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Peptide enantiomer separations: Influence of sequential isomerism and the introduction of achiral glycine moieties on chiral recognition

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Abstract

The influence of sequential isomerism and the introduction of achiral, conformationally flexible glycine moieties into a peptide chain on the chiral recognition mechanism of a cinchona alkaloid based chiral selector has been evaluated. For this purpose, enantiomers of N-terminally protected alanine–glycine di- and tripeptides were separated by liquid chromatography–mass spectrometry on a corresponding chiral stationary phase (CSP). To obtain complementary information, the reversed phase retention behaviour of the various peptides was also evaluated and subsequently used to further elucidate the chromatographic characteristics of the CSP. For peptides that contained glycines in the N-terminal region chiral recognition was compromised, while glycines located at the C-terminus had no or little negative effect.

Keywords: Peptides; Chiral recognition; Enantiomers; Sequence isomers; Glycine; Liquid chromatography–mass spectrometry

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1. Introduction

α-Amino acids and peptides as their oligomers form an important group of analytes in the area of enantiomer separations. While the only structural variability of α-amino acids lies in their side chains, peptides offer an additional possibility for variation based upon sequential isomerism, i.e. identical amino acid composition but different arrangement of the amino acid residues within the peptide chain. Each sequence isomer may influence the chiral recognition process in a specific way and consequently affect the respective enantiomer separation result.

This dependence of the enantiomer discrimination on the arrangement of given amino acid residues in the peptide sequence has been noted and discussed in several studies dealing with the separation of peptide enantiomers. In two early investigations using gas chromatography and Chirasil-Val® as chiral stationary phase (CSP) the enantiomer elution order was reversed between Leu-Gly and Gly-Leu [1,2]. Regarding peptide enantiomer separations by HPLC, a pronounced influence of sequential isomerism on the chiral recognition process has been described in several publications. Markedly differing enantioselectivities were observed for the two pairs of enantiomers of Ala-Leu and Leu-Ala on a crown ether based CSP [3] and for the Ala-Gly/Gly-Ala and Leu-Gly/Gly-Leu isomers on teicoplanin [4], teicoplanin aglycone [5] and crown ether [6] based CSPs. In enantiomer separation studies of di- and tripeptides consisting of one or two glycine moieties, respectively, plus an alanine or leucine residue employing different cyclodextrin derived CSPs separability and elution order varied depending on the sequences [7,8]. In a study of the pairs of enantiomers of Leu-X and X-Leu, where X was Phe, Tyr or Trp, on a chymotrypsin CSP the magnitude of enantiomer resolution was affected depending on the location of the aromatic amino acid and a reversal of the enantiomer elution order occurred in some cases [9]. Turning to investigations where capillary zone electrophoresis (CZE) was used as separation technique, differences between sequence isomers regarding enantiomer separation results were observed in a number of studies by

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Scriba and co-workers. They employed various native and modified cyclodextrins and studied the chiral recognition of Gly-Asp-Pro/Pro-Asp-Gly [10], Ala-Phe/Phe-Ala [11–14], Ala-Leu/Leu-Ala [13] and Gly-Ala-Phe/Ala-Gly-Phe [14]. In the studies dealing with Ala-Phe/Phe-Ala and Ala-Leu/Leu-Ala the (*R*,*R*)- and (*S*,*S*)-enantiomers were resolved less often for the peptides with Ala at the C-terminus than for those with Ala at the N-terminus [11–13]. Moreover, a reversal in the enantiomer migration order was observed in some cases for Ala-Phe/Phe-Ala [11,14] and Gly-Ala-Phe/Ala-Gly-Phe [14].

A lot of the peptides investigated in enantiomer separation studies contain a glycine residue. The incorporation of an achiral glycine moiety into a peptide chain with chiral amino acid units does not only reduce the number of possible stereoisomers but also adds an increment of conformational flexibility due to the lack of a side chain. Consequently, this change in the peptide's steric properties and behaviour can be expected to also affect its chiral recognition. Therefore, a systematic variation of the position(s) and the number of glycine residues that are introduced into a peptide chain offers the possibility of assessing the role a specific amino acid residue within a peptide plays in the stereoselective recognition process of the latter. The effects of replacing a chiral amino acid by glycine or inserting/adding a glycine moiety within/at the end of a peptide chain have been investigated in some HPLC and CZE studies of peptide enantiomer separations. The elongation of Ala-Gly to Ala-Gly-Gly was found to increase enantioselectivity in HPLC using a crown ether based CSP [6]. In HPLC investigations using cyclodextrin derived CSPs the addition of one or two glycine moieties to an amino acid or dipeptide increased the retention of the analytes and the separation of the enantiomers was often enhanced [7,8,15]. In some cases, a reversal of elution order occurred. The position(s) of the glycine residue(s) relative to that of the chiral amino acid significantly altered the chiral recognition process [8]. Pirkle et al. utilised the introduction of a glycine moiety between the amino acids of a dipeptide to assess whether the stereochemistry of the second amino acid residue still manifested itself in the chromatographic behaviour of the peptide enantiomers on various “Pirkle-type” CSPs, if it was moved away from the principle interaction site [16]. Finally, the effects of either replacing a chiral amino acid by glycine or adding/inserting an extra glycine residue in Ala-Phe on the migration order of the (all-*S*)- and (all-*R*)-enantiomers in CZE were studied by Sabbah and Scriba and marked differences were observed [11].

The family of cinchona alkaloid derived chiral selectors has been shown to be highly suited for the discrimination of N-protected peptide enantiomers [17–23]. Several studies showing successful HPLC separations, where CSPs containing immobilised versions of the selectors were used, have been reported in the recent years [18,19,21,22]. In HPLC applications that employ a reversed-phase type (hydro-organic) mobile phase the enantioselective discrimination of this class of selectors is based on a weak anion-exchange functionality, which makes acidic compounds the prime analyte target group. The primary ion-pairing process is non-enantioselective and mainly serves the purpose of bringing the selector and the analyte close enough together so that secondary enantioselective interactions that include hydrogen-bonding, π–π-stacking and steric interactions can come into effect and yield the desired enantiomer separation [18]. Besides their ion-exchange characteristic and the resultant retention behaviour, the cinchona alkaloid based CSPs also exhibit a reversed phase (hydrophobic) retention increment, which is non-enantioselective [24]. Therefore, the overall retention of an N-terminally protected peptide (and of any other acidic analyte) is a combination of the ion-exchange process and the hydrophobic interactions. Thus, an examination of the differences between the enantiomer separation behaviours of peptides with different sequences on a cinchona alkaloid based CSP will be aided and enhanced by a deconvolution of these two contributions, which can be accomplished by measuring the reversed phase retentions of the peptides.

In the present study, the influence of introducing one or two glycine residue(s) into an oligoalanine di- or tripeptide chain at different positions on the chiral recognition by a cinchona alkaloid chiral selector is investigated. To this end, the enantiomers of the various peptides were separated by HPLC employing an immobilised version of the selector in the form of a CSP. Arising from the considerations discussed above, these experiments were accompanied by an analysis of the reversed phase behaviour of the peptide set.

2. Experimental

2.1. Materials

The amino acids and most of the peptides were purchased from Sigma–Aldrich (Steinheim, Germany) or Bachem (Bubendorf, Switzerland). Those peptides that were not commercially available were synthesized as *N*-3,5-dinitrobenzoyl derivatives by piChem (Graz, Austria) according to standard protocols. 3,5-Dinitrobenzoyloxysuccinimide was prepared from 3,5-dinitrobenzoyl chloride (Sigma–Aldrich) and hydroxysuccinimide (Fluka, Buchs, Switzerland) by Hünig base coupling. Ammonium acetate, acetic acid, sodium hydrogencarbonate and sodium carbonate were obtained from Fluka. HPLC grade methanol (Merck, Darmstadt, Germany) and doubly distilled water were used throughout.

The preparation of the *tert*-butylcarbamoylquinine chiral selector has been described elsewhere [25]. The corresponding chiral stationary phase was obtained by subsequent coupling of the selector to thiol-modified silica gel (Kromasil 100-5 μm, Eka Chemicals, Bohus, Sweden) [25]. The 5 μm CSP particles were slurry-packed into a 150 mm × 4.0 mm I.D. stainless steel column (Austrian Research Centers, Seibersdorf, Austria). A 150 mm × 4.6 mm I.D. BetaBasic-18 column containing a C_{18} stationary phase with 3 μm