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Review

Enantiomeric Ratio of Amino Acids as a Tool for Determination of Aging and Disease Diagnostics by Chromatographic Measurement

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Abstract: Occurrence of D-amino acids in living organisms is a useful indicator of various changes, diseases, or disorders. Determination of amino acid enantiomers, namely the enantiomeric ratio of amino acids or excess of certain D-amino acids, represents a useful tool in the studies of aging processes or biomarkers in disease/disorder diagnosis in humans. The amount of D-amino acids is usually very low. Therefore, suitable sample pretreatment, often derivatization, and highly selective and sensitive separation methods are essential for D-amino acid analysis in this field. Chromatographic techniques offer appropriate choices for solving these tasks. This review covers the advances in methodology and development of improved instrumental chromatographic methods focused on D,L-amino acid separation and determination. New findings in the area of possible D-amino acid biomarkers are also included.

Keywords: chromatography; amino acids; enantiomeric ratio; aging; biomarkers

1. Introduction

Formerly, it was believed that the proteins in living organisms are composed exclusively of L-amino acids (AAs). Later on, it was recognized that some tissues also contain D-AAs [1,2]. In the living body, AAs that constitute proteins are normally composed of the L-enantiomers, although there are some exceptions that are biologically synthesized using D-enantiomers [3]. With increasing age, a gradual transformation of the L-enantiomers in proteins into D-enantiomers (racemization) occurs; this transformation is influenced by various factors (e.g., temperature, pH) [4]. The racemization also occurs in metabolically inactive tissues such as teeth, eye lenses, vertebral discs, and parts of the brain. The presence of some D-AAs in the human body can indicate various diseases or disorders. D-aspartic acid (D-Asp) belongs to the most often occurring D-AA. The formation of D-Asp was explained as a result of spontaneous racemization in different tissues.

An interesting study dealing with tryptophanase activity was published by Akihiko et al. [5]. Tryptophanase displays no activity to D-tryptophan under usual conditions. Nevertheless, the authors found out that in the presence of diammonium hydrogen phosphate the tryptophanase catalyzes synthesis of L-tryptophan from D-serine. The enantiomeric form of tryptophan was verified by HPLC with circular dichroism detection—see Figure 1.

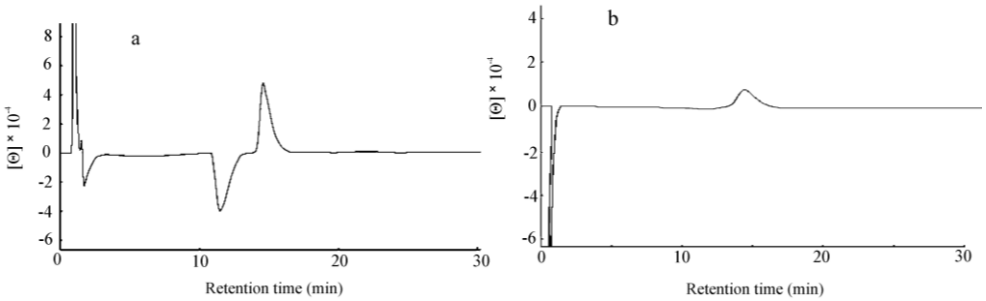

Figure 1. (**a**) Chromatogram of enantioseparation of D,L-tryptophan; (**b**) chromatogram of product of synthesis of tryptophan from D-serine catalyzed by tryptophanase in the presence of diammonium hydrogen phosphate, i.e., L-tryptophan. Crownpack CR (+) (chiral crown ether coated on silica gel) column, mobile phase (MP): aqueous solution of perchloric acid, pH 2.0; flow rate 1 mL/min, circular dichroism-1595 detector from Jasco [5]. Reproduced with permission from [5]. Copyright MDPI, 2009.

Since D-AAs often occur in trace levels in biological materials, it has been a great challenge to develop new fast and sensitive separation methods. Among the possible methods used for the enantioselective separation/determination of AAs in different matrices, gas chromatography (GC), micellar electrokinetic chromatography (MEKC), and high- or ultra-performance liquid chromatography (HPLC, UPLC) mostly prevail [6–12]. Other methods are used less frequently. Direct or indirect chromatographic methods can be used for the separation of AA enantiomers [13]. Advantages and disadvantages of direct and indirect methods as kinetic resolution or possibility of racemization are clearly summarized in the work of Ilisz et al. [14]. In order to improve the detection sensitivity and separation selectivity, AAs are often derivatized [13,15]. The recent review by Baghdady and Schug clearly presents the commonly used in situ derivatization techniques coupled to liquid chromatography with mass spectrometry-based bioanalysis [16].

The aim of this work is to show and explain the importance of determination of D- and L-AAs in humans, which represents a useful and irreplaceable tool in the studies of aging processes or biomarkers in disease/disorder diagnosis. Since the chromatographic methods are appropriate for this purpose, detailed description of chromatographic separation conditions are included. Focus is placed on methodological aspects relating advances in chromatographic methods applicable for separation and determination of AA enantiomers in biological tissues/fluids. Another field where the determination of D-AAs or D-/L-ratio plays an important role is the dating of geological samples. Also, dietary control requires reliable methods of D-/L-AAs determination. However, these areas have not been targeted in this review.

2. D/L Amino Acid Ratio as a Tool for the Determination of Age of Humans

The amino acids of vital tissue are L-AAs, but D-AAs converted from L-AAs accumulate with aging in petrified bone [17–19], teeth [1], eye lenses [20,21], and the brain [22], which show slow metabolism [23]. The D-Asp to L-Asp ratio in proteins obtained from dentine from teeth can be used for the estimation of the age at death of humans, since these materials are well preserved from additional racemization. Methods for the determination of Asp racemization are discussed in the review by Yekkala et al. [4]. Many of these methods used GC analysis. However, in this review special attention was given to the efficiency and sensitivity of HPLC coupled with fluorescence detection, since this method revealed higher ratios of Asp racemization. The effects of sample preparation and buffer conditions were studied. The usual approach to estimating the age from teeth is by D/L-Asp ratio from hydrolyzed dentin [23–27]. A general problem that must be taken into account is possible AA racemization during acidic hydrolysis of proteins or peptides [28,29]. This procedure is a usual part of sample pretreatment. Realization of more exact D/L-analysis, which eliminates racemization generated by hydrolysis, was demonstrated [30] and subsequently optimized [31]. Hydrolysis

should be performed in deuterated solvents, usually in deuterated hydrochloric acid [30,32,33]. Any molecule that is inverted during this step automatically becomes labeled with deuterium and thus, differing by one mass unit, is detected by MS. This method differentiates D-isomers formed during protein acid hydrolysis from the initial contribution of the sample. Many papers dealing with the estimation of the age from teeth were published. Some articles concentrate just on the determination of D/L-Asp using a previously described method [24]. AAs from dentin were derivatized with *o*-phthalaldehyde-*N*-acetyl-L-cysteine and the formed diastereomers could be separated by achiral reversed-phase (RP) HPLC with fluorescence detection. Advantages of this method are 15 min short analysis time (including derivatization and chromatographic analysis) and good sensitivity of detection (1 pmol). Another group of papers deals with the method improvement and proper evaluation of the results. The authors tested different separation conditions, e.g., effect of pH [23] and temperature [27], in order to improve method accuracy and reliability. Consequently, conditions for the analysis under which no additional racemization occurs were optimized. The racemization rate can differ depending on the protein structure [21]. The reliability of age estimation based on Asp enantiomeric ratio from different tissues depends on the methods of sample preservation, preparation, and analysis. Racemization must not proceed during the analysis. Collagen is very resistant to physico-chemical degradation processes. However, long postmortem intervals or unfavorable postmortem conditions may provoke an acceleration of degradation. The authors described some additional racemization at higher temperatures [27]. Benešová et al. performed an interesting study [26] in which two well-established, routinely used methods were compared. (i) AAs from hydrolyzed collagen from dentin derivatized with *o*-phthaldialdehyde-*N*-acetyl-L-cysteine (OPA-NAC) were separated by RP HPLC on C8 column; (ii) AAs for GC analysis were converted into trifluoroacetic acid isopropyl esters. Chiral XE-60-S-VAL-SA-PEA fused silica column was used for GC measurement. The following equation was used for the calculation of the age [26,34]:

$$KR = a \times \text{age} + b \quad (1)$$

where a represents the rate constant of the racemization reaction of aspartic acid in dentin and b is the intercept. Coefficients a and b are determined from samples of known age and obtained by linear regression with KR as the dependent variable.

KR is a coefficient of racemization defined as:

$$KR = \ln\left(\frac{1 + D/L}{1 - D/L}\right) \quad (2)$$

Both methods were described as accurate and reliable. However, the HPLC method provided lower KR values for all the samples tested. The authors attributed these results to the derivatization procedure in GC, where racemization can occur and cause higher KR values. When comparing estimated age and actual age, the HPLC method gave slightly lower values while the GC method showed much higher values.

A new capillary GC method for the enantioselective separation of derivatized AAs was introduced using newly prepared chiral selectors based on *S*-(-)-*t*-Leu derivatives. Chiral selector *S*-(-)-*t*-Leu-cyclooctylamide proved to be applicable for the age estimation from the Asp racemization extent in human dentin [25]. Increased detection sensitivity for the determination of polar AAs was achieved by the selective ion monitoring (SIM) mode (m/z 134) with zero needle potential [35]. The utility of zero needle voltage electrospray ionisation (ESI) was demonstrated on the age determination of human teeth by the Asp racemization method. HPLC enantioseparation of Asp from tooth extract was performed on a teicoplanin-based Chirobiotic T column with a mobile phase (MP) composed of 90% methanol, 9% water, and 1% of a 30% NH_3 (by volume) and detection by zero voltage ESI-MS. The gain in signal-to-noise ratio was 40–50 times higher than detection at 4 kV.

3. D-Amino Acids as Biomarkers of Diseases/Disorders

Proteins are subjected to numerous forms of damage during aging. McCudden et al. reviewed the biochemistry of AA racemization in proteins and its clinical application to musculoskeletal diseases [36]. Since the racemization occurs slowly, the quantification of D-AAs is particularly useful when directed at long-lived proteins, such as those found within musculoskeletal tissues. As many of these diseases are caused by the dysregulation of protein turnover rates, it is supposed that the determination of the racemization level in particular biomarkers could improve the diagnostic and prognostic information value. The concentrations of many D-AAs in organs and tissues change during various diseases. Chervyakov et al. summed up the role of D-AAs in normal aging and pathogenesis of neurodegenerative diseases [37]. The distribution and physiological role of D-AAs during normal aging, occurrence of D-AAs in pathology, relation between D-AAs and conformational diseases of brain, and some methods for the determination of D-AAs and their metabolites are also included. D-aspartyl residues were detected in various proteins from diverse tissues of elderly individuals [38,39]. Moreover, detection of D-β-Asp is associated with some age-related diseases. Fujii et al. described a method for the detection of D-β-Asp at specific sites in particular proteins, proposed a mechanism by which Asp residues invert and isomerize to D-β-form with age under physiological conditions, and discussed factors that favor such a reaction [38]. The comprehensive and quantitative data of Asp isomer formation (Lα, Lβ, Dβ, Dα) in age-related proteins might be used as a reliable biomarker for age-related diseases such as Alzheimer's disease (AD), cataracts, etc. [39,40]. Ultra-high performance liquid chromatography methods for the determination and analysis of disease biomarkers including some chiral methods for AAs were clearly reviewed [41]. Several review papers focused on the presence of D-AAs in mammals, their function, their diagnostic value as biomarkers, and also HPLC methods for their determination [42–47]. Examples of D-AAs related to some diseases are summarized in Table 1.The enantioseparation of D,L-AAs continues to be a subject of immense study due to its significant importance in various areas. Despite recent improvements in analytical techniques that facilitated the accuracy of AA enantiomer analysis at the trace level, there is still a need to develop new, more sensitive, rapid, and robust methods [7]. The next section focuses on chromatographic methods developed for the determination of L- and D-AAs in physiological fluids and tissues. Some of these methods were applied for the determination of D,L-AAs in samples from patients with different diseases. Certain new findings were made in the role of D,L-AAs in various diseases/disorders.

Table 1. D-AAs related to some diseases described previously [43]. Ala: alanine; Asp: aspartic acid; Ser: serine; Asx: asparagine/aspartic acid; Pro: proline.

D-AAs	Disease	Source	Ref.
D-Ala	Alzheimer's disease	brain	[48]
D-Asp		brain	[48,49]
		cerebrospinal fluid	[50]
D-Ser	Parkinson's disease	brain	[51]
	Schizophrenia	brain	[52]
D-Ala	Renal disease	plasma	[53]
		serum	[54]
D-Asx		serum	[55]
D-Pro		serum	[53]
D-Ser		plasma, serum	[53,55]

3.1. Chromatographic Methods for AA Determination in Biological Samples

Recent discovery of specific functions of D-AAs in humans will inevitably lead to the elucidation of D-AA abnormalities in various diseases/disorders. Therefore, high-throughput analysis techniques are necessary to determine D-AAs in biological fluids and tissues [56]. Fuchs et al. developed and validated two chromatographic methods, i.e., GC-MS and LC-MS for the determination of D-Ser,

L-Ser, and glycine (Gly) in cerebrospinal fluid (CBF) [56]. Pentafluoropropionic anhydride was used for sample derivatization before CG analysis. Analysis was carried out on a Chirasil-L-Val (*N*-propionyl-L-valine-*tert*-butylamide polysiloxane) fused-silica capillary column under programmed temperature elution with helium as a carrier gas. Samples were derivatized with Marfey's reagent [57] before LC analysis. Gradient elution of AA derivatives was performed on Atlantis dC18 column, linear gradient composed of 100% MP A (250 mg ammonium formiate in 1 L of water; pH adjusted to 4.6 with formic acid) to 50% MP B (acetonitrile (ACN)) in 15 min. Using these methods, age-dependent concentration ranges of studied AAs in humans were determined. MEKC with laser-induced fluorescence (LIF) detection was developed for the determination of chiral AAs in CBF and urine [58]. AAs were derivatized with a chiral reagent, i.e., (+)- or (−)-1-(9-anthryl)-2-propyl chloroformate, before analysis. Separation electrolyte consisted of 20 mM borax buffer pH 9.8. The micellar system consisted of 20 mM sodium dodecyl sulfate (SDS) and 7.5 mM sodium deoxycholate (SDC). D-Ala, D-glutamine (D-Gln), and D-Asp were detected in CBF and D-Ala and D-glutamic acid (D-Glu) in urine. Grant et al. developed a simple and versatile methodology using HPLC with fluorimetric detection to simultaneously determine D-Ser along with other metabolically related neuroactive AAs in the glutamatergic system: L-Ser, L-Glu, L-Gln, and Gly in human plasma [59]. AAs were derivatized by a combination of two chiral thiol reagents, *o*-phthaldialdehyde and *N*-isobutyryl-L-cysteine. Separation was carried out on a Symmetry C18 column (Waters Corp., Milford, MA, USA) by a concave MP gradient. This methodology is proposed for studying the role of AAs in the glutamatergic system in the pathophysiology and treatment of neurological and psychiatric disorders. A straightforward approach for the determination of D-AA trace levels in mammals utilizes a two-dimensional (2D) on-line column-switching system consisting of non-enantioselective and enantioselective separation parts in combination with sensitive pre-column or post-column derivatization [60–62]. Hamase et al. [60] described a 2D HPLC method for the comprehensive analysis of small quantities of branched aliphatic D-AAs in the presence of large amounts of their L-congeners in mammalian tissues and physiological fluids. Target analytes were determined as their fluorescent derivatives, pre-column labeled with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F). The authors established a multi-loop 2D column-switching HPLC system, combining on-line a reversed-phase system (Capcell pak C18 MG II column) (Shiseido Co., Tokyo, Japan) as the first dimension with an enantioselective column (Chiralpak QN-AX or Chiralpak QD-AX) (Chiral Technologies Europe, Illkirch, France) in the second dimension for the simultaneous determination of D-valine (D-Val), D-*allo*-Ile, D-isoleucine (D-Ile), and D-leucine (D-Leu). Chiral columns contain weak anion exchange-type chiral stationary phase (CSP) with quinine (or quinidine) *tert*-butylcarbamate moiety as chiral selectors and provide reversal elution order of the enantiomers. Significant amounts of D-Leu were found in rats in all the tissues and physiological fluids tested. High amount of D-*allo*-Ile was determined in urine. D-*allo*-Ile was also found in the urine of dogs and mice. This scientific group also developed a micro 2D HPLC method for the determination of hydrophilic AA enantiomers (histidine (His), asparagine (Asn), Ser, Gln, arginine (Arg), Asp, allo-threonine (allo-Thr), Glu, and Thr) in mammalian tissues and physiological fluids [63]. This method combines a microbore-monolithic octadecylsilica (ODS) column in the first dimension and a narrowbore Pirkle-type chiral column (Sumichiral OA-2500S or Sumichiral OA-2500R) (self-packed columns, material was from Sumika Chemical Analysis Service, Osaka, Japan) in the second dimension. Derivatization of AAs with NBD-F was performed before analysis. D-isomers of all investigated AAs were found in rat urine but at various enantiomeric ratios. The developed procedure was also successfully used for the determination of D- and L-Ser in urine and serum of mice after renal ischemia-reperfusion injury (IRI), known as a mouse model of acute kidney injury [64]. The level of D-Ser gradually increased in serum after renal IRI in parallel with creatinine, whereas the L-Ser level decreased sharply in the early phase after IRI. The work provides a novel understanding of the AA imbalance during renal failure and offers a new potential biomarker for the early detection of acute kidney injury. A column-switching chiral HPLC system using similar column types and derivatization agent, as mentioned above, was formerly applied for the determination of minute

amounts of D-Ala in rat tissues [65]. It was revealed that the amount of D-Ala is highest at the age of six weeks and then significantly decreases, and the amount of D-Ala is significantly higher during the daytime than the nighttime. Han et al. used the 2D HPLC method for the determination of D-AAs in biological samples [66]. This method combines columns and derivatization agents mentioned above; monolithic ODS column in the first dimension, Chiralpak QD-1-AX column in the second dimension, and NBD-F for derivatization. This method was applied to analyze the aging model senescence accelerated mouse prone 1 (SAMP1) mice, which had low immunocompetence. There was an obvious increase of D-Asp in the thymus and spleen of SAMP1 mice compared to the accelerated senescence-resistant mice. Visser et al. developed and validated a sensitive, fast, and simple UPLC/MS/MS method for the quantification of both enantiomers of proteinogenic AAs in body fluids [67]. Seven different chiral derivatization agents for the D-AA analysis were compared. The (*S*)-NIFE (*N*-(4-nitrophenoxycarbonyl)-L-phenylalanine 2-methoxyethyl ester) reagent offered an outstanding performance in the terms of sensitivity and enantioselectivity. The diastereoisomers were separated on an Acquity BEH C18 column (Waters Corp., Milford, MA, USA) under gradient elution. MP composed of ACN and 10 mM ammonium hydrogencarbonate, pH 9.5. Baseline separation ($R > 2.45$) was achieved for all 19 chiral proteinogenic AA isomers. It was shown that D-AAs in stable isotope tracers could result in erroneous estimates of enrichment, particularly if urine is used as a surrogate for plasma enrichment [68]. To describe the effects of D-AA content of less than 0.2% in 3 different AA tracers on the isotope enrichment in urine and plasma, Arg, Pro, and Phe tracers were given enterally to human neonates. The enrichment was measured in urine and plasma by HPLC with teicoplanin-based Chirobiotic T column and tandem MS. Labeled D-Arg resulted in an enrichment overestimate of 20% in plasma and 87% in urine. Smaller effect was obtained for D-Phe, where the overestimate was 5% in plasma and 40% in urine. D-Pro had no significant effect. The same Phe tracer was tested on children and adults. A reduction in the overestimate in children compared with infants and no effect on enrichment in adults were observed. Stable isotope-labeled AA studies should be performed, particularly in children, to ensure that this potential error is identified and eliminated. Reischl et al. developed an enantioselective LC-MS/MS method applicable for the analysis of proteinogenic AAs in urine [69]. This method utilizes a derivatization step on the amino group with an iron ferrocenyl propionate hydroxy succinimide ester followed by enantioselective anion exchange chromatography with cinchona alkaloid-based CSP. Homemade HALO-QD-AX-CSP contains *t*-butyl-carbamoylquinidine immobilized on a highly efficient fused core chromatographic support material. MP was composed of 90% methanol (MeOH) with 10% water and 100 mM ammonium formate/0.1% formic acid (*v*/*v*). Baseline enantioseparation for all proteinogenic AAs except for Pro, Arg and His was achieved. Cinchona alkaloid-based zwitterionic ion-exchange type enantioselective column, Chiralpak ZWIX(+) (for the structure see Figure 2 [70]) was successfully used for a simultaneous analysis of D-Ala, D-Asp, and D-Ser besides their L-forms in rat plasma and tissues [71]. Sensitive and selective chiral LC-MS/MS method was developed and validated for this purpose. AAs were derivatized with 6-aminoquinolyl-*N*-hydroxysuccinimidylcarbamate or *p*-*N*,*N*,*N*-trimethylammonioanilyl *N*′-hydroxysuccinimidyl carbamate iodide before analysis. MP composed of MeOH/aqueous solution containing 50 mM ammonium formate and 50 mM formic acid 98/2 (*v*/*v*) ratio. Three D-AAs and their L-enantiomers were simultaneously determined within 20 min in biological samples. Selective and high-throughput LC-MS/MS method using a surrogate analyte and an authentic matrix to determine D-Ser in the mouse brain was developed and validated [72]. To ensure the validity of D-Ser determination, [2,3,3-^{2}H]D-serine and [^{15}N]D-serine were used as a surrogate analyte and an internal standard, respectively. Enantiomeric separation was performed within 6 min on a chiral crown ether column (CROWNPAK CR(+)), MP composed of 0.3% trifluoroacetic acid at 5 °C without derivatization. Results demonstrated that the surrogate analyte method is valid for the accurate measurement of D-Ser in the mouse brain and is time-saving in sample preparation and analysis. Furthermore, method successfully provided D-Ser levels in the brain of normal mice. Sugimoto et al. developed a new, highly sensitive, and specific LC/ESI-MS/MS

method with the same chiral column for the determination of L- and D-Ser in human plasma [73]. Phosphate buffered saline (PBS) was used as a surrogate matrix. D- and L-Ser in human plasma and PBS were treated by cationic exchange solid phase extraction (CE SPE) and no sample derivatization. Elution was isocratic, MP was composed of 0.3% trifluoroacetic acid in 10% ACN, and the separation temperature was below 4 °C. This method offers a direct enantioseparation of D- and L-Ser without a time-consuming and/or poor qualitative derivatization step. The same scientific group also developed LC/ESI-MS/MS method for the determination of L- and D-Leu in human and animal plasma [74]. PBS was used as a surrogate matrix for the preparation of calibration curves and quality control samples. CE SPE was applied for the extraction of D- and L-Leu from plasma. The enantioseparation was performed on a Chiralpak ZWIX(−) column (for the structure see Figure 2 [70]), MP composed of MeOH/ACN/1 M ammonium formate/formic acid 50/50/2.5/0.2 ($v/v/v/v$). Baseline enantioseparation was accomplished without a derivatization step to accurately determine the D- and L-Leu in plasma. The fragmentation at m/z 43 for D- and L-Leu enabled the discrimination of D,L-Ile and D,L-*allo*-Ile by MS. Mochizuki et al. synthesized new chiral labeling reagents, i.e., L-pyroglutamic acid succinimidyl ester (L-PGA-OSu) and its isotopic variant (L-PGA[d5]-OSu), for the derivatization and consequent enantioseparation of AA derivatives by UPLC/ESI-MS/MS [75]. Nine pairs of proteolytic AAs were completely separated by RP chromatography using Acquity UPLC BEH C18 column under isocratic elution conditions; MP was composed of an ACN–water mixture containing 0.1% (v/v) formic acid. Method efficiency was demonstrated by analyzing AAs in human serum samples. Proposed procedure using L-PGA-OSu and L-PGA[d5]-OSu was intended for D,L-AA chiral metabolome study. A novel triazine-based chiral derivatization agent (*S*)-2,5-dioxopyrrolidin-1-yl-1-(4,6-dimethoxy-1,3,5-triazin-2-yl) pyrrolidine-2-carboxylate was synthesized and applied for the determination of chiral amines and AAs in the saliva of healthy volunteers by UPLC/ESI-MS/MS [76]. Diastereomers derived from proteolytic AAs except serine were well separated under isocratic elution conditions by RP chromatography using an Acquity UPLC BEH C18 column. D,L-Ser was separated on a Capcell Core ADME (core-shell type column with adamantyl functional group bonded on the polymer coated silica). Although the simultaneous separation of proteolytic D,L-AAs by a single chromatographic run was not possible, the enantiomeric separations of 19 AAs were performed using several elution profiles and different columns. The amount of the D-isomer in saliva was negligible for most AAs, except Ala and Pro. An interesting method of trace analysis optimization was considered in the paper by Péter et al. Replacing the Cinchona alkaloid-based CSPs, quinine, and quinidine-based anion exchangers resulted in changing the elution order of enantiomers and thus decreasing the limit of detection (LOD) value of the minor enantiomer if eluted before the major component. The method allowed quantification of less than 0.01% of minor N_α-Fmoc-Amino-acid enantiomer in the presence of the major one [77].

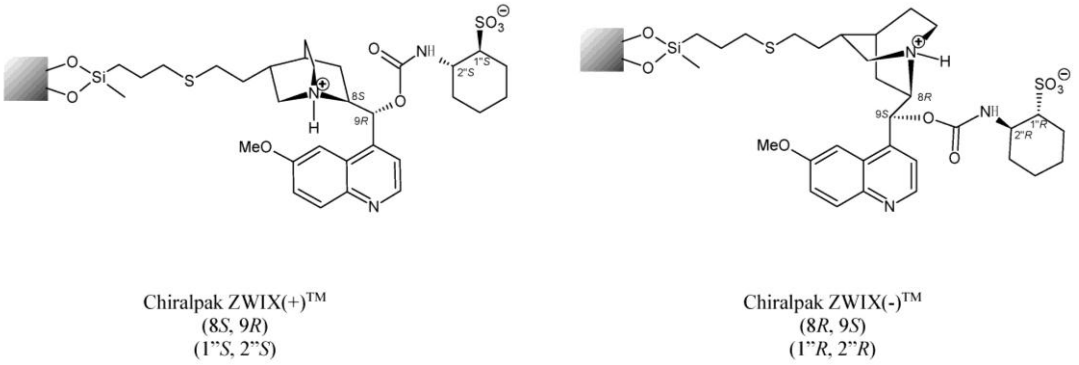

Figure 2. Structures of quininine-[ZWIX(+)™] and quinidine-based [ZWIX(−)™] chiral stationary phases [70] Reproduced with permission from [70]. Copyright MDPI, 2015.

3.2. Chromatographic Determination of AAs as Possible Biomarkers of Diseases/Disorders

Nowadays, the importance of chiral AA analysis for the screening of new biomarkers is arising in diagnostic applications. *N*-methyl-D-aspartate (NMDA) receptors are involved in learning and memory processes. It was shown that in AD there is a reduction of NMDA receptors [49]. NMDA also plays an important role in the pathophysiology of schizophrenia. D-Ser may function as an endogenous agonist of the Gly site in the NMDA receptor [78,79]. A reduction in serum levels of D-Ser, an endogenous co-agonist of the NMDA receptor, was reported in schizophrenia [78]. D'Aniello et al. supposed there is a relation between reduced levels of D-Asp in the brain and reduction of NMDA receptor signal transduction system, since D-Asp is an endogenous agonist of NMDA receptor [49]. Using an HPLC method, regional distribution of free D-Asp levels in post-mortem brain samples from patients with AD were determined. Significantly lower D-Asp levels were found in Alzheimer's patients compared to controls. Thorsén et al. focused on AA enantiomeric composition of β-amyloid peptides from deceased AD patients [80]. Peptides were hydrolyzed with mineral acid, free AAs were derivatized with chiral reagent, i.e., (+)- or (−)-1-(9-anthryl)-2-propyl chloroformate, and subsequently separated using MEKC-LIF method. The separation electrolyte consisted of 20 mM borate buffer adjusted to pH 9.8 with 0.1 M NaOH. The micellar system consisted of 20 mM SDS and 7.5 mM SDC. Method allowed simultaneous determination of nine AA enantiomers. Samples revealed impurities that could overshadow or dilute racemization events inherent to the β-amyloid peptides. Thus, more rigorous peptide purification protocols must be designed. The role of D-Ser in the pathophysiology of AD was also studied [81]. The reason of the study was that D-Ser may function as an endogenous agonist of the Gly site on the NMDA receptor that was implicated in the pathophysiology of AD. A previously described HPLC column-switching method with fluorimetric detection was used [82]. AAs were derivatized with 4-nitro-7-piperazino-2,1,3-benzoxadiazole fluorescent reagent. At first, step gradient elution was performed on ODS column; initial MP composed of ACN, MeOH and 0.1% trifluoroacetic acid. Then the analysis method was switched to chiral, using phenylcarbamoylated β-cyclodextrin (CD) column and MP composed of ACN/MeOH/water 75/20/5 (*v*/*v*/*v*). D-Ser serum levels determined in patients with AD were slightly lower than those of controls. In contrast, serum levels of L-Ser in patients were slightly higher than those of controls. This study suggested that reduced activity of serine racemase, an enzyme catalyzing the formation of D-Ser from L-Ser, can play a role in the pathophysiology of AD. Samakashvili et al. used the chiral MEKC-LIF method for the determination of D- and L-AA content in CBF samples related to different AD stages [83]. Samples were derivatized with fluorescein isothiocyanate (FITC) before analysis. The running buffer was composed of 100 mM sodium tetraborate, 80 mM SDS, and 20 mM β-CD at pH 10.0. Using this method, L-Arg, L-Leu, L-Gln, γ-aminobutyric acid, L-Ser, D-Ser, L-Ala, Gly, L-Lys, L-Glu, and L-Asp were detected in all the CBF samples. Some of the results shown in this work seem to disagree with those shown in the literature (e.g., an insignificant variation in the D-Ser level was observed depending on the AD stage), while some of them agree with the results obtained by others (e.g., L-Asp occurs at significantly lower concentrations in AD CBF than normal CBF). The post-translational AA racemization (AAR) and AA isomerisation (AAI) are typical markers of protein aging and could significantly impact the density and localization of plaque deposition in brain tissues [84]. AD is related to the formation and aggregation of amyloid-β peptide plaques in the human brain. Inoue et al. developed a covalent chiral derivatized ultra performance liquid chromatography tandem mass spectrometry (CCD-UPLC-MS/MS) method for the determination of post-translational AAR and AAI of *N*-terminal amyloid-β peptide (*N*-Aβ_{1-5}) in human brain tissue [84]. Covalent chiral derivatization reagent (*R*)-(−)-4-(*N*,*N*-dimethylaminosulfonyl)-7-(3-isothiocyanatopyrrolidin-1-yl)-2,1,3-benzoxadiazole was used for the derivatization of amyloid-β peptides. Separation was performed by Acquity UPLC BEH C18 column; MP was composed of 50 mM aqueous ammonium formate with 0.01% formic acid and 0.01% formic acid in MeOH. The CCD-UPLC/MS/MS assay of potential *N*-Aβ_{1-5}discovered the presence and ratio levels of these *N*-Aβ_{1-5} sequences with L-Asp, D-Asp, L-isoAsp, and D-isoAsp

in AD patients. The representative chromatograms of the *N*-Aβ_{1-5} standard solution are shown in Figure 3.

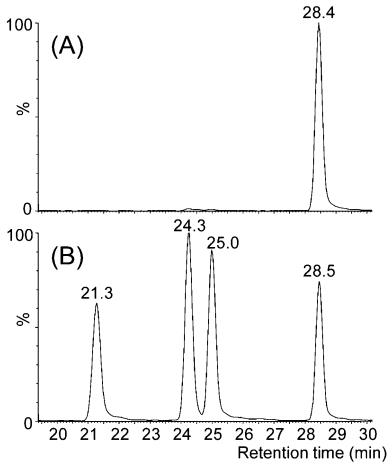

Figure 3. Selected reaction monitoring (SRM) chromatograms of *N*-Aβ_{1-5} standard solution. (**A**) SRM chromatogram (m/z 989.6 → m/z 637.0/678.9) for the isotope stable [^{15}N]-labeled *N*-terminal peptide from Aβ_{1-40} (retention time (RT): 28.4 min); (**B**) SRM chromatogram (m/z 989.6 → m/z 637.0/678.9) for the *N*-Aβ_{1-5} with L-Asp (RT: 28.5 min), D-Asp (RT: 24.3 min), L-isoAsp (RT: 25.0 min), and D-isoAsp (RT: 21.3 min). This separation of the derivatized *N*-Aβ_{1-5} fragment included with L-Asp, D-Asp, L-isoAsp, and D-isoAsp was achieved using the UPLC BEH C18 column with a mobile phase consisting of 0.1% formic acid in water/methanol [84] Reproduced with permission from [84]. Copyright American Chemical Society, 2014.

A novel UPLC/ESI-MS/MS method for the determination of biomarker candidates, chiral amines, and carboxyls in AD brain homogenates was developed [85]. Solutions of carboxyls or amines were derivatized with a pair of enantiomers of chiral derivatization reagents, i.e., (*S* and *R*)-1-(4,6-dimethoxy-1,3,5-triazin-2-yl)pyrrolidin-3-amine or (*S* and *R*)-2,5-dioxopyrrolidin-1-yl-1-(4,6-dimethoxy-1,3,5-triazin-2-yl) pyrrolidine-2-carboxylate. Derivatives were separated by gradient elution using water/ACN containing 0.1% formic acid on Acquity UPLC BEH C18 column. As a result, only L-Phe and L-lactic acid were identified as decreased and increased biomarker candidates in the AD brain, respectively. This strategy should be helpful for the identification and extraction of chiral metabolomics in samples from different environments. The determination of chiral metabolites in different sample groups is currently in progress in the authors' laboratory. Xing et al. developed and validated the UPLC/MS/MS method with pre-column derivatization with (*S*)-NIFE for the rapid and simultaneous determination of 18 D-AAs in complex rat plasma [86]. Derivatization in combination with MS/MS detection provided excellent sensitivity. Separations of derivatives were performed at 50 °C by gradient elution on Acquity UPLC BEH C18 column; MPs contained ACN and 8 mM ammonium hydrogen carbonate. The method was applied for the determination of endogenous levels of D-AAs in AD rat plasma and normal controls. The concentrations of D-Ser, D-Asp, D-Ala, D-Leu, and D-Pro in AD rat plasma were significantly decreased compared with those in normal controls, while D-Phe levels increased. The authors revealed that some of these D-AAs would be potential diagnostic biomarkers for AD.

Xie et al. developed and fully validated LC/MS/MS method for the determination of D-Ser levels in human plasma [87]. The method was successfully applied to sequential changes in plasma D-Ser and L-Ser levels in six CRPS (complex regional pain syndrome) patients receiving a continuous five-day intravenous infusion of (*R*,*S*)-ketamine. The method utilizes pre-column derivatization using (*R*)-1-Boc-2-piperidine carbonyl chloride. Separation was performed on a Zorbax Eclipse XDB-C18 column (Agilent Technologies, Waldbroon, Germany) under gradient elution; MP was composed of

water with 0.3% trifluoroacetic acid (TFA) (eluent A) and MeOH with 0.3% TFA (eluent B). Interesting results were observed. In three patients, (*R*,*S*)-ketamine administration produced a continuous drop in D-Ser levels with a maximum decrease of 20%. This treatment also produced increased D-Ser levels in two patients, with a 35% and 21% increase, respectively. However, one patient had an initial 17% increase in circulating D-Ser levels on the third day, followed by 8% reduction at the end of the treatment. These results indicate the importance of determining the D-Ser levels in plasma and their potential role in physiological response. The above mentioned method was also applied for the determination of D-Ser plasma levels in patients with (*R*,*S*)-ketamine treatment-resistant depression [88]. Baseline (before infusion of ketamine) D-Ser and also L-Ser plasma concentrations were significantly lower in ketamine treatment responders than in ketamine treatment non-responders. The obtained data suggested that the pre-treatment determination of baseline D-Ser levels in major depressive disorder patients could be used as an effective, rapid, and simple method for the prediction of antidepressant response to ketamine treatment.

Pålsson et al. used previously reported HPLC methods with fluorescence detection [89–91] to explore the glutamate hypothesis of bipolar disorder by examining peripheral and central levels of AAs related to glutamate signaling [92]. The study included 215 patients with bipolar disorder and 112 healthy controls. Serum levels of Glu, Gly, and D-Ser were significantly higher, whereas L-Ser levels were lower in patients as compared to controls. No differences between the patient and control group in AA levels were observed in CBF. The obtained results could be interpreted as a systemic aberration in AA metabolism that affects several AAs related to glutamate signaling.

Trace analysis of homocysteine, methionine, and cysteine enantiomers in the serum of healthy volunteers and patients with multiple sclerosis was performed in 2D HPLC with electrochemical detection [93]. AAs were separated from each other by achiral column (Purospher RP-18) in the first dimension and their enantiomers were separated on teicoplanin-aglycone based column (Chirobiotic TAG) in the second dimension with an on-line system. MP was composed of ACN/MeOH/25 mM phosphate buffer, 1 mM 1-octanesulfonic acid sodium salt, pH 2.7 3/3/94 (*v*/*v*/*v*). The LOD values ranged between 0.05 and 0.50 $\mu g \cdot mL^{-1}$. D-enantiomers of all tested AAs were not detected in serum samples. Preliminary results showed that patients with multiple sclerosis had higher levels of Met in comparison to healthy volunteers. Nevertheless, no generalization can be made, since only three samples from patients were tested. In order to obtain a reliable correlation between higher levels of Met in patients with multiple sclerosis further studies involving more patients must be carried out. Sulfur-containing amino acids were tested as possible indicators of human health or certain diseases. However, little attention was paid to the enantiomeric content related to this issue [94].

GC/MS method employing RT-γDEXsa (2,3-di-acetoxy-6-*O*-tert-butyl-dimethylsilyl γ-CD doped into 14% cyanopropylphenyl/86% dimethyl polysiloxane) column for AA determination in human serum and urine was developed and applied to urine samples from patients with renal insufficiency [95]. The method required protein removal by precipitation before AA derivatization with methyl chloroformate/methanol and reaction performance at neutral pH. In comparison to healthy volunteers, D-ratios of Ala, Val, Pro, Thr, Asp, and Asn significantly increased in patients. The differences in D-AA ratios were mainly result of significant decrease in L-AA concentrations normalized by creatinine levels.

Free D-AAs can also serve as biomarkers of diabetes mellitus (DM). Min et al. developed UPLC/ESI-TOF-MS method for the determination of D,L-AAs derivatized with *R*(−)-4-(3-isothiocyanatopyrrolidin-1-yl)-7-(*N*,*N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazole in nails of diabetic patients [96,97]. Separations of derivatives were performed by gradient elution on an Acquity UPLC BEH C18 column, MPs contained water and ACN with 0.1% formic acid or 5 mM ammonium acetate. The method provided trace detection of D,L-AAs and enabled the detection of D-Ala, D-Pro, D-Val, D-Ile, and D-Leu from nails of diabetic patients and healthy volunteers. No significant difference in the content of L-AAs was observed. However, a statistically significant and strong correlation between the D/L-AA concentration ratios for Ala, Val, Ile, and Leu was observed. A chiral GC-MS

method for the determination of free D-AAs in urine was developed, validated, and applied to a gestational DM study [98]. Pentafluoropropionic anhydride was used for sample derivatization before analysis. Chiral separations were carried out on a Chirasil-L-Val fused-silica capillary column under programmed temperature elution with helium as a carrier gas. % D-relative amounts were determined for Ala, Val, Thr, Ser, Leu, Asx (Asp + Asn), Glx (Glu + Gln), Met, Phe, Tyr, Orn, and Lys. Statistically significant differences were observed only for D-Phe and higher values were found in the gestational DM group in comparison with pregnant women with normal glucose tolerance. Kimura et al. performed a chiral amino acid metabolomic profiling of patients with advanced chronic kidney disease (CKD) [99]. The authors used previously reported 2D HPLC method for D-AAs determination [63,100]. The chromatograms obtained by labeling of the AAs with 4-fluoro-7-nitro-2,1,3-benzoxadiazole are shown in Figure 4. Sixteen out of 21 D-AAs were found in plasma from 108 CKD patients. The levels of D-Ser, D-Pro, and D-Asn were strongly associated with kidney function (estimated glomerular filtration ratio). The presence of D-Ala and D-Pro were associated with age, and D-Asp and D-Pro were associated with the DM. Unfortunately, a reference group of healthy volunteers was not used in this study. Nevertheless, this work determined some D-AAs as potential biomarkers in kidney diseases.

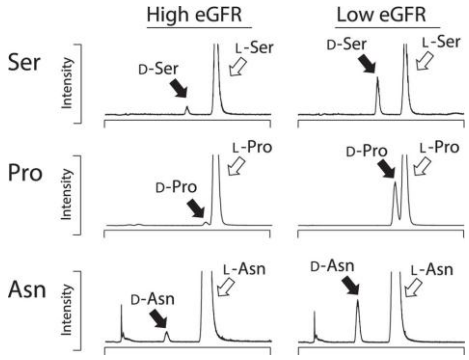

Figure 4. Detection of 4-fluoro-7-nitro-2,1,3-benzoxadiazole labeled amino acid enantiomers in the plasma of patients with chronic kidney disease using a 2D-HPLC-MS/MS system [99] Reproduced with permission from [99]. Copyright Macmillan Publishers Limited, 2016.

4. Conclusions

This review article gives an overview of the application of AA enantiomeric ratio and/or D-amino acids contents for determination of age at death and as possible biomarkers of various diseases/disorders. The improvement in chromatographic methods suitable for the determination of chiral AAs in biological samples is described in detail. The growing interest in the analysis of chiral AAs in biological samples is obvious from the number of papers dealing with the development and/or improvement of chromatographic methods. The problem is that D-AAs occur at trace levels in biological fluids and tissues. The progress in the quantitation of trace levels of D-AAs was enabled substantially by the introduction of 2D HPLC and fast and sensitive UPLC/MS methods. Both direct and indirect chiral separation methods are used. Indirect LC methods require appropriate chiral derivatization agent, while direct methods require a suitable chiral stationary phase. Also, non-chiral derivatization agents are frequently used in direct methods to increase the selectivity and sensitivity of analyses. Further advances in the development of chromatographic methods with very low detection limits, in combination with biochemical and clinical approaches, focused on improved trace level analysis are necessary in this field.

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Abbreviations

The following abbreviations are used in this manuscript:

AA	amino acid
GC	gas chromatography
MEKC	micellar electrokinetic chromatography
HPLC	high performance liquid chromatography
UPLC	ultra performance liquid chromatography
MP	mobile phase
OPA-NAC	*o*-phthaldialdehyde-*N*-acetyl-L-cysteine
SIM	selective ion monitoring
AD	Alzheimer's disease
CBF	cerebrospinal fluid
ACN	acetonitrile
LIF	laser-induced fluorescence
SDS	sodium dodecyl sulfate
SDC	sodium deoxycholate
2D	two dimensional
NBD-F	4-fluoro-7-nitro-2,1,3-benzoxadiazole
CSP	chiral stationary phase
ODS	octadecylsilica
IRI	renal ischemia-reperfusion injury
(*S*)-NIFE	*N*-(4-nitrophenoxycarbonyl)-L-phenylalanine 2-methoxyethyl ester
MeOH	methanol
PBS	phosphate buffered saline
CE SPE	cationic exchange solid phase extraction
L-PGA-OSu	L-pyroglutamic acid succinimidyl ester
NMDA	*N*-methyl-D-aspartate
CD	cyclodextrin
FITC	fluorescein isothiocyanate
AAR	AA racemization
AAI	AA isomerization
CCD-UPLC-MS/MS	covalent chiral derivatized ultra performance liquid chromatography tandem mass spectrometry
DM	diabetes mellitus
CKD	chronic kidney disease
RP	reversed-phase
ESI	electrospray ionization
LOD	limit of detection
SRM	selected reaction monitoring
TFA	trifluoroacetic acid
CRPS	complex regional pain syndrome

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