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State-of-the-art enantioseparations of natural and unnatural amino acids by high-performance liquid chromatography

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ABSTRACT

This review discusses recent publications on the separation and analysis of natural and unnatural amino acid enantiomers by liquid chromatography. Focus is placed on methodological aspects relating chiral stationary phases and chiral columns which can cope with the challenge. Conceptually, amino acids can be enantioseparated in free form, which refers to the resolution of polar ampholytes, or as *N*-protected amino acids, which can be regarded as acidic commodities. Such synthons are used, for instance, in peptide synthesis protocols. Amino groups are frequently tagged with highly fluorescent or MS/MS active labels in order to generate sensitive and simultaneously stereoselective assays of diverse amino acids in complex matrices.

It is our intention to present the state of the art of enantioselective amino acid analysis by HPLC concepts and to pinpoint practical aspects.

Contents

Abbreviations: AA, amino acid; Ac, acetyl; AcOH, acetic acid; ADAM, 1-aminoadamantane; AQC (AccQ-Tag), 6-aminoquinoyl-*N*-hydroxysuccinimidyl carbamate; BA, butylamine; *t*-Boc, *t*-butyloxycarbonyl; BSA, bovine serum albumin-based CSP; Bz, benzoyl; CAD, corona discharge detector; CBZ (Z), benzyloxycarbonyl; CD, cyclodextrin; CLEC, chiral ligand-exchange chromatography; CSP, chiral stationary phase; *R*(−)-DBD-PyNCS, *R*(−)-4-(3-isothiocyanatopyrrolidin-1-yl)-7-(*N*,*N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazole; DCT, dichloro-s-triazine; 3,5-DCIB, 3,5-dichlorobenzoyl; DEA, diethyl amine; 2D-HPLC, two dimensional HPLC; 3,5-DNB, 3,5-dinitrobenzoyl; DNP, 2,4-dinitrophenyl; Dns, dansyl; EA, ethylamine; ELSD, evaporative light scattering detector; Exc/Em, excitation/emission wavelength; EtOH, ethanol; FA, formic acid; Fl, fluorescence detection; FLEC, (+)-1-(9-fluorenyl)ethyl chloroformate; FMOC, 9-fluorenylmethyl-chloroformate; FP, succinimidyl ferrocenyl propionate; GITC, 2,3,4,6-*tetra*-*O*-acetyl-*ß*-D-glycopyranosil isothiocyanate; HFBA, heptafluorobutyric acid; HPLC, high-performance liquid chromatography; IBLC, *N*-isobutyryl-L-cysteine; LOD, limit of detection; LOQ, limit of quantitation; Marfey reagent (FDAA, MR, FDNP-L-Ala-NH_2), 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide; MBIC, (*R*)-methyl benzyl isothiocyanate; MeCN, acetonitrile; MCT, monochloro-s-triazine; MQD, *O*-9-[2-(methacryloyloxy)-ethylcarbamoyl]-10,11dihydroquinidine; MS, mass-spectrometry; NAC, *N*-acetyl-L-cysteine; Nap-Btz, (*S*)-1-[H-benzo(d) (1,2,3)triazol-1-yl)-2[6-methoxynaphthalen-2-yl-propan-1-one]; NBD-Cl, 7-chloro-4-nitro-2,1,3-benzoxadiazole; NBD-F, 4-fluoro-7-nitro-2,1,3-benzoxadiazole; NBD-PyNCS, 4-(3-isothiocyanatopyrrolidin-1-yl)-7-nitrobenzofurazan; NEIC, (*S*)-1-(1-naphthyl) ethyl isothiocyanate; (*S*)-NIFE, (*S*)-*N*-(4-Nitrophenoxycarbonyl)phenylalanine methoxyethyl ester; *N*-NPTH, *N*-naphthoyl; (*S*)-OBS, *O*-benzyl-(*S*)-serine; OPA, *o*-phthalaldehyde; L-PGA-OSu, L-pyroglutamic acid succinimidyl ester; L-PGA[d(5)]-OSu, isotopic variant of L-pyroglutamic acid succinimidyl ester; Phg, phenylglicine; PA, propylamine; *N*-PTH, *N*-phthaloyl; QD, quinidine; QN, quinine; QN-AX and QD-AX, *t*-butyl carbamoylated QN and QD CSPs; L-STC, (*S*)-trityl-L-cysteine; TAHS, *p*-*N*,*N*,*N*-trimethylammonioanilyl *N*′-hydroxysuccinimidyl carbamate iodide; TBA, tributylamine; TEA, trimethylamine; TEAA, triethylammonium acetate; *N*-TCPHT, *N*-tetrachlorophthaloyl; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TPA, tripropylamine; UHPLC, ultra-high-performance liquid chromatography; ZWIX(+)™, sulfocyclohexyl carbamoylated QN-based zwitterionic CSP; ZWIX(−)™, sulfocyclohexyl carbamoylated QD-based zwitterionic CSP.

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1. Introduction

Chirality caused by molecular asymmetry has special effects on physical, chemical, biological and pharmacological properties appearing at a molecular level. Amino acids (AAs), and carbohydrates (sugars), and the peptides, proteins, glycoproteins, polysaccharides etc. composed of such chiral building blocks are stereochemically defined. In biological systems, chiral entities (e.g. receptors) often display different biologically relevant responses to the enantiomers of chiral compounds such as drugs, agrochemicals, food additives, fragrances, etc., and also natural compounds including the D- and L-enantiomers of proteinogenic and non-proteinogenic AAs.

As a result of the awareness of the importance of stereoselective bioactivity phenomena such as pharmacodynamics and pharmacokinetics, a strong demand has arisen in life and pharmaceutical sciences for efficient tools with which to separate the individual stereoisomers of a large body of chiral compounds *per se*, but also in trace amounts in biological matrices.

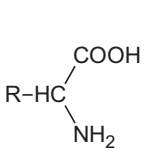
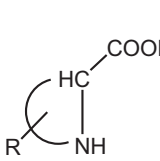
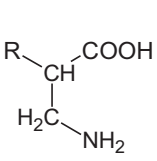
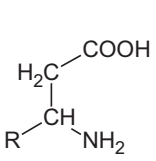
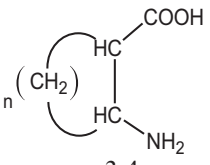
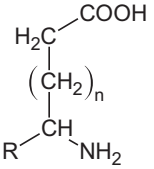
At an analytical level, liquid-phase separation techniques [liquid chromatography (LC) and capillary electrophoresis (CE)] are currently most widely applied; high-performance LC (HPLC) and supercritical fluid chromatography (SFC) have become very popular, though CE is also very powerful analytical technology [1,2]. For preparative enantiomer separations, HPLC and particularly SFC are the methods of choice [3]. Gas chromatography (GC) provides highly efficient enantioselective analysis systems for compounds which fulfill specific chemical requirements. Very useful GC separation systems exist for fully derivatized AAs [4]. However, the subject of this review is rather a critical overview of publications of the last 5 years dealing with LC methodologies for the stereoselective resolution of AAs and some peptides.

Various strategies have been developed for the differentiation of enantiomers by chromatography: (*i*) *indirect methods* involving derivatization of a mixture of target enantiomers by a chiral derivatizing agent (CDA) and the generation of diastereomeric compounds which are physicochemically different and should therefore be chromatographically resolvable on achiral chromatographic columns; (*ii*) *direct methods* in which chiral mobile phase additives are used together with standard achiral stationary phases; and (*iii*) *direct methods* in which chiral separations are achieved by using a chiral stationary phase (CSP). The direct methods are based on the intermediate formation of diastereomeric associates between the chiral selector (SO) moiety and the two enantiomers of the chiral analytes, the selectands (SAs). When the stability constants of the two diastereomeric associates, symbolized by [(*R*)-SO ↔ (*R*)-SA] and [(*R*)-SO ↔ (*S*)-SA], are sufficiently different from each other, direct enantioseparation can be achieved. In order to stabilize such associates, multiple intermolecular interactions between the SO and the SAs must come into play.

Over the years, HPLC and SFC with diverse types of CSPs have become the preferred methodologies to resolve mixtures of enantiomers and racemates. Where applicable, GC remains a powerful analytical tool for chiral separations due to the exceptionally high efficiency. For preparative enantiomer separations, HPLC and SFC are increasingly more often the method of choice thanks to a number of convincing arguments often associated with “green chemistry” [3,5].

This review discusses only the status and recent developments in the analytical separation of enantiomers of free and *N*-derivatized (tagged) natural and unnatural AAs, focusing on the direct HPLC methods. A recent review concentrated on indirect AA enantioseparations [4].

As depicted in Scheme 1, the structural variability of AAs is quite high, in view of (*i*) *α*-, *β*- and *γ*-AAs, (*ii*) cyclic AAs, (*iii*) constraint AAs and (*iv*) structural isomers, including the insertion of an additional chiral center besides the one to which the amino group is attached to. Further, it should be emphasized that AAs are

***α*-amino acids**		***β*-amino acids**			***γ*-amino acids**
primary	secondary	β^2-amino acids	β^3-amino acids	cyclic β^3-amino acids	
				 n= 3,4	 n= 1,2

R_1: alkyl, cycloalkyl, aromatic, substituted aromatic, hetero aromatic

R_2: H, alkyl

Scheme 1. Several structures of investigated amino acids.