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Flow Injection Determination of Alloxan

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Abstract
The present communication reports two simple flow injection (FI) methods for the determination of trace alloxan in biological matrices. The FI method for the amperometric determination of alloxan combines its chemical derivatization into dialuric acid by an amino thiol (L-cysteine, glutathione) and detection of dialuric acid at a glassy carbon electrode. The FI spectrophotometric method is based on the ability of alloxan to act as a ROS generator in the presence of unithiol followed by sequential detection of free radicals by means of formation of colored formazan from tetrazolium derivatives. The both proposed methods permit the automatic determination of ALX with good reproducibility and frequency (50 h^{-1}). Validation was performed by recovery tests on synthetic samples.

Keywords Flow injection, spectrophotometry, physiologically active substances, alloxan, dialuric acid, free radicals

1. Introduction

During the last years there is the growing interest to problems of developing methods for the determination of physiologically active substances in biological liquids, food products and pharmaceutical preparations. According to the literature and our own experience, the methodology of the flow injection (FI) analysis or its newest versions is one of the most promising tools for these purposes [1,2].

Alloxan (2,4,5,6-[1H,3H]-pyrimidinetrone) is selectively toxic substance that induces as a potent diabetogenic agent, selectively destroying the insulin-producing β-cells of pancreas [3,4]. The cytotoxic action of alloxan (ALX) is mediated by the formation of reactive oxygen species (ROS) during the interaction between ALX and sufficient suitable reducing biocompounds [5].

At present, however, little information is available on the content of ALX in a human organism. According to the data received by the american researchers [6], the level of ALX in blood plasma of the healthy man or dog is within 0.1 - 0.2 mg %, and this level remarkably increased for diabetics. Main reason for the difficulties for in vivo experiments with ALX lies in its chemically instability under physiological conditions.

The presence of O=C-C=O group in a molecule of ALX gives the basis to consider that this compound can have electroactive properties. Thus, for example P.I. Veksler has shown that ALX can be determined polarographically in whole blood up to 0.3 mM [7]. Afterwards, the voltammetric method for the determination of ALX was developed by measuring a current of its reduction ($E_{1/2}$~ 0.0 V) at a graphite electrode in acidic solutions (1.0 M HCl) [8].

The HPLC method has been proposed for UV-detection of ALX as a product of electrochemical oxidation of oxypurinol in a weakly acidic media [9]. One other chromatographic method for the determination of ALX (0.6 - 95 mM) was based on the hydroxylation of barbituric acid [10].

A few spectrophotometric methods for ALX are based on using its decomposition reaction in the presence of cyanide ions [11-13]. Among them, the more convenient method has been developed by using photometric reaction with phosphotungstic acid [14]. In this case, limit of detection of ALX was found to be 0.05 mg %. The photometric method for the determination of ALX (0.05 - 1.0 mg, 4 mL) in blood has been proposed by using its ability to reduce hexacyanoferrate (III)-ion at room temperature (pH 6.5–8.0) [15]. Most of the described methods are rather insensitive and subject to interference from coexisting compounds or have a long sampling frequency. Therefore, more suitable methods for determination of ALX are desired.

This study aimed to develop FI methods for the determination of ALX by using the ability of this compound to act as a generator of ROS in the presence of electron donors.

2. Experimental

2.1. Chemicals and solutions

Alloxan monohydrate, dialuric acid, reduced glutathione, L-cysteine and unithiol were purchased from Sigma Chemical Company. A stock solution of alloxan (10 mM or 1.6 mg/mL) was in N_2 sparged 1.0 mM HCl and kept on ice.

All chemicals were of analytical reagent grade and were used without further purification. Deionized distilled water was used throughout the whole experiment.

2.2. Apparatus and procedures

All FI experiments were conducted using a flow injection analyzer FIAStar-5010 (Tecator, Hoganas, Sweden) equipped by Personal computer. A 5023 single-beam spectrophotometer with 5032 controller was used for spectrophotometric measurements. Cyclic voltammetric (CV) and chronoamperometric experiments were made with the aid of an electrochemical analyzer "Ecotest-VA" (Econix-Expert, Moscow, Russia). A three-electrode cell consisted of a glassy carbon (GC) disk electrode (5 mm in diameter) as a working electrode, an Ag/AgCl reference electrode and a platinum auxiliary electrode.

Spectral changes during the decomposition of ALX and its interaction with amino thiols were monitored on an UV/VIS spectrophotometer TU-1800 (DPG Inst. Co. Ltd., Beijing,

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Chine) with 10 mm quartz cells.

A pH-meter OP-110 (Radelkis, Budapest, Hungary) was employed for the pH measurements.

Schematic illustrations of the proposed FI systems are shown in Fig.1.

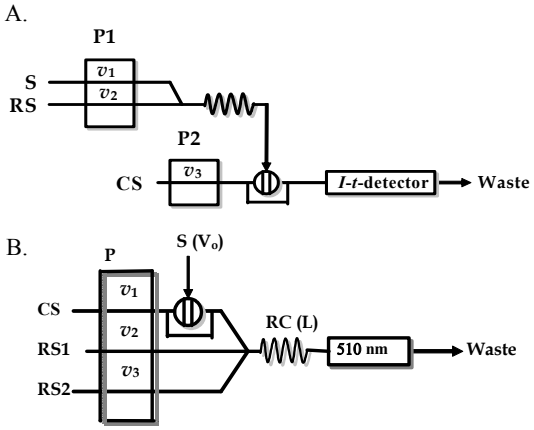


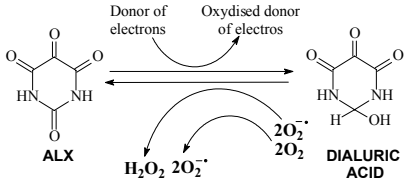
Fig.1. FI manifolds for the determination of ALX with amperometric (A) and spectrophotometric (B) detection.

3. Results and Discussion

3.1. Preliminary study

3.1.1. Behavior of alloxan in the presence of amino thiols

Results from many experiments indicate that ALX and its reduced derivative, dialuric acid (DUA), act as a redox couple, driven by amino thiols such as reduced glutathione or L-cysteine, generating in vitro both superoxide radicals and hydrogen peroxide [16-18]:



(1)

The activity of this redox couple is limited only by the availability of ALX, reducing agent and suitable electron acceptors (e.g. oxygen).

In contrast, in the absence of suitable electron donors, the pH shift of a solution containing ALX from pH 3.0 to pH 7.2 leads to a rapid disappearance of the alloxan absorption at 245 nm (Fig.2).

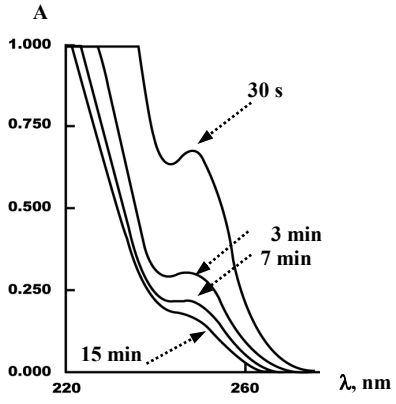


Fig.2. Spectral changes during spontaneous decomposition of 0.1 mM ALX (pH 7.2).

On the other hand, addition of ALX to a neutral solution of an amino thiol (RSH) at a molar ratio of 1: 10 (and more) led to an immediate increase in absorption at 275 nm, characteristic of DUA (Fig.3).

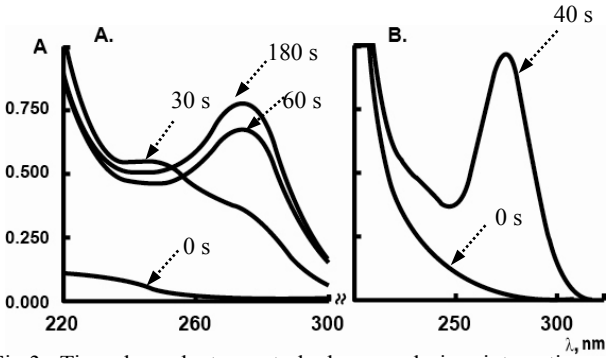


Fig.3. Time-dependent spectral changes during interaction of ALX with L-cysteine (A) and unithiol (B) at 25° C. Reaction mixtures contain 0.1 mM of ALX and 1.0 mM of RSH in a Tris-HCl buffer solution (20 mM, pH 7.2).

According to the literature [15, 18, 19], the letter compound is formed as a result of the following reactions:

ALX + RSH ⟶ ALX* + RS*

ALX* + RSH ⟶ DUA + RS*,

where ALX* - alloxan-radical.

Our findings showed that the rate of the reduction of ALX was faster in the presence of excess of unithiol than in case of its reduction by glutathione or L-cysteine under the same conditions. At 40 s after reaction initiation, more than 95% of ALX reduced to the DUA.

3.1.2. Electrochemical activity of alloxan and dialuric acid

Both ALX and DUA are electroactive and a variety of experiments have been carried out for investigation of their electrochemical properties. Earlier polarographic investigations of the alloxan – alloxantin - dialuric acid were shown that ALX exhibits two well-defined cathodic waves at a dropping mercury electrode [20]. The first wave was attributed to the kinetically controlled 2e-reduction to DUA. The second alloxan reduction wave was only observed in the narrow pH range.

We have investigated the voltammetric behavior of ALX and DUA at a GC electrode in the wide pH range. As it can be seen from the Fig.4A, two peaks appeared in CV response curve of ALX in neutral aqueous solutions, but only one anodic peak appeared in acidic solutions.

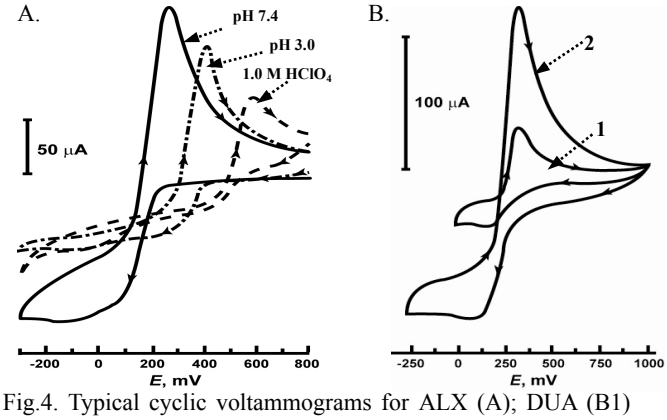


Fig.4. Typical cyclic voltammograms for ALX (A); DUA (B1) and the reaction mixture of ALX and L-cysteine at pH = 7.4 (B2) at a GC electrode. Concentration of ALX and DUA – 5.0 mM, scan rate - 100 mV/s.

The wide cathodic peak at – 0.083 V (v = 25 mV/s) is shifted in a negative direction with increasing scan rate (v) and it is due to the reduction of ALX. The sharp anodic peak at + 0.258 V (v = 25 mV/s) is slightly shifted in a positive direction with increasing v from 2 to 100 mV/s and it is due to oxidation of DUA. The plots of peak currents I_{pc} or I_{pa} vs. $v^{½}$ give a light downward curvature. This behavior is typical for diffusion-kinetic control for the overall electrode process.

It was found that the anodic peak current recorded for ALX in the presence RSH was considerably more than that for electrooxidation of DUA to ALX (Fig.4B). This fact leads to the conclusion that this peak can be due to "catalytic" effect caused by the chemical reaction coupled with an electron transfer.

Based on these chemical considerations and experiments, FI procedures for rapid and simple procedures for determination of ALX in a physiological medium were developed.

3.2. Flow injection determination of alloxan

3.2.1. FI electrochemical procedure

According to the electrochemical results described previously, we GC electrode can be used for indirect amperometric detection of ALX. In this case, the analysis is based on the chemical derivatization of ALX into dialuric acid by glutathione (GSH) followed by amperometric detection of dialuric acid at a GC electrode (Fig.5).

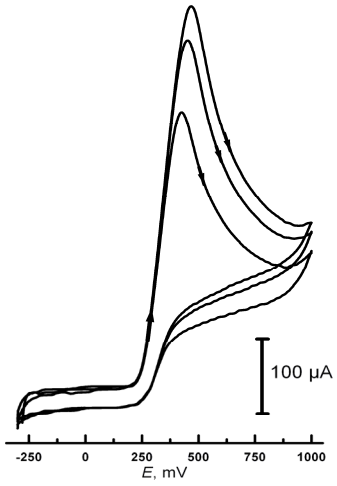


Fig.5. Cyclic voltammograms of a GC electrode (100 mV/s) in the buffered solution of 10.0 mM and 50 mM GSH (pH 7.14).

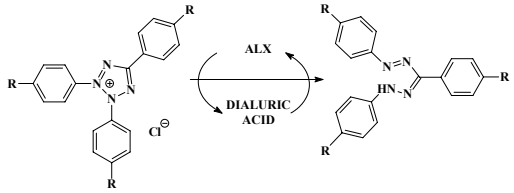
The FI manifold used for this purpose is shown in Fig.1A. In the first step, a sample solution stream (S) was mixed with a stream of GSH solution (RS) and this mixture filled the sample loop of a six-way injection valve. In the second step, after switching the position of the valve, an aliquot (400 μL) of the chemical mixture was injected into the carrier stream (CS). Further, the injected zone of an analyzed solution went to the amperometric detector cell (applied potential: + 0.35 V).

The recorded peak height is influenced by flow rate of the streams. Under the chosen flowing conditions (v_1 = 1.2 mL/min, v_2 = 0.6 mL/min, v_3 = 0.8 mL/min), a calibration graph with a good linear relationship (r = 0.9987, n = 6, P = 0.95) was obtained up to 0.2 mM. The 3σ limit of detection (LOD) was 50 μM.

3.2.2. Spectrophotometric procedure

The second rapid procedure for the determination of ALX was carried out by using a tree-channels manifold with spectrophotometric detection (Fig.1B).

Due to the fact that the rate of the ALX-DUA reactions (1) was dependent on amino thiol, unithiol (UNSH) was applied to the generation of ROS. The sequential spectrophotometric detection of these radicals was performed by means of formation of blue-colored 1,3,5-triphenylformazan from 2,3,5-tri-phenyltetra -zolium ion (TPhTZ) at λ_{max} = 510 nm:



Experiments were conducted to establish the optimum conditions of analysis. As shown in Fig.6, maximum signal was observed at the carrier flow-rate of 0.6 - 0.8 mL/min.

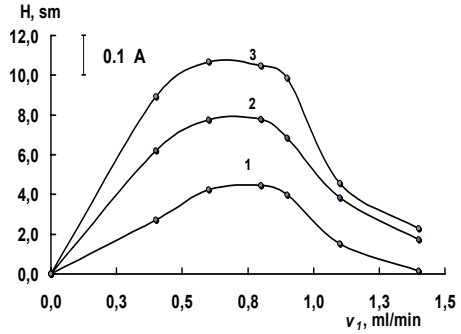


Fig.6. Effect of the carrier flow rate on the peak height H for the following concentration of ALX (mM): 1- 0.10, 2- 0.25, 3- 0.50.

The maximum and constant signal height was recorded when sample solution was injected over range 200 - 400 μL (Fig.7). A length of reaction coil of 240 - 300 sm (0.7 mm ID) was found to be sufficient in order to attain a maximum signal peak height (Fig.7).

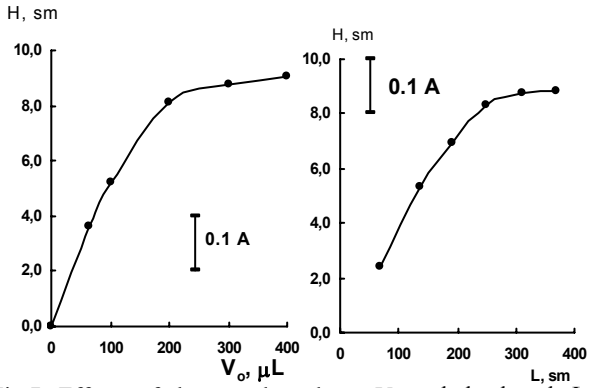


Fig.7. Effects of the sample volume V_o and the length L of reaction coil on the peak height of ALX.

Under the fixed FI variables (V_o = 200 мкл, v_1 = 0.6 mL/min, v_2 = 0.6 mL/min; v_3 = 1.0 mL/min; L = 240 sm) и chemical conditions within the reaction zone (C_{TPhTZ} = 2.5 мМ; C_{UNSH} = 3.8 мМ), the calibration graph was characterized by two linear plots in the concentration range of 0.1 – 2.5 mM ALX, and the 3σ LOD was 36 μM ALX (Tabl.1).

Table 1. Analytical characteristics of FI method for the indirect spectrophotometric determination of ALX

Linear concentration . range, мМ	Regression equation (r)	LOD, μM
0.1 – 1.0	H = ΔA = 0.835 *C* (0.9997)	36
1.0 – 2.5	H = ΔA = 0.464 C + 0.372 (0.9995)	

The accuracy of the proposed method was tested by analysis of model solutions of human serum (Tabl.2).

Table 2. The recovery of ALX added to the model solutions of human serum* (n = 4, P = 0.95).

Added (mM)	Found (mM)	R.S.D. (%)	Recovery (%)
0.15	0.16 ± 0.01	6	107
0.25	0.26 ± 0.01	4	104
0.75	0.76 ± 0.02	3	101
1.25	1.24 ± 0.03	2	99
1.60	1.62 ± 0.03	2	101
2.25	2.24 ± 0.03	1	100

***** Content: human albumin (5 g/L), glucose (2.5 mM), NaCl (30 mM), KCl (1 mM), ascorbic acid (0.01 M).

5. Conclusion

Novel flow injection methods were developed for investigation and fast quantitative determination of a redox-active alloxan – a biocompound playing a potential role in human metabolism. From the above results it could be concluded that the proposed FI methods are expected to be useful in the routine determination of micro amounts of ALX in biological fluids with good reproducibility and frequency (50 h^{-1}).

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