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A Colorimetric Method to Measure the Amount of Hydroquinone in Liposomal Formulations

Rabea Khoshneviszadeh[1], Bibi Sedigheh Fazly Bazzaz[2,3], Omid Rajabi[4,*]

[1]Department of Biochemistry & Biophysics Sciences Faculty, Varamin-Pishva Branch, Islamic Azad University, Varamin, Iran.

[2]Biotechnology Research Center, Institute of Pharmaceutical Technology, Mashhad University of Medical Sciences, Mashhad, Iran.

[3]School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran.

[4]Department of Drug and Food Control, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran.

Abstract

A method for determination of concentration of solute depended on its chemical properties, degree of dilution and matrix that takes it is developed. Formulation of liposomal hydroquinone, a drug delivery system, is developed to increase its efficacy. In this study, a colorimetric method based on the method of diphenyl picryl hydrazyl (DPPH) reduction was used to measure the concentration of hydroquinone in the liposomal matrix. Hydroquinone is an ingredient with antioxidant activity that is able to reduce DPPH molecules. the samples were dissolved in methanol for testing, and after an hour of mixing with DPPH, the absorption intensity of each sample was measured at the wavelength of 516 nm, then validation parameters such as linearity and range, precision, accuracy, specificity, LOQ and LOD were measured. This method can be used to determine the concentration of liposomal hydroquinone samples in the concentration range of 0.5 to 2 µg/ml, with a regression rate of 0.999, precision 2.1% for intraday and 2.8% for inter-day, recovery 96.2% and 100.8% for 80% and 120% main concentration, respectably. LOQ 0.149 µg/ml and LOD 0.04 µg/ml were obtained. This method is precise, accurate specific and linear for determination of hydroquinone in liposomal matrix.

Keywords: Colorimetric method, Determination of concentration, DPPH, Hydroquinone, Liposome, Validation.

1. Introduction

A liposome is a drug delivery system that increases the treatment efficiency by slowly releasing the drug. It reduces the toxic effects of the drug and increases its ability to target a specific cell or organ (1-3). A liposomal formulation is an appropriate strategy to increase the crossing of the dermatological drugs through the stratum corneum barrier (4). Hydroquinone (HQ) has depigmentation properties that inhibit the skin's ability to produce melanin by inhibiting the enzyme tyrosinase (5). It is effective in treating freckles, melasma, and chloasma (6). The production of a liposomal formulation of hydroquinone is a way to increase the effectiveness of the treatment with low concentrations of the drug.

So far, various pharmaceutical formulations of hydroquinone have been provided, where the amount of hydroquinone was measured by

Corresponding Author: Omid Rajabi, Department of Drug and Food Control, Faculty of Pharmacy, Mashhad University of Medical Science, Mashhad, Iran
Email: rajabio@mums.ac.ir

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spectrophotometry (7-8), high-performance liquid chromatography (HPLC) (9-12), micellar electrokinetic chromatography (MEKC) (13), capillary electrochromatography (CEC)(14-15) and thin layer chromatography (TLC) (16). But the measurement of hydroquinone in the liposomal matrix has been reported by these authors first time with the help of the spectrophotometric technique (17).

Hydroquinone has a powerful antioxidant property because it is susceptible to oxidation. After being in the water environment, it turns into a sustainable free radical called semiquinone (18). Diphenyl picryl hydrazy (DPPH) is a stable radical that acts as an oxidizing agent in oxidation-reduction reactions (Figure 1). There are some antioxidant compounds that have the potential to reduce a higher DPPH; they can restore it and change its color from purple to yellow (19). Thus, the antioxidant concentration can be evaluated by reducing the absorption intensity of DPPH at 516 nm (20). This article has described a way to do so and reports the validation parameters of hydroquinone measurement in a liposomal matrix by using DPPH.

2. Experimental

2.1. Reagents

Phosphatidylcholine (PC) from soybean lecithin (S100) was purchased from Lipoid Company. Diphenyl picryl hydrazil is provided from Sigma. Hydroquinone, Benzoquinone, cholesterol, sodium metabisulfite, chloroform, mono sodium dihydrogen phosphate (analytical grade) from Merck were used. Methanol and alpha ketopherol acetate from Chemical Pars (analytical grade) and Applichem were obtained, respectively.

2.2. Preparation of standard and sample solutions

2.2.1. Preparation of the DPPH stock solution

Some of the materials were dissolved in methanol so that its absorption, at a wavelength of 516 nm, was one against a blank methanol.

2.2.2. Preparation of the control solution

Three ml of the DPPH stock was mixed with 2 ml of methanol. After an hour, its absorption was measured at 516 nm by a Cecil 9500 Double Beam Spectrophotometer.

2.2.3. Preparation of standard hydroquinone solutions

Fifty mg of hydroquinone was dissolved in 100 ml water to obtain a concentration of 500 µg/ml. This solution was diluted with methanol to prepare a hydroquinone stock solution with a concentration of 10 µg/ml. Then the hydroquinone standard solutions were prepared in the concentration range of 0.5-2 µg/ml, with the help of the methanol and the DPPH stock solution (with 3 times repetition).

2.2.4. Preparation of liposomal hydroquinone solutions

Liposomal samples were prepared by evaporation of solvent phase method (22). First, cholesterol (1.5%), HQ (0.5%), S100 (7.78%) and alpha ketopherol (0.17%) w/v were dissolved in 15 ml of chloroform and, 5 ml of methanol. The oraganic solvent was eliminated by Buchi rotary evaporator to formed thin film phospholipid; then flushed with nitrogen gas for 1 min, phosphate buffer (pH 6, 0.01 M) with sodium metabisufite (0.1%) was added slowly to the thin film, and swelled by shaking, vortexed and was homog-

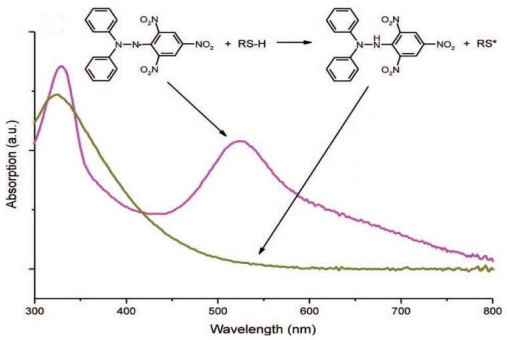

Figure 1. Intensity of absorption of DPPH is decreased at 516 nm after reduction (21).

enized for 5 min at 2000 rpm (IKA T10 homogenizer). According to this process the particles of liposome is formed (23), and spherical structure of liposomal particles was seen by Olympus optical microscope-equipped camera (Figure. 2). Fresh liposomal hydroquinone samples were diluted initially up to 1,000 times with methanol. Then this concentration was diluted five more times with the DPPH stock solution and methanol until finally the dilution was conducted 5,000 times on the hydroquinone liposomes.The final volume of each sample was 5 ml, 3 ml of which contained the DPPH stock.

2.2.5. Preparation of empty liposome

The same method described for the liposomal formulation preparation of hydroquinone was used, with the difference that hydroquinone was not used in this preparation. Hydroquinone free liposome samples were diluted of the same liposomal hydroquinone samples. HQ-free of liposome was as solution (placebo) were diluted 5000 times, the same as preparation of liposomal hydroquinone samples, by methanol and DPPH stock.

2.3. Method validation

For validation studying, the International Conference on Harmonization (23) and AOAC International Guidelines for Validation of Analytical methods were used (24).

2.3.1. Linearity

The calibration curve was obtained at ten concentration levels of HQ solutions (0.5-2 µg/ml) by the least square regression method. The linearity was evaluated with triplicate determinations at each concentration level.

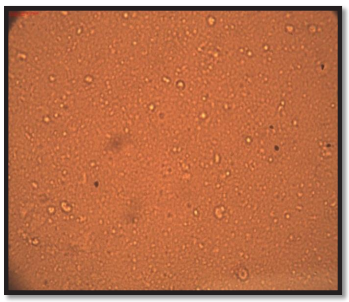

Figure 2. The spherical shape of particles of liposome (100x).

2.3.2. Precision

The degree of aggregate among test results was checked when a method was applied repeatedly. Variation in intra-day and inter-day was analyzed. The intra-day and inter-day precision was determined by analyzing the same concentration of HQ (1 µg/ml). Six samples of hydroquinone (1 µg/ml) were prepared in three days. The concentrations obtained were based on the obtained equations of the calibration diagram.

2.3.3. Accuracy

The HQ liposome solution (0.5%) was diluted 500 times by methanol (100 µg/ml), then 5 ml of this solution and 3, 5 and 7 ml of a HQ stock solution (100 µg/ml) were transferred into 100 ml volumetric flasks, separately, and methanol was added to make up the volume to give concentrations of 8, 10 and 12 µg/ml. one ml of each sample was transferred into tube and 3 ml of the DPPH stock solution and sufficient methanol were added to provide 0.8, 1 and 1.2 µg/ml final concentrations. All solutions were prepared in triplicate and analyzed.

2.3.4. Specificity

The specificity was studied by analyzing HQ-free liposome, where in the sample matrix was analyzed without the analyte. The result was examined for the presence of interferences or overlaps with the HQ result. HQ-free liposome solution (placebo) were diluted 5000 times, the same as preparation of liposomal hydroquinone samples, by methanol and DPPH stock.

2.3.5. Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The limit of detection (LOD) of HQ was evaluated from the slope (S) of calibration curve and the standard deviation of the control (d) using equation as: LOD=3.3 d/S.

The minimum quantity of drug that can be quantified by the instrument is defined was LOQ. The LOQ were evaluated from the slope(s) of calibration curve and the standard deviation of the blank (d) using equation as: LOQ=10 d/S.

Then, according to the slope of the calibration line equation and the standard deviation ob-

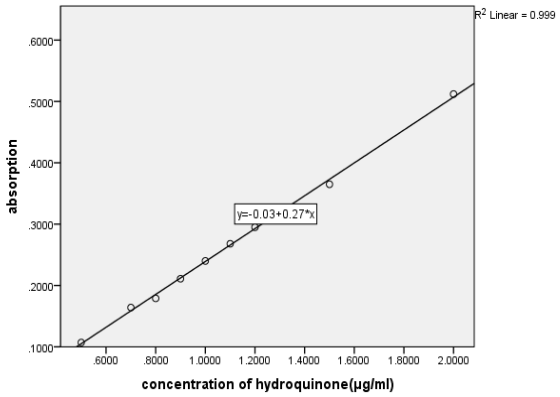

Figure 3. The calibration curve and correlation coefficient of HQ solutions (0.5-2 µg/ml).

tained from multiple measurements of the blank, LOD and LOQ were obtained.

2.4. measurment

All of samples were prepared in foil-covered tubes with a lid, and one hour was given for the reaction of the DPPH on the samples. Thenaborbance was measured after one hour, at 516 nm, with the methanol at zero. Then the absorption intensity of the samples was measured and subtracted from the amount of the control absorption.

3. Results and Discussion

3.1. Linearity and Range

The differences of absorption of control sample with hydroquinone at a concentration range of 0.5-2 µg/ml is shown in Table 1, and it is applied to draw a standard diagram (Figure 3) where the most selective concentrations were at a concentration of 1 µg/ml. The value of the correlation coefficient was 0.999; it was obtained after drawing the best line in the concentration diagram against the absorbance, indicating a good matching between the concentration value and the reduction of DPPH. In a previous study (17), the lower point of the concentration slope was 1 mg/ml; in this study, that has been extended to lower values.

3.2. Precision

One mg/ml hydroquinone was used to assess the reproducibility rate of responses resulting from a laboratory process of repeated measurements for concentration. The hydroquinone samples were prepared at a concentration of 1 µg/ml and their concentrations were measured in accordance with the described laboratory process in three consecutive days according to the ICH, 6 protocol. After conducting statistical calculations, the RSD amount obtained was 2.1% in one day and 2.8% between days, which represents the dispersion of answers for the conducted tests. The

Table 1. Experimental data of calibration curve of Hydroquinone.

Concentration (µg/ml)	Differences of absorption between control and standard solutions in 516 nm			Mean
2	0.510	0.513	0.513	0.512
1.5	0.365	0.364	0.366	0.365
1.3	0.316	0.317	0.321	0.318
1.2	0.297	0.296	0.292	0.295
1.1	0.266	0.271	0.267	0.268
1	0.24	0.241	0.239	0.24
0.9	0.211	0.209	0.213	0.211
0.8	0.182	0.173	0.182	0.179
0.7	0.167	0.159	0.166	0.164
0.5	0.103	0.114	0.104	0.107

Table 2. Accuracy results for hydroquinone plus liposome.

Liposomal hydroquinone µg/ml	0.5	0.5	0.5
Hydroquinone added µg/ml	0.3	0.5	0.7
Final concentration µg/ml	0.8	1	1.2
Differences of absorption between control	0.175	0.245	0.301
and solutions in 516nm	0.176	0.239	0.290
	0.183	0.235	0.302
Found concentration µg/ml	0.76	1.02	1.23
	0.76	0.99	1.18
	---	0.98	1.23
Mean µg/ml	0.77	0.99	1.21
Recovery	96.2%	99%	100.8%
Standard Deviation	0.017	0.020	0.028
Relative Standard Deviation%	2.25	2.09	2.38

low amount of RSD obtained in this study indicates that the reproducibility of the test is good. In the same study (17), a concentration of 10 µg/ml of hydroquinone was measured in a liposomal formulation, and here a concentration of up to 10 times less was measured.

3.3. Accuracy

This parameter was used to evaluate the closeness of results of the laboratory process with the real values. The accuracy obtained was between 100.8% and 96.2%, which was close to the real value (Table 2).

3.4. Specificity

The presence of other ingredients in the formulations may affect the accuracy of the reported concentration in medicinal products. In this formulation, in addition to hydroquinone, there were other materials present such as phospholipids, cholesterol, and sodium metabisulfite, so that they may interact in the process, and the effects could be tested. The liposomal formulation was used to evaluate the effects of the other ingredients without hydroquinone. A concentration equivalent to the absorption of this sample was calculated according to the standard equation. The results of showed concentrations less than the LOD, which shows-non-interference of other molecules in the DPPH reduction, and only hydroquinone is able to reduce it.

3.5. LOD and LOQ

The lowest concentration of hydroquinone that can be measured by the process is 0.149 µg/ml, and the lowest concentration of hydroquinone that is detectable in the liposomal matrix is 0.04 µg/ml. In a study, where the spectrophotometric technique was used, the LOQ and LOD obtained were 0.72 and 0.24 µg/ml (17), respectively. The present study has proved the possibility of evaluating smaller amounts.

4. Conclusion

The antioxidant property of HQ can be used to measure its concentration in the liposomal matrix with the help of DPPH at concentrations lower than the spectroscopic technique. The presence of sodium metabisulfite that has an antioxidant behaviour showed no effect on the DPPH radicals because the mixture of water and methanol causes phase separation between the molecules of water and methanol. Therefore, the interaction between the sodium metabisulfite dissolved in water and the DPPH solution in methanol decreases. But since hydroquinone is well dissolved in methanol, it can still interact with DPPH and reduction of DPPH radicals. The use of fresh samples has been one of the crucial points in the use of this technique because the reproducibility of old discolored samples is low.

Conflict of Interest

None declared.

5. References

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