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Fesoterodine stress degradation behavior by liquid chromatography coupled to ultraviolet detection and electrospray ionization mass spectrometry

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ABSTRACT

In the present study, a rapid validated stability-indicating LC method was established and comprehensive stress testing of fesoterodine was carried out according to ICH guidelines. Fesoterodine was subjected to stress conditions of acid and basic hydrolysis, oxidation, photolysis and thermal decomposition. The degradation products formed under stress conditions were investigated by LC-UV and LC-ESI-MS. Successful separation of the drug from its degradation products was achieved on a monolithic C_{18} column (100 mm × 4.6 mm i.d.) maintained at 45 °C using acetonitrile-methanol-0.03 mol L^{-1} ammonium acetate (pH 3.8) (30:15:55, v/v/v) as the mobile phase. The flow rate was 2.4 mL min^{-1} and the detection wavelength was 208 nm. Validation parameters such as specificity, linearity, precision, accuracy, and robustness were evaluated. Chromatographic separation was obtained within 2.5 min and it was suitable for high-throughput analysis. Fragmentation patterns of degradation products formed under different stress conditions were studied and characterized through LC-ESI-MS fragmentation. Based on the results, a drug degradation pathway was proposed, and the validated LC method was successfully applied to the quantitative analysis of fesoterodine in tablet dosage forms, helping to improve quality control and to assure therapeutic efficacy.

1. Indroduction

Liquid chromatography (LC) is usually the analytical method of choice for pharmaceutical quality control [1]. As a result of the ever increasing need for speed and efficient use of time in the pharmaceutical industry, it is currently necessary to develop faster high throughput analytical procedures [2,3]. Different strategies can be implemented in order to perform faster procedures, while maintaining acceptable chromatographic performance [4–6]. A very interesting alternative to particulate-based LC columns are monolithic columns. Monolithic columns prepared from organic and silica monomers can potentially provide a high performance close to that of ultra high-performance LC or capillary electrophoresis by using common LC instrumentation [7–9].

The parent drug stability test guideline Q1A (R2) issued by the International Conference on Harmonization (ICH) [10] suggests that stress studies should be carried out on a drug to establish its inherent stability characteristics, leading to identification of degradation products and, hence to better understanding of the drug molecule stability. It also requires that analytical test procedures for stability samples should be stability-indicating and fully validated [11].

Traditional methods involving process scale-up, isolation, and purification of trace components such as degradants and impurities are expensive and time-consuming. Besides, the identification of the chemical structures of the compounds is not possible by LC alone. Therefore, the use of LC coupled to an electrospray ionization mass spectrometry (LC-ESI-MS) profiling method is an attractive alternative to rapidly characterize degradation products and impurities [12].

Overactive bladder (OAB) syndrome is defined as urinary urgency, with or without urgency urinary incontinence, usually accompanied by increased micturition frequency and nocturia [13]. The condition is chronic with an overall prevalence of approximately 12% in the general population [14]. Fesoterodine (Fig. 1), chemically described as 2-[(1R)-3-(diisopropylamino)-1-phenylpropyl]-4-(hydroxymethyl)phenyl isobutyrate, is a novel nonselective oral antimuscarinic agent that acts functionally as a prodrug. It is rapidly and extensively hydrolyzed by nonspecific esterases to the active metabolite 5-hydroxymethyltolterodine (5-HMT) [15–18]. Fesoterodine is an ester which is susceptible to substantial degradation after administration *in vivo* as well as during storage under stress conditions, e.g., in a humid environment and at high temperatures. It is believed that hydrolyzation and oxidation are among the major mechanisms resulting in degradation.

LC coupled to tandem mass spectrometry methods for the quantitative determination of 5-HMT in pharmacokinetic studies of fesoterodine in healthy volunteers has been reported using

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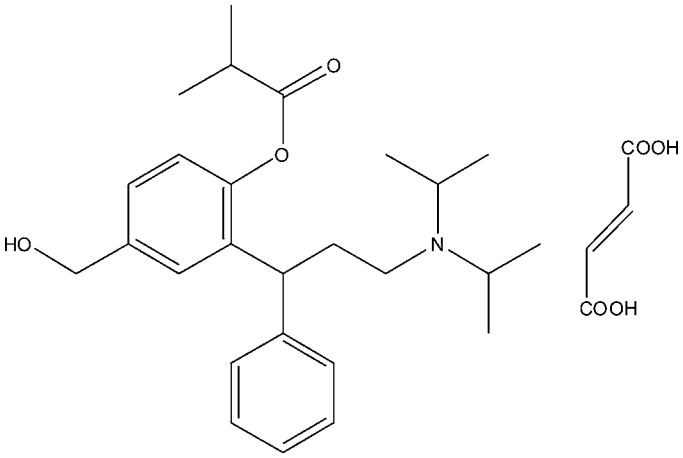

Fig. 1. Chemical structure of fesoterodine fumarate.

liquid–liquid extraction with hexane–ethyl acetate (1:1, v/v) and acetonitrile-0.01 mol L^{-1} ammonium acetate pH 3.0 (35:65, v/v) as mobile phase at a flow rate of 0.2 mL min^{-1} [19,20]. These methods were developed to determine a low level of active metabolite, thus they are not suitable for routine analysis of formulated product. Fesoterodine is commercially available, but is currently not included in any pharmacopoeia, and no analytical method has been reported for quantitative analysis of the drug in the presence of its degradation products. There is lack of a comprehensive LC-UV and LC-ESI-MS study of the degradation behavior of fesoterodine under various ICH prescribed stress condition. Therefore, the aim of the present article was to develop a selective, simple, rapid and validated stability-indicating LC method on a monolithic reversed-phase column to perform forced decomposition studies, contributing to improve the quality control and ensuring therapeutic efficacy. An integral aim of the study was to identify degradation products, and to postulate a drug degradation pathway.

2. Experimental

2.1. Chemical and reagents

The fesoterodine fumarate reference substance was purchased from Toronto Research Chemicals Inc (Toronto, ON, Canada). Tablets containing 8 mg of fesoterodine fumarate (Toviaz®, Pfizer Inc, Zwickau, Germany) were obtained from commercial sources. Fumaric acid was acquired from Merck KGaA (Darmstadt, Germany). Phosphoric acid 85% and LC-grade acetonitrile and methanol were obtained from Tedia Company Inc (Fairfield, OH, USA). Ammonium acetate was purchased from Spectrum Chemical Corp (Gardena, CA, USA). Ultrapure water (Milli Q Gradient System, Millipore Corp., Bedford, MA, USA) was used for all the analyses.

2.2. Instrumentation

2.2.1. LC-UV

Two different LC systems equipped with UV detectors were used for the study. The corresponding specifications are presented below:

- LC system 1: the LC 1 apparatus was a Shimadzu LC system (Shimadzu Corp, Kyoto, Japan) equipped with an SCL-$10A_{VP}$ system controller, an LC-$10AD_{VP}$ binary pump, a SIL-$10AD_{VP}$ autosampler, a CTO-10ACvp column oven, and an SPD-$M10A_{VP}$ photodiode array (PDA) detector. The peak areas were integrated automatically by computer using Class VP software (v 6.12).
- LC system 2: the LC 2 apparatus was an Agilent 1200 series (Agilent Technologies Inc, Santa Clara, USA) consisting of a G1311A quaternary pump, G1322A vacuum degasser, G1316A thermostat column compartment, G1329A standard autosampler and G1315B diode array detector. The peak areas were determined using the tools of the Agilent Chemstation software.

The experiments were performed on a reversed-phase Phenomenex Inc (Torrance, CA, USA) Onyx C_{18} monolithic column (100 mm × 4.6 mm id). A guard column was used to protect the analytical column.

2.2.2. LC-ESI-MS

The LC-ESI-MS method was performed on a Shimadzu LC system (Shimadzu) equipped with an SCL-$10A_{VP}$ system controller, an LC-$10AD_{VP}$ quaternary pump, a DGU-14A degasser, a SIL-$10AD_{VP}$ autosampler, and a CTO-$10AD_{VP}$ column oven. The triple quadrupole mass spectrometer (Micromass UK Ltd., Manchester, UK), model Quattro LC, was equipped with an ESI source in positive mode. Data acquisition and analysis were performed using the Masslynx software (v 3.5). A syringe pump (Bioanalytical Systems Inc, West Lafayette, IN, USA) was used to infuse the solutions.

2.3. Preparation of reference solution

The stock solution of fesoterodine was prepared by accurately weighing, 25 mg of fesoterodine fumarate (purity 98%) diluted to volume with methanol, obtaining a concentration of 1 mg mL^{-1} of fesoterodine base. The stock solution was stored at 2–8 °C, protected from light and daily diluted to an appropriate concentration in mobile-phase.

2.4. Preparation of sample solution

Tablets containing 8 mg of fesoterodine fumarate (6.2 mg fesoterodine base) were accurately weighed and crushed to a fine powder to prepare the sample solutions. Appropriate amounts were transferred into individual 50 mL volumetric flasks. After adding 30 mL of methanol, the flasks were vortex mixed for 3 min. Then the samples were made up to volume with methanol, transferred to appropriate tubes and centrifuged at 3000 × *g* for 10 min. An aliquot of the clear supernatant liquid at a final concentration of 200 μg mL^{-1} of the active pharmaceutical ingredient was filtered through a 0.45 μm membrane filter (Millipore Corp). Working sample solutions were prepared daily by diluting the stock solution to an appropriate concentration with mobile-phase.

2.5. LC-UV conditions

The optimized chromatographic conditions for the fesoterodine determination and all the validation tests were obtained using the Shimadzu LC system 1. The LC system 1 and LC system 2 were operated isocratically at controlled temperature (45 °C) using a mobile phase of acetonitrile-methanol-0.03 mol L^{-1} ammonium acetate (pH 3.8) (30:15:55, v/v/v), run at a flow rate of 2.4 mL min^{-1} and detected at 208 nm. The injection volume was 10 μL.

2.6. LC-ESI-MS conditions

Firstly, the mass spectrometer conditions were optimized with a preliminary direct injection of fesoterodine reference solution (2 μg mL^{-1}) into the system. The best response was obtained with capillary voltage, extractor voltage, RF lens voltage, cone voltage, source temperature and ESI probe temperature of 3.5 kV, 3 V, 0.4 V, 45 V, 120 and 500 °C, respectively. Degradation samples were injected directly into a mass spectrometer and full scan spectra

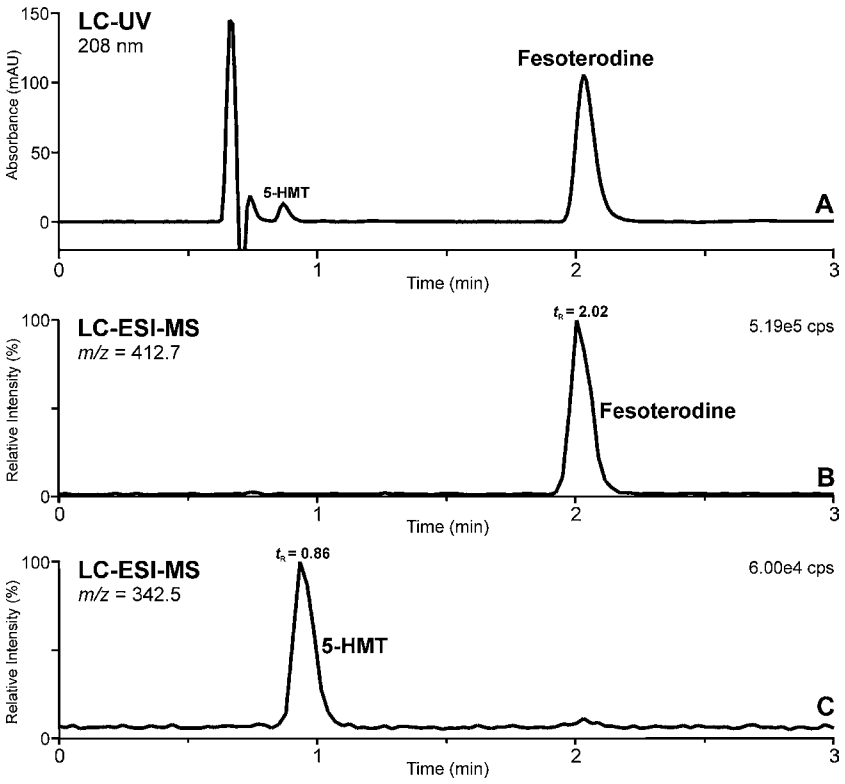

Fig. 2. LC-UV and LC-ESI-MS chromatograms of fesoterodine tablet solution: (A) UV detection at 208 nm (50 μg mL^{-1}); (B) ESI-MS detection of fesoterodine at *m*/*z* 412.7 (2 μg mL^{-1}) and (C) 5-HMT (5-hydroxymethyltolterodine; *m*/*z* 342.5). Chromatographic conditions: Phenomenex Onyx C_{18} monolithic column (100 mm × 4.6 mm id), 45 °C; mobile phase: acetonitrile-methanol-0.03 mol L^{-1} ammonium acetate (pH 3.8) (30:15:55, v/v/v); flow rate: 2.4 mL min^{-1} for LC-UV and 2.4 mL min^{-1} (split 1:10 post column) for LC-ESI-MS.

were acquired in the *m*/*z* range between 100 and 600 amu. The selected ions were monitored in single-ion recording (SIR) mode by the LC-ESI-MS system, carried out under the same analytical LC conditions, with eluent split at approximately 1:10 post-column. The following protonated molecular ions were acquired in SIR mode: *m*/*z* 412.7, *m*/*z* 393.4, *m*/*z* 365.7, *m*/*z* 356.6, *m*/*z* 342.5, *m*/*z* 284.5, and *m*/*z* 175.2.

2.7. Statistical software

The experimental designs and statistical analysis of the data were performed by the MINITAB 14 (Minitab Inc, State College, PA, USA) data analysis software system.

2.8. Forced degradation studies

The stability-indicating capability and specificity of the method were determined by subjecting a tablet solution (200 μg mL^{-1}) to accelerated degradation by acidic, basic, thermal, oxidative, and photolytic conditions to evaluate interference in the quantitation of fesoterodine. A sample solution prepared in 2 mol L^{-1} hydrochloric acid (HCl) was used for acidic hydrolysis, and a sample solution in 0.01 mol L^{-1} sodium hydroxide (NaOH), for basic hydrolysis evaluation. Both solutions were maintained at ambient temperature for 6 h and 10 min, respectively, and neutralized with acid or base, as necessary. For thermal stress testing, the tablet solution was diluted in water, sealed in a glass vial and placed in the oven at 60 °C for 36 h. The oxidative study was induced by storing the solution in 10% hydrogen peroxide (H_2O_2), at ambient temperature for 6 h, protected from light. Photodegradation was induced by exposing the sample in the photostability chambers to near UV-A (365 nm) and UV-C (254 nm) light for 6 h and 45 min, respectively. After subjecting the samples to stress studies, they were withdrawn at suitable time intervals and diluted with the mobile phase to final concentrations of 50 μg mL^{-1} before LC injection, and diluted to 2 μg mL^{-1} with methanol for LC-ESI-MS studies. Peak purity testing of degradation products was done by PDA detector.

2.9. Validation of the LC-UV method

Analytical method development and validation play a major role in the discovery, development, and manufacture of pharmaceuticals [21]. The present method was validated using samples of tablet dosage forms following the ICH guidelines [10,11].

In order to determine the method specificity, a solution of analytical placebo (in-house mixture of all the tablet excipients) was prepared in accordance with the sample preparation procedure and injected. To evaluate the absence of interference from the formulation excipients on the fesoterodine peak, the mixture of inactive ingredients (placebo), reference solution, and the commercial pharmaceutical preparation were analysed by the developed method. The specificity of the method was also established by determining purity for the fesoterodine peak in a mixture of stressed samples using LC-ESI-MS and PDA detector. The resolution factor of the drug peak from the nearest resolving degradation product peak was also studied.

The linearity was determined by constructing three independent analytical curves, each one with seven concentrations of fesoterodine, in the range of 5–150 μg mL^{-1} (5 μg mL^{-1}, 10 μg mL^{-1}, 25 μg mL^{-1}, 50 μg mL^{-1}, 75 μg mL^{-1}, 100 μg mL^{-1}, and 150 μg mL^{-1}), prepared in mobile-phase. Before the solutions were injected, the column was equilibrated for at least 20 min with

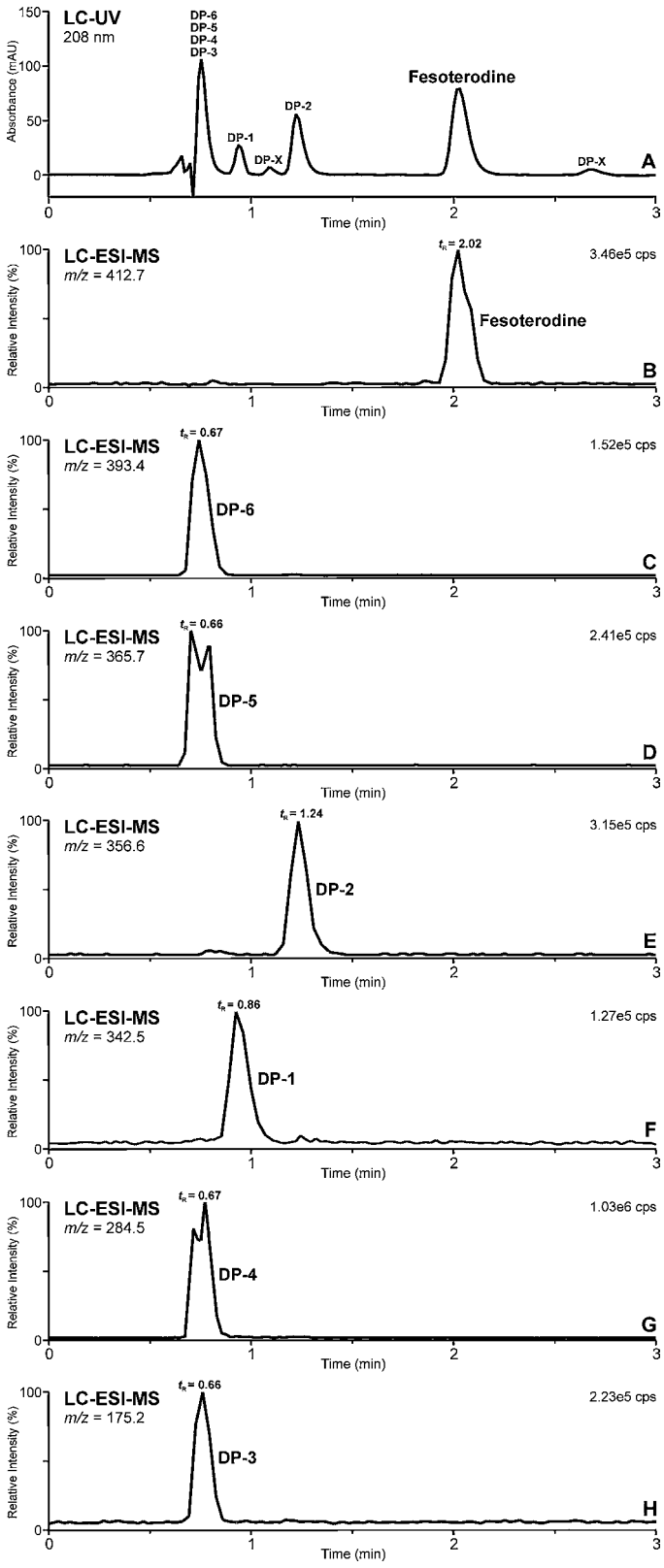

Fig. 3. LC-UV and LC-ESI-MS chromatograms of fesoterodine acidic degradation in 2 mol L^{-1} HCl (t = 6 h); detected by UV at 208 nm (A); detected by ESI-MS: m/z 412.7 for fesoterodine (B), m/z 393.4 for DP-6 (C), m/z 365.7 for DP-5 (D), m/z 356.6 for DP-2 (E), m/z 342.5 for DP-1 (F), m/z 284.5 for DP-4 (G), and m/z 175.2 for DP-3 (H). Chromatographic conditions were described in Fig. 2. DP: degradation product. DP-X: unidentified degradation product.

the mobile phase flowing through the system. The peak areas of the chromatograms were plotted against the respective concentrations of fesoterodine to obtain the analytical curves. The results were subjected to regression analysis by the least squares method to calculate the calibration equation and the determination coefficient, and by analysis of variance (ANOVA) for compliance of the linear model.

The method precision was determined by repeatability and intermediate precision, with a nominal concentration of 50 μg mL^{-1}. Repeatability was examined by six independent sample preparations of the same concentration of fesoterodine, on the same day, under the same experimental conditions. Intermediate precision is obtained when the assay is performed by multiple analysts, using multiple instruments, on multiple days, in one laboratory [11,22]. A multivariate approach was used to study these effects simultaneously. The considered variables included analysts (1 and 2), equipment (LC 1 and 2) and days (1 and 2). The considered response was the drug assay found (%). A linear model ($y = b0 + b1 \times 1 + b2 \times 2 + b3 \times 3$) was postulated and a 2^3 full factorial design was employed to estimate the model coefficients [23]. Each experiment was repeated three times in order to evaluate the experimental error variance.

Accuracy was evaluated by applying the proposed method to the analysis of an in-house mixture of the excipients with known amounts of the drug, to obtain solutions at concentrations of 40, 50 and 60 μg mL^{-1}, equivalent to 80, 100 and 120% of the nominal analytical concentration, respectively. Accuracy criteria for an assay method are that the mean recovery will be 100 ± 2% at each concentration over the range of 80–120% of the target concentration [24,25]. The samples of each concentration were also analysed for 3 consecutive days as part of inter-day validation. The results were expressed as the percentage of fesoterodine reference substance recovered from the formulation matrix

The LoQ was taken as the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy, and the LoD was taken as the lowest absolute concentration of analyte in a sample that can be detected but not necessarily quantified. LoD/LoQ parameters are not a requirement for drug assay; however, it is always useful to demonstrate that the analyses are being conducted in a region which is above the LoQ value. LoD and LoQ were calculated based on the standard deviation of the response (y-intercepts of regression lines) and the slope using three independent analytical curves, as defined by the ICH guideline. LoD and LoQ were calculated as $3.3\sigma/S$ and $10\sigma/S$, respectively, where σ is the standard deviation of the response and S is the slope of the calibration curve. Experimentally, the background noise was obtained after injection of the blank, observed over a distance equal to 20 times the width at half-height of the peak in a chromatogram obtained by the injection of 50 μg mL^{-1} of the reference substance. The signal-to-noise ratio applied was 10:1 for LoQ and 3:1 for LoD.

In order to study the simultaneous variation of the factors in the considered responses, a multivariate approach using design of experiments is recommended in robustness testing. A Plackett–Burman design was used to examine the effects of five factors at two levels in 21 experiments [26]. The ranges examined were small deviations from the method settings and the corresponding responses in the drug assay, peak symmetry (tailing factor), retention factor, and theoretical plates were observed. A half-normal probability plot for the effects was used to estimate the error and identify significant effects.

In order to demonstrate the stability of both reference solutions and tablet sample solutions during analysis, both solutions were maintained at 2–8 °C for 48 h and also put into the autosampler, at room temperature, for 24 h. The stability of these solutions was studied by performing the experiment and observing any change

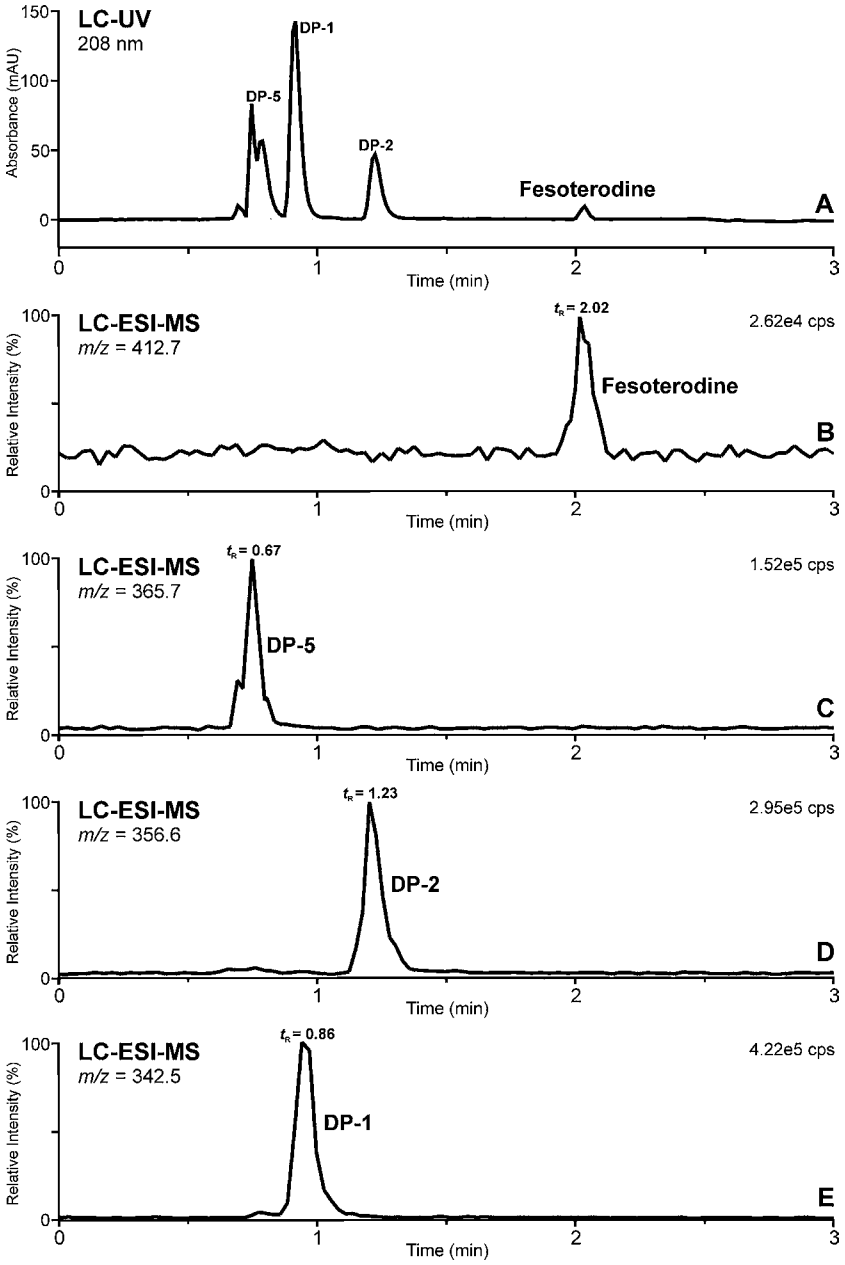

Fig. 4. LC-UV and LC-ESI-MS chromatograms of fesoterodine basic degradation in 0.01 mol L^{-1} NaOH (t = 10 min); detected by UV at 208 nm (A); detected by ESI-MS: m/z 412.7 for fesoterodine (B), m/z 365.7 for DP-5 (C), m/z 356.6 for DP-2 (D), and m/z 342.5 for DP-1 (E). Chromatographic conditions were described in Fig. 2. DP: degradation product.

in the chromatographic pattern, compared with freshly prepared solutions.

System suitability tests were performed in accordance with USP 32 to confirm that the equipment was appropriate to the analysis to be performed [27]. The test was carried out by injecting six replicates of the reference solution containing 50 μg mL^{-1} of fesoterodine.

2.10. Sample analysis

For fesoterodine quantitation in the tablet formulations, the respective stock solutions were diluted to an appropriate concentration (50 μg mL^{-1}) with mobile-phase, filtered, injected in triplicate and the percentage recoveries of the drug calculated against the reference substance.

3. Results and discussion

3.1. Method optimization

In order to develop a rapid stability-indicating LC method with efficient chromatography, high sensitivity, and suitable electrospray interface to perform LC-ESI-MS studies, the mobile phase was composed of acetonitrile, methanol and a volatile buffer (ammonium acetate).

The suitable mobile phase should control the selectivity and achieve reproducible separations with an acceptable peak shape. Thus accurate pH control is essential as well as a high buffer capacity [21]. The buffer capacity of the mobile-phase was suitable and the fesoterodine molecule was fully protonated and properly eluted in the column. Ammonium acetate has a pKa of 4.8 and its buffer range (pKa ± 1 unit) is 3.8 and 5.8. Therefore, the pH value

of 3.8 was chosen due to a better tailing factor and high sensitivity. Ammonium acetate solution had an adequate buffering and volatilization capacity to maintain the chosen pH and to obtain suitable electrospray ionization. The use of acetonitrile resulted in better sensitivity, short analysis time, improving the tailing factor (about 1.24), and the use of methanol was important to increase the retention factor and to obtain a better resolution of drug and degradation products. The column temperature was set at 45 °C to improve the repeatability between runs, reduce the analysis time and decrease the back pressure, which is important to extend the column lifetime. Moreover, the best chromatographic separation of degradation products was achieved in the monolithic column used.

The monolithic columns represent an approach that provides high rates of mass transfer at lower pressure drops as well as high efficiencies even at elevated flow rates [28]. Due to its inherent characteristic, the fact that consumption of mobile phase per unit time is higher than for conventional methods is compensated by the much shorter run time of each analysis, which means that the total solvent consumption per analysis is lower than the conventional LC methods with particle-packed columns. Therefore, the use of monolithic columns allows a significant decrease in time and costs per analysis required in method validation and routine procedures [29,30].

For analytical methods in routine quality control analysis, especially if it is applied to processing a large number of samples in a short time, it is extremely important to demonstrate column stability and work life [31]. Column stability and work life were demonstrated by performing a great number of injections (at least 450 injections) of the tablet formulation samples and also by pumping high volumes of mobile phase through the column. Non-significant changes were detected in chromatographic pattern, retention times and peak areas.

The optimal wavelength was established experimentally after measuring all spectra in mobile phase and testing the detector response of analyte using UV absorbance scanned over the range of 190–360 nm. As observed, fesoterodine shows a maximum at 200 nm. No interference with other components of the formulation and mobile phase composition was presented at 208 nm, and it was chosen as the optimal wavelength.

The optimized conditions of the LC method were validated for the analysis of fesoterodine in tablet dosage forms, and a typical chromatogram obtained by the proposed method, demonstrating the resolution of the symmetrical peak corresponding to fesoterodine, is shown in Fig. 2A. The retention time (t_R) observed (2.02 min) allows a fast determination of the drug, which is suitable for the quality control laboratories. The peak detected at 0.86 min (m/z 342.5) was chemically characterized as 2-(3-(diisopropylamino)-1-phenylpropyl)-4-(hydroxymethyl)phenol and identified by LC-ESI-MS studies as the last intermediate product of fesoterodine synthesis, 5-HMT impurity (Fig. 2C), the same product as the fesoterodine active metabolite [20,32]. In this method, fesoterodine, a hydrophilic basic drug, and corresponding acidic counter-ion (fumarate) were separated in the same run on a reversed-phase monolithic column. Fumaric acid solution was injected to prove the elution at 0.66 min.

3.2. Degradation behavior

Stress testing is the main tool used to predict stability-related problems, develop analytical methods, and identify degradation products and pathways. Understanding the degradation pathways enables the assessment of sites in the compound that are susceptible to degradation under different conditions. This information is essential to understand the intrinsic stability characteristics of a drug compound [33].

The emphasis today is on techniques that allow characterization of very low quantities of degradation products instead of conventional processes of isolation and spectral analysis [34,35]. The hyphenated techniques are considered for this purpose, among which LC-MS tools have been explored more strongly due to their potential to directly characterize small quantities of degradation products. Applications of LC-MS are extensive, with retention time and molecular weight emerging as essential analytical features [36].

The following degradation behavior of the drug under different stress conditions was investigated using the developed stability-indicating LC method and LC-ESI-MS studies.

3.2.1. Acidic conditions

Initially, 1 mol L^{-1} HCl was maintained at ambient temperature for 6 h but only 10% degradation was observed. The reaction in 2 mol L^{-1} HCl at an ambient temperature around 32% of the drug was degraded in 6 h. As shown in the LC chromatogram, degradation of the drug resulted in the rise of one major degradation product at 1.23 min and two minor products at 1.12 and 2.71 min. An increase in peak area at 0.86 min was also observed. The study of mass values (Fig. 3) indicated two degradation products as having a mass-to-charge ratio (m/z) of 342.5 (t_R = 0.86 min) and 356.6 (t_R = 1.23 min). The intensity of m/z 342.5 ion product was higher than that observed in the tablet solution (Fig. 2C), indicating the formation of a degradation product of fesoterodine due to hydrolyzation of the ester bond, identified as DP-1. The product ion at m/z 356.6 was attributed to 3-(3-(diisopropylamino)-1-phenylpropyl)-4-hydroxybenzoic acid (DP-2). Besides the organic acid peak (fumaric acid) at 0.66 min, co-eluted ions at m/z 175.2 (DP-3), m/z 284.5 (DP-4), m/z 365.7 (DP-5) and at m/z 393.4 (DP-6) were identified by the LC-ESI-MS method. Moreover, it was not necessary to perform chromatographic resolution of co-eluting or unresolved components to obtain product ion data for structural analysis, due to the mass resolving capability of mass spectrometry [12]. Due to the very small amounts, the identification of the degradation products eluted at 1.12 and 2.71 min was unsuccessful [35]. Complete degradation of the drug was observed in 36 h when exposed to 2 mol L^{-1} HCl at ambient temperature.

3.2.2. Basic conditions

The rate of degradation of the base was faster compared to that of acid. The drug was found to be highly labile to basic degradation. Complete degradation of the drug was observed in 0.01 mol L^{-1} NaOH at ambient temperature within 15 min. After 10 min of basic hydrolysis, approximately 95% drug degradation were observed. As shown in the LC chromatogram, degradation of the drug resulted in the rise of one major degradation product at 0.86 min and a minor product at 1.23 min. The study of mass values indicated the two ions at m/z 342.5 and 356.6, respectively (Fig. 4). A more detailed LC-ESI-MS study performed in 1 mol L^{-1} NaOH demonstrated that the fesoterodine was fully degraded to DP-1. After 1 h, 11% DP-1 degradation was observed, and the obtained product was converted to DP-2 due to the oxidation of the hydroxymethyl group. This proposed fragmentation was confirmed in accordance with the metabolic pathway of fesoterodine in which DP-2 is its inactive carboxy metabolite [20,37,38].

3.2.3. Oxidative conditions

Fesoterodine was found to be stable in 2% H_2O_2 at room temperature. However, almost 35% drug degradation was seen on exposure to 10% for 6 h at room temperature. An additional peak at 0.86 min was seen in the chromatogram (Fig. 5) and the LC-ESI-MS investigation demonstrated the respective degradation product, which corresponds to DP-1.

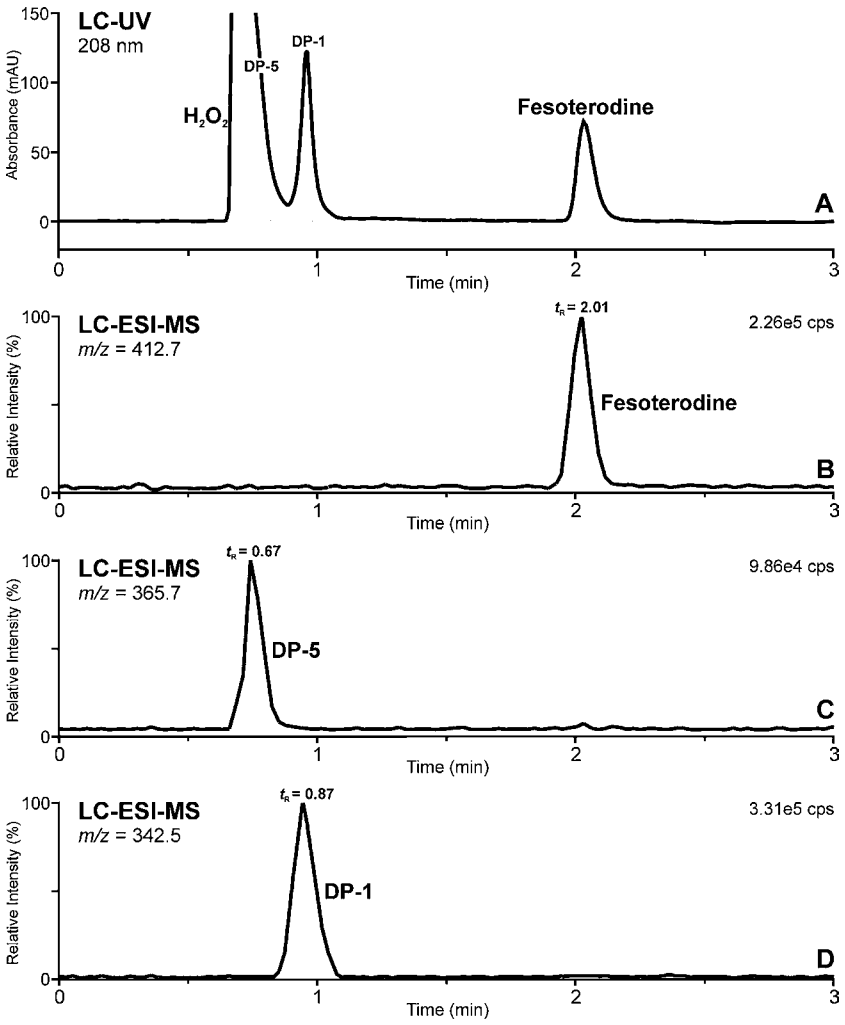

Fig. 5. LC-UV and LC-ESI-MS chromatograms of fesoterodine oxidative degradation in 10% H_2O_2 (t = 6 h); detected by UV at 208 nm (A); at 2 μg mL^{-1} detected by ESI-MS: m/z 412.7 for fesoterodine (B), m/z 365.7 for DP-5 (C), and m/z 342.5 for DP-1 (D). Chromatographic conditions were described in Fig. 2. DP: degradation product.

3.2.4. Thermal conditions

Under thermal conditions, approximately 17% degradation of the drug was seen after heating for 36 h at 60 °C with an increased peak area of DP-1 (t_R = 0.86 min) and forming one peak at 1.23 min (m/z 356.6), attributed to DP-2 (Fig. 6).

3.2.5. Photolytic conditions

There was no significant photodegradation of fesoterodine solution on exposure to UV-A light (365 nm) at ambient temperature for 6 h. However, the exposure of drug to UV-C (254 nm) light for 6 h resulted in full degradation of the drug. Exposing the drug for 45 min, around 60% degradation was seen and one new additional peak was detected at 3.30 min, besides an increase in DP-1 peak area. The LC-ESI-MS investigation has shown that co-eluted peaks were detected at 0.66 min, corresponding to DP-4 (m/z 284.5) and DP-5 (m/z 365.7), as represented in Fig. 7. No new peak was seen in mass spectra at 3.30 min, indicating that the drug was degraded to non-chromophoric compound, very low molecular weight or non-ionizable product [35].

Under all conditions, an ion product at m/z 365.7 (t_R = 0.66 min) was detected by LC-ESI-MS. The supposed product (DP-5) undergoes beta-cleavage of the diisopropylamine group to form a piperidine ring as well as loss of the hydroxymethyl group. A proposal for degradation pathways is presented in schematic form (Fig. 8).

3.3. Method validation

3.3.1. Specificity

The presence of degradants and impurities in pharmaceutical formulations can result in change in chemical, pharmacological and toxicological properties affecting their efficacy and safety. Therefore, the adoption of stability-indicating methods is always required to control the quality of pharmaceuticals during and after production. This greatly contributes to the possibility of improving drug safety [39,40]. Specificity studies should be the first step in method validation. Specificity of the method was established by verifying purity of the drug peak in a mixture of stressed samples by LC-ESI-MS and PDA analyses [41]. The studies showed that the fesoterodine peak was free from any coeluting peak, with values of a peak purity index higher than 0.9999, indicating the absence of any co-eluting peak in the drug peak. The resolution factor for the drug peak was >2 from the nearest resolving peak. Additionally, the evaluation of the main peak present in the LC method showed only an m/z of 412.6 attributable to fesoterodine, and no other mass determinations were observed. No interference from formulation excipients was found using PDA detection, showing that the peak was free from any coeluting peak, thus demonstrating that the proposed method is specific for the rapid analysis of fesoterodine.

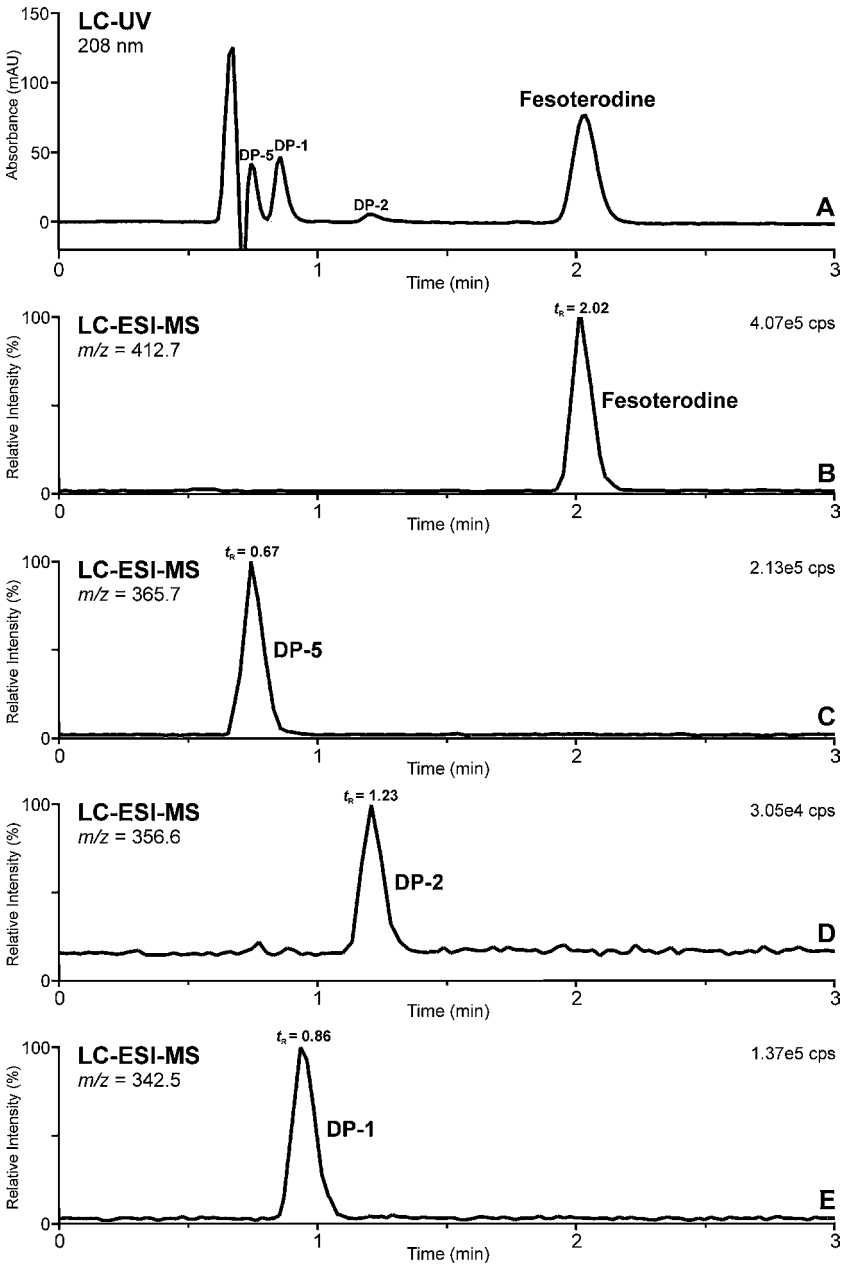

Fig. 6. LC-UV and LC-ESI-MS chromatograms of fesoterodine thermal degradation in the oven at 60 °C (t = 36 h); detected by UV at 208 nm (A); detected by ESI-MS: m/z 412.7 for fesoterodine (B), m/z 365.7 for DP-5 (C), m/z 356.6 for DP-2 (D), and m/z 342.5 for DP-1 (E). Chromatographic conditions were described in Fig. 2. DP: degradation product.

3.3.2. Linearity and range

The analytical curves constructed for fesoterodine were found to be linear in the 5–150 μg mL^{-1} range. This range corresponded to 10–300% of the intended test concentration of 50 μg mL^{-1} for the pharmaceutical quality control of fesoterodine and the drug in its formulation. The value of the determination coefficient calculated (r^2 = 0.9995, y = 11711.55 x + 17019.76), where x is concentration and y is the peak absolute area) indicated the linearity of the analytical curve for the method. The validity of the assay was also verified by means of ANOVA, which demonstrated significant linear regression (P > 0.05) and non-significant linearity deviation (P < 0.05).

3.3.3. Precision

The precision evaluated as the method repeatability resulted in a relative standard deviation (RSD) value of 1.48% (n = 6). The analyses of intermediate precision were carried out in a randomized order according to the experimental plan. The normal plot of residuals for the response evaluated (assay) is demonstrated in Fig. 9. The residuals of a good model are centered around zero with a high proportion (about 95%) within ±2, and no pattern to the residuals. The respective graph shows random scatter around zero with all points inside the ±2 limits. No considered factor was found significant for the regression model used. The mean values were found to be 99.27% with an RSD value of 0.87% (n = 24). The results were acceptable, indicating an excellent precision of the analytical procedure.

3.3.4. Accuracy

Accuracy was assessed from three replicate determinations of three different solutions containing 40, 50 and 60 μg mL^{-1} of fesoterodine for 3 consecutive days, equivalent to 80, 100 and 120% of the nominal analytical concentration, respectively, as shown in Table 1. Recoveries of intra-day accuracy were in the range of 99.06 and 100.51%

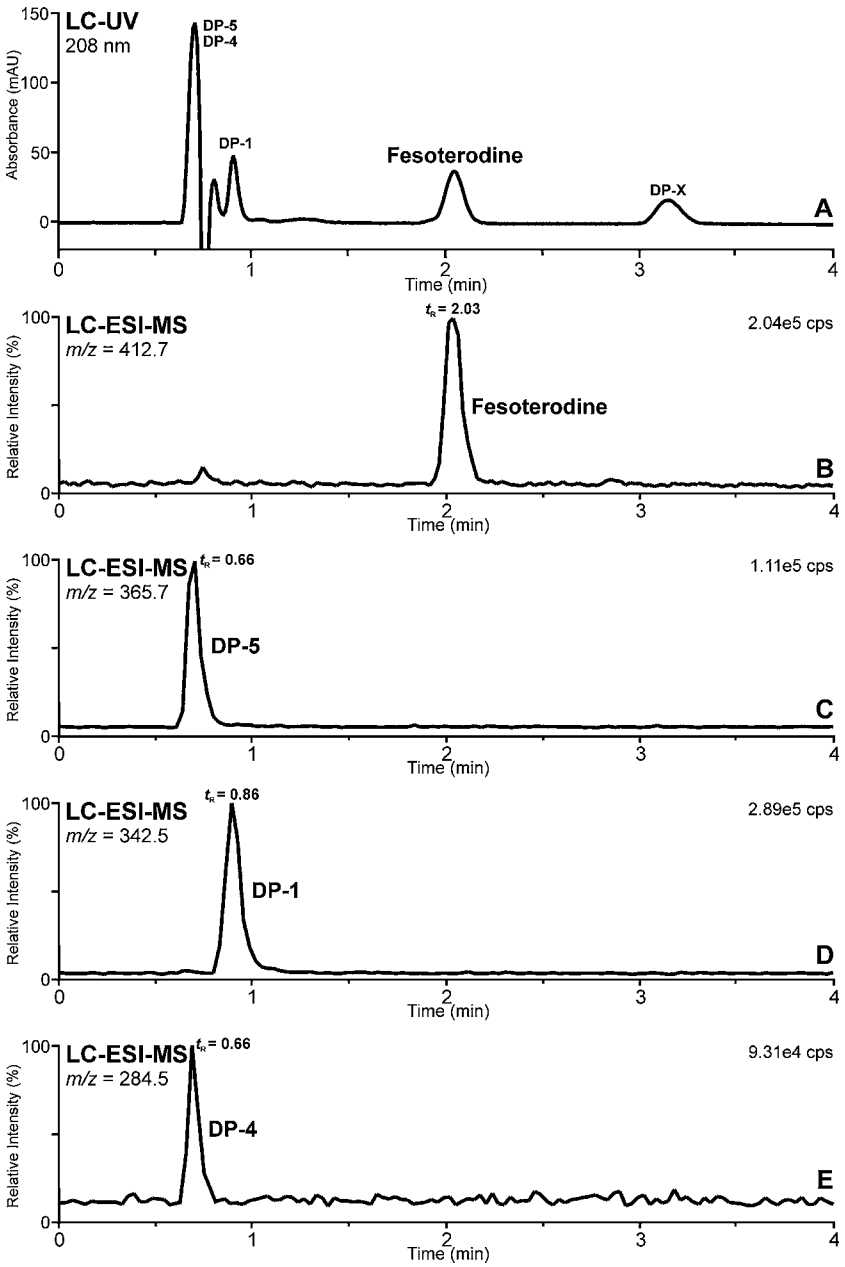

Fig. 7. LC-UV and LC-ESI-MS chromatograms of fesoterodine photolytic degradation in the photostability chambers to near UV-C (254 nm) (t = 45 min); detected by UV at 208 nm (A); detected by ESI-MS: m/z 412.7 for fesoterodine (B), m/z 365.7 for DP-5 (C), m/z 342.5 for DP-1 (D), and m/z 284.5 for DP-4 (E). Chromatographic conditions were described in Fig. 2. DP: degradation product. DP-X: unidentified degradation product.

with RSDs lower than 1.14% (n = 9). The value of inter-day accuracy (n = 27) was found to be 99.83% (RSD = 1.19%), demonstrating that the method is accurate within the desired range.

Table 1
Intra-day and inter-day accuracy data for proposed LC method.

Nominal concentration ($\mu g\,mL^{-1}$)	Recovery[a] (%)		
	Day 1	Day 2	Day 3
40	98.97	98.16	99.50
50	99.95	98.88	100.48
60	100.85	100.15	101.54
Intra-day accuracy[b] (n = 9)	99.92 ± 0.95	99.06 ± 1.11	100.51 ± 1.14
Inter-day accuracy[b] (n = 27)	99.83 ± 1.19		

[a] Mean of three replicates.
[b] Mean ± RSD (Relative standard deviation).

3.3.5. LoD and LoQ

LoD and LoQ were obtained by using the mean of the slope, 11711.55 ± 286.64 and the standard deviation of the intercept of the independent curves, determined by a linear regression line as 1603.05. The LoD and LoQ calculated were 0.41 and 1.37 $\mu g\,mL^{-1}$, respectively. Experimentally LoD was 0.49 $\mu g\,mL^{-1}$. LoQ, with the precision lower than 5% and accuracy within ±5%, was found to be 1.50 $\mu g\,mL^{-1}$.

3.3.6. Robustness

The susceptibility of the developed analytical method to changes was tested in order to evaluate the robustness. For this purpose a Plackett–Burman design was employed. The experimental plan and the corresponding responses are summarized in Table 2. The responses are the percentages of fesoterodine in the commercial tablets (relative to their label claimed concentration) obtained

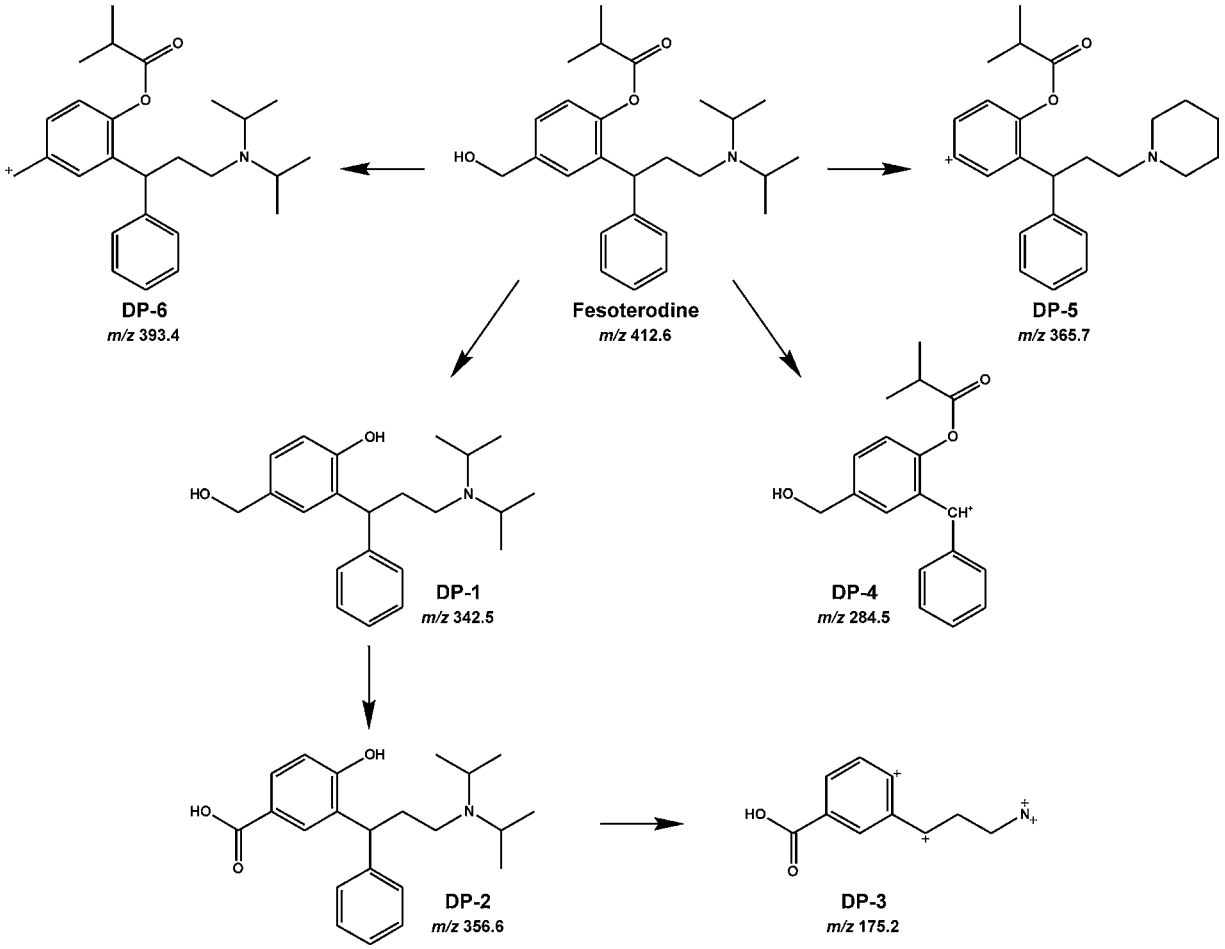

Fig. 8. A proposal for degradation pathways of fesoterodine through forced degradation using acidic, basic, oxidative, thermal and photolytic conditions obtained by LC-ESI-MS studies. DP: degradation product.

Table 2
Selected Plackett–Burman design for the robustness testing of fesoterodine.

Experiment	Flow rate (mL min^{-1})	Buffer (mM)	pH	Buffer (%)	Temperature (°C)	Assay (%)	T_f[a]	k'[b]	N[c]
1	2.5	28	4.0	57	43	100.02	1.35	2.64	3673
2	2.3	32	4.0	57	47	97.94	1.17	1.80	3588
3	2.5	28	4.0	53	47	98.24	1.37	1.68	3306
4	2.5	28	3.6	57	47	101.12	1.32	2.53	3219
5	2.5	32	3.6	53	47	99.56	1.35	1.66	2928
6	2.3	28	3.6	57	47	102.38	1.34	1.68	3797
7	2.3	28	3.6	53	47	100.56	1.32	1.04	3236
8	2.5	28	4.0	57	47	102.00	1.32	2.52	3488
9	2.3	32	3.6	57	43	100.40	1.34	1.77	3651
10	2.5	32	4.0	57	43	100.94	1.21	2.79	3444
11	2.5	32	4.0	53	47	98.15	1.26	1.73	2807
12	2.3	32	4.0	53	43	98.02	1.26	1.19	2967
13	2.5	32	3.6	53	43	97.86	1.37	1.73	3054
14	2.5	28	3.6	53	43	98.41	1.36	1.71	3006
15	2.3	28	3.6	53	43	100.61	1.39	1.10	3300
16	2.3	28	4.0	57	43	99.78	1.39	1.80	3931
17	2.5	32	3.6	57	43	99.78	1.33	2.58	3623
18	2.3	28	4.0	53	43	101.49	1.37	1.12	3433
19	2.3	32	4.0	53	47	98.71	1.23	1.11	2860
20[d]	2.4	30	3.8	55	45	99.72	1.24	1.68	3428
21	2.3	32	3.6	57	47	100.74	1.29	1.67	3765

[a] Tailing factor.
[b] Retention factor. $k' = (t_R - t_0)/t_0$; t_R = retention time, t_0 = void time.
[c] Theoretical plates.
[d] Optimal conditions.

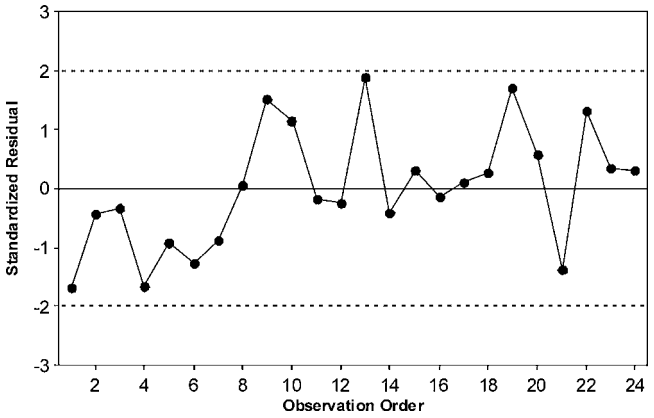

Fig. 9. Normal plot of residuals for intermediate precision data (assay) obtained by 2^3 full factorial design ($n = 24$) performed by analysts 1 and 2, equipment LC 1 and LC 2, and days 1 and 2.

compared to the reference solution in each experiment, tailing factor, retention factor, and theoretical plates. All experiments were performed in randomized order to minimize the effects of uncontrolled factors that may introduce a bias into the response.

After calculating the effects for each parameter, the statistical interpretation, through the ANOVA, allowed defining which one is significant and which is not. Non-significant factors were detected for all analyses ($P > 0.05$). Moreover, other chromatographic responses were analysed. Peak areas had less variability and retention time was between 1.78 and 2.39 min. There were non-significant changes in the chromatographic pattern when the modifications were made in the experimental conditions, thus showing the method to be robust.

3.3.7. Solution stability

The stability of the both reference and sample solutions was studied and the data obtained showed the stability during 24 h in the autosampler and during 48 h when maintained at 2–8 °C. The retention time and peak area of fesoterodine remained almost unchanged and non-significant degradation was observed within the indicated period, which was sufficient for the whole analytical process.

3.3.8. System suitability

The system suitability test carried out for retention time, peak area and tailing factor showed RSD values of 0.26, 0.50, and 0.67%, respectively. The number of theoretical plates was about 3440 (RSD = 0.86%). The experimental results showed that the parameters tested were within the acceptable range (RSD < 2.0%), indicating that the method is suitable for the analysis intended.

3.4. Application of the proposed method to pharmaceutical analysis

Acceptable results of application of the proposed method in tablet dosage forms (Table 2) were obtained, demonstrating the quality of the pharmaceutical samples and the applicability of the validated method for quality control analysis of fesoterodine. It should be considered that excipients did not interfere in the determination of the active compound.

4. Conclusion

The high-throughput assay is an extremely important advantage of LC methods using monolithic columns, especially during new product development and validation of manufacturing processes. The results of the validation studies showed that the stability-indicating LC method is specific, accurate, robust, and possesses significant linearity and precision, without any interference from the excipients and degradation products, according to ICH requirements. The method is fast, selective, and requires a simple sample preparation procedure. In this study, the intrinsic stability of fesoterodine was established using various ICH recommended stress conditions, and degradation products were identified based on their chromatographic relative retention times using validated LC conditions combined for molecular mass information obtained during LC-ESI-MS studies. Thus, the proposed LC method was successfully applied for the quantitative analysis of fesoterodine in tablet dosage forms and can be employed for drug analysis during stability studies, contributing to the improvement of quality control.

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