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Determination of diphencyprone and its photo-degradation product incompounded preparations using HPLC

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Abstract: Diphencyprone (DPCP) is frequently used as a compounded preparation in dermatology for the treatment of alopecia and recalcitrant warts based on the immune reaction of skin allergy. However, DPCP is a non-recognized agent in Pharmacopoeia, because there are no criteria or analytical method for quality control of its powder and formulation. DPCP is unstable under light irradiation because as it easily decomposes to diphenylacetylene (DPA). This study aims to develop a simultaneous HPLC analytical method for analyzing DPCP and DPA in the raw materials and compounded preparation. The method required a C18 column (250 × 4.6 mm, 5 µm) at 40 °C with a mobile phase of (A) 0.01 M phosphoric acid in water and (B) acetonitrile at UV 220 nm. DPA conversion to DPCP in the powder and compounded preparations was accelerated after light exposure for 60 min. In addition, this resulted in different patterns depending on the wavelength of light and the formulation. That is, DPCP in compounded preparation was more unstable than that in the powder. However, the DPCP formulation in amber bottles was observed to remain stable, although the measured concentrations of DPCP were somewhat different from the nominal concentration of the compounded preparations. The control of the exact concentration is required for effective disease treatment, depending on the state of the patient. In conclusion, these results will be useful for the recognition of DPCP in Pharmacopoeia and new DPCP formulation development to prevent photodecomposition.

Key words: Diphencyprone, Diphenylacetylene, Compounded preparation, Photodecomposition, High performance liquid chromatography

1. Introduction

Diphencyprone (diphenylcyclopropenone, DPCP) is commonly used to treat alopecia totalis,[1,2] alopecia areata[3] and recalcitrant wart disease[4] caused by human papillomavirus (HPV). In the treatment of alopecia areata, DPCP acts as an irritant and sensitizer that induces allergic contact dermatitis. This triggers an immune response against autoreactive cells

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that cause hair loss.[5,6] DPCP was shown to induce significant hair regrowth in 40 % of 41 patients with alopecia areata after 6 months. Two-thirds of these patients exhibited sustained hair regrowth after 12 months.[7] Several studies have also shown the bioactivity of DPCP in treatments for alopecia and recalcitrant wart diseases.[8-10] Recent case studies describe the use of a DPCP complex for the immuno-therapeutic treatment of alopecia areata.[11,12] Nevertheless, DPCP is not recognized as official medicines by the Ministry of Food and Drug Safety. Because it has uncertainties surrounding the metabolites of DPCP and the difficulties in identifying the short-lived and the decomposed adducts of DPCP in alcoholic solutions. Furthermore, DPCP is unstable and easily photo-decomposes to diphenylacetylene (DPA) under ultraviolet (UV) irradiation or sunlight.[13] Therefore, DPA can serve as an indicator of the stability of DPCP under illumination. The photostability of DPCP is a primary concern in safety investigations. To freely prescribe DPCP, it must be formally recognized as a therapeutic agent by the regulatory agencies. To facilitate such recognition, a simple and effective analytical method for quantitating DPCP and its metabolites is required. Previous studies have not quantitated the conversion ratio of DPCP to DPA, although changes in the UV absorbance spectrum of DPCP while undergoing photolysis have been reported.[14,15] A quantitative method for examining the photo-stability of DPCP has not been reported. This study describes a high-performance liquid chromatography (HPLC) method for the simultaneous quantitation of DPCP and its photo-metabolite, DPA, and estimates the photodecomposition ratios of pure, powdered DPCP and mixtures of DPCP in medications.

2. Experimental

2.1. Chemical and reagents

DPCP and DPA were of 99.0 % purity and purchased from Sigma-Aldrich Co. (Merck, Germany). Water was deionized to 18 MΩ using an Optimos SHRO-UP purifier (Shinhan Science Tech, Korea). All other solvents were of HPLC grade or comparable quality. The chemical structures of reference standards are shown in *Fig*. 1. Samples of DPCP taken from compounded preparations were obtained from the Department of Dermatology of Inha University Hospital. Test samples for photostability were prepared directly from DPCP standard powder. All samples were stored at 4 ºC.

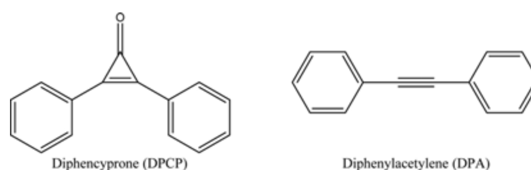

Fig. 1. The chemical structures of diphencyprone (DPCP) and diphenylacetylene (DPA).

2.2. Sample preparation for photostability tests

Aliquots of DPCP powder (100 mg) and compounded preparations were spread onto aluminum dishes (ø 5 cm) and exposed to ultraviolet light (254 or 365 nm) at 6 W using a VL-6.L lamp (Vilber Lourmat, France) or sunlight for 0, 5, 10, 30, 60, 90 and 120 min. After every exposure time, samples for photostability test were taken each 10 mg out of DPCP powder (100 mg). And they were dissolved in 1 mL of acetone, and filtered through a syringe membrane filter (PTFE, 0.45 μm; SmartPor-II, Woonki Science, Korea) prior to being injected into the HPLC system. The injected volume was 10 μL. The conversion ratios of DPCP to DPA were calculated as follows: DPCP conversion ratio (%) = {mole of DPA / (mole of DPCP + mole of DPA)} × 100.

2.3. Standard preparation

Samples of DPCP and DPA powders (10 mg) were dissolved in 10 mL of acetone to generate 1 mg/mL standard stock solutions. These were mixed in equal proportions to generate a standard mixture containing 0.5 mg/mL of each compound. Standard mixtures were diluted to 4, 8, 16, 32, 64, 128, 250 and 500 μg/mL with acetone.

2.4. HPLC analysis

HPLC data were collected with an LC-20A system (Shimadzu, Japan) equipped with an SPD-20A ultraviolet/visible (UV-Vis) detector, two LC-20AD pumps, a CTO-10ASvp column oven, and a Sil-20A auto-sampler driven by LabSolutions software (Ver. 1.25; Shimadzu). Sample separation was performed on a Hector-M C18 column (250 × 4.6 mm, 5 μm; RStech Co. Ltd., Korea) at 40 ^{o}C under gradient conditions with a mobile phase consisting of 0.01 M phosphoric acid in water and acetonitrile. The gradient program was as follows: an increase from 55 % B to 60 %

B over the initial 20 min, an increase of 60 % B to 100 % B over the next 5 min, and then hold at 100 % B for the final 10 min of elution. Eluate absorbance was monitored at 220 nm.

2.5. Method validation

The analytical method was developed in accordance with the guidelines of the Ministry of Food and Drug Safety of Korea. The limit of detection (LOD) and limit of quantitation (LOQ) were determined by serially diluting stock standard solutions of DPCP down to the lowest detectable concentration. The LOD was defined as the concentration corresponding to the lowest detectable peak with a signal-to-noise (S/N) ratio of 3.3 on the HPLC chromatogram. LOQ was defined as the concentration corresponding to an S/N of 10. Intra- and inter-day precision and accuracy were determined by measuring five identical injections within a single day, or by repeating one injection per day for 5 consecutive days at three different analyte concentrations (16, 70 and 300.0 μg/mL).

2.6. Quantitative and statistical analysis of DPCP in DPCP medications

The contents of DPCP and DPA in a DPCP-based medications were determined using the HPLC method described above. All samples were examined in triplicate. Data are shown as mean ± standard deviation (SD). The statistical analysis was estimated by the t-test in 95 % confidence interval ($p < 0.05$) using Microsoft Excel program (Ver. 2016, Microsoft Co. Ltd., Bellevue, Washington, USA).

3. Results and Discussion

3.1. Optimization of HPLC conditions

A protocol for HPLC analysis of DPCP was provided by Sigma-Aldrich. However, this was deemed unsuitable due to significant peak overlap between DPCP and acetone in the compounded preparation. The method was therefore modified as section 2.4. This method yielded baseline-resolved chromatograms of DPA and DPCP with peak symmetry factors of 1.18 and 1.38, respectively (*Fig.* 2).

3.2. Method validation

Validation of the above method was performed in partial accordance with the guidelines of the MFDS. LOD and LOQ levels of DPCP were 0.94 and 2.85 μg/mL, and those of DPA were 0.64 and 1.95 μg/mL, respectively (*Table* 1); this indicates that the method described herein is suitable for analyses of DPCP and DPA. The precision and accuracy of DPCP quantitation were 0.54~1.23 % and 99.3~100.6 %, (intra-day) and 3.63~6.45 % and 102.7~104.5 % (inter-day), respectively. The precision and accuracy of DPA analyses were 2.31~5.15 % and 94.1~101.8 % (intra-day), and 2.65~5.14 % and 101.1~104.6 % (inter-day), respectively. These data satisfy the analytical criteria stated in the MFDS guidelines (*Table* 2).

3.3. Photostability tests of DPCP

The instability of DPCP, both as a pure powder and

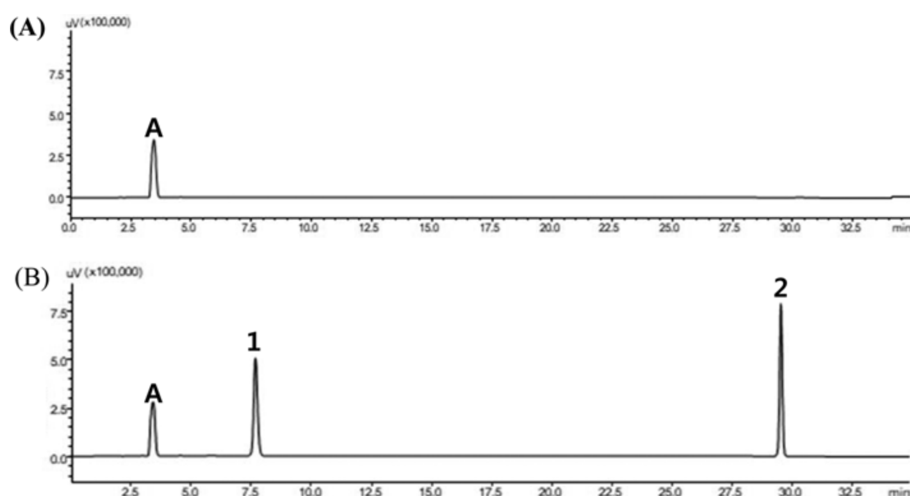

Fig. 2. HPLC chromatograms of (A) blank and (B) compounded preparation at UV 220 nm; Peaks: A. acetone, 1. DPCP, 2. DPA.

blended into a medication, was evaluated following exposure to sunlight and UV irradiation at 254 nm and 365 nm. However, direct comparison for the effects of sunlight versus UV irradiation was not possible, because to control the strength of sunlight was very difficult. DPA concentration produced via the photolysis of pure powder was 2.25 times higher under UV irradiation at 365 nm than at 254 nm, and 10.32 times higher in the medication in 2 h irradiation (*Table* 3). At discrete wavelengths of 254 nm and 365 nm, the yield of DPA was 2.06 and 9.43 times higher in the medication than in pure DPCP powder, respectively, while in sunlight, the yield of DPA was similar between the pure DPCP powder and that detected in medication, with a relative DPA yield factor of 1.03 (*Table* 3). The higher degree of photolysis at the longer UV wavelength is due to the inherent thermal fragility of DPCP. The acetone used to prepare medications is known to accelerate the energy absorption of DPCP at 350 nm.[14] In DPCP powder, the conversion ratio during irradiation at 254 nm slowly increased from 0.00 % to 0.35 % within 60 min, and then rapidly increased up to 1.23 % after 120 min. At 365 nm, the conversion ratio increased rapidly from 0.0 % to 1.89 %

Table 1. Regression equation, linearity, limits of detection (LODs) and limits of quantification (LOQs) for DPCP and DPA

Compounds	Range (μg/mL)	Regression equation (y (peak area) /10^4)	Linearity (r^2)	LODs (μg/mL)	LOQs (μg/mL)
DPCP	4 ~ 500	y = 0.0234x + 0.0741	0.9996	0.94	2.85
DPA	4 ~ 500	y = 0.0287x + 0.1137	0.9992	0.64	1.95

Table 2. Intra- / inter-day precision and accuracy of DPCP and DPA (*n*=5)

Compounds	Concentration (μg/mL)	Intra-day		Inter-day	
		Accuracy (%)	Precision (%RSD)	Accuracy (%)	Precision (%RSD)
DPCP	16	99.3	1.23	103.8	6.45
	70	100.5	0.54	104.5	3.63
	300	100.6	1.19	102.7	3.81
DPA	16	94.1	5.15	104.6	5.14
	70	100.9	2.31	103.1	2.65
	300	101.8	2.85	101.1	3.37

Table 3. DPCP-to-DPA ratios in powder and compounded preparation after the light exposure of UV 254 nm, UV 365 nm and sunlight

Light exposure time (min)	DPCP-to-DPA ratios in powder (%)[a, b]			DPCP-to-DPA ratios in compounded preparation (%)[a, b]		
	UV 254 nm	UV 365 nm	Sunlight	UV 254 nm	UV 365 nm	Sunlight
0	0.00±0.00	0.00±0.00	0.94±0.08	0.24±0.02	0.32±0.09	0.45±0.13
5	0.03±0.01	0.12±0.02	1.47±0.28	0.33±0.04	1.10±0.17	1.00±0.22
10	0.07±0.01	0.26±0.06	1.82±0.42	0.43±0.04	1.98±0.50	1.15±0.22
15	0.10±0.02	0.37±0.08	2.61±0.33	0.55±0.01	3.10±0.88	1.82±0.46
30	0.18±0.02	0.80±0.11	3.27±0.32	0.89±0.05	5.75±0.77	3.68±0.12
60	0.35±001	1.89±0.29	3.90±1.11	1.51±0.10	13.55±0.22	6.20±0.17
90	0.74±0.05	2.33±0.08	17.15±0.19	1.94±0.09	19.89±1.79	22.68±0.29
120	1.23±0.13	2.77±0.23	41.99±0.07	2.53±0.12	26.11±3.63	43.38±0.68

[a]The conversion ratio of DPCP to DPA were calculated as follows: DPCP conversion ratio (%) = {mole of DPA / (mole of DPCP + mole of DPA)} × 100.
[b]Quantification levels were same as LOQs of DPCP (2.85 μg/mL) and DPA (1.95 μg/mL) and their corresponding ratios were 2.85×10^{-7} % and 1.95×10^{-7} %, respectively.

within 60 min. And it relatively slowed from 1.89 % to 2.77 % over the next 60 min. In the medication, the conversion ratio increased linearly from 0.24 % to 2.53 % over 120 min at UV 254 nm. Also it constantly increased from 0.32 % to 26.11 % with exposure at 365 nm. However, the DPA content of the compounded preparation under sunlight increased slowly from 0.45 % to 6.20 % within 60 min. And it rapidly increased from 6.20 % to 43.38 % after an additional 60 min (*Table* 3). These data show that the photodecomposition of DPCP is not only dependent on heat, substitution, and solvent polarity, but also on the wavelength of light and the physical composition (*i.e.*, pure compound or mixed).[15]

3.4. The quality of DPCP in compounded preparations

Using the above HPLC-based method, the DPA content in a medication containing DPCP was quantified by HPLC and estimated by non-paired t-test analysis in 95 % confidence interval ($p < 0.05$) (*Table* 4). The actual concentration of 0.01 % and 1.0 % DPCP solution among the measured concentration of DPCP differed significantly from the nominal concentration. No DPA was observed in the compounded preparation, indicating that DPCP in acetone is photostable when kept in an amber bottle.

4. Conclusions

The conversion ratio of DPCP to DPA, in powder form and in compounded preparations, was dependent on the duration of sunlight exposure, and on the UV wavelength and exposure time. The degree of photo- decomposition was greater at 365 nm than at 254 nm. DPCP is thermally fragile and its decomposition when irradiated at 365 nm was accelerated in an acetone solvent. However, no DPA was observed in a DPCP-containing compounded preparation that had been kept in an amber bottle. Measured concentrations of DPCP differed from the nominal concentration detected in the medication. To control the exact concentration is needed for the effective treatment of disease depending on the state of the patient. In conclusion, the method described herein will be useful for the quality control of DPCP-containing compounded preparation, and for the development of new DPCP formulations.

Table 4. Quality evaluation of the DPCP preparation compounded by a medical center

Nominal concentration (%)	Measured concentration (%, n = 3)	
	DPCP	DPA
0.001	0.001±0.000	N.D.
0.01	0.018±0.001$^{***, a}$	N.D.
0.1	0.094±0.004	N.D.
1	1.182±0.003$^{***, a}$	N.D.

***Non-paired t-test ($p < 0.05$).
^{a}The measured concentration of DPCP differed significantly from the nominal concentration.

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