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Short Communication

Kinetic–fluorimetric determination of pilocarpine in ophthalmic solutions

M. CARMEN GUTIERREZ, AGUSTINA GOMEZ-HENS and MIGUEL VALCARCEL*

Department of Analytical Chemistry, Faculty of Sciences, University of Córdoba, Córdoba, Spain

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Introduction

Pilocarpine is a compound of ophthalmological interest for which different separation and determination procedures have been described. Among the separation techniques, chromatography has been used most frequently, especially high-performance liquid chromatography (HPLC), which has been shown to be the most suitable for the specific determination of pilocarpine [1, 2] and for the resolution of mixtures of pilocarpine and other similar compounds [3–6].

A variety of methods have been described for the determination of pilocarpine, including photometric [7–10], volumetric [11, 12], potentiometric [13, 14], amperometric [15], conductimetric [16, 17] and thermometric [18] procedures. However, fluorimetry rarely has been used in the determination of pilocarpine. A general method for assaying alkaloids containing a tertiary amine group has been reported [19], involving the use of the malonic acid–acetic anhydride system, which results in the formation of highly fluorescent condensation products of the alkaloids. Although the reaction is selective for tertiary amines, it does not distinguish between different tertiary amines. Two kinetic–spectrophotometric methods based on the catalytic activity of the imidazole portion of pilocarpine on the hydrolysis of 2,4-dinitrophenyl acetate [20, 21] are among the few methods for pilocarpine using the kinetic principle.

This paper describes the first kinetic method with fluorimetric detection for the determination of pilocarpine. It is based on the enhancement of the fluorescence intensity of the 1,1,3-tricyano-2-amino-1-propene (TRIAP)–hydrogen peroxide–Cu(II) system which previously has been used to determine copper in serum [22] and to resolve histidine–histamine mixtures [23]. Although the method proposed for pilocarpine suffers interference from other imidazole derivatives and, therefore, is less selective than the methods using HPLC, it is very fast, simple and inexpensive. The method has been

*To whom correspondence should be addressed.

applied satisfactorily to the analysis of pilocarpine in ophthalmic solutions and represents one of the few applications of kinetic non-enzymic analysis to organic compounds.

Experimental

Apparatus

Fluorescence measurements were made on a Perkin–Elmer fluorescence spectrophotometer (Model MPF-43A) fitted with a xenon-arc source and a device for direct recording of fluorescence–time graphs at fixed excitation and emission wavelengths. The temperature of the spectrofluorimeter cell compartment was kept at 60°C by circulating water from a waterbath. The instrumental conditions used were: sensitivity ×3; excitation and emission slits, 6 nm. A set of fluorescent polymer samples was used daily to adjust the spectrofluorimeter to compensate for changes in source intensity.

Reagents

A 1 g l^{-1} (free base) stock solution of pilocarpine hydrochloride (Aldrich) was prepared in distilled water and stored at 0–4°C. Standard solutions of lower concentration were prepared daily by diluting the stock solution with distilled water.

A 1,1,3-tricyano-2-amino-1-propene (TRIAP) (Aldrich) solution (4×10^{-3}M) was prepared by dissolving 26.5 mg of reagent in distilled water and diluting to 50 ml.

A copper(II) nitrate solution (10 μg ml^{-1} Cu), a 0.1 M K_2HPO_4–KH_2PO_4 buffer solution (pH 7.0) and 1.25 M hydrogen peroxide were also prepared.

All chemicals used were analytical reagent grade.

Procedure

A volume of sample containing between 0.02 and 1.25 mg of pilocarpine, 1.5 ml of 0.1 M phosphate buffer (pH 7.0), 1.3 ml of 4×10^{-3}M TRIAP solution, 1 ml of 1.25 M hydrogen peroxide and 0.5 ml of 10 μg ml^{-1} Cu(II) solution, were mixed in a 10-ml standard flask. A stop-clock was started and the solution was made up to the mark with distilled water. A portion of the reaction mixture was immediately transferred to a 1-cm cell in the cell compartment thermostatted at 60 ± 0.1°C. The variation of fluorescence intensity (λ_{ex} 350, λ_{em} 450 nm) with time was measured starting exactly 2 min after the addition of the copper reagent. The net fluorescence intensity was calculated by subtracting the measurements obtained for a blank solution prepared in a similar manner but containing no pilocarpine. Calibration graphs were constructed from the fluorescence–time curves by using one of the standard procedures indicated below.

Determination of pilocarpine in ophthalmic solutions

The sample solution without any pretreatment was diluted with distilled water to give a concentration of pilocarpine within the range of the calibration graph. The diluted solutions were treated as described above under the section entitled *Procedure*.

Results and Discussion

Fluorescence reaction

In the presence of hydrogen peroxide and copper the TRIAP–pilocarpine system yields an emission spectrum (λ_{ex} 350 nm) with a maximum at about 450 nm, as shown in Fig. 1. The fluorescence intensity of this band is greater than that obtained for

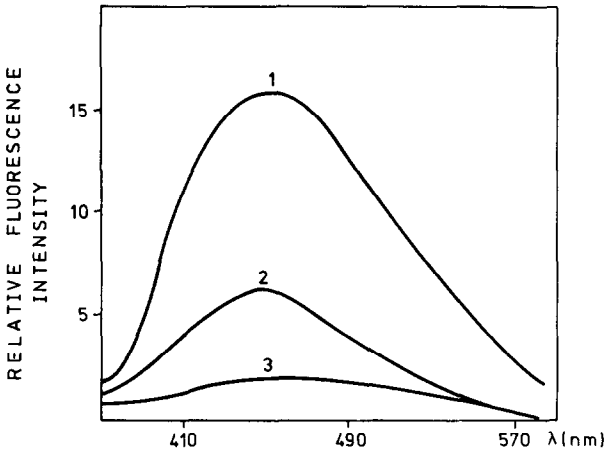

Figure 1
Emission spectra (λ_{ex} 350 nm): (1) pilocarpine–TRIAP–H_2O_2–Cu(II); (2) pilocarpine–H_2O_2–Cu(II); (3) TRIAP–H_2O_2–Cu(II). Conditions: 6×10^{-4} M TRIAP; 0.2 M H_2O_2; 0.5 μg ml^{-1} Cu(II); 50 μg ml^{-1} pilocarpine; reaction time 6 min; 60°C.

pilocarpine in the absence of TRIAP. Earlier studies [22, 23] showed that the fluorescence characteristics of the oxidation product of TRIAP are markedly affected by the presence of imidazole or one of its derivatives such as histidine or histamine. In the absence of imidazole, the oxidation product of TRIAP shows a fluorescence band around 500 nm (λ_{ex} 370 nm) in acidic or neutral solution. However, in the presence of imidazole (or histidine, histamine or pilocarpine) and in a neutral medium, a transient fluorescence band appears at 400–450 nm, depending on the imidazole derivative. When the solution is acidified, this maximum disappears as the band at 500 nm appears. As TRIAP possesses a charge-deficient carbon atom and the imidazole ring has a nitrogen atom with a lone electron-pair, the transient fluorescence can be attributed to the formation of a condensation product between TRIAP and imidazole, which is destroyed in acidic solution. The oxidation of this condensation product by hydrogen peroxide is favoured by the presence of copper, which results in the formation of an imidazole–copper complex [24, 25].

Effect of the reaction variables

The system was optimized by changing each variable in turn whilst keeping all the others constant. The optimum values were those yielding the minimum relative standard deviation for the initial-rate measurements, under conditions in which the reaction order with respect to the variable concerned was zero or close to zero.

An increase in temperature in the range 40–80°C caused an increase in the initial rate of the reaction and a decrease in the maximum fluorescence intensity. A temperature of 60 ± 0.1°C was selected.

Figure 2A shows that the initial rate of the reaction is at a maximum value in the pH range 6.8–7.8. A K_2HPO_4–KH_2PO_4 buffer solution (pH 7.0) was chosen to adjust the pH of the samples. The reaction rate does not depend significantly on the concentration of this buffer, at least in the range 10^{-2} to 6×10^{-2}M.

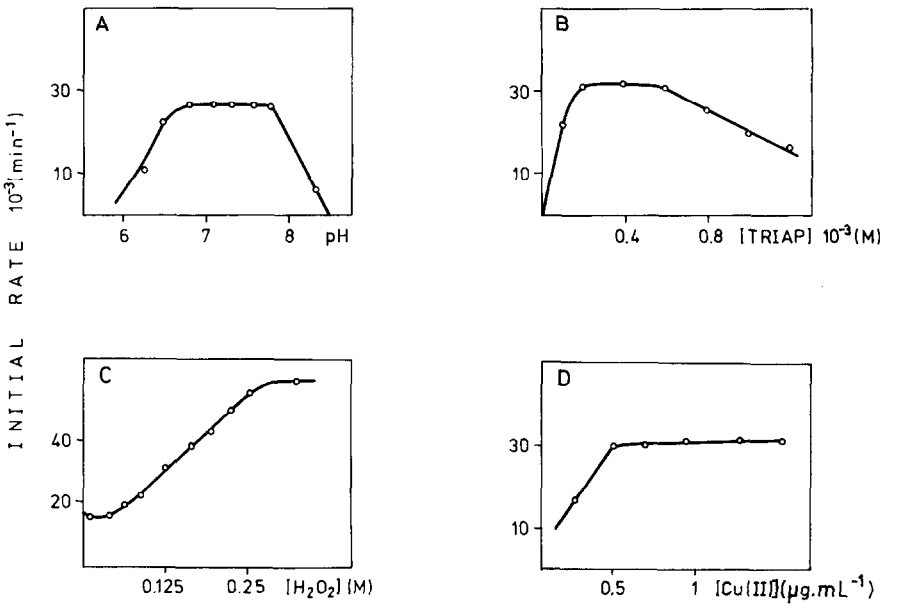

Figure 2
Effect of variables on reaction rate: (A) pH; (B) TRIAP concentration; (C) hydrogen peroxide concentration and (D) Cu(II) concentration.

The variation of the reaction rate with the change of the TRIAP concentration is shown in Fig. 2B. The reaction rate is constant and maximum in the concentration range 2×10^{-4} to 6×10^{-4}M. An increase in the hydrogen peroxide concentration in the range 3.7×10^{-2} to 0.25 M results in a gradual increase in the reaction rate and at higher concentrations the rate remains constant (Fig. 2C). However, when the concentration of hydrogen peroxide is high, the duration of the linear ratio between fluorescence intensity and time is very short and this results in decreased precision in the initial-rate measurements. A concentration of hydrogen peroxide 0.125 M was thus chosen.

The concentration of copper(II) in the range 0.3–1.5 μg ml^{-1} does not affect the reaction rate as shown in Fig. 2D.

A decrease in the reaction rate is observed when the dielectric constant of the solution is lowered by using organic solvents such as ethanol or dimethylformamide. The best results are obtained in an entirely aqueous medium. The variation of the ionic strength of the solution up to a value of 0.4 does not affect the reaction rate when potassium nitrate or sodium perchlorate is used although a decrease in the rate was observed when potassium chloride was added. Finally, the order of addition of reagents has no effect on the reaction development.

The kinetic equation suggested for the reaction, based on the results of the above experiments, is as follows:

$$d\,[\text{TRIAP}]_{ox}/dt = k\,[H_2O_2]^{2/3}\,[\text{Pilocarpine}],$$

where $[\text{TRIAP}]_{ox}$ is the concentration of oxidized reagent and k is the conditional rate constant.

Table 1
The concentration ranges and the relative standard deviations in the kinetic–fluorimetric determination of pilocarpine

Method	Concentration range of pilocarpine ($\mu g\ ml^{-1}$)	RSD (%)*
Tangent	10–100	±3.78
Fixed-time	2–100	±3.57
Maximum fluorescence intensity	20–125	±5.61

* Concentration of pilocarpine = 50 $\mu g\ ml^{-1}$.

Table 2
Results of the assay of pilocarpine hydrochloride in ophthalmic solutions (2% m/v)

Sample	Pilocarpine hydrochloride found (%)*		
	Tangent	Fixed-time	Maximum fluorescence intensity
Oculos (Frumtost-Prem S.A.)	2.09	2.02	2.05
Colircusi (Cusi S.A.)	1.94	1.92	1.96
Llorens (Llorens S.A.)	2.00	2.03	1.96

* Mean of three determinations.

Table 3
Recovery of pilocarpine added to ophthalmic solutions

Sample	Pilocarpine ($\mu g\ ml^{-1}$)			
	Added	Found*		
		Tangent	Fixed-time	Maximum fluorescence intensity
Oculos (Frumtost-Prem S.A.)	20.0	17.5	17.4	18.2
	40.0	40.4	37.6	37.8
	60.0	56.1	58.7	56.1
Colircusi (Cusi S.A.)	20.0	21.7	20.4	21.7
	40.0	39.6	40.6	42.0
	60.0	61.4	58.2	58.2
Llorens (Llorens S.A.)	20.0	20.6	22.5	22.5
	40.0	42.3	39.1	42.0
	60.0	58.2	63.6	62.6

* Mean of three determinations.

Assessment of the analytical methods

The fluorescence intensity versus time curves for solutions containing different concentrations of pilocarpine under optimum conditions were analysed by the tangent, fixed-time and maximum fluorescence intensity methods. For the fixed-time method, measurements were made 5 min after the sample preparation. The calibration graphs were linear over the concentration ranges indicated in Table 1, which also gives the corresponding relative standard deviations (n = 11). The maximum fluorescence intensity method was the least precise and the other two gave similar precision.

Applications

The three methods described above have been satisfactorily applied to the determination of pilocarpine in ophthalmic solutions from Spanish pharmaceutical laboratories, containing 2% pilocarpine hydrochloride. The results obtained are summarized in Table 2. The measurement of recovery was carried out by adding different amounts of pilocarpine to the ophthalmic solutions and subtracting from the measurements obtained those for the ophthalmic solution prepared in a similar manner but with no added pilocarpine. The recovery values obtained are summarized in Table 3.

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