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Short communication

Total homocysteine levels in plasma: high-performance liquid chromatographic determination with electrochemical detection and glassy carbon electrode

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Abstract

An isocratic high-performance liquid chromatography with electrochemical detection (HPLC–ED) method for the determination of total plasma homocysteine [H(e)] has been developed. The electrochemical detection is performed using a glassy-carbon electrode that is not specific for thiol groups. We have tried to solve the problem of specificity focusing our work on chromatographic resolution and have obtained good results without coelution of other thiol compounds or any substances mentioned as common interferences for carbon electrode methods: uric acid, ascorbic acid and salicylates. Thirty samples a day can be assayed for total homocysteine with a lower limit of detection of 2 pmol, and a limit of quantification of 1.0 μmol/l, with a coefficient of variation (C.V.) <20%. For a concentration of total plasma homocysteine of 9.36 μmol/l, the intra- and inter-assay C.V.s were of 3.86% and 5.55% respectively. The analytical recovery achieved in the preparation of the samples ranged from 85.0% to 98.3% and the electrochemical response was linear up to 100 μmol/l.

Keywords: Vascular disease; Homocysteinemia

1. Introduction

Homocysteine (Hcy), a sulfhydryl amino acid derived from the metabolism of methionine, is readily oxidized in body fluids to the disulfides, homocystine and cysteine–homocysteine mixed disulfide. The protein bound and three free forms have been referred to as total homocysteine (H(e)). Hyperhomocysteinemia has recently received considerable attention, especially because it represents a risk factor for early-onset of occlusive vascular disease.

In homocystinuric patients, H(e) is markedly increased. Severe abnormalities in enzymes controlling Hcy metabolism have been described [1–3]. Patients presenting vitamin B_{12} and folate deficiency show an increase in H(e) values [4,5]. Furthermore, a moderate increase in H(e) concentration has been considered as a risk factor for coronary disease, independent from other factors related to lipid metabolism [6–12].

There are several techniques for H(e) determi-

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nation, including simple chemical assays for measuring high urine Hcy concentration [13], plasma assays using amino acid analyzers [14], radioenzymatic methods [15–17], fluorimetric methods employing ammonium 7-fluorobenzofurazan-4-sulfonate (SBD-F) [18–20] or monobromobimane (mBrB) derivatization [21]. Biological thiols determination [22,23] including Hcy [24,25] as HPLC coupled to an electrochemical detector, is also widespread. This technique has been improved for H(e) determination by Malinow et al. [26] who modified the method of Smolin and Sneider [27]. In this method, $NaBH_4$ [28] is used as a reductant, and no derivatization of the sample is required. Thiols are detected in the column effluent by a single gold–mercury electrode, which affords great specificity towards sulfhydryl components. Thus the electrochemical assay offers several attractive features: simple sample processing (it does not include derivatization), specificity, short run time, large sample throughput, and the possibility of determination of other thiols like cysteine. In the assays including reduction of all forms into a unique species, redistribution between free and protein-bound Hcy, does not affect the results, and H(e) can be measured in stored samples, because of the fact that H(e) is not altered when plasma is kept frozen [29].

The purpose of the present report is a presentation of a method of suitable sensitivity and reliability for H(e) determination without derivatization, using HPLC–ED with a glassy-carbon electrode.

2. Experimental

2.1. Reagents

DL-Homocysteine, L-homocystine, L-cysteine, β-glutamylcysteine (βGluCys), L-cysteinylglycine (CysGly), glutathione, methionine, caffeine, ascorbic acid, uric acid, sodium salicylate, monochloroacetic acid, and sodium octylsulfate were obtained from Sigma (St. Louis, MO, USA). Water for use in HPLC was prepared with a Milli-Q reagent water system (Millipore, Milford, MA, USA). All other chemicals were of analytical grade.

2.2. HPLC apparatus

The HPLC instrument, consisting of a Model 2150 pump with a 20-μl sample loop, a Model 2220 integrator and a Model 2143 electrochemical detector with a glassy-carbon electrode (spacer 2.2 FEP) was obtained from LKB (Bromma, Sweden) and the injector was a Rheodyne Model 7125 (Rheodyne, Berkeley, CA, USA). Chromatography was performed on an Adsorbosphere C_{18} column 150×4.6-mm I.D., 3-μm particle size, Alltech Associates (Deerfield, IL, USA), that was protected with an Alltech C_{18} 7.5×4.6-mm I.D., 5-μm particle size guard-column.

2.3. Preparation of standards

The standard curve was prepared adding L-homocystine dissolved in 0.1 *M* HCl to a plasma pool, taking into account that, after reduction, one mole of homocystine gives two moles of Hcy. Values were obtained by subtracting the Hcy peak area corresponding to the plasma pool from the Hcy peak area corresponding to the plasma pool plus homocystine. The standards were fractionated and stored at −20°C.

Aqueous standards were prepared using Hcy also dissolved in 0.1 *M* HCl, but dilutions were immediately used, and kept on ice between runs.

2.4. Preparation of samples

Whole blood from fasting patients was drawn into tubes containing EDTA as anticoagulant. The samples were immediately centrifuged at 3000 *g* for 15 min. Plasma was separated and stored at −20°C until analysis. All samples were processed by duplicate. The procedure for reduction of samples was based on Fiskerstrand et al. [30] with minor modifications: in 1.5-ml eppendorf tubes 60 μl of plasma was mixed with 30 μl of 4 mol/l $NaBH_4$ in 0.066 mol/l NaOH and 333 ml/l dimethyl sulfoxide, 10 μl of 75 mg/ml EDTA, 10 μl of 1-octanol and 20 μl of 1.8 *M* HCl following this particular order. The tube was gently shaken and left at room temperature for 10 min. The reaction was stopped by the slow addition of 200 μl of cold 20% trichloroacetic acid. The samples were

centrifuged for 10 min at 10 000 *g* to separate precipitated protein, and the supernatants were filtered through 0.45-μm filters type HV (Millipore, Bedford, MA, USA), then 20 μl were injected into the HPLC apparatus.

2.5. Mobile phase

The mobile phase consisted of 0.1 *M* monochloroacetic acid and 3.6 m*M* sodium octylsulfate, adjusted to pH 3.2 with sodium hydroxide. It was filtered through a 0.45-μm Millipore HV membrane, sparged with N_2 during 30 min to eliminate dissolved oxygen, and afterwards, the solution was degassed under vacuum (generated by an aspirator) for 10 min to eliminate most dissolved gases. Before use, the mobile phase was recirculated overnight at 0.5 ml/min at +0.80 V to decrease background currents.

2.6. Running conditions

Chromatographic separation was performed at room temperature with a flow-rate of 1.0 ml/min, at 0.80 V with N_2 sparging to prevent reoxidation of the reduced Hcy.

The reference electrode used (LKB 2143) was based upon the electrolytical H_2/H^+ ion couple. This type of reference electrode has been chosen for its maintenance-free usage. It is continuously stabilized by the buffering function of the passing eluent. This kind of electrode allows a reduction of approximately 0.2 V the voltage suggested for Ag/AgCl reference electrodes. With graphite paste electrochemical detector versus Ag/AgCl 1 V was suggested for thiols [31]. In our system, we observed that for a 200 pmol Hcy injection no response was obtained up to 0.5 V, then the signal arose with the increasing voltage and reached the plateau at 0.8 V.

Under these conditions, the Hcy peak eluted at about 3.60 min, and total run time was 15 min. This time is sufficient to allow all the oxidizable compounds to appear, and the system to be ready for a new run. The retention times varied slightly with variations in room temperature and upon column aging.

Hcy peak area was automatically determined by an integrator, and the concentration was calculated by interpolating on the standard curve.

3. Results

Fig. 1 illustrates the chromatographic profiles of $NaBH_4$-treated plasma, generated by HPLC–ED with a glassy-carbon electrode. The Hcy peaks corresponding to the plasma pool, and the same plasma pool containing 12 μmol Hcy exogenously added as homocystine, are shown.

In the Smolin and Sneider method [27], the preparation of the sample includes a 30-min incubation at 50°C. In the present method, the preparation of the sample needs only 15 min and room temperature. The addition of dimethyl sulfoxide to the media reveals thiol groups which are conformationally inaccessible in macromolecules dissolved in the usual aqueous buffers.

For the preparation of the mobile phase we assayed different concentrations of sodium octylsulfate, and concluded that 3.6 mmol/l leads to a better separation of the Hcy peak. Bibliography refers to interferences [32] like ascorbic acid, uric acid and sodium salicylate, but they do not coelute with Hcy in our method. We assayed other compounds, mostly

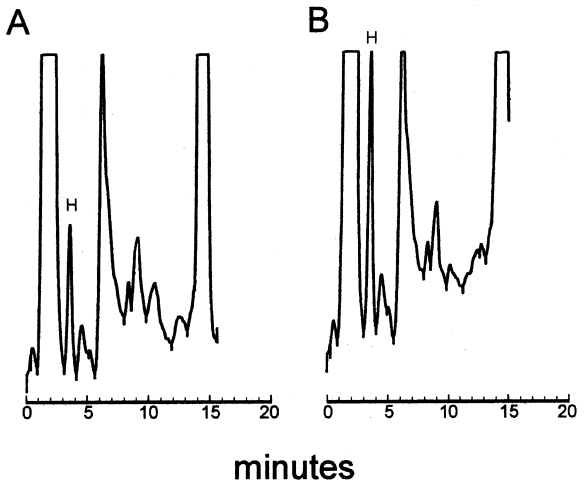

Fig. 1. A: Chromatogram of plasma pool. B: Chromatogram of the same pool spiked with homocystine (6 μmol/l). H, homocysteine.

Table 1
Retention times of common biological thiols and other compounds (in aqueous solution) mentioned as potential interferences

Compound	Retention time (min)
Ascorbic acid	1.96
L-Cysteine	2.25
Uric acid	2.37
β-Glutamylcysteine (β-GluCys)	2.48
Reduced glutathione	2.80
L-Homocysteine	3.80
L-Cysteinylglycine (CysGly)	6.60
Sodium salicylate	15.92
Methionine	N.D.[a]
Caffeine	N.D.

[a] N.D.: Non detectable

thiols, and none of them interfered with the Hcy peak (Table 1).

The calibration curve for an aqueous solution of Hcy was linear over the concentration range of 0–100 μmol/l ($y=2.51x+10.39$; $r=0.9828$), and for homocystine added to a plasma pool and processed according to the present method, was linear up to the addition of 100 μmol/l ($y=2.50x+13.34$; $r=0.9985$).

Under our conditions, the detection limit for a Hcy aqueous standard was approximately 2 pmol (signal-to-noise ratio of 3). In human plasma, this lower limit of detection was 1.8 μmol/l (6.5 pmol per injection) (signal-to-noise ratio of 3), and this is sufficient for H(e) determination in clinical samples [33].

Routinely, we cleaned the electrodes with acetone and methylene chloride and changed the spacer after 90–100 samples because at this point the detector response begins to decay.

Standard curves of pooled plasma with added homocystine at concentrations of 2.5, 5, 7.5, 10 and 15 μmol/l were obtained by duplicate at the beginning, middle, and end of each daily run of about thirty samples.

Recovery experiments were carried out by adding homocystine equivalent to 6–12–24 μmol/l of Hcy to three different plasma samples. Then the concentrations in plasma samples and in plasma samples spiked with standards were determined by triplicate. The average recovery was 91.1% with a coefficient of variation (C.V.) of 4.56% (Table 2).

Table 2
Recovery of homocysteine added to plasma as homocystine

Homocysteine (μmol/l)			Recovery (%)
Added	Calculated	Observed[a]	
Plasma 1			
0	–	10.5	–
6	16.5	16.3	96.7
12	22.5	20.9	86.7
24	34.5	32.3	90.8
Plasma 2			
0	–	9.4	–
6	15.4	14.7	88.3
12	21.4	21.2	98.3
24	33.4	31.2	90.8
Plasma 3			
0	–	8.5	–
6	14.5	14.1	93.3
12	20.5	18.7	85.0
24	32.5	30.0	89.6
Average (and C.V., %)			91.1 (4.56)

[a] Mean of three determinations.

This method was compared with the HPLC and fluorescence detection method developed by Fiskerstrand et al. [30] using five different plasma samples (Table 3). The two methods gave similar values; their ratios ranged from 0.88 to 1.07.

The intra-assay C.V. was performed at two levels of concentration for total plasma homocysteine: 9.36 μ*M* ('normal') and 18.38 μ*M* ('high'). We assayed six replicates of each level in one run. C.V.s of 3.86% and 4.55% respectively were obtained.

Between-day precision was obtained by determining the same biological samples over a period of five consecutive days. For 9.36 μ*M* H(e), the C.V. inter-

Table 3
Comparison of two different methods for the determination of total homocysteine in blood samples[a]

Total homocysteine (μmol/l plasma)			ED/FD
Sample	Electrochemical detection (ED)	Fluorimetric detection (FD)	
1	4.92±0.15	5.56±0.20	0.88
2	7.10±0.22	6.83±0.32	1.04
3	13.24±0.52	12.35±0.76	1.07
4	13.41±0.23	14.22±0.71	0.94
5	25.30±0.73	26.98±1.14	0.94

[a] Results are expressed as means±S.E. of each sample analyzed for triplicate.

Table 4
Limit of quantification

Homocysteine (μmol/l)		Standard deviation	Coefficient of variation (%)
Calculated	Observed (average[a])		
5.0	4.45	0.38	8.54
4.0	3.58	0.36	10.06
3.0	2.78	0.33	11.87
2.0	1.78	0.27	15.17
1.0	0.92	0.18	19.56
0.5	0.46	0.15	32.61

[a] Mean of three determinations.

assay was 5.55%, and for 18.38 μ*M* H(e), the C.V. inter-assay was 7.37%.

The limit of quantification was tested by measuring Hcy aqueous standards ranging from 5.0 μmol/l to 0.5 μmol/l. Each of the standards were assayed in triplicate. The quantification limit of this HPLC method was 1.0 μmol/l with a C.V. $<$ 20% (Table 4).

4. Discussion

H(e) determination by HPLC–ED was first developed with gold–mercury electrode that possesses specificity for thiols [26]. In this paper we present the results of H(e) determination by HPLC–ED with a glassy-carbon electrode. Glassy-carbon is a hard, amorphous graphite, highly conductive material capable of being polished to a mirror-like finish, that is firmly imbedded in a block to form the detector cell. On glassy carbon electrodes, it has been shown that disulfides are the usual product of thiol oxidation:

$$2\,RSH \rightarrow RSSR + 2H^{+} + 2e^{-}$$

Conversely, it has been mentioned that carbon paste or glassy-carbon electrodes, being nonspecific, oxidize other substances than those containing sulfhydryl groups [32]. In our research, we centered the solution to the problem of specificity in the chromatographic separation.

The presented method may be useful for laboratories that have an electrochemical detection system available.

The method is adequate for laboratories processing low work volume, because once the mobile phase is prepared and recirculated, it can be stored at 4°C and reused.

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