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Stability-indicating spectrophotometric and spectrofluorimetric methods for determination of alfuzosin hydrochloride in the presence of its degradation products

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Validated stability-indicating spectrophotometric and spectrofluorimetric assays (SIAMs) were developed for the determination of alfuzosin hydrochloride (ALF) in the presence of its oxidative, acid, and alkaline degradation products. Three spectrophotometric methods were suggested for the determination of ALF in the presence of its oxidative degradation product; these included the use of zero order (^{0}D), first order (^{1}D), and third order (^{3}D) spectra. The absorbance was measured at 330.8 nm for (^{0}D) method, while the amplitude of first derivative (^{1}D) method and that of third derivative (^{3}D) method were measured at 354.0 and 241.2 nm, respectively. The linearity ranges were 1.0–40.0 μg/ml for (^{0}D) and (^{1}D) methods, and 1.0–10.0 μg/ml for (^{3}D) method. Two spectrofluorimetric methods were developed, one for determination of ALF in the presence of its oxidative degradation product and the other for its determination in the presence of its acid or alkaline degradation products. The first method was based on measuring the native fluorescence of ALF in deionized water using $\lambda_{excitation}$ 325.0 nm and $\lambda_{emission}$ 390.0 nm. The linearity range was 50.0–750.0 ng/ml. This method was also used to determine ALF in human plasma with the aid of a suggested solid phase extraction method. The second method was used for determination of ALF via its acid degradation product. The method was based on the reaction of fluorescamine with the primary aliphatic amine group produced on the degradation product moiety. The reaction product was determined spectrofluorimetrically using $\lambda_{excitation}$ 380.0 nm and $\lambda_{emission}$ 465.0 nm. The linearity range was 100.0–900.0 ng/ml. All methods were validated according to the International Conference on Harmonization (ICH) guidelines, and applied to bulk powder and pharmaceutical formulations.

1. Introduction

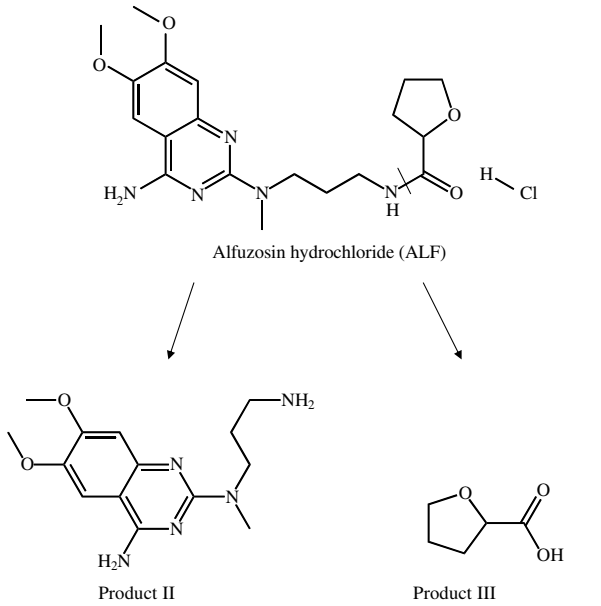
Alfuzosin hydrochloride (ALF), *N*-{3-[4-amino-6,7-dimethoxyquinazolin-2-yl) (methyl)amino]propyl}tetrahydro-2-furancarboxamide hydrochloride, belongs to the class of second generation alpha$_1$-adrenoceptor antagonists. This group of drugs is used for the treatment of benign prostatic hyperplasia (BPH). The pharmaco-therapeutic effect is achieved by blockade of the alpha$_1$-adrenergic receptors which causes relaxation of the smooth muscles in the bladder neck, allowing urine to flow through the prostate, and decreasing the symptoms of BPH (Rhodes et al. 1999; Barry et al. 2001; Ahtoy et al. 2002).

Reversed phase HPLC methods have been suggested for determination of ALF in plasma using fluorescence (Guinebault et al. 1986; Carlucci et al. 1994; Yao et al. 2002), or MS (Weisner et al. 2003) detection. Electro-chemical oxidation of ALF has been investigated by various voltammetric methods (Uslu 2002).

The International Conference on Harmonization document on "Stability testing of new drug substances and products" requires stress testing to be carried out to elucidate the inherent stability characteristics of the active substance (ICH 1993), and oxidative, acid and alkaline stability studies are required. In previous work in our laboratory, validated HPLC and HPTLC stability-indicating assay methods were developed to determine ALF in the presence of its degradation products (Fayed et al. 2006). No spectrophotometric or spectrofluorimetric methods are mentioned in the literature for determination of ALF either alone or in the presence of its degradation products.

The aim of this work is to follow the oxidative degradation of ALF under accelerated and stressed conditions according to the ICH guidelines, and develop stability-indicating spectrophotometric and spectrofluorimetric methods for determination of ALF in the presence of its oxidative degradation product. In addition, a spectrofluorimetric stability-indicating method is proposed for determination of ALF via its acid or alkaline degradation products. The methods are alternatives and comparable in specificity and accuracy to chromatographic methods, which although highly specific and accurate, are more time consuming, are performed in several steps, use environmentally hazardous solvents, and are rather expensive.

Scheme 1



Structures of alfuzosin HCl, and its alkaline and/or acid degradation products

2. Investigations, results and discussion

In a previous study, ALF (Scheme 1) was subjected to different stress conditions, namely alkaline, acid, oxidative, thermal, and photo-degradation. To study the effect of acids and alkalis, ALF was heated with different concentrations of hydrochloric acid and sodium hydroxide. The products of hydrolysis were the same and were identified by FT-IR and NMR. The hydrolysis pathway was suggested to be via hydrolysis of the amide group with the production of a primary aliphatic amine product and a carboxylic acid product. It was also observed that the drug underwent oxidation upon refluxing with 3.0% H_2O_2, while it showed stability when heated for 6 h in a dry oven or on exposure to sunlight (Fayed et al. 2006).

In this work, validated, simple, sensitive, and rapid spectrophotometric and spectrofluorimetric methods are proposed for the determination of ALF in the presence of its oxidative degradation product. Also a spectrofluorimetric SIAM is proposed for determination of ALF in the presence of its acid or alkaline degradation products.

Oxidative degradation of ALF was performed under accelerated conditions by incubating ALF with 3.0% H_2O_2 at room temperature for 35 d; and it was also performed under stress conditions by refluxing ALF with 3.0% H_2O_2 at 100 °C. The oxidation was monitored by HPLC using the previously suggested method (Fayed et al. 2006). One main peak was produced within the first 5 d at 2.8 min, while that of the intact drug was at 10.9 min and the degradation percentage was 7.3%, it reached 33.0% after 35 d.

(Chromatographic conditions were: Xettra RP^{TM} C_{18} column; mobile phase acetonitrile/phosphate buffer (pH 3.5) in a ratio of 20 : 80 (v/v); flow rate 1.0 ml/min and detection at 247 nm). In order to develop SIAMs, stressed oxidation was performed by refluxing with 3.0% H_2O_2 for 2 h such that the peak corresponding to the intact drug completely disappeared. The solution obtained from oxidative degradation was subjected to ESI-MS, where one oxidative degradation product (I) was identified which was apparently that which separated at 2.9 min in the HPLC

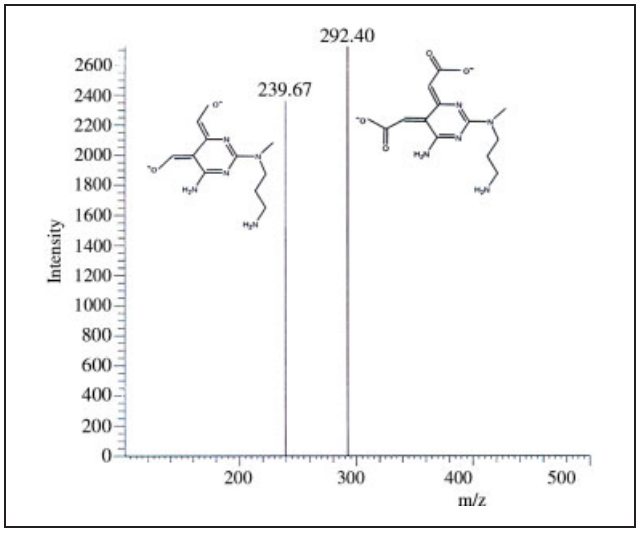


Fig. 1: ESI-MS of alfuzosin oxidative degradation product

method. The negative-ion ESI-MS spectrum (Fig. 1) of (**I**) showed a deprotonated molecular ion at m/z 292 and another ion fragment at m/z 239. The suggested molecular structure of (I) and the second fragment formed are also shown in Fig. 1. The oxidative degradation pathway is proposed to be through demethoxylation with the quinazoline ring opening and the formation of a dicarboxylate product accompanied by cleavage of the amide bond. The

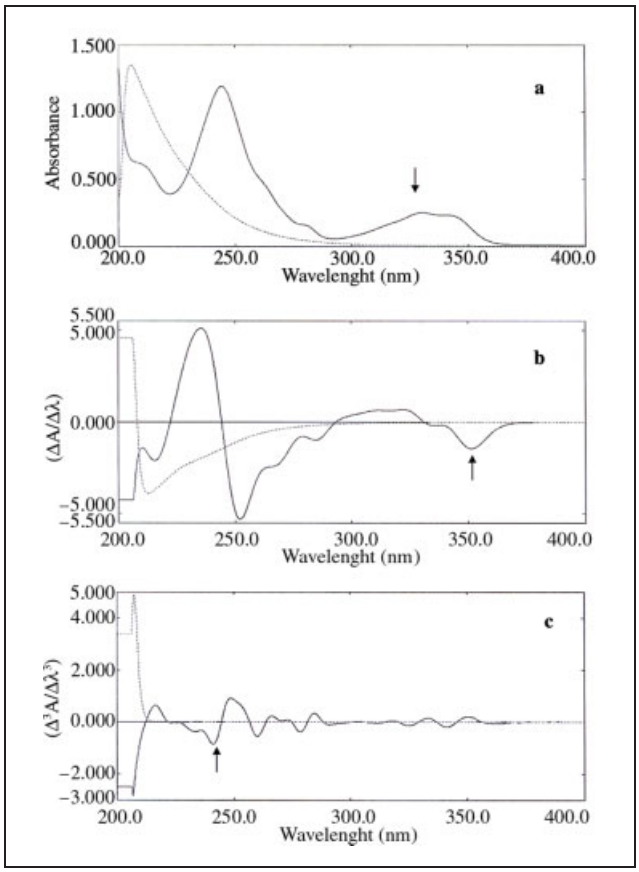


Fig. 2: (a) Zero order absorption (^{0}D) spectra of alfuzosin HCl 10 μg/ml (——) and its oxidative degradation product 10 μg/ml (-----)
(b) First derivative (^{1}D) spectra of alfuzosin HCl 10 μg/ml (——) and its oxidative degradation product 10 μg/ml (-----)
(c) Third derivative (^{3}D) spectra of alfuzosin HCl 10 μg/ml (——) and its oxidative degradation product 10 μg/ml (-----)

Table 1: Results for laboratory prepared mixtures of alfuzosin hydrochloride with its degradation products by the proposed spectrophotometric and spectrofluorimetric methods

Sample No.	(%) of oxidative degradate	Recovery (%) of ALF					
		Methods				Fluorescamine	
		^{0}D	^{1}D	^{3}D	Native fluorescence	(%) of acid degradate	Recovery (%) of ALF
1	10	102.10	102.29	99.21	101.36	10	100.62
2	20	100.75	98.32	99.17	101.33	20	101.34
3	30	101.42	101.97	100.97	100.97	30	98.62
4	50	98.88	100.00	102.33	100.01	50	102.65
5	70	101.71	100.46	99.07	101.66	70	102.28
6	80	101.00	99.25	98.15	99.76	80	102.10
7	90	115.02*	99.58	98.15	100.26	90	114.94*
Mean		100.98	100.26	99.58	100.76		101.26
%RSD		1.13	1.42	1.53	0.73		1.46

* rejected

fragment obtained at m/z 239 indicates the elimination of the two carbonyl groups of the two carboxylate groups.

The suggested spectrophotometric SIAMs were based on direct measurement of ALF at 330.8 nm, as the absorption of (**I**) is negligible at this λ (Fig. 2). A linear correlation was obtained in the concentration range of 1.0–40.0 μg/ml. To assess the validity for determination of ALF in the presence of (**I**), mixtures in solution were prepared with different ratios. ALF could be determined in the presence of up to 80% of (**I**) (Table 1). To increase the selectivity of the method, ^{1}D and ^{3}D methods were developed. Different parameters were studied to achieve the best selectivity and correlation, namely Δλ, smoothing and choice of zero-crossing analytical λ. The best results were obtained when using $\Delta\lambda = 8$ nm without smoothing for both ^{1}D and ^{3}D methods, while amplitudes of 354.0 nm and 241.2 nm [zero-crossing for (**I**)] gave the best results (Fig. 2). The linearity ranges were 1.0–40.0 μg/ml and 1.0–10.0 μg/ml for the ^{1}D and ^{3}D methods, respectively. Both methods could determine ALF in the presence of up to 90% of (**I**) (Table 1).

A spectrofluorimetric SIAM was also suggested depending on measuring the native fluorescence of ALF; (**I**), on the other hand, did not show any fluorescence characteristics. Scans of the excitation and emission spectra of ALF showed $\lambda_{excitation}$ at 325.0 nm and $\lambda_{emission}$ at 390.0 nm in deionized water (Fig. 3). The effects of different solvents, namely, 0.1 M NaOH, 0.05 M H_2SO_4, acetonitrile, methanol, and water, were studied. The highest intensity was obtained when using water or methanol as the diluting solvent. A linear correlation was obtained between fluorescence intensity (I_f) and concentration in the range of 50.0–750.0 ng/ml. To assess the validity and specificity of the method as a SIAM, solution mixtures of ALF with (**I**) were prepared. ALF could be determined up to 90% of (**I**) (Table 1). The high sensitivity of the method allowed its use for determination of ALF in plasma which was performed after a suggested solid phase extraction method (SPE).

During development of the SPE method, different washing and eluting systems were tried. When using acetonitrile, no elution occurred; while by using methanol slightly alkalinized using ammonia, turbidity of the sample solutions was observed. The best recovery was obtained by using 2.0 ml of methanol twice in succession for elution.

Reviewing the C_{max} (maximum plasma concentration of ALF on drug administration) (Martindale 34th Ed. 2005) showed that the method could be applied for bioavailability studies, as SPE minimized the plasma background and

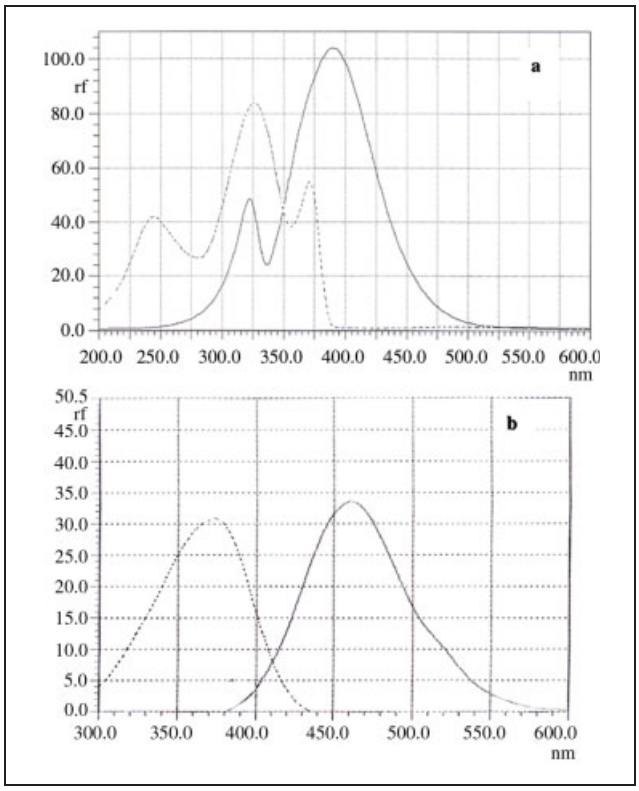


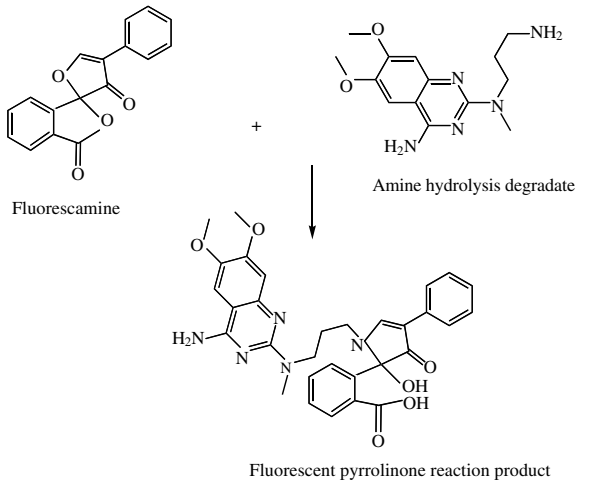
Fig. 3: (a) Excitation (-----) and emission (——) curves of alfuzosin HCl 400 μg/ml
(b) Excitation (-----) and emission (——) curves of fluorescamine-alfuzosin hydrolysis degradation product 400 μg/ml

Table 2: Results of alfuzosin hydrochloride assay in human plasma using the proposed native fluorescence method

Added (ng/ml)	Found (ng/ml)	Recovery (%)*
50	50.39	100.78
100	100.81	100.81
150	148.80	99.20
200	205.68	102.84
250	256.00	102.40
300	303.60	101.20
400	408.24	102.06
Mean		101.32
SD		1.22
RSD%		1.20

* Mean of three separate determinations

Scheme 2



Suggested reaction mechanism of fluorescamine and alfuzosin hydrolysis degradation product

matrix interference, where blank plasma recorded fluorescence intensity in the range of 0.1–0.4. Good results were obtained when applying the method to spiked plasma as shown by the % recovery and relative standard deviation % RSD presented in Table 2.

On the other hand, a SIAM was developed for determination of ALF via reaction of its acid or alkaline degradation amine product (Scheme 1, **II**) with fluorescamine. Fluorescamine is a non-fluorescent compound that reacts with primary amines to produce a fluorescent compound (Bernardo et al. 1974). It has been used for determination of procainamide (Henry et al. 1975), amphetamines (Klein et al. 1986), and procaine (Cerretero et al. 1999). However, some aromatic amines such as 2-aminopyridine or p-aminobenzoine do not undergo the mentioned reaction (Baeye et al. 1991). Both ALF and its hydrolysis degradation product contain primary aromatic amine. In addition, the degradation product also contains primary aliphatic amine. Due to the similarity of the aromatic amino group present in ALF and its amine hydrolysis product (**II**) to that found in 2-aminopyridine (attached to the methylene group adjacent to the cyclic N-atom in the ring) it was expected that they would not react with fluorescamine, while the aliphatic group present in the degradation product will react. Scanning of the excitation and emission curves of the reaction product of fluorescamine with (**II**) showed $\lambda_{excitation}$ at 380.0 nm and $\lambda_{emission}$ at 465.0 nm, where as ALF did not show any emission at this wavelength. The experimental conditions, namely pH, buffer type and volume, and diluting solvent, were studied. The best results were obtained upon using 2.0 ml universal buffer (pH 7.5), water as the diluting solvent, and 1.0 ml of 0.1 % fluorescamine. The reaction was instantaneous and the product remained stable for at least 3 h. The stoichiometry of the reaction was determined by the limiting logarithm method (Rose 1964) where a 1 : 1 ratio was obtained. A suggested reaction in accordance with the general reaction of fluorescamine with primary amines is shown in Scheme 2. To assess the validity and specificity of this SIAM, solution mixtures of ALF and (**II**) were prepared in different ratios. Up to 80% of (**II**) could be determined (Table 1). The method was applied to the determination of ALF in dosage forms by first subjecting it to acid hydrolysis then carrying out the assay while using the same amount of intact drug without hydrolysis in the blank so that the difference in fluorescence intensity obtained will be equivalent to the ALF in the dosage form.

The five suggested SIAMs were validated according to the ICH guidelines (ICH 1996) and the results are shown in Table 3. The range, linearity, and regression data for the calibration curves (n = 8) of all the suggested methods showed good linear relationships as shown by the correlation coefficients. Descriptive statistics of the regression showed low values of standard deviation of residuals ($S_{x/y}$) and standard error of the intercept (SE[a]) and slope (SE[b]) which indicated high accuracy with minimum deviations and low scattering of the calibration point (Miller and Miller 2000) (Table 3). The accuracy of the methods was tested by analyzing freshly prepared solutions of the intact drug in triplicate at concentrations of 10.0, 20.0, and 30.0 μg/ml for the ^{0}D method; and of 5.0, 15.0, and 25.0 μg/ml for the ^{1}D method; and of 3.0, 5.0, and 7.0 μg/ml for the ^{3}D method. In addition, analyses were performed at concentrations of 200.0, 400.0, and 600.0 ng/ml for the native fluorescence method; and of 300.0, 500.0, and

Table 3: Regression equation parameters and validation of the proposed spectrophotometric and spectrofluorimetric methods for the determination of alfuzosin hydrochloride

Parameters	Method				
	^{0}D	^{1}D	^{3}D	Native fluorescence	Fluorescamine
Linearity range	1–40 μg/ml	1–40 μg/ml	1–10 μg/ml	50–750 ng/ml	100–900 ng/ml
Intercept (a)	0.0025	−0.0374	−0.0070	1.6159	16.778
SE[a]	0.0018	0.0088	0.0034	0.5310	0.3424
Slope (b)	0.0248	0.1405	0.0891	0.2479	0.1333
SE[b]	0.0001	0.0005	0.0006	0.0011	0.0006
Correlation coefficient (r)	0.9999	0.9999	0.9999	0.9999	0.9999
Accuracy*	99.84 ± 0.84	100.03 ± 1.23	99.85 ± 1.12	99.91 ± 0.87	100.33 ± 0.82
Precision					
Repeatability*	99.74 ± 0.65	100.75 ± 1.48	100.18 ± 0.92	100.50 ± 1.12	100.24 ± 0.45
Intermediate precision*	99.73 ± 0.59	99.84 ± 1.08	99.51 ± 1.28	100.73 ± 0.47	100.12 ± 1.29
LOD**	0.07 μg/ml	0.09 μg/ml	0.03 μg/ml	1.60 ng/ml	9.04 ng/ml
LOQ**	0.22 μg/ml	0.30 μg/ml	0.08 μg /ml	4.86 ng/ml	27.39 ng/ml

Linear equation for all methods: y = b x + a; where y = response and x = concentration
[a] Standard error of intercept
[b] Standard error of slope
* Mean % recovery ± %RSD (n = 9)
** LOD = (SD of intercept/Slope) · 3.3; LOQ = (SD of the intercept/Slope) · 10

Table 4: Determination of alfuzosin hydrochloride in pharmaceutical dosage forms using the proposed spectrophotometric and spectrofluorimetric methods

Pharmaceutical formulation	Recovery (%) ± SD of the claimed mean (mg/tablet)*					
	^{0}D	^{1}D	^{3}D	Native fluorescence	Fluorescamine	HPLC
Xetral® SR tablets (Batch no: 117901[B]) (labeled content 5mg/tablet)	99.70 ± 1.69	99.51 ± 1.72	99.87 ± 1.45	100.09 ± 0.78	99.83 ± 1.17	100.37 ±1.25
V (Variance)	2.853	2.952	2.103	0.517	1.49	1.562
t (2.776)**	0.47	0.033	0.412	0.974	0.33	
F (9.276) **	1.05	1.913	1.350	3.021	2.89	

* n = 4 tablets each mean of two experiments
** Values between parentheses are critical "t" and "F" at p = 0.05

Table 5: Statistical comparison of accuracy of the proposed spectrophotometric and spectrofluorimetric methods and a reported method* for determination of alfuzosin hydrochloride**

Comparison items	Method					
	^{0}D	^{1}D	^{3}D	Native fluorescence	Fluorescamine	HPLC
Mean*	99.84	100.03	99.85	99.91	100.33	101.15
SD	0.84	1.23	1.13	0.87	0.83	1.10
RSD%	0.84	1.23	1.12	0.87	0.82	1.08
V (Variance)	0.71	1.50	1.23	0.76	0.69	1.22
t (2.13)**	0.83	0.16	0.65	0.65	0.76	
F (3.44)**	1.72	1.24	1.04	1.60	1.77	

* n = 9
** Values between parentheses are critical "t" and "F" at p = 0.05
*** Fayed et al. 2006

700.0 ng/ml for the fluorescamine method. The % recovery and %RSD revealed excellent accuracy. The four methods suggested for determination of ALF in the presence of (II) were compared statistically using one way ANOVA. The data showed that there was no significant difference between them where the F value was (0.067) less than the tabulated one (2.9) (p = 0.976). The intra- and interday precision was evaluated by assaying freshly prepared solutions in triplicate on the same day and on three successive days, respectively. Percent Recovery and %RSD showed the precision and ruggedness of the methods. The selectivity of the methods was tested by analyzing different mixtures of ALF and its degradation product (Table 1). Satisfactory results were obtained, indicating the high selectivity of the methods.

The ICH guidelines (ICH 1996) for calculation of LOD and LOQ were followed, where the method based on the standard deviation of the response and the slope using the calibration curve was followed (Table 3).

The five proposed methods were applied to the determination of ALF in commercial tablets. The results shown in Table 4 were satisfactory and in good agreement with the labeled amount. To assess the validity of the methods, the tablets were analyzed by HPLC (Fayed et al. 2006), and statistical comparison of the results showed that with 95% confidence there were no significant difference regarding accuracy and precision between the proposed methods and that of the HPLC method (Table 5). As a conclusion, the methods are suitable for quality control of the drug.

3. Experimental

3.1. Instrumentation

A "La-Chrom" HPLC instrument (HITACHI- MERCK, Germany), was used for HPLC analysis, under conditions as described by Fayed et al. (2006).

The oxidative degradation product was identified by Electrospray-Mass Spectrometry (ESI-MS). The analysis was carried out on a Thermo-Finnegan ion trap quadruple mass spectrometer, Model Advantage Max (USA-UK), with data acquired in the flow ionization analysis (FIA) mode using a Finnegan surveyor LC pump. The capillary temperature was kept at 267 °C. Ion spray voltage was 4.52 kV and the current was 22.70 μA. The capillary voltage was maintained at 10.76 V for negative-ion analysis. Nitrogen sheath gas flow rate was 79.4 L/h. Helium auxiliary sweep gas flow rate was 19.3 L/h. The X-calibur Finnegan "LCQ" advantage version 1.3 SRI SP3 software provided with the instrument and running under Windows NT was used for instrument control and data acquisition. The aqueous solution of the oxidative degradation product was analyzed by FIA-ESI-MS in negative mode.

A double-beam UV-visible spectrophotometer (Shimadzu, Japan) Model UV-1601 connected to an IBM compatible PC and HP 800 inkjet printer was used. The bundled software was UVPC personal spectroscopy software version 3.7. The spectral bandwidth was 2 nm and wavelength-scanning speed was 2800 nm/min.

A Model SFM25 spectrofluorimeter (BIO-TEK Kontron, Switzerland) connected with an IBM compatible PC and HP 1020 Laserjet printer was used. The bundled software was WIND25 personal spectroscopy software.

Solid phase extraction (SPE) was done using a Chromabond® vacuum manifold fitted with Chromabond® C_{18} columns 3 ml/500 mg and a Laboport® (N811KN.18) vacuum pump [KNF (GmbH), Machery-Nagel, Germany].

A pH-meter (Jenway 3505, UK), equipped with combined glass electrode, was used for pH measurements.

3.2. Materials and reagents

Alfuzosin hydrochloride was supplied by FARMAK S.A., Czech Republic and certified to have a purity of 100.70%; The commercial Xetral® SR tablets (Batch no: 117901[B]) used were manufactured by Amryia Company for Pharmaceutical Industries, Alexandria, Egypt under license of Laboratories Synthelabo-Synthelabo Groupe, Paris, France and labeled to contain 5 mg alfuzosin HCl per tablet. Plasma was purchased from JMS Singapore Ltd., Singapore, Batch no.: 05091002. Fluorescamine was from Aldrich, Germany, Lot no.: 60307-09525AE. All other materials were of HPLC and analytical reagent grades.

Fluorescamine solution was prepared at a 0.1 % concentration in acetone and was left for at least 24 h for aging before use. Universal buffer solutions having different pH values were prepared according to Lurie (1978). Phosphate buffer solutions of pH 3 and 6 were prepared by adjusting the

pH of 0.05 M KH_2PO_4 either with 0.2 M NaOH or 0.5 M H_3PO_4; all buffer solutions were checked by pH meter.

3.3. Standard solutions

A standard stock solution of ALF (400.0 μg/ml) in water was prepared. Working standard solutions of 40.0 μg/ml for spectrophotometry and 4.0 μg/ml for spectrofluorimetry were prepared by appropriate dilution with deionized water.

A standard solution of the oxidative degradation product was prepared by refluxing 20 mg of ALF powder with 20.0 ml of 3.0% H_2O_2 for 2 h. The solution was then aerated by carbon dioxide at 60 °C for 1 h to remove oxygen and decompose surplus H_2O_2. Finally, the solution was transferred into a 50-ml volumetric flask and the volume was made up with water to obtain a concentration of 400.0 μg/ml. Complete oxidative degradation was checked by the HPLC method (Fayed et al. 2006). Working standard solutions of 40.0 μg/ml for spectrophotometry and 4.0 μg/ml for spectrofluorimetry were prepared by appropriate dilution with deionized water.

A standard solution of the acid degradation product was prepared by refluxing 20 mg of ALF powder with 20.0 ml of 2.0 M HCl for $2\frac{1}{2}$ h; the solution was neutralized by a calculated volume of 2.0 M NaOH, then transferred into a 50-ml volumetric flask and the volume made up with water to obtain a concentration of 400.0 μg/ml. The solution was checked for complete degradation by HPLC (Fayed et al. 2006). 4.0 μg/ml solutions for spectrofluorimetry were prepared by appropriate dilution with deionized water.

3.4. Construction of calibration curves

3.4.1. Calibration curves for ^0D, ^1D, and ^3D spectrophotometric methods

Aliquots of ALF working standard solution were transferred to a series of 10-ml volumetric flasks, and diluted to volume with water to obtain solutions in the concentration range of 1.0–40.0 μg/ml for the ^0D and ^1D methods and 1.0–10.0 μg/ml for the ^3D method. The absorption spectra of the solutions were scanned in the range of 200.0–400.0 nm. ^1D curves were computed at $\Delta\lambda = 8$ nm using 100 as scaling factor; and ^3D curves at $\Delta\lambda = 8$ nm and scaling factor of 400. The absorbance value of ^0D at 330.8 nm, the amplitude of ^1D at 354.0 nm, and the amplitude of ^3D at 241.2 nm were recorded. Calibration curves for each method were constructed by plotting values of each of ^0D, ^1D, and ^3D *versus* concentration, and the regression equations were computed.

3.4.2. Calibration curve for spectrofluorimetric method using native fluorescence

Aliquots of ALF working standard solution 4.0 μg/ml were transferred to a series of 10-ml volumetric flasks, and diluted to volume with water to obtain solutions in the concentration range of 50.0–750.0 ng/ml. The fluorescence intensity (I_f) was recorded using $\lambda_{excitation}$ 325.0 nm and $\lambda_{emission}$ 390.0 nm. A plot of I_f *versus* concentration was constructed and the regression equation was computed.

2.4.3. Calibration curve for spectrofluorimetric method using fluorescamine

Aliquots of ALF acid degradate working standard solution 4.0 μg/ml were transferred to a series of 10-ml volumetric flasks, and 2.0 ml of universal buffer (pH 7.5) followed by 1.0 ml fluorescamine (0.1%) were added. Solutions were shaken for 5 min and volumes were completed with water to obtain a concentration range of 100.0–900.0 ng/ml. The I_f was recorded using $\lambda_{excitation}$ 380.0 nm and $\lambda_{emission}$ 465.0 nm. The I_f values were plotted against concentration and the regression equation was computed.

3.5. Assay of the pharmaceutical formulation

Twenty Xetral® SR tablets were accurately weighed and finely powdered. An accurately weighed amount of the powdered tablets equivalent to four tablets was transferred to a 50-ml volumetric flask, to which 35.0 ml methanol was added. The sample was sonicated for 30 min, made up to volume with methanol, and filtered. Solutions in the calibration ranges were prepared by dilution with water and used for determination of the drug by the proposed methods as described under calibration curve.

3.6. SPE and assay of ALF in plasma

1.0 ml of human plasma in a series of Wassermann tubes, was spiked with different concentrations of ALF to provide final concentrations of 50.0–400.0 ng/ml. 0.5 ml of 0.05 M KH_2PO_4 buffer (pH 6) was added to the samples, and vortexed for 10 s. C_{18} 3 ml/500 mg columns were fitted to the SPE manifold. The columns were conditioned by 2.0 ml methanol followed by 0.5 ml of 0.05 M KH_2PO_4 buffer (pH 6) without vacuum. Samples were then applied under vacuum at a rate of 1.0 ml/min. The washing step was carried out with 2.0 ml followed by 1.0 ml of 0.05 M KH_2PO_4 buffer (pH 6). Elution was carried out using 2.0 ml of methanol two successive times. The methanolic solutions were transferred into a series of 10-ml volumetric flasks and the volume was made up with water. The I_f was then recorded as described under calibration curve.

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