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Spectrofluorimetric and Spectrophotometric Determination of Troxerutin in Pharmaceutical Preparations

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Two new, sensitive and simple spectrofluorimetric and spectrophotometric methods have been developed for the determination of troxerutin in pharmaceutical preparations. Troxerutin inhibits the activity of hemoglobin on the catalyzed reaction of H_2O_2 with rhodamine B, which is a highly sensitive fluorogenic and chromogenic reagent used in many investigations. The reaction product was measured by spectrofluorimetry at 575 nm after excitation at 550 nm. The percentage inhibition was directly proportional to the concentration over the range 0.04-1.5 μg/mL for the spectrofluorimetric method. The relation between the percentage inhibition of the absorbance at 550 nm and the concentration is rectilinear over the range 0.2-30 μg/mL. This method was applied successfully to the determination of troxerutin in pharmaceutical dosage form.

Keywords: Troxerutin, Spectrofluorimetry, Spectrophotometry.

INTRODUCTION

Troxerutin is a trihydroxyethylated flavonoid derivative of rutin present in coffee, cereal grains and many kinds of vegetables and fruits[1,2]. It has antithrombotic, antierythrocytic, fibrinolytic, oedema-protective and rheological activity[3] and has been used therapeutically to treat chronic venous insufficiency[4], varicose veins[5] and haemorrhoids[6]. In recent years, a few methods have been reported on the determination of ethamsylate in dosage forms and biological fluids such as ultraviolet spectrophotometry[7], spectrofluorimetry[8], chemiluminescence[9] and high performance liquid chromatography[10].

Haemoglobin, a necessary vehicle for oxygen carriage in body, has the natural quaternary structure as enzymes. It contains four subunits of polypeptide and each polypeptide chain contains a heme group that may be able to serve as the active center[11]. In a recent paper, haemoglobin was used as biological catalyst[12].

This study presents two new spectrofluorimetric and spectrophotometric methods for the assay of troxerutin. The methods are based on the inhibitory of hemoglobin on the catalyzed reaction of H_2O_2 with rhodamine B, which is a highly sensitive fluorogenic and chromogenic reagent. The applicability of the developed methods was evaluated through the determination of troxerutin in pharmaceutical dosage form.

EXPERIMENTAL

Fluorescence spectra and measurements were taken on a FP-750 spectrofluorimeter (JASCO). Excitation and emission wavelengths were set at 550 and 574 nm, respectively.

The spectrophotometric detection was carried out on a V-530 UV-visible spectrophotometer (JASCO). The temperature was controlled by using a TB-85 thermostat bath (Shimadzu) and the pH values were measured with a pHS-3C precision pH meter (Shanghai, China).

Hemoglobin (bovine erythrocytes) solution was prepared by dissolving certain amount of hemoglobin (Shanghai Aobo Institute of Biochemistry, Shanghai, China) in distilled water and stored below 4 °C. Rhodamine B (Beijing Chemical Plant, Beijing, China) stock solution was prepared to the concentration of 10^{-3} M in rhodamine B and diluted appropriately before use. H_2O_2 solution was prepared by appropriately diluting 0.01 mL of 30 % H_2O_2 (standardized by titration with $KMnO_4$) to 100 mL. It was stored in a brown bottle in a refrigerator. A stock solution of troxerutin (Shanghai Xinmeng Institute of Biochemistry, Shanghai, China) containing 1 mg/mL was prepared in water and diluted further with the water to obtain standard solution of 100 μg/mL. NH_3·H_2O-NH_4Cl buffer solutions of different pH was used throughout the present study. Doubly distilled water was used throughout. All other chemicals were of analytical-reagent grade.

For the spectrofluorimetric study, 2 mL pH 8.9 NH_3·H_2O-NH_4Cl buffer solutions was mixed with 2.4 mL of 1×10^{-5} M rhodamine B, an aliquot of 0.004-0.15 mL from standard or sample solution, 0.6 mL of 1×10^{-3} M H_2O_2 and 1 mL of 1×10^{-5} M hemoglobin and then diluted with water to 10 mL. After being equilibrated in room temperature for 20 min, the fluorescence intensities were measured at 575 nm while exciting at 550 nm. The percentage inhibition (I %) was then calculated by the following equation:

$$\text{Inhibition}\,(\%) = \frac{(F_2 - F_1)}{(F_0 - F_1)} \times 100$$

where F_0, substrate absorbance alone; F_2, substrate absorbance in the presence of hemoglobin and inhibitor and F_1, substrate absorbance in the presence of hemoglobin only.

For the spectrophometric study, 2 mL pH 8.9 NH_3·H_2O-NH_4Cl buffer solutions was mixed with 2 mL of 1×10^{-4} M rhodamine B, an aliquot of 0.02-3 mL from standard or sample solution, 0.8 mL of 1×10^{-3} M H_2O_2 and 1 mL of 1×10^{-5} M hemoglobin and then diluted with water to 10 mL. After being equilibrated in room temperature for 20 min, the absorbance was monitored at the selected maximum absorption wavelength of 550 nm. The percentage inhibition (I %) was calculated on the basis of the following equation:

$$\text{Inhibition}\,(\%) = \frac{(A_2 - A_1)}{(A_0 - A_1)} \times 100$$

where A_0, substrate absorbance alone; A_2, substrate absorbance in the presence of hemoglobin and inhibitor and A_1, substrate absorbance in the presence of haemoglobin only.

RESULTS AND DISCUSSION

Rhodamine B is a highly sensitive fluorogenic and chromogenic reagent used in many investigations. In this redox reaction between H_2O_2 and rhodamine B, different amounts of troxerutin had inhibitory effects on hemoglobin-catalyzed reaction. Under the described experimental conditions, the fluorophore exhibits its highest fluorescence intensities at λ_{ex} of 550 nm and λ_{em} of 575 (Fig. 1A) nm. The absorbance was measured at λ_{max} 550 nm (Fig. 1B).

The different experimental parameters affecting the fluorescence intensity were studied and optimized to obtain maximum inhibitory effect. First, the influence of pH on the fluorescence intensity was studied. The pH was varied over 8.6-10.4 using NH_3·H_2O-NH_4Cl buffer where the maximum relative fluorescence intensity was obtained at pH 8.9.

It is noted that troxerutin has less effect in assay involving higher concentrations of hemoglobin. The relative fluorescence intensity increased with increase in hemoglobin concentration at first, but decreased above 8×10^{-6} M. So 8×10^{-6} M of hemoglobin was selected for further work.

The effect of H_2O_2 concentration on the spectrofluorimetric system was studied. The relative fluorescence intensity increased with the increase in H_2O_2 up to 6×10^{-5} M, above which it had little effect. Thus 6×10^{-5} M was selected for further study. Considering the fluorescence intensity getting too weak at very low rhodamine B concentration, 2.4×10^{-6} M rhodamine B was chosen for further study.

The time needed to reach equilibrium after 20 min. Due to the decomposition of H_2O_2 at high temperature, room temperature was chosen and the measurements were carried out after 20 min.

As can be seen from Table-1, linear relationship was found between the relative fluorescence intensity and the concentration of troxerutin in the range of 0.04-1.5 µg/mL. Linear regression analysis of the concentration-relative fluorescence intensity date gave the following equation:

$$I\,\% = 22.4348C + 34.1650$$

where C is the concentration in µg/mL and I % is the percentage inhibition (n = 8). The correlation coefficient (r) was 0.9923 showing excellent linearity. The detection limit (LOD), calculated according to the $3S_b/k$ criterion (in which "k" is the slope over the range of linear used and "S_b" is the standard deviation (n = 11) of the signal from the blank), was found to be 5 ng mL^{-1}. Under the optimum reaction conditions the percentage inhibition (I %) was found to be linearly correlated to troxerutin concentration over the range of 0.2-30 µg/mL. The regression equation was found as:

$$I\,\% = 0.8660C + 18.7038 \; (r = 0.9985, n = 9)$$

where I % is the percentage inhibition of troxerutin at λ_{max}, a is the slope, b is the intercept and C is the concentration of the measured solution in µg mL^{-1}). The obtained results are summarized in Table-1.

The current method was applied to determine troxerutin in tablets and injection by using the procedure described in the experimental section. For analysis of tablets, accurate measured amounts of powered tablets were dissolved in ethanol

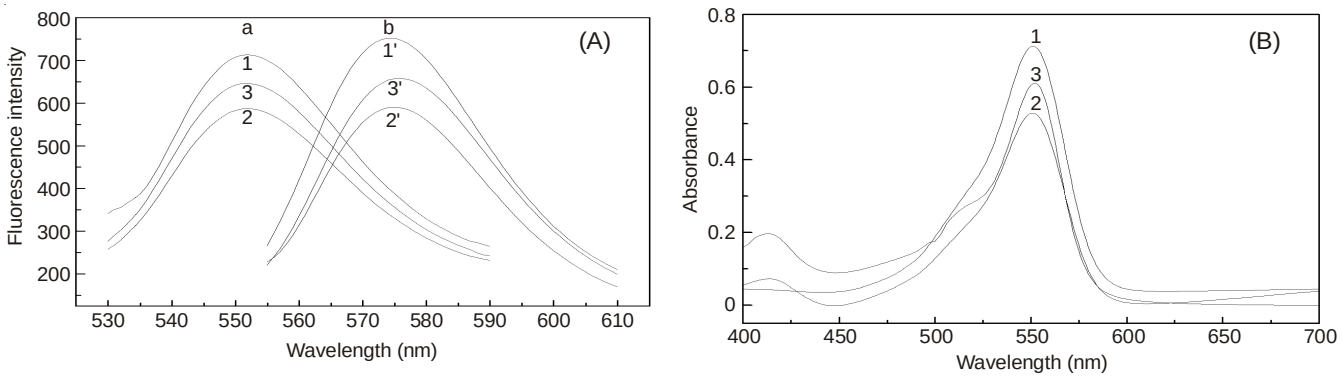

Fig. 1. (A) Excitation and emission spectrum (B) Absorption spectrum

TABLE-2
ANALYTICAL RESULTS FOR TROXERUTIN IN TABLETS AND INJECTIONS (n = 5)

Sample	Labeled (mg)	Founded mass (mg)		
		Spectrofluorimetric method	Spectophotometric method	UV method
Tablet	60	59.7 ± 0.4	59.3 ± 0.3	59.8 ± 0.2
Injection[a]	300	297.9 ± 0.3	296.1 ± 0.5	298.5 ± 0.2

[a]10 mL of the ampoule to 300 mg of troxerutin

TABLE-1
RELATED PARAMETER OF TWO METHODS

Parameter	Spectroflurimetric study	Spectrophotometric study
pH	8.9	8.9
Haemoglobin (M)	8.0×10^{-7}	1.0×10^{-6}
H_2O_2 (M)	6.0×10^{-5}	8.0×10^{-5}
Rodamine B (M)	2.4×10^{-6}	2.0×10^{-5}
Temperature (°C)	Room temperature	Room temperature
Time (min)	20	20
Beer's law limit (μg/mL)	0.04-1.5	0.2-30
Regression equation		
Slope ± S.D.	22.4348 ±1.1441	0.8660 ± 0.0180
Intercept ± S.D.	34.1650 ±1.0621	18.7038 ± 0.2350
Correlation coefficient (r^2)	0.9923	0.9985
LOD (μg/mL)	0.005	0.014

with the aid of ultrasonication. Certain volumes of the above solution were diluted with buffer solution. The filtered solutions of troxerutin were diluted to different concentrations with double distilled water, so the final concentration was in the working range for further sample analysis. The injection solutions of troxerutin were appropriately diluted with distilled water so that the final concentration was in the working range for further sample analysis. In order to evaluate the validity of the proposed method, UV method was also used for the determinations by closely following a reported procedure[7]. The results obtained by the two different methods were statistically compared in Table-2. It can be seen that no significant differences were found between them. It is indicated that the method is able to determine troxerutin in pharmaceutical preparations.

Conclusion

The suggested spectrofluorimetric and spectrophotometric methods have the advantage of being simple, accurate, sensitive, selective and suitable for routine quality control of pure drug and in pharmaceutical formulations. The spectrofluorimetric method exhibits the highest sensitivity that makes it possible to measure concentrations down to 0.04 μg/mL. The applicability of the method to the biological samples is now under investigation.

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