Determination of Homocysteine by HPLC with Pulsed Integrated Amperometry, Jovan Evovski,1 Michael Callaghan,2 and David E. C. Cole1,2,4 (Depts. of 1 Clin. Biochem., 2 Med., and 2 Paediatr. (Genetics), Univ. of Toronto, Banting Institute, Toronto, ON, Canada; 3 Dionex Canada Ltd., 4-586 Argus Rd., Oakville, ON, Canada; 4 address for correspondence: Rm. 415, Dept. of Clin. Biochem., Banting Institute, 100 College St., Toronto, ON M5G 1L5, Canada: fax 416-978-5650)

Determination of total homocysteine can be a useful adjunct in the diagnosis of cobalamin deficiency and a potentially important predictive test for premature cardiac and other vascular occlusive disease (1). However, methodologic considerations limit the use of this analyte assay in routine clinical chemistry. Electrochemical methods have the advantage of increased selectivity without extensive pretreatment and should make this assay more accessible; until recently, however, the use of electrochemical detection has been restricted because of problems associated with electrode fouling and flow cell contamination (1).

Pulsed integrated amperometry (PIA) offers a potential solution to these problems. By using a multistep potential–time (E-t) waveform that rapidly alternates between an amperometric detection mode with oxidative cleaning/reductive reactivation potentials (inset, Fig. 1), degradation of the electrode surface is minimized (2). Application of PIA to the detection of sulfur-containing species—at least those with a minimum of one pair of unbonded outer electrons—at a solid gold electrode has the advantage of high sensitivity and selectivity without prior derivatization and does not require special modification of the gold surface, since the electrode is self-cleaning (3).

We have adapted this method for determination of total homocysteine in serum or plasma with the Dionex DX-500 Ion Chromatograph (Dionex Canada, Oakville, ON) equipped with a ED40 electrochemical detector and gold working electrode, as described by Vandeberg and Johnson (3). Disulfide reduction, converting all homocysteine to its free thiol form (4), was achieved by diluting 200 μL of plasma with 100 μL of water (or homocysteine calibrator) and mixing with 300 μL of 9 mol/L urea (pH 9.0), 50 μL of NaBH₄ (100 g/L) in 0.1 mol/L NaOH, and 50 μL of amyl alcohol (all solvents HPLC-grade) as an antifoaming agent (5). Samples were incubated at 50°C for 30 min and then cooled to 4°C in an ice bath; the reaction was stopped by adding 500 μL of HClO₄ (200 mL/L). After centrifugation at 10,000 × g for 5 min, the supernate was passed through a C₁₈ Sep-Pak™ solid-phase extraction cartridge (Millipore Waters, Mississauga, ON, Canada) to extend the life of the column. A 50-μL volume of filtrate was used to fill a 25-μL injection loop. All homocysteine calibrators and additions were prepared from L-homocysteine thiolactone (Sigma Chemical Co., St. Louis, MO) (6).

The chromatograph was outfitted with two pumps (in parallel), valves, and two columns (an OmniPac PCX-500 (4 × 50 mm) precolumn and an OmniPac PCX-500 analytical (4 × 250 mm) column), which were plumbed in series so as to permit "heart-cut" trapping of reduced homocysteine (7). The eluent, consisting of HClO₄ (0.10 mol/L), NaClO₄ (0.15 mol/L), and CH₃CN (50 mL/L), was filtered through a 0.45-μm (pore-size) nylon membrane, deoxygenated with helium, and supplied to both pumps. The flow rate was set at 1 mL/min for the auxiliary pump and 1 mL/min for the primary pump. Column switches were set for 0.2 and 1.5 min after injection. The initial eluate from the precolumn, containing void volume and

Fig. 1. Typical serum chromatograms: Homocysteine elutes as a single fairly symmetrical peak at 7.86 min (arrow). Bottom tracing, control (nominal homocysteine concentration 18.3 μmol/L); middle tracing, a patient with hyperhomocystinemia (homocysteine 38 μmol/L); and upper tracing, a patient with symptomatic cobalamin (B₁₂) deficiency (homocysteine 118 μmol/L). Inset: Time–potential (E-t) waveform used for homocysteine assay. Amperometric integration, equivalent to the area bounded by the triangle, is directly proportional to the concentration of oxidizable sulfur in the eluant stream. GSH, reduced glutathione; CySH, cysteine.
early-eluting analytes, was passed to waste. At 0.2 min, the column switch redirected the precolumn eluate to the analytical column, and analytes moderately adsorbed to the precolumn (including homocysteine) were further separated before passing to the detector. At 1.5 min, the precolumn eluate, containing only species strongly adsorbed to the precolumn, was switched back to waste. Although some contaminants were removed by solid-phase extraction prior to chromatography, we found that carryover (presumably from late-eluting species) was reduced substantially by this last cut to waste.

The electrochemical detection cycle of 980 ms (inset, Fig. 1) was only slightly modified from that described by Vandeberg and Johnson (3). This form of electrochemical detection—originally called potential-sweep pulsed coulometric detection—depends on transient formation of a gold hydroxide electrode surface, which is the proximal catalyst for amperometric oxidation and reduction of the organosulfur species (8). Detector output is analyzed with Peaknet™ (Dionex) software and generates a display of chromatograms that can be autoscaled to show full-scale deflection between 10 and 1000 nC for widely varying strengths of signal from the electrochemical cell.

An autoscaled composite of three serum chromatograms is shown in Fig. 1. Calibrators between 0 and 100 μmol/L give a linear calibration curve (r = 0.99977), and the assay could detect <1 μmol/L, with appropriate adjustment of the full-scale deflection. In our hands, within-run CV was 6.03% (n = 11) at a serum concentration of 5.1 μmol/L, and between-run CV was 9.8% (n = 16) at 12.2 μmol/L. Recoveries of 5, 10, and 20 μmol/L authentic homocysteine added to a serum sample with a nominal homocysteine concentration of 7.9 μmol/L were 95%, 97%, and 99%, respectively. Further improvements in precision might be achieved by incorporation of an internal standard but we did not examine this possibility. Our reference mean (± SD) was 12.9 ± 1.1 μmol/L (n = 31); range, 10.2–14.8 μmol/L. These are very close to reported values (9).

In summary, this method appears to offer a reasonable alternative to previously published methods, but with less preparation. An additional potential advantage of this method is thecodetermination of other important sulfur-containing species. With automated serum pretreatment and column injection, such as that described by Fiskerstrand et al. (9), precision might be improved, reagent costs minimized, and sample throughputs of 10/h might be possible without any sacrifice in accuracy.

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References


Homocysteine in Plasma: Stabilization of Blood Samples with Fluoride, Jan Møller and Karsten Rasmussen (Dept. of Clin. Biochem., Univ. Hosp. of Aarhus, Skejby Sygehus, DK 8200 Aarhus N, Denmark; 1 author for correspondence: fax 45 89 49 60 18)

The concentration of total homocysteine (HCY) in plasma (P-HCY) is increased in cobalamin and folate deficiency and decreases after treatment with the relevant vitamin. Thus, P-HCY is of value in the diagnosis and follow-up of these deficiency states (1). Furthermore, increased P-HCY appears to be a significant independent risk factor for premature cardiovascular diseases (1, 2), suggesting a large future demand for P-HCY determinations.

After sampling, the blood cells still produce and release HCY into the plasma (3, 4), causing an artificial increase of P-HCY by ~10%/h. Cooling the blood on ice immediately after collection and separating the blood cells from the plasma as soon as possible or at least within 1 h inhibits the release of HCY by the cells (3, 4). However, this procedure is inconvenient and a source of error, and it will be impossible in many clinical settings.

Ubink et al. (5) demonstrated that sodium fluoride partially inhibited the release of HCY by the blood cells, but no attempts were made to find the optimal concentration or to investigate a practical application. In the present work, we explored the dose–response effect on the release of HCY by the blood cells of adding fluoride to the sample, and we propose a blood sampling procedure with standard heparin–fluoride tubes.

We measured P-HCY by a modification of the gas chromatography–mass spectrometric techniques developed by Stabler et al. (6). The amount of d₈-homocystine (internal standard) added to plasma was chosen so as to result in the smallest imprecision and analytical inaccuracy at borderline HCY concentrations (15 μmol/L), where the method might provide the most useful clinical information, i.e., from high normal to moderately above normal. After reduction of the disulfide bonds between HCY and other thiols or albumin with dithiothreitol, we quantitatively extracted onto a small anion-exchange column total HCY from plasma, together with added internal standard, as described previously (7) for the determination of cystathionine. The method was linear from 0.5 to 300 μmol/L. The total analytical precision was 0.18 and 0.26 μmol/L (SD) at concentrations of 7.0 and 23.1 μmol/L, respectively (n = 9).

In agreement with Ueland et al. (1), a 0.95 central reference interval of 4.6–12.3 μmol/L was obtained from samples donated by 24 members of the laboratory staff after informed consent in accordance with the Helsinki Declaration. From each of the 24 volunteers, blood was drawn in as many as 10