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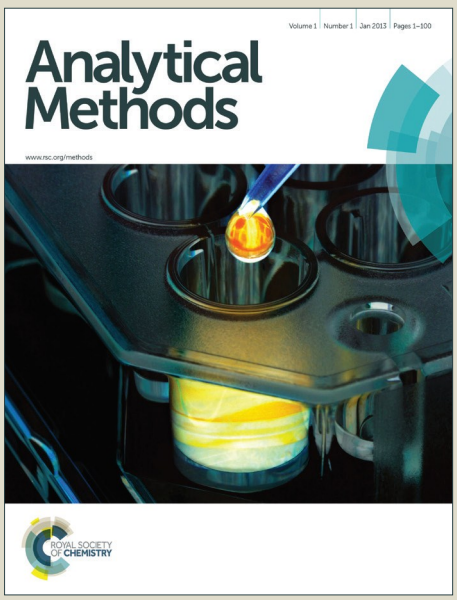

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Development of Electrochemical Method for Determination of Bicalutamide at SWCNT/CPE in Pharmaceutical Preparations and Human Biological Fluids

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Abstract

A modified Single Walled Carbon Nanotube (SWCNT) Carbon Paste Electrode (CPE) was prepared to investigate the electrochemical behavior of an anticancer drug Bicalutamide (BIC) by Cyclic Voltammetry and Differential Pulse Voltammetry (DPV). It was found that the drug selectively adsorb by diffusion-controlled process at the modified electrode surface with high efficacy compared to bare carbon paste electrode and undergoes an irreversible reduction process in 0.1M phosphate buffer at pH 7.0. The electrochemical behavior of the drug was further studied in terms of varying scan rates (ν), *p*H, deposition potential and deposition time, to optimize the experimental conditions and to develop a simple, selective, sensitive, economical and time-saving method for the determination of under considered drug. Under the optimized experimental conditions, the reduction peak was found linearly dependent on the concentration of Bicalutamide in the range 1 x 10^{-8} to 1 x 10^{-6} M. The limit of detection (LOD) and limit of quantification (LOQ) for the drug was found to be 5.20 x 10^{-8} M ($\pm$0.005µM) and 1.74 x 10^{-6} M ($\pm$0.005µM) respectively. The optimized method was successfully applied for the determination of Bicalutamide in Pharmaceutical preparations and Human Biological samples.

Keywords: Bicalutamide, Cyclic Voltammetry, Single walled Carbon Nanotube, Carbon Paste Electrode.

1. Introduction

Electrochemical techniques are inevitable tool for the analysis of biological and environmental samples. Their wide application is attributed with comparatively cheap instrumentation, very good sensitivity, rapid analysis and simultaneous determination of several analytes[1-3]. With the advancement of chemically modified electrodes (CMEs) in the electrochemical determinations has increased the sensitivity and selectivity for the detection of trace amounts of biologically important compounds and environmentally toxic pollutants. One such CMEs are Carbon Paste Electrode (CPE), which are widely applicable in both electrochemical studies and electroanalysis mainly due to properties like very low background current (compared to solid graphite or metal electrodes), low cost, large potential window, simple surface renewal processes and easiness of miniaturization. Further modification of these CPEs has been achieved since the report of carbon nanotube (CNT) in 1991 by S. Iijima. Carbon nanotubes (CNT's) have gained a considerable focus to be used as electrode materials due to their large electroactive surface area, remarkable mechanical and electrical properties. The restrained electronic behavior of carbon nanotubes reveals that they have the ability to promote electron-transfer reactions when used as an electrode material in electrochemical reactions.These materials hold great promises for increasing selectivity, sensitivity and reproducibility in voltammetric measurements.[4-6].

Bicalutamide (BIC), N-(4-cyano-3-trifluoromethyl-phenyl)-3-(4-fluoro-phenylsulfonyl)-2-hydroxy-2-methyl-propionamide (*Scheme 1*) is an orally active potent, well-tolerated, non-steroidal[7] pure Antiandrogen with negligible gastrointestinal intolerance[8]. BIC binds to androgen receptor (AR) which is essential for the development of male characters and is also a key factor for development and progression of prostate cance [9-10]. BIC is one of the newest nonsteroidal antiandrogenic drug traded as Caluran, Casodex, Bicaluran *etc*.

BIC binds to prostrate AR of rat, dog and human and is having approximately four times higher affinity for rat prostate AR than hydroxyl Flutamide, the active metabolite of Flutamide[11]. AR antagonists such as BIC are effective in blocking AR-activity and tumor growth in primary prostate cancer but are not much effective at blocking the reactivated AR in prostate cancer that recures after androgen deprivation therapy. Recent studies have indicated that the AR antagonist activity of BIC may be mediated by nuclear receptor co-repressor (NCoP) recruitment and that

BIC can function as an AR agonist in response to high level AR expression or removal of NCoR from the AR complex[7, 12-17].

A thorough literature survey has revealed that BIC has been reported by few analytical procedures *viz*. HPLC, LC assay method, UV-spectrophotometric method, HPTLC, LC-MS/MS method[18-24]. All these analytical procedures have one or the other demerits *e.g*. HPLC, UV-spectrophotometry, HPTLC requires longer time of analysis although are economical, on the other hand LC-assay and LC-MS/MS are costlier. The electrochemical techniques are time saving, economical, sensitive compared to other analytical procedures[25-30].

In this view an attempt has been made to develop a simple, sensitive, economical and time saving electrochemical procedure for the determination of BIC in pharmaceutical formulations and Biological samples.

2. Experimental Section

2.1 Reagents and Chemicals

Bicalutamide was purchased from Sigma-Aldrich and used without further purification. A standard stock solution of BIC was prepared by dissolving 1mg/10ml BIC in pure ethanol. 0.1M phosphate buffer with pH ranging from 3.0-9.0 was prepared. All other chemicals and reagents of analytical grade used were purchased from Merck India. Doubly distilled water was used throughout the experiment for preparation of solutions and reagents. Caluran tablets (Ranbaxy Laboratories Ltd. India) labelled 50mg Bicalutamide were purchased locally.

2.2 Preparation of Modified Carbon paste Electrode

CPE was prepared by hand-mixing Graphite powder and mineral oil (paraffin oil) in 70:30 ratio. Modified SWCNT/CPE was prepared in the same way in the ratio 10:60:30 (SWCNT: Graphite: Paraffin oil). The resulting paste was filled in Teflon well having a pre-inserted copper wire connected to establish an external electric contact. New electrode surface was formed by mechanically pressing the paste from top. A smooth clean glass rod was rolled at the electrode surface for smoothening of the electrode surface. Finally the electrode was carefully washed with distilled water[25,31]. Prior to each measurement the paste was pressed from the top and smoothened as before to provide fresh electrode surface.

2.3 Instrumentation

All the electrochemical measurements were carried out with Ω Metrohm model 797 VA Computrace (ion Analyzer, Swiss made), employing a three electrode cell with hand-made bare CPE and modified SWCNT/CPE as working electrode, an Ag/AgCl (saturated KCl) reference electrode and a platinum wire as counter electrode. *p*H measurements were performed with Systronics digital μpH meter model-361. All experiments were performed at ambient temperature of 298 K (25^{o}C). Pure nitrogen gas was purged through test solutions for oxygen free atmosphere.

2.4 General Analytical Procedure

For electrochemical measurements 5ml of 0.1M phosphate buffer pH 7.0 supporting electrolyte and a specific aliquot of sample was transferred to the Voltammetric cell and purged with pure nitrogen for 300s to remove oxygen. The parameters for voltammetric were:

Start potential: -1.50 V. Final potential: -0.30 V. Voltage step: 0.05V.

Equilibration time: 5 s. Scan rate: 200 mVs^{-1}. Deposition time: 90s

2.5 Sample Preparation

Ten Caluran tablets, labelled as 50mg BIC were weighed and manually homogenized to fine powder. An adequate amount of this powder equivalent to 1mM was weighed and dissolved in pure ethanol. The solution was sonicated for 15 minutes to achieve complete dissolution. The solution was filtered and stored for preparing working solutions of pharmaceutical formulations by taking suitable aliquots and diluting them with same solvent and phosphate buffer.

2.6 Biological Sample Preparation

Human Blood (for serum) and Urine samples were collected from a healthy volunteer. Blood samples were allowed to stand for 0.30 to 1 hour to coagulate at room temperature. After an hour the samples were centrifuged for 10 minutes at 1500 rpm. The supernatant serum generated was carefully separated using clean pipette. Both serum and urine samples were diluted 100 times with 0.1M phosphate buffer and stored in refrigeration for further analysis.

3. Results and Discussion

3.1 Surface Study

The area of bare and modified electrodes where obtained by performing cyclic Voltammetric measurements using 1.0 mM $K_3Fe(CN)_6$ probe in 0.1M KCl electrolyte at

different scan rates. $K_3Fe(CN)_6$ exhibited a pair of reversible peaks at bare and modified electrodes. Randles-Sevick[27] equation for a reversible system can be described as

$$\boldsymbol{I_{pa} = 0.4463\left(\frac{F^3}{RT}\right)^{1/2} n^{3/2} A_0 D_0^{1/2} C v^{1/2}} \quad 1$$

Where $\boldsymbol{I_{pa}}$ is anodic peak current, *n* is number of electrons transferred, $\boldsymbol{A_0}$ is surface area of electrode, $\boldsymbol{D_0}$ is diffusion coefficient and *C* is concentration of $K_3Fe(CN)_6$ respectively, υ is scan rate and other terms have their usual meanings. For 1.0 mM $K_3Fe(CN)_6$ in 0.1M KCl at T = 298 K, *n* = 1 and $\boldsymbol{D_0}$ = 7.6 x 10^{-6} cm^2 s^{-1}. The surface area was calculated from the slope of the plot of $\boldsymbol{I_{pa}}$versus $\boldsymbol{v^{1/2}}$. In present study the surface area of bare electrode was calculated to be 0.057 cm^2 and for modified electrode the microscopic surface area was found to be 0.270 cm^2.

3.2 Cyclic Voltammetric Behavior of Drug

The electrochemical behavior of 1 x 10^{-6}M BIC in 0.1M phosphate buffer (pH 7.0) yielded a single low but well-defined, irreversible, Cathodic peak in the potential range from -0.30 V to -1.5 V at bare CPE. However the peak current intensity of the drug increased at Modified SWCNT/CPE as shown in Fig. 1 (curve a and b). The enhancement in peak current is fairly due to the high electroactive surface area and excellent electrodic conductivity of Carbon nanotubes[25].

The Cathodic peak is attributed to the reduction of Nitrile group (N≡C) [32-33]. No peak was observed in the reverse scan, indicating the irreversible nature of reduction process. At extended potential ranges no anodic peak of the reduced product was observed at modified SWCNT/CPE.

3.3 Effect of deposition potential and time

Cyclic voltammetric method has been employed to investigate the effect of deposition potential and deposition time. The maximum adsorption of the drug was achieved at-1.1 V, hence applied for subsequent analysis.

Influence of deposition time on the reductive process of 1 x 10^{-6}M BIC was studied from 20-120 s and is shown in Fig. 2. The peak current increase linearly with deposition time upto 90 s, afterward the current becomes constant indicating that adsorptive equilibrium of the drug has been achieved at the deposition time of 90 s.

3.4 Effect of pH.

The electrocatalysed reduction of 1 x 10^{-6}M BIC was investigated in 0.1M phosphate buffer in the pH range of 3.0 to 9.0 by cyclic voltammetric measurements. For the under considered drug, it was found that the reduction peak shifted towards more negative potential with increasing pH as shown in Fig. 3, the peak current also increases from lower pH values upto pH 8.0 and decreases at higher pH as displayed in insert graph in Fig 4. However at higher pH (8 and above) there was a change in the shape of voltammograms. Hence *p*H 7.0 was applied for all voltammetric measurements. The plot of peak potential [E_p] *vs* pH (Fig. 4) gave a slope of 0.054 $mVpH^{-1}$, which is very close to the expected theoretical value of 59 $mVpH^{-1}$, which reflects that equal number of electrons and protons take part in electrode reaction. Thus the *p*H of the buffer exerts a significant effect on the reduction of BIC, hence *p*H 7.0 was selected as the optimum *p*H for further measurements.

3.5 Effect of Scan rate

Useful information *e.g*. reduction mechanism of the drug at modified SWCNT/CPE can be acquired from the relationships of peak current and scan rate. The effect of scan rate on peak current and peak potential has been studied at the modified SWCNT/CPE in scan range of 25-200 mVs^{-1}. Fig. 5 illustrates the effect of varying Scan rates (ν mVs^{-1}) on reduction peak current (I_p) and peak potential (E_p), It was observed that with an increase in scan rate the reduction peak potential shifts to more negative values and shows a linear relationship with increasing scan rate. The relationships of peak current and peak potential with varying scan rates (Shown in Fig. 6) illustrates whether the process is diffusion controlled or adsorption controlled at the electrode surface. A plot of logarithm of I_p against logarithm of scan rate gives a straight line described by the equation

$$\log I_p\ (\mu A) = 0.357 \log \nu + 0.13 \qquad 2$$

with a slope of 0.357 close to the theoretical values of 0.5 confirming that the electrode processes is a typical diffusion controlled process [26]. For an irreversible reduction process the half peak width ($W_{1/2}$) is calculated from Laviron equation[34].

$$W_{\frac{1}{2}} = \frac{2.44RT}{\alpha nF} = \frac{62.5}{\alpha n}\ (at\ 298K) \qquad 3$$

Where α *is* electron transfer coefficient and n is number of electron transferred in the electrode process. According to Bard and Faulkner[35], α can be calculated from the difference between peak potential (E_p) and half peak potential ($E_{\frac{p}{2}}$) as:

$$\alpha = \frac{47.7}{E_p - E_{\frac{p}{2}}} \qquad 4$$

From the equations (3) and (4) n was calculated to be 4.13 indicating that the drug undergoes $4e^-$ $4H^+$ irreversible reduction at modified SWCNT/CPE in 0.1 M phosphate buffer (*p*H 7.0).

3.6 Possible reaction mechanism

The irreversible reduction of BIC is accompanied by $4e^-$ $4H^+$ transfer process suggesting a possible reduction of Cyano (N ≡ C) group of the substituted Benzonitrile part of the compound[32-33]. The possible reduction mechanism of the electrode process is shown in *scheme 2*.

3.7 Calibration Graph and Detection Limit

Differential pulse Voltammetry (DPV) was employed to study an effect of concentration on the peak current, as DPV is highly sensitive and selective technique at lower concentration as compared to CV. Under the optimized experimental conditions linear calibration curve was obtained for BIC in the concentration range 1 x 10^{-7} M to 1 x 10^{-6}M shown in Fig. 7 and the calibration plot is shown in Fig. 8. The linear equation in this range was

$$I_p = 5.783C(\mu M) + 5.84 \qquad r = 0.982 \qquad 5$$

The results showed deviation from linearity at more higher and lower concentrations. This may be due to surface adsorption of BIC/or reduction product at electrode surface. The limit of detection (LOD) and Limit of Quantification (LOQ) were calculated using the equations (6) and (7)

$$LOD = \frac{3s}{m} \qquad 6$$

$$LOQ = \frac{10s}{m} \qquad 7$$

Where *s* is the standard deviation of peak current for blank (n=5) under same experimental conditions and *m* is the slope of calibration curve. The analytical performance results, LOD and LOQ are presented in **Table 1**.

The LOD and LOQ for BIC were found to be 5.20 x 10^{-8} M (±0.005µM) and 1.74 x 10^{-7} M (±0.005µM) respectively. The detection limits of BIC by other methods[22-23] compared with the present method are listed in **Table 3**.

4 Analytical Applications

4.1 Recovery test

To validate and check the accuracy of the proposed method intraday and interday recovery measurements were performed at two different concentrations (n=5) employing standard addition method. The results observed are listed in the **Table 2**.

4.2 Determination of Bicalutamide in pharmaceutical formulations

The validation of the proposed method was further confirmed by the determination of BIC concentration in the pharmaceutical formulation of Caluran (tablets). SWCNT/CPE was directly employed to determine the drug concentration of caluran tablets through DPV. The tablet analysis was followed from section 2.5 described earlier. The results exhibit a good agreement with manufactures label claim and are shown in **Table 3**.

4.3 Detection of Bicalutamide in Spiked Biological samples

The determination of spiked BIC in human serum and urine samples was followed from the procedure given in section 2.6 through DPV method at modified SWCNT/CPE. The recoveries from human serum and urine samples were measured by spiking drug free samples with known concentration of BIC.

The determined results obtained for the biological samples are listed in **Table 4** for serum samples and **Table 5** for urine samples. Recovery results were in the range from 99.00% to 99.83% for serum and 99.62% to 101.6% for urine samples.

5 Conclusion

A modified SWCNT/CPE was employed to investigate the electrochemical behaviour of an anti-cancer drug BIC in 0.1M phosphate buffer pH 7.0. The modification of electrode proceeded with a remarkable peak current enhancement and excellent electrocatalytic properties. Simultaneously a simple, sensitive, rapid and economical method has been developed and

validated for the determination of BIC in pharmaceutical preparations and biological samples. It was shown that BIC undergoes a $4e^-$ $4H^+$ reduction at the electrode surface. The electrocatalyzed reduction process was found to be irreversible and diffusion controlled. Owing to the simple and economical nature of the proposed method, it can be successfully applied for various quality control and assurances.

Acknowledgments

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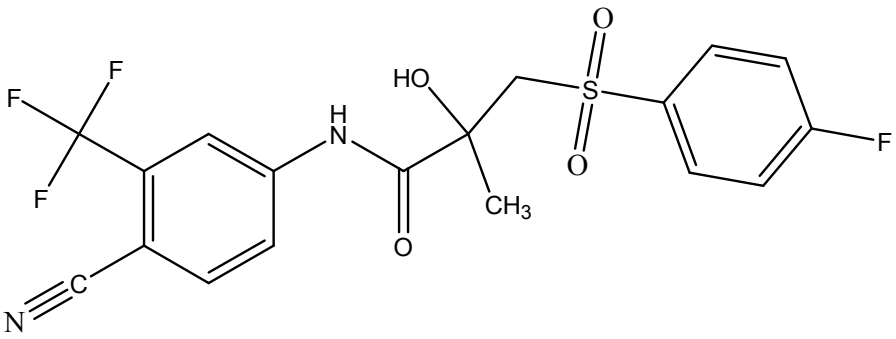
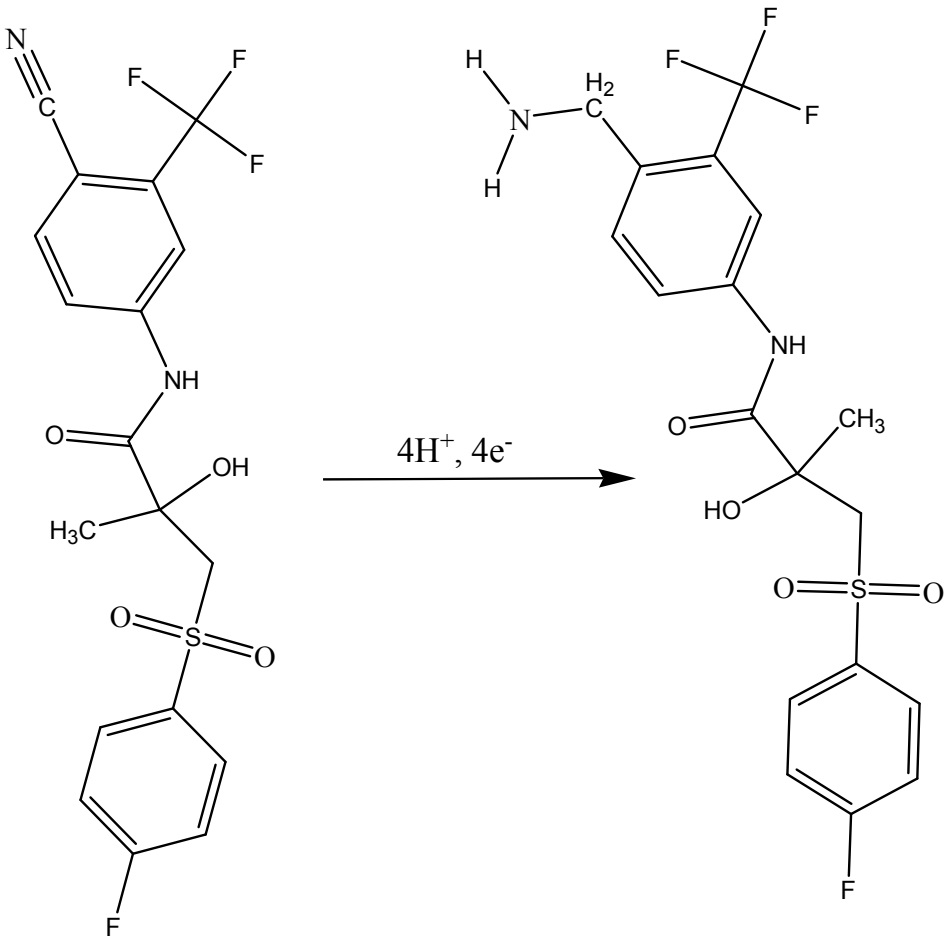


N-(4-Cyano-3-trifluoromethyl-phenyl)-3-(4-fluoro-benzenesulfonyl)-2-hydroxy-2-methyl-propionamide

Scheme 1



Scheme 2

Plausible reduction mechanism

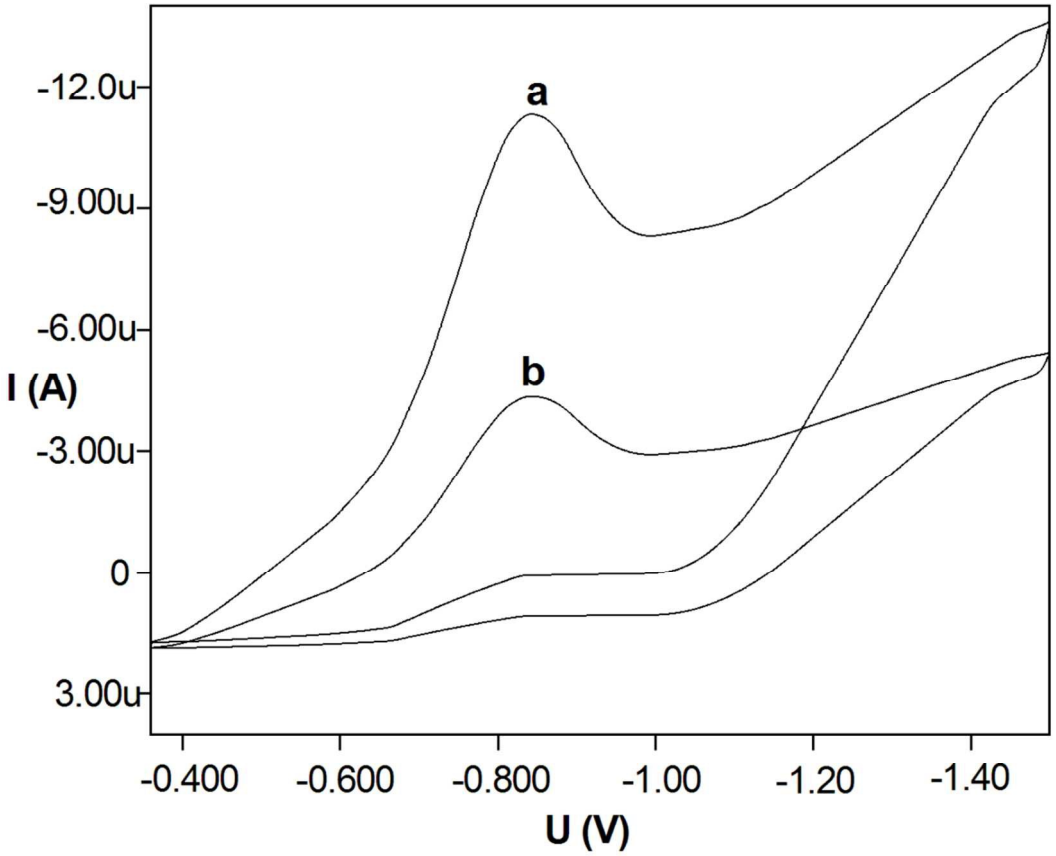


Fig. 1 Cyclic Voltammograms of 1 x 10^{-6} M Bicalutamide at a) Modified SWCNT-CPE and b) at bare CPE

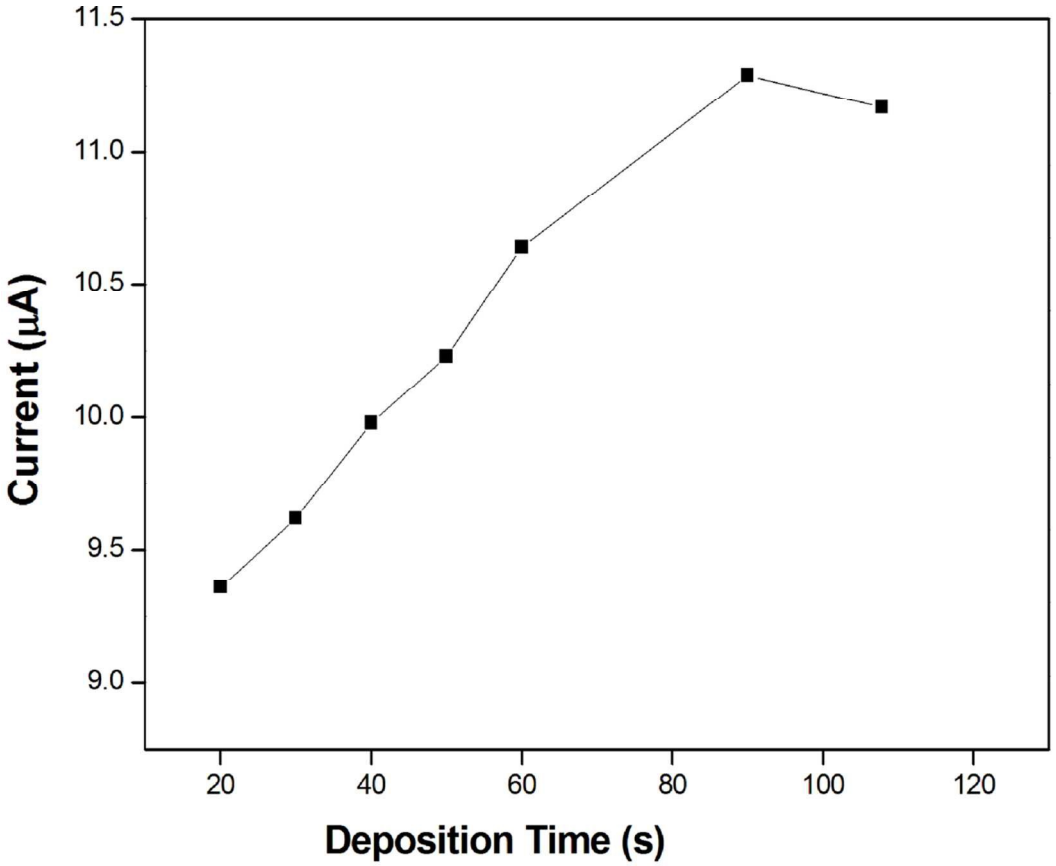


Fig. 2 Influence of Deposition time on Peak Current I_p (μA) of 1 x 10^{-6} M Bicalutamide

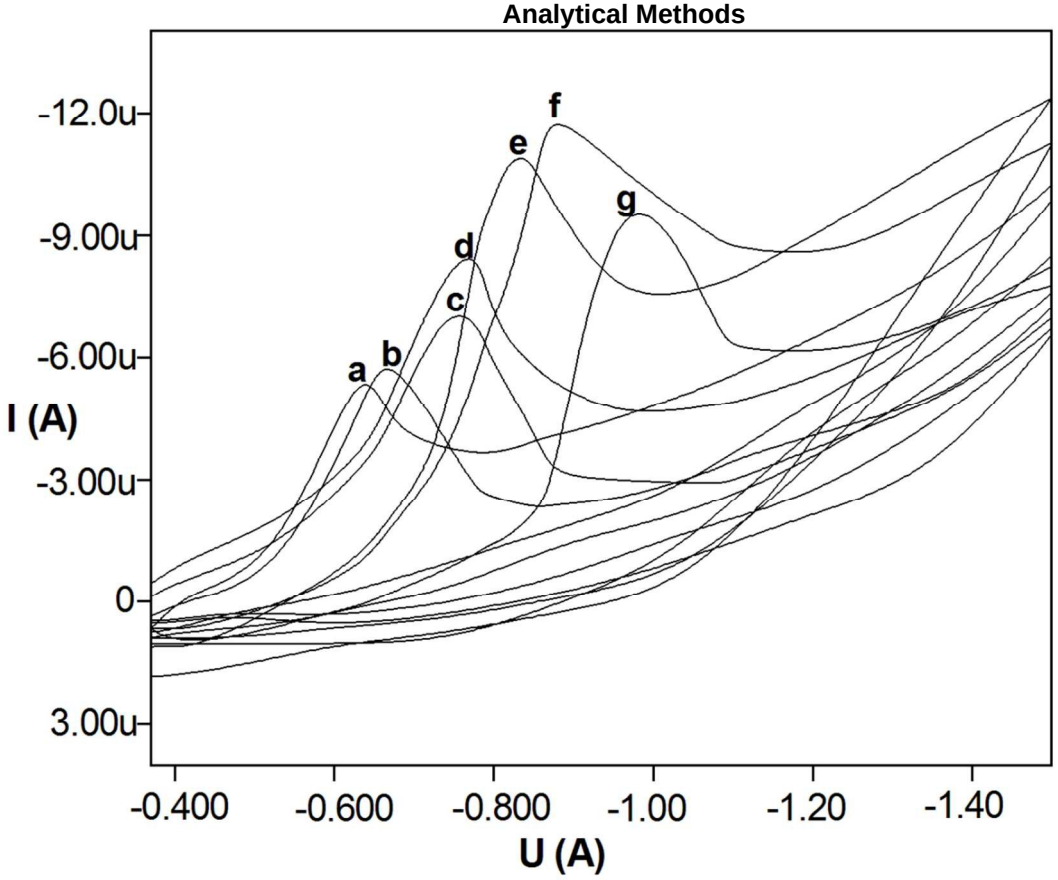


Fig. 3 Influence of *p*H on peak potential and peak current of 1 x 10^{-6} M Bicalutamide at *p*H a) 3.0 b) 4.0 c) 5.0 d) 6.0 e) 7.0 f) 8.0 g) 9.0

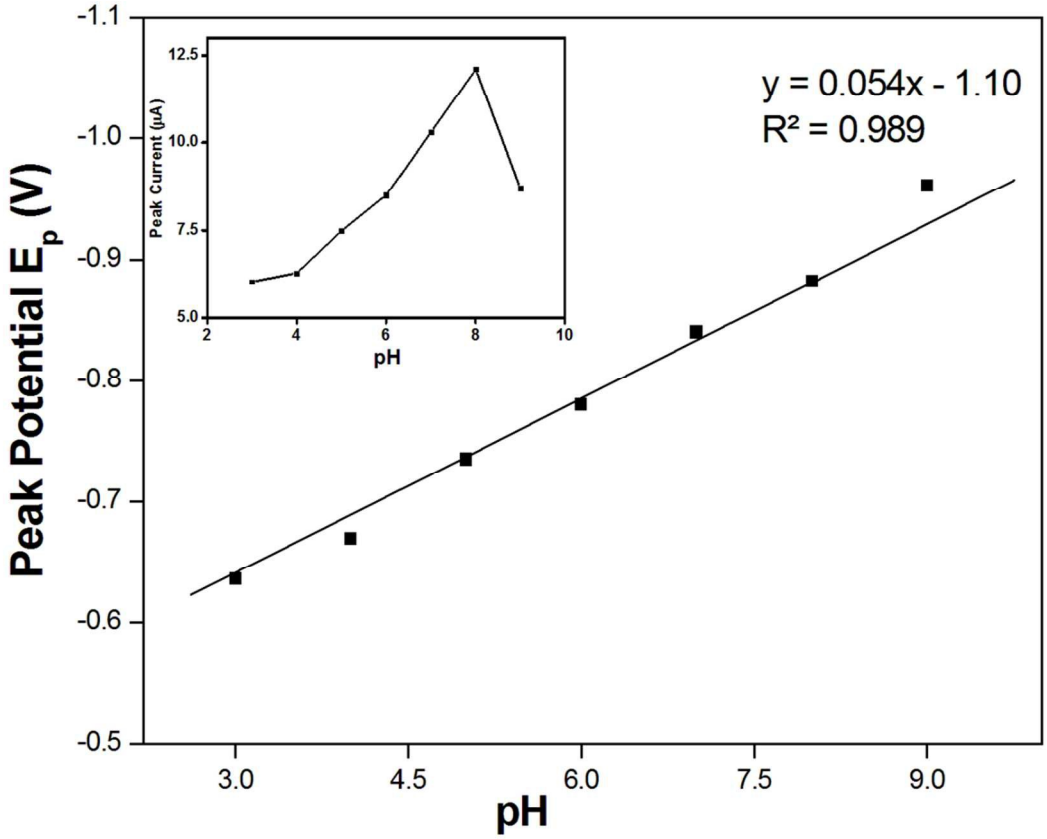


Fig. 4 Variation of peak potential (E_P) of 1 x 10^{-6} M Bicalutamide with pH. Insert: Variation of peak current (I_P) with pH.

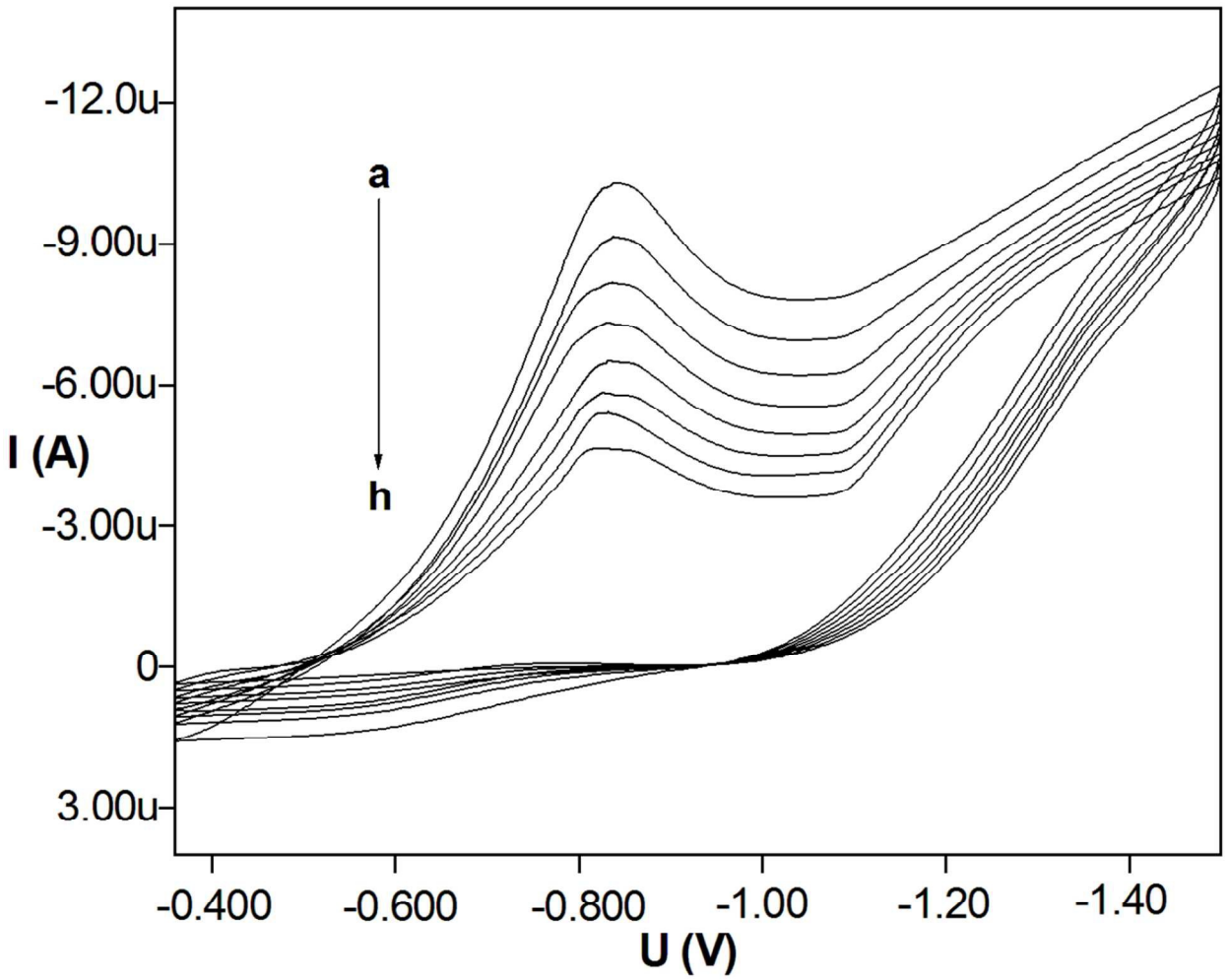


Fig. 5 Cyclic Voltammagrams of 1 x 10^{-6} M Bicalutamide at different scan rates (a-h: 200, 175, 150, 125, 100, 75, 50, 25. mVs^{-1})

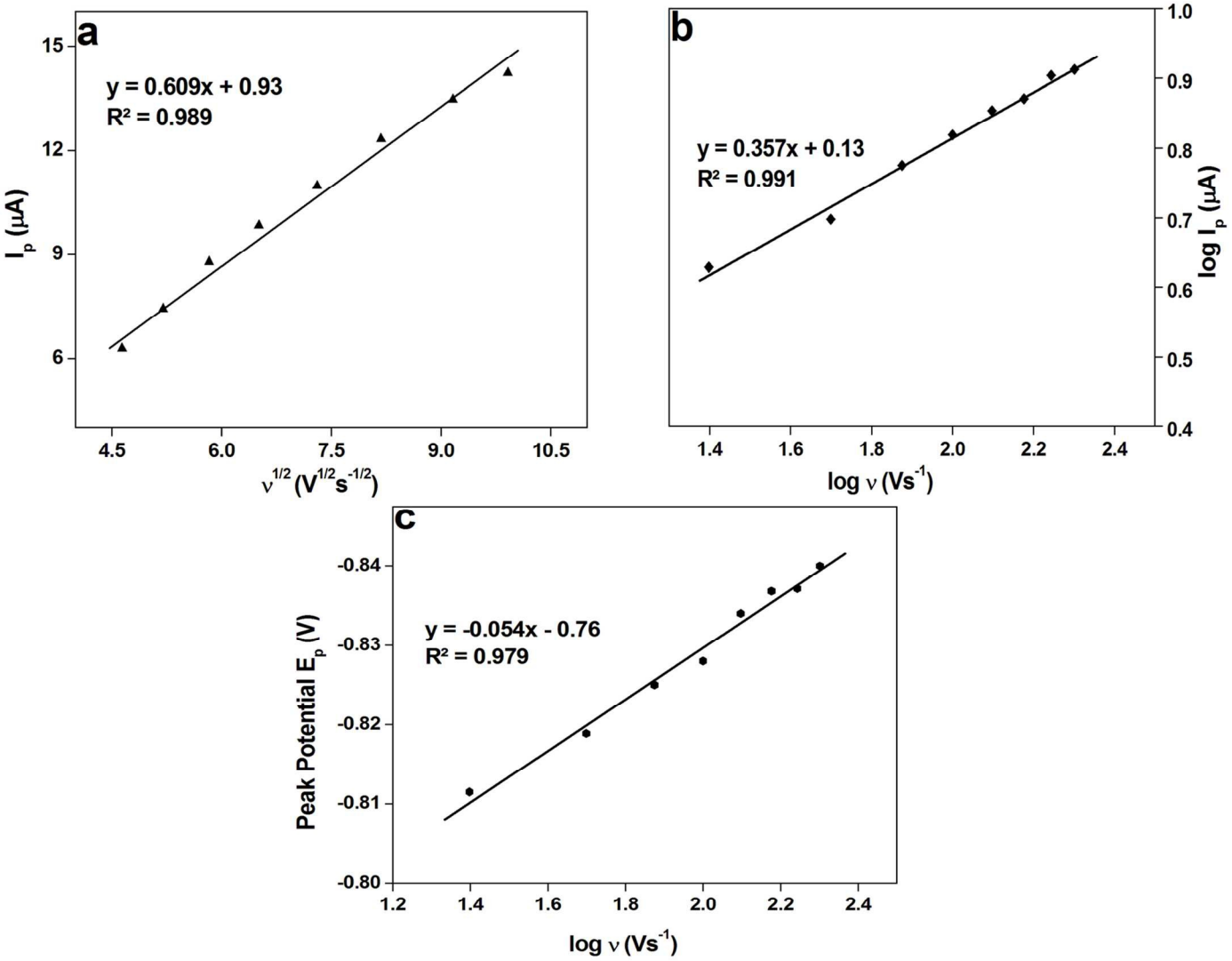


Fig. 6 (a) Peak current dependence on square root of scan rate. (b) Dependence of logarithm of Peak current on logarithm of scan rate (c) Relationship between peak potential and logarithm of scan rate

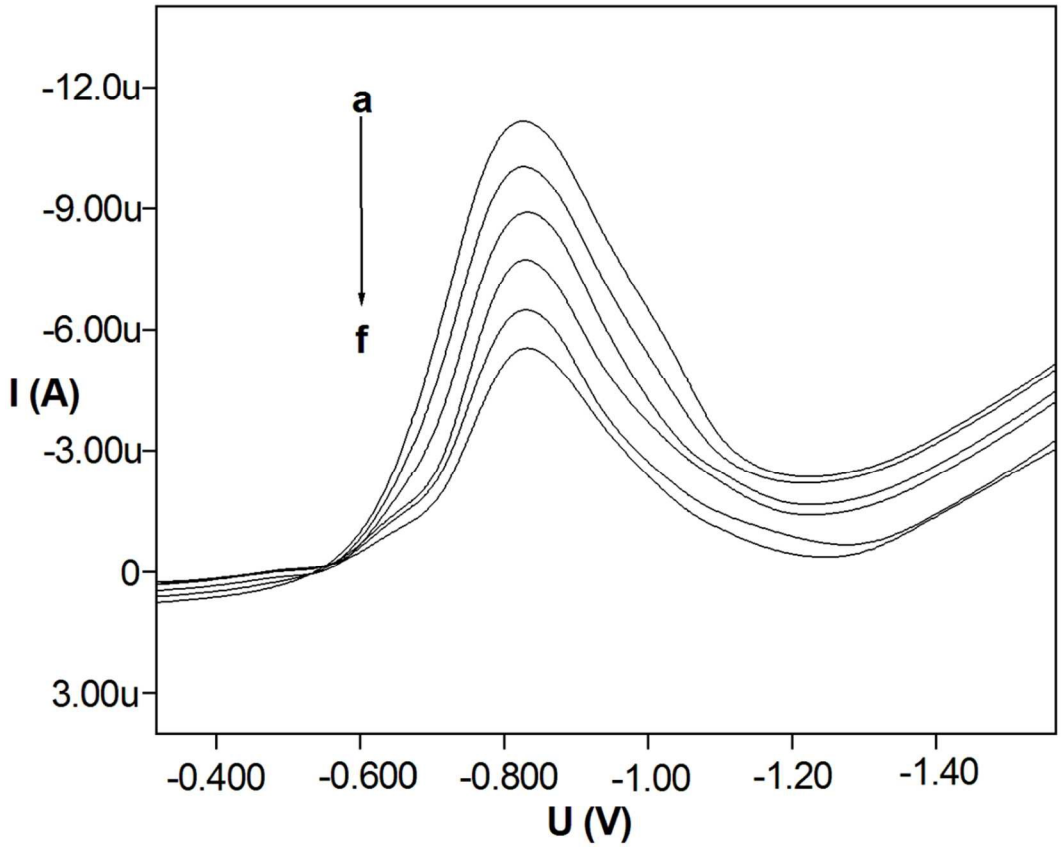


Fig. 7 Differential pulse voltammograms with decreasing concentration of Bicalutamide in 0.1M phosphate buffer (pH 7.0) (a-f: 1µM, 0.8 µM, 0.6 µM, 0.4 µM, 0.2 µM, 0.01 µM)

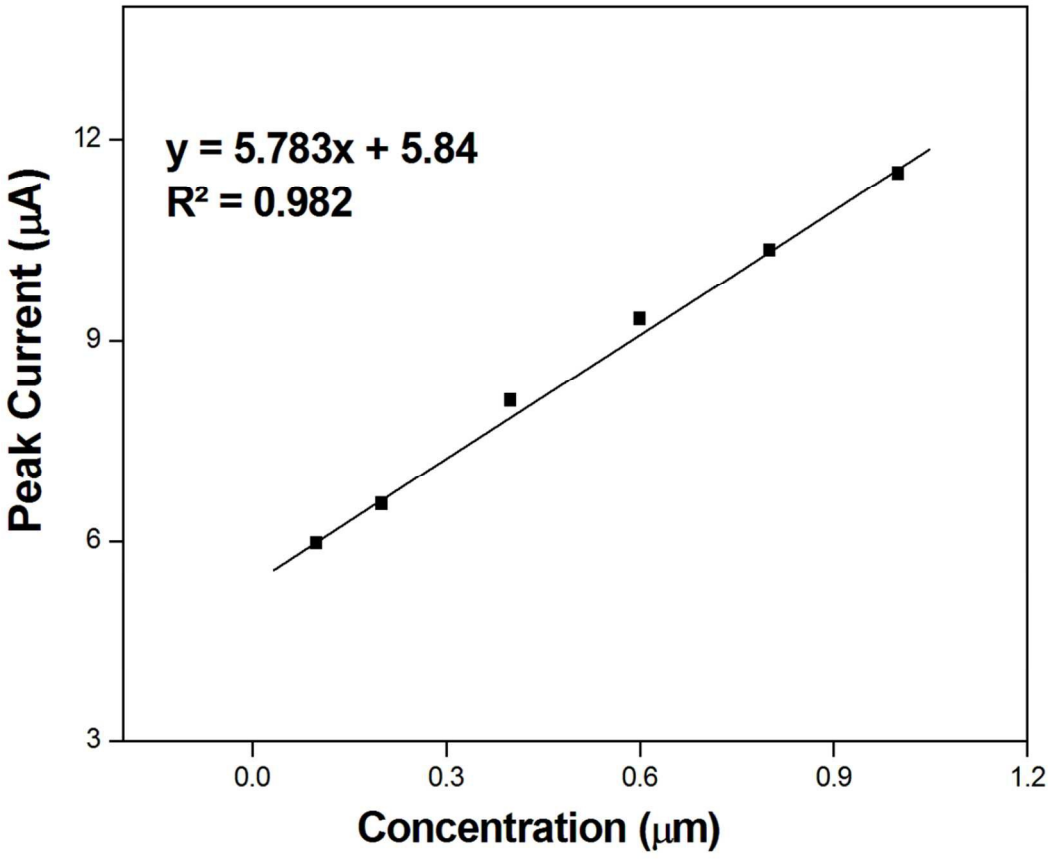


Fig. 8 Plot of peak current against the concentration of Bicalutamide.

Tables

Table 1 Regression data for calibration curve using DPV.

Parameters	Cyclic Voltammetry
Peak potential E_p (V)	-0.840
Linearity range (μM)	0.01-1
Slope (μA/μM)	5.783
Intercept	5.841
Correlation Coefficient (R^2)	0.982
LOD (μM)	0.052 (±0.005)
LOQ(μM)	0.174 (±0.005)
Repeatability of peak current (RSD%)	1.21
Repeatability of peak potential (RSD%)	0.82

Table 2 Analytical precision and Accuracy Recovery experiment data

	Added[a] (μM)	Found (μM)	SD	RSD%
Intraday	1.00	0.9889	0.011	1.16
	0.01	0.00986	0.167	1.70
Interday	1.00	0.9819	0.015	1.53
	0.01	0.00973	0.01	0.67

[a] mean of five (n=5)

Table 3 Results of recovery test of Bicalutamide in pharmaceutical formulations

	DPV[a]	LC[23]	Spectroscopic [22]
Label Claim (mg)	50	50	50
Amount Found (mg)	49.46[b]	37.43[c]	49.41
Recovery (%)	99.24	74.85[c]	98.63
RSD%	0.71	<1	0.28
LOD (μM)	0.052 (±0.005)	0.03	0.10
LOQ (μM)	0.174 (±0.005)	0.93	0.40

[a]Present method. [b]average of five (n=5). [c]average of four (n=4)

Table 4 Determination of Bicalutamide in Human Serum

Sample	Spiked[a] (μM)	Detected (μM)	Recovery (%)	RSD%
1	1	0.99	99.00	0.27
2	2	1.998	99.90	0.13
3	4	3.975	99.37	0.26
4	6	5.99	99.83	0.12
5	8	7.98	99.75	0.17

[a] average of five (n=5)

Table 5 Determination of Bicalutamide in Human Urine

Sample	Spiked[a] (μM)	Detected (μM)	Recovery (%)	RSD%
1	1	1.016	101.6	2.82
2	2	2.006	100.3	0.81
3	4	3.991	99.77	0.18
4	6	5.996	99.93	0.43
5	8	7.97	99.62	0.96

[a] average of five (n=5)

Graphical Abstract