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# Development and Validation of HPLC–Photodiode Array Method for Detecting Steroids in Skin Whitening Products Simultaneously

**Yustina*** **and Alfi Sophian**

National Quality Control Laboratory of Drug and Food, Jalan Percetakan Negara No 23, Jakarta Pusat 10560

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*Corresponding author
Yustina

Email:
yustina@pom.go.id

**ABSTRACT**

Since steroids are prohibited to be present in cosmetic products, thus identification of these outlawed compounds intentionally incorporated in the skin whitening product is sufficient for market surveillance purposes. Simultaneous methods for detecting prednisone (PRN), triamcinolone acetonide (TCA), hydrocortisone acetate (HCA), clobetasol propionate (CBP), and mometasone furoate (MTF) have not been developed. The goal of this study is to develop and validate the screening of these five steroid compounds in a cosmetic product sing reverse phase (RP) HPLC equipped with a photodiode array (PDA) detector. The sample was prepared by diluting it with methanol. Furthermore, the sample solution was vortexed, sonicated and then centrifuged at 4500 rpm. The filtered solution was then put into a chromatographic system. A validation method was applied including the system suitability test, determination of selectivity and detection limit. In the optimization step, we succeeded in distinguishing and identifying five steroid compounds with different retention times.The retention times of PRN, TCA, HCA, CBP and MTF were detected at 3.267, 4.260, 4.910, 15.360, and 16.707 min, respectively. The method performed good selectivity with a resolution of more than 1.5. While for determination of the limit of detection (LOD) was carried out by calibration curve approach over the range 0.5-5 µg/mL and resulted in LOD values for PRN, TCA, HCA, CBP and MTF were 0.36, 0.44, 0.41, 0.47, and 0.42 µg/mL, respectively. The developed method can be applied for routine analysis of PRN, TCA, HCA, CBP and MTF in various types of cosmetic products.

**Keywords:** Steroids, Cosmetic, qualitative, validation, HPLC PDA

## INTRODUCTION

Steroids, or formerly known as corticosteroids, have been prohibited in Indonesia based on Indonesia FDA (Badan POM)'s regulatory No. 17/2022 about technical requirements for cosmetic ingredients (BPOM, 2022). According to it, topical use of steroids should be under doctor's prescription. But, in most marketed whitening cosmetic products, steroids have been illegally adulterated by manufacturers in order to bring whitening and glowing effects to facial skin. Long-term use of steroids will cause the skin to become sensitive, inflamed and thinned. If the user stops the cream suddenly, acne usually occurs and the skin will turn red (Giaccone et al, 2017). Commonly abused steroids in cosmetics include prednisone (PRN), triamcinolone acetonide (TCA), hydrocortisone acetate (HCA), clobetasol propionate (CBP), and mometasone furoate (MTF). Steroids are classified based on their potential strength (Spada et al., 2018). The least potent is occupied by PRN and HCA (Class VII). The moderate class (class III) was occupied by TCA and the high potency class was occupied by MTF. CBP occupies the ultra-potent position of all steroids (class I).

Steroids are widely applied in the field of dermatology to treat several skin diseases, including psoriasis and eczema (Giaccone et al., 2017). Further use of steroids can lead to serious effects, for instance, some systematic-related diseases (Giaccone et al., 2017). Therefore, commercial cosmetic products should not contain steroids, either to be a decorative tool or to provide a therapeutic effect. Consumers are at high risk of consuming illegal products in the long term without adequate medical supervision, for instance some systematic-related diseases (Giaccone et al., 2017).

Many studies have been reported to determine steroids, both as a single or simultaneous analyte, for instance, analysis of triamcinolone acetonide in nasal spray formulation by HPLC (Sudsakorn et al., 2006), analysis of clobetasol propionate in pharmaceutical formulation using HPLC with PDA detector (Marika et al., 2008), and simultaneous analysis of hydrocortisone acetate, dexamethasone, beta-methasone, betamethasone 17 valerate and triamcinolone acetonide in cosmetic products by using TLC and HPLC with UV detection (Indonesian Food and Drug Administration, 2011). In addition, the simultaneous analysis of 39 steroid compounds was carried out and then divided into several standard solution mixtures using HPLC with UV Vis Detector. Among those steroid compounds were clobetasol propionate, triamcinolone acetonide, mometasone furoate and hydrocortisone acetate (Gimeno *et al.,* 2016). Last, dexamethasone, triamcinolone acetonide, hydro-cortisone acetate, betamethasone valerate were analyzed simul-taneously with hydroquinone and retinoic acid in cream formula by HPLC PDA (Rahmayuni et al.,2018). According to the literature review above, there has been no simultaneous analysis of prednisone (PRN), triamcinolone acetonide (TCA), hydrocortisone acetate (HCA), clobetasol propionate (CBP) and mometasone furoate (MTF) in cosmetic products, particularly in whitening products, reported. Therefore, it is necessary to develop a fast and efficient analytical method to increase sensitivity and selectivity in the simultaneous qualitative testing of PRN, TCA, HCA, CBP and MTF on skin-lightening products. The method to be made is validated with validation parameters for category IV, namely specificity and limit of detection (LOD) according to the USP 40 and ICH Q2 (R1) guidelines (ICH, 1995; U.S. Pharmacopeia, 2016).

## MATERIAL AND METHODS

The materials used in this study include reference standards and chemicals. The reference standards used were prednisone (BPFI), triamcinolone acetonide (BPFI), hydrocortisone acetate (BPFI) and mometasone furoate (BPFI) and clobetasol propionate (Sigma). The solvents used were formic acid (Merck), acetonitrile (Merck) and 18.2MΩ Milli-Q® water. The sample matrices used were water-based, solid and liquid lotion of the commercial cosmetic products.

### Instrumentation and conditions of chromatographic analysis

The method was carried out on the Thermo Scientific Dionex Ultimate 3000 HPLC chromate-graphy system equipped with a quaternary pump which has back pressure 9000 psi, a four-channel solvent degasser, and a detection system with a PDA detector Thermo Scientific DAD-3000 (RS). The analysis of the data used the Chromeleon® software. Chromatographic separation was carried out using a Waters XBridge C18 column (250x4.6 mm i.d. and 5µm particle size) with the oven temperature maintained at 35°C. The component mobile phase used was acetonitrile and formic acid 0.1% solution (50:50) with a flow rate of 1.0mL/min through isocratic elution system. The mobile phase was filtered using a 0.45µm filter membrane and aspirated using ultrasound. Detection was carried out at 200-400nm using a PDA detector. The injection volume used was 10µL.

### Preparation of standard solution

The method was modified and developed from the method that was proposed by (Gimeno *et al.*, 2016). PRN, TCA, HCA, CBP and MTF standards were first weighed 5 mg each into a 5mL volumetric flask. After that, they were prepared by dissolving, diluting, vortexing and fication sonicating them for 5min, then filtered it using a 0.45µm PVDF membrane filter. A standard mixed steroid solution was obtained consisting of PRN, TCA, HCA, CBP and MTF with a final concentration of 10g/mL each analyte. The preparation of a standard preservative solution with the same concentration was also carried out to prove that this method can separate steroids from preservatives which are generally contained in cosmetic products and provide good resolution. Preservative solutions used were methyl paraben and propyl paraben.

### Preparation of sample and spiked sample

For the preparation of the sample, 0.5gof the matricess were weighed in a 15 mL centrifuge e tube and 10 mL of solvent was added. Furthermore, the sample solution was vortexed and sonicated for a minute, respectively. In addition, the solution centrifuged for at least 10min at 4000 rpm. Lastly, the supernatant was filtered through a 0.45µm PVDF membrane filter. Meanwhile, for the preparation of spiked sample solution, 0.5g of the sample matrices was weighed and put into a 15 mL centrifuge tube, then 1 mL of standard stock solution and 9 mL of solvent were added.

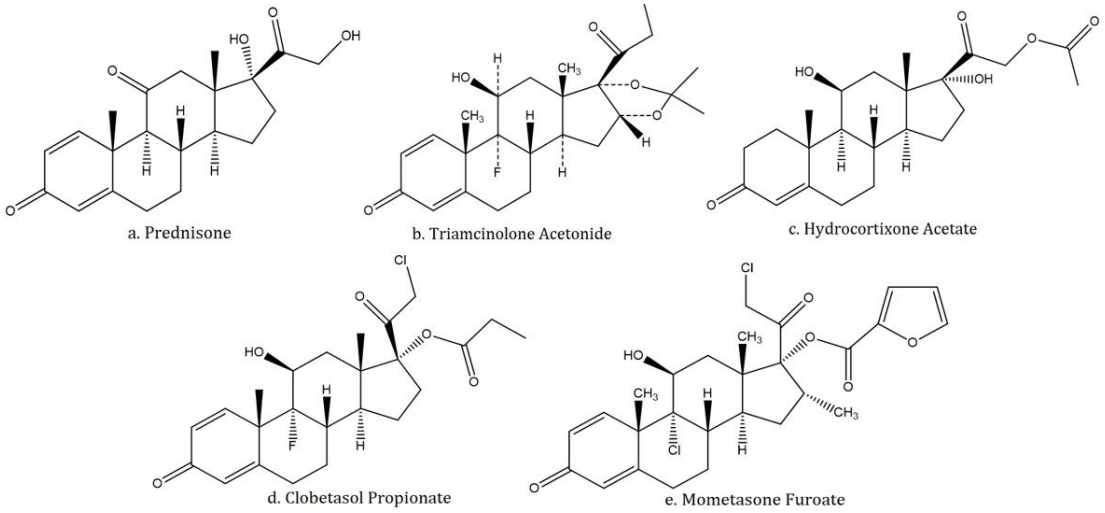



Figure 1. Chemical structures of (a) Prednisone, (b) Triamcinolone acetonide, (c) Hydrocortisone acetate, (d) Cobetasol Propionate, (e) Mometasone Furoate. (Source: PubChem, https://pubchem.ncbi.nlm.nih.gov)

Table I. Relative Standard Deviation Value of System Suitability Test

| No. | Steroid | Relative Standard Deviation (%) | |
|---|---|---|---|
| | | Retention Time | Minimum Area |
| 1. | Prednisone | 0.05 | 0.96 |
| 2. | Triamcinolone Acetate | 0.07 | 0.96 |
| 3. | Hydrocortisone Acetate | 0.08 | 0.73 |
| 4. | Clobetasol Propionate | 0.08 | 1.38 |
| 5. | Mometasone Furoate | 0.08 | 0.80 |

Thus, a spiked sample solution containing PRN, TCA, HCA, CBP and MTF with each concentration 10 g/mL was obtained.

**Method Optimization**

Optimization of the chromatographic conditions was carried out by changing the mobile phase's composition, injection volume and flow rate of the mobile phase. Optimal conditions for chromatography analysis were defined by some parameters, eg. retention time (Rt), peak shape, tailing factor and best resolution (Rs) >1.5.

**Mobile phase composition**

Optimization of the analytical conditions was carried out by varying the mobile phase's composition (Table I).

**Injection volume**

The aim of injection volume's optimization was to gain the highest response of each analyte. The volume of the injection was optimized for 15, 10 and 5µL.

**Flow rate**

Optimization of the mobile phase's flow rate is another way to achieve maximal performance and fast separation. Before implemented to the chromatography system, the mobile phase was sonicated for 10min. Optimization of the mobile phase flow rate at 0.7-1.0 mL/min range.

**Method validation**

**System suitability test (SST)**

SST was done by injecting a 10µg/mL steroid standard solution six times and calculating the obtained %RSD. Some parameters to evaluate SST were the number of theoretical plate (N), tailing factor, asymmetry and resolution.

**Specificity and selectivity**

Determination of specificity and selectivity was done by multiply injecting a mixture standard solution and sample solution. Specificity and

selectivity measurements were carried out to prove that PRN, TCA, HCA, CBP and MTF were well separated, marked with a resolution of 1.5.

**Determination of detection limit**

The detection limit is determined to see how small this method can detect the analyte. We used the calibration curve approach for LOD determination. This work was the same as the determination of specificity and selectivity. Series of mixed standard solutions with concentrations of 0.5, 1, 2, 3, 4, and 5µg/mL were injected and analyzed. Furthermore, the value of Sy/x and $S_A$ was calculated and the value of LOD = 3 $S_A$/b was gained by extrapolating an acquired calibration curve (Gimeo et al., 2016).

## RESULT AND DISCUSSION

**Method optimization**

In this study, five steroid analytes PRN, TCA, HCA, CBP and MTF were analyzed by using reverse phase (RP) HPLC. RP HPLC is suitable for analyzing steroid compounds because steroid compounds are classified as non-volatile compounds. HPLC provides optimum separation results compared to other methods. The key feature of steroids is the ring system of three cyclohexanes and one cyclopentanes fused together (Figure 1). Steroid has an unsaturated group of 3-oxo, thus we could obtain maximum absorption at wavelength between 240 to 245 nm. Analysis of an analyte at its maximum wavelength will provide better sensitivity. The advantage of PDA detector over UV Vis detector is that the spectra of the peaks are eluted, thus PDA are generally suitable for qualitative analysis. In addition, with PDA, peak uniformity or purity can also be monitored, therefore this technique is suitable for use in the method development step.

The Types of columns for RP HPLC analysis are varied. But, in this study, over the other type of column, C18 column (dimension 250x4.6mm i.d. and 5µm particle size) was preferred. For qualitative analysis, resolution is the most important parameter in defining specificity. In this step we preferred using longer column size over short columns to achieve better resolution. It is expressed by resolution value more than 2.0, tailing factor value less than 2.0 and its peak sharp and gaussian shape. According to its chemical structure (Figure 1), we can infer that the analytes that we analyzed have a wide range of polarity. PRN has the simplestmole cule structure than the other four steroid compounds. Thus, its nature makes PRN the most polar compound. It is proven that PRN eluted first than TCA, HCA, CBP and MTF. On the contrary, MTF which has the longest hydrocarbon bond, MTF is the least analyte eluted in the column.

Optimization methods that have been carried out for the simultaneous analysis of PRN, TCA, HCA, CBP and MTF were a variety of optimization, e.g mobile phase composition, injection volume and flow rate. In initial mobile phase optimization, we performed trials with isocratic elution and resulted in good repeatability in retention time. This finding confirmed study from Schellinger and Carr[14] that isocratic elution has advantages in lesser regular instrument maintenance and better resolution than gradient elution since the elution power of mobile phase was stable during analysis. In other words, the eluate behavior gave effect to the peak sensitivity. For optimization of the mobile phase composition, we tried a variety of composition ratios of acetonitrile and formic acid 0.1%solution, eg. 45:55 and 50:50. In the first trial, the resolution of CBP was acquired below the requirement (resolution less than 1.5), while the analytes separation according to the condition of the mobile phase composition of acetonitrile and formic acid 0.1% were 50:50 resulted in separation between the CBP and MTF with better shapes (resolution more than 1.5).

Furthermore, to optimize the injection volume, optimization has been carried out at the injection volume conditions of 15, 10 and 5 µL. Based on those conditions, the best separation results for five analytes PRN, TCA, HCA, CBP and MTF were obtained from the condition with injection volume of 10 µL (CBP resolution > 1.5). Injection volume has affected both peak minimum area and resolution. Reducing injection volume made decreasing efficiency.

Finally, the optimization of flow rates 0.7, 0.8, 0.9 and 1.0mL/min is as follows. Under conditions of flow rate optimization of 0.7 mL/min, the resolution of CBP was 2.71 and the retention time of PRN was 4.660. The total retention time required to separate the five analytes was 24 minutes. Under optimization conditions of the flow rate of 0.8 mL/min, the resolution of CBP was 2.83 and the retention time of PRN was 4.083. The total retention time required to separate the 5 analytes was 21mins. Under optimization conditions of 0.9mL/min flow rate, the resolution of CBP was 2.70 and the retention time of PRN was 3.633. The total retention time required to separate the five analytes was about 18.5min.

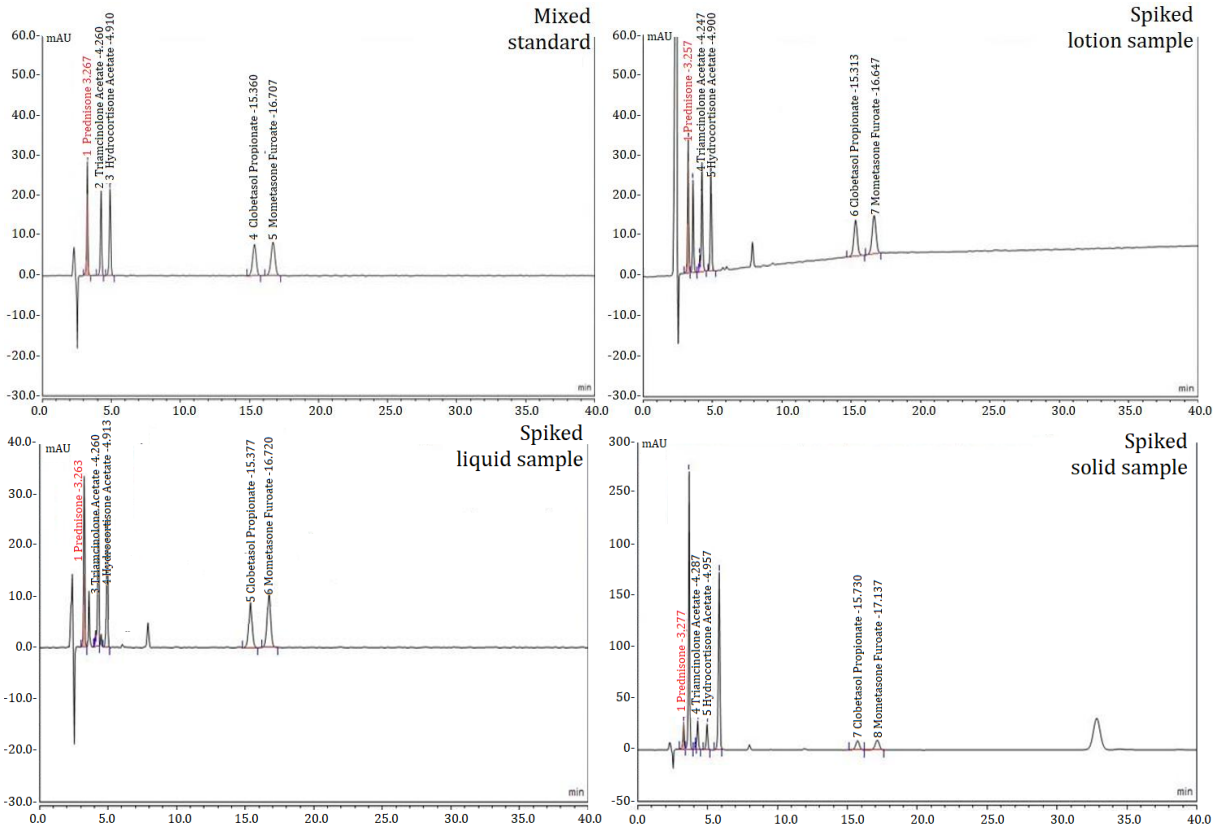



Figure 2. Chromatograms of reference standard and spiked sample in lotion, liquid and solid matrices. Peak in matrice chromatograms gave the same retention time with reference standard.

Table II. Standard Solution Chromatogram Parameters[a]

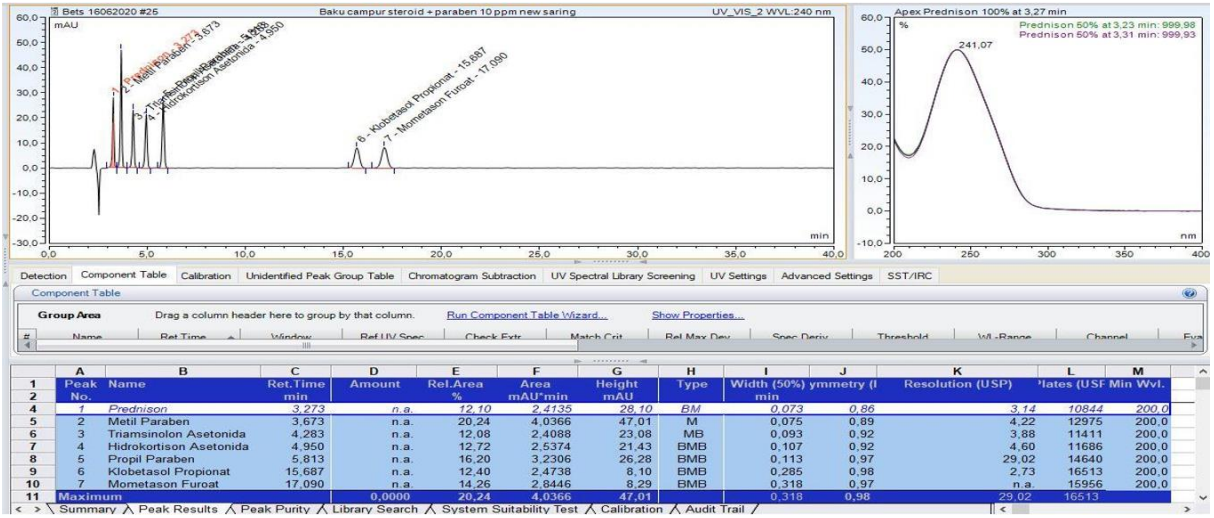
| No. | Steroid | Analyte | $t_R$ | Area (mAu*min) | Rs | Tf | N | Wavelength (nm) |
|---|---|---|---|---|---|---|---|---|
| 1. | Prednisone | PRN | 3.267 | 2.5265 | 6.89 | 0.85 | 10529 | 241 |
| 2. | Triamcinolone Acetonide | TCA | 4.260 | 2.2766 | 3.77 | 0.90 | 11222 | 240 |
| 3. | Hydrocortisone Acetate | HCA | 4.910 | 2.6085 | 31.26 | 0.91 | 11386 | 244 |
| 4. | Clobetasol Propionate | CBP | 15.360 | 2.4235 | 2.64 | 0.95 | 16077 | 240 |
| 5. | Mometasone Furoate | MTF | 16.707 | 2.8427 | n.a. | 0.98 | 15641 | 249 |

[a] tR: retention time; Rs: resolution among adjacent peaks; Tf: peak asymmetry factor N: theoretical plate number

Meanwhile, under optimization conditions of 1.0mL/min flow rate, the resolution of CBP was 2.76 and the retention time of PRN was 3.277. The total retention time required to separate the five analytes was 17mins. Based on the flow rate optimization data above, the optimum flow rate is 1.0 mL/minute, with the separation of the five analytes having good resolution and the a nalysis time required is not too long.

**System Suitability Test**

System suitability test (SST) was carried out by injecting steroid mixed standard solution in six replicates. Based on the requirements set by USP 40 and ICH Q2 (R1), the system suitability test acceptance is stated as a percentage of RSD less than two percent. The system suitability test was conducted to ensure that the chromatographic system has good performance to be used during analysis (Table II).




| | A | B | C | D | E | F | G | H | I | J | K | L | M |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Peak No. | Name | Ret.Time min | Amount | Rel.Area % | Area mAU*min | Height mAU | Type | Width (50%) min | ymmetry (I | Resolution (USP) | Plates (USF | Min Wvl. |
| 4 | *1* | *Prednison* | *3,273* | *n.a.* | *12,10* | *2,4135* | *28,10* | *BM* | *0,073* | *0,86* | *3,14* | *10844* | *200,0* |
| 5 | 2 | Metil Paraben | 3,673 | n.a. | 20,24 | 4,0366 | 47,01 | M | 0,075 | 0,89 | 4,22 | 12975 | 200,0 |
| 6 | 3 | Triamsinolon Asetonida | 4,283 | n.a. | 12,08 | 2,4088 | 23,08 | MB | 0,093 | 0,92 | 3,88 | 11411 | 200,0 |
| 7 | 4 | Hidrokortison Asetonida | 4,950 | n.a. | 12,72 | 2,5374 | 21,43 | BMB | 0,107 | 0,92 | 4,60 | 11686 | 200,0 |
| 8 | 5 | Propil Paraben | 5,813 | n.a. | 16,20 | 3,2306 | 26,28 | BMB | 0,113 | 0,97 | 29,02 | 14640 | 200,0 |
| 9 | 6 | Klobetasol Propionat | 15,687 | n.a. | 12,40 | 2,4738 | 8,10 | BMB | 0,285 | 0,98 | 2,73 | 16513 | 200,0 |
| 10 | 7 | Mometason Furoat | 17,090 | n.a. | 14,26 | 2,8446 | 8,29 | BMB | 0,318 | 0,97 | n.a. | 15956 | 200,0 |
| 11 | Maximum | | | 0,0000 | 20,24 | 4,0366 | 47,01 | | 0,318 | 0,98 | 29,02 | 16513 | |

Figure 3. Chromatogram showing separation betw een steroids and parabens

Table III. Mix Standard Solution Chromatogram Parameters

| No. | Steroid | $t_R$ | Area (mAu*min) | Rs | Tf | N | Wavelength (nm) |
|---|---|---|---|---|---|---|---|
| 1. | Prednisone | 3.273 | 2.4135 | 3.14 | 0.86 | 10844 | 241 |
| | | 3.673 | 4.0366 | 4.22 | 0.89 | 12975 | 255 |
| 2. | Triamcinolone Acetonide | 4.283 | 2.4088 | 3.88 | 0.92 | 11411 | 240 |
| 3. | Hydrocortisone Acetate | 4.950 | 2.5374 | 4.60 | 0.92 | 11686 | 244 |
| | | 5.813 | 3.2306 | 29.02 | 0.97 | 14640 | 256 |
| 4. | Clobetasol Propionate | 15.687 | 2.4738 | 2.73 | 0.98 | 16513 | 240 |
| 5. | Mometasone Furoate | 17.090 | 2.8446 | n.a. | 0.97 | 15956 | 249 |

[a] tR: retention time; Rs: resolution among adjacent peaks; Tf: peak asymmetry factor N: theoretical plate number

The essential characteristics of SST include the number of theoretical plates (N), asymmetry, tailing factor, and resolution.

**Selectivity**

Method validation refers to USP 40 and ICH Q2 (R1) Guidelines which require specificity and determination of detection limit data should be present for identification purpose (category IV). This method was specified by complete separation of PRN, TCA, HCA, CBP and MTF and there was no excipient peak that interfered the analyte peaks (Figure 2). The average retention times for PRN, TCA, HCA, CBP and MTF 3.267, 4.260, 4.910, 15.360 and 16.707 minutes, respectively. In the solvent chromatogram (not shown) no peaks with the same retention time were found. The solvent chromatogram found several solvent peaks, but no peaks with the same retention time or adjacent to the analyte were found. Both the sample solvent and the mobile phase solvent can affect the separation based on their polarity and selectivity. The more polar the solvent, the longer the analyte is retained in the column. Selectivity is influenced by the polarity of the solvent and the position of the solvent in the solvent-selectivity triangle[16]. In the matrices chromatogram of liquid, lotion and solid samples (not shown) were also found no peaks that have the potential to interfere at analytes' retention time (Figure 2a and Table II). Based on the guidelines issued by the USP 40 and ICH Q2 (R1), the acceptance of a good chromatogram peak must meet several criteria, including the asymmetry factor (Tf), resolution factor (Rs) and the number of theoretical plates (N). The asymmetry factor for five peaks has met the recommendation ($Tf \leq 2$), the same result was also obtained for the resolution factor value which is above the recommendation ($Rs>2$) and the theoretical plate number value ($N>2000$). The five steroid analytes PRN, TCA, HCA, CBP and MTF have the highest intensity at its maximum wavelengths at 241, 240, 244, 240 and 249 nm, respectively.

Table IV. Parameters for determining detection limits

| No | Steroid | Linearity Range | Slope | Intercept | r | LOD (µg/mL) | LOD (%) | LOD (%)[a] |
|---|---|---|---|---|---|---|---|---|
| 1 | Prednisone | 0.5-5 µg/mL | 0.299 | 0.0642 | 0.997 | 0.36 | 0.036 | n.a. |
| 2 | Triamcinolone Acetonide | 0.5-5 µg/mL | 0.282 | 0.0790 | 0.995 | 0.44 | 0.044 | 0.01-0.05 |
| 3 | Hydrocortisone Acetate | 0.5-5 µg/mL | 0.325 | 0.0319 | 0.996 | 0.41 | 0.041 | n.a. |
| 4 | Clobetasol Propionate | 0.5-5 µg/mL | 0.271 | 0.0547 | 0.996 | 0.47 | 0.047 | 0.01-0.05 |
| 5 | Mometasone Furoate | 0.5-5 µg/mL | 0.336 | 0.0883 | 0.996 | 0.42 | 0.042 | 0.01-0.05 |

a : Data obtained from research conducted by Gimeno, *et al* (2012)

Some of each chromatogram of spiked sample liquid, lotion, and solid matric gave the same retention time with standard (Figure 2).

In this study, we have proved that the method of separating steroids and parabens produces a method that meets several parameter requirements (Figure 3 and Table III). All peaks have Rs, Tf and N value under the requirement. Thus, we can infer that paraben compounds would not interfere in the analyte peaks' retention time.

**Determination of Detection Limit**

The detection limit parameters as presented in Table 3 showed that from the linearity range of 0.5-5 g/mL, the analyte tested has a slope value ranging from 0.2705 to 0.3359 where the lowest slope is obtained from the CBP analyte and the highest slope is obtained from the MTF analyte, while for the intercept value, obtained values in the range of 0.0319–0.0883 with the lowest intercept value obtained from the HCA analyte and the highest intercept value obtained from the MTF analyte (Table IV).

The spiked sample solution calibration curve shows the equation of the line y = 0.2994x + 0.0642 for PRN, y = 0.2824x + 0.0790 for TCA, y = 0.3250x + 0.0319 for HCA, y = 0.2705x + 0.0547 for CBP and y = 0.2994x + 0.0642 for MTF, with correlation values for all five analytes above 0.995. The curve is said to be linear and can be used to determine the detection limit. The detection limit value obtained from this method for TCA, CBP and MTF is smaller than the study conducted by Gimeno, *et al* (2012) which was at 0.01-0.05%. Meanwhile, PRN and HCA are not stated. A smaller detection limit value means this method is more sensitive.

One of the critical factors in the validation of an analytical method's process is defining the limit of detection (LOD). The limit of detection is the smallest amount or concentration of analyte in a sample that can be reliably distinguished from zero under research condition (US Pharmacopeia, 2021).

**CONCLUSION**

A rapid, simple, efficient and sensitive simultaneous HPLC PDA method to identify prednisone, triamcinolone acetonide, hydrocortisone acetate, clobetasol propionate, and mometasone furoate in skin whitening product need to be done in order to strengthen the surveillance of cosmetic products in Indonesia. Analytical method of PRN, TCA, HCA, CBP and MTF has been developed and validated simultaneously using HPLC-PDA. The method meets the validation criteria for the identification type analysis method according to USP 40 and ICH Q2 (R1) by showing that the method is specific and selective towards the analyte target. The validation results proved reliable of the proposed method by revealing that the method is simple, precise, sensitive and accurate. The method could be successfully applied for routine analysis for market surveillance purposes in Indonesia coverage. The method gave a lower LOD value than other study. Analysis for more than one steroid compound using sophisticated instruments is suggested.

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**AUTHORS' CONTRIBUTION**

**Yustina:** conceptualization, data curation, data analysis, writing-original draft & editing. Alfi Sophian: review & editing.

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