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A RAPID QUANTIFICATION OF SERUM FREE METHIONINE BY HPLC IN RELEVANCE TO POULTRY INDUSTRY

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ABSTRACT

A sensitive and specific high-performance liquid chromatography (HPLC) method for the separation and quantification of methionine in serum has been developed. After derivatization of serum amino acids with PITC (Phenyl isothiocyanate), a 20 µl sample was loaded on a reversed-phase x-terra MS C18 column (particle size 3.5um, 3.0x150mm). An isocratic program using tetrahydrofuran/methnol/0.1M sodium acetate, pH 7.2 and acetonitrile was used with detection by UV detector. The elution time was 15 minutes. Calibration curves suitable for the analysis of serum were linear (r2 > 0.981) with limits of detection (LOD) was 3.6 pmol/ml. Intraday relative standard deviation and interday were both lower <15.81%and <3.74%. The sensitivity and rapidity of this HPLC method is particularly applicable to provide a more sensitive assessment of protein nutritional status and diet quality of Broiler chicks. This method is applicable to plasma, serum and other complex biological fluids and is suitable for biochemical diagnostic and therapeutic purposes.

KEYWORDS

Quantification, Methionine, HPLC, Serum, free amino acid.

INTRODUCTION

Methionine (MET) is one of the most important amino acid, 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]. As homocysteine research has gained impulsion, the evaluation of plasma methionine concentrations has acquired importance. Cancer cells have elevated MET requirements[5] which are being targeted by a MET degrading enzyme, L-methionine a-deamino-mercapto- methane lyase (methioninase) [EC 4.4.1.111 cloned from *Pseudomonas putida*[6]. Methionine measurement generally has been performed by HPLC after PITC derivatization. Its separation from other amino acids is time-consuming. Profiles of free amino acid concentrations in serum and muscle tissue have been used successfully to evaluate the protein nutritional status of laboratory animals[7] and of humans[8]. Concentration of free amino acids in serum and tissue represent a balance between amino acids intake in food, rate of their use in protein synthesis, and in muscle catabolism or amino acid oxidation[9]. It was hypothesized that clinical evaluation incorporating concentrations of free amino acids in serum would provide a more sensitive assessment of nutritional status. Profiles of selected amino acids in

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serum can be used for assessing protein nutritional status and diet quality of poultry birds. With many applications requiring serum MET measurement, a rapid and sensitive serum MET assay is needed. Such a rapid, sensitive and high-resolution method is described in this report.

MATERIAL AND METHODS

Reagents and chemicals

PITC (Phenyl isothyocyanate) derivatization reagent solution, amino acid standard solution (own synthesized). L-methionine (Loba chemicals pvt ltd, Mumbai, India),sodium acetate (Qualigens fine chemicals, India), acetonitrile, methanol, tetrahydrofuran and HPLC grade water (Merck specialties pvt ltd) were used. This experimental design is approved by Research and Development Committee of CSJM University, Kanpur.

Apparatus

An Water L-07515901A/910A Pump model 515, an Water 2998 photodiode array UV detector (Waters, Milford, MA, USA), a reversed-phase x-terra MS. C18 column (particle size 3.5 um, 3.0x150 mm, Waters, Milford, MA, USA), a water bath and an eppendorf centrifuge (Beckman. San Diego. CA, USA) were used.

Chromatography and separation conditions

The chromatographic system used to perform the analyses consisted of two Waters model 515 pumps (Waters, Milford, MA, USA), a manual injector (Rheodyne, Cotati, CA, USA) equipped with a 20 µl loop, and a model 2998 UV detector (Waters); it was operated by means of the Empower 2 software (Waters). The chromatographic separation was performed in reversed-phase mode using a reverse-phase x-terra MS C18 column for free amino acid analysis (particle size 3.5 um, 3.0x150 mm, waters Pvt. ltd, Milford, USA) operating at room temperature; the detection wavelength used was 254 nm. The analyses were carried out applying an isocratic gradient profile to the mobile phase composition. Eluent A was HPLC-grade water containing 0.1 M sodium acetate, tetrahydrofuran, methanol (v/v/v=900/5/95, adjusted to pH 7.2 with 10% acetic acid) and eluent B was HPLC-grade acetonitrile, water, and methanol (9:8:3,v:v:v). The isocratic elution programme was run at a constant flow rate of 0.60 ml/min and was set with 1:1 ratio. The total duration of a single run, including column re -equilibration was 15 min.

Preparation of standard solution

MET standard solution for MET peak identification was prepared by dissolving 14.921 mg of L-MET (MW 149.21) in 10 ml distilled water to obtain a concentration of 10 mmol/ml which was then diluted with different quantities of distilled water to obtain a varied range in concentration required for calibration.

Serum sample collection and storage

Blood samples were collected from four groups of chicks (5 in each group fed with different protein percentage for 4 weeks) by exsanguinated via the jugular vein in vacuum tubes which were allowed to clot, then centrifuged at 10^3 × g for 15 min. Serum thus obtained were decanted and stored frozen at -20 °C for methionine and other amino acid analysis. MET is stable for one month at 4°C and approximately 12 months at -20°C or -80°C.

Deproteinization of serum samples

Serum from each bird was deproteinized by filtering through a cut-off ultra filtration membrane filter (Ultra free-MC, Millipore, Bedford, MA 01730) by centrifuging at 10^3×g for 20 min [10].

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Derivatization of serum sample with PITC

Fifty µl of deproteinized specimen and standard solution of amino acids were dried under vacuum (<45 mm Hg). The samples were reconstructed with 20 µl of solution I, dried at 40 mm Hg, and dissolved in 40 µl of derivatization solution II. Derivatization of amino acids occurred within 30 min at 40-45 °C to produce the corresponding phenylthiocarbamyl (PTC) derivatives. The samples were then dried until a constant vacuum of 45 mm Hg was obtained. Finally, the dried samples were dissolved in 150 µl of sample eluent A, filtered through 0.22 um syringe filter and injected. Solution I and II were prepared from 1 M sodium acetate-methanol-TEA (2:1:1) solution and methanol– water–TEA–PITC mixture in the ratio (7:1:1:1) respectively.

Data analysis and calibration

The methionine peak was identified by the addition of the methionine standard solution to the PITC derivatization solution. The calibration curve was constructed with the concentration of methionine added to the standard solution as the x-axis, and the area of the methionine peak as the y-axis. The methionine levels in the serum samples were calculated from the calibration curve.

Limit of MET quantification

A calibrator of 10 pmol/L MET was diluted with normal saline to obtain concentrations of 0.1,0.5 and 1 pmol/L. Each sample had four replicates, The limit of quantification of the rapid MET HPLC assay was 36.0 pmol/L, defined as the lowest concentration having a coefficient of variation (CV) ~20%. Linearity and the limit of detection were estimated by injecting methionine standard solutions and determination of limit of detection was 3.6 pmol/L . Average data and dilution recovery were calculated. Regression analysis was performed by using the Statistical Analysis System Software (Originpro 7.5 of Microcol Software Inc Procedure. USA). Pearson correlation coefficients (r) and 95% confidence intervals (CI) for slope values were calculated.

RESULT AND DISCUSSION

Figure-1 presents the analysis chart for standard methionine (10 pmol/ml) with the retention time of methionine as 6.910 min under the present experimental conditions. Figure 2 (A,B, C, and D) show an example of chromatogram of 8%, 15%, 23% and 35% diet containing chicks serum respectively. The calibration curve showed a linear response over the concentrations tested with a regression coefficient (r2) of 0.981. The coefficients of variation (CVs) of the slope and intercept of the calibration curve were 1.76% and 19.38% respectively. Injection reproducibility was calculated by injecting the same standard solution 5 times consecutively. Within-run precision (intra assay) of the method was evaluated by measuring the same biological sample, independently prepared, in the same sample set 5 times. Precision tests indicated a good repeatability of the method for both migration times (CV<1.12%) and areas (CV<5.72%). Moreover, a good reproducibility values of intra-assay and inter-assay tests were obtained (CV<15.81% and CV<3.74%, respectively).

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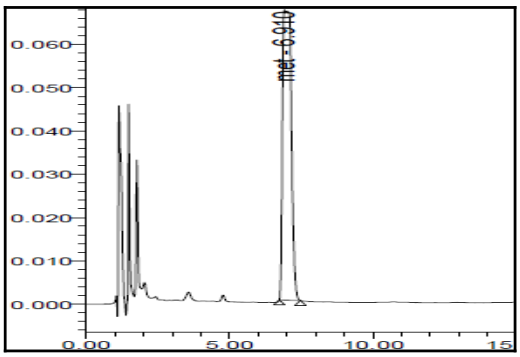

Figure1. ***Chromatograms of standard methionine solution***

The limit of quantification was about 36.0 pmol/L of methionine. The limit of detection was 3.6pmol/L of methionine. Linear regression of the observed MET vs. calculated MET. The average recovery rate was more than 95%. 1ml serum was collected from each animal of every group, and was derivatized followed by injecting for its analysis and the result showed 0.1, 0.9, 2 and 700 pmol/ml or 0.00707 ,0.56569, 1.58114 and 187.08287 methionine (mean value and standard deviation) in 8, 15, 23and 35% protein fed chicks respectively. Therefore, this method can be used not only for measuring the methionine content in serum but also to monitor methionine concentrations in serum after derivatization with PITC.

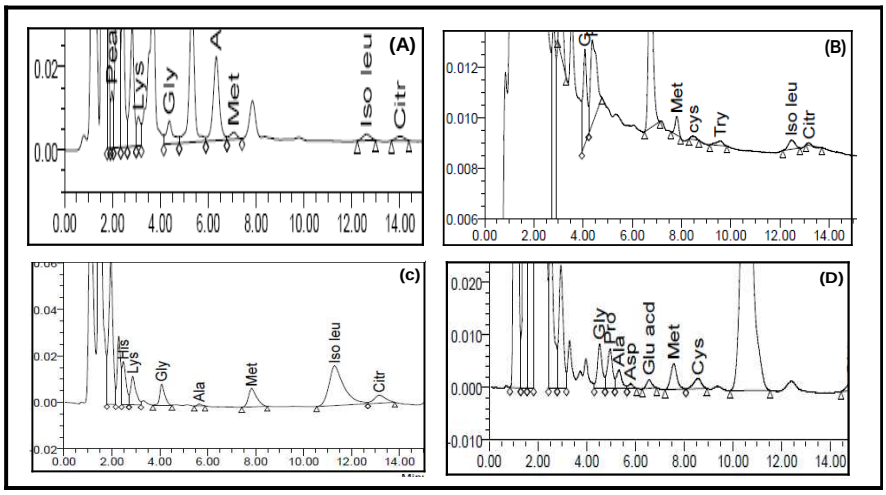

Figure 2. ***Chromatograms of chick's serum sample of different protein Containing fed (a) 8% (b) 15% (c) 23% and (d) 35%.***

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CONCLUSIONS

The RP-HPLC application described in this work can be used to determine the concentration of the methionine in serum by analyzing the derivatized serum. The method's analytical performance showed good reliability over a wide number of bulk and individual serum samples drawn from different protein fed chicks. A significant operational advantage can be achieved as the usual result-affecting and time-consuming sample preparation procedures can be replaced by changing the ratio of eluent composition. In addition, this protocol can be easily adopted in the food and nutritional industries as it requires only common instrumentation to work. This gives major advantages in speed, ease of handling and accuracy. Altered nutritional states in birds often yield rapid adjustments in physiological homeostasis, resulting in measurable changes in blood chemistry [11, 12]. The poorly defined sensitivity of most measures of physiological responses to alterations in diet quality limits their value in nutritional assessment, especially with regard to protein nutrition, in Broiler chicks.

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