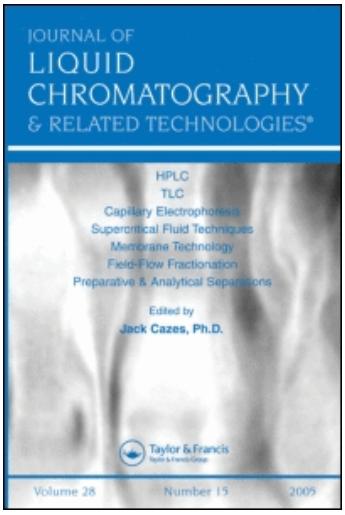

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C. Hunter[a]; K. Sugden[a]; J. G. Lloyd-jones[a]
[a] Pharmaceutical Division, Danson, Reckitt and Colman Ltd., Lane, Great Britain

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HPLC OF PEPTIDES AND PEPTIDE DIASTEREOISOMERS ON ODS- AND CYANOPROPYL-SILICA GEL COLUMN PACKING MATERIALS

C. Hunter, K. Sugden and J. G. Lloyd-Jones,

Reckitt and Colman Ltd.,
Pharmaceutical Division,
Dansom Lane,
Kingston-upon-Hull,
Great Britain

ABSTRACT

The use of hplc for the separation of various peptide diastereoisomers without the need for sample pre-treatment is described. The effect of the concentration of acetonitrile in the mobile phase on the retention of peptides on ODS- and Cyanopropyl-bonded phases is discussed and compared with the effect of methanol. The use of temperature and pH to control the relative retention of peptides on cyano phase is also discussed.

INTRODUCTION

With the continuing development of synthetic peptides for use as potential drug substances the need for rapid and accurate methods of analysis has become increasingly apparent. The advent of chemically

bonded octadecyltrichlorosilane silica gel (ODS) column packings has enabled peptide analysis to be transferred from the amino acid analyzer to more versatile and rapid high performance liquid chromatographic (hplc) systems[1-4].

Since the degree of optical isomerisation of the amino acid residues within the peptide molecule can have a profound effect on the pharmacological activity of the compound, it is imperative that the chiral character of the molecule can be accurately determined. Prior to the development of hplc the method[5] used involved derivatisation of the peptide acid hydrolysate with an enantiomeric reagent such as the N-carboxyanhydride of L-leucine or L-glutamic acid, with subsequent analysis on the amino acid analyzer. Other similar procedures using gas chromatographic[6-9] and hplc[10-11] analysis have also been described. With these derivatisation procedures however, care must be taken to avoid either racemisation of amino acid residues or the production of interfering artefacts via side reactions. Recently[12] it has been shown that hplc analysis can be used to resolve peptide diastereoisomers, without the need for derivatisation.

It is the purpose of this report to describe some of the work that has been carried out in this laboratory concerning the separation of diastereoisomers and also to compare the performance of chemically bonded 3-cyanopropyl silica gel (CN) column packing material with ODS silica gel.

MATERIALS

Apparatus

The hplc system used consisted of a Waters 6000A constant flow pump to provide mobile phase flow and a Pye Unicam LC3 ultra violet detector to monitor column eluent. Sample injection was via a Rheodyne 7120 valve fitted with a 20µl loop.

All columns were packed in our laboratory into stainless steel tubes via a methanol slurry at approximately 5000 psi using a Magnus P5000 column packing pump. A glass walled water jacket heated and fed by a Shandon Water pump was used to control the column temperature.

Chemicals

The mobile phases were prepared with Hplc Grade S acetonitrile (Rathburn Chemicals Ltd.), Analar Grade

methanol (Hopkins and Williams Ltd.) and glass distilled water. All peptide samples were synthesized in the Medicinal Chemistry Department of Reckitt and Colman Ltd., Pharmaceutical Division.

RESULTS AND DISCUSSION

The applicability of hplc analysis to the separation of peptide diastereoisomers is demonstrated by the rapid and efficient resolution that may be achieved between L-histidyl-L-prolyldiketopiperazine, an *in vivo* metabolite of L-pyroglutamyl-L-histidyl-L-prolineamide (TRH), and its D-His-L-ProDkp isomer (Figure 1): Using conventional methods of analysis involving acid hydrolysis, unpredictable racemisation of the histidyl moiety invariably occurs making quantification difficult. With hplc, however no time consuming pre-treatment of the sample is required and racemization is minimal. The detection limit of one isomer within the other is $\leqslant 1\%$ w/w when monitored at 210nm.

The utility of reverse-phase hplc for the separation of diastereoisomers is also illustrated by the resolution of L-Tyr-D-Ala-Gly-L-Phe-L-Met from the L-Ala^{2} and D-Met^{5} isomers and by the separation of

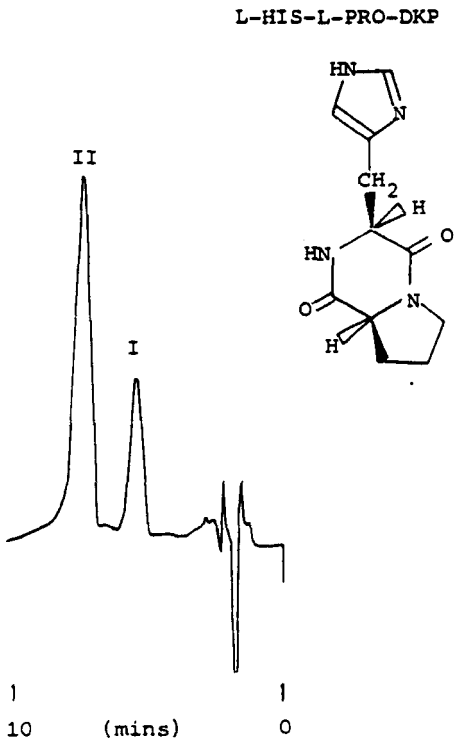


FIGURE 1

Separation of D-His-L-Pro-Diketopiperazine (I) from the L-His-L-Pro Isomer (II). Column: Partisil 10µm ODS, 25 x 0.46 cm i.d. Mobile Phase: 0.1% (W/W) Ammonium acetate in acetonitrile:water (10:90), 2 ml/min. Detection: UV at 210 nm.

a racemic mixture of L-Tyr-D-Ala-Gly-L/D-MePhe-D-Leu (Figures 2 and 3). Resolution of the D-Met^5 and L-Met^5 isomers can also be achieved by a ligand exchange technique using the CN-silica gel phase modified by complexation with Cu(II) ions (Figure 4). Previous work[13] in this laboratory has demonstrated

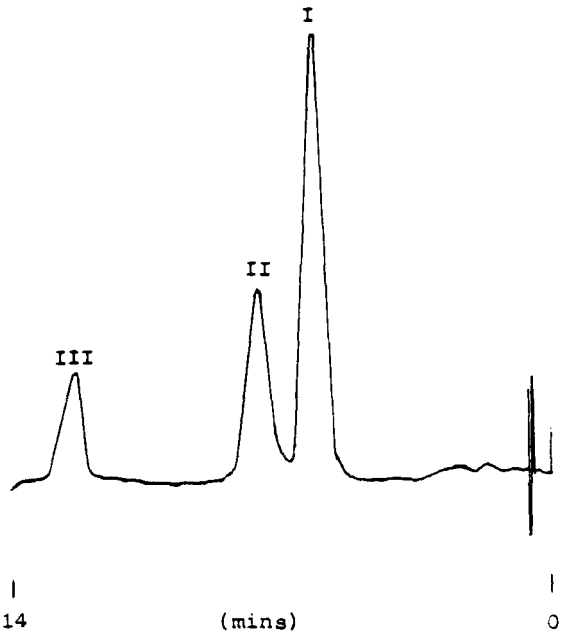


FIGURE 2

Separation of L-Tyr-D-Ala-Gly-L-Phe-L-Met (I) from the L-Ala$_2$ (II) and D-Met$_2$ (III) Isomers. Column: Spherisorb 5µm ODS, 10 x 0.46 cm i.d. Mobile Phase: 0.1% (W/V) Ammonium acetate in acetonitrile:water (10:90), 3 ml/min. Detection: UV at 215 nm.

how a similar technique can be used to separate L-pyroglutamyl-L-histidyl-L-3,3-dimethylprolineamide from the D-L-L, L-D-L and L-L-D isomers.

The effect of changing both the nature and concentration of the organic modifier in the mobile phase on the retention of the peptide L-Tyr-D-Ala-Gly-L-MePheNH$(CH_2)_2NMe_2$ on ODS-silica gel is given

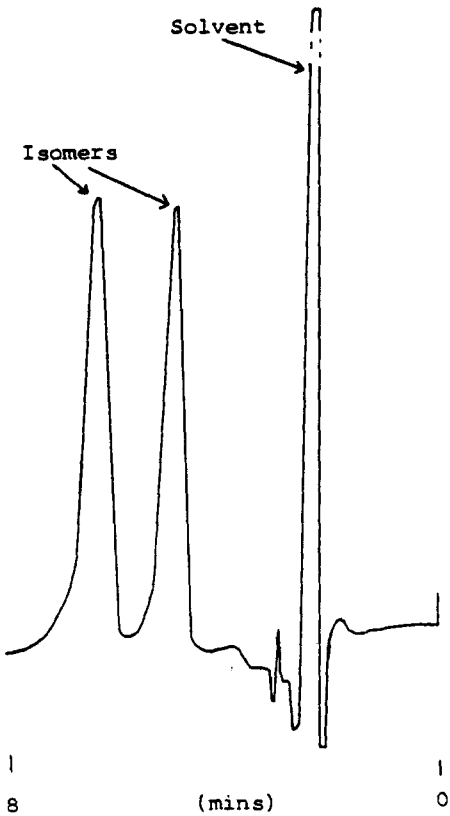


FIGURE 3

Separation of L-Tyr-D-Ala-Gly-L-MePhe-D-Leu from the D-MePhe[4] Isomer. Column: Spherisorb 5µm ODS, 10 x 0.46 cm i.d. Mobile Phase: 0.1% (W/V) Ammonium acetate in acetonitrile: 0.025% (V/V) acetic acid (70:30), 0.5 ml/min. Detection: UV at 215 nm.

in Figure 5; with methanol as the modifier k' decreases exponentially with increasing concentration and the system behaves in a typical reverse-phase manner, but with acetonitrile present as the

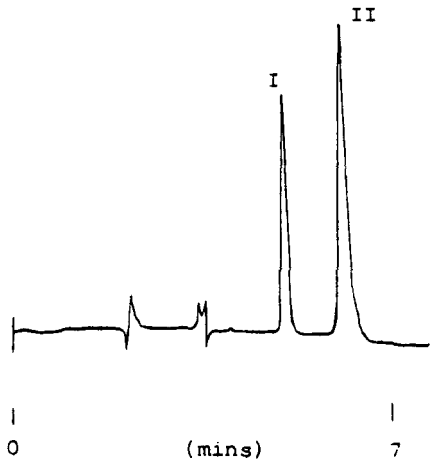
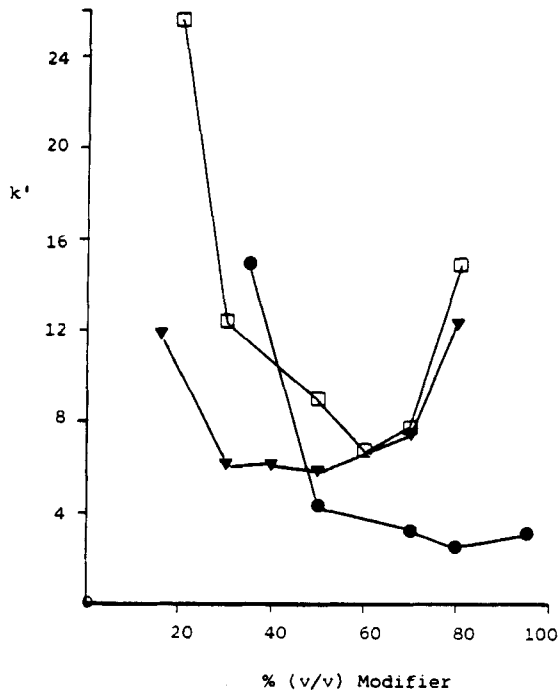


FIGURE 4

Separation of L-Tyr-D-Ala-Gly-L-Phe-L-Met (I) and the D-Met[5] Isomer (II) by Ligand Exchange Chromatography. Column: Spherisorb 5µm CN, 25 x 0.46 cm i.d., 87°C. Mobile Phase: 0.035 mM Cupric acetate in acetonitrile:water (30:70) 1 ml/min. Detection: UV at 210 nm.

modifier the decrease in retention is reversed at and above a concentration of approximately 60% v/v to give an increase in retention. The reason for this change in mechanism is unclear, but it may be due to modification of the stationary phase by absorption of acetonitrile and result in partition of the solute into a solvent rich surface.[14] Similar trends in retention are also observed with increasing acetonitrile concentration on the CN column packing, but since the solvent is very similar to the



□: Spherisorb 5μm ODS ; 0.1% (w/v) Ammonium acetate + 0.01% (v/v) acetic acid in aqueous acetonitrile.

●: As for □ with aqueous methanol in the mobile phase.

▼: As for □ on Spherisorb 5μm CN.

FIGURE 5

Effect of the Nature and Conc'n of the Organic Modifier in the Mobile Phase on Retention Time. Substrate: L-Tyr-D-Ala-Gly-L-MePheNH $(CH_2)_2$ NMe_2.

stationary phase surface adsorption of acetonitrile is less likely to cause drastic changes in retention. With this column it is possible that the retention mechanism changes from reverse phase partition to an adsorption mode as the polarity of the mobile phase

decreases. The relative efficiencies of the two column packing materials are given in Table I. With methanol in the mobile phase, the efficiency of the ODS column is very poor and peak shape unacceptable from a quantitative point of view. Some improvement in column efficiency is observed with acetonitrile in the mobile phase, but transfer to a system using a CN-column and acetonitrile provides the best efficiency. With aqueous methanol on CN-silica gel the peptide has a strong affinity for the stationary phase and capacity ratios are extremely high. In our experience CN phase is generally more efficient in peptide chromatography than is the ODS phase, and this is well illustrated by the separation of TRH from its 3,3-dimethylprolineamide analogue (Figure 6). The ODS column shows greater affinity for the TRH analogue than does the CN column, but the latter is by far the more efficient and allows for more rapid analysis whilst still maintaining adequate resolution.

The effect of the pH of the mobile phase on the retention of L-Tyr-D-Ala-Gly-L-MePheNH$(CH_2)_2NMe_2$ and the L-Ala[2] isomer is illustrated in Figure 7; below pH = 5.4 capacity ratio increases with decreasing hydrogen ion concentration but above this pH retention decreases rapidly and the order of

TABLE I

COMPARATIVE EFFICIENCIES OF ODS- AND CN- SILICA GEL COLUMNS

Substrate: Tyr-D-Ala-Gly-MePheNH$(CH_2)_2NMe_2$

Mobile phase: 0.1% (w/w) Ammonium acetate + 0.01% (v/v) orthophosphoric acid in organic modifier : Water delivered at 2ml min^{-1}.

% ORGANIC MODIFIER IN MOBILE PHASE	PLATE HEIGHT (μm)		
		ODS	CN
Organic Modifier:	MeOH	CH_3CN	CH_3CN
30	-	275	103
35	637	-	-
50	826	245	97
70	1471	210	85
80	2381	142	70

elution of the isomers is reversed. The reason for this dramatic change in retention mechanism is unclear but may be due to either a change in molecular conformation or be related to the pK_a value of the compound in the mobile phase, and both of these possibilities require further investigation.

Retention time can also be controlled by variation of the column temperature, the effects of which are inter-

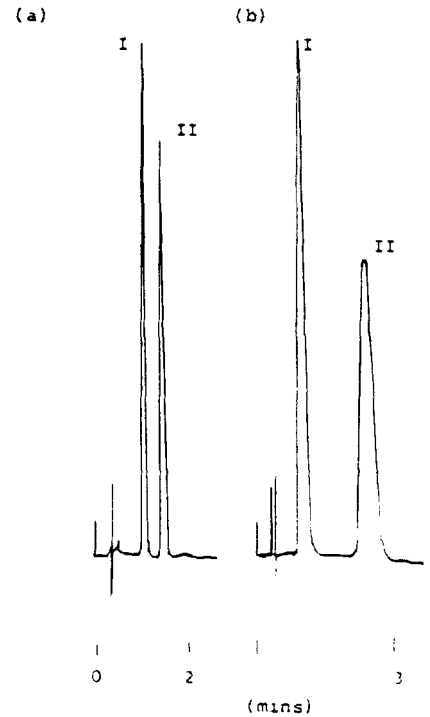


FIGURE 6

Separation of TRH (I) and the 3,3-Dimethyl-Prolineamide Analogue (II) on (a) Spherisorb 5µm CN and (b) Spherisorb 5µm ODS. Mobile Phase: (a) 0.1% (W/V) Ammonium acetate in acetonitrile:water (8:92), 3ml/min.; (b) 0.1% (W/V) Ammonium acetate in acetonitrile:water (10:92), 3 ml/min.

dependent with the pH of the mobile phase (Figure 8); at pH = 2.6 the retention and resolution of the two isomers increase with increasing temperature, but above pH = 5.4 the capacity ratios are inversely related to temperature. Similar effects of pH and

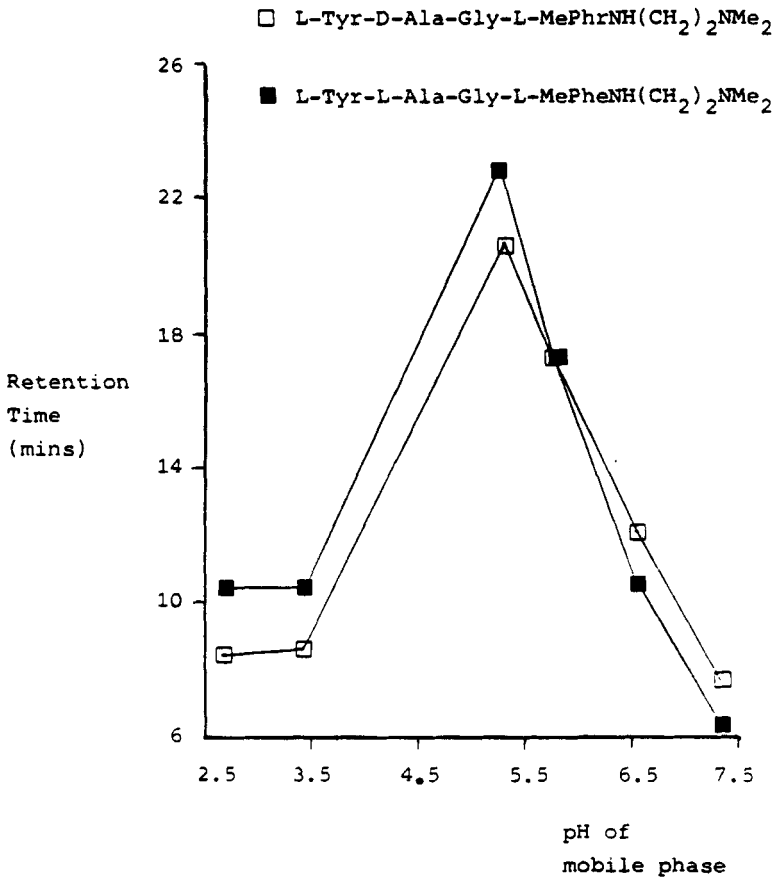


FIGURE 7

Effect of pH of the Mobile Phase on Retention Time on Cyanopropyl-Silica Gel Column Packing. Column: Spherisorb 5µm CN, 25 x 0.46 cm i.d. Mobile Phase: 0.01M Triethylammonium phosphate in acetonitrile:water (80:20), 2 ml/min. Detection: UV at 215 nm.

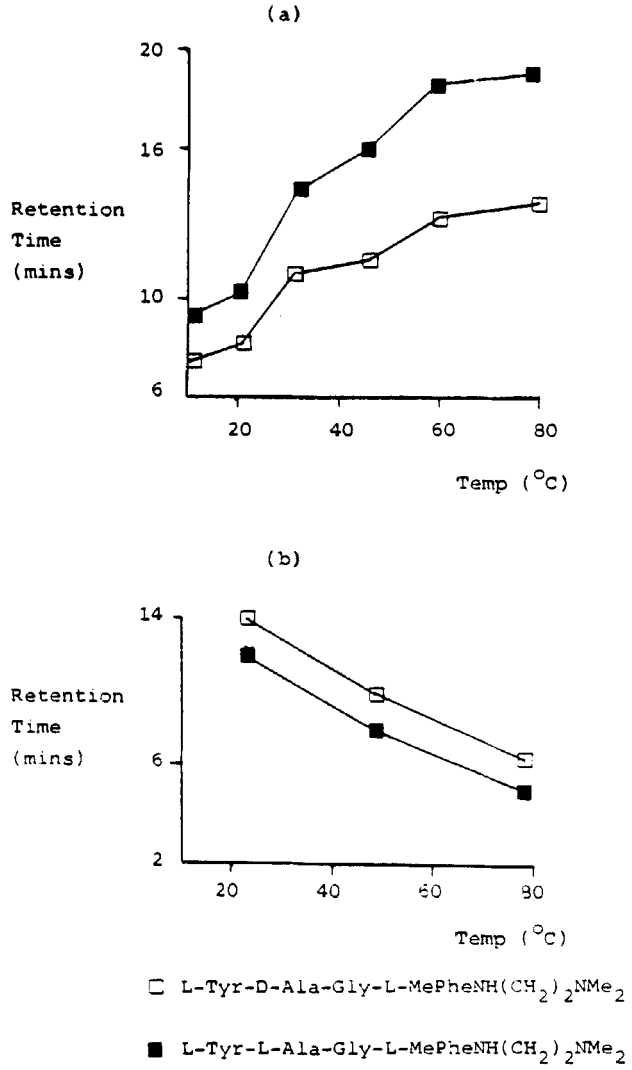


FIGURE 8

Effect of temperature on Retention Time on Cyanopropyl-Silica Gel Column Packing. Column: Spherisorb 5μm CN, 25 x 0.46 cm i.d. Mobile Phase: 0.01M Triethylammonium phosphate in acetonitrile:water (80:20), 2 ml/min. (a) pH=2.6, (b) pH=6.6. Detection: UV at 215 nm.

temperature are observed with other diastereoisomers of the peptide and are useful from a practical point of view in controlling retention and resolution.

CONCLUSION

Peptide diastereoisomers can be rapidly separated by hplc without the need for sample pre-treatment.

Chemically bonded 3-cyanopropyl silica gel tends to have less affinity for peptides than does ODS-silica gel, but its greater efficiency allows resolution to be maintained whilst analysis time is reduced. By variation of the concentration of acetonitrile in the mobile phase unusual effects on retention time on both CN- and ODS- column packings can be achieved and by careful selection of mobile phase pH and column temperature both absolute and relative retention can be effected and optimised.

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