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Square wave and elimination voltammetric analysis of azidothymidine in the presence of oligonucleotides and chromosomal DNA

Libuše Trnková[a,*], René Kizek[b], Jan Vacek[b]

[a] Department of Theoretical and Physical Chemistry, Faculty of Science, Masaryk University Brno, Kotlářská 2, 611 37 Brno, Czech Republic
[b] Department of Chemistry and Biochemistry, Faculty of Agronomy, Mendel University of Agriculture and Forestry, Zemědělská 1, 613 00 Brno, Czech Republic

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Abstract

Azidothymidine (AZT, 3′-azido-3′-deoxythymidine, Zidovudine, Retrovir) is an approved and widely used antiretroviral drug for the treatment of human immunodeficiency virus (HIV) infection. Dynamic electrochemical methods have been employed for the fast and inexpensive determination of this drug in natural samples. The electrochemical signal of AZT, resulting from the reduction of azido group, was studied by square wave voltammetry (SWV), linear sweep voltammetry (LSV) and elimination voltammetry with linear scan (EVLS) using a hanging mercury drop electrode (HMDE). This paper explores the possibility of determining AZT in the presence of native (dsDNA) or denatured calf thymus DNA (ssDNA), and/or some synthetic oligodeoxynucleotides (ODNs). The detection limit of AZT in the absence and in the presence of ssDNA (10 μg/ml) is 1 and 250 nM, respectively. It was found that the signal of AZT is not substantially affected by the presence of DNA. We can therefore assume that the electrons are transferred through the adsorption layer of nucleic acids. By using the elimination procedure, both irreversible reduction signals of AZT and DNA are augmented. Moreover, the elimination signal in the peak–counterpeak form may indicate the adsorption of the analytes on the electrode surface preceding an electron transfer.

Keywords: Azidothymidine (AZT); Square wave voltammetry (SWV); Linear sweep voltammetry (LSV); Elimination voltammetry with linear scan (EVLS); Hanging mercury drop electrode (HMDE); Oligonucleotides; PCR product; Chromosomal DNA

1. Introduction

Azidothymidine (3′ -azido-3′ -deoxythymidine, AZT, Zidovudine, Retrovir, Fig. 1A) is a synthetic nucleoside analogue which inhibits human immunodeficiency virus type 1 (HIV-1) [1,2]. AZT is derived from thymidine where the hydroxyl group at carbon 3 of the sugar moiety is replaced by the azide. The azido group is responsible not only for the in vivo antiviral activity but also for the electrochemical reduction signal of AZT on a mercury electrode. Recently, the reduction signal of azido group in AZT measured by square wave voltammetry (SWV) has been employed for the determination of AZT in natural body samples (urine, serum, whole blood) and cell cultures, such as the HaCat line of keratinocytes without the necessity of mineralization and/or purification. The mechanism of the AZT reduction on mercury electrodes has been studied but the results are rather ambiguous. It was concluded that the reduction of AZT in acid as well as alkaline solutions is probably a two-electron [3] or four-electron process [4–7]. The toxic side effects of AZT [8,9] spurred interest in examining its chemical, biochemical, and electrochemical properties.

It is known that native and denatured DNAs measured by voltammetric methods using the negatively polarized mercury electrode provide a cathodic signal caused by the reduction of adenine and cytosine residues [10,11]. The signal is common for both bases and the peak height is larger for ssDNA than for dsDNA [12]. In order to obtain a well-developed reduction peak of DNA on a hanging mercury drop electrode (HMDE), an adsorptive stripping technique was applied in both SWV [13] and elimination voltammetry with linear scan (EVLS) [14].

The EVLS enables elimination of some of the currents from the results obtained using linear scan voltammetry.

* Corresponding author. Tel.: +420-541-12-92-97; fax: +420-541-211214.
E-mail address: libuse@chemi.muni.cz (L. Trnková).

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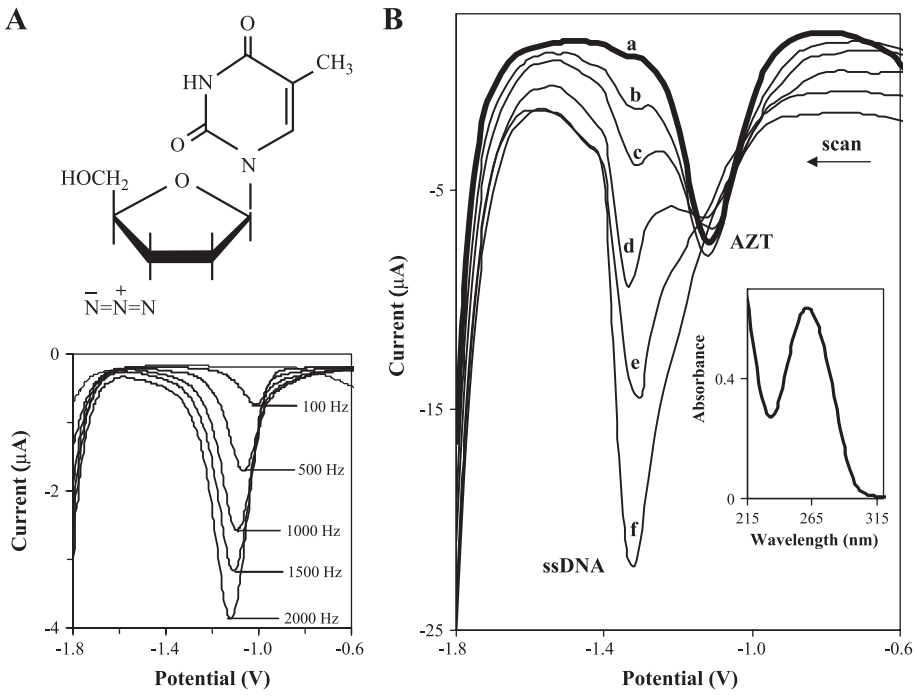

Fig. 1. (A) Structure of 3′-azido-3′-deoxythymidine (AZT); SWV peaks of 2 μM AZT measured at the frequency of 100, 500, 1000, 1500, and 2000 Hz; (B) Square wave voltammograms of 5 μM AZT in a mixture containing 500 ng/ml ssDNA measured in phosphate buffer (pH 8.0) at different accumulation time (in seconds): (a) 0, (b) 30, (c) 60, (d) 120, (e) 240, (f) 360 at the deposition potential of −0.1 V. Arrows indicate the direction of scan. Experimental SWV parameters: initial potential −0.1 V, end-point potential −1.8 V, step potential 5 mV, amplitude 20 mV, frequency 2000 Hz, equilibration time 5 s. Inset: absorption spectrum of the solution containing AZT (5 μM) and 10 μg/ml denatured single stranded DNA (ssDNA) in phosphate buffer (pH 8.0), λ_{max}(AZT) = 267 nm and λ_{max}(DNA) = 260 nm.

This elimination can be achieved by using an elimination function formed by a linear combination of total currents measured at different scan rates. The basic idea of the elimination of chosen currents (for example the charging current, I_c, the diffusion current, I_d, and the kinetic current, I_k) consists of the different dependence of the current on the scan rate [15]. The function, which eliminates both kinetic and charging currents and conserves the diffusion current, was calculated for an adsorbed electroactive substance and verified experimentally on DNA samples [14,16].

The application of EVLS in the study of nucleic acids on the mercury and silver electrodes was described in Refs. [14,16–18]. The above-mentioned elimination function provides the peak–counterpeak signal for the chromosomal ssDNA. The nucleic acid is strongly adsorbed on the surface of mercury electrode and the elimination of the reduction peak of adenine and cytosine residues keeps this form. Thanks to this elimination signal the sensitivity of the measurement increases by 13 times.

The main aim of the paper is to provide a superior method of AZT analysis in solutions, where chromosomal calf thymus ssDNA, dsDNA and/or some synthetic oligonucleotides are present. For this purpose, square wave voltammetry and elimination voltammetry with linear scan have been employed.

2. Experimental

2.1. Chemicals

Azidothymidine was purchased from Fluka BioChemika (purity by TLC, >99%). Chemicals were prepared by Sigma Aldrich, USA (purity of ACS). Phosphate buffer with components 0.1 M NaH_2PO_4 + 0.1 M Na_2HPO_4 (pH 8.0) was used. All solutions were prepared using deionized ACS water (Sigma Aldrich). The pH of the solutions was checked by pH-meter Präcitronic (type MV870, Germany). The isolation and thermal denaturation of calf thymus DNA were carried out as described previously [19]. Two synthetic oligonucleotides (ODNs): 5′-$(A)_{12}$-3′ and 5′-$(TTC)_{24}(A)_{25}$-3′ were purchased from VBS-GENOMICS, Austria. The PCR-product with a random sequence of all nucleic acid bases (226 bp) was kindly provided by Dr. Jan Paleček, Institute of Biophysics, AV ČR, Czech Republic. Supercoiled pBluescript (Pvu II) was isolated and purified using Qiagen kits (Qiagen, Germany) [19].

2.2. Procedure

2.2.1. Voltammetric measurements

Square wave voltammetry (SWV) and/or linear sweep voltammetry (LSV) were performed with the AUTOLAB analyzer (EcoChemie, Utrecht, Netherlands) connected to

the VA-Stand 663 (Metrohm, Zurich, Switzerland) using an electrochemical cell in standard three electrode arrangement. A Hanging Mercury Drop Electrode (HMDE) with an area of 0.4 mm^2 was used as a working electrode. The Ag/AgCl/3M KCl served as a reference electrode and a platinum wire was used as an auxiliary electrode. Two milliliter of analyte were deoxygenated by purging with 99.99% argon gas for at least 5 min prior to analysis. All experiments were carried out at room temperature in phosphate buffer (pH 8.0) under the following experimental parameters, common to both methods: initial potential of − 0.1 V, end-point potential of − 1.8 V, and equilibration time of 5 s. The additional SWV parameters, amplitude 20 mV, frequency 2000 Hz, and step potential of 5 mV were used. For LSV the scan rates of 80, 160, 320, and 640 mV/s at the constant potential step of 2 mV were employed. The raw LSV data were treated using the Savitzky and Golay filter (level 4) [20] integrated into GPES software (EcoChemie).

2.2.2. Elimination procedure

Linear sweep voltammetric data obtained at the scan rate of $1/2v$, v and $2v$ were exported into Microsoft Excel (Microsoft, USA). The particular elimination function was calculated using Microsoft Visual Basic 6.0.

2.2.3. UV–Vis spectrophotometry

AZT, DNA, and their mixtures were measured by UV–Vis spectrophotometer (Hewlett Packard, Model 8452A, USA) equipped with a diode array detector using a temperature-controlled cell (1 cm).

3. Results and discussion

Firstly, we measured the square wave voltammetric curves of AZT on the HMDE at different frequencies. The pH value of phosphate buffer (pH 8.0) was chosen according to results published in Ref. [21]. With increasing frequency (100, 500, 1000, 1500, and 2000 Hz) the peak height increased and the peak potential shifted to negative values (Fig. 1A). The frequency change of 1000 Hz induced a potential shift of about 35 mV. Secondly, the reduction signals of AZT, ssDNA and their mixtures were measured at different accumulation times. The SWV measurements were repeated in phosphate buffer (pH 8.0) with the following experimental parameters: initial potential − 0.1 V, end-point potential − 1.8 V, step potential 5 mV, amplitude 20 mV, frequency 2000 Hz, equilibration time 5 s. Both AZT and DNA yield voltammetric signals on the mercury electrode. While AZT provides an irreversible reduction peak at a potential near − 1.1 V (vs. Ag/AgCl/3M KCl), the DNA gives the reduction peak due to adenine and cytosine residues at a potential of ca. − 1.4 V. The detection limit of AZT in the absence and in the presence of ssDNA (10 μg/ml), 1 and 250 nM, respectively, was confirmed [21]. It was shown that the accumulation process does not have a substantial effect on the increase of AZT peak height, in fact, rather the opposite. The adsorption step is suitable for ssDNA and at longer accumulation times the increasing peak of ssDNA overlaps the AZT peak (Fig. 1B). Overlapped absorption spectral responses of AZT and ssDNA in mixtures can also be expected, because in UV–Vis spectra the absorption maxima of both substances are very similar (λ_{max} 267 nm for AZT and λ_{max}260 nm for ssDNA). The spectrum of one mixture containing AZT (5 μg/ml) with ssDNA (10 μg/ml) recorded in the range from 215 to 315 nm is shown in the set of Fig. 1B [19].

On the basis of a sufficiently large difference in peak potential between the common voltammetric signal of adenine and cytosine in nucleotides and the reduction signal of azido group in AZT, we assumed that AZT in mixtures with oligo- and polynucleotides can be measured. This possibility is illustrated in Fig. 2 where solutions with the constant concentration of AZT were titrated by ssDNA (Fig. 2A), dsDNA (Fig. 2B), and oligodeoxynucleotides [5′ -$(TTC)_{24}(A)_{25}$-3′] (Fig. 2C). The AZT concentration was 0.5 μM; the ssDNA and dsDNA concentrations varied from 250 ng/ml to 60 μg/ml; the ODN concentration ranged from 250 ng/ml to 10 μg/ml. The current responses of DNAs increased with the increasing concentrations and the AZT response did not change significantly. While the height of AZT peak and ODNs in solutions with dsDNA decreased by about 5% (top Fig. 2B and C), a 10% decrease of the height of AZT peak was observed in solutions with ssDNA during the initial additions of DNA (top Fig. 2A). The saturation of the surface layer on a mercury electrode by ssDNA was achieved at the concentration of ca. 40 μg/ml. In the case of dsDNA and ODNs, this concentration did not suffice to produce the same effect.

The application of elimination voltammetry (EVLS) to the system studied is demonstrated in Fig. 3. The function $f(I)$ which eliminates the charging and kinetic currents (I_c, I_k) and conserves the diffusion current (I_d) and using for the integer 2 (the scan rate of $(1/2)v$, v and $2v$, where v is the reference scan rate) is expressed as the linear combination: $f(I) = -11.657I_{1/2} + 17.485I - 5.8284I_2$, where I is the reference current measured at the reference scan rate, $I_{1/2}$ and I_2 are the total currents measured at a half and double reference scan rate [14,15,22]. For adsorbed electroactive substance this elimination function creates a characteristic signal, i.e. a peak (I_p) which passes directly to a large and sharp opposite peak (I_{cp}) and creates the characteristic peak–counterpeak signal ($I_p + I_{cp}$) [14,16–18]. The elimination function results in a significant increase of sensitivity in the determination of DNA [14]. Thanks to elimination, the signal of adenine and cytosine residues in ssDNA is 13 times higher than the respective LSV signal (Fig. 3A and B). Linear sweep voltammetric measurements were performed in phosphate buffer (pH 8.0) at different scan rates (40, 80, 160, 320, 640, 1280

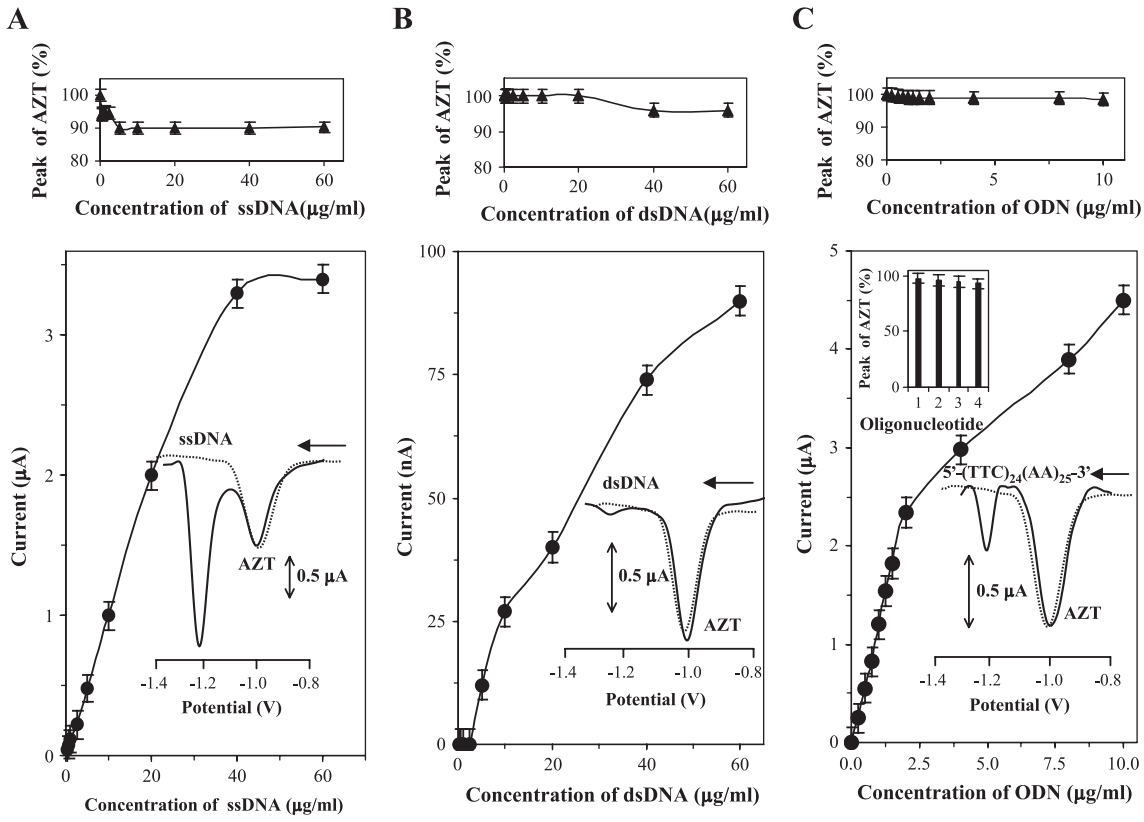

Fig. 2. The dependences of peak heights (top plots) of AZT and of adenine and cytosine (bottom plots) on the concentration of (A) ssDNA, (B) dsDNA, (C) ODN: 5′-$(TTC)_{24}(A)_{25}$-3′. The peak height of 0.5 μM AZT was taken as 100% (top figures). Insets in A, B, C: square wave voltammograms of a mixture of 0.5 μM AZT in mixture with ssDNA (20 μg/ml), dsDNA (20 μg/ml), and ODN (0.5 μg/ml). Top inset (C): (1) pBluescript (Pvu II), (2) PCR-product (Materials and methods), (3) 5′-$(TTC)_{24}(A)_{25}$-3′, (4) 5′-$(A)_{12}$-3′, the concentration of DNAs was 5 μg/ml. The peak height of 5 μM AZT was taken as 100%. Arrows indicate the scan direction. Other conditions as in Fig. 1.

mV/s). The scan rate of 80 mV/s was taken as a reference scan rate in the elimination procedure. The currents, $I_{1/2}$ and I_2, were measured at half and double reference scan rates, i.e. at 40 and 160 mV/s. The theoretical value of the ratio $I_p/(I_p+I_{cp})$, where I_p and I_{cp} represent the heights of the current peak and counterpeak is 0.4097 [23]. For the reduction adenine and cytosine residues in ssDNA this ratio corresponds to the value of 0.453 (Fig. 3B). Linear sweep and elimination voltammetric curves of AZT are shown in Fig. 3C and D. From the significant difference of the elimination function course for AZT and ssDNA it could be assumed that the reduction process of the azido group in AZT has a different character than the reduction process of adenine and cytosine in ssDNA. The irreversible reduction of the azido group is probably accompanied by a slow chemical reaction preceding the electron transfer and the adsorption did not play a significant role. In the case of the reduction process of adenine and cytosine residues in ssDNA the adsorption is important for the electron transfer. Using EVLS the sensitivity of LSV increased and the elimination of AZT signal is nine times higher than the original voltammetric signal. Fig. 3 (E and F) illustrates elimination results for a mixture of ssDNA (10 μg/ml) and AZT (10 μg/ml). For both components, the EVLS signals have a peak–counterpeak shape, indicating that the electron transfer proceeds in the adsorbed state. Moreover, the different course of the elimination curves of AZT in the absence and in the presence of DNA indicates the change in the electrode mechanism of AZT. It means that electron transfer can proceed through the DNA adsorbed layer. One interesting finding is that in the comparison with LSV signals EVLS signals of ssDNA and AZT increased, for ssDNA 20-fold and for AZT 10-fold. The ratio $I_p/(I_p+I_{cp})$ in both cases is ca. 0.55 which exceeds the theoretical value. However, it does not differ significantly from that calculated for individual components.

With respect to the possible interaction of AZT with genetic material, the effect of ODNs and DNAs on the voltammetric signals of AZT is helpful. Using a mercury electrode, the detection of the reduction signal of the azido group of azidothymidine (~ -1.1 V) in mixtures with DNAs or ODNs was possible because the common reduction of adenine and cytosine in nucleic acid proceeds at more negative potentials (~ -1.4 V) in comparison with AZT. It was found that in the case of the square wave voltammetric analysis of AZT it is more suitable to perform the detection of AZT in these solutions without any

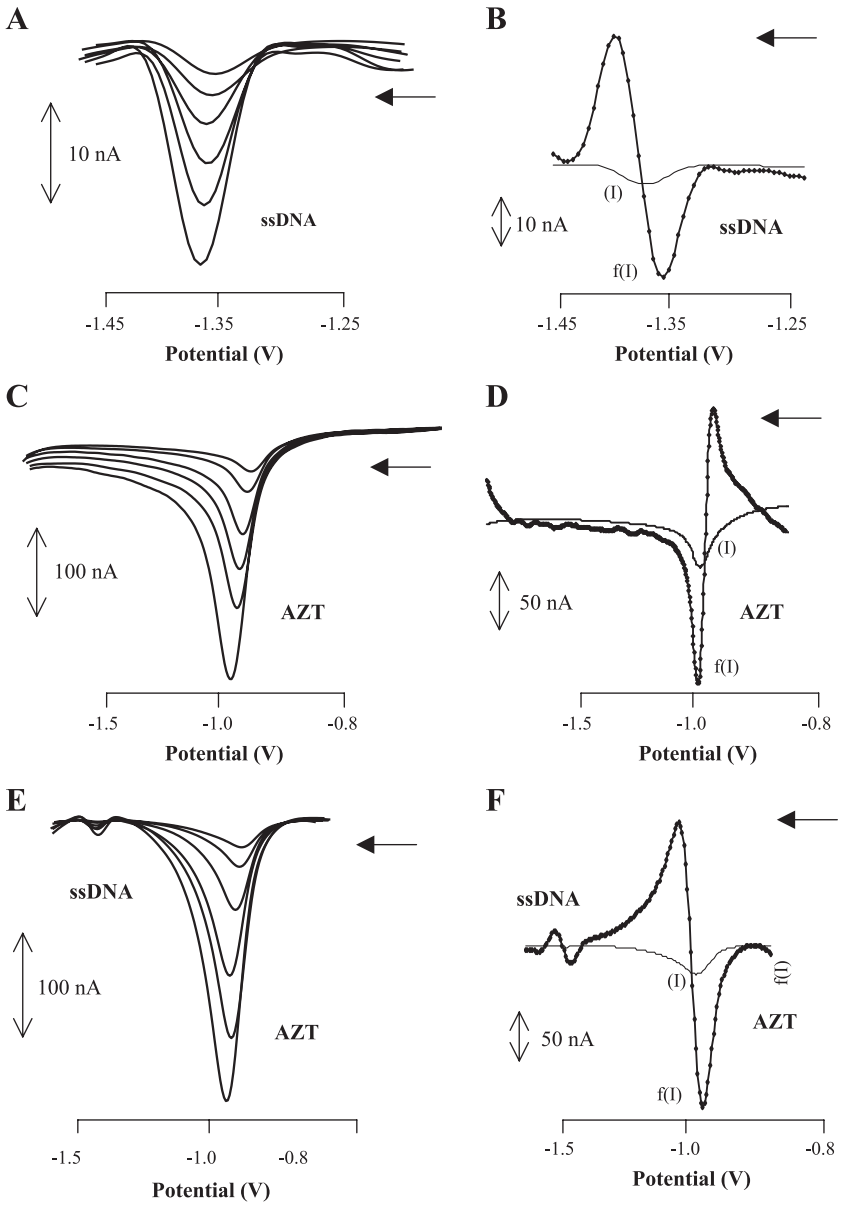

Fig. 3. LSV and EVLS voltammograms: (A, B) 10 μg/ml ssDNA, (C, D) 10 μM AZT, and (E, F) the mixture of 10 μg/ml ssDNA and 10 μM AZT. LSV measurements (A), (C), and (E) were performed in phosphate buffer (pH 8.0) using the following experimental parameters: initial potential − 0.1 V, end potential − 1.8 V, step potential 2 mV, scan rates 40, 80, 160, 320, 640, and 1280 mV/s, equilibration time 2 s. EVLS procedures (B), (D), and (F) were used for the reference scan rate 80 mV/s. The $I_{1/2}$ and I_2 currents were 40 and 160 mV/s, respectively. Arrows show the direction of scan.

accumulation of electroactive material on the mercury electrode surface. The presence of nucleotides, as depolarizers competitive with AZT, does not have a significant effect on the peak height of AZT. Furthermore, the electron transfer in the reduction process of the azido group proceeds through the adsorption layer of ssDNA, dsDNA and/or ODNs. We have demonstrated that SWV and LSV in connection with EVLS are useful and effective tools for the determination of AZT. Using EVLS, it is possible to increase the sensitivity of LSV. Moreover, this method may be used in the detailed study and classification of electrode processes. The SWV and EVLS determination of AZT in a mixture with DNAs or ODNs has some advantages. Both procedures are fast, accurate, and inexpensive. Only a small amount of a sample is required. Preliminary sample mineralizations are not necessary.

We consider the results of this research to be very important for the study of possible incorporation of AZT into an oligonucleotide chain.

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References

[1] M.S. Hirsch, R.T. D'Aquila, Therapy for human immunodeficiency virus infection, N. Engl. J. Med. 328 (1993) 1686–1695.
[2] R.M.W. Hoetelmans, D.M. Burger, P.L. Meenhorst, J.H. Beijnen, Pharmacokinetic individualisation of zidovudine therapy. Current state of pharmacokinetic–pharmacodynamic relationships, Clin. Pharmacokinet. 30 (1996) 314–327.
[3] B. Czochralska, B. Sapok, D. Shugar, Electrochemical analysis 3′-azidothymidine (AZT), Nucleos. Nucleot. 9 (1990) 443–444.
[4] G.C. Barone, H.B. Halsall, W.R. Heineman, Electrochemistry of azidothymidine, Anal. Chim. Acta 248 (1991) 399–407.
[5] G.C. Barone, A.J. Pescine, H.B. Halsall, W.R. Heineman, Electrochemical determination of azidothymidine in human whole-blood, Anal. Biochem. 198 (1991) 6–9.
[6] W. Kawczynski, B. Czochralska, D. Shugar, Electrochemical reduction of azido nucleosides—mechanism and synthetic and analytical applications, Bioelectrochem. Bioenerg. 26 (1991) 441–455.
[7] W. Kawczynski, P.F. Torrence, J.E. Kinjo, B. Czochralska, Electrochemical study of pyrimidine analog of 3′ azido-3′-deoxythymidine (AZT) related to AIDS dementia, Bioelectrochem. Bioenerg. 33 (1994) 171–179.
[8] E.M. Cretton, D.V. Waterhous, R. Bevan, J.P. Sommadossi, Glucuronidation of 3′-azido-3′-deoxythymidine by rat and human liver-microsomes, Drug Metab. Dispos. 18 (1990) 369–372.
[9] E.M. Cretton, M.Y. Xie, R.J. Bevan, N.M. Goudgaon, R.F. Schinazi, J.P. Sommadossi, Catabolism of 3′-azido-3′-deoxythymidine in hepatocytes and liver-microsomes, with evidence of formation of 3′-amino-3′-deoxythymidine, a highly toxic catabolite for human bone-marrow cells, Mol. Pharmacol. 39 (1991) 258–266.
[10] E. Palecek, M. Fojta, V. Jelen, V. Vetterl, Electrochemical analysis of nucleic acids, Encycl. Electrochem. 9 (2002) 365–429.
[11] E. Palecek, From polarography of DNA to microanalysis with nucleic acids-modified electrodes, Electroanalysis 8 (1996) 7–14.
[12] E. Palecek, F. Jelen, L. Trnkova, Cyclic voltammetry of DNA at a mercury electrode: an anodic peak specific for guanine, Gen. Physiol. Biophys. 5 (1986) 315–329.
[13] F. Jelen, M. Tomschik, E. Paleček, Adsorptive stripping square wave voltammetry of DNA, J. Electroanal. Chem. 423 (1997) 141–148.
[14] L. Trnkova, R. Kizek, O. Dracka, Application of elimination voltammetry to adsorptive stripping of DNA, Electroanalysis 12 (2000) 905–911.
[15] O. Dracka, Theory of current elimination in linear scan voltammetry, J. Electroanal. Chem. 402 (1996) 19–28.
[16] L. Trnkova, Electrochemical elimination methods, Chem. Listy 95 (2001) 518–527.
[17] L. Trnkova, Electrochemical behavior of DNA at a silver electrode studied by cyclic and elimination voltammetry, Talanta 56 (2002) 887–894.
[18] L. Trnkova, R. Kizek, O. Dracka, Elimination voltammetry of nucleic acids on silver electrodes, Bioelectrochemistry 55 (2002) 131–133.
[19] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: a Laboratory Manual, 1987.
[20] M.U.A. Bromba, H. Ziegler, Application hints for Savitzky–Golay digital smoothing filters, Anal. Chem. 53 (1981) 1583–1586.
[21] J. Vacek, Z. Andrysik, L. Trnkova, R. Kizek, Determination of azidothymidine—an antiproliferative and virostatic drug by square-wave voltammetry, Electroanalysis 16 (2004) 224–230.
[22] L. Trnkova, O. Dracka, Elimination voltammetry. Experimental verification and extension of theoretical results, J. Electroanal. Chem. 413 (1996) 123–129.
[23] L. Trnkova, I. Postbieglova, F. Jelen, Application of elimination voltammetry to the resolution of adenine and cytosine signals in oligonucleotides: I. Homooligonucleotides A9 and C9, Electroanalysis 15 1529–1535.