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METHOD OF THE SIMULTANEOUS DETERMINATION OF L-PHENYLALANINE AND L-TYROSINE BY MEANS PHENYLALANINE – AMMONIA-LYASE FROM *RHODOTORULA AURANTIACA* KM-1

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An enzymatic method of simultaneous determination of L-phenylalanine (Phe) and L-tyrosine (Tyr) in blood and other biological liquids by phenylalanine – ammonia-lyase of *Rhodotorula aurantiaca* KM-1 was developed. Differences between optical absorptions of trans-cinnamic, p-hydroxy-cinnamic acids, Phe and Tyr at 250 nm and 310 nm wavelengths allow for simultaneous quantitative detection of these aromatic amino acids in investigated samples. The linear model of the dependence of concentration measured from prepared samples is statistically significant for Phe (calculated by SPSS 11.5). Similarly, the linear model of the dependence of measured concentration from prepared samples is statistically significant for Tyr. Besides, the measured concentrations of Phe and Tyr are mutually independent. The regression analysis of measurements of Phe concentration in dried spots of blood taken from normal and sick children by offered method and by means of amino acid analyzer reveals strong correlation.

Разработан метод одновременного определения концентраций L-фенилаланина (L-фен) и L-тирозина (L-тир), в пробах крови и других биологических жидкостях, посредством фенилаланин – аммиак-лиазы *Rhodotorula aurantiaca* KM-1. Различие оптических поглощений транс-коричной кислоты и п-гидроксикоричной кислоты при длинах волн 250 нм и 310 нм, а также низкое оптическое поглощение L-фен и L-тир в указанном диапазоне можно использовать для одновременного количественного определения этих ароматических аминокислот в исследуемых образцах. Как для L-фен, так и для L-тир линейная модель зависимости измеренных концентраций от приготовленных статистически значима (рассчитанно по программе SPSS 11.5). В то же время измеренные значения L-фен и L-тир взаимонезависимы. Регрессионный анализ измерений концентраций L-фен в высушенных пятнах крови нормальных и больных детей данным методом и при помощи аминокислотного анализатора выявляет сильную регрессию.

Մշակվել է *R. aurantiaca* KM-1-ից անջատված և մաքրած ՖԱԼ-ի կիրառմամբ արյան մեջ և այլ կենսաբանական հեղուկներում L-ֆենիլալանինի (L-Ֆեն) և L-թիրոզինի (L-թիր) խտությունների միաժամանակյա չաձման մեթոդ: Տրանս-դարչնաթթվի և պիհիդրոքսիդարչնաթթվի օպտիկական խտությունների տարբերությունը 250 և 310 նմ ալիքի երկարության պարագայում, ինչպես նաև տվյալ ալիքների տիրույթում L-Ֆեն և L-թիր ցածր օպտիկական խտությունը կարելի է կիրառել հետազոտվող նմուշներում նշված արոմատիկ ամինոթթուների միաժամանակյա չափման համար: Ինչպես L-ֆեն, այնպես էլ L-թիր համար չափված խտությունների կախվածության գծային մոդելը պատրաստվածներից -վիճակագրորեն հավաստի է (հաշվարկը կատարվել է SPSS 11.5 ծրագրով): Մի առ ժամանակ L-ֆեն և L-թիր չափումները կախյալ չեն իրարից: L-ֆեն խտությունների չաթման ռեգրեսիոն վերլուծումը հիվանդ և առողջ երեխաների արյան չորացած բծերում առաջարկվող մեթոդով և ամինաթթվային անալիզատորի օգնությամբ ցուցաբերում է հստակ ռեգրեսիա:

L-phenylalanine, L-tyrosine, L-phenylalanine – ammonia-lyase, Rhodotorula aurantiaca KM-1, differential spectrophotometry, phenylketonuria.

Phenylketonuria (PKU) is an inborn error of Phe methabolism. Mutations in L-phenylalanine hydroxylase (PAH) gene result in enzyme deficiency leading to the hyper phenylalaninemia (HPA) [4], i.e. to the accumulation in blood and other liquids of organism of Phe (600-1800 μmol L^{-1} instead of 180-480 μmol L^{-1} as normal), phenylpyruvic, phenylacetic, ortooxyphenylacetic acids and other agents, which are excreted with urine. The consequences of metabolic disorder are mental retardation, microcephaly, epileptic seizures, and, without the curing of the disease, the patients become a deep invalids for the whole life.

Two types of PKU are known:

1. Classical PKU, which is the result of just PAH deficiency owing to the mutation in the enzyme coding gene.
2. The group of atypical forms of PKU determined by the PAH cofactor - tetrahydrobiopterine deficiency owing to the mutant genes inheritance. Among these genes are ones coding for 5'GTP-cyclohydrolase, 6-pyruvoiltetrahydropterinsyntase, seriapterinreductase, dihydropterin-reductase, etc.

The disease is inherited through autosome-recessive type. The probability of mutant gene appearance is approximately 1-2 per 100 persons, but the disease develops only in the case when both mother and father are the carriers of the risk gene and the child inherits it in double set. That is why the disease occurs much more seldom. The morbidity of PKU has ethnic and race variability. The average frequency of occurrence among Europeans is 1:10000; the range is varies between1:4000 – 1:25000. According to the data of Ministry of Health of Republic of Armenia the number of registered PKU children in the age up to one year old was: in 2004 – 3, in 2005 – 2. In 1998 in RA 76 patients (1975-1998 years of birth) with PKU diagnosis have been registered and cared.

Obviously, the timely revelation of disease and immediate realization of necessary medical measures (diet therapy and other methods of treatment) might promote patients recovery and minimize or completely prevent disability. This will allow for achieving their social rehabilitation and integration into the society.

The attempts were made to cure PKU by the Phe removal from the blood. In such way the mice were injected by *Rhodosporidium toruloides* phenylalanine – ammonia-lyase (PAL): the purified enzyme was intramuscularly injected to mice with PAH mutation. It was shown, that injections decreased Phe concentration in blood plasma of treated mice to physiological value [3].

By all its characteristics PKU corresponds to inherited metabolic defects, which are subjected to the total screening. The microbiological, chromatographic, fluorometric and enzymatic methods are used for urine and blood screening in PKU diagnostics [8].

The disadvantages of microbiological methods are: semi quantitative results, long duration of analyses (no less than 24 h.) and possibility of the influence of antibiotics on the results of analysis, which can be a cause of false-negative results. Chromatographic and fluorometric methods are inaccessible for majority of clinics because of necessity of expensive apparatuses. For developing countries like Armenia, enzymatic photo colorimetric methods for quantitative determinations of Phe in dried blood samples are more convenient.

The enzymatic method of Phe determination by phenylalanine dehydrogenase (PDH) is often used in Armenian clinics. This method is quite specific, fast and automation friendly [3]. The method of simultaneous determination of Phe and Tyr by PAL of *Rhodotorula glutinis* was also described [5].

The micro method of quantitative determination of Phe for PKU diagnostics by using *Proteus rettgeri* was described too. Authors referred to the ability of this strain to deaminate Phe, but the true mechanisms of reaction were not described. Despite to the statistical validity of this method, it is complicated, prolonged and the mechanism is not clear [7].

For Phe determination the method of sensitive (0.03-3.00 mM) enzymatic electrode preparation on the base of recombinant PDH from *Bacillus badius* is known. However required working volume of electrode - 5 ml is too large for PKU diagnostics [9].

In the healthy organism Tyr is non essential amino acid, synthesized from Phe under the influence of PAH. Its concentration depends on the condition of organism and varies during the day (35 to 100 $\mu mol\ L^{-1}$). PKU patients, subjected to strong anti-phenylalanine diet, have a problem of hypo tyrosinemia. In dietary products, prescribed to the treated PKU patients, the quantity of Tyr varies in the range of 30 to 80 $\mu mol\ L^{-1}$. But sometimes in the blood of such patients' one can observe the Tyr concentration's increase up to 340 $\mu mol\ L^{-1}$ [6]. The increased concentration of Tyr, in its turn, exerted toxic influence on the organism. Thus, Tyr determination in the blood of PKU patient is of great importance too for scientists and physicians.

The purpose of this work is developing the method, convenient for clinical application, which allows the simultaneous determination of Phe and Tyr in blood samples and other biological liquids by PAL of *Rhodotorula aurantiaca* KM-1.

Materials and Methods. PAL. The purified PAL o*f Rhodotorula aurantiaca* KM-1 with 2.5-3.0 U/mg specific activity was used in this study [1]**.**

Spectral researches. Spectral researches were carried out on UV-VIS spectrophotometer Perkin-Elmer 550S (Bodenbeewerk, Germany). Trans-cinnamic and p-hydroxy-cinnamic acid, Phe, Tyr were purchased from Sigma-Aldrich-Fluka. Molecular extinction coefficients of studied compounds at 250 nm and 310 nm were determined by single point mode.

Analytical methods. Phe and Tyr concentrations were measured in the reaction mixture, which contained in 200 μl of final volume: 100 μl of model mixture extract of amino acids after precipitation of blood plasma enzymes with 3% trichloroacetic acid (TCA), and 100 μl of mixture of 500 mM Tris-HCl buffer (pH 8.9) and 0.1 U PAL. The model mixture of amino acids contained 60 % (V/V) human blood plasma. Two reaction mixtures were prepared for each model system. In the first mixture the reaction was stopped after 5 min incubation in a boiling water bath just after PAL addition (control), and in the second mixture the reaction was stopped by the same way after 4 h incubation at 30°C. The method of calculation of amino acids' concentrations is given with results.

The measurement of Phe concentrations in dried blood spots of normal and sick children by means of PAL was carried out using above described way, after extraction of amino acids in round pieces of a filter paper (I cm diameter). The extraction of amino acids was realized by shaking pieces of filter paper on a round rocking chair B. ITH1 (Braun, Germany) in tubes containing 0.5 ml of 3 %TCA during 30 min at a room temperature. Blood proteins were removed by centrifugation. The volume of the blood, by which was impregnated the piece of filter paper, was counted by the quantity of distilled water, sucked in with the filter paper piece of the same size.

Measurement of Phe concentration in dried spots of blood of normal and sick children was carried out by amino acid analyzer AAA T339 (Microtechna, Praha). The above described amino acid extracts were used for these analyses.

Statistics. The regression analyses were carried out by software package SPSS 11.5.

Results and Discussions. Dependences of extinction coefficients on wavelengths for trans-cinnamic and p-hydroxy-cinnamic acids, Phe and Tyr are shown in Chart 1.

$$D^{K}_{250} = \varepsilon^{P}_{250} \times C^{P}_{250} + \varepsilon^{T}_{250} \times C^{T}_{250} + D_{250}$$
$$D_{250} = \varepsilon^{Cin}_{250} \times C^{Cin}_{250} + \varepsilon^{HC}_{250} \times C^{HC}_{250} + D_{250} \quad (1)$$

$$D^{K}_{310} = \varepsilon^{P}_{310} \times C^{P}_{310} + \varepsilon^{T}_{310} \times C^{T}_{310} + D_{310}$$
$$D_{310} = \varepsilon^{Cin}_{310} \times C^{Cin}_{310} + \varepsilon^{HC}_{310} \times C^{HC}_{310} + D_{310} \quad (2)$$

Where

D^{K}_{250} - optical dencity of control at 250 nm,

D_{310} - optical dencity of sample at 310 nm after PAL action,

ε^{P}_{250} - extinction coefficient of L-phenylalanine at 250 nm,

P, T, Cin, HC - L-phenylalanine, L-tyrosine, t-cinnamic acid and p-hydroxy-cinnamic acid

$$C_P = \frac{(D_{250} - D^{K}_{250}) \times (\varepsilon^{HC}_{310} - \varepsilon^{T}_{310}) - (D_{310} - D^{K}_{310}) \times (\varepsilon^{HC}_{250} - C^{T}_{250})}{(\varepsilon^{Cin}_{250} - \varepsilon^{P}_{250}) \times (\varepsilon^{HC}_{310} - \varepsilon^{T}_{310}) - (\varepsilon^{Cin}_{310} - \varepsilon^{P}_{310}) \times (\varepsilon^{HC}_{250} - C^{T}_{250})} \quad (3)$$

$$C_T = \frac{(D_{310} - D^{K}_{310}) \times (\varepsilon^{Cin}_{250} - \varepsilon^{P}_{250}) - (D_{250} - D^{K}_{250}) \times (\varepsilon^{Cin}_{310} - \varepsilon^{P}_{310})}{(\varepsilon^{Cin}_{250} - \varepsilon^{P}_{250}) \times (\varepsilon^{HC}_{310} - \varepsilon^{T}_{310}) - (\varepsilon^{Cin}_{310} - \varepsilon^{P}_{310}) \times (\varepsilon^{HC}_{250} - C^{T}_{250})} \quad (4)$$

Where

C_P - L-phenylalanine concentration

C_T - L-tyrosine concentration

Chart 1. Formulas for calculation of Phe and Tyr concentrations.

The results obtained show that differences between optical absorptions of trans-cinnamic and p-hydroxy-cinnamic acids at 250-260 nm and 300-310 nm wavelengths, as well as low optical absorptions of Phe and Tyr in the mentioned diapason can be used for simultaneous quantitative determination of these aromatic amino acids in investigated samples.

Experimental estimation of combinations of different wavelength pairs allows choosing a pair 250 and 310 nm as optimal in this respect. The coefficients of molecular extinctions for mentioned wavelengths are presented in Table 1.

Table 1. Molecular extinction coefficient for trans-cinnamic and p-hydroxy-cinnamic acids, L-Phe and L-Tyr

Agent	Extinction coefficient, $M^{-1}cm^{-1}$	
	250 nm	**310 nm**
L-Phe	158±15	12±01
L-Tyr	2630±56	208±10
Trans-cinnamic acid	7640±67	220±11
p-hydroxy-cinnamic acid	3733±45	14550±96

The idea of simultaneous measurement of Phe and Tyr concentrations in unknown biological solutions is based, according to Ber's law, on the measurement of initial optical absorption of solution (at 1 cm light path) depending on the concentrations of Phe, Tyr and other agents (the formulas 1 and 3 on the Fig. 1) and the absorption of the same solution after PAL treatment depending, in this time, on the absorption of trans-cinnamic, p-hydroxy-cinnamic acids and not reacted agents (the formula 2 and 4 on the chart 1) synthesized during enzymatic deamination.

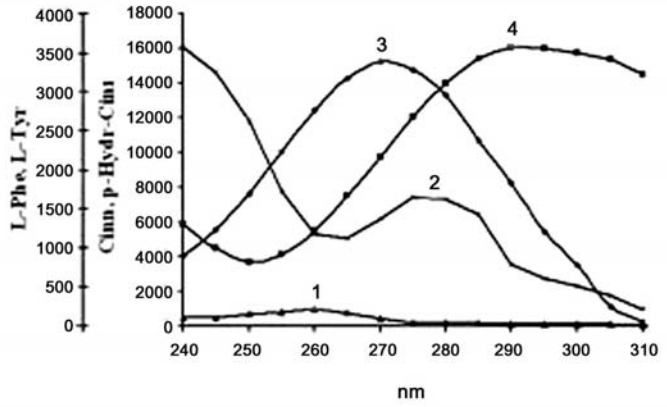

Fig. 1. Dependences of extinction coefficients on wavelength for trans-cinnamic (3) and p-hydroxy-cinnamic (4) acids, Phe (1) and Tyr (2).

Then unknown concentrations of Phe and Tyr were determined by solving the system of two linear equations consisting of differences of equations (2) and (1), on the one side, and equations (4) and (3), on the other side. They were calculated by formulas (5) and (6) respectively (chart 1). The data of simultaneous measurement of Phe and Tyr concentrations in model solutions, which were prepared on human blood plasma, are presented in Table 2.

Table 2. Measurement of Phe and Tyr concentrations in model solutions prepared from human blood plasma

Probe	Measured. mM		Prepared. mM	
	Phe	Tyr	Phe	Tyr
1	16.04	2.12	15.0	2.0
2	5.53	2.05	5.0	2.0
3	2.39	2.08	2.0	2.0
4	0.63	2.29	0.5	2.0
5	0.29	2.23	0.2	2.0
6	16.15	0.95	15.0	1.0
7	5.58	0.99	5.0	1.0
8	2.54	1.11	2.0	1.0
9	0.63	1.21	0.5	1.0
10	0.24	1.22	0.2	1.0
11	16.63	0.19	15.0	0.2
12	5.49	0.24	5.0	0.2
13	2.16	0.24	2.0	0.2
14	0.65	0.22	0.5	0.2
15	0.25	0.24	0.2	0.2

The regression analysis of correlation between Phe concentration measured by offered method and the prepared concentrations of this amino acid, calculated by SPSS 11.5, reveals the linear dependence shown on the chart 2 (the formula 7). Similar regression dependence for Tyr is presented by the formula 8 on the chart 2.

$$[Phe_{measured}] = a_1 + b_1 \times [Phe_{prepared}] \qquad (5)$$

Where

$a_1 = 0.118 \pm 0.053$

$b_1 = 1.078 \pm 0.007$

$$[Tyr_{measured}] = a_2 + b_2 \times [Tyr_{prepared}] \qquad (6)$$

Where

$a_2 = 0.017 \pm 0.041$

$b_2 = 1.071 \pm 0.032$

Chart 2. Dependence of Phe (7) and Tyr (8) concentrations measured by PAL on prepared concentrations of these amino acids.

The linear model of concentration dependence measured from prepared samples is statistically significant for Phe, since R = 1.000; R^2 = 0.999; F = 21439.118, Sig F = 0.000; t = 146.421, Sig t = 0.000 (where R – coefficient of correlation, R^2–coefficient of determination, Sig F–consequence of F-statistics, Sig t – consequence of t-statistics) [2]. Similarly the linear model of dependence of measured concentration from prepared samples is statistically significant for Tyr too, since in this case R = 0.994; R2 = 0.989; F = 1150.510, Sig F = 0.000; t = 33.919, Sig t = 0.000. Besides the measured concentrations of Phe and Tyr are mutually independent, since calculated by SPSS 11.5 correlation coefficient between them R = 0.068.

It is necessary to note also, that initial concentrations of Phe – a_1=0.118 mM and Tyr – a_2=0.017 mM (chart 2) specify concentrations of corresponding amino acids, included in model system with human blood plasma.

The results of measurement of Phe concentrations in dried blood spots of normal and sick children by offered method and by amino acid analyzer are presented in Table 3.

Table 3. Measurement of Phe concentration in dried blood spots of normal and sick children by PAL and the amino acid analyzer

Probe	Phe concentration, mM	
	Offered method	Amino acid analyzer
1	0.11	0.10
2	0.12	0.13
3	0.09	0.10
4	0.17	0.15
5	0.07	0.08
6	0.31	0.35
7	0.38	0.37
8	1.09	1.01
9	0.78	0.81

The regression analysis of measurements of Phe concentration in dried spots of blood taken from normal and sick children by offered method and by means of amino acid analyzer reveals strong regression with following parameters: R = 0.996; R^2 = 0.992; F = 886.183, Sig F = 0.000; t = 29.769, Sig t = 0.000.

Discussion. The method is based on the ability of *Rhodotorula aurantiaca* KM-1 PAL at pH 8.9 to transform completely Phe to trans-cinnamic acid, and Tyr - to p-hydroxy-cinnamic acid accordingly [8] and on essential differences of absorption spectra of trans-cinnamic, p-hydroxy-cinnamic acids and corresponding amino acids. This allows to measure quantitatively trans-cinnamic and p-hydroxy-cinnamic acids, derived after enzymatic hydrolysis, by the differences of optical absorption of initial and final solutions (before and after enzymatic hydrolysis) at two different wavelengths (250 and 310 nm).

This method favorably differs from other enzymatic methods of PKU diagnosis, as it allows simultaneous determination of Phe and Tyr [1, 3-7]. Tyr determination is necessary for correct PKU therapy. The known method of Phe and Tyr simultaneous determination by Shen and Abell, though being rather simple, is less precise [4]. It does not take into account absorptions of Tyr and other blood components in employed wavelength areas. The proposed method of differential spectrophotometric determination of Phe and Tyr allows solving this problem. The apparent difficulties of calculation of concentrations with using advised formulas does not comprise any separate difficulty as they can be done, for example, by Excel software package.

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