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(54) **METHOD OF ASSAYING ENDOCRINE SUBSTANCE IN SPECIMEN**

VERFAHREN ZUM TESTEN ENDOKRINER SUBSTANZ IN PROBEN

PROCÉDÉ D'ANALYSE DE SUBSTANCE ENDOCRINE DANS UN SPÉCIMEN

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Description

TECHNICAL FIELD

5 **[0001]** The present invention relates to a method for the measurement of an endocrine substance in an analyte, in particular a feline blood analyte and so on. More particularly, the present invention relates to a method for the measurement of the endocrine substance such as insulin and so on in an analyte such as a feline blood analyte or the like in order to diagnose and treat diseases including, for example, feline diabetes and so on.

10 BACKGROUND TECHNOLOGY

[0002] For the diagnosis and treatment of diseases, it is required to identify the diseases, for example, by identifying the kind of substances causing the diseases or associated therewith and measuring a quantitative variation in such substances or investigating susceptibility thereto. Among those diseases, metabolic syndromes are becoming a great social issue because they may be caused by a change of life style including a habit of eating food, that is, a habit of eating a high caloric food with a high content of proteins, carbohydrates, and/or fats...and, in addition thereto, by a lack of exercise due to a change of life environment. Particularly, diabetes is causing a serious problem because a number of patients with diabetes mellitus as well as potent people with a risk of causing diabetes are increasing rapidly. In order to diagnose and treat human diabetes mellitus, an amount of human insulin, an endocrine substance of diabetes-associated substances, in blood has to be measured, and the measurement of amounts of human insulin is conducted as a routine check-up at hospitals, clinics and so on.

[0003] Recently, in addition to the human being, pet animals such as cats, dogs and so on are causing to occur diseases corresponding to metabolic syndromes like the human being, and a number of pet animals with such diseases is also increasing rapidly due to a change in raising circumstances. For example, diabetes mellitus and so on is increasing among pet animals, which may be caused by feeding food with a high content of proteins, carbohydrates, and/or fats... and high calories, such as pet food or the like, and by a lack of exercise due to raising in a narrow room space. It is needless to say that an amount of feline insulin in a feline blood analyze has to be measured in order to diagnose and treat feline diabetes mellitus.

[0004] Now, one of the issues is, however, that currently there is no practical method for measuring feline insulin itself in a feline blood analyte. Therefore, feline insulin is currently measured in accordance with a method for the measurement of human insulin (see Patent Publication 1 for example). As a matter of course, this human insulin measurement method cannot measure an accurate amount of the feline insulin because feline insulin is not used as a standard substance so that a difference in reactivity with an antibody can be observed due to a difference in the amino acid sequence between feline insulin and human insulin. Moreover, the current human insulin measurement method has the big problem because it uses a radioactive substance so that there is a concern about a pollution of environment due to radioactive contamination and a disposal of the radioactive substances after the use for measurement. In addition, as the feline insulin cannot be measured directly by this current system, the diagnosis of feline diabetes is conducted simply by a close-up of conditions and measurement of a sugar level of blood and urine. In such a current situation, it is practically impossible to diagnose whether the feline diabetes is caused to occur due to a problem with the function of the organ or a problem with the function of an insulin carrier or an insulin receptor in blood. Therefore, the big problem encountered with the current diagnosis and treatment of feline diabetes is that an appropriate treatment of feline diabetes cannot be carried out because an accurate cause of the feline diabetes cannot be determined. Particularly, in order to appropriately diagnose and treat diseases of animals, the method for directly measuring endocrine substances associated with such diseases has to be determined.

[0005] In this description, for brevity of explanation of the subject matter of the present invention, feline is illustrated as an object to be analyzed and feline insulin is illustrated as an endocrine substance in an analyte of the object to be analyzed. It is to be understood, however, that the object to be analyzed and the endocrine substance are not interpreted as being limited to the feline and the feline insulin and it is needless to say that any object to be analyzed and endocrine substance can be applied to the present invention as long as they are appropriate for the subject matter of the present invention.

[0006] As the method for detecting an antigenic substance in a body fluid such as blood serum and so on or measuring concentrations of the antigenic substance, an immunological measurement is recently being applied increasingly. These methods are well known to the art, and an antigenic substance to be measured can be detected or a concentration of the antigenic substance can be measured, for instance, by labeling the antigenic substance or an antibody with a radioactive substance, forming a complex between the antigenic substance and the antibody, and measuring the labeled component.

(Patent Publication #1) Japanese Patent Publication No. H11-326,322.

[0007] A. Casesnoves et al., "influence of anti-insulin antibodies on insulin immunoassays in the antoimmune insulin

syndrome", Annals of Clinical Biochemistry, Vol. 35, No. 6, pages 768 to 774 discloses a method for measuring a total Insulin by, as pre-treatment process, adding acid and ethanol to a sample, centrifuging the sample and then measuring the Insulin in the resulting supernatant.

5 [0008] H. Arnqvist et al., "Free and total insulin as determined after precipitation with polyethylene glycol, analytical characteristics and effects of sample handling and storage", Clinical Chemistry, Vol. 33, No. 1, 1987, pages 93-96 relates to a method for measuring a total insulin by, as pre-treatment processes, adding acid to a sample, adding polyethylene glycol (PEG) thereto, centrifuging the sample and then measuring the insulin in the resulting supernatant.

[0009] US 4,855,242 and M. Hoenig et al., "Beta cell and insulin antibodies in treated and untreated diabetic cats", Veterinary Immunology and Immunopathology, Vol. 77, No. 1-2, pages 93 to 102 relate to methods for measuring an autoantibody to insulin: These prior art references are silent about any pre-treatment process at all.

10 [0010] According to US 2005/026826 A, it is essential to use a series of steps to collect all insulin in a sample for measuring the Insulin quantitatively.

SUMMARY OF INVENTION

15 [0011] As a result of extensive studies and review on methods for measuring endocrine substances of animals, it has been found by the present inventors that an endocrine substance such as feline Insulin or the like present in an analyte of an animal such as feline or the like can be measured in the analyte treated previously to remove an autoantibody with the endocrine substance bound thereto from the analyte. The present invention has been completed from this finding.

20 [0012] Therefore, the present invention has the object to provide a method for measuring an endocrine substance in an analyte, which comprises measuring the endocrine substance such as feline insulin in a blood analyte of an animal such as feline or the like accurately and rapidly by removing an autoantibody bound to the endocrine substance such as feline insulin or the like.

25 [0013] The present invention has another object to provide a method for removing an autoantibody which comprises removing the autoantibody bound to the endocrine substance from the analyte by adding an acid to the analyte.

[0014] In order to achieve the above objects, the present invention provides a method for the pre-treatment of feline blood sample, which comprises removing the autoantibody bound to the endocrine substance from the analyte.

30 [0015] In a further aspect, the present invention provides a method for removal of the autoantibody, which comprises separating the autoantibody bound to the endocrine substance in the analyte and removing the autoantibody bound thereto from the analyte by adding an acid to the analyte.

[0016] The method according to the present invention is defined by the steps mentioned in claim 1.

35 [0017] The method for the measurement of substances such as human insulin has so far been applied to measurement of endocrine substances such as feline insulin and so on in an analyte of an animal such as feline and so on. It is now to be noted here that the present invention provides a method that can directly measure an endocrine substance such as feline insulin or the like accurately and rapidly. Further, the endocrine substance measurement method according to the present invention is provided with the great advantages in terms of handling with great conciseness because it can utilize an enzyme immune measurement method ordinarily utilized in the art involved without using any radioactive substance.

40 BRIEF DESCRIPTION OF THE DRAWINGS

[0018]

45 Fig. 1 is a flow chart showing a method for determining an anti-insulin autoantibody in a feline blood sample.

Fig. 2 is a graph showing the result of measurement of anti-feline insulin autoantibody (an IgG-insulin complex).

Fig. 3 is a graph showing the result of measurement of a value of an insulin-bound insulin autoantibody and a value of an anti-mouse-IgG antibody.

Fig. 4 is a graph showing the result of measurement by a feline serum-insulin-sepharose chromatography.

Fig. 5 is a graph showing the result of measurement of a value of an insulin autoantibody.

50 Fig. 6 is a graph showing a value of an antibody to a mouse rheumatoid factor (RF).

Fig. 7 is a graph showing the result of measurement of a strength of an anti-feline insulin of a gas-chromatography eluate.

Fig. 8 is a graph showing a standard curve of feline insulin.

55 Fig. 9 is a graph showing a correlation of a standard feline insulin with a standard rat insulin.

BEST MODES FOR CARRYING OUT THE INVENTION

[0019] In order to diagnose the presence or absence of a certain disease and treat it, endocrine substances associated

with the disease are required to be measured. For example, in order to diagnose human diabetes mellitus, it is needed to measure an amount of human insulin, an endocrine substance that is closely associated with the human diabetes mellitus. Likewise, it is essential to measure an amount of feline insulin in a blood analyte of an animal such as feline for diagnosing and treating feline diabetes mellitus.

5 **[0020]** An autoantibody to an endocrine substance such as insulin or the like may be secreted in blood of an animal such as feline or the like, and a portion of the autoantibody is present in such a state that it is bound to the endocrine substance. In order to measure the endocrine substance in a blood analyte, it is needed to remove the autoantibody bound to the endocrine substance from the blood analyte before measuring the collected blood analyte. In other words, the endocrine substance may be measured by using blood as an analyte in which the endocrine substance is separated from the autoantibody bound to the endocrine substance. At this end, for example, an anti-insulin feline antibody and an anti-mouse IgG (rheumatoid factor) antibody by measuring a value of an insulin-bound insulin autoantibody and a value of anti-mouse IgG (rheumatoid factor) autoantibody.

10 **[0021]** On the other hand, a portion of the autoantibody to the endocrine substance such as feline insulin or the like secreted in blood is present in a state in which it is not bound to the endocrine substance. In order to measure the non-bound autoantibody, the autoantibody is adsorbed on an appropriate adsorbent such as a chromatography column or the like and a value of the autoantibody non-bound to the endocrine substance in an eluate from the adsorbent is measured.

15 **[0022]** Therefore, the measurement of the value of the autoantibody to the endocrine substance such as feline insulin or the like can be applied to, for example, a method for selection of the method for the treatment of a feline with a high blood sugar level or a method for determination of the effect of the treatment thereof or a method for selection of the method for the treatment of a feline with an autoimmune disease or the like or a method for determination of the effect of the treatment thereof.

20 **[0023]** In order to apply such methods, it is needed to measure an amount of insulin of an individual object to be measured, such as feline or the like. For instance, the measurement of an amount of insulin of a feline individual allows a diagnosis of the kind and type of diseases such as diabetes mellitus or the like. It is to be noted here, however, that the feline insulin has to be free from other interfering substances in a blood analyte in order to measure an accurate amount of the insulin of the feline individual. At this end, it is required to measure a value of the autoantibody of insulin to determine whether a substance bound to insulin is present or not.

25 **[0024]** Further, in the event that the autoantibody to the endocrine substance such as insulin or the like is detected, the autoantibody thereto has to be removed. In other words, the analyte of an object to be analyzed is to be pre-treated to remove the autoantibody from the analyte. Moreover, it is preferred to confirm the autoantibody to insulin has already been removed from the analyte by measuring a value of the autoantibody to insulin. After the confirmation of the removal of the autoantibody thereto, an amount of insulin in a feline blood analyte is then measured.

30 **[0025]** Moreover, the present invention can be applied to a selection of an insulin preparation and a route of administration of the insulin preparation by measuring the anti-insulin autoantibody non-bound to insulin before administration of the insulin preparation. In addition, it can also be applied to a determination of diagnosis and treatment effects of insulin preparations by measuring an insulin autoantibody bound to insulin of a feline individual.

35 **[0026]** Furthermore, the measurement of an antibody value of the autoantibody to insulin and an antibody value of a mouse antibody to rheumatoid factor as a marker for an autoimmune disease can also be applied to the determination of effects of diagnosis and treatment of the autoimmune diseases.

40 **[0027]** The analyte to be used for the present invention is a feline blood sample. The blood analyte may be total blood or serum or plasma separated from blood, and such serum or plasma analytes may be prepared by separation from the blood analyte by usual techniques.

45 **[0028]** The endocrine substance to be used as an object to be measured in accordance with the present invention may include, for example, substances which are produced in the organs and tissues, and secreted into body fluids of pet animals such as feline and so on and which act as a marker useful for diagnosis of a morbid state of a certain disease and so on or for treatment of the disease. In the case of feline diabetes mellitus, the endocrine substance may include feline insulin useful for diagnosis and treatment of feline diabetes mellitus.

50 **[0029]** As a result of review on the possibility of measuring the endocrine substance derived from an animal species as the object to be measured in comparison of the amino acid sequence of the endocrine substance with an amino acid sequence of the endocrine substance of another animal species, it was found that feline insulin can be measured by utilizing insulin of rat as another animal species. In this case, the measurement can be conducted by sandwich method using antibodies to cat insulin A chain and rat insulin B chain.

55 **[0030]** The amino acid sequences of feline insulin and rat insulin are as follows:

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Table 1 (Cat Insulin A chain):

Animal	1	6	11	16	21
Cat	GIVEQ (SQ ID #1)	CCASV (SQ ID #2)	CSLYQ (SQ ID #3)	LEHYC (SQ ID #4)	N (SQ ID #5)
Rat I	GIVDQ (SQ ID #6)	CCTSI (SQ ID #7)	CSLYQ (SQ ID #8)	LENYC (SQ ID #9)	N (SQ ID #10)
Rat II	GIVDQ (SQ ID #11)	CCTSI (SQ ID #12)	CSLYQ (SQ ID #13)	LENYC (SQ ID #14)	N (SQ ID #15)

Table 2 (Rat Insulin B chain):

Animal	1	6	11	16	21	26
Cat	FVNQH (SQ ID #16)	LCGSH (SQ ID #17)	LVEAL (SQ ID #18)	VLVCG (SQ ID #19)	ERGFF (SQ ID #20)	VTRKA (SQ ID #21)
Rat I	FVKQH (SQ ID #22)	LCGPH (SQ ID #23)	LVEAL (SQ ID #24)	VLVCG (SQ ID #25)	ERGFF (SQ ID #26)	VTRKS (SQ ID #27)
Rat II	FVKQH (SQ ID #28)	LCGSH (SQ ID #29)	LVEAL (SQ ID #30)	VLVCG (SQ ID #31)	ERGFF (SQ ID #32)	VTPMS (SQ ID #33)

[0031] The reference symbols referred in the above tables are: A is alanine; C is cysteine; D is aspartic acid; E is glutamic acid; F is phenylalanine; G is glycine; H is histidine; I is isoleucine; K is lysine; L is leucine; M is methionine; N is asparagine; P is proline; Q is glutamine; R is arginine; S is serine; T is threonine; V is valine; and Y is tyrosine.

[0032] As a result of comparison between the amino acid sequences of feline insulin and rat insulin, it can be presumed that feline insulin can be measured by utilizing rat insulin. Therefore, it can be considered that feline insulin can be measured using a rat insulin ELISA kit.

[0033] As described above, for instance, an autoantibody to the endocrine substance is produced in blood of an animal such as feline and so on, and the autoantibody thereto is present in such a state that all the autoantibody is not bound to the endocrine substance while a portion of the autoantibody is bound thereto. The presence of the autoantibody bound to the endocrine substance in a blood analyte makes it difficult to measure the endocrine substance itself in the analyte. Therefore, in order to measure an accurate amount of the endocrine substance in the analyte, the endocrine substance has to be present in the analyte in such a state that the endocrine substance is not bound to the autoantibody. At this end, it is needed, accordingly, to detect the presence or absence of the autoantibody bound to the endocrine substance and measure a value of the autoantibody to the endocrine substance. If the autoantibody bound to the endocrine substance were detected in the analyte, the autoantibody bound to the endocrine substance has to be removed from the analyte.

[0034] First, a description will be made regarding the method for the detection of the presence of the substance bound to the endocrine substance by taking ELISA method conventionally used in the art as an example. In this example, feline serum was measured by ELISA using insulin-bound insulin autoantibody and anti-mouse IgG autoantibody.

[0035] The insulin-bound autoantibody to insulin may be measured, for example, by adding a feline serum analyte to an immobilized anti-insulin antibody on a plate and reacting the anti-insulin antibody with an antigen in the analyte by an antigen-antibody reaction, and reacting the antibody-antigen complex with a labeled enzyme-conjugated goat anti-feline IgG. After the completion of the reaction, an enzyme substrate is allowed to develop a color and the color development is measured at a given absorbency. On the other hand, the measurement of the anti-mouse IgG autoantibody can also be carried out in substantially the same manner as that of the insulin-bound insulin autoantibody.

[0036] As the enzyme used for labeling in ELISA, there may be used generally any enzyme conventionally used for ELISA, and it may include, but may not be limited to, peroxidase, alkali phosphatase, β -D-galactosidase, glucose oxidase, luciferase, esterase, β -D-glucuronidase, and so on. Among these enzymes, peroxidase or alkali phosphatase is preferred from the point of view to achieve a highly sensitive and stable detection. On the other hand, the substrate may appropriately be selected according to the enzyme to be used and include, but may not be limited to, 3,3',5,5'-tetramethylbenzidine or the like in the case of peroxidase, and sodium p-nitro phenyl phosphate or the like in the case of alkali phosphatase.

[0037] The detection and quantitative measurement of a product from the above enzyme may be conducted by measuring an absorbency of the product. For instance, the reaction product of 3,3',5,5'-tetramethylbenzidine as the substrate

can be measured at absorbency of 450 nm.

[0038] The purification of the endocrine substance will then be described by taking feline insulin as an example. For instance, feline insulin in a feline pancreas analyte can be purified by adding a hydrochloric acid solution to the analyte to form a homogenate and purifying the resulting homogenate by usual purification procedures. Feline insulin can be purified in accordance with the known purification procedures for human insulin.

[0039] More specifically, feline insulin can be purified, for example, by adding a hydrochloric acid solution to an analyte from the feline pancreas or the like to form a homogenate, separating a supernatant by centrifugation of the resulting homogenate, and subjecting the resulting supernatant to conventional purification procedures such as chromatography or the like. For this purification, it is preferred to use a carrier for purification of biopolymers such as proteins or nucleic acids, e.g., fractogel for example. This carrier is particularly preferred for purification of a large amount of proteins and antibodies because it has a higher capacity for binding proteins and so on, as compared with usual carriers for purification of proteins and so on.

[0040] The eluate obtained in the manner as described above can further be subjected to conventional purification procedures such as chromatography or the like and feline insulin can be purified by separating and collecting a fraction having a given peak from the fractions of the eluate. For the chromatography to be used herein, there may be preferably used, for example, a high-performance reverse-phase chromatography column suitable for purification of biomolecules.

[0041] The purified feline insulin may then be freeze-dried after confirmation of its molecular weight by means of SDS-PAGE or the like and weighed for use as a first standard product of a kit for measurement of feline insulin.

[0042] The anti-insulin autoantibody in a feline blood analyte can be confirmed by means of the method as shown in Fig. 1. As a result of SDS-PAGE electrophoresis of the eluate fraction (2ME+), two bands were confirmed nearby at the molecular weights of approximately 50,000 and 25,000. This is confirmed that the fraction comprises an IgG-type antibody.

[0043] A description will then be made regarding the measurement of an analyte such as a feline serum or plasma analyte. As described above, feline insulin can be measured using a rat insulin ELISA kit on the basis of the presumption from a comparison between the amino acid sequences of feline insulin and rat insulin. In the event that the rat insulin ELISA kit reacts with feline insulin extracted directly from feline pancreas but it does not react with feline insulin in the serum or plasma analyte, it may be considered that the reaction of the rat insulin ELISA kit with the antibody is obstructed by the autoantibody to feline insulin due to its binding to the feline insulin in the blood analyte. This is considered to happen because the autoantibody to feline insulin is present in feline blood.

[0044] It is needed therefore to confirm the presence of the autoantibody to insulin in a feline serum or plasma analyte. The insulin autoantibody can be confirmed by using a monoclonal antibody to rat insulin in accordance with the procedures as have been described above.

[0045] In the event that the presence of the autoantibody bound to the endocrine substance such as insulin or the like in the analyte is confirmed, the autoantibody bound to the endocrine substance is to be removed from the analyte. After the autoantibody bound to the endocrine substance has been removed from the analyte, the endocrine substance can be extracted and then measured accurately and rapidly.

[0046] A description will now turn to the procedures for removing the autoantibody bound to the endocrine substance from a blood analyte in which the autoantibody bound thereto is present.

[0047] In order to remove the autoantibody bound to the endocrine substance from the blood analyte, first, the blood analyte is made acidic to terminate an antigen-antibody reaction for the binding of the endocrine substance to the autoantibody, thereby allowing a separation of the endocrine substance from the antibody. The analyte may be acidified with an acidic substance including, but being not limited to, an inorganic acid such as hydrochloric acid or sulfuric acid or an organic acid such as acetic acid. Among the acids, the inorganic acid is preferred and, more specifically, it is preferred to use 0.5 M to 2 M hydrochloric acid or 0.25 M to 1 M sulfuric acid. An amount of the acid to be added to the analyte may be from a 1/50-fold to 10-fold amount, preferably a 1/20-fold to one-fold amount, with respect to the total amount of the analyte, although the amount of the acid is not restricted to a particular one and it may vary with the amount of the analyte.

[0048] After the binding of the autoantibody to the endocrine substance by means of the antigen-antibody reaction is terminated by making the analyte acidic in the manner as described above, the analyte is then added with a protein-precipitation agent to precipitate the autoantibody. The precipitation of the autoantibody bound to the endocrine substance permits a separation of the autoantibody non-bound to the endocrine substance. The protein-precipitation agent may include, but may not be limited to, saturated ammonium sulfate, saturated sodium sulfate and polyethylene glycol. Among the protein-precipitation agent, there may be preferably used saturated ammonium sulfate in a concentration of approximately 30% to 60%, preferably approximately 33% to 55%, or polyethylene glycol 3000 - 6000 in a concentration of approximately 0.5% to 20%. An amount of the protein-precipitation agent to be added is not limited to a particular one as long as it can precipitate the above protein and may be from approximately 30% to 70%, preferably from approximately 40% to 60% as a final concentration of the protein-precipitation agent. Further, the pH of the analyte may be in the range of approximately pH 3.0 to pH 2.0.

[0049] After the addition of protein-precipitation agent, the addition of an ethanolic solution to the analyte can recover

the endocrine substance co-precipitated with the autoantibody bound to the endocrine substance. The recovery of the endocrine substance can lead to a simultaneous separation of the autoantibody from the analyte. The ethanolic solution may be in a concentration of from approximately 60% to 95%, preferably from approximately 65% to 80%. The final concentration of ethanol may be from approximately 60% to 80%, preferably from approximately 65% to 75%. Further, the ethanolic solution may be added as a mixture with an acid such as hydrochloric acid.

[0050] Undissolved matters are then to be separated from the analyte prepared in the manner as described above. The separation may be effected to separate the undissolved matters (e.g., auto antibodies including the autoantibody bound to the endocrine substance and so on) from a supernatant centrifuged or filtrate filtered by conventional separation procedures including, for example, centrifugation or filtering under given conditions. The resulting supernatant or filtrate can be used as an analyte to be measured. Where the analyte so pre-treated in the above-mentioned manner to remove the autoantibody bound to the endocrine substance therefrom is used as an analyte for analysis, the endocrine substance as the object to be measured present in the analyte can be measured with high accuracy and rapidity.

[0051] It is preferred to re-confirm whether the autoantibody bound to the endocrine substance is yet left in the pre-treated analyte from which the autoantibody bound to the endocrine substance has been removed by re-measuring a value of the autoantibody. In the event where the autoantibody bound thereto still remains in the once pre-treated analyte, the remaining autoantibody bound to the endocrine substance is to be removed again from the analyte by repeating the procedures for removing the autoantibody. Once it has been confirmed that no antibody bound to the endocrine substance remained in the analyte, the endocrine substance is detected by measuring the endocrine substance in the analyte.

[0052] In accordance with the present invention, the endocrine substance such as insulin and so on present in the pre-treated analyte can be measured and detected by conventional techniques including, but being not limited to, sandwich ELISA method customarily used in the art.

[0053] More specifically, for instance, a monoclonal antibody to rat insulin is immobilized on a plate and a biotin-bound anti-insulin monoclonal antibody is added to the plate. Then, a feline blood analyte (a pre-treated analyte) is added to the plate to cause an antigen-antibody reaction with an antibody corresponding to the endocrine substance such as insulin or the like in the analyte to form an antigen-antibody complex. Thereafter, avidin bound with an enzyme such as peroxidase or the like is added to the resulting complex and a color is developed with the above enzyme substrate, and the endocrine substance such as insulin or the like as an object for measurement is measured at a given absorbency.

[0054] Therefore, the present invention can briefly and accurately measure the endocrine substance such as feline insulin and so on in the blood analyte of an animal such as feline and so on in a quantitative manner by pre-treating the analyte in accordance with the pre-treatment method according to the present invention.

[0055] The present invention further allows a diagnosis and treatment of diseases associated with the endocrine substance, such as diabetes mellitus and so on, by measuring a value of the autoantibody to the endocrine substance such as feline insulin in an analyte such as a feline blood analyte or the like by means of the autoantibody value measurement method. A combination of the autoantibody value measurement method with the endocrine substance measurement method enables a diagnosis and treatment of diseases associated with the endocrine substances.

[0056] In addition, the present invention provides a kit for use in measurement of the endocrine substance, which is capable of measuring the endocrine substances including, but being not limited to, feline insulin and so on. A kit for use in measurement of feline insulin may comprise, for example, an antibody-immobilized plate, a feline insulin standard solution, a buffer, biotin-bound antibody to insulin, avidin-bound peroxidase, a color developing solution (e.g., TMB), a reaction terminator (e.g., sulfuric acid), a rinsing liquid and so on.

[0057] The following is a description regarding working examples in accordance with the present invention. It is to be noted here, however, that the present invention is not interpreted in any respect as being restricted to the example which follow below and that the examples are described hereinafter merely as a specific illustration of the present invention.

EXAMPLES

Example 1: Purification of Feline Insulin

[0058] To a feline pancreas was added 40 ml of a mixture solution of 750 ml of ethanol, 15 ml of concentrated sulfuric acid and 235 ml of DW (4 ml per gram of the tissue), the mixture was homogenized and stirred at 37°C for 2 hours and centrifuged at 10,000 x g for 20 minutes. The resulting supernatant was collected and evaporated to a half volume. The concentrated supernatant was again centrifuged at 10,000 x g for 20 minutes. The resulting supernatant was collected and eluted with 50 mM aqueous ammonia solution through fractogel (EMD-SO3-650, Merck). The resulting eluate was adjusted to pH 2.0 with TFA and purified through a reverse-phase chromatography column (RESOURCE RPC; Buffer A: 0.1% TFA (pH 2.0); Buffer B: 0.1% TFA + 60% AcCN; Linear Gradient: A → B; Abs. 215 nm). The resulting fraction having a peak at nearby 50% was collected as an insulin fraction and stored as a purified feline insulin solution.

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Example 2: Test for confirmation of Reactivity of Antibody with Feline Insulin

[0059] The purified feline insulin solution prepared by Example 1 was subjected to a qualitative test by means of dot blot method as will be described hereinafter. A piece of nitro cellulose membrane, 0.5 cm long and 1.5 cm wide, was blotted with 3 μ l of physiological saline on the left side, 1.5 μ l of purified feline insulin solution at the center, and 3 μ l of the supernatant of the feline pancreas homogenate on the right side. The membrane was blocked with 0.05% Block Ace® and then allowed to stand at room temperature for 2 hours. Thereafter, the membrane was washed three times with a mixture of 0.05% Tween® 20 and 10 mM PBS and then reacted with an anti-insulin monoclonal (D3E7) antibody-biotin conjugate (rat insulin ELISA kit) at 4 °C for 15 to 18 hours. After the membrane was re-washed three times with a mixture of 0.05% Tween® 20 and 10 mM PBS, avidin-HRP (horse radish peroxidase) was added and reacted at room temperature for 30 minutes, followed by re-washing three times with a mixture of 0.05% Tween® 20 and 10 mM PBS. The membrane was then subjected to a color development with a diethylene glycol solution of 4-chloro-1-naphthol at room temperature for 90 minutes. The test results indicated that physiological saline caused no reaction, while the purified feline insulin solution and the feline pancreas homogenate supernatant caused a positive reaction.

Example 3:

Preparation of Feline Serum/Plasma Samples

[0060] Blood samples were collected from feline at 10 minutes each before and after feeding of standard pet food, and serum or plasma was separated by conventional procedures and used as a feline serum sample and a feline plasma sample, respectively.

Measurement of Feline Serum/Plasma Samples

[0061] From a result of a comparison of the amino acid sequence of feline insulin with the amino acid sequence of rat insulin (See Table 1: Insulin A chain; Table 2: Insulin B chain), it is presumed that feline insulin can be measured using a rat insulin ELISA kit.

[0062] As a control, a feline blood sample without pre-treatment was measured for an insulin concentration (ng/ml), a C-peptide concentration (pg/ml), and albumin concentration (mg/ml) (TIA method). The test results are shown in Table 3-1. Insulin was measured using Lbis® insulin kit, C-peptide using Lbis® C-peptide kit, and albumin using Lbis® albumin kit. On the other hand, the feline sample pre-treated in the manner as will be described in detail hereinafter was measured for an insulin concentration (ng/ml) using Lbis® insulin kit. The results are shown in Table 3-1:

Sample ID#	State	Insulin Concentration (ng/ml)	C-Peptide Concentration (pg/ml)	Albumin Concentration (mg/ml)
A	Before Meal	Not Detected	Not Detected	89.3
	After Meal	Not Detected	Not Detected	84.8
B	Before Meal	Not Detected	Not Detected	78.5
	After Meal	Not Detected	Not Detected	87.5
C	Before Meal	Not Detected	Not Detected	85.5
	After Meal	Not Detected	Not Detected	56.5

Table 3-2:

Sample ID#	State	Insulin Concentration (ng/ml)
B	Before Meal	1.69
	After Meal	2.56
C	Before Meal	3.60
	After Meal	5.82

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Example 4: Measurement of Autoantibody to Feline Insulin (IgG-Insulin complex)

[0063] To each well of a 96-well plate immobilized with a monoclonal antibody to rat insulin was added 50 μ l of a feline serum sample which was diluted at seven dilution steps from a 10-fold dilution, and the plate was allowed to react at room temperature for 1 hour followed by washing with a mixture of 0.05% Tween® 20 and 10 mM PBS. To each well of the plate prepared so was then added 50 μ l of HRP-bound goat anti-feline IgG, and the plate was reacted at room temperature for 1 hour followed by washing with a mixture of 0.05% Tween® 20 and 10 mM PBS. To each well was added TMB as a color developer in the amount of 50 μ l and the plate was reacted at room temperature for 10 minutes, followed by addition of 50 μ l of 1 M sulfuric acid to terminate the reaction. The wells were measured for absorbency at 450 nm (620 nm) and the results are shown in Table 4 and Fig. 2.

Table 4:

Dilution Rate	Absorbency 450 (Δ 620) nm (n=2)		
	Sample C (Before Meal)	Sample C (After Meal)	Sample C (Before Meal), Pre-treated
x 10	2.439	2.424	0.102
x 10 ²	1.605	1.693	0.087
x 10 ³	0.371	0.427	0.052
x 10 ⁴	0.079	0.096	0.033
x 10 ⁵	0.037	0.039	0.029
x 10 ⁶	0.020	0.016	0.026
x 10 ⁷	0.026	0.030	0.027
Blank	0.018	0.018	0.015

Example 5: Measurement of Values of Insulin-Bound Insulin Autoantibody and Anti-Mouse-IgG Antibody in non-pre-treated blood samples.

[0064] Each well of a plate was coated with anti-insulin antibody (Plate A) and with mouse IgG (Plate B). To each well of the plates was added 50 μ l of a non-pretreated feline blood sample (10 samples), and the plates were allowed to stand at room temperature for 1 hour, followed by three-time washing with a buffer solution (10 mM PBS). Then, 50 μ l of HRP-bound goat anti-feline IgG was added to each well and the plate was then allowed to stand at room temperature for 1 hour, followed by three-time washing with 10 mM PBS. Then, 50 μ l of 3,3',5,5'-tetramethyl benzidine (TMB) was added to each well and thereafter 1 M sulfuric acid was added by 50 μ l to each well to terminate the reaction. The plate was then measured for absorbency at 450/620 nm. The results are shown in Table 5 and Fig. 3.

Example 6: Measurement of Values of Insulin-Bound Insulin Autoantibody and Anti-Mouse-TgG Antibody in pretreated blood samples corresponding to those of Example 5.

[0065] In substantially the same manner as Example 5, each well of a plate was coated with anti-insulin antibody (Plate A) and with mouse IgG (Plate B). To each well of the plates was added 50 μ l of a pretreated feline blood sample (10 samples), and the plates were allowed to stand at room temperature for 1 hour, followed by three-time washing with a buffer solution (10 mM PBS). Then, 50 μ l of HRP-bound goat anti-feline IgG was added to each well and the plate was then allowed to stand at room temperature for 1 hour, followed by three-time washing with 10 mM PBS. Then, 50 μ l of 3,3',5,5'-tetramethyl benzidine (TMB) was added to each well and thereafter 1 M sulfuric acid was added by 50 μ l to each well to terminate the reaction. The plate was then measured for absorbency at 450/620 nm. The results are shown in Table 5 and Fig. 3.

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Table 5:

Sample ID#	Plate A		Plate B (*NT=Not Tested)	
	Not Pre-treated	Pre-treated	Not Pre-treated	Pre-treated
1	1.618	0.070	0.485	NT
2	2.184	0.086	NT	NT
3	2.701	0.084	1.661	NT
4	2.533	0.069	NT	0.019
5	1.630	0.067	NT	NT
6	2.180	0.106	NT	NT
7	0.982	0.114	0.348	0.020
8	1.871	0.093	NT	NT
9	2.616	0.089	1.414	NT
10	1.840	0.098	NT	NT
(n = 2)				

Example 7: Measurement by Feline Serum Insulin-Sepharose Chromatography

[0066] Three samples used in Examples 5 and 6 (Sample #5 (as referred to as Sample 264 in the drawing); Sample #7 (as referred to as Sample R-1 in the drawing); and Sample #8 (as referred to as Sample R-2 in the drawing)) were used for measurement.

[0067] Each sample (10 µl) was diluted with buffer (10 mM PBS) to 10 ml. The resulting solution was injected into a bovine insulin-bound Sepharose column and chromatography was carried out using 10 mM PBS as a binding buffer solution and 0.1 M glycine-HCl solution (pH 2.5) as an eluting buffer solution at a rate of 1 ml/cycle. A pass fraction and an eluate fraction was each collected and measured for a value of anti-insulin antibody and RF-IgG after a pH adjustment to a neutral range. The results are shown in Table 6-1 (Sample #5), Table 6-2 (Sample #7) and Table 6-3 (Sample #8). Table 4 is a graph showing the above results. In Fig. 4, Sample #5 is represented as Sample 264, Sample #7 as Sample R-1, and Sample #8 as Sample R-2.

Table 6-1:

Fraction #	1	2	3	4	5
280 nm	0.013	0.028	0.772	1.565	1.371
Fraction #	6	7	8	9	10
280 nm	0.754	0.410	0.228	0.134	0.083
Fraction #	11	12	13	14	15
280 nm	0.054	0.037	0.031	0.017	0.020
Fraction #	16	17	18	19	20
280 nm	0.019	0.017	0.024	0.090	0.052
Fraction #	21	22			
280 nm	0.033	0.021			
(Fraction #4 & #5: Pass solution pool; Fraction #19: Eluate pool)					

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Table 6-2:

Fraction #	1	2	3	4	5
280 nm	0.012	0.190	1.751	2.113	0.953
Fraction #	6	7	8	9	10
280 nm	0.332	0.141	0.079	0.056	0.042
Fraction #	11	12	13	14	15
280 nm	0.034	0.021	0.017	0.018	0.015
Fraction #	16	17	18	19	20
280 nm	0.020	0.076	0.052	0.026	0.015
(Fraction #4 & #5: Pass solution pool; Fraction #17: Eluate pool)					

Table 6-3:

Fraction #	1	2	3	4	5
280 nm	0.145	1.294	1.936	1.532	0.857
Fraction #	6	7	8	9	10
280 nm	0.475	0.235	0.143	0.100	0.075
Fraction #	11	12	13	14	15
280 nm	0.063	0.057	0.048	0.046	0.027
Fraction #	16	17	18	19	20
280 nm	0.114	3	1.968	1.981	1.135
Fraction #	21	22	23	24	25
280 nm	0.482	0.257	0.176	0.137	0.117
Fraction #	26	27	28	29	30
280 nm	0.100	0.087	0.084	0.076	0.073
Fraction #	31	32	33	34	35
280 nm	0.057	0.054	0.052	0.047	0.042
(Fraction #2 - #4: Pass solution pool; Fraction #17 - #20: Eluate pool)					

[0068] Figs. 5 and 6 show the results of measurement for a value of an insulin autoantibody and a value of mouse rheumatoid factor (RF), respectively, as a marker for an autoimmune disease. In Fig. 5, Sample #5 is represented as Sample 264, Sample #7 as Sample R-1, and Sample #8 as Sample R-2.

Example 8: Tests for Reactivity of Eluate with Insulin by Feline Serum Insulin-Sepharose Chromatography

[0069] Bovine insulin was added in the amount of 50 μ l to each well of a plate and the plate was allowed to stand at room temperature for two hours, followed by washing three times with a buffer solution. Then, 250 μ l of 25% Block Ace® was added to each well and the wells were washed three times with buffer, after immobilization. Thereafter, three samples used in Examples 5 and 6, i.s., feline serum-insulin-Sepharose eluates (Sample #5, #7 and #8), were diluted to a given concentration and added by 50 μ l to each well, and allowed to react followed by washing three times with buffer. Further, 50 μ l of goat anti-feline IgG-HRP was added to each well and the well was washed three times with buffer. The sample was then mixed with 50 μ l of TMB and allowed to stand at room temperature for 30 minutes, followed by adding 50 μ l of 1 M sulfuric acid to each well and measuring for absorbency at 450/620 nm. As a control, a sample (blank) with no eluate added thereto was used. The results are shown in Table 7-1 (Sample #5), Table 7-2 (Sample #7) and Table 7-3

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(Sample #8) and in Fig. 7. In Fig. 7, Sample #5 is represented as Sample 264, Sample #7 as Sample R-1, and Sample #8 as Sample R-2.

Table 7-1:

Dilution	Absorbency 450 ($\Delta 620$) nm		Mean
0.200	0.678	0.592	0.635
0.100	0.374	0.262	0.318
0.050	0.260	0.125	0.192
0.025	0.124	0.047	0.085
0.013	0.063	0.024	0.043
0.006	0.047	0.017	0.032
0.003	0.024	0.014	0.019
Blank	0.012	0.010	0.011

Table 7-2:

Dilution	Absorbency 450 ($\Delta 620$) nm		Mean
0.200	0.285	0.253	0.269
0.100	0.113	0.103	0.108
0.050	0.055	0.049	0.052
0.025	0.027	0.025	0.026
0.013	0.018	0.018	0.018
0.006	0.015	0.014	0.014
0.003	0.012	0.013	0.012
Blank	0.009	0.010	0.010

Table 7-3:

Dilution	Absorbency 450 ($\Delta 620$) nm		Mean
0.200	0.859	0.826	0.843
0.100	0.428	0.391	0.410
0.050	0.183	0.175	0.179
0.025	0.067	0.075	0.071
0.013	0.040	0.046	0.043
0.006	0.023	0.023	0.023
0.003	0.017	0.017	0.017
Blank	0.011	0.011	0.011

Example 9: Pre-treatment of Feline Blood Sample

[0070] To a feline blood sample (serum or plasma) was added a 1/10 (v/v) volume of 1 M hydrochloric acid, and the mixture was stirred. Then, a saturated ammonium sulfate solution (pH 2.5) was added to reach a final concentration of 50%, and the mixture was stirred at room temperature for 10 minutes, followed by adding an ethanol-10 mM HCl solution to reach a final ethanol concentration of 70%. The resulting mixture was stirred at room temperature for 10 minutes and

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centrifuged at 2,800 rpm at 4 °C for 15 minutes or filtered to remove undissolved matters. The resulting supernatant or filtrate was collected as a sample for use in measurement. This process removed the autoantibody to insulin from the feline blood sample.

5 Example 10: Recovery of Sample after Treatment

[0071] To a feline serum sample was added insulin extracted from feline pancreas and purified, and the sample was pre-treated in the same manner as above. A recovery rate of insulin was measured (see Table 8). The measured value was a value translated into a solution amount in unit of pg/ml.

10 Table 8:

Amount of Insulin Added	Measured Value	Recovered Amount	Recovery Rate (%)
0	777	-	-
250	978	201	80
500	1,159	382	76
1,000	1,535	758	76
(n = 5)			

15 Example 11: Comparison of Feline and Rat Insulin Standards

25 1. Preparation of standard feline Insulin Sample Solution

[0072] The feline insulin preparation purified from the pancreas was diluted with 10mM PBS to make up solutions with insulin concentration of 2,500pg/ml, 1,250pg/ml, 625pg/ml, 313pg/ml, 156pg/ml, 78pg/ml, and 39pg/ml. The rat insulin standard solutions were also prepared in the same manner.

30 2. Preparation of 96-Well Plate Immobilized with Monoclonal Antibody to Rat Insulin

[0073] A 96-well plate was washed three times with a mixture of 0.05% Tween® 20 and 10mM PBS, and 100µl of anti-rat insulin monoclonal antibody was added to each well of the plate to prepared a 96-well plate immobilized with the monoclonal antibody to rat insulin.

35 3. Method for Measurement

[0074] To each well of the 96-well plate immobilized with the monoclonal antibody to rat insulin were added 100µl of biotin-bound anti-rat insulin monoclonal antibody and 10µl of standard feline insulin solution or rat insulin standard solution, and the mixture was allowed to react at 20 to 25 °C for 2 hours. After washing the mixture three times with 10mM PBS containing 0.05% Tween® 20, 100µl of HRP-bound streptavidin solution was added to each well, and allowed to react at 20 to 25°C for 30minutes. After washing the well-plate for three times with the washing solution described above, 100µl of TMB as a color developer was added to each well and the plate was allowed to react at 20 to 25 °C for 30 minutes. Thereafter, 100µl of 1M sulfuric acid was added to terminate the reaction. Each well was measured for absorbency at 450/620nm, and the result is shown as a standard curve in Fig. 8. Further Fig. 9 shows a correlation of a standard feline insulin preparation with a standard rat insulin preparation.

40 Example 12: Measurement of Feline Blood Samples

[0075] In substantially the same manner as Example 11, to each well of a plate immobilized with the monoclonal antibody to rat insulin were added 100µl of biotin-bound anti-rat insulin monoclonal antibody and 10µl of standard solution prepared by serial dilution of feline standard insulin starting from 10,000pg/ml or feline sample serum (Sample #1-#10) with or without pretreatment. Then following the assay procedure described above, Assay values of feline insulin in the feline serum samples was calculated from their absorbency using the standard curve. The results are shown in Table 9-1 (standard insulin solution) and in Table 9-2 (feline serum).

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Table 9-1:

pg/ml	Absorbency 450	(Δ620) nm	Mean	SD	CV (%)
10,000	2.166	2.125	2.145	0.029	1.35
5,000	1.048	0.980	1.014	0.048	4.77
2,500	0.476	0.445	0.450	0.022	4.68
1,250	0.200	0.187	0.193	0.009	4.87
625	0.085	0.083	0.084	0.002	1.85
313	0.044	0.041	0.042	0.002	4.88
156	0.029	0.030	0.030	0.001	2.85
0	0.022	0.021	0.021	0.001	4.63
(n = 2)					

Table 9-2:

Sample ID #	Measurement Results (pg/ml)	
	Not Pre-treated	Pre-treated
1	ND	332
2	ND	424
3	ND	524
4	ND	501
5	ND	417
6	ND	622
7	ND	562
8	ND	629
9	3.36	546
10	ND	430
(n = 2)		

INDUSTRIAL APPLICABILITY

[0076] The method for the measurement for human insulin has so far been applied, for example, to measuring endocrine substances themselves such as feline insulin and so on in an feline blood analyte of an animal. The present invention has the merit that the endocrine substances themselves such as feline insulin and so on can be measured more accurately and instantly. The present invention has also the merit that it can be handled with an extreme brevity because no radioactive substance is used and an enzymatic immunological method ordinarily used in the art involved can be applied.

[0077] The method for the measurement of the endocrine substances according to the present invention can accurately, rapidly and briefly measure the endocrine substances such as feline insulin and so on in an analyte, e.g., a blood analyte, of an animal such as cat, etc., in order to diagnose and treat diseases associated with the endocrine substance, e.g., diabetes mellitus. In addition, the method of the present invention is very convenient because it uses established procedures of the measurement methods conventionally used in the art involved.

Claims

1. A method for the pre-treatment of a feline blood sample comprising:

treating the blood sample with an acid to separate the feline insulin and an autoantibody bound thereto:

5 adding the autoantibody with saturated ammonium sulfate, saturated sodium sulfate or polyethylene glycol to the feline blood sample to precipitate the autoantibody non-bound thereto and the feline insulin co-precipitated therewith;
adding an alcohol to the blood sample to extract the feline insulin co-precipitated therewith;
removing the autoantibody bound thereto to collect a total feline insulin.

10 **Patentansprüche**

1. Verfahren zur Vorbehandlung einer Katzenblutprobe, umfassend:

15 Behandeln der Blutprobe mit einer Säure zur Abtrennung des Katzeninsulins und eines daran gebundenen Autoantikörpers;
Zugeben des Autoantikörpers mit gesättigtem Ammoniumsulfat, gesättigtem Natriumsulfat oder Polyethylen-glycol zu der Katzenblutprobe zur Präzipitation des nicht daran gebundenen Autoantikörpers und des damit kopräzipitierten Katzeninsulins;
20 Zugabe eines Alkohols zu der Blutprobe zur Extraktion des damit kopräzipitierten Katzeninsulins;
Entfernen des daran gebundenen Autoantikörpers, um ein gesamtes Katzeninsulin zu sammeln.

Revendications

25 1. Procédé pour le traitement préalable d'un échantillon de sang félin comprenant les étapes suivantes :

30 traiter l'échantillon de sang avec un acide pour séparer l'insuline féline et un auto-anticorps y associé ;
ajouter l'auto-anticorps avec du sulfate d'ammonium, du sulfate de sodium saturé ou du polyéthylène glycol à l'échantillon de sang félin pour précipiter l'auto-anticorps non associé et l'insuline féline co-précipitée ;
ajouter de l'alcool à l'échantillon de sang pour extraire l'insuline féline co-précipitée ;
enlever l'auto-anticorps associé pour recueillir une insuline féline totale.

Fig. 1

Weigh 1 mg of CNBr-activated Sepharose™ 4B (Amersham)



Allow to stand overnight in 1 mM HCl to swell



Wash with 0.1 M CB (pH 8.5)



Couple 50 mg of bovine insulin (Sigma) having an amino acid sequence different from that of feline insulin by one amino acid on gel using 10 ml of 0.1 M CB (pH 9.6) after dissolving in 70% ethanol-1 mM



Block with 0.1 M Tris-HCl (pH 7.8)



Wash with 10 mM PBS



Prepare Insulin-conjugated affinity column

Prepare a dilute solution of feline plasma (100 µl) with 10 mM PBS (10 ml)



Apply said dilute solution to said insulin-conjugated affinity column

↓ using 10 mM PBS as conjugation buffer; and

↓ using 0.1 M glycine - HCl (pH 2.5) as elution buffer

Recover pass fractions and elution fractions



Adjust pH close to neutral



Recover elution fraction



Subject non-insulin-conjugated feline autoantibody and bovine insulin to antigen-antibody reaction in the elution fraction to confirm antigen-antibody reaction with anti-feline IgG (H+L)

Fig. 2

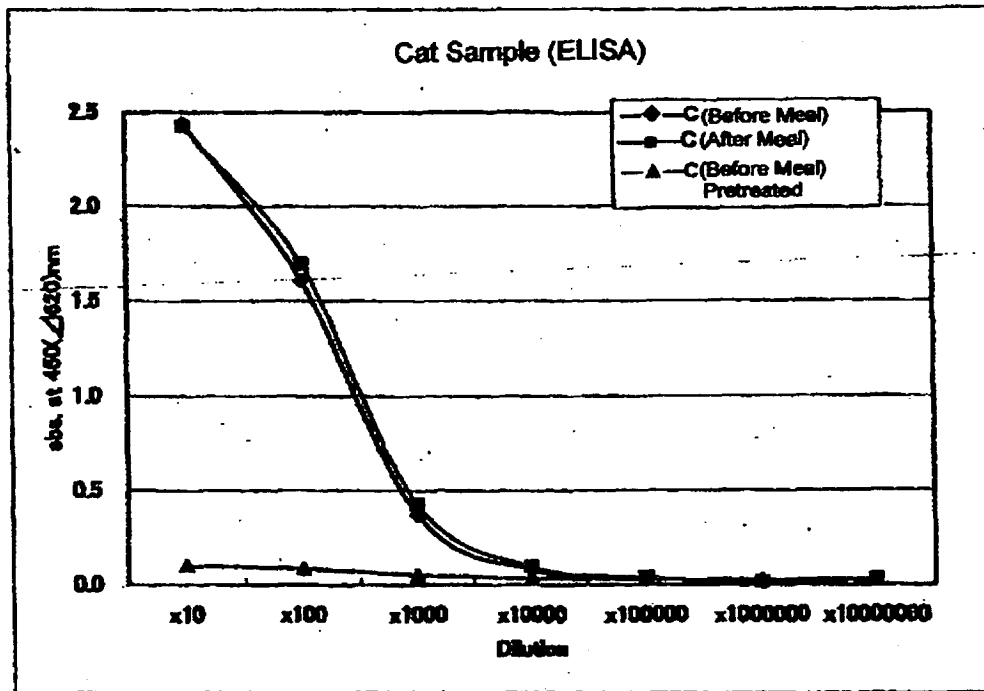


Fig. 4

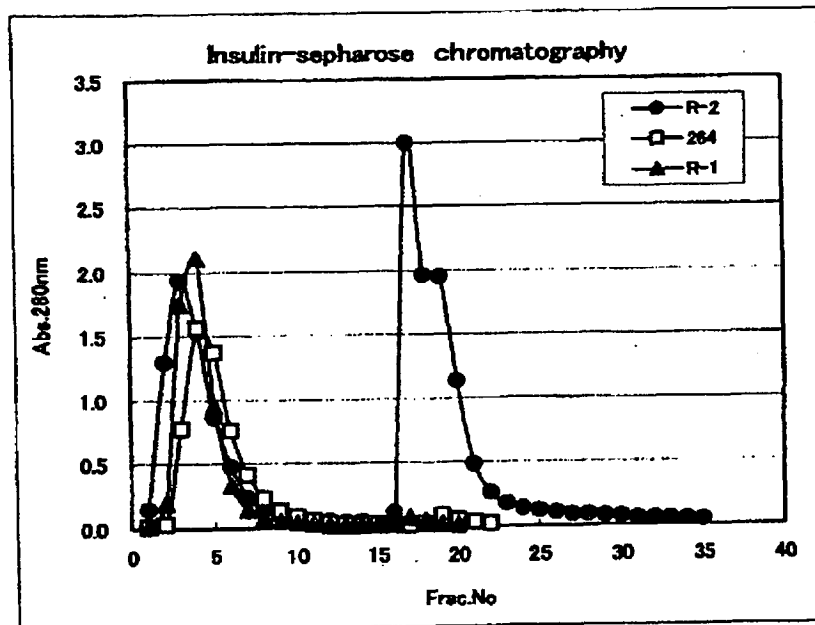


Fig. 3

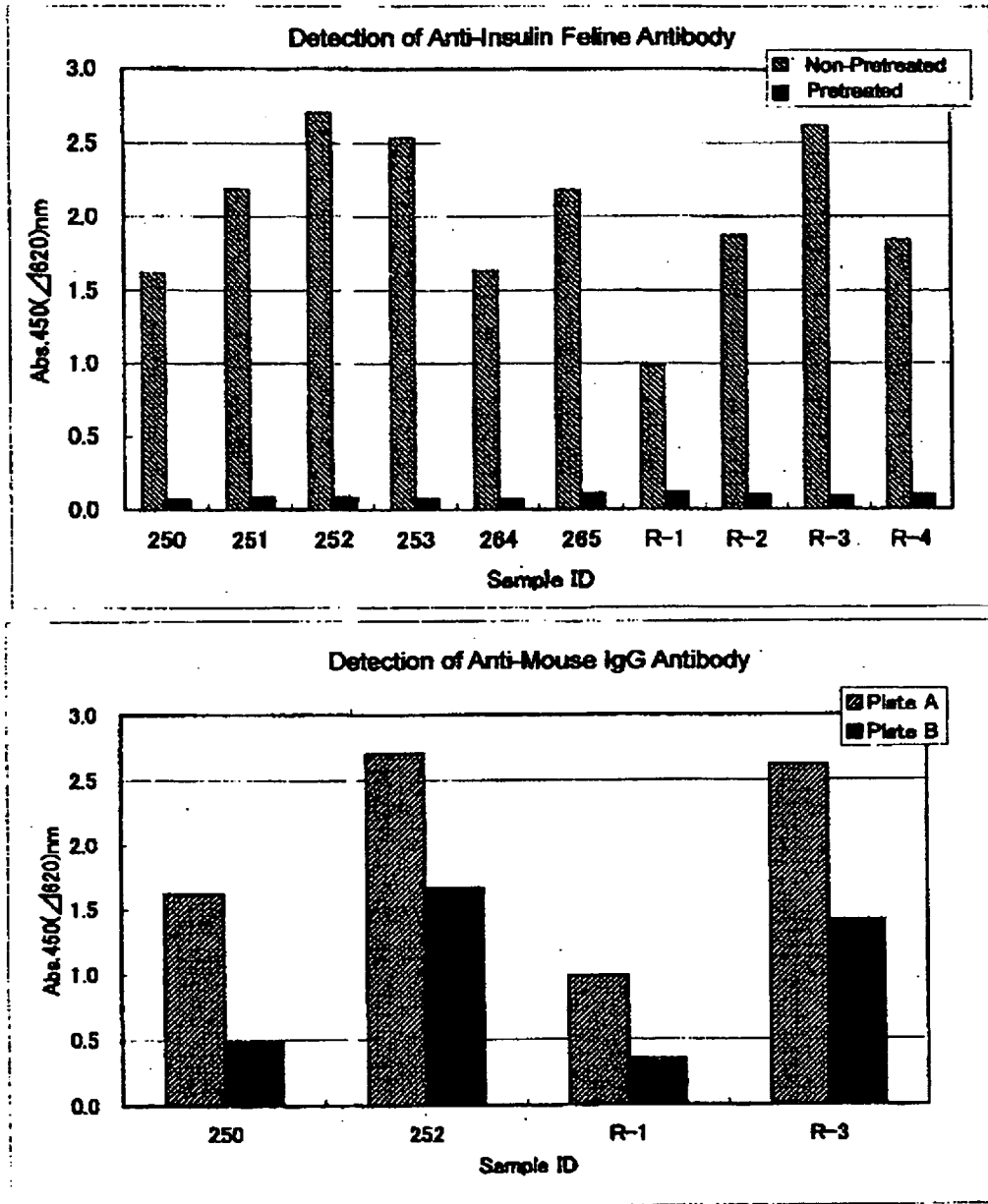


Fig. 5

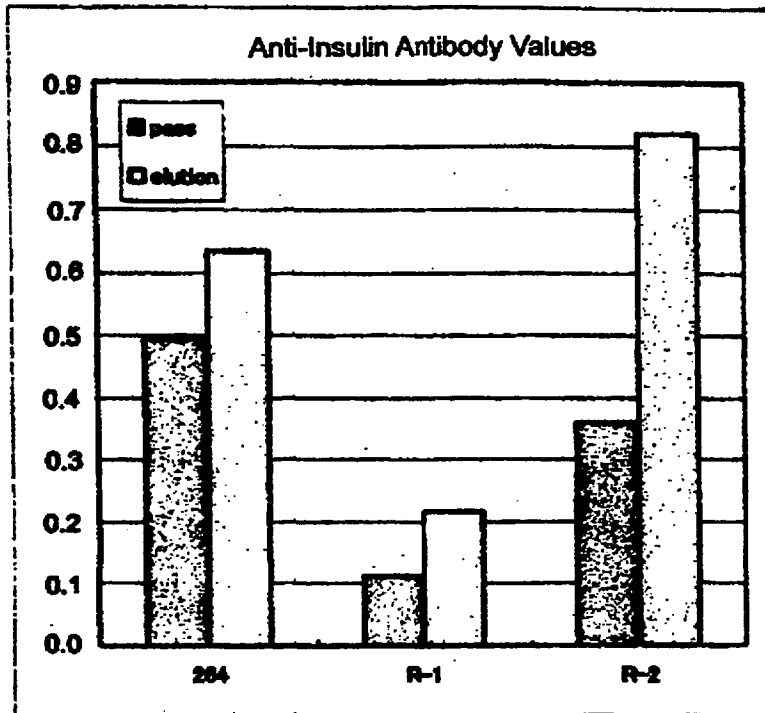


Fig. 6

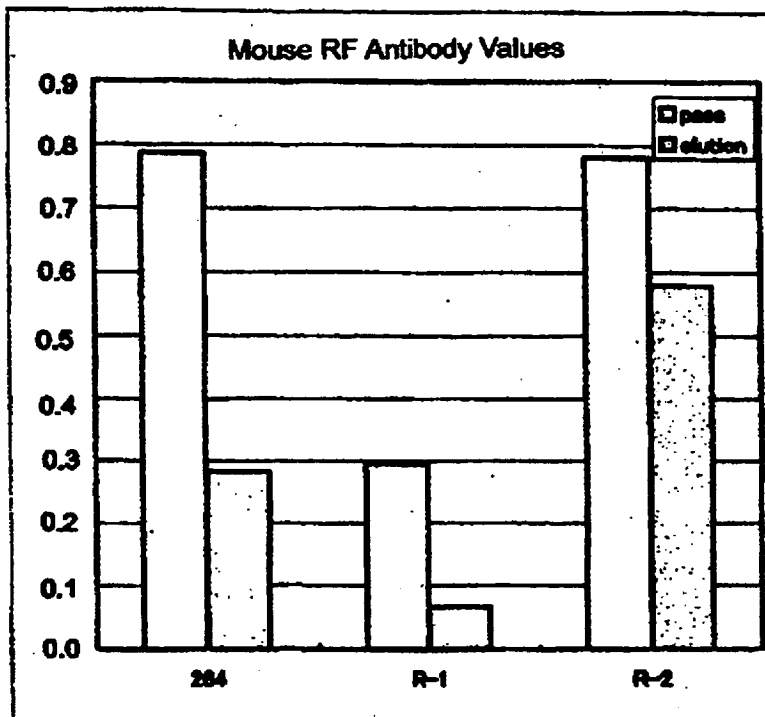


Fig. 7

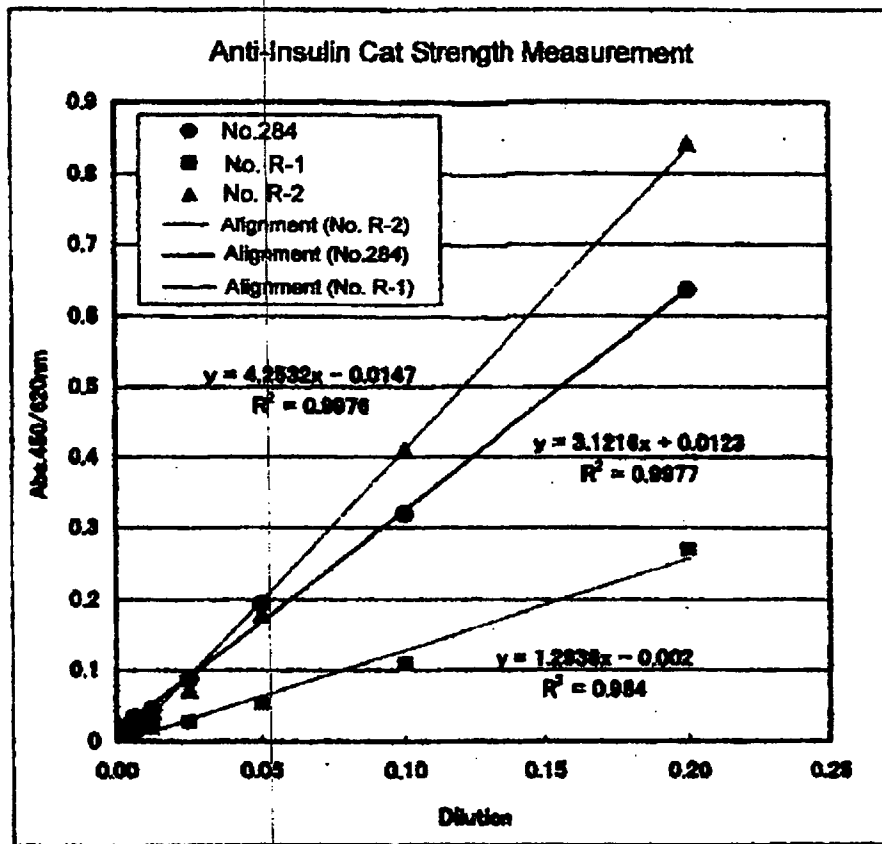


Fig. 8

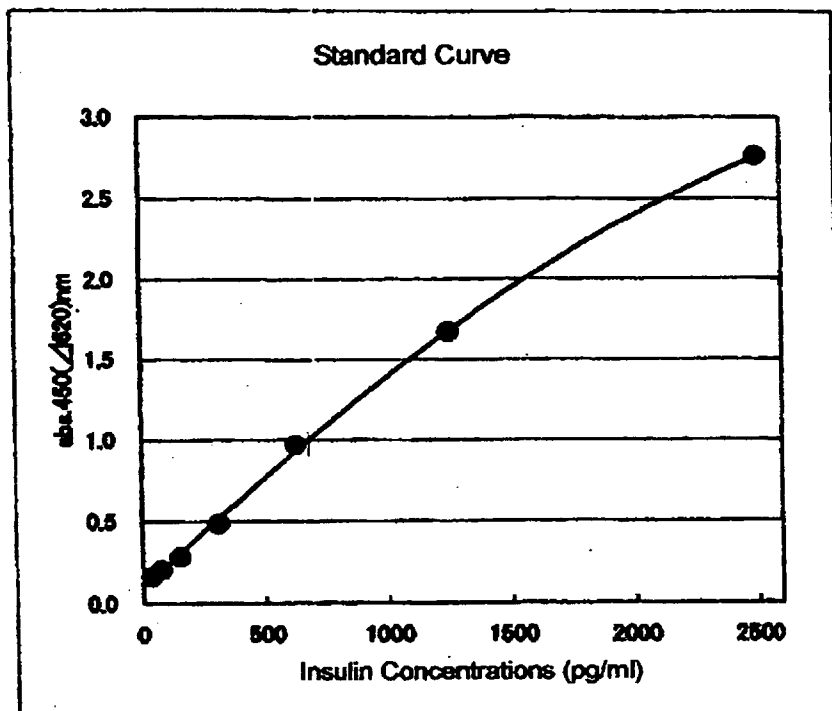
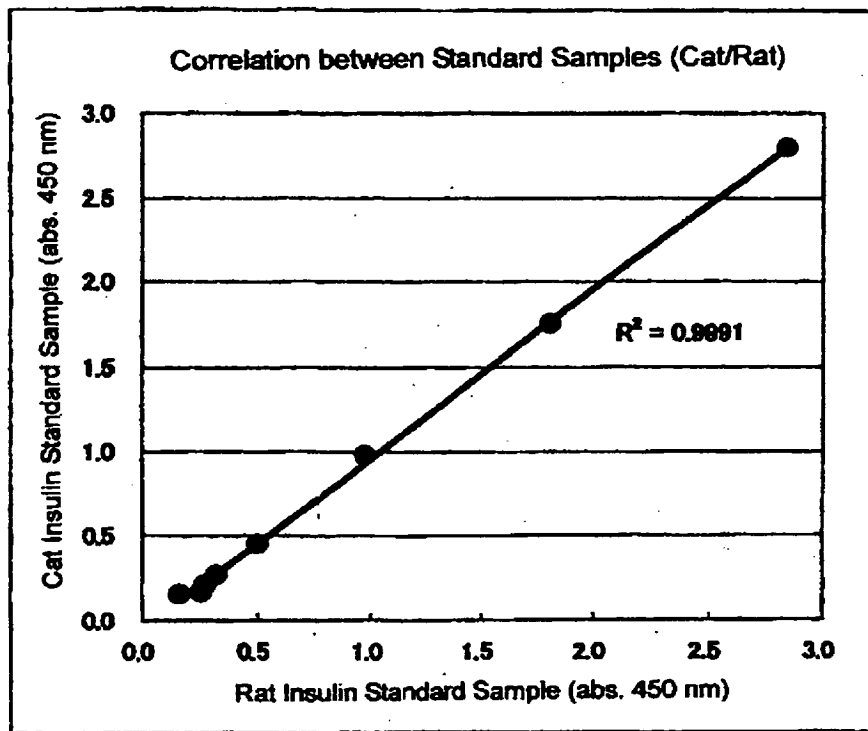


Fig. 9



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REFERENCES CITED IN THE DESCRIPTION

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专利名称(译)	测定标本中内分泌物质的方法		
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申请号	EP2005809384	申请日	2005-11-22
[标]申请(专利权)人(译)	SHIBAYAGI		
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IPC分类号	G01N33/53		
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代理机构(译)	GILLE HRABAL STRUCK NEIDLEIN PROP ROOS		
优先权	2005190003 2005-06-29 JP		
其他公开文献	EP1898215A4 EP1898215A1		
外部链接	Espacenet		

摘要(译)

本发明提供一种测量方法，该方法能够以高精度，快速和简洁的方式测量动物如猫等的分析物中的内分泌物质，例如猫胰岛素。根据本发明的用于测量分析物中的内分泌物质的方法包括通过去除与分析物中存在的内分泌物质结合的自身抗体并且测量来自动物如猫科动物等的预分析物。预处理分析物中的内分泌物质。此外，本发明可以测量与分析物中的内分泌物质结合的自身抗体的值。另外，本发明通过测量分析物中内分泌物质的自身抗体值，可以诊断和治疗与内分泌物质有关的疾病。

Fig. 1

Weigh 1 mg of CNBr-activated Sepharose™ 4B (Amersham)
↓
Allow to stand overnight in 1 mM HCl to swell
↓
Wash with 0.1 M CB (pH 8.5)
↓
Couple 50 mg of bovine insulin (Sigma) having an amino acid sequence different from that of feline insulin by one amino acid on gel using 10 ml of 0.1 M CB (pH 9.8) after dissolving in 70% ethanol-1 mM
↓
Block with 0.1 M Tris-HCl (pH 7.8)
↓
Wash with 10 mM PBS
↓
Prepare Insulin-conjugated affinity column
↓
Prepare a dilute solution of feline plasma (100 µl) with 10 mM PBS (10 ml)
↓
Apply said dilute solution to said insulin-conjugated affinity column
↓ using 10 mM PBS as conjugation buffer; and
↓ using 0.1 M glycine - HCl (pH 2.5) as elution buffer
Recover pass fractions and elution fractions
↓
Adjust pH close to neutral
↓
Recover elution fraction
↓
Subject non-insulin-conjugated feline autoantibody and bovine insulin to antigen-antibody reaction in the elution fraction to confirm antigen-antibody reaction with anti-feline IgG (H+L)