A New Approach how to Define the Coefficient of Electroactivity of Adenine and Its Twelve Derivatives Using Flow Injection Analysis with Amperometric Detection

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Abstract
We studied the electrochemical behaviour of adenine derivates (adenosine, 2-aminopurine, 2,6-diaminopurine, 6-benzyl-aminopurine, adenosine monophosphate, cyclic adenosine monophosphate, nicotinamide adenine dinucleotide, adenosine triphosphate, *S*-adenosyl-L-methionine, and synthetic derivatives AD-3, AD-6 and AD-9) using flow injection analysis with electrochemical detection using a glassy carbon electrode. The influences of pH, flow rate and potential on the signal height of the studied derivates were tested. The optimal pH was 3, the flow rate of the mobile phase 0.75 mL min^{-1} and the potential 1100 mV. Further, we attempted to characterize each of the studied derivatives by mathematical equations and classic analytical parameters. The lowest detection limit was estimated for adenine as 0.9 nM and 2-aminopurine as 0.5 nM.

Keywords: Adenine derivatives, Flow injection analysis, Amperometric detection, Glassy carbon electrode, Electroactivity

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1 Introduction

Since its discovery, deoxyribonucleic acid (DNA), has been attracting attention of numerous researches in the world [1,2]. Its function, structure, interactions with other biologically important molecules even its isolation and determination in a real sample are still of great interest [3]. Electrochemical interest is also paid to particularly built purine-based and pyrimidine-based blocks of this molecule called nucleic acid bases as adenine (A), cytosine (C), guanine (G) and thymine (T) [4]. In many cases of genetic disorders or metabolic diseases we can observe different levels of nucleic acid bases in body liquids [5]. Besides level, detection of derivatives of nucleic acid bases is also of clinical importance because they might be markers of some diseases like methylation of DNA [6,7]. Additionally, some derivatives of nucleic acid bases such as azidothymidine can be used as therapeutic agents in treatment of viral diseases including human immunodeficiency virus [8–10]. Small amounts of free DNA circulate in both healthy and diseased human plasma/serum, and increased concentrations of DNA are present in the plasma of cancer patients [11,12]. Thus, this can be used as a marker of a tumour disease [13]. High performance liquid chromatography (HPLC) [14–16] or capillary electrophoresis (CE) [17,18] belong to the most commonly used techniques for derivatives determination.

Professor Palecek as a pioneer in electrochemistry of nucleic acid discovered that nucleic acids gave two types of signal: i) redox signals of adenine and cytosine, and ii) oxidative signals of guanine [19]. Since than, many milestones in this promising area including development of elimination voltammetry used for the resolution of the reduction signal of adenine and cytosine [20–25] and/or detection of all nucleic acid bases at carbon electrodes [26–28] have been reached. This great development tends to the suggestion of numerous methods and protocols

mainly focused on DNA sequence-specific sensors and biosensors [4, 29–37].

In this study, we investigate the basic electrochemical behaviour of adenine and the large family of its derivatives (adenosine, 2-aminopurine, 2,6-diaminopurine, 6-benzyl-aminopurine, adenosine monophosphate, cyclic adenosine monophosphate, nicotinamide adenine dinucleotide, adenosine triphosphate and *S*-adenosyl-L-methionine, and three synthetic adenine derivatives called AD3 (1-[4-(6-amino-purine-9-yl)-butyryl]-pyrrolidine-2-one), AD6 (1-[4-(6-amino-purine-9-yl)-butyryl]-piperidine-2-one) and AD9 (1-[4-(6-amino-purine-9-yl)-butyryl]-azepan-2-one) using flow injection analysis with amperometric detection (Coulochem III detector). The whole system is also connected to UV-vis-detector as a reference technique. The results obtained are treated with advanced mathematical approaches to improve characterization and description of behaviour of adenine and its derivatives.

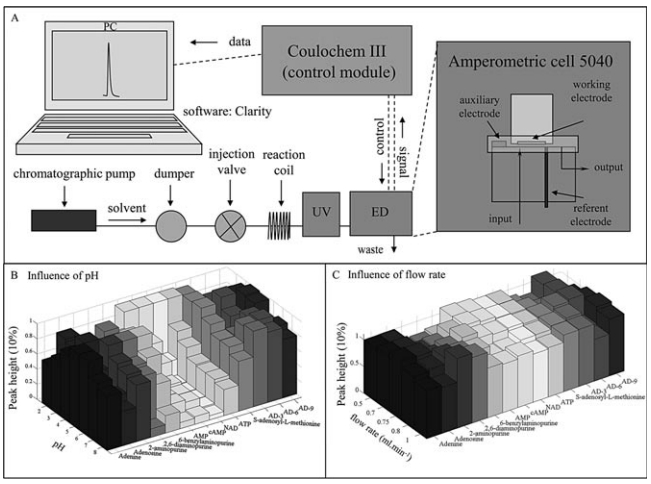

Fig. 1. (A) Scheme of flow injection analysis coupled with UV-vis and amperometric detector. (B) Influence of pH of the mobile phase (Britton–Robinson buffer, pH from 2 to 8) on the height of adenine and its derivatives. (C) Influence of the flow rate of the mobile phase on the peak height of adenine and its derivatives in a concentration of 100 μg/mL. Experimental conditions: guard cell potential: 0 V, working electrode potential: 1000 mV, mobile phase: Britton–Robinson buffer pH 3 and its flow rate 0.5 mL min^{-1}.

2 Experimental

2.1 Flow Injection Analysis Coupled with Coulochem III Detector

The instrument for flow injection analysis with electrochemical detection (FIA-ED) consisted of a solvent delivery pump operating in the range from 0.001 to 9.999 mL min^{-1} (Model 582 ESA Inc., Chelmsford, MA, USA), a guard cell (Model 5020 ESA, USA), a reaction coil (peak capillary 1 m), UV-vis detector (Model 528, ESA, USA) and the electrochemical detector Coulochem III. The closed electrochemical detector includes one low volume flow-through analytical cell (Model 5040, ESA, USA), which consists of a glassy carbon working electrode, a hydrogen-palladium electrode as reference electrode and auxiliary electrode, and Coulochem III as a control potenciostat module. Britton–Robinson buffer from acetic acid (0.04 M), phosphoric acid (0.04 M) and boric acid (0.04 M) adjusted addition of NaOH (0.2 M) was used at different pH (pH 2–8) as mobile phase. Stock solutions of all standards in amount of 1 mL were placed in a cooled sample rack at 8 °C and the sample (20 μL) was injected by autosampler (Model 542, ESA, USA). Analysis of each injection took 1 minute. The data obtained were treated by CSW 32 software (Version 1.2.4, Data Apex, Czech Republic). The experiments were carried out at room temperature. Guard cell potential was set as 0 V. UV detector was set to 260 nm. A glassy carbon electrode was polished mechanically by 0.1 μm alumina (ESA Inc., USA) and sonicated at room temperature for 5 min using a Sonorex Digital 10 P Sonicator (Bandelin, Berlin, Germany) at 40 W [38–40]. A scheme of the instrument is shown in Figure 1A.

2.2 Chemicals and pH Measurements

Synthetic adenine derivatives were prepared and donated by the Department of Natural Drugs, University of Veterinary and Pharmaceutical Sciences, Czech Republic. All other chemicals used in this study were purchased in ACS purity (chemicals meet the specifications of the American Chemical Society) from (Sigma-Aldrich, USA) unless noted otherwise. The stock standard solutions of adenines (1 mM) were prepared with ACS water and stored in the dark at −20 °C. Working standard solutions were prepared daily by dilution of the stock solutions with ACS water. The pH value was measured using inoLab Level 3 with terminal Level 3 (Wissenschaftlich-Technische Werkstätten – WTW, Weilheim, Germany), controlled by the personal computer program (MultiLab Pilot; WTW). The pH-electrode (SenTix-H, pH 0–14/3 M KCl) was calibrated by a set of buffers (WTW). Deionized water underwent demineralization by reverse osmosis using the instruments Aqua Osmotic 02 (Aqua Osmotic, Tisnov, Czech Republic) and then it was subsequently purified using Millipore RG (Millipore Corp., USA, 18 MΩ) – MiliQ water.

2.3 Mathematical Treatment of Data and Estimation of Detection Limits

Mathematical analysis of the data and their graphical interpretation was realized by software Matlab (Version 7.11.). Results are expressed as mean ± standard deviation (*SD*) unless noted otherwise (EXCEL). The detection limits (3 signal/noise, *S*/*N*) were calculated according to Long and Winefordner [41], whereas *N* was expressed as

standard deviation of noise determined in the signal domain unless stated otherwise.

3 Results and Discussion

The electrochemical behaviour of adenine and its derivatives (adenosine; 2-aminopurine, 2,6-diaminopurine; 6-benzyl-aminopurine; adenosine monophosphate (AMP); cyclic adenosine monophosphate (cAMP); nicotinamide adenine dinucleotide (NAD); adenosine triphosphate (ATP) and *S*-adenosyl-L-methionine) including three structurally very similar synthetic derivatives AD3 (1-[4-(6-amino-purine-9-yl)-butyryl]-pyrrolidine-2-one), AD6 (1-[4-(6-amino-purine-9-yl)-butyryl]-piperidine-2-one) and AD9 (1-[4-(6-amino-purine-9-yl)-butyryl]-azepan-2-one), which can be considered as potential viral drugs, was studied using flow injection analysis with UV-vis and electrochemical detection (FIA-UV-ED, Figure 1A). To investigate basic electrochemical behaviour, it is necessary to carry out measurements under optimal conditions. Therefore, two fundamental experimental conditions for flow injection analysis as influence of pH of mobile phase (Britton–Robinson buffer) and its flow rate were optimized. Primarily, the influence of pH of Britton–Robinson buffer within the scale from 2 to 8 was investigated (Figure 1B). Based on the preliminary results obtained, we selected 1000 mV as suitable for sensitive detection of all derivates. cAMP, NAD, ATP and *S*-adenosyl-L-methionine exhibited the highest signal under the most acidic pH (from 2 to 4). This phenomenon can be associated with the fact that all mentioned substances contain several moieties with pK_a lower than 3 [42]. These moieties are not charged under this condition and, therefore, they can be easily oxidized using amperometric detection. Moreover, their signal decreased with increasing pH of the mobile phase for more than 90% in the case of cAMP and NAD at pH 8, for more than 70% in the case of ATP at pH 8 and for more 30% in the case of *S*-adenosyl-L-methionine at pH 8. The second group of similarly behaving substances included adenine, adenosine, 2-aminopurine, 2,6-diaminopurine and 6-benzyl-aminopurine. These compounds have their signal maxima within the pH range from 4 to 5. This can correspond with higher pK_a of their moieties compared to the previous group. Similar to previous adenine derivatives, their signal decreased with increasing pH of the mobile phase up to 55%, 35%, 64%, 11% and 22% of their highest height for adenine, adenosine, 2-aminopurine, 2,6-diaminopurine and 6-benzyl-aminopurine, respectively. The behaviour of synthetic derivatives of adenine differed from the other group markedly. Their electrochemical activity varied within the studied pH interval (Figure 1B). To determine the most suitable mobile phase pH, for simultaneous determination of all target molecules, we have standardized the influence of pH on the signal height. Standard curves are calculated as ratio of maximum to mean square deviation for each course separately. To obtain the optimum pH for all substances, we then calculated the sample variance of all waveforms and selected those values that differed least from it. The most suitable pH for detection of all derivatives was pH 3 covering 72% of the maximum values (relative standard deviation of the measurement was 3.7%; $n=5$).

We also tested the influence of the flow rate on adenine derivates signal height. In these electrochemical analyses the optimum pH of the mobile phase was 5 and flow rates were studied within the range from 0.5 to 1.0 mL min^{-1} (Figure 1C). The signal of AMP, cAMP, NAD and ATP decreased with increasing flow rate, however, the decrease of the signal was not so sharp compared to pH influence. The electrochemical response of adenine and adenosine behave similarly (differences between signal heights were lower than 5%). In contrary, 2-aminopurine, 2,6-diaminopurine and *S*-adenosyl-L-methionine exhibited enhanced signals (app. 8% per 0.1 mL min^{-1}) with increased flow rate. The rest of the molecules of interest had their maxima within the range of the tested interval. To find the optimal value data were treated with the abovementioned procedure. The optimized flow rate was found as 0.75 mL min^{-1} covering 95% of the maximum values (about relative standard deviation of measurement 2.6%; $n=5$).

3.1 Electrochemical Behaviour of Adenine and Its Derivatives

Under optimized experimental conditions (Britton-Robinson buffer (pH 5) as mobile phase and its flow rate: 0.75 mL min^{-1}), adenine and its derivatives were measured at 260 nm using an UV detector to confirm the stability of a stock standard solution of the target molecule (Figures 2, 3 and 4). Then, we continued with studying the electrochemical behaviour of adenine and its derivatives in a flow system, what has not been done yet. The influence of the potential applied on the glassy carbon working electrode within the interval from 200 to 1300 mV in 100 mV steps on the height of adenine and its derivatives oxidation signals was studied. All derivatives were measured under each potential three times for calculation of relative standard deviation, which was not higher than 4.5%. Besides the standard hydrodynamic voltammogram the cumulative hydrodynamic voltammogram was also determined and is shown in Figures 2, 3 and 4 (●). This type of voltammogram is defined as the sum of signal heights measured at the given potential and at all previous lower potentials. We also attempted to compare and show these two different approaches for determining HDV. It clearly follows from the results obtained that classical HDV is more accurate to find change in a small potential range but the cumulative HDV is more valuable for the overall value. The detailed description of electrochemical behaviour of single derivatives follows.

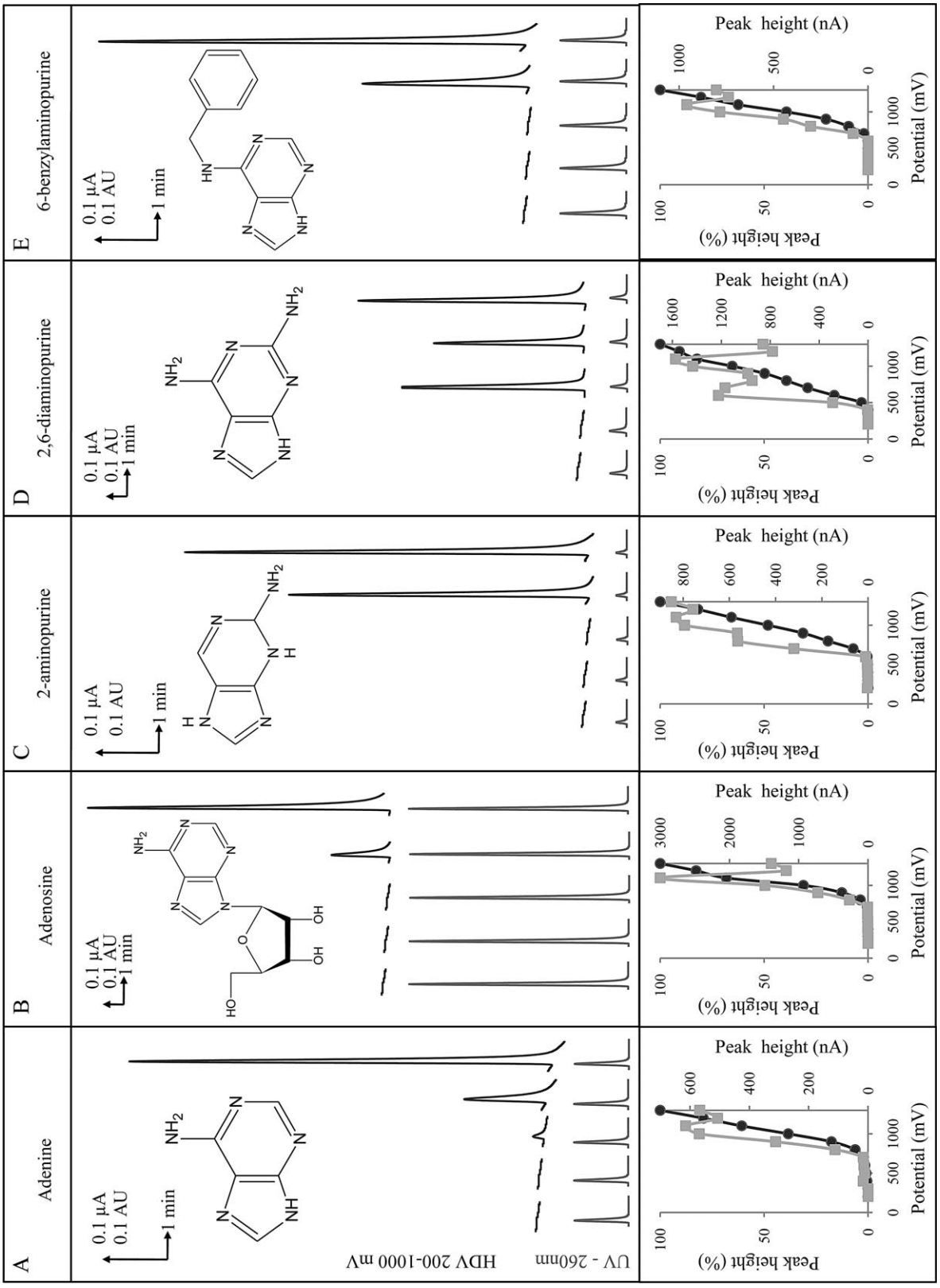

Fig. 2. Structures, FIA-ED and FIA-UV signals, and standard and cumulative hydrodynamic voltammograms of (A) adenine, (B) adenosine, (C) 2-aminopurine, (D) 2,6-diaminopurine and (E) 6-benzyl-aminopurine. Bottom figures belong to the hydrodynamic voltammograms (HDV). Left vertical axes express peak height in % because of better comparison between all studied derivates (peak height of 100 % corresponds to 615 nA for adenine, 3012 nA for adenosine, 852 nA for 2-aminopurine, 1577 nA for 2,6-diaminopurine and 959 nA for 6-benzyl-aminopurine). Right vertical axes express the cumulative HDV. The lower horizontal axes represent the potential which has been applied to all studied derivates. Experimental conditions: guard cell potential: 0 V, working electrode potential: 200–1300 mV, mobile phase: Britton–Robinson buffer pH 3 and its flow rate 0.75 mLmin^{-1}, wavelength: 260 nm.

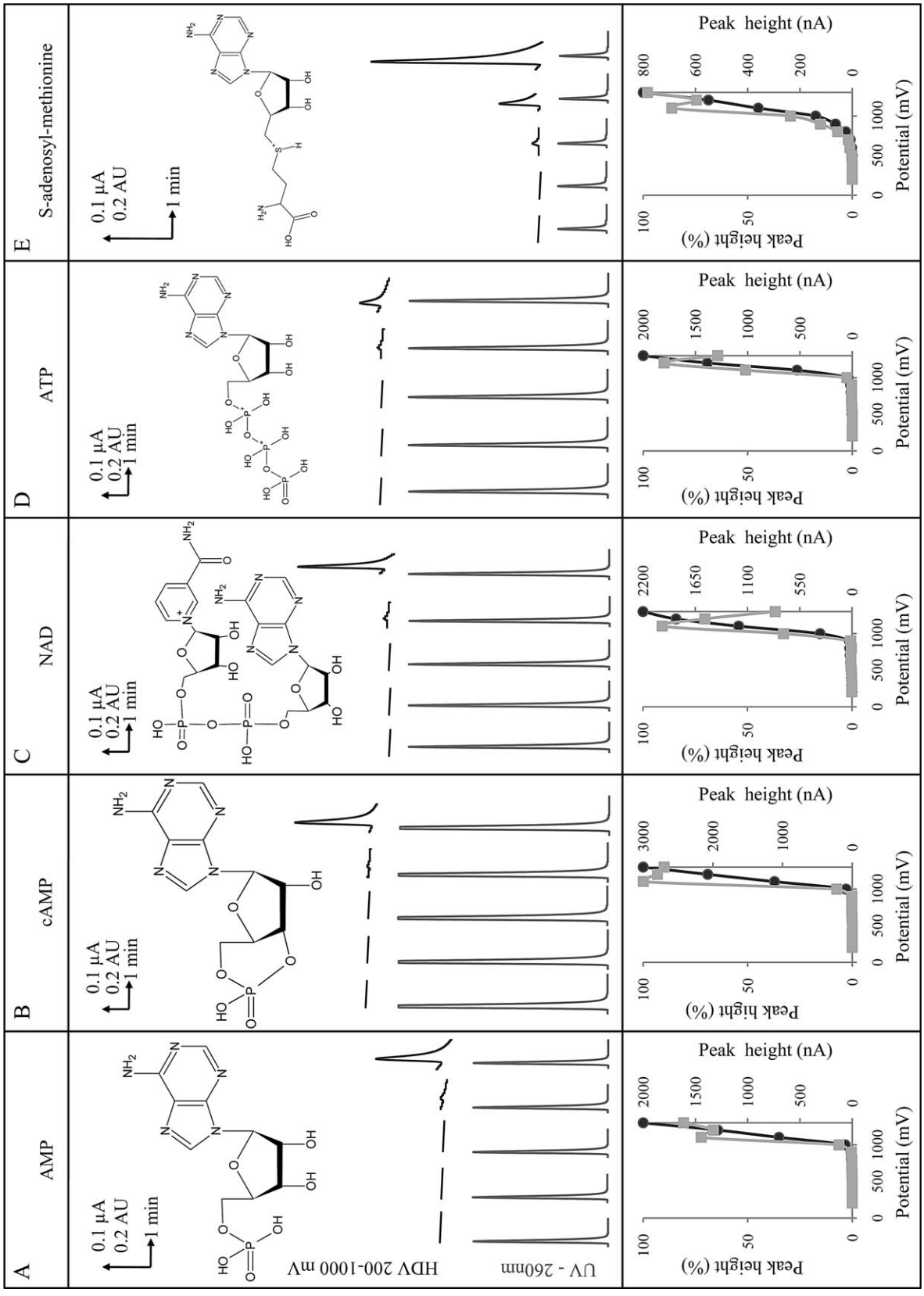

Fig. 3. Structures, FIA-ED and FIA-UV signals, and standard and cumulative hydrodynamic voltammograms of (A) adenosine monophosphate, (B) cyclic adenosine monophosphate, (C) nicotinamide adenine dinucleotide, (D) adenosine triphosphate and (E) *S*-adenosyl-L-methionine. Bottom figures belong to the hydrodynamic voltammograms (HDV). Left vertical axes express peak height in % because of better comparison between all studied derivates (peak height of 100% corresponds to 1617 nA for adenosine monophosphate, 3052 nA for cyclic adenosine monophosphate, 2042 nA for nicotinamide adenine dinucleotide, 1812 for adenosine triphosphate and 785 nA for *S*-adenosyl-L-methionine). Right vertical axes express the cumulative HDV. The lower horizontal axes represent the potential which has been applied to all studied derivates. Experimental conditions are same as in Figure 2.

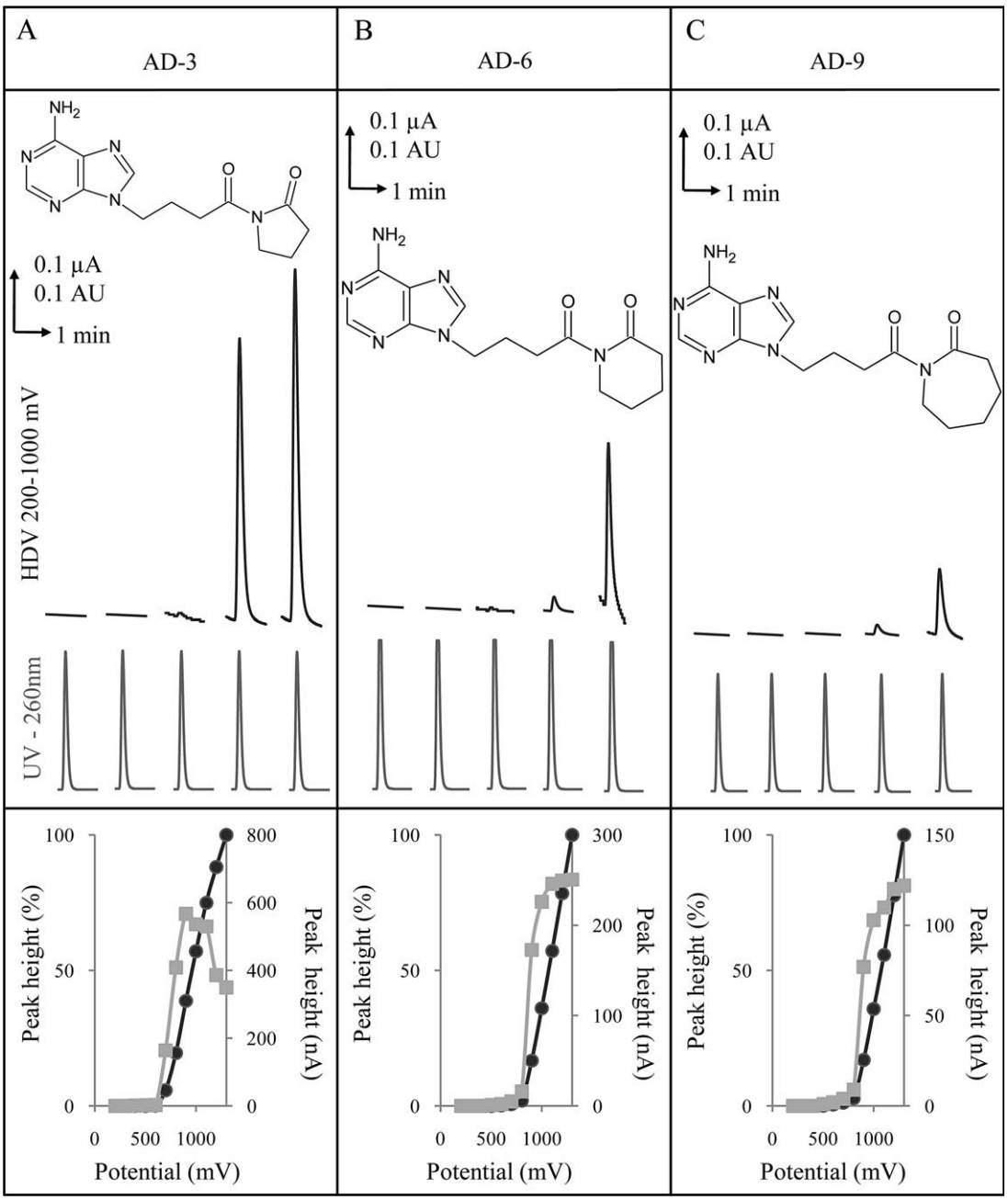

Fig. 4. Structures, FIA-ED and FIA-UV signals, and standard and cumulative hydrodynamic voltammograms of (A) AD-3, (B) AD-6 and (C) AD-9. Bottom figures belong to the hydrodynamic voltammograms (HDV). Left vertical axes express peak height in % because of better comparison between all studied derivates (peak height of 100% corresponds to 567 nA for AD-3, 251 nA for AD-6 and 122 nA for AD-9). Right vertical axes express the cumulative HDV. The lower horizontal axes represent the potential which has been applied to all studied derivates. Experimental conditions are same as in Figure 2.

3.1.1 Adenine

Adenine also called aminopurine is a heterocyclic compounds with great biological importance due to the fact that it belongs to nucleic acid bases forming DNA. Adenine can be detected using ion chromatography with conductivity detection [5], reverse-phase liquid chromatography with UV or electrochemical detection [43], or glassy pencil electrode [33]. The determined hydrodynamic voltammogram (a dependence of signal height on the potential of the working electrode under flow system) is shown in Figure 2A (■). The first detectable signal was registered at 400 mV. However, the sharp increase in the signal height for more than 1000% was determined at 800 mV compared to the signal measured at 700 mV. The highest signal was determined at 1100 mV. Besides the standard hydrodynamic voltammogram, the cumulative hydrodynamic voltammogram was also determined and is shown in Figure 2A (●).

3.1.2 Adenosine

Adenosine is composed of adenine and ribose attached via glycosides bond. The –OH group of ribose can bind one to three phosphates. This leads to the formation of important compounds adenosine mono-, di- and triphosphate (AMP, ADP and ATP). In addition to adenosine, which occurs in RNA, there is deoxyadenosine, which contains the sugar deoxyribose and is found in DNA. High performance liquid chromatography with UV-vis detection was utilized for determination of adenosine in extracts from *Isatis tinctoria* (also known as *Radix isatidis*) [44]. It is also possible to determine the electrochemical activity of adenosine deaminase by cyclic voltammetry [45]. Both hydrodynamic voltammograms (standard ■ and cumulative ● curve) are shown in Figure 2B. The first detectable signal was registered at 800 mV, followed by a sharp increase in the signal height. The highest signal was determined at 1100 mV similar to adenine, but the height of the signal of adenosine was app. 15 % lower compared to the adenine one.

3.1.3 2-Aminopurine

2-Aminopurine is a structural analogue of adenine. Therefore, 2-aminopurine is being used as a fluorescent probe to study conformational changes in DNA caused by proteins [46–48]. To determine this molecule itself, a piezoelectric chemosensor [49] or a electrochemical device [33] can be used. Both hydrodynamic voltammograms (standard ■ and cumulative ● curve) are shown in Figure 2C. The first detectable signal was registered at 600 mV, which was followed by a sharp increase in the signal height. However, the signals measured at 800 and 900 mV were similar. The interesting phenomenon can be probably associated with the presence of amino moiety. The highest signal was determined at 1300 mV similar to previous target molecules.

3.1.4 2,6-Diaminopurine

2,6-Diaminopurine is a structural analogue of adenine. It is used in leukaemia treatment [50] and for the studying of mechanisms of the HIV action [51]. Electrochemical oxidations of aminopurines (adenine. 2-aminopurine. 2,6-diaminopurine) and their complexes with Cu(I) on a pencil graphite electrode were investigated by means of linear sweep voltammetry and elimination voltammetry with linear scan [33]. Both hydrodynamic voltammograms (standard ■ and cumulative ● curve) are shown in Figure 2D. The first detectable signal was registered at 500 mV, which was followed by a sharp increase in the signal height to 600 mV. Then, the steady decrease of the signal was observed up to 800 mV followed by an increase up to 1100 mV. The increasing/decreasing in the signal can be probably associated with the presence of two amino moieties (Figure 1), which can cause structural changes in the derivative under various potentials and, thus, mask some electroactive centres.

3.1.5 6-Benzylaminopurine

6-Benzylaminopurine is one of the firstly artificial synthesized cytokinins. It stimulates cell division of plant cells and thus plant growth [52]. Using capillary electrophoresis, 6-benzylaminopurine was separated from other plant hormones in banana leaf extracts [53]. Another option of the detection is reverse-phase column with liquid chromatography mass spectrometry with chemical ionization at atmospheric pressure [54]. Both hydrodynamic voltammograms (standard ■ and cumulative ● curve) are shown in Figure 2E. The first detectable signal was registered at 700 mV, which was followed by asharp increase in the signal height. The highest signal was determined at 1100 mV similar to previous target molecules. On the other hand, we did not detect any decrease of the signal with increase of the potential of the working electrode in spite of the fact that 6-benzylaminopurine contains amino moiety so as 2-aminopurine and 2,6-diaminopurine. This can be related to the presence of benzyl as another possible target for redox reactions.

3.1.6 Adenosine Monophosphate

Adenosine monophosphate (AMP) has a fundamental role in energy metabolism. It is also one of the building blocks for the formation of RNA nucleotides. The content of AMP and its derivatives can be determined using hydrophilic interaction liquid chromatography (HILIC) with UV detection at 254 nm [55]. Both hydrodynamic voltammograms (standard ■ and cumulative ● curve) are shown in Figure 3A. The first detectable signal was registered at 1000 mV, followed by a sharp increase in the signal height up to 1100 mV. Then the signal decreased. The highest signal was determined at 1300 mV, which was markedly different compared to the previous target molecules. This phenomenon differs from the previously determined molecules probably due to the presence of sugar-phosphate structure.

3.1.7 Cyclic Adenosine Monophosphate

Cyclic adenosine monophosphate (cAMP) is a derivative of ATP. The molecule plays an important role in transmitting information in a number of intracellular signalling pathways in both prokaryotic and eukaryotic cells. It is considered to play a role of the second messenger and acts as an activator of enzymes, mostly kinases. Determination of cAMP can be carried out with combination of HILIC separation and tandem mass spectrometry (MS/MS) detection [56]. Both hydrodynamic voltammograms (standard ■ and cumulative ● curve) are shown in Figure 3B. The first detectable signal was registered at 1000 mV, followed by a sharp increase in the signal height

up to 1100 mV. At higher potentials, the signal of the derivative decreased.

3.1.8 Nicotinamide Adenine Dinucleotide

In an organism nicotinamide adenine dinucleotide can be found in several forms of NAD^+, NADH, $NADP^+$ and NADPH and is very important for the transport of protons in energy metabolism and also as a proton acceptor in other biochemical reactions. Its various forms have different absorption peaks in the UV region; therefore, UV spectrometry is used in the study of enzyme kinetics and for determination the enzyme activity [57]. The content of NAD can be, thus, determined using HILIC chromatography with UV detection [55]. Both hydrodynamic voltammograms (standard ■ and cumulative ● curve) are shown in Figure 3C. The first detectable signal was registered at 900 mV, followed by a sharp increase in the signal. The highest signal was determined at 1100 mV.

3.1.9 Adenosine Triphosphate

It is one of the most important energy compounds. It arises under the influence of a proton gradient on the enzyme ATP synthase in mitochondria. It was found that ATP might also have signalling function in plants besides energy metabolism [58,59]. The content of ATP and its derivatives can be determined using reverse-phase HPLC with UV detection [60] or by separation using HILIC stationary phase [55,61]. Both hydrodynamic voltammograms (standard ■ and cumulative ● curve) are shown in Figure 3D. The first detectable signal was registered at 1000 mV, followed by a sharp increase in the signal height. The highest signal was determined at 1300 mV, which is different from all other compounds. This shift in the signal maximum compared to AMP can be probably associated with the presence of three phosphate moieties.

3.1.10 S-Adenosyl-L-Methionine

S-Adenosyl-L-methionine is one of the main cosubstrates of transferring methyl moiety. It forms during the metabolism of methionine by reaction with ATP. Its biochemistry is studied in connection with the development of chemotherapeutic agents [62] and/or in connection with the study of cirrhosis [63]. Its content can be determined both by HPLC-UV [64] and by capillary electrophoresis [65]. Both hydrodynamic voltammograms (standard ■ and cumulative ● curve) are shown in Figure 3E. The first detectable signal was registered at 700 mV, followed by a sharp increase in the signal height. The highest signal was determined at 1300 mV.

3.1.11 New Synthesized Adenine Derivatives – AD-3, AD-6 and AD-9

FIA-ED-UV was also used for electrochemical characterization of synthesized adenine derivatives called AD-3, AD-6 and AD-9. Their minor structural difference caused marked change in the electrochemical behaviour and their electroactivity. Both hydrodynamic voltammograms (standard ■ and cumulative ● curve) of these derivatives are shown in Figure 4A,B,C. The first detectable signal was registered at 700 mV, 800 mV and 900 mV for AD-3 (Figure 4A), AD-6 (Figure 4B) and AD-6 (Figure 4C), respectively. The highest signal was determined at 900 mV for AD-3 and, then, the signal decreased gradually with increasing potential for more than 30 % at 1300 mV. AD-6 and AD-9 showed the highest signals at 1300 mV. This phenomenon is associated with the enlarging of the carbon ring in the adenine derivative (AD-3, AD-6 and AD-9).

3.2 Mathematical Evaluation of Hydrodynamic Voltammograms

To find the optimal potential for the detection of all target molecules and to describe the electroactivity of all measured substances, a more sophisticated mathematical treatment was used compared to pH and flow rate evaluation. Therefore, the area under HDV for a single molecule was integrated (Figure 5). Table 1 shows the calculation of the integration area from the left of the limited value (x), which is just as 10 % of the highest measured signal, to the right as 1300 mV. The integrations were determined using trapezoidal numerical integration. To calculate the integration and to show the courses of the single HDV, cubic spline calculation using one-dimensional interpolation was used to ensure uniformity of the signal. Derived from the interpolated curves, we found maxima signals for the individual substances, e.g., 1070 mV for adenine, 1100 mV for adenosine and 1220 mV for AMP (Table 1), where the values on the x axis correspond to the potential, at which the maxima of signals on the y axis were determined. The maxima were calculated as the local values free of prediction for the measured range. The spectral character of the individual waveforms is very complex. For their description at a statistical level ($p < 0.05$), we had to use a sixth order polynomial. The individual coefficients are shown in Table 2.

This mathematical approach was used for estimation of an optimal detection potential and for description of the electrochemical behaviour of all target molecules. Based on the results presented in Table 1, we estimated the optimal potential for detection of all substances as app. 1100 mV. This value is important for the determination of these substances, but their electroactivity under the defined conditions still remains unclear. Therefore, we were interested in the issue whether we could use the obtained data for description of electroactivity of adenine and its derivatives. This could be of great interest not only for fundamental electrochemical research but also for bio-application of these substances as a way how to mimic their behaviour in a cell, because they may have the ability of scavenging/producing reactive oxygen species [66]. New coefficients of electroactivity were defined by using the

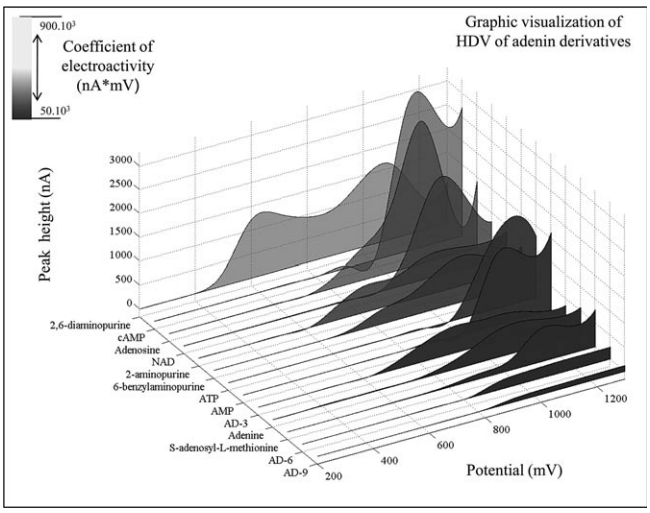

Fig. 5. Comparison of visualized HDV of adenine derivatives obtained by mathematical analysis using cubic spline.

mathematical treatment of data measured by flow injection analysis and are shown in Figure 5. This value shows the tendency of the substance to be oxidized at the surface of the working electrode, which means the ability to scavenge reactive oxygen species or to undergo their adverse effects, if the substance is a part of an important biomolecule. Based on this criterion, 2,6-diaminopurine was the most electroactive substance with a coefficient of electroactivity above 900×10^3 followed by cAMP and adenosine. On the other hand, the lowest electroactive substance was AD-9 followed by AD-6 and *S*-adenosyl-L-methionine.

If we look on the biological purpose of these substances, we can find interesting relations between coefficients of electroactivity of adenine and its derivatives and their biological roles. Adenine belongs to the least electroactive compounds from the set of the studied substances showing the stability of the basic part of DNA. Rapid increase in the electroactivity of adenosine as structurally close derivative of adenine as the third most electroactive compound can be associated with the presence of sugar in this molecule, which can be, thus, easily oxidized. The oxidation of sugars in DNA is not concern compared to the oxidation of the nucleic acid base itself. One of the interesting results is also the enhancement of the electroactivity of adenine with one shifted amino (2-aminopurine) or two amino moieties (2,6-diaminopurine). 6-Benzylaminopurine as a substance with important biological role has lower electroactivity compared to two previously described adenine derivatives but it is more active compared to adenine. Lower coefficients of electroactivity were also determined in AMP and ATP, which are of great importance in the energy metabolism. However, cAMP as a signalling molecule, which means a molecule with limited life-time, has the second higher coefficient of electroactivity. This indicates the fact that molecules with crucial roles in various metabolic pathways have a much lower electroactivity coefficient, thus, much higher stability in the cell environment compared to those with limited lifetime and/or of artificial origin. Electroactivity coefficients of NAD and *S*-adenosyl-methionine support this presumption due the fact that NAD is important in the redox metabolism and must be easily oxidized. Based on the discussed results, we attempted to predict the fate of newly synthesized adenine derivatives AD-3, AD-6 and AD-9 in a cell. It can be assumed that these derivatives can not be easily oxidized and, thus, they would be stable in the cell environment. From the biological point of view, their electroactivity is similar to adenine. This is a good feature to use these derivatives as a base for an antiviral drug due to the possibility to exchange adenine and stop DNA or RNA replication, as well as withstand the oxidative stress.

Table 1. Mathematical expression of parameters of hydrodynamic voltammograms.

Substance [a]	Area of scan [b]	Maximum [c]	
	(nA mV)	*X* (potential, mV)	*Y* (current, nA)
Adenine	236442	1070	627
Adenosine	693773	1100	3000
2-Aminopurine	424625	1060	846
2,6-Diaminopurine	869005	1070	1638
6-Benzylaminopurine	365037	1090	962
AMP	364832	1220	1830
cAMP	725616	1140	3292
NAD	467264	1120	2057
ATP	359075	1130	1566
S-Adenosyl-L-methionine	199735	1120	717
AD-3	275577	920	570
AD-6	103595	1170	250
AD-9	47956	1240	123

[a] studied adenine derivatives; [b] integration of area of hydrodynamic voltammogram of adenine derivative; [c] maximum of dependence of current on applied potential – hydrodynamic voltammogram (HDV).

Table 2. HDV progress characterized by polynomial regression.

Substance [a]	Mathematical model of the HDV [b]
Adenine	$y=-9.04\times10^{-13}x^6+5.71\times10^{-9}x^5-1.48\times10^{-5}x^4+2.02\times10^{-2}x^3-15.291216x^2+6096.31x-1000621.4$
Adenosine	$y=-1.14\times10^{-12}x^6+1.50\times10^{-8}x^5-5.89\times10^{-5}x^4+1.07\times10^{-1}x^3-102.08327x^2+49188.30x-9517801.3$
2-Aminopurine	$y=4.79\times10^{-13}x^6-2.57\times10^{-9}x^5+5.61\times10^{-6}x^4-6.39\times10^{-3}x^3+3.9869803x^2-1283.13x+165115.235$
2,6-Diaminopurine	$y=1.33\times10^{-12}x^6-6.76\times10^{-9}x^5+1.39\times10^{-5}x^4-1.49\times10^{-2}x^3+8.59x^2-2550.95x+303081.957$
6-Benzylaminopurine	$y=2.82\times10^{-13}x^6-1.14\times10^{-9}x^5+1.46\times10^{-6}x^4-2.15\times10^{-4}x^3-1.0274752x^2+828.79x-197004.25$
AMP	$y=6.32\times10^{-12}x^6-5.25\times10^{-8}x^5+1.78\times10^{-4}x^4-0.31637x^3+311.73784x^2-161595.07x+34465155.1$
cAMP	$y=1.24\times10^{-11}x^6-1.03\times10^{-7}x^5+3.49\times10^{-4}x^4-6.21\times10^{-1}x^3+612.42855x^2-317620.16x+67784721.9$
NAD	$y=-2.99\times10^{-11}x^6+2.00\times10^{-7}x^5-5.53\times10^{-4}x^4+8.12\times10^{-1}x^3-666.9842x^2+290426.32x-52401231$
ATP	$y=2.06\times10^{-12}x^6-1.53\times10^{-8}x^5+4.73\times10^{-5}x^4-7.79\times10^{-2}x^3+72.330124x^2-35836.42x-197004.25$
S-Adenosyl-L-methionine	$y=-1.46\times10^{-12}x^6+1.11\times10^{-8}x^5-3.38\times10^{-5}x^4+5.36\times10^{-2}x^3-46.647124x^2+21226.65x-3955086$
AD-3	$y=5.08\times10^{-13}x^6-2.98\times10^{-9}x^5+7.24\times10^{-6}x^4-9.28\times10^{-3}x^3+6.6143775x^2-2483.25x+382744.783$
AD-6	$y=7.55\times10^{-13}x^6-4.80\times10^{-9}x^5+1.26\times10^{-5}x^4-1.76\times10^{-2}x^3+1.37\times10x^2-5639.68x+959524.473$
AD-9	$y=3.04\times10^{-13}x^6-1.96\times10^{-9}x^5+5.23\times10^{-6}x^4-7.39\times10^{-3}x^3+5.83x^2-2430.01x+418321.549$

[a] studied adenine derivatives; [b] parametrical expression of hydrodynamic voltammograms of adenine derivatives

3.3 Analytical Results

Besides defining of the new coefficient of electroactivity, we also optimized the experimental conditions for the most sensitive detection of adenine and its derivatives. Under the optimized parameters (Britton–Robinson buffer (pH 3) as mobile phase and its flow rate (0.75 mL min^{-1}), potential of the working electrode of 1100 mV) calibration dependences were determined. Dose-response curves were measured within the range from 1 to 100 μM for adenine, 2-aminopurine, from 1 to 500 μM for AD-3 and AD-6, from 1 to 750 μM for AD-9 and for other analytes from 1 to 1000 μM (Table 3). These ranges are appropriate from the point of view of concentration of the analytes in real samples. All measured calibration curves were linear and/or strictly linear depending on their R^2, which indicates a good applicability of FIA-ED under the above mentioned conditions for analysis of real samples. The lowest detection limit (3 *S*/*N*) was estimated for adenine as 0.9 nM and for 2-aminopurine as 0.5 nM. On the other hand, the highest detection limit of 100 nM was estimated for ATP. Nevertheless, all detection limits are below the real concentration level of some target molecules in live organisms. *RSD* of dose-response measurements were within the range from 2.5 to 5.2 % ($n=5$).

4 Conclusions

Flow injection analysis coupled with an electrochemical detector can be considered as easy-to-use and low cost instrument to investigate electroactivity and biological stability of biologically important molecules, as it was demonstrated on adenine and its derivatives (adenosine, 2-aminopurine, 2,6-diaminopurine, 6-benzyl-aminopurine, adenosine monophosphate, cyclic adenosine monophosphate, nicotinamide adenine dinucleotide, adenosine triphosphate, *S*-adenosyl-L-methionine, and similar synthetic derivatives AD-3, AD-6 and AD-9). From the analytical point of view, our developed method and obtained results might be useful for another method modification, as em-

Table 3. Validation of experimental parameters for studied adenine derivates.

Analyte [a]	Regression equation	Linear dynamic range (μM)	Linear dynamic range (μg/mL)	R^2	*LOD* [b] (nM)	*LOD* (ng/mL)	*LOQ* [c] (nM)	*LOQ* (ng/mL)	*RSD* [d] (%)
Adenine	$y=10.22x-11.98$	0.1–100	0.014–13.513	0.998	0.9	0.1	3	0.4	2.5
Adenosine	$y=0.350x-10.04$	1–1000	0.267–267.2	0.990	70	20	200	60	4.1
2-Aminopurine	$y=14.22x+10.73$	0.1–100	0.135–13.513	0.996	0.5	0.1	2	0.2	3.3
2,6-Diaminopurine	$y=0.641x-0.107$	1–1000	0.151–151.13	0.998	9	1	30	5	3.5
6-Benzylaminopurine	$y=0.287x-5.078$	1–1000	0.225–225.0	0.997	30	6	90	20	4.8
AMP	$y=0.025x+4.499$	1–1000	0.324–324.22	0.987	30	10	100	30	4.6
cAMP	$y=0.044x+2.955$	1–1000	0.329–329.2	0.988	20	5	50	20	5.2
NAD	$y=0.043x+3.546$	1–1000	0.663–663.4	0.956	30	20	80	60	4.4
ATP	$y=0.010x+0.451$	1–1000	0.505–505.14	0.984	100	60	300	200	4.7
S-Adenosyl-L-methionine	$y=0.254x+17.44$	1–1000	0.398–398.44	0.971	6	2	20	8	4.3
AD-3	$y=2.837x+17.80$	1–500	0.546–273.0	0.996	3	1	9	5	3.6
AD-6	$y=1.911x+16.96$	1–500	0.588–294.0	0.997	1	0.6	4	2	4.1
AD-9	$y=0.954x-1.815$	1–750	0.63–472.5	0.996	6	4	20	10	3.2

[a] studied adenine derivatives; [b] limits of detection (3 *S*/*N*); [c] limits of quantification (10 *S*/*N*); [d] relative standard deviations.

ployment of HPLC separation, to which the tested FIA-ED system was complementary. Another methods as stationary electrochemistry or microfluidic system with miniaturized electrochemical systems should use results showed in this study.

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