Plasma Homocysteine Measurement with Ion Exchange Chromatography (Jeol Aminotac 500): A Comparison with the Abbott IMx Assay

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Conclusion: The IEC method for tHcy measurement shows adequate precision and correlates highly with the IMx assay. The IEC method is more time-consuming but less expensive in reagent cost and allows simultaneous determination of plasma methionine concentration which may help to explain the underlying mechanism responsible for hyperhomocysteinemia.

Introduction

Increased plasma total homocysteine (tHcy), a sulfur-containing amino acid, is recognized as an independent risk factor for cardiovascular disease. Studies also showed a link with neuropsychiatric disorders, pregnancy complications, or birth defects [1]. Commonly, hyperhomocysteinemia is a consequence of folate or cobalamine deficiencies, the vitamins involved in Hcy metabolism, but may also be linked to enzyme defects in the metabolism of sulfur-containing amino acids. Numerous techniques have been developed to assay tHcy in plasma or serum [2]. Among the automated methods, fluorescence polarization (FP) is widely used in routine clinical chemistry laboratories [3] and correlates well with high-performance liquid chromatography [4, 5]. However, this immunoassay is limited solely to tHcy determination and cannot...
quantify other amino acids levels, like methionine (Met), involved in Hcy metabolism. Jeol Aminotac 500 is an automated amino acid analyzer based on ion exchange chromatographic (IEC) separation followed by ninhydrin detection and provides the basis for quantification of all amino acids. A ‘short’ program allows determination of Hcy, and possibly also Met, in the same run. We have evaluated the analytical performance of the Jeol Aminotac 500 for tHcy determination and compared it with the IMx tHcy assay.

Materials and Methods

Blood Sampling

Whole blood was collected in tubes containing lithium heparin, kept cold on ice after collection, and quickly centrifuged (2,500 g, 10 min). During a 2-month period, plasma samples of patients scheduled for routine tHcy determination (n = 55) were stored in two aliquots at −20°C until assayed by the two methods. Clinical practice complied with the ethical rules of our institution.

Calibrators and Sample Treatment

IEC Method. All reagents were obtained from Sigma (Saint-Quentin-Fallavier, France). A solution with 25 µmol/l L-Hcy and 50 µmol/l L-Met prepared in pH 7 citrate buffer was used as calibrator. Then, a 2-step determination was performed using a 500 µl volume of plasma or calibrator solution: (1) reduction using a 12% dithiothreitol solution in pH 7 buffer (5 min at 20°C, 10/1 v/v), and (2) deproteinization using a 30% sulfosalicylic acid solution (10 min at 20°C; 10/1 v/v). After centrifugation (10,000 g, 5 min), treated samples were mixed (1:1 v/v) with Jeol sampling buffer containing 50 µmol/l L-norvaline (internal standard). The injection volume for the IEC assay was 50 µl.

FP Method. A plasma volume of 150 µl was used and no sample treatment was required.

Instrumentation and Reagents

IEC Method. Aminotac 500 was supplied by Jeol (Tokyo, Japan). The elution program was optimized for tHcy analysis using two lithium citrate buffers, i.e. ref. P-12 (pH 3.28, 35%) and ref. P-13 (pH 3.46, 65%) (flow rate 0.56 ml/min, temperature 55°C). After reaction with ninhydrin (135°C, flow rate 0.25 ml/min), amino acids were detected at 570 nm. In these conditions, tHcy eluted at 13.7 min and Met at 14.7 min, allowing automated analysis of up to 40 plasma samples per 24 h. Data acquisition and calculations were made using the Jeol Workstation software version 3.08E.

FP Method. IMx analyzer, disposables and reagents were provided by Abbott (Abbott Park, Ill., USA). The IMx tHcy assay was performed as described previously [3] and according to the manufacturer’s instructions. This is a totally automated FP immunoassay with no sample pretreatment. Dithiothreitol reduces Hcy bound to albumin and to other molecules, and tHcy is converted to S-adenosyl-L-homocysteine (SAH) with SAH hydrolase. A monoclonal antibody that recognizes SAH and a fluoresceinated SAH analogue tracer constitute the FP immunoassay detection system.

Method Validation

Validation of the tHcy assay on Aminotac 500 (linearity, precision, limit of detection, interference) was carried out according to the French Biology Society (Société Française de Biologie Clinique) guidelines [6].

Statistical Analysis

Statistical analyses were done using Prism 4 (GraphPad Software, San Diego, Calif., USA). The linearity of the IEC technique and the correlation between the two methods were studied using Pearson’s test and Deming’s regression, respectively. Deming’s regression makes the assumption that the data plotted on the x-axis have errors and more accurately represent those obtained in clinical laboratory. Results were also compared by plotting the difference for tHcy measurements (Aminotac 500 – IMx) versus average concentration according to Bland and Altman [7]. A p value below 0.05 was considered significant.

Results

IEC Method Validation

Linearity. A 7-point (5, 15, 24, 52, 98, 144, 190 µmol/l) standard curve was prepared. The linear regression equation curve was y = x – 0.10 with r = 0.999, and linearity was confirmed up to 190 µmol/l (not tested beyond).

Limit of Detection

The limit of detection (LD) is defined as LD = 3.3 · SD/a, where SD is the standard deviation of the response and a is the calibration curve slope. Regarding chromatography methods, SD is the standard deviation of y-intercepts of calibration curve regression lines [8]. Then, 5 linearity curves were built in 5 successive assays to calculate SD. LD was found at 1.6 µmol/l (with a = 1.00 and SD = 0.50 µmol/l).

Precision. Three plasma levels containing low (6 µmol/l), medium (15 µmol/l) and high (30 µmol/l) concentrations of tHcy were studied. To determine between-day imprecision, each level was analyzed over 15 consecutive days. Inter-assay coefficients of variation (CVs) were 9.6% (low), 5.1% (medium) and 4.9% (high). To determine within-run imprecision, each level was analyzed in a replicate of 14. Intra-assay CVs were 8.2% (low), 3.1% (medium) and 2.3% (high).

Interference. The peaks of other sulfur amino acids, i.e. L-Met retention time (RT) (14.7 min) and L-cysteine (RT 7.8 min), were well separated from L-Hcy (RT 13.7 min) and L-norvaline (RT 15.5 min) peaks and did not interfere with L-Hcy quantification.
Methods Correlation

Deming’s regression (fig. 1) showed an excellent correlation between the two methods: \( y = 0.99x – 1.23 \) \( (r = 0.97; p < 0.001) \). The Bland-Altman plot of the difference in tHcy measurements obtained with the two analyzers as a function of their mean value (fig. 2) showed an average discrepancy of \(-1.3 \mu mol/l\) with no trend of the discrepancy getting bigger (or smaller) as the average increases.

Discussion

The IEC tHcy assay is validated according to the standardized protocol for the evaluation and validation of the techniques [6]. The physiopathological range (approximately 3–40 \( \mu mol/l \) in the 0.5th to 99.5th percentiles in the general population) [2] fits in the analytical range. The precision is acceptable, and there is no interference from other sulfur-containing amino acids. Thus, our study shows that the IEC technique can be a valuable option in a routine laboratory, giving a wider analytical range reaching 190 \( \mu mol/l \) compared with 50 \( \mu mol/l \) for the IMx. The precision is good with a CV of around 5% at the usual upper reference limit of 15 \( \mu mol/l \); FP assays, however, have a better precision [3–5]. The Jeol Aminotac 500 also correlated well with the IMx tHcy immunoaassay which is commonly used in clinical biochemistry laboratories. Although calibrators were different, no proportional error was observed and the results obtained by the two methods agreed well, with less than 5% of values differing by 2 SD. The fact that analytical principles are totally different may account for the systematic error of \(-1.3 \mu mol/l\) observed between the methods. Thus, as reported by other authors who used HPLC assay [5], tHcy concentrations measured with IEC tended to be lower than those measured from the FP assay.

**Fig. 1.** Correlation of the IEC tHcy assay on the Jeol Aminotac 500 with the IMx assay (Deming’s plot): study of 55 plasma samples.

**Fig. 2.** Bland-Altman plot analysis of the tHcy values obtained by two methods: the y-axis represents the difference between Aminotac 500 and IMx values and the x-axis represents the average of the Aminotac 500 and IMx values. The solid line represents the mean value. Dotted lines are 2 SD. The mean difference is \(-1.3 \mu mol/l\).
The positive points of this IEC method are: (1) a wide analytical range, avoiding re-run of samples with high tHcy concentration; (2) a lower reagent/consumable cost (EUR 4.2 vs. 9.2 per sample; (3) provision for simultaneous determination of the concentration of other sulfur-containing amino acids which can help with the study of mechanism underlying hyperhomocysteinemia [1]. However, IEC requires skilled staff and time-consuming treatment of samples. Conversely, analyzers using FP are more user friendly [3], less labor-intensive, have a lower imprecision [3–5] and may have a random/continuous mode allowing rapid tHcy quantitation.

**Conclusion**

Aminotac 500 fulfills all requirements for validation of tHcy measurement and its routine use. However, this method is best suited for laboratories with experience of such a technology and the need for simultaneous determination of plasma Hcy as well as other amino acids such as L-Met using optimized experimental conditions.

**References**