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ARTICLES

Racemization Kinetics of Enantiomeric Oxazepams and Stereoselective Hydrolysis of Enantiomeric Oxazepam 3-Acetates in Rat Liver Microsomes and Brain Homogenate

SHEN K. YANG[x] AND XIANG-LIN LU

Received November 11, 1988, from the *Department of Pharmacology, F. Edward Hébert School of Medicine, Uniformed Services University of the Health Sciences, Bethesda, MD 20814-4799.* Accepted for publication February 27, 1989.

Abstract □ Enantiomers of oxazepam and of 3-*O*-acyl, 1-*N*-acyl-3-*O*-acyl, and 3-*O*-methyl ether derivatives of oxazepam were resolved on HPLC columns packed with Pirkle's chiral stationary phases [CSP; (*R*)-*N*-(3,5-dinitrobenzoyl)phenylglycine or (*S*)-*N*-(3,5-dinitrobenzoyl)leucine] bonded either ionically or covalently to spherical particles of γ-aminopropylsilanized silica, and on a column packed with poly-*N*-acryloyl-(*S*)-phenylalanine ethyl ester bonded covalently to silica gel (Chiraspher). Resolution was achieved, with several mobile phases of different solvent compositions and with varying chromatographic resolutions, on all of the chiral stationary phases tested. Resolved enantiomers of oxazepam undergo racemization, whereas enantiomers of 3-*O*-acyl and 3-*O*-methyl derivatives are stable. Racemization half-lives of oxazepam enantiomers were determined by monitoring changes in ellipticity as a function of time on a spectropolarimeter immediately (within 30 s) following resolution of enantiomers and were found to substantially vary, depending on the solvents used. Rates of hydrolysis of racemic and enantiomeric 3-*O*-acyl-oxazepams by esterases in liver microsomes and brain homogenate of rats were determined by a simple and sensitive CSP-HPLC method. The relative rate of hydrolysis was 3*R* > racemate >> 3*S* by rat liver microsomes and 3*S* > racemate >> 3*R* by rat brain homogenate.

Oxazepam is among the therapeutically used 1,4-benzodiazepines that have a hydroxyl group at the asymmetric C3 carbon. Oxazepam is an active metabolite of diazepam, which is one of the most frequently prescribed drugs[1] for the treatment of anxiety and insomnia and as an adjuvant for anesthesia.[2] 3-*O*-acyl and 3-*O*-methyl derivatives of oxazepam are also pharmacologically active, with activities comparable to that of oxazepam in several animal tests.[3] (+)Enantiomers of benzodiazepines containing asymmetric carbons at C3 have been found to possess higher potency in displacing [^{3}H]diazepam binding than the respective (−)enantiomer in synaptosomal preparations from rat cerebral cortex.[4]

Maksay et al.[5] reported that esterases in liver and brain homogenates of mice have opposite stereoselectivity; esterases in liver homogenate preferentially hydrolyze (−)3*R*-oxazepam acetate, whereas esterases in brain homogenate preferentially hydrolyze (+)3*S*-oxazepam acetate. Oxazepam acetate, when administered intraperitoneally to mice, provides a higher brain:blood concentration ratio of oxazepam than the underivatized oxazepam.[6] It was proposed that, on the basis of the brain:blood concentration ratio of oxazepam following intraperitoneal administration of oxazepam acetate to mice, hydrolysis precedes brain penetration.[6]

Enantiomers of oxazepam were first resolved on a poly-*N*-acryloyl-(*S*)-phenylalanine ethyl ester coated onto silica gel;[7] a covalently bonded version[8] is recently available commercially (Chiraspher). Enantiomers of 3-*O*-acyl-oxazepam were partially separated by an ultrafiltration method, making use of differential stereoselective binding activities of enantiomers to human serum albumin.[9] Enantiomers of 3-*O*-acyl-oxazepam were also separated on immobilized human serum albumin.[10,11] Enantiomers of 3-*O*-acyl-oxazepam and of 3-*O*-methyl-oxazepam have been more efficiently resolved on chiral stationary phase (CSP) columns with covalently bonded (*R*)-*N*-(3,5-dinitrobenzoyl)phenylglycine and (*S*)-*N*-(3,5-dinitrobenzoyl)leucine; ionic columns afford similar results but with lower separability factors.[12]

It is known that enantiomers of oxazepam undergo racemization in aqueous medium.[7,13] Equilibrium with an open aldehyde form was proposed to be the mechanism responsible for racemization.[14,15] However, kinetics of racemization and possible ways to stabilize the enantiomers from racemization have heretofore not been reported.

This paper reports CSP-HPLC resolution of enantiomers of oxazepam (3-*O*-acyl-oxazepam, 1-*N*-acyl-3-*O*-acyl-oxazepam, and 3-*O*-methyl-oxazepam) with several mobile phases of different solvent compositions. Half-lives of racemization of oxazepam enantiomers in several solvents were determined. Rates of hydrolysis of racemic and enantiomeric 3-*O*-acyl-oxazepams by liver microsomes and brain homogenate of rats were studied by a simple and sensitive CSP-HPLC method.

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Experimental Section

Materials—Oxazepam (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). The molar extinction coefficient of oxazepam (in methanol) was determined to be 34160 $cm^{-1} M^{-1}$ at 230 nm. 3-*O*-Acyl-oxazepam (oxazepam 3-acetate) and 1-*N*-acyl-3-*O*-acyl-oxazepam (oxazepam 1,3-diacetate) were prepared by reaction of oxazepam with acetic anhydride in pyridine overnight at room temperature, followed by normal-phase HPLC separation (see below). Molar extinction coefficients of oxazepam 3-acetate and oxazepam 1,3-diacetate, which have UV absorption spectra closely similar to that of oxazepam, are assumed to be the same as that of oxazepam. 3-*O*-Methyloxazepam was converted from oxazepam 3-acetate in methanol containing 3.4 M HCl at 50 °C for 40 min, followed by normal-phase HPLC purification.

Liver microsomes and brain homogenate were prepared from livers of male Sprague-Dawley rats weighing 80–100 g. Liver microsomes and brain homogenate contained 20.6 and 104.5 mg protein/g tissue, respectively. Protein contents were determined by the method of Lowry et al.,[16] with bovine serum albumin as the protein standard.

High-Performance Liquid Chromatography—The HPLC was performed using a Waters Associates (Milford, MA) liquid chromatograph consisting of a model 6000A solvent delivery system, a model M45 solvent delivery system, a model 660 solvent programmer, and a Kratos Analytical Instruments (Ramsey, NJ) model Spectraflow 757 UV-VIS variable wavelength detector. Samples were injected via a Valco model N60 loop injector (Valco Instruments, Houston, TX). Retention times and area under chromatographic peaks were determined with a Hewlett-Packard model 3390A integrator.

Normal-Phase High-Performance Liquid Chromatography—Normal-phase HPLC was carried out on a Zorbax SIL column (9.4 mm i.d. × 25 cm, Dupont). The eluant (2.5 or 3 mL/min) used for separation of oxazepam acetates was hexane:ethanol:acetonitrile (93:4.67:2.33, v/v). Purified oxazepam acetates (racemic as well as enantiomeric) are more stable when the solvents are evaporated and stored at 4 °C.

Chiral Stationary-Phase High-Performance Liquid Chromatography—Chiral stationary-phase enantiomeric resolutions of oxazepam and its 1-*N*- (and/or) 3-*O*-acyl and *O*-methyl derivatives were achieved on CSP columns (4.6 and 10 mm i.d. × 25 cm, Regis Chemical, Morton Grove, IL) packed with spherical particles (5 μm diameter) of γ-aminopropylsilanized silica to which either (*S*)-*N*-(3,5-dinitrobenzoyl)leucine (*S*-DNBL) or (*R*)-*N*-(3,5-dinitrobenzoyl)phenylglycine (*R*-DNBPG) was bonded ionically (I) or covalently (C). A column (4 mm i.d. × 25 cm) packed with poly-*N*-acryloyl-(*S*)-phenylalanine ethyl ester bonded covalently to silica gel (Chiraspher)[8] was purchased from Bodman Chemicals (Stone Mountain, GA). Several mobile phases were used. Eluants EA5, EA7, EA10, EA15, and EA20 are 5, 7, 10, 15, and 20% ethanol:acetonitrile (2:1, v/v) in hexane, respectively; eluant P10 is hexane:2-propanol (9:1, v/v); eluant D17EA3 is hexane:dioxane:ethanol:acetonitrile (80:17:2:1, v/v); and eluant D17P3 is hexane:dioxane:2-propanol (80:17:3, v/v).

Kinetics of Racemization—Within 30 s of separation by CSP-HPLC, changes of ellipticity (ΔΦ) of an enantiomeric oxazepam (1 to 2 A_{230}/mL) were recorded at 255 nm (peak of a Cotton effect) as a function of time. The half-life of racemization ($t_{1/2}$) was determined by plotting log (ΔΦ) versus time. Racemization of enantiomeric oxazepams in all solvents tested follows first-order kinetics. Hence, the rate constant (k) of racemization is $0.693/t_{1/2}$. The solvents used for determining $t_{1/2}$ are the mobile phases used in CSP-HPLC (see above).

Enzymatic Hydrolysis of Racemic and Enantiomeric Oxazepam 3-acetates—Racemic or enantiomeric oxazepam 3-acetates (29, 58, 146, or 292 nmol dissolved in 25–50 μL of methanol 5 to 10 min before use) were incubated with rat liver microsomes or brain homogenate in a 1-mL incubation mixture containing 250 mmol of Tris-HCl (pH 7.5) and 2.67 to 24.8 mg tissue equivalent of rat liver microsomes (or brain homogenate). The reaction mixture was pre-incubated at 37 °C for 2 min in a water shaker bath before the addition of oxazepam 3-acetate. At the end of the incubation period (5 to 30 min), the reaction was stopped by the addition of 1 mL of acetone. Oxazepam 3-acetate and its hydrolysis product (oxazepam) were extracted by the addition of 3 mL of chloroform. Extraction efficiency was higher (≥98%) using chloroform than using ethyl acetate or ethyl ether. The organic phase was evaporated to dryness under a stream of nitrogen in a 50 °C water bath. The residue was redissolved in 25–100 μL of hexane:ethanol:acetonitrile (80:13.34:6.66, v/v) for CSP-HPLC analysis on a covalently bonded *S*-DNBL column. Hydrolysis of oxazepam 1,3-diacetate was not studied.

Spectral Analysis—Mass spectral analysis was performed on a Finnigan model 4000 gas chromatograph-mass spectrometer-data system with a solid probe by electron impact or chemical ionization, with methane as the ionization gas, at 70 eV and 250 °C ionizer temperature. Ultraviolet-visible absorption spectra of samples were determined using a 1-cm path length quartz cuvette with a Varian model Cary 118C spectrophotometer. The CD spectra of samples in a quartz cell of 1-cm path length at room temperature were measured using a Jasco model 500A spectropolarimeter equipped with a model DP500 data processor. The concentration of the sample is indicated by $A_{\lambda 2}$/mL (absorbance units at wavelength λ2 per milliliter of solvent). The CD spectra are expressed by ellipticity ($\Phi_{\lambda 1}/A_{\lambda 2}$, in millidegrees) for solutions that have an absorbance of $A_{\lambda 2}$ unit per milliliter of solvent at wavelength λ2 (usually the wavelength of maximal absorption). Under the conditions of measurements indicated above, the molecular ellipticity ($[\theta]_{\lambda 1}$, in deg·cm^2·$dmol^{-1}$) and ellipticity ($\Phi_{\lambda 1}/A_{\lambda 2}$, in millidegrees) are related to the molar extinction coefficient ($\epsilon_{\lambda 2}$, in $cm^{-1} M^{-1}$) as follows:

$$[\theta]_{\lambda 1} = 0.1\epsilon_{\lambda 2} (\Phi_{\lambda 1}/A_{\lambda 2}) \quad (1)$$

and

$$A_{\lambda 2} = C\epsilon_{\lambda 2} \quad (2)$$

where C is the concentration of sample (in mol/L).

It is apparent from the above equations that molar ellipticity of an enantiomer (or diastereomer) must be reported along with its molar extinction coefficient. Unfortunately, molar ellipticities of enantiomers/diastereomers are quite often reported in the literature without the molar extinction coefficients.

Results and Discussion

Acetylation of oxazepam in pyridine with acetic anhydride produced a 3-*O*-acyl-oxazepam ($M^+ + 1$ at *m/z* 329, CI) and a 1,3-diacetate (1-*N*-acyl-3-*O*-acyl-oxazepam, $M^+ + 1$ at *m/z* 371, CI), in addition to two minor products (see Figure 1 for normal-phase HPLC separation). Identities of two minor products have not been characterized. 3-*O*-Methyl-oxazepam ($M^+ + 1$ at *m/z* 301, CI) is converted from oxazepam 3-acetate in HCl:methanol; the retention time on normal-phase HPLC is shown in Figure 1. Oxazepam 3-acetate slowly undergoes

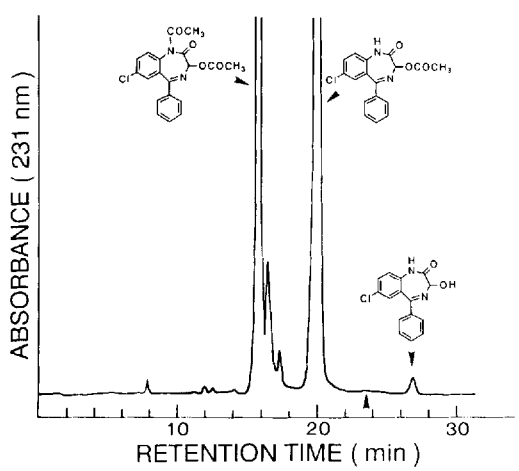

Figure 1—Normal-phase HPLC separation of acyl derivatives of oxazepam. The column used was DuPont Zorbax SIL (9.4 mm i.d. × 25 cm), and the eluant was ethanol:acetonitrile:hexane (10:5:85, vol ratio) at a rate of 2.5 mL/min. The retention time of oxazepam 3-*O*-methyl ether is indicated by an arrow at 23.5 min. Chromatographic peaks that eluted between 1,3-diacetate and 3-acetate have not been characterized.

Table I—CSP-HPLC Resolution of Enantiomers of Oxazepam and Its 1-*N*- (and/or) 3-*O*-Acyl and 3-*O*-Methyl Derivatives

Chemical	CSP[b]	Eluant[c]	Retention Time, min[a]		α[d]	R_s[d]
			E_1	E_2		
Oxazepam	*R*-DNBPG-I	EA7	68.9 (*R*)	70.8 (*S*)	1.03	0.34
	R-DNBPG-C	EA7	29.2 (*R*)	32.6 (*S*)	1.13	1.56
		P10	60.8 (*R*)	73.4 (*S*)	1.22	1.30
		P10	(*R*)	(*S*)	1.20[e]	
		D17EA3	24.2 (*R*)	27.2 (*S*)	1.13	1.46
		D17P3	34.7 (*R*)	39.8 (*S*)	1.17	1.37
	S-DNBL-I	EA7	42.4 (*S*)	42.9 (*R*)	1.01	~0.1
	S-DNBL-C	EA5	39.6 (*S*)	45.4 (*R*)	1.15	1.45
		EA7	27.1 (*S*)	30.8 (*R*)	1.15	1.48
		EA10	15.5 (*S*)	17.2 (*R*)	1.13	1.41
		EA15[f]	31.2 (*S*)	36.0 (*R*)	1.15	2.07
		E10	20.5 (*S*)	23.5 (*R*)	1.16	1.31
		P10	47.5 (*S*)	55.9 (*R*)	1.18	1.22
		P10	(*S*)	(*R*)	1.13[e]	
		D17EA3	19.3 (*S*)	21.9 (*R*)	1.15	1.53
		D17P3	31.5 (*S*)	35.8 (*R*)	1.15	1.06
	Chiraspher	EA7	34.8 (*S*)	37.0 (*R*)	1.07	0.95
		D17EA3	20.0 (*S*)	22.5 (*R*)	1.15	1.58
		D17P3	23.8 (*S*)	27.5 (*R*)	1.17	1.39
Oxazepam 3-acetate	*R*-DNBPG-I	EA7	16.7 (*R*)	18.0 (*S*)	1.27	1.26
	R-DNBPG-C	EA7	17.2 (*R*)	19.6 (*S*)	1.16	1.58
		P10	20.3 (*R*)	25.7 (*S*)	1.31	2.18
		P10	(*R*)	(*S*)	1.27[e]	
		D17EA3	9.9 (*R*)	11.4 (*S*)	1.20	2.01
		D17P3	13.5 (*R*)	16.1 (*S*)	1.23	2.03
	S-DNBL-I	EA7	12.2 (*S*)	12.6 (*R*)	1.05	0.28
	S-DNBL-C	EA7	11.1 (*S*)	14.1 (*R*)	1.35	3.14
		EA15[f]	17.6 (*S*)	21.6 (*R*)	1.23	4.90
		P10	14.0 (*S*)	20.3 (*R*)	1.55	2.30
		P10	(*S*)	(*R*)	1.62[e]	
		D17EA3	8.1 (*S*)	10.4 (*R*)	1.41	2.85
		D17P3	11.7 (*S*)	16.2 (*R*)	1.49	2.73
	Chiraspher	EA7	15.9 (*S*)	16.8 (*R*)	1.07	0.96
		D17EA3	10.0 (*S*)	11.0 (*R*)	1.13	1.11
		D17P3	11.5 (*S*)	13.1 (*R*)	1.17	1.09
Oxazepam 1,3-diacetate	*R*-DNBPG-I	EA7	16.5 (*R*)	17.8 (*S*)	1.09	1.41
	R-DNBPG-C	EA7	16.1 (*R*)	18.5 (*S*)	1.16	2.16
		P10	18.8 (*R*)	23.9 (*S*)	1.31	1.52
		D17EA3	8.8 (*R*)	10.0 (*S*)	1.19	1.53
		D17P3	13.2 (*R*)	16.0 (*S*)	1.25	1.89
	S-DNBL-I	EA7	12.3 (*S*)	12.9 (*R*)	1.06	0.63
	S-DNBL-C	EA7	10.6 (*S*)	13.4 (*R*)	1.35	2.75
		EA15[f]	17.2 (*S*)	21.1 (*R*)	1.22	3.84
		P10	12.7 (*S*)	18.4 (*R*)	1.54	1.69
		D17EA3	8.2 (*S*)	10.5 (*R*)	1.53	2.63
		D17P3	11.6 (*S*)	16.0 (*R*)	1.48	2.37
	Chiraspher	EA7	15.7 (*S*)	16.6 (*R*)	1.07	0.88
		D17EA3	10.0 (*S*)	11.0 (*R*)	1.14	1.12
		D17P3	11.6 (*S*)	13.2 (*R*)	1.18	1.17
Oxazepam 3-*O*-methyl	*R*-DNBPG-I	EA7	27.6 (*R*)	29.0 (*S*)	1.05	1.15
	R-DNBPG-C	EA7	21.4 (*R*)	23.9 (*S*)	1.13	1.92
		P10	41.6 (*R*)	50.6 (*S*)	1.23	1.68
		P10	(*R*)	(*S*)	1.33[e]	
		D17EA3	24.0 (*R*)	26.8 (*S*)	1.13	1.50
		D17P3	37.5 (*R*)	42.6 (*S*)	1.14	1.27

(Continued)

Table I—Continued

Chemical	CSP[b]	Eluant[c]	Retention Time, min[a]		α[d]	R_s[d]
			E_1	E_2		
	S-DNBL-I	EA7	17.1 (*S*)	17.3 (*R*)	1.01	<0.2
	S-DNBL-C	EA7	15.5 (*S*)	18.2 (*R*)	1.21	2.19
		EA15[f]	21.6 (*S*)	25.0 (*R*)	1.16	3.19
		P10	32.3 (*S*)	37.1 (*R*)	1.16	1.07
		P10	(*S*)	(*R*)	1.19[e]	
		D17EA3	19.2 (*S*)	21.2 (*R*)	1.12	1.51
		D17P3	12.6 (*S*)	17.3 (*R*)	1.47	2.55
	Chiraspher	EA7	15.1 (*S*)	17.7 (*R*)	1.21	2.06
		D17EA3	13.7 (*S*)	18.0 (*R*)	1.39	2.98
		D17P3	17.2 (*S*)	24.4 (*R*)	1.49	2.88

[a] See text for the assignment of absolute configurations of resolved enantiomers; enantiomers are designated by E_1 and E_2 according to their elution order. [b] CSPs are described in the *Experimental Section*. [c] Eluants EA5, EA7, EA10, and EA15 are 5, 7, 10, and 15% of ethanol:acetonitrile (2:1, v/v) in hexane, respectively; eluant P10 is hexane:2-propanol (9:1, v/v); eluant D17EA3 is hexane:dioxane:ethanol:acetonitrile (80:17:2:1, v/v); eluant D17P3 is hexane:dioxane:2-propanol (80:17:3, v/v); unless otherwise noted, flow rate of eluant is 2 mL/min. [d] α and R_s are selectivity and resolution, respectively. [e] Data taken from ref 12 for comparison. [f] A 9.4 mm i.d. × 25 cm column was used; flow rate of eluant was 3 mL/min.

spontaneous hydrolysis in solution to form oxazepam. Oxazepam 1,3-diacetate slowly hydrolyzes in solution to form oxazepam 3-acetate and oxazepam. Both the 3-acetate and 1,3-diacetate of oxazepam are more stable when stored dried in a refrigerator.

The enantiomeric pairs of oxazepam, oxazepam 3-acetate, oxazepam 1,3-diacetate, and 3-*O*-methyl-oxazepam were separated on five CSPs, with varying chromatographic resolutions (Table I). Four mobile phases were used; eluant EA (various percentages of ethanol:acetonitrile (2:1, v/v) in hexane), eluant P10 (hexane:2-propanol, 9:1, v/v), eluant D17EA3 (hexane:dioxane:ethanol:acetonitrile, 80:17:2:1, v/v), and eluant D17P3 (hexane:dioxane:2-propanol, 80:17:3, v/v). By using eluant EA7 [7% ethanol:acetonitrile (2:1, v/v) in hexane], the ionically bonded *R*-DNBPG and *S*-DNBL columns resolve enantiomeric pairs less efficiently than the covalently bonded *R*-DNBPG and *S*-DNBL columns, respectively (Table I). These data are consistent with the results reported by Pirkle and Tsipouras[12] who used eluant P10 in their study. Because of poorer resolutions on the ionically bonded *R*-DNBPG and *S*-DNBL columns, enantiomeric resolutions with other eluants were not attempted.

Enantiomeric pairs of oxazepam and oxazepam 3-acetate may be resolved on the covalently bonded *S*-DNBL column in one chromatographic run (Table I). We have taken advantage of this simple chromatographic system (i.e., simultaneous resolution of two pairs of enantiomers) to study the rates of stereoselective hydrolysis of racemic and enantiomeric oxazepam 3-acetate (see below).

For the purpose of comparison, resolutions of enantiomeric pairs on covalently bonded *R*-DNBPG and *S*-DNBL columns using eluant P10 were also investigated in this study (Table I); similar or higher values of α and lower values of R_s were obtained than with eluant EA10. The polarity of eluant EA10 is greater than that of eluant P10. Consequently, retention times of an enantiomeric pair are shorter using eluant EA10. Thus, eluant EA10 is preferred over eluant P10 for shorter retention times (smaller k' values) and better chromatographic resolution (larger R_s values).

When an identical eluant (eluant EA7) was used, resolution of enantiomeric pairs was less efficient on Chiraspher than on covalently bonded *R*-DNBPG and *S*-DNBL columns (Table I). Elution orders of enantiomeric pairs on Chiraspher (a covalently bonded *S*-CSP) are the same as those on the covalently bonded *S*-DNBL column.

Enantiomeric pairs of oxazepam and its 3-acetate and 1,3-diacetate were efficiently resolved (R_s = 1.46–1.58) on covalently bonded Chiraspher, *R*-DNBPG, and *S*-DNBL columns by using eluant D17EA3 (Table I). Enantiomers of 3-*O*-methyl-oxazepam can be efficiently resolved on Chiraspher and covalently bonded *R*-DNBPG and *S*-DNBL columns by choosing one of several possible eluants (Table I). When the 3% ethanol:acetonitrile (2:1, v/v) in eluant D17EA3 was replaced by 3% 2-propanol (eluant D17P3), retention times of enantiomers were much longer and the enantiomeric pairs were all less efficiently resolved (with smaller R_s values). Based on the results of Table I, eluant D17EA3 is the mobile phase of choice and provides the best overall resolution of enantiomeric pairs of compounds in this study. It should be noted that retention times of enantiomers were sensitive to the exact composition of eluant and were different when different columns with the same CSP were used. Retention

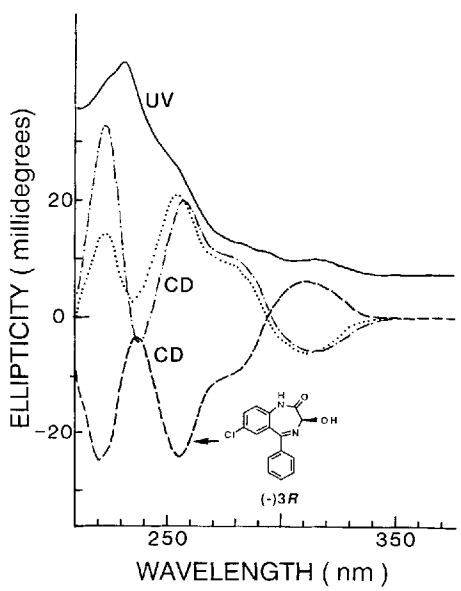

Figure 2—Ultraviolet absorption spectrum of oxazepam (———) and CD spectra of oxazepam enantiomers [(+)3*S* (. . ., *S*/*R* > 20/1) and (−)3*R* (---, *R*/*S* > 30/1); concn. A_{230}/mL = 1.0] and the less strongly retained enantiomer of 3-*O*-methyl-oxazepam [(+)3*S* (-•-•-); optically pure, concn. A_{230}/mL = 1.0; Φ_{223} = 41.5, Φ_{257} = 25.1, and Φ_{315} = −7.1 mdeg, respectively] on the covalently bonded *S*-DNBL column. The UV absorption spectrum of 3-*O*-methyl-oxazepam is similar to that of oxazepam. The actual scale of ellipticity for 3-*O*-methyl-oxazepam in this figure should be multiplied by 1.25.

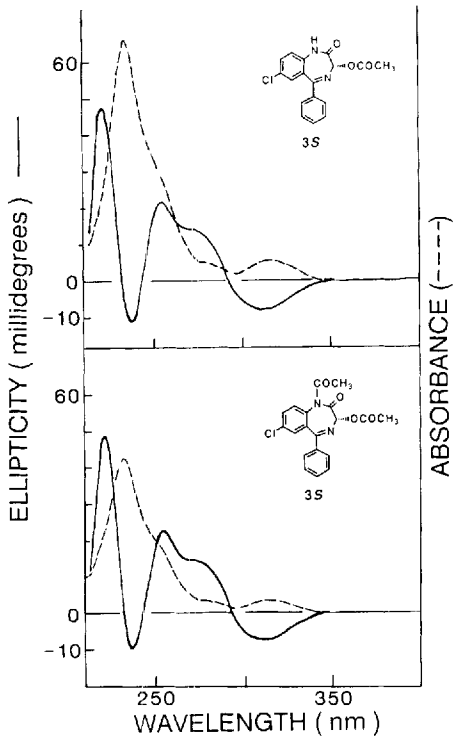

Figure 3—Ultraviolet absorption spectra (------) and CD spectra (——) of the 3*S* enantiomer of oxazepam 3-acetate (upper panel, concn. A_{230}/mL = 1.0, optically pure; Φ_{222} = 47.5, Φ_{255} = 21.8, Φ_{314} = −7.7 mdeg, respectively) and 1,3-diacetate (lower panel, concn. A_{230}/mL = 1.0, optically pure; Φ_{222} = 48.5, Φ_{255} = 22.8, Φ_{314} = −7.7 mdeg, respectively). Both are the less strongly retained enantiomers on the covalently bonded *S*-DNBL column (see Table I).

Table II—Racemization Half-lives of Enantiomeric Oxazepam and 3-*O*-Acyl and 3-*O*-Methyl Derivatives

Enantiomer[a]	Eluant[b]	Racemization Half-life of Enantiomers ($t_{1/2}$), min[a]	$k \times 10^2$, min^{-1}[a]
Oxazepam	EA5	29 ± 1	2.39
	EA7	28 ± 1	2.48
	EA10	23 ± 1	3.01
	EA15	21 ± 1	3.30
	EA20	19 ± 1	3.65
	E10	20 ± 1	3.47
	P10	56 ± 1	1.24
	D17EA3	290 ± 5	0.24
	D17P3	510 ± 5	0.14
Oxazepam 3-acetate	EA7	∞	0
Oxazepam 1,3-diacetate	EA7	∞	0
Oxazepam 3-*O*-methyl	EA7	∞	0

[a] Racemization half-life ($t_{1/2}$, in min), average of two or three determinations, and rate constant (k, in min^{-1}) of enantiomers in the solvent indicated were determined by recording changes of ellipticity of either 3*R* or 3*S* enantiomer at 255 nm as a function of time. [b] Compositions of various eluants are indicated in the legend of Table I.

times were also slightly different on different days, even when an identical CSP column was used. Data in Table I were obtained on the same day by using the identical mobile phase preparation and CSP column.

Because of rapid racemization,[7,12] the CD spectra of oxazepam enantiomers have heretofore not been reported.

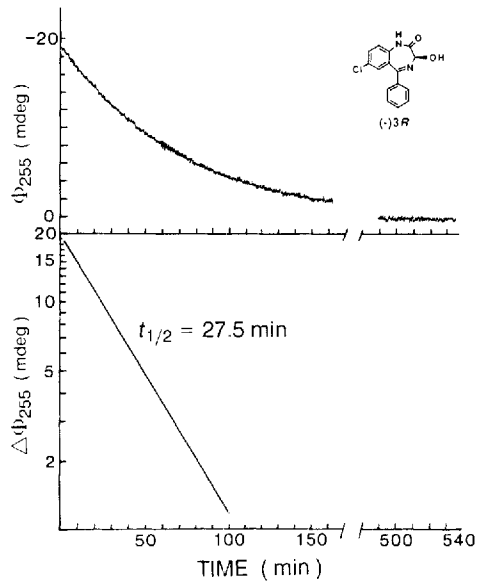

Figure 4—Kinetics of racemization of (−)3*R*-oxazepam in hexane:ethanol:acetonitrile (93:4.67:2.33, vol ratio). Racemization was monitored by recording the increase of ellipticity (in millidegrees) at 255 nm as a function of time. Change in ellipticity of the (−)3*R*-oxazepam was monitored within 20 s of its isolation by CSP-HPLC (see Figure 2).

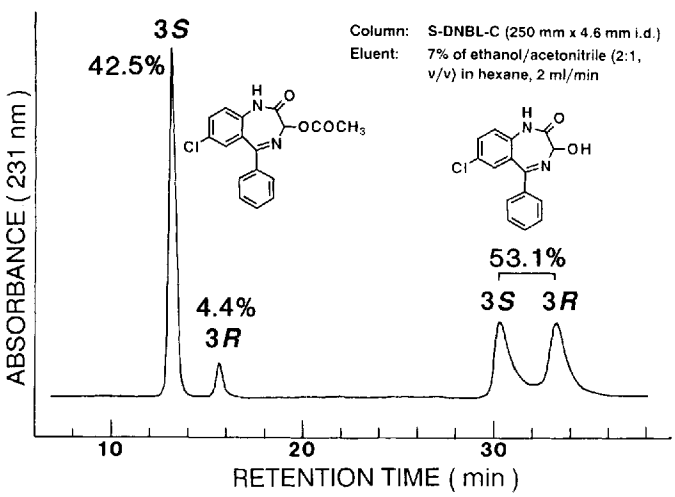

Figure 5—Stereoselective hydrolysis of (±)-3-*O*-acyl-oxazepam by rat liver microsomes. Racemic 3-*O*-acyl-oxazepam (42 nmol/mL of incubation mixture) was incubated with rat liver microsomes (equivalent to 2.67 mg of liver tissue/mL of incubation mixture) for 15 min. Unhydrolyzed 3-*O*-acyl-oxazepam (46.9%) and the hydrolyzed product (oxazepam, 53.1%) were analyzed on a covalently bonded *S*-DNBL column. See the *Experimental Section* and Table III.

However, optical rotation data of enantiomerically impure oxazepam enantiomers have been reported.[7] We were able to measure the CD spectra of oxazepam enantiomers (Figure 2) within 30 s of their separation on CSP-HPLC. The (−) and (+)enantiomers were assigned to have 3*R* and 3*S* absolute configurations, respectively.[7,12] The UV absorption and CD spectra of 3*S*-*O*-methyl-oxazepam, 3*S*-*O*-acyl-oxazepam, and 3*S*-*O*-acyl-1-*N*-acyl-oxazepam of known enantiomeric purities are shown in Figures 2 and 3, respectively. The CD spectral data of 3*S*-*O*-methyl-oxazepam[12] and 3-*O*-acyl-oxazepam enantiomers[5] were previously reported, although spectral data–enantiomeric purity relationships were not established. The CD Cotton effects of enantiomeric oxazepam

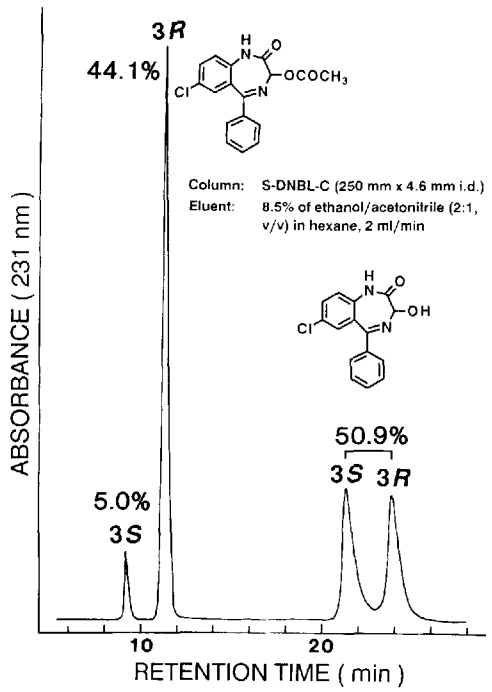

Figure 6—Stereoselective hydrolysis of (±)-3-*O*-acyl-oxazepam by rat brain homogenate. Racemic 3-*O*-acyl-oxazepam (84 nmol/mL of incubation mixture) was incubated with rat brain homogenate (equivalent to 24.8 mg of brain tissue per milliliter of incubation mixture) for 10 min. Unhydrolyzed 3-*O*-acyl-oxazepam (49.1%) and the hydrolyzed product (oxazepam, 50.9%) were analyzed on a covalently *S*-DNBL column. See the *Experimental Section* and Table III.

3-acetates are similar to those of enantiomeric oxazepam hemisuccinates.[17]

The kinetics of racemization of oxazepam enantiomers in various solvents were studied (Table II). The solvents were the eluants used in CSP-HPLC separation of enantiomers. Racemization of an enantiomer was studied by monitoring changes (either increase or decrease) of ellipticity at 255 nm as a function of time (Figure 4) immediately (within 30 s) of its isolation by CSP-HPLC. As shown in Table II, increase of the percentages of ethanol:acetonitrile (2:1, v/v) in eluant EA results in a decrease of the $t_{1/2}$ of racemization. Thus, the rate of racemization is progressively faster in solvents of increasing polarity. Replacing 10% ethanol:acetonitrile in eluant EA10 by 10% 2-propanol in eluant P10 caused $t_{1/2}$ to increase by 2.4-fold (Table II). Similarly, replacing 3% ethanol:acetonitrile in eluant D17EA3 by 3% 2-propanol in eluant D17P3 caused $t_{1/2}$ to increase from 290 to 520 min. Replacing 10% ethanol in eluant E10 by 10% 2-propanol in eluant P10 slowed down the $t_{1/2}$ of racemization from 20 to 56 min. These results suggest that oxazepam enantiomers are more stable (against racemization) in a nonpolar environment. The racemization half-life of oxazepam enantiomers in physiological media is probably shorter than in any of the solvents used in this study. Enantiomers of 3-*O*-acyl, 1-*N*-acyl-3-*O*-acyl, and 3-*O*-methyl derivatives of oxazepam did not undergo racemization in the solvents studied.

Racemization of oxazepam enantiomers was proposed to be due to equilibrium with a tautomer with an open aldehyde form.[14,15] It appears that the open aldehyde tautomer of oxazepam forms more easily as the polarity of solvent increases. Preliminary results indicate that enantiomers of temazepam (with a CH_3 group at N1 of oxazepam) and 3-hydroxyhalazepam (with a CF_3CH_2 group at N1 of oxazepam) also undergo racemization in solvent EA7, with half-lives (~230 and ~580 min, respectively) considerably longer than that of an enantiomeric oxazepam. These results suggest that it is possible to substantially stabilize enantiomers of oxazepam by substituting either a strong electron-donating group or a strong electron-withdrawing group at the N1 position, and possibly by substituents at aromatic ring positions as well. Steric factors of substituents may also influence the stability of enantiomers against racemization.

Table III—Hydrolysis of Racemic and Enantiomeric Oxazepam 3-Acetate by Liver Microsomes (LM) and Brain Homogenate (BH) of Rats

Expt. No.	Oxazepam 3-acetate, nmol/mL	Source of Esterases, mg tissue[a]	Time of Incubation, min	AUC at 231 nm, %[b]			Specific Activity[c]	
				Oxazepam 3*S*-acetate	Oxazepam 3*R*-acetate	Oxazepam (3*S* + 3*R*)	nmol/ min/mg protein	nmol/ min/g tissue
1	± (146)	LM (2.67)	5	50.5	37.6	11.9 (11.0)[d]	58	1203
	± (146)	LM (2.67)	10	50.0	32.6	17.4 (15.9)		
	± (146)	LM (2.67)	15	50.2	27.2	22.6 (21.7)		
	± (146)	LM (2.67)	20	49.9	22.2	27.9 (27.1)		
	± (146)	LM (2.67)	30	49.8	17.8	32.4 (31.2)		
	3*S* (58)	LM (2.67)	10	92.0	0	8.0 (3.7)	4	80
	3*R* (292)	LM (2.67)	5	0	84.9	15.1 (11.3)	120	2471
2	± (29)	LM (2.67)	15	44.3	3.7	52.0 (48.8)		
	± (58)	LM (2.67)	15	48.0	13.0	39.0 (36.2)		
	± (58)	LM (26.7)	15	24.1	0.1	75.8 (73.4)		
3	± (146)	BH (4.65)	5	42.9	49.5	7.6 (6.6)	4.0	414
	± (146)	BH (4.65)	10	36.7	50.0	13.3 (12.1)		
	± (146)	BH (4.65)	15	32.9	49.9	17.2 (16.2)		
	± (146)	BH (4.65)	20	30.0	49.6	20.4 (19.3)		
	± (146)	BH (4.65)	30	19.7	49.3	31.0 (29.8)		
	3*S* (292)	BH (4.65)	10	80.7	0	19.3 (15.1)	9.1	948
	3*R* (58)	BH (4.65)	10	0	96.2	3.8 (2.1)	0.2	26
4	± (58)	BH (8.25)	10	20.4	47.5	32.1 (30.5)		
	± (58)	BH (24.8)	10	5.7	43.9	50.4 (48.9)		

[a] Liver microsomes: 20.6 mg protein/g tissue; brain homogenate: 104.5 mg protein/g tissue. [b] Average values of triplicate samples. [c] Specific activity is calculated only for samples that are closest in the linear range of enzyme kinetics; the values represent lower estimates due to competitive inhibition of the hydrolysis of oxazepam 3-acetate by the product oxazepam (ref 19). [d] Number in parentheses indicates the net percentage of oxazepam 3-acetate hydrolyzed by esterases after subtraction of spontaneously hydrolyzed oxazepam 3-acetate in the control samples that do not have LM or BH.

Rates of hydrolysis of racemic and enantiomeric oxazepam 3-acetates by rat liver microsomes and brain homogenate were studied by CSP-HPLC on a covalently bonded *S*-DNBL column (Figures 5 and 6). The hydrolysis product (oxazepam), enantiomeric composition of unhydrolyzed substrate, and percentage of enzyme-catalyzed hydrolysis can all be determined in one chromatographic run. Although hydrolyses of 3*R*-oxazepam acetate and 3*S*-oxazepam acetate result in 3*R*-oxazepam and 3*S*-oxazepam, respectively, equal amounts of oxazepam enantiomers are always detected (Figures 5 and 6). Oxazepam enantiomers are rapidly racemized in the incubation medium and the solvents used for extraction and CSP-HPLC analysis. The concentrations of substrate and enzyme indicated in Table III were chosen from results of preliminary experiments.

When <32% of the oxazepam 3-acetate is hydrolyzed by rat liver microsomes (experiment 1, Table III), essentially all of the racemic oxazepam 3-acetate hydrolyzed was derived from (3*R*)-oxazepam acetate. Similarly, when <30% of the racemic oxazepam 3-acetate is hydrolyzed by rat brain homogenate, essentially all of the oxazepam 3-acetate hydrolyzed was derived from (3*S*)-acetate (experiment 3, Table III). If these were the only experiments performed, one would have concluded that only (3*R*)-oxazepam acetate is hydrolyzed by liver microsomes and only (3*S*)-oxazepam acetate is hydrolyzed by brain homogenate (i.e., the hydrolysis is stereospecific). However, when the hydrolysis reaction is carried out by using a lower concentration of racemic substrate and/or a higher concentration of esterases, hydrolysis of (3*S*)-oxazepam acetate by rat liver microsomes and hydrolysis of (3*R*)-oxazepam acetate by rat brain homogenate became apparent (experiments 2 and 4, Table III). Hence, the hydrolysis reaction is not stereospecific, but rather stereoselective. Specific activities in the hydrolysis of enantiomerically pure oxazepam 3-acetates by rat liver microsomes were found to differ by ~30-fold; 120 and 4 nmol/min/mg protein for 3*R*-acetate and 3*S*-acetate, respectively (Table III). Thus, when 3*R*-acetate in the substrate pool is exhausted because of its high rate of hydrolysis by rat liver microsomes, 3*S*-acetate is then hydrolyzed, but at a much lower rate. In comparison, 3*S*-oxazepam acetate is hydrolyzed 36-fold faster than 3*R*-oxazepam acetate in brain homogenate. On a per gram of tissue basis, the activity of esterases in brain homogenate in the hydrolysis of racemic oxazepam 3-acetate is ~2.9-fold lower than that in liver microsomes (Table III). Thus, the stereoselectivity in the hydrolysis of oxazepam 3-acetate in brain homogenate is opposite to that in liver microsomes.

The results described above are similar to those reported by Maksay et al.[5] who showed CD spectra of a racemic oxazepam 3-acetate partially hydrolyzed by mouse liver and brain homogenates. The results shown in Table III indicate that the CSP-HPLC method described in this report is considerably simpler and more sensitive than the titrimetric method described previously.[5] The sensitivity of this CSP-HPLC method can be substantially increased by using radiolabeled oxazepam 3-acetate. The results described in this report and those reported by Maksay et al.[5] are in contrast to those reported by Salmona et al.;[17] oxazepam 3-hemisuccinate is hydrolyzed only by esterases in the soluble fraction of cells and (+)-(3*S*)-hemisuccinate is hydrolyzed faster than (−)-(3*S*)-hemisuccinate by soluble esterases of liver and kidney of rats and mice, whereas (−)-(3*R*)-hemisuccinate is hydrolyzed faster by soluble brain esterases of guinea pigs.[18] It appears that various esterases have different catalytic activity and stereoselectivity toward esters of different benzodiazepine structures.

Conclusions

Enantiomers of oxazepam and of 1-*N*- (and/or) 3-*O*-acyl and 3-*O*-methyl ether derivatives of oxazepam can be resolved by several CSP columns. Racemization half-lives of oxazepam enantiomers are longer in solvents of lower polarity. Chromatographic resolutions vary depending on the solvent composition of eluants. Rates of racemization of oxazepam enantiomers in various solvents can be determined by spectropolarimetry. Oxazepam enantiomers may be stabilized against racemization by introducing either an electron-donating alkyl group or a electron-withdrawing group at the N1 position, and possibly by substituents at aromatic ring positions as well. Hydrolysis rates of 3-*O*-acyl-oxazepam enantiomers by esterases are determined by a simple and sensitive CSP-HPLC method. Esterases in liver microsomes and brain homogenate of rats have opposite stereoselectivity in catalyzing the hydrolysis of enantiomeric 3-*O*-acyl-oxazepam; liver microsomes is stereoselective for 3*R*-*O*-acyl-oxazepam and brain homogenate is stereoselective for 3*S*-*O*-acyl-oxazepam.

References and Notes

1. *Pharmacy Times*, April 1987, pp 32–40.
2. *Benzodiazepines—A Handbook: Basic Data, Analytical Methods, Pharmacokinetics and Comprehensive Literature*; Schütz, H., Ed., Springer-Verlag: New York, 1982.
3. Bell, Stanley C.; McCaully, Ronald J.: Gochman, Carl: Childress, Scott J.; Gluckman, Melvyn I. *J. Med. Chem.* **1968**, *11*, 457–461.
4. Möhler, H.; Okada, T. *Science* **1977**, *198*, 849–851.
5. Maksay, Gabor; Tegyey, Zsuzsanna; Otvos, Laszlo *J. Pharm. Sci.* **1978**, *67*, 1208–1210.
6. Maksay, Gabor; Tegyey, Zsuzsanna; Otvos, Laszlo *J. Med. Chem.* **1979**, *22*, 1443–1447.
7. Blaschke, Gottfried; Markgraf, Hildegunde *Chem. Ber.* **1980**, *113*, 2031–2035.
8. Kinkel, J. N.; Fraenkel, W.; Blaschke, G. *Kontakte (Darmstadt)* **1987**, *1*, 3–14.
9. Simonyi, Miklos; Fitos, Ilona; Tegyey, Zsuzsanna *J. Chem. Soc. Chem. Commun.* **1980**, 1105–1106.
10. Fitos, Ilona; Simonyi, Miklos; Tegyey, Zsuzsanna; Otvos, Laszlo; Kajtar, Judit; Kajtar, Marton *J. Chromatogr.* **1983**, *259*, 494–498.
11. Fitos, Ilona; Tegyey, Zsuzsanna; Simonyi, Miklos; Sjoholm, Ingvar; Larsson, Thomas; Lagercrantz, Carl *Biochem. Pharmacol.* **1986**, *35*, 263–269.
12. Pirkle, William H.; Tsipouras, Athanasios *J. Chromatogr.* **1984**, *291*, 291–298.
13. Corbella, Attilio; Gariboldi, Poerluigi; Jommi, Giancarlo; Forgione, Angelo; Marcucci, Franca; Martelli, Paola; Mussini, Emilio; Mauri, Francesco *J.C.S. Chem. Commun.* **1973**, 721–722.
14. Stromar, M.; Sunjic, V.; Kovac, T.; Klasinc, L.; Kajfaz, F. *Croat. Chem. Acta* **1974**, *46*, 265–274.
15. Lhoest, G.; Frigerio, A. In *Advances in Mass Spectrometry in Biochemistry and Medicine*, Vol. II; Frigerio, A., Ed.; Spectrum Publications: London, England, 1976; pp 339–349.
16. Lowry, O. H.; Rosebrough, N. H.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 265–275.
17. Salmona, Mario; Saronio, Carlo; Bianchi, Roberto; Marcucci, Franca; Mussini, Emilio *J. Pharm. Sci.* **1974**, *63*, 222–225.
18. Müller, Walter E.; Wollert, Uwe *Mol. Pharmacol.* **1975**, *11*, 52–60.
19. Maksay, Gabor; Otvos, Laszlo *Drug Metab. Rev.* **1983**, *14*, 1165–1192.

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