Capabilities of MS for Analytical Quantitative Determination of the Ratio of α− and βAsp7 Isoforms of the Amyloid−β Peptide in Binary Mixtures

ARTICLE *in* ANALYTICAL CHEMISTRY · MARCH 2011

Impact Factor: 5.64 · DOI: 10.1021/ac103213j · Source: PubMed

8 AUTHORS, INCLUDING:

Maria I Indeykina

8 PUBLICATIONS **50** CITATIONS

SEE PROFILE

Sergey A Kozin

Engelhardt Institute of Molecular Biology (EI…

54 PUBLICATIONS **609** CITATIONS

SEE PROFILE

Alexey Kononikhin

Russian Academy of Sciences

76 PUBLICATIONS **332** CITATIONS

SEE PROFILE

Evgeny (Eugene) N Nikolaev

Russian Academy of Sciences

190 PUBLICATIONS **1,944** CITATIONS

SEE PROFILE

analytical chemistry

TECHNICAL NOTE

pubs.acs.org/ac

Capabilities of MS for Analytical Quantitative Determination of the Ratio of α- and βAsp7 Isoforms of the Amyloid-β Peptide in Binary Mixtures

Maria I. Indeykina,[†,‡,§] Igor A. Popov,[†,§] Sergey A. Kozin,[‡,⊥] Alexey S. Kononikhin,[†,§] Oleg N. Kharybin,[⊥] Philippe O. Tsvetkov,[‡] Alexander A. Makarov,[‡] and Evgenij N. Nikolaev*[,†,§]

[†]Emanuel Institute of Biochemical Physics of the Russian Academy of Sciences, Kosygina Street 4, Moscow, Russia, 119334

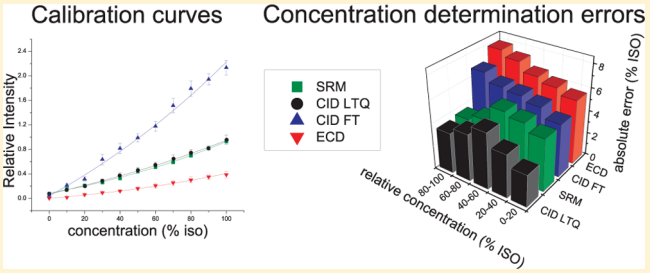
[‡]Engelhardt Institute of Molecular Biology of the Russian Academy of Sciences, Vavilova Street 32, Moscow, Russia, 119991

[§]Institute for Energy Problems of Chemical Physics of the Russian Academy of Sciences, Leninsky Prospekt 38 Bldg 2, Moscow, Russia, 119334

[⊥]Orekhovich Institute of Biomedical Chemistry of the Russian Academy of Medical Sciences, Pogodinskaya Street 10, Moscow, Russia, 119121

Supporting Information

ABSTRACT: There is strong evidence that the amyloid-β peptide (Aβ) plays a crucial role in the pathogenesis of Alzheimer's disease (AD), a lethal neurodegenerative disorder of the elderly. During pathology development, the peptide as well as its various chemically modified isoforms is accumulated in specific brain tissues as characteristic proteinaceous deposits, the so-called amyloid plaques, which are the pathomorphological mark of AD, although the level of Aβ in the blood is the same for healthy individuals and for AD patients. Earlier, it has been shown that isomerization of aspartate 7, the most abundant post-translational modification of the Aβ peptide, is tightly involved in a set of molecular processes associated with AD progression. Therefore, the isoAsp 7-containing Aβ isomer (isoAβ) is assumed to be a potential biomarker of AD that can be identified in the blood. Here, we present an analytical mass spectrometric method for quantitative determination of the ratio of normal and isomerized Aβ fragments 1−16 in their binary mixtures, and all analytical capabilities, such as accuracy, detection limits, and sensitivity of the presented method, are determined and thoroughly discussed. On the basis of this method, an analytical approach for quantitative determination of this modification in the blood will be developed in further studies.



Isomerization of aspartate and asparagine residues, resulting in the formation of isoaspartate (isoAsp, β-Asp), is a widely spread chemical modification of damaged and/or long-living proteins both in vitro and in vivo.[1] The isomerization process occurs in peptides and proteins spontaneously with time and appears to be a consequence of the degradation process during aging.[2] The isoaspartate, β-Asp, differs from the normal aspartate, α-Asp, structurally, but has the same molecular weight as the latter. The transformation of normal Asp or Asn residues into isoAsp occurs via a cyclic succinimide stage through a nonenzymatic intramolecular rearrangement of the peptide bond following the residue.[3] As a result, the main chain of the peptide skeleton is lengthened by one $-CH_2-$ group, and correspondingly, the side chain is shortened by the same group, thus leading to substantial changes in the structure of the modified protein.[4] Usually, after the isomerization of aspartate residues, the protein loses its normal function and structure and is degraded. In vivo there is a special enzymatic system which returns the β-aspartate into its normal α-Asp form, but this system works only inside the cell and cannot repair extracellular proteins.[1] Thus, isoAsp-containing proteins may be accumulated in the organism as it happens in Alzheimer's disease (AD).

According to one of the main hypotheses of involvement of protein aging in AD development, the central role in the initiation of the pathogenic oligomerization process of amyloid-β (Aβ) is played by aspartate isomerization and spontaneous transformation of the Aβ peptide into its isomerized form, which triggers the rest of the pathogenic cascade of AD.[5] Indeed, it has been shown that in the amyloid plaques of AD patients about 75% of all aspartates within the Aβ are isomerized.[6,7] It should be noted that the native Aβ has three aspartates, Asp1, Asp7, and Asp23; however, it has been established that only isomerization of the aspartate residue in the seventh position crucially influences such processes as zinc and copper ion

Received: December 8, 2010
Accepted: February 21, 2011
Published: March 16, 2011

chelation by the metal-binding domain of Aβ,[8,9] zinc dependent oligomerization of Aβ,[10] and hydrolysis of Aβ by the angiotensin converting enzyme.[11] Since each of these molecular processes is closely related to the aggregation ability of Aβ and, thus, plays a potentially crucial role in AD progression, it has been supposed that the Aβ isoform carrying the isoAsp7 residue (isoAβ) would be a candidate biomarker of AD. However, in order to validate isoAβ as a biomarker, it is first necessary to have an analytical method which would allow one to make accurate quantitative estimations of the ratio of intact Aβ (norAβ) and isoAβ in biological fluids, particularly, in the blood.

Currently, there are several confirmed experimental techniques appropriate for determining the presence of isoaspartate residues in proteins,[12] but only mass spectrometry has a low enough detection limit for working with isoAsp-containing species extracted from in vivo sources.[13] Recently, it has been shown that quantitative label-free analysis of the relative abundance of peptide isomers is possible using differences in the fragmentation mass spectra of peptides with different Asp isoforms.[14,15] If these differences are reproducible and stable for different concentrations, it is possible to construct a method for the determination of relative concentrations of peptides with a different isomeric form of aspartic acid in solution. Nevertheless, such a quantitative method has not been developed yet, and it has not been verified as accurate and sensitive enough to analyze the proteins isolated from biological samples.

In the presented study, the differences in the fragmentation spectra of the two isoforms of fragments 1−16 of Aβ were analyzed and an analytical mass spectrometric method for quantitative determination of the relative abundance of isomeric substitutions of the aspartyl residues at the seventh position in Aβ in their binary mixtures was introduced. The obtained results have demonstrated that detection and sensitivity limits of the presented method are sufficient to perform isoAβ quantification in the low nanomolar concentration range, thus allowing the method to be applied in further clinical trials in order to validate the role of the isoAsp7-containing Aβ species as biomarkers of Alzheimer's disease.

MATERIALS AND METHODS

For quantifying the relative abundance of isoAβ in human plasma, the Aβ fragment 1−16 has been chosen as the analytical target. This was necessary because unlike the full-size Aβ this fragment is stable, soluble, and monomeric in aqueous solutions and does not aggregate spontaneously, thus making it a convenient model for investigations with modern physicochemical methods, including electrospray ionization (ESI) mass spectrometry.[16,17] Also, the fragment can be efficiently derived from intact Aβ using lysilendopeptidase (Lys C, LEP),[18] contains the site of interest (Asp 7), and is present in all prominent Aβ forms (Aβ1−40, Aβ1−42, and others), thus allowing one to determine the total isoaspartate 7 content in all of the peptide forms.

Reagents and Peptides. All chemicals and solvents used throughout this study were of HPLC-grade or better and were obtained from Sigma-Aldrich (St. Louis, MO, USA). The normal and isoAsp7-containing Aβ1−16 synthetic peptides (norAβ1−16 [$CH_3CO-D_1AEFR_5HDSGY_{10}EVHHQ_{15}K\text{-}NH_2$] and isoAβ1−16 [$CH_3CO-D_1AEFR_5H[isoAsp]SGY_{10}EVHHQ_{15}K\text{-}NH_2$], purity >95%) were bought from Sigma-Genosys (Woodlands, TX, USA). For details on sample preparation procedures, see Supporting Information.

Instrumentation. All of the experiments were performed on a Finnigan LTQ FT (Thermo Electron, Bremen, Germany) mass-spectrometer with an Ion Max (ESI) ion source by direct infusion. Fragmentation spectra were obtained using collision-induced dissociation (CID) and electron capture dissociation (ECD) methods. For detailed technical information on the parameters of the instrument, see Supporting Information.

Mathematical Analysis. Comparative analysis of the fragmentation spectra was done using Microsoft Excel 2002 (Microsoft Corporation) and a piece of in-house written software ML-Aligner. All of the calibration curves were graphed, and statistical analysis of their parameters was conducted using the software package OriginPro 8 (OriginLab Corporation, USA).

RESULTS AND DISCUSSION

Qualitative Analysis of Differences in Mass Spectra of norAβ1−16 and isoAβ1−16. Since isomerization of aspartate 7 leads only to a change in the space structure of Aβ1−16, while its molecular weight remains the same, there is no difference in the primary mass spectra of the peptides carrying different aspartate 7 isoforms (Figure S-1, Supporting Information), but the significant difference in the peptide structure caused by this transformation allows one to distinguish the two peptide isomers using MS/MS analysis. In the primary mass spectra, one of the charge states of the molecular ion, namely, the triply charged $(M + 3H)^{3+}$ ion (m/z 666), was isolated and fragmented using different dissociation techniques, CID and ECD (Figure S-2, Supporting Information). The triply charged species was selected for fragmentation analysis, because it has a high enough intensity to be efficiently detected, isolated, and fragmented, and it gives many different singly and doubly charged fragments in the dissociation spectra in a wide m/z range. The other charge states are less convenient, since the doubly charged ions are of low intensity and cannot be effectively fragmented at low absolute concentrations, while the 4+ charge state, though highly intensive, mostly gives multiply charged fragment ions all in a very narrow m/z range, what significantly complicates the analysis.

Analysis of the differences in the fragmentation spectra of the two isomers was conducted. The analysis was carried out using in-house written software. After normalizing the intensities of the signals in the spectra obtained from binary mixtures of the two Aβ1−16 isomers, analysis of the behavior of the normalized intensity of each peak as a function of the relative concentration of isoAβ1−16 in the binary mixture was carried out. If the formation of an ion is affected by the isomerization process, then the intensity of the corresponding peak will be a function of the relative abundance of the isomerized peptide in the mixture; otherwise, random changes in the intensity of this peak will be observed, and no concentration dependence will be evident. As a result, the peaks were divided into two main categories: (i) those whose relative intensity changed as the relative concentration of isoAβ1−16 in the mixture changed, and (ii) relatively stable peaks, whose intensity varied only insignificantly and independently from the changes in the relative abundance of the isomers in the mixture with no single direction or an obvious trend. The most significant of the detected differences are shown in Table 1 (for a more complete list of the found differences, see Table S-2 of the Supporting Information).

Table 1. List of the Main Differences in the MS/MS Fragmentation Spectra of the Two Peptide Isoforms Obtained Using Different Dissociation Methods

(A) CID

no.	m/z	fragment	behavior[a]	the difference in peak intensities in the spectra of the two isoforms
1	399.68	b_6^{2+}	increases	factor of 2−4
2	408.69	b_6+H_2O	increases	marker ion[b]
3	457.19	b_7^{2+}	increases	factor of 2−3
4	599.78	y_{10}^{2+}	increases	factor of 5−6
5	798.35	b_6	increases	factor of 3−4
6	816.36	b_6+H_2O	increases	marker ion[b]
7	1083.52	y_9	increases	factor of 3−4
8	1198.54	y_{10}	increases	factor of 2−4

(B) ECD

no.	m/z	fragment	behavior[a]	the difference in peak intensities in the spectra of the two isoforms
1	815.37	c_6	increases	factor of 1.5−2
2	872.37	c_6+57	increases	marker ion[b]
3	1125.53	z_{10}-57	increases	marker ion[b]
4	1182.53	z_{10}	increases	factor of 2

[a] With the increase in the relative abundance of the isoAβ1-16 in the mixture. [b] Marker ions are not observed in the fragmentation spectra of the peptides with normal aspartate residues.

The most important difference in the fragmentation spectra of the Aβ1−16 isomers is the presence of "marker ions", which are only found in the spectra of one of the peptide forms and are absent in the spectra of the other, thus allowing one to qualitatively determine the presence of the exact form in the mixture. Also, among the peaks common for both isoforms, there is a number of "characteristic" peaks, whose intensities differ significantly for the two isomers (their intensity in the spectra of one form is several times greater than in the spectra of the other), and though these changes are not helpful for the qualitative analysis, since their presence in the spectra does not allow one to definitely determine the presence or absence of a specific isomer of Aβ1−16, they are quite useful for quantitative analysis.

In order to minimize the concentration measurement error, a set of several characteristic and marker ions should be used. Those marker and characteristic ions which are convenient for practical analysis will be referred to as diagnostic ions and used further in the study. For CID fragmentation, the b_6+H_2O ion (m/z 816.36) can be used as a marker ion for the FT spectra, since it is only present in the spectra of isoAβ1−16 and is usually not found in the spectra of norAβ1−16 (Figure 1). For the linear ion trap, this ion cannot be used as a marker ion of the isomerized form but only as a characteristic ion, since (although its intensity significantly increases with the increase in the concentration of isoAβ1−16) it can also be found in the spectra of the normal peptide at a very low intensity (Figure S-4, Supporting Information). This might be due to higher sensitivity of the ion trap in comparison to the FTICR. The presence of this marker ion for the isoaspartate containing peptides was first reported for high-energy CID MS/MS spectra of peptides with basic amino acids at the N-end in the early 1990s and, then, also found in low-energy CID MS/MS spectra.[19−21] Among the characteristic ions, the most significant variations in the intensities of the ions are observed for those fragments, which are formed by bond cleavage near the seventh aspartate during CID fragmentation, such as, for example, an almost 3-fold increase in the intensity of the b_6 (m/z 798.35) (Figure 1) and a 4-fold increase of the y_9 (m/z 1083.53) ions (Figures S-4 and S-5 Supporting Information).

For ECD fragmentation spectra, the complementary c_6+57 (m/z 872.38) and $z_{10}-57$ (m/z 1125.55) ions, which are present only in the spectra of isoAβ1−16, can be used as marker ions (Figure 1; Figure S-6, Supporting Information). These results are in good compliance with the results of P. O'Connor's group on other isoaspartate containing peptides, including other fragments of the amyloid-β peptide.[14,15,22]

Quantitative Determination of the Ratio of α- and β-Asp7 Isoforms of Aβ1-16 in Model Binary Mixtures. Since only the relative abundances of the peptides are of interest, there is no need in any isotope labels or other complicated methods for absolute quantitation. A rather inexpensive approach to determine the fraction of each peptide in their binary mixture can be developed based on the differences in the fragmentation spectra of norAβ1−16 and isoAβ1−16 that have been described in the previous section. It is worth noting that the absolute intensity of the diagnostic ions cannot be used for quantitation since it depends on the absolute peptide concentration and on various discriminating factors at every stage of the measurement cycle. However, a standard approach in quantitative label-free mass spectrometric studies is normalization of the intensities of the ions of interest on the intensities of some other ions from the same spectra, the base ions. For our case, the relatively stable peaks present in the spectra of both isomers, whose intensities did not depend on the relative abundance of the peptides in the mixture, were selected to be used as base ions, i.e., internal reference points and intensity normalization standards. For each of the diagnostic ions, a corresponding stable base ion was selected so that its m/z ratio was as close as possible to the m/z of the diagnostic ion in order to minimize ion discrimination processes during detection and ion transport inside the mass spectrometer. The following peaks were selected as base ions for CID fragmentation: b_{13}^{2+} (m/z 793.34), y_{13}^{2+} (m/z 819.9), y_8 (m/z 996.5), and b_{10} (m/z 1220.5) (Figure 1; Figures S-4 and

dx.doi.org/10.1021/ac103213j |Anal. Chem. 2011, 83, 3205–3210

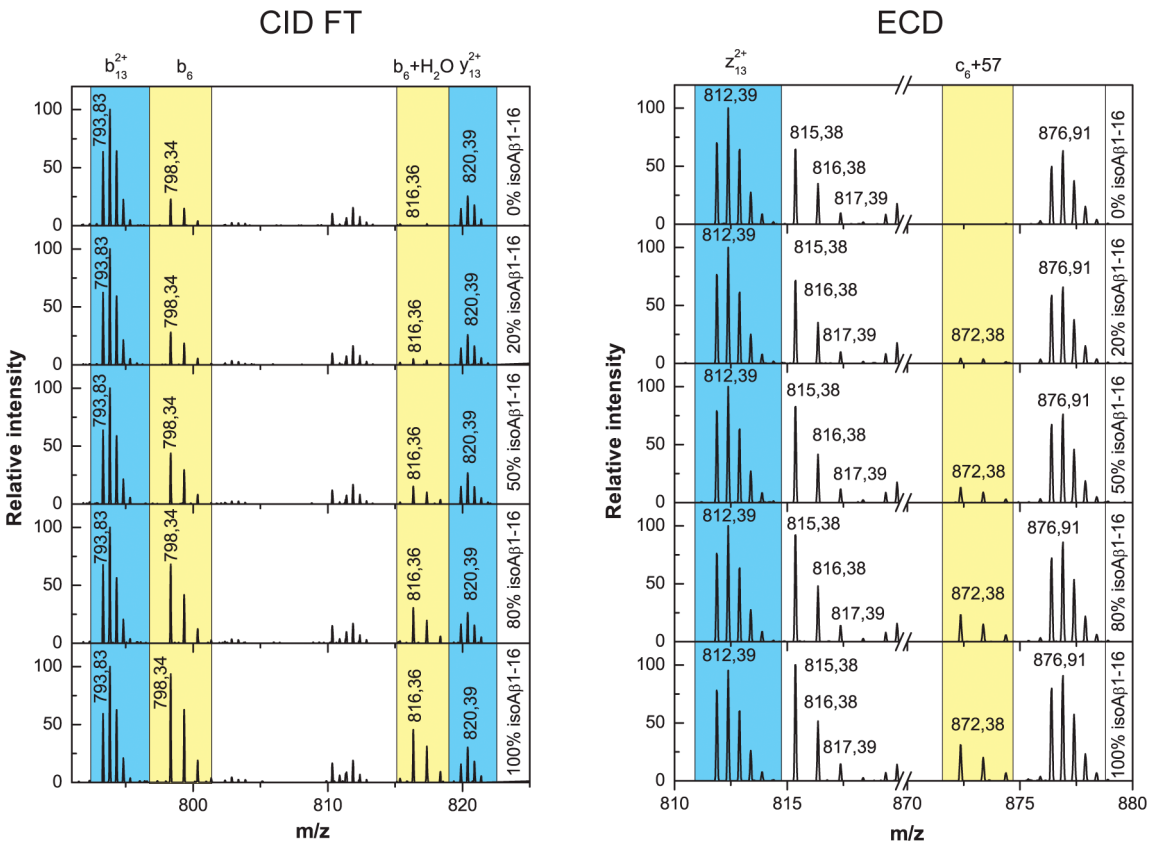


Figure 1. Marker and base ions in CID FT (right) and ECD (left) mass spectra (yellow, marker ions; blue, base peaks).

S-5, Supporting Information); and z_{13}^{2+} (m/z 811.88) and c_{12} (m/z 1465.63) for ECD fragmentation (Figure 1; Figure S-6, Supporting Information).

Then, using sets of binary mixtures, in which the relative concentration of the peptide with the isomerized aspartate residue was varied from 0 to 100% in 10% increments, calibration curves for each pair of diagnostic and corresponding base ions were obtained. For this, the intensities of all of the diagnostic and base ions were extracted from the corresponding mass lists and calibration curves of the dependence of the intensity ratios of the diagnostic ions to the corresponding base ions from the relative abundance of the isomerized peptide in the mixture were plotted.

These calibration curves were fitted with various functions. Initially, it was assumed that the two isoforms have identical physicochemical properties and are ionized and transported inside the mass spectrometer with the same efficiency. This assumption, though almost never stated, is used in most of the works on mass-spectrometry and especially on quantitative mass-spectrometry, of isoAsp-containing peptides and proteins.[13,14,23] In this case, the behavior of intensities of the b_6+H_2O, z_{10}−57, and c_6+57 ions (which are not formed during normal aspartate fragmentation) should follow the same trend as the changes in the concentration of isoAβ1−16; i.e., their intensities should increase linearly with the linear increase of the relative concentration of isoAβ1−16. However, this was not confirmed by our experiments; the best fit was obtained using nonlinear functions, such as the parabolic or exponential functions (R^2 = 0.996 for the exponential function, 0.995 for the parabolic function, and only 0.966 for the linear function; Figure 2; Figures S-7, S-8, and S-9, Supporting Information).

In order to check the nonartificial character of the nonlinearity of the calibration curves, additional experiments were conducted in which the following parameters were varied: the number of spectrum averages, the number of ions (AGC parameters), and the absolute peptide concentration. Nevertheless, independent of these parameters, the calibration curves retained their form and place (Figure S-10, Supporting Information). Thus, the nonlinearity of the calibration curves is not caused by the experimental conditions and is a feature of the process, which requires future investigations, which would also allow one to determine the real fitting formula for the calibration curves. As for practical use of the calibration curves, the exponential fit gives a very close approximation and the concentration measurement error caused by the deviation from the real curve is insignificant in comparison with the error from the inaccuracy of the intensity measurements, which was shown to be almost independent from the peptide concentrations and made up about 5−10% of the relative intensity.

Also, during the experiments with the variations of the absolute peptide concentrations of the binary mixtures, the quantitation limits for each of the mass detectors and fragmentation methods were determined. The smallest total peptide concentration at which a valid calibration curve in the full range of relative concentrations could be obtained was considered as the lower quantitation limit. For each of the methods, it was equal to (1) CID LTQ in the SRM mode, 5 nM; (2) CID LTQ in the full MS mode, 20 nM; (3) CID FT, 50 nM; and (4) ECD, 100 nM.

Besides the absolute peptide concentration, there is also a quantitation limit for the relative concentration of the isomerized

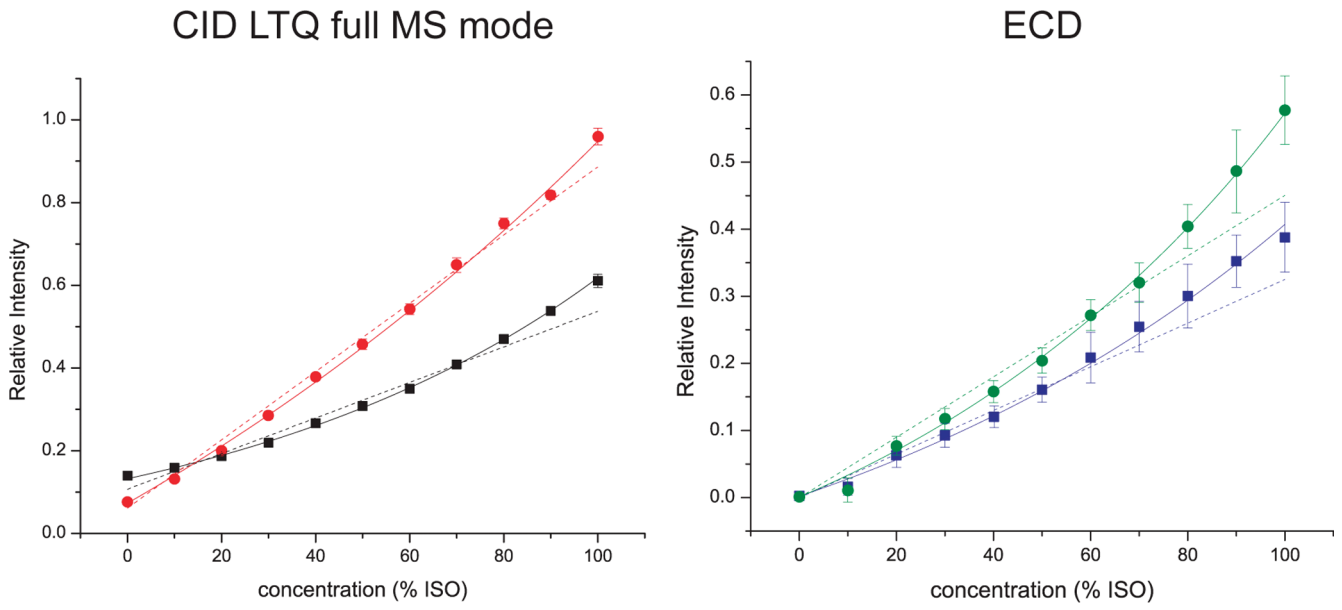


Figure 2. Calibration curves obtained for CID LTQ in full MS mode (left) and ECD (right) mass spectra (black, b_6 (m/z 798.35)/b_{13}^{+2} (m/z 793.34); red, b_6+H_2O (m/z 816.35)/y_{13}^{+2} (m/z 819.91); blue, c_6+57 (m/z 872.38)/z_{13}^{+2} (m/z 811.89); green, z_{10}−57 (m/z 1125.55)/c_{12} (m/z 1465.63); solid line, exponential fit of data; dashed line, linear fit of data).

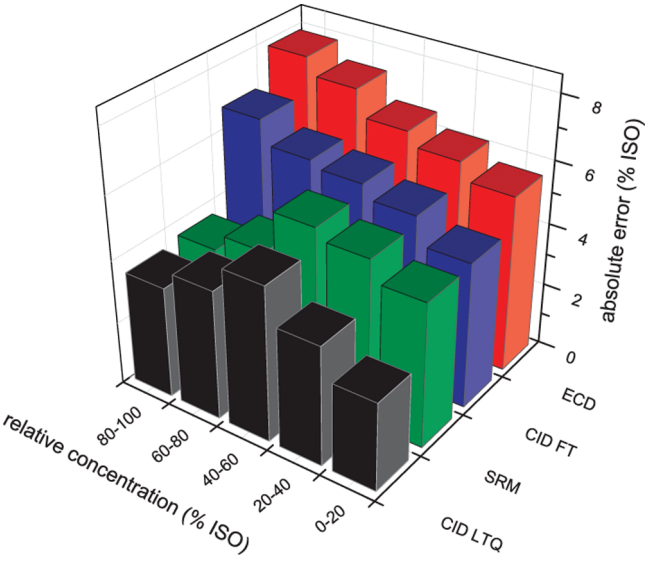


Figure 3. Accuracy of determination of the relative abundances of the isoforms depending on the percentage of the isomerized form in the binary mixture in case of different measurement techniques (black, CID LTQ full MS mode; green, CID LTQ SRM mode; blue, CID FT; red, ECD).

form, i.e., the smallest relative concentration of the isomerized form of the peptide which can be determined. For the CID fragmentation, this limit was at the level of method accuracy (accuracy of determination of relative concentration), while for ECD it was about 15%, that is if the relative concentration of the isomerized form was below 15% it was almost impossible to detect, especially at low absolute concentrations of the peptides.

The method accuracy was determined during "blind" experiments, when the stock solutions of the pure norAβ1−16 and isoAβ1−16 with the same absolute concentrations were mixed in random proportions, ciphered, and analyzed. During these experiments, more than a 100 of such "blind" samples were analyzed with different relative and absolute peptide concentrations. It was shown that the accuracy of the concentration determination depends on the fragmentation method, mass analyzer, and scan range used and makes up about (Figure 3): (1) for CID LTQ in full scan mode, 3.8%; (2) for CID LTQ in the SRM mode, 4.6%; (3) for CID FT, 5.8%; (4) for ECD, 6.7%.

CONCLUSIONS

A mass spectrometric method for quantitative determination of the relative abundance of synthetic peptides with isomerized aspartate residues in the seventh position, corresponding to the fragment 1−16 of Aβ, was developed using relative intensities of diagnostic ions characteristic for this isoform in MS/MS spectra. Despite the nonlinearity of the obtained calibration curves, this method can be used for quantitative analysis of the abundance of the isomerized form in binary mixtures at low peptide concentrations. Though this approach was developed on a model system, it appears to be adequate to be further used to get a reliable estimation of the level of isoAβ in human blood plasma samples, thus allowing the method to be employed in further clinical trials to validate the role of the isoAsp7-containing Aβ species as biomarkers of Alzheimer's disease. This method can also be adapted for quantitative analysis of the amounts of isomerized aspartate residues in other positions of Aβ or other peptides, extracted from biological samples or genetically engineered.

ASSOCIATED CONTENT

Supporting Information. Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*E-mail: ennikolaev@rambler.ru. Tel/Fax: (499)-939-73-70. Address: Kosygina Street 4, Moscow, Russia, 119334.

ACKNOWLEDGMENT

This work was supported by the "Molecular and Cellular Biology" and "Fundamental Sciences for Medicine" programs of the Russian Academy of Sciences, by the Russian Foundation of Basic Research (Grants 09-04-12130, 09-04-12225, 09-04-00725, 09-03-92500, 07-02-01482, 10-04-13306), by ICGEB CRP/RUS08-02, by the Russian Federal program (State Contracts 02.512.11.2329, 02.512.11.2197, 16.512.11.2081, 16.740.11.0369, 14.740.11.0755), and by the "Proteomics in medicine and biotechnology" program of the Russian Academy of Medical Sciences.

REFERENCES

(1) Shimizu, T.; Matsuoka, Y.; Shirasawa, T. *Biol. Pharm. Bull.* **2005**, *28*, 1590–1596.

(2) Reissner, K. J.; Aswad, D. W. *Cell. Mol. Life Sci.* **2003**, *60*, 1281–1295.

(3) Geiger, T.; Clarke, S. *J. Biol. Chem.* **1987**, *262*, 785–794.

(4) Hsu, Y. R.; Chang, W. C.; Mendiaz, E. A.; Hara, S.; Chow, D. T.; Mann, M. B.; Langley, K. E.; Lu, H. S. *Biochemistry* **1998**, *37*, 2251–2262.

(5) Orpiszewski, J.; Schormann, N.; Kluve-Beckerman, B.; Liepnieks, J. J.; Benson, M. D. *FASEB J.* **2000**, *14*, 1255–1263.

(6) Roher, A. E.; Lowenson, J. D.; Clarke, S.; Wolkow, C.; Wang, R.; Cotter, R. J.; Reardon, I. M.; Ziircher-Neely, H. A.; Heinrikson, R. L.; Ball, M. J.; Greenberg, B. D. *J. Biol. Chem.* **1993**, *268*, 3072–3083.

(7) Roher, A. E.; Lowenson, J. D.; Clarke, S.; Woods, A. S.; Cotter, R. J.; Gowing, E.; Ball, M. J. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 10836–10840.

(8) Tsvetkov, F. O.; Makarov, A. A.; Archakov, A. I.; Kozin, S. A. *Biofizika* **2009**, *54*, 197–201.

(9) Zirah, S.; Kozin, S. A.; Mazur, A. K.; Blond, A.; Cheminant, M.; Ségalas-Milazzo, I.; Debey, P.; Rebuffat, S. *J. Biol. Chem.* **2006**, *281*, 2151–2161.

(10) Tsvetkov, P. O.; Popov, I. A.; Nikolaev, E. N.; Archakov, A. I.; Makarov, A. A.; Kozin, S. A. *ChemBioChem* **2008**, *9*, 1564–1567.

(11) Toropygin, I. Y.; Kugaevskaya, E. V.; Mirgorodskaya, O. A.; Elisseeva, Y. E.; Kozmin, Y. P.; Popov, I. A.; Nikolaev, E. N.; Makarov, A. A.; Kozin, S. A. *Rapid Commun. Mass Spectrom.* **2008**, *22*, 231–239.

(12) Aswad, D. W.; Guzzetta, A. W. In *Deamidation and Isoaspartate Formation in Peptides and Proteins*; Aswad, D. W., Ed.; CRC Press Inc.: Boca Raton, FL, 1995; pp 7−30.

(13) Yang, H.; Fung, E. Y.; Zubarev, A. R.; Zubarev, R. A. *J. Proteome Res.* **2009**, *8*, 4615–4621.

(14) Cournoyer, J. J.; Lin, C.; Bowman, M. J.; O'Connor, P. B. *J. Am. Soc. Mass Spectrom.* **2007**, *18*, 48–56.

(15) Sargaeva, N. P.; Lin, C.; O'Connor, P. B. *Anal. Chem.* **2009**, *81*, 9778–9786.

(16) Kozin, S. A.; Zirah, S.; Rebuffat, S.; Hoa, G. H.; Debey, P. *Biochem. Biophys. Res. Commun.* **2001**, *285*, 959–964.

(17) Zirah, S.; Rebuffat, S.; Kozin, S. A.; Debey, P.; Fournier, F.; Lesage, D.; Tabet, J. C. *Int. J. Mass Spectrom.* **2003**, *228*, 999–1016.

(18) Mori, H.; Takio, K.; Ogawara, M.; Selkoe, D. J. *J. Biol. Chem.* **1992**, *267*, 17082–17086.

(19) Papayannopoulus, I. A.; Biemann, K. *Pept. Res.* **1992**, *5*, 83–90.

(20) Schindler, P.; Muller, D.; Marki, W.; Grossenbacher, H.; Richter, W. J. *J. Mass Spectrom.* **1996**, *31*, 967–974.

(21) González, L. J.; Shimizu, T.; Satomi, Y.; Betancourt, L.; Besada, V.; Padrón, G.; Orlando, R.; Shirasawa, T.; Shimonishi, Y.; Takao, T. *Rapid Commun. Mass Spectrom.* **2000**, *14*, 2092–2102.

(22) Cournoyer, J. J.; Pittman, J. L.; Ivleva, V. I.; Fallows, E.; Waskell, L.; Costello, C. E.; O'Connor, P. B. *Protein Sci.* **2005**, *14*, 452–463.

(23) Ni, W.; Dai, S.; Karger, B. L.; Zhou, Z. S. *Anal. Chem.* **2010**, *82*, 7485–7491.