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Development of the Method for Determination of Related Impurities in a New Anti-Epileptic Drug Dibamk

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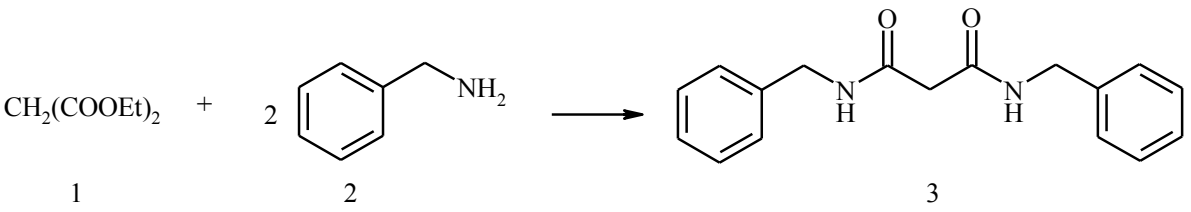
ABSTRACT

According to statistics every hundredth inhabitant of the planet suffers from epilepsy. Treatment of epilepsy involves taking anticonvulsants, and sometimes lifelong. Typically, each of the groups of drugs used to treat epilepsy acts only for certain attacks, such as partial, generalized myoclonic, tonic and atonic. The dibenzyl amide of malonic acid synthesized under the conditional name dibamk has shown the ability to counteract different convulsive poisons in experiments on animals, and it is valuable in the pharmacotherapy of epilepsy in case of spasms of various origins. The article presents data on development of the method for determination of related impurities of the synthesis (benzylamine, diethyl malonate) in the dibamk substance using the method of high-performance liquid chromatography (HPLC). Determination was carried out on a liquid chromatograph with a diode array detector using the chromatographic column with the size of 150 × 4.6 mm filled with octadecylsilyl silica gel for chromatography R Symmetry® C18 with the particle size of 3.5 μm. The chromatographic separation was performed in the gradient elution using the mobile phases of 0.1% solutions of trifluoroacetic acid in water and in acetonitrile on the column filled with octadecylsilyl silica gel. The gradient program for chromatography was as follows: time (min)/%; mobile phase A: 0/90; 5/90→65; 15/65; 25/65→90; 30/90; the time of chromatography-35 min; the flow rate of the mobile phase-1 ml/min, the injection volume-50 μm, the column temperature -25°C; detection was performed at the wavelength of 210 nm. In the chromatographic conditions proposed the complete resolution of dibamk peaks and peaks of related impurities of the synthesis was achieved.

Keywords: Standardization, Dibamk, Related impurities, HPLC method

INTRODUCTION

Dibamk (dibenzyl amide of malonic acid) proposed as an anticonvulsant for treating seizures of different etiologies ï is a promising biologically active substance to create medicines [1,2]. Dibamk was included in the research plan of "Farmak" JSC as a promising pharmaceutical development due to its attractiveness for industrial synthesis. The substance (3) is synthesized in one step with high yields from the available chemical substances–diethyl malonate (1) and benzylamine (2) (Scheme 1) [3].



Scheme 1: Substance

The State Pharmacopoeia of Ukraine (SPhU) [4] recommends to determine related impurities, which can have a significant impact on human health due to possible teratogenic, mutagenic or carcinogenic effects, in substances to be analyzed [4,5]. Control and monitoring of impurities is a critical issue when developing and manufacturing medicines [6-10]. Based on Scheme 1 the starting products of the synthesis–benzylamine and diethyl ester of malonic acid can be impurities of dibamk. To control related impurities of dibamk the method of sorbent thin-layer chromatography in the system of solvents of *n*-butanol–glacial acetic acid–water (40:10:1) is described in the literature. The authors propose this method for determination of related impurities of dibamk both in the substance and in finished dosage forms [2]. Unlike the method of thin-layer chromatography (TLC), the method of liquid chromatography is more sensitive and selective instrumental method of analysis, and it is proposed by the SPhU for determination of related impurities.

Dibamk and such related impurities as benzylamine (impurity A) and diethyl malonate (impurity B) have different hydrophilic properties. Therefore, the aim of our study is to develop the method for determining related impurities in the substance by the HPLC method, and it can provide a complete chromatographic separation and the necessary accuracy and reproducibility of the results when conducting the quality control.

MATERIALS AND METHODS

A "ProStar" liquid chromatograph with a diode array detector (Varian, USA), Mettler Toledo AB-204/A analytical balance (UK), measuring glassware of class A, and reagents meeting the requirements of the SPhU were used.

The method of determination

Test solution. Place 50 mg (accurately weight) of the dibamk substance into a 25 ml volumetric flask, add 15 ml of acetonitrile *R*, treat for 5 min in an ultrasonic bath until complete dissolution, dilute the solution to the volume with the same solvent, and mix thoroughly. Filter through a 0.45 μm membrane filter.

Reference solution (a): Place 1.0 ml of the test solution into a 100 ml volumetric flask, dilute the solution to the volume with acetonitrile *R* and mix. Reference solution (b): Place 5.0 ml of the reference solution (a) into a 50 ml volumetric flask, dilute the solution to the volume and mix. Reference solution of impurity A. Place 1 ml of benzylamine *R* into a 50 ml volumetric flask, add 30 ml of ml of acetonitrile *R*, dilute the solution to the volume with the same solvent and mix. Reference solution of impurity B. Place 1 ml of diethyl malonate *R* into a 50 ml volumetric flask, add 30 ml of acetonitrile *R*, dilute the solution to the volume with the same solvent and mix. Solution for the system suitability test. Place 5.0 ml of the reference solution (a), 5.0 ml of the reference solution of impurity A and 5.0 ml of the reference solution of impurity B into a 20 ml volumetric flask, dilute the solution to the volume with acetonitrile *R* and mix. All solutions were used freshly prepared.

Stationary phase

The chromatographic column with the size of 150 × 4.6 mm filled with octadecylsilyl silica gel for chromatography *R* Symmetry® C18 with the particle size of 3.5 μm. Mobile phase A: 0.1% solution of trifluoroacetic acid *R* in water *R*; Mobile phase B: 0.1% solution of trifluoroacetic acid *R* in acetonitrile *R*.

The gradient program for chromatography was as follows

Run time, min	Mobile phase A, %	Mobile phase B, %
0-5	90	10
5-15	90→65	10→35
15-25	65	35
25-30	65→90	35→10
30-35	90	10

The flow rate of the mobile phase: 1 ml/min; The column temperature: 25°C; Detection: at the wavelength of 210 nm; the injection volume: 50 μm. The solvent (blank chromatogram) and the solution for the system suitability test were chromatographed.

To identify impurities the chromatogram of the solution for the system suitability test and the relative retention times (the retention time of dibamk is approximately 21.6 min) were used. The elution order and the relative retention times were benzylamine (impurity A–0.16); diethyl malonate (impurity B–0.79). The reference solution (b), reference solution (a) and the test solution were chromatographed at least 3 times.

The chromatographic system is considered to be suitable if: the symmetry factor of the main peak should not be less than 0.8 and not more than 1.5; the relative standard deviation (RSD, %) calculated for the area of the main peak of the reference solution (a) should not exceed the values specified [2]:

The number of parallel injections	2	3	4	5	6	7	8
RSD, %	0.32	0.84	1.20	1.48	1.72	1.93	2.11

Normalization

Impurity A: the peak area should not exceed 1.5 of the area of the main peak on the chromatogram of the reference solution (b) (0.15%); impurity B: the peak area should not exceed 1.5 of the area of the main peak on the chromatogram of the reference solution (b) (0.15%); any other impurity: the peak area should not exceed 1.0 of the area of the main peak on the chromatogram of the reference solution (b) (0.1%); total impurities: the total areas of all peaks should not exceed 1.0 of the area of the main peak on the chromatogram of the reference solution (a) (1.0%); do not take into account: peaks with the area less than 0.2 of the area of the main peak on the chromatogram of the reference solution (b). Peaks that coincide with the corresponding peaks on the blank chromatogram by their retention times are also not considered.

Methods for determining dissolution of dibamk in the stressful conditions

Acid: Place 50 mg of the dibamk substance into a 50 ml volumetric flask, dissolve in 15 ml of acetonitrile R, add 5 ml of 0.1 M solution of hydrochloric acid (HCl). Heat the resulting solution at 80°C for an hour. Cool the solution, add 5 ml of 0.1 M solution of sodium hydroxide, and dilute the solution to the volume with acetonitrile R and mix.

Alkaline: Place 50 mg of the dibamk substance into a 50 ml volumetric flask, dissolve in 15 ml of acetonitrile R, add 5 ml of 0.1 M solution of sodium hydroxide (NaOH). Heat the resulting solution at 80°C for an hour. Cool the solution, add 5 ml of 0.1 M solution of hydrochloric acid, and dilute the solution to the volume with acetonitrile R and mix.

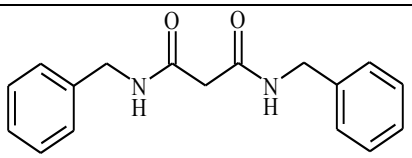
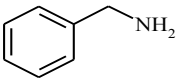
Peroxide: Place 50 mg of the dibamk substance into a 50 ml volumetric flask, dissolve in 15 ml of acetonitrile R, add 2 ml of solution of concentrated hydrogen peroxide R. Heat the resulting solution at 80°C for an hour. Cool the solution; dilute the solution to the volume with acetonitrile R and mix.

Thermal: Place 50 mg of the dibamk substance into a 50 ml volumetric flask, dissolve in 15 ml of acetonitrile R, heat at 80°C for an hour. Cool the solution; dilute the solution to the volume with acetonitrile R and mix.

RESULTS AND DISCUSSION

Quantitative determination of related impurities in the dibamk substance was carried out by the HPLC method. It included preparation of the test solution, as well as the reference solutions (a) and (b) by diluting the test solution with the subsequent alternate chromatography of the solutions. Calculation of the quantitative content of impurities was performed in relation to the peak areas calculated for the reference solutions (a) and (b) using the analytical system for HPLC in the gradient elution with the mobile phases of 0.1% solutions of trifluoroacetic acid in water and in acetonitrile on the column filled with octadecylsilyl silica gel. The gradient elution program was as follows: time (min) /%; mobile phase A: 0/90; 5/90→65; 15/65; 25/65→90; 30/90; the time of chromatography–35 min; the flow rate of the mobile phase–1 ml/min, the injection volume–50 μm, the column temperature–25°C; detection–at the wavelength of 210 nm. According to the scheme of the synthesis the substance may contain impurities that are given in Table 1.

Table 1: Determination of impurities in the dibamk substance

Substance	**Dibamk**	
Impurity A*	Benzylamine	
Impurity B*	Diethyl malonate	$CH_2(COOEt)_2$
Impurity C*	–	Unidentified
Impurity D*	–	Unidentified

*Impurities A and B are impurities of the synthesis of the dibamk substance, impurities C and D are decomposition products of the substance

Comparison of the chromatograms (Figures 1-7) shows that in the conditions of the method proposed the solvent, the mobile phase or the active substance interfere determinations of impurities. Thus, it indicates the specificity of the method. Under the conditions specified the retention time of the peak of the active substance were approximately 21.5 min (Figure 7), the peak of impurity A–approximately 3.4 min (Figure 5), and the peak of impurity B–approximately 17.0 min (Figure 6).

To elute impurity A the mobile phase with the acetonitrile content of approximately 10% is optimal. However, for peak resolution of impurity B and dibamk it is necessary to increase elution of the mobile phase by increasing the content of acetonitrile up to 35%. Therefore, it is suggested to carry out the separation of impurities and the active substance in the gradient elution [11].

The symmetry factor of the active substance in dibamk is 0.8, and it meets the requirements of the chromatographic system suitability.

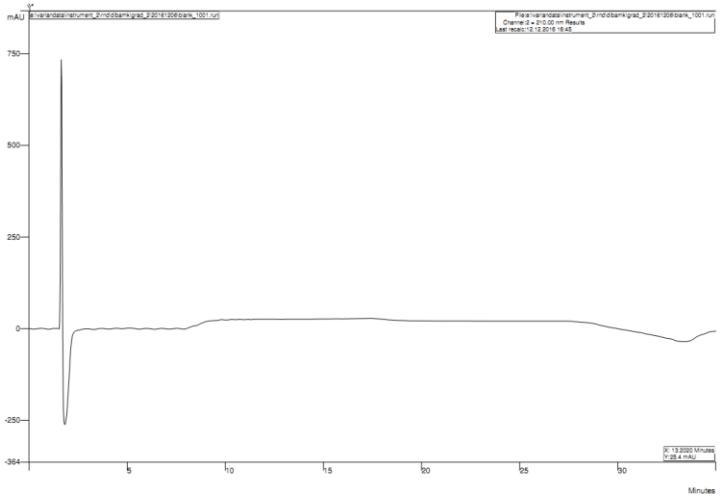


Figure 1: The chromatogram of the solvent (blank chromatogram)

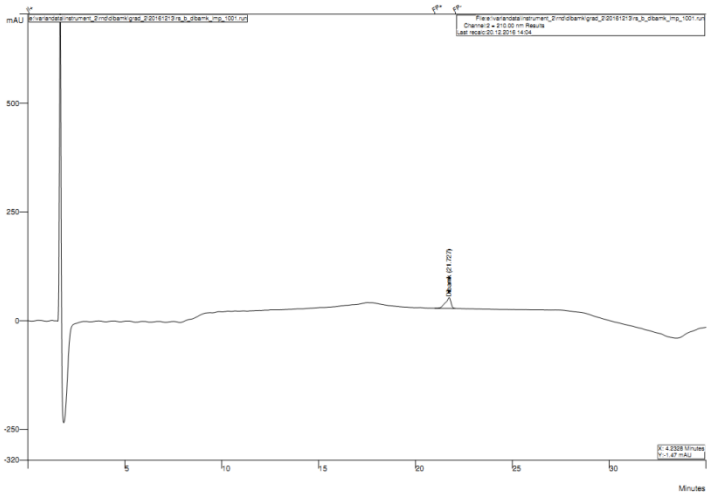


Figure 2: The chromatogram of the reference solution (b)

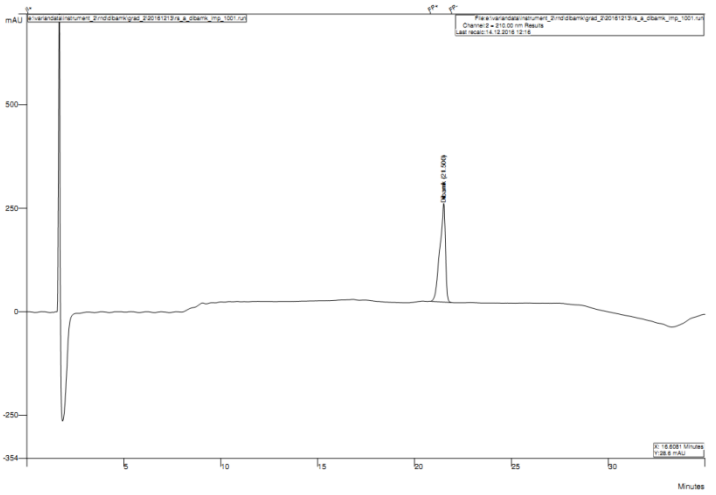


Figure 3: The chromatogram of the reference solution (a)

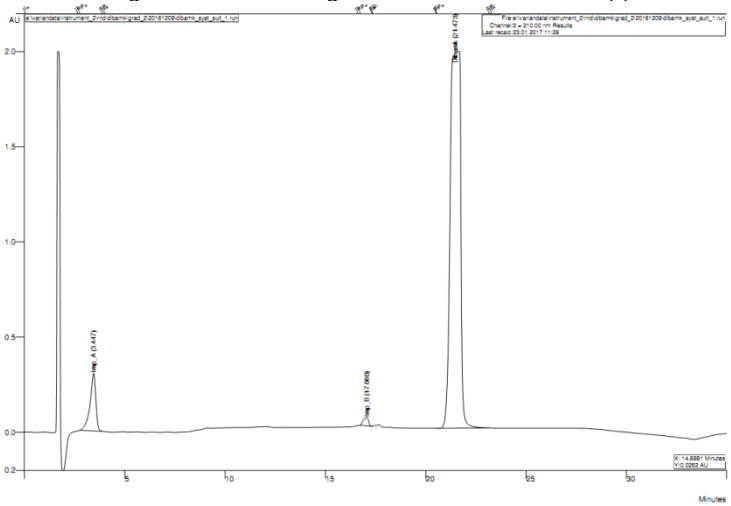


Figure 4: The chromatogram of the solution for the system suitability test

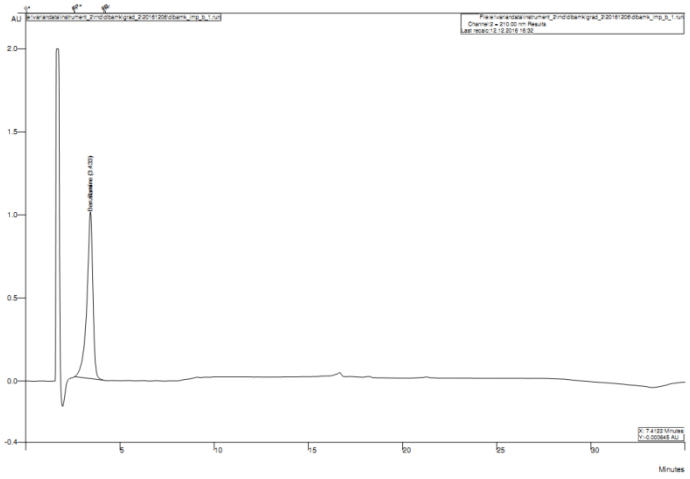


Figure 5: The chromatogram of the reference solution of impurity A (benzylamine)

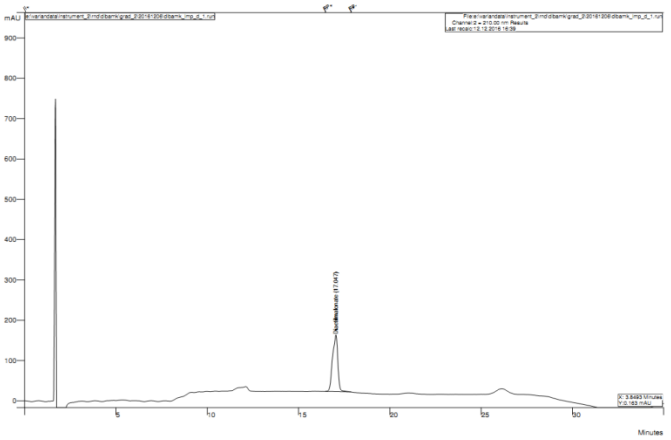


Figure 6: The chromatogram of the reference solution of impurity B (diethyl malonate)

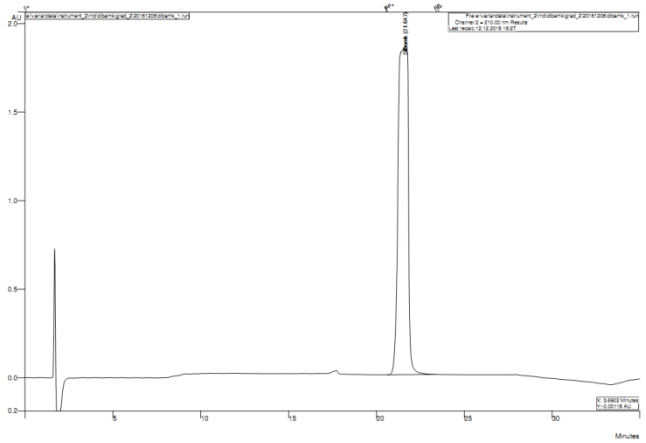


Figure 7: The chromatogram of the test solution

The quantitative content of impurities was determined in relation to the peak areas of the reference solution (a) and the reference solution (b) (Figure 2). Calculation of the quantitative content of impurities in the dibamk substance is given in Table 2.

Table 2: Calculation of the quantitative content of impurities in the synthesis of the dibamk substance

	Reference solution (a)	Reference solution (b)	Impurity A	Impurity B
The peak areas	24576586	2559024	–	3446191
	24453164	2544724	–	3364557
Mean	24514875	2551874	–	3405374
% of the impurity	1.0	0.1	–	0.13
Individual impurity	–	–	Unidentified	<0.15%
Total impurities	–	–	0.13%	

According to these data it can be concluded that the content of related impurities in the dibamk substance meets the requirements for regulated limits of the quantitative content of impurities.

The dibamk substance was subjected to accelerated dissolution under the stressful conditions. As can be seen from the chromatograms of the test solution (Figures 8-11), the dibamk substance is resistant to the stressful conditions.

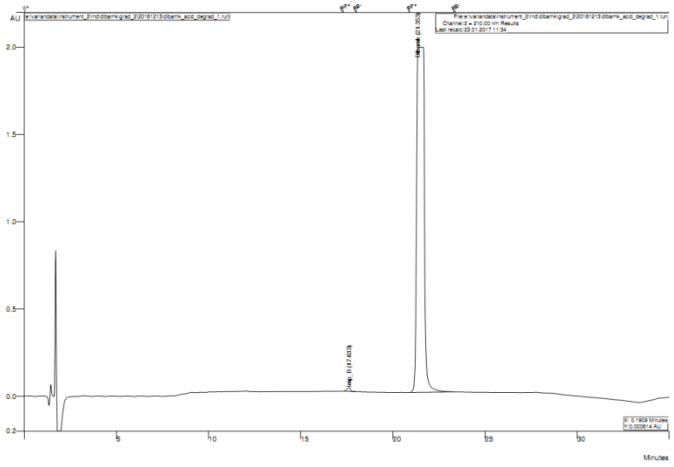


Figure 8: The chromatogram of the dibamk solution after acid dissolution

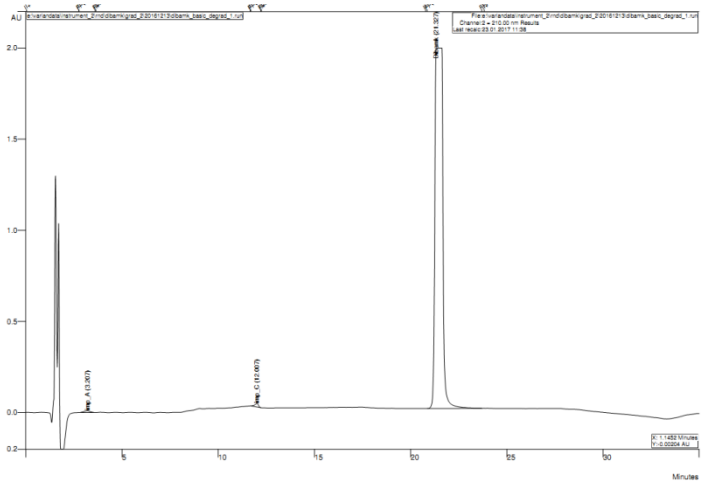


Figure 9: The chromatogram of the dibamk solution after alkaline dissolution

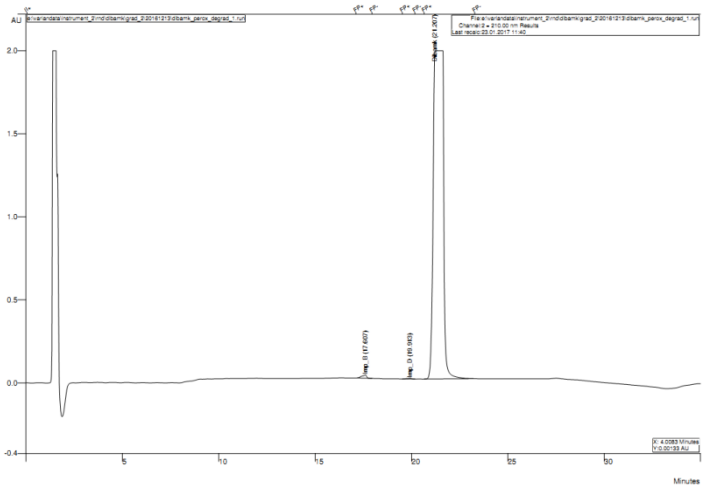


Figure 10: The chromatogram of the dibamk solution after peroxide dissolution

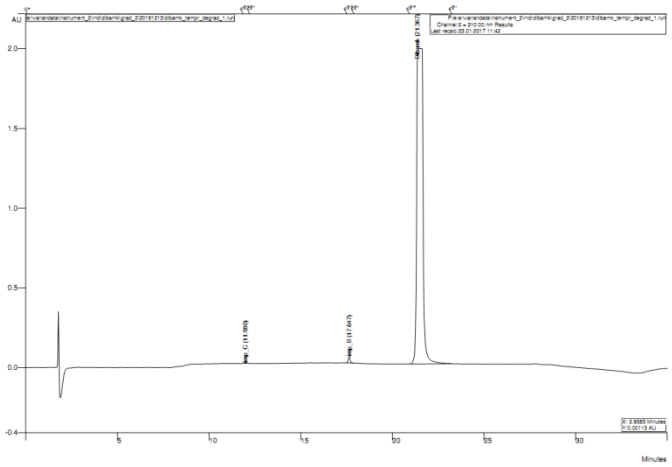


Figure 11: The chromatogram of the dibamk solution after thermal dissolution

In acid dissolution (Figure 8) the substance studied was decomposed forming impurity B. Under conditions of alkaline hydrolysis (Figure 9) formation of impurity A and appearance of the peak of an unidentified impurity with the relative retention time approximately 12 min (impurity C) were observed. Under conditions of peroxide dissolution (Figure 10), in addition to impurity B, there was the peak of an unidentified impurity with the relative retention time approximately 19.9 min (impurity D). The thermal dissolution of dibamk (Figure 11) led to formation of impurity B and an unidentified impurity with the relative retention time approximately 12 min (impurity C).

CONCLUSION

The new method for determination of related impurities in the dibamk substance has been developed using HPLC. It provides the complete separation of the active substance and peaks of related impurities due to the conditions of the gradient chromatography, allows determining impurities quantitatively by comparing their areas of peaks with the peak areas of the reference solutions (a) and (b) with high precision, and it is promising for determining the purity level of the dibamk substance. It has been found that the dibamk substance is resistant to the stressful conditions.

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