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Hydrolysis and epimerization kinetics of pilocarpine in basic aqueous solution as determined by HPLC

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Summary

A high-performance liquid chromatographic method is described which allows the simultaneous determination of pilocarpine and the products of its degradation, isopilocarpine, pilocarpic acid and isopilocarpic acid. Using this method the degradation pattern of pilocarpine in basic aqueous solution was determined. The epimerization of pilocarpine to isopilocarpine was found to be a reversible reaction although the equilibrium is strongly in favor of isopilocarpine. Isopilocarpine was readily detected during the overall degradation of pilocarpine and was found to possess the same stability as its diastereoisomer. The relative importance of epimerization to hydrolysis of pilocarpine increased with increased temperature, ranging from 12% at 18°C to 20% at 66°C.

Introduction

Pilocarpine (I) is widely used as a topical miotic in the treatment of glaucoma. Its stability in aqueous solution has been the subject of several investigations, showing that the principal reactions contributing to the instability are hydrolysis of the γ-lactone moiety to yield pilocarpic acid (III) and epimerization at the α-carbon of the lactone ring resulting in the formation of isopilocarpine (II). The latter can degrade further by hydrolysis to isopilocarpic acid (IV). These reactions are summarized in Scheme 1, which is adapted from the schemes proposed by Chung et al. (1970), Nunes and Brochmann-Hanssen (1974) and Neville et al. (1976).

The hydrolysis of the γ-lactone ring is a cyclic equilibrium process which is catalyzed by hydrogen ions and hydroxide ions (Chung et al., 1970). The equilibrium position depends on pH; in neutral and basic aqueous solutions pilocarpinate is the

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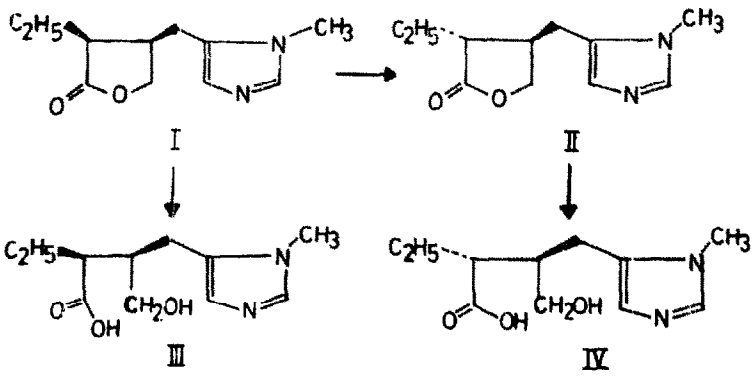

Scheme 1

predominant or sole species while at low pH the equilibrium position shifts to pilocarpine (Chung et al., 1970; Neville et al., 1976). Somewhat less is known about the epimerization reaction. Nunes and Brochmann-Hanssen (1974) studied the epimerization of pilocarpine in alkaline aqueous solution using NMR spectroscopy and pH-stat titrimetry and found it to be hydroxide-ion catalyzed like the hydrolysis reaction. It was further reported that the relative importance of these two degradation reactions was dependent on the temperature due to different energies of activation of the processes. The rate of hydroxide-ion catalyzed epimerization increased more rapidly with temperature than did the rate of hydrolysis; at 25°C epimerization accounted for 20–21% of the total degradation, increasing to 32% at 55°C. Using a [^{13}C]NMR spectroscopic method Neville et al. (1976) reported that the epimerization accounted for 28 ± 3% of the total degradation at 30°C and pH 10–14 and that the relative dominance of hydrolysis and epimerization did not vary with the temperature which is contradictory to the finding based on kinetic studies (Nunes and Brochmann-Hanssen, 1974). Both groups of investigators reported that the epimerization of pilocarpine to isopilocarpine was irreversible at any pH, the detection limit being about 3%, and that the two carpic acids (i.e. pilocarpic and isopilocarpic acids) are unable to epimerize whether in the anion or in the free acid form, as is also reported by others (Döpke and d'Heureuse, 1968).

In these studies reporting on the epimerization of pilocarpine the extent and relative importance of the reaction were deduced from analysis of the final product of epimerization, isopilocarpic acid; no analysis was made of isopilocarpine, the compound being postulated to be more susceptible to hydrolysis than pilocarpine and therefore to have only a transitory existence (Nunes and Brochmann-Hanssen, 1974). Similarly, Repta and Higuchi (1971) have assumed that degraded solutions of pilocarpine contain only pilocarpic and isopilocarpic acid.

The purpose of this investigation was to provide a detailed interpretation of the degradation pattern of pilocarpine in alkaline aqueous solution and to resolve the contradictory findings described above. To this end an unambiguous high-performance liquid chromatographic method for the simultaneous quantitative determination of pilocarpine and its degradation products II–IV was developed.

Materials and methods

Chemicals

Pilocarpine hydrochloride (Ph. Nord. 63) and isopilocarpine nitrate (Aldrich Chemicals) were used without further purification. All other chemicals or solvents used were of reagent grade.

Equipment

A Waters liquid chromatograph equipped with a high-pressure pump (M-6000A), a 710A WISP autoinjector, a 441 absorbance detector (214 nm), a 730 data module and a 720 system controller were used with a Knaur 25 cm × 4.6 mm i.d. column packed with 5 μm silica (LiChrosorb Si 60, E. Merck, F.G.R.). The column was thermostated in a LC 250/3 Kratos oven.

HPLC analysis conditions

The normal-phase column was eluted isocratically with a mobile phase consisting of methanol–2 M phosphoric acid–water (3:5:92 v/v) containing 3% of anhydrous sodium sulphate. The flow rate was 1.2 ml min^{-1} and the column effluent was monitored at 214 nm. The column was operated at 40 ± 0.5°C.

Standard solutions of pilocarpine hydrochloride or isopilocarpine nitrate were prepared in water and those of the acids in 0.1 M sodium hydroxide (see below) over a concentration range of 0.1–1 mg ml^{-1}. The solutions (5 μl) were chromatographed and calibration lines were constructed on the basis of peak-area measurements.

Degradation studies

The degradation of pilocarpine or isopilocarpine was studied in aqueous buffer solutions at a constant temperature. The reactions were initiated by adding 100 μl of aqueous stock solutions of the compounds to 25.00 ml of pre-heated buffer solution in screw-capped test-tubes, the final concentration of pilocarpine or isopilocarpine being about 1 mg ml^{-1}. At appropriate intervals, samples were taken and mixed with a pre-determined amount of 2 M hydrochloric acid to give a pH of 6 ± 0.5. The mixtures were analyzed within 0.5 h for pilocarpine and the degradation products II–IV by the HPLC assay described above.

Results and discussion

Chromatography

Recently, high-performance liquid chromatography has been utilized to separate and quantitate pilocarpine and its degradation products. Using derivatization with *p*-nitrobenzyl bromide, Mitra et al. (1980) described the separation of pilocarpine and its diastereoisomer, but no mention was made of detection or estimation of the two carpic acids III and IV. Similarly, Dunn et al. (1981) have reported the separation of pilocarpine and isopilocarpine in a normal-phase system with a silica column but the procedure did not allow analysis of the carpic acids due to their

retention on the column. Noordam et al. (1968) succeeded in separating pilocarpine and its 3 degradation products on a C_{18} column using a mobile phase of water–methanol (97:3) containing 5% of potassium dihydrogen phosphate adjusted to pH 2.5 with phosphoric acid. Refractive index detection was used but increased sensitivity can be achieved by using UV detection at 215–216 nm (O'Donnell et al., 1980; Noordam et al., 1981). Recently, Kennedy and McNamara (1981) modified this procedure by replacing the octadecylsilane column with a μBondapak phenyl column and operating the column at 40°C. This modification reduced analysis times and improved peak shapes and resolution [1].

As also experienced by Kennedy and McNamara (1981) and Dunn et al. (1981) our attempts to repeat the method by Noordam et al. (1978, 1981) gave relatively poor resolution and excessive tailing. We found, however, that increasing the column temperature from ambient to 40°C resulted in significantly improved resolution and peak shape.

The present method utilizing a column packed with 5 μm silica particles was found to afford satisfactory separation of pilocarpine and the degradation products II–IV within an analysis time of 15 min as can be seen from the chromatogram

[1] The paper by Kennedy and McNamara (1981) appeared after the completion of this work and their method has not yet been tried.

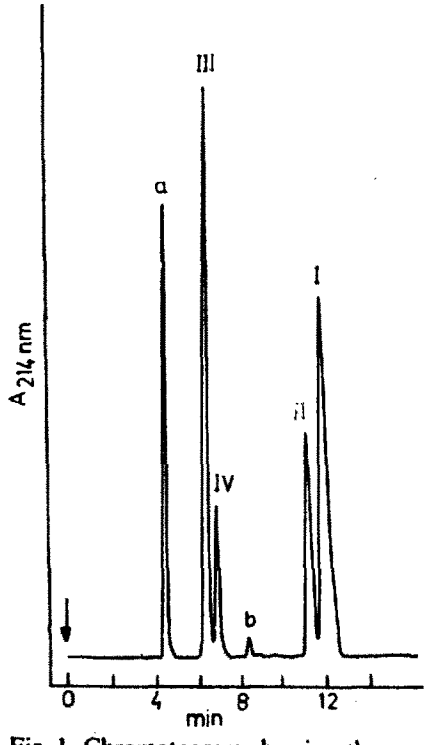

Fig. 1. Chromatogram showing the separation of pilocarpine (I), isopilocarpine (II), pilocarpic acid (III) and isopilocarpic acid (IV). The peaks denoted a and b represent nitrate and an unknown degradation product of pilocarpine, respectively.

shown in Fig. 1. Poor separation of pilocarpine and isopilocarpine was observed by operating the column at 20–25°C and a temperature of 40°C was found to be optimal.

For all the compounds linear standard curves passing through the origin were observed for concentrations up to at least 2 mg ml^{-1}. The reproducibility of the method was good with relative standard deviations of 1–2% and an internal standard was not required. Pilocarpic and isopilocarpic acid elute early and can be determined in the presence of pilocarpine in concentrations less than 0.01% of the pilocarpine concentration while the lower limit of detecting isopilocarpine in the presence of pilocarpine is about one part in 100 parts of pilocarpine.

Since pilocarpic and isopilocarpic acid are not available it has been common to prepare standard solutions of the acids by hydrolyzing known quantities of pilocarpine or isopilocarpine in 0.1 M sodium hydroxide at room temperature (Repta and Higuchi, 1972; Noordam et al., 1978 and 1981; O'Donnell et al., 1980; Kennedy and McNamara, 1981). However, such a procedure does not lead, as apparently supposed in these reports, to pure solutions. Hydrolysis of isopilocarpine in 0.1 M sodium hydroxide gave almost exclusively isopilocarpic acid, the pilocarpic acid produced amounting to 5.5% as estimated by assuming equal peak areas of equimolar amounts of the acids. Having an almost pure standard solution of isopilocarpic acid, the composition of a hydrolyzed solution of pilocarpine in 0.1 M sodium hydroxide (kept for 2–4 h at 20°C) could then be determined and was found to be 85% of pilocarpic acid and 15% of isopilocarpic acid.

Thus, for calculation of the carpic acid concentration in samples to be analyzed standard solutions corrected in this way should be used. It was confirmed that the two carpic acids in identical concentrations gave the same peak areas (within ± 2%).

Degradation of pilocarpine in basic solution

The HPLC method described was used to determine the degradation pattern of pilocarpine in alkaline aqueous solution. Fig. 2 shows the time courses for pilocarpine and its degradation products in a 0.1 M carbonate buffer solution ($\mu = 0.5$) of pH 10.90 at 37°C. At any time the sum of the concentrations of pilocarpine and the compounds II–IV corresponds to 100 ± 5% in relation to the initial pilocarpine concentration. As is apparent from the figure isopilocarpine is formed in significant amounts during the degradation, the maximum concentration reached being 8%. It is also seen that there is a marked induction period in the formation of isopilocarpic acid, in agreement with its production from the initially formed isopilocarpine. The overall reactions may be described by the following scheme of parallel and consecutive reactions:

$$\begin{array}{l} \mathrm{I} \xrightarrow{k_1} \mathrm{II} \xrightarrow{k_2} \mathrm{IV} \\ \quad\downarrow k_3 \\ \mathrm{III} \end{array}$$

Scheme 2

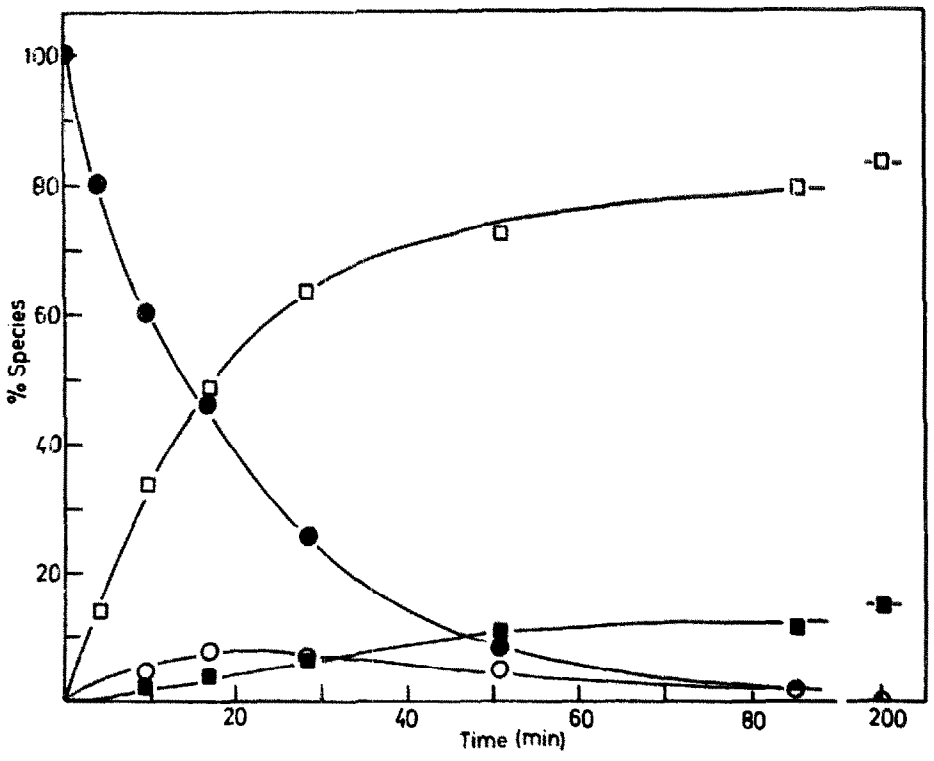

Fig. 2. Time courses for pilocarpine (●), isopilocarpine (○), pilocarpic acid (□) and isopilocarpic acid (■) in the degradation of pilocarpine hydrochloride (1 mg ml^{-1}) in 0.1 M carbonate buffer of pH 10.90 at 37°C. The concentrations at various times, expressed as mol% in relation to the initial pilocarpine concentration, were determined by HPLC.

where k_1–k_3 are pseudo-first-order rate constants for the depicted hydrolysis and epimerization reactions.

The observed pseudo-first-order rate constant for the overall degradation of pilocarpine (k_{obs}) was calculated from the slope of a linear plot of the logarithm of residual pilocarpine against time and found to be 0.047 min^{-1}. The appearance of pilocarpic acid also followed strict first-order kinetics and the rate constant (i.e. k_{obs}) obtained from its time course was identical to the value for k_{obs} derived from the pilocarpine curve. The amount of pilocarpic acid formed at the end of degradation was 86% and consequently, the value for k_3 is equal to $0.86 \cdot k_{obs} = 0.040$ min^{-1}. Using the identity of $k_{obs} = k_1 + k_3$, k_1 was determined to be 0.007 min^{-1}.

The remaining rate constant k_2 was most accurately obtained by following the degradation of isopilocarpine in a separate experiment at the same reaction conditions. The overall reaction followed first-order kinetics over at least two half-lives with a rate constant of 0.047 min^{-1}. As seen from Fig. 3 the chromatographic analysis of the reaction solution revealed the continuing formation of pilocarpic acid reaching a final concentration of 5.5%. The remaining acid predominant product of degradation corresponding to 94.5% was isopilocarpic acid while traces (< 1%) of pilocarpine were detected during the reaction. Both pilocarpic and isopilocarpic acid were found to be stable in the basic solution and analysis of the isopilocarpine

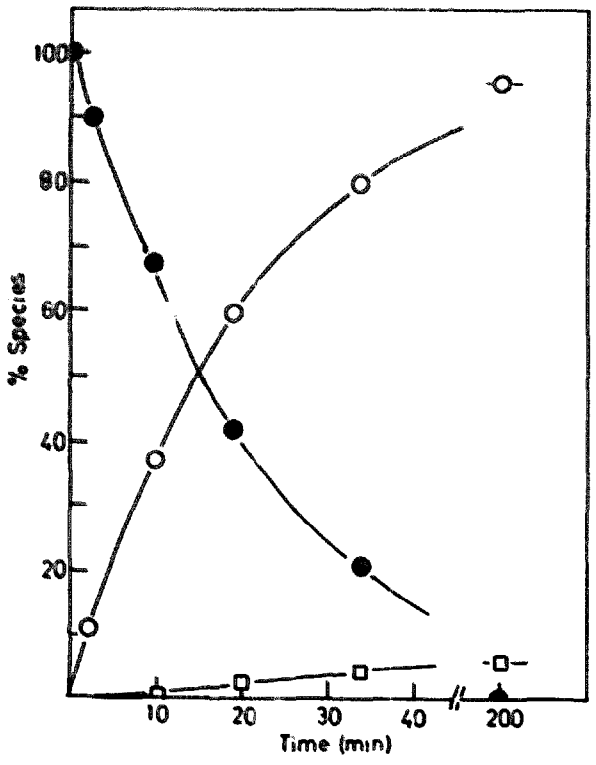

Fig. 3. Time courses for isopilocarpine (●), isopilocarpic acid (○) and pilocarpic acid (□) in the degradation of isopilocarpine nitrate (1 mg ml^{-1}) in 0.1 M carbonate buffer of pH 10.90 at 37°C. The concentration at various times, expressed as mol% in relation to the initial isopilocarpine concentration, were determined by HPLC.

sample used showed that the occurrence of pilocarpine as an impurity in the preparation was minimal (< 1%). Thus, the results obtained clearly demonstrate that isopilocarpine epimerizes to pilocarpine in basic aqueous solution concurrently with undergoing hydrolysis to isopilocarpic acid. Identical results were obtained in 0.1 M sodium hydroxide at 25°C. The epimerization is a minor process compared with the hydrolysis, and although the equilibrium between pilocarpine and isopilocarpine strongly favours the former, the data provide for the first time evidence for the reversible nature of the epimerization. Taking the slight epimerization of isopilocarpine into account the value of the rate constant k_2 was determined to be 0.044 min^{-1}. According to these results the reaction I → II in Scheme 2 should be formulated as a reversible reaction to be strictly correct.

The degradation of pilocarpine at other basic pH values at 37°C showed similar patterns to that at pH 10.90 which is in accord with the previous finding of hydrolysis and epimerization both being specific base-catalyzed reactions (Nunes and Brochmann-Hanssen, 1974).

The overall stability of pilocarpine and isopilocarpine in alkaline solution is seen to be exactly the same. The rate constants for hydrolysis at pH 10.90 and 37°C are also almost equal (0.040 min^{-1} for pilocarpine and 0.044 min^{-1} for isopilocarpine) which is in contrast to the statement by Nunes and Brochmann-Hanssen (1979) of

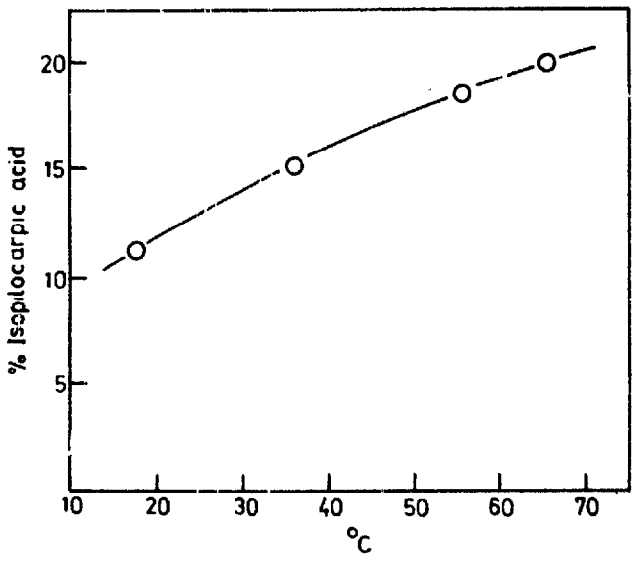

Fig. 4. Effect of temperature on the percentage amount of isopilocarpic acid formed upon degradation of pilocarpine hydrochloride (1 mg ml^{-1}) in 0.1 M carbonate buffer of pH 10.90 (measured at 37°C).

greater hydrolytic susceptibility of isopilocarpine.

The chromatographic monitoring of the degradation of pilocarpine revealed the progressive formation of a minor product in addition to the compounds II–IV. As seen from Fig. 1 this unknown compound eluted between the carpic acids and isopilocarpine. This product was, however, formed in amounts less than 1%.

The influence of temperature on the relative importance of hydrolysis and epimerization of pilocarpine was investigated by analyzing completed reaction solutions (1 mg ml^{-1} of pilocarpine hydrochloride in 0.1 M carbonate buffer of pH 10.9) for pilocarpic and isopilocarpic acid. As seen from Fig. 4 the relative importance of epimerization increases with increasing temperature within the range 19–66°C. This result is in contrast to the statement made by Neville et al. (1976) but in agreement with the conclusion reached by Nunes and Brochmann-Hanssen (1974) although the percentage amounts of isopilocarpic acid reported by these authors are somewhat higher (20 and 32% at 25 and 55°C, respectively) than those found in the present investigation.

Acknowledgement

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