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Development and Validation of Voltammetric Method for Quantitation of New Antiviral Drug Triazavirin using Bare Carbon Screen-Printed Electrodes

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Abstract- Bare carbon screen-printed electrode based on carbon ink Electrodag 407C was used for quantitation of the new Russian anti-influenza drug Triazavirin. For the first time it was established that an electrochemical activity of an active substance of the drug (sodium salt of 2-methylthio-6-nitro-1,2,4-triazolo[5,1-c][1,2,4]triazin-7-one, dihydrate, triazavirin) is caused by electrochemical reduction of a nitro group bonded to a conjugated aromatic system. The effect of voltammetric mode, pH of electrolyte, scan rate, time and potential of preliminary accumulation on the current of triazavirin reduction were studied. A quick, simple and "green" method for triazavirin determination using a direct square wave cathodic voltammetry was developed. The peak current obtained was linearly related to triazavirin concentration in the range of 0.1 to 180 mg L^{-1} with correlation coefficient of 0.9996. Good analytical performance was achieved with a detection limit of 0.04 mg L^{-1}, a repeatability of 1.0% and an intermediate precision <2%. The developed method was validated and applied for the analysis of Triazavirin capsules. Found values are close to the claimed content and are in good agreement with the results of the HPLC method.

Keywords- Triazavirin, Bare carbon screen-printed electrode, Square wave voltammetry, Validation

1. INTRODUCTION

The new Russian antiviral drug Triazavirin belongs to the class of non-nucleoside antiviral etiotropic agents of the azoloazine family. The drug was proved to be a highly effective anti-influenza agent that works at any stage of the infectious process [1]. Triazavirin is effective against infections caused by influenza A and B viruses, parainfluenza and a number of other infections. The main mechanism of Triazavirin action is the inhibition of the synthesis of viral RNA and the replication of genomic fragments. In terms of a therapeutic effect, it surpasses many Russian and foreign analogues [2].

There is a method for Triazavirin (TZ) determination in the Triazavirin substance using HPLC in strongly acidic and strongly alkaline media with a spectrophotometric detection using a diode array and a wavelength of 215 nm with a slit of 8 nm [3]. The best results were shown by basic buffer solutions on the chromatographic column Phenomenex Synergimax-RPC 12. The method allows measuring the content of Triazavirin in a concentration range from 0.0002 to 2 g L^{-1}.

Frequently, voltammetry (VA) is not inferior in sensitivity and selectivity to the HPLC method in determining the active substance in pharmacy objects. In addition, the VA analysis is quick, simple, low-cost and uses portable instrumentation, which does not require toxic organic solvents and the involvement of expensive staff for its service [4-6].

TZ refers to the class of pharmaceutical nitro compounds. The most useful signal for quantitative purposes is the four-electron signal due to the reduction of the nitro group to produce the hydroxylamine derivative. The advantages of this signal are both the high quantity of current produced per mol of electroactive compound and the relatively low energy requirements for the nitro reduction. Consequently we can obtain high current / concentration ratios and low reduction potentials. These advantages have permitted the development of a great quantity of electrochemical methods applied to nitro compounds of biological significance [7].

Mercury, carbon paste and glassy carbon (bare and modified) electrodes were generally used in voltammetry of pharmaceuticals from 1996 until 2006 [7-9]. According to the Scopus database (during the period 1996-2006) only about 10 works relating to pharmaceutical nitro compounds on carbon screen printed electrodes (CSPE) were published. Over the last decade, the number of publications increased up to 40.

Screen printing technology is a simple, quick and very cheap method for mass production of disposable carbon containing electrodes for the VA with a very high degree of precision and with a wide range of configurations. Single use of CSPE makes it possible to prevent contamination of an electrode surface by reaction products and eliminate the problem of a sensor sensitivity loss during operation [10]. The use of such electrodes is cost effective, because they can be operated by unqualified personnel on inexpensive equipment.

CSPE's preliminary modified with nanomaterials, graphene, carbon black, mercury films, etc. are used for determination of biologically active nitro compounds [11-16]. Main problems of using such electrodes are the complexity of the modifying procedure, the toxicity of some components, the short shelf life of nanomaterials and organic composites. These problems do not occur with bare CSPE. Such electrodes are kept for a long time without changing of their surface activity, and they are compatible with "green analytical chemistry" approach [17]. We have not found any publications describing a use of bare CSPE in voltammetry of pharmaceutical nitro compounds. In our work, we tried to get the first successful experience of voltammetric determination of pharmaceutical nitro compounds using bare CSPE. The new Russian highly effective antiviral drug Triazavirin was chosen as a target.

The aim of this work is to study the voltammetric behavior of TZ for development and validation of a quick and simple "green" method for quantitation of the active substance in the antiviral drug Triazavirin using bare CSPE.

2. EXPERIMENTAL

2.1. Reagents and materials

Acids, salts and alkali of C.P. grade purchased from Russian manufacturers were used without further purification. 3-Methylthio-5-amino-1,2,4-triazole (an identified impurity) was purchased from Sigma-Aldrich. A standard sample (SS) of the Triazavirin (registration number 9875-2011 in the State Register of the Russian Federation) was dried to a constant weight at a temperature of 95 to 99 °C. TZ stock solution of 10 $gL^{-1}$was kept in the dark at room temperature. Working solutions of TZ were prepared by diluting a stock solution with water. The commercial samples used for TZ determination (capsules Triazavirin from OOO "Plant Medsintez", Ekaterinburg, containing 250 mg of the active substance) were purchased from a local pharmacy. Deionized water obtained on device DVS-M/1HA(18)-N ("Mediana filter", Russia) was used throughout. The pH-measurements were done with ionomer "Expert-pH" ("Econics Expert", Russia).

2.2. Instrumentation

Electrochemical measurements were performed in a standard three-electrode cell using a potentiostat/galvanostat μAutolab Type III complete with magnetic stirrer and 693 VA Processor/694 VA Stand (Metrohm, Switzerland). All electrochemical experiments were carried out in a conventional three electrode cell. Ag/AgCl (3 M KCl) as reference electrode and a glassy carbon rod as auxiliary electrode were used in the measurement. A glassy carbon disc with a diameter of 3 mm pressed into Teflon from Metrohm (type 1), carbon screen-printed electrodes (CSPE) based on Circalok ink from Lord Corporation, USA (type 2) and

Electrodag 407C from Acheson, Netherlands (type 3) were used as working electrodes. CSPE were laboratory-made using a TIC-50B machine for screen printing (China). The carbon containing ink was applied through a mesh stencil onto a fiberglass polymer substrate of 0.035 cm thickness (ZAO "Elektroizolit", Russia) in the form of strips measuring 0.2×3.8 cm and with a layer of about 40 μm thickness. The strips were heat-treated in a drying cabinet in accordance with the regulations of the ink manufacturer and insulated. The working surface area of the working electrodes was about 0.07 cm^2.

Before use, the glassy carbon electrode (GC) surface was polished using polishing set (kit 6.2802.010): aluminum oxide with 0.3 μm particle size and polishing cloth and then rinsed with 0.1 M nitric acid and deionized water. CSPE (type 2, 3) were used without any preparation. The pH was measured on ionomer "Expert-pH" ("Ekonics-Expert", Russia)

2.3. Preparation of the drug form for analysis

About 0.025 g of the capsule Triazavirin content (a precisely weighed amount) were dissolved in deionized water in a 25 mL volumetric flask, the volume of the solution was made up to the mark with deionized water and thoroughly mixed [3]. An aliquot of the sample solution placed in an electrochemical cell was calculated in accordance with the regression equation from the calibration curve of TZ.

3. RESULTS AND DISCUSSION

3.1. Electrochemical behavior of TZ at carbon containing electrodes

The study of electrochemical transformation of TZ (Figure 1, **I**) was carried out by the cyclic voltammetry in 0.1 M nitric acid solution without and with addition of 5mM of its sodium salt. To determine the nature of the signal-forming process under the specified conditions, the electrochemical behavior of a number of substances of the class of 2-R-6-X-4,7-dihydro-1,2,4-triazolo[5,1-c][1,2,4]triazines-7-ones (compounds **II,III**) containing and not containing a nitro group was studied. The results obtained are presented in Fig. 1.

As can be seen from Fig. 1, a reduction peak is observed on the cyclic voltammograms of compounds having the nitro group in their structure. At the same time, this peak is absent on the cyclic voltammograms of compound **III** that does not contain the nitro group. Consequently, the electrochemical activity of TZ is caused by the process of electrochemical reduction of the nitro group bonded to the conjugated aromatic system.

This allowed using the value of the maximum current of TZ reduction as the response in the present work.

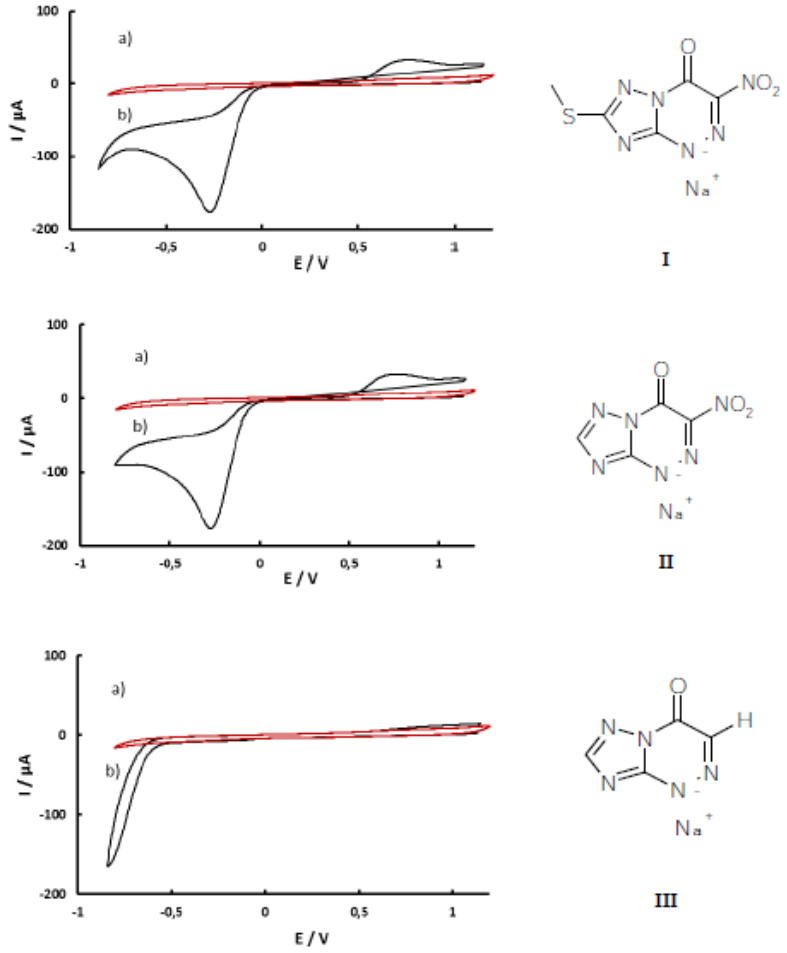

Fig. 1. Cyclic voltammograms at glassy carbon electrode in 0.1 MHNO_3 registered at a scan rate of 0.1 Vs^{-1}without addition (1) and after addition (2) of 5mM compounds **I - III**

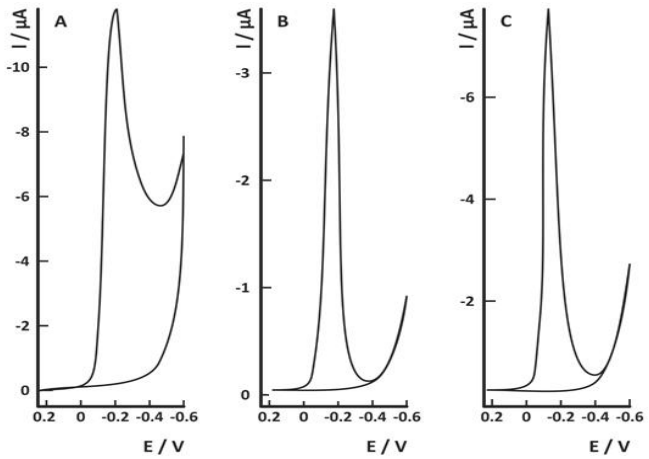

Fig. 2. Cathodic voltammograms at CSPE (type 3) in 0.1 M HNO_3 registered without addition and after addition of 40 mg L^{-1} TZ at different VA modes: linear (a); differential pulse (b); square wave (c). Scan rate=60 mVs^{-1}, pulse step=6 mV, pulse amplitude=20 mV, frequency=50 Hz

3.2. Optimal conditions for Triazavirin determination

3.2.1. Voltammetric mode

Fig. 2 shows voltammograms of TZ reduction recorded on the CSPE (type 3) at a linear (a), differential pulse (b) and square wave (c) potential sweep. Comparison of different voltammetric modes at the same scan rate presented in Fig. 2 allowed concluding that the square wave voltammetry (SqW) is the optimum one. In this case, the peak of TZ is symmetrical and can be measured with high precision in contrast to the linear recording mode with asymmetrical shape. At the same time, the response is twice as high as compared to the differential pulse mode. Therefore, further studies were carried out in the SqW mode.

3.2.2. Working electrode

Comparison of the value, width and the reproducibility of the TZ response for different electrodes (Table 1) showed that its highest value and the best reproducibility are achieved when using the type 3 electrode. This electrode was chosen as the working one.

Table 1. Comparison of TZ responses recorded for different type of electrodes using the SqW mode. Conditions: 0.1 M HNO_3+40 mg L^{-1} TZ; scan rate=60 mVs^{-1}, pulse step=6 mV, pulse amplitude=20 mV and frequency=50 Hz

Response	**Electrode type**		
	1	**2**	**3**
Peak potential, mV	–182±1	–246±3	-150±2
The peak width at half height, mV	67±1	107±2	69±1
Peak current, μA (*n*=18, *P*=0,95)	4.16±0.005	3.91±0.015	7.05±0.007
RSD for peak current,%	0.3	0.8	0.2

A preliminary holding of the electrode type 3 in a stirred solution for possible adsorptive accumulation of TZ on the CSPE surface within 5-120 s in the range of potentials of 0.2-1.0 V, in which TZ does not undergo electrochemical conversions, does not have any significant effect on the TZ response. This allows to exclude the stage of the preliminary accumulation of the analyte and to use the direct VA for its detection.

3.2.3. Effect of pH

The effect of the pH of the supporting electrolyte on TZ reduction was evaluated in the pH range from0 to 12 (Fig. 3). With increasing pH from 0 to 5, the peak potential shifts toward negative potential values and then is stabilized in neutral and alkaline solutions (Fig. 3A). The TZ response is sufficiently stable at pH 0-2. The decrease in the current of TZ reduction is observed in solutions with pH>2 (Fig. 3B). The obtained results indicate a

possible participation of hydrogen ions in the process of electrochemical reduction of the compound. Here, we chose pH 1 (0.1 M solution of HNO_3) for analytical purposes.

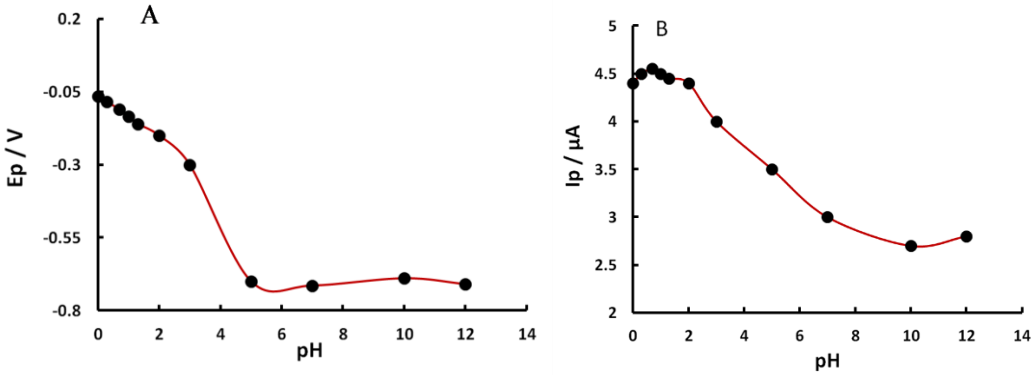

Fig. 3. Plot of peak potential (A) and peak current (B) versus pH for 40 mg L^{-1} TZ, Supporting electrolyte: 1 $MHNO_3$ (pH=0); 0.5 $MHNO_3$ (pH=0.3), 0.2 $MHNO_3$ (pH=0.7); 0.1 $MHNO_3$ (pH=1); 0.05 $MHNO_3$ (pH=1.3); 0.1 M $NaNO_3$+xM HNO_3 (pH=2,3), 0.1 M $NaNO_3$ (pH=5), 0.1 M $NaNO_3$+xMNaOH (pH=7, 10, 12). SqW sweep parameters are as given in Table 1

3.2.4. Optimization of SqW parameters

The response in the SqW mode essentially depends on certain instrument parameters (pulse frequency, pulse step and pulse amplitude). The TZ response at a frequency of 50 Hz and a pulse step of 6 mV linearly increases with an increase in a pulse amplitude in the range of 2-50 mV. The linear dependence of the current of TZ reduction on the pulse frequency at the pulse amplitude of 20 mV and the pulse step of 6 mV is observed in the region of 15-150 Hz. After that the increase in the response stops. At the same time, the increase in the signal in absolute magnitude within the frequency range from 50 to 150 Hz is insignificant (it does not exceed 9%). Whereas, the base line of the residual current increases by an order of magnitude. In this way the ratio useful signal/residual current decreases significantly, which complicates the TZ peak recording and worsens the reproducibility of the measurement results. The frequency of 50 Hz was used in further studies as the most acceptable one.

The scan rate effect on the TZ response at the concentration of 40 mg L^{-1} was studied in the range of 2-240 mV s^{-1} at the frequency of 50 Hz, the pulse amplitude of 50 mV and the pulse step of 6 or 12 mV (Fig. 4A).

The scan rate of 120 mV s^{-1} is optimal for routine measurements. The analysis time increases with scan rate reducing. The ratio useful signal/residual current worsens with a further increase of the scan rate.

The linear dependence of the current value on the square root of the scan rate (Fig. 4B) indicates the diffusion control of TZ reduction process on the surface of the CSPE based on Electrodag 407C ink.

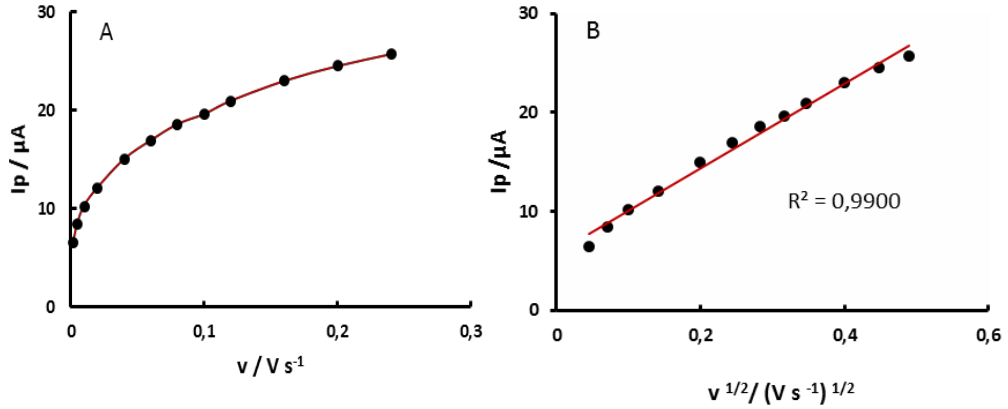

Fig. 4. A) Plot of peak current versus scan rate; B) Plot of peak current versus square root of scan rate for 40 mg L^{-1} TZ in 0.1 M HNO_3; pulse amplitude=50 mV, pulse step=6 and 12 mV, frequency=50 Hz

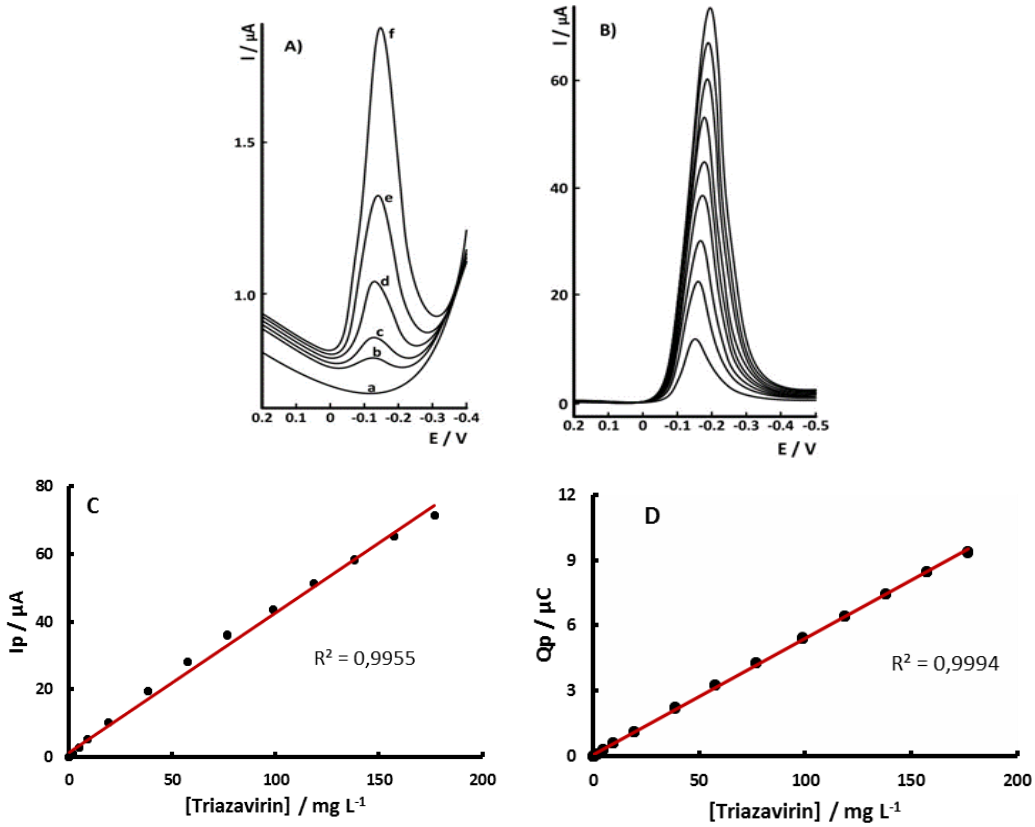

Fig. 5. SqW voltammograms (A) for low concentrations of TZ: (a) blank solution, (b) 0.1, (c) 0.2, (d) 0.5, (e) 1, (f) 2 mg L^{-1}; (B) for 20 – 180 mg $L^{-1}$of TZ with a step of 20 mg L^{-1} and plots of height (C)/area (D) of TZ reduction peak versus its concentration (obtained from 3 independent measurements for each one). SqW sweep parameters: scan rate=120 mV s^{-1}, pulse step=12 mV, pulse amplitude=50 mV, frequency=50 Hz

3.2.5. Calibration curve

SqW voltammograms for different TZ concentrations in 0.1 M HNO_3 solution registered under optimal experimental conditions are presented on Fig. 5 A, B. Well-defined response for 0.1 mg L^{-1} TZ can be seen at Fig. 5A.

The peak current and the peak area are directly proportional to the TZ concentration in the range of 0.1 to 80 mg L^{-1} (Fig. 5C) and 0.1-180 mgL^{-1} (Fig. 5D), respectively. It is more preferable to use the peak area as the response for TZ determination with a standard addition method. The detection limit calculated from the calibration curve [18] with regression equation Q (μC)=(0.0677±0.0003) C-(0.0106±0.0008) is 0.04 mg L^{-1}. The quantitation limit (0.12 mg L^{-1}) is almost 2 times lower as compared to the HPLC method with spectrophotometric detection [3].

3.3. Validation of voltammetric method for TZ determination

The validation of the method was carried out taking into account the recommendations [18].

3.3.1. Specificity

In order to test the specificity of the method, we compared the results of the voltammetric determination of TZ obtained by an analysis of standard TZ solutions: **A** (pure) and **B** (a placebo) is containing 100% of the expected level of concentrations of the substance to be determined (50 mg L^{-1}). 0.1 M $HNO_3$was used as the solution **A**.

Table 2. Evaluation of the method specificity

Specificity	***(n = 6, P = 0.95)***	
Average value of recovery (R), %	**Solution A**	**Solution B**
	100.1	99.8
SD of R	0.56	0.77
RSD, %	0.6	0.8

The solution **B** contained an auxiliary agent (calcium stearate) and the identified impurity in concentrations corresponding to the formulation of the dosage form (0.8% and 1%, respectively). Metrological characteristic of the results obtained are given in Table 2. Comparison of the results obtained for both solutions using *F*- and *t*-test showed that calculated *F* (*1.89*) and *t* (*0.67*) values do not exceed tabulated (theoretical) values of *5.05* and *2.23*, respectively. This confirms the specificity of the developed method.

3.3.2. Accuracy

Accuracy was evaluated on solutions of the standard TZ sample containing 50 (Level 1), 100 (Level 2) и 150% (Level 3) of an expected level of concentrations of the substance to be determined by the analysis of spiked samples. An accuracy index was calculated for each level of TZ concentrations (25, 50 и 75 mg L^{-1}). As a result, 3 concentrations of solutions were tested in a 3-fold determination for each concentration. The accuracy index (R,%) for different concentration levels is close to 100%: 100.4, 98.8, 99.2 (Level 1); 100.4,99.0, 100.6 (Level 2); 100.4, 99.7, 100.3 (Level 3). The evaluation was carried out by calculating the accuracy index and a confidence interval of its mean value, which is 99.9±0.5, a standard deviation SD=0.70, a relative standard deviation RSD, %=0.7 *(n=9, P=0.95)*. The RSD is less than 1.0%. The confidence interval of an average analysis result includes 100%.

3.3.3. Precision

Repeatability of the method was evaluated using the results of 6 determinations for the sample with the analyte content close to the nominal value. Each test aqueous solution of the contents of Triazavirin capsules was prepared from the precisely weighed amount.

Table 3. Evaluation of the method precision

Precision	*(n=6, P=0.95)*	Recommended value [18]
Repeatability		
Found, g L^{-1}	1,00±0,01	
SD	0,011	
RSD,%	1,0	≤1
Intermediate Precision		
Found, g L^{-1}	1,01±0,02	
SD	0,018	
RSD,%	1,8	≤2

The intermediate precision of the method was evaluated under the working conditions of one laboratory in different days by different performers. The result of a single determination was obtained by a standard addition method. The results are given in Table 3.

The developed method for TZ quantitative determination meets the precision criteria for the electroanalysis [18]. The method is accurate and selective. It may be used for the analysis of dosage forms of Triasavirin for determination of main substance content.

3.4. Determination of Triazavirin in pharmaceutical formulation

The developed method was successfully used for the analysis of Triazavirin capsules with a minimal sample preparation (dissolution of the precisely weighed amount in water). The

analysis was carried out according to the procedure described in the experimental part. The results of the dosage form analysis obtained by the voltammetric and chromatographic methods are given in Table 4. The found values are close to the claimed content. Comparison of the results obtained by both methods using F- and t-test showed that the calculated F (*1.10*) and t (*0.26*) values do not exceed the tabulated (theoretical) values of *5.05* and *2.23*, respectively. This confirms the absence of any significant differences between the results of both methods. There is no significant difference in the precision between these methods. The RSD does not exceed 1%. However, the VA analysis is more sensitive, simple and quick. It does not require the use of an expensive equipment, inert gases and toxic organic solvents.

Table 4. Determination of TZ in capsules

(*n=6, P=0.95*)

Labeled amount (mg per capsule)	VA	HPLC
	250	250
Found (mg per capsule)	249±2	251±2
RSD, %	0.9	0.9

4. CONCLUSION

In this work, for the first time we have used the bare carbon screen-printed electrode based on carbon ink Electrodag 407C for voltammetric determination of pharmaceutical nitro compound. The new Russian highly effective antiviral drug Triazavirin was chosen as a target. The square wave cathodic voltammetry without the preliminary accumulation was chosen as the optimum mode for Triazavirin response. An electrochemical regeneration of the electrode surface during subsequent recording voltammograms of TZ is not required due to the high reproducibility of the response (the RSD value does not exceed 0.2%). In terms of a limit of quantification (0.12 mg L^{-1}), the voltammetric method is not inferior to a known method for determination of Triazavirin using HPLC. The proposed method was validated and used for determination of Triazavirin in real samples.

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