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Original Article

# SIMULTANEOUS IDENTIFICATION AND QUANTIFICATION OF HYDROQUINONE, TRETINOIN AND BETAMETHASONE IN COSMETIC PRODUCTS BY ISOCRATIC REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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**ABSTRACT**

**Objective:** The objective of this study was to obtain a simple and selective analysis method for determination of hydroquinone, tretinoin and betamethasone in whitening creams using reversed-phase high-performance liquid chromatography (HPLC).

**Methods:** Reverse Phase HPLC was used for method development, validation studies, and sample analysis. The method was optimized by evaluating several parameters that affects the extraction of the sample, composition and types of mobile phase and also flow rate. Chromatographic separation was optimized on a C18 column [Sunfire, 250 x 4.6 mm, 5 µm] utilizing a mobile phase consisting acetonitrile, methanol (90:10 v/v) and slightly addition of glacial acetic acid to reach pH 5in the ratio of 30: 50:20 v/v at a flow rate of 0.8 ml/min with UV detection at 270 nm and 350 nm.

**Results:** The analytical methods fulfilled the validation requirements including accuracy, precision, linearity, selectivity, detection limits, and quantitation limits. The results showed the mean levels of hydroquinone, tretinoin and betamethasone in samples A and B were 1.78%; 0.07%; 0.12% and 2.00%; 0.07%; 0.13% respectively.

**Conclusion:** The method was successfully applied for the determination of cosmetic formulation containing hydroquinone, tretinoin and betamethasone simultaneously. There were seven samples analyzed and two samples were positive containing hydroquinone, tretinoin, and betamethasone.

**Keywords:** hydroquinone, tretinoin, betamethasone, whitening cream, HPLC reversed phase, optimization, validation


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## INTRODUCTION

Skin whitening cream is a type of cosmetic product that is now popular in many countries in Africa and Asia [1-3]. For most women in Indonesia, beauty defined with clear, spotless, white and bright skin. Therefore, the presence of brownish spots is considered to interfere with the concept. Although the spot is not harmful to health, many people often try to eliminate it. Some skin whitening products are claimed to contain a variety of highly toxic active ingredients especially after prolonged applications, such as hydroquinone, tretinoin, and corticosteroids [2][4-5]. The whitening agent may trigger harmful local effects such as skin burning, ochronosis, irritant contact dermatitis, leukoderma (vitiligo), post-inflammatory hyperpigmentation, and systemic toxicity, especially for the liver and kidneys [6]. In 2008, the Indonesian Food and Drug Administration also prohibited the use of hydroquinone, tretinoin, and betamethasone in cosmetic whitening products [7].

Several methods have been developed for the analysis of hydroquinone, tretinoin and betamethasone in illegal whitening creams. Analysis of hydroquinone in whitening cream has been developed using thin layer chromatography (TLC) and UV-Vis spectrophotometry [8-10]. Furthermore, high-performance liquid chromatography or HPLC methods have also been developed to analyze the hydroquinone and tretinoin content in cosmetics of physician prescription cream [11, 12]. Gas chromatography-mass spectrometry (GC-MS) method also have been developed to determine skin whitening agent in cosmetic products [13]. Another HPLC method also has been described by Desmedt (2013) to screen and quantify legal and illegal skin bleaching agent [6].

None of the above research methods performed simultaneous hydroquinone, tretinoin and betamethasone analyze using a simple isocratic HPLC method. So, this study was aimed to develop simultaneous, rapid, and selective analysis method towards these three harmful active substances in whitening cream using HPLC with some optimization. This HPLC method was preferred because of its high sensitivity, relatively simple implementation, efficient, good separation, analysis can be done in a relatively short time and also reproducible [14].

## MATERIALS AND METHODS

### Instrumentation

High Performance Liquid Chromatography LC 20AT (Shimadzu, Japan) equipped with pump, SunFireTM C18 column, UV-VIS detector SPD-10A (Shimadzu, Japan), manual injector, and data processor (LC-Solution), Syringe HPLC (SGE, Australia), UV-Vis Spectrophotometry (Jasco V-530), centrifuge (Labofuge 5100), vortex (Thermo Scientific), Micropipette (Eppendorf), pH meters (Eutech Instruments pH 510), sonicator UltrasoniC, hotplate (IKA® C-MAG HS 7), Whatman filter membrane diameter 0.45 µm, Whatman filter paper no. 41, ovens, desiccators, analytical scales, volumetric pipettes, rubber balloons, and glassware.

### Materials

Hydroquinone, tretinoin, and betamethasone (Merck). Methanol and acetonitrile HPLC Grade (Merck), aquabidestilata (Ikapharmindo). Seven samples of whitening cream used are listed below table 1

**Table 1: Skin whitening cream sample information**

| Sample | Origin | Source |
|---|---|---|
| A | No name and country of manufacturer information | Social media (*Instagram*) |
| B | No name and country of manufacturer information | Social media (*Instagram*) |
| C | Local manufacturer | Public market |
| D | Local manufacturer | Traditional market |
| E | Local manufacturer | Department store |
| F | Local manufacturer | Department store |

**Standard solution preparation**

Standard solution of hydroquinone, betamethasone, and tretinoin were prepared using acetonitrile to obtain a final concentration of 1000 µg/ml. The mixture was filtered through a 0.45 µm membrane filter and stored in the refrigerator until to be used.

**Sample solution preparation**

Approximately, 1 g of cream sample was extracted using 10 ml of acetonitrile and add 0.1% glacial acetic acid P into acetonitrile. The mixture was put into heating over the water bath at 40-60 °C and stirred thoroughly using magnetic stirrer until perfectly dissolved. Centrifugation was performed at 4000 rpm for 15 min. The supernatant was filtered through a 0.45 µm filter membrane to separate the undissolved matter. The filtrate was collected in a 25 ml volumetric flask wrapped in aluminum foil and ad with acetonitrile.

**Optimization of the analysis condition**

The analysis parameters that need to be optimized were combination and composition of mobile phase, flow rate and wavelength of analysis. The optimization was done by injecting 20 µl of standard solution into HPLC. Table 2 showed the variation of mobile phase combination. Flow rate variation were ranged between 0,8-1,2 ml/min. The optimum analysis conditions were assessed by the separation between two adjacent peaks or resolution (R), peak sharpness, analytical area, Tf, retention time, column efficiency (HETP) and the number of theoretical plates (N).

**Table 2: Selection of optimal mobile phase**

| No | Mobile phase | Composition |
|---|---|---|
| 1 | Acetonitrile–Phosphate buffer pH 6 | (90:10) v/v |
| 2 | Acetonitrile–Water–TEA | (90:10:0,5) v/v/v |
| 3 | Acetonitrile–Methanol–Ethyl acetate | (60:25:15) v/v |
| 4 | Acetonitrile–Methanol | (50:50) v/v |
| 5 | Acetonitrile–Methanol | (70:30) v/v |
| 6 | Acetonitrile–Methanol | (90:10) v/v |

Note: No. 4-6 added glacial acetic acid (pH modifier) until the solution reaches pH 5.

**System suitability test**

A standard mixed solution (10 µg/ml) was injected as much as 20 µl into the HPLC with selected analysis conditions. 6 repeated injection was done to ensure a constant peak area. The injection result was recorded and the coefficient of variation was counted (% CV). The % CV value should be ≤ 2%. The parameters were based on the separation between two adjacent peaks (R), tailing factor, retention time, column efficiency (HETP) and the number of theoretical plates (N).

**Validation of analysis methods**

**Preparation of calibration curve and linearity test**

The calibration curve test solution was prepared by diluting the standard solution of hydroquinone, tretinoin and betamethasone with the range of 75-300µg/ml for hydroquinone; 0.5-5 µg/ml for tretinoin and 1-20 µg/ml for betamethasone. 20 µl of each concentration level was injected into HPLC with selected analysis conditions. The ICH requirement value of correlation coefficient is ≥ 0.999 as shown in fig 1-3.

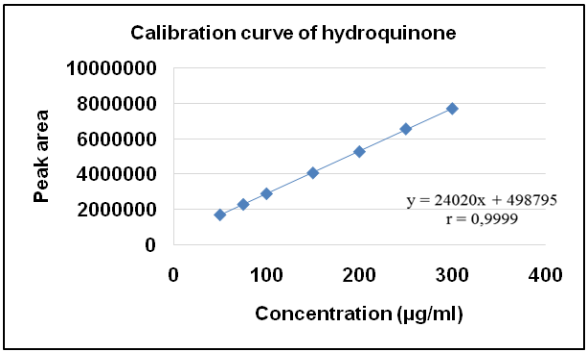


**Fig. 1: Hydroquinone calibration curve**

**Detection limit test (LOD) and quantitation limit (LOQ)**

From the obtained calibration curve, calculated the least detectable concentration (LOD) and quantitatively detected (LOQ) using a statistical calculation of the linear regression equation of the calibration curve. Limit detection and quantitation limits were calculated by measuring the blank response several times then calculated the standard deviation of the blank. The standard deviation of the blank (Sb) is equal to the residual standard deviation (Sy/x) [15].

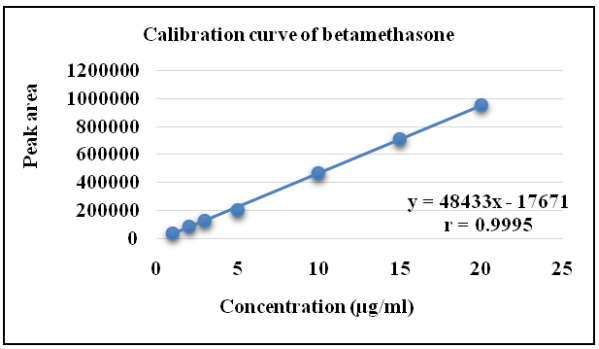


**Fig. 2: Betamethasone calibration curve**

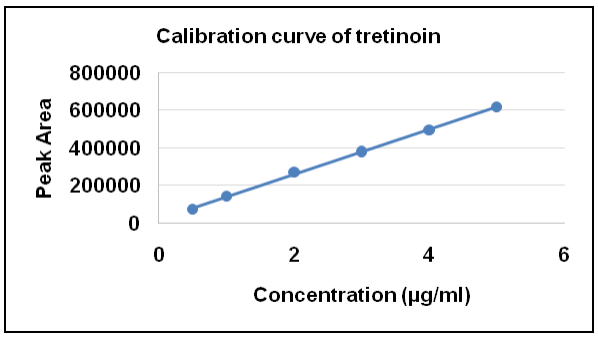


**Fig. 3: Tretinoin calibration curve**

**Selectivity test**

20 µl of 100 µg/ml standard solution, Standard mixed solutions of hydroquinone, tretinoin and betamethasone (100 µg/ml) were injected 20 µl into HPLC with selected analysis conditions.

Interferences were evaluated by comparing chromatogram of matrix solution and standard solution.

**Accuracy and precision**

20 µl of 100 µg/ml standard solution, Standard mixed solutions of hydroquinone, tretinoin and betamethasone (100 µg/ml) were injected 20 µl into HPLC with selected analysis conditions. The accuracy is determined from the recovery of at least three different concentrations with three repetitions per concentration [16]. Precision was determined using a minimum of 6 determinations at 100% of the test concentration.

**RESULTS**

**Optimization of chromatographic analysis**

Combination of an acetonitrile-phosphate buffer as a mobile phase produced chromatogram with uneven baseline, the peak was not symmetrical and sharp. The area of the compound were small and not separated well. This chromatogram also occurred in combination 2, 3 and 4. The use of acetonitrile-methanol (90:10 v/v) as mobile phase resulted in the best chromatogram with better, sharper peak, larger area, faster retention time, and good resolution. Thus, this combination was used as a mobile phase for sample analysis.

The starting wavelength value for the analysis of hydroquinone, betamethasone, and tretinoin was determined using Spectrophotometer. Maximum wavelength values obtained for hydroquinone was 294 nm, tretinoin 352 nm, and betamethasone 254 nm. The maximum wavelength of tretinoin was far different from hydroquinone and betamethasone. The way to analysis simultaneously was by changing the maximum wavelength. The first wavelength used was 270 nm to determine hydroquinone and betamethasone. After both peak appeared, the maximum wavelength was changed into 352 nm for tretinoin determination. This method was successfully determined the three ingredients simultaneously.

**Flow rate selection**

The search for optimum conditions of flow rate was carried out at three different flow rates, ie 0.8 ml/min; 1.0 ml/min; and 1.2 ml/min. the results of the optimization is shown in table 4:

**Table 3: Optimized wavelength optimization results of hydroquinone, tretinoin, and betamethasone**

| Wavelength (nm) | Compound | Area (µV/s) | RT (min) | Tf | HETP | N | R |
|---|---|---|---|---|---|---|---|
| 260 | Hydroquinone | 22671 | 2.687 | 1.289 | 21.772 | 6889.449 | 0.000 |
| | Betamethasone | 211589 | 3.085 | 1.220 | 25.027 | 5993.424 | 2.369 |
| | Tretionin | 1202236 | 6.321 | 0.871 | 12.590 | 11913.976 | 16.990 |
| 270 | Hydroquinone | 50145 | 2.690 | 1.133 | 22.674 | 6615.575 | 0.000 |
| | Betamethasone | 88567 | 3.041 | 1.188 | 20.251 | 7407.101 | 2.514 |
| | Tretionin | 1206024 | 6.521 | 0.858 | 12.834 | 11687.345 | 17.784 |
| 280 | Hydroquinone | 137321 | 2.702 | 1.242 | 18.951 | 7915.032 | 0.000 |
| | Betamethasone | 28470 | 3.024 | 1.512 | 29.523 | 5080.735 | 2.311 |
| | Tretionin | 1208625 | 6.450 | 0.862 | 12.467 | 12032.063 | 16.488 |

**Table 4: Optimized flow rate analysis of hydroquinone, tretinoin, and betamethasone**

| Flow rate (ml/min) | Compound | Area (µV/s) | RT (min) | Tf | HETP | N | R |
|---|---|---|---|---|---|---|---|
| 0.8 | Hydroquinone | 58116 | 3.376 | 1.210 | 19.942 | 7521.742 | 0.000 |
| | Betamethasone | 93130 | 3.783 | 1.180 | 17.164 | 8739.449 | 2.649 |
| | Tretionin | 1270690 | 8.072 | 0.811 | 11.477 | 13069.717 | 19.202 |
| 1.0 | Hydroquinone | 50145 | 2.690 | 1.133 | 22.674 | 6615.575 | 0.000 |
| | Betamethasone | 88567 | 3.041 | 1.188 | 20.251 | 7407.101 | 2.514 |
| | Tretionin | 1206024 | 6.521 | 0.858 | 12,834 | 11687.345 | 17.784 |
| 1.2 | Hydroquinone | 42543 | 2.249 | 0.000 | 36.075 | 4158.008 | 0.000 |
| | Betamethasone | 78786 | 2.531 | 0.000 | 35.120 | 4271.032 | 1.914 |
| | Tretionin | 1105425 | 5.371 | 0.881 | 13.824 | 10851.056 | 15.730 |

Increasing the flow rate can result in faster retention times for all three compounds, but the resulting area is smaller and column pressure becomes higher. The faster the flow rate used then the resulting area will be smaller because the separation has not happened perfectly. In this study, a flow rate of 0.8 ml/min was chosen as the optimum flow rate because it resulted in a larger peak area, better resolution, column pressure at an optimum range, small HETP values, and the number of large theoretical plates.

**System suitability test**

Before the selected analysis is used for a study, it is necessary to first test the conformity of the system because of the possible variations in the equipment and the analytical techniques used. The results of repeatability test with injection as much as six times, obtained by follow-up factor of 1.403, HETP 18.381, number of theoretical plates 8160.723, and a coefficient of 0.46% variation in hydroquinone compounds. In the tretinoin, compound obtained a factor of 0.854, HETP 11.185, the number of theoretical plates 13410.664, the coefficient of variation 0.75%. Furthermore, in the compound betamethasone obtained element follow up of 1.100, HETP 20.434, the number of theoretical plates 7340.564, the coefficient of variation 0.35%. The data table 5 meet the requirements of the system suitability test because the value of repetition or coefficient of variation is below 2%.

**Table 5: System suitability result**

| | Tf | HETP | N | R | Standard deviation (SD) | Coefficient of variation (% CV) |
|---|---|---|---|---|---|---|
| Hydroquinon | 1.40 | 18.38 | 8160.72 | 0.000 | 271.62 | 0.46 |
| Bethametasone | 1.10 | 20.43 | 7340.56 | 2.651 | 331.11 | 0.35 |
| Tretinoin | 0.85 | 11.18 | 13410.66 | 18.654 | 9647.61 | 0.75 |

**Validation of analysis methods**

**Calibration curve and linearity test**

Based on linear regression calculation, the equation of calibration curve line on hydroquinone compound y = 24020x+498795, tretinoin compound y = 119722x+19932, and betamethasone y = 48433x-17671, where x is the concentration of hydroquinone, tretinoin or betamethasone and y is the area peak hydroquinone, tretinoin or betamethasone. Linearity is a measurement method to see how well the response relationship of various concentrations on a calibration curve to produce a straight line. The result of linearity test of hydroquinone solution in standard with range 50-300 µg/ml yielded correlation coefficient (r) equal to 0.9999, while the tretinoin linearity test within the standard with a range of 0.5-5 µg/ml yielded a correlation coefficient (r) of 0.9994, and betamethasone linearity test in standard with range 1-20 µg/ml yielded correlation coefficient (r) equal to 0.9995. It can be concluded that the three calibration curves meet the linearity test as it yields a correlation coefficient (r) greater than or equal to 0.999 (≥ 0.999).

The purpose of the development of the method mainly is to determine the misuse of these ingredients in cosmetics products. The need to develop low LOD and LOQ is because the usual dose of this ingredient in cosmetics is relatively low. Smaller LOD and LOQ indicate that this method is able to identify and measure even in low concentrations. The LOD and LOQ for hydroquinone was 6.86 µg/ml and 22.89 µg/ml respectively, while LOD and LOQ for tretinoin was 0.18 µg/ml and 0.61 µg/ml respectively, and the LOD and LOQ for the betamethasone was 0.71 µg/ml and 2.39 µg/ml respectively. The summary is shown in table 6.

**Table 6: Summary of validation**

| Parameter | Hydroquinone | Betamethasone | Tretionin |
|---|---|---|---|
| Linearity | y = 24020x+498795<br>r = 0.9999 | y = 48433x–17671<br>r=0.9995 | y = 119722x+19932<br>r = 0.9995 |
| Recovery (%) | 99.63-99.92 | 99.51-100.02 | 99.62-100.08 |
| Precision (%CV) | 0.20-0.49 | 0.27-0.55 | 0.23-0.47 |
| LOD (µg/ml) | 6.86 | 0.71 | 0.18 |
| LOQ (µg/ml) | 22.89 | 2.39 | 0.61 |

The retention times of hydroquinone, tretinoin and betamethasone respectively were 3.3 min, 8.0 min, and 3.8 min. The injection of 20.0 µl of placebo solution (cream matrix) treated as in the preparation of the sample showed no interference at the time of retention of hydroquinone, tretinoin or betamethasone. This may prove that the method was selective to determine these three ingredients.

Based on the results of the analysis, the average recovery value (% recovery) on three different concentrations for hydroquinone is 80% (99.76%), 100% (99.63%), 120% (99.92%), tretinoin is 80% (100.08%), 100% (99.82%), 120% (99.62%), and for betamethasone that is 80% (99.51%), 100% (99.69%), 120% (100.02%). This result showed that the method was met the requirement in ICH, which with each of six replicas at each concentration meeting the 98-102% criterion.

Furthermore, the value of the coefficient of variation (CV) for hydroquinone, tretinoin and betamethasone was approximately 0.20-0.49%, 0.23-0.47%, and 0.27-0.55%. The precisions of the method were found to be satisfactory as the RSD values determined by repeatability and intermediate precision studies were all less than 2.0%.

**Analysis of whitening cream sample**

From 7 samples that was collected from the market an social media, two of them were positive contains hydroquinone, tretinoin, and betamethasone.

**Table 7: Determination of hydroquinone, betamethasone and tretinoin levels in 1 mg skin whitening cream**

| Sample | Analyte | Response detector (µV/s) | Concentration of detected analyte (µg/ml) | % |
|---|---|---|---|---|
| A | Hydroquinone | 3923712 | 142.58 | 1.78 |
| | Betamethasone | 448923 | 9.63 | 0.12 |
| | Tretinoin | 699876 | 5.67 | 0.07 |
| B | Hydroquinone | 4325306 | 159.30 | 1.99 |
| | Betamethasone | 488161 | 10.44 | 0.13 |
| | Tretinoin | 797094 | 6.49 | 0.08 |

**DISCUSSION**

Hydroquinone, betamethasone and retinoic acid are forbidden to be used in cosmetics preparation due to its harmful effect on the skin. Despite that fact, those illegal ingredients are often found in cosmetics, especially, cosmetics that are not legally registered. These cosmetics are often found in the traditional market or sold via online shop [16]. Thus, the simple and rapid method to identify the presence of these illicit compounds is needed to be developed for market survey control. In the present study, HPLC was used for the qualitative and quantitative determination of hydroquinone, betamethasone, and tretinoin simultaneously from skin whitening cream.

The choice of composition and mobile phase combination is essential in the initial step of optimizing the analysis conditions. The objective of this optimization was to obtain good separation characteristics, resolution, selective and generates a good area of the analyte. Generally, In the separation with reversed phase HPLC, the mobile phase used tends to be more polar (more hydrophilic) than the surface of the stationary phase. Based on the separation system used, the most polar hydroquinone will elute first and then be followed by betamethasone and the last is tretinoin which is the most non-polar [18-19]. Experiments with the mobile phase Acetonitrile-Methanol (90:10 v/v) resulted in better separation, sharp peaks, larger areas, faster retention times, and resolutions meeting the requirements. pH was adjusted to pH 5 by adding glacial acetic acid P to generates sharper peak. Selection of glacial acetic acid P as a pH modifier due to its better performance than other types of acids and is often used with acetonitrile and methanol [20]. In addition, glacial acetic acid resulted in better efficiency to increase the resolution between three compounds and sharpen the peak [20].

The results of the validation assay of the mixture are within acceptable limits. Good linearity was observed in the concentration range employed in the test with a regression coefficient of more than 0.9990. The coefficient correlation of those three active substances was low, being less than CV max. Thus the method is precise. Selectivity test was carried out by injecting 20 µl of the standard solution of hydroquinone, tretinoin, and betamethasone and

compared to the blank matrix. The results obtained did not show any interference at the retention time of hydroquinone, tretinoin, and betamethasone. The developed, validated method was applied for the 6 commercial cosmetic products. According to the Indonesian Ministry of Food and Drug safety, does not allow for the existence of hydroquinone, tretinoin, and betamethasone in whitening cosmetics [21-22]. As compiled in table 8, From 7 samples that were collected from the market and social media, two of them were positive contains hydroquinone, tretinoin, and betamethasone. Overall, the results showed that the proposed method could be successfully applied for the determination of cosmetic formulation containing hydroquinone, tretinoin, and betamethasone simultaneously.

## CONCLUSION

Optimum conditions for the analysis of hydroquinone, tretinoin and betamethasone in cosmetic preparations using Reverse Phase Chromatography with UV-Vis detector, SunFireTM C18 column (column length 250 mm, inner diameter 4.6 mm, 5 µm particle size), using mobile phase composition acetonitrile-ethanol (90:10 v/v), wavelengths of 270 nm and 350 nm, and flow rate of 0.8 ml/min. An injection volume of 20.0 µl. The analysis method used fulfilled all validation criteria of analysis method consisting of selectivity test, linearity test, accuracy test, and precision test so that the method used is valid. Of the seven samples of whitening cream analyzed, two samples of them positively contained hydroquinone, tretinoin and betamethasone. In sample A, we have obtained hydroquinone, tretinoin and betamethasone levels respectively, 1.78%; 0.07% and 0.12%. In sample B, we have obtained the levels of hydroquinone, tretinoin and betamethasone respectively, 2.00%; 0.07% and 0.13%. Both samples were obtained from social media.

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## AUTHORS CONTRIBUTIONS

All the author have contributed equally

## CONFLICT OF INTERESTS

Authors declare no conflicts of interest

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