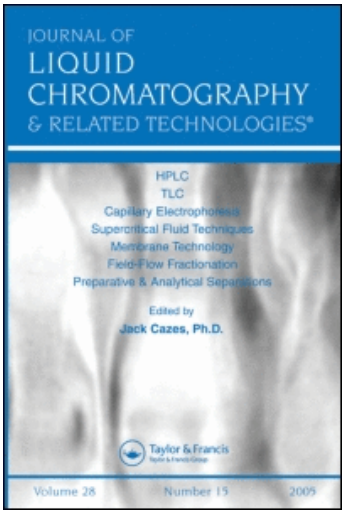

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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF 3-*O*-ACYLOXAZEPAM AND ITS HYDROLYSIS AND ALCOHOLYSIS PRODUCTS

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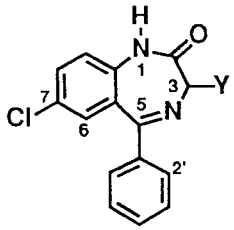
ABSTRACT

A reversed-phase high performance liquid chromatographic system was developed to separate oxazepam and its 3-*O*-acyl and 3-*O*-alkyl (alkyl = methyl, ethyl, isopropyl, n-propyl, sec-butyl, isobutyl, n-butyl, and n-pentyl) derivatives. The chromatographic conditions established a simple analytical method for monitoring the kinetics in the hydrolysis and alcoholysis of 3-*O*-acyloxazepam (oxazepam 3-acetate) under a variety of experimental conditions.

INTRODUCTION

Oxazepam (OX) is among the therapeutically used 1,4-benzodiazepines that has a hydroxyl group at the C3 position (Figure 1). OX is an active metabolite of diazepam; the latter is one of the most frequently prescribed drugs (1) for the treatment of anxiety and insomnia, and as an adjuvant for anesthesia (2). Among 3-*O*-alkyl derivatives of OX, both 3-*O*-methyl-OX (MeO-NDZ) and 3-*O*-ethyl-OX (EtO-NDZ) were



No.	Abbreviation	Y
	NDZ	H
1	OX	OH
2	MeO-NDZ	OCH_3
3	OXA	$OCOCH_3$
4	EtO-NDZ	OC_2H_5
5	iPrO-NDZ	$OCH(CH_3)_2$
6	nPrO-NDZ	$O(CH_2)_2CH_3$
7 & 8	sBuO-NDZ	$OCH(CH_3)CH_2CH_3$
9	nBuO-NDZ	$O(CH_2)_3CH_3$
10	iBuO-NDZ	$OCH_2CH(CH_3)_2$
11	nPeO-NDZ	$O(CH_2)_4CH_3$

FIGURE 1. Structures and abbreviations of nordiazepam (NDZ), oxazepam (OX) and its 3-*O*-acyl and 3-*O*-alkyl derivatives. Compound numbers correspond to the peak numbers in Figure 2. Compounds 7 and 8 are diastereomeric to each other.

found to be ~tenfold pharmacologically less potent than OX in test animals (3). Interestingly, 3-*O*-methyl-temazepam (temazepam = 1-methyloxazepam) is pharmacologically more potent than temazepam (3). The chemical and pharmacological properties of either 3-*O*-alkyl-OX's with an alkyl substituent more bulkier than an ethyl group or 3-*O*-alkyl-temazepam's with an alkyl substituent more bulkier than a methyl group have not been reported.

OX is conveniently prepared by hydrolysis of 3-*O*-acyloxazepam (OXA); the latter is efficiently prepared by rearrangement of nordiazepam (NDZ) 4-oxide in acetic anhydride (4, 5). Ether derivatives of OX can be prepared by acid-catalyzed alcoholysis of OXA. Although many chromatographic methods for the separation of OX and related compounds have been published (6), no method has been described for the separation of OX and its alkyl ether derivatives. A simple analytical method has heretofore unavailable to monitor the kinetics in the hydrolysis and alcoholysis of OXA under a variety of experimental

conditions. For kinetic studies of hydrolysis and alcoholysis reactions of OXA, a simple reversed-phase HPLC method was developed and is the subject of this report.

MATERIALS

Racemic OX (7-chloro-1,3-dihydro-3-hydroxy-5-phenyl-2*H*-1,4-benzodiazepin-2-one) was generously provided by Dr. Yvon Lefebvre of Wyeth-Ayerst Research (Princeton, NJ). Demoxepam (7-chloro-1,3-dihydro-5-phenyl-2*H*-1,4-benzodiazepin-2-one 4-oxide or NDZ 4-oxide) was generously provided by Dr. Peter F. Sorter of Hoffmann-La Roche, Inc. (Nutley, NJ). (S)-(+)-2-Butanol [(S)-(+)-*sec*-butyl alcohol; 99%] was purchased from Aldrich Chemical Co. (Milwaukee, WI). Racemic 3-*O*-acyloxazepam (OX 3-acetate, OXA) was prepared from demoxepam according to the procedure described by Bell and Childress (4). 3-*O*-Alkyl derivatives were each prepared by reaction of racemic OXA with an alcohol containing 5 mM H_2SO_4 at room temperature. Alcoholysis reaction was monitored until the reaction has reached >99% completion, as revealed by the result of an HPLC analysis (see method described below).

METHODS

Reversed-phase HPLC. HPLC was performed using a liquid chromatograph consisting of a Waters Associates (Milford, MA) Model M45 solvent pump, a Model 717 autosampler, and a Model 441 absorbance (254 nm) detector. Retention time and area under chromatographic peaks at 254 nm were recorded with an Apple Macintosh SE computer (Apple Computer, Inc., Cupertino, CA) using Dynamax HPLC Method Manager (Rainin Instrument Co., Woburn, MA). Retention times reported were averages of 5 to 7 chromatographic analyses and were reproducible within ~1%.

A Vydac C18 column (5 μ particles, 4.6 mm i.d. x 25 cm, catalog no. 201TP54; The Separations Group, Hesperia, CA) was used and the analysis was conducted at ambient temperature (22±1°C). The mobile

phase consisted of various volume ratios of acetonitrile (40-70%) and 20 mM phosphate buffer (pH 7.0) at a flow rate of 1 ml/min.

KINETICS OF HYDROLYSIS AND METHANOLYSIS OF OXA

Hydrolysis: OXA (100 μg) was dissolved in 1 ml of acetonitrile:0.05 M NaOH (4:1, v/v) at ambient temperature (22±1°C). Following the addition of solvent, aliquots (10 μl) were injected at various times for analysis by reversed-phase HPLC using acetonitrile:0.02 M phosphate buffer (pH 7.0) (55:45, v/v) as the mobile phase at 1 ml/min.

Methanolysis: OXA (100 μg) was dissolved in 1 ml of methanol containing 5 mM H_2SO_4 at ambient temperature (22±1°C). Following the addition of solvent, aliquots (10 μl) were injected at various times for analysis by reversed-phase HPLC using acetonitrile:0.02 M phosphate buffer (pH 7.0) (45:55, v/v) as the mobile phase at 1 ml/min.

SPECTRAL ANALYSIS

Absorbance of samples in acetonitrile was determined using a 1 cm path length quartz cuvette on a Model DW2000 spectrophotometer (SLM Instruments, Urbana, IL). CD spectra of samples in acetonitrile were determined on a JASCO Model 500A spectropolarimeter (Japan Scientific Co., Easton, MD). CD spectra of samples are expressed in ellipticity (in millidegrees) for a solution that contains 1.0 absorbance unit at 230 nm per ml of acetonitrile.

RESULTS

REVERSED-PHASE HPLC

During the initial phase of this study, OX, OXA and most of its alcoholysis products were analyzed one compound at a time in order to establish the retention time of each compound. These initial experiments indicated that OX, OXA and most of its alcoholysis products can be separated among one another by using 45% to 55% (vol ratio) of

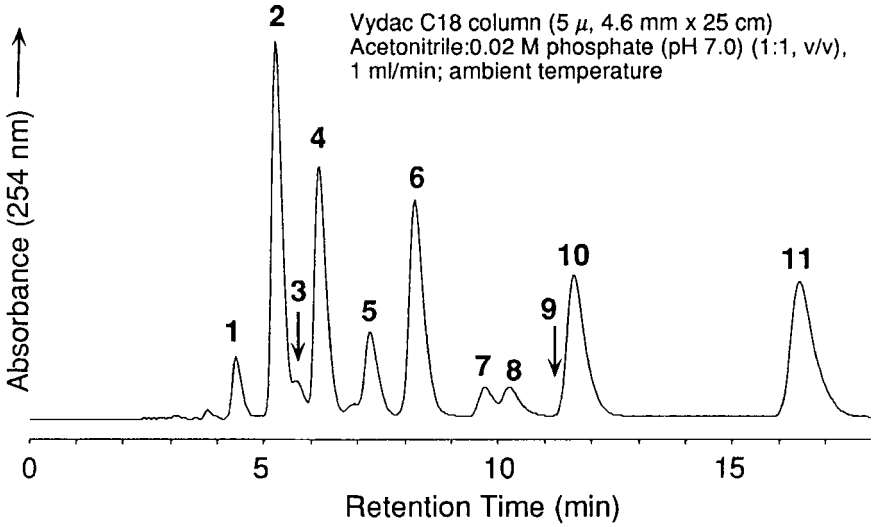


FIGURE 2. Reversed-phase HPLC separation of OX and its derivatives. The mobile phase was acetonitrile:0.02 M phosphate buffer (pH 7.0) (1:1 v/v) at 1 ml/min. The identities of various chromatographic peaks are: **1**, OX; **2**, MeO-NDZ; **3**, OXA; **4**, EtO-NDZ; **5**, iPrO-NDZ; **6**, nPrO-NDZ; **7**, sBuO-NDZ #1; **8**, sBuO-NDZ #2; **9**, nBuO-NDZ; **10**, iBuO-NDZ; **11**, nPeO-NDZ. Peaks **7** (sBuO-NDZ #1) and **8** (sBuO-NDZ #2) are diastereomeric to each other. Due to close elution with iBuO-NDZ (peak **10**), retention time of peak **9** (nBuO-NDZ; indicated by an arrow) was determined by a separate chromatographic run.

acetonitrile in 0.02 M phosphate buffer (pH 7.0) as the mobile phase. An example of a chromatogram consisting of 11 analytes, using 1:1 volume ratio of acetonitrile and 0.02 M phosphate buffer (pH 7.0) as the mobile phase, is shown in Figure 2. nBuO-NDZ and iBuO-NDZ were poorly separated from each other under all chromatographic conditions tested (Figure 2).

MeO-NDZ and OXA were eluted increasingly closer to each other as the volume percentage of acetonitrile in the mobile phase increased (Figure 2). MeO-NDZ and OXA were better resolved than that indicated in Figure 2 by using either 45% acetonitrile (α = 1.18 and R_S = 1.34) or

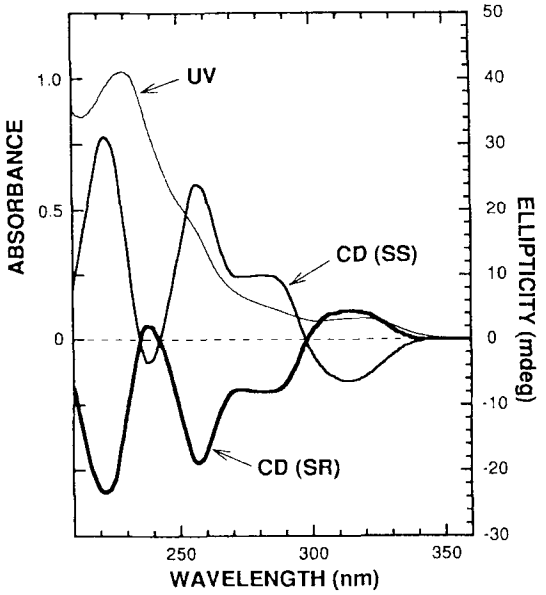


FIGURE 3. Uv absorption and CD spectra of (SS)-sBuO-NDZ (98% enantiomeric excess) and (SR)-sBuO-NDZ (87% enantiomeric excess) in acetonitrile. The retention times of (SS)-sBuO-NDZ and (SR)-sBuO-NDZ were the same as those of peaks 7 and 8 in Figure 2, respectively.

40% acetonitrile (α = 1.25 and R_S = 2.35) in the acetonitrile-0.02 M phosphate buffer (pH 7.0) mixture.

Two diastereomeric sBuO-NDZ's (peaks 7 and 8 in Figure 2), resulting from alcoholysis of racemic OXA in racemic *sec*-butyl alcohol, were better resolved from each other by using lower volume percentages of acetonitrile in the mobile phase; R_S values were 0.95 and 1.50 by using 45% and 40% acetonitrile in the mobile phase, respectively.

Acid-catalyzed alcoholysis of rac-OXA in (S)-*sec*-butanol yielded (SS)-sBuO-NDZ and (SR)-sBuO-NDZ. These two enantiomers, which are diastereomeric to each other, were purified for further characterization by repetitive chromatography using 40% acetonitrile in

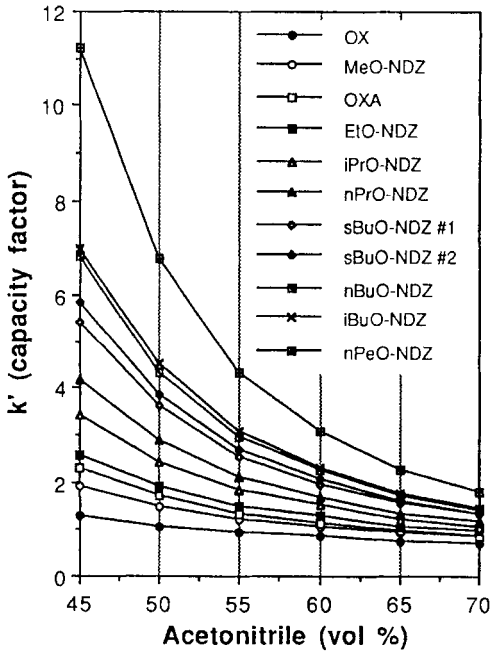


FIGURE 4. Relationship of k' (capacity factor) and percentage of acetonitrile in the mobile phase. See Figure 1 for structures and abbreviations.

the mobile phase. The enantiomer eluted earlier was purified to contain an enantiomeric excess of 98% and the second enantiomer was purified to contain an enantiomeric excess of 87%. The enantiomer eluted earlier had a CD spectrum (Figure 3) with similar Cotton effects to those of either (S)-OX or (S)-OXA (7, 8). The second enantiomer had CD Cotton effects opposite in signs to those of the enantiomer with a shorter retention time. Hence the two enantiomers were established to be (SS)-sBuO-NDZ (early-eluting) and (SR)-sBuO-NDZ (late-eluting) respectively. These results established that peak 7 of Figure 2 contained SS and RR enantiomers and peak 8 contained SR and RS enantiomers.

The results shown in Figure 4 indicated that the k' value (capacity factor) of each compound decreased exponentially as the volume

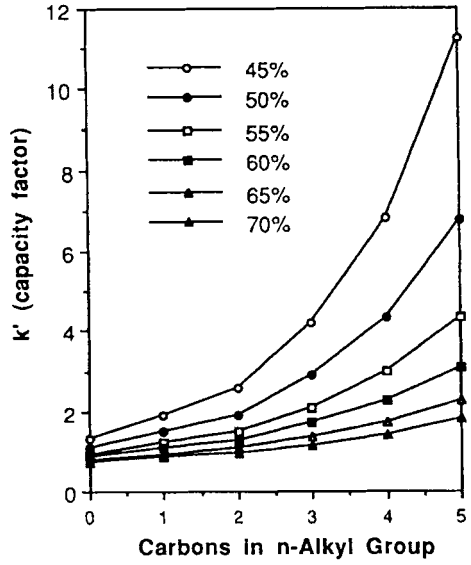


FIGURE 5. Relationship of k' (capacity factor) and carbon number in 3-*O*-n-alkyl derivatives of OX. The percentages of acetonitrile in each mobile phase for determining the k' values are indicated. The abscissa indicates the number of carbon (0 for OX, 1 for MeO-NDZ, 2 for EtO-NDZ, 3 for nPrO-NDZ, 4 for nBuO-NDZ, and 5 for nPeO-NDZ) in the n-alkyl group of 3-*O*-alkyl derivatives of OX.

percentage of acetonitrile in the mobile phase increased. k' values of compounds using mobile phases containing intermediate acetonitrile concentrations can be fairly accurately estimated from the curves in Figure 4.

The data in Figure 4 indicated that the k' value increased as the size and/or length of *O*-alkyl group in 3-*O*-alkyl-OX increased. The trend became apparent when the k' values were plotted against the number of carbons in the n-alkyl group of 5 n-alkoxy-NDZ's (Figure 5). It appeared that, at every mobile phase composition tested, k' increased exponentially as a function of the number of carbons in the n-alkyl group of n-alkoxy-NDZ's.

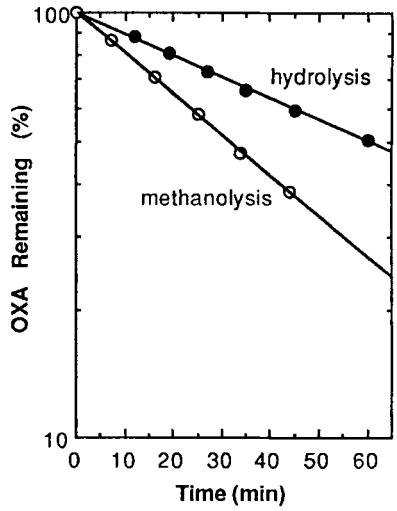


FIGURE 6. Kinetics of base-catalyzed hydrolysis ($t_{1/2}$ = 60.2 min and k = 0.0115 min^{-1}) and acid-catalyzed methanolysis ($t_{1/2}$ = 31.6 min and k = 0.0219 min^{-1}) of OXA at ambient temperature. See METHODS for experimental detail.

KINETICS OF HYDROLYSIS AND METHANOLYSIS OF OXA

Results of two kinetic studies utilizing the reversed-phase HPLC method described above are indicated in Figure 6. Base-catalyzed hydrolysis of OXA was conducted in acetonitrile:0.05N NaOH (4:1, v/v) at ambient temperature (22±1°C). Acid-catalyzed methanolysis of OXA was conducted in methanol containing 5 mM H_2SO_4 at ambient temperature (22±1°C).

The chromatographic data in Figure 4 indicated that a mobile phase composition [acetonitrile:0.02 M phosphate buffer (pH 7.0); 55:45, v/v] could be chosen so that OXA and its hydrolysis product (OX) were well resolved and an HPLC analysis could be completed every 6 min. With the chosen mobile phase composition, the sampling interval was 7 min or longer. These experimental conditions allowed the determination

of half-life (60.2 min) and rate constant (0.0115 min^{-1}) of the base-catalyzed hydrolysis of OXA (Figure 6). The half-life (31.6 min) and rate constant (0.0219 min^{-1}) of the acid-catalyzed methanolysis of OXA (Figure 6) were determined by using 45:55 volume ratio of acetonitrile:0.02 M phosphate buffer (pH 7.0) as the mobile phase in the separation of MeO-NDZ and OXA.

DISCUSSION

Results of hydrolysis and methanolysis studies illustrate that the reversed-phase HPLC method described in this report is useful in general for kinetic studies of hydrolysis and alcoholysis of OXA under a variety of experimental conditions. The effects of variables such as temperature, pH, solvent composition and ionic strength may be studied by using the analytical system described. Because samples can be directly injected for analysis, the need for extraction procedures using organic solvents is avoided.

The chromatographic data in Figure 4 can be used to serve as a guide in choosing an appropriate mobile phase composition to analyze OXA and its hydrolysis/alcoholysis product. When the product, formed in either hydrolysis or methanolysis of OXA, is eluted ahead of OXA, the use of 45% acetonitrile in the mobile phase is suitable for monitoring the kinetics of the reaction. Products formed in the alcoholysis of OXA with ethanol and higher alcohols are all eluted later than OXA (Figure 2). Hence mobile phases containing ≥45% acetonitrile are suitable for monitoring the kinetics of these alcoholysis reactions. For example, the mobile phases containing 60% and 70% acetonitrile in 0.02 M phosphate buffer (pH 7.0) are suitable for studying n-propanolysis and n-butanolysis of OXA, respectively (see k' values in Figure 4). It is not desirable to employ a chromatographic condition that produces a larger k' difference between OXA/alkoxy-NDZ pair because it unnecessarily increases the width of chromatographic peaks, analysis time, and sampling interval.

By using the experimental conditions employed in this study, the lower limit of sampling interval was ~3 min. Hence reactions with half-

lives <10 min are difficult to determine accurately. However, it may be possible to employ columns of smaller size and higher efficiency in order to increase the sampling frequency for studying reactions with faster kinetics.

ACKNOWLEDGMENTS

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