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Measurement of total plasma and cerebrospinal fluid homocysteine by fluorescence following high-performance liquid chromatography and precolumn derivatization with *o*-phthaldialdehyde

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ABSTRACT

Precolumn derivatization of amino acids with *o*-phthaldialdehyde followed by high-performance liquid chromatographic separation and fluorescence detection is used in many clinical and experimental laboratories for the measurement of primary amino acids. This technique was adapted for the measurement of homocysteine in plasma and cerebrospinal fluid (CSF) following alkylation of the free sulphydryl group with iodoacetate. The minimum detection limits are less than 1 μM in plasma and 80 nM in CSF. Within-day and between-day coefficients of variation for plasma and for CSF are less than 10%. Values for normal plasma homocysteine range from 6.04 to 16.2 μM and for CSF from 0.28 to 0.66 μM.

INTRODUCTION

Homocysteine can be metabolized by three separate pathways. The first two involve methylation to methionine, either via the 5-methyltetrahydrofolate- and vitamin B_{12}-dependent methionine synthetase (5-methyltetrahydrofolate-homocysteine methyltransferase; EC 2.1.1.13) or via the cofactor-independent betaine homocysteine S-methyltransferase (EC 2.1.1.5). In the third, homocysteine is catabolized to cysteine using the pyridoxal 5'-phosphate (vitamin B_6)-dependent enzymes, cystathionine β-synthase (EC 4.2.1.22) and cystathyionase (EC 4.4.1.8).

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An increase in plasma homocysteine concentration has been reported in dietary deficiency of vitamin B_{12}, folate [1,2], or pyridoxine [3], in inborn errors of metabolism which affect the concentration of these cofactors [4], and in those leading to decreased activity of 5,10-methylenetetrahydrofolate reductase (EC 1.1.1.68) [5], cystathionine β-synthase [5] or methionine synthetase [6]. Elevated levels of homocysteine have also been found in humans treated with methotrexate [7]. In addition, recent evidence suggests that total plasma homocysteine can act as an independent risk factor for premature vascular disease [8–10] especially after a methionine load [11]. Plasma homocysteine levels can therefore be used to help in the diagnosis and monitoring of treatment in the above conditions. Whether homocysteine measurement within cerebrospinal fluid (CSF) has any predictive or diagnostic implica-

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tions has not yet been determined, essentially as methods to date have not had suffcient sensitivity to accurately measure the low levels that are present.

Several methods exist for the measurement of total plasma homocysteine. These include enzymatic conversion to S-adenosylhomocysteine using labelled adenosine with subsequent separation of the labelled product by thin-layer chromatography or high-performance liquid chromatography (HPLC) [12], direct electrochemical measurement following HPLC separation [13,14], the use of thiol-specific fluorogenic derivatizing agents followed by HPLC separation [15–18] and the use of gas chromatography–mass spectrometry (GC–MS) with stable isotope internal standard [19].

Turnell and Cooper [20] demonstrated that it was possible to measure the sulphur amino acids by fluorescence following *o*-phthaldialdehyde (OPA) derivatization, provided that the free thiol groups were first blocked using iodoacetic acid. OPA derivatization of primary amino acids followed by HPLC separation and fluorescence detection is a standard method for analysis of amino acids in many clinical and experimental laboratories; therefore we examined the possibility of adapting this methodology for the measurement of homocysteine in human plasma. The method established is also the first to allow the accurate measurement of total CSF homocysteine.

EXPERIMENTAL

Chemicals

β-Mercaptoethanol (MSH), iodoacetic acid, homocysteic acid, OPA, DL-homocysteine, and cysteine were from Sigma (St. Louis, MO, USA). Acetonitrile was from Baxter (McGraw Park, IL, USA). All other chemicals were of analar grade.

Sample collection

Blood was collected in potassium EDTA tubes, plasma separated within 15 min and stored at −20°C until analysed. CSF was collected at the bedside onto dry ice and stored at −70°C until analysed.

OPA reagent

OPA (3 mg) was dissolved in 50 μl of methanol in a foil-wrapped glass autosampler tube. To this were added 400 μl of 1.5 *M* borate buffer pH 11.0 and 50 μl of MSH. The tube was sealed with a PTFE screw cap. This solution could be kept at room temperature for up to a week without any loss in potency.

Sample preparation

To 150 μl of plasma were added 20 μl of 1 m*M* homocysteic acid (internal standard) and 10 μl of a 10% (v/v) solution of MSH. The sample was mixed and the protein immediately removed by centrifugation for 3 min at 10 000 *g* through a Millipore Ultrafree-MC 10 000 mass cut-off filter (Millipore, Bedford, MA, USA). A 20-μl aliquot of the filtrate was then immediately added to 100 μl of iodoacetic acid reagent (1.7 g of iodoacetic acid in 100 ml of 0.5 *M* boric acid, pH 11.0), mixed and 10 μl of OPA reagent were added. After exactly 1 min 20 μl of 1 *M* HCl were added and 30 s later 5 μl were injected onto the HPLC column. CSF was treated in a similar manner except 10 μl of internal standard were added, the filtration step was omitted and 20 μl of the final reaction mixture were injected onto the column.

Chromatography

The chromatographic system consisted of two Waters Model 581 pumps controlled by a Waters Model 680 gradient controller, a Rheodyne 7125 injector (Cotati, CA, USA), and a 5-μm Spherisorb ODS (25 cm × 0.45 cm I.D.) reversed-phase column (Phase Separations, Queensferry, Clwyd, UK) protected by a 5-μm ODS guard column (1 cm × 0.45 cm I.D.) (Upchurch, Oak Harbour, WA, USA). Detection of the OPA-derivatized homocysteine was achieved using either a Perkin-Elmer LS3B or an LS4 spectrofluorimeter (Norwalk, CT, USA). Excitation and emmission wavelengths were set at 335 and 455 nm, respectively. Peak areas were determined using a Spectra-Physics Chromjet integrator.

Mobile phase

Mobile phase A was 0.05 *M* sodium acetate

containing 8% acetonitrile pH 6.8 and mobile phase B was acetonitrile. The mobile phases were degassed with helium before use.

Separation

The flow-rate was 1.3 ml/min and the column temperature was maintained at 35°C using a column oven. Separation of internal standard, cysteine and homocysteine was accomplished isocratically using mobile phase A. Due to the "lag" in the gradient system the wash procedure required commencement of the gradient after 6.0 min. The wash consisted of a linear gradient from 100% A to 70% B from 6.0 to 8.0 min. 70% B was held for 2 min and the original conditions were returned to using a linear gradient over the next 2 min. Allowing for re-equilibration, a sample could be injected every 25 min.

RESULTS

Method development: initial considerations

The optimum conditions for the reduction and derivatization reactions were established for plasma homocysteine measurement. As compared to the original method described by Turnell and Cooper [20], this required (a) determination of the optimum MSH concentration required for reduction, (b) an increase in iodoacetic acid in order to provide sufficient to block the thiol groups on the homocysteine in the presence of the added MSH and (c) an increase in the MSH concentration in the final OPA reagent sufficient to remove the excess iodoacetic acid and leave adequate free thiol groups to allow the OPA derivitization to proceed.

The total thiol concentration in normal plasma is about 400 μM [15] and this may rise to approximately 1 mM in homocysteinemia. An MSH concentration was therefore used that would provide an approximate 50- to 100-fold excess over the highest possible total plasma thiol content. The MSH concentration chosen (approximately 70 mM) was picked for convenience (10 μl of 10% MSH added to the plasma sample) and because it fell within the range which had previously been shown to free 100% of the homocysteine [19]. The conditions for the rest of the assay were determined based on this initial concentration of thiol groups.

The amount of iodoacetic acid was adjusted to give a final concentration approximately five times that of the total of both the endogenous plasma thiols and those present in the added MSH. The concentration of the MSH in the final OPA derivatization step was also adjusted to provide an excess after "mopping up' the remaining iodoacetic acid.

Several problems became apparent during the initial method development. The homocysteine concentration measured in aliquots from a single plasma sample was extremely variable (not shown). This was found to be caused by the inability of the reaction buffers to maintain a constant pH due to the slight variation in the MSH concentration in the final OPA derivatization reaction. The fluorescence response of homocysteine was extremely pH-dependent below pH 9.5. Above this pH, it increased slowly in a linear fashion up to at least pH 12 (Fig. 1). To overcome this problem the molarity of the reaction buffers was increased from the suggested 0.5 M to 1.5 M and the pH adjusted from 9.5 to 11.0. Homocysteic acid was also added to the plasma samples and used as an internal standard. Although the fluorescence response of the homocysteic acid was not dependent on iodoacetate derivatization, it was pH-sensitive in a similar manner to that of homocysteine (Fig. 1) and its inclusion corrected for variation in injection volume and sample handling.

Chromatography: plasma homocysteine measurement

Separation of homocysteine from all other OPA-derivatizable materials was obtained using an isocratic mobile phase (Fig. 2b). Subsequently a rapid gradient to 70% acetonitrile was necessary in order to wash off the rest of the amino acids. Cysteine eluted earlier than the homocysteine and was also well resolved from other peaks (Fig. 2b). Although not validated, it is likely that this amino acid could also be quantitated using this method. Peaks were not observed at the re-

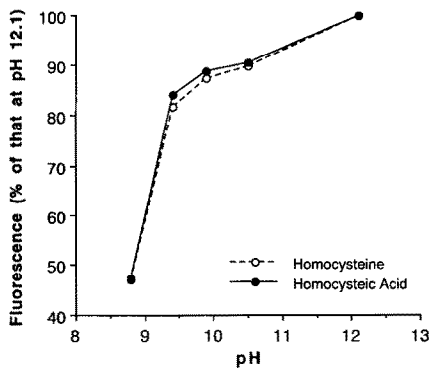

Fig. 1. Effect of pH on the fluorescence response of homocysteine and internal standard. Derivatization was performed as described in the Experimental section except that the pH of the iodoacetic acid–borate buffer solution was varied between 8.8 and 12.1.

tention times of either homocysteine or cysteine if the iodoacetic acid step was omitted from the assay (Fig. 2c). In the absence of the MSH reduction step, the concentration of free homocysteine and cysteine could be determined if required (Fig. 2d).

A small peak was always found in plasma which co-eluted with the internal standard and could not be separated by altering the chromatographic conditions (Fig. 2e). To remove the possibility of interference from this endogenous peak the sensitivity of the fluorescence detector was decreased until the peak essentially disappeared. The concentration of the internal standard was adjusted to give an appropriate size peak. After elution of the internal standard the sensitivity of the system was increased to allow for quantitation of the homocysteine. When using the LS4 spectrofluorimeter the sensitivity was increased four-fold automatically during the run. With the LS3B this was not possible, and the attenuation of the integrator was decreased to provide a good visual chromatogram. Even using the LS3B the limits of detection were less than 1 μM. By manipulating the system in this manner the magnitude of the endogenous peak was never greater than 3% of that of the internal standard. This was the case even in homocystinuric patients where homocysteic acid is thought to accumulate

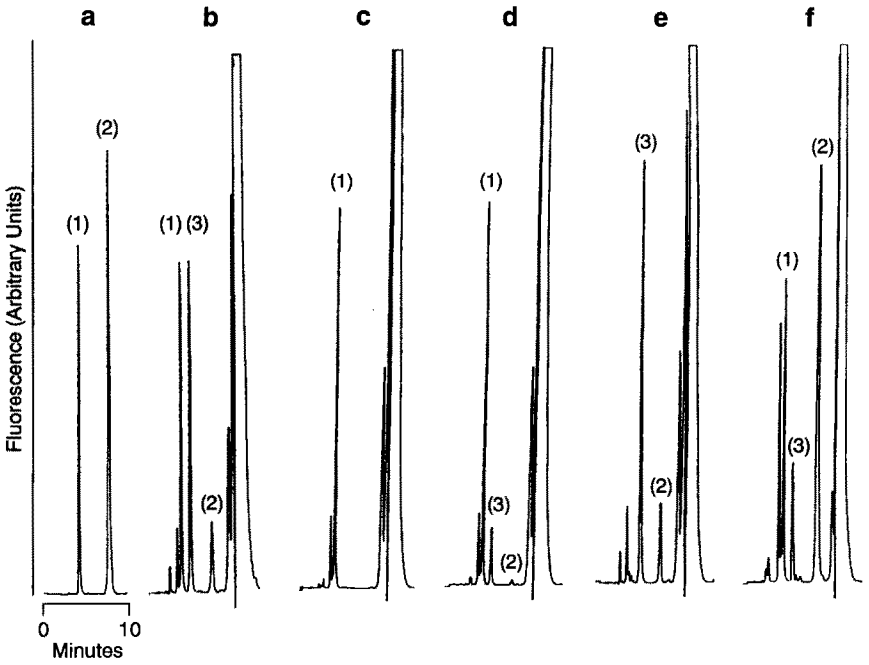

Fig. 2. Fluorescence chromatograms from plasma: (a) Standard (homocysteine = 100 μM); (b) normal plasma; (c) normal plasma without iodoacetic acid; (d) normal plasma without MSH reduction; (e) normal plasma without internal standard; (f) plasma from a patient with cystathionine synthetase deficiency. Peaks: 1 = internal standard; 2 = homocysteine; 3 = cysteine.

as a result of the oxidation of homocysteine [21] (not shown). The large increase in homocysteine and the decrease in cysteine seen in plasma from a patient with cystathionine synthetase deficiency is readily demonstrable using the system (Fig. 2). The minimum detection limit for plasma homocysteine was less than 1 μM with a signal-to-noise ratio of 3. These limits could be greatly increased either by increasing the sensitivity of the fluorescence detector or by injection of a larger volume of sample. A 100-μl aliquot of sample could be injected without loss in resolution of the homocysteine peak (not shown).

Validation

Reduction with mercaptoethanol. Using the final assay conditions, the measured homocysteine was independent of the concentration of the MSH used for he initial reduction step in the range from 17.5 to 140 mM. MSH concentrations below or above these values led to a reduction in the concentration of homocysteine measured (not shown).

To determine the time course of the release and the stability of the homocysteine in the MSH-treated plasma extract, samples of MSH-treated plasma filtrate were left at room temperature and analysed over a 24-h period. The maximum release of plasma homocysteine occurred within the 4 min required for centrifugation of the sample to remove protein. After this period, there was a slow decline ($<10\%$/h) in the homocysteine concentration measured. For this reason, samples were processed and analysed immediately after addition of the MSH and removal of the protein.

Iodoacetic acid reaction. The reaction with iodoacetate was virtually instantaneous, there being no difference in the homocysteine concentration measured on incubation from 10s to 5 min (not shown). Incubation of plasma or plasma filtrate with iodoacetic acid for times greater than 5 min led to the slow appearance of two peaks on either side of the homocysteine peak (Fig. 3). If chromatographic conditions were not perfect, these peaks occasionally interfered with the measurement of the homocysteine. For this reason

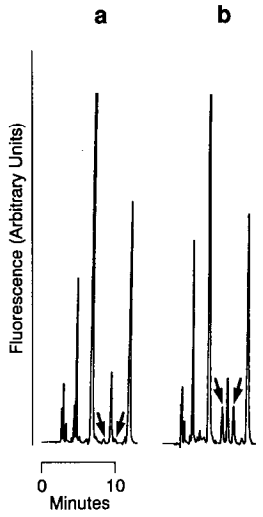

Fig. 3. Chromatograms demonstrating the formation of additional peaks (arrows) adjacent to homocysteine. Samples were mixed with iodoacetic acid and left for (a) 10 min and (b) 30 min before the addition of OPA.

derivatization with OPA was always performed immediately after the addition of the iodoacetate. Under these conditions formation of the additional peaks did not occur (Fig. 2b).

Precision

Aqueous solutions of homocysteine were treated exactly as for plasma. The standard curve for homocysteine in aqueous solution was essentially linear from below 1 μM to 1000 μM (slope = 16.74 ± 0.215; r = 0.9993). The within- and between-day coefficients of variation (C.V.) were determined using a single plasma sample which was separated into 150-μl portions and kept frozen at −20°C. The within-day C.V. was 6.07 (n = 6) and the between-day C.V. was 8.97 (n = 12). To ensure recovery from plasma, aliquots from a single plasma sample were spiked in duplicate with homocysteine to give an additional homocysteine concentration ranging from 3 to 330 μM. Recoveries across the whole range lay between 95.9 and 106.6%.

Normal values: plasma

Samples of plasma were obtained from a random population aged from 1 to 38 years. These consisted of volunteers and patients who were

admitted to hospital for conditions other than those likely to affect homocysteine metabolism. Samples were obtained at random times and no account was taken of feeding status. The mean (± 1 S.D.) for homocysteine in plasma was 9.1 ± 3.0 μM (range = 6.0–16.2 μM; n = 16).

Homocysteine measurement in CSF

Total and free CSF homocysteine could be easily measured using the LS4 spectrofluorimeter (Fig. 3). The low levels required that the sensitivity of the detector be increased by a factor of 10 compared to that used for the measurement of plasma homocysteine and the injection volume increased to 20 μl. Under these conditions the lower limit of detection for CSF homocysteine was 80 nM with a signal-to-noise ratio of 3. This could be lowered further by increasing the volume of sample injected. Peaks were not seen at the retention time of homocysteine if either iodoacetate or MSH were omitted from the sample procedure (Fig. 4). The within-day and between-day C.V.s were determined using a single CSF sample which was separated into 150-μl portions and kept frozen at −70°C. The within-day C.V. was 9.3 (n = 5) and the between-day C.V. was 7.8 (n = 6).

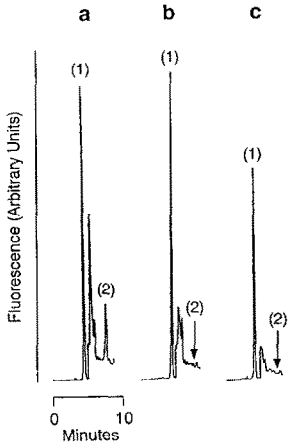

Fig. 4. Fluorescence chromatograms from cerebrospinal fluid. The retention time of homocysteine is indicated by the arrow. The sensitivity of the detector was automatically increased by a factor of 50 just after the elution of the internal standard. (a) Normal CSF, homocysteine concentration = 0.4 μM; (b) normal CSF without mercaptoethanol; (c) normal CSF without iodoacetate. Peaks: 1 = internal standard; 2 = homocysteine.

Normal values: cerebrospinal fluid

Samples of CSF were obtained from neurological controls consisting of patients with motor neurone disease and peripheral neuropathies in whom serum vitamin B_{12} and red cell folate concentrations were normal. The mean (± 1 S.D.) value for homocysteine in CSF was 0.46 ± 0.13 μM (range = 0.28–0.66 μM; n = 9).

DISCUSSION

The initial method described by Turnell and Cooper [20] for the measurement of homocysteine in plasma required two steps: the blocking of the free thiol group using iodoacetic acid followed by derivatization with OPA in the presence of excess thiol. This method was designed only to measure the free homocysteine; however, most of the homocysteine in plasma is bound to albumin or present as a mixed disulphide with other thiol-containing compounds [13]; a reducing agent is therefore required to liberate the homocysteine. Agents used to date have included dithioerythritol [12], MSH [19], sodium borohydride [15,16], and tri-*n*-butylphosphine [17,18]. It was hoped that the present method would eventually be suitable for automation, therefore MSH was used because of the virtual instantaneous reduction achieved with this agent. Although DTE also provides rapid reduction [12], it was found to contain contaminants which yielded several fluorescent peaks that eluted, at, or near, the retention time of homocysteine.

Using GC–MS with a stable isotope internal standard, it has been shown that MSH–homocysteine mixed disulfides can form following addition of MSH to plasma [19]. The sample preparation required for the GC–MS method was long and it is not clear at what stage the formation of the MSH–homocysteine mixed disulfide occurred. Using the current method, the measured concentration of homocysteine slowly decreased following the addition of MSH. The decrease was less than 10% over a period of 1 h and was therefore considered negligible during the 4 min required for the removal of protein. Unfortunately iodoacetic acid could not be added prior

to the centrifugation, as this led to additional peak formation on either side of the homocysteine which occassionally interfered with the homocysteine quantitation. The use of sodium borohydride as the reducing agent [15,16] would have eliminated the possibility of MSH–homocysteine mixed disulfide formation. It was not used, as it increased the complexity of the sample handling procedure. Specifically: (1) a 30-min incubation was required for complete recovery of plasma homocysteine; (2) it caused excessive and variable amounts of frothing, both on first addition to the plasma and on addition of the PCA which was required to destroy the residual sodium borohydride and to precipitate the protein; (3) additional buffer had to be added to the PCA-treated sample to neutralize the acid.

Despite the fact that OPA derivatizes all primary amino acids, the chromatography allowed good separation of this amino acid from all other fluorescent compounds. There are two other major compounds found in plasma which are likely to be derivatized both by the iodoacetic acid and OPA, these being glutathione and cysteinylglycine. Glutatione eluted earlier than cysteine and homocysteine (not shown). Although the elution time of cysteinylglycine was not checked, it is unlikely that it co-elutes with the homocysteine given that it is present in relatively high concentrations in plasma [17,18] and that the normal range (6.4–16.2 μM) and mean (9.07 μM) values found for homocysteine using the current method were very similar to those reported by others [12,15,17,19].

Previous methods have been able to detect elevated concentrations of CSF homocysteine but have not had the necessary sensitivity to measure normal values [22]. Using the current methodology we have been able to measure normal values and establish reference ranges. This will allow subtle changes in homocysteine within CSF to be detected. It remains to be seen whether such changes may reflect early signs of intracellular central nervous system vitamin B_{12} or folate deficiency.

Several other methods are now available for the measurement of homocysteine [12–17,19,23]. The present method is currently unsuitable for automation, however, it has major advantages in that it is extremely sensitive and it provides a rapid technique which can be performed using a simple modificaton of methodology already available in many clinical and experimental laboratories.

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