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# Improved HPLC method for total plasma homocysteine detection and quantification

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**Recent clinical research has pointed at hyperhomocysteinemia as an independent risk factor in a number of cardiovascular and neurological diseases. We have improved a chromatographic method of total plasma homocysteine measurements in order to obtain higher sensitivity, reliability and reproducibility. The method demonstrates excellent linearity (R = 0.999), range (< 2–100 μM), precision (instrumental RSD 0.06 and method RSD 1.17), accuracy (recovery of 99.92 and RSD 1.27), reproducibility, quantification limit and ruggedness (e.g. pH from 2.0 to 2.5). Because even a small increase in homocysteine level can be a significant risk factor of cardiovascular diseases, such a precise method is required. The constructed method allows the measurement of plasma pyridoxal phosphate, PLP, the co-enzyme form of vitamin $B_6$, on the same column and similar reagents. The developed method has been successfully applied to measure both total plasma and serum homocysteine in a group of acute stroke patients.**

**Keywords**: homocysteine measurement, HPLC, stroke risk factors, validation

## INTRODUCTION

Homocysteine is an amino acid with a free sulphydryl group which does not occur in the natural human diet but is an essential part of the metabolism of methionine (Finkelstein & Martin, 2000) and it is produced as a result of methylation reactions (Brosnan *et al.*, 2004). Its other metabolic functions include: being part of choline metabolism, recycling of tissue folates and being a precursor for cystathionine, cysteine and further metabolites (Finkelstein, 1990). Increased concentration of homocysteine in blood, called hyperhomocysteinemia, can be caused by various factors, both endogenous and exogenous. The most important endogenous factors are polymorphisms of the genes coding for the main enzymes involved in homocysteine metabolism, e.g. a common C677T transition in the gene coding for methylenetetrahydrofolate reductase (MTHFR) (Lievers *et al.*, 2001), resulting in a thermolabile variant of the enzyme. The most significant exogenous factors are connected with the composition of the diet, e.g. dietary deficiency of folate (Selhub *et al.*, 1999). Hyperhomocysteinemia is considered to be an independent risk factor in cardiovascular diseases (Mayer *et al.*, 1996) and high homocysteine concentration has also been shown to be present in patients with cerebrovascular diseases, like brain stroke or dementia (Seshadri *et al.*, 2002). It has recently been found that hyperhomocysteinemia is linked with cancer, which

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**Abbreviations**: MTHFR, methylenetetrahydrofolate reductase; NAC, *N*-acetylcysteine; PLP, pyridoxal phosphate; PN, pyridoxine; SBD-F, 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonic acid; TCAA, trichloroacetic acid; TCEP, tris(2-carboxyethyl)phosphine hydrochloride.

leads to the suggestion that homocysteine can also be regarged as a tumour marker (Sun *et al.*, 2002). The main enzymes of homocysteine metabolism have vitamin cofactors, like vitamin $B_6$ or $B_{12}$. This is why vitamins' level is often an important factor to be considered in homocysteine research and treatment (Moriyama *et al.*, 2002), even though vitamins cannot be usually treated as strict determinants of homocysteine (Pietrzik & Bronstrup, 1998).

Since homocysteine levels are elevated in several diseases, a lot of research has been directed at methods of its measurement. Homocysteine exists in blood in three forms — protein-bound (about 80%), oxidised (homocystine or homocysteine–cysteine, about 18%) or free (about 1%). Even though the relative composition of these three fractions of homocysteine carries certain information (Iciek *et al.*, 2004), in human clinical practice total homocysteine is the most important diagnostic indicator (Gilfix *et al.*, 1997). There are two main groups of methods of total plasma homocysteine measurements: chromatographic separation methods and immunoassays (Nekrassova *et al.*, 2003). These methods implement various reducing agents, as well as various derivatizing agents (Bald *et al.*, 2000). Recently, 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonic acid (SBD-F) has been used more often as the derivatizing agent, due to the simplicity of its use, as well as the accuracy and convenience of fluorescence as the detection technique (Garcia & Apitz-Castro, 2002). There have been indications that even a slight excess of homocysteine in blood can result in increased risk of cardiovascular diseases (Refsum & Ueland, 1998), therefore a successful method of plasma homocysteine measurement must be characterised by high accuracy and precision. The main problems connected with methods used so far are low specificity due to a lack of complete separation of cysteinyl-glycine from homocysteine and loss of both long-term stability and specificity due to insufficient column regeneration. Some of the methods were also very difficult in terms of laboratory practice, because of the nature of reagents used. We have modified and improved the existing procedures of total plasma homocysteine measurement and validated the resulting method, taking special care to ensure sufficiently high accuracy and precision, while keeping the procedure and experimental conditions as simple and convenient as possible. We have also tested the application of our method to measuring homocysteine concentration in serum, because using serum instead of plasma greatly simplifies the initial stage of the whole procedure, which is an essential factor in smaller laboratories and clinics. Our method also allows for subsequent measurements of homocysteine and vitamin $B_6$ on the same column, with a similar set of buffers.

## MATERIALS AND METHODS

**Chemicals and equipment.** In the course of the experiments we have used the following chemicals: TCEP, TCAA, sodium borate, SBD-F, D,L-homocysteine, L-cysteine, cysteinyl-glycine, glutathione and PLP, NAC, pyridoxamine, PMP, pyrodoxal, pyridoxine and pyridoxic acid. All these reagents were purchased from Sigma (Warszawa, Poland). The chromatographic analysis was performed using a Waters Millenium system (Waters 600) with a Waters 474 Scanning Fluorescent Detector and a Supelcosil LC 18 DB analytical column (250 mm × 4.6 mm, 3 μm particle size, Supelco, Warszawa, Poland), used together with a pre-column (Supelguard LC-18-DB, 20 × 4.6 mm, Supelco).

**Sample preparation.** The initial stages of homocysteine sample collection were analogous to those described in previously published papers (Minniti *et al.*, 1998; Frick *et al.*, 2003). The first part of the procedure took place in the clinic. Immediately after blood collection, the blood was placed on ice and about 500 μL was taken into an Eppendorf tube. This portion was centrifuged at 2000 × *g* for 15 min, as soon as possible. Then, two portions of plasma were obtained by taking 90 μL of the supernatant into separate Eppendorf tubes. Both plasma samples were then frozen at –20°C for transport to the laboratory for further steps of the assay. Long-term storage of any remaining plasma or samples from other stages of the procedure required –70°C. All patient samples were taken from patients in the acute phase of ischemic stroke, hospitalised in the Department of Neurology of the Medical University of Gdańsk. The study was approved by the local ethics committee.

During the initial step of sample preparation, 10 μL of an internal standard, 0.2 mM NAC, was added to each sample. Then, 10 μL of reducing agent, 10% TCEP, was added in order to cleave all sulphur bridges. Successful action of TCEP required 30 min of incubation at 4°C. The precipitation of proteins was achieved by the addition of 100 μL of cold 10% TCAA with 1 mM EDTA and subsequent centrifugation for 5 min at 21 000 × *g*. Clear supernatant (100 μL) was then taken into a fresh Eppendorf tube. Labelling with the fluorescent marker required addition of 20 μL of 1.55 M NaOH, 250 μL of 0.125 M borate buffer (pH = 9.5) with 4 mM EDTA and 10 μL of the fluorescent marker, SBD-F, 10 mg/mL solution in 0.125 M borate buffer, pH = 9.5. The whole resultant solution was incubated at 60°C for 60 min and then cooled on ice for subsequent HPLC analysis. For HPLC analysis samples of 20 μL were used.

We measured the level of vitamin $B_6$ as pyridoxal 5′-phosphate (PLP) present in human plasma following a previously published method (Deitrick *et*

*al.*, 2001). In brief, the initial steps of sample preparation were identical to those used for homocysteine level measurement, but the obtained plasma sample size was 100 μL. We added 100 μL of 0.8 M perchloric acid to 100 μl of plasma, vortexed it vigorously and centrifuged at 1700 × *g* for 10 min. Supernatant (50 μL) was directly analysed by HPLC.

**HPLC analysis.** Chromatographic analysis of the sample used for homocysteine measurement was carried out at 25°C, in a column thermostat module, using two buffers: 0.1 M potassium dihydrogen phosphate buffer, pH = 2.1 (buffer A) and 0.1 M potassium dihydrogen phosphate buffer, pH = 2.1, mixed with acetonitrile, at the ratio 1 : 1 (buffer B, final potassium buffer concentration is 0.05 M). The flow rate was 1 mL/min. The specific gradient composition is 12% of buffer B (88% of buffer A) from start for 6 min, 9 min of a linear gradient until 30% of buffer B is reached, 2 min of regenerative 30% buffer B, 1 min of a linear gradient until 12% of buffer B (88% of buffer A) is reached again, 7 min of 12% buffer B. Fluorescence detection was performed at 385 nm excitation and 515 nm emission wavelengths.

In order to successfully analyse the homocysteine chromatogram, the ratio of area of each individual peak to the area of the peak of the internal standard was measured and compared against the calibration curve. This was performed for the homocysteine peak, as well as for all other peaks on the chromatogram. The concentrations used to obtain five points at the calibration curve were chosen to cover the whole physiological range, as well as the expected pathological fluctuations: 50, 100, 200, 400 and 600 μM for cysteine, 12.5, 25, 50, 125 and 200 for cysteinyl-glycine, 2.5, 5, 10, 35 and 100 for homocysteine and 2.5, 5, 10, 25 and 50 for glutathione. NAC concentration was 200 μM in each case.

In the case of PLP measurement, we used two HPLC buffers, A (0.1 M potassium dihydrogen phosphate containing 0.1 M sodium perchlorate and 0.5 g/l sodium bisulphite, adjusted to pH 3.0 with *o*-phosphoric acid) and B (30% acetonitrile in water). Specific gradient composition is 100% buffer A for 8 min, then 1 min gradient to 100% buffer B, 6 min of 100% buffer B, 1 min gradient to 100% buffer A and 9 min of 100% buffer A. The calibration curve was based on the following points: 25, 50, 100, 150 and 200 nM PLP.

**Method validation.** After determining suitable chromatographic conditions we carried out validation of the method for homocysteine level measurement. Specificity was determined by chromatographic analysis of homocysteine and other sulphur-containing amino acids expected to be found in human plasma and serum. They included cysteine, cysteinyl-glycine and glutathione. The retention time of each compound was studied individually, using dilutions from 0.5 M stock solutions. Linearity was assured by calculating the regression coefficients for the calibration curves. Range of the method was determined by assuring that all the points on the calibration curve were characterised by acceptable precision and accuracy. Precision of the validated method was checked by 10 independent repeats of the measurement of one sample and by determining the standard deviation of the obtained data, separately for each amino acid studied. Reproducibility was obtained by performing another series of 10 measurements of a particular sample, by the same person, on the next day and then comparing the precision obtained for each series. Accuracy was assured by the addition of a known amount of homocysteine to the sample and measuring the change in the response (fluorescence). Such a measurement was repeated 6 times. Quantification limit was determined by analysing a sample in which the amino acid was present at a concentration 20 times lower than that expected under physiological concentration, and the ratio of the obtained signals to the noise level was analysed. Analysis of the ruggedness of the method included controlled changes at certain points of the procedure. We checked ruggedness both during labelling of the sample with SBD-F and during HPLC analysis. The same standard sample was measured several times, and each time the concentration of one of the reagents was first decreased, and then increased by 20%. The next series of measurements of the same sample included extending and shortening all the incubation times by 20%. As for the HPLC analysis, we checked the three most important parameters. First, we performed an analysis of the same standard sample in varying temperatures (20°C, 25°C, 30°C). Second, we checked the influence of the mobile phase composition on the analysis results (varying the slope and total duration of the acetonitrile gradient, as well as the regeneration time). Third, we checked the influence of the pH of the phosphate buffer on the analysis outcome (pH = 2, 2.05, 2.1, 2.2 and 2.5).

**Measurement of homocysteine levels in serum.** We also studied the applicability of the method to total homocysteine determination in serum. In order to achieve this, we prepared both plasma and serum samples from 10 patients in parallel. Plasma samples were prepared as described above. Serum samples were obtained by collecting blood, dividing it into 3 portions of approx. 1 mL each, and allowing them to clot for 30 min, 60 min or 90 min, respectively. Serum was then drawn from each such sample and 90 μL of such serum was later treated in exactly the same way as the plasma samples.

## RESULTS AND DISCUSSION

We intended to develop a method of homocysteine detection and quantification which would be highly accurate, but at the same time not so time-consuming. After obtaining satisfying preliminary parameters, the next step in full method development is validation, essential in the case of all methods connected with medical trials. A full validation process ensures that a method can be applied with a satisfying degree of certainty and assurance. Fulfilling all the criteria is extremely important, especially if the method is used over an extended period of time. An example of obtained chromatograms of standards and samples is shown in Fig. 1. The validation parameters obtained are summarised in Table 1.

The critical steps in the whole procedure are fast sample preparation and fluorescent labelling. The crucial part of the sample preparation stage is reduction of the disulphide bridges. Even though various reducing agents can be used during the sample preparation phase (Minniti *et al.*, 1998), TCEP is the most convenient one. TCEP has high long-term stability at –20°C, does not require oxygen-free conditions and is easy to use, which leads to procedure simplification and therefore shortening of the time required for the assay. The efficiency of TCEP action as a reducing agent can be checked by measuring a standard sample containing oxidised form of homocysteine — homocystine. We recommend checking the general efficiency of the assay by measuring a standard mixture sample, which should be done in every series. Additionally, every several series of measurements and every time when a new TCEP solution is prepared, a mixture of standard oxidised forms should be measured in order to ensure TCEP activity.

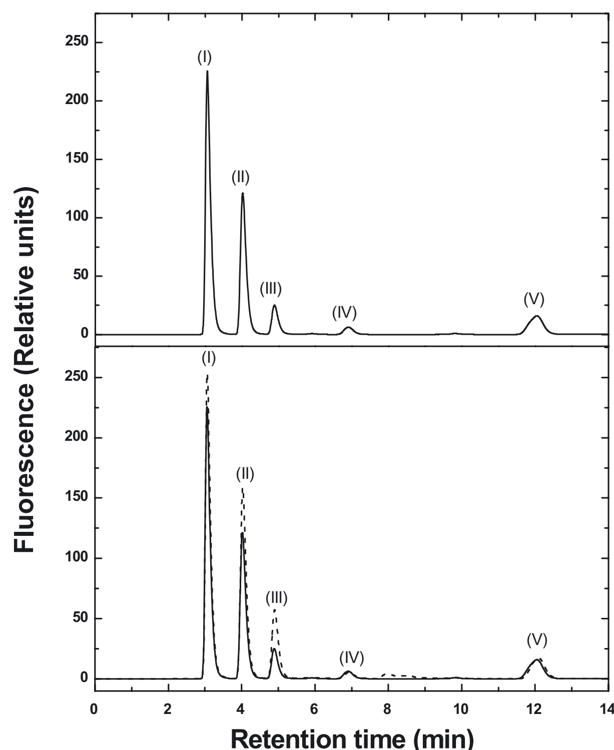


**Figure 1. Sample HPLC chromatograms of homocysteine. Top panel.** Chromatogram of a standard mixture. The peaks represent: (I) cysteine, (II) cysteinyl-glycine, (III) homocysteine, (IV) glutathione, and (V) NAC. **Bottom panel.** Chromatogram of two patient samples. Solid line represents a patient with normal level of total plasma homocysteine (9.19 μM), while dashed line represents a patient with mild elevation of homocysteine level (18.15 μM).

**Table 1. Validation parameters of the method.**

Linearity was checked for the whole range of the calibration curve R is the coefficient of correlation of the obtained curve, intercept and slope are the variables of the linear equation (concentration in mM against area ratio of homocysteine peak to NAC peak). The curve was based on five calibration points: 2.5, 5, 10, 35 and 100 μM homocysteine. All relative standard deviation values (RSD) are given in percent of the average value. Instrumental precision was obtained by studying ten HPLC assays of the same sample, while method precision was studied by analyzing ten standard samples of the same concentration processed independently. Mean recovery was calculated basing on five samples with an addition of 10 μM of homocysteine. The quantification limit was studied by diluting the physiological concentration of standard compounds 20-fold and calculating the signal to noise ratio. A satisfying value was assumed to be 20. Similar studies were conducted for cysteine, cysteinyl-glycine and glutathione, giving similar results, with the exception of quantification limit for glutathione, which was too low to fulfill the criteria.

| Linearity | |
|---|---|
| R | 0.999 |
| Intercept | –0.0165 (± 0.0094)(± S.E.) |
| Slope | 77.9 (± 0.391)(± S.E.) |
| Accuracy | |
| Mean recovery | 99.92 |
| RSD (%) | 1.27 |
| Quantification limit and range | |
| Quantification limit | 0.5 μM |
| Range | < 2–100 μM |
| Precision, RSD (%) | |
| Instrumental | 0.05 |
| Method | 0.84 |

There are several factors influencing the efficiency of the method at the HPLC analysis stage. The first is the mobile phase composition, as it influences the peak retention times. Acetonitrile concentration must not be too low nor too high (an optimum between retention time and peak width must be found). The influence of the fraction of buffer B in the mobile phase on the retention time of peaks is presented in Fig. 3. Considering the total time of the

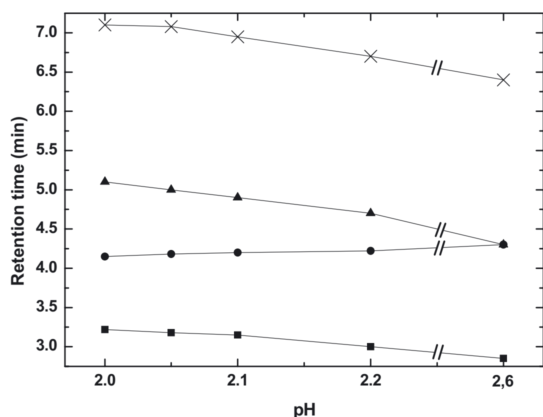


**Figure 2. Dependence of the capacity factor of peaks on pH.**
The lines represent the relative capacity factors of the respective peaks. Squared line represents the cysteine peak, circled line represents cysteinyl-glycine, triangled line represents homocysteine and crossed line represents glutathione.

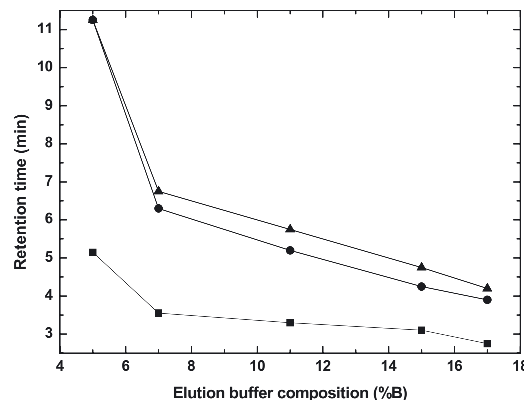


**Figure 3. Influence of elution buffer composition on retention times.**
Squared line represents the cysteine peak, circled line represents cysteinyl-glycine and triangled line represents homocysteine. Glutathione peak is not presented on the graph, since it is separated completely from homocysteine regardless of the elution buffer composition.

assay and peak separation, we found the optimal composition to be 12% of buffer B. Although there have been suggestions on performing the run in isocratic conditions only (Minniti *et al.*, 1998; Accinni *et al.*, 1998), we found that a precise analysis requires the regenerative phase. Performing the measurements at isocratic conditions only, without any kind of a column regenerating step, leads to the appearance of baseline disturbances between individual samples (especially in the case of samples with abnormally high amounts of one of the labelled compounds), decreases the accuracy of the assay and causes retention time to differ between subsequent analyses. Therefore, it is highly recommended to include some kind of a regeneration step in the run. In our experience, 2 min of 15% acetonitrile flow are sufficient to eliminate any unwanted interference between samples. Apart from the within-run regeneration, the method requires full regeneration at the end of the series. Such a full regeneration is achieved by flushing with 50% acetonitrile. This form of regeneration provides long-term stability of the column and it is even more important in the case of subsequent PLP and homocysteine measurements. It would also be very hard to shorten the overall time required for the HPLC analysis without loss of specificity. The duration of a single measurement is 25 min, and it would be hard to shorten. The initial isocratic part of the HPLC analysis is essential in order to obtain separate cysteinyl-glycine and homocysteine peaks. A gradient of at least 9 min is also inevitable, because a shorter gradient results in a poorly separated peak of glutathione. Moreover, the final isocratic period is also essential in order to equilibrate the column for a next run. Careful determination of the isocratic step and gradient is the first element leading to the obtained precision, which is critical considering the significance of small differences in total plasma homocysteine level.

The most important parameter studied while determining the ruggedness of the method turned out to be pH. The influence of pH on the capacity factor of peaks of the substances studied is shown in Fig. 2. We have discovered that the method is very sensitive to pH changes. An increase of pH brings the cysteinyl-glycine and homocysteine peaks closer together, and at pH higher than 2.1 they tend to merge, which is especially problematic in the case of abnormally high cysteinyl-glycine or homocysteine levels, where even partial overlapping with a high peak of one compound can render the quantification of the other compound peak problematic. On the other hand, pH cannot be reduced below 2–2.05, because it is the lower pH limit of the column. We have found pH of 2.05 to 2.1 to be optimal for specific measurements of total plasma homocysteine level. Therefore very strict pH determination is extremely important, as it ensures that problems with peak overlapping, which was the case in previous methods (Minniti *et al.*, 1998; Frick *et al.*, 2003), are avoided. Careful attention to pH is another factor influencing the overall precision of the method. The influence of pH on the relative positions of the peaks must be controlled very strictly, preferably by performing a test measurement of a standard mixture sample every time a new portion of HPLC buffer is prepared, with attention paid especially to homocysteine and cysteinyl-glycine peaks' positions. Furthermore, such a check allows for the evaluation of the column stability and resolution.

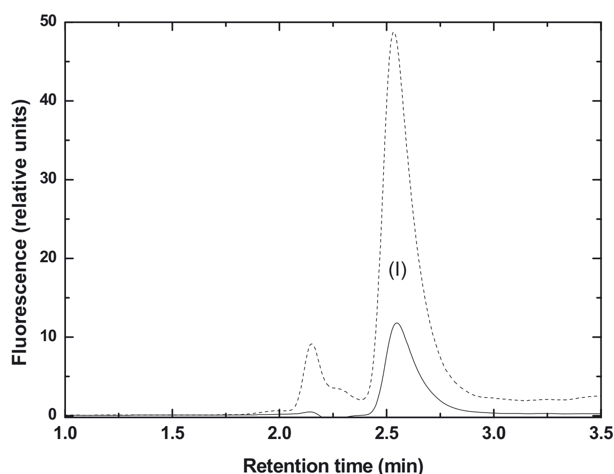


**Figure 4. Sample HPLC chromatogram of plasma PLP.** Solid line represents a standard mixture chromatogram (PLP is peak marked I), while the dashed line represents a plasma sample of a patient with abnormally high plasma PLP.

**Table 2. Influence of clotting time on the result of measurement.**

Average ratio of serum measurements compared to results for plasma for the same samples are shown. Five independent samples were studied. A blood sample was immediately separated into a portion used to obtain plasma and a portion for serum. Both samples were processed independently, keeping the plasma preparation time the same as clotting time during preparation of serum

| Clotting time (min) | 30 | 60 | 90 |
|---|---|---|---|
| Average ratio serum to plasma result (%) | 105.3 | 106.7 | 108.1 |

Changing the temperature was found to increase the retention time of all peaks, without any major changes in their positions relative to each other. Generally, every 3°C changes the retention time of homocysteine by 0.1 min. We chose the temperature of 25°C, which was kept by a column heater module. In thermally stable environments, however, such equipment might not even be necessary. It is only important to conduct all the experiments at a constant temperature, in order to avoid peak drift.

Another point which must be considered carefully is the use of an internal standard. Although there have been indications that an internal standard does not increase, or sometimes even decreases, the within-run precision of the assay (Accinni *et al.*, 1998), in our experience the internal standard is the factor which ensures high precision and reproducibility of the method. The amount of NAC added must be strictly controlled and a portion of NAC cannot be used over an extended period of time, but rather should be prepared freshly. However, adding NAC at the very beginning ensures that any external factors affecting the labelling procedure or other occurrences will also influence the NAC peak area, which can be observed on the chromatogram. Basing on it, regular control of the internal standard area stability is an additional way of controlling the whole stage of sample labelling. The use of NAC is also a crucial factor influencing the precision of the method, which is the most important aspect in consideration of total plasma homocysteine level as a risk factor in a number of diseases.

The method also allows total homocysteine measurements in serum, which eliminates the necessity of centrifugation and keeping the sample on ice during the initial stages of the procedure required for measurements in the plasma, even though during serum preparation there is a possibility of homocysteine migrating to serum from erythrocytes, which may lead to falsely high results. The obtained results of total serum homocysteine measurements are presented in Table 2. The measured amount of homocysteine in serum is higher than the respective value for plasma, as expected. However, the 5% difference between plasma and serum levels after 30 min of clotting is significantly lower than reported previously (Minniti *et al.*, 1998). It can also be seen that increasing the clotting time does not significantly increase the difference between plasma and serum results. This is quite important, because longer clotting time allows a better clot, and therefore easier separation of serum from the clot. Therefore, we recommend 30 min as optimal. Since the difference between the readings for serum and plasma is known, it can be subtracted from the results obtained for serum if rapid centrifugation of samples to obtain plasma is not convenient.

Our method has been successfully used for homocysteine measurements in a group of patients in the acute phase of ischemic stroke, hospitalised in the Department of Neurology of the Medical Academy of Gdańsk. We measured total plasma homocysteine level in 135 patients and 65 members of a control group, matched by age and sex. We have discovered a statistically significant difference between total plasma homocysteine level in both groups, which is an evidence of an association between homocysteine level and the risk of ischemic brain stroke. It also confirms the crucial importance of an exact determination method.

In studies published to date, measuring homocysteine and vitamin $B_6$ were determined in two separate chromatographic systems (Kelly *et al.*, 2004). Measuring both homocysteine and vitamin $B_6$ on the same column is far more convenient. An example of a PLP chromatogram obtained by the described method is shown in Fig. 4. The buffer used for measuring homocysteine and PLP levels differ

mainly in pH, which makes switching between the two systems easy. Measuring total plasma homocysteine and PLP is especially important in the case of medical units, which aim at supplementing their patients with vitamins in order to lower their homocysteine level. It is also very convenient in the case of less advanced medical facilities, where performing measurements in two separate chromatographic systems is not an option. Plasma PLP level is three orders of magnitude smaller, therefore care must be taken to regenerate the column well in order to obtain stable baseline. This method of subsequent homocysteine and PLP measurements has been successfully used with a group of patients hospitalised in the Department of Neurology of the Medical University of Gdańsk.

In conclusion, our modified method of total plasma homocysteine measurement has been successfully validated. The most important characteristic of the method is high precision, but at the same time it is very fast and convenient. We achieved this mainly through careful choice of pH and its strict control, careful choice of reagents and the use of an internal standard. Our method is also suitable for homocysteine measurements in serum. The duration of the HPLC assay ensures high specificity and also enables analysis of other sulphur-containing amino acids present in plasma or serum. The convenience of use of this method is additionally increased when homocysteine needs to be measured together with vitamin $B_6$, which can be done subsequently, using similar sets of reagents.

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