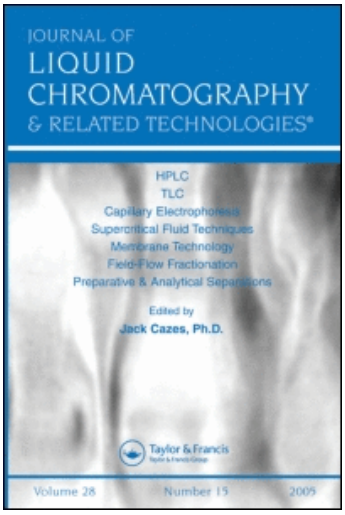

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Development and Validation of Stability-Indicating High Performance Liquid Chromatographic (HPLC) and DD_1-Spectrophotometric Assays for Etodolac in Bulk Form and in Pharmaceutical Dosage Form

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Development and Validation of Stability-Indicating High Performance Liquid Chromatographic (HPLC) and DD_1-Spectrophotometric Assays for Etodolac in Bulk Form and in Pharmaceutical Dosage Form

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Abstract: A stability-indicating reversed phase high performance liquid chromatographic assay procedure has been developed and validated for etodolac. The liquid chromatographic separation was achieved isocratically on C_{18} Zorbax ODS using a mobile phase containing methanol: water: acetic acid (70:30:0.1%, v/v/v), at a flow rate 1 mL/min and UV detection at 254 nm. The method was linear over the concentration range of 2.4–16 μg/mL ($r = 0.9999$) with a limit of detection and quantitation of 0.03 and 0.10 μg/mL, respectively. Another method was applied for the determination of etodolac in the presence of its degraded products using the first derivative ratio spectrophotometry (DD_1) at 293 nm using 1000 μg of degraded etodolac as a divisor. The method was linear over the concentration range of 10–50 μg/mL ($r = 0.9999$). Both methods have been found to have the required accuracy, selectivity, sensitivity, and precision to assay etodolac in bulk form and in pharmaceutical dosage form. Degradation products resulting from the stress studies did not interfere with the detection of etodolac, which indicates that the assays are stability-indicating assays.

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Keywords: Etodolac, HPLC and DD_1-Spectrophotometry, Stability-indicating

INTRODUCTION

Etodolac, 1,8-diethyl-1,3,4,9-tetrahydropyrano[3,4-b]indole-1-acetic acid, Figure 1, is of considerable interest for its nonsteroidal anti-inflammatory, analgesic, and antiarthritic activities.[1–7] Determination of enantiomeric impurity of etodolac was reported by capillary electrophoresis using (2-hydroxy propyl)-β-cyclodextrin,[8] by precolumn derivatization RP-HPLC and application to drug protein binding in human plasma.[9] Moreover, etodolac was determined in human serum or plasma using HPLC with a 0.2 µg/mL limit of detection,[10] in addition to HPLC tandem mass spectrometry.[11] The sequential injection analysis was also used for determination and evaluation of the antioxidant activity of etodolac as anti-inflammatory drug,[12] while, Ficarra et al. reported the use of a reversed phase high performance liquid chromatographic (RP-HPLC) procedure for etodolac determination in tablet formulation using diazepam as an internal standard.[13]

This article describes a simple, selective, stability-indicating HPLC and the first derivative ratio spectrophotometry (DD_1) assays for the analysis of etodolac in the presence of its degradation products in bulk and in tablet formulation.

EXPERIMENTAL

Chemicals and Reagents

Etodolac standard bulk powder was purchased from Pharco Pharmaceuticals Alexandria. Etodolac® tablets containing 300 mg etodolac were produced by European Egyptian Pharm. Ind. Alexandria, Egypt. Acetyl salicylic acid from Bayer Company (Germany), methanol, and acetic acid

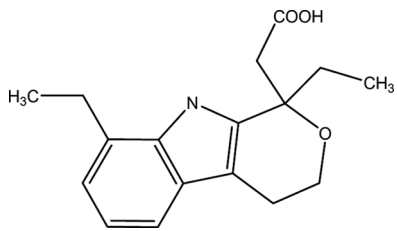

Figure 1. Etodolac.

were obtained from Merck (Darmstadt, Germany). All chemicals were of analytical grade. Purified HPLC grade water was obtained by reverse osmosis and filtration through a Milli-Q system (Millipore, Milford, MA, USA) and was used as a mobile phase.

Instrumentation and Conditions

The HPLC unit was an Agilent 1100 series apparatus equipped with a quaternary pump, a vacuum degasser, a column oven, a diode array UV-detector, and a HP Chemstation software. The column used was symmetry C_{18} Zorbax ODS (150 mm × 4.6 mm, i.d., 5 μm particle size, Agilent Technologies, USA). Separation was achieved using a mobile phase consisting of methanol: water: acetic acid (70:30:0.1%, v/v/v) at a flow rate 1 mL/min. The column was maintained at ambient temperature and an injection volume of 20 μL was used. The mobile phase was filtered through 0.45 μm Chrom Tech Nylon-66 filter and degassed in an ultrasonic bath. For analysis of forced degradation samples, the photodiode array detector was used in scan mode with a scan range of 200–500 nm. Peak homogeneity was expressed in terms of peak purity values, and was obtained directly from spectral analysis report obtained using the instrument software. All absorption spectra and derivatives were recorded with ultraviolet 1601 UV-Visible double beam Spectrophotometer with 1 cm quartz cuvetts, (Shimadzu Corporation, Kyoto, Japan).

The LC/MS consisted of an HPLC system, which was equipped with a high pressure pump model Agilent G 1311-A with Degasser Model Agilent G 1379-A and Autosampler Model Agilent G1367-B, using methanol: water: acetic acid (30:70:0.1%, v/v/v) at flow rate of 1 mL/min min with splitting (4:1). The MS instrument was an Applied Biosystems Model AP13200Q. The MS parameters were declustering potential (−40 v), entrance potential (−3 v), ion source voltage (−4500 v), ion source temperature 450°C, curtain gas 50 psi, and Q1 was maintained at unit resolution. The liquid chromatography mass was carried out in the negative ion electrospray mode of ionization. The mass range was m/z 150–300 and the run time was 10 min.

Preparation of Stock and Standard Solutions

A stock solution of etodolac (0.2 mg/mL) was prepared in HPLC grade methanol. The stock solution was protected from light using aluminum foil and stored for 1week at 4°C, and was found to be stable during this period. In the HPLC assay, aliquots of the standard stock solution of

etodolac were transferred using A-grade bulb pipettes into 25 mL volumetric flasks, adding 1 mL acetyl salicylic acid (1 mg/mL) as an internal standard (I.S.), and the solutions were made up to volume with methanol to give final concentrations of 2.4, 4, 8, 12, and 16 μg/mL. In spectrophotometric assay, aliqutes of the standard stock solution of etodolac were transferred using A-grade bulb pipettes into 10 mL volumetric flasks and the solutions were made up to volume with the methanol to give final concentrations of 10, 20, 30, 40 and 50 μg/mL.

Preparation of Tablets for Assay

Ten tablets were weighed and ground to a fine powder. A portion of the powdered tablets equivalent to 5 mg was accurately weighed into 25 mL A-grade volumetric flask and 15 mL methanol was added. The volumetric flask was sonicated for 20 min to ensure complete dissolution of the etodolac, then, the solution was made up to the volume with methanol. Suitable aliquots of solution were filtered through a 0.45 μm nylon filter and completed as previously mentioned under preparation of stock and standard solutions for both HPLC and spectrophotometric assays.

Forced Degradation Studies of Authentic Etodolac and Its Tablet Formulation

In order to determine whether the proposed analytical methods are stability-indicating, etodolac tablets and powder were stressed under various conditions to conduct forced degradation studies.[12] Regulatory guidance in ICH Q2A, Q2B, Q3B, and FDA 21 CFR section 211 require the development and validation of stability indicating potency assays. Unfortunately, the current guidance documents did not indicate detailed degradation conditions in stress testing. The optimization of such conditions was based on trial and error. As etodolac is practically soluble in methanol, methanol was used as cosolvent in degradation studies tests.[15]

Oxidation

Solutions for oxidation studies were prepared by dissolving 5 mg of authentic etodolac and powdered tablets containing an equivalent to 5 mg etodolac in methanol and 3% H_2O_2 (20:80%, v/v), protected from light and stored at room temperature for 3 days.

Acid Degradation Studies

Solutions for acid degradation studies were achieved by refluxing 50 mg authentic etodolac and powdered tablets containing an equivalent to 50 mg of etodolac in 2 M hydrochloric acid at 100°C in an oil bath for 24 hrs. The reaction mixture was then diluted with water and extracted with ethyl acetate, dried (Na_2SO_4 anhydrous), and evaporated. The residue was dissolved in methanol (50 mL). The solution was diluted with methanol to reach the concentration of 0.2 mg/mL.

Alkali Degradation Studies

Solutions for alkali degradation studies were performed by refluxing 50 mg authentic etodolac and powdered tablets containing an equivalent to 50 mg of etodolac in 1 M sodium hydroxide at 100°C in an oil bath for 24 hrs. The reaction mixture was then neutralized with 2 M hydrochloric acid, extracted with ethyl acetate, dried, Na_2SO_4 anhydrous, and evaporated. The residue was dissolved in methanol (50 mL). The solution was diluted with methanol to reach the concentration of 0.2 mg/mL.

Neutral Degradation Studies

Solutions for neutral degradation studies were prepared in methanol and water (20:80%, v/v), protected from light, and stored at room temperature for 3 days.

Temperature Stress Studies

Standard etodolac and its powdered tablets were exposed to dry heat (90°C) in an oven for 3 days.

Photostability-Degradation Assay

Bulk etodolac powder, its powdered tablets, and their methanolic solutions were exposed to light to determine the effect of irradiation on the stability of etodolac in the solid state and in the solution form. Approximately, 50 mg of standard etodolac powders were spread on a glass dish in a layer that was less than 2 mm in thickness. Solutions of standard etodolac powders (0.2 mg/mL) were prepared in methanol.

All samples for photostability testing were placed in a light cabinet (Thermo lab, India) and exposed to light for 40 h resulting in an over all illumination $\geq$200 wh/m^2 at 25°C with UV radiation at 320 – 400 nm. Control samples, which were protected from light with aluminum foil, were also placed in the light cabinet and exposed concurrently following the removal from the light cabinet.

RESULTS AND DISCUSSION

HPLC Method Development and Optimization

C_{18} Zorbax ODS was used for the separation and the method validated for the determination of etodolac in bulk and in pharmaceutical dosage form. The stressed samples were initially analyzed using a mobile phase consisting of methanol:water:acetic acid (70:30: 0.1%, v/v/v) at a flow rate of 1 mL/min and UV detection at 254 nm using 1 mg/mL acetyl salicylic acid as an internal standard.

Validation

The method was validated with respect to parameters including linearity, limit of quantitation (LOQ), limit of detection (LOD), precision, accuracy, selectivity, and recovery.

Linearity

The calibration curves constructed for etodolac were linear over the concentration range of 2.4–16 μg/mL. Peak areas of etodolac were plotted versus etodolac concentration and linear regression analysis performed on the resultant curve. Typically, the regression equation for the calibration curve was found to be

$$y = 0.0707x - 0.0031 \quad r = 0.9999.$$

where y is the area under the peak, x is the concentration in μg/mL, and r is the correlation coefficient.

Limit of Quantitation (LOQ) and Limit of Detection (LOD)

The LOQ and LOD were determined based on signal to noise ratios. The LOQ was found to be 0.1 μg/mL with a resultant % R.S.D of 0.4% (n = 5), where the LOD was found to be 0.03 μg/mL.

Precision

Precision of the assay was investigated with respect to both repeatability and reproducibility. Repeatability was investigated by injecting nine replicate samples of each of the 2.4, 4, and 16 μg/mL standards where the mean concentrations were found to be 2.45, 4.08, and 16.08 with associated % R.S.D values of 2.1, 1.3, and 0.22, respectively. Inter-day

precision was assessed by injecting the same three concentrations over 3 consecutive days, resulting in mean concentrations of etodolac of 2.47, 4.09, and 16.2 μg/mL and associated % R.S.D of 2.35, 2.45, and 0.34%, respectively. The ruggedness of the method and the % R.S.D values were assessed by comparison of the intra-and inter-day assay results for etodolac that has been performed by two analysts in the same laboratory, and it was found that they did not exceed 3%, thus indicating the ruggedness of the method. The retention time (R_t) of etodolac was 8.714 min with % R.S.D of 0.814%.

Accuracy

Accuracy of the assay was determined by interpolation of replicate ($n = 6$) peak areas of three accuracy standards (2.4, 4, and 16 μg/mL) from a calibration curve prepared as previously described. In each case, the percent relevant error was calculated. The resultant concentration, were 2.440 ± 0.010 μg/mL (mean ± S.D.), 4.100 ± 0.064 μg/mL, and 16.07 ± 0.05 μg/mL with percent relevant errors of 2.58, 2.47, and 0.26%, respectively.

Selectivity

The results of stress testing studies indicated a high degree of selectivity of this method for etodolac. The degradation of etodolac was found to be similar for both the tablets and bulk powder. Typical chromatograms obtained following the assay of pure bulk sample and stressed samples are shown in Figures 2–4.

Photodiode array detection was also used as an evidence of the selectivity of the method, and to evaluate the homogeneity of the drug peak. Chromatographic peak purity data was obtained from the spectral analysis report and a peak purity value greater than 0.999 indicates a homogenous peak. The peak purity values of etodolac in chromatograms of stressed samples were in the range of 0.999–1 for both the tablets and bulk powder, indicating homogenous peaks and thus establishing the selectivity of assay method.

Recovery

A known amount of etodolac standard bulk powder was added to aliquots ($n = 20$) of tablet contents, mixed, and the powder was extracted and diluted to yield a starting concentration of 12 μg/mL. This solution was analyzed and the assay was repeated ($n = 9$) over 3 consecutive days to obtain intermediate precision data. The observed concentration of etodolac was found to be 11.91 ± 0.23 μg/mL (mean ± S.D.). The resultant

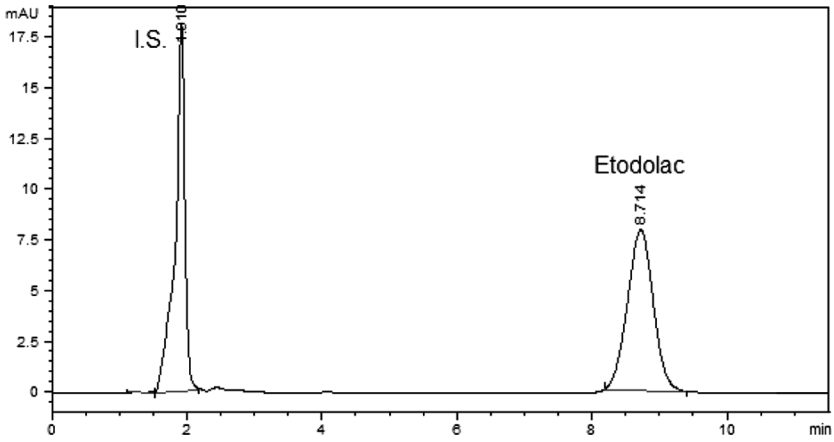

Figure 2. Typical chromatogram of Etodolac 16 μg/mL on a C_{18} Zorbax ODS (150 mm × 4.6 mm, i.d., 5 μm particle size, Agilent Technologies, USA) using a mobile phase consisting of methanol:water:acetic acid 70:30: 0.1%, v/v/v at a flow rate 1 mL/min at 254 nm.

% R.S.D for this study was found to be 0.219% with a corresponding percentage recovery value of 100.27%.

Stability Studies

All stressed samples in both solid states and in solution forms remained colorless. No decomposition was observed on exposure of etodolac in

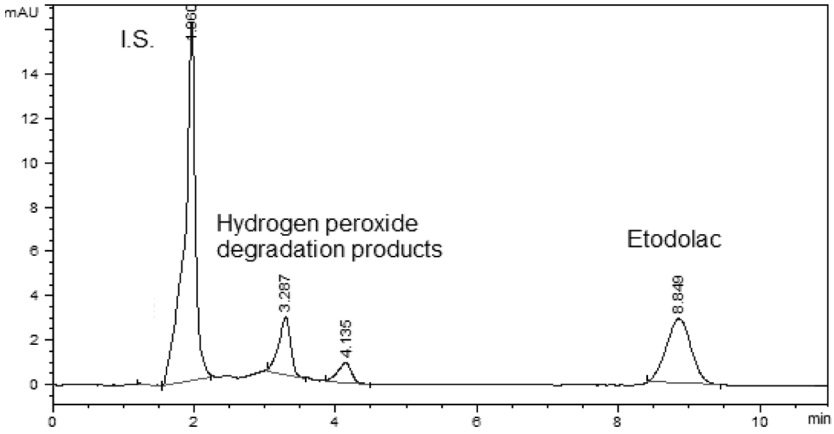

Figure 3. Chromatogram of hydrogen peroxide degradation product of Etodolac. Chromatographic conditions as in Figure 2.

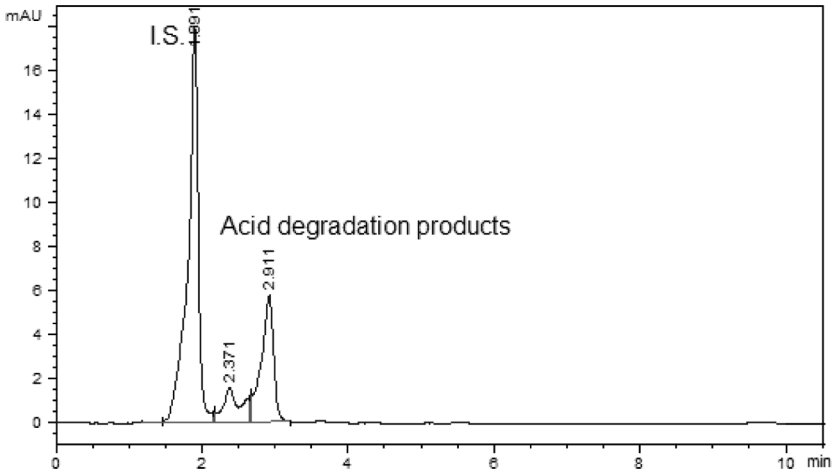

Figure 4. Chromatogram of acid degradation product of Etodolac. Chromatographic conditions as in Figure 2.

solid states and in solution forms to light in a photostability chamber at room temperature. In alkaline and neutral media, etodolac was stable and no degradation was observed under the studied conditions. On the other hand, it was found that about 30% of the drug was degraded on treating with 3% H_2O_2 for 3 days as shown in Figure 3.

In acid media 100% degradation occurred as shown in Figure 4. The retention time R_t of degraded products of etodolac were 2.371 and 2.911 min, while R_t of acetyl salicylic acid (I.S.) was 1.991 min.

In this study, the identification of degradation products of etodolac under acidic condition by LC/MS/MS revealed the detection of the degradation products **1a** and **1b** (m/z 243), namely 7-ethyl-2-(1-methylenepropyl)-1H-indole-3-ethanol and 7-ethyl-2-(1-methyl-1-propenyl)-1H-indole-3-ethanol, respectively. In addition to another decarboxylated product namely: 1,8-diethyl-1-methyl-1,3,4,9-tetrahydropyrano-[3,4-b] indole (**2**) (m/z 243) and 7-ethyltryptophol (**3**) (m/z 189) were identified (Figure 5). These results were in agreement with the results of Lee et al.[16]

Assay

The proposed method was applied for the determination of etodolac in the tablet formulations. The results of these assays yielded 100.04% (% R.S.D = 1.250%) of expected amount of the tablets. These results indicated that the method is selective for the assay of etodolac without interference from the excipients used in those dosage forms.

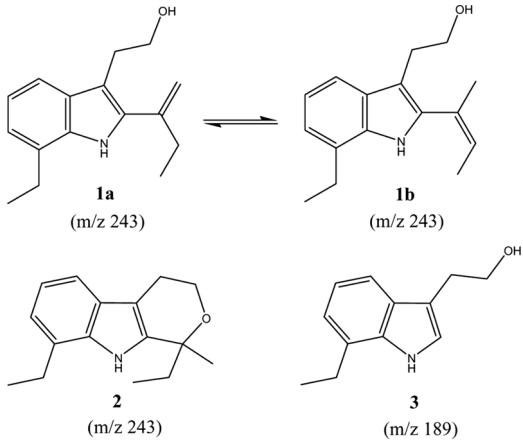

Figure 5. Chemical structures of Etodolac acid degradation products.

Stability Indicating Method for Analysis of Etodolac in Presence of Its Acid Degraded Products by HPLC

A simple HPLC method was adopted for the simultaneous determination of etodolac and its acid degraded products in bulk powder. To optimize the proposed HPLC method, all of the experimental conditions were investigated.

To optimize the mobile phases, different systems were tried for chromatographic separation of the two components by combining homogenous design and solvent polarity optimization. The best resolution was achieved using a mobile phase consisting of methanol:water:acetic acid (70:30: 0.1% v/v/v), which gave good resolution and sensitivity of all components.

A linear relationship was obtained between peak area and the concentration of etodolac in the presence of its acid degraded products in the range of 2.4–16 μg/mL. The linear regression equation was computed as:

$$y = 0.0707x - 0.003 \quad r = 0.9999$$

where y is the area under the peak, x is the concentration in μg/mL, and r is the correlation coefficient.

Results obtained by applying the HPLC procedure showed that etodolac can be simultaneously analyzed in the presence of its acid degraded with mean recoveries of $100.19 \pm 0.816\%$, Table 1.

Table 1. Determination of pure bulk Etodolac by HPLC and first derivative ratio spectrophotometry (DD_1)

HPLC				DD_1 spectrophotometry			
Mixture No.	Concentration taken (μg/mL)	Found (μg/mL)	Found (%)	Mixture No.	Concentration taken (μg/mL)	Found (μg/mL)	Found (%)
1	2.4	2.434	101.42	1	10.0	9.9	99.00
2	4.0	4.004	100.10	2	20.0	20.18	100.90
3	8.0	7.979	99.74	3	30.0	29.88	99.60
4	12.0	11.910	99.25	4	40.0	39.98	99.95
5	16.0	16.069	100.43	5	50.0	49.98	99.96
Mean ± S.D.			100.19 ± 0.816	Mean ± S.D.			99.88 ± 0.690

Table 2. Application the standard addition technique to the analysis of Etodolac by HPLC and first derivative ratio spectrophotometry (DD_1) in its tablet dosage form

Product	Found (%)	Pure added (μg/mL)	Found (μg/mL)	Recovery (%)
Etodolac Tablets (Batch No. 501501)	HPLC 99.87 ± 0.451	1.500	1.504	100.27
		3.000	3.018	100.60
		4.500	4.514	100.31
		6.000	6.001	100.02
		7.500	7.510	100.13
		Mean ± S.D.		100.27 ± 0.219
	DD_1-spectrophotometry 99.83 ± 0.624	15.00	14.85	99.00
		20.00	19.89	99.45
		25.00	25.26	101.04
		30.00	29.75	99.17
		35.00	34.93	99.80
		Mean ± S.D.		99.69 ± 0.812

The proposed method has been applied to the assay of etodolac in its tablet form without any interference from the excipients. The validity of the suggested procedure was further assessed by applying the standard addition technique with mean recoveries $100.27 \pm 0.219\%$, Table 2.

Stability Indicating Method for Analysis of Etodolac in Presence of Its Acid Degraded Products by First Derivative Ratio (DD_1) Spectrophotometeric Method

The theory of derivative ratio spectrophotometry[17] was taken into consideration to solve the problem of overlapping absorption spectra for etodolac in presence of its acidic degraded products as shown in Figure 6. Etodolac can be assayed in the presence of its acidic degraded by dividing the absorption spectra of different concentration of etodolac in the range of 10–50 μg/mL by the absorption spectrum of 1000 μg/mL acid degraded etodolac.

The obtained first derivative ratio spectra were differentiated with respect to wavelength as shown in Figure 7, DD_1 value showed good linearity and accuracy at λ 293.0 nm.

The linear regression equations were found to be at λ 293.0 nm:

$$y = -0.0051x - 0.0005 \quad r| = 0.9999$$

where y is the peak amplitude of the DD_1 value at λ 293.0 nm, x is the concentration in μg/mL, and r is the correlation coefficient. Results

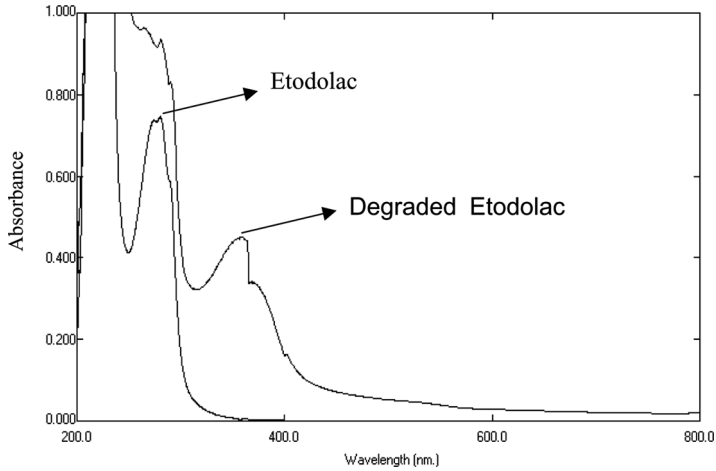

Figure 6. Zero-order absorption spectra of etodolac (30 μg/mL) and its degraded (400 μg/mL) in methanol.

obtained by applying the DD_1 procedure showed that etodolac can be simultaneously analyzed in the presence of its acid degraded products with mean recoveries of $99.88 \pm 0.690\%$, Table 1.

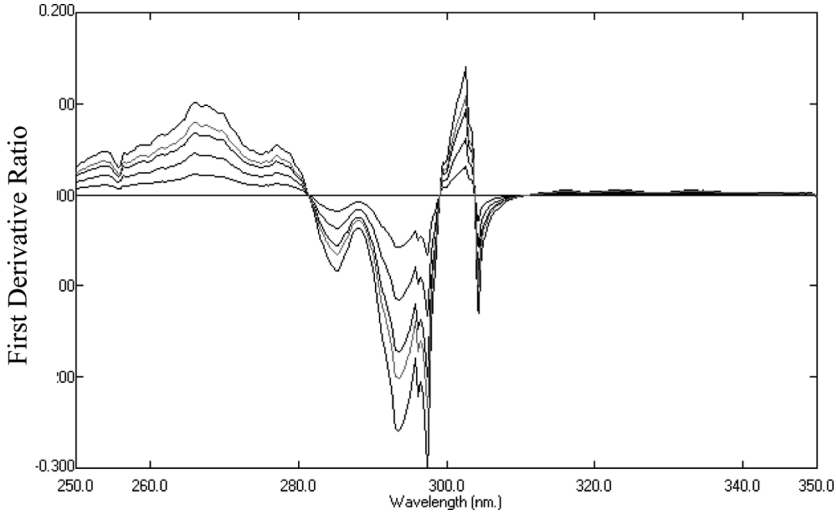

Figure 7. First-derivative Ratio Spectra (10–50 μg/mL) etodolac using (1000 μg/mL) degraded etodolac as a divisor.

Table 3. Statistical analysis of the results obtained by applying the proposed and UV spectrophotometric method[18] for the analysis of pure Etodolac

Parameter	Proposed methods		UV spectrophotometric method[18]
	HPLC	DD_1 Spectrophotometry	
Mean ± S.D.	100.19 ± 0.816	99.88 ± 0.690	99.99 ± 0.388
n[a]	5	5	5
T	0.408(2.306)[b]	0.237(2.306)[b]	–
F	4.411(6.390)[b]	3.152(6.390)[b]	–

[a]n = Number of determinations.
[b]The values in parentheses are corresponding to the theoretical values of t and F at (P = 0.05).

The proposed method has been applied to the assay of etodolac in its tablet form without any interference from the excipients. The validity of the suggested procedure was further assessed by applying the standard addition technique with mean recoveries 99.69 ± 0.812%, Table 2.

A statistical comparison of the results obtained by the proposed methods (HPLC and DD_1-spectrophotometry) and the Clarke procedure that depends on UV spectrophotometric determinations of etodolac[18] in a pure drug is shown in Table 3. The calculated t- and F- values were less than the tabulated values,[19] which revealed that there is no significant difference with respect to accuracy and precision determined through assay of variables. Blind samples of pure etodolac of different concentrations were calculated from their regression equations. The degree of method repeatability was measured by analyzing each concentration of etodolac four times by the proposed methods, and calculating the relative standard deviation (R.S.D) values between the proposed methods and the UV spectrophotometric procedure procedure.[18]

The results of the proposed methods showed that they are accurate, precise, specific, and rugged according to the R.S.D values of intra-day and inter-day determinations.

CONCLUSION

Validated stability-indicating HPLC and DD_1-spectrophotometric analytical methods have been developed for the determination of etodolac in pure form and in tablet dosage formulation. The results of stress testing were undertaken according to the international conference on

harmonization (ICH) guidelines, which revealed that the studied methods are selective and stability-indicating. The proposed methods are simple, accurate, precise, specific, and have the ability to separate the drug from its degradation products and excipients found in the tablet dosage forms. The achieved methods are suitable for the routine analysis of etodolac in either bulk powder or in pharmaceutical dosage forms.

The simplicity of the applied methods allows their application in quality control laboratories that lack sophisticated analytical instruments, such as LC-MS, or GC-MS. These methods are complicated and expensive, rather than simple HPLC and DD_1-spectrophotometric procedures.

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