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A liquid chromatography mass spectrometry method for the measurement of cystathionine β-synthase activity in cell extracts

Desirée E.C. Smith[a], Marisa I.S. Mendes[a,b], Leo A.J. Kluijtmans[c], Mirian C.H. Janssen[d], Yvo M. Smulders[e,f], Henk J. Blom[a,f,*]

[a] Department of Clinical Chemistry, VU University Medical Center, Amsterdam, The Netherlands
[b] Research Institute for Medicines and Pharmaceutical Sciences, University of Lisbon, Portugal
[c] Department of Laboratory Medicine, Laboratory of Genetic, Endocrine and Metabolic Diseases, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands
[d] Department of General Internal Medicine, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands
[e] Department of Internal Medicine, VU University Medical Center, Amsterdam, The Netherlands
[f] Institute for Cardiovascular Research, ICaR-VU, VU University Medical Center, Amsterdam, The Netherlands

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ABSTRACT

Background: In order to correctly assess the efficacy of therapy or diet in intervention studies on the activity of cystathionine β-synthase (CBS) a sensitive analytical method is necessary. *Methods*: An electrospray LC–MS/MS method preceded by a solid phase extraction step was developed for the measurement of CBS activity in cell extracts. Nonafluoropentanoic acid was used as an ionpair to provide the underivatized cystathionine the desired retention on a C18 column. *Results*: A detection limit of 50 pmol cystathionine/h/mg protein was achieved. In fibroblasts, intra- and inter-assay CVs for the CBS activity were 5.2% and 14.7%, respectively. A K_m value of 8 μmol/L for homocysteine, and 2.5 μmol/L for serine was calculated. In fibroblasts wildtype, heterozygous, and homozygous CBS activity ranges measured were 8.5–27.0, 4.2–13.4, 0.0–0.7 nmol/h × mg protein, respectively. The method was applied to a study where rats were fed 2 diets. Increase of dietary methionine (7.7 versus 3.8 mg/kg methionine) significantly increased the CBS activity in rat liver lysates from a median of 58.0 to a median of 71.5 ($P = 0.037$) nmol/h × mg protein. In a lymphoblasts cell culture experiment, the addition of Hcy to the culture media increased the activity of CBS 3 fold. *Conclusion*: This LC–MS/MS is able to diagnose CBS deficiency at the enzyme level, and can accurately measure the effect diets or therapy might have on the CBS activity in a variety of cell types.

1. Introduction

In one-carbon metabolism the enzyme cystathionine β-synthase (CBS) condenses homocysteine (Hcy) and serine to form cystathionine (Cysta) (Fig. 1). When gene mutations disturb the function of this enzyme, Hcy accumulates resulting in severe hyperhomocysteinemia and homocystinuria. CBS deficient patients display a wide range of clinical manifestations such as ectopia lentis, skeleton malformations, mental retardation, and suffer from early thromboembolic events [1,2].

The majority of individuals heterozygous for CBS deficiency are considered to have no clinical manifestations, but they may have elevated Hcy, in particular after methionine loading [3]. While CBS polymorphisms may not relate directly to hyperhomocysteinemia [4], a combination of the CBS 844ins68 polymorphism and the methylenetetrahydrofolatereductase 677TT polymorphism does show an increased risk of cardiovascular disease [5]. Additionally, a 31 bp VNTR in the CBS gene affects plasma Hcy levels [6,7]. Since mildly elevated plasma levels of Hcy have been correlated to the aforementioned disorders like cardiovascular disease and neural tube defects, it is necessary to understand the link between increased plasma Hcy levels, cellular CBS function and these types of disorders [8].

The association between CBS gene variants and the clinical entities mentioned, raises the question whether CBS could be a valid target for homocysteine-lowering therapies. Hcy is located on an intersection of two pathways in the 1C metabolism. It can remethylated to form methionine (and subsequently to methyl group donor S-adenosylmethionine (SAM)), or it can be irreversibly converted to Cysta by CBS. Either of these steps could be disturbed, however

Abbreviations: CBS, cystathionine β-synthase; Hcy, homocysteine; Cysta, cystathionine; SAM, S-adenosylmethione; EDTA, ethylenedinitrilotetraacetic acid; PLP, pyridoxal-5-phosphate; SAH, S-adenosylhomocysteine.

* Corresponding author at: Department of Clinical Chemistry, VU University Medical Center, De Boelelaan 1117, 1081 HV Amsterdam, The Netherlands. Tel.: +31 204442880; fax: +31 204440305.
E-mail addresses: d.smith@vumc.nl (D.E.C. Smith), h.blom@vumc.nl (H.J. Blom).

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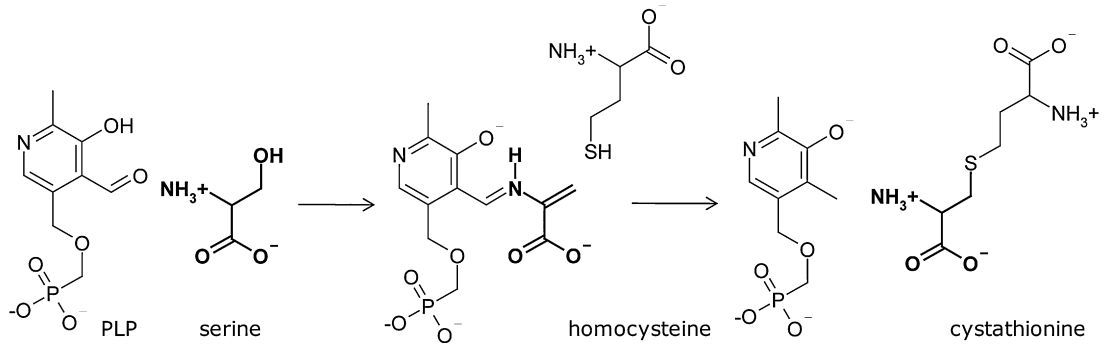

Fig. 1. Chemical formation of cystathionine.

only the conversion to Cysta removes Hcy completely from the 1C cycle. CBS is responsible for about 40–50% of the conversion of Hcy [9]. A mutation in the C-terminal regulatory domain of the CBS gene is able to increase its activity and, hence, lower plasma Hcy [10]. This may provide an option for future Hcy-lowering therapies.

CBS is mainly active in liver, kidney and pancreas [11]. The activity in other cell types is low. Whether CBS activity can influence Hcy levels in cell types like human umbilical vein endothelial cells, a well-established cell model to study vascular disease, remains to be investigated. However, one study did not observe any difference in Hcy export between control and CBS deficient endothelial cells, indicating that CBS is most likely hardly expressed in this cell type [12]. The fact that CBS activity in most cell types is low, warrants a sensitive method for measuring the enzyme activity. To date several methods have been published for the measurement of CBS activity in cell extracts, ranging from radioisotopes to LC–MS/MS [13–18]. However, most of them have proven to be rather laborious and not sensitive or specific enough. Methods using radioisotopes are very sensitive. However, they lack an internal standard which lowers their precision and makes them less suitable to detect subtle changes during cellular manipulations or due to diet. The same applies for spectrophotometric and HPLC methods. Due to the availability of labeled internal standards, LC–MS/MS has become a very specific and reliable analytical technique. It has already been successfully used for the determination of the CBS activity in plasma and cells [14,15].

This paper describes a sensitive straightforward LC–MS/MS method for the assessment of the CBS activity in cell extracts, useful in diagnosis of CBS deficiency and cellular studies on CBS function. As an example we demonstrate the efficacy of the method by measuring CBS activity in liver of rats on a high methionine diet and in lymphoblasts exposed to homocysteine.

2. Materials and methods

2.1. Materials

L-Homocysteine thiolactone hydrochloride (purity >98%), L-serine (purity >99%), S-(5′-adenosyl)-L-methionine p-toluenesulfonate (purity >80%), pyridoxal 5′-phosphate monohydrate (purity >97%), DL-Cysta (purity >90%) and DL-dithiotreitol (purity >98%) were purchased from Sigma (Deiselhofen, Germany). Potassium dihydrogen phosphate, di-potassium hydrogen phosphate trihydrate, ammonia 25%, tris(hydroxymethyl)aminomethane, Titriplex® III (ethylenedinitrilotetraacetic acid disodium salt dihydrate, and sodiumhydroxide were purchased from Merck (Darmstadt, Germany). Hydrochloric acid 37%, acetonitrile, and methanol were purchased from VWR (West Chester, USA). Lubrol was purchased from MP Biomedicals (OH, USA). Nonapentafluoric acid (purity >97%) was purchased from Acros (Geel, Belgium). [D_4]-Cysta (purity >98%) was purchased from C/D/N Isotopes Inc. (Quebec, Canada).

L-Homocysteine was prepared by incubating L-homocysteine thiolactone in 4 mol/L sodiumhydroxide at 37 °C for 5 min. After incubation an equimolar concentration of dithiotreitol was added, and the pH of the solution was adjusted to 7–8.

2.2. Fibroblast cultures

Human skin fibroblasts taken from confirmed CBS patients, parents and unrelated controls. The fibroblasts were grown in Ham-F10 Medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Invitrogen), and 1% (v/v) penicillin-streptomycin (Invitrogen). Cultures were grown in 175 cm^2 culture flasks (Greiner Bio One, Frickenhausen, Germany) and maintained at 37 °C in an atmosphere of 5% CO_2. The cells were harvested with trypsin (Invitrogen) after reaching confluence, washed twice with Hank's buffered salt solution (Invitrogen). Cell pellets were stored at −80 °C.

2.3. Lymphoblast cultures

A human control lymphoblast cell line was exposed to medium containing either 5 μmol/L or 50 μmol/L Hcy for 4 passages. Lymphoblasts were grown in RPMI medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Invitrogen), and 1% (v/v) penicillin–streptomycin (Invitrogen). Cultures were grown in 175 cm^2 culture flasks (Greiner Bio One, Frickenhausen, Germany) and maintained at 37 °C in an atmosphere of 5% CO_2. The cells were harvested by centrifuging the cells for 6 min at 500 × *g*. Subsequently, the cells were washed twice with Hank's buffered salt solution (Invitrogen). Cell pellets were stored at −80 °C.

2.4. Rat liver homogenates

Hyperhomocysteinemia in rats was induced by increasing the methionine content of their food [19]. At the age of 4 weeks female Wistar rats were divided in 2 groups. The control group was fed a standard rodent chow (containing 3.8 mg/kg methionine). The second group was fed a diet containing 7.7 mg/kg methionine. The diets were matched for kilocalories, and the mice were allowed free access to food and water. The mice were sacrificed after 8 weeks on the diet. Approximately 10 mg of liver was homogenized on ice in 50 mmol/L phosphate buffer (pH = 7.4) using Potter-Elvehjem tubes.

2.5. Preparation of enzyme extracts

Frozen cell pellets were suspended in a 50 mmol/L potassium phosphate buffer (pH = 7.4) containing 0.1% lubrol, and allowed to lyse on ice for 30 min [20]. The cell suspension was subsequently centrifuged for 10 min at 10,000 × *g*. The supernatant was used for the enzyme assay. The protein concentration was determined by the bicinchoninic acid protein assay (Sigma, Deiselhofen, Germany).

2.6. Enzyme assay

The enzyme assay was based on a previously published method [11]. Fifty microliter of enzyme extract was placed on ice, and 12.5 μL 0.8 mol/L Tris/HCl buffer pH = 8.6, 12.5 μL 64 mmol/L L-serine, 10 μL 50 mmol/L ethylenedinitrilotetraacetic acid (EDTA), and 10 μL of water (in case of stimulation by PLP or SAM this amount was replaced by either 5 μL 20 mmol/L pyridoxal 5-phosphate or 5 μL 2 mmol/L S-adenosyl-L-methionine). In order to start the reaction 15 μL of 20 mmol/L L-homocysteine was added. The samples were mixed, and placed in a water bath. The samples were allowed to incubate at 37 °C for 4 h under atmospheric pressure. After 4 h the samples were placed directly on ice. Subsequently 50 μL of 6 mol/L hydrochloric acid and 500 μL of water were added. [D_4]-Cysta (0.25 nmol) was added as an internal standard. The samples were mixed thoroughly.

2.7. Sample clean-up

For sample clean-up 60 mg Mixed-Mode Cation Exchange Oasis cartridges (Waters, Milford, MA, USA) were used. The cartridges were conditioned with 500 μL of methanol, and 1 mL of water. After conditioning, the enzyme extract was applied to the cartridges. The cartridges were washed with 1.5 mL of water and subsequently eluted with 500 μL of 6 mol/L ammonia into vials. The ammonia fraction was evaporated under nitrogen at 45 °C, and redissolved in 500 μL of 5 mmol/L nonafluoropentanoic acid. The samples were stored at −20 °C until analysis.

2.8. Liquid chromatography–tandem mass spectrometry

All analyses were performed on an API 3000 triple quadrupole tandem mass spectrometer (Applied Biosystems, Foster City, CA, USA) with a Perkin-Elmer Series 200 HPLC pump and a Perkin-Elmer Series 200 auto sampler (operated at 4 °C). Using an Xterra MS C18 analytical column (2.9 mm × 100 mm; 3.5 μm; Waters) 5 μL of the sample was separated using a mobile phase containing 5 mmol/L nonafluoropentanoic acid. In 9 min the acetonitrile content of the mobile phase was increased from 10% to 50%. The turbo ion electrospray was operated in positive ion mode, the cone temperature was set to 450 °C and the cone voltage was 5000 V. Nitrogen was used as the turbo ion gas at a flow rate of 8 L/min. Collision induced dissociation was initiated using nitrogen as the collision gas at a pressure of 0.06 kPa. The collision energy was set to 21 V, the focusing potential to 260 V, and the declustering potential to 41 V. All MS/MS experiments were performed using unit resolution. For each precursor fragment transition, a dwell time of 150 ms was applied. The mass spectrometer was optimized for Cysta using constant infusion with a Harvard Apparatus Pump 11 infusion pump (Harvard Apparatus, Inc., MA, USA). The LC–MS/MS data were acquired and processed using Analyst software (Applied Biosystems).

2.9. CBS genotype of the fibroblasts

Genomic DNA was isolated from fibroblast and the mutations in the CBS gene were assessed as described previously [21].

2.10. Method validation

The sensitivity of the method was assessed by estimating the limit of quantification (signal-to-noise >10) for Cysta in a cell lysate sample with a protein concentration of 1 mg/mL. The precision of the method was determined by obtaining intra- ($n = 5$) and inter-assay ($n = 8$) variation for a control fibroblast cell line and a control lymphoblast cell line. The accuracy of the method was determined by spiking a cell lysate with 5 different known amounts (0.1, 0.3, 3.4, 7.9, and 16.9 nmol) of Cysta and calculating recovery estimates. Linearity was assessed by construction of a calibration curve. The stability of the enzyme was assessed by 3 freeze–thaw cycles of the cell pellet, and during storage in a −80 °C freezer for 2 years. Furthermore, the CBS activity was assessed in high passage (over 20) fibroblasts cultures.

2.11. Calculations and statistics

CBS activity (nmol/h × mg protein) was assessed using stable isotope dilution calibration curves. The ratio analyte peak area/internal standard peak area was plotted against the concentration. Least-squares linear regression analysis was used to fit a line to the data points. CBS activity was calculated by dividing the measured Cysta concentration by the incubation time (4 h) and the protein concentration. SPSS 17.0 was used for all statistical calculations. In order to establish whether the a high methionine diet diets yielded an altered CBS, differences were analyzed using one-way ANOVA.

3. Results

3.1. Liquid chromatography tandem mass spectrometry

Cysta exhibited an intense protonated molecular ion under positive turbo electrospray conditions. After collision-induced dissociation Cysta showed the neutral loss of the ($COOHC(CH_2)HNH_2$) moiety. This was confirmed by the product ion produced by the D_4 labeled Cysta. The following transitions were used: 223.1 → 134.0 and 227.1 → 138.0 for Cysta and [D4]-Cysta, respectively.

Since the cation exchange clean-up used is not very specific, and large quantities of serine and homocysteine were added to the incubation mixture, it was investigated whether Cysta suffered from ion suppression in the different cell types [22]. This was however not the case.

3.1.1. Sensitivity, precision, and accuracy

The quantification limit (signal/noise >10) for the CBS assay is 0.05 nmol/h × mg protein (for a sample with a protein concentration of 1 mg/mL). The intra- ($n = 5$) and inter-assay ($n = 6$) CVs for a control fibroblast cell line were <5.2% and <14.7%, respectively. Intra- and inter-assay CVs for a control lymphoblast cell line were <6.0% and 19.3%, respectively over the time period of 2 years. The average recovery (five different concentrations) in relation to the internal standard was 99.7 ± 2.1%.

3.1.2. Linearity

The calibration curve for Cysta was linear over a concentration range of 0–40 nmol/L. Correlation coefficients for these calibration curves were 0.9998 ± 0.0002 (inter-assay, $n = 5$).

3.2. Enzyme assay parameters

For the optimization of all enzyme assay parameters, a control fibroblast cell line was used.

3.2.1. pH, time and protein dependence

The CBS activity at pH values between 7 and 10 was assessed. The enzyme reached maximum activity at a pH of 8.6. This correlates well with previously reported methods [11,23]. The assay was linear up to 300 min of incubation at 37 °C. The assay showed linearity for a protein concentration of de cell lysate between 0.4 and 3 mg/mL (20–150 μg protein per assay).

3.2.2. K_m for serine and homocysteine

Serine concentrations were varied from 0.1 to 22 mmol/L in the assay mixture. For serine a K_m of 2.5 mmol/L was calculated using a Lineweaver–Burke plot. This value corresponded well with previously published methods, in which 2–5 mmol/L was estimated [24–26]. Homocysteine concentrations were varied from 0.1–9 mmol/L in the assay mixture. For homocysteine a K_m of 8 mmol/L was calculated using a Lineweaver–Burke plot. This also corresponded well with previously published results, that indicated a K_m of ~1–15 mmol/L [24–26].

3.2.3. PLP stimulation

In the concentration range of 0.03–3.7 mmol/L pyridoxal-5-phosphate (PLP), no effect on the CBS activity was observed.

3.2.4. Blank value

Even with no protein present, Cysta was formed when PLP was present. The addition of EDTA (optimal concentration 9 mmol/L in the assay mixture) prevented this.

3.3. Stability of the enzyme

3.3.1. General stability

The addition of protease inhibitor phenylmethylsulfonyl fluoride did not contribute to the stability of CBS in the cell extracts. Cell pellets stored at −80 °C are stable for at least 2 years. The first freeze–thaw cycle diminished the CBS activity by approximately 20%. Additional freeze–thaw cycles did not lower the CBS activity any further.

3.3.2. CBS activity in higher passage number

CBS activity in fibroblast cell lines up to 20 passage was constant. Fibroblasts cell lines showed a marked linear decrease in CBS activity in passages over 20. Passage number 35 showed only one third of the original CBS activity.

3.4. Regulation by SAM and SAH

As shown in Fig. 2, the addition of SAM to the incubation mixture increased the CBS activity. The activity reached a plateau at concentration of 0.4 mmol/L. Addition of concentrations of S-adenosylhomocysteine (SAH) higher than 0.4 mmol/L to the incubation mixture decreased the CBS activity (Fig. 2).

3.5. Enzyme activity in fibroblasts

The range of CBS activities in fibroblast extract (wild type, heterozygous and homozygous CBS deficient) with and without the addition of PLP are listed in Table 1.

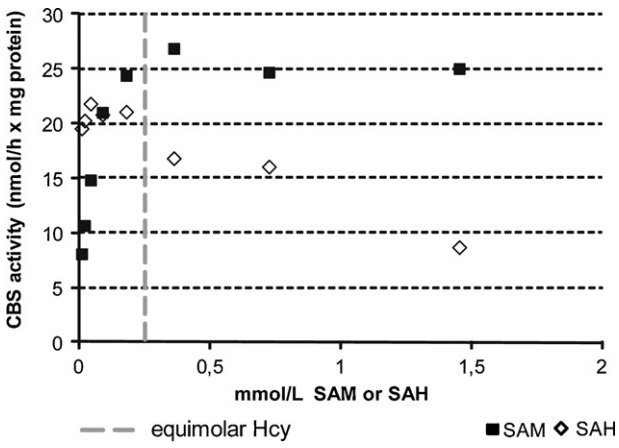

Fig. 2. Regulation of CBS activity by SAM and SAH.

Table 1
CBS activity in fibroblast cell lines according to their genotype.

Genotype	CBS activity (nmol/h × mg protein)			
	−PLP		+PLP	
	Median	Range	Median	Range
CBS wild type (n = 10)	16.3	8.5–27.0	17.4	8.4–29.9
CBS heterozygous (n = 7)	5.1	4.2–13.4	5.7	4.7–13.1
CBS homozygous (n = 10)	0.2	0.0–0.7	0.3	0.0–0.8

3.6. Effect of homocysteine in the medium on CBS activity and Cysta concentrations in lymphoblasts

Exposure of a control lymphoblast cell line to 5 μmol/L of Hcy in the medium for 4 passages increased the CBS activity from 2.9 to 10.6 nmol/h × mg protein. Exposure of the same cell line to 50 μmol/L of Hcy in the medium for 4 passages increased the CBS activity of 3.7–13.6 nmol/h × mg protein (Fig. 3). Cysta levels in the medium increased from 3.3 to 5.3 μmol/L for cells exposed to 5 μmol/L Hcy for 4 passages, and from 4.5 to 21.3 μmol/L for cells exposed to 50 μmol/L Hcy for 4 passages (Fig. 3). Intracellular Cysta levels increased from 4.2 μmol/L to 36.0 nmol/mg protein for cells exposed to 5 μmol/L Hcy for 4 passages, and from 7.0 to 45.2 nmol/mg protein for cells exposed to 50 μmol/L Hcy for 4 passages (Fig. 3).

3.7. Effect of dietary methionine on CBS activity in rat liver

Increase of dietary methionine (3.8 versus 7.7 mg/kg methionine) significantly increased the plasma Hcy levels from a median of 7.1 (range 5.7–8.5) to 11.3 (range 9.7–14.6) (P < 0.001). The CBS activity in rat liver lysates significantly increased (P = 0.037) from a median of 58.0 (range 18.2–81.6) to a median of 71.5 (range 46.9–90.0) nmol/h × mg protein (Fig. 4).

4. Discussion

A sensitive LC–MS/MS method was developed to assess the CBS activity in different kinds of cell extracts in order to diagnose CBS deficiency at the enzyme level and to evaluate the effects of diet or other manipulations on CBS activity in different cell types. Secondly, it might be used in newborn screening for homocystinuria, since a faster and more sensitive method is required for an accurate diagnosis.

Traditionally, CBS activity in cell extracts is measured by using radioisotopes. This type of method is characterized by high sensitivities with a limit of quantification ~30 pmol/h/mg

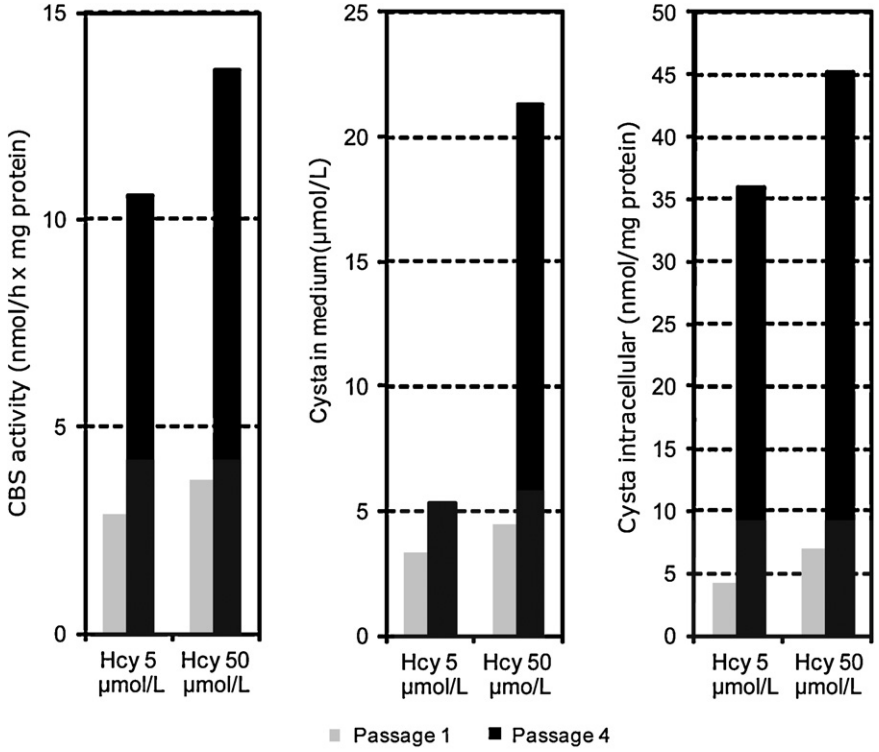

Fig. 3. CBS activity and Cysta concentration in lymphoblasts after exposure for 1 and 4 passages to two different concentrations of Hcy in the medium.

protein.[18] Our LC–MS/MS method and 2 other recently published methods offer comparable sensitivity [14,15]. However, to be able to measure small changes due to for example therapy or diet, a good precision is also required. Since LC–MS methods use stabile isotope dilution, this method usually offers the highest precision of the available techniques. Our intra-assay variation is higher than a previously published LC–MS/MS method (1.4%), however those activities were measured in plasma and expressed as nmol/h × L [14]. In our study, the CBS activity in cell extracts is expressed per mg protein, which makes the rather inaccurate spectrophotometric protein determination (intra-assay CV 6%) the dominant factor in variation. In addition, the overall analysis time was reduced from 2 days to only one for LC–MS/MS methods. Another benefit of a LC–MS/MS method is the possibility of up scaling it to 96-wells plates, which enables high-throughput analyses. For second-tier testing in newborn screening, LC–MS/MS methods offer the possibility of measurement of CBS activity in plasma or cells exhibiting low CBS activity, like peripheral blood mononuclear cells. This would considerably lower diagnosis time and costs since cell culture would not be required.

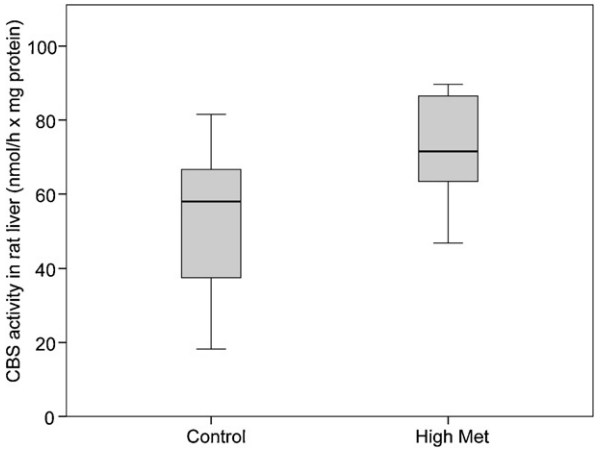

Fig. 4. CBS activity in rat liver.

In the diagnostics of inborn errors of homocysteine metabolism at enzyme level, CBS activity is usually measured in extracts of fibroblasts cell lines, so our method was validated using this cell type. The method was able to distinguish between CBS activity in controls and samples from homozygous deficient patients. Samples from obligate heterozygotes showed an overlap with controls, as published previously [27]. We observed that the addition of EDTA to the incubation mixture drastically decreased the blank values in samples containing PLP, suggesting that Cysta is also formed chemically without any enzyme present. Our finding may explain why another group found CBS activity to be 22% lower in EDTA plasma than in heparinized plasma [14]. This could also explain our observation of a the lack of difference between the CBS activity in cell extracts with and without PLP added (Table 1) which is in contrast with previously reported increased CBS activity on PLP addition to the incubation mixture [28,29]. This phenomenon may also be explained by the fact that the cell culture medium contains an excess of vitamin B6 and therefore CBS may be already saturated with PLP.

As could be concluded from the stability data, one freeze-thaw cycle influences CBS activity. Therefore, all samples should be treated in the same way to be able to compare them accurately.

The 1C metabolism is a well-regulated system. The universal methyl group donor SAM is one of its main regulators. One way is via activation of the enzymatic CBS activity [30,31]. When SAM levels rise, CBS activity is increased as is shown in Fig. 2. The effect of SAH on CBS is less defined. One report describes activation while another describes no effect at low concentrations [23,32]. In this paper, we demonstrated that only at concentrations above 250 µmol/L (when SAH equals Hcy concentrations in the incubation mixture) SAH inhibits CBS activity. One explanation may be a competition with available Hcy, which may be physiologically

relevant since intracellular concentrations of SAH are higher than Hcy (unpublished observations), and elevated plasma SAH levels are measured in conditions like hyperhomocysteinemia [33].

The value of our method is demonstrated by measuring significant changes of CBS activity in livers of rats with hyperhomocysteinemia induced by a variation in methionine content of their diet. From this animal experiment, it could be concluded that a relative modest increase of 2 fold in dietary methionine results in a significant elevation of CBS activity in liver. This corresponds well with previously published results [19]. An additional subject of potential relevance is that serine is needed for the conversion of Hcy to Cysta. When an overload of methionine induces CBS activity, serine pools could be depleted, which could affect other serine dependent reactions in the body as well. However, we did not observe a decrease in liver serine levels in rats on a high methionine diet (data not shown).

To show that this method is also able to detect changes in cell types with low CBS activity, we mimicked the previous experiment in lymphoblasts by culturing them for 1 and 4 passages on different Hcy concentrations. We observed that even addition of low concentrations of Hcy (5 μmol/L and 50 μmol/L) in the culture medium increased CBS activity. Lymphoblasts are not one of the cell types in which CBS is primarily expressed, however low concentrations of Hcy were able to induce CBS activity. The underlying mechanism for this phenomenon requires further study.

Small changes in plasma Hcy levels are associated with detrimental effects. Elevations of fasting plasma Hcy level as small as 3 μmol/L are associated with an increased incidence of ischemic heart disease (11%) and stroke (19%) [8,34]. To study whether small changes in CBS enzymatic activity could play a part in these conditions, a sensitive, straightforward and high throughput method is necessary. In this paper we describe such a method. We show that the method can be applied to different cell types, and is capable of detecting small changes in CBS activity due to dietary changes.

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