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DEVELOPMENT AND EVALUATION OF HPLC METHOD FOR ARGININE ASSAY IN BIOLOGICAL SAMPLES

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We developed a method for monitoring L-arginine content in cell culture media, human blood plasma and tumor cells lysates using reverve-phase high-performance liquid chromatography (HPLC) with phenyl isothiocyanate (PITC) derivatization. The biological fluids were deproteinized by acetonitrile solvent. Tumor cells were homogenized by 60% aqueous acetonitrile quenching solution. PITC solution was used as a derivatization reagent and a gradient elution was carried out. The peak of amino acid L-arginine was completely separated in all tested biological samples. The linearity of the assay for L-arginine ranged from 5 to 2500 μmol/L. The method is characterized by its simplicity, the use of relatively inexpensive Supelco C_{18} column, simple gradient and small amount of solvents needed for the analysis. The developed and validated procedure is a reliable analytical method suitable for routine arginine detection in scientific laboratories.

Keywords: amino acid analysis, HPLC, L-Arginine, PITC.

Amino acid analysis is an important technique for detection of different disorders in organism. Amino acid arginine and its metabolites play an important role in physiology, including cellular proliferation, vasodilation, neurotransmission, calcium release, and immunity [21]. Arginine is involved in the synthesis of a wide range of peptides and proteins, production of nitric oxide (NO), citrulline, ornithine, urea, creatine, polyamines, proline, agmatine and other biological compounds via the citrulline – NO pathway and the urea cycle [15]. Recent studies showed that arginine levels were significantly altered in the cerebrospinal fluid (CSF) of patients with Alzheimer's disease (AD) and in different types of cancer [14, 20].

Metabolic enzymotherapy based in arginine starvation is a promising approach for cancer treatment effective for growth inhibition and decreasing viability of human hepatocellular carcinoma, malignant melanoma, renal carcinoma and leukemic cells [2, 8, 13, 19, 20]. Recombinant arginine-degrading enzymes are now in phase II–III of clinical trials and have demonstrated strong anticancer activities [1, 13]. Therefore, it is important for the ongoing scientific research and in clinical practice to develop a simple and accurate analytical method for analysis arginine level in cultured cells, biological fluids or tissues.

Since the end of 1980s, the classical ion-exchange chromatographic method for the analysis of amino acids has been replaced by reversed-phase high-performance liquid chromatographic (HPLC) methods that are faster, more sensitive, simple in instrumentation and inexpensive. One of the two most widely used HPLC methods involves pre-column derivatization of the amino acids with phenyl isothiocyanate (PITC) [3, 12]. Different derivatization reagents were utilized that included 9-fluorenylmethyl chloroformate (FMOC-Cl), ortho-phthalaldehyde (OPA), phenyl isothiocyanate (PITC), 1-fluoro-2,4-dinitrobenzene, 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide, and dansyl chloride [7, 10, 16–18]. Each of these reagents has specific advantages and

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limitations [6, 11]. Only PITC, FMOC-Cl, and OPA are currently used for analysis of amino acids, but PITC derivatization methods are less sensitive as compared with those based on fluorimetric detection; OPA does not react with secondary amino acids and FMOC-C1 reacts rapidly [6]. During last years, many researchers used the "Waters Pico Tag" system for the quantification of amino acids in food and biological samples with PITC derivatization.

In this study we describe a modified HPLC method that utilizes derivatization with phenyl isothiocyanate for the analysis of L-arginine in Supelco LC18 column. Previously, we analysed amino acids by pre-column derivatization with dabsyl chloride (DABS) in Supelco LC18 column. But this method gave poor yields and resolution of amino acid peaks such as arginine. Next, we used alternative derivatization procedure with PITC reagent and derivatives separated in Supelco LC18 column. Our method is overall similar to Cohen's "Waters Pico Tag" system and we proposed it for analyzing L-arginine content in different biological samples such as cell culture medium, tumor cells extracts and blood plasma.

Materials and methods

Reagents

HPLC grade acetonitrile, methanol, triethylamine (TEA), PITC, and amino acids were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade water was used from a Milli-Q water system (Millipore, Bedford, MA, USA). Amino acid standards were produced freshly by diluting the stock of standard solutions with ultra-pure water for each assay. The concentration series of each standard were made at 5, 10, 25, 50, 100, 250, 500, 1000, 2500, 5000 μmol/L, respectively along a blank control sample. The qualitative analysis was conducted using the method of retention time and the quantitative analysis was performed using the method of external standardization. The calibration curves were evaluated by plotting the L-arginine peak area values against the respective concentrations of L-arginine standards.

Cell culture and amino acids extraction

Monolayer human melanoma SK-MEL-28 and suspension leukemic Jurkat cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) or RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 300 mg/L glutamine and 50 μg/ml gentamicine solution. Cell cultures were maintained in a humidified atmosphere of 5% CO_2 at 37°C. For intracellular arginine analysis, cells were washed three times in ice-cold PBS and frozen or lysed. Briefly, $5x10^6$ cells in dish were lysed in 200 μl of 60% ice-cold acetonitrile quenching solution, vortexed and incubated on ice for 10 min. The extracts were centrifuged at 13,000 x g for 15 min and 50 μl of supernatant was used for amino acids derivatization.

Sample Preparation

Normal blood samples were collected in heparinized tubes and immediately centrifuged at 3000 rpm for 10 min to separate the plasma. Before analysis, plasma samples (50-100 μl) were deproteinized with 200-400 μl of 100 % acetonitrile, containing 0.2 mmol/l D-nor-valine as internal standard. The 200 μL of supernatant was transferred into another tube and evaporated for derivatization. Cell culture medium – RPMI-1640 with 10 % FBS deproteinized analogously to blood samples.

Pre-column derivatization

200 μL of each deproteinized biological sample and 50 μL of arginine standard were transferred into another tube and dried under vacuum. Next, the dried matrix of samples was dissolved in 25 μL of a 2:2:1 mixture of ethanol : water : TEA (v/v), by vortexing and then evaporated. Derivatization was performed by adding into tube 25 μL of a mixture of 7:1:1:1 ethanol : water :

TEA : PITC (v/v), which was fresh-made and mixing in vortex. The reaction between PITC and the sample to produce phenylthiocarbamyl (PTC) amino acids was allowed to continue for 20 min at room temperature. Samples were then completely dried-out under vacuum and stored in a freezer.

Apparature and chromatographic conditions

The HPLC system from PerkinElmer Series 200 (PerkinElmer, USA) was used in this work. The system consisted of binary pump, vacuum degassator, variable-wavelength uv-visible range detector (UV/VIS) and TotalCrom analytical software. The separation of PITC-amino acids was performed using a 3 µm Supelcosil LC-18 column (150 mm x 4.6 mm i.d.) protected with a 5µm Supelcosil LC-18 guard column (20 mm x 4.6 mm i.d.) (Supelco, Bellefonte, PA).

The PITC amino acids were separated using a multistep linear gradient with two phases. The mobile phase A consisted of 70 mM sodium acetate buffer with 2.5 vol. % acetonitrile (pH 6.5), while the mobile phase B consisted of 15% methanol, 45% acetonitrile, and 40% water. Prior to the use, the mobile phase solutions were filtered using 0.40-µm membrane. The flow rate was 1 mL/min throughout, and the gradient consisted of the following profiles: 100% A at start, 97% A at 13.5 min, 94% A at 10.5 min (concave curve), 91% A at 6 min (convex curve), 66% A at 5 min, 0% A at 1 min, 0% A hold at 3 min and reequilibration at 100% A. The wavelength of detection was 254 nm, column temperature 46 C°. Vacuum-dried samples with PITC-amino acids were dissolved in 250-µL of phase A pH 7.5 containing 5% acetonitrile by vortex mixing. The new and freshly reconstituded samples were filtered through a 0.2-µm and 20 microliters of sample were injected by manual injector.

Statistical analysis

All statistical analyses were performed using Microsoft Excell software. Aginine content in biological fluids was 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.

Results and discussion

At the first stage, we prepared standard solutions of free amino acid L-arginine and analyzed them versus a blank sample under the conditions described in Materials and Methods. Comparison of chromatograms allowed to identify a peak that corresponds to L-arginine with 20.4 min retention time point (Fig. 1, A). To confirm the identity of a presumable arginine peak, we have used arginine-degrading enzyme – recombinant human arginase (rhARG) which converts L-arginine to L-ornitine and urea (at concentration of 2 U/mL). The chromatogram of the sample supplemented with rhARG is shown on Fig. 1, B and the peak presumed to be that corresponding to L-arginine was not observed. Thus,the peak corresponding to free L-arginine was correctly identified and clearly separated.

The reaction of protein amino groups with PITC is the basis of the Edman degradation of polypeptides, one of the famous methods in classic biochemistry [3]. Phenylthiocarbamilation of the amino groups of amino acids was conducted under the conditions described in Materials and Methods. We analyzed the stability of derivatized products under different conditions. We found that after the derivatization reagent is removed under vacuum, the derivatized amino acids can be stored dry and frozen for several days with no significant degradation. Dissolved in solution prior to injection into the liquid chromatographic system, degradation will occur if the samples are not kept cold. After 6 h of incubation at the room temperature, a 5–10% drop in the response occured for arginine. Therefore, we propose to inject only a freshly-prepared samples for quantitative analysis of L-arginine.

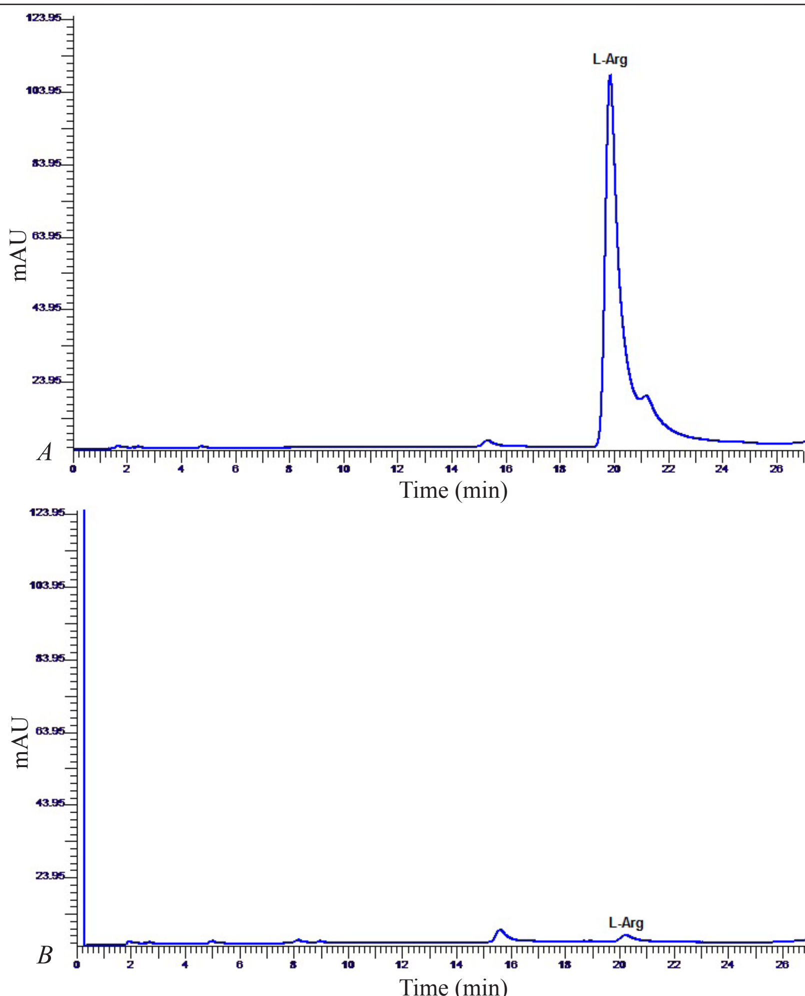

Fig. 1. Chromatograms of the standard L-arginine solution *(A),* and of the same L-arginine solution supplemented with rhARG in concentration of 2 U/mL *(B).* L-Arg – arginine (retention time was 20.4 min).

In many chromatographic analyses, it is important to achieve a linear regression of an analysed compound in the range of analytical interest. L-arginine standards varying from 5 to 5000 μM/L were derivatized and analyzed. Samples were run in triplicate and the corresponding peak areas were calculated. The data are presented on Fig. 2 and demonstrate the linear response in the range of 5-2500 μM/L. Correlation coefficients for these data exceeded 0.999. The poor linearity was obtained in the range 2 mM/L and higher. The lower detection limit was 0.5 μM/L (corresponding to 2 pmoles in the injected sample).

As the next experimental step, we monitored arginine content in selected biological samples of practical interest. First, we used for the analysis RPMI-1640 cultural medium for tumor cells which a defined mixture of different amino acids. There are different deproteinization methods for detecting amino acid in biological samples, including strong acids, organic solvents, ultrafiltration, high-speed centrifugation and dialysis, etc. [11, 18]. We tested different deproteinization agents and have chosen acetonitlile, which is more suitable for our chromatographic conditions but requires larger volumes and dilutes a sample.

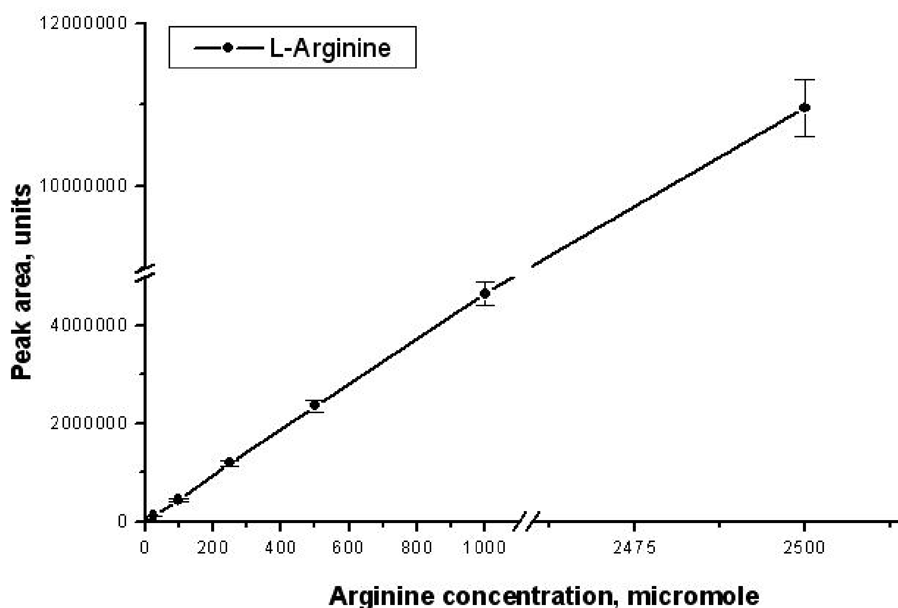

Fig. 2. Linearity of the detector response to PITC derivatized L-arginine standard solutions.

Fig. 3 shows a chromatographic profile of cell cultural medium sample where, as predicted, significant number of peaks was detected. The representative chromatogram exhibits good peak resolution. For the optimization of elution conditions, we investigated the effect of changing the pH of the sodium acetate buffer, and finally selected pH 6.5 as the optimum. The peak of L-arginine present in RPMI-1640 medium at high concentration was identified. According to our calibration curve, the L-arginine concentration in this medium without FBS was estimated as 1020±30 μM/L what is in agreement with a datasheet specification. In order to verify the specific peak of arginine, we analyzed culture medium with administrated recombinant human arginase (rhARG) and compared the obtained chromatograms. The arginine peak was clearly separated and disappeared in the sample treated with rhARG.

For the next analysis, we utilized a more complex biological sample, blood plasma, which contains all amino acids and other amino-compounds. We deproteinized blood plasma samples analogously to the culture medium. The work with blood plasma must be conducted in cold because the arginase from erythrocytes can be released into plasma and decrease physiological arginine level. The chromatogram of the separated blood plasma amino acids is provided on Fig. 3, **C**. The arginine peak was found to be clearly separated too. It has to be mentioned that arginine analysis in blood plasma is very important for clinical medicine to control effectiveness of administered rhARG as an antitumor agent in cancer patients.

As the last step, we examined the alterations in free arginine level in selected tumor cells cultured under different conditions. It was especially interesting to establish, whether a predicted drop in free intracellular arginine correlates with sensitivity of different model tumor cells to arginine deprivation. These data may also provide new insight into question, what intracellular mechanisms and when may be induced in tumor cells in response to alterations in free intracellular arginine pool.

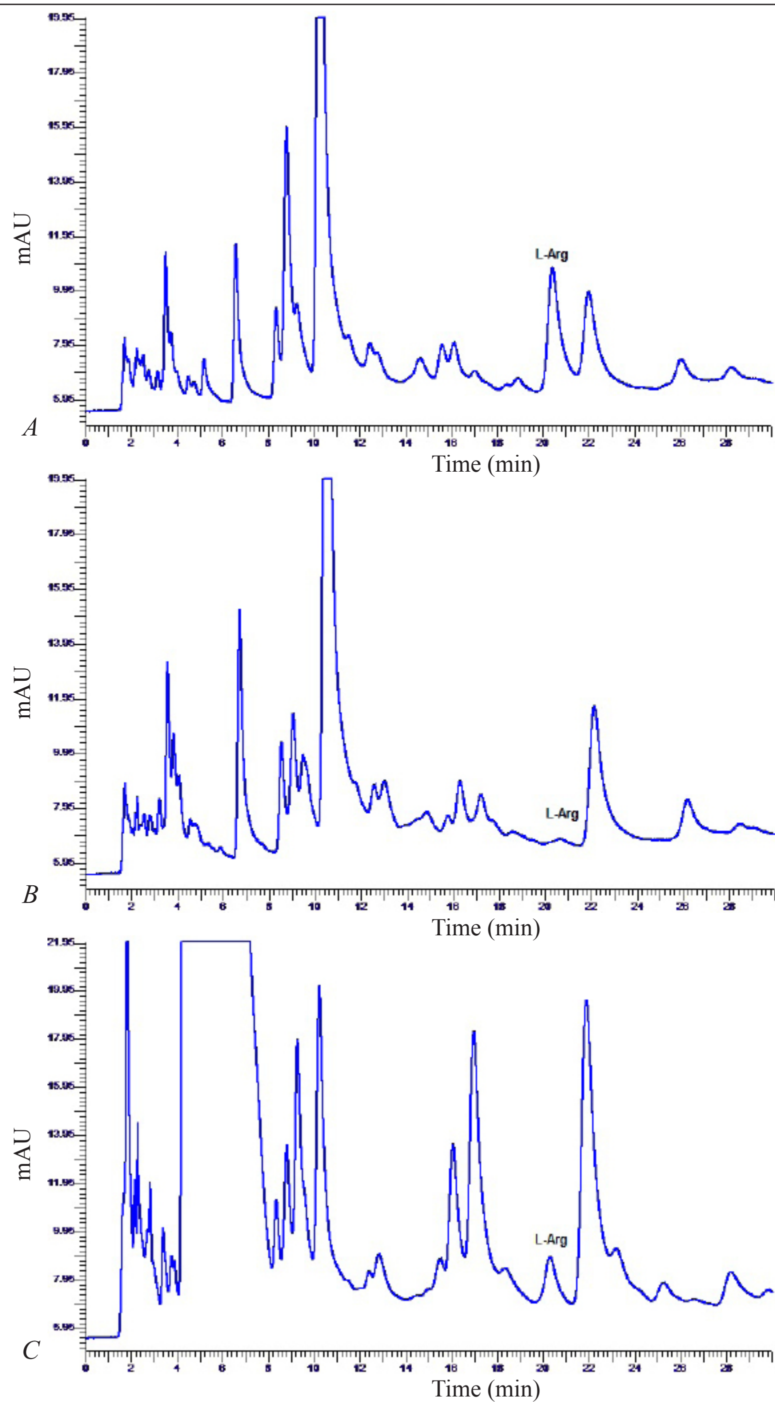

Fig. 3. Chromatograms of PITC derivatized amino acids by reverse-phase HPLC. *A* – RPMI-1640 cell culture medium; *B* – RPMI-1640 culture medium with added rhARG (2 U/ml); *C* – deproteinized normal human plasma.

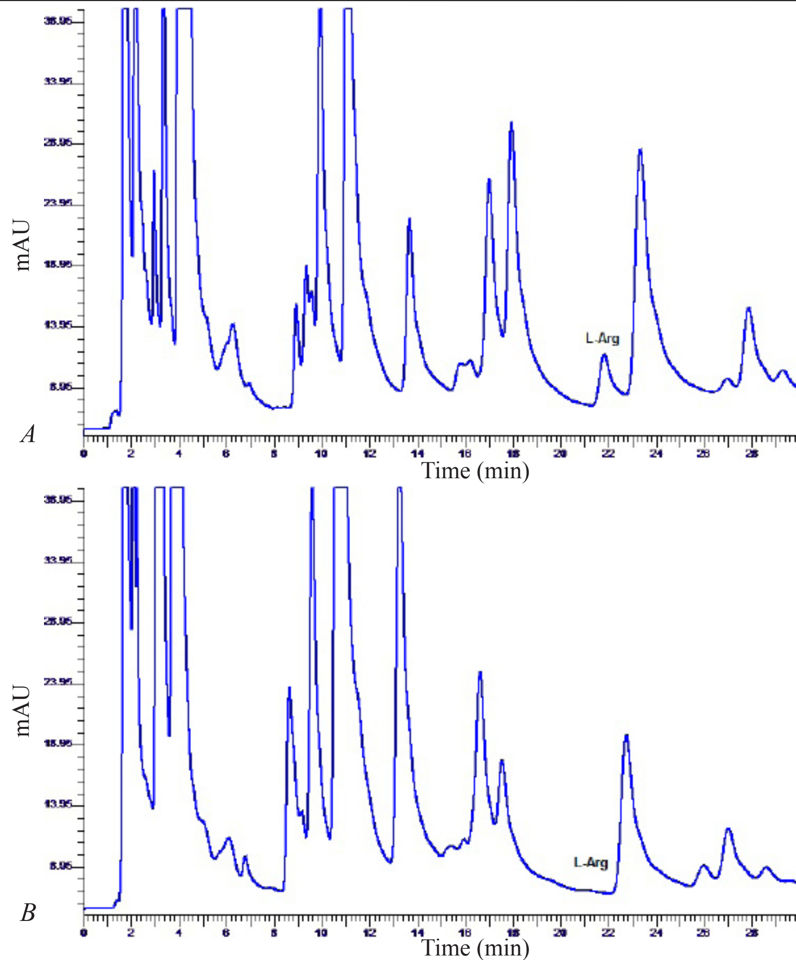

Fig. 4. Chromatograms of PITC derivatized amino acids by reverse-phase HPLC from Jurkat cell extracts: *A* – Control cell extract (arginine-sufficient medium); *B* – Cell extract after 30 min of full arginine deprivation.

Determination of amino acids levels in the cell is a difficult process, because there are many omnipresent metabolites that may interfere with the efficient separation. A key challenge in cell metabolite analysis is the design of a metabolite extraction protocol for different initial samples, which have very diverse chemical and physical properties (e.g., size, weight, polarity, volatility, solubility, stability). According to the literature data, the most suitable for amino acids analysis is 60 % cold acetonitrile quenching solution, which quantitatively yields higher amino acids titres. We analyzed the intracellular content of arginine in two cancer cell lines: cultured as a monolayer human melanoma SK-MEL-28 and in grown in suspension human leukemic Jurkat cells, both in complete or the formulated arginine-deficient media. Fig 4 shows typical chromatograms of extracts from Jurkat cells. Quantitative data of intracellular arginine pool in the tested cancer cells are represented in Table 1. The results are expressed as nmole/mg cellular protein. The basal level of the arginine in Jurkat cells (2.5±0.45 nM/mg) was higher then in SK-MEL-28 cells (1.32±0.5 nM/mg) Our experiments demonstrate dramatical and fast reduction of free intracellular arginine already after 30 min of arginine starvation. This observation is very important

for elucidating early signalling mechanisms of cancer cells response to arginine deficiency, both *in vitro* and *in vivo*.

Table 1

Intracellular L-arginine concentration in tumor cells cultivated in standard and arginine-deficient media (in nmole/mg of cellular protein; M±m; n=3)

Cell lines	Control	30 min of starvation
Jurkat	2.5±0.45	0.23±0.07
SK-MEL-28	1.32±0.5	0.36±0.09

In conclusion, the described here elaborated RT-HPLC method for monitoring of L-arginine in different biological samples is characterized by relative simplicity, the use of inexpensive C_{18} column, exhibits good precision, linearity and satisfactory recovery from different samples. We propose this method for utilization in laboratory and clinical practice.

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РОЗРОБКА І ОЦІНКА ВЕРХ МЕТОДУ ДЛЯ ВИЗНАЧЕННЯ АРГІНІНУ В БІОЛОГІЧНИХ ЗРАЗКАХ

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Розроблено метод аналізу концентрації аргініну в культуральному середовищі, плазмі крові та у зразках із екстрактів пухлинних клітин за допомогою зворотно-фазової високоефективної рідинної хроматографії (ВЕРХ) шляхом дериватизації з фенілізотіоціанатом (ФІТЦ). Біологічні зразки депротеїнізували ацетонітрилом. Пухлинні клітини гомогенізували у 60% водному розчині ацетонітрилу. Використовували градієнтну елюцію та розчин ФІТЦу як дериватизуючий реагент. Пік аргініну повністю розділявся у всіх протестованих біологічних зразках. Лінійність аналізу L-аргініну спостерігалась у діапазоні 0–2500 мкмоль/л. Метод характеризується простотою, використанням відносно недорогої Supelco C_{18} колонки, простим градієнтом і невеликою кількістю розчинників, необхідних для аналізу. Розроблений метод пропонується як аналітичний для рутинної детекції аргініну в наукових лабораторіях.

Ключові слова: амінокислотний аналіз, ВЕРХ, L-аргінін, ФІТЦ.

РАЗРАБОТКА И ОЦЕНКА ВЭЖХ МЕТОДА ДЛЯ ОПРЕДЕЛЕНИЯ АРГИНИНА В БИОЛОГИЧЕСКИХ ОБРАЗЦАХ

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Разработан метод анализа концентрации аргинина в культуральной среде, плазме крови и в образцах из экстрактов опухолевых клеток при помощи обращенно-фазовой высокоэффективной жидкостной хроматографии (ВЭЖХ) путем дериватизации с фенилизотиоцианатом (ФИТЦ). Биологические образцы депротеинизировали ацетонитрилом. Опухолевые клетки гомогенизировали в 60% водном растворе ацетонитрила. Использовали градиентную эллюцию и раствор ФИТЦа в качестве дериватизирующего реагента. Пик аргинина разделялся полностью во всех протестированных биологических образцах. Линейность анализа L-аргинина наблюдалась в диапазоне 0–2500 мкмоль/л. Метод характеризуется простотой, использованием относительно недорогой Supelco C_{18} колонки, простым градиентом и небольшим количеством растворителей, необходимых для анализа. Разработанный метод предлагается в качестве аналитического для рутинной детекции аргинина в научных лабораториях.

Ключевые слова: аминокислотный анализ, ВЭЖХ, L-аргинин, ФИТЦ.