Journal of Chromatography, 204 (1981) 195–200
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,154

SEPARATION OF THE DIASTEREOISOMERS OF PYROGLUTAMYL-HISTIDYL-3,3-DIMETHYLPROLINEAMIDE BY LIGAND-EXCHANGE CHROMATOGRAPHY

K. SUGDEN*, C. HUNTER and J. G. LLOYD-JONES
Reckitt and Colman, Pharmaceutical Division, Dansom Lane, Hull (Great Britain)

SUMMARY

A novel high-performance ligand-exchange technique based upon complexation with Cu^{2+} for the rapid and direct separation of L-pyroglutamyl-L-histidyl-L-3,3-dimethylprolineamide from its D-L-L, L-D-L and L-L-D isomers is described and the effect of various chromatographic parameters on retention and on bonding of copper to peptide is discussed.

INTRODUCTION

The conventional method for the determination of the chiral integrity of a peptide involves conversion of the acid hydrolysate amino acid mixture into diastereomeric dipeptides by reaction with the N-carboxyanhydride of an optically pure amino acid, usually L-leucine or L-glutamic acid, with subsequent analysis on an amino acid analyzer[1]. This method has several disadvantages in that it can lead to erroneous results via either racemisation of the amino acids on hydrolysis or by the production of interfering artefacts on derivatisation. Moreover, a single analysis on the amino acid analyzer can take many hours to complete and total analysis time may be of the order of several days.

Other more recent methods also require hydrolysis prior to analysis and involve the direct separation of the optical isomers of amino acids by liquid chromatography on optically active metal chelate stationary phases[2–5].

This report describes how sample pretreatment can be avoided to directly resolve the diastereoisomers of the tripeptide pyroglutamylhistidyl-3,3-dimethylprolineamide by use of a simple and rapid ligand-exchange technique.

EXPERIMENTAL

A Waters 6000A constant flow pump (Waters Assoc., Northwich, Great Britain) was used to provide mobile phase flow and either a Pye LC3 (Pye Unicam, Cambridge, Great Britain) or an Applied Chromatography Systems 750/11 (Applied Chromatography Systems, Luton, Great Britain) UV detector was used to monitor column eluent.

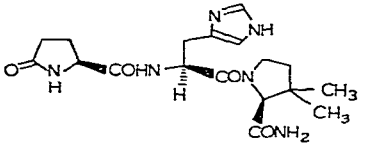
0021-9673/81/0000–0000/$02.50

The chromatographic columns were prepared by packing either Partisil–ODS (10 μm) (Whatman, Maidstone, Great Britain) or Spherisorb-CN (5 μm) (Phase Separations, Queensferry, Great Britain) into a 25 cm $\times$ 0.46 cm I.D. stainless-steel tube via a methanol slurry and a Magnus P5000 slurry packing unit (Magnus Scientific, Sandbach, Great Britain). The temperature of the column was controlled by a surrounding water-jacket thermostatically heated and fed by a Shandon water pump (Shandon, London, Great Britain).

Sample injection was achieved via a Rheodyne 7120 valve fitted with a 20-μl loop.

The isomers of pyroglutamylhistidyl-3,3-dimethylprolineamide were synthesised in the Medicinal Chemistry Laboratory of the Pharmaceutical Division of Reckitt and Colman. All solutions of the peptides used in this work were prepared in water.

RESULTS AND DISCUSSION



Pyroglutamylhistidyl-3,3-dimethylprolineamide

Previous work in this laboratory[6] and elsewhere[7] has shown that the diastereoisomers of certain enkephalins can be resolved on either 3-cyanopropyl- or octadecylsilylated high-performance silica gel. However, a preliminary investigation of the liquid chromatographic properties of L-pyroglutamyl-L-histidyl-L-3,3-dimethylprolineamide and its D-L-L, L-D-L and L-L-D isomers on both of these columns showed some of the diastereosiomers to co-chromatograph to give partial resolution only (Figs. 1 and 2). To achieve the desired separation it was necessary to develop a ligand-exchange technique based upon a cyano phase modified by complexation with Cu^{2+} added to the mobile phase. The elemental composition of such a phase (Table I) indicates that few of the surface cyano sites are available for complexation, but nonetheless by careful control of the various parameters it was possible to resolve all four isomers within 25 min (Fig. 3), with a detection limit of approximately 60 ng on column when the eluent is monitored at 210 nm. The effect of the concentration of Cu^{2+} in the mobile phase on the retention of the four isomers is given in Fig. 4. The retention of all the isomers increases as the Cu^{2+} ionic strength is increased from $1 \cdot 10^{-3}$ mM to $5 \cdot 10^{-2}$ mM but above this concentration the retention time is seen to

TABLE I

ELEMENTAL ANALYSIS OF THE Cu^{2+}-LOADED CYANO PHASE

Carbon (μmoles/g)	Hydrogen (μmoles/g)	Nitrogen (μmoles/g)	Copper (μmoles/g)
480	440	440	5.2

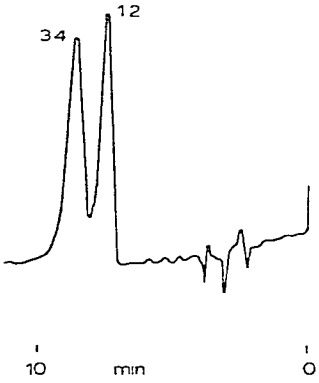


Fig. 1. Chromatogram of L-pyroglutamyl-L-histidyl-L-3,3-dimethylprolineamide and its isomers. Peaks: 1, L-L-D isomer; 2, L-D-L isomer; 3, L-L-L isomer; 4, D-L-L isomer. Conditions: column, Partisil-ODS (10 μm), 25 × 0.46 cm I.D. at 87°C; mobile phase, acetonitrile–0.1 m*M* sodium acetate (30:70), flow-rate 1 ml/min; detection, UV absorption at 210 nm.

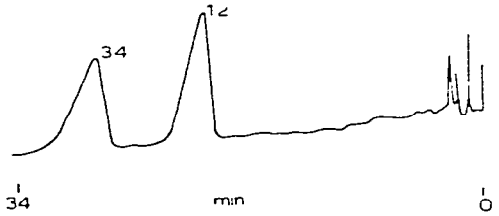


Fig. 2. Chromatogram of L-pyroglutamyl-L-histidyl-L-3,3-dimethylprolineamide and its isomers. Peaks: 1, L-L-D isomer; 2, L-D-L isomer; 3, L-L-L isomer; 4, D-L-L isomer. Conditions: column, Spherisorb-CN (5 μm), 25 × 0.46 cm I.D. at 87°C; mobile phase, acetonitrile–0.01 m*M* sodium acetate (30:70), flow-rate 2 ml/min; detection, UV absorption at 210 nm.

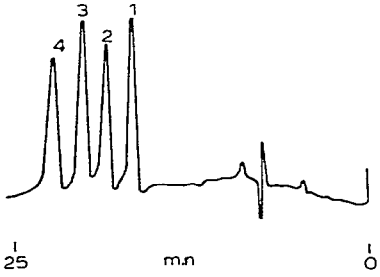


Fig. 3. Chromatogram of L-pyroglutamyl-L-histidyl-L-3,3-dimethylprolineamide and its isomers. Peaks: 1, L-L-D isomer; 2, L-D-L isomer; 3, L-L-L isomer; 4, D-L-L isomer. Conditions: column, Spherisorb-CN (5 μm), 25 × 0.46 cm I.D. at 87°C; mobile phase, acetonitrile–0.01 m*M* copper(II) acetate (30:70), flow-rate 0.5 ml/min, detection, UV absorption at 210 nm.

decrease. Cooke *et al.*[8] have noticed similar trends in the retention of sulpha drugs on a diamine phase modified by the presence of Cd^{2+} in the mobile phase. When the concentration of Cu^{2+} in the mobile phase is kept low (0.01 m*M*), to ensure that all active sites remain complexed, and ionic strength is adjusted by the addition of a non-complexing ion, such as Na^+, then trends in retention similar to those observed in the

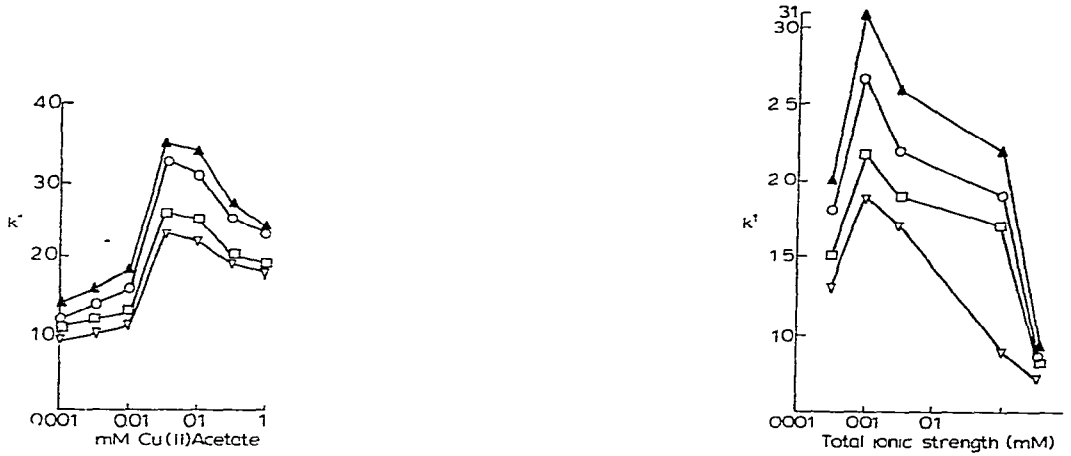


Fig. 4. Effect of Cu^{2+} concentration (mM) in the mobile phase on capacity ratio (k') on the cyano column. Conditions: mobile phase, acetonitrile–copper(II) acetate (30:70); column, Spherisorb-CN, 25 × 0.46 cm I.D. at 87°C. Symbols: ▲, D-L-L isomer; ○, L-L-L isomer; □, L-D-L isomer; ▽, L-L-D isomer.

Fig. 5. Effect of Na^+ concentration (mM) in the mobile phase on the capacity ratio (k') on the cyano column modified by complexation with Cu^{2+}. Conditions: mobile phase, acetonitrile–0.01 mM copper(II) acetate + sodium acetate (30:70); column, Spherisorb-CN, 25 × 0.46 cm I.D. at 87°C. Symbols: ▲, D-L-L isomer; ○, L-L-L isomer; □, L-D-L isomer; ▽, L-L-D isomer.

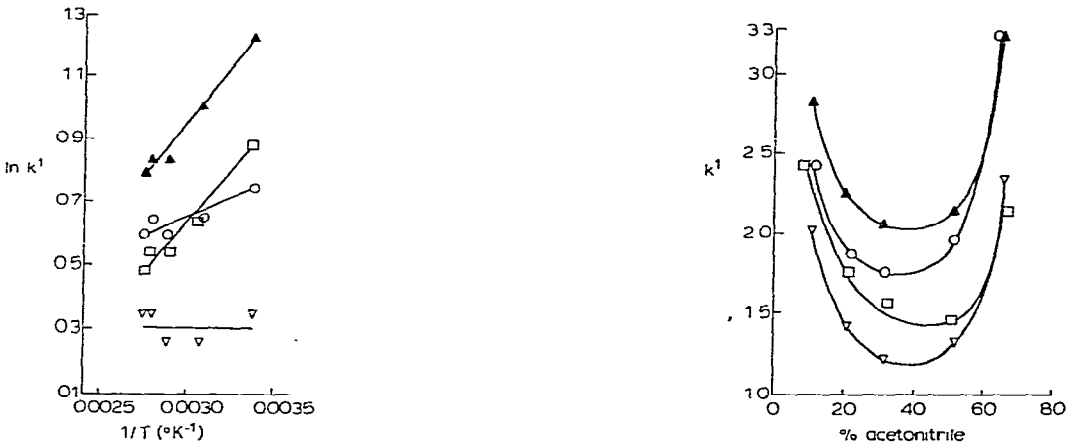


Fig. 6. Effect of column temperature (T) on capacity ratio (k'). Conditions: mobile phase, acetonitrile–0.01 mM copper(II) acetate (30:70); column, Spherisorb-CN, 25 × 0.46 cm I.D. at 87°C. Symbols: ▲, D-L-L isomer; ○, L-L-L isomer; □, L-D-L isomer; ▽, L-L-D isomer.

Fig. 7. Effect of acetonitrile concentration of the mobile phase on capacity ratio (k'). Conditions: mobile phase, acetonitrile–0.01 mM copper(II) acetate (30:70); column, Spherisorb-CN (5 μm), 25 × 0.46 cm I.D. at 87°C. Symbols: ▲, D-L-L isomer; ○, L-L-L isomer; □, L-D-L isomer; ▽, L-L-D isomer.

presence of varying concentrations of Cu^{2+} only are observed (Fig. 5). Although with the presence of Na^+ the total ionic strength required to achieve maximum retention time is shifted from $5 \cdot 10^{-2}$ M to $1 \cdot 10^{-2}$ M, total removal of Cu^{2+} from the mobile phase results in slow deterioration in column performance, due to sequestration of

the copper from its binding site, and it appears that the significant role of Cu^{2+} is to initially modify and then maintain the copper loading on the column.

Increasing the temperature of the column greatly reduces the retention times of the D-L-L and L-D-L isomers but has less effect on L-L-L and negligible effect on L-L-D (Fig. 6). This suggests that the rate of ligand exchange is more sensitive to changes in the chirality of the glutamyl and histidyl moieties than in the 3,3-dimethylprolineamide moiety. Hence the role of the latter in the separation mechanism is less significant. Initial Cu^{2+}–peptide binding studies using proton NMR tentatively indicate by proton broadening that it is the histidyl moiety which bonds predominantly to Cu^{2+}. Other workers[9] have confirmed this observation by similar studies with thyrotropin releasing hormone (TRH). From a practical viewpoint control of the temperature is very advantageous since it enables both the absolute and relative retention of the diastereoisomers to be changed, and provides an improvement in the resolution.

The acetonitrile concentration of the mobile phase has a significant effect on both the absolute and relative retention of the isomers (Fig. 7). The role of the solvent is unclear but it does have a more marked effect upon a conformational change in the histidyl moiety than on any other. A similar study carried out with the L-L-D isomer only in systems free of Cu^{2+} but containing Na^{+} shows similar trends in retention behaviour and suggested that a mixed chromatographic mechanism is involved. At low acetonitrile concentrations, such that the polarity of the mobile phase is greater than that of the stationary phase, reversed-phase partition occurs and at high acetonitrile concentration an absorption mechanism characteristic of conventional silica gel chromatography seems to be involved.

CONCLUSION

The chirality of pyroglutamylhistidyl-3,3-dimethylprolineamide can be determined by high-performance ligand-exchange chromatography. The technique overcomes the problems inherent in the conventional method in that it is simple to perform, rapid and minimises the possibility of racemisation.

Various chromatographic parameters have been shown to have a significant effect on the relative retention of the diastereoisomers and it is foreseen that the technique will prove to be useful to resolve the isomers of other peptides capable of association with metals.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. B. A. Morgan who synthesised the peptides and to Dr. R. Henson for carrying out the NMR studies.

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