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Determination of Total Homocysteine in Human Plasma by Isocratic High-Performance Liquid Chromatography

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Summary: A simple, sensitive and precise isocratic HPLC method for the determination of total homocysteine in human plasma is described. The thiol compounds were liberated from plasma proteins by reduction with tri-*n*-butylphosphine and derivatized with a thiol-specific fluorogenic marker, 7-fluoro-benzo-2-oxa-1,3-diazole-4-sulphonate. The derivatives were separated isocratically within 7 min by reversed-phase HPLC using a Superspher 100 RP-18 column as stationary phase.

By using this approach more than 200 samples a day can be assayed for total homocysteine. The method was linear up to 100 µmol/l and proved to be sensitive with a detection limit of 0.1 µmol/l and the lowest limit of reliable quantification of 0.5 µmol/l for homocysteine in buffer. Intra- and inter-assay coefficients of variation were both <4% at a concentration of 10 µmol/l homocysteine. Similar results were obtained for homocysteine concentrations between 0.5 and 100 µmol/l. The analytical recovery for these concentrations ranged from 94.9 to 117.0%.

As compared to other protocols published so far, this modified method is less complicated but equally sensitive and reproducible and allows a rapid determination of total homocysteine and cysteine in human plasma under routine conditions.

Introduction

Even mildly elevated concentrations of plasma homocysteine, referred to as hyperhomocyst(e)inaemia, are considered to be an independent risk factor for premature atherosclerosis resulting in coronary heart disease, stroke and peripheral vascular disease (1–4). Underlying causes for elevated plasma concentrations of homocysteine may be either deficiencies of enzymes of the degradation pathway of sulphur containing aminoacids like cystathionine β-synthase[1)] and 5-methylenetetrahydrofolate-homocysteine *S*-methyltransferase[1)] or of their respective cofactors such as vitamin B_6 , vitamin B_{12}, folic acid or betaine (5). Recently, a 677 C-T mutation in the methylenetetrahydrofolate reductase[1)] gene, leading to a thermolabile form of the enzyme, was shown to be an independant risk factor for neural tube defects (6).

The detection of homozygous patients with cystathionine β-synthase deficiency (homocystinuria) will not present difficulties in most cases due to the typical clinical symptoms and the markedly elevated basal concentrations of total homocysteine in plasma and urine (7).

However, the identification of patients with mildly elevated concentrations of homocysteine due to heterozygous deficiencies of cystathionine-β-synthase or 5-methyltetrahydrofolate-homocysteine S-methyltransferase or their respective cofactors is considered to be more difficult. In these cases fasting plasma levels of total homocysteine may not differ significantly from healthy subjects. Recent work demonstrates that these patients can only be reliably assessed by an oral methionine loading test (8).

In plasma, homocysteine mainly occurs as a disulphide conjugated to albumin (~70%), to a lesser extent as homocystinyl moiety or mixed disulphides and only a small amount exists as free homocysteine. It has been found that the small amount of free homocysteine does not reflect the diagnostically relevant total homocysteine pool (9,10). Moreover, free homocysteine escapes detection when the plasma is stored, since it is spontaneously bound to protein molecules (9,10). Routine amino acid analysis, however, usually measures free amino acids

1) Enzymes

Cystathionine β-synthase = α-serine hydro-lyase (adding homocysteine): EC 4.2.1.22

5-Methylenetetrahydrofolate-homocysteine *S*-methyltransferase = 5-methyltetrahydro pteroyl-α-glutamate : α-homocysteine S-methyltransferase: EC 2.1.1.14

Methylenetetrahydrofolate reductase (NAD^+) = 5,10-methylenetetrahydrofolate : NAD^+ oxidoreductase: EC 1.5.1.15

γ-Glutamyltransferase = (5-α-glutamyl)-peptide : amino-acid 5-glutamyltransferase: EC 2.3.2.2.

and is therefore not suitable for the detection of mild hyperhomocyst(e)inaemia. In addition, the commonly used post-column derivatization with ninhydrin does not seem to be sensitive enough for the reliable quantitation of physiological plasma concentrations of free or total homocysteine (1, 11).

Thus, a variety of high performance liquid chromatography (HPLC) methods have been established for the detection of total homocysteine in plasma (2,5,12–14). Most techniques, however, have proven to be difficult for routine application e. g. due to the requirement of gradient systems, demanding derivatization procedures or interferences. Therefore, the aim of this study was to establish a modified protocol allowing simple and high throughput analysis for routine purposes.

Materials and Methods

Reagents

7-Fluoro-benzo-2-oxa-1.3-diazole-4-sulphonate (SBD-F), tri-n-butylphosphine and N,N-dimethylformamide were obtained from Fluka (Ulm, Germany). *L*-Cysteine, *L*-homocysteine, glutathione, γ-glutamyltransferase[1]) and Na_2EDTA were purchased from Sigma (Deisenhofen, Germany). All other reagents were of analytical grade and obtained from commercial sources. Cysteinylglycine was prepared according to l.c. (15) with slight modifications.

HPLC equipment and chromatographic conditions

The HPLC system consisted of a M 480 pump connected to a GINA 160 autosampler with cooled racks (both Gynkotek, Germering, Germany) and a fluorescence spectrophotometer (F 1000, Merck/Hitachi, Darmstadt, Germany). As stationary phase a Superspher 100 RP-18 endcapped column (250×4 mm, 4 µm) protected by a guard-column LiChrospher 60 RP-select B, 5 µm (both Merck, Darmstadt, Germany) was used.

The mobile phase consisted of a 30 mmol/l ammonium-formiate – 40 mmol/l ammonium-nitrate buffer (adjusted to pH 3.65 with formic acid) and acetonitrile (95 + 5, by vol.). The mobile phase was filtered (0.2 µm, Costar, Cambridge, USA) and degassed prior to use. Flow rate was 1 ml/min and column temperature was maintained at 35 °C. The column effluent was monitored by fluorescence detection with an excitation wavelength of 385 nm and an emission wavelength of 515 nm. Data were recorded and processed using an integration software (Gynkosoft, Gynkotek, Germering, Germany).

Sample collection

Blood from healthy subjects was collected into prechilled EDTA-containing tubes (Vacuette, Greiner, Solingen, Germany) by venipuncture and immediately centrifuged at 2000 *g* for 10 min at 0 °C. Plasma was pooled and stored at −20 °C until analysis.

Sample preparation

Plasma (200 µl) was treated on ice with 20 µl 100 ml/l tri-*n*-butylphosphine in dimethylformamide for 30 min at 0 °C in order to reduce thiols and decouple them from the plasma proteins. Perchloric acid (200 µl, 0.5 mol/l, containing 1 mmol/l Na_2EDTA) was added to the sample, and the mixed sample was incubated for 10 min on ice. Protein was removed by centrifugation at 10 000 *g* for 10 min at 0 °C. Hundred (100) µl of the supernatant were mixed with 250 µl 2 mol/l borate buffer (containing 5 mmol/l Na_2EDTA, pH 10.5) and 100 µl 7-fluoro-benzo-2-oxa-1.3-diazole-4-sulphonate (1.0 g/l in 0.1 mol/l borate buffer, containing 2 mmol/l Na_2EDTA, pH 9.5) and incubated at 60 °C for 60 min. To terminate the reaction, the solution was snap-cooled on ice and transferred to a cooled autosampler vial. An aliquot of 20 µl was injected onto the column.

Preparation of standards and assessment of variability

To determine the intra-assay and inter-assay variability standard samples of 13 different concentrations of *L*-homocysteine (0.1 to 1000 µmol/l) and *L*-cysteine (0.3 to 3000 µmol/l) were prepared by serial dilution in 0.1 mol/l borate buffer, containing 2 mmol/l Na_2EDTA, pH 9.5. In a similar way plasma samples spiked with 11 different concentrations of *L*-homocysteine (0.5 to 1000 µmol/l) and *L*-cysteine (1.5 to 3000 µmol/l) were prepared.

To assess the intra-assay (within day) variability 10 replicates of these serial dilutions as well as blank plasma were measured. Samples of the same concentrations were stored at − 20 °C and one sample of each concentration was analysed on 10 different days over a period of five months to assess inter-assay (day to day) variability.

The concentration of total homocysteine in plasma samples was calculated from a peak area of 50 µmol/l homocysteine in buffer as external standard.

Recovery

L-Homocysteine and *L*-cysteine were either dissolved in 0.1 mol/l borate buffer, containing 2 mmol/l Na_2EDTA, pH 9.5, or in pooled plasma to give a concentration of 6000 µmol/l and 3000 µmol/l, respectively. These stock solutions were further diluted with buffer or pooled plasma to known concentrations. The concentration of thiols determined in 10 replicates of a water matrix (standards, S), was used to adjust the system. Then the concentrations of homocysteine in pooled plasma (P) and in plasma supplemented with standards (PS) were determined in 10 replicates and analytical recovery was calculated as follows:

$$\text{Recovery (\%)} = ((PS - P)/S) \times 100.$$

Results

Chromatography

Under the chromatographic conditions described retention times proved to be stable with a coefficient of variation for homocysteine of 0.5% (within run) and 4.6% (between run) for a period of five months. In addition to homocysteine also other substituents of importance like cysteine, cysteinylglycine or glutathione are clearly separated in the same run. Baseline separation of homocysteine from these other thiol containing peaks could be achieved and maintained for a column life of more than 500 injections of plasma samples (fig. 1).

Linearity and sensitivity

The determination of homocysteine was linear up to 100 µmol/l both for samples prepared in buffer (correlation coefficient $r = 0.99$, $p < 0.001$) and in plasma (correlation coefficient $r = 0.99$, $p < 0.001$) as shown in figure 2. At higher concentrations the fluorescence intensity exceeded the upper limit of the detector used. With another fluorescence spectrophotometer (F 1080, Merck/Hitachi, Darmstadt, Germany) the response was found to be linear up to 1000 µmol/l (data not shown).

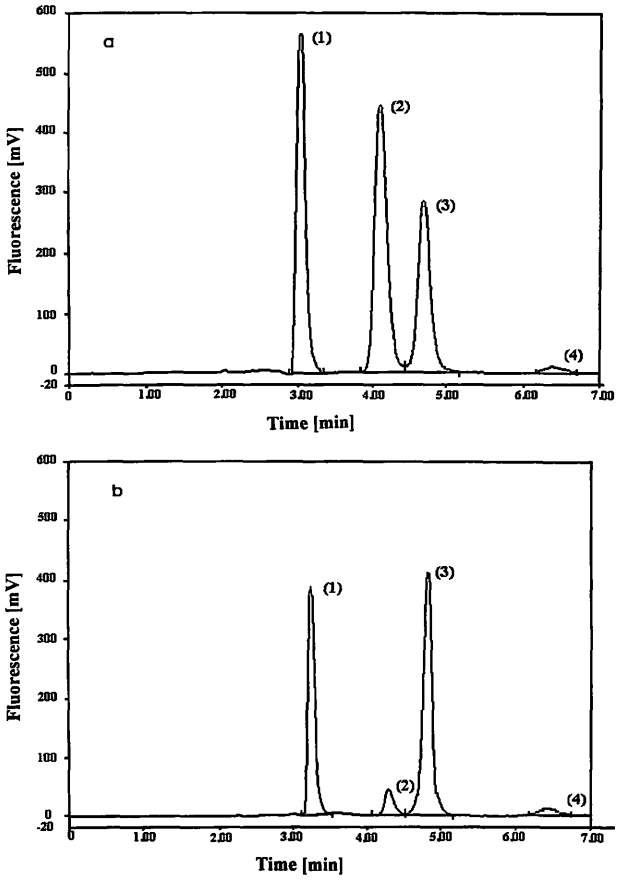

Fig. 1 Representative chromatograms of 7-fluoro-benzo-2-oxa-1.3-diazole-4-sulphonate-derivative of thiols in plasma
(a) Plasma sample spiked with 50 µmol/l of homocysteine and 150 µmol/l of cysteine.
(b) Plasma sample of a person with a homocysteine concentration in the physiological range (homocysteine 5.4 µmol/l, cysteine 132.3 µmol/l).
Chromatogram (a) was recorded with a new column, chromatogram (b) after injection of around 300 plasma samples.
Peaks are (1) cysteine, (2) homocysteine, (3) cyteinylglycine and (4) glutathione.

The detection limit of the method for homocysteine standards was 0.1 µmol/l and the lowest limit of reliable quantification was 0.5 µmol/l. For homocysteine plasma samples the lowest concentration of homocysteine which resulted in an area increase that could be reliably distinguished from the blank plasma values (>2.57 SD, n = 10) was 0.69 µmol/l.

Precision and recovery

The within day (intra-assay) coefficient of variation for homocysteine standards ranged from 5.5% for 0.5 µmol/l to 0.7% for 100 µmol/l. The respective values for standards diluted in pool plasma were 4.9% and 1.5%. The day to day (inter-assay) coefficient of variation was 8.4% for 0.5 µmol/l and 4.1% for 100 µmol/l for standards in buffer and 6.3% and 5.2% for standards in plasma, respectively.

The analytical recovery after adding known concentrations of *L*-homocysteine (0.5 to 100 µmol/l) to human plasma ranged from 94.9 to 117.0%. Data are shown in table 1.

With the method described we subsequently studied total homocysteine plasma levels in 30 presumably healthy male individuals (mean age: 38.3 ± 16.6 years) after an overnight fast. Mean total homocysteine concentration in plasma in this group was 10.5 ± 4.3 µmol/l and thus in good agreement with previous published data (8).

Discussion

Blood samples were collected following the method of *Fiskerstrand* et al. (12) and *Refsum* et al. (13). They could show that it is important to collect the blood in prechilled EDTA-containing tubes followed by immediate centrifugation. Under these conditions the separation of plasma and blood cells will prevent false positive results which otherwise may reflect the intracellular erythrocyte homocysteine synthesis and release.

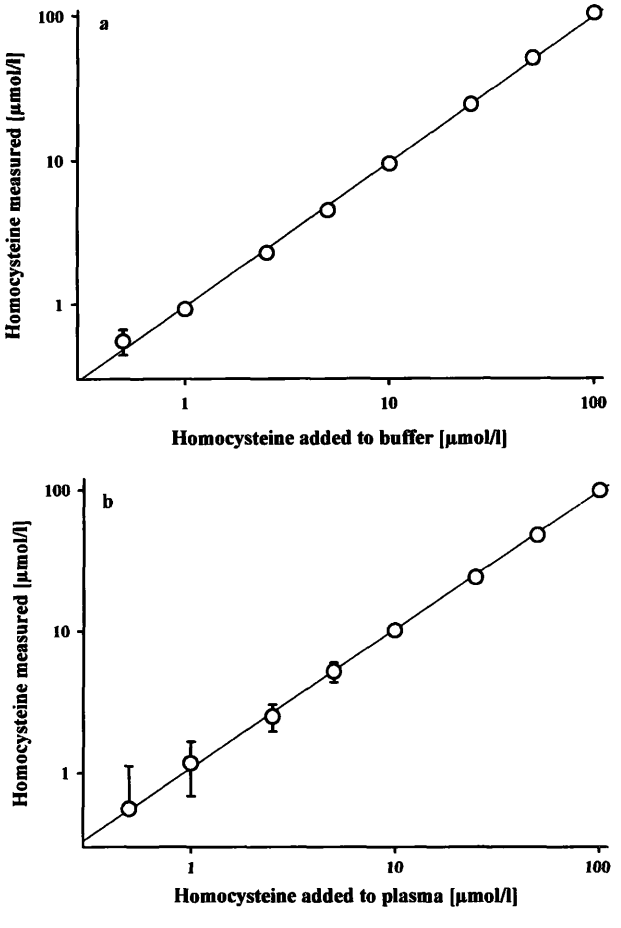

Fig. 2 Correlations between added and measured homocysteine concentrations in buffer (a) and pooled plasma (b) for a concentration range from 0.5 to 100 µmol/l. The plasma blank was subtracted from the measured values. Means ± standard deviations each of ten measurements were shown. Standard deviations not depicted are within the symbols

Tab. 1 Inter-assay recovery studies of homocysteine added to plasma and homocysteine added to buffer (n = 10). Plasma values are given after subtracting the plasma blank (8.3 ± 0.8 µmol/l).

Homocysteine added (µmol/l)	Homocysteine found			
	in buffer (µmol/l)	recovery (%)	in plasma (µmol/l)	recovery (%)
100	101.8	101.8	98.3	98.3
50	50.0	100.1	47.5	94.9
25	24.2	96.8	24.0	95.8
10	9.4	94.2	10.1	100.9
5	4.5	89.6	5.1	102.4
2.5	2.3	90.1	2.5	98.8
1.0	0.9	92.0	1.2	117.0
0.5	0.6	110.0	0.6	112.0

Very recent studies have demonstrated that thiol-containing amino acids are more stable in tubes containing heparin as anticoagulant with sodium fluoride (16) which may represent an alternative method for sample stabilization.

Determination of total homocysteine requires the reduction of several species of homocysteine disulphides in plasma. Several reducing reagents for liberating homocysteine and other sulphur compounds from plasma proteins have been described: dithiothreitol (17), tri-*n*-butylphosphine (18), mercaptoethanol (19) and sodium borohydride (12, 13). However, in our opinion, the sodium borohydride concentration used in these reports was not appropriate. It was not sufficiently potent to reduce all disulphide bonds and to prevent the re-oxidation of homocysteine. At higher concentrations we noticed strong foam formation despite the addition of octanol leading to partial loss of the sample and poor recovery (data not shown).

We also experienced difficulties with monobromobimane as thiol-specific derivatization reagent (12, 13) due to a contamination resulting in an overlap of the relevant peaks. In addition, this method is disadvantageous as it requires a high cost programmable autosampler equipment allowing eight derivatization-steps.

Therefore we decided to use a previously described manual derivatization method by *Araki & Sako* (18) with slight modifications. Smaller sample volumes were used in order to economize reagents and to reduce sample size. Plasma was deproteinized with perchloric acid instead of trichloracetic acid. Tri-*n*-butylphosphine was selected as reducing agent, allowing complete thiol liberation within 30 minutes. 7-Fluoro-benzo-2-oxa-1.3-diazole-4-sulphonate as fluorescence agent requires 60 min for incubation at 60 °C for reacting with the free thiols. This step is obviously inconvenient as compared to the method of *Refsum & Fiskerstrand* (12, 13). It increases, however, the specificity of the derivatization step since this reagent does not react with alcohols, phenols or amino groups. As another thiol-specific derivatisation reagent, 7-fluoro-benzo-2-oxa-1.3-diazole-4-sulphonamide (ABDF) was proposed by *Cornwell* (20). It allows complete formation of the corresponding thiol complex within 10 min. However, this sulphonamide complex with homocysteine shows a markedly higher retention time and thus would require to increase the amount of acetonitrile up to 10% of the mobile phase in order to achieve reasonably short running times.

Most methods published so far have used gradient systems that require rather long total running times beyond 10 min (12–14, 18). In addition, gradient systems are more susceptible to irregularities as compared to isocratic systems. An isocratic method has previously been described by *Ubbink* et al. (21). As compared to the conditions described here a rather high flow rate (2 ml/min) is necessary to elute the sulphonate homocysteine complex from the column that also results in somewhat higher retention times. Moreover, as outlined in this report the separation of homocysteine from cysteinylglycine is highly sensitive to pH changes of the mobile phase. We have selected a column with a high carbon load. Therefore, hydrophilic interactions of the analyte with free silica of the column matrix are less important and rendered our separation much less pH-dependent.

The measurement of homocysteine was linear over the physiologically and diagnostically relevant range of concentrations. We observed higher concentrations only in untreated homozygous homocystinuria and in this case samples were diluted fivefold. However, this limitation for high concentrations was not due to the derivatization procedure itself but only to the fluorescence detector range. With another fluorescence-detector the response was linear at least up to 1 mmol/l homocysteine (data not shown).

As compared to other amino acids the normal amount of homocysteine in human plasma is low. Therefore, methods for homocysteine determination need to be feasible, especially in the low concentration range. Our method proved to be sufficiently precise and sensitive to detect even small changes in homocysteine concentrations which is considered a prerequisite for monitoring therapeutic interventions. The results obtained for intra- and inter-assay variability were compatible with those of other chromatographic assays. The analytical recovery for homocysteine added to plasma was nearly complete over the entire concentration range tested. The recovery for known concentrations of homocysteine added to buffer, however, was somewhat more variable, especially in the low concentration range. This might be due to interferences and/or absorption effects which are suppressed by the plasma matrix.

Determination of homocysteine is receiving increasing attention in medical diagnostics. Case-control (22), cross-sectional (23) and prospective epidemiological

studies (24) showed that moderately elevated homocysteine concentrations are associated with an increased risk of atherosclerotic vascular disease. In a recent study of 1100 patients, *Selhub* et al. (25) reported that 29.3% of their cohort exhibited hyperhomocyst(e)inaemia, 67% of those were found to have vitamin B_6 -deficiency. This study has led to emphasize adequate dietary intake of folate and vitamin supplements. Taken together, the method devised, seems to be a procedure applicable to the sensitive and precise measurement of total homocysteine in plasma in such instances and will potentially benefit clinical researchers and nutritionists. The method would be equally suitable for the determination of total cysteine if needed for special purposes.

Diagnosis of this independent risk factor for atherosclerosis has the potential for reducing the incidence of cardiovascular diseases by intervention with an inexpensive vitamin therapy not only in elderly patients but also in the general population.

Fully automated fluorescence polarisation immunoassays for homocysteine have already been published but not introduced (26). Therefore an accurate, rapid and cheap HPLC-method for quantifying total homocysteine will help to identify patients who might benefit from the intervention.

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