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(54) Titre : METHODE DE DETERMINATION D'HOMOCYSTEINE
(54) Title: A METHOD FOR THE DETERMINATION OF HOMOCYSTEINE

(57) **Abrégé/Abstract:**

The invention relates to a method for determining homocysteine concentration in samples in which homocysteine is condensed using an enzyme cystathionine β synthase to form cystathionine. Pyruvate and/or ammonia are released from cystathionine by action of an enzyme, cystathionine β lyase, and homocysteine is regenerated. The release of pyruvate and/or ammonia can be correlated to the concentration of homocysteine present in the sample.



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(57) Abstract

The invention relates to a method for determining homocysteine concentration in samples in which homocysteine is condensed using an enzyme cystathionine β synthase to form cystathionine. Pyruvate and/or ammonia are released from cystathionine by action of an enzyme, cystathionine β lyase, and homocysteine is regenerated. The release of pyruvate and/or ammonia can be correlated to the concentration of homocysteine present in the sample.

A METHOD FOR THE DETERMINATION OF HOMOCYSTEINE

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BACKGROUND OF THE INVENTION

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Total concentration of homocysteine in body fluids, such as plasma or serum, is an important marker for disease. For example, homocysteine quantification can be an important risk indicator for cardiovascular disease, can be a sensitive marker of cobalamin and folate deficiencies, and can be used to diagnose in-born errors in metabolism known as homocystinuria. Homocysteine quantification has also been reported as useful in assessing birth defects in pregnant women and cognitive impairment in the elderly. See Frantzen, et al., Enzyme Conversion Immunoassay for Determining Total Homocysteine in Plasma or Serum, Clinical Chemistry 44:2, 311-316 (1998). Current assays, such as those using HPLC or GC-MS, are expensive and require highly skilled technical staff. An efficient and accurate assay, that can be carried out without necessity for highly skilled personnel or complex analytical chemistry equipment, has been needed.

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SUMMARY OF THE INVENTION

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The present invention provides an assay for homocysteine found in plasma, serum or other body fluids of a patient. According to this assay, a homocysteine containing sample is condensed using an enzyme, cystathionine β -synthase, to form cystathionine. This cystathionine is subjected to another enzyme, cystathionine β -lyase, to release pyruvate and ammonia, and homocysteine. The total homocysteine concentration in the patient sample can be determined based on the detection and correlation of the pyruvate and/or ammonia released.

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In another embodiment of the invention, the patient sample is subjected to treatment by dithiothreitol or other reducing agent, in appropriate amounts to produce free homocysteine in the sample.

5 In a still further embodiment, the enzymes cystathionine β -synthase and cystathionine β -lyase may be treated with a phosphorylated form of vitamin B6 in order to optimize their function in the assay of the invention.

10 A still further embodiment of the present invention is a kit for determining homocysteine concentration in a sample that includes the enzymes cystathionine β -synthase and cystathionine β -lyase, and serine. Such kit may further include a reducing agent such as dithiothreitol (DTT), and an enzyme co-factor such as pyridoxal 5' phosphate (PLP).

15 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates an assay method for homocysteine according to the invention.

20 Figure 2 illustrates the calculated linearity of homocysteine concentration as measured by pyruvate release according to assay method of the invention.

DETAILED DESCRIPTION OF THE INVENTION

25 The measurement of total plasma homocysteine from plasma, serum, or other body fluids can be performed by condensing homocysteine with serine to form cystathionine, by a beta substitution reaction, using the enzyme cystathionine β -synthase (CBS) (enzyme classification [EC] 4.2.1.22). The cystathionine then undergoes a beta elimination reaction catalyzed by cystathionine β -lyase (CBL) (EC 30 4.4.1.8), releasing pyruvate and/or ammonia, and regenerating homocysteine. The regenerated homocysteine will then be free to be condensed with another serine molecule by CBS, EC 4.2.1.22, and again to form cystathionine, and again release pyruvate and ammonia by beta elimination with CBL, EC 4.4.1.8.

This recycling will continue at a rate determined by the concentration of total homocysteine in the plasma or serum sample. At the low concentrations of homocysteine found in plasma and serum, the reaction rate is slow and very linear to homocysteine concentration. The Michaelis-Menten constants (K_m) for the two enzyme activities described exist in nature in the 10^{-3} moles/liter range, while plasma and serum tend to be in the 10^{-5} moles/liter range. This dictates the slow linear correlation between reaction rate and plasma concentration. By measuring the production of pyruvate and/or ammonia over a fixed length of time, the reaction rate can be determined and related to the total homocysteine concentration in the sample.

There are many ways of determining the presence of ammonia and/or pyruvate by standard commercially available diagnostic reagent methods. Although homocysteine occurs in very low concentrations in plasma, for example, sensitivity can be overcome by cycling the reaction in the presence of serine until an adequate amount of pyruvate and/or ammonia is produced to be accurately measured by the commercially-available, selected method(s).

It will be appreciated by those of skill in the art that the assay of the invention may be conducted without recycling the enzyme reactions. This may be accomplished by the selection of an appropriate chromophore, coloring assay reagent, or the selection of sensitive methods of measuring ammonia and/or pyruvate. Selection of these elements would be within the capability of those of skill in the art.

Homocysteine is found in body fluids mostly in the form of molecules covalently disulfide-linked to another compound. In this regard, homocysteine may be found bound to another homocysteine molecule (forming homocystine), to a cysteine molecule, or a cysteine residue in a protein molecule. Seventy percent of homocysteine is disulfide linked to a cysteine residue on an albumin molecule. Only one percent (1%) of all homocysteine exists as unbound molecules that are not disulfide-linked to another molecule. Therefore, to optimize the enzyme reactions described above, homocysteine molecules should be liberated from their disulfide interactions and permitted to exist as free homocysteine. This can be achieved by reducing the disulfide bonds. One of the easiest ways to facilitate this is with a

chemical reducing agent, such as dithiothreitol (DTT), although there are many such chemical agents available that would also reduce the disulfide bonds adequately. This compound should be present in the lowest possible concentration to disrupt the disulfide bonds without interfering with the functions of enzymes in the enzyme cycle
5 or the assays to determine pyruvate and/or ammonia. High levels of DTT, for example, can interfere with analytical reagents and prevent the conduct of the assay. In this regard, the homocysteine recycling enzymes can generally tolerate concentrations of DTT up to approximately 30 millimolar. More preferably, and depending on the detection method used, the reduction of homocysteine disulfide
10 interaction may be accomplished with an amount of DTT approximately up to 10 millimolar, and most preferably 2 to 10 millimolar DTT.

Optimal function of the enzymes that recycle homocysteine, as described above with respect to CBS, EC 4.2.1.22, and CBL, EC 4.4.1.8, can be obtained with
15 the use of low concentrations of an enzyme co-factor, such as pyridoxal 5' phosphate (PLP), a phosphorylated form of vitamin B6. These enzymes have a Km for PLP of 5×10^{-6} moles/L and therefore, about a 5 micromolar concentration of PLP can typically be used. However, it will be understood that other amounts of PLP, and other enzyme co-factors having similar characteristics, may be used in order to carry out the assay of
20 this invention.

Figure 1 illustrates the cyclic reaction that permits the measurement of homocysteine according to the invention.

25 In one embodiment of the invention, free homocysteine is condensed with serine to form cystathionine using CBS, EC 4.2.1.22. The cystathionine is then treated with CBL, EC 4.4.1.8 to release pyruvate and ammonia and regenerate homocysteine.

30 In another embodiment, the use of DTT, or other reducing agent, in an appropriate amount facilitates the production of free homocysteine, protein and cysteine from bound protein samples. This homocysteine is then available for condensation with serine and treatment with CBS, EC 4.2.1.22. Preferably, PLP may also be added to this reaction mixture. Optimally, PLP may further be added to the

cystathionine and CBL, EC 4.4.1.8. An example of these optimized reactions may be written as follows:

(1) (a sample containing any or all of homocysteine disulfide linked to protein, homocystine, homocysteine and homocysteine bound to cysteine) + DTT = free homocysteine + protein + cysteine + oxidized DTT;

(2) free homocysteine + serine + PLP + CBS = cystathionine + PLP + CBS;

(3) cystathionine + PLP + CBL = free homocysteine + pyruvate + ammonia.

These steps are repeated to produce sufficient amounts of pyruvate and/or ammonia to permit measurement.

After a fixed period of time, pyruvate and/or ammonia are measured using a commercially available assay. In this regard, pyruvate and/or ammonia detection may be accomplished with pyruvate oxidase, lactate dehydrogenase or an ammonia reagent. It will be understood that other methods for detecting pyruvate and/or ammonia concentrations could also be used.

By conducting these reactions of the invention cyclicly, measurements may be made of pyruvate and/or ammonia production over time, which can then be correlated to the total homocysteine concentration in the sample.

Detection of total homocysteine can be accomplished as follows:

To a 100 microliter sample of plasma, serum, or other body fluid, is added 10 millimoles of dithiothreitol, one microunit of CBS, one microunit of CBL, 5 micromoles of PLP and 1 millimole serine. Also added are the commercially available reagents for the colorimetric detection of pyruvate and/or ammonia. At time zero, pyruvate and/or ammonia are measured according to specifications of the commercial reagents. Then, the sample is allowed to incubate in a temperature controlled chamber (at approximately 37°C) for 10 minutes, and then pyruvate and/or ammonia are again measured. This procedure may need to be performed several

times until an adequate amount of pyruvate and/or ammonia has accumulated for an accurate reading. Once a length of time has been established that will reproducibly measure an amount of pyruvate and/or ammonia that is suitable for the sensitivity of the respective commercial kits, then samples will always be incubated for that length
5 of time. A correlation between homocysteine and pyruvate and/or ammonia will be established with a series of standards of known homocysteine values in plasma and a linear curve will be established. The unknown sample will be determined by linear regression.

10 The present invention also provides a diagnostic kit for determining homocysteine concentration in a sample, which contains the enzymes cystathionine β -synthase and cystathionine β -lyase, and serine. The kit may further include a reducing agent, such as DTT, and enzyme co-factor, such as PLP.

15 **EXAMPLES:**

I. The CBL Enzymes:

A. Procedure for Cloning CBL

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The pLC4-14 plasmid carrying the metC gene was isolated from ATCC Clone #37384 (J.R. Uren, Proc. Natl. Acad.Sci. USA 68: 367-371, 1971) using techniques described in Maniatis et al., Molecular Cloning, A Laboratory Manual, 2d, Cold Spring Harbor Laboratory Press (1989). The metC gene was cloned from the plasmid
25 by PCR using primers synthesized according to the published DNA sequence. In addition, restriction enzyme sites were added at the N-and C-termini that were suitable for cloning the metC cDNA into an appropriate expression vector. PCR primers were synthesized and had the following 5' - 3' sequences: Forward Primer (added an Nde I site at the ATG start codon of metC) GGG AAT TCC ATA TGG
30 CGG ACA AAA AGC TTG ATA CTC Reverse Primer - (added a BamH1 site after the stop codon of metC) CGC GGA TCC AAA AGT GGC AAT GTT ATA CAA TTC GCG C. The PCR products were digested with Nde I and BamH I and ligated into a lac promoter expression vector (pGB3Nde of Genzyme Corporation, Cambridge, MA) which had been digested with the same enzymes. The resulting

plasmid was then transformed into Top 10 *E. Coli* cells (Invitrogen) following the protocol provided with the cells. The transformants were plated on LB agar + 50µg/ml carbenicillin + 0.5% glucose and grown overnight. Colonies were screened for the presence of the appropriate plasmid and enzyme activity.

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The pGB3Nde vector of Genzyme Corporation is described, in part, as "pGB3" in U.S. Patent No. 5,236,838. The described pGB3 vector has been modified to add an Nde site, forming the presently employed vector designated pGB3Nde. Methodology for modification of a vector in this manner may be found in T. Maniatis, 10 E. Fritsch, and J. Sambrook, *Molecular Cloning, A Laboratory Manual*, 2d ed., Cold Spring Harbor Press (1989), and F.M. Ausubel et al., *Current Protocols in Molecular Biology*, Green Publishing Associates and Wiley-Interscience (1989). It will be appreciated that other similar base vectors, such as may be available from Stratagene, La Jolla, CA, can also be employed.

15

B. Procedure for Purification of CBL

The purification procedure used for CBL is an adaptation of a published procedure (Dwivedi, C. M., *Biochemistry* 1982, 21, 3064-3069). Cultures from a 20 CBL clone were grown and expression of CBL was induced with isopropyl thiogalactoside (IPTG). The cell suspension was centrifuged and the resulting cell pellet was lysed using 1mg/ml lysozyme on ice for 15 minutes, followed by sonication. The lysate was loaded onto a Q Sepharose FF (Pharmacia) column and eluted with a salt gradient (0-0.5 M sodium chloride). Active fractions were purified 25 on a hydroxyapatite column (Macro-Prep Ceramic Hydroxyapatite TYPE 1 40 µM from BioRad) and eluted with a phosphate gradient (10-400mM potassium phosphate, pH 6.8). The eluate was loaded onto a Blue Sepharose CL-6B column (Pharmacia) and the CBL eluted with a salt gradient (0-1.0 M sodium chloride). A Mono Q column (Pharmacia) was used to concentrate the enzyme. The fractions with activity 30 were pooled, diluted with glycerol to 50% and stored at -20° C.

An alternative procedure for cloning and purification of the CBL enzyme is described below.

C. Procedure for Cloning CBL-MBP Protein Fusion

The CBL gene was cloned from an expression vector (pGB3Nde) into the pMAL-c2X vector (New England Biolabs) to create an MBP (maltose binding protein) Fusion Protein. PCR primers were designed and synthesized to clone the CBL open reading frame and add a Sma I site at the N-terminus. The Forward Primer had the following 5' - 3' sequence: TGG CCC GGG GCG GAC AAA AAG CTT GAT ACT CAA CTG. The addition of this site resulted in the first methionine in the CBL sequence to be replaced by a glycine. The Reverse Primer had the following 5' - 3' sequence: GGG GGA TCC AAA AGT GGC AAT GT. The PCR products were digested with Sma I and Bam HI and ligated into the pMAL-c2X vector which had been digested with Xmn I and Bam HI. The resulting plasmid was then transformed into Top 10 *E. coli* cells (Invitrogen) following the protocol provided with the cells. The transformants were plated on LB agar + 50µg/mL carbenicillin + 0.5% glucose and grown overnight. Colonies were screened for the presence of the appropriate plasmid and CBL activity.

D. Procedure for Purification of CBL-MBP

Cultures from a CBL-MBP clone were grown and expression of CBL-MBP was induced with IPTG. The cell suspension was centrifuged and the resulting cell pellet was lysed using 1 mg/mL lysozyme and 150 U/mL of DNase I. The lysate was loaded onto an Amylose column (New England Biolabs) and eluted with a buffer containing 10mM maltose. Active fractions were pooled, diluted with glycerol and stored at -20°C.

E. DTNB Assay for Cystathionine β Lyase

Assay Principle:

Cysteine, homocysteine or similar thiols are produced by enzymatic cleavage of cystathionine (CTT), djenkolic acid (DJA) or other thioether substrates. The thiols undergo a disulfide exchange with the aromatic disulfide 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) which releases one equivalent of the chromophore 2-nitrobenzoic acid-5-mercaptide (TNB). The release of TNB is followed

spectrophotometrically and the reaction rate is calculated from the slope of the absorbance at 410nm over time using a molar extinction coefficient of $13,200 \text{ M}^{-1} \text{ cm}^{-1}$ for TNB:

5 Reagents:

- 5,5'-dithio-bis(2-nitrobenzoic acid) (Sigma D-8130)
 L-(+)-cystathionine (Sigma C-7505)
 L-djenkolic acid (Sigma D-9255)
 Tris (base) (J.T. Baker 4109-02)
 10 dithiothreitol (DTT) (EM Science 11474-4)
 pyridoxal 5' phosphate (PLP) (Sigma P-9255)
 bovine serum albumin (BSA) (Sigma A-3059)
 Hydrochloric acid (HCl) reagent grade
 Mono-and dibasic potassium phosphates, reagent grade

15

Stock Solutions:

Assay buffer: 0.1 M Tris-HCl buffer, pH 9.0.

Store sterile at room temperature.

20

Substrate solution: 10mM L-djenkolic acid or L-(+)-cystathionine in 0.01 N HCl. Suspend solid in HCl, warm container in hot water briefly, mix or sonicate until dissolved, let cool to room temperature. Store at -20°C , reheat after thawing for complete dissolution.

25

DTNB Solution: 10 mM DTNB in 0.1 M potassium phosphate buffer, pH 7.0. Store at -20°C . Enzyme dilution buffer: 10mM potassium phosphate buffer, pH 7.0, 100 μM DTT, 10 μM PLP, 10 g/L BSA.

Assay Procedure:

30

Make up the following amount of reagent cocktail at room temperature for one 96-well plate:

- | | |
|---------|--------------------|
| 15.6 mL | assay buffer |
| 4.0 mL | substrate solution |
| 0.4 mL | DTNB solution |

Dilute the enzyme samples to be assayed as required with enzyme dilution buffer and keep on ice. The enzyme concentration should be between 0.02 and 25 U/mL. Aliquot 200 μ L of reagent cocktail into each well of a flat-bottom 96-well assay plate. Set up microwell plate reader for 10 min. kinetic reading at 410 nm, OD range 0-2. Rapidly add 1 to 10 μ L of prediluted enzyme samples to assay plate and mix. Immediately transfer plate into reader and start kinetic program.

Unit definition:

For experimental simplicity an enzyme unit is defined as the amount of enzyme that catalyzes the formation of one mole of free thiol per minute from L-djenkolic acid at room temperature. Cystathionine is cleaved by most lyases at similar rates to L-djenkolic acid but some enzyme specific differences occur. A difference in activity can result from assaying at 37°C as described in some published procedures.

Detection Limits:

The minimum amount of enzyme to be added to the assay is estimated as about 10 μ L of a 0.01 U/mL solution, which would result in a measurement of 10 mOD/min. Conversely, the estimated maximum photometer rate of 150 mOD/min over 10 minutes would be achieved by adding 1 μ L of a 14 U/mL stock. Adjustments to minimum and maximum amounts with vary according to the detection method employed.

II. The CBS Enzymes:

A. Procedure for cloning CBS:

The CBS gene was cloned from *Saccharomyces cerevesiae* genomic DNA. PCR was performed using primers which encoded an Nde I site on the 5' end and a BamH I site on the 3' end. The amplicon was digested with these restriction enzymes and then ligated into an expression vector (pGB3Nde) which was digested with the same restriction enzymes. The plasmid was then transformed into Top 10 *E. coli* cells

(Invitrogen) and plated on selective media. The colonies were selected that contained a plasmid of the appropriate size.

An expression experiment was performed to determine if CBS activity was present in the clones. The cultures of the clones were grown in selective media with thiamine and aminolevulinate. IPTG was added for 4 hours and 21 hours to induce protein production. The cell pellets were lysed with lysozyme and DNase and the lysate supernatant was assayed for CBS activity using the radioactive assay described by Jan Kraus in Kraus, JP, Cystathionine β -Synthase (human), Methods Enzymol. 143, 388-394 (1987) (hereinafter "Kraus publication"), and the ninhydrin assay to detect if cystathionine (CTT) was formed by incubating the lysate with serine and homocysteine. By both assays, lysate expressed CBS activity.

B. Procedure for Purification of CBS:

15

Based on the sequencing and expression results, the clone was selected for purification which has activity and has no error in the sequence side by side comparing with the Kraus publication.

20

The CBS clone was grown in Terrific Broth (TB) with 50 μ g carbenicillin, 0.001% thiamine and 0.3 mM aminolevulinic acid (ALA) at 30°C. 1mM IPTG was added and the culture was incubated for 21 hours at 30°C. Cells were pelleted by centrifugation for eight minutes at 8000 RPM. The cell pellets were resuspended in 50 mM Tris-HCl, pH 8.0 containing 10 μ M pyridoxal 5' phosphate (PLP) and spun again. The cell pellet was frozen until ready for lysis and purification.

25

The cell pellet was thawed quickly in a 37°C waterbath and resuspended in the lysis buffer containing 50 mM Tris-HCl, pH 8.0, 10 μ M PLP, 1 mM MgCl₂, 0.1 mM DTT, 1 mg/ml lysozyme and 100 units/ml DNase 1. To the lysate, 2mM EDTA was added and it was centrifuged. The supernatant was placed in ice and while stirring, ammonium sulfate was added slowly to a final concentration of 30%. The suspension was stirred for 30 minutes and centrifuged. Ammonium sulfate was added to the supernatant to a final concentration of 70% and after stirring for 1 hour in ice, the suspension was centrifuged. The pellet was resuspended in the buffer containing 50 mM Tris-HCl, pH 7.5 and 10 μ M PLP and dialyzed against the same buffer. The

30

dialysate was loaded onto a MonoQ column which was equilibrated with the same dialysis buffer. The CBS was eluted off the column with a gradient from 0 - 1 M NaCl. Fractions were assayed for CBS activity using the radioactive assay described in the Kraus publication, and also using the results of the cyclic method of the invention. Those containing activity were pooled and stored at -20°C.

An alternative procedure for cloning and purification of the CBL enzyme is described below.

10 C. Procedure for Cloning CBS-MBP Protein Fusion

The CBS gene was subsequently cloned from an expression vector (pGB3Nde) into the pMAL-c2X vector (New England Biolabs) to create an MBP (maltose binding protein) Fusion Protein. PCR primers were designed and synthesized to clone the CBS open reading frame and add a Sma I site at the N-terminus. The Forward Primer had the following 5' - 3' sequence: TGG CCC GGG ACT AAA TCT GAG CAG CAA GCC GAT TCA. The addition of this site resulted in the first methionine in the CBS sequence being replaced by a glycine. The Reverse Primer had the following 5' - 3' sequence: GGG GGA TCC TTA TGC TAA GTA GCT CAG. The PCR products were digested with Sma I and Bam HI and ligated into the pMAL-c2X vector which had been digested with Xmn I and Bam HI. The resulting plasmid was then transformed into Top 10 *E. coli* cells (Invitrogen) following the protocol provided with the cells. The transformants were plated on LB agar + 50µg/mL carbenicillin + 0.5% glucose and grown overnight. Colonies were screened for the presence of the appropriate plasmid and CBS activity.

D. Procedure for Purification of CBS-MBP

Cultures from a CBS-MBP clone were grown and expression of CBS-MBP was induced with IPTG. The cell suspension was centrifuged and the resulting cell pellet was lysed using 1 mg/mL lysozyme and 150 U/mL of DNase I. The lysate was loaded onto an Amylose column (New England Biolabs) and eluted with a buffer containing 10mM maltose. Active fractions were pooled and stored at -80°C.

E. Cystathionine β Synthase Assay1. A first embodiment:

5 Reagents:

Tris-HCl, 1 M, p H 8.6

Bovine Serum Albumin, 25 mg/ml

Serine, 0.1 M

Pyridoxal 5' phosphate (PLP), 10mM

10 Dithiothreitol, 0.2 M

NaOH, 5 M

HCl, 5 M

Homocysteine Mixture

Homocysteine thiolactone 30.72 mg

15 NaOH (5M) 245 μ l

Dissolve homocysteine thiolactone in NaOH. Incubate 5 min at 37°C to break the ring. Next add:

Tris (1M, pH 8.6) 100 μ l20 DTT (0.2 M) 100 μ lH₂O 355 μ lHCl (5N) 200 μ l

Check pH with pH paper and adjust to 8.5 if necessary.

This solution is stable for 24 hours at 4°C.

25

Assay Mixture

Stock	Amount
1 M Tris pH 8.0	10.0 μ l
BSA 25 mg/ml	2.0 μ l
0.1 M Serine	5.0 μ l
10 mM PLP	10.0 μ l
0.2 M hcy	7.5 μ l
C ¹⁴ Serine	1.0 μ l
dH ₂ O	59.5 μ l

30

Total 95.0 μ l

Multiply each amount times the total number of samples to be assayed. Store the assay mixture on ice prior to use.

5

Enzyme Sample

Dilute enzyme sample to CBS specific protein concentration of 1 mg/ml with 10 mM Tris pH 8.0. Glycerol may also be used in place of Tris.

10 With glass tubes (\approx 5 ml volume) on ice, add 95 μ l of assay mixture to each tube, including the blank.

Add 5 μ l of enzyme sample to each tube (or amount to equal 2-5 μ g) except the blank, add 5 μ l dH₂O to blank.

15

Place the rack containing the tubes in a 37°C water bath and shake slowly for 30 minutes.

20 While the reaction is running, prepare a sheet of Whatman #3 chromatography paper, marking lanes for each sample, 3 cm wide.

Terminate the reaction by chilling the tubes in ice water.

25 Take a 5 μ l sample of 3 random assays, spot each onto a small square of assay paper, put these into scintillation vials, set aside until chromatogram is done. Spot 20 μ l of the reaction on the paper in the middle 1 cm of each lane. Let the paper dry. Spot 5 μ l of cystathionine/serine standard on each end lane of the paper. Place the papers in a chromatography tank.

30 Develop the chromatogram overnight (or appropriate time for size of the paper) using the following solvents:

Isopropanol	160 ml
Water	40 ml
Formic acid	12 ml

Dry the chromatogram until smell of formic acid is gone. Cut off the end lanes of the chromatogram, soak the strips in ninhydrin solution and dry briefly. Amino acid spots of serine and cystathionine will develop.

5

Align the stained strips to the chromatogram, mark the cystathionine spot (spot closer to start), including about 1/2 cm of unstained area around the cystathionine spot. Cut out areas in each lane corresponding to the cystathionine standard put into a scintillation vial, add 5 ml of scintillation fluid and count, also include previous 5 μ l samples.

10

The activity is calculated as follows:

$[S], \mu\text{mole} = \% \text{ Substrate consumed} \times \text{serine concentration in assay mixture}$

$\% \text{ Substrate consumed} = \text{CPM} - \text{blank} / \text{Total Counts}$

15 Activity, U = $[S] \mu\text{mole} / \text{incubation time (hour)}$

Specific Activity, U/mg = Activity/mg of enzyme in reaction mixture

2. An alternative embodiment:

In an alternative embodiment in which ninhydrin is used instead of scintillation, the assay may be conducted as follows.

20

Procedure

<u>Enzyme Dilution Buffer</u>	0.1M Tris pH 8.3
	240 micromolar PLP
<u>Reaction Cocktail</u>	0.154M Homocysteine
	0.099M Serine

25

Dilute these components in 0.1M Tris at pH 8.3.

<u>Ninhydrin Reagent</u>	333 mg Ninhydrin
	33.3 ml Acetic Acid Glacial
	11.1 ml Phosphoric Acid

30

Stir well until Ninhydrin is completely dissolved.

Cystathionine (CTT) Standards

31.25mM CTT (range from 31.25 mM to
0.4883 mM)

Make the dilution in 0.1N HCL

- 1.) Make serial dilution of the enzyme.
- 5 2.) Pipette 50 μ L of serially diluted enzyme, followed by 50 μ L of the reaction cocktail into wells of a micro titer plate (first plate), cover the plate.
- 3.) Incubate for 45 mins. at 37°C while shaking.
- 4.) On a second micro titer plate (the final plate), prepare enzyme blanks by adding 200 μ L of the ninhydrin reagent to wells followed by 6 μ L of the reaction cocktail and 6 μ L of
10 the diluted enzyme.
- 5.) Remove the first plate from incubator and pipette 12 μ L of each sample into the final plate. Also pipette 12 μ L of serially diluted 31.25 mM CTT standard into the final plate.
- 6.) Immediately add 200 μ L of the ninhydrin reagent to the samples and standards.
- 7.) Incubate the final plate for 10 minutes in a plate warmer at 95°C.
- 15 8.) Remove plate from plate warmer and place on ice for 5 minutes.
- 9.) Read the end point absorbance at 450nm.

Calculations:

Draw the standard curve for the CTT standard samples. The equation for calculating the
20 amount of CTT formed in micromoles/hr is:

$$[(S-B) / (A \times \text{Volume of sample in ml} \times \text{Reaction time in hr})] \times \text{DF}$$

where

S= absorbance of the sample

B= absorbance of the blank

25 A= absorbance of 1 micromole of standard cystathionine.

DF= the dilution factor of the enzyme

Enzyme Units

An enzyme unit is defined as the micromoles of cystathionine formed per hour under
the assay conditions.

30

III. Homocysteine Assay Method**A. A first embodiment:**

Trinder Reagent:

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PCT/US99/26989

	Component	Concentration*
	Tris (pH 8.0)	0.1M
	4-aminoantipyrine (4-AAP)	1.6mM
	N-ethyl-N-(2 hydroxy-3-sulfopropyl)	
5	-m-toluidine (TOOS)	1.6mM
	Thiamine pyrophosphate (TPP)	0.77mM
	MgCl ₂	2.6mM
	KH ₂ PO ₄	19.3mM
10	Enzymes & Cofactors:	
	Component	Concentration*
	BSA	0.012mg/mL
	Pyridoxal 5' phosphate (PLP)	0.1mM
	Peroxidase	0.45 U/mL
15	pyruvate oxidase	10.7 U/mL
	CBL	0.4 U/mL
	CBS	90 U/mL
	Serine:	
	Component	Concentration*
20	Serine	5mM

*Concentration is the final concentration in the Reagent

Enzymes and Cofactors are added to the Trinder reagent just prior to use. Enzymes
25 and Cofactors are stored as stocks.

5 μ L serine stock (230 mM) and 25 μ L homocysteine containing Sample are added to
reaction well.

30 200 μ L of reagent (Trinder+Enzymes & Cofactors) is added to the serine and
homocysteine containing sample.

Reaction is incubated at 37°C (with mixing) for 20 minutes.

Color development is measured at 570 nm.

The calculated linearity of homocysteine concentration is shown in Figure 2. In this figure, the absorbance of released pyruvate is shown against the concentration of homocysteine.

B. A second embodiment:

Plasma and/or serum samples containing homocysteine were reduced using 2.5 mM DTT in the buffer containing 12.5 mM sodium citrate buffer at pH 7.8, 1% Triton, and 0.5 mM EDTA. The treated solution was capped and incubated for 20 minutes at 25°C then was uncapped and placed on the analyzer wheel. 20 microliters of sample and 5 microliters of water were added to 72 microliters of Reagent 1 and 92 microliters of Reagent 2. Reagent 1 was added at time point T1 (0 minutes), and Reagent 2 was added at T2 (about 20 seconds afterwards). Read points were at 25 and 50 (about 8 minutes). The assay was read at 550nm, using the Cobas Mira analyzer.

Reagent composition was as follows:

20	<u>R1 Reagent Composition</u>	<u>R2 Reagent Composition</u>
	2.56 mM serine	2.42 U/mL CBL
	1.15 U/mL peroxidase	0.2 mM PLP
	27.3 U/mL pyruvate oxidase	360 U/mL CBS
	10 mg/mL BSA	10 mg/mL BSA
25	127.8 mM Tris buffer at pH 7.8	100 mM Tris buffer at pH 7.8
	6.6 mM MgCl ₂	3.2 mM TOOS
	3.4 mM 4-AAP	80 mg/ml Gentamycin
	49.3 mM KH ₂ PO ₄	
	1.97 mM TPP	
30	80 mg/ml Gentamycin	

Results obtained with the above-described assay, as compared to the use of HPLC and standardized samples of homocysteine, are as follows. The automated HPLC procedure for measuring homocysteine was conducted in accordance with T.

- Fiskerstrand, H. Refsum, G. Kvalheim, and P.M. Ueland, Clin Chem 39:263-271 (1993). As these results show, the assay of the invention provides accurate determinations of homocysteine concentration in patient samples, as compared to the HPLC automated procedure that has been used in the art as a standard for
- 5 homocysteine quantification analysis.

**Homocysteine
Cobas Mira vs HPLC**

- 10 Mira was calibrated with homocysteine (HCY) Control (BioRad 10) at 10 micromoles/L. All samples were reduced before assay. For the purposes of the chart below, "umol/L" shall be understood to mean micromoles per liter.

Sample	HCY (umol/L)	HCY (umol/L)	Difference (umol/L)	%Difference
	Genzyme MIRA	PBI HPLC	Genzyme - HPLC	Genzyme - HPLC
4	7.63	6.60	-1.03	-15.6%
5	10.54	11.40	0.86	7.5%
10	8.16	9.50	1.34	14.1%
16	9.30	9.70	0.40	4.1%
17	7.47	7.10	-0.37	-5.2%
18	7.23	8.50	1.27	15.0%
24	6.73	8.10	1.37	16.9%
26	7.93	9.60	1.68	17.4%
29	6.72	6.70	-0.02	-0.4%
30	6.96	8.20	1.24	15.1%
35	7.37	5.80	-1.57	-27.1%
38	8.28	9.70	1.42	14.6%
44	7.57	8.70	1.13	13.0%
45	7.40	8.10	0.71	8.7%
49	7.06	7.50	0.45	5.9%
50	5.25	5.20	-0.05	-0.9%
57	6.15	7.90	1.76	22.2%
BIORAD10	9.69	10.00	0.31	3.1%
Mean	7.64	8.24	0.60	6.0%

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing
5 from the scope of the invention encompassed by the appended claims.

What is claimed is:

1. A method for determining homocysteine concentration in a sample comprising the steps of:
 - 5 a) condensing homocysteine in the sample using serine and an enzyme cystathionine β -synthase to form cystathionine;
 - b) releasing at least one of pyruvate and ammonia from cystathionine and regenerating homocysteine using an enzyme cystathionine β -lyase; and
 - 10 c) cycling steps a and b to release said at least one of pyruvate and ammonia at rate that can be correlated to the concentration of homocysteine in the sample.
2. The method of Claim 1 wherein the rate of release of said at least one of
15 pyruvate and ammonia is correlated with standard homocysteine values of concentration.
3. The method of Claim 1 wherein the condensing step further includes
20 subjecting the sample to a reducing agent in a sufficient amount to produce free homocysteine.
4. The method of Claim 3 wherein the reducing agent is dithiothreitol.
5. The method of Claim 4 wherein said dithiothreitol is provided in a
25 concentration of up to approximately 30 millimolar.
6. The method of Claim 1 wherein at least one of the enzyme cystathionine β -
30 synthase and the enzyme cystathionine β -lyase is subjected to an enzyme co-factor.
7. The method of Claim 6 wherein said enzyme co-factor is pyridoxal 5'
phosphate provided in a concentration of at least approximately 5 micromolar.

8. A method for determining homocysteine concentration in a sample, comprising the steps of:
- a) condensing homocysteine in the sample using serine and an enzyme cystathionine β -synthase to form cystathionine,
 - 5 b) releasing an amount of at least one of pyruvate and ammonia from cystathionine and regenerating homocysteine by use of an enzyme cystathionine β -lyase, and
 - c) measuring the amount of at least one of pyruvate and ammonia released and correlating that amount to the concentration of homocysteine present
- 10 in the sample.
9. The method of Claim 8 wherein the measuring step further includes measuring the released amounts of at least one of pyruvate and ammonia over time.
- 15 10. The method of Claim 8 wherein the rate of release of said at least one of pyruvate and ammonia is correlated with standard homocysteine values of concentration.
- 20 11. The method of Claim 8 wherein the condensing step further includes subjecting the sample to a reducing agent in a sufficient amount to produce free homocysteine.
12. The method of Claim 11 wherein the reducing agent is dithiothreitol.
- 25 13. The method of Claim 12 wherein said dithiothreitol is provided in a concentration of up to approximately 30 millimolar.
14. The method of Claim 8 wherein at least one of the enzyme cystathionine β -synthase and the enzyme cystathionine β -lyase is subjected to an enzyme co-
- 30 factor.
15. The method of Claim 14 wherein said enzyme co-factor is pyridoxal 5' phosphate provided in a concentration of at least approximately 5 micromolar.

16. A method according to claim 1 for measuring homocysteine concentration in a patient sample, comprising the steps of:
- a) condensing homocysteine in the sample using serine and an enzyme cystathionine β -synthase to form cystathionine;
 - 5 b) releasing pyruvate and ammonia from cystathionine and regenerating homocysteine by a reaction using an enzyme cystathionine β -lyase; and
 - c) cycling steps a) and b) to release pyruvate and ammonia at a rate that can be correlated to the concentration of homocysteine in the patient sample.
- 10
17. A kit for the determination of homocysteine concentration in a sample comprising an enzyme cystathionine β -synthase, an enzyme cystathionine β -lyase and serine.
- 15
18. The kit of Claim 17 that further comprises pyridoxal 5' phosphate.
19. The kit of Claim 17 that further comprises a reducing agent.
- 20 20. The kit of Claim 19 wherein said reducing agent is dithiothreitol.

CYCLIC ASSAY FOR HOMOCYSTEINE

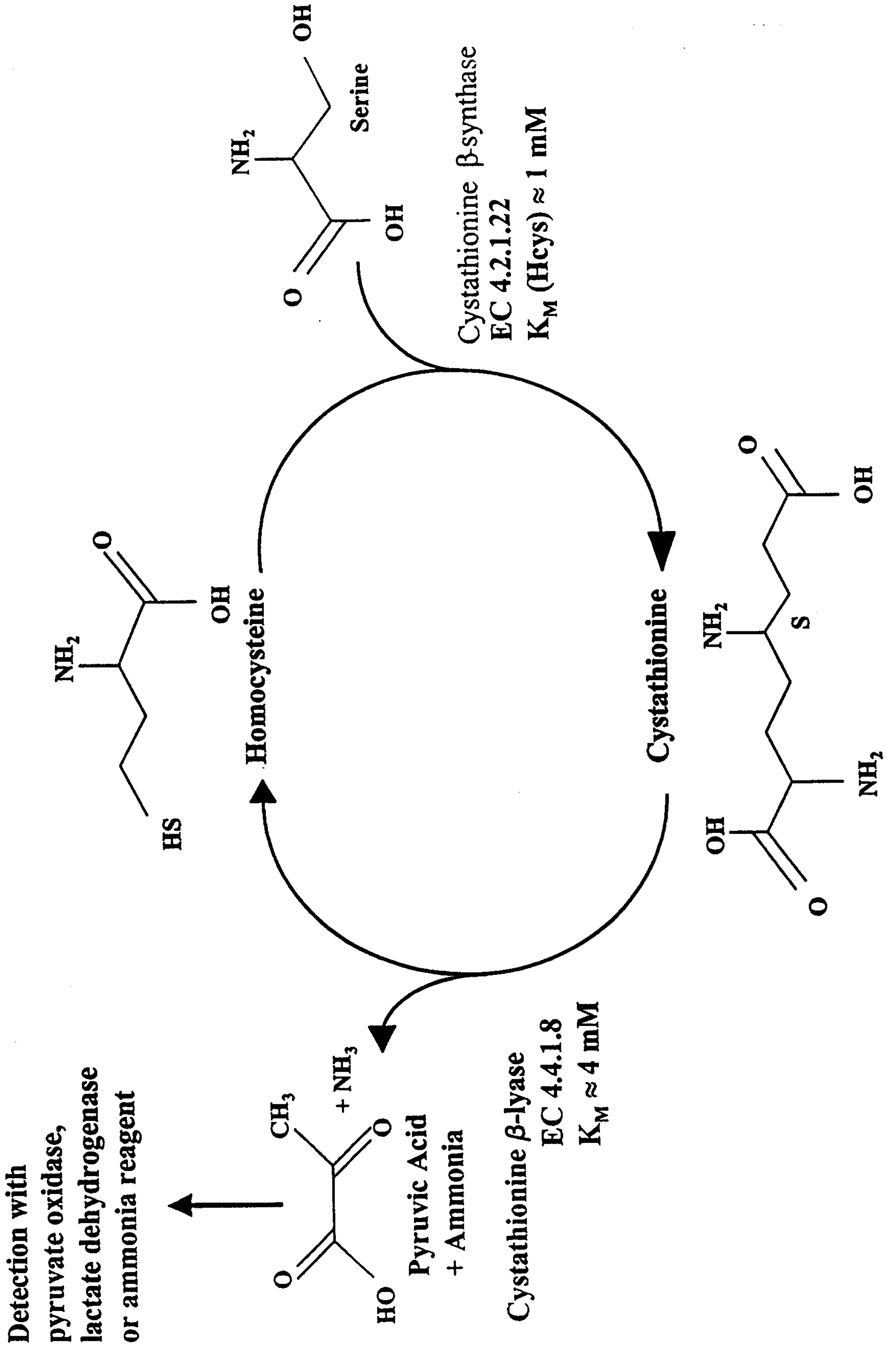


FIGURE 1

SUBSTITUTE SHEET (RULE 26)

Linearity of Homocysteine

Components Concentration : BSA, 0.012 mg/ml; pyridoxal 5 phosphate (PLP), 0.1 mM;
Peroxidase, 0.45 units/ml; Pyruvate oxidase, 10.7 units/ml; CBL, 0.4 units/ml;
CBS, 90 units/ml; Serine, 5mM.

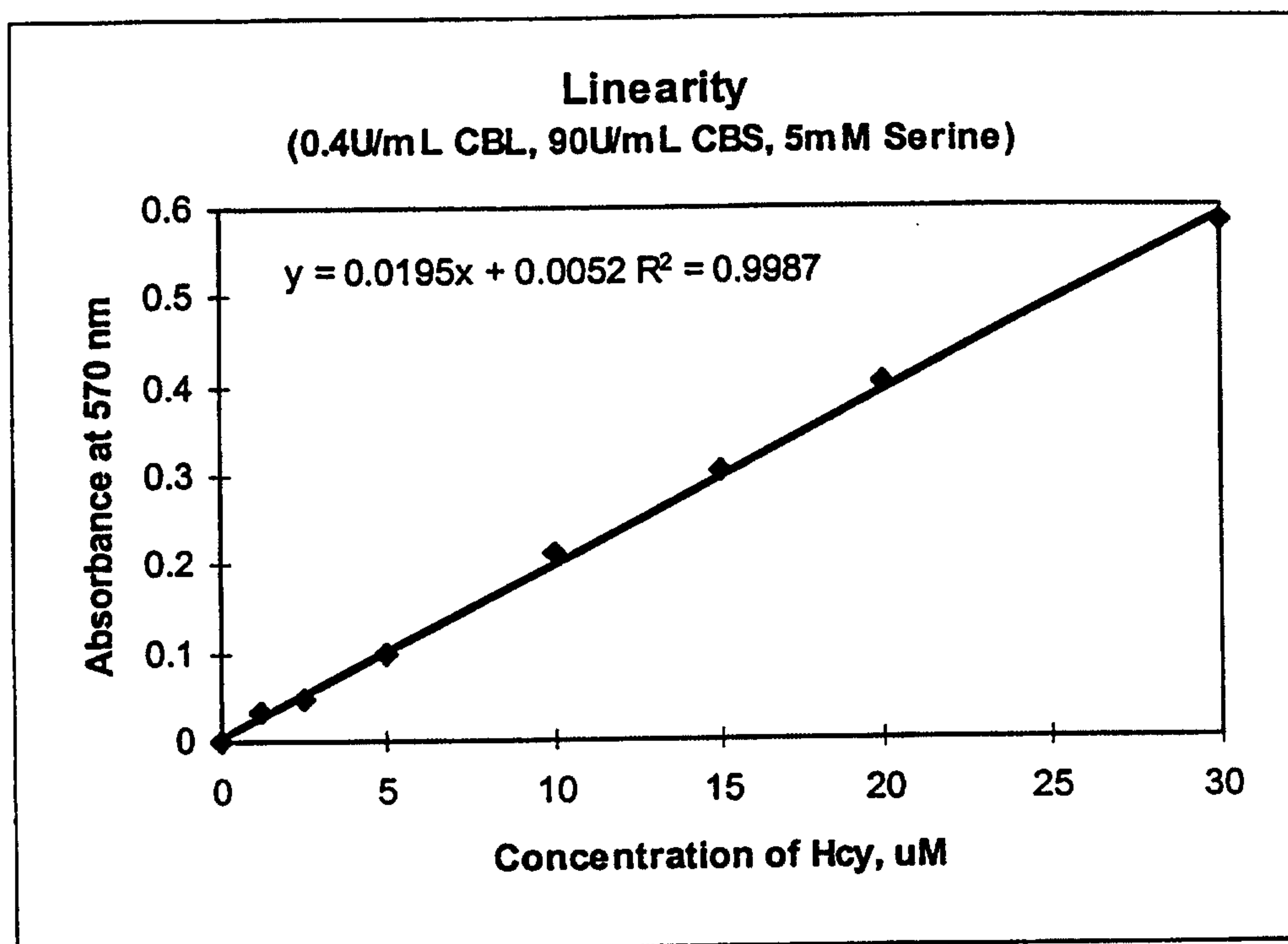


FIGURE 2