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## Note

# Capillary gas—liquid chromatographic determination of the benzodiazepine triazolam in plasma using a retention gap

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Triazolam [8-chloro-6-(*o*-chlorophenyl)-1-methyl-4H-*s*-triazolo[4,3-α][1,4]-benzodiazepine; Halcion®] is a triazolobenzodiazepine with particular interest for clinical pharmacological research because of its very short half-life of about 2.5 h in healthy volunteers [1, 2]. Due to its high potency, the recommended hypnotic doses are 0.25 or 0.125 mg [3], and concentrations of triazolam in plasma are low, for example between 0.5 and 5 ng/ml after a single dose of 0.25 mg.

Jochemsen and Breimer [4] described a gas—liquid chromatographic (GLC) method for the assay of triazolam using a solid injection system, a support-coated open-tubular (SCOT) column and electron-capture detection (ECD). Since their solid injection system was not available commercially, an on-column injector together with a glass capillary column and ECD was used in a recent

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investigation [2]. However, endogenous compounds in the plasma extract were deposited in the capillary column, producing absorption and/or decomposition of triazolam and necessitating frequent changes to new capillary columns. Therefore, an uncoated, persilanized fused-silica retention gap was used for this study, as recommended by Grob [5], in order to improve the lifetime of capillary columns during analysis of triazolam in human plasma.

## EXPERIMENTAL

### *Chemicals*

All reagents were of analytical grade. *n*-Pentane, dichloromethane, ethyl acetate, and dichlorodimethylsilane were purchased from E. Merck (Darmstadt, F.R.G.). Unlabelled triazolam, [$^{14}$C]triazolam (specific activity 108 $\mu$Ci/mg) and tablets containing 0.25 mg of triazolam (Halcion) were kindly supplied by the Upjohn Company (Kalamazoo, MI, U.S.A.). Unlabelled clonazepam and [$^{14}$C]clonazepam (specific activity 31 $\mu$Ci/mg) were gifts from F. Hoffmann-La Roche (Basle, Switzerland).

All glassware was silanized with a solution of 10% dichlorodimethylsilane in toluene for 1 h. The reagent was removed by washing with toluene and methanol. Thereafter the glassware was oven-dried at 100°C for at least 2 h.

### *Extraction procedure*

The assay procedure of Jochemsen and Breimer [4] was applied with slight modifications. Clonazepam (10 ng in 100 $\mu$l of ethanol) was added to 2 ml of plasma as internal standard and the sample was alkalinized with 0.5 ml of 0.2 *M* sodium borate buffer (pH 9.4). It was then extracted with 5.5 ml of *n*-pentane—dichloromethane (4:3) for 20 sec on a whirl mixer, and centrifuged at 3500 rpm (2000 *g*) (Labofuge I; Heraeus Laborgeräte, Zurich, Switzerland) for 10 min. The upper organic layer was transferred to a conical tube and a few drops of toluene were added. After evaporation to dryness at 40°C under a gentle stream of nitrogen, the tubes with the extracts were kept under high vacuum for several hours. Thereafter they were dissolved in 20 $\mu$l of ethyl acetate and volumes of 0.2—0.3 $\mu$l were injected into the gas chromatograph.

### *Apparatus and chromatographic conditions*

A Carlo Erba gas chromatograph Model 4160 was used equipped with a $^{63}$Ni electron-capture detector (Model HT-25/251) and a Grob-type on-column injector (Model 416). A 15 m × 0.32 mm I.D. glass capillary column was persilanized with 1,3-divinyl-1,1,3,3-tetramethyldisilazane (Fluka, Buchs, Switzerland) and coated with OV-1701 (Silicone 1701; Supelco, Gland, Switzerland; 0.4% static coating; 0.32 $\mu$m film thickness). The stationary phase was immobilized with 1% dicumyl peroxide (Elfa-Oxychemie, Zurich, Switzerland) [6]. To avoid accumulation of condensed by-products in the initial part of the column, a 1.5 m × 0.32 mm persilanized uncoated fused-silica piece (Brechbühler, Schlieren, Switzerland) was attached to the column inlet with the aid of a butt connector (Supelco) [5]. A portion of about 20 cm of this retention gap was cut off after every ten injections. The column temperature was increased from 80°C to 260°C by 30°C/min. Hydrogen was used as the

carrier gas at a flow-rate of 4 ml/min (at 260°C, 100 kPa). The settings of the electron-capture detector were as follows: temperature 275°C; constant-current mode; pulse width 1 μsec; pulse amplitude 50 V; make-up gas argon—methane (95:5). The retention times of clonazepam (internal standard) and triazolam were 12 and 15 min, respectively.

*Calibration*

For calibration 0.5, 1.25, 2.5 and 5.0 ng/ml triazolam in ethanol were added to 2 ml of normal human plasma, followed by 10 ng of clonazepam as internal standard. Extraction and analysis were performed as described above. The ratios of the peak heights of triazolam and clonazepam were plotted against the plasma concentrations of triazolam standards.

*Determination of recovery*

The recoveries of [$^{14}$C]triazolam and [$^{14}$C]clonazepam were assessed six times by adding 10 ng (specific activity 108 μCi/mg) and 13 ng (specific activity 31 μCi/mg) of the labelled substances to 2 ml of normal human plasma, respectively. As described above, the spiked plasma samples were extracted with *n*-pentane—dichloromethane (4:3). An aliquot of 500 μl of the organic phase was counted after addition of 10 ml of scintillation cocktail containing toluene (800 ml), Triton X-100 (200 ml), 2,5-diphenyloxazole (5 g, PPO, Merck) and 1,4-bis(5-phenyloxazole-2-yl)benzene (100 mg, POPOP, Merck). A Packard Tri-Carb® 2660 liquid scintillation system (Packard Instrument International, Zurich, Switzerland) with the external channels ratio was used.

## RESULTS AND DISCUSSION

The recoveries of [$^{14}$C]clonazepam and [$^{14}$C]triazolam through the extraction procedure described above were 88 ± 6% (S.D.) and 96 ± 2%, respectively (Table I). The extraction with *n*-pentane—dichloromethane (4:3) yielded an optically clear extract, compared to the extraction with diethyl ether, which was also tested.

The attachment of a piece of an uncoated persilanized fused-silica column (retention gap [5]) to the inlet end of the coated glass capillary column proved to be very useful in preventing peak broadening and in maintaining optimal separation efficiency. Fig. 1 shows three chromatograms obtained after

TABLE I

RECOVERIES OF [$^{14}$C]TRIAZOLAM AND [$^{14}$C]CLONAZEPAM (INTERNAL STANDARD) EXTRACTED FROM SPIKED NORMAL HUMAN PLASMA

| Compound | Amount added to 2 ml of plasma | | Percentage recovered* |
|---|---|---|---|
| | ng | nCi | |
| [$^{14}$C]Triazolam | 19 | 2 | 96 ± 2 |
| [$^{14}$C]Clonazepam | 13 | 0.4 | 88 ± 6 |

*Mean ± S.D. of six determinations.

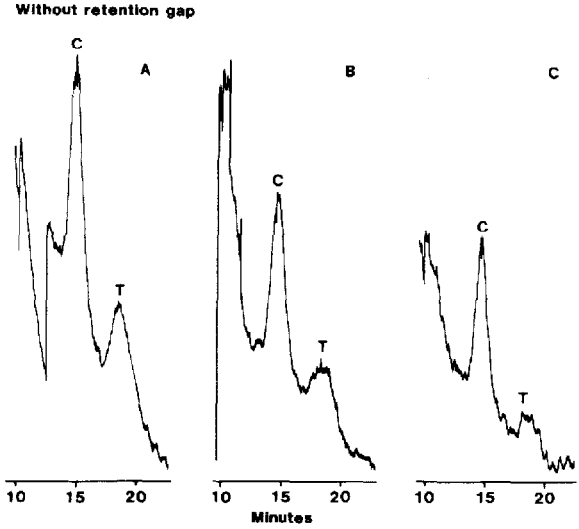


Fig. 1. Consecutive injections (A, B, C) of the same plasma extract spiked with 2.5 ng/ml triazolam (T) and 5 ng/ml clonazepam (C) without use of a retention gap. The ratios of the peak heights T/C are 0.37, 0.32 and 0.24 in chromatograms A, B and C, respectively.

consecutive on-column injections of 0.3-$\mu$l aliquots of the same plasma extract (spiked with 2.5 ng/ml triazolam) into the gas chromatograph without retention gap. Both the triazolam and the clonazepam peaks are broad and the peak heights are declining. The calculated peak height ratios are 0.37, 0.32 and 0.24 for the first, the second and the third injection, respectively. Consequently, a reduction in peak height ratios, presumably due to alteration of the column by retention of condensed by-products, led to an underestimation of triazolam concentrations in plasma with each successive injection. The retention gap solved this problem. Fig. 2 shows two chromatograms of another plasma sample spiked again with 2.5 ng/ml triazolam. Four additional injections of serum extracts were made between Fig. 2A and Fig. 2B. The latter chromatogram shows neither peak broadening, splitting, nor a reduction in peak height ratio. Presumably non-volatile compounds extracted from plasma are held back by the retention gap and in this way cannot contaminate the coated glass capillary column. By cutting a portion (20 cm) of the retention gap every ten injections, it was possible to maintain the appearance of peaks and the separation efficiency constant.

Calibration curves obtained with this method passed through the origin and were linear within the concentration range 0.5—5.0 ng/ml triazolam. Peak height ratios varied between 0.08 and 1.0. Intra-day reproducibility of triazolam determination as expressed by the coefficient of variation was 9.1% ($n$ = 5; 4.4 ± 0.4 ng/ml in spiked normal human plasma). Inter-day reproducibility was 10.0% ($n$ = 26; 1.8 ± 0.2 ng/ml in pooled normal human plasma from six healthy volunteers; blood samples were taken 2 h after ingestion of 0.25 mg of triazolam).

A representative plasma concentration—time curve obtained from a normal volunteer after ingestion of 0.25 mg of triazolam is shown in Fig. 3. The maximal plasma concentration ($c_{max}$) and the time it took to achieve it ($t_{max}$)

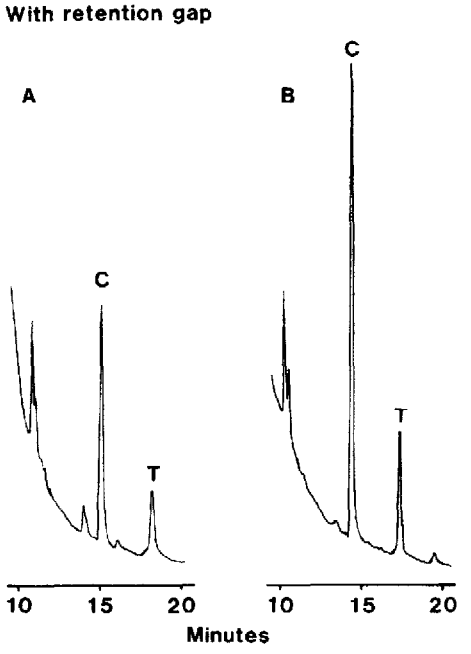


Fig. 2. Two chromatograms from a normal human plasma sample spiked with triazolam (T, 2.5 ng/ml) and clonazepam (C, 5 ng/ml), using the same extraction procedure and chromatographic conditions as in Fig. 1. In addition, a retention gap was included. Four injections of other plasma extracts were made between A and B. The peak height ratio T/C is 0.27 in both chromatograms.

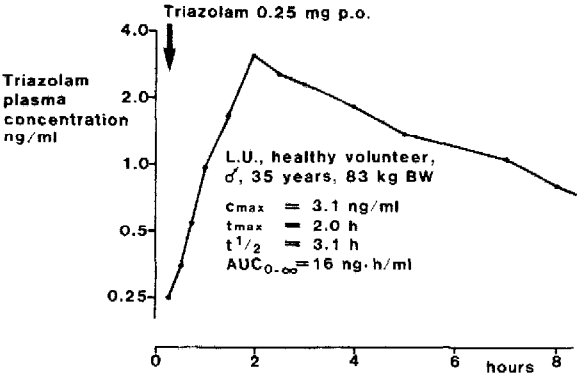


Fig. 3. Plasma concentration—time curve obtained from a young healthy volunteer after ingestion of 0.25 mg triazolam per os. The ordinate shows the total (bound and free) plasma concentration of triazolam. Each point represents one sample analysed on the same day. Since the first two samples yielded very distinct chromatograms, the values were interpolated on the standard curve although they are below the lowest standard.

were 3.1 ng/ml and 2.0 h, respectively. The half-life of triazolam obtained graphically from a semilogarithmic plot of the plasma concentration—time curve was 3.1 h and the area under the curve (AUC) extrapolated to infinity was 16 ng h $ml^{-1}$. These results are in good agreement with previous reports on the pharmacokinetic parameters of triazolam [1—4].

This methodology opens the door for clinical pharmacological studies of concentration—effect relationships with a benzodiazepine having a very short half-life. Consequently, investigations over a large range of concentrations are possible within a short period of time. In view of the sensitivity of the assay it is now possible to study subjects exposed to an ordinary hypnotic dose of 0.25 mg.

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