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Short communication

Total plasma homocysteine determination by liquid chromatography before and after methionine loading Results in cerebrovascular disease

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Abstract

Elevated homocysteine (HCY) levels in tissues and blood are associated with premature occlusive diseases. A number of techniques have been developed to assay HCY, including high-performance liquid chromatography (HPLC) with fluorimetric or electrochemical detection, and radioenzymatic methods. The present study evaluated the adaptation of a liquid chromatographic, ion-exchange technique with postcolumn derivatization using ninhydrin. Fasting and moreover post-methionine load total plasma HCY were assayed in 50 patients three months after a stroke and in 20 age-matched controls. Ion-exchange liquid chromatography was performed on an amino acid analyzer using a modified procedure to improve methionine and HCY separation. HCY values in the fasting state were moderately but significantly increased ($P<0.05$) in the patients compared to the controls: 10.5 ± 3.4 versus 9.3 ± 2.3 μmol/l. The difference between the two groups was amplified in post-load HCY results, which were significantly increased ($P<0.05$) in the patients: 41.6 ± 17.8 versus 29.2 ± 5.5 μmol/l in controls. The relationship between cerebrovascular disease and impaired HCY metabolism has previously been emphasized by other investigators. Our findings suggest that certain inherited and/or acquired HCY disorders observed in the fasting state (14%) and especially in post-methionine load conditions (32%) may occur during acute disease, and that total plasma HCY can be determined by ion-exchange chromatography even after oral methionine loading.

Keywords: Homocysteine; Methionine

1. Introduction

The hyperhomocysteinemia accompanying homocystinuria, a rare inherited disorder, may be responsible for the premature vascular disease with atherosclerosis observed in the pathology [1,2]. Hyperhomocysteinemia has a number of causes, including enzymatic deficits and deficiencies in the vitamin cofactors of homocysteine (HCY) metabolism (folates, vitamins B12 and B6). Based on the frequency of the homozygote form of cystathionine β-synthase deficiency (CBS), the most frequent

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cause of homocystinuria, the heterozygote form, is estimated to occur at between 1:70 and 1:200 in the normal population and is a putative cause of vascular accidents [3–5]. A recently identified enzyme defect on the remethylation pathway of HCY (thermolabile methylenetetrahydrofolate reductase) and an identified genetic mutation may also be implicated in vascular disease [6].

Total plasma HCY is generally assayed for study purposes. The majority of plasma HCY is protein-bound (70%); the remainder is spontaneously oxidized to homocystine or forms the homocysteine–cysteine disulfide, and only 2–3% remains as free HCY [7]. Procedures for determination of total plasma HCY include reduction of the protein-bound HCY and reduction of homocystine and homocysteine–cysteine mixed disulfide. Reduction may be followed by deproteinization. Several methods may be used to measure HCY. The high-performance liquid chromatographic technique (HPLC) developed by Refsum et al. [8] utilizes sodium borohydride as the reducing agent. HCY is measured by the detection of the fluorescence of the compound obtained by its condensation with monobromobimane. Interfering substances are eliminated by an initial column switch. This method has since been simplified and automated [9]. Other fluorophores, such as ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (SBD-F) [10] and OPA [11], can also be used for HPLC. HPLC with electrochemical detection, using a gold–mercury amalgam as the working electrode, is another highly specific technique for HCY assays, although optimization is complex [12]. HCY determination by gas chromatography has also been described [13]. A method with a low coefficient of variation featuring isotopic dilution has also been developed [14]. All of these methods are time-consuming. We determined total plasma HCY by ion-exchange chromatography and postcolumn derivatization with ninhydrin modifying a previously published method [15] to improve HCY and methionine separation, which is difficult after methionine loading. Adaptation was performed on an amino acid analyzer. This method allowed us to investigate HCY metabolism in cerebrovascular disease (CVD). Homocysteinemia was measured after oral methionine loading in 50 patients under 56 years of age 3 months after a stroke. Results were compared with values established for 20 age-matched controls.

2. Experimental

2.1. Method

Blood samples were drawn in tubes containing EDTA, placed immediately on ice, and centrifuged within 1 h at 3000 *g* for 10 min at +4°C. Heparin and sodium citrate are also acceptable anticoagulants. The plasma must be rapidly separated from the formed elements of the blood. Whereas, in vivo, the majority of circulating HCY is of hepatic origin, the erythrocytes and leukocytes in blood samples release HCY. Samples must, therefore, be placed on ice and centrifuged within 4 h [9]. Total HCY is stable for six months in plasma frozen at −20°C [16].

Total plasma HCY was measured on a Beckman Model 2300 amino acid analyzer (Beckman, Gagny, France) after reduction by dithiothreitol, according to Brattström et al. [17]. Calibration was performed using an authentic 12.5 μmol/l homocystine standard prepared in parallel with the samples by dilution of 1 mmol/l homocystine in water, corresponding to 25 μmol/l HCY. The absence of homocystine is checked regularly (reduction must be total in every assay serum); a sample of pooled plasma prepared monthly and kept frozen at −20°C was analyzed. Pooled plasmas spiked with three levels of homocystine, corresponding to loads of 12.5, 25 and 50 μmol/l HCY, were also analyzed.

The program of the amino acid analyzer was modified to improve separation of HCY and methionine. Samples were injected at a column temperature of 32°C and eluted by a buffer consisting of 2% lithium citrate, 1% lithium chloride and 1% hydrochloric acid in bidistilled water (Beckman Li-D buffer). The pH was adjusted from pH 3.0 to pH 2.8 with 1% hydrochloric acid. After 5 min, the column temperature was raised to 47°C (temperature gradient 2°C/min). Following a total program time of 36 min after the elution of methionine the column was regenerated for 2 min at high temperature (70°C), followed by a 14 min equilibration period with the pH-modified Li-D buffer.

2.2. Patients

Fifty patients (27 men, 23 women) with CVD under 56 years of age (mean 42.3; range 22–55) who

had been hospitalized for strokes affecting the carotid or vertebrobasilar territories were entered in the study. All patients underwent brain CT and/or MR; ultrasonography was performed systematically, sometimes completed by angiography. The possibility of an emboligenic etiology, a coagulopathy or non-atherosclerotic vascular disease was formally ruled out in all patients.

Total plasma HCY was assayed in all 50 patients in fasting and post-methionine load conditions. Post-load blood samples were obtained 6 h after an oral methionine load (0.1 g/kg). Fasting and post-methionine load homocysteinemia were also measured in 20 controls without vascular disease (8 men, 12 women) aged 23–54 years (mean 42.9). None of the subjects had diabetes or hepatic or renal disease; cholesterolemia was under 7.2 mmol/l in all subjects. Serum folate and vitamin B12 levels determined by microbiological assays were within normal ranges (respectively 3–20 mg/l and 200–1000 μg/l).

The statistical significance of differences between patient and control values was assessed with Student's *t*-test.

The study was approved by an Ethics Committee.

3. Results

Determination of total plasma HCY by ion-exchange liquid chromatography is simple, but the method described in the literature [17] was not applicable in our laboratory and required a number of modifications to be used with our amino acid analyzer. The external standard was diluted in water rather than in acid solution, because the latter gave much too high HCY-loaded plasma recoveries. In addition, the eluting buffer was acidified (pH 2.80 in our modified method versus pH 3.38 in the literature) to improve separation of HCY from methionine after oral methionine loading. The temperature gradient was 2°C/min. Recovery of 12.5, 25 and 50 μmol/l HCY added to a pooled plasma which initially contained 18 μmol/l was, respectively, 100%, 101% and 103% ($n=15$). When a post-load plasma sample exhibiting an increased methionine concentration was spiked with 50, 75 and 100 μmol/l HCY, recovery was, respectively, 104%, 106% and 111%. The coefficient of variation calculated for a pooled plasma sample (mean HCY 14.5 μmol/l, $n=30$) was 7.8%. Fig. 1 shows the chromatograms of fasting (A) and postmethionine load (B) homocysteine in plasma extracts.

3.1. Fasting homocysteinemia

Results are expressed as means ±1 S.D. for patients and controls. The mean fasting HCY concentration of the patients was moderately yet significantly increased ($P<0.05$) compared to the controls (10.5±3.4 μmol/l; range 3.3–20.0 versus

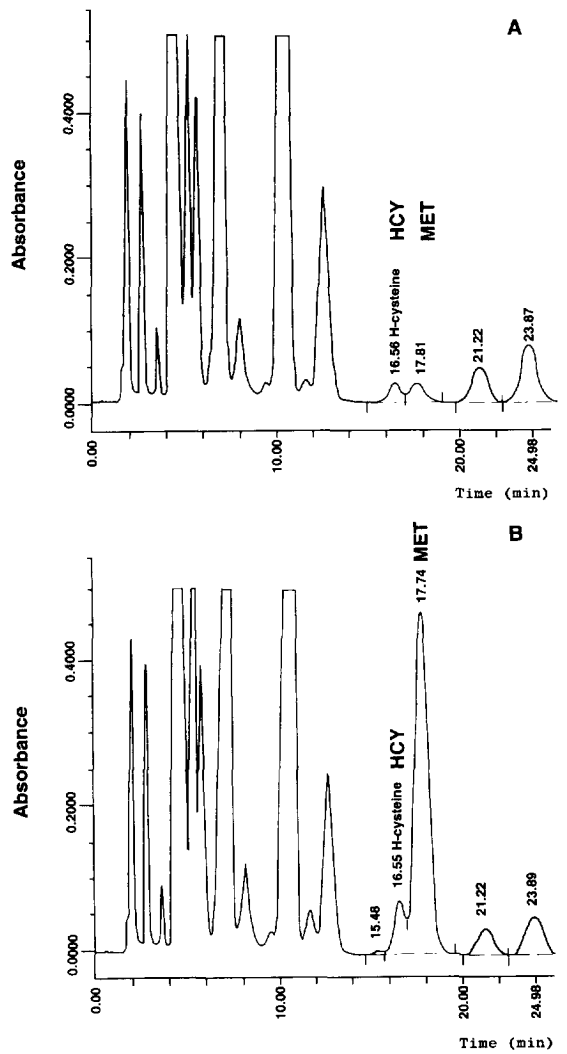

Fig. 1. Chromatograms of (A) fasting plasma HCY (24.6 μmol/l) and (B) post-methionine load HCY (69.7 μmol/l). Peaks: HCY= homocysteine; MET=methionine.

9.3±2.3; range 5.0–15.0 μmol/l). Seven patients (14%) had concentrations over 15 μmol/l (respectively, 15.5, 16.0, 16.4, 16.8, 17.0, 18.4 and 25.0 μmol/l). 15 μmol/l was the upper value of the controls and was similar to the literature data [18]. Three other patients had a HCY concentration equal to our control mean +1 S.D. (14.0 μmol/l).

3.2. Post-methionine load homocysteinemia

Mean HCY was significantly higher ($P \leq 0.05$) in the patients 6 h post-load: 41.6±17.8 μmol/l (range 15–110) versus 29.2±5.5 μmol/l (range 17–55) in the controls. Sixteen patients (32%) had an abnormally increased HCY response to methionine challenge: >45.6 μmol/l, according to the literature [19] (>40 μmol/l, HCY mean +2 S.D. in our control series), but 14/16 had a normal fasting HCY concentration (<15 μmol/l).

4. Discussion

Use of the previously published methodology failed to provide satisfactory results for plasma HCY in our laboratory. The chromatographic conditions described in this report allowed good separation between HCY and methionine. Under these conditions, excess HCY concentrations can be detected in pooled plasma, especially after an oral methionine load. Of course, all laboratories must establish their own normal values. While our values are similar to those in the literature, only 14% of our stroke patients had fasting HCY levels higher than those of the controls versus nearly 30% in the literature [20–22]. In contrast, our postmethionine load results were similar to those of Coull et al. [21], who observed an abnormal increase in HCY after methionine loading in 36% of patients with cerebral and peripheral occlusive vascular disease. Investigations of this type are valuable because they may demonstrate the therapeutic value of lowering HCY levels by innocuous means such as administration of folates and vitamins B6 and B12 [17,23].

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