# VOLTAMMETRIC DETERMINATION OF FLUTAMIDE AND ITS METABOLITE 4-NITRO-3-TRIFLUOROMETHYLANILINE AT A HANGING MERCURY DROP MINIELECTRODE

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Received June 22, 2011
Accepted August 3, 2011
Published online January 23, 2012

*To the memory of Doc. RNDr. PhMr. Jiří Volke, DrSc.*

Optimum conditions were found for direct current and differential pulse voltammetric determination of anticancer drug Flutamide and its main metabolite 4-nitro-3-trifluoromethylaniline on a hanging mercury drop minielectrode in mixed Britton–Robinson buffer (pH 12.0)–methanol (9:1) or 0.01 M NaOH–methanol (9:1) media with limit of quantitation ca. $10^{-7}$ mol $l^{-1}$. It was proved that the newly developed method is applicable for the determination of Flutamide in pharmaceutical formulations and for the determination of both Flutamide and 4-nitro-3-trifluoromethylaniline in urine either directly with limit of quantitation ca. $10^{-5}$ mol $l^{-1}$ or after solid phase extraction with limit of quantitation in the $10^{-7}$ mol $l^{-1}$ concentration range. The sufficient peak potential difference of the two substances suggests the possibility of the analysis of their mixtures.

**Keywords**: Voltammetry; Analytical methods; Antitumor agents; Flutamide; 4-Nitro-3-trifluoromethylaniline; Hanging mercury drop minielectrode; Solid phase extraction; Urine.

Flutamide (FLA; 4-nitro-3-trifluoromethylisobutylanilide; Fig. 1) is a synthetic antiandrogen which is used for the treatment of prostatic cancer[1]. Its main metabolite is 2-hydroxyflutamide in plasma and 4-nitro-3-trifluoromethylaniline (NTMA; see Fig. 1) in urine[2,3]. Many analytical methods were described in the literature for the determination of FLA and its metabolites in bulk form, pharmaceutical formulations, and human plasma, mainly



doi:10.1135/cccc2011127

based on spectrophotometry[4–8] or high performance liquid chromatography (HPLC)[9–13]. The chromatographic determination of NTMA (hydrolysis product)[13] as well as 2-hydroxyflutamide[10] in the presence of FLA has been described. HPLC/MS/MS tandem technique was used for detailed investigation of FLA metabolism[2]. Impurities contained in FLA have been determined by planar chromatography[14] and FLA in pharmaceutical forms was determined by gas chromatography[15]. Flow injection analysis with direct spectrophotometric detection succeeded in dissolution studies of pharmaceutical tablets[16].

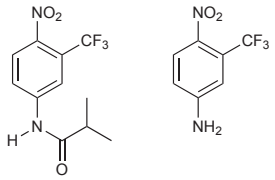


FIG. 1
Structural formulas of Flutamide (FLA) and 4-nitro-3-trifluoromethylaniline (NTMA)

Spectrophotometric methods rely on the formations of chromophores by reaction of FLA or its reduction products with promethazine hydrochloride, resorcinol[4,7], NEDA (*N*-(1-naphthyl)ethylenediamine dihydrochloride)[4,5], chromotropic acid[7] or *p*-dimethylamino cinnamaldehyde[5].

However, these methods are time consuming and expensive, they are not too suitable for fast screening purposes, they require several sample preparation steps, including reduction, extraction and clean-up procedures in order to obtain a final extract fully compatible with chromatographic and/or spectrophotometric determination. Further, they generate a large amount of waste containing organic solvents, which makes the procedure more complicated and expensive. Therefore, attention was paid to fast and inexpensive polarographic and voltammetric methods[1,17–19]. The polarographic reduction of FLA was investigated by direct current polarography (DCP), alternating current polarography (ACP), normal pulse polarography (NPP), and differential pulse polarography (DPP) at a dropping mercury electrode (DME). The supporting solution was phosphate buffer in limited pH range 6–8 containing 5 to 95% ethanol. The reduction process at the DME was diffusion controlled and irreversible. Extraction with 95% ethanol followed by polarographic reduction was selected as the basis of a method for the determination of FLA as a pure compound and in tablets[17]. More detailed study[1] resulted in the determination of FLA in tablets using DPP at DME down to $2 \times 10^{-6}$ mol $l^{-1}$. Three adsorptive cathodic stripping voltammetric

procedures were optimized for determination of FLA in bulk, tablets, and human serum applying linear-sweep, differential-pulse, and square-wave waveforms. The achieved limits of detection of the bulk drug were $1.9 \times 10^{-7}$, $8.7 \times 10^{-8}$, and $9.7 \times 10^{-9}$ mol $l^{-1}$ using the optimized differential-pulse, linear-sweep, and square-wave adsorptive stripping voltammetric procedures, respectively[18]. Differential pulse voltammetry (DPV) at a non-toxic mercury meniscus modified silver solid amalgam electrode was used for the determination of FLA in micromolar concentration range and it was proved to be applicable for the determination of the active substances in Apo-Flutamide tablets[19].

However, so far no attempt has been made to use modern voltammetric methods, namely direct current voltammetry (DCV) and DPV at a hanging mercury drop electrode (HMDE) for the determination of FLA in the presence of its metabolites and the applications of these techniques for the determination of FLA and its main metabolite NTMA in urine at micromolar concentration levels. Therefore, the aim of this work was to find optimum conditions for voltammetric determination of FLA and NTMA at a miniaturized pen type hanging mercury drop minielectrode (HMDmE)[20] and to verify their applicability using model samples of urine spiked with tested substances. Further, an electroanalytical method for quantitation of FLA in commercially available pharmaceutical formulations using HMDmE is proposed and compared with the UV spectrophotometric method recommended by the Czech Pharmacopoeia[21].

## EXPERIMENTAL

### Instrumentation

Voltammetric measurements were carried out using a computer driven Eco-Tribo Polarograph with software PolarPro version 5.1 (Eco-Trend Plus, Prague, Czech Republic) in a three electrode system. A miniaturized pen type hanging mercury drop minielectrode (HMDmE) of the UMμE type (Eco-Trend Plus, Prague, Czech Republic), with the maximum drop size attainable obtained by opening the valve for 200 ms, with a surface of 1.36 $mm^2$ was used as a working electrode. The Ag|AgCl electrode (1 M KCl) of the RAE 113 type was the reference electrode and the platinum wire electrode was the auxiliary electrode (both Electrochemical Detectors, Turnov, Czech Republic). Polarization rate of 20 mV $s^{-1}$ (for both DCV and DPV) and the modulation amplitude in DPV of –50 mV with pulse duration of 100 ms was used. pH measurements were carried out using a Jenway 4330 conductivity and pH meter (Jenway, Dunmow, Essex, UK) with combined glass electrode of the 924005 type. pH of mixed buffer–methanol solutions measured with this combined electrode calibrated using standard aqueous buffers is further denoted as $pH^f$.

Materials

Stock solutions of Flutamide (C.A.S. Name Propanamide, 2-methyl-*N*-[4-nitro-3-(trifluoromethyl)phenyl]-; C.A.S. Registry number 13311-84-7; Sigma Aldrich; $c = 1 \times 10^{-3}$ mol $l^{-1}$), and 4-nitro-3-trifluoromethylaniline (C.A.S. Name 4-nitro-3-(trifluoromethyl)aniline; C.A.S. Registry number 393-11-3; Sigma Aldrich; $c = 1 \times 10^{-3}$ mol $l^{-1}$) were prepared by dissolving of 0.02762 and 0.02061 g of FLA and NTMA, respectively, in 100 ml of methanol p.a. (Merck). Stock solutions of FLA and NTMA ($c = 1 \times 10^{-4}$ mol $l^{-1}$) in water were prepared by dissolving 0.00276 g of FLA and 0.00206 g of NTMA in 100 ml of deionized water (Millipore Q-plus System, Millipore, USA). All solutions were kept in glass vessels in dark at refrigerator. The Britton–Robinson (BR) buffer solutions were prepared in a usual way by mixing a 0.04 M solution of phosphoric, acetic and boric acid with an appropriate amount of 0.2 M sodium hydroxide, using analytical-reagent grade chemicals (Merck). Lichrolut EN (40–120 μm, 200 mg, 3 ml, Merck) columns were used for solid phase extraction (SPE) separation and preconcentration of FLA and NTMA from model urine samples. The analyzed pharmaceutical formulations were Apo-Flutamide (Chanelle Medical, Ireland) tablets with declared FLA content 250 mg.

Procedures

The general procedure to obtain DC or DP voltammograms was as follows: The volume of the measured solutions was 10 ml, the ratio 9:1 of the aqueous phase/methanol was kept. The solutions were prepared by addition of aqueous phase to 1 ml of methanol containing tested analytes in 10 ml volumetric flask. Oxygen was removed from the measured solutions by bubbling with nitrogen for 5 min. A prebubbler containing a water–methanol mixture in the same ratio as in the measured solution was placed prior to the voltammetric vessel. The height of DCV peak was measured from the straight line constructed as the prolongation of the residual current before the onset of the peak. The height of the DPV peak was measured from the straight line connecting minima on both sides of the peak in the case of individual substance or from the straight line connecting the minimum before first peak and after second peak in the case of the mixture of FLA and NTMA. The calibration curves were measured in triplicate and their statistical parameters (e.g., slope, intercept, limit of quantitation) were calculated using Microcal Origin 6.0 software (Microcalsoftware, USA). The significance of the intercepts of linear calibration dependences was tested by statistic software ADSTAT [22]. Limit of quantitation ($L_Q$) was calculated using a 10σ/slope ratio, where σ is the standard deviation of the mean value for 10 voltammograms of the blank, determined according to IUPAC recommendations[23]. All statistical data are calculated for the level of significance $\alpha = 0.05$.

For the determination of FLA in Apo-Flutamide tablets 250 mg, ten tablets were weighed and homogenized in a mortar. The average weight of one tablet was 678.4 ± 4.9 mg. The quantitation of FLA by voltammetric methods was performed as follows: 25 mg of the powder mass from the tablets was accurately weighed ($n = 4$) and dissolved in 25.00 ml of methanol; 4.00 ml of thus prepared solution were diluted with methanol to 100 ml; 1.00 ml of resulting solution was diluted with 0.01 M NaOH to 10 ml, transferred into voltammetric vessel and DC and DP voltammograms were recorded. Exact concentration of FLA was determined applying one standard addition of 1.0 ml from the FLA standard stock solution ($c = 9.09 \times 10^{-6}$ mol $l^{-1}$) in methanol, and plotting resulting analytical curve. For the UV spectrophotometric quantitation, the method prescribed by the Czech Pharmacopoeia[21] was used

measuring the absorbance at absorption maximum of FLA at λ = 295 nm using the following procedure: 25 mg of the powder mass from the tablets was accurately weighed ($n$ = 4) and dissolved in 25.00 ml of methanol; 2.00 ml of thus prepared solution were diluted with methanol to 100 ml and the absorbance was measured.

For direct DPV determination of FLA and NTMA and their mixtures in model samples of urine, required amounts of FLA and NTMA solutions in methanol were transferred into 10 ml volumetric flask, methanol was added to total volume of 2 ml and the volume was filled up to the mark with urine; 1 ml of thus prepared model samples was afterwards diluted to 2 ml with 0.01 M NaOH, transferred into voltammetric vessel and DP voltammograms were recorded.

For DPV and DCV determination of FLA and NTMA in urine after SPE, Lichrolut EN (Merck) columns based on styrene–divinylbenzene copolymer were used. At first, the column was activated according to supplier recommendations[24] by 3 ml of ethyl acetate, 3 ml of methanol and 3 ml of water. Afterwards, 100 ml of model sample of urine spiked with FLA and NTMA were applied at flow rate 5 ml $min^{-1}$. After washing the cartridge with 1 ml of deionized water, FLA and NTMA were eluted with 10 ml of methanol, the eluate was evaporated under decreased pressure to dryness, the residue was dissolved in 1 ml of methanol using sonication and filled up to 10 ml with 0.01 M NaOH. The solution was transferred into voltammetric vessel and after removing oxygen by 5 min bubbling with nitrogen, DC and DP voltammograms were recorded.

## RESULTS AND DISCUSSION

### *Direct Voltammetric Determination of Flutamide and 4-Nitro-3-trifluoromethylaniline at a Hanging Mercury Drop Minielectrode*

At first, the influence of pH on DC and DP voltammograms of tested substances was investigated (Fig. 2). 1–2 peaks were observed in dependence on pH, the second one being always much worse developed and not useful for analytical purposes. The observed linear dependence of peak currents on the square root of the scan rate confirms the diffusion control of the observed processes. Potential of all peaks moves towards negative values with increasing pH. Obviously, these peaks correspond to the well known four-electron reduction of nitro group to hydroxylamino group, or further two-electron reduction to corresponding amine in acidic media at mercury electrodes[25–27]. The highest and best developed peaks were obtained at $pH^f$ 12.0, similarly as in other studies on reduction of nitro aromatics at mercury electrodes[28–31]. To simplify the base electrolyte, BR buffer can be substituted by 0.01 M NaOH and, thus, all calibration dependences were subsequently measured in the mixture 0.01 M NaOH–methanol (9:1, $pH^f$ 11.3). The parameters of obtained linear dependences calculated by linear regression method are summarized in Table I. Lower $L_Q$s were obtained for DPV compared with DCV, i.e., $5 \times 10^{-8}$ and $3 \times 10^{-7}$ mol $l^{-1}$ for FLA and NTMA,

TABLE I
Parameters of calibration straight lines for voltammetric determination of FLA and NTMA at HMDmE

| Analyte | Method | Concentration mol $l^{-1}$ | Slope mA l $mol^{-1}$ | Intercept nA | Correlation coefficient | $L_Q$ mol $l^{-1}$ |
|---|---|---|---|---|---|---|
| Direct determination in 0.01 M NaOH–methanol (9:1, $pH^f$ 11.3) mixed medium | | | | | | |
| FLA | DCV | (2–10) × $10^{-5}$ | –9.3 | –10.8 | –0.9974 | – |
| | | (2–10) × $10^{-6}$ | –12.5 | –3.0 | –0.9997 | – |
| | | (2–10) × $10^{-7}$ | –10.5 | –1.3 | –0.9949 | 1 × $10^{-7}$ |
| FLA | DPV | (2–10) × $10^{-5}$ | –8.5 | –143.2 | –0.9902 | – |
| | | (2–10) × $10^{-6}$ | –14.9 | –9.3 | –0.9982 | – |
| | | (2–10) × $10^{-7}$ | –14.1 | –1.7 | –0.9937 | 5 × $10^{-8}$ |
| NTMA | DCV | (2–10) × $10^{-5}$ | –4.2 | 2.8 | –0.9986 | – |
| | | (2–10) × $10^{-6}$ | –4.0 | 1.1 | –0.9999 | – |
| | | (2–10) × $10^{-7}$ | –4.1 | 0 | –0.9997 | 5 × $10^{-7}$ |
| NTMA | DPV | (2–10) × $10^{-5}$ | –6.3 | 8.2 | –0.9998 | – |
| | | (2–10) × $10^{-6}$ | –5.6 | 1.6 | –0.9998 | – |
| | | (2–10) × $10^{-7}$ | –4.4 | –0.1 | –0.9971 | 3 × $10^{-7}$ |
| Direct determination in model samples of urine (DPV in 0.01 M NaOH–urine (1:1)) | | | | | | |
| FLA | DPV | (2–10) × $10^{-5}$ | –1.2 | –2.2 | 0.9941 | 2 × $10^{-5}$ |
| NTMA | DPV | (2–10) × $10^{-5}$ | –1.4 | –0.1 | –0.9914 | 1 × $10^{-5}$ |
| Determination in model samples of urine after SPE from 100 ml of urine (DPV in 0.01 M NaOH–methanol (9:1, $pH^f$ 11.3)) mixed medium | | | | | | |
| FLA | DPV | (2–10) × $10^{-6}$ | –33.1 | 5.8 | –0.9940 | – |
| | | (2–10) × $10^{-7}$ | –31.6 | –32.8 | –0.9846 | 8 × $10^{-7}$ |
| NTMA | DPV | (2–10) × $10^{-6}$ | –28.6 | –1.2 | –0.9976 | – |
| | | (2–10) × $10^{-7}$ | –44.5 | 5.1 | –0.9255 | 7 × $10^{-7}$ |

respectively. For such low concentrations, the peak height repeatabilities are 8.3–32% as listed in the Table II.

It can be seen from Fig. 3 that both DCV and DPV is applicable for the determination of the mixture of the two substances because of sufficient difference in their peak potentials, which is caused by electronic influence of *para* substituents of nitro group. In the case of NTMA, an amino group in *para* position with +M effect increases electron density at the nitro group thus shifting its peak potential towards negative values compared to FLA in which +M effect of –NH(C=O) group is diminished by the presence of carbonyl group.

*Direct Voltammetric Determination of Flutamide in Apo-Flutamide Tablets at a Hanging Mercury Drop Minielectrode*

Standard addition method using DCV and DPV at HMDmE was developed for the determination of FLA in tablets Apo-Flutamide containing 250 ±

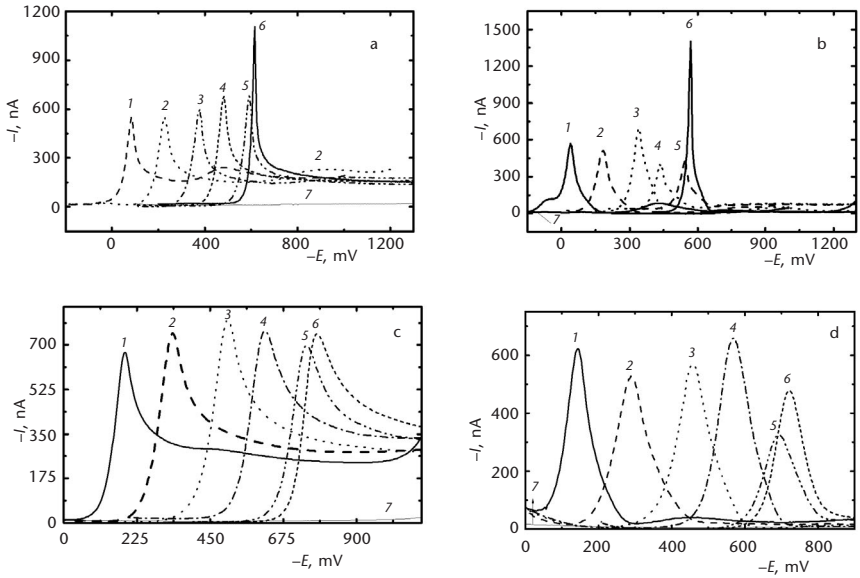


FIG. 2

DC (a, c) and DP (b, d) voltammograms of Flutamide (a, b) and 4-nitro-3-trifluoromethylaniline (c, d) ($c = 1 \times 10^{-4}$ mol $l^{-1}$ for both analytes) in BR buffer–methanol (9:1) mixture with resulting $pH^f$ 2.1 (*1*), 4.2 (*2*), 6.2 (*3*), 8.3 (*4*), 10.1 (*5*), 12.0 (*6*) and voltammograms of the supporting electrolyte at $pH^f$ 2.1 (*7*)

12.5 mg FLA according to the manufacturer and compared to spectrophotometric method recommended by the Czech Pharmacopoeia[21].

The obtained mean values of FLA are summarized in Table III together with standard deviations $s$, relative standard deviations $s_r$ and confidence intervals $L_{1,2}$. The mean value for the percentage content referred to the value declared by manufacturer is in all cases between 99 and 106.1%. This

TABLE II
Peak height repeatability (relative standard deviation $s_r$, $n$ = 10) of FLA and NTMA for their voltammetric determination in 0.01 M NaOH–methanol (9:1, pH$^f$ 11.3) mixed medium

| Analyte | Method | Concentration mol l$^{-1}$ | $s_r$, % |
|---|---|---|---|
| FLA | DCV | $1 \times 10^{-4}$ | 3.8 |
| | | $6 \times 10^{-8}$ | 8.3 |
| | DPV | $1 \times 10^{-4}$ | 1.3 |
| | | $2 \times 10^{-8}$ | 14 |
| NTMA | DCV | $1 \times 10^{-4}$ | 0.6 |
| | | $2 \times 10^{-7}$ | 32 |
| | DPV | $1 \times 10^{-4}$ | 1.6 |
| | | $2 \times 10^{-7}$ | 22 |

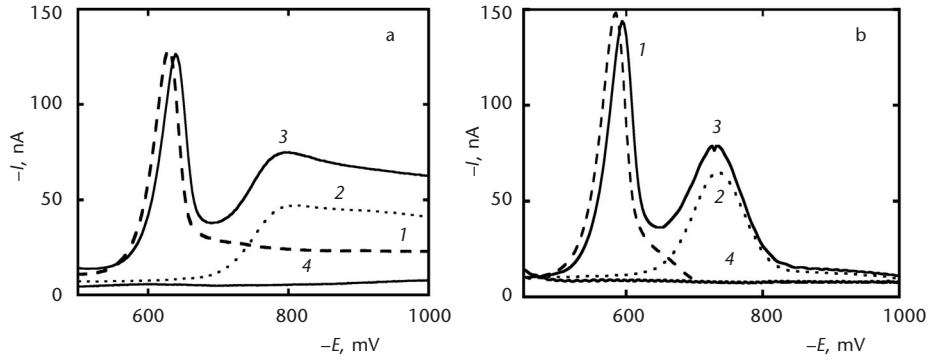


FIG. 3
DC (a) and DP (b) voltammograms at HMDmE of FLA (*1*), NTMA (*2*) and their mixture (*3*) ($c$ = $1 \times 10^{-5}$ mol l$^{-1}$) in a 0.01 M NaOH–methanol (9:1, pH$^f$ 11.3) mixture (*4*)

highest value obtained for DCV lies outside the tolerance allowed by the Czech Pharmacopoeia protocol, which is 97–103% of the analytical standard target. This may be caused by the way of evaluation of the DCV peak height relying on the straight line constructed as the prolongation of the residual current before the onset of the peak, which might be not very precise.

The accuracy of all tested methods is comparable as follows from relative standard deviations $s_r$. No interferences of excipients were observed in the analysis of the tablets. It can be concluded that the developed voltammetric

TABLE III

Mean values $m_A$, standard deviations $s$, relative standard deviations $s_r$, limits of confidence $L_{1,2}$ ($n$ = 4, $a$ = 0.05) of percentage content of FLA in Apo-Flutamide 250 mg tablets determined by voltammetric methods at HMDmE in NaOH–methanol (9:1, pH[f] 11.3) mixed media and UV spectrophotometry (λ = 295 nm)

| Method | $m_A$, % | $s$, % | $s_r$, % | $L_{1,2}$, % |
|---|---|---|---|---|
| DCV | 106.1 | 7.4 | 6.9 | 11.8 |
| DPV | 99.0 | 6.7 | 6.8 | 10.7 |
| Spectrophotometry | 100.9 | 8.7 | 8.6 | 13.8 |

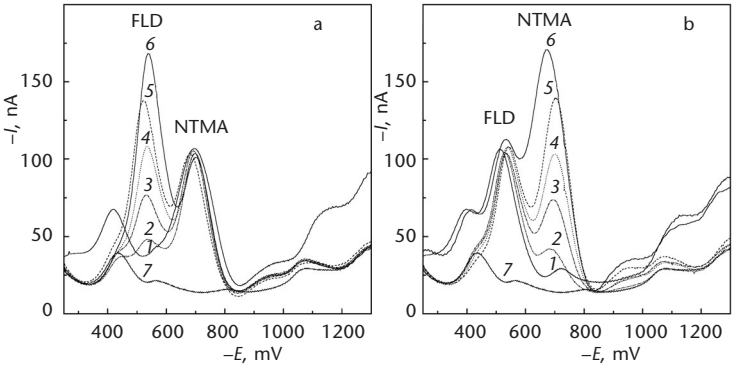


FIG. 4

DP voltammograms of the mixture of FLA and NTMA at HMDmE in 0.01 M NaOH–urine model sample (1:1): $c_{FLA}$ = 0 (*1*), 20 (*2*), 40 (*3*), 60 (*4*), 80 (*5*), 100 (*6*) μmol $l^{-1}$, $c_{NTMA}$ = 60 μmol $l^{-1}$ (a) and $c_{NTMA}$ = 0 (*1*), 20 (*2*), 40 (*3*), 60 (*4*), 80 (*5*), 100 (*6*) μmol $l^{-1}$, $c_{FLD}$ = 60 μmol $l^{-1}$ (b); urine model sample: urine–methanol (8:2; *7*)

methods offer a suitable alternative to the spectrophotometric method prescribed in the Czech Pharmacopoeia[21].

*Direct DPV Determination of Flutamide and 4-Nitro-3-trifluoromethylaniline in Urine*

Using the procedure described in Experimental, it is possible to determine FLA and NTMA in urine in $10^{-5}$ mol $l^{-1}$ concentration range; parameters of

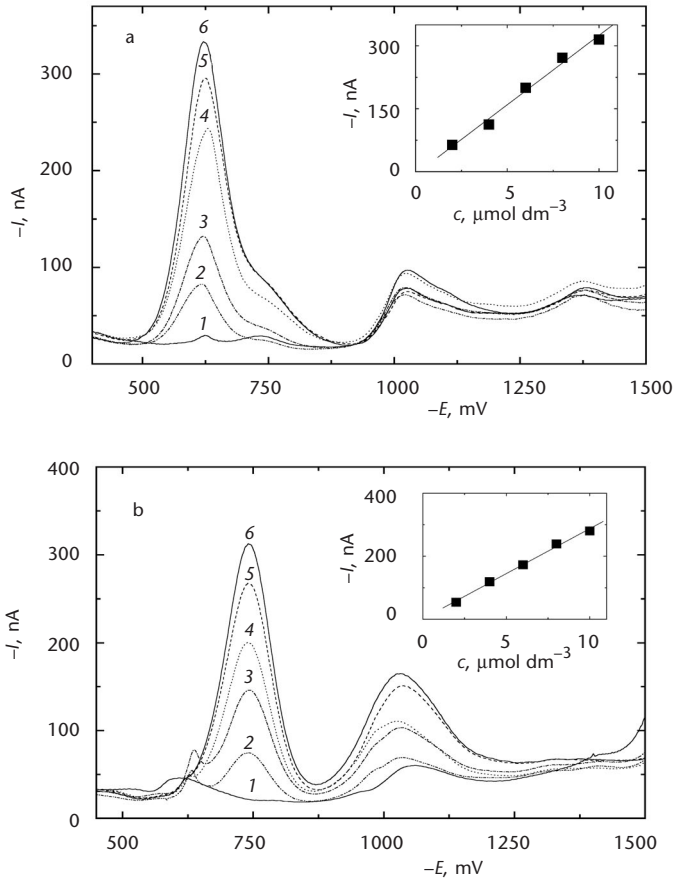


FIG. 5
DP voltammograms of FLA (a) and NTMA (b) at HMDmE in 0.01 M NaOH–methanol (9:1) mixture after SPE from 100 ml of urine model sample containing 0 (*1*), 2 (*2*), 4 (*3*), 6 (*4*), 8 (*5*), and 10 (*6*) μmol $l^{-1}$ of the test substance. Inset: The dependence of peak heights $I_p$ vs *c*

obtained linear concentration dependences are summarized in Table I. The peak height repeatability for the lowest measurable concentration $2 \times 10^{-5}$ mol $l^{-1}$ is satisfactory – 6.5 and 3.5% for FLD and NTMA, respectively (relative standard deviation, $n = 10$). Moreover, it is possible to determine both compounds simultaneously in the mixture, each in the concentration range (2–10) $\times 10^{-5}$ mol $l^{-1}$ as documented on the example of the DP voltammograms in Fig. 4. For all these measurements, the model samples were diluted with supporting electrolyte (0.01 M NaOH) in the ratio 1:1. Dilution with lower content of supporting electrolyte is inexpedient due to relative decrease of the peak height of the analytes and complex character of the baseline caused by the ingredients of urine. As result, it is impossible to determine micromolar and lower concentrations of FLA and NTMA in urine. Because their expected therapeutical concentrations are in micromolar concentration range, it is necessary to use solid phase extraction (SPE) for preliminary separation and preconcentration.

### *DPV Determination of Flutamide and 4-Nitro-3-trifluoromethylaniline in Urine after Solid Phase Extraction*

Using the procedure described in Experimental, it is possible to determine both FLA and NTMA in urine in micromolar concentration range, i.e., at expected therapeutical levels. The recovery of the extraction procedure was 100.3 ± 4.9% and 86.6 ± 6.1% ($n = 4$) for model urine samples containing FLA and NTMA, respectively. The parameters of obtained linear concentration dependences are summarized in Table I. They were measured over two concentration ranges of magnitude (2–100) $\times 10^{-7}$ mol $l^{-1}$. For the sake of illustration, DP voltammograms corresponding to the higher concentration range are depicted in Fig. 5.

## CONCLUSIONS

It was proved that DC and DP voltammetry at a hanging mercury drop minielectrode is suitable for the determination of micromolar concentrations of anticancer drug Flutamide in pharmaceutical formulations and together with its main metabolite 4-nitro-3-trifluoromethylaniline for the direct determination in urine. Determination of both analytes in submicromolar concentrations is possible after their preliminary separation and preconcentration using solid phase extraction. Further study on the possibility of simultaneous determination of these two analytes in biological fluids is under progress.

*J. F. thanks the Czech Science Foundation (Grant P206/10/P087), the work was further financially supported by the Ministry of Education, Youth and Sports of the Czech Republic (projects LC 06035, MSM 0021620857, and RP 14/63), and by the Czech Science Foundation (Grant P206/12/G151).*

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