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(54) HUMAN sCD14-ST ASSAY METHOD

(57) An analysis method capable of accurately measuring human sCD14-ST when using a whole blood sample is provided. In the analysis method, human sCD14-ST in a whole blood sample is analyzed within 6 hours of the collection of the sample.

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Description

TECHNICAL FIELD

[0001] The present invention relates to a method of analyzing human sCD14-ST, which is known as a diagnostic marker of sepsis.
The term "analysis" as used herein includes a detection to judge the presence or absence of human sCD14-ST as a substance to be analyzed, and a measurement to quantitatively or semi-quantitatively determine the amount of the substance to be analyzed.

BACKGROUND ART

[0002] The CD14 molecule was named as a protein identified by a family of antibodies which recognize a glycoprotein expressed on the membrane surface of monocytes at the Third Leukocyte Typing Conference in 1986. In 1990, Wright et al. elucidated that the CD14 molecule was a receptor for an endotoxin, LPS (non-patent literature 1). The CD14 molecule is a glycoprotein having a molecular weight of 53 to 55 kDa, and analyses on cDNA revealed that 1.4kb mRNA has a coding sequence of 356 amino acids (non-patent literature 2).

[0003] Human CD14 molecules include soluble CD14 as well as membrane-bound CD14, and it is known that a plurality of soluble CD14 subtypes having different molecular weights is present in blood. As these soluble CD14 subtypes, two soluble CD14 of about 55 kDa and 49 kDa (soluble high molecular weight cD14) and a soluble low molecular weight CD14 subtype of about 13 kDa (hereinafter referred to as human sCD14-ST) are known, and human sCD14-ST is clinically useful as a diagnostic marker of sepsis (patent literature 1).

[0004] Regarding human sCD14-ST, patent literature 1 discloses that when serum samples are repeatedly frozen and thawed, or allowed to stand at room temperature for a long time, it is assumed that human sCD14-ST in the serum samples is decomposed and cannot be measured in some cases, or it is assumed that high molecular weight CD14 in the serum samples is decomposed to human sCD14-ST or its similar structures and leads to incorrect measured values (paragraph [0151]). Patent literature 1 also discloses that human sCD14-ST is generated by digesting the full length of CD14 with proteases such as elastase, and that the generating sCD14-ST is stably present in the living body (in serum) at least in detectable quantities (paragraph [0126]).
As described above, the cases where incorrect measured values are obtained when serum samples are frozen and thawed, or allowed to stand at room temperature for a long time are assumed in patent literature 1, but it is general information about storage conditions of specimens, and does not caution any special handling. Patent literature 1 rather discloses that human sCD14-ST is stable in the living body and in serum.

CITATION LIST

NON-PATENT LITERATURE

[0005]

[Non-patent literature 1] Science (the United States), 1990, vol. 249, p. 1431-1433
[Non-patent literature 2] Nucleic Acids Research (the United Kingdom), 1988, vol. 16, p. 4173

PATENT LITERATURE

[0006]

[Patent literature 1] Japanese Patent No. 4,040,666

SUMMARY OF INVENTION

TECHNICAL PROBLEM

[0007] Under the circumstances where it has been known that human sCD14-ST is relatively stably present in serum, the present inventors have conducted intensive studies to develop a more accurate method of analyzing human sCD14-ST contained in samples including plasma samples and whole blood samples, and as a result, unexpectedly found that the measured values of human sCD14-ST were affected when whole blood samples were used.
The present invention has been made based on this finding. An object of the present invention is to provide an analysis

method capable of accurately measuring human sCD14-ST, even when a whole blood sample is used.

SOLUTION TO PROBLEM

[0008] The problem may be solved by the present invention, that is, a method of analyzing human sCD14-ST, characterized by analyzing human sCD14-ST in a whole blood sample within 6 hours of the collection of the sample.

[0009] The term "human sCD14-ST" (also referred to as Presepsin (registered trademark)) as used herein means the "soluble CD14 antigen of the first aspect" disclosed in Japanese Patent No. 4,040,666, and more particularly, is a soluble CD14 antigen having the following characteristics:

1) Molecular weight of 13 ± 2 kDa, as measured by SDS-PAGE under nonreducing conditions;
2) Having the amino acid sequence of SEQ ID NO: 1 at the N-terminal sequence; and
3) Binding specifically to an antibody prepared using a peptide consisting of 16 amino acid residues of SEQ ID NO: 2 as the antigen.

[0010]

SEQ ID NO: 1:

Thr Thr Pro Glu Pro Cys Glu Leu Asp Asp Glu
1 5 10

SEQ ID NO: 2:

Arg Val Asp Ala Asp Ala Asp Pro Arg Gln
1 5 10
Tyr Ala Asp Thr Val Lys
15

ADVANTAGEOUS EFFECTS OF INVENTION

[0011] According to the present invention, human sCD14-ST, which is stable in serum or plasma samples, but measured values of which is fluctuating with the progress of time in whole blood samples, can be accurately analyzed using a whole blood sample, without preparing a serum or plasma sample from the whole blood sample.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012]

FIG. 1 is a graph showing the results of the measurement of human sCD14-ST in whole blood samples and plasma samples (specimen 1) which were prepared using heparin blood collection tubes and were allowed to stand at room temperature for predetermined periods of time (0, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 8 hours, and 10 hours).

FIG. 2 is a graph showing the results of the measurement of human sCD14-ST in whole blood samples and plasma samples (specimen 2) which were prepared using heparin blood collection tubes and were allowed to stand at room temperature for predetermined periods of time (0, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 8 hours, and 10 hours).

FIG. 3 is a graph showing the results of the measurement of human sCD14-ST in whole blood samples and plasma samples (specimen 3) which were prepared using heparin blood collection tubes and were allowed to stand at room temperature for predetermined periods of time (0, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 8 hours, and 10 hours).

FIG. 4 is a graph showing the results of the measurement of human sCD14-ST in whole blood samples and plasma samples (specimen 1) which were prepared using EDTA blood collection tubes and were allowed to stand at room temperature for predetermined periods of time (0, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 8 hours, and 10 hours).

FIG. 5 is a graph showing the results of the measurement of human sCD14-ST in whole blood samples and plasma samples (specimen 2) which were prepared using EDTA blood collection tubes and were allowed to stand at room

temperature for predetermined periods of time (0, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 8 hours, and 10 hours).

FIG. 6 is a graph showing the results of the measurement of human sCD14-ST in whole blood samples and plasma samples (specimen 3) which were prepared using EDTA blood collection tubes and were allowed to stand at room temperature for predetermined periods of time (0, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 8 hours, and 10 hours).

FIG. 7 is a graph showing the results of the measurement of human sCD14-ST in serum samples (specimens 1 to 3) which were allowed to stand at room temperature for predetermined periods of time (0, 1 hour, 2 hours , 3 hours, 4 hours, 5 hours, 6 hours, and 8 hours).

FIG. 8 is a graph showing the results of the measurement of human sCD14-ST in samples which were allowed to stand under various conditions (conditions 1 to 3), in order to examine the factors of instability of whole blood samples.

FIG. 9 is a graph showing the results of the measurement of CRP in whole blood samples (specimens 1 to 3) which were prepared using heparin blood collection tubes and were allowed to stand at room temperature for predetermined periods of time (0, 2 hours, 4 hours, and 8 hours).

FIG. 10 is a graph showing the results of the measurement of CRP in whole blood samples (specimens 1 to 3) which were prepared using EDTA blood collection tubes and were allowed to stand at room temperature for predetermined periods of time (0, 2 hours, 4 hours, and 8 hours).

DESCRIPTION OF EMBODIMENTS

[0013] The analysis method of the present invention may be carried out in accordance with known analysis methods for human sCD14-ST, except that a whole blood sample is used as the sample to be analyzed, and that its analysis is carried out within 6 hours of the collection of the sample.

[0014] Human sCD14-ST may be analyzed using various known analysis methods for proteins, for example, immunological analysis methods using one or more antibodies, or biochemical methods such as electrophoresis, and an automatic analyzer for clinical testing may be used.

For example, Japanese Patent No. 4,040,666 discloses a method of measuring human sCD14-ST, more particular, a sandwich EIA system [Example 7-(1) of Japanese Patent No. 4,040,666] using the combination of a polyclonal antibody (S68 antibody) or a monoclonal antibody (F1146-17-2 antibody) prepared using a peptide (S68 peptide described in Japanese Patent No. 4,040,666) consisting of 16 amino acid residues of SEQ ID NO: 2 as the antigen, and an anti-CD14-antigen monoclonal antibody (for example, F1031-8-3 antibody or F1106-13-3 antibody), and it may be applied to the analysis method of the present invention.

In the analysis method used in the Examples described below, human sCD7_4-ST contained in each sample is bound to magnetic latex coated with an anti-sCD14-ST mouse monoclonal antibody (F1106-13-3 antibody) and an ALP (alkaline phosphatase)-labeled anti-sCD14-ST rabbit polyclonal antibody (S68 antibody) to form an immunocomplex by an antigen-antibody reaction; the excess ALP-labeled anti-sCD14-ST rabbit polyclonal antibody is removed from the immunocomplex; and the ALP enzyme activity of the immunocomplex is measured by a chemiluminescent enzyme immunoassay (CLEIA) using a luminescent substrate (CDP-Star; Tropix) to determine the concentration of human sCD14-ST contained in the sample.

[0015] In the analysis method of the present invention, a whole blood sample is used as the sample to be analysis. The expression "a whole blood sample is used" as used herein means that the sample used is a sample which has been stored in the form of whole blood for a period of time from the collection of whole blood to the beginning of the analysis of the sample (hereinafter referred to as the storage period after blood collection) or most of that period. The whole blood sample may be collected by a normal operation, i.e., using a blood collection tube containing an anticoagulant such as heparin, EDTA, or citric acid.

The whole blood sample collected using a blood collection tube may be stirred by inversion several times, and then, may be directly used for measurement or may be used after plasma separation. The expression "used after plasma separation" includes the case where whole blood is centrifuged to separate two layers of plasma and blood cells and the plasma component is used in that state, and the case where the plasma component is used after further dispensing the plasma component. In the analysis method of the present invention, the whole blood sample is used as the sample to be analyzed, and the case where whole blood after the storage period after blood collection is separated into plasma and the other components is used is included in embodiments of "a whole blood sample is used". According to the findings of the present inventors including the Examples below, when human sCD14-ST contained in a whole blood sample is measured, the measured values may fluctuate with the progress of time in some cases, and excessive physical shock when preparing the sample or transporting the sample may be a factor which affects the measured values in some cases.

[0016] In the analysis method of the present invention, the analysis is carried out within 6 hours (preferably within 4 hours) of the collection of the sample. The expression "the analysis is carried out within 6 hours" as used herein means

that the analysis begins within 6 hours.
More particularly, it is preferable that when the whole blood sample is collected using an EDTA blood collection tube, the analysis is carried out within 6 hours, and it is preferable that when the whole blood sample is collected using a heparin blood collection tube, the analysis is carried out within 4 hours.
As shown in the concrete experimental data described in the Examples below, an increase in the measured values of human sCD14-ST was observed in whole blood samples from healthy people, even when the measurement was after 6 to 8 hours from the blood collection, in which period no changes in measured values are observed in plasma samples or serum samples. It is known that the measured value of human sCD14-ST is different by 50-fold between healthy people and sepsis patients, and the concentration of human sCD14-ST contained in the blood of healthy people is extremely low. This enables sepsis to be diagnosed with high sensitivity and high specificity by setting an appropriate cut-off value (Example 12 of patent literature 1). However, where the measured values of human sCD14-ST in healthy people increase with the progression of time after collecting a whole blood sample, there is a possibility that the judgment of whether the measured value is lower or higher than the cut-off value is different depending on the measuring time. Under these circumstances, the present inventors found that the measured values of human sCD14-ST in a whole blood sample did not fluctuate within 6 hours (preferably within 4 hours) of the collection of a sample. Since the analysis method of the present invention is carried out within 6 hours of the collection of a whole blood sample, human sCD14-ST can be accurately measured without an increase in measured values.

EXAMPLES

[0017] The present invention now will be further illustrated by, but is by no means limited to, the following Examples.

«EXAMPLE 1: Method of measuring human sCD14-ST in samples»

[0018] Each sample to measure human sCD14-ST was measured using reagents for measuring human sCD14-ST. These reagents included a solution of magnetic latex coated with an anti-sCD14-ST mouse monoclonal antibody (F1106-13-3 antibody; Japanese Patent No. 4,040,666) as the first antibody solution, a solution of an ALP (alkaline phosphatase)-labeled anti-sCD14-ST rabbit polyclonal antibody (S68 antibody) as the second antibody solution, a washing solution for B/F separation, a substrate solution, and the like, and were packaged in a cartridge applicable to an automatic luminescent immunoanalyzer described below.
An automatic luminescent immunoanalyzer (PATHFAST; Mitsubishi chemical Medience Corporation), which is similar to that disclosed in Japanese Patent No. 3, 115, 501 and which can automatically perform immunoassay using magnetic particles, was used for the measurement. This apparatus is capable of efficiently performing B/F separation by magnetic force in a tip arranged as a unit of liquid suction and discharge, and exhibits high efficiency in washing. The measurement steps of the apparatus are as follows.
A chemiluminescent substrate '°CDP-Star" (Tropix) was used as the substrate, and emission counts detected by a photomultiplier tube (PMT) were regarded as the measurement results.
[0019] Each cartridge for an automatic measurement was filled with each sample, a sample diluent solution, the solution of magnetic particles (coated with the first antibody), the washing liquid for B/F separation, the solution of the second antibody, the substrate solution, and the like, and loaded to the automated analyzer. The following steps were carried out in accordance with the normal procedure:

(1) The sample solution previously adjusted to predetermined dilution ratio with a sample diluent solution, the solution of magnetic particles, and the solution of the second antibody were mixed to generate an immunocomplex by an antigen-antibody reaction.
(2) A B/F separation was carried out to remove unreacted substances as follows. The resulting reaction solution was aspirated into the tip arranged as a unit for aspirating a solution, and the magnetic particles were trapped by contact with a magnet on the outer wall of the tip. The solution was discharged from the tip while the magnetic particles were trapped on the inner wall of the tip. After separation, the washing liquid for the B/F separation held in another reaction vessel was aspirated and discharged to wash the magnetic particles in the tip.
(3) The magnet was removed from the outer wall of the tip. The substrate solution was aspirated and discharged to disperse the magnetic particles trapped on the inner wall of the tip and carry out an enzyme reaction.
(4) The amount of luminescence was measured by PMT.

«EXAMPLE 2: Examination of measured values in whole blood, plasma and serum samples after short-term storage»

[0020] Whole blood samples, serum samples, and plasma samples were allowed to stand at room temperature for predetermined periods of time, and then used to measure human sCD14-ST in accordance with the measuring method

described in Example 1.

More particularly, whole blood samples, serum samples, and plasma samples, which had been collected from three healthy people using blood collection tubes containing an anticoagulant (heparin or EDTA), were allowed to stand at room temperature for 0, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 8 hours, or 10 hours (only for the whole blood samples and the plasma samples), and each measurement was immediately carried out at each time point to confirm the time course of the measured values for each sample. As the serum samples, whole blood samples were collected using plain blood collection tubes (Terumo) and centrifuged and separated into sera, and these serum samples were used. EDTA plasma samples were prepared using EDTA blood collection tubes (Terumo), and heparin plasma samples were prepared using heparin blood collection tubes (Terumo). The whole blood samples were collected using the above blood collection tubes, and directly used without plasma separation.

[0021] The results of the whole blood samples and the plasma samples prepared using heparin blood collection tubes are shown in Figures 1 to 3 (specimens 1 to 3), and the results of the whole blood samples and the plasma samples prepared using EDTA blood collection tubes are shown in Figures 4 to 6 (specimens 4 to 6). When the value measured after the samples were collected and immediately treated is regard as 100%, each increase or decrease from the initial value is indicated as percentage in Figures 1 to 6. The values within the range of 90% to 110% are evaluated as "stable". The measured values in the whole blood samples tended to increase after 5 to 8 hours from the blood collection, but no changes in the measured values were observed in the plasma samples which had been allowed to stand under the same conditions. The measured values in the whole blood samples prepared using EDTA blood collection tubes were stable for 6 hours, and the measured values in the whole blood samples prepared using heparin blood collection tubes were stable for 4 hours.

In addition, when excessive physical shock, such as stirring, was applied to the whole blood samples, a remarkable increase in the measured values was observed (data not shown).

The results of the serum samples are shown in Figure 7. No changes were detected in the serum samples which had been allowed to stand under the same conditions.

Further, it was confirmed that the measured values were sufficiently stable in the serum samples and the plasma samples, even when they had been allowed to stand at room temperature for at least 24 hours (data not shown).

«EXAMPLE 3: Examination of stability of whole blood samples»

[0022] The cause of the instability of the measured values of the whole blood samples was examined.

Condition 1: Whole blood sample
Condition 2: Plasma sample prepared by allowing the whole blood sample to stand at room temperature for predetermined periods of time and performing plasma separation before the measurement
Condition 3: Plasma sample

The preparation and the measurement of the samples were carried out, using heparin blood collection tubes, in accordance with Example 2. The results are shown in Figure 8. The measured values in the whole blood samples (Condition 1) increased after 4 or more hours from the blood collection, as shown in Example 1, whereas the measured values in the plasma samples (Condition 3) were stable for 8 hours. In contrast, an increase in the measured values was observed in the plasma samples of Condition 2, which had been separated from the whole blood samples stored for predetermined periods of time, as similar to the whole blood samples (Condition 1).

It was suggested from these results that the increase in the measured values was caused by factor(s) not from the plasma component but from the blood cell component. It was necessary to measure a whole blood sample directly within 4 hours of the blood collection, and further, unless plasma separation was carried out within 4 hours of the blood collection of a whole blood sample, an increase in the measured values after the plasma separation was observed, and therefore, it was found that the plasma separation within 4 hours of the blood collection was necessary to obtain accurate measured values.

«Referential Example 1>>

[0023] Whole blood samples were collected from three healthy people using blood collection tubes containing an anticoagulant (heparin or EDTA), and were allowed to stand at room temperature for 0, 2 hours, 4 hours, and 8 hours, and each measurement was immediately carried out at each time point, using a commercially available CRP measuring reagent (PATHFAST hs CRP measuring reagent; Mitsubishi chemical Medience Corporation) to confirm the time course of the measured values for each sample. The preparation and the measurement of the samples were carried out in accordance with Example 2, except that the CRP measuring reagent was used.

The results are shown in Figure 9 (heparin blood collection tube) and Figure 10 (EDTA blood collection tube). Unlike

the case of human sCD14-ST in Example 2, the measured values did not tend to increase after 4 or more hours from the blood collection, and it was confirmed that CRP was stable in whole blood samples.

INDUSTRIAL APPLICABILITY

[0024] The analysis method of the present invention may be used in, for example, the diagnosis of sepsis.
Although the present invention has been described with reference to specific embodiments, various changes and modifications obvious to those skilled in the art are possible without departing from the scope of the appended claims.

SEQUENCE LISTING

<110> Mitsubishi Chemical Medience Corporation
Mochida Pharmaceutical Co., Ltd.

<120> Method of analyzing human sCD14-ST

<130> MCM-870

<150> JP 2010-018340
<151> 2010-01-29

<160> 2

<170> PatentIn version 3.5

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<212> PRT
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<400> 1

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1 5 10

<210> 2
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<400> 2

Arg Val Asp Ala Asp Ala Asp Pro Arg Gln Tyr Ala Asp Thr Val Lys
1 5 10 15

Claims

1. A method of analyzing human sCD14-ST, **characterized by** analyzing human sCD14-ST in a whole blood sample within 6 hours of the collection of the sample.

2. The method according to claim 1, wherein human sCD14-ST in the whole blood sample is analyzed within 4 hours of the collection of the sample.

3. The method according to claim 1 or 2, wherein the analysis is carried out using an untreated whole blood sample.

4. The method according to any one of claims 1 to 3, wherein the whole blood sample is collected using a heparin blood collection tube or an EDTA blood collection tube.

Figure 1

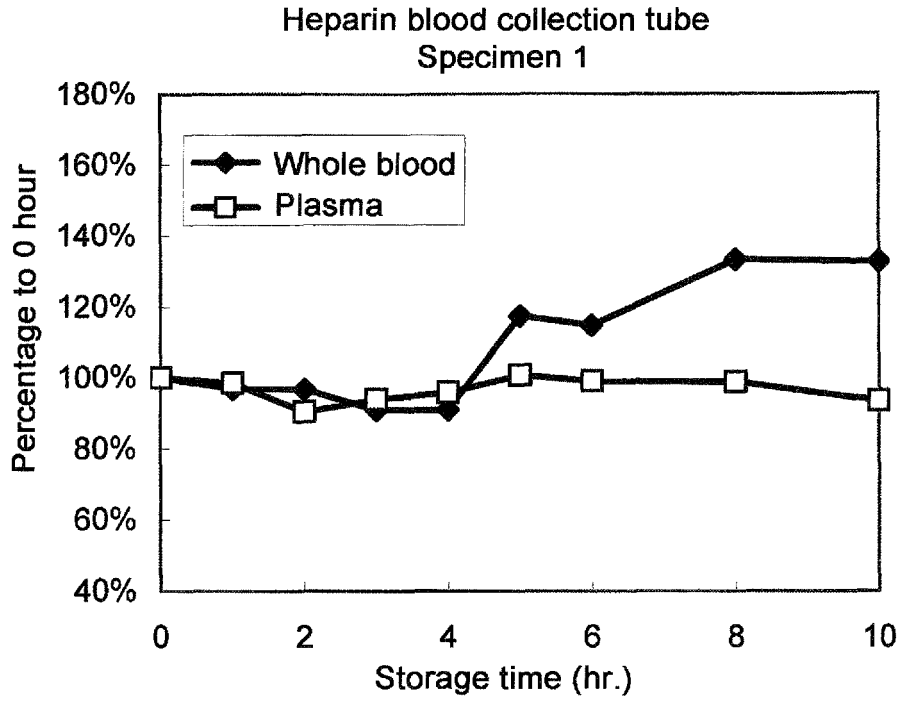


Figure 2

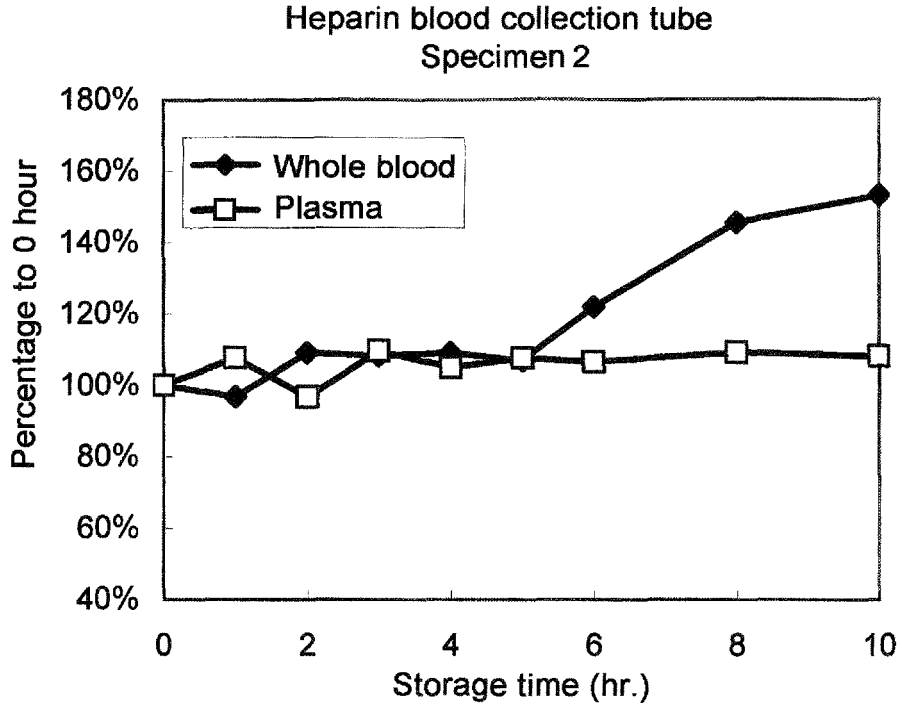


Figure 3

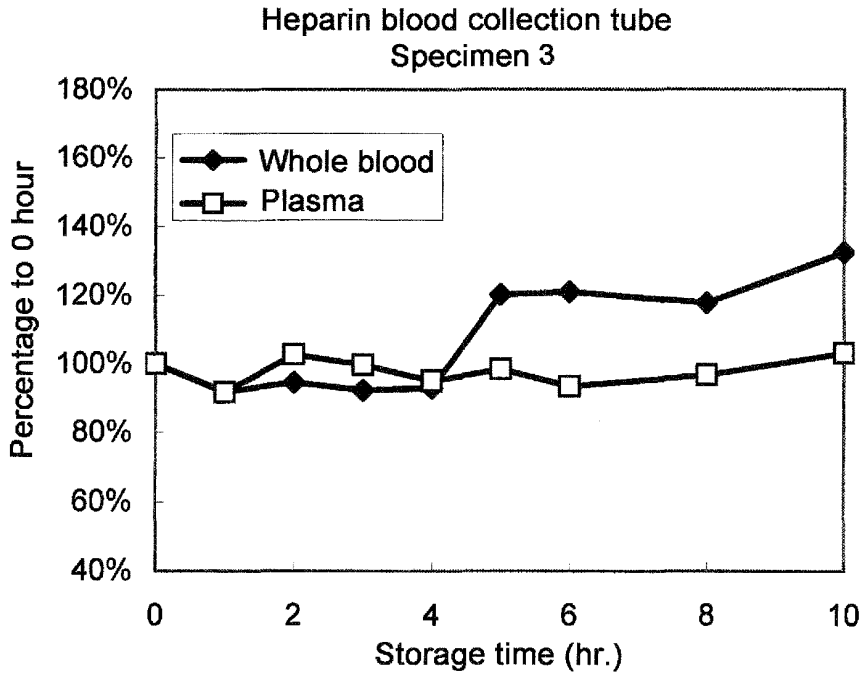


Figure 4

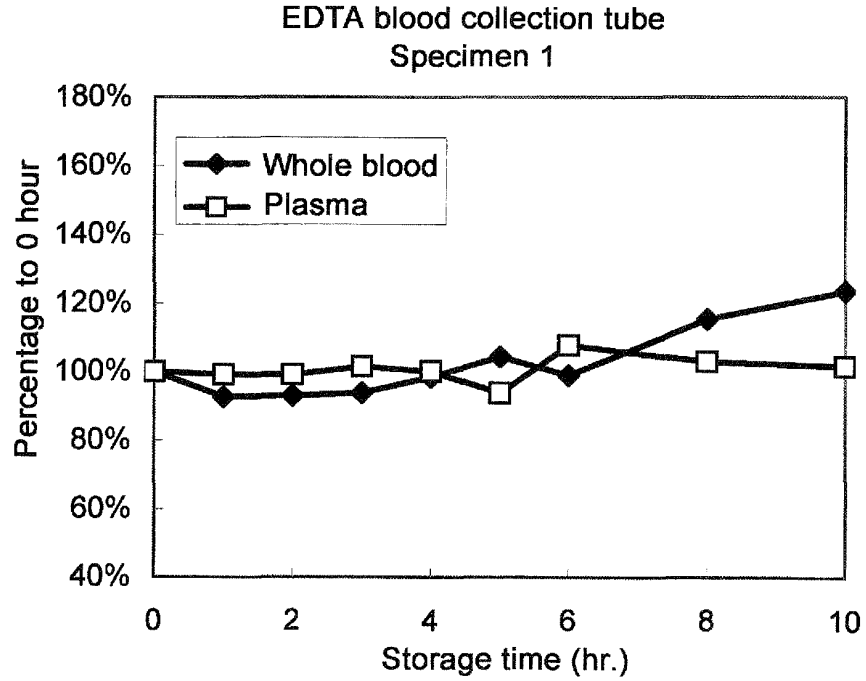


Figure 5

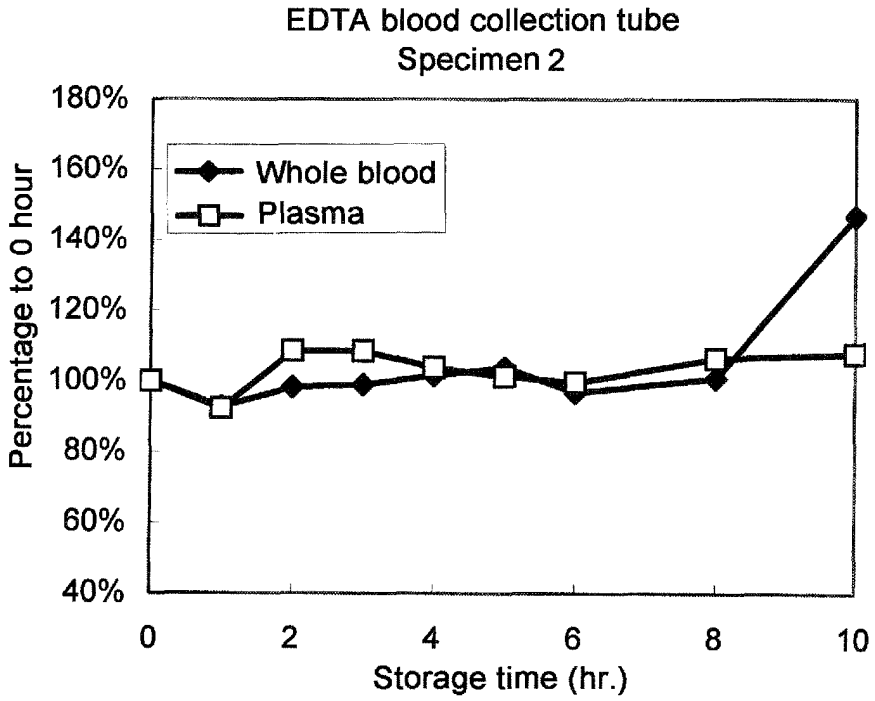


Figure 6

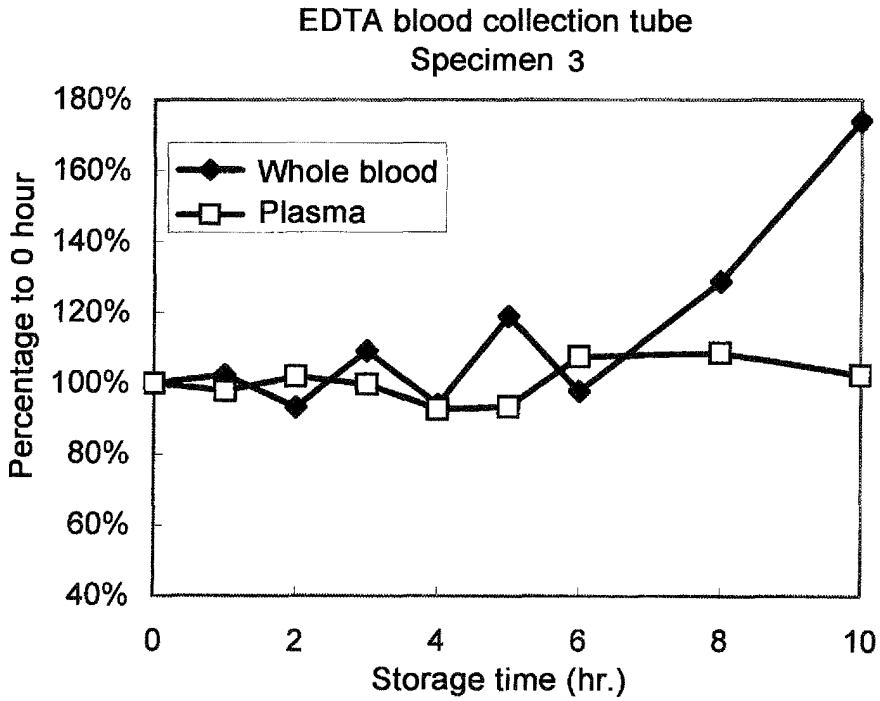


Figure 7

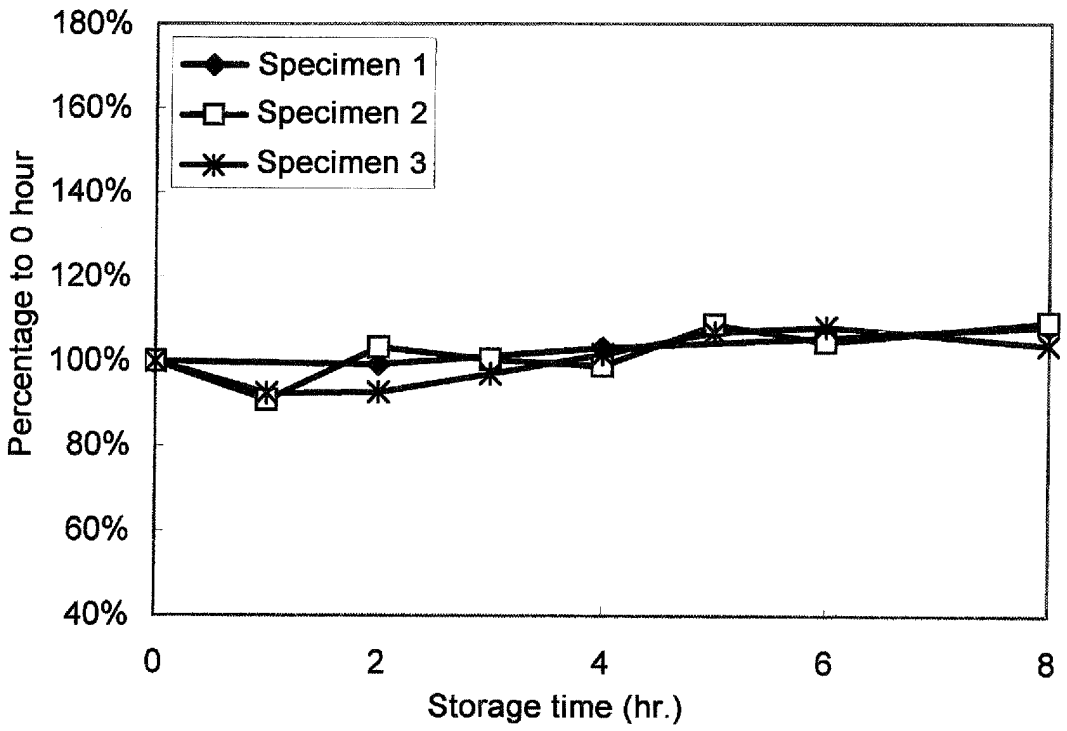


Figure 8

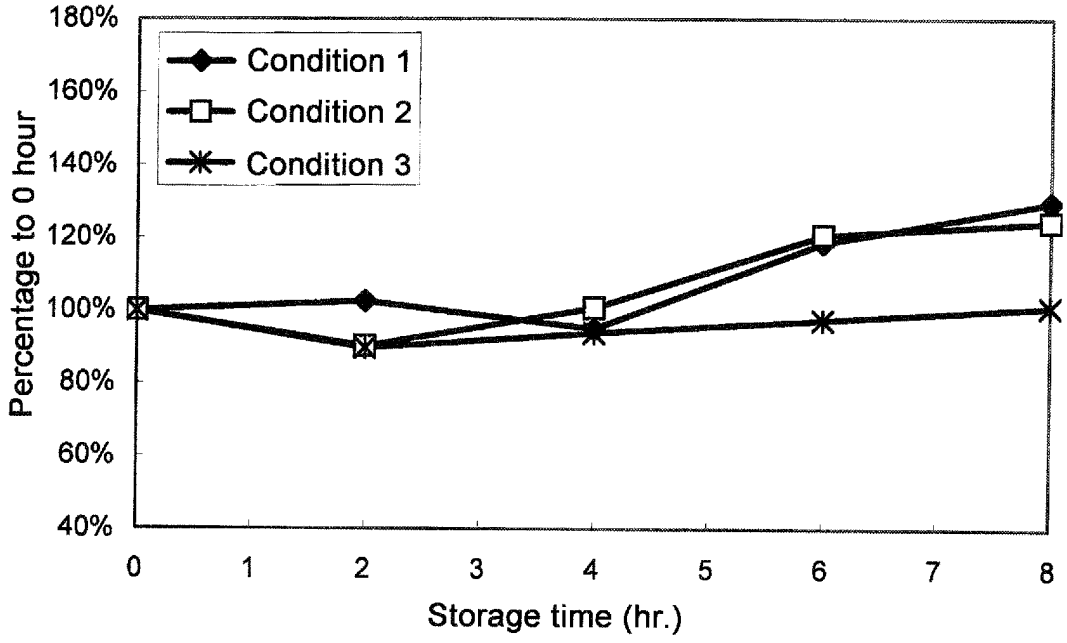


Figure 9

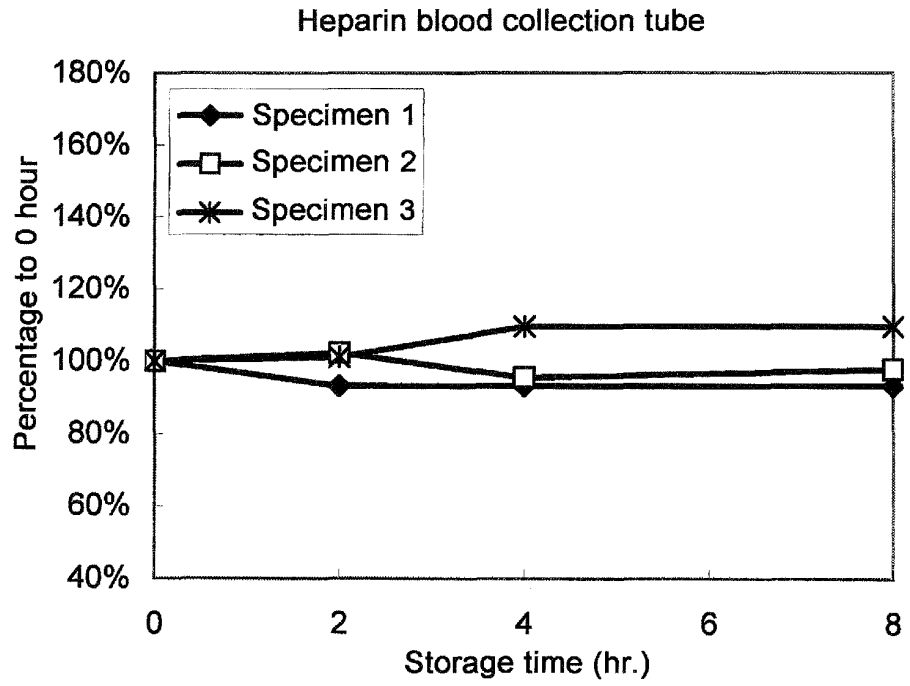
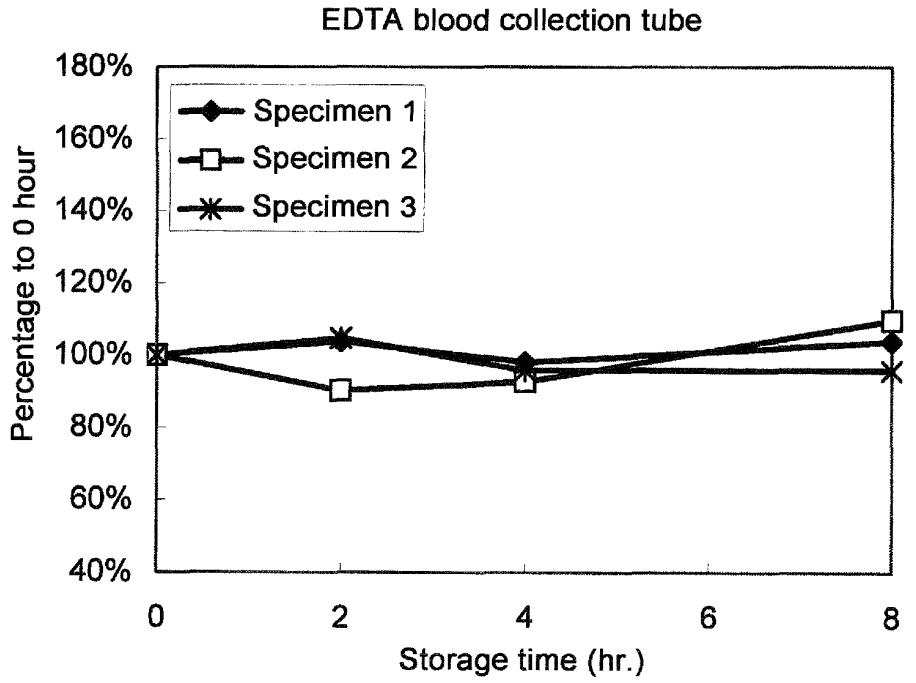


Figure 10



INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2011/051778

A. CLASSIFICATION OF SUBJECT MATTER
G01N33/68(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N33/68

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Jitsuyo Shinan Koho 1922-1996 Jitsuyo Shinan Toroku Koho 1996-2011
Kokai Jitsuyo Shinan Koho 1971-2011 Toroku Jitsuyo Shinan Koho 1994-2011

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
JSTPlus/JMEDPlus/JST7580(JDreamII)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JP 4040666 B2 (Mochida Pharmaceutical Co., Ltd.), 30 January 2006 (30.01.2006), paragraphs [0147], [0148] & US 2006/0068445 A1 & EP 1746104 A1 & WO 2005/108429 A1 & CA 2566101 A	1-4
Y	Hideaki SHIRAI et al., "Tokutei Kenko Shinsa ni Okeru Kentai no Toriatsukai Part 2: Kentai Saishu kara Enshin Bunri made no Hochi Jikan", Japanese Journal of Clinical Laboratory Automation, 01 September 2007 (01.09.2007), vol.39, no.4, page 426	1-4

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
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"P" document published prior to the international filing date but later than the priority date claimed
"T" 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
"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
"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
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 March, 2011 (25.03.11)	05 April, 2011 (05.04.11)
Name and mailing address of the ISA/ Japanese Patent Office Facsimile No.	Authorized officer Telephone No.

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2011/051778

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Yasushi TAKAGI, "Koredake wa Shitte Okitai Kensa no Point (sixth series) Rinsho Kensa Soron Kentai Saishu Teishutsu-ji no Chuiten", Medicina, 30 October 1999 (30.10.1999), vol.36, no.11, pages 16 to 19	1-4
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专利名称(译)	人sCD14-ST分析方法		
公开(公告)号	EP2530468A4	公开(公告)日	2013-08-21
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优先权	2010018340 2010-01-29 JP		
其他公开文献	EP2530468B8 EP2530468B1 EP2530468A1		
外部链接	Espacenet		

摘要(译)

提供了一种能够在使用全血样品时精确测量人sCD14-ST的分析方法。在分析方法中，在收集样品的6小时内分析全血样品中的人sCD14-ST。