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Note

Enantiomeric resolution of an optically active guanine derivative by high-performance liquid chromatography with phenylalanine–Cu(II) in the mobile phase

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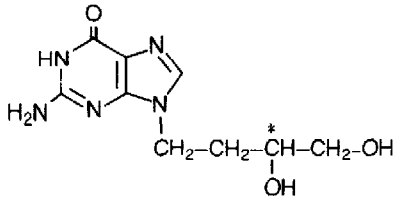
9-(3,4-Dihydroxybutyl)guanine (I), which has antiherpes activity[1], contains one optically active centre located in the side-chain. The two enantiomers exhibit antiherpes activity of different magnitudes, with the *R*-form being considerably more potent. For determination of the optical purity of I a method for separating the two enantiomers is required.



(I)

Liquid chromatographic separation of the *R*- and *S*-forms of I could possibly be performed by a pre-column derivatization in order to form a diastereomer. However, I contains two hydroxy groups and one amino group, all three groups being potential targets for derivatization agents. The possibility of a mixture of products that would aggravate the analysis is therefore apparent. Resolution of the *R*- and *S*-isomers without the need for pre-column derivatization is naturally preferred.

Optical resolution of amino acids with the aid of on-column formation of diasteromeric complexes is now an established technique[2–6]. A complex between an optically active amino acid and a metal ion, usually Cu(II), is used in the mobile phase. The diastereomeric complexes are then formed between the sample amino acid, the amino acid of the mobile phase and the metal ion. So far, this approach has been employed for the separation of amino acids and amino acid derivatives. However, other types of compounds with functional groups capable of complexing with metal ions should be potential candidates for this type of optical separation. This approach was therefore investigated for the resolution of *R*- and *S*-forms of I. The results obtained by using L-phenylalanine (L-Phe) and Cu(II) in a molar ratio of 2:1 in the mobile phase are reported here.

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EXPERIMENTAL

Instrumentation

The chromatograph consisted of a Waters M-45 pump and a Shimadzu SPD-2A UV detector. Recording and peak area integration were performed with a Pye Unicam PU 4810 computing integrator. A Rheodyne 7125 injector valve with a 20-μl sample loop was used. The columns used were Ultrasphere C_{18}, 5 μm, 250 × 4.6 mm I.D. and 150 × 4.6 mm I.D., supplied by Beckman, Nucleosil C_{18}, 5 μm, 150 × 4.6 mm I.D., from Macherey, Nagel & Co. and LiChrosorb C_8, 10 μm, 150 × 4.6 mm I.D., which was home-packed.

Reagents

(*R*)- and (*S*)-9-(3,4-Dihydroxybutyl)guanine were synthesized at Astra Läkemedel, Research and Development Laboratories, Södertälje, Sweden.

L-Phenylalanine, L-proline (Janssen), D-phenylglycine (Ward Blenkinsop), L-tryptophan (Fluka) N-L-aspartyl-L-phenylalanine (Searle) and copper(II) sulphate pentahydrate (analytical-reagent grade) (Merck) were used as received.

Procedure

UV detection was performed at 280 nm and a flow-rate of 1.0 ml/min was used throughout.

RESULTS AND DISCUSSION

Mobile phase concentration

The resolution obtained depends to some extent on the concentration of L-phenylalanine and Cu(II) in the mobile phase. In Table I, the resolution factor, R_s [$R_s = 2(t_2 - t_1)/(W_2 + W_1)$, where t_1 and t_2 are retention times and W_1 and W_2 are peak widths at the base], and capacity factors (k') are listed for three different concentrations of L-Phe–Cu. The retention was affected in such a way that a decrease in retention time resulted from an increase in concentration. The resolutions obtained

TABLE I

RETENTION (k') AND RESOLUTION (R_s) WITH DIFFERENT MOBILE PHASE COMPOSITIONS

Sample concentration: $2 \cdot 10^{-4}$ *M* of each enantiomer. Column: Ultrasphere C_{18}, 5 μm (150 × 4.6 mm I.D.).

Mobile phase composition	k'		R_s
	S-form	*R-form*	
3 m*M* L-phenylalanine–1.5 m*M* $CuSO_4$	28.8	30.4	1.14
6 m*M* L-phenylalanine–3 m*M* $CuSO_4$	25.3	27.0	1.43
12 m*M* L-phenylalanine–6 m*M* $CuSO_4$	13.3	14.3	1.08

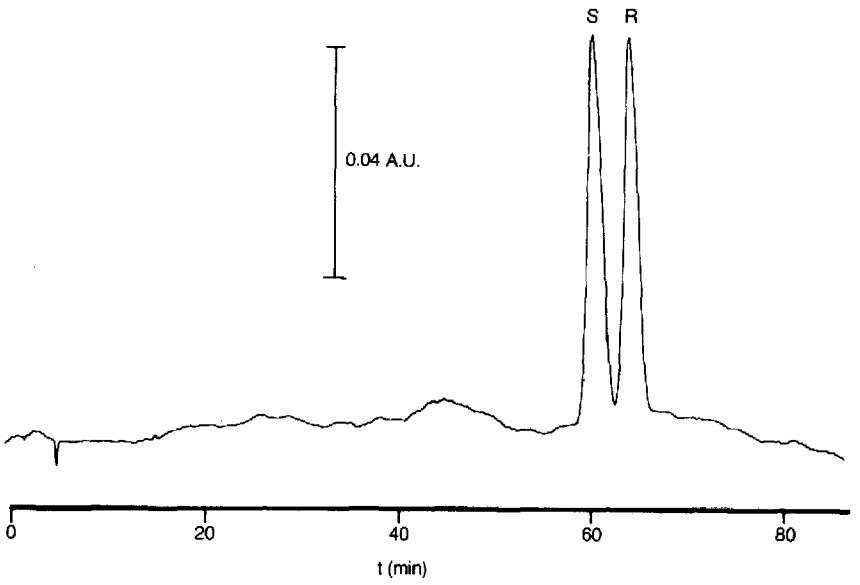

Fig. 1. Separation of $2 \cdot 10^{-4}$ *M* each of *R*- and *S*-forms of I with 6 m*M* L-phenylalanine–3 m*M* $CuSO_4$ as mobile phase. Column: Ultrasphere C_{18}, 5 μm (250 × 4.6 mm I.D.).

with the weakest and strongest mobile phases were about the same, but the retention time found for the strongest one was less than half that of the weakest. The resolution per unit retention time therefore increased markedly with increasing concentration of L-Phe–Cu in the mobile phase. The optimum resolution, disregarding the retention time, was found at an intermediate mobile phase concentration, however. A typical chromatogram obtained with 6 m*M* L-phenylalanine and 3 m*M* $CuSO_4$ is shown in Fig. 1. The enantiomers are almost baseline separated. The noisy background signal is due to the high absorptivity of the mobile phase. The chromatograms were run without temperature stabilization. It is possible that an improved baseline would result from better temperature control.

Sample concentration

An increased signal-to-noise ratio was obtained by increasing the sample concentration. On the other hand, a decrease in separation factor also resulted. By increasing the concentration of *R*- and *S*-forms from $2 \cdot 10^{-4}$ *M* (Fig. 1) to $1 \cdot 10^{-3}$ *M*, R_s decreased to 1.1. At a $2 \cdot 10^{-3}$ *M* concentration of each isomer, R_s was 0.9. Further, at this level, the latest eluted enantiomer (with L-Phe this is the *R*-form) became slightly distorted. In spite of this, the resolution was sufficient to detect small amounts of the *S*-form in the presence of large amounts of *R*-form. Fig. 2 shows chromatograms of $2 \cdot 10^{-3}$ *M* of pure *R*-form both without and with the addition of $2 \cdot 10^{-5}$ *M* of the *S*-form. Integration of the peak areas gave for this addition a 0.96% content of the *S*-form. The detection limit of the *S*-form in the *R*-form was approximately 0.3%. It should be noted that by using D-Phe in the mobile phase, the elution order is reversed and determination of small amounts of the *R*-form in the presence of large amounts of the *S*-form becomes possible. The retention times in Fig. 2 are shorter than those in Fig. 1. Increasing the sample concentration decreases

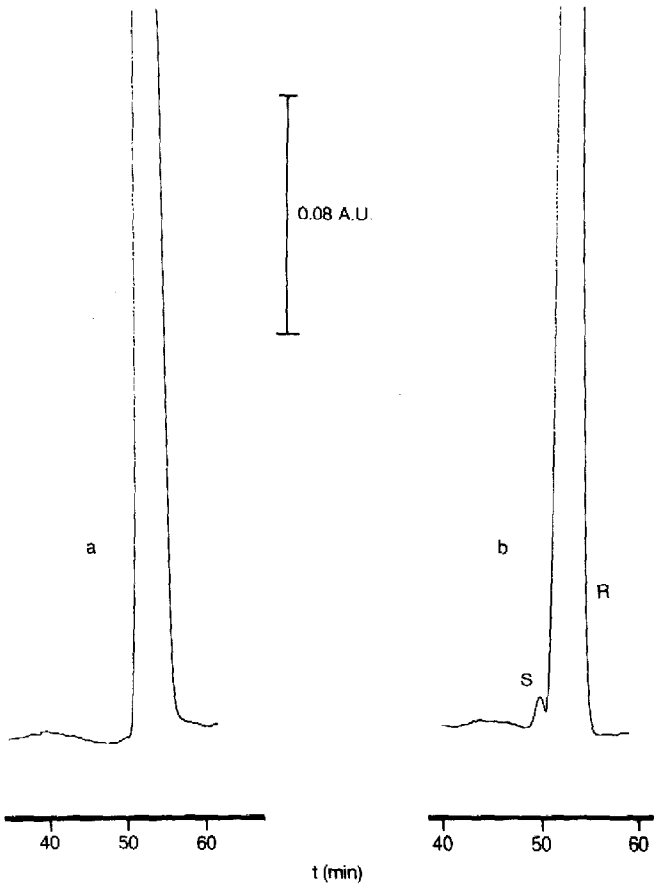

Fig. 2. (a) $2 \cdot 10^{-3}$ *M* *R*-form of I; (b) $2 \cdot 10^{-3}$ *M* *R*-form and $2 \cdot 10^{-5}$ *M* *S*-form of I. Mobile phase: 6 m*M* L-phenylalanine–3 m*M* $CuSO_4$. Column: Ultrasphere C_{18}, 5 μm (250 × 4.6 mm I.D.).

retention slightly. Further, the chromatograms in Figs. 1 and 2 were run on different occasions with the column having been exposed to several mobile phases in between. The retention time in a certain mobile phase does vary between occasions. When the influence of the mobile phase composition on the retention was investigated (Table I), all three mobile phases were run in a row on the same occasion.

Choice of column

Columns with different packing materials were investigated with respect to their influence on resolution and retention and the results are summarized in Table II. The best separation was achieved with the 250 × 4.6 mm I.D. Ultrasphere C_{18} column, which gave a plate number of 18,700 when the measurement was performed using anisole with methanol–water (60:40) as the mobile phase (k' = 3.6). As a comparison, the Nucleosil and the LiChrosorb columns gave 8200 and 3100 plates, respectively, in this test system (k' = 2.1 and 1.7, respectively). Selection of a high-resolution column is thus of importance for the successful separation of the *R*- and *S*-forms of I.

TABLE II

RETENTION (k') AND RESOLUTION (R_s) OBTAINED WITH DIFFERENT COLUMNS

Sample concentration: $2 \cdot 10^{-4}$ *M* of each enantiomer.

Column	k'		R_s
	S-form	*R-form*	
Ultrasphere C_{18}, 5 μm (250 × 4.6 mm I.D.)	25.3	27.0	1.43
Ultrasphere C_{18}, 5 μm (150 × 4.6 mm I.D.)	20.6	21.9	0.92
Nucleosil C_{18}, 5 μm (150 × 4.6 mm I.D.)	18.9	19.5	0.43
LiChrosorb C_8, 10 μm (150 × 4.6 mm I.D.)	6.6	6.6	–

Other amino acids in the mobile phase

Amino acids other than phenylalanine were investigated for use in the mobile phase. Aspartame (N-L-aspartyl-L-phenylalanine), phenylglycine and proline were used at 5 m*M* concentrations with 2.5 m*M* Cu(II). Tryptophan was investigated at 1 m*M* and 0.5 m*M* Cu(II). Only aspartame gave any resolution of the *R*- and *S*-forms of I. The resolution factor obtained with the 150 × 4.6 mm I.D. Ultrasphere column was 0.5, which should be compared with 0.9 obtained with L-Phe with this column (Table II).

CONCLUSIONS

The successful resolution of the *R*- and *S*-forms of I by using a complex between L-phenylalanine and Cu(II) in the mobile phase illustrates that substances other than (±)-amino acids and their derivatives can be resolved in this way. If the resolution mechanism for I follows the same principle as at discussed for amino acids[2,3], it seems as if I competes successfully with L-Phe for complex formation with Cu(II). The investigation of other optically active substances with complex-forming properties, and the employment of cations other than Cu(II), is naturally of interest.

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