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ORIGINAL PAPER

Voltammetric determination of leucovorin in pharmaceutical preparations using a boron-doped diamond electrode

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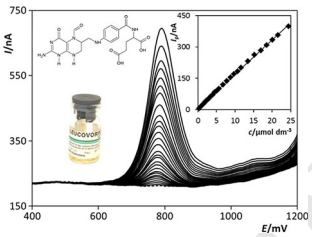
Abstract

Method for voltammetric determination of leucovorin, a drug frequently applied to decrease some unfavorable effects of anticancer drugs such as methotrexate or to increase the therapeutic effect of 5-fluorouracil, has been developed employing a bare boron-doped diamond electrode. It is the first method for leucovorin determination based on its electrochemical oxidation. Although at least three anodic and three cathodic voltammetric peaks could be recorded under the used conditions, only the anodic response situated at about + 900 mV (vs. saturated Ag|AgCl electrode) was suitable, namely due to its shape and position, for analytical purposes. Using differential pulse voltammetry with optimized parameters and supporting electrolyte of pH 3, the linear dynamic range of leucovorin determination was recorded from 0.15 to 25 μmol dm^{-3}. Under such conditions, low limit of quantification of 0.050 μmol dm^{-3} and limit of detection of 0.015 μmol dm^{-3} as well was reached. Relative standard deviation calculated from 11 repeated measurements amounted to 0.7% and calculated from five repeated determinations amounting less than 3.0%. Applicability of the developed method was verified by repeated analysis of the pharmaceutical preparation with excellent results (recovery 98.7–102.8%, relative standard deviation 1.81%).

Graphical abstract



Keywords Boron-doped diamond electrode · Determination · Folinic acid · Leucovorin · Pharmaceutical samples · Voltammetry

Introduction

Leucovorin (LV) known as folinic acid (5-formyltetrahydrofolate, 5-formyl-H_4folate), which is target analyte of the present paper, is depicted in Fig. 1. It occurs as a racemic mixture, but only its L-form is pharmaceutically active. LV is formed by reduction of folic acid (FA). From a biochemical point of view, LV is a 5-formyl derivative of tetrahydrofolic acid [1, 2]. It has been applied as a drug

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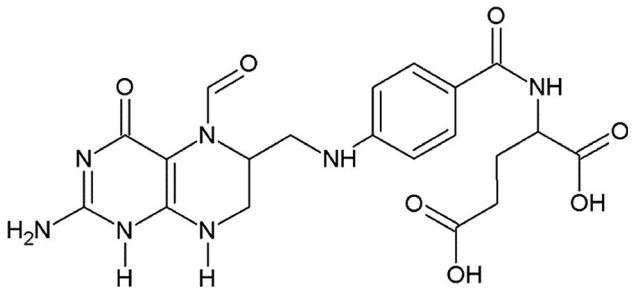

Fig. 1 Structural formula of leucovorin

which is able to decrease unfavorable effects of pyrimethamine or immune system suppressant methotrexate (MTX) [3]. Furthermore, LV in high doses can find its utilization in simultaneous administration with 5-fluorouracil to treat gastric and colorectal carcinoma [4, 5].

As it is evident from the above-mentioned information, it is highly important to determine LV in pharmaceutical products and in body fluids. Various analytical methods have been used for these purposes up to now. From non-electrochemical methods application of mass spectrometry for these purposes can be mentioned [6]. As in other cases, different separation methods have been used most frequently, e.g., high performance liquid chromatography (HPLC) with UV detection [7], with fluorescence detection [8], or with gradient elution with following dual UV–fluorescence detection [9, 10]. Pre-separation of an analyzed sample using solid-phase extraction has been described in literature as well [6, 7, 11]. LV levels in urine or serum samples without pre-separation steps have been also analyzed using spectrophotometric techniques [12, 13]. Some authors have reported the application of capillary zone electrophoresis [14, 15] or of kinetic fluorimetry [16] for LV determination.

On the other hand, only a little attention has been recently paid to the application of electroanalytical methods of LV determination. Using these methods (mainly voltammetry and polarography) FA and its derivatives and metabolites can be easily determined. Because these compounds are electrochemically reducible and oxidizable, respectively, voltammetric techniques could be employed for their analysis. Utilization of the different electrodes have been described in literature sources, e.g., modified carbon electrodes [17, 18], multi-walled carbon nanotube-modified gold electrodes [19], single-walled carbon nanotube–ionic liquid paste electrode [18, 20], mercury electrodes [21], as well as amalgam electrodes [22, 23]. MTX can be analyzed non-electrochemically (e.g., using HPLC [9] as well as electrochemically using different electrodes (e.g., amalgam or boron-doped diamond electrodes) [24, 25] too. Electrochemical behavior and determination of LV was described in details many years ago using dropping mercury electrode (DME) [26] and all of the following works deal also with the utilization of mercury [27] or silver solid amalgam electrodes (AgSAE) [28].

LV reaction mechanisms were described in detail in, e.g., [26–28]. Three oxidation signals were recorded on DME [26]. Heyrovský et al. [27] observed a peak pair (anodic peak at about − 800 mV, cathodic peak at about − 950 mV) on hanging mercury drop electrode (HMDE). Two voltammetric signals corresponding to the oxidation of tetrahydropteridine ring was registered at potentials of − 150 and of 0 mV. The oxidation products, which are adsorbable at the electrode surface, can be reduced at about − 400 mV. This signal was successfully used for LV determination on HMDE [27] as well as on AgSAE [28].

All the above-mentioned voltammetric methods of LV determination are based on its reduction. In the present paper, electrochemical oxidation of LV was studied and the procedure of LV determination on boron-doped diamond electrode (BDDE) was developed. Electrodes based on boron-doped diamond film have been so far successfully applied in the voltammetric analysis of various biologically active compounds, e.g., [29–32]. In the past, there was published the determination of FA [33] and MTX [25] on a bare BDDE using differential pulse voltammetry (DPV). Therefore, this paper focuses on development and verification of an electrochemical method of LV determination on this electrode too. Optimum conditions for DPV determination of LV were found and proposed and this sensitive method was tested by analysis of LV in a commercially available pharmaceutical preparation.

Results and discussion

Voltammetric behavior of leucovorin in dependence on pH

First, cyclic voltammetry (CV) on a bare BDDE [supporting electrolyte Britton–Robinson buffer (BRB)] was utilized to characterize recordable and evaluable voltammetric signals of LV and influence of pH on the shape, position, and number of CV peaks or more correctly waves (Fig. 2). It was found that LV provides two anodic (oxidation) peaks [at pH 5 at about + 900 mV (Fig. 2, peak 1) and at about + 1500 mV (Fig. 2, peak 2)] and two cathodic (reduction) peaks (at pH 5 at about + 800 mV, peak 1′, and + 1300 mV, peak 2′) in a wide range of pH 1–10. Differences between peak potentials of the more positive as well as of the more negative pair of peaks have confirmed their quasi-reversible characters. Presence of these two oxidation and two reduction pairs, respectively, corresponds with earlier published results recorded on DME [26], HMDE [27], or on two modifications of AgSAE

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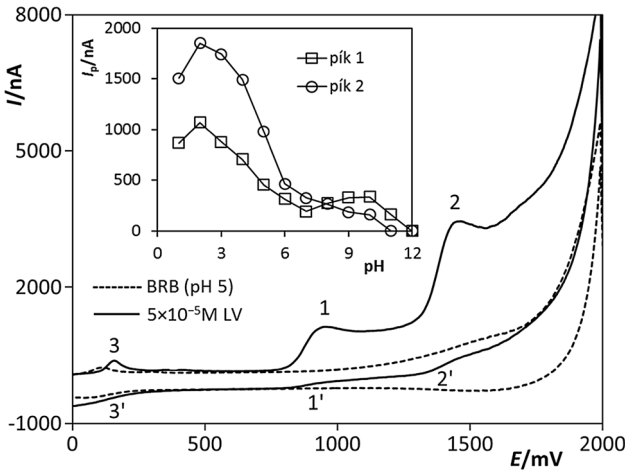

Fig. 2 Cyclic voltammograms of LV recorded on BDDE. Method: CV, supporting electrolyte: BRB (pH 5.0) (dashed line), initial potential (E_{in}) = 0 mV, switch potential (E_{sw}) = + 2000 mV, scan rate (v) = 100 mV s^{-1}, c_{LV} = 50 µmol dm^{-3} (solid line); inset: dependences of chosen anodic peak heights on supporting electrolyte pH values

[28]. Moreover, one pair of small and hardly evaluable peaks (Fig. 2, peak 3 and 3′) was located at about + 150 mV and at about 0 mV, respectively.

It is obvious that the cathodic signals were much smaller than the anodic ones (Fig. 2) independently on tested pH of the supporting electrolyte. Therefore, the anodic signals seemed to be more suitable for analytical purposes. Moreover, peaks 1 and 2 were much higher than signal 3; therefore, we paid attention to them in all subsequent studies. From the evaluation of the dependences of anodic peak heights (I_p) on pH of the supporting electrolytes (Fig. 2, inset), it could be concluded that the highest signal was recorded in BRB of pH 2 for both peaks (BRB was used as the supporting electrolyte in the pH range from 2 to 12 and the H_2SO_4 solution as the supporting electrolyte of pH 1). On the other hand, the repeatability of the anodic peak located at about + 900 mV was not sufficient in this medium (relative standard deviation (RSD) of I_p values evaluated from 11 repeated measurements of 50 µmol dm^{-3} LV achieved 23%). Therefore, BRB with pH of 5 was used for the following experiments focused on the voltammetric behavior of LV in dependence on scan rate. Furthermore, the attention has been paid to the finding a suitable supporting electrolyte pH during the optimization of DPV again.

The influence of scan rate on voltammetric behavior of leucovorin

In the following step, the controlling processes of the registered LV signals were investigated. Therefore, the dependences of peak heights (registered using CV) on applied scan rates (v) was investigated and the obtained curves are displayed in Fig. 3. In the case of all anodic LV signals, almost ideal linear dependences of I_p on the square root of the scan rate (in the range from 25 to 500 mV s^{-1}) were obtained [correlation coefficients (r) = 0.997, 0.996, and 0.998; Eqs. (1)–(3)]. According to these results, it was possible to conclude that all observed processes were diffusion controlled.

$$I_p\ [\mathrm{nA}] = (20.60 \pm 0.39)v^{1/2}[(\mathrm{mV/s})^{1/2}] + (342.5 \pm 6.4), \quad r = 0.997 \tag{1}$$

$$I_p\ [\mathrm{nA}] = (59.6 \pm 1.1)v^{1/2}[(\mathrm{mV/s})^{1/2}] + (258 \pm 19), \quad r = 0.996 \tag{2}$$

$$I_p\ [\mathrm{nA}] = (13.83 \pm 0.21)v^{1/2}[(\mathrm{mV/s})^{1/2}] - (14.0 \pm 3.5), \quad r = 0.998 \tag{3}$$

The realized log–log analyses were linear too (r = 0.997, 0.998, and 0.999), but they revealed that the value 0.5 was not included in any of all calculated slopes of these log–log dependences. In the case of signals 1 and 2, the slope values [0.2117 ± 0.0042 log(nA s mV^{-1}), Eq. (4) and 0.3747 ± 0.0054 log(nA s mV^{-1}), Eq. (5)] were between 0 and 0.5. Therefore, some kinetically controlled process which was independent of scan rate and which participated in a controlling of both registered processes should be taken into account. The slope value of peak 3 [0.5326 ± 0.0068 log(nA s mV^{-1}), Eq. (6)] is very close to the theoretical value 0.5, which can imply simple diffusion controlled process.

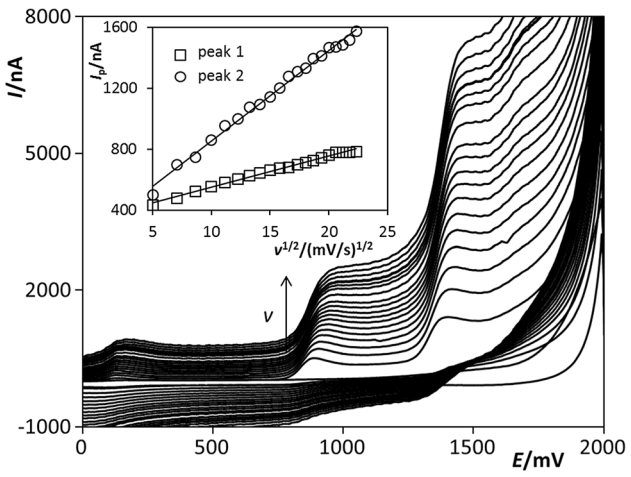

Fig. 3 Cyclic voltammograms of leucovorin obtained on BDDE in dependence on scan rate. Method: CV, supporting electrolyte: BRB (pH 5.0), E_{in} = 0 mV, E_{sw} = + 2200 mV, v = 25–500 mV s^{-1}, c_{LV} = 50 µmol dm^{-3}; inset: dependences of peak heights on square root of scan rates for LV peak 1 and 2, respectively

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$$\log(I_p\ [\mathrm{nA}]) = (0.2117 \pm 0.0042)\log(v[(\mathrm{mV/s})]) + (2.3230 \pm 0.0010), \quad r = 0.997 \tag{4}$$

$$\log(I_p\ [\mathrm{nA}]) = (0.3747 \pm 0.0054)\log(v[(\mathrm{mV/s})]) + (2.185 \pm 0.013), \quad r = 0.998 \tag{5}$$

$$\log(I_p\ [\mathrm{nA}]) = (0.5326 \pm 0.0068)\log(v[(\mathrm{mV/s})]) + (1.031 \pm 0.016), \quad r = 0.999 \tag{6}$$

Determination of leucovorin in model solutions

Finally, for purposes of LV determination, DPV method was applied due to the generally know higher sensitivity of the pulse voltammetric techniques. The anodic DPV peak located at about + 850 mV was used in this respect considering its favorable position and shape. Firstly, it was confirmed that I_p dependence on pH brought us the same conclusions as it was found in the case of CV and the obtained curves are depicted in Fig. 4. Considering clarity of Fig. 4, voltammograms recorded in media of pH values from 1 to 5 are displayed. The highest current peak 1 was observed in BRB of pH 2. Probably due to the higher DPV sensitivity, we were able to reveal that on the positive shoulder of the investigated peak, small and a bit positively situated peak was registered in the most acidic solutions (Fig. 4). This small peak decreased with increasing pH value of the supporting electrolyte and in solutions of pH ≥ 3 completely disappeared. The presence of this peak affected negatively repeatability of recorded signals. Therefore, contrary to the widely accepted theory that most of the compounds are hardly adsorbable on the surface of a BDDE, in our case, presumably, some of the reaction intermediate was adsorbed on the used polycrystalline diamond surface in acidic media (pH < 3) [34, 35].

The obtained findings were confirmed by experiments, with the results depicted in Fig. 5. The most significant DPV anodic peak 1 decreased monotonously with an increasing number of repetitions. Simultaneously, smaller and about 130 mV more positively situated peak, increased monotonously with an increasing number of repetitions. However, no such positively situated peak was observed at pH 3 or higher and peak 1 exhibited almost constant height (Fig. 5, inset). Nevertheless, the small difference between background current of supporting electrolyte and background current under LV presence (Figs. 2, 5) indicated hypothetical adsorption on the diamond surface. The results of repeatability of LV peak current (c_{LV} = 10 mol dm^{-3} in BRB with pH values from 2 to 5) are summarized in Table 1. While the repeatability of the signal was poor in the BRB of pH 2 (RSD_{11} = 8.3%), the results proved to be significantly improved in less acidic media. In the case of pH 3, RSD_{11} of I_p values amounted to 1.9%, and the decrease of average I_p was about 15% only. Therefore, this pH value of the supporting electrolyte was chosen as the most suitable for the analytical purposes, i.e., for LV determination.

The following experiments were focused on the optimization of basic parameters of DPV and are illustrated in

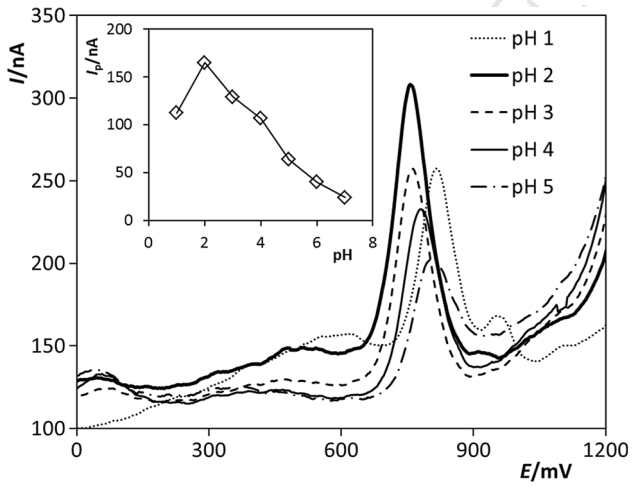

Fig. 4 DP voltammograms of LV obtained on BDDE in dependence on pH. Method: DPV, supporting electrolyte: BRB (pH 1–5), E_{in} = 0 mV, E_{fin} = + 1300 mV, v = 25 mV s^{-1}, pulse height = + 50 mV, pulse width = 70 ms, c_{LV} = 10 µmol dm^{-3}; inset: dependence of I_p (of the DPV anodic peak located at about + 850 mV) on pH of supporting electrolyte

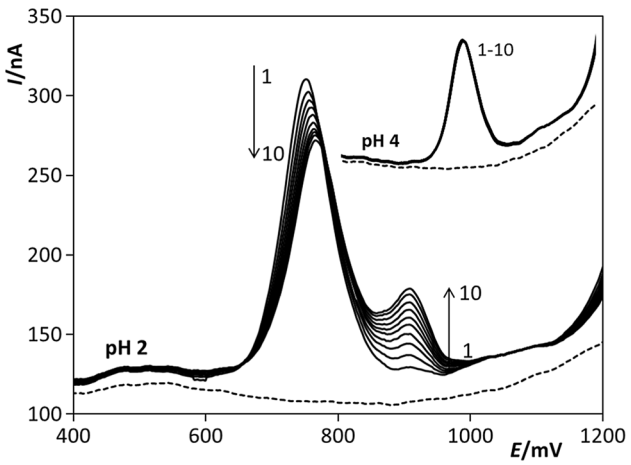

Fig. 5 10 times repeated DP voltammograms of LV recorded using BDDE in BRB (pH 2.0). Inset: 10 times repeated voltammograms of LV recorded using BDDE in BRB (pH 4.0). Method: DPV, E_{in} = 0 mV, E_{fin} = + 1200 mV, v = 25 mV s^{-1}, pulse height = + 50 mV, pulse width = 50 ms, c_{LV} = 10 µmol dm^{-3} (solid lines), supporting electrolyte of pH 2 and 4, respectively (dashed lines)

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Table 1 Repeatability of DPV measurement of 10 μmol dm^{-3} LV in dependence on pH

PH	I_p/nA	RSD$_{10}$/%
2	161.4 ± 8.8	8.3
3	132.8 ± 1.7	1.9
4	107.09 ± 0.62	0.9
5	64.53 ± 0.83	1.9

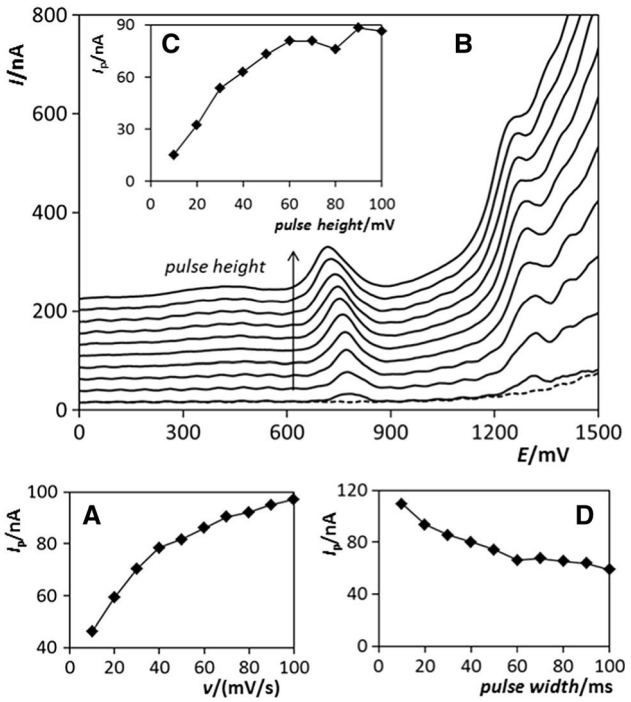

Fig. 6 Optimization of DPV parameters: **a** dependence of I_p(LV) on v, **b** DP voltammograms of LV in dependence on pulse height, **c** dependence of I_p(LV) on pulse height, **d** dependence of I_p(LV) on pulse width. Method: DPV, supporting electrolyte: BRB (pH 3), E_{in} = 0 mV, E_{fin} = + 1500 mV, v = 10–100 mV s^{-1} (**a**), 40 mV s^{-1} (**b**, **c**, **d**), pulse height = + 50 mV (**a**, **d**), + 10–100 mV (**b**, **c**), pulse width = 50 ms (**a**, **b**, **c**), 10–100 ms (**d**), c_{LV} = 5.0 μmol dm^{-3}

Fig. 6. All measurements were realized in LV solution with concentration of 5.0 μmol dm^{-3}. Tested parameters were changed in these ranges: v—10–100 mV s^{-1}, pulse height—+ (10–100) mV, pulse width—10–100 ms and were optimized as follows: v = 40 mV s^{-1}, pulse height = + 50 mV, pulse width = 20 ms (where the current values were registered and averaged in last 20 ms). These parameters were used for all subsequent DPV measurements.

The linear dynamic range of LV determination was found from 0.15 to 25 μmol dm^{-3}. The concentration dependences were linear in different smaller subranges too (summary in Table 2, example in Fig. 7). Reached correlation coefficients were higher than 0.9991 in all cases and the slope values were almost identical. From the registered parameters, it was possible to calculate limit of detection

Table 2 Statistical parameters of LV concentration dependences registered under conditions given in the legend for Fig. 7

c/μmol dm^{-3}	Slope/nA dm^3 μmol^{-1}	Intercept/nA	r
1.0–11.0	16.389 ± 0.062	0.61 ± 0.42	0.9999
0.25–2.8	17.19 ± 0.25	0.55 ± 0.42	0.9991
0.15–1.7	17.201 ± 0.084	0.089 ± 0.086	0.9999
0.3–24.5	16.392 ± 0.03)	5.4976 ± 4.1	0.9996

Confidence intervals calculated at the level of significance α = 0.05

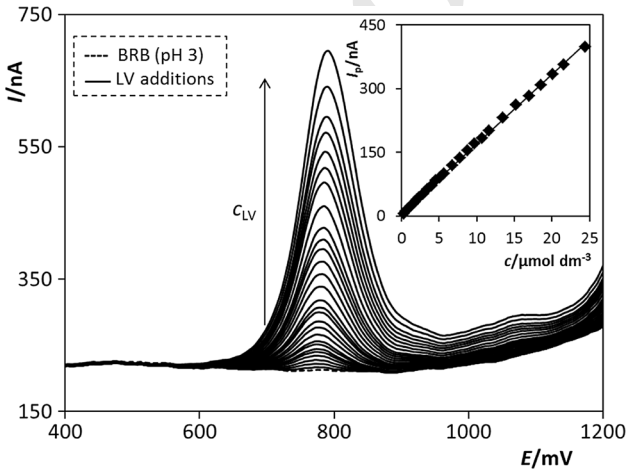

Fig. 7 DP voltammograms of LV obtained on BDDE in dependence on LV concentration. Method: DPV, supporting electrolyte: BRB (pH 3), E_{in} = 0 mV, E_{fin} = + 1200 mV, v = 40 mV s^{-1}, pulse height = + 50 mV, pulse width = 20 ms, c_{LV} = 0.30–24.5 μmol dm^{-3}; inset: dependence of I_p on LV concentration

(LOD) = 0.015 μmol dm^{-3} and limit of quantification (LOQ) 0.050 μmol dm^{-3}, respectively. The values confirmed applicability of the proposed technique also for detection and determination of LV on the low concentration level.

To confirm the applicability of the suggested method for LV determination in a simple model solution of BRB, three solutions of different concentration levels were prepared: 10.0, 3.0, and 0.3 μmol dm^{-3}. Each determination was five

Table 3 Results of five repeated LV determinations in model BRB solutions

Added/μmol dm^{-3}	Found/μmol dm^{-3}	Recovery/%	RSD$_5$/%
10.0	10.10 ± 0.17	98.0–105.0	2.57
3.0	3.030 ± 0.035	98.3–102.6	1.74
0.3	0.3000 ± 0.0029	99.0–102.6	1.46

Used parameters are given in the legend for Fig. 7. Confidence intervals calculated at the level of significance α = 0.05

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times repeated. The achieved results are summarized in Table 3. It could be concluded that all found LV concentrations corresponded to added LV amounts ($\alpha = 0.05$), reached LV recovery amounted to from 98.0 to 105.0% and RSD calculated from all five repeated determinations (RSD_5) was in all of the tested concentration levels < 2.6%.

Determination of leucovorin in pharmaceutical preparation

Finally, the applicability of the above described and developed DPV method of LV determination was verified by analysis of this analyte in a commercial preparation "Leucovorin CA LACHEMA 10". This preparation was an injection powder with declared LV content of 10 mg per vial. The analyzed solution was prepared by dissolving of LV powder in distilled water according to the producer instructions and as it is described in the "Experimental" part of this manuscript in the chapter "Pharmaceutical sample analysis". The LV determination was realized using the standard addition method and repeated five times (Fig. 8). The determined amount of LV 10.08 ± 0.12 mg of LV per vial was in good agreement with declared LV content of 10 mg per vial ($\alpha = 0.05$). RSD of five repeated determinations reached 1.81% and recovery 98.7–102.8%. Therefore, it could be summarized that the suggested method is suitable for analysis of pharmaceutical samples without insertion of any preparation technique. The determination has not been disturbed by the presence either of sodium chloride, sodium hydroxide (present in this preparation in approximately comparable amounts with LV, i.e., 10 and 8 mg, respectively, cf. 10 mg of LV), or of any of other pharmaceutical fillers used.

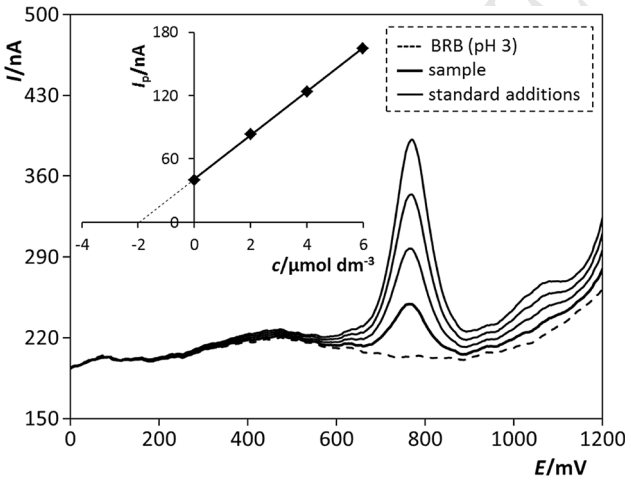

Fig. 8 DPV determination of LV in a pharmaceutical preparation sample using BDDE. Method: DPV, supporting electrolyte: BRB (pH 3), E_{in} = 0 mV, E_{fin} = + 1200 mV, v = 40 mV s^{-1}, pulse height = + 50 mV, pulse width = 20 ms, standard additions: V = 20 mm^3, c_{LV} = 1 mmol dm^{-3}; inset: graphical evaluation of standard addition method

Conclusion

It was confirmed that a bare BDDE, as a working electrode, could be used for voltammetric detection and determination of LV based on its electrochemical oxidation. BRB, particularly of pH 3, proved to be suitable supporting electrolyte. Using either CV or DPV, two anodic and two cathodic significant and well developed voltammetric LV peaks could be recorded (at about + 850 and + 1450 mV) and one pair of small and hardly evaluable peaks (at about + 150 mV). Finally, the DPV anodic peak located at about + 850 mV was found to be suitable for analytical purposes. Its height was the most sensitive to LV concentration changes, it was the best developed and reproducible under optimized conditions. The highest and simultaneously the most reproducible peak was recorded in BRB of pH 3, which was chosen for all other analysis. The DPV method was applied for determination of LV in deionized water (linear dynamic range from 0.15 to 25 µmol dm^{-3}, LOQ 0.050 µmol dm^{-3}, and LOD 0.015 µmol dm^{-3}). Similarly, determination of LV in a commercial pharmaceutical preparation "LEUCOVORIN CA LACHEMA 10" was found to be successful considering the achieved results, which were consistent with the declared LV content (recovery 98.7–102.8%).

It could be concluded, that our proposed method represent simple but very precise and sensitive tool for determination of the important bioactive compound LV in the pharmaceutical samples. It is the first voltammetric method for LV determination based on its oxidation and simultaneously the first described method using non-mercury working electrode.

Experimental

Chemicals

The 1 mmol dm^{-3} solution of LV was prepared by dissolving of the appropriate amount of calcium folinate, European Pharmacopoeia (EP) Reference Standard (Sigma-Aldrich, Czech Republic) in distilled water and stored in the dark at + 4 °C. The analyzed solutions were prepared daily fresh by dilution of the BRB stock solution.

All chemicals used to prepare stock solutions and basic electrolytes were of p.a. purity. BRBs of pH value from 2.0 to 12.0 were prepared from an alkaline component of 0.2 mol dm^{-3} NaOH and an acidic component consisting of 0.04 mol dm^{-3} H_3PO_4, 0.04 mol dm^{-3} H_3BO_3, and

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0.04 mol dm^{-3} CH_3COOH (all these chemicals Lachema, Czech Republic). Solutions of H_2SO_4 were prepared by dilution of concentrated 96% H_2SO_4, p.a. (Ing. Petr Švec-PENTA, Czech Republic) by deionized water. Deionized water (conductivity < 0.05 μS cm^{-1}) produced by Milli-Q-Gradient, Millipore, Prague, Czech Republic, was used for all described measurements.

The pharmaceutical preparation in powder form for injection solution preparation "LEUCOVORIN CA LACHEMA 10" was purchased from Pliva-Lachema, Brno. Declared content of calcium folinate pentahydrate was 12.7 mg (corresponding to 10 mg of LV in 1 cm^3 of prepared injection solution). Moreover, this preparation contained sodium chloride (10 mg) and sodium hydroxide (8 mg).

Instrumentation

The Eco-Tribo Polarograph (Polaro-Sensors, Czech Republic) controlled by POLAR.PRO software (version 5.1, Polaro-Sensors, Czech Republic) and by Multielchem software (version 3.1, J. Heyrovský Institute of Physical Chemistry of the Czech Academy of Sciences, Czech Republic) was used for voltammetric measurements. They were carried out in a three-electrode arrangement where commercially available BDDE (Windsor Scientific, UK, active surface area of 7.07 mm^2, inner diameter of 3 mm, resistivity of 0.075 Ω cm with a *B*/*C* ratio during deposition 1000 ppm) was used as a working electrode. A saturated argent chloride electrode (Ag|AgCl(KCl), sat.) served as a reference electrode and a platinum wire (diameter 1 mm) (both Monokrystaly, Czech Republic) served as an auxiliary electrode.

Accumet pH-meter AB150 (Fisher Scientific, Czech Republic) was used for the pH measurements. All realized experiments were performed at laboratory temperature (23 ± 2 °C).

Voltammetric measurements

At the beginning of every series of measurements, BDDE was activated in 0.5 mol dm^{-3} H_2SO_4 solution by insertion of − 1000 mV for 60 s and of + 2000 mV for 60 s. Then, the electrode surface was rinsed with deionized water. Subsequently, 20 cyclic voltammograms were realized in the potential range from − 1000 to + 2000 mV. A positive regeneration potential (E_{reg}) of + 2000 mV for a regeneration time (t_{reg}) of 5 s was inserted on the used BDDE before the start of each measurement. This step provided the O-terminated surface of the BDDE for the realized measurement and, at the same time, ensured oxidation of the most of the impurities trapped on the electrode surface.

Elucidations of the supporting electrolyte effect (pH) (v = 100 mV s^{-1}) and of the scan rate effect were realized using CV from E_{in} = 0 mV to E_{fin} = + 2000 mV and reversely. Supporting electrolyte was represented either by the solution of H_2SO_4 (pH 1) or by BRB (pH 2–12). The dependence of cyclic voltammograms of LV (c_{LV} = 5 × 10^{-5} mol dm^{-3}) on the scan rate was investigated from 25 to 500 mV s^{-1} in BRB (pH 5).

DPV was applied with the following parameters (if not stated otherwise): E_{in} = 0 mV, E_{fin} = + 1200 mV, v = 40 mV s^{-1}, pulse height = + 50 mV, pulse width = 70 ms (where the current values were registered in last 20 ms), BRB of pH 3, which was chosen based on the study, where supporting electrolyte of pH from 1 to 7 was employed.

The values of LOD and of LOQ were calculated as three times and ten times, respectively, a standard deviation of the blank solution divided by the calculated slope of the calibration curve [36]. The parameters of the calibration curves (i.e., slope, intercept, correlation coefficients) were calculated and all of the graphical dependences were constructed using MS Excel 365 software (Microsoft, USA). All confidence intervals were calculated at the level of significance α = 0.05.

Pharmaceutical sample analysis

A commercially available pharmaceutical preparation "LEUCOVORIN CA LACHEMA 10" (in the powder form), representing a real sample of LV, was after dissolving analyzed using DPV. The declared content was 10 mg of LV per vial. The sample was prepared for analysis according to the manufacturer's instructions. i.e., by dissolving of the vial content in 1 cm^3 of distilled water and further diluted ten times. 10 mm^3 of sample solution thus prepared was added to 10 cm^3 of BRB (pH 3). All quantitative analyses were performed by the standard addition method (1 addition = 20 mm^3 of the standard solution of 1 mmol dm^{-3} LV). The LV determination was repeated 5 times.

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