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Changes in chiral selectivity with temperature for an ovomucoid protein-based column

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ABSTRACT

The effect of temperature on the direct HPLC separation of drug enantiomers was studied for the commercially available ovomucoid protein chiral stationary phase. We confirm that temperature is a valuable experimental parameter for optimizing separations of racemic drugs. The resolution of certain acidic and basic drugs responds differently with temperature change. However, these resolution changes involve more than the effect of temperature on mobile phase viscosity that influences column plate numbers. A simple strategy involving the selection of pH, organic modifier and temperature is presented for optimizing separations of drug enantiomers with this column.

INTRODUCTION

Chiral HPLC separations are of special concern to pharmaceutical and agrichemical industries because of increased regulatory pressure. Currently there are several types of columns used to separate racemic mixtures into their component enantiomers for analytical and preparative applications. Separation columns include the "Pirkle"-type, inclusion-formation systems such as the cyclodextrins, various cellulose-based materials, ligand exchangers, and those based on various immobilized proteins. The utility of these columns now has resulted in some systematic studies that define applications and the manner in which these systems should be operated for satisfactory results [1–5].

Previous studies have shown that a silica-based column packing with an ovomucoid protein stationary phase (Ultron ES-OVM) permits excellent separations of many racemic drugs [6–8]. A large variety of acidic, basic and neutral racemic drugs have been separated into their enantiomers in about 15 min with this column [9]. We focused our attention on this protein-based column because of its high stereoselectivity, broad applicability and compatibility with the aqueous buffered mobile phases widely used in many biological systems. A simple procedure was proposed for optimizing separations with this column by adjusting aqueous mobile phase pH and the type and concentration of the organic modifier [9].

Columns with stationary phases of bovine serum albumin (BSA) [10], cellulase [11], and "Pirkle"-type phases [12] previously have shown enantioselectivity changes with variation in the separation temperature. This study concerns the influence of temperature on band spacing and resolution of enantiomers separated on the ovomucoid protein column. We find that the selectivity factor, α, and separation resolution, R_s, is significantly influenced by column temperature for the ovomucoid column. Certain results obtained during this work with model drugs and proprietary compounds not herein described are somewhat different from those previously reported for protein-based columns [13]. These studies include a data base of various racemates, representing acids, bases and neutral drugs.

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Characteristic patterns regarding temperature effects now permit the integration of this parameter into the previously proposed scheme for rapidly optimizing the resolution of drug enantiomers.

EXPERIMENTAL

Apparatus and column

The HPLC apparatus used a Model LC-600 pump (Shimadzu, Columbia, MD, USA). A Model 7125 loop injector valve (Rheodyne, Cotati, CA, USA) and a Spectraflow 783 variable-wavelength UV detector (ABI, Ramsey, NJ, USA) completed the chromatographic apparatus. The UV detector was set at the wavelength of maximum absorption for each compound. A Multichrom Version 2.0 data system (VG Laboratory Systems, Manchester, UK) acquired and analyzed the data. Sample injections were 2 μl of a 1 mg/ml solution of each drug in ethanol. Column temperatures of 10–35°C were controlled by placing the column in a Model RBC-3 circulating bath (Neslab Instruments, Newington, NH, USA). At least 30 min were allowed for re-equilibration after each temperature change.

Ovomucoid protein-based analytical and guard columns (Ultron ES-OVM, 15 × 0.60 cm, and Zorbax-Diol, 1.0 × 0.4 cm, respectively) were obtained from Mac-Mod Analytical (Chadds Ford, PA, USA). The ovomucoid protein (molecular mass 28 800; isoelectric point 3.9–4.0) is immobilized on 5-μm silica particles with pores of 12 nm. The protein structure contains 0.3 sialic acid residues, 9 disulfide bridges and 30% carbohydrate per molecule.

Reagents and chemicals

Reagent-grade potassium dihydrogenphosphate, potassium hydroxide, HPLC-grade water, methanol and acetonitrile were obtained from J.T. Baker (Phillipsburg, NJ, USA). Absolute ethanol was from Quantum Chemicals (Cincinnati, OH. USA). Lorglumide was synthesized by ZENECA Pharmaceuticals (Wilmington, DE, USA). Dr. I.W. Wainer of McGill University (Montreal, Canada) kindly provided the halofantrine hydrochloride. Warfarin, 8-OH-DPAT [(±)-8-hydroxydipropylaminotetralin] hydrobromide, verapamil, pindolol, disopyramide, lorazepam and SKF 83566 [(±)-7-bromo-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1-H-3-benzapine hydrochloride] were obtained from Research Biochemicals (Natick, MA, USA) or Sigma (St. Louis, MO, USA). Structures of the non-proprietary racemic drugs studied are given in Fig. 1.

Chromatographic conditions and procedures

Phosphate buffers of pH 5.0–7.5 were prepared from 10 m*M* potassium dihydrogenphosphate adjusted to the desired pH with 1.0 *M* potassium hydroxide. The organic modifier was acetonitrile or ethanol. The desired pH for the mobile phase was established at 10°C, then an organic modifier was appropriately added to give solute k' values in the 4–10 range. Sample injections containing 1–2 μg of solute were from stock solutions of the test racemates at 1 mg/ml in ethanol. The effects of pH and temperature on the separation of halofantrine were studied at the optimum and two additional pH values.

RESULTS AND DISCUSSION

Table I summarizes chromatographic data from an ovomucoid protein column with basic, acidic and neutral drugs, respectively. Results were obtained at different pH values with temperatures of 10–35°C (recommended range for this column). Included in this table are the retention time (t_R) and column plate number (N) values for the first-eluting enantiomer. Also included are selectivity factors (α) and resolution values (R_s) for the two drug enantiomers.

Fig. 2A graphically represents the effect of temperature on plate number for all of the drugs at various pH values. It is well known that higher temperatures increase column efficiency because of decreased mobile phase viscosity and increased solute diffusion rates for improved mass transfer. This pattern of temperature effect is general for all drug types studied. Limited data suggest that plate numbers are changed less at lower pH values as temperature varies.

Fig. 2B shows the effect of temperature on α values for the drugs studied. Solutes generally

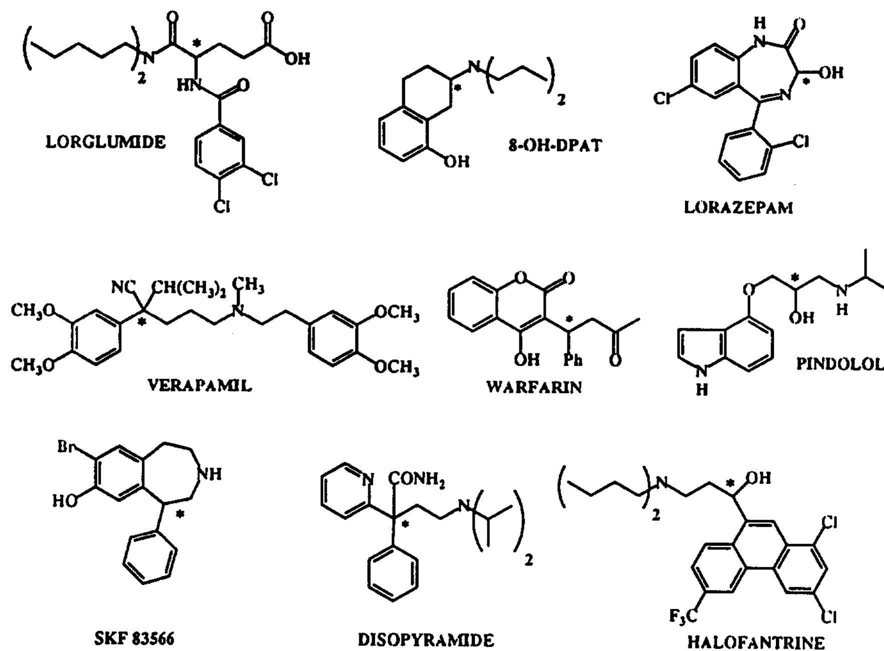

Fig. 1. Structures of racemic drugs studied.

exhibit larger α values as temperature decreases. The exception is 8-OH-DPAT · HBr that exhibits no variation in α with temperature change. For the basic drug, halofantrine, α values are less influenced by temperature change at lower pH values.

Fig. 2C summarizes the effect of temperature on resolution of the drugs. Neutral compounds show higher resolution at lower temperatures. Conversely, the resolution for basic drugs generally improves with increasing temperature, reaching optimum, then decreases as temperature increases. Increased retention and enantioselectivity with increasing column temperatures were reported for racemic propranolol with another silica-immobilized protein, cellulase [11]. As found for neutral drugs with the ovomucoid column, an acidic drug shows decreased resolution with temperature increase.

Changing the ovomucoid column temperature normally produces a linear ln k' *vs.* $1/T$ relationship (the so-called Van 't Hoff plot; see ref. 10). This linear temperature effect also has been observed in this laboratory for several applications involving acidic and other drugs. However, the basic drug, halofantrine, shows an entirely different pattern with temperature change, as illustrated in Fig. 3. Van 't Hoff plots for this drug at pH 6.0 and 7.0 show the usual close linear fit. However, in Fig. 3A the Van 't Hoff plots for the two enantiomers at pH 5.5 show much greater deviation from linearity than usual ($r^2 = 0.45$ and 0.86). The data at pH 5.5 more logically fits a curved line relationship. As given in Fig. 3B, α values for pH 6.0 and 7.0 again show a linear decrease ($r^2 > 0.95$) with temperature increase. At pH 5.5, however, the change in the α value is not linear: α is constant from about 10 to 20°C, then decreases with temperature increase. At pH 5.5 this drug should be highly ionized. We speculate, therefore, that since interaction of the ionized form with the protein stationary phase is significantly affected by temperature, this effect largely is due to modifications in the ability of the protein stationary phase to interact chirally. Similar effects were reported in the chiral recognition of tryptophan by silica-immobilized albumin [10]. These results suggest a thermally induced change in the conformation of the bound protein under pH 5.5 conditions.

The three-dimensional plot in Fig. 4 shows the

TABLE I

THE EFFECT OF TEMPERATURE ON THE CHIRAL SEPARATION OF DRUGS ON OVOMUCOID PROTEIN COLUMN

Compound	pH	Temperature (°C)	α	R_s	N^a	t_R (min)
Acidic						
Lorglumide	6.5	10	2.4	6.0	1666	6.5
		20	2.0	5.5	2851	5.5
		25	2.2	4.6	1787	4.8
		35	1.9	4.3	2921	4.3
Neutral						
Verapamil	6.2	10	1.2	1.0	1090	20.2
		20	1.1	0.83	1637	14.3
		25	1.1	0.72	2560	12.1
		35	1.1	0.7 (estimated)	N/A[b]	10.2
Warfarin	6.0	10	1.6	4.1	1560	15.7
		20	1.4	2.8	2389	12.0
		25	1.3	2.9	3095	10.5
		35	1.2	1.8	3335	8.2
Basic						
Halofantrine	5.5	10	2.7	4.9	865	7.5
		20	2.7	5.9	1312	7.2
		25	2.6	6.7	1600	7.0
		35	2.4	6.1	2114	6.0
	6.0	10	2.5	3.6	891	4.7
		20	2.3	4.9	1751	5.0
		25	2.2	4.4	1438	5.1
		35	2.0	4.3	1537	4.9
	7.0	10	3.0	5.4	820	6.1
		20	2.7	6.5	1476	6.3
		25	2.5	6.3	2078	6.2
		35	2.2	5.3	2420	5.6
8-OH-DPAT	6.0	10	1.3	2.0	2850	6.4
		20	1.3	2.5	2844	6.3
		25	1.3	2.3	3782	6.4
		35	1.3	2.6	4538	6.5
Disopyramide	7.0	10	1.2	1.3	2138	16.3
		20	1.2	1.4	2508	16.4
		25	1.1	1.4	2678	16.3
		35	1.1	1.4	3191	16.1
Pindolol	5.0	10	1.4	2.7	1523	20.1
		20	1.3	2.7	2052	15.4
		25	1.3	2.5	1777	13.7
		35	1.3	2.4	2205	10.4

[a] Calculations for first-eluting enantiomer based on peak width.
[b] Not available.

effect of both pH and temperature on the resolution of the basic drug, halofantrine. This presentation clearly illustrates the advantage of separations at either pH 5.5 or 7.0 at an optimum temperature of about 25°C.

These results suggest that the decrease in chiral resolution of an acidic drug with increasing temperature depends on the strong chiral interactions affecting α values. Column efficiency improves at higher temperatures, as indicated in Fig. 2A. (Similar results have been obtained in this laboratory with other proprietary acidic chiral drugs.) However, this increased column efficiency is more than overcome by the decrease in α values with higher temperatures, as shown in Fig. 2B. These results suggest that highest resolution for neutral and acidic drugs are at lower temperatures.

The effect with basic drugs can be quite different. Chiral resolution apparently can in-

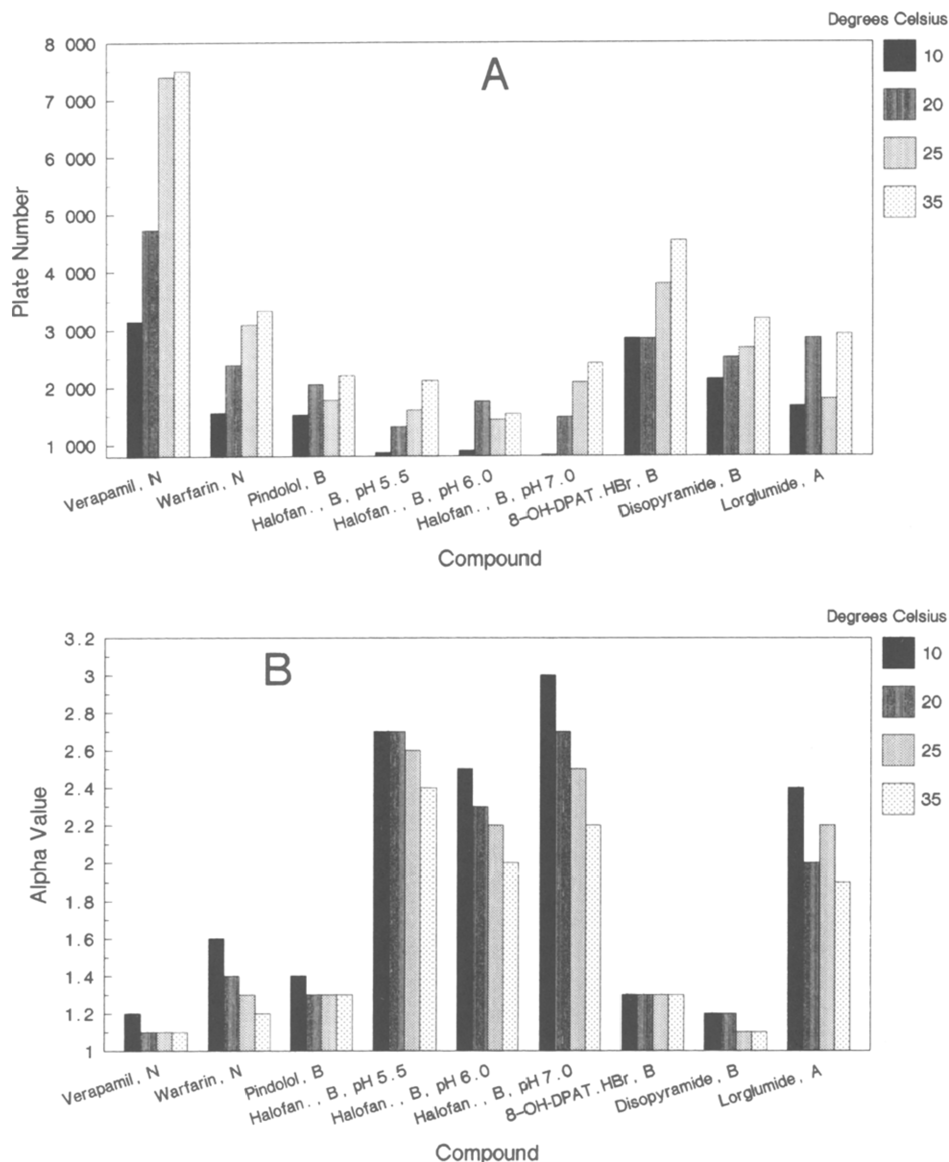

Fig. 2.

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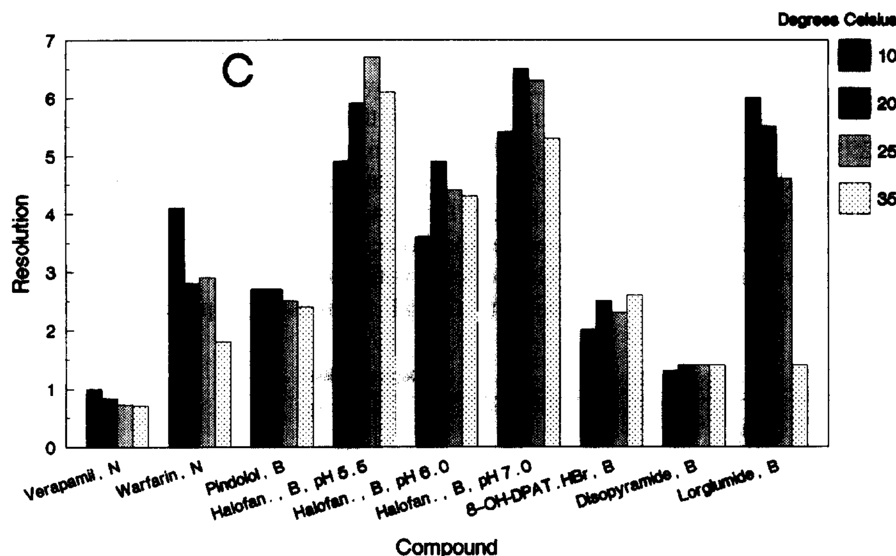

Fig. 2. Effect of temperature on separation of racemic drugs. Column: 15 × 0.60 cm Ultron ES-OVM; mobile phase and operating conditions as described in Experimental. N = Neutral, B = basic and A = acidic drugs. Drug identities given in Fig. 1. (A) Effect on column plate number. (B) Effect on separation factor, α. (C) Effect on separation resolution, R_s. Halofan. = Halofantrine.

crease with increasing temperature, reach optimum, then decrease. Depending on the pK_a value of the drug, this effect may be influenced by a change in the ionic state of the molecules. Alteration in the ionic equilibrium by temperature change might well influence the enantioselectivity that is favored by the non-ionized form of the chiral molecules. However, when chiral resolution changes with pH and temperature for fully ionized basic drugs, one might suspect a conformational change in the ovomucoid protein bound to the packing surface. Such induced changes in protein conformation then could determine the type and extent of chiral interaction possible with enantiomeric compounds of different structures.

Fig. 5 shows the effect of temperature on the resolution of the basic dopamine antagonist drug, SKF 83566, with the ovomucoid protein column. Complete overlap of enantiomers occurred at 35°C; unit resolution was obtained by reducing the temperature to 10°C. The effect of improved resolution at lower temperatures has been reported previously for several racemic mixtures on α-acid glycoprotein (AGP) and BSA protein-based columns [10,13]. However, with halofantrine and other basic drugs, operation at lower temperatures may not produce the highest chiral resolution, as discussed above.

For maximum resolution, the effect of temperature should be investigated for all separations performed on the ovomucoid protein column. At times, however, separations attempted at higher temperatures can cause problems, as illustrated in Fig. 6 for the basic drug lorazepam. At 35°C odd-shaped ("Batman") peaks were found for the enantiomeric mixture, as shown in Fig. 6A. Lowering the temperature to 10°C produced the separation in Fig. 6B. The raised baseline pattern between the peaks for the two enantiomers probably is because of chiral isomer interconversion during passage through the column. The interconversion problem for lorazepam is much greater at the higher temperature, but is still just apparent at 10°C. The on-column enantiomerization of benzodiazopinones during chromatography also has been noted by others [14].

Strategy for optimizing enantiomer separations

In a previous publication, we suggested a simple, but effective, strategy for optimizing the

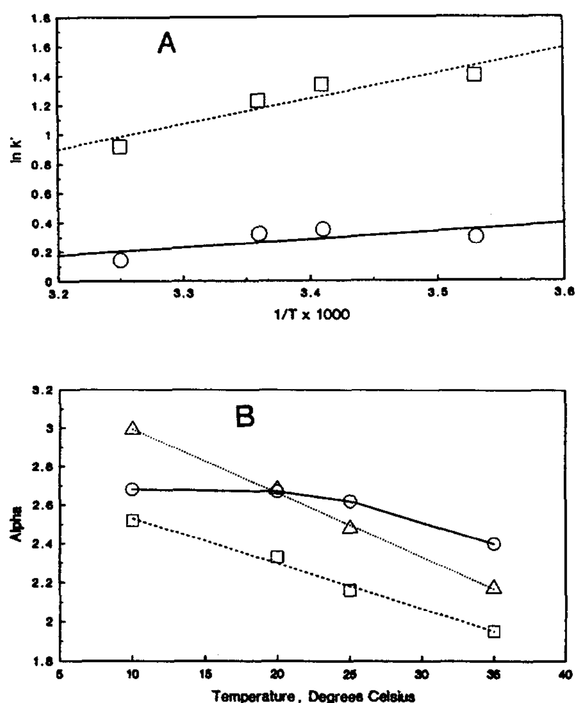

Fig. 3. Temperature effects on the basic drug, halofantrine. (A) Van 't Hoff plots for enantiomers; column: 15 × 0.60 cm Ultron ES-OVM; mobile phase: acetonitrile–0.01 *M* phosphate buffer, pH 5.5 (40:60); UV detector: 258 nm. (B) Effect of temperature on selectivity factor, α; conditions as in Fig. 3A, except 0.01 *M* phosphate buffers at different pH values. ○ = pH 5.5; □ = pH 6.0; △ = pH 7.0.

enantiomer separations on the ovomucoid protein column [9]. Because of the present study on chiral resolution, we have modified this scheme to include the influence of temperature changes on the separation. An updated flow sheet summarizing the modified strategy is given in Fig. 7. We suggest that initial separations should be attempted at pH 6.0 and 25°C, using acetonitrile as the mobile phase organic modifier. The con-

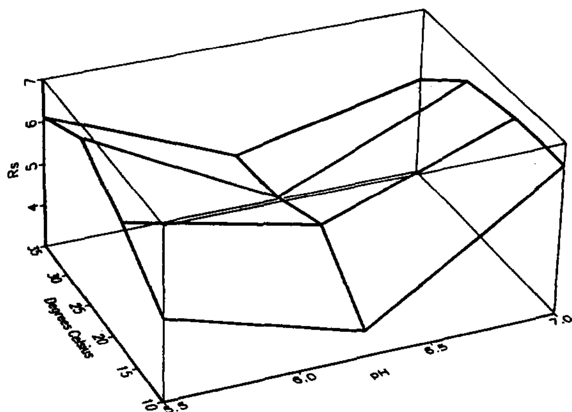

Fig. 4. Effect of pH and temperature on the resolution of halofantrine enantiomers. Conditions as in Fig. 3B.

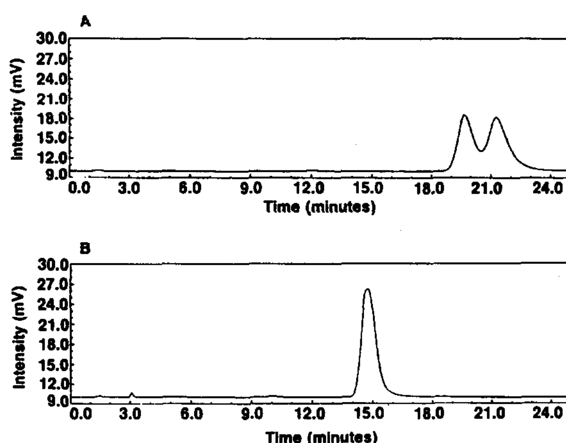

Fig. 5. Effect of temperature on the separation of SKF 83566 enantiomers. Column: 15 × 0.60 cm Ultron ES-OVM; mobile phase: acetonitrile–0.01 *M* phosphate buffer, pH 7.0 (20:80); flow-rate: 1.0 ml/min; detector: UV detector: 230 nm. (A) 10°C. (B) 35°C.

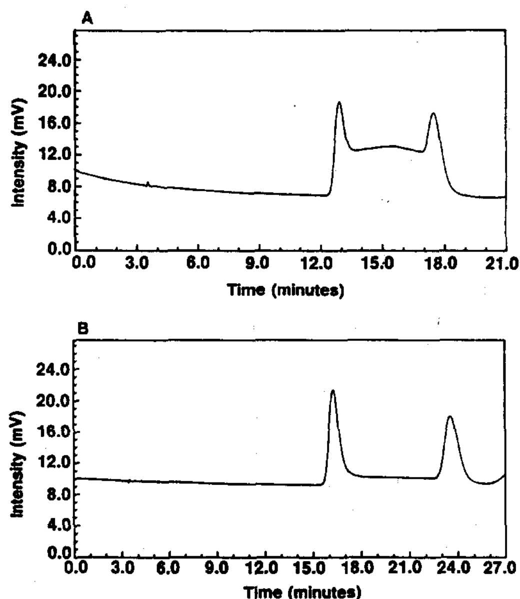

Fig. 6. Effect of temperature on the separation of lorazepam enantiomers. Condition as in Fig. 6, except acetonitrile–0.01 *M* phosphate buffer, pH 6.0 (12:88); UV detector: 228 nm. (A) 35°C. (B) 10°C.

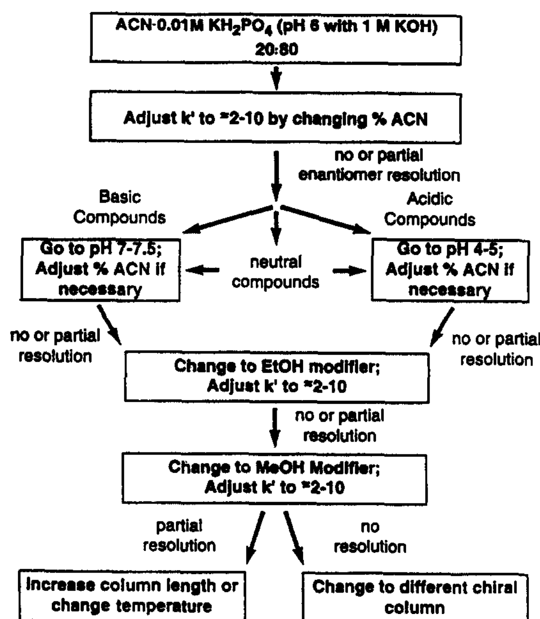

Fig. 7. Suggested strategy for optimizing drug enantiomer separations on ovomucoid protein column. ACN = Acetonitrile.

centration of acetonitrile first is appropriately adjusted to position the peaks of interest in the usual $k' = 2–10$ range. If no or only partial resolution occurs for basic compounds, the mobile phase then should be adjusted to pH 7–7.5 (changing the percent acetonitrile, if necessary, to maintain the proper k' range).

When no or partial resolution persists, the mobile phase modifier should be changed to ethanol (adjusting the organic concentration to maintain the desired k' range, if necessary). If no or partial resolution still occurs, the mobile phase modifier should be changed to methanol (again adjusting the concentration to maintain $2 < k' < 10$, if required). If partial resolution is still a problem, resolution can be increased by increasing column length. However, if no resolution occurs, increasing or decreasing the column temperature should be attempted to seek additional resolution. Usually, a 5–10°C initial change will indicate whether this approach will be helpful (see Fig. 5). If a temperature change has no beneficial effect, it is likely that a different chiral column and separating system are required.

If no or partial resolution of acidic compounds is seen after the initial separation at pH 6 with acetonitrile, the mobile phase should be adjusted to pH 4–5 (while maintaining the proper k' range by changing the percent acetonitrile). If no or partial resolution persists, change first to ethanol, then to methanol modifier, as for basic drugs above. Increasing column length, or varying column temperature can be helpful when these steps are unsuccessful. Prevailing insufficient resolution usually indicates the need for a different chiral column and separating system.

The strategy given in Fig. 7 for optimizing separating conditions with the ovomucoid column has proved useful with many difficult drug chiral separations in this laboratory. The scheme has been helpful for separating enantiomers with structures that were similar to those already successfully resolved. However, we also find this approach especially helpful in establishing useful separation procedures for chiral structures that have not been reported or studied previously.

CONCLUSIONS

Selectivity factor, α, and separation resolution, R_s, sometimes are significantly improved by temperature changes when attempting separations with the ovomucoid column, Ultron ES-OVM. The tertiary structure/conformation and potential enantioselectivity of the bound protein probably are influenced by mobile phase pH and organic modifier composition. These parameters in turn are affected by temperature. Drug structure also determines the effect of temperature, particularly for ionic or ionizable species.

Based on results from this study, chiral resolution of acidic drugs decreases with increasing temperature. This suggests a strong dependence on the selective interactive forces affecting α values. Chiral resolution of basic drugs can increase with increasing column temperature, reaching an optimum before decreasing. This effect indicates that these solutes are responsive to differences in mass transfer that influence column efficiency. An upgraded procedure is proposed to help in rapidly developing useful methods for separating drug and agrichemical enantiomers with the ovomucoid column. This

study suggests that the temperature of the ovomucoid protein column should be carefully controlled for optimum reproducibility of retention and quantitative data.

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