

A Rapid HPLC Method for the Measurement of Ultra-low Plasma Methionine Concentrations Applicable to Methionine Depletion Therapy

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Abstract. A rapid, sensitive and specific high-performance liquid chromatographic (HPLC) method for the separation and quantification of L-methionine in plasma has been developed. After derivatization of plasma amino acids with *o*-phthalaldehyde (OPA), a 50 μ l sample was loaded on a reversed-phase Supelcosil LC-18-DB column (particle size 5 μ m, 25 cm x 4.6 mm, 120Å pores). A customized gradient program using tetrahydrofuran/methanol/0.1 M sodium acetate, pH 7.0, v/v/v=5/95/900 and methanol was used with detection by fluorescence. The elution time was 15 minutes, a 3-fold improvement over existing methods. The linearity was 1-100 μ M. The limit of detection was 0.5 μ mol/L, a 10-fold improvement over existing methods. The inter-assay CVs were 2-5%, and the intra-assays CVs were 4-8%. The sensitivity and rapidity of this HPLC method is particularly applicable to determine the efficacy of methionine depletion therapy of cancer.

Methionine (MET) is one of the most important amino acids, as it is necessary for both protein synthesis and cellular transmethylation reactions (1, 2). MET is also the precursor of homocysteine, a major risk factor for cardiovascular disease and other diseases (3, 4). Cancer cells have elevated MET requirements (5) which are being targeted by a MET degrading enzyme, L-methionine α -deamino- γ -mercapto-methane lyase (methioninase) [EC 4.4.1.11] cloned from *Pseudomonas putida* (6). With many applications requiring serum MET measurement, a more rapid and sensitive serum MET assay is needed. A rapid, sensitive and high-resolution method is described in this report.

MET measurement originally involved separation of amino acid mixtures using classical ion-exchange chromatography

followed by post-column derivatization with ninhydrin or a fluorogenic reagent (7). This technique resolved most amino acids with good detection limits, especially if either fluorescamine or *o*-phthalaldehyde (OPA) was used as the post-column derivatizing reagent. However, this procedure generally required specialized equipment and an analysis time of up to 3-4 h for physiological fluids.

MET has been measured with OPA as a precolumn derivatizing reagent. OPA in the presence of either 2-mercaptoethanol or ethanethiol reacts rapidly with primary amino acids to form highly fluorescent, thio-substituted isoindoles (7, 8). These derivatives were measured by reversed-phase high-performance liquid chromatography (HPLC) with reasonably high specificity and sensitivity (8). This HPLC method reduced the analysis time of physiological fluids by decreasing the length of the ODS columns filled with 3-mm particles. A methanol gradient in 0.1 M aqueous sodium acetate, pH 7.2, was used for elution. Mixtures which contained as many as 48 components were resolved with an analysis time of less than 50 min (8).

We describe here a more rapid, sensitive, and high-resolution HPLC method to measure MET with pre-column OPA derivitization using a modified sodium acetate tetrahydrofuran and methanol gradient. This method is applicable to plasma, serum and other complex biological fluids and is suitable for biochemical, diagnostic, and therapeutic purposes.

Materials and Methods

Equipment. An Hitachi L-6200A Intelligent Pump (Hitachi, Ltd., Tokyo, Japan), an Hitachi F 1000 fluorescence detector (Hitachi, Ltd.), a reversed-phase Supelcosil LC-18-DB column (particle size 5 μ m, 25 cm x 4.6 mm, 120 Å pores) (Supelco Park, Bellefonte, PA, USA) a water bath and an Eppendorf centrifuge (Beckman, San Diego, CA, USA) were used.

Reagents. Fluoraldehyde™ (*o*-phthalaldehyde(OPA) reagent solution (Pierce, Rockford, IL, USA), amino acid standard H (Pierce), L-methionine (Sigma, St. Louis, MO, USA), sodium acetate (Sigma),

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acetonitrile (Fisher Scientific, Springfield, NJ, USA), methanol (Fisher Scientific), and tetrahydrofuran (Fisher Scientific) were used.

Preparation of HPLC working solutions. Distilled water (DW) was obtained from a Milli-Q PLUS ultra-pure water system (Millipore, Bedford, MA, USA) and used for dilutions. 0.1 M sodium acetate (NaAC), pH 7.0, was prepared by dissolving 8.2 g NaAC in 800 ml DW. The pH was adjusted with acetic acid to 7.0 with DW added to a final volume of 1L. Solution A for HPLC was prepared with tetrahydrofuran/methanol/0.1 M sodium acetate (pH 7.0), v/v/v=5/95/900. Solution B for HPLC was methanol.

Preparation of standard solutions. Amino acid standard H solutions for MET quantitation were prepared as follows: 4 μ l amino acid standard H solution (containing each amino acid at 2.5 μ moles/ml) was added to 96 μ l DW to obtain 100 pmoles/ μ l of each amino acid. The amino acid solution was then diluted to 100, 50, 10 and 1 pmoles/ μ l with DW. MET standard solution for MET peak identification was prepared by adding 15 mg of L-MET (MW 149.21) in 10 ml DW to obtain 10 mmoles/ml, then diluted with DW to obtain 50 pmoles/ μ l MET.

Plasma sample collection and storage. Blood was collected by orbital puncture from mice into a Vacutainer Tube (Becton Dickinson, Rutherford, NJ, USA) containing EDTA. Samples were immediately stored at 0-4°C. Plasma was separated within one hour by centrifuging at 3,000 rpm for 5 min at 4°C. MET is stable for one month at 4°C and approximately 6 months at -20°C or -80°C.

Derivatization with OPA. Ten μ l plasma samples were rapidly defrosted and maintained on ice. Thirty μ l acetonitrile was added to the plasma sample and mixed well, then centrifuged at 13,000 rpm for 2 min at 4°C. Ten μ l of the supernatant was collected into an Eppendorf tube. Five μ l OPA solution was added, mixed well, and incubated at room temperature for 90 sec. The remaining supernatant was treated with acetonitrile and stored at -70°C. One-hundred and fifty μ l 0.1 M sodium acetate (pH 7.0) was added and mixed well. The OPA-derivatized samples were loaded immediately on the HPLC column.

Thirty μ l acetonitrile was added to the 10 ml amino acid standard and mixed well. Ten μ l of the mixture was added to an Eppendorf tube. Five μ l of OPA solution was added and mixed well, and then incubated at room temperature for 90 sec. One-hundred and fifty μ l 0.1 M sodium acetate (pH 7.0) was added and mixed well. The OPA-derivatized samples were loaded immediately on the HPLC column.

HPLC protocol. A Supelcosil LC-18 DB column (particle size 5 μ m, 25 cm x 4.6 mm, 120 Å pores) was used with a matched guard column.

The gradient program for HPLC was as follows:

- 0-5 min: 46% Solution B, flow rate 2 ml/min.
- 5.1-10 min: 46-48% Solution B, flow rate 1 ml/min.
- 10.1-15 min: 100% Solution B, flow rate 2 ml/min.
- 15.1-20 min: 46% Solution B, flow rate 2 ml/min.

The sample loading volume was 50 μ l. Running time was a total of 15 min. The column was kept at 20-25°C. Detection wavelengths were excitation at 350 nm and emission at 450 nm.

Data analysis. The MET peak was identified by the addition of the MET standard solution to the amino acid standard H. The

calibration curve was constructed with the concentrations of MET added to the amino acid standard H solution as the x-axis, and the area of the MET peak as the y-axis. The MET levels in the plasma samples were calculated from the calibration curve.

Precision. The precision of the rapid MET HPLC assay was determined based on the NCCLS EP5-T2 guideline "Evaluation of Precision Performance of Clinical Chemistry Devices". Three plasma samples, containing low, medium and high levels of MET, respectively, were measured. In each run, aliquots of low, medium, and high MET calibrators were used. The average and standard deviation were calculated: CV (%) = standard deviation / average x 100%.

Linearity. The linearity of the rapid MET HPLC assay was determined by measuring 3 plasma samples with MET concentrations of 100, 20 and 5 μ mol/L serially diluted 2-, 4- and 8- fold with saline. The samples were analyzed in four replicates in a single run. Average data and dilution recovery were calculated.

Results and Discussion

Elution time. The elution time of the rapid MET HPLC assay was 15 min compared to 40 min for the previous method (8). The retention times of the MET peak (Figure 1) in the rapid MET HPLC assay was 7.5-8.0 min compared to 24-25 min for the previous method (8).

Linearity of MET measurements. A calibration curve for MET of concentrations between 1 and 100 μ mol/L was obtained as described in Materials and Methods. Four calibrators (1, 5, 10 and 100 μ mol/L) were used. The calibration curve was linear and repeatable between 1-100 μ mol/L.

Limit of MET quantification. A calibrator of 10 μ mol/L MET was diluted with normal saline to obtain concentrations of 0.1, 0.5 and 1 μ mol/L. Each sample had four replicates. The limit of quantification of the rapid MET HPLC assay was 0.5 μ mol/L, defined as the lowest concentration having a coefficient of variation (CV) <20%. The limit of quantitation of the previous MET HPLC assay was 5 μ mol/L (8).

Peak resolution (Figure 1). The resolution of the MET peak of the rapid MET HPLC assay is similar to the previous HPLC method (8) with the MET values between 10-100 μ mol/L. However, only the rapid MET HPLC assay could clearly identify a MET peak below 5 μ mol/L.

MET recovery rate. A serial dilution of a sample with high MET concentration (120 μ mol/L) was determined by the rapid MET HPLC assay. Linear regression of the observed MET vs. calculated MET resulted in a recovery of 100 \pm 5%. Recovery of known amounts of MET added to a plasma sample with a MET of 58 μ mol/L was determined. The average recovery rate was more than 95%.

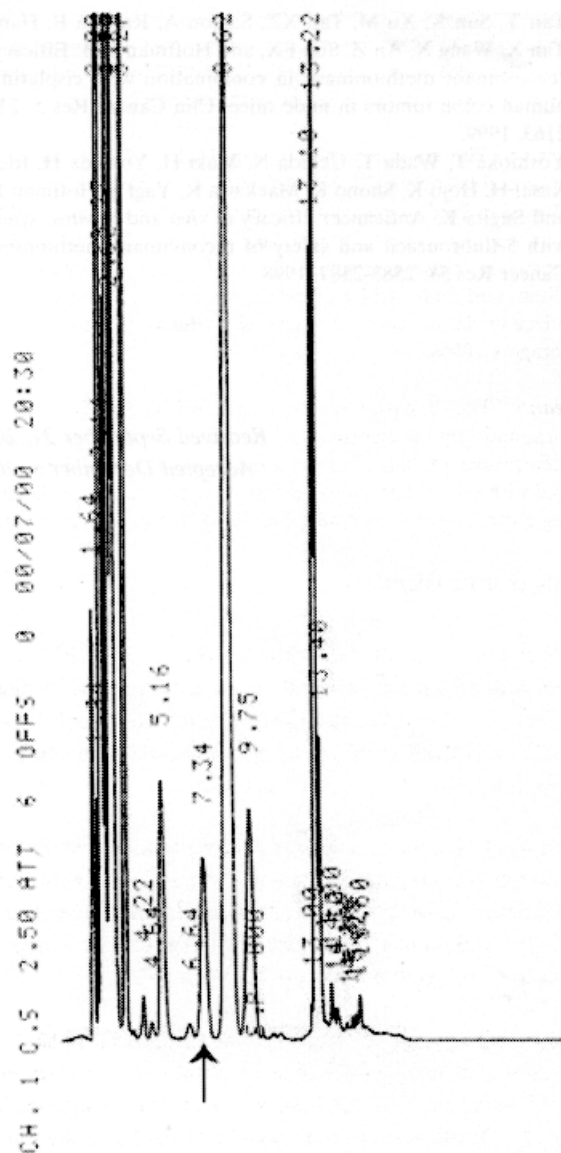


Figure 1. Elution profile of OPA-derivatized amino acids from mouse plasma. Y-axis is fluorescence and X-axis is time. Chromatography was on a reversed phase Supelcosil LC-18-DB column, 26 cm x 4.6 mm with 5 μ m particle size. Elution time was 15 min. See Materials and Methods for gradient conditions and flow rates for each segment of the gradient. Arrow indicates methionine peak.

Precision of MET measurements. The precision of the rapid MET HPLC assay was determined after analyzing three plasma samples containing low, medium and high MET in parallel assays. The within-assay variation was determined from results from 8 parallel analytical set-ups. The between-assay precision was calculated in 10 successive assays carried out in 10 days. The CVs for within-assay and between-assays were less than 5% and 8%, respectively.

Table I. Comparison of the rapid MET HPLC assay and the previous HPLC method (8) for MET determination.

	Linearity (μ mol/L)	Limit of quantitation (μ mol/L)	Running time (minutes)	MET peak elution time (minutes)
Previous HPLC MET assay (8)	10-100 μ mol/L	5 μ mol/L	40 minutes	24-25 minutes
Rapid MET HPLC assay	1-100 μ mol/L	0.5 μ mol/L	15 minutes	7.5-8.0 minutes

In conclusion, we have developed a rapid, sensitive, high-resolution and precise HPLC method for the separation and quantification of MET in plasma. See Table I for comparison of present and previous methods and results: The running time of the improved method is only 15 min, less than one-third the time of the previous method (8, 9). The linearity is 1-100 μ mol/L. The limit of quantitation is 0.5 μ mol/L, a 10-fold improvement over the previous method. This method is highly useful for determining the efficacy of methionine depletion methods such as methioninase treatment (9-11).

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