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Note

Use of mobile phase modifiers to alter retention and stereoselectivity on a bovine serum albumin high-performance liquid chromatographic chiral stationary phase

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Bovin serum albumin (BSA) is a globular, hydrophobic protein which has been shown to stereoselectively bind small enantiomeric molecules[1]. The enantioselectivity of BSA has been utilized in the development of chiral stationary phases (CSPs) for use in liquid chromatography. For example, in 1973, Stewart and Doherty[2] immobilized BSA on succinoylaminoethyl-Sepharose and used the resulting CSP to stereochemically resolve D,L-tryptophan. In 1983, Allenmark *et al.*[3] reported the covalent immobilization of BSA on 10-μm silica. His latter work was used as the basis for the development of a commercially available high-performance liquid chromatographic (HPLC) CSP (BSA-CSP).

A wide variety of enantiomeric solutes have been stereochemically resolved on the BSA-CSP[4]. However, while the stereoselectivity of the BSA-CSP is impressive, the routine application of this column to large-scale clinical and pharmacological studies is often hampered by the CSP's chromatographic properties, *i.e.* high retention and low efficiency.

Allenmark and co-workers[4–7] have altered the retention (k') and stereoselectivity (α) on the BSA-CSP by manipulating the composition of the mobile phase. The standard mobile phase used with the BSA-CSP is composed of an aqueous solution of phosphate buffer modified with 1-propanol. The parameters which have been studied by Allenmark and co-workers include the concentration of the buffer[4,5], the pH[6,7] and the 1-propanol content[6].

This is not the only possible approach to the alteration of k' and α on the BSA-CSP. Another method has been suggested by the work of Schill *et al.*[8,9] with the HPLC CSP based upon immobilized α_1-acid glycoprotein (AGP-CSP). The retention and stereoselectivity of solutes on the AGP-CSP can be changed through the addition of neutral or ionic compounds to the mobile phase. These mobile phase modifiers affect k' and α through a variety of mechanisms including direct competition with the solute for binding sites on the protein; alteration of the affinity of the protein for the solute; and formation of ion pairs with the solute.

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The potential also exists for using mobile phase modifiers with the BSA-CSP. This possibility is suggested by *in vitro* and *in vivo* studies which have demonstrated the competitive displacement from BSA binding of a variety of substances including warfarin[1,10–12]. It was assumed that the compounds which displace warfarin from serum albumin could be used as mobile phase modifiers to reduce the retention of warfarin on the BSA-CSP.

In this study, we have used three compounds (trichloroacetic acid, cyclamic acid and lauric acid) which are known to displace warfarin from serum albumin as mobile phase modifiers and have confirmed that these modifiers alter the retention and stereoselectivity of warfarin on the BSA-CSP. Three compounds (lorazepam, D- and L-tryptophan) which do not displace warfarin from serum albumin in *in vitro* studies were also investigated and their affects on k' and α varied. The effect of trichloroacetic acid and cyclamic acid on k' and α of other solutes was also investigatd. It appears that these modifiers offer a valuable method for improving the chromatographic properties of the BSA-CSP.

EXPERIMENTAL

Apparatus

The chromatography was performed with a modular liquid chromatograph composed of a Beckman 110B solvent module pump (Beckman Instruments, Houston, TX, U.S.A.), a Spectra-Physics Spectraflow 980 or a Spectra-Physics 8480 scanning UV detector (Spectra-Physics, Santa Clara, CA, U.S.A.), a Shimadzu C-R6A Chromatopac integrator (Shimadzu Scientific Instruments, Columbia, MD, U.S.A.) and a commercially packed chiral phase HPLC column composed of bovine serum albumin immobilized on 10-μm spherical silica (Resolvosil, Alltech Assoc., Deerfield, IL, U.S.A.). The column temperature was maintained within ± 0.1°C of the desired setting with a Forma Scientific Model 2006 circulating water bath (VWR Scientific, Chicago, IL, U.S.A.) and an Alltech HPLC column water jacket.

Chemicals

(*R*,*S*)-Warfarin, (6*R*,6*S*)-leucovorin, N-benzoyl-(*S*,*R*)-alanine, N-benzoyl-(*R*)-alanine, N-benzoyl-(*S*,*R*)-phenylalanine, N-benzoyl-(*R*)-phenylalanine and cyclamic acid (sodium salt) were purchased from Sigma (St. Louis, MO, U.S.A.). (*R*,*S*)-Benzoin and (*S*)-(+)-benzoin were purchased from Aldrich (Milwaukee, WI, U.S.A.). Trichloroacetic acid was purchased from J. T. Baker (Phillipsburg, NJ, U.S.A.) and lorazepam was obtained from Wyeth Labs. (Princeton, NJ, U.S.A.). The remaining chemicals were reagent grade and used as purchased.

Chromatographic conditions

The basic mobile phases were composed of an aqueous solution of sodium phosphate and 1-propanol. The molarity and pH of the phosphate buffer and the 1-propanol content were varied according to the solute. However, once these parameters were set for a particular solute, they remained constant throughout the study. In particular, the pH of the mobile phase was adjusted after the addition of the mobile phase modifier to the phosphate buffer and before the addition of the 1-propanol.

RESULTS AND DISCUSSION

The structures of solutes used in this study are presented in Fig. 1. During the chromatographic studies, the solute concentrations averaged 10^{-6} *M*. The actual concentrations were selected by plotting *k' versus* on column solute molarity to determine the binding capacity of the BSA-CSP. This process was repeated for each of the BSA-CSPs used in this study ($n = 5$) and the calculated binding capacities ranged from $2 \cdot 10^{-6}$ *M* to $8 \cdot 10^{-5}$ *M*. A solute molarity which was five-fold lower than the observed saturation concentration was then used. A 10^{-4} *M* lorazepam solution was used because of difficulties in the detection of this compound.

Where possible, the enantiomeric elution order was determined for each of the solutes. The enantiomeric elution orders on the BSA-CSP for warfarin[13] and leucovorin[14] have been previously determined as *S* before *R*. The enantiomeric elution orders for benzoin, N-benzoyl-alanine and N-benzoyl-phenylalanine were determined during the cours of this study using unequal mixtures of the resolved enantiomers. In each case, the isomer with the *S* configuration at the chiral center eluted first. The resolved enantiomers of lorazepam were not available and the enantiomeric elution order was not determined.

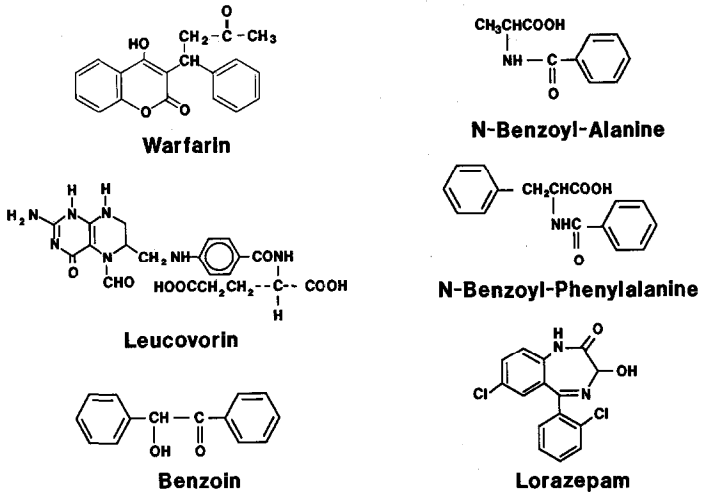

Fig. 1. The structures of the solutes used in this study.

The effect of mobile phase modifiers on retention, stereoselectivity and stereochemical resolution

Warfarin. Table I summarizes the maximum observed effect of the mobile phase modifiers used in this study on the retention (*k'*), stereoselectivity (α) and stereochemical resolution (R_s) of warfarin. Trichloroacetic acid, cyclamic acid and lauric acid were chosen as modifiers based on previous work by Sellers and Koch-Weser[10] in which these compounds were shown to displace warfarin from serum albumin binding. The effect of lorazepam and D- and L-tryptophan on *k'* and α were also examined.

TABLE I
EFFECT OF MOBILE PHASE MODIFIERS ON THE RETENTION AND STEREOCHEMICAL RESOLUTION OF WARFARIN

k'_1 = capacity factor of the first elued enantiomer [(*S*)-warfarin], k'_2 capacity factor of the second eluted enantiomer [(*R*)-warfarin], α = stereoselectivity (k'_2/k'_1), and R_s = stereochemical resolution = $(t_{R2} - t_{R1})/(W_1 + W_2)$ (where t_R = retention time and W = peak width).

Modifier	*Concentration (mM)*	k'_1	k'_2	α	R_s
Trichloroacetic acid	0.0	18.1	21.8	1.20	1.32
	5.0	9.4	10.9	1.16	1.09
Cyclamic acid	0.0	23.9	28.6	1.20	1.23
	7.0	18.3	21.2	1.16	0.79
Lauric acid	0.0	20.7	24.8	1.20	1.18
	0.5	6.1	6.1	1.0	0.0
Lorazepam	0.0	23.6	28.3	1.20	1.29
	0.4	19.4	20.1	1.04	0.45
L-Tryptophan	0.0	19.8	26.0	1.31	1.62
	3.0	18.3	24.0	1.31	1.570
D-Tryptophan	0.0	18.2	23.	1.26	1.48
	3.0	17.3	22.3	1.29	1.39

The consecutive addition of trichloroacetic acid to the mobile phase up to a concentration of 5.0 m*M* resulted in a decrease in the measured parameters (Table I, Fig. 2A). The addition of 5.0 m*M* trichloroacetic acid resulted in a 50% reduction of the k' values of (*S*)- and (*R*)-warfarin while α and R_s were diminished by 3% and 17%, respectively. Similar results were obtained through the addition of cyclamic acid to the mobile phase although the magnitude of the effect on k' was not as large (Table I, Fig. 2B). A 7.0 m*M* concentration of cyclamic acid reduced the k' for both enantiomer by an average of 25%, α was reduced by 3% and R_s was diminished by 36%.

Dramatically different results were obtained with either lauric acid or lorazepam as the mobile phase modifier. The addition of 0.5 m*M* lauric acid to the mobile phase reduced the k' of both enantiomers by more than 70% with an accompanying loss of stereoselectivity (Table I). When 0.4 m*M* lorazepam was added to the mobile phase the effect is similar to the result obtained with lauric acid. There was an 18% reduction in k' for (*S*)-warfarin, a 29% reduction in k' for (*R*)-warfarin, α fell from 1.20 to 1.04 and R_s was reduced from 1.29 to 0.45. Higher concentrations of lorazepam were not studied due to the lack of solubility in the mobile phase.

The effects of D- and L-tryptophan on the measured chromatographic parameters of warfarin on the BSA-CSP were not significant (Table I). A 3.0 m*M* concentration of L-tryptophan in the mobile phase produced an 8% reduction in k' for both warfarin enantiomer and no change in α. With the same concentration of D-tryptophan, the k' of (*S*)-wafarin was reduced by 5%, the k' of (*R*)-warfarin was increased by 2% and α increased from 1.26 to 129. The calculated R_s values also remained essentially constant.

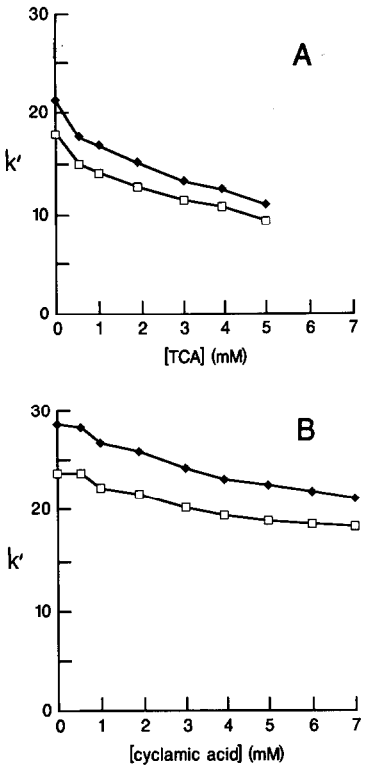

Fig. 2. The effect of (A) trichloroacetic acid and (B) cyclamic acid on the k' of warfarin. Chromatographic conditions: mobile phase, 0.2 *M* phosphate buffer (pH 7.5)–1-propanol (97.3, v/v); temperature, 30°C; flow-rate, 1 ml/min. □ = (*S*)-warfarin; ◆ = (*R*)-warfarin.

Leucovorin, benzoin, N-benzoyl-alanine and N-benzoyl-phenylalanine. In order to assess the general applicability of trichloroacetic acid and cyclamic acid as mobile phase modifier, their effect on the retention and stereoselectivity of the four above-mentioned solutes was investigated. The results of these studies are summarized in Table II. As observed with warfarin, the mobile phase modifiers produced a reduction in k' and α for each solute although the magnitude of the effect varied. The R_s value were also decreased except when N-benzoyl-phenylalanine was chromatographed using 5 m*M* trichloroacetic acid as the mobile phase modifier (Fig. 3).

Lorazepam. The effect of the trichloroacetic acid concentration in the mobile phase on the retention of the two enantiomer of lorazepam is presented in Fig. 4. Since the resolved enantiomer were not available, the elution order is unknown. The addition of up to 3.0 m*M* trichloroacetic acid had very little effect on the k' of the first eluted enantiomer. However, the k' of the second eluted enantiomer passed through a maximum between 0.5 and 1.0 m*M* trichloroacetic acid and then returned to approximately the initial value at 3.0 m*M* trichloroacetic acid. As a result, the stereoselectivity also passed through a maximum rising from α = 1.48 (0 m*M* trichloroacetic acid) to 1.95 (1.0 m*M*) to 1.62 (3.0 m*M*).

When the concentration of trichloroacetic acid in the mobile phase was raised

TABLE II

EFFECT OF TRICHLOROACETIC ACID AND CYCLAMIC ACID ON THE RETENTION AND STEREOCHEMICAL RESOLUTION OF LEUCOVORIN, BENZOIN, N-BENZOYL-ALANINE AND N-BENZOYL-PHENYLALANINE

k'_1, k'_2 = capacity factors of first and second eluted enantiomer, respectively.

Solute	*Modifier*	*Concentration (mM)*	k'_1	k'_2	α	R_s	*Mobile phase**
Leucovorin	Trichloroacetic acid	0	4.01	5.99	1.49	1.57	A
		5	3.18	3.65	1.15	0.40	A
	Cyclamic acid	0	5.12	7.35	1.44	1.5	A
		7	3.94	5.24	1.33	0.91	A
Benzoin	Trichloroacetic acid	0	4.75	8.0	1.68	2.15	B
		5	3.40	3.60	1.06	0.20	B
	Cyclamic acid	0	4.5	7.50	1.67	2.15	B
		7	3.43	4.75	1.39	1.31	B
N-Benzoyl-alanine	Trichloroacetic acid	0	6.00	15.58	2.55	4.74	C
		5	1.00	1.33	1.33	0.69	C
	Cyclamic acid	0	5.5	15.50	2.45	5.12	C
		7	1.20	1.63	1.36	0.97	C
N-Benzoyl-phenylalanine	Trichloroacetic acid	0	20.25	46.00	2.27	3.96	C
		5	6.50	12.75	1.96	4.29	C
	Cyclamic acid	0	21.00	46.75	2.23	4.00	C
		7	6.25	9.75	1.56	2.71	C

* A = 0.2 *M* phosphate buffer (pH 5.0), B = 0.05 *M* phosphate buffer (pH 7.5)–1-propanol (98:2, v/v), C = 0.05 *M* phosphate buffer (pH 6.5)–1-propanol (99:1).

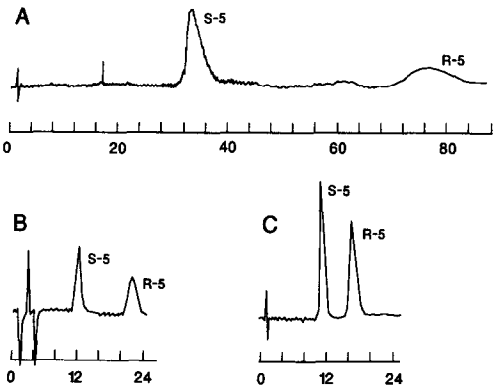

Fig. 3. The effect of mobile phase modifier on the chromatography of N-benzoyl-phenylalanine. Mobile phase, 0.05 *M* phosphate buffer (pH 6.5)–1-propanol (99:1, v/v); temperature, 40°C; flow-rate, 1 ml/min. (A) No mobile phase modifier, (B) 5.0 m*M* trichloroacetic acid added to mobile phase, (C) 7.0 m*M* cyclamic acid added to mobile phase. S-5 = (*S*)-N-benzoyl-phenylalanine; R-5 = (*R*)-N-benzoyl-phenylalanine Injected: 3.7 μM in phosphate buffer (0.05 *M*, pH 7.5). (A + C) 0.02 a.u.f.s. (B) 0.03 a.u.f.s.

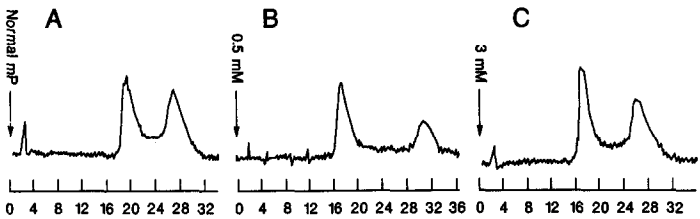

Fig. 4. The effect of trichloroacetic acid on the chromatography of lorazepam. Mobile phase, 0.01 *M* phosphate buffer (pH 6.6)–1-propanol (99:1, v/v); temperature, 40°C; flow-rate, 1 ml/min. (A) No mobile phase modifier, (B) 0.5 m*M* trichloroacetic acid added to mobile phase, (C) 3.0 m*M* trichloroacetic acid added to mobile phase.

from 3.0 m*M* to 5.0 m*M*, the k' of the first eluted enantiomer increased from 9.3 to 15.8 (70% increase) and the stereoselectivity was lost. An additional rise in trichloroacetic acid concentration from 5.0 m*M* resulted in an increase in the k' of both enantiomers to 18.5.

CONCLUSION

This study has demonstrated that mobile phase modifiers can be used to improve the chromatographic performance on the BSA-CSP. Trichloroacetic acid and cyclamic acid are now regularly used as modifiers in this laboratory and have been utilized in the development of assays for large-scale clinical studies[13,14].

The results also suggest that the effect of the modifiers on k' and α is a function of where the solute and the modifier bind to the BSA and the strength of that binding. The results of an investigation of the relationship between HPLC retention on the BSA-CSP and *in vitro* studies of BSA protein binding will be reported elsewhere.

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