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Simultaneous analysis of citrulline and arginine in serum and tissue

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ABSTRACT

We developed a method for simultaneously determining L-citrulline and L-arginine levels in serum and tissue samples using RP-HPLC with ultraviolet (UV) detection. The serum was deproteinized by trichloroacetic acid and heat; the tissue was homogenized by trichloroacetic acid and deproteinized as the same as serum. Phenylisothiocyanate (PITC) solution was used as derivatization reagent and a gradient elution was carried out. The linearity for L-arginine and L-citrulline ranged from 0 to at least 1000 μmol/L. R^2 values were above 0.9999 for both. LODs for L-arginine and L-citrulline were 0.0462 μmol/L and 0.0195 μmol/L, respectively, while LOQs were 0.530 μmol/L and 0.417 μmol/L, respectively. Intra- and inter-day CVs were less than 3.5% and 7.5% in serum, respectively. The average recovery was from 85.5% to 116.5% in serum. Intra-assay CVs were 3.8% and 10.3%, and inter-assay CVs were 13.7% and 10.7% for L-arginine and L-citrulline respectively in tissue. The average recovery was from 92.8% to 113.5% in tissue. This is a reliable and convenient analytical method which is suitable for most clinical laboratories.

1. Introduction

In humans, the endogenous L-arginine is synthesized from L-citrulline by the collaboration between the proximal tubules of the kidney and the epithelial cell of the small intestine, and a small quantity of L-arginine can been produced via the urea cycle in liver. Arginine can produce nitric oxide (NO), citrulline, ornithine, urea, creatine, polyamines, proline, agmatine and other biological substances via the citrulline –NO pathway and the urea cycle. Apart from the synthesis of protein in vivo, arginine and its metabolites play an important role in biology, including cellular proliferation, vasodilation, neurotransmission, calcium release, and immunity. The amino acid citrulline, which is a byproduct of the NO pathway catalysed by NO synthase (NOS), plays a key role in the urea cycle. The metabolites of L-arginine are involved in many physiological and pathological processes [1–5]. Present researches show that arginine and its major catabolic product citrulline have relationship with hepatocellular carcinoma, renal cell carcinoma, pancreatic cancer and melanoma [6–10]. The citrulline concentration increased evidently in the cerebrospinal fluid (CSF) from patients with acute hydrocephalus, and the arginine/citrulline molar ratio decreased [11]. Citrulline strongly correlated with short bowel syndrome, small bowel absorptive capacity, and septic, trauma as well as idiopathic pulmonary arterial hypertension [2,12,13]. The recent study showed that citrulline and arginine levels were significantly augmented in the CSF of patients with Alzheimer's disease (AD) compared to those with mild cognitive impairment (MCI) [14]. Therefore, it is important in clinical medicine to develop a simple and accurate analytical method for simultaneously determining arginine and citrulline in body fluids or tissues.

Because arginine and citrulline do not contain aromatic ring and other chromophore, in general, the amino acids ought to be derivatized before measuring. Several techniques are available to measure the amino acids in biological body fluids. These techniques include amino acid analyzers, capillary electrophoresis (CE) and high performance liquid chromatography (HPLC), LC-MSMS and ultra performance liquid chromatograph tandem mass-spectrometry (UPLC-MSMS) etc. In amino acid analyzers method, amino acids are derivatized by post-column with ninhydrin or o-phthalaldehyde (OPA), it is suitable for measuring unknown complex samples. However, the amino acid analyzers method is complex and the equipment is expensive. The CE method has many advantages, including only needs micro-samples, and has high column effectivity, however, when some detecting methods were used, such as UV detector, the sensitivity will be reduced. LC-MSMS and especially recentry UPLC-MSMS have many advantages such as ultra resolution, ultra speed, ultra sensitivity and robustness, as well as, even no need for derivatization, however, these equipments are very expensive. Amino acids are determined simultaneously by precolumn derivatization with different reagents, followed by HPLC separation mainly. At present, precolumn derivatization with OPA has been used predominantly for analysis of arginine and citrulline in physiologic fluids using fluorescence detector [1,11,15–18]. Nevertheless, most of clinical laboratory use HPLC system with a UV–visible detector.

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Therefore, we developed an analytical method for the simultaneous analysis of arginine and citrulline contents in serum and tissue by reverse phase high performance liquid chromatography (RP-HPLC) using UV detection, in which PITC solution was used as precolumn derivatization reagent. In this experiment, serum was deproteinized by trichloroacetic acid and heat; tissue was homogenized by trichloroacetic acid and deproteinized as the same as serum. We measured the arginine and citrulline levels in the serum and tissue of patients with colorectal cancer by using this new method for the first time.

2. Materials and methods

2.1. Reagents

HPLC grade acetonitrile was purchased from the Shanghai Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). The PITC and the standards of L-arginine and L-citrulline were purchased from Sigma-Aldrich (St. Louis, MO, USA). The HPLC grade water used was purchased from Merck (Darmstadt, Germany). Other reagents used were of analytical grade and obtained from the Shanghai Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

The L-arginine and L-citrulline standard solutions were made by exactly weighing each standard of amino acid, and dissolving them in ultra-pure water (HPLC grade). Each standard was diluted to 1.0 mmol/L with ultra-pure water and was stored at 4 °C as a stock solution. Assay standards were produced freshly by diluting the stock standard solutions with ultra-pure water for each assay. The derivatization reagent solution consisted of methanol/ethanol/triethylamine/ultra-pure water/PITC (6/1/1/1/1, v/v/v/v/v), which was made freshly. The mobile phase A consisted of 0.12 M sodium acetate and 2.5 μM EDTA buffer with 2.5vol. % acetonitrile (pH 6.5), while the mobile phase B consisted of 15% methanol, 45% acetonitrile, and 40% ultra-pure water. Prior to use, the mobile phase solutions were filtered using 0.40-μm films and then degassed ultrasonically.

2.2. Apparatus

The HPLC system consisted of a high performance liquid chromatograph LC-10A (Shimadzu, Kyoto Japan), including LC-10AT binary pump, SIL—10AD automatic injection, SCL—10A controller, CTO—10A column oven, SPD—10A UV–visible detector, and CLASS—VP chromatogram analytical software.

2.3. Samples

2.3.1. Patients

A total of 22 patients with colorectal cancer, males 12, females10, (mean age 59 ± 13 years, ranging from 37 to 79 years) were recruited from the Departments of Surgery at Shanghai Changzheng Hospital and Intensive Care Unit at Shanghai Gongli Hospital, from Mar 2009 to Aug 2009. The patients included 5 with sigmoid cancer, 2 with colon descendens cancer, 6 with rectal cancer, 4 with colon ascendens cancer, 5 with colon transversum cancer.

2.3.2. Control subjects

A total of 25 healthy volunteers (males 16, females 9) matched for age and sex with the patients (no significant difference statistically, compared with the patients) were recruited from the Department of Health Screen at Tongji University Affiliated East Hospital. The mean age of them was 56 ± 10 years , ranging from 38 to 74 years.

2.3.3. Collection of samples

All blood samples were collected in the morning after the subjects had fasted overnight. The blood samples were centrifuged for 10 min at 4000 × *g*, and the serum were stored at −80 °C until analyzed. Tissue samples were from the patients with colorectal cancer. When the patients were operated, the tumour tissue and the side normal tissue of tumour were obtained at the same time. After washing away blood with normal saline, the tissue samples were subsequently stored at −80 °C until analyzed.

2.4. Samples processing

200 μL serum was mixed with 80 μL trichloroacetic acid (0.1 g/mL) and allowed to react for 30 s with stirring prior to incubation at 75–80 °C for 15 min and then centrifugated at 10,000 × *g* for 15 min. The 50 μL supernatant was transferred into another test tube and mixed with 100 μL of derivatization reagent, namely, phenyl-isothiocyanate (PITC) solution which consisted of methanol/ethanol/triethylamine/ultra-pure water/PITC (6/1/1/1/1, v/v/v/v/v). The mixture was allowed to react for 20 min at room temperature. After derivatization, we used freeze drying to evaporate the mixture to dryness and reconstituted the sample in mobile A, the solution was filtered through a 0.20-μm needle shaped colander before loading onto the HPLC column for analysis.

The tumour tissue and the side normal tissue of tumour were weighed exactly, and 0.1 g of tissue was homogenized in 0.5 mL of trichloroacetic acid (0.1 g/mL) solution in an ice bath. The homogenate was transferred to Eppendorf centrifuge tube. Next, the operation was the same as that of serum sample.

The protein concentration in the serum and the supernatant after centrifugation was measured by ultraviolet spectrophotometry.

2.5. Chromatographic conditions

The chromatographic column used was a PICO-TAG (300 × 3.9 mm i.d., 5 μm particle size, Waters USA). Gradient elution was selected at 0–13.5 min, 0%A–97%A; 13.5–20 min, 97%A–96%A; 20–20.5 min 96% A–20% A; 20.5–22.5 min, 20% A–0%A; 22.5–30.01 min, 0%A. The temperature of the column was kept at 46 °C, the mobile phase solutions were pumped at a flow rate of 1.0 mL/min, and 20 μL samples were injected. The wavelength of detection was 254 nm.

2.6. Standard processing

The stock standard solutions were diluted with ultra-pure water. The concentration series of each standard were made at 0, 7.81, 15.62, 31.25, 62.5, 125, 250, 500, 1000 μmol/L, respectively. To each standard, the operation was carried out as described above serum sample processing before loading to the HPLC column. The qualitative analysis was completed using the method of retention time, and the quantitative analysis was completed using the method of external standardization. Analyte concentrations were determined using a calibration curve freshly prepared in ultra-pure water. The calibration curves were evaluated by plotting the L-citrulline and L-arginine peak area values against the respective concentrations of L-citrulline and L-arginine standards.

2.7. Statistical analysis

All statistical analyses were performed using SPSS 14.0 software. Aginine or citrulline contents in healthy controls and patients were expressed as the mean ± standard deviation (mean ± SD). Differences of average values between groups were assessed using the Student's t-test, and p-values less than 0.05 were considered statistically significant.