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Stability-indicating chromatographic determination of hydroquinone in combination with tretinoin and fluocinolone acetonide in pharmaceutical formulations with a photodegradation kinetic study†

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Two sensitive and selective stability-indicating methods were developed for simultaneous determination of the active pharmaceutical ingredient hydroquinone in combination with tretinoin and fluocinolone acetonide in their pure forms and within the pharmaceutical formulation. Method A was based on a gradient elution liquid chromatographic (HPLC) determination of the active ingredients, their degradation products (hydroquinone polymer, 1,4-benzoquinone, isotretinoin and fluocinolone acetonide photodegradation) and in the presence of the preservatives methyl and propyl parabens found in pharmaceutical formulations. Method B was a thin layer chromatography (TLC)-densitometry method using a chiral developing system for the separation and determination of the active ingredients, isotretinoin, the preservatives and 1,4-benzoquinone. The molecular weight of the hydroquinone polymer formed from its alkali degradation was characterized by gel permeation chromatography. The mechanism of fluocinolone acetonide photodegradation in acetonitrile at 254 nm was studied using single crystal X-ray diffraction. The degradation products, hydroquinone polymer and isotretinoin, were found in one batch of the pharmaceutical formulation analyzed near its expiry date. The proposed HPLC method was also used for a comparative kinetic study of the photodegradation of the active ingredients. Hydroquinone showed reversible zero order kinetics, tretinoin and fluocinolone acetonide followed complex kinetic reactions in acetonitrile within two hours. The results obtained were statistically analyzed and compared with those obtained by applying the manufacturers method.

1. Introduction

The combination of hydroquinone (HQ), tretinoin (TRT) and fluocinolone acetonide (FLA) is used as an external cream for the treatment of melasma. The chemical name of HQ is benzene-1,4-diol[1] and it works by blocking the synthesis of melanin *via* the inhibition of tyrosinase.[2] Tretinoin (all-*trans* retinoic acid) in this combination accelerates cell turnover. Fluocinolone acetonide chemically known as 6α,9α-difluoro-11β,16α,17,21-tetrahydroxy-pregna-1,4-diene-3,20-dione cyclic-16,17-acetal with acetone[1] reduces the irritation and inflammation of the combined drugs.[2] Two HPLC methods were reported for the determination of HQ in combination with TRT and FLA.[3,4] Many HPLC methods were published for studying the stability of TRT or FLA in single forms.[5–14] Kinetic studies were performed on tretinoin photodegradation.[6,9] A high performance thin layer chromatographic method was reported as a stability indicating determination of single HQ in pharmaceutical formulations.[15]

The scientific novelty of this work was developing and validating simple, rapid and sensitive stability indicating HPLC and TLC-densitometric methods as there were no stability indicating methods reported for the simultaneous determination of this mixture in presence of their degradation products, in raw materials and pharmaceutical formulation. The alkali degradation pathway of hydroquinone by sodium carbonate was studied. The molecular weight of the formed polymer, which was not calculated before, was characterized by gel permeation chromatography. This work studied the mechanism of the photodegradation of FLA in acetonitrile at 254 nm. The degradation product structure was elucidated using single crystal X-ray diffraction.† A chiral developing system was used in the proposed TLC-densitometric method for separation and quantitation of TRT and its isomer isotretinoin (ISO) simultaneously with the other compounds that were not reported before. The

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HPLC method was also used to perform a comparative kinetic study on the active ingredients photodegradation. The kinetic study was performed because the drugs are very susceptible to degradation, so the rate, the order of their degradation and the formation of the degradation products was important to be studied. The HQ degradation product, 1,4-benzoquinone (BQ), is very toxic and if it is present in the pharmaceutical formulation, it will be absorbed through skin. The degradation products of HQ and TRT were found in one batch of the pharmaceutical formulation analyzed near its expiry date.

2. Experimental

2.1. Instruments

HPLC Agilent model 1260 was equipped with a quaternary pump, Rheodyne injector with a 20 μL loop and UV detector (California, USA). Separation and quantitation were made on column Agilent Eclipse plus C18, 100 × 4.6 mm, 3.5 μm particle size.

TLC aluminum sheets, 20 × 20 cm precoated with silica gel F_{254}, 0.25 mm thickness, Merck (Darmstadt, Germany) were used. The samples were applied to the plate using 25 μL Hamilton Analytical Syringe. CAMAG dual wavelength flying spot densitometer was used (Muttenz, Switzerland). The measuring mode was absorbance, slit dimensions: 4 × 0.3 mm. Scanning speed: 20 mm s^{-1}. Data resolution: 100 μm per step. Optimize optical system for maximum: light. Band width: 20 mm. The peak area under curve was integrated.

UVC (G8T5) lamp 8 Watt ozone-free was used. Its irradiance was 24.0 μW cm^{-2} (GE Lighting, Japan).

The pH meter used was Jenway 3510 (Essex, UK).

For gel permeation chromatography, HPLC Agilent model 1100 was equipped with a quaternary pump and refractive index detector (California, USA). Separation was made on column PL aquagel–OH 7.5 mm, 30 μm pore type, 8 μm particle size.

Single Crystal Diffractometer, Bruker-Nonius KappaCCD, equipped with a charge-coupled device (CCD) detector and a liquid-nitrogen low-temperature device, on a Bruker-Nonius FR590 X-ray generator with a molybdenum sealed tube. The CCD detector allows many diffraction spots to be collected simultaneously.

2.2. Materials and chemicals

Pure form. Hydroquinone, tretinoin, fluocinolone acetonide, methyl and propyl parabens (MET and PRP) and were kindly supplied by T3A pharmaceutical company, Cairo Egypt; their purity were 99.74, 100.03, 99.95, 100.00 and 99.94, respectively, according to the manufacturer method. Isotretinoin 99.90% was supplied by Chongqing Huapont Pharm. Co., Ltd., China and 1,4-benzoquinone 98.00% was supplied by Sigma-Aldrich. Butylated hydroxyltoluene 99.50 was supplied by T3A pharmaceutical company.

Pharmaceutical formulation. Melanofree® cream was labeled to contain HQ 4%, TRT 0.05% and FLA 0.01% as active ingredients. The preservative MET and PRP were in concentration of 0.1% and 0.01%. Sodium metabisulfite 0.5%, butylated hydroxyl toluene 0.2% and butylated hydroxyl anisole 0.02% were found besides the bases for oil in water cream. It was manufactured by T3A Pharmaceutical Company, Cairo, Egypt. Batch no. 130054.

Chemicals. All chemicals used were of analytical grade, and the solvents were of HPLC and spectroscopic grade. Sodium dihydrogen phosphate dihydrate, disodium hydrogen phosphate dihydrate Sodium carbonate, hydrochloric acid 37%, hydrogen peroxide 30% (Adwic), acetonitrile, dichloromethane (SDFCL), ethyl acetate, acetone, acetic acid (Riedel-de Häen), petroleum ether, sodium azide and β-cyclodextrin (Sigma-Aldrich). The water for HPLC was prepared by double distillation and filtration through a 0.45 μm membrane filter.

2.3. Degraded samples

Preparation of HQ alkali degradation product. A 100 mg of HQ was dissolved in 100 mL of 0.02 M aqueous sodium carbonate solution and left in dark in air for ten days. The solution became dark brown and its pH was about 7.5. The solution was evaporated under boiling water bath. The degradation product was identified by gel permeation chromatography.

Preparation of HQ acid degradation product. A 100 mg of HQ was dissolved in 100 mL of 0.1 M HCl and left in dark place for ten days.

Preparation of HQ oxidative degradation product. It was prepared using 50 mg of HQ and 4 mL hydrogen peroxide 10%, the solution was diluted to 50 mL water and protected from light for 1 hour.

Preparation of HQ, TRT and FLA photo degradation products. Solutions of HQ, TRT and FLA were prepared in concentrations of 8.0 mg mL^{-1}, 100.0 μg mL^{-1} and 20.0 μg mL^{-1}, respectively, in acetonitrile and subjected to UV light source at 254 nm for 30 minutes.

The degradation products were tested for complete degradation by the proposed HPLC and TLC methods.

The solution of FLA photodegradation was applied in bands to the TLC plates using the described TLC method. The band at R_f 0.2 was scratched, dissolved in acetonitrile, filtered and evaporated to dryness at room temperature. Colorless needle crystals were formed representing the FLA photodegradation product. It was identified by single crystal X-ray diffraction.

2.4. Standard solutions

All standard solutions must be freshly prepared and protected from light.

For HPLC method. Stock standard solutions of HQ (4.0 mg mL^{-1}), FLA (100.0 μg mL^{-1}), HQ polymer (1.0 mg mL^{-1}), BQ (100.0 μg mL^{-1}), FLA photodegradation product (100.0 μg mL^{-1}), MET (100.0 μg mL^{-1}), PRP (100.0 μg mL^{-1}) were prepared in solvent mixture (acetonitrile : water 50 : 50, v/v). Stock standard solutions of TRT and ISO (100.0 μg mL^{-1} of each) were prepared in acetonitrile.

Working standard solutions of HQ polymer (140.0 μg mL^{-1}), BQ (10.0 μg mL^{-1}) and ISO (20.0 μg mL^{-1}) were prepared in solvent mixture.

For TLC-densitometric method. Stock standard solutions of HQ (6.0 mg mL^{-1}), TRT (500.0 μg mL^{-1}), ISO (500.0 μg mL^{-1}), FLA photodegradation product (1.0 mg mL^{-1}), MET (1.0 mg mL^{-1}), PRP (100.0 μg mL^{-1}) were prepared in acetonitrile. Stock standard solution of FLA (1.0 mg mL^{-1}) was prepared in dichloromethane.

Laboratory prepared mixtures. For HPLC method different aliquots of HQ, TRT, FLA were accurately transferred into series of 10 mL volumetric flasks then the degradation products and the preservatives were added to prepare different mixtures. The volume was completed by the solvent mixture.

For TLC-densitometric method, aliquots of HQ, TRT, FLA, were accurately transferred into series of 10 mL volumetric flasks then ISO and the preservatives were added to prepare different mixtures. The volume was completed by acetonitrile.

2.5. Chromatographic conditions

For HPLC method. A step gradient elution of two mobile phases was used. Mobile phases A and B were buffer and acetonitrile in ratios of 65 : 35, v/v and 40 : 60, v/v, respectively. The buffer was a mixture of 0.04 M sodium dihydrogen phosphate dihydrate and 0.01 M disodium hydrogen phosphate dihydrate (pH 6.1 ± 0.1). The elution was performed using 100% of mobile phase A for 7.00 min then 100% of mobile phase B from 7.01 min to 20.00 min. The mobile phase was filtered using 0.45 μm nylon disposable filter (Millipore, Milford, MA) and degassed by ultrasonic vibrations prior to use. The samples were also filtered using 0.45 μm disposable filters. The flow rate of the mobile phase was 1.0 mL min^{-1}. A volume of 20 μL of each sample solution was injected. The wavelength was changed in the run as follows:

Time in minutes	Wavelength (nm)
0.00	365
1.05	315
1.60	235
8.40	365
8.75	235
12.00	365

For TLC-densitometric method. The analysis was performed on 20 × 20 cm TLC aluminum sheets precoated with silica gel F_{254}, 0.25 mm thickness. A volume of 10 μL of different concentrations of the solutions was applied as spots to the plate using 25 μL Hamilton Analytical Syringe. The spots were spaced 10 mm apart and 15 mm from the sides and bottom of the plate. The chromatographic chamber was lined internally with normal cellulose paper or filter paper 9 ± 0.5 cm height and was protected from light. The plate was developed by ascending chromatography immediately using petroleum ether–ethyl acetate–acetone–acetic acid–β-cyclodextrin 1.0 mg mL^{-1} in water (70 : 20 : 5.6 : 4 : 0.4 by volume) as a developing system to distance of 12 ± 0.5 cm. The plate was air dried at room temperature, detected under UV lamp and scanned at 235 nm (for HQ, FLA, BQ, MET and PRP) and 365 nm (for TRT and ISO), respectively, as under the described instrumental parameters.

3. Procedures

3.1. Construction of calibration curves

For HPLC method. Aliquots of standard solutions of the active ingredients (equivalent to 0.4–10.0 mg of HQ, 10.0–200.0 μg of TRT and 10.0–200.0 μg of FLA) were separately transferred into a series of 10 mL volumetric flasks, the volume was completed to the mark with the solvent mixture. Triplicate 20 μL injections were made for each concentration using the chromatographic conditions described. The peak area of each concentration was plotted against the corresponding concentration to obtain the calibration curves then the regression equations were computed. Aliquots of solutions of the degradation products (equivalent to 56.0–280.0 μg of HQ polymer, 2.0–20.0 μg of BQ, 2.0–100.0 μg of ISO and 20.0–200.0 μg of FLA photodegradation product) and preservatives (equivalent to 10.0–250.0 μg of MET and 10.0–200.0 μg of PRP) were separately transferred into a series of 10 mL volumetric flasks and followed as under chromatographic conditions.

For TLC-densitometric method. Into a series of 10 mL volumetric flasks, aliquots of standard solutions of the active ingredients (equivalent to 1.2–30.0 mg of HQ, 500.0–1750.0 μg of TRT and 0.4–1.4 mg of FLA) were separately transferred into a series of 10 mL volumetric flasks, the volume was completed to the mark with acetonitrile for HQ and TRT and dichloromethane for FLA. The chromatographic conditions were followed and the peak areas were recorded. The calibration curves were constructed and the regression equations were computed. Aliquots of solutions of ISO equivalent to 500.0–1750.0 μg, MET equivalent to 0.4–1.4 mg and PRP equivalent to 40.0–140.0 μg were separately transferred into a series of 10 mL volumetric flasks, completed to the mark with acetonitrile and the procedure was followed as under chromatographic conditions.

3.2. Application to pharmaceutical formulation

For HPLC method. Into a 100 mL beaker, an amount of 2.0 g cream was accurately weighed and dissolved in 50 mL acetonitrile with aid of stirring and ultrasound for 5 minutes. The solution was transferred into a 100 mL volumetric flask and the volume was completed with water by aid of sonication for about 3 minutes. The solution was transferred into the refrigerator for 15 minutes to enhance filtration by aggregation of the cream base and filtered through filter paper. The procedure under construction of calibration curves was followed for determination of HQ, TRT, FLA, MET and PRP, respectively.

For TLC-densitometric method. Into a 100 mL beaker, an amount of 10.0 g cream was accurately weighed and dissolved in about 40 mL acetonitrile with aid of stirring and ultrasound for 5 minutes. The solution was transferred into a 50 mL volumetric flask and the volume was completed with acetonitrile by aid of sonication for about 3 minutes. The solution was transferred into the refrigerator for 15 minutes and filtered through filter paper. Then, the procedures were followed as under construction of calibration curve. This solution was used for determination of TRT. Further dilution was done (15.0 mL in 50.0 mL acetonitrile) for determination of HQ, MET and PRP.

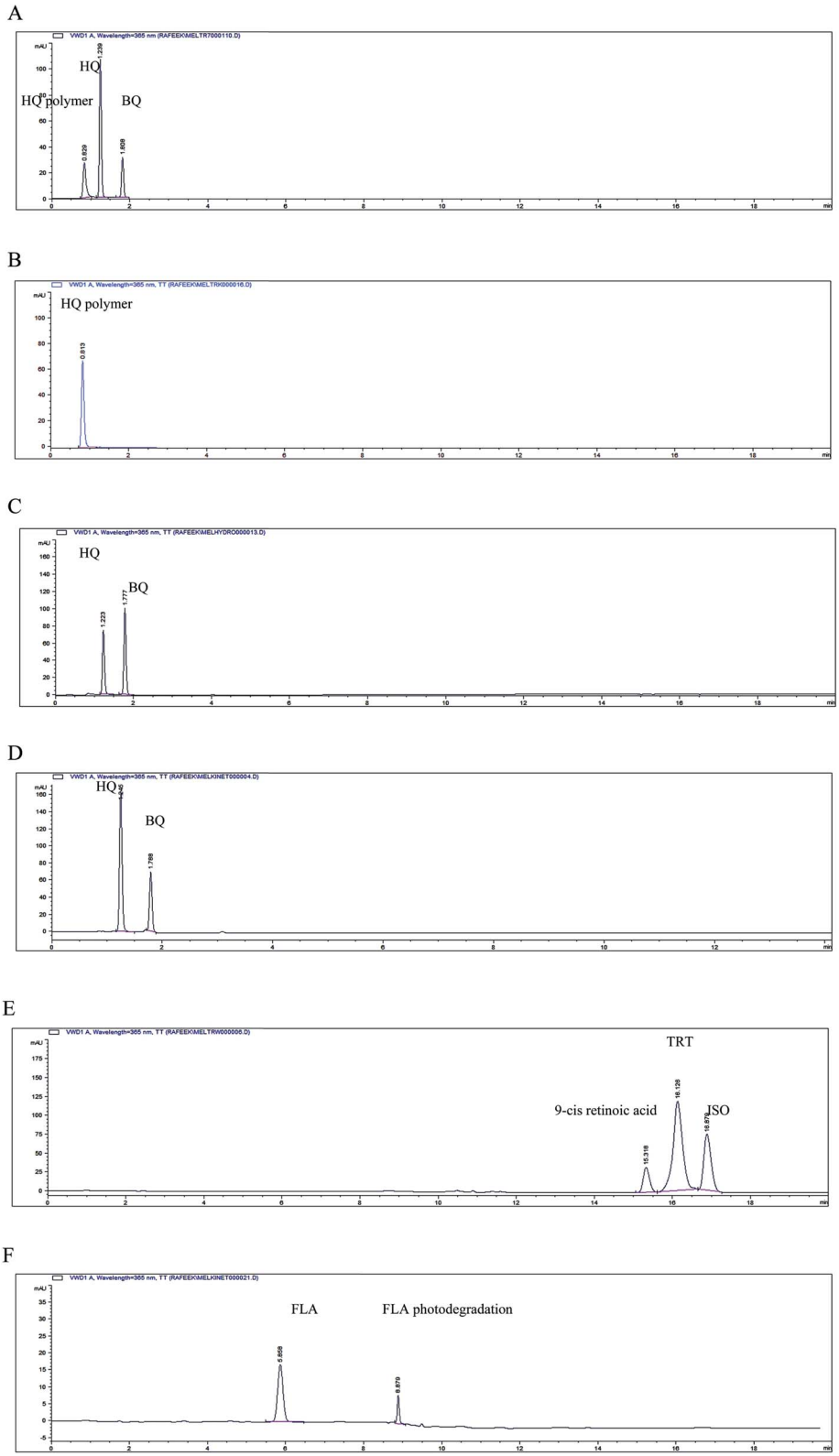

Fig. 1 HPLC chromatogram of: (A) alkali degradation of hydroquinone 1.00 mg mL^{-1} in 0.02 M sodium carbonate after one day. (B) Alkali degradation of hydroquinone 1.00 mg mL^{-1} in 0.02 M sodium carbonate after ten days evaporated till residue, weighed and diluted with acetonitrile : water 50 : 50, v/v to final concentration of 28.00 μg mL^{-1}. (C) Oxidative degradation of hydroquinone 1.00 mg mL^{-1}. (D) Hydroquinone 800.0 μg mL^{-1} after photodegradation for 15 minutes (from the kinetic study). (E) Tretinoin 45.00 μg mL^{-1} after photodegradation for 10 minutes (from the kinetic study). (F) Fluocinolone acetonide 9.06 μg mL^{-1} after photodrgradation for 30 minutes (from the kinetic study).

For determination of FLA: into a 25 mL beaker, an amount of 6.0 g cream was accurately weighed and dissolved in about 8 mL dichloromethane with aid of stirring and ultrasound for 1 minute. The solution was transferred into a 10 mL volumetric flask and the volume was completed with dichloromethane. Then, the procedure was followed as under HPLC method and construction of calibration curves.

Standard addition technique. Different known concentrations of pure form HQ, TRT, FLA, MET and PRP were added to pharmaceutical formulation. The general procedures for HPLC and TLC-densitometric methods described under construction of calibration curves were followed and the concentration of HQ, TRT, FLA, MET and PRP were calculated from the corresponding regression equations.

3.3. Kinetic study of the photodegradation of hydroquinone, tretinoin and fluocinolone acetonide

Studying the kinetic order of the reaction starting with concentrations ratio used in pharmaceutical formulation. Amounts of 2.0 g, 25.0 mg and 5.0 mg of HQ, TRT and FLA were accurately weighed and separately transferred into three 250 mL volumetric flasks, respectively; the volume was completed to the mark with acetonitrile. The initial concentrations C_0 were 8000.0, 100.0 and 20.0 μg mL^{-1} for HQ, TRT and FLA, respectively. From each solution, 10 mL was transferred into several 100 mL beakers for each time interval. The solutions were subjected to UV light from 2 to 120 minutes. Each solution was transferred into 10 mL volumetric flask and the volume was completed by acetonitrile if necessary due to evaporation of the solvent. Aliquots of 1.0, 4.5 and 5.0 mL from HQ, TRT and FLA solutions were separately transferred into 10 mL volumetric flasks and the volume was completed by the solvent mixture. A volume of 20 μL was injected from each solution (800.0, 45.00 and 10.00 μg mL^{-1} for HQ, TRT and FLA) using the chromatographic conditions described above. The concentrations of HQ, TRT and FLA were calculated from their corresponding regression equation. The log of the remaining concentration against time was plotted.

Studying the kinetic order of the reaction starting with equimolar concentrations. Solutions of HQ, TRT and FLA were prepared in acetonitrile of initial concentrations C_0 each of 10 mmol. The procedure was followed as under 3.3.1. Aliquots of 5.0, 1.5 and 0.2 mL from HQ, TRT and FLA solutions were separately transferred into 10 mL volumetric flasks and the volume was completed by the solvent mixture. The solutions (550.0, 45.0 and 9.06 μg mL^{-1} for HQ, TRT and FLA, respectively) were injected using the chromatographic conditions described above. The concentrations of HQ, TRT and FLA were calculated from the corresponding regression equation. The log of the remaining concentrations against time was plotted.

4. Results and discussion

It is important to study the stability and determine the photosensitive drugs HQ, TRT and FLA in a mixture applied topically to the skin. Hydroquinone concentration was high in this formula and its degradation product 1,4 benzoquinone formed is irritant and can be absorbed through skin. It is very toxic and possible carcinogen.[16]

The reported HPLC methods[3,4] determined HQ, TRT and FLA not in presence of their degradation products and preservatives. The manufacturer HPLC method determined HQ, FLA and the preservatives MET and PRP in one run but another HPLC method was used for determining TRT. The reported methods on FLA[10–12] did not mention its photodegradation pathway.

Hydroquinone was subjected to alkali, acid hydrolysis, oxidation and UV light as it was formulated in higher concentration compared to TRT and FLA in ratio of 400 : 5 : 1. Hydroquinone and BQ were separated by the proposed HPLC method at retention times 1.21 and 1.76 min, respectively. The alkali solution of HQ was turned dark by time. After one day, the HQ alkali solution showed three peaks by the proposed HPLC method corresponding to HQ, BQ and another peak at retention time 0.84 min, Fig. 1A. The HQ alkali solution showed complete degradation after ten days, confirmed by the proposed HPLC method, showing only one peak at retention time 0.84 min and disappearance of the peaks corresponding to HQ and BQ as shown in Fig. 1B. The complete alkali degradation of HQ was also confirmed by the proposed TLC method (dissolved in water and acetonitrile mixture 50 : 50, v/v) showing one brown spot at the starting line, while no spot was observed at R_f 0.20 or 0.65 corresponding to the intact drug and BQ. The alkali degradation product residue formed was dark brown colored and soluble in mixture of water and acetonitrile. Hydroquinone can easily undergo oxidative polymerization in alkali solution but the reaction product molecular weight was not identified.[17–21]

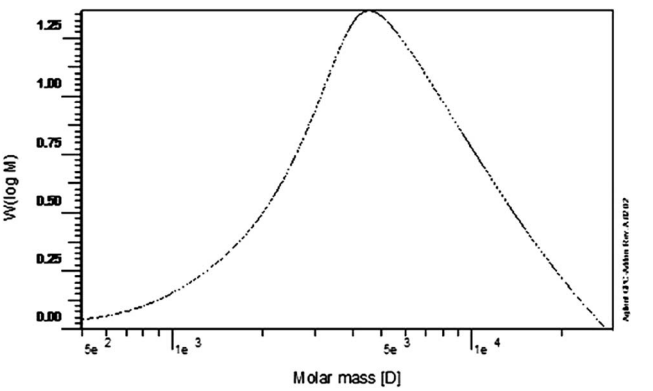

Fig. 2 Gel permeation chromatogram report of the polymer from hydroquinone alkali degradation showing its number average molecular weight and weight average molecular weight. M_n: 3.6370 × 10^3 g mol^{-1}, M_w: 6.1701 × 10^3 g mol^{-1}, M_z: 9.3810 × 10^3 g mol^{-1}, M_v: 0.000000 g mol^{-1}, D: 1.6965 × 10^0, [n]: 0.000000 mL g^{-1}, V_p: 8.1935 × 10^0 mL, M_p: 4.5612 × 10^3 g mol^{-1}, A: 2.6265 × 10^4 ml V. 10% 1.8513 × 10^3 g mol^{-1}, 30% 3.4399 × 10^3 g mol^{-1}, 50% 4.9007 × 10^3 g mol^{-1}, 70% 7.1263 × 10^3 g mol^{-1}, 90% 1.2361 g mol^{-1}. Baseline was from 0.000 to 19.962 min. integration was from 7.183 to 9.388 min, aquisation interval: 0.430 s. MHK-A (Cal.): 0.000000 × 10^{+0}, MHK-K (Cal.): 1.000000 × 10^{+0} mL g^{-1}, injection volume: 100.00 μL, delay volume: 0.000 mL, sample concentration: 0.01 gm in 2 mL, mobile phase and solvent: 0.2 g L^{-1} sodium azide in water.

Gel permeation chromatography was used for this polymer characterization.[22] The molecular mass of a polymer differs from typical molecules, in that polymerization reactions produce a distribution of molecular weights and shapes. The distribution of molecular masses can be summarized by the number average molecular weight, weight average molecular weight, and polydispersity.[23] Fig. 2 showed the number average molecular weight (M_n) to be 3637 and the weight average molecular weight (M_w) was 6170.1. The distribution of molecular weights in a polymer sample is the polydispersity index, which found to be 1.7. Hydroquinone was stable in acidic medium. It showed incomplete degradation upon oxidation and photodegradation resulting in formation of BQ, Fig. 1C and D. The formed BQ was confirmed by comparing it with pure BQ solution using the proposed HPLC and TLC methods separating it at retention time 1.76 min and R_f 0.65, respectively.

The photodegradation of TRT showed two degradation products which were separated and confirmed by the proposed HPLC method as shown in Fig. 1E. The first degradation product was isotretinoin (13-*cis* retinoic acid) separated at retention time 16.96 min which is the same retention time of pure ISO injected using the proposed HPLC method. The

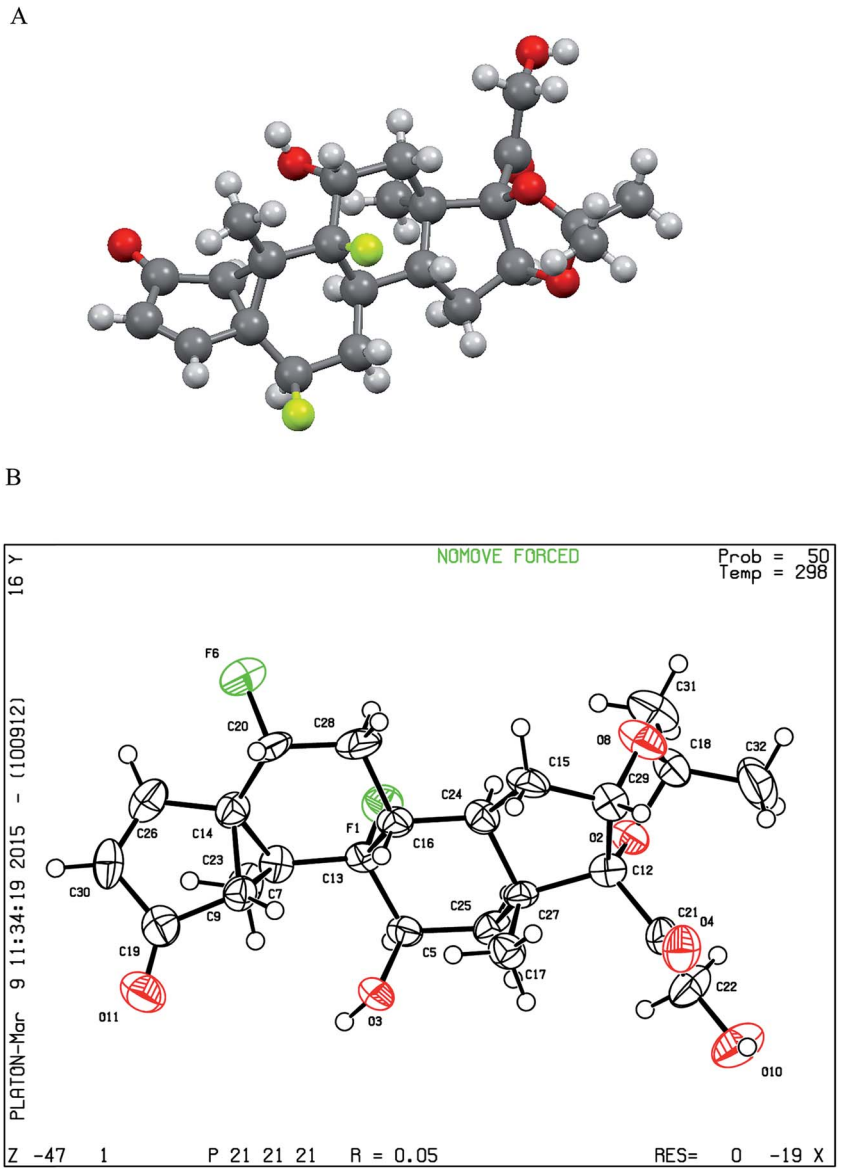

Fig. 3 (A) Molecular structure of flucinolone acetonide photodegradation product (that crystallized from acetonitrile) in Mercury® software determined by X-ray diffraction showing hydrogen atoms. (B) Oak Ridge Thermal Ellipsoid Plot (ORTEP) of flucinolone acetonide photodegradation product determined by X-ray diffraction.† Crystal data: $C_{24}H_{30}F_2O_6$, $M = 452.494$, orthorhombic, space group $P2_12_12_1$, $a = 9.1420$ (3)Å, $b = 11.9074$ (4)Å, $c = 20.3303$ (10)Å, $V = 2213.1$ (2)Å^3, $Z = 4$, $D_x = 1.358$ Mg m^{-3}, density measured by: not measured, fine-focus sealed tube, Mo Kα radiation, $\lambda = 0.71073$, $\mu = 0.11$, $T = 298$ K, 3848 measured reflections, 3059 independent reflections, 815 observed reflections, $R_{int} = 0.038$. Refinement on F^2: full matrix least squares refinement, $R_{(all)} = 0.180$, $R_{(gt)} = 0.033$, $wR_{(ref)} = 0.067$, $wR_{(all)} = 0.120$, $wR_{(gt)} = 0.067$, $S_{(ref)} = 1.315$, $S_{(all)} = 1.370$, $S_{(gt)} = 1.315$. Flack parameter = 0 (2).

second degradation product was eluted at retention time 15.50 min which may be 9-*cis* retinoic acid.[5–8]

Fluocinolone acetonide was subjected to photodegradation at 254 nm in acetonitrile showing one degradation product. This drug was sensitive to light and its degradation pathway was affected by the wavelength and the solvent used. In the reported studies[13,14] the photodegradation pathways differ when using wavelengths 365 and 312 nm. The degradation product of FLA was separated at retention time 8.89 min and R_f 0.20 by the proposed HPLC and TLC methods, respectively. This FLA degradation product is colorless needle crystals could be easily identified by single crystal X-ray diffraction. The product showed chemical formula $C_{24}H_{30}F_2O_6$ the same as the intact drug and its structure was shown in Fig. 3. The X-ray analysis established the structure and relative stereochemistry of FLA degradation product that its absolute structure could then be inferred from the known stereochemistry of the intact FLA.[24] The mechanism of this product formation was a kind of a photochemical reaction similar to santonin lumisantonin rearrangement[25,26] and the photodegradation of triamcinolone acetonide.[27] The C-30 carbonyl group was moved to C-19 and the C-7 carbon was inverted. The suggested IUPAC nomenclature of this new compound was (2*S*,5a*S*,5b*R*,5c*R*,6*S*,7a*S*,7b*S*,10a*R*,11a*S*,11b*S*)-2,5c-difluoro-6-hydroxy-7b-(2-hydroxyacetyl)-5b,7a,9,9-tetramethyl-1,5a,5b,5c,6,7,7a,7b,10a,11,11a,11b-dodecahydrocyclopenta[2″,3″]cyclopropa-[1″,2″:3′,4′]-benzo-[1′,2′:4,5]-indeno-[1,2-*d*]-[1,3]-dioxol-5(2*H*)-one. As shown in Fig. 4, a beta–beta bond is formed. Subsequent to this, radiationless decay was found lead to a zwitterion ground state.[28]

4.1. For HPLC method

Several trials were carried out to obtain a good resolution between the selected drugs and their degradation products. These trials involved the use of different mobile phases with different ratios, different pH and wavelengths. The best resolution with sharp and symmetric peaks was obtained using the chromatographic conditions described. The retention times for the intact drugs, their degradation products and the preservatives were shown in Fig. 5.

A gradient mobile phase program was developed starting with more aqueous solution and ending with more organic one because the polarity of the separated compounds varied a lot from very polar compounds (HQ) to very non polar ones (TRT

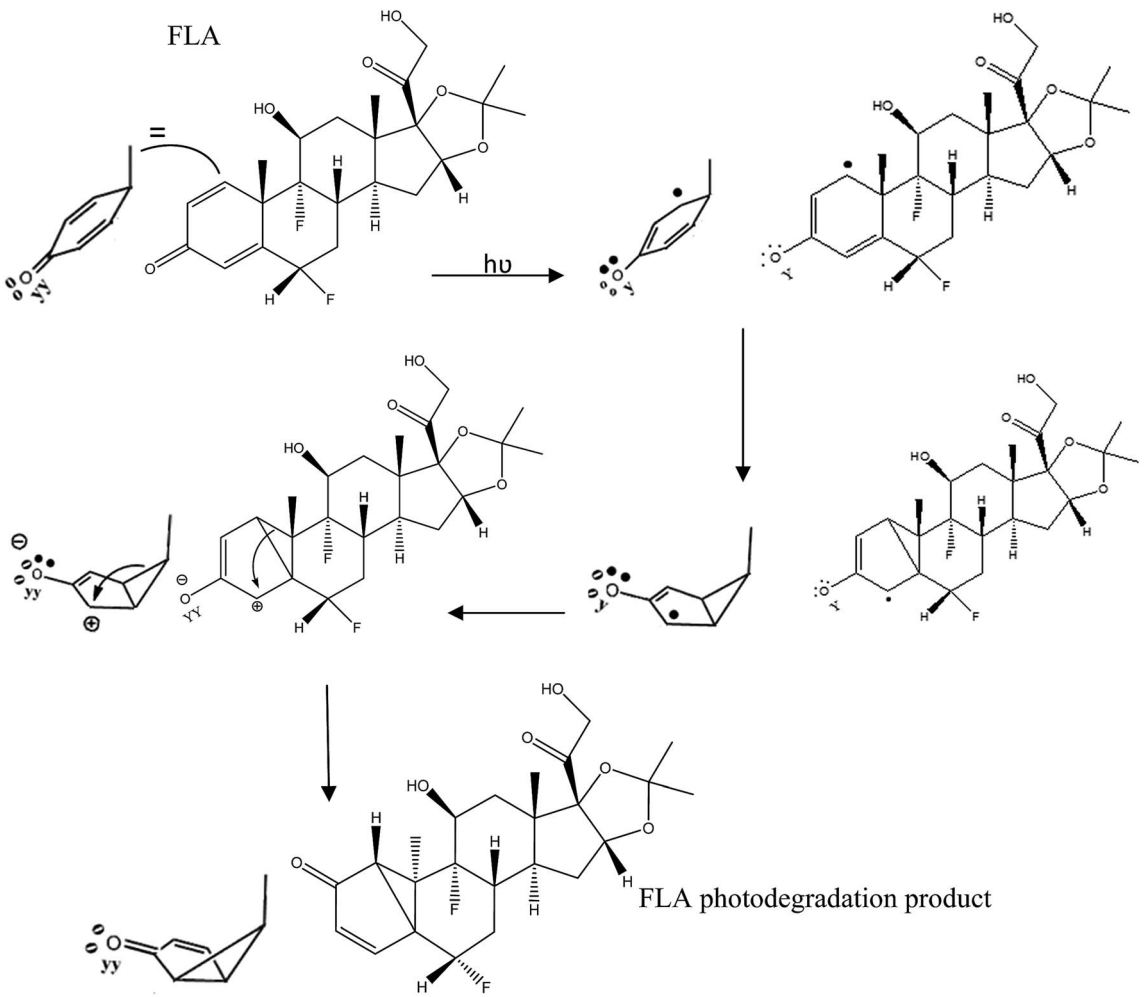

Fig. 4 The proposed pathway of photodegradation of fluocinolone acetonide.

and ISO). The solvent mixture of acetonitrile : water (50 : 50, v/v) dissolved the selected compounds and gave best peak symmetry for HQ, MET and FLA.

The pH of the mobile phase was very critical affecting the retention time of the compounds specially TRT and ISO. At pH 5.5, the retention times of TRT and ISO were delayed to 23 and 24 min, by increasing the pH to 6.8, TRT and ISO were eluted at

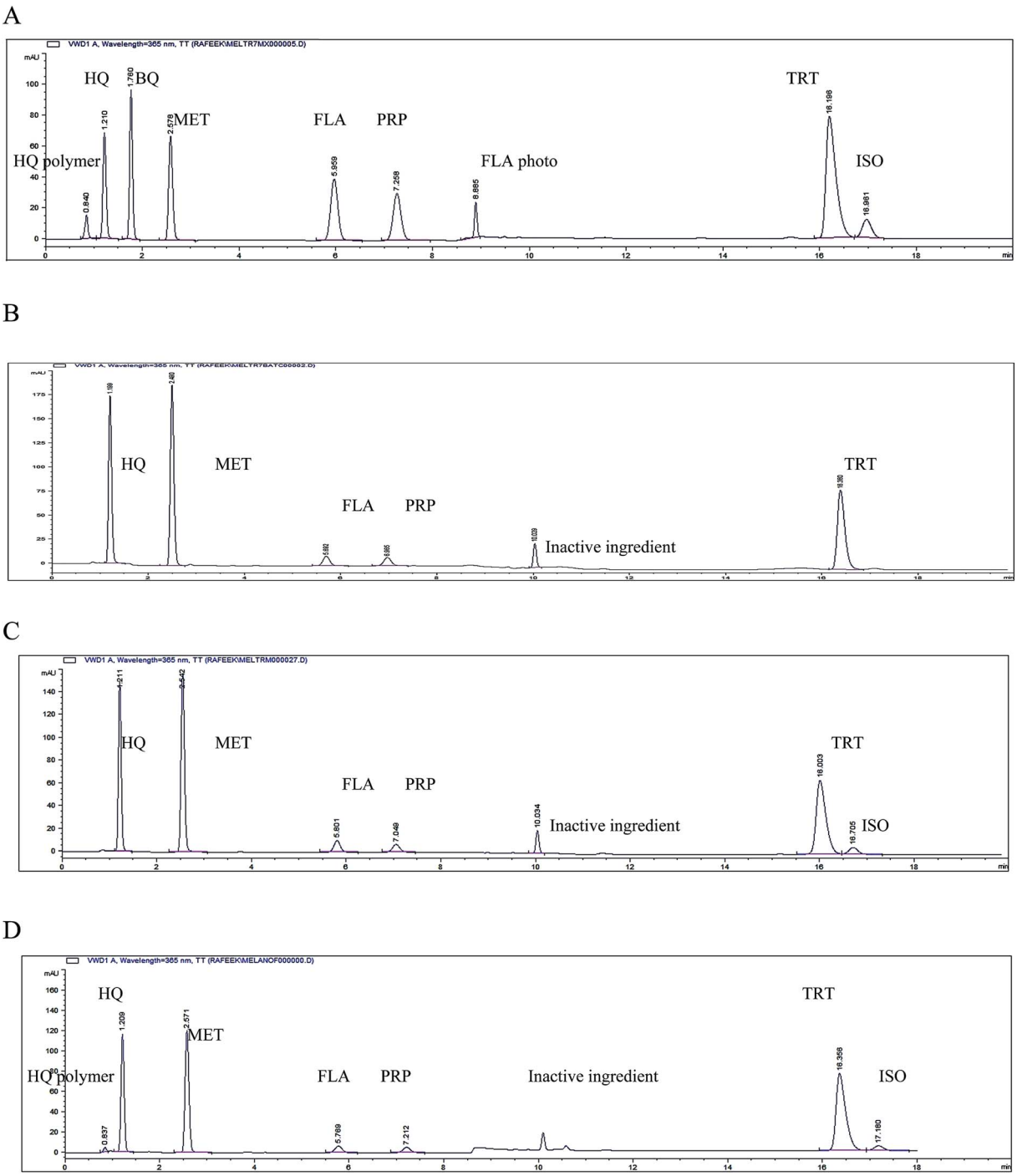

Fig. 5 HPLC chromatograms of: (A) the laboratory prepared mixture of hydroquinone, tretinoin, fluocinolone acetonide, hydroquinone polymer, benzoquinone, isotretinoin, fluocinolone acetonide photodegradation product, methyl and propyl paraben 400.00, 10.00, 10.00, 14.00, 1.00, 1.00, 10.00, 10.00 and 10.00 μg mL^{-1}, respectively. (B) The pharmaceutical formulation Melanofree® cream batch no. 140078 for the assay showing hydroquinone, tretinoin, fluocinolone acetonide, methyl and propyl paraben 800.00, 10.00, 2.00, 20.00, 2.00 μg mL^{-1}, respectively. (C) The pharmaceutical formulation Melanofree® cream batch no. 130054 for the assay showing hydroquinone, tretinoin, fluocinolone acetonide, methyl and propyl paraben 800.00, 10.00, 2.00, 20.00, 2.00 μg mL^{-1}, respectively with presence of isotretinoin 0.53 μg mL^{-1}. (D) The pharmaceutical formulation Melanofree® cream batch no. 130054 with detection of hydroquinone polymer.

Table 1 System suitability parameters of the proposed HPLC method

Parameter	Obtained value									Reference value
	HQ polymer	HQ	BQ	MET	FLA	PRP	FLA photo degradation	TRT	ISO	
Resolution (R_s)		3.18	4.71	6.32	15.82	4.49	8.12	30.83	2.07	$R > 1.5$
Selectivity (α)		1.44	1.45	1.47	2.31	1.22	1.22	1.82	1.05	>1
Tailing factor (T)	1.10	0.86	0.87	0.84	0.94	0.91	0.79	0.75	0.86	~1
Number of theoretical plates (N)	959	1534	4145	4772	7377	9347	126 045	30 097	34 473	Increase with efficiency of separation
HETP height equivalent to theoretical plate (cm per plate)	0.01	6.5×10^{-3}	2.4×10^{-3}	2.1×10^{-3}	1.4×10^{-3}	1.1×10^{-3}	7.9×10^{-5}	3.3×10^{-4}	2.9×10^{-4}	The smaller the value the higher the column efficiency
Retention time min ± 0.2	0.84	1.21	1.76	2.58	5.96	7.30	8.89	16.19	16.96	

retention time 12 min with poor separation, therefore the optimum pH was 6.1 ± 0.1.

A wavelength time table was developed for maximum detection of the selected compounds. It was started with 365 nm to detect HQ polymer as it was colored and to eliminate the noise peaks found in pharmaceutical formulation. Hydroquinone could be detected at 315 nm to give response in the same scale of the other compounds. A wavelength 235 nm was selected to determine BQ, MET, PRP, FLA photodegradation and specially FLA as it was found in small concentration and the wavelength selected was its maximum wavelength. From 8.40 till 8.75 min the detector was set at 365 to diminish the baseline noise due to changing of mobile phase. The maximum wavelength for detection of TRT and ISO was 365 nm.

System suitability parameters of the proposed HPLC method were calculated showing good resolution, selectivity and symmetrical peaks according to the USP,[29] Table 1. When changing the wavelength ± 2 nm, the system suitability parameters did not change giving the same good results indicating the robustness of the proposed method.

4.2. For TLC-densitometric method

This proposed method could determine HQ, TRT, FLA, ISO, MET and PRP, Fig. 6. 1,4-Benzoquinone (BQ) could only be detected at R_f 0.65 without any determination because of its gradual sublimation[30] giving non reproducible results. The HQ polymer was remained at the starting line when dissolved in a mixture of equal volumes of water and acetonitrile. The photodegradation product of FLA could only be determined if present in the raw material of FLA, Table 2. It could not be determined with the cited components in pharmaceutical formulation as it was separated at the same R_f of HQ and the concentration of the intact FLA in pharmaceutical formulation was smaller 400 times than HQ. The experimental condition for TLC-densitometric method as developing system, scan mode and wavelength of the detection were optimized to provide accurate and precise results for determination of the cited components. The wavelength of the scanning 235 nm could determine all the cited components but TRT and ISO were best detected at 365 nm for maximum sensitivity. The best separation of the studied compounds was obtained under the chromatographic conditions described.

Using β-cyclodextrin aqueous solution in a small amount (4 μg mL^{-1}) as a chiral material in developing system enabled the separation of TRT and its isomer ISO without increasing its amount to be compatible with the rest of the developing system components. Both acetic acid and acetone had a role in separation and developing of TRT, ISO from BQ and developing FLA.

Method validation was performed according to ICH[31] guidelines for the two proposed methods. Tables 2 and 3 showed results of accuracy, repeatability and intermediate precision of the methods. Robustness of the proposed methods was determined from small changes in some of conditions, as shown in Tables 2 and 3, showing small changes in recovery (98.00–102.00%) and retention time (±0.2 min) or R_f (±0.03). Characteristic parameters for the regression equations of the

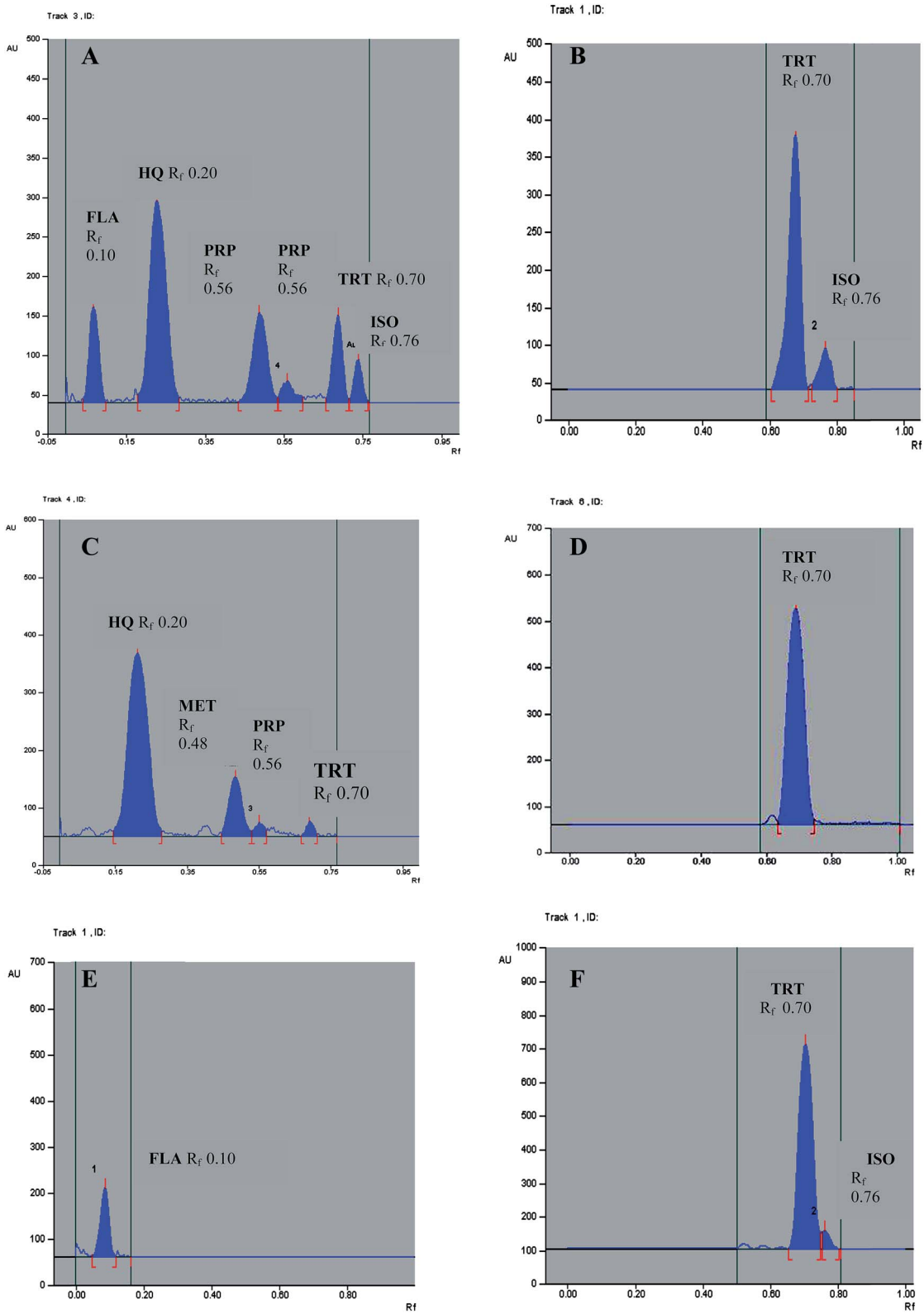

Fig. 6 Densitogram of: (A) laboratory prepared mixture of hydroquinone, tretinoin, fluocinolone acetonide, isotretinoin, methyl and propyl paraben 12.00, 1.50, 0.60, 1.25, 0.40 and 0.060 μg per spot, respectively at 235 nm. (B) Laboratory prepared mixture showing tretinoin and isotretinoin of concentrations 1.75 and 0.50 μg per spot at 365 nm. (C) Hydroquinone, methyl and propyl paraben (24.00, 0.60 and 0.060 μg per spot) in pharmaceutical formulation batch no. 140078 for their determination at 235 nm. (D) Tretinoin (1.00 μg per spot) in pharmaceutical formulation batch no. 140078 for its determination at 365 nm. (E) Fluocinolone acetonide (0.60 μg per spot) in pharmaceutical formulation batch no. 140078 for its determination at 235 nm. (F) Tretinoin (1.00 μg per spot) in pharmaceutical formulation batch no. 130054 for its determination at 365 nm with detection of isotretinoin.

Table 2 Characteristic parameters of hydroquinone, tretinoin, fluocinolone acetonide, hydroquinone polymer, benzoquinone, isotretinoin, fluocinolone acetonide photodegradation, methyl and propyl paraben by the proposed HPLC method

Characteristic parameter	HQ	TRT	FLA	HQ polymer	BQ	ISO	FLA photo degradation	MET	PRP
Range μg mL^{-1}	40.0–1000.00	1.00–50.00	1.00–20.00	5.60–28.00	0.20–2.00	0.20–10.00	2.00–20.00	1.00–25.00	1.00–20.00
Linearity									
Slope	0.7834	111.2300	45.1730	5.2489	398.1800	151.3400	14.1630	38.8230	35.2430
Intercept	0.5680	2.5182	0.0728	−0.4020	−7.1667	2.2774	1.2677	3.5183	8.2558
Correlation coefficient (r)	0.9998	0.9998	0.9999	0.9998	0.9998	0.9998	0.9996	0.9997	0.9999
SE of the slope[a]	0.0065	0.9067	0.1750	0.0422	3.9064	1.4005	0.1979	0.4839	0.2547
CL of the slope[b]	0.7652–0.8015	108.7137–113.7482	44.6876–45.6591	5.1320–5.3661	387.3341–409.0262	147.4475–155.2243	13.6127–14.7118	37.4796–40.1666	34.5354–35.9498
SE of the intercept	3.9524	27.4527	2.1200	0.7707	4.9619	8.4812	2.5141	7.3279	3.0864
CL of the intercept	−10.4041 to 11.5432	−73.7004 to 78.7417	−5.8149 to 5.9574	−2.5428 to 1.7370	−20.9428 to 6.6098	−21.2717 to 25.8236	−5.7082 to 8.2524	−16.8270 to 23.8642	−0.3122 to 16.8261
Accuracy (mean ± SD)	99.76 ± 1.16	100.54 ± 1.25	100.24 ± 1.60	99.47 ± 0.70	99.57 ± 0.44	100.15 ± 1.33	99.48 ± 0.81	100.36 ± 1.17	99.72 ± 0.96
Precision (% RSD)									
Repeatability[c]	0.24	0.44	1.08	1.26	0.59	1.45	0.33	0.88	1.07
Intermediate precision[d]	0.90	1.12	1.59	1.48	0.63	1.71	0.97	0.96	1.16
Specificity and selectivity	100.29 ± 1.08	100.13 ± 1.23	99.23 ± 1.00	99.21 ± 1.28	99.73 ± 1.57	100.49 ± 1.59	100.09 ± 1.36	100.57 ± 1.15	99.92 ± 1.11
Robustness[e]	100.80 ± 0.98	100.95 ± 1.30	100.82 ± 0.78	101.03 ± 1.53	101.45 ± 0.57	101.07 ± 0.95	100.73 ± 1.21	101.14 ± 0.88	100.90 ± 1.28
LOD[f] μg mL^{-1}	5.39	0.20	0.18	1.16	0.05	0.04	0.64	0.22	0.22
LOQ[f] μg mL^{-1}	16.34	0.59	0.56	3.52	0.14	0.13	1.94	0.67	0.66

[a] Standard error. [b] Confidence limit. [c] The intraday (n = 9), average of three different concentrations repeated three times within day. [d] The interday (n = 9), average of three different concentrations repeated three times in three successive days. [e] Average in the change of pH (±0.1), flow rate (±0.1 min) and ratio of mobile phase (±2%). [f] Limit of detection and quantitation are determined *via* calculations, LOD = (SD of regression residuals/slope) × 3.3; LOQ = (SD of the response/slope) × 10.

Table 3 Characteristic parameters of hydroquinone, tretinoin, fluocinolone acetonide, isotretinoin, methyl and propyl paraben by the proposed TLC-densitometric method

Characteristic parameter	HQ	TRT	FLA	ISO	FLA photo degradation	MET	PRP
Range μg per spot	1.20–30.00	0.50–1.75	0.40–1.40	0.50–1.75	1.00–3.50	0.40–1.40	0.040–0.140
Linearity							
Slope	650.75	18 025.00	3996.20	8346.70	2133.40	5088.10	10 269.00
Intercept	3691.00	−7604.10	575.49	−2780.00	323.56	2329.50	3339.73
Correlation coefficient (r)	0.9996	0.9994	0.9995	0.9996	0.9991	0.9996	0.9996
SE of the slope[a]	9.2614	269.1465	61.5205	111.6972	44.1816	72.9537	135.5337
CL of the slope[b]	625.0320–676.4597	−8498.66 to −6700.29	3825.3931–4167.0098	8036.5396–8656.7816	2010.7665–2256.1020	4885.5470–5290.6510	9892.7981–10 645.4019
SE of the intercept	168.3032	323.8624	59.2218	134.4045	106.3269	70.2278	13.0470
CL of the intercept	3223.7120–4158.2811	17 291.62–18 786.16	411.0642–739.9165	−3153.1599 to −2406.8264	28.3454–618.7670	2134.506–2524.473	303.5019–375.9501
Accuracy (mean ± SD)	99.70 ± 1.23	101.17 ± 0.53	99.32 ± 1.09	100.54 ± 1.45	100.67 ± 1.27	99.86 ± 1.54	100.82 ± 1.47
Precision (% RSD)							
Repeatability[c]	0.79	0.36	0.51	1.63	1.74	1.12	1.71
Intermediate precision[d]	1.32	0.75	1.14	1.76	1.91	1.42	1.75
Specificity and selectivity	100.90 ± 0.52	101.15 ± 1.13	99.80 ± 1.16	100.40 ± 1.80	—	100.51 ± 1.32	99.72 ± 1.33
Robustness[e]	100.63 ± 0.88	100.85 ± 0.81	100.13 ± 0.89	101.17 ± 0.85	101.01 ± 0.63	100.83 ± 0.88	100.42 ± 1.03
LOD[f] μg per spot	0.24	0.05	0.12	0.04	0.13	0.04	0.003
LOQ[f] μg per spot	0.72	0.14	0.04	0.13	0.39	0.11	0.010

[a] Standard error. [b] Confidence limit. [c] The intraday ($n = 9$), average of three different concentrations repeated three times within day. [d] The interday ($n = 9$), average of three different concentrations repeated three times in three successive days. [e] Average in the change of developing system composition ± 3.0, 2.0, 0.4, 0.2, 0.1 mL of petroleum ether–ethyl acetate–acetone–acetic acid–β-cyclodextrin solution and distance development (±0.5 cm). [f] Limit of detection and quantitation are determined *via* calculations, LOD = (SD of regression residuals/slope) × 3.3; LOQ = (SD of the response/slope) × 10.

proposed methods were also given in Tables 2 and 3. The proposed HPLC and TLC-densitometric methods were selective and accurate for determination of the cited components in laboratory prepared mixtures, Tables 4 and 5.

The suggested methods were valid and applicable for the analysis of HQ, TRT, FLA, MET and PRP in their pharmaceutical formulation Melanofree® cream, Tables 6 and 7. The limit of ISO in TRT must not exceed 5.0% as stated in USP.[29] Isotretinoin was present above the limit in pharmaceutical formulation batch no. 130054 near the expiry date; its amount was determined by the proposed HPLC method to be 5.3%, Fig. 5C, and could be detected by the proposed TLC-densitometric method, Fig. 6F. Also, in this batch brown spots were found due to excessive opening of the cream tube detecting HQ polymer by the proposed HPLC method, Fig. 5D.

The validity of the proposed methods was assessed by applying the standard addition technique, which showed accurate results and there is no interference from excipients as shown in Tables 6 and 7. Butylated hydroxyltoluene, one of the inactive ingredients, was separated at retention time 10 min and at the front line by the proposed HPLC and TLC-densitometric methods. This was confirmed by applying pure butylated hydroxyltoluene.

Statistical comparison of the results of the compounds analysis obtained by the proposed methods and the manufacturer one was also done using student's t-test and the F-ratio at 95% confidence level (Tables 8 and 9). It was clear that there is no significant difference between the proposed and the manufacturer methods with regard to accuracy and precision. The proposed HPLC method had advantages of being more sensitive, stability indicating for simultaneous determination of the cited drugs and faster elution than the reported one.[4]

4.3. Kinetics of the photodegradation

In this work, a comparative kinetic investigation of HQ, TRT and FLA photodegradation was done. Calculations were based on the measurement of the concentration of intact drug using the proposed HPLC method. This study was performed twice starting with concentrations ratio used in pharmaceutical formulation and using equimolar concentration of the three drugs. The order of the photodegradation rate of the reaction was determined by following the decrease in concentration of each drug within two hours at certain time interval.

The photodegradation rate of HQ was independent on its concentration predicted to be zero order reaction. The rate constant of the degradation (K) and the half life of the reaction ($t_{1/2}$) were calculated from the equations: slope = $-K$ and $t_{1/2}$ =

Table 4 Determination of hydroquinone, tretinoin, fluocinolone acetonide, hydroquinone polymer, benzoquinone, isotretinoin, fluocinolone acetonide photodegradation, methyl and propyl paraben in laboratory prepared mixtures by the proposed HPLC method

Mixture number		HQ	TRT	FLA	HQ polymer	BQ	ISO	FLA photo degradation	MET	PRP
1[a]	Concentration (μg mL^{-1})	800.00	10.00	2.00	14.00	0.50	2.00	10.00	20.00	2.00
	Recovery[b] %	100.81	101.19	100.27	98.07	98.20	102.00	101.72	99.36	100.37
	% of the degradation products	—	—	—	1.72	0.06	20.00	83.33	—	—
2[a]	Concentration (μg mL^{-1})	800.00	10.00	2.00	5.60	0.20	0.20	2.00	20.00	2.00
	Recovery[b] %	98.84	100.32	99.17	100.03	101.50	98.20	101.43	99.44	99.89
	% of the degradation products	—	—	—	0.70	0.02	19.61	50.00	—	—
3	Concentration (μg mL^{-1})	400.00	10.00	10.00	14.00	1.00	1.00	10.00	10.00	10.00
	Recovery[b] %	101.72	101.33	98.35	98.07	101.34	102.01	99.13	101.18	98.39
	% of the degradation products	—	—	—	3.38	0.25	9.09	50.00	—	—
4	Concentration (μg mL^{-1})	1000.00	20.00	20.00	5.60	0.20	0.20	2.00	10.00	1.00
	Recovery[b] %	100.30	99.37	98.17	98.89	98.99	100.40	99.08	102.04	99.53
	% of the degradation products	—	—	—	0.56	0.02	0.99	9.09	—	—
5	Concentration (μg mL^{-1})	40.00	50.00	1.00	28.00	2.00	0.20	2.00	1.00	1.00
	Recovery[b] %	99.77	98.43	100.19	100.97	98.64	99.85	99.08	100.84	101.42
	% of the degradation products	—	—	—	41.18	4.76	0.40		—	—
	Mean ± SD	100.29 ± 1.08	100.13 ± 1.23	99.23 ± 0.99	99.21 ± 1.27	99.73 ± 1.57	100.49 ± 1.60	100.09 ± 1.36	100.57 ± 1.16	99.92 ± 1.11

[a] Ratio of HQ, TRT, FLA, MET and PRP in pharmaceutical formulation. [b] Average of 3 determinations.

Table 5 Determination of hydroquinone, tretinoin, fluocinolone acetonide, isotretinoin, methyl and propyl paraben in laboratory prepared mixtures by the proposed TLC-densitometric method

Mixture number		HQ	TRT	FLA	ISO	MET	PRP
1	Concentration (μg per spot)	12.00	1.50	0.60	1.25	0.40	0.060
	Recovery[b] %	101.68	99.37	101.30	99.39	98.80	100.21
	% of the degradation products	—	—	—	45.45	—	—
2	Concentration (μg per spot)	9.00	1.75	1.20	0.50	1.20	0.120
	Recovery[b] %	100.44	100.65	100.68	102.56	99.78	98.11
	% of the degradation products	—	—	—	22.22	—	—
3	Concentration (μg per spot)	1.20	0.50	1.00	1.75	1.00	0.100
	Recovery[b] %	100.61	101.74	99.32	98.45	101.90	98.64
	% of the degradation products	—	—	—	77.78	—	—
4[a]	Concentration (μg per spot)	24.00	0.50	0.40	0.50	0.60	0.060
	Recovery[b] %	100.58	101.98	98.42	102.08	101.77	101.37
	% of the degradation products	—	—	—	50.00	—	—
5	Concentration (μg per spot)	30.00	0.50	0.50	0.75	0.80	0.050
	Recovery[b] %	101.18	101.99	99.29	99.52	100.28	100.29
	% of the degradation products	—	—	—	60.00	—	—
	Mean ± SD	100.90 ± 0.52	101.15 ± 1.14	99.80 ± 1.16	100.40 ± 1.81	100.51 ± 1.33	99.72 ± 1.33

[a] Ratio of HQ, MET and PRP in pharmaceutical formulation. [b] Average of 3 determinations.

initial concentration in molar/2K.[32] The K forward values were nearly the same and the $t_{1/2}$ values were different when starting with different concentrations, Fig. 7. Hydroquinone was photodegraded to BQ (Fig. 1D) and its concentration remained constant due to reversibility between HQ and BQ.[33] Hydroquinone is more stable than BQ because it is more aromatic.[34] Therefore the photodegradation of HQ to BQ was reversible zero order reaction.

The photodegradation of TRT to ISO was reversible besides the formation of 9-*cis* retinoic acid (Fig. 1E) and their concentrations were decreased by time. Fig. 1F showed the photodegradation of FLA. The photodegradation rates of TRT and FLA

Table 6 Determination of hydroquinone, tretinoin, fluocinolone acetonide, methyl and propyl paraben in pharmaceutical formulation by the proposed HPLC method

	Hydroquinone		Tretinoin		Fluocinolone acetonide		Methyl paraben		Propyl paraben	
Parameter	HPLC method	Manufacturer method[d]	HPLC method	Manufacturer method[e]	HPLC method	Manufacturer method[d]	HPLC method	Manufacturer method[d]	HPLC method	Manufacturer method[d]
Mean ± SD[a] of Melanofree®[b]	100.76 ± 1.26	100.23 ± 1.04	96.46 ± 1.23	96.47 ± 1.23	99.54 ± 1.75	99.54 ± 1.65	104.33 ± 1.11	105.35 ± 0.83	90.15 ± 1.18	90.80 ± 1.55
RSD[a]	1.25	1.04	1.28	1.28	1.76	1.66	1.06	0.79	1.31	1.71
Variance	1.59	1.08	1.51	1.51	3.06	2.72	1.23	0.69	1.39	2.40
Student's *t*-test (2.306)[c]	0.726		0.013		0.000		1.647		0.747	
F-value (6.39)[c]	1.47		1.00		1.13		1.34		1.73	
Standard addition technique	100.58 ± 1.78		100.98 ± 0.68		99.87 ± 1.00		101.67 ± 0.30		100.31 ± 1.76	

[a] Standard deviation and percentage relative standard deviation for 5 determinations. [b] Labelled to contain 4% hydroquinone, 0.05% tretinoin, 0.01% fluocinolone acetonide, 0.1% methyl paraben and 0.01% propyl paraben batch no: 140078. [c] Tabulated values for t and F at $P = 0.05$ and $n = 5$. [d] Manufacturer HPLC method using ODS 250 × 4.6 mm, 5 μm column, mobile phase mixture of 320 mL acetonitrile and 680 mL 1.8 g sodium heptanes sulfonate in water pH 3.5 by phosphoric acid, flow rate 1.2 mL min^{-1} and detection at 254 nm. [e] Manufacturer HPLC method using ODS 250 × 4.6 mm, 5 μm column, mobile phase mixture of 800 mL acetonitrile and 200 mL 0.5% acetic acid in water, flow rate 1.5 mL min^{-1} and detection at 360 nm.

Table 7 Determination of hydroquinone, tretinoin, fluocinolone acetonide, methyl and propyl paraben in pharmaceutical formulation by the proposed TLC-densitometric method

	Hydroquinone		Tretinoin		Fluocinolone acetonide		Methyl paraben		Propyl paraben	
Parameter	TLC-densitometric method	Manufacturer method[d]	TLC-densitometric method	Manufacturer method[e]	TLC-densitometric method	Manufacturer method[d]	TLC-densitometric method	Manufacturer method[d]	TLC-densitometric method	Manufacturer method[d]
Mean ± SD[a] of Melanofree®[b]	99.10 ± 0.90	100.23 ± 1.04	95.83 ± 1.01	96.47 ± 1.23	98.23 ± 1.41	99.54 ± 1.65	104.53 ± 1.05	105.35 ± 0.83	91.06 ± 1.50	90.80 ± 1.55
RSD[a]	0.91	1.04	1.05	1.28	1.44	1.66	1.00	0.79	1.65	1.71
Variance	0.81	1.08	1.02	1.51	1.99	2.72	1.10	0.69	2.25	2.40
Student's *t*-test (2.306)[c]	1.839		0.900		1.351		1.371		0.270	
F-value (6.39)[c]	1.33		1.48		1.37		1.59		1.07	
Standard addition technique	98.91 ± 0.87		100.78 ± 0.68		99.03 ± 0.88		99.03 ± 1.99		100.97 ± 0.86	

[a] Standard deviation and percentage relative standard deviation for 5 determinations. [b] Labelled to contain 4% hydroquinone, 0.05% trtinoin, 0.01% fluocinolone acetonide, 0.1% methyl paraben and 0.01% propyl paraben batch no: 140078. [c] Tabulated values for t and F at $P = 0.05$ and $n = 5$. [d] Manufacturer HPLC method using ODS 250 × 4.6 mm, 5 μm column, mobile phase mixture of 320 mL acetonitrile and 680 mL 1.8 g sodium heptanes sulfonate in water pH 3.5 by phosphoric acid, flow rate 1.2 mL min^{-1} and detection at 254 nm. [e] Manufacturer HPLC method using ODS 250 × 4.6 mm, 5 μm column, mobile phase mixture of 800 mL acetonitrile and 200 mL 0.5% acetic acid in water, flow rate 1.5 mL min^{-1} and detection at 360 nm.

Table 8 Statistical comparisons of the proposed HPLC and the manufacturer methods for determination of pure hydroquinone, tretinoin, fluocinolone acetonide, isotretinoin, methyl and propyl paraben

	Hydroquinone		Tretinoin		Fluocinolone acetonide		Methyl paraben		Propyl paraben	
Parameter	HPLC method	Manufacturer method[c]	HPLC method	Manufacturer method[d]	HPLC method	Manufacturer method[c]	HPLC method	Manufacturer method[c]	HPLC method	Manufacturer method[c]
Mean ± SD[a]	99.76 ± 1.16	99.74 ± 1.15	100.54 ± 1.25	100.03 ± 1.26	100.24 ± 1.60	99.95 ± 1.59	100.36 ± 1.17	100.00 ± 1.13	99.72 ± 0.96	99.94 ± 1.23
RSD[a]	1.16	1.15	1.24	1.26	1.60	1.59	1.17	1.13	0.96	1.23
Variance	1.35	1.32	1.56	1.59	2.56	2.53		1.28	0.92	1.51
Student's *t*-test (2.306)[b]	0.027		0.643		0.288		0.164		0.316	
F-value (6.39)[b]	1.02		1.02		1.01		1.85		1.64	

[a] Standard deviation and percentage relative standard deviation for 5 determinations. [b] Tabulated values for t and F at $P = 0.05$. [c] Manufacturer HPLC method using ODS 250 × 4.6 mm, 5 μm column, mobile phase mixture of 320 mL acetonitrile and 680 mL 1.8 g sodium heptanes sulfonate in water pH 3.5 by phosphoric acid, flow rate 1.2 mL min^{-1} and detection at 254 nm. [d] Manufacturer HPLC method using ODS 250 × 4.6 mm, 5 μm column, mobile phase mixture of 800 mL acetonitrile and 200 mL 0.5% acetic acid in water, flow rate 1.5 mL min^{-1} and detection at 360 nm.

Table 9 Statistical comparisons of the proposed TLC-densitometric and the manufacturer methods for determination of pure hydroquinone, tretinoin, fluocinolone acetonide, isotretinoin, methyl and propyl paraben

	Hydroquinone		Tretinoin		Fluocinolone acetonide		Methyl paraben		Propyl paraben	
Parameter	TLC-densitometric method	Manufacturer method[c]	TLC-densitometric method	Manufacturer method[d]	TLC-densitometric method	Manufacturer method[c]	TLC-densitometric method	Manufacturer method[c]	TLC-densitometric method	Manufacturer method[c]
Mean ± SD[a]	99.70 ± 1.23	99.74 ± 1.15	101.17 ± 0.53	100.03 ± 1.26	99.32 ± 1.09	99.95 ± 1.59	99.86 ± 1.54	100.00 ± 1.13	100.82 ± 1.47	99.94 ± 1.23
RSD[a]	1.23	1.15	0.52	1.26	1.10	1.59	1.54	1.13	1.46	1.23
Variance	1.51	1.32	0.28	1.59	1.19	2.53	2.37	1.28	2.16	1.51
Student's *t*-test (2.306)[b]	0.053		1.865		0.731		0.164		1.028	
F-value (6.39)[b]	1.14		5.68		2.13		1.85		1.43	

[a] Standard deviation and percentage relative standard deviation for 5 determinations. [b] Tabulated values for t and F at $P = 0.05$. [c] Manufacturer HPLC method using ODS 250 × 4.6 mm, 5 μm column, mobile phase mixture of 320 mL acetonitrile and 680 mL 1.8 g sodium heptanes sulfonate in water pH 3.5 by phosphoric acid, flow rate 1.2 mL min^{-1} and detection at 254 nm. [d] Manufacturer HPLC method using ODS 250 × 4.6 mm, 5 μm column, mobile phase mixture of 800 mL acetonitrile and 200 mL 0.5% acetic acid in water, flow rate 1.5 mL min^{-1} and detection at 360 nm.

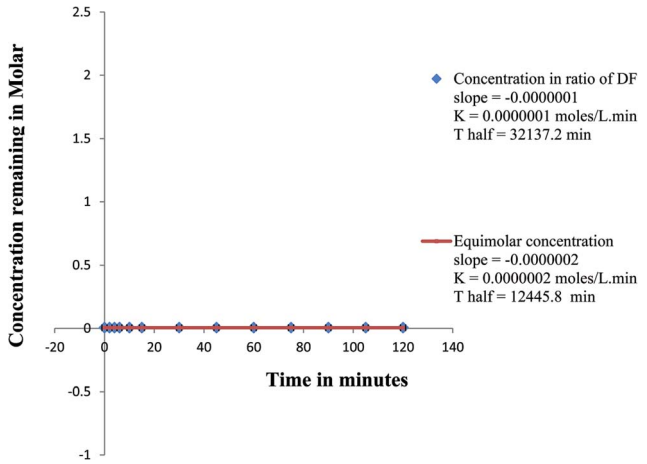

Fig. 7 Zero order kinetic plot of the photodegradation of hydroquinone starting with concentrations ratio used in pharmaceutical formulation (initial concentrations 8.0 mg mL^{-1} in acetonitrile) and equimolar concentration (10 mmoL in acetonitrile).

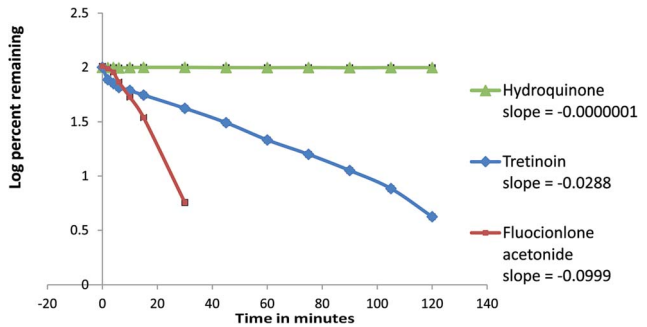

Fig. 8 Kinetic plot of the photodegradation of hydroquinone, tretinoin and fluocinolone acetonide starting with concentrations ratio used in pharmaceutical formulation (initial concentrations 8.0 mg mL^{-1}, 100.0 μg mL^{-1} and 20.0 μg mL^{-1} in acetonitrile, respectively).

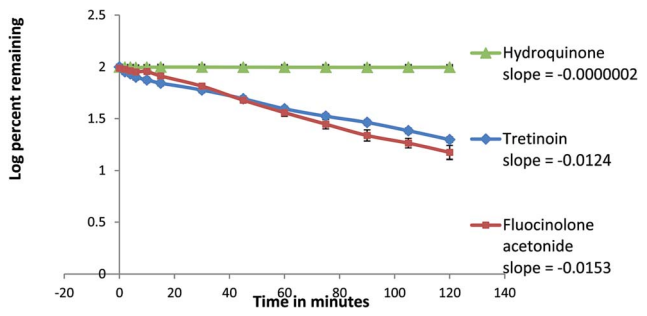

Fig. 9 Kinetic plot of the photodegradation of hydroquinone, tretinoin and fluocinolone acetonide starting with equimolar concentrations (initial concentrations 10 mmol in acetonitrile of each).

could not be expressed by simple first order equations because their rates changed with different concentrations. The photodegradation rates of TRT and FLA were proposed to be complex reactions.[32]

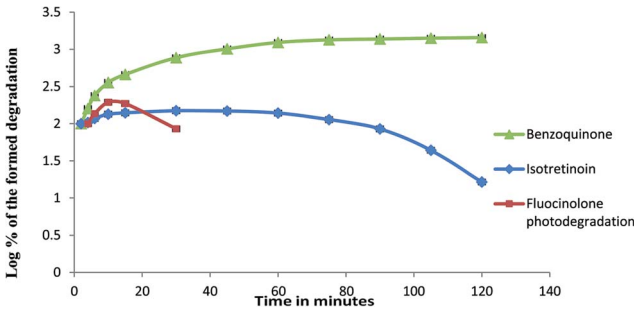

Fig. 10 Kinetic plots of the photodegradation products (benzoquinone, isotretinoin and fluocinolone acetonide photodegradation product) formed from hydroquinone, tretinoin and fluocinolone acetonide when starting with concentrations ratio used in pharmaceutical formulation.

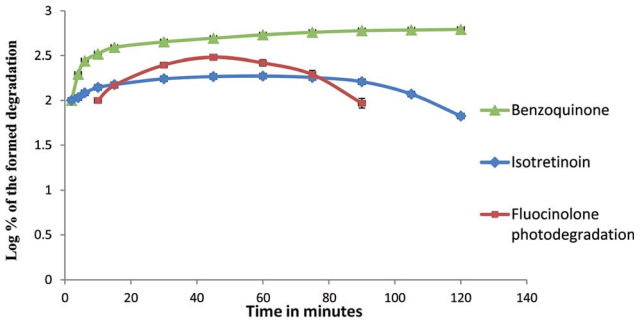

Fig. 11 Kinetic plots of the photodegradation products (benzoquinone, isotretinoin and fluocinolone acetonide photodegradation product) formed from hydroquinone, tretinoin and fluocinolone acetonide when starting with equimolar concentrations.

Starting with concentrations ratio used in pharmaceutical formulation, FLA was degraded faster than TRT. While, both TRT and FLA showed nearly the same rate of degradation when starting with equimolar concentrations (Fig. 8 and 9).

A comparison to the formed photodegradation products was shown in Fig. 10 and 11. 1,4-Benzoquinone formed was increased gradually and remained constant. Isotretinoin formed was increased gradually then remained stable and started in disappearing. The formed FLA photodegradation product was faster in disappearance.

5. Conclusion

The proposed HPLC and TLC-densitometric methods provided simple, sensitive, selective and accurate simultaneous determination of HQ, TRT, FLA, the preservatives MET and PRP in pure form and pharmaceutical formulation, without any interference from the excipients and in presence of the drugs degradation products. The proposed methods were validated and could be used for routine analysis in quality control laboratories, where economy and time are essential.

The HPLC method was found to be more sensitive than the TLC-densitometric one, The HPLC method could simultaneously determine nine components (the three active

ingredients, the preservatives, HQ polymer, BQ, ISO and FLA photodegradation product). Separation of TRT photodegradation product; 9-*cis* retinoic acid was done by the proposed HPLC method only.

The TLC-densitometric method could simultaneously determine six components (the three active ingredients, the preservatives and ISO) and separate BQ as it is liable to sublimation. The advantage of this method was the separation of TRT and its isomer ISO using chiral developing system besides the other components in one run. Fluocinolone photodegradation product could be determined by the proposed TLC-densitometric method if present with the intact drug in raw material only. This method had also the advantages of short run time, large sample capacity and the use of minimal value of solvent.

The degradation products HQ polymer and ISO were found in pharmaceutical formulation near expiry date. Also, the proposed HPLC method had the advantage of lower limit of detection of ISO, 0.04 $\mu g\ mL^{-1}$.

The proposed HPLC method was used in comparative determination of HQ, TRT and FLA photodegradation kinetic rate in acetonitrile within two hours. Hydroquinone showed reversible zero order kinetics. Tretinoin and fluocinolone acetonide followed complex kinetic reactions.

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