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A METHOD FOR ASSESSING THE QUALITY OF RECOMBINANT HUMAN MILK PEPTIDE ANALOGUES

Aynura Teibovna Mekhtieva[1], Angelina Sergeevna Martynyuk[2], Alzhanat Jalalovna Ilyasova[3], Georgy Konstantinovich Tatonov[4*], Maryam Rezvanovna Pogorova[5], Milena Aslanovna Beremukova[5], Madina Aslanovna Malkarova[5], Yulia Alekseevna Korneeva[6]

1. ***Department of Therapy, Faculty of Medicine, Stavropol State Medical University, Stavropol, Russia.***
2. ***Department of Therapy, Faculty of Medicine, Rostov State Medical University, Rostov-on-Don, Russia.***
3. ***Department of Therapy, Faculty of Therapeutic and Prophylactic, Rostov State Medical University, Rostov-on-Don, Russia.***
4. ***Department of Therapy, Faculty of Pediatrics, North-Ossetian State Medical Academy, Vladikavkaz, Russia.***
5. ***Department of Therapy, Faculty of Medicine, Kabardino-Balkarian State University, Nalchik, Russia.***
6. ***Department of Therapy, Faculty of Pediatrics, Stavropol State Medical University, Stavropol, Russia.***

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ABSTRACT

A genetically engineered analog of human kappa casein was developed and obtained. It has been found to have the ability to induce apoptosis of human cancer cells in culture and inhibit the growth and metastasis of animal and human tumors. Based on the recombinant protein obtained, a new antitumor drug RAHMP (recombinant analogs of human milk peptides) has been developed, which is a concentrate for the preparation of an infusion solution. The main function of this drug is prevention and treatment of human breast cancer. The purpose of this work was to develop and validate a technique for the quantitative determination of impurities in the RAHMP substance by reverse-phase high-performance liquid chromatography. Criteria for assessing the quality of the substance were the area of the main peak, which should be at least 95% of the total area of all detected peaks, and the sum of the areas of additional peaks should be no more than 5% of the total area of all peaks. The permissible content of foreign impurities in the substance should be no more than 5%. The relative standard deviation of the impurity concentration at all levels of the concentrations under consideration does not exceed 2.0%, which indicates satisfactory convergence of the results.

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Introduction

Recently researchers isolated and characterized proteolytic fragments of human kappa casein with a molecular weight of ~8.6 kDa from human milk [1]. Its genetically engineered analog was obtained, which, like a natural peptide, can induce apoptosis of human cancer cells in culture and inhibit the growth and metastasis of animal and human tumors [2, 3].

Based on the recombinant protein obtained, a new antitumor drug RAHMP (recombinant analogs of human milk peptides) has been developed, which is a concentrate for the preparation of an infusion solution. As part of preclinical studies, it has been shown that the developed drug is safe, exhibits antitumor and antimetastatic activity in studies on laboratory animals, and can be recommended for clinical trials. The purpose of the drug is the treatment of human breast cancer [4, 5].

Corresponding Author: Georgy Konstantinovich Tatonov; Department of Therapy, Faculty of Pediatrics, North-Ossetian State Medical Academy, Vladikavkaz, Russia. E-mail: ruslankalmykov777@yandex.ru.

The active substance of the drug is the RAHMP protein, a recombinant analog of the human milk peptide produced by a genetically modified culture of the Escherichia coli strain, located in 0.9% sodium chloride solution [6-9]. Appearance – frozen solution (dense solidified white mass), after defrosting – colorless liquid, transparent or slightly opalescent.

To ensure the safety and effectiveness of recombinant protein-based medicines, careful quality control of the purified protein using validated techniques is necessary [10-12]. The level of specific and non-specific impurities in the substance is determined by the source of its production, and the technological process and is the most important component of the quality of the drug [13-15]. The content of impurities should be strictly regulated.

The purpose of this work was to develop and validate a technique for the quantitative determination of impurities in the RAHMP substance by reverse-phase high-performance liquid chromatography.

Materials and Methods

To determine the regulatory quality parameters of the RAHMP substance, a technique for the quantitative determination of foreign impurities in a drug by reverse-phase high-performance liquid chromatography was developed [16, 17]. The development and validation of the technique was carried out on 5 experimental series of the RAHMP substance (recombinant analog of human milk peptides).

The quantitative content of impurities in the substance was determined using a microcolumn liquid chromatograph Milichrome A-02 (Econova, Russia), which has a gradient eluent supply system and a UV spectrophotometric detector. Chromatographic data processing was carried out using the MULTICHROME software (Ampersand, Moscow).

Solutions and reagents: acetonitrile, concentrated phosphoric acid, purified water. Validation of the methodology for the quantitative determination of impurities in the RAHMP substance was carried out according to the following characteristics: selectivity, linearity, correctness, and precision.

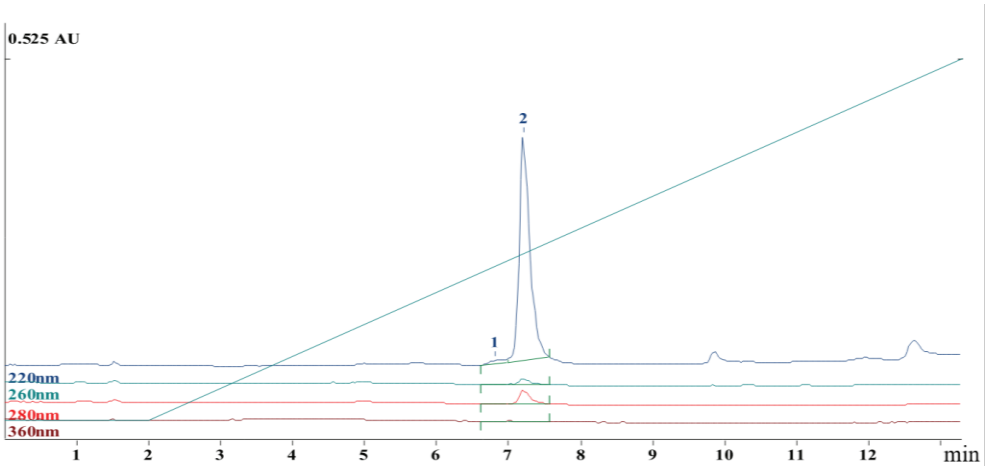
To check the linearity of the technique, a sample of the substance was used, and subjected to partial degradation to accumulate impurities in it by exposure to extreme conditions (temperature 45°C for 48 hours). The experimental sample containing 6.85% impurities after thermal destruction was further diluted in 0.9% sodium chloride solution to an impurity concentration of 1.71% and analyzed.

Results and Discussion

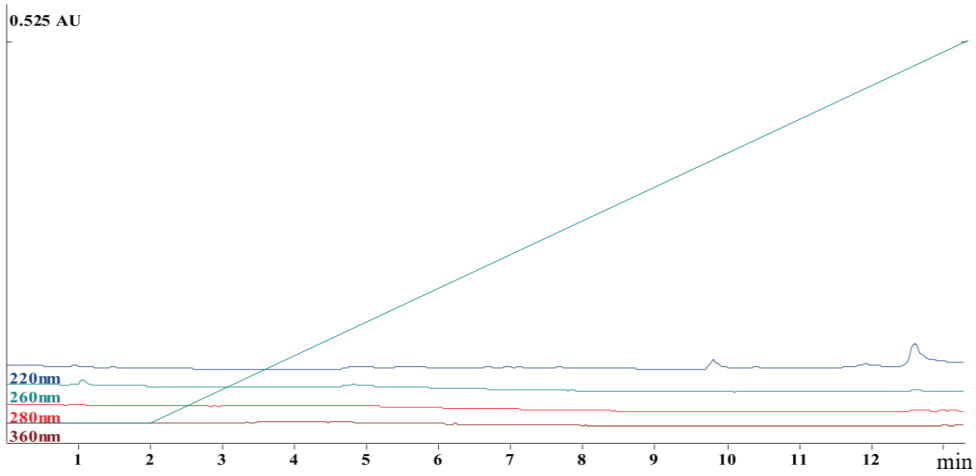
To quantify impurities in the RAHMP substance, we have developed a method of reverse-phase high-performance liquid chromatography: Protnto "SIL" chromatographic column-120-5- C18 AQ" (Econova CJSC, Russia) 2.0×75 mm in size, filled with a C18 type sorbent with a particle size of 5.0 microns (Pronto SIL 120-5-C18); mobile phase – eluent A: 0.1 vol.% orthophosphoric acid in water; eluent B: 0.1 vol.% orthophosphoric acid in acetonitrile; flow rate 150 µl/min; column thermostat temperature 35 °C; volume of injected sample 5 µl; chromatography time 13 min; the detector is spectrophotometric, base λ = 220 nm.

To prepare a working solution of the test substance, 5 µl of the substance and 120 µl of the mobile phase were placed in a microprobe, mixed, and then 25 µl of the resulting solution was transferred to a test tube for an autoclave and chromatographed. The suitability of the chromatographic system was confirmed by six consecutive analyses of the standard RAHMP sample.

Figure 1, shows a chromatogram for the determination of impurities in a standard sample of the RAHMP substance enterprise using the developed methodology. On the chromatogram, the peaks of the RAHMP protein and impurities are well separated from each other and do not interfere with their determination. The average retention time of the RAHMP protein is 7.2 minutes, impurities are 6.9 minutes. At the same time, no peaks are detected on placebo chromatograms with a retention time that coincides with the retention time of the RAHMP peak **(Figure 1b)**.



a)



b)

Figure 1. Chromatogram: a) a standard sample of RAHMP (1 – peak of high molecular weight impurities, 2 – peak of RAHMP); b) placebo

The efficiency of the chromatographic column, calculated from the peak of the RAHMP protein, is 21,000 theoretical plates, and the average resolution of the peaks of the RAHMP protein and impurities is 1.16, which satisfies the criterion of the suitability of the chromatographic system for the analysis of high-molecular impurities (average peak resolution ≤ 2.0 and the number of theoretical plates ≥ 5000) [18]. The relative standard deviation (RSD) of the peak areas of the RAHMP protein for six consecutive chromatograms was 0.87%. The criteria for assessing the quality of the substance were the area of the main peak, which should be at least 95% of the total area of all detected peaks, the sum of the areas of additional peaks should be no more than 5% of the total area of all peaks [19, 20].

The linearity of the technique was determined at five levels of estimated concentrations of concomitant impurities in the concentration range from 1.71% to 6.85%. Model solutions were prepared by diluting an experimental sample of a substance containing 6.85% impurities after thermal destruction **(Table 1)**. The graph of the dependence of the total area of the impurity peaks on the concentration is linear and is expressed by the equation y = 0.033x – 0.0129 **(Figure 2)**.

Table 1. The results of the analysis of the studied samples for the linearity indicator

Sample number	The content of impurities, %	The sum of the peak areas of the accompanying impurities, rel. units.			Average area, rel. units	RSD
4	6.86	0.217	0.212	0.217	0.215	1.10
5	4.92	0.145	0.146	0.147	0.146	0.54
6	3.41	0.103	0.101	0.102	0.102	1.27
7	2.29	0.064	0.063	0.064	0.064	0.78
8	172	0.042	0.042	0.041	0.042	1.19

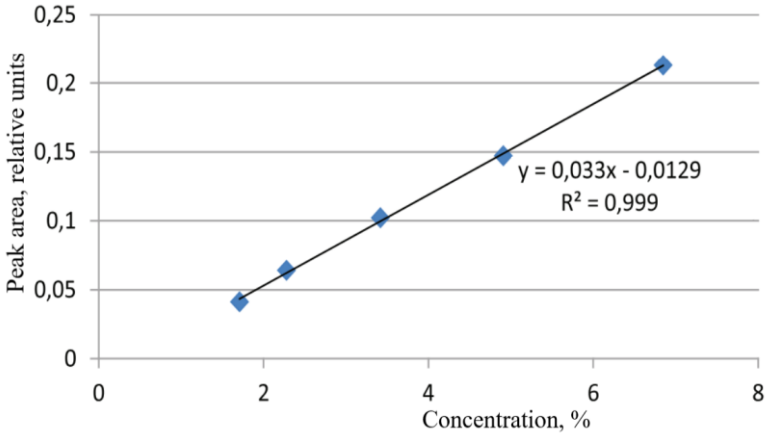


Figure 2. Dependence of the total area of impurity peaks (relative units) on concentration (%)

The reliability of the linear relationship between the values of the impurity peak area and the concentration in the range of studied concentrations is shown by the value of the correlation coefficient r = 0.999. Since the permissible content of impurities

in the substance should be no more than 5%, therefore, the range of experimental data satisfying the linear model in the concentration range from 1.71% to 6.85% can be considered as an analytical area [21]. At the same time, the lowest concentration of impurities in the sample, which can be determined using a validated technique with the required accuracy and precision, is 1.71% and meets the acceptance criterion (the value of the limit of quantitative determination of the analytical technique is below the controlled limit) [22].

The correctness of the technique was evaluated based on the results of the analysis of model solutions of an experimental sample of a substance subjected to partial degradation at five levels of impurity content [23]. The acceptance criterion is the average value of % recovery (R), which should be in the range of 98 to 102%. The average recovery percentage is 100.24% and meets the acceptance criterion **(Table 2)**.

The repeatability (convergence) of the technique was confirmed by the results of the analysis of 6 samples prepared from one batch of RAHMP substance, as well as the results of the analysis of model solutions prepared from an experimental sample of a substance subjected to partial degradation at three levels of impurity concentration. The relative standard deviation of the impurity concentration at all levels of the concentrations under consideration does not exceed 2.0%, which indicates satisfactory convergence of the results **(Table 3)**.

Table 2. Determination of the correctness of the methodology

The specified content of impurities, %	Measured impurity content, %	R	R av.
6.84	6.93	101.3	100.21
4.90	4.84	98.8	
3.41	3.47	101.8	
2.27	2.32	102.2	
1.70	1.65	97.1	

Table 3. Results of sample analysis for the repeatability indicator

Name of the sample	The content of impurities in the sample, % Sample number						The average content of impurities, %	RSD
	1	2	3	4	5	6		
Substance Series	2.07	2.04	2.05	1.95	2.04	1.96	1.97	0.52
Model solution 1	6.82	6.93	6.98	6.84	6.99	6.98	6.93	1.09
Model solution 2	4.81	4.84	4.85	4.84	4.85	4.90	4.86	0.60
Model solution 3	3.55	3.46	3.43	3.43	3.46	3.55	3.49	0.61

The evaluation of in-laboratory precision was carried out on the results obtained in determining repeatability, together with a set of data obtained by a second chemist when analyzing the same series of substances on another day, on different equipment, and with other reagents. Statistical processing of the data obtained by the first laboratory assistant and the second laboratory assistant was carried out, and the relative standard deviation for impurities in the substance was calculated, which was 1.9% and meets the acceptance criterion (no more than 2%) **(Table 4)**.

Table 4. The results of the analysis of the studied samples of the substance for the indicator intra-laboratory precision

Conducting research	The content of impurities in the sample, % Sample number					
	1	2	3	4	5	6
The first laboratory assistant	2.07	2.04	2.05	1.95	2.04	1.96
The second laboratory assistant	1.98	2.00	2.03	1.99	1.99	2.02
The relative standard deviation for impurities is 1.9%						

The analysis of five experimental series of the RAHMP substance showed that the content of impurities in them does not exceed 5.0%.

Conclusion

Thus, a method of reverse-phase high-performance liquid chromatography for the quantitative determination of foreign impurities in the RAHMP substance (a recombinant analog of human milk peptides) has been developed and validated. Criteria for assessing the quality of the substance were the area of the main peak, which should be at least 95% of the total area of all detected peaks, and the sum of the areas of additional peaks should be no more than 5% of the total area of all peaks. The

permissible content of foreign impurities in the substance should be no more than 5%. The relative standard deviation of the impurity concentration at all levels of the concentrations under consideration does not exceed 2.0%, which indicates satisfactory convergence of the results. Thus, the results obtained satisfy the acceptance criteria and allow us to conclude that the technique is reproducible in the laboratory and the results obtained using this technique are reliable.

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