DRUG FORMULATIONS AND CLINICAL METHODS

Different Techniques for the Determination of Tofisopam

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Five simple and sensitive methods were developed for the determination of tofisopam (TF). The first four are stability-indicating depending on the determination of TF in the presence of its degradation product, while the fifth depended on the determination of TF via its degradation product. Method A was based on first and second derivative spectrophotometry, ^{1}D and ^{2}D, measuring the amplitude at 298 and 332 nm in the case of ^{1}D and at 312 and 344 nm in the case of ^{2}D. Method B depended on measuring the peak amplitude of the first derivative of the ratio spectra ^{1}DD at 336 nm. Method C was based on difference spectrophotometry by measuring ΔA at 366 nm. Method D was a TLC method using silica gel 60 F_{254} plates, the optimized mobile phase ethyl acetate–methanol–ammonium hydroxide 10% (8.5 + 1.0 + 0.5, v/v/v), and quantification by densitometric scanning at 315 nm. In method E, spectrofluorometry was applied for the determination of TF via its degradation product; maximum emission was 383 nm when excitation was 295 nm. Linearities were obtained in the concentration range 2–20 µg/mL for methods A, B, and C and 2–20 µg/band and 0.2–1.6 µg/mL for D and E, respectively. In method A, the mean recoveries were 99.45±0.287 and 100.28±0.277% at 298 and 332 nm, respectively, in the case of ^{1}D and 99.40±0.245% and 99.50±0.292% at 312 and 344 nm, respectively in the case of ^{2}D. The mean recovery was 100.03 ± 0.523% at 366 nm in method B. Method C showed mean recovery of 100.20±0.642%. Recoveries for methods D and E were 98.98±0.721 and 100.25±0.282%, respectively. The degradation product was obtained in acidic stress condition, separated, and identified by IR and mass spectral analysis, from which the degradation product was confirmed and the degradation pathway was suggested. The first four methods were specific for TF in the presence of different concentrations of its degradation product. The five proposed methods were successfully applied for the determination of TF in Nodeprine tablets. Statistical comparison among the results obtained by these methods and that obtained by the official method for the determination of the drug was made, and no significant differences were found.

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Tofisopam (TF) is a 2,3-benzodiazepine related structurally to 1,4 benzodiazepines (Figure 1) such as diazepam and sharing some of the same actions. It is reported, however, to be largely lacking in the selective anticonvulsant and muscle relaxant properties of conventional benzodiazepines. TF has been given orally in the short-term treatment of anxiety disorders. The R-(+)-isomer dextofisopam is under investigation for the treatment of irritable bowel syndrome (1).

The determination of TF is described in the Japanese Pharmacopoeia (2) by a nonaqueous titration method. It was also determined by methods involving spectrofluorometry with the fluorescence measured at 488 nm (3), HPLC using different kinds of columns and different mobile phases (4–14), and capillary electrophoresis on a phenyl silica stationary phase with detection at 220 nm (15). GC with a nitrogen-phosphorus detector (16) and a polarographic method (17) have also been reported for determination of TF.

No stability-indicating methods have been cited in the literature for determination of TF and its degradation product. None of the reported methods was concerned with either the degradation of TF or its determination in the presence of its degradation product formed under stress conditions mentioned in International Conference on Harmonization (ICH) guidelines (18). Therefore, this paper presents a study of the degradation of TF and the development of four stability-indicating methods for its determination and a fifth for TF determination via its degradation product. The five methods are based on first and second derivative spectrometry (method A), derivative ratio spectrometry (method B), difference spectrometry (method C), TLC (method D), and spectrofluorometry (method E).

Experimental

Reagents

Methanol, sodium hydroxide, HCl, ethyl acetate, methanol, and ammonium hydroxide 10% were Prolabo from VWR International, West Chester, PA.

Instrumentation

The instruments used were a UV-Vis spectrophotometer

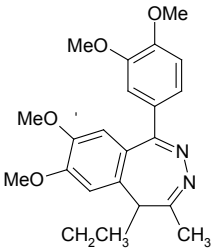

Figure 1. Structure of TF; molecular formula $C_{22}H_{26}N_2O_4$.HCl; MW = 382.46.

UV 300 (Unicam, Kyoto, Japan), IR spectrophotometer Vector 22 (Bruker Optics, Ettlingen, Germany), mass spectrometer GC-MS-QP1000EX quadrupole spectrometer equipped with electron multiplier detector (Shimadzu, Tokyo, Japan), Rf-1501 spectrofluorometer, and Shimadzu CS-9301 PC dual wavelength flying spot scanning densitometer.

Reference Sample

Pure TF was kindly supplied by Acapi Pharmaceuticals (Cairo, Egypt), Batch No. 200706180184. It was assayed for its purity according to a pharmacopoeial method (2) and found to contain 99.6 ± 0.300% TF.

Pharmaceutical Formulation

Nodeprine tablets, manufactured by Acapi Pharmaceuticals, Batch No. 21081, labeled to contain 50 mg TF in each tablet.

Degradation of the Sample

TF (100 mg) was refluxed with 100 mL 3 M HCl solution in a 250 mL round-bottom flask for 5 h and tested for complete degradation by TLC using ethyl acetate–methanol–ammonium hydroxide 10% (8.5 : 1.0 : 0.5, v/v/v) as the mobile phase. Only one spot was observed not corresponding to TF. The degraded solution was then cooled and neutralized with 5 M NaOH until the pH was 7. The solution was evaporated nearly to dryness, cooled, and transferred into a 100 mL volumetric flask with methanol. Then the volume was completed to the mark with methanol and filtered. The degraded solution was used for the IR and mass spectral scans and for testing the four stability-indicating methods.

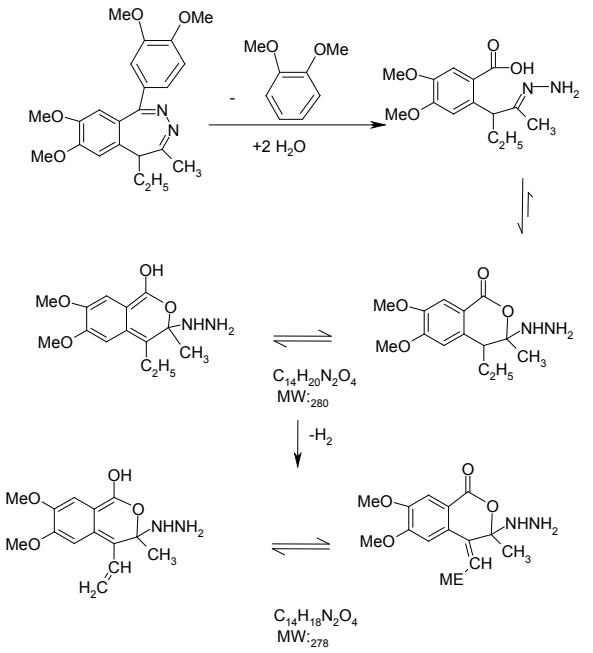

Figure 2. Degradation pathway of TF.

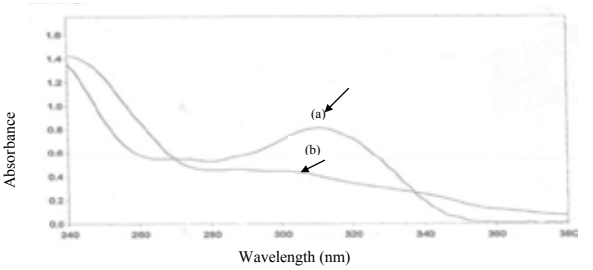

Figure 3. Zero-order absorption spectra of TF in methanol (a) and its degradation product in methanol (b); concentration of each was 18 µg/mL.

Standard and Degradation Product Solutions

(**a**) *TF stock standard solution (1 mg/mL) in methanol for methods A–E.*—Prepared by dissolving 25 mg TF in a 25 mL volumetric flask and then completing the volume to the mark with methanol.

(**b**) *Working standard solution (0.2 mg/mL) in methanol for methods A–C.*— Prepared by transferring 20 mL TF stock standard solution (1 mg/mL) into a 100 mL volumetric flask and then completing the volume to the mark with methanol.

(**c**) *Degradation product stock solution (1 mg/mL) in methanol for stability-indicating methods A–D.*—Prepared by refluxing 100 mg TF with 100 mL 3 M HCl solution in a 250 mL round-bottom flask for 5 h. The degraded solution was then cooled and neutralized with 5 M NaOH until pH was 7. The solution was evaporated nearly to dryness, cooled, and transferred into a 100 mL volumetric flask with methanol. Then the volume was completed to the mark with methanol and filtered.

(**d**) *Working degradation product solution [TF deg (1)] (0.2 mg/mL) in methanol for methods A–C.*—Prepared by transferring 20 mL degradation product stock solution (1 mg/mL)

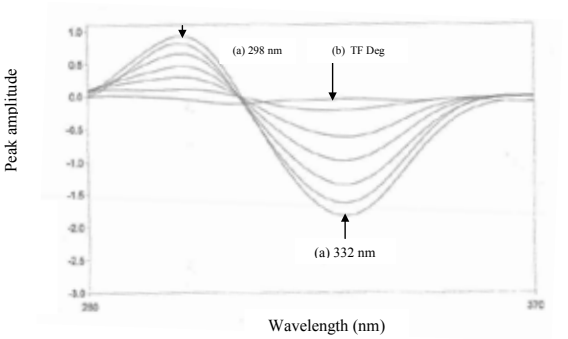

Figure 4. First derivative absorption spectra of TF (a) in methanol (2–20 µg/mL) and 2 µg/mL degradation product (b).

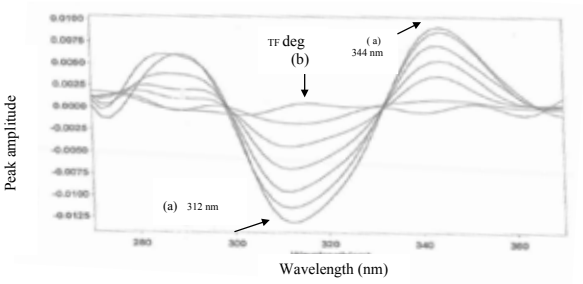

Figure 5. Second derivative absorption spectra of TF (a) in methanol (2–20 µg/mL) and 2 µg/mL degradation product (b).

into a 100 mL volumetric flask and then completing the volume to the mark with methanol.

(**e**) *Working degradation product solution [TF deg (2)] (0.02 mg/mL) in methanol for method E.*—Prepared by transferring 2 mL degradation product stock solution (1 mg/mL) into a 100 mL volumetric flask and then completing the volume to the mark with methanol.

Laboratory-Prepared Mixtures Containing Different Ratios of TF and its Degradation Product

(**a**) *Methods A and B.*—Aliquots (0.9–0.1 mL) of TF were accurately transferred from its working standard solution (0.2 mg/mL) equivalent to 180–20 µg into a series of 10 mL volumetric flasks. Aliquots (0.1–0.9 mL) of TF deg (1)] (0.2 mg/mL) equivalent to 20–180 µg were added, and the volume was completed with methanol to prepare mixtures containing 10–90% of the degradation product.

(**b**) *Methods C.*—Aliquots (0.9–0.1 mL) of TF were accurately transferred from its working standard solution (0.2 mg/mL) equivalent to 80–20 µg into a series of 10 mL volumetric flasks. Aliquots (0.1–0.9 mL) of TF deg (1) (0.2 mg/mL) equivalent to 20–180 µg were added, and the volume was completed with 1 M HCl to prepare mixtures containing 10–90% of the degradation product.

For blanks, aliquots (0.9–0.1 mL) of TF were accurately transferred from its working standard solution (0.2 mg/mL) equivalent to 180–20 µg into a series of 10 mL volumetric flasks. Aliquots (0.1–0.9 mL) TF deg (1) (0.2 mg/mL) equivalent to 20–180 µg were added, and the volume was completed with 1 M NaOH to prepare mixtures containing 10–90% of the degradation product.

(**c**) *Methods D.*—Aliquots (4.5–0.5 mL) of TF stock standard solution (1 mg/mL) equivalent to 4.5–0.5 mg were accurately transferred into a series of 5 mL volumetric flasks. Aliquots (0.5–4.5 mL) of degradation product stock solution (1 mg/mL) equivalent to 0.5–4.5 mg were added to prepare mixtures containing 10–90% of the degradation product.

Construction of the Calibration Graphs for Method A (^{1}D and ^{2}D)

Aliquots (0.1, 0.3, 0.5, 0.7, 0.9, and 1.0 mL) of the drug working standard solution (0.2 mg/mL) were transferred into a series of 10 mL volumetric flasks. The volume was then completed to the mark with methanol.

The zero-order spectra were recorded, then ^{1}D spectra of each solution were obtained with $\Delta\lambda$ = 4 nm and scaling factor 10. The peak amplitudes at 298 and 332 nm were measured. Linear calibration graphs were constructed relating the peak amplitudes at 298 and 332 nm to the corresponding concentrations of TF, and the corresponding regression equations were computed.

^{2}D spectra of the recorded zero-order spectra of each solution were also obtained with $\Delta\lambda$ = 4 nm and scaling factor 10. The peak amplitudes at 312 and 344 nm were measured. Linear calibration graphs were constructed relating the peak amplitudes at 312 and 344 nm to the corresponding concentrations of TF, and the corresponding regression equations were computed.

Construction of the Calibration Graph for Method B (^{1}DD)

Alternatively, the zero-order spectra of the prepared solutions for method A were divided by the spectrum of 2 µg/mL of its degradation product, and the ratio spectra were obtained. Then the first derivative of the ratio spectra ^{1}DD with $\Delta\lambda$ = 4 nm and scaling factor 10 were obtained. The peak amplitudes of the first derivative of the ratio spectra at 336 nm ($^1DD_{336}$) were measured. A linear calibration graph was constructed relating the peak amplitude of the first derivative of the ratio spectra at 336 nm ($^1DD_{336}$) to the corresponding concentrations of TF, and the regression equation was computed.

Construction of the Calibration Graph for Method C (ΔA)

Aliquots (0.1, 0.3, 0.5, 0.7, 0.9, and 1.0 mL) of drug working standard solution (0.2 mg/mL) were transferred into a series of 10 mL volumetric flasks. The volumes were then completed to mark with 1 M HCl.

For blanks, other aliquots (0.1, 0.3, 0.5, 0.7, 0.9, and 1.0 mL) of drug working solution (0.2 mg/mL) were transferred into a series of 10 mL volumetric flasks. The volumes were then completed to mark with 1 M NaOH. The ΔA spectra were obtained, and the absorbance difference at 366 nm was measured. A linear calibration graph was constructed relating the absorbance difference (ΔA) at 366 nm to the corresponding concentrations of TF, and the corresponding regression equation was computed.

Construction of the Calibration Graph and Chromatographic Conditions for Method D (TLC)

Aliquots (0.5, 1, 2…5 mL) of stock standard solution (1 mg/mL) were transferred into a series of 5 mL volumetric flasks. The volumes were then completed to the mark with methanol. A 20 µL amount of each solution was spotted onto TLC plates using a CAMAG (Muttenz, Switzerland) Linomat autosampler with a microsyringe (25 µL), and the chromatographic conditions given below were adopted. The scanning profile for TF was obtained. The calibration graph relating the area under the peak to the corresponding concentration was constructed, and the regression equation was computed.

The plates were prewashed by development with the mobile phase ethyl acetate–methanol–ammonium hydroxide 10% (8.5+1.0+0.5, v/v/v) and then activated for 15 min by placing in an oven at 100°C before use. For detection and quantitation, 20 µL of prepared standard solutions was applied 20 mm apart and 15 mm from the bottom of the plates, with 2 mm band length and 150 nL/s dosage speed. The chromatographic tank was saturated with the mobile phase for 15 min. The plates were

Table 1. Results of validation parameters of the responses and the regression equations obtained by the proposed methods

	Method A				Method B	Method C	Method D	Method E
	^{1}D		^{2}D		^{1}DD	ΔA	TLC	Fluorometry
Parameters	298 nm	332 nm	312 nm	344 nm	336 nm	366 nm		
Slope[a]	0.0456	0.0962	0.0007	0.0005	1.9017	0.0388	0.0539	116.79
Intercept[a]	0.0184	0.0288	0.0007	0.0001	0.0320	0.0347	0.0119	2.7500
r	0.9993	0.9991	0.9995	0.9993	0.9991	0.9998	0.9993	0.9996
Concn range, µg/mL	2–20	2–20	2–20	2–20	2–20	2–20	2–20, µg/band	0.2–1.6, mg/mL
Average accuracy, %	99.45	100.28	99.40	99.50	100.03	100.20	98.98	100.25
SD	0.285	0.278	0.244	0.291	0.523	0.643	0.714	0.283
RSD, %	0.287	0.277	0.245	0.292	0.522	0.642	0.721	0.282
Repeatability, %[b]	100.50 ± 0.361	99.39 ± 0.316	98.91 ± 0.616	99.75 ± 0.478	100.23 ± 0.373	100.12 ± 0.620	99.45 ± 0.685	100.15 ± 0.269
Intermediate precision, %[c]	100.63 ± 0.153	99.75 ± 0.478	98.60 ± 0.537	99.77 ± 0.668	100.43 ± 0.501	100.10 ± 0.707	99.17 ± 0.904	99.43 ± 0.406

[a] Results of five determinations.

[b] $n = 3 \times 3$.

[c] $n = 3 \times 3$.

developed over a distance of 15 cm in the ascending direction, air-dried, and scanned under the following conditions: source of radiation: deuterium lamp; scan mode: zigzag; slit dimensions: 3 × 0.45 mm; scanning speed: 20 mm/s; output: chromatogram and integrated peak area; and wavelength: 315 nm.

Construction of the Calibration Graph for Method E (Fluorometry)

Aliquots (0.1, 0.2,… 0.8 mL) of TF deg (2) (0.02 mg/mL) were transferred into a series of 10 mL volumetric flasks. The volumes were then completed to the mark with methanol. The fluorescence intensity was measured using excitation and emission wavelengths of 295 and 383 nm, respectively, against methanol as a blank. A linear calibration graph was constructed relating the fluorescence intensity at 383 nm to the corresponding concentrations of TF, and the regression equation was computed.

Application of the Proposed Methods to the Analysis of Laboratory-Prepared Mixtures of Intact Drug and its Degradation Product

(**a**) *Methods A–C.*—The absorption spectra of the laboratory-prepared mixtures were recorded. Then the procedures were completed as described in the *Construction of the Calibration Graphs* subsection. The concentrations of TF were calculated by substituting in the corresponding regression equations.

(**b**) *Method D.*—Aliquots (20 µL) of laboratory-prepared mixtures were spotted onto the TLC plates, the procedure was completed as described in the *Construction of the Calibration Graph* subsection, and the concentration was calculated from the corresponding regression equation.

Application of the Proposed Methods to the Analysis of TF in the Pharmaceutical Preparation

(**a**) *Methods A–C.*—Five tablets were weighed accurately and powdered. An amount of powder equivalent to 25 mg of TF was accurately weighed into a 25 mL volumetric flask and extracted with 15 mL methanol in an ultrasonic bath for 30 min, diluted to volume with methanol, and filtered. Suitable dilutions were made using methanol to prepare a tablet solution containing 0.2 mg/mL; 0.4 mL of the solution was accurately transferred to a 10 mL volumetric flask and diluted to volume with methanol for methods A and B or with 1 M HCl for method C. Then the procedures were completed as described under *Construction of the Calibration Graphs*. The concentration of TF was calculated by substituting in the corresponding regression equations.

(**b**) *Method D.*—An amount of powdered tablets equivalent to 25 mg TF was accurately weighed into a 25 mL volumetric flask and extracted with 15 mL methanol in an ultrasonic bath for 30 min, diluted to volume with methanol, and filtered. A 2 µL amount of the filtrate was accurately transferred to a 5 mL volumetric flask and diluted to volume with methanol. Then the procedure was completed as described under *Construction of the Calibration Graph*. The concentration of TF was calculated by substituting in the corresponding regression equation.

(**c**) *Method E.*—An amount of powdered tablets equivalent to 100 mg TF was accurately weighed and refluxed with 100 mL 3 M HCl solution in a 250 mL round-bottom flask for 5 h. The degraded solution was then cooled and neutralized with 5 M NaOH until the pH was 7. The solution was evaporated nearly to dryness, cooled, and transferred into a 100 mL volumetric flask with methanol. Then the volume was completed to the mark with methanol and filtered. Suitable dilutions were made using methanol to prepare a tablet solution containing 0.02 mg/mL.

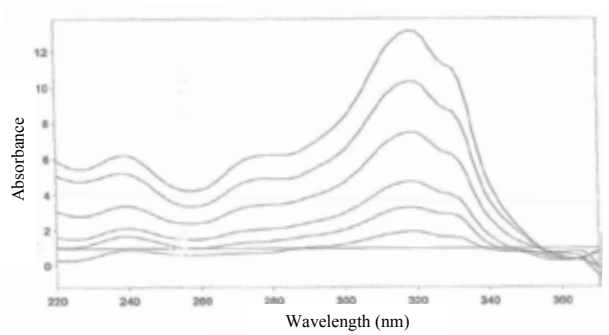

Figure 6. Zero-order absorption spectra of the ratio spectra of TF in methanol (2–20 µg/mL) using 2 µg/mL of its degradation product as a divisor.

Then 0.3 mL of the prepared solution was accurately transferred to a 10 mL volumetric flask and diluted to volume with methanol. The procedure was completed as described under *Construction of the Calibration Graph*. The concentration of TF was calculated by substituting in the corresponding regression equation.

Results and Discussion

The stability of TF was studied according to ICH guidelines for:

(**a**) *Acid and alkaline stress*.—1 M HCl/1 M NaOH for 16 h, 2 M HCl/2 M NaOH for 16 h, and 3 M HCl/3 M NaOH for 4 and 5 h.

(**b**) *Oxidation*.—3% H_2O_2 for 2, 4, 6, and 10 h.

(**c**) *Thermal degradation*.—At 100°C in an oven for 2, 4, and 6 h.

(**d**) *Photo degradation*.—UV lamp producing UVB radiation for 6, 8, and 10 h.

The degradation process under the previously mentioned conditions was followed using TLC, and TF was found to be liable to acid degradation, forming a single component that was confirmed by TLC as indicated by the appearance of only one spot after complete degradation.

Because this work was concerned with the development of different methods for the determination of TF in presence of its degradation product or via its degradation product, the degradation product was laboratory-prepared, as mentioned in the *Degradation of the Sample* section. The structure of the isolated acid degradation product was confirmed using IR and MS.

The IR spectrum of intact TF is characterized by the NH band at 3434.6 cm^{-1}, C = C aromatic and NH-bending at 1600 cm^{-1}, O-R ether function at 1099 cm^{-1}, aromatic C-H bending at 802 cm^{-1}, and the absence of C = O above 1600 cm^{-1}. On the other hand, the IR spectrum of the degradation product of TF revealed an NH band and broad band of a hydrogen bond at 3448 cm^{-1}, C = C aromatic and NH-bending at 1597 cm^{-1}, and O-C aryl and alkyl bonds at 1257 and 1026 cm^{-1}. Moreover, the spectrum showed the characteristic C = O at 1647 cm^{-1}. A mass spectrum of degraded TF shows the mass of $C_{14}H_{18}N_2O_4$ at *m/z* 278; this may indicate M-2 of $C_{14}H_{20}N_2O_4$ and/or $C_{14}H_{18}N_2O_4$. These two compounds give the same fragmentation pattern. These findings suggested the degradation pathway and indicate the structure of the degradation product of TF, as illustrated in Figure 2.

Methods A and B

Derivative spectrophotometry has become a well-established technique for the assay of drugs in mixtures and pharmaceutical dosage forms (19). The zero-order spectra of TF and its degradation product show an overlap (Figure 3) that prevents the use of direct spectrophotometric analysis of the drug in the presence of its degradation product. In an attempt to resolve this overlap, a derivative method was applied. Upon examining the first-derivative spectra of the drug and its degradation (Figure 4), it was noticed that TF can be determined at 298 and at 332 nm, where the degradation product has no contribution and shows zero reading.

Linearities of the peak amplitudes of the relationship of the first derivative curves to the corresponding concentrations of the drug were examined at the selected wavelengths. The proposed method was valid in the range of 2–20 µg/mL, and the regression equations were:

$$^1D_{298} = 0.0456C + 0.0184$$
$$r = 0.9993$$

$$^1D_{332} = 0.0962C + 0.0288$$
$$r = 0.9991$$

where $^1D_{298}$ and $^1D_{332}$ are the peak amplitudes at 298 and 332 nm, respectively, C is the concentration of TF in µg/mL, and r is the correlation coefficient.

Upon examining the second-derivative spectra of the drug and its degradation (Figure 5), it was noticed that TF can be determined at 312 and 344 nm, where the degradation product has no contribution and shows zero reading. Linearities of the peak amplitudes of the relationship of the second derivative curves to the corresponding concentrations of the drug were examined at the selected wavelengths. The proposed method was valid in the range of 2–20 µg/mL, and the regression equations were:

$$^2D_{312} = 0.0007C + 0.0007$$
$$r = 0.9995$$

$$^2D_{344} = 0.0005C + 0.0001$$
$$r = 0.9993$$

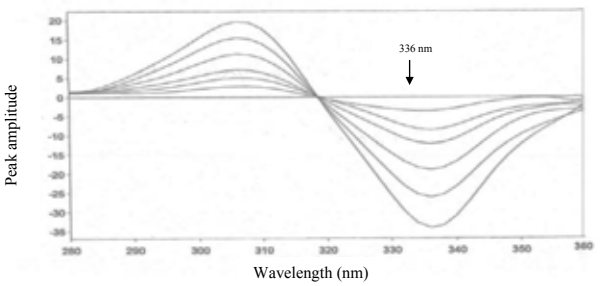

Figure 7. First order derivative ratio spectra of TF in methanol (2–20 µg/mL) using 2 µg/mL of its degradation product as a divisor.

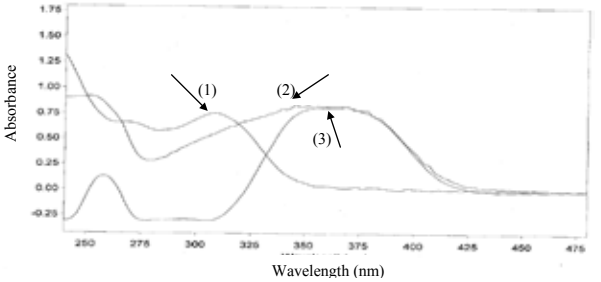

Figure 8. Zero order absorption spectra of intact TF in 1 M NaOH (1) and in 1 M HCl (2), each concentration 20 µg/mL, and their ΔA spectrum (3).

where $^2D_{312}$ and $^2D_{344}$ are the peak amplitudes at 312 and 344 nm, C is the concentration of TF in µg/mL, and r is the correlation coefficient.

The proposed method was successfully applied for determination of the drug in pure powder form with mean recoveries of 99.45 ± 0.287% and 100.28 ± 0.277% at 298 and 332 nm, respectively, in the case of ^1D, and 99.40 ± 0.245% and 99.50 ± 0.292% at 312 and 344 nm, respectively, in the case of ^2D (Table 1).

The derivative ratio spectra method is a successful and widely used application of derivative spectrophotometry for the resolution of some binary or ternary pharmaceutical mixtures (20). Therefore, derivative ratio spectrophotometry can also be applied. The zero-order absorption spectra of TF were divided by the spectrum of a 2 µg/mL solution of its degradation product. This gave the best compromise in terms of sensitivity, repeatability, and S/N. The first derivative of the ratio spectra at 336 nm with Δλ = 4 nm and scaling factor 10 ($^1DD_{336}$) were obtained. The peak amplitudes were measured at 336 nm (Figures 6 and 7).

The linearity between the concentrations of the drug and peak amplitudes at 336 nm ($^1DD_{336}$) was studied. Linear relationship was obtained in the range of 2–20 µg/mL TF; the regression equation was:

$$^1DD_{336} = 1.9017C + 0.0320$$
$$r = 0.9991$$

where $^1DD_{336}$ is the peak amplitude at 336 nm, C is the concentration of TF in µg/mL, and r is the correlation coefficient. The proposed method was successfully applied for

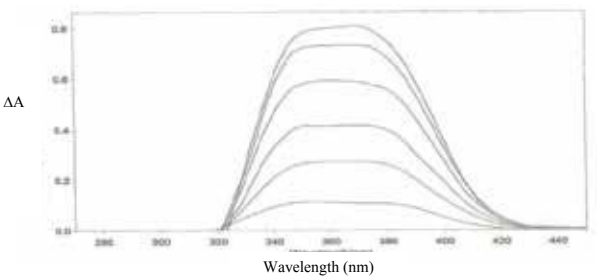

Figure 9. Difference absorption (ΔA) spectra of TF and its degradation product (2–20 µg/mL).

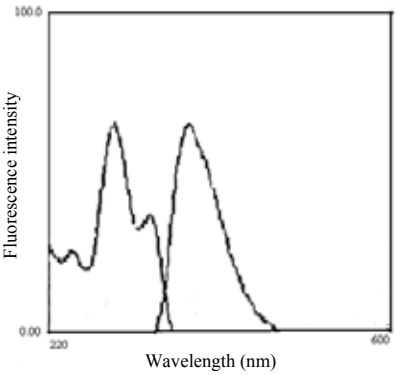

Figure 10. Excitation and emission spectra of TF degradate in methanol (0.6 µg/mL); λ_{max} excitation = 295 nm and λ_{max} emission = 383 nm.

the determination of the drug in pure powder form with mean recovery of 100.03 ± 0.522% at 336 nm (Table 1).

Method C

To study the effect of pH of the medium on TF and its degradate, the spectrum of each was recoded in 1 M NaOH and 1 M HCl in addition to their ΔA spectra between the acidic and alkaline media (Figure 8). From these spectral data it is clear that a bathochromic shift in the peak of the intact molecule takes place from 310 nm in NaOH to 366 nm in HCl together with a small hyperchromic effect. However, such a bathochromic shift was not observed in the spectrum of its degradate, which exhibits nearly the same absorption bands at 364 nm in both media at which their ΔA exhibits nearly zero absorbance.

Accordingly, the ΔA peak at 366 nm for intact TF between 1 M NaOH and 1 M HCl could be considered as the λ_{max} most suitable for adopting the ΔA technique for the selective determination of intact drug in the presence of its degradate; at this maximum, ΔA for the latter reads about zero. The ΔA method (intact drug in 1 M HCl versus intact drug in 1 M NaOH) has been applied for the determination of the drug in its pure powder form and in pharmaceutical dosage forms in the presence of its degradation product.

Linearity between the absorbance difference (ΔA) and the corresponding concentrations of the drug at 366 nm was examined (Figure 9). A linear relationship was obtained in the range of 2–20 µg/mL, and the regression equation was:

$$\Delta A = 0.0388C + 0.0347$$
$$r = 0.9998$$

where ΔA is the absorbance difference at 366 nm, C the concentration of TF in µg/mL and r the correlation coefficient. The proposed ΔA method was successfully applied for the determination of the drug in pure powder form with mean recovery of 100.20 ± 0.642% (Table 1).

Method D

Densitometry is a simple method of quantitation directly on a TLC plate by measuring the optical density of the separated spots (21). The proposed method is based on the difference in the R_f values between the intact drug and its degradation product.

Table 2. Results of analysis of TF in laboratory-prepared mixtures containing different ratios of TF and its degradation product in pure powder form by the first four methods

			Recovery, %						
			Concn, μg/mL						Concn, μg/band
			Method A				Method B	Method C	Method D
Degradation, %	TF	Degradation product	298 nm ^{1}D	332 nm ^{1}D	312 nm ^{2}D	344 nm ^{2}D	336 nm ^{1}DD	366 nm ΔA	
10	18	2	99.40	98.50	101.00	101.00	99.4	98.20	99.50
20	16	4	98.90	99.00	100.00	100.00	100.20	98.60	100.30
30	14	6	98.50	100.30	101.00	100.00	98.90	98.20	99.40
40	12	8	99.30	100.50	100.00	100.50	101.30	99.94	100.40
50	10	10	98.90	100.70	100.00	100.00	98.50	99.30	99.11
60	8	12	99.40	—	101.10	—	100.25	101.10	101.10
70	6	14	99.20	—	100.20	—	99.30	99.80	101.13
80	4	16	—	—	100.35	—	—	—	98.90
90	2	18	—	—	100.65	—	—	—	100.60
Mean			99.09	99.90	100.48	100.30	99.96	99.31	100.05
RSD, %			0.336	0.985	0.464	0.446	0.955	1.072	0.843

A sensitive, accurate, and precise densitometric TLC method is described for the quantitative determination of TF in bulk powder and in a dosage form in the presence of its degradation product.

The mobile phase was selected to achieve the best separation of the drug from its acid degradation product; other necessary conditions have also been established. Different mobile phases were tried; separation of TF from its degradation product was achieved using ethyl acetate–methanol–ammonium hydroxide 10% (8.5 : 1.0 : 0.5, by volume). The instrumental conditions for densitometric measurement, such as scan mode and wavelength of detection, were optimized. The scan mode chosen was zigzag, and the scanning wavelength was 315 nm. TF was completely resolved from its degradation product, and its R_f value was 0.73; the spot obtained from acid degradation had an R_f value of 0.94. This permitted quantitative determination of TF in the presence of its acid degradation product.

The relation between the concentration of TF and the integrated area under the scan peak of the bands was investigated. The linear relationship was tested, resulting in an r value of 0.9993 for the concentration range of 2–20 μg/band, with mean recovery of 98.98 ± 0.721% (Table 1).

Method E

Spectrofluorometric methods have an advantage over spectrophotometric methods because they offer much greater selectivity and sensitivity. Thus, procedures based upon fluorescence should be considered when measuring techniques are being chosen for small quantities of materials, e.g., in the analysis of trace impurities in drug substance or in unit dose assays of certain drugs such as alkaloids and steroids, which are administered at very low doses (22).

The method developed in the present investigation for the spectrofluorometric determination of TF via its degradate depends upon the native fluorescence of the methanolic solution of the degradate solution and the absence of fluorescence of

Table 3. Quantitative determination of TF in a pharmaceutical formulation by the proposed methods, and results of application of the standard addition technique

	Nodeprine tablets B.N. 21081							
	Method A				Method B			
	^{1}D		^{2}D		^{1}DD	Method C	Method D	Method E
	298 nm	332 nm	312 nm	344 nm	336 nm	ΔA	TLC	Fluorometry
Found, %[a]	100.61 ± 0.236	100.90 ± 0.566	98.59 ± 0.370	99.96 ± 1.433	100.63 ± 1.211	99.42 ± 0.558	99.83 ± 0.478	99.68 ± 0.880
Recovery of standard added, %[b]	100.80 ± 0.198	100.38 ± 0.303	99.83 ± 0.352	99.25 ± 0.423	99.88 ± 0.593	100.43 ± 0.592	100.69 ± 0.342	99.85 ± 1.004
	Nodeprine tablets B.N. 07137188 (expired batch)							
Found, %[c]	98.01 ± 0.211	97.90 ± 0.164	96.79 ± 0.099	97.45 ± 0.114	97.74 ± 0.371	97.62 ± 0.297	97.69 ± 0.401	—

[a] Average of five determinations.

[b] Average of three determinations.

[c] Average of three determinations (claimed amount 8 mg/mL in methods A, B, and C, and 8 mg/band in method D).

Table 4. Statistical analysis between the results obtained for the determination of TF in pure samples by the proposed methods and that obtained by the official method (Ref. 2)

	Method A				Method B				
	^{1}D		^{2}D		^{1}DD	Method C	Method D	Method E	Official method[a]
	298 nm	332 nm	312 nm	344 nm	336 nm	ΔA	TLC	Fluorometry	
Mean, %	99.45	100.28	99.40	99.50	100.03	100.20	98.98	100.25	99.60
SD	0.285	0.278	0.244	0.291	0.523	0.643	0.714	0.238	0.300
RSD, %	0.287	0.277	0.245	0.292	0.522	0.642	0.721	0.282	0.301
Variance	0.081	0.077	0.060	0.085	0.273	0.413	0.510	0.079	0.090
n	6	6	6	6	6	6	6	8	5
Student's *t*	0.371 (2.262)[b]	2.093 (2.262)	0.637 (2.262)	0.304 (2.262)	1.090 (2.262)	0.943 (2.262)	1.405 (2.262)	2.117 (2.201)	
F test	1.111 (5.19)	1.169 (5.19)	1.500 (5.19)	1.059 (5.19)	3.033 (6.26)	4.589 (6.26)	5.667 (6.26)	1.139 (4.12)	

[a] Non aqueous titration method.

[b] Figures in parentheses are the corresponding tabulated values at $P = 0.05$.

the drug solution. Figure 10 shows the excitation and emission maxima of TF degradate in methanol; the degradate shows a maximum emission at 383 nm when excited at 295 nm, while a similar concentration of TF has no fluorescence emission at the same excitation wavelength. This permits the determination of TF intact molecule via its TF degradate.

The fluorescence intensity of different concentrations of TF degradate in methanol was recorded against methanol as a blank, using 295 and 383 nm as excitation and emission wavelengths, respectively. The fluorescence intensity was a linear function of the concentration of TF degradate in the range of 0.2–1.6 μg/mL; the regression equation was:

$$I_f = 116.79\ C + 2.7500$$
$$r = 0.9996$$

where I_f is the fluorescence intensity, C the concentration of TF degradate in μg/mL and r the correlation coefficient. The proposed spectrofluorometric method was successfully applied for the determination of the drug via its degradate with mean recovery of 100.25 ± 0.282% (Table 1).

Several factors affecting the fluorescence intensity were studied, and it was found that using methanol as the solvent and removing the surfactant gave satisfactory results. Stability of the fluorescence intensity was also studied, and it was found that the intensity of the fluorescence was stable up to 1 h.

The specificity of methods A–D was proved by analysis of a laboratory-prepared mixture containing different percentages of the degradation product. In method A, ^{1}D was specific for TF in presence of up to 50 and 70% of its degradation product at 298 and 332 nm, respectively, while ^{2}D at 312 nm was specific in presence of up to 90% of its degradation product but only up to 50% at 344 nm. Methods B and C were specific for TF in presence of up to 70% of its degradation product. Specificity of method D was achieved in presence of up to 90% of its degradate (Table 2).

The usefulness of the five proposed methods for the analysis of TF in a pharmaceutical preparation was studied by assaying Nodeprine tablets (Table 3). The standard addition technique was applied to assess the validity of the proposed methods (Table 3). Also, the four proposed stability-indicating methods A–D have been successfully applied to assay TF in Nodeprine expired tablets in which very minor degradation had occurred (Table 3).

Results obtained by the proposed methods for the determination of pure samples of the drug were statistically compared to those obtained by the official method (2) of the drug. No significant difference was observed (Table 4).

Linearity of each method was assessed by determination of the same concentration range as the calibration graphs. The mean accuracies are given in Table 1.

The repeatability and interday precision were evaluated by assaying freshly prepared solutions of the drug in triplicate on the same day and on 3 successive days, respectively, at concentrations within the linearity range for the five proposed methods. RSD results (Table 1) showed the precision of the methods.

Validation of methods A–D was performed by measuring range, accuracy, precision, repeatability, interday precision, linearity, and specificity. Validation of method E was done using all the previously mentioned parameters, except specificity. Results obtained are given in Tables 1 and 2. These data indicate the applicability of the proposed methods for the QC of the drug formulations.

The stability of TF in methanol was determined by keeping one sample in a refrigerator and another in a tightly capped volumetric flask at ambient temperature under normal lighting conditions. The samples were assayed over 3 successive days of storage and compared with freshly prepared sample by the proposed methods. The RSD values of the assay were below 2.0% in both cases, indicating that TF is stable in the solution.

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