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SENSITIVE VOLTAMMETRIC METHOD FOR DETERMINATION OF CHEMOTHERAPEUTIC DRUG METHOTREXATE USING LIQUID MERCURY FREE SILVER SOLID AMALGAM ELECTRODE

Lenka BANDŽUCHOVÁ[1], Renáta ŠELEŠOVSKÁ and Jaromíra CHÝLKOVÁ
Department of Environmental and Chemical Engineering,
The University of Pardubice, CZ–532 10 Pardubice

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Voltammetric behaviour of a wide-spread used chemotherapeutic drug methotrexate (MTX) was investigated on a polished silver solid amalgam electrode (p-AgSAE) employing various voltammetric methods like cyclic voltammetry, direct current voltammetry and differential pulse voltammetry. It was found that MTX provided two reduction and one oxidation peak in an acidic medium, and the positively situated reduction signal (E_p = –340 mV), due its favorable analytical properties, was studied more in detail. 0.05 mol l^{-1} acetate buffer of pH 5 was selected as an optimum medium for monitoring of the MTX reduction on p-AgSAE. Operating parameters of differential pulse voltammetry were optimized and proposed voltammetric method provided high repeatability (relative standard deviation of 11 repeated measurements at concentration level of methotrexate of 1×10^{-7} mol l^{-1} equaled to 1.7 %), linear concentration range

[1] To whom correspondence should be addressed.

from 5×10^{-10} to 3×10^{-6} mol l^{-1} and low limit of detection (LD = 1.5×10^{-10} mol l^{-1}). The applicability of proposed method was verified by analysis of real sample of chemotherapeutic preparation.

Introduction

Methotrexate (MTX, Amethopterin) is chemical compound based on pteridine and its structure is very similar to the structure of another important biologically active substance — folic acid (FA). MTX differs only in a methyl group connected to the amino group of amino benzoic acid (N(10)) and the amino group substituted on C(4) on the pteridine cycle (comparison of their structures is shown in Fig. 1). MTX, often called antifolate, competitively inhibits the enzyme dihydrofolate reductase (E.C. 1.5.1.3.), which leads to a disruption of the metabolism of FA and reducing of its biological active derivatives (e.g., tetrahydrofolates) concentration [1,2]. This effect is used for treatment of various diseases and MTX has demonstrated effective antineoplastic activity. It is used in the treatment of certain forms of cancer, psoriasis, rheumatoid arthritis, lupus, inflammatory bowel disease or scleroderma, e.g., described in Refs [2-8]. MTX is less toxic than other chemotherapeutic drugs. Moreover, MTX is the only cytostatic, which has an antagonist – tetrahydrofolate leucovorin (LV), which can minimize side-effects of MTX and, therefore, MTX can be administrated even in high dosage by particular dosing schedule, e.g., Refs [1,2,7,8].

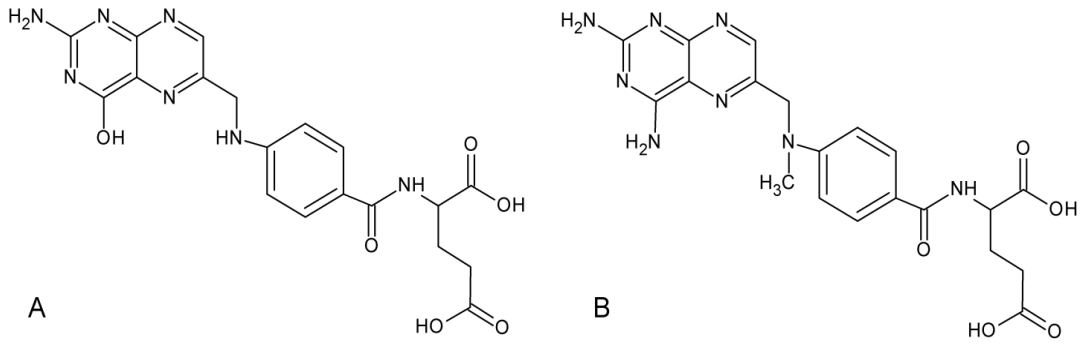

Fig. 1 Chemical structure of folic acid (A) and methotrexate (B)

Various analytical methods for determination of MTX have already been developed due its biological significance. The most widely used analytical method for MTX determination is liquid chromatography (LC) and high-performance liquid chromatography (HPLC) with various detections like UV [11-14], fluorimetry [15-17] or mass spectrometry detection [18,19]. Other analytical techniques like spectrophotometry [20], capillary zone electrophoresis [21] or various immunoassay methods [22,23] have been successfully used for MTX

analysis as well.

Electrochemical methods represent a good alternative to the above mentioned analytical approaches, especially due to the low costs of instrumentation, fast and sensitive analysis and possibility of miniaturization. MTX is electrochemically active compound, and its polarographic and voltammetric behaviour was studied mainly with mercury electrodes. Firstly, Asahi focused on the polarographic behaviour of MTX using dropping mercury electrode (DME) in 1959 [24]. More than 20 years later, Gurira and Bowers clearly described electrochemical processes of MTX and its main metabolite 7-hydroxymetothrexate (7-OHMTX) on DME and hanging mercury drop electrode (HMDE) [25,26]. Afterwards, several papers dealt with the voltammetric determination of MTX on mercury electrodes especially on HMDE in the conjunction with cyclic (CV) and differential pulse voltammetry (DPV) [27] or adsorptive stripping (AdS) DPV [28,29]. Ordieres *et al*. applied also a.c. polarography for the analysis of MTX on DME [30]. Recently, some papers have been also focused on the voltammetric determination of MTX using solid or paste working electrodes, e.g., carbon based electrodes [28,31-35] and bismuth film electrode [36]. The development of new electrode materials, which can compete with mercury electrodes, is highly current trend in the modern electroanalytical chemistry. However, mercury is still one of the most commonly used electrode material due to its excellent electrochemical properties, e.g., high hydrogen overvoltage, renewing of a working surface after every analysis by making of a new drop, etc. It has also some disadvantages like poor mechanic stability and alleged toxicity of the liquid mercury [37]. The silver solid amalgam electrodes (AgSAE) represent an intermediate step between mercury and solid electrode and combine advantages of mercury and solid working electrodes [38-40]. Various types of AgSAE based on the modification of the working surface exist: polished (p-AgSAE), mercury meniscus modified (m-AgSAE) or mercury film modified (MF-AgSAE). The polished modification, which was used as a working electrode in the present paper, is totally liquid mercury free modification of AgSAE and it represents "green" alternative to the mercury electrodes with very similar electrochemical properties [41]. In last years, our research group focused on the voltammetric analysis of bioactive compounds including the group of folates (FA and LV) or structurally similar compounds (MTX) with m-AgSAE [42-44] and p-AgSAE [43,45], respectively. It was found, that AgSAEs introduce suitable alternative tool for voltammetric analysis of the bioactive compounds.

The possibility of the voltammetric analysis of MTX with liquid mercury free p-AgSAE is firstly described in the present paper. Working conditions of DPV were found and proposed method was successfully applied for the determination of MTX content in a pharmaceutical preparation.

Experimental

Materials

All the chemicals used for preparation of the standard solutions, supporting electrolytes and other stock solutions were of p.a. purity. All the solutions were prepared in distilled water. Methotrexate (Lachema, Brno, the Czech Republic) was dissolved in 0.01 mol l^{-1} solution of NaOH (Lachema, Brno, the Czech Republic) and then was stored in the dark in a refrigerator. The analyzed solutions were prepared by a dilution of the stock solution with distilled water daily. Britton–Robinson buffer (BR) of a pH value from 3 to 12 was prepared from an alkaline component of 0.2 mol l^{-1} NaOH (Lachema, Brno, the Czech Republic) and an acidic component consisting of H_3PO_4, H_3BO_3 and CH_3COOH (all Lachema, Brno, the Czech Republic) of the same concentration (0.04 mol l^{-1}). 0.05 mol l^{-1} acetate buffer was prepared by mixing 0.5 mol l^{-1} sodium acetate and 0.5 mol l^{-1} acetic acid (both Sigma Aldrich) and diluted with distilled water to the required concentration. "Methotrexate Lachema 2.5" tablets were produced by Lachema, Brno, the Czech Republic. Solution of KCl required for the activation of the working electrode was prepared by dissolving of the suitable amount of KCl powder (Lachema, Brno, the Czech Republic) in distilled water.

Instrumentation

Voltammetric measurements were performed with the computer controlled Eco-Tribo Polarograph (Polaro-Sensors, Prague, the Czech Republic), equipped with a POLAR.PRO software for Windows XP, version 5.1. All experiments were carried out in the three electrodes set up, where p-AgSAE with the working surface of 0.28 mm^2 (Eco-Trend Plus, Prague, the Czech Republic) served as a working electrode, Ag/AgCl/saturated KCl electrode was used as a reference and platinum wire as an auxiliary electrode (both from Monokrystaly, Turnov, the Czech Republic). Oxygen was removed from the measured solution by bubbling with nitrogen (purity class 4.0; Linde, Prague, the Czech Republic) for 5 min. The values of pH were measured using pH-meter Hanna 221 (Hanna Instruments, Inc., USA) and solution of MTX sample was prepared by applying an ultrasonic bath Bandelin Sonorex (Schalltec GmbH, Germany). All the measurements were performed at laboratory temperature (23 ± 2 °C).

Voltammetric Measurements

Cyclic (CV) and direct current voltammetry (DCV) were used for the examination of the influence of pH value and scan rate, respectively, on the MTX responses recorded on p-AgSAE. DPV with pulse width of 80 ms, pulse height of –50 mV and with a scan rate of 20 mV s^{-1} was used for determination of MTX. Other parameters of DPV were optimized. 0.05 mol l^{-1} acetate buffer (pH 5) was used as the supporting electrolyte for measurements. Purified nitrogen had been passed through the solution for a period of 300 s prior to the measurements and then nitrogen atmosphere was maintained above the solution in the cell. DPV as well as DC peaks were evaluated from the straight line connecting the minima before and after the peak (tangent to the curve joining the beginning and end of a given peak).

The limits of decision (*LC*), detection (*LD*) and quantification (*LQ*) were calculated using K*Sigma method [46] and the parameters of calibration curves (e.g., slope, intercept) were calculated using software Excel 2010 (Microsoft, USA).

Preparation of Silver Solid Amalgam Electrode for Measurements

A silver solid amalgam electrode was abraded on a soft emery-paper at first and then polished using the polishing kit (Elektrochemické detektory s.r.o., Turnov, the Czech Republic) consisting of polishing polyurethane pad, Al_2O_3 suspension (particle size 1.1 mm), and soft polishing Al_2O_3 powder (particle size 0.3 mm). It is suitable to polish the working surface once per week in the case of long term measurements. Before beginning of the work, as well as after every pause longer than 1 hour, the electrode surface was activated in the solution of 0.2 mol l^{-1} KCl by applying –2200 mV vs Ag/AgCl/sat. KCl electrode for 300 s while the solution was stirred. The regeneration step of the electrode surface was inserted directly into the particular measuring method used in the software Polar 5.1, and it consisted of the 30 regeneration cycles between 0 and –1600 mV (the limiting potentials were kept for 0.3 s). The regeneration was realized directly in the analyzed medium.

Real Sample Solution Preparation

One tablet of "Methotrexate Lachema 2.5" was powdered in a grinding mortar, and then the sample was quantitatively transferred into a 100 ml standard flask and was dissolved in 0.01 mol l^{-1} NaOH. It was filtered after previous sonication and the aliquot (9 μl) of the clear filtrate was filled up with the supporting electrolyte

to 10 ml. The supposed methotrexate concentration in the analyzed solution was 5×10^{-8} mol l^{-1}. The content of MTX in tablets was evaluated by the standard addition method, when 50 μl of 1×10^{-5} mol l^{-1} standard solution of MTX was added.

Results and Discussion

Effect of Supporting Electrolyte on Voltammetric Behavior of MTX

Generally, the supporting electrolyte plays an important role in an electrode reaction of studied analyte, because it can modify the thermodynamics and kinetics of electrochemical processes and charge transfer within the cell. The influence of pH of the supporting electrolyte on the voltammetric behaviour of 1×10^{-5} mol l^{-1} MTX was examined using CV in BR buffer of pH values varied from 3 to 12. It was found that MTX provided 2 reduction (cathodic) signals (E_{p1} = –350 mV, E_{p2} = –760 mV in medium of pH 5) in acidic and neutral solutions (pH from 3 to 7) and at higher pH values, the second peak rapidly decreased and disappeared. The only one oxidation (anodic) signal ($E_{p1'}$ = –320 mV; pH 5) could be recorded in the whole tested range of pH values. The reversible electrode process can be supposed, from the difference between positions of the first reduction and belonging oxidation peak and assumption that the electrode process involves $2e^-/2H^+$ exchange based on the reference [25]. These results are consistent with the literature, where Gurrira *et al*. described that MTX provided three $2e^-/2H^+$ reduction signals in a neutral and an acidic medium and simple reduction response in alkaline solutions on mercury electrodes. The oxidation signal was also recorded in the whole tested range of pH [25]. The third reduction peak obtained on mercury electrodes [25] or electrodes with mercury surface [44] could not be recorded due to the narrower potential window of p-AgSAE and it was probably overlapped by signal of the decomposing of the supporting electrolyte. The cyclic voltammogram of MTX recorded in medium of pH 5 (bold line) and pH 8 (thin line) is depicted in Fig. 2.

Recorded reduction signals of MTX were strongly dependent on the pH value, which is obvious from Fig. 3A. The highest current response of peak 1, which was selected for further analytical examination due to its good shape and easy evaluability, was recorded in medium of pH 5 (Fig. 3A). Therefore, BR of pH 5 and 0.05 mol l^{-1} acetate buffer (pH 5) was chosen for further examination and it was ascertained that the followed reduction peak of MTX was more stable and intensive in the acetate buffer, which was used as a supporting electrolyte for all the subsequent experiments. The positions of the reduction signals also varied with changing pH value. The peaks linearly moved to the more negative potentials with increasing pH values, and these dependences are illustrated in Fig. 3B with particular equations.

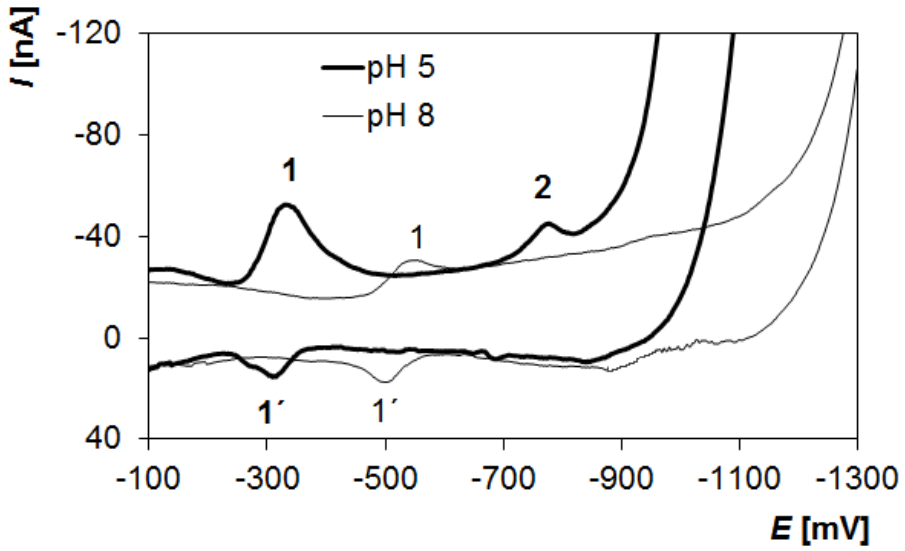

Fig. 2 Cyclic voltammogram of 1×10^{-5} mol l^{-1} MTX in BR buffer of pH 5 and 8 recorded on p-AgSAE. Method: CV; parameters: E_{in} = 0 mV, E_{fin} = –1300 mV, v = 100 mV s^{-1}. Legend: 1, 2 – reduction signals of MTX, 1´ – oxidation signal of MTX

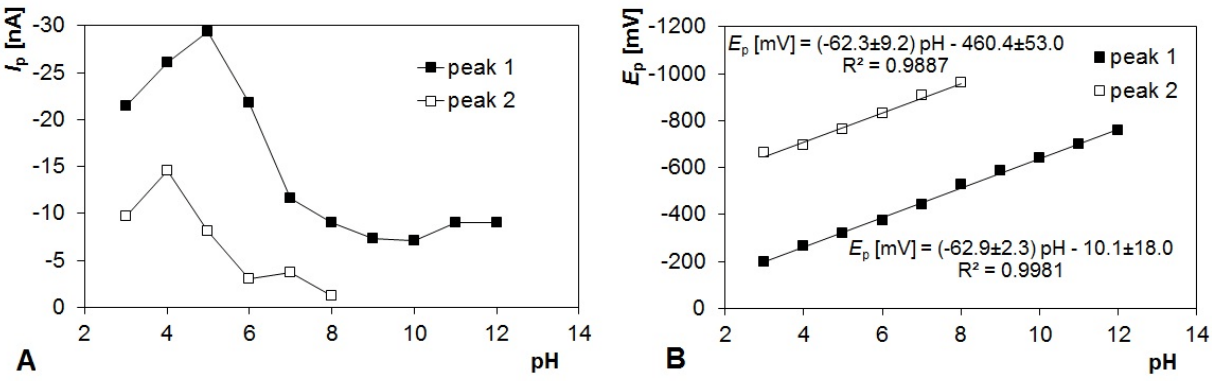

Fig. 3 Dependence of current response (A) and peak position (B) of 1×10^{-5} mol l^{-1} MTX on pH value of supporting electrolyte recorded on p-AgSAE. Method: CV; parameters: E_{in} = 0 mV, E_{fin} = –1300 mV, v = 100 mV s^{-1}. Supporting electrolyte: BR buffer of pH from 3 to 12

Scan Rate Effect on Voltammetric Behaviour of MTX

Useful information including the electrochemical mechanism (rate-limiting step) may be obtained from the dependence of the peak current on the scan rate. The effect of scan rate on the voltammetric behaviour of 5×10^{-6} mol l^{-1} MTX was examined employing DCV in the range of scan rates (v) from 10 to 300 mV s^{-1} and the obtained voltammetric curves are shown in Fig. 4. From the inset of Fig. 4, it is obvious that the reduction peak 1 increased linearly with a growing scan rate

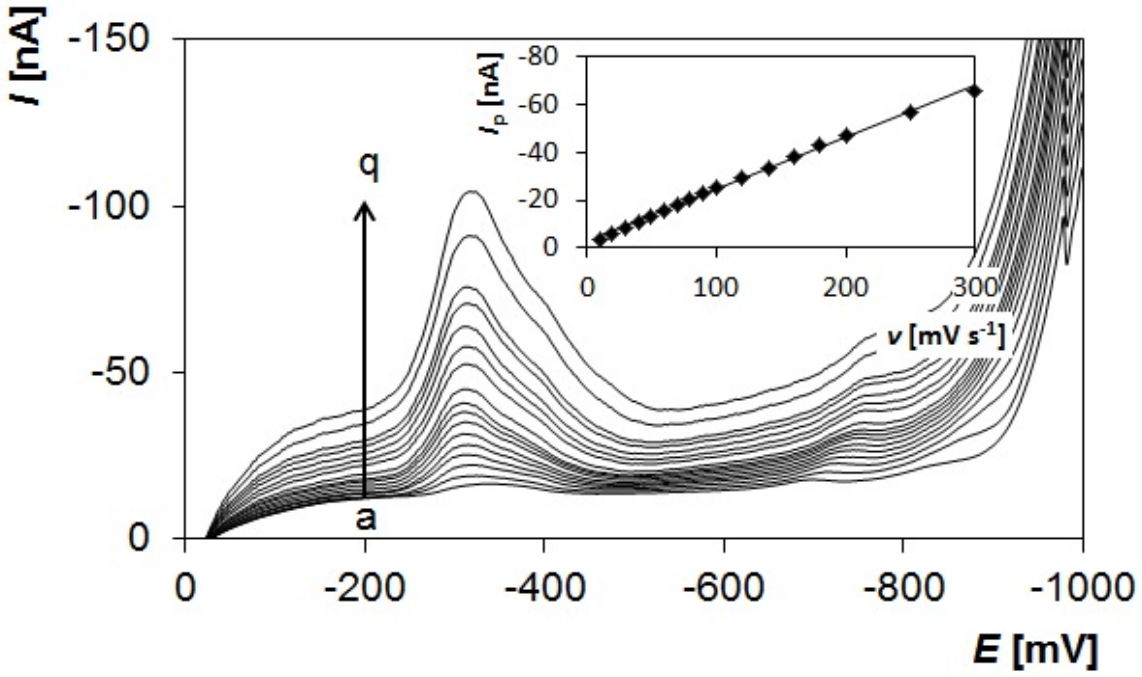

Fig. 4 Voltammetric curves of 5×10^{-6} mol l^{-1} MTX at different scan rates obtained on p-AgSAE. Method: DCV; parameters: E_{in} = 0 mV, E_{fin} = –1000 mV, v = 10-300 mV s^{-1}; supporting electrolyte: 0.05 mol l^{-1} acetate buffer (pH 5). Legend: a – 10, b – 20, c – 30, d – 40, e – 50, f – 60, g – 70, h – 80, i – 90, j – 100, k – 120, l – 140, m – 160, n – 180, o – 200, p – 250 and q – 300 mV s^{-1}

and this dependence could be approximated by Eq. (1) with a coefficient of determination $R^2 = 0.9974$. This result suggested an adsorption-control electrode process. The "log(I_p) - log(v)" analysis was applied for the elucidation of controlling processes of the reduction as well. The linear dependence between log(I_p) and log(v) was obtained and the slope of the equation $k = (0.8770 \pm 0.0079)$ is not equal to 1 or 0.5 with $p \leq 0.05$. Therefore, it can be assumed that the investigated process is more complicated and cannot be characterized as an adsorption controlled only or as diffusion controlled only.

$$I_p[\text{nA}] = (-0.210 \pm 0.012)v[\text{mV s}^{-1}] - 3.3 \pm 1.2 \quad (1)$$

Analysis of Model Solutions

DPV is considered to be an effective electrochemical technique, which has already been applied to analysis of numerous biologically and electrochemically active compounds. The optimization of DPV working parameters influencing the current response of analyte is an important step in the development of electroanalytical methodology. Accordingly, the instrumental parameters such as initial potential (E_{in}), potential of accumulation (E_{acc}), time of accumulation (t_{acc}) and parameters of electrochemical regeneration of the p-AgSAE working surface were examined

in order to optimize the experimental set-up for determination of MTX. All the experiments (except investigations of accumulation time) were carried out at 1×10^{-7} mol l^{-1} MTX in 0.05 mol l^{-1} acetate buffer.

To ensure the ideal reproducibility of the registered current signals on p-AgSAE, it was necessary to optimize the parameters of the regeneration process at the beginning. The regeneration step was inserted directly into the controlling software before every analysis and it was performed right in the analyzed solution. Two mechanisms of the surface regeneration were tested: application of a constant negative potential for a given time and few tens of polarizing cycles. The second mechanism proved higher efficiency for regeneration of the electrode surface of p-AgSAE and the optimum regeneration process included the application of 30 cycles between –1600 mV and 0 mV, when the limiting potentials were kept for t_{reg} = 0.3 s. The efficiency of the surface regeneration was confirmed by repeated measurements and the relative standard deviation of 11 repeated measurements (RSD_M(11)) on the same p-AgSAE surface was calculated as 1.68 %. The achieved value was fully comparable with that obtained for HMDE (described in our previous paper [44]), where the surface regeneration was realized by removing of the old and formation of the new mercury drop. The RSD_M(11) of 1×1^{-10} mol l^{-1} MTX measured on HMDE amounted to 1.75 %. Thus, it can be concluded that the supposed regeneration mechanism provided high efficiency for MTX voltammetric analysis on p-AgSAE.

Subsequently, the influence of the initial potential was examined in the range of potentials from 200 to –200 mV. The change of this parameter did not influence the followed response significantly (±3 %) and thus E_{in} = 0 mV was chosen for all next experiments, especially due to the duration of the analysis. It could be supposed, due to the fact, that the reduction of MTX is partly influenced by an adsorption (discussed more in detail in the previous chapter), that MTX could be adsorbed on the p-AgSAE working surface at suitable accumulation potential. Thus, the effect of E_{acc} on the response of MTX was investigated, while the parameter varied from 0 to –600 mV. The highest current response was found, when E_{acc} = 0 mV was utilized, and application of lower accumulation potentials caused decline of the current response. Thereby, this value of E_{acc} was employed for all next experiments. The possibility of preconcentration and thus increasing sensitivity of the method was also confirmed, when the accumulation time was extended and the followed peak was increased, even linearly in some range of accumulation time. As an example should serve Eq. (2), which describes linear dependence between current response of 1×10^{-7} mol l^{-1} MTX and accumulation time, when the t_{acc} = 0-40 s. Further prolongation led to a non-linear growth of the current response and the dependence reached some limiting current value (in this case the limiting value is reached by application of t_{acc} about 80 s). A suitable accumulation time was searched for every determined concentration of MTX

$$I_p\,[\mathrm{nA}] = (-0.181 \pm 0.010)\,t_{acc}\,[\mathrm{s}] - 2.69 \pm 0.28 \qquad R^2 = 0.9940 \qquad (2)$$

Table I Results of repeated determinations of MTX in model solutions

Added, mol l^{-1}	Found*, mol l^{-1}	Recovery, %	$RSD_D(5)$, %
1.000×10^{-8}	$(1.001 \pm 0.010)\times10^{-8}$	99.1-101.1	1.9
5.000×10^{-9}	$(4.990 \pm 0.120)\times10^{-9}$	97.4-102.2	0.4
1.000×10^{-9}	$(1.020 \pm 0.020)\times10^{-9}$	100.0-104.0	3.4

* From 5 repeated determinations

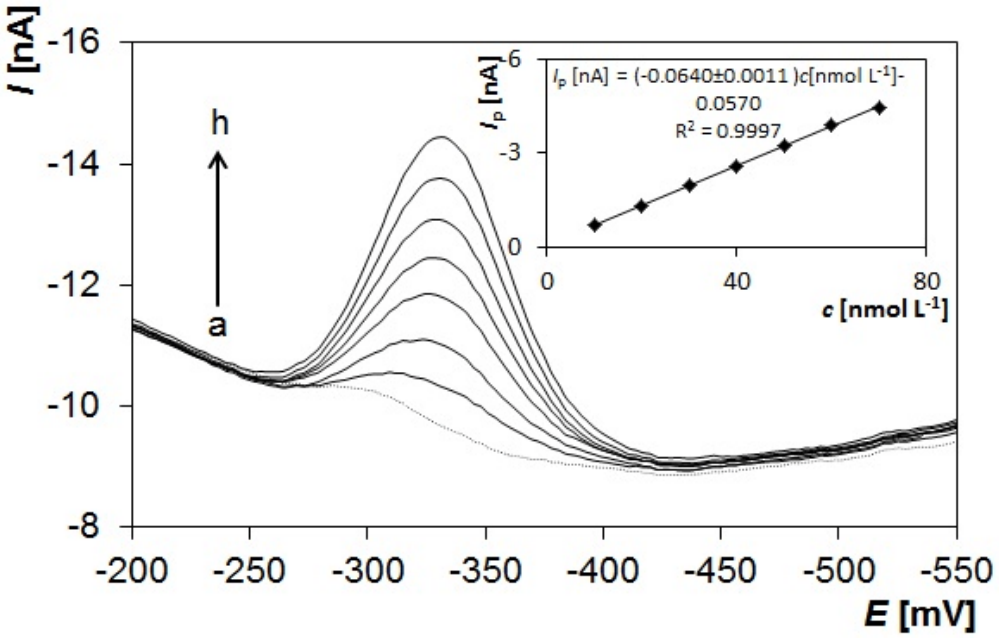

Fig. 5 Voltammetric curves of various concentrations of MTX recorded on p-AgSAE. Method: DPV; parameters: $E_{in} = 0$ mV, $E_{fin} = -1000$ mV, $E_{acc} = 0$ mV, $t_{acc} = 20$ s, $v = 20$ mV s^{-1}, No. of regeneration cycles: 30, $E_{reg1} = 0$ mV, $E_{reg2} = -1600$ mV, $t_{reg1,2} = 0.3$ s, peak height: –50 mV, peak width: 80 ms; supporting electrolyte: 0.05 mol l^{-1} acetate buffer (pH 5). Legend: a – 0, b – 1×10^{-8}, c – 2×10^{-8}, d – 3×10^{-8}, e – 4×10^{-8}, f – 5×10^{-8}, g – 6×10^{-8} and h – 7×10^{-8} mol l^{-1}. Inset: Dependence of height of MTX peak (I_p) on its concentration (c)

The found operating parameters of DPV were applied to determination of various concentrations of MTX in the model solutions. The obtained results and particular relative standard deviations of 5 repeated determinations ($RSD_D(5)$) are summarized in Table I. It is obvious that the obtained results are correct and accurate. An example of the concentration dependence of 1×10^{-8}-7×10^{-8} mol l^{-1} MTX recorded on p-AgSAE is shown in Fig. 5. It is evident, from the inset of Fig. 5, that the I_p grew linearly with increasing concentration of MTX in the analyzed

solution over the whole tested concentration extent. The concentration range, in which the height of the followed peak increased linearly (linear dynamic range (*LDR*)), was found for p-AgSAE from 5×10^{-10} to 3×10^{-6} mol l^{-1}. Due to the application of various accumulation times, it was not possible to measure this dependence in one analysis.

Other statistical parameters like limit of decision (*LC*), limit of detection (*LD*) and limit of quantification (*LQ*) were calculated and are as follows: $LC = 8.0\times10^{-11}$ mol l^{-1}, $LD = 1.5\times10^{-10}$ mol l^{-1} and $LQ = 2.2\times10^{-10}$ mol l^{-1}. The low value of *LD* confirms the high sensitivity of proposed method, and it can be concluded that the proposed procedure proved to be suitable for a precise detection and quantification of MTX.

Table II Comparison of various voltammetric methods published in literature with our proposed technique for the voltammetric determination of MTX

Working electrode	Voltammetric technique	Supporting electrolyte	E_p, mV
DME	a.c. polar.	BR (pH 4)	–500 (vs. Ag/AgCl/sat. KCl)
HMDE	DPV	BR (pH 9.2)	–820 (vs.SCE)
HMDE	AdSDPV	PBS (pH 2.5)	–510 (vs. Ag/AgCl/sat. KCl)
HMDE	AdSDPV	ABS (pH 5.5)	–650 (vs. Ag/AgCl/3 mol l^{-1} KCl)
HMDE	AdSDPV	BR (pH 8)	–500 (vs. Ag/AgCl/sat. KCl)
CPE	AdSDPV	PBS (pH 4)	+750 (vs. Ag/AgCl/sat. KCl)
GCE	SWV	ABS (pH 3.6)	+860 (vs. SCE)
DNA-modif. GCE (ox.)	SWV	BR (pH 2.0)	+900 (vs. SCE)
PABSA/Q-MWNTs/GCE	DPV	PBS (pH 7.0)	+700 (vs. SCE)
nano-Au/LC/GCE	SWV	BR (pH 2.0)	+920 (vs. SCE)
BiFE	AdSDPV	PBS (pH 6.0)	–600 (vs. Ag/AgCl/3 mol l^{-1} KCl)
m-AgSAE	DPV	BR (pH 5)	–350 (vs. Ag/AgCl/sat. KCl)
p-AgSAE	AdSDPV	ABS (pH 5)	–340 (vs. Ag/AgCl/sat. KCl)
DME	0.005-40.000	0.300 (in serum)	human serum
HMDE	0.010-10.000	0.002	-
HMDE	0.025-0.250	0.002	human urine

Table II – Continued

Working electrode	*LDR* μmol l^{-1}	*LD* μmol l^{-1}	Analyzed samples	Ref.
HMDE	0.044-1.590	0.008	human serum	[29]
HMDE	-	0.00044	pharmaceutical preparation	[44]
CPE	-	0.010	-	[28]
GCE	0.80-20.00	0.350	human urine	[32]
DNA-modif. GCE (ox.)	0.020-4.000	0.005	pharmaceutical preparation, human urine	[33]
PABSA/Q-MWNTs/GCE	0.10-8.00	0.015	human urine	[34]
nano-Au/LC/GCE	0.040-2.000	0.010	tablets, human serum	[35]
BiFE	0.012-1.650	0.0009	pharmaceutical preparation	[36]
m-AgSAE	0.002-1.000	0.0018	pharmaceutical preparation	[44]
p-AgSAE	0.001-3.000	0.00015	pharmaceutical preparation	present paper

Abbreviations: DME – dropping mercury electrode, a.c. polar. – alternating current polarography, BR – Britton–Robinson buffer, sat. – saturated, HMDE – hanging mercury drop electrode, DPV – differential pulse voltammetry, SCE – saturated calomel electrode, AdSDPV – adsorptive stripping differential pulse voltammetry, CPE – carbon paste electrode, PBS – phosphate buffer solution, SWV – square wave voltammetry, ABS – acetate buffer solution, DNA-modif. GCE (ox.) – pretreated glassy carbon electrode modified by deoxyribonucleic acid, PABSA/Q-MWNTs/GCE – *p*-aminobenzene sulfonic acid on quaternary amine functionalized multi-wall carbon nanotubes modified glassy carbon electrode, nano-Au/LC/GCE – glassy carbon electrode modified by L-cysteine and gold nanoparticles, BiFE – bismuth film electrode, m-AgSAE – mercury meniscus modified silver solid amalgam electrode, p-AgSAE – polished silver solid amalgam electrode

Comparison of Proposed Method with Other Voltammetric Methods

On the basis of literature survey, the mercury electrodes [27-30,44], carbon based working electrodes [28,32-34] and bismuth film electrode [36] were successfully applied in voltammetric analysis of MTX. Our research group also described possibilities of m-AgSAE in the determination of this chemotherapeutic drug [44].

The comparison between the analytical performance of our proposed method and other electrochemical methods for the determination of MTX is summarized in Table II. It is obvious that p-AgSAE in combination with DPV provides the lowest *LD* from the compared performances. Moreover, p-AgSAE is made of non-toxic silver amalgam and does not contain liquid mercury [41]. It is also mechanically and chemically stable and its preparation and pretreatment is very simple and time saving in comparison with working electrodes described, e.g., in Refs [33-35]. All these facts confirm excellent electrochemical properties of p-AgSAE and could be convenient for the determination of MTX in real samples.

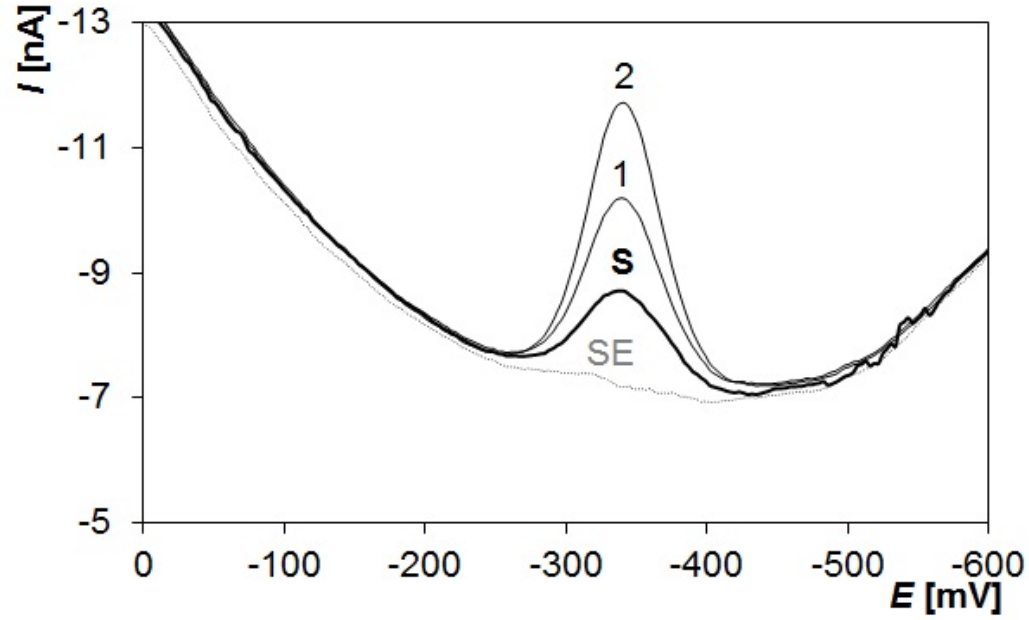

Fig. 6 Example of voltammetric determination of MTX in pharmaceutical preparation "Methotrexate Lachema 2.5". Method: DPV; parameters: E_{in} = 0 mV, E_{fin} = –1000 mV, E_{acc} = 0 mV, t_{acc} = 5 s, v = 20 mV s^{-1}, No. of regeneration cycles: 30, E_{reg1} = 0 mV, E_{reg2} = –1600 mV, $t_{reg1,2}$ = 0.3 s, peak height: –50 mV, peak width: 80 ms; supporting electrolyte: 0.05 mol l^{-1} acetate buffer (pH 5). Legend: SE – curve of the supporting electrolyte, S – curve after addition of the sample solution (9 μl), 1, 2 – curves after addition of 50 μl 1×10^{-5} mol l^{-1} MTX standard solution

Analysis of Real Sample

The applicability of the proposed method was verified by analysis of tablets "Methotrexate Lachema 2.5" with declared content of MTX of 2.500 mg per tablet. The sample solution was prepared as described in Experimental and the standard addition method was utilized for the determination of MTX. The determined content of MTX was (2.499 ± 0.012) mg per tablet (99.48-100.44 %

recovery) and this result was obtained as an average value from the 5times repeated determinations with $RSD_D(5)$ amounted to 0.7 %, which demonstrates an accurate and precise result of the determination. An example of the obtained voltammetric curves is illustrated in Fig. 6.

Conclusion

The polished silver solid amalgam electrode, the liquid mercury free modification of AgSAE, was firstly used for the voltammetric analysis and determination of important chemotherapeutic drug — methotrexate. It was proved that this working electrode in the combination with DPV provided a suitable electrochemical tool for the determination of MTX. Moreover, the sensitivity could be increased by insertion of accumulation step before the analysis and thus it was possible to reliably and rapidly detect and determine nanomolar amounts of MTX. The obtained *LD* is much lower or fully comparable with those previously described in the literature (summary in Table II). Thereby, it can be concluded that the proposed voltammetric method can undoubtedly be considered as an effective, sensitive and environmentally acceptable tool in the analysis of MTX and other folates as well as it may represent the electrochemical alternative to mercury electrodes.

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