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Note

Separation of pyridone carboxylic acid enantiomers by high-performance liquid chromatography using copper(II)–L-amino acid as the eluent

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Pyridone carboxylic acids such as ofloxacin (OFLX)[1] are important substances in the field of medical treatment. Related compounds are widely used as chemotherapeutic agents in clinical medicine, and OFLX, for instance, is administered clinically as a racemic mixture[2]. When the pharmacological properties of enantiomers differ from each other, there is considerable interest in the chromatographic resolution of the enantiomers for analytical and preparative purposes. A variety of approaches have been used for the resolution of enantiomers by high-performance liquid chromatography (HPLC), which is of especial pharmaceutical interest, but no studies on the chromatographic resolution of the enantiomers of pyridone carboxylic acids have yet been reported. A useful method for the analysis of these isomers is required for the determination of optical purity in pharmaceutical preparations and in biological fluids for pharmacokinetic purposes.

Various methods have been reported for the separation of enantiomeric mixtures[3]. These involves derivatization with chiral reagents[4,5], the use of chiral stationary phases[6] and of chiral eluents[7–9]. Chiral mobile phase methods are particularly simple and can be used with conventional HPLC columns, but their applications have been limited to the separation of amino acids, hydroxy carboxylic acids and their derivative compounds[10].

In this paper, we describe a new, simple and convenient HPLC method for the resolution of the enantiomers of pyridone carboxylic acids using a chiral mobile phase.

EXPERIMENTAL

Reagents

The ofloxacin enantiomers (+)- and (−)-9-fluoro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-2,3-dihydro-7H-pyrido(1,2,3-*de*)-1,4-benzoxazine-6-carboxylic acid and their derivatives were kindly donated by the Chemical Research Center of Daiichi Seiyaku.

The amino acids were from Kishida Kagaku (Osaka, Japan). All other chemicals and reagents used were obtained from the usual commercial sources. Water (HPLC grade) was obtained by purification through a Milli Q Purification System (Millipore, Bedford, MA, U.S.A.).

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TABLE I

RESOLUTION OF OFLX ENANTIOMERS WITH VARIOUS LIGANDS

Copper(II) concentration: 1/2 mol of ligand concentration. Methanol concentration: 15%. The pH was not adjusted.

Ligand	*Concentration (mM)*	*Separation coefficient, α*
L-Phenylalanine	6	1.26
L-Isoleucine	6	1.24
L-Leucine	6	1.16
Aspartame*	2	1.14
N-Methyl-L-phenylalanine	2	1.13
L-Valine	6	1.10
L-Tryptophan	6	1.07
L-Methionine	6	1.04
L-Tyrosine	6	1.06
D-Phenylglycine	6	1.00**
Toluenesulphonyl-L-phenylalanine	1	1.00**

* Aspartame is N-L-α-aspartyl-L-phenylalanine 1-methyl ester.
** Not separated.

Chromatographic conditions

The mobile phase contained between 15 and 40% of methanol in water or a 10 m*M* phosphate buffer containing one of nine amino acids (see Table I) and copper sulphate, the concentration of which was 1/2 mol of amino acid (these concentrations are indicated in Table I and the figures). The buffer was adjusted to various pH with phosphoric acid. The flow-rate was 1.0 ml/min and the detection wavelength was 300 nm (UV), or 330 (excitation) and 505 nm (emission) (fluorescence), respectively.

Instrumentation

The chromatographic system consisted of an high-pressure pump (Oriental Motor Model RLD-150) equipped with a Model 7125 injector (Rheodyne, Berkeley, CA, U.S.A.), a YMC AM-312 ODS column (15 cm × 6 mm I.D., particle size 5 μm; Yamamura Chemical, Kyoto, Japan), an UVIDEC-100-II spectrophotometric detector (Nihon Bunko, Tokyo, Japan) and a F-1100 fluorescence detector (Hitachi Seisakushyo).

RESULTS

The enantiomers of OFLX and their derivatives were resolved as mixed complexes of Cu(II) and amino acids on a reversed-phase column. The chromatograms are shown with the (+)-(*R*)-isomers more strongly retained. Table I shows the correlation between the ligands added and the separation coefficients, α, of the OFLX enantiomers. Good separation was obtained with L-phenylalanine, but not with phenylglycine and toluenesulphonyl-L-phenylalanine, which have been used for enantiomer analysis[11].

Fig. 1 shows the fluorescence spectra of OFLX in the L-phenylalanine chiral mobile phase (maximum excitation and emission wavelengths were about 330 and 505 nm).

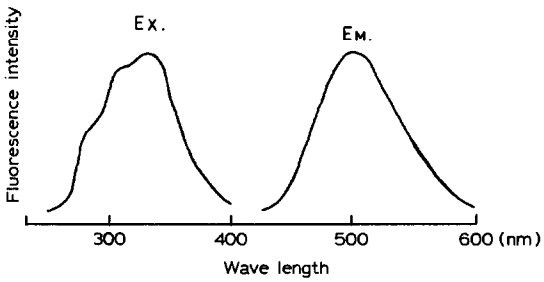

Fig. 1. Fluorescence spectra of OFLX in the mobile phase: 6 m*M* L-Phe, 3 m*M* copper sulphate; the pH level was not adjusted.

The retention and separation behaviour are dependent on the ligand concentration and mobile phase pH (Figs. 2 and 3). Above pH 4.5, copper was deposited as a milky precipitate, and at high ligand concentrations the column was easily damaged. However at higher pH values and ligand concentrations, better resolution was obtained. As in other reversed-phase systems, lowering of the methanol concentration usually resulted in longer retention of the solutes and slightly better resolution of the optical isomers when the percentage of methanol was varied from 12.0 to 18.0%. We therefore decided that the optimum HPLC conditions were as shown in Fig. 4. The detection limit of the optical purity was about 0.1%.

The derivatives of OFLX can also be separated by this method (Fig. 5).

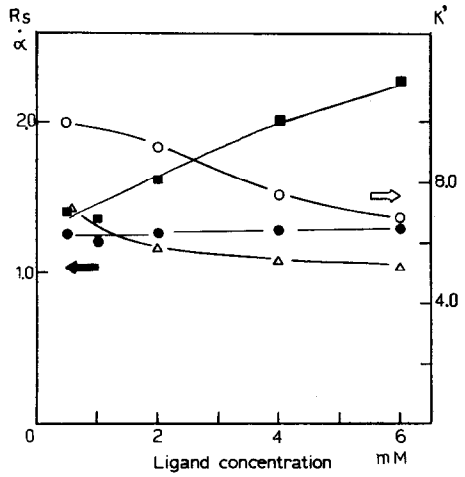

Fig. 2. Effects of the ligand concentration (L-Phe) on the resolution, R_s, separation coefficient, α, and capacity factor, k'. The pH levels were not adjusted and the copper(II) concentration was changed in proportion to the increasing ligand concentration. ■ and ●, R_s and α; ○ and △, (+)-(*R*)- and (−)-(*S*)-isomer.

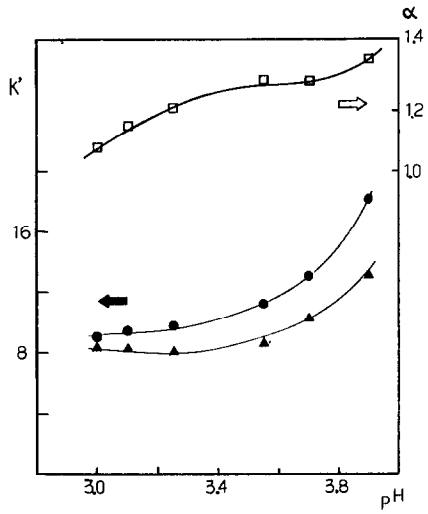

Fig. 3. Effect of the pH of the mobile phase on α and k' (6 mM L-Phe, 3 mM copper sulphate). □, α; ● and ▲, (+)-(R)- and (−)-(S)-isomer.

DISCUSSION

It has long been known that the carboxylate and nitrogen of an amino acid can chelate copper(II) ions in a bidentate manner to form bis(amino acidato) complexes; this technique for the isomeric resolution of amino acids is also applicable to keto-carboxylic acids. Table I shows the results of resolution of OFLX enantiomers with various ligands. Among the chiral ligands we studied, both aromatic amino acids

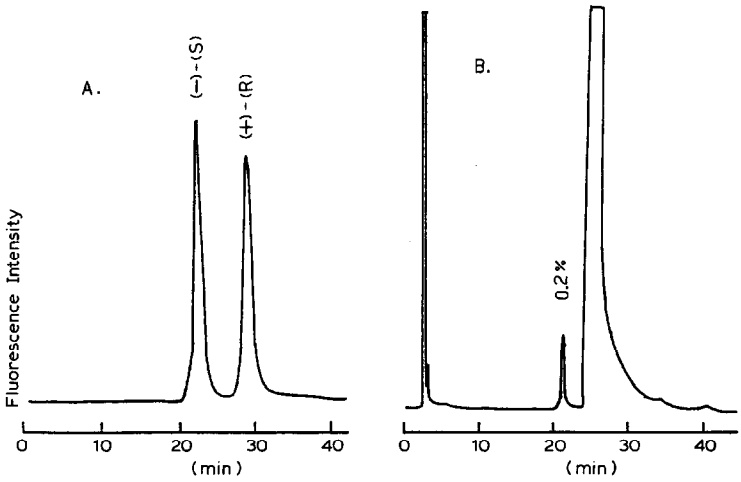

Fig. 4. Typical chromatograms of OFLX enantiomers under the optimum HPLC conditions. Mobile phase: 6 mM L-Phe, 3 mM copper sulphate (pH 3.5). Detection: fluorescence. Sample amount: (A) about 200 ng as a mixture; (B) about 3 μg as the (+)-enantiomer containing 0.2% (−)-enantiomer.

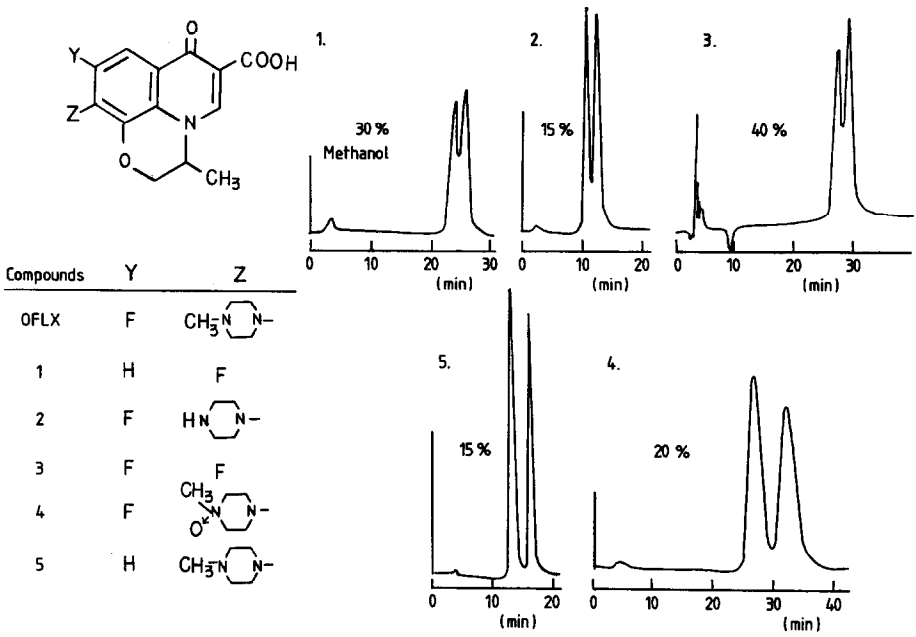

Fig. 5. Chromatograms of OFLX enantiomer derivatives. HPLC conditions: 6 m*M* L-Phe, 3 m*M* copper sulphate; the pH was not adjusted. Methanol concentrations as indicated in the figure. Sample amount: about 200 ng as a mixture.

(*e.g.*, L-Phe) and aliphatic amino acids (*e.g.*, L-Ile) exhibited good separation. With the D-phenylglycine and toluenesulphonyl-L-phenylalanine system, however, less stereoselectivity is observed with the isomeric pairs of OFLX. This seems to be the result of steric hindrance between these ligands and OFLX, which possesses a tricyclic structure. We suggest that ternary complexes of amino acids and OFLX isomers with Cu(II) possess simple structures, as shown in Fig. 6.

OFLX derivatives, which have an asymmetric carbon in the position C-3, are also separated with L-Phe. The substituents of these derivatives do not affect the resolution of the optical isomers.

Since the stereoselectivity is dependent on the stability and the hydrophobicity of the diastereomeric metal complex in the ODS stationary phase, the pH value (Fig. 3), ligand concentration (Fig. 2) and methanol concentration would thus affect both the

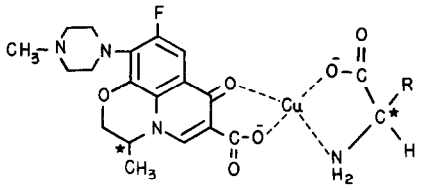

Fig. 6. Proposed structure of the ternary complex of OFLX and amino acid with Cu(II).

capacity factor, k', and selectivity, α, R_s. The extent of complex formation is increased and the resolution of the optical isomers is improved by raising the pH value and ligand concentration. On the other hand, lowering the methanol content in the mobile phase results in longer retention and slightly improved resolution.

In conclusion, a procedure has been described for the separation of pyridone carboxylic acid enantiomers by ligand-exchange chromatography. The compounds of pyridone carboxylic acid enantiomers are directly detectable with UV or fluorescence spectroscopy. This method will be useful and convenient for the simple determination of such enantiomers, for example OFLX, in pharmaceutical preparations and biological fluids.

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