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Chiral separations for D-amino acid analysis in biological samples

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ABSTRACT

It is widely accepted that some of the free D-amino acids play important biological role. D-Aspartate and D-serine formed in the central nervous system of higher vertebrates have neurotransmitter/neuromodulator function. Together with D-alanine they are distributed in various tissues and biological fluids. Studying their physiological and pathological significance requires their sensitive and accurate determination in biological samples.

The various separation and detection methods used for their analysis are overviewed in the present paper. Our focus is mainly the quantitative performance and the analysis of real biospecimens.

1. Introduction

Since the discovery that D-enantiomers of the proteinaceous amino acids are present in various tissues of mammals, there is a continuous interest in their sensitive and selective analysis in various biological samples. Quantitative determination is needed to explore the way and control of their formation and degradation, as well as for understanding their biological significance. These studies mainly have focused on their importance in neuroscience and clinical chemistry, while they can also serve as indicators in food chemistry, as well. The various studies either focused on determination of certain D-amino acids, mainly D-alanine, D-serine and D-aspartate, in order to get more detailed information on their biological significance, or on the simultaneous analysis of several D-amino acids for screening relevant changes in various pathological conditions.

Since the first reports on the presence of D-aspartate in mammalian brain [1] and D-serine in mammalian tissues [2,3], there was considerable progress in the development of the analytical techniques, providing better enantioseparations and more precise quantification. In this paper we overview the commonly used separation and detection methods in chiral amino acid analysis focusing on determinations of their D-enantiomers in biological samples (tissues, serum, cerebrospinal fluid, urine) and the quantification performance of the methods.

2. Common difficulties of chiral amino acid analysis of biological samples

Chiral amino acid analysis is rather challenging especially in case of complex biological samples. The separation suffers from various difficulties such as the need of high efficiency for resolution of enantiomers present in considerably different concentration. While high level of proteinogenic L-amino acids can be found in the tissues, their D-enantiomers are usually present in one or two orders of magnitude lower concentration. As amino acids are rather hydrophilic, ionic compounds electromigration techniques are generally better suited to their separation. In case of chromatographic methods derivatization can be applied to improve their properties; lipophilicity and/or volatility in case of liquid and gas chromatography, respectively. Derivatization on the other hand is also required for their optical detection, because the most amino acids lack either chromophore or fluorophore moiety. A further goal of the derivatization can be the synthesis of diastereomeric compounds from the enantiomers allowing their separation in an achiral environment. This latter approach is called indirect chiral analysis.

However, derivatization is not only time consuming but is the source of several analytical bias. Conventional derivatization pro-

Abbreviations: CBQCA, 3-(4-carboxybenzoyl)-2-quinoline-carboxaldehyde; DM-β-CD, heptakis(2,6-di-*O*-methyl)-β-cyclodextrin; DOPA, dihydroxyphenylalanine; FITC, fluorescein isothiocyanate; HPA-β-CD, 6-monodeoxy-6-mono(3-hydroxy)-propylamino-β-cyclodextrin; HP-β-CD, hydroxypropyl-β-cyclodextrin; NBD-F, 4-fluoro-7-nitrobenzofurazan; NDA, naphthalene dicarboxaldehyde; OPA, orthophtalaldehyde; (*S*)-NIFE, (*S*)-*N*-(4-nitrophenoxycarbonyl)-l-phenylalanine-2-methoxyethyl ester; TM-β-CD, heptakis(2,3,6-tri-*O*-methyl)-β-cyclodextrin.

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cedures for GC analysis e.g. perfluorinated acylation reaction are usually performed at high temperature and in acidic condition that can result in racemization of the amino acids leading to overestimation of the minor enantiomer [4]. However optimization of the derivatization conditions could minimize the extent of enantiomer inversion [5]. Furthermore this acidic environment also induces hydrolysis of the amide group of asparagine and glutamine converting them to aspartate and glutamate, respectively. These methods thus can only determine the sum of the acidic amino acids and their respective amide analogues [4,6].

In case of biological samples containing high amounts of proteins and peptides their hydrolysis during derivatization can also compromise the accurate quantification of free amino acids thus a prior deproteinization step is necessary [4].

Another possible source of analytical bias is the non-quantitative conversion of the analytes to their derivatives. The efficiency of labeling reaction thus primarily determines the accuracy, the quantification performance of the method. In submicromolar analyte concentration the derivatization is often not reliable due to the competing simultaneous reactions, e.g. hydrolysis of the label that results in loss of linearity. Due to this phenomenon the typical LOQ values of various correctly validated methods using sample derivatization are all around 0.1 μM, while detection sensitivity (i.e. LOD) is usually much lower and can go down to the low nanomolar-subnanomolar range. The precise determination of LOQ thus should be based on the accuracy data. The regression coefficient of the calibration curve by itself can be misleading, since it is determined by the absolute deviation of the data points from the regression line and is thus less sensitive to the errors in the low concentration range [7–10].

3. Liquid chromatography

HPLC is the most frequently used separation technique in bioanalysis. However, in chiral separation due to its limited efficiency and peak capacity other separation techniques may have advantages. Chiral stationary phases usually possess poor chemical selectivity, thus separation and analysis of complex biological samples face some difficulties. Direct chiral separation thus usually uses orthogonal analytical approaches. This can be the hyphenation of chiral LC to MS detection or using 2D separation mode, when a conventional reversed-phase column is coupled to a chiral one. These latter coupled column systems are rather complicated and require special instrumentations [11–13].

Indirect chiral separations are more popular due to the better efficiency of reversed-phase columns that are used for resolution of the diastereomer derivatives. However they also suffer from several possible drawbacks especially when trace amount of one of the enantiomers should be determined. The purity of the derivatization agent is crucial for precise measurements.

3.1. Direct chiral HPLC analysis

Although there are several commercially available chiral stationary phases with different characteristics it is still difficult to predict, which of them can provide enantioseparation for a certain analyte. Derivatization is a characteristic step in sample pretreatment even in direct chiral analysis, in order to improve both chromatographic and detection properties of amino acids. 4-Fluoro-7-nitrobenzofurazan (NBD-F) is one of the most widely used labeling reagent as it provides appropriate retention on a reversed-phase column and can be sensitively detected by both fluorescence [11,12,14] and mass spectrometry [15]. Earlier methods mainly based on fluorescent detection with the typical quantification limit around 0.1 μM. Recently mass spectrometry and tandem mass spectrometry gained more importance, although significant improvement in quantitative sensitivity was not achieved [13,15], probably because LOQ is determined by the efficiency of the derivatization rather than the detection mode, as it was discussed earlier. An advantage of this detection method is that there is no need for baseline separation of various amino acids as they can be specifically detected in selective ion monitoring or selective reaction monitoring mode allowing 1D chiral separation. Recently some new derivatization agents were introduced providing rapid derivatization reaction and/or allowing separation of more amino acid enantiomers in a single run. However these tags failed to significantly improve quantification sensitivity [16–18].

There are a few reports when underivatized amino acids were directly separated on chiral crown ether columns. These methods were used for determination of D-serine level in mouse brain [19] and human plasma [20]. Although in this case the bias from derivatization is not an issue the reported quantification limits show that the intrinsic sensitivity of tandem mass spectrometry detection in case of amino acids is not superior.

3.2. Indirect chiral HPLC analysis

Indirect chiral separation mode is more commonly used in LC analysis because conventional reversed-phase columns can be applied for separation of diastereomeric derivatives and there is no need for the more complicated coupled column arrangement. In the early methods orthophtalaldehyde (OPA) + chiral thiol reagents were used to provide optically detectable diastereomers of amino acids [3,21]. Besides OPA, 4-(3-isothiocyanatopyrrolidin-1-yl)-7-(*N*,*N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazole was also used as a fluorescent chiral tagging reagent with similar detection sensitivity. It was demonstrated to react equally with the amino acid enantiomers and cause no racemization during derivatization. The authors identified several D-amino acids in human urine samples [22,23].

Recent advance in this field was also the introduction of mass spectrometry and tandem mass spectrometry detection. In addition to the previously used fluorescent tags [24,25] other reagents are also applied in this setting. Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide) and its analogues are commonly used as they react under relatively mild conditions and have good chromatographic properties allowing enantioseparation of several amino acids on conventional reversed-stationary phases [26]. Berna and Ackermann used this derivatization for LC–MS/MS measurement of serine enantiomers in rat brain microdialysate. The method was properly validated in the 10–7500 ng/mL concentration range corresponding to about 0.1 μM quantitation sensitivity, comparable to that previously reported with fluorescent detection methods [27]. Serine enantiomers were also determined in human cerebrospinal fluid with similar sensitivity [28]. Plasma D-serine level was also measured by using another derivatization agent, (*R*)-1-Boc-2-piperidine carbonyl chloride by LC–MS/MS. The method was validated and detection sensitivity of 0.19 μM LOQ was reported [29].

Visser et al., compared seven chiral derivatization agents in terms of sensitivity and enantioselectivity using three model amino acids (i.e. alanine, serine and methionine). Regarding peak area (*S*)-*N*-(4-nitrophenoxycarbonyl)-L-phenylalanine-2-methoxyethyl ester ((*S*)-NIFE) was found superior to the other studied reagents. However the comparison was performed only at a relatively high analyte concentration of 5 μM, which does not necessarily prove a better sensititvity, because only shows the detector response but does not provide information about the reaction efficiency and reliability at low, submicromolar analyte concentration. Furthermore, contrary to the impressive subnanomolar-nanomolar LODs of the optimized method using (*S*)-NIFE, accuracy was only

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