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Analytical Methods Validation of Retinoic Acid and Hydroquinone Using Ultra High Performance Liquid Chromatography in Medicinal Cream

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Abstract: Hydroquinone and retinoic acid have been widely used as whitening agents in cosmetic creams and have a negative impact if used without a medical prescription. In this study, development of analytical method of retinoic acid (REA) and hydroquinone (HYQ) were carried out using Ultra High Performance Liquid Chromatography (UHPLC) with a PDA detector, Phenomenex Luna® ODS / C_{18} column (150 mmx4.6 mm), methanol-acetic acid 0.1% (85:15) as mobile phase, flow rate of 1.0 mL/min and detected at 295 nm for HYQ and 341 nm for REA. The result of the validation method showed a retention time of HYQ at 1.55 minutes and REA at 3.73 minutes. Validation parameters performed are selectivity, range, linearity, LOD, LOQ, accuracy, and precision. Linear detector response in the concentration range of 75-250 ppm with a calibration curve equation. The calibration curve equation for HYQ is $y = 0.4607x + 1.9014$ (r = 0.999), mean while REA is $y = 2.3138x + 21.0429$ (r = 0.999). LOD and LOQ values of HYQ are 1.615 ppm; 13.98 ppm respectively and REA 1.02 ppm and 3.11 ppm, respectively. The precision (% RSD) $\leq \pm 2\%$ and accuracy (% recovery) in the range of 99.0% - 100.6%. This method is valid to be used to analyze HYQ and REA in commercial cosmetic cream samples.

1. INTRODUCTION

Retinoic acid (REA) or tretinoin is extensively used as anti-aging non-peeling and it is forbidden in all cosmetic preparation (Kligman, Duo and Kligman, 1984; Badan Pengawasan Obat dan Makananan, 2003). REA is teratogenic and can cause skin irritation in excessive using. Hydroquinone (HYQ) is one of the components used as a whitening agent in cream products. It is working as inhibitor melanin formation by inhibiting tyrosinase enzymes (Yang *et al.*, 2010). In medicine, HYQ was used to prevent hyperpigmentation such as melasma. However, it can cause hypopigmentation if used excessively and in a long time. The HYQ that has a concentration of more than 4% can cause irritation immediately (Badan Pengawasan Obat dan Makanan, 2008; Pengawasan Obat dan Makanan, 2011).

Many studies have been reported in analyzing REA and HYQ as a single component (Martono, Febriani and Rohman, 2018; Rahmayuni, Harmita and

Suryadi, 2018). This study aimed to develop a rapid, simple, less expensive, sensitive and validated method in determining simultaneously REA and HYQ in cream using ultrahigh-performance liquid chromatography (UHPLC). Validation parameters that used to validate the proposed method were: specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), precision and accuracy in accordance with USP guidelines (The United States Pharmacopeia, 2016). The validated method was further used to analyze cream containing REA and HYQ whether as medicinal preparations or cosmetics cream.

2. MATERIAL AND METHODS

2.1 Material

The chemical used in this research was methanol grade for liquid chromatography (Merck), acetic acid 98-100% pro analysis (Merck), aqua pro injection (Widatra Bhakti), hydroquinone (Sigma-Aldrich), retinoic acid (Hangzou Rebtech Novel), cream base

oil-in-water (PT. Ika Pharmindo Putra). Commercial whitening topical cream containing both HYQ and REA concentration more from online store X in Indonesia.
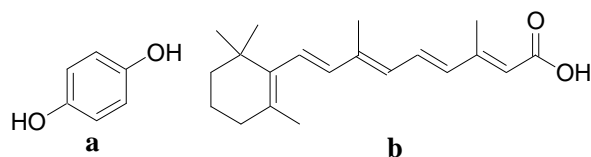

Figure 1. Chemical structures of hydroquinone (a) and retinoic acid (b).

2.2 Instrumentation and Chromatographic Conditions

All separations in this research were performed on the UHPLC Dionex 3000 with a photodiode array (PDA) detector. The data were obtained and analyzed using Chromeleon software. Chromatographic separation was carried out on Phenomenex Luna® 10 C18 (150 mm x 3.9 mm). The mobile phase used for stationary elution was methanol-acetic acid 0.1% which was filtered through a 0.45 μm membrane filter and degassed by ultrasonic wave (Elmasonic). The flow rate was maintained at 1.0 mL/min with a column temperature of 40°C. The injection volume was 20 μL, and detection was performed at 295 nm HYQ and 341 nm REA using PDA detector.

2.3 Preparation of Standard Stock Solutions

The standard stock solutions of hydroquinone and retinoic acid were prepared by accurately weighing and diluted in volumetric flask. The drugs were dissolved in methanol that used for eluent and the solution was diluted to volume. The nex step dilutions both were prepared from these stock solutions become 75, 100, 150, 200 and 250 ppm.

2.4 Preparation of Simulation Cream

The oil in water cream base was made with saponification surfactant mechanism. Furthermore, hydroquinone and retinoic acid were added into the cream base. An approximately 0.5 g was accurately

weighted using the micro-analytical balance. Added with methanol in 10 ml volumetric flask, put in vortex mixer about 15 minutes then extracted with ultrasonic in 15 minutes, then filtered with PTFE 0.45 um.

2.5 Method Validation

a. Specificity

The specificity for this UHPLC method was resoluted by the complete separation of hydroquinone and retinoic acid without any disruption of a chromatogram from excipients peak with analyte peaks by analyzing the resolution (R), tailing factor (Tf) and theoretical plates (N) parameters of each peak.

b. Linearity and Range

In linearity determining, calibration curves have been plotted over a range of 75-250 μg/mL both respectively. A 20 μL of sample solution was injected into the chromatographic system. The determined concentration then made a comparison between each peak area of analyte versus the corresponding drug concentration. All measurements were repeated three times for each concentration. %RSD ≤ 2 and R-value (≥ 0.999) showed the good linearity of the method Limits of detection and quantification: from the same sample with linearity test, the limit of detection (LOD) and limit of quantification (LOQ) were counted with formula. The result was counted from the standard deviation (SD) of response and the slope of the curve (S) by means of the equations. the aquation for LOD = 3.3 (SD/S) and for LOQ = 10 (SD/S), where SD: standard deviation response of the detector; and (S) mean: slope of the calibration curve (Harmita 2004, Rahmayuni, Harmita and Suryadi, 2018).

c. Precision

The precision of this method was calculated by prepare three different concentrations from both standard at low, medium, and high in simulation cream, which freshly prepared then soon to be analyzed. The precision % relative standard deviation was calculated by analyzing standard drug solutions in the calibration range. The precision of the method was expressed as %RSD.

d. Accuracy

Accuracy means the difference between the theoretical added amount and the practical result amount. Accuracy of the method was performed in triplicate by standard addition at 80%, 100%, and 120%. The known amount of standard HYQ and REA were added in simulation cream to analyzed trial cream and intended for the proposed method.

2.6 Analysis of Marketed Cream Preparation

Some sample used in this test is a medicine cream. The sample was extracted with the eluent methanol, the sample dugs dissolution were helped by vortex mixer and ultrasonication for 15 minutes, then filtered using a 0.45 μm PTFE filter.

3. RESULTS AND DISCUSSION

3.1 Optimization of Procedures

Initially, the optimization of the mobile phase was conducted by using acetonitrile-water (85:15). The chromatogram showed separated peaks, but the resolution value was not qualified (≤1.5). Next, the mobile phase changed to the water with 0.1% acetic acid glacial, and the result still not qualify for resolution and asymmetry factor. The next optimization was conducted by replacing acetonitrile with methanol, hence the mobile phase used was methanol-water (85:15). Without adding acid the result that given by chromatogram were symmetry, but resolution factor still under-qualified. The last, methanol-acetic acid glacial 0.1 % (85:15) was used as a mobile phase, and the chromatogram showed the best separation and peak (Figure 2).

The separation was conducted in the isocratic program, a flow rate of 1.0 mL/min with a column temperature of 40°C. PDA was used as a detector using Chromeleon Chromatography software with a time wavelength procedure. The HYQ was read at 295 nm, and REA was read at 341 nm.
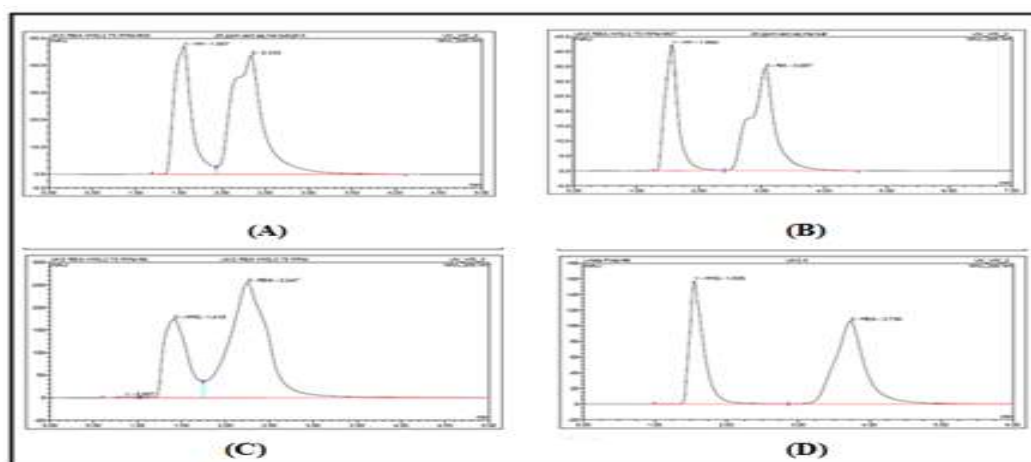

Figure 2: Chromatogram HYQ and REA (A) ACN-water (85:15). (B) ACN-acetic acid glacial 0.1% (85:15). (C) Methanol-water (85:15). (D) Methanol-acetic acid glacial 0.1% (85:15).

The optimum condition was given in table 1 and suitable parameters were given in tables 2 and 3. The average retention times for HYQ and REA were found to be 1.5 and 3.7 respectively (Figure 3).
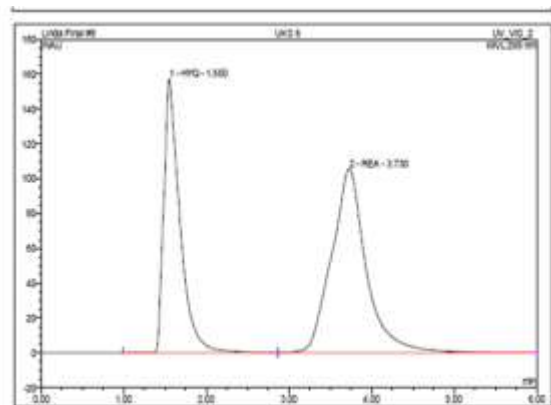

Figure 3. Chromatogram standard of HYQ and REA

3.2   Validation Methods

The UHPLC method was identified and determined by the perfect separation of HYQ and REA without any interference of peak from excipients within the analyte peaks (Figure 3). The resolution (R), asymmetric parameters and plate count (PC) of each peak, fulfilled the requirement. The R-value was more than 2, asymmetric factor less than 2 and Plate Count more than 2000 (Tables 2 and 3).

Table 1: UHPLC conditions for analysis

Parameter	Condition
Column	Phenomenex Luna 10 C18 (150 mm X 3,9 mm) 5 um.
Mobile Phase	Methanol-acetic acid glacial 0.1% (85:15)
Flow Rate	1,0 mL/min
Detection	295 nm, 341 nm
Pump Mode	Isocratic
Injection Volume	20 μl
Run Time	5 min
Temperature	40ºC
Retention Time	1.5 for HYQ and 3.9 for REA

Linear regression of the calibration curve showed a good linear relationship between response and correlation. The linear regression formula were $y = 0.4607x + 1.9014$ for HYQ, and $y = 2.3138x + 21.0492$ for REA (Table 4). LOD and the LOQ value from this simultaneous analysis were found 4.61μg/ml and 13.9μg/ml for HYQ, 1.02μg/ml and 3.11μg/ml for REA, respectively. For precision evaluation of HYQ the RSD values and REA were in the precision studies, these values indicate that the repeatability of this method is satisfactory. The accuracy was evaluated by simulation cream and the recovery percentage was used as accuracy indication. The average of % recovery obtained. The values of recovery indicate the proposed method is accurate (Harmita, 2004; Supandi *et al.*, 2018).

Table 2. System suitability testing parameters HYQ

Ret.Time (min)	Area (mAU)	Theoretical Plates	Asymmetry factor	Resolution
1.551	35.283	3863	1.87	4.15
RSD = 0.078%	RSD = 1.84%	RSD = 0.82%	-	-
Requirement	RSD ≤ 2.0%	RSD ≤ 2.0%	<2	≥1.5

*n=6

Table 3. System suitability testing parameters REA

Ret. Time (min)	Area (mAU)	Theoretical Plates	Asymmetry factor	Resolution
3.735	179.230	3851.5	1.19	1.53
RSD = 0.179%	RSD = 1.70%	RSD = 1.7%	-	-
Requirements	RSD ≤ 2.0%	RSD ≤ 2.0%	<2	≥1.5

*n=6

Table 4. Specificity, range, LOD and LOQ, recission, accuracy and statistic parameters.

Parameter	HYQ	REA
Linearity range, µg/ml	75 – 250	75 – 250
Regression equation	y = 0.4607x + 1.9014	y = 2.3138x + 21.0492
R	0.9993	0.9998
Slope	0.4607	2.3138
Y-intercept	1.9014	21.0492
LOD (µg/ml)	4.615	1.02
LOQ (µg/ml)	13.98	3.11
Precision (%RSD)	≤0.524	≤0.810
Acuracy (% recovery)	98.09 – 100.64	99.00 – 99.20

3.3 Application Analysis of Marketed Formulation

Based on the validation result, it can be applied to commercial samples analysis. The figure revealed UHPLC chromatogram of commercial samples containing HYQ and REA. The result of this study revealed this method was valid to analyze both components in commercial samples. The creams are not supposed to buy without any prescription. The result of the measurement is presented in Table 5.

Table 5. Measurement of commercial creams

Cream	Containing	
	HYQ* (%)	REA* (%)
A	4.2 ± 0.5	0.7 ± 0.1
B	2.9 ± 0.3	0.4 ± 0.1
C	1.5 ± 0.2	0.4 ± 0.1
D	3.8 ± 0.4	5.1 ± 0.6

*n=3.

4. CONCLUSION

The proposed UHPLC method is accurate, precise, sensitive, selective, and rapid for the simultaneous determination of hydroquinone and retinoic acid and it can be applied in a cream formulation.

5. ACKNOWLEDGMENT

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