. P. Juhl, *Drug Intell. Clin. Pharm.*, 17 (1983) 495.
3 G. Baktir, H. U. Fisch, P. Huguenin and J. Bircher, *Clin. Pharmacol. Ther.*, 34 (1983) 195.
4 H. Friedman, D. J. Greenblatt, E. S. Burstein, J. S. Harmatz and R. I. Shader, *Br. J. Clin. Pharmacol.*, 22 (1986) 639.
5 J. M. Scavone, D. J. Greenblatt, H. Friedman and R. I. Shader, *Clin. Res. CNS Drugs*, 26 (1986) 208.
6 J. S. Schou, *Pharmacol. Toxicol.*, 64 (1989) 6.
7 R. Jochemsen and D. D. Breimer, *J. Chromatogr.*, 223 (1981) 438.
8 P. Coassolo, C. Aubert, and J. P. Cano, *J. Chromatogr.*, 274 (1983) 161.
9 G. Baktir, J. Bircher, H. U. Fisch and G. Karlaganis, *J. Chromatogr.*, 339 (1985) 192.
10 A. Johnson and R. C. Woollard, *J. Pharmacol. Methods*, 9 (1983) 193.
11 A. Johnston, *J. Pharmacol. Methods*, 14 (1985) 323.
12 K. Grob and K. Grob, Jr., *J. Chromatogr.*, 94 (1974) 53.
13 G. Bakti, H. U. Fisch, G. Karlaganis, C. Minder and J. Bircher, *Hepatology*, 7 (1987) 629.
14 P. D. Kroboth, R. B. Smith, M. R. Silver, R. Rault, M. I. Sorkin, J. B. Puschett and R. P. Juhl, *Br. J. Clin. Pharmacol.*, 19 (1985) 839.
15 H. Kitagawa, Y. Esumi, S. Kurosawa, S. Sekine and T. Yukoshima, *Xenobiotica*, 9 (1979) 415.