

EXPERIMENTAL / LABORATORY STUDIES

A Comparison of Three High Performance Liquid Chromatographic (HPLC) Methods for Measurement of Plasma Total Homocysteine

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Abstract: Homocysteine (tHcy) has been in routine use in many centers after this amino acid was reported as an independent risk factor for cardiovascular, cerebrovascular and peripheral vascular diseases. Many high-performance liquid chromatographic (HPLC) methods including a commercially available kit (Chromsystems) were reported for measuring tHcy in biologic samples. Although the commercial kits are believed to have higher precision, sensitivity and accuracy as well as practical and fast, it may cause high prices by increased test ordering. In this study we aimed to compare two different low-cost methods which had similar advantages with commercial kit as a reference method. In the first method the sample was derivatized with 7-fluoro-2,1,3-benzoxa sulfonamide (ABD-F) following the reduction with tris-(2-carboxylethyl) phosphine (TCEP). Homocysteine peak was obtained at 4.7 min and total run time was 7 min. In the second method, samples were derivatized with 7-Fluorobenzo-2-oxa-1,3-diazole-4-sulfonic acid (SBD-F). Homocysteine peak was obtained at 2.9 min and total run time was 5 min. In both methods cysteamine hydrochloride was added as an internal standard and C18 (ODS) (150 X 4.6 mm, 5 µm) column was used. Intraassay CV's of SBD-F and ABD-F methods was 0.98% and 1.83%, respectively. Interassay CV values were 2.22% and 2.49%, respectively. Both methods show linearity up to 200 µmol /L. Recovery was 99.37% and 98.10% in SBD-F method after additions of 7-14 µmol / L homocysteine standard. Similarly, for ABD-F method, recovery was 99.7% and 100.7% following the addition of homocysteine standard (6.7-13.4 µmol/L). A significant correlation was found between both methods and reference method (P < 0.001). In conclusion, SBD-F and ABD-F methods were reproducible, rapid, and easy to use for the quantification of plasma tHcy, with similar advantages of the commercially available kit. Moreover, the SBD-F method may be the method of choice among these assays because of the lowest cost per test and better precision.

Key Words: Homocysteine, HPLC, Method, SBDF, ABDF

Introduction

Several studies have revealed that elevated levels of total homocysteine (tHcy (1)) in plasma in the fasting state is associated with an increased risk for atherosclerotic and tromboembolic vascular disease (2-5). This non-essential, sulfur-containing amino acid is formed as an intermediate, during the essential amino acid methionine metabolism, which is at the intersection of two main metabolic pathways: re-methylation and trans-sulfuration, regulated by methylentetrahydrofolate reductase (MTHFR) and cystathionine β-synthase (CBS) (6). In the last decade the growing interest in so called 'emerging risk factors' has led to the development of several methods for the determination of tHcy (7-12).

Modern measurement procedures appropriate for assays of plasma homocysteine concentrations in large numbers of clinical samples are based on HPLC separation and electrochemical detection of homocysteine, fluorometric detection of homocysteine derivatives, or on gas chromatography-mass spectrometry methodology (13). Reduction and derivatization followed by HPLC separation and fluorescent detection is the most widely applied technique (14). The most frequently used derivatizing agents are the halogen sulfonyl benzofurans (7-benzo-2-oxa-1,3-diazole-4-sulfonic acid (SBD-F) and 4-aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole (ABD-F)) because of their good tHcy-adduct stability and high HPLC resolution (15). These derivatizing agents are not

fluorescent, their thiol adducts are stable, and no fluorescent hydrolysis products are formed. Thus, their use allows isocratic separation, resulting in clean chromatograms with no reagent peaks (16). Among the trialkylphosphines widely used as reducing agents, however, tri-*n*-butylphosphine (TBP) is the most commonly used reducing agent, it is an irritant with an unpleasant odor and is poorly soluble in water. Thus it was recently proposed to replace TBP by tris-(2-carboxyl-ethyl)-phosphine (TCEP), which is nonvolatile, stable, and soluble in aqueous solutions (17). Among the compounds using as internal standards, cysteamine was reported as the most suitable internal standard for tHcy measurements (18).

Analytical procedures, which use HPLC to separate homocysteine, are by far the most commonly used ones. Unfortunately, a wide variability in the manipulation of samples, chromatographic conditions, sample detection and quantification has made standardization impossible to date. Some manufacturers have developed HPLC kits in order to optimize HPLC separation and to make this assay less laborious and also to improve sample throughput. Among them, the Chromsystems HPLC kit (Chromsystems GmbH, Germany) is one of the latest developed commercial kits. Additionally, a preferred tHcy determination method for routine clinical laboratories was described as cost-effective, fast, robust, easy to perform, and stable over time.

In this study using patient samples, we evaluated HPLC fluorescence assays using SBD-F and ABD-F as derivatizing agents by comparing them with the commercial HPLC kit (Chromsystems GmbH, Germany).

Materials and Methods

Chemicals

L-Homocysteine, L-cystine, cysteineglycine (reduced form), glutathione (reduced form), cysteamine, tris-(2-carboxyl-ethyl)-phosphine (TCEP), 7-benzo-2-oxa-1,3-diazole-4-sulfonic acid (SBD-F) and 4-aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole (ABD-F) were purchased from Sigma (St. Louis, MO, USA). Potassium dihydrogen phosphate, dipotassium hydrogen phosphate, ethylenediaminetetraacetic acid (EDTA) sodium salt, sodium hydroxide, ammonium hydroxide, borate, potassium chloride, phosphate buffered saline (PBS; pH 7.4), perchloric acid, isopropyl alcohol, acetonitril,

methanol, orthophosphoric acid, acetic acid and sodium acetate were obtained from Merck (Darmstadt, Germany). All chemicals and solvents were of analytical-reagent grade. Ultra pure water and solvents were filtered through 0.20 µm filters from Millipore (Bedford, MA, USA).

Equipment

The HPLC System was a Hewlett-Packard Model 1050 consisting of a quadratic pump, a degasser and an auto sampler. The fluorescence detector was a Hewlett-Packard Model 1100 operating at an excitation wavelength of 385 nm and emission wavelength of 515 nm.; the data obtained were analyzed with the HP-chemstation program for windows 3.1 (Hewlett-Packard).

Method

After overnight fasting, blood was collected into a vacutainer tube (Becton Dickinson) containing EDTA and cooled on ice water; the plasma was separated by centrifugation at 2000 g for 10 min. at 4 °C within 30 min. and stored at -70 °C until used.

For SBD-F assay a mixture of 50 µL of plasma, 25 µL of internal standard (20 µmol/L cysteamine solution), and 25 µL of phosphate buffered saline (PBS, pH 7.4) was incubated with 10 µL of 100 g/L tris-(2-carboxyl-ethyl)-phosphine for 30 min. at room temperature. Ninety µL of 100 g/L trichloroacetic acid containing 1 mmol/L EDTA was added for deproteinization. After the sample was centrifuged for 10 min at 10 000 g, 50 µL of the supernatant was added to an autosampler vial containing 10 µL of 1.55 mol/L NaOH; 125 µL of 0.125 mol/L borate buffer containing 4 mmol/L EDTA, pH 9.5; and 50 µL of 1 g/L SBDF in the borate buffer (0.125 mol/L pH 9.5). The mixture was then incubated for 60 min. at 60 °C. A 10 µL aliquot was later injected into the HPLC system.

For ABD-F assay sample preparation was performed as follows: reduction with tris-(2-carboxyl-ethyl)-phosphine, derivatization with ABD-F, and precipitation of proteins. The derivatization was performed before the precipitation to avoid the realkalinization step. To 50 µL of the sample, we added 100 µL of 250 mmol/L borate buffer at pH 10.5. We added an internal standard (40 µL

of a 50 $\mu\text{mol/L}$ cysteamine solution), and then 30 μL of tris-(2-carboxyl-ethyl)-phosphine (9.4 mmol/L pH 10.5) followed by 30 μL of ABD-F (9.2 mmol/L in 1 mol/L ammonium hydroxide pH 10.5). Samples were incubated at 55 $^{\circ}\text{C}$ for 15 min. and then cooled at room temperature. Protein was precipitated by adding 50 μL of 60% perchloric acid. Precipitated HCY-ABD-F derivatives are stable 24 h at room temperature, avoiding sunlight. After the sample was centrifuged for 10 min at 10 000 g, a 20 μL aliquot was later injected into the HPLC system.

For commercial kit (Chromsystems GmbH) the procedure was carried out as follows: A 100 μL volume of plasma was mixed with 25 μL internal standard (Chromsystems) and 25 μL reduction reagent (Chromsystems) in an eppendorf tube and incubated for 5 min. at room temperature. Then, 100 μL precipitation reagent (Chromsystems) were added. After centrifugation at 9 000 g for 7 min., 50 μL supernatant were mixed with 100 μL derivatization mix (Chromsystems). The samples were mixed and then incubated at 50-55 $^{\circ}\text{C}$ for 10 min. A 20 μL aliquot was later injected into the HPLC system. Internal standard, reduction reagent, precipitation reagent and derivatization mix of commercial kit were not explained in the instruction manual of kit.

L-Homocysteine and other standards were prepared in PBS, pH 7.4. For internal calibration lyophilized plasma calibration standard (Chromsystems) was used and was subjected to the same preparation processes as the samples.

Column Liquid Chromatography

Column

Derivatized plasma samples performed on a Hypersil C18 analytical column (150 X 4.6 mm, 5 μm particle-size, Phenomenex), protected with an Alltech C18 guard-column (7.5 X 4.6 mm, 5 μm particle-size).

Mobile Phase

For SBD-F assay the mobile phase was 1 mol/L acid-acetate buffer, pH 5.0, containing 30 mL/L methanol at a flow rate 0.7 mL/L . For ABD-F assay the mobile phase was 5 mmol/L potassium dihydrogen phosphate buffer containing 30 mL/L acetonitril and 30 mL/L isopropyl

alcohol, pH 1.9, at a flow rate of 1.5 mL/L . For commercial kit we used its own mobile phase at a flow rate 1.7 mL/L .

Comparison of Methods

For method comparisons, 30 plasma samples in different tHcy concentrations (4.2-52.94 $\mu\text{mol/L}$) were used. tHcy levels of the plasma samples were determined by three methods. SBD-F and ABD-F methods were compared with commercial kit (as a reference method) by linear regression analysis and correlation coefficients. We have also evaluated the difference plots (19) to show the differences of SBD-F and ABD-F methods from reference method.

Results

The chromatograms of the thiol components of a plasma sample for 3 assays are shown in Figure 1. In both SBD-F and ABD-F methods, in addition to homocysteine also other substances of importance such as cysteine, cysteinylglycine or glutathione and cysteamine as an internal standard are clearly separated in the same run. In Chromsystems method homocysteine and internal standard are well separated. Although the SBD-F and ABD-F methods were well correlated with commercial assay (Figure 2), there were relatively less discrepancies between SBD-F method and commercial kit especially below the 25 $\mu\text{mol/L}$ of tHcy levels (Figure 3). The main features of tHcy determination methods were listed in Table 1. The chromatographic time for all SBD-thiols derivatives were shorter than 5 min in SBD-F method. In ABD-F method elution of peaks was complete in only 7 min. For the Chromsystems assay run time was 4 min.

For the SBD-F method; calibration curves for homocysteine were linear up to 200 $\mu\text{mol/L}$ for samples prepared in PBS ($r^2 = 0.999$). The limit of detection for homocysteine was 0.16 $\mu\text{mol/L}$. Imprecision of the method was evaluated using two different serum pools, one with high (21.75 $\mu\text{mol/L}$), the other low concentrations (12.06 $\mu\text{mol/L}$). After sample preparation, aliquots of probes were frozen at -20°C . CVs were assayed by measuring four replicates of each pool serum over 10 days. CVs of the within-run assays were 0.93% and 1.3%. CVs of the between-day assays were 1.74%

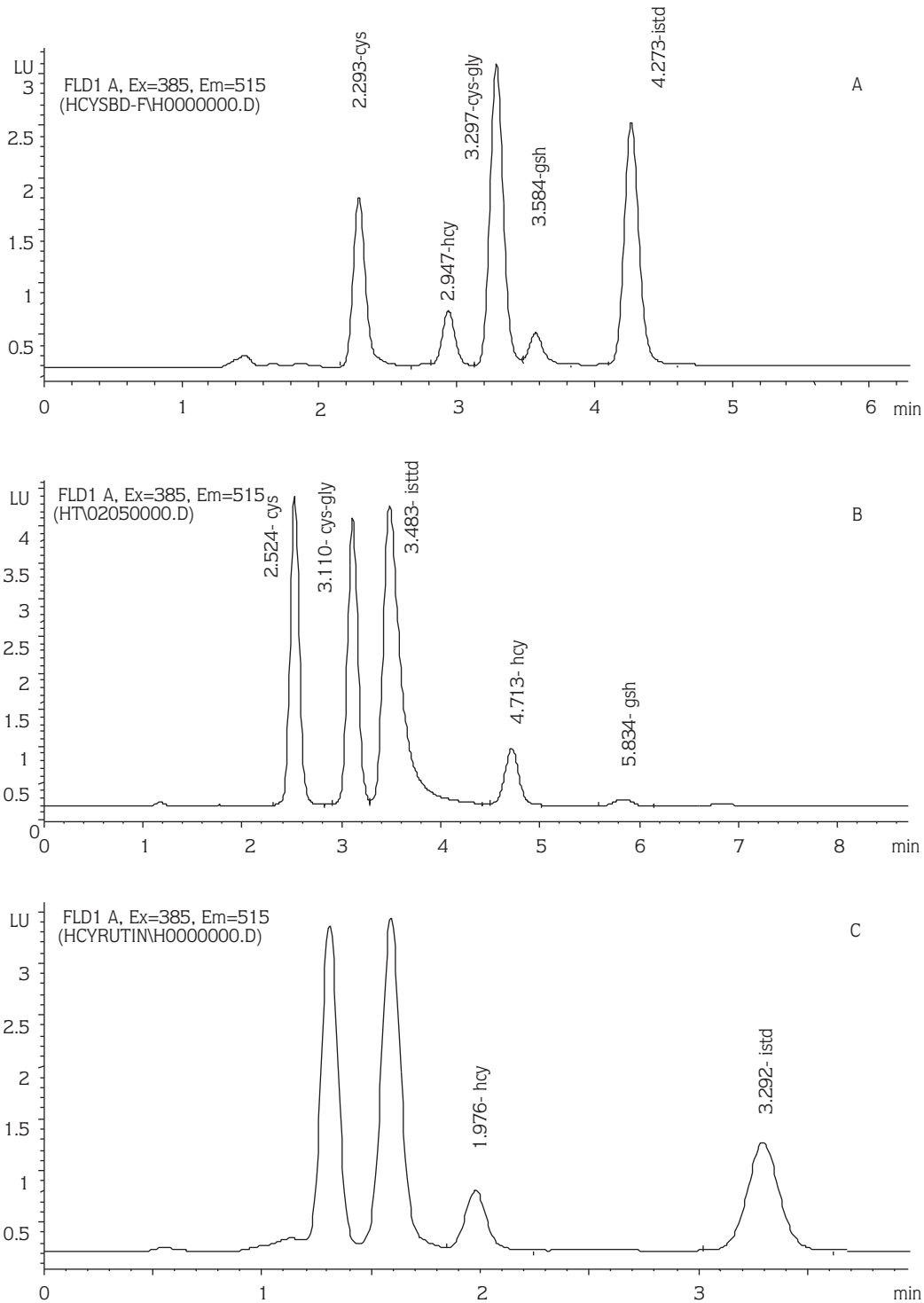


Figure 1. Chromatograms of plasma sample (~ 10 µmol/L) (A) SBD-F, (B) ABD-F and (C) Commercial assay.

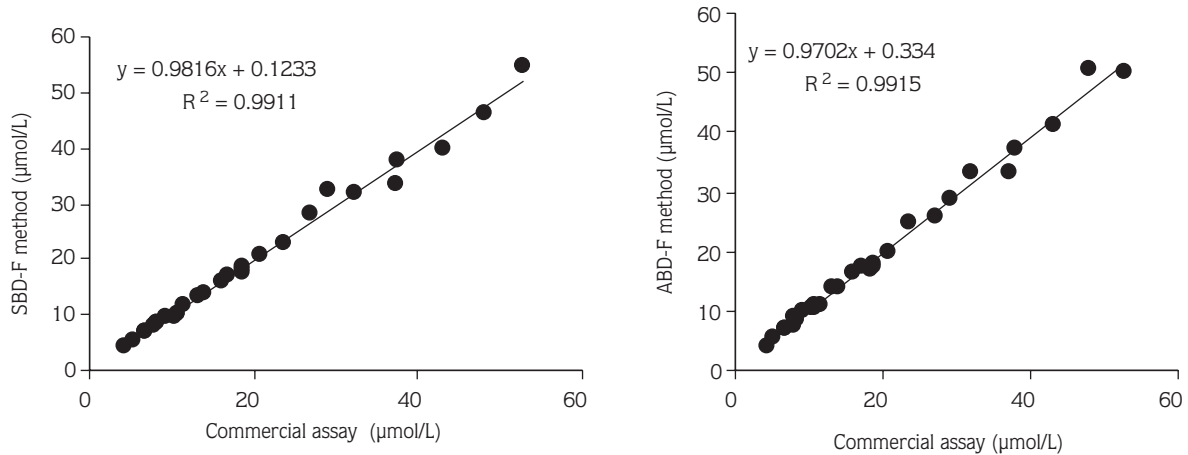


Figure 2. Comparisons of SBD-F and ABD-F methods with Commercial kit using linear regression analysis.

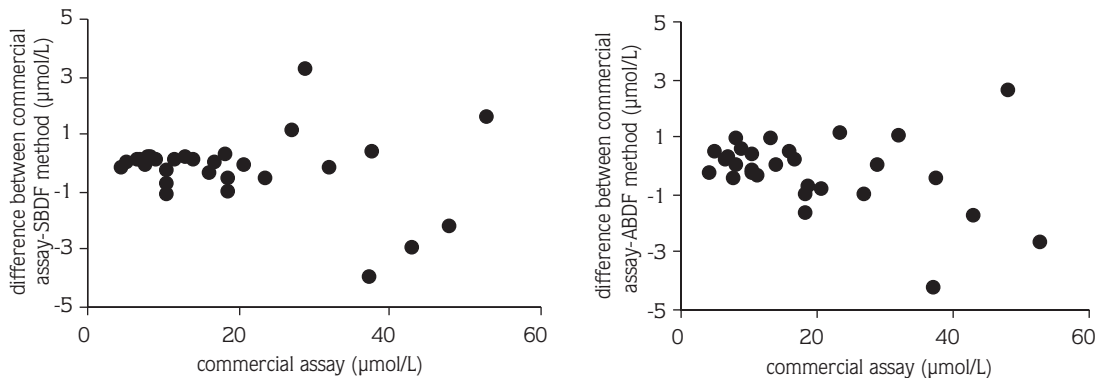


Figure 3. Comparisons of SBD-F and ABD-F methods with Commercial kit using difference plots.

and 2.69%. The analytical recovery of the method was tested by adding known Hcy concentrations (7-14 µmol/L) to aliquots of a plasma specimen (6.12 µmol/L homocysteine concentrations). The mean recovery determined in 10 days, was $98.73\% \pm 0.37$.

For the ABD-F method; the determination of homocysteine was linear up to 200 µmol/L both for samples prepared in PBS ($r^2 = 0.999$). The detection limit of this method was 0.20 µmol/L. The within-run reproducibility of the method was determined by successively injecting aliquots ($n = 10$) of two plasma samples with low and high concentrations (13.4 and 17.4 µmol/L, respectively). The CV values were 1.99 and 1.68% respectively. CVs of between-day assays for 10

days were 2.76 and 2.21 µmol/L, respectively. The analytical recovery of the method was tested by adding known Hcy concentrations (6.7-13.4 µmol/L) to aliquots of a plasma specimen (6.12 µmol/L homocysteine concentrations). The mean recovery determined in 10 days, was $99.72\% \pm 0.01$.

For Chromsystems method; this test was linear up to 200 µmol/L. The detection limit was 0.5 µmol/L. Determination of the intraassay-precision was made ten times by quantification of the homocysteine concentration within a normal-ranged plasma pool. The CV values were 1.8% for 8.99 µmol/L. Interassay CV was 4.2% for 14.11 µmol/L. The mean recovery was $98.6\% \pm 1.67$.

Table 1. Main features of SBD-F method, ABD-F method and Commercial Kit (CV% values are expressed as mean of CVs% for different concentrations).

| | Commercial Kit | SBD-F Method | ABD-F Method |
|---------------------------------------|----------------|--------------|--------------|
| Cost | ~20\$ | ~4\$ | ~7\$ |
| Run time (min) | 4.0 | 5.0 | 7.0 |
| Time for treatment procedures (min) | 30 | 110 | 40 |
| Recovery% (mean) | 98.6 | 98.73 | 99.72 |
| Detection limit ($\mu\text{mol/L}$) | 0.16 | 0.16 | 0.20 |
| CV% (intrassay) | 1.80 | 0.98 | 1.83 |
| CV% (interassay) | 4.20 | 2.22 | 2.49 |
| pH of mobile phase | 5.0 | 5.0 | 1.9 |
| Flow rate (mL/min) | 1.7 | 0.7 | 1.5 |
| Injection Volume (μL) | 20 | 10 | 20 |

Discussion

The methods described above are well suited for a rapid and reliable analysis of homocysteine in serum or plasma. Our estimates of within-run and between-run reproducibility of three methods are acceptable at both apparently healthy and high tHcy concentrations. Although, assay reproducibility and linearity of the SBD-F, ABD-F and commercial methods are clinically acceptable, the SBD-F method had the best method validation parameters among them. The column life was shorter in the ABD-F method compared with SBD-F method due to low pH (1.9) of its mobile phase. Although there are some commercial kits for determination of tHcy levels based on the Fluorescence Polarization Immunoassay (FPIA) principle, we especially focused on HPLC methods which is known as the method of choice for tHcy determination.

The reduction and thiol derivatization steps are cumbersome and laborious among HPLC methods and are sources of intermethod variability. We used the same reducing agent in SBD-F and ABD-F methods to minimize intermethod variability and to show the effect of derivatization agent on differences in results. For example, the use of the more water-soluble reducing agent TCEP in our assays instead of the tri-n-butylphosphine (TBP) enabled faster reduction, generally within 5–7 min instead of 30 min, respectively (17,20). Gilfix et al, (17) introduced TCEP as a novel and more suitable reductant for the routine determination of plasma tHcy. In a small method comparison study, they

found that the TCEP method yielded values ~21% higher than the TBP method. Pfeiffer et al (21) previously directly compared results obtained with these two reducing agents and found that, although the use of TCEP produced higher relative fluorescence intensities, the calculated concentrations of plasma tHcy were not significantly different if calibration was performed in plasma and cysteamine was used as the internal standard.

ABD-F reacts 30 times faster with thiols than SBD-F (22), a shorter incubation time—7 min instead of 60 min at 60 °C, respectively—was required. In addition, the efficiency of SBD-F thiol derivatization can be matrix-dependent (18) and substantially affected by lot-to-lot variations and by differences among manufacturers (20) The best between-run replicate sample measurements among the three methods were consistent with the SBD-F method although the ABD-F method requires less technologist handling and specimen transfer steps when compared with the other two assays. For example, with the ABD-F assay, reduction and derivatization occur in the same tube progressively with short incubation times. The SBD-F and commercial methods require reduction before protein precipitation, the subsequent removal of the plasma proteins, and the transfer of an aliquot of the resulting supernatant to another tube for derivatization.

In conclusion, SBD-F and ABD-F methods were reproducible, rapid, and easy to use for the quantification of plasma tHcy, with similar advantages to the commercially available kit. Moreover, despite longer duration of treatment procedures, the SBD-F method

may be the method of choice among these assays because of the lowest cost per test and better precision. Alternatively, because of shortest turnaround time of ABD-F method, clinical laboratories could prefer the ABD-F method according to their laboratory conditions such as the shorter working time, higher test order counts, and lack of laboratory technicians etc.

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