

The influence of pH and temperature on the stability of flutamide. An HPLC investigation and identification of degradation product by EI⁺-MS

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Abstract

The chemical stability of flutamide (FLT) was investigated using a new validated stability-indicating HPLC method. Separation of FLT from its degradation product was achieved on a C₁₈ column using a mobile phase of methanol-phosphate buffer (0.04 M, pH 4.0) (75:25, v/v) with UV-detection at 240 nm. The method exhibited excellent linearity for FLT over the concentration range of 0.2-25.0 µg/mL. FLT was found to be labile to degradation in buffered, acidic and alkaline solutions. The degradation kinetics of FLT in aqueous solutions was evaluated as a function of pH and temperature. Degradation of FLT followed first-order kinetics and Arrhenius behavior over the temperature ranges of 70-100 and 60-90 °C under acidic and alkaline conditions. The pH-rate profile was studied over the pH range of 2–12 with a maximum stability at pH 3.0-5.0. The activation energy for hydrolysis of FLT was calculated as 79.4 and 52.0 kJ/mol at pH 0.5 (0.3 M HCl) and 12.5 (0.03 M NaOH), respectively. 4-Nitro-3-trifluoromethyl aniline was identified by mass spectrometry to be the degradation product resulted from the hydrolysis of FLT. The proposed HPLC method was validated according to ICH guidelines and applied for the quality control of FLT in commercial tablets with mean percentage recovery of 100.09±0.20%.

Keywords: Flutamide, HPLC-UV, stability study, degradation kinetics, pH-rate profile.

Introduction

Flutamide (FLT), propanamide, 2-methyl-N-[4-nitro-3-trifluoromethyl)-phenyl] [1], is a non-steroidal drug with anti-androgenic properties which appears to act by inhibiting the uptake and/or binding of androgens in target tissues. It is used, usually with gonadorelin analogues, in the palliative treatment of prostatic carcinoma. It is also used in combination with testolactone for the treatment of congenital adrenal hyperplasia [2].

FLT is an official drug in the United States Pharmacopoeia (USP) [1] that recommends an HPLC method for its determination in pure state and capsules. Also, it is authorized in the British Pharmacopoeia (BP) [3] that describes a direct spectrophotometric assay at 295 nm for its determination in pure form. Reviewing the literature revealed some analytical methods for the determination of FLT such as spectrophotometry [4-7], HPLC [8-10], voltammetry [11-13], and GC-electron capture detection [14]. Few analytical methods studied the stability of FLT [15, 16]. Nevertheless, such methods did not study the kinetics of the degradation process or elucidate the chemical structure of FLT degradant. So far, no study elucidated the pH-rate profile of FLT despite it provides useful information for the optimal formulation and storage conditions of pharmaceutical products [17]. This encouraged us to develop a simple, rapid and accurate stability-indicating HPLC method for FLT with main targets of (i) studying its degradation kinetics, (ii) identification of possibly formed degradation products under different International Conference on Harmonisation (ICH)-outlined stress conditions [18, 19], and (iii) elucidation of its pH-rate profile curve.

Experimental

Instruments

Chromatographic separation was achieved with a Hitachi HPLC system (Tokyo, Japan) equipped with 655A-11 liquid chromatograph, a high sensitivity series L-4000 H UV-detector, D-2500 chromato-integrator, LC-organizer and a Rheodyne injector valve with a 50- μ L sample loop. Positive electron ionization mass spectra (EI⁺-MS) were recorded using JMS DX-303 mass spectrometer (Joel Ltd., Japan). SK-620 pH/mV meter (Sato Keiryoki MFG Co. Ltd, China) was used for pH adjustment. A BT-15 Yamato thermostatically controlled water bath (Tokyo, Japan) was used in the stability studies. Asonic ultrasonic bath (Osaka, Japan) was used for sonication of solutions and mobile phase.

Chemicals and reagents

A gift sample of flutamide with a certified purity of 98.82% (batch # 9007) was kindly provided by Schering-Plough Co. (Cairo, Egypt). Cytomid[®] tablets labeled to contain 250 mg FLT/tablet (product of Cipla Ltd., Mumbai, India) was purchased from a local Egyptian pharmacy. Methanol (HPLC grade) was obtained from Kanto Chemical Co., INC. (Tokyo, Japan). Orthophosphoric acid (85% w/v) and potassium dihydrogen phosphate were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Hydrochloric acid (35-37%) and sodium hydroxide were obtained from Chameleon Reagent (Osaka, Japan). Hydrogen peroxide (30% w/v) was obtained from Nacalai Tesque, Inc. (Kyoto, Japan). Purified water was obtained using Millipore direct-Q 3UV water purification system (Molsheim, France). Aqueous solutions of 0.3 M HCl and 0.03 M NaOH were prepared. Phosphate buffer of pH 4.0 was prepared by adjusting the pH of 0.04 M potassium dihydrogen phosphate with few drops of orthophosphoric acid (85% w/v). Britton-Robinson buffer (BRB) containing 0.04 M boric acid, 0.04 orthophosphoric acid, and 0.04 M acetic acid was prepared and the pH was adjusted to 2-12 with 0.2 M NaOH [20].

Standard solution

A stock solution of FLT containing 2000.0 µg/mL was prepared in methanol. A standard solution (200.0 µg/mL) was prepared by dilution with the same solvent. The solutions were stable for one week when stored in a refrigerator at 4 °C.

Chromatographic conditions

Separation was performed on a Cosmosil 5C₁₈-MS column (150 mm x 4.6 mm id, 5-µm particle size) from Nacalai Tesque, Inc. A mobile phase consisted of methanol-phosphate buffer (0.04 M; pH 4.0) (75:25, v/v) was pumped at a flow rate of 1 mL/min and UV-detection was set at 240 nm.

Calculation of degradation kinetics parameters

Kinetics parameters for the degradation of FLT were calculated [21]. The observed first-order degradation rate constants (*k*) were calculated from the slopes of semi-logarithmic plots of $\log \frac{a}{a-x}$ versus time (*t*) in accordance with Eq. (1):

$$kt = 2.303 \log \frac{a}{a-x} \quad (1)$$

Where (*a*) is the initial drug concentration and (*a-x*) is the remaining drug concentration.

Half-life times ($t_{1/2}$), for the first order degradation reactions, were calculated according to Eq. (2):

$$t_{1/2} = \frac{0.693}{k}. \quad (2)$$

Activation energy (E_a) was calculated from the slope of Arrhenius plot of $\log k$ versus $1/T$ in accordance to Eq. (3):

$$\log k = \log A - \frac{E_a}{2.303 RT}. \quad (3)$$

Where: R is the gas constant = 8.314 J/mol/K and T is the absolute temperature ($^{\circ}\text{K} = 273 + ^{\circ}\text{C}$).

General recommended procedures

Calibration graph. Accurately measured volumes of FLT standard solution (200.0 $\mu\text{g/mL}$) were diluted with the mobile phase to obtain final concentrations over the range of 0.2-25.0 $\mu\text{g/mL}$. Twenty μL aliquots were injected (triplicate) and eluted with the mobile phase under the optimum chromatographic conditions. The average peak areas of FLT were plotted *versus* the corresponding drug concentrations ($\mu\text{g/mL}$) and the regression equation was derived.

Assay of tablets. Ten tablets were accurately weighed, finely pulverized and thoroughly mixed. An accurately weighed amount of the powder equivalent to 10.0 mg FLT were transferred into 100 mL volumetric flask and diluted to the mark with methanol. The solution was sonicated for 30 min then filtered. Accurately measured volumes of the filtrate were diluted with the mobile phase and chromatographed under the optimum conditions. Nominal contents of FLT were calculated using the regression equation.

Degradation protocol. 0.5 mL aliquots of FLT standard solution (200.0 $\mu\text{g/mL}$) were transferred into a series of 10 mL glass vials followed by 2 mL of 0.3 M HCl or 0.03 M NaOH. Solutions were then incubated in a thermostatically controlled water bath at different temperature settings (70-100 and 60-90 $^{\circ}\text{C}$ for acidic and alkaline degradation, respectively). Samples were taken at appropriate times (10-40 min), neutralized and made up to 5.0 mL with the mobile phase and mixed well. Samples were eluted under the optimum chromatographic conditions and the remaining drug concentrations were calculated using the regression equation. A plot of $\log \frac{a}{a-x}$ *versus* time (t) was constructed. The observed first-order degradation rate constants (k) and half-lives ($t_{1/2}$), were calculated using Eqs. (1) and (2), respectively. Arrhenius plot was constructed by plotting $\text{Log } k$ *versus* $1/T$ ($^{\circ}\text{K}$) and the activation energy (E_a) for FLT degradation was calculated from the slope according to Eq. (3).

Oxidative stress study was also carried out as follow: 2 mL of H₂O₂ (30, w/v) were added to 50 µL of FLT stock solution and heated for 2 h at 80°C. For the photo-degradation study; 50 µL of FLT stock solution was transferred into a series of glass vials and diluted with 2 mL of methanol, water or methanol-water mixture (1:1, v/v), then exposed to UV-lamp emitting radiation at a wavelength of 254 nm for 24 h. Then, the solutions were made up to 5 mL with the mobile phase, mixed well and eluted under the optimum chromatographic conditions.

pH-rate profile for FLT. 1 mL of FLT stock solution (2000.0 µg/mL) was transferred into a series of 10 mL glass vials, 9 mL of BRB (pH = 2-12) were added and mixed well. Solutions were heated under reflux in a boiling water bath. 1 mL aliquots were taken at appropriate times (10-40 min), neutralized and diluted to 10 mL with the mobile phase then analyzed. The remaining drug concentrations were calculated from the regression equation. A plot of $\log \frac{a}{a-x}$ versus time (*t*) was constructed and the observed reaction rate constants (*k*) were calculated using Eq. (1). The pH-rate profile curve was constructed by plotting log *k* versus pH of BRB.

Results and Discussion

HPLC degradation studies of FLT revealed its stability under neutral, oxidative and photolytic conditions. Meanwhile, it was found susceptible to degradation under acidic and alkaline conditions yielding the same degradation product (DP). Hence, a kinetic study was conducted to explore the degradation process and pH-rate profile curve was also constructed.

Method development

The pH, composition, and flow rate of the mobile phase as well as detection wavelength were optimized to achieve good separation of FLT from its stress induced degradation product (DP) with high sensitivity. For optimization of HPLC conditions, stressed samples of FLT under acidic (0.3 M HCl) and alkaline (0.03 M NaOH) conditions at 80 °C for 30 min were chromatographed.

The pH of phosphate buffer was tested over the range of 3.0-7.0. It was found that, changing the pH exhibits a negligible effect on the retention of the drug and its degradation product. Hence, this study was conducted using phosphate buffer of pH 4.0 to confirm maximum stability and durability of the column. The percentage of methanol in the mobile phase was also studied over the range of 60-80 %, v/v. A mobile phase containing 75%, v/v methanol was selected as the optimal yielding good resolution between FLT and DP within a short analysis time (less than 5 min). At lower concentrations of methanol, the retention times of both FLT and DP increased with a distinct effect on FLT.

Meanwhile, on increasing the percentage of methanol to 80%, v/v, the retention of both components decreased (especially of FLT) and the resolution between FLT and DP was adversely affected. Studying the influence of ionic strength of phosphate buffer over the range of 0.01-0.05 M revealed no significant effect on the separation process, 0.04 M phosphate buffer was used in the present study. Flow rate of the mobile phase was also studied (0.8-1.2 mL/min) and a flow rate of 1.0 mL/min was selected as optimal giving good resolution within short analysis time. Detector wavelength was investigated by monitoring the eluents responses at different wavelength settings (215, 240, 254 and 295 nm). Best sensitivities for both FLT and degradation product were attained at 240 nm. A summary of the influence of different chromatographic parameters on system suitability is illustrated in Table 1. Under the optimum chromatographic conditions, good resolution was achieved between the drug ($t_R = 4.0$ min) and its degradation product ($t_R = 2.44$ min) within a short analysis time.

A comparison between the analytical performance of the proposed HPLC method and methods reported in the literature for the determination of FLT is presented in Table 2.

Method validation

A validation procedure was conducted according to ICH guidelines [22] to evaluate linearity, range, limit of detection, limit of quantification, accuracy, precision, selectivity, robustness, system suitability, and stability of standard solution and mobile phase.

Linearity and range. Eight concentration levels were considered to study the linearity. Regression analysis of the data [23] proved the excellent linearity of the proposed method over a wide dynamic range (0.2-25.0 $\mu\text{g/mL}$) as revealed by high value of correlation coefficient ($r=0.9999$), small values of the standard deviations of the residuals ($S_{y/x}$), slope (S_b), and intercept (S_a), and the % relative error. Table 3 illustrates the linear regression analysis data for FLT.

Limit of detection (LOD) and limit of quantification (LOQ). Both LOD and LOQ were calculated as *per* ICH guidelines [22] according to the following equations:

$$\text{LOQ} = 10S_a/b \quad (4)$$

$$\text{LOD} = 3.3S_a/b \quad (5)$$

Where S_a = the standard deviation of the intercept of regression line and b = the slope of regression line. The results are also presented in Table 3.

Accuracy. Accuracy of the proposed method was determined by recovery studies. The method was demonstrated to be accurate giving average recovery of $100.63 \pm 1.40\%$. Statistical comparison of the obtained results with those of the reference HPLC method [1] using the Student's *t*-test and the variance ratio *F*-test was done [23]. Both the *t*- and the *F*- values obtained at the 95% confidence level, did not exceed the theoretical tabulated values [23]. Therefore, there is no significant difference between the proposed and reference methods [1] regarding the accuracy and precision (Table 4).

Precision .The repeatability (intra-day precision) studies were performed by analysis of FLT at three concentration levels at three successive times on the same day, whereas, intermediate precision (inter-day precision) was checked by repeating the analysis of the three drug concentrations on three consecutive days. The precision was expressed as %RSD. Results of precision studies summarized in Table 5 confirm the precision of the method.

Selectivity. Results of the forced degradation studies revealed the ability of the proposed method to entirely separate the drug from its degradation product with a good resolution factor (R_s). In addition no interference from common pharmaceutical excipients in the tablets with the peak of the drug was observed. These results indicated the selectivity of the proposed method.

Robustness. To determine the robustness of the proposed method with deliberated minor changes in chromatographic conditions, four parameters were varied: pH of the mobile phase (4.0 ± 0.2), ratio of methanol (75 ± 2 %, v/v), molarity of phosphate buffer (0.04 ± 0.001 M) and detection wavelength (240 ± 2 nm). Introducing these small but deliberate variations didn't significantly affect the resolution or peak area of the analyte. These results demonstrated the robustness of the developed method.

System suitability. The system suitability parameters including R_s , number of theoretical plates (N), retention factor (k), selectivity factor (α) were calculated according to USP guidelines [1]. The results are illustrated in Table 6.

Stability of standard solution and mobile phase. Comparing the analytical response of freshly-prepared standard solution of FLT with that of an aged solution (24 h at room temperature) revealed the stability of the standard solution over this period. Similarly, the mobile phase was found to be stable up to 48 h.

Application

Degradation behavior of FLT under ICH conditions. FLT exhibited a high stability under photolytic, neutral hydrolysis and oxidative stress conditions, while it underwent rapid degradation under acidic and alkaline conditions yielding the same degradation product (DP). Figure 1 illustrates representative chromatograms of FLT after acidic and alkaline treatments. Degradation of FLT in acidic and alkaline conditions followed first-order kinetics (Fig 2), and the degradation process obeyed Arrhenius behavior over the temperature ranges of 70-100 and 60-90 °C under acidic and alkaline conditions, respectively. Linear plots for Arrhenius equations for the acidic and alkaline degradation were obtained as illustrated in Fig. 3. Table 7 represents a summary of the calculated k , $t_{1/2}$, E_a and Arrhenius equations for FLT degradation in 0.3 M HCl and 0.03 M NaOH. The activation energies E_a for the acidic and alkaline degradation of FLT were found to be 79.4 and 52 KJ/mol. These values are within the range of 50–96 KJ/mol most commonly reported and represents an intermediate sensitivity of the degradation process to temperature [21].

The liability of the drug to hydrolysis under alkaline condition is very high compared with its behavior under acidic condition, as revealed from k and E_a values (Table 7). FLT is an anilide derivative, in which the amide nitrogen is linked to an aromatic ring which can "pull" electron density from this atom, weakening the amide C-N bond. This ring can also stabilize the leaving group by delocalization of electron pair on nitrogen. As a result, the amide nitrogen could be attacked easier by the hydroxide ion ($\bar{O}H$) than hydronium ion (H_3O^+), which rational the liability of the drug to hydrolysis under basic conditions more than under acidic conditions.

pH-rate profile of FLT. The degradation of FLT in BRB over pH range of 2.0-12.0 at boiling temperature resulted in the appearance of DP as a degradation product. All the correlation coefficients (r) of the semi-logarithmic plots of the drug remaining *versus* time were > 0.98 with most of them > 0.99 indicating that, the degradation of FLT followed first-order kinetics. The pH-rate profile of FLT over the pH range of 2.0–12.0 is illustrated in Fig. 4, showing maximum stability over pH range of 3.0-5.0. Degradation occurred more rapidly in basic solution with increased rate of degradation upon increasing the pH. At pH 3.0-5.0, the estimated degradation rate constant was in the range of 1.2×10^{-6} – $1.3 \times 10^{-6} \text{ min}^{-1}$ giving an estimated half-life ($t_{1/2}$) of 13.4-12.3 months at 100 °C. In more acidic solution (pH 2.0) the degradation rate constant increased to $3.3 \times 10^{-6} \text{ min}^{-1}$.

The rapidness of the proposed method (separation was achieved within 5 min) together with its sensitivity, selectivity, and accuracy, permitted the analysis of large number of samples generated from pH-rate profile studies in a short time.

EI⁺-MS study of degradation product of FLT. HPLC analysis showed that hydrolysis of FLT in BRB and in 0.3 M HCl as well as 0.03 M NaOH solutions produced the same degradation product with a constant retention time (2.44 min). The corresponding EI⁺-MS spectrum of this compound yielded an *m/z* ratio of 206 which is consistent with 4-nitro-3-trifluoromethyl aniline (C₇H₅F₃N₂O₂) formed as a result of hydrolysis of the amide linkage of FLT (Fig 5). Figure 6 illustrates the obtained EI⁺-MS spectra for FLT and its hydrolytic degradation product.

Quality control of flutamide tablets. The suggested method was successfully applied for the determination of FLT in Cytomid[®] tablets. The results shown in Table 3 were satisfactory and with good agreement with the labeled amount. The obtained results indicated stability of FLT under storage conditions since no degradation product was detected. The results were statistically compared with those obtained by the official HPLC method [1] using Student *t*- and Variance ration F-tests [23]. There was no significant difference between the two methods in terms of the mean values and standard deviation at the 95% confidence level (Table 4). The obtained results indicated the suitability of the proposed method for the quality control of FLT without interferences from common tablets excipients.

Conclusion

We developed a sensitive, accurate, precise, and rapid HPLC method able to discriminate between FLT and its degradation product. FLT was found to be very sensitive to alkaline and acidic media. The proposed method was managed to study the degradation kinetics of FLT under alkaline and acidic conditions and to elucidate its Arrhenius plots. In addition, the proposed method was applied to derive the pH-rate profile curve for FLT in BRB which exhibited highest stability over pH 3.0-5.0.

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References

- [1] The United States Pharmacopeia, The United States Pharmacopeial Convention, NF 29, 34th ed., Rockville, MD, 2011.
- [2] S.C. Sweetman, Martindale: The Complete Drug Reference, 36th ed., the Pharmaceutical Press, London, 2009, p.725.
- [3] The British Pharmacopoeia, British Pharmacopoeial Commission, Her Majesty Stationery Office, London, 2010.
- [4] P. Nagaraja , K.R. Sunitha and M.F. Silwadi, *J. Pharm. Biomed. Anal.* 2000, 23, 617.
- [5] P. Nagaraja, H.R. Arun Kumar, R.A. Vasantha and H.S. *Int. J. Pharm.*, 2002, 235, 113.
- [6] P. Nagaraja, H.S. Yathirajan, H.R.A. Kumar and R.A. Vasantha, *Ind. J. Pharm. Sci.*, 2002, 64, 272.
- [7] H. N. Deepakumari and H. D. Revanasiddappa, *ISRN Spectroscopy*, 2012, Article ID 728594, doi:10.5402/2012/728594.
- [8] H. R. N. Salgado, M. de Menezes and M. P. B. Storti, *Acta Farm. Bonaerense*, 2005, 24, 246.
- [9] A. A. Smith, R. Manavalan, K. Kannan and N. Rajendiran, *Int. J. Pharm.Tech. Res*, 2009, 1, 360.
- [10] N. Bujji Babu and R.Ramesh Raju, *J. Pharm. Res.*, 2011, 4, 4556.
- [11] P. K. Brahman, R. A. Dar, S. Tiwari and K. S. Pitre, *Colloids Surf. A*, 2012, 396, 8.
- [12] E. Hammam, H. S El-Desoky, K. Y. El-Baradie and A. M. Beltagi, *Can. J. Chem.*, 2004, 82, 1386.
- [13] K. Pecková, M. Průchová, J. C. Moreira, J. Berek, J. Fischer and V. Vyskočil, *Collect. Czech. Chem. Commun.*, 2011, 76, 1811.
- [14] M. Schulz, A. Schmoltdt, F. Donn and H. Becker, *Eur. J. Clin. Pharmacol.*, 1988, 34, 633.
- [15] A. Miranda , I. Caraballo and M. Millán, *Drug Dev. Ind. Pharm.*, 2002, 28, 413.
- [16] A. Alvarez-Lueje, C. Peña, L.J. Núñez-Vergara and J.A. Squella, *Electroanalysis*, 1998, 10, 1043.
- [17] O.A.G.J. van der Houwen, M.R. de Loos, J.H. Beijnen, A. Bult and W.J.M. Underberg, *Int. J. Pharm.* 1997, 155, 137.

- [18] ICH Harmonised Tripartite Guideline, Stability Testing of New Drug Substances and Products, Q1A (R2), Geneva, 2003.
- [19] ICH Harmonised Tripartite Guideline, Stability Testing: Photostability Testing of New Drug Substances and Products, (Q1B), Geneva, 1996.
- [20] H.T.K. Britton and R.A. Robinson, *J. Chem. Soc.*, 1931, 1456-1462.
- [21] A.T. Florence and D. Attwood, *Physicochemical Principles of Pharmacy*, 4th Ed., the Pharmaceutical Press, London, 2006, 93-138.
- [22] ICH Harmonised Tripartite Guideline, Validation of analytical procedures: text and methodology, Q2(R1), Geneva, 2005.
- [23] J. N. Miller and J. C. Miller, *Statistics and Chemometrics for Analytical Chemistry*, 5th Ed., Pearson Education Limited, Harlow, England, 2005, P.P. 39-73, 107-149, 256..

Table 1. Effect of different chromatographic conditions on system suitability parameters of the proposed method^a

Parameter	No of theoretical plates (<i>NTP</i>)		Capacity factor (<i>k'</i>)		Resolution (<i>R_s</i>)	Relative retention (<i>α</i>)
	FLT	DP	FLT	DP		
Ratio of methanol (% v/v)						
60	11507	14578	10.47	2.23	21.83	4.70
65	9132	6180	4.11	1.10	13.66	3.74
70	4282	4343	2.50	0.76	11.04	3.29
75*	8864	3972	1.52	0.52	6.50	2.92
80	5744	2633	1.03	0.37	4.24	2.78
pH						
3.0	8132	3271	1.54	0.52	6.04	2.96
4.0*	8864	3972	1.52	0.52	6.50	2.92
5.0	8383	3164	1.27	0.40	6.00	3.18
6.0	8732	3191	1.34	0.41	5.71	3.27
7.0	8680	3462	1.49	0.49	6.72	3.04
Ionic strength of phosphate buffer (M)						
0.01	7659	3380	2.1	0.87	4.63	2.41
0.02	9540	3463	1.95	0.79	6.04	2.47
0.04*	10007	3631	2.38	1.03	6.26	2.31
0.05	8864	3972	1.52	0.52	6.50	2.92
Flow rate (mL/min)						
0.8	5697	5053	1.61	0.64	5.11	2.52
1.0*	10010	3642	2.41	1.00	6.30	2.30
1.2	5816	2305	1.59	0.63	4.53	2.52

^a System suitability parameters were calculated as *per* USP [1].

*Conditions selected as optimum.

Table 2 Comparison between the proposed and reported methods for FLT.

Method	Linearity range ($\mu\text{g/mL}$)	LOD ($\mu\text{g/mL}$)	Remarks	Ref
spectrophotometry	0.25-7 0.25-6	N/A	harsh conditions, multiple derivatization steps, time consuming, not stability indicating assays	4
spectrophotometry	0.8-6 0.8-14	0.132 0.288	harsh conditions, multiple derivatization steps, time consuming, not stability indicating assays	5
spectrophotometry	N/A	N/A	harsh conditions, multiple derivatization steps, time consuming, not stability indicating assay	6
spectrophotometry	0.5–12 0.5–10 0.3–10	0.1197 0.1047 0.2587	harsh conditions, multiple derivatization steps, time consuming, not stability indicating assays	7
HPLC-UV	2.9-11.6	N/A	poor sensitivity, narrow linearity range, not stability indicating assay	8
HPLC-FL	0.1-0.6	8.697×10^{-3}	very narrow linearity range, harsh and multiple steps derivatization conditions, not stability indicating assay	9
HPLC-UV	2.0-12.0	0.02	narrow linearity range, not stability indicating assay	10
Voltammetry	20.0-160.0	0.05	needs special polymer film modified carbon paste electrode, not stability indicating assay	11
Voltammetry	N/A	0.052 0.024 0.003	not stability indicating assays	12
Voltammetry	0.06-0.28	N/A	not stability indicating assay	13
GC- electron capture (^{63}Ni) detection	0.002-0.3 0.003-0.5	N/A	not stability indicating assay, require special instrumentation and expertise, high cost	14
HPLC-UV	0.5-20.0	N/A	longer retention time, alkaline, oxidative, photolytic degradation, and pH-rate profile were not studied, DP wasn't identified	15
HPLC-diode array detection spectrophotometry voltammetry	0.138-138.0 0.276-276.0 0.524-276.0	N/A	time consuming, degradation kinetics and pH-rate profile were not studied, DP was not identified	16
HPLC-UV	0.2-25.0	0.05	short retention time, wide linearity range, high sensitivity, stability indicating assay, degradation under different ICH-conditions was conducted, degradation kinetics was studied, pH-rate profile was constructed.	present method

N/A: data are not available.

Table 3 Linear regression analysis data for the determination of FLT by the proposed method

Parameter	Result
Concentration range ($\mu\text{g/mL}$)	0.2-25.0
Limit of detection (LOD) ($\mu\text{g/mL}$)	0.05
Limit of quantification (LOQ) ($\mu\text{g/mL}$)	0.16
Correlation coefficient (r)	0.9999
Slope	9.83×10^3
Intercept	1.22×10^2
Standard deviation of the residuals ($S_{y/x}$)	6.65×10^2
Standard deviation of the intercept (S_a)	1.61×10^2
Standard deviation of the slope (S_b)	26.01
% RSD	1.39
% Error ($\% \text{RSD}/\sqrt{n}$)	0.5

Table 4 Application of the proposed and reference methods for the determination of FLT in pure form and tablets

Matrix	Proposed method			Reference method [1]
	Conc. taken ($\mu\text{g/mL}$)	Conc. found ($\mu\text{g/mL}$)	% Found ^a	% Found ^a
Pure form	0.2	0.202	101.00	99.05
	0.4	0.407	101.75	100.16
	1.0	1.027	102.70	100.25
	4.0	4.033	100.83	
	8.0	7.892	98.65	
	15.0	14.882	99.21	
	20.0	20.296	101.48	
	25.0	24.863	99.45	
Mean $\bar{\pm}$ SD			100.63 \pm 1.40	99.82 \pm 0.67
t			0.942(2.262) ^b	
F			4.40(19.35) ^b	
Cytomid [®] tablets (250 mg FLT/tablet)	1.0	1.003	100.30	99.68
	15.0	14.987	99.91	98.25
	25.0	25.365	101.46	100.25
Mean $\bar{\pm}$ SD			100.09 \pm 0.20	99.39 \pm 1.03
t			1.54(2.776) ^b	
F			1.63(19.00) ^b	

^aEach result is the average of three separate determinations.

^bValues between parentheses are the tabulated *t*- and *F*- values, respectively [23].

Table 5 Precision data of the proposed method for determination of FLT in pure form.

Conc. ($\mu\text{g/mL}$)	% Found \pm SD	% RSD	% Error
Intra-day precision			
0.4	101.20 \pm 0.34	0.34	0.20
10.0	100.54 \pm 1.34	1.33	0.77
25.0	99.87 \pm 1.50	1.50	0.90
Inter-day precision			
0.4	99.54 \pm 0.92	0.93	0.53
10.0	100.20 \pm 1.35	1.08	0.62
25.0	100.54 \pm 0.53	0.53	0.30

Table 6 Final system suitability test parameters for FLT and DP by the proposed method^a

Parameter	No of theoretical plates (<i>NTP</i>)		Capacity factor (<i>k'</i>)		Resolution (<i>R_s</i>)	Relative retention (<i>α</i>)
	FLT	DP	FLT	DP		
Result	10010	3642	2.41	1.00	6.30	2.30

^a System suitability parameters were calculated as *per* USP [1].

Table 7 kinetic parameters for the degradation of FLT under acidic and alkaline conditions

Condition	Temperature (°C)	k (min⁻¹)	t_{1/2} (min)	E_a (kJ/mol)	Arrhenius equation
HCl (0.3 M)	70	2.78×10 ⁻³	249	79.4	Log k= 9.60 – (4.15/T)
	80	9.06×10 ⁻³	77		
	90	1.37×10 ⁻²	51		
	100	2.89×10 ⁻²	24		
NaOH (0.03 M)	60	1.03×10 ⁻²	68	52.0	Log k= 6.14 – (2.72/T)
	70	1.63×10 ⁻²	43		
	80	3.25×10 ⁻²	22		
	90	4.46 ×10 ⁻²	15		

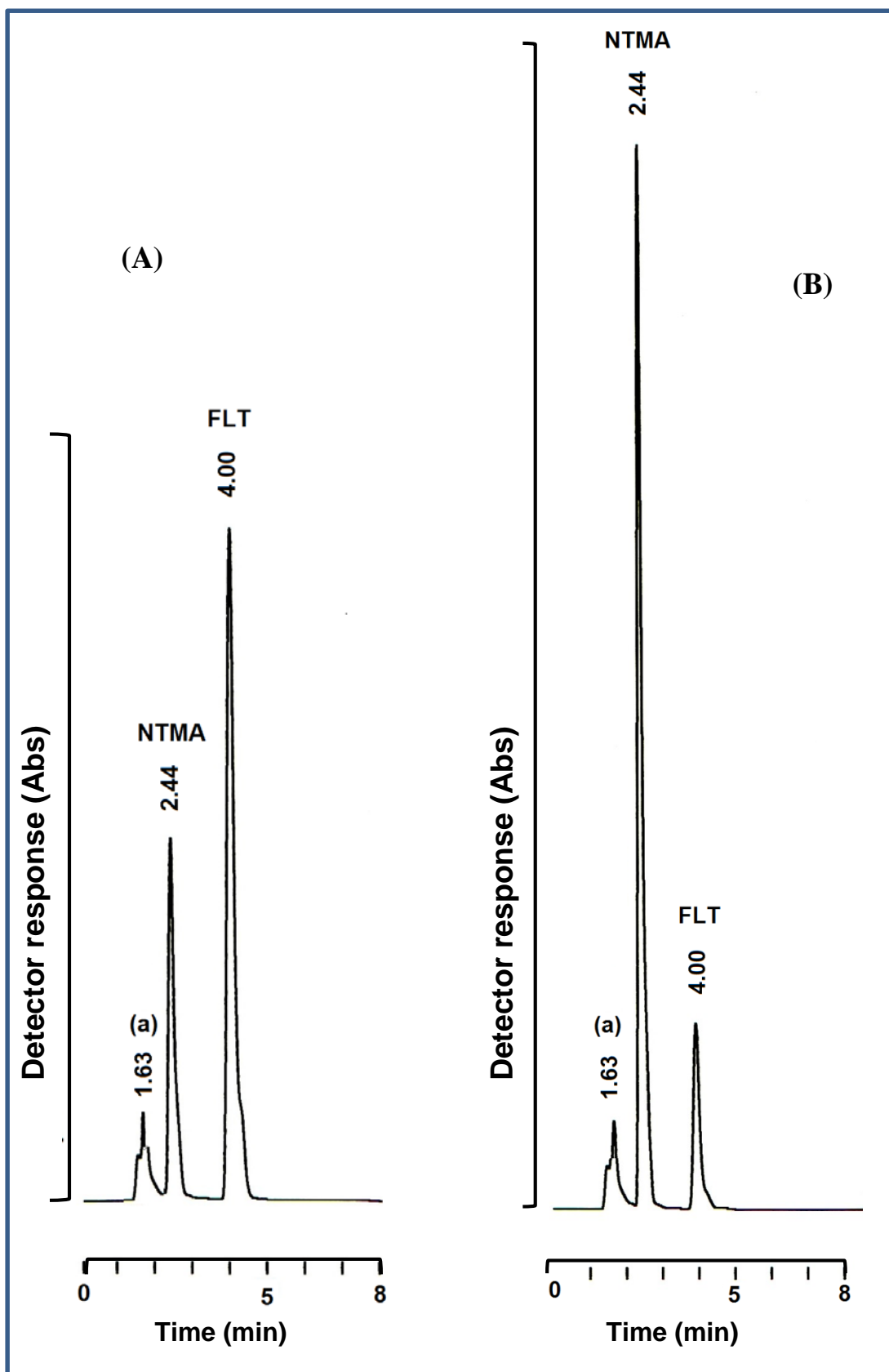


Fig. 1 Representative chromatograms for FLT (20.0 μg/mL) after exposure to (A) alkaline degradation (0.03 M NaOH at 90 °C for 30 min) and (B) acidic degradation (0.3 M HCl at 90 °C for 30 min), where: (a) is the solvent front, NTMA is 4-nitro-3-trifluoromethyl aniline (DP).

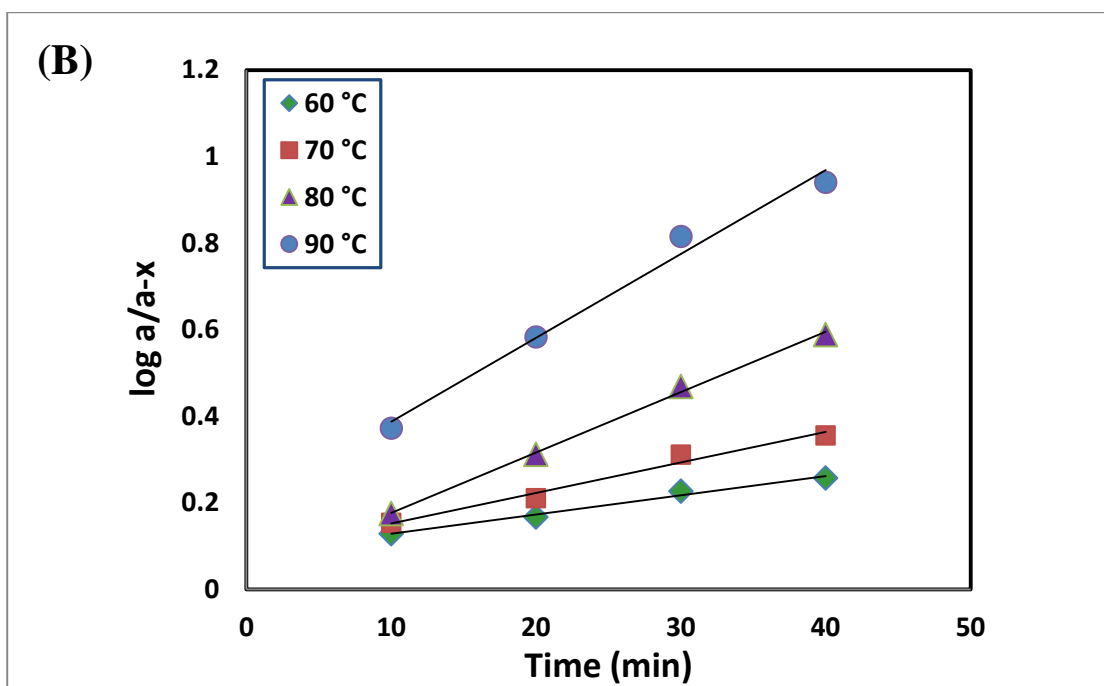
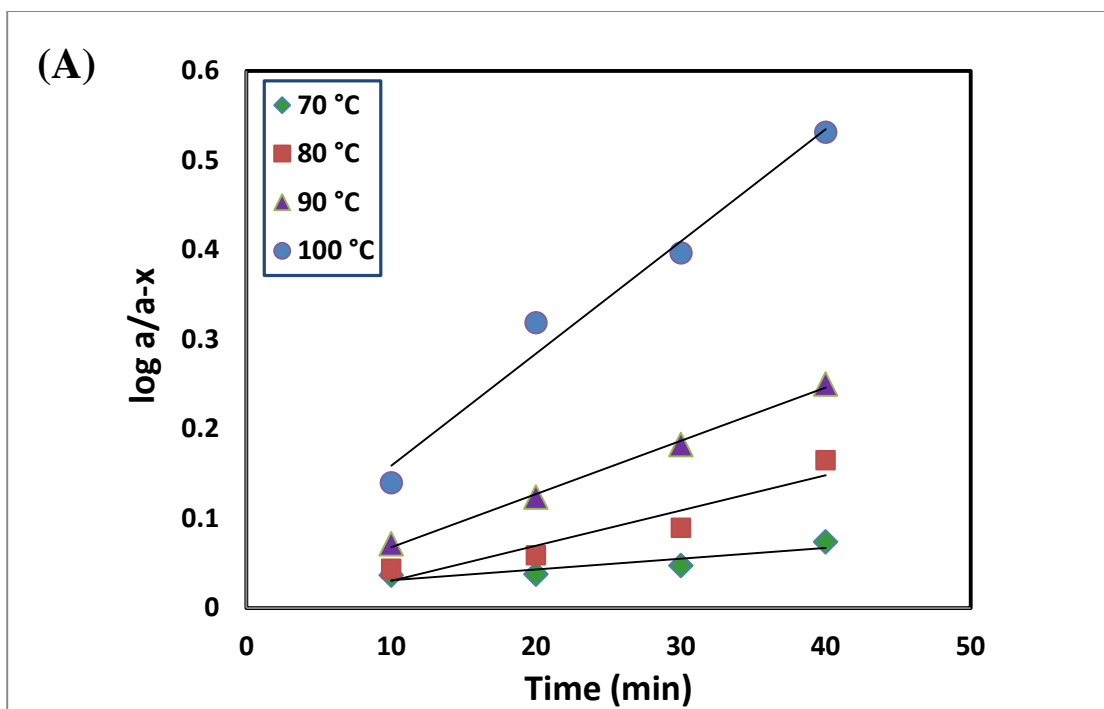


Fig. 2 Semi-logarithmic plots for the first-order degradation of FLT under (A) acidic (0.3 M HCl) and (B) alkaline (0.03 M NaOH) conditions at different temperature settings.

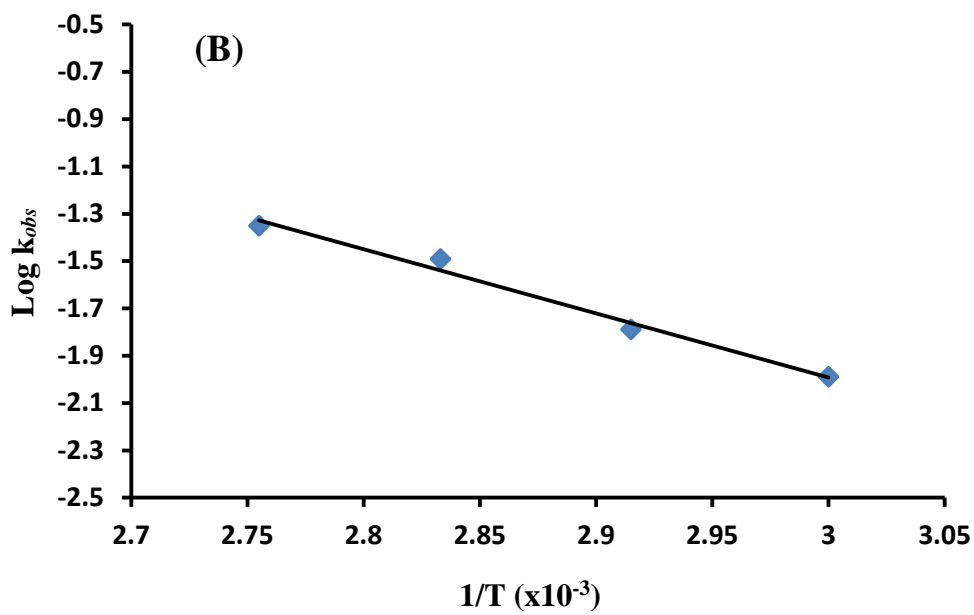
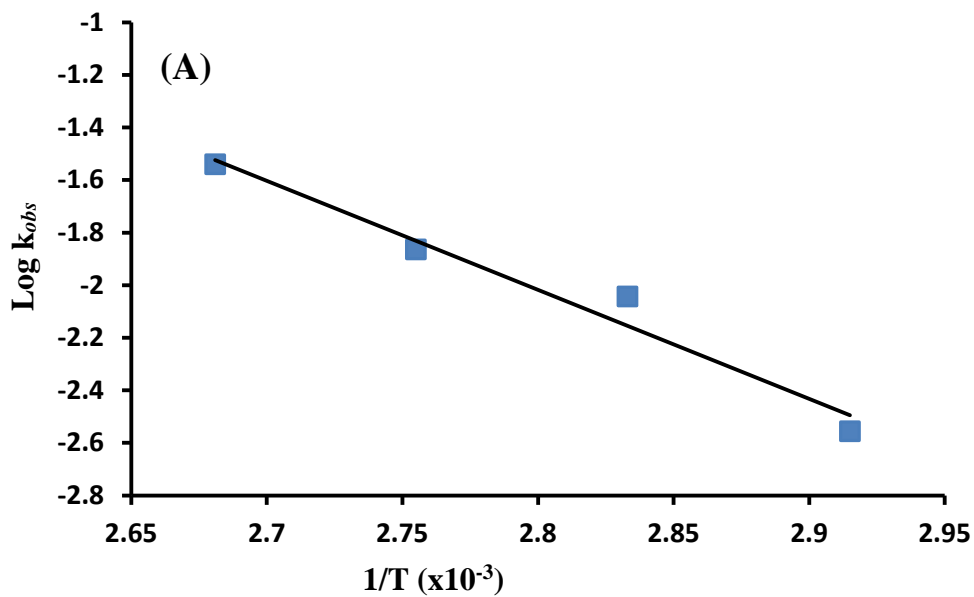


Fig. 3 Arrhenius plots for (A) acidic (0.3 M HCl) and (B) alkaline (0.03 M NaOH) degradation of FLT.

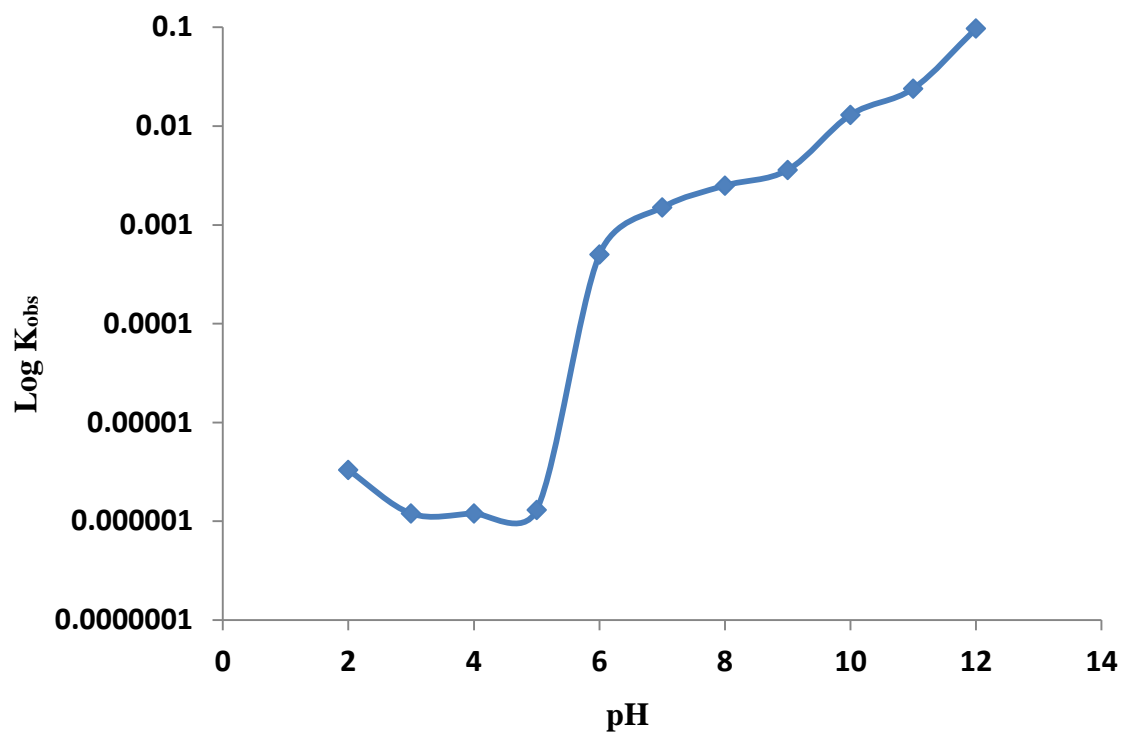
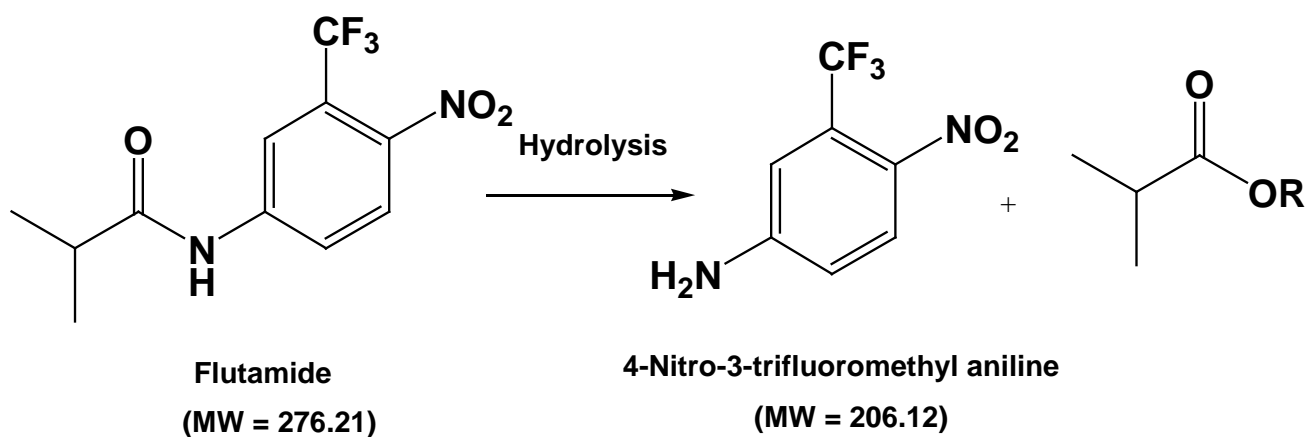


Fig. 4 pH-rate profile curve for FLT in BRB (0.04 M) at 100°C.



R = H⁺ in case of acidic hydrolysis
R = Na⁺ in case of alkaline hydrolysis

Fig. 5 Degradation pathway of FLT under hydrolytic conditions.

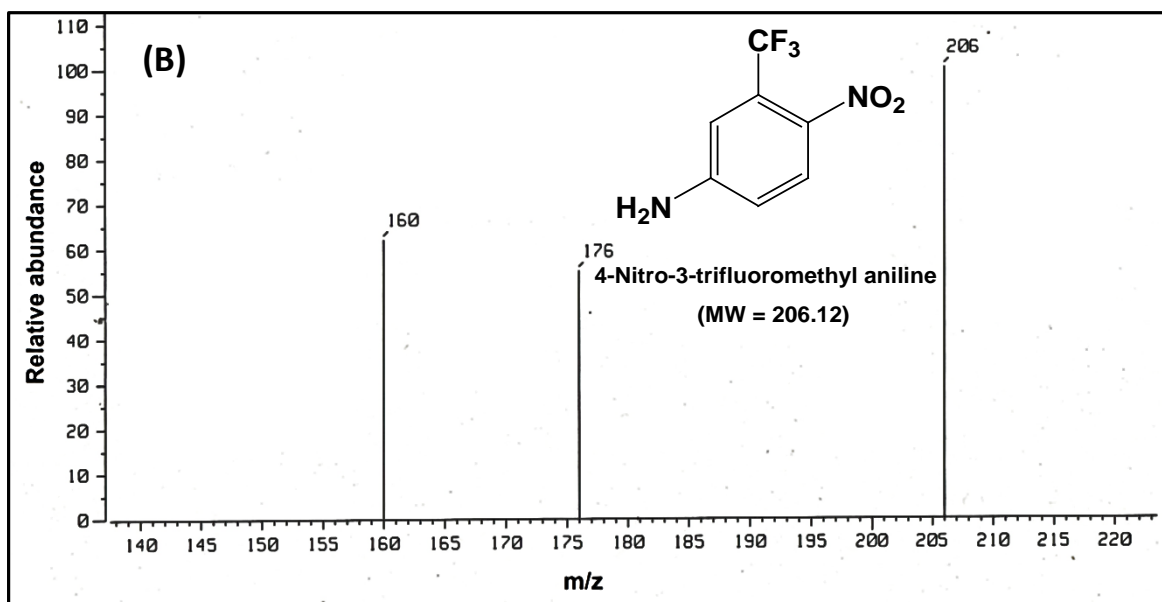
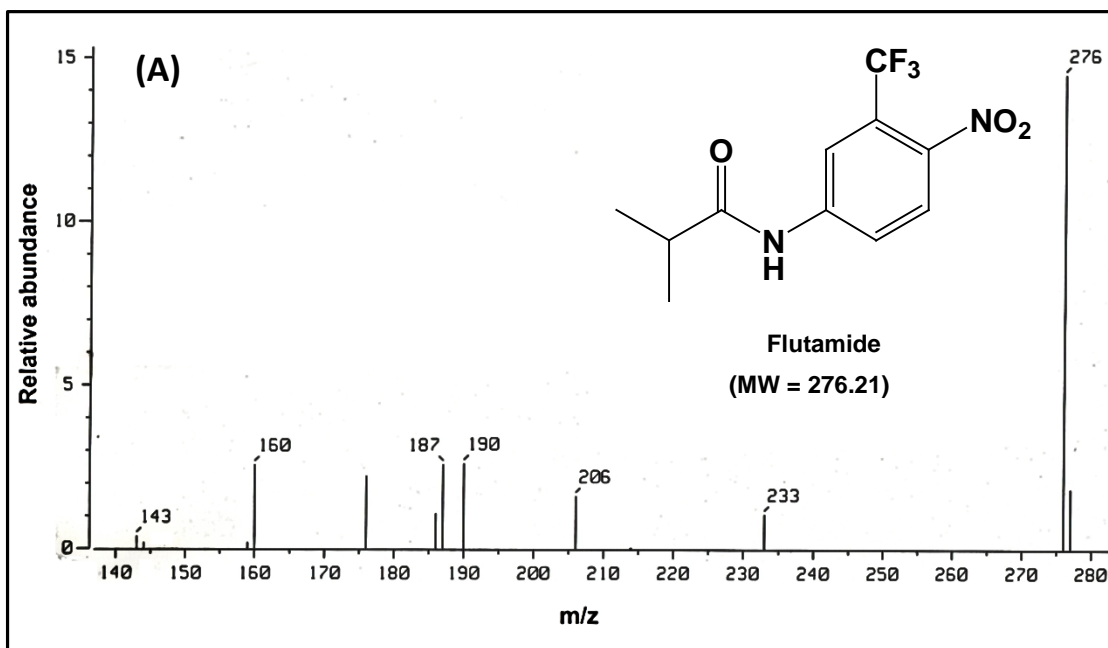


Fig. 6 EI⁺-MS spectra of (a) flutamide intact drug and (b) its hydrolytic degradation product.