High-Performance Liquid Chromatography of Biologically Important, Small Epimeric Peptides and their L, D-Amino Acid Content

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Abstract: Since most of the proteinogenic α-amino acids contain a chiral carbon atom, the stereoisomers of both these amino acids and the peptides in which they are to be found may possess differences in biological activity in living systems. The important analytical task of the separation of optical isomers is achieved mainly by chromatographic methods. This special review surveys direct and indirect HPLC separations of biologically important, small epimeric peptides and their L,D-amino acid content.

Keywords: Chirality, enantiomers, peptide epimers, amino acids, high-performance liquid chromatography, chiral derivatizing agents (CDAs), chiral stationary phases (CSPs).

1. INTRODUCTION

The existence of enantiomers has been known for many years and the importance of chirality with respect to biological activity is nowadays clearly recognized. Chirality exists throughout the universe and plays a vital role in our lives. With the exception of Gly, all of the 20 proteinogenic α-amino acids contain a chiral carbon atom. This chiral center allows the existence of enantiomers, *i.e.* two chemically identical molecular species differing only in optical activity (*i.e.* their ability to rotate the plane of polarized light).

The genetically encoded amino acids comprise the building blocks of proteins, which are the most important constituents of all living systems. It is well known that the L- and D- forms of amino acids in peptides can differ significantly with respect to stability and biological activity. Evaluation of the degree to which racemization occurs in the peptides produced is regularly required. Other consequences of chirality are concerned with the metabolic processes. Various transformations, such as prochiral to chiral, chiral to chiral, chiral to diastereoisomer, chiral to nonchiral and chiral inversion, can occur in a living organism. Aspects of chirality are also very important in the environment, in the pharmaceutical, food and beverages, agrochemical and petrochemical industries.

Initially chiral analyses were relatively difficult and most separations were carried out with derivatization prior to analysis. The first chiral stationary phase (CSP) for gas chromatography was described in 1966 [1]. Davankov and Rogozhin [2] introduced chiral ligand-exchange chromatography (LEC) in 1971, and LEC became the first direct high-performance liquid chromatographic (HPLC) method for the resolution of amino acids. The breakthrough in HPLC technology in the 1970s led to the commercial availability of liquid chromatographs appropriate for fast, efficient and reliable separations. The development of the hardware techniques was accompanied in the 1970s and 1980s by advances relating to packing materials. Polymeric materials are not suitable for high-pressure operation, while the relative instability of the physical coating of the support materials proved to be one of the main drawbacks of the early CSPs. With increasing experience and knowledge and continuous development in the field, the disadvantageous properties were progressively overcome. The appearance of mechanically stable, porous, small-diameter silica particles led to commercialization of the chemically bonded CSPs. The chiral separation techniques have subsequently become a very sophisticated field of analytical chemistry. The number of CSPs now exceeds 200, and studies focusing on the development of new types of chiral selectors are published virtually daily. The most important classes currently applied are ligand exchangers, polysaccharides, cyclodextrins (CDs), crown ethers, proteins, antibiotics, and π-donor-acceptor-type (Pirkle) CSPs.

The main methodologies utilized for enantioseparation are enzymatic degradation, crystallization, membrane-based, spectroscopic, capillary electrophoretic (CE) and HPLC methods. In chromatographic methods, two main strategies have evolved for chiral separation: *indirect methods*, based on the formation of diastereomers by the reactions of chiral compounds with a chiral derivatizing agent (CDA) and separation of the diastereomeric derivatives on an achiral stationary phase; and *direct methods*, based on the formation of diastereomers on a CSP, or with a chiral selector in the mobile phase on an achiral stationary phase. Both approaches have their advantages and disadvantages.

This review has the aim of the discussion of the papers appeared recently in the field of direct and indirect HPLC separations of biologically important, small epimeric peptides and their L,D-amino acid content. It does not discuss the chiral separations of different amino acids and their derivatives and analogous procedures for chiral gas chromatographic, thin-layer chromatographic, supercritical fluid

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1389-5575/10 $55.00+.00

chromatographic and CE separations. Even in this restricted field, the limitations of this section do not permit a survey of the extremely large number of publications from the beginning. Early information on this subject may be found in the original papers cited in numerous previous reviews and monographs [3-12].

2. INDIRECT SEPARATION OF AMINO ACID ENANTIOMERS IN PEPTIDES

The reaction of enantiomers with homochiral reagents to form diastereomeric derivatives that could be separated on achiral columns was the first widely used general method. Following the introduction of new chromatographic and CE techniques, the importance of chiral derivatization has naturally decreased to some extent. However, this general method is still a method of choice that is widely used. The reasons include the large number of commercially available homochiral reagents, well-established reactions and (if the *R*- and *S*-forms of the reagent are available), the peak of the enantiomeric impurity can be eluted before the main peak.

For determination of the chiral amino acid composition of peptides or biological matrices, peptides are hydrolyzed and the liberated amino acids are then converted to diastereomers by derivatization with different CDAs. To differentiate racemization occurring in the course of peptide synthesis or hydrolysis, hydrolysis in a deuterated acid solvent and mass spectrometric (MS) analysis are sometimes necessary. Peptide hydrolysis in deuterated acids circumvents the problem by labeling each amino acid that racemizes with one deuterium on the α-carbon.

Many biochemically important compounds have at least one functional group in their structure. The major types of reactions for chiral amines, including amino acids, are mainly based on the formation of amides, carbamates, ureas and thioureas.

The reactions of acid chloride and chloroformate reagents proceed rapidly to furnish the corresponding amides and carbamates. The most important chloroformate possessing a reactive functional group is 1-(9-fluorenyl)ethyl chloroformate (*FLEC*), developed by Einarsson *et al.* [13] (Fig. (**1**)).

An example of the application of *FLEC* is the determination of the enantiomers of 2-amino-1,2,3,4-tetrahydronaphthalene-1-carboxylic acid and 1,2,3,4-β-carboline-1-carboxylic acids in oxytocin analogs (Table **1**) [14]. New (+)- and (-)-4-(6-methoxy-2-naphthyl)-2-butyl chloroformates (*NAB-C*) have been compared with other reagents containing a 6-methoxy-2-naphthol moiety and found to be suitable for the analysis of nanogram amounts of β-adrenoreceptors [15].

Chiral isocyanates and mainly isothiocyanates are good labels with which to produce stable ureas and thioureas. Among the chemically most selective CDAs are isothiocy-

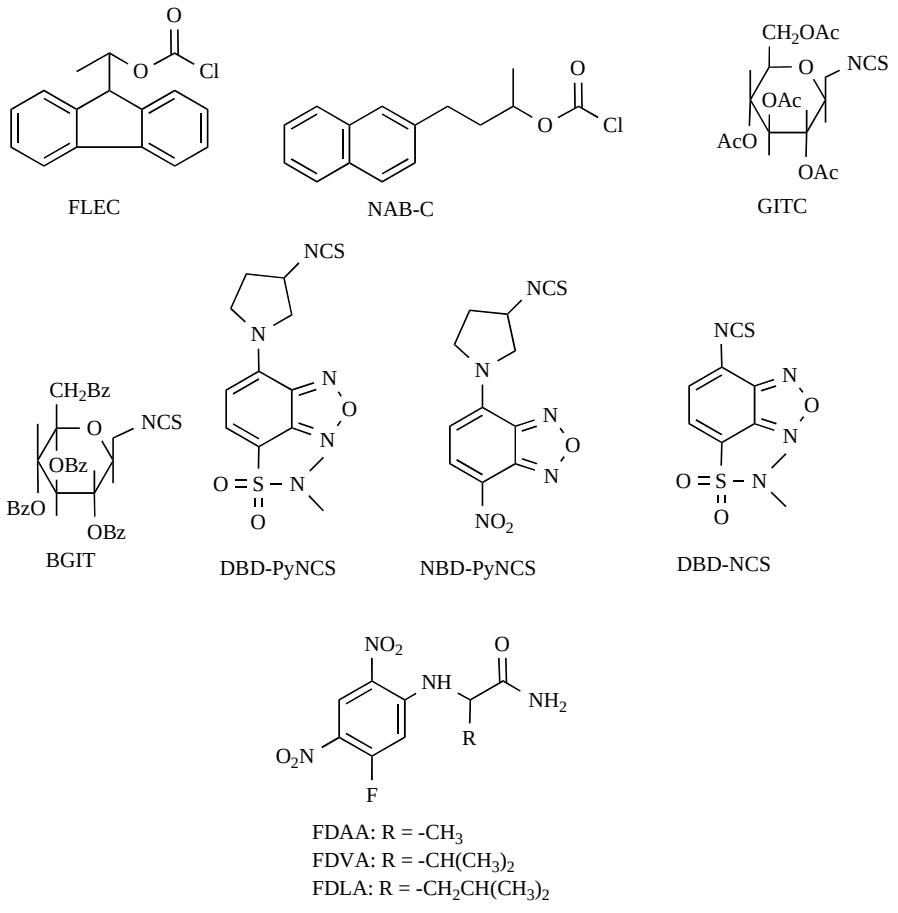

Fig. (1). Chemical structures of the most important derivatizing agents.

anates such as 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (*GITC*) and 2,3,4,6-tetra-*O*-benzoyl-β-D-glucopyranosyl isothiocyanate (*BGIT*) (Fig. (**1**)). Kinoshita [16] reviewed the application of *GITC* in the enantioseparation of proteinogenic amino acids and peptides. Szabó *et al.* [17] used various isothiocyanates for the separation of epimeric mixtures of oxytocin and other peptides containing Cys or cystine [18].

Toyo'oka *et al.* [19] developed fluorescent Edman-type reagents bearing an -NCS function, *i.e. R*(-)- and *S*(+)-4-(3-isothiocyanatopyrrolidin-1-yl)-7-(*N,N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazole [(*R*)(-)- and (*S*)(+)-*DBD-PynCS*], and *R*(-)- and *S*(+)-4-(3-isothiocyanatopyrrolidin-1-yl)-7-nitro-2,1,3-benzoxadiazole [(*R*)(-)- and (*S*)(+)-*NBD-PynCS*] (Fig. (**1**)). The D-amino acid contents were determined in milk, cream, fermented dairy products, fermented beverages and human urine, and the D-amino acids were identified by using both isomers of *DBD-PyNCS* and online HPLC-ESI-MS detection [20].

These chiral reagents were applied to determine the stereochemical purities of peptides such as D,L-Ala and D,L-Leu-containing di- and tripeptides [21]; neurotensin, [D-Ala^2,D-Leu^5]enkephalin and morphine tolerance peptide [22]; [D-Ala^2]Leu-enkephalin [23]; [D-Ala^2]Leu-enkephalin, [D-Ala^2]deltorphin II, D-Phe-Met-Arg-Phe-NH_2, Phe-D-Met-Arg-Phe-NH_2, β-lipotropin, Asp-Ser-Asp-Pro-Arg and Pro-Asp-Val-Asp-His-Val-Phe-Leu-Arg-Phe-NH_2 [24]; and [L-Ala^2]Leu-enkephalin and [D-Ala^2]-Leu-enkephalin [25] (Table **1**). The results of the application of *DBD-PynCS* and *NBD-PynCS* are reviewed in papers by Toyo'oka [5,9].

Marfey's reagent [26], 1-fluoro-2,4-dinitrophenyl-5-L-Ala amide (*FDAA*), and its chiral variants, in which the L-Ala amide is replaced by some amino acid amide [27], are among the most important CDAs (Fig. (**1**)). B'Hymer *et al.* [6] and Bhushan and Brückner [7] have published excellent background reviews on the application of Marfey's reagent. Marfey's reagent has been used for the determination of amino acid isomer residues in Arg-Lys-Lys-Asp-Val-Tyr [28], for homocysteic acid, cysteic acid and Cys residues in peptides [18], and for the chiral purity analysis of seleno amino acids in selenium-inoculated yeast and pharmaceutical preparations [29]. The chiral compositions of Cys in somatostatin analogs [30], the synthetic decapeptide Suc-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-Glu-OH [31] and Woc(114-108) [32] have also been determined by the use of *FDAA*.

Another general reaction for the chiral derivatization of amino acids is with *OPA* and chiral thiols to form fluorometrically highly active isoindole derivatives (Table **1**). Rajewski *et al.* [33] derivatized a protein kinase C inhibitor (SPC-100270) with *OPA* and *N*-acetyl-D-penicillamine (*NAP*). The D-amino acid contents in peptide antibiotics such as bacitracin, gramicidins A and B, polymyxin B, metanicin C, peptide toxin malformin A and the peptide drugs D-Arg-[Hyp^3-Thi$^{5-8}$-D-Phe^7]bradykinin, β-casomorphin and α_{s1}-exorphin [34] were analyzed through the application of *OPA* and *N*-isobutyryl-L-Cys (*IBLC*) or *N*-isobutyryl-D-Cys (*IBDC*). Further, *OPA* and *N*-isobutyryl-L-Cys or *N*-isobutyryl-D-Cys derivatization was used for the enantiomeric determination of the D-Glu, D-*erythro*-β-MeAsp, D-Ala, L-Ala and L-Leu contents in microcystins and nodularin peptides [35]. A novel chiral thiol reagent, *N*-(*tert*-butylthiocarbamoyl)-L-Cys ethyl ester (*BTCC*), was developed by Nimura *et al.* [36] and applied together with *OPA* for determination of the enantiomers of the amino acids Asp, Glu, Ser, Ala and Phe in the Asp racemase assay. A new strategy for the selective determination of D-amino acids was developed by Oguri *et al.* [37]. The D enantiomers of Ala, Leu, Met, Phe and Val in *Ruditapes philippinarum* were analyzed after enzymatic digestion with D-amino acid oxidase and derivatization with *o*-phenylenediamine in the presence of 2-mercaptoethanol, giving fluorescent quinoxalinol derivatives.

Indirect methods play lesser and lesser important role in enantiomeric separations due to the dynamic improvement in CSP technology, but they are still in routine use in the pharmaceutical industry. However the direct methods will develop, the indirect methods will always be a method of choice in chiral analysis. Further details of indirect separations of biologically and pharmaceutically important enantiomers can be found in a review paper from our group [38].

3. DIRECT SEPARATION OF SMALL PEPTIDE EPIMERS AND AMINO ACID ENANTIOMERS

The *direct method* based on the application of a CSP is divided into two categories: one is the direct enantioseparation of underivatized amino acids or peptides on a suitable CSP, while the other is the derivatization with an achiral reagent before the enantioseparation. (The latter one is another choice if the aim is to achieve better separability or higher detection sensitivity.) The direct method with chiral mobile phase additives (applying achiral stationary phase) has a drawback relating to the chiral purity of the chiral selectors.

Since Davankov and Rogozhin [2] introduced chiral LEC for the direct separation of amino acid enantiomers, metal chelate additives have been frequently used in the mobile phase. With the chiral chelate L-2-isopropyl-4-octyldiethylenetriamine-Zn(II) or -Ni(II) in the mobile phase, common amino acids have been resolved as their *Dns* derivatives and Gly-containing dipeptides [39]. Various racemic mixtures of underivatized amino acids and Gly- containing dipeptides were resolved when a C_{18} HPLC column was dynamically coated with the chiral selector N^{ε}-*n*-decyl-L-spinacine and then loaded with Cu(II) ions [40]. The chiral resolution of Gly-containing dipeptides has been carried out on different chemically bonded LEC phases by binding L-Pro or L-Hyp to silica gel and using Cu(II) sulfate in a mobile phase [41,42]. The enantiomeric and diastereoisomeric dipeptides Gly-Val, Gly-Leu, Gly-Phe, D,L-Leu-Gly, Leu-D,L-Ala and D,L-Leu-D,L-Phe were separated by RP-HPLC methods when coordinated in ternary Co(III) complexes [Co(*R,R*-*N*-benzyl-*N*'-(2-picolyl-1,2-diaminocyclohexane)(peptidato)]$^+$ on Amex Prepsil column [43].

Since the introduction CDs as CSPs, they have been successfully applied for the separation of short-chain epimeric peptides. Dipeptides, insulin chain B and [D-Ala^2]deltorphin II have been coupled with the Edman-type reagent *DBD*-isothiocyanate (*DBD-NCS*) and converted to *DBD*-carbamoyl

Table 1. Chiral Derivatizing Agents Applied for the Determination of D-Amino Acid Content

Reagent(s)	Investigated analytes	Detection	References
FLEC	2-amino-1,2,3,4-tetrahydronaphthalene-1- and 1,2,3,4-β-carboline-1-carboxylic acids	Fl, exc: 260 nm em: 315 nm	[14]
NAB-C	β-adrenoreceptors	Fl, exc: 230 nm em: 270 nm	[15]
GITC *BGIT*	D-Cys or cystine in oxytocins	UV, 250 nm UV, 231 nm	[17]
GITC, FDAA, FDVA	Cys or cystine in oxytocins	UV, 340 nm	[18]
[(*R*)(-)- and (*S*)(+)-*DBD-PynCS*] and [(*R*)(-)- and (*S*)(+)-*NBD-PynCS*]	D-amino acids in milk, cream, fermented dairy products, fermented beverage D- and L-amino acids in peptides	Fl, ex: 460-470 nm, em: 530-570 nm ESI-MS	[19-25]
FDAA	D-amino acids in peptide Arg-Lys-Lys-Asp-Val-Tyr	UV, 340 nm	[28]
FDAA	seleno-amino acids	ICP-MS	[29]
FDAA	Cys in somatostatin analogs	UV, 340 nm	[30]
FDAA	Suc-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Ala-Cha-Glu-OH	UV, 340 nm	[31]
FDAA	Peptide: Woc-114-108	UV, 340 nm	[32]
OPA + *N*-acetyl-D-penicillamine	protein kinase C inhibitor (SPC-100270)	UV, 330 nm	[33]
OPA + *N*-isobutyryl-L- and D-Cys (*IBDC and IBLC*)	protein amino acids in bacitracin, gramicidins A and B, polymyxin B, metanicin C, peptide toxin malformin A and the peptide drugs D-Arg-[Hyp^3-Thr^{5-8}-D-Phe^7]-bradykinin, *β*-casomorphin and α_{s1}-exorphin	Fl, exc: 230 nm em: 445 nm	[34]
OPA + *N*-isobutyryl-L- and D-Cys (*IBDC and IBLC*)	D-Glu, D-*erythro*-β-MeAsp, D-Ala, L-Ala and L-Leu content in microcystins and nodularin peptides	Fl, exc: 330 nm em: 445 nm	[35]
OPA + *N*-(*tert*-butylthiocarbamoyl)-L-Cys ethyl ester (BTCC)	Asp, Glu, Ser, Ala, Phe in the aspartate racemase assay	Fl, exc: 335 nm em: 420 nm	[36]
o-phenylenediamine + 2-mercaptoethanol	D-amino acids of Ala, Leu, Met Phe and Val in *Ruditapes philippinarum*	Fl, exc: 341 nm em: 413 nm	[37]

amino acid derivatives, which were then separated on ß-CD-based columns [44] and their D/L configurations were determined (Table **2**). The same ß-CD-based columns were applied by Iida *et al.* [45] for determination of the D/L configuration of phenyl isothiocyanate (*PITC*)-derivatized ß-amyloid(1-16) and different dipeptides. The D/L amino acids in [D-Ala^2D-Leu^5]enkephalin and in the peptide (D-Ala-Gly-L-Phe-D-Leu) have also been determined as *PITC* derivatives on a phenylcarbamoylated ß-CD column [46].

Armstrong *et al.* [47,48] performed a systematic study with 9-fluorenylmethyl chloroformate (*FMOC-Cl*) and its Gly and ß-Ala analogs for the derivatization of Gly-containing di- and tripeptides. All these derivatives were chromatographed on native *α*-, ß- and *γ*-CD columns. Gly and ß-Ala groups situated between the *FMOC* and amino acid moieties enhanced the chiral selectivities of the amino acid derivatives. Chen *et al.* [49] separated a number of di- and tripeptides on different *α*-, ß- and *γ*-CD columns after their pre-column derivatization with the fluorescence derivatizing agent 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (*AQC*). A variety of selective interactions on ß-CD-bonded silica have been used to separate the *cis* and *trans* conformers of Pro-containing peptides [50]. For the monitoring of traces of D-Ala and D-Glu in peptides, the protein hydrolyzate was dansylated, and the chiral resolution was performed on a C_{18} column, with ß-CD as chiral mobile phase additive [51]. Examples of dansyl-derivatized amino

acid or peptide chiral separations can also be found in the literature [52,53].

For the mechanism of the separation on CD selectors it is generally accepted that under reversed phase conditions liphophilic solutes interact with CD selectors via inclusion complexation; the hydrophobic part of the guest molecule penetrates into the CD cavity and leads to the release of solvent molecules. Van der Waals interactions inside the cavity and additional hydrophilic interactions may also take place. For aromatic derivatives π-π stacking increments may exist as well. Various reviews covering the applications and mechanism of CD-based enantioseparations are available in the literature [4,11].

Crown ethers can be used to resolve enantiomers that contain a primary amine functional group. The generated chiral ammonium ions can bind to the macrocyclic crown by inclusion complexation. Enantioselectivity is governed probably by steric factors of the substituents of the chiral ammonium ions and the residues attached to the chiral moieties incorporated into the crown ether. (Applying polar organic eluents this CSP may act as chiral cation exchanger for the separation of secondary amines.) The chiral crown ether CSP Crownpak CR(+) was applied by Esquivel *et al.* [54] and Hilton *et al.* [55] for the separation of underivatized di- and tripeptides. Besides an attractive interaction between the ammonium functional group of the peptide and the oxygens of the crown ether, a combination of steric and hydrophobic interactions influence the extent of chiral recognition. A CSP based on (-)-(18-crown-6)-2,3,11,12-tetracarboxylic acid has been evaluated as concerns the direct resolution of the enantiomers of di- and tripeptides [56]. From the aspect of the elution sequence, with a few exceptions the LL and LD enantiomers interacted more strongly than the corresponding DD or DL enantiomers with the CSP. Examples of HPLC applications of chiral crown ethers were reviewed by Hyun *et al*. [57].

Cellulose- or amylose-based CSPs have also been applied for the direct separation of peptide epimers. Eight sets of enantiomeric protected dipeptides, *Z*-Ala-Phe-OBzl, *Z*-Phe-Phe-OBzl, *etc*. were separated on cellulose-based Chiralcel OD, but Pirkle-type 1-A, Sumipax OA-1100 and OA-2200 were also tested [58]. A direct preparative purification of all 4 isomers of the unnatural amino acid *ß*-MePhe was achieved as *Cbz*-Me ester derivatives on an amylase-based Chiralpak AD-H column [59].

Newly developed cinchona alkaloid derivative-based CSPs have been widely applied for the separation of peptide epimers with MS detection. *N*-Terminally protected Ala-Gly di- and tripeptides [60], oligoalanine di-, tri-, tetra- and hexapeptides [61], and 11 different *N*-terminal-protected (*Ac, Bz, FMOC, etc*.) oligo-Ala peptide enantiomers containing up to 6 amino acids [62] exhibited excellent separations on cinchona alkaloid derivative-based CSPs. The separation of peptide enantiomers containing up to 6 amino acid residues on micro-HPLC columns packed with a quinine-based CSP demonstrates that miniaturized HPLC separation is possible without sacrifice of the separation power [63]. Further, a CSP based on 1,4-bis(9-*O*-quinidinyl)phthalazine was able to distinguish between the all-*R* and all-*S* enantiomers of hepta- to deca-Ala peptides [64]. The enantiomers of *N-Boc*-phosphinic pseudodipeptides, their *N*-Boc-α-aminophosphinic acid precursors and various other structural analogs have been successfully separated on a set of cinchona alkaloid-derived CSPs [65].

Macrocyclic antibiotic-based CSPs have been effectively applied for the separation of short-chain epimeric peptides. Di- and tripeptide epimers were separated on teicoplanin by Berthod *et al*. [66], while *Pht*-protected Gly-dipeptide epimers were successfully resolved by Ekborg-Ott *et al.* [67]. Schmid *et al.* [68] used teicoplanin aglycone in micro-HPLC for the separation of Gly-dipeptide epimers. Desai *et al.* [69] utilized teicoplanin-based CSP and atmospheric pressure chemical ionization (APCI) and ESI-MS for the separation and detection of amino acid and Gly-, Leu and Phe-peptide enantiomers. APCI demonstrated an order of magnitude better sensitivity relative to ESI for free amino acids and low molecular mass peptides, but as the peptide chain length increased ESI proved to be more suitable.

Armstrong *et al*. [70] applied teicoplanin, teicoplanin aglycone and ristocetin A as selectors for the separation of forty-two polymorphic and epimeric peptides (up to 13 amino acids in length) and teicoplanin and its aglycone analogue [71] as stationary phases for even larger peptides (up to 36 amino acids in length) with MS detection. They found that the smaller di- and tripeptides can still bind to the stationary phase in a mechanism similar to that of the single amino acids. However, once the peptides reach the size of the enkephalin peptides, this may not always be possible.

The determination of MK-0974 (a novel calcitonin gene-related peptide receptor antagonist with two chiral centers) has been achieved by Xu and Musson [72] in human plasma by MS detection. Direct separation of its four stereoisomers has been investigated both on macrocyclic glycopeptide and polysaccharide-based CSPs. Among the tested columns, a cellulose-based CSP (Chiralcel OJ-RH) exhibited the best separation for all four isomers under reversed-phase condition.

Macrocyclic antibiotics have proved to be an exceptionally useful class of chiral selectors for the separation of enantiomers of biological and pharmacological importance. Enantioseparation may be possible via several different mechanisms, including inclusion in a hydrophobic pocket, π-π complex formation, dipole stacking, hydrogen-bonding, electrostatic, short-distance van der Waals interactions, steric/rigidity effects, or combinations thereof. The possible interactions strictly depend on how the enantiomers fit into the aglycone cavity which is determined by the structural geometry. However, the mobile phase composition does control the nature (and the strength) of the interactions between the chiral selector and analyte. Their applications as well as the chiral recognition mechanism were subject of numerous review papers appeared recently [10,11].

N-3,5-Dinitrobenzoyl derivatives of 12 dipeptide methyl esters and 2 tripeptide esters have been separated on 3 different Pirkle-type CSPs [73]. A series of dipeptide *tert*-butylamide-bonded CSPs have been used for the resolution of racemic derivatives of α-amino acids and dipeptides by

Table 2. Direct Separation of Peptide Epimers and Amino Acid Enantiomers

Investigated analytes	*N*-protecting groups	Selector/column	Ref.
Gly-Ala, Gly-Leu, Gly-Val, Gly-Met, Gly-Thr, Gly-Nleu, Gly-Nval, *etc*.	*Dns*	L-2-isopropyl-4-octyldiethylenetriamine-Zn(II) or -Ni(II)	[39]
Gly containing dipeptides	--	N^{τ}-*n*-decyl-L-spinacine-Cu(II)	[40]
Gly-Ala, Gly-Leu, Gly-Nleu, Gly-Val, Gly-Nval, Gly-Met, Gly-Thr, Gly-Asp, Gly-Phe, Gly-Trp	--	L-Pro or L-Hyp-Cu(II)	[41,42]
Gly-Val, Gly-Leu, Gly-Phe, D,L-Leu-Gly, Leu-D,L-Ala and D,L-Leu-D,L-Phe	--	(*R*,*R*-*N*-benzyl-*N*'-(2-picolyl-1,2-diamino-cyclohexane)-Co(III)	[43]
Dipeptides; insulin chain B; [D-Ala^2]-deltorphin II	*DBD-NCS*	ß-CD /Ultron ES-CD; phenylcarbamoylated ß-CD/ Ultron ES-1/4PhCD	[44]
ß-amyloid(1-16); dipeptides	*PITC*	ß-CD /Ultron ES-CD; phenylcarbamoylated ß-CD/ Ultron ES-1/2PhCD	[45]
[D-Ala^2,D-Leu^5]-enkephalin; D-Ala-Gly-L-Phe-D-Leu	*PITC*	phenylcarbamoylated ß-CD/ Ultron ES-1/2PhCD	[46]
Gly-Ala, Ala-Gly, Gly-Gly-Ala,, Ala-Gly-Gly, Gly-Leu, Leu-Gly, *etc*.	*FMOC-Cl, FMOC-Gly-Cl, FMOC-ß-Ala-Cl*	α-, ß- and γ-CD-based CSP/Cyclobond III, I and II	[47,48]
Leu-Ala, Gly-Leu, Gly-Nleu, Glyy-Val, Ala-Leu-Gly, *etc.*	*AQC*	*α-*, *ß-* and *γ-*CD	[49]
Ala-Pro, Ile-Pro, Leu-Pro,,Phe-Pro, Ile-Pro-Ile, Val-Pro-Leu, Tyr-Pro-Phe, *etc*.	--	ß-CD	[50]
D-Ala and D-Glu containing peptides	*Dns*	ß-CD in mobile phase	[51]
Leu-Leu, Ala-Ala, Leu-Ala, Ala-Leu, Ala-Ala-Ala, *etc*.	--	Crown-ether-based CSP/ Crownpak CR(+)	[54]
Gly-Ala, Ala-Gly, Gly-Leu, Leu-Gly, Gly-Nval, Gly-Nleu, Ala-Gly-Gly, *etc*.	--	Crown-ether-based CSP/ Crownpak CR(+)	[55]
Ala-Ala; Ala-Trp, Ala-Tyr, Asp-Phe, Gly-Ala-Phe, Leu-Ala, Lys-Phe, Phe-Ala, *etc*.	--	(-)-(18-crown-6)-2,3,11,12-tetracarboxylic acid based CSP /ChiroSil SCA(-)	[56]
Z-Ala-Phe-OBzl, *Z*-Phe-Phe-OBzl, Z-Ala-Ala-O*Bzl*, *etc*.	*Z*	Cellulose-based CSP/ Chiralcel OD	[58]
Cbz-ß-MePhe-OMe	*Cbz*	Amylose-based CSP/ Chiralpak AD-H	[59]
Ala-, Phe-containig di-, tri-, and oligopeptides	*Ac*, *Piv*, *Bz*, *DNB*, *Dns*, *Boc*, *Z*, *DNZ*, *Fmoc*, *DNP*, *etc*.	cinchona alkaloid derivative-based CSPs	[60-65]
Di- and tripeptides	--	Teicoplanin/Chirobiotic T	[66]
Gly-containing dipeptides	*Pht*	Teicoplanin/Chirobiotic T	[67]
Gly-containing dipeptides	--	Teicoplanin aglycone	[68]
polymorphic and epimeric peptides	--	teicoplanin, teicoplanin aglycone and ristocetin A /Chirobiotic T, TAG and R	[70]
Gly-, Leu- and Phe-peptides; di-, tri-, tetra-, penta-, hexa-Gly, -Leu and -Phe	--	Teicoplanin/Chirobiotic T	[71]
MK-0974 calcitonin gene-related peptide receptor antagonist	--	macrocyclic glycopeptide, polysaccharide-based CSP	[72]

(Table 2). Contd…..

Investigated analytes	*N*-protecting groups	Selector/column	Ref.
Dipeptide and tripeptide esters; Ala-Ala, Ala-Leu, Ala-Met, Leu-Ala, Leu-Leu, Leu-Gly-Phe, Leu-Gly-Met, *etc.*	*DNB*	Pirkle-type phases	[73]
Dipeptides	--	dipeptide *tert*-butylamide bonded CSPs	[74]
Leu-Phe, Phe-Leu, Leu-Tyr, Tyr-Leu, Leu-Trp, Trp-Leu	--	α-chymotrypsin	[75]
Leu-Phe-OMe	*Cbz, Boc,*	poly(L-Leu) and poly(L-Phe)	[76]
Dipeptide Me esters: Leu-Ala, Leu-Val, Leu-Leu, Val-Ala, Val-Leu, Ala-Val, *etc.*	*DNB*	(*S*)-1-(6,7-dimethyl-1-naphthyl)isobutylamine	[77]
Ala-Phe, Phe-Ala, Val-Phe, Phe-Val	--	L-Val-L-Val-L-Pro	[78]
Dipeptide Me ester	*Boc*	*N*-acyl-L-Val	[79]
H-Gly-L-Phe-OH; H-Gly-D-Phe-OH; H-D-Leu-L-Leu-OH; H-L-Leu-D-Leu-OH; H-L-Leu-L-Leu-OH; H-L-Leu-D-Leu-OH, *etc.*	--	Chiral plate (TLC)	[80]

HPLC. All the bonded CSPs were effective for the resolution of one pair of the racemic dipeptide derivatives [74].

A series of 24 enantiomeric and diastereomeric dipeptides (D,D- and L,L-dipeptides and D,D-/L,L- and L,D-/D,L-dipeptides) were chromatographed on a CSP based upon immobilized α-chymotrypsin (ACHT) [75]. The results suggested that binding interactions between the dipeptides and the ACHT-CSP occur at the active site of the ACHT and at other hydrophobic sites on the ACHT molecule. Polymer-bonded CSPs, containing poly(L-Leu) and poly(L-Phe) gave high enantioselectivity for the resolution of various derivatives of Leu-Phe-OMe [76]. The HPLC resolution of 15 enantiomeric dipeptide Me esters as their *N*-3,5-dinitrobenzoyl derivatives was investigated on a CSP derived from (*S*)-1-(6,7-dimethyl-1-naphthyl)isobutylamine [77]. The 4 stereoisomers present in each dipeptide derivative were separated quite well, with the *R,R* isomer being eluted first. A CSP prepared by bonding a tripeptide, L-Val-L-Val-L-Pro, to silica gel through 1-(dimethylchlorosilyl)-2-(4-chloro-3-methylphenyl)ethanesilane was used for the separation of dipeptide isomers [78]. Racemic Boc-dipeptide Me esters were separated on *N*-acyl-L-Val-based CSPs (*N*-acyl: *N*-formyl-, *N*-acetyl-, *N*-propionyl-, *N*-*n*-butyryl- and *N*-*n*-valeryl-L-valyl) [79]. Dipeptide enantiomers, *e.g.* H-Gly-L-Phe-OH and H-Gly-D-Phe-OH or H-D-Leu-L-Leu-OH and H-L-Leu-D-Leu-OH, and diastereoisomers, *e.g.* H-L-Leu-L-Leu-OH and H-L-Leu-D-Leu-OH, have been separated by thin-layer chromatography on a Chiral plate [80].

4. BIOLOGICAL ASPECTS OF D-AMINO ACID CONTENT OF SYNTHETIC AND NATURALLY OCCURRING PEPTIDES

The L- and D-forms of amino acids in peptides can differ significantly with respect to stability and biological activity. Loew *et al.* [81] investigated the effects of modification of the Tyr residue on the receptor affinities, selectivities and opioid analgesic activities of enkephalin-like tetrapeptides of the form X-D-Ala-Gly-*N*(Me)Phe-NH_2 (X = *p*-OH-Tyr, *m*-OH-Tyr or ß-CH_3-*m*-OH-Tyr. The enkephalin-type tetrapeptides exhibited greatly enhanced μ-receptor selectivities and efficacies as analgesic agonists as compared with the corresponding [D-Ala^2]Met-enkephalin-NH_2 analogs. Okuyama *et al.* [82] reported that pradimicin analogs designed with the D-α-amino acid derivatives (except for the D-Pro analog) retained the antifungal activity.

The fractioned peptide of chicken egg white lysozyme(98-112) (IVSDGNGMNAWVAWR) displays antimicrobial activity against both Gram-positive and Gram-negative bacteria. Pellegrini *et al.* [83] found that the replacement of Asp^{106} by Arg (RAWVAWR) increased the bactericidal activity considerably and the D enantiomer of RAWVAWR was as active as the L-form against 5 tested bacteria. Fractionation of clostripain-digested lysozyme yielded a pentadecapeptide with antimicrobial activity, but without muramidase activity.

Bovine lactoferricin is an antimicrobial, cationic peptide generated by the gastric pepsin cleavage of bovine lactoferrin. The bactericidal effects of native lactoferricin [Lfcin B(17-41)], a shortened derivative [Lfcin B(17-31)] and the all-D-amino acid counterpart of Lfcin B(17-31) against *Escherichia coli* and *Staphylococcus aureus* were investigated by Ulvatne *et al.* [84,85]. D-Lfcin B(17-31) was more efficient against *E. coli* and *S. aureus* than the L-form, but not than Lfcin B(17-41).

Avrahami *et al.* [86] synthetized a series of model amphipathic all-L-amino acid peptides and their diastereomers with the sequence KX(3)KWX(2)KX(2)K, where X = Gly, Ala, Val, Ile or Leu. A direct correlation for the diastereomers between hydrophobicity and propensity indicated the

formation of a helix/distorted-helix and activity (induced membrane leakage and antibacterial activity), despite the fact that they contained 30% D-amino acids.

In order to investigate the structure-activity relationships for the terminal peptidic moiety in the novel natural antibiotics pyloricidin A, B and C, which possess potent and highly selective antibacterial activity against *Helicobacter pylori*, the L-amino acids were changed to α-D-, β- and γ-amino acids or peptidemimetics [87]. The result was a drastically decreased activity, but on the other hand, the derivatives containing α-L-amino acids were found to retain activity.

The conjugation of undecanoic acid and palmitic acid, with the advantage of using diastereomers versus all-L-amino acid peptides, magainin containing 4 D-amino acids ([D]-4-magainin), and a diastereomeric lytic peptide containing Lys and Leu ([D]-K(5)L(7)), led to the design of a new group of potent antifungal peptides [88].

The cyclic beta-sheet structure possessed by the 10-residue antibiotic peptide gramicidin S was taken as the structural framework for the *de novo* design of biologically active peptides with membrane-active properties by Lee *et al.* [89,90]. The effect of the ring size on the antimicrobial activity and hemolytic activity of peptides with from 4 to 16 residues was tested. A series of peptide diastereomers were created that were substituted only at position 4 by a D- or L-amino acid (Leu, Phe, Tyr, Asn, Lys and achiral Gly). The enantiomeric substitutions all disrupted the ß-sheet structure in benign medium and decreased the peptide amphipathicity. Interestingly, on increase of the ring size of the peptide to 14 residues, the hemolytic activity and antimicrobial activity were dissociated (peptide GS14). Furthermore, increased specificity for microbial membranes, with decreasing toxicity to red blood cells, was observed when D-amino acids were substituted for L-amino acids (and *vice versa*) into the GS14 sequence.

The adoption of a helical conformation in a membrane environment effectively increases the "apparent hydrophobicity" of a peptide segment by satisfying the backbone H-bonding potential, thereby stabilizing it in this environment. In order to uncouple peptide hydrophobicity from helicity, Maeda *et al.* [91] used the prototypic peptide KKAAAAAAAAAAAAWAAAAAAKKKK-NH_2 as a template, and performed pairwise DD-scanning mutagenesis over the length of the sequence. Studies on this library of 13 peptides indicated that the DD replacements at positions near the center of the peptide sequence had the most significant effects on the retention times of the peptides. The decreased retention times correlated well with the decreased helicity. The overall findings supported the notion that a distinction should be made between the hydrophobicity of the individual residues and the apparent hydrophobicity of the peptide as a whole.

Two peptides that mimic the action of neuroprotective proteins derived from *astrocytes*, NAPVSIPQ and SALLRSIPA, prevent the neuronal cell death induced by electric blockade. The all-D-amino acid peptides of NAP-VSIPQ and SALLRSIPA furnished similar potency and efficacy for neuroprotection as observed for their respective L-amino acid peptides [92]. The combined administration of neuroprotective peptides revealed that they can act through a mechanism independent of chiral recognition.

Braunstein *et al.* [93] reported that a diastereomer (containing 33% D-amino acids) of an antimicrobial peptide, K6L9 (LKLLKKLLKKLLKLL-NH2), but not the all-L-amino acid parental peptide, cures neutropenic mice infected with the gentamycin-sensitive bacterium *Pseudomonas aeruginosa* and the gentamycin-resistant *Acinetobacter baumannii*. The results of various biophysical experiments suggested a membranolytic-like effect.

The invariant Gly^{17} residue in human neutrophil α-defensin 2 (HNP2) was replaced by L-Ala or one of the D-amino acids Ala, Glu, Phe, Arg, Thr, Val or Tyr [94]. Although L-Ala^{17}-HNP2 could not be folded, leading to massive aggregation, all of the D-amino acid-substituted analogs folded with high efficiency. The 7 D analogs of HNP2 exhibited highly variable bactericidal activity against the Gram-positive and Gram-negative test strains.

Cyclic peptides with an even number of alternating D,L-α-amino acid residues, such as (SWFKTKSK-), have been shown to be stable upon protease treatment, membrane-active and bactericidal, and to exert antimicrobial activity against *Staphylococcus aureus* and other Gram-positive bacteria [95]. Some of these peptides were efficacious against methicillin-sensitive *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA).

Panizzutti *et al.* [96] conducted the first examination of the existence of a significant amount of free D-Ala in *Leishmania amazonensis* and of Ala racemase activity present in cell lysates; using a HPLC method they observed the restriction of D-Ala to bacteria, some fungi and *Leishmania amazonensis*.

Three cyclic peptolides were isolated from 2 different fungal species by Boros *et al.* [97]. Icosalides A1, A2 and B each contain 2 Ser and 2 Leu residues and incorporate 2 fatty acid moieties as part of the central 20-member ring. A1 contains L-Ser and both D- and L-Leu residues, while A2 and B contain only L-amino acid residues. Icosalide A1 displayed antimicrobial activity against *Streptococcus pyogenes*, *Streptococcus pneumoniae* (Felton), and *Enterococcus faecalis.*

Many frog species produce amphipathic α-helical peptides, and studies with model α-helical peptides such as temporin-1DRa (HFLGTLVNLAKKIL-NH_2) have shown that an increase in cationicity promotes antimicrobial activity, whereas increases in hydrophobicity, helicity and amphipathicity promote hemolytic activity and the loss of selectivity for microorganisms. The cytolytic activities of analogs of temporin-1DRa in which each amino acid is replaced by L-Lys or D-Lys have been tested against a range of microorganisms and human erythrocytes [98]. The data suggest a strategy of selective increases in cationicity concomitant with decreases in helicity and hydrophobicity in the transformation of naturally-occurring antimicrobial peptides into non-toxic therapeutic agents.

Jung *et al.* [99] found an antifungal effect on human pathogenic fungi of the cell-penetrating peptide Tat(47-58)

derived from HIV-1. Following substitution of the L-amino acid for the D-amino acid, Tat(47-58) also exhibited a broad spectrum of antibacterial activity and, unlike L-Tat(47-58), D-Tat(47-58) displayed a significant proteolytic resistance against all proteases tested and antimicrobial activities in the presence of trypsin. Moreover, D-Tat(47-58) inhibited MRSA infection in human HeLa cells.

G10KHc, a specifically targeted antimicrobial peptide (KKHRKHRKHRKH-GGSGGS-KNLRRIIRKGIHIIKKYG) developed by Eckert *et al.* [100], exerts rapid and selective killing activity against *Pseudomonas aeruginosa* in culture medium. It was found that robust G10KHc activity could be maintained in expectorated sputum if the Ser protease-dependent digestion associated with this fluid was inhibited, either by chemical antagonists or by the construction of a D-amino acid enantiomer of G10KHc.

Via parallel toxicity assays on both prokaryotic and eukaryotic organisms, Fletcher *et al.* [101] discovered examples of 6-residue cyclic D,L-α-peptide sequences [KWFFFH] with either broad-spectrum or highly selective biocidal activities toward both MRSA and *E. coli* and also the marine algae *Ulva linza* and *Navicula perminuta*. Peptides with D-amino acids behaved as new biofouling tools which are not generally toxic to all organisms, but rather specifically target microbial agents of interest.

Non-cytotoxicity and proteolytic stability of protease-resistant cell-penetrating peptides (CPPs) was obtained by chiral inversion of the residues of a known self-assembling CPP (from all-L-amino acids to all-D-amino acids) and then assessed against trypsin and human serum [102]. The results of uptake experiments indicated that the protease-stable (*i.e.*, D-amino acid) analog of the peptide is internalized by cells to the same extent as the protease-susceptible (*i.e.*, L-amino acid) parent peptide.

ACKNOWLEDGEMENTS

This work was supported by OTKA grant K 67563. I. I. wishes to express his thanks for a Bolyai János Postdoctoral Research Scholarship to support his work.

ABBREVIATIONS

APCI = Atmospheric pressure chemical ionization

AQC = 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate

BGIT = 2,3,4,6-tetra-*O*-benzoyl-β-D-glycopyranosyl isothiocyanate

Boc = *tert*-butyloxycarbonyl

BTCC = *N*-(*tert*-butylthiocarbamoyl)-L-cysteine ethyl ester

Bz = Benzoyl

Bzl = Benzyl

Cbz=Z = Benzyloxycarbonyl

CD = Cyclodextrin

CDA = Chiral derivatizing agent

CSP = Chiral stationary phase

DBD-NCS = 7-[(*N*,*N*-dimethylamino)sulfonyl]-2,1,3-benzoxadiazol-4-yl isothiocyanate

DBD-PynCS = 4-(3-isothiocyanatopyrrolidin-1-yl)-7-(*N*,*N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazole

DNB = 3,5-dinitrobenzoyl

DNP = 2,4-dinitrophenyl

Dns = 5-dimethylaminonaphthylsulfonyl (dansyl)

DNZ = 3,5-dinitrobenzyloxycarbonyl

ESI-MS = Electrospray ionization mass spectrometry

FDAA = 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide

FDLA = 1-fluoro-2,4-dinitrophenyl-5-L-leucine amide

FDVA = 1-fluoro-2,4-dinitrophenyl-5-L-valine amide

FLEC = 1-(9-fluorenyl)ethyl chloroformate

Fmoc = 9-(fluorenylmethyl)oxycarbonyl

GITC = 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate

HPLC = High-performance liquid chromatography

IBDC = *N*-isobutyryl-D-cysteine

IBLC = *N*-isobutyryl-L-cysteine

MRSA = Methicillin-resistant *Staphylococcus aureus*

MSSA = Methicillin-resistant *Staphylococcus aureus*

NAB-C = 4-(6-methoxy-2-naphthyl)-2-butyl chloroformate

NAC = *N*-acetyl-L-cysteine

NAP = *N*-acetyl-D-penicillamine

NBD-PynCS = 4-(3-isothiocyanatopyrrolidin-1-yl)-7-nitro-2,1,3-benzoxadiazole

OPA = *o*-phthalaldehyde

Pht = Phthaloyl

PITC = Phenylisothiocyanate

Piv = *tert*-butylcarbonyl (pivaloyl)

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Received: December 05, 2009 Revised: March 06, 2010 Accepted: March 07, 2010