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A stability indicating RP-HPLC-UV assay method for the simultaneous determination of hydroquinone, tretinoin, hydrocortisone, butylated hydroxytoluene and parabens in pharmaceutical creams

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ABSTRACT

Multicomponent drugs are medications that combine two or more active pharmaceutical ingredients in a single dosage form. These dosage forms improve the patient compliance, reduce the risk of drug interactions, and simplify dosing regimens. However, quality control of these multicomponent dosage forms can be challenging, especially if the final product contains four or more ingredients that are active (comprise stabilizers, preservatives, excipients, and other components). This problem can be more pronounced if the excipients can interfere with the analysis. In this work, a stability indicating assay method was developed and validated (according to the ICH International Guidelines) for the simultaneous determination of hydroquinone (HQ), tretinoin (TRT), hydrocortisone (HCA), butylated hydroxytoluene (BHT), methyl paraben (MP) and propyl paraben (PP) in commercially available pharmaceutical creams. The proposed method is based on gradient elution using X-Bridge C18 (150 × 4.6 mm, 5 μm) column with a flow rate of 1 mL/min. The linear ranges (μg/mL) were 240–560 for HQ, 24–56 for MP, 132–308 for HCA, 6–14 for PP, 12–28 for BHT, 6.6–15 for TRT. During the validation process, the intra- and interday precision and trueness (evaluated as recovery) were found to be below 2.0% and between 100–102%, respectively. System suitability tests (SST) allow validating the herein proposed procedure specifically for pharmaceutical and industrial applications. SST test shows that the reported procedure fulfill with the Guidelines, allowing excellent separation of the analytes with very sensitive, accurate (precise and true) and reproducible quantitation of each analytes. The method was successfully applied in forced degradation studies of the six analytes. Specifically, acid degradation slightly affected HCA and BHT (91% recovery), while alkaline degradation drastically reduced HCA recovery (5.5%) and moderately affected BHT (85%). Photodegradation primarily influenced TRT quantity, and oxidative degradation intensified the BHT peak (130%).

1. Introduction

Topical pharmaceutical formulations that contain a combination of hydroquinone (HQ), corticosteroids, and tretinoin (TRT) are used to treat melasma, a specific skin condition that affects the face, precisely the cheeks, forehead, and upper lip, and is generally characterized by

Abbreviations: As, asymmetry factor; BHT, Butylated hydroxytoluene; HCA, hydrocortisone acetate; HQ, Hydroquinone; SST, System suitability tests; ICH, International Conference of Harmonization: k', retention factor; LOD, limits of detection; LOQ, limits of quantitation; MP, Methyl paraben; N, number of theoretical plates; PP, propyl paraben; RP-HPLC, Reversed-phase high-performance liquid chromatography; Rs, resolution; S/N, signal-to-noise ratio; t_r, retention time; TRT,, Tretinoin; α, selectivity.

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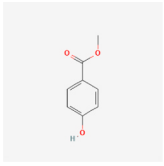
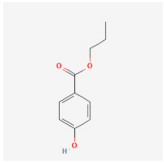
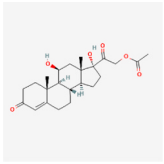
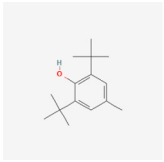
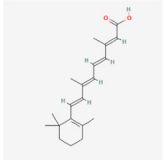
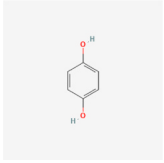
Table 1
Chemical structures and properties of the investigated analytes.

Compound	Chemical structure	Molecular Weight (g/mol)	Protein Binding	Half life	LogP	pKa (Strongest Acidic)
Methyl paraben (MP)		152.15	n.a.	n.a.	2.17	8.5
Propyl paraben (PP)		180.20	n.a.	n.a.	3.24	8.5
Hydrocortisone acetate (HCA)		404.50	95%	6-8 h	2.31	12.61
Butylated hydroxytoluene (BHT)		220.35	n.a.	n.a.	5.25	11.6
Tretinoin (TRT)		300.40	≥ 95%	0.5-2 h	5.66	4.76
Hydroquinone (HQ)		110.11	n.a.	n.a.	0.71	9.68

n.a. not available

abnormal pigmentation. This disease is more prevalent in women, accounting for 90% of reported cases [1,2]. Hydroquinone (benzene-1, 4-diol; HQ) is the main depigmenter used and it acts through the inhibition of the tyrosinase enzyme which prevents the conversion of DOPA to melanin. Tretinoin (all-trans retinoic acid; TRT) is used to enhance and improve the cell regeneration while hydrocortisone acetate (HCA) reduces associated UV-induced inflammation. The use of the three agents together is known as “triple combination therapy” which was shown to be more effective in treating melasma [3]. Methyl and propyl parabens (MP and PP) are often used in pharmaceutical formulations as preservatives, especially for their low toxicity and wide antibacterial and antifungal activities. Butylated hydroxytoluene (BHT) was added to the formulation to benefit from its antioxidant effects. The chemical structures for all these components are reported in Table 1.

The assay used for HQ, as reported in the official USP, is widely reported alone [4–6], in combinations [7–14], as well as in the presence of its main degradation products [15–17]. In all these procedures the mainly used instrument configuration was high performance liquid chromatography (HPLC), although spectrophotometric [4,5,12,14,17], and chemiluminescence [6] were also applied. The second component, HCA, was similarly widely quantified using various techniques alone or in combinations of topical formulations. Several methods were also reported for the quantitation of TRT in dermatological preparations in the presence of other compounds [18,19], degradation products [16,

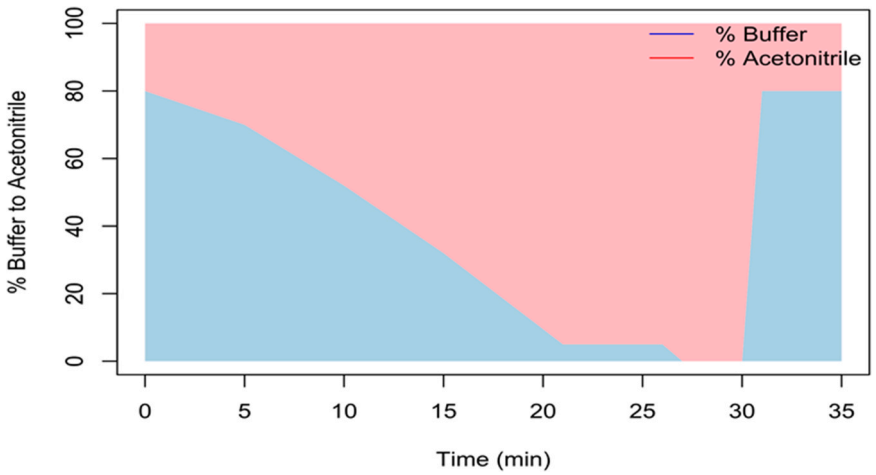


Fig. 1. : Mobile phase gradient composition.

18–23], or metabolites [22,24,25].

By a systematic literature survey, only few works report simultaneous separation and quantitation of all active principles of the triple combination therapy formulations (HQ, TRT, and a corticosteroid) [7–9, 26,27] let alone preservatives too [15]. In addition, the study of the stability is crucial for the final commercial product before releasing in the market, so forced degradation studies must be performed. The process of forced degradation entails subjecting drug substances and products to harsher conditions than those used in accelerated conditions [28]. This leads to the formation of degradation products, which can be analyzed to assess the stability of the molecule. Also help in developing stability indicating methods which must demonstrate high level of specificity, while also offering a better understanding of the degradation pathways and products of the drug substance [29].

To the best of our knowledge, the herein proposed method is the first to successfully quantify all six components simultaneously in a commercially available topical formulation. The inclusion of preservatives in the analysis is a novel aspect, as previous methods have mostly focused on the quantification of the active ingredients alone. The challenge faced during method development was to successfully separate all six components which varied so much in polarity from the polar HQ (Octanol/Water Partition Coefficient; LogP= 0.71) to highly non-polar TRT (Octanol/Water Partition Coefficient; LogP= 5.66). What was even more challenging was that some of the constituents' polarities were very close (HCA/MP and BHT/TRT). Another important challenge was the staggering difference in components' concentrations, where HQ was almost 40 times that of TRT, highlighting that this multianalytes method allows the quantification of the compounds in a wide large concentration range.

2. Materials and methods

2.1. Materials and solvents

All chemicals used throughout this study were of analytical grade. The solvents were HPLC grade (Fisher Scientific, NJ, USA). Global NAPI Pharmaceuticals (Cairo, Egypt) kindly gifted high purity reference material of HQ, TRT, HCA, MP, PP, BHT as well as Tritospot® cream (labeled to contain 3% HQ, 0.02% TRT, 1% HCA and 4% eusolex).

2.2. Instrument and software

The employed HPLC system was a Waters 2695 HPLC system (Waters Corp., Milford, USA) connected to VWD-3400RS UV detector set at 280 nm and WPS-3000TPLRS autosampler, using Empower v.2 software for data acquisition and processing. The HPLC column was an X-Bridge C18 (4.6 ×150 mm, 5 μm, Waters Corp., Milford, USA). The buffer's pH was adjusted using Hanna HI 8314 pH-meter (Hanna, Padua, Italy).

2.3. Procedures

2.3.1. Standard solutions

The diluent used was a mixture of methanol: acetonitrile: tetrahydrofuran: phosphoric acid (ratio 50:30:20:0.2). Stock standard solution was prepared by transferring accurately: 22.0 mg TRT reference material, 40.0 mg BHT reference material, 80.0 mg MP reference material and 20.0 mg PP reference material into 100 mL dark colored volumetric flask. The powders were dissolved, and the volume completed to the mark with the diluent.

The Working standard solutions were prepared by transferring accurately 40.0 mg HQ reference material and 22.0 mg HCA reference material into 100 mL volumetric flask to be dissolved with 50 mL diluent. From the stock standard solution, 5.0 mL were added, mixed, and the volume completed to the mark with diluent. Aliquots from the working standard solution were diluted to produce the different concentrations of HQ (240–560 μg/mL), MP (24–56 μg/mL), HCA (132–308 μg/mL), PP (6–14 μg/mL), BHT (12–28 μg/mL), and TRT (6.6–15 μg/mL). 10 μL of the sample was injected and chromatographed using the specified chromatographic conditions. The peak responses of all six components were recorded simultaneously and plotted against their corresponding concentration.

2.3.2. Chromatographic conditions

Different mobile phases, gradient profiles, pH, and detection wavelengths were tried. More stable baselines were observed when phosphate buffer was employed instead of formate buffer. Better peak shapes were obtained when acetonitrile was employed as a mobile phase modifier, compared with methanol. The resolution between peaks improved by decreasing the buffer pH. Accordingly, the use of phosphate buffer (pH 2.1)-acetonitrile as a mobile phase resulted in better separation with improved peak sharpness, larger area, quicker retention time, and enhanced resolution. Using these phases, different gradient elution profiles were evaluated, starting from high polarity to lower, in order to resolve the analytes, obtain good peak symmetries, and to obtain an adequate total runtime (important in pharmaceutical industry and related to the concept of the high throughput).

For the detection wavelength, 210, 254 and 280 nm were investigated. Parabens (MP and PP) had high absorptivity at shorter wavelengths while TRT did not, which made both 210 and 254 nm not suitable for TRT quantification especially at such low concentration compared to other five constituents. The wavelength of 280 nm, on the other hand, gave satisfactory response for all six analytes. In addition,

Table 2
Characteristic parameters of the calibration equations for the proposed HPLC method.

	Range μg/mL	R (*n* = 7)	a	b	S_a	S_b	LOD μg/mL
Hydroquinone	240 - 560	0.99944	-210619.47	11570026.78	94008.30	222043.66	26.813
Methyl paraben	24 - 56	0.99946	-30184.79	16280665.29	12711.50	307500.82	2.577
Propyl paraben	6 - 14	0.99948	-7735.3414	13510701.08	2612.97	251433.37	0.638
Hydrocortisone acetate	132 - 308	0.99942	-3030.1379	583315.978	2544.45	11409.15	14.395
Butylated hydroxytoluene	12 - 28	0.99960	-6972.8770	8515586.90	2844.79	138252.70	1.10
Tretinoin	6.6 - 15.4	0.99920	-5617.7836	14094409.25	3701.82	323825.34	0.867

Table 3
System suitability parameters for the proposed HPLC method for simultaneous determination of Tritospot components.

	t_r	*k'*	α	Rs	As
HQ	2.29	21.92			1.45
MP	7.16	70.74	3.23	19.02	1.13
PP	12.12	70.74	1.05	2.61	1.12
HCA	11.54	114.35	1.62	18.09	1.16
BHT	21.88	21.78	1.8	45.15	0.98
TRT	23.19	23.09	1.06	6.54	1.01

Table 4
Intraday and interday precision (RSD%) and trueness (Mean % found).

Analyte	Intraday			Interday		
	Added (μg/mL)	Found (μg/mL)	% found	Added (μg/mL)	Found (μg/mL)	% found
HQ	400.0	409.8	102.4	400.0	405.0	101.3
	400.0	415.0	103.7	400.0	406.6	101.7
	400.0	402.6	100.7	400.0	392.4	98.1
		Mean	**102.3**			**100.3**
		RSD%	**1.5**			**1.9**
MP	40.0	41.1	102.6	40.0	40.3	100.8
	40.0	40.4	101.0	40.0	40.6	101.5
	40.0	40.4	100.9	40.0	40.0	99.9
		Mean	**101.5**			**100.8**
		RSD%	**0.9**			**0.8**
PP	10.0	10.4	104.1	10.0	10.2	101.5
	10.0	10.5	105.4	10.0	10.1	101.4
	10.0	10.3	102.6	10.0	9.8	98.4
		Mean	**104.0**			**100.4**
		RSD%	**1.4**			**1.7**
HCA	220.0	223.4	101.5	220.0	223.7	101.7
	220.0	220.7	100.3	220.0	220.7	100.3
	220.0	220.5	100.2	220.0	218.8	99.5
		Mean	**100.7**			**100.5**
		RSD%	**0.7**			**1.1**
BHT	20.0	20.6	102.8	20.0	20.2	100.9
	20.0	20.5	102.7	20.0	20.6	103.0
	20.0	20.1	100.4	20.0	20.3	101.5
		Mean	**101.9**			**101.8**
		RSD%	**1.3**			**1.1**
TRT	11.0	11.2	101.6	11.0	11.0	100.2
	11.0	11.2	101.9	11.0	10.8	98.1
	11.0	11.0	100.2	11.0	11.2	101.7
		Mean	**101.2**			**100.0**
		RSD%	**0.9**			**1.8**

using different detection wavelengths for different phases of gradient/separation led to unacceptable noise/drifting of the baseline.

Gradient elution (Fig. 1) (from high polarity to lower) was preferred to ensure best separation at reasonable time. Flow rate was chosen at 1 mL/min. Faster flow rates resulted in overlap of BHT and TRT peaks, which were very sensitive to changes in organic phase percent. Because of the proximity of their polarities, they required enough time to interact with the stationary phase in a way that resolve them completely (higher selectivity factor). Slower flow rates on the other hand were not advantageous since they led to deteriorating peak shapes and prolonged analysis time for no improvement in resolution (1 mL/min showed resolution of 2.6 that was more than enough for a successful complete separation). The injection volume was 10 μL. The employed buffer was prepared by mixing 10 mL glacial acetic acid and 10 mL phosphoric acid 85% in 2 L of water. Then, 2 mL triethylamine were added, and the pH was adjusted to 2.1 by adding ammonia. Before using the mobile phase, it was filtered and degassed.

2.3.3. Validation

2.3.3.1. Specificity. The ability to assess unequivocally the analyte in the presence of components which might be expected to be present by determining and calculating system suitability parameters including retention time (t_r), retention factor (k'), selectivity (α), resolution (Rs), and asymmetry factor (As).

2.3.3.2. Linearity and range. Several concentrations were prepared and used to create a calibration curve for each component. Each concentration was injected three times and the area under the curve is plotted against the concentration. After that, the regression equation was calculated for each ingredient.

2.3.3.3. Trueness. The mean percentage recovery of three replicates for three different concentration levels within the linear range (nine measurements) were calculated for all six analytes.

2.3.3.4. Precision repeatability. The relative standard deviation was calculated for three different concentrations of all ingredients by injecting each concentration three times. On the same day under the same experimental conditions.

2.3.3.5. Intermediate precision. The RSD was calculated using the same procedure of mentioned in Precision repeatability except for that injections are done on three different days.

2.3.3.6. Limit of quantitation and limit of detection. The limit of detection (LOD) and limit of quantification (LOQ) were validated by means of signal-to-noise ratio (S/N) equal to 3 and 10, respectively [30,31]. Additionally, the LOQs were validated considering the back-calculated concentrations and evaluating their BIAS% with respect to the theoretical concentration.

2.3.4. Forced degradation

Forced degradation are carried out to achieve to generate a degradation profile that is comparable to what would be seen under normal stability study.

2.3.4.1. Acid degradation. Samples of the working standard solution were treated with 1 M HCl at 60 °C for 1 h. During that time, every 20 min a 10.0 mL aliquot was pipetted out of the mix, neutralized and assayed using the proposed HPLC method.

2.3.4.2. Base degradation. The stock standard and materials mixture were dissolved in 5 M NaOH and heating the solution to 60 °C for 1 h. Every 20 min, 10 mL of the mixture was taken, neutralized with 5 M HCl, and analyzed by the proposed HPLC method.

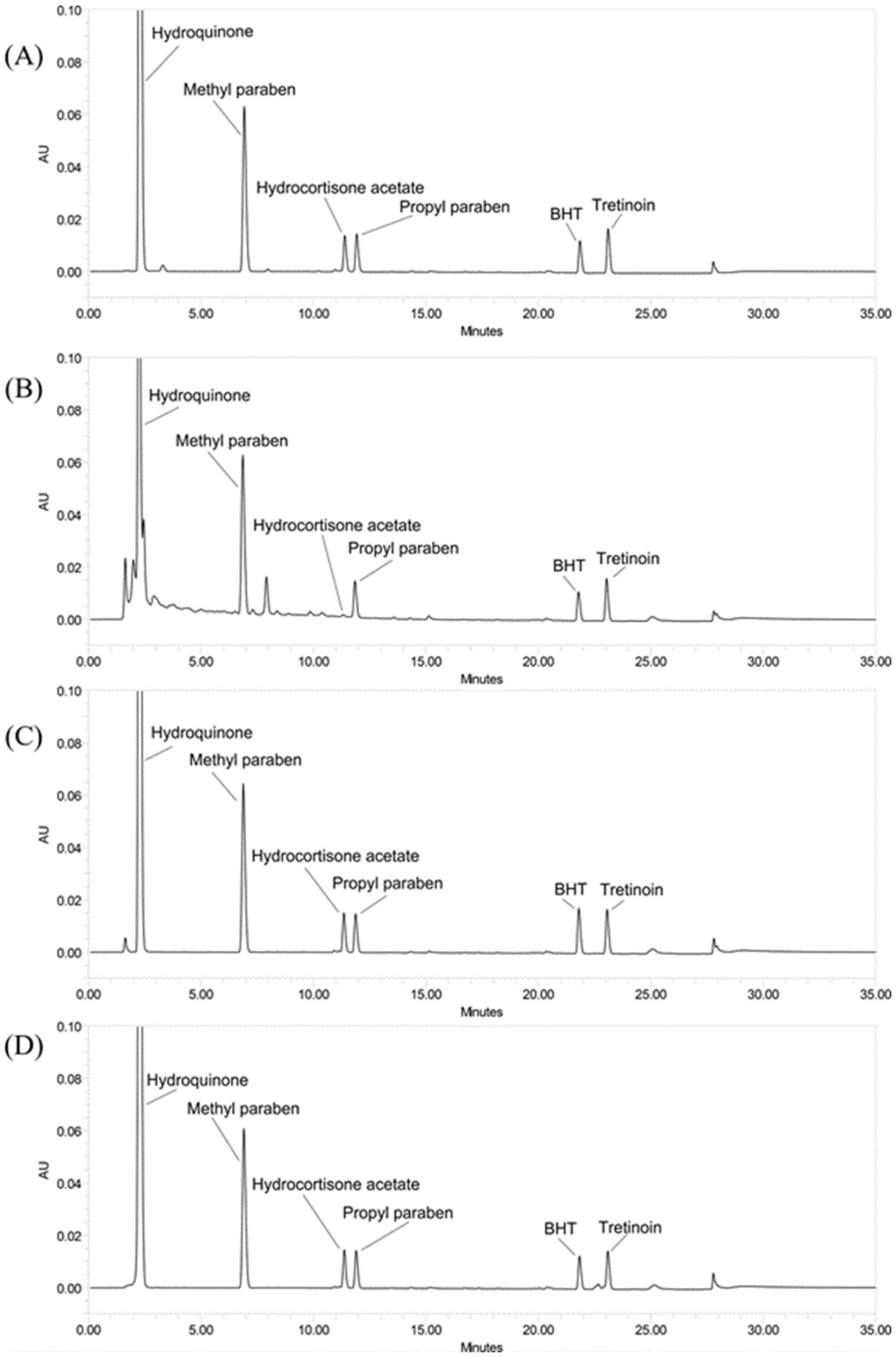


Fig. 2. : Chromatogram of photodegradation of all six components of Tritospot® cream (A) Acid degradation (B) Alkaline degradation (C) Oxidative degradation (D) Photodegradation.

2.3.4.3. Oxidative degradation. The oxidative degradation was investigated by treating the standard solution of raw material with 3% H_2O_2 and heating the solution for 1 h at 60 °C. The area under curve of the peaks was monitored using the same chromatographic conditions.

2.3.4.4. Photodegradation. Was studied by applying the radiation of a UV lamp to the working standard solution in a quartz cell for 24 h and similarly taking samples every 20 min to be assayed.

2.3.5. Application to pharmaceutical preparation

Accurately weighed 2.0 gm of test sample (Tritospot cream) were transferred to an amber conical flask. 70 mL diluent were then added,

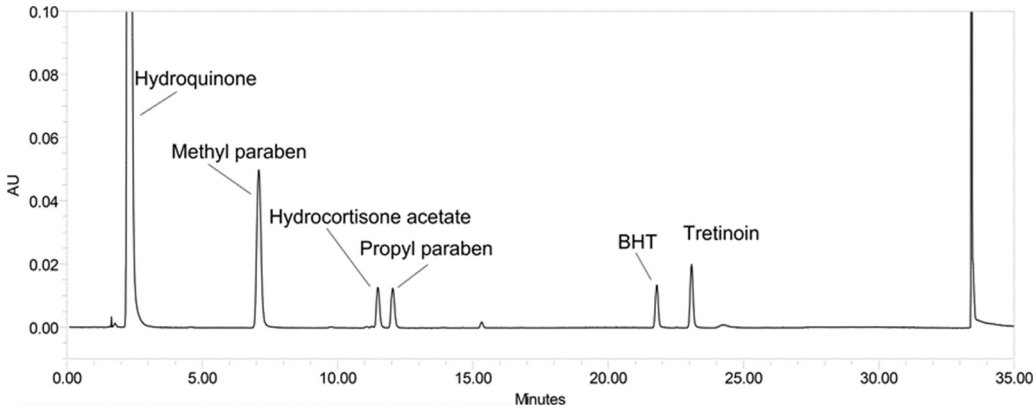


Fig. 3. : Chromatogram of all six components of Tritospot® cream in a real sample analysis.

Table 5
A comparison between our proposed method and previous published works.

Analyte (s)	Instrument configuration	Elution mode	Range (μg/mL)	LOD/LOQ	Ref.
HQ	HPLC-UV	Isocratic	50-250	0.4208/ 1.2750	[7]
TRT			1-5	0.2866/ 0.8686	
HQ	HPLC-DAD	Isocratic	25-150	2.123/ 7.077	[8]
HCA			25-150	2.003/ 6.676	
HQ	HPLC-DAD	Isocratic	10–200	2.01/ 6.11	[9]
HCA			5–100	1.13/ 3.41	
TRT			1–40	0.28×10^{-3}/ 0.87×10^{-3}	
HQ	HPLC-UV	Isocratic	50-300	6.86/ 22.89	[26]
TRT			0.5-5	0.18/ 0.61	
HQ	UHPLC	Gradient	N.R	0.2993/ 0.8982	[27]
HCA				0.0557/ 0.1115	
TRT				0.1116/ 0.3348	
HQ	HPLC-UV	Isocratic	100–300	3.75/ 11.37	[32]
TRT			0.625 to 1.875	0.02/ 0.07	
HQ	HPLC-UV	Isocratic	100-300	1.80/ 5.44	[33]
TRT			0.625-1.875	0.02/ 0.07	
HQ	HPLC-UV	Gradient	240 – 560	26.813/240	Current method
TRT			6.6 - 15.4	0.867/6.6	
HCA			132 – 308	14.395/132	
BHT			12 – 28	1.10/12	
MP			24 – 56	2.577/24	
PP			6 - 14	0.638/6	

shaken by mechanical means for 15 min, and Sonicated for 30 min at 40 °C. The contents were left to cool down to room temperature before they were filtered into 100 mL volumetric flask where volume was completed to the mark with diluent.

3. Results and discussion

RP-HPLC was an obvious answer to the challenges imposed by this pharmaceutical preparation because of its high separation power, versatility, sensitivity and reproducibility. The wide range of polarities and concentrations encompassed together with the proximity of the polarities of (HCA & PP) and (BHT & TRT) meant that separation conditions had to be carefully tuned to achieve best separation at reasonable time with acceptable sensitivity and reproducibility.

3.1. Method validation

The developed method was validated in accordance with the ICH guidelines [31]. The results of the validation study showed that the method is accurate and precise, as summarized in Table 2.

3.1.1. Specificity

Various system suitability parameters were calculated to ensure its specificity and complete separation of all six components. They were summed up in Table 3. Resolution of all peaks was greater than 1.5, which indicated complete separation. The peaks symmetry was within acceptable range. Understandably, HQ peak symmetry factor was the largest because of its very high concentration compared to other components.

3.1.2. Linearity and range

To test the linearity of the method, six different concentrations were utilized. The correlation coefficients obtained for all six components were > 0.9992 as shown in Table 2.

3.1.3. Precision and trueness

The mean percentage recovery used to evaluate the trueness of the herein proposed method were calculated for all six analytes and summed in Table 4. All these values were between 100 – 102% for all analytes except PP (104%) which was still acceptable for routine quality control work (admitted values must be within ± 15%). The relative standard deviation percentages (RSD%) for replicate injections (intra and inter-day) was always below 2.0%. These results in terms of precision and trueness (both intra- and interday) were very useful for quality control requirements, which require such precision and trueness to release products to the market with confidence in their test results.

3.1.4. Limit of detection and limit of quantitation

The signal-to-noise ratio (S/N) was employed to establish the limits of detection (LOD) and limits of quantitation (LOQ) in the chromatographic techniques, coupled also with the evaluation of the BIAS% at these concentration levels. The LOD values were 26.813 μg/mL, 2.577 μg/mL, 0.638 μg/mL, 14.395 μg/mL, 1.10 μg/mL, and 0.867 μg/mL for HQ, MP, PP, HCA, BHT, and TRT, respectively. LODs were very suited to the method's use for pharmaceutical preparation analysis.

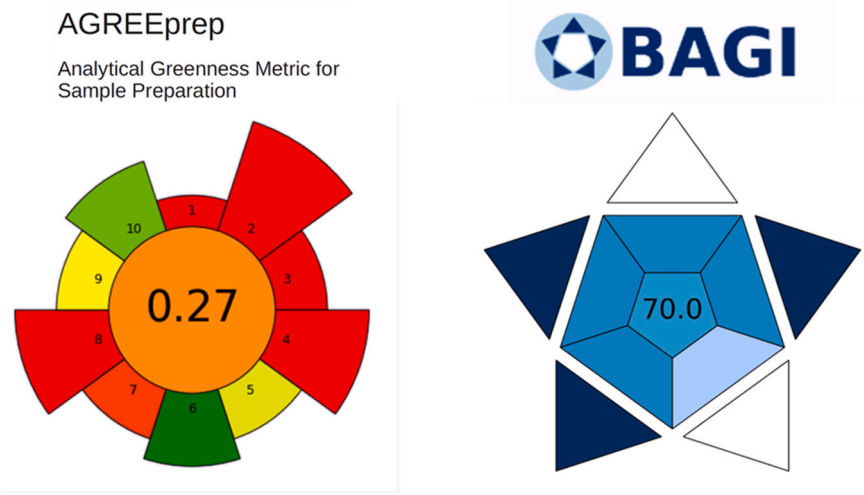


Fig. 4. : AGREEprep and BAGI pictogram for the herein reported method.

3.2. Forced degradation

The focus was on all components using the same proposed chromatographic conditions and following the changes in peaks response and shape. Acid degradation (Fig. 2A) slightly affected HCA and BHT (mean percent recovery 91%), while alkaline degradation (Fig. 2B) showed massive decrease in percent recovery of HCA (5.5%) and too much lesser extent BHT (85%). Oxidative degradation (Fig. 2C) intensified the BHT peak (130%), while photodegradation (Fig. 2D), as expected, mainly affected TRT.

3.3. Application to pharmaceutical preparation

The proposed method was used to analyze the commercial product Tritospot®. Mean percent recoveries for all studied six components were reasonable and RSD of six replicates was less than 2% for all studied analytes indicating the applicability of the proposed method to pharmaceutical dosage form analysis with satisfactory trueness and precision Fig. 3.

3.4. Comparison with other reported methods

The simultaneous separation of six analytes in one HPLC run is quite challenging. A few reports have been found for the concurrent analysis of HQ, HCA, and TRT [9,27]. Other methods could determine two of the active ingredients [7,8,26,32,33], as shown in Table 5. To the best of our knowledge, no analytical method has been reported yet for the simultaneous analysis of these six analytes. Determination of multiple analytes using a single method in a single run is congruent with the current trend of developing analytical methods that save time and reduce organic solvents consumption, without sacrificing the method reliability. The developed method is more suitable for the routine chemical analysis of these compounds in dosage forms.

3.5. AGREEprep and BAGI evaluation

The method proposed and validated here was evaluated using the AGREEprep tool [34,35] relating to the evaluation of its green profile and environmental compatibility.

Furthermore, it was also evaluated using the BAGI tool [36] in order to evaluate practicality of an analytical method, and it can be combined with the most common about Green Chemistry.

The pictograms relating to the AGREEprep and BAGI tools are shown in Fig. 4, highlighting how this procedure responds to the main applicability requirements.

Certainly, based on the AGREEprep pictogram, the method lends itself to many possible improvements in terms of environmental impact. In fact, for its evaluation, only 1 sample per hour was considered (criterion 6) prepared ex situ (criterion 1) by manual procedure (criterion 7) with high sample quantities (criteria 2, 4, and 5).

A possible improvement consists in modifying everything through an online approach on (proportionately) smaller quantities and with the aid of automatic preparation systems (automatic preparers).

The evaluation using BAGI certainly went better. In fact, with this tool its applicability appears in terms of practicality to further improve and to compare the performance of different methods.

4. Conclusion

The proposed method successfully separated and simultaneously quantified all six components of a topical formulation used for treatment of hyperpigmentation for the first time. The analysis procedure was relatively simple and fast, even if its transferability could be challenging due to the gradient elution mode. Various validation parameters were calculated and found to meet the specified ICH standards allowing the application of the herein proposed method to clinical and pharmaceutical fields. The proposed method, assessed through AGREEprep and BAGI tools, exhibits potential for environmental enhancements and practicality. It proves excellent for quality control and serves as a promising foundation for further improvements, particularly in terms of eco-compatibility and high throughput.

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CRediT authorship contribution statement

Hamed Mahmoud: Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Locatelli Marcello:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Investigation, Data curation, Conceptualization. **Mansour Fotouh R.:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Khairy Mostafa A.:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data

curation, Conceptualization. **Hamad Amal:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpba.2024.116021.

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