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Der Pharmacia Lettre, 2012, 4 (5):1599-1606
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ISSN 0975-5071
USA CODEN: DPLEB4

Electrochemical behaviour and Validated Determination of the Anticancer Drug Tamoxifen

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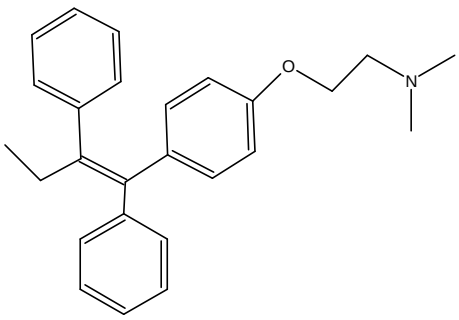
ABSTRACT

The electrooxidative behaviour of tamoxifen (Tam) and 4-hydroxytamoxifen (TamOH) was investigated by cyclic (CV), differential-pulse adsorptive anodic stripping (DPAdAS) and square-wave adsorptive anodic stripping (SWAdAS) voltammetric techniques. The anodic oxidation peak of Tamoxifen was attributed to the cyclization reaction to form the corresponding phenanthrene derivative and the mechanism of oxidation was postulated on the basis of controlled potential electrolysis and isolation of the oxidative product. Oxidative stripping analysis was successfully applied to the determination of tamoxifen in a bulk pharmaceutical formulation, and sensitivity in human urine and serum was validated. The achieved limits of detection (LOD) of bulk tamoxifen were 1.8 × 10^{-6} mol L^{-1} and 2.4 × 10^{-6} mol L^{-1} for DPAdAS and SWAdAS, respectively. The LOD values for tamoxifen in human urine and serum sample analysis were 4.75 × 10^{-7} mol L^{-1} and 2.63 × 10^{-7} mol L^{-1} and 1.98 × 10^{-7} mol L^{-1} and 3.28 × 10^{-7} mol L^{-1} for DPAdAS and SWAdAS, respectively. 4-hydroxytamoxifen is oxidised at more positive potentials than Tamoxifen, separated from the Tamoxifen stripping peak, and its adsorption to the glassy carbon electrode is less pronounced. This affects the ability to determine this important phase I metabolite in serum and urine samples.

Key words: tamoxifen; 4-hydroxytamoxifen; anodic adsorptive stripping voltammetry.

INTRODUCTION

Tamoxifen, [Z]-2-[4-(1,2-diphenyl-1-butenyl)-phenoxy]-N,N-dimethylethylamine (Tam), a nonsteroidal anti-estrogen, has been the most important hormonal agent for treatment of breast cancer for more than two decades, and recently has been approved as a long-term chemo preventive agent for breast cancer in healthy women at high risk for developing breast cancer.[1-4] Tamoxifen undergoes chemical transformation to its phase I metabolites in vivo, resulting in a series of modified species, predominately through methylation or hydroxylation of the benzene rings on the tamoxifen structure, to structures such as 4-hydroxytamoxifen.[1]



There are a variety of bioanalytical methods that have been developed to determine the concentrations of Tam in biological fluids and pharmaceutical preparations. Methods developed for Tamoxifen analysis include capillary electrophoresis,[5] and chromatography used in conjunction with a range of detection techniques.[6] Liquid chromatography coupled to detection by mass spectrometry is particularly gaining acceptance.[7-8] Electrochemical techniques have received significant attention in analysis of pharmaceuticals, due to their low detection limits and rapid time frame. They offer the analyst a technique for the analysis of drugs that is rapid, simple and low cost.[9] Electrochemical studies of Tamoxifen has centred upon the properties of Tamoxifen by constant current potentiometric stripping at a glassy carbon electrode,[10] and voltammetric analysis at a carbon paste electrode [11], however due to the importance of the drug there is a desire to have a validated method for rapid determination of Tamoxifen in pharmaceutical preparations, and also increase our understanding of the electrochemistry in biological fluids not only of Tamoxifen, but of its phase I metabolites.[6] Many organic compounds exhibit surface-active properties that are manifested by their adsorption from solution onto the surface of a solid phase.[9] This phenomenon forms the basis for adsorptive stripping voltammetry (AdSV), where the species to be determined are accumulated on the electrode by adsorption. Adsorptive stripping voltammetry has been demonstrated as a sensitive analytical method for a wide range of pharmaceutical compounds that can be adsorbed onto an electrode surface. [12-22] The present work is concerned with the validation of the determination of Tamoxifen by adsorptive stripping analysis in pharmaceutical formulation and also urine and plasma samples. The electrochemical behaviour of a phase I metabolite 4-hydroxytamoxifen (TamOH) is also reported and contrasted to the pure drug. Anodic adsorptive stripping analysis is shown to be a dependable technique for determination of tamoxifen.

MATERIALS AND METHODS

All chemicals used were of analytical reagent grade quality and were employed without further purification. Bulk form Tamoxifen was obtained from Dabur pharma Ltd. Mumbai, India, and was used as received. Tablets containing Tamoxifen citrate (Sensival) labelled 10 mg Tam were purchased from commercial sources. Pure samples of Tamoxifen and 4-Hydroxytamoxifen were obtained from Sigma-Aldrich. Britton–Robinson (BR) buffers of pH 2–10 (mixtures of 0.04 mol/L acetic, orthophosphoric, and boric acids; adjusted to the required pH with 0.1M sodium hydroxide solution or 0.1M hydrochloric acid) were prepared and used as supporting electrolytes with 15 % methanol added on pure samples to ensure drug solubility.

For bulk analysis, ten tablets were weighed and the average mass per tablet was determined and then ground to a homogeneous fine powder in a mortar. A portion of the finely ground material equivalent to 10 mg of TAM was used for analysis**.** Drug-free human blood, obtained from healthy volunteers was centrifuged (4000 rpm) for 30 minutes at room temperature, and separated serum samples were stored frozen until assay. An aliquot of serum sample was prepared with tamoxifen citrate dissolved in double distilled water to achieve a final concentration of 1 x 10^{-3} M. After vortexing for 30 seconds, the mixture was then centrifuged for 10 min. at 4000 rpm in order to eliminate serum protein residues. Appropriate volumes of these samples were transferred into the voltammetric cell and diluted with Britton Robinson (BR) universal buffer (pH 4.2) and subsequently analyzed according to the general analytical procedure. A blank experiment was carried out adopting the above procedure. An aliquot of human urine sample was collected and analyzed in the same way as the serum samples.

A CHI model no. 1230 A or 920C electrochemical analyzer (CHI Instruments, USA) was employed for electrochemical experiments. A three-electrode cell system was used with a commercial glassy carbon electrode as the working electrode, Ag/AgCl (3 M KCl) as the reference electrode, and a platinum wire as the auxiliary electrode. Surface pre-concentration steps were performed while stirring at 400 rpm at constant potential as described in the text. Bulk electrolysis was performed in the potentiostatic mode using ruthenium oxide (RuO_2) doped on Ebonex as the working electrode and a Pt wire as the counter electrode. All electrolytes were purged for 10 min with purified nitrogen gas, and kept under a blanket of nitrogen. The glassy carbon working electrode was polished to a mirror finish using 0.2 um alumina on a felt polishing pad, washed with distilled water, sonicated for 2 minutes in methanol, and then dried under a nitrogen purge, before use in stripping experiments.

*Typical analytical procedure***:-** Britton Robinson (BR) universal buffers of pH 4.2 (8.5 ml) plus methanol (1.5 mL) and an appropriate small volume aliquot of the drug sample were introduced into the voltammetric cell, through which a pure nitrogen stream was passed. An accumulation potential (vs. Ag/AgCl, KCl) was applied at the GCE for a selected time period (typically 20 seconds) while the solution was stirred at 400 rpm. At the end of the accumulation time period, the stirring was stopped and 10 s were allowed for the solution to become quiescent. Then, the voltammograms were recorded by scanning the potential toward the positive direction using the cyclic voltammetric (CV), differential pulse (DP) or square wave (SWV) waveform. All data was obtained at room temperature. The conditions used in this paper, which were shown to give well resolved peaks that are later validated are for DPAdAS; scan rate ν (10 mV s^{-1}), pulse height a (5 to 50 mV), pre-concentration potential

(E_{acc}=0.8) and pre-concentration time (t_{acc} 20 sec.), SWAdAS; pre-concentration potential (E_{acc}=0.8), pre-concentration time (t_{acc} 20 sec.), pulse-height a (5 to 50 mV), frequency f (40 Hz) and scan increment ΔEs (10 mV).

RESULTS AND DISCUSSION

Voltammetry

For all the electrochemical methods employed, Tam gave one well-defined oxidation peak in Britton Robinson (BR) universal buffer (pH 4.2):15 % methanol at glassy carbon. Cyclic voltammograms for Tam were recorded at different pH, scan rate and concentration values. The effect of pH value on the oxidation peak current of TAM was examined in the range of pH 2-10 in Britton Robinson (BR) universal buffer using a test concentration of $1x10^{-4}$ mol L^{-1} tamoxifen. The maximum peak height was seen at pH 4.2, leading to the choice of this pH for the analytical experiments. Pre-concentration times of between 10 and 120 seconds indicated that a maximum peak current was obtained after 20 seconds at 0.8 V vs Ag/AgCl, representing a significantly higher peak than studies involving no pre-concentration, and this was chosen as the standard conditions in all subsequent voltammetric studies where only Tam is present. Figure 1 shows the voltammogram at different scan rates after pre-concentration (with an inset of the effect of pH on peak height). The peak potentials are shifted to more positive values, as expected for an irreversible process with a contribution from diffusion control.

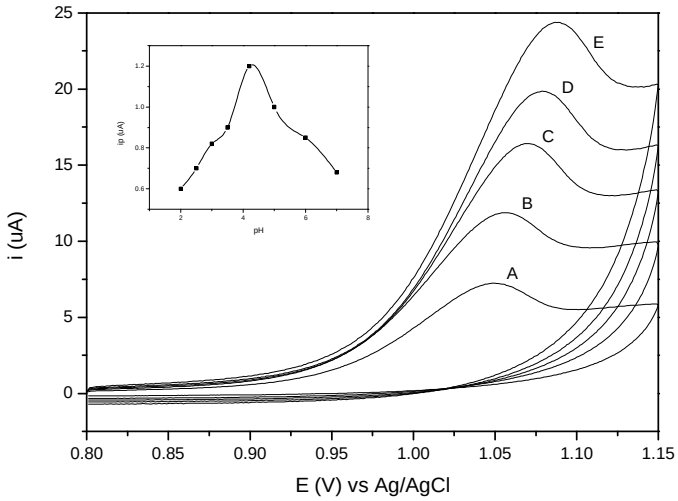


Figure1. Voltammogram of 1 x 10^{-4} M Tamoxifen at scan rates of 100 (A), 200 (B), 300 (C), 400 (D) and 500 (E) mV/s at pH 4.2. Inset plot of ip vs pH for 50 mV/s scan rate

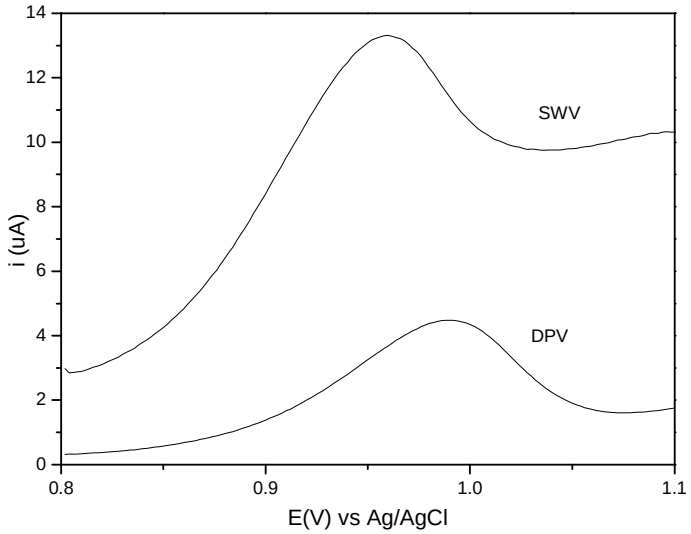


Figure 2. Differential pulse (DPAdAS) and the square wave voltammogram (SWAdAS) of 1 x 10^{-4} M Tamoxifen, pH 4.2 BRB.

Analysis of the peak height (i_p) with increasing scan rate indicates that the height of the oxidation peak i_p is not directly proportional to v over the scan rates 50 to 500 mV/s, with deviations from linearity also observed for a plot of i_p with $v^{1/2}$, confirming that adsorptive stripping is a major contribution to peak current, in addition to some diffusive contribution. Successive scans lead to a significant decrease in peak height (i_p). The voltammogram shows the linear dependence of peak height with concentration. In order to obtain the best level of detection for the Tamoxifen, differential pulse and square wave voltammetry were undertaken, once again using the same conditions of pre-concentration used for the linear voltammetry. Figure 2 shows both the Square wave and differential pulse anodic adsorptive voltammograms (SWAdAS and DPAdAS) for the pre-adsorbed Tam.

In all cases a single oxidative peak is observed. These conditions were applied firstly to pharmaceutical bulk formulation to determine the quality of the analysis possible.

Validation of the Procedure

Validation of the proposed procedure for assay of the drug at trace levels was examined via evaluation of the limit of detection (LOD), limit of quantization (LOQ), reproducibility, recovery, selectivity, robustness and ruggedness. The Limits of detection (LOD) and quantification (LOQ) of Tam were calculated using the following equations:

LOD= 3s/b

LOQ=10s/b

s is the standard deviation of the intercept and b is the slope of the calibration curve. Reproducibility, accuracy and precision of results applying the described stripping voltammetric methods were examined by performing five replicate analysis of standard solutions of Tam. The mean percentage recovery (%R) had been calculated for the found concentrations as a percent of the nominal concentrations in the standard solutions. Accuracy was expressed as relative error (RE %) while precision was assessed from the relative standard deviation in percentage (RSD %) of the mean recovery. The obtained results confirmed the reliability of the described stripping voltammetric methods. The robustness was examined by evaluating the influence of small variations on some of the most important procedure variables including pre-concentration potential (E_{acc}) and pre-concentration time (t_{acc}). The obtained result provided an indication of the reliability of the proposed procedure for the assay of TAM, and hence it can be considered robust. The obtained mean percentage recoveries based on the average of five replicate measurements were not significantly affected within the studied range of variations of some operational parameters, and consequently the proposed procedure can be considered robust. The ruggedness test of the analytical assay method is defined as the degree of reproducibility of assay results obtained by the successful applications of the assay over time and multiple laboratories and analysts. Two analysts analyzed the same standard with SWAdAS and DPAdAS methods using the same instrument. The methods were found to be rugged with the results of variation coefficients for SWAdAS and DPAdAS methods for first and second analysts, respectively. The results show no statistical differences between different analyses.

Table I. Stripping voltammetric determination of Tamoxifen urine using SWAdAS and DPAdAS modes.

Techniques	SWAdAS	DPAdAS
Linearity range (mol cm^{-3})	$0.2x10^{-6}$– $1.5x10^{-6}$	$0.2x10^{-6}$– $1.5x10^{-6}$
Slope (µA/M)	$3.5x10^{-6}$	$1.68x10^{-6}$
Intercept (µA)	0.43	0.16
Correlation coefficient	0.996	0.999
t- test	0.29	0.27
Variance ratio (F)	1.08	1.06
LOD (M)	$1.98x10^{-7}$	$4.75x10^{-7}$
LOQ (M)	$6.54x10^{-7}$	$1.3x10^{-8}$
Repeatability of peak current (RSD%)	0.74	0.62
Repeatability of peak potential (RSD%)	0.19	0.20
Reproducibility of peak current (RSD%)	0.71	0.68
Reproducibility of peak potential (RSD%)	0.11	0.15

Assay of Tam in spiked human urine

Tamoxifen in spiked human urine was successfully analyzed by both DPAdAS and SWAdAS without the necessity for extraction of the drug prior to the analysis. Representative DPAdAS and SWAdAS voltammograms of Tam in spiked human urine recorded under the optimum operational conditions of the described stripping voltammetric

methods are shown in Figure 4. No major interfering peaks for Tam were observed in the blank human urine within the studied potential range. Linear variations of the peak current (i_{pa}) with concentration of TAM in spiked human urine were obtained within the concentration ranges of 0.2×10^{-6} to 1.5×10^{-6} mol L^{-1} (DPAdAS) and 0.2×10^{-6} to 1.5×10^{-6} mol L^{-1} (SWAdAS) following the regression equations: (i_{pa} (µA) = 41.80 C (µmol L^{-1}) + 0.466; r = 0.994 and n = 6), and (i_{pa} (µA) = 55.5 C (µ mol L^{-1}) + 0.169; r = 0.999 and n =6), respectively. Detection limits of 4.75×10^{-7} and 1.98×10^{-7} mol L^{-1} and quantitation limits of 1.3×10^{-8} and 6.54×10^{-7} mol L^{-1} TAM were thus achieved by the DPAdAS and SWAdAS methods respectively. Mean percentage recoveries and relative standard deviations of 101.14 ± 2.38 (DP-AAdSV) and 101.16 ± 1.91 (SW-AAdSV) were achieved based on replicate measurements of 5×10^{-6} mol L^{-1} (Tables I and II) TAM in spiked human urine. These results confirmed the reliability of the described stripping voltammetric methods for assay of Tam in human urine.

Table II. Application of the stripping voltammetric determination of Tamoxifen in urine using SWAdAS and DPAdAS modes.

Techniques	SWAdAS	DPAdAS
Added (µġ cm^{-3})	0.2 0.35 0.5 0.75 0.9 1.5	0.2 0.35 0.5 0.75 0.9 1.5
Found (µġ cm^{-3})	0.21 0.351 0.499 0.751 0.91 1.51	0.212 0.352 0.498 0.751 0.899 1.51
n	6	6
Average recovery %	105.00 100.28 099.80 100.13 101.11 100.66	106.00 100.57 099.60 100.13 099.88 100.66
Mean S.D RSD %	101.16 1.93 1.91	101.14 2.41 2.38
Bias %	-0.16	-0.14

Assay of Tam in spiked human serum

Tamoxifen in spiked human serum was successfully analyzed by DPAdAS and SWAdAS without the necessity for extraction of the drug prior to analysis. Representative DPAdAS and SWAdAS voltammograms of tamoxifen in spiked human serum are shown in Figure 3. No significant interfering peaks for Tam were observed in the blank human serum within the studied potential range.

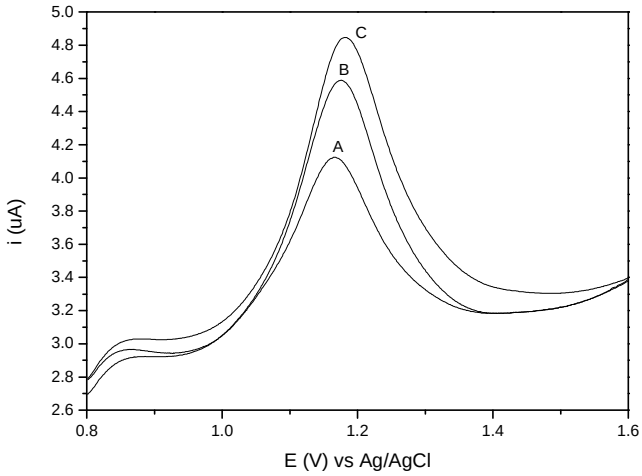


Figure 3. DPAdAS (top) and SWAdAS (bottom) of Tam in blood sample at spiked concentrations of 5.0 x 10^{-7} M (A), 8.0 x 10^{-7} M (B) and 1.0 x 10^{-6} M (C). ; $E_{acc.}$=0.8 V, $t_{acc.}$=20 s, pulse amplitude 50 mV, pulse width 30 ms, ΔE=10 mV and BR buffer of pH 4.2.

Linear variations of the peak current (i_{pa}) with concentration of TAM in spiked human serum were obtained within the concentration ranges of 0.3×10^{-6} to 1×10^{-6} mol L^{-1} (DPAdAS) and 0.3×10^{-6} to 1×10^{-6} mol L^{-1} (SWAdAS)

following the regression equations: (i_{pa} (µA) = 41.80 C (µ mol L^{-1}) + 0.466; r = 0.998 and n = 8), and (i_{pa} (µA) = 55.5 C (µ mol L^{-1}) + 0.169; r = 0.998 and n = 8), respectively. Detection limits of $2.63\times10^{-7}$and 3.2×10^{-7} mol L^{-1} and quantitation limits of 8.6×10^{-7} and 1×10^{-8} mol L^{-1} for Tam were achieved by the DPAdAS and SWAdAS methods respectively. Mean percentage recoveries and relative standard deviations of 100.33 ± 0.556 (DP-AAdSV) and 101.68 ± 2.07 (SW-AAdSV) were achieved based on replicate measurements of 1×10^{-6} mol L^{-1} (Tables III and IV) tamoxifen in spiked human serum. These results confirmed the reliability of the described stripping voltammetric methods for assay of TAM in human serum.

Table III. Stripping voltammetric determination of Tamoxifen serum using SWAdAS and DPAdAS modes.

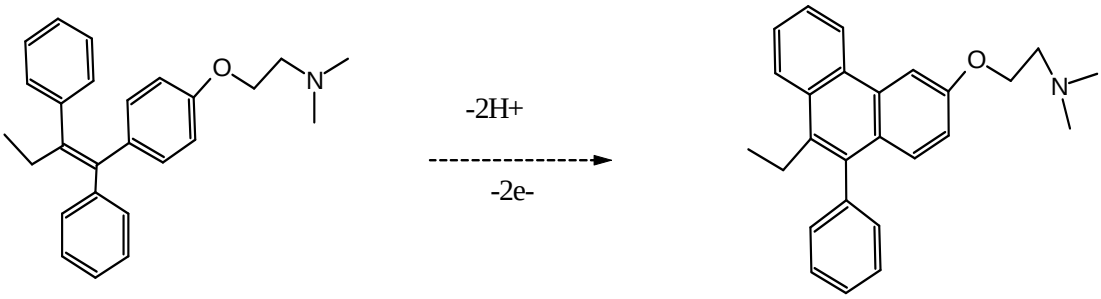
Techniques	SWAdAS	DPAdAS
Linearity range (mol cm^{-3})	$0.3x10^{-6} – 1x10^{-6}$	$0.3x10^{-6} – 1x10^{-6}$
Slope (µA/M)	$1.98x10^{-6}$	$2.4x10^{-6}$
Intercept (µA)	0.14	0.43
Correlation coefficient	0.998	0.998
t-test	0.37	0.31
Variance ratio (F)	1.20	1.13
LOD (M)	$3.28x10^{-7}$	$2.63x10^{-7}$
LOQ (M)	$1x10^{-8}$	$8.6x10^{-7}$
Repeatability of peak current (RSD%)	0.62	0.77
Repeatability of peak potential (RSD%)	0.16	0.18
Reproducibility of peak current (RSD%)	0.54	0.59
Reproducibility of peak potential (RSD%)	0.12	0.17

Table IV. Application of the stripping voltammetric determination of Tamoxifen in serum using SWAdAS and DPAdAS modes.

Techniques	SWAdAS	DPAdAS
Added (µġ cm^{-3})	0.3 0.4 0.5 0.6 0.7 0.8 0.9 1	0.3 0.4 0.5 0.6 0.7 0.8 0.9 1
Found (µġ cm^{-3})	0.31 0.42 0.499 0.601 0.698 0.81 0.902 1.04	0.302 0.398 0.501 0.599 0.701 0.802 0.91 1.01
n	8	8
Average recovery %	103.33 105.00 99.80 100.16 099.71 101.25 100.22 104.00	100.66 099.50 100.20 099.83 100.14 100.25 101.11 101.00
Mean S.D RSD %	101.68 2.11 2.07	100.33 0.556 0.554
Bias %	-0.68	-0.33

Controlled potential Electrolysis Behavior (CPE)

Controlled potential electrolysis was carried out at -1.12 V on a ruthenium oxide (RuO_2) doped on Ebonex electrode in 0.04 M Britton Robinson (BR) universal buffer (pH 4.2). Literature reports have identified that the following reaction occurs in compounds of a similar structure.[10, 23]



(Scheme-1)

During electrolysis, it was confirmed that 2 electrons were consumed for each mole of tamoxifen dissolved in solution, confirming a two electron process. After electrolysis, the catholyte was cooled to 278 – 283K in ice and neutralized with sodium hydroxide solution. This solution was then filtered, and the filtrate was treated with diethyl ether to extract the highly viscous white product. The IR spectrum showed a sharp spike at 3050 cm^{-1}, characteristic of the cyclisation to form the phenanthrene, with the disappearance of the peak at 1600 cm^{-1} corresponding to the C=C stretch in the original Tamoxifen compound.

Electrochemical behaviour of 4-Hydroxytamoxifen

An important phase I metabolite for Tam is 4-hydroxytamoxifen (TamOH), and as it is important to understand the stripping behaviour of this copound. Figure 6 shows the SWAdAS and DPAdAS of a mixture of pure Tam and TamOH in pH 4.2 BRB buffer after an accumulation potential of 0.4 V vs Ag/AgCl (20 s). The curves show that the adsorbed TamOH is oxidised at a more positive potential than the Tam. As such, in analyses of urine and plasma samples where phase I metabolites would be present, the peak used for validation would only include the Tam drug. The adsorption of TamOH to the surface of glassy carbon is less pronounced than Tam, as seen by the lower stripping peak for TamOH relative to Tam, and this affects its determination in urine and plasma samples. The presence of TamOH in comparable quantities to Tam would be able to be observed in urine and plasma samples at a potential of 0.5-0.6 V vs Ag/AgCl, however its limited adsorption and presence of interfering species in this region has limited a validated technique being established in this study.

CONCLUSION

The electrochemical investigation of Tam at the glassy carbon electrode and ruthenium oxide (RuO_2) doped on Ebonex electrode in phosphate buffered solution has been studied, based on the adsorption behavior of TAM at the glassy carbon electrode surface. The Cyclic voltammetric behavior show well defined irreversible anodic peak at 1.12V, so to the cyclization reaction to form the corresponding phenanthrene derivative. A fully validated, simple, sensitive, selective, fast and low-cost differential pulse and square wave adsorptive anodic stripping voltammetric methods were developed for determination of Tam in bulk form, and in spiked human urine and serum. The described methods could be recommended for use in trace analysis, quality control and clinical laboratories. The phase I metabolite TamOH was shown to be significantly separated from Tamoxifen, being oxidised at a lower potential, but its lower adsorptive character and interfering species at ots stripping potential limits our ability to validate a method for its determination.

Acknowledgement

Dr. D. K. Sharma is thankful to University Grants Commission, New Delhi, for financial support for this. Dr. Lathe Jones (RMIT University) thanks his institution for receipt of a Vice-Chancellors (Industry) Research Fellowship.

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