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 gave a linear calibration curve ($r = 0.9997$), 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 99%, 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 (5).

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 the codetermination 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.

We thank Peter J. Vandeberg and Dennis C. Johnson for their help in the development of this method. This work was supported in part by a grant from the PSI Foundation of Ontario.

References


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 at ~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 $d_3$-homocysteine (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
different blood-collecting tubes, incubated at room temperature, and centrifuged after specified lengths of time. After centrifugation (5 min, 1800g), plasma was separated and kept frozen at −18°C until analyzed. Samples were analyzed in duplicate.

The effect of adding fluoride to the samples was investigated by using tubes with added heparin only, with heparin plus 2 g (47.6 mmol/L) of sodium fluoride, and with heparin plus 4 g (95.2 mmol/L) of sodium fluoride per liter of blood. Changes in concentration were calculated relative to reference samples collected on ice and centrifuged immediately.

The time dependence of the changes in P-HCY is shown in Fig. 1. There is a negative dose–response effect with added sodium fluoride but no significant difference between the change in P-HCY in collection tubes with a concentration of sodium fluoride of 2 or 4 g/L blood. However, the difference between using sodium fluoride or not is highly significant (analysis of variance, confidence level = 0.05). Adding sodium fluoride produced no significant increase in P-HCY after the first 2 h of incubation—in contrast to the P-HCY from unsupplemented heparinized blood, where storage at room temperature resulted in an average increase of P-HCY of 0.9 μmol/L per hour during the first hours.

The analytical method has proved to be well suited to perform routine determinations of P-HCY. The method is certainly accurate, precise, and practical enough to diagnose cobalamin or folate deficiency, and to be of value in investigating the role of homocysteine in the pathogenesis of cardiovascular disease. Using the deuterated disulfide homocystine as the internal standard corrects for both reoxidation of endogenous homocysteine in its reduced state during sample processing and the variable recovery through the various steps in the procedure. The precision and specificity of the method are superior to those of most other methods published (i.e., HPLC). The error introduced in the determination of P-HCY by the release of HCY after blood sampling must be evaluated in relation to the analytical and biological variance. The need for controlled sample handling, particularly when investigating the role of slightly increased P-HCY as a risk factor in atherosclerotic disease, was illustrated by the report by Wu et al. (8), who found a significant mean difference of <2 μmol/L between proband cases of early coronary artery disease and controls. Comparing this with the results of Vester and Rasmussen (3), Andersson et al. (4), and Ubbink et al. (5), who documented release of HCY to plasma from the erythrocytes equivalent to between 1 and 2 μmol/L per hour at room temperature, makes evident the need for utmost care in blood collection and sample treatment. The general availability of standard blood-collection tubes with fluoride added makes the use of fluoride addition to the collection tubes quite practicable. The addition of fluoride does not interfere with the analytical method used in the present investigation.

In conclusion, we suggest that, for the determination of homocysteine in plasma, the collection tube should contain heparin as an anticoagulant with sodium fluoride added to a final concentration of 4 g/L blood. This will inhibit the release of homocysteine from the blood cells, and will for the first 2 h prevent a significant increase in the resulting concentration of homocysteine in the plasma.

References