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**DEVELOPMENT AND VALIDATION OF METHODS OF QUANTITATIVE DETERMINATION OF THE NEW ANTIDIABETIC DRUG IN THE BLOOD PLASMA OF RATS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH MASS SPECTROMETRIC DETECTION**

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**Abstract.** We developed a method of quantification of the new antidiabetic drug 3-(1h-benzimidazole-2-yl)-1,2,2-trimethyl-cyclopentane-carbonic acid (C7070) in the blood plasma of rats by high-performance liquid chromatography with mass-spectrometric detection. The analytical range of the method was 0.02-3876.0 µg in 1 ml of blood plasma. The research was partially supported by the grant of the President of the Russian Federation № MD-4711.2015.7 and MK-6135.2016.4.

**Keywords:** antidiabetic agent; C7070; the blood plasma of rats; high-performance liquid chromatography; mass spectrometric detection; validation.

According to the State Register as of January 1, 2012, more than 3 million 540 thousand patients with diabetes mellitus (DM), 90% of them – with type 2 diabetes were registered in the Russian Federation. However, results of control and epidemiological studies of the Endocrinology Research Center, conducted in 2005-2010, as well as WHO's data for 2010 indicate that the actual number of diabetic patients exceeds the registered one by more than 3 times [1].

Considering this, the development of new and effective drugs for the treatment of DM is one of the urgent problems of modern medicine. As it is known, the development itself is impossible without studying the pharmacokinetics, which accurate assessment requires a sensitive and highly-selective method. Currently, the reference method for studying the pharmacokinetics is a high-performance liquid chromatography with mass spectrometry [1, 3, 4].

Subject to the above, the objective of the study is to develop and validate a method of quantification of C7070 by high-performance liquid chromatography with mass-spectrometry (HPLC-MS/MS).

#### *Experimental part.*

Reagents used in this study are the follows: C7070 (CJSC "VladMiVa", Belgorod), fabomotizole (Sigma), formic acid (Panreac), ammonium acetate (Panreac), methanol (Merk) acetonitrile for gradient

chromatography (Merk), water purified and deionized with "Gene Pure" system (Thermo Scientific, USA).

Identification of C7070 in rat plasma was carried out with a liquid chromatograph UltiMate 3000 LC (Thermo Fisher Scientific, USA) equipped with a thermostated automatic dispenser, vacuum degasser, gradient pump, and column thermostat. Detection of the analyte was carried out with a mass-spectrometer Velos Pro (Thermo Scientific, USA) under ionization in the heated electrospray (H-ESI-II).

#### **Sample preparation.**

Preparation of C7070 solutions involved several stages. At the first stage, stock solution of C7070 was prepared in methanol at a concentration of 0.2%. At the second stage, C7070 solutions with methanol were prepared by a series of dilutions of stock solution to be further added to standard solutions and control solutions at a concentration of 0.00002%, 0.002% and 0.011%.

Fabomotizole solutions (internal standard) in methanol for introducing into standard and test solutions were prepared at the same concentration level – 0.1% solution.

The study used standard samples of C7070 (CJSC "VladMiVa", Belgorod), and fabomotizole (Sigma).

The solutions for the construction of calibration curve, in accordance with modern requirements [5, 6, 7], were prepared at seven concentration levels (0.02 µg, 0.2 µg, 2.00 µg, 1.94 µg, 193.80 µg, 1938.00 µg and 3876.00 µg in 1 ml plasma). For this purpose, 100 µl of plasma were placed in 1.5 ml Eppendorf tubes, then added aliquots of stock solutions, 100 µl of internal standard solution and 100 µl of acetonitrile. Then the analyte was extracted in an ultrasonic bath for three minutes. The samples were then frozen at -70°C. After defrosting, samples were centrifuged at 13,000 rev/min and a temperature of 4°C for 25 minutes. The supernatant was decanted and analyzed.

Test solutions were prepared similarly to the solutions for the calibration curve based on four levels of concentration in five repetitions: 0.02 µg (lower limit of quantification – LLOQ), 0.20 µg

(lower quality control – LQC), 19.38 µg (mean control quality – MQC) and 1938.00 µg (upper quality control – UQC) in 1 ml of plasma.

Solutions for the determination of matrix effect were prepared similarly to test solutions, but with replacing plasma with water [8].

Chromatographic separation was performed on a 150×3.0 mm column filled with converted-phase sorbent Zorbax Eclipse XDB C18 with particle size of 3.0 µm and with a 12.5×3.0 mm guard column Zorbax Eclipse XDB C18 with a particle size of 5.0 µm at a temperature of 40°C. The chromatographic analysis was performed with the use of UltiMate 3000 LC system coupled with a mass spectrometric detector under the following chromatographic conditions:

Sample volume:	2 µl				
Mobile phase:	Time, min	flow, ml/min	5 mM Ammonium acetate + 0.1% formic acid	Acetonitrile, %	Methanol, %
	0	0.4	80	20	0
	5.0	0.4	80	20	0
	8.5	0.4	65	10	10
	9.0	0.4	20	70	10
	11.0	0.4	20	70	10
	11.1	0.4	80	20	0
	12.5	0.4	80	20	0
Approximate retention times:	C7070 – about 4.7 min; Internal Standard (Fabomotizole) – about 8.5 min.				
Injection time:	12.5 min				
Ionization type:	H-ESI				
Polarity:	C7070 “+” Internal Standard (Fabomotizole) “+”				
Mass transition:	C7070 – 272.35→255.15; Internal Standard (Fabomotizole) – 307.41→114.				
Collision energy:	C7070 – 42; Internal Standard (Fabomotizole) – 19				
Source voltage (V):	C7070 – 3000 Internal Standard (Aminophylline) – 3000				
Source temperature (°C):	C7070 – 300 Internal Standard (Fabomotizole) – 300				
Capillary temperature (°C):	C7070 – 350 Internal Standard (Fabomotizole) – 350				
Sheath gas pressure (Arb):	C7070 – 60 Internal Standard (Fabomotizole) – 60				
Aux gas pressure (Arb):	C7070 – 20 Internal Standard (Fabomotizole) – 20				

### Results and discussion:

We developed a highly-selective method with a wide range of linearity (0.02 to 3876.00 µg in 1 ml of plasma) for carrying out qualitative pre-clinical studies. The width of the range is due to the nature of the operations (dose adjustment and evaluation of the

response linearity). Use of an internal standard ensured minimization of sample preparation errors and improved the accuracy of the determination. Since the pre-clinical studies in animals involve a large number of control points that need to be analyzed strictly in a particular analytical cycle, the method used a simple

and express sample preparation – protein precipitation with acetonitrile, followed by ultrasound extraction [9, 10, 11]. To ensure a satisfactory sample purity and stable detection, the method of chromatographic separation on an analytical column filled with sorbent Zorbax Eclipse XDB C18 was used.

### Chromatographic system suitability parameters.

To determine the chromatographic system suitability, the solutions for calibration curve were analyzed, the solution at a concentration of 19.38 µg in 1.0 ml of plasma was chromatographed six times, the equation of the calibration curve was calculated, and the obtained amounts were subject to reverse recalculation. The chromatographic system was considered suitable when meeting the following generally accepted criteria [9]:

- errors in the reverse recalculation should not exceed: for a point equal to the LLOQ not more than 20%; for all other points – not more than 15%;
- relative standard deviation of the ratio of C7070 peaks area to the internal standard peak areas calculated on 6 consecutive chromatograms of solution at a concentration of 19.38 µg in 1.0 ml of plasma should not exceed 7%;
- C7070 peak asymmetry factor at a

concentration of 19.38 µg in 1.0 ml of plasma and the internal standard should not exceed 2.2%;

– signal-to-noise ratio for the C7070 peak in the chromatogram of solution equal to the LLOQ must be at least 10:1.

### Method validation.

Validation of the method for quantification of C7070 in rat plasma was conducted in accordance with the Guidelines for the examination of medicines [5], as well as Guidelines for the validation of bioanalytical FDA [8] and EMA practices [7] under the following characteristics: selectivity (specificity) linearity, accuracy and precision (within a series and between series), the limit of quantification, the sample stability, the matrix effect and the transferable residue.

### Selectivity (specificity).

To determine the selectivity (specificity) a pure rat plasma sample was analyzed, as well as pure plasma samples with the addition of the standard solutions C7070 and internal standard in the expected range of concentrations.

The chromatograms of pure plasma samples showed no peaks with retention time corresponding to retention time of C7070 and the internal standard (Fig. 1 (A-B)).

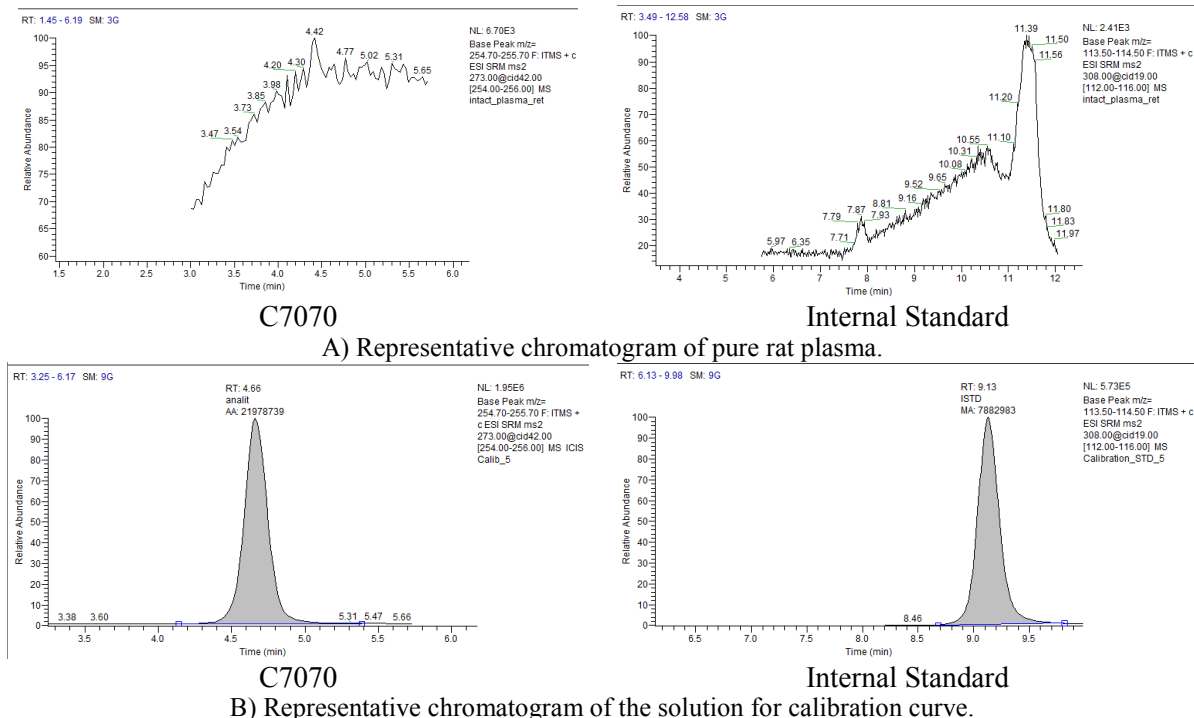


Figure 1. Representative chromatograms in determining the selectivity.

### Transferable residue.

To determine the presence of transferable residue the pure sample of rat plasma was analyzed after administration of maximum concentration solution (solution for calibration curve, level 7).

The chromatograms of pure plasma samples after administration of maximum concentration solution

showed no peaks with retention time corresponding to retention time of C7070 and the internal standard. This was achieved by satisfactory flushing of the input unit (syringe and needle were flushed with a 3 ml solution of water, acetonitrile, methanol, isopropanol and formic acid in a volumetric ratio of 24:25:25:25:1 prior to each administration).

### Matrix Effect

The said indicator was studied in six samples of biological matrix from different donors. The values of the coefficient of variation (CV) of the normalized

matrix factors for six different rat matrices on the lower and upper concentration levels did not exceed 15%. The calculation of the normalized matrix factor is presented in Table 1. None of the results exceeds allowable limits.

Table 1

Determination of the normalized matrix factor in rat plasma.

Solution	S <sub>i0</sub>	S <sub>is0</sub>	S <sub>i</sub>	S <sub>is</sub>	Normalized matrix factor	CV, %	Acceptance Criteria
LQC	1286284	3328954	1398451	3288954	0.94	14.1	NMT 15 %
	1326741	3326544	1398754	3326841	0.95		NMT 15 %
	1358744	3421587	1339587	3521478	1.05		NMT 15 %
	1385655	3321478	1346744	3658421	1.08		NMT 15 %
	1258741	3185647	1828974	3268741	0.71		NMT 15 %
	1247841	3125877	1389751	3654871	1.05		NMT 15 %
UQC	112254871	3236544	143528974	3265471	0.83	5.1	NMT 15 %
	112684512	3328741	146875421	3388744	0.84		NMT 15 %
	123654123	3265877	144658971	3326952	0.84		NMT 15 %
	122657458	3128741	143289568	3297451	0.84		NMT 15 %
	119547877	3326877	144651847	3544326	0.90		NMT 15 %
	120745216	3184544	141286544	3641288	0.94		NMT 15 %

### Limit of Quantitation (LOQ):

To find LOQ, a series of dilutions was prepared (from 1 to 25 ng in 1 ml of plasma). Based on the obtained results, the concentration with the signal/noise ratio for C7070 peak 10 and a coefficient of variation (CV) between six parallel definitions of not more than 10% was selected. LOQ was 20 ng in 1 ml of plasma.

### Linearity

According to the obtained values, the calibration

curve was constructed, shown in Fig. 2. To assess the acceptability of the obtained values the reverse calculation of C7070 amounts from those entered by the equation of the calibration curve was performed. None of the values exceeded the allowable limits (for LOQ not more than 20%; for all other points – not more than 15%). Thus, the linear range of the method was 0.020 – 3876.0 µg in 1 ml of plasma.

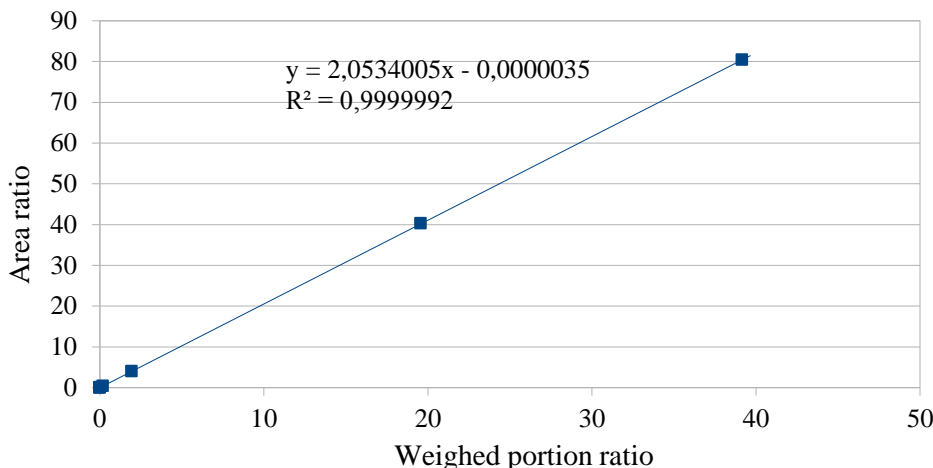


Figure 2. The linear dependence of peak area C7070 on its concentration normalized to the internal standard.

### Accuracy and precision (within a series and between series).

Accuracy and precision of the method was evaluated on rat plasma samples with the addition of known quantities of C7070 at four concentration levels (LLOQ, LQC, MQC and UQC), which were prepared separately from the solutions prepared to confirm the linearity of the method. Each level was prepared five-fold. The accuracy was expressed:

within the series, as a percentage of a nominal content of C7070 (Recovery) and between the series in the form of CV of the obtained values for each level on different days, in percentage. The precision was expressed with CV between parallel determinations of each concentration level within the series, in percentage. The results are presented in Tables 2-3.

Table 2

The accuracy (between the series) of the determination of C7070 in rat plasma.

Solution	C <sub>aver.</sub> , µg/ml	C <sub>aver.</sub> , µg/ml	C <sub>aver.</sub> , µg/ml	C <sub>aver.</sub> , µg/ml	CV, %	Acceptance criterion
	cycle 1	cycle 2	cycle 3	cycle 4		
LLOQ	0.0216	0.0199	0.0211	0.0209	3.5	NMT 20 %
LQC	0.1899	0.1934	0.1904	0.1905	0.8	NMT 15 %
MQC	19.0575	18.9885	18.5546	19.4093	1.8	NMT 15 %
UQC	1839.50	1945.11	1861.32	1889.58	2.4	NMT 15 %

Table 3

The accuracy and precision (within the series) of the determination of C7070 in rat plasma.

Solution concentration on level	C <sub>intr.</sub> , µg/mL	Cycle 1		Cycle 2		Cycle 3		Cycle 4	
		Recovery, %	CV, %	Recovery, %	CV, %	Recovery, %	CV, %	Recovery, %	CV, %
LLOQ	0.0205	117.5	9.7	81.9	10.2	106.2	4.7	98.2	8.4
		113.1		100.3		102.2		102.9	
		95.9		106.8		98.4		99.6	
		106.3		103.1		98.1		99.3	
		94.3		92.6		109.2		106.1	
LQC	0.1938	99.3	9.0	99.5	3.3	103.9	6.5	106.3	3.3
		100.2		100.5		95.8		87.4	
		110.6		100.3		101.3		93.7	
		87.3		103.8		88.2		90.8	
		92.5		94.7		101.9		98.8	
MQC	19.38	100.7	3.7	101.6	3.6	95.1	6.6	96.7	5.9
		97.6		100.7		99.6		99.1	
		97.3		94.4		84.9		108.4	
		93.4		99.1		99.3		99.6	
		102.8		94.0		99.8		95.0	
UQC	1938.0	102.7	5.0	101.1	6.0	92.9	5.2	99.2	6.7
		93.3		107.3		102.7		97.4	
		92.8		102.6		98.9		100.5	
		95.6		90.8		89.9		106.0	
		90.2		100.1		95.7		105.1	

*Solution stability.*

Stability of the solutions was assessed on two criteria – stability upon analysis in the automatic

dispenser and stability at defreezing/freezing. The results are presented in Tables 4-5.

Table 4

Solution stability upon freezing/defrosting.

Solution	C <sub>aver.</sub> , µg/ml	C <sub>aver.</sub> , µg/ml	C <sub>aver.</sub> , µg/ml	C <sub>aver.</sub> , µg/ml	CV, %	Acceptance Criterion
	cycle 0	cycle 1 of freezing	cycle 2 of freezing	cycle 3 of freezing		
LQC	0.0216	0.0202	0.0238	0.0206	7.5	NMT 15 %
UQC	1839.50	1842.88	1857.321	1947.99	2.7	NMT 15 %

Table 5

Solution stability upon storage in the autosampler

Solution	C <sub>aver</sub> , µg/ml			CV, %	Acceptance Criterion
	0 hours of storage	8 hours of storage	16 hours of storage		
LQC	0.0216	0.02031	0.01978	4.5	NMT 15 %
UQC	1839.50	1799.31	1899.48	2.7	NMT 15 %

Representative chromatograms of both C7070 and the internal standard (fabomotizole) at a MQC

concentration in rat plasma (19.38 µg in 1 ml of rat plasma) are shown in Fig. 3.

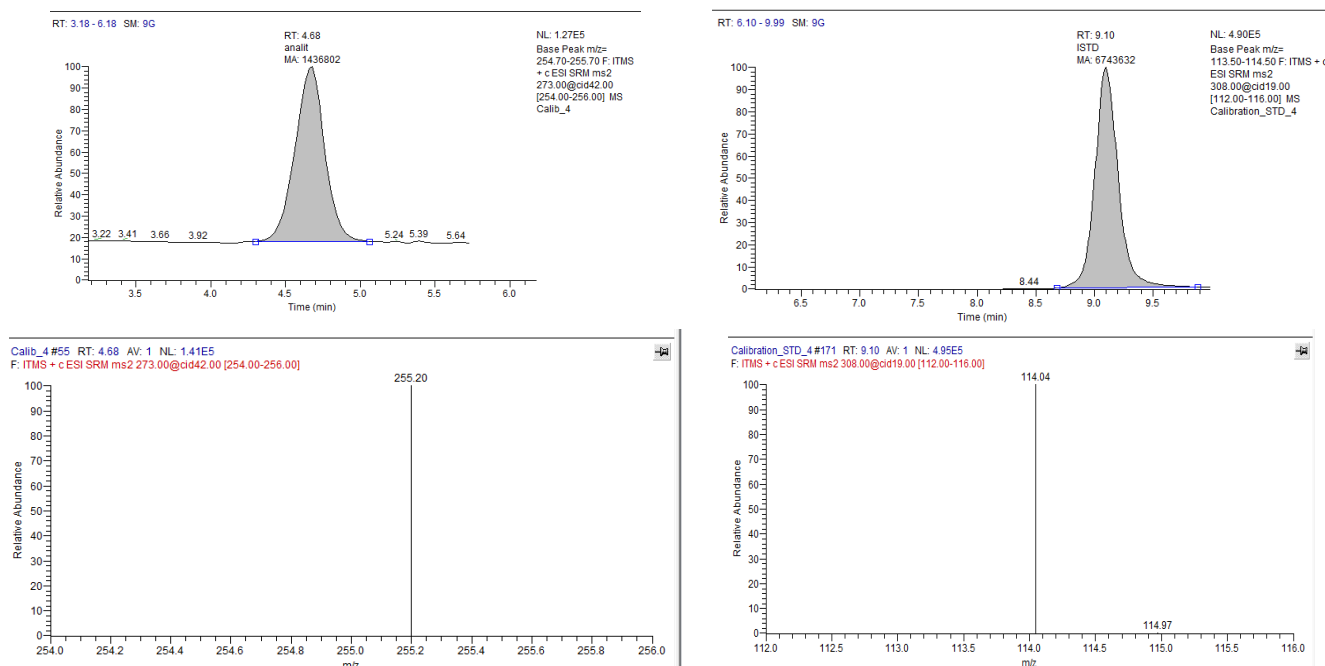


Figure 3. Chromatography and mass spectra of both C7070 and internal standard (fabomotizole) in rat plasma.

Thus, the developed method of quantification of C7070 in rat plasma by high-performance liquid chromatography with mass spectrometry is easy to implement, meets the requirements of the validation characteristics, and ensures reliable and accurate determination of C7070 in rat plasma in the concentration range of 0.02 µg/ml to 3876.00 µg/ml.

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