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Separation of 1,4-Benzodiazepines and Analogues Using Cholesteryl-10-Undecenoate Bonded Phase in Microcolumn Liquid Chromatography

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Abstract

In this work, 18 1,4-benzodiazepines and derivatives are analyzed using the novel bonded phase cholesteryl-10-undecenoate in microcolumn liquid chromatography. The elution order is found to be remarkably different from that of the ODS phase, which is influenced to a larger extent by the hydrophobicity of the samples. It appears that the retention mechanism of the cholesteryl phase is based on its ability to recognize specific structures found in the molecules. A mixture of benzodiazepines with different basic structures based on modifications of the 1,4-benzodiazepine nucleus is successfully resolved.

Introduction

The novel bonded phase cholesteryl-10-undecenoate has been previously evaluated in our laboratory to determine its characteristic retention mechanism and its molecular recognition capability with regard to the analysis of polycyclic aromatic hydrocarbons. The resulting retention data have already been reported (1), and it was shown that this novel phase was comparable with the ODS phases and that activity for molecular recognition was intermediate to that of the monomeric and polymeric phases.

In the continuing evaluation of this bonded phase, the main focus is on its applicability to the analysis of the 1,4-benzodiazepines and some of its derivatives using microcolumn liquid chromatography. Analyses by gas chromatography (2–7), high-performance liquid chromatography (HPLC) (8–11), thin-layer chromatography (TLC) (10,12), reversed-phase liquid chromatography (13), and other methods (3,7,14–16) have already been reported for this group of compounds. Although these methods offer different degrees of sensitivity and versatility, the search for newer methodologies still continues to address the problems and difficulties in existing methods. Analysis by gas chromatography–mass spectrometry (GC–MS), which is the widely accepted method, presents serious limitations with compounds that are not volatile and/or decomposed by high temperatures. Basically for GC–MS, the benzodiazepines are converted to the corresponding benzophenones to make them volatile for this kind of determination. However, some do not form specific benzophenones, as in the case of the triazolo derivatives, and some form similar benzophenones that make identification of parent benzodiazepines impossible. Nevertheless, GC–MS is still a highly recommended method for positively identifying the presence of such compounds after recovery from biological matrices. In TLC, some benzophenones cannot be detected by the conventional TLC method. The ease of hydrolysis of most of these benzodiazepines with the mobile phases used also presents some problems such as a longer required analysis time. Analysis with HPLC has been performed using fluorometric detection by first converting the benzodiazepines to corresponding acridanones or quinazolines, although direct ultraviolet (UV) detection has been successful. The main problem with HPLC (especially reversed-phase HPLC) is peak tailing, which creates the need to deactivate residual silanols with some organic modifiers and/or deactivators. For other methods, such as capillary electrophoresis (17), the peak height, peak width, and overall separation is influenced by the organic additives in the sample solvent matrix, making it harder to manipulate experimental parameters.

The benzodiazepines are a group of compounds that, as psychotropic agents, are widely used for their varied therapeutic effects either as anxiolytic, anticonvulsant, sedative, or mild

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hypnotic drugs. They are widely used to aid patients with anxiety and sleeping disorders and, hence, have potential for abuse. Many cases of poisoning, accidental and otherwise, have already been reported. For most countries, the benzodiazepines are classified as controlled drugs, and yet they are frequently encountered in clinical and forensic toxicological analyses involving intoxication, overdosage, and traffic accidents and are sometimes implicated in the commitment of crimes. Therefore, the availability of reliable, sensitive, specific, and fast analytical methods for their determination is deemed important.

Experimental

The microcolumn LC system consisted of a microfeeder (MF-2, Azuma Electric, Tokyo, Japan), which served as a pump with a flow rate set at 2 μL/min, a UV detector (UVIDEC-100-III, Jasco, Tokyo, Japan) set at wavelengths of maximum absorbances for the corresponding benzodiazepines, and a microloop injector (model 7619, Rheodyne, Cotati, CA) for sample injection.

The microcolumns used were laboratory-made, fused-silica capillaries (150 × 0.53-mm i.d.) packed with the novel phase cholesteryl-10-undecenoate using a conventional slurry method. The bonding density of this phase was 1.4 μmol/m^2. The microbore LC setup utilized a Shiseido (Tokyo, Japan) Nanospace SI-1 LC system and a Shiseido Superiorex (ODS) column (250 × 1.5-mm i.d.). A Rheodyne 7125 injector was used with a 1-μL sample loop. The flow rate was set at 100 μL/min, and the detection wavelength was 220 nm.

Benzodiazepine samples were kindly provided by Dr. M. Hayashida (Nippon Medical School, Tokyo, Japan). Mobile phases were prepared from chromatographic-grade acetonitrile and deionized water obtained from a Milli-Q water system (Millipore, Tokyo, Japan). Samples were dissolved in methanol at concentrations of 100 ppm. Other solvents used were reagent-grade. Uracil was used as a column dead-volume marker. Results were reported as averages of triplicate determinations.

In the gradient mode using microcolumn LC, the teflon tubing (0.5-mm i.d.) connecting the column to the microfeeder was calibrated so that it was made to contain the required volume of solvent A needed for the first group of peaks to elute. The microfeeder contained solvent B for the next group of peaks. Molecular modeling was carried out using Chem 3D Plus software (Cambridge Scientific Computing, Cambridge, MA).

Results and Discussion

The 1,4-benzodiazepines are considered weak bases. Although they have certain degrees of hydrophobicity, they can form ionic moieties easily and are thus classified as ionogens. The weakly basic properties are due to the nitrogen at position 4 (Figure 1), which can undergo protonation. The protonation at N-4 of the 1,4-benzodiazepines can occur at pK_a values between 1.5 and 3.5 (18). The presence of a nitro group at position 7 (e.g., nitrazepam) can create an acidic character at N-1 unless it is substituted as in the case of flunitrazepam. The 3-hydroxyl derivatives (e.g., oxazepam) can be deprotonated at high pH values, which makes it acidic at this condition. Therefore, the 1,4-benzodiazepines can exhibit acid–base characteristics depending on substituent groups present in the molecule and likewise on pK_a or pH changes. The presence of these groups can therefore change the physicochemical properties of these drugs and may cause specific differences in retention during chromatographic analysis.

A number of 1,4-benzodiazepine analogues have been synthesized over the years. The basic chemical structures of the 1,4-benzodiazepines have been modified in a variety of ways. In this study, the 1,4-benzodiazepines were used together with the following derivatives and analogues (see Figure 2): group A, keto derivatives (including the 3-hydroxyl derivative); group B, triazolo derivatives (triazolam, estazolam, and alprazolam); group C, 4-N-oxide derivative (chlordiazepoxide); group D, thienotriazolodiazepines (brotizolam and etizolam); group E, thienodiazepine (clotiazepam); group F, oxazolo derivatives (oxazolam, cloxazolam, and haloxazolam); and group G, deoxy derivative of diazepam (medazepam).

Retention behavior of the benzodiazepines

For the analysis of benzodiazepine retention behavior in the cholesteryl phase, 18 1,4-benzodiazepines and analogues were used. Relative hydrophobicity (log P values) and the corresponding retention factors at different solvent concentrations are given in Table I. The more hydrophobic samples were expected to be retained over the less hydrophobic ones. Medazepam, which has the highest log P value, registered the longest retention, as expected. However, the relationship was not linear for most of the samples. Some samples with higher log P values eluted ahead of samples with lower log P values. The relationship showed a lower correlation (r^2) as compared with the ODS phase. For zolams, r^2 with ODS was 0.798, and r^2 with cholesteryl was 0.250; for zepams, r^2 with ODS was 0.871, and r^2 with cholesteryl was 0.751. It was apparent that the retention was not primarily dependent on the hydrophobic effects but seemed to be greatly dependent on the presence of specific contributing structures in the molecule.

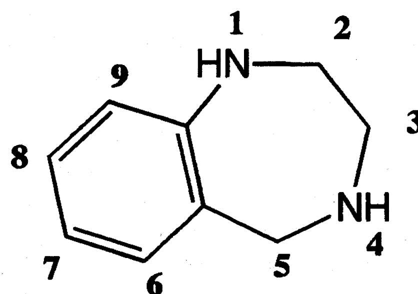

Figure 1. The benzodiazepine parent structure is characterized by the presence of a benzene ring fused to a saturated seven-membered ring with nitrogens at positions 1 and 4.

Comparison of the retention data for the cholesteryl phase of the benzodiazepines with the ODS phase showed a remarkable difference in the elution order. Further inspection of the data seems to suggest that the cholesteryl phase could recognize the basic structure of the parent 1,4-benzodiazepine and could discriminate subsequent modification in the structure. Benzodiazepines having characteristic molecular modifications elute almost as a group within a specific time range. Elution within the group seems to be likewise affected by additional substituents on the parent structure. The following groups were noted based on the retention factor (Table I): group A, the 2-keto derivatives (nitrazepam and clonazepam, oxazepam, flunitrazepam and nimetazepam, and diazepam and fludiazepam); group B, the triazolo derivatives (estazolam,

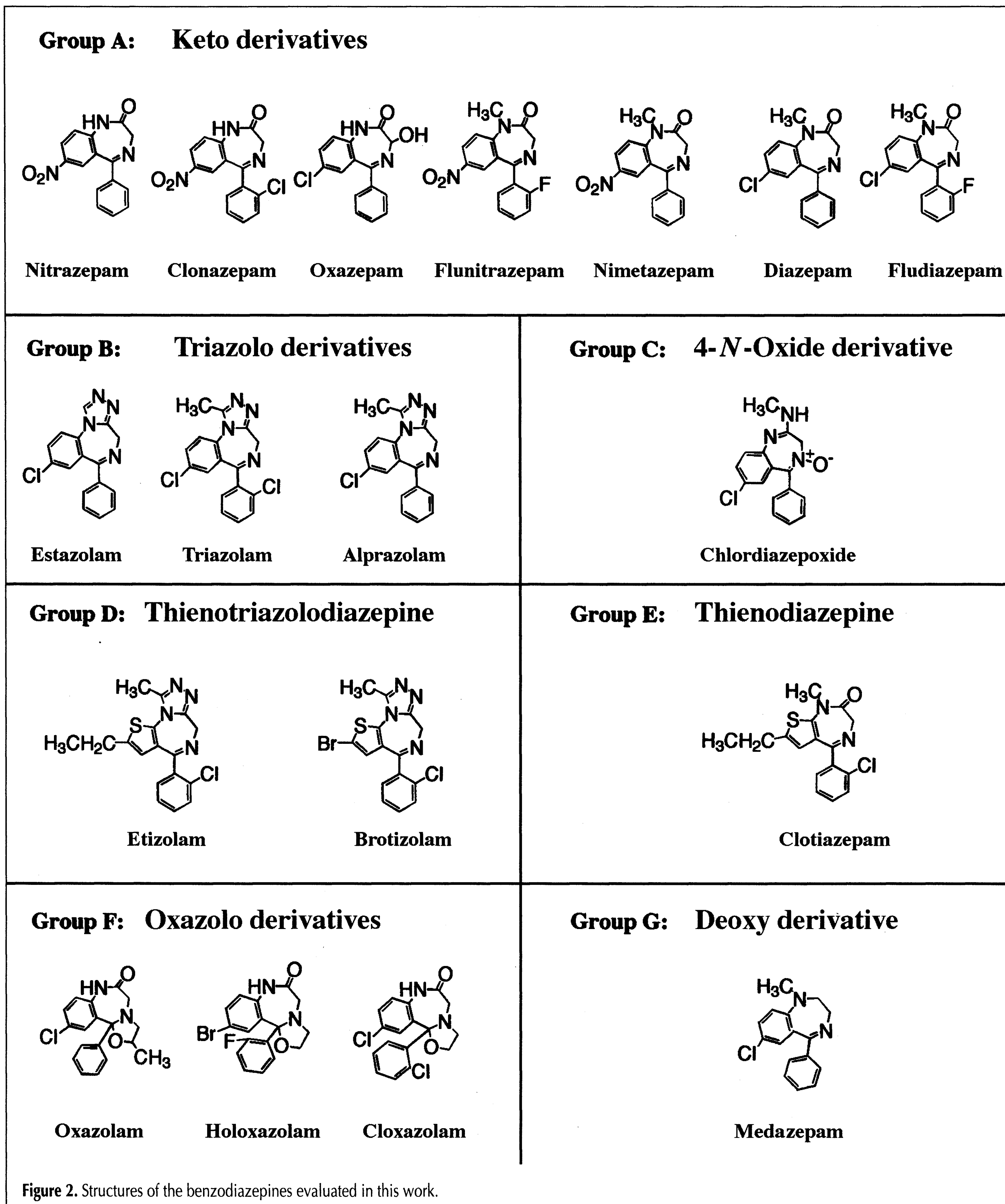

Figure 2. Structures of the benzodiazepines evaluated in this work.

Table I. Molecular Weight, Log *P*, and Retention Factors of 20 Benzodiazepines at Different Mobile Phase Compositions in Cholesteryl and ODS Phases Grouped According to Structure

Group/no.		Benzodiazepines	MW	log *P**	Retention factors					
					Acetonitrile concentration in water (%)					
					35	40	45	50	70	35
					Cholesteryl					ODS
Group A	1	Nitrazepam	281	2.54	0.74	0.61	0.32	0.23	0.04	4.06
	2	Clonazepam	316	2.84	0.84	0.67	0.33	0.25	0.03	4.72
	3	Oxazepam	287	2.10	0.92	0.67	0.35	0.26	0.07	3.76
	4	Flunitrazepam	313	2.35	0.95	0.78	0.39	0.27	0.04	6.54
	5	Nimetazepam	295	2.21	1.04	0.84	0.42	0.31	0.05	7.28
	6	Diazepam	285	3.18	1.38	1.3	0.62	0.41	0.08	13.6
	7	Fludiazepam	303	2.70	1.42	1.20	0.53	0.37	0.07	11.1
Group B	8	Estazolam	295	2.79	1.30	1.01	0.52	0.43	0.14	3.84
	9	Triazolam	342	3.20	1.63	1.27	0.63	0.50	0.16	4.68
	10	Alprazolam	309	3.20	1.66	1.30	0.65	0.54	0.19	4.8
Group C	11	Chlordiazepoxide	300	2.44**	1.71	1.27	0.72	0.63	0.20	4.84
Group D	12	Etizolam	343	3.10	1.75	1.35	0.65	0.51	0.15	3.84
	13	Brotizolam	394	2.83	1.76	1.38	0.66	0.52	0.15	6.34
Group E	14	Clotiazepam	319	3.20	2.26	1.75	0.76	0.53	0.10	23.7
Group F	15	Oxazolam	329	4.32	2.70	2.24	1.06	0.67	0.08	14.82
	16	Haloxazolam	377	3.50	3.67	2.60	1.52	1.12	0.24	29.14
	17	Cloxazolam	349	3.90	15.84	12.03	8.21	4.58	1.79	17.05
Group G	18	Medazepam	271	4.47	20.08	14.10	8.34	5.84	1.91	46.1

* Calculated log *P* values taken from *Comprehensive Medicinal Chemistry*, Volume 6, Pergamon Press, UK, 1990.
** Experimentally determined, also taken from *Comprehensive Medicinal Chemistry*, Volume 6, Pergamon Press, UK, 1990.

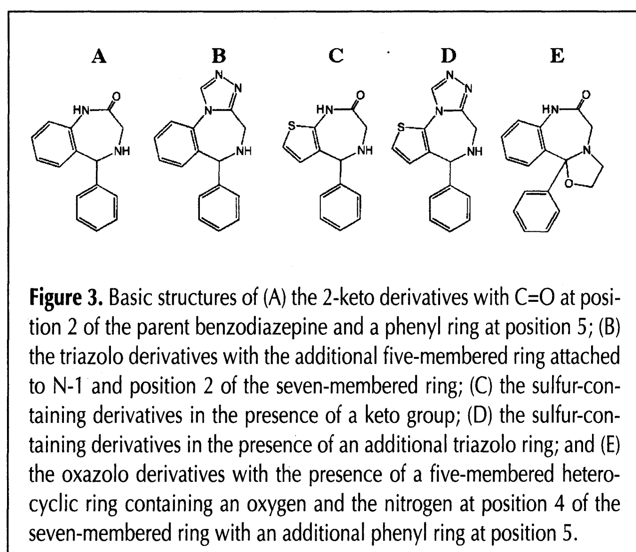

Figure 3. Basic structures of (A) the 2-keto derivatives with C=O at position 2 of the parent benzodiazepine and a phenyl ring at position 5; (B) the triazolo derivatives with the additional five-membered ring attached to N-1 and position 2 of the seven-membered ring; (C) the sulfur-containing derivatives in the presence of a keto group; (D) the sulfur-containing derivatives in the presence of an additional triazolo ring; and (E) the oxazolo derivatives with the presence of a five-membered heterocyclic ring containing an oxygen and the nitrogen at position 4 of the seven-membered ring with an additional phenyl ring at position 5.

triazolam, and alprazolam); group C, the 4-*N*-oxide derivative (chlordiazepoxide); group D, the thienotriazolodiazepines (etizolam and brotizolam); group E, the thienodiazepines (clotiazepam); group F, the oxazolo derivatives (oxazolam, haloxazolam, and cloxazolam); and group G, the deoxy derivative (medazepam).

Benzodiazepines in group A were all found to be 2-keto derivatives of the 1,4-benzodiazepines characterized by the presence of a ketone (C=O) at position 2 (Figure 3A). Nitrazepam and clonazepam both contain an additional nitro group at position 8. However, clonazepam differs from nitrazepam only in the presence of chlorine at position 2 of the phenyl ring attached to position 5 of the seven-membered ring, whereas oxazepam contains a hydroxyl substituent at position 3, and instead of a nitro group, chlorine is attached to position 8. Flunitrazepam and nimetazepam are characterized by the presence of the methyl group attached to N-1 and differs only at the 5-phenyl ring where the presence of fluorine is found at position 5 for flunitrazepam. Diazepam and fludiazepam contain a methyl group at N-1, and instead of a nitro group, a chlorine is found at position 8. The difference is the presence of fluorine at position 2 in the five-phenyl ring.

Group B belongs to the triazolo derivatives characterized by the presence of an additional five-membered ring (Figure 3B) that includes N-1 and C-2 of the parent benzodiazepine and two additional nitrogen atoms at positions 3 and 4 relative to N-1. Position 8 contains chlorine. This group includes estazolam, triazolam, and alprazolam. Triazolam and alprazolam are characterized by an additional methyl group at position 2 of the five-membered ring. These two differ only in the presence of chlorine at position 2 of the five-phenyl ring.

It was apparent from the retention data of the first two groups that substitution of a methyl group at N-1 increased the retention. However, this effect was less when there was a nitro group present at position 8 and enhanced with the presence of a chlorine. Likewise, the presence of a methyl group at position 2 of the triazolo ring also increased retention (i.e., estazolam eluted before triazolam and alprazolam). In the case of estazolam, the presence of the triazolo ring without the methyl group enabled it to elute almost at the same time if not ahead of diazepam and fludiazepam, which belong to the 2-keto derivatives.

Group C was a 4-*N*-oxide derivative modified at position 4, as exemplified by chlordiazepoxide. It was also characterized by the presence of a secondary amine at position 2, instead of the keto group, with a methyl group attached to it. Elution was intermediate to the triazolo derivatives and the thienotriazolodiazepines.

Groups D and E were typical examples of S-containing benzodiazepine analogues. Brotizolam and etizolam are thienotriazolodiazepines, whereas clotiazepam is a thienodiazepine. In clotiazepam, instead of a six-membered ring attached to the seven-membered ring, a five-membered ring containing sulfur at position 9 is found (Figure 3C). It is also a 2-keto derivative with a methyl group attached to N-1. The thienotriazolodiazepines, aside from the five-membered sulfur ring, contain the triazolo ring similarly found in group B (Figure 3D). The thienotriazolodiazepines eluted ahead of the thienodiazepine. This can be attributed to the presence of the triazolo ring; the presence of the methyl group at N-1 in clotiazepam contributed to its increased retention. The presence of the ethyl group in position 8 of the sulfur ring in etizolam possibly decreased its retention as compared with brotizolam, which contains a bromine in the same position. But this difference is not very remarkable. They eluted almost at the same time.

Group F was oxazolo derivatives with the presence of an additional five-membered ring containing an oxygen attached to the N-4 and position 5 of the seven-membered ring. Oxygen is found at position 4 relative to the N-4 (Figure 3E). Increased retention was noted with the presence of this heterocyclic ring. In this case, it was also noted that the presence of the methyl group at position 3 of the oxazolo ring relative to N-4 did not have the same effect as in the 2-keto derivative and the triazolo ring (an increase in retention). In this case, the orientation and/or attachment of the halogen greatly affected retention. Cloxazolam, characterized by the presence of two chlorine atoms, was greatly retained as compared with oxazolam and haloxazolam. It seems that the presence of a halogen on the phenyl ring attached to position 5 of the seven-membered ring at the same side of the oxazolo ring affected the retention. The fluorine in the phenyl ring attached to position 5 on the opposite plane of the oxazolo ring also affected the retention of haloxazolam but to a lesser degree than that of cloxazolam. Oxazolam's phenyl ring at position 5 does not contain any substituent, and this probably caused the early elution as compared with the two former compounds.

Group G was medazepam, which is a deoxy derivative of diazepam. It was apparent that the absence of the ketone at position 2 of the benzodiazepine nucleus was responsible for the marked increased in elution time. The medazepam was retained very strongly by the cholesteryl phase. This was also due to the fact that medazepam has considerable hydrophobicity. Increasing the acetonitrile concentration decreased the elution time and improved the peak shape.

By looking at the two-dimensional structure of the cholesteryl-10-undecenoate bonded phase (Figure 4A), it can be seen that it has three important structures that could influence the molecular recognition capability: the carbonyl group, the cholesteryl moiety, and the long alkyl chain. Furthermore, the cholesteryl phase is also characterized by a flat surface on one side and a slightly curved surface on the other side, as demonstrated in Figure 4B using a three-dimensional structure obtained by Chem3D software. Some additional basic characteristics of this phase, as compared with the ODS phase, are given in Table II.

Molecular modeling by Chem3D Plus using nitrazepam and medazepam to illustrate possible sample and phase interactions showed that nitrazepam had less interaction with the stationary phase than medazepam. For both samples, their hydrophobic portions were oriented toward the hydrophobic and planar portion of the phase. The nitrazepam was characterized by the presence of a polar keto group and a nitro group that interacted with the polar solvent to a greater extent than with the phase and was thus least retained (Figure 5A). It was also noted that, compared with nitrazepam, medazepam had a larger surface area that came into contact with the cholesteryl phase (Figure 5B). As it passed through the phase, it was retained along the alkyl chain to a greater extent than nitrazepam, due to hydrophobic interaction. The carbonyl group found between the alkyl chain and the cholesterol moiety also had an influence on the retention of medazepam because it could interact with the N-4 of the seven-membered ring, which was made more reactive by the presence of an electron-withdrawing group (CH_3) found at N-1.

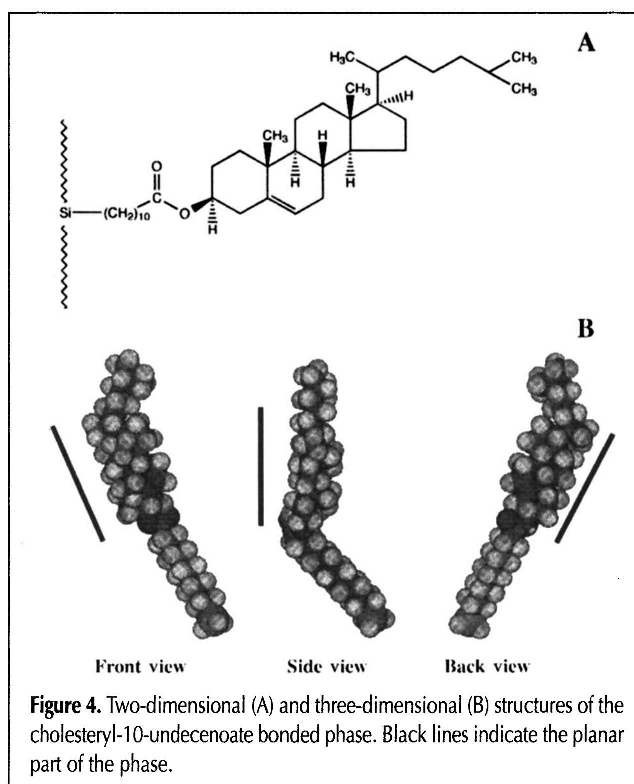

Figure 4. Two-dimensional (A) and three-dimensional (B) structures of the cholesteryl-10-undecenoate bonded phase. Black lines indicate the planar part of the phase.

Table II. Basic Characteristics of the Cholesteryl and ODS Stationary Phases

Stationary phase	Pore size (Å)	Carbon content (%)	Surface coverage (μmol/m²)
Cholesteryl	300	6.5	1.5
ODS	100	24	3.5

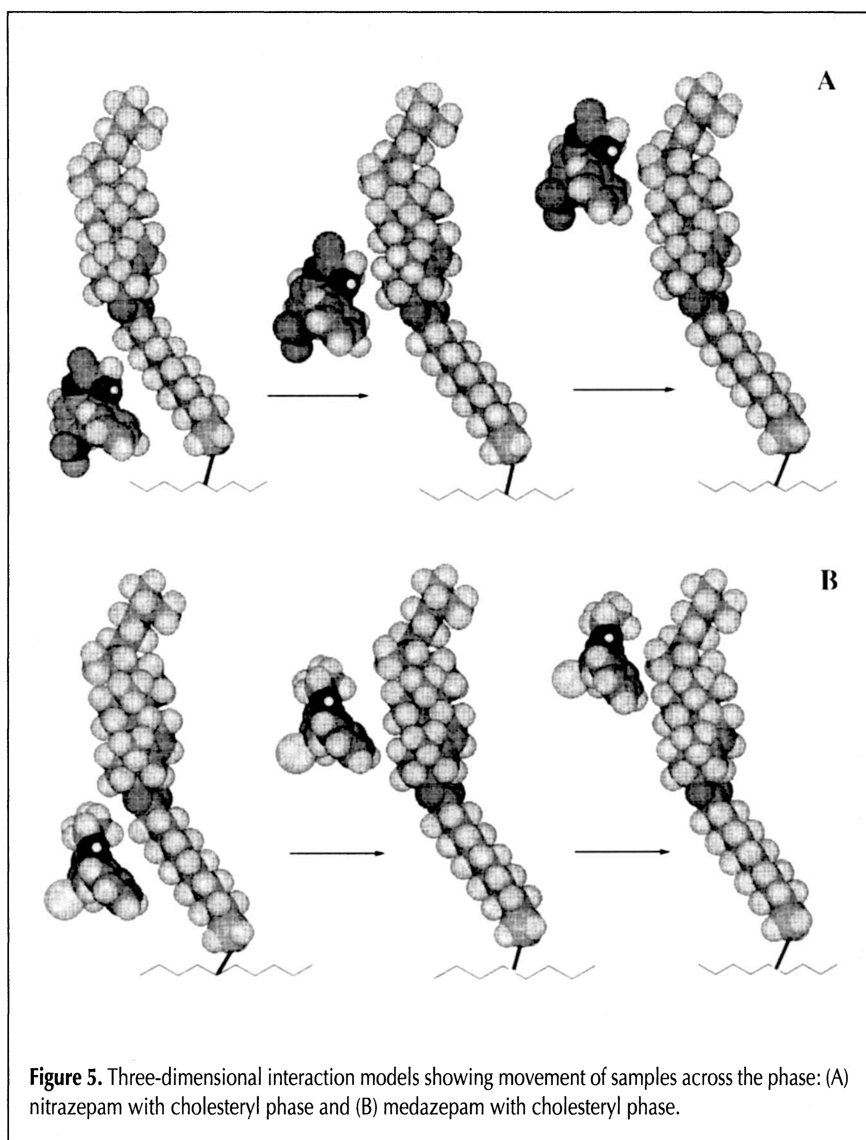

Figure 5. Three-dimensional interaction models showing movement of samples across the phase: (A) nitrazepam with cholesteryl phase and (B) medazepam with cholesteryl phase.

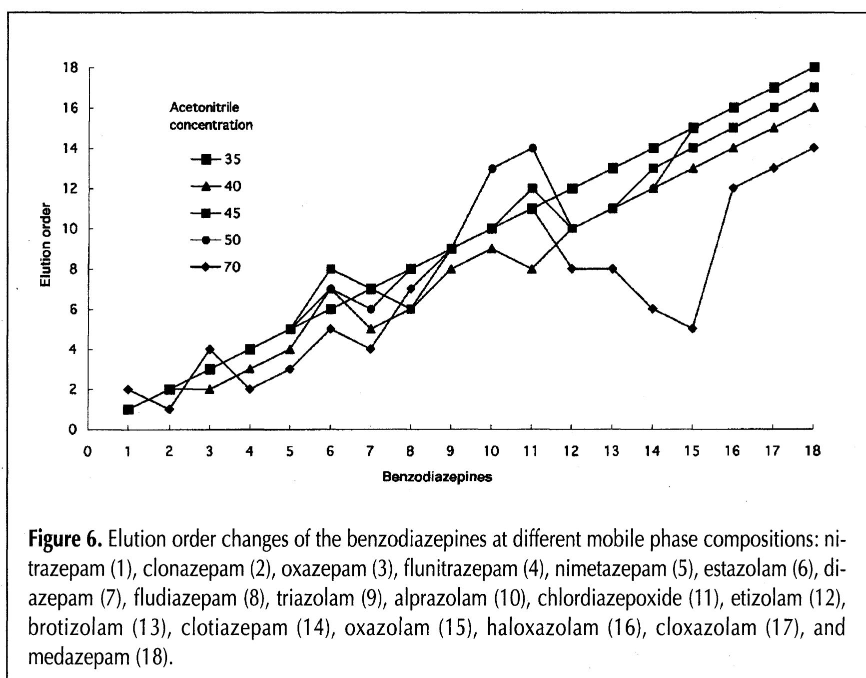

Figure 6. Elution order changes of the benzodiazepines at different mobile phase compositions: nitrazepam (1), clonazepam (2), oxazepam (3), flunitrazepam (4), nimetazepam (5), estazolam (6), diazepam (7), fludiazepam (8), triazolam (9), alprazolam (10), chlordiazepoxide (11), etizolam (12), brotizolam (13), clotiazepam (14), oxazolam (15), haloxazolam (16), cloxazolam (17), and medazepam (18).

Effect of increasing acetonitrile concentration

Increasing the acetonitrile concentration did not seem to affect the elution order except to further differentiate them into groups (Figure 6). Estazolam, which eluted ahead of diazepam and fludiazepam, now eluted after the two, and the retention value was now closer to those of triazolam and alprazolam, which have the same basic structure at about 50% acetonitrile concentration. The most notable change can be observed with oxazolam. At higher acetonitrile concentrations up to 70%, the retention was reduced considerably, and the peak shape was improved. The rest of the benzodiazepines became weakly retained as the acetonitrile concentration increased.

Isocratic and gradient elution of a mixture of benzodiazepines

A mixture of nine benzodiazepines were separated successfully by the cholesteryl phase in the isocratic elution mode (Figure 7A) using 35% acetonitrile in water. It contained nitrazepam, a keto derivative (group A); nimetazepam, an N-1-methylated keto derivative (group A); estazolam, a triazolo derivative (group B); brotizolam, a thienotriazolodiazepine (group D); clotiazepam, a thienodiazepine (group E); oxazolam, haloxazolam, and cloxazolam, three oxazolo derivatives (group F); and medazepam, a deoxy derivative of diazepam (group G). These results again showed that the cholesteryl phase could discriminate basic modifications in the 1,4-benzodiazepine structure, and such modifications become the basis for the successful separation in microcolumn LC. The ODS likewise successfully separated these benzodiazepines, although with a different elution order (Figure 7B). It became more apparent that the elution mechanisms governing the two stationary phases were remarkably different.

However, we observed markedly increased retention times for the last two peaks and performed separation by gradient elution to reduce the separation time. This also improved the resolution of the last two peaks by the increased percentage of the organic solvent, acetonitrile (Figure 8). Complete separation by gradient elution was successful using 30% acetonitrile in water as solvent A and 60% acetonitrile in water as solvent B.

Conclusion

This study has shown that the cholesteryl-10-undecenoate bonded phase has a remarkable capability to recognize basic structures of molecules and can subsequently recognize modified derivatives of such structures, as in the case of the 1,4-benzodiazepines and some of its analogues. This will find considerable application in the analysis of samples that differ only in the presence of additional substructures or substituent groups. This elution mechanism differs greatly from that exhibited by the ODS phase. This difference is probably due to the presence of the carbonyl group, which can interact with a number of different substituents in the molecule and the long alkyl chain that gives added mobility to the stationary phase. The cholesteryl moiety also enhances the selectivity of the phase through possible π–π interactions and steric effects.

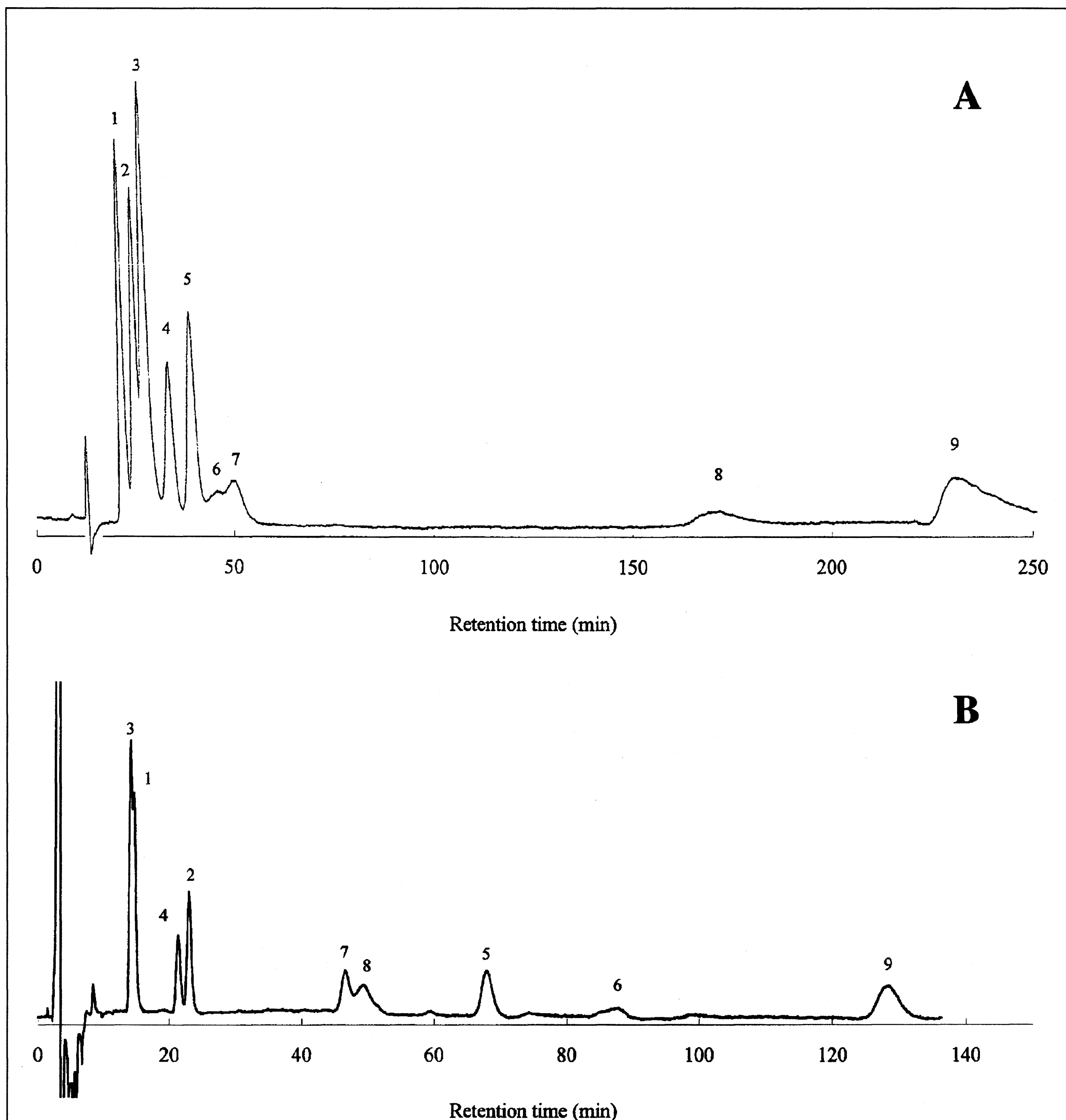

Figure 7. Chromatograms of mixture of benzodiazepines in (A) cholesteryl and (B) ODS phases at 35% acetonitrile concentration in water as the mobile phase. Peaks: 1, nitrazepam (group A); 2, nimetazepam (group A); 3, estazolam (group B); 4, brotizolam (group D); 5, clotiazepam (group E); 6, oxazolam (group F); 7, haloxazolam (group F); 8, cloxazolam (group F); and 9, medazepam (group G).

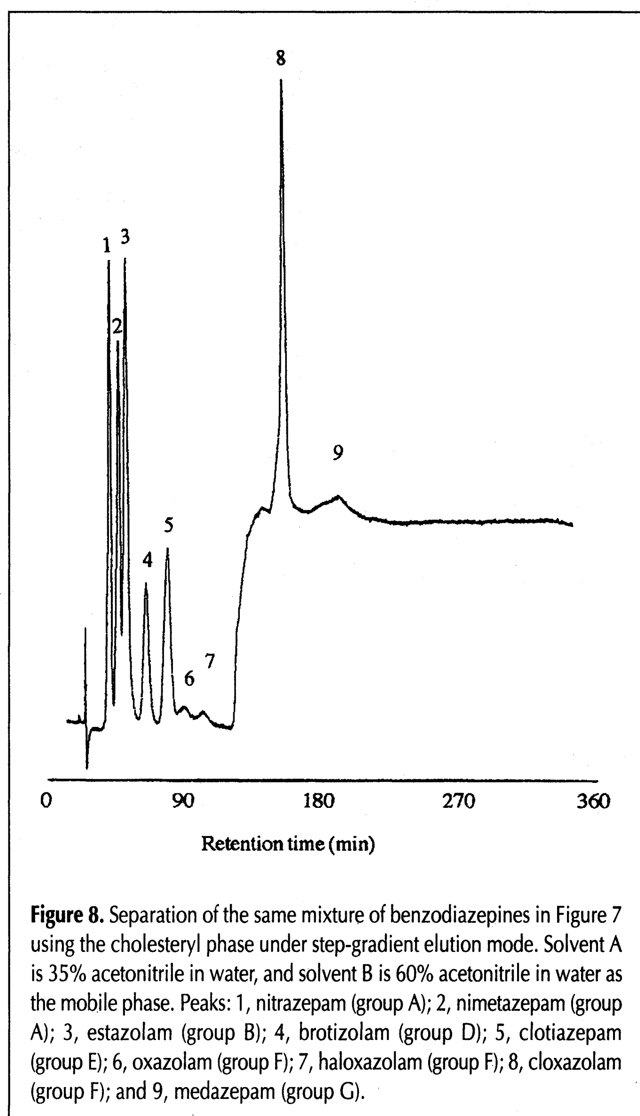

Figure 8. Separation of the same mixture of benzodiazepines in Figure 7 using the cholesteryl phase under step-gradient elution mode. Solvent A is 35% acetonitrile in water, and solvent B is 60% acetonitrile in water as the mobile phase. Peaks: 1, nitrazepam (group A); 2, nimetazepam (group A); 3, estazolam (group B); 4, brotizolam (group D); 5, clotiazepam (group E); 6, oxazolam (group F); 7, haloxazolam (group F); 8, cloxazolam (group F); and 9, medazepam (group G).

These varied structures in the cholesteryl phase enhances its molecular recognition capability. It is therefore suggested to further evaluate this unique retention mechanism using other samples within an analogous series.

Acknowledgments

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