Electrochemical Study of Flutamide, an Anticancer Drug, and Its Polarographic, UV Spectrophotometric and HPLC Determination in Tablets

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Abstract
Flutamide, 4-nitro-3-trifluoromethyl-isobutylanilide, a synthetic antiandrogenic with therapeutic use in prostatic cancer, is electrochemically studied to propose a new electroanalytical alternative for its quantitative determination in pharmaceutical forms. Flutamide is shown to be electrochemically reducible in aqueous and mixed media, due to the presence of the nitroaromatic moiety in the molecule. From the mixed media studies, a nitro radical anion derivative from flutamide is described. From the pH dependence of the polarographic peak an apparent pK_a of 4.8 was obtained and corroborated by means of UV spectroscopy. Based on the linear relation between the peak current and the flutamide concentrations a differential pulse polarographic method was developed. For comparative purposes spectrophotometric and $HPLC_{UV}$ methodologies were developed. Furthermore the developed methods were checked for selectivity using hydrolysis, photolysis, thermolysis and chemical oxidation.

Keywords: Flutamide, Differential pulse polarography, UV-spectrophotometry, HPLC, Acidic hydrolysis, Alkaline hydrolysis

1. Introduction

Flutamide, 4-nitro-3-trifluoromethyl-isobutilanilide (Fig. 1), is a synthetic antiandrogenic agent devoid of hormonal agonist activity. It seems to have antiandrogenic specificity only in genitalia organs androgen-dependent, and its therapeutic use is in prostatic cancer [1, 2]. Flutamide is an unusual example of an antiandrogenic drug lacking a steroidal structure.

After human oral administration flutamide is quickly metabolized, producing about 10 metabolites, mainly 2-hydroxyflutamide and 3-trifluoromethyl-4-nitroaniline [2].

Flutamide and its hydroxylated metabolite have been determined by high performance liquid chromatography, with detection limits of 11.27 and 5.67 mg/mL, respectively [3]. On the other hand, impurities contained in samples of flutamide have been determined by sobrepresurized liquid chromatography and evaluated by densitometry at 237 nm [4]. The chromatographic determination of 4-nitro-3-trifluoromethylaniline (hydrolysis product) in presence of flutamide also has been described [5]. Related to the determination of flutamide in pharmaceutical forms a gas chromatographic method has been reported [6].

From the electrochemical point of view, flutamide it has been studied only in a narrow pH range (6.1 to 7.9) including different electrochemical techniques as cyclic voltammetry, differential pulse and tast polarography. From this study, extraction with 95% ethanol followed by polarographic reduction was selected as the basis of a method for the determination of flutamide in tablets [7].

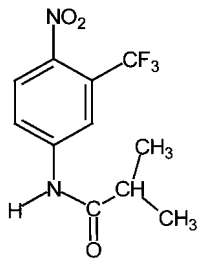

Fig. 1. Chemical structure of flutamide.

Taking into account that some aspects of the polarographic behavior of flutamide has been previously described [7], the main goals of this article will be focused on the investigation of the electrochemical behavior in all the pH range (2–12) including aqueous and mixed media by different voltammetric techniques. Emphasis in the cyclic voltammetric behavior, in order to obtain a detailed understanding of the different steps of its reduction process will be considered. Special attention to the feasibility of the high reactive nitro anion radical formation, which could be responsible for toxicity of flutamide in patients under treatment with this drug [8] will be investigated. In a previous report, an analogue drug, nilutamide, has shown that its toxicity can be partially explained by the formation of an electrophilic metabolite, presumable, the nitro radical anion [9].

On the other hand, due to the fact that this drug it not included in the Pharmacopoeia, this work evolved in the development of a sensitive, precise, selective and accurate procedure for the determination of flutamide without an extraction procedure being necessary before the voltammetric measurement. Moreover, since a comparative method of analysis was desirable, both an UV-spectrophotometric and HPLC method for the determination of the drug in commercial tablets was also developed.

2. Experimental

2.1. Reagents and Solutions

Flutamide (100% chromatographically pure) was obtained from Schering Plough Laboratories (Santiago, Chile) and commercial tablets of Drogenil (amount declared 250.0 mg flutamide per tablet) were obtained commercially. All other reagents employed were of analytical grade.

For HPLC the solutions under study were buffered using a 0.05 M buffer phosphate solution adjusted at pH 3 with phosphoric acid. For spectrophotometry and voltammetric techniques 0.04 M Britton-Robinson buffer adjusting the ionic strength at 0.1 M with KCl were used.

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2.2. Apparatus

A 25-mL thermostated Metrohm measuring cell, with dropping mercury electrode as a working electrode, a platinum wire counter electrode and an Ag/AgCl, Metrohm type reference electrode (SCE) were employed. The operating conditions were: sensitivity 5–10 μA; drop time 1 s; potential range 0 to –1700 mV; $\Delta E_p = -5$ mV; pulse retard 40 ms; pulse height –50 mV.

Spectrophotometric measurements were carried out with an UV-vis spectrophotometer ATI Unicam Model UV3, using 1 cm quartz cell and equipped with a 486 computer with Vision acquisition and treatment program.

Cyclic voltammetry experiments were carried out on a totally automated Inelecsa assembly, similar to one previously described [10].

HPLC measurements were carried out by using a Waters assembly equipped with a Model 600 Controller pump and a Model 996 Photodiode Array Detector. The acquisition and treatment of data were made by means of the Millenium version 2.1 software. As chromatographic column a μBondapak/μPorasil C-18 column of 3.9 mm × 150 mm was used. As precolumn a C18 μBondapak (30 mm × 4.6 mm) was employed. The injector was a 20 μL Rheodine valve.

Stock solutions of flutamide were prepared at a constant concentration of 2.5×10^{-3} M in ethanol.

An aliquot of stock solution was taken and diluted with ethanol/0.04 M Britton-Robinson buffer mixture (20/80) to obtain a final working solution concentration of 1×10^{-4} M.

2.3. Calibration Plot Preparation

A series of ten solutions were prepared containing flutamide concentration ranging between 1×10^{-6} and 1×10^{-5} M in ethanol/0.04 M Britton-Robinson buffer mixture (20/80) at pH 8.0 (for UV and PPD) or in methanol/0.05 M phosphate buffer (50/50), pH 3.0 (for HPLC).

2.4. Sample Preparation

Excipients (cornstarch, magnesium stearate, lactose, sodium lauryl sulfate and talc) were added to the drug for recovery studies, according to manufacturer's batch formulas for 250.0 mg flutamide per tablet.

2.5. Tablets Assay Procedure

2.5.1. Polarography

Ten series of one tablet of Drogenil (amount declared 250.0 mg flutamide per tablet) were suspended in ethanol, sonicated and diluted to 50 mL. A 2.5 mL aliquot of each solution was taken and diluted to 50 mL with ethanol/0.04M Britton-Robinson buffer mixture (20/80), pH 8.0. Each sample solution was transferred to a polarographic cell, degassed with nitrogen for 5 min and recorded at least twice from –300 mV to –800 mV. The mg amount of flutamide in the sample solution was calculated from a prepared standard calibration plot.

2.5.2. Spectrophotometry

The same general procedure described above was employed to obtain the 50 mL ethanol solution. Then, this solution was centrifuged for 10 min at 4000 rpm and a 0.3 mL aliquot was taken and diluted to 50 mL with ethanol/0.04 M Britton Robinson buffer mixture (20/80), pH 8.0. The obtained solutions were measured at 304 nm, and the mg flutamide in the sample solution were calculated from a prepared standard calibration plot.

2.5.3. HPLC

An aliquot of the solution described in Section 2.5.2. was taken but a methanol/0.05 M phosphate buffer (50/50) mixture was used as diluent. A 20 μL volume of this solution was injected into the chromatographic system. The photodiode array detector was operated at 302 nm for quantification. The mobile phase flow [methanol/0.05 M phosphate buffer (50/50), pH 3.0] was maintained at 1 mL/min and a helium sparging (10 mL/min) was applied to remove dissolved gases. The temperature was keep constant at 35 °C.

2.5.4. Apparent pK_a Determination (pK'_a)

For this purpose, both the 302 and 402 nm UV bands were used. The pH solution was changed each 0.5 units and near the pK_a zone it was varied each 0.25 units pH. The temperature was kept constant at 25 °C. The used flutamide concentration was 5×10^{-5} M for all the pH range.

2.6. Selectivity Study [11, 12]

2.6.1. Degradation Trials

Hydrolysis: Three different solutions of 2.5×10^{-3} M flutamide (ethanol/water, 50/50 for neutral hydrolysis, ethanol/1 M HCl for acid hydrolysis and ethanol/1 M NaOH for basic hydrolysis) were transferred to a 25 mL-distillation flask and boiled for one hour at reflux. Then, the solution was kept at –20 °C and protected from light.

Chemical oxidation: A 2.5×10^{-3} M flutamide solution (ethanol/20 % H_2O_2, 90/10) was transferred to a 25 mL-distillation flask and boiled for one hour at reflux. Then, the solution was kept at –20 °C and protected from light.

Photolysis of pure drug: 7.0 mg flutamide was dissolved in the minimal quantity of ethanol for total dissolution. From this solution a uniform layer was put onto a glass, placed in a thermoregulator bath at 50 °C to accelerate the ethanol evaporation and to obtain a uniform monolayer of drug on the glass. The glass was transferred to a black box and irradiated with UV light at a distance of 15 cm for 2 h. Then, the sample was dissolved with 10 mL of ethanol and kept at –20 °C and protected from light.

Photolysis in solution: 10 mL of a 2.5×10^{-3} M flutamide ethanol solution was bubbled 2 min with nitrogen and transferred to a black box and irradiated with UV light at a distance of 15 cm for 2 h. Then, the solution was kept at –20 °C and protected from light.

Thermolysis: 7.02 mg flutamide was heated at 105 °C for 5 h and then dissolved in ethanol and the resulting solution was kept at –20 °C and protected from light.

Each solution obtained from the degradation trials was assayed by DPP, UV and HPLC techniques. All the degradation trials were carried out in duplicate.

2.6.2. Statistic Analysis

Comparison between different techniques, as well as the comparison with standard deviations, was carried out by means of the *t*-Student test, and using significance limits between 95 % and 99 % of confidence [13, 14].

3. Results and Discussion

Flutamide in hydroalcoholic solution (0.04 M Britton-Robinson buffer/ethanol, 80/20), shows a cathodic response in a wide range of pH (2–12), by differential pulse and tast polarographic techniques.

In relationship to the pH effect (Fig. 2), a well-defined cathodic peak (i_1) in all the pH range studied was observed. This peak was shifted toward more cathodic potential upon increasing the pH. Furthermore a second signal (i_2) at more cathodic potential, that appears only at acidic pHs is seen. The peak potential (E_p) versus pH plot is shown in Figure 3. In this figure different linear zones that correspond to changes in the reduction mechanism can be observed. Probably these changes are due to balances of protonation-deprotonation in the molecule of flutamide. The break in the E_p–pH curve at approximately 4.8 is related with the polarographic pK_a of the molecule. The pH-independent zone above pH 9.5 means

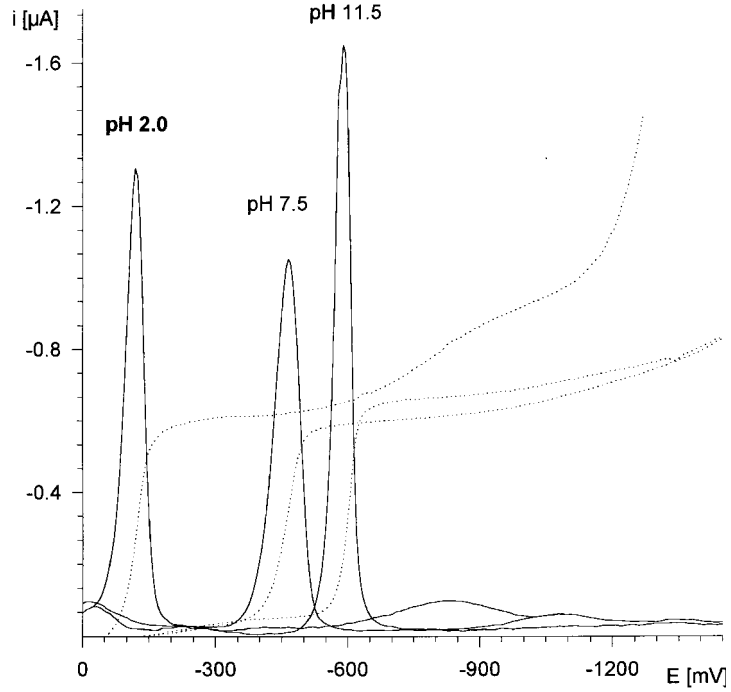


Fig. 2. Differential pulse (solid line) and tast (dashed line) polarograms of 0.1 mM flutamide solution at different pH (ethanol/Britton-Robinson buffer; 20/80).

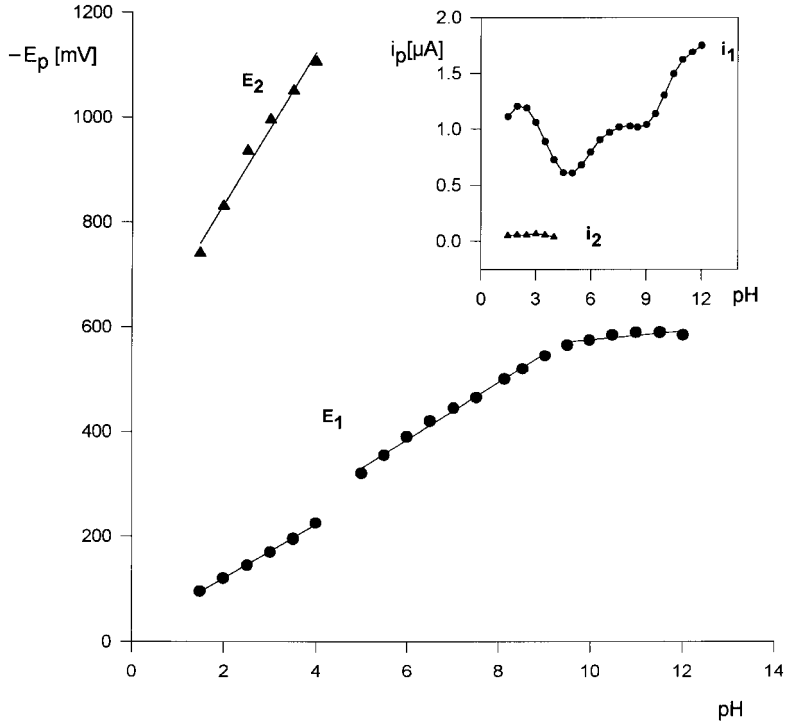


Fig. 3. Differential pulse peak potential and peak current (inset) dependence of 0.1 mM flutamide solution with pH.

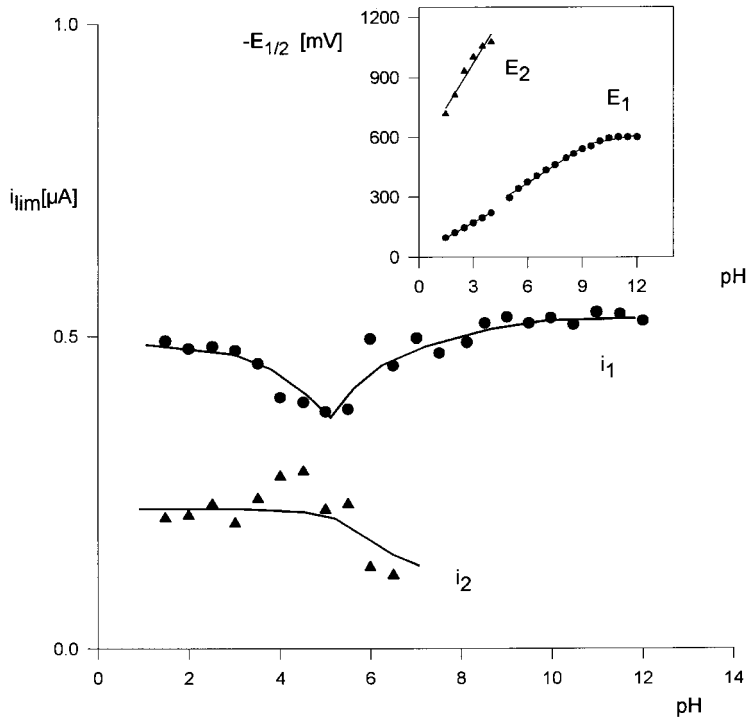


Fig. 4. Limiting current by tast polarography and half-wave potential (inset) dependence of 0.1 mM-flutamide solution with pH.

that there are no proton transfer steps before the electron transfer rate determining step. By using tast polarography a totally analogous behavior for the half-wave potential was obtained.

The evolution of the limiting current (i_l) with pH (Fig. 4) can be interpreted as the disappearance of one wave and the appearance of another thus giving account of a possible acid-base balance with a polarographic apparent pK_a value of 4.75, coincident with the minimum in the curve. Also it was observed that the second signal in acid pHs disappeared at neutral pH. Based on previous results, peak i_1 would account for the reduction of the nitro group contained in the 4-position (Fig. 1) as the most easily electroreducible group, and peak i_2 accounts for the hydroxylamine protonated derivative to give the amino derivative [15].

CV studies in protic media (0.04 M Britton-Robinson buffer/ethanol, 70/30) reveals a well-defined cathodic peak (E_p = −350 mV) at pH 2, that would account for the nitro group reduction. Also, a more cathodic wave was observed that would account for the hydroxylamine protonated derivative reduction, according to the well-known behavior of nitroaromatics [15–17]:

$$ArNO_2 + 4H^+ + 4e^- \rightarrow ArNHOH + H_2O$$

$$ArNHOH_2^+ + H^+ + 2e^- \rightarrow ArNH_2 + H_2O$$

At pH 7 an irreversible peak, due to the nitro group reduction can be observed. At pH 12 a similar peak was observed, but in the anodic sweep (approximately at −350 mV) a peak due to the hydroxylamine derivative oxidation was observed, according to the following equation

$$ArNHOH \rightleftharpoons ArNO + 2H^+ + 2e^-$$

Using CV in mixed media (Fig. 5), a well-defined irreversible peak at pH 2 is observed, similar to that observed in protic media. However, at pH 12 a reversible couple (E_{pa} = − 720, E_{pc} = − 790 mV) was observed (Fig. 5), which corresponds to the one-electron reduction of the nitro group to the nitro radical anion, according to

$$ArNO_2 + e^- \rightleftharpoons ArNO_2^{\bullet -}$$

The appearance of this reversible one-electron wave in mixed basic media is a well-known fact for several nitroaromatics compounds [18], but up to now nobody had described the formation of a free radical for flutamide. The existence of this species can have important consequences from the metabolic point of view and will be object of further studies in our laboratory. In fact, in another antiandrogenic related compound, nilutamide, the nitro anion free radical formation in rat microsomes under anaerobic conditions has been demostrated [9].

Furthermore, at more cathodic potentials (E_{pc} = − 1100 mV) a peak appears that accounts for the later reduction of the radical (Fig. 5) according to the following equation

$$ArNO_2^{\bullet -} + 3e^- + 4H^+ \rightarrow ArNHOH + H_2O$$

Finally in the anodic sweep a peak (approximately at −200 mV), that accounts for the hydroxylamine derivative oxidation appears.

With the aim of making a comparison between different techniques and to further the knowledge of the chemistry in solution of flutamide, an UV-visible spectrophotometric study was conducted. UV spectra at different pHs (Fig. 6a) reveals two absorption signals for flutamide in all the pH range. The first one presents a pH-dependent maximum at a wavelength of 230 nm. The second one presents a pH-independent maximum at a wavelength of 304 nm, which was used to quantify the drug. The sensitivity of the first band with pH was used to determine the spectrophotometric apparent pK_a (Fig. 6b and c). The value of pK_a' obtained for flutamide was 4.83 and was calculated by the linear regression method. It is in agreement with the break in the E_p *versus* pH plot observed by DPP and tast. The corresponding dissociation equilibrium is shown in Scheme 1.

According to the obtained results by electrochemical and spectroscopic techniques, it is possible to apply these techniques to the quantitative analysis of flutamide. Consequently a HPLC method using electrochemical or spectrophotometric detection will be also possible. As working pH pH 8 for DPP and UV techniques was selected and pH 3 for HPLC.

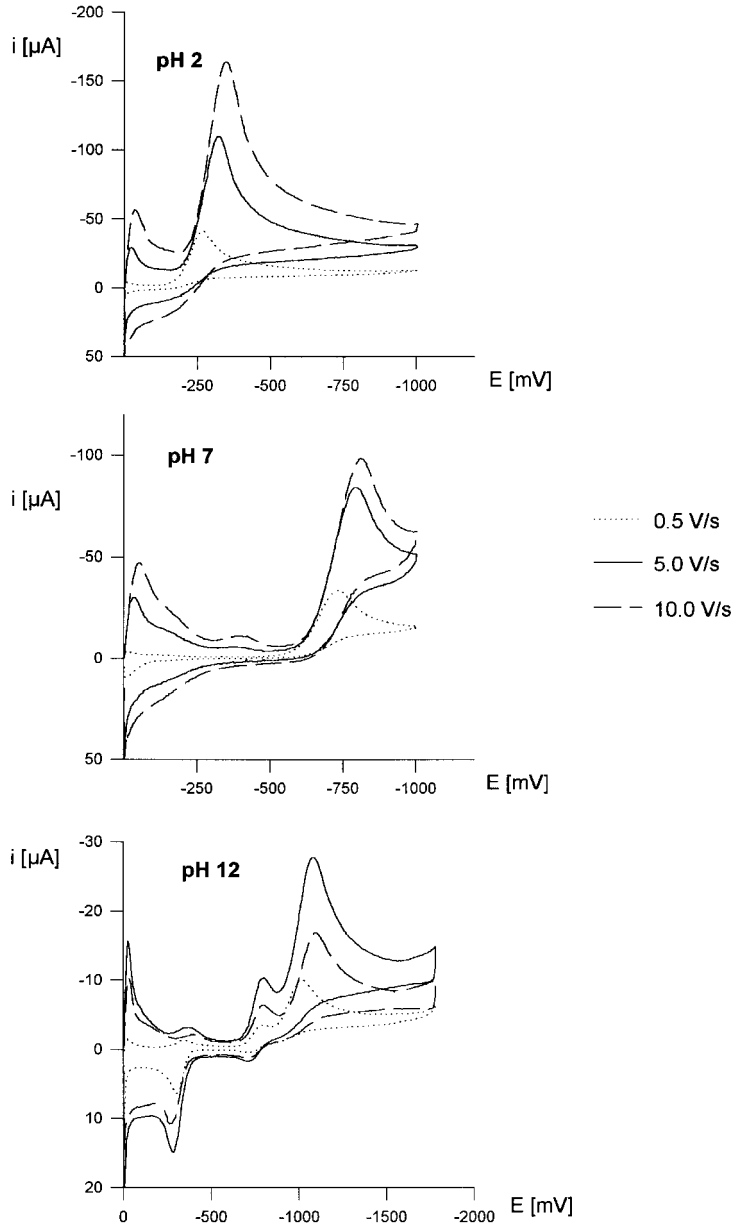


Fig. 5. Cyclic voltammograms of 1 mM flutamide solution in acidic, neutral and alkaline media at different sweep rates.

In order to provide a DPP quantitative procedure the dependence between the flutamide concentration and peak current (i_p) was conducted. A linear relation in the concentration range between 1×10^{-3} M and 1.9×10^{-6} M was found indicating that the response was diffusion controled in this range [19].

On the other hand, with the UV spectrophotometric technique the concentration study showed a linear response in the concentration range between 1×10^{-3} M and 1×10^{-6} M. While in HPLC only with the photodiode array detector was it possible to obtain a linear response between the concentration range 5×10^{-7} M and 5×10^{-4} M. In order to quantify flutamide samples the calibration plot method was applied.

From the repeatability study, variation coefficients of 1.65 %, 0.08 % and 1.04 % were obtained by DPP, UV and $HPLC_{UV}$ techniques, respectively. These results show that the three techniques present adequate repeatability. From the reproducibility study (changing both operator and equipment) the following variation coefficients were obtained: 1.93 % (DPP), 0.28 % (UV) and 1.75 % ($HPLC_{UV}$). On the other hand, by using $HPLC_{ED}$ a variation coefficient of 13.6% was obtained, therefore this detector was not selected for later analytical applications.

In order to check our proposed methods for selectivity we tried different degradation pathways for flutamide. Selectivity is a parameter that gives account of the capacity of the method of producing a signal due to only to the presence of the analyte (flutamide), and consequently free of interference of other components, as degradation products, metabolites and pollutants. This study was carried out by using the following trials: hydrolysis (acid, basic and neutral), photolysis (solid pure drug and in solution), thermolysis and chemical oxidation [11]. Then all the obtained solutions in the degradation trials were evaluated by the different techniques and compared with the standard pure drug. In the neutral hydrolysis, by DPP, UV and HPLC techniques did not reveal changes. According to the obtained results, we concluded that under these conditions, the developed methods did not deliver information that permit one to establish a degradation of the flutamide molecule. However, when acid hydrolysis was performed, the initial DPP peak obtained for

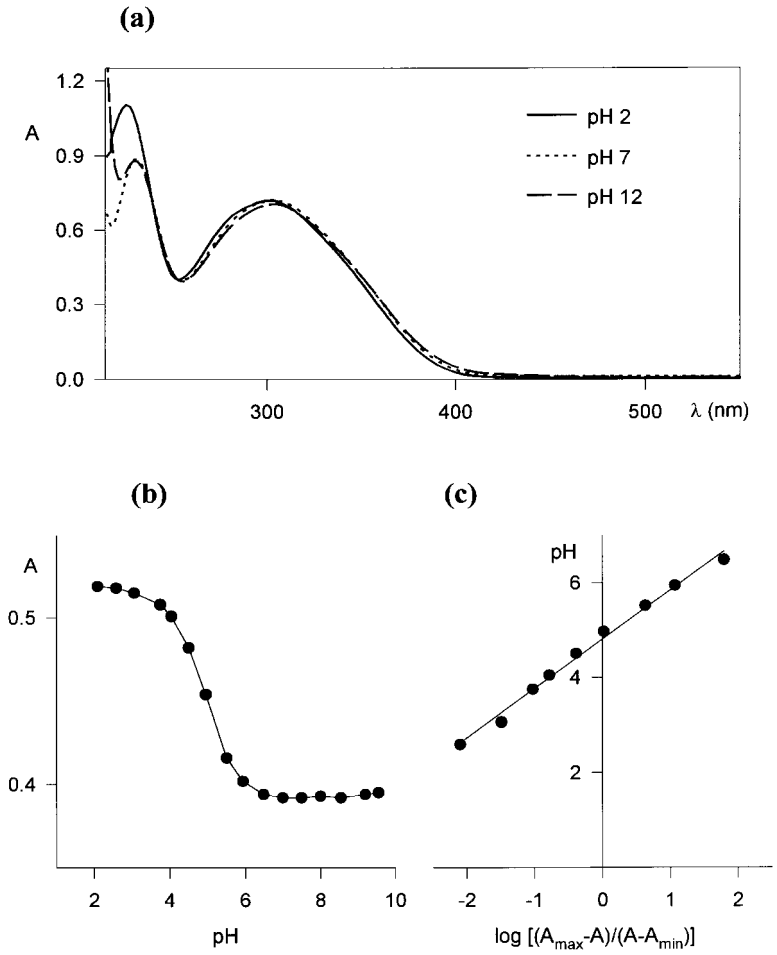
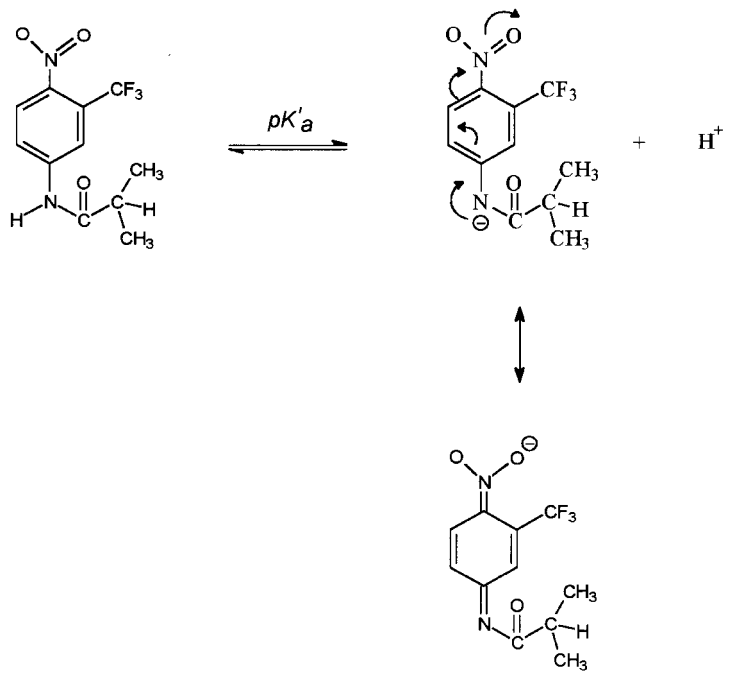


Fig. 6. a) UV spectra of 0.1 mM flutamide solution at different pH, b) absorbance dependence of the wave at $\lambda = 230$ nm with pH of a 0.05 mM flutamide solution and c) pk'_a calculation plot at $\lambda = 230$ nm.



Scheme 1. Dissociation equilibrium of flutamide.

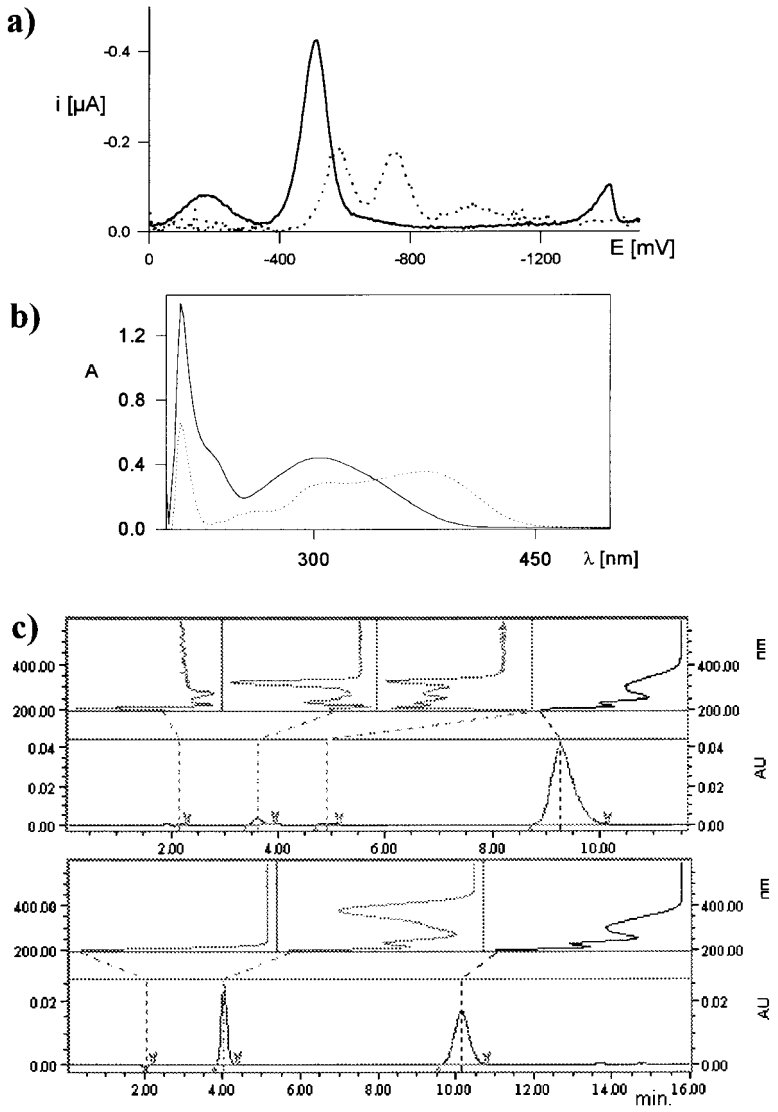


Fig. 7. Acidic hydrolytic degradation of flutamide examined by: a) differential pulse polarography; solid line; without hydrolysis, dashed line: after hydrolysis. b) UV spectroscopy; solid line: without hydrolysis, dashed line: after hydrolysis. c) HPLC-UV spectroscopy combined, upper chromatogram: without hydrolysis and lower chromatogram: after hydrolysis.

flutamide showed modifications (Fig. 7a), one main additional peak appearing at a more cathodic potential (E_{p_1} = −740 mV) with the consequential decrease of the initial peak of flutamide without hydrolysis. In UV spectra (Fig. 7b), a new band at 374 nm and a decrease of the signal at 300 nm in respect to the standard without hydrolysis were observed. The chromatogram (Fig. 7c) shows an increase of the signal with a retention time of 4 min, which also appears in the standard as traces. Also, the signal of the flutamide with retention time of 10.17 min was reduced.

In the alkaline hydrolysis total disappearance of the flutamide PPD signal (Fig. 8a), and the appearance of one peak at a more cathodic potential (E_p = −740 mV) was observed. The UV absorption spectrum (Fig. 8b) revealed the appearance of a new absorption band, approximately at 374 nm and an increase of the band at 210 nm. By HPLC the total degradation of flutamide was confirmed by the disappearance of the signal at a retention time at 10 min but still maintaining and increasing the signal at a retention time of 4 min (Fig. 8c).

The photolysis did not reveal large changes in DPP, only a decrease of the peak current of the drug signal and a displacement of the peak potential toward more cathodic zones (E_p = −600 mV). In UV spectrophotometry, the intensity of the absorption bands diminished, mainly in the photolysis of the solid, and the appearance of new bands was not observed. In HPLC only a decrease of the initial signal of flutamide was observed, with no apparition of new signals in the chromatogram, maintaining the retention time in both cases (photolysis of the solid and of the liquid solution).

In the thermolysis degradation experiments a mild decrease of the peak current existed with respect to the standard. In HPLC new signals were not detected, revealing only a decrease of the chromatographic peak, maintaining the same retention time with respect to the standard.

The chemical oxidation analyzed by DPP did not permit one to visualize the changes, due to the signal of the hydrogen peroxide. But by UV spectrophotometry the following effects were observed: a clear increase in the absorption band at 216 nm, a disappearance of the signal at 304 nm and the appearance of a broader signal at 310 nm. For HPLC only one signal appeared with a retention time of 1.83 min. These results show that the strong conditions of the oxidation test produced a total destruction of the molecule.

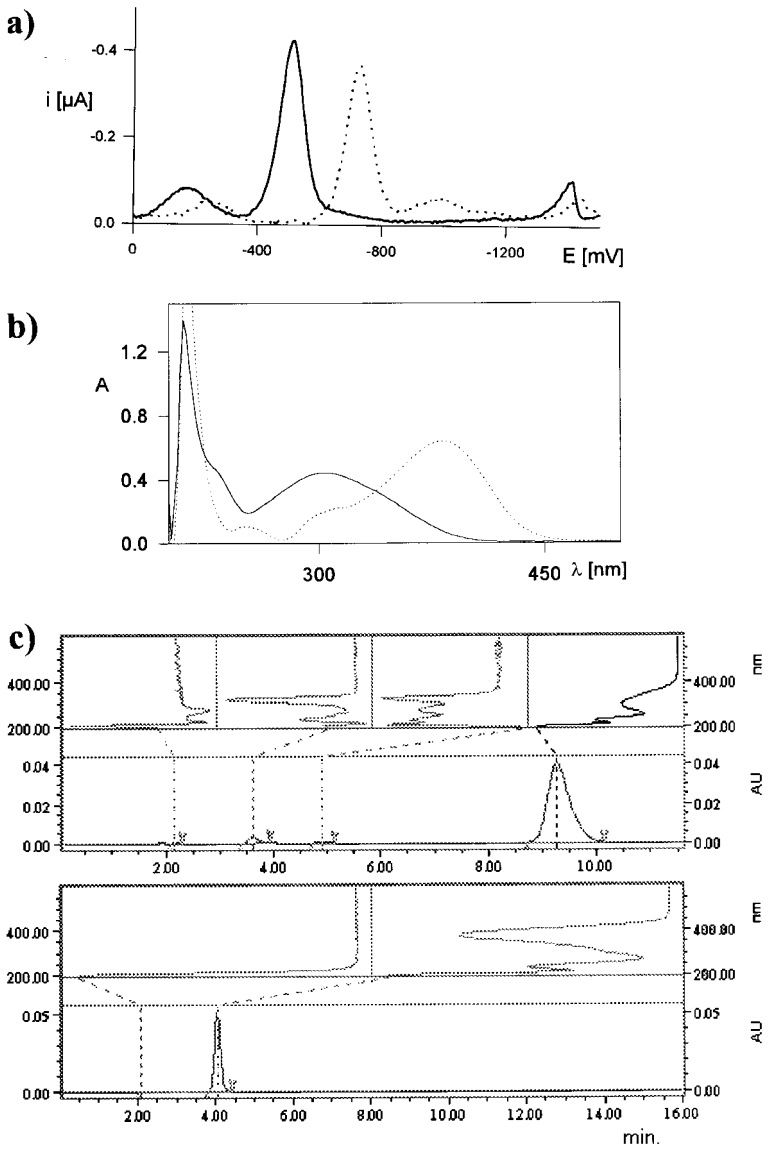


Fig. 8. Alkaline hydrolytic degradation of flutamide examined by: a) differential pulse polarography; solid line: without hydrolysis, dashed line: after hydrolysis. b) UV spectroscopy; solid line: without hydrolysis, dashed line: after hydrolysis. c) HPLC-UV spectroscopy combined, upper chromatogram: without hydrolysis and lower chromatogram: after hydrolysis.

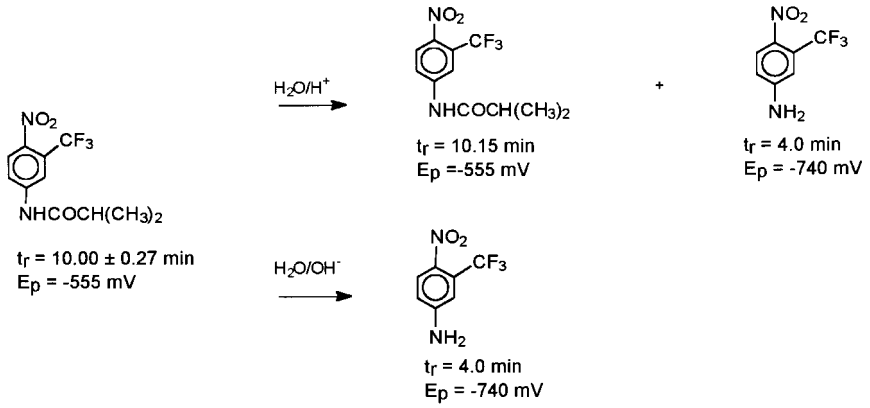
It can be concluded that the molecule of flutamide was thermo- and photorresistent, but showed degradation by acid and basic hydrolysis and oxidation. Also, HPLC and PPD techniques were able to discriminate between the degradation products and the intact flutamide thus showing adequate selectivity. The UV spectrophotometric method results were totally unselective for degradation products. According to the above chromatographic and polarographic results only one main degradation product detected which was characterized with an $E_p = -740$ mV by DPP and a retention time of 4.0 min by HPLC. By comparison with a standard solution, the degradation product would be 4-nitro-3-trifluormethylaniline as shown in Scheme 2. The difference of about 185 mV in the peak potential of flutamide ($E_p = -555$ mV) with respect to the amine derivative ($E_p = -740$ mV) is explained by considering the electronic effects of the *para*-substituent. When NH_2 is the *para*-substituent the electron density on the nitroaromatic group is higher than when the amide group is the *para*-substituent, due to the strong electron donor character of the NH_2 group. The DPP method also can permit the determination of the hydrolysis product in the presence of flutamide as has been previously described for the HPLC method [5].

In order to obtain the precision and accuracy of the developed method we performed a recovery study for the three different techniques. The results are summarized in Table 1. These results reveal that all three methods have adequate precision and accuracy and consequently can be applied to the determination of flutamide in tablets. The results obtained in the analysis of flutamide tablets are shown in Table 2. From these results we can conclude that for all these methods, the requirements of content uniformity are

Table 1. Results of the recovery study of flutamide by DPP, UV and HPLC.

Assay[a]	*DPP*	*UV*	*HPLC$_{UV}$*
Average recovery	101.09	101.21	100.18
Standard deviation	1.86	2.80	3.14
Coefficient of variation [%]	1.82	2.77	3.14

[a] Each average was calculated from ten independent samples.



Scheme 2. Acidic and alkaline hydrolytic degradation pathway for flutamide.

Table 2. Individual tablet assay results from commercial tablets of flutamide. Declared amount: 250.0 mg flutamide per tablet.

Assay	*PPD*		*UV*		*HPLC$_{UV}$*	
	mg/tablet	*%found*	*mg/tablet*	*%found*	*mg/tablet*	*%found*
Average	263.52	105.40	259.90	103.88	243.51	97.40
s.d	8.60	3.40	7.80	3.07	4.30	1.73
CV [%]	3.26	3.26	3.00	2.95	1.78	1.78

fulfilled. In fact, the percentage of flutamide was found within range, 85–115 %, of the declared quantity and the variation coefficient was smaller to the 6 %, in agreement with the general criterion of the Pharmacopoeia [20]. Considering the three used techniques in the analysis of flutamide, we recommend the DPP technique as a good analytical alternative. The preparation of the sample was easy and since the excipients did not interfere in the DPP determination, separation was not necessary, as in the UV and HPLC methods. Furthermore the analysis time was not long and the procedure had adequate precision and accuracy.

From the statistical analysis of each applied method it could be concluded that no significant differences among them was found and that they were statistically equivalent, on comparing means recoveries by applying the *t*-Student test ($p < 0.05$, $n = 20$).

4. Acknowledgements

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