*Portugaliae Electrochimica Acta 2015, 33(6), 323-330*

DOI: 10.4152/pea.201506323

**PORTUGALIAE ELECTROCHIMICA ACTA**
ISSN 1647-1571

# Synthesis Characterization and Anticancer Activity of Co(II)- Flutamide Complex

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Received 20 January 2015; accepted 13 October 2015

**Abstract**

Cobalt complex with flutamide was synthesized and physico-chemically characterized by amperometry, polarography, elemental analysis and IR spectrometry. After synthesizing the metal complex, it was evaluated for antibacterial and antifungal activities against various pathogenic microorganisms such as *Streptococcus aureus, Prosteus mirabilis, Klebsiella pneumonia and Aspergillus niger, Nigrosporan sp Fungi*. B16-F10 melanoma cell and C-57BL/6 mice have been used for anticancer screening of the metal complex for in vitro and in vivo study. The result of pharmacological studies with M:L revealed that the complex is more potent as compared to the pure drug as regards to its anticancer activity.

***Keywords****:* Anticancer drug, Cobalt Complex, Polarography, Antibacterial activity, Pharmacology.

## Introduction

Flutamide 2-methyl-N-[4-nitro-3-(trifluoromethyl) phenyl] propanamide is used as antineoplastic and antiandrogen drug [1]. It is a powerful nonsteroidal androgen antagonist which has treated prostate cancer and is believed to block androgen receptor sites. This drug and its primary hydroxyl metabolite decrease metabolism of C-19 steroid by the cytochrome P-450 system at the target cells in the secondary sex organ [2]. Prostate cancer is the most common cancer in men in western countries and it is the second leading cause of cancer death [3].
Cobalt ion is present in cobamide, which is a derivative of vitamin B12 and one of the most extraordinary in all biologically active substances. Cobamide is

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important in the *in-vivo* synthesis of amino acids used to make proteins. Vitamin B12 is the first metallo complex in living systems being studied in great depth [4-5]. Cobalt is a life essential element; it must be supplied in diet in its physiologically activity from vitamin B12. The complexation behavior of Co (II) for medicinal purposes has been widely published [6].

The study on cobalt complex of anticancer flutamide drug has been carried out physico-chemically, microbially and pharmacologically. The metal ligand complexation equilibrium has been studied using amperometric titration polarographic studies and elemental analysis. IR spectral analysis has been worked out, giving the probable formula for the complex as being 1:1. Various pathogenic bacteria like *Streptococcus aureus, Prosteus mirabilis, Klebsiella pneumonia, Aspergillus niger, and Nigrosporan sp*, have been used to microbial study using the disc diffusion method.

B16-F10 melanoma cell and C-57BL/6 mice were used for the *in vitro* and *in vivo* anticancer study of the complex, respectively. The results of the physicochemical method, and of microbial and pharmacological studies with Co(II)-flutamide complex may be suggested to the therapeutic experts for their possible use as a more potent anticancer drug.

## Experimental

The entire chemicals used were of analytical "grade"; the flutamide drug was purchased from Sigma Chemical Company®, USA. Standard solutions of Co(II) 1 mM, flutamide 1 mM, and potassium chloride 0.1 M, were 5% of 95% ethyl alcohol and prepared according to our choices.

Polarographic / voltammetric measurement was carried out using a Metrohm® instrument, Model 797A Computrace, which stands three electrodes containing a DME dropping mercury electrode as a working electrode, a coiled platinum wire as an auxillary electrode, and a saturated calomel electrode (SCE) as a reference electrode.

### *Electrochemical studies of Co(II)- flutamide complex*

For the study of the metal : ligand (M:L) complexation equilibrium, experiment sets were prepared by keeping overall Co(II) and potassium chloride (supporting electrolyte) concentrations fixed at 1 mM and 0.1M, respectively. The ligand concentration was varied until 15 mM. The pH of the test solution was adjusted to 7.0 ±0.1 using HCl/NaOH solution. The test solutions were deaerated by bubbling nitrogen gas for 15 min before recording the polarogram.

The amperometric titrations were performed on a manually operated set up equipped with a polyflex galvanometer (sensitivity $8.1 \times 10^{-9}$ amp per div) and an Agco Vernier potentiometer. The capillary characteristics of DME had $m^{2/3} t^{1/6}$ value of 2.5 $mg^{2/3} S^{-1/2}$ at 50 cm effective height of mercury column. A systronics digital pH meter- 335 was used for the pH measurements.

Experimental sets each having different but known amount of Co(II) were prepared in appropriate quantity of the supporting electrolyte (KCl) and the pH was adjusted to 7.0 ±0.2 and titrated separately against the standard solution of

the titled flutamide whose pH was also adjusted to that of the titrate ( 7.0± 0.1 using NaOH / HCl) at -1.20 V versus SCE (the plateau potential of Co(II)). The current offered by each addition of the titrant was read, and a curve was plotted between current against volume of the titrant added.

***Synthesis procedure of the solid complex***

Cobalt chloride and flutamide were prepared separately in water and were mixed in 1:1 molar "ratio". The mixture was then refluxed in a round bottom flask for 2 h. The complex was marked by precipitation; after reducing (complex) it was filtered and washed thoroughly to remove any unreacted material; then it was dried at low temperature and stored over $P_4O_{10}$.

The results of elemental (C, H, N) and F analysis on the drug and Co(II)-flutamide complex were furnished by CDRI Lucknow, India. The gravimetric method was used for the estimation of cobalt in the complex [7].

***Antimicrobial screening***

The microorganisms used in this study were *Klebsiella pneumonia, Proteus mirabilis, Stretococus aureus, fungi Asperginus niger and Nigrosporas SP*. All strains were obtained from the microbiological department Govt. Motial Vigyan mahavidyalal Bhopal (M.P.) India. Each microorganism was maintained on Mueller–Hinton (MH) agar medium at 4 ºC.

Kirby-Baller et al. disc diffusion was followed for the antimicrobial activity screening of the complex against various microorganisms: *Klebsiella pneumonia, Proteus mirabilis*, *Streptococcus aureus, fungi Asperginus niger* and *Nigrosporas S.P.* [8]. The number of replicates in each case was three; the percentage of inhibition was calculated using the following formula [9]:

$$\text{percentage inhibition} = \frac{a-b}{a} \times 100$$

where *a* represents the diameter of the zone of inhibition for flutamide control, and *b* represents the zone of inhibition for the complex (Co(II)- flutamide).

***Pharmacological studies***

*In-vitro* and *in vivo* studies of anticancer activity of the prepared metal drug complex have been done using the following procedure [10-12].

*In-vitro* B16-F10 melanoma cell line strains were obtained from Jawaharlal Nehru Cancer Hospital and Research Centre, Idgah Hills Bhopal M.P, India, as a monolayer culture in Roux battles (Corning® plastics U.S.A.).

***Cell Culture***

The cells B16-F10 (13) melanoma obtained were in culture in 5 mL 24 well culture plates (corning® plastics, USA). The cells were seeded in $2\times10^5$ cell per well, grown in 1.0 mL dulbecco's modified Eagles medium (DMEM Gibco®), supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acid, 1mM sodium pyruvate, 100 µg/mL penicillin, 100 µg/ mL streptomycin, and 5% v/v heat inactivated fetal calf serum. The B16F10 cell line was growth at 37 °C. The cells were kept in an incubator at 37 °C for 8 h in 5% $CO_2$ atmosphere and

95% humidity. The cell counter was made on Neubaur's Chamber (Fine optic®, Germany).
Two dilutions viz 1 μm, 10 μm of pure drug and its complex were made and then the cells were treated as follows:

| Column | Free drug | Metal complex |
|---|---|---|
| A | 1μM (1ML) | 1 μM (1ML) |
| B | 10 μM (1ML) | 10 μM (1ML) |

After addition of the respective solutions, the culture plate was incubated at 37 °C for 8 hours. Finally, the cell counts were made where appropriate to be shown. These are compared with the cell cultured in DMEM without treatment.

***Cells vialibility counts***
Cell vialibility counts were made by trypan blue dye exclusion test. Two drops of trypan blue were added to each cell culture well and kept for 15 minutes. Now a drop of culture was added to hemocytometer (Neubaurs chambers) and the number of stained, unstained, and the total numbers of cells were counted; then the % inhibition was calculated using the equation

[(Nº viable cells – Nº viable cells after treatment) / Nº viable cells without treatment]×100

The experiment of each concentration of the drug and the complex was repeated thrice and statistical conclusions were drawn.

***In vivo***
The comparative efficiency of pure and complex forms of flutamide drug was evaluated from the difference in response after treatment with two forms of the drug.

Animal modle : C57BL/6 mice weight 25 – 30 gm
Tumor modle : B16-F10 melanoma cell line
Drug : flutamide and its cobalt complex

Cells growing in nutrient medium (DMEM) were obtained from NCCS Pune. They were brought into a single cell suspension by trypsinization (0.2% trypsin). The cell suspension was centrifuged to obtain a concentrated suspension ($2\times10^5$ cell/mL); approximately $10^5$ cells of tumor were injected on the dorsal skin of an adult C57BL/6 mice and allowed the tumor grow; palpable size was reached by 6-8 days.
The time required to double the tumor volume (volume doubling time (VDT)) from 100 to 200 $mm^3$ was taken as a criterion to assess the antitumor efficiency of pure and complex drug in B16F10 tumor bearing mice. The treatment was started after tumor size reached 100 ±10 $mm^3$. Indicated doses (0.2 mg) of free drug and drug complex were injected intravenously and the tumor growth was monitored. The tumor size was calculated by the formula $V=(\pi/6)\ D_1D_2D_3$, where

$D_1D_2D_3$ = diameters in three perpendicular planes, measured using a vernier caliper, and V=volume of tumor [14].

## Results and discussion

### *Polarographic behaviour of flutamide with Co(II)*

In 0.1 M KCl at pH 7.0±0.1 the Co(II) and its complex with the ligand under study were found to be reversible, and the diffusion controlled polarographic wave, which was revealed by the log plot slop id versus √h (effective height of mercury column) respectively on gradual addition of ligand the $E_{1/2}$ of metal shift more electronegative value indicating the formation of complex (Fig. 1). Lingane's treatment [15] of the observed polarographic data revealed 1:1 [M:L] complex formation in solution with log $\beta_1$=5.2.

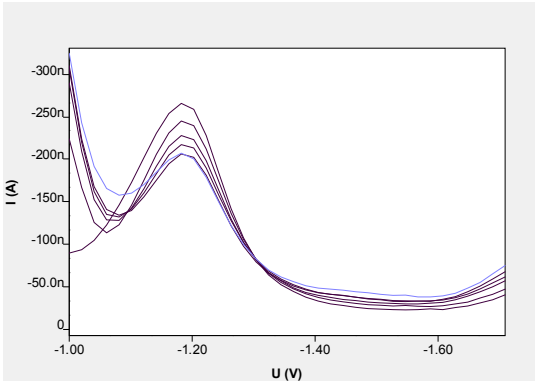


**Figure 1.** Polarogram of Co(II) (1.0 mM) in 0.1 M KCl supporting electrolyte at pH 7.0±0.1 and 1.0 mM flutamide

### *Amperometric determination of flutamide with Co(II)*

Co (II) with flutamide gives a well defined polarographic waves peak in 0.1 M KCl; at 7.0±0.1 pH the diffusion current was found to be proportional to the concentration of Co(II). The plateau potential for the polarographic wave of Co (II) (-1.25V) vs. Hg Pool was applied for carrying out amperometeric titration.

The current goes on decreasing to minimum and then attends a constant value. The plot of id versus volume (V+vV) of the titrant added revealed L shaped curve (Fig. 2). The end point was indicated by the intersection of the two lines, which confirmed 1:1 [M: L] complex formation.

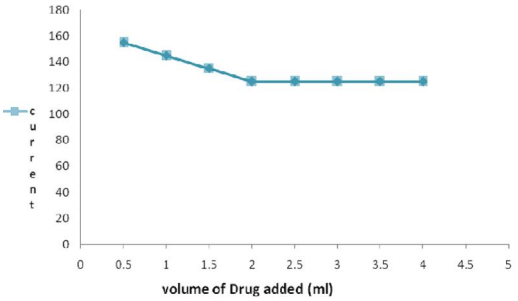


**Figure 2.** Amperometric titration of (2.0 mM/25mL) flutamide (2.0 mM/mL) Co(II) solution in 0.1 M KCl solution.

### *Elemental analysis*

Percentage of C, H, O, N, F and Co found and calculated in the complex and flutamide drug are summarized in Table 1.

**Table 1.** Result of elemental analysis (%)

| Element | Metal | C | H | O | N | F |
|---|---|---|---|---|---|---|
| Flutamide | - | 47.83 (47.87) | 4.01 (3.75) | 17.38 (17.49) | 10.14 (10.06) | 20.63 (20.80) |
| Co(II)-Flutamide | 16.8 (16.86) | 39.76 (39.76) | 3.33 (3.34) | 14.44 (14.48) | 8.43 (8.48) | 17.15 (17.18) |

### *Spectroscopic measurement*

A comparison of IR data for the flutamide drug and its complexes reveals that the bond at 3356 $cm^{-1}$, 1717 $cm^{-1}$, 1513 $cm^{-1}$ and 1243 $cm^{-1}$ in the drug are shifted to 3350 $cm^{-1}$, 1656 $cm^{-1}$, 1540 $cm^{-1}$ and 1280 $cm^{-1}$ respectively in the spectrum of the drug complex display nitrogen and (C=O) vibration in the spectrum of the free ligand suggesting ketoform of ligand and nitrogen indicating the coordination.

On the basis of the above discussion, a tentative of structure to the flutamide and its complexes may be assigned as shown in Fig. 3.

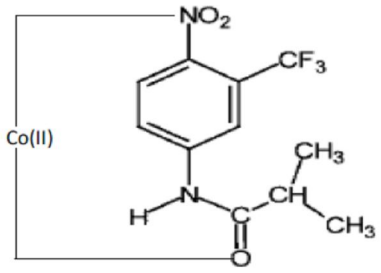


**Figure 3.** Co(II)- flutamide complex.

### *Antimicrobial activity*

Antimicrobial behavior of Co(II)-flutamide complex against various pathogenic bacteria and fungi is reported in Table 2. A perusal of data in the table reveals that the complex shows increased antimicrobial activity against all the pathogenic bacteria under study, as compared to the parent drug flutamide.

**Table 2.** Result of antibacterial activity of flutamide and its Co(II)-flutamide complex.

| Test organism | Zone of inhibition | | % Inhibition |
|---|---|---|---|
| (A-*Bacteria)* | Control (mm) | Complex (mm) | |
| *Streptococcus aureus* | 8 | 14 | -75 |
| *Klebsiella pneumania* | - | 14 | - |
| *Prosteus.mirabilis* | 6 | 11 | -83.3 |
| *(*B-*Fungi) Aspergillus niger* | 7 | 13 | -85.71 |
| *Nigrosporan sp* | 6 | 10 | 66.6 |

### *Pharmacological studies*

*In vitro*

The result of *in-vitro* experiments of the pure drug and its complex is shown in Table 3. Perusals of results show that cobalt flutamide complex was found to be more effective than the pure drug. The complex under study showed an increased inhibition against the melanoma cell line B16F10 at all the test concentrations, i.e., 1, 10, µM/ML. The increased inhibition activity of the complex was 47.34±1.56%, 90.12±1.10% as against 26.87±1.21%, 56.79±1.60% shown by the drug, respectively. The statistical treatment of the observed inhibition data, i.e., standard deviation and coefficient of variance, which never exceeded 0.9 and 18 %, respectively, speaks the reliability of the observed inhibition data.

**Table 3.** *In-vitro* cytotoxicity of flutamide and Co(II)-flutamide complex against B16-F10 melanoma cell.

| Compound | Concentration (µM/ML) | % inhibition after 8 h |
|---|---|---|
| Flutamide | 1.0 | 26.87 ± 1.21 (a) (b) |
| | 10 | 56.79 ± 1.60 |
| Co (II)- flutamide complex | 1.0 | 47.34 ± 1.56 |
| | 10 | 90.12 ± 1.1 |

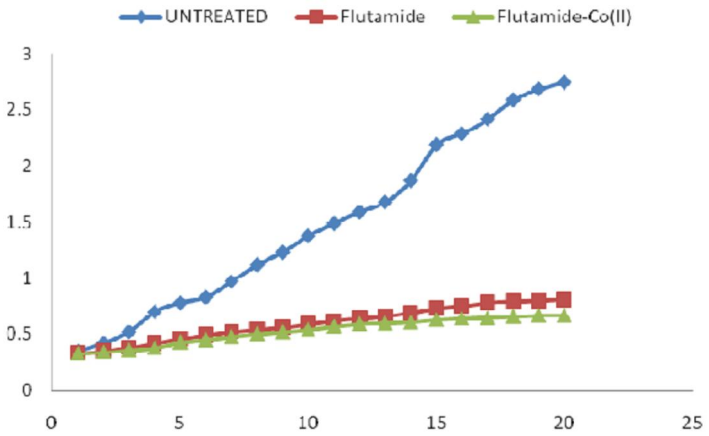


**Figure 4**. *In-vivo* cytotoxicity of flutamide and Co(II)-flutamide complex against B16-F10 melanoma cell.

*In vivo*

The results of the average of mice tumor against flutamide drug and cobalt complex under study are shown in Fig. 4. The results indicated that the tumor volume was 0.2 $cm^3$ on the mice tumor cells injected without administering drug or complex; 20 days after administering the flutamide drug in the mice tumor injected, the tumor size was reduced 0.81± 0.63 $cm^3$. The inhibition rate was found to be 70.54%. However, in the case of Co(II) flutamide, the administrated mice (tumor cell injected) show significant decrease in the tumor volume of 0.67 ± 0.73 $cm^3$, and the inhibition rate was 75.63%, thus indicating the increasing *in vivo* of the tumor inhibition power of the complex.

## Conclusions

To investigate the structure and behavior of complex of flutamide with life essential metal ion Co(II) some physicochemical method i.e. IR spectral analysis, elemental analysis, amperometry and polarography have been successfully used. The obtained results suggest that the complexes are more stable as compared to the pure drug. On the basis of the observed results we can conclude that Co(II) with flutamide complex is more effective and non toxic in nature as compared to the parent drug. Thus, polarographic and amperometric methods may be recommended to study more potent drugs in lieu of the drug taken for present study, having excellent potential for clinical application.

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