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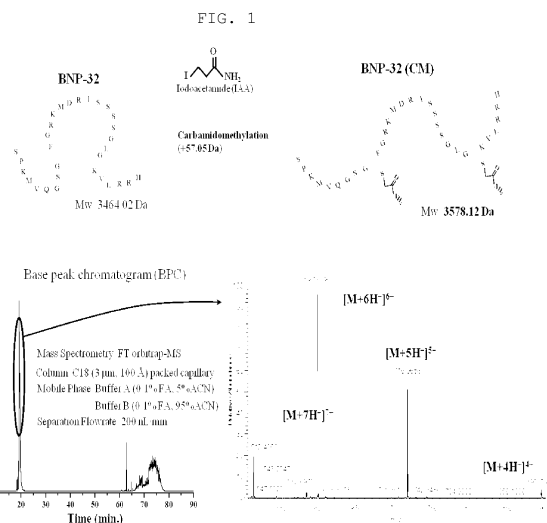
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(54) **MASS SPECTROMETRY OF BRAIN NATRIURETIC PEPTIDE IN BLOOD USING CHEMICAL ISOTOPIC SUBSTITUTION**

(57) The present invention relates to a quantitative analysis method on the basis of an isotope-dilution mass spectrometry using a chemical isotope labelling method for a brain natriuretic peptide which is widely used as a biomarker for diagnosis and prognostic evaluation of cardiovascular-associated diseases. The quantitative analysis method of a brain natriuretic peptide in blood using a chemical isotope labelling technique is a brain natriuretic peptide-specific quantitative analysis technique which is more accurate than a conventional immunoassay using an antibody.



Description

[Technical Field]

[0001] The present invention relates to a mass spectrometry specific to a brain natriuretic peptide which is a cardiovascular disease diagnostic biomarker, using chemical isotopic substitution, and a method for determining occurrence of the cardiovascular disease and evaluating a prognosis thereof through experimental results obtained by the mass spectrometry.

[Background Art]

[0002] In accordance with the recent domestic and international development of analysis techniques converged with tandem mass spectrometry (MS/MS), research into characteristics of various proteins present in cells and research into proteomics capable of conducting qualitative and quantitative analysis have been actively conducted. In particular, these researches aim to define causes of diseases associated with improvement of the quality of human life at protein levels. Particularly, it is possible to conduct quantitative comparison of protein samples between normal subjects and patients through development of a protein-specific and stable isotope-labeling method with regard to a biological sample such as cells, tissue, blood serums, etc., which contribute to the discovery of biomarkers for early diagnosis of various diseases and clinical diagnosis fields. However, the quantitative analysis of proteins using the stable isotope-labeling method has a problem in that cell-related experts or amino acids substituted with high-priced isotopes and complicated pre-treatment techniques such as SILAC (stable isotope labeling with amino acids in cell culture), MeCAT (metal-coded affinity tags), TMT (tandem mass tags), etc., are required.

[0003] Due to the above problem, an immunoassay using a protein marker-specific antibody has been the most widely used in most of the clinical diagnosis evaluation. In particular, for diagnosis and prognostic evaluation of cardiovascular diseases, quantitative changes of a brain natriuretic peptide (BNP) protein marker are diagnosed through the immunoassay. However, there are disadvantages in that uncertainty of quantitative measurement of the brain natriuretic peptide is large due to low reproducibility of antigen-antibody non-specific reaction and a fluorescein labeling technique of an immunoassay, high-priced enzymes are used, long periods of reaction time are needed, etc., which is not effective in conducting the diagnosis and the prognostic evaluation, and as a result, another alternative is required.

[0004] Therefore, the present inventors recognized the need for development of a sample pre-treatment technique and quantitative analysis that are more accurately and easily conducted for early detection and verification of cardiovascular diseases, developed mass spectrometry of brain natriuretic peptide in blood using chemical

isotopic substitution, and completed the present invention.

[Disclosure]

[Technical Problem]

[0005] An object of the present invention is to provide an isotope-coded carbamidomethylation (iCCM) isotope labeling capable of solving a antigen-antibody non-specific reaction and low reproducibility of fluorescein labeling technique of an immunoassay, and simultaneously solving a complicated sample pre-treatment process of an isotope labeling technique, and a quantitative analysis method specific to a brain natriuretic peptide on the basis of a mass spectrometer using the same.

[Technical Solution]

[0006] In one general aspect, a mass spectrometry includes: a) adding and reacting a protein reducing agent to a brain natriuretic peptide, Troponin I protein or Troponin T protein; b) reacting protein mixtures of step a) with iodine acetamide (IAA) and with an isotope of iodine acetamide, respectively; and c) obtaining mass spectra of two kinds of proteins obtained by step b) by using a mass spectrometer.

[0007] Troponin I is not found in normal skeletal muscle, but exists only in adult myocardium. Accordingly, under a situation in which creatine kinase isoenzymes (CK-MB) is increased in addition to myocardial damage, Troponin I is not detected in blood, which is very specific to the myocardial damage, and is associated with a myocardial infarct size. In addition, it is known that when acute cardiovascular diseases occur, an expression level thereof is rapidly increased, and detection of this protein is significantly important for early diagnosis of the acute cardiovascular diseases. Meanwhile, when myocardial damage occurs, Troponin T is continuously dissociated in blood, and a concentration thereof is increased. After heart disease, Troponin T has the highest value within 24 hours, and is continuously present for 9 days or more as the highest value or more, such that diagnosis time thereof is broader than those of creatine kinase and creatine kinase isozyme. Accordingly, Troponin T has high sensitivity and specificity, which is useful for early diagnosis and subsequent diagnosis of myocardial infarction and is advantageous to estimate an infarct size.

[0008] The protein reducing agent in step a) is not limited, but for example, dithiothreitol, dithioerythritol, tris(2-carboxyethyl)phosphine, or tributylphosphine may be used as the protein reducing agent. Preferably, dithiothreitol may be used. The reacting in step b) may change reactivity of a thiol group of cysteine into an irreversible form in order to prevent cysteine-cysteine refolding. For improving the enzyme treatment with regard to the protein in the enzyme treatment process, disulfide bonds formed by cysteine-cysteine bonds of the protein are first-

ly subjected to protein denaturation by using the reducing agent such as dithiothreitol (DTT), and iodine acetamide which is an alkylating agent for a thiol group (-SH) of the cysteine is added in order to prevent the cysteine-cysteine refolding, such that reactivity of the thiol group of the cysteine is changed into the irreversible form. In accordance with the chemical modification by iodine acetamide, improvement in the efficiency of enzyme treatment with regard to protein samples and reproducibility may be finally secured. The cysteine-specific CM (carbamidomethylation) process by iodine acetamide results in an increase of 57.05 Da to the cysteine group. The present invention is characterized by an isotope labeling method for BNP quantitative analysis using the CM-based chemical modification.

[0009] The isotope of iodine acetamide in step b) is not limited, but may be iodine acetamide- $^{13}\text{C}_2$, D_2 . In order to conduct the isotope labeling for BNP quantitative analysis, iodine acetamide and the isotope of iodine acetamide, i.e., iodine acetamide- $^{13}\text{C}_2$, D_2 are reacted with two standard BNP protein solutions each having a purity of 97% or more, respectively. Accordingly, a molecular weight of the BNP having two cysteine groups is increased by two spots of CM reactions. Finally, BNP- CM_2 and BNP-iCCM $_2$ produced by iodine acetamide and the isotope of iodine acetamide, i.e., iodine acetamide- $^{13}\text{C}_2$, D_2 have molecular weights of 3578.12 Da and 3586.12 Da, respectively. That is, a difference in molecular weight between two BNP protein samples may be 8 Da. Therefore, a difference in molecular weight between the iodine acetamide and the isotope of iodine acetamide may be preferably 3 to 5 Da.

[0010] The mass spectrometer of step c) is not limited, but for example, a liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) may be used.

[0011] Further, in another general aspect, a method for diagnosis and prognostic evaluation of cardiovascular diseases includes: steps a) to c) as described above; and d) determining whether the protein and an isotope of protein are appropriate as a material for diagnosis or prognostic evaluation of cardiovascular diseases, from the mass spectra obtained by step c).

[Advantageous Effects]

[0012] The quantitative analysis method of the brain natriuretic peptide according to the present invention is capable of minimizing time loss and economic loss caused from quantitative analysis by a high-priced isotope-substituted peptide labeling pre-treatment method or a complicated protein isotope labeling pre-treatment method, and is capable of reducing uncertainty in quantitative measurement and side reactions caused during the protein isotope labeling pre-treatment method. According to the quantitative analysis method on the basis of the brain natriuretic peptide isotope-labeling using iCCM as described in the present invention, low-priced io-

dine acetamide (IAA) and iodine acetamide- $^{13}\text{C}_2$, D_2 may be used, the isotope labeling pre-treatment method for quantitative analysis of the brain natriuretic peptide protein may be performed within 30 minutes, which is significantly applicable to a more accurate diagnosis for cardiovascular diseases as compared to a conventional quantitative analysis method.

[Description of Drawings]

[0013]

FIG. 1 shows an overview of a carbamidomethylation (CM) reaction of a brain natriuretic peptide (BNP) protein using iodine acetamide (IAA), and analysis results through LC-ESI-FT orbitrap-MS.

FIG. 2 shows structural difference of BNP- CM_2 and BNP-iCCM $_2$ using CM-based IAA and isotope IAA- $^{13}\text{C}_2$, D_2 and molecular weights thereof measured through mass analysis.

FIG. 3 shows base peak chromatogram (BPC) and mass spectrum of a mixture of BNP- CM_2 and BNP-iCCM $_2$, detected through LC-ESI-FT orbitrap-MS analysis.

FIG. 4 shows base peak chromatogram (BPC) and BNP- CM_2 -specific and BNP-iCCM $_2$ -specific mass spectra with regard to a blood serum sample to which 400 pg BNP-iCCM $_2$ is added.

FIG. 5 shows BNP- CM_2 -specific and BNP-iCCM $_2$ -specific extracted ion chromatogram (XIC) analysis results with regard to a blood serum sample to which 400 pg BNP-iCCM $_2$ is added.

[Best Mode]

[0014] Hereinafter, exemplary embodiments of the present invention will be described in detail with reference to the following Examples and the accompanying drawings. However, the detailed description is to help a specific understanding of the present invention, and the protection scope of the present invention is not limited to Examples below.

(Example 1) Brain natriuretic peptide (BNP)-specific carbamidomethylation (CM) and mass spectrometer (MS) spectrum using iodine acetamide (IAA)

[0015] BNP (molecular weight of 3464.02 Da), DTT, IAA, and its isotope IAA- $^{13}\text{C}_2$, D_2 were purchased from Sigma-Aldrich®. In order to verify CM reactivity and label efficiency with regard to a standard BNP protein, BNP protein (1 mg) was dissolved in 50 mM NH_4CO_3 (1 mL) solution, and DTT (1 mg) was added thereto, followed by gently shaking and reacting at 37°C for 2 hours. 40 mM IAA solution (20 μL) was added to the NH_4CO_3 solution, and reacted in a darkroom at room temperature for 30 minutes. Mass spectra with regard to the BNP- CM_2 protein obtained by the cysteine-specific CM proc-

ess by the IAA was examined by using FT orbitrap-MS, and schematized in FIG. 1.

[0016] FIG. 1 shows BPC (base peak chromatogram) and mass spectrum detected at 19.2 minutes of the BNP protein (1 ng) obtained by using FT orbitrap-MS, after the CM-pretreated standard BNP protein (1 ng) was separated by using a capillary tube (internal diameter of 75 μm and external diameter of 360 μm) filled with C8(5 μm , 200 \AA). It was confirmed that 99% or more of BNP- CM_2 having a molecular weight of 3578.12 Da was detected, which is 114.10 Da larger than a unique molecular weight of the BNP protein, i.e., 3464.02 Da, in accordance with two CM reactions. In particular, multiple hydride ions such as $[\text{M}+4\text{H}^+]^{4+}$, $[\text{M}+5\text{H}^+]^{5+}$, $[\text{M}+6\text{H}^+]^{6+}$, $[\text{M}+7\text{H}^+]^{7+}$, etc., could be confirmed.

(Example 2) Comparison in efficiency between BNP-specific CM and BNP-specific iCCM isotope-labeling using IAA and isotope IAA- $^{13}\text{C}_2$, D_2

[0017] BNP- CM_2 and BNP-iCCM $_2$ each having different molecular weight were prepared through CM reaction using IAA and IAA- $^{13}\text{C}_2$, D_2 on the BNP proteins, respectively, and mass spectra of the BNP- CM_2 and the BNP-iCCM $_2$ obtained by using a mass spectrometer were shown in FIG. 2.

[0018] BNP- CM_2 produced by the IAA and BNP-iCCM $_2$ produced by the isotope IAA- $^{13}\text{C}_2$, D_2 were mixed at a weight ratio of 1:1, and BPC was measured by LC-ESI-FT orbitrap-MS analysis method, and mass spectrum obtained by elution at 19 minutes was secured, and shown in FIG. 3.

[0019] As a result of comparison with mass/charge amount values, i.e., m/z values with regard to $[\text{M}+6\text{H}^+]^{6+}$ ion among precursor ions for BNP- CM_2 (3578.12 Da) and BNP-iCCM $_2$ (3586.12 Da) produced by IAA as shown in FIG. 2, it could be confirmed that the m/z values of the BNP- CM_2 (3578.12 Da) and BNP-iCCM $_2$ (3586.12 Da) were 597.6393 and 598.9790, respectively, and a difference between two proteins was about 1.333 Da (+6). Based on the result above, it was confirmed that the difference in molecular weight between two proteins was 8 Da, which is the same efficiency as the result by the CM reaction. In particular, BNP- CM_2 products of which a m/z value shown by IAA is 597.6393 was not found like the mass spectrum of BNP-iCCM $_2$ using IAA- $^{13}\text{C}_2$, D_2 , from which it indicates that an efficiency of producing the isotope labeling material of BNP-iCCM $_2$ was made close to 100%.

[0020] As shown in FIG. 3, a difference in m/z between the BNP- CM_2 protein and the BNP-iCCM $_2$ protein in a state in which two of the proteins were mixed with each other was 1.333 Da. In addition, quantitative changes of two of the proteins were not found even in the mixing pretreatment process of the protein produced by the different chemical reactions, from which it could be appreciated that this method is an isotope labeling method that is able to applicable to a quantitative comparative study on pro-

tein mixture samples present in various media such as blood serum, urine, etc., without mutual side reactions.

(Example 3) Quantitative analysis for blood serum BNP protein using CM-based isotope labeling method

[0021] BNP protein actually included in a blood serum sample was quantified on the basis of the BNP-specific isotope labeling pre-treatment methods of Examples 1 and 2. The blood serum sample (500 μL) of a cardiovascular patient was CM-treated by IAA, and mixed with 400 pg BNP-iCCM $_2$. Then, BPC and mass spectrum of the sample eluted by a solution in which ACN:H $_2$ O is mixed at a ratio of 10:90 (v/v) using C18 SPE cartridge were secured by LC-ESI-FT orbitrap-MS.

[0022] It was confirmed that mixtures of proteins and peptides in various shapes were detected as shown in FIG. 4. Particularly, within a range at which m/z is 592 to 606 in mass analysis spectrum detected at 24 minutes, ions of which m/z values produced by BNP- CM_2 and BNP-iCCM $_2$ are 597.6393 and 598.9790, respectively, were confirmed.

[0023] FIG. 5 shows extracted ion chromatogram (XIC) obtained in a single ion observation (SIM) mode with respect to ions of which the m/z values detected by BNP- CM_2 present in blood serum are 597.6393 (+6) and 716.9556 (+5), and the m/z values shown by 400 pg BNP-iCCM $_2$ added to the blood serum are 598.9790 (+6) and 718.5556 (+5). On the basis of results of FIG. 5, it was confirmed that according to the mass spectrometry of the present invention, quantitative evaluation with regard to the BNP protein actually present in blood serum was possibly performed through quantitative information of isotope-labeled BNP-iCCM $_2$ protein. In addition, it was confirmed that the mass spectrometry of the present invention is significantly applicable to isotope-dilution mass spectrometry (ID-MS) which is the top-level quantitative analysis method.

Claims

1. A mass spectrometry comprising:
 - a) adding and reacting a protein reducing agent to a brain natriuretic peptide, Troponin I protein or Troponin T protein;
 - b) reacting protein mixtures of step a) with iodine acetamide and with an isotope of iodine acetamide, respectively; and
 - c) obtaining mass spectra of two kinds of proteins obtained by step b) by using a mass spectrometer.
2. The mass spectrometry of claim 1, wherein the protein reducing agent in step a) is dithiothreitol, dithioerythritol, tris(2-carboxyethyl)phosphine or tributylphosphine.

3. The mass spectrometry of claim 1, wherein the reacting in step b) changes reactivity of a thiol group of cysteine into an irreversible form in order to prevent cysteine-cysteine refolding. 5
4. The mass spectrometry of claim 1, wherein the isotope of iodine acetamide in step b) is iodine acetamide- $^{13}\text{C}_2$, D_2 . 10
5. The mass spectrometry of claim 4, wherein a difference in molecular weight between the iodine acetamide and the isotope of iodine acetamide is 3 to 5 Da. 15
6. The mass spectrometry of claim 1, wherein the mass spectrometer of step c) is LC-ESI-MS/MS. 20
7. A method for diagnosis and prognostic evaluation of cardiovascular diseases comprising:
 - steps a) to c) of claim 1; and 25
 - d) determining whether the protein and an isotope of protein are appropriate as a material for diagnosis or prognostic evaluation of cardiovascular diseases, from the mass spectra obtained by step c). 30

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FIG. 1

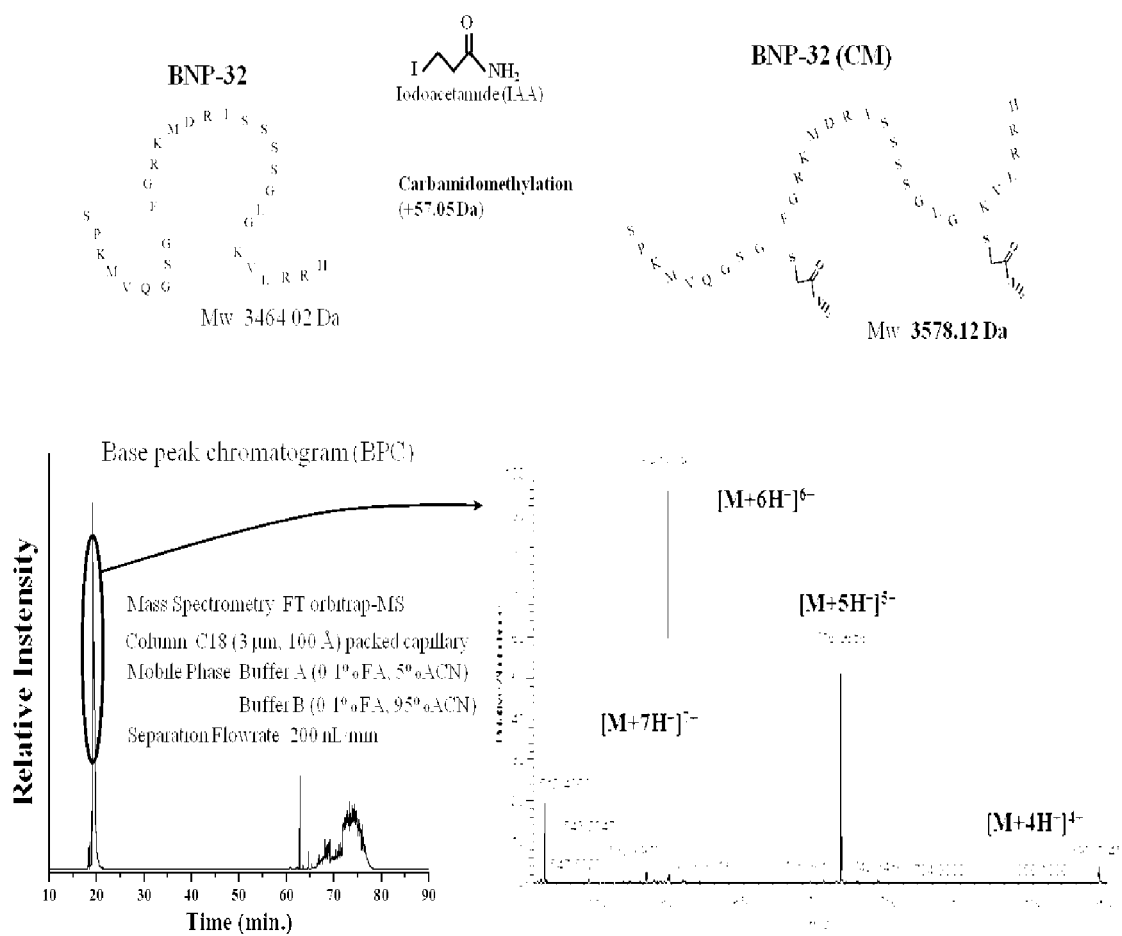


FIG. 2

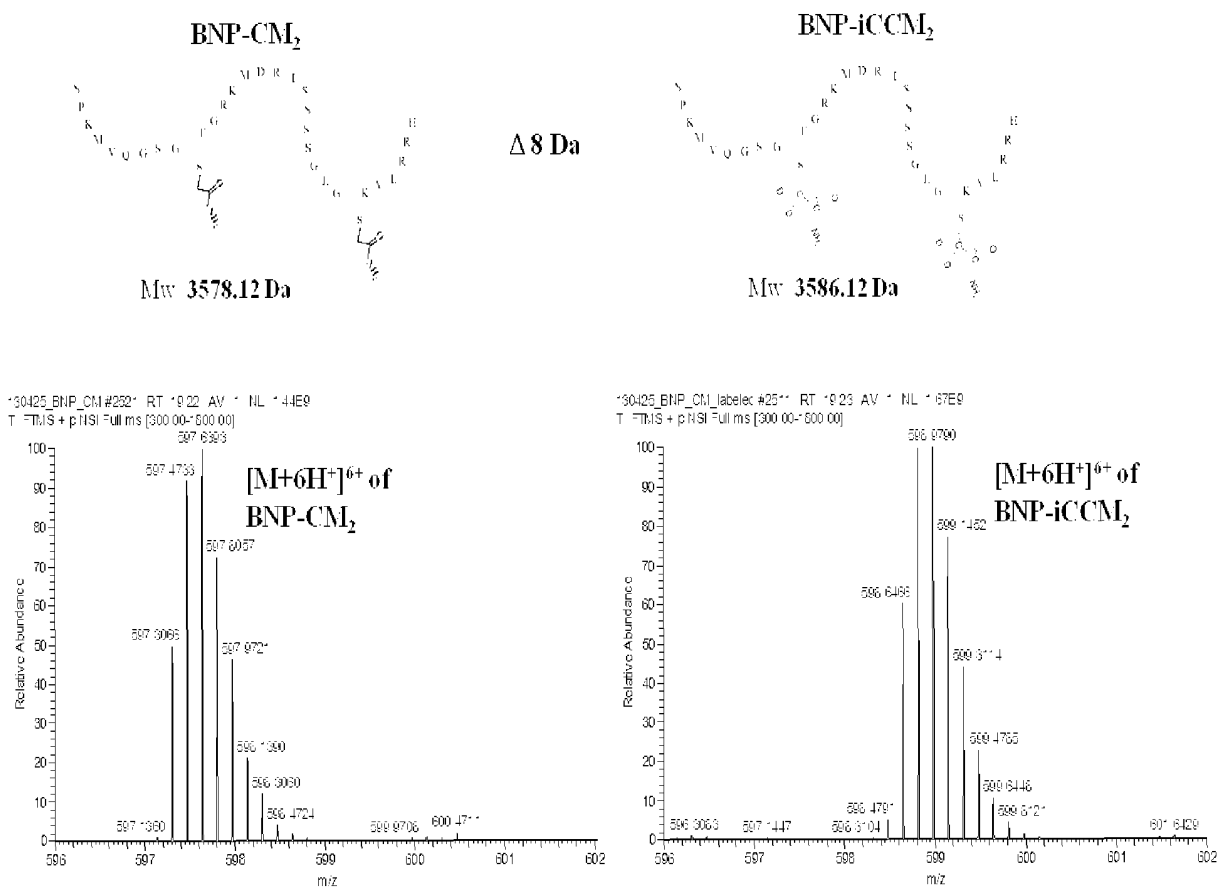


FIG. 4

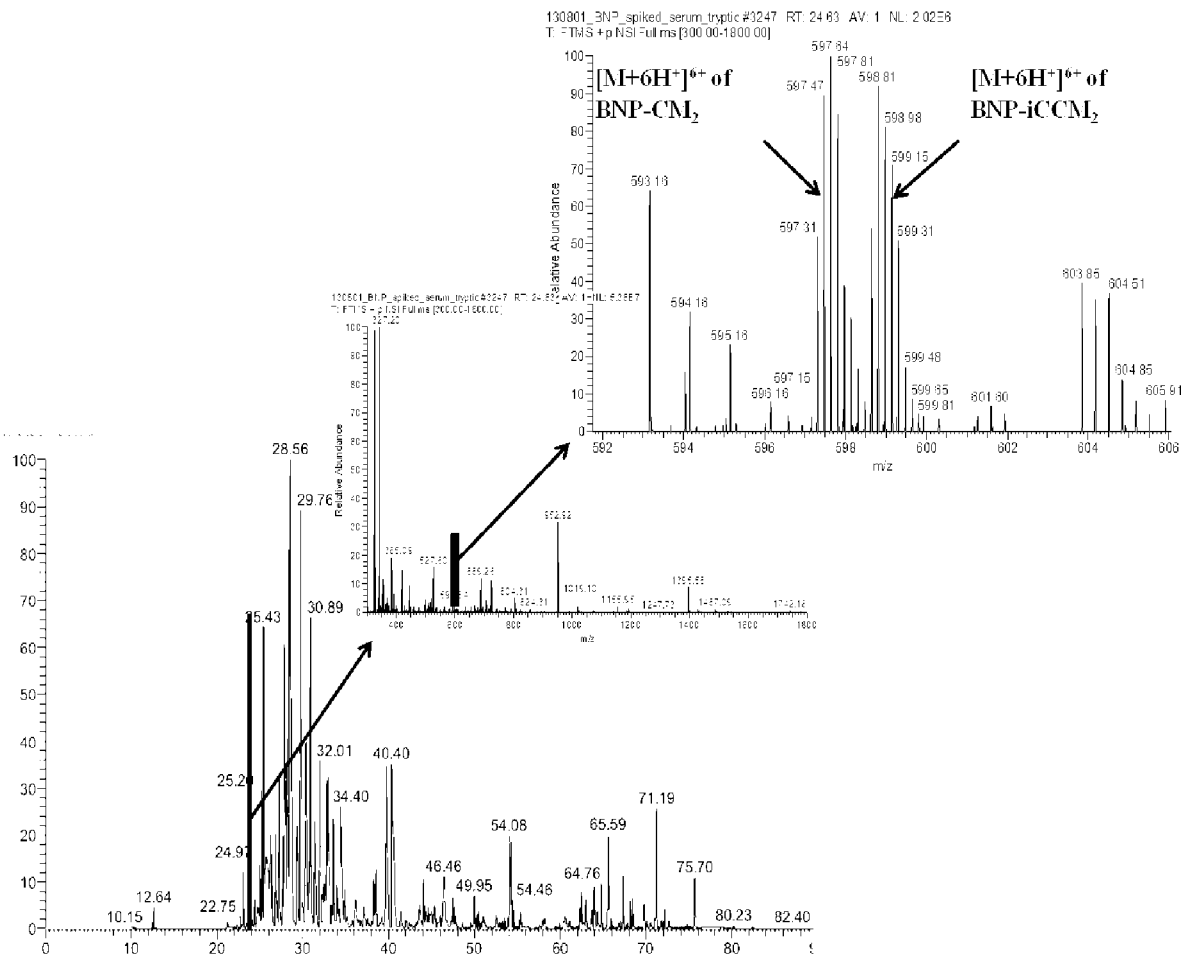
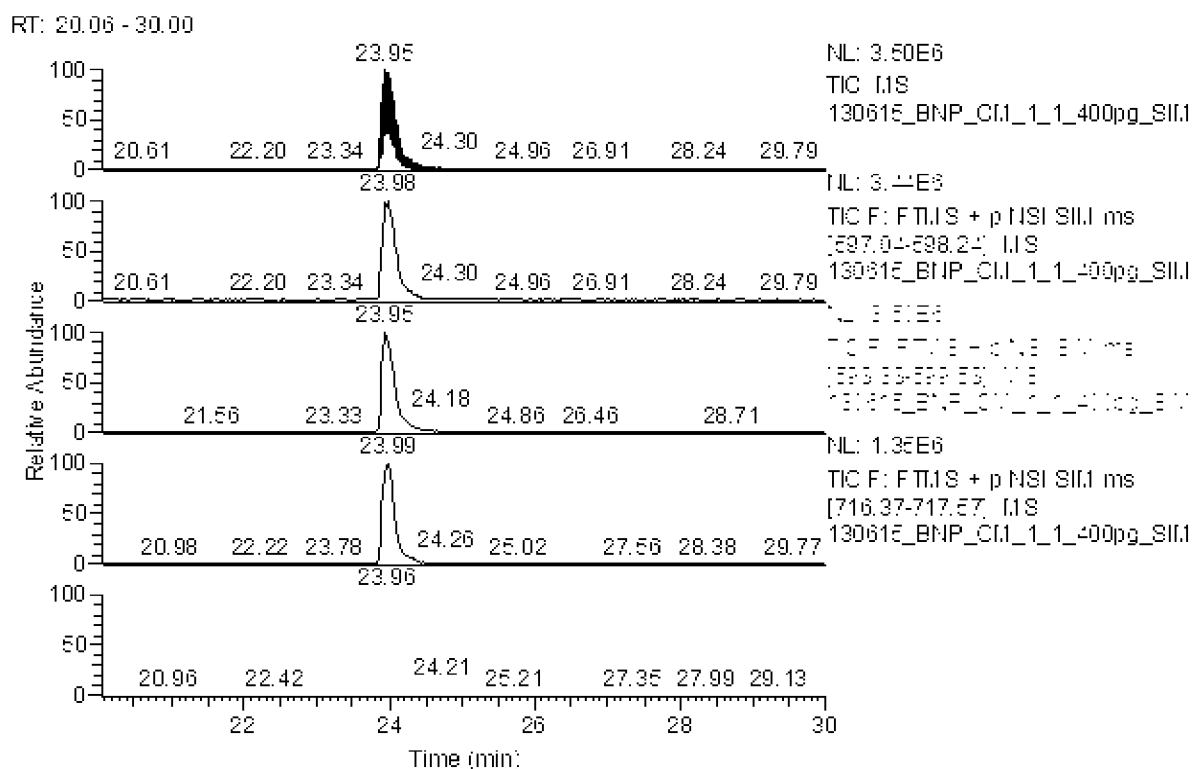



FIG. 5



INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR2014/008684

5	A. CLASSIFICATION OF SUBJECT MATTER	
	<i>G01N 33/68(2006.01)i, G01N 33/53(2006.01)i, G01N 27/62(2006.01)i, G01N 30/72(2006.01)i</i>	
	According to International Patent Classification (IPC) or to both national classification and IPC	
	B. FIELDS SEARCHED	
10	Minimum documentation searched (classification system followed by classification symbols) G01N 33/68; C12Q 1/00; G01N 27/62; G01N 33/566; G01N 33/53; C12M 1/34; G01N 30/72	
	Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Korean Utility models and applications for Utility models: IPC as above Japanese Utility models and applications for Utility models: IPC as above	
15	Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) cKOMPASS (KIPO internal) & Keywords: natriuretic, troponin, disulfide bond, isotope, BNP, mass spectrometry	
	C. DOCUMENTS CONSIDERED TO BE RELEVANT	
20	Category*	Citation of document, with indication, where appropriate, of the relevant passages
		Relevant to claim No.
	A	US 2012-0303083 A1 (AGNETTI, Giulio et al.) 29 November 2012 See abstract, claims 16, 22-24.
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35		
40	<input type="checkbox"/> Further documents are listed in the continuation of Box C. <input checked="" type="checkbox"/> See patent family annex.	
	* Special categories of cited documents:	"I" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
	"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
45	"E" earlier application or patent but published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
	"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
	"O" document referring to an oral disclosure, use, exhibition or other means	
	"P" document published prior to the international filing date but later than the priority date claimed	
50	Date of the actual completion of the international search 22 DECEMBER 2014 (22.12.2014)	Date of mailing of the international search report 22 DECEMBER 2014 (22.12.2014)
55	Name and mailing address of the ISA/KR  Korean Intellectual Property Office Government Complex-Daejeon, 189 Seonsa-ro, Daejeon 302-701, Republic of Korea Facsimile No. 82-42-472-7140	Authorized officer Telephone No.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR2014/008684

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Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

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1. Claims Nos.: 7
because they relate to subject matter not required to be searched by this Authority, namely:
Claim 7 pertains to a method for treatment of the human body by surgery or therapy, or to a diagnostic method, and thus pertains to subject matter on which the International Searching Authority is not required to carry out an international search under the provisions of PCT Article 17(2)(a)(i) and PCT Rule 39.1(iv).

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2. Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

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3. Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

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1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

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2. As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

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3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

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4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

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- Remark on Protest**
- The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.

PCT/KR2014/008684

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INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/KR2014/008684

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专利名称(译)	利用化学同位素替代法对血液中脑利钠肽进行质谱分析		
公开(公告)号	EP3059593A4	公开(公告)日	2017-06-07
申请号	EP2014854826	申请日	2014-09-18
[标]申请(专利权)人(译)	韩国标准科学研究院		
申请(专利权)人(译)	韩国研究院标准和科学		
当前申请(专利权)人(译)	韩国标准科学研究院		
[标]发明人	KANG DUKJIN KIM SOOK KYUNG LEE SUN YOUNG		
发明人	KANG, DUKJIN KIM, SOOK-KYUNG LEE, SUN YOUNG		
IPC分类号	G01N33/68 G01N33/53 G01N27/62 G01N30/72		
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外部链接	Espacenet		

摘要(译)

本发明涉及基于同位素稀释质谱法的定量分析方法，所述同位素稀释质谱法使用脑利尿钠肽的化学同位素标记方法，其广泛用作心血管相关疾病的诊断和预后评估的生物标志物。使用化学同位素标记技术对血液中脑利钠肽进行定量分析的方法是脑利钠肽特异性定量分析技术，其比使用抗体的常规免疫测定更准确。