Optical Resolution of a Series of Potential Cholecystokinin Antagonist 4(3*H*)-Quinazolone Derivatives by Chiral Liquid Chromatography on α_1-Acid Glycoprotein Stationary Phase

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Abstract

Optical resolution of the enantiomers of new 4(3*H*)-quinazolone derivatives is investigated using the α_1-acid glycoprotein chiral stationary phase (Chiral-AGP). Stereoselective separation of the model compounds can be controlled by varying the pH and adding uncharged organic modifiers (acetonitrile and 2-propanol) to the mobile phase. For the majority of quinazolone derivatives, Chiral-AGP is proved to be an excellent enantioselector, because optimized chromatographic conditions allow for the baseline separation of the enantiomers. Separation factors between 1.19 and 1.85 are obtained. The effects of acetonitrile and 2-propanol on the chromatographic behavior of the model compounds are quite different because of their different hydrophobic- and hydrogen-bonding properties. The eluent pH and organic modifier concentration also contributes to the chiral recognition by altering the protein environment. The analysis of the experimental results leads to new information about the chromatographic mechanism on a Chiral-AGP surface.

Introduction

Quinazolones are versatile nitrogen-containing heterocyclic compounds that display a broad spectrum of biological and pharmacological activities in humans. The chemistry and pharmacology of these molecules have been of great interest to medicinal chemists. The substitutions in the quinazolone nucleus at various positions have profound influence on the pharmacological effect of quinazolones (1–3). Recently, a series of new chiral 4(3*H*)-quinazolone derivatives have been synthesized as potential cholecystokinin antagonists in our laboratory (4).

The aim of this study was to optimize the liquid chromatographic resolution of the racemic compounds, because the pharmacological activity or toxicity of the enantiomers or both may depend on their stereochemistry (5).

Because the binding of quinazolones to the plasma protein is known (6), we expected that enantioselectivity might be achieved by using an α_1-acid glycoprotein (AGP)-bonded chiral stationary phase (Chiral).

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The Chiral-AGP column has a wide applicability to the resolution of chiral drugs with different structures (i.e., acidic, basic, and nonprotolytic samples) (7–9). The reversed-phase character of the stationary phase gives many possibilities to regulate the retention and the enantioselectivity as well as the possibility to use an aqueous mobile phase that is compatible with biological fluids.

AGP contains numerous ionizable side chains with strongly different pK_a values (e.g., arginine, lysine, glutamic, and sialic acid), which explains the sensitivity of the AGP surface to the pH changes of the eluent (10). As a consequence, the protonation of the protein is quite different at various pHs, which may influence the conformation and thus the binding properties of the stationary phase (11).

At pH 4.0, certain acidic groups such as aspartic, glutamic, and sialic acid units are mainly protonated, and the basic groups (i.e., histidine, lysine, and arginine units) are fully protonated, which results in the enhancement of the hydrogen-bonding ability of the selector apart from the hydrophobic interactions.

However, at pH 7.0, the acidic side chains are deprotonated and some basic units are still in the protonated state, which is favorable for electrostatic interactions as well as dipole–dipole, induced dipole, and π–π interactions.

This separation study of the six quinazolone derivatives may give further insight into the chiral-binding properties of the Chiral-AGP column. In the case of model compounds bearing no protonable groups, any change in the retention time obtained by varying the pH should be attributed to the alteration of the chiral-binding sites.

It is well-known that the type of organic modifier added to the mobile phase may influence the enantioselectivity to a great extent (12,13). In this study, the interaction between the solutes and AGP was studied by screening the effects of acetonitrile, 2-propanol, and the eluent pH on the retention and enantioselectivity.

Experimental

Chemicals

The model compounds were synthesized in our laboratory according to previously described procedures (14). The chem-

ical structures of 4(3*H*)-quinazolone derivatives used in this study are shown in Figure 1. Melting points were determined on a Boetius hot plate. The purity was controlled by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) (normal and reversed phase).

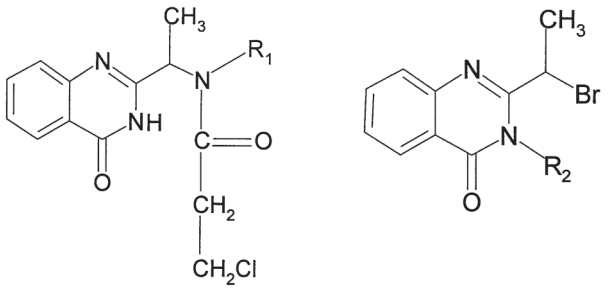
Spectral (infrared, nuclear magnetic resonance, and ultraviolet) and X-ray crystallographic data obtained for the test compounds were in agreement with the assigned structures (15).

Acetonitrile, 2-propanol, and *n*-hexane obtained from Chemolab (Budapest, Hungary) were of HPLC grade. Double distilled water was used for all measurements. Sodium dihydrogen phosphate, sodium hydrogen phosphate, sodium hydroxide, and phosphoric acid purchased from Reanal (Budapest, Hungary) were of analytical grade.

Instrumentation

HPLC

The HPLC system consisted of an ISCO M 2350 pump (Lincoln, NE), a Rheodyne M 125 (Cotati, CA) injector unit with a 10-µL loop, and an ISCO V4 (Lincoln, NE) variable wavelength UV detector set at 225 nm.



	R_1		R_2
1	H	**5**	$CH_2CH_2COOC_2H_5$
2	C_6H_6	**6**	CH_2CH_2COOH
3	C_6H_5-OCH_3		
4	C_6H_5-OC_2H_5		

Figure 1. Structure of the model compounds.

A Chiral-AGP column equipped with a guard column (150- × 4.0-mm i.d. and 10- × 3.0-mm i.d., 5 µm, respectively) was used as the stationary phase (ChromTech, Hägersten, Sweden).

The temperature was kept at 23 ± 0.1°C using a Jones Chromatography (Hengoed, UK) M 7955 column thermostat.

The experimental data were collected and analyzed on the Barspec Data System.

Chromatographic conditions

The mobile phases were 0.01M phosphate buffers to which organic modifiers were added. The buffer solutions were prepared by mixing the proper volume of 0.01M aqueous solutions of sodium dihydrogen phosphate and sodium hydrogen phosphate. The pH was adjusted to the desired level by the addition of 1M sodium hydroxide or phosphoric acid.

The mobile phases were freshly prepared, filtered, degassed in an ultrasonic bath, and thermostatted to ambient temperature (23°C) before use. A constant flow rate of 0.9 mL/min was maintained during all experiments. The sample concentration was 0.01 mg/mL.

The column was washed with 200 volumes of double distilled water between the experiments. All chromatographic data reported are the mean of three parallel measurements.

The 4(3*H*)-quinazolones used were racemic compounds. The racemates were resolved by semipreparative HPLC on a Chiralcel OD column (1- × 25-cm i.d.) (Daicel Chemical Industries, New York, NY), because the latter's loading capacity was much higher than that of Chiral-AGP. The optical rotation of the isomers was determined by measuring the activity of the respective enantiomers eluted from the semipreparative Chiralcel OD column using an eluent containing hexane/2-propanol (99:1). The flow rate was 3 mL/min. The separated optical isomers were reinjected onto the Chiral-AGP column, and the peaks were identified by their retention times. It is worth noting that the eluted fractions gave the same elution order on Chiral-AGP and Chiralcel OD columns (i.e., the first eluted peak showed levorotatory activity).

Table I. Influence of the Nature and the Concentration of Organic Modifiers on Retention and Enantioselectivity*

	1		2		3		4		5	
Modifier	k_1'	**Separation factor**	k_1'	**Separation factor**	k_1'	**Separation factor**	k_1'	**Separation factor**	k_1'	**Separation factor**
Acetonitrile										
14% (v/v) (2.67M)	0.30	1.42	5.46	1.09	2.72	1.33	3.21	1.46	4.67	1.19
10% (v/v) (1.91M)	0.69	1.46	15.34	1.13	8.34	1.30	9.95	1.49	11.35	1.17
7% (v/v) (1.33M)	1.36	1.61	46.93	1.25	24.22	1.22	29.22	1.48	28.67	1.13
2-Propanol										
10% (v/v) (1.33M)	0.35	1.40	5.82	1.37	4.36	1.42	5.01	1.33	4.21	1.26
7% (v/v) (0.94M)	0.57	1.58	12.45	1.33	8.98	1.38	10.66	1.28	8.92	1.23
4% (v/v) (0.53M)	1.10	1.85	34.29	1.19	22.99	1.33	28.36	1.20	20.94	1.19

* Mobile phase: 0.01M phosphate buffer (pH 7.0) with the addition of an organic modifier.
† k_1', the retention factor of the first eluted enantiomer.

The optical rotation dispersion measurements were carried out by using a Jasco J-720 (Tokyo, Japan) spectropolarimeter employing a 10-cm thermostatted cylindrical cell, a response of 8 s, and a step resolution of 0.2 nm. The bandwidth was 1.0 nm.

Results and Discussion

In this study, the enantiomer separation ability of the Chiral-AGP stationary phase for several racemic 4(3*H*)-quinazolone derivatives was investigated.

In our previous study (11), we described that acetonitrile as an organic additive was adsorbed on a Chiral-AGP surface at both pH 4.0 and 7.0 to a large extent. However, the shape of the adsorption isotherms was quite different at the two pH levels. At pH 7.0, by increasing the percentage of acetonitrile in the mobile phase, a saturation was obtained in the adsorbed amount, but at pH 4.0 at a defined modifier concentration, the saturation was followed by a net break. These results indicate that the binding properties of the AGP surface were dramatically affected by changing the eluent pH in the presence of the organic additive with a given concentration.

Effect of organic modifier on retention and enantioselectivity

The adsorption of the organic solvent on the protein surface may influence the retention and enantioselectivity. In general, both the retention (k') and separation factors decrease by increasing the modifier concentration (16). However, for certain compounds, it is possible to improve the enantioselectivity on a protein-based column by rising the modifier content in the mobile phase (17). This effect is also highly dependent on the type of modifier (shown in Table I). The results show that the enantiomers of the model compounds can be easily resolved on the Chiral-AGP column. The retention of the antipodes is strongly reduced by enhancing the modifier concentration in all instances. However, the enantioselectivity for compounds 5 and 3 was enhanced by increasing the acetonitrile concentration from 7 to 14 % (v/v) in a 0.01M buffer solution at pH 7.0. The chromatographic resolution of sample 5 is given in Figure 2, in which baseline separation could be achieved by rising the acetonitrile percentage. However, by increasing the acetonitrile content of the eluent (from 7 to 14%, v/v), the separation factors for solutes 1 and 2 were significantly decreased from 1.61 to 1.42 and 1.25 to 1.09, respectively. At the same time, the chiral selectivity of compound 4 was virtually unchanged in the whole concentration range studied.

Whereas using 2-propanol in the eluent as the organic additive, the chiral selectivity (except sample 1) was highly improved for all model compounds by increasing the modifier content.

There are several plausible explanations for these observations. (*a*) The modifier can compete with the solute for adsorption on chiral and nonchiral binding sites via hydrophobic- or hydrogen-bond interactions or both. (*b*) As a result of the modifier adsorption, the solute can be bound together with the organic solvent onto the surface, which may cause an increase in the selectivity. (*c*) However, the organic additives can also be bound to the solutes and by solvation could influence the

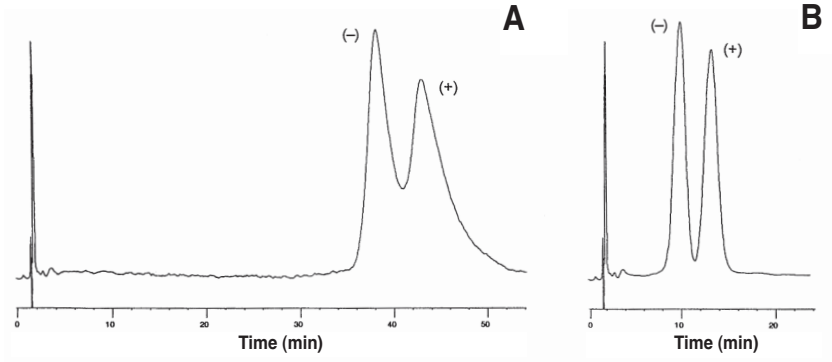


Figure 2. Separation of the enantiomers of compound 5 in Figure 1. Mobile phase: phosphate buffer (pH 7.0) containing 7% (v/v) of acetonitrile (A) and 14% (v/v) of acetonitrile (B) ($[\alpha]^{23°C}_{589.3\ nm}$ = (–) 55.81 and (+) 56.53° cm²/g, respectively).

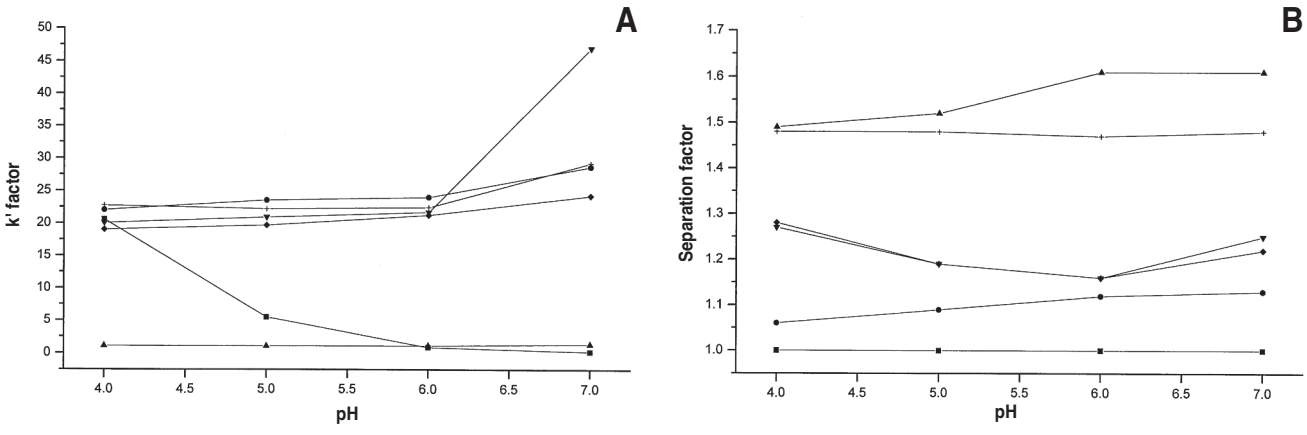


Figure 3. Influence of the eluent pH on k' (A) and separation factors (B). Mobile phase: 7% (v/v) (1.33M) acetonitrile in 0.01M phosphate buffer. Samples:

stereoselective binding to the selector. (*d*) Additionally, conformational changes (included by the organic modifier) may take place in the protein structure by altering the protein environment, causing the exploration of new chiral binding sites (18). Therefore, it is quite understandable that modifiers with different chemical characteristics alter the chiral selectivity in different ways.

Influence of pH on the retention and enantioselectivity

Figure 3 shows the effect of the eluent pH on the retention and chiral selectivity of the test solutes, in which the eluent used is a 0.01M phosphate buffer containing 7% (v/v) (1.33M) of the acetonitrile as the organic additive. As expected, the k' factors of the acidic compound 6 strongly decreased by increasing the mobile phase pH. Taking the carboxylic pK_a value of this type of compound (pK_a is approximately 3.5) (19) and the isoelectric point of AGP into account (p1 = 2.7) (20), it seems that electrostatic interactions played a key role in the retention of sample 6 (Figure 3A). However, these interactions are insufficient over the whole pH range studied in order to result in the chiral recognition (Figure 3B). It can be observed that the k' values of the other quinazolone substances were almost unaffected in the pH range of 4.0 to 6.0. However, between pH 6.0 and 7.0, an increase in the retention took place. Regarding the nonprotolytic character of the model substances in the pH range investigated—the pK_a of the aromatic amine was approximately 2.4 (19) and the aliphatic and cyclic amido nitrogens were approximately neutral—any changes observed in the k' factors by varying the pH should be a result of structural changes in certain binding groups of the selector. It has been described (21) that because of the unfolding process of the initially buried, mainly aromatic amino acid residues can appear on the protein surface and act as new binding sites. This assumption is supported by the fact that the increase of the retention induced by pH change was rather extensive for compound 2 (unsubstituated phenyl ring in the R_1 group). Its k' value was doubled by increasing the pH from 6.0 to 7.0, which indicates the dominance of π–π interactions between the protein and the phenyl ring of the sample at pH 7.0. However, introducing an electron-donating substituent at position 4 of the aromatic ring—i.e., methoxy (compound 3) or ethoxy group (compound 4)—the k' values rose only moderately (from 19.73 to 24.22 and 22.44 to 29.22, respectively), because these substituents affect the electron density of the ring system, and consequently, they can alter the strength of π–π interactions.

Regarding the enantioselectivity shown in Figure 3B, it can be observed that no enantioseparation was achieved for sample 6 at all pH levels; however, its ester derivative (compound 5) was well-resolved in the same systems. This result reflects that the ester function of compound 5 should contribute to the chiral resolution to a large extent. The separation value for the enantiomers of sample 5 increased linearly with pH from 1.06 to 1.13. All the other solutes were baseline-resolved over the whole pH range tested. Although compound 4 gave almost the same enantioselectivity through pH 4.0 to 7.0, the separation factors of the other solutes were highly dependent on the eluent pH. Solutes 3 and 4 had maximum enantioselectivity at pH 4.0 and a minimum value at pH 6.0. However, for compound 1, the chiral selectivity was improved by increasing the pH of the mobile phase, but remained fairly constant between pH 6.0 and 7.0. Taking into account that the highest selectivity was obtained for compound 1 bearing an amide function in the

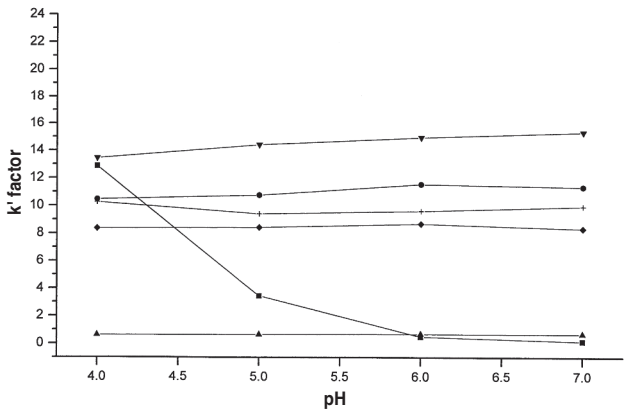


Figure 4. Influence of the eluent pH on k' factors. Mobile phase: 10% (v/v) (1.91M) acetonitrile in phosphate buffer. Samples: s = 1; t = 2; u = 3; + = 4; • = 5; n = 6. For other conditions, see Experimental section.

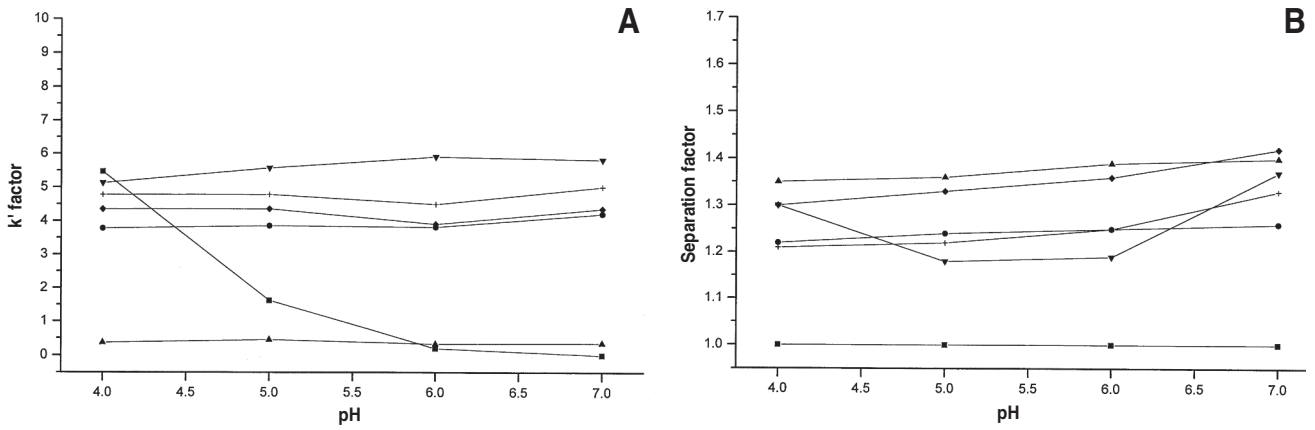


Figure 5. Effects of the mobile phase pH on k' (A) and separation factors (B). Mobile phase: 10% (v/v) (1.33M) 2-propanol in phosphate buffer. Samples: s = 1; t = 2; u = 3; + = 4; • = 5; n = 6. For other conditions, see Experimental section.

side chain at position 2 of the quinazolone nucleus (as shown in Figure 1), it seems that hydrogen-bond interactions should play an important role in the chiral discrimination.

However, as Figure 4 shows, by increasing the acetonitrile content up to 10% (v/v) (1.91M), the structural changes observed in the binding sites mentioned previously might not have taken place, because the k' values of the neutral compounds were almost unaffected over the whole pH range. Accordingly, it should be emphasized that the chromatographic parameters of the solutes were determined by an overall effect of the mobile phase variables (i.e., pH, chemical character, and percentage of organic modifier).

To evaluate the effects of the two types of organic modifiers on the chromatographic parameters, Figure 3 should be compared with Figure 5. Figure 5 shows the effect of the eluent pH on the k' and separation value when the 2-propanol concentration is equimolar (1.33M) with 7% (v/v) of acetonitrile. The lower dielectric constant of 2-propanol lead to a dramatic decrease in the k' factor for all the enantiomers. The influence of the eluent pH on the enantioselective retention was quite different from that obtained in the presence of equimolar acetonitrile. The difference was particularly large in the case of compound 2, because the break in the k' values at pH 7.0 could not be observed in the presence of 2-propanol. Consequently, the hydrophobic and hydrogen donor/acceptor properties of the organic solvent also had a crucial effect on the protein structure determining both the retention and selectivity.

Regarding the enantioselectivity concerns (Figure 5B), sample 1 and 4 had higher separation factors with the use of acetonitrile, and for compounds 3 and 5, it was strongly enhanced by using 2-propanol. The separation factors for solute 2 were almost the same by using 2-propanol instead of acetonitrile. Because 2-propanol had both a hydrogen donor and acceptor property and acetonitrile was a weak hydrogen acceptor, it is expected that these should alter the binding sites of the surface in a different way. In accordance with this assumption, the influence of 2-propanol and acetonitrile on the chromatographic behavior of the model compounds was quite different. The data revealed that depending on the composition of the mobile phase (pH, type, and concentration of the organic modifier), even small changes in the substituents of the quinazolone model compounds result in a significant alteration of the enantioselectivity.

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