Journal of Chromatography, 268 (1983) 92–98
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

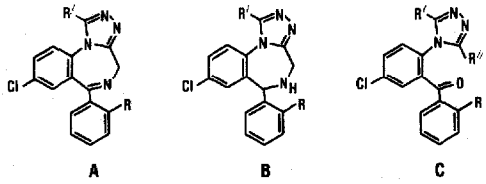
CHROM. 15,990

Note

Development of a liquid chromatographic method for the determination of triazolo-benzodiazepines

DON L. THEIS* and PHIL B. BOWMAN
Control Analytical Research and Development Laboratories, The Upjohn Company, Kalamazoo, MI 49001 (U.S.A.)
(Received May 13th, 1983)

Alprazolam (8-chloro-1-methyl-6-phenyl-4H-s-triazolo[4,3-*a*][1,4]benzodiazepine) and triazolam {8-chloro-6-(*o*-chlorophenyl)-1-methyl-4H-s-triazolo[4,3-*a*][1,4]benzodiazepine} are two new drugs exhibiting central nervous system (CNS) activity[1–3]. Xanax® brand of alprazolam (The Upjohn Company) has been approved by the U.S. Food and Drug Administration (FDA) as an antianxiety agent. Halcion® brand of triazolam (The Upjohn Company) is a short-duration hypnotic also recently approved by the U.S. FDA. Both are formulated as compressed tablets. Related compounds of alprazolam are estazolam (I) (desmethylalprazolam), 5-chloro-2-(3-methyl-4H-1,2,4-triazol-4-yl)benzophenone (II), 5-chloro-2-[3-(hydroxymethyl)-5-methyl-4H-1,2,4-triazol-4-yl]benzophenone (III) and dihydroalprazolam (IV). Triazolam related compounds are desmethyltriazolam (V) and dihydrotriazolam (VI). Structures of these compounds are shown in Fig. 1. Several liquid and gas chromatographic systems have been reported[4,5] for other 1,4-benzodiazepines. These methods, however, did not provide either adequate selectivity or suitable chroma-



	R	R′	R″
A — Alprazolam (6.7)	H	CH_3	—
A — Triazolam (4.4)	Cl	CH_3	—
A — Estazolam (2.6)	H	H	—
A — desmethyltriazolam (2.1)	Cl	H	—
B — dihydroalprazolam (8.1)	H	CH_3	—
B — dihydrotriazolam (6.7)	Cl	CH_3	—
C — Compound II (2.1)	H	CH_3	H
C — Compound III (4.4)	H	CH_3	CH_2OH

Fig. 1. Structures of benzodiazepines and related compounds used in this study. Capacity factors are shown in parentheses.

tography of alprazolam and triazolam. This is probably due to the electron withdrawing triazolo ring altering the chromatographic behavior of triazolo-benzodiazepines as compared to other 1,4-benzodiazepines. Differential pulse polarographic methods have been reported for 1,4-benzodiazepines in serum, plasma and urine[6,7] and for triazolam in compressed tablets[8]. These methods generally lacked the specificity needed to adequately differentiate closely related benzodiazepines and potential process impurities. Liquid chromatographic[9,10], radioimmunoassay[11] and thin-layer chromatographic methods[4] for triazolam and other 1,4-benzodiazepines in serum have also been reported. However, for routine testing of pharmaceutical products a method was desired with a simple sample preparation, isocratic chromatography and selectivity for alprazolam and triazolam. A stability indicating, easily automated method meeting these requirements is described here.

During the development of this method various columns and mobile phases were utilized. From these results it appears the selectivity of the system for the triazolo-benzodiazepines is due to adsorption effects onto a modified silica surface.

EXPERIMENTAL

Alprazolam, triazolam and I–VI (all from Upjohn, Kalamazoo, MI, U.S.A.) were used as received. Acetonitrile, chloroform and 1-butanol (all from Burdick & Jackson Labs., Muskegon, MI, U.S.A.) were distilled in glass. The glacial acetic acid (Mallinckrodt, St. Louis, MO, U.S.A.) was analytical reagent grade. Deionized water was used in the mobile phase.

Mobile phases were prepared by mixing acetonitrile, chloroform, 1-butanol, water and glacial acetic acid, at the ratio of 850:80:50:20:0.5, respectively. Other mobile phases were prepared as described with varying percentages of these components. Mobile phases were degassed by vacuum and sonication after mixing.

Instrumentation

A modular liquid chromatographic system consisting of a Model 110A pump equipped with a pulse dampener (Altex, Palo Alto, CA, U.S.A.), a Model 7125 low dead volume injector (Rheodyne, Cotati, CA, U.S.A.) with a 20-μl loop, a 30 cm $\times$ 3.9 mm I.D. column containing microparticulate silica (Waters Assoc., Milford, MA, U.S.A.), a 254-nm fixed-wavelength detector (LDC, Riviera Beach, FL, U.S.A.), and recorder (Sargent-Welch, Skokie, IL, U.S.A.). The detector was interfaced to a PDP 11/40 computer (Digital Equipment, Maynard, MA, U.S.A.) system for data collection[12]. The flow-rate was adjusted to 2.0 ml min^{-1} resulting in a pressure of about 1000 p.s.i. The 254-nm detector was set at a range of 0.064 absorbance units full scale and the chart speed was 0.5 cm min^{-1}. The system was operated at ambient temperature.

Internal standard preparations

A solution of about 0.03 mg ml^{-1} of triazolam in mobile phase was used as an internal standard for the determination of alprazolam. A solution of about 0.025 mg ml^{-1} of alprazolam in mobile phase was used as an internal standard for the determination of triazolam.

Standard preparations

Separate solutions of alprazolam (0.025 mg ml^{-1}) and triazolam (0.030 mg ml^{-1}) bulk drug were prepared having an accurately known concentration in the respective internal standard solutions.

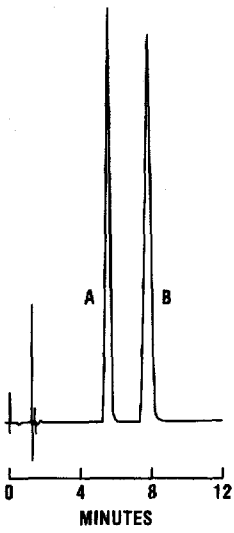
Sample preparations

Bulk drug for assay was prepared as in the standard preparation. Compressed tablets were first dispersed with a small amount of water (about 0.1 ml per tablet). The samples were then diluted in internal standard solution to about 0.03 mg drug per ml. The amount of alprazolam (triazolam) in the sample was determined by comparing the ratio of the alprazolam (triazolam) peak response to the internal standard peak response in the sample preparation to their ratio in the standard preparation.

RESULTS AND DISCUSSION

Initial attempts to develop a liquid chromatographic assay were made on a μBondapak C_{18} (octadecylsilane) column (Waters Assoc.). While good chromatography (*i.e.*, peak shape, retention time) was achieved with acetonitrile–water mobile phases, inadequate specificity for related compounds was obtained. At low water concentrations (< 10%, v/v) better specificity was observed, but unacceptable tailing resulted. Addition of acid did not improve tailing nor did ammonium ions. Substitution of other modifiers such as tetrahydrofuran or methanol for acetonitrile resulted in loss of retention.

Alprazolam and triazolam were strongly retained when injected into a silica



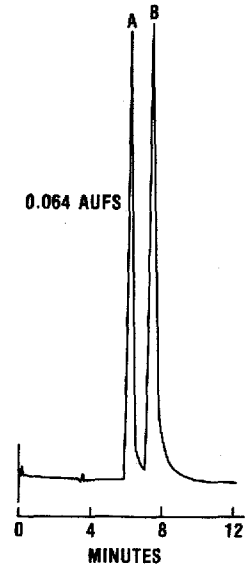


Fig. 2. Chromatogram of triazolam (A) and alprazolam (B) on a silica column, 2% water in mobile phase.

Fig. 3. Chromatogram of triazolam (A) and alprazolam (B) on a C_{18} column, 2% water in mobile phase.

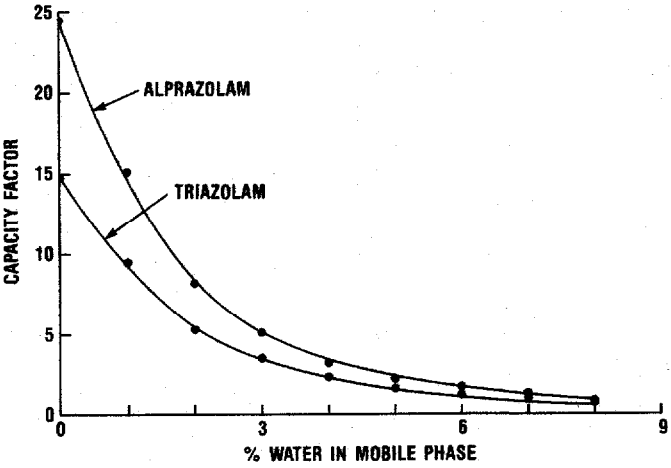


Fig. 4. Relationship between capacity factor and amount of water in the mobile phase; silica column.

column with relatively non-polar mobile phases such as those containing hexane, butyl chloride and/or chloroform. When a high percentage of acetonitrile in the mobile phase was used, similar retention characteristics to those observed with a C_{18} column were obtained. The addition of acetic acid and chloroform improved peak shape on the silica column. Fig. 2 shows a chromatogram of a mixture of alprazolam and triazolam on a silica column using the mobile phase described in the Experimental section. With this system, increased water content decreased the capacity factors, k', for alprazolam and triazolam. Fig. 3 shows a chromatogram of these compounds on a C_{18} column with the same mobile phase. As the water content of the mobile phase increased the k's for the compounds decreased, contrary to expected partition behavior on a C_{18} column. When the water concentration was increased to about 20% (v/v) the k's began increasing. At higher concentrations of water (about

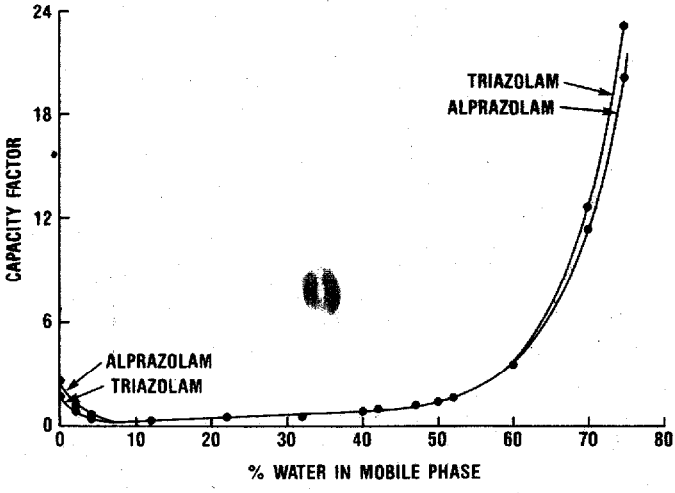


Fig. 5. Relationship between capacity and amount of water in the mobile phase; bonded phase column.

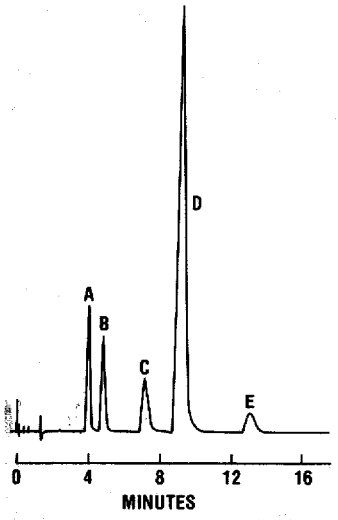
65%) alprazolam and triazolam are separated, with elution order reversed from the low water concentration separation. In the development of a serum assay for alprazolam and triazolam, Adams[10] observed a similar phenomenon. This effect was not observed for other benzodiazepines such as ketazolam and diazepam, nor was it observed on the silica column. Plots k' of alprazolam and triazolam *vs.* water concentration on silica and C_{18} columns are shown in Figs. 4 and 5, respectively. These observations indicated silanophilic attractions of the triazolo-benzodiazepines for the underivatized sites of the C_{18} column. Residual silanols have been reported in the majority of bonded phase silica columns[13,14].

As shown in Fig. 5 by the minimum in the k' *versus* water level curve, the mechanism of retention on the bonded phase columns undergoes a change. At low water levels it appears that absorption on the free silanol sites is controlling the separation. The low capacity factors observed for triazolam and alprazolam on the bonded phase columns at low water levels support the hypothesis of an adsorption retention mechanism. As the water level is increased, the free silanols are deactivated and partitioning becomes the controlling factor in the separation. However, on silica columns the adsorption mode predominates and, as shown in Fig. 4, the capacity factor continually decreases as the water level increases.

These results indicated that better specificity would be obtained on a silica column. Concentrations of chloroform, acetic acid and 1-butanol in the mobile phase were optimized for the separation and reduction of tailing. Chromatography of alprazolam, triazolam and related compounds are shown in Figs. 6 and 7, respectively. Capacity factors are shown in Fig. 1 along with the structures.

Linearity and recovery

The linearity of this system was demonstrated by determining the ratio of the



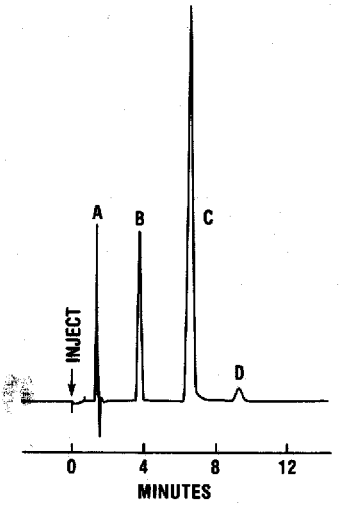


Fig. 6. Chromatogram of alprazolam with added compounds: A, compound II; B, estazolam (Compound I); C, compound III; D, alprazolam; E, dihydroalprazolam.

Fig. 7. Chromatogram of triazolam with added related compounds: A, solvent; B, desmethyltriazolam; C, triazolam; D, dihydrotriazolam.

TABLE I

RECOVERY OF ALPRAZOLAM FROM SPIKED PLACEBO (0.25 mg)

Alprazolam added (mg)	*% Label*	*Alprazolam found (%)*
0.050	20	102.0
0.099	40	100.7
0.149	60	99.9
0.198	80	100.8
0.223	90	102.0
0.248	100	100.6
0.297	120	100.4
0.347	140	101.3
0.397	160	98.3
0.446	180	101.7
0.496	200	100.6
		$\bar{x}$ (n=11) = 100.7
		S.D. = 1.06%
		R.S.D. = 1.05%

alprazolam or triazolam to the internal standard over a concentration range of 5–50 μg ml^{-1}. The equation of the resulting lines were found to be $y = 45.2\,x - 0.006$ for alprazolam and $y = 34.5\,x - 0.012$ for triazolam; correlation coefficients of 0.9996 and 0.9997 respectively were obtained. These results indicate acceptable linearity over the range tested. The relative standard deviation of the peak response ratios of six injections of the same standard was 0.26%.

The accuracy and recovery of the method were determined by spiking a series of tablet placebo mixtures with alprazolam or triazolam from 20 to 200% of label.

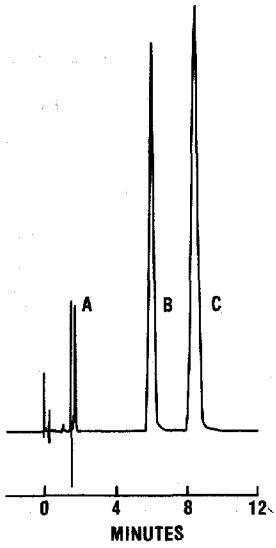


Fig. 8. Chromatogram of a Xanax tablets sample preparation: A, sodium benzoate; B, internal standard (triazolam); C, alprazolam.

TABLE II

RECOVERY OF TRIAZOLAM FROM SPIKED PLACEBO (0.125 mg)

Triazolam added (mg)	*% Label*	*Triazolam found (%)*
0.025	20	100.9
0.050	40	101.7
0.076	61	98.8
0.101	81	101.6
0.114	91	98.9
0.126	100	99.1
0.151	121	99.4
0.177	142	99.8
0.202	162	101.2
0.227	182	99.2
0.252	202	102.7
		$\bar{x}$ (n = 11) = 100.2%
		S.D. = 1.3%
		R.S.D. = 1.3%

No interferences were observed when the placebos were chromatographed. Sodium benzoate, a tablet excipient, exhibited a peak at a k' of less than 0.1 and did not interfere. The drugs were quantitated by comparing peak response ratios to the appropriate standard ratio. Table I shows recovery of alprazolam from tablets averaged 100.7%. Fig. 8 is a typical chromatogram for Xanax® tablet sample preparation. Preparations from Halcion® tablets produced comparable chromatograms. Similar results were obtained when recovery studies were performed for triazolam formulations as shown in Table II.

Further precision data were obtained by assaying replicate samples from the same lot of tablets on different days and on different columns. For both drugs, the relative standard deviations of the results were less than 1.0%.

REFERENCES

1 J. B. Hester, A. D. Rudzik and B. V. Kamdar, *J. Med. Chem.*, 14 (1971) 11.
2 R. B. Moffett, *Lect. Heterocyl. Chem.*, 3 (1976) S-123.
3 F. S. Eberts, Jr., V. Philopoulos, L. M. Reineke and R. W. Vliek, *Clin. Pharmacol. Ther.*, 29 (1981) 81.
4 I. M. Clifford and W. F. Smyth, *Analyst (London)*, 99 (1974) 1178.
5 J. D. Wittwer, Jr., *J. Liquid Chromatogr.*, 3 (1980) 1713.
6 M. R. Hackman, M. A. Brooks, J. A. F. de Silva and T. S. Ma, *Anal. Chem.*, 46 (1974) 1075.
7 M. A. Brooks and J. A. F. de Silva, *Talanta*, 22 (1975) 849.
8 P. B. Bowman and P. A. Hartman, *Fourth Annual Meeting of the Federation of Analytical Chemistry and Spectroscopy Societies, Detroit, 1977*, Abstract 267.
9 W. J. Adams, U. M. Rykert and P. A. Bombardt, *Anal. Lett.*, 13 (1980) 149–161.
10 W. J. Adams, *Anal. Lett.*, 12 (1979) 657–671.
11 H. Ko, M. E. Royer, J. B. Hester and K. T. Johnston, *Anal. Lett.*, 10 (1977) 1019–1049.
12 L. E. Fox, R. A. Johnson and N. D. Young, *Ind. Res. Develop.*, 20 (1978) 64.
13 R. C. George and C. Patel, *Pharm. Technol.*, 1 (1982) 88.
14 L. R. Snyder and J. J. Kirkland, *Introduction to Modern Liquid Chromatography*, Wiley-Interscience, New York, 2nd ed., 1979.